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CHLORANILIC ACID AS A REAGENT IN THE PAPER CHROMATOGRAPHY OF THE SODIUM SALTS OF ORGANIC ACIDS

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INTRODUCTION

Chloranilic acid (2,5-dichloro-3,6-dihydroxy-*p*-quinone) was first applied in analytical chemistry by BARRETO[1,2], who used it for the determination of inorganic compounds such as calcium, barium, strontium and zinc. Later on the same author used it as a reagent in organic chemistry, obtaining good results with nitrogenous compounds such as caffeine, theobromine and theophylline[3], coniine[4] and nicotinamide[5].

We have already applied chloranilic acid in the paper chromatography of inorganic compounds[6], whereby a high sensitivity was obtained together with a very low specificity, so that it can be classified as an efficient chromatographic reagent. This efficiency was maximal for sodium, of which amounts as low as 0.2 μg could be detected.

The two main problems in the paper chromatography of organic acids are the "tailing" of the spots and the loss of material due to volatilization (in the case of the two lower members of the fatty acids), which can both be avoided by using salts instead of the free acids. The ammonium derivatives are most frequently used[7,8], as they react readily with the pH indicators commonly employed as sprays[8,9]. They are not so efficient, however, from the point of view of preventing volatilization. In this respect sodium salts are much more effective; they were used by BROWN[10], BROWN AND HALL[9] and HISCOX AND BERRIDGE[11].

In any case, the use of pH indicators for the localization of the isolated spots is not very satisfactory, as the colors are transient. By using the sodium salt in order to stabilize the spots, and localizing them by means of the reaction of chloranilic acid with the cation, a good sensitivity and a permanent record were obtainable.

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MATERIALS AND METHODS

Reagent. Chloranilic acid (0.1 % in ethyl ether) kept in a dark bottle.

Samples. Stock solutions of the sodium salts of the organic acids were prepared so that they contained 1 % of the cation. They were kept in the refrigerator and diluted as required.

Paper chromatography. Macherey-Nagel No. 261 filter paper was used, together with the solvent system of ISHERWOOD AND HANES[7] (propanol–conc. ammonium hydroxide (70:30)). The chromatograms were run by the descending technique for 24 h (displacement of about 30 cm), or by the "dripping" technique for 48 h, in which case formate was taken as reference standard ($R_F' = 1.0$).

Identification of the spots. The thoroughly dried chromatogram (120°/15 min) was put in an enamel tray and covered with a thin layer of the reagent. After a few seconds the reagent was returned to the bottle and the paper was washed twice with ethyl ether, dried (with hot air or in the oven at 120°) and observed under the U.V. lamp.

Sensitivity evaluation. As stated[6], the sensitivity limit of chloranilic acid for the sodium ion is 0.2 μg. A sufficient volume of the samples was applied to the paper to yield that amount of the cation. It was then run as a descending chromatogram as described. In some cases the sensitivity was higher than the theoretical value, and smaller amounts were tested to ascertain the limit.

RESULTS AND DISCUSSION

The results obtained with the described technique are summarized in Table I. The R_F values found by us are not the same as those obtained by ISHERWOOD AND HANES[7]. This is partly due to differences in procedure (they used Whatman No. 1 filter paper), but mainly to the use of different cations (they used NH_4^+ and not Na^+).

TABLE I

SENSITIVITY OF CHLORANILIC ACID TOWARDS THE SODIUM SALTS OF SOME ORGANIC ACIDS IN DESCENDING PAPER CHROMATOGRAMS

The theoretical values are given between parentheses.

Sample	*μg salt*	*μg acid*	R_F	R_F'*
Carbonate	0.4	0.2	0.11	0.68
Formate	0.6	0.4	0.19	1.00
Acetate	0.7	0.5	0.14	0.87
Citrate	0.7	0.5	0.05	0.31
Oxalate	0.6	0.3	0.00	0.00
Tartrate	0.6	0.4	0.02	0.12
Pyruvate	1.0	0.7	0.14	0.77
Lactate	1.0	0.7	0.16	0.87
α-Ketoglutarate	0.8	0.6	0.13	0.68
Glycerophosphate	1.3	1.0	0.12	0.66
Nicotinate	1.0 (1.3)	0.8 (1.1)	0.13	0.90
p-Aminobenzoate	1.0 (1.3)	0.8 (1.1)	0.13	0.86

* With formate as reference substance in "dripping" chromatograms.

As has been stated, we preferred sodium because we had a very sensitive reaction for its identification, which not only yields a permanent record but is also simple and rapid. We also checked the ammonium salts (the sensitivity of chloranilic acid towards ammonium salts is about the same as for sodium salts), but some loss by volatilization was observed in the case of the formate and acetate spots.

Under the conditions we used, paper chromatograms of the sodium salts of organic acids show a single spot for each sample, with the exception of some impure batches of sodium acetate and sodium lactate, which presented a fake spot besides the true one. This led us to the assumption that no dissociation of the salt occurred during development.

Should dissociation occur, then in a medium containing such a high concentration of ammonium hydroxide as the solvent we used, at least three spots would have been found: one for the sodium salt itself, another for the newly formed ammonium derivative and yet another one for the resulting NaOH.

The R_F values for such possible contaminants differ considerably from those of the original samples, as can be seen in Table II.

TABLE II

COMPARISON OF THE R_F VALUES OF THE SODIUM SALTS OF SOME ORGANIC ACIDS WITH THOSE OF POSSIBLE CONTAMINANTS

Salt	Na^+	NH_4^+
Acetate	0.14	0.78
Citrate	0.05	0.11
Oxalate	0.00	0.12
Tartrate	0.02	0.21
Hydroxide	0.13	—

As is to be expected, the sensitivity is proportional to the number of neutralized carboxyls and inversely proportional to the number of carbon atoms in the molecule. On the other hand, the sensitivity of chloranilic acid towards the sodium salts of nitrogen-containing organic acids, such as nicotinic and *p*-aminobenzoic acid, is higher than that expected from their sodium content. This is due to coupling of the reagent with the pyridine nucleus, in the first case, and with the aromatic amine, in the second.

The sensitivity of chloranilic acid towards carbonate was included in Table I, because of the occurrence of carbonate in the metabolic pathways of micro-organisms, and because this is the case in which the chromatographic separation of organic acids is most frequently used.

The high reactivity of chloranilic acid forbids the use of buffered papers and solvents, as well as of chelating agents such as EDTA (ethylenediaminetetraacetic acid, versene). The chromatograms must be thoroughly dried before development, in order to avoid ghost spots (due to traces of moisture). This is especially necessary when using ammonia-containing solvents, which are bound to cause heavily coloured

backgrounds. It must also be borne in mind that only neutral or basic solvent systems can be used, as acid solvents would cause decomposition of the sodium salts.

From what has been said it can be inferred that the main advantages of the use of the sodium salts for the paper chromatography of organic acids are the stabilization of the spots of the lower members (formic and acetic acids) as well as of carbonic acid, which frequently accompanies them, and the prevention of "tailing". Chloranilic acid is a very sensitive reagent for the localization of the spots; the procedure is rapid and easy, and a permanent record of the results is obtained.

ACKNOWLEDGEMENTS

The present work was carried out in the Department of Biochemistry of the Central Laboratory of Tuberculosis, with the assistance of the Institute of Phthisiology and Pneumology of the University of Brazil and in collaboration with the Department of Agricultural Chemistry of the Rural University. One of us (R.C.R.B.) received a grant from the National Council of Research. We are very much indebted to Prof. A. BARRETO for his suggestions regarding the behaviour of chloranilic acid towards the nitrogen-containing organic acids.

SUMMARY

The authors studied the use of chloranilic acid for the localization of sodium salts of organic acids separated by paper chromatography. Besides avoiding "tailing" of the spots and loss by volatilization of the lower members of the series, the use of sodium salts permits a very high sensitivity, as amounts as low as 0.2 μg of sodium are detectable. Sensitivity in relation to the acids was found to be proportional to the number of neutralized carboxyls and inversely proportional to the number of carbon atoms in the molecule. In the case of nitrogen-containing organic acids the sensitivity was higher than that expected, because chloranilic acid reacts with the N atom.

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CHLORANILIC ACID AS A REAGENT IN THE PAPER CHROMATOGRAPHY OF INORGANIC COMPOUNDS

II. HEAVIER METALS

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INTRODUCTION

We have already studied the use of chloranilic acid as a reagent in the paper chromatography of alkali and alkali earth metals[1], with extremely good results. Its application to the identification of chromatographic spots of heavier metals was the next logical step, since BARRETO[2] had already used it in the determination of zinc and VOIGT[3] in that of zirconium.

In order to make a general approach to the subject we took samples from all the remaining analytical groups, and examined their chromatographic behaviour and the sensitivity of chloranilic acid towards each one of them. In the present paper the results of this work are presented.

MATERIALS AND METHODS

Reagent

As in the other instances[1], chloranilic acid was used as a 0.1 % (w/v) solution in ether (kept in a dark bottle in the refrigerator), and the detection of the spots was carried out by dipping the dry paper for a few seconds in the reagent, and afterwards washing it twice with ether. The cations appear as dark spots under U.V. light, or as coloured zones, by daylight, in the case of higher concentrations.

Sample solutions

Samples were prepared by dissolving a sufficient amount of the acetate in water to obtain a solution containing 0.1 % of the cation, or, in some cases, by dissolving a calculated amount of the oxide in glacial acetic acid. These 0.1 % solutions were diluted as required.

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Sensitivity evaluation

The sensitivity was evaluated first by means of a spot-test on filter paper[1] and then by running paper chromatograms with increasing concentrations, starting at the limits formerly established.

Paper chromatography

Macherey-Nagel No. 261 filter paper was used, and the first runs were carried out by the ascending technique, using ERLENMEYER's[4] solvent system (96% ethanol–2 *N* acetic acid, 80:20). Later on, many other solvent systems were investigated, as we wished to find the best means for a two-dimensional separation. Moreover, ERLENMEYER's solvent gave tailing spots with ferric iron, which disturbed the identification of the other cations.

We had either to obtain a compact Fe^{3+} spot or to eliminate it by elution, and therefore we tried the following solvent systems: acetic acid–methanol[5], acetone–acetic acid–methyl ethyl ketone (50:10:40), acetone–ethanol–acetic acid (95:4:1), dioxan–water (90:10 and 80:20) and a modification of ALMASSY's solvent[6] containing ethanol–acetic acid–ether (17:3:80). The best results, however, were obtained with acetone–8 *N* acetic acid (90:10). In this case the descending technique was used, iron being displaced with the solvent front.

When silver samples were spotted the chromatograms were run in the dark room, in order to avoid reduction.

RESULTS

The results obtained with the described technique are summarized in Table I. Sensitivity was expressed as the minimum amount of the cation (in μg) detectable after chromatographic separation.

TABLE I

SENSITIVITY OF CHLORANILIC ACID TOWARDS SOME OF THE HEAVIER METALS, SEPARATED BY PAPER CHROMATOGRAPHY WITH (1) ETHANOL–ACETIC ACID AND WITH (2) ACETONE–ACETIC ACID

Cation	*Sensitivity* μg	R_F		*Colour of spot*
		1	*2*	
Fe^{3+}	0.01	0.08*	1.00	brownish
Ni^{2+}	0.10	0.34	0.07	greenish
Co^{2+}	0.20	0.42	0.07	yellow
Cu^{2+}	0.10	0.27	0.20	light green
Zn^{2+}	5.00	0.78	0.31	greenish
Ag^{+}	4.00	0.37	0.08	brownish red
Cd^{2+}	2.00	0.71	0.20	brownish
Hg^{2+}	5.00	0.00	1.00	brownish
Pb^{2+}	0.10	0.10	0.09	brownish
U^{4+}	1.00	0.83	0.86	brownish

* Tailing.

As can be seen, there was a large variation in the sensitivity of chloranilic acid towards the heavier metals. It was extremely high towards ferric iron, where 0.01 μg was detectable, and only moderate with respect to zinc, mercury (II) and silver, where 4–5 μg were necessary. The values for the other metals lay between these extremes, 0.1–0.2 μg of nickel, copper (II), lead and cobalt (III) and 1.0–2.0 μg of uranium (as uranyl acetate) and cadmium, being easily identifiable.

The R_F values were determined after 18-h runs (displacement 30 cm). From these a two-dimensional map was drawn (Fig. 1) and then checked by running a

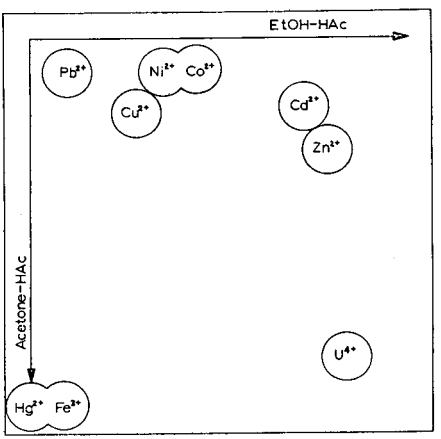

Fig. 1.

chromatogram. The first run was carried out by the descending technique, using acetone–acetic acid, and the second by capillary ascension, with ethanol–acetic acid. Good separations were obtained and well defined spots were found with concentrations that were about five times those considered as the limits of sensitivity (Table I).

DISCUSSION

As has been stated before[1], the main advantages of the use of chloranilic acid as a reagent in the paper chromatography of inorganic compounds are its low specificity and high sensitivity, as well as the short time required for the reaction and the ease with which it can be performed. By using ether as a solvent the risk of eluting material from the spot is completely eliminated, the spot does not diffuse and the chromatogram can be rapidly dried.

The main drawback of the method is that the cations must be run as salts of organic acids (we used acetates), as the reagent seems to be unable to displace the stronger inorganic ones. This limits the choice of the solvent to be used, and excludes HCl-containing mixtures, which are the solvents most frequently applied.

By using acetone–acetic acid for the separation of samples containing iron it is possible to prevent interference from this element, because it is carried to the front

of the solvent (tailing was observed with most of the proposed mixtures). This was specially true in the case of two-dimensional separations, when the first run with this solvent is essential.

ACKNOWLEDGEMENTS

The present work was carried out in the Dept. of Biochemistry of the Central Laboratory of Tuberculosis, with the help of the Institute of Phthisiology and Pneumology of the University of Brazil and in collaboration with the Institute of Agricultural Chemistry. One of us (R.C.R.B.) received a grant from the National Council of Research.

SUMMARY

The authors studied the use of chloranilic acid as a reagent in the paper chromatography of some of the heavier metals. Sensitivity was found to vary from 0.01 μg for ferric iron, to 5.0 μg for mercury (II) and zinc. It was found that iron gave tailing spots with most of the solvent systems, which interfered with the separation of the other cations. This interference was avoided by using acetone–acetic acid, which displaces iron to the solvent front. Efficient two-dimensional separations were obtained by carrying out a second run with ethanol–acetic acid.

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CHROMATOGRAPHIC BEHAVIOUR OF SOME STEREOCHEMICALLY INTERRELATED FLAVONOID COMPOUNDS IN AQUEOUS MEDIUM

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INTRODUCTION

The most versatile pair of solvent systems used for studying the complexity of plant extracts by two-dimensional paper chromatography consists of a partitioning mixture for the first direction followed by dilute acetic acid for the second. This combination, due to ROBERTS AND WOOD[1], might not be ideal for all complex mixtures of flavonoid compounds, but offers the advantage of separation based on different principles for each direction.

In the partitioning systems, for example, butyl alcohol–water mixtures often containing acetic acid, separation depends principally on the number of hydroxyl groups substituent on the C_{15} skeleton, and also on the configuration, *i.e. cis-* or *trans-* arrangement, of the main substituent groups at C atoms 2 and 3 in the heterocyclic ring (see BATE-SMITH AND WESTALL[2], BRADFIELD AND BATE-SMITH[3], ROBERTS[4,5], ROUX AND EVELYN[6] and ROUX AND MAIHS[7]).

During "adsorptive" separations in water, the mobility of the C_{15} compound is apparently dependent on the non-planar nature of the compound (see ROUX[8], ROBERTS, CARTWRIGHT AND WOOD[9], ROUX AND EVELYN[6] and ROBERTS[10]), and separations of the optical isomers of catechins and epicatechins have been shown to occur (ROBERTS AND WOOD[1]). ROUX AND EVELYN[6] showed that structural differences are also responsible for variations of R_F in water or dilute acetic acid. These factors may be evaluated more accurately now that the stereochemical interrelationship between many flavonoid compounds has been established as a result of the work of KING, CLARK-LEWIS AND FORBES[11], FREUDENBERG[12], BIRCH, CLARK-LEWIS AND ROBERTSON[13] and WEINGES[14,15].

EXPERIMENTAL AND RESULTS

Origin of substances

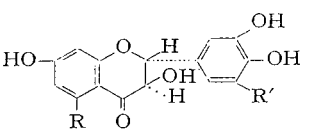
(—)-7,3′,4′-Trihydroxyflavan-3,4-diol from *Schinopsis* spp.[16,17,18] was hydrogenated under conditions established by WEINGES[14] to give (+)-7,3′,4′-trihydroxyflavan-3-ol [(+)-fisetinidol]. (—)-Fustin from *Cotinus coggygria*[15] was a gift from Dr. K. WEINGES. (+)-Trihydroxyflavan-3,4-diol was similarly hydrogenated to (—)-7,3′,4′-trihydroxyflavan-3-ol[19]. (+)-Fustin was isolated from the heartwood of *Acacia mollissima*[20]. (+)-Dihydrorobinetin was obtained from the heartwood of *Robinia pseudacacia*[14]

and hydrogenated under conditions established by FREUDENBERG AND ROUX[21] to (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol[14]. (—)-Robinetinidol was isolated from the bark of *A. mollissima*[22]. The R_F of (—)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol was determined by using the racemate obtained by the hydrogenation of (±)-dihydrorobinetin[23]. (+)-Catechin, (—)-epicatechin, (—)-epicatechin gallate, (+)-gallocatechin, (—)-epigallocatechin and (—)-epigallocatechin gallate were isolated from air-dried tea leaves and from the bark of *Acacia pycnantha*[24].

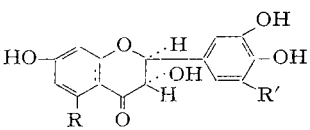
Chromatographic methods

The pure substances were applied within the concentration range 10–20 μg on Whatman No. 1 chromatographic paper and the chromatograms mounted on a stainless steel frame. Chromatograms were developed simultaneously with 2% aqueous acetic acid by upward migration to a point 13–14 inches (33–36 cm) from the starting line over a period of about 6–7 h. After drying the solvent front was located under ultra-violet light and accurately marked. R_F values were calculated and the average of at least three values obtained in the presence on each sheet of the reference compounds (+)-catechin (R_F, 0.35) and (—)-robinetinidol (R_F, 0.42).

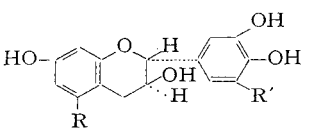
When different substances are applied to the same spot, a variable amount of mutual interference often occurs during migration resulting in slightly anomalous R_F values[7]. Only pure crystalline materials run singly from individual spots were



(Ia)



(Ib)



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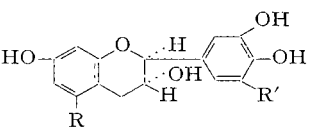

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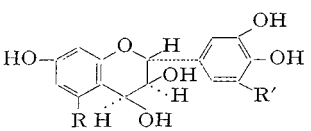

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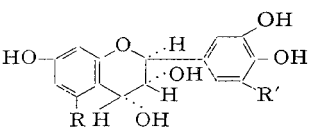

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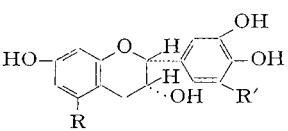

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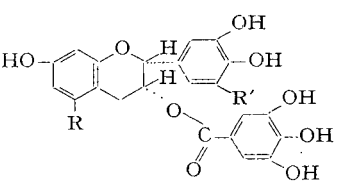

(V)

compared, exceptions being the pure racemate (±)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol and the (±)-2,3-dihydroflavonols. The R_F values of compounds having the formulae I–V are given in Table I. Values of the (±)-2,3-dihydroflavonols are the average for the optical isomers, which appear to have close values as shown for the enantiomorphous (+)- and (—)-fustins (R_F, 0.37 and 0.35 respectively).

TABLE I

R_F VALUES IN 2 % ACETIC ACID OF FLAVAN-3-OLS, FLAVAN-3,4-DIOLS, 2,3-DIHYDROFLAVONOLS, AND FLAVAN-3-GALLATES

Compound	R_F
(*a*) *Resorcinol series*	
(*1*) $R = R' = H$	
(+)-Fustin (Ia)	0.37
(—)-Fisetinidol (IIa)	0.48
(+)-7,3′,4′-Trihydroxyflavan-3,4-diol (IIIa)	0.52
(—)-Fustin (Ib)	0.35
(+)-Fisetinidol (IIb)	0.43
(—)-7,3′,4′-Trihydroxyflavan-3,4-diol (IIIb)	0.47
(*2*) $R = H$; $R' = OH$	
(+)-Dihydrorobinetin (Ia)	0.34
(—)-Robinetinidol (IIa)	0.42
(+)-7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol (IIIa)	0.46
(—)-7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol (IIIb)	0.40
(*b*) *Phloroglucinol series*	
(*1*) $R = OH$; $R' = H$	
(±)-Dihydroquercetin (I)	0.28
(+)-Catechin (IIa)	0.35
(—)-Epicatechin (IV)	0.30
(—)-Epicatechin gallate (V)	0.23
(*2*) $R = OH$; $R' = OH$	
(±)-Dihydromyricetin (I)	0.24
(+)-Gallocatechin (IIa)	0.32
(—)-Epigallocatechin (IV)	0.24
(—)-Epigallocatechin gallate (V)	0.20

DISCUSSION

Those mobile flavonoid compounds which have been studied in Table I have the absolute configurations illustrated in formulae I to V. Of these, the members of the group (+)-fustin, (—)-fisetinidol and (+)-7,3′,4′-trihydroxyflavan-3,4-diol, and of the group (+)-dihydrorobinetin, (—)-robinetinidol and (+)-7,3′,4′,5-tetrahydroxyflavan-3,4-diol are interconvertible, and have been shown by WEINGES[14,15] to have the same absolute configuration at C atoms 2 and 3 as (+)-catechin, whereas (—)-fustin, (+)-fisetinidol and (—)-7,3′,4′-trihydroxyflavan-3,4-diol correspond to (—)-catechin. The (+)- and (—)-fustins[19], (+)- and (—)-fisetinidols[20], (+)- and (—)-7,3′,4′-trihydroxyflavan-3,4-diols[25,26] and presumably also the (+)- and (—)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diols are enantiomorphous pairs of compounds. These compounds

as well as (+)-catechin[11,12], (+)-gallocatechin[27], (±)-dihydroquercetin[28] and presumably (±)-dihydromyricetin have a 2,3-*trans* configuration of substituent groups, whereas (—)-epicatechin, (—)-epigallocatechin and their gallates have a 2,3-*cis* configuration[11,12]. Comparison of compounds with the same or with different configurations, under the following headings, is of interest:

Separation of enantiomorphs

ROBERTS AND WOOD[1] first showed that the optical antipodes of catechin and gallocatechin and their epimers could be resolved using water as irrigant. The optical antipodes of 7,3′,4′-trihydroxyflavan-3-ol (fisetinidol) (ΔR_F, 0.05), and of the flavan-3,4-diols, leuco-fisetinidin (ΔR_F, 0.05) and leuco-robinetinidin (ΔR_F, 0.06) may similarly be separated in water or 2 % acetic acid (Table I). Also the 2,3-dihydroflavonols, (+)- and (—)-fustin show differences in R_F (0.37 and 0.35) but the difference between the enantiomeric fustins is small (ΔR_F, 0.02) compared with enantiomeric flavan-3-ols and flavan-3,4-diols (ΔR_F, 0.05–0.06), and effective resolution occurs only after prolonged irrigation. For catechin, gallocatechin, fustin, 7,3′,4′-trihydroxyflavan-3,4-diol and 7,3′,4′,5′-tetrahydroxyflavan-3,4-diol the (+)-form has a higher R_F in water or 2 % acetic acid than the (—)-form. This sequence is reversed for epicatechin and epigallocatechin (see ROBERTS AND WOOD[1]) and also for the fisetinidols (Table I) and will evidently also apply to the robinetinidols of which only the (—)-form is known. This reversed sequence for the fisetinidols confirms the stereochemical interrelationship between (+)-fisetinidol, (—)-fustin and (—)-7,3′,4′-trihydroxyflavan-3,4-diol shown by WEINGES[14] and between those of their mirror-images[19,20]. The same must apply to (—)-robinetinidol in relation to (+)-dihydrorobinetin and (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol.

Cis–trans relationship

(+)-Catechin and (—)-epicatechin have the same configuration (2 R) at C atom 2 but differ at C atom 3 with (3 S) and (3 R) configurations respectively[15,29]. The effect of R_F on the *trans*-configuration of the 2-phenyl and 3-hydroxyl groups as in (+)-catechin as opposed to their *cis*-configuration as in (—)-epicatechin is shown in the pairs (+)-catechin (0.35) and (—)-epicatechin (0.30) (ΔR_F, 0.05), (+)-gallocatechin (0.32) and (—)-epigallocatechin (0.24) (ΔR_F, 0.08). Similar relative behaviour in the water direction is shown by the pairs (—)-catechin and (+)-epicatechin, (—)-gallocatechin and (+)-epigallocatechin on the two-dimensional chromatograms illustrated by ROBERTS AND WOOD[1]. Catechins (flavan-3-ols) of the "phloroglucinol series" therefore have higher R_F values in aqueous medium compared with the corresponding epicatechins in which the 3-hydroxyl group is inverted. (+)- and (—)-Catechins also have higher R_F values than (+)- and (—)-epicatechins in partitioning mixtures (see BRADFIELD AND BATE-SMITH[3], and ROBERTS[4,5]) and these effects may be due to disparity in their molecular shape[5,30] and possibly to the nearly planar nature of the epicatechins, with their 2-aryl group presumably in equatorial position[5,30].

Galloyl groups in 3-position

Comparison of the R_F values of the pairs (—)-epicatechin (0.30) and (—)-epicatechin gallate (0.25), (—)-epigallocatechin (0.24) and (—)epigallocatechin gallate (0.22) shows that in both instances (ΔR_F, 0.05 and 0.02 respectively) galloylation in the 3-position reduces the R_F slightly. Similar deductions may be made from the R_F values in water for these substances by ROBERTS, CARTWRIGHT AND WOOD[9].

Carbonyl group in the 4-position

Comparison of the 2,3-dihydroflavonols with their stereochemically related flavan-3-ols (catechins), for example, the pairs (+)-fustin (R_F, 0.37) and (—)-fisetinidol (R_F, 0.48) (ΔR_F, 0.11), and (—)-fustin and (+)-fisetinidol (0.35 and 0.43) (ΔR_F, 0.08), shows that introduction of a carbonyl group in the 4-position reduces the R_F considerably. Similar conclusions may be drawn from the comparison of (±)-dihydrorobinetin (0.35) and (—)-robinetinidol (0.42) (ΔR_F, 0.07), (±)-dihydromyricetin (0.24) and (+)-gallocatechin (0.32) (ΔR_F, 0.08), and (±)-dihydroquercetin (0.28) and (+)-catechin (0.35) (ΔR_F, 0.07). The considerable reduction in R_F is evident for both phloroglucinol and resorcinol series of compounds, although in the former group hydrogen bonds between the 5-hydroxyl and 4-carbonyl almost certainly exist[7]. The lower R_F of the dihydroflavonols compared with the catechins may be due to the equatorial arrangement of the bulky 2-phenyl group and also of the 3-hydroxyl group as suggested by MAHESH AND SESHARDI[31]. These equatorial arrangements will confer a nearly planar structure to 2,3-dihydroflavonols, resulting, as in the (—)-epicatechins, in a reduced R_F in water, compared with catechins where the 2-phenyl group is likely to have an axial arrangement[30].

Hydroxylation in the 4-position

The suggestion made by ROUX AND EVELYN[6] that hydroxylation in the 4-position increases the R_F, is confirmed by the comparison of stereochemically related flavan-3-ols and flavan-3,4-diols. Examination of the pairs (—)-fisetinidol (0.48) and (+)-7,3′,4′-trihydroxyflavan-3,4-diol (0.52) (ΔR_F, 0.04), (+)-fisetinidol (0.43) and (—)-7,3′,4′-trihydroxyflavan-3,4-diol (0.47) (ΔR_F, 0.04), (—)-robinetinidol (0.42) and (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol (0.46) (ΔR_F, 0.04) shows that the increase in R_F is smaller than originally anticipated[6]. This effect may be expected as introduction of an aliphatic hydroxyl on the heterocyclic ring should contribute to the solubility of the C_{15} unit as a whole.

Hydroxylation in the 5-position

Comparison of the stereochemically related pairs (+)-catechin (0.35) and (—)-fisetinidol (0.48) (ΔR_F, 0.13), and (+)-gallocatechin (0.32) and (—)-robinetinidol (0.48) (ΔR_F, 0.16) shows that for the flavan-3-ols introduction of a (phenolic) hydroxyl in the 5-position causes a large reduction in R_F. This also applies to the 2,3-dihydroflavonols *e.g.* (±)-dihydromyricetin (0.24) and (±)-dihydrorobinetin (0.35) (ΔR_F,

0.11), and (±)-dihydroquercetin (0.28) and (±)-fusin (0.36) (ΔR_F, 0.08) although the 5-hydroxyl is probably hydrogen-bonded with the 4-carbonyl in this group of compounds[7].

Hydroxylation in the 5'-position

ROBERTS, CARTWRIGHT AND WOOD[9] have shown that with water as irrigant, introduction of a hydroxyl group in the 5'-position causes a slight reduction in the flavan-3-ols of the phloroglucinol series. Similar behaviour is shown in 2 % acetic acid by these compounds, for example (+)-catechin (0.35) and (+)-gallocatechin (0.32) (ΔR_F, 0.03), (—)-epicatechin (0.30) and (—)-epigallocatechin (0.24) (ΔR_F, 0.06), (—)-epicatechin gallate (0.25) and (—)-epigallocatechin gallate (0.22) (ΔR_F, 0.03), and also by flavan-3-ols of the resorcinol series (—)-fisetinidol (0.48) and (—)-robinetinidol (0.42) (ΔR_F, 0.06). 2,3-Dihydroflavonols show the same behaviour, for example (±)-fustin (0.36) and (±)-dihydrorobinetin (0.33) (ΔR_F, 0.03), (±)-dihydroquercetin (0.28) and (±)-dihydromyricetin (0.24) (ΔR_F, 0.04). The above data show that catechins (flavan-3-ols) of the "phloroglucinol" and "resorcinol" series with 2,3-*trans* configuration of substituent groups, those of the "phloroglucinol" series with 2,3-*cis* configuration and their gallates, and 2,3-dihydroflavonols of both series (all 2,3-*trans* configuration[31]) show similar small reductions of R_F (0.03–0.06) with the introduction of a hydroxyl group in the 5'-position, using 2 % acetic acid as chromatographic irrigant.

Correlation between mobility, planarity and solubility

Flavonoid compounds which are completely planar, for example anthocyanidins, flavonols, flavones, aurones and chalcones, do not migrate in water[6,8,9,10]. Although this apparent affinity for the cellulose has been ascribed to planarity[8–10] and to special adsorption effects associated with the planar structure[6], it appears more likely that planarity and low solubility are also associated properties in the C_{15} group. The above groups of compounds all have low solubilities in cold water, and flavonols, although not mobile in cold water, migrate on cellulose columns developed with hot water. Furthermore the addition of formic or acetic acid to the aqueous irrigant, allows for the migration of all these substances on a cellulose substrate in a regular manner[32]. Low solubility associated with a planar structure is therefore almost certainly responsible for the zero R_F in water or 2 % acetic acid. Solubility might also be a predominant single factor affecting the migration of those flavonoids which are mobile in predominantly aqueous systems.

Summarised conclusions

Comparisons of some stereochemically related flavonoid compounds as detailed above, has shown that the enantiomers of flavan-3-ols, flavan-3,4-diols and 2,3-dihydroflavonols may be separated in water, sometimes with a reversal in the sequence of migration of the (+)- and (—)-forms. Amongst the flavan-3-ols, those of 2,3-*trans* configuration of substituent groups always have an appreciably higher R_F than the related epimer of 2,3-*cis* configuration in which the 2-hydroxyl group is inverted.

Regarding the functional groups, it was shown that a galloyl group in the 3-position slightly reduces the R_F of the corresponding 2,3-*cis*-flavan-3-ol (ΔR_F, —0.02 to —0.05). The presence of a carbonyl group in the 4-position (2,3-dihydroflavonols) introduces a pronounced reduction in R_F compared with the corresponding flavan-3-ol (ΔR_F, —0.07 to —0.11). Hydroxylation in the 4-position (aliphatic hydroxyl) produces a small increase of R_F, (R_F, +0.04) whereas the introduction of hydroxyl groups in positions 5 and 5′ (both phenolic hydroxyls) produces large (ΔR_F, —0.08 to —0.16) and very small (ΔR_F, —0.03 to —0.06) reductions in R_F values respectively. The solubility of flavonoid compounds in water appears to be a predominant factor in determining their R_F on cellulose substrates.

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SUMMARY

Factors affecting the chromatographic behaviour of some flavonoid compounds in aqueous medium may be evaluated more accurately now that their stereochemical interrelationships have been established. The effect of the following factors on R_F have been examined: (a) the separation of enantiomorphous flavan-3-ols, flavan-3,4-diols and 2,3-dihydroflavonols; (b) the *cis-trans* relationship of substituents in the heterocyclic ring of flavan-3-ols, (c) the introduction of a carbonyl group in the 4-position, hydroxyl groups in the 4, 5 and 5′-positions, and a galloyl group in the 3-position. The zero R_F of flavonoid compounds of planar structure is likely to be due to their low solubility in water. Solubility appears to be one of the predominant factors affecting the R_F of flavonoid compounds in aqueous medium.

NOTE ADDED IN PROOF

Hydroxylation in the 3-position

(±)-Butin (3-deoxyfustin) (0.22) prepared from butein by alkali isomerization[33] has a lower R_F in 2 % acetic acid (ΔR_F, 0.14) than (±)-fustin (0.36). Similarly (±)-7,3′,4′-trihydroxyflavan-4-ol (0.33, 0.28) obtained from the reduction of (±)-butin[34], has a lower R_F (ΔR_F, 0.19) than either (±)- or (—)-7,3′,4′-trihydroxyflavan-3,4-diols (0.52, 0.47). Substitution of hydroxyls (aliphatic) in 3-position in both flavanones and flavans produces large R_F increases in 2 % acetic acid (ΔR_F, 0.14–0.19) contrasting with the small increase (ΔR_F, 0.04) accompanying hydroxylation (aliphatic) in the 4-position.

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PLANT PHENOLS

III. SEPARATION OF FERMENTED AND BLACK TEA POLYPHENOLS BY CELLULOSE COLUMN CHROMATOGRAPHY

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INTRODUCTION

It is well known that the most important step in the manufacture of black tea is the fermentation during which the organoleptic qualities of the final product appear.

A chemical study of tea fermentation was undertaken by HARRISON AND ROBERTS in 1939[1]. It was rapidly recognised that the so-called fermentation was in fact an enzymic oxidation of some polyphenolic compounds followed by non-enzymic rearrangements and polymerisations. In 1944 BRADFIELD AND PENNEY[2] found that the lower polymers, which are the most important from an organoleptic point of view, could be extracted from the infusion by ethyl acetate. Two years later, BRADFIELD[3], using dialysis, gave further evidence of the presence of substances of relatively high molecular weight in a tea infusion. Further work concerning the research in this field before 1952 is reported by ROBERTS[5].

Studying Assam tea extracts by paper chromatography, ROBERTS[4-6] has shown that the only polyphenols undergoing enzymic oxidation were (—)-epigallocatechin and its gallic ester. An increase in gallic acid during fermentation was noticed. More recently and still by paper chromatography, ROBERTS has detected a series of polyphenolic substances which he called A, B, C, P, Q, S_I, S_{Ia}, S_{II}, X, Y and Z. He has shown that all these compounds are derived from the two aforementioned flavanols[7-9]. Tentative structures have been proposed for some of them and hypotheses made regarding their formation during fermentation[10-12].

The composition of the raw material used in our study is given in Table I (percentages calculated for dry weight) for:

(a) fresh tea leaf as reference[13],

(b) fermented but unfired tea,

(c) black tea.

As in the case of tea leaf phenolics[13], black tea compounds may be divided into three fractions containing:

(A) substances extracted from their aqueous solution by ethyl acetate,

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(B) substances remaining in the water layer and precipitating as lead salts in a weakly acidic medium (pH about 5.5),

(C) substances remaining in the water layer and precipitating as lead salts only in a weakly alkaline medium (pH about 8.5).

TABLE I

COMPOSITION OF THE RAW MATERIAL

Percentages calculated for dry weight; a = fresh tea leaf as reference; b = fermented but unfired tea; c = black tea.

	a	*b*	*c*
Substances not extractable by 80% ethanol:			
proteins (Kjeldahl N × 6.25)	15.25	—*	—
fibres (by difference)	30.25	—	—
polyphenols lost during fermentation or firing, mainly by condensation with proteins	—	8.8	11.1
Substances extractable by 80% ethanol:			
insoluble in water			
pigments (chloroformic extract minus caffeine)	5.55	—	—
soluble in water			
caffeine (according to MSDA** 1939)	4.65	—	—
polyphenols soluble in ethyl acetate (A)	26.70	14.0	11.0
polyphenols precipitated by lead acetate at pH 5.5 (B)	3.60	7.4	8.1
polyphenols precipitated by lead acetate at pH 8.5 (C)	1.60	1.7	1.7
amino acids (Kjeldahl N × 6.25)	4.15	—	—
ash	4.40	—	—
sugars (by difference)	3.85	—	—

* These determinations have not been carried out because the substances do not take part in the fermentation.

** "Manuel Suisse des Denrées Alimentaires".

As fermented tea contains more complex polyphenolic mixtures than tea leaves, it is advisable to subject less complex fractions than (A) or (B) to the chromatographic runs. This can be achieved:

(i) by extracting decaffeinated tea extract with ethyl propionate or the mixture ethyl propionate–petroleum ether (9:1) prior to the treatment with ethyl acetate,

(ii) by separating the polyphenols in (A) and (B) according to their solubility in cold water.

This refined scheme gives rise to the following six groups:

(1) substances extracted with ethyl propionate,

(2) cold water-soluble substances extracted with ethyl acetate,

(3) cold water-insoluble substances extracted with ethyl acetate,

(4) cold water-soluble substances precipitated with lead acetate at pH 5.5,

(5) cold water-insoluble substances precipitated with lead acetate at pH 5.5,

(6) substances precipitated with lead acetate at pH 8.5.

In the following the term "group" of polyphenols will always refer to this fractionation.

EXPERIMENTAL SECTION

I. Isolation of fermented tea polyphenols

(*a*) *Raw material.* Part of the tea leaves mentioned previously[13] has been subjected to:

(i) withering (18 h at 23° and 60 % relative humidity, moisture loss about 40 %)

(ii) rolling (40 min)

(iii) fermentation (2 h at 28°).

This material is referred to as fermented tea.

(*b*) *Extraction.* Immediately after fermentation, 240 g of tea (containing 100 g solids) are homogenized with 560 ml ethanol (yielding with the tea moisture an 80 % ethanolic solution) and 1 l of 80 % ethanol. To avoid oxidation 100 mg $K_2S_2O_5$ in a 10 % aqueous solution are added. After homogenization the mixture is stirred (20 min, 40°), then filtered. The residue is extracted twice more with 750 ml of 80 % ethanol (20 min stirring at 40°). The three filtrates are combined and concentrated in a rotating evaporator under reduced pressure. The concentrated extract (about 500 ml) should contain at least 10 % ethanol in order to keep the polyphenol–caffeine complex, which is sparingly soluble in cold water, in solution. The caffeine and the pigments are extracted with $CHCl_3$ using a rotating flask to avoid emulsion formation.

(*c*) *Separation of the polyphenols.* The aqueous phase is freed of the residual $CHCl_3$ by vacuum evaporation, then extracted three times for 5 min with 1-1 volumes of ethyl propionate, and three times for the same length of time with ethyl acetate. Both organic extracts are separately concentrated in a rotating evaporator under reduced pressure to 100 ml. About 100 ml of 20 % ethanol are added and the concentration is continued until organic solvents are removed. The first extract is freeze-dried. The second one is kept in about 100 ml water at 4° for several hours, then filtered. The precipitate is dried in a desiccator and the filtrate is freeze-dried after concentration to a few ml.

The aqueous phase remaining after ethyl acetate extraction is freed from dissolved organic solvent by vacuum evaporation. A few ml ethanol are added to dissolve the sparingly soluble polyphenols. The final pH is about 5.5. A saturated solution of $Pb(OAc)_2$ is added. The precipitate is filtered and washed with 3 l distilled H_2O and kept in suspension by continuous gentle stirring.

On gradually adding Dowex-50 (H-form) to the suspension, the Pb^{++} is bound to the cation exchanger and the free polyphenols go into solution. 0.2 vol. ethanol are added to dissolve the polyphenols. The mixture is filtered and the resin thoroughly washed with 20 % ethanol. The filtrate is concentrated to 100 ml under vacuum in a rotating evaporator, the concentrate kept at 4° for several hours, then filtered. The precipitate is dried in a desiccator and the filtrate freeze-dried after concentration to a few ml.

The polyphenols precipitated as lead salts at pH 8.5 are obtained as described previously[13] under I(d).

A quantity of fermented tea corresponding to 100 g dry weight gives the following yields for the six groups:

Group (1)	3.6 g	Group (4)	5.0 g
(2)	7.1 g	(5)	2.4 g
(3)	3.3 g	(6)	1.7 g

The substances present in each of these six groups are shown in Fig. 1. Those designated by numbers are identical with the main components of tea leaf[13]. The substances indicated by letters are formed during fermentation. When it is certain that a substance corresponds to one already detected by ROBERTS in Assam tea we have used the terminology of this author[8,9].

II. Isolation of black tea polyphenols

(*a*) *Raw material.* Part of the fermented tea described in Section Ia has been fired (20 min, 110°, air current). This material is referred to as black tea.

(*b*) *Extraction.* 105 g of black tea (containing 100 g solids) are homogenized with 1500 ml 80 % ethanol, then stirred (20 min, 40°) and filtered. The residue is extracted twice more with 750 ml of 80 % ethanol. The rest of the process is as in Section I.

The yields of the six groups of polyphenols are:

Group (1)	2.8 g	Group (4)	5.1 g
(2)	5.9 g	(5)	3.0 g
(3)	2.4 g	(6)	1.7 g

The substances present in each of these six groups are shown in Fig. 1.

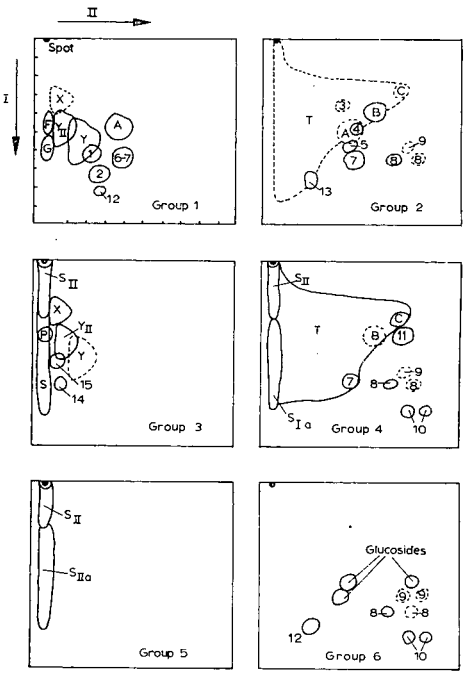


Fig. 1. Two-dimensional paper chromatograms. Paper: Whatman No. 1. Solvent systems for first dimension: butanol–acetic acid–water (4:1:2.2); second dimension: 2% acetic acid in water.

III. Chromatographic technique

(*a*) *Paper chromatography*. As described previously[13] under IId.

(*b*) *Adsorption column chromatography*. As described previously[13] under IIa to IIc, with exception of the following modifications:

(i) Washing the cellulose with 2 % citric acid (to remove traces of iron), then with alcohol and acetone prior to the treatment with distilled water.

(ii) Addition of substance mixture by blending the freeze-dried polyphenols with three to four times their weight of cellulose powder, moistening the mixture with water and placing it on the top of the column which is still covered with a small layer of water.

When the *total* polyphenols are subjected to adsorption chromatography, they are eluted according to Fig. 2. The end of Fraction VI corresponds to the arrival of

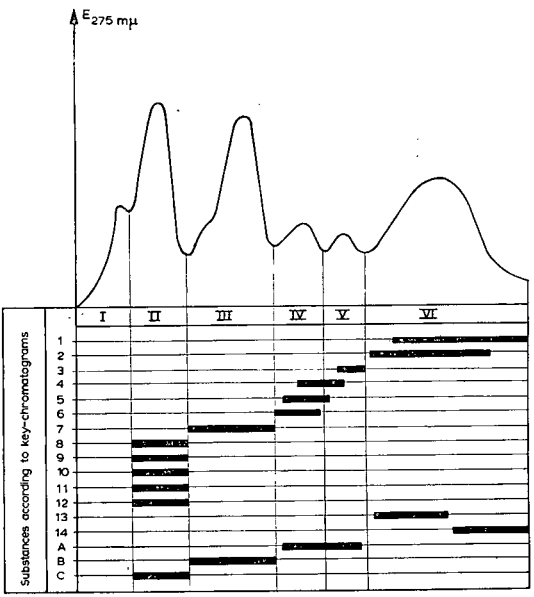


Fig. 2. Adsorption chromatography. Total polyphenols. Substances T which are present in each fraction are not indicated in this figure. Fraction I only contains substances which are not polyphenolic. These substances remain at the top of the partition columns.

the coloured substances at the bottom of the column. The approximate location of the substances remaining on the column is indicated in Fig. 3.

The cellulose may be removed from the glass column and cut into three pieces according to Fig. 3. Each piece is treated with 80 % ethanol. The extracts are concentrated under vacuum and freeze-dried.

By adsorption, the polyphenols are therefore divided into two groups, colourless and coloured compounds. With exception of T, 13 and 14, all the substances present in the six fractions of Fig. 2 are colourless.

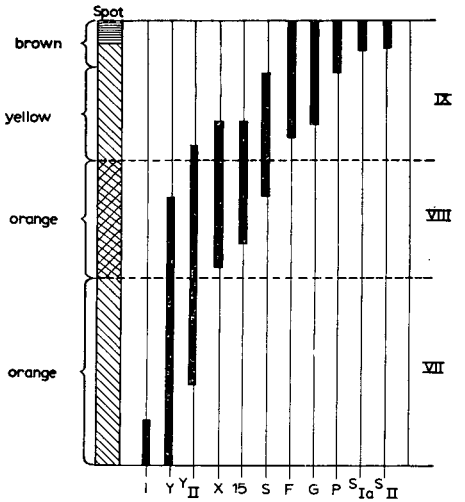


Fig. 3. Adsorption chromatography of the total polyphenols. Approximate location of the substances remaining on the column at the end of the elution.

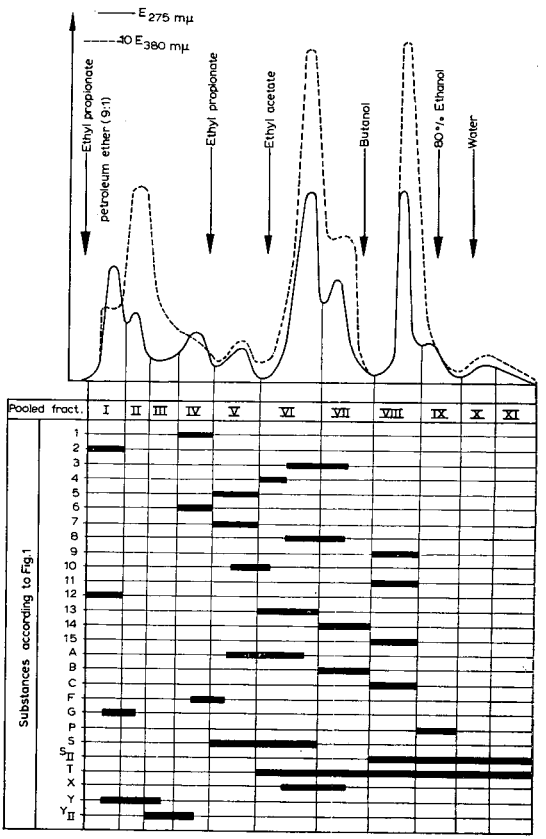


Fig. 4. Partition chromatography. Polyphenols soluble in ethyl acetate (Groups 1, 2 and 3).

(c) *Partition column chromatography.* Fig. 4 shows how the polyphenols extractable with ethyl acetate (Groups 1, 2 and 3) are eluted when the technique described previously[13] under IIIa to IIId is used.

Fig. 5 shows the behaviour of the substances precipitating as Pb salts at pH 5.5 (Groups 4 and 5).

Figs. 1 to 5 apply to fermented (unfired) tea polyphenols. The firing step does not have an important effect on the shape of the elution curves. With black tea

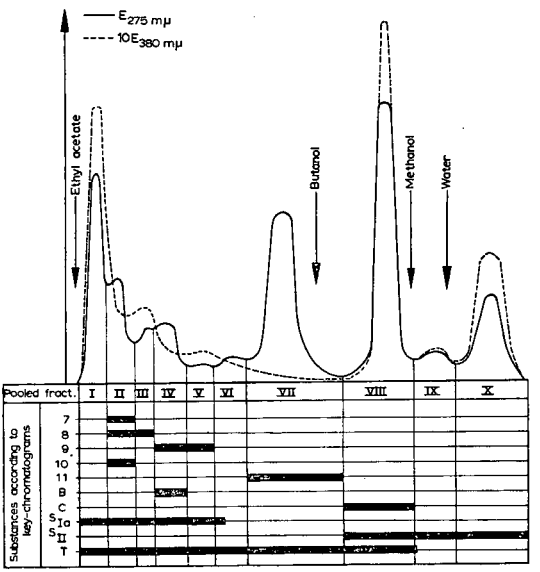


Fig. 5. Partition chromatography. Polyphenols precipitated as Pb salts at pH 5.5 (Groups 4 and 5).

polyphenols, Fig. 4 would show higher peaks for Fractions V and X, Fig. 5 for Fractions VIII and X.

Firing does not yield new substances, only quantitative changes occur (see Table I and the weights of the six groups of polyphenols).

IV. Isolation of pure polyphenols

(1) *Isolation of (—)-epigallocatechin gallate and (—)-epicatechin gallate.* If the polyphenols in Group 1 are subjected to adsorption chromatography at a very slow flow-rate, it is possible to have all the (—)-epigallocatechin gallate in Fraction VI. After partition chromatography with the mixture ethyl propionate–petroleum ether (9:1) the two flavanol gallates may be obtained quantitatively from this fraction.

(2) *Isolation of substance A.* Group I is subjected to adsorption chromatography. The fractions corresponding to Fractions IV and V of Fig. 2 are pooled and submitted to partition chromatography with ethyl propionate. Small amounts of (+)-catechin, (—)-epicatechin, gallic acid, (—)-epicatechin gallate and (—)-epigallocatechin gallate

may be present. However, they are eluted prior to substance A with ethyl propionate.

Substances T and some glucosides (not indicated in Fig. 1) remain at the top of the partition column.

Substance A has been crystallized from ether (see Table III for physical data).

(3) *Isolation of substance B.* Group II is subjected to adsorption chromatography. The two main constituents of Fraction III (Fig. 2) are readily separated with ethyl acetate. Fraction III should not be contaminated with the acids of Fraction II. However, small amounts of (+)-catechin, (—)-epicatechin and substance A do not interfere because they are eluted prior to substance B with ethyl acetate.

Substances T remain at the top of the partition column.

Substance B has been crystallized from water (see Table III for physical data).

(4) *Isolation of substance C.* Groups II and IV are pooled and subjected to adsorption chromatography. In addition to the acids mentioned in Fig. 2, Fraction II also contains some water-soluble glucosides, presumably of the 3-rhamnoglucoside type.

By partition chromatography with ethyl acetate the acids and most of the glucosides are separated from C which moves very slowly. Substances T remain at the top of the column. When the elution of the acids is complete, the cellulose is removed from the glass column and cut into 3 or 4 pieces, each of which is eluted with alcohol. In general, substance C is in the middle of the column.

Substance C has been crystallized from water (see Table III for physical data).

(5) *Isolation of coloured substances.* Substances Y, Y_{II} and X are obtained by pooling Groups 1 and 3, subjecting them to adsorption chromatography, pooling Fractions VII and VIII (Fig. 3) and submitting them to partition chromatography, using the gradient technique of Fig. 4.

From Fraction IX of the same adsorption chromatogram it is possible to get substances F and G which are eluted with ethyl propionate. With ethyl acetate substance P migrates very slowly (pink band). When this latter zone is completely separated from the brown substances S_{II} remaining at the top of the column, it may be eluted from the cellulose (removed from the glass column) with isoamyl alcohol

RESULTS AND DISCUSSION

The polyphenolic substances present in fermented or in black tea are listed in Table II. From the substances already present in tea leaves, only those acting as possible substrates in the enzymic oxidation are considered in the following discussion.

The substances formed during fermentation have been classified according to their visible and U.V. absorption spectra.

(*a*) *Colourless substances exhibiting only one absorption band (between 270 and 280 mμ)**

* In the following we are dealing only with the spectrum range from 220 to 600 mμ.

TABLE II

POLYPHENOLIC SUBSTANCES PRESENT IN FERMENTED OR IN BLACK TEA

Code to Fig. 1	*Name*	*Group where substance is to be found*	*Colour of substance*
Main polyphenols already present in tea leaf[13]			
1	(—)-epigallocatechin gallate	1	white
2	(—)-epicatechin gallate	1	white
3	(—)-epigallocatechin	2	white
4	(+)-gallocatechin	2	white
5	(—)-epicatechin	2	white
6	(+)-catechin	1,2	white
7	gallic acid	1,2,4	white
8	chlorogenic acid	2,4,6	white
9	neochlorogenic acid	2,4,6	white
10	*p*-coumaryl-quinic acid	4,6	white
11	theogallin	2,4	white
12	caffeic acid	1,6	white
13	kaempferol-3-glucoside	2	yellow
14	quercetin-3-glucoside	3	yellow
15	myricetin-3-glucoside	3	yellow
Polyphenols formed during fermentation			
A	bisflavanol[12]	1,2	white
B	bisflavanol[12]	2,4	white
C	bisflavanol[12]	2,4	white
X	theaflavin[8,11,12]	1,3	orange
Y	theaflavin gallate[8,11,12]	1,3	orange
Y_{II}		1,3	orange-red
S		3	orange
F		1	orange-yellow
G		1	orange-yellow
P		3	pink
T		2,4	brown
S_{Ia}	thearubigin[8,11,12]	2,5	brown
S_{II}	thearubigin[8,11,12]	3,4,5	brown

(1) *Monomers.* (—)-Epigallocatechin gallate and (—)-epicatechin gallate can be obtained quantitatively as described in Section IV 1. On the basis of dry weight, the two substances are present in the following percentages:

	Tea leaves[13]	*Fermented tea*
(1) (—)-Epigallocatechin gallate	10.55 %	0.5 %
(2) (—)-Epicatechin gallate	2.15 %	0.3 %

95 % of (1) and 89 % of (2) disappear in 2 hours of fermentation. We do not know what happens to (2) which is reported by ROBERTS as not taking part in the fermentation of Assam tea. It might be possible that the enzymic systems of Assam and Ceylon

Substance A
(from ether)

Substance B
(from water)

Substance C
(from water)

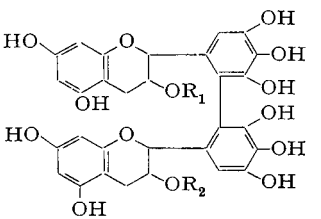
Fig. 6. The figures on the scale indicate mm.

teas are different. Furthermore, it has been reported by DZHEMUKHADZE AND MILESHKO[14] that (—)-epicatechin gallate is oxidized during processing of Georgian tea leaf.

Although we do not have any quantitative results with regard to the other four flavanols (which are much more difficult to isolate from fermented tea than from fresh tea leaves) we can say that (—)-epigallocatechin is almost completely oxidized whereas (+)-catechin, (+)-gallocatechin and (—)-epicatechin seem to escape oxidation.

(2) *Presumed dimers (biflavanols)*. Substances A, B and C have been obtained in a crystalline state, A from ether, B and C from water (see Fig. 6).

ROBERTS suggests[12] that substances A, B and C have the structures (I).



(I)

$R_1 = R_2 =$ galloyl : substance A
$R_1 =$ galloyl, $R_2 =$ H : substance B
$R_1 = R_2 =$ H : substance C

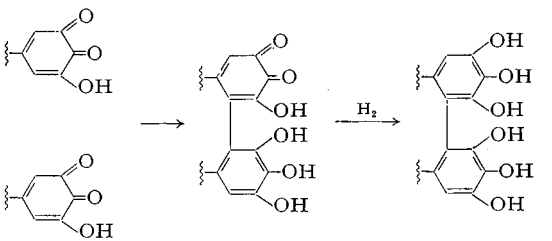
They would be formed by dimerisation of:

2 molecules of (—)-epigallocatechin gallate

1 molecule of (—)-epigallocatechin gallate and 1 molecule of (—)-epigallocatechin

2 molecules of (—)-epigallocatechin,

respectively, in the quinone form, the dimer being reduced in the course of a coupled reaction.



Physical data are reported in Table III.

The surprisingly high hydrogen content of substance A could be explained by the assumption that this substance crystallizes with two molecules of ether. This is supported by the fact that at 215° the crystals decompose very violently. From the carbon and hydrogen contents of B, it cannot be ascertained whether this substance crystallizes with one molecule of water or not.

TABLE III

PHYSICAL DATA

		Substances		
		A	*B*	*C*
Crystallized from		ether	water	water
Formula*		$C_{44}H_{34}O_{22}$	$C_{37}H_{30}O_{18}$	$C_{30}H_{26}O_{14}$
		914.71	762.61	610.50
Analysis				
calc. for anhydrous product	% C	57.90	58.27	59.01
	% H	3.75	3.97	4.29
calc. with 2 molecules cryst. ether for A and with 1 molecule cryst. H_2O for B	% C	58.76	56.93	
	% H	5.12	4.13	
found (for A and B two different crystalline preparations were analysed)	% C	58.24 59.61	56.98 57.45	59.06
	% H	5.74 5.40	4.22 4.06	4.71
M.p.**		decomp. at around 215° without melting	decomp. at around 200° without melting	can be heated to 300° without decomp. or melting
$[\alpha]_D^{25°}$***		−247° ± 2°	−183° ± 2°	−66° ± 2°
λ_{max}***		277 mμ	274 mμ	270 mμ
ε_{max}†		20,500	15,500	4,500

* According to ROBERT's assumption[12].

** The substances have been dried over P_2O_5–NaOH under 0.01 mm Hg at 60° for 4 h. The crystals turned slightly brown. M.p. measured with Kofler block.

*** For the determination of $[\alpha]_D$ and for the U.V. spectra we have used white crystals dried over P_2O_5–NaOH under 0.01 mm Hg at room temperature for 1 h. $[\alpha]_D$ determined in ethanol.

† Calculated on the basis of the molecular weights given under "Formula". The spectra have been taken in ethanol with a Beckman DK-2 spectrophotometer.

If we compare the $[\alpha]_D$, λ_{max} and ε_{max} with the corresponding values of (—)-epigallocatechin and of its gallic ester[13]:

	(—)-Epigallocatechin gallate	*(—)-Epigallocatechin*
$[\alpha]_D^{25°}$	−185° ± 2°	−59.5° ± 2°
λ_{max}	275 mμ	271 mμ
ε_{max}	11,500	1,450

we see that ROBERTS' assumption is consistent with our physical data.

In addition to that, the I.R. spectra (to be published elsewhere) show a strong ester band at 5.9 μ for A. This band is reduced in B and absent in C.

It is interesting to note that A cannot be crystallized from water because its aqueous solutions become too viscous on cooling (for instance a 1.5 % solution at 4°).

Substances A and B are very sensitive. The dry crystals turn slightly brown within a few days, even at 4°. Substance C is stable.

(*b*) *Coloured substances*

(1) *Polyphenols with three absorption bands**. Fig. 7 shows the spectra of substances Y, Y_{II} and X. Our $E_{1\,cm}^{0.002\,\%}$ values for X and Y do not agree too well with ROBERTS' values[11,12].

Substance Y_{II} has not been reported by ROBERTS in Assam tea. In the dry state, it is a light red powder, whereas X and Y are orange.

These three substances have a great tendency to precipitate from their solution in an amorphous state (although these precipitates seem to be sometimes microcrystalline). So far all our preparations have consisted of approximately 50 % of crystalline and 50 % of amorphous material, as can be seen with the polarising microscope.

(2) *Polyphenols with two absorption bands**. We have been able to isolate four compounds belonging to this class.

It is certain that our substance P corresponds to that of ROBERTS[10]: their paper-chromatographic behaviour is the same and both have an E_{max} at 520 mμ.

Substances F, G and S have not been obtained chromatographically pure. They all have an E_{max} between 370 and 380 mμ. G is bright yellow whereas F and S are rather orange.

Our substances F and G do not seem to correspond to ROBERTS' substances Q and Z.

(3) *Polyphenols with one absorption band**. This class is made up of substances S_{Ia} and S_{II} which are brown and have been called thearubigins by ROBERTS[8]. According to this author[11], they are oxidation products of the theaflavins. They have an E_{max} at around 275 mμ and a more or less pronounced shoulder at about 370 mμ.

Thearubigins (which must be viewed as a mixture of different substances) make up the whole Group 5 of polyphenols (see Fig. 1). Our thearubigins contain 0.55 %N which cannot be accounted for either by caffeine or amino acids. Hydrolysis with 5 *N* HCl at 120° for 12 hours (sealed tube containing 250 mg thearubigin and 10 ml 5 *N* HCl) gives rise to the following free amino acids which have been detected by paper chromatography: alanine, arginine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine.

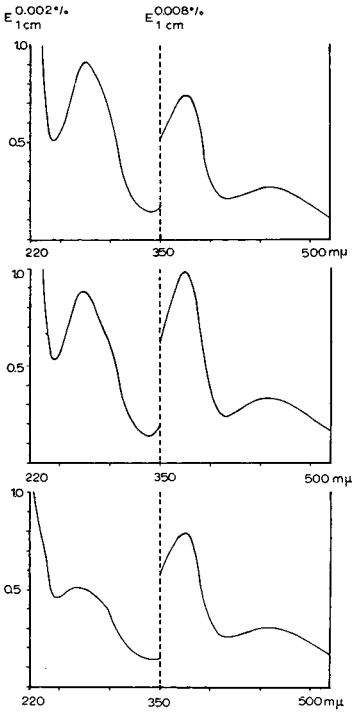
This could indicate that thearubigin is partly composed of substances related to humic acids.

The *o*-quinone forms of the 3',4'-dihydroxy- and the 3',4',5'-trihydroxyflavan derivatives are known to be highly reactive. One of their fates is to dimerise to substances A, B, C and theaflavins. But they might also condense with amino acids or with proteins, yielding humic acid-like substances. It is likely that the polyphenols condensing with proteins lose their extractability by 80 % alcohol. This would account for the drop in total polyphenols occurring during fermentation (see Table I). The polyphenols condensing with amino acids could give rise to the N-containing substances present in Group 5.

* See the footnote on p. 24.

Partition chromatography of Group 5 according to Fig. 5 yields four fractions. S_{Ia} which is eluted with ethyl acetate does not contain N, whereas the three other fractions (eluted with butanol, methanol and water respectively) all contain N.

About 50% of the N-containing substances remain adsorbed on the cellulose and can be eluted with alkaline solutions.



Substance Y

λ_{max}	$E_{1\,cm}^{0.002\%}$
275 mμ	0.980
372 mμ	0.223
453 mμ	0.074

Substance Y_{II}

λ_{max}	$E_{1\,cm}^{0.002\%}$
270 mμ	0.850
373 mμ	0.272
455 mμ	0.096

Substance X

λ_{max}	$E_{1\,cm}^{0.002\%}$
270 mμ	0.520
375 mμ	0.197
458 mμ	0.076

Fig. 7. Substances Y and Y_{II} are paper-chromatographically pure. Substance X is contaminated with very small amounts of glucoside 15 and substance S.

(4) *Substances without absorption bands**. Substances T seem to be oxidative breakdown products of thearubigin. They have lost their polyphenolic characteristics and do not even possess an E_{max} at about 270 mμ.

ACKNOWLEDGEMENTS

We are grateful to Mr. H. FROHOFER, Chemical Institute, University of Zürich, for carrying out the microanalyses. We should also like to thank Miss N. DESPLAND and Mr. G. VODOZ for their efficient technical assistance.

SUMMARY

The polyphenolic constituents of fermented (unfired) and black (fermented and fired) Ceylon tea have been fractionated by column chromatography, using procedures

* See the footnote on p. 24.

recently described for the preparation of (unfermented and unfired) tea leaf polyphenols[13].

(—)-Epigallocatechin gallate and (—)-epicatechin gallate, the two main polyphenols of tea leaf, have been isolated quantitatively. The amount of both compounds is much lower than in tea leaves, which indicates that not only (—)-epigallocatechin gallate but also (—)-epicatechin gallate disappear during enzymic oxidation.

The fermentation products have been classified according to their absorption spectra in the U.V. and visible region. Some of them have been purified chromatographically and characterised physically:

(a) Three colourless substances possessing one U.V. maximum only, which had already been detected paper-chromatographically by ROBERTS[12], could be prepared in a crystalline state. Their analytical data support the view[12] that these compounds are biflavanols resulting from oxidative dimerisation of (—)-epigallocatechin and/or its gallic ester.

(b) Three coloured substances possessing three absorption maxima have been prepared paper-chromatographically pure. Two of them, orange in colour, seem to be identical with the compounds described by ROBERTS[8,11,12]. The third one is light red and has, to our knowledge, not yet been reported.

(c) Four coloured substances possessing two absorption maxima have been obtained. Only one of them has been detected by ROBERTS in Assam tea.

(d) The fraction called thearubigin by ROBERTS[8,11,12] possesses only one maximum in the U.V. This brown fraction is a very complex mixture. It has been found to yield 14 amino acids on hydrolysis with HCl.

NOTE ADDED IN PROOF

Since this paper was submitted, substance C has been subjected to alkaline degradation. The presence of phloroglucinol and ellagic acid as main degradation products clearly indicates that the two molecules of (—)-epigallocatechin are linked by a 2′–2′ C–C linkage as was postulated by ROBERTS[12]. This work which is to be published elsewhere, has been carried out by Mr. J. MONNIN.

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THE DETECTION OF TRITIUM LABELLED COMPOUNDS IN VAPOUR PHASE CHROMATOGRAPHY

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INTRODUCTION

In recent years a variety of methods have been developed for the preparation of tritium labelled compounds[1–7]. In those methods that employ exchange and irradiation techniques the desired tritiated compound is almost invariably accompanied by tritium labelled impurities[8,9], and rigorous purification and analysis is essential in most cases to ensure that the product is *radiochemically* pure. Vapour phase chromatography has proved a very useful technique both for the purification and the analysis of labelled compounds. In vapour phase chromatography it is desirable to measure continuously the activity of the gas coming from the column to avoid the misleading results that may be obtained by condensing fractions and measuring their activities individually. The low maximum energy (18 KeV) of tritium β particles prohibits the use of end window counters that can be used for detecting β particles with higher energies such as those from carbon-14[10,11]. Suitable detectors which may be used with tritium are discussed below.

SCINTILLATION AND PROPORTIONAL DETECTORS

LOWE AND MOORE[12,13] have used a liquid scintillation technique for measuring ^{14}C-labelled vapours from a gas–liquid chromatography column; the same method can also be used for detecting tritium labelled compounds[14]. FUNT AND HETHERINGTON[15] used a hollow plastic scintillator in the form of a spiral for measuring ^{14}C in the gas phase and CAMERON, BOYCE AND TAYLOR[16] have used a similar plastic scintillator in the form of a hollow cylinder (volume about 0.5 c.c.) for detecting tritium in a flowing gas.

WOLFGANG AND ROWLAND[17] have developed a gas flow proportional counter that can be used at temperatures up to 200°. In their method methane is injected into the gas stream to make a suitable "counting" gas after it has passed through a thermal conductivity mass detector, and the mixture fed into a specially designed gas flow proportional counter.

IONISATION CHAMBER DETECTOR

A gas flow ionisation chamber can be used as a high temperature detector[18,20] and this paper describes a chamber that has been used at temperatures up to 190°.

The maximum current that can be measured in an ionisation chamber is given by:

$$I = \frac{N \cdot E \cdot e}{w}$$

where I = current (amp.)
N = number of disintegrations per second
E = average energy per disintegration (electron volts)
w = number of electron volts required, to produce one ion pair in the gas. This varies with the nature of the gas in the cell; for air the value is 32.5 eV and for argon it is 27.8 eV[19].
e = electronic charge (coulombs)

For 1 μC of T gas in an ionisation chamber filled with argon, the maximum current due to the T is about $1.2 \cdot 10^{-12}$ amp. This theoretical ionisation current will not be obtained in practice due to absorption of the β particles in the chamber walls. The proportion of β particles lost by this effect is reduced by increasing the size of the cell, thus its dimensions are determined by compromising the increase in efficiency obtained by increasing its size with the decrease in chromatographic resolving power.

EXPERIMENTAL

A design that has been used in this laboratory for detecting tritiated water in a gas flow at temperatures up to 190° is shown in Fig. 1. Several designs for the central collecting electrode were tested but the concentric steel rod shown in the diagram proved to be the most efficient. The chamber (volume 3.0 c.c.) was connected to the amplifier by a length of screened co-axial Teflon cable fitted with a jockey adaptor.

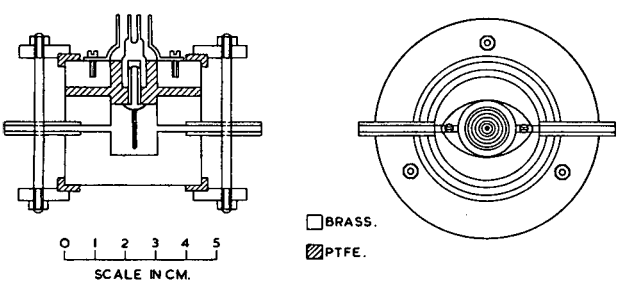

Fig. 1. A gas flow ionisation chamber for use at high temperatures.

To determine the characteristics of the design, the ion chamber was housed in an oven and connected to a D.C. amplifier (A.E.R.E. type 1388B); all experiments were carried out using the minimum time constant setting. The amplifier was

connected to a Honeywell Brown recorder and the voltage to the cell was supplied by dry batteries. The efficiency of the ionisation chamber was tested using the arrangement shown schematically in Fig. 2. The gas flow rate was determined by a calibrated flow meter (Rotameter) and tube T contained tritiated water of known

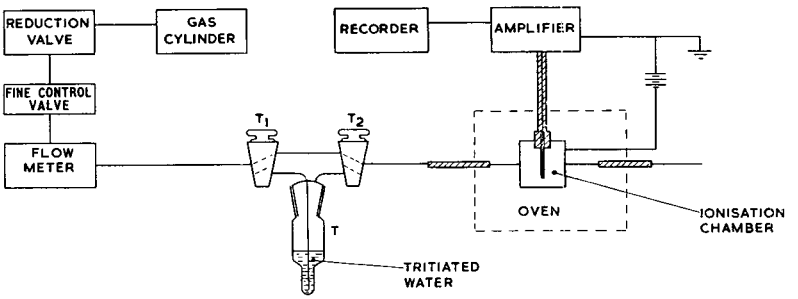

Fig. 2. The arrangement used for determining the characteristics of the ionisation chamber

specific activity. By turning taps T_1 and T_2 simultaneously the stream of gas of predetermined flow rate was bypassed through the water for any desired period thus becoming saturated with water vapour and delivering a known amount of tritium to the chamber. To prevent variations of the vapour pressure of the tritiated water, T was immersed in a thermostated water bath. The gas after bubbling through T was shown to be saturated with tritiated water vapour by condensing out several fractions in a liquid nitrogen trap and counting them individually in a calibrated liquid scintillation counter. The measured count rates agreed with calculated values.

RESULTS

The graph (Fig. 3) was obtained by measuring the peak heights on the recorder chart produced by passing nitrogen (flow rate 0.1 l/min) through the water for 10-sec intervals at different voltages at 25°. An almost identical curve was obtained at 180°.

The areas under the peaks obtained by passing nitrogen gas of flow rate 0.1 l/min through the tritiated water for different periods of time with a polarising potential of 72 V are shown by the broken line in Fig. 4. The areas were measured by cutting out the peaks from the recorder chart and weighing the paper. The variation of peak height shown by the continuous line were measured directly from the recorder chart. From this graph one can conclude that the area under the peaks is linearly related to the amount of tritium passing through the cell for vapours of constant activity. To test the variation of the area under the peaks with different specific activities, the tritiated water in T (Fig. 2) was diluted with known amounts of inactive water and the gas stream bypassed for 10-sec periods. Fixing the activity of the original water at the arbitrary value of 10, the results obtained are shown in Fig. 5. From Figs. 4 and 5 it is evident that there is a linear relationship between

the total activity passing through the cell and the area under the peaks at constant flow rate. The efficiency of the chamber, with a polarising potential of 72 V, was determined as 37 % by comparing the current obtained when the chamber contained a known amount of tritium (as water vapour) in nitrogen carrier gas with the value calculated from the equation given earlier.

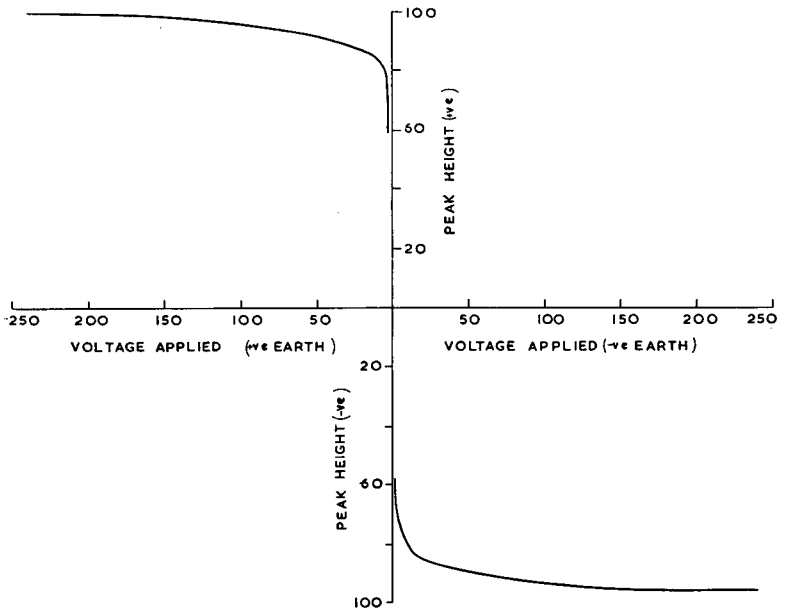

Fig. 3. The effect of variation of voltage on peak height.

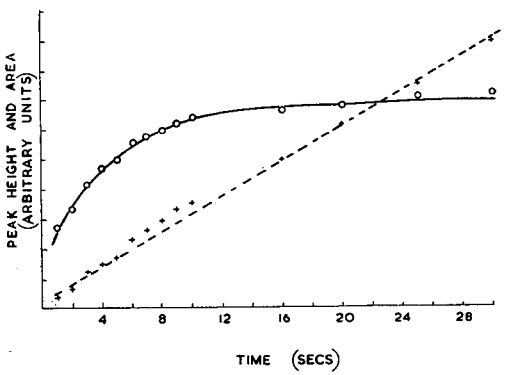

Fig. 4. The effect of bypassing the gas stream through tritiated water on peak height (continuous line) and area under the peaks (broken line) for different periods of time.

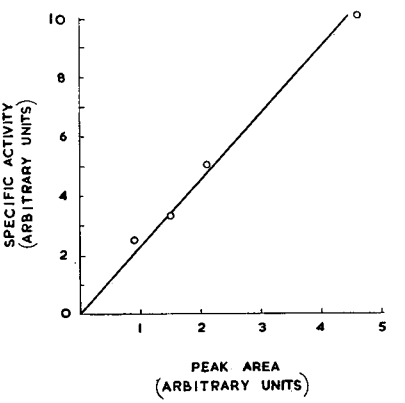

Fig. 5. The effect of bypassing the gas stream through water containing varied concentrations of T_2O before passing it through the ionisation chamber.

COMPARISON OF DETECTORS

Ionisation chambers are comparatively simple and robust detectors capable of measuring a very wide range of activities but are less sensitive than other methods. The one described in this paper has been used at temperatures up to 190°, and gives a good peak with about 0.5 μC of tritium; this sensitivity could probably be improved using a vibrating reed amplifier. The upper temperature limit at which it could be used was not determined; PTFE is chemically stable up to about 300° although other factors may prohibit its use at such high temperatures. Plastic scintillators are limited to use at room temperature; a design using a channelled inorganic scintillator and a suitable light guide could possibly be developed as a high temperature scintillation detector. The arrangement of CAMERON, BOYCE AND TAYLOR had a background count of 5 c.p.s. at 20° and the amount of activity needed to give a good peak (about 50 c.p.s.) at an efficiency of 2 % is about 0.07 μC of tritium. The methods of LOWE AND MOORE, and WOLFGANG should give a tenfold increase in efficiency on this but have drawbacks. The LOWE AND MOORE method gives an integrated record and it would be difficult to detect fractions containing a small amounts of activity if they were to follow a fraction of high activity. In the WOLFGANG method the position of the counting plateau varies with the proportion of methane in the counting mixture; it would therefore be necessary to exercise careful control over this factor, especially at high temperatures where the plateau length is reduced.

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SUMMARY

With the increase in the use of tritium as a radioactive tracer, several methods for measuring tritiated materials as they come off a vapour phase chromatography column have been developed. The author gives a very brief review of scintillation and proportional methods and a more detailed account of a gas flow ionisation chamber that has been used at temperatures up to 190°.

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PAPER ELECTROPHORESIS: TEMPERATURE, PAPER WETNESS AND SERUM COMPONENT MOBILITY WITH THE KUNKEL SYSTEM*

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Paper electrophoresis has been used as a means to separate a heterogenous mixture of charged particles into separate groups, each of more homogeneous nature. When applied to thoroughly studied mixtures, such as human serum proteins, the identity of any of the separated fractions, once established by independent means, is most readily achieved by observation of its position in relation to other fractions of the specimen rather than by measurement of its absolute displacement from the point of application. Measurement of the absolute displacement (or mobility) of a homogeneous group as a means of identification of that group assumes greater importance when the mixtures under study are ones which have not been examined as rigorously as human serum, *e.g.* in the study of proteins of "unknown" plant or animal species.

To extend the usefulness of paper electrophoresis as a basic tool, an investigation was made of some of the factors which control the mobility of serum proteins. McDONALD[1,2] has indicated the importance of solvent movement, moisture content, conductivity and temperature of the paper strip as some of the variables affecting the mobility of the migrating particles. These observations related particularly to a horizontal open system and the observations did not extend to multiple separations on the same strip. Comparisons for qualitative analysis of substances of similar mobilities are conveniently carried out on one sheet of paper[3]. KUNKEL AND TISELIUS[4] have described an electrophoretic apparatus which effects appreciable control of gross movements of solvent and in which multiple samples of a given substance can be run in parallel on a single strip with apparently identical mobilities. The description of this apparatus did not include data concerning reproducibility or the effect of wetness or temperature. This study was undertaken to investigate the effects of temperature and wetness in a rapid Kunkel system and to determine the variability of mobility of multiple separations on the same strip. Comparison is made with separations carried out on a DURRUM type apparatus[5].

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METHODS

The electrophoretic apparatus was similar to the horizontal, closed system described by KUNKEL AND TISELIUS[4]. The lower plate was 4 × 10 × 3/4 in., the upper was 4 × 10 × 1/4 in. All surfaces in contact with the paper were lightly siliconed. Whatman 3MM paper 2 ¼ × 11 in., and barbital buffer, pH 8.6, ionic strength 0.05 were used in all cases. After the paper was positioned between the plates 12 bottles filled with mercury were placed upon the top plate to give a total pressure of 1.25 lbs./sq. in. The level in each reservoir was maintained 1 ½ in. below the horizontal paper and the electrolyte was changed when appropriate.

The voltage gradient was measured by a separate voltmeter by inserting platinum micro electrodes 1–2 mm into the strip 10 cm each side of the origin. All runs were for 60 min at a constant voltage of 15 V/cm as measured in the area in which the proteins were migrating and voltage drops in other portions of the system were ignored.

Heat dissipation from the strip was by conduction to the heavy glass plates which surrounded the paper and thence by convection into the air of a water-cooled, thermostatically controlled cabinet. The glass plates and other portions of the system were temperature equilibrated before each run.

The effects of temperature and wetness were studied at 5°, 16°, and 25°. To obtain measurements of paper wetness, each paper was weighed, then placed in a tared, covered tray made level with a poured layer of paraffin. A known volume of buffer was applied along the paper and allowed to equilibrate 50 min. Three 2 λ samples of serum were applied as spots equispaced along a previously marked line midway between the ends of the strip. After the specimens were applied the strip was immediately transferred to the Kunkel apparatus and the tray was reweighed and the increase in weight due to residual buffer was used to correct the value of buffer applied to the strip. The physical demands of the system limited wetness to values between 1 g buffer/g paper (1/1) and 2 g buffer/g paper (2/1). Less than this amount of buffer would not diffuse uniformly throughout the paper in 50 min and more than 2 g buffer/g paper resulted in an excess which was visibly squeezed out of the paper when pressure was applied. At a lesser pressure, 1.00 lb./sq. in., variability of results is greater, resolution is poorer and increase in mobility with increasing temperature does not show a straight line relationship.

In separate runs, 3 % dextran was applied at the origin, and 5 and 10 cm to each side to measure capillary and endosmotic electrolyte movement.

At the end of the run the paper was dried and stained with bromophenol blue. Movement of the albumin and γ-globulin fractions was measured from the origin to the points of greatest dye concentration. All separations were made on a single pool of human serum stored in 2 ml aliquots at —10°. Fifty-six runs were made, a total of 168 separations.

Comparative data were obtained on a Durrum type apparatus (Spinco model Beckman Instruments), at room temperature (18–21°) with barbital buffer pH 8.6, ionic strength 0.05, on six parallel strips of Whatman 3MM paper at a constant

current of 4.5 mA. The wet strips were equilibrated 30 min and then 10 λ portions of serum were applied at the apex. In separate runs an equal volume of 3 % dextran was applied at the apex and 5 cm to either side.

RESULTS

With the Kunkel system described, the observed (apparent) mobility of albumin and γ-globulin was found to increase with both wetness and temperature. The increase in mobility was in the direction of migration, anodal for albumin and cathodal for globulin. All measurements are based on constant conditions of time and voltage gradient and are presented in terms of actual displacement rather than the equivalent mobility values in terms of cm/sec/V/cm. At a 1/1 wetness value, observed albumin mobility increased 0.063 cm/°C over the entire temperature range investigated and at wetness 2/1 this mobility increased 0.087 cm/°C. At a 1/1 wetness value, observed γ-globulin mobility increased 0.0172 cm/°C and at 2/1 this mobility increased 0.0315 cm/°C (Table I). The data on dextran displacement given in Fig. 1 indicate the net

TABLE I

RELATIONSHIP OF OBSERVED ALBUMIN AND γ-GLOBULIN MOBILITY (y) TO WETNESS (x) AT THREE TEMPERATURES: VARIABILITY WITH KUNKEL TECHNIQUE USING HUMAN SERUM

Component	°C	*Least squares line*	*Standard deviation (S.D.)*	*N**	*Variability of migration*			
					Wetness			
					less than 1.5		*1.5 or greater*	
					***Cm*	****CVx(%)*	***Cm*	****CVx(%)*
Albumin	5	$y = 0.747 + 0.534x$	0.23	20	0.09	6.4	0.12	7.2
	16	$y = 1.35 + 0.73x$	0.19	12	0.15	6.6	0.22	8.2
	25	$y = 1.7 + 0.95x$	0.21	32	0.15	5.2	0.19	5.6
γ-Globulin	5	$y = 0.153 + 0.108x$	0.10	20	0.06	22.3	0.07	16.8
	16	$y = 0.16 + 0.19x$	0.11	12	0.09	23.4	0.12	24.2
	25	$y = 0.21 + 0.304x$	0.10	20	0.10	16.9	0.07	9.4

* Number of runs.
** Cm = Average difference between most and least mobile on same strip.
*** CVx = (S.D./mean observed mobility) × 100 = coefficient of variation, value as percent.

flow to be toward the center on dry strips and toward the ends on wetter ones. One example of albumin mobility corrected for dextran movement is given in Table II.

On the Durrum apparatus the mean albumin mobility was 6.15 cm, standard deviation (S.D.) 0.65, coefficient of variation (CVx) 10.6 %. The γ-globulin mobility was 0.49 cm, S.D. 0.183, CVx 37.4 % (Table III). Movement of dextran on the Durrum apparatus was in a negative direction and displaced maximally on the positive limb. The dextran movement observed after an 8 h run was the same as that after a run of 16 h (Fig. 2).

With the Kunkel apparatus three specimens were placed in parallel on the same strip. For albumin, the average variability in the distance migrated, from most to

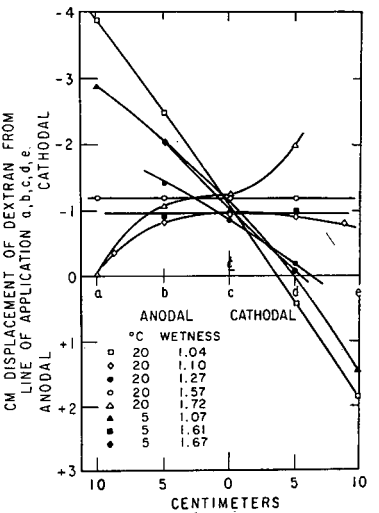

Fig. 1. Dextran displacement from origin, Kunkel type apparatus. The dextran movement is toward the center on dry strips and toward the ends on wetter ones.

TABLE II

OBSERVED AND CORRECTED MOBILITY VALUES (+, —)* ON HUMAN SERUM COMPONENTS, KUNKEL TECHNIQUE

	Albumin					*γ-Globulin*			
°C	5°		20°			5°		20°	
Wetness	1.04	1.72	1.04	1.72		1.04	1.72	1.04	1.72
	Mobility		*Mobility*			*Mobility*		*Mobility*	
Observed**	+1.30	+1.75	+2.68	+3.33	Observed**	—0.26	—0.33	—0.51	—0.73
Marker (dextran)	—1.70	—1.00	—1.70	—1.00	Marker (dextran)	—0.70	—1.50	—0.70	—1.50
Corrected	+3.00	+2.75	+4.38	+4.33	Corrected	+0.44	+1.17	+0.19	+0.77

* + = movement toward anode; — = movement toward cathode.
** From least squares data $y = a + bx$ (*cf.* Table I).

TABLE III

STATISTICAL CONSTANTS DERIVED FROM OBSERVED MOBILITY DATA OBTAINED AT ROOM TEMPERATURE, DURRUM TECHNIQUE, HUMAN SERUM

Component	$\bar{x}$ = *average mobility (cm)*	*Total strips*	*Standard deviation (S.D.)*	*CVx* (%)*	*Average difference in mobility***		
					Cm	*Number of runs*	*CVx(%)*
Albumin	***+6.15	36	0.65	10.6	0.49	6	6.9
γ-Globulin	—0.49	34	0.18	37.4	0.25	6	68.0

* CVx = (S.D./mean observed mobility) × 100 = coefficient of variation, value as percent.
** From greatest to least value for mobility in same run of six strips.
*** + anodal; — cathodal movement.

least mobile on the same strip, was 6.5 % of the distance migrated (range 5.2–8.2 %) (Table I). For the γ-globulin fraction the average difference was 18.8 % (range 9.4–24.2 %) (Table I).

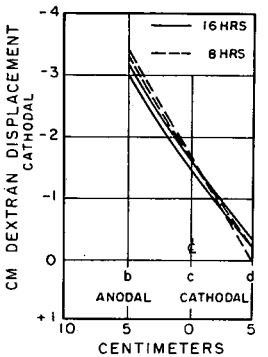

Fig. 2. Dextran displacement from origin, Durrum type apparatus. Movement is always cathodal and more pronounced on the anodal limb.

The average variability in the distance migrated, from most to least mobile among the six strips in the Durrum chamber, was (Table III) for albumin, 6.9 % of the distance migrated (range 5.0–8.6 %). For the γ-globulin fraction for any one run the average difference was 68 % (range 40–100 %).

DISCUSSION

Equilibration

The equilibration time of 50 min was sufficient to allow an apparently even distribution of electrolyte throughout the paper. In order to maintain a constant potential (as measured in the area of the strip in which the specimens were migrating) it was necessary occasionally to make minor readjustments in the voltage during the first 20 min of the run. EDWARD AND CRAWFORD[6] have found equilibration for 20 min adequate in a Kunkel system. Using a modified Kunkel, CRESTFIELD AND ALLEN[7] found an equilibration period of 30–45 min resulted in a constant field strength. MOINAT AND TULLER[8] point out the desirability of measuring voltage on the strip and not only at the power supply.

Temperature

The findings for albumin and γ-globulin show an increase in mobility with an increase in temperature (Table I). Similar increases occur in open strip ionophoresis[9]. FORBES AND TAYLOR[10] found the migration rate of β-lipoprotein but not that of albumin and globulin to increase with increasing temperature. ENGELKE *et al.*[11] concluded from data on migration of markers that temperature changes between 12° and 20° did not affect the migration of the markers. SCHULZ AND WEGNER[12], working with a horizontal type apparatus with no applied pressure, detected a slight increase in apparent mobility at lower temperatures. WHITEHEAD[3] has indicated that heating causes increased electrolyte flow.

This variety of findings suggests that temperature measurements alone are not adequate to account for variations in migrant mobility.

Wetness

McDONALD *et al.*[13] mention that the uncorrected electromigration velocity of a migrant on an open strip decreases to practically zero as the wetness approaches zero, and increases up to the velocity in free solution as the wetness increases.

Choice of a mobility marker and a wetness ratio

The assumption is made that the albumin and the dextran marker are moved equally by the electrolyte flow in the Kunkel system employed. For albumin, a dextran marker probably is correct in size, since the corrected albumin mobility is $6 \cdot 10^{-5}$ cm/sec/V/cm at 5°. This value is in good agreement with free flow mobility measurements of TISELIUS and others. The measured mobility of the dextran marker also is of a magnitude similar to that reported by others, about $2 \cdot 10^{-5}$ cm/sec/V/cm at a wetness of 1.5.

McDONALD *et al.*[9] found paper wetness to stabilize at 1.58/1 in an open system and Whatman No. 1 paper. EDWARD AND CRAWFORD[6] found a value of 1.55/1 with a Kunkel system and Whatman No. 3MM paper. In the Kunkel system described, with evaporation controlled, at a wetness of 1.57/1 the flow is practically uniformly toward the cathode (Fig. 1). This probably represents the minimum flow due to endosmosis upon which siphoning effects are superimposed and is considered to be the wetness ratio of choice by McDONALD[9]. At greater wetness values the flow tends to be from the center outward. The absolute value of the rate of electrolyte flow at various locations on the strip is independent of temperature. Thus, in the Kunkel system at a given pressure, corrected albumin mobility appears to be a function of temperature only and starting wetness merely determines what the rate of electrolyte flow is likely to be. To assess the effect of electrolyte flow will be more complex for the Durrum type apparatus, however, since after a given displacement (which depends upon starting point) the dextran will stop, presumably indicating an equilibrium position between evaporative changes and true endosmosis although buffer equilibrium is never achieved[14]. This was indicated here by the identical dextran displacement on Durrum strips run for 8 or for 16 h (Fig. 2).

If dextran is an acceptable marker for albumin, a different marker may be needed for γ-globulin. This is suggested by the negative temperature coefficient obtained by correcting observed γ-globulin mobility values for dextran movement. McDONALD[15,9] suggests that no single electro-osmotic rate flow indicator is satisfactory for migrants of different molecular volumes. In contrast, little or no variance in flow values has been obtained by others[11] with a variety of markers, including hydrogen peroxide. KUNKEL AND TRAUTMAN[16] express similar disregard for the size and nature of the marker. If the molecular size is unimportant, search must be made for some other factor to account for the variability of apparent mobility values for γ-globulin obtained here with both the Durrum and the Kunkel methods and

elsewhere by McDONALD *et al.*[9] with the open strip method at wetness values giving a cathodal electrolyte flow which is constant throughout the strip.

Distance migrated, variability

Measurement of the distance migrated by albumin and γ-globulin is warranted after a 60 min run in the Kunkel system employed. The elapsed time, including equilibration, staining and drying is less than three hours, the interval designated for rapid ionophoresis by the technique of WERUM, GORDON AND THORNBURG[17].

Findings both for the Durrum and for the Kunkel show an intra-run variation in albumin mobility of about 6.5 %. With the open strip at 15° McDONALD *et al.*[9] have detected equivalent variability. For γ-globulin, the average variability with the Kunkel was 18.8 %, a value of the same magnitude as established by the open strip method[18].

In terms of absolute albumin displacement in the Kunkel system the average variation from most to least mobile on the same strip is 0.15 cm. This variability may represent the sum of the errors inherent in applying the sample, drying the paper and measurement[18–20]. The remaining variability may result from variation in structure of the supporting medium with consequent small, localized variations in paper wetness. Equipment for measurement of voltage and temperature at nine spots on the paper during the run has been designed here and may detect such localized variations. Until such measurements are made it will be difficult to extend the usefulness of paper electrophoresis to a qualitative analysis of components of similar mobilities by a simple characterization in terms of mobility. The described system has been applied in this laboratory to the separation of the whole blood proteins of closely related bird species. Within limits of the variability designated, pattern inspection for specimen purity is satisfactory and in some cases identification of specimens can be made on the basis of mobility alone.

For similar specimens of nearly identical mobility, starch block electrophoresis may yield better differentiation at peak concentration but the yield on elution is low[21]. As a preparative technique none of the methods discussed is likely to be as satisfactory for serum proteins as are continuous flow techniques[22]. The cellulose ion-exchange columns, in use here, offer hope for specific reproducible fractionation in greater volume or in single drop elution aliquots[23].

SUMMARY

A rapid Kunkel type electrophoretic system was used to determine the effect of wetness and temperature upon the mobility of serum components. Comparison is made with a Durrum type apparatus. Variability averaged 6.5 % for albumin with both techniques.

Observed component mobility increases both with wetness and temperature with the Kunkel system. When corrected by use of dextran, observed mobility values are independent of wetness, but increase directly with temperature.

Comment is made on mobility markers and a wetness ratio, on specimen identification by mobility alone and on the usefulness of zone electrophoresis in qualitative analysis.

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THE ROLE OF LATERAL DIFFUSION AS A RATE-CONTROLLING MECHANISM IN CHROMATOGRAPHY

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A great number of kinetic and diffusion processes influence the resolution obtainable in chromatographic separations. These processes serve to establish local nonequilibrium[1] within the column or paper and consequently smear individual zones. The effect increases as the processes become slower. A first step in improving resolution has followed the basic understanding of the role of these processes as established by GLUECKAUF[2], THOMAS[3], and many others. Until recently, however, no method was available for understanding the role of complex processes involving more than one, or at most two, steps. The basis of the method to be used here for diffusion-controlled processes was evolved for use with complex reaction schemes composed of many simultaneous single-step reactions[1]. Application has been made to adsorption chromatography in which the surface is heterogeneous and consists of n different kinds of sites, consecutive reactions (this being an approximation to simultaneous partition and adsorption), the sorption of large molecules, and the simultaneous occurrence of chemical reaction and sorption in chromatography[4]. The solutions obtained by this method are exactly valid only at infinite time, a characteristic which they share with the VAN DEEMTER[5], GOLAY[6], and other equations. Fortunately, in chromatography, the equations which apply strictly only at infinite times are usually excellent approximations during most of the running time of an experiment.

As a special case of this method applied to diffusion-controlled processes, results have already been calculated for the effects of bending chromatographic columns[7]. In this case lateral diffusion across the column is the important controlling process. The tubewise nonequilibrium found in that case is in contrast to the nonequilibrium extending over only one or so particle diameters as discussed here.

THEORY

The flow of the mobile phase fluid in chromatography is responsible for establishing nonequilibrium by continually bringing new fluid with varying solute concentration into contact with the fixed bed and its associated stagnant layers. The sorption and desorption processes are opposing this trend by leveling off the concentration excesses and deficiencies. If these processes are rapid, and it is one of the goals in chromatography to make them so, they succeed in establishing a condition with very little

departure from equilibrium soon after a run begins. The nonequilibrium discussed here is always local nonequilibrium, especially that established between mobile stationary phases due to the above processes. This is in contrast to the nonequilibrium always associated with gross concentration gradients such as found in chromatographic zones and boundaries. Assuming simple geometries, the nonequilibrium is established laterally with respect to the flow direction, but with complex media the nonequilibrium is more complicated. Nevertheless the word "lateral" will be used in describing this nonequilibrium and the diffusion acting against it.

If attention is centered on a small volume element in the chromatogram with its coordinates fixed to the stationary support, it can be established that the concentration changes in that element due to three potential sources; the flow of new material directly into the element (operative in the mobile phase only), the longitudinal diffusion into the element due to the gross concentration gradients, and the flux of material due to local nonequilibrium. The equation for these changes in phase i is

$$\frac{\partial m_i}{\partial t} = s_i - v_i \frac{\partial m_i}{\partial z} + D_i \frac{\partial^2 m_i}{\partial z^2} \tag{1}$$

where z is the net flow direction, t the time, m_i the concentration in that phase, D_i the diffusion coefficient of the solute in phase i, and v_i the velocity of phase i at that point. The velocity, of course, must be written as a function of the lateral coordinates since it may vary from point to point across the channel as with parabolic flow. The rate of solute accumulation per unit volume due to local nonequilibrium is designated as s_i. If one assumes the departure from equilibrium to be small, the derivatives of the actual concentration m_i can be replaced by the derivatives of the equilibrium concentration, m_i^*. Thus s_i can be written to a good approximation as

$$s_i = \frac{\partial m_i^*}{\partial t} + v_i \frac{\partial m_i^*}{\partial z} - D_i \frac{\partial^2 m_i^*}{\partial z^2} \tag{2}$$

The first term on the right-hand side can be replaced by the following, provided we again assume conditions near to equilibrium

$$\frac{\partial m_i^*}{\partial t} = -\bar{u} \frac{\partial m_i^*}{\partial z} + \frac{m_i^*}{\sum A_j m_j^*} \sum D_j A_j \frac{\partial^2 m_j^*}{\partial z^2} \tag{3}$$

where A_j is the fraction of the cross sectional area occupied by phase j, and the summations extend over all phases. The average downstream velocity of the solute molecules is $\bar{u}$. This equation can be substituted back into eqn. (2). It can be shown, however, that the longitudinal diffusion terms appearing at the end of both eqns. (2) and (3) are ordinarily negligible[1]. Furthermore the two terms tend to cancel one another when the equations are combined. In fact when all values of D_j are equal, the combined term vanishes altogether. We are thus able to write

$$s_i = (v_i - \bar{u}) \frac{\partial m_i^*}{\partial z} \tag{4}$$

An alternate expression for s_i can be written in terms of the "lateral" diffusional transport which it represents (diffusional processes, only, are considered in this paper):

$$s_i = D_i \nabla^2 m_i \tag{5}$$

where the Laplacian operator ∇^2 is to be applied only to local, and not to gross, concentration gradients. The equations are simplified by defining the equilibrium departure term, ε_i, by the equation

$$m_i = m_i^* (1 + \varepsilon_i) \tag{6}$$

Rewriting eqn. (5), and keeping in mind the fact that m_i^* is the local equilibrium concentration and is thus locally invariant, we have

$$s_i = m_i^* D_i \nabla^2 \varepsilon_i \tag{7}$$

Eliminating s_i between eqns. (4) and (7), we have

$$\nabla^2 \varepsilon_i = (v_i - \bar{u}) \frac{1}{D_i} \frac{\partial \ln c}{\partial z} \tag{8}$$

where c is the overall concentration in terms of a unit volume containing a proportionate amount of all the phases. The differential $\partial \ln m_i^*$ has been replaced by $\partial \ln c$ since m_i^* is proportional to c for the linear equilibrium considered here.

A major part of the chromatographic problem is solved if a solution for ε_i can be obtained as a function of the coordinates for each phase i. Eqn. (8) is, mathematically, the well known Poisson's equation, a fact which may be of some use in obtaining solutions to some complex problems. The solutions must be compatible with certain boundary conditions related to the geometry and physical characteristics of the system. The principle types of boundary conditions applying to eqn. (8) are as follows.

(1) The condition that m_i^* results from the local equilibration of all concentrations m_i in the column, and therefore the integral of m_i^* over the total cross sectional area is equal to the integral of m_i over the area. The difference between the terms, $m_i^* \varepsilon$, must integrate to zero, $\sum_i \int m_i^* \varepsilon_i \mathrm{d}A_i = 0$.

(2) The boundary condition at a non-connecting interface (an interface across which no material flux is possible), $\partial \varepsilon_i / \partial x = 0$.

(3) The boundary condition expressing the equality of fluxes at a connecting interface, $D_i (\partial \varepsilon_i / \partial x) = D_j K_{ij} (\partial \varepsilon_j / \partial x)$, where K_{ij} is the partition coefficient, m_j^*/m_i^*.

(4) The condition expressing equilibrium at an interface (this is usually applicable), $\varepsilon_i = \varepsilon_j$.

(5) Symmetry conditions, which generally lead to expressions of the form $\partial \varepsilon_i / \partial x = 0$ when applied to the center of a symmetrical channel or to the center of a spherical ion exchange bead, etc.

The local nonequilibrium, described above, is responsible for the smearing of zones following the equations of diffusion. The development of this idea is analogous

to that used previously[1], but is summarized here because the equations must be rewritten when the rate-controlling processes are diffusional.

The flux of material through a unit area normal to the flow direction is given by

$$q = \sum_i \int m_i v_i \mathrm{d}A_i \tag{9}$$

Using eqn. (6) this rearranges to

$$q = \sum_i \int m_i^* v_i \, \mathrm{d}A_i + \sum_i \int m_i^* \varepsilon_i v_i \mathrm{d}A_i \tag{10}$$

where the first term on the right-hand side represents the mean convective drift of the zone and the second term on the right-hand side is responsible for zone dispersion and can be equated to $-D_c \partial c/\partial z$.

$$D_c = -\frac{\sum_i \int m_i^* \varepsilon_i v_i \mathrm{d}A_i}{\partial c/\partial z} \tag{11}$$

Since the ε_i terms, obtained from the integration of eqn. (8), are proportional to $\partial \ln c/\partial z$, D_c becomes a diffusion coefficient independent of position and the overall concentration, and proportional to the velocity squared. This coefficient is to be added to the coefficients of ordinary molecular diffusion and eddy diffusion. These terms contribute directly to the flux and when multiplied by the negative of the concentration gradient can, if desired, be added directly on to eqn. (9) as a starting point.

The calculated results for zone spreading can be expressed either in terms of diffusion or plate height. The contribution to plate height due to nonequilibrium effects is $H_c = 2D_c/\bar{u}$. The method outlined above is a very general method for obtaining plate height expressions which can be applied to several special cases now in the literature in addition to numerous other practical cases. Some of these will now be calculated by way of illustration.

CALCULATIONS

Most of the useful models employed in connection with chromatography consist of just two phases; a fluid mobile phase and a fixed stationary phase. While diffusion through these phases is often regarded as the rate-controlling process for mass transfer, it is likely that many actual cases also involve a contribution from single-step reactions. This has been investigated in ion-exchange chromatography, but not in gas and paper chromatography. In the latter cases adsorption, simultaneously with the partitioning process, must often occur in a manner that increases plate height. The methods used in previous papers[1,4] must be employed to account for single-step reactions of this kind.

The model used to represent the complex geometry of the fixed support and the interstitial fluid is open to some choice, and will ordinarily vary with the kind of

support chosen. The fixed support in ion-exchange chromatography is usually considered to consist of spherical beads, and in gas chromatography, a liquid film of uniform thickness. Randomly oriented cylindrical fibers may be assumed in this role in paper chromatography. The interstitial space, for which order of magnitude estimations are made, is usually not equated to a geometrical model. A model is quite important here, however, since the occurrence of a flow pattern that is essentially parabolic determines the rate-controlling process at high R values. The parabolic nature of laminar flow must consequently be considered in this region. The method used here can easily be applied to a number of simple models, with and without parabolic flow. It is possible to extend the calculations to more complicated models, as shown below, by using a liquid film of variable thickness for gas chromatography. It is anticipated that very realistic results can be obtained by numerical methods applied to a three-dimensional, periodic-lattice model of the fixed phase.

Sationary film of uniform thickness

In this case diffusion in the mobile phase (phase 1) will be considered to be very rapid, and ε_1 will therefore be constant. The geometry of the mobile phase is immaterial in this case. The stationary film is of thickness d_2. Applying eqn. (8) to this phase we obtain

$$\frac{\partial^2 \varepsilon_2}{\partial x^2} = -\frac{\bar{u}}{D_2}\frac{\partial \ln c}{\partial z} \tag{12}$$

where x is the distance through the film. Integrating with respect to x we get

$$\varepsilon_2 = -\frac{\bar{u}x^2}{2D_2}\frac{\partial \ln c}{\partial z} + g_{02}x + g_{12} \tag{13}$$

where g_{02} and g_{12} are integration constants. The type (1) boundary condition, considering the fact that ε_1 is constant, appears as

$$\varepsilon_1 A_1 = -\frac{KA_2}{d_2}\int_0^{d_2} \varepsilon_2 \mathrm{d}x \tag{14}$$

where K is the partition coefficient $K_{12} = (m_2^*/m_1^*)$. The type (2) boundary condition requires that $\partial\varepsilon_2/\partial x$ must equal zero at the non-connecting interface, $x = d_2$. The type (4) boundary condition requires that the constant ε_1 must equal ε_2 at $x = 0$; $\varepsilon_1 = g_{12}$. These three are sufficient to determine the three constants, ε_1, g_{02} and g_{12}. Only ε_1 is required in the integral in eqn. (11); ε_2 is multiplied by the velocity $v_2 = 0$, and hence drops out. The constant ε_1 is

$$\varepsilon_1 = \frac{-K\bar{u}d_2^2}{3D_2}\frac{\partial \ln c}{\partial z}\frac{A_2}{A_1 + KA_2} \tag{15}$$

Using this in the integral of eqn. (11)

$$D_c = -\frac{m_1^* A_1 \varepsilon_1}{d_1 c \partial \ln c/\partial z}\int_0^{d_1} v_1 \mathrm{d}x \tag{16}$$

we obtain $$D_c = \frac{1}{3}R^2(1-R)v^2\frac{d_2^2}{D_2}, \qquad H_c = \frac{2}{3}R(1-R)v\frac{d_2^2}{D_2} \tag{17}$$

where v is the average mobile phase velocity given by $\int_0^{d_1}(v_1 dx)/d_1$; R is the ratio of zone to mobile phase velocity, $\bar{u}/v$, and is given by $R = A_1/(A_1 + A_2 K)$; m_1^*/c is given by R/A_1; and K is obtained from the expression for R as $K = A_1(1-R)/A_2R$. These expressions will be used in subsequent sections also.

Homogeneous spheres

Again we assume that diffusion in the mobile phase is rapid enough to eliminate lateral concentration gradients in that phase. Consequently each part of the bead's spherical surface will be bathed at the same concentration level. This introduces spherical symmetry into the problem, and we can write eqn. (8) as

$$\frac{1}{r^2}\frac{\partial}{\partial r}r^2\frac{\partial \varepsilon_2}{\partial r} = -\frac{Rv}{D_2}\frac{\partial \ln c}{\partial z} \tag{18}$$

integration with respect to r gives

$$\varepsilon_2 = -\frac{Rvr^2}{6D_2}\frac{\partial \ln c}{\partial z} - \frac{g_{02}}{r} + g_{12} \tag{19}$$

Boundary conditions of type (1), type (4), and type (5) are applied to evaluate the constants ε_1, g_{02}, and g_{12}. The value of ε_1 is

$$\varepsilon_1 = -\frac{RvKb^2}{15D_2}\frac{\partial \ln c}{\partial z}\frac{A_2}{A_1 + KA_2} \tag{20}$$

where b is the radius of the bead. When used in connection with eqn. (11), this equation leads to

$$D_c = \frac{1}{15}R^2(1-R)v^2\frac{b^2}{D_2}, \qquad H_c = \frac{2}{15}R(1-R)v\frac{b^2}{D_2} \tag{21}$$

Cylindrical rods

Diffusion into cylindrical paper fibers in paper chromatography must proceed by means of a tortuous path, following the amorphous pathways between the crystalline regions of the fiber. Due to the obstructions the diffusion coefficient is reduced. Since the amorphous and crystalline regions are apparently small compared to the fiber diameter, the diffusion equation still applies. The reasonable assumption is made that the fiber length is great enough to neglect longitudinal gradients in the fiber. Diffusion in the mobile phase is again assumed to be comparatively rapid.

Eqn. (8), written in cylindrical coordinates with the above assumptions, appears as

$$\frac{1}{r}\frac{\partial}{\partial r}r\frac{\partial \varepsilon_2}{\partial r} = -\frac{Rv}{!D_2}\frac{\partial \ln c}{\partial z} \tag{22}$$

This equation integrates to

$$\varepsilon_2 = -\frac{Rvr^2}{4D_2}\frac{\partial \ln c}{\partial z} + g_{02}\ln r + g_{12} \tag{23}$$

The constant ε_1 is obtained with the application of type (1), (4), and (5) boundary conditions

$$\varepsilon_1 = -\frac{RvKb^2}{8D_2}\frac{\partial \ln c}{\partial z}\frac{A_2}{A_1 + KA_2} \tag{24}$$

where b is the cylinder radius. Use of eqn. (24) with eqn. (11) gives

$$D_c = \tfrac{1}{8}R^2(1-R)v^2\frac{b^2}{D_2}, \qquad H_c = \tfrac{1}{4}R(1-R)v\frac{b^2}{D_2} \tag{25}$$

Pores of nonuniform depth

In gas chromatography liquid solvent covers rough granular particles and forms a film of nonuniform thickness. The following calculation reveals the nature of the influence of these small, closely spaced heterogeneous regions. Our model calls for $n-1$ pores (each considered to be a different phase) of uniform cross section throughout their length and liquid depth d_i, and each occupying a respective fraction A_i of the total column cross section (or volume). The mobile phase, in which diffusion is assumed to be rapid, is phase 1 whereas the liquid-filled pores are phases 2 through n. For each pore we have, using eqn. (8)

$$\frac{\partial^2 \varepsilon_i}{\partial x^2} = -\frac{Rv}{D_2}\frac{\partial \ln c}{\partial z} \tag{26}$$

where x is the distance of penetration into the pore and D_2, as previously, is the diffusion coefficient in the stationary phase. This integrates to

$$\varepsilon_i = -\frac{Rvx^2}{2D_2}\frac{\partial \ln c}{\partial z} + g_{0i}x + g_{1i} \tag{27}$$

The type (1) boundary condition appears as

$$\varepsilon_1 A_1 = -K\sum_{i=2}^{n}\frac{A_i}{d_i}\int_0^{d_i}\varepsilon_i \mathrm{d}x \tag{28}$$

The type (2) boundary condition applies at depth d_i and gives

$$g_{0i} = \frac{Rvd_i}{D_2}\frac{\partial \ln c}{\partial z} \tag{29}$$

The type (4) boundary condition gives

$$\varepsilon_1 = g_{1i} \tag{30}$$

These equations can be solved for ε_1, eliminating the integration constants

$$\varepsilon_1 = -\frac{KRv}{3D_2}\frac{\partial \ln c}{\partial z}\frac{\sum_2^n A_i d_i^2}{A_1 + K\sum_2^n A_i} \tag{31}$$

This equation is analogous to eqn. (15) for a uniform film. Substituting into eqn. (11) and rearranging, we have

$$D_c = \tfrac{1}{3}R^2(1-R)v^2\frac{\sum A_i d_i^2}{D_2 \sum A_i} \tag{32}$$

where the summations run from 2 to n. The ratio of summations is simply the mean square depth, $\overline{d^2}$, averaged over cross-sectional area, or, equivalently, over the total liquid content. It is not the ordinary mean square pore depth as illustrated by the following calculation. If we are considering just two pores of equal radius, and one twice as deep as the other (h and $2h$), the ordinary mean square pore depth is $5h^2/2$, but the proper mean square depth is $3h^2$.
Substituting $\overline{d^2}$ into eqn. (32), we have

$$D_c = \tfrac{1}{3}R^2(1-R)v^2\frac{\overline{d^2}}{D_2}, \qquad H_c = \tfrac{2}{3}R(1-R)v\frac{\overline{d^2}}{D_2} \tag{33}$$

These equations are of the same form as eqn. (17). The introduction of a nonuniform film does not greatly complicate the picture, but does lead to a different interpretation of the film thickness. A similar simplicity has been found to exist with adsorption chromatography on heterogeneous surfaces[4].

MOBILE PHASE

Calculations for the mobile phase proceed in much the same manner as for the stationary phase. The problem is more complicated, however, for two reasons. First, the geometry is usually more complex as illustrated by comparing the interstitial space with the individual spherical beads in an ion exchange column. Second, the fluid motion in the interstitial space is complicated by viscous forces. For simple geometries, *i.e.*, circular cross sections or flow between parallel faces, the fluid motion is parabolic. It is reasonable to assume that flow of a basically similar type (parabolic) occurs in more complex interstices. Beyond this, the understanding of the non-equilibrium in complex interstitial spaces will probably not be much advanced until numerical solutions to the Navier-Stokes equation and then to eqn. (8) are obtained for some reasonable two and three dimensional lattices. Until then we can only assume the basic correctness of the simpler models.

The problem arising when diffusion in both phases is partially controlling is also important. The contributions to D_c and H_c add*, as will be shown, for some

* Additive terms are those contributing a fixed amount to the plate height irrespective of the rate (diffusion coefficient) in the other phase (see eqn. (49)).

simple geometries. It is questionable if the terms are additive for complex geometries as found in chromatographic columns. This premise is supported by calculations on a model, to be presented shortly.

Mobile-phase fluid with constant thickness and velocity

The simplest assumption for the mobile phase is flow at constant velocity between parallel faces each covered with a stationary partitioning layer. The constant velocity pattern is being used here primarily for comparison with the parabolic profile to be evaluated next. Rapid diffusion is assumed for the stationary phase. The calculations proceed in the same way as shown for the stationary film of uniform thickness. We obtain

$$D_c = \tfrac{1}{3}R(1-R)^2v^2\frac{d_1^2}{D_1}, \qquad H_c = \tfrac{2}{3}(1-R)^2v\frac{d_1^2}{D_1} \tag{34}$$

where $2d_1$ is the distance between the faces and D_1 is the diffusion coefficient of solute molecules in the mobile phase. A comparison with eqn. (17) shows a symmetry in D_c such that one is converted into the other if the mobile phase quantities R, d_1, and D_1, are exchanged with $(1-R)$, d_2 and D_2. No such symmetry is exhibited by H_c.

Mobile fluid with constant thickness and parabolic velocity

Laminar flow between parallel faces a distance $2d_1$ apart occurs with a velocity

$$v_1 = \tfrac{3}{2}v\left(1-\frac{x^2}{d_1^2}\right) \tag{35}$$

where x is the distance from the center and v, as before, is the average mobile phase velocity. Substituting eqn. (35) into eqn. (8), we obtain

$$\frac{\partial^2\varepsilon_1}{\partial x^2} = \frac{v}{D_1}\frac{\partial \ln c}{\partial z}\left[\left(\frac{3}{2}-R\right)-\frac{3x^2}{2d_1^2}\right] \tag{36}$$

Integration with respect to x gives

$$\varepsilon_1 = \frac{v}{2D_1}\frac{\partial \ln c}{\partial z}\left[\left(\frac{3}{2}-R\right)x^2-\frac{x^4}{4d_1^2}\right] + g_{01}x + g_{11} \tag{37}$$

Application of type (1), (4) and (5) boundary conditions to this gives

$$\varepsilon_1 = \frac{v}{2D_1}\frac{\partial \ln c}{\partial z}\left[\left(\frac{3}{2}-R\right)x^2-\frac{x^4}{4d_1^2}-\frac{d_1^2}{60}(40R^2-108R+75)\right] \tag{34}$$

Eqns. (35) and (38) are substituted into eqn. (11) yielding an equation similar to eqn. (16) except that the term ε_1 is under the integral sign. Evaluation of the integral leads to

$$D_c = \tfrac{1}{3}R\left(R^2-\frac{12}{5}R+\frac{51}{35}\right)v^2\frac{d_1^2}{D_1}, \qquad H_c = \tfrac{2}{3}\left(R^2-\frac{12}{5}R+\frac{51}{35}\right)v\frac{d_1^2}{D_1} \tag{39}$$

These expressions differ from those in eqn. (34), especially at R values approaching unity. An expression equivalent to eqn. (39) has been derived by GOLAY for capillary columns[6].

Mobile fluid with circular cross section and constant velocity

This problem proceeds along the same lines as the diffusion into cylindrical rods. The results are

$$D_c = \tfrac{1}{8}R(1-R)^2v^2\frac{r_0^2}{D_1}, \qquad H_c = \tfrac{1}{4}(1-R)^2v\frac{r_0^2}{D_1} \tag{40}$$

where r_0 is the tube radius. If R and $(1-R)$ are interchanged, this D_c becomes identical in form to the one in eqn. (25).

Mobile fluid with circular cross section and parabolic velocity

This case is analogous to parabolic flow between parallel faces. The important equations are

$$v_1 = 2v\left(1-\frac{r^2}{r_0^2}\right) \tag{41}$$

$$\frac{1}{r}\frac{\partial}{\partial r}\,r\frac{(\partial\varepsilon_1)}{\partial r} = \frac{v}{D_1}\frac{\partial \ln c}{\partial z}\left[(2-R)-\frac{2r^2}{r_0^2}\right] \tag{42}$$

$$\varepsilon_1 = \frac{v}{4D_1}\frac{\partial \ln c}{\partial z}\left[(2-R)r^2-\frac{r^4}{2r_0^2}-\frac{r_0^2}{6}(3R^2-10R+9)\right] \tag{43}$$

These equations take the place of eqns. (35), (36) and (38) for flow between parallel faces. The effective diffusion coefficient and plate height expressions are

$$D_c = \tfrac{1}{48}R(6R^2-16R+11)v^2\frac{r_0^2}{D_1}, \qquad H_c = \tfrac{1}{24}(6R^2-16R+11)v\frac{r_0^2}{D_1} \tag{44}$$

This expression for plate height is equivalent to one derived earlier for the influence of lateral diffusion in capillary columns[6].

The results obtained above for both stationary and mobile phases are summarized in Table I.

TWO OR MORE RATE PROCESSES

The method outlined above can be applied when two or more lateral diffusion processes influence the plate height. However, as already indicated, the calculations become excessively difficult for realistic models of packed columns. It is necessary to choose simple models that still exhibit some of the principle characteristics of the chromatographic nonequilibrium. When dealing with two phases, it is not possible to choose a model for each of the phases independently. It is unreasonable to assemble a model that is geometrically incompatible. Thus one would not assume cylindrical rods for the stationary phase and tubes of circular cross section for the mobile phase since

TABLE I

THE CONTRIBUTION OF VARIOUS GEOMETRIES AND FLOW PATTERNS TO THE PLATE HEIGHT

Rate-controlling diffusion process	*Plate height, H_c*
Stationary phase	
$D_2 = \infty$, geometry irrelevant	0
Flat film of uniform thickness d_2	$\frac{2}{3}R(1-R)v\frac{d_2^2}{D_2}$
Homogeneous spheres of radius b	$\frac{2}{15}R(1-R)v\frac{b^2}{D_2}$
Cylindrical rods of radius b	$\frac{1}{4}R(1-R)v\frac{b^2}{D_2}$
Pores of nonuniform depth (mean square depth $\overline{d^2}$)	$\frac{2}{3}R(1-R)v\frac{\overline{d^2}}{D_2}$
Mobile phase	
$D_1 = \infty$, geometry irrelevant	0
Constant velocity and thickness $2d_1$	$\frac{2}{3}(1-R)^2v\frac{d_1^2}{D_1}$
Parabolic velocity and constant thickness $2d_1$	$\frac{2}{3}\left(R^2-\frac{12}{5}R+\frac{51}{35}\right)v\frac{d_1^2}{D_1}$
Constant velocity in circular tube of radius r_0	$\frac{1}{4}(1-R)^2v\frac{r_0^2}{D_1}$
Parabolic velocity in circular tube of radius r_0	$\frac{1}{24}(6R^2-16R+11)v\frac{r_0^2}{D_1}$

these two geometries are incompatible. The question as to whether all the terms for the stationary phase in Table I are additive to all the terms for the mobile phase when both processes are rate-controlling is meaningless since many of the geometries are incompatible. One cannot tell if the two terms are additive for an ion exchange column of spherical beads since no one has evaluated the nonequilibrium in the interstitial space. Previous assumptions to this effect are unjustified. Hence many of the results in Table I are useful only if the process referred to is rate-controlling. More will be said about rate-controlling processes in the next section.

Simple models, such as flat, adjacent mobile and stationary phases of constant thickness, can be easily evaluated. These models should reveal the nature of *some* of the processes in real columns. The method will be illustrated by a simple model in which fluid of thickness $2d_1$ flows with constant velocity between plates each possessing a retentive layer of thickness d_2. Integration of eqn. (8) for each phase gives

$$\varepsilon_1 = \frac{v(1-R)x^2}{2D_1}\frac{\partial \ln c}{\partial z} + g_{01}x + g_{11} \qquad (45a)$$

$$\varepsilon_2 = \frac{vRx^2}{2D_2}\frac{\partial \ln c}{\partial z} + g_{02}x + g_{12} \qquad (45b)$$

In order to evaluate any or all of the constants, g_{01}, g_{11}, g_{02}, and g_{12}, four boundary

conditions are used: types (1), (2), (4), and (5) are most easily applied. From these, ε_1 is obtained as

$$\varepsilon_1 = v(1 - R)\frac{\partial \ln c}{\partial z}\left[\frac{x^2 - d_1^2}{2D_1} + \frac{Rd_1^2}{3D_1} - \frac{Rd_2^2}{3D_2}\right] \tag{46}$$

and, with the help of eqn. (11)

$$D_c = \tfrac{1}{3}R(1 - R)^2v^2\frac{d_1^2}{D_1} + \tfrac{1}{3}R^2(1 - R)v^2\frac{d_2^2}{D_2} \tag{47}$$

These two expressions are the sum of the respective contributions from eqns. (17) and (34). It can similarly be shown that H_c and D_c are composed of additive terms if the flow is parabolic. GOLAY has obtained the latter result, and also finds gas and liquid diffusion terms additive in capillary columns of circular cross section[6].

In packed chromatographic columns the interstitial space is complicated by the presence of pockets and fissures extending into the support. The diffusion into these will compete with diffusion through the stationary phase to the same general area. That this destroys the additive make-up of plate-height expressions can be shown by the following model, which, while not entirely realistic, does allow for the competition between diffusion in the two phases in getting solute to remote areas of the stationary phase (the high symmetry of the previous cases has disallowed this). Our model consists of deep (depth d_2) narrow pores, alternate ones filled with the mobile phase. These pores each terminate at the boundary of the flat channel occupied by moving fluid. For mathematical convenience it will be assumed that solute exchanges freely back and forth between the pores, and that the pores are sufficiently narrow to prevent any "sideways" nonequilibrium from developing. The relative pore volumes occupied by mobile and stationary phases are a_1 and a_2 respectively. An analysis of this case leads to the following result

$$\begin{aligned} H_c = {} & \tfrac{2}{3}(1 - R)^2v\frac{d_1^2}{D_1}\left[\left(\frac{d_1 + a_1d_2}{d_1}\right)\left(\frac{Ka_2 + a_1}{a_2K}\right)^2\right] \\ & + \tfrac{2}{3}R(1 - R)v\frac{d_2^2}{D_2}\left[\left(\frac{Ka_2 + a_1}{a_2K}\right)\left(\frac{Ka_2D_2 + a_1D_2}{Ka_2D_2 + a_1D_1}\right)\right] \end{aligned} \tag{48}$$

where R now equals $(d_1 + a_1d_2)/(d_1 + a_1d_2 + a_2Kd_2)$. The terms in brackets are corrections to eqn. (47). As a_1 and a_2 approach zero and unity, respectively, the bracketed terms approach unity in value. Eqn. (48) is no longer additive in the sense that the kinetics in each phase are responsible for a single term to be added to give H_c. Thus the equality

$$H_c(D_1, D_2) = H_c(D_1, \infty) + H_c(\infty, D_2) \tag{49}$$

is no longer true.

RATE-CONTROLLING PROCESSES

Even though the plate height is not always an additive quantity, an approximate evaluation of the relative importance of the two diffusion processes in the two phases can be obtained by comparing the separate contributions.

Using Table I, and assuming the mobile phase to have parabolic flow between parallel faces, we find the relative contribution to H_c due to the stationary and mobile phases as

$$\frac{H_c\,(\text{stat})}{H_c\,(\text{mob})} = \frac{R(1-R)\tau_2}{\left(R^2 - \frac{12}{5}R + \frac{51}{35}\right)\tau_1} = r\frac{\tau_2}{\tau_1} \qquad (50)$$

where τ_1 and τ_2 are approximate diffusional relaxation times in the two media; $\tau_1 = d_1^2/D_1$ and $\tau_2 = \bar{d}^2/D_2$ if the nonuniform pore model is being used. It is important to note that the above ratio can be strongly influenced by r as well as the ratio of relaxation times. The quantity r goes to zero as R approaches both zero and one. The limit at $R = 1$ is caused entirely by parabolic flow since if we assume constant flow velocity with the same geometry, we have

$$\frac{H_c\,(\text{stat})}{H_c\,(\text{mob})} = \frac{R}{1-R}\frac{\tau_2}{\tau_1} \qquad (51)$$

This ratio goes to infinity at $R = 1$. It is clear that it is absolutely necessary to consider the parabolic nature of flow in evaluating the rate-controlling processes at high R values. This result is due to the ability of parabolic flow to cause a dispersion by itself even if absorption is absent. Eqn. (50) must be considered as a reasonably good approximation for actual columns, reflecting the basic nature of viscous flow. The ratio r and $R/(1-R)$ are compared in Fig. 1.

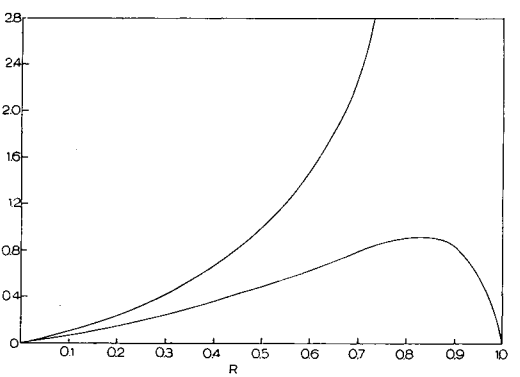

Fig. 1. Relative value of the plate height of the stationary phase to the plate height of the mobile phase for uniform flow (upper curve) and parabolic flow (lower curve).

TABLE OF SYMBOLS

A_i	fraction of cross sectional area occupied by phase i.
a_1, a_2	relative pore volumes occupied by mobile and stationary phases.
b	radius of spherical bead and of rod.
c	overall concentration, with all phases included.
d_1	one half the distance between parallel faces.

d_2 thickness of the stationary film.
$\overline{d^2}$ mean square pore depth averaged over cross sectional area, eqn. (32).
D_i diffusion coefficient of solute in phase i.
D_c nonequilibrium contribution to the diffusion coefficient describing zone spreading.
ε_i equilibrium departure term for phase i, eqn. (6).
g_{02}, etc. integration constants (see eqn. (13)).
H_c nonequilibrium contribution to plate height.
K_{ij} partition coefficient, m_j^*/m_i^*.
K partition coefficient, m_2^*/m_1^*.
m_i concentration in phase i.
m_i^* concentration in phase i under conditions of local equilibrium.
q material flux for unit area and time.
R ratio of zone to mobile phase velocity, $\bar{u}/v$.
r distance from center of spheres, rods, circular tubes (see also eqn. (50)).
s_i incoming flux due to lateral diffusion in phase i.
t time.
τ_1, τ_2 diffusional relaxation times, d_1^2/D_1 and $\overline{d^2}/D_2$.
u average downstream velocity of solute molecules.
v_i local downstream velocity in phase i.
v average downstream velocity of mobile phase.
x lateral (or local) coordinate.
z longitudinal coordinate.

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SUMMARY

A general method is outlined for calculating lateral nonequilibrium when the rate-controlling steps involve lateral diffusion only. The nonequilibrium is responsible for a contribution to the diffusion, or to the plate height, of a zone in a column. The latter is easily calculated from the nonequilibrium. The stationary phase models chosen for calculation include a flat film of uniform thickness, homogeneous spheres, cylindrical rods and pores of nonuniform depth. These models are useful in describing gas, ion exchange and paper chromatography. Mobile phase models include uniform and parabolic flow; both between parallel faces and in circular tubes.

The role of two simultaneous diffusion processes is considered, and the conditions under which these contribute additive terms are discussed. Examples of both additive and nonadditive cases are shown. Finally, the rate-controlling influences of stationary phase and mobile phase diffusion are compared. The importance of parabolic flow, in contrast to uniform flow, is shown at R values near unity.

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LATERAL DIFFUSION AND LOCAL NONEQUILIBRIUM IN GAS CHROMATOGRAPHY

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In the previous paper[1] a general equation describing local nonequilibrium in chromatographic media is derived and applied to certain models. The equation relating nonequilibrium to zone spreading (plate height) is also obtained. The application of these concepts to gas chromatography is especially interesting because of the mushrooming interest and intensive theoretical effort applied to the technique. The valid comparison of theoretical and experimental work has proceeded further in this area than in any of the fields of chromatography.

We will be concerned here with the effect of nonequilibrium in gas chromatographic columns. An equation for plate height will be obtained and the contributing terms will be compared to find the rate-controlling step. Of particular interest is the contribution of the controversial "eddy" diffusion term, to be discussed shortly.

THE RATE-CONTROLLING STEP

The relative contribution of diffusion in the stationary phase compared to the mobile phase is given approximately by eqn. (50) of ref. [1]. In gas chromatography, where ordinarily $R \ll 1$, r approaches $35R/51$. In view of the approximate nature of this result, equal confidence can be established for the simpler expression, $r = 2R/3$. Thus

$$\frac{H_c\,(\text{stat})}{H_c(\text{mob})} = \frac{2R\tau_2}{3\tau_1} \qquad (1)$$

An evaluation of τ_2 and τ_1 is more difficult than it appears since it is not clear how the liquid distributes itself over the common solid supports. It is often assumed that the liquid surrounds the support particle with a layer of nearly uniform thickness, but other evidence[2] shows the importance of deep narrow pores which may easily penetrate to the center.

The ratio τ_1/τ_2 has been estimated at $3.3 \cdot 10^6$ and $4 \cdot 10^4$ for two different cases by VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[3]. For R values in the neighborhood of 0.01, the ratio in eqn. (1) becomes $2.2 \cdot 10^4$ and $2.7 \cdot 10^2$ for the two cases, indicating the dominant influence of stationary phase diffusion.

If one assumes that the liquid distributes itself as a layer of constant thickness on the outside of a support particle of radius r_0, then the layer depth is approximately

$d_2 = r_0 f/3$, where f is the volume fraction of liquid referred to the support. This will differ only by a numerical constant from the weight fraction (usually given as weight percent). The depth d_1 (effective thickness of the mobile phase) will be assumed to equal r_0. While the channels are actually smaller than this, the effect of some non-equilibrium over one or more particle diameters must be allowed for. With the above assumptions the relative contribution to plate height, eqn. (1), becomes

$$\frac{H_c\,(\text{stat})}{H_c(\text{mob})} = \frac{2RD_1f^2}{27D_2} \tag{2}$$

This clearly depends on the amount of liquid present as well as the other parameters. Assuming $R = 10^{-2}$, $D_1/D_2 = 10^4$, and $f = 0.3$, the above ratio equals 2/3. This would indicate an even balance between the two diffusion processes.

If now we assume that the pores penetrate to the center of the support particle, and that these are gradually filled up by capillary forces from smallest to largest, then it is reasonable to let both $d_1 = r_0$ and $d_2 = r_0$. Using the same R and D_1/D_2 values, we obtain the ratio of plate heights as 67. This result is independent of the amount of stationary liquid diffusion. It is likely that the actual behavior of stationary liquid is intermediate between the two models given. Thus the plate height ratio increases with f, but not as rapidly as the square of f. Because of the dependence on both R and diffusion coefficients, different solutes in the same run might exhibit the predominance of different diffusion processes. Evidence accumulated in this laboratory indicates the predominance of liquid diffusion by a six to one ratio under typical conditions.

THE PLATE HEIGHT EQUATION

The equation given by VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[3] can be written as

$$H = 2\lambda d_p + \frac{2\gamma D_1}{v} + \frac{8}{\pi^2} R(1 - R)v\frac{d_f^2}{D_2} \tag{3}$$

where d_p is the particle diameter and d_f is an effective film thickness. It is assumed here that lateral diffusion into the liquid is rate-controlling. It must also be assumed that longitudinal diffusion in the liquid is not important. This assumption is usually valid with gas chromatography. The eddy diffusion term, $2\lambda d_p$, is assumed to be independent of velocity.

Several questionable points have arisen with respect to eqn. (3). Foremost among these is the repeated appearance of small and even negative eddy diffusion terms[4]. The rate of lateral diffusion in the gas has also been questioned. Several investigators have suggested adding another term for this effect. This at first seems reasonable if we can assume that gas and liquid contributions add. Even though this assumption is fairly good, the assumption that the eddy diffusion and lateral gas-phase diffusion terms add is erroneous. In order to establish this, the respective roles of the two processes will be examined.

The assumption that lateral gas-phase diffusion contributes an additive term

to the plate height, independent of other processes, is based on the picture of a simple, symmetrical interstice model with straight parallel walls. The theoretical behavior of such a model is approached in practice by capillary columns. If a molecule is started in a given streamline within a capillary column, the only mechanism by which it can change its location relative to the wall is lateral diffusion. If the diffusion coefficient, D_1, were zero, the molecule would remain the same distance from the wall, and due to the lack of lateral equilibration, the plate height would approach infinity.

A different situation exists in a packed column where the streamlines follow tortuous paths. A given streamline will alternately pass near to and far away from the adjacent walls (of the support particles). The distance of nearest approach to a wall can be made arbitrarily small by waiting a sufficient time. Hence there is an effective lateral equilibration even if the extent of lateral diffusion is negligible (since the stream velocity approaches zero at the walls, it may be necessary to postulate the existence of lateral diffusion for a few mean free paths at this location). As a result of this, the plate height remains at a finite value even though the diffusion coefficient approaches zero. It is consequently impossible to have an additive plate height term proportional to d^2_1/D_1.

The tortuous streamlines just referred to are responsible for the effect known as "eddy diffusion". Two adjacent streamlines may have quite different histories, each one changing randomly from a high to a low axial velocity depending on the configuration of the neighboring support particles. A low velocity, for instance, may be caused by the nearness of the streamline to a particle boundary, or by the presence of a predominating lateral component of the velocity. Each new change in velocity persists for a distance the order of the particle diameter, and this may be considered the length of step in a random walk process[5]. The eddy diffusion is proportional to the length of step as discussed here, and consequently proportional to the particle diameter. At this point it is useful to again consider the role of lateral diffusion. Lateral diffusion serves to shuttle molecules back and forth from one streamline to another. Thus a velocity bias for an individual molecule may not persist for an entire particle diameter, particularly at low velocities where sufficient time exists for several diffusional transfers. This reduction in the length of step causes a reduction in the coefficient of eddy diffusion. The eddy diffusion effect, from this argument, would not contribute a constant, velocity-independent term to the plate height.

The two interrelated effects discussed above, lateral diffusion in the gas phase and eddy diffusion, can both be accounted for in the following picture. In any chromatographic medium, packed or capillary, the axial streamline velocity varies widely from point to point. This variation tends to establish nonequilibrium. Both lateral diffusion and the effective exchange of streamlines in a packed medium act to reestablish equilibrium by redistributing solute molecules relative to the gas–liquid interface. These processes compete with one another in this role. The contributions to plate height by competing processes add as resistances in parallel rather than in series (this is shown, for example, in the calculations on the chromatographic sorption of large molecules where the attachment to the surface can occur in different ways[6]),

this can explain the disappearance of the eddy diffusion term when it is large compared to the lateral gas-phase diffusion term.

The above problem reduces to the correct calculation of local nonequilibrium, in common with the examples of ref. [1]. While considerably more difficulty might be encountered in an accurate analysis of this problem, much can be gained by a closer look at the processes involved. It has been established that two distinct processes are responsible for lateral equilibration in the gas phase. Lateral diffusion itself operates equally near channel boundaries or in the center of channels where the velocity is much larger. The lateral equilibration due to the effective exchange of streamlines operates more rapidly in the higher velocity regions since, to an approximation, a new velocity is assumed after the passage of a constant distance (the particle diameter). There is very little interchange due to this effect in the stagnant layers immediately adjacent to the channel walls. Consequently a situation can exist in which lateral diffusion controls the equilibration rate in the slow moving regions, and the effective exchange of streamlines predominates in the faster moving regions. Although we have assumed that the processes compete throughout, and thus add in parallel, we see that the actual situation is more complicated.

We are now in a position to comment on the name given to the dispersion of solute due to tortuous paths in a packed medium; namely, "eddy diffusion". The name is usually justified because of the tortuous path of flow in analogy to true eddy diffusion. The name has also been attacked as misleading since the Reynold's number is ordinarily far below that necessary for turbulent flow. The preceding discussion has revealed another similitude between true eddy diffusion and the chromatographic process. In certain heterogeneous reaction studies the liquid contents of a vessel are vigorously stirred in order to facilitate equilibration throughout the liquid. It has been found necessary, however, to postulate a diffusion layer adjacent to the surface where molecular diffusion rather than eddy diffusion controls the rate of mass transfer[7]. The thickness of the diffusion layer is inversely proportional to the stirring velocity. In the analysis of chromatography just presented, it was determined that molecular diffusion was controlling the equilibration in a slow moving zone that may likewise be called a diffusion layer. When the layer is shallow compared to the channel dimensions, this diffusion layer, as before, is inversely proportional to the flow velocity. At very low velocities the diffusion layer expands to fill the entire channel, in close analogy to the transition from turbulent to laminar flow. All of these chromatographic processes, of course, occur without the actual presence of turbulence.

Although the picture of eddy diffusion just given might suggest the conclusion that the two mass-transfer resistance terms add in series, it is still believed, when the velocity dependence of the diffusion layer thickness is considered, that the addition as parallel resistances is the best approximation available at this time. Using this approximation and several of the expressions from Table I of ref. [1] (assuming parabolic flow between parallel faces and nonuniform pore depths), we obtain the following plate height equation:

$$H = \frac{1}{\frac{1}{2\lambda d_p} + \frac{1}{\frac{2}{3}\left(R^2 - \frac{12}{5}R + \frac{51}{35}\right)v\frac{d_1^2}{D_1}}} + \frac{2\gamma D_1}{v} + \frac{2}{3}R(1-R)v\frac{\overline{d_2^2}}{D_2} \qquad (4)$$

which can be written as

$$H = \frac{1}{1/A + 1/Epv} + \frac{B}{pv} + Cv \qquad (5)$$

The terms A, B, C and E can be obtained by direct comparison of the two equations. The pressure p arises from the inverse pressure dependence of D_1. The expression for C is admittedly an approximation describing nonequilibrium in a complex system. No matter how this nonequilibrium manifests itself (as long as it remains small), the general equations show us that the contribution to plate height is always proportional to velocity.

The first term of the equation shows several expected characteristics. At high velocities where molecules exchange from fast to slow stream paths almost entirely by following the flow, the term approaches A, the usual eddy diffusion expression. At low velocities where the exchange is due to lateral diffusion, the term approaches Epv. It is very possible that the average experiment is run in this regime[5] and that the measured values of $A = 2\lambda d_p$, using eqn. (3), would be near zero. This observation has been often verified. Much careful experimentation is needed before these results can be stated conclusively, however.

SOME UNSOLVED PROBLEMS

While the gross features of the chromatographic process are well understood, many of the finer details, which may involve subtle influences making better separations possible, lack a theoretical interpretation. One need only look at the field of gas–solid chromatography (GSC) where surface adsorption forces predominate, or to gas–liquid chromatography (GLC) where adsorption forces often contribute, to realize that many areas have not been explored. This brief discussion of unsolved problems, along with possible theoretical approaches, deals mainly with kinetics and nonequilibrium in gas chromatography.

One of the unsolved problems in the area of GLC concerns the effect of the kinetics of adsorption at the stationary phase–solid support interface. This is known to occur particularly with polar solutes and nonpolar stationary phases. Adsorption of this kind is usually assiduously avoided even though it might be profitably used if understood. Surface adsorption is potentially capable of offering the most versatile and selective characteristics of any of the known retentive mechanisms. With the advent of extremely sensitive detectors, the problem of overloading adsorption columns is no longer a serious one.

Another problem in this area is the increase in plate height due to using a mixed mesh support[8]. This increase occurs partly in the rate term, H_c. Such an effect is

possibly due to a nonequilibrium extending over several particle diameters caused by an excessive heterogeneity established in the flow and absorption pattern. It is felt that calculations on a reasonable model, compared to experimental data, might be an important clue here. Finally, in GLC (as in all other chromatography), it is important to acquire more realistic models showing the influence of nonequilibrium in complex media and how this departs from the simple models commonly employed. A two or three dimensional lattice model is proposed for this purpose.

Gas–solid chromatography has received much recent attention. The rate effects include single-step reactions at the surface and lateral diffusion in the gas phase. While these have been treated singly[1,6], a combined approach is desired. With highly porous media like charcoal, diffusion into deep narrow pores with adsorption on the pore walls must be accounted for. If the adsorption–desorption process at the wall is rapid enough to essentially eliminate sideways nonequilibrium in the pore, the narrow, adjacent pore model (eqn. (48) of ref. [1]) should be used as a first approximation. Again a major challenge occurs in adequately representing a complex geometry—this time, the pore structure of an adsorbent particle.

The influence of "nonuniform" adsorption sites in gas–solid chromatography has been discussed in several places[9,10]. Nonuniformity is often considered to be objectionable without a proper qualification of the reasons. It is true that if some sites are extremely tenacious, tailing (not related to nonlinearity) may result. Furthermore, the average exchange rate should be maintained as large as possible consistent with good adsorption. Otherwise nonuniformity, *per se*, is not to be avoided (see ref. [6]), and may sometime be used as another degree of freedom leading to adsorbent versatility.

Several other techniques introduce complications into the calculation of nonequilibrium. Gas-adsorption layer chromatography, described by CREMER[11], must be treated analogously to simultaneous adsorption–partition, as discussed earlier. The use of two or more stationary liquids, especially if these are immiscible, means that up to three phases share the diffusion and nonequilibrium. Nonlinear kinetics, while making the calculation of zone profiles difficult, does not greatly increase the difficulty in calculating local nonequilibrium.

Large columns, used for preparative work, inherit in magnified form some of the nonequilibrium that is unimportant in ordinary columns. The bending of such columns, for instance, introduces a large lateral nonequilibrium that can seriously impair resolution[12]. The extent of nonequilibrium is almost negligible in small columns since equilibration occurs rapidly. If the column packing is not uniform in large columns, either with respect to its permeability to gas flow or its relative solute retentivity (R value), nonequilibrium can be serious. Suppose, for example, that a column is uniformly packed except that numerous fine particles accumulate on one side. That side is less permeable and more retentive (lower R value) than the other if it is assumed that the same weight fraction of stationary phase is present on all support particles. A large nonequilibrium will develop since the equilibration is slow. In order to theoretically predict the nonequilibrium, a detailed knowledge of the

packing would have to be available. Information on column packing, realizing that it is not entirely reproducible from one laboratory to the other, might come from visual observations, density and grain size measurements in individual sections, the visual tracking of colored substances flowing through the column (a suggestion by MARTIN[13]), and by measuring the peak spreading due to nonequilibrium and tracing this back to the original nonuniformity. An assumed model of nonuniform packing has been treated by GOLAY[14]. His suggestion of speeding up equilibration with mixing washers might not be necessary if more effort were applied to finding uniform packing methods.

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SUMMARY

The occurrence of local nonequilibrium in gas chromatography is investigated. The question as to whether liquid or gas-phase lateral diffusion is the rate-controlling step is discussed in the light of theoretical equations. It is found that this depends, to a large extent, on the distribution of stationary liquid over the solid support. Allowing for both liquid and gas diffusion, and also for the influence of tortuous streamlines leading to eddy diffusion, a new, approximate plate height equation is obtained. Finally, some of the important unsolved problems in the field of gas chromatography are discussed.

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CHROMATOGRAPHIC STUDIES ON THE OXIDATION OF SULPHUROUS ACID BY FERRIC IRON IN AQUEOUS ACID SOLUTION

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We have previously indicated[1] that chromatographic procedures can be of great value in kinetic studies, and in this paper which discusses the oxidation of sulphurous acid by ferric iron, the ferrous and ferric iron is separated and estimated by chromatography whilst dithionate is isolated from cations which would otherwise have interfered with its estimation.

Using non-chromatographic techniques, BASSETT[2,3] showed that dithionate is formed in a number of reactions where sulphurous acid is oxidized, thus:

(a) Metal ions, easily reducible to a lower valency state or the metal itself, yield dithionate as a result of complex-ion formation with sulphite ion followed by auto-oxidation or reduction of the complex. With ferric chloride solution, the complex-ion $FeCl_4^-$ is sufficiently stable to prevent the formation of the ferrisulphite anion and consequently little dithionate is formed, whereas with sulphate and nitrate no stable complex-ion is formed so that in the presence of sulphurous acid, the ferrisulphite is formed with ease. The auto-oxidation of the latter leads to dithionate, *viz*:

$$2[Fe(SO_3)_2]^- + H_2O = SO_4^{2-} + 2H^+ + 2Fe^{2+} + 3SO_3^{2-}$$
$$2[Fe(SO_3)_2]^- = S_2O_6^{2-} + 2Fe^{2+} + 2SO_3^{2-}$$

(b) Both sulphate and dithionate can be formed in the oxidation of sulphurous acid by the oxides of manganese. Dithionate can only be formed when an oxide is reduced in one-electron steps. In either the first step $Mn^{4+} \longrightarrow Mn^{3+}$ or in the second step $Mn^{3+} \longrightarrow Mn^{2+}$, a single electron would be adsorbed from a SO_3^{2-} ion which could then lose its second electron, giving sulphate, or combine with another singly charged SO_3^- ion to give $S_2O_6^{2-}$. The reaction probably occurs on the surface of the oxide and depends on the structure and the location of the active centres.

Some kinetic aspects and stoichiometries of the oxidation of sulphurous acid are described by HIGGINSON AND MARSHALL[4] and the results are compared with those obtained in the oxidation of hydrazine. The results discussed are almost identical to those of BASSETT and co-workers[3] in that all oxidizing agents should lead to the formation of sulphate, but dithionate should only be formed in oxidations with one-equivalent reagents. The copper-catalysed reaction between ferric iron and sulphurous acid[5] was studied, being more suitable for accurate kinetic interpretation. The following mechanism was postulated:

$$Fe(III) + H_2SO_3 \xrightarrow{k_1} Fe(II) + HSO_3$$

$$Fe(II) + HSO_3 \xrightarrow{k_{-1}} Fe(III) + H_2SO_3$$

$$2HSO_3 \xrightarrow{k_2} H_2S_2O_6$$

$$Fe(III) + HSO_3 \xrightarrow{k_3} Fe(II) + SO_3(H_2SO_4)$$

$$Cu(II) + HSO_3 \xrightarrow{k_4} Cu(I) + SO_3(H_2SO_4)$$

$$Cu(I) + Fe(III) \xrightarrow{\text{rapid}} Cu(II) + Fe(II)$$

EXPERIMENTAL SECTION

Reagents

1. A stock solution of iron (III) sulphate, (0.4 *M* with respect to iron) was prepared by oxidation of the corresponding iron (II) sulphate with "100 volume" hydrogen peroxide. Sufficient sulphuric acid was added to make the hydrogen-ion concentration 1.0 *M* and the iron was estimated gravimetrically as the oxide.

2. A 0.2 *M* solution of sodium sulphite was prepared before each run and standardized using iodine (acid conditions).

3. A 0.2 *M* solution of ferrous sulphate in 1.0 *M* sulphuric acid was prepared by weighing the AnalaR salt.

4. A stock solution of 1.0 *M* sulphuric acid, AnalaR grade. The final reaction mixture was so arranged to be of constant hydrogen-ion concentration, 0.5 *M*, by the addition of sulphuric acid. All reactions were carried out in a thermostat at 25.0 $\pm$ 0.1° and solutions were allowed to equilibrate overnight before reaction was started by the addition of sulphite.

As the reaction proceeded, samples were withdrawn for analysis at convenient intervals (usually every 3 h). Iron (II) and iron (III) analyses were carried out as described in a previous paper[6] using anion-exchange techniques to separate iron (II) from iron (III). Ferrous iron was first eluted with 4 *M* hydrochloric acid followed by ferric iron using 0.5 *M* hydrochloric acid. Colorimetric estimation then followed using 2-nitroso-1-naphthol-4-sulphonic acid.

The analysis of dithionate presents a somewhat different problem than that used for dithionic acid or dithionate alone. In the aliquot taken from the reaction mixture, the estimation is rendered impossible by the presence of ferrous iron and sulphurous acid. HIGGINSON AND MARSHALL[4] used a titration technique followed by an oxidation of dithionate with dichromate[7].

$$Cr_2O_7^{2-} + 3S_2O_6^{2-} + 2H^+ = 2Cr^{3+} + 6SO_4^{2-} + H_2O$$

In order to avoid this procedure for the oxidation of ferrous iron to ferric iron, all interfering cations were removed from the aliquot of the reaction mixture with a cation exchange resin Zeo-Carb 225. The column technique was used with a 14 cm bed of resin in the hydrogen form. A 10.0 ml aliquot of the reaction mixture was

adsorbed on the top of the column and elution commenced with distilled water until approximately 120 ml of eluant had been collected. This procedure effectively arrested reaction and left the dithionate, sulphate and sulphite as the free acids. The solution was made acid by the addition of 5 ml of concentrated sulphuric acid and the sulphur dioxide was removed with a stream of carbon dioxide. Excess $M/300$ potassium dichromate was added and the solution boiled for 30 min. A further 50 ml of 2 N sulphuric acid was added and the solution boiled for 30 min. After cooling, excess ferrous iron was added and the excess was estimated volumetrically with potassium dichromate.

Several determinations were carried out on synthetically prepared mixtures similar to those to be analysed from the reaction mixture. Varying amounts of $1.06 \cdot 10^{-2}$ M dithionate were used (Table I).

TABLE I

Sample number	*ml of* $S_2O_6^{2-}$ *taken*	*ml of* $K_2Cr_2O_7$ *used*	*Molarity of* $S_2O_6^{2-} \times 10^2$
1	2.5	2.70	1.08
2	2.5	2.80	1.12
3	5.0	5.48	1.09
4	5.0	5.27	1.05
5	5.0	5.37	1.07
6	10.0	10.58	1.06
7	10.0	11.01	1.10
8	10.0	11.30	1.13

RESULTS AND DISCUSSION

The advantage of using chromatographic methods of analysis in the Fe(III)–H_2SO_3 reaction is that unfavourable ratios of Fe(III) to Fe(II) may be measured more accurately than by standard estimations. Also the cation exchange treatment prior to the estimation of dithionate effectively arrests the reaction and leaves the dithionate in a suitable form for analysis. It was for these reasons that the copper was omitted from the reaction.

A plot of reaction rate showed that initially the reaction was bimolecular, but as more ferrous iron was formed, deviation from bimolecularity occurred. Fig. 1 shows a plot of

$$\log_{10} \frac{[\mathrm{Fe(III)}]_0 \, [\mathrm{H_2SO_3}]_t}{[\mathrm{H_2SO_3}]_0 \, [\mathrm{Fe(III)}]_t}$$

against time in hours for a reaction mixture of $[\mathrm{Fe(III)}]_0 = 0.200$ M and $[\mathrm{H_2SO_3}]_0 = 0.0468$ M. The initial reaction velocity k_1 was obtained from the log plot (*cf.* Table II). Assuming that the mechanism postulated by HIGGINSON AND MARSHALL[4] for the

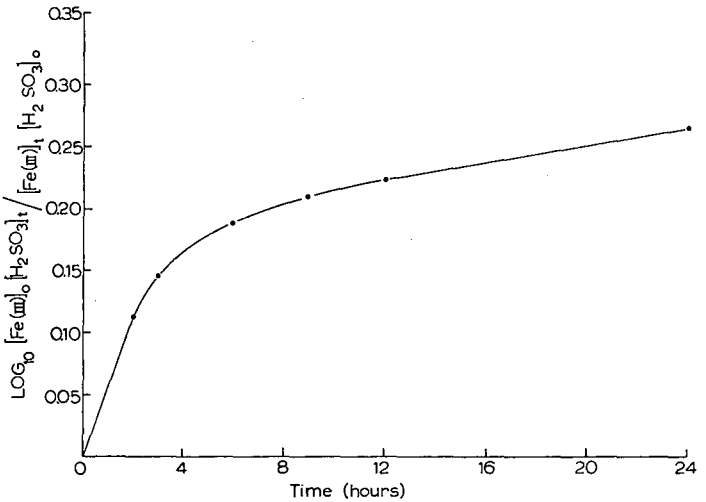

Fig. 1. The effect of time upon the initial reaction rate.

TABLE II

$[Fe(II)]_0 = 0$

Run number	$[Fe(III)]_0$ *mole·l⁻¹*	$[H_2SO_3]_0$ *mole·l⁻¹*	$R(Fe) \times 10^2$ *mole·l⁻¹·h⁻¹*	$R(H_2S_2O_6) \times 10^2$ *mole·l⁻¹·h⁻¹*	*Value of slope*	k_1 *l·mole⁻¹·h⁻¹*
1	0.0400	0.0850	0.42	0.20	0.0240	1.23
2	0.0200	0.0895	0.22	0.11	0.0403	1.33
3	0.2000	0.0468	0.85	0.35	0.0565	0.85
4	0.2000	0.0189	0.45	0.16	0.0680	0.87
5	0.2000	0.0915	1.40	0.65	0.0330	0.70
6	0.1000	0.0920	0.85	0.40	0.0030	0.86

Average value $k_1 = 0.97 \pm 0.20$ l·mole^{-1}·h^{-1}.

copper-catalysed reaction between sulphurous acid and ferric iron is correct

$$Fe(III) + H_2SO_3 \xrightarrow{k_1} Fe(II) + HSO_3 \quad (1)$$

$$Fe(II) + HSO_3 \xrightarrow{k_{-1}} Fe(III) + H_2SO_3 \quad (-1)$$

$$2HSO_3 \xrightarrow{k_2} H_2S_2O_6 \quad (2)$$

$$HSO_3 + Fe(III) \xrightarrow{k_3} Fe(II) + SO_3(H_2SO_4) \quad (3)$$

For convenience, all sulphur species are shown as uncharged molecules or radicals.

It was found that, on adding increasing amounts of ferrous iron to the reaction mixture deviation from bimolecularity occurs very quickly after the initial rate. From the suggested mechanism and making the usual stationary-state assumption that $R(HSO_3) = 0$, an equation relating the products and reactants with the initial velocity constant k_1 may be derived:

$$\frac{[Fe(III)]\,[H_2SO_3]_0}{R(Fe)} = \frac{1}{2k_1} + \frac{k_{-1}}{2k_1k_3}\frac{[Fe(II)]_0}{[Fe(III)]_0} \qquad (4)$$

as long as $R(Fe) \gg R(H_2S_2O_6)$. This equation did not hold, particularly where $[H_2SO_3]_0 > [Fe(III)]_0$ when almost quantitative oxidation of sulphurous acid to dithionate occurred (Table III).

TABLE III

Run number	*11*	*12*	*7*	*8*	*9*	*10*
$[Fe(III)]_0$ mole·l^{-1}	0.1049	0.1026	0.1008	0.1010	0.0433	0.0417
$[H_2SO_3]_0$ mole·l^{-1}	0.0425	0.0460	0.0895	0.0900	0.0860	0.0860
$[Fe(II)]_0$ mole·l^{-1}	0.04523	0.01740	0.04920	0.01900	0.04671	0.01835
$[Fe(II)]_0/[Fe(III)]_0$	0.4311	0.1695	0.4882	0.1881	1.078	0.440
$R(Fe) \times 10^3$ mole·l^{-1}·h^{-1}	1.75	3.15	3.50	5.67	1.9	2.5
$R(H_2S_2O_6) \times 10^4$ mole·l^{-1}·h^{-1}	8.0	13.5	10.5	24.0	8.5	12.5
$\frac{[Fe(III)]_0[H_2SO_3]_0}{R(Fe)}$	2.548	1.498	2.577	1.603	1.960	1.434
$\frac{[Fe(III)]_0^2[H_2SO_3]_0^2}{[Fe(II)]_0^2} \times 10^3$	9.72	73.62	33.60	228.8	6.36	38.18

If the rate of disappearance of HSO_3 radicals by reaction (—1) is sufficiently greater than the sum of the rates of reaction (2) and (3), we have as an approximation

$$[HSO_3] = K\frac{[Fe(III)]\,[H_2SO_3]}{[Fe(II)]}$$

where

$$K = k_1/k_{-1}$$

From reaction (2):

$$R(H_2S_2O_6) = \tfrac{1}{2}\,k_2K^2\frac{[Fe(III)]^2\,[H_2SO_3]^2}{[Fe(II)]^2} \qquad (5)$$

and a plot of $R(H_2S_2O_6)$ against $[Fe(III)]^2\,[H_2SO_3]^2/[Fe(II)]^2$ should be a straight line and pass through the origin. This is not the case in Fig. 2 and it may be that dithionate is formed in some other way.

From the scheme suggested[4], copper salts should speed up the reaction with the production of more ferrous iron and less dithionate

$$Cu(II) + HSO_3 \xrightarrow{k_4} Cu(I) + SO_3(H_2SO_4) \qquad (6)$$
$$Cu(I) + Fe(III) \xrightarrow{\text{rapid}} Cu(II) + Fe(II)$$

The initial rate was obtained for an equal stoichiometric mixture of ferric iron and sulphurous acid with 0.01 *M*, 0.005 *M* and 0.002 *M* copper sulphate solution and, as expected, less HSO_3 radicals were available for dimerisation with the production of less dithionate and more ferrous iron.

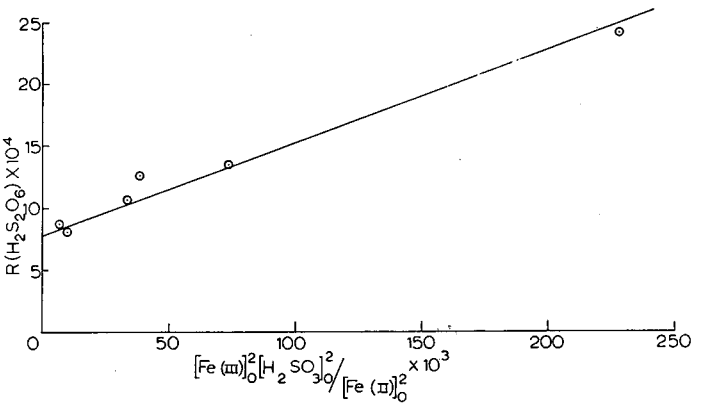

Fig. 2. The test of equation (5).

SUMMARY

Chromatographic techniques have been employed for the separation of ferrous and ferric iron and also in the isolation of dithionate prior to analysis.

The mechanism postulated by HIGGINSON AND MARSHALL[4] has been used to explain the results obtained in the reaction between sulphurous acid and ferric iron in the absence of copper. Almost quantitative oxidation of sulphurous acid to dithionate occurred with increasing H_2SO_3/Fe^{3+} ratios.

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ZUR CHROMATOGRAPHIE VON FLAVONOLEN IM FLIESSMITTELSYSTEM CHLOROFORM–EISESSIG–WASSER

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Zahlreiche Fliessmittelsysteme sind zur chromatographischen Analyse von Flavonolen mit Erfolg erprobt und beschrieben worden. Sie erlauben die Lösung der meisten auftretenden Probleme. Zu den noch unbefriedigend gelösten Aufgaben gehören die Trennung der 3-Rhamnoside von Myricetin, Quercetin und Kaempferol, eine sichere Abtrennung der Monomethyläther von den homologen Hydroxylverbindungen sowie ein sicherer Nachweis des Kaempferols neben seinen häufigsten Begleitern bei möglichst mittleren R_F-Werten.

Mit dem im Folgenden beschriebenen Fliessmittelsystem konnten bei geeigneter Variation des Mischungsverhältnisses brauchbare analytische Ergebnisse erzielt werden. Um einen raschen Überblick über die Anwendbarkeit in speziellen Fällen zu ermöglichen, wird die vorangegangene kurze Darstellung[1] ergänzt durch die Wiedergabe des Verhaltens einiger Flavonole im gesamten Bereich der Fliessmittelfolge, die durch die Begrenzung der Mischungslücke des ternären Systems Chloroform–Eisessig–Wasser definiert wird (Fig. 1). Die graphischen Darstellungen ent-

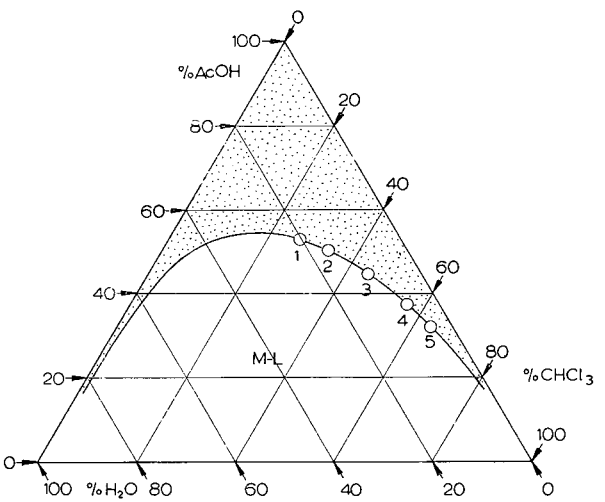

Fig. 1. Diagramm des ternären Systems $CHCl_3$–AcOH–H_2O. Die Begrenzung der Mischungslücke (M.-L.) definiert die hier beschriebene Fliessmittelfolge. 1–5 die bevorzugten Gemische, für die in Tabelle I R_F-Werte angegeben sind. Gemisch 1 : $CHCl_3$-AcOH = 1 : 2, Gem. 2 = 2 : 3, Gem. 3 = 1 : 1, Gem. 4 = 3 : 2, Gem. 5 = 2 : 1, jeweils wassergesättigt.

halten nur die wichtigsten Verbindungen, weitere Ergebnisse sind in der Tabelle I zusammengefasst.

TABELLE I

R_F-WERTE VON 24 FLAVONOLEN IN FÜNF VERSCHIEDENEN GEMISCHEN VON CHLOROFORM, EISESSIG UND WASSER

Name	*Substitution*	*Chloroform–Eisessig: Mischungs-verhältnis*				
		1	*2*	*3*	*4*	*5*
		1:2	*2:3*	*1:1*	*3:2*	*2:1*
Rhamnocitrin	3,5,4′-OH 7-OCH_3	98	97	97	96	96
Kaempferid	3,5,7-OH 4′-OCH_3	96	95	94	94	93
Galangin	3,5,7-OH	95	94	92	91	91
Datiscetin	3,5,7,2′-OH	95	95	87	81	68
Rhamnetin	3,5,3′,4′-OH 7-OCH_3	86	80	72	63	51
Isorhamnetin	3,5,7,4′-OH 3′-OCH_3	79	75	68	59	50
Kaempferol	3,5,7,4′-OH	68	62	50	38	28
Morin	3,5,7,2′,4′-OH	61	52	31	21	15
Patuletin	3,5,7,3′,4′-OH 6-OCH_3	59	52	37	28	19
Nortangeretin	3,5,6,7,4′-OH	40	34	21	15	09
Quercetin	3,5,7,3′,4′-OH	40	34	22	13	08
Quercetagetin	3,5,6,7,3′,4′-OH	46	34	16	07	03
Myricetin	3,5,7,3′,4′,5′-OH	19	14	06	03	03
Norauranetin	3,6,7,8,4′-OH	18	13	06	03	02
Glykoside						
Afzelin	K-3-rh	75	64	38	19	13
Kaempferol-3-arabinosid	K-3-arab	74	60	36	20	13
Astragalin	K-3-gluk	69	54	31	15	08
Quercitrin	Q-3-rh	61	48	24	10	05
Robinin	K-3-rhgal-7-rh	65	51	20	07	03
Hyperosid	Q-3-gal	55	43	18	07	03
Nicotiflorin	K-3-rhgluk	59	48	14	07	03
Rutin	Q-3-rhgluk	52	41	13	05	02
Myricitrin	My-3-rh	44	33	13	04	02
Quercimeritrin	Q-7-gluk	34	27	10	03	02

Erläuterungen: K = Kaempferol, Q = Quercetin, -rh = -rhamnosid, -arab = -arabinosid, -gluk = -glukosid, -gal = -galaktosid. Mischungsverhältnisse in Volumenteilen. Wanderungswerte in $R_F \times 100$.

Zur Darstellung. Bei der graphischen Darstellung der Ergebnisse muss über die Einteilung der Abszisse willkürlich verfügt werden. Die in den Fig. 3–6 verwendete Skala stellt die linear gestreckte Begrenzung der Mischungslücke dar, welcher willkürlich die Länge 10 zugeschrieben wird. Die Mischungsverhältnisse, die den Skalenteilen im einzelnen zuzuordnen sind, lassen sich der Fig. 2 entnehmen. Ab Skalenteil 5 ist die Abszisse annähernd linear bezüglich des Gehaltes der Gemische an Chloroform, von 0–5 dagegen annähernd linear bezüglich des Wassergehaltes. Auf diese Weise kommt der Einfluss aller drei Komponenten des Gemisches gleichmässig zur Geltung, was nicht der Fall ist, wenn auf der Abszisse nur % H_2O oder % $CHCl_3$ linear abgetragen werden. So erkennt man auch klar die Abhängigkeit der R_F-Werte von der Essigsäure-Konzentration: die maximalen R_F-Werte liegen im Gebiet maximalen Essigsäure-Gehaltes.

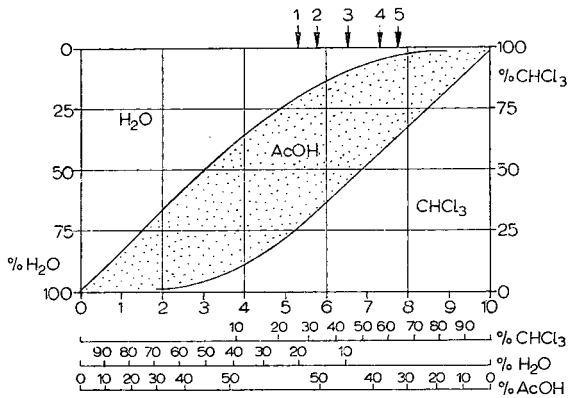

Fig. 2. Diagramm der an der Begrenzung der Mischungslücke liegenden Gemische des Systems $CHCl_3$–AcOH–H_2O. Der Rand der Mischungslücke ist linear abgetragen und mit einer Dezimalskala versehen. 1–5 die bevorzugten Gemische, vgl. Fig. 1. Der Fig. können die den einzelnen Skalenwerten zuzuordnenden Mischungsverhältnisse entnommen werden.

METHODE

Die Ergebnisse wurden bei aufsteigender Entwicklung der Chromatogramme teils in einfachen Glastöpfen, teils in der Kammer nach EGGER UND ENSSLIN[2] gewonnen. Die Laufzeit beträgt etwa 13–18 Stunden bei einer Laufstrecke von 17–20 cm. Als Lösungsmittel dienten 20 verschiedene Gemische (gleichmässig über den Rand der Mischungslücke verteilt) von Chloroform, Eisessig und Wasser. Die fertigen getrockneten Chromatogramme wurden mit 5 %iger saurer methanolischer Lösung von Zirkonoxychlorid gesprüht. Es wurde das sehr harte Papier 263 von Macherey, Nagel & Co. verwendet. Die Temperatur im Chromatographierraum betrug 19 ± 1°.

Die starke Flüchtigkeit des Chloroforms erfordert gut geschlossene Kammern und Temperaturen möglichst nicht über 18–20°. Da die R_F-Werte sehr stark vom Chloroform-Gehalt abhängen (steiler Kurvenverlauf gerade im bevorzugten Gebiet), muss sogar berücksichtigt werden, dass nach dem Einbringen des Gemisches ein beträchtlicher Anteil an Chloroform an die Kammeratmosphäre abgegeben wird. Im Falle ungenügend geschlossener Kammern wird die Reproduzierbarkeit der Werte sehr schlecht. Die in der vorangegangenen Mitteilung[1] beschriebenen R_F-Werte sind noch durchweg zu hoch und entsprechen einem Gemisch mit niedrigerem Chloroform-Gehalt als angegeben. Die Entwicklung darf auch nicht zu lange dauern, da stets etwas Lösungsmittel vom Papier verdampft und die R_F-Werte damit verfälscht werden.

Die Laufgeschwindigkeit hängt stark von der Zusammensetzung des Gemisches ab. Sie geht bei maximaler Essigsäure-Konzentration durch ein Minimum, steigt zur Wasserseite hin mässig, zur Chloroformseite hin sehr stark an. Chloroformreiche Gemische steigen gut doppelt so rasch als Butanolgemische. Die Entwicklung erfolgt bei den meist verwendeten weichen Papieren (Schleicher und Schüll 2043, Whatman No. 1) so rasch, dass die optimale Trennung nicht mehr erreicht wird. Bei den sehr harten Papieren (Macherey, Nagel & Co., No. 263, Schleicher und Schüll 2045a) jedoch

geht die Entwicklung hinreichend langsam, um die hohe Trennfähigkeit voll auszunutzen, gleichzeitig aber noch hinreichend schnell, um die oft auftretenden Störungen durch Temperaturgefälle gering zu halten.

Innerhalb der Gruppe der Tri- bis Hexa-oxyflavone, ihrer Glykoside und Monomethyläther wird eine sehr scharfe Differenzierung erreicht. Die Fleckenzeichnung ist im Bereich 3.5–7.5 sehr gut, ausserhalb stört vor allem bei den Aglykonen Schweifbildung.

Zur photographischen Wiedergabe wurde das Rundfilterchromatogramm mit Glykosiden unbehandelt gegen eine blau fluoreszierende Unterlage gepresst und im auffallenden U.V.-Licht photographiert. Die Glykosidzonen erscheinen dabei dunkel auf blauem Grund. Fig. 7 stellt somit ein Positiv dar. Das aufsteigend entwickelte Chromatogramm mit Aglykonen wurde nach dem Sprühen mit Zirkon unter Verwendung eines gelbdurchlässigen Filters im auffallenden U.V.-Licht photographiert. Es erscheinen helle Flecken auf dunklem Grund. Fig. 8 stellt also ebenfalls ein Positiv dar.

DISKUSSION DER R_F-WERTE

(1) Glykoside. Das Verhalten der Glykoside sei am Beispiel der 3-Rhamnoside von Myricetin, Quercetin und Kaempferol gezeigt. Diese Verbindungen werden in Butanol–Essigsäure–Wasser-Gemischen zwar noch gut getrennt, besitzen dort aber unangenehm hohe Wanderungswerte, die vor allem ein präparatives Arbeiten mit Zellulose-Säulen erschweren. In wasserreichen Mischungen dagegen ist die Trennung sehr schlecht. Fig. 3 zeigt die R_F-Werte in der Chloroform-Fliessmittelfolge. Im Bereich

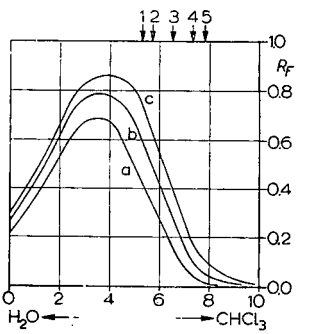

Fig. 3. Abhängigkeit der R_F-Werte der Glykoside Myricitrin (a), Quercitrin (b) und Afzelin (c) von der Zusammensetzung des Fliessmittels. Erläuterungen vgl. Fig. 1 und 2.

4.5–7.5 verlaufen die Kurven nahezu parallel. Das erlaubt, beliebig niedrige Wanderungswerte durch geeignete Wahl der Chloroform-Konzentration einzustellen. Bei Gemischen mit über 45 % $CHCl_3$ wird zweckmässigerweise auf Durchlauf entwickelt. Das Verfahren lässt sich gut auf Zellulose-Säulen übertragen[3].

Die 3-Hexoside verhalten sich ähnlich wie die Rhamnoside. Im Bereich 4–8 verlaufen ihre R_F-Kurven so, dass gilt:

$$R_F(\text{My} - \text{hex}) < R_F(\text{My} - \text{rh}) < R_F(\text{Q} - \text{hex}) < R_F(\text{Q} - \text{rh}) < R_F(\text{K} - \text{hex}) < R_F(\text{K} - \text{rh}) \quad (1)$$

wobei rh = 3-rhamnosid, hex = 3-hexosid. Diese Beziehung (1) wird durch das in Fig. 7 wiedergegebene Rundfilterchromatogramm veranschaulicht. Es ist mit dem Gemisch Chloroform–Eisessig 1:1, wassergesättigt, entwickelt worden.

(2) Die analytisch besonders wichtigen Aglykone, Myricetin, Quercetin, und Kaempferol werden im Bereich 4–8 sehr gut getrennt. Die R_F-Werte zeigt Fig. 4.

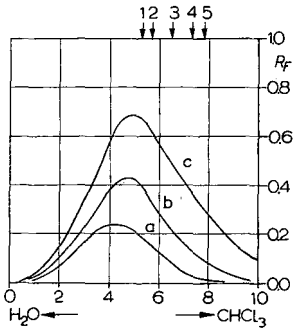

Fig. 4. Abhängigkeit der R_F-Werte der Aglykone Myricetin (a), Quercetin (b) und Kaempferol (c) von der Zusammensetzung des Fliessmittels. Erläuterungen vgl. Fig. 1 und 2.

Insbesondere um 6–7.5 ist die Bestimmung von Quercetin und Kaempferol wesentlich sicherer als bei Entwicklung in Butanol-Gemischen, wo die zu hohen Wanderungswerte und die häufige Überlagerung mit fluoreszierenden Begleitern strören[4]. Fig. 8 zeigt die Abtrennung des Kaempferols von dem sehr viel tiefer liegenden Quercetin, entwickelt mit dem Gemisch Chloroform–Eisessig 2:3, wassergesättigt.

(3) Überraschend ist das Verhalten der Methyläther, das die Fig. 5 und 6 zeigen. Im Bereich 5–10 bewirkt die Methoxylgruppe nicht, wie beim Partridge-Gemisch,

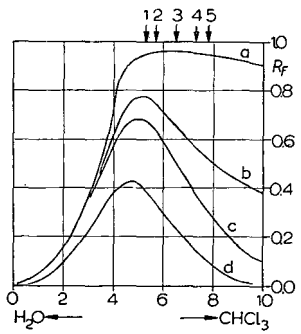

Fig. 5. Abhängigkeit der R_F-Werte der Methyläther Kaempferid (a) und Isorhamnetin (b) sowie der zugehörigen Hydroxylverbindungen Kaempferol (c) und Quercetin (d) von der Zusammensetzung des Fliessmittels. Erläuterungen vgl. Fig. 1 und 2.

eine geringe Senkung des R_F-Wertes, sondern erhöht diesen im Gegenteil so stark, dass der Methyläther im Chromatogramm höher zu liegen kommt als die um einen Substituenten ärmere Hydroxylverbindung. Das ergibt die Beziehung:

$$R_F(\mathrm{R} - \mathrm{OH}) < R_F(\mathrm{R} - \mathrm{H}) < R_F(\mathrm{R} - \mathrm{OCH_3}) \qquad (2)$$

die für Tri- bis Hexa-oxyflavone gültig ist. Im Partridge-Gemisch gilt dagegen:

$$R_F(\mathrm{R-OH}) < R_F(\mathrm{R-OCH_3}) < R_F(\mathrm{R-H}). \quad (3)$$

Das gestattet, Methyläther im zweidimensionalen Chromatogramm gut zu charakterisieren. Zusammen mit den korrespondierenden Hydroxylverbindungen der Beziehungen (2) und (3) entwickelt, verraten sie sich durch eine charakteristische Dreiecks-Anordnung der Flecke. Isorhamnetin und Rhamnetin lassen sich eindimensional neben Quercetin und Kaempferol sicher bestimmen (Fig. 8, Chromatogramm aufsteigend in $CHCl_3$–AcOH 2:3, wassergesättigt, entwickelt). Die Beziehung (2) ist auch für Glykoside von Methyläthern gültig.

(4) Morin und Datiscetin haben in der gesamten Folge auffallend hohe R_F-Werte, Fisetin, Robinetin, 3,6,7,8,4′-Pentahydroxyflavon sowie 3,6,7,8,3′,4′-Hexahydroxy-

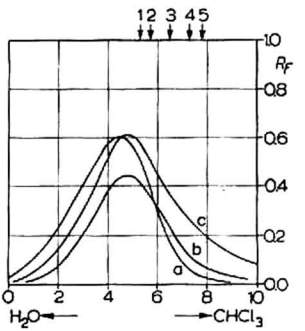

Fig. 6. Abhängigkeit der R_F-Werte der Aglykone Quercetagetin (a), Nortangeretin (b) und Patuletin (c) von der Zusammenzetzung des Fliessmittels. Erläuterungen vgl. Fig. 1 und 2.

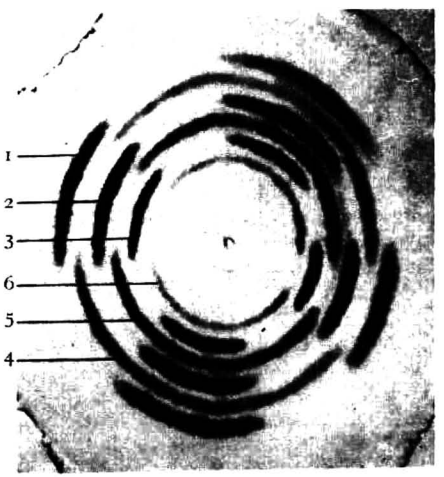

Fig. 7. Rundfilterchromatogramm, das die R_F-Beziehung (1) veranschaulicht, mit den Glykosiden: 1 = Afzelin, 2 = Quercitrin, 3 = Myricitrin, 4 = Astragalin, 5 = Hyperosid, 6 = unbekanntes Myricetin-3-hexosid. Fliessmittel $CHCl_3$–AcOH 1:1, wassergesättigt. Papier von Macherey, Nagel & Co., No. 263.

flavon wandern dagegen sehr langsam. Ihr R_F-Wert konvergiert mit steigender $CHCl_3$-Konzentration rasch gegen jenen der Verbindungen, die in 5-Stellung zusätzlich eine OH-Gruppe tragen. Galangin wandert geringfügig langsamer als Kaempferid. Robinin hat in der ganzen Folge höhere R_F-Werte als Rutin, die Werte konvergieren stetig mit steigendem $CHCl_3$-Gehalt der Gemische.

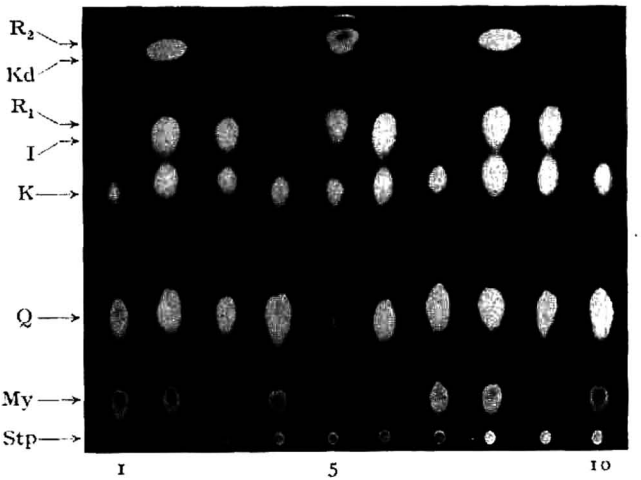

Fig. 8. Aufsteigend entwickeltes Chromatogramm mit Aglykonen. Über den Startpunkten 1–4 und 6–10 verschiedene Mischungen von: My = Myricetin, Q = Quercetin, K = Kaempferol, I = Isorhamnetin, Kd = Kaempferid. Laufspur 5 trägt ein natürliches Gemisch der Aglykone aus Rhamnusbeeren mit Rhamnetin (R_1) und Rhamnocitrin (R_2). Stp = Startlinie mit nachträglich markierten Startpunkten.

DANK

Herrn Prof. G. BARGELLINI, Rom, danke ich für die Überlassung von 3,6,7,8,4'-Pentahydroxyflavon, Herrn Prof. T. R. SESHADRI, New-Delhi, für die Zusendung von Nortangeretin. Herrn Prof. Dr. L. HÖRHAMMER, München, gilt mein besonderer Dank für die Überlassung von Astragalin, Kaempferol-3-rhamnoglukosid und Kaempferol-3-arabinosid. Meinem ehemaligen Institutskollegen Dr. W. ENSSLIN danke ich für Proben von Patuletin und Quercetagetin.

Die Untersuchungen sind durch Zuwendungen der Deutschen Forschungsgemeinschaft ermöglicht und gefördert worden, wofür an dieser Stelle gedankt sei.

ZUSAMMENFASSUNG

An einer Reihe von Flavonolen wurde die Abhängigkeit des R_F-Wertes von der Zusammensetzung des Lösungsmittels (System Chloroform–Eisessig–Wasser) gezeigt. Dabei ergibt sich: Gemische im Bereich von 25–70 % $CHCl_3$ sind zur Chromatographie von Flavonolen vorzüglich geeignet. Bei guter Trennschärfe liegen einige günstige R_F-Beziehungen vor, die gestatten:

(1) Kaempferol sehr sicher und getrennt von störenden Begleitern zu bestimmen,

(2) Methyläther glatt von zugrunde liegenden Hydroxylverbindungen zu trennen und sie—insbesondere im zweidimensionalen Chromatogramm—schnell als solche zu erkennen,

(3) eine sehr scharfe Trennung der Rhamnoside, Glukoside und Gemische beider von Myricetin, Quercetin und Kaempferol durchzuführen.

Besonders günstig sind Gemische von Chloroform und Eisessig im Verhältnis von 1:2 bis 2:1, jeweils wassergesättigt und einphasig. Es werden die R_F-Werte von 24 Flavonolen in 5 Gemischen dieses Bereichs gegeben.

SUMMARY

The dependence of the R_F value on the composition of the solvent system (chloroform–acetic acid–water) is demonstrated in the case of a number of flavonols. It was established that mixtures containing 25–70 % $CHCl_3$ are well suited for the chromatography of flavonols and that the R_F relations make it possible:

(1) to obtain kaempferol free from troublesome impurities,

(2) to separate methyl ethers clearly from the corresponding hydroxyl compounds and, especially in the case of two-dimensional chromatography, to rapidly recognize the methyl ethers as such, and

(3) to obtain a very sharp separation of the rhamnosides, glucosides and mixtures thereof from myricetin, quercetin and kaempferol.

Water-saturated and mono-phase mixtures of chloroform and acetic acid with ratios ranging from 1:2 to 2:1 are particularly favourable. The R_F values of 24 flavonols obtained with five mixtures within this range are given.

LITERATUR

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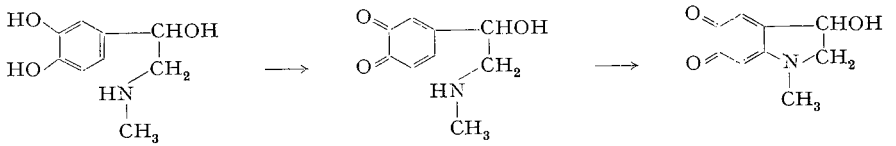
[4] H. REZNIK UND K. EGGER, *Z. Naturforsch.*, 15 b (1960) 247.

Short Communications

Detection of biogenic amines on paper chromatograms

The detection of adrenaline and noradrenaline on paper chromatograms can be carried out by the method proposed by JAMES[1,2], in which the chromatograms are sprayed with a 0.44% potassium ferricyanide solution in phosphate buffer of pH 7.8.

A red brown or light red spot is formed, due to the formation of an indole derivative, adrenochrome:



This reaction is given by other arylaliphatic amines that possess a secondary amino group in β position in the side chain, have no substituents in the *ortho* position and contain a catechol group.

By means of this reaction not only adrenaline but also several related amines can be detected on paper chromatograms as already reported by CRAWFORD[3].

GOLDENBERG *et al.*[4] observed that this reaction could be enhanced if the chromatograms were subsequently sprayed with a solution of ferric chloride. In this case the brown spots turn blue, the sensitivity becoming much higher and visualization easier.

This reaction does not depend on the formation of adrenochrome, but only on the ability of the various substances to reduce the potassium ferricyanide to ferrocyanide at room temperature and on paper. The ferrocyanide can then be easily detected with ferric salts, with which it forms an insoluble and deeply coloured precipitate (prussian blue).

We have observed that, owing to its great sensitivity, this second reaction can be used to detect a series of amines and amino acids of biological and pharmaceutical interest that cannot be revealed by the adrenochrome reaction, *viz.* 1-(3-hydroxyphenyl)-2-ethylamino-ethanol (Effortil), 1-(4-hydroxyphenyl)-2-methylamino-ethanol (Sympathol), tyramine, tyrosine, tryptamine, 5-hydroxytryptamine (Serotonin), tryptophan, 5-hydroxytryptophan.

Furthermore this reaction enables us to distinguish rapidly the amines that on oxidation yield products of the adrenochrome type (adrenaline, noradrenaline, isoprenaline(1-(3,4-dihydroxyphenyl)-2-isopropylamino-ethanol), 3-hydroxytyramine, 3-hydroxytyrosine (DOPA), etc.) from those that do not give such products.

We have found that the use of an unbuffered solution of potassium ferricyanide before the treatment with the ferric salt solution makes it possible to detect many amines that are not revealed with the ferric salt if the buffered spray reagent is used (Tables I and II).

TABLE I

REACTION OF AMINES WITH UNBUFFERED FERROCYANIDE FOLLOWED BY FERRIC CHLORIDE

Solvent: butanol–acetic acid–water (4:1:5); development time: 12 hours.

Substance	R_F	*Red colour with* $K_3Fe(CN)_6$ *0.5% in* H_2O	*Sensitivity* μg	*Blue colour with* $FeCl_3$ *0.5%*	*Sensitivity* μg
Adrenaline	0.36	+	2	+	0.2
Noradrenaline	0.29	+	5	+	0.1
Sympathol*	0.53	—		+	0.2
Effortil	0.72	—		+	0.2
Isoprenaline	0.58	+	0.3	+	0.1
Tyramine	0.63	—		+	0.5
3-Hydroxytyramine	0.39	+	1	+	0.1
Tyrosine*	0.35	—		+	0.3
3-Hydroxytyrosine	0.22	+	0.5	+	0.2
Tryptamine	0.73	—		+	0.1
5-Hydroxytryptamine	0.43	—		+	0.1
Tryptophan	0.41	—		+	0.3
5-Hydroxytryptophan	0.19	—		+	0.1

* After being dipped in $K_3Fe(CN)_6$, the chromatograms were dried in an oven or at room temperature.

TABLE II

REACTION OF AMINES WITH BUFFERED (pH 7.8) FERROCYANIDE FOLLOWED BY FERRIC CHLORIDE

Solvent: butanol–acetic acid–water (4:1:5); development time: 12 hours.

Substance	R_F	*Red colour with* $K_3Fe(CN)_6$ *0.44% in phosphate buffer pH 7.8*	*Sensitivity* μg	*Blue colour with* $FeCl_3$ *0.5%*	*Sensitivity* μg
Adrenaline	0.36	+	2	+	0.3
Noradrenaline	0.29	+	5	+	0.1
Sympathol	0.53	—		—	
Effortil	0.72	—		—	
Isoprenaline	0.58	+	0.3	+	0.1
Tyramine	0.63	—		—	
3-Hydroxytryptamine	0.39	+	1	+	0.3
Tyrosine	0.35	—		—	
3-Hydroxytyrosine	0.22	+	0.5	+	0.1
Tryptamine	0.73	—		—	
5-Hydroxytryptamine	0.43	—		+	0.2
Tryptophan	0.41	—		—	
5-Hydroxytryptophan	0.19	—		+	0.3

The sensitivity of the reaction was established by chromatographing solutions of each substance in the following concentration range: 5, 2, 1, 0.5, 0.3, 0.2, 0.1 μg per spot.

To carry out the reaction the chromatogram was dipped for a few seconds in a solution of potassium ferricyanide and after 10 minutes the sheet was rapidly

dipped in a solution of ferric chloride (0.5 %). Blue spots formed on a yellow background; the chromatograms are not stable due to the diffusion of the blue colour, which is less rapid if the sheets are dried before being dipped in ferric chloride solution.

The reaction is positive also with other substances, such as pyrocatechol, resorcinol, hydroquinone[5] and ascorbic acid, and may be used for detecting other reducing compounds.

Further applications of this reaction will be reported in due course.

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[1] W. O. JAMES, *Nature*, 161 (1948) 851.
[2] W. O. JAMES, N. KILBEY, *Nature*, 166 (1950) 67.
[3] T. B. B. CRAWFORD, *Biochem. J.*, 48 (1951) 203.
[4] M. GOLDENBERG, M. FABER, E. J. ALSTON AND E. C. CHARGAFF, *Science*, 109 (1949) 534.
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A sample-solvent evaporator for paper chromatography

In preparing a chromatogram before development in a suitable solvent system, one applies a liquid sample along a starting line, or as a spot, with a pipette held in one hand and with the tube containing the sample, as well as a source of air, usually a glass pipette connected to a compressed air line, for evaporating solvent as the sample is applied to the paper, held in the other hand. This procedure is unwieldy and offers several possible sources of difficulty. For one thing, there may occur excessive spread of the starting line band resulting in wide bands, or excessively spread spots, on the finished chromatograms making analysis difficult if not impossible. When one attempts to restrict the spread of the starting line band by applying the air jet to the paper immediately after applying the sample, there is danger that the air jet will cause the spray of sample from the tip of the application pipette to the paper. This results in annoying extraneous spotting of the chromatogram. At best, it is difficult to apply such liquid samples in a uniform manner and without excessive spread of the starting line band.

This report describes an apparatus which circumvents the difficulties described by allowing efficient continuous evaporation of the solvent contained in a sample which is applied to the starting line of various widths of chromatograms. This unit is presented in Fig. 1. It is made of a section of ¼ in. (O.D.) brass tubing plugged at one end and into which are drilled holes of 0.040 in. diameter, spaced ¼ in. apart. Sliding sleeves of ¼ in. (I.D.) permit any number of holes to be exposed depending upon the width of chromatogram to be processed. The open end of the tubing is connected to a compressed air line, or to a nitrogen line, and air is thus allowed to

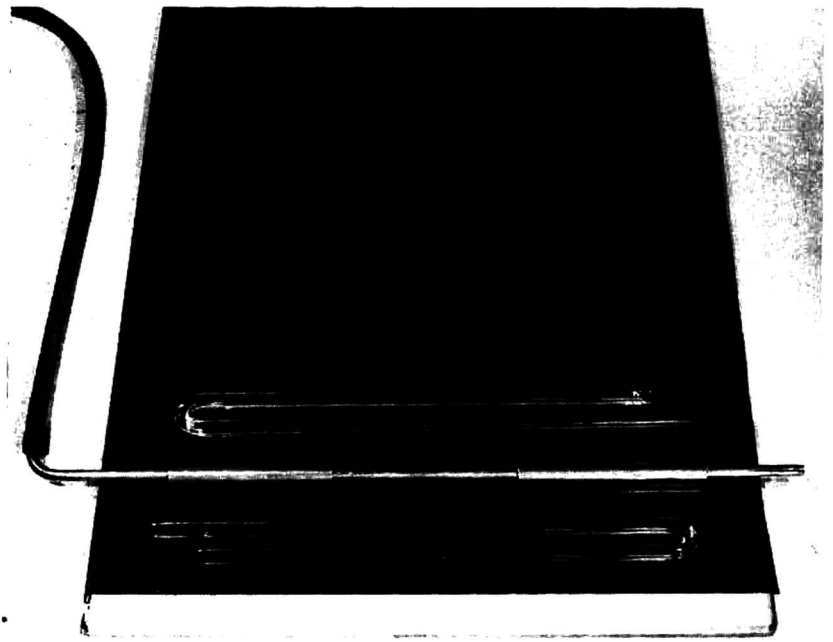

Fig. 1. Continuous sample-solvent evaporator.

pass through only those holes not covered by the sleeves.

The paper chromatogram upon which the sample is to be applied is placed across the glass rods so that the starting line is directly over, but not touching, the exposed holes of the unit. The sleeves are adjusted to accommodate the width of the chromatogram, the air stream is started, and the sample is applied directly to the paper. As the sample is applied, the stream of air coming from the unit beneath the paper, evaporates the solvent in the sample. This procedure allows the sample to be applied to the paper more rapidly and with less difficulty. At the same time the unit prevents excessive spread of solvent at the starting line.

This unit is economical and offers the advantages of simplicity in design, construction, and operation.

Acknowledgement

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A device for spraying paper chromatograms

Various methods have been tried in order to find appropriate and time-saving procedures for large series of paper chromatographic analyses. A variety of chromatographic solvent systems must often be used for the identification and recording of substances from plant extracts, metabolic products from living organisms, substances from culture media of microorganisms and when testing organic syntheses or when examining pharmacological preparations as to their quality and purity. In order to establish more reliable characteristics for the substances in question, it is in most cases necessary to use several chromatographic solvent systems, as well as a number of more or less selective colour reagents when spraying paper chromatograms. For this reason convenient methods had to be elaborated in order to evaluate a great number of paper chromatograms so as to be able to compare different series of substances investigated.

One method of solving the problem was described by the author[1], in which six paper chromatograms, 24 cm wide and 48 cm high, are sprayed simultaneously on a single frame. The spraying device has been developed further and now 20 series of six chromatograms each can be sprayed consecutively. When chromatograms of smaller dimensions than those mentioned above are used, even more than 120 chromatograms can be sprayed consecutively.

Fig. 1 gives an overall view of the spraying arrangement. As can be seen in the figure, the chromatograms are fastened with the aid of winged clips on glass frames that are fixed on a rotating band system. The glass frames (height 48 cm, width 144 cm) are moved, with foot control or manual control, upwards or downwards with the aid of a servo motor. The frames move in front of an illuminated screen (equipped with five Philips 65 W light-tubes of 1.5 m length). This gives an even illumination area for two frames at a time. A rack, which can be moved both horizontally and vertically, is placed at a constant distance from the frames. On the rack there are three sprayers for reagents. When the spraying is performed one frame is moved into a suitable working position. It is hereby preferable to use the foot control and thus leave the hands free to perform the spraying. The colour reagent is sprayed onto the chromatogram in a narrow line along the areas, where the samples have previously been applied. The device on which the sprayers are fixed is thereby moved up and down in front of the chromatograms at a constant distance from them. Spot chromatograms as well as line chromatograms are treated in this way.

For spraying, retouching air brushes, type II B Grapho are used, which are coated with gold on the inside and outside in order to minimize corrosion from the reagents. Sprayers operated with compressed air produce a thin spray cone through narrow slits. The slits are movable in front of and along with the sprayers and they also prevent the reagents from contaminating other areas than those actually being sprayed. On the glass frames the spots can be drawn directly on the chromatograms with a soft pencil. This is a great advantage, since very often a number of spots decolorize rapidly, and may gradually disappear. Another possibility is to spray

several different reagents adjacent to each other on different line chromatograms within the same frame. Furthermore, one can obtain large analytical series of chromatograms, developed in different solvent systems, by spraying these on several frames in the order desired.

The storing of chromatograms of earlier analyses—until now a considerable problem—has been solved in a satisfactory way, since 20 frames are now available.

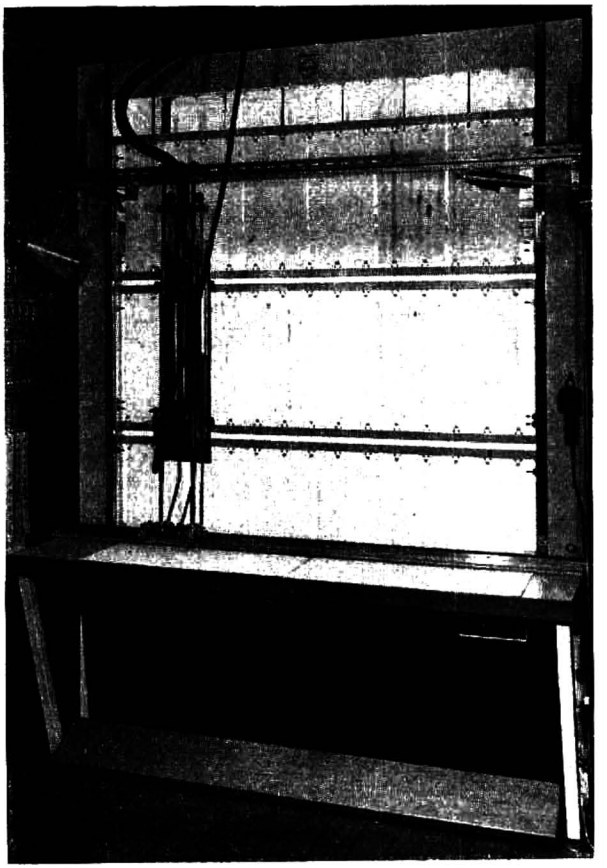

Fig. 1. Spraying arrangement. For further explanation, see text.

In order to save space, glass frames that are not in use are stored under the working table in front of the device. Chromatograms from substances analysed earlier that have been found useful for comparative purposes are left on their frames, which are moved upwards or downwards from the actual working position until the comparative studies have been terminated. Owing to the even illumination the coloured spots are well discernible, also those obtained with substances of low concentrations, and coloured slides from freshly sprayed chromatograms can easily be made for recording purposes.

Close to the movable spray-holder two R_F-meters made from elastic bands are fixed. These graduated bands can be adjusted by means of a screw, so that they follow the variations of the solvent front on the chromatogram. The R_F values of the single spots are read off by moving the spray-holder horizontally adjacent to the spots. On the glass frames there are several horizontally movable nylon threads. If required, one thread at a time can be adjusted to the appropriate R_F-level in order to obtain an R_F-network. This is very useful, provided that the solvent fronts on all chromatograms placed on one and the same frame have migrated the same distance.

Movable U.V.-lamps, placed in front of the glass frames allow the fluorescing spots to be studied in the darkroom before and after the spraying of the reagents.

In order to facilitate the exchange of the spraying solutions, a rack with flasks containing reagents is placed near the apparatus. These flasks, which have a capacity of *ca.* 15 ml, can be fixed directly on the sprayers.

A ventilation arrangement is connected with that part of the rack where the sprayers are placed.

The dimensions of the whole device are: height 255 cm, width 183 cm and depth 94 cm.

The device for spraying paper chromatograms has also proved to be a useful observation frame for radioautographic films, X-ray plates etc., without any rearrangement.

The author is greatly indebted to Svenska Aktiebolaget Philips, The X-ray Department, Stockholm, for placing their alternator at his disposal for this work.

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[1] L. REIO, *J. Chromatog.*, 1 (1958) 338.

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Water-cooled sample injection port for high temperature gas chromatography

In conventional gas chromatography instruments the liquid sample injection port is attached directly to the flash heater and both are at about the same temperature. When the flash heater is operated above 250°, ordinary rubber septums used in the sample injection port gradually decompose. This causes leaks in the carrier gas system and contamination problems in the chromatographic column. Further complications are encountered with capillary columns. Small fragments of decomposed septum material are swept into the carrier gas stream. These particles lodge at the entrance of the capillary column, restricting or even completely cutting off the flow

of the carrier gas. High temperature rubber or silicide septums have other undesirable features. Relatively thick material must be used, and the septums formed are inherently stiff. Often the hypodermic needle will bend or kink before it penetrates the septum.

To eliminate these difficulties, a water-cooled adapter for the liquid sample injection port has been developed. While the adapter was designed specifically for either the Barber-Colman Model 10 or Model 20 gas chromatograph, with minor modifications it can be used with most commercial gas chromatographic instruments.

The adapter consists of a threaded fitting that replaces the knurled knob furnished with the instrument (Fig. 1). The fitting is fabricated from a 1-in. diameter bar of stainless steel $1^3/_8$ in. long. A portion of the fitting is machined out and sealed with a sleeve. The machined portion is used as a water jacket; $^1/_4$-in. copper tubing is welded to the sleeve for the water inlet and outlet. A soft rubber septum is held

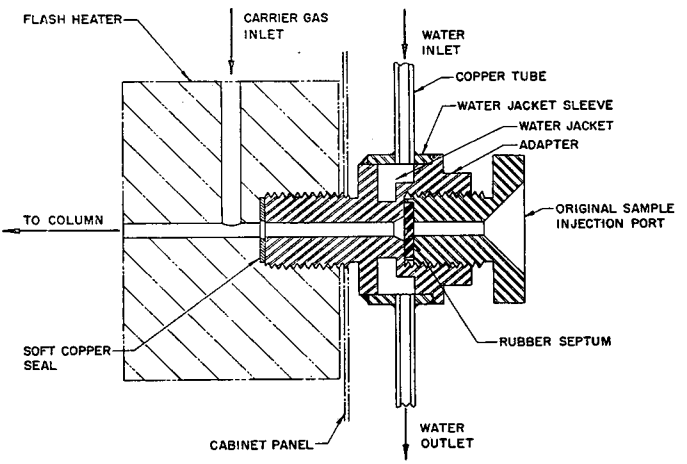

Fig. 1. Water-cooled adapter for sample injection port.

in place by threading the original knurled knob into the front end of the fitting. The effective overall increase in length of the liquid sample injection port is somewhat less than 1 in., so that Hamilton syringes with sealed needles reach well into the heated zone of the flash heater.

With the water-cooled adapter, a soft thin rubber septum can be used since it is located in the cool portion of the adapter. The flash heater may be operated at full heat without danger of disintegrating the rubber. A coolant water flow of 2 or 3 ml per min is sufficient to maintain the adapter at room temperature when the flash heater is heated to 350°.

The water-cooled adapter does not affect the retention times or the peak shapes of compounds having boiling points even in excess of 400°.

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Received August 3rd, 1960

Simple techniques for starch gel electrophoresis

Most published techniques for starch gel electrophoresis involve the use of somewhat complicated apparatus and techniques to provide forms for the gel and to prevent evaporation from and undue temperature changes in the gel during electrophoresis. The techniques described below have the advantage of simplicity while overcoming the difficulties already enumerated.

(1) A plexiglass frame (Fig. 1, C) is made in two identical U-shaped parts (Fig. 1 A and B and Fig. 2) which fit on top of each other. A strip of Whatman No. 1 filter paper is placed over the transverse bar at the base of the U (marked D. 1 in Fig. 1). The frame is slipped into a 250 mm length of 43 mm wide transparent dialysing tubing (Fig. 1, E.3). The dialysing tubing is then clamped transversely just outside the transverse bar of the plastic frame so that the greater part of the strip of filter paper projects from it (Fig. 1, F.1). With the dialysing tubing and plastic held between two glass plates, starch solution (13 % solution of Pfanstiehl hydrolysed starch in borate buffer at pH 8.5 or in other suitable buffers) in its sol form is poured in. When the level of the starch sol is close to the top of the frame, a strip of filter paper (similar to that at the lower end of the frame) is placed in position across the upper part of the frame (Fig. 1, D.2) so that a short length of it is covered with starch sol. The open end of the tubing is then clamped so that the greater part of

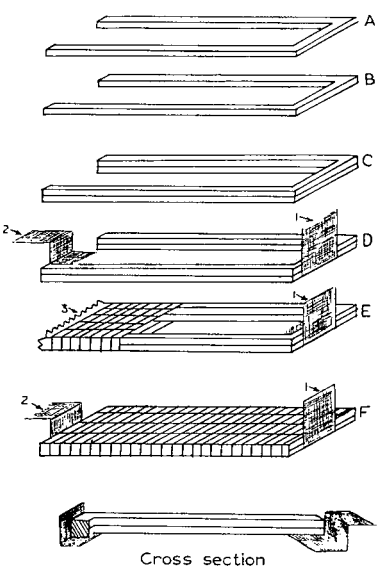

Fig. 1. Diagrammatic representation of the assembly of the apparatus.

this second strip of filter paper projects from it (Fig. 1, F.2). When the starch solution gels, the tube is laid on its side, the upper glass plate removed, a slit made to receive

the sample, the free ends of the filter paper dipped into the electrolyte solution carrying the electrical charge and the electrophoresis carried out in the usual manner.

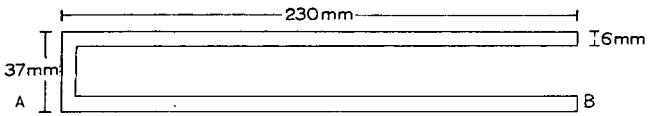

Fig. 2. Detail of the plastic form used in the apparatus.

Once the electrophoresis is complete, the dialysing membrane can be cut and peeled off. If desired, the starch gel can be slit lengthwise into two sections by inserting a razor blade between the two parts of the plastic frame—one part can then be stained to show the distribution of the proteins and the other saved for further investigations.

(2) Chromatographic tubes with ground-glass joints and an internal diameter of 10 mm can be used. The short end with the fritted disc (the inner member) is filled with starch sol and a length of Whatman No. 1 filter strip inserted into its open end. When the starch solution has gelled in this part of the column, it is fitted into the longer piece of the column (the packing column) which is filled with starch sol and a length of Whatman No. 1 filter strip inserted into its open end. After the starch solution has gelled in the longer column, the two pieces of the column are parted and the sample pipetted onto a small disc (7 mm diameter) of filter paper which is laid on the fritted disc so that, when the complete column is reassembled, the sample will lie directly against the starch gel column. The filter paper strips projecting from the ends of the assembled column are allowed to dip into the electrolyte solutions carrying the electrical charge and the electrophoretic operation is begun.

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Contact prints of starch gel electrophoresis patterns

The method of electrophoresis in starch gel first introduced by SMITHIES[1] in 1955 is now used extensively for many purposes. One problem associated with this technique is that of obtaining a satisfactory laboratory record of the separations

obtained. Photography of every gel is time-consuming and tedious, whilst drawing, even when accurate, is not satisfactory as the intensity of the bands is not easily reproduced.

SMITHIES[2] reports that gels may be rendered transparent by heating in glycerol or by dehydrating in benzyl alcohol, but in the latter solvent they become rigid and brittle. It has been found that by soaking the stained gels in ethyl alcohol followed by a mixture of benzyl alcohol and glycerol (2:1 v/v) a flexible, transparent gel is obtained. This can be pressed on to photographic paper and will give a satisfactory contact print.

The stained surface of the gel is placed in contact with the photographic paper in a pool of the benzyl alcohol–glycerol mixture. More of the mixture is poured on to the back of the gel and a piece of plate glass placed on it, avoiding air bubbles. It is necessary to have both faces of the gel in contact with the mixture to render it completely transparent. Extra hard contact paper is used and is developed in a contrast developer.

Whilst these contact prints are not suitable for reproduction, it is felt that this technique will enable others using starch gel electrophoresis to make rapid permanent records.

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[1] O. SMITHIES, *Biochem. J.*, 61 (1955) 629.
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NEW BOOK

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AN APPARATUS FOR LARGE SCALE PREPARATIVE CHROMATOGRAPHY WITH ESPECIAL APPLICATION TO THE SEPARATION OF A LONG CHAIN FLUORO-FATTY ACID

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In recent work[1] a new apparatus has been used for the separation of a long chain unsaturated fluoro-fatty acid by reversed phase chromatography.

It was developed when it was found that cylindrical columns of a diameter greater than 1 cm were unsuitable for the isolation of the fluoro-fatty acid from the seed fat of *Dichapetalum toxicarium*. The apparatus is described here because it is likely that wider uses could be found for it than that of the particular research for which it was constructed. The description given of its use is for the separation of the fluoro-fatty acid.

The essential feature of the apparatus was the utilization of a large narrow block of supporting medium impregnated with the stationary phase, and held together by plates of glass and "Tufnol", a fabric-based synthetic resin.*

THE APPARATUS

This consisted of a sheet of $^3/_8$ in. or ½ in. thick plate glass (not Triplex), 21¾ in. long by 11 in. wide, with a sheet of ¼ in. thick "Tufnol-carpbrand" of similar size separated by four glass or "Tufnol" strips 1½ in. wide and of a thickness varying from a ¼ in. to 1½ in. according to requirements, and bolted together by O BA chromium-plated brass bolts with grooved heads and wing nuts. Two strips were placed in a vertical position along the length of the plates and two shorter base strips were set at 45° angles to meet each other, forming an arrow with a $^1/_8$ in. gap through the point. Thus a narrow wedge-shaped vessel was constructed. In the tip was a perforated block of "Tufnol" covered by a piece of filter paper. This block was of the same thickness as the strips separating the main plates. Before assembly the surfaces of the "Tufnol" or glass strips were smeared liberally with a paste of 7 % w/v paraffin wax in liquid paraffin.

The threads of the brass bolts were covered by thin translucent rubber sleeves and between the wing nuts and the glass plates were placed, first a hard rubber

* The apparatus can be obtained as described from the Shandon Scientific Co. Ltd., 6 Cromwell Place, London, S.W. 7.

washer 1 in. in diameter with a $^5/_{16}$ in. hole, then a brass or duralumin washer of similar size.

It was important to tighten the nuts and bolts evenly so that they were just "finger-tight", this being sufficient to effect a seal between the plates and strips. The excess sealing paste was easily removed from the inside of the apparatus by rubbing untreated cellulose powder along the edges of the strips and removing it with a thin length of wood. All the nuts, bolts and washers were lightly smeared with the sealing paste.

The apparatus prepared for a descending separation is shown in Fig. 1, and detailed diagrammatically in Fig. 2.

Packing and loading the column for ascending chromatography

The column was packed with treated ashless "Whatman" cellulose powder (Standard grade) in amounts of about 50 g at a time and compressed tightly with a flat rectangular stainless steel plate at the end of a suitable handle. If the column was required to be 1 in. thick, about a kg of paper was required. Packing was continued to within 2 in. of the open end. There is a full description elsewhere of the preparation of the cellulose powder by treatment with 85% v/v acetic acid–water followed by impregnation with liquid paraffin[1].

To load a 1 in. thick column, 10 g of an oil fraction soluble in acetone to 1% w/v at —20° (as free fatty acids) were mixed with 25 ml of 0.1% Oil Red 4B (George Gurr Ltd.) in ether as a marker and to this were added 80 g of the treated cellulose. Oil Red 4B was chosen for the marker because it contains an orange coloured component which travels chromatographically to the same position as the fluoro-fatty acid. The whole was mixed together thoroughly with a pestle and mortar, and put on to the column as a compressed band. The remaining ½ in. of the column was then filled with treated cellulose. A piece of thick filter paper was wrapped over the end to prevent the cellulose from falling out and kept in position with cellulose adhesive tape. The column was developed at room temperature for 18 h with a solvent consisting of acetic acid, formic acid and water in the proportions of 2:2:1 by volume.

It was convenient to stand the apparatus on its open end in a large glass chromatography tank containing about 1 in. depth of the solvent. After development, a broad orange band could be seen half way up the column. This band contained the fluoro-fatty acid. Oleic and linoleic acids remained near the place of application in a bright red band.

The components were isolated by cutting out ½ in. or 1 in. wide sections of the pad and extracting these with 200 ml of 85% v/v acetic acid. The cellulose was separated on a sintered glass funnel and the acetic acid removed by distillation under reduced pressure ensuring that the temperature did not rise above 30°. Fatty acids were extracted from the residue by shaking with 10–20 ml of methanol which left any liquid paraffin undissolved. Demonstration of the fluoro compound in the clear solutions was achieved by paper chromatography already described[1]. Those fractions which contained only fluoro-fatty acid were pooled and further purified by

precipitation as the calcium salt[1]. Fractions containing traces of other fatty acids were pooled until several grams were available and refractionated on a fresh column.

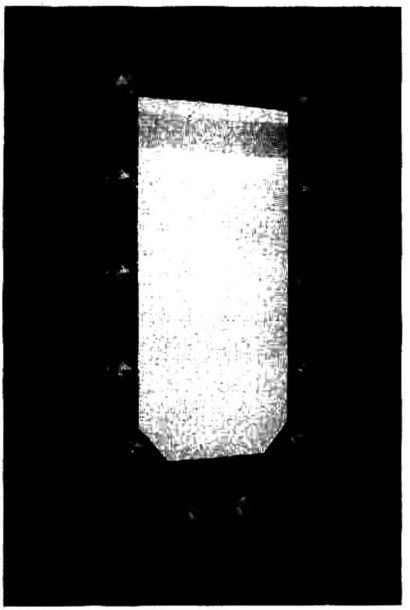

Fig. 1. The apparatus prepared for the separation of long chain fatty acids by descending technique.

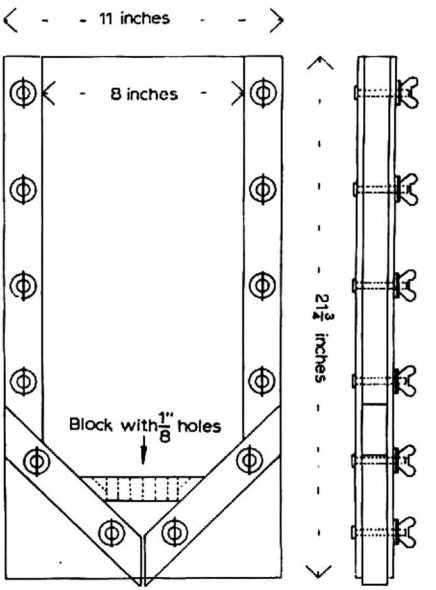

Fig. 2. Diagrammatic detail of the apparatus.

For descending chromatography

More discrete separations were obtained by descending technique which had the further advantage of being quicker. This mode of use, however, necessitated constant attention to the apparatus throughout the developing period. The apparatus was packed with the treated cellulose to within 4 in. of the end. The oil, mixed with dye and cellulose, was applied as for ascending development and then covered with 1 in. or so of packed treated cellulose. The apparatus was supported on blocks and held by clamps fitted to a retort stand on each side of it. The developing reagent was supplied from two 500 ml stoppered separating funnels having 8 mm delivery tube stems with 5 mm holes in the stop-cocks. The funnels, with their stoppers inserted, are fixed with their stems just below the surface of a 1 in. layer of solvent on top of the cellulose. When the level of the solvent fell below the tip of the stems it was automatically replaced from the reservoirs.

The first eluate usually came through after approximately 2 h at the rate of about 5 ml/min. Successive eluates varying from 20 ml to 200 ml were collected from which the solvent was removed by distillation under vacuum. Latterly, it was found adequate to extract the eluates with small volumes of light petroleum (boiling range 40–60°). The long chain fatty acids and some dye went into the petroleum leaving a large amount of dye in the acid solvent. The petroleum could be evaporated off with a

stream of compressed air in a fume cupboard, and the oil subsequently redissolved in methanol to remove traces of liquid paraffin.

DISCUSSION

Techniques of reversed phase column chromatography for long chain fatty acids have been described by HOWARD AND MARTIN[2] and CROMBIE, COMBER AND BOATMAN[3], separating the components of no more than 60 mg of mixed acids.

The present apparatus is described for the separation of 10 g of mixed fatty acids on a 1 in. thick column, and it has been successfully used as a $1^5/_8$ in. thick column when 15 g of oil were put on. There seems no reason to suppose that the overall dimensions of the apparatus could not be varied, but larger models might require thicker glass front plates. The high concentrations of acetic and formic acids had no effect upon the "Tufnol" but other plastics such as "Perspex" were softened after a short time. Experience has shown that whilst the chromium-plated wing nuts and bolts resisted corrosion by the vapours of acetic and formic acids for a long time, it would probably be better for them to be made of stainless steel. Small cylindrical glass columns still proved useful for the isolation of a small quantity of material, but, when large cylindrical columns were tried, components tended to separate initially and then run into each other during elution in the form of a series of concentric cones.

BÉKÉSY[4] observed the same effect during studies on the chromatographic separation of plant extracts with circular columns, and concluded that a larger surface area was required to overcome the problem. He constructed a very small apparatus consisting of lengths of thin sheet glass held only 5 mm apart by cork strips, the whole assembly being maintained by clamps. Although BÉKÉSY's apparatus was very different from that described in this paper, the principles and reasons involved in its design were similar. Such an apparatus, however, could not be constructed on a larger scale. In this laboratory two attempts to make the large apparatus described in this paper entirely of ¼ in. plate glass were unsuccessful; the glass plates rapidly developed lateral cracks. By using a "Tufnol" sheet in conjunction with plate glass this problem was overcome. It is not clear why a block of supporting medium should function so well and not a large round column of it. It is possible that more uniform packing is achieved with a narrow rectangular column.

Experiments in this laboratory with cellulose powder impregnated with silicone MS200/16CS, the reagent employed by SCHLENK *et al.*[5] with filter paper sheets, showed that this system could give a more satisfactory separation of the long chain fluoro-fatty acids from oleic, linoleic and linolenic acids than did silane-treated kieselguhr[2]. Partition was effected with 85 % v/v acetic acid. The same reagents were employed with Whatman No. 531 filter paper sheets for the identification of the fluoro-acid. Later work showed that better results could be obtained by employing liquid paraffin instead of silicone and for the preparative technique, substituting the acetic–formic acid mixture for 85 % acetic acid. If, however, 85 % acetic acid was employed as the

developing solvent, complete separation of oleic, linoleic, linolenic, ricinoleic and fluoro-oleic acids was achieved, and these were collected chromatographically pure. By this method about 2 g of chromatographically pure fluoro-oleic acid were prepared as well as large quantities of oleic acid.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the constant encouragement given by Sir RUDOLPH PETERS F.R.S.

I should like to thank Mr. D. V. BARKER and Mr. C. C. INGREY of the Instrument Workshop for their help in the construction of the apparatus and Mr. D. HARBIDGE for valuable technical assistance.

SUMMARY

An apparatus is described for the separation and purification by reversed phase chromatography on cellulose powder treated with liquid paraffin, of 10 g or more of mixed long chain fatty acids. Its application to the isolation of a long chain unsaturated fluoro-fatty acid is described and it is suggested that the apparatus may find wider uses in other preparative chromatographic separations.

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AN IMPROVED TECHNIQUE FOR RECORDING CHROMATOGRAMS ANALYSIS OF CRESOL MIXTURES

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(Received May 20th, 1960)

In the course of an investigation of new counter current procedures, a rapid method suitable for series analyses of cresol mixtures was required. Since *o*- and *m*-cresol cannot be determined by U.V. spectrometry, because their spectra are too similar, a chromatographic analysis method was developed, which was an improvement of already known procedures[1–3].

Composition of the column

The celite column method described by WHITE AND VAUGHAN[2] gives excellent separation, but the elution takes too long (one hour) for our purpose. Increasing the flow rate by applying pressure finally reduced both the degree of separation and the lifetime of the column. This disadvantage of celite does not occur with silicagel (0.08–0.1 mm) and a phosphate buffer as stationary phase. A narrow grade in particle size is important for high column efficiency.

10 g of silicagel are suspended in about 50 ml of buffer (0.06 *M* sec. phosphate and 0.14 *M* tert. phosphate for cresol mixtures), and boiled a few minutes with rapid stirring. The suspension is allowed to cool, the bulk of supernatant buffer decanted to eliminate the fines and the gel is collected on a suction filter. The silicagel is again suspended in 50 ml of fresh buffer and the described procedure is repeated twice more. Finally the silicagel is collected on a fritted glass filter which can be placed in a centrifuge. The centrifuge is run at 1500 r.p.m. to remove the excess of buffer. The silicagel is then stirred into a slurry in 2,2,4-trimethylpentane and packed in a column (9 mm inner diam.) according to the method of MARTIN[3].

Column and flow-cell

The recording chromatographic procedure developed in our laboratory[1] was improved. The new sample introduction assembly is shown in Fig. 1.

The introduction of the samples (0.01–1 mg of the mixture in 0.1–1 ml solvent) is carried out as in gas–liquid chromatography with a hypodermic syringe through a rubber capsule without interrupting the flow of eluant (Fig. 1). The purpose of tube C (7 × 2 cm) filled with silicagel impregnated with stationary phase is to equilibrate the eluting solvent with the buffer phase before it reaches the separating column.

This device increases the lifetime of the column and acts as a buffer against pressure changes when samples are injected.

To introduce the sample in a minimum volume, stopcock A is closed, some air is injected and when the eluant-level just reaches the top of the silicagel column the sample is injected. The capillary tube B is rinsed by injecting fresh solvent (about 0.2 ml) and stopcock A is opened when the level again reaches the silicagel surface. The excess of pressure is automatically released through stopcock A. All these actions must be carried out rapidly since suction of air into the silicagel column must be avoided.

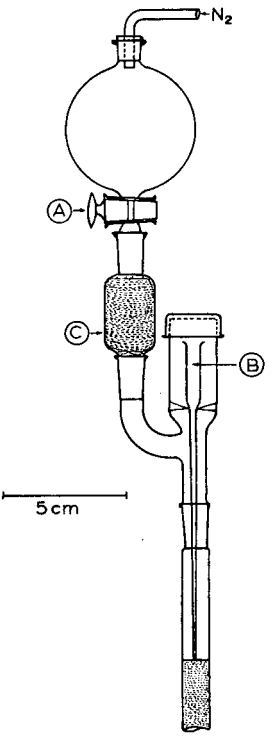

Fig. 1. Sample introduction. See text.

For series work the eluant is maintained at a level of 1-2 cm above the packing and the sample is injected as a "plug" without interrupting the flow of eluant. The eluant-level can be adjusted by pressing or sucking air with a syringe through the rubber capsule. The injection of sample and rinsing can be done simultaneously by carefully filling up the syringe first with fresh eluant and then with the sample. The required dexterity can be obtained by repeating all these actions with a coloured solution.

By using coloured solutions we could see that material could escape detection in the flow-cell, unless all parts of the cell are accessible to the light beam during the photometric measurement. To this end the cell is made as small as possible, the

quartz windows being actually somewhat smaller than the light beam of the photometer in the U.V. region. This does not affect the results if light scattering is avoided by blackening all cell parts, except of course the windows. The improved flow-cell is shown in Fig. 2. The quartz windows are fixed with an appropriate glue. For the solvent 2,2,4-trimethylpentane, a polyepoxyester (Stabilit) is satisfactory. The connec-

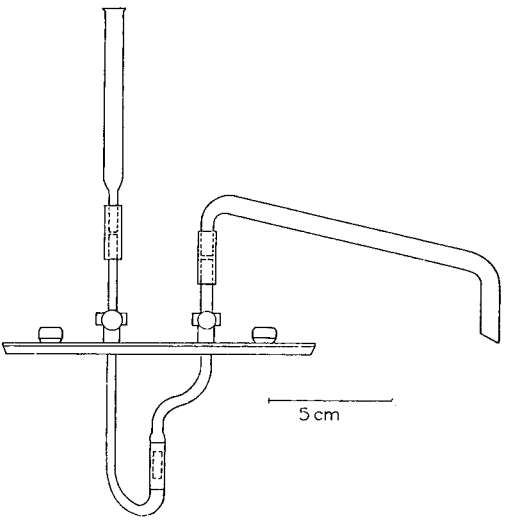

Fig. 2. Flow-cell showing introducing funnel, perforated photometer box lid, quartz windows etc.

tion tubes as shown are made of polyvinyl chloride. After a very short time all extractable material interfering with the light absorption is eliminated and no difficulties arise from the use of this connecting material.

Recording of the chromatograms

A Beckman D.U. spectrophotometer, mains fed by a Beckman Power Supply unit and provided with an energy-recording adapter (ERA 5800) and a 10 mV graphic recorder (G. 10 Varian Associates California), was used[1].

Transmission is recorded and the band surface is not proportional to the amount of material. However, the maximum extinction of each peak can be found by conversion of the transmission-extinction as found on the scale of the spectrometer. The relative concentrations can then be calculated by a modified triangle method, the base of the triangle being multiplied by the maximum extinction instead of the height of the triangle.

Of course the most reliable method consists in collecting each separated fraction with the recorder as a guide, and measuring its volume and extinction.

When the chromatography is run under a pressure of 50 cm water, the flow rate is about 150 ml/h for a column of 8 cm length. *o*- and *m*-Cresol mixtures are then separated in 10 to 20 minutes time (Fig. 3). With flow speeds of 300 ml/h no appreciable difference is noted in the chromatogram. The three cresols can be separated on a

column of 12 cm length (Fig. 4). These columns are suitable for up to fifty chromatograms.

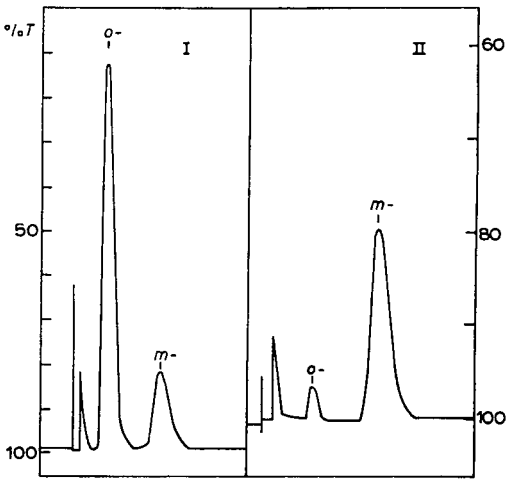

Fig. 3. Recording of the chromatographic separation of *o*- and *m*-cresol. Column length 8 cm, wave-length 276 mμ. The sample in II is about ten times smaller than in I. For other details see text.

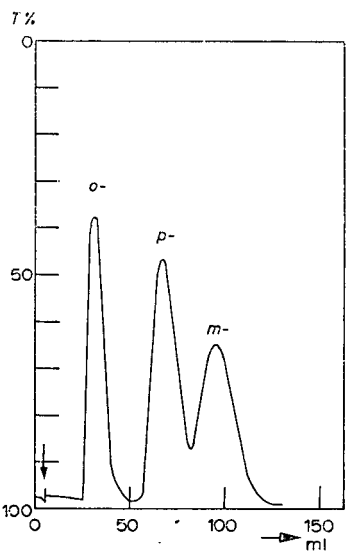

Fig. 4. Recording of the chromatographic separation of *o*-, *m*- and *p*-cresol (0.5 mg of each). Column length 12 cm, wave-length 276 mμ.

The sensitivity of the method can be increased by adjusting the amplification of the phototube-current until about 30 mV difference between "dark-current" and "open-slit" is obtained. In this way the recorder-pen goes off scale at a transmission of about 70 and relatively weak signals can be detected (Fig. 3 II).

As can be calculated from Fig. 3 the HETP for these columns is around 1 mm[4].

SUMMARY

A new device for the introduction of samples without interruption of the eluant flow in liquid chromatography is described. This, together with the described continuous U.V. recording technique of the outflow, makes rapid series analysis by partition chromatography as convenient as in gas chromatography.

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METHODOLOGICAL ASPECTS OF GEL FILTRATION WITH SPECIAL REFERENCE TO DESALTING OPERATIONS

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The ability of porous bodies to distinguish between molecules of different sizes has been repeatedly observed. The regular structure of zeolites results in an abrupt change in adsorptive properties at a definite molecular size[1]. This has been utilized to obtain separations among gases and organic substances with a small number of atoms. The porous structure of the synthetic ion-exchange resins is due to the swollen three-dimensional network in which the size of the meshes determines the porosity. According to the nature of the synthetic procedure they vary in size and their structure is much less defined than in the crystalline zeolites. Nevertheless they are quite useful for separations according to size as has been shown by WHEATON AND BAUMAN[2]. The molecular sieve effect has been utilized to increase the selectivity in ion-exchange operations, *e.g.* in the separation of cellulose xanthate from small ions[3], to separate polygalacturonic acid from galacturonic acid[4] and to fractionate peptides of different sizes[5].

Networks of polar character and devoid of charged groups do not exchange or exclude ions and thus solutes behave in a similar manner no matter if they are charged or not. In columns packed with swollen starch LINDQVIST AND STORGÅRDS[6] separated peptides of different sizes and LATHE AND RUTHVEN[7] demonstrated that for a large number of solutes the elution volume varied with the size of the solute.

The use of particular dextran gels for separations according to size was introduced by PORATH AND FLODIN[8]. The method was named gel filtration as suggested by TISELIUS. A study of the conditions for separation of amino acids, peptides and proteins has been published by PORATH[9]. Two gels with different degrees of cross-linkage were compared. This was also done by FLODIN AND GRANATH[10] with fractions of dextran as solutes. As expected the range of separation increased with decreasing degree of cross-linkage. A fractionation of enzymes from snake venoms has been made with the aid of highly swelling gels[11]. The purification of pepsin on a preparative scale[12] and the fractionation of extracts from pituitary posterior lobes[13] have been described. By using dextran gels of small particle size it was possible to separate molecular species that differed only slightly in size, as was shown for cellodextrins, where the oligosaccharides up to cellohexaose appeared as well-separated components[14]. Effects due to adsorption onto the gel matrix have sometimes been observed. A thorough study of a large number of solutes[15] revealed certain regularities.

With distilled water as eluant the behaviour of many solutes was irregular, but as soon as salt was present some of these disappeared. Some solutes of aromatic character displayed adsorption effects independent of the presence of salt.

The purpose of this paper is to study some factors influencing the removal of salts from colloids, notably proteins, by gel filtration. The conditions are chosen so as to give a hint as to the optimal conditions for transferring a protein to a new salt medium. This type of operation must often be used prior to electrophoresis or ion-exchange chromatography.

EXPERIMENTAL

Materials

The dextran gel used was Sephadex G-25 (Pharmacia, Uppsala, Sweden) lots number 125 and 161. Their water regain was 2.3 g of water per g of dry material. The particle size was 50–270 mesh on the U.S. Standard screen series, as measured by analysis of the dry material. In most experiments fractions with a narrower size distribution were used. They were obtained by dry screening.

The haemoglobin was prepared by haemolysis of washed bovine erythrocytes and lyophilization. It contained an appreciable amount of methaemoglobin. Carbon-monoxy-haemoglobin was prepared from bovine erythrocytes according to the method of PRINS[16]. All other chemicals were *pro analysi* or of a comparable grade of purity.

Methods

Preparation of gels. The dry Sephadex powder was suspended in tap water and stirred for a few minutes to allow it to swell. After a sedimentation time of ½ to 1 hour the fines remaining in the supernatant were removed by decantation. The procedure was repeated at least five times. The volume of water was chosen so that the ratio supernatant to sediment was at least 10:1.

Packing. The columns used were cylindrical glass tubes of 2 and 4 cm diameter and 40 to 100 cm in length. At the bottom they were joined to a 5 cm long capillary with a 1 mm bore.

The procedure used was essentially that described by FLODIN AND KUPKE[17] for cellulose columns.

Before packing, the column was mounted vertically and filled with tap water at room temperature. A small piece of glass wool was laid over the outlet capillary, and above it a 2 cm layer of glass beads (diameter 0.5 mm). The top of the column tube was connected to a 1 l funnel through a 100 cm long glass tube with half the column diameter and inserted through a rubber stopper. The arrangement is shown in Fig. 1. The system was then filled with water up to the funnel. Care was taken that no air bubbles were present in the columns.

The water-swollen Sephadex was then added to the funnel and agitated with a motor-driven stirrer during the packing procedure. The bottom outlet from the column was opened to allow a flow rate of 5 to 20 ml per minute. When the suspended particles reached the bottom of the column the flow was stopped for a long enough time for a 1 to 2 cm high bed to be formed, whereupon the flow was started again.

A rising horizontal boundary of packed material was considered evidence of good packing. To compensate for the increasing resistance to flow with proceeding packing the outlet tube was progressively opened wider. After all the material had been packed, the funnel and connecting tube were removed and a filter paper with a

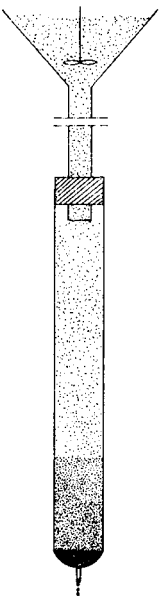

Fig. 1. Arrangement for packing of the columns.

diameter slightly smaller than the bore of the column tube was placed on the horizontal even surface of the bed. To let the bed stabilize it was percolated for at least a couple of hours, preferably overnight, with the eluant to be used.

Application of the sample. Most of the liquid over the bed was removed and the last few ml were allowed to pass into it. At the moment when the surface was about to dry out the sample was carefully added dropwise from a pipette. Then the flow was started and the sample allowed to enter the bed. At the moment it disappeared a few ml of the eluant were added to wash the surface. Finally, the space above the bed was filled with eluant.

Elution. The top end of the column tube was closed by a rubber stopper through which the eluant was fed. When low viscosity samples were run, a constant flow rate was obtained by means of a Mariotte flask. A constant-feeding pump (Sigma-motor) was used when the viscosity of the samples was high or when a more precise control of flow rate was necessary. The eluates were collected in a fraction collector. In most experiments a time-regulated collector (Stålprodukter, Uppsala, Sweden) was used. In the study of the influence of particle size and flow rate a volume-regulated collector was used (Radi Rac, LKB-Produkter, Stockholm, Sweden). All experiments were made at room temperature.

Control of packing. The homogeneity of packing was checked by passing a zone of haemoglobin through the bed. The column was repacked if the zone became skew during the passage. All experiments with haemoglobin were made with an eluant containing salt to avoid adsorption of denatured protein. In the cases where no electrolytes were present in the eluant the column was checked by passing a zone of dilute india ink as described by LATHE AND RUTHVEN[7].

To determine the void volume the elution curve for a narrow zone of haemoglobin was taken. Since haemoglobin is completely excluded from the gel particles the position of the maximum of the curve represented the void volume. A quantitative check of the column performance was sometimes made by eluting a sample the volume of which was 10% of the bed volume. It was considered satisfactory when dilution during the passage was less than twofold.

Preservation of the beds. To avoid microbial growth the buffers were stored over chloroform.

Analytical methods. The ultraviolet light absorbing substances were analysed in a Unicam SP 500 spectrophotometer. The chloride ion determinations were made by the Volhard titration procedure. The conductivity measurements were made with a Conductolyzer (LKB-Produkter, Stockholm, Sweden).

RESULTS

Flow rate and particle size

To investigate the influence of flow rate and particle size on the efficiency of a column, experiments were made with uridylic and hydrochloric acids. They are retained to the same degree by the column, but differ in molecular weight and thus their diffusion coefficients are different. In order to make the experiments strictly comparable they were performed in columns with as nearly equal dimensions as possible and with narrow sieve fractions from the same batch of dextran gel. The dimensions of the columns and the sieve fractions are given in Table I. The volume of the sample was 3 ml throughout and the concentrations were 0.358 and 237 mg per ml for uridylic acid and hydrochloric acid, respectively. Fractions of 2 ml were taken and analysed by U.V.-absorption at 260 mμ and by titration with sodium hydroxide, respectively.

TABLE I

COLUMN DIMENSIONS

Column No.	*Sieve fraction mesh*	*Test substance*	*Column dimensions cm × cm*
1.	50– 80	uridylic acid	2 × 65
2.	140–200	uridylic acid	2 × 65
3.	270–400	uridylic acid	2 × 67
4.	50– 80	hydrochloric acid	2 × 67
5.	270–400	hydrochloric acid	2 × 70.5

From the elution curves the number of theoretical plates was calculated according to GLUECKAUF[18]. For curves having the shape of gaussian error curves the plate number is

$$N = 8\left(\frac{V_{max}}{\varepsilon}\right)^2$$

where ε is the band width at the height c_{max}/e and V_{max} is the elution volume for the maximum concentration (c_{max}). The equivalent height per theoretical plate (EHTP) was obtained by dividing the height of the column by the number of theoretical plates. In Tables II and III the values obtained under varying experimental conditions are given.

TABLE II

EHTP VALUES IN mm FOR URIDYLIC ACID

v ml/h	*Sieve fraction, mesh*		
	50–80 (column 1)	*140–200 (column 2)*	*270–400 (column 3)*
10	0.39	0.102	0.15
24	0.72	0.182	0.11
51	1.49	0.21	
90	2.62		
130	4.14		
190	5.49		

In Fig. 2A are shown three of the elution curves obtained with uridylic acid in column 1 when the flow rate was varied. At a rate of 24 ml per hour in columns 1 to 3 the curves given in Fig. 2B were obtained.

For uridylic acid the EHTP in column 1 was proportional to the rate of flow. With hydrochloric acid the increase with flow rate was small and the efficiency was practically constant within the range investigated. Higher values for the EHTP were obtained at the lowest flow rates indicating longitudinal diffusion or convection. Decreasing particle size decreased the EHTP for both solutes. The values in Tables II

TABLE III

EHTP VALUES IN mm FOR HYDROCHLORIC ACID

v ml/h	*Sieve fraction, mesh*	
	50–80 (column 4)	*270–400 (column 5)*
14	1.05	0.23
24	0.69	0.26
48	0.44	
70	0.45	
96	0.51	

and III show that the most efficient way to increase the performance of a column is to decrease the particle size of the dextran gel.

A few experiments with smaller sample volumes were made in column 3 in order to measure the accuracy of the EHTP. However, the observed changes in the EHTP were small.

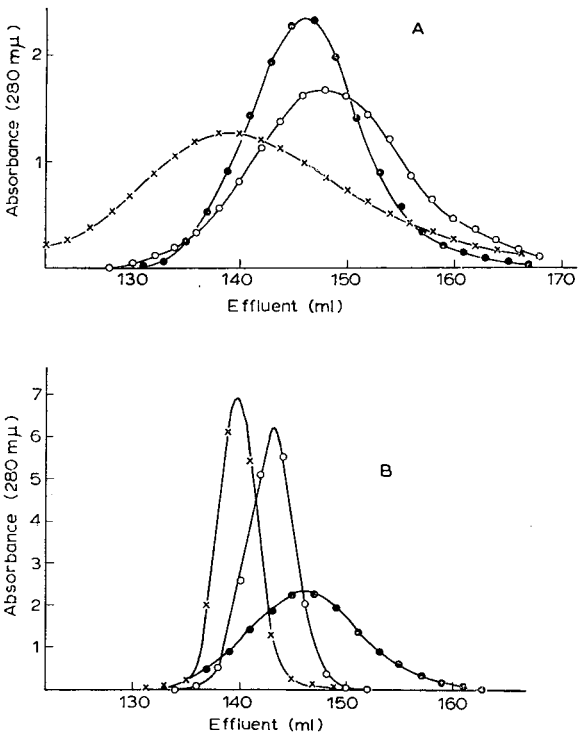

Fig. 2. Elution curves for uridylic acid. (A) From a column packed with the 50–80 mesh fraction and the elution rates 24 ml (●—●—●), 90 ml (o—o—o), and 190 ml (×—×—×) per h. (B) At a constant elution rate of 24 ml/h from columns packed with the 50–80 mesh (●—●—●), 140–200 mesh (o—o—o), and 270–400 mesh (×—×—×) sieve fraction.

Viscosity

In a column with an original height of 85 cm and 4 cm in diameter a series of experiments was made in order to evaluate the influence of the viscosity of the sample on the efficiency of the column. The 100 ml samples contained 0.1 g haemoglobin and 1 g of sodium chloride and varying amounts of a dextran fraction with limiting viscosity number (η) equal to 0.68 (M_w = 1,800,000). In Table IV the compositions of the solutions and their viscosities are shown. The eluant was 0.1 M phosphate buffer of pH 7.0. The bed was eluted at a rate of 180 ml per hour. The elution curves for haemoglobin and sodium chloride were measured and can be seen in Fig. 3A, B and C. The dextran distribution is closely similar to that of the haemoglobin.

In experiment 1 the peaks were very unsymmetrical and there was considerable overlapping. With lower sample viscosity the peaks became more symmetrical

TABLE IV

DEXTRAN CONCENTRATION AND VISCOSITY OF SAMPLES

Experiment No.	*Dextran %*	*$\eta_{rel.}$ centipoise*
1.	5	11.8
2.	2.5	4.2
3.	1	2.0
4.	0,	1.0

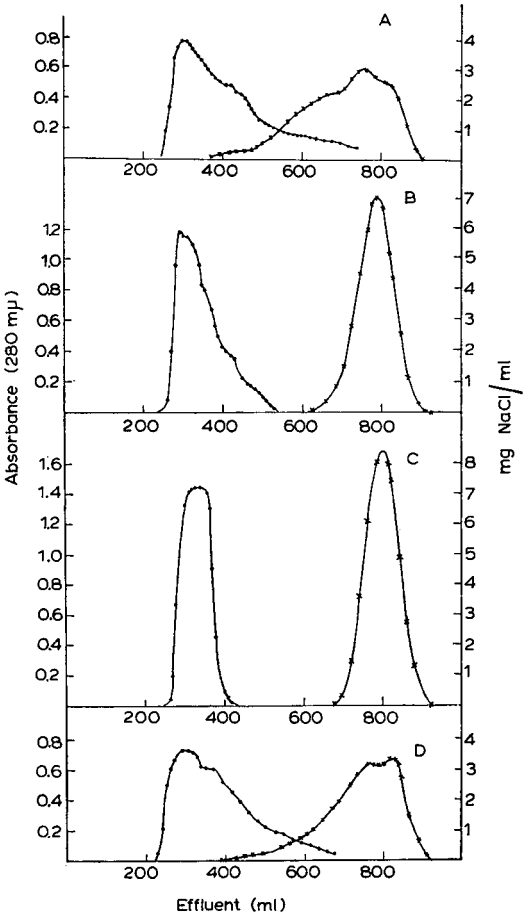

Fig. 3. Influence of viscosity. Elution curves for haemoglobin (o—o—o) and sodium chloride (×—×—×) in the presence of varying amounts of dextran. At an elution rate of 180 ml/h with (A) 5% dextran, (B) 2.5% dextran, and (C) without dextran. (D) 5% dextran and an elution rate of 36 ml/h.

(Fig. 3B) and in the absence of dextran (Fig. 3C) they were almost perfectly symmetrical. An interesting feature is that the fast component has a sharp front and a diffuse rear, while the opposite is true for the slow one. A partial explanation for this is that the bed was compressed while the viscous samples were in the column.

An experiment was made with the most viscous solution ($\eta_{rel.}$ = 11.8 cp) and a flow rate of only 31 ml per hour. From Fig. 3D it is evident that the resolution was somewhat improved, although the difference was small. Reducing the viscosity was much more efficient.

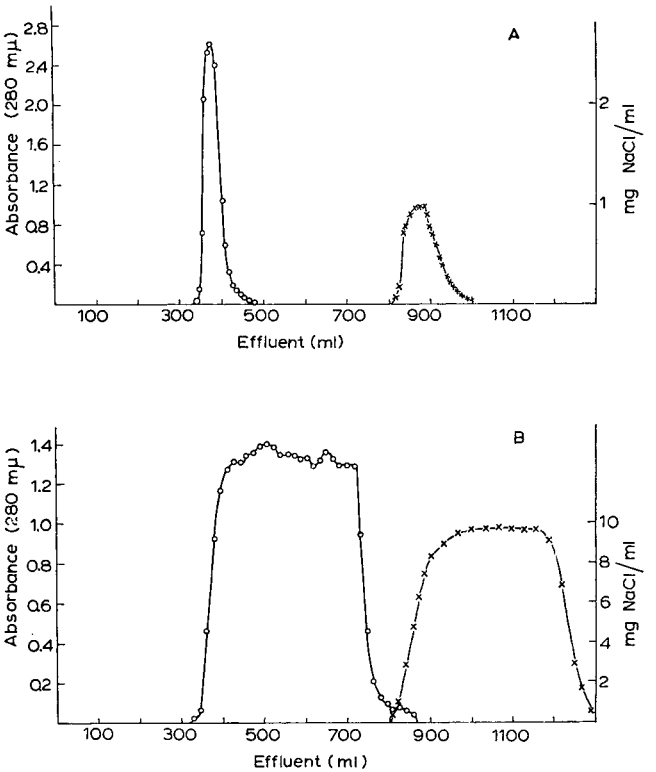

Fig. 4. Elution curves for haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 240 ml/h. (A) Sample volume 10 ml. (B) Sample volume 400 ml.

Sample volume

In Figs. 4A and B the elution curves for two experiments are shown, in which the volume of the sample differed by a factor of forty. They were performed in a 4 cm × 85 cm bed (volume = 1070 ml) packed with 100–200 mesh dextran gel. In the first experiment 10 ml of a solution containing haemoglobin and sodium chloride (100 mg of each) were applied and in the second one a 400 ml sample containing 400 mg haemoglobin and 4 g sodium chloride. In both experiments the haemoglobin appeared in the effluent at the same breakthrough volume. A complete separation

was not obtained in the second experiment although about 99 % of the haemoglobin was free from salt. It is of interest to compare the dilution of the protein in the experiments. In the first one it was diluted 10 times and in the second 1.25 times. In the second experiment the salt was diluted 1.35 times.

Buffer concentration

The influence of the amount of buffering salts present in the eluant was studied with samples of the same concentration of CO-haemoglobin. The sample volume was kept to approximately 10 % of the bed volume. The experimental conditions are given in Table V. The columns were packed with the 100–200 mesh sieve fraction.

TABLE V

EXPERIMENTAL CONDITIONS

Experiment No.	*Column dimensions cm × cm*	*Eluant*	*Sample volume ml*
1.	4 × 36	0.1 *M* phosphate, pH 5.9	50
2.	4 × 36	0.05 *M* phosphate, pH 6.0	50
3.	4 × 75	0.01 *M* phosphate, pH 6.2	100

The samples were prepared by diluting a 10 % stock solution of CO-haemoglobin containing sodium chloride with an equal volume of the eluant in experiments 1 and 3 and with an equal volume of water in experiment 2. The elution rate was 120 ml per hour and the effluent collected in 15 ml fractions. The protein and chloride ion concentrations were measured in each fraction and the pH and conductivity in every second one. Experiments 1 and 2 gave similar results and in Fig. 5 experiment 2 is illustrated.

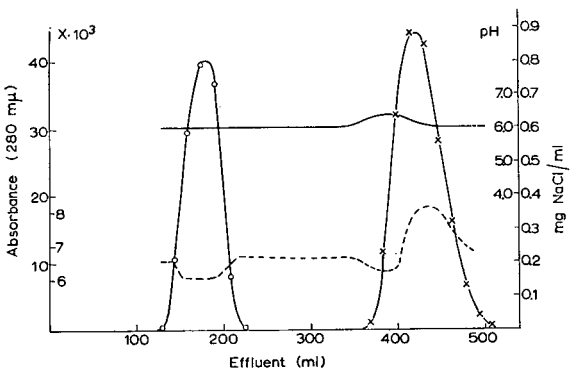

Fig. 5. Elution curves for CO-haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 120 ml/h. Eluant: 0.05 *M* sodium phosphate buffer. The solid horizontal line represents the pH and the broken line the conductivity.

It is seen that the buffer was of sufficiently high concentration to shift the pH to that of the buffer. A zone of higher pH appears immediately before the chloride zone and corresponds to the amount of hydrogen ions consumed. The minimum in

conductivity in the protein peak shows that a Donnan equilibrium has been established. In Fig. 6, illustrating experiment 3, the zone of higher pH is much broader and has just separated from the protein peak. The excess of electrolytes was removed as usual but the titration of the protein was evidently not finished until it had travelled down the greater part of the column. The conductivity curve was correspondingly more irregular in this experiment.

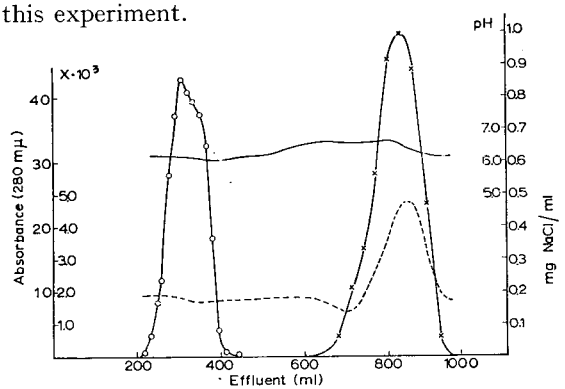

Fig. 6. Elution curves for CO-haemoglobin (o—o—o) and sodium chloride (×—×—×) at an elution rate of 120 ml/h. Eluant: 0.01 *M* sodium phosphate buffer. The horizontal solid line represents the pH and the broken line the conductivity.

DISCUSSION

As is the case in liquid–liquid partition chromatography, in gel filtration the solutes are distributed between a mobile and a stationary phase. However, the composition of the phases is the same in the latter method and the stabilizing substance is not passive as it is supposed to be in partition chromatography, but has a decisive influence on the process. The function of the swollen dextran gel is not only to stabilize the stationary phase, but also to provide a defined three-dimensional network having the property to sort molecules according to their size.

In analogy with the liquid–liquid partition chromatographic systems, it is possible to define a parameter for a solute, the distribution coefficient (K_D), which is independent of the geometry of the column. In the following the calculation and significance of this coefficient is discussed.

The total volume of the gel bed is

$$V_t = V_0 + V_i + V_g$$

where V_0 is the void volume, V_i the volume of the stationary phase and V_g the volume of the gel matrix. The weight of the stationary phase is calculated from the known amount of dry gel material (a) in grams and its water regain (W_r), defined as the number of grams of water held by one gram of dry material. The latter quantity is constant over a wide range of pH and ionic strength in the presence of many common salts and buffer substances. Notable exceptions are solutions containing borate or hydroxyl ions. If the density of water is taken as unity

$$V_i = aW_r$$

As a consequence of this definition V_i includes the water of hydration, which presumably is inactive as solvent and tends to lower the values for the distribution coefficients.

The void volume is easily determined by passing through the column a zone of a solute known to be completely excluded from the gel grains.

Sometimes the volume of the stationary phase must be calculated indirectly, for instance, when some gel has been lost during the back-wash. It is given by the formula

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

where d is the true wet density of the swollen gel particles.

The distribution coefficient is given through the relationship

$$K_D = \frac{V_e - V_0}{V_i}$$

where V_e is the elution volume of the solute.

In partition chromatography the distribution coefficient is defined as the ratio of the concentrations in the mobile and stationary phases. For a molecular sieve mechanism, such as the one described in the present paper, some parts of the stationary phase are available to the solute while others are not. The coefficients are thus a measure of the part of the phase that contains solute of the same concentration as the mobile phase.

A zero value for the distribution coefficient means complete exclusion from the gel particles. Values between zero and unity mean either partial penetration of the particles or that adsorption occurs, or both. Adsorption is indicated when the value is higher than unity.

The elution volume of a solute is

$$V_e = V_0 + K_D V_i$$

and two solutes with the distribution coefficients K_D' and K_D'' appear in the effluent separated by a volume equal to

$$V_i(K_D' - K_D'')$$

To obtain a complete separation the sample volume must be less than this value. How much less depends on a number of factors, among which the particle size, the flow rate and the viscosity of the sample are the most important ones.

The volume of the stationary phase is generally in the range of 40–60% of the total bed volume. It is therefore of great importance that the packing is made carefully. Even a moderately skewed zone reduces the separation efficiency considerably. The method of packing described in this paper gives good results, but is not entirely reliable and checking the column by passing a coloured zone is therefore recommended.

If the packing of the column and the application of the sample and the removal of the zones could be made perfectly, the shape of the column would be of minor importance. However, experience has shown that the best results are obtained when the ratio of height to diameter is large. With the type of equipment used in this investigation, a ratio of at least 10 to 1 gives excellent results. Satisfactory separations have been obtained with a 4 to 1 ratio, although disturbances due to uneven packing are less easily suppressed.

The variations in particle size and flow rate prove the former factor to be the most important one for the efficiency of the column. A large number of theoretical plates, and thus improved column efficiency, is best obtained by using a small particle size. However, a small particle size increases the pressure drop over the column and hence decreases the flow rate. A compromise between the time to be allowed for an experiment and the particle size is therefore necessary. For the type of separations described in this paper, the 100–200 mesh fraction gives excellent results. The experiment may be completed in less than one day, in many cases in one to two hours. Since a completely excluded solute remains in the column only about one third of the time for the total experiment, an experiment with a protein may often be made at room temperature without risk of denaturation. However, if desirable, the experiments can be conveniently made close to the freezing point.

The importance of the particle size and flow rate strongly indicates diffusion as the rate-determining factor in the gel filtration process. The fact that uridylic acid is much more sensitive to variations than hydrochloric acid is explained by their different diffusion rates. The spreading of solutes incapable of penetrating the gel phase is due to eddy formation, channelling and wall effects.

The gel filtration procedure is limited by the viscosity of the sample. A viscous zone spreads as a result of irregularities in the flow pattern. In the experiments described in this paper, varying amounts of dextran were added to the samples in order to vary this parameter. The haemoglobin and the dextran appeared simultaneously in the effluent and the spreading of the former substance was a consequence of the presence of dextran. The pressure drop over the bed increased and resulted in a compression of the bed. This was only manifested in a decrease of the void volume and not of the stationary phase. Thus it is not a true compression of the gel particles but a deformation of them with denser packing as a result. Similar phenomena probably cause disturbances also in other chromatographic processes.

In all separations reported in this paper the values of the distribution coefficient have been zero for the large molecular species and about 0.8 for the small ones. Thus the sample volume has to be smaller than $0.8\,V_i$. In the experiment illustrated in Fig. 4B the sample volume was $0.75\,V_i$ and an almost complete separation was still obtained. It should be observed, however, that the viscosity of the sample was very near to that of the eluant. With large sample volumes the dilution of the components becomes small. In the cited experiment the protein was diluted 1.25 times and the salt 1.35 times.

When the viscosity is low the concentration of the sample appears to be of little importance. Observations in favour of this view have been reported[8,12].

In the experiments reported the solutes separated differed very much in molecular size, and the results are comparable to those obtained with dialysis. In fact, the process may be considered as a multistage counter-current dialysis, in which the gel particles replace the dialysis bags. It was therefore of interest to see whether the effect on the excluded solute was similar to that obtained in dialysis. In all experiments with low-viscosity samples the salt present was completely removed. The conductivity and pH were also studied and it was found that the pH was adjusted to that of the buffer, provided that the stationary phase contained enough buffer ions to titrate the protein. The effects on the conductivity indicated that a Donnan equilibrium had been established. Experiments with polyelectrolytes and distilled water as eluant have shown that extraneous ions are removed, but the counter-ions remained with the polyelectrolyte. Thus all evidence hitherto collected indicates that the results are equivalent to an exhaustive dialysis. An important difference from the latter procedure is that a gel filtration experiment may be performed in a much shorter time.

A complete removal of salts from a protein is possible if water is the eluant. In many cases, for instance with blood serum a precipitate may develop which clogs the column. To prevent such complications a volatile buffer is advantageously used since it may be removed by lyophilization or evaporation.

SUMMARY

1. A study of the gel filtration method has been made in order to find the optimal conditions for removing salts from proteins.

2. The column efficiency increases with decreasing particle size and flow rate.

3. The viscosity of the sample rather than the concentration is a limiting factor.

4. Gel filtration of a protein solution is equivalent to an exhaustive dialysis but it can be carried out in a much shorter time.

5. The mechanism of the process is discussed.

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A SIMPLIFIED FRACTION COLLECTOR FOR GRADIENT ELUTION CHROMATOGRAPHY

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Gradient elution, as contrasted to the "step-wise" elution technique, is best executed with the aid of a fraction collector. This instrument is becoming an essential part of the equipment of many research laboratories, but unfortunately, the high prices of the apparatus available commercially are very often a deterrent to this acquisition. A relatively simple and inexpensive fraction collector has been constructed in this laboratory, and, although it does not have all the advantages which the commercial apparatus offers, it has found wide application in both the protein and virus fields.

DESCRIPTION OF THE APPARATUS

In Fig. 1 is given a fairly detailed sketch of the fraction collector. A is an Erlenmeyer flask of 250 c.c. capacity connected via a capillary tube and stopcock to a similar flask B which contains a tiny bar magnet. B is held firmly on a magnetic stirrer C.

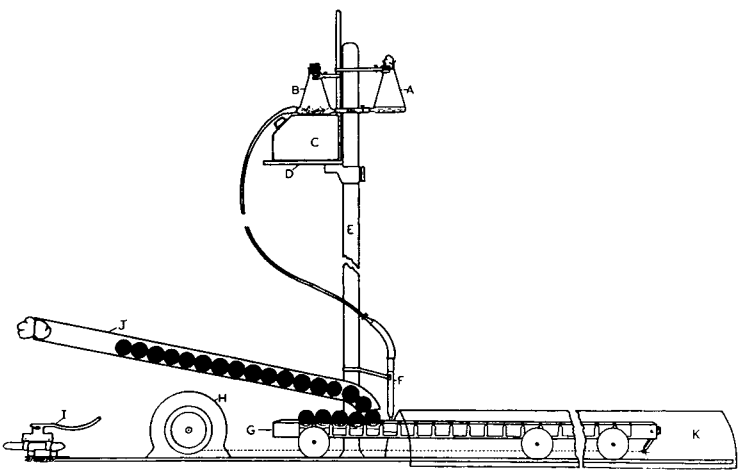

Fig. 1. The fraction collector. (For details see text).

C is supported on a metal platform D, the altitude level of which can be varied along a rigid metal rod E (2 m in length) and which is fixed onto a heavy metal plate.

A latex tube of 1 mm bore joins B onto the ion exchange column F. G is a perspex bar, of dimensions 2 × 2 × 100 cm, into which 50 cavities of uniform capacity have been drilled. The upper 2 mm of the cavities were drilled in such a way that they overlap slightly to produce sharp ridges between the cups. The perspex bar is supported by three pairs of meccano wheels which fit into grooves cut into a perspex sheet of dimensions 5 × 200 cm. The perspex carriage or "collector bar" is pulled along the tracks by a cotton thread which is wound up on a pulley attached to the shaft of an electric clock motor H. By attaching pulleys of different diameters onto the shaft, the speed by which the collector bar is pulled along the tracks may be varied. When the front of the carriage reaches the end of the tracks it is stopped automatically when it pushes over a mercury switch I, which, in turn disconnects the current supply to the motor.

A means is provided for minimizing the time of exposure of the samples collected in the cups to the atmosphere. A glass tube J, 100 cm long and 2 cm internal diameter, has its end bent slightly and its periphery cut to form an elipsoidal orifice, so that when the tube is mounted on a stand the elipsoidal orifice will be parallel to the surface of the collector bar. Prior to mounting, the tube is filled with glass marbles of approximately 15 mm diameter and autoclaved. The tube is mounted above the collector bar in such a way that each cavity is covered by a marble as it moves past the ion exchange column. The tube is held obliquely in order to reduce the weight of the column of marbles on the collector bar.

The salt or pH gradient, whichever it may be, is produced by means of the arrangement of flasks A and B and the magnetic stirring mechanism in a similar manner to that described previously by POLSON AND CRAMER[1]. The gradient curve is established from refractive index measurements on droplets taken from the various samples collected. An Abbe refractometer is well suited for the purpose.

Sterilization

Autoclaving as a means of sterilization of the collector bar was avoided on account of the harmful effect which excessive heating has on perspex. The cavities were therefore sterilized by washing with a 10% formalin solution followed by thorough rinsing with cold sterile distilled water. Prior to use the bar is kept under the perspex hood K shown in Fig. 1.

RESULTS

Turnip yellow mosaic virus (TYMV)

2 ml of a 1% virus prepared according to the method of MARKHAM AND SMITH[2] suspension in 0.01 *M* phosphate buffer of pH 7.2, to which was added a trace of rabbit haemoglobin, was adsorbed onto a DEAE anion exchange column and eluted by a gradient of sodium chloride in 0.01 *M* phosphate buffer. The haemoglobin served as a control for the process. The virus antigen contents of the different fractions collected were assayed by the double gel diffusion technique described by POLSON[3], after they had been dialysed against saline and their volumes adjusted to 2 ml.

In Fig. 2 the results of the experiments are given. The percentages of original virus in the different cups are plotted as ordinates against the sample number as abscissae. The salt gradient curve is shown as a separate line running obliquely across the diagram. It will be noticed that the virus antigen was eluted as a single component

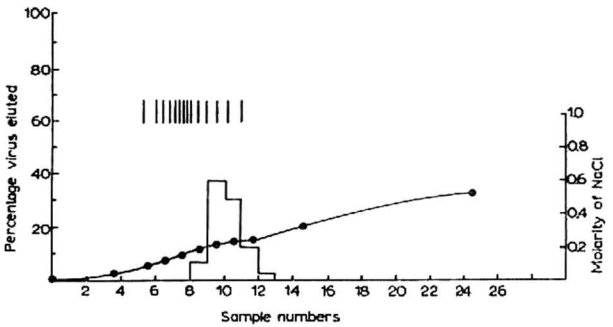

Fig. 2. Elution diagram of TYMV from a DEAE column. The ordinates present the percentage of original virus antigen present in each sample. The position of the haemoglobin is indicated by vertical stripes, sample 8 containing the highest concentration. The oblique line presents the sodium chloride concentration of the fractions as determined by light refraction measurements. The bulge in the line between sample 9 and 12 is due to the contribution of refractive index by the virus antigen.

from the column and that there was only very slight tailing on the elution diagram. In Fig. 3 is given a photograph of the gel precipitin apparatus taken during the formation of the precipitin bands in the test on the antigen contents of the fractions. Preliminary tests indicated that well defined precipitin bands may be produced by TYMV suspensions containing as little as 10^{-5} g per ml. It therefore follows that the virus contents of the fractions which failed to produce bands contained less than this amount of antigen.

Fig. 3. Double gel precipitin tests on the different samples collected. The photograph is a composite picture of two separate perspex apparatuses, the break occurring between tubes 10 and 11. The samples were placed in the top compartments of the apparatus and the antiserum in the bottom cups. 0.5% neutral agar in saline was placed in the central cavities. The first two tubes are standards showing bands formed by the original antigen and by the antigen diluted 1 in 50 respectively. The remaining tubes are numbered with the appropriate sample numbers and the second series of precipitin bands are formed by the antigen in the fractions.

MEF_1 strain of type II poliovirus

Suckling mouse-adapted MEF_1 virus was partially purified by three cycles of high and low speed ultracentrifugation of 10 ml of a 10% brain suspension in the manner commonly used. The final pellet was resuspended in 2 ml, 0.01 *M* phosphate buffer

of pH 7.2 and, after final clarification, was subjected to DEAE adsorption elution chromatography. A trace of haemoglobin was again used as a control. The fractions obtained were titrated in 3–4 weeks old mice using six mice per tenfold dilution. The animals were observed for 3 weeks. In Fig. 4 the results of an experiment are

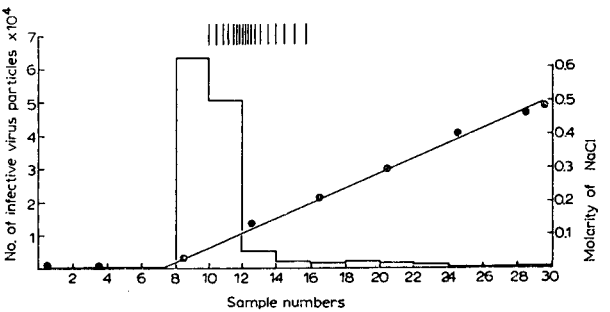

Fig. 4. Elution diagram of MEF_1 strain of Type II poliovirus. The ordinates present the titres of the different samples expressed as number of infective particles per 0.03 ml. The peak of the haemoglobin, the position of which is indicated by vertical strips, appeared in samples 12 and 13. The oblique line presents the NaCl concentration of the fractions as determined by refractive index measurements. The amount of crude virus placed on the column was too small to have any effect on the refractive index.

given. It will be seen from the elution diagram that, unlike TYMV, this virus did not behave as a single entity, but that it showed a main sharp peak which was eluted before the haemoglobin and a broader second region of activity which made its appearance after the haemoglobin had moved out of the column. Adsorption elution chromatography has been done previously on poliovirus using ECTEOLA cellulose anion exchanger by HOYER *et al.*[4].

ACKNOWLEDGEMENTS

The author would like to express his gratitude to Professor A. KIPPS for his continued interest in this work and to Miss D. DEEKS for very valuable technical assistance.

SUMMARY

An inexpensive collector is described which is easy to construct and to operate. The rate of flow is regulated and the fractions collected in cups drilled into a perspex bar. The cups are shaped in such a way that there is slight overlapping of their peripheries to form sharp ridges between them. The volumes of the fractions may be varied by altering the hydrostatic pressure in the adsorption column or by varying the speed with which the perspex bar is pulled past the adsorption column. The eluting gradient curves were established from refractive index measurements on droplets of the different fractions. The method is demonstrated on two viruses, TYMV and on the suckling mouse-adapted MEF_1 strain of Type II poliovirus. TYMV behaved as a single entity whereas MEF_1 poliovirus showed evidence of heterogeneity.

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ÉLECTROPHORÈSE SUR PAPIER DE QUELQUES IONS DE MÉTAUX DANS DES SOLUTIONS DE SULFATES

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(Reçu le 31 mai 1960)

Un article récent publié par ce laboratoire[1] décrit des expériences d'électrophorèse sur les ions hexaquo-rhodium(III) et hexaquo-chrome(III) dans des solutions de sulfates. Alors que ces deux ions se déplacent comme des cations dans l'acide sulfurique dilué, ils semblent neutres ou légèrement anioniques dans des solutions suffisamment concentrées de sulfates alcalins (solutions environ molaires).

La comparaison de différents sulfates utilisés comme électrolytes montra que les sulfates alcalins produisent ce retard alors que le sulfate de magnésium et le sulfate de zinc, à la même normalité, permettent un mouvement cationique des ions hydratés du Cr(III) et du Rh(III).

HARMELIN ET DUVAL[2], dans de récentes recherches sur l'alun de chrome et de potassium conclurent d'études thermogravimétriques et aux infra-rouges que six des douze molécules d'eau de $KCr(SO_4)_2 \cdot 12H_2O$ devaient être attribuées à un anion contenant aussi le Cr(III) et deux ions sulfate; ils proposent la formule $[Cr(SO_6H_4)_2(H_2O)_2]K \cdot 6H_2O$.

Il semble ainsi que dans une solution de sulfate, il se produit une certaine association des anions sulfate et des cations trivalents tels que Rh(III) et Cr(III) sans perte des six molécules d'eau d'hydratation des ions métalliques comme on peut le mettre en évidence par la couleur et le spectre de ces solutions qui diffèrent considérablement de ceux, bien connus, des complexes sulfatés.

On peut trouver dans les manuels[3] que les aluns peuvent se ranger parmi les complexes et la formation de paires d'ions entre anions polyvalents et cations polyvalents (par ex. sulfate et $Co(NH_3)^{3+}$) a été très étudiée.

Il nous a semblé intéressant d'étudier le mouvement des ions des métaux, en électrophorèse, dans des solutions de sulfates car peu de travaux semblent avoir été faits sur la chimie des solutions en milieu sulfate, à part quelques expériences d'échange d'ions de KRAUS ET NELSON[4].

1. *Comparaison du mouvement en électrophorèse de Al(III), Cr(III) et Fe(III) dans des solutions de divers sulfates*

Nous nous occupons principalement, dans cette étude, des cations trivalents (tels que Cr, Fe, et Al) qui forment des aluns. Dans des expériences comparatives, il fut nécessaire de faire une distinction entre les électrolytes de molarité identique et les

électrolytes de conductivité identique, parce que les conductivités du sulfate de magnésium et du sulfate de potassium diffèrent considérablement. Le sulfate de magnésium conduisant beaucoup moins que K_2SO_4.

Dans l'article de SHUKLA ET LEDERER[1] des solutions équimolaires furent comparées et, effectivement, des différences furent trouvées entre les sulfates alcalins et le sulfate de magnésium et le sulfate de zinc.

Cependant, si on utilise des solutions de même conductivité comme électrolyte, le mouvement par électrophorèse des ions Fe(III), Cr(III) et Al(III) est le même dans les différents sulfates. Des résultats caractéristiques sont donnés au Tableau I.

TABLEAU I

MOUVEMENT EN ÉLECTROPHORÈSE DE Al(III), Cr(III), ET Fe(III) DANS DES SOLUTIONS DE DIFFÉRENTS SULFATES

360 V (voltage initial) pendant une heure. Électrolytes: toutes les solutions furent préparées par dissolution d'une quantité suffisante du sel dans H_2SO_4 $N/10$ pour avoir une solution ayant une conductivité de $9.3 \cdot 10^{-2}$ mho/cm, à l'exception de l'acide sulfurique préparé 1 N. La conductivité de $9.3 \cdot 10^{-2}$ mho/cm correspond à Na_2SO_4 $M/2$ contenant assez de H_2SO_4 pour avoir une concentration en ions H^+ de $N/10$.

Électrolyte	*Mouvement en mm de*				
	CrO_4^{2-}	*Cu(II)*	*Al(III)*	*Fe(III)*	*Cr(III)*
Na_2SO_4	—66	14	5	—14	3
K_2SO_4	—66	22	6	—12	12
$(NH_4)_2SO_4$	—70	16	5	—15	4
$MgSO_4$	—49	13	6	— 8	4
$CuSO_4$	—58			—14	5
H_2SO_4 N	—50	40	28	9	38

Toutes les solutions de sulfates sont amenées à pH 1 par addition de H_2SO_4 pour éviter l'hydrolyse.

Dans H_2SO_4 N, utilisé comme électrolyte, les cations trivalents migrent plutôt rapidement et se comportent comme des cations. Ici, évidemment, la concentration en SO_4^{2-} est très basse, la plupart du H_2SO_4 se dissociant seulement en HSO_4^-.

Pour corriger les différences possibles dans le déplacement électroosmotique, on a fait migrer parallèlement aux substances examinées les spots de CrO_4^{2-} et Cu(II), sur toutes les feuilles. La technique de la plaque de verre[5] utilisant des plaques de 32.4 cm × 8 cm fut tout le temps utilisée.

Il est intéressant de noter que Fe(III) a une tendance plus forte à se complexer avec les sulfates que Al(III) ou Cr(III) (comme KRAUS l'a aussi observé sur les résines échangeuses d'ions) et que tous trois, ils se déplacent plus lentement que Cu(II).

Aucune interprétation de l'association des cations trivalents et des sulfates n'est préférable à l'autre (effet Debye-Hückel ou complexation); il est évident que ces ions trivalents forment des particules de très faible mobilité.

Nous aimerons faire quelques remarques spéculatives sur le genre de liaison qui peut se produire. Les ions sulfate sont hydratés dans de nombreux composés solides. Par suite, il est possible que le même genre de liaison (liaison hydrogène) relie l'ion sulfate à une molécule d'eau d'hydratation du complexe métallique trivalent.

Une liaison de ce type serait favorisée par la charge électrostatique du cation hydraté. Les particules les plus stables seraient naturellement celles ayant la charge totale la plus basse, telles que $[M(H_2O)_6SO_4]^+$ ou $[M(H_2O)_6(SO_4)_2]^-$. Cette dernière se formerait de préférence en présence de fortes concentrations de sulfates et semble être la particule dont les sels alcalins forment les séries connues sous le nom d'alun. En accord avec ce schéma, les aluns ne se formeraient pas si l'autre métal dans la formule $M(I) \cdot M(III)(SO_4)_2 \cdot 12H_2O$ a aussi tendance à s'associer avec les ions sulfate. Les aluns ne peuvent également se former si l'eau d'hydratation n'est pas fortement liée au cation trivalent, car, à la concentration convenable en sulfate, les complexes sulfatés se formeraient de préférence.

2. Mouvement de plusieurs ions métalliques dans le sulfate de sodium M/2

Pour donner une image du comportement des cations trivalents par rapport aux autres ions métalliques en solution sulfate, nous avons examiné une série de sulfates de métaux dans le sulfate de sodium molaire utilisé comme électrolyte (pH amené à 1 par H_2SO_4 et la force exacte ajustée à une conductivité de $9.3 \cdot 10^{-2}$ mho/cm). Ag(I) et Tl(I) se meuvent beaucoup plus vite que les cations bivalents. Les métaux de transition bivalents tels que Fe(II), Co(II), Ni(II), Mn(II), Cu(II) se meuvent plus vite que les cations trivalents, mais pas aussi vite que Mg(II). On note un faible mouvement pour Ga(III), In(III), Cr(III), Al(III), V(IV) et Ge(IV). Les terres rares se meuvent légèrement comme des cations. Des séparations définies sont obtenues avec le mélange Sc (anion), La et Y comme le montre la Fig. 1. On observe un com-

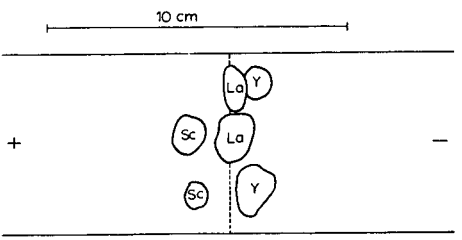

Fig. 1. Séparation de mélanges de La–Y, Sc–La et Sc–Y par Na_2SO_4 $M/2$–H_2SO_4 $N/10$ comme électrolyte sous 360 V pendant une heure. Les terres rares se déplacent à la même vitesse que La. Réactif: 8-hydroxyquinoline en solution alcoolique ammoniacale.

portement anionique pour UO_2^{2+}, Th(IV), Mo(VI), Ti(IV), Bi(III). $Co(NH_3)_6^{3+}$ se déplace un peu plus lentement que Co(II).

Le Tableau II montre le mouvement des ions métalliques pendant une heure sous un potentiel de 360 V. Pour corriger les effets d'adsorption possibles sur le papier, tous les ions métalliques furent aussi chromatographiés en utilisant l'électrolyte

comme solvant. Comme le montre le Tableau III, seuls quelques ions s'adsorbent; la plupart se déplacent sur le front du liquide.

TABLEAU II

MOUVEMENT D'IONS MÉTALLIQUES DANS Na_2SO_4 $M/2$

pH 1 par H_2SO_4, conductivité $9.3 \cdot 10^{-2}$ mho/cm; une heure sous 360 V. En raison de l'électro-osmose et de l'évaporation, le mouvement varie un peu d'une expérience à l'autre. Les valeurs données ici sont celles de plusieurs ions métalliques testés ensemble sur la même feuille pour éviter ces erreurs.

Ion métallique	*Longueur du déplacement en mm*	*Ion métallique*	*Longueur du déplacement en mm*
Ag(I)	39*	In(III)	—14
Tl(I)	50	Ga(III)	— 6
Fe(III)	21	Bi(III)	—12
Co(II)	22	$Co(NH_3)_6^{3+}$	12
Ni(II)	21	Sc(III)	—14
Zn(II)	23	Y(III)	9
Cd(II)	19	La(III)	— 6
Cu(II)	23	Th(IV)	—30
Mn(II)	13	Zr(IV)	—19***
Mg(II)	26	Ti(IV)	—24
Be(II)	7	V(IV)	—11
Pb(II)	0**	Ge(IV)	— 4
Hg(II)	0	CrO_4^{2-}	—70
Fe(III)	—9	MoO_4^{2-}	—30
Al(III)	9	UO_2^{2+}	—44
Cr(III)	4		

* Avec une comète due aux traces de Cl du papier.
** Précipité.
*** Hf est légèrement plus lent.

TABLEAU III

VALEURS DES R_F DES CATIONS MÉTALLIQUES

Technique ascendante avec Na_2SO_4 $M/2$ (pH 1) sur papier Whatman No. 1. Les autres électrolytes du Tableau I donnent essentiellement les mêmes résultats.

Ion métallique	*Valeur de R_F*	*Ion métallique*	*Valeur de R_F*
Fe(III)	0.95	Al(III)	0.96
Co(II)	0.94	Ga(III)	0.87
$Co(NH_3)_6^{3+}$	0.98	In(III)	0.88
Ni(II)	0.95	Tl(I)	0.82
Cr(III)	0.92	Ti(IV)	0.95
CrO_4^{2-}	0.57, 0.89	Zr(IV)	0.91
MoO_4^{2-}	0.51, 0.88	Hf(IV)	0.90
Th(IV)	0.95	V(IV)	0.97
UO_2^{2+}	0.96	Bi(III)	0.82
Be(II)	0.95	Sm(III)	0.94
Mg(II)	0.96	Eu(III)	0.97
Mn(II)	0.88	Gd(III)	0.97
Zn(II)	0.91	Dy(III)	0.98
Cd(II)	0.92	Ge(IV)	0.87
Cu(II)	0.91	Sn(IV)	0.70
Sc(III)	0.96	Pb(II)	0
Y(III)	0.97	Hg(II)	0.89
La(III)	comète	Ag(I)	comète

Si nous groupons les cations suivant leur mobilité, nous avons dans l'ordre: les cations monovalents (les plus rapides) puis les cations bivalents, puis les cations tri- et tétravalents. Ainsi l'interaction entre sulfate et cations métalliques inverse complètement l'ordre trouvé pour les sels ionisés à dilution infinie.

Il y a plusieurs séparations intéressantes qui peuvent être effectuées en utilisant le sulfate de sodium comme électrolyte. Quelques unes ont été confirmées en faisant l'électrophorèse de mélanges convenables d'ions de métaux (à concentration approximativement égales). Des résultats types sont donnés à la Fig. 2.

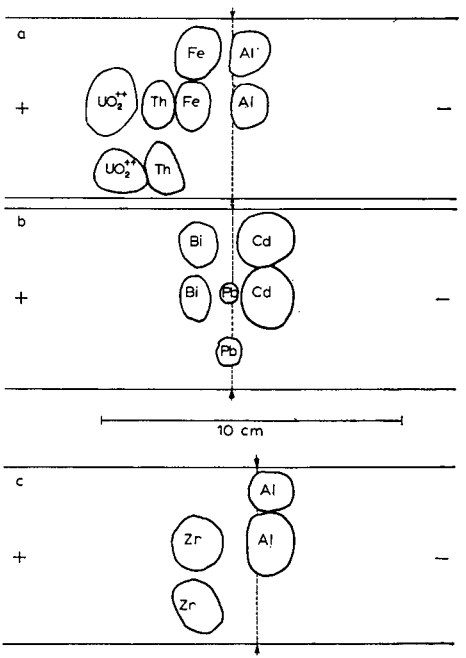

Fig. 2. Plusieurs séparations de mélanges par Na_2SO_4 $M/2$–H_2SO_4 $N/10$ comme électrolyte sous 360 V pendant une heure. (a) Fe–Al, UO_2–Th–Fe–Al et UO_2–Th sur la même bande de papier. Les taches furent révélés par la 8-hydroxyquinoline en solution alcoolique ammoniacale. (b) Bi–Cd, Bi–Pb–Cd et Pb sur la même bande de papier. Réactif comme pour (a). (c) Al, Al–Zr et Zr sur la même bande de papier. Réactif comme pour (a).

RÉSUMÉ

L'électrophorèse sur papier de quelques ions métalliques dans des solutions de sulfates a été étudié. L'ordre de mouvement en sulfate $M/2$ est pratiquement l'inverse de l'ordre de leur charges.

SUMMARY

The paper electrophoresis of some metal ions in sulphate solutions was studied. The order of movement in $M/2$ sulphate was practically the reverse of that of the charges.

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PAPER CHROMATOGRAPHIC SEPARATION OF MULTI-COMPONENT ANTIBIOTIC MIXTURES

THE ACETYLATED OLEANDOMYCINS*

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The use of paper chromatography in separating, classifying and identifying biological products has in the last ten years become a well established technique. When used in conjunction with bioautographic methods it has proven particularly fruitful in the field of antibiotics. Recently, two exceptionally challenging antibiotic mixtures, the quinocycline complex of six components[1] and the group of seven acetylated oleandomycins[2,3,4] have been examined by CELMER *et al.* We wish to report the details of the paper chromatographic methods that have proven successful in our laboratories for resolving these mixtures.

The use of formamide-impregnated paper along with a non-polar mobile phase has been described by ZAFFARONI[5] for the separation of steroids and by SCHINDLER[6] for the separation of alkaloids. The utility of this technique for the separation of the same materials has been confirmed in our laboratories and also it has proven very useful in separating many solvent-soluble antibiotics, especially those of the macrolide group[9]. These methods described more fully here have been particularly successful in resolving naturally occurring mixtures such as the quinocyclines, and closely related derivatives such as the acetylated oleandomycins. The toxicity of formamide for some of the bacteria used in preparing bioautographic plates may have discouraged other workers from using these systems; however, there are several micro-organisms that can be employed.

The use of paper chromatography in following the preparation and recovery of the six members of the quinocycline complex, along with the solvent system** and a table of R_F's has been mentioned by CELMER *et al.*[1]. In the following procedure we shall elaborate on the specific techniques of paper chromatography employed using as an example separation of the acetylated oleandomycins achieved by these systems.

METHODS

Whatman No. 4 paper is cut into 6½ in. × 19 in. strips with the long axis in the machine direction of the paper. A 1-in. hem is sewn on one end of the paper to

* Triacetyloleandomycin is commercially available from the J. B. Roerig Division of Chas. Pfizer and Co., Inc. The registered trademark for this material is TAO.

** Chloroform–ethyl acetate (3:1) saturated with formamide.

serve as a holder for a solid glass anchor rod, which also serves as a convenient grip in handling the sheets in later steps. The strips are impregnated by passing them through a 50% solution of formamide in methanol and then blotted on clean white blotters. Spots of the material* to be examined are then placed on the sheets. Normally we place on a sheet five or six evenly spaced 10-λ spots containing from 1 to 10 γ of antibiotic material per spot; the concentration depending upon the potency and number of components. Usually one control spot containing oleandomycin base and its seven acetylated derivatives is used on each sheet.

The sheets are then developed by a descending solvent in a 12 in. × 24 in. cylindrical jar using an 8 in. × 3 in. × 1½ in. porcelain tray to contain the developing solvent. No anti-siphon rods are needed with these trays. Solvents used were of analytical grade not further purified. The solvent is allowed to run to about 1 or 2 in. from the end of the paper, usually requiring between 1 and 2 hours. Longer runs, wherein the solvent drips off the sheets, will achieve greater separation of the components.

The bioautographic plate is prepared from a sheet of Pyrex plate glass 19 in. × 15 in. × $^3/_{16}$ in. to which are cemented** two strips 13½ in. × ¾ in. × ¼ in. and two strips 19 in. × ¾ in. × ¼ in. to make a shallow glass tray. A second sheet of glass can be used as a lid for the plate. Each plate is filled with 300 ml of Bacto Streptomycin assay agar*** inoculated with *B. subtilis* (ATCC No. 6633). After the agar has set, the chromatogram sheet, previously air dried for about 30 min to remove the volatile solvents but not the formamide, is gently pressed onto its surface by means of a lipless test tube, and left in contact from 15 to 30 min. Similar bioautographic techniques have been reported by other workers[7,8]. The reverse side of the plate can be marked with the point of origin, and suitable sample identification marks. The sheets are removed, the plates incubated overnight, and on the following day the outlines of the inhibition zones are delineated with a glass marking crayon. By using heavier inoculum and allowing the plates to preincubate while the chromatogram sheets are developing, a sample can be run and visualized within 6 to 8 hours. Photographs of the plates can be made either with a camera or by direct contact on Kodagraph paper, but for routine work, copies made on tracing paper are more rapid and less expensive.

Great care must be taken to avoid contaminating the chromatogram sheet with antibiotic particles such as might be floating in the air and the same precautions must be taken with the bioautographic plates. Airborne bacterial contamination is seldom a serious problem providing the surface of the agar is allowed to dry slightly before applying the chromatogram sheet. It is a characteristic of the formamide systems that at different temperatures and also in different chambers the R_F and degree of separation of the components may vary considerably. The separation of 1,2-diacetyl-

* Antibiotic samples were supplied by Dr. W. D. CELMER and his co-workers.
** At present we are using Flintkote Rubber Adhesive No. 979 obtained from the Flintkote Co., 30 Rockefeller Plaza, New York 20, N.Y.
*** Difco Laboratories, Inc., Detroit, Mich.

oleandomycin (1,2-DAO) and triacetyloleandomycin (TAO) is especially sensitive to temperature. By altering the proportions of the solvents in the system or by using a more or less polar solvent combination the desired resolution can be obtained. Two general precautions should be observed: the first is not to overload the chromatogram spots; the other is to use control spots and not depend entirely on R_F values.

RESULTS

In Table I the R_F's of the various acetylated oleandomycins are shown for the four most useful solvent combinations arranged in order of increasing polarity. These values were obtained from a series of runs made at the same time; chromatograms run at other times may show different R_F's but the components will appear in the same order. Figs. 1 and 2 illustrate bioautographs of the separations obtainable by this method. Fermentation broths, broth isolation samples, reaction mixtures, urines, and solvent extracts of blood have all been examined by using these paper chromatographic systems.

TABLE I

R_F VALUES OF ACETYLATED OLEANDOMYCINS*

Compound	*R_F in solvent system****			
	A	*B*	*C*	*D*
Oleandomycin base	0.02	0.05	0.05	0.30
3-MAO	0.05	0.10	0.15	0.50
2-MAO	0.10	0.25	0.40	0.70
2,3-DAO	0.20	0.35	0.70	0.95
1-MAO	0.30	0.50	0.95	0.95
1,3-DAO	0.40	0.65	0.95	0.95
1,2-DAO	0.80	0.90	0.95	0.95
TAO	0.95	0.95	0.95	0.95

* The nomenclature of the acetylated oleandomycins is that of CELMER[3,4]: 3-MAO = 3-monoacetyloleandomycin; 2,3-DAO = 2,3-diacetyloleandomycin; TAO = triacetyloleandomycin, etc.

** Solvent system: A = benzene–cyclohexane (1:1); B = benzene–cyclohexane (2:1); C = benzene–chloroform (3:1); D = benzene–chloroform (1:1); all systems saturated with formamide.

Several trials were made to see if quantitative results could be determined from the size of the inhibition zones. Reasonably accurate values could be obtained for most of the derivatives if the following conditions were observed: (A) uniform spot volumes were used; (B) reference standards of the same derivative to be measured were used; and (C) the component to be measured must be in the region of R_F 0.2 to 0.5. Plots of area *versus* logarithm of concentration usually gave smooth curves. However, a sample containing several components required a multiplicity of standards and more than one solvent system, resulting in a cumbersome and error-prone procedure. It was usually satisfactory, and also simpler, to make an estimate of the relative amounts of the components by visual inspection of the bioautographic plates.

Samples of blood and urine taken at various intervals after the ingestion of

TAO have been examined and the appearance of the several de-acetylated products can be readily detected. The metabolic fate of other derivatives was similarly investigated[4]. In some blood samples an unidentified component is often noted. This material is slightly more polar than TAO and is characterized by a less distinct zone edge on the bioautographic plates. It is thought to be a conjugate or additive compound composed of one of the oleandomycins and some blood constituent.

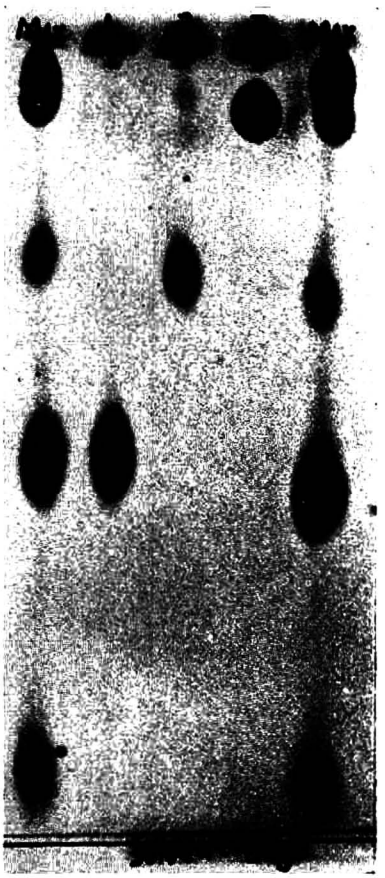

Fig. 1. *B. subtilis* bioautographic plate showing separation of the MAO's by solvent system B. The mix also contains oleandomycin base and TAO.

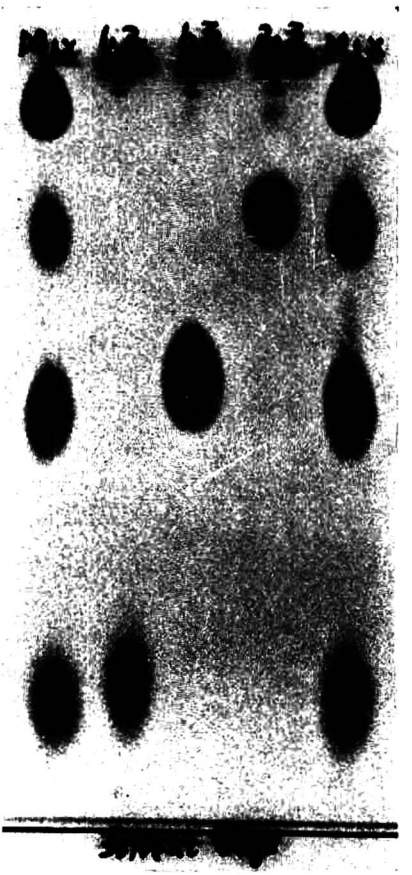

Fig. 2. *B. subtilis* bioautographic plate showing separation of the DAO's by solvent system A. The mix also contains oleandomycin base.

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SUMMARY

A paper chromatographic procedure has been described which has proved to be a powerful tool for separating the solvent-soluble antibiotics such as the macrolides and the quinocyclines. The method is rapid, versatile and capable of handling a wide variety of sample types. It has proven extremely valuable in following the synthesis of the several acetylated derivatives of oleandomycin and their appearance and interconversions in biological fluids.

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MOLECULAR WEIGHT DETERMINATION WITH THE MARTIN DENSITY BALANCE

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A frequent problem in gas-chromatography is the identification of compounds giving rise to peaks on the chromatogram, when they cannot be characterised by their retention times. The gas-density balance, designed by MARTIN[1] for use as a gas-chromatographic detector, gives a response which is a simple function of the densities of the carrier gas and vapours which pass through it, and so it can be used to determine the molecular weights of small amounts of vapours as an aid to their identification.

When a weight q of a vapour of molecular weight M is passed through the density balance in a stream of carrier gas, molecular weight m, an integral response (normally the peak area) A is obtained at constant flow rate such that

$$q = kA \frac{M}{M - m} \tag{1}$$

where k is a constant for a particular instrument. LIBERTI *et al.*[2] have determined molecular weights with the balance by measuring its change in response to a vapour in the presence of carrier gases of different molecular weights. By injecting separate portions of the same mixture of volatile compounds, containing one compound of known molecular weight, on to a chromatographic column before the balance, two sets of responses are obtained, so that

$$\frac{A}{A_1} \frac{M_1 - m}{M - m} = \frac{A'}{A_1'} \frac{M_1 - m'}{M - m'} \tag{2}$$

where M and M_1 are the molecular weights for the known and unknown compounds, m and m' are the molecular weights of the carrier gases, and A, A' and A_1, A_1' are the peak areas with the two carrier gases of the known and unknown compound. The method is very attractive in that it allows the molecular weights of all the peaks in a chromatogram to be determined without prior separation, provided that the molecular weight of one is known. LIBERTI *et al.* claimed an accuracy of 4 % in their molecular weight determinations. From our own experience we are able to substantiate this claim, but we have found that there are a number of difficulties which are not immediately apparent in the method. In the first place the calculation is such that small percentage errors in peak areas become considerably magnified in the final

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molecular weight. Secondly it is essential that the ratio of the two components (unknown and known molecular weights) in the two portions used with different carrier gases should be constant to within 1%. This may be obtained by injection with a microsyringe, the method used by LIBERTI *et al.*, but it is necessary to flush out the needle of the syringe with some of the sample immediately before injection. Capillary injection[3] and stream splitting devices in which the sample was divided into two in the gas phase were not found to provide adequate reproducibility. The latter was found to be within $\pm$ 2% in most cases, and molecular weight determinations correspondingly $\pm$ 5 to 10%, using an average of about four determinations for each mixture.

We have therefore developed an alternative method for finding molecular weights with the density balance. Equation (1) can be arranged to

$$q/M = kA/(M - m)$$

or

$$PV = KA/(M - m) \qquad (3)$$

where P and V are the pressure and volume of a vapour, and K a new constant. Thus by making pressure-volume measurements on a vapour and then passing it through the density balance, the molecular weight M can be found. Unlike LIBERTI's method, pure vapours are required, but these are readily obtained from a mixture in sufficient amounts by gas–liquid chromatography. The constant K in equation (3) is evaluated with a compound of known molecular weight.

EXPERIMENTAL

The density balance was a copper-block instrument constructed by Dr. A. J. P. MARTIN (Abbotsbury Laboratories, Elstree, Herts). Whenever possible it was used at room temperature, when it is about twice as sensitive as it is at 100°. The electrical response from the balance was fed to a 2 mV Honeywell-Brown potentiometric recorder. A transmitting slidewire on the recorder could be used to operate an electrical integrator (proportional counter motor) for peak area measurements, but this was found less accurate and reliable than cutting out the peaks from the recorder chart paper and weighing them.

The Pressure-Volume apparatus measured pressure at almost constant volume. Fig. 1 shows one form (Apparatus I), incorporating a PEARSON manometer[4] and designed so that both it and the density balance were operated at room temperature. A small correction was made for the variation in sensitivity of the balance with variation in room temperature. The whole apparatus could be evacuated, the connecting tubes between T_1 and T_3 all being of 4 mm bore, which gave a reasonable compromise between a low dead volume and a high pumping speed. The trap B was constructed of quill-tubing. The vapour contained in the volume E (110 ml) could be isolated from the rest of the system by means of the mercury cut-off C. The widening of the tube in this cut-off at the Y-junction made it possible to open the cut-off

satisfactorily (by withdrawing mercury through the tap) even when there was a small pressure difference across it.

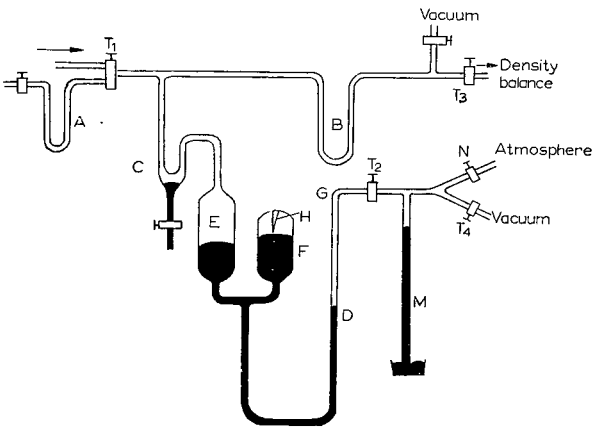

Fig. 1. The Pressure-Volume system (Apparatus I).

The operating procedure was as follows:

(1) The sample (which might correspond with a chromatogram peak) was trapped in A at liquid air temperature. The whole of the Pressure-Volume system was evacuated, a rotary oil-pump being found quite sufficient for this purpose. The sample was allowed to evaporate slowly from A until its pressure in the system (as measured roughly by the depression of the mercury in E) was sufficient. T_1 was then closed and A recooled to retain the rest of the sample.

(2) B was now cooled in liquid air, and after one to two minutes the system evacuated again. This indirect transfer from A to B has been found essential in order to displace adsorbed non-condensible gas from the walls of the system[5]. If this procedure was not adopted, very slow transfer from E to B occurred when the sample was finally retrapped into B (see paragraph (6) below).

(3) By operation of T_4, connected to vacuum, air was withdrawn from G, thus lowering the mercury level F below the pointer H. Operation of the needle valve N (connected to atmosphere) now allowed the mercury to be raised slowly in F until it just touched H. The mercury level in the precision-bore tubing D was now read. This cycle of operations (paragraph (3)) was repeated until a constant value of the mercury level in D was obtained.

(4) The sample in B was allowed to warm up and fill E. The valve C was closed and the rest of the system thoroughly evacuated (in our apparatus this involved approximately 10% of the sample in B being run to waste).

(5) By repeating the operations in paragraph (3), a new reading of the mercury level in D was obtained. The difference between this reading and the previous reading was equal to the pressure of the vapour in E multiplied by the ratio

cross-sectional area of E/cross-sectional area of D,

which was 55 in our apparatus. The pressures measured were normally 1–6 mm of mercury, corresponding to about 5–30 cm movement in D.

The volume of vapour in E varies very slightly with its pressure, the correction from constant volume being of the order of 1 %. (For measurements above room temperature when D was inside a vapour jacket it was found more convenient to measure the movement of the mercury in D as a change in pressure of the gas in G, registered on the manometer M.)

(6) B was cooled in liquid air and the vapour transferred to it from E by opening C. Transfer normally took about 20 min, and was checked for completeness by measuring the vacuum in E as in paragraph (3) above.

(7) Tap T_2 was closed and nitrogen (or other carrier gas) was introduced slowly (otherwise the top of F is shattered) into the system through T_1. (Tap T_2 may be replaced by a tap in the narrow tube immediately below E. Less care is then necessary as F and G are protected, but small errors can arise from tap-grease rising in the mercury into E.) A flow of nitrogen through T_3 into the density balance was then established, and when the flow and the electrical output from the balance were steady, the gas flow was measured and the sample introduced by allowing B to warm up. The flow was checked after the sample had passed through the balance. A soap-film flow-meter, accuracy $\pm$ 0.5 %, was used.

(The tube connecting T_3 to the density balance may contain conveniently a gas–liquid chromatographic column. This has the advantage that if the sample contains some impurity then this is detected and allowance made. If T_3 is connected directly to the density balance, then B must only be allowed to warm up slowly so that a sufficiently spread-out "peak" is obtained on the recorder.)

The room-temperature apparatus has been found satisfactory for substances with boiling points up to 150°. For higher boiling substances, it was necessary to raise the temperature of the Pressure-Volume system in order to produce sufficiently high vapour pressures. Measurements have been made with the Pearson manometer (E, F and D) and the density balance maintained at 100° with a steam-jacket, and with the connecting tubes electrically heated above this temperature (Apparatus II). In this case each of the unheated taps was protected by mercury cut-offs similar to C. T_3 was warmed for the passage of the sample into the density balance. At 100° loss of mercury from the Pearson manometer by distillation becomes a serious factor. It was reduced by keeping C at 100° and closed as much as possible.

For compounds boiling below 90°, the Pearson manometer may be replaced by a bulb of about 10 ml capacity, the vapour pressures of such compounds at room-temperature being sufficient for them to be measured accurately by a simple manometer (Apparatus III).

RESULTS AND DISCUSSION

Table I shows results obtained with various forms of the Pressure-Volume apparatus. The values of the determined molecular weights are mostly the averages of two or three measurements with each compound. The maximum errors in the individual

determinations were 1.5 % for Apparatus I and II and 1.9 % for Apparatus III.

The constant K (equation (3)) was determined using benzene for Apparatus I and III, and chlorobenzene in Apparatus II. The standard deviations for seven of these calibration runs with each apparatus were 0.7 %, 1.0 % and 1.7 % for Apparatus I, II and III respectively.

TABLE I

MOLECULAR WEIGHT DETERMINATIONS

Compound	*Boiling point* °C	*Molecular weight*	*Determined molecular weight*	*Error* %
		Apparatus I		
Diethyl ether	35	74.1	74.0	—0.1
Ethyl propionate	99	102.1	102.2	+0.1
Toluene	110	92.1	92.6	+0.5
p-Xylene	138	106.2	107.1	+0.8
Tetrachloroethane	146	167.9	168.0	+0.1
Silicon tetrachloride	58	169.9	170.9	+0.6
Trisilane	53	92.3	92.4	+0.1
Tetrasilane	*ca.* 108	122.4	122.2	—0.2
		Apparatus II		
Mesitylene	165	120.2	120.1	—0.1
Dimethylaniline	193	121.2	120.8	+0.5
		Apparatus III		
Ethyl bromide	38	109.0	108.3	—0.6
Methyl ethyl ketone	80	72.1	72.4	+0.4
Trichloroethylene	87	131.4	131.2	—0.2

A total of 1 to 6 mg of each compound was used to measure the molecular weights given for Apparatus I and II and slightly more for Apparatus III. With Apparatus I and II, the quantity used could readily be reduced by a factor of 10 by suitable amplification of the density-balance response (the noise level is low enough for this to be possible) and reduction of the volume of E. About 1 h was required for a single molecular weight determination with Apparatus I and II, but only 35 to 40 min with Apparatus III as this was simpler to operate.

Compounds with boiling points greater than 200° have not been determined successfully with Apparatus II, although this was designed for compounds boiling up to 250°. In experiments with higher boiling compounds the measured molecular weights were too high, probably due to inadequate heating of the apparatus and significant adsorption of the compounds in the Pressure-Volume system.

The initial measurements of the molecular weights of the silanes and silicon tetrachloride using Apparatus I gave low results. In each case, however, after four measurements the molecular weight had risen to the expected value. This effect was traced to decomposition of part of the silane or of silicon tetrachloride by reaction with the internal surface of the density balance. Once the surface had been conditioned by exposure to the appropriate vapour, all the sample from the Pressure-Volume

system could pass through the balance without reaction. When traces of moisture or air were allowed into the balance the conditioning was destroyed and low molecular weight results obtained. This defect has been overcome by the use of a glass density balance[7]. There was no decomposition of the reactive compounds in the Pressure-Volume apparatus.

ACKNOWLEDGEMENTS

We wish to thank Imperial Chemical Industries for the loan of the density balance and recorder, and to express our appreciation to Dr A. J. P. MARTIN, F.R.S., and to Dr B. W. BRADFORD for their continued interest in our work.

SUMMARY

Vapour samples, which may be trapped out from a gas-chromatographic analysis, are measured in a Pressure-Volume apparatus and weighed in a Martin gas-density balance. Molecular weights may thus be determined to an accuracy of better than 1 %.

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THE ISOLATION AND EXAMINATION OF THE ESSENTIAL OIL OF THE KUMQUOT (*F. MARGARITA* (LOUR.) SWINGLE)

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There is a large body of information concerning the botanical and horticultural aspects of the kumquots[1-4], but there is little reported in the literature concerning analyses of the chemical composition of the fruit itself. There are a few reports on the vitamin content of kumquots[5,6], especially vitamin C distribution[6], and some reports on the technology of preserving and utilization of kumquots[7]. Only one group, that of FESTER[8,9] and his co-workers in South America, has reported on the physical properties of the essential oil obtained from the epicarp (flavedo) of the kumquot fruit. FESTER[8] has reported the density, refractive index, optical rotation, acid number, and ester number of the essential oils of a number of South American plants; the physical properties of kumquot oil were included in this publication[8].

Kumquot trees belong to the plant family *Rutaceae* which include two genera, *Citrus* and *Fortunella*. The latter genus includes the kumquot. The kumquot fruit bears a close similarity to citrus fruit. When ripe they are small ovoid or oblong fruits with a bright orange skin that contains large oil sacs, and when split open, present the typical segmented appearance of a citrus fruit. Fruits are generally ½ to ¾ in. in diameter and ¾ to 1–½ in. in length. It is interesting to note the albedo (mesocarp) and flavedo (epicarp) of the kumquot are quite sweet; and the central portion of the fruit, even when ripe, has a rather sour, acid taste. Kumquots are used principally as decorative fruits and in preserves and jelly-making[1,2].

EXPERIMENTAL

The kumquots used in this study were of the variety *margarita* and grown on the University farm located in Winters (about 7 miles west of Davis), California. They were harvested just after the fruit had reached a rich golden orange color (it is difficult to determine the exact state of maturity of these fruits). Immediately after harvest, the kumquots were brought to the laboratory where they were chilled, thoroughly rinsed in cold water, drained, rinsed in a cool solution of a commercial detergent, rinsed twice with fresh cool water, and dried with clean cloths. Two kilograms of the fruit were selected and the epicarp (flavedo) was carefully stripped from each fruit by means of a stainless steel knife; the mesocarp (albedo) and segment mem-

branes of the fruit were discarded. The epicarp was rinsed in cool distilled water to remove any contamination by the acid present in the juice of the mesocarp, segment membranes, or juice sacs. The weight of the resulting epicarp obtained from 2000 g of whole fruit was 815 g.

The epicarp was added to 500 ml distilled water in a blendor and homogenized for 15 min. The homogenate was transferred to a 1-l round bottom distillation flask and the contents steam-distilled in the conventional manner through a 30 cm bubble-plate column fitted with a Kjeldahl trap and a 50 cm ice water-cooled condenser. The steam distillation was conducted at atmospheric pressure. Distillation was discontinued 15 min after the last drop of oil appeared in the receiver and when the latter contained about 450 ml of total distillate. The aqueous phase was saturated with sodium chloride, and the upper oil phase separated from the water phase in a separatory funnel. A colorless, light oil with a pleasant, fruity aroma resulted. The oil remaining in the aqueous phase was extracted with 3–80 ml portions of *n*-pentane; the extracts and the oil combined, and dried over anhydrous sodium sulfate for 24 h. The combined extracts and oil were filtered, and placed in a rotary flash evaporator where the *n*-pentane was removed under vacuum at 26°. A condenser using dry ice and alcohol (—77°) was used to collect the *n*-pentane and prevent loss of volatile components. The yield of oil above the aqueous phase was 7.57 g and that recovered from the *n*-pentane extractions was 1.13 g, making a total yield of 8.70 g (1.07 % of total peel weight and 0.435 % of the total fresh fruit weight).

A small amount of the oil was placed in a pycnometer and the density at 25° was determined. The density of this steam-distilled kumquot oil was 0.8389. The oil had an optical rotation of $\alpha_D^{25°}$ + 15.76°. The refractive index at 20° was 1.4758. An ultraviolet absorption spectrum of the oil was taken, and this curve revealed none of the sharp peaks, or bands generally associated with the coumarin compounds found in typical citrus oils. It is interesting to compare the results obtained from this study with those obtained by FESTER[8] in his study of South American kumquot oil. FESTER found a density at 15° of 0.8505, and a refractive index of 1.4769 (temperature not specified), $\alpha_D^{17.5°}$ + 93°20′, an acid number of 2.0, and an ester number of 4.4 (we did not make a determination of acid number or ester number in this study). The differences between these data are quite probably due to differences in species and possibly varietal differences. FESTER[8] did not indicate what species or variety his group examined and reported.

The gas–liquid chromatographic equipment used to separate the components of the essential oil was an Aerograph model A-90-C, equipped with a ¼ in. O.D. stainless steel column. An improved four-cell catharometer assembly was employed for detection. The exact experimental parameters of operation, *i.e.*, temperature, flow rate, etc., accompany the figures and tables.

The kumquot oil was deterpenated by a modified KIRCHNER procedure[10] as follows: 15 g of dried (heated at 125° for 3 h immediately before use), 100 mesh silicic acid were added to a solution of 3.0 g of the essential oil in 25 ml of *n*-pentane with shaking, and the mixture was allowed to stand at room temperature with occasional

shaking for 2 h. The silicic acid was filtered off on a sintered glass crucible under reduced pressure, and the pentane solution dried over anhydrous sodium sulfate for 1 h. The terpenoid fraction was eluted from the adsorbent by placing the silicic acid in 25 ml of absolute ethanol and allowing the mixture to stand with occasional swirling at room temperature for 2 h. The adsorbent was filtered off as above and discarded. The ethanol was dried over anhydrous sodium sulfate. When dry, the pentane and ethanol solutions were each filtered, and the respective solvents removed by means of a rotary flash evaporator under moderate vacuum (*ca.* 1.0 mm Hg) at 26°. The terpene and the terpenoid fractions were then stored at —10° until used.

RESULTS AND DISCUSSION

When kumquot oil was examined by means of gas–liquid chromatography employing a stationary liquid phase consisting of LAC-2-R446 (the adipate polyester of diethylene glycol partially cross-linked with pentaerythritol)[11,12], 14 major peaks were evident on the chromatogram. The assignment of various peak identities was made by determination of the corrected retention volumes (V_R°)[13] of known compounds and comparison with those for the unknown peaks. In this manner, a tentative identification of a large number of the components present was achieved. Confirmation of these results was obtained by an enrichment procedure in which known compounds were added, one at a time, to fresh portions of the kumquot oils and re-examined by gas–liquid chromatography. Data are presented in the form of relative retention

TABLE I

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-2-R446*
(*n*-Decanal = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.0387	—	—
2	0.0663	—	—
3	0.141	0.148	(α-Pinene)
4	0.213	0.227	(Myrcene)
5	0.301	0.298	*d*-Limonene
6	0.843	0.840	*n*-Octyl acetate
7	1.00	1.00	*n*-Decanal
8	1.50	1.47	*n*-Undecanal
		1.50	Bornyl acetate
9	1.75	—	—
10	2.03	—	—
11	2.35	2.38	Citronellol
12	2.71	—	—
13	3.26	3.25	Geranyl propionate
14	3.78	3.63	(*trans*-Carveol)

* Temperature 150°; helium flow rate 90 ml/min.

volumes, $(V_{\_}/V_R^\circ)$[13] (Table I). It should be noted that it is experimentally impossible to distinguish between two compounds whose relative retention volumes differ by 7 % or less since they will appear on the chromatogram as a single, united peak. Differences of 10 to 15 % in relative retention volumes will show peaks that are united, *e.g.*, shoulders, or doublets; and differences of 20 % or more are necessary for complete separation of zones or peaks[14,15].

In order to be well within the bounds of experimental error, an arbitrary limit of agreement for corrected relative retention volumes not to exceed 2 % was established. Compounds with values for corrected relative retention volumes not agreeing to within 2 % of each other are enclosed in parentheses. In Table I, those peaks that differ by more than the 2 % limit are: peak (3) α-pinene (4.7 % difference); peak (4) myrcene (6.2 % difference); peak (14) *trans*-carveol (4.1 % difference). These differences are still well within the 7 % limit found by BERNHARD[14] and JAMES[15].

As a further check on identity, relative retention volumes were evaluated on a

TABLE II

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-4-R777*
(*n*-Decanal = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.0572	—	—
2	0.0960	—	—
3	0.142	0.136	(α-Pinene)
4	0.221	0.217	Myrcene
5	0.301	0.294	(*d*-Limonene)
6	0.618	—	—
7	0.725	0.715	Methyl heptenone
8	0.789	0.791	3-Hepten-1-ol
9	0.846	0.849	*n*-Octyl acetate
10	0.902	—	—
11	1.00	1.00	*n*-Decanal
12	1.15	1.15	Linalool
		1.18	(Linalyl acetate)
13	1.27	—	—
14	1.41	1.41	*n*-Undecanal; Linalyl propionate
15	1.75	1.71	(Decyl acetate)
16	1.85	1.80	(Citronellyl acetate)
17	2.08	2.10	*n*-Decanol
18	2.35	2.31	Terpinyl acetate
19	2.45	2.41	Geraniol; α-Terpineol
		2.46	Citronellol
20	2.76	2.74	Geranyl acetate
21	2.86	—	—
22	3.23	3.22	Citral
23	4.08	4.01	*trans*-Carveol
		4.05	Linalyl butyrate
		4.06	Geranyl butyrate
24	4.51	—	—

* Temperature 150°; helium flow rate 90 ml/min.

second stationary liquid phase LAC-4-R777 (the succinate polyester of diethylene glycol)[12] (Table II). The data supplied by the use of another stationary liquid phase lend credence to the tentative identification of the compounds present in the oil. Employing a liquid phase of LAC-4-R777, kumquot oil showed 24 major peaks on the chromatogram. The identities were assigned on the basis of agreement of corrected relative retention volumes for the unknown peaks with those values for known compounds. Those peaks that differed by more than the 2 % limit of agreement are: peak (3) α-pinene (4.4 % difference); peak (5) *d*-limonene (2.4 % difference); peak (12) linalyl acetate (2.5 % difference); peak (15) decyl acetate (2.3 % difference); peak (16) citronellyl acetate (2.8 % difference). Once again these differences are well within the 7 % limit[14,15].

The choice of the two LAC liquid stationary phases was most certainly not an arbitrary one. While it was possible to demonstrate the presence of only 5 distinct peaks in whole lemon oil[16] using non-polar stationary liquid phases, *e.g.*, D.C. Silicone Fluids, the use of these polyester stationary liquid phases increased this resolution to some 20–30 compounds[17]. Thus it appeared advisable to use these materials to separate the components of kumquot oil. Reference to Fig. 1 shows a typical chro-

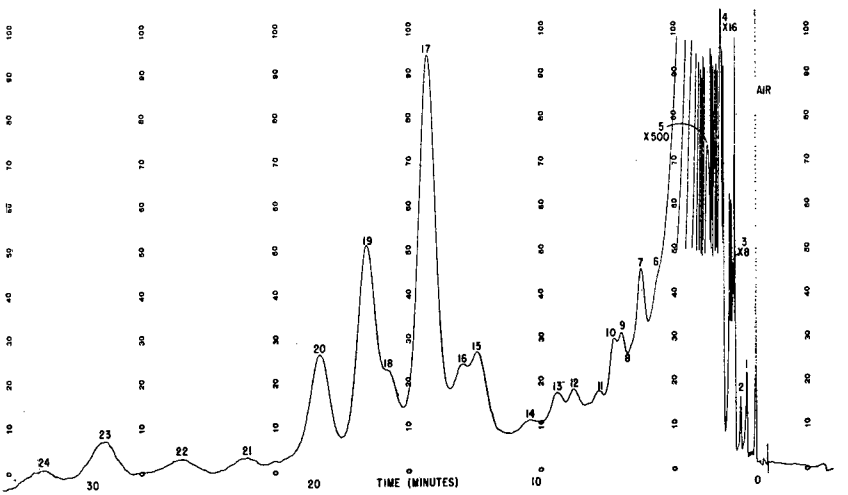

Fig. 1. Gas–liquid chromatogram of the components of the essential oil of kumquot. Sample size: 40 μl; temperature: 150°; helium flow rate: 90 ml/min; stationary phase: LAC-4-R777 on a support of Sil-O-Cel C-22 (42–60 mesh), 20 % by weight; stainless steel column 10 ft. by ¼ in. O.D.; 1 mV recording potentiometer; chart speed: 30 in./h. Peak identities are presented in Table II.

matogram for whole kumquot oil employing a LAC-4-R777 column. The curve indicates a wide divergence in the boiling points of the components present, and this makes efficient separation of these constituents by gas–liquid chromatography at a fixed temperature inconvenient. A sure sign of this wide boiling point range is indicated by the crowding of the peaks at the initial or starting point of the curve and the distention of the peaks near the terminal portion of the curve. A possible solution

to this problem would be to resort to temperature programming or multi-stage chromatographic columns[18]. Since this equipment was unavailable to us, a preliminary separation of the components present was deemed desirable. Such a separation must be conducted under the mildest of conditions, and it was decided to employ the chromatographic deterpenation procedure of KIRCHNER AND MILLER[10] for this purpose. By this technique the kumquot oil was separated into two major fractions: one, the hydrocarbon fraction (terpenes) with a boiling range of some 60°, and the other the terpenoid fraction (the compounds bearing oxygen functions, *e.g.*, esters, aldehydes, ketones, etc.) with a boiling range of some 80°. This now permitted the chromatographic examination of the two fractions at different column temperatures. The first temperature selected would be the optimum for the separation of the terpenes, and the second temperature selected would be the optimum column temperature for the separation of the terpenoid compounds.

Examination of the terpene fraction from kumquot oil revealed that there are six distinct peaks in the chromatograms when the oil is separated on a LAC-2-R446 column. The data for these separations are presented in Table III. Once again two peaks differed by more than the 2 % limit of agreement; these were peak (3) α-pinene (4.0 % difference) and peak (4) camphene (4.2 % difference).

TABLE III

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE TERPENE FRACTION OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-2-R446*
(*d*-Limonene = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.148	—	—
2	0.213	—	—
3	0.365	0.379	(α-Pinene)
4	0.497	0.519	(Camphene)
5	0.745	0.742	Myrcene
6	1.00	1.00	*d*-Limonene

* Temperature 100°; helium flow rate 145 ml/min.

When the terpene fraction was examined by means of a stationary liquid phase consisting of LAC-4-R777, 9 distinct peaks were detected. Reference to Table IV shows that the corrected relative retention volumes for the unknowns agree well with the corrected relative retention volumes of the knowns. Only two peaks, peak (5) camphene (4.9 % difference) and peak (8) γ-terpinene (2.4 % difference) exceeded the arbitrary 2 % limit of agreement. The chromatogram for this separation employing LAC-4-R777 is presented in Fig. 2. The capital S near the origin of the chromatogram indicates some residual solvent, from the deterpenation step, that remained in the terpene fraction.

TABLE IV

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE TERPENE FRACTION OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-4-R777*
(*d*-Limonene = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.214	—	—
2	0.285	—	—
3	0.374	0.372	α-Pinene
4	0.382	—	—
5	0.553	0.527	(Camphene)
6	0.767	0.776	Myrcene
7	1.00	1.00	*d*-Limonene
8	1.28	1.25	(γ-Terpinene)
9	1.45	—	—

* Temperature 100°; helium flow rate 145 ml/min.

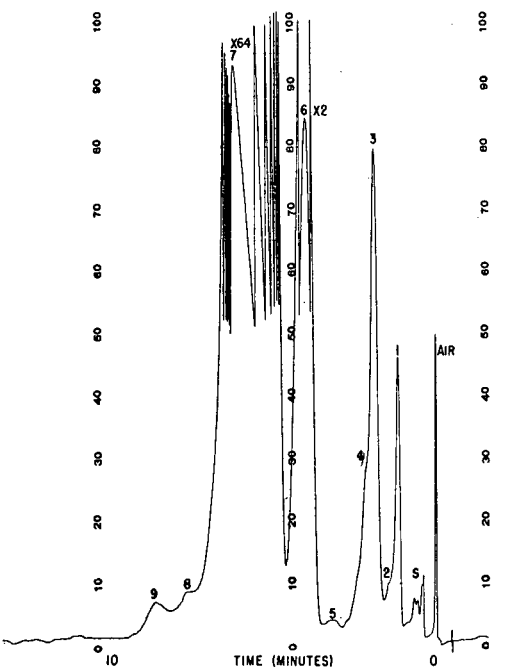

Fig. 2. Gas–liquid chromatogram of the components of the terpene hydrocarbon fraction of the essential oil of kumquot. Sample size: 20 μl; temperature: 100°; helium flow rate: 145 ml/min; stationary phase: LAC-4-R777 on a support of Sil-O-Cel C-22 (42–60 mesh), 20% by weight; stainless steel column 10 ft. by ¼ in. O.D.; 1 mV recording potentiometer; chart speed: 30 in./h. Peak identities are presented in Table IV.

The terpenoid fraction was examined by means of a LAC-2-R446 column and the chromatogram revealed 11 distinct peaks. Only one peak, peak (6), geranyl formate (3.1 % difference) exceeded the arbitrary 2 % limit of agreement. These data are presented in Table V. The terpenoid fraction was next examined employing a LAC-4-R777 column and the chromatogram revealed 21 distinct peaks (Table VI). Those peaks that differ by more than the 2 % limit of agreement are: peak (2) *d*-limonene (2.7 % difference); peak (6) citronellal (2.8 % difference); peak (8) linalyl

TABLE V

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE TERPENOID FRACTION OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-2-R446*
(*n*-Decanal = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.150	0.148	α-Pinene
2	0.301	0.298	*d*-Limonene
3	0.668	0.676	*n*-Nonanal
4	0.835	0.840	*n*-Octyl acetate
5	1.00	1.00	*n*-Decanal
6	1.66	1.61	(Geranyl formate)
7	1.93	1.95	Borneol
8	2.28	2.28	α-Terpineol
9	2.61	2.61	Geranyl acetate
10	3.10	3.10	*d*-Carvone
11	3.60	3.63	*trans*-Carveol

* Temperature 150°; helium flow rate 90 ml/min.

propionate and undecanal (2.1 % difference); and peak (14) terpinyl acetate (2.1 % difference). A chromatogram for this separation using a LAC-4-R777 column is presented in Fig. 3.

Examination of Fig. 1 shows a very large amount of *d*-limonene (peak 5) to be present in the whole oil, and there are also relatively large amounts of *n*-decanol (peak 17), citronellol and/or α-terpineol–geraniol (peak 19), and geranyl acetate (peak 20).

Fig. 2 indicates that α-pinene (peak 3), myrcene (peak 6), and *d*-limonene (peak 7) are the principal terpene hydrocarbons found in kumquot oil. It is interesting to note that no β-pinene was detected in kumquot oil. This appears to be an analogous situation to that of navel orange oil, for here, little evidence has been found for the presence of this compound[19]. However, β-pinene is present in lemon oil[20], grapefruit, lime, mandarin, and bergamot oils[19].

The terpenoid fraction (Fig. 3) reveals relatively large amounts of *n*-decanal and citronellal (peaks 5 and 6), bornyl acetate (peak 9), decyl acetate (peak 10), geraniol and/or α-terpineol (peak 15), geranyl acetate (peak 16) and *trans*-carveol (and quite possibly the butyrates of geraniol and linalool) (peak 20).

In contrast to the citrus oils, kumquot oil appears to contain less of the lower

TABLE VI

CORRECTED RELATIVE RETENTION VOLUMES OF THE COMPONENTS OF THE TERPENOID FRACTION OF THE ESSENTIAL OIL OF KUMQUOT

Stationary phase: LAC-4-R777* (*n*-Decanal = 1.00)

Peak	V_R°/V_R°		*Compound*
	Unknown	*Known*	
1	0.201	—	—
2	0.302	0.294	(*d*-Limonene)
3	0.832	0.849	*n*-Octyl acetate
4	0.879	—	—
5	1.00	1.00	*n*-Decanal
6	1.10	1.07	(Citronellal)
7	1.20	1.18	Linalyl acetate
8	1.38	1.41	(Linalyl propionate; *n*-Undecanal)
9	1.59	1.57	Bornyl acetate
10	1.73	1.71	Decyl acetate
11	1.80	1.80	Citronellyl acetate
12	1.94	—	—
13	2.09	2.10	*n*-Decanol
14	2.26	2.31	(Terpinyl acetate)
15	2.42	2.41	Geraniol; α-Terpineol
16	2.73	2.74	Geranyl acetate
17	3.18	3.22	Citral
18	3.54	—	—
19	3.68	3.68	*d*-Carvone
20	4.04	4.01	*trans*-Carveol
		4.05	Linalyl butyrate
		4.06	Geranyl butyrate
21	4.45	—	—

* Temperature 150°; helium flow rate 90 ml/min.

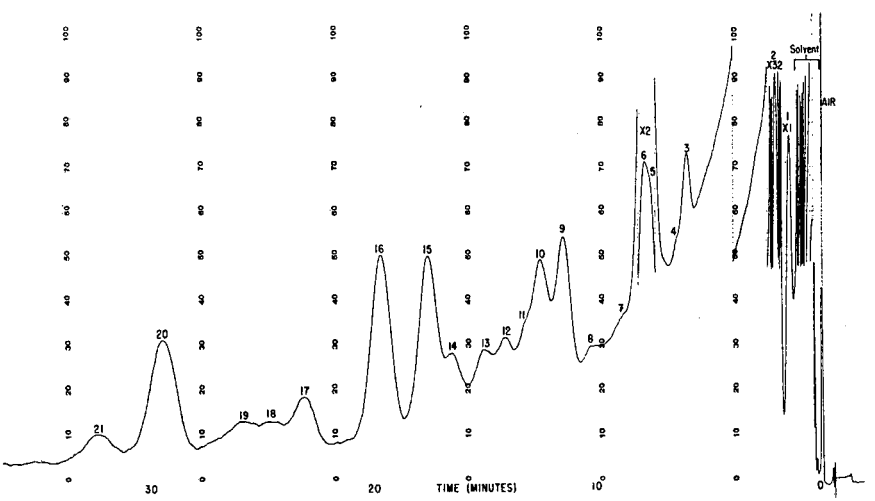

Fig. 3. Gas–liquid chromatogram of the components of the terpenoid fraction of the essential oil of kumquot. Sample size: 20 μl; temperature: 150°; helium flow rate: 90 ml/min; stationary phase: LAC-4-R777 on a support of Sil-O-Cel C-22 (42–60 mesh), 20% by weight; stainless steel column 10 ft. by ¼ in. O.D.; 1 mV recording potentiometer; chart speed: 30 in./h. Peak identities are presented in Table VI.

straight-chain aldehydes from C_8–C_{12}. No octanal was detected in this study. The citral content of the oil is low and is generally comparable to the citral level found in orange oils. It is noticeably lower than that level found in lemon oils. The absence of large amounts of these aldehydes may account, at least in part, for the milder, less penetrating aroma of kumquot oil.

We were unable to detect any sesquiterpenes[20] in the oil. It may well be that the method of isolation precludes their occurrence in the fractions examined.

The complex nature of this oil will require further studies and it is hoped in the future, we will be able to report the results of a more detailed examination of the oil.

SUMMARY

1. The essential oil of the kumquot was isolated from the epicarp of the ripe fruit by steam distillation.

2. The whole oil was examined by means of gas–liquid chromatography and a number of the components present were tentatively identified.

3. The whole oil was deterpenated and the terpene and terpenoid fractions were examined individually by gas–liquid chromatography.

4. The data for gas–liquid chromatographic examinations are presented in the form of corrected relative retention volumes (V_R°/V_R°) employing two stationary liquid phases, LAC-2-R446 and LAC-4-R777.

5. The oil was found to contain large amounts of *d*-limonene, some α-pinene, myrcene, terpene esters, aldehydes, a ketone, and some free alcohols.

6. The physical constants for the oil, *i.e.*, refractive index, optical rotation, etc., are presented.

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ACTINIUM: RADIOCOLLOIDAL BEHAVIOUR

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A number of heavy element hydroxides and sulphides are known to exhibit radiocolloidal properties[1]. Although many lanthanides have these properties[2,3], the existence of actinium hydroxide or sulphide as a radiocolloid does not appear to have been reported. During the course of extraction and separation of MsTh-1 and MsTh-2, it was found that the hydroxide of the latter formed a radiocolloid in solutions of high pH.

In addition to the usual methods of investigating radiocolloids, a paper chromatographic technique has been developed for the study of these colloidal systems. The method is based on the principle that a colloid when adsorbed on chromatography paper does not move with the eluant, while the ionic form does. The colloid formation can be readily studied by this method.

EXPERIMENTAL

MsTh-1 was separated from 2 kg of thorium nitrate by coprecipitating it with barium sulphate. The sulphates were fused with sodium carbonate and the fused mass was extracted with water and filtered. The residue was dissolved in the minimum amount of hydrochloric acid.

The radioactivity was assayed with the help of a scintillation counting set-up with a NaI(Tl) well-type crystal, and that on the paper strip was determined with the Tracer-lab TGC.2 GM counter in conjunction with a scaler.

(*a*) *Adsorption of MsTh-2 on sintered glass at various pH values*

1 ml of the original solution containing MsTh-1 in equilibrium with MsTh-2 was diluted to 10 ml and the pH adjusted to the desired value by the addition of ammonium hydroxide. This solution was immediately filtered through a sintered glass funnel under suction and washed with 5 ml of water adjusted to the same pH. This operation took less than three minutes. The matter asdorbed on the sintered glass was removed by washing it with 5 ml of concentrated nitric acid, which completely removed the adsorbed matter, as no activity was detected in further washings. The samples at different pH values were counted for a week and MsTh-2 estimated in each sample. The results are shown in Fig. 1.

The coagulation isotherm at pH 9.5 is obtained by determining the percentage adsorption at different concentrations of MsTh-2 and plotting the logarithm of the

amount adsorbed *versus* the logarithm of the initial concentration. The results are given in Table I and are plotted in Fig. 2.

In order to study the effect of foreign electrolytes on the adsorption of MsTh-2 on sintered glass, increasing concentrations of KCl were used and the adsorption of MsTh-2 was determined at pH 9.5. The data are presented in Table II.

The effect of aging of the solutions on the adsorption behaviour of MsTh-2 was determined as follows:

1 ml of the original solution diluted to 10 ml was adjusted to the desired pH. The solution was then transferred to 20 ml glass-stoppered pyrex bottles and allowed to age for a desired length of time. Two series of experiments were conducted with aging times of 50 and 120 hours. The adsorption and elution from sintered glass was carried out as described above and the samples were followed for 3 to 4 days to obtain equilibrium values. Curves 2 and 3 in Fig. 1, refer to solutions aged for 50 and 120 hours respectively.

(b) Centrifugation studies on the radiocolloid

In each of five centrifuge tubes 5 ml of the MsTh-2 solution, adjusted to a definite pH value, was placed and then centrifuged for over 2 hours at 4000 r.p.m. The tubes were removed without disturbing the solutions. 3 ml of the solution from the top were very carefully removed to a vial and counted. The remaining solution was transferred to another vial along with the 2 ml of conc. HCl used for rinsing the centrifuge tube, and then counted. The results are given in Table III.

(c) Paper chromatographic experiments

The behaviour of a known radiocolloid, UX_1, on paper chromatography was first studied. A drop of a solution of uranyl nitrate in equilibrium with UX_1 was applied at one end of a paper strip and chromatographed with water at pH 3. A clear separation of UX_1 from uranium was achieved. UX_1, which forms a radiocolloid, does not move while the uranyl ions move along with the water front as verified by means of ferrocyanide solution. Similar studies with MsTh-2 have been carried out. 1 ml of the original solution was diluted to 10 ml and the pH adjusted to the desired value. 0.5 ml of this solution was applied with a micro-pipette to a strip of Whatman No. 3 MM paper and eluted with water adjusted to the same pH as the test solution. The results, plotted in Figs. 3–6, are discussed below.

RESULTS AND DISCUSSION

The original solution contains MsTh-1 and MsTh-2 in equilibrium. The radioactivity detected either by scintillation or with the GM counter is mainly due to the presence of MsTh-2, since MsTh-1 emits only weak β and γ rays. MsTh-2 is assayed by following its decay or growth in the solutions.

Curve 1 in Fig. 1 gives the plot of pH against the percentage adsorption of MsTh-2 on the sintered glass, when the solutions are filtered immediately after adjusting the

pH. There is no significant adsorption up to pH 5, after which there is a sharp increase in adsorption. This adsorption continues to rise with the pH till a limiting value of 75 % is reached at pH 9.5.

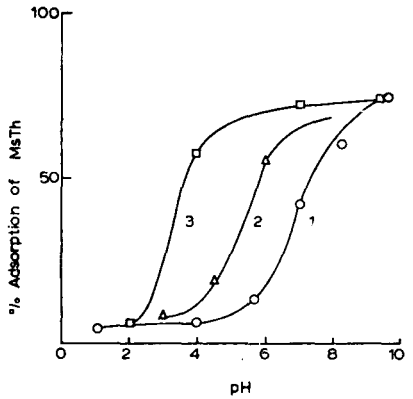

Fig. 1. Adsorption of MsTh-2 on sintered glass as a function of pH and time. Curve 1: zero hour; Curve 2: 50 hours; Curve 3: 120 hours.

When the solutions are aged, the curve shifts to the left, the shift being greater the longer the period of aging as indicated in curves 2 and 3 in Fig. 1, for 50 and 120 hours aging time respectively. This aging effect, however, cannot be interpreted in the same way as proposed by KURBATOV AND KURBATOV[4–6] and BOUISSIÈRES *et al.*[7] for the radiocolloidal formation of Y, Zr, Th and Ba in trace quantities. The mean life of MsTh-2 atoms in the solution is 8.86 hours, and so aging of its solutions for periods greater than 40 to 50 hours cannot be expected to have any effect on its adsorption behaviour. It is, therefore, surprising to find that an aging period greater than 50 hours (curve 3, Fig. 1) does influence the adsorption of MsTh-2.

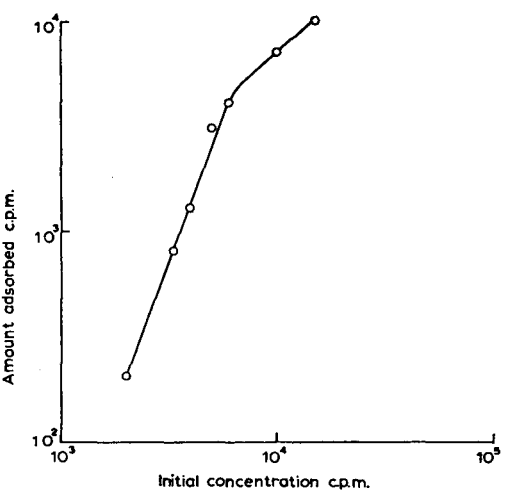

Fig. 2. Coagulation isotherm of MsTh-2 at pH 9.5.

The adsorption isotherm of MsTh-2 on sintered glass at pH 9.5, given in Fig. 2, shows that the percentage adsorption increases with the concentration becoming constant at higher concentrations of MsTh-2 (see also Table I).

TABLE I

ADSORPTION OF MsTh-2 ON SINTERED GLASS
(pH 9.5; volume of solution 10 ml)

Initial concentration c.p.m.	*Amount adsorbed c.p.m.*	*% adsorption*
2,000	211	10.5
3,300	810	24.6
4,000	1,300	32.5
5,000	3,106	62.0
6,000	4,140	69.0
10,000	7,200	72.0
15,000	10,780	71.49

The effect of concentration on the adsorption of radiocolloids on filter paper has been studied by KURBATOV *et al.*[4,5,8,9] for zirconium and thorium. They observed an increase in the adsorption with decreasing concentration of these substances, other variables remaining constant. However, in all these cases the concentration range lay between 10^{-6} and 10^{-12} g.atoms/l, whereas in our studies still lower concentrations were used. It appears from the results that in the case of MsTh-2, the (maximum) limit for the percentage adsorption is reached at concentrations as low as 10^{-12} g.atoms/l.

No variation in the adsorption of MsTh-2 on sintered glass was observed in the presence of varying amounts of KCl, although KCl was added up to an overall concentration of 0.5 *M*, as shown in Table II.

The decrease in the adsorption of zirconium and thorium reported by KURBATOV and others[4,5,8,9] was observed in the presence of a maximum concentration of 0.05 *M* KCl. The solutions of MsTh-2 used in the present series of experiments, contained comparable amounts of $BaCl_2$ (*vide infra*) and therefore further addition of KCl does not appear to influence the adsorption.

TABLE II

ADSORPTION OF MsTh-2 ON SINTERED GLASS IN THE PRESENCE OF KCl
(pH of the solution 9.5)

Concn. of KCl M	*MsTh-2 adsorbed c.p.m.*
0.00	6,619
0.02	6,969
0.5	6,800
0.10	6,647
0.20	6,728
0.50	6,791

The results shown in Table III clearly indicate that as the pH of the MsTh-2 solution increases, a correspondingly larger percentage of the activity settles at the bottom when the solutions are centrifuged; this is strong evidence for the formation of radiocolloid in MsTh-2 solutions.

TABLE III

CENTRIFUGATION STUDIES ON MsTh-2

pH	*Activity in the lower portion c.p.m.*	*Activity in the upper portion c.p.m.*	*% activity in lower portion*
1.5	5,630	8,450	40.0
2.5	5,180	8,780	37.1
4.0	7,900	6,462	55.2
6.0	9,160	4,110	69.0
7.5	11,021	1,203	90.1
9.0	13,331	1,072	92.5
9.8	12,569	1,071	92.1

The results obtained above clearly indicate that MsTh-2 in actinium behaves like a radiocolloid at pH 5. Further striking evidence for its behaviour as radiocolloid is obtained by paper chromatography. The distribution of Ba and the radioactivity due to MsTh-2 in the filter paper strip at different pH values are shown in Figs. 3–6. At pH 1.5 (Fig. 3), MsTh-2 moves with the solvent front and is detected only in the last zone; Ba also moves along with MsTh-2 since at this pH both of them are present in the ionic form. At pH 3 (Fig. 4), MsTh-2 moves only up to the second zone and at

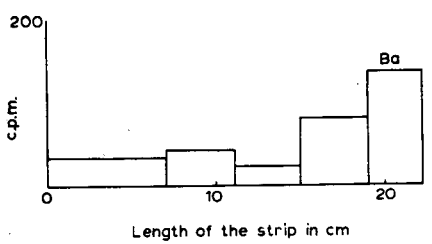

Fig. 3. Paper chromatogram of MsTh-2 and Ba at pH 1.5.

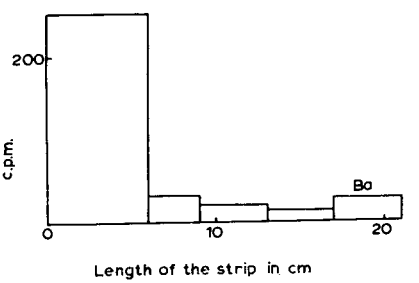

Fig. 4. Paper chromatogram of MsTh-2 and Ba at pH 3.0.

higher pH values it does not move out of the initial zone (Figs. 5 and 6). Ba moves along with the solvent front in all these cases and has been detected only in the last two zones, as shown in Figs. 3–6. The radiocolloid formation as indicated by these experiments seems to start at pH 3.0. This supports the observations made on the aged solutions of MsTh-2. Furthermore the paper chromatography strips appear to be better adsorbents than sintered glass.

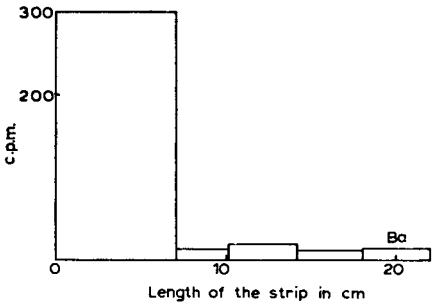

Fig. 5. Paper chromatogram of MsTh-2 and Ba at pH 8.0.

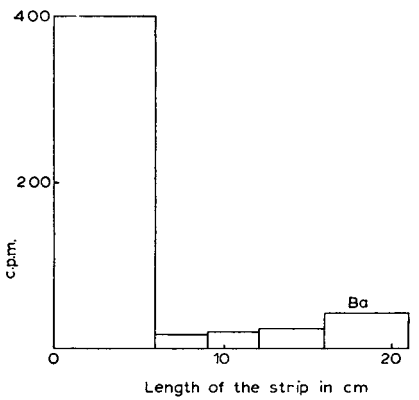

Fig. 6. Paper chromatogram of MsTh-2 and Ba at pH 9.5.

ACKNOWLEDGEMENT

The authors express their sincere thanks to Dr. JAGDISH SHANKAR for his encouragement and great interest during the course of this investigation.

SUMMARY

A report is given of the radiocolloidal behaviour of actinium as studied by adsorption on sintered glass and on paper.

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THE SEPARATION OF GLYCERIDES OF MIXED FATTY ACID CHAIN LENGTH BY GLASS PAPER CHROMATOGRAPHY

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INTRODUCTION

Acetoglycerides, or acetin fats as they are sometimes called, are synthetic mixed glycerides in which acetic acid is substituted for a portion of the long chain fatty acids. They are being considered as possible lubricants for food machinery, spreads, and as lower melting food fats[1,2]. There are presently no published reports on their separation and characterization by means of paper chromatography.

The following report describes a method which separates several glycerides of fatty acids of mixed chain length on glass paper impregnated with silicic acid.

MATERIALS

Glycerides

1,3-Distearin, diolein adipate, 1-mono-palmitin, 1-aceto-3-stearin, 1-butyro-3-palmitin, 1,3-diolein, 1-aceto-3-olein, 1,2-diaceto-3-stearin, 2-aceto-1,3-distearin, 1-monoölein, 1-monostearin, and 1,2-diaceto-3-olein were synthesized by first preparing acetone glycerol by the method of FISCHER AND PFAHLER[3], then acylating according to the method of AVERILL, ROCHE AND KING[4]. Triolein, tristearin, stearic acid, oleic acid, 1,2-dipalmitin, 1,3-dipalmitin, and palmitic acid were commercial products recrystallized to constant melting point. All compounds were shown by chemical methods to be 98–99 % pure.

Solvents

The developing solvents employed were 2:98 and 5:95 (v/v) ether in isoöctane.

METHODS

Preparation of paper

Glass fiber filter paper, type 934-AH (H. Reeve Angel and Co.)**, was cut into 4 × 7 in. sheets and heated in a 600° oven for one hour to remove the organic binder present.

* One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

** Trade names are given as part of the exact experimental conditions and not as an endorsement of the products over those of other manufacturers.

The sheets were impregnated with silicic acid by the method of DIECKERT *et al.*[5] and stored in a dust-free container until used.

Chromatographic procedure

The chromatograms were prepared essentially by the technique developed by DIECKERT AND REISER[6]. About 5 μg of material per spot was applied to the sheets of paper as approximately 0.05 % solutions in commercial hexane (Skellysolve B)*. The chromatograms were developed for 18 min, dried, then sprayed with sulfuric acid, and heated until the chromatographed materials charred. The charred spots were outlined in pencil and the R_F values determined.

RESULTS AND DISCUSSION

For this investigation the following three classes of glycerides were selected for comparison:

1. Stearins in which the stearic acid is partially replaced by acetic or butyric acids.
2. Oleins in which oleic acid is similarly replaced by acetic acid.
3. Palmitins in which palmitic acid is similarly replaced by acetic or butyric acids.

Figs. 1 and 2 represent tracings from typical chromatograms obtained using the solvent system: 2 % ether in isoöctane. The results given in Table I (A, B, and C) have been calculated from such chromatograms and are reproducible averages of

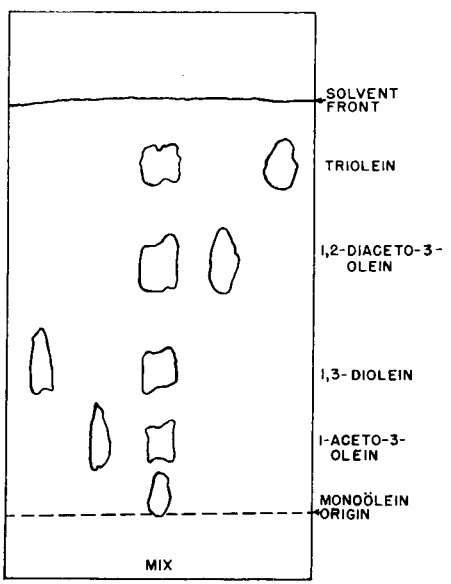

Fig. 1. Separation of oleins using 2 % ether–isoöctane as developing solvent.

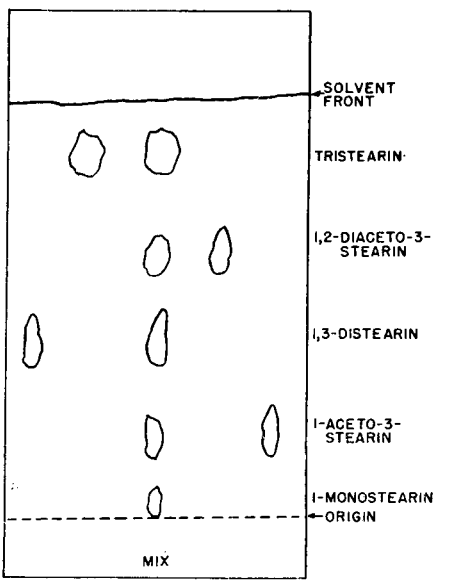

Fig. 2. Separation of stearins using 2 % ether–isoöctane as developing solvent.

* Trade names are given as part of the exact experimental conditions and not as an endorsement of the products over those of other manufacturers.

from 4–10 values each. Table IA lists the results obtained in the separation of fats containing stearic acid. With 5% ether in isoöctane as the developing solvent, the triglycerides moved so close to the solvent front that differences in R_F were not evident when only one acetyl group replaced a higher acyl group. However, development with 2% ether in isoöctane and/or replacement of a stearyl group with an

TABLE I

SEPARATION OF SUBSTITUTED GLYCERIDES IN ETHER–ISOÖCTANE SOLUTIONS

Glyceride	*Solvent, ether–isoöctane*	
	2% ether	*5% ether*
A. Stearins		
1-monostearin	0.05	0.14
1,3-distearin	0.33	0.65
tristearin	0.82	0.93
1,2-diaceto-3-stearin	0.58	0.85
2-aceto-1,3-distearin	0.76	0.94
1-aceto-3-stearin	0.21	0.40
stearic acid	0.77	0.90
1-butyro-3-stearin	0.29	0.55
B. Oleins		
1-monoölein	0.05	0.17
1,3-diolein	0.24	0.66
triolein	0.78	0.95
1,2-diaceto-3-olein	0.50	0.84
1-aceto-3-olein	0.18	0.41
oleic acid	0.67	0.87
diolein adipate*	0.08	0.21
C. Palmitins		
1-monopalmitin	0.03	0.15
1,2-dipalmitin	0.20	0.55
1,3-dipalmitin	0.26	0.68
1-aceto-3-palmitin	0.21	0.46
palmitic acid	0.75	0.89
1-butyro-3-palmitin	0.30	0.56

* The ester of 1,3-diolein and adipic acid.

acetyl group caused a decrease in the relative movement of the triglyceride. Substitution of two stearyl with acetyl groups lessened the movement still more, reducing the R_F from 0.82 to 0.76 to 0.58 for tristearin, 2-aceto-1,3-distearin, and 1,2-diaceto-3-stearin, respectively (Fig. 2). A similar separation of 1,2-diaceto-3-olein and triolein is shown in Fig. 1.

With the series of 1,3-diglycerides, the better separations were made with 5% ether in isoöctane. When 1-aceto- and 1-butyro-substituted diglycerides were chromatographed concurrently with the long chain diglycerides (Table IA and C), R_F values increased with increasing chain length in the order aceto-, butyro-, and palmito-(stearo- or oleo-)glycerides. In this system, it would seem that as fatty acid chain length increases (within each class of glycerides), there is an increase in movement of the material up the chromatogram.

There is little or no difference between the R_F values of the monoglycerides of oleic, stearic, and palmitic acids, nor between their di- or triglycerides. However, ORY *et al.*[7] separated saturated and unsaturated fatty acids by bromination at the double bond, followed by methylation, and subsequent chromatography. Since the R_F values for mono-, di-, and triglycerides are notably different, as shown in Table I and by others[6,8,9], the mono-, di-, and triglycerides of oleic and stearic acids may be conveniently separated by developing a mixture in one direction, bromination directly on the chromatogram by exposure to bromine vapors in a closed jar, and redevelopment in a second direction. This was done and, as expected, the brominated glycerides moved slower than their saturated counterparts.

This technique rapidly separates the members of a mixture of mixed glycerides based on the different mobilities of the short and long chain fatty acid components. FRANC AND JOKL[10] showed that increasing the length of the chain by increments of a methylene group in some aliphatic mono- and dicarboxylic acids resulted in different mobilities on the chromatogram.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. J. W. DIECKERT for his helpful suggestions, and T. L. WARD, N. V. LOVEGREN, and A. T. GROS for some of the glycerides used here.

SUMMARY

A chromatographic separation on glass fiber paper of mixed glycerides of acetic, butyric, stearic, palmitic, and oleic acids in solvents consisting of 2 % and 5 % ether in isoöctane has been described. In general, glycerides containing fatty acids of increasing chain length tend to move further up the chromatograms than those of shorter chain length.

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PAPER CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS
A SOLVENT TO REPLACE PHENOL*

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Good resolution of mixtures of *some* of the common amino acids can usually be obtained by a sensible choice of the various conventional solvent systems employed in two-dimensional paper chromatography. Yet, while most of the amino acids can be identified, these solvent systems fail to resolve complex mixtures of *all* of the common amino acids. Phenol has traditionally been employed as one of the solvents that enable separation of complex mixtures of the common amino acids by two-dimensional chromatography. However, the continued use of phenol is probably due to historical prominence rather than effectual attributes.

Some of the disadvantages of phenol are: (a) Phenol of sufficient purity is difficult to obtain, either in liquid or loose crystalline form. A chemical firm that supplied phenol in loose crystal form, and which we found to be of sufficient purity, has recently ceased to offer this product. (b) The purification of obtainable phenol is laborious. (c) Even with pure phenol, a solution of sodium cyanide, 8-hydroxyquinoline, or other preservatives must be placed in the chromatographic chamber to prevent phenolic decomposition during a run. (d) In our hands the amino acid spots have a tendency to "tail" and are less discrete in phenol than are the spots obtained with the reported solvent system. (e) Some of the common amino acids (*e.g.*, leucine and isoleucine) are not separated by phenol.

Because of these disadvantages we began a search for a solvent system which would replace phenol. After many trials, a solvent system consisting of *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.) proved to be the most promising. It did separate the leucines and most of the common amino acids. Nevertheless, with this system (in combination with *n*-butanol, acetic acid and water as the other dimension), glycine and serine ran together. WOLFE[1] used an *n*-butanol–methyl ethyl ketone–water (5:3:1 by vol.) solvent system containing 1 part of 17 *N* ammonia (in combination with *n*-butanol, acetic acid, water as the second dimension) to separate a mixture of all of the common amino acids. Although WOLFE's alkaline solvent system separates glycine and serine, he reported that asparagine partly overlapped lysine and arginine. We therefore endeavored to explore the possibilities of an *n*-butanol–methyl ethyl ketone–water system containing an alkaline component other than ammonia.

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HARDY *et al.*[2] reported good separation with such a system using the organic base, cyclohexylamine. Moreover, they reported that the introduction of cyclohexylamine had additional value in that it imparted variations in the usual color of several amino acids after treatment with ninhydrin. But, they also noted that solvents containing cyclohexylamine tended to turn the entire chromatographic sheet blue during the final drying unless the drying and development with ninhydrin were performed at a high temperature and for a short time (75° for 5 min). We found their findings to be essentially true; however, we experienced difficulty in preventing bluing of the entire paper even when the drying and development were performed in the suggested manner.

However, if the cyclohexylamine was placed in a separate vessel in the chamber, the vapors were sufficient to permit resolution and diverse coloration of the amino acids without the undesirable bluing of the paper. Even at lower temperatures of drying and development with ninhydrin (45° for 15 min) the blue discoloration did not occur. We noticed that if the completed chromatogram was exposed to strong light for a long period of time the paper would then develop a light blue cast. Since this would occur only after several hours of exposure to strong light, it posed no problem.

Our system consisted of *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.); and a beaker of cyclohexylamine (1 ml for every 25 ml of solvent mixture) was placed in the chamber to provide cyclohexylamine vapors. Further tests confirmed the value of this solvent system; it resolved most of the amino acids *and* also imparted distinctive colors to some of them. Because of the distinctive colors, this system or similar systems can also be used for single-dimensional chromatographic resolution of amino acids in mixtures that could not be resolved by phenol or other one phase systems[2]. Furthermore, when employed in quantitative two-dimensional chromatography, this type of system yields much better amino acid recoveries than phenolic systems[1].

The following procedure was employed for qualitative two-dimensional chromatography. A mixture of amino acids was spotted on Whatman No. 1 filter paper, 46 × 57 cm, and subjected to descending development in the first dimension (30 h) with *n*-butanol–acetic acid–water (25:6:25 by vol., WOIWOD[3]), and in the second dimension (20 h) with our solvent system*. The development took place at a temperature of 21° ± 1°. After final development the chromatograms were dried for 30 min at 45° and then dipped in a 0.25 % w/v solution of ninhydrin in acetone containing 7 % v/v glacial acetic acid[2], and finally heated for 15 min at 45°. The colors reported in Table I could be intensified, when necessary, if a hair drier was held directly over the spots soon after the final heating. R_F values (Table I) were determined in the usual manner. Since better resolution was obtained when the solvent front was allowed to run off the bottom of the paper, the distances traveled by the amino acids are also expressed as ratios of the distance between the origin and phenylalanine, *i.e.*, R_φ values (Table I).

* These times (30 and 20 h) permitted the solvent fronts to run off the bottom of the paper.

TABLE I

R_F AND R_φ VALUES OF AMINO ACIDS AND THE COLORS THEY DEVELOP IN *n*-BUTANOL–METHYL ETHYL KETONE–WATER PLUS CYCLOHEXYLAMINE VAPORS AFTER NINHYDRIN TREATMENT

(See text for details)

No.	*Amino acid*	R_F	R_φ	*Color*
1	Phenylalanine	0.63	1.00	Blue-grey
2	Tryptophan	0.60	0.96	Brown-grey
3	Leucine	0.57	0.91	Purple
4	Isoleucine	0.54	0.86	Purple
5	Threonine	0.52	0.82	Purple
6	Methionine	0.46	0.75	Purple
7	Tyrosine	0.43	0.69	Violet-grey
8	Valine	0.42	0.67	Purple
9	Serine	0.26	0.41	Purple
10	Histidine	0.25	0.40	Green-grey
11	Cysteic acid	0.24	0.39	Purple
12	Proline	0.24	0.38	Yellow
13	α-Alanine	0.22	0.35	Purple
14	Hydroxyproline	0.19	0.30	Carmine → beige†
15	Glycine	0.18	0.28	Purple → violet-grey†
16	Cystine	0.16	0.25	Brown-grey
17	β-Alanine	0.16	0.25	Royal blue
18	Asparagine	0.15	0.24	Beige
19	Glutamine	0.15	0.24	Purple
20	Cysteine	0.14	0.21	Violet-grey
21	Glutamic acid	0.12	0.19	Purple
22	Aspartic acid	0.11	0.18	Royal blue
23	Lysine	0.09	0.15	Purple
24	Arginine	0.07	0.11	Purple

† Original colors change to the indicated color after a few hours.

The photograph (Fig. 1) of a two-dimensional chromatogram illustrates the excellent resolution of a mixture of the common amino acids*. The only spots that lie somewhat close to one another are; 6 (methionine, purple) and 7 (tyrosine, violet-grey); and, 12 (proline, yellow) and 13 (alanine, purple). These spots are readily distinguished by their obvious color differences.

* Comparable chromatograms, using hydrolyzed and unhydrolyzed tissues, are currently being obtained in our studies of the amino acid composition of regenerating newt limbs.

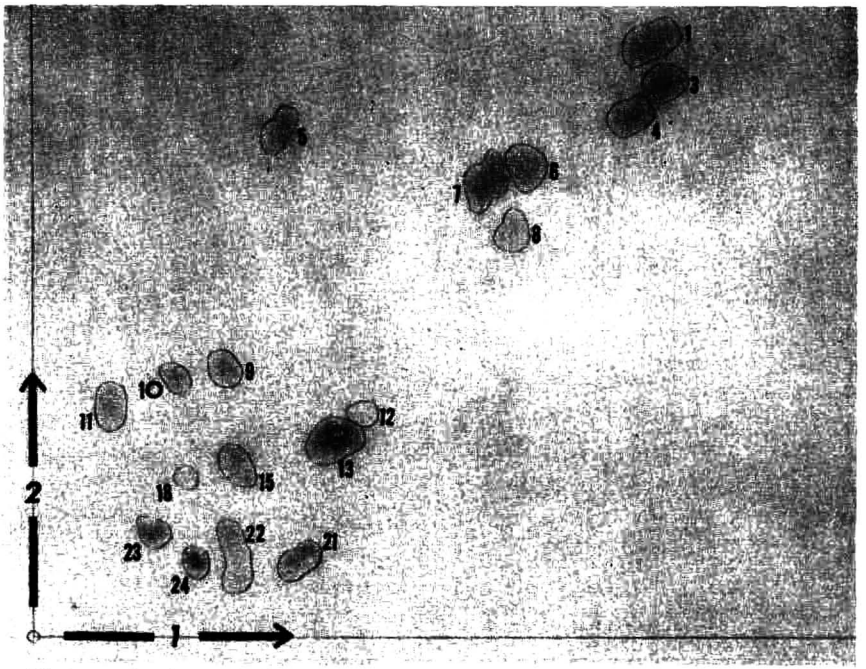

Fig. 1. Photograph of a typical two-dimensional chromatogram of a composite mixture of the common amino acids. Tryptophan and cystine were not spotted for this particular run; however they separate well and do not overlap any of the other amino acids (see R_F values in Table I). First dimension, *n*-butanol–acetic acid–water (25:6:25 by vol.), second dimension, *n*-butanol–methyl ethyl ketone–water (2:2:1 by vol.) plus cyclohexylamine vapors. See Table I for identity of spots.

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SUMMARY

The solvent *n*-butanol–methyl ethyl ketone–water (2:2:1) with an atmosphere saturated with cyclohexylamine yields excellent separation of mixtures of all the common acids, is readily reproducible, lacks the capriciousness of phenolic systems, and furthermore, imparts distinctive colors to many of the amino acids after ninhydrin treatment. These characteristic colors serve to confirm identification based on control runs for these amino acids and also serve as valuable "landmarks" facilitating the identification of adjacent amino acids that have the customary purple ninhydrin color.

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SEPARATION OF HEAVY RARE EARTHS BY REVERSED-PHASE PARTITION CHROMATOGRAPHY

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In our previous paper[1] it was shown that small amounts of some light rare earths can be clearly separated by means of reversed-phase partition chromatography, using tributyl phosphate (TBP) as the stationary phase and HNO_3 as the mobile phase. The separation of heavy rare earths by this method appears to be more difficult, since their separation factors are generally smaller than those of the light rare earths[2,3]. Nevertheless, it seems that the effect of the smaller separation factors can be outweighed by using columns of better separating power. The present paper describes the results obtained with the heavy rare earths from Tb to Lu using HNO_3 and HCl as eluting agents.

EXPERIMENTAL

The radioactive rare earths were prepared by irradiation of the appropriate oxides in the Polish reactor "EWA".

The columns used in the course of this work were of the same size as previously described[1] and were prepared by the same method. Special attention was paid to the packing of the beds in order to obtain columns with the highest separating power. The flow rate was 4 drops per minute or 0.4 $ml.cm^{-2}.min^{-1}$. The total amount of each of the rare earths introduced into the column was less than 0.01 mg, and the volume of the radioactive solution was 0.04 ml.

RESULTS AND DISCUSSION

Elution with HNO_3

The separation of Sm, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu with 11.5 *M* HNO_3 as eluting agent is shown in Fig. 1. With this concentration of HNO_3 there is only slight overlapping of the Tb and Dy curves. Better separation can be achieved with 12.3 *M* HNO_3 (Fig. 2), and still better with 13.0 *M* HNO_3 (Fig. 3), but in the latter case the time needed for elution of all the heavy rare earths is too long, since the partition coefficients are too high. The positions of the maxima of heavy rare earths, when eluted with 12.3 *M* HNO_3, relative to the position of Tb, and the partition coefficients at the same acid concentration relative to the partition coefficient of Tb are given in Table I. The partition coefficients with 12.3 *M* HNO_3 were taken from the paper published by HESFORD *et al.*[3]. Since in the calculation of the position of

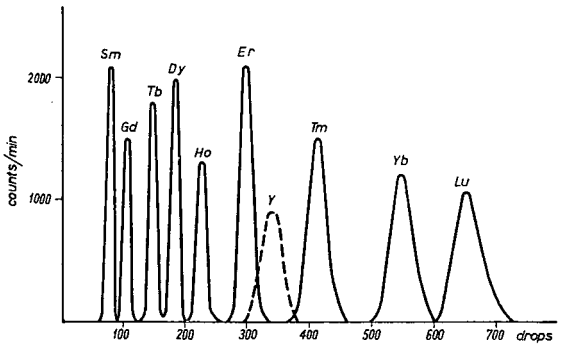

Fig. 1. Separation of Sm–Gd–Tb–Dy–Ho–Er–Tm–Yb–Lu with 11.5 M HNO_3.

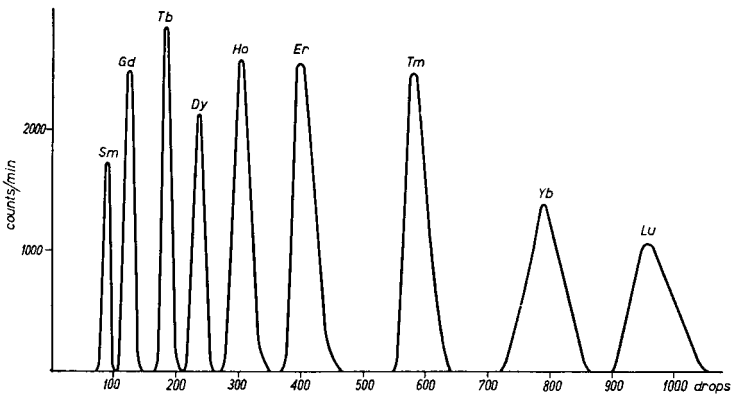

Fig. 2. Separation of Sm–Gd–Tb–Dy–Ho–Er–Tm–Yb–Lu with 12.3 M HNO_3.

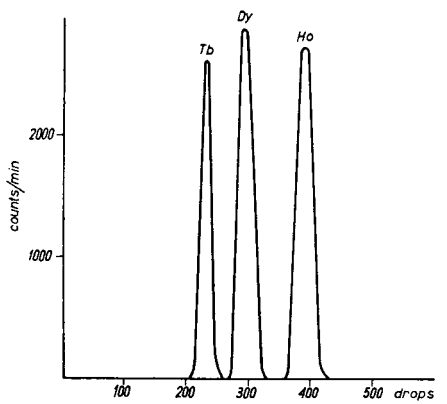

Fig. 3. Separation of Tb–Dy–Ho with 13 M HNO_3.

the maximum, the first free volume was always subtracted, the two sets of values can be directly compared. This comparison shows that the differences between the values obtained by dynamic and static methods are not great and can be caused by experimental errors occurring in both methods. The separation factor defined as the

TABLE I

POSITIONS OF THE MAXIMA AND THE PARTITION COEFFICIENTS OF HEAVY RARE EARTHS RELATIVE TO THOSE OF Tb

Eluting agent: 12.3 *M* HNO_3.

	Tb	*Dy*	*Ho*	*Er*	*Tm*	*Yb*	*Lu*
Position of the maximum	1.0	1.5	2.0	2.8	4.3	6.0	7.4
Partition coefficient	1.0	1.6	2.1	2.9	4.4	5.9	7.2

ratio of the positions of the two maxima is given in Table II. It appears from these data, that the separation factor for the Yb–Lu pair is exceptionally small and that this pair plays the same role among the heavy rare earths as the Eu–Gd pair does among the light rare earths. The position of Y when eluted with 11.5 *M* HNO_3 is between Er and Tm (see Fig. 1).

TABLE II

SEPARATION FACTORS OF PAIRS OF NEIGHBOURING HEAVY RARE EARTHS

Eluting agent: 12.3 *M* HNO_3.

Tb–Dy	*Dy–Ho*	*Ho–Er*	*Er–Tm*	*Tm–Yb*	*Yb–Lu*
1.5	1.33	1.4	1.53	1.4	1.23

Elution with HCl

It has been shown by PEPPARD *et al.*[4], that rare earths are extracted by TBP from HCl solutions. It therefore seemed worthwhile to find out whether HCl can be used as eluting agent instead of HNO_3. In Figs. 4 and 5 two examples of elution of some

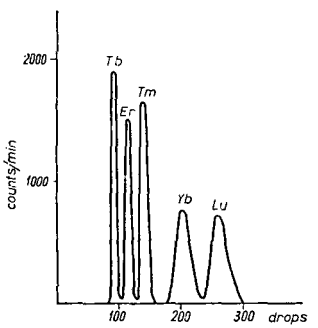

Fig. 4. Separation of Tb–Er–Tm–Yb–Lu with 11.8 *M* HCl.

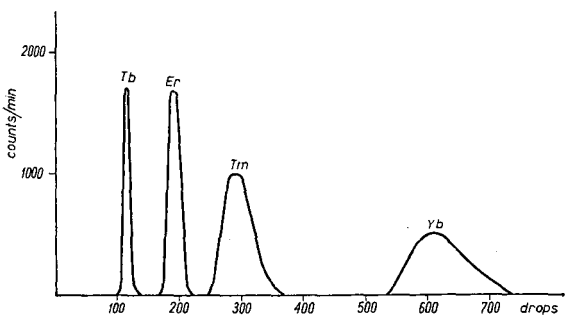

Fig. 5. Separation of Tb–Er–Tm–Yb with 12.3 *M* HCl.

heavy rare earths with 11.8 *M* and 12.3 *M* HCl are shown. As can be readily seen the elution curves are not much less favourable than those obtained with HNO_3 as eluting agent. The separation factor in the system HCl–TBP calculated from the positions of the maxima (see Tables III and IV) is even greater than in the system HNO_3–TBP. Nevertheless, only the heaviest rare earths can be separated by elution

TABLE III

POSITIONS OF THE MAXIMA OF SOME HEAVY RARE EARTHS RELATIVE TO THE POSITION OF THAT OF Tb

Eluting agent: HCl.

Concentration of HCl M	*Tb*	*Er*	*Tm*	*Yb*	*Lu*
11.8	1.0	2.0	3.2	6.2	9.2
12.3	1.0	2.9	5.4	13.6	—

TABLE IV

SEPARATION FACTORS OF SOME PAIRS OF HEAVY RARE EARTHS

Eluting agent: HCl.

Concentration of HCl M	*Tb–Er*	*Er–Tm*	*Tm–Yb*	*Yb–Lu*
11.8	2.0	1.6	1.94	1.48
12.3	2.9	1.86	2.52	—

with HCl, since the partition coefficients for lighter rare earths are too small even in the most concentrated solutions of HCl available. It should be noted that the influence of the HCl concentration on the positions of the maxima is very strong, and that the separation factor for the Yb–Lu pair is smaller than that for the Tm–Yb pair, as in the case of HNO_3 as eluting agent.

The effect of the amount of rare earth and of the volume of the solution introduced into the column

It has been observed in the course of the work with light rare earths that the shape of the elution curve of gadolinium and the position of its maximum depend on the amount of gadolinium introduced into the column. In order to examine this effect more closely two sets of experiments with the Tb–Tm pair were carried out. In the first set of experiments the amount of Tm was kept constant and very small (below 0.01 mg) and the amount of Tb was gradually increased up to 0.8 mg. In the presence of more than about 0.1 mg of Tb carrier a change in the shape of the elution curve of Tb was noted. The curve became broad and asymmetric and the position of its maximum was shifted towards the left. This effect increased with the amount of added carrier. The influence on the elution curve of Tm was less pronounced, although

a broadening of the curve and the formation of a tail were also observed. In the second set of experiments the amount of Tb was kept small and constant, and increasing amounts of Tm up to 0.4 mg were successively added. In that case the position and shape of the elution curve of Tb did not change, but the elution curve of Tm was shifted towards the left, so that the initial separation factor of 3.3 for small amounts of both rare earths with 10.6 M HNO_3 decreased to 1.85 when the amount of Tm was increased to 0.4 mg. The shape of the elution curve of Tm was changed too, the curve becoming very asymmetric and broad. The experiments described above show that the addition of carrier does change the position and shape of its own curve. The effect on the curve of another substance present in the system in microamounts depends on whether the partition coefficient of this substance is smaller or greater than that of the substance added, the effect being greater in the second case.

The effect of the volume of the radioactive solution introduced into the column on the shape of the curve was investigated. No effect was observed with volumes smaller than 0.07 ml. With larger volumes the curves became broad, but the position of the maximum did not change.

SUMMARY

Reversed-phase partition chromatography with tributyl phosphate as the stationary phase was applied to the separation of heavy rare earths from Gd to Lu. As eluting agent 11.5 M, 12.3 M and 13.0 M HNO_3 were used. The heaviest rare earths can also be separated with HCl as eluting agent. The influence of the amount of the rare earth introduced into the column on the shape of the elution curve was investigated.

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PAPER CHROMATOGRAPHY OF NITRATE AND TOSYLATE ESTERS

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Several paper chromatographic solvent systems containing methanol have been reported for the separation of hydrophobic substances. RICE, KELLER AND KIRCHNER[1] described the paper chromatography of 3,5-dinitrobenzoates of various alcohols using 2% methanol in petroleum ether. MEIGH[2] followed up this work but used *n*-heptane saturated with methanol. BUSH[3], apparently independently, used methanol-containing solvents for the paper chromatography of steroids. These procedures used conventional filter paper without pretreatment as the support medium and the solvent was irrigated downwards after allowing the spotted paper to equilibrate in the vapours of the two-phase organic solvent for 4 to 18 hours. In the present work several nitrate and tosylate esters of polyhydroxy compounds were found to chromatograph well in this type of system. Although neither of these two types of compounds could be chromatographed successfully on paper with aqueous-organic solvents it was possible to obtain good separation within a group of diastereoisomers with the methanol–alkane system.

Paper chromatography, as generally practised, is only suitable for substances which are more or less hydrophilic. The stationary phase in these systems consists of the aqueous part of the solvent sorbed on the cellulose and will be hydrophilic. A compound must be at least partially soluble in the stationary phase in order for it to move with an R_F value of less than 1.0. In the case of the solvent systems containing methanol, hydrocarbon, and very little water it is probable that the stationary phase will consist mainly of methanol and hence will be less hydrophilic.

Several methanol–hydrocarbon solvent systems (Table I) were tested with the nitrate and tosylate esters of the three diastereoisomeric 1,4:3,6-dianhydrohexitols (isohexides). *n*-Hexane or petroleum ether, b.p. 60–75°, saturated with methanol was found to be the most useful system for the chromatography of the isohexide dinitrates and clear separations were obtained. The corresponding tosylate esters did not move from the starting line in this solvent but could be separated when benzene was added to the system. Three series of variations in the solvent system were tested:

(a) Variation of the hydrocarbon in the methanol–alkane system was found to affect the R_F values of the nitrate esters (Table II). An increase in the length of the

TABLE I

COMPOSITIONS OF SOLVENT SYSTEMS (v/v) *

System	*Alkane***	*Benzene*	*Methanol*	*Water*
A	20	–	10	–
B	20	–	20	–
C	20	–	20	–
D	20	–	9	1
E	20	–	8	2
F	20	–	7	3
G	18	2	8	2
H	16	4	8	2
I	16	4	9	1

* The proportions of hydrocarbon to methanol–water are not critical provided two layers are obtained. The proportions of alkane to benzene and of methanol to water are critical.
** *n*-Hexane or petroleum ether, b.p. 60–75°, except for B (*n*-heptane) and C (*n*-octane).

TABLE II

EFFECT OF HYDROCARBON ON R_F VALUES OF ISOHEXIDE DINITRATES

Solvent system	*Alkane*	*Isomannide dinitrate*	*Isosorbide dinitrate*	*Isoidide dinitrate*
A	*n*-Hexane	0.20	0.26	0.42
B	*n*-Heptane	0.12	0.175	0.31
C	*n*-Octane	0.07	0.10	0.20

alkane chain caused a decrease in R_F value although similar separation patterns for the isomers were obtained in each solvent.

(b) Absolute methanol was completely miscible with petroleum ether but addition of very small amounts of water caused separation into two layers. Since it was more convenient to have water present in the system a series of runs was made to determine the effect of the addition of different amounts of water (Table III). As the proportion of water in the methanol was increased, the R_F values of the nitrate esters were increased.

TABLE III

EFFECT OF WATER ON R_F VALUES OF ISOHEXIDE DINITRATES

Solvent system	*Ratio water: methanol*	*Isomannide dinitrate*	*Isosorbide dinitrate*	*Isoidide dinitrate*
A	0:1	0.20	0.26	0.42
D	1:9	0.18	0.26	0.43
E	2:8	0.25	0.34	0.54
F	3:7	0.50	0.59	0.72

(c) Addition of benzene to the solvent system increased the R_F values for both types of esters (Table IV). While the tosylate esters did not chromatograph in the methanol–alkane system, the addition of benzene brought about a clear separation. The effect of water on the benzene-containing system was the same as for the methanol–alkane system.

TABLE IV

EFFECT OF ADDED BENZENE ON R_F VALUES

Ratio water: methanol	*Ratio benzene: alkane*	*Solvent system*	*Isomannide dinitrate*	*Isosorbide dinitrate*	*Isoidide dinitrate*	*Isomannide ditosylate*	*Isosorbide ditosylate*	*Isoidide ditosylate*
0:1	0:1	A	0.20	0.26	0.42	0.0	0.0	0.0
2:8	1:9	G	0.34	0.45	0.60	0.18	0.36	0.58
2:8	2:8	H	0.49	0.57	0.68	0.43	0.58	0.66
1:9	2:8	I	0.44	0.51	0.63	0.24	0.40	0.55

The effects of the variations in the solvent system described above could be readily explained in terms of the solubilities of the esters in the stationary and mobile phases. The nitrate esters were less soluble in the higher alkanes and so less soluble in the mobile phase and a lower R_F was found. Addition of water, which would go mainly to the stationary phase, decreased the solubility in this phase and thus increased the R_F value. Benzene increased the solubility in the mobile phase and again the R_F was increased.

It is apparent that this type of solvent system is capable of being varied to suit a fairly wide range of hydrophobic substances. Since standard filter paper may be used without pretreatment or impregnation, a considerable saving in time and material may be realised. For the nitrate esters solvent systems D and E (Table I) are recommended, and for tosylate esters system H. The R_F values were reproducible to $\pm$ 0.02 with these systems.

KITCHEN[4] has studied the petroleum ether–methanol system using ascending irrigation. This method gave lower R_F values than the descending method and poorer separation; however, variations in the solvent system were not studied.

The two groups of compounds were detected on the developed and dried chromatograms by spraying with 1% alcoholic solution of diphenylamine, drying and exposing to short wave u.v. light for 10 min. The nitrate esters were observed as yellow spots in daylight or black spots under the u.v. light[7]. The tosylate esters were observed as strongly fluorescent spots under the u.v. light. The nitrate esters could also be detected by spreading a solution of diphenylamine in concentrated sulphuric acid over the paper supported on a glass plate. Deep blue spots[8] were readily observed at nitrate locations but the paper disintegrated after about 15 min. In contrast, the photochemical detection method provided a permanent record.

The photochemical reaction may also be used to detect nitrate ion[9] and diphenylamine and is being further investigated.

EXPERIMENTAL

Materials

All solvents were C.P. grade and were used without further purification. The nitrate and tosylate esters were identical with samples described previously[5,6]. Whatman No. 1 filter paper was used throughout.

Procedure

Dishes containing the two layers of the solvent mixture were placed at the bottom of the chromatographic tank and the top trough was filled with the hydrocarbon layer. The filter paper sheet was spotted with the materials to be tested and fastened to a glass rod which was then suspended directly below the lid of the tank by strings passing through a hole in the lid or by a glass rod in a device similar to that described by POTTER, LINDAY AND CHAYEN[10]. The paper was held over the top trough for at least 4 h, then lowered into the solvent so that descending irrigation could take place. The solvent was fast running (about 20 cm/h) and after a suitable development time the paper was removed and the solvent front marked quickly before the solvent evaporated.

The paper was allowed to dry, sprayed with 1 % alcoholic diphenylamine, dried and exposed to u.v. light for 5 to 10 min. The nitrate esters were detected as yellow spots on a white background in daylight or black on white under u.v. light. The tosylate esters were strongly fluorescent under the u.v. light.

ACKNOWLEDGEMENT

This work was supported by the Defence Research Board of Canada.

SUMMARY

Three water-insoluble diastereoisomeric nitrate esters were cleanly separated on untreated filter paper by the use of alkane–methanol solvents. Addition of benzene to the solvent system permitted a similar separation of the corresponding ditosylates. The effects on the R_F values of variations in the solvent compositions were readily explained on the basis of the ester solubilities. The method should be applicable to a wide range of hydrophobic substances.

A photochemical reaction of diphenylamine with the esters has been developed as a convenient method for detecting them on chromatograms.

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A SIMPLE METHOD OF PLOTTING THEORETICAL CURVES OF NET IONOPHORETIC MOBILITY AS A FUNCTION OF pH

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CONSDEN, GORDON AND MARTIN[1] derived a formula relating the net ionophoretic mobility of an organic electrolyte to the pH of the buffer solution present in the paper strip. They also gave formulas for the pH value at which the difference in net mobilities of two electrolytes is maximal, and worked out a chart by means of which this pH value can be found. It must be pointed out, however, that in certain cases the position of the maximum does not correspond to the optimum separation conditions and larger net mobility differences are obtained when both electrolytes are completely ionized. Such a case is illustrated in Fig. 1. Moreover, when the mixture

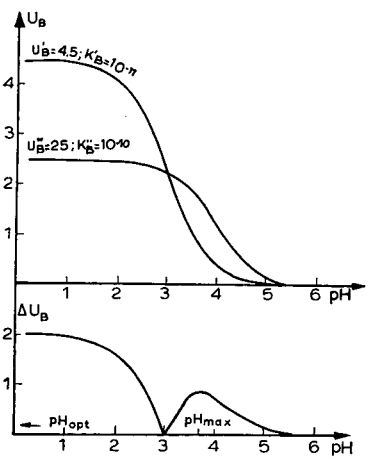

Fig. 1.

to be separated is composed of three or more components, a different pH_{opt} is obtained for each pair of components. Therefore, it would be more convenient to know the graphical dependence of the net mobilities of the components in the whole pH range; plotting of such a dependence is a much simpler task than it appears to be at first sight, if some mathematical properties of the function $U = f(pH)$ are taken into account.

The formulas for net mobility given by CONSDEN *et al.*[1] can be represented as follows:

$$U_A = \frac{u_A}{1 + \frac{[H^+]}{K_A}} \text{ (acid)} \qquad U_B = \frac{u_B}{1 + \frac{[OH^-]}{K_B}} \text{ (base)} \tag{1}$$

where u is the ionic mobility and U is the observed, net mobility (at a given pH). The indices A, B denote acid and base, respectively.

$U = f(pH)$ curves are S-shaped ;the height of a curve is equal to u, the ionic mobility; the inflexion point of a curve is its center of axial symmetry, and lies at half-height of the curve ($\frac{u}{2}$), and at a pH equal to the pK_A of the acid (or $pK_A = 14 - pK_B$ of a base). It can easily be shown that the position of a curve depends solely upon the ionization constant, and the shape solely upon the ionic mobility u.

Denoting anionic net mobility as positive, and cationic net mobility as negative, the relationship between net mobility and pH can be represented as shown in Fig. 2.

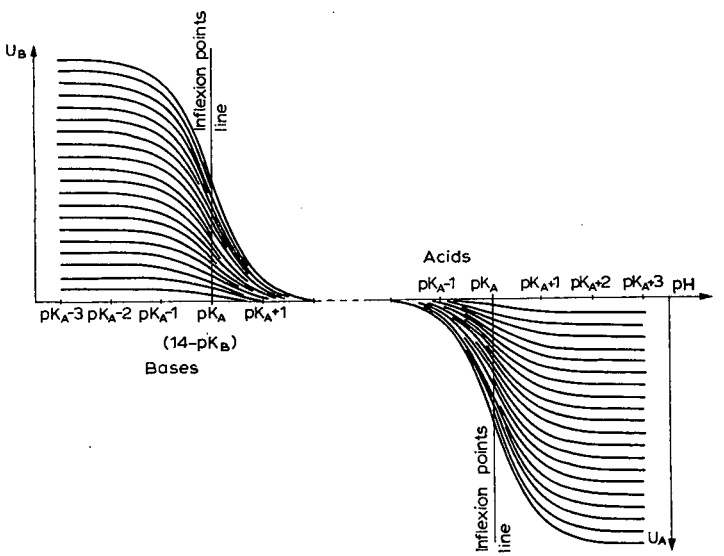

Fig. 2.

The diagram can be used to plot the theoretical curve $U = f(pH)$ in the whole pH range for a substance of any ionization constant and ionic mobility u. First, on a sheet of tracing paper a system of coordinates is drawn with the pH axis in the same scale as in Fig. 2, and the net mobility (U) axis in such a scale that the ionic mobility u of the substance does not exceed the highest curve of Fig. 2. The sheet of tracing paper is then laid upon Fig. 2 (right hand side if the substance is an acid, left hand side if the substance is a base) and then shifted horizontally, with the pH axes overlapping, until the line of inflexion points of Fig. 2 coincides with the pH value on the tracing paper equal to the pK_A of the substance. The correct position of the

TABLE I

NET IONOPHORETIC MOBILITY U

pH* \ u	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
$pK_A \pm 3$	5.00	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0	55.0	60.0	65.0	70.0	75.0	80.0	85.0	90.0	95.0	100.0
$pK_A \pm 2.5$	5.0	10.0	14.9	19.9	24.9	29.9	34.9	39.9	44.9	49.8	54.8	59.8	64.7	69.7	74.7	79.6	84.6	89.6	94.6	99.6
$pK_A \pm 2$	5.0	9.9	14.8	19.8	24.8	29.7	34.6	39.6	44.5	49.5	54.4	59.4	64.3	69.2	74.2	79.2	84.1	89.0	94.0	99.0
$pK_A \pm 1.5$	4.9	9.7	14.5	19.4	24.2	29.1	33.9	38.8	43.6	48.5	53.3	58.2	63.0	67.8	72.7	77.5	82.4	87.2	92.1	97.0
$pK_A \pm 1$	4.5	9.1	13.6	18.2	22.8	27.3	31.8	36.4	40.8	45.5	50.0	54.5	59.1	63.7	68.2	72.8	77.3	81.8	86.4	90.9
$pK_A \pm 0.5$	3.8	7.6	11.4	15.2	19.0	22.8	26.6	30.4	34.2	38.0	41.8	45.6	49.3	53.3	57.0	60.8	64.7	68.5	72.2	76.0
pK_A	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0	37.5	40.0	42.5	45.0	47.5	50.0
$pK_A \mp 0.5$	1.2	2.4	3.6	4.8	6.0	7.2	8.4	9.6	10.8	12.0	13.2	14.4	15.7	16.7	18.0	19.2	20.4	21.6	22.8	24.0
$pK_A \mp 1$	0.5	0.9	1.4	1.8	2.3	2.7	3.2	3.6	4.1	4.6	5.0	5.5	5.9	6.4	6.8	7.3	7.7	8.2	8.6	9.1
$pK_A \mp 1.5$	0.15	0.31	0.46	0.61	0.77	0.92	1.07	1.23	1.38	1.53	1.69	1.84	2.00	2.15	2.30	2.46	2.61	2.76	2.92	3.07
$pK_A \mp 2$	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.64	0.70	0.74	0.79	0.84	0.89	0.94	0.99
$pK_A \mp 2.5$	0.01	0.03	0.05	0.06	0.08	0.09	0.11	0.13	0.14	0.15	0.17	0.19	0.21	0.22	0.23	0.25	0.27	0.28	0.30	0.32
$pK_A \mp 3$	0.00	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05	0.06	0.06	0.07	0.07	0.08	0.08	0.09	0.09	0.10	0.10

* The upper sign refers to acids, the lower sign to bases.

plot having thus been found, the curve of which the height is equal to the u of the substance is copied. If experimental points have been plotted on the tracing paper and one of the theoretical curves of Fig. 2 is made to fit these points, then the line of inflexion points of Fig. 2 will indicate a pH value on the experimental plot on the tracing paper, that is equal to the pK_A of the substance (*cf.* SILLÉN[4]).

For plotting theoretical curves in any scale, Table I may also be used.

The method is similar to that of plotting theoretical $R_F = f(pH)$ curves in buffered paper chromatography[2] and $K = f(pH)$ curves[3] (K = partition ratio).

Example

Three bases have the following ionic mobilities and ionization constants:

(1) $u'_B = 5$ $K'_B = 10^{-10}$

(2) $u''_B = 4.5$ $K''_B = 10^{-11}$

(3) $u'''_B = 2.5$ $K'''_B = 10^{-8}$

The plotted theoretical curves are given in Fig. 3. It can be seen that the resolution of all three components is obtained when pH = 3.5. At this pH substance 1 is the fastest and substance 2 the slowest.

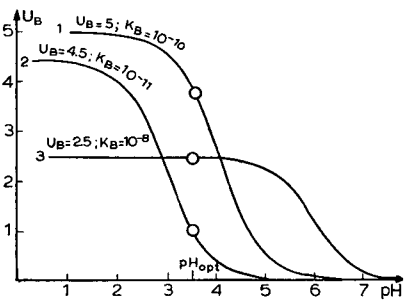

Fig. 3.

Of course, in formula (1) adsorption and other phenomena have not been taken into account, so that deflections from the theoretical curves are to be expected in practice.

SUMMARY

A graphical method for the estimation of separation efficiency as a function of pH in paper ionophoresis of organic acids and bases is described.

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CHROMATOGRAPHISCHE UNTERSUCHUNGEN ZUR ISOLIERUNG UND REINIGUNG VON TRYPSIN

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(Eingegangen den 30. Juni 1960)

Über die Trennung und Reinigung von Proteinen insbesondere von Enzymen mittels chromatographischer Methoden ist bereits mehrfach berichtet worden[1–13].

Wir verwendeten zur chromatographischen Isolierung und Reinigung von Trypsin aus handelsüblichen Präparaten und Pancreasextrakten als stationäre Phase CAM-Pulver (Schleicher und Schüll), einen Kationenaustauscher auf Cellulosebasis. Das CAM-Pulver wurde in 0.2 *N* Na-Phosphatlösung, pH 6.8[14], aufgeschlämmt und ohne weitere Vorbehandlung in die chromatographischen Kolonnen eingefüllt. Es wurden für analytische Untersuchungen Säulen von 15 × 0.9 cm verwendet. Der beste Trenneffekt konnte mit der genannten Pufferlösung erreicht werden, nachdem mit anderen Eluierungsmitteln mit pH-Werten zwischen pH 4.25 und 9.00 keine Adsorption erreicht werden konnte, die bei einer nachfolgenden Eluierung eine geeignete Trennung der einzelnen Komponenten gewährleistet hätte. Die Durchflussgeschwindigkeit der Pufferlösungen ist auf 10 ml/h bemessen worden. Die Chromatographie erfolgte bei Raumtemperatur. Der Nachweis der getrennten Substanzen er-

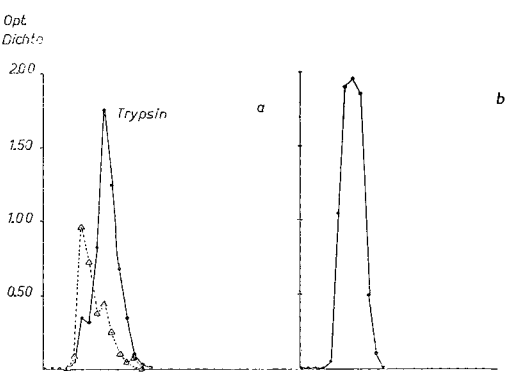

Fig. 1. Chromatographische Trennung von Trypsin an 15 × 0.9 cm CAM-Säulen im analytischen Masstab. (a) ●———● Verlauf der Elution von Trypsin (Merck), der mit "Trypsin" bezeichnete Gipfel enthielt Trypsinaktivität; △———△ Chromatogramm von Pancreatin (Merck). Elutionsmittel: 0.2 *N* Na-Phosphatlösung pH 6.8. Volumen der Eluatfraktionen: 2 ml. (b) Chromatogramm von Trypsin (Merck). Elutionsmittel: 0.2 *N* Na-Citratlösung, pH 4.25. Volumen der Eluatfraktionen: 2 ml.

* Direktor: Prof. Dr. E. SCHÜRMANN.

folgte mit Hilfe eines modifizierten Ninhydrin-Reagenzes[15]. Die für die analytischen Arbeiten verwendeten Mengen an Trypsin-Präparaten (10 mg Protein) sind in der Pufferlösung pH 6.8 auf die Säulen aufgetragen worden.

Die Ergebnisse der Chromatographie von trypsinhaltigen Präparaten im *analytischen Masstab* sind in Fig. 1 dargestellt.

Zur Trennung und Reinigung von trypsinhaltigen Präparaten (Trypsin (Merck), Pancreatin (Merck) sowie Pancreatin (Schwab)) im *präparativen Rahmen* verwendeten wir CAM-Säulen von 15 cm Länge und Durchmesser von 2 cm. Die Kolonnen sind ebenfalls mit dem Puffer pH 6.8 äquilibriert worden. Die je Analyse auf die Säulen aufzutragenden Mengen sind auf 100–200 mg Protein bemessen worden. Das aus den Säulen austretende Eluat wurde in Portionen von 5 ml aufgefangen; ein aliquoter Teil jeder Eluatfraktion ist nach der Ninhydrin-Methode analysiert worden. Nur der in der Fig. 2 als "Trypsin" bezeichnete Gipfel besass Trypsin-Aktivität.

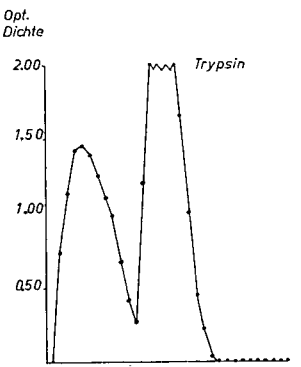

Fig. 2. Chromatographische Reinigung von Trypsin an 15 × 2 cm CAM-Säulen. Nur der als "Trypsin" bezeichnete Gipfel enthielt Trypsinaktivität. Elutionsmittel: 0.2 *N* Na-Phosphatlösung pH 6.8. Volumen der Eluatfraktionen: 5 ml.

ZUSAMMENFASSUNG

Es wird eine Methode zur Chromatographie von Trypsin an Cellulose-Austauschern (CAM) für analytische und präparative Zwecke beschrieben.

SUMMARY

A method for the chromatography of trypsin on cellulose-exchangers (CAM) for analytical and preparative work is described.

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Short Communications

Ein Verhältnismasstab zur schnellen Bestimmung des R_F-Wertes

Im Laufe der letzten Jahre wurden verschiedene Geräte zur direkten Messung des R_F-Wertes auf Papierchromatogrammen entwickelt. Durchsichtige Arbeitsschablonen gestatten beim Auflegen auf das Chromatogramm mit Hilfe von Schlüssellinien, den R_F-Wert abzulesen. Solche Schlüsselblätter sind im Handel zu haben. ROCKLAND UND DUNN[1] schlugen ein durchsichtiges, liniertes Dreieck—Partogrid—für diesen Zweck vor. Auch mit einem geeigneten Proportionalzirkel kann man den R_F-Wert sofort abgreifen[2]. CLEMENTS[3] konstruierte eine mechanische Vorrichtung bei der ein Winkel von einer diagonalen Zunge bestrichen wird. Ein elektrisches Auswertegerät, das nach dem Prinzip der Analogierechenmaschine arbeitet, baute BERBALK[4].

Bei unserem Verhältnismasstab griffen wir auf den Grundgedanken von PHILLIPS[5] zurück, der schon 1948 ein elastisches Band zur schnellen R_F-Wert-Bestimmung benutzte (siehe auch[6]). Wir montierten auf eine 2 cm breite, linealähnliche Leiste von etwa 50 cm Länge ein 20–25 cm langes, 0.5–1 cm breites (mit Stoff durchwirktes) Gummiband, das mit Hilfe eines Läufers beliebig gedehnt werden kann. Die Leiste trägt eine Zentimetereinteilung, das Gummiband eine Skala von 0–100; dabei ist es so fixiert, dass die beiden Nullpunkte zusammenfallen. Weiterhin liegt es etwa 3 mm vom Rand der Leiste entfernt, so dass beide Skalen gleichzeitig abzulesen sind. Eine Vorstellung vermittelt Fig. 1.

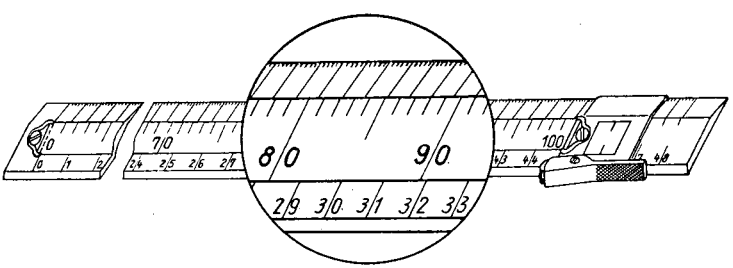

Fig. 1.

Mit diesem Gerät ist es möglich, die Laufstrecke in cm und den R_F-Wert abzulesen. Man legt den gemeinsamen Nullpunkt an den Startfleck und dehnt das Gummiband bis zur Lösungsmittelfront, bzw. bei R_X-Werten bis zum Kontrollfleck. Eine Feder drückt den Läufer an die Leiste, damit das elastische Band nicht zurückrutschen kann. Die Proportionalität des Bandes bei der Ausdehnung wurde überprüft. Sie ergab eine Fehlergrenze von höchstens $\pm 2\,\%$ für den R_F-Wert, was für die papierchromatographische Methode praktisch bedeutungslos ist.

Für kleinere Laufstrecken benutzt man ein kürzeres (etwa 15 cm langes) Gummiband, das man leicht mit Hilfe von zwei Halteschrauben mit dem ursprünglichen auswechseln kann. Selbstverständlich lässt sich der Verhältnismasstab insgesamt auch in anderer, z.B. kleinerer Grösse anfertigen. Dehnt sich nach längerem Gebrauch das elastische Band oder wird es "müde", dann muss man es durch ein neues ersetzen.

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J. Chromatog., 5 (1961) 177–178

Contribution to the paper chromatographic separation of *Withania somnifera* alkaloids

A recent report[1] implied the existence of eight alkaloids in the root of *Withania somnifera*. Our investigations[2] have established the occurrence of eleven bases. Seven of these have been isolated as white crystalline compounds; one as an amorphous white powder. An admixture of three alkaloids has not been resolved. Qualitative data on the isolated alkaloids will be published; preliminary data on the nature of each base is presented in Table I.

TABLE I

Compound	R_F
I. Tertiary nitrogen alkaloid	0.07
II. Quaternary ammonium base	0.10
III. Tertiary nitrogen alkaloid	0.14
IV. Quaternary ammonium base	0.20
V. Tertiary nitrogen alkaloid	0.23
VI. Quaternary ammonium base	0.35
VII. Unqualified alkaloid	0.43
VIII. Unqualified alkaloid	0.53
IX. Unqualified alkaloid	0.69
X. Neutral alkaloid	0.84
XI. Neutral alkaloid	0.92

All alkaloids in a semi-pure extract of the root are revealed on one chromatogram although the quaternary ammonium bases are minor components of the admixture. The average R_F values of an alkaloid mixture, as a root extract, chromatographed

on Whatman No. 1 paper impregnated with 0.5 *M* KCl solution, dried, and developed 25–35 cm with *n*-butanol–HCl (98:2 v/v), water-saturated, are reported in Table I. Revelation was achieved by spraying the dried paper with modified Dragendorff reagent.

The major alkaloids, in the several root samples investigated, are III and V; the major quaternary base is VI. Compound II is choline. None of these alkaloids is nicotine and this compound has not been observed in root and leaf samples investigated.

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[2] C. K. ATAL, *Ph. D. Dissertation*, University of Connecticut, 1958, and unpublished data.

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Gas chromatographic analysis in fractional distillation of multi-component systems

The course of fractional distillation of a multi-component system is commonly followed by noting the changes in boiling points and/or refractive indices. These techniques are not completely adequate in those cases in which the boiling points are very close and the differences in refractive indices are very small or non-existent. Gas chromatography offers a very useful and indispensable tool for following fractional distillations of this type. Gas chromatographic analysis of each fraction as the distillation proceeds permits one to make a more judicious choice in effecting the best separation of the components and therefore obtaining a maximum amount of each component in high purity.

In this note, the utilization of gas chromatography in the purification of 2,2,4,6,6-pentamethylheptane (PMH) by fractional distillation is described.

Experimental

PMH was prepared by catalytic hydrogenation of commercially available triisobutylene[1]. The crude PMH, after washing and drying, was charged to a total condensation, intermittent take-off-type distillation column. The packed section, 1 in. by 4 ft., was packed with Podbielniak Heli-Pak No. 2117 stainless steel packing. The column and take-off valve were designed so that the distillate did not come in contact

with stopcock grease at any point. The distillation was carried out at 18 mm pressure. A chaser, 200 g of eicosane, was added. After proper equilibration of the column, fractions of 25 ml or less were taken. The take-off valve was operated by a solenoid and a 30-sec cycle timer arrangement so that the valve was open 5 % of every 30-sec cycle. Refractive indices and chromatograms were obtained on each fraction.

The chromatograms were obtained on a 26-ft. column. The packing consisted of Dow-Corning high vacuum silicone grease, 20 % by weight, on 30–60 Chromosorb. The conditions for the chromatograms are given in Fig. 1.

Results and discussions

WHITMORE *et al.*[2] reported that triisobutylene is composed of four components: (a) 35 % 2,2,4,6,6-pentamethylheptene-3, (b) 55 % 2-neopentyl-4,4-dimethylpentene-1, (c) 5 % 2,4,4,6,6-pentamethylheptene-1, and (d) 5 % 2,4,4,6,6-pentamethylheptene-2. Hydrogenation of this mixture would yield two saturated hydrocarbons: 2,2,4,6,6-pentamethylheptane (PMH) and 2,2,4,4,6-pentamethylheptane. The purification of PMH, obtained by hydrogenating triisobutylene, is mainly a problem of separating the PMH from 2,2,4,4,6-pentamethylheptane. A chromatogram of the crude PMH is shown in Fig. 1. This purification was accomplished by careful fractional distillation of the crude PMH at reduced pressure.

Gas chromatography served as a valuable analytical aid in following the course of the distillation. The gas chromatograms provided the information necessary to decide which fractions were pure PMH. The chromatograms of the first six fractions showed that impurities, more volatile than PMH, were present. The refractive indices and boiling points for fractions 2 through 6 indicated that these fractions were essentially pure PMH. A representative chromatogram of those obtained for each of the first six fractions is shown in the chromatogram for fraction 2 in Fig. 1.

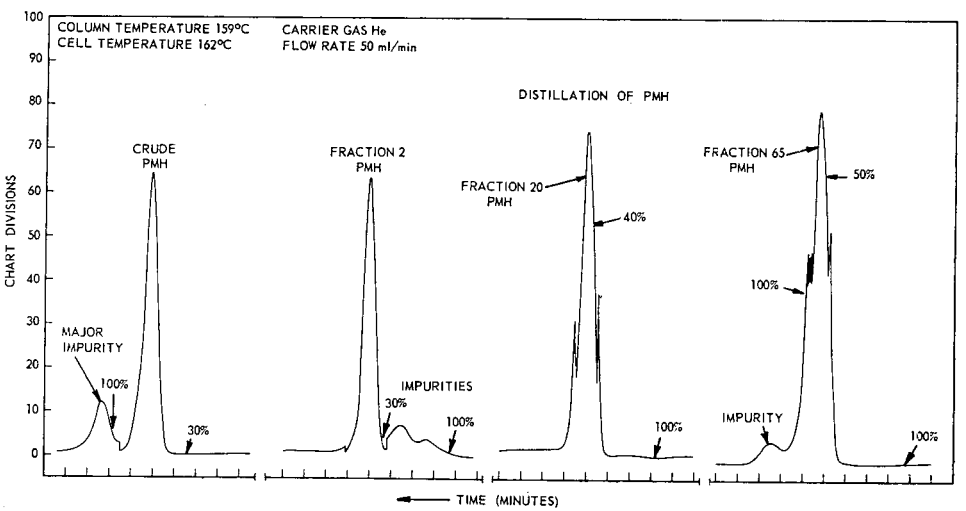

Fig. 1. Representative chromatograms. The relative responses (sensitivities) are given in percentages along the chromatograms.

The impurities, of course, decreased in amount with each fraction through fraction 6 and were not present starting with fraction 7. The chromatogram for fraction 20, Fig. 1, is representative of those obtained for each of the fractions 7 through 63. These fractions represent the pure PMH obtained by the fractional distillation of the crude material. Starting with fraction 64, a small peak was present immediately after the PMH peak, even though there was no apparent change in the boiling point or refractive index. This showed that the higher boiling impurity, 2,2,4,4,6-pentamethylheptane, was present, and the amount increased with each succeeding fraction. The chromatogram for fraction 65 is representative of the chromatograms for these fractions, with the higher boiling impurity increasing with each fraction. The utilization of gas chromatography in this particular case provided the necessary information as to which fractions were pure PMH, and, therefore, a maximum amount of pure PMH was obtained.

Gas chromatography offers a valuable analytical tool in following the course of the fractional distillation of multi-component systems. In those cases where differences in boiling points are small and differences in refractive indices are non-existent, gas chromatography is virtually indispensable.

Acknowledgement

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J. Chromatog., 5 (1961) 179–181

Use of 2-methylpiperazine diformamide in gas chromatography

In the course of a research project being carried out in this laboratory, an experimental quantity of 2-methylpiperazine diformamide was prepared from 2-methylpiperazine and formic acid. This compound proved to be very polar and showed good thermal stability. It distilled at 200° at about 5 mm pressure without decomposition and melted at about 36°.

Because of its high polarity, low melting point, and thermal stability, this compound appeared to be a logical choice as a stationary phase in gas chromatography as a substitute for dimethylformamide or dimethylsulfolane, especially under

conditions in which these materials would have an undesirably high vapor pressure. Dimethylformamide has a vapor pressure of about 1 mm at 0°, so that its use is limited by a short column life, even at this temperature. Dimethylsulfolane is recommended for use only at 40° and below, again because of a relatively high vapor pressure. Since the methylpiperazine diformamide has a vapor pressure of only about 5 mm at 200°, it should be usable as a stationary phase at least to 100°.

A comparison was made between 2-methylpiperazine diformamide and sulfolane, a highly polar compound known to give large deviations from perfect solution behavior for the various classes of hydrocarbons. This comparison is given in Table I.

TABLE I

RETENTION VOLUMES

	2-Methylpiperazine diformamide ml	*Sulfolane* ml
2,2,4-Trimethylhexane	38	42
Methylcyclohexane	35	36
n-Decane	192	202
Octene	65	74
Toluene	339	386

In each case, the stationary phase was 15 % by weight on 35–80 mesh firebrick, and the column was 1 m long by 0.2 in. diameter. The temperature was 68.5° in both cases. Helium was used as the carrier gas.

The octene (practical grade) was partially resolved into 3 components by the diformamide, and was resolved less well by the sulfolane.

A comparison of the separation factors for close-boiling pairs of different types is given in Table II.

TABLE II

SEPARATION FACTORS

	2-Methylpiperazine diformamide	*Sulfolane*
Octene/trimethylhexane	1.69	1.74
Toluene/methylcyclohexane	9.8	10.8

It is seen that the separation factors for the two stationary phases are quite similar, with sulfolane giving slightly better results. However, since the vapor pressure of the diformamide is much lower than that of sulfolane, the use of 2-methylpiperazine diformamide would be preferable.

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BOOK REVIEWS

Die Papierelektrophorese. Methoden und Ergebnisse, by CH. WUNDERLY, 2nd Ed., Verlag H. R. Sauerländer & Co., Aarau and Frankfurt a.M., 1959, 202 pages, 72 figs., price Sw.Frs./DM 18.80.

The rapid expansion of paper electrophoresis, which has brought electrophoretic analysis within the reach of even small hospitals, was accelerated by the readiness of the founders and pioneers of moving boundary electrophoresis to accept the paper technique; they were fortunately free from prejudices against new methods, so often encountered in narrower minds. WUNDERLY, one of the co-authors of the classical manual *Die Bluteiweisskörper des Menschen*, belongs to these pioneers and he was able to incorporate the experience of many years already in the first edition of his *Papierelektrophorese*. The second edition (1959) covers a substantial part of the literature that has appeared since 1954; although it includes more than three times as many references (1665), the number of pages has only been doubled. The book has been entirely rewritten, but characteristic features of the first edition have been retained and in some cases emphasized; the book deals mainly with proteins, especially blood proteins (only 12 pages of the special part are devoted to non-protein substances), and it has been the aim of the author to give a maximum of information in a minimum of space (this is facilitated by a clear and distinct print and excellent paper); the sections on analytical techniques and reviews of biological applications are well balanced.

The second edition is introduced by a preface by P. KÖNIG*. Although the format and size as well as the numerous practical hints it contains correspond to a laboratory manual, the book also gives an authoritative report on recent progress. When seeking a reference, the reader is saved the trouble of turning over the pages by the system of numbered references on every page, which is extremely useful for a book of this type. Limitations imposed by the size of the book made it impossible for the author to cover the formidable amount of literature completely and to discuss all the results obtained by the authors quoted. For a more exhaustive treatment, the reader should consult a monograph such as *Electroforesis em papel* by RIBEIRO *et al.*

Among the points of technical interest it is worth noting that the book mentions the application on dry paper as the sole method even in the case of protein-containing samples. The suggestions on staining technique and on the use of polyethyleneimine

* We are informed in a footnote that this is one of the last documents written by Dr. PAULO KÖNIG, who died in São Paulo on November 8th, 1958. The reviewer would like to take this opportunity of commemorating this founder of paper electrophoresis of proteins, with whom he had occasion to exchange several letters. Although Dr. KÖNIG gave up this kind of work many years ago, his ideas of a chromatographic arrangement based on the molecular sieve effect showed acuteness of intellect similar to that which had manifested itself in the first communication on paper electrophoresis of proteins in 1937.

in sodium phenylmonosulfonate solution as a standard for protein staining and of a triglyceride mixture for lipoprotein staining are based on personal experience. An excellent discussion is devoted to a comparison of the proportions of blood proteins found by various methods and modifications. A frequently checked "own value" may be considered the most reliable reference standard for a given laboratory. The chapter on lipoproteins is not only twice as long as that in the first edition, but differs from it in the selection of valuable information it contains. The wide acceptance of paper electrophoresis for the study of serum proteins has established the merits of this method so firmly that more space is devoted to warnings against uncritical interpretation of electrophoretic data than to the enumeration of the advantages of the method. Experimental workers will appreciate the chapter devoted to animal serum proteins.

Misprints in authors' names do not occur very frequently. The subject and author indexes are satisfactory though far from complete. Both cover and typography are worth mentioning for their esthetic merits. The publisher and printer are to be congratulated for the short production time which has made it possible to account for all important papers up to 1958.

I. M. HAIS (Prague)

J. Chromatog., 5 (1961) 183–184

Quantitative Paper Chromatography of Steroids (Memoirs of the Society for Endocrinology, No. 8), edited by D. ABELSON AND R. V. BROOKS, Cambridge University Press, London, 1960, 103 pages, price 30 s.

The 100 or so pages of this publication deal with the proceedings of a symposium on the quantitative paper chromatography of steroids held in July 1958. Although the reader may well wonder why it should have taken two whole years for this book to appear, it nevertheless constitutes an up-to-date contribution on the subject, for the simple reason that today the problems involved have still not been solved. The $\pm$ 20 % degree of accuracy attained so far, on which even expensive equipment has failed to achieve any appreciable improvement, may be adequate for many biochemical purposes; moreover, where a high degree of specificity is required of a determination, the question of quantitative accuracy is generally of less importance. Considered in the absolute, however, a $\pm$ 20 % margin of error is too high. Try as one may to solve this problem, the factor paper = cellulose invariably plays a decisive and often critical role—so much so that for certain purposes the watchword "back to the column!" (*e.g.* celite) would appear justified. The various papers read at the symposium, and the contributions to the discussions, not only shed light on numerous aspects of this question but also deal with complete "quantitative" methods. Readers who have to tackle such problems will derive a great deal from this compilation, containing as it does a wealth of interesting details and personal experiences. The index at the back of this compendium adds further to its usefulness.

R. NEHER (Basel)

ANALOG INTEGRATION TECHNIQUES IN CHROMATOGRAPHIC ANALYSIS

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INTRODUCTION

The need is often encountered in instrumental analysis for recording the integral of the instrument output signal. Especially frequent use has been made of this procedure in recent years in gas chromatography. In another category of analytical techniques, including paper chromatography, it is often desirable to integrate the density of a developed pattern on paper, film or the like. In either case, the signal or pattern to be integrated comprises a series of more or less discrete "events", such as peaks or spots, and it is of interest mainly to know the integral only within the time (or position) intervals defining these separate events. In practice, the integral value has been either (a) continuously recorded, with provision made for correlating the value with the beginning and end of each event, or (b) recorded only at the instant of beginning or end (or both) of each event by use of suitable means for sensing these occurrences.

The method most widely used has been to mark the abscissa axis of the record continuously with a series of pips at a frequency proportional to the signal, the pip count being thereby a measure of the integral between selected abscissa values. The counting of such pips is, however, often laborious, and subject to error. Further, it is difficult to select a pipping rate that will both give sufficient precision on small peaks and avoid the merging of pips on large peaks. In another type of apparatus*, the integrator drives a print-out numerical counter which records the integral value automatically at the start of each new peak (and, by manual triggering, at the end of last peak). A difficulty here is that the trigger circuit must distinguish the occurrence of new peaks from base line noise or zero drift.

The present paper discusses several related analog methods that eliminate both pip counting and the need for any trigger circuitry. Indication of the beginning and end of "events" is automatic and inherent in the techniques. One of these methods is particularly simple and especially suited to gas chromatography. In a modified form, this technique may be applied to the automatic integration of an array of spots of variable shape and density, as in paper chromatography.

* Perkin-Elmer Corp., Norwalk, Conn.

"INTEGRAM" RECORDING

The authors have discussed in an earlier paper[1] a continuous analog integration method in which, in lieu of the conventional chromatogram, a transformed record or "integram" is presented. This record has the property that the base width of each transformed peak is proportional to the integral of the original peak.

Given a typical gas chromatograph output signal as shown in Fig. 1(a), the corresponding integram appears as shown in Fig. 1(b). Here the base width of the pentane peak is proportional to the area under the corresponding peak in Fig. 1(a);

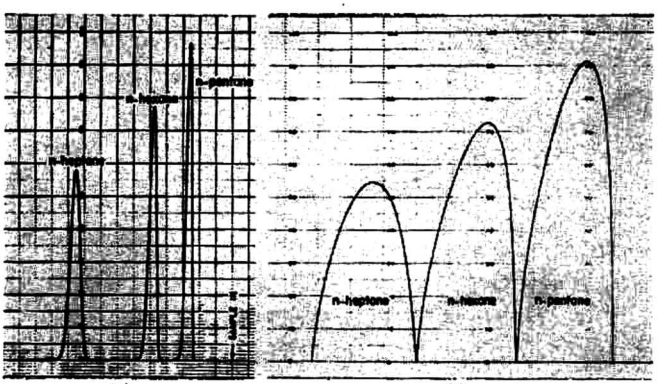

Fig. 1. Chromatogram *vs.* integram. Sample: *n*-pentane, *n*-hexane and *n*-heptane, equal volumes. Chromatogram chart speed: 0.5 in./min. Integram chart speed: 8 in./min/100% signal. Column: 6 ft., silicone 550, 40/60 C-22 firebrick. Temperature: 91°. Detector: thermal conductivity cell. Sample size 0.02 ml.

similarly for the other peaks. The requirement for producing the transformed curve is essentially a simple one: the chart is caused to be driven at a rate *proportional to the signal magnitude.* The pen is servo-driven in the ordinate direction in the usual way. It may be seen that the mechanism needed to drive the chart is an integrator, and that the length of chart paper fed from the recorder between any time limits is proportional to the time integral of the signal between those limits.

"STRIP-INTEGRAM" RECORDING

The authors have now developed a related technique that permits retaining the conventional presentation of signal against a uniform time scale. This offers certain advantages to be mentioned later. At the same time, the system appears to be the simplest yet devised for continuous integration recording in gas chromatography or other techniques such as paper electrophoresis that may have a similar requirement.

From the vantage point of the operator, the manner of presentation is the following: An accessory integrating unit is mounted on or alongside the recorder. From this unit, during the chromatogram run, a narrow paper tape is fed out on which at varying intervals appears a series of lines. On completion of the run, the

line recorded on the strip is somewhat broadened. Since the length of paper fed past the slit in any time interval is proportional to the integral of the signal during that interval, distances between successive dark lines are a measure, respectively, of successive peak areas.

The precise photometric characteristics of the sensitive paper are of no consequence to the measurement, of course, since only these inter-linear distances are of interest rather than the darkness of any portion of the strip. By the same token, nominal drifts in source intensity and moderate fogging of the strip are without effect on the readings obtained.

Fig. 3 shows the appearance of an actual strip-integram and the corresponding

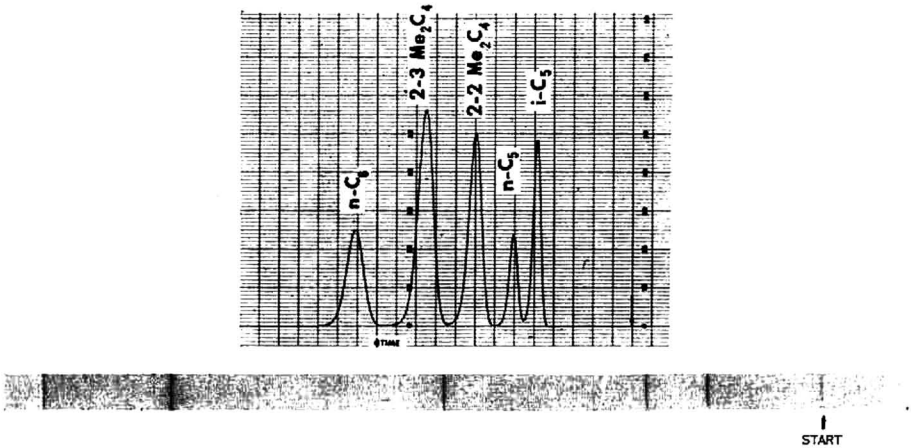

Fig. 3. Chromatogram and strip-integram. Resolved peaks. Sample: isopentane, *n*-pentane, 2,2-dimethylbutane, 2,3-dimethylbutane, and *n*-hexane, respectively 1.0, 0.5, 1.5, and 2.0 and 1.0 parts by volume. Chart speed: ½ in./min. Strip speed: 7.5 in./min/100% signal. Column: same as in Fig. 1. Temp.: 70°. Detector: hydrogen flame. Attenuation: 300,000. Sample size: 0.0025 ml.

conventional chromatogram obtained on a mixture of hydrocarbons. The peaks here are essentially fully resolved. Duration of analysis was about 16 min, and the length of the strip fed out during this time at a rate of 15 in./min per 100 % signal was about 11 in. Reading from right to left, the distance between the first two lines is proportional to the area of the isopentane peak; distance between the second and third lines is a measure of the *n*-pentane peak area, etc. The largest of the peak areas indicated here may be read against a steel rule to within about 0.1 %, the smallest to within about 0.5 %. If desired, a longer strip of paper may be fed out for the same total amount of sample (for example, by driving the integrator disk at higher speed) and reading accuracy thereby increased. This is useful, of course, only so long as the accuracy of the integrating device (or other limiting factor in the system) has not been exceeded.

In Fig. 4 we have deliberately run the same sample mixture at higher column temperature (100° instead of 70°). We obtained in this way varying degrees of resolu-

strip is torn off and laid next to an ordinary measuring rule. The distance between the first and second lines is proportional to the area of the first peak; distance between the second and third lines measures the second peak area, etc. We have called this taped record a "strip-integram".

The apparatus is shown schematically in Fig. 2. The signal source, *e.g.* the output of the gas chromatograph, drives the pen of a conventional recorder. The pen servo also drives the input of an integrating device which may as shown be the convenient,

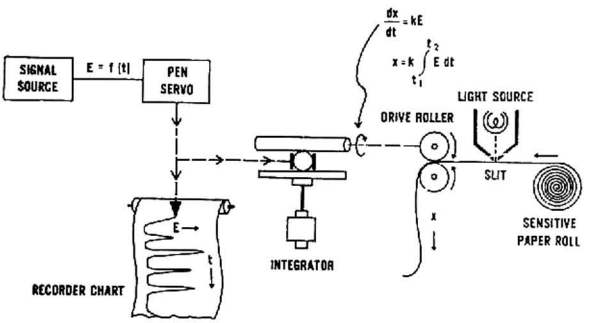

Fig. 2. Apparatus for strip-integram recording.

relatively inexpensive ball-and-disk type integrator. Actually, in the work subsequently shown, the authors used a velocity-servo type of integrator[2] already in their possession and which they described in their earlier paper[1]. The electrical input for integrators of this type is derived either from a re-transmitting potentiometer on the recorder or directly from the signal source. The integrator in any event is of a type that provides a mechanical output, preferably as a rotating shaft, the angular excursion of which is proportional to the integral of the input. The output shaft of the integrator drives a strip of light-sensitive paper past a constantly illuminated slit. The slit, about 0.005 in. wide in the present work, was directly illuminated by a small, commercially available pencil-type mercury vapor source*. The simple power supply furnished with the lamp was connected directly to the a.c. power line.

The paper is a dry, self-developing type that darkens directly on exposure to light and requires no wet development. It is commercially available** and has recently come into wide use in a number of recording oscilloscopes.

It is characteristic of the integrator, as indicated by the equations in Fig. 2, that its output shaft turns at a rate always proportional to the signal magnitude. Thus, at the start of a run or between peaks, where signal level is zero, the sensitive paper is stationary and receives a relatively strong exposure at the slit. At such times a dark, sharp line is impressed on the paper. When a peak appears, the paper is set in motion and exposure thereby much reduced. Lighter background tones are therefore presented between the dark lines. Where a pair of adjoining peaks is not fully resolved, the sensitive strip does not come to a full stop between peaks; hence the

* Ultraviolet Products, Inc., San Gabriel, Calif.
** Kodak Linagraph Direct-Print Paper, Eastman Kodak Company, Rochester, 4 N.Y.

tion between the peaks. To avoid too short a strip-integram on this run (with sample size reduced about three-fold) we doubled the factor of strip speed *vs.* signal level as compared with Fig. 3. The resulting strip length was about 7 ½ inches. The contrast of the lines is seen to be reduced and the line width increased as resolution is diminished. The reading error introduced by the line broadening appears, however, to be always appreciably less than the uncertainty inherent in the fact of incomplete resolution. Thus, whereas in the chromatogram about 15 % of the true area of the second (*n*-C5) peak overlaps the area of the first peak, and a low-contrast, broad line appears on the strip-integram between these peaks, the area of the second peak is nevertheless readable on the strip to about 1 %. The areas of the somewhat

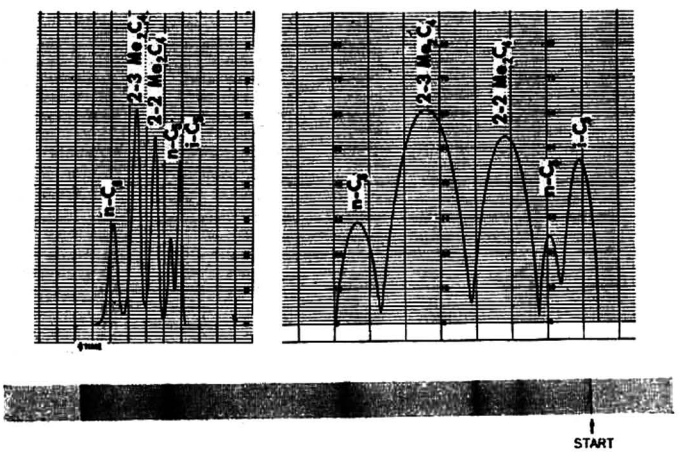

Fig. 4. Chromatogram, integram and strip-integram. Incompletely resolved peaks. Sample: same as in Fig. 3. Chart speed: 1 in./min. Strip speed: 15 in./min/100 % signal. Column: same as Figs. 1 and 3. Temperature: 100°. Detector and attenuation as in Fig. 3. Sample size: 0.0008 ml.

better-resolved peaks, such as the third and fourth (2,2- and 2,3-dimethyl-butane), are readable to within 0.2 to 0.3 %.

In cases such as the preceding, where somewhat poorly resolved peaks are encountered, it may be desirable to increase the exposure level on the strip to bring out the boundaries between such peaks. Fortunately, the sensitive paper has a sufficient latitude of exposure that this will not unduly overexpose and broaden the lines between well-resolved peaks in the same record. Should it prove necessary, however, the latitude of exposure may in effect be easily increased by a large factor. By illuminating the slit non-uniformly, *i.e.* more intensely at one end than the other, there will always be a portion of the slit where exposure is an optimum, whether the peaks be poorly or well resolved.

For purposes of comparison, Fig. 4 includes at the right the corresponding integram run concurrently with the chromatogram and strip-integram. The integram offers the advantage of presenting, in a single record, information on peak height and resolution; at the same time it provides, in the base widths, a rapid and convenient

measure of the time integral of each peak. It does not, however, indicate elapsed time (useful for peak identification), though we have shown[1] that a second pen, driven at constant speed in the ordinate direction, will easily provide this information.

In the strip-integram technique, on the other hand, we retain the familiar, conventional record; and merely by supplementing the recorder with an integrator to drive a sensitive strip, provide an extremely simple and convenient means for continuous integration recording.

A certain advantage that the strip-integram shares with the integram is worth noting. If we consider unresolved peaks, then in contrast to the conventional chromatogram, the extrapolated contours of adjoining peaks in the integram do not overlap. Instead, they may be extrapolated to a common, single point on the axis. This follows from the fact that total integram base width for adjoining peaks is proportional to the sum of their separate integrals, independent of the resolution, on the assumption that detector response is linearly additive for superposed components. This assumption has appeared reasonable at least for the thermal conductivity and hydrogen flame type detectors. Where departure from such behavior is observed, extrapolation to a single point still causes only slight error, because the ratio of overlapping to non-overlapping areas will generally be quite small.

The strip-integram shares the convenience of the integram in the sense that adjoining integration limits for any pair of unresolved peaks may be fairly represented by a single position on the strip, this position being in most cases very close to the center of the broadened line presented on the strip.

The need to change signal attenuation during a strip-integram run presents no particular difficulty. For accurate integration, such change would be made between peaks, when the sensitive strip is stationary or nearly so. Appropriate multiplying factors are of course then applied in reading the successive strip lengths, in a manner analogous to that of other methods of integration.

The sensitive paper may be handled for appreciable lengths of time in ambient light, following exposure in the instrument, without loss of the recorded image. Darkening is, however, more rapid under fluorescent lighting than incandescent lighting. Nevertheless, the line patterns persist for several days of continuous exposure to ordinary ambient fluorescent light levels. Although developing solutions that will permanently fix the images are available from the paper manufacturer, we found in the present use that this results in serious mottling and loss of tonal gradation. There appears to be no compelling need, however, to make the records fully permanent.

The integration accuracy of strip-integram will generally be limited only by the accuracy of the integrating device used. However, in the case of a high-quality ball-and-disk integrator driven by a recorder pen-servo, performance may be limited by the linearity of the recorder slidewire. There appears to be no difficulty, in any event, in obtaining peak area values to $\pm$ 0.2 % or better on peaks for which the length of strip fed from the instrument is about 3 in. or more.

INTEGRATION OF PAPER CHROMATOGRAM SPOTS

The principle of the strip-integram may be extended to the integration of an array of paper chromatogram spots of variable shape and density. An advantage compared with previously reported scanning techniques of spot integration[3] is that the spots need not be presented individually and manually to the instrument. Instead the whole array of spots, regardless of their disposition, is scanned as an ensemble.

Assume an array such as *A*, *B*, *C*, *D* in Fig. 5(a), that may be scanned in a raster pattern for optical density, fluorescence, or radioactivity, etc. The scanning spot may

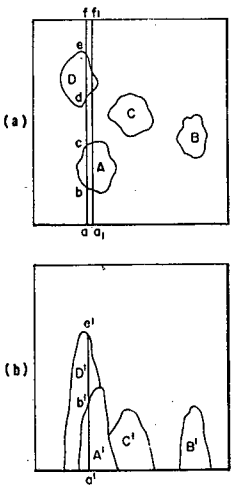

Fig. 5. Chromatogram and transformed chromatogram.

for instance start at the lower left and cover the chromatogram in a series of upward directed scanning strokes af, a_1f_1, etc. Simultaneously, a small spot of light is caused to scan or "write" upon a sheet of light-sensitive paper, represented in Fig. 5(b). Lateral displacement of this writing spot is made synchronous with the lateral displacement of the scanning site on the chromatogram. In the vertical direction, however, the writing spot moves at a variable rate: starting always at the lower margin of the sensitive sheet at the start of each vertical scan on the chromatogram, the writing spot is made to move upward at a rate proportional to the concentration of sample on the chromatogram (as derived from optical density, fluorescence, etc.). After a vertical scan on the chromatogram is completed, the writing spot is brought back to the lower margin for the next scan.

Assuming we scan the chromatogram of Fig. 5(a) for optical density by transmitted light, an electrical signal may be derived proportional to the density. An adjustment or compensation may be made in this signal for the background density of the paper. The corrected density may be integrated, as by a ball-and-disk integrator, and the integrator output caused to drive the writing spot with respect to the paper. Consider scanning of the line af on the chromatogram. The corrected density signal

is zero between points *a* and *b*, hence the writing spot is initially stationary at the lower margin of the sensitive paper and registers a dark mark there. From *b* to *c* the density signal is of finite value. In this interval the writing spot moves from *a'* to *b'*. Since from *c* to *d* on the chromatogram the corrected density is again zero, a dark spot is again recorded at *b'*. The action is repeated as the chromatogram is scanned from *d* to *f*, following which the writing spot is returned rapidly from *e'* to the starting line. Scanning the total array of spots in this manner results in an array of transformed spots *A'*, *B'*, *C'*, *D'*. Since the length of any ordinate such as *a'b'* on the transformed spot is the integral of the density along the corresponding ordinate segment such as *bc* on the original spot, it follows that the total area of any transformed spot is proportional to the total integrated density of sample on the original spot, to the extent that Beer's law applies. It is necessary now only to measure the areas of the transformed spots to obtain values proportional to the amount of sample in each spot. This may be done rapidly with a planimeter, or by cutting out the spots and weighing.

An apparatus that may be used to transform the spots in the case of optical density scanning is shown in Fig. 6. The chromatogram is mounted on a transparent drum I rotated at constant speed by motor *d*. An opaque band *e* bridges the beginning and end of the paper. A source *a* projects a spot of light on the paper, and *b* is a photodetector unit that may include a wavelength-selective filter. Units *a* and *b*

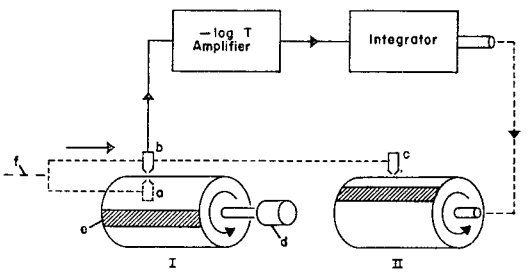

Fig. 6. Apparatus for transforming the chromatogram.

are driven together across the paper at constant speed from a common driving source *f*. The speed of *f* is such that during one revolution of drum I the scanning elements *a*,*b* are displaced laterally an amount equal to the separation of successive scanning lines, such as *af* and a_1f_1 in Fig. 5. A logarithmic amplifier, with output adjustable to compensate for paper background, derives a density signal that is applied to an integrator with a mechanical output. The integrator drives a drum II which carries the sensitive paper. Unit *c*, mechanically coupled to the driving source *f*, projects a small writing spot on the sensitive paper.

At the beginning of each scan, the scanning elements *a*, *b* are aligned with the starting edge of the chromatogram, and unit *c* is aligned with the starting edge of the sensitive paper. When *a*, *b* have completed a scan, as from *a* to *f*, Fig. 5(a), the writing spot at *c* has in general only partially traversed the sensitive sheet, as from

a' to *e'*, Fig. 5(b). As drum I continues to rotate, bringing band *e* under the scanning site, the starting line on the sensitive sheet is again brought into alignment with the writing spot, in readiness for the next scan, as follows: Opaque band *e*, moving under the scanner, drives the amplifier to saturation and causes the integrator to drive drum II at relatively high speed. This speed is such as to cause negligible exposure of the paper. The speed is high enough moreover that, if need be (at abscissa positions where there is no chromatogram spot), drum II may turn a full revolution before opaque band *e* completely traverses the scanning site. A mechanical stop arrests drum II when it reaches the starting line position. At the moment, however, that band *e* just clears the scanning site, a switch actuated by drum I releases this stop (freeing drum II for another revolution), and so synchronizes the starting time for ensuing scans on both drums.

SUMMARY

Some new analog methods of integration recording are discussed that eliminate the need for pip counting, or for trigger circuitry to sense the beginning and end of "events" in the signal, such as signal peaks. One version especially suited to gas chromatography appears to be the simplest yet devised, requiring only a ball-and-disk integrator driving a light-sensitive paper past a constantly illuminated slit. The paper is a dry, self-developing, commercially available type. By modification of the technique, an array of spots of variable shape and optical density, as in paper chromatography, may be transformed entirely automatically into a configuration from which the integrated density of the original spots may be more directly and rapidly determined.

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AN APPARATUS FOR HIGH-VOLTAGE PAPER ELECTROPHORESIS

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Paper electrophoresis is based on the differential migration of charged ions or particles in an electrical field. Since migration rates within a given time are proportional to the field strength, it is desirable to be able to use as high a voltage as experimentally possible in order to obtain the following immediate advantages: (1) economy in time, (2) high resolving power, (3) easier detection of components present in low concentration.

The definition of high-voltage technique implies the application of a potential gradient of, at least, 50 V/cm[1]. To obtain the full benefit of the technique it is advisable, however, to aim at the use of potential gradients of 100 V/cm and more, though the difficulties in designing suitable equipment for this range become progressively greater with increasing voltage.

Several designs of high-voltage equipment have been described in recent years. Some are based on direct cooling of the paper strip by an organic solvent, immiscible with the sample and the aqueous background electrolyte solution, with and without cooling of the organic solvent, as suggested by MICHL[2], HEILMEYER *et al.*[3], and TURBA *et al.*[4]. Others are based on cooling of the paper strip by plates of metal, glass or plastics which in turn are cooled by refrigerated brine or ordinary tap water. Designs incorporating one cooling plate, generally the bottom plate, have been described by BERBALK AND SCHIER[5], WERNER AND WESTPHAL[6], WIELAND AND PFLEIDERER[7], and DOSE[8]. Designs incorporating two cooling plates (sandwich technique) for greatly enhanced efficiency have been proposed by MICHL[9] and GROSS[10-12].

THEORETICAL CONSIDERATIONS

The movement of ions in an electric field is proportional to field strength and net charge carried:

$$\text{Field strength} = X = \frac{V}{l} \tag{1}$$

$$\text{Mobility} = u = \frac{d/t}{V/l}, \text{ or} \tag{2}$$

$$d = \frac{u \cdot V \cdot t}{l}$$

where d = distance travelled by ion, l = length of field, V = voltage, t = time.

The equations apply primarily to free electrophoresis and a certain correction factor for tortuous path in the stabilising medium has to be introduced for paper electrophoresis. Although the net charge can be altered by complexing agents and changes in pH, it is more convenient to increase migration rates by the application of higher voltage.

Diffusion

As a function of temperature, concentration, time and molecular weight, diffusion plays a significant part in paper electrophoresis, mainly by counteracting and reducing the separating efficiency of the technique. With a considerable reduction in time at high voltage, the diffusion effects are greatly repressed and the separating power increased, particularly for low-molecular weight compounds. This is helped by the maintenance of a steady, moderately low temperature. Small differences in mobilities are not obscured by the spreading and overlapping of zones. The sensitivity of the method is increased, as the detection and estimation of compact zones by colour reaction are extended to lower levels of concentration.

Heat dissipation

The passage of electric current through the moist, conducting filter paper is proportional to the voltage applied and causes generation of heat. The heat, according to Joule's law, increases with the square of the current and is given in calories per second by the formula:

$$Q = \frac{RI^2}{A} = \frac{VI}{4.185} \tag{3}$$

where Q = heat, R = electrical resistance of paper strip, I = current, and A = mechanical heat equivalent. ($A = 4.185 \cdot 10^7$ erg/cal.)

If the heat is not dissipated, the ensuing rise of temperature affects detrimentally the experimental conditions, since conductance and pH (to a lesser degree) of the background electrolyte solution, and mobilities of the ions under investigation are all temperature-dependent. To obtain steady conditions, it is essential that generated heat should be effectively dissipated so that there be no significant temperature rise during the experiment. The level of applied voltage is thus limited only by the efficiency of heat dissipation or cooling.

Cooling

Cooling can take place by evaporation, conduction or radiation, which latter plays no significant part in the designs discussed. Evaporation is the usual system, particularly in simple apparatus of the "moist chamber" type found so useful in low-voltage electrophoresis, but it leads to serious complications above a potential gradient of 10 V/cm. It is thus desirable that heat dissipation should occur predominantly by conduction. Metal plates in close contact with the heated surface (paper) are excellent conductors of heat and the coolant circulating in the plates provides a

suitable means of dissipation. The efficiency is somewhat reduced by the need for electrical insulation of the plates by glass, Polythene or other plastics. The heat exchange mechanism in this case is complicated because the heat has to pass the narrow air gap between paper and insulating layer, the thickness of the layer, another air gap, and finally the distance from metal surface to coolant, before it can be carried away. By choosing the thinnest possible insulating layer, yet one of sufficient dielectric strength, by cooling both surfaces of the paper and compressing the paper between two cooling plates under sufficient pressure to ensure close contact, a high degree of heat transfer efficiency can be achieved.

The cooling efficiency will also depend greatly on the choice of suitable materials and the design of plates, particularly with regard to flatness of surfaces.

Choice of materials of construction

The material chosen for cooling plates must fulfil several conditions. It must be of high thermal conductivity and, preferably, of high dielectric strength, *i.e.* afford excellent electrical insulation. However, these two properties cannot be found together, since, as a rule, a material of good thermal conductivity is also one of good electrical conductivity, and vice versa. Glass is almost as good an insulator as any of the plastic materials. Its dielectric strength per 0.001 in. is about 700 V against 500 V for P.V.C. (polyvinylchloride), 1000 V for Teflon (polytetrafluoroethylene) and 1000 V for Polythene (polyethylene). However, its mechanical weakness in thin layers puts it at a serious disadvantage in comparison with the plastics which can be used as insulating films. Its thermal conductivity at $1.7 \cdot 10^{-3}$ cal/cm^2/°C/cm is admittedly better than that of Polythene (or similar material) at $7 \cdot 10^{-4}$, but still very far below the conductivity of a metal such as aluminium at $5 \cdot 10^{-1}$.

In view of the contrasting properties, a combination of the high insulating strength of a film of plastics with the good heat-conducting property of a metal plate would appear to offer the best solution, provided the thinness of the film is kept to a minimum limited only by the insulating capacity under the voltage conditions required. It must be remembered, however, that the dielectric strength of plastics is dependent on the temperature and time of exposure and is usually decreasing with increasing temperature. Weakening or failure of insulation can often be caused by mechanical or chemical breakdown of the material. It is therefore imperative to maintain the temperature of the film at a moderate level and avoid local overheating leading to ultimate breakdown of the insulation. If the cooling is efficient, the temperature of the film can be kept within 1–2° of that of the coolant.

Experience has shown that a 0.01 in. thick film of Polythene (British Visqueen Ltd.), or similar material, is suitable for experiments employing potential gradients of 100–160 V/cm. It must be added, however, that the energy throughput and the type of background electrolyte solution used are important factors in deciding the performance of insulating films. A strong, highly-dissociated buffer, like ammonium carbonate or ammonium citrate solution, with consequent relatively high current consumption at a given voltage puts a heavier demand on insulation than

e.g. a mixed acetic acid–formic acid solution. In this case, the energy which can be dissipated per unit area (W/cm²) without leading to a significant temperature rise will be the governing factor.

Cooling efficiency

Water or refrigerated brine can be used to carry away the heat from the plates, the most effective way being circulation of the coolant in a system of channels milled out of the plates. If a refrigerating unit is used, the coolant is best re-circulated by a suitable pump.

The present design uses as a coolant tap water without refrigeration and re-circulation. The quantity of water required to dissipate the average energy throughput can be calculated from the following considerations: supposing the temperature of the cooling water to be 12°, the energy throughput 1500 W and the amount of energy converted to heat as 20%, *i.e.* 300 W or 258 kcal/h, then a flow of 720 l/h will be sufficient to prevent any significant rise in temperature. Measurements have shown the average temperature differences between inflow and outflow to be within 1°. With a lower current consumption and water temperature, the conditions will be even more favourable.

The effect of efficient cooling is shown by the constancy of current consumption during the run, giving thus a certain indication of constancy of temperature, ionic strength, moisture and potential difference, all important factors ensuring steady conditions in the paper strip.

A vital point in the calculation mentioned is the assumption that heat transfer from the heated surface (paper) to the coolant is uninterrupted and almost instantaneous. This is, however, not the case and thus the results can only be approximate.

Air pressure

The pressure applied to the top plate need be only moderate. Measurements have shown that a pressure of 1.5 lb./sq.in. is sufficient to ensure good contact between paper, Polythene film, and cooling surface, without undue interference with the proper moisture level of the paper strip. Increasing pressure leads to less moisture, lower current consumption, lower migration rates, and deterioration of cooling efficiency. At pressures of 6–8 lb./sq.in., lack of sufficient moisture leads to inefficient cooling of the cellulose fibres instead of the solution between the fibres. A good control of air pressure will ensure high reproducibility of the results.

Cooling plates

A very important point is the quality of the plate surfaces. Utmost attention to the finishing of the surfaces to an overall flatness of 0.001 in. is essential for uniform cooling and avoidance of warm spots responsible for distortion of migrating zones and breakdown of the insulation. Systematic temperature gradients over the whole area must also be avoided. This is best done by the choice of an effective channelling system for the coolant, preferably a double-countercurrent flow, smoothing out any differences in temperature between inflow and outflow.

Mechanism of hydrodynamic flow

With evaporation reduced to a negligible minimum, shifts of liquid in the paper are due to electro-osmosis and capillary flow caused by small differences in temperature along the length of the strip. There is a certain non-uniformity of flow, with rate of flow increasing slightly from cathode to anode, due to this composite effect. The liquid flow gradient is dependent on several factors such as pH, ionic strength, time, temperature, potential gradient, and moisture content of the paper. In the interest of high reproducibility of results it is advisable to minimise the hydrodynamic flow in the paper, although the inherent electrokinetic phenomenon of electro-osmosis cannot be eliminated. The choice of a suitable moisture content in the paper and the use of cellophane sleeves are helpful in this respect.

Measurements have shown that a moisture content of 130–140% of electrolyte solution on oven-dry paper and uniform wetting produce dependable conditions. The use of cellophane sleeves[13] to restrict the influx of liquid from the buffer vessels into the separating area, greatly assists in stabilising the liquid level in the paper for the relatively short duration of the high-voltage experiment.

DESIGN OF APPARATUS

The considerations set out above and the need for a heavy-duty, compact, safe, efficient, yet simple, apparatus led to the adoption of the design of a double-cooled, pressure-regulated sandwich-type of model.

The apparatus consists of the following parts: (1) 2 cooling plates, (2) pneumatic uniform-pressure device, (3) lifting device for top plate, (4) buffer vessels containing the electrodes, (5) safety cage. In addition, there are various instruments, 2 safety electrical cut-outs and an acoustic warning system.

Fig. 1 shows diagrammatically a view of the apparatus in the closed and operating position. The cooling plates, D and E, are made of aluminium alloy, NP5/6M, (British Aluminium Co. Ltd.) and their overall dimensions are 22 × 13 × 1 in. The cooling faces are precision-ground to a flatness with a tolerance of 0.001 in. over the whole area. The ends of the plates are cut out so as to form on closure a cavity on either end. Two thick pads, H, of buffer-soaked filter paper are pressed into the cavity at either end and, by sandwiching the emerging strip, G, help to minimise temperature gradients arising from less efficient cooling at these points. One of the pads forms the electrical bridge to the power supply, by being extended into the buffer vessel, Q. It is advisable to wrap the pads tightly in cellophane so as to reduce evaporation and minimise the hydrodynamic flow from the buffer vessels into the strip. This is also helped by the level of the strip being considerably higher than that of the electrolyte solution in the vessels. A minimum plate thickness of 1 in. is governed by the necessity for a system of ¼ in. wide and ⅜ in. deep grooves in the plates to allow for a rapid and efficient water circulation, and by the rigidity required to avoid warping or bending of the plates on compression. A double-countercurrent system of flow has been chosen for each plate so as to avoid any temperature gradients. Total water flow is 12 l/min.

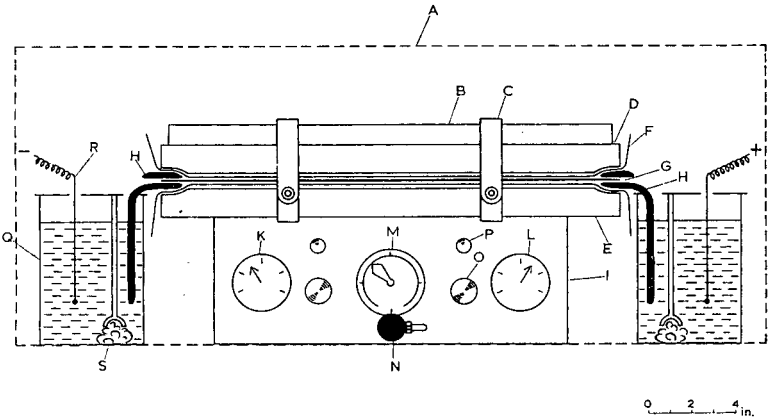

Fig. 1. Diagram of apparatus, front view. Key: A = Safety cage; B = Pneumatic pressure device; C = Retaining bracket; D = Top cooling plate; E = Bottom cooling plate; F = Insulating film; G = Paper strip; H = Thick paper pad; I = Instrument panel; K = Air pressure gauge; L = Water flow gauge; M = Timing device; N = Knob for plate locking device; O = Pressure reducing valve; P = Neon indicator light; Q = Buffer vessel; R = Platinum electrode; S = Cotton wool plug.

In addition, the channelling systems in the two plates are arranged counter-current to one another so that the smoothing-out of any still existing temperature differences is brought about in this way as effectively as possible, though the heat transfer between top and bottom plates is appreciably diminished by the interposed insulation. The channelling system adopted is shown in Fig. 2.

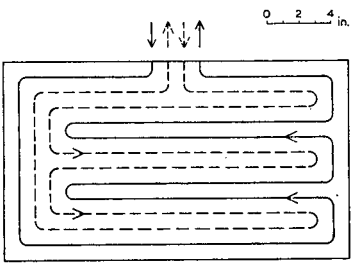

Fig. 2. Channelling system in cooling plate; arrows indicate direction of water flow.

Pressure control

The uniform-pressure control device, B, (Fig. 1) rests on top of the upper plate and consists of an inflatable plastic air cushion approx. 20 × 13 in. (Pneumatic Tent Co. Ltd., Dorking, Surrey), contained in a casing made of steel sheet, ¼ in. thick, and joined through an inlet tube to a compressed air supply. On closure of the top plate the two retaining brackets, C, are engaged by suitable studs and the cushion inflated to the desired pressure so as to produce a uniform, strictly reproducible pressure over the whole area of the paper strip. Incorporated instruments such as an air pressure gauge, K, pressure reducing valve, O, and taps enable strict control over the pressure.

Buffer vessels

The buffer vessels are made by heat welding of Polythene sheet, ¼ in. thick, and 13 × 5½ × 6 in. in dimensions, each holding about 4.5 l of electrolyte solution. The big volume was chosen so as to minimise the effects of pH changes due to electrolytic action. Covers are made of Perspex for easy fitting of electrodes. The vessels stand on narrow drip trays (Polythene) to prevent spilled electrolyte solution from making contact with the metal casing.

The vessel is divided into two compartments of uneven dimensions, the smaller one to accommodate the paper pad, H, acting as electrical bridge, and the larger one to hold the electrode, R. Communication between the two halves is afforded by a narrow horizontal, ½ in. wide slit, ¼ in. from the bottom, plugged with cotton wool and acting only as a physical barrier.

Electrodes

Platinum wire, 18 S.W.G., 6 in. long, horizontally extended between 2 vertical holders made of Perspex, dipping to about 2 in. below the liquid level in the vessel and connected to water-proof sealed terminals in the cover, was found to provide a clean and durable electrode. Dropping of the liquid level below the electrodes during an experiment must be avoided. Filling the liquid in each vessel to the marked required height before each experiment makes the use of a special levelling device superfluous.

Lifting and arresting mechanism

The heavy weight of the plate (28 lb.) makes the provision of some lifting arrangement both for speed and convenience of operation necessary. The design evolved is shown diagrammatically in Fig. 3.

The movable top plate, D, is fixed to a horizontal, U-shaped levering device, U, the curved frontal portion of which is formed by a rubber-insulated stainless steel handle for convenient and safe manipulation. One pair of strong (0.19 in. diam.,

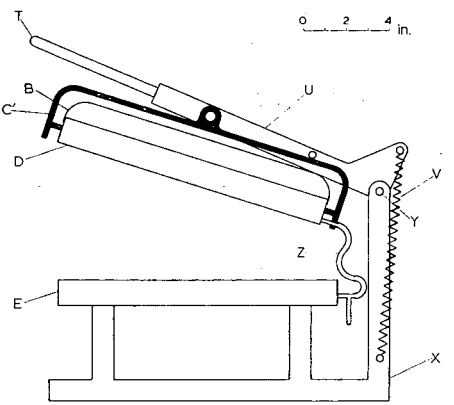

Fig. 3. Diagrammatic side view of the lifting device. Key (see also Fig. 1): T = Handle; U = Lever; V = Steel spring; X = Stand; Y = Pivoting pin; Z = Heavy rubber tubing; C′ = Levelling bracket.

65 lb./in. extension) non-fatiguing steel springs, V, (International Spring & Engineering Co. Ltd., London) is attached eccentrically to either open end which turns about a pivoting pin, Y, set in a rigid pillar, X, forming part of the steel stand. Overtilting is prevented by a fixed stop.

Upward movement of the plate is assisted by the counterbalancing action of the moderately extended springs, whilst in the downward movement any abrupt drop due to gravity is braked and smoothed out by the resistance of the slightly more extended springs, the difference in extension between the two positions being not more than ¼ in. Oil-filled dashpots assist in making the movement smooth.

Safety devices

The use of high voltage requires strict precautions to eliminate the risk of fatal accidents. To ensure the safety of the operator the apparatus is enclosed in a safety cage consisting of a base and back of steel sheet, sides of expanded metal (metal mesh) and front and cover of Perspex for visibility. The cage is suitably earthed and incorporates two micro-switches connected to the input side of the electrical circuit to ensure that the apparatus can be used only when the cover is closed.

In addition, there is a device combining an acoustic warning system (buzzer) with an automatic electrical cut-out. This is activated by the pressure of the cooling water and set so that below a certain pressure it comes into operation, draws the attention of the operator to insufficient cooling, prevents overheating, and thus saves the experiment and equipment even if the water supply should fail.

Insulation

The choice of Polythene film of appropriate thickness (0.01 in.) for electrical insulation in preference to other plastic films such as P.V.C., Teflon etc. was prompted by the excellent electrical properties, pliability, lack of moisture absorption and inexpensiveness, which made handling and replacement in case of a breakdown simple and convenient. An occasional breakdown of the insulation, however, cannot be entirely prevented, since repeated mechanical handling, creasing, and overheating, particularly at points of less efficient cooling, *i.e.* ends of plates, under conditions of high current density reduce the dielectric strength of the material and thus bring about a breakdown in the form of pinholes in the weakest places. This is usually accompanied by a rattling noise, which serves as a warning to switch off the current supply and replace the damaged film before continuing the experiment. If the warning is unheeded and the situation allowed to continue for several minutes, the pinholes are widened to sizable slits and may finally lead to a short circuit and significant damage. This is the main reason why direct insulation of the plates by coating was rejected. Damage to the insulation in this case means a tedious and expensive repair, putting the equipment out of use for some time.

Electrical power unit

The power requirements, rather than the maximum attainable voltage, determine the design. The convenience of working with wide strips of paper entails the need

for a powerful electrical supply, as the current consumption increases proportionally with the width of the strip. A compromise solution has been found whereby an effectively high voltage could be produced together with the sufficient amount of current required for use with buffer systems of varying ionic strength, composition and degree of dissociation.

The power unit, the circuit diagram of which is shown in Fig. 4, is capable of supplying 500 mA at 5 kV, although in practice a current consumption of over 350 mA would present some difficulties in heat dissipation.

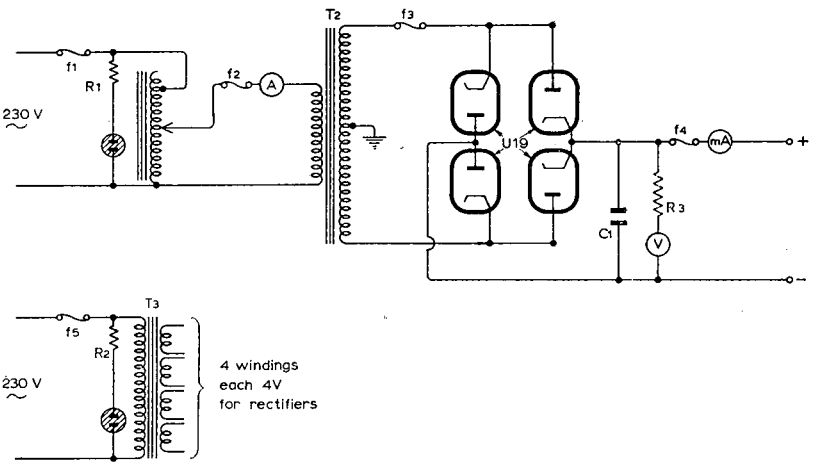

Fig. 4. Circuit diagram for a 5 kV power unit. Meters: A = 0–10 A, A.C. 50 ~ ; mA = 0–500 mA, m/c, V = 0–5000. Resistances: R1 = 220 kΩ; R2 = 220 kΩ; R3 = 5 MΩ 10 W. Transformers: T1 = 100R Variac; T2 = 230 V in, 2250 0 2250 V, 500 mA out; T3 = 230 V in, 4 V 4A, four times, high insulation. Condenser: C1 = 3 mfd, 8000 V, D.C. Fuses: f1 = 10A; f2 = 10A; f3 = 500 mA; f4 = 500 mA; f5 = 1A.

A stand-by unit of a maximum rating of 150 mA, 10 kV has in the past proved to be insufficient for experiments using strips wider than 8 in., even in weak electrolyte solutions such as used for the separation of amino acids and peptides.

The ratings of the component parts given in the diagram are not necessarily the best, but were often selected on account of availability and convenience of price. A Variac of a higher output rating would be advisable. The rectifying valves, transformers and condensers have performed well over more than two years and only few replacements were required.

A special smoothing unit was tested but finally omitted as not essential. The set of fuses placed in several critical positions in the circuit has proved to be ample protection against sudden damage. The power unit is, of course, carefully earthed.

RESULTS

With efficient cooling, uniform pressure, and evaporation reduced to insignificant proportions, current constancy and thus steady conditions of temperature, ionic

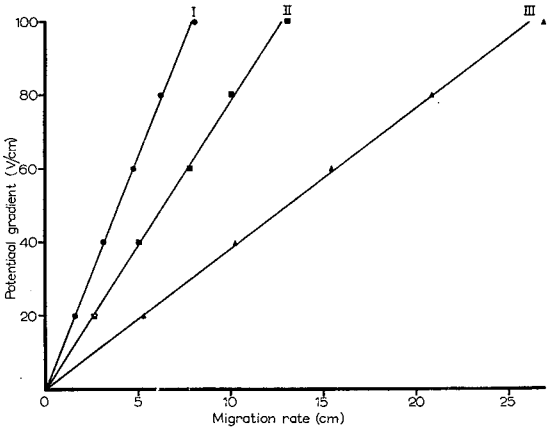

Fig. 5. Migration rates of amino acids as a function of potential gradient. Experimental conditions: Whatman No. 3 MM paper, 12 × 22½ in., pH 2.0, 0.75 *M* formic acid solution, 10°, 20–100 V/cm, 1 lb./sq.in., 30 min. I = tryptophan; II = leucine; III = lysine.

strength, conductance, pH, moisture and potential gradient, are attained after a very short time (a few minutes). Under these conditions a linear relationship should obtain between migration rate and voltage applied, and likewise with respect to time of electrophoresis. The constancy of the current is a good indication of steady conditions prevailing in the paper strip. Figs. 5 and 6 show the results obtained, using the equipment at up to 5 kV and an energy throughput of up to 0.8 W/cm². The values plotted are migration rates corrected for the electro-osmotic and hydrodynamic effects established by measuring the displacement of various sucrose spots across the whole area of the strip. Cellophane barriers were used to minimise fluid flow.

The three amino acids chosen cover a wide range of mobilities. Linear movement with respect to potential gradient, in this case up to 100 V/cm, seems clearly establish-

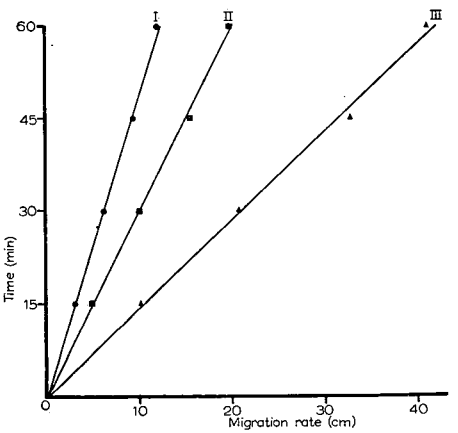

Fig. 6. Migration rates as a function of time. Experimental conditions: as in Fig. 5, 80 V/cm, 160 mA.

ed. In another series of experiments, using narrower strips and potential gradients up to 160 V/cm, a linear relationship could still be found.

The experiment carried out at 80 V/cm shows a substantially linear migration with time although the slight scattering of the later points on the lysine line may indicate some temperature disturbance near the cathodic end of the strip. The time limit of one hour is set by the length of the strip and the migration rate of the fastest compound. Distances linear with time can be assumed to indicate a uniform potential gradient along the length of the strip.

It would therefore appear that the conditions essential for the measurement of electrophoretic mobilities, even at high potential gradients, are amply provided by the general design of the apparatus, and that for ordinary separation experiments a high degree of reproducibility can be expected, even under less strictly controlled conditions. The latter has, in fact, been achieved in separations of complex mixtures of amino acids[12,14], of organic acids[15] and aliphatic amines[16] with a reproducibility for individual runs within 3%. The good reproducibility and high resolution aid substantially in the isolation and identification of unknown constituents.

DISCUSSION

There are several advantages in using high-voltage electrophoresis. Since the rate of diffusion is independent of the applied voltage and dependent on time, a significant reduction in the time of the electrophoretic run, for the same distance of migration, is bound to minimise the adverse effects on resolution of diffusion, particularly in the case of low-molecular weight compounds. The control of hydrodynamic flow in the strip over short periods is also much easier. The economy in time is substantial.

Attempts to apply high voltages are met, however, by a serious limitation, *viz.* the generation of heat. Efficient heat dissipation is the most important factor in solving the problem and any useful design of high-voltage equipment must be judged primarily by this criterion. The voltage and current level is limited by the heat-dissipating capacity of the apparatus.

Liquid heat exchangers can only dissipate energy[1] up to 0.2 W/cm^2 and have certain objectionable features such as evaporation of solvent with resultant fire and health hazards. Solid heat exchangers, on the other hand, are far more effective, provided they are good thermal and bad electrical conductors. However, as a rule, material of good thermal conductivity is also of good electrical conductivity. Glass is a good insulator but a poor heat conductor, especially when used in a thickness to impart sufficient mechanical strength. The same applies to plastics. Cooling plates made of plastics are probably able to cope satisfactorily with an energy throughput of up to 0.1 W/cm^2, depending on the design.

A combination of an excellent heat-conductor such as a metal plate with a very thin film of highly insulating plastic material such as Polythene (polyethylene) gives satisfactory results, particularly when the heated paper strip is compressed between two plates for good contact. An apparatus designed on this principle and

described above has proved to be of high cooling efficiency, making the dissipation of 1 W/cm^2 possible by using a flow of tap water of 12 l/min at 12°. This design makes it essential to have uniform, reproducible, moderate pressure exerted on the paper strip, since several important conditions such as moisture, conductance, uniform potential gradient and current consumption are governed by the pressure. The advantage of this arrangement over single-plate cooling is quite considerable. A recently described apparatus[8] based on bottom plate (copper) cooling with insulating film and a refrigerated coolant, or water, was described as capable of dissipating only about 0.1 W/cm^2.

It is often desirable to use the highest possible potential gradient allowed by the heat-dissipating capacity in order to obtain the maximum benefit from the technique. Very small differences in mobilities are insufficient for separations, unless a fairly large distance is travelled within a short time. Good examples to prove this point were the separation of sorbitol from mannitol[17] and of the alkali metal ions K, Rb, Cs from one another[18] carried out on a similar, though smaller apparatus.

The present dimensions, though suitable both for analytical and preparative work (using a thick sort of filter paper), impose certain limitations on the important two-dimensional work. It is thought that square cooling-plates, 22 × 22 in., would be more convenient, though the power requirements may present a difficult problem.

The method of using a coolant refrigerated to a temperature of just above 0°, as stipulated by designs employing glass or plastics for solid heat-exchangers, is not without serious disadvantages. Although the cooling is improved by the lower temperature of the coolant, the viscosity of the electrolyte solution is increased and the mobilities of the ions are significantly reduced. Consequently, the migration time or the applied voltage will have to be increased to produce the same path length within the given time of experiment, thus reducing the intrinsic value of the technique. Experience over several years with tap water has given convincing proof that, even with seasonal temperature fluctuations from 5–20°, water provides an inexpensive, ample and convenient means of cooling. It is, of course, necessary to compare mobilities at the same temperature, and for this purpose provision for measuring the temperature of the water entering and leaving the cooling system has to be made. The estimated temperature in the strip is about 1–2° higher, as measurements with inserted thermocouples have indicated.

The smooth performance of the apparatus is also dependent on the dielectric strength of the isulating film under the experimental conditions. At present, using Polythene film, it is found that occasional breakdowns due to overheating, and mechanical wear at the edges of the cooling plates (mostly the anodic side) cannot be entirely eliminated. Occasional flaws in the material may also contribute to it. It is, however, hoped that in the not too distant future improvements in the quality and dielectric strength, or the appearance of new plastics, will considerably reduce the risk of insulation failure.

ACKNOWLEDGEMENTS

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contribution to the design, to Mr. E. W. UNDERWOOD for construction of the apparatus, and to the Directors of Tate and Lyle Ltd. for permission to publish this communication.

SUMMARY

1. An apparatus for high-voltage (> 50 V/cm) paper electrophoresis has been developed, with efficient cooling and high degree of reproducibility of results as the major features of the design.

2. A sandwich-type with double-cooling of the paper strip by a system of thinly insulated solid heat-exchangers (aluminium alloy plates), with tap water as coolant, was found most efficient with respect to heat-dissipating capacity.

3. A special double-countercurrent channelling system in the plates minimises the possibility of temperature gradients.

4. Polythene (polyethylene) film of 0.01 in. thickness has proved itself as a suitable, convenient and inexpensive insulation.

5. The apparatus is provided with a pneumatic uniform-pressure device for evenness of cooling and control of moisture.

6. A lifting device is incorporated for convenient handling and safety devices ensure both the safety of the operator and the prevention of damage to the equipment

7. The results obtained at potential gradients of 100 V/cm and energy throughputs of up to 1 W/cm^2 show a straight-line relationship of migration rate with respect to potential gradient and time. The degree of reproducibility of individual runs is high and usually within 3%. The steady-state conditions obtainable in the apparatus make the measurement of migration rates, even in background electrolyte solutions of relatively high ionic strength (> 0.2), both convenient and reliable. Difficult separations of complex mixtures of various groups of low-molecular weight compounds such as amines, amino acids, sugars, inorganic cations, organic acids etc. can be accomplished with a high degree of resolution within a comparatively very short time.

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A SAMPLE APPLICATOR FOR CHROMATOGRAPHIC PAPER AND ITS USE FOR 5-HYDROXYMETHYL-2-FURALDEHYDE AND LEVULINIC ACID ANALYSIS

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INTRODUCTION

In a recent investigation into the kinetics of the decomposition of glucose in aqueous acidic media and its conversion to 5-hydroxymethyl-2-furaldehyde and levulinic acid[1], it was necessary to develop experimental techniques for the quantitative analysis of these compounds. To assure that isothermal conditions were obtained in these reactions where maximum yields were reached in a few seconds, it was necessary to work with reactant volumes as small as 0.03 ml, using paper chromatography to separate the materials of interest prior to quantitative measurement. Since the wide range of variables of interest in the kinetics study demanded the analysis of hundreds of samples, a new applicator was developed which was rapid, precise, and permitted the loading of paper to the limit of its capacity.

The design of this applicator is described below and, in addition, the data obtained through its use for the analysis of 5-hydroxymethyl-2-furaldehyde and levulinic acid are summarized.

DESIGN OF APPLICATOR

One of the main criteria of an applicator for quantitative paper chromatography is that it yields a reproducibly uniform streak of the sample solution on the paper. In order to extend its range of usefulness, it should also be capable of applying a different volume per lineal dimension to separate sheets. An applicator which satisfies these requirements and has the additional desirable feature of ease of operation is shown in Fig. 1.

This model was constructed from two $\frac{1}{4} \times 2 \times 24$ in. machinist's straight edges. These were joined at their ends as shown by two cross pieces which were fastened to the lower straight edge permanently and to the upper straight edge by close fitting removable bolts. The angle between these two edges could then be varied in

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** Maintained in cooperation with the University of Wisconsin.

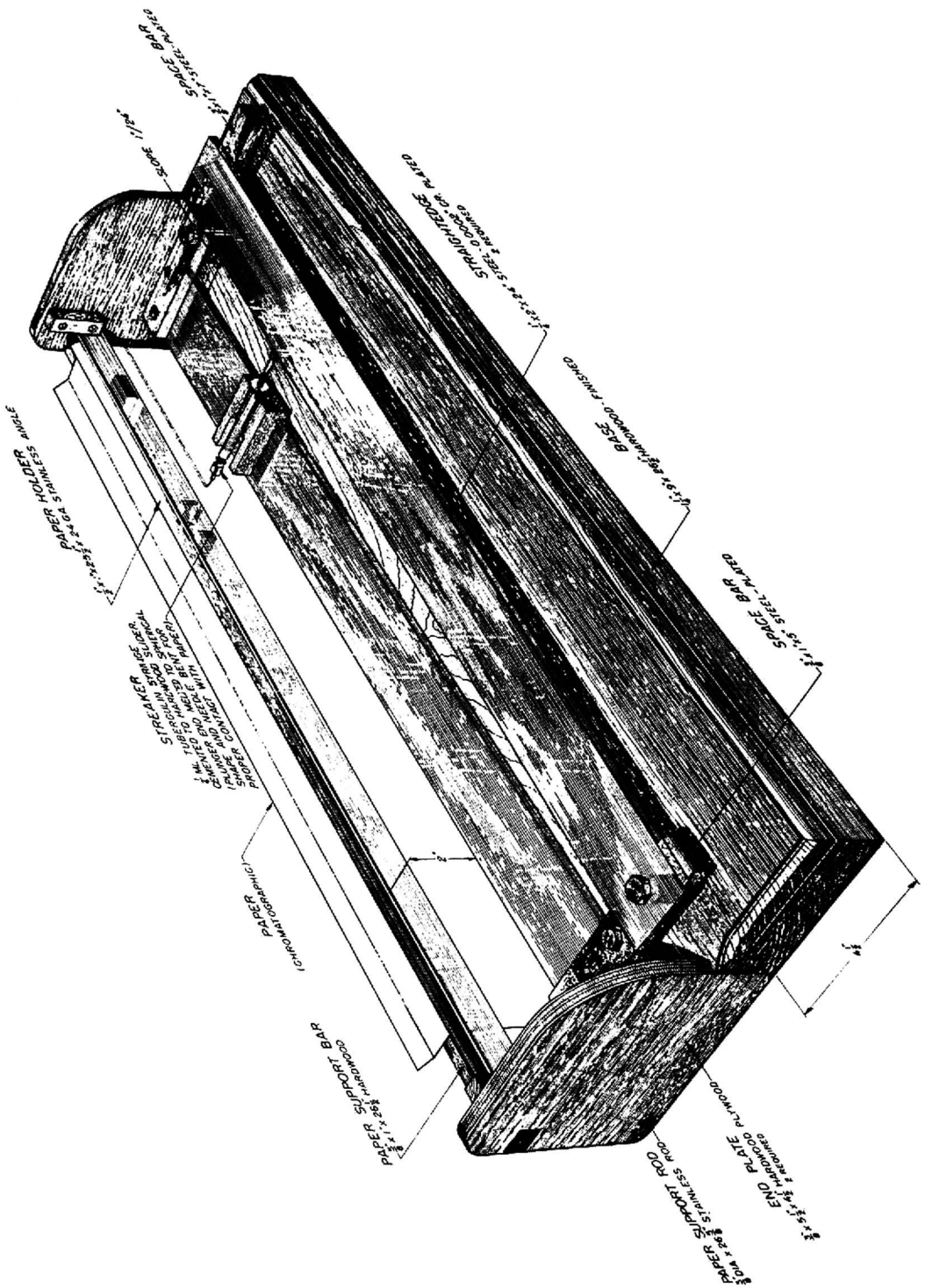

Fig. 1. Sample applicator for chromatographic paper.

discrete steps. All pieces of this model had been chrome-plated to reduce any corrosion and cleaning problems.

The second part of this applicator was made by securing a precision hypodermic syringe with adhesive to a maple sled as shown in Fig. 1. There is an overhang on the back of the sled to act as a guide when it is moved along the lower machinist's edge. The back of the syringe plunger had been given a hemispherical shape where it touched the upper bar. The hypodermic needle tip had been cut to an appropriate length, ground smooth, and given a slight backward curve to avoid tearing the paper. The whole assembly was then mounted on a wooden base and fitted with a bracket to hold the paper sheets as shown in the figure.

The operation of the applicator is carried out as follows: A sheet of chromatography paper is first placed on the bracket of the applicator and the sample solution loaded into the syringe. The sled with the plunger maintained in its extended position is then placed on the lower bar and moved slowly along until the back end of the plunger contacts the upper bar and forces out some solution. The needle tip is then wiped dry and the paper moved into position so that one edge touches the needle tip. The paper is then held firmly in position. As the sled is moved toward the apex of the angle of the two straight edges, the plunger is forced into the syringe and the solution is applied uniformly to the sheet.

The volume of solution applied per lineal dimension may be varied in three ways. The angle between the two bars may be changed, syringes calibrated to different sizes may be used, and multiple streaks may be applied. If multiple streaks are used, it is advisable to dry each application before a subsequent streak is made to prevent the formation of an unduly wide starting line.

In an effort to measure the linearity or uniformity of the applied streak of solution, the following trial was made. The syringe was filled with water and placed in streaking position on the lower straight edge. A stop was then clamped securely 1.5 in. down the bar. As the sled was moved slowly toward the stop, the ejected water was taken up in a tared glass capillary tube. The stop was then moved 1.5 in. further down the bar and the process repeated. This was continued in nine steps which reached from one end of the machinist's edge to the other.

The results of two such trials are summarized in Table I. A linear regression analysis of each data set was made in which the volume delivered per 1.5 in. increment was measured as a function of position on the applicator. The very small random values obtained for the slopes of these two regression lines, $-1.50 \cdot 10^{-5}$ and $+3.33 \cdot 10^{-6}$ ml/1.5 in. per increment, corroborate the linearity of the applied streak over the length of the applicator. A measure of the precision of this method may also be obtained from these data from the magnitude of the standard deviation, 0.00023 ml/1.5 in., of all the individual observations from the overall average. When replicate samples from one streak are averaged, as will normally be done, the standard deviation will, of course, be smaller (*e.g.*, the standard deviation of the average of each set in Table I from the overall average is 0.00006 ml/1.5 in.). It must be pointed out that a portion of this error can be ascribed to the collection and weighing of the delivered liquid.

TABLE I

TESTS FOR APPLICATOR LINEARITY

Sample position	*Volume delivered ml/1.5 in.*	*Deviation from average ml/1.5 in.*
1	0.0260	—0.00008
2	0.0262	+0.00012
3	0.0260	—0.00008
4	0.0263	+0.00022
5	0.0260	—0.00008
6	0.0262	+0.00012
7	0.0263	+0.00022
8	0.0256	—0.00048
9	0.0261	+0.00002
Average	0.02608	
1	0.0260	+0.00004
2	0.0261	+0.00014
3	0.0254	—0.00056
4	0.0263	+0.00034
5	0.0259	—0.00006
6	0.0259	—0.00006
7	0.0261	+0.00014
8	0.0261	+0.00014
9	0.0258	—0.00016
Average	0.02596	

Quantitative tests were also made to measure the effect of the rate of sample application. In these tests, streaks of 2*N* potassium iodate solution were applied to sheets of chromatographic paper very rapidly in one case, very slowly in another, and alternately rapidly and slowly in still another. When the streaks had dried, quadruplicate samples precisely 3 in. long were cut from each streak, placed in individual 40 ml beakers of distilled water and titrated with a standardized sodium thiosulphate solution. In Table II are summarized the average recoveries for each trial which fail to indicate a significant dependence on the rate of application. A measure of the precision of this method of sample application, including the analytical errors of the potassium iodate titration, may also be obtained from these data in

TABLE II

TESTS FOR DEPENDENCE ON RATE OF APPLICATION

Sample	*Average potassium iodate concentration recovered from paper*		
	Rapid streaking mequiv./3 in.	*Slow streaking mequiv./3 in.*	*Intermittent streaking mequiv./3 in.*
1	6.893	6.842	6.840
	6.862	6.855	6.775
2	7.430	7.478	7.385
	7.470	7.475	7.465

which the standard deviations from the overall average of each sample set are both approximately 0.034 mequiv. per 3 in.

EXPERIMENTAL

Analysis for 5-hydroxymethyl-2-furaldehyde

An extensive investigation of various solvent systems for irrigating the papers did not result in one system which could handle both 5-hydroxymethyl-2-furaldehyde and levulinic acid satisfactorily. Consequently, separate systems were employed in the analysis of each. The solvent system used in the analysis of 5-hydroxymethyl-2-furaldehyde was suggested by BUCH, MONTGOMERY AND PORTER[3] and was the organic layer which results from the mixing of equal volumes of 5 *M* formic acid and pentanol. This irrigating system resulted in a compact band of 5-hydroxymethyl-2-furaldehyde with an R_F value of 0.75. Quantitative tests performed to determine its distribution over the entire sheet indicated that less than 1 % of the material was located outside of the indicated band.

The analytical procedure employed involved streaking the solutions onto 18 by 22 in. sheets of Whatman No. 3 MM chromatographic paper, irrigating them at 30° for 15 hours with the formic acid–pentanol solvent, drying them, and locating the band of 5-hydroxymethyl-2-furaldehyde with the aid of an ultraviolet lamp. Four samples, each 75 mm wide, were then cut from this band and placed into individual beakers containing predetermined quantities of distilled water to elute the 5-hydroxymethyl-2-furaldehyde. Since solutions of this material follow Beer's law, their concentrations were determined from the optical absorbence with a Beckman Model DU spectrophotometer at 284 mμ. It should be noted that the very dilute solutions of 5-hydroxymethyl-2-furaldehyde required by the spectrophotometer show a tendency to degrade when stored in sunlight at room temperature. This degradation will not occur significantly if they are kept under refrigeration and in the dark. In practice, direct sunlight and long delays in analysis should be avoided.

This method was calibrated with carefully recrystallized samples of 5-hydroxymethyl-2-furaldehyde prepared in this Laboratory and the results of this calibration are shown in Fig. 2. A linear regression analysis made on these data employing the method of least squares indicated a correlation coefficient of 0.99 (significant well below the 1 % level) with a standard error of estimate from the regression line of 0.031 mg per 75 mm. The slope of this line is 0.919 and the intercept value of 0.006 agreed closely with the experimentally determined paper blank.

From these results it is evident that approximately 8 % of the material applied was not covered. A detailed investigation into the possible sources of this loss, including several chromatographic separations of the same sample of 5-hydroxymethyl-2-furaldehyde, seemed to indicate that the loss is due to its volatilization during the removal of water after streak application, prior to irrigation. Since the loss is constant over a several-fold change in concentration and is satisfactorily

compensated for, the indicated calibration may be used with confidence over the range indicated.

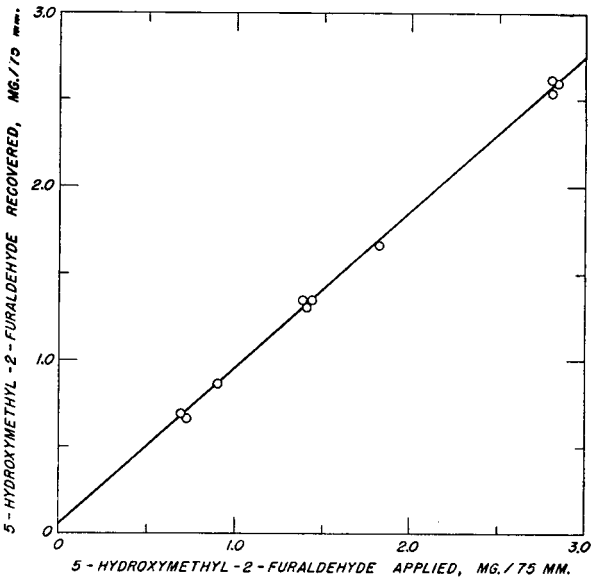

Fig. 2. Calibration curve for chromatographic recovery of 5-hydroxymethyl-2-furaldehyde.

A study was also made of several additional similar compounds which might result from the acid-catalyzed degradation of glucose. Since the chromatographic and spectroscopic behavior of these materials may be of value to those working in this field, the properties measured in this investigation are summarized in Table III and Fig. 3. It will be noted that the chromatographic behavior is quite similar for all the compounds, but that fortunately the molecular absorption spectra differ sufficiently to allow differentiation. Quantitative calibrations for these other materials were not established.

TABLE III

PROPERTIES OF 5-HYDROXYMETHYL-2-FURALDEHYDE AND RELATED COMPOUNDS

Compound	*Melting point* °C	*Wave length at major peak* mμ	*Molecular extinction coefficient* l/mole/cm	R_F*
5-Hydroxymethyl-2-furaldehyde	33.4	284	16,920	0.75
Oxy-bis-(5-methylenefurfural)	114.5	282	28,600	0.84
5-Hydroxymethyl-2-furoic acid	160–161 (dec.)	251	12,420	0.74
2-Hydroxyacetylfuran	82.5	276	14,090	0.73
Levulinic acid	33.5	270	25.1	0.75

* Irrigation solvent system is the organic layer from an equi-volumetric mixture of 5 *M* formic acid and pentanol.

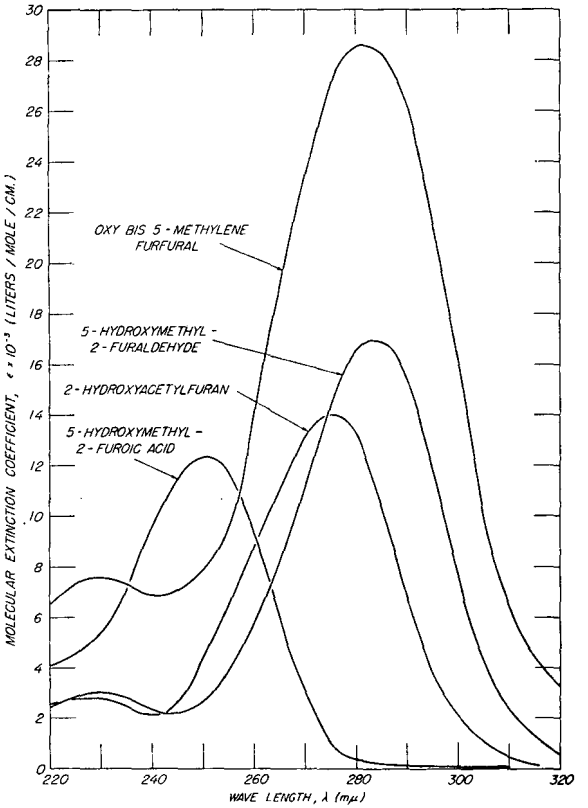

Fig. 3. Ultraviolet absorption spectra for 5-hydroxymethyl-2-furaldehyde and related compounds.

Analysis for levulinic acid

Free levulinic acid is too volatile to yield a reproducible, quantitative chromatographic separation. Therefore, the possibility of separating it as a salt was considered and it was found that ammonium levulinate would chromatograph into compact, quantitative bands when a mixture of ethanol (95 %), aqueous ammonia (29 %), and water in a volumetric ratio of 100:5:5, respectively, was used as the irrigating solvent. This system resulted in the following R_F values for these major components of the reacted glucose solutions:

Component	R_F
Glucose	0.30
Ammonium formate	0.35
Ammonium levulinate	0.40
5-Hydroxymethyl-2-furaldehyde	0.75

The method employed to measure the amount of the separated levulinic acid was suggested by PLOETZ AND BARTELS[4] and FROST AND KURTH[5], and depends on the

presence of the acetyl group which undergoes the iodoform reaction in the presence of alkaline iodine solutions. Since the reaction is not stoichiometric, a standardization was made by analyzing aqueous levulinic acid solutions of known concentrations varying from 0 to 5 mg/ml. 1 ml aliquots of these solutions were analyzed by adding 5 ml of a 0.1 *N* iodine solution followed by 5 ml of 1.0 *N* potassium hydroxide. They were then swirled, stoppered, and placed in a thermostated water bath for 10 min, during which time a yellow flocculent precipitate of iodoform was produced. The samples were then removed, 5 ml of 1.1 *N* hydrochloric acid added to render the solution acid, and the free iodine remaining in the samples titrated with a standardized 0.1 *N* sodium thiosulfate solution, using a starch solution as an indicator. A blank sample containing only water was also run through this procedure as a measure of the amount of iodine originally added. Since this analysis is somewhat sensitive to reaction conditions, the specified procedure must be carefully followed. From the above standardization, the calibration of Fig. 4 was obtained. It may be noted that

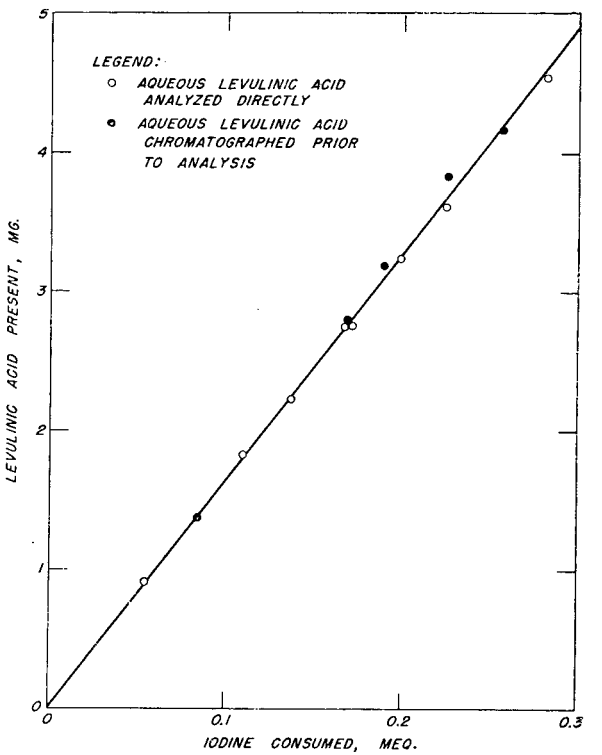

Fig. 4. Standardization curve for analysis of levulinic acid.

7.18 moles of iodine are consumed per equivalent of levulinic acid indicating that the overall reaction is not a simple iodoform reaction.

To determine if the separation step was also quantitative, the calibration was repeated on samples of levulinic acid recovered from 18 × 24 in. sheets of Whatman

No. 3 MM chromatography paper. In these trials, a streak of 1.0 *N* ammonium carbonate was first applied to the paper and dried. The solution of levulinic acid was then applied over the first streak and thus converted to ammonium levulinate *in situ*. After a 15 hours irrigation with the designated solvent, the papers were dried and four 75 mm vertical strips removed for analysis. The remaining guide strips were sprayed with a 2,4-dinitrophenyl-hydrazine indicator (2 g dissolved in 400 ml of 10% hydrochloric acid) to locate the position of the levulinate band. This position was marked on the 75 mm strips and the corresponding area containing the levulinate removed for analysis.

The ammonium levulinate was eluted from the paper tabs in a modification of the method described by SAEMAN *et al.*[2]. To accommodate the thick 3 mm paper, the lower glass plate was substituted for by a stainless steel plate with a notch 0.125 in. wide by 0.008 in. deep milled into the front edge. In this method, the material is eluted from the paper tab and collected completely in a 0.75 ml capillary pipette. The solution was then transferred to a 50 ml Erlenmeyer flask for analysis. Since the ammonium ions interfere with the iodometric analysis, it was necessary to remove them by adding 0.25 ml of 0.5 *N* sodium carbonate to the flask and evaporating the solution to dryness. This was done at room temperature under a reduced pressure aspirator in a vacuum manifold arrangement capable of handling several samples at one time[2]. The dried sodium levulinate was then put back into solution with 1 ml of distilled water and the analysis carried out as described above. The results of these analyses are also shown in Fig. 4 where the amount of levulinic acid applied to the paper is plotted as a function of the iodine consumed.

The data of Fig. 4 indicate that the chromatographic separation and recovery of levulinic acid in the manner described is quantitative and that the proposed technique may be employed with confidence within the range studied. A linear regression analysis of all the data shown in Fig. 4 was made and a correlation coefficient of 0.99 was obtained. The slope of the line is 16.27 mg of levulinic acid per mequiv. of iodine consumed and the data show a standard error of estimate from the regression line of 0.059 mg.

Application of chromatographic techniques

The techniques described have served effectively in an extensive study of the acid-catalyzed decomposition of glucose. The sample applicator aided significantly in analytical scheme by reducing the time-consuming step of sample application, and the analysis schemes for 5-hydroxymethyl-2-furaldehyde and levulinic acid proved very reliable.

SUMMARY

In the course of a kinetics study into the production of 5-hydroxymethyl-2-furaldehyde and levulinic acid from aqueous acid solutions of glucose, a sample applicator was developed for use in paper chromatographic separations. This applicator is capable of delivering a continuous, uniform streak of the sample solution to the paper

and has the additional advantages of versatility of range and ease of operation. Also included are the analysis schemes and standardizations developed for the determination of 5-hydroxymethyl-2-furaldehyde and levulinic acid. These materials are first separated chromatographically from the other reaction products and then the concentration of 5-hydroxymethyl-2-furaldehyde is measured by its ultraviolet absorption and the levulinic acid concentration determined by means of the iodoform reaction.

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ÜBER TRITERPENE

VII. DÜNNSCHICHT- UND IONENAUSTAUSCHERPAPIER-CHROMATOGRAPHIE VON TRITERPENOIDEN*

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I. EINLEITUNG

Während die Papierchromatographie der Steroide sehr intensive Bearbeitung erfahren hat (vgl. z.B. die Übersicht von NEHER[2]), ist über die Trennung von Triterpenoiden bisher in der Literatur nur sehr wenig berichtet worden. NISHIOKA[3] hat die Papierchromatographie von einigen Triterpenoiden und Steroiden systematisch behandelt, wobei er Paraffin oder Methylstearat als stationäre Phase verwendete. Als Laufmittel erwiesen sich wässrige Alkohole und die leichten Phasen von Kohlenwasserstoff–Methanol–Wasser-Gemischen als geeignet. Wir konnten die Ergebnisse NISHIOKAS an einigen Beispielen bestätigen, es gelang uns aber nicht, Gemische von isomeren oder verwandten Triterpenoiden zu trennen, da sich die langgezogenen Flecke überdeckten.

HASHIMOTO UND CHATANI[4] arbeiteten ebenfalls nach der "reversed phase"-Methode, indem sie das Papier (Toyo Roshi, No. 52) mit einer Lösung von Dow Corning Silicone No. 1107 imprägnierten und als Laufmittel Alkohole, wässrige Essigsäure oder Toluol–Ammoniak-Gemische (leichte Phase) verwendeten. Auch hier gelang es uns nicht (auf Schleicher & Schüll, sowie Whatman-Papieren), Triterpenoidgemische in diskrete Flecke aufzulösen. TSCHESCHE UND POPPEL[5] erzielten mit dem System Isooctanol–Pentanol–10 % wässr. Pyrrolidin–Formamid (6:2:1:4) in Analogie zu der Papierchromatographie von Herzgiften[6] (Imprägnieren des Papiers mit der leichten Phase, Entwickeln mit der wässrigen) eine gute Auftrennung saurer Triterpenoide, wobei man aus dem R_F-Wert die Anzahl der O-Funktionen abschätzen kann, während isomere Verbindungen hier gleich weit liefen. Triterpen-dicarbonsäuren liessen sich auch sehr gut mit den Gemischen Isooctanol–Pentanol–10 % wässr. Morpholin (5:5:7) und Butanol–2 *N* wässr. Ammoniak trennen[1], wobei in letzteren Fällen die organische Phase als Laufmittel diente.

II. DÜNNSCHICHT-CHROMATOGRAPHIE

Die ausgezeichneten Erfolge, die wir bei Anwendung der Dünnschicht-Chromatographie an Kieselgel G (Merck)[7] auf die Triterpensäuren aus *Bredemeyera floribunda*

* VI. Mitteilung siehe [1].

** Neue Anschrift: Bonn, Chemisches Institut der Universität, Meckenheimerallee 168.

Willd.[1] und bei einigen Steroiden[8,9] erzielen konnten, veranlassten uns, dieses sehr einfache Verfahren systematisch auf seine Brauchbarkeit zur schnellen Analyse von Triterpenoiden zu untersuchen. Wie aus den Tabellen I und II hervorgeht, erhielten wir unter Verwendung einfacher Lösungsmittelkombinationen sehr brauchbare Trennungen (siehe auch Fig. 1 und 2). Durch Zusatz von Pyridin oder Diäthylamin

TABELLE I

DÜNNSCHICHT-CHROMATOGRAPHIE DER SÄUREN

Das System I war am allgemeinsten anwendbar und diente als repräsentatives Gemisch. Säuren, die darin Schwänze gaben, wurden in den Systemen II bis V geprüft; auch die übrigen Verbindungen lassen sich darin meist trennen, wobei die R_F-Werte eine ähnliche Reihenfolge einnehmen, wie im System I.

In dieser und in Tabelle II sind jeweils nur die R_F-Werte des Hauptflecks angegeben, falls sich die Substanzen als nicht völlig rein erwiesen. Die R_F-Werte im System I sind immer auf den der Oleanolsäure = 0.68 bezogen. S bei der Angabe der R_F-Werte bedeutet Schwanzbildung, eingeklammerte Zahlen gelten für Nebenflecke.

Substanz	*R_F-Werte in Gemisch**				
	I	*II*	*III*	*IV*	*V*
Oleanolsäure	0.68	0.64	0.50	0.48	0.96
Ursolsäure	0.68	0.64	0.50		
Betulinsäure	0.68				
Säure aus *Alphitonia excelsa*	0.68				
Morolsäure	0.59				
Oleanonsäure	0.68				
Masticadienonsäure	0.47				
Isomasticadienonsäure	0.47				
Cochalsäure	0.18 S	0.15 S	0.45	0.29	0.55 S
Bredemolsäure	0.59 S	0.62			0.82
Siaresinolsäure	0.66				
Machaerinsäure	0.23 S	0.27	0.25	0.23	0.54
Guaijavolsäure	0.35				
Dihydroxysäure-Gemisch aus *Crataegus oxyacantha* L.	0.42 0.54 0.63				
Acantholsäure	0.29				
Bayogenin	0.31				
Sapogenin aus *Sideroxylon Pohlm.*	0.37	0.34 S	0.35		
Chinovasäure	0.55				
Medicagensäure	0.29 S	0.38 S	0.35	0.10 S	0.71
Acetylursolsäure	0.76				
Acantholsäureacetat	0.66				
Emmolsäure	0.59				
Rohe Nimbsäure			Start 0.07 (0.37) S (0.56) S 0.71		

* Gemische: I: Diisopropyläther–Aceton (5:2); II: Diisopropyläther–Aceton (5:2) mit Zusatz von 5% Pyridin; III: Essigester–Methanol–Diäthylamin (14:4:3); IV: Chlorbenzol–Eisessig (9:1); V: Methylenchlorid–Pyridin (7:2).

TABELLE II

DÜNNSCHICHT-CHROMATOGRAPHIE VON NEUTRALEN TRITERPENOIDEN

Die angegebenen R_F-Werte stellen Mittelwerte aus mehreren Chromatogrammen dar. Die Substanzen wurden nur immer in den Systemen untersucht, in denen keine zu kleinen oder zu grossen R_F-Werte auftreten.

Substanz	*R_F-Werte in Gemisch**				
	I	*VI*	*VII*	*VIII*	*IX*
β-Amyren			0.82		
β-Amyrin				0.12	0.38
β-Amyrin-acetat				0.45	
α-Amyrenon				0.31	
Lanosterin		0.75		0.14	0.40
Dihydro-lanosterin-acetat			0.03	0.43	
Ursolsäure-methylester-acetat				0.26	0.77
Oleanolsäure-methylester-acetat				0.24	0.77
Ursonsäure-methylester		0.85			0.51
Crataegolsäure-methylester-monoacetat	0.80	0.40			0.13
Crataegolsäure-methylester-diacetat	0.92	0.72			0.37
Dehydro-crataegolsäure-methylester-diacetat		0.69			0.39
11-Keto-crataegolsäure-methylester-monoacetat	0.77				
Acantholsäure-methylester-monoacetat	0.73				
Echinocystsäure-methylester		0.59			0.15
Emmolsäure-dimethylester		0.73			

* Gemische: VI: Diisopropyläther; VII: Cyclohexan; VIII: Benzol; IX: Methylenchlorid.

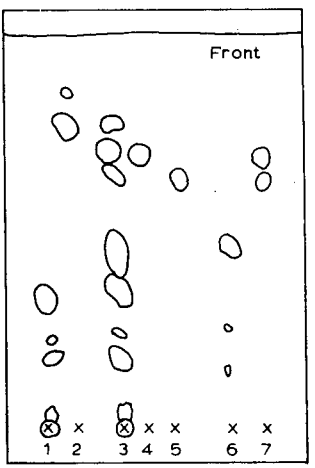

Fig. 1. Dünnschicht-Chromatographie von Triterpen-carbonsäuren im Gemisch I. (1) Nimbsäure (roh); (2) Acetyl-ursolsäure; (3) Gemisch von (1), (2), (4), (5) und (6); (4) Oleanolsäure; (5) Morolsäure; (6) Guaijavolsäure; (7) Gemisch von (4) und (5).

liessen sich bei einigen Substanzen, die auch im Dünnschicht-Chromatogramm zur Schwanzbildung neigten, diskrete Flecke erzielen.

Der Vorteil gegenüber den bisher beschriebenen Verfahren liegt erstens darin, dass die gesamte Zeitdauer für ein Chromatogramm auf 20–30 Minuten reduziert ist; zweitens liessen sich Verbindungen mit verschiedenem R_F-Wert auch in Vielkomponenten-Gemischen gut trennen; drittens ist diese Methode ebenfalls auf neutrale Triterpenoide (Ester, Alkohole, Kohlenwasserstoffe) anwendbar und viertens

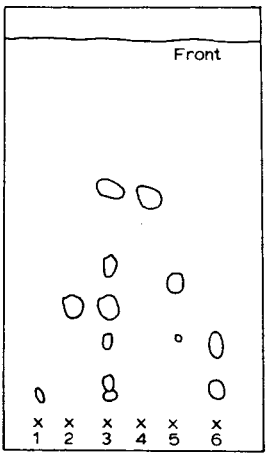

Fig. 2. Dünnschicht-Chromatogramm von Triterpencarbonsäure-estern im Gemisch IX. (1) Crataegolsäure-methylester-monoacetat; (2) Crataegolsäure-methylester-diacetat; (3) Gemisch von (1), (2), (4), (5) und (6); (4) Ursolsäure-methylester-acetat; (5) Ursonsäure-methylester; (6) Echinocystsäure-methylester (Hauptfleck mit kleinerem R_F-Wert).

ist die Empfindlichkeit der zum Nachweis verwendeten Farbreaktion mit Chlorsulfonsäure[8] ausserordentlich gross. So ist im entwickelten Chromatogramm noch 0.02 γ Oleanolsäure bequem nachweisbar. Dieses Verfahren ist daher auch zur raschen Kontrolle von Reaktionsansätzen und zur Prüfung auf Reinheit sehr geeignet. Die Reproduzierbarkeit der R_F-Werte ist von der jeweils verwendeten Kieselgel-Charge und von der Dicke der Schicht abhängig. Es ist daher ratsam, eine Standard-Substanz (z.B. Oleanolsäure) auf jeder Platte mitlaufen zu lassen und die R_F-Werte darauf zu beziehen.

III. IONENAUSTAUSCHERPAPIER-CHROMATOGRAPHIE

Wie aus Tabelle I hervorgeht, sind die isomeren Verbindungen Oleanol-, Ursol- und Betulinsäure im Dünnschicht-Chromatogramm nicht voneinander zu trennen, während die Morolsäure einen kleineren R_F-Wert aufweist. Da es uns nicht gelang, durch Variation des Laufmittels (auch nicht durch Verwendung von Al_2O_3 G (Merck) oder durch Herstellen saurer oder basischer "Ionenaustauscher-Platten" durch Anteigen mit Salzsäure oder Ammoniak) hier eine Trennung zu erreichen, versuchten wir eine solche auf Ionenaustauscherpapier zu erzielen. MYHRE UND SMITH[10]

berichteten bereits 1958 über die erfolgreiche Trennung verschiedener stark polarer organischer Säuren, wie Gluconsäure, Glucuronsäure, Ascorbinsäure und verschiedener Aminosäuren. Unter Verwendung der Angaben von DAVIES UND OWEN[11], die an Ionenaustauscher-Säulen nahe verwandte Säuren mithilfe von organischen Lösungsmitteln trennen konnten, versuchten wir diese Methode auf die schwach polaren Triterpen-carbonsäuren zu übertragen.

Zur Verwendung gelangte das Anionenaustauscherpapier der Firma Schleicher & Schüll. Als Laufmittel erwiesen sich nach einer Reihe von Vorproben mit 99%iger Ameisensäure gesättigte Lösungsmittel wie Cyclohexan (mit 20 bis 40% Toluol- oder Xylol-Zusatz) und Methylcyclohexan–Chloroform-Gemische als am besten geeignet (siehe Tabelle III). Es wurde dabei nach der aufsteigenden Methode gearbeitet. Die Anfärbung geschah mit dem Zinntetrachlorid-Reagens[5], bei damit nur schlecht reagierenden Triterpensäuren mit $SbCl_3$–$SbCl_5$-Gemischen. Im System X treten allerdings diskrete Flecke erst bei mehrstündigem Liegen nach der $SnCl_4$-Anfärbung hervor. Fig. 3 zeigt die Trennung von Oleanol-, Ursol- und Betulinsäure im Gemisch XI. Zur Identifizierung diente ausser dem R_F-Wert auch noch die unterschiedliche Anfärbbarkeit mit $SbCl_3$–$SbCl_5$; die ersten beiden Säuren werden dabei violett, Betulinsäure gelbbraun. Auf diese Weise konnte nach Anreicherung durch mehrmaliges Umkristallisieren in der "Crataegussäure" die Oleanolsäure neben der Ursolsäure nachgewiesen werden (Gemisch X)[12].

TABELLE III

IONENAUSTAUSCHERPAPIER-CHROMATOGRAPHIE SAURER TRITERPENOIDE

Die angegebenen R_F-Werte beziehen sich auf die Mittelpunkte der etwas länglichen Flecke, "S" bedeutet längere Schwanzbildung.

Substanz	*R_F-Werte in Gemisch**		
	X	*XI*	*XII*
Oleanolsäure	0.30	0.30	
Ursolsäure	0.12	0.13	
Betulinsäure	0.45 S	0.43	
Dihydroxysäure-Gemisch aus *Crataegus oxyacantha* L.			0.28 0.40 0.51
Acantholsäure			0.18
Bayogenin			0.30

* Gemische: X: Cyclohexan–Toluol (4:1), gesättigt mit Ameisensäure; XI: Methylcyclohexan–Chloroform (4:1), gesättigt mit Ameisensäure; XII: Methylcyclohexan–Chloroform (1:1), gesättigt mit Ameisensäure.

Die Ionenaustauscherpapier-Chromatographie lässt sich auch zur Untersuchung von polareren Triterpencarbonsäuren verwenden. So konnten im rohen Dihydroxymonocarbonsäure-Gemisch aus *Crataegus oxyacantha* L.[5] im System XII drei Substanzen nachgewiesen werden, welche der Crataegolsäure, Neotaegolsäure und einer noch unbekannten Säure zukommen müssen (vgl. Fig. 4). Von den Trihydroxymonocarbonsäuren konnten aus Materialmangel nur Acantholsäure und Bayogenin

geprüft werden. Auch diese liessen sich unter Verwendung von System XII auftrennen.

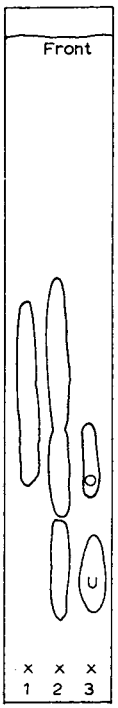

Fig. 3. Ionenaustauscherpapier-Chromatographie. Monohydroxy-triterpen-carbonsäuren im Gemisch XI. (1) Betulinsäure; (2) Gemisch von (1) und (3); (3) Ursolsäure (U) + Oleanolsäure (O). Bei Anfärbung mit $SbCl_3$–$SbCl_5$ erhält man von der Ursol- und der Oleanolsäure rotviolette Flecke; die Betulinsäure erscheint gelbbraun und ist damit trotz des Ineinanderfliessens der Flecke von Oleanol- und Betulinsäure gut identifizierbar.

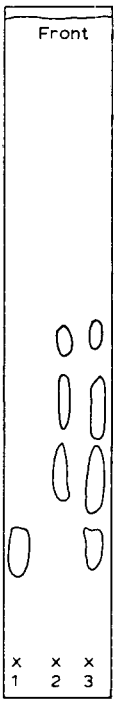

Fig. 4. Stärker hydroxylierte Triterpen-carbonsäuren im Gemisch XII. (1) Acantholsäure; (2) Dihydroxysäure-Gemisch aus *Crataegus oxyacantha* L.; (3) Gemisch von (1) und (2).

IV. EXPERIMENTELLER TEIL

(*a*) *Dünnschicht-Chromatographie*

Auf die Glasplatten wurde in der früher beschriebenen Weise[8] ein Gemisch von Kieselgel G "zur Dünnschicht-Chromatographie" (Merck) und Wasser 1:3 mit einem breiten Spatel gleichmässig ausgestrichen und bei 130° getrocknet. Zur Anfärbung wurde nach dem Trocknen der entwickelten Platten mit einer Lösung von Chlorsulfonsäure in Eisessig (1:2) besprüht, 5 Minuten lang bei 130° gehalten und bei Tageslicht und unter der U.V.-Lampe betrachtet. Die Flecken waren meist violett bis bräunlich gefärbt, Nimbsäure[13] erschien grün. Die Anfärbung kann aber ebensogut mit den üblichen sauren Triterpenreagenzien ausgeführt werden.

An Tagen mit hoher Luftfeuchtigkeit können die R_F-Werte besonders dann, wenn die Platten längere Zeit der Luft ausgesetzt waren, unverhältnismässig gross werden. Denselben Effekt kann man auch durch Einstellen der fertigen Platten in Gefässe mit wassergesättigter Atmosphäre erzielen. Um ihn auszuschliessen, trocknet man zweckmässigerweise vor dem Einbringen in die Chromatographiegefässe 2 Minuten lang bei 100°, um das vom Kieselgel adsorbierte Wasser zu entfernen.

(*b*) *Ionenaustauscherpapier-Chromatographie*

Anionenaustauscherpapier der Firma Schleicher & Schüll wurde in Streifen von 3 × 20 cm geschnitten, auf die 1 cm vom Rand entfernt bis zu 3 Flecken nebeneinander aufgetragen werden konnten. Die Laufmittel wurden durch kräftiges Durchschütteln der angegebenen Lösungsmittelgemische mit 20 % ihres Volumens an käuflicher 99 %iger Ameisensäure bereitet. Nach Abtrennung der schweren Phase im Schütteltrichter wurde die leichte zur Entwicklung der Chromatogramme verwendet.

Es wurde nach der aufsteigenden Methode in entsprechend grossen Reagenzgläsern gearbeitet, anschliessend wurden die Streifen 15 Minuten lang bei 100° getrocknet. Eine vorherige Überführung des Papiers in die Formiat-form brachte keine Vorteile. Auf absteigenden Chromatogrammen wurden sehr langgezogene Flecke erhalten.

Meist wurden die Substanzen nach TSCHESCHE UND POPPEL[5] mit einer Lösung von 10 ml $SnCl_4$ in 160 ml eines Eisessig–Tetrachlorkohlenstoff-Gemisches (1:1) sichtbar gemacht, wobei nach 5-minutiger Erwärmung auf 100° rotviolette Tönungen erhalten werden. Im System X scheinen dabei die einzelnen Flecke zunächst ineinander überzugehen, nach mehrstündigem Liegen treten aber auch hier die Hauptmaxima deutlich hervor. Auf diese Weise nur schwer anfärbbare Verbindungen, die auch mit $SbCl_3$ oder $SbCl_5$ allein kaum nachgewiesen werden konnten, erschienen aber deutlich nach aufeinanderfolgender Behandlung mit $SbCl_3$ (10 %ige Lösung in Chloroform, Streifen 2 Minuten lang auf 100° erhitzen) und $SbCl_5$ (40 %ige Lösung in Chloroform, bei Zimmertemperatur entwickeln). Stattdessen lässt sich auch ein Gemisch von $SbCl_3$–$SbCl_5$ (10:1) (10 %ige Lösung in Chloroform, Streifen 1–2 Minuten lang erhitzen auf 100°) verwenden. Auch hierbei tritt meist eine rotviolette Farbe auf, während aber z.B. Betulinsäure gelbbraun erscheint.

DANK

Für die Überlassung einiger der in den Tabellen erwähnten Substanzen möchten wir auch hier den Herren Prof. Dr. H. R. ARTHUR (Hongkong), Prof. Dr. D. H. R. BARTON (Glasgow), Prof. Dr. C. DJERASSI (Stanford), Dr. N. S. NARASIMHAN (Poona) und Dr. J. J. H. SIMES (Sydney) bestens danken.

ZUSAMMENFASSUNG

Es werden verschiedene Lösungsmittelsysteme angegeben, mit denen es gelingt, auf Dünnschichtplatten saure und neutrale Triterpenoide auch in komplexeren Mischun-

gen zu trennen. Oleanol-, Ursol- und Betulinsäure, die damit nicht unterscheidbar sind, können aber an basischem Ionenaustauscherpapier voneinander getrennt werden. Auch für andere polarere Triterpencarbonsäuren ist dieses Verfahren brauchbar.

SUMMARY

Some solvent systems are described for the separation of acidic and neutral triterpenoids on chromatoplates, even in the case of complex mixtures. Oleanolic, ursolic and betulinic acids, which cannot be distinguished by this method, are separable on basic ion-exchange paper. This technique is also useful for other more polar acidic triterpenoids.

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GAS–LIQUID CHROMATOGRAPHY OF VOLATILE METAL HALIDES

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In general, gas chromatography has largely been concerned with the separation of volatile organic compounds and very little has been done with metals. Development of high temperature techniques has now opened up this area. Two trends are apparent in the results reported so far. One involves the use of fused salts as the immobile phase while the other is the use of slightly volatile liquids such as silicones or vacuum greases usually encountered in high temperature work. Both have advantages and disadvantages. The former, while useful at very high temperatures, are difficult to prepare on the support, especially in anhydrous form, and are subject to reaction with the solutes. The latter are lost from the column, redistribute on the support, may be thermally unstable, and are capable of reaction with the solutes.

DE BOER[1] chromatographed a zinc–cadmium alloy on lithium chloride at 620°. There was evidence that separation was not due to simple distillation but involved interaction between the solute and the immobile phase. JUVET AND WACHI[2] used a eutectic mixture of $BiCl_3$–$PbCl_2$ at 240° in a separation of titanium(IV) chloride and antimony(III) chloride to avoid volatile organic partitioners and undesirable reactions between solute and immobile phase. With regard to fused salts, it is interesting to note that HANNEMAN, SPENCER AND JOHNSON[3] chromatographed C_{15} to C_{28} *n*-paraffins and polyphenyls on a eutectic of alkali metal nitrates at 300°. On the basis of the variation of HETP with flowrate, the linear relation between the log retention time and carbon number for homologous series, and symmetrical peaks, they concluded that molten salt phases behave in the same manner as organic liquids. Apparently small samples were necessary to obtain these results. The results indicate only linear kinetics and not necessarily solution in the immobile phase. Consideration of adsorption of the solute at the liquid–gas or liquid–liquid interface has been neglected in all accounts of chromatographic theory[4,5] and this phenomenon may prove to be of major importance with fused salts. The short retention times reported by HANNEMAN *et al.* may be an indication of this since such an adsorption might not involve appreciable diffusion into and out of the immobile phase thus reducing the period of fixation by this phase.

DUSWALT[6] suggested that since the acetyl acetonates of Mn, Pb, Zn, Co, Ni, Fe, Cu, Be, Hg, Al, In, U, Th, and Cr are thermally stable, they ought to be considered as solutes for gas chromatographic (GC) separation since a number of them are known to be volatile. He prepared and tested the acetyl acetonates of Al, Be, Cd, Hg, Sc,

and Zn obtaining peaks for Be, Sc, and Zn on silicone oil and propylene glycol–adipic acid polymer supported by Celite at 225°. Failure to obtain peaks for Al and Hg was attributed to too low a temperature of the sample injection unit. Very broad peaks were traced to this same source. We feel this same deficiency in the work reported here.

FREISER[7] separated tin(IV) chloride from titanium(IV) chloride at 102° using *n*-hexadecane (31% by weight) on Chromosorb as the immobile phase. The ratio of the vapor pressures of tin(IV) chloride to titanium(IV) chloride (1.91) was nearly the inverse of the ratio of retention times (2.32). The peaks were very nearly symmetrical and well resolved. A small minor peak, very close to the air peak, was observed with the sample containing titanium(IV) chloride (see Fig. 4A for this same result in the present work). Whenever a large quantity of either of these solutes was used, the peak was preceded by a "foot" never exceeding 1 mV on a 10 mV range (see Fig. 4B); the recorder went off scale with the major peak in these cases. The foot or shoulder is very similar in appearance and formed under the same circumstances as those observed by WEURMAN AND DHONT[8] in the chromatography of alcohols on polyethylene glycol. They suggested that the immobile phase decomposed to give formic acid which then esterified with the alcohol to give the faster moving ester. KIESER AND SISSONS[9] attributed the effect to reaction with formaldehyde and formic acid present as impurities in the polyethylene glycol used to prepare the partitioner. It would seem that in either case the ester would continually be produced in the region of the column containing the migrating alcohol and appear as a shoulder rather than as a well defined peak as it is eluted from the alcohol zone. In our case, a decomposition product might be produced in the zone containing the inorganic halide and the product then moving out of this region to give a shoulder. This phenomenon might well be discussed from the viewpoint of KELLER AND GIDDINGS[10] as a special case of multiple-zone formation. We have also observed the aforementioned peak superimposed on these shoulders resulting in some rather unusual concentration profiles indeed. These secondary effects were always minor compared to the major peaks and there was no confusion in identifying the major constituent. Recognizing that the column was being operated appreciably above the recommended maximum temperature of 40° for the *n*-hexadecane[11], little more was done with this partitioner, it having been established that separations were feasible. In spite of this higher temperature, there was no drift in the base line or condensation of partitioner in the outlet tube which are generally the symptoms of loss of immobile phase. An extension of this investigation has already been reported[12].

WACHI[13] attempted the chromatography of tin(IV) chloride and titanium(IV) chloride on silicone grease and Apiezon M grease supported on firebrick in stainless steel columns with the sample port at 150° and the column at 125°. Failure to obtain peaks was attributed to complete reaction of the chlorides with the greases and the walls of the column. Chromatograms of iron(III) chloride on these two partitioners at 325° also failed. WACHI then turned to fused inorganic eutectics in seven foot glass columns (the fused salt partitioners reacted with stainless steel) and reported:

(1) A chromatogram of tin(IV) chloride on a 41% by weight cadmium chloride–potassium chloride eutectic (m.p. 383°) at 464° (sample injector at 210°). (2) Partial resolution of tin(IV) chloride and antimony(III) chloride on this eutectic at 474°. Base line drift was attributed to temperature variation of the furnace. (3) Chromatograms of tin(IV) chloride and titanium(IV) chloride on 37% by weight aluminum chloride–sodium chloride at 145° and 143° respectively (sample injector at 109°). (4) Partial resolution of a mixture of tin(IV) chloride and titanium(IV) chloride on this eutectic at 145°. Poor results were attributed to the insolubility of the latter compound in the eutectic. (5) Failure of nitrate eutectics to separate the transition metal chlorides due to the oxidizing properties of the nitrates. (6) GSC chromatograms of tin(IV) chloride and mixtures of it with titanium(IV) chloride on a 12 ft. column of C-22 firebrick at 141°. Resolution was very poor and the retention time was short. In all these cases flowrates were either 20 or 30 ml of nitrogen per min and retention times were all very nearly 5 min which is considerably less than we have observed. WACHI concludes that the eutectics act as a partition medium. We do not substantiate his conclusion that metal columns cannot be used with volatile inorganic materials because of reaction with the metal. We do not contest the statement that the eutectics react with the metal. The preparation of coiled glass columns, packing them, and coating of the firebrick with an anhydrous eutectic salt present some considerable challenges in technique. WACHI's use of a flash vaporizer as the sample injection unit is an improvement on DUSWALT's and our own apparatus.

We estimate the vapor pressures of titanium(IV) chloride and antimony(III) chloride to be 6530 mm and 1197 mm respectively at 240°[14]. The ratio of the vapor pressures of the first to the second is 5.46 while the inverse ratio of retention times is 6.0 for the fused salt immobile phase (JUVET AND WACHI[2]). Here, as with the *n*-hexadecane, emergence is ordered according to vapor pressure and little or no chemical interaction or, at least, the same degree of interaction of solute and immobile phase is indicated.

TABLE I

VOLATILITY OF CERTAIN METAL HALIDES

Metal	*Boiling point (°C)*			*Metal*	*Boiling point (°C)*		
	Cl	*Br*	*I*		*Cl*	*Br*	*I*
Boron(III)	12.5	90.1[a]	210	Antimony(III)	223	280	401
Silicon(IV)	57.6	153	290	Niobium(V)	240.5	270	—
Germanium(IV)	83.1	186.5	—	Tantalum(V)	242	320	—
Tin(IV)	114.1	202[b]	341	Gold(III)	265[s]	—	—
Arsenic(III)	130.2	221	403	Molybdenum(V)	268	—	—
Titanium(IV)	136.4	230	> 360	Tungsten(V)	275.6	333	—
Vanadium(IV)	149.5[c]	—	—	Mercury(II)	302	322	354
Antimony(V)	140	—	400.6	Iron(III)	315	—	—
Aluminum(III)	177.8[s]	263.3[d]	382	Zirconium(IV)	300[s]	—	—
Gallium(III)	201.2	278.8	345[s]	Tungsten(VI)	346.7	—	—

[a] 740 mm Hg pressure [b] 734 mm Hg pressure [c] 755 mm Hg pressure
[d] 747 mm Hg pressure [s] Sublimes

That inorganic halides are likely solutes for GC separation is demonstrated by Table I[14].

The investigation described here involves tin(IV) chloride, titanium(IV) chloride niobium(V) chloride, and tantalum(V) chloride on several partitioners at different temperatures.

APPARATUS

Analyzer

Analyses were made with a Cenco No. 70130 Vapor Phase Analyzer (Central Scientific Co., Chicago, Ill.) equipped with a katharometer detector. Column, katharometer, and sample injection unit were all at the same temperature. Concentration profiles were recorded on a Leeds and Northrup Speedomax Model S, variable range, variable sensitivity recorder of 1 sec response time and 30 in./h chart speed. A dry box containing a drying agent and through which dry nitrogen was passed, was placed above the sample port to permit sample introduction under anhydrous conditions. Fuming was still observed, however. This same dry box was used in the preparation of solutions. Sample introduction was by a 0.25 ml hypodermic syringe. Driving pressure was measured by a mercury manometer placed at the source of the helium carrier and flowrates were measured by means of a soap-film flowmeter[11] and stopwatch.

Columns

The columns were all of coiled 0.25-in. copper tubing, 1.60 m in length (actual length of packing).

Partitioning agent

The partitioning agents were supported by Red Chromosorb (Johns-Manville, New York, N.Y.) dried at 125° for 48 h before application of the immobile phase. The immobile liquid was applied by dissolving it in diethyl ether and then evaporating off the solvent while in contact with the support with constant stirring. The material was then kept in a vacuum desiccator at reduced pressure for two days to insure removal of the ether and size graded to 30–60 mesh. Quantities of the packing were weighed, the column packed, and the remaining packing weighed to determine the actual weight of material in the column. Weighed samples of the materials were also extracted with diethyl ether using a Soxhlet extractor to determine the quantity of immobile phase present. Table II summarizes the important characteristics of each column. Squalane column I was used in the chromatography of the individual solutes at the three different temperatures while column II was used in the chromatography of a mixture of the four at 200°. The columns were conditioned by passing helium through them for 4–5 h at 100° before analyses were made.

Solutes

Tin(IV) chloride (J. T. Baker Chemical Co., Phillipsburg, N.J., C.P.) and titanium(IV) chloride (Kahlbaum, Chemische Fabrik, Adlershof bei Berlin) were used as the liquids (sample sizes of 0.015 to 0.02 ml). Niobium(V) chloride (Stauffer Chemical

Co., New York, N.Y.) and tantalum(V) chloride (Stauffer Chemical Co.), solids at room temperature, were dissolved in redistilled carbon tetrachloride and saturated solutions used in the chromatography of the individuals. These liquids and solutions were stored in glass-stoppered flasks in a desiccator while not in use.

TABLE II

COLUMN CHARACTERISTICS

Immobile phase	*Per cent by wt.*	*Total weight of immobile liquid phase in the columns (g)*	*Column density (g/m)*
n-Octadecane[a]	33.9	6.41	11.81
Squalane[b]			
Column I	34.4	6.67	12.12
Column II	34.4	6.68	12.13
Apiezon T[c]	28.6	3.74	8.18[f]
Silicone oil[d]	36.0	6.82	11.84
Paraffin[e]	38.1	4.09	6.71[f]

[a] Eastman Organic Chemicals, D.P.I. (Practical), Rochester, N.Y.
[b] Eastman Organic Chemicals, D.P.I. (White Label), Rochester, N.Y.
[c] Apiezon Products Limited, James G. Biddle Co., Philadelphia, Pa.
[d] Silicone Oil 81705, General Electric, Waterford, N.Y.
[e] "Fruitmaster" Paraffin Wax, Penola Oil Co., Bayonne, N.J.
[f] The immobile phases did not "flow" well because of the cohesive properties of the liquid and they gave a less dense packing.

The obvious objection to the procedure for the solutions is that the sample volume is quite large (about 0.15 to 0.20 ml) so that migration is not initiated from a narrow zone. Fortunately the solute and solvent peaks are widely separated. The recorder was set at 20 mV until the solvent peak had passed and then changed to 5 mV to pick up the smaller solute peaks. Secondly, the temperature of the sample injection unit does not insure immediate vaporization of the solute. Niobium(V) chloride melts at 194° while tantalum(V) chloride melts at 221°[14] which means the sample port temperature is in the region of the melting point. Obviously the material does not enter the column as a "plug" and excessive peak broadening is to be expected. A high temperature sample injector is needed.

Finally, niobium(V) chloride and tantalum(V) chloride were added to a 50–50 (by volume) mixture of tin(IV) chloride and titanium(IV) chloride to give a saturated solution. This mixture was chromatographed on squalane (column II) at 200°. The sample volume was such that the amounts of tin and titanium chlorides would be the same as those used in the chromatography of the pure solutes. In spite of this adjustment, the recorder response was much less with the mixture. This, coupled with the appearance of a precipitate on making up the solution, indicated interaction of these solutes in an as yet undetermined manner. Fig. 3 is the concentration profile of this chromatogram.

RESULTS

Flowrates were measured with a stopwatch every 10 min for short duration chromatograms and every 30 min for longer chromatograms. Average flowrates were used when the individual values showed a random variation about the mean. If, however, there was a trend in the flowrate due to a drop in the driving pressure, a least squares line was fitted to a plot of flowrate *vs.* time, and the retention volume determined from the empirical equation for the variation of flowrate with time. Where this was done, the average driving pressure was used in correcting for the pressure drop across the column. Uncertainties in the retention volumes are based on average deviations from the mean or average deviation from the least squares line. Temperature variations of the column are maximum.

Measurements, terminology, and symbols are those recommended by AMBROSE, KEULEMANS AND PURNELL[15] and JOHNSON AND STROSS[16]. V_g is the retention volume of the solute per gram of immobile phase, relative to the air peak, measured in terms of dry carrier gas at standard conditions. Table III gives the values of log V_g at various

TABLE III

LOG SPECIFIC RETENTION VOLUMES

	$\log V_g$	$1/T \times 10^4$		$\log V_g$	$1/T \times 10^4$
	Squalane column I			*n-Octadecane*	
Sn	2.127	26.80	Sn	2.169	26.80
	1.534	23.64		1.531	23.63
	1.099	21.12			
			Ti	2.549	26.77
Ti	2.177	26.77			
	1.651	23.64		*Silicone*	
	1.255	21.12	Sn	1.777	26.77
				1.786	26.77
Nb	2.909	23.61		1.169	23.66
	2.534	21.14		0.702	21.15
Ta	2.922	21.14		*Paraffin*	
	Squalane column II		Sn	2.075	26.71
				1.512	23.62
Sn	0.775	21.13		1.029	21.13
Ti	1.127	21.13	Ti	2.508	26.78
				2.473	26.70
Nb	2.513	21.13		1.915	23.65
				1.496	21.13
Ta	2.732	21.13			

temperatures and partitioners. Fig. 1 is a plot of log V_g *vs.* the reciprocal of the absolute temperature for all the solutes on squalane. Fig. 2 is a similar plot for tin(IV) chloride and titanium(IV) chloride only on different partitioners at different temperatures. In Table IV we report column inlet and outlet pressures and flowrates of dry gas at the column temperature and outlet pressure since the actual operating condi-

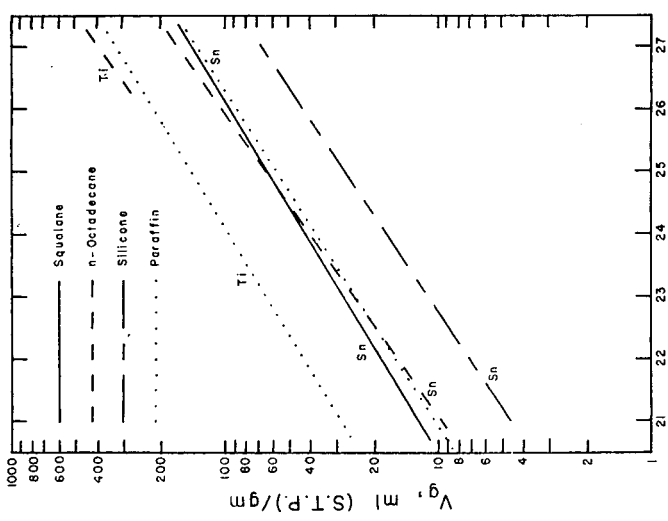

Fig. 2. Log of the retention volume (ml of dry helium at 1 atm. and 0° corrected for the pressure drop) per g of partitioning liquid *vs.* the reciprocal temperature for tin(IV) chloride and titanium(IV) chloride on columns of *n*-octadecane, squalane, paraffin wax, and silicone oil supported by firebrick.

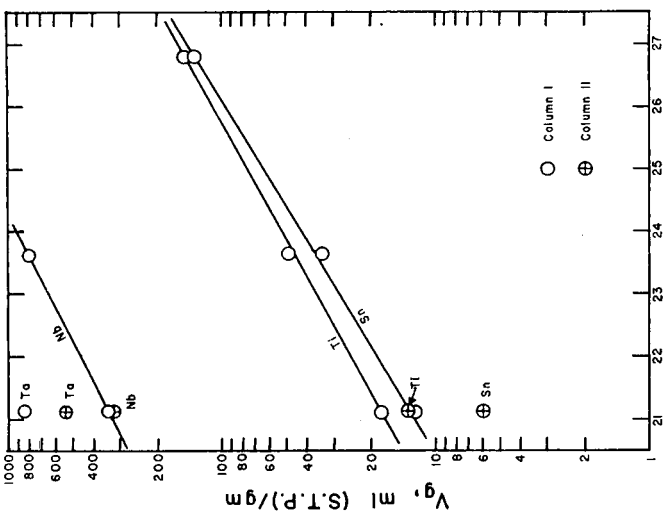

Fig. 1. Log of the retention volume (ml of dry helium at 1 atm. and 0° corrected for the pressure drop) per g of partitioning liquid *vs.* the reciprocal temperature for tin(IV) chloride, titanium(IV) chloride, niobium(V) chloride, and tantalum(V) chloride on two different columns of squalane supported by firebrick.

TABLE IV

OPERATING CONDITIONS

	Temperature[a] (°C)	Inlet pressure (cm Hg)	Outlet pressure (cm Hg)	Flowrate[b] (ml/min)	V_g (ml(STP)/g)
			Squalane column I		
Sn	99.8 ± 0.6	81.2	69.7	36.5 ± 0.4	134 ± 2
	149.7 ± 0.5	84.1	69.6	40.0 ± 0.4	34.2 ± 0.3
	200.1 ± 0.3	91.8	69.6	60.3 ± 0.5	12.56 ± 0.09
Ti	100.3 ± 0.9	83.3	69.8	41.6 ± 0.2	150.2 ± 0.6
	149.7 ± 0.7	84.0	69.6	39.9 ± 0.2	44.8 ± 0.1
	200.2 ± 0.4	91.7	69.6	58.9 ± 0.3	18.0 ± 0.1
Nb	150.3 ± 0.5	88.4	69.6	52.8 ± 0.4	812 ± 9
	199.7 ± 0.5	92.1	69.7	58.0 ± 0.7	342 ± 4
Ta	199.8 ± 0.8	94.9	70.2	65.2 ± 0.4	836 ± 6
			Squalane column II		
Sn	199.9 ± 0.5	85.1	70.1	40.2 ± 0.2	5.96 ± 0.03
Ti	199.9 ± 0.5	85.1	70.1	40.2 ± 0.2	13.4 ± 0.1
Nb	199.9 ± 0.5	85.1	70.1	40.2 ± 0.2	326 ± 2
Ta	199.9 ± 0.5	85.1	70.1	40.2 ± 0.2	539 ± 3
			n-Octadecane		
Sn	99.9 ± 0.5	97.8	70.1	38.7 ± 0.2	147.6 ± 0.6
	149.9 ± 0.3	105.2	70.3	42.2 ± 0.5	34.0 ± 0.3
Ti	100.3 ± 0.7	97.7	70.2	36.9 ± 0.2	354 ± 3
			Silicone		
Sn	100.3 ± 0.3	78.0	70.2	28.6 ± 0.2	59.8 ± 0.6
	100.3 ± 0.7	81.9	70.4	44.6 ± 0.6	61.1 ± 0.9
	149.4 ± 0.4	80.2	70.2	35.2 ± 0.2	14.76 ± 0.07
	199.6 ± 0.4	81.7	70.1	37.1 ± 0.6	5.04 ± 0.09
			Paraffin		
Sn	101.1 ± 0.3	70.6	69.9	51.3 ± 0.1	118.8 ± 0.2
	150.0 ± 0.8	70.5	69.7	57.0 ± 0.8	32.5 ± 0.5
	200.0 ± 0.2	70.9	69.8	72.8 ± 0.7	10.7 ± 0.1
Ti	100.2 ± 0.6	70.5	69.9	51.1 ± 0.3	322 ± 2
	101.2 ± 0.6	70.6	69.8	68.1 ± 0.6	297 ± 3
	149.6 ± 0.4	70.5	69.7	57.8 ± 0.3	82.3 ± 0.2
	200.0 ± 0.2	70.9	69.8	72.3 ± 0.6	31.3 ± 0.2

[a] Deviations are the maximum observed.

[b] Flowrates are for the dry gas at the outlet pressure and temperature of the column and are not corrected for the pressure drop. Deviations are the average deviations from the mean or the least squares plot.

tions dictate column performance. Table V gives the values of the constants of the equation

$$\log V_g = a + \frac{b}{T} \times 10^4$$

TABLE V

CONSTANTS OF THE LEAST SQUARES PLOT FOR

$$\log V_g = a + \frac{b}{T} \times 10^4$$

Immobile phase	*Solute*	*a*	*b*
Squalane (column I)	$SnCl_4$	—2.60	0.176
	$TiCl_4$	—2.18	0.161
	$NbCl_5$	—0.814	0.158
n-Octadecane	$SnCl_4$	—3.22	0.201
Silicone	$SnCl_4$	—3.29	0.189
Paraffin wax	$SnCl_4$	—2.92	0.187
	$TiCl_4$	—2.29	0.178

as determined by the fit of a least squares line to the data of Table III.

Two attempts were made to chromatograph tin(IV) chloride on Apiezon grease at 100° with no success, which agrees with WACHI's results[13]. This solute did chromatograph on the silicone oil but titanium(IV) chloride did not appear either at 100° or 150°. We assume the latter reacted with the partitioning agent. Two results are reported at 100° ($V_g = 59.8 \pm 0.6$ and 61.1 ± 0.9) which are within experimental error of one another and differ by 2 %.

Fig. 1. shows only fair reproducibility between squalane columns I and II. Figs. 3 and 4 are excellent demonstrations of difficulties to be encountered in this work. Fig. 4A is the concentration profile of tin (IV) chloride on paraffin at 100°. The first peak, attributed to some artifact in the original sample, probably hydrogen chloride formed in the solution on standing, is well defined and typical of the results expected in

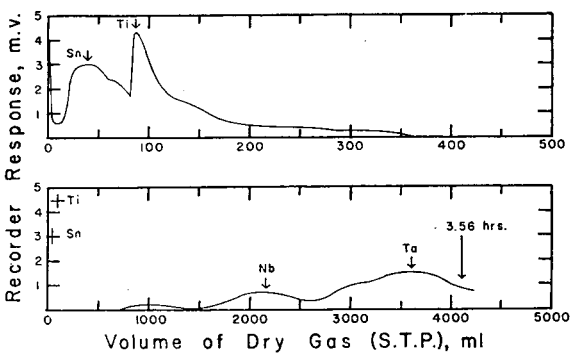

Fig. 3. Concentration profile of a chromatogram of a mixture of tin(IV) chloride, titanium(IV) chloride, niobium(V) chloride, and tantalum(V) chloride on squalane at 200° and helium flowrate of 40.2 ml/min. The volume scale and flowrate is for ml of dry gas at 1 atm. and 0° corrected for the pressure drop.

good chromatography. Fig. 4B shows the concentration profiles of titanium(IV) chloride on this same column. Each peak is preceded by the aforementioned shoulder, and, as can be seen from the peak positions, this shoulder would interfere with the peak for tin(IV) chloride. This may explain the poor resolution of tin(IV) chloride

and titanium(IV) chloride shown in Fig. 3. The shoulder is more predominant and the major peak smaller at the slower flowrate than at the faster. We ascribe this to an increased opportunity for reaction between solute and immobile phase at the slower flowrate. Since the sample sizes are probably different for the two runs, the

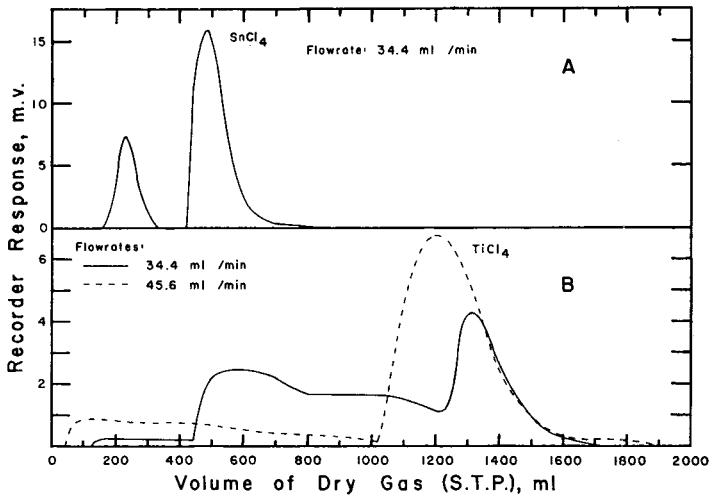

Fig. 4. Concentration profile of tin(IV) chloride and titanium(IV) chloride on paraffin wax at 100° and different flowrates of helium. The volume scale and flowrate is for ml of dry gas at 1 atm. and 0° corrected for the pressure drop. A. Tin(IV) chloride at 34.4 ml/min. B. Titanium(IV) chloride at 34.4 and 45.6 ml/min.

ratio of the areas under the shoulder to that under the entire curve was taken as a measure of the extent of the reaction. At the lower flowrate this ratio is 0.64 while at the faster it is 0.22.

CONCLUSIONS

It is apparent from Fig. 2 that hydrocarbon phases are satisfactory in the separation of the metal halides. In so far as tin(IV) chloride is concerned, the three paraffins used behave in very much the same manner. Apiezon grease and silicone oil react with the solutes. The normal alkanes appear more satisfactory than branched chain compounds. If paraffin wax is assumed to be more branched than squalane, the latter having six methyl branches per molecule, then one may explain the increased predominance of the shoulder with increased branching by assuming the solutes to act on the tertiary hydrogen of the partitioning liquids to remove a hydrogen atom or, as a Lewis acid, to remove a hydride ion. The remaining free radical or carbonium ion could rearrange in a number of ways. The union of the solute and the hydrogen might then decompose to release hydrogen chloride and a reduced form of the solute. As mentioned earlier, this artifact could interfere with preceding solute peaks (Fig. 4). Asymmetry of the solute peaks seems to increase as one goes to higher molecular weight alkanes or with branching. We conclude that normal alkanes are the most

satisfactory liquids, but, unfortunately, they are generally unsuitable at high temperature because of their volatility.

It is obvious from Fig. 3 that this separation is ripe for attack by temperature-programmed techniques. Tin(IV) chloride and titanium(IV) chloride appear in the first 100 ml of carrier while the chromatogram is not completed until about 4 l of carrier.

The greatest difficulty in this work, especially if quantitative results are desired, is the preparation and introduction of measured samples under anhydrous conditions. We are presently engaged in filling glass ampoules with weighed quantities of solute and using a "crusher" type injection system. Obviously a prevaporization sample chamber is also required.

ACKNOWLEDGEMENT

We gratefully acknowledge the financial assistance of the Research Corporation and the National Science Foundation and thank the Stauffer Chemical Co. for samples of the metal halides.

SUMMARY

The literature on the gas chromatography of volatile metal compounds is reviewed.

Results are reported for the gas–liquid chromatography of tin(IV) chloride, titanium(IV) chloride, niobium(V) chloride, and tantalum(V) chloride on *n*-octadecane, squalane, silicone oil, paraffin wax, and Apiezon grease at 100°, 150°, and 200°. Results indicate normal alkanes to be the best partitioners and that branched alkanes lead to reaction on the column. Suggestions are made concerning anhydrous conditions in handling samples, sample prevaporization, and the use of temperature programming.

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CHROMATOGRAPHY OF SOME CATIONS BY MEANS OF PAPER TREATED WITH A LIQUID ANION EXCHANGER

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It is well known that anionic resins, such as IRA-400, Dowex-1, etc., have been used very successfully to retain and/or separate the cations that form anionic complexes with the most common acids. Hydrochloric acid forms chloro-complexes of different strength with the majority of cations; the behaviour of such complexes with anionic resins has been widely investigated and the adsorption curves have been reported for nearly every element of the periodic system[1].

Later, SMITH AND PAGE[2] found that when some amines are dissolved in organic solvents that are immiscible with water, they can be used to extract the anionic complexes of many cations from acid solutions, the behaviour of these amines being similar to that of liquid anion exchangers. Furthermore, long chain tertiary amines, such as tri-*n*-octylamine, tri-iso-octylamine, methyl-dioctylamine, etc., showed a good selectivity for the different cations.

As has been pointed out elsewhere, comparison of the data[3–11] published on the extraction of anionic complexes with anionic resins[1] with that on amines, shows that their behaviour is generally very similar.

Because of the great selectivity of these amines for many cations, it seemed worthwhile to investigate the possibility of applying them to paper-chromatographic separations.

Some interesting chromatographic separations were carried out by LEDERER[12–14], who used papers impregnated with solid anion-exchange resins; these papers were obtained by dipping the chromatographic paper into a colloidal form of the resin. WITKOWSKI AND LEWANDOWSKI[15–17] also prepared some papers impregnated with ion-exchange resins; later PETERSON[18] separated uranium from bismuth with ion-exchange papers impregnated with IRA-400 and IR-45, as directly furnished by Rohm and Haas Co. KEMBER[19] and KNIGHT[20] obtained some interesting results with a chemically modified cellulose: the papers were prepared from cotton fabrics that had been treated either with urea phosphate or with 2-aminoethyl sulphuric acid.

However, all these methods involve solid resins, and are sometimes rather tedious; furthermore the wet strips are generally weak and difficult to handle. It was therefore our aim to develop a reliable and simple method in which the chromatographic paper was treated with an amine solution.

EXPERIMENTAL

Circular paper chromatography

Hydrochloric acid as eluting agent

In order to get rapid and clear results, circular chromatography was used. From 0.02 to 0.05 ml of hydrochloric solution containing 20 to 50 μg of cations was deposited at the center of a paper disc (Whatman No. 1) with a diameter of about 10 cm. When the spot was completely dry, the paper was immersed in a solution of tri-*n*-octylamine (Fluka Co.) 0.2 *M* in kerosene 30–50°, and after elimination of the excess of the solvent with warm air, it was placed horizontally on the top of a glass crystallizing dish. The spot was then eluted by slowly dropping the acid on the centre of the disc with the aid of a very thin capillary tube. When the diameter of the wet surface was 7–8 cm, the paper was dried with warm air and then the different elements were developed with their specific reagents. In this manner it was possible to separate two or more elements in 15–20 minutes, and to obtain many indications for a preliminary investigation.

From the adsorption curves given in ref.[1] it can be seen that there are great differences in the behaviour of iron, cobalt and nickel in hydrochloric acid solutions. Therefore an attempt was made to separate the three elements by means of the above-mentioned technique using 4 *N* HCl. As expected on the basis of the considerations mentioned above, nickel actually followed the solvent front, not being complexed by the hydrochloric acid and therefore not retained by the amine; cobalt gave an R_F of 0.5 because of its partial complexation, whilst iron was completely complexed and therefore retained by the amine at the centre of the paper (Table I). A blank with a paper not treated with the amine did not show any separation, the three elements being found together at the solvent front ($R_F = 1$).

TABLE I

SEPARATION OF Fe^{3+}–Co^{2+}–Ni WITH 4 *N* HCl

Element	E_a° *(from ref.*[1]*)*	R_F	*Developer*
Fe^{3+}	1000	0	KCNS
Co^{2+}	< 1	0.50	8-Hydroxyquinoline
Ni	0	0.97	Dimethylglyoxime

Thus, by means of this chromatographic procedure, it is possible to separate two or more elements on the basis of the indications given by the adsorption curves of the anionic resins, that is by eluting the spots with a hydrochloric acid solution of suitable molarity. As a consequence of the treatment with the amine solution, the paper behaves like an anionic resin film, with the advantage that it is possible to develop the spots as in normal paper chromatography. Thus alkali metals, alkaline earth metals, aluminium, rare earths, yttrium, scandium, actinium, thorium, titanium, nickel, etc., can be separated from every element that gives complexes with hydrochloric acid. Generally the following rule holds: $R_F \rightarrow 0$ when $E_a^\circ \rightarrow \infty$, and $R_F \rightarrow 1$ when

$E_a^\circ \rightarrow 0$, where E_a° is the distribution coefficient of the anionic resin, or better, the extraction coefficient of the amine.

The separations obtained with the procedure described above are summarized in Tables I–X; the E_a° values are taken from the paper of KRAUS AND NELSON[1].

TABLE II

SEPARATION OF U^{6+}–Zr–Th WITH 10 *N* HCl

Element	E_a°	R_F	*Developer*
U^{6+}	1000	0	$K_4Fe(CN)_6$
Zr	100	0.35	Quercetin
Th	0	0.95	8-Hydroxyquinoline

In the same manner as for the separation of U^{6+}–Zr–Th (Table II) it was possible to separate U^{6+}–Zr–Ti^{4+}; in fact titanium (developed with hydrogen peroxide) showed an R_F value of 0.85.

Table III shows that the separation of copper from iron and aluminium becomes better if the molarity of the acid is increased from 1 to 1.5, in which case copper is more easily complexed and retained by the amine.

TABLE III

SEPARATION OF Fe^{3+}–Cu–Al WITH 1–1.5 *N* HCl

Element	E_a°	R_F (1 *N* HCl)	R_F (1.5 *N* HCl)	*Developer*
Fe^{3+}	10	0	0	$K_4Fe(CN)_6$
Cu	< 1	0.80	0.42	$K_4Fe(CN)_6$
Al	0	0.98	0.98	Alizarin

The separation of U^{6+}–V^{5+}–Ti^{4+} is reported in Table IV, and the separations involving zinc, cobalt, manganese and nickel in Tables V–VII.

Furthermore, Cr^{6+} and Fe^{3+} can be separated from Al, and Fe^{3+} from Cr^{3+}, with 1–12 *N* HCl; in fact the R_F value for Cr^{6+} and Fe^{3+} is about zero, and that for Al and Cr^{3+} about 1 at every molarity of the acid.

TABLE IV

SEPARATION OF U^{6+}–V^{5+}–Ti^{4+} WITH 3 *N* HCl

Element	E_a°	R_F	*Developer*
U^{6+}	> 10	1	$K_4Fe(CN)_6$
V^{5+}	< 1	0.38	Hydrogen peroxide
Ti^{4+}	0	0.95	Hydrogen peroxide

TABLE V

SEPARATION OF Zn–Mn^{2+} WITH 3 N HCl

Element	E_a°	R_F	*Developer*
Zn	>100	0.33	8-Hydroxyquinoline
Mn^{2+}	0	0.96	8-Hydroxyquinoline

TABLE VI

SEPARATION OF Zn–Co^{2+}–Mn^{2+}(Ni) WITH 4 N HCl

Element	E_a°	R_F	*Developer*
Zn	>100	0.15	8-Hydroxyquiline
Co^{2+}	<1	0.45	8-Hydroxyquinoline
Mn^{2+}	$\ll 1$	0.82	8-Hydroxyquinoline
(Ni)	0	0.97	Dimethylglyoxime

TABLE VII

SEPARATION OF Zn–Mn^{2+}–Ni WITH 10 N HCl

Element	E_a°	R_F	*Developer*
Zn	>10	0.17	8-Hydroxyquinoline
Mn^{2+}	<10	0.50	8-Hydroxyquinoline
Ni	0	0.96	Dimethylglyoxime

The chromatographic method described here was successfully used also to separate zirconium and hafnium in spite of the difficulty of separating these so similar elements. A solution of 8 N HCl containing 5 % conc. HNO_3 was used as eluting agent, and quercetin as developer. This type of solution had given very good separation factors for Zr and Hf in the extraction with tri-*n*-octylamine[11]. In the experiments, zirconium and hafnium were first considered separately, and afterwards together. The experimental results are reported in Table VIII.

TABLE VIII

SEPARATION OF Zr AND Hf WITH 8 N HCl + 5 % CONC. HNO_3

Element	E_a° *(from ref.* [11]*)*	R_F	*Developer*
Zr	>2	0.25	Quercetin
Hf	<0.01	0.80	Quercetin

From the data given so far it is clear that many interesting chromatographic separations can be carried out by this simple method, provided a suitable eluting agent can be found.

Sulphuric acid as eluting agent

Tertiary amines have also been very successfully used to extract uranium from sulphuric leaches[21–26], as only U^{6+} ($E_a^\circ \sim 100$), Mo^{6+} ($E_a^\circ \sim 1000$), Zr ($E_a^\circ \sim 200$), and V^{5+} ($E_a^\circ \sim 20$) are extracted in 0.1–1 *M* H_2SO_4.

By using the chromatographic method described here, it was possible to separate U^{6+} from Fe^{3+}, Cu and Ni with 0.2 *M* H_2SO_4, whilst Mo^{6+} was separated from the same elements with 0.5 *M* H_2SO_4, as reported in Tables IX and X.

TABLE IX

SEPARATION OF U^{6+} FROM Fe^{3+}, Cu AND Ni WITH 0.2 *M* H_2SO_4

Element	E_a° *(from ref. [21])*	R_F	*Developer*
U^{6+}	>100	0.08	$K_4Fe(CN)_6$
Fe^{3+}	<0.1	0.66	$K_4Fe(CN)_6$
Cu	<0.1	0.68	$K_4Fe(CN)_6$
Ni	<0.1	0.97	Dimethylglyoxime

TABLE X

SEPARATION OF Mo^{6+} FROM Fe^{3+}, Cu AND Ni WITH 0.5 *M* H_2SO_4

Element	E_a° *(from ref. [24])*	R_F	*Developer*
Mo^{6+}	>1000	0	8-Hydroxyquinoline
Fe^{3+}	<0.1	0.85	$K_4Fe(CN)_6$
Cu	<0.1	0.87	$K_4Fe(CN)_6$
Ni	<0.1	0.97	Dimethylglyoxime

In the same way U^{6+}, Mo^{6+}, V^{5+} and Zr can be separated from any other element.

Descending chromatography

Hydrochloric acid as eluting agent

Good results have also been obtained by descending chromatography. The only drawback in our laboratory trials was that the eluted spots and the solvent front were sometimes a little irregular. This trouble occurred especially when the amount of amine was too large, and the molarity of the eluting acid too low, but it was possible

TABLE XI

SEPARATION OF Fe^{3+}–Co^{2+}–Ni WITH 3 *N* HCl

Element	E_a°	R_F	*Developer*
Fe^{3+}	100	0	$K_4Fe(CN)_6$
Co^{2+}	<1	0.37	8-Hydroxyquinoline
Ni	0	0.89	Dimethylglyoxime

to eliminate it by treating the paper with an amine solution pre-equilibrated with the acid that was to be employed as eluting agent. The results of the different separations obtained by descending chromatography with HCl are summarized in Tables XI–XVIII.

TABLE XII

SEPARATION OF Zn–Co^{2+}–Mn^{2+}(Ni) WITH 3 N HCl

Element	E_a°	R_F	*Developer*
Zn	>100	0.03	8-Hydroxyquinoline
Co^{2+}	<1	0.44	8-Hydroxyquinoline
Mn^{2+}(Ni)	0	0.90	8-Hydroxyquinoline (dimethylglyoxime)

TABLE XIII

SEPARATION OF Fe^{3+}–Cu AND Al WITH 2 N HCl

Element	E_a°	R_F	*Developer*
Fe^{3+}	100	0.03	$K_4Fe(CN)_6$
Cu	<1	0.21	$K_4Fe(CN)_6$
Al	1	0.90	Alizarin

TABLE XIV

SEPARATION OF U^{6+}–Zr AND Th WITH 8 N HCl

Element	E_a	R_F	*Developer*
U^{6+}	>100	0	$K_4Fe(CN)_6$
Zr	<10	0.32	Quercetin
Th	0	0.82	8-Hydroxyquinoline

TABLE XV

SEPARATION OF U^{6+}–Zr AND Ti^{4+} WITH 8 N HCl

Element	E_a°	R_F	*Developer*
U^{6+}	>100	0	$K_4Fe(CN)_6$
Zr	<10	0.32	Quercetin
Ti^{4+}	<1	0.76	Hydrogen peroxide

TABLE XVI

SEPARATION OF U^{6+}–Ti^{4+} AND Th WITH 9 N HCl

Element	E_a°	R_F	*Developer*
U^{6+}	>100	0	$K_4Fe(CN)_6$
Ti^{4+}	>1	0.67	Hydrogen peroxide
Th	0	0.82	8-Hydroxyquinoline

TABLE XVII

SEPARATION OF Zr–Ti^{4+}–Th WITH 9 N HCl

Element	E_a°	R_F	*Developer*
Zr	>10	0.1	Quercetin
Ti^{4+}	>1	0.67	Hydrogen peroxide
Th	0	0.82	8-Hydroxyquinoline

TABLE XVIII

SEPARATION OF U^{6+}–V^{5+}–Ti^{4+} WITH 3 N HCl

Element	E_a	R_F	*Developer*
U^{6+}	>10	0.01	$K_4Fe(CN)_6$
V^{5+}	<1	0.69	Hydrogen peroxide
Ti^{4+}	0	0.90	Hydrogen peroxide

Finally, as can be seen in Table XIX, a good separation of zirconium and hafnium was also obtained with HCl containing some HNO_3.

TABLE XIX

SEPARATION OF Zr AND Hf WITH 8 N HCl + 5 % CONC. HNO_3

Element	E_a° *(from ref.* [11]*)*	R_F	*Developer*
Zr	>2	0.17	Quercetin
Hf	<0.01	0.50	Quercetin

Sulphuric acid as eluting agent

Uranium and molybdenum were separated from iron, copper, and nickel by descending chromatography with sulphuric acid, as show in Tables XX and XXI.

TABLE XX

SEPARATION OF U^{6+} FROM Fe^{3+}, Cu AND Ni WITH 1 M H_2SO_4

Element	E_a° *(from ref.* [24]*)*	R_F	*Developer*
U^{6+}	>100	0.19	$K_4Fe(CN)_6$
Fe^{3+}	<0.1	0.56	$K_4Fe(CN)_6$
Cu	<0.1	0.58	$K_4Fe(CN)_6$
Ni	<0.1	0.76	Dimethylglyoxime

TABLE XXI

SEPARATION OF Mo^{6+} FROM Fe^{3+}, Cu, AND Ni WITH 0.2 M H_2SO_4

Element	E_a° *(from ref.* [24]*)*	R_F	*Developer*
Mo^{6+}	>1000	0.01	8-Hydroxyquinoline
Fe^{3+}	<0.1	0.27	$K_4Fe(CN)_6$
Cu	<0.1	0.30	$K_4Fe(CN)_6$
Ni	<0.1	0.77	Dimethylglyoxime

CONCLUSIONS

The results of this work show that a chromatographic paper treated with tri-*n*-octylamine behaves like an anion exchanger, and that many separations that are possible with the aid of solid resins or liquid exchangers, can also be achieved with this type of chromatography.

Moreover, this technique provides a simple and rapid method for the isolation of traces of complexed elements from large quantities of other elements not retained by the amine. After a kind of "stripping" of the spot with dilute nitric acid, it is possible to determine the isolated element quantitatively. Thus, a few μg of iron were separated quantitatively from mg quantities of nickel by filtering the hydrochloric solution containing the two elements through a filter paper treated with tri-*n*-octylamine.

SUMMARY

Chromatographic paper treated with a liquid anion exchanger (tri-*n*-octylamine) behaves like an anionic resin film; consequently many separations of cations that form anionic complexes can also be carried out by means of this new chromatographic procedure. Many examples are reported, among which the separation of the very similar elements zirconium and hafnium is of great significance.

It is anticipated that it will be possible to isolate traces of complexed metals from large quantities of non-complexed elements.

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EFFECT OF THE DRYING TEMPERATURE ON THE ION-EXCHANGE PROPERTIES OF ZIRCONIUM PHOSPHATE

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A considerable amount of study has been carried out recently on the ion-exchange properties of synthetic inorganic materials such as zirconium phosphate, titanium phosphate, zirconium oxide, thorium oxide, etc.[1-11]. The chief interest of such exchangers lies in the fact that they are more highly resistant to strong doses of ionizing radiations and to high temperatures than organic resins, which makes them particularly suitable for tackling problems of ion separation arising in reactor operation.

In two earlier papers we reported on certain preliminary experiments regarding the possibility of improving the separation of Sr^{2+} and Cs^{+} on zirconium phosphate dried at various temperatures. The separation can be considerably improved either by first applying ion adsorption at room temperature and afterwards drying the exchanger at 180°,[12] or by drying the exchanger first at temperatures ranging from 180° to 260° and adsorbing and eluting the ions afterwards[13].

In this paper the effect of the drying temperatures on the ion-exchange properties of zirconium phosphate (ZP) are studied.

EXPERIMENTAL

The ZP was prepared as indicated by GAL AND GAL[10]. 200 ml 4 *N* HCl containing 30 g $ZrOCl_2 \cdot 8H_2O$ were poured slowly into 500 ml of a 4 *N* HCl solution containing 64 g H_3PO_4. The high PO_4/Zr ratio (7:1) in the reagents is necessary in order to have an exchanger with many acid phosphate groups. The product thus obtained is dried at 50° for 24 h and finely ground and sieved. In the experiments described here, the fraction corresponding to a particle size of 0.1–0.2 mm was used. To heat the sample a furnace thermoregulated within $\pm$ 1° was used.

The ions Na^{+}, K^{+} and Li^{+} were determined by flame photometry. The ions Cs^{+}, Rb^{+}, Tl^{+}, Ba^{2+}, Sr^{2+} were determined radiometrically, using the radioactive isotopes ^{137}Cs, ^{86}Rb, ^{204}Tl, ^{140}Ba and ^{89}Sr as tracers.

Effect of drying temperature

(*a*) *Weight loss.* When the ZP prepared as described above is heated, it can be observed that as the drying temperature increases the weight decreases rapidly at

first (up to temperatures of about 250°) and more slowly from 250° to 800°. From 800° to 1,000° there is no further appreciable dehydration. Fig. 1 shows the weight loss of ZP, measured on a Stanton thermobalance, as a function of the temperature. For drying temperatures above 150° the dehydration was also observed to be partly irreversible.

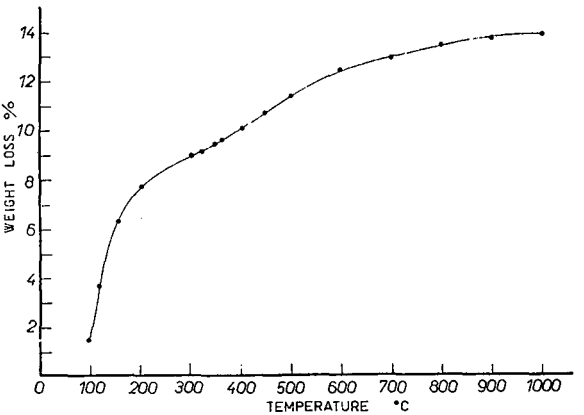

Fig. 1. Weight loss of ZP as a function of drying temperature. Heating rate: 4°/min.

(*b*) *Exchange capacity*. For drying temperatures in the 50–110° range, the specific exchange capacity of the ZP (mequiv./g ZP) increases, at a given pH, in proportion to the loss in weight of the exchanger. For drying temperatures in the 110–300° range, as AMPHLETT has pointed out[6], there are changes in properties as compared with the undried ZP, which depend on the pH value at which the determinations are carried out. The values are now no longer the same, even with the exchange capacity value corrected for weight loss of the exchanger.

Fig. 2 shows the exchange capacity of the ZP dried at 50° for 24 h and at 260° for 1 h 15 min as a function of the pH. As the drying temperature increases, the exchange capacity of the ZP decreases for pH values between 8 and 11, while for pH values below 7 the exchange capacity remains the same, or increases slightly.

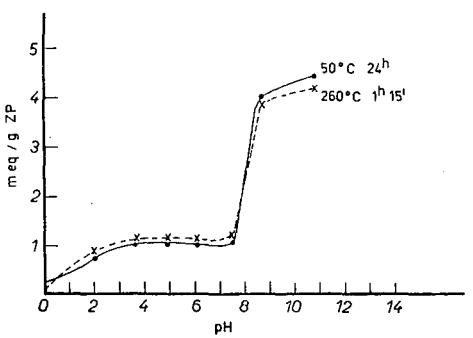

Fig. 2. Cation exchange capacity for K^+ ion of ZP dried at 50° for 24 h and at 260° for 1 h 15 min.

Table I shows the exchange capacity values obtained for certain ions in a solution at pH 2 on ZP dried at 50° for 24 h and at 260° for 1 h 15 min. It can be seen that the exchange capacity values also depend on the ion under consideration, and that

TABLE I

EXCHANGE CAPACITY AT pH 2 OF ZP DRIED AT DIFFERENT TEMPERATURES
(mequiv./g ZP referred to original weight)

Drying temperature	Li^+	K^+	Cs^+	Sr^{2+}
50° (24 h)	0.184	0.75	0.9	0.6
260° (1 h 15 min)	0.113	0.92	0.976	0.538
500° (8 h)	—	0.405	—	—

the ZP does not lose its exchange capacity, even after prolonged drying at high temperatures (500° for 8 h).

At this point we must mention that the exchange capacity of the ZP also depends on the PO_4/Zr ratio in the reagents[6].

(*c*) *Titration curves.* The ZP titration curves also depend on the drying temperature. Fig. 3 gives curves for ZP dried at 50° for 24 h and at 260° for 1 h 15 min.

(*d*) *Mass distribution coefficients.* Table II gives the mass distribution coefficients (mequiv./g exchanger per mequiv./ml solution) at equilibrium for alkali metal ions and Tl^+, Sr^{2+}, Ba^{2+}, Th^{4+} and UO_2^{2+} ions as obtained at different pH values on ZP dried at 50° for 24 h and at 260° for 1 h 15 min. The values were determined by equilibrating weight amounts of exchanger (0.5 g) with known volumes of suitable solutions (30 ml 0.008 *N* solution of the ion under investigation).

When equilibrium is reached the ZP is separated by centrifugation. The pH of the solution and the concentration of the ion in question are then measured. As can be seen from the values given in Table II, the temperature at which the ZP is dried has a considerable influence on the mass distribution coefficients. This influence is due only in small part to the loss in weight of the exchanger (14 %).

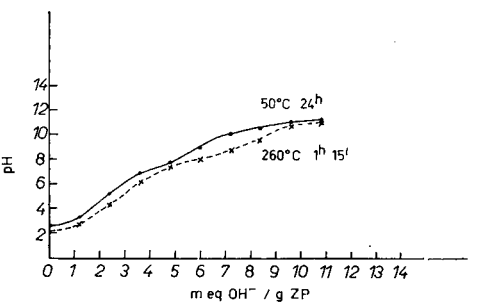

Fig. 3. Titration curve for ZP dried at 50° for 24 h and at 260° for 1 h 15 min. Mole ratio of phosphate to zirconium in reagents: $PO_4/Zr = 7:1$.

TABLE II

MASS DISTRIBUTION COEFFICIENTS ON ZP (mequiv./g ZP per mequiv./ml soln.)
A: ZP dried at 50° for 24 h. B: ZP dried at 260° for 1 h 15 min.

	Li^+		Na^+		K^+		Rb^+		Cs^+		Tl^+		Sr^{2+}		Ba^{2+}		Th^{4+}		UO_2^{2+}	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
HCl 4 *N*							< 1	15	6	35										
HCl 2 *N*					< 1	9	< 1	21	7	45										
HCl 1 *N*					< 1	11	7	39	18	70	< 1 ($HClO_4$ 1 *N*)	30	< 1	< 1	< 1	< 1	703	625		
HCl 0.1 *N*					18	140			60	480										
pH 1.6							38	222	1501 (carrier free)	> 10^5										
pH 2	< 1	< 1	< 1	< 1		220	75	370					116	120		171				
pH 2.4				44		240				2670									230	9
pH 2.5	9	7																		
pH 2.6			40		91															
pH 2.7				68					322											
pH 3															59				> 5000	468
pH 3.2			44																	

It is interesting to note that the increase in the mass distribution coefficients obtained by heating the ZP is not constant for all the ions studied. Thus, while there are considerable differences between the mass distribution coefficients for K^+, Rb^+ and Cs^+ obtained on ZP dried at 50° for 24 h and at 260° for 1 h 15 min, the differences obtained with Li^+ and Sr^{2+} are negligible.

For the monovalent and bivalent ions studied, the effect of the temperature on the mass distribution coefficients decrease in the following order: $Tl^+ > K^+ \geqslant Rb^+ \geqslant Cs^+ > Na^+ > Li^+$ and $Ba^{2+} > Sr^{2+}$. The separation coefficients between any two ions obtained on ZP dried at high temperature will therefore be different from those obtained on ZP dried at a temperature of 50°. This may be of considerable practical interest in view of the possibility of improving a number of separations on ZP dried at temperatures above 150°.

The measurements that were made further demonstrated that between 100° and 260° the value of the mass distribution coefficient increases with the rise in the drying temperature. For drying temperatures above 300° this value decreases progressively (Table III).

TABLE III

MASS DISTRIBUTION COEFFICIENT ON ZP DRIED AT DIFFERENT TEMPERATURES

(Exchanger in hydrogen form equilibrated with 0.25 mequiv. Cs^+ in 30 ml 2 *N* HCl)

Drying temperature	$K_D\left(\frac{\text{mequiv./g ZP}}{\text{mequiv./ml soln.}}\right)$
50° (24 h)	7
180° (1 h 30 min)	11
260° (1 h 15 min)	45
260° (13 h)	45
500° (8 h)	1
850° (8 h)	< 1

(*e*) *Rates of exchange.* Determinations of the exchange rate on ZP dried at different temperatures have shown that the rate decreases as the drying temperature increases, a fact which is very noticeable in the case of ions with larger radii, such as complex ions. Fig. 4 shows the variation in the mass distribution coefficient as a function of time for the zinc–ammonia complex (0.5 mequiv. Zn^{2+} in 30 ml 1 *N* NH_4OH) on ZP dried at 50° for 24 h and at 260° for 1 h 15 min.

(*f*) *Column elutions and separations.* For elution experiments small glass columns (diam. 0.6 cm) filled with 0.5 g ZP dried at a given temperature were used[12,13]. 0.0125 mequiv. of a given ion was then adsorbed on each column. Elution was carried out with HCl of different concentrations. For each ion a series of elution curves is thus obtained (several examples are given in Figs. 5, 6, 7) from which it can be seen that K^+, Rb^+ and Cs^+ ions have a greater resistance to elution the higher the temperature at which the ZP is dried. In the case of other ions, *e.g.* Na^+ and Ba^{2+}, the increase in resistance is very small, while the process is negligible and even reversed slightly for Li^+ and Sr^{2+}. The mass distribution coefficient values are thus confirmed. It was expected that a number of separations would be easier if ZP dried

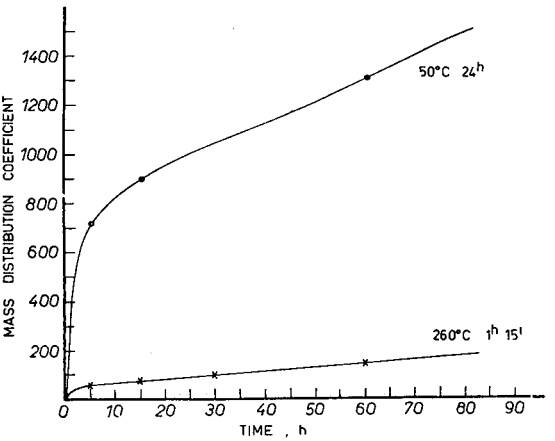

Fig. 4. Initial variations in the mass distribution coefficient (K_D) of zinc–ammonia complex on ZP dried at 50° for 24 h and at 260° for 1 h 15 min. Conditions: 0.5 mequiv. Zn^{2+} in 30 ml 1 *N* NH_4OH on 0.5 g ZP.

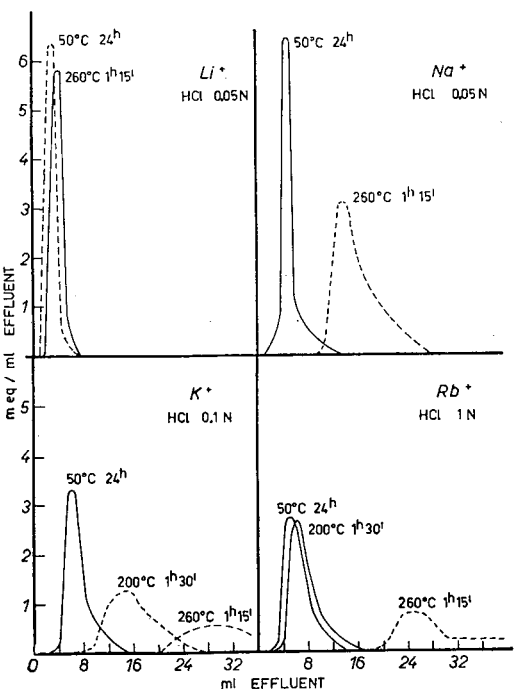

Fig. 5. Elution curves for Li^+, Na^+, K^+, Rb^+ ions, with various concentrations of HCl, on ZP dried at different temperatures. Conditions: 0.0125 mequiv. of each ion are adsorbed on 0.5 g ZP. Flow rate: 0.8 cm/min.

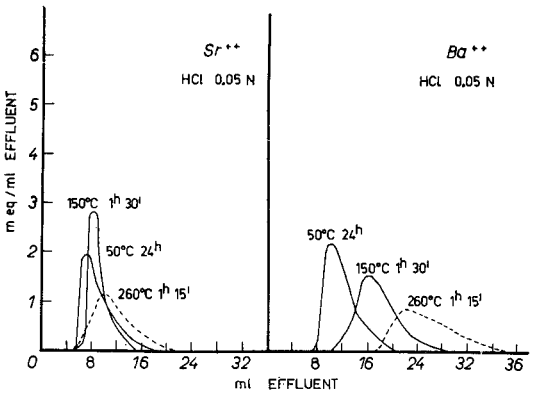

Fig. 6. Elution curves for Sr^{2+} and Ba^{2+} ions, with various concentrations of HCl, on ZP dried at different temperatures. Conditions: 0.01 mequiv. of each ion are adsorbed on 0.5 g ZP. Flow rate: 0.8 cm/min.

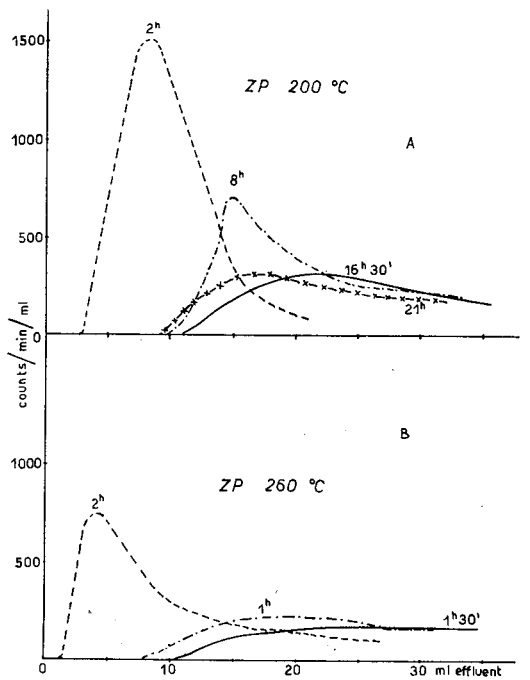

Fig. 7. Elution curves with 2 *N* HCl of 0.025 mequiv. Cs^{+} adsorbed on 0.5 g ZP dried at 200° (A) and at 260° (B) for varying drying times. Flow rate: 0.8 cm/min.

at temperatures in the 150–300° range was used; this was in fact subsequently confirmed. In Figs. 8 and 9 separations are shown of 0.025 mequiv. Cs^+ from 0.025 mequiv. Sr^{2+} and of 0.0125 mequiv. Li^+ from 0.0125 mequiv. Na^+ on ZP dried at both high and low temperatures.

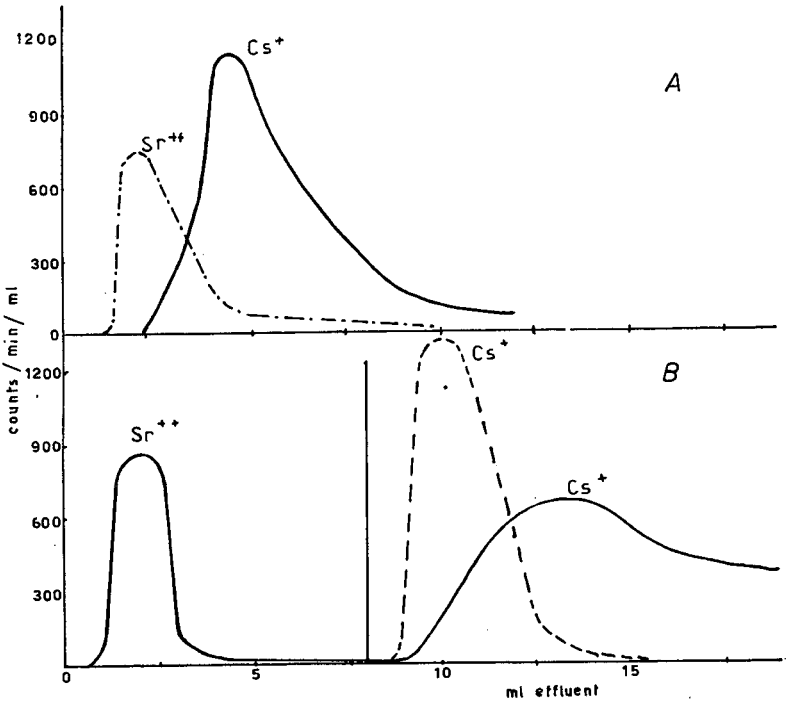

Fig. 8. Separation of 0.025 mequiv. Sr^{2+} from 0.025 mequiv. Cs^+: (A) on 0.5 g ZP dried at room temperature; (B) on 0.5 g ZP dried at 260° for 1 h 15 min. ——— elution with 2 *N* HCl. – – – – – elution with 5 *N* NH_4Cl–1 *N* HCl. Flow rate: 0.8 cm/min.

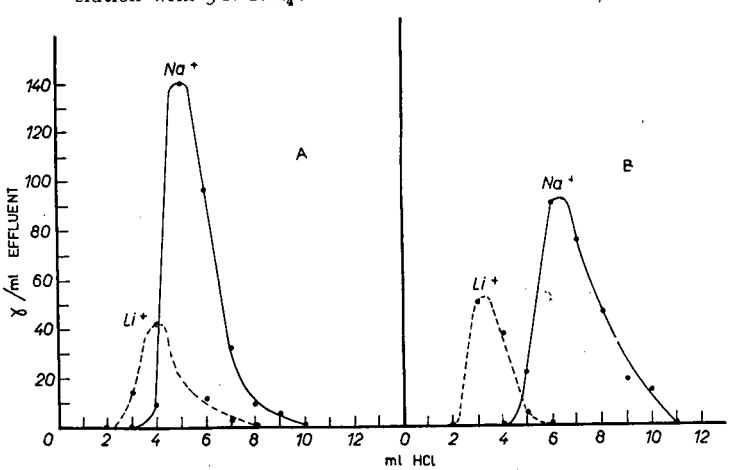

Fig. 9. Separation of 0.0125 mequiv. Li^+ from 0.0125 mequiv. Na^+: (A) on 0.5 g ZP dried at 50° for 24 h; (B) on 0.5 g ZP dried at 260° for 1 h 15 min. Elution with 0.05 *N* HCl.

DISCUSSION

These experiments show that as the drying temperature of ZP, precipitated in excess phosphoric acid, is raised, a considerable alteration in its ion-exchange properties occurs in addition to an irreversible dehydration.

The most important variations are those found for the mass distribution coefficients of the various ions. From Table II it can be seen that for monovalent ions the ratios of the mass distribution coefficients obtained on ZP dried at 260° and 50°, decrease in the following order:

$$Tl^+ > K^+ \geqslant Rb^+ \geqslant Cs^+ > Na^+ > Li^+$$

This order is the same as that obtained by ARGERSINGER, DAVIDSON AND BONNER[14] for organic resins with high and low degrees of cross-linking. In our case it was further observed that on ZP dried at high temperature the exchange rate decreases, especially in the case of ions with larger radii, *e.g.* complex ammonia ions. As is known[15,16], the same decrease takes place with resins with high degrees of cross-linking. Analogies between the ion-exchange properties of ZP dried at high temperature and such properties of organic resins with high degrees of cross-linking could then be easily explained by the assumption that an increase in the drying temperature causes a greater condensation in the structure of the ZP. This is corroborated by the irreversible loss of water, a fact which led AMPHLETT *et al.*[6] to propose the hypothesis that at high temperature "the process taking place may involve condensation of acid phosphate groups to form condensed P–O–P structures and further cross-linking". The phenomena observed with ZP dried at different temperatures would then be analogous to those studied by several authors with resins of varying degrees of cross-linking[17–19].

The structure and chemical composition of ZP precipitated in excess phosphoric acid and dried at both high and low temperatures are being investigated in order to obtain further confirmation of the above hypothesis.

ACKNOWLEDGEMENT

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SUMMARY

The ion-exchange properties of zirconium phosphate, dried at temperatures ranging from 150° to 850°, are compared with those of zirconium phosphate dried at 50°. In addition to partially irreversible dehydration, permanent changes in certain properties of zirconium phosphate dried at temperatures above 150° could be observed. Variations in selectivity for certain ions of ZP dried at high temperature can be employed to improve the separation of these ions from each other. All phenomena observed can be explained by the assumption that an increase in drying temperature causes a greater condensation in the structure of the exchanger.

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ÜBER DIE GASCHROMATOGRAPHISCHE TRENNUNG VON ORGANISCHEN SULFIDEN

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Im Zusammenhang mit den Studien über die Identifizierungsmöglichkeiten organischer Thioäther[1] befassten wir uns mit der Analyse dieser Stoffe in Gemischen. Wir lösten dieses Problem mit Hilfe der Papierchromatographie[2] der Thioäther nach vorheriger Überführung in geeignete nichtflüchtige Derivate (S,S-Dialkyl-N-*p*-nitrobenzolsulfonylsulfilimine)[3]. Auf diese Weise erzielten wir eine gute Trennung der homologen Sulfide, die Isomere und Metamere konnten jedoch nicht getrennt werden. Da die Sulfide flüchtige Verbindungen sind, bot sich die Möglichkeit an, ihre Trennung durch die Gas-Flüssigkeit Chromatographie zu schaffen. Durch diese Methodik wurden bereits einige niedrig siedende Sulfide getrennt[4–6]. In unserer Arbeit wird die Trennung und Identifizierung von Dialkylsulfiden mit Alkylen C_1 bis C_4 und von Alkylbenzylsulfiden C_1 bis C_4 beschrieben.

EXPERIMENTELLER TEIL

Apparatur

Unsere Versuche führten wir mit einer selbstgebauten Apparatur durch. Die U-förmige Kolonne aus Glas von der Gesamtlänge 125 cm und Innendurchmesser von 4 mm wurde mit 20 Gew. % der betreffenden stationären Phase auf Kieselgur (50–60 Maschen) gefüllt. Die Detektion der Stoffe wurde nach ihrer Verbrennung und Konversion des entstandenen Wassers auf Wasserstoff[7] durch thermische Leitfähigkeit ermittelt. Die Verbrennung erfolgte in einem an die Kolonne anschliessenden Quarzrohr (30 × 0.8 cm) auf einer Schicht von CuO (9 cm) und Co_3O_4 (3 cm) bei 700–750°. Das Quarzrohr enthielt weiter eine 4 cm Schicht von Silberwolle zur Absorption der Schwefeloxyde (450–550°) und eine 14 cm Schicht von Fe-Spänen (1–1.5 mm) auf der bei 450–550° Wasser zu Wasserstoff reduziert wurde. CO_2 wurde im anschliessenden Röhrchen durch Natronasbest absorbiert. Der resultierende Wasserstoff wurde durch die Leitfähigkeitszelle bei Zimmertemperatur entdeckt.

Als Trägergas wurde Stickstoff benutzt. Die Elutionskurven wurden mit einem 5 mV-Kompensationsschreiber EPP 09 (MAW Magdeburg) registriert.

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Stationäre Phasen

Siliconöl: Methylphenylsiliconöl wurde durch Molekulardestillation fraktioniert und die Fraktion 245–275°/5·10^{-5} mm Hg angewendet. 5;6- und 7;8-Benzochinoline wurden aus β- bzw. α-Naphthylamin durch die Skraupsche Synthese dargestellt[8] und durch die Adsorptionschromatographie auf Al_2O_3 gereinigt. 5;6-Benzochinolin schmolz bei 90–91° (Sdp. 359°), der Schmelzpunkt des 7;8-Derivats lag bei 48.5–49.5° (Sdp. 351°). Die Darstellung von 2,4-Dimethyl-7;8-benzochinolin erfolgte durch Kondensation von α-Naphthylamin mit Acetylaceton[9]. Schmp. 49.5°. Phenanthren reinst. Schmp. 99°, Sdp. 340°.

ERGEBNISSE UND DISKUSSION

Da die organischen Sulfide Verbindungen von nichtpolarem Charakter sind, wählten wir als stationäre Phase nichtpolares Siliconöl. Diese Trennflüssigkeit ermöglicht eine gute Trennung von homologen Reihen der Dialkyl- und Alkylbenzyl-sulfide und zwar ihren Siedepunkten nach. Eine Ausnahme bildete nur Diisopropylsulfid, das gemeinsam mit Methyl-*sec.*-butylsulfid eluiert wurde, trotzdem der Siedepunkt des letztgenannten Thioäther um 7° niedriger liegt. Wie es auch bei anderen homologen Reihen der Fall ist, besteht in den homologen Reihen von Dialkyl- und Alkylbenzyl-sulfiden eine lineare Abhängigkeit zwischen den Logarithmen der Retentionsvolumina und der Anzahl der Kohlenstoffatome. Dieselben Gesetzmässigkeiten fanden wir bei Anwendung anderer unpolarer Phasen, z.B. bei Paraffinöl.

In der Reihe der aliphatischen Dialkylsulfide existiert eine grosse Anzahl isomerer und metamerer Verbindungen. Viele von diesen haben nahe beieinander liegende oder sogar dieselben Siedepunkte. Bei Anwendung nichtpolarer stationärer Phasen konnten Verbindungen wie z.B. Propyl-isopropyl- und Äthyl-*sec.*-butyl-sulfid, Isopropyl-isobutyl- und Äthyl-butyl-sulfid, Isobutyl-*sec.*-butyl- und Propyl-butyl-sulfid (siehe Fig. 1) und andere nicht getrennt werden. Deshalb versuchten wir für diese Verbindungen eine geeignete selektive Phase zu finden. Die von ZLATKIS[10]

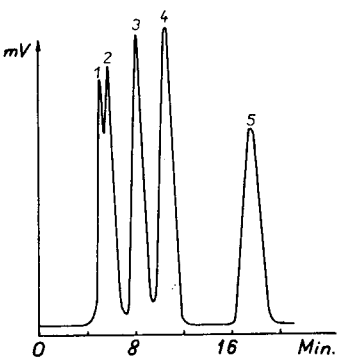

Fig. 1. Trennung isomerer Dialkylsulfide auf Siliconöl bei 100° und 25 ml N_2/min. (1) Äthyl-isopropyl-; (2) Diisopropyl-, Methyl-isobutyl-, Methyl-*sec.*-butyl-; (3) Propyl-isopropyl-, Äthyl-*sec.*-butyl; (4) Isopropyl-isobutyl-, Äthyl-butyl-; (5) Isobutyl-*sec.*-butyl-, Propyl-butyl-sulfid.

beschriebene Trennung von isomeren Hexanen unter Anwendung von Chinolin und Isochinolin als stationäre Phasen gab uns die Anregung zur Verwendung heterocyklischer Basen als Trennflüssigkeiten für isomere Thioäther. Um bei höheren Temperaturen arbeiten zu können richteten wir unsere Aufmerksamkeit auf 7;8-Benzochinolin, das einen hohen Siedepunkt und relativ niedrigen Schmelzpunkt aufweist. Wir konnten beobachten, dass bei Anwendung von 7;8-Benzochinolin die

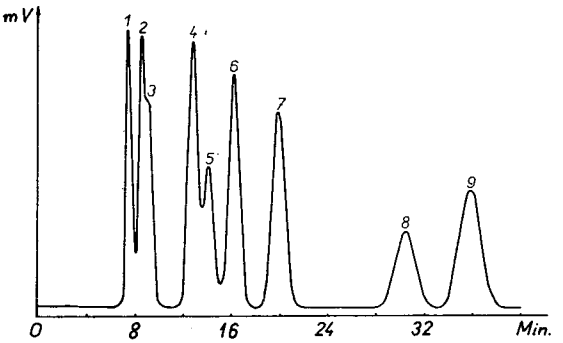

Fig. 2. Trennung eines Gemisches isomerer Dialkylsulfide auf 7;8-Benzochinolin bei 100° und 25 ml N_2/min. (1) Äthyl-isopropyl-; (2) Methyl-isobutyl-; (3) Diisopropyl-, Methyl-*sec.*-butyl-; (4) Propyl-isopropyl-; (5) Äthyl-*sec.*-butyl-; (6) Isopropyl-isobutyl-; (7) Äthyl-butyl-; (8) Isobutyl-*sec.*-butyl-; (9) Propyl-butyl-sulfid.

TABELLE I

RELATIVE RETENTIONSVOLUMINA DER DIALKYLSULFIDE AUF VERSCHIEDENEN TRENNFLÜSSIGKEITEN

(t = 100°, 25 ml N_2/min., V_{rel} Benzol = 1.00)

Sulfid	*Sdp. °C*	*A*	*B*	*C*	*D*	*E*
Dimethyl-	38	0.34	—	—	—	—
Methyl-äthyl-	67	0.66	—	—	—	—
Diäthyl-	92	1.17	—	—	—	—
Äthyl-isopropyl-	107	1.62	1.74	1.34	1.53	1.91
Methyl-isobutyl-	113	1.96	2.05	1.58	1.80	2.28
Methyl-*sec.*-butyl-	113	1.96	2.19	1.70	1.89	2.34
Äthyl-propyl-	118		—	—	—	—
Diisopropyl-	120	1.96	2.19	1.58	1.89	
Propyl-isopropyl-	132	3.08	3.12	2.43	2.96	3.63
Äthyl-isobutyl-	133	3.08	3.45	2.70	3.29	4.02
Äthyl-*sec.*-butyl-	133	3.08	3.45	2.70	3.29	4.02
Dipropyl-	143	4.35	4.98	3.88	4.64	5.91
Äthyl-butyl-	143	4.35	4.98	3.88	4.64	5.91
Isopropyl-isobutyl-	143	4.35	4.01	3.10	3.96	4.79
Isobutyl-*sec.*-butyl-	166	8.25	7.75	5.71	7.83	9.51
Propyl-butyl-	167	8.25	9.19	6.94	8.74	11.2
Dibutyl-	182	15.2	—	—	—	—
V_g Benzol		37	46	44	65	39
Separationsfaktor Äthyl-butyl/ Isopropyl-isobutyl-sulfid		1.00	1.24	1.25	1.17	1.23

A = Siliconöl; B = 7;8-Benzochinolin; C = 5;6-Benzochinoli ; D = 2,4-Dimethyl-7;8-benzochinolin; E = Phenanthren.

Sulfide mit verzweigten Alkylen von *n*-Alkylsulfiden getrennt werden, wobei erstere kürzere Elutionszeiten haben (Fig. 2). Um die Selektivität dieser Phase zu erklären, versuchten wir auch von 5;6-Benzochinolin, bzw. 2,4-Dimethyl-7;8-benzochinolin Gebrauch zu machen. Diese Phasen zeigten dieselbe Selektivität. Der Separationsfaktor von Äthyl-butyl- und Isopropyl-isobutyl-sulfid war der gleiche auch bei Anwendung von Phenanthren als stationäre Phase (Tabelle I). Aus dieser Tatsache erfolgt, dass bei allen diesen Trennflüssigkeiten die grösste Rolle die Struktur der aromatischen Kerne spielt. Die Trennungsselektivität wird also durch sterische Faktoren dirigiert. Aus dem Vergleich der Benzochinoline mit Phenanthren ist ersichtlich, dass der Stickstoff im heterocyklischen Ring keinen Einfluss auf die Trennung ausübt. Durch die Anwesenheit des Stickstoffatoms wird jedoch der Schmelzpunkt erniedrigt und der Siedepunkt erhöht, sodass die Benzochinoline gegenüber Phenanthren als stationäre Phasen bessere Eigenschaften besitzen.

TABELLE II

RELATIVE RETENTIONSVOLUMINA DER ALKYLBENZYLSULFIDE

(Phase Siliconöl, $t = 170°$, 25 ml N_2/min, $V_{rel.}$ Naphthalin = 1.00)

Sulfid	*Sdp. C°*	*$V_{rel.}$*
Methyl-benzyl-	197–198	0.89
Äthyl-benzyl-	218–220	1.23
Isopropyl-benzyl-	223–224	1.44
Propyl-benzyl-	235	1.81
Butyl-benzyl-	250	2.76
V_g Naphthalin		129

Die Selektivität der Benzochinoline als Trennflüssigkeiten für aliphatische Verbindungen mit verzweigten Alkylen scheint eine allgemeine Eigenschaft zu sein. Ähnliche Wirkung wie bei Sulfiden zeigten sie bei der Trennung der Dialkyläther[11].

Für praktische Zwecke sind die 5;6- und 7;8-Benzochinoline am geeignetsten. Das erste kann bei Temperaturen bis 120° benützt werden, der zweite bis 110°; bei höheren Temperaturen verflüchtigen sie sich schon teilweise.

DANK

Herrn Dr. VEČEŘA danke ich für sein Interesse und Anregung zu dieser Arbeit.

ZUSAMMENFASSUNG

Es wird eine Methode zur gaschromatographischen Trennung von C_1–C_4 Dialkylsulfiden und C_1–C_4 Alkylbenzylsulfiden beschrieben. Auf Siliconöl als stationärer Phase werden die Sulfide ihren Siedepunkten nach getrennt, die Anwendung von 5;6- und

7;8-Benzochinolinen ermöglicht die Trennung der Sulfide mit verzweigten Alkylen von Sulfiden mit normaler Kette. Diese Selektivität der Benzochinoline wird diskutiert.

SUMMARY

A method is described for the separation of C_1–C_4 dialkyl sulphides and C_1–C_4 alkyl benzyl sulphides by gas chromatography. With silicone oil as the stationary phase, the sulphides are separated according to their boiling points. By using 5;6- and 7;8-benzoquinolines, sulphides with branched alkyl chains can be separated from those with normal chains. This selectivity of the benzoquinolines is discussed.

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ÜBER DAS VERHALTEN MEHRBASISCHER SÄUREN UND IHRER ALKALOIDSALZE AN ALUMINIUMOXYD-SÄULEN

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(Eingegangen den 19. August 1960)

Die chromatographische Zerlegung von Salzen der China-alkaloide an Aluminiumoxyd-Säulen ist vielfach untersucht worden und verläuft ohne Komplikationen[1]. Da bei derartigen, für analytische Zwecke brauchbaren Spaltungen die Verwendung von Aceton–Wasser-Gemischen als Lösungsmittel gelegentlich praktische Vorteile bringen kann[2], interessierte uns das Verhalten einiger Chininsalze unter diesen Bedingungen*. Als wir im Verlauf dieser Untersuchungen die Kapazität der verwendeten Aluminiumoxyde für Chinin·HCl und Chinin·H_2SO_4 zu ermitteln suchten, ergaben sich einige zunächst nicht erwartete Befunde. Bei der Zerlegung werden im Sinne der früher dargelegten Auffassung[3] die auf Grund des Solvolysegleichgewichtes

$$[\text{Chinin}\cdot\text{H}]^{\oplus} + \text{H}_2\text{O} \rightleftharpoons \text{Chinin} + [\text{H}_3\text{O}]^{\oplus}$$

gebildeten Hydroxoniumionen auf der Säule fixiert, und im Filtrat tritt die freie Alkaloidbase auf. Die gleiche Menge Aluminiumoxyd sollte damit dem 2 Hydroxoniumionen liefernden Chinin·H_2SO_4 gegenüber ein nur halb so grosses Neutralisationsvermögen zeigen, wie dem nur 1 Hydroxoniumion liefernden Chinin·HCl gegenüber.

Diesbezügliche Versuche wurden in der Weise durchgeführt, dass auf Säulen von jeweils 4.0 g Aluminiumoxyd "Woelm basisch" oder "Woelm neutral" eine Lösung von 1 mmol. Chininsalz in 100 ml 80 %igem Aceton gegeben wurde. Diese Salzmenge liegt über der Durchbruchsbelastung der Säule; man findet infolgedessen im Filtrat nebeneinander Chininbase und Chininiumionen. Durch potentiometrische Titration mit 0.1 *N* Salzsäure bzw. 0.1 *N* Schwefelsäure bis zum Äquivalenzpunkt der 1. Stufe wurde anschliessend die Menge der Chininbase im Eluat bestimmt, die ein Masstab für die Spaltungskapazität des Aluminiumoxyds sein musste. Die unter übereinstimmenden Versuchsbedingungen erhaltenen Werte sind in Tabelle I gegenübergestellt.

Zunächst seien die Verhältnisse beim basischen Aluminiumoxyd diskutiert.

* Chinin enthält zwei Stickstoffatome, ist also eine zweisäurige Base. Die übliche Nomenklatur bezeichnet jedoch die mit 1 Äquivalent Säure entstehenden Salze als neutrale, z.B. Chininsulfat (2 Chinin·H_2SO_4) und die mit 2 Äquivalenten entstehenden als saure Salze, z.B. Chinin-hydrogensulfat (Chinin·H_2SO_4). Um jegliche Zweifel auszuschliessen, sollen im Folgenden an Stelle der üblichen Bezeichnungen die Bruttoformeln angegeben werden.

TABELLE I

ZERLEGUNG VON CHININSALZEN AN ALUMINIUMOXYD-SÄULEN

Versuch Nr.	*Säule von 4.0 g Al_2O_3 Woelm Akt. Stufe 1*	*1 mmol. (= 324 mg) Chininbase gelöst mit*	*In der Lösung vorliegendes Salz*	*Lösungsmittel Aceton Vol. %*	*Erforderliche ml 0.1 N Säure zur Neutralisation des Eluates*	*Im Eluat enthaltene mmol. Chininbase*
1	basisch	10 ml 0.1 *N* HCl	Chinin·HCl	80	5.23	0.52
2	basisch	20 ml 0.1 *N* H_2SO_4	Chinin·H_2SO_4	80	9.40	0.94
3	basisch	20 ml 0.1 *N* HCl	Chinin·2HCl	80	3.18	0.32
4	basisch	10 ml 0.1 *N* HCl	Chinin·HCl	90	5.40	0.54
5	neutral	10 ml 0.1 *N* HCl	Chinin·HCl	80	3.78	0.38
6	neutral	20 ml 0.1 *N* H_2SO_4	Chinin·H_2SO_4	80	8.09	0.81
7	neutral	20 ml 0.1 *N* HCl	Chinin·2HCl	80	1.88	0.19
8	neutral	10 ml 0.1 *N* HCl	Chinin·HCl	90	4.42	0.44

4.0 g Aluminiumoxyd "Woelm basisch" setzten aus 1 mmol. Chinin·HCl in acetonischer Lösung 0.52 mval. Base in Freiheit (Versuch 1). Die gleiche Menge Adsorbens machte hingegen aus 1 mmol. Chinin·H_2SO_4 0.94 mval. Base frei, also etwa 80 % mehr (Versuch 2). Dieses Ergebnis war überraschend. Wir hatten erwartet, dass weniger Base in Freiheit gesetzt würde als beim Chlorid; Chinin·H_2SO_4 kann ja die doppelte Menge Protonen an den Austauscher abgeben als die gleich molare Menge Chinin·HCl. Versuch 3 zeigt auf der anderen Seite, dass 4.0 g Aluminiumoxyd aus 1 mmol. Chinin·2HCl tatsächlich eine geringere Menge Alkaloidbase in Freiheit setzt, und zwar nur 60 % der beim Chinin·HCl gefundenen Menge.

Ähnliche Verhältnisse wurden bei Säulen von Aluminiumoxyd "Woelm neutral" gefunden. Auch hier verhalten sich die Mengen der durch die gleiche Menge Adsorbens freigesetzten Alkaloidbase beim Chinin·HCl, Chinin·H_2SO_4 und Chinin·2HCl etwa wie 1:2:0.5. Schliesslich lehren die Versuche 4 und 8, dass die Durchbruchsbelastung der beiden untersuchten Aluminiumoxyde gegenüber Chinin·HCl bei Verwendung von 90 %igem Aceton als Lösungsmittel etwas höher liegen als bei 80 %igem Aceton, dass aber grundsätzliche Unterschiede nicht bestehen.

Die in Tabelle I wiedergegebenen Befunde zeigen somit, dass die Spaltungskapazität der Aluminiumoxyde Alkaloidsalzen gegenüber im hohen Masse von der Natur des jeweiligen Anions abhängig sind. Das Bindungsvermögen der Aluminiumoxyde für verschiedene Anionen ist verschieden gross. Es entsprechen unter den gewählten, übereinstimmenden Versuchsbedingungen 4.0 g Aluminiumoxyd "Woelm basisch" 0.94 mmol. Schwefelsäure bzw. 0.52 mmol. Chlorwasserstoff und 4.0 g Aluminiumoxyd "Woelm neutral" 0.81 mmol. Schwefelsäure bzw. 0.38 mmol. Chlorwasserstoff.

Wir haben daraufhin das Verhalten der Lösungen einiger anorganischer und

organischer Säuren in 90 %igem Aceton gegenüber den beiden Aluminiumoxyden untersucht. Das Neutralisationsvermögen der Oxyde ist freien Säuren gegenüber grösser, als wenn diese in Form von Alkaloidsalzen vorliegen. Man erkennt dies, wenn man eine Säule von Aluminiumoxyd "Woelm neutral" zunächst mit der die Spaltungskapazität überschreitenden Menge von 1 mmol. Chininhydrochlorid in 90 %igem Aceton behandelt, mit dem gleichen Lösungsmittel wäscht und anschliessend eine Lösung von Salzsäure in 90 %igem Aceton aufgibt; ausser den aus dem Chininsalz stammenden 0.4 mmol. Chlorwasserstoff werden sodann weitere 3.1 mmol. von der Säule neutralisiert. Sehr unterschiedlich ist auch hier wieder das Verhalten der Aluminiumoxyde verschiedenen Säuren gegenüber. Unter Berücksichtigung der bisherigen Befunde gingen wir, um vergleichen zu können, von gleichen Molaritäten aus und brachten jeweils Gemische von 10 ml 0.5 mol. Säure und 90 ml Aceton auf Säulen von 4.0 g Aluminiumoxyd. Wieder wurden Mengen verwandt, die über der Durchbruchsbelastung der Säulen lagen. Die erhaltenen Eluate wurden zunächst qualitativ auf die jeweils interessierenden Ionen geprüft und anschliessend die wünschenswerten quantitativen Bestimmungen durchgeführt. In Tabelle II sind die wichtigsten Ergebnisse gegenübergestellt. Vergleicht man zunächst die einbasischen Säuren, so erkennt man, dass die Säurestärke für das Ausmass der Fixierung

TABELLE II

BINDUNG FREIER SÄUREN AN ALUMINIUMOXYD-SÄULEN

Versuch Nr.	*Säule 4.0 g*	*Aufgegeben in 100 ml 90 Vol. % Aceton gelöst 5 mmol.*	*Kationen*						*Anionen*		
			Zur Neutralisation des Filtrats erforderlich 0.1 N NaOH		*Durch die Säule neutralisierte Säure*[a]		*Sonstige Kationen i. Filtrat*		*Art*	*Im filtrat gefunden mval.*	*Auf der Säule geblieben mval.*[a]
			Indikator	*ml*	*mmol.*	*mval.*	*Art*	*mval.*			
1	Aluminiumoxyd "Woelm basisch"	$HClO_4$	Dimethylgelb	29.6	2.04	2.04	Al	0.83[b]	ClO_4	3.89[c]	1.11
			Methylrot	36.8			Na	0.10[a]			
2		HCl	Dimethylgelb	15.0	3.50	3.50	Al	1.20[b]	Cl	3.10[d]	1.90
			Methylrot	27.0			Na	0.40[a]			
3		H_2SO_4	Methylrot	32.2	3.39	6.78	Al, Na		SO_4	3.22[e]	6.78
4		$(COOH)_2$	Phenolphthalein	61.6	1.92	3.84	nicht		$(COO)_2$	6.16[f]	3.84
5		H_3PO_4	Phenolphthalein	34.2	3.29	9.87	nachweis-		PO_4	5.16[g]	9.84
6		CH_3COOH	Phenolphthalein	37.7	1.23	1.23	bar		CH_3COO	3.77[a]	1.23
7	Aluminiumoxyd "Woelm neutral"	$HClO_4$	Dimethylgelb	30.4	1.96	1.96	Al	1.01[b]	ClO_4	4.20[c]	0.80
			Methylrot	39.8			Ca	0.30[b]			
8		HCl	Dimethylgelb	14.5	3.55	3.55	Al	1.20[b]	Cl	3.30[d]	1.70
			Methylrot	27.0			Ca	0.50[b]			
9		H_2SO_4	Methylrot	44.0	2.80	5.60	Al, Ca		SO_4	4.40[e]	5.60
10		$(COOH)_2$	Phenolphthalein	65.6	1.72	3.44	nicht		$(COO)_2$	6.56[f]	3.44
11		H_3PO_4	Phenolphthalein	38.8	3.06	9.18	nachweis-		PO_4	5.88[g]	9.12
12		CH_3COOH	Phenolphthalein	41.3	0.87	0.87	bar		CH_3COO	4.13[a]	0.87

[a] Werte nicht experimentell bestimmt, sondern aus der Differenz berechnet.
[b] Komplexometrisch.
[c] Als Nitron-perchlorat.
[d] Argentometrisch.
[e] Als $BaSO_4$.
[f] Permanganometrisch.
[g] Als $P_2O_5 \cdot 24MoO_3$.

nicht entscheidend zu sein scheint. Die Mengen der von 4.0 g Aluminiumoxyd "Woelm basisch" neutralisierten Protonen betragen bei Perchlorsäure 2.0 mmol., bei Chlorwasserstoff 3.5 mmol. und bei Essigsäure 1.2 mmol.; bei Aluminiumoxyd "Woelm neutral" sind die entsprechenden Zahlen 2.0, 3.6 und 0.9 mmol. Vielleicht ist zur Deutung dieser Befunde die räumliche Ausdehnung der Anionen heranzuziehen, über die Aussagen aber schwierig sind, weil sich der Austausch in Aceton–Wasser-Gemischen abspielt, wo über die Umhüllung der Ionen mit Lösungsmittelmolekeln kaum etwas bekannt ist; erschwerend tritt hinzu, worauf HAYEK[4] hingewiesen hat, dass bei der Verwendung von Lösungsmittelgemischen die Oberfläche des Adsorbens von einer Molekelschicht bedeckt sein kann, die nicht der Zusammensetzung des homogenen Lösungsmittel entspricht. Von Bedeutung ist in unserem Zusammenhang aber vor allem, dass die Mengen der von beiden Aluminiumoxyden fixierten mehrbasischen Säuren etwa gleich gross, zum Teil aber auch grösser sind als bei den einbasischen Säuren. 4.0 g Aluminiumoxyd "Woelm basisch" neutralisieren 3.4 mmol. Schwefelsäure, 1.9 mmol. Oxalsäure und 3.3 mmol. Phosphorsäure. Aluminiumoxyd "Woelm neutral" hat meist ein etwas geringeres Neutralisationsvermögen als das basische Präparat, im übrigen sind die Unterschiede bei den einzelnen Säuren aber weitgehend analog.

Die in Tabelle II wiedergegebenen Befunde weisen somit gleichfalls daraufhin, dass dem Anion eine sehr wesentliche Rolle im Hinblick auf die Austauschkapazität der Aluminiumoxyde zukommt. Noch deutlicher wird dies, wenn man an Stelle der Millimole neutralisierter Säure die Millivale betrachtet oder wenn man die Menge der auf der Säule verbliebenen Anionen ermittelt. Die Annahme wird nahegelegt, dass die Anionen mehrbasischer Säuren nur monovalent an das Aluminiumoxyd gebunden werden. Im Sinne von D'ANS, HEINRICH UND JÄNCHEN[5] besteht bei der Bindung eines zweiwertigen Anions auf dem Austauscher die Möglichkeit, dass entweder die zweite Wertigkeit als Ion freibleibt, oder dass sie—falls es die räumlichen Verhältnisse zulassen—gleichfalls vom Aluminiumoxyd neutralisiert wird. Experimentell zeigten diese Autoren, dass verdünnte Salzsäure eine grössere Menge Aluminiumoxyd verbraucht als die äquivalente Menge verdünnter Schwefelsäure, damit die Anschlämmung in beiden Fällen das gleiche pH zeigt; sie zogen daraus den Schluss, dass von den beiden Wasserstoffen der Schwefelsäure nur eins von dem Austauscher gebunden wird. Die maximale Kapazität von Aluminiumoxyd gegenüber verschiedenen Anionen haben auch NYDAHL UND GUSTAFSSON[6] untersucht; ein Vergleich mit unseren Befunden ist wegen der durch andere Fragestellung ganz verschiedenen Versuchsanordnung aber nicht möglich.

Wenn mehrbasische Säuren nur monovalent an das Aluminiumoxyd gebunden werden, so sollten die entstehenden Oberflächenverbindungen den Charakter von Säuren haben. Der Nachweis dafür liess sich tatsächlich erbringen. Wenn wir eine Aluminiumoxyd-Säule zunächst mit der Lösung einer mehrbasischen Säure, z.B. Schwefelsäure oder Phosphorsäure in 90 %igem Aceton behandelten, so liess sich die auf der Säule fixierte Säure mit wasserfreiem Aceton nicht herunterwaschen. Gaben wir aber anschliessend eine Lösung von Lithiumchlorid in wasserfreiem Aceton auf,

so wurden die Protonen der Oberflächenverbindung durch Lithiumionen verdrängt und wir fanden in Eluat äquivalente Mengen Hydroxoniumionen und Chlorionen. Diese Elution verlief zwar relativ langsam, sie lehrte jedoch, dass es grundsätzlich möglich ist, Aluminiumoxyd durch Behandeln mit mehrbasischen Säuren in einen Kationenaustauscher zu verwandeln, der so stark sauer ist, dass Alkali-ionen in acetonischer Lösung gegen Hydroxoniumionen ausgetauscht werden.

Die Annahme, dass mehrbasische Säuren nur monovalent vom Aluminiumoxyd gebunden werden, ist für die Deutung der experimentellen Befunde allerdings nicht ausreichend, denn sonst sollten in allen Fällen äquimolare Mengen der Säuren neutralisiert werden. Auch hier sind also noch spezifische Beziehungen zwischen Adsorbens und Anion zu berücksichtigen, wie solche auch bei den einwertigen Anionen ganz deutlich sind. SCHWAB UND DATTLER[7] sowie KUBLI[8] haben für die Anionenadsorption des Aluminiumoxyds eine Reihe aufgestellt und ein der Löslichkeit der entsprechenden basischen Aluminiumsalze paralleles Verhalten gefunden. Dass unsere Ergebnisse eine teilweise abweichende Reihenfolge ergeben, scheint nicht verwunderlich, da wir nicht mit Wasser, sondern mit 90 %igem Aceton als Lösungsmittel arbeiteten. Für die Tatsache, dass Aluminiumoxyd verschiedenen Anionen gegenüber ein unterschiedliches Austauschvermögen zeigt, sind möglicherweise räumliche Gründe massgebend. Genauere Aussagen sind wegen der unbekannten Oberflächengestaltung des Adsorbens nicht möglich. Unterschiedlich ist aber nicht nur der Flächenbedarf der Anionen sondern auch der der Kationen, wie dies aus dem verschiedenen Neutralisationsvermögen des Aluminiumoxyds gegenüber Hydroxoniumionen und Chininiumionen zu erkennen ist. Die Austauschvorgänge sind also durch eine gewisse Selektivität gekennzeichnet in Abhängigkeit von der Grösse und zusätzlich der Ladung der beteiligten Ionen. Dass bei Austauschvorgängen an Aluminiumoxyd Tartrat-, Oxalat- und Phosphationen besonders festgehalten werden, ist ausser von SCHWAB UND DATTLER[7] und KUBLI[8] auch von SINHA UND CHOUDHURY[9], von THIES[10] sowie von GRASSHOF[11] gezeigt und durch die Entstehung von Oberflächenverbindungen erklärt worden.

EXPERIMENTELLES

Zu Tabelle I

Das abgewogene Aluminiumoxyd wurde mit Hilfe von 50 ml wasserhaltigen Acetons —der Gehalt in Vol. % ist in der Tabelle jeweils angegeben—in ein mit einer G3-Filterplatte versehenes Chromatographierohr eingeschlämmt und völlig abtropfen lassen. Anschliessend wurden 100 ml Chininsalzlösung der jeweils angegebenen Konzentration auf die Säule gegeben. Nach erfolgtem Durchlauf wurde portionsweise mit etwa 50 ml Lösungsmittel-gemisch nachgewaschen* und anschliessend auf dem Wasserbad vorsichtig abgedampft. Der Rückstand wurde in einer Mischung von 2 Vol. Methanol und 1 Vol. Wasser gelöst und die freie Base potentiometrisch titriert bis zum Äquivalenzpunkt des neutralen Chininsalzes.

* Diese Menge Lösungsmittel war in allen Fällen ausreichend. Es wurde stets anschliessend weiteres Lösungsmittel aufgegeben und festgestellt, dass dann im ablaufenden Filtrat keine nichtflüchtigen Stoffe mehr enthalten waren.

Zu Tabelle II

In der zuvor beschriebenen Weise wurden zunächst 4.0 g Aluminiumoxyd mit Hilfe von 50 ml 90 Vol. % Aceton in ein mit G3-Filterplatte versehenes Rohr eingeschlämmt und völlig abtropfen gelassen. Auf diese Säule wurde jeweils ein Gemisch von 10 ml 0.5 *M* Säure und 90 ml Aceton, also eine 0.05 *M* Säure in 90 %igem Aceton, gegeben und wieder jeweils völlig abtropfen gelassen. In keinem Fall wurde mit Lösungsmittel nachgewaschen, da hierdurch die Reproduzierbarkeit verschlechtert wird. Das Eluat wurde beim Vorliegen nichtflüchtiger Säuren auf dem Wasserbad vorsichtig vom Aceton befreit und anschliessend alkalimetrisch titriert; bei flüchtigen Säuren wurde hingegen nach dem Verdünnen mit Wasser zunächst alkalimetrisch titriert und erst dann zur Entfernung des Acetons eingeengt. Die hinterbleibenden wässrigen Lösungen wurden mit Wasser zu 200 ml aufgefüllt und in aliquoten Teilen dieser Lösung die jeweils interessierenden Ionen nachgewiesen bzw. quantitativ bestimmt.

ZUSAMMENFASSUNG

Das Verhalten acetonischer Lösungen von freien Säuren oder ihren Alkaloidsalzen an Aluminiumoxyd-Säulen lässt sich verstehen unter der Annahme, dass

(1) verschiedene Ionen in unterschiedlichen Ausmass die Oberfläche des Austauschers beanspruchen,

(2) mehrbasische Säuren monovalent am Aluminiumoxyd gebunden werden.

Mit Schwefelsäure sauer formierte Aluminiumoxyd-Säulen vermögen Alkalikationen in acetonischer Lösung gegen Hydroxoniumionen auszutauschen.

SUMMARY

The behaviour of acetone solutions of free acids or their alkaloid salts on aluminium oxide columns can be explained by assuming that:

(1) different ions occupy the surface of the exchanger to a different extent,

(2) polybasic acids are bound to aluminium oxide with only one valency.

Acid aluminium oxide columns that have been prepared with sulphuric acid can exchange alkali cations in acetone solution for hydroxonium ions.

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AUTOMATIC RECORDING OF THE RADIOACTIVITY OF ZONES ELUTED FROM THE GAS–LIQUID CHROMATOGRAM

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The high resolving power of the gas–liquid chromatogram has prompted many attempts at designing counters capable of continuously recording the radioactivity of vapours eluted from the column. Flow proportional counters working at column temperature have been used[1–3], as has a proportional counter at room temperature[4]. In the latter case condensation of the zones was prevented by dilution with a large volume of gas introduced into the train after the vapour detector. Scintillation counting has been adopted by KARMEN AND TRITCH[5] by condensing fractions in tubes containing a crystalline scintillator coated with a silicone oil. POPJAK and his collaborators[6] designed an ingenious trap containing a solution of a scintillator in toluene held between two photomultiplier tubes. All the zones accumulate in the trap and an automatic range-changing device keeps the record of count rate on the scale of the recorder. In our experience these methods possess some disadvantages. Apart from the general difficulties of construction of proportional counters capable of operating at temperatures up to 250°, expansion of the centre wire can change the operating characteristics. A major problem is the poisoning of the centre wire caused by stripping of stationary phase from the column. Dilution of the gas stream from the detector with cold gas when long-chain fatty acids are being separated can lead to deposition within the cell.

Trapping of individual fractions followed by counting either with an end-window counter[7] or with a scintillation counter[5] is laborious and necessary only when substances of very low specific activity are to be detected. Furthermore, fractions can be conveniently collected only when the vapour detector records a peak; radioactive intermediates present in very small amounts can be missed. The apparatus of POPJAK[6] is efficient but suffers from the disadvantage that all the zones are trapped in the cell so that fast moving materials of high specific activity can cause the instrument to switch to low sensitivity unless the cell is emptied and refilled during the separation.

We have adopted the alternative approach of using argon as the carrier gas of the chromatogram and burning all organic compounds (whether derived from the stationary phase or an eluted zone) over heated copper oxide. Water is removed by passing the gas stream over coarsely powdered magnesium perchlorate; carbon dioxide is injected to give a final concentration of 5 % and the gas is passed, at room temperature,

into a proportional counter of the simplest construction. To detect and record the pulses from the proportional counter a linear amplifier, ratemeter and pen recorder are needed. The amplifier (for ^{14}C) has a gain of 200 and can conveniently be a "ring of three" in a small case which also contains an E.H.T. filter, adjacent to the counter. This unit derives its electrical power from the ratemeter. The ratemeter, which includes an E.H.T. supply, has a pulse-height discriminator, followed by the usual pulse shaping circuits, diode pump integration and valve voltmeter stages. For the presentation of pulses occurring at rates less than a few pulses per second the "rate" presentation is unsuitable because the relatively long integration times, which are necessary to maintain a slowly changing value for recording, limit the resolution. A more satisfactory record is obtained by integrating the peaks (Fig. 6B). The ratemeter used in this apparatus was modified to give four ranges of rate (1, 10, 100 and 1000 pulses/sec) and three integrating ranges (50, 500 and 5000 pulses) with provision for any intermediate setting. In addition, a circuit was provided allowing automatic shorting of the integrating capacitor when full-scale deflection was reached.

The apparatus is cheap and easy to construct, reliable in operation, the counter has almost 4 π geometry and can be used with conventional commercial counting equipment.

EXPERIMENTAL

Construction of gas train

A silica tube ½ in. diameter and 2 in. long is attached to the vent of the vapour detector by a P.T.F.E. connector. Around the silica tube is placed a small furnace Fig. 1) of the type used to vaporise the sample in a Dumas combustion train. A

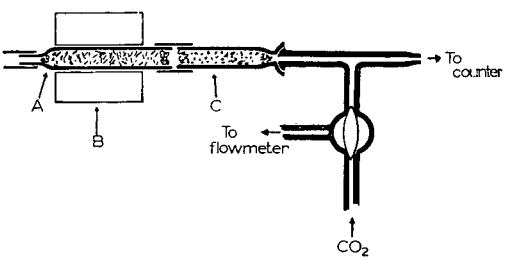

Fig. 1. General arrangement of the gas train for combustion and drying of the vapour eluted from the gas chromatogram. A: Silica tube packed with copper oxide (wire form). B: Furnace. C: Drying tube containing anhydrous magnesium perchlorate.

furnace temperature of approximately 700° is suitable. The cool end of the silica tube is attached with silicone rubber tubing to a ¼ in. diameter glass tube containing coarsely powdered anhydrous magnesium perchlorate. The end of this tube is fitted with a spherical ground glass joint allowing a flexible attachment to a capillary T-piece in which the CO_2 is introduced. The gas stream then passes through a short length of silicone rubber tubing to the entry port of the counter. A trap containing soda-lime is attached at the exit tube of the counter to absorb all the CO_2.

Construction of the counter

The body of the counter consists of a short length (most frequently 4 in.) of ½ in. copper water pipe. The pipe possesses a very smooth interior finish and requires no treatment beyond acid dipping. The end plugs are machined from polythene, the centre of each is tapped 2 B.A. thread to take the brass anode supports, which also serve to introduce the gas (Fig. 2). Contact between the anode wire (0.002 in. diameter tungsten) and the support, is achieved by bending the wire over the tube and

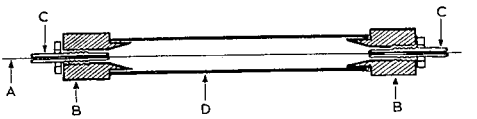

Fig. 2. Section of proportional counter. A: Tungsten wire 0.002 in. diameter. B: Polythene plug. C: Brass insert. D: Copper tube ½ in. diameter.

forcing on a short length of silicone rubber tubing. The anode connection to the amplifier is soldered to one of the anode supports. The counter is assembled as follows: one of the polythene caps is inserted, the tungsten wire is drawn through and held in place by the rubber tubing. The wire is drawn through the counter tube and then through the other cap, the cap is forced into place, the wire is pulled tight, bent over the brass insert and silicone rubber tubing pushed over the insert. The counter is contained in a 1 in. thick lead sandwich to which is attached the amplifier.

Circuitry

Amplifier. Many commercial amplifiers are suitable, two known to the authors are as follows:

Burndept BN 106 and Dynatron N 640, with the gain adjusted to be × 200.

Ratemeter. The ratemeter used was an A.E.R.E. type 1037b, the commercial equivalent is the Dynatron N 522. The higher ranges of rate originally provided on the instrument were not required for this work and these positions were used to

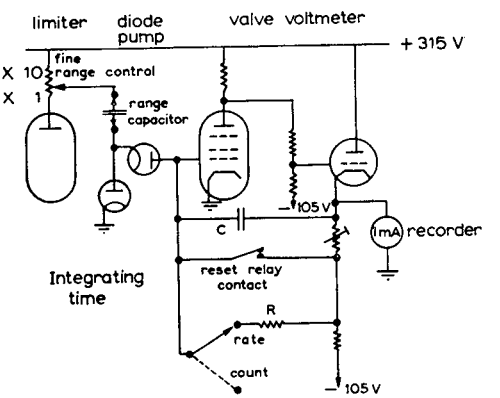

Fig. 3. Circuit illustrating range switching in the ratemeter.

provide three integrating ranges of 50, 500 and 5,000 counts. A fine control provided a continuous change of range sensitivity between the coarse steps (Fig. 3). The integrating capacitor is automatically shorted by a relay contact when full scale deflection is reached, the relay being operated for a time sufficient to discharge the condenser via a flip-flop (Fig. 4) to ensure that the capacitor is fully discharged. The relay can be operated by switch contact in the case of a servo recorder or with many coil recorders the pointer can intercept a light beam falling on a photo-transistor.

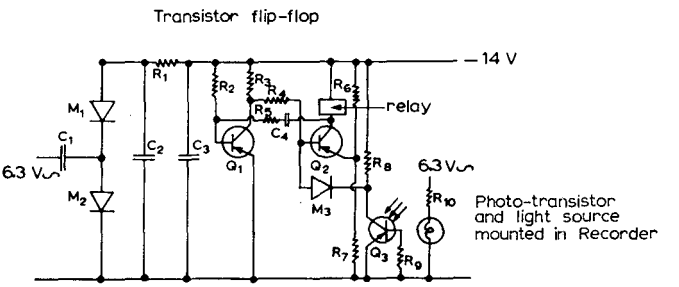

Fig. 4. Circuit for automatic shorting of integrating capacitor actuated by full scale deflection of the recorder.

M1, 2 and 3	Mullard OA71	R1	470 Ω	all ¼ W 10%	R6	12 kΩ	all ¼ W 10%
Q1, Q2	Mullard OC71	R2	560 kΩ		R7	1.5 kΩ	
Q3	Mullard OCP71	R3	10 kΩ		R8	47 kΩ	
C1, 2 and 3	25 μF 50 V electrolytic	R4	47 kΩ		R9	10 kΩ	
C4	2 μF 25 V electrolytic	R5	56 kΩ		R10	33 Ω	3 W vitreous

Relay 14,000 Ω 0.8 mA operating current.

Calibration

(a) *Counter plateau.* An external radiation source can be used to determine the plateau of the counter. The ratemeter and recorder are set to record count rate while the E.H.T. voltage is increased in small steps until the discharge point is reached. Inspection of the record will show the position and length of the plateau; the operating point corresponds to the voltage at the centre of the plateau. This procedure should be repeated occasionally to check on counter performance.

(b) *Counter efficiency.* Stearic acid of known specific activity was diluted with inactive stearate and the absolute activity was determined by combustion according to the procedure described by BRADLEY *et al.*[8]. Samples of the methyl ester of the acid were then run on the gas chromatogram and the counts obtained from known quantities compared with the known absolute count.

RESULTS AND DISCUSSION

Typical recorder diagrams are shown in Figs. 5 and 6. The rate record (Fig. 5, curve B) is useful when scanning a group of acids for the occurrence of radioactivity but less useful when an accurate measurement of amount of radioactivity is required. The

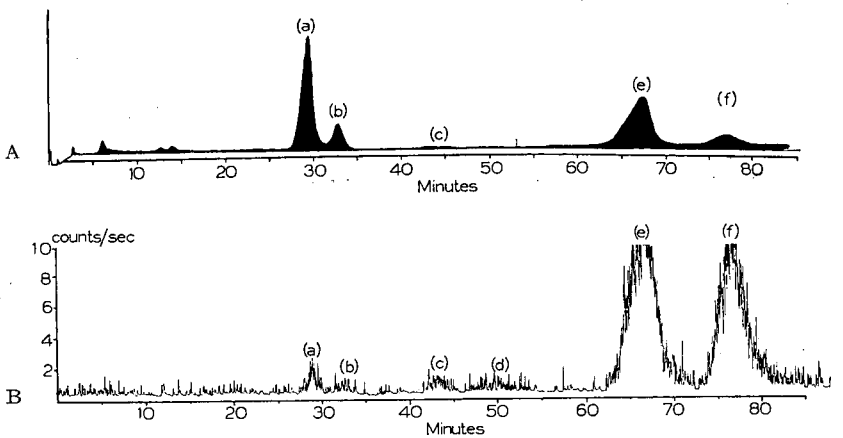

Fig. 5. Comparison of simultaneous record of vapour detector (gas density meter) and proportional count rate, from analysis of fatty acids isolated from yeast grown in the presence of 2-^{14}C-stearic acid, containing labelled palmitic and heptadecanoic contaminants. Peak identification: (a) Palmitoleic acid; (b) Palmitic acid; (c) *n*-Heptadecenoic acid; (d) *n*-Heptadecanoic acid; (e) Oleic acid; (f) Stearic acid. Labelled palmitic and *n*-heptadecanoic acids were minor impurities in the labelled stearic acid. Column: 4 ft. Apiezon L on Celite 100–120 mesh at 197°. Argon flow rate 10 c.c. in 5.5 sec. CO_2 flow rate 0.5 c.c. in 5.5 sec. Load 1.0 mg.

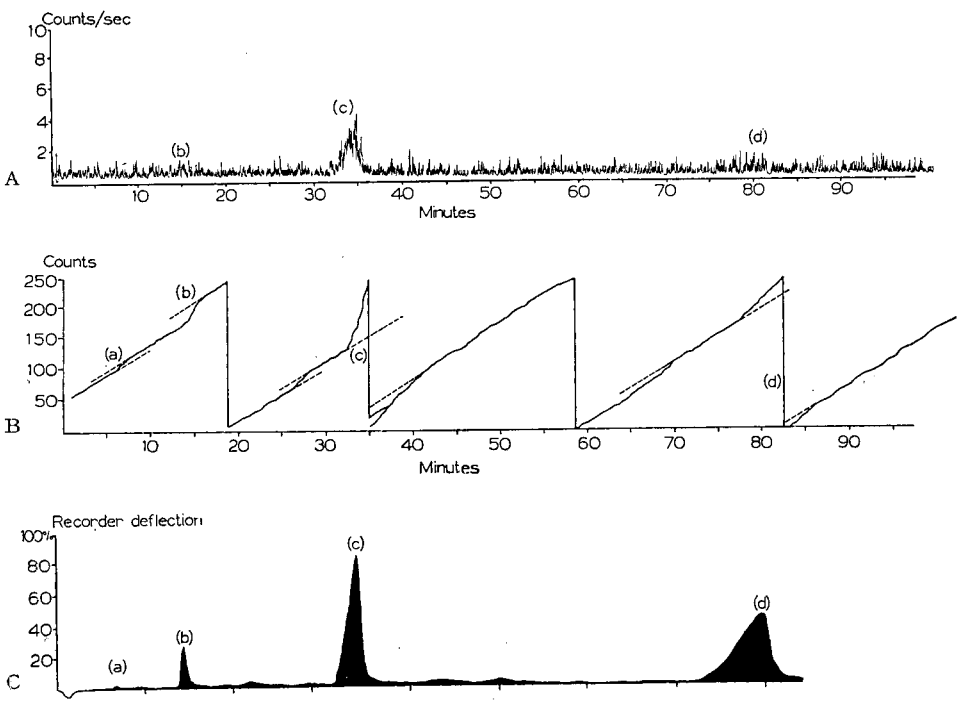

Fig. 6. Comparison of records of integrated count and count rate for a mixture of labelled lauric, myristic, palmitic and stearic acid. A: Count rate measurement; B: Total count measurement; C: Vapour concentration record. (a) Lauric acid; (b) Myristic acid; (c) Palmitic acid; (d) Stearic acid.

integrated count (Fig. 6, curve B) is best for accurate measurements since the count less background can be read off directly. The base line slopes upwards because of the accumulation of background count.

Sensitivity. The sensitivity of the counter varies inversely with the flow rate. With a volume of 10 c.c. the average residence time of each molecule at a flow rate of 2 c.c./sec will be 5 sec. This is adequate for specific activities of the order of 600 counts/min/mg and greater. The minimum detectable amount will vary with the retention volume of the peaks, slow peaks emerging over 10 min or more will require larger loads. The volume of the counter should not be greater than that of the vapour detector or peak resolution will suffer.

If used in conjunction with ionisation vapour detectors such as the hydrogen flame ionisation and argon ionisation instruments, a gas splitting device must be placed at the end of the column in order to allow the use of large (1 mg upward) column loads without overloading the detectors. In many biochemical experiments the amount of labelling is small so that a few mg of material will be necessary to obtain sufficient counts. It will often be an advantage to use column conditions (*i.e.* polar stationary phases and/or higher than usual column temperatures) to decrease the retention volume of the components so that low flow rates can be used and yet allow reasonable times of analysis.

The system should also be applicable to tritium counting. In this case the magnesium perchlorate should be replaced by a reagent such as calcium carbide to generate a permanent gas from the water produced by combustion of the sample. Greater amplification of the pulses would be necessary.

Counter efficiency. Comparison of the absolute count from a sample of labelled methyl stearate showed that 90 % of the counter volume was effective. By plotting counts for a known sample weight obtained at a variety of flow rates against the flow rate a straight line was obtained indicating complete mixing within the counter volume.

SUMMARY

A simple proportional flow counter for use with the gas chromatogram is described, which is operated at room temperature by burning all eluted material to CO_2 over heated copper oxide. The gas stream is dried, 5 % CO_2 is injected and the mixture passed into the counter. Details are given of the necessary circuitry. Examples are provided of its use with long chain fatty acids.

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Short Communications

Comptage de radiochromatogrammes par scintillation liquide

La mesure de la répartition de la radioactivité sur des chromatogrammes de substances marquées par le tritium peut être effectuée par différentes méthodes. Celle que nous utilisons depuis le plus longtemps[1] consiste en un découpage des sections du chromatogramme, élution dans les flacons de comptage de la radioactivité, évaporation du solvant d'élution, solubilisation du résidu dans le mélange scintillant précédée ou non de l'addition d'un sel d'ammonium quaternaire tel que l'hyamine*. Cette technique est quantitative: elle permet de compter la totalité des coups déposés; mais elle est extrêmement laborieuse.

Deux publications plus récentes[2,3] proposent d'immerger le segment de papier dans le mélange scintillant, soit dans un flacon spécial qui le maintient rigide, soit en l'introduisant simplement dans le flacon.

Compte tenu du prix du mélange scintillant, nous avons essayé de limiter le volume de celui-ci et sommes parvenus à la conclusion que ce volume n'est pas critique: le comptage dépend uniquement de l'imbibition du papier et de la transparence qu'il acquiert de ce fait. Les papiers sont donc introduits simplement** dans la fiole et imbibés de 2 ou 3 gouttes de mélange scintillant; le comptage est effectué dans un compteur à scintillation liquide***. Nous avons également vérifié que la différence de taille du segment de papier n'entraîne pas de modification du nombre de coups comptés. Les essais effectués ont permis de préciser la validité de la méthode. Le Tableau I reproduit quelques chiffres qui résument ces conclusions. La Fig. 1 illustre

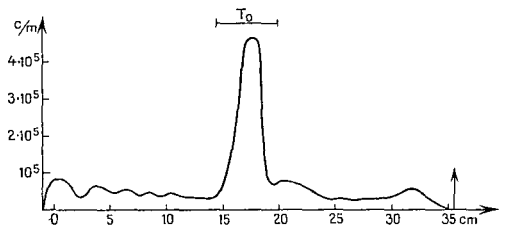

Fig. 1. Comptage par scintillation liquide d'un chromatogramme (solvant *n*-butanol–acide acétique) du produit brut de synthèse de la L-thyronine, marquée en 3,5 par ^3H (T_0).

* L'hyamine est utilisée chaque fois que la substance éluée du chromatogramme est insoluble dans le mélange scintillant. On utilise en général 0.2 ml d'une solution *M* d'hyamine dans le méthanol.

** La bandelette de papier, introduite dans le flacon s'applique contre la paroi cylindrique de celui-ci.

*** Packard Tri-Carb.

les résultats du comptage réalisé par cette nouvelle méthode d'un chromatogramme d'une préparation brute de thyronine marquée en 3,5 par le tritium. La proportionnalité est respectée à $\pm$ 3.5 %.

Nous avons pu, à partir de ces chiffres, calculer le rendement du comptage par rapport à la radioactivité d'un corps soluble dans le mélange scintillant, correction faite du "quenching". Dans le cas du papier immergé, le rendement est de 66 % alors

TABLEAU I

COMPTAGE PAR SCINTILLATION LIQUIDE
DE CORPS MARQUÉS PAR LE TRITIUM DANS DIFFÉRENTES CONDITIONS

Échantillon	*Témoins*	*Papier imprégné*		*Papier immergé*	
		trouvé	*calculé*	*trouvé*	*calculé*
1	8160	4203	—	5286	—
2	15977	8353	8406	10411	10572
3	24200	13054	12609	15393	15858

Influence de la taille du papier

Échantillon	*Surface du papier*	*Coups comptés*
1	2 × 1 cm	4272
2	3 × 1 cm	4186
3	5 × 1 cm	4152

qu'il est de 52 % pour le papier imprégné. Le rendement final du comptage est, compte tenu du compteur utilisé, de 13.2 % dans le premier cas et de 11.4 % dans le second.

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Reçu le 21 novembre 1960

Chromatography of some lipids on polytetrafluoroethylene

Chromatographic separations of vitamins A and D and cholesterol have previously been reported from this laboratory on columns of silaned kieselguhr[1] and polyethylene powder[2]. These studies have been extended using polytetrafluoroethylene as column packing.

Teflon (obtained from E.I. Du Pont, 60–100 mesh) was packed in methanol into a column 20 cm in length and 1.1 cm diameter using a perforated disc[3]. When packed, the column was held in a state of tension by a filter paper disc on top held down by a close fitting polyethylene ring. The column was then washed with 100 ml of degassed methanol. The methanol was displaced by water before applying the sample for analysis dissolved in 1 ml of methanol. The column was run using the gradient pump described by ARCUS[4] with 100 ml methanol in the reservoir and a mixture of 30 ml methanol and 20 ml water in the mixer. All solvents were carefully degassed before use. The column effluent was collected in 1.3 ml fractions. The ultraviolet absorption of each fraction was measured in the Beckman DU spectrophotometer at 325 mμ for vitamin A and at 265 mμ for vitamin D. Cholesterol and coprosterol were determined by the nephelometric method[2].

The separations obtained with 0.2 mg each of vitamins A and D and with 0.5 mg each of cholesterol and coprosterol are shown in Figs. 1 and 2.

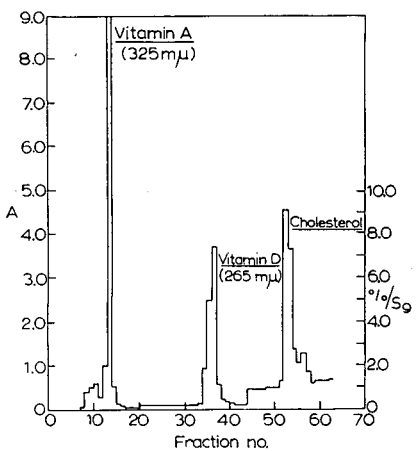

Fig. 1. Elution curve of vitamin A, vitamin D and cholesterol.

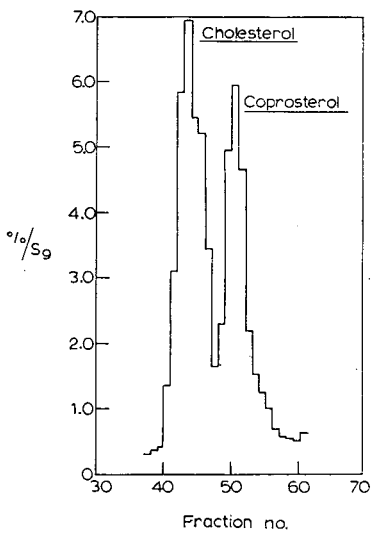

Fig. 2. Separation of cholesterol and coprosterol.

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Konzentrierung des Tropffleckes bei der Papierchromatographie von Corticosteroiden

Vom Gesichtspunkte der Gestaltung des Papierchromatogramms der aus biologischem Material (z.B. Lebergewebe, Nebenniere) stammenden Corticosteroide ist die Art und Weise des Auftragens des zu untersuchenden Tropfens äusserst wichtig. Beim Auftragen grösserer Tropfen ist die Separation der Substanzen eine unvollständige und die entstehenden Flecke sind verzerrt, z.B. halbmond- oder ringförmig, bzw. gestreckt. Die zur Konzentrierung des Fleckes verwendeten Methoden[1,2] sind ziemlich umständlich, da sie einer vorangehenden Chromatographie bedürfen. Es schien daher angebracht, nach neueren, einfachen Methoden zu suchen.

Sehr gute Ergebnisse erhielten wir bei der Chromatographierung der Corticosteroide—besonders aus biologischem Material—bei Anwendung eines Filtrierpapierringes. Wird dieser Ring—mit dem entsprechenden Solvens durchtränkt—so auf das Chromatographierpapier gelegt, dass der Fleck genau in seinem Mittelpunkt zu liegen kommt, so bewirkt das radiär in Richtung des Zentrums sich fortbewegende Solvens gleichmässige Verdichtung des Fleckes. Der Ring wird nur solange auf dem Papier belassen, bis die Front des Lösungsmittels den Mittelpunkt des Kreises erreicht, da das Lösungsmittel nur bis zu diesem Zeitpunkt strömt. Das Solvens ist richtig gewählt, wenn es das Untersuchungsmaterial (z.B. Steroide) während des Laufes auf dem Papier in der Front mit sich nimmt. Im Falle von Corticosteroiden dient als Solvens ein Gemisch von Äthylacetat–Methanol, 2:1. Hat das Solvens den Mittelpunkt des Kreises erreicht, so wird der Papierring entfernt und der Fleck mit Hilfe eines warmen Luftstromes (40°) getrocknet. Nun wird die Konzentrierung wiederholt, bis der Fleck homogen und klein ist.

Es ist ratsam, den Filtrierpapierring der leichteren Handhabung halber zwischen konisch verjüngte Glasröhren zu klemmen. Der Ring wird aus 2 × 40 cm langen, zuvor extrahierten (Äthylacetat) Filtrierpapierstreifen angefertigt.

Das mitgeteilte Verdichtungsverfahren gestattet auch kleinere Substanzmengen nachzuweisen, als es bisher möglich war und erlaubt eine exaktere Bestimmung der R_F-Werte, da in dem Chromatogramm die Flecken konzentriert und regelrecht kreisförmig angeordnet liegen.

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Eingegangen den 1. November 1960

BOOK REVIEW

Elektroforesa na papíře a v jiných nosičích (Electrophoresis on paper and in other carriers), by ČESTMÍR MICHALEC, JAROSLAV KOŘÍNEK, JAN MUSIL AND JIŘÍ RŮŽIČKA, Nakladatelství Československé akademie věd, Praha, 1959, 315 pages, 98 figs., price Kčs 34.50.

The value of this book lies in the well-balanced contents. It would be difficult to find a technique or a group of substances of some importance that are not included. This is the main respect in which it differs from some books of similar nature and size in which certain aspects of theory, technique or application predominate owing to personal preference of the author. For example, in this book only about 35 pages are devoted to plasma proteins (including a bibliography of selected papers concerning certain groups of diseases), so that it can be considered as being complementary to the more specialized works that deal mainly with plasma proteins, such as those of WUNDERLY, DITTMER and ENSELME AND DREYFUS. In the emphasis that is laid upon applications outside the protein field, this book resembles to a certain extent the earlier introduction by M. LEDERER, but inorganic separations are treated less extensively in the present book.

In the historical introduction, credit is given to KÖNIG for having published the first paper on this subject (1937); this seems to be correct as far as proteins are concerned, but in 1931 a report on inorganic separations by a sort of paper ionophoresis had already been published by DYACHKOVSKIĬ AND ISAENKO (*cf. Chem. Abstr.*, 26 (1932) 48).

The theoretical chapter by RŮŽIČKA (45 pp.) starts from the general principles of electrophoresis (conductivity of solutions, ionic charge, etc.) and proceeds to the main features specific for carrier electrophoresis, such as the effects of stabilizing media (electro-osmosis, tortuosity, "barrier effect", adsorption) and complications due to heating and evaporation. In the concise chapter on the general technique (25 pp.), MICHALEC discusses various types of equipment for low- and high-voltage electrophoresis, selection of paper and electrolytes, application of samples, detection and determination of substances, and some special techniques such as electrorheophoresis, counter-current continuous electrophoresis, two-dimensional separations, and radial electrophoresis.

Due attention is paid to the separation of low-molecular substances (MUSIL, 65 pages). Synthetic drugs are dealt with under a rather loose heading "Substances with biological activity". Eighty-seven pages are devoted to the separation of proteins (MICHALEC) under the title "Electrophoresis of high-molecular substances", although nucleic acids and most polysaccharides were included in the preceding chapter. Veterinary surgeons and experimental biologists would perhaps welcome more

detailed data in the article on animal sera, but it may at least serve as a bibliographic source. The author does not mention that the addition of mercuric chloride to ethanolic bromophenol blue staining solution for proteins is not necessary.

KOŘÍNEK has contributed a very interesting and stimulating chapter on electrophoresis in other carriers than paper (starch suspensions, starch gel, agar gel, etc.) and on preparative electrophoresis, which includes mainly column electrophoresis and the continuous descending technique (about 50 pp.). An excellent discussion is devoted to immunoelectrophoresis: both the technical details and the biological interpretations are dealt with critically in a very lucid way. The wide acceptance that KOHN's membrane filter electrophoresis has gained, seems to have come too late for it to be included.

In making a choice between alternative procedures, the authors have relied partly on their own experience, and partly on that of other workers. They have always taken pains to remain fair and objective and to avoid making a definite judgment upon questions that have not yet been unambiguously settled. Examples of this attitude may be found in all the chapters. Thus, for example, several methods for the estimation of corrected mobility are presented on equal terms, various modifications of column electrophoresis are described without preference being given to any of them, and none of the various methods of protein staining and quantitative evaluation are favoured, the choice of the procedure and the introduction of more or less individual correction factors for a given procedure being left to the individual worker.

Of the applications, only those that were considered to be of essential value are mentioned; the authors themselves acknowledge that such a choice is open to objections, but they have mostly hit the mark.

Bibliographic lists are given at the end of relatively short sections, the page on which they appear being indicated at the foot of every page, which facilitates their use. The total number of references amounts to about 1300 including some duplications. The literature up to the middle of 1958 has been covered. Subject and author indexes are included. Both printing and binding are excellent and the drawing on the dust-jacket is both technically correct and artistically competent. The price is moderate. Incorrect spelling, such as Draggendorf, Bräunniger or Natrii diethylbarbiturici, is an exception.

The present book is a well-balanced and systematically arranged account of techniques and applications, written in a clear, logical, and didactically competent manner.

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J. Chromatog., 5 (1961) 275–276

NEW JOURNAL

Zeitschrift für Chemie, published by VEB Deutscher Verlag für Grundstoffindustrie, Leipzig. Vol. 1 begins in 1960, 12 issues per year, price D.M. 3.00 per issue. This new journal contains review articles and short communications.

REVIEW

CHROMATOGRAPHY OF PORPHYRINS AND METALLOPORPHYRINS

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(Received December 12th, 1960)

CONTENTS

1. INTRODUCTION

In recent years, chromatographic methods have played an essential role in many of the diverse aspects of pyrrole pigment research: in the elucidation of the structure of haem *a*, the prosthetic group of cytochromes *a* and a_3; in the elucidation of the structure of haem a_2, the iron-chlorin prosthetic group of bacterial cytochrome a_2; in the definition of a number of the intermediate steps in porphyrin and haem biosynthesis and in the elucidation of the structure and isomeric form of a number of porphyrins including hepta-, hexa- and penta-carboxyl porphyrins, which have long been known to occur both in pathological materials and in biosynthetic experiments.

The chromatography of porphyrins was reviewed by the author in 1954[1].

Considerable advances have since been made, and the time seems opportune for a more detailed account of the techniques available. In view of the rapid expansion of research in this field, and of the needs of the non-specialist, an account is included below of some of the properties of these pigments, of methods for their isolation from natural sources and of various treatments which are often necessary preliminaries to their chromatography.

Some other review articles and books may be found useful: the chemistry and biochemistry of the porphyrins and metalloporphyrins is discussed fully by FALK[4] and their physical chemistry by PHILLIPS[5]; BÉNARD, GAJDOS AND GAJDOS-TÖRÖK[2] have treated in some detail the porphyrias and other aspects of the clinical pathology of these compounds; the comprehensive treatise of LEMBERG AND LEGGE[6] is invaluable in all aspects of the field up to 1949, and lastly, the organic chemical aspects of most of the pigments mentioned, and many others, are treated by FISCHER AND ORTH[7] and FISCHER AND STERN[8].

The chromatography of chlorophylls was reviewed in this Journal by ŠESTÁK[3] in 1958.

2. PROPERTIES, METHODS OF ISOLATION AND PRELIMINARY TREATMENTS

(a) *Acid–base properties and solubility*

1. *Free porphyrins*

The naturally-occurring porphyrins all possess carboxyl side-chains, and are thus ampholytes, soluble in alkali by virtue of the carboxyl groups and in mineral acids by virtue of the ring nitrogens. The isoelectric points of most common porphyrins lie between pH 3 and 4.5; in this pH range they may be precipitated from aqueous solutions. All porphyrins are soluble in glacial acetic acid, strong mineral acids, pyridine and related solvents and in dioxan, and less soluble in methyl and ethyl alcohols, acetone and ether. They are freely soluble in polar organic solvents containing acids, *e.g.*, acetone–HCl, ether–acetic acid or ethyl acetate–acetic acid mixtures, and they are soluble in aqueous solutions of anionic, cationic or neutral detergents. Porphyrins do not dissolve readily in ether from the solid state, and transference from aqueous phases to ether depends on vigorous dispersion with the ether immediately after bringing to the isoelectric point, before flocculation occurs. They may be extracted from ether by aqueous alkali or by aqueous mineral acids. Of the common porphyrins, uroporphyrins are the most water-soluble, and in fact can be brought into organic solvents under special conditions only (see Section 2. b).

HCl number or "Willstätter number". FISCHER AND ORTH[7] have recorded HCl numbers for many porphyrins. The HCl number is defined as that concentration of HCl (in % w/v) which from an equal volume of an ether solution of a porphyrin extracts two thirds of the porphyrin[10].

The "pH" number is defined as the pH of a buffer solution which extracts half of a porphyrin from four volumes of its ether solution[9].

The HCl number depends on both the dissociation of the porphyrin as a base,

and on the distribution coefficient of the free porphyrin and its hydrochloride between water and the organic solvent[11]. HCl numbers are commonly used to separate mixtures of porphyrins, and some approximate values are given in Table I.

TABLE I

HCl NUMBERS

	Free porphyrin	*Methyl ester*
Uro		1.5
Copro	0.08	1.5
Haemato	0.1	—
Deutero	0.3	2.0
Meso	0.5	2.5
Proto	2.5	5.5
Aetio	3.0	—
Chlorocruoro	4.6	—

* Uroporphyrins are not soluble in ether.

Some studies have been made[12–14] of the behaviour of porphyrins in countercurrent distribution between organic solvents and dilute hydrochloric acid solutions. Distributions coefficients have been determined for several porphyrins. These values are more precise than the HCl numbers.

2. *Porphyrin esters*

Porphyrins with carboxyl side-chains are readily esterified, and the esters hydrolysed, without degradation. The esters are more lipophilic than the free porphyrins, as indicated by their HCl numbers and their solubility in organic solvents. Unlike the free porphyrins, they are soluble in $CHCl_3$, CCl_4, C_6H_6, CS_2, etc. Purification of porphyrins is often carried out via the methyl esters, particularly when column chromatography is required. The esters crystallise well, having characteristic, though high, melting-points[7]. Mixed melting-point curves are available for the methyl esters of coproporphyrins I and III[15] and uroporphyrins I and III[16].

3. *Metal complexes*

The iron complexes of porphyrins have no basic nitrogen function and are thus insoluble in aqueous acids. They are soluble in alkali, pyridine, dioxan, and less so in alcohol; they can be brought into strong solution in ethyl acetate or ether via their ready solubility in acidified organic solvents. Protohaem and protohaemin (ferro- and ferri-protoporphyrin respectively) dissolve readily in cold concentrated sulphuric acid, which removes the iron. This treatment does not alter the porphyrin nucleus, but causes hydration of the vinyl side-chains to hydroxyethyl; thus protohaemin is converted to haematoporphyrin. Copper is removed from Turacin (copper-uroporphyrin) by similar treatment.

Esters of metalloporphyrins may be prepared in two ways: the carboxyl side-

chains of metalloporphyrins may be esterified, or the metal may be introduced into the esterified porphyrin. The esters have lost their alkali-solubility, have gained solubility in $CHCl_3$, CCl_4, etc., but otherwise have similar solubilities (*e.g.* pyridine, dioxan) to the unesterified compounds.

(*b*) *Extraction from natural materials*

Though metal-free porphyrins may associate with proteins, the binding is usually by electrostatic and van der Waals forces, and is easily broken; acidified organic solvents, such as mixtures of ethyl acetate or ether with glacial acetic acid, acetone with HCl, extract most free porphyrins quantitatively from tissues, at the same time precipitating the protein in a form which may be removed by filtration or at the centrifuge. The bonds between the apoprotein and the haem iron are also broken by these solvents, and most haems are extracted from tissues in the same way as porphyrins. Covalent bonds between haem and apoprotein, such as the thioether bonds in mammalian cytochrome *c*, are of course not broken by these treatments and other methods are required (*cf.* ref.[4]).

The grinding of biological material with glacial acetic acid, followed by admixture with ether and removal of the solids, was used extensively by FISCHER[7] and is particularly useful for extraction of porphyrins from tissues such as bile or faeces. For extraction of both haems and porphyrins from blood, body tissues and plant tissues, the extracting agents most widely used are either acetone containing from 1 to 5 % (v/v) of concentrated HCl, or ethyl acetate mixed with 25 % (v/v) of glacial acetic acid. The latter method was introduced by SCHWARTZ AND WIKOFF[17] and has been used extensively. The isolation of uro-, copro- and protoporphyrins, and haem, following initial extraction with ethyl acetate–acetic acid has been described in detail by DRESEL AND FALK[18]. Briefly, the protein-free extract is washed free of acetic acid, buffering during the washings with sodium acetate. If the pH of the aqueous phase is not permitted to drop below about 4, so that porphyrins of low HCl number (*e.g.* coproporphyrin) are not extracted, copro- and proto- and related porphyrins remain in the organic phase. Uroporphyrins are insoluble in most neutral organic solvents, and are found in the aqueous phase, from which they are recovered by extraction with cyclohexanone after acidification to pH 1.8[18,19]. They are eventually extracted from the cyclohexanone with aqueous HCl, and may then be chromatographed after evaporation of the HCl. The porphyrins remaining in the original organic phase are removed by extraction with HCl, transferred to ether, and removed from this stepwise with increasing concentrations of HCl, according to their Willstätter numbers. These fractions may then be transferred again to ether, when they are ready for chromatography. It is important that the ether used for these purposes be peroxide-free. Any haems extracted remain in the original organic phase after these treatments, and may be recovered, by evaporation, in a relatively pure state. Thus from blood, protohaem recovered in this way may be crystallized[20] without further purification, as may protoporphyrin dimethyl ester prepared from it[18].

(c) *Removal of iron from haemins*

A variety of methods[6,7] is available for the removal of iron; they all depend upon the fact that in these complexes, ferrous iron is more readily replaced by protons than is ferric iron. Most iron–porphyrin complexes and their derivatives are readily autoxidized, so that most processes for the removal of iron feature a reducing agent (*e.g.* iron powder, stannous chloride, sodium amalgam) and a proton-source (formic, oxalic, acetic, hydrochloric acids). On the small scale, a convenient and widely-used method is that of WARBURG AND NEGELEIN[21], in which the solvent is glacial acetic acid, the reducing agent ferrous acetate, and the proton-source concentrated HCl. The process has been modified and much improved by making use of ferrous sulphate in place of ferrous acetate[22].

(d) *Esterification and saponification*

Porphyrins readily form esters with alcohols; of these, methanol is most commonly used. The porphyrin is dissolved in anhydrous methanol saturated with gaseous HCl or, much more conveniently, in anhydrous methanol to which 5 % (v/v) concentrated sulphuric acid has been added. The solution is allowed to stand in the dark for about 24 hours at room temperature. This time and temperature are required for full esterification of, *e.g.*, uro- and coproporphyrins, but protoporphyrin in particular is fully esterified in 24 hours, and suffers less decomposition, at 0°. To the solution an equal volume of crushed ice is added, and as quickly as possible the ester is extracted into chloroform and the chloroform solution washed with water, with 2 *N* ammonia and finally with water until the washings are neutral. The chloroform is removed *in vacuo* and the porphyrin ester crystallized from a methanol–chloroform mixture. Traces of unesterified porphyrin, though they should be extracted by ammonia, sometimes persist. Free porphyrins do not move from the origin in paper chromatography under some conditions (see Section 3.a.2) and may thus be detected, as may also partly esterified porphyrins, which are sometimes soluble in chloroform. The methyl esters may be prepared also very conveniently, by reaction with diazomethane in ethereal solution.

The esters are saponified by standing at room temperature for about 40 hours in 7 *N* HCl.

(e) *Detection and determination*

1. *Spectra*

Some extinction coefficients for the more common compounds are given in Table II.

The spectrum of most common porphyrins in neutral solvents consists of four relatively sharps bands, increasing stepwise in intensity from band I, in the region of 620 mμ, to band IV, in the region of 500 mμ. Between bands I and II a very small band, Ia, occurs in some porphyrins. This type of spectrum in the visible region is called aetio-type. In addition, a band much more intense than band IV is found in the region of 400 mμ; this is the "Soret" band, characteristic of all conjugated

TABLE II

a. POSITIONS AND EXTINCTION COEFFICIENTS OF SORET BANDS

In chloroform Porphyrin ester	*Soret max. (mμ)*	$10^{-5}\varepsilon M$	*Mol. wt.*
Uroporphyrin III octamethyl ester	405–6	2.17	942
Coproporphyrin III tetramethyl ester	399.5	1.80	710
Deuteroporphyrin dimethyl ester	399	1.75	538
Protoporphyrin dimethyl ester	407.5	1.71	590

In aqueous HCl Porphyrin	*Concn. of HCl (N)*	*Soret max. (mμ)*	$10^{-5}\varepsilon M$	*Mol. wt.*
Uroporphyrin III	0.5	405.5	5.41	830
Coproporphyrin III	0.1	399.5	4.89	654
Deuteroporphyrin	0.1	398	4.33	510
Protoporphyrin	1.37	408	2.75	562

The values of λ and ε for uro- and copro-porphyrins I are identical to those of the corresponding isomers of series III. The data quoted are from RIMINGTON[23], who gives also correction factors, for use when porphyrins in tissue extracts are determined by measurement at the Soret band. The position and intensity of the Soret band vary with HCl concentration[24,25].

b. POSITIONS AND EXTINCTION COEFFICIENTS OF BANDS IN THE VISIBLE REGION (NEUTRAL SPECTRA)

Porphyrin	*Band: I*		*Ia*		*II*		*III*		*IV*	
	λmax (mμ)	$10^{-4}\varepsilon M$	λmax (mμ)	$10^{-4}\varepsilon M$	λmax (mμ)	$10^{-4}\varepsilon M$	λmax (mμ)	$10^{-4}\varepsilon M$	λmax (mμ)	$10^{-4}\varepsilon M$
Uroporphyrin	624	0.412	596	0.137	569	0.711	532	0.957	499	1.567
Coproporphyrin	621	0.515	595	0.132	567	0.672	529	0.997	497	1.470
Deuteroporphyrin	618	0.433	593	0.129	565	0.680	525	0.859	495	1.595
Protoporphyrin	630	0.558	603	0.141	575	0.678	537	1.158	503	1.464
Mesoporphyrin	620	0.541	594	0.133	567	0.659	528	0.982	496	1.424

The values given, for the methyl esters in dioxan solution, are from the papers of STERN AND WENDERLEIN (*cf.*[26]). For use with porphyrins dioxan must be purified[26].

tetrapyrroles, but lacking when the conjugation is broken as in the bile pigments.

Solutions of porphyrins in aqueous mineral acids have two bands in the visible region, characteristic of the porphyrin dication. The Soret band also is present, its maximum being found at longer wavelengths as the proton concentration increases[24,25].

Complexes of porphyrins with metals such as Ni, Co, Cu, have a Soret and two visible bands, with intensities I>II, while in complexes such as those with Zn, Cd, Mg, the relative intensities are reversed (*cf.* ref.[27]).

2. *Fluorescence*

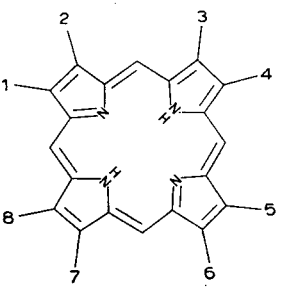
On irradiation with light at about 400 mμ (*e.g.* u.v. light filtered through Woods glass) porphyrins have a characteristic, very intense orange to red fluorescence. The fluorescence is quenched by some organic solvents, and by a variety of biological

compounds, and thus for quantitative determinations strict control of the conditions is necessary. The fluorescence is best observed in aqueous HCl solutions, in which less than 0.1 μg/ml is quite visible to the eye; very much lower concentrations are detectable by fluorimetry[28]. Metal complexes such as those with Cd, Zn, Mg fluoresce in some solvents, but the Co, Ni, Fe, Cu complexes, for example do not[5,27].

The detection[48] of spots on paper containing as little as 0.005 μg of porphyrins and 0.04 μg of non-fluorescent metallo-porphyrins, is described in Section 3.a.2, and the detection[82] of $3 \cdot 10^{-4}$ μg of haemins, by the use of a special benzidine spray, in Section 4.a.

TABLE III

THE SIDE-CHAINS OF SOME COMMON PORPHYRINS



	Side-chains							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Aetioporphyrin I	M	E	M	E	M	E	M	E
Coproporphyrin I	M	P	M	P	M	P	M	P
Coproporphyrin II	M	P	P	M	M	P	P	M
Coproporphyrin III	M	P	M	P	M	P	P	M
Coproporphyrin IV	P	M	M	P	M	P	P	M
Uroporphyrin I	A	P	A	P	A	P	A	P
Uroporphyrin III	A	P	A	P	A	P	P	A
Protoporphyrin IX	M	V	M	V	M	P	P	M
Deuteroporphyrin IX	M	H	M	H	M	P	P	M
Haematoporphyrin IX	M	B	M	B	M	P	P	M
Mesoporphyrin IX	M	E	M	E	M	P	P	M

Side-chain abbreviations: A = $-CH_2COOH$; B = $-CH(OH)CH_3$; H = $-H$; M = $-CH_3$; P = $-CH_2CH_2COOH$; V = $-CH=CH_2$.

The structures of many other porphyrins are given by FISCHER AND ORTH[7] and of chlorophyll derivatives by FISCHER AND STERN[8].

3. CHROMATOGRAPHY OF PORPHYRINS

There exist now a variety of methods for column- and paper-chromatography of porphyrins and haems. In some methods the free porphyrins are used, and in some the porphyrin esters. Column chromatograms serve most commonly for separations on the preparative scale, and paper chromatograms for identifications; the capacity of paper for porphyrin separations is rather low—spots of from 0.005 to 0.5 μg are

usually employed and are quite easily visible under ultraviolet light filtered through Wood's glass.

(a) *Paper chromatography*

1. *Free porphyrins*

One method of basic importance is the separation of free porphyrins in lutidine–water mixtures, when their R_F's bear a more or less linear relationship to the number of carboxylic acid side-chains they possess. The method, which was introduced by NICHOLAS AND RIMINGTON[29] in 1949, may be used with crude materials (blood fluids, urine, tissue and plant extracts; FALK, unpublished observations) and is thus useful for preliminary surveys of porphyrin content, though for more critical identifications isolation and some purification of the porphyrins is required. The solvent system used originally by NICHOLAS AND RIMINGTON[29] was the organic phase obtained by saturating a mixture of 2,4- and 2,5-lutidines (dimethylpyridines) with water at 21°, and the atmosphere was saturated with ammonia vapour (but see ref.[30]). The virtually linear relationship of R_F to the number of carboxyl groups in a porphyrin is illustrated in Fig. 1a. 2,4-Lutidine has been used similarly[33,34].

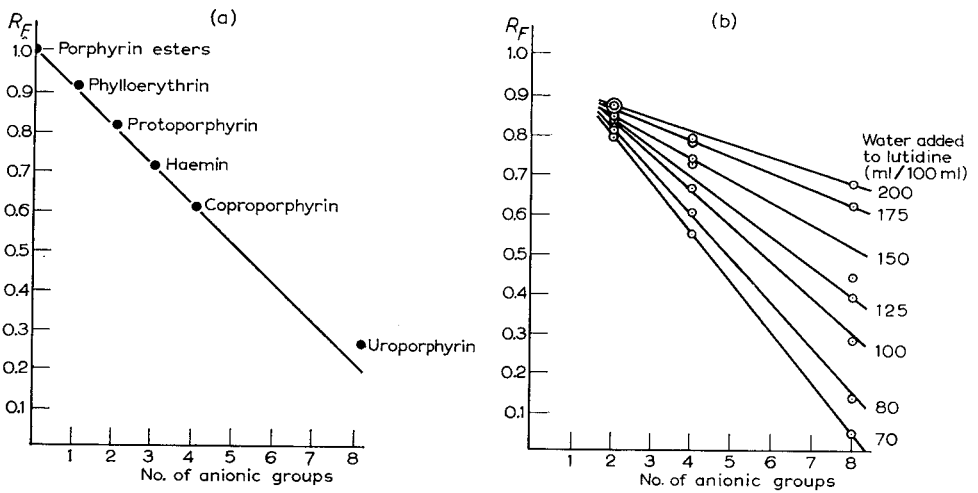


Fig. 1. Paper chromatography with 2,4-,2,5-lutidine–water systems. (a) Relationship between R_F and number of dissociable anionic groups per molecule, at 21°. (b) Effect of quantity of water present upon R_F, at 5°.

NICHOLAS AND RIMINGTON[30] have studied the effect of water content in this developing solvent, at a temperature (5°) below that at which 2,4-, 2,5-lutidine becomes miscible with water (17°). The relationship of R_F to number of carboxyl groups holds good over a wide range of water concentrations, R_F decreasing as water concentration decreases (Fig. 1b).

For the use of this method at temperatures around 20°, well-controlled constant temperature rooms are essential, since the solubility of water in the lutidine phase is very temperature-sensitive. This difficulty was overcome by KEHL AND STICH[31]

TABLE IV

R_F VALUES IN 2,4-, AND 2,4-,2,5-LUTIDINE–WATER SYSTEMS

	R_F*	R_F**
Uroporphyrin	0.3	0.19
Coproporphyrin	0.6	0.47
Haemin	0.68	0.63
Protoporphyrin	0.8	0.75
Haematoporphyrin	0.8	0.77
Deuteroporphyrin	0.8	0.79
Mesoporphyrin	0.8	0.81
Phylloerythrin	0.8	—
Porphyrin esters	1.0	1.0

* NICHOLAS AND RIMINGTON[29], 2,4-,2,5-lutidine–water, Whatman No. 1 paper, 19°, descending, NH_3 vapour (but *cf.* ref. [30]).
** KEHL AND STICH[33], 2,4-lutidine–water, Schleicher & Schüll paper 2043b, 15°, ascending, NH_3 vapour.

who in 1951 introduced the use of 2,6-lutidine, which is miscible with water up to 40°. The method was studied further by ERIKSEN[32]; ascending chromatograms were used, at 21°, with NH_3 vapour in the atmosphere, and the solvent was a mixture of 5 vols. of 2,6-lutidine with 3 vols. of water. The R_F values found by KEHL AND STICH and by ERIKSEN are shown in Table V.

TABLE V

R_F VALUES IN 2,6-LUTIDINE–WATER SYSTEMS

	R_F*	R_F**
Uroporphyrin	0.26	0.06
Pentacarboxylic porphyrin	—	0.42
Coproporphyrin	0.54	0.56
Haemin	0.7	—
Tricarboxylic porphyrin	—	0.68
Protoporphyrin	0.84	0.83
Mesoporphyrin	0.86	—
Haematoporphyrin	0.87	—
Deuteroporphyrin	0.88	—
Aetioporphyrin	1.0	—
Porphyrin esters	1.0	—

* KEHL AND STICH[31], Schleicher & Schüll paper 2043b, 25°, ascending, NH_3 vapour.
** ERIKSEN[32], Whatman No. 1 paper, ~ 20°, ascending, NH_3 vapour.

It was found by FALK AND BENSON[49] that the 2,6-lutidine method, in addition to separating porphyrins in accordance with the number of carboxyl groups, is capable of separating mixtures of the coproporphyrin isomers (Fig. 2), and it has been used for this purpose[1,14,35,36]. The method has been modified further by ERIKSEN[37]. Isomers I and III, which occur in nature, and isomer II are well separated. Isomer IV which, like isomer II, is not known to occur in nature, has an R_F identical to that of isomer III.

Similar isomer-separation was then found with the NICHOLAS AND RIMINGTON method; these separations, though intrinsically very useful, of course preclude strict interpolation of the R_F's of unknown porphyrins in the curve of R_F against number of carboxyl groups. Happily, the isomers of uroporphyrins I and III do not separate under these conditions, and isomers of dicarboxylic (proto-, deutero-) and of tricarboxylic porphyrins, other than those related to coproporphyrin III, are not known

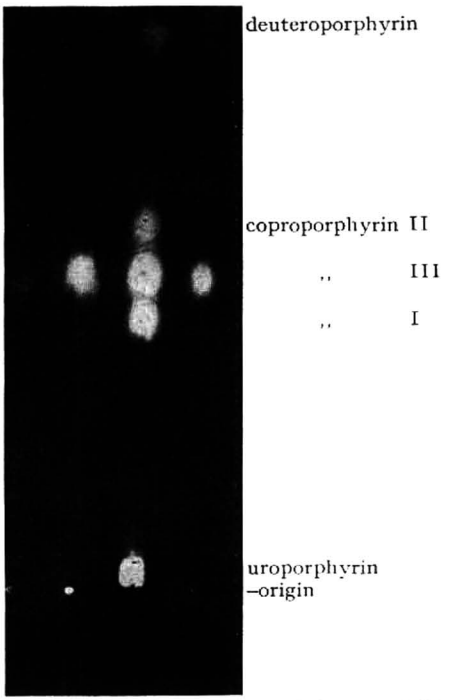


Fig. 2. Separation of porphyrins, including coproporphyrin isomers I, II and III, by 2,6-lutidine[14]. A photograph of a chromatogram under u.v. light.

to occur in nature. The region of the chromatograms in which interpretations must be made with most caution is thus where R_F values appear to correspond to penta-, hexa- or heptacarboxyl porphyrins; the identification of isomers among these porphyrins is discussed in Sections 3.a.2 and 3.b, below.

The paper used most widely for these separations has been Whatman No. 1; a number of investigators in Europe have found that Schleicher and Schüll paper 2043b gives similar results. Spots containing about 0.1 to 0.3 μg of porphyrin are applied to the paper by a micropipette, usually from a solution in dilute NH_4OH, though solutions in ether or acetone are equally suitable. High salt concentrations interfere with the chromatography.

WITH[38] has used instead of lutidine–water mixtures, neutral aqueous salt solutions as developing solvents. In an ascending method, with Whatman No. 1 paper,

using 0.1 *M* LiCl as developing solvent with an atmosphere containing NH_3 vapour, he has found the R_F's shown in Table VI. The separation according to number of carboxyl groups occurs in the reverse order to that found with lutidine developments and the times required are much shorter.

TABLE VI

R_F VALUES IN A LiCl–WATER SYSTEM

	R_F*
Haematoporphyrin	0.05–0.09
Coproporphyrin	0.20–0.30
Hexacarboxylic porphyrin	0.45–0.55
Uroporphyrin	0.85–0.95

* WITH[38], Whatman No. 1 paper, 0.1 *M* LiCl, ~ 20°, ascending, NH_3 vapour.

Some other methods have been reported. Thus absolute methanol has been used as developing solvent by NISHIKAWA[39], who reported separation of coproporphyrins I and III, and that the logarithm of the concentration was proportional to the area and diameter of the porphyrin spots.

A simplified procedure, adapting the lutidine method for use with paper strips in test tubes, and using simple HCl-eluates of lead acetate precipitates of urines, has been described by CORWIN AND ORTEN[40]. Several authors have adapted the methods to radial (circular) paper chromatography, for which a very convenient apparatus has been described by RAPPOPORT, CALVERT, LOEFFLER AND GAST[41]; R_F values have been given (Table VII) for a number of chlorophyll derivatives chromatographed in this way with mixtures of acetone, benzene and hexane, by HENDRICKSON, BERUEFFY AND MCINTYRE[42].

The paper chromatographic identification of some porphyrins from a petroleum aggregate has been described by DUNNING AND CARLTON[43]. A mixture of butanol, glacial acetic acid and water (4:1:1) has been found useful by URATA AND KIMURA[44] for paper chromatographic studies of the porphyrins formed enzymically from porphobilinogen. An apparatus for paper chromatography of porphyrins has been described by KENNEDY[45]; a number of paper strips may be run simultaneously, and the positions of spots may be monitored, during development, by their fluorescence in u.v. light.

2. *Porphyrin esters*

In 1951, CHU, GREEN AND CHU[46], using the porphyrin methyl esters and development first by a chloroform–kerosene mixture, then by a mixture of kerosene and *n*-propanol, achieved the first separation of porphyrin isomers on paper. The coproporphyrins I and III separated as shown in Table VIII, and by the same method the esters of uroporphyrin I, protoporphyrin and mesoporphyrin also were separated. As in the separation of the free porhyrins by lutidine (above) there is again an approximate

TABLE VII

CHROMATOGRAPHIC CONSTANTS OF SELECTED CHLOROPHYLL DERIVATIVES*

Compound	*Solvent***	R_R *(3000 sec)*	R_F *(3000 sec)*
Pheophytin *a*	10:20:70	0.966	0.933
Pheophytin *b*	10:20:70	0.855	0.731
Pheophorbide *a*	30:70	0.784	0.614
Pheophorbide *a* methyl ester	10:20:70	0.908	0.824
Pheophorbide *b*	30:70	0.570	0.324
Pheophorbide *b* methyl ester	10:20:70	0.753	0.568
Chlorin e_6	30:70	0.355	0.126
Chlorin e_6 trimethyl ester	10:20:70	0.933	0.871
Chlorin p_6 trimethyl ester	10:20:70	0.939	0.881
Cu-chlorin p_6 trimethyl ester	10:20:70	0.879	0.772
Purpurin 7*a* trimethyl ester	10:20:70	0.878	0.771
Purpurin 7*b*	30:70	0.613	0.375
Purpurin 7*b* trimethyl ester	10:20:70	0.642	0.412
Cu-purpurin 7*b* trimethyl ester	10:20:70	0.584	0.341
Purpurin 18*a*	30:70	0.755	0.569
Cu-purpurin 18*a* methyl ester	10:20:70	0.722	0.521
"Unstable" rhodin (rhodin *k*) trimethyl ester	10:20:70	0.852	0.725
Rhodin *g* trimethyl ester	10:20:70	0.771	0.595

* HENDRICKSON *et al.*[42], circular chromatography, Whatman No. 3 paper, ~ 23°. For definitions of R_R and R_F see original paper. For the structures of the chlorophyll derivatives referred to here and in Table IX, the original papers, and also FISCHER AND STERN[8] should be consulted.
** 10:20:70, proportions of acetone–benzene–hexane. 30:70, proportions of acetone–hexane.

TABLE VIII

R_F VALUES OF PORPHYRIN METHYL ESTERS*

	Second run, n-propanol with:				
	*kerosene***	*n-decane****	*n-dodecane****	*n-tetradecane****	*n-hexane****
Uroporphyrin I	0.17	0.14	0.20	0.13	0.15
Coproporphyrin I	0.47	0.42	0.52	0.45	0.47
Coproporphyrin III	0.67	0.70	0.76	0.74	0.66
Protoporphyrin IX	0.84	0.86	0.92	0.92	0.89
Mesoporphyrin IX	0.89	0.92	0.96	0.95	0.93

Whatman No. 1 paper, 24°, ascending; the paper was dried at 105–110° for about 4 min after completion of the first run. The atmosphere was saturated with the same solvents as used for development.
* CHU *et al.*[46].
** First run, chloroform–kerosene, 2.6:4.0.
*** First run, chloroform with the same alkane as used in the second run. Propanol–alkane, 1:5.

inverse relationship between R_F and the number of carboxyl groups present. In place of kerosene, *n*-decane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane were all found to effect satisfactory separations, but the intensity of fluorescence of the spots was found to be less than when kerosene was used. It was found that isopropanol may be used in place of the *n*-propanol, that changes in temperature affect the R_F values

but not the relative sequence of R_F's of the different porphyrin esters, and that the porphyrin free acids do not move with these organic developing solvents. The papers were not dried before use, and in the writer's experience it is not necessary to dehydrate the solvents rigorously—ordinary distilled, pure solvents are suitable. It is thus possible that the separations involve both partition on the hydrated cellulose and adsorption. CHU *et al.* used Whatman No. 1 paper; the method has been found[47] to work similarly with paper 2043b of Schleicher & Schüll.

This method has been modified by BLUMER[48], for the purpose of studying the porphyrin compounds occurring in petroleum and in sedimentary rocks. BLUMER found that using Whatman No. 3 paper, running with the grain, and developing with a mixture of iso-octane (3 vols.) and carbon tetrachloride (7 vols.), the preliminary development introduced by CHU *et al.* was not necessary for good resolution. Porphyrin esters and porphyrin metal complexes, and porphyrins lacking carboxyl groups (*e.g.* aetioporphyrin) all move away from the origin and separate adequately. Porphyrins with free carboxylic acid groups (*e.g.* proto-, meso-porphyrins) remain at the origin; this spot may be esterified on the paper with diazomethane, and then developed in the second dimension with the same solvent. Some R_F values found by BLUMER are shown in Table IX. It was found also that spraying the completed

TABLE IX

R_F VALUES OF FREE PORPHYRINS, THEIR METAL COMPLEXES AND METHYL ESTERS*

	R_F
Aetioporphyrin III	0.63
Deoxophylloerythroaetioporphyrin–nickel	0.54
Deoxophylloerythroaetioporphyrin	0.47
Deoxophylloerythroaetioporphyrin–vanadium	0.20
Deoxophylloerythrin ester–nickel	0.23
Deoxophylloerythrin ester	0.15
Deoxophylloerythrin ester–vanadium	0.08
Mesoporphyrin ester–nickel	0.12
Mesoporphyrin ester	0.10
Protoporphyrin ester–copper	0.13
Protoporphyrin ester–nickel	0.10
Protoporphyrin ester	0.07

* BLUMER[48]; horizontal development on Whatman No. 3 paper, with carbon tetrachloride–iso-octane, 70:30.
See also footnote* to Table VII.

chromatograms with iso-octane before observation under u.v. light increased markedly the sensitivity of the fluorescence, 0.005 μg being observable. For spots of non-fluorescent porphyrin metal complexes, it was found that on spraying with a solution of fluoranthene in *n*-pentane, and then illuminating with light at 366 mμ, the porphyrins and their metal complexes showed as dark spots against a fluorescent background, about 0.04 μg being observable. The maximum amount of porphyrin or metal complex that could be chromatographed without tailing was 0.5 μg. It was found that with care, determinations by measuring the area of spots could be made to

an accuracy of ± 10%, and a calibration curve for the determination of mesoporphyrin in this way is given.

FALK AND BENSON[49] found that isomers I and III of the uroporphyrins (methyl esters) did not separate under the conditions of CHU *et al.*[46], and introduced the use of dioxan for this purpose. Again a double development, kerosene–chloroform followed by kerosene–dioxan, with Whatman No. 1 paper at 22°, was used. As far as the uroporphyrins are concerned, the main function of the preliminary development is to move the esters away from the origin, leaving behind both impurities and any non-esterified materials; the paper is cut off above this level before the second development. This procedure, a single development with kerosene–chloroform, serves incidentally as a convenient test for the presence of unesterified porphyrins in preparations of the esters. Separation by the dioxan method of mixtures of uroporphyrins I and III permits a certain assessment of the proportions of the isomers present (see Fig. 2 of ref.[49]); it was apparent, however, that uroporphyrin I tends to be carried along with isomer III when considerable proportions of the latter are present. It has been found[50] that the resolution is much improved if the amount of porphyrin ester analysed does not exceed about 0.3 μg. Using approximately 1:1 mixtures of ^{14}C-labelled uroporphyrins I and III, BOGORAD AND MARKS[51] have found that not only is uroporphyrin I entrained with uroporphyrin III, but that isomer III is held up in the spot of isomer I, though when chromatographed separately the two isomers moved cleanly to their respective R_F positions. There has long been evidence for some kind of "molecular compound" formation between porphyrins of this type[16], and some controversies in the literature have resulted. While entrainment in paper chromatograms of a slower-moving substance in the spot of a faster-moving one is not uncommon, the virtually equal partition between the two positions found by BOGORAD AND MARKS appears to be a new phenomenon. The method of FALK AND BENSON[49] has been used widely and successfully, but there is real need for a new method which does not suffer from this complication. In addition, there is need for a method which separates the uroporphyrin isomers II and IV; under the conditions described above, isomers I and II have identical R_F values, as do isomers III and IV, and no conditions have yet been found to separate them. Though it is unlikely that isomers II and IV should occur in nature, this is not impossible (*cf.* ref.[52]).

The kerosene–dioxan chromatograms have proved valuable for the study of a porphyrin which may be important for the elucidation of the biosynthetic pathway[14,53], and which has been called *pseudo*uroporphyrin because of its chemical similarity to a uroporphyrin[14,36]. Chromatographically, it differs from all four uroporphyrin isomers; further decarboxylation of it led to coproporphyrin III, thus establishing its isomeric form[36]. A porphyrin behaving similarly, and occurring in certain porphyric urines, has been studied by CANIVET AND RIMINGTON[54], and another by GRINSTEIN, SCHWARTZ AND WATSON[55]. The methyl ester of the latter has a melting point of 208°, and chromatography has shown that it differs from *pseudo*uroporphyrin[36,56]. A recent study[57] suggests that these porphyrins may be mixtures of isomeric heptacarboxylic porphyrins.

3. *Dicarboxylic porphyrins*

While most of the methods described above separate porphyrins or their esters according to the number of carboxyl side-chains they possess, they do not effect very useful separations from each other of the common dicarboxylic compounds such as meso-, proto-, haemato- and deutero-porphyrins. A method which serves this purpose, found by CHU AND CHU[58], involves development of the porphyrin esters in a first direction, against the grain of Whatman No. 1 paper, with a mixture of kerosene, tetrahydropyran and methyl benzoate. The paper is then dried, treated with silicone in petroleum ether solution, dried again, and developed in the second direction with a mixture of water, acetonitrile, *n*-propanol and pyridine. The second development is "reverse phase" chromatography, silicone serving as the stationary phase and the atmosphere being saturated with water vapour. For some purposes, the second development may be carried out with a mixture of water, acetonitrile and dioxan. Very good separations of a number of dicarboxylic porphyrins were effected, as shown in Table X.

TABLE X

R_F VALUES OF THE METHYL ESTERS OF SOME DICARBOXYLIC PORPHYRINS*

Methyl esters of	R_F			*Minimal detectable amount*	
	*KTM***	*WAPP****	*WAD*†	*Two-dimensional development* μg	*Single development* μg
Haematoporphyrin IX dimethyl ether	0.59	0.63	0.46		
Deuteroporphyrin IX	0.59	0.45	0.46	0.01	0.005
Mesoporphyrin IX	0.59	0.23	0.23	0.01	0.005
Protoporphyrin IX	0.51	0.23	0.24	0.02	0.01
Monovinylmonohydroxyethyl-deuteroporphyrin IX	0.20	0.74	0.61		
Haematoporphyrin IX	0.03	0.80	0.71	0.005	0.003

* CHU AND CHU[58], Whatman No. 1 paper, 22°, ascending. For vapour phases, see original paper.
** KTM, 5:1.4:0.35, kerosene–tetrahydropyran–methyl benzoate.
*** WAPP, 3.8:1:2:0.5, water–acetonitrile–*n*-propyl alcohol–pyridine (with silicone as stationary phase).
† WAD, 2.3:2.8:0.8, water–acetonitrile–dioxan (with silicone as stationary phase).

4. *Porphyrins with hydroxyl groups in the side-chains*

This method was devised by BARRETT[59] for studies of porphyrin *a* and chlorin a_2, obtained by removal of iron from the prosthetic groups of the cytochromes *a* and a_2 respectively, and its use revealed that each of these porphyrins contains one side-chain with a hydroxyl group. The methyl esters of the porphyrins, and the same compounds after acetylation, were chromatographed by the method of CHU, GREEN AND CHU[46]. As may be seen in Table XI, the differences in R_F between free hydroxyl compounds and their acetylation products give some indication of the number of hydroxyl side-chains.

TABLE XI

R_F VALUES OF HYDROXYLATED PORPHYRINS AND CHLORINS, AND THEIR ACETYLATED PRODUCTS*

	Chloroform–kerosene, 2.6: 4		*Propanol–kerosene, 1: 5*	
	Alcohol	*Acetate*	*Alcohol*	*Acetate*
Monohydroxyethyl deuteroporphyrin	0.34	0.64	0.38	0.68
Monovinyl-monohydroxyethyl deuteroporphyrin	0.29	0.60	0.34	0.66
Haematoporphyrin	0.03	0.56	0.18	0.57
Monohydroxymethyl deuteroporphyrin	0.19	0.54	0.24	0.57
Monohydroxymethyl monovinyl deuteroporphyrin	0.22	0.54	0.31	0.56
Dihydroxymethyl deuteroporphyrin	0.01	0.56	0.14	0.56
2-Formyl-4-hydroxyethyl deuteroporphyrin	0.16	0.58	0.26	0.64
Porphyrin *a*	0.10	0.56	0.26	0.62
2-Ethyleneglycol deuteroporphyrin	0.30	0.64	0.32	0.61
2-α-Hydroxymesorhodochlorin	0.31	0.80	0.34	0.78
2-α-Hydroxymesochlorin p_6	0.33	0.82	0.35	0.80
2-α-Hydroxymesophaeophorbide *a*	0.10	0.78	0.34	0.72
Chlorin a_2	0.30	0.65	0.40	0.60

* BARRETT[59]; chromatography by the method of CHU *et al.*[46]. R_F values for some further chlorins are given by BARRETT[60].

(*b*) *Column chromatography*

Talc columns were used for purification of porphyrin esters by FISCHER AND HOFMANN[61], and for free porphyrins by COMFORT[62], who used aqueous HCl for the elution.

Columns of Al_2O_3 were used with some success for the separation of porphyrin esters by WALDENSTRÖM[63], PRUNTY[64], LEDERER AND TIXIER[65] and MCSWINEY, NICHOLAS AND PRUNTY[66], while $CaCO_3$ columns were used by GRINSTEIN, SCHWARTZ AND WATSON[55], WATSON, SCHWARTZ AND HAWKINSON[67] and GRAY AND HOLT[68].

The technique was studied systematically by NICHOLAS[69] in 1951. The adsorbants Al_2O_3, MgO, $MgCO_3$ and $CaCO_3$ were each prepared in several grades of adsorptive power, classified by the method of BROCKMANN AND SCHODDER[70] (*cf.* WILLIAMS [71]). The separation upon such columns of the methyl esters of uroporphyrins, coproporphyrins, protoporphyrin and some other dicarboxylic porphyrins was shown to be reproducible provided that the conditions are standardized. As in paper chromatography, porphyrins separate on columns in order of the number of carboxyl side-chains they possess. Thus on Al_2O_3 or $CaCO_3$, the ease of elution decreases from proto- to copro- to uroporphyrin (2-, 4-, 8-carboxyl groups respectively). This order is reversed on MgO or $MgCO_3$ columns. It is very convenient in practice to have the choice of reversal of order of elution; thus with a mixture of porphyrins, it is convenient to choose for the first separation that column from which the porphyrin(s) required pure are eluted easily, leaving others behind; the fractions so obtained are then purified separately on columns on which they are more strongly adsorbed, so that traces of easier-eluted impurities may be removed. A wide choice of organic solvent systems is available for the different columns, as indicated in Fig. 3.

Columns of $CaCO_3$ were used by GRINSTEIN *et al.*[55] for the isolation of the porphyrins from pathological urines, including their "208" porphyrin. The same sample of

$CaCO_3$ (kindly made available by Dr. C. J. WATSON) allowed the separation from uroporphyrin III of the *pseudo*uroporphyrin of DRESEL AND FALK[53], but no other samples tried were effective. The constitution of these two porphyrins has been discussed in Section 3.a.2.

Calcium carbonate columns, developed with benzene, are used also in a standard purification of coproporphyrin III tetramethyl ester[72].

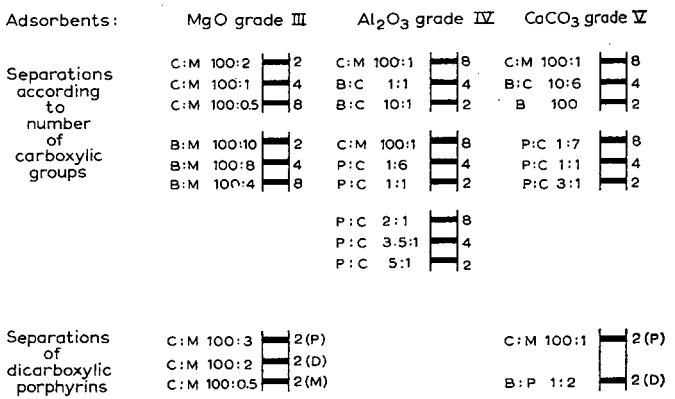


Fig. 3. Column chromatography of porphyrin methyl esters, from the data of NICHOLAS[69]. *Abbreviations.* Solvents: B, benzene; C, chloroform; M, methanol; P, light petroleum. Porphyrins: 8, 4, 2 represent the octa-, tetra- and di-carboxylic uro-, copro- and proto-porphyrins respectively. P, D and M represent the dicarboxylic proto-, deutero- and meso-porphyrins.

It was found by NICHOLAS[69] that the esters of dicarboxylic porphyrins may be separated from each other either on MgO grade III or on $CaCO_3$ grade V, the ease of elution increasing from proto- to deutero- to meso-porphyrin (Fig. 3).

Preparations of porphyrin esters are often contaminated by small amounts of the free (non-esterified) porphyrins, which remain at the top of columns developed with these organic solvents and may be recovered and re-esterified.

It has been found by the writer that metal complexes of the porphyrin esters may be purified conveniently on columns of Al_2O_3 or of $MgCO_3$, on which they are readily separated from metal-free porphyrins. Certain metal complexes, however (*e.g.* Zn, Cd) lose their metal on Al_2O_3 columns (*cf.* ref.[27]).

LEMBERG AND PARKER[73] have reported the separation, on columns of BDH "alumina for chromatographic separation", of proto- (2,4-divinyl-), chlorocruoro- (2-formyl-4-vinyl-) and 2,4-diformyl-deuteroporphyrins, and of the very closely related 2- and 4-monoformyl-deuteroporphyrins. The columns were packed as a slurry in a mixture of equal volumes of chloroform and ether, and the same mixture used for development.

Separations on the above columns are clearly due to adsorptive forces. A method of partition chromatography, in which porphyrin esters are chromatographed on columns of hydrated silica gel, with development by mixtures of chloroform and ligroin, has been worked out by LUCAS AND ORTEN[74]. When 1 mg of a mixture con-

taining equal amounts of the methyl esters of proto-, meso-, copro- and uro-porphyrins was applied to a column made from 1 g of silica gel, complete elution of the protoporphyrin was achieved with 100 ml of ligroin containing 20% (v/v) of chloroform. A further 50 ml of the same mixture then removed the mesoporphyrin; 100 ml of 30% chloroform in ligroin eluted the coproporphyrin, and finally 100 ml of 50% chloroform in ligroin eluted the uroporphyrin.

The order of elution is similar to that described by NICHOLAS[69] (see above) for columns of Al_2O_3 or $CaCO_3$, and again unesterified material remains at the top of the column. The silica gel columns have the advantage, however, of much higher capacity than the adsorption columns; for chromatography of 1 mg of a porphyrin mixture on the latter, a column size of the order of 2 × 20 cm at least, is required.

Silica gel columns, and also cellulose powder columns developed with ether, petroleum ether and acetone, have been used successfully by LEMBERG AND STEWART[75] for the purification of porphyrin *a* from heart muscle.

None of the methods of column chromatography so far described is capable of separating mixtures of porphyrin isomers. Several such separations claimed in the earlier literature have not been confirmed, but recently CHU AND CHU[50,76] have found that on columns of celite (Hyflo supercel) not only may porphyrin esters be separated in order of their carboxyl groups but also, under appropriate conditions, uroporphyrins I and III, and also penta- hexa- and hepta-carboxylic porphyrins of isomer-types I and III may be separated.

For the preliminary separation according to carboxyl groups, a solution of about 0.5 g of a mixture of the porphyrin esters is mixed with a little supercel and dried; this is then packed on the top of a column of supercel of dimensions 1.8 × 14 cm. Development is carried out, with suction, with a mixture of 1 vol. of chloroform and 2 vols. of petroleum ether (b.p. 30°–60°). The fluorescent zones are then removed by spatula from the column and analysed by paper chromatography. The lower the number of carboxyl groups, the faster the migration. For the isomer-separation, a column of about 3 × 45 cm of supercel is used for 2 to 3 mg of porphyrin ester mixture; the column is developed with benzene, then with a mixture of chloroform (1 vol.) and benzene (2 vols.) with increasing additions of ethanol, until the porphyrin front almost reaches the bottom of the column. The fluorescent zone is then removed by spatula in equal arbitrary fractions, the porphyrin eluted from the supercel with chloroform, and analysed by paper chromatography by the method of FALK AND BENSON[49] with some modifications[50]. Pure uroporphyrin III was found towards the top of the column, and pure uroporphyrin I at the bottom, separated by a graded mixture of the two isomers. The purity of the uroporphyrins I and III so obtained was confirmed[77] by their melting points, by melting points and chromatography (CHU *et al.*[46]) of the coproporphyrins obtained from them by decarboxylation and by the use of infra-red spectra.

As has long been evident (see refs. 1–12 of ref.[78]) porphyrins with 3, 5, 6 and 7 apparent carboxyl groups occur in various pathological materials as well as in biosynthetic preparations. CHU AND CHU[57,77,78] have been able to prepare, by stepwise

decarboxylation of uroporphyrins I and III, reference samples of hepta-, hexa- and penta-carboxylic porphyrins of both isomeric types. Natural materials isolated from the urine of patients with porphyria cutanea tarda have been compared with the reference substances in respect to behaviour on the supercel columns, paper chromatography, melting points and infra-red spectra, all the evidence confirming the existence in the natural samples of isomeric types I and III in these "intermediate" porphyrins.

Cellulose powder columns have been used by ERIKSEN[79] for the large-scale separation of porphyrins. A mixture of uro-, copro- and meso-porphyrins, as the free acids, was dissolved in a small volume of acetone containing NH_4OH; the solution was mixed with a little cellulose powder (Whatman "for Chromatography") and dried in a desiccator. The dry powder was packed on top of a 15 cm column of the same cellulose powder, packed dry by tamping. Development by 2,6-lutidine–water (6:2) separated the porphyrins with the approximate R_F values: meso-, 0.95; copro-, 0.53; uro-, 0. Further development with the same solvent, under slight positive pressure, caused elution of the mesoporphyrin. On development now with 2,6-lutidine–water (6:4) the coproporphyrin was eluted, and the uroporphyrin was finally eluted as a narrow, concentrated band, when a drop of concentrated NH_4OH was added to 25 ml of the same solvent. The behaviour of the porphyrins and the R_F values found on these columns are very similar to those found on paper chromatography with similar solvents (see above). Attemps at isomer-separation on such columns have not been reported.

4. CHROMATOGRAPHY OF IRON COMPLEXES OF PORPHYRINS (HAEMS)

(a) *Paper chromatography*

CHU AND CHU[80] have described methods for the separation of haemins or their methyl esters, in reverse phase systems in which Whatman No. 1 paper coated with silicone furnishes the stationary phase. A number of solvent systems were found to be useful (Table XII), the most interesting involving development of the free haemins with a mixture of water, *n*-propanol and pyridine. This system gave good separation of uro-, copro- and proto-porphyrins according to the number of carboxyl groups, and in addition separated from each other the dicarboxylic haemato-, deutero-, meso- and proto-haemins. Spots as small as 0.05 μg were detectable under u.v. light.

Using a similar silicone-treated Whatman No. 1 paper, MORRISON AND STOTZ[81] were able to separate protoporphyrin, its mono-and di-methyl esters, haem *a* and haematohaem *c*. The two latter were prepared from the prosthetic groups of cytochromes *a* and *c* respectively, and each separated, on the paper chromatograms, into two components. The best solvent system of the four mixtures used by these authors appears to have been a mixture of chloroform, hexane and formic acid, though they found basic solvents (*e.g.* pyridine–water or pyridine–isopropanol–ammonia) to be useful for particular purposes. In 1958, CONNELLY, MORRISON AND STOTZ[82] reported

the use of several other solvent systems for similar separations, a mixture of 8 vols. of glacial acetic acid in 100 vols. of water-saturated toluene having particularly good resolving power for heart muscle haemins.

TABLE XII

R_F VALUES OF HAEMINS*

Haemin	R_F**	R_F***
Proto-	0.34	0.76
Meso-	0.45	0.77
Deutero-	0.62	0.76
Haemato-	0.72	0.77
Copro-	0.88	0.56
Uro-	0.96	0.20

* CHU AND CHU[80], Whatman No. 1 paper treated with silicone, 22°, ascending.
** Developing solvent mixture: water–*n*-propanol–pyridine, 5.5:0.1:0.4; atmosphere, water and pyridine vapours.
*** Developing solvent mixture: 2,6-lutidine–water, 3.3:2.7; atmosphere, water vapour.

By the use of a special benzidine spray, CONNELLY *et al.*[82] were able to detect as little as $3 \cdot 10^{-4}$ μg of haemin. The spray is made up, not more than 3 hours before use, as follows: 25 ml of absolute methanol are shaken for 1 min with excess of benzidine hydrochloride, the solution is decanted, and to it is added 12.5 ml of water, 5.0 ml of glacial acetic acid, 2.5 ml of 3% hydrogen peroxide and 0.5 ml of pyridine.

(*b*) *Column chromatography*

KIESE AND KURZ[83] were able to separate the haemin *a* of heart muscle from protohaemin on columns of Al_2O_3. For the separation of heart-muscle haemins, MORRISON AND STOTZ[84] used partition chromatography on silicic acid columns. After washing, the silicic acid was mixed with 0.15 *N* HCl, chloroform added, and a column packed from the slurry. The haemins were applied in chloroform–hexane solution and the columns developed with this mixture to remove lipides before elution of the haems with chloroform.

For his purification of haem a_2, the iron-chlorin prosthetic group of bacterial cytochrome a_2, BARRETT[60] used silica gel columns. The following procedure was found to leave the particles of the gel well dispersed, giving smoothly working columns: 9 g of silica gel were shaken, under N_2, with 4 ml of a mixture of 7 vols. of methanol with 3 vols. of water. The silica gel particles were then suspended in 60 ml of petroleum ether (b.p. 68°), and a column packed from the slurry. The haemins were applied to the column in benzene solution, lipides were eluted with petroleum ether (b.p. 68°), and development followed by petroleum ether equilibrated with an equal volume of a mixture of 7 vols. of methanol with 3 vols. of water, then by wet benzene to which increasing amounts of methanol were added. The green haem a_2 was eluted when the methanol concentration was 0.5 to 1.0%.

5. ELECTROPHORESIS OF PORPHYRINS

Electrophoresis has been applied to porphyrins both on paper and on other supporting media, by several investigators. While both methods are capable of separating porphyrin free acids with more than 3 carboxyl groups from each other, the dicarboxylic porphyrins are not separated, and no isomer-separations have been achieved.

(*a*) *Media other than paper*

The first report of the application of this method to the separation of porphyrins appears to be that of KENCH AND PAPASTAMATIS[85], who used agar gel as the supporting medium with 0.06 *M* phosphate buffer of pH 8.0 and 4 V/cm. Uro- copro- and proto-porphyrins were found to separate, and 60 to 80% recoveries were obtained at the 1.0 μg level. No separation was found of uro- or copro-porphyrin isomers I and III, or of the dicarboxylic deutero-, meso- and protoporphyrins.

ERIKSEN[86] has tried a number of supporting media, including starch paste, starch gel, paper powder and glass powder, all of which were found to be inferior to agar gel. Troughs 50 × 18 × 1.5 cm were used, with 0.5 or 1.0% agar gel, prepared in veronal buffer of pH 8.6 and ionic strength 0.05. The porphyrins were applied in highly concentrated solution in small holes in the gel, and electrophoresis was carried out at 4°; for a film thickness of 2 to 5 mm, 6 V/cm were applied, and separation was complete after 10 to 12 hours. Excellent separation was found of octa-, hexa-, penta-, tetra- and tri-carboxylic porphyrins.

(*b*) *Paper electrophoresis*

The electrophoretic separation on paper of uro-, copro- and proto-porphyrins, as the free carboxylic acids, was studied by LARSEN, MELCER AND ORTEN[87] in 1955. Separation according to the number of carboxyl groups was achieved in veronal or borate buffers of pH 8.5. The porphyrin precursors δ-aminolaevulic acid and porphobilinogen, were separated from porphyrin mixtures in borate buffer at pH 11 and ionic strength 0.2 to 0.3, and were stable under these conditions. Isomers I and III of copro- and uro-porphyrins did not separate. In the same year HEIKEL[88] published a similar method. The method was studied further by WITH[89], who used Whatman No. 1 paper, 0.05 *M* barbiturate buffer of pH 8.6, and 7.5 to 8 V/cm for 1 to 3 hours. WITH[89] also found that no isomer-separations could be achieved by this method. The distances moved from the origin (near the anode end) under WITH's conditions were: uroporphyrin 10–15 cm, coproporphyrin 0.5–2.0 cm and protoporphyrin 0–0.3 cm.

STERLING AND REDEKER[90] used 30 × 3 cm strips of Whatman No. 3 paper with an EDTA buffer of pH 8.6, and ran for 4 h at 7 V/cm at a temperature of 5°. The distances moved were 6–6.5 cm, 0–0.2 cm and zero for uro-, copro- and proto-porphyrins respectively. Within certain stated limits the amount of porphyrin applied

did not affect the separation or the recovery. After the electrophoresis the fluorescent areas were leached from the paper with aqueous HCl solutions, and recoveries for uro-, copro- and proto-porphyrins were 96 ± 3%, 87 ± 1% and 87 ± 2.5% respectively.

WITH[91] applied the method to investigate whether the commonly-occurring porphyrins are bound to serum proteins, as is bilirubin. Electrophoresis at 400 V, 20 mA, in barbiturate buffer of pH 8.6, on Whatman No. 1 paper, showed that proto- and haemato-porphyrins, coproporphyrins I and III and uroporphyrins I and III move quite independently of the proteins of normal human serum to which they had been added. Bilirubin added to serum in the same way is immediately attached to the albumin.

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GAS–LIQUID CHROMATOGRAPHY IN QUALITATIVE ANALYSIS

PART I. AN INTERPOLATION METHOD FOR THE PREDICTION OF RETENTION DATA

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INTRODUCTION

The use of retention data in qualitative analysis is impeded by the difficulty of preparing authentic reference compounds. A logarithmic interpolation method of calculating the retention times of unsymmetrical molecules from those of the symmetrical counterparts has been investigated and is described in this paper. The compounds investigated include *n*-paraffins, olefins, sulphides, disulphides, thiols, *sec.*-alcohols, halides, ketones, ethers and selenides.

EXPERIMENTAL

Apparatus and procedure

Chromatograms were obtained using an apparatus consisting of glass columns (5 ft. long and 4 mm I.D.) with a modified flame ionisation detector[1,2]. The carrier gas was a 3:1 (by volume) mixture of hydrogen and nitrogen. The columns were packed, with the stationary phases silicone fluid 704 (Hopkin and Williams), squalane (B.D.H.) and polyethylene glycol 400 (Union Carbide) supported on acid-washed (1% phosphoric acid) 60–72 mesh celite (J.J.'s Ewell, Surrey) and in certain cases on 60–72 mesh C.22 brick dust (J.J.'s), by conventional techniques. Samples (approximately 10 γ) for analysis were introduced by means of stainless steel capillary pipettes[3]. The column efficiencies were between 400 and 500 theoretical plates per foot of column.

Calculation of the relative retention times

Retention times were measured from the time of injection of the sample to the time of appearance of the peak maxima on the recorder chart, and were corrected for the calculated "dead-time"[4] of the apparatus. *n*-Paraffins were chosen as internal standards for the determination of the relative retention times on silicone fluid and squalane whilst *n*-alcohols were chosen as secondary internal standards for polyethylene glycol (PEG). All values quoted in this paper are relative to theoretical *n*-nonane[5].

Materials

The compounds used in this work were either purified commercial materials or were prepared by members of N.R.P.R.A. staff. Details of the synthesis of the 2-methyl-pent-2-en-1-yl and 4-methyl-pent-3-en-2-yl sulphides will be published shortly[6].

RESULTS AND DISCUSSION

The logarithm of the absolute retention volume for a particular substance is directly related to the free energy of solution of the solute molecules in the stationary liquid phase. As the free energy of solution is an approximately additive function of the groups constituting the molecule, linear homologous series plots (log R against N the carbon number) have been observed for many classes of organic compounds[7,8]. These plots have been used to predict the retention times of unavailable members of a series. However, it is often neither convenient nor possible to obtain sufficient members of a series in order to determine the required retention time by this method. Retention times can also be calculated by the method of "summation of group contributions"[9,10], but except in the most favourable cases the results are not sufficiently precise for qualitative analysis.

A consequence of the additivity of group values of free energy of solution is that, if a molecule can be represented as R-X-R', then

$$\log_{10} R_{1,2} = \tfrac{1}{2} \{ \log_{10} R_{1,1} + \log_{10} R_{2,2} \}$$

where $R_{1,2}$, $R_{1,1}$ and $R_{2,2}$ are the retention times of R-X-R', R-X-R and R'-X-R', respectively.

The agreement between the observed and calculated values may be expected to be good provided that:

(i) there is no conjugation across the group X
(ii) none of the molecules are sterically hindered
(iii) neither R nor R' are strongly polar
(iv) the bonds linking R and X and R' and X are symmetrical in polar character or that these bonds are separated by a number of non-conjugated atoms in group X, and
(v) the retention times of the two symmetrical compounds can be measured with comparable and good accuracy.

The virtues of this method, where applicable, are that only two substances are required to calculate the retention time of the unknown and since only two terms are used in the calculation accumulative errors are minimised. We have found the method particularly useful in cases where the symmetrical compounds are easier to synthesise than the unsymmetrical counterparts, for instance mono- and di-sulphides.

n-Paraffins

Since specific interactions are very weak in this series, excellent agreement may be expected and is indeed obtained between the observed and calculated values, as reflected by a standard deviation of 0.83 % (Table I).

TABLE I

Stationary phase and concentration w/w	*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
						R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
Squalane 5 %	Celite	20	*n*-propyl	*n*-butyl	$-(CH_2)_2-$	0.41	2.55	1.00	1.02
Squalane 5 %	Celite	20	*n*-propyl	*n*-butyl	$-CH_2-$	0.17	1.00	0.41	0.41
Squalane 5 %	Celite	65	*n*-butyl	*n*-amyl	$-CH_2-$	1.00	5.29	2.30	2.30
Squalane 20 %	C22	65	*n*-propyl	*n*-butyl	$-CH_2-$	0.18	1.00	0.42	0.42
PEG 20 %	Celite	65	*n*-amyl	*n*-hexyl	$-CH_2-$	3.71	13.66	7.09	7.12
PEG 20 %	Celite	65	*n*-amyl	*n*-hexyl	$-(CH_2)_2-$	7.09	26.40	13.66	13.68

Alkyl-aryl hydrocarbons

This series has not been widely investigated, however, preliminary results indicate that *para*-substituted compounds give good agreement (2.7 % R.M.S. deviation) whilst the *ortho* compounds give poor agreement (15 % error), as shown in Table II.

TABLE II

Stationary phase and concentration w/w	*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
						R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
PEG 20 %	Celite	65	H	*o*-methyl		1.92	9.11	3.58	4.18
PEG 20 %	Celite	65	H	*p*-methyl		1.92	6.73	3.58	3.59
PEG 20 %	Celite	65	H	*p*-ethyl		1.92	19.84	6.40	6.17

Δ-1 Olefins

The retention times of alk-1-enes may be calculated from those of suitable *n*-alkanes and alka-α,ω-dienes, for instance that of oct-1-ene is obtained using those of *n*-decane and hexa-1,5-diene.

The agreement between calculated and observed values may be expected to be slightly inferior compared with *n*-paraffins owing to the somewhat polar nature of the double bond. As can be seen from Table III a R.M.S. deviation of 2.4 % is observed for the three *Δ*-1 olefins measured. In the cases of hept-1-ene and oct-1-ene this

deviation may in part arise from the large difference in retention time of the two symmetrical compounds.

TABLE III

Stationary phase and concentration w/w	*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
						R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
Squalane 5 %	Celite	65	vinyl	ethyl	$-(CH_2)_2-$	0.053	0.074	0.062	0.063
Squalane 5 %	Celite	65	vinyl	*n*-propyl	$-(CH_2)_2-$	0.053	0.411	0.152	0.148
Squalane 5 %	Celite	65	vinyl	*n*-butyl	$-(CH_2)_2-$	0.053	2.45	0.350	0.360

Alkyl and alkenyl monosulphides

The method gives excellent results for alkyl and alkenyl sulphides, provided that *tert.*-alkyl groups are not present, as illustrated in Table IV. The disagreement between observed and calculated values for *tert.*-alkyl sulphides appears to be about 12 % with the unsymmetrical compound emerging sooner than calculated. This may be due to steric hindrance. We propose to investigate the anomalous behaviour of *tert.*-butyl sulphides further.

TABLE IV

Stationary phase and concentration w/w	*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
						R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
Silicone 20 %	Celite	160	cyclohexyl	cyclohex-2-enyl	—S—	53.6	75.6	63.6	63.7
PEG 20 %	Celite	65	ethyl	allyl	—S—	1.42	7.28	3.25	3.22
Silicone 20 %	Celite	65	ethyl	allyl	—S—	0.34	1.42	0.69	0.69
PEG 20 %	Celite	65	methyl	*n*-butyl	—S—	0.66	14.07	3.08	3.05
PEG 20 %	Celite	65	methyl	allyl	—S—	0.66	7.28	2.20	2.19
Silicone 20 %	Celite	65	methyl	allyl	—S—	0.075	1.42	0.35	0.33
PEG 20 %	Celite	65	ethyl	*tert.*-butyl	—S—	1.42	3.39	1.90	2.19
Silicone 20 %	Celite	65	ethyl	*tert.*-butyl	—S—	0.34	1.63	0.67	0.74

R.M.S. deviation (excluding anomalous *tert.*-butyl cases) 2.47 %.

Alkyl and alkenyl disulphides

The overall picture of agreement with these compounds is similar to that of the mono-sulphides (Table V).

TABLE V

Stationary phase and concentration w/w		*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
							R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
PEG	20%	Celite	100	isopropyl	allyl	—S·S—	9.61	31.85	17.55	17.50
Silicone	20%	Celite	100	isopropyl	allyl	—S·S—	4.13	7.79	5.75	5.67
PEG	20%	Celite	100	*n*-butyl	allyl	—S·S—	46.65	31.85	38.32	38.55
Silicone	5%	Celite	138	*n*-butyl	allyl	—S·S—	15.38	5.21	8.87	8.95
Silicone	5%	Celite	138	ethyl	I	—S·S—	2.2	44.2	9.9	9.9
Silicone	5%	Celite	138	*tert.*-butyl	I	—S·S—	5.3	44.2	14.6	15.3
Silicone	5%	Celite	138	I	II	—S·S—	44.2	75.8	58.2	57.9

R.M.S. deviation excluding *tert.*-butyl compound 0.76%.
I = 4-methylpent-3-en-2-yl.
II = 2-methylpent-2-en-1-yl.

Alkane-1-thiols

The retention times of alkane-1-thiols have been calculated from those of suitable *n*-paraffins and propane-1,3-dithiol and butane-1,4-dithiol. The somewhat poor agreement between the observed and calculated values may be due to the slightly polar nature of the thiol group and also the difference in the retention times of the paraffin and dithiol, particularly on polyethylene glycol stationary phase. (Table VI).

TABLE VI

Stationary phase and concentration w/w		*Solid support*	*Temperature °C*	*R*	*R'*	*X*	*Relative retention times*			
							R-X-R	*R'-X-R'*	*R-X-R' observed*	*R-X-R' calculated*
Silicone	5%	Celite	65	*n*-propyl	$—CH_2·SH$	$—CH_2—$	0.21	4.49	0.95	0.97
Silicone	5%	Celite	65	*n*-butyl	$—CH_2·SH$	$—CH_2—$	1.00	4.49	2.18	2.12
PEG	20%	Celite	65	*n*-butyl	$—CH_2·SH$	$—CH_2—$	0.95	48.57	6.64	6.79
Silicone	5%	Celite	65	*n*-amyl	$—CH_2·SH$	$—CH_2—$	5.29	4.49	5.08	4.87
PEG	20%	Celite	65	*n*-amyl	$—CH_2·SH$	$—CH_2—$	3.79	48.57	12.56	13.56
Silicone	5%	Celite	65	*n*-hexyl	$—CH_2·SH$	$—CH_2—$	27.8	4.49	11.60	11.18
PEG	20%	Celite	65	*n*-hexyl	$—CH_2·SH$	$—CH_2—$	13.99	48.57	23.70	26.07
Silicone	5%	Celite	65	ethyl	$—CH_2·SH$	$—(CH_2)_2—$	0.11	11.96	0.95	1.15
Silicone	5%	Celite	65	*n*-propyl	$—CH_2·SH$	$—(CH_2)_2—$	0.45	11.96	2.18	2.32
Silicone	5%	Celite	65	*n*-butyl	$—CH_2·SH$	$—(CH_2)_2—$	2.30	11.96	5.08	5.24

R.M.S. deviation 8.0%.

Ketones

Data for these materials are very limited. The results indicate that observed and calculated values should agree to within about 5 % (Table VII).

TABLE VII

Stationary phase and concentration w/w	Solid support	Temperature °C	R	R′	X	Relative retention times			
						R-X-R	R′-X-R′	R-X-R′ observed	R-X-R′ calculated
PEG 20 %	Celite	65	methyl	ethyl	>C=O	1.09	2.87	1.78	1.77
PEG 20 %	Celite	65	methyl	*n*-propyl	>C=O	1.09	6.68	2.80	2.70
PEG 20 %	Celite	65	methyl	*n*-butyl	>C=O	1.09	25.64	5.69	5.28

Selenides

To date only one selenide system has been investigated. The result suggests reasonable agreement should be obtainable (Table VIII).

TABLE VIII

Stationary phase and concentration w/w	Solid support	Temperature °C	R	R′	X	Relative retention times			
						R-X-R	R′-X-R′	R-X-R′ observed	R-X-R′ calculated
PEG 20 %	Celite	65	methyl	allyl	—Se—	0.92	12.98	3.63	3.45

Aromatic ethers

Only one ether system of this type has been studied. The result suggests reasonable agreement in the case of *p*-substituted ethers (Table IX).

TABLE IX

Stationary phase and concentration w/w	Solid support	Temperature °C	R	R′	X	Relative retention times			
						R-X-R	R′-X-R′	R-X-R′ observed	R-X-R′ calculated
PEG 20 %	Celite	100	H	*p*-methoxy		1.95	131.5	16.38	16.02

sec.-Alcohols, sec.-thiols, and sec.-sulphides

In the two *sec.*-alcohol systems studied excellent agreement between observed and calculated values has been obtained. The retention times of *sec.*-thiols and *sec.*-sulphides can be calculated similarly (Table X).

TABLE X

Stationary phase and concentration w/w	Solid support	Temperature °C	R	R'	X	Relative retention times			
						R-X-R	R'-X-R'	R-X-R' observed	R-X-R' calculated
PEG 20%	Celite	65	methyl	ethyl	—CH(OH)—	2.84	8.54	5.21	4.92
Silicone 5%	Celite	65	methyl	ethyl	—CH(OH)—	0.16	0.53	0.29	0.29
PEG 20%	Celite	65	methyl	ethyl	—CH(SH)—	0.61	2.17	1.18	1.15
PEG 20%	Celite	65	methyl	ethyl	—CH(S·C_2H_5)—	1.74	5.15	3.16	2.99

prim.-Halides

In this series, if one of the symmetrical compounds contains two halogen atoms, poor agreement may be expected and Table XI confirms this.

TABLE XI

Stationary phase and concentration w/w	Solid support	Temperature °C	R	R'	X	Relative retention times			
						R-X-R	R'-X-R'	R-X-R' observed	R-X-R' calculated
Silicone 20%	Celite	65	*n*-butyl	—CH_2·Br	—CH_2—	1.00	4.54	2.45	2.13

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SUMMARY

A logarithmic interpolation method for the prediction of the retention times of unsymmetrical molecules from those of the symmetrical counterparts is described. The classes of organic compounds to which the method has been successfully applied include *n*-paraffins, olefins, sulphides, disulphides, thiols, *sec.*-alcohols, halides, ketones, ethers and selenides.

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THE EFFECT OF pH ON THE R_F VALUES OF ORGANIC ACIDS IN PAPER CHROMATOGRAPHY WITH NEUTRAL SOLVENTS

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INTRODUCTION

In enzymology the substances taking part in a reaction are generally suspended in a buffer solution, and at the final stage the proteins are precipitated with trichloroacetic acid (TCA). When studying the paper chromatographic behaviour of the products resulting from such a reaction, we observed that the R_F values of some substances alter considerably when the pH is altered.

These observations led us to carry out an investigation with the object of determining which substances behave in this manner and the conditions under which these changes occur. As our attention had first been drawn to this problem when investigating an enzymic reaction involving amino- and keto-acids, our research was directed to the study of the behaviour of organic acids in general.

EXPERIMENTAL

The organic acids were used in 0.1 % or 1.0% solutions according to the pH we wished to obtain. Each sample was investigated at an acid and an almost neutral pH, the solutions being prepared in water, in 0.2 *M* phosphate buffer of pH 8.0, and in the same buffer treated with 30 % trichloroacetic acid, or *N* hydrochloric acid, or *N* sulphuric acid until the pH reverted to the acid zone.

Ascending paper chromatograms were run on Macherey-Nagel No. 261 paper at room temperature for 20 hours in several neutral solvent systems. In order to find the system that gives the best results, the following neutral mixtures were tested [1,2]:

(a) 1-Butanol–water (85:15).
(b) Acetone–water (80:20).
(c) 2-Butanol–amyl alcohol–water (4:4:1).
(d) Water-saturated amyl alcohol.
(e) 1-Propanol–water (70:30).
(f) 95 % ethanol.
(g) 77 % ethanol.

For aliphatic dicarboxylic, hydroxy- and keto-acids, the best results were obtain-

ed with 1-propanol–water (70:30), whereas 77% ethanol was most convenient for amino acids.

For locating the compounds on the paper, the following spraying reagents were used [1, 3]:

(a) 0.1 % ninhydrin solution in isopropanol for amino acids.
(b) Bromocresol green solution in ethanol for acids in general.
(c) Ammoniacal silver nitrate for reducing compounds.
(d) 2 % ferric chloride for keto-acids.
(e) 1 % potassium permanganate for hydroxy-acids.

RESULTS

The effect of using trichloroacetic acid, hydrochloric acid and sulphuric acid to adjust the pH of the various compounds was investigated and the R_F values obtained are given in Table I.

TABLE I

INFLUENCE OF TCA, HCl AND H_2SO_4 ON THE R_F VALUES OF AMINO ACIDS

Amino acids adjusted to pH = 1.0	R_F		
	TCA	*HCl*	H_2SO_4
Alanine	0.51	0.50	0.47
Valine	0.65	0.63	0.62
Glycine	0.34	0.35	0.35
Leucine	0.60	0.60	0.58
Glutamic acid	0.54	0.54	0.56
Aspartic acid	0.40	0.39	0.40

Since in our experiments it was often necessary to dissolve some of the acids in a phosphate buffer of pH 8.0, in order to obtain a final pH between 5.0 and 6.0, the possible influence of the buffer upon the migration rate of the compounds tested had to be examined.

TABLE II

INFLUENCE OF PHOSPHATE BUFFER ON THE R_F VALUES OF AMINO ACIDS

Amino acid at pH = 5.0	R_F		*Amino acid at pH = 1.0*	R_F	
	H_2O	*Buffer*		*TCA*	*Buffer + TCA*
Aspartic acid	0.22	0.24	Aspartic acid	0.41	0.42
Glutamic acid	0.32	0.30	Glutamic acid	0.54	0.55

As it was found that under our working conditions the buffer did not interfere at all with the migration of the spots on paper, we could proceed with our experiments on the different types of organic acids.

Amino acids

The complete series of the most common mono- and dicarboxylic amino acids, as well as the amides of the latter, were investigated as regards their R_F values when the pH of the solution was altered (Table III).

TABLE III

INFLUENCE OF pH ON THE R_F VALUES OF AMINO ACIDS

Amino acids	R_F		
	pH 5.0	*pH 3.0*	*pH 1.0*
Glycine	0.37	0.37	0.37
Alanine	0.47	0.49	0.50
Valine	0.60	0.60	0.61
Leucine	0.58	0.59	0.61
Serine	0.41	0.41	0.42
Threonine	0.53	0.53	0.54
Cysteine	0.10	0.11	0.12
Lysine	0.32	0.32	0.35
Glutamic acid	0.29	0.41	0.57
Aspartic acid	0.22	0.25	0.40
Glutamine	0.27	0.27	0.33
Asparagine	0.12	0.13	0.17

Aliphatic acids

Dicarboxylic aliphatic acids, as well as some keto- and hydroxy-acids, were tested as to their behaviour on paper chromatography when the pH of the solution is altered (Table IV). The monocarboxylic aliphatic acids with a short carbon chain were excluded from our work because they could not be tested in neutral solvents owing to their volatility.

TABLE IV

INFLUENCE OF pH ON THE R_F VALUES OF ORGANIC ACIDS

Organic acid	R_F	
	pH 5.0-6.0	*pH 1.0*
Oxalic	0.21	0.31
Malonic	0.48	0.59
Succinic	0.78	0.81
Adipic	0.90	0.89
Lactic	0.34	0.68
Malic	0.16	0.64
Tartaric	0.22	0.55
Citric	0.12	0.57
Pyruvic	0.46	0.69
Oxaloacetic	0.12	0.49
α-Ketoglutaric	0.15	0.63

The R_F values at pH 3.0 were omitted from Table IV, because they are always intermediate values and do not contribute any further information.

DISCUSSION

From Tables I and II it is evident that when the pH of the sample is adjusted to 1.0 the R_F values of the samples remain constant, irrespective of whether trichloroacetic, hydrochloric or sulphuric acid is used, or whether the initial dilution is made in water or phosphate buffer.

From the series of amino acids (Table III) it can be seen that a variation of the R_F is evident only for the dicarboxylic acids, and does not occur with the monocarboxylic acids even when other groups are present in the molecule. It should be noted that for glutamine and asparagine, which are amides of dicarboxylic amino acids, the magnitude of the variation is considerably diminished.

The differences in the R_F values can be accentuated still more if an excess of the auxiliary acid is used, and this may even lead to slight alterations in the cases that were considered as negative here.

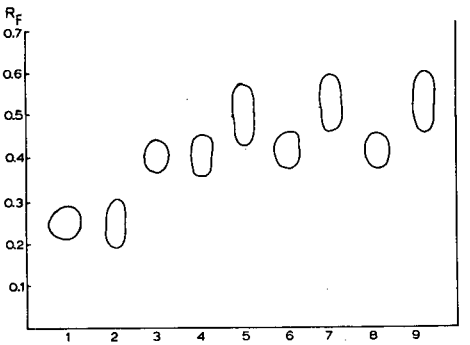

Fig. 1. Chromatogram of aspartic acid in: (1) water, pH = 4.0; (2) buffer, pH = 4.5; (3) TCA, pH = 1.0; (4) buffer and TCA, pH = 1.0; (5) buffer and an excess of TCA; (6) buffer and HCl, pH = 1.0; (7) buffer and an excess of HCl; (8) buffer and H_2SO_4, pH = 1.0; (9) buffer and an excess of H_2SO_4. Solvent: 77% ethanol.

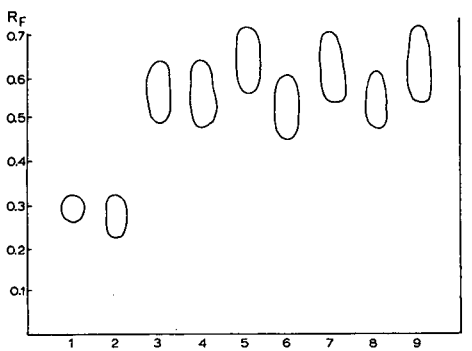

Fig. 2. Chromatogram of glutamic acid in: (1) water, pH = 4.0; (2) buffer, pH = 5.0; (3) TCA, pH = 1.0; (4) buffer and TCA, pH = 1.0; (5) buffer and an excess of TCA; (6) buffer and HCl, pH = 1.0; (7) buffer and an excess of HCl; (8) buffer and H_2SO_4, pH = 1.0; (9) buffer and an excess of H_2SO_4. Solvent: 77% ethanol.

The results obtained with the dicarboxylic aliphatic acids were unexpected because of the lack of regularity. Only the first two acids of the series, those with two and three carbon atoms, behaved in this fashion. On the other hand, there is strong evidence for believing that the presence of hydroxyl and carbonyl groups is responsible for the change in the R_F values when the pH is altered (Table IV). For instance, the influence of the hydroxyl groups of tartaric acid can be clearly seen in Fig. 3, where this acid has been chromatographed together with succinic and malonic acid.

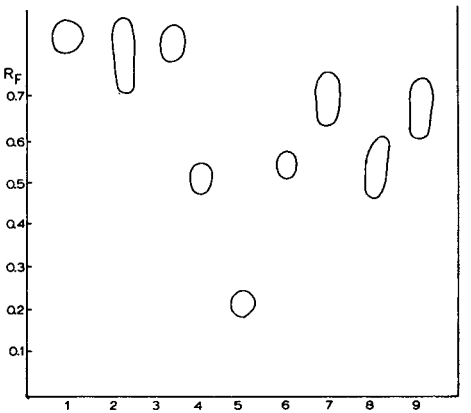

Fig. 3. Chromatogram of: (1) succinic acid in water, pH = 1.0; (2) succinic acid in buffer, pH = 6.0; (3) succinic acid in buffer and TCA, pH = 1.0; (4) tartaric acid in water, pH = 2.0; (5) tartaric acid in buffer, pH = 6.0; (6) tartaric acid in buffer and TCA, pH = 1.0; (7) malonic acid in water, pH = 1.0; (8) malonic acid in buffer, pH = 6.0; (9) malonic acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

On comparing the results obtained with those for the amino acids, it can be observed that the R_F values of the hydroxy-monocarboxylic acids alter considerably when the pH is altered. Not only the hydroxyl-group but also the carbonyl-group in mono- and dicarboxylic acids seems to influence the R_F values. This can be easily seen when succinic acid, which shows no differences, is compared with oxaloacetic acid for which the R_F changes from 0.12 to 0.49 when the pH is altered from 6.0 to 1.0. Fig. 4 represents one of the chromatograms of the keto-acids studied.

To explain these facts we first thought of the possibility of cyclization products being formed, although our working conditions were not the same as those applied for the preparation of these products. Thus, in the case of glutamic acid the lactam, pyrrolidone-carboxylic acid could be formed, which is not detected by ninhydrin. The spot obtained after altering the pH was, however, located on the paper by means of the ninhydrin spray[4]. Further support for abandoning this supposition came from the observation that when our sample was dissolved in water at a concentration sufficient to obtain a pH of 1.0, its R_F was the same as that obtained when the sample was dissolved in the buffer solution and the pH then adjusted to 1.0 with one of the auxiliary acids, a treatment which could lead to the formation of a new compound (Fig. 5).

We are, therefore, inclined to believe that all the variations observed are a conse-

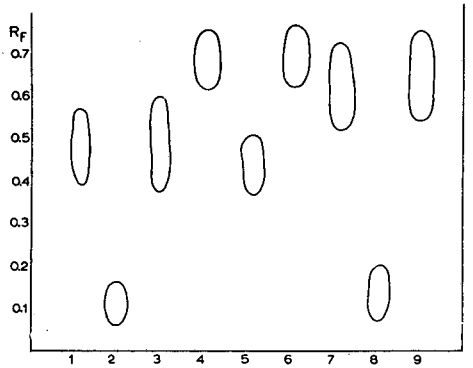

Fig. 4. Chromatogram of: (1) oxaloacetic acid in water, pH = 1.0; (2) oxaloacetic acid in buffer, pH = 6.0; (3) oxaloacetic acid in buffer and TCA, pH = 1.0; (4) pyruvic acid in water, pH = 1.0; (5) pyruvic acid in buffer, pH = 5.0; (6) pyruvic acid in buffer and TCA, pH = 1.0; (7) α-ketoglutaric acid in water, pH = 1.0; (8) α-ketoglutaric acid in buffer, pH = 6.0; (9) α-ketoglutaric acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

quence of alteration of the pH. The irregularities of the phenomena must still be considered. The results obtained for the dicarboxylic aliphatic acids furnish strong evidence for the assumption that the dissociation constant of the compounds studied is responsible for the variations of the R_F values. In view of the fact that at a very acid pH almost no

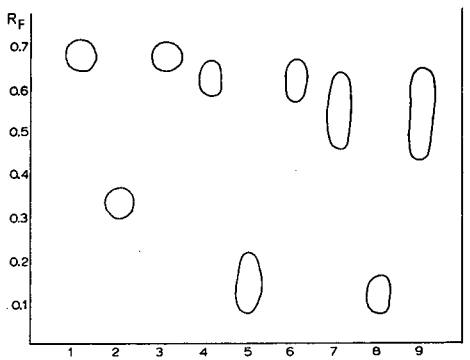

Fig. 5. Chromatogram of: (1) lactic acid in water, pH = 1.0; (2) lactic acid in buffer, pH = 6.0; (3) lactic acid in buffer and TCA, pH = 1.0; (4) malic acid in water, pH = 1.0; (5) malic acid in buffer, pH = 6.0; (6) malic acid in buffer and TCA, pH = 1.0; (7) citric acid in water, pH = 1.0; (8) citric acid in buffer, pH = 6.0; (9) citric acid in buffer and TCA, pH = 1.0. Solvent: 1-propanol–water (70:30).

dissociation occurs, oxalic and malonic acids, which have higher dissociation constants than succinic and adipic acids, must undergo a greater change in dissociation between pH 6.0 and pH 1.0, which is the cause of the R_F variations observed. The results obtained with hydroxy-acids provide further support for this hypothesis, since it is a well-established fact that a hydroxyl group at the alpha position increases the dissociation constant.

CONCLUSIONS

From what has been stated above, it may be concluded that:

(a) The acids used to adjust the pH do not influence the migration of the samples spotted on the paper.

(b) The use of a phosphate buffer does not affect the results.

(c) In the series of amino acids studied, only the dicarboxylic acids show a variation in their R_F values when the pH of the solution is altered.

(d) Blocking of one of the carboxyl groups by another amino group reduces the magnitude of the variation considerably.

(e) In the series of dicarboxylic aliphatic acids, only the first two members of the series show some variations.

(f) Hydroxy- and keto-, mono- and dicarboxylic acids exhibit considerable variations.

(g) The variations observed are the result of alterations in the pH of the solutions and not of the formation of new compounds; they are probably due to dissociation phenomena.

Finally, it should be noted that in chromatographic analysis these observations are of importance, especially when a comparison of reaction products with standard solutions might lead to erroneous interpretations if the variations due to alteration of the pH during the reaction are not taken into account. This is precisely the case in enzymic reactions in which minute concentrations of the reagents are suspended in a buffer solution and very often treated with trichloroacetic acid which is not always completely eliminated.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Dr. RAYMUNDO MONIZ DE ARAGÃO for valuable advice during this work and to Miss NORMA G. DE OLIVEIRA for technical assistance.

SUMMARY

The variations of the R_F values of organic acids due to changes in the pH of the solution were studied, as well as the influence upon the R_F of the use of trichloroacetic acid, hydrochloric or sulphuric acids for the adjustment of the pH.

Amino acids, as well as two of their amides, aliphatic dicarboxylic acids, hydroxy- and keto-, mono- and dicarboxylic acids were examined in various neutral solvent systems and their chromatographic behaviour in relation to pH variations was registered.

Practical applications of the results obtained, as well as possible theoretical explanations of the phenomena are discussed.

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ELECTROPHORETIC BEHAVIOUR OF HAEMOGLOBINS IN AGAR GEL

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INTRODUCTION

Starch and agar gels are now widely used as supporting media for the zone electrophoresis of proteins. Agar gel below neutral pH was found by ROBINSON *et al.*[1] to be of particular value for the study of human haemoglobin variants. The patterns obtained exhibit certain features, however, which suggest that the mechanism of separation is not simply electrophoretic, but that other factors are involved. Agar gel electrophoresis of proteins at conventional pH (8–9) has been widely used, and gives results which are often superior to those obtained on paper[2–4], for instance, with serum proteins. In this pH range the separation of haemoglobins is comparable with their behaviour on paper[5–7], in particular with respect to the sequence of migration, which reflects the isoelectric points. Thus, Hb-S*, Hb-C, etc. separate on the cathodic side of Hb-A, and Hb-F is not resolved from the latter. If, however, the pH is reduced to 6–6.5, Hb-F is widely separated from Hb-A[1] and Hb-S and Hb-C separate clearly on the anodic side. In addition Hb-D—which on paper and other electrophoretic media is indistinguishable from Hb-S—does not separate from Hb-A. Other features are the characteristic curvature of the pigment zones, other than Hb-F, and the fact that only this haemoglobin appears to have a constant mobility.

EXPERIMENTAL

The following procedure has been used in the present work for the routine separation of haemoglobins. A 1% gel of Difco Bacto Agar in *M*/2 citrate buffer (pH 6.2), is prepared by boiling and poured on a thin glass plate (10 in. × 4 in.) to give a 1 mm layer. Strips of Whatman 3 MM filter paper, slightly narrower than 1 mm, are impregnated with haemolysates, lightly blotted and inserted into slits made with a razor blade, about 3 in.–4 in. from one end of the gel. The plate rests on the rims of plastic boxes containing buffer solution, with which contact is made by filter paper bridges resting on the surface of the gel. The latter is protected against evaporation

* Abbreviations: Hb-A, human normal adult haemoglobin; Hb-F, human foetal haemoglobin; Hb-S, Hb-C etc. are the various genetically-determined variants of human haemoglobin.

with a sheet of polythene film lightly smeared with paraffin oil. Contact between the buffer reservoirs and reversible electrodes is made with strips of plastic sponge soaked in buffer. Optimal separation is achieved in *ca.* 4 h, with a potential of 40 V across the gel (total applied voltage 200 V), with a current of 20 mA. After completion of the electrophoresis the pattern is photographed by contact, and may then be stained with a protein or haem-specific stain. For the former, the requisite part of the gel is transferred to a smaller glass plate, the protein zones fixed by immersion in methanol–acetic acid mixture for some minutes, and the gel allowed to dry overnight in air. It is then stained with amidoblack or azocarmine by the procedure of URIEL AND GRABAR[8]. Benzidine staining is best carried out immediately after electrophoresis and the stabilised reagent of SWARUP *et al.*[9] gives excellent results. The direct spectroscopic examination of separated zones of native haemoglobin in agar gel has already been described[10].

The unusual zone shapes (Fig. 1) preclude the estimation of haemoglobin components by simple densitometry, but reliable results can be obtained by dye-elution. After staining and differentiation with a glycerol-containing reagent[8] the zones can be excised with a razor blade, peeled from the plate, the dye eluted in a known volume of $N/50$ aqueous alkali (*e.g.* 4 ml) and estimated spectrophotometrically (at 620 mμ for amidoblack).

RESULTS

Human haemoglobin variants

Fig. 1 shows typical separations of several human haemoglobins. The curvature of all the zones, other than that of Hb-F, is characteristic. It is also apparent that the relative mobilities of the various species cannot be compared in different samples. The haemoglobin variants, the behaviour of which in agar gel electrophoresis at low

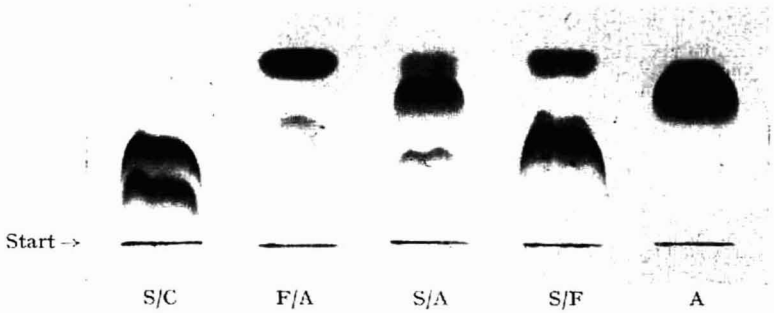

Fig. 1. Typical agar gel electrophoresis patterns of human haemoglobin mixtures; $M/20$ citrate buffer, pH 6.2; contact photograph of unstained zones.

pH has previously been described, are Hb-S, Hb-C[1,11] (all slower than Hb-A) and the following, which behave identically with Hb-A: Hb-D[1,12], Hb-E[13], Hb-I, Hb-J, "Chernoff-N", Hopkins-I, Hopkins-II, Lepore[12]. The relative mobilities of haemoglobins in agar electrophoresis at pH 6, compared with conventional zone and free-

boundary electrophoresis, are as indicated in Fig. 2. Several samples containing Hb-H were examined, and this variant was found to migrate more slowly than Hb-A, but unlike the other variants, with a straight front (Fig. 3).

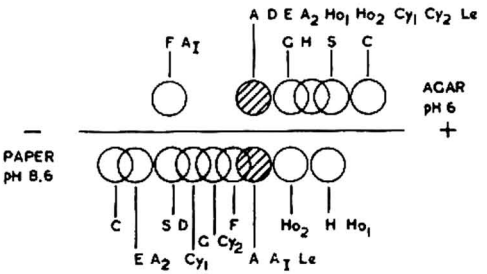

Fig. 2. Migration sequences of human haemoglobins in agar gel (*ca.* pH 6) and paper (pH 8.6) electrophoresis.

All samples of Hb-A also show a minor component (Hb-A_I) running in the position associated with Hb-F, from which, however, it has been distinguished[13], and a further small component[12, 13] which appears to arise from ageing of the haemolysate. Other minor components of Hb-A are indistinguishable from the main component[13].

Experimental variables

(*a*) *Type of agar.* Unlike agar electrophoresis at high pH, the results with haemoglobins below neutrality are critically dependent on the agar used. Of seven commercial preparations tested, Difco Bacto Agar and B.D.H. Japanese agar give separations

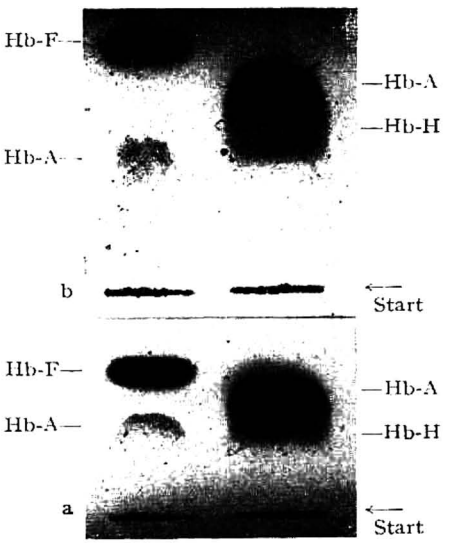

Fig. 3. Agar gel electrophoresis of an Hb-A/Hb-H mixture, compared with an Hb-F/Hb-A mixture; (a) 4 h, (b) 6 h migration.

of the kind shown in Fig. 1 (the former being the more satisfactory), while the remainder gave no separation whatever. Fig. 4 shows typical results for the same samples as used in Fig. 1, examined in Difco Noble Agar (a purified form of Bacto Agar) and Light's Ionagar II respectively. Different batches of Difco Bacto Agar all gave satisfactory results, and a sample purified by the procedure described by BUSSARD AND PERRIN[14] showed unchanged behaviour.

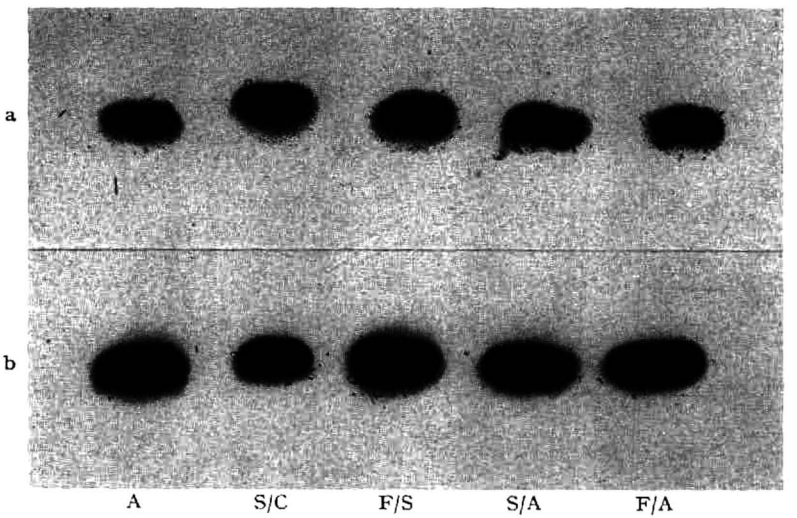

Fig. 4. Agar gel electrophoresis of human haemoglobin mixtures in 1 % gels of Difco Noble Agar and Light's Ionagar II, showing absence of separation.

(*b*) *Thickness.* It has been found important to restrict the gel thickness to *ca.* 1 mm in order to minimize heating effects. In thicker layers spurious zones appear, many of which can be suppressed by incorporation of cyanide (*ca.* *M*/100) in the buffer, and may therefore be presumed to be methaemoglobin and intermediate oxidation stages[15].

(*c*) *Voltage across gel.* Any appreciable increase in the potential gradient above about 2 V/cm leads to rapid heating, again with formation of spurious zones. The temperature at the surface of a 1 mm layer under the standard conditions is *ca.* 27° and must therefore be considerably higher at the centre of a thicker layer. Since on relatively brief exposure, even to 35°, haemoglobin undergoes considerable changes in properties, including electrophoretic mobility[16], the joint restrictions on gel thickness and working voltage are of great importance.

(*d*) *Duration of electrophoresis.* For Hb-F and the minor component Hb-A_1 migration distance in the gel is directly proportional to time (see below). With haemolysates of the usual concentration (*ca.* 10–15 %) the distribution of zones is optimal after about 4 h. With the slow-moving haemoglobins migration ceases after about this period, and in some cases the zone actually begins to move back towards the cathode (Fig. 5).

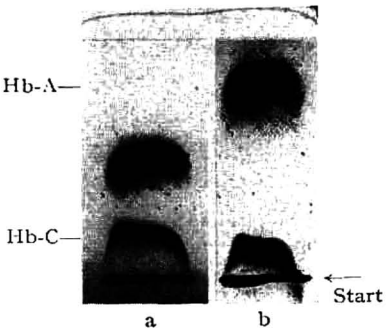

Fig. 5. Agar gel electrophoresis of an Hb-A/Hb-C mixture, photographed (a) 4 and (b) 6 h after start of experiment.

(*e*) *Condition of samples.* The mobility of a pigment in the gel depends on its concentration, except in the case of Hb-F and Hb-A_1. Fig. 6 shows the electrophoresis of serial dilutions of an Hb-A/Hb-F mixture. Whereas the mobility of the Hb-F remains constant, the Hb-A component is progressively retarded with dilution. This effect occurs only below a limiting concentration and it is therefore desirable to use concentrated haemolysates. Retardation cannot be avoided, however, when a component is present in low proportion, such that its own partial concentration is low. The discrepancy between the mobility of Hb-A in a normal adult haemolysate and in cord blood haemoglobin (*cf.* Fig. 1) is explicable in these terms, and must be regarded as a fundamental drawback of the method.

Equilibration of the samples is unimportant, as prior dialysis against buffer has no effect on the separations or the zone shapes.

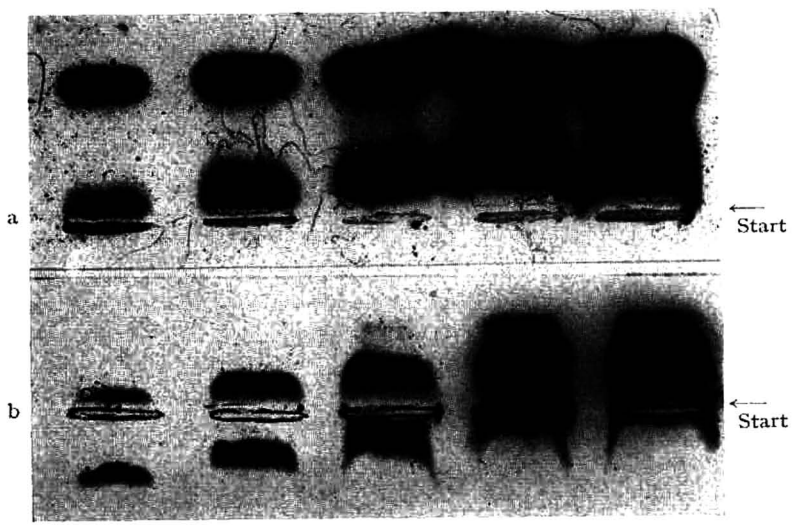

Fig. 6. Agar gel electrophoresis of serial dilutions of: (a) an Hb-A/Hb-F mixture; (b) an Hb-A/Hb-C mixture; benzidine stain. Note fast-running minor component (Hb-A_1).

(*f*) *Application of samples.* The possibility was considered that the zone curvature and back-streaming arise from the presence of the filter-paper strips in the sample slits, or the slits themselves. Removal of the strips after the pigment has entered the gel, and sealing the slit with molten agar do not affect the results, however. No improvement is found when the samples are applied differently, *e.g.* by pipetting a just molten mixture of agar and sample into a slot, or by impregnation of broader filter paper strips lying on the surface of the gel, as proposed by MARDER AND CONLEY[12].

(*g*) *Buffers.* The nature of the separation depends on pH, ionic strength and the anionic species present. The ionic strength is not very critical, but any considerable increase leads to excessive heating under the requisite potential gradient. At a citrate ion concentration of less than *ca.* $M/30$ zone curvature and back-streaming become very pronounced.

A number of buffers cause precipitation of haemoglobin near the start line. These include pyridine acetate, phthalate, and, to some extent, cacodylate. With other buffers, back-streaming and the dependence of mobility on pigment concentration are very marked, *e.g.* phosphate and maleate. In an unbuffered system ($M/20$ sodium chloride), where the local pH is presumably determined by the protein, reasonable separations are achieved.

Good results, comparable with those in citrate, are achieved by the use of $M/25$ ethylenediaminetetraacetic acid (EDTA) buffer, and indeed the zones are somewhat sharper (Fig. 7). The effects of variation in pH are discussed below.

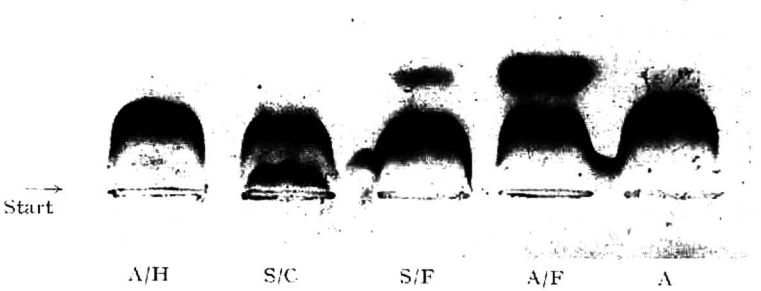

Fig. 7. Agar gel electrophoresis patterns of human haemoglobins in $M/25$ EDTA buffer, pH 6.4.

MECHANISM OF SEPARATION OF HAEMOGLOBINS

Sequence of migration

In electrophoresis in other supporting media, including agar gel at higher pH, the separation of human haemoglobins is governed by differences in their overall charge. In a group of such closely related species a simple relationship of this kind is to be expected, and the degree of separation of any two pigments is in fact closely related to their relative mobilities in free-boundary electrophoresis[16]. The sequence of separation of a series of haemoglobins:

H F A S C

will be independent of the direction of migration (except possibly over an extreme pH range, insofar as their mobility/pH curves may not be parallel) and will represent the order of isoelectric points. In agar electrophoresis under the stated conditions the sequence is:

C S H A F

This alteration indicates that the separation is not primarily dependent on the charge, but on factors which must reflect some other features of the protein structure. Since the above species have isoelectric points between the approximate limits[16] of pH 5.6 (Hb-H) and 7.3 (Hb-C), these factors must be of predominant importance.

Specificity for haemoglobins

The mechanism in question evidently applies only to the haemoglobins. When serum proteins are run in agar gel under the same conditions (pH 6–6.5) the separation is somewhat similar to that obtained on paper, and certainly no better; the characteristic curved fronts are not observed.

Any form of "molecular sieve" mechanism, such as is believed to operate in starch gel electrophoresis[17] can be excluded, as GORDON *et al.*[2] found that retardation with increasing agar concentration occurred only for proteins of very large molecular weight. Furthermore the behaviour of serum proteins in agar gel suggests that only a conventional electrophoretic process is operating. In any case the identical molecular weights, and presumably closely similar shapes, of the haemoglobin variants would render a molecular sieve mechanism irrelevant.

Similarity to ion-exchange columns

Some remarkable similarities exist between the migration sequences of haemoglobins in agar gel electrophoresis and their order of elution from ion-exchange columns at the same pH[18]. This, in increasing order of R_F value is:

C D S E G A F H

compared with the migration sequence

C S H G A D E F

for the same species on agar.

Hb-F is scarcely adsorbed on the resin and Hb-E moves only slightly slower than Hb-A[19]; both are therefore quite similar on columns and in agar gel by contrast with their normal electrophoretic behaviour. The behaviour of Hb-H can presumably be explained by its outstandingly low isoelectric point. The position of Hb-D is a real anomaly. It seems possible, however, that the different forms of this variant[20], though electrophoretically identical on paper, might differ in agar electrophoresis and column chromatography.

A situation may now be envisaged in which the haemoglobins are "eluted" in the cathod c direction by the electroendosmotic buffer flow, Hb-F being essentially unadsorbed. Haemoglobins, A, S and C will be desorbed in the same order as on ion-exchange columns. The "R_F" value of Hb-F is less than unity, however, (*v.i.*) and this can be explained in terms of its retardation by the superimposed field. From available isoelectric point data[16] most of the haemoglobins would be expected to be positively charged at the working pH, but PRINS[21] states that their isoelectric points are lower by two pH units in citrate (μ 0.1) compared with phosphate. Differences in the same sense were earlier observed for horse haemoglobin[22]. The field will then be opposing the "elution" towards the cathode. This situation is entirely consistent with the behaviour of the pigments at still lower pH (*v.i.*).

Dependence of adsorption on pH

By analogy with ion-exchange columns[23] the degree of adsorption should decrease with increasing pH, and if the slowest-moving zones in agar are the most strongly adsorbed, they should be successively desorbed by progressive increases in buffer pH.

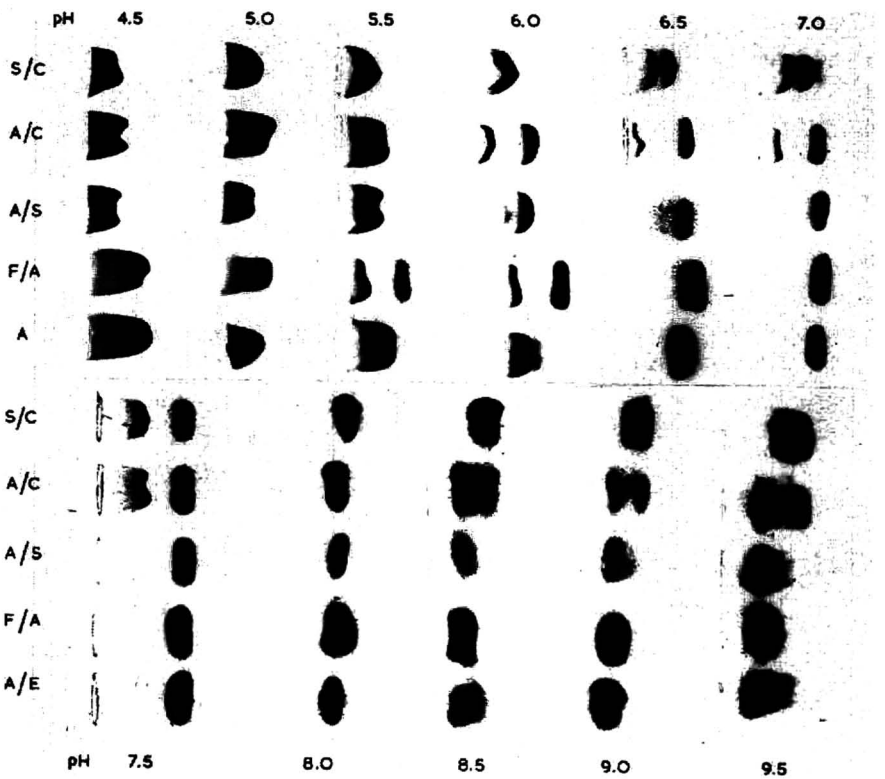

Fig. 8. Effect of pH variation on separation of human haemoglobins in Difco Bacto Agar gel, using acetate, citrate, triethanolamine or barbital buffers of constant ionic strength. An Hb-A/Hb-E mixture was used instead of Hb-A over the pH range 8.0–9.5.

Examination of a typical agar electrophoresis pattern (*e.g.* Fig. 1; see also MARDER AND CONLEY[12]) shows that zone curvature and trailing is most marked with the slowest-moving haemoglobins. The desorption which accompanies an increase in pH should eliminate the manifestations of adsorption. The results of agar gel electrophoresis over the pH range 4.5–9.0 are shown in Fig. 8, for buffers of constant ionic strength. At the lowest pH severe trailing, with probable denaturation, is occurring, but in the range 5.0–5.5 separations in the above-mentioned sequence are observed. The Hb-F zone, however, has the curved shape of the other haemoglobins and may therefore be considered to be adsorbed. At pH 6.0 the Hb-F zone is straight, and the Hb-A zone is the next to lose its curved shape with further increase in pH; by pH 7.0 it is completely straight and resembles that of Hb-F. Moreover, the mechanism responsible for the separation of these two pigments is now suppressed, and they migrate in mixtures as a single zone. Hb-S is next desorbed and by pH 7.5 it too has a straight zone shape and is not resolved from Hb-A. Hb-C is the last to retain a curved front, and this is only entirely lost by pH 8.0. At this point there is little separation of any of the variants. With still further increase in pH, the usual region for the electrophoretic separation of approximately neutral proteins is reached, and agar gel is then operating as a normal supporting medium. The samples shown in Fig. 8 include a specimen of Hb-E trait haemoglobin, and this variant, which is unresolved at the lower pH value, is now separated. The resolution of Hb-C is clear, and it will be noted that the relative positions of Hb-A and Hb-C with respect to polarity are reversed, compared with the lower pH. Thus the electrophoretic mechanism at high pH is conventional and depends on charge differences. Because of the large magnitude of the electroendosmosis the displacement is always cathodic.

Chromatography in agar gel

If the mechanism of fractionation of haemoglobins in agar gel at low pH is adsorptive, it should be possible to dispense with the potential gradient if a liquid flow can be maintained.

Accordingly a mixture of Hb-A and Hb-F (*ca.* 10 mg) was rapidly mixed with some just-molten agar gel and poured on the surface of a column of agar gel (10 cm × 1 cm diameter). After this had set, another layer of just-molten agar was added, and the column eluted with $M/20$ citrate buffer. Elution was slow (less than 1 ml in 24 h) but the haemoglobin migrated down the column. The first eluate fractions were yellow due to an impurity in the agar. The pigment was eluted after some days at 4° as a moderately concentrated solution. No separation of zones on the column was observed, possibly because of diffusion, but examination of the first pigment fraction by the moving-plate spectrograph[24] showed it to be almost pure Hb-F, indicating that the adsorption mechanism was operative.

Dilution effect and adsorption isotherm

An explanation of the dilution effect (Fig. 6) is now evident. It is known[25] that the adsorption of proteins often follows the empirical Freundlich isotherm: $c_a \propto c_s^{1/n}$

where c_a and c_s are the respective concentrations of the adsorbate in the adsorbent and the continuous phase and n a constant (the adsorption constant). This behaviour is a consequence of the progressive occupation of sites on the adsorbent as the adsorbate concentration increases. An asymptotic maximum is thus approached. That such an effect is operating here is shown by the plot (Fig. 9) of the concentration of Hb-A applied to the gel (in arbitrary units) *vs.* migration distance in a constant time; the latter should be simply related to the proportion of unadsorbed pigment.

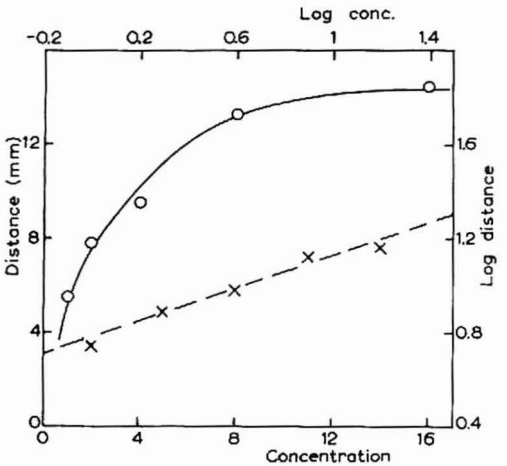

Fig. 9. Direct and logarithmic plots of migration distance (mm) of Hb-A zones *vs.* pigment concentration (arbitrary units).

A marked resemblance to a Freundlich isotherm is evident. The logarithmic plot is linear and its slope is 0.36 ($n = 2.8$), which is comparable with values reported for various adsorbing systems involving proteins[26,27]. The increased back-streaming and decreased mobility, which accompany a reduction in ionic strength, are also in accord with the results of adsorption experiments on the majority of proteins, whereby adsorption is repressed by high salt concentration[25].

Retardation of zones with time

The progressive retardation of zones other than that of Hb-F culminating, in the slowest-moving electrophoresis variants, in reversal of migration, might be thought to be due to a change in electroendosmotic flow with time. To test this possibility, hydrogen peroxide was used as an uncharged marker in an electrophoresis at pH 6.2. At intervals filter-paper prints of the gel surface were taken and stained with ammoniacal silver hydroxide to detect the marker, followed by amidoblack to locate the haemoglobins. Fig. 10 is a plot of migration of marker and Hb-F *vs.* time, showing that over the duration of the experiment the electroendosmotic flow remained constant at 1.3×10^{-4} cm/sec.

The only possible explanation of the retardation, therefore, is the effective

decrease in concentration of protein in the gel as the zones diffuse. Because of the dilution effect described above the degree of adsorption of the pigment then increases, with consequent progressive retardation of the zones.

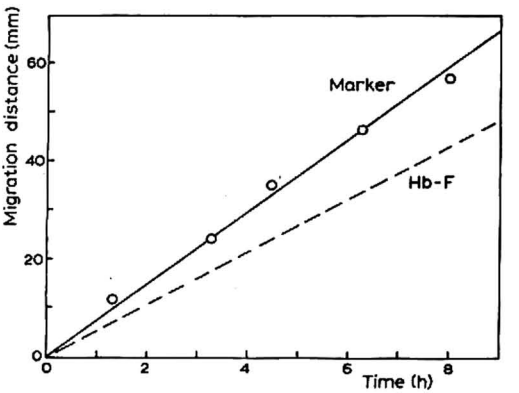

Fig. 10. Plot of migration distance (mm) *vs.* time (h) of uncharged marker (hydrogen peroxide) compared with Hb-F zone.

Reversal of strongly adsorbed zones

The actual reversal of the slowest-moving zones is less easily explicable. It might, however, be expected that the more dilute the protein the greater its degree of anion-binding, on a stoichiometric basis. A point might thus be reached where the additional negative charge so acquired would be sufficient to cause anodic migration. It seems likely that strong specific binding, of the kind known to occur between some proteins and small anions[28], is occurring here. It may also be envisaged that the mechanisms of desorption of proteins under a potential gradient on the one hand and by a buffer flow on the other, may differ. A dilution effect has also been observed for human serum albumin, its mobility changing with concentration both in paper[29] and free-boundary[30] electrophoresis.

The different results obtained with various buffers may also be explicable in terms of anion binding. Ions of high polarisability should be especially strongly bound, but the addition of thiocyanate or cyanide does not affect the electrophoresis patterns in agar. It appears to be generally true, however, that the greater the valency of the ion, the greater its interaction with proteins[31]. The superiority of the results obtained with citrate and EDTA buffers may be due to this effect. In particular, citrate ion, which at pH 6.0–6.5 is (unlike phosphate) at the upper limit of its buffering range, carries a high charge and might be expected to interact strongly with haemoglobins.

Zone curvature

The remaining phenomenon in agar gel electrophoresis to be explained is the distinctive zone curvature which accompanies adsorption. It has already been shown

that this is not caused by the sample slit nor is it in any way dependent on the manner in which the sample is applied. It must therefore arise directly from the influence of the protein itself on the ionic strength and pH in its vicinity, and is a zone boundary effect. An analogy with the behaviour of a protein on a cation-exchange column may again be drawn: as the protein zone moves, cations are displaced with a measurable heat of desorption[32] and a front of increased ionic strength precedes the zone through the column, accompanied by a readily detectable pH ripple (see *e.g.* GUTTER *et al.*[33]). At the same time an ionic strength and pH discontinuity must develop behind the protein zone. A similar process may be envisaged in agar gel. Here, however, an extra dimension has to be considered, since the zone also has boundaries parallel to the direction of migration. The ionic strength discontinuity at the zone boundaries should be associated with an increase in adsorption of the protein, resulting in retardation of protein transport by the buffer flow, *i.e.* zone curvature and "back-streaming".

Differences between agar preparations

It remains to consider in what structural respect Difco Bacto and B.D.H. Japanese agars may differ from other agar preparations which are not effective as supporting media under the standard conditions. The difference seems likely to reside in variations in the degree of ionization, number, or steric disposition of the acidic groups, or a combination of these factors. If the difference is related to the total electrostatic charge in the gel structures, there should be corresponding differences in zeta-potential, which will be reflected in the magnitudes of the electroendosmosis. An indication that this might be so came from a comparison of serum protein patterns after electrophoresis in the same experiment in Difco Bacto Agar and in Light's Ionagar II gels of the same concentration. Fig. 11 shows the striking differences between the positions of the zones, which must be caused by differences in electroendosmosis.

Gels containing identical concentrations of the seven available types of agar in the same buffer were loaded with electroendosmosis markers (hydrogen peroxide, glucose or dextran). At hourly intervals during electrophoresis filter-paper prints of

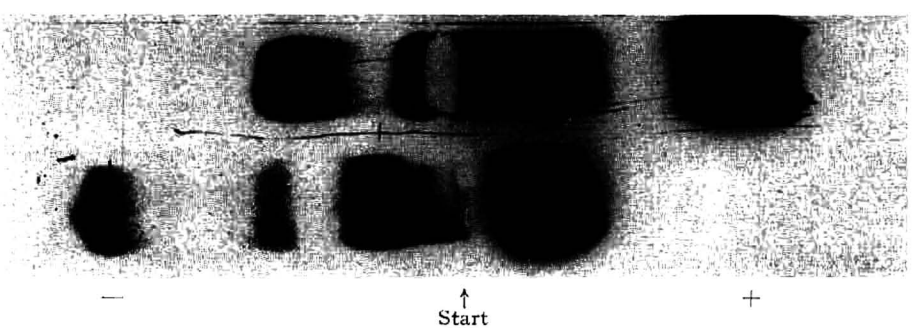

Fig. 11. Simultaneous agar-gel electrophoresis of human serum in (a) Light's Ionagar II and (b) Difco Bacto Agar; 1.25% gels; barbital buffer, pH 8.6; azocarmine stain.

the gel surfaces were taken and stained with ammoniacal silver hydroxide for hydrogen peroxide, or with aniline hydrogen phthalate[34] for glucose. Dextran was often directly visible as a zone of greater transparency. These experiments were carried out with $M/20$ citrate buffer (pH 6.2) and barbital buffer (pH 8.6, μ 0.03), and plots of marker migration distance *vs.* time in all cases gave good straight lines. The electroendosmotic flow rates are listed in Table I, from which it is clear that the values for the two effective types of agar are greater by a factor of two than those for the remainder, with only Difco Noble Agar giving an intermediate value.

TABLE I

Agar	*Electroendosmotic flow rate (cm/sec/V/cm × 10^4)*		*Electrophoretic performance with haemoglobins*
	Barbital pH 8.6, μ 0.03	*Citrate pH 6.2, M/20*	
Light's Ionagar II	1.5	0.53	— —
Oxo	1.5	0.53	— —
Light's New Zealand	1.5	0.49	— —
Gurr's Bacteriological	1.65	—	— —
Difco Noble	2.1	0.75	—
Difco Bacto	3.05	1.3	+ + +
B.D.H. Japanese	3.1	1.2	+ +

Thus the effective gels presumably have a higher effective charge density. The responsible groups may be either polysaccharide carboxyl or sulphate[35], of which the former should be titratable in an accessible pH range. Titrations on suspensions of agars showed no acidic or basic dissociations over the pH range 5–10. The charged groups responsible for the differences in electroendosmosis are therefore not titrated in this range, and it seems likely that they are sulphate groups.

If the concentration of charged groups in a Difco Noble Agar gel was increased by making the agar concentration 3 %, normal electrophoretic patterns were obtained. With the other types of agar a concentration of 5 % is necessary for any separations, but the results remain poor. With Difco Bacto Agar, on the other hand, its concentration could be halved by admixture with an ineffective agar, without appreciable effect on its performance.

Relation of adsorption to haemoglobin structure

From an analysis of the ion-exchange chromatography of haem proteins, BOARDMAN AND PARTRIDGE[23] concluded that in their working pH range (below 7) adsorption did not arise from simple Coulombic forces, since the haemoglobin molecule carries little if any negative charge under these conditions, but rather from short-range interactions of a hydrogen-bonding character between undissociated carboxyl groups on the resin and acceptor groups on the protein. The degree of adsorption is thus likely to be largely dependent on the steric disposition of charge on the protein

molecule. Another treatment[36] indicates that adsorption on ion-exchange resins is mainly governed by the distances separating the carboxyl and amino groups of the protein.

The entirely different behaviour of Hb-F from Hb-A in agar gel and other adsorptive systems suggests, if their α-polypeptide chains are identical[37], that the β^A-chains contain the structural elements responsible for adsorption, whereas the α^A- and the γ^F-chains do not. The reported structure[38] of Hb-H, *viz.* β^A, is not in these terms in accord with its low adsorption. The different extent of adsorption of haemoglobins A, D and E from haemoglobins G, S and C, and these latter from each other, should be reconcilable with their known structural differences. These may be summarised in terms of the one varying amino acid unit in each β-chain, *viz.* Hb-A[39], glutamic acid; Hb-S[39], valine-; Hb-C[39], lysine-; Hb-G[40], glycine-; Hb-E[41], lysine- (for a different glutamic acid residue).

In the case of Hb-S the loss of the negative groups (carboxyls of glutamic acid, when replaced by valine) is responsible for a large increase in adsorption, and the same situation obtains in Hb-G. In Hb-C, with lysine replacing glutamic acid, the degree of adsorption increases still further. Thus the carboxylate group presumably opposes interaction with the negatively charged groups in the agar, whereas the addition of a further amino group (present as NH_3^+) promotes the interaction. A similar substitution occurs in Hb-E, but with no changes in the adsorptive behaviour. The groups involved in the substitutions in haemoglobins A, S, C and G must therefore be near the surface of the molecule, whereas the group responsible for the difference between Hb-A and Hb-E (and presumably Hb-D and other variants not separating on agar gel) must be remote from it. These results are consistent with the position near the N-terminus of the β-chain, which the aberrant residues in haemoglobins S, C and G are believed to occupy[39,40], by contrast with Hb-E, in which the difference lies in another part of the chain[41].

CONCLUSIONS

It would appear that agar gel electrophoresis under the conditions discussed above might have applications beyond its evident analytical use. The simple reasoning given above suggests that it could, when used in conjunction with conventional electrophoretic techniques, provide further correlations with structural studies. Certainly, as indicated by Figs. 8 and 9, it offers a method of greater interest for adsorption studies than the use of, for instance, alumina[42], which like most media of this kind, is essentially a denaturing agent[43]. The adsorption isotherms may be of value for the characterisation of individual haemoglobin variants.

SUMMARY

Experimental variables in the electrophoresis of human haemoglobins in agar gel have been investigated. The migration of haemoglobins in this medium below neu-

trality, including the separation of the adult and foetal pigments, is explicable in terms of an adsorptive mechanism. Differences in the behaviour of some haemoglobin variants in agar gel electrophoresis have been tentatively correlated with their known structural differences.

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A SIMPLIFIED MULTICHAMBER GRADIENT MIXER

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A previous report[1] described a mixer capable of producing an almost infinite number of systematically variable gradients, simple or compound, for use in chromatography. It comprised a ring of nine serially connected cylindrical chambers, the contents of which were stirred magnetically or by rotating paddles as liquid flowed from one to another to maintain hydrostatic equilibrium in response to the removal of liquid from one end of the series. The shape of the gradient was determined by the concentration initially introduced at each position, and any number of different solutes could be given independent gradients in the emerging liquid. This communication presents a more compact version that is simpler and less expensive to construct, although identical in its theoretical aspects. In the new design, nine rectangular chambers are arranged linearly, and mixing is accomplished by the oscillation of a line of stirrers in a manner similar to that of a windshield wiper.

Fig. 1 provides end and top views of a mixer constructed of Lucite and capable of holding 1200 ml. The sides and ends of the tank are 1/4 in. thick, the bottom 3/16 in. Eight partitions of 1/8 in. Lucite divide the interior into chambers measuring

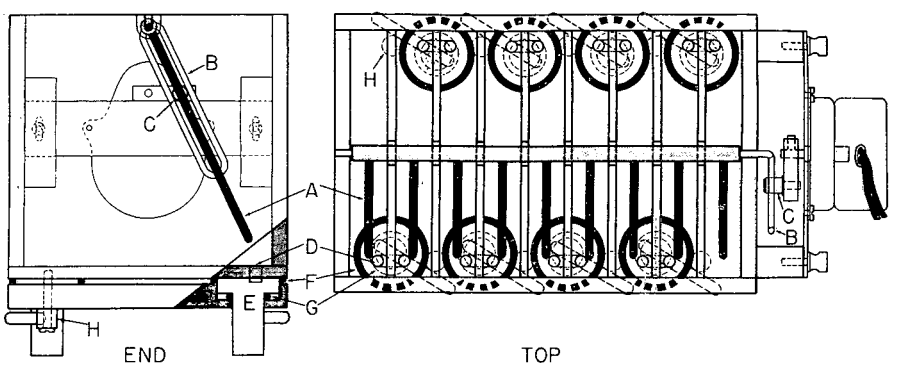

Fig. 1.

10 × 10 × 1.5 cm. The stirring unit is a comb-like assembly of nine Lucite rods (A), 1/8 in. in diameter, mounted in a 1/4 in. rod that is supported at its ends by inserts of 3/32 in. stainless steel wire that rest in 1/8 in. slots in the end walls of the tank. One of these inserts extends beyond the end of the tank and is bent and soldered to

form a flat loop (B) in a plane perpendicular to the axis of the 1/4 in. rod. Within this loop moves a brass sleeve (C), rotating freely on a steel pin fixed to a steel arm, which is in turn attached to the shaft of a 60 r.p.m. Cramer double torque (60 in. oz. at 1 r.p.m.) synchronous motor. As the motor shaft turns, the pin moves in a 1.5 in. circle, imparting a back and forth motion to the stirring assembly. A marked difference between the forward and backward speeds prevents the building up of sizable waves and aids mixing by causing a vertical circulation of the liquid in the direction, at the bottom of the chamber, of the more rapid motion. The 1/8 in. stirring rods used in this model have proved adequate for mixing 1 *M* NaCl with water, but liquids differing greatly in density will require the use of paddles instead of rods. Paddles would be needed, also, for larger mixers. In such cases the stirring unit can be cut as a single piece from a sheet of Lucite.

Entrance and exit holes (D) are 3/16 in. in diameter and are located at opposite ends of the floor of each chamber. The Teflon stopcock plugs (E) are 1 in. in diameter, with 1/2 in. shanks. Off center channels, 3/16 in. wide and 1/16 in. deep, in the flat, upper surfaces of the plugs connect exit and entrance holes of adjacent chambers when the stop-cocks are in the open position. In the closed position only one of the holes of a given pair remains connected with the channel. The rounded ends of each channel, corresponding also to the positions of the holes, are centered on radii of the Teflon plug 120° apart. Neoprene "O" rings (F), 1 in. in diameter, prevent leakage to the exterior. The plugs are supported in a 3/8 in. Lucite plate and are pressed against the holes in the bottom of the tank by 1/2 in. "O" rings (G) that are compressed by tightening the ten screws (only one is shown) that fasten the stopcock assembly to the tank. These screws pass through the centers of the sections of 5/8 in. Lucite rod that serve as stops (H) for the stopcock handles. The tank is covered with a 1/8 in. Lucite plate (not shown) containing one or more holes to accommodate a glass or plastic outlet tube (see ref. [1]). A leveling bubble should be mounted on the top surface of the cover and two small Lucite studs on its bottom surface to fit into diagonally opposite corners of the tank. A 1/2 in. steel rod fastened along the bottom of the stopcock plate provides a means of mounting the apparatus on a vertical supporting rod.

The mixer can be constructed with fixed channels instead of stopcocks, in which case the holes are stoppered with removable plugs while the chambers are being filled[1]. However, this will result in a significant loss in operating convenience.

SUMMARY

An improved design for a nine chambered variable gradient mixer, employing a single oscillating stirrer unit and easily made stopcocks, is described.

REFERENCE

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DETERMINATION OF STEROIDS BY PAPER STRIP ELUTION CHROMATOGRAPHY

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Despite the widespread application of paper chromatography to almost every field of biochemistry, the quantitative analysis of the separated compounds presents problems which are still to some extent unsolved. Measurements are usually undertaken either *in situ* on the paper, or in solution after the area containing the compound has been cut out and eluted. The first method, whether it involves visual, densitometric or fluorometric[1] comparison with standards chromatographed at the same time, is subject to greater error than most other laboratory analytical procedures. The second method involves an extra step, with consequent loss of time and material. In addition, other compounds in the near vicinity may be included in the area cut out for elution.

The method of fractional analysis of the eluate, commonly used in column chromatography, has not, to our knowledge, been applied to descending paper chromatography except by SOLMS[2], who employed a rotating paper cylinder provided with saw teeth along its lower border. This arrangement was convenient for preparative purposes. In the present work we used a paper strip, which is preferable for analytical purposes because of the smaller volume of effluent collected. Consideration was also given to the importance of minimizing vapour losses, so that systems employing volatile solvents[3] could be used.

EXPERIMENTAL

The apparatus is shown diagrammatically in Fig. 1. The chromatographic chamber is a glass cylinder 12 in. in height and 6 in. in diameter, and is provided with a hole 1 in. in diameter in the center of the base. This hole is sealed with a hollow polyethylene stopper ("Teflon" would probably be more satisfactory, because it is more resistant to organic solvents). The chromatographic paper is a strip approximately 27 cm long, pointed at its lower end. The distance from origin to tip is 19 cm. Mobile phase drips off the end of the paper and through a glass funnel into the fraction collector. The stem of the funnel passes through a small hole in the polyethylene stopper and is thereby held securely. A small drop of fluid is normally retained at the tip of the funnel, thus minimizing exchange of gases with the outside atmosphere. For equilibration purposes the usual arrangements appropriate for chromatography

with volatile solvents[3] are used. The trough containing mobile phase is supported on a wire basket adapted from a test tube rack. Delivery of mobile phase into the trough may be effected automatically by a time clock, if desired, using the apparatus previously described[4].

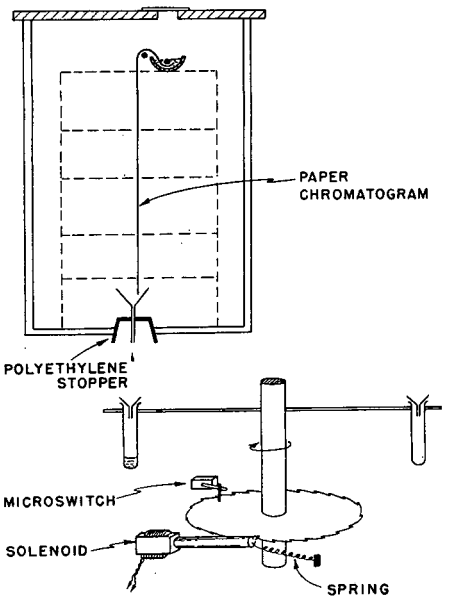

Fig. 1. Apparatus for paper strip elution chromatography.

The fraction collector comprises a circular steel plate 11 in. in diameter, carrying twenty-four 75 × 10 mm Pyrex test tubes. The plate is rotated by a solenoid acting on a toothed wheel at intervals preset by an electronic timer. After tube 24 has been reached, the turning mechanism can be switched off by a lever mounted vertically on the wheel, which actuates a micro-switch.

The parameters of the method were examined using mixtures containing 5 or 10 μg of each of the standard steroids (corticosterone, cortisone and cortisol), chromatographed at 25° in the toluene–75 % methanol system of BUSH[5], after 4 hours' preliminary equilibration. In some experiments the paper was first washed with boiling methanol–ethyl acetate (20:1) for 72 hours in a Nolan extractor.

In operating the turntable an interval was selected which allowed approximately 0.5 ml of effluent to collect in each tube. Timed operation was found to be simpler than drop-counting and weight- or volume-actuated devices and possessed the additional advantage, when chromatography was carried out in a temperature-controlled room using flammable solvents, that a minimum of electronic equipment was situated in the immediate vicinity of the tank. Such apparatus as was required was located outside the chromatography room and included a revolution counter, which recorded each movement of the turntable and hence the number of tubes filled.

The eluted fractions were dried *in vacuo*, and the Δ^4-3-ketosteroid content estimated in the same tubes by alkaline fluorometry in potassium *tert.*-butoxide[6].

RESULTS AND DISCUSSION

Fig. 2(a) shows the elution profile obtained with a mixture of 10 μg each of corticosterone, cortisone and cortisol, using a strip of unwashed Whatman No. 3MM paper 2 in. wide. Fig. 2(b), (c) and (d) shows the results of experiments using mixtures of

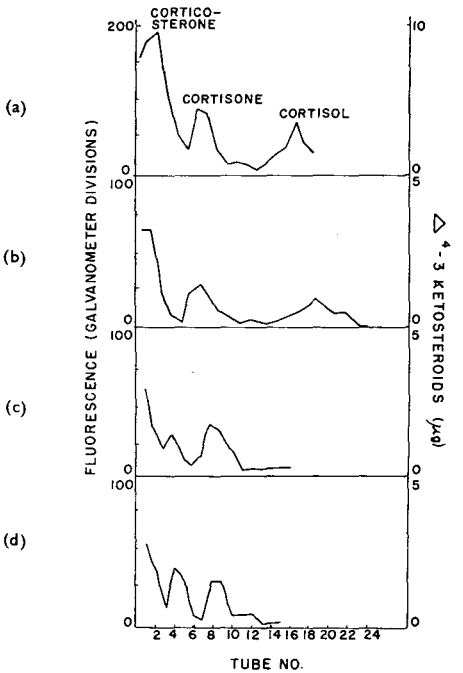

Fig. 2. Elution profiles of mixtures of corticosterone, cortisone and cortisol.

5 μg of each steroid chromatographed on washed paper. In (b) a 2-in. strip of No. 3MM paper was used, in (c) a 1-in. strip of No. 3MM paper, and in (d) a 2-in. strip of Whatman No. 1 paper.

It will be seen that the separation and measurement of steroids in amounts as small as 5 μg are readily possible. At these levels the use of washed paper is recommended, for with the fluorometric technique employed the blank was reduced to negligible proportions (approximately 0.15 μg). The recovery of cortisol in the four experiments was 88 %, 74 %, 104 % and 102 % respectively.

When 2-in. No. 1 paper was substituted for 2-in. 3MM paper, the steroids were eluted in smaller volume. A similar effect was produced when 1-in. 3MM paper was substituted for 2-in. 3MM. In both instances the effect can be attributed to alteration of the cross-sectional area of the strips.

The technique is readily adaptable to other solvent systems, groups of compounds, or detection methods (including liquid scintillation counting).

ACKNOWLEDGEMENTS

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SUMMARY

A technique is described for the separation and quantitative analysis of Δ^4-3-ketosteroids using paper strip elution chromatography. The method is sensitive to quantities of steroid of the order of 5 μg.

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CHROMATOGRAPHIE D'ADSORPTION SUR PAPIER DES ACÉTATES DE STÉROÏDES PEU POLAIRES

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De nombreux systèmes de solvants ont été proposés pour réaliser la chromatographie des mono-acétates des stéroïdes présentant au moins 3 oxygènes et des diacétates des stéroïdes ayant au moins 4 oxygènes[1-4].

En revanche, les monoacétates des stéroïdes dioxygénés et les diacétates de stéroïdes trioxygénés ne peuvent être séparés dans les systèmes aussi lents que: méthylcyclohexane/propanediol, ligroïne/méthanol–eau, heptane/propanediol, triméthyl-2,2,4 pentane/méthanol–eau; car l'expérience prouve qu'ils migrent avec le front du solvant ou au voisinage de celui-ci, c'est ainsi que nous n'avons pas réussi à séparer les acétates des isomères de la 3ξ,21-diol dihydroxy 5ξ pregnane 20-one dans ces systèmes.

Il en est de même pour les acétates des stéroïdes dioxygénés en C_{19}; toutefois, NEHER ET WETTSTEIN[5] et SAVARD[6] en utilisant comme phase stationnaire le phénylcellosolve ont réussi à séparer les acétates d'androstérone et d'épiandrostérone avec des R_F voisins: 0.34 et 0.29 respectivement.

La difficulté de la séparation des acétates des isomères peu polaires, nous a amenés à les déposer sur du papier Whatman No. 1 non imprégné par une phase stationnaire et à tenter leur séparation en faisant passer les solvants suivants: ligroïne, heptane, triméthyl-2,2,4 pentane, méthylcyclohexane et décaline. Cela revenait, en fait, à utiliser le papier comme un adsorbant et à réaliser une véritable chromatographie d'adsorption sur papier avec des solvants peu polaires.

Les acétates des stéroïdes utilisés dans ce travail sont:

4 diacétates de stéroïdes trioxygénés*:
(1) 5β pregnane 3α,21-diol, 20-one
(2) 5α pregnane 3β,21-diol, 20-one
(3) 5β pregnane 3β,21-diol, 20-one
(4) pregna 5-ène 3β,21-diol, 20-one

5 monoacétates de stéroïdes dioxygénés:
(5) 5α androstane 3α-ol, 17-one
(6) 5β androstane 3α-ol, 17-one
(7) 5α androstane 3β-ol, 17-one
(8) 5β androstane 3β-ol, 17-one
(9) androsta 5-ène 3β-ol, 17-one

* Le stéroïde 5β pregnane 3α,21-diol, 20-one a été envoyé par le Dr. W. TAYLOR, le stéroïde 5α pregnane 3β,21-diol, 20-one par le Dr. A. ZAFFARONI, le stéroïde 5β pregnane 3β,21-diol, 20-one par le Dr. S. K. FIGDOR et le pregna 5-ène 3β,21-diol, 20-one par le Dr. W. KLYNE; nous les remercions tous vivement.

MODE OPÉRATOIRE

Acétylation

200 μg de chaque stéroïde ont été acétylés en présence de 0.20 ml de pyridine et 0.20 ml d'anhydride acétique pendant 18 h à température ambiante. La solution est évaporée à sec et les traces des réactifs éliminés par addition et évaporation de benzène, puis de méthanol.

Dépôt des acétates de stéroïdes

Dans nos premières expériences, nous avons dissous les acétates dans l'éthanol et nous avons constaté qu'une grande partie demeurait sur la ligne de départ; cet inconvénient était en grande partie supprimé en utilisant le mélange acétone–acétate d'éthyle 1:1 v/v; dans nos essais plus récents, nous avons réussi à chromatographier la totalité du dépôt en procédant de la façon suivante: au niveau de la ligne de départ, le papier est imbibé avec un mélange de méthanol–propanediol 2:1 v/v sur une hauteur de 2 cm et l'acétate est déposé dans cette région, de sorte qu'on a au démarrage une chromatographie de partage à laquelle succède une chromatographie d'adsorption sur le papier.

On dépose de 3 à 8 μg de chaque acétate dans 4–10 μl. Les cuves sont équilibrées avec les différents solvants pendant 24 h à 24° $\pm$ 1°.

La chromatographie descendante se poursuit selon le cas pendant 3 à 4 h pour les quatre premiers solvants et pendant 8 h pour la décaline.

Les réactions suivantes sont utilisées pour la détection des taches:

réaction au bleu de tétrazolium pour les stéroïdes α-cétoliques;

réaction de Zimmermann pour les 17-cétostéroïdes.

RÉSULTATS

Nous n'avons pas constaté de traînées pour les différents acétates lorsque les quantités chromatographiées sont de 3 à 8 μg; la tache est circulaire, ayant un diamètre de 1.5 à 3 cm.

Le Tableau I donne les résultats de la chromatographie pour cinq solvants utilisés avec trois isomères de diacétates de tétrahydro-désoxycorticostérone: 5β

TABLEAU I

Diacétate	*Structure*	R_T 5β,3α TH-DOC-diacétate				
		Ligroïne	*Heptane*	*Méthyl-cyclohexane*	*Triméthyl-pentane*	*Décaline*
5β,3α TH-DOC	3α,5β	1 (8 cm/h)	1 (9 cm/h)	1 (7.5 cm/h)	1 (4 cm/h)	1 (3.5 cm/h)
5α,3β TH-DOC	3β,5α	0.56	0.55	0.85	0.42	0.77
5β,3β TH-DOC	3β,5β	0.54	0.59	0.82	0.40	0.75
Pregna 5-ène 3β,21-diol, 20-one	3β,Δ^5	0.58	0.55	0.77	0.41	0.69

pregnane 3α,21-diol, 20-one (5β,3α TH-DOC), 5α pregnane 3β,21-diol, 20-one (5α,3β TH-DOC), 5β pregnane 3β,21-diol, 20-one (5β,3β TH-DOC), et le pregna 5-ène 3β,21-diol, 20-one.

On constate que les deux stéroïdes isomères ayant un hydroxyle équatorial sont nettement séparés dans ces systèmes; le stéroïde de structure 3α,5β est moins polaire que l'isomère 3β,5α.

Les vitesses de migration des stéroïdes de structure 3β,5β (axiale), 3β,5α (équatoriale) et du stéroïde non saturé sont très voisines.

Le Tableau II indique les résultats obtenus dans les différents solvants avec les monoacétates des 17-cétostéroïdes suivants: androstérone (5α androstane 3α-ol, 17-one); épiandrostérone (5α androstane 3β-ol, 17-one); étiocholanolone (5β androstane 3α-ol, 17-one); et déhydroépiandrostérone (androsta 5-ène 3β-ol, 17-one).

TABLEAU II

Monoacétate	*Structure*	*R_T étiocholanolone-monoacétate*				
		Ligroïne	*Méthyl-cyclohexane*	*Heptane*	*Décaline*	*Triméthyl-pentane*
Androstérone	3α,5α	0.97	1.03	0.98	0.97	0.91
Étiocholanolone	3α,5β	1 (8 cm/h)	1 (9 cm/h)	1 (6 cm/h)	1 (3 cm/h)	1 (5 cm/h)
Épiandrostérone	3β,5α	0.72	0.85	0.73	0.79	0.51
3β Étiocholanolone	3β,5β	0.73	0.83	0.85	0.80	0.59
Déhydroépi-androstérone	3β,Δ^5	0.69	0.79	0.84	0.80	—

On constate le même phénomène que précédemment, à savoir que les deux isomères ayant un hydroxyle équatorial sont bien séparés l'un de l'autre. Le stéroïde de structure 3β,5α est plus polaire que son isomère 3α,5β. Les deux isomères axiaux sont également bien séparés l'un de l'autre.

La migration du stéroïde de structure 3β,5α est voisine de celle de son isomère 3β,5β et de celle de la déhydroépiandrostérone (3β,Δ^5).

La migration du stéroïde de structure 3α,5α est voisine de celle de son isomère 3α,5β.

DISCUSSION

Les résultats précédents indiquent qu'il est possible de réaliser sur une feuille de papier Whatman une chromatographie d'adsorption qui est assez lente pour séparer des substances aussi peu polaires que les monoacétates des stéroïdes dioxygénés et les diacétates des stéroïdes trioxygénés, pour lesquelles les systèmes habituels ne conviennent pas.

Il est bien établi depuis les travaux de SAVARD[6,7] et NEHER[8] que la chromatographie de partage permet de bien séparer les deux stéroïdes isomères axiaux des isomères équatoriaux à l'état libre. Cette séparation est également possible dans le

cas des acétates de 11-oxo-étiocholanolone (3α,5β) et de 11-oxo-androstérone (3α,5α) ou encore dans des diacétates de tetrahydrocortisol (3α,5β) et allo-tetrahydrocortisol (3α,5α) et de diacétate de tetrahydrocortisone (3α,5β) et allo-tetrahydrocortisone (3α,5α)[9].

Il est intéressant de comparer l'ordre de migration des acétates des isomères de la TH-DOC dans notre méthode (Tableau I) avec l'ordre de migration des mêmes stéroïdes libres dans les différents systèmes de chromatographie de partage (Tableau III).

Nous pouvons faire les remarques suivantes:

(1) La migration de l'isomère 3β,5β par rapport à celle de l'isomère 3α,5β est inversée dans les deux systèmes. En chromatographie de partage, l'isomère 3α,5β est plus polaire que l'isomère 3β,5β à l'état libre, alors que dans les systèmes d'adsorption pour les acétates, c'est le stéroïde de structure 3β,5β qui est plus polaire que son isomère 3α,5β.

(2) En chromatographie de partage des stéroïdes libres, il est difficile, voire impossible, de séparer les deux isomères axiaux et les deux isomères équatoriaux l'un de l'autre. Dans la méthode proposée, on obtient une bonne séparation des acétates correspondant aux deux isomères axiaux et aux deux isomères équatoriaux.

(3) En revanche, dans la méthode décrite, l'isomère 3β,5β et l'isomère 3β,5α à l'état d'acétates ont une migration très voisine, de même les isomères 3α,5α et 3α,5β; c'est le contraire dans le cas de la chromatographie de partage des stéroïdes libres.

Le schéma suivant montre la différence de polarité des stéroïdes axiaux et équatoriaux à l'état libre et acétylé.

Polarité croissante →

Stéroïde — Libre	3α,5α ≅ 3β,5β < 3α,5β ≅ 3β,5α axiaux équatoriaux	(systèmes de chromatographie de partage)
Stéroïde — Acétylé	3α,5α ≅ 3α,5β < 3β,5β ≅ 3β,5α axial équatorial axial équatorial	(systèmes de chromatographie décrits)

TABLEAU III

Stéroïde	R_T 5β,3α TH-DOC				
	Ligroïne/ propanediol	*Heptane/ formamide*	*Tétraline/ propanediol*	*Décaline/ propanediol*	*Décaline/ méthanol-eau*
5β,3α TH-DOC	1.00	1.00	1.00	1.00	1.00
5α,3β TH-DOC	0.92	0.85	0.94	0.87	1.17
5β,3β TH-DOC	1.66	1.83	1.17	1.91	2.00
Pregna 5-ène 3β,21-diol, 20-one	0.68	0.65	0.84	0.62	0.93

CONCLUSION

En utilisant une méthode de chromatographie d'adsorption sur feuille de papier pour monoacétates et diacétates de stéroïdes peu polaires, on obtient des R_F qui sont plus faibles que ceux des systèmes de chromatographie de partage les plus lents. En outre, les vitesses de migrations relatives des diacétates de différents steroïdes sont très différentes de celles que l'on trouve dans les systèmes de chromatographie de partage.

En associant la chromatographie de partage de stéroïdes libres à la chromatographie d'adsorption sur papier de leurs acétates, on dispose d'un moyen qui permet de séparer et par suite d'identifier des stéroïdes isomères ayant un hydroxyle axial ou équatorial.

Dans une première étape on sépare par chromatographie de partage dans des systèmes appropriés les isomères axiaux (3α,5α et 3β,5β) des isomères équatoriaux (3α,5β et 3β,5α). Dans une deuxième étape ces stéroïdes sont acétylés et leurs acétates sont à nouveau chromatographiés dans les systèmes d'adsorption sur papier, ce qui permet la séparation des deux isomères de chaque catégorie.

RÉSUMÉ

Les auteurs décrivent une méthode de chromatographie d'adsorption sur papier avec des solvants peu polaires permettant la séparation des mono- et diacétates de stéroïdes di- ou trioxygénés.

Ce procédé permet de séparer les deux isomères ayant en C_3 un hydroxyle axial ainsi que les deux isomères ayant un hydroxyle équatorial en C_3.

SUMMARY

A method for the separation of mono- and diacetates of di- and trioxygenated steroids is described. This procedure involves chromatographic adsorption on paper with less polar solvents.

Of the steroids that have a C_3 hydroxyl group it was possible to separate the two isomers with an axial configuration as well as the two isomers with an equatorial configuration.

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CHROMATOGRAPHIE PAR ADSORPTION SUR PAPIER

V. SUR LES COMPLEXES DU RHÉNIUM ET DU TECHNÉTIUM AVEC LA THIOURÉE

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(Reçu le 27 août 1960)

Nous avons récemment étudié la chromatographie par adsorption sur papier de quelques complexes avec la thiourée[1] et nous avons pensé qu'il serait intéressant d'étendre ces études aux complexes formés avec le rhénium et le technétium.

Les références sur de tels complexes sont fort rares; RYABCHIKOV ET LAZAREV[2] ont mentionné un composé jaune du rhénium, JASIM et ses collaborateurs[3] ont, pour leur part, décrit un composé orange du technétium.

Ces deux complexes présentent un certain nombre de points communs: même valeur de R_F en chromatographie de partage ou d'adsorption, spectres relativement similaires, valences apparemment identiques. Ceci semble en contradiction avec les observations de JASIM *et al.*[3] qui ne purent obtenir le complexe Tc–thiourée qu'à partir de TcO_4^-. D'autre part les complexes formés par le rhénium ou le technétium sont stables et peuvent être chromatographiés avec des solvents ne contenant pas de thiourée, ce qui est assez inattendu, RYABCHIKOV ET LAZAREV[2] ayant bien noté que le complexe du rhénium n'obéit pas à la loi de Beer.

La présente étude avait donc pour but de clarifier ces quelques points. Nous avons utilisé dans nos travaux la spectrophotométrie, la potentiométrie et la chromatographie par adsorption sur papier. Les résultats obtenus nous permettent de présenter une méthode rapide de séparation du Re et du Tc, méthode dont le besoin a été encore souligné récémment[4]. Les techniques utilisées auparavant en électrophorèse et chromatographie sur papier se révèlent plus lentes et plus difficiles à appliquer pour des macroquantités[5].

EXPÉRIENCES

Les complexes Re–thiourée

RYABCHIKOV ET LAZAREV[2] ont obtenu leurs complexes par mélange de thiourée, de perrhénate, et de chlorure stanneux en solution chlorhydrique. Ils ont suivi la réaction par photométrie. Ils ont obtenu un maximum d'intensité de coloration pour un rapport molaire perrhénate/chlorure stanneux de 1:1, c'est-à-dire une réduction affectant deux électrons et ils ont conclu que le composé formé devait être pentavalent;

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de plus selon eux, le développement total de la couleur ne peut être atteint qu'après 30 minutes d'attente au moins. Enfin ils ont noté que la loi de Beer n'est pas applicable pour la couleur obtenue mais que les déterminations colorimétriques de microquantités avec des courbes étalons donnent des résultats bien reproductibles. Ils ont également noté l'influence de la température et de la concentration en HCl.

Nous avons mélangé qualitativement dans nos expériences préliminaires perrhénate, $SnCl_2$ et thiourée excès; nous avons observé le développement d'une coloration pourpre évoluant progressivement au brun. Les chromatogrammes de ces solutions réalisées avec HCl 2 *N* comme solvant, donnaient généralement trois bandes bien distinctes: le perrhénate, un composé pourpre et un composé jaune intense. Les trois composés réagissent avec un mélange $SnCl_2$–KCNS en donnant des taches rouge-brun.

CROUTHAMEL[6] a étudié des complexes à peu près identiques formés entre Tc et thiocyanate; il a observé un composé rouge de Tc(V) et un composé jaune de Tc(IV).

Nous avons réalisé deux séries d'expériences afin de déterminer les états de valence des différents complexes du rhénium avec la thiourée:

1. Par titrage potentiométrique. Les réactions sont trop lentes pour pouvoir être concluantes. La Fig. 1 montre la courbe de titrage obtenue en espaçant de 30

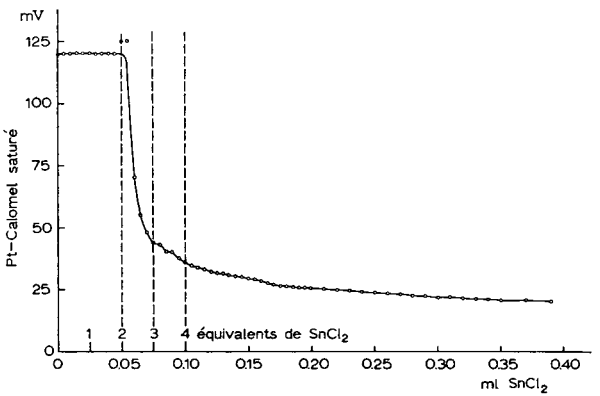

Fig. 1. Titrage de 0.1 mmoles de perrhénate–20 mg thiourée dans 2 ml de HCl 2 *N* avec *M*/2 $SnCl_2$ (precisément *M*/2 × 0.94) (dans HCl 2 *N*). Électrode: Pt/calomel saturé.

secondes deux additions consécutives de réactif. La chute de potentiel n'a lieu qu'après addition de deux équivalents de réducteur, la stabilisation n'intervient qu'après addition de trois équivalents.

2. Par titrage avec analyse chromatographique après chaque addition du réactif. La solution a été obtenu par dissolution de 0.1 mmole de perrhénate dans 2 ml de HCl 2 *N* avec un large excès de thiourée (1 mmole = 76 mg). Le réactif est $SnCl_2$ 1 *N* (très exactement 0.94) dans le HCl 2 *N*. Nous avons ajouté le réactif par des quantités correspondant à 0.5, 1, 2, 3 et 4 équivalents et nous avons chromatographié après chaque addition des prélèvements de l'ordre de 0.01 ml.

La Fig. 2 montre les chromatogrammes révélés par pulvérisation sur le papier d'une solution chlorhydrique de $SnCl_2$–KCNS.

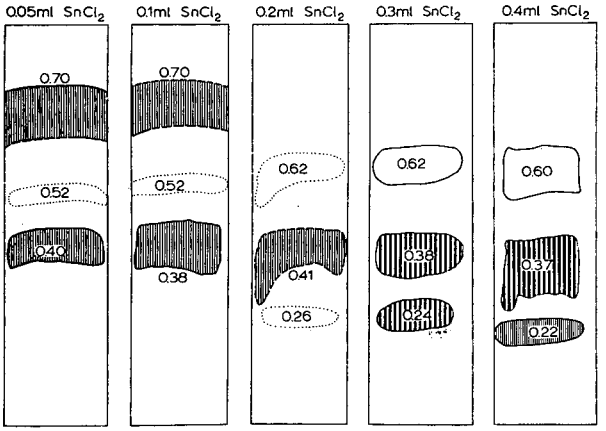

Fig. 2. Les chromatogrammes des mélanges d'une solution perrhénate–thiourée comme dans Fig. 1 avec 0.5, 1, 2, 3 et 4 équivalents de $SnCl_2$ (dans HCl 2 *N*).

La tache à R_F 0.7 est due au perrhénate, elle ne disparaît qu'en présence de deux équivalents de $SnCl_2$. Le perrhénate est alors accompagné d'une faible tache (R_F 0.52) et d'une tache pourpre (R_F 0.41). La tache à R_F 0.52 peut être du Re (VI) instable et peut être rapprochée de celle du Tc décrite par CROUTHAMEL[6]. Avec deux équivalents de réactif, la tache pourpre devient prépondérante; elle est accompagnée de deux taches secondaires, la première jaune à R_F 0.26, la seconde incolore à R_F 0.62. L'addition de 3 équivalents se traduit par un accroissement très net en intensité de la tache jaune au dépens de la tache pourpre avec persistance (à l'état de trace) de la substance incolore à R_F 0.62.

Il ne reste pratiquement que le composé jaune après l'addition d'un quatrième équivalent de réactif, bien que persiste encore quelques faibles traces du composé incolore.

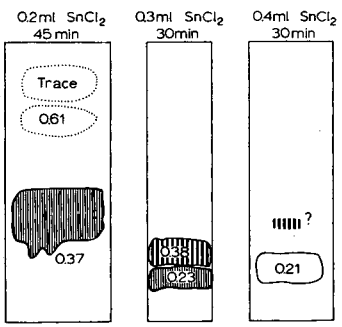

Fig. 3. Les chromatogrammes de mélanges de perrhénate–thiourée avec 2, 3 et 4 équivalents de $SnCl_2$, chromatographiés après un repos de 45, 30 et 30 minutes (respectivement).

La Fig. 3 montre les chromatogrammes réalisés avec des solutions contenant 2, 3 et 4 équivalents de réactif, ces solutions ont été laissées au repos pendant 30 minutes avant d'être chromatographiées. Avec 2 équivalents, le principal composé est pourpre,

avec 3 équivalents, le composé prédominant est jaune, avec un peu de complexe pourpre, enfin avec 4 équivalents l'ensemble est entièrement converti à des traces près en un complexe jaune. La tache incolore à R_F 0.62 n'apparaît qu'après réduction totale de ReO_4^- et disparaît progressivement sauf en présence de quantités importantes de complexe pourpre. Il semble donc que ce soit une forme non-complexée de Re (V).

L'analyse chromatographique de mélanges en proportions variables de ReO_4^- et Sn (II) permet de conclure à la pentavalence du complexe pourpre et à la tétravalence du complexe jaune. Elle montre également toute la complexité de la réaction de réduction, celle-ci pouvant même mettre en jeu du Re (VI) et du Re (V) non-complexé.

L'attente de 30 minutes, dans nos conditions expérimentales, ne semble pas particulièrement efficace et n'a pas amené de réduction totale à la valence la plus basse en présence d'un excès de chlorure stanneux. Nous avons observé le même complexe pourpre pour des échantillons conservés pendant plusieurs jours. Nous avons également effectué un certain nombre d'expériences préliminaires afin d'étudier l'influence du chauffage. Un chauffage au bain-marie amène un certain nombre de complexes bruns entièrement différents de ceux observés à la température ambiante. La thiourée se décomposant à la chaleur, il est fort probable qu'il s'agit d'un mélange de complexes thio-, thiocyanato et thiouréiques.

Les spectres des complexes Re–thiourée

Une solution de complexe pourpre, absolument pure de toute autre forme colorée du rhénium, a été préparée par réduction partielle du perrhénate par chlorure stanneux (voir ci-dessus) et le tout dilué avec une solution HCl 0.5 *N* à 1 % de thiourée. La coloration disparaît avec la dilution en l'absence de thiourée.

La Fig. 4 montre le spectre de cette solution pourpre dont la coloration peut être quelque peu accentuée par addition d'une très faible quantité de $SnCl_2$, le pic à 520 mμ devient alors plus prononcé (Fig. 4b).

Le complexe jaune a été préparé par addition d'un excès de $SnCl_2$ à une solution 2 *N* HCl de thiourée et de perrhénate. La séparation a été réalisée par chromatographie sur papier et l'élution avec une solution HCl 0.5 *N* à 1 % de thiourée.

La Fig. 5 montre des spectres obtenus à deux concentrations différentes. Ce complexe obéit à la loi de Beer (Fig. 6, longueur d'onde 390 mμ).

Les complexes Tc–thiourée

JASIM *et al.*[3] ont obtenu un complexe orange par mélange de pertechnétate et de thiourée en milieu 2 *N* HNO_3 et après un léger chauffage. Selon eux il est indispensable pour l'obtention du complexe que le technétium soit à l'état de pertechnétate.

Nous avons opéré de même[3] en partant d'une solution de 1 mg de pertechnétate fourni par le C.E.A.-Saclay. Le spectre observé est très proche de celui du complexe tétravalent Re–thiourée (voir Fig. 7). La chromatographie en milieu HCl aqueux sur papier Whatman No. 1 donne des valeurs de R_F presque identiques à celles obtenues avec le complexe jaune du rhénium à toutes concentrations chlorhydriques

(Fig. 8). Ces similitudes nous amènent tout naturellement à conclure que le technétium forme un complexe tétravalent avec la thiourée. Ceci n'est guère surprenant car la

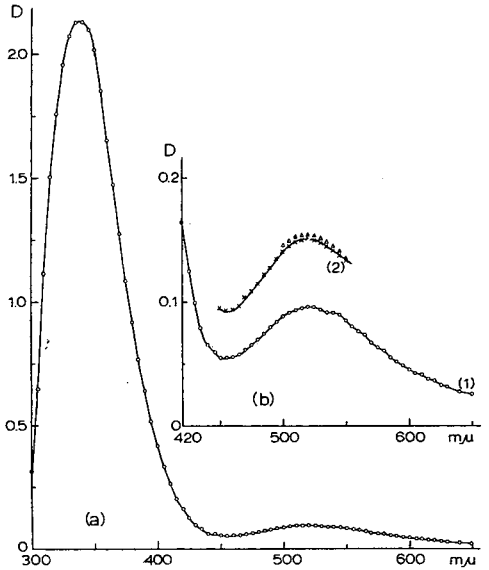

Fig. 4. Le spectre d'absorption du complexe pourpre rhénium–thiourée formé à partir de 0.97 mg de Re avec 4 mg de thiourée et une addition de 0.004 ml de $SnCl_2$ $M/2$. La solution a été dilué à 3.5 ml avec HCl 0.5 N contenant 1 % de thiourée. Fig. 4b (1) est le pic du spectre de Fig. 4a à une plus grande échelle. Fig. 4b (2) la solution avec une addition de 0.006 ml de $SnCl_2$ $M/2$. Spectrophotomètre: Unicam SP 500 avec les cellules en verre de 1 cm épaisseur.

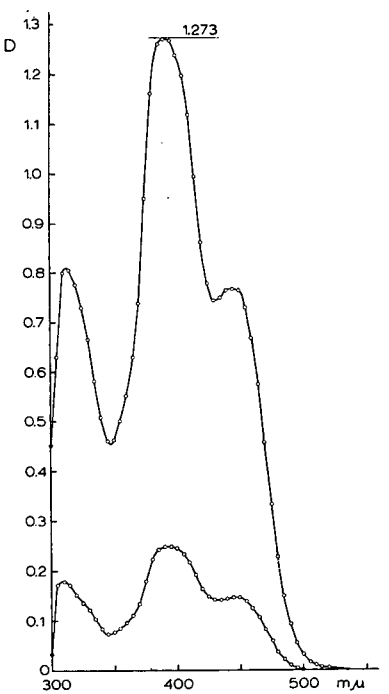

Fig. 5. Le spectre d'absorption du complex jaune du rhénium avec la thiourée dans HCl 0.5 N contenant 1 % de thiourée dans deux dilutions différents.

thiourée est un réducteur suffisamment puissant pour amener Cu (II) à la monovalence et le pertechnétate est plus facilement réduit que le perrhénate.

Nous pourrions également montrer que les solutions de Tc réduit par chauffage avec HCl conc. (GUERLIT[7]) donnent des complexes oranges à valeurs de R_F et à spectre identiques à ceux préparés à partir de TcO_4^- (Fig. 9).

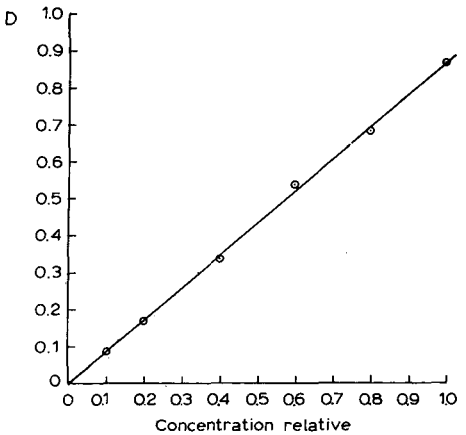

Fig. 6. Graphique de la densité optique contre la concéntration (relative) (vérification de la loi de Beer). Substance: complexe jaune Re–thiourée. Longueur d'onde 390 mμ, aperture de 0.036 mm, cellule de verre d'une épaisseur de 1 cm.

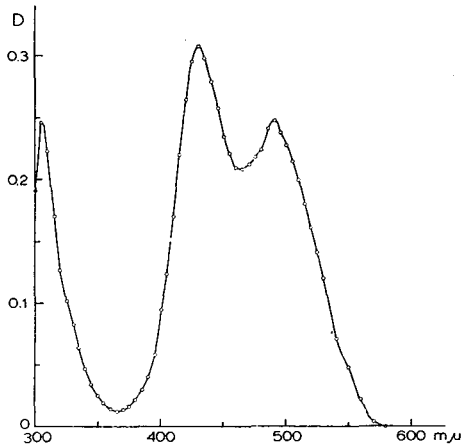

Fig. 7. Spectre d'absorption du complexe Tc–thiourée dans HCl 0.5 *N* contenant 1 % de thiourée. Conditions comme dans Fig. 4.

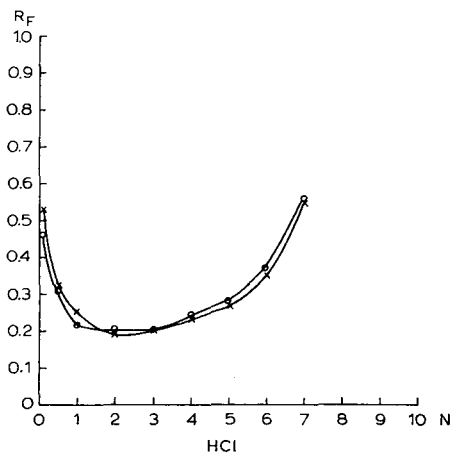

Fig. 8. Valeurs de R_F du complexe jaune de Re–thiourée et du complexe Tc–thiourée sur papier Whatman No. 1 avec comme solvant HCl aqueux. ×—× Re; O—O Tc.

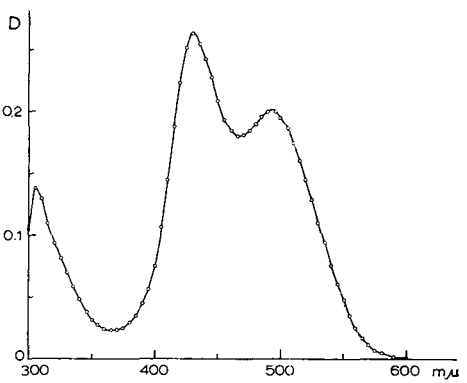

Fig. 9. Spectre d'absorption d'une solution de Tc (IV) (préparé par réduction avec HCl conc.) avec la thiourée dans HCl 2 *N*.

Séparation du rhénium et du technétium

JASIM *et al.*[3] ont noté que le perrhénate ne réagit pas à froid avec la thiourée en milieu nitrique, ni même avec un léger chauffage. Le perrhénate a un R_F de 0.7 en milieu 2 *N* chlorhydrique, celui du complexe Tc–thiourée étant de 0.2. Il en résulte que toutes les conditions d'une bonne et rapide séparation sont réunies à condition que le Re reste quantitativement à l'état heptavalent. On peut aussi obtenir une séparation totale même avec le rhénium partiellement réduit (Re(V)), le rhénium se trouvant sur plusieurs taches.

Nous avons voulu examiner le comportement du rhénium en solution nitrique en présence de TcO_4^- et de thiourée. Nous avons procédé aux expériences suivantes:

12 mg de Re sont dissous dans 2 ml environ de HNO_3 conc. et chauffés jusqu'à disparition complète des vapeurs nitreuses. La solution est alors diluée avec un égal volume d'eau. On prélève une partie de cette solution et on la dilue jusqu'à ce qu'elle soit environ *N* nitrique; on ajoute la thiourée (50 mg environ) et on laisse au repos toute la nuit. Cette solution ne change pas de couleur et la chromatographie opérée après 16 heures avec HCl 2 *N* ne laisse apparaître qu'une seule bande d'acide perrhénique. De même un chauffage de 2 minutes au bain-marie ne modifie pas la coloration et la chromatographie ne révèle également que la seule forme ReO_4^-.

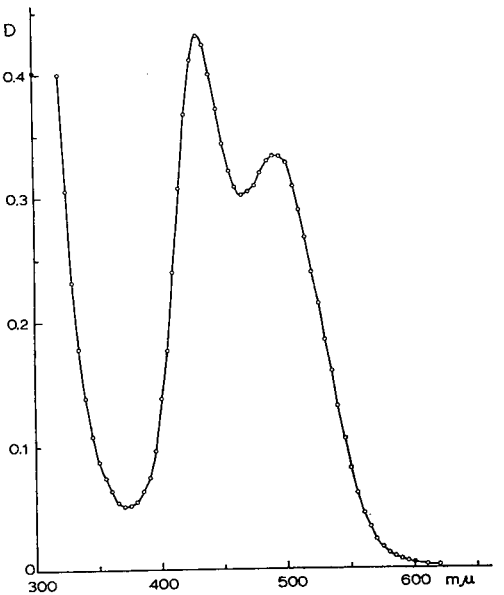

Fig. 10. Spectre d'absorption d'un mélange TcO_4^-–ReO_4^- dans HNO_3 2 *N* avec une addition d'un excès de thiourée. Le spectre a été mésuré après une dilution avec 0.5 *N* HCl contenant 1 % de thiourée.

Nous avons également fait réagir l'acide perrhénique en milieu 6 *N* HNO_3 avec du pertechnétate et un excès de thiourée et chauffage de 2 minutes environ au bain-marie. Nous avons obtenu une solution orangée dont la chromatographie avec

HCl 2 *N* donne une bande orange, lente, correspondant au complexe Tc–thiourée nettement distincte de la bande plus rapide de perrhénate (Fig. 11). Le spectre de la solution est identique au spectre du complexe pure du Tc avec la thiourée (Fig. 10).

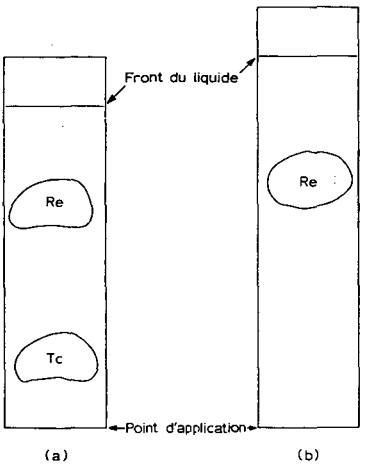

Fig. 11. (a) Un chromatogramme d'une mélange TcO_4^-–ReO_4^- en HNO_3 2 *N* avec un excès de thiourée, développé avec HCl 2 *N*. (b) Un chromatogramme de ReO_4^- seul mélangé avec la thiourée dans HNO_3 2 *N* et laissé au repos pendant 16 heures.

Séparation du $^{99*}Tc$ (5.9 h) de Mo, Re et Mn

Nous avons naturellement été amenés à observer le comportement du Tc en quantité traceur avec la thiourée. Nous l'avons étudié avec du $^{99*}Tc$ sans entraîneur, obtenu à Saclay par irradiation à la pile de molybdène. Des échantillons de $^{99*}TcO_4^-$ (sans entraîneur) ont été mélangés avec quelques cristaux de thiourée et quelques gouttes de HCl dilué, chauffés, puis déposés sur papier et chromatographiés. Le chromatogramme présente un pic d'activité dans la région de R_F 0.2 et un autre très faible dans la région de R_F 0.7. Ce dernier correspond à une période de 65 heures environ et est probablement dû à des traces de molybdène.

Nous avons également pu effectuer des séparations après avoir mélangé du $^{99*}Tc$, le perrhénate (ou du molybdate ou encore des sels manganeux) puis ajouté la thiourée, ces différents éléments migrant avec un R_F de l'ordre de 0.7. Une réduction occasionelle se traduit par deux bandes bien distinctes du pic de technétium. La Fig. 12 montre la répartition des activités sur les chromatogrammes.

La structure des complexes avec la thiourée

RYABCHIKOV ET LAZAREV[2] ont proposé la formule $ReO_2[SC(NH_2)_2]_4Cl$. Il se sont appuyés:

(1) sur l'analyse du complexe insoluble de diphénylthiourée, d'où la détermination des pourcentages de Re et S;

(2) sur l'adsorption sur résine échangeuse de cation de la substance colorée.

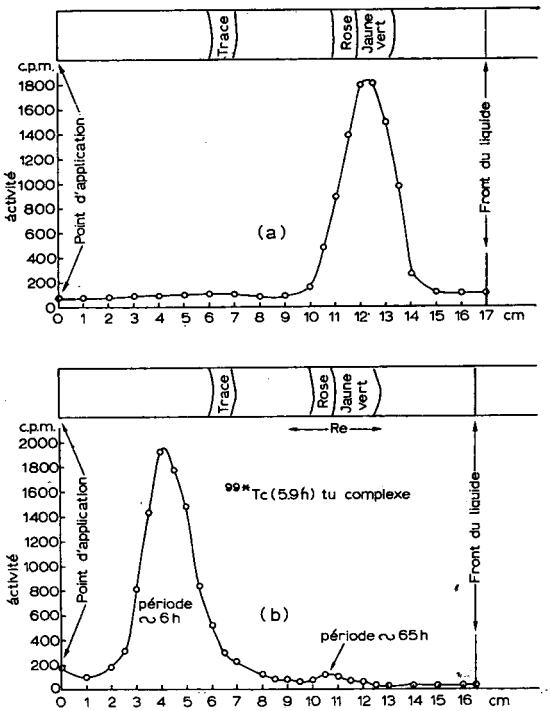

Fig. 12. Distribution de l'activité et des taches révélées avec $SnCl_2$–KCNS: (a) d'une solution de $^{99m}TcO_4^-$ (avec du Mo comme impureté) et (b) la même solution après addition de quelques mg de thiourée. L'endroit ou se trouve le Re est indiqué sur la figure.

Nous avons pu montrer par électrophorèse sur papier (électrolyte 0.5 *N* HCl à 1 % de thiourée, tension 160 V, durée 1 h) que les complexes du rhénium et du technétium sont réellement tous cationiques. Par contre nous n'avons pas pu utiliser les valeurs de mobilité pour estimer les charges car l'adsorption sur le papier est relativement forte. Nos travaux nous ont toutefois permis de conclure à la valence 4 pour les complexes stables du Re et du Tc et, de ce fait, la formule de RYABCHIKOV ET LAZAREV demande à être revisée.

Nous avons noté lors de la plupart de nos expériences de réduction un précipité brun foncé formé soit au repos soit déjà pendant la réduction. Ce précipité, séparé par filtration et dissous dans HCl 2 *N* donne une solution jaune. Déposé directement sur papier et chromatographié, il se dissout très rapidement au développement. Ce solide a toujours donné une seule et unique bande jaune par chromatographie avec HCl 2 *N*. Nous pensons qu'il s'agit soit du complexe peu soluble du Re (IV) avec la thiourée, soit d'un sel double de ce complexe avec $SnCl_2$ ou $SnCl_4$. Nous avons parfois obtenu des cristaux de quelques millimètres en laissant au repos des solutions du complexe Re(IV)–thiourée. Nous nous proposons de reprendre ultérieurement cette question.

DISCUSSION

Nous avons été surpris des différences considérables entre nos résultats et ceux publiés antérieurement. Cependant nous n'avons procédé à la réduction uniquement de l'acide

perrhénique préparé par dissolution de l'élément dans l'eau régale puis évaporation à sec, mais également avec du perrhénate de potassium, spectrochimiquement pur, préparé par Johnson, Matthey & Co. Les résultats ont toujours été rigoureusement identiques. Par contre nos résultats ont été entièrement différents avec de l'acide perrhénique préparé par attaque du métal avec H_2O_2.

JASIM *et al.*[3] ont mentionné lors de la préparation des complexes Tc–thiourée, deux états de valence: valence 7 et valence 4, à ce dernier stade le Tc ne réagit pas en donnent de composés colorés. Notre réduction par chauffage pendant une heure à 100° avec HCl 12 *N* devrait donner, selon GUERLIT[7], une solution de Tc (IV) sous forme de $TcCl_6^{2-}$ or notre solution ainsi préparée s'est révélée aussi réactive avec la thiourée que le pertechnétate d'origine. Nous ne pouvons, pour le moment, fournir d'explications aux observations de JASIM *et al.*

En conclusion, nous voudrions mentionner que la chromatographie d'adsorption sur papier d'ions minéraux est encore peu employée bien que nous en ayons récemment montré certaines possibilités[8]. Nous pensons également que l'étude de réactions de complexation, du genre de celles étudiés ci-dessus, par des méthodes chromatographiques relève d'une technique nouvelle qui peut s'avérer très intéressante dans les recherches sur d'autres réactions ou les équilibres sont très lents à se réaliser.

RÉSUMÉ

Une séparation du Re et du Tc par chromatographie sur papier en présence de la thiourée est décrite. La nature des complexes avec la thiourée de ces éléments était étudiée.

SUMMARY

A separation of Re and Tc by paper chromatography in the presence of thiourea is described. The nature of the complexes formed between these elements and thiourea, was studied.

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DÜNNSCHICHT-CHROMATOGRAPHIE

VI. MITTEILUNG. SPURENANALYSE VON ZUCKERGEMISCHEN AUF KIESELGUR G-SCHICHTEN

EGON STAHL UND ULRICH KALTENBACH

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(Eingegangen den 22. August 1960)

Bisher wurde über die adsorptionschromatographische Trennung lipophiler Gemische auf Kieselgel G-Schichten berichtet und eine Reihe von Arbeitstechniken beschrieben (STAHL, I.–V. Mitteilung)[1-5]. Es schien nun von Interesse, den Anwendungsbereich über die Aminosäuren[6,7] hinausgehend in das Gebiet der hydrophilen Zucker auszudehnen, ohne allerdings die rein anorganischen Trennschichten zu verlassen.

SORPTIONSMITTEL UND ELUTIONSMITTEL

Auf den bisher von uns bevorzugten Kieselgel G- und Aluminiumoxyd G-Schichten lassen sich auch bei Verwendung entsprechend stark polarer Elutionsmittel keine brauchbaren Zuckertrennungen durchführen. Erst der Einsatz von Kieselgur G brachte Erfolge, die sich durch eine schwache Pufferung mit Natriumazetat verbessern liessen. Von den zahlreichen, bisher beschriebenen Lösungsmittelsystemen konnten wir keines direkt übernehmen. Durch stufenweise Variation eines Gemisches von Äthylazetat–Isopropanol–Wasser gelangten wir zu den optimalen Trennbedingungen.

Bei diesen Versuchen beobachteten wir eine u.U. interessante Erscheinung, die

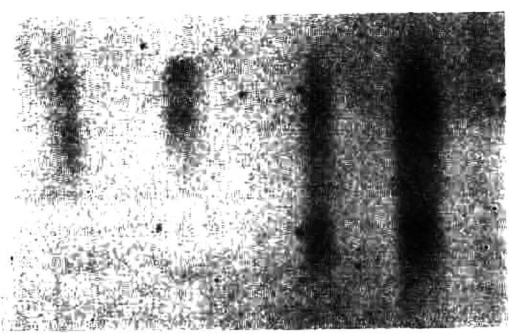

Fig. 1. Ausschnitt aus einem Dünnschicht-Chromatogramm auf ungepuffertem Kieselgur G. Bei der Glucose (links), 0.5 und 1 μg, und der Galaktose (rechts), 0.5 und 1 μg, treten 2 Zonen deutlich hervor.

auf ungepufferten Kieselgur G-Schichten bei Verwendung eines Gemisches Methylazetat (18 Volumenteile)–Isopropanol (1 Volumenteil)–Wasser (1 Volumenteil) besonders deutlich hervortrat: Die in Pyridin gelösten Monosaccharide, am deutlichsten Glucose und Galaktose, trennten sich in 2 Zonen (Fig. 1). Ob es sich hier um eine Trennung in die α- und β-Form oder Aldehyd- (Keto-) und Lactol-Form handelt, bleibt offen. Fig. 1 lässt erkennen, dass dieser Effekt nicht durch einen Entmischungsvorgang des Elutionsmittels zustande kommt. Dieses Phänomen tritt nicht auf, wenn wir ein Gemisch von 65 Volumenteilen Äthylazetat und 35 Volumenteilen Isopropanol 65 % verwendeten. Hiermit lassen sich auf einer Strecke von 10 cm 8 Zucker in 25–30 Minuten trennen (Fig. 2). Durch Variation des Isopropanol–Wasser-Anteils im Gemisch lassen sich die R_F-Werte erhöhen beziehungsweise senken. Interessieren zum Beispiel mehr die Disaccharide, so wird man diesen Anteil auf 35–40 % erhöhen, und ihn bei Pentosen u.U. auf 25–30 % verringern.

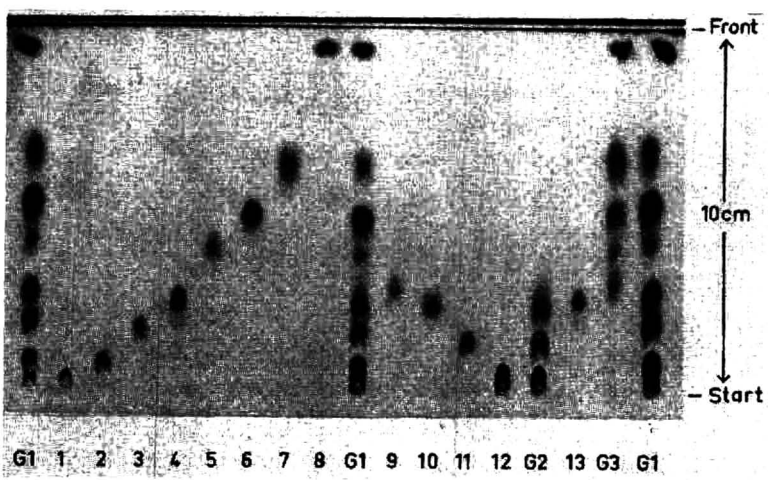

Fig. 2. Dünnschicht-Chromatogramm von Zuckern (je 0.5 μg) auf einer gepufferten Kieselgur G-Schicht. Die Sichtbarmachung erfolgte mit dem beschriebenen Anisaldehyd-Schwefelsäure-Reagenz. G 1 = Gemisch von No. 1–8; G 2 = Gemisch von No. 9–12; G 3 = Gemisch von No. 5–9. In der Tabelle I sind unter den Nummern 1–13 die verschiedenen Zucker aufgeführt.

SICHTBARMACHUNG DER GETRENNTEN ZUCKER

Von den zahlreichen im letzten Jahrzehnt beschriebenen Reagenzien zur Sichtbarmachung der getrennten Zucker (s. z.B. bei MERCK[8] und HAIS UND MACEK[9]) ergab das Anilinphtalatreagenz die brauchbarsten Ergebnisse. Als ein wesentlich empfindlicheres Sprühreagenz erwies sich die für Steroide empfohlene KÄGI-MIESCHER-Reaktion mit Anisaldehyd–Schwefelsäure. Beim Erhitzen der hiermit besprühten Chromatogramme treten deutliche Farbdifferenzierungen auf, die sich neben den R_F-Werten zur Identifizierung heranziehen lassen (Tabelle I).

Das Reagenz ist allerdings nicht spezifisch, wie schon aus seiner bisherigen Verwendung zum Steroidnachweis hervorgeht. Nach unseren Erfahrungen möchten wir

es in die Reihe der Universalreagenzien für Naturstoffe einreihen. Es hat uns zum Beispiel auch bei der Charakterisierung der einzelnen Komponenten ätherischer Öle gute Dienste erwiesen.

TABELLE I

R_F-WERTE UND FARBREAKTIONEN DER ZUCKER

No. (Fig. 2)	*Zucker*	*R_F-Werte**	*Farbreaktion: 0.5 µg Zucker und Anisaldehyd–Schwefelsäure 100°*
1	Lactose	0.04	grünlich
2	Saccharose	0.08	violett
3	Glucose	0.17	hellblau
4	Fructose	0.25	violett
5	D(+)-Xylose	0.39	grau
6	D(—)-Ribose	0.49	blau
7	L(+)-Rhamnose	0.62	grün
8	D(+)-Digitoxose	0.94	blau
9	L(+)-Arabinose	0.28	gelbgrün
10	D(+)-Mannose	0.23	grün
11	D(+)-Galaktose	0.18	grüngrau
12	Maltose	0.06	violett
13	L(—)-Sorbose	0.26	violett

* Die R_F-Werte gelten für das System 65 Teile Äthylazetat – 35 Teile Isopropanol (65%ig) für 20 × 20 cm Kieselgur G-Schichten und "Kammerübersättigung". Sie liegen auf 5 × 20 cm Schichten in runden Trennkammern höher.

DISKUSSION

Über papierchromatographische Zuckertrennungen liegen zahlreiche Arbeiten vor. Als optimale Auftragmenge wird der Bereich zwischen 100 und 500 µg empfohlen und die untere Nachweisgrenze pro Zucker um 5 µg angegeben. Auf Kieselgur G-Schichten liegt bei Verwendung des Anisaldehyd–Schwefelsäure-Reagenzes die untere Erfassungsgrenze bei 0.05 µg, der optimale Bereich um 0.5 µg und die maximale Auftragemenge bei 5 µg pro Zucker (Fig. 3). Die beschriebene Methode ist gegenüber den bisherigen Verfahren um 2 Zehnerpotenzen empfindlicher, ein Vorteil, der bei der Untersuchung von Stoffwechselvorgängen kleiner Zellkomplexe (z.B. von pflanzlichen Drüsen, Siebröhren, Nektarien) von Wert ist.

Die Kieselgur G-Schichten sind den gepufferten Glasfaserpapieren überlegen*, bei denen JAYME UND KNOLLE[10] als besonderen Vorteil die kurze Laufzeit von 2 Stunden (Papier *ca.* 8 Stunden) hervorheben. Wenn GRÜNE[11] zu den Zuckertrennungen auf Glasfaserpapieren schreibt "Hier und da versagt die Methode noch aus bislang nicht erklärbaren Ursachen", so wollen wir annehmen, dass das Kieselgur G "MERCK"[12] in gleichbleibender Güte herstellbar ist. Da es sich im Gegensatz zu den von uns bisher beschriebenen adsorptionschromatographischen Trennungen lipophiler Substanzen[1–5] hier um einen Verteilungsvorgang handelt, machen sich Störungen durch grössere Mengen von Fremdionen deutlich bemerkbar. Dieser Effekt lässt sich jedoch herab-

* Dies gilt wohl auch für die von DIECKERT UND MORRIS[13] zum gleichen Zweck vorgeschlagenen mit Kieselsäure imprägnierten Glasfaserpapiere.

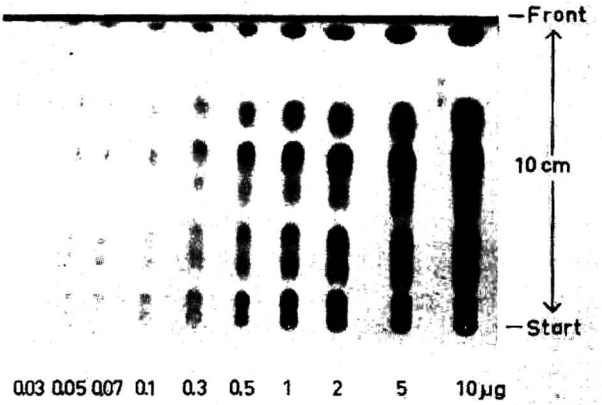

Fig. 3. Dünnschicht-Chromatogramm des Zuckergemisches G 1 (siehe Text Fig. 2). Die aufgetragene Menge pro Zucker steigt von links (0.03 µg) nach rechts (10 µg) an.

drücken, wenn etwa wie bei den zuckerhaltigen Fruchtsäften die Möglichkeit einer starken Verdünnung (z.B. 1:100) mit Pyridin besteht.

Der Anwendungsbereich der Dünnschicht-Chromatographie erstreckt sich nun von den lipophilen bis zu den hydrophilen Gemischen, und es ist nur noch eine Frage der Zeit, die verbliebenen Lücken zu füllen, und die Vor- und Nachteile der Verfahren gegeneinander abzuwägen.

EXPERIMENTELLER TEIL

Kieselgur G-Schichten

Die Herstellung der Trennschichten 200 × 200 mm erfolgte in bekannter Weise mit der DESAGA-Grundausrüstung No. 600*. Als Sorptionsmittel wurden für je 5 Platten 30.0 g Kieselgur G für Dünnschicht-Chromatographie nach STAHL (Hersteller E. MERCK, Darmstadt) mit 60 ml einer 0.02 *M* wässrigen Natriumazetatlösung gleichmässig gemischt. Nach dem Aufstreichen der Schicht wurden die Platten 30 Minuten bei 100° getrocknet und danach die Startpunkte 15 mm vom unteren Rand entfernt durch Einstiche markiert. Die Trennstrecke betrug in allen Fällen 100 mm.

Elutionsmittel

65 ml Essigsäureäthylester "zur Chromatographie" + 35 ml einer Mischung aus 2 Volumenteilen Isopropanol p.a. und 1 Volumenteil dest. Wasser. Das Gemisch wurde täglich frisch bereitet.

Es wurde nur mit "Kammerübersättigung" (s. STAHL[4]) gearbeitet. Die Raumtemperatur lag um 20°.

Zucker

Neben der Zuckerkollektion "Merck" (Tabelle I, No. 1, 2, 3, 4, 5, 9, 11, 13) wurden die uns freundlicherweise von Fa. HOFFMANN LA ROCHE, Basel, überlassenen Zucker (Tabelle I, No. 6, 7, 8, 10, 12) verwendet.

* Hersteller: DESAGA, Heidelberg, Hauptstr. 60.

Die Zucker wurden 0.1 %ig in Pyridin p.a. gelöst und hieraus die entsprechenden 0.01 und 0.005 %igen Verdünnungen hergestellt. Zuckergemische wie Honig, Malzextrakt wurden 0.5 %ig in Pyridin gelöst, Fruchtsäfte wurden 1:100 mit Pyridin verdünnt.

Sichtbarmachung

Besprühen der Schicht nach dem Chromatographieren mit 10 ml eines jeweils frisch bereiteten Gemisches bestehend aus: 9 ml Äthanol 95 % + 0.5 ml konz. Schwefelsäure p.a. + 0.5 ml Anisaldehyd "MERCK". Danach 5–10 Minuten auf 90–100° erhitzen: Farben siehe Tabelle I.

Bei nicht mit Azetat gepufferten Schichten wurde dem vorstehenden Reagenz einige Tropfen Eisessig zugefügt.

ZUSAMMENFASSUNG

Die Dünnschicht-Chromatographie ermöglicht bei Verwendung von Kieselgur G-Schichten mit einem Gemisch von 65 Volumenteilen Essigsäureäthylester + 35 Volumenteilen Isopropanol (65 %ig) die Trennung einer Reihe von Zuckern in 25–30 Minuten. Die untere Erfassungsgrenze der Zucker liegt bei zusätzlicher Verwendung eines Anisaldehyd–Schwefelsäure-Reagenzes um 2 Zehnerpotenzen unter der Papierchromatographie.

SUMMARY

By means of thin-layer chromatography on Kieselguhr G layers it is possible to separate a number of sugars in 25–30 minutes with a mixture (65:35 v/v) of ethyl acetate + isopropanol (65 %). Using an anisaldehyde–sulphuric acid reagent the minimum amount of sugar that can be detected is 2 orders of magnitude lower than in paper chromatography.

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SÉPARATION RADIOCHIMIQUE DES LANTHANIDES DANS LES PRODUITS DE FISSION

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Notre but étant d'isoler les produits de fission du groupe des lanthanides ou terres rares, afin de les doser par analyse et mesure physiques de leurs rayonnements, il nous était nécessaire de connaître le rendement chimique de la séparation, avec précision. Nous avons donc utilisé la chromatographie par échange d'ions qui permet de travailler avec des éléments entraîneurs en quantité pondérable.

Nos séparations ont porté, jusqu'à présent, sur le cérium, le praséodyme, le néodyme, le samarium, l'europium et l'yttrium, afin de pouvoir mesurer leurs isotopes radioactifs, ^{141}Ce, ^{144}Ce, ^{143}Pr, ^{147}Nd, ^{154}Sm, ^{156}Eu, ^{91}Y, ^{93}Y; cependant, la méthode que nous proposons, peut être utilisée pour la séparation de tous les éléments des terres rares dans les produits de fission.

MODE OPÉRATOIRE

Il se divise en trois parties:

I. Séparation et purifications préliminaires du groupe des lanthanides.

II. Séparation chromatographique par échange d'ions.

III. Précipitation des éléments et montage des sources.

I. Séparation et purifications préliminaires

La séparation chromatographique doit être précédée de l'isolement du groupe des terres rares de la solution nitrique des produits de fission, puis d'une purification, pour éliminer le plutonium, l'uranium et les autres produits de fission.

1. Principe

On précipite les fluorures de terres rares en milieu oxydant (bromate), en présence d'entraîneur zirconium pour éliminer l'uranium, le plutonium et la plupart des autres produits de fission[1]. Après redissolution du précipité par NO_3H en présence d'ions borate, une précipitation des hydroxydes de lanthanides en présence d'entraîneur baryum permet d'éliminer les activités baryum et strontium[1].

Les hydroxydes redissous par HCl concentré sont passés sur des colonnes de

résine échangeuse d'anions (Dowex II) qui retiennent les autres impuretés radioactives[2]; les terres rares passent dans l'éluat.

2. Technique

Dans un tube à centrifuger de 100 ml, on met successivement:

(a) Les entraîneurs des activités dues aux produits de fission du groupe des terres rares que l'on veut séparer. On utilise des solutions étalons à 10 mg/ml pour chaque élément envisagé. Nous avons utilisé des quantités voisines de 10 mg par élément, exactement connues. On ajoute 50 mg d'entraîneur zirconium.

(b) La prise d'essai de la solution nitrique de produits de fission.

(c) 5 ml d'une solution de BrO_3K 0.15 *M*.

On porte au bain-marie bouillant pendant 30 minutes (oxydations de Pu à la valence VI pour qu'il ne soit pas entraîné par le précipité de fluorures de lanthanides).

(d) 5 ml environ d'HF concentré.

On porte au bain-marie bouillant pendant 5 minutes.

On centrifuge. On lave deux fois le précipité par 10 ml d'HF 0.1 *M*.

Le précipité est alors dissous dans la quantité minimum de NO_3H et de BO_3H_3 en solution saturée. Après dissolution on dilue à 20 ou 30 ml environ, on ajoute 5 mg d'entraîneur baryum. On précipite à chaud les hydroxydes de lanthanides par l'ammoniaque à pH 9, après addition de NH_4Cl.

Après centrifugation et lavages, le précipité est redissous dans le minimum d'HCl concentré. La solution chlorhydrique est passée sur une colonne (diamètre = 6 mm; longueur = 30 cm) de résine anionique Dowex II (100–200 mesh) préalablement lavée par HCl concentré.

On passe au moins un volume de colonne de HCl concentré de façon à sortir toutes les terres rares. L'éluat est évaporé partiellement puis traité par un courant de gaz ammoniac après nouvelle addition de 5 mg d'entraîneur.

Après centrifugation et lavage, le précipité d'hydroxydes est dissous par HCl 6 *N*; la solution est passée sur une colonne anionique Dowex II (100–200 mesh) en milieu HCl 6 *N* ayant les mêmes dimensions que la première.

L'éluat est partiellement évaporé, les hydroxydes sont précipités par le gaz ammoniac. Le précipité centrifugé et lavé est dissous dans le minimum de HCl 0.1 *N*. Cette solution de terres rares est prête à subir la séparation chromatographique.

II. Séparation chromatographique par échange d'ions

Nous n'en rappellerons pas le principe. Nous utilisons une résine Dowex 50 de 200 à 400 meshs. La qualité de la séparation dépendant des conditions opératoires de l'élution, nous avons été amenés à mettre au point un appareillage permettant de faire varier les différents paramètres dont dépend l'élution, température, pression, débit, concentration de l'agent éluant en cours d'élution. L'agent éluant utilisé est le lactate d'ammonium en solution à pH 5; il permet d'obtenir d'excellentes séparations par simple variation de la concentration des solutions éluantes [1,3,4].

1. Description de l'appareillage

Il comprend deux parties (Fig. 1):

la colonne de résine entourée d'un manchon chauffant,

un dispositif d'alimentation sous pression en éluant, permettant de faire varier la concentration en agent complexant.

Il faut ajouter aussi un collecteur de fractions de type commercial.

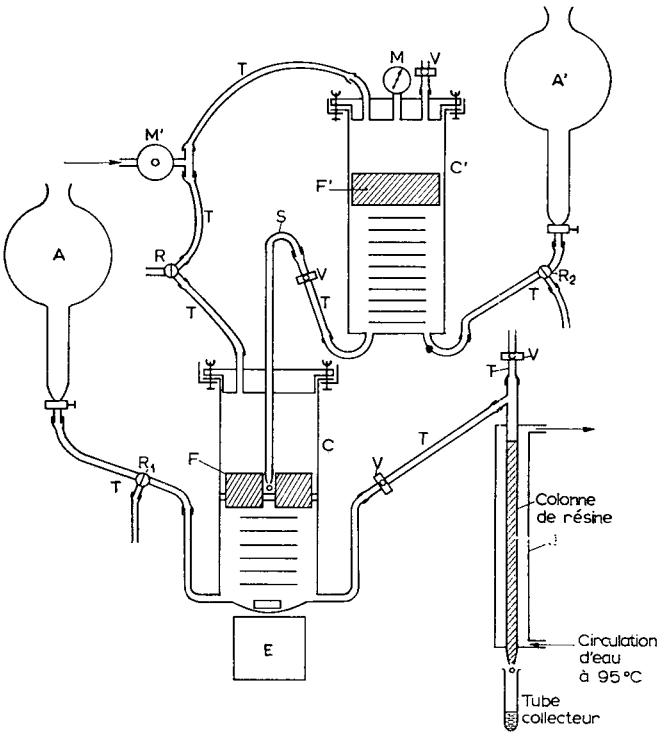

Fig. 1. Schéma de l'appareil à élution. A et A': Réservoirs de remplissage. C et C': Réservoirs des solutions éluantes. E: Agitateur électromagnétique. F et F': Flotteurs. J: Manchon chauffant. M: Manomètre de contrôle. M': Manodétendeur. R, R_1 et R_2: Robinets à 3 voies. S: Siphon. T: Tube de caoutchouc. V: Pince à vis.

A. La colonne de résine mesure 60 cm de long et 7 mm de diamètre intérieur. Elle est entourée d'un manchon chauffant où circule de l'eau provenant d'un bain thermostaté. Toutes nos expériences ont été effectuées à 95°.

La résine utilisée est une Dowex 50 de 200 à 400 meshs dont nous avons sélectionné la fraction qui sédimente dans 15 cm d'eau en 2 à 15 minutes. Cette fraction est débarrassée du fer par lavage avec une solution 6 *M* de thiocyanate d'ammonium jusqu'à ce qu'il ne se produise plus de coloration rouge (thiocyanate de fer). On lave ensuite à l'eau distillée puis par HCl 6 *N* et encore par l'eau distillée. La résine est enfin mise sous forme ammonium en la traitant à chaud par du lactate d'ammonium moléculaire. On la fait bouillir ensuite pendant une heure dans l'eau distillée.

Le remplissage de la colonne s'effectue en versant la suspension de résine bouillante dans la colonne préalablement portée à 95°. Ce remplissage doit se faire en une seule fois et on doit veiller à ce que la résine soit toujours surmontée d'une couche liquide.

La connaissance de la densité de la résine sèche, de son gonflement dans la solution éluante utilisée et du volume de résine humide utilisée pour remplir la colonne permet de déterminer le volume libre de la colonne selon la méthode de TOMPKINS[5]. Ce volume libre est de 16.28 ml pour les colonnes que nous utilisons; il a été retranché sur les abscisses des courbes expérimentales que nous reproduisons.

B. Le dispositif d'alimentation en éluant comprend: deux réservoirs cylindriques en verre C et C' qui communiquent par un siphon S permettant au liquide de C' de s'écouler goutte à goutte dans C. Dans chaque réservoir se trouve un flotteur en verre pour diminuer la surface de contact entre le liquide et l'air comprimé utilisé pour avoir un débit suffisamment rapide à la sortie de la colonne de résine. Les extrémités inférieures du siphon S et de la colonne de résine ont le même diamètre intérieur de façon à donner des gouttes de même grosseur ce qui facilite le réglage de leurs débits relatifs.

A l'intérieur de C se trouve le barreau aimanté d'un agitateur électromagnétique qui assure le mélange des solutions. Les récipients auxiliaires A et A' servent au remplissage de C et C' en solution éluante.

Si nous désignons par S_1 et S_2 les solutions contenues respectivement dans C et C'; C_1 et C_2 étant leurs concentrations moléculaires en agent éluant, la concentration molaire de l'éluant arrivant sur la colonne de résine sera fonction:

(a) de C_1; du volume V_1 initial et du volume v_1 écoulé de S_1;

(b) de C_2 et du volume v_2 écoulé de S_2 dans S_1.

Dans nos expériences d'élution à gradient de concentration, nous avons fait $v_1 = v_2 = v$. Dans ces conditions, on a, si C_x = concentration en lactate dans V_1 à un instant donné:

$$V_1 C_x - C_x \mathrm{d}v + C_2 \mathrm{d}v = V_1 (C_x + \mathrm{d}C_x)$$

$$(C_2 - C_x)\, \mathrm{d}v = V_1 \mathrm{d}C_x$$

d'où

$$\frac{\mathrm{d}C_x}{\mathrm{d}v} - \frac{C_2 - C_x}{V_1} = 0$$

équation différentielle linéaire du 1er ordre dont la solution est:

$$C_x = C_2 - (C_2 - C_1)\, \mathrm{e}^{-v/V_1}$$

(C_1 étant la concentration en lactate dans V_1 au commencement de l'élution), relation qui permet de calculer la concentration de l'éluant arrivant sur la colonne de résine.

L'élution avec gradient de concentration permet d'effectuer des séparations chromatographiques avec le minimum d'éluant et d'avoir des courbes d'élution symétriques[6].

2. Technique

(*a*) *Fixation des terres rares sur la colonne de résine.* La colonne de résine est d'abord mise en équilibre avec la solution de lactate qui va être utilisée au début de l'élution, en faisant passer un volume égal à cinq fois le volume libre de la colonne. On introduit alors à la partie supérieure de la colonne 5 ml environ d'eau distillée bouillante que l'on fait passer sur la résine, puis la solution chlorhydrique 0.1 *N* des terres rares obtenue dans la séparation préliminaire. On passe ensuite 5 ml d'eau distillée bouillante et on commence l'élution.

(*b*) *Élution d'un mélange Y, Eu, Nd, Pr, Ce* (Fig. 2). On utilise une solution de lactate d'NH_4 0.2 *M* et une solution de lactate d'NH_4 0.6 *M* préparées toutes les deux par dilution d'une solution moléculaire de lactate d'NH_4 ajustée à pH 5 par addition d'ammoniaque. On fait $V_1 = 500$ ml avec $C_1 = 0.2\ M$; $V_2 = 600$ ml avec $C_2 = 0.6\ M$.

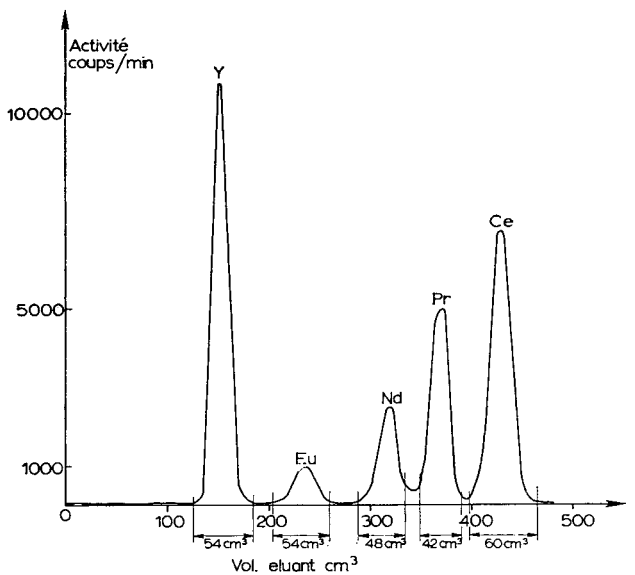

Fig. 2. Courbe d'élution d'un mélange Y, Eu, Nd, Pr et Ce, par le lactate d'ammonium à concentration variable suivant la relation: $C_x = C_2 - (C_2 - C_1)\ e^{-v/V_1}$; $C_1 = 0.2\ M$; $C_2 = 0.6\ M$; $V_1 = 500$ ml.

La température de la colonne est réglée à 95°. On amorce S et on règle le débit du liquide s'écoulant dans C à la même valeur que celui de l'éluat sortant de la colonne de résine. On obtient une bonne séparation avec un débit de 0.5 ml à 1 ml/min pour un diamètre de colonne de 7 mm. On recueille des fractions de 3 ml.

(*s*) *Élution d'un mélange Y, Eu, Sm* (Fig. 3). Cette séparation est délicate car l'europium et le samarium ont des propriétés très voisines. Pour avoir une bonne séparation, nous avons été obligés d'éluer à une concentration constante par du lactate 0.24 *M* à pH 5; la température de la colonne étant de 95°. On recueille des fractions de 3 ml.

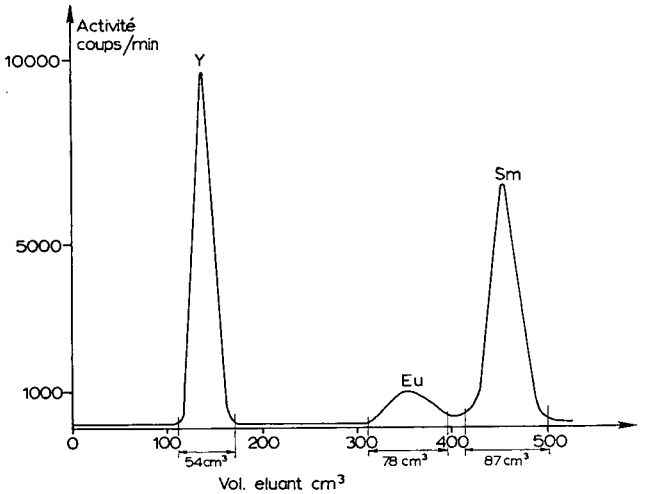

Fig. 3. Courbe d'élution d'un mélange Y, Eu et Sm, par le lactate d'ammonium 0.24 *M*.

III. Précipitation et confection des sources

Les lanthanides sont précipitées sous forme d'oxalate en milieu acide. La précipitation s'effectue dans les tubes collecteurs par 2 ml d'acide oxalique en solution saturée. En s'effectuant pendant l'élution, la précipitation a l'avantage de matérialiser aussitôt la sortie d'un élément. Les tubes correspondant à un même élément sont ensuite portés 5 minutes au bain-marie bouillant. Le précipité est recueilli sur filtre millipore à l'aide d'un Buchner démontable, puis calciné à 850° en creuset de silice. A cette température, les oxalates de terres rares donnent des oxydes de composition bien définie[7,8]: Y_2O_3; Eu_2O_3; Sm_2O_3; Nd_2O_3; Pr_6O_{11}; CeO_2.

Après calcination, les oxydes sont repris par quelques ml d'eau distillée, broyés délicatement à l'aide d'une baguette de silice, puis déposés sur filtre millipore préalablement séché à 100° et taré. Les filtres millipores sont des membranes filtrantes à base d'esters de cellulose ou de cellulose régénérée de porosité bien définie. Ils absorbent difficilement l'humidité atmosphérique; un séchage d'une demi-heure à 100° permet de les amener à poids constant. Leur surface rigoureusement lisse permet d'avoir des dépôts de précipité bien plans. L'oxyde est pesé sur son filtre après séchage à 100°. On en déduit le poids de l'oxyde qui permet de déterminer le rendement de la séparation chimique et dont la valeur doit être connue afin de faire la correction d'auto-absorption pour les éléments dont on mesure le rayonnement β.

Nous avons choisi un diamètre de 10 mm pour les dépôts d'oxyde pour avoir une bonne géométrie. Les quantités d'entraîneur utilisées sont telles qu'on ait une bonne précision à la pesée et un dépôt suffisamment mince pour que l'auto-absorption soit faible.

Les sources sont montées suivant la technique classique qui consiste à inclure le disque millipore supportant le précipité entre deux feuilles minces de Mylar.

RÉSUMÉ

La méthode que nous venons d'exposer permet de réaliser avec de très bons rendements (80 à 85 %) des séparations chromatographiques des produits de fission du groupe des lanthanides en vue de leur dosage par mesure physique. Pour arriver à ce résultat nous avons utilisé un appareil qui permet de faire varier à volonté les conditions de l'élution. Nous donnons deux exemples de séparation pour illustrer son fonctionnement.

SUMMARY

A method is described that permits the chromatographic separation in good yields (80–85 %) of fission products of the lanthanide group, for purposes of determination by physical methods. An apparatus was used with which the conditions of elution can be altered at will. Two examples of such a separation are given to illustrate the operation of the apparatus.

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Short Communications

A chromatographic artefact involving iodide-131

CRITCHLOW AND GOLDFINCH[1] described a radioactive component which was present in extracts of plasma, obtained from a thyrotoxic patient who had been treated with 7 mC of iodine-131. The R_F value of this material on chromatography in butanol–dioxane–ammonia was 0.22 in the presence of potassium iodide carrier, and 0.33 in the absence of carrier. The R_F of potassium iodide in carrier quantities was found to be 0.22, in contrast to the value given by GROSS[2] for this solvent system (0.4). We have also observed R_F values of about 0.4. CRITCHLOW AND GOLDFINCH did not report having carried out radioautography of their chromatograms, and their results were obtained by direct counting of the paper strips. It seemed possible that interference with the chromatography of iodide by traces of metal impurities, such as is known to occur in the case of phosphate esters[3], could have accounted for their findings.

A solution of carrier-free iodide-131 was applied to untreated Whatman No. 1 paper, and to Whatman No. 1 paper previously treated with ethylenediaminetetraacetic acid (EDTA) as described by EGGLESTON AND HEMS[3]. Five portions of solution each containing 0.5 μC of carrier-free sodium iodide-131, were successively dried onto any one spot. Carrier potassium iodide (50 μg) was subsequently added to half the spots. Each chromatogram was developed for 16 h by the descending method in *n*-butanol–dioxane–2 *N* NH_4OH (4:1:5 by vol.), allowing the solvent to drip off the end of the paper in order to obtain a longer run. The chromatogram was then dried and placed in contact with "Ilfex" double-coated X-ray film for 24 h.

Fig. 1a shows that the point of maximum density of the carrier iodide spot on untreated paper has a smaller R_F than that of the carrier-free iodide spot. Fig. 1b shows that, on paper treated with EDTA the region of maximum spot density has the same R_F value whether carrier iodide is present or not. The different shape of the spots in the presence of iodide carrier can be explained by saturation of the stationary phase with iodide. Treatment of the paper with EDTA would be expected to remove traces of metal impurities[3]. Part of the iodide might combine with heavy metal ions, present in trace amounts in the paper, to form poorly soluble salts, thereby lowering its R_F value. However, a portion of iodide, corresponding to the solubility product of the least soluble salt, would remain free, and this portion would presumably run in the leading part of the spot, whose R_F on untreated paper corresponds to that of the greater part of the carrier-free iodide-131. When iodide is present as carrier-free iodide-131, the solubility product is probably not reached, and the iodide spot remains homogeneous even on untreated paper. The use of an alkaline solvent system might be expected to favour the formation of insoluble iodides. There is a small amount of

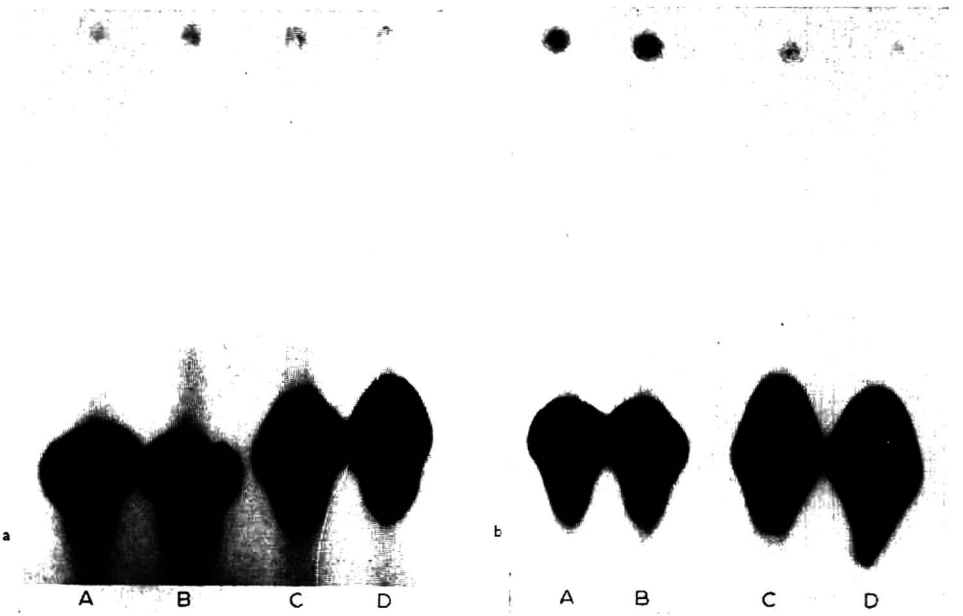

Fig. 1. Radiochromatograms of iodide-131 on untreated (a) and EDTA-treated (b) Whatman No. 1 paper. In each case, spots A and B contained iodide-131 only, while potassium iodide carrier (50 μg) was added to spots C and D.

"tailing" extending on both sides of the iodide spot, more marked in the untreated than in the treated paper. It might be attributable to an iodination reaction catalysed by trace quantities of metals.

It seems, therefore, that trace amounts of metal impurities in the chromatography paper may cause changes in the R_F of iodide. Tissue extracts would also contain metals which might be expected to cause a similar interference. This effect might account for the observations of CRITCHLOW AND GOLDFINCH[1].

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A method for the elution of paper chromatograms*

There has been a need for a simple method for eluting substances from paper chromatograms, especially when there are a large number of samples to be handled. Many types of apparatus have been described which are either expensive or are time consuming. The following simple apparatus is offered for eluting chromatograms on a large scale with small volumes of many volatile and non-volatile solvents.

Fig. 1. shows the materials that are needed: (a) An eluter composed of 0.75 mm internal diameter thick walled capillary connected to 1 cm glass tubing of suitable length. (b) A suitable receiver; in this case, a 20 × 150 mm test tube is used. (c) A paper clip; a 90° bend is made in the paper clip approximately 5 mm from the

Fig. 1.

largest end clipped into the test tube as shown. The eluter is then set into the test tube through the seat provided by the paper clip. The paper to be eluted is then folded and placed into the eluter. Solvents are now added as desired. The draining time will depend on the size of the capillary and the type of solvent. Using an 0.75 mm capillary 5 cm long with 2 ml of ethanol, a draining time of 4 minutes is observed.

* This study was supported by grants from the National Institutes of Health and the G. D. Searle Company

10, 15, 20, 25, 30 and 50 μg quantities of dehydroepiandrosterone in the following solvent systems, toluene–propylene glycol, heptane–propylene glycol, and heptane–phenyl cellosolve were spotted on Whatman No. 1 paper; eluting these papers with the above method using successive 2 ml volumes of ethyl alcohol and methyl alcohol to a total volume of 10 ml resulted in a recovery of 94 $\pm$ 3.5 %.

The main advantages of this method are: (1) The individual eluters are cheaply and quickly constructed. (2) The apparatus is easily handled and requires a minimum of attention. (3) Large numbers of samples may be eluted simultaneously; 100 samples can be processed easily. (4) A variety of solvents may be used in succession on the same sample to ensure recovery. The apparatus works equally well with H_2O, methanol, ethanol, chloroform, methylene chloride, ether, etc. (5) The apparatus is easily assembled, disassembled, and maintained.

This eluter was made to our specifications by Labtician Products Company, 190–04, 99th Avenue, Hollis 23, New York.

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Séparation de radioéléments par chromatographie sur papier imprégné d'un échangeur d'ion minéral

La séparation de divers cations par chromatographie sur papiers imprégnés d'échangeurs d'ions minéraux a été signalée récemment par ALBERTI ET GRASSINI[1,2], et CABRAL[3]. Ces auteurs précipitent sur le papier des composés minéraux dont les propriétés d'échange ionique ont été signalées par KRAUS[4] (phosphate et molybdate de zirconium) et SMIT[5] (phosphomolybdate d'ammonium).

Nous avons utilisé avec succès ces techniques en vue de séparer quelques radioéléments naturels et artificiels. La méthode est très souple par le choix du composé d'imprégnation, de la nature de l'éluant, de sa concentration. Nous avons fixé les conditions expérimentales conduisant à la séparation la plus rapide, en raison de la courte période de certains radioéléments étudiés.

Les papiers imprégnés de phosphate de zirconium (PZ) ont été préparés selon la technique décrite par ALBERTI[1], les papiers chargés d'oxyde de zirconium hydraté (OZ) et de tungstate de zirconium (WZ) ont été préparés par des méthodes similaires: traitement du papier imprégné d'oxychlorure de zirconium par de l'ammoniaque ou du tungstate de sodium.

Les papiers lavés et séchés sont découpés en bandes (largeur 2 cm, longueur

12 cm); toutes les chromatographies sont descendantes et ont lieu dans une atmosphère de vapeur d'eau à 60°. Le développement des chromatogrammes se fait selon la méthode classique de déplacement d'un compteur G.-M. devant la bande de papier; ce balayage est effectué à intervalles réguliers pour identifier les composés de courte période.

Les séparations suivantes ont été obtenues (Tableau I).

TABLEAU I

Produit de départ	*Imprégnation du papier*	*Éluant (M)*	*Durée de la chromatographie (min)*	R_F	
^{211}Pb (AcB, t = 36 min)	PZ	ClH 0.5	5	Tl 0.1	Pb 1
en équilibre avec					
^{207}Tl (AcC″, t = 4.76 min)	OZ	NO_3NH_4 0.1	12	Tl 0.6	Pb 0
$^{204}Tl^+$–$^{204}Tl^{3+}$	PZ	ClH 0.5	30	Tl^+ 0.2	Tl^{3+} 0.9
	OZ	NO_3NH_4 0.1	30	Tl^+ 0	Tl^{3+} 0.8
^{212}Pb (ThB, t = 10.6 h)		ClH 0.1			
en équilibre avec		ou			
^{212}Bi (ThC, t = 1 h)	OZ	NO_3NH_4 0.05	30	Bi 0	Pb 0.4
^{140}Ba–^{140}La	PZ	$ClNH_4$ 0.5	20	La 0	Ba 0.9
	OZ	NO_3NH_4 0.1	30	La 0	Ba 0.9
^{90}Sr–^{90}Y	PZ	ClH 0.5	30	Y 0.3	Sr 1
	OZ	NO_3NH_4 0.1	30	Y 0	Sr 0.95
^{228}Ra (MTh_1)					
en équilibre avec					
^{228}Ac (MTh_2, t = 6.13 h)	PZ	$ClNH_4$ 0.5	30	Ra 0.85	Ac 0.1
^{223}Ra (AcX) – ^{223}Fr	OZ	NO_3NH_4 0.1	20	Fr 0	Ra 0.85
(AcK, t = 21 min)	WZ	$ClNH_4$ 0.1	20	Ra 0	Fr 0.5
^{137}Cs–^{223}Fr	WZ	$ClNH_4$ 1	20	Fr 0.5	Cs 0.6

La séparation Tl^+–Tl^{3+} est particulièrement intéressante et est utilisée pour la détermination quantitative des deux états de valence de cet élément; la séparation francium–radium a été utilisée pour purifier le francium: on précipite du carbonate de lanthane actinifère, le francium reste en solution mais contaminé par du radium 223, dont on le sépare par chromatographie.

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Reçu le 21 décembre 1960

A new method for the detection of diglycerides on a microscale

Work involving the isolation and identification of diglycerides from natural sources can be simplified by the use of paper chromatography. This is well exemplified in the phospholipid field where acid hydrolysis of glycerophosphatides usually yields diglycerides. A new method is described for the detection of diglycerides in microgram quantities by paper chromatography.

It is well known that hydroxylamine reacts with fatty acid esters under alkaline conditions to yield hydroxamates and the free alcohols. When treated in this way, diglycerides give rise to glycerol which can be detected in amounts of ~ 2 μg using the periodate–Schiff's spray reagent as modified for α-glycols by BADDILEY *et al.*[1]. The experimental procedure described allows 5–10 μg of diglyceride (DG) to be detected on paper chromatograms. As such small amounts of DG are detected, reactions liberating if can be followed on a microscale. It is also possible to distinguish between monoglyceride (MG) and DG without the need for standards, since, due to the presence of an α-glycol grouping, MG is detected when sprayed directly with the modified periodate–Schiff's spray and DG is not.

Experimental

Samples of monostearin (MG) and distearin (DG) (*e.g.*, 20 μg in 20 μl) were spotted in duplicate on Whatman No. 1 paper (40 × 15 cm) impregnated with formaldehyde according to HÖRHAMMER *et al.*[2]. Chromatograms were developed for 12 h by the ascending technique in the solvent[2] for phospholipid separation. The solvent front moved about 25 cm at room temperature (22°). Chromatograms were dried and divided lengthwise into two strips (A and B) to obtain spots of MG and DG on each strip. Strip A was sprayed with aqueous alkaline hydroxylamine solution. The spray reagent was prepared by mixing equal volumes of aqueous solutions of 2.5 *M* sodium hydroxide and 2 *M* hydroxylamine hydrochloride. After 30 min, A was sprayed with 3 *M* hydrochloric acid and dipped for 5 sec in water and then allowed to dry. A and B were sprayed with 1 % aqueous sodium metaperiodate solution and after 6 min were treated with sulphur dioxide gas until the liberated iodine was reduced, giving a white background. Immediate spraying with a decolorised, 1 % aqueous solution of pararosaniline hydrochloride, followed by treatment with sulphur dioxide gas gave rise to mauve spots due to DG and MG on A and a spot due only to MG on B. The detection limit for DG was 5–10 μg.

The fact that the glycerol produced by the hydroxylamine treatment was not dissolved out of the paper by the washing indicated that it was probably complexed to unreacted DG or to the paper.

In the system described, the R_F of distearin was 0.95–0.99, spotting 50–5 μg, whereas that of monostearin was 0.75–0.85. In comparison, the R_F values of glycerol and myoinositol were 0.3–0.4 and 0.02 respectively. The myoinositol showed up as a greyish purple spot 15 min after the last sulphur dioxide treatment, whilst the mauve spots appeared in less than 3 min.

When MG and DG were spotted together, the MG R_F rose to $\sim$ 0.86. If $>$ 50 μg of DG were spotted, slight streaking was observed.

Discussion

The described technique identified and distinguished between microgram amounts of DG and MG. It has certain advantages over other published paper chromatographic detection methods. The sensitivity of diglyceride detection is of the same order as that obtained by the use of Rhodamine 6G[3] and is greater than in the pancreatin–α-dextrin–I_2 method[4]. The method is less laborious, it is quicker, and needs less precautions than the system used by MANGOLD *et al.*[4]. Whereas the detection method used by RENKONEN AND RENKONEN[3] depends on the lipid nature of the whole molecule, our method indicates an atomic grouping within the molecule. It can be extended to any fatty acid esters which yield an α-glycol on treatment with alkaline hydroxylamine provided that the appropriate chromatographic paper and solvent system are chosen.

Application

Simple monophosphoinositides, when heated with 99 % acetic acid[5], yield diglycerides and inositol phosphates. This reaction was applied to horse liver monophosphoinositide (1 mg). Evaporation of the acetic acid yielded material, easily identified as DG by the above technique. This enabled the position of the fatty acid groups to be determined, since it excluded the possibility of a fatty acid being esterified to the myoinositol residue. In conjunction with other work[6], this knowledge confirmed the structure of horse liver monophosphoinositide to be a di-O-acyl glycerol-1-(L-myo-inositol-1-phosphate). The probable migration of the acyl group in the acetic acid reaction did not influence the R_F of the resultant diglyceride. Distearin was used as the standard DG and after treatment under the conditions of the reaction its R_F had not altered.

This work was carried out during the tenure of a D.S.I.R. Maintenance Award.

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Chromatography of lipids on silicic acid: infrared spectrophotometric elution curves

Direct spectrophotometric measurements on eluates greatly facilitate preparative column chromatography. They give elution curves that are often as useful as gravimetric elution curves, and make it possible to isolate compounds constituting specific peaks rapidly, under chemically mild conditions.

Measurement of electronic absorption is not appropriate for lipid eluates because most lipids only absorb selectively at frequencies at which the eluents are opaque. But measurement of vibrational absorption is potentially more useful, if eluent composition is constant or not subject to abrupt change. Thus we have been able to obtain spectrophotometric elution curves for silicic acid columns developed[1,2] with a continuous concave gradient of methanol in chloroform.

Results of measurements at 1745 cm^{-1} (the approximate fundamental frequency of C:O stretching in esters) were very satisfactory: most features of gravimetric curves were reproduced qualitatively until just before lecithins were eluted (see Fig. 1). Rising eluent opacity, caused by the gradient of methanol concentration, tended to submerge peaks in the spectrophotometric elution curves. The tendency was not very significant in the experiment described here, but it was much more significant in an experiment in which less lipids and less silicic acid were used with the same elution device, *viz.*, when lipid concentrations were roughly quartered. Attempts were made to correct for eluent opacity by subtracting functions (D_{eluent}) of the fraction number (n) from each absorbance reading (D). Several functions of the form, $D_{\text{eluent}} = An + Bn^2 + Cn^3$, were tried, but one of the simplest, $D_{\text{eluent}} = 10^{-4}n^2$, proved quite satisfactory.

Other spectrophotometric curves were plotted for hydrocarbon and peptide absorptions (2850 cm^{-1}, 1540 cm^{-1}). The results were not so good as those for ester absorption, though they were of limited use: a sterol peak, for example, was located by its hydrocarbon absorption.

Method

A sample cell of path length 2.5 mm, with barium fluoride windows, was used*. The spectrophotometer (Perkin-Elmer Infracord, Model 137E) was fitted with a logarithmic chart reading absorbance, and was adjusted to the polystyrene peak at 1745 cm^{-1}. The reference beam was attenuated with sufficient polyethylene film to balance roughly the absorption of the sample cell filled with the initial eluent (pure chloroform).

Tubes were removed from the fraction collector up to 60 h after they had been filled. The contents of each in turn were stirred with a small glass piston, and samples were removed by syringe for measurement of D. Since chart readings of D greater than 0.6 are very inaccurate it was sometimes necessary to "subtract" a part of D by putting more polyethylene film in the reference beam and then restoring sensitivity

* Sodium chloride windows are eroded by $>10\%$ methanol in chloroform.

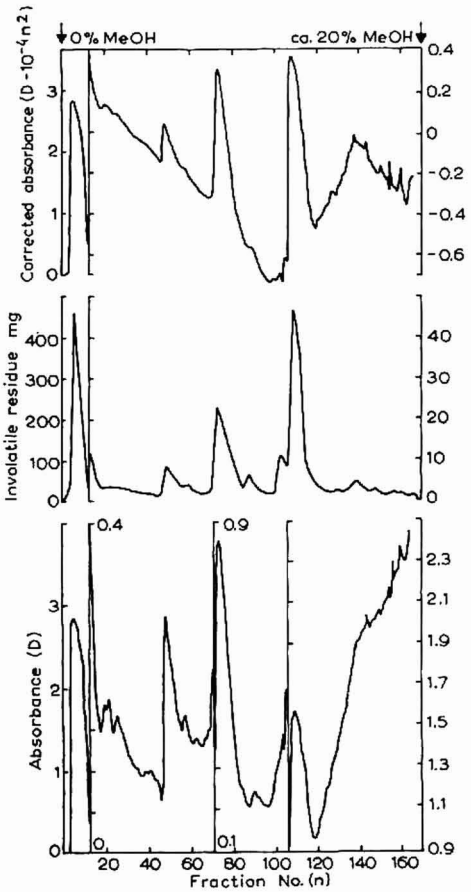

Fig. 1. Continuous, concave gradient elution of a wheat flour lipid preparation (6 g) from a column of silicic acid (170 g, diameter 33 mm). Lower curve: spectrophotometric (1745 cm^{-1}). Middle curve: gravimetric (every third 20 ml fraction taken). Upper curve: spectrophotometric, simple correction applied for eluent opacity.

by increasing the slit width; then two readings on a single sample were necessary to show the increment subtracted. It was sometimes necessary to reverse this procedure at the tail of a large peak.

Eventually, when they contained about 20% methanol, eluates became too opaque for measurement. The nominal values of slit width and D were then 450 μ and 2.5 respectively.

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1 J. J. WREN, *Nature*, 184 (1959) 816.
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A simple apparatus for rapid drying of paper strip chromatograms

The apparatus described was developed for rapid drying of paper strip chromatograms at room temperature. Several strips of various length can be simultaneously dried in this simple apparatus. The model used in this laboratory is shown in Fig. 1 and consists of a glass tube, 6 cm in diameter and 30 cm in length, clamped vertically in a stand and fitted at the lower end with a glass Buchner funnel of 6 cm bottom diameter. The tube is tightly fitted to the funnel by means of a rubber tube about 8 mm in diameter. A special device for hanging paper strip chromatograms is placed

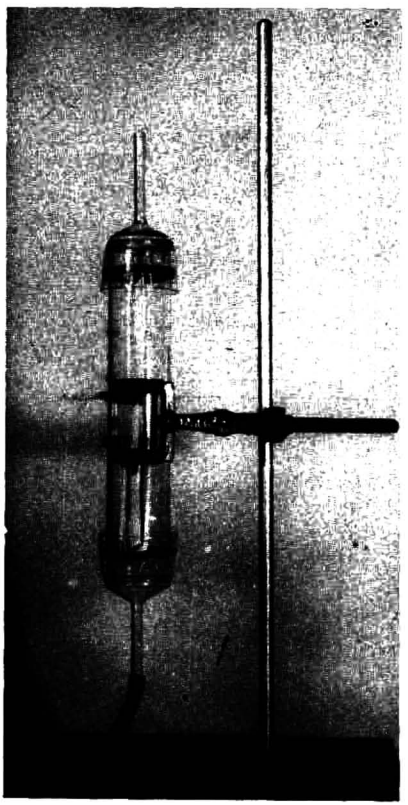

Fig. 1.

on top of the tube. The construction and the dimensions of this device are shown in Fig. 2. It consists of a ring 3 cm in diameter made of stainless steel wire (1 mm), to which two crossed stainless steel strips 3 mm wide and 0.5 mm thick, are attached. The ends of the strips rest on the top of the tube and the device with chromatograms suspended from the ring by wire hooks at suitable intervals, is kept in position by the bends in the strips, as can be seen in Fig. 3. After the chromatograms have been attached to the ring, the top of the tube is covered with another funnel, the connection between

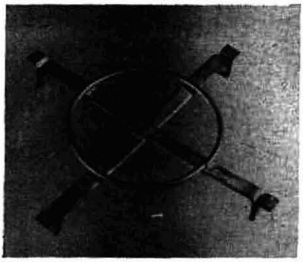

Fig. 2.

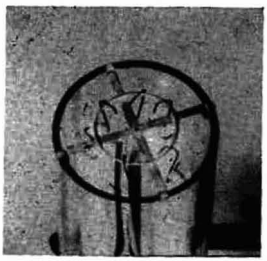

Fig. 3.

the tube and the funnel being made in the same way as for the lower funnel. The outlet of the lower funnel is connected by means of a rubber tube to a water jet pump. With the aid of this pump a continuous stream of air is made to flow through the device. The air stream flows around the strip chromatograms and increases the rate of drying.

The apparatus can be easily adapted for drying paper strip chromatograms in inert gases and at elevated temperature.

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The separation of C_6-C_{12} dibasic acids in the presence of monobasic acids A simple procedure by paper chromatography

The separation by paper chromatography of dicarboxylic acids has been described by FINK AND FINK[1], KALBE[2] and SEHER[3]. These methods necessitate the preparation of derivatives or salts, or the use of swamp acids. They are unsuitable when monobasic acids are present because their R_F values lie close to those of the dibasic acids which it is desired to separate. In such cases the technique of ZBINOVSKY[4] using silica gel columns is available. The procedure described is a modification of this method which allows the separation to be carried out readily on paper.

A mixture of 9 parts of redistilled technical grade methyl cellosolve, b.p. 124°, and 1 part of distilled water was shaken with an equal volume of reagent grade *n*-butyl ether and the two layers separated. The upper layer was retained for the mobile phase and the lower for the stationary phase. A sheet of Whatman No. 1 paper (57 cm × 46 cm) was passed once through the methyl cellosolve water layer contained in a shallow dish and allowed to drain for 5 min at room temperature (20°). The mixed acids were dissolved in acetone and aliquots of 0.02 ml containing 10–50 μg of acid

were spotted 8 cm from the end of the paper which was immediately transferred to a sealed cabinet containing dishes of each of the mobile and stationary phases. The paper was developed without equilibration in a descending direction for 18–24 h using the *n*-butyl ether layer as the developing solvent. It was then dried at 105° for 5 min and sprayed with a 0.1 % solution of bromocresol green in 0.05 % aqueous sodium bicarbonate which produced yellow spots on a blue background.

TABLE I

R_F VALUES OF DIBASIC ACIDS

Acid	*R_F value*
Adipic	0.10
Pimelic	0.14
Suberic	0.17
Azelaic	0.23
Sebacic	0.33
1,9-Nonamethylene-dicarboxylic	0.39
1,10-Decamethylene-dicarboxylic	0.52

The monobasic acids move close to the solvent front well ahead of any of the dibasic acids. The R_F values at 20° of the dibasic acids are shown in Table I.

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BOOK REVIEWS

Phenolics in Plants in Health and Disease, par J. B. PRIDHAM, Éditeur, Pergamon Press, London, 1960, 140 pages, prix 42 s.

Ce livre contient des rapports présentés à un Symposium tenu à l'Université de Bristol en avril 1959. Il est divisé en quatre parties; la première, d'ordre général, contient les articles suivants:

The distribution of phenolic compounds in apple and pear trees (A. H. WILLIAMS)
The formation and possible function of phenolic glycosides (J. B. PRIDHAM)
The mobilization of betanin in beetroot (S. P. SPRAGG)
Germination inhibitors in plant material (C. F. VAN SUMERE)
The inhibitory substances contained in sugar beet glomerules (J. DE ROUBAIX ET O. LAZAR).

La seconde partie contient trois articles (de T. SWAIN; F. A. ISHERWOOD; et G. BULOCH) sur les rapports entre leuco-anthocyanines et lignine et sur la formation de lignine.

La troisième partie contient cinq articles (de D. WOODCOCK; A. E. FLOOD ET D. S. KIRKHAM; A. C. HULME ET K. L. EDNEY; R. J. W. BYRDE, A. H. FIELDING ET A. H. WILLIAMS; et C. H. CADMAN) concernant les rapports entre substances phénoliques et résistance à l'infection chez les plantes.

La quatrième partie contient trois articles (de J. B. HARBORNE; R. C. PECKET; et W. J. FEENSTRA), sur des aspects génétiques de la formation de substances phénoliques.

Plusieurs de ces articles contiennent des données sur des méthodes chromatographiques utilisées pour la séparation des substances phénoliques.

Ce livre est très bien présenté, illustré de nombreuses photographies et sera étudié avec profit par tous ceux qui s'intéressent à la biochimie des substances phénoliques des végétaux.

E. LEDERER (Paris)

J. Chromatog., 5 (1961) 374–375

Protides of the Biological Fluids, Proceedings of the Seventh Colloquium, Bruges, 1959. Edited by H. PEETERS, published by Elsevier Publ. Co., Amsterdam, 1960, x + 420 pages, price 76 s.

Sint Jans Hospital in Bruges, which is very well known to art enthousiasts because of its collection of paintings by HANS MEMLINK, has in recent years become equally well known among clinical chemists on account of the annual international colloquia on protides (*i.e.* proteins, peptides and amino acids) of the biological fluids, that are organized there.

The previous two volumes of these Proceedings have already been reviewed by MARINI-BETTÒLO in this Journal, Vol. 3 (1959) pp. 98 and 203. In the present volume, 20 papers appear in the form of summaries onlys, as they have already been published in *Clin. Chim. Acta* or elsewhere. The rest of the volume contains 66 original papers, an introductory review on antibody synthesis by SCHULTZE, and an account of a round table conference under the chairmanship of PEETERS, which deals briefly with a variety of subjects. There were two sections specifically devoted to electro-

phoresis and chromatography, but other sections are also interspersed with papers concerning these topics. The section on immunoelectrophoresis deals with specific applications to perchloric acid-soluble serum protein (DE VAUX ST. CYR), organ proteins (KESSEL), salivary proteins (GABL), and proteins of tears, saliva, milk and labyrinthine fluid as compared with serum (KOHN). Agar immunoelectrophoresis (as well as electrophoresis on paper) was also the method most commonly used in other sections of the Colloquium. By interposing a thin agar layer between an anti-serum-soaked paper and a two-dimensional "star" paper electropherogram of serum, PEETERS AND VUYLSTEKE obtained a kind of immunoelectrophoretic two-dimensional pattern. ORIOL-BOSCH and VOIGT reported on their experiences with KOHN's method of cellulose acetate foil electrophoresis. A preliminary description of an interesting new method of free zone electrophoresis was given by HJERTÉN, in which a horizontal tube rotates on its long axis in order to suppress the disturbing effects of convection.

In the section on chromatography, MEIJERING and HUISMAN showed that normal haemoglobin can be separated into six fractions on CM-cellulose columns, HOLMBERG and WESTLUND investigated fractions obtained on DEAE-cellulose by means of electrophoresis on paper and starch gel, PENARANDA studied amino acids of exudates and transudates, using paper chromatography. Chromatography on cellulosic ion exchangers was described in papers of other sections also. A very sensitive method of measuring the absorption of proteins at 210 mμ has been adapted for the eluates of DEAE-cellulose columns by TOMBS *et al.*

The section entitled "Biochemistry" includes an article by BODMAN, which is a cross between a brilliant essay and a useful technical account on the electrophoretic investigation of foetal proteins. BIGWOOD poses the question whether any other amino acids besides β-amino-*n*-butyric acid are ninhydrin-negative in solution. BLOEMENDAL and BOSCH employ starch-block electrophoresis and ion-exchange chromatography in the study of "soluble" RNA. Agar-gel electrophoresis has proved to be very efficient for the investigation of haemolymph proteins of several insect species (MISSELIJN *et al.*).

The section on carnitine deals with various facets of the remarkable and comparatively little known biological properties of this betaine. Similar sections dealing with a particular topic, *e.g.* macroglobulins and urinary proteins, are included in the chapter on protein pathology. Further sections were devoted to the binding properties of proteins and general problems of protein nutrition.

The level of the contributions is high and the quality of paper and typography are up to the usual Elsevier standards. The illustrations are well produced as exemplified by the very numerous immunoelectrophoretic patterns. Misprints are not infrequent, but they are compensated for by the promptness of publication and are not disturbing.

I. M. HAIS (Prague)

REVIEW

QUANTITATIVE RADIO PAPER CHROMATOGRAPHY

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CONTENTS

The various techniques utilised in the preparation and measurement of a radio paper chromatogram are commonly referred to as quantitative radio paper chromatography. The scope and application of this technique, which combines paper chromatography and the use of radioactive tracers, have increased considerably during the past decade. This is primarily due to the fact that the measurement of the activity is performed directly by scanning the paper with an appropriate measuring device, which is now usually entirely automatic. Consequently, a rapid comparison of the activity of the separated substances is possible.

1. PREPARATION OF RADIOCHROMATOGRAMS

A radio paper chromatogram is obtained when radioactive substances are separated or when non-radioactive substances are separated and subsequently labelled, either with a radioactive reagent or by activation.

(a) *Chromatography of radioactive substances*

In the chromatography of radioactive substances, particular care and precautions must be taken over and above the usual procedures which chromatography involves.

Because of the high sensitivity with which the radio isotopes can be detected and the chemical non-specificity of this detection, it is necessary to take the utmost care to avoid contamination.

The radioactive solution should preferably come into contact only with glass, since certain radioactive ions partially attach themselves to metallic parts, such as

the needle of the micro-syringe with which the solution is placed on the paper, and are subsequently liberated by ion exchange. For example, in the chromatography of a solution of ^{14}C-labelled substances containing phosphate ions, the authors observed a spurious radioactive spot on the chromatogram, which was identified as ^{32}P-phosphate. This originated from ion exchange in the needle of a syringe that had previously been used for a solution containing ^{32}P-phosphate ions; evidently washing with water had not been sufficient to eliminate this contamination.

In the chromatography of a substance labelled with radioactive isotopes emitting soft beta rays (^{14}C, ^{35}S, etc.) it is necessary to keep in mind that the self-absorption of this radiation may not be uniform, either because of irregularity in the thickness of the paper or because of irregularity in the distribution of the substance through the paper.

WINTERINGHAM, HARRISON AND BRIDGES[1] have found that the irregularity in the thickness of a sheet of Whatman No. 1 paper is about 4 %. In chromatograms containing substances labelled with ^{14}C or ^{35}S, this irregularity produces an error of some 2 or 3 % in the value of the radioactivity.

Another significant error that may occur in some cases arises from the irregular distribution of the substance within the paper, caused by the method used for the evaporation of the sample to be chromatographed. It is known that in chromatography, evaporation of the successive drops of the solution can be speeded up by applying a stream of hot air from a standard hair dryer. This stream of hot air causes a partial accumulation of the substance on the lower surface of the sheet, because evaporation is more rapid there. This results in a non-homogeneous distribution of the substance through the paper. The authors have found[2] that, owing to this accumulation on the lower surface (R) of the paper, the amount of radioactivity measured on the upper surface of the paper (F) is decreased, while that on the lower surface (R) is increased (Table I). Such irregularities in distribution will gradually be eliminated

TABLE I

EFFECT OF VARIOUS CONDITIONS OF EVAPORATION ON THE MEASURABLE ACTIVITY OF ^{14}C-GLUCOSE ABSORBED ON WHATMAN NO. 1 PAPER

The measurements, made with a Geiger counter on the upper (F) and the lower (R) surfaces of the sheet, are expressed in c.p.m. and are the means ± s.e.m. of 8 samples of 40 μl each, applied 2 μl at a time. The current of air was directed at the lower surface of the sheet.

Evaporation conditions	*F*	*R*	*Mean*	*R/F*	*R'/F'**
Without current of air	3135 ± 45	3045 ± 45	3095 ± 35	0.97 ± 0.01	0.96 ± 0.01
With current of cold air at 50 cm	2350 ± 30	3970 ± 75	3160 ± 30	1.69 ± 0.05	0.98 ± 0.01
With current of cold air at 5 cm	2025 ± 35	5415 ± 35	3720 ± 25	2.67 ± 0.05	0.97 ± 0.01
With current of hot air at 5 cm	1995 ± 25	5745 ± 35	3870 ± 35	2.88 ± 0.05	0.97 ± 0.01

* R'/F' is the ratio of the activities measured after development of the chromatogram (according to POCCHIARI AND ROSSI[2]).

during further chromatographic development, and it is therefore only a source of error in the measurement of the radioactivity of the substances that remain in the initial position ($R_F = 0$). A similar error occurs if a comparison is required of the activity of the sample before chromatographic development with that of the different substances separated on the chromatogram after development. To eliminate the errors resulting from irregularity in the distribution of the substance, it is necessary to take as the true value of the activity the mean of the values measured on both sides of the paper; if this ratio is greater than 1.5, a correction factor must be applied[2].

(b) *Labelling of separated substances with a radioactive reagent or by activation*

Up to the present these methods of labelling have had limited application, and they are mentioned here only on account of their originality and because they are applicable in the case of special problems.

In order to measure certain amino acids directly on chromatograms, WINTERINGHAM *et al.*[1] exposed the chromatograms to the vapours of ^{131}I methyl iodide. They found that the amount of the radioactivity remaining on the radiochromatograms after pumping away the excess of methyl iodide was proportional to the quantity of amino acids present. In order to estimate substances containing bromine and chlorine, the same authors[1] activated the latter by irradiating the chromatogram with neutrons in an atomic pile.

An interesting method, which permits the application of radio paper chromatography to a substance that has been labelled with a stable isotope, was developed by FÖGELSTROM-FINEMAN *et al.*[3]. They were able to detect a substance labelled with ^{18}O by activating the substance with protons, whereby ^{18}O is transformed into radioactive ^{18}F. Since the paper contains oxygen, which normally has a content of 0.2 % of ^{18}O, it is necessary to transfer the separated substances to a metal sheet before activation. This can be done by eluting the spots with a special device in a direction perpendicular to the strip, so that the relative positions of the spots are not altered.

2. MEASUREMENT OF RADIOCHROMATOGRAMS

(a) *Measuring devices*

An approximate evaluation of the radioactivity of the spots on a chromatogram can be made by autoradiography (FINK, DENT AND FINK[4]). The radiochromatogram is brought into contact with an X-ray film for a sufficient length of time to produce darkened areas on the film after photographic development. To obtain an optical density of 0.6 with the β-rays of ^{14}C, it is sufficient that about 10–20 million particles per cm^2 bombard the emulsion during the entire period of exposure. Some examples of autoradiography are shown in Figs. 11, 12 and 13.

On the other hand, quantitative determination of the radioactivity present in a radiochromatogram may be achieved by direct scanning of the paper with measuring

devices, such as an end-window Geiger counter*, a flow counter**, an ionization chamber or a scintillation counter. The proper choice of one of these devices is dependent on the type of radiation to be measured and the degree of efficiency desired.

TOMARELLI AND FLOREY[11] were the first to construct a simple apparatus for scanning strips of paper. These were mounted on a sliding base, which was moved manually at regular intervals across a Geiger counter. The movement of the chromatogram, the control of the measuring period and the recording of the number of impulses can be done effectively by hand. Devices of similar types have been used by numerous investigators; an example is shown in Fig. 1.

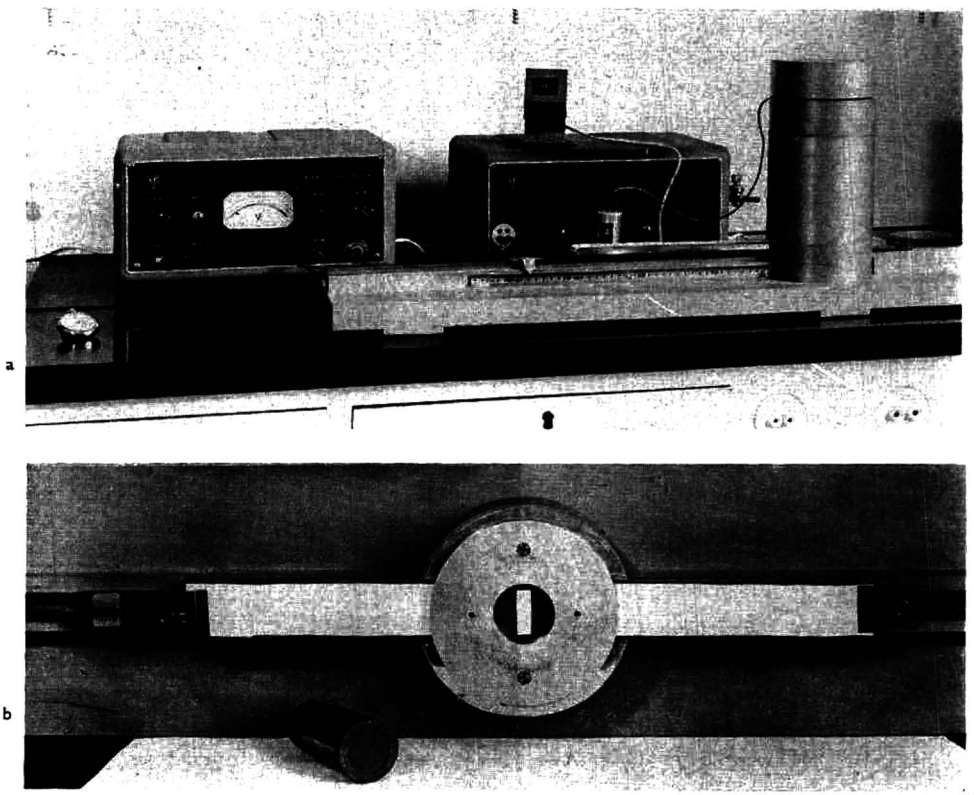

Fig. 1. Apparatus for manual measurement of monodimensional radiochromatograms. (a). General view. (b). Detail showing the slide which carries the chromatogram and the scanning slit (view from above after removing the lead shield and the Geiger counter).

* FULLER[6] has described a counter with an end-window of Mylar, 0.5 thousandths of an inch thick. This window has a lower absorption than one of mica and thus permits the measurement of ^{14}C with an absolute efficiency of about 10%.

** Flow counters have been suitably adapted for the scanning of radiochromatograms. Some investigators have made the contact between the counter and the chromatogram as airtight as possible (GRAY *et al*[6]; BANGHAM[7]; LÖWENSTEIN AND COHEN[8]; OSINSKI[51]). DEMOREST AND BASKIN[9], on the other hand, have modified the counter by incorporating into it a chamber with a sliding base carrying the chromatogram. A similar counter without a window but completely airtight has been constructed by HARRISON AND WINTERINGHAM[10].This counter has a geometrical efficiency of 4π.

It is evident that although the measurement of a radiochromatogram with such a simple apparatus is accurate, it is inconvenient because of the considerable amount of work and time involved. Various ways of simplifying this technique have therefore been suggested.

The most simple method is localization of the spots by autoradiography, followed by quantitative determination of their activity. Such measurement is improved by the use of a counter larger than those usually employed, whereby it is possible to determine the activity of an entire spot in only one measurement (see, *e.g.*, KATZ AND CHAIKOFF[12] and STEENBERG AND BENSON[13]). In another method all the zones of the chromatogram are measured simultaneously with a multiple measuring device. GILBERT AND KEERNE[14] have constructed such an apparatus containing 30 adjoining counters each with a rectangular window 8 × 25 mm. The impulses transmitted by these counters are accumulated in a series of condensers. The potential of each of these condensers, which is determined with an electrometer at the end of the measuring period, gives the value of the activity present in the corresponding zone of the chromatogram.

In measuring the activity of radiochromatograms containing isotopes emitting soft radiation (^{3}H, ^{14}C, ^{35}S), the efficiency in the measurement can be increased, and thus the counting time reduced, by elimination of the self-absorption of the radiation from the paper. This has been achieved by ROUCAYROL, OBERHAUSEN AND SCHULER[15] who impregnated the chromatogram with a solution of phenyl-biphenyl-oxadiazole in toluene and afterwards measured the light coming from the paper with a multiplier phototube. Recently, FUNT AND HETHERINGTON[16] have found that the use of a solvent less volatile than toluene, such as monoisopropylbiphenyl, guarantees a better reproducibility of the measurements. SELIGER AND AGRANOFF[17], using a solution of anthracene in toluene, carried out the measurements after evaporation of the solvent.

However, the method most often applied to reduce the manual work involved has been the automatic advancement of the chromatogram and automatic recording of the radioactivity.

(*b*) *Automatic apparatuses*

Up to the present about 30 such apparatuses have been described; these can be divided into two groups according to the system used for advancement of the chromatogram and for recording the radioactivity of the spots. The type of recording can be graphic or numerical, and the mode of advancement of the chromatogram can be continuous or discontinuous, but for practical purposes the continuous advancement of the chromatogram is generally combined with graphic recording and the discontinuous method with numerical recording.

(1) *Apparatuses with continuous advancement of the chromatogram and graphic recording of the radioactivity*

With apparatuses of this type, the statistical sequence of impulses coming from the

counter is transformed by a ratemeter into a continuous flow of current which drives a graphic recorder*.

The first such devices were described in 1951 by MÜLLER AND WISE[19], WILLIAMS AND SMITH[20] and FRIERSON AND JONES[21].

Fig. 2 shows, for example, the plan of the apparatus used by MÜLLER AND WISE, together with a typical recording obtained. The activity of each spot is calculated

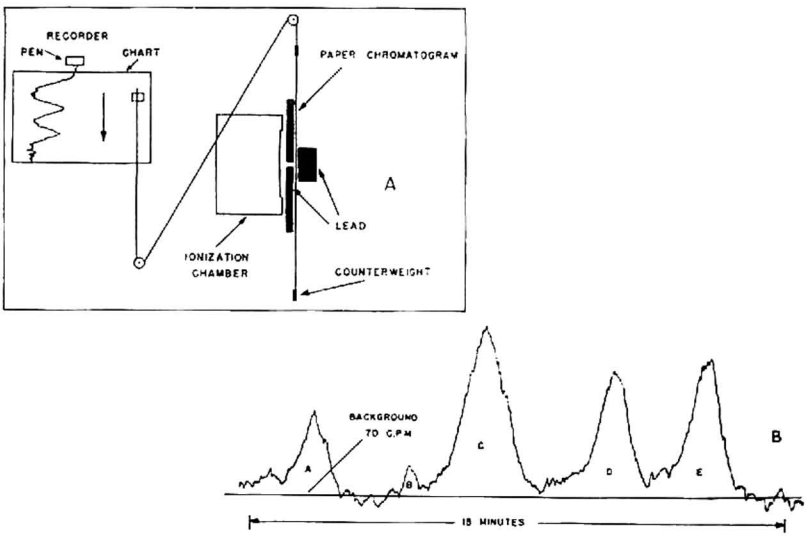

Fig. 2. (A). Scheme of the arrangement for the automatic advancement of a monodimensional radiochromatogram. (B). A typical record obtained with this apparatus. (According to MÜLLER AND WISE[19].)

by measuring the area enclosed between the corresponding peak and the base line; the error in this calculation is about 1 %.

Other devices based on the same general principle, but with small differences in the system of advancement of the chromatogram, have successively been described by various authors (JONES[22]; BRADLEY[23]; CARLESON[24]; LERCH AND NEUKOMM[25]; DEMOREST AND BASKIN[9]; PINAJIAN AND CHRISTIAN[26]; COHN, BUCKALOO AND CARTER[27]; STERNBERG[28]; VIGNE AND LISSITZKY[29]. Devices of this type have also been produced commercially for the past few years.

The factors that influence the profile of a curve are: the size of the slit; the speed of advancement of the chromatogram and of the recording paper; the electrical and mechanical inertia of the ratemeter and the recorder; and the statistical fluctuation of the radioactivity (BONET-MAURY[30]). These variables are strictly correlated. For example, WILLIAMS AND SMITH[20] have shown that in order to obtain an accurate recording, the slit width (S), the time constant (T) of the ratemeter and the speed (V)

* SOLOWAY, RENNIE AND STETTEN[18] have described a device in which the graphic registration, instead of being a continuous curve, is a series of parallel segments, their height being proportional to the number of impulses counted during a pre-set time interval of integration.

of movement of the chromatogram should conform to the relationship $S = 4\ TV$.

A drawback to the system of graphic recording is that the degree of sensitivity of the instrument has to be selected according to the most active zone. This obviously causes a relatively greater percentage of error in the zone with the weakest radioactivity. In order to eliminate this factor, BERTHET[31] devised an apparatus which changes the degree of sensitivity of the recorder automatically when the radioactivity reaches pre-set values. By this means the error of the apparatus is kept constant and, for radioactivity varying between 300–30,000 c.p.m., is always under 2 %.

The areas on the graphic recording corresponding to the radioactivity of the spots on the chromatogram are generally measured with a planimeter. ROBERTS AND CARLETON[32] obtained under specific experimental conditions a proportional relationship between the height of the peak of the curve and the radioactivity of the whole spot. To achieve this, however, it was necessary to chromatograph, besides the sample, a standard series of the same substances. DURRUM AND GILFORD[33] avoided using a planimeter for the integration by making a device whereby registration of the integral curve is obtained. The activity of the spot is measured by making two successive chromatographic scannings; the first registers the radioactivity of each point and the second recording gives the integral curve, the two curves being superimposed. The difference between the ordinates of the second graphic recording drawn at the minima at each side of a peak of the first recording, gives a direct indication of the total radioactivity of the corresponding spot (Fig. 3). A similar apparatus has been constructed by SMILJANIČ AND RABUZIN[52].

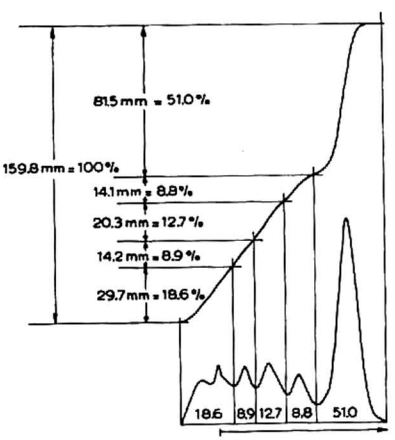

Fig. 3. Recorded curve of radioactivity of a scanned radiochromatogram and the corresponding integral curve (according to DURRUM AND GILFORD[33]).

Two automatic graphic devices described by WINGO[34] and ARONOFF[35] can be included in this group. These apparatuses, however, instead of recording one measurement, make a facsimile (Fig. 4) of the distribution of the radioactive spots on the chromatogram, the result being similar to an autoradiograph but obtained in a much shorter time. The device described by WINGO consists of a rotating cylinder on whose

wall the chromatogram and the recording paper are wrapped next to each other. The impulses from the Geiger counter are fed through a scaler. This has a relay which controls the pen-recorder so that each impulse is registered. A succession of scannings produces a series of parallel recordings, which constitute a facsimile of the radioactive spots. In the apparatus of ARONOFF, on the other hand, the Geiger counter and pen-recorder move continuously in a direction parallel to the axis of the cylinder, which rotates continuously carrying the chromatogram and recording paper with it.

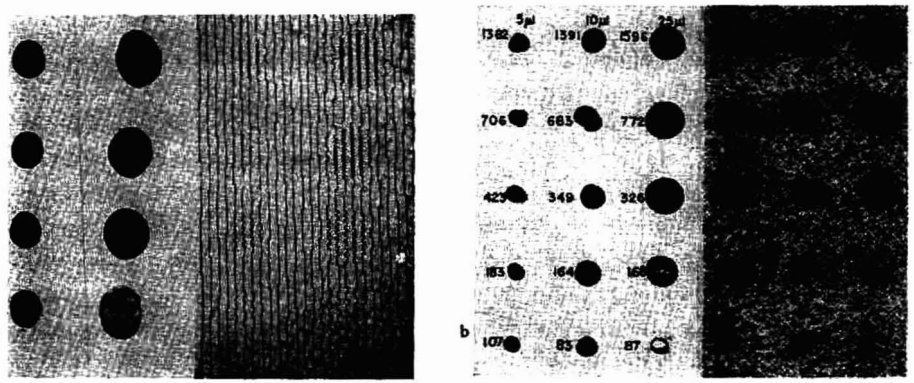

Fig. 4. Autoradiograph together with the corresponding facsimile record of two artificial chromatograms (a. according to WINGO[34], b. according to ARONOFF[35]).

The impulses from the counter, suitably modified, pass through the stylus and are registered on the sensitive paper at the point of contact. Thus, a combination of the two movements, namely the rotation of the drum and the advancement of the counter and pen-recorder, provides a complete scanning of the chromatogram. The tracing that appears on the paper is a facsimile of the radioactive spots, in the form of clusters of points.

(2) *Apparatuses with discontinuous movement of the chromatogram and numerical recording of the radioactivity**

Some apparatuses of this type record the number of impulses counted in a pre-set time period, while others record the time required to reach a pre-fixed number of impulses. ROCKLAND, LIEBERMAN AND DUNN[37] were the first to make an apparatus with discontinuous advancement of the radiochromatogram. The sliding platform with the chromatogram is moved in increments under an end-window Geiger counter by means of an advancing device. This latter comprises an interchangeable ratchet and slit, a spring and an electro-magnet. The movement of the sliding platform (with the attached chromatogram) is regulated by the dimensions of the ratchet (paired with the slit). The latter are interchangeable and can vary the increment from $^1/_{16}$ in. to 1 in. The impulses coming from the counter are accumulated on a binary scale. When

* WINTERINGHAM *et al.*[1] and PIPER AND ARNSTEIN[36] have described apparatuses with a system of graphic recording and discontinuous advancement of the chromatogram.

the pre-set number of impulses is reached, the scaler (by means of a suitable circuit) transmits an electric impulse to a timer which registers the elapsed time. Immediately afterwards, the scaler and time marker are reset and the chromatogram is advanced to the next position. The value of the activity of each zone scanned can easily be obtained from the recording time. Such a system of counting with a pre-set number of impulses has, however, the disadvantage that much of the time required for scanning the entire chromatogram is spent in measuring only the background.

On the other hand, recording the number of impulses counted in a pre-set time presents a major construction difficulty, in that the mechanical recorder lacks the rapidity necessary to accumulate the statistical impulses coming from a highly active zone of a chromatogram.

HELLSTRÖM[38] has solved this problem by means of a "robot scaler" in which the impulses are accumulated in a series of decadic tubes and the recording is done at the end of each measuring period by means of an electromechanical read-out and a digital printing unit. The chromatographic strips to be scanned can be joined together, end to end, in one long strip with a maximum length of 40 m (Fig. 5). The strip moves

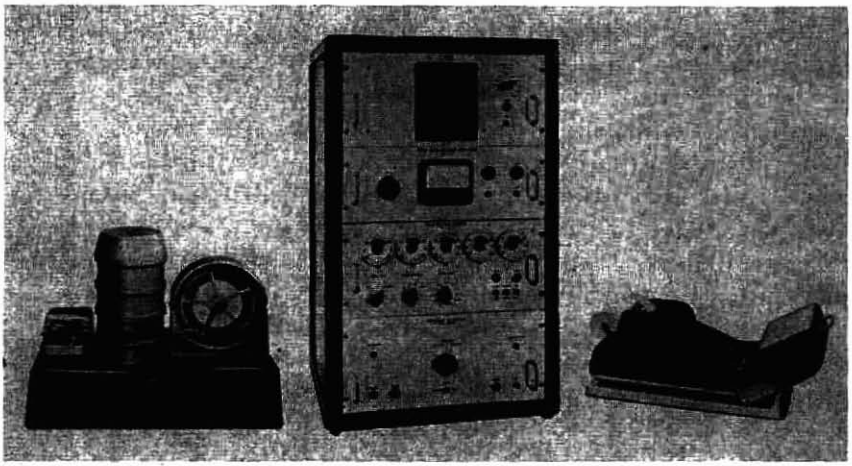

Fig. 5. Robot scaling equipment with chromatogram scanner, robot scaler and digital printing unit (according to HELLSTRÖM[38]).

step-wise under the Geiger tube of the scanning device, the step length varying between 3 and 7 mm. The impulses coming from the counter are counted by a decadic scaler and after a pre-set time interval the digits indicated on the decadic tubes are transferred by a read-out and a series of solenoid-operated plugs to the keys of an adding machine, which prints the digits of counted pulses on a strip of paper. Immediately afterwards the decadic tubes become reset and the chromatogram is automatically advanced to the next position.

The instruments discussed thus far (with the exception of that described in a short communication by PIPER AND ARNSTEIN[36]) can only scan strips of paper. Therefore, bidimensional chromatograms have to be cut into strips for measurement. With

this method it often happens that the most radioactive zone of the spot is situated at the edge of the paper strip. It is, therefore, necessary that all points be measured with the same efficiency. This can be done by keeping the width of the strips to about 1 cm, which is less than that of the usual (3 cm) monodimensional radiochromatograms. In measuring the latter, less efficiency is in fact permissible along the edges of the strips, because the spots occur along the central axis of the chromatogram. If the width of the strip is reduced, even a better resolving power will be achieved. If the width is the same as the distance of advancement, the zone to be scanned will be a square and, therefore, the resolving power will be the same in both directions. In any event, measurement of bidimensional chromatograms by the above-mentioned system involves considerable handling. In the devices with graphic registration furthermore, when the adjacent graphs corresponding to each strip are assembled, the map obtained is expanded in one direction and consequently the distribution of the spots is less evident.

From all that has been said above, it is evident that to obtain the best results a map of the radioactive distribution of a bidimensional radiochromatogram should be made, and this involves designing a device specifically for that purpose.

In 1956 CHAIN *et al.*[39] described a series of apparatuses of this type. The chromatogram is mounted on a frame which moves in a vertical plane between two mica end-window Geiger counters, having a diaphragm with an open square 1 cm by 1 cm; the frame moves horizontally at pre-set time intervals. The scanning begins at the lower edge of the paper and when the whole width of the paper has been scanned, the frame returns automatically to the original position and at the same time the chromatogram is lowered 1 cm. The horizontal scanning commences again and the sequence of movements is repeated until the whole chromatogram has been scanned. The movements of the frame are controlled by an electromechanical timer (which is activated by means of a motor) and by a series of microswitches. These devices have different methods of recording the impulses coming from the Geiger counter: a photographic system, a system of counter stamping and an electronic system. *Photgraphic system*: In this method the impulses coming from the counter, after being divided in a scaler, are counted in a mechanical register (Fig. 6). The numbers appearing are automatically photographed at the end of each scanning period. To obtain a map of the numbers representing the distribution of the radioactivity on a bidimensional radiochromatogram, it is necessary to cut the photographic film into strips corresponding to each horizontal scanning and place these parallel to each other. The manual work involved in this operation can be avoided by using a stamping counter moved by a pantograph. *Stamping counter system*: The impulses are counted by a special mechanical stamping register (UGOLINI[40]), which is moved by means of a pantograph synchronized with the movement of the frame (Fig. 7). At the end of each measuring period, the number of impulses are reproduced on a sheet of paper in positions corresponding to those of the scanned zone of the radiochromatogram. It must be borne in mind, however, that the stamping counter of necessity has a mechanical inertia greater than that of the usual counter, and therefore the coincidence

loss increases rapidly if the necessary counting rate is to be maintained to count some hundreds of impulses per minute (CHAIN *et al.*[39]). On the other hand, it is evidently not possible to augment the scaling factor noticeably, because then even the missed

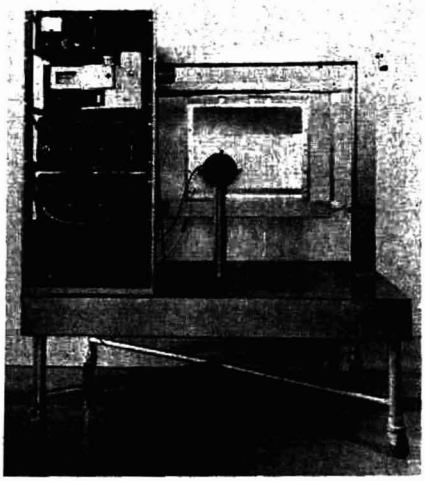

Fig. 6. Automatic apparatus for the measurement of bidimensional radiochromatograms with photographic recorder (according to CHAIN *et al.*[39]).

remainder increases and hence the error in the measurement of the zone with the least radioactivity becomes excessive. An *electronic system* in which the afore-mentioned disadvantage is eliminated was finally adopted by these investigators[39]. The impulses are counted on a scale comprising a series of decadic tubes, and at the end of each measurement the numbers indicated on each tube are transferred by a completely electronic read-out unit (FRANK[41]) and a series of solenoid-operated plugs to the keys of an

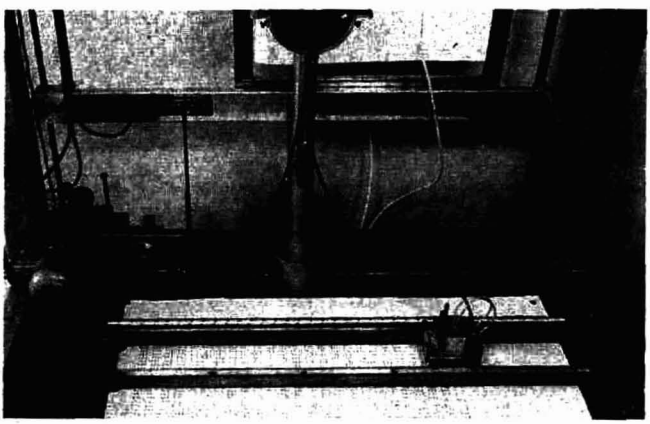

Fig. 7. Automatic apparatus for the measurement of bidemensional radiochromatograms with stamping recorder (according to CHAIN *et al.*[39]).

electric typewriter whose carriage advances and rotates in synchronization with the horizontal and vertical movement of the frame carrying the chromatogram. As with the system of the mechanical stamping register, the number of impulses recorded by the typewriter corresponds in value and in position to the zone scanned on the chromatogram. Fig. 8 shows the numerical map thus obtained with a bidimensional radio-

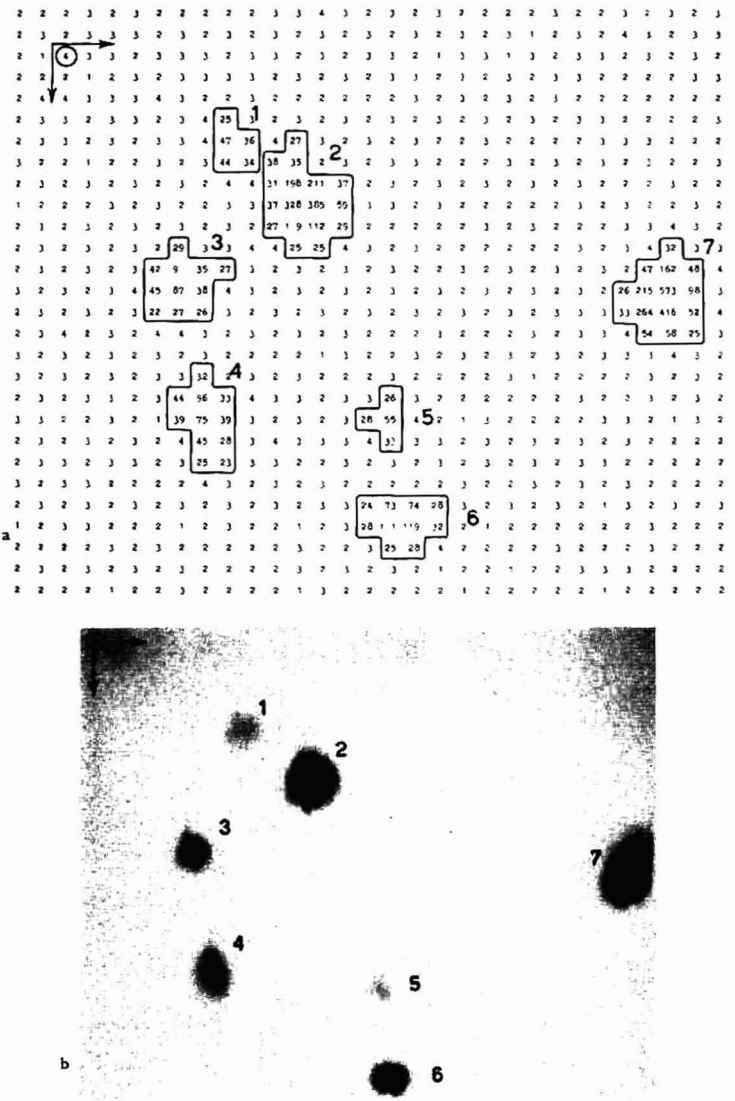

Fig. 8. (a) Numerical map obtained by electronic registration; (b) the corresponding radioautograph. In order to avoid the appearance on the map of figures with many zeros preceding the final digit such as 001, 002 etc., which would obscure the localisation of the active zones, the pulse from the read-out corresponding to the digit 0 is diverted to the solenoid of the advancement key. (According to CHAIN *et al.*[39]).

chromatogram and, for comparison, also the autoradiograph of this example. It should be noted that the numerical map is obtained in 18 hours, whereas the autoradiograph requires 170 hours. With these devices CHAIN *et al.*[39] obtained an optimum reproducibility in the measurement of radioactivity present on a radiochromatogram. In fact, the standard deviation, even including the errors due to the apparatus, was found to be only slightly greater than that occurring with the statistical fluctuations of the radioactivity, which is equivalent to the square root of the number of impulses counted (Table II, line 1). Furthermore, it has been shown that the mode of mounting the chromatogram does not influence the value found for the total radioactivity of each spot (Table II, line 2).

TABLE II

REPRODUCIBILITY OF THE MEASUREMENTS OF A RADIOCHROMATOGRAM CONTAINING SPOTS OF DIFFERENT INTENSITIES*

The radioactive spots are those of a radiochromatogram similar to that of Fig. 8. The results are expressed as c.p.m. ± standard deviation observed and, in parentheses, standard deviation calculated.

Spot	1	2	3	4	5	6	7
1. c.p.m. ± s.d. (mean of 8 expts.)	2190 ± 119 (47)	480 ± 40 (22)	1120 ± 79 (34)	490 ± 40 (22)	8870 ± 475 (94)	950 ± 53 (31)	1630 ± 79 (40)
2. c.p.m. ± s.d. (mean of 4 expts.)	2200 ± 132 (47)	460 ± 53 (22)	1110 ± 79 (34)	490 ± 40 (22)	8900 ± 449 (94)	950 ± 53 (31)	1600 ± 66 (40)

* According to CHAIN *et al.*[39].

Recently, an apparatus has been described by FRANK, CHAIN, POCCHIARI AND ROSSI[42], which is mechanically much simpler than the foregoing, in that the electromechanical devices for the horizontal and vertical movements of the radiochromatogram are eliminated. The radiochromatogram is simply attached by adhesive tape to one edge of a blank sheet of paper which is inserted into the carriage of an electric typewriter (Fig. 9). The chromatogram is then moved between the two counters by the automatic movement of the carriage. The decadic scale, the autoprogramme unit and the electronic read-out are the same as described heretofore[39,41]. The authors have also designed a similar apparatus for measuring monodimensional chromatograms. The chromatogram is mounted on a small frame joined at one end to the recording paper of an adding machine and at the other end to a counter balance (Fig. 10). After the number of pulses counted in each zone has been recorded, the recording paper advances and moves the chromatogram between the two counters. The scanning window is 5 × 30 mm.

All the apparatuses described above[39,42] also contain a device for automatic selection between two pre-set counting periods, one a multiple of the other. If, during the measurement of the radioactive zone of the chromatogram the number of impulses counted in a given time t (usually 45 seconds) is less than the pre-set threshold, this is considered as the background, and the apparatus moves on to scan the next zone. If, on the other hand, the number of pulses counted exceeds the value of the threshold, this indicates that the zone being scanned is radioactive, and the device continues

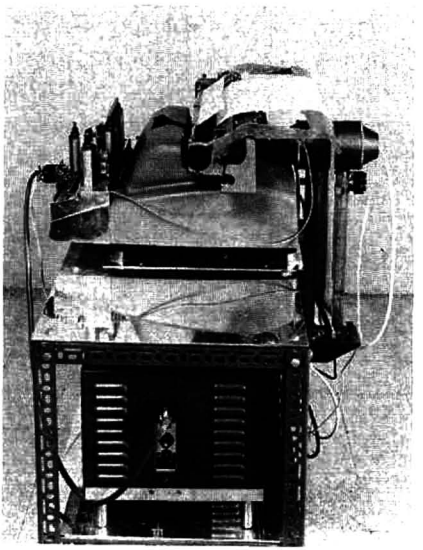

Fig. 9. Automatic apparatus for the measurement of bidimensional radiochromatograms, in which an electrical typewriter is used both for moving the chromatogram and for recording the pulses (according to FRANK *et al.*[42]).

to measure this zone for a period T equal to a pre-set multiple (usually 5 times) of the t time period. This autoprogramme is necessary for measuring bidimensional chromatograms, because in these the radioactive zones *in toto* represent only a small percentage of the entire area scanned.

The above-mentioned devices[39,42] are generally used for measuring different radioactive isotopes emitting β particles. The suitability of the apparatus depends mainly on the type of measuring device used (as already discussed on pp. 380, 381), and on the collimation system. With regard to the latter, it should be remembered that when using ^{32}P it has been found[42] that the error in collimation exceeds that observed with radioactive isotopes emitting less penetrating rays, as for example ^{14}C

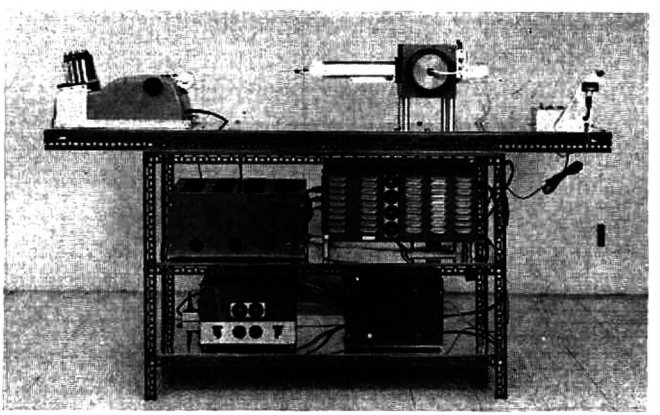

Fig. 10. Automatic apparatus for measuring monodimensional radiochromatograms.

and ^{35}S. While the total relative intensity of the spots is not much altered by this error, it does cause an apparent enlargement on the numerical map and hence, a lower resolving power.

(c) Use of the various measuring techniques in radio paper chromatography

Up to the present radio paper chromatography has been utilized mainly in the field of biochemistry. In fact, in the study of intermediate metabolism with labelled substances it has provided a complete picture of the distribution of the tracer in the various metabolites. Such a picture can be obtained semiquantitatively by autoradiography. Examples are shown in Figs. 11, 12 and 13. Fig. 11 shows the autoradiograph of a

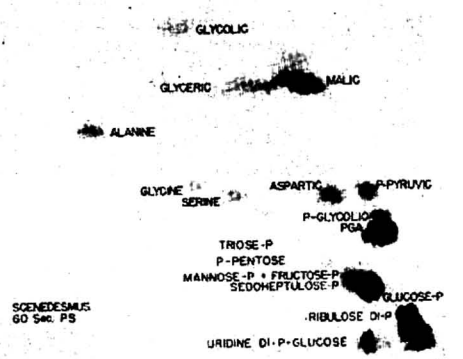

Fig. 11. Radioautograph of a bidimensional chromatogram of an 80% alcoholic extract of *Scenedesmus* after 60 sec photosynthesis in radioactive CO_2 (according to CALVIN[44]).

bidimensional chromatogram of an alcoholic extract of *Scenedesmus alga* kept for 60 seconds in air containing $^{14}CO_2$. Identification of the various intermediate metabolites together with a kinetic study of the incorporation of ^{14}C into these compounds enabled CALVIN and his co-workers to suggest a cycle for the reduction of carbon in photosynthesis and to connect this cycle to other metabolic pathways (for bibliography see refs.[43,44]). Figs. 12 and 13 show autoradiographs of chromatograms of aqueous ethanolic extracts of rat diaphragm (BELOFF-CHAIN *et al.*[45]) and of rat brain (BELOFF-CHAIN *et al.*[46]) after incubation with uniformly-labelled ^{14}C-glucose. Such autoradiographs reveal that there is a distinct difference in the metabolism of glucose in the two tissues; in the muscle tissue the ^{14}C-labelled glucose was converted into oligo- and polysaccarides and in the brain into amino acids; in both instances glucose is also converted into lactate.

In order to determine a balance in the distribution of the tracer in the various metabolites, it is necessary to measure exactly the activity of a single spot. For this purpose, apparatuses for the manual advancement of the chromatogram and recording of the values were used by BELOFF-CHAIN *et al.*[45], who were able to study the effect of insulin on glucose metabolism in the isolated diaphragm of a rat. Considerable time and labour were required to accomplish such a study by this method.

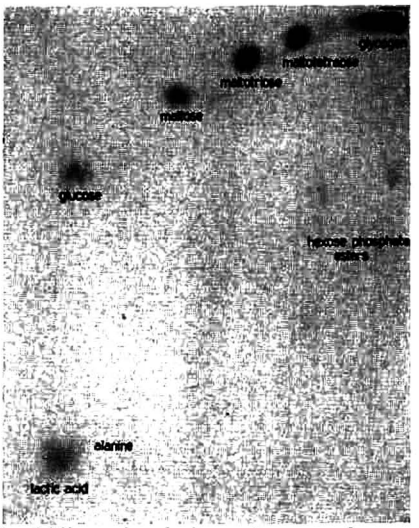

Fig. 12. Radioautograph of a bidimensional chromatogram of an aqueous ethanolic extract of rat diaphragm muscle incubated with uniformly labelled ^{14}C-glucose. Development of the chromatogram: ⟶ butanol–ethanol–NH_3–water; ↓ picric acid–*tert.*-butyl alcohol–water. (According to BELOFF-CHAIN *et al.*[45]).

Automatic devices for continuous advancement of the chromatogram and graphic registration of the activity have a large scope because they can easily be made in the laboratory and simple commercial types are readily available. Thus, the literature contains many reports of the use of such devices. Because of their limitations (see pp. 385, 386) such devices have only been used in experiments in which a small number of samples has to be measured, or in semiquantitative determinations. None has been used for the measurement of bidimensional radiochromatograms.

Automatic apparatuses with numerical registration of the type described by HELLSTRÖM[38] for the exploration of monodimensional radiochromatograms have been used by LINDBERG AND ERNSTER[47], who were able to determine the turnover rate of phosphorus in mitochondria.

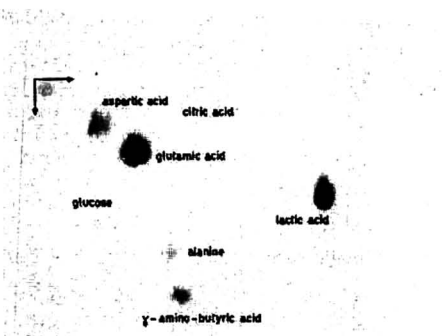

Fig. 13. Radioautograph of a bidimensional chromatogram of an aqueous ethanolic extract of rat brain incubated with uniformly labelled ^{14}C-glucose. Development of the chromatogram: ⟶ *sec.*-butyl alcohol–formic acid–water; ↓ phenol–water. (According to BELOFF-CHAIN *et al.*[46]).

Finally, by using apparatuses with numerical registration for bidimensional radiochromatograms with autoprogrammes, which make possible the rapid measurement of a great number of chromatograms with well-defined spots, CHAIN and his collaborators have been able to examine critically the entire problem of the mechanism of the action of insulin[48] and to compare quantitatively the metabolism of glucose and other substrates in various types of animal tissue, such as diaphragm, liver, brown adipose tissue, brain and pituitary (for references see CHAIN[49]).

As we have seen from this brief survey of the use of various techniques for measuring radiochromatograms, the introduction of automatic devices perfected to such a point that a numerical map of the activity exactly superimposed on bidimensional radiochromatogram is obtained, provides new ways (not easily accessible by other means) for the resolution of specific biochemical problems, such as the mechanism of the action of hormones and pharmacologically active substances.

While this manuscript was in preparation a review on the use of radioisotopes in chromatography was published by ROCHE, LISSITZKY AND MICHEL[50].

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PAPER PARTITION CHROMATOGRAPHY OF ALKYL-SUBSTITUTED 1-PHENYLAZO-2-NAPHTHOLS

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Although paper chromatography of water-soluble dyes has received a considerable amount of attention, very little has been published to date on the application of this technique to problems of separation and identification of oil-soluble dyes. The first use of paper chromatography for oil-soluble dye mixtures was reported by THALER AND SCHELER[1] who separated two simple dye mixtures on untreated paper. Separations of more complex dye mixtures by paper chromatography, again on untreated paper, have been reported by JAX AND AUST[2] and by FUJII[3] using a variety of solvent mixtures for development. The experiments of THALER AND SCHELER[1] and those of JAX AND AUST[2], using untreated paper, were repeated by VERMA AND DASS[4] and by MARK AND MCKEOWN[5] and in each case it was reported that poor resolution was obtained due to a considerable amount of tailing of the spots. The use of reversed-phase paper chromatography would be expected to lead to improved resolution of mixtures of oil-soluble dyes, and indeed, LINDBERG[6], VERMA AND DASS[4], and MARK AND MCKEOWN[5] all have reported excellent separations by using paper impregnated with a variety of lipophilic substances and development with a number of aqueous solutions of organic solvents.

This paper is concerned with the reversed-phase paper chromatographic behaviour of a series of alkyl-substituted 1-phenylazo-2-naphthols, all of which are oil-soluble and either isomers or homologues of one another. The specific compounds chosen for study were 1-phenylazo-2-naphthol, all three isomers of 1-(methylphenylazo)-2-naphthol, all six isomeric 1-(dimethylphenylazo)-2-naphthols, 1-(2-ethylphenylazo)-2-naphthol, 1-(2,4,5-trimethylphenylazo)-2-naphthol, and 1-(2,4,6-trimethylphenylazo)-2-naphthol. Data reported previously by other workers[4-6] on the reversed-phase paper chromatography of two homologous series of dyes indicate that the R_F values of the dyes within each series differ sufficiently to allow at least the lower members of each series to be separated from one another. One of these groups of dyes consisted of commercial samples of Sudan I, Orange SS, and Oil Red XO which are mainly 1-phenylazo-2-naphthol, 1-(2-methylphenylazo)-2-naphthol, and a mixture of 1-(2,4-dimethylphenylazo)-2-naphthol and 1-(2,5-dimethylphenylazo)-2-naphthol respectively; these compounds have been included in the work reported here. The other group of homologous dyes was comprised of commercial

samples of Oil Yellow AB and Oil Yellow OB which are respectively 1-phenylazo-2-naphthylamine and 1-(2-methylphenylazo)-2-naphthylamine. Although Oil Red XO is known to be a mixture of two isomeric dyes, resolution of this mixture was not reported in the work referred to above. One might conclude from this that the difference in R_F values of isomeric dyes that differ only in the relative positions of methyl groups is too small to permit their separation; however, it has been observed in this laboratory[7] that resolution of the two isomeric dyes comprising Oil Red XO could be accomplished under appropriate conditions of development. Thus the most interesting aspect of the work reported here is the chromatographic behaviour of the isomeric dyes within the group of compounds which were chosen for study.

EXPERIMENTAL

1. *Amine intermediates*

The following amines were purchased from Eastman Kodak Company, Rochester, N.Y., and were used without further purification.

2-Methylaniline. Reagent grade, Cat. No. 253; b.p. 83–85°/15 mm.

3-Methylaniline. Reagent grade, Cat. No. 862; b.p. 92–93°/15 mm.

4-Methylaniline. Reagent grade, Cat. No. 254; m.p. 43–45°.

2-Ethylaniline. Practical grade, Cat. No. P3066; b.p. 90–92°/8 mm.

2,3-Dimethylaniline. Practical grade, Cat. No. P5952. This amine was received in the form of the hydrochloride salt and was used as such without liberation of the free amine. Since this salt decomposes on heating, a purity check was done by liberating a small quantity of the amine, b.p. 220–221°/atm.

3,5-Dimethylaniline. Reagent grade, Cat. No. 2324; b.p. 104–105°/14 mm.

Pure samples of four other amines were obtained as follows:

Aniline. Reagent grade aniline (Fisher Scientific Company, New York, N.Y., Cat. No. A-740) was distilled from zinc dust in an atmosphere of nitrogen. The distillation, which was conducted at atmospheric pressure and without fractionation, gave a centre-cut distillate which boiled at 184°.

3,4-Dimethylaniline. This amine was synthesized from fenchone (technical grade, Cat. No. T4571, Eastman Kodak Company, Rochester, N.Y.), b.p. 190–197°/atm, according to the procedure described by ZAUGG[8]. The product was obtained in white crystalline plates, b.p. 97–98°/10 mm; b.p. 225–226°/atm; m.p. 49–50°. Reported for 3,4-dimethylaniline, b.p. 224°/atm; and m.p. 48°[9]; m.p. 50–51°[8].

2,4,5-Trimethylaniline (*Pseudocumidine*). 100 g of commercial Ponceau 3R (F.D. & C. Red No. 1) (Calco Chemical Co., Inc., Boundbrook, N.J.) was dissolved in 2 l of water and made alkaline by the addition of 10 g of sodium hydroxide. The Ponceau 3R was reduced at 60° by the dropwise addition, with vigorous stirring, of a saturated aqueous solution (*ca.* 30 %) of sodium hydrosulphite. Addition of the hydrosulphite was continued until the reaction mixture was no longer red in colour; at the end of the reduction the reaction mixture was murky and yellow-brown in colour. The substituted anilines liberated during the reduction were isolated from the reaction mixture by steam distillation and extraction of the distillate with ether. The ethereal

extract was washed with water, dried over potassium hydroxide pellets, and the ether removed by distillation. The oily liquid residue was then fractionally distilled under reduced pressure in an atmosphere of nitrogen. The fraction which boiled at 104–105°/10 mm was collected and most of this fraction crystallized when chilled in a refrigerator. The small quantity of uncrystallized material was separated from the solid by pressing the mixture between pads of filter paper. Recrystallization of the solid material from ethanol gave colourless needles, m.p. 65–66°. Reported for 2,4,5-trimethylaniline, m.p. 66°[10].

2,4,6-Trimethylaniline (*Mesidine*). Reagent grade acetone was converted to mesitylene using the procedure described by ADAMS AND HUFFERD[11]. Nitration of the mesitylene as described by POWELL AND JOHNSON[12], gave 2-nitromesitylene and this was then reduced with tin and hydrochloric acid using a procedure similar to that described by FIESER[13] for the reduction of nitrobenzene. When the reduction had been completed, the reaction mixture was made strongly alkaline with sodium hydroxide and then steam-distilled to isolate the amine. The steam distillate was extracted with ether and the ether extract was dried over potassium hydroxide pellets. Distillation of the ether extract gave a colourless liquid, b.p. 232–233°/atm. Reported for 2,4,6-trimethylaniline, b.p. 232–233°/atm[14].

2. Azo dyes

Each of the amines described above was diazotized and coupled with 2-naphthol using the following procedure:

A solution containing 0.050 mole of the amine and 8.4 ml (0.100 mole) of concentrated hydrochloric acid in 150 ml of water was prepared and cooled to 0–5° in an ice bath. To this solution was added, dropwise with vigorous stirring, a solution of 3.5 g (0.051 mole) of sodium nitrite in 50 ml of water. After addition of the nitrite had been completed, stirring was continued at 0–5° for 15 min and then 10 % aqueous sulphamic acid solution was added dropwise until the excess nitrous acid had been destroyed (negative test with starch–iodide paper).

Concurrent with the diazotization of the amine, a solution containing 7.9 g (0.055 mole) of 2-naphthol (technical grade, m.p. 119–122°, Cat. No. T171, Eastman Kodak Company, Rochester, N.Y.), 4.0 g of sodium hydroxide, and 8.0 g of sodium acetate in 150 ml of water was prepared and cooled to 0–5° in an ice bath. The diazonium salt solution was then added dropwise, with vigorous stirring, over 30 min and was maintained at 0–5° by the addition of small quantities of ice. The ice bath was removed and stirring was continued for 1 h. The azo dye was collected by extraction of the reaction mixture with chloroform. The chloroform extract was washed with water and then evaporated to dryness on a steam bath. The residual, crude azo dye was recrystallized repeatedly from mixtures of ethanol and benzene until the product had a constant melting point.

In the case of the 2,3-dimethylaniline, which was used in the form of the hydrochloride salt, the above procedure was modified slightly to correct for the hydrogen chloride which was added via the salt. In this case, 0.050 mole of the salt and 4.2 ml

(0.050 mole) of concentrated hydrochloric acid were used in the diazotization step, the remainder of the procedure being unchanged.

The following pure azo dyes were prepared:

1-Phenylazo-2-naphthol. Dark red needles, m.p. 133.5–134.5°. Reported m.p. 132–133°[15].

1-(2-Methylphenylazo)-2-naphthol. Dark red needles, m.p. 131.9–132.4°. Reported m.p. 130.5–131.5°[16].

1-(3-Methylphenylazo)-2-naphthol. Dark red rods, m.p. 141.3–141.7°. Reported m.p. 139–140°[16].

1-(4-Methylphenylazo)-2-naphthol. Dark red rods, m.p. 135.3–135.8°. Reported m.p. 133–135° [15]; m.p. 135.5–137.0°[16].

1-(2-Ethylphenylazo)-2-naphthol. Dark red needles, m.p. 123.0–123.5°. No melting point has been reported previously for this compound although DOLINSKY AND JONES[17] have reported its infrared spectrum.

1-(2,3-Dimethylphenylazo)-2-naphthol. Dark red needles, m.p. 187.5–188.0°. Reported m.p. 125–130°[18].

1-(2,4-Dimethylphenylazo)-2-naphthol.*

1-(2,5-Dimethylphenylazo)-2-naphthol.*

1-(2,6-Dimethyphenylazo)-2-naphthol.*

1-(3,4-Dimethylphenylazo)-2-naphthol. Dark red needles, m.p. 149.6–150.1°. Reported m.p. 146°[19].

1-(3,5-Dimethylphenylazo)-2-naphthol. Dark red needles, m.p. 196.4–196.9°. This is the first synthesis reported for this compound.

1-(2,4,5-Trimethylphenylazo)-2-naphthol. Dark orange needles, m.p. 164.4–164.9°. Reported m.p. 160–161°[19].

1-(2,4,6-Trimethylphenylazo)-2-naphthol. Dark red clusters of needles, m.p. 135.3–135.8°. Reported m.p. 134–135°[15].

3. Reversed-phase paper chromatography

A paper chromatographic apparatus supplied by Canadian Laboratory Supplies Limited, Montreal (Cat. Nos. 49–730, 18–310, and 18–316) was used for the development of the chromatograms. It consisted of a cylindrical glass tank (h. 24 in.; d. 12 in.) with a close fitting plate glass cover and was equipped with a stainless-steel rack that supported two 8 in. glass solvent troughs. The troughs were positioned for descending development of the chromatograms. Since the chromatograms being run were of the "Durchlauf" type, where the solvent front is allowed to run off the end of the paper and the running time is rather long, the tank was fitted with a constant-head apparatus. This consisted of a 2 l Erlenmeyer flask closed by a one-hole rubber stopper through which there extended a short piece of 8 mm glass tubing. A second short piece of 8 mm glass tubing was connected, by means of rubber tubing, to the one through the stopper such that the total length of tubing extending from the flask

* The samples of 2,4-, 2,5-, and 2,6-dimethyl-derivatives were obtained from Mr. W. PRZYBYLSKI of this Laboratory, to whom the authors wish to express their gratitude.

was about 2 in. From 1 l to 1.5 l of developing solvent was placed in the flask and the delivery tube was closed by a pinch-clamp applied to the rubber tubing. The flask was suspended in an inverted position above the tank with the delivery tube extending through a 0.5 cm hole in the glass cover and into one of the solvent troughs. The end of the delivery tube was positioned in the trough at the desired solvent level and, if necessary, the position of the rubber tubing was adjusted so that it closed off the small hole in the cover. The pinch-clamp was removed from the delivery tube so as to allow solvent to run into the trough and solvent was transferred to the second trough by a siphon made from glass tubing. When the level of liquid in the troughs fell below the tip of the delivery tube, air was admitted to the flask thus allowing fresh solvent to flow into the troughs until the original level was restored. Before developing chromatograms with a new solvent, the tank was equilibrated by placing 200 ml of the solvent in the bottom of the tank and allowing it to stand overnight. It was not necessary to equilibrate the tank again until the composition of the developing solvent was altered.

The paper used for the chromatograms was Whatman No. 3MM cut into strips 6 in. × 22.5 in. and serrated at one end. Two pencil lines were drawn 2.25 in. and 2.75 in. respectively from the opposite end of the paper with the latter being marked every 19 mm. Impregnation of the paper with non-polar stationary phase was accomplished by passing it, at uniform speed, through a trough containing a solution of 5 g white mineral oil, U.S.P. XII (Fisher Scientific Co., New York, N.Y.; Cat. No. O–119) dissolved in 100 ml ether. The paper was then suspended in a vertical position and allowed to dry at room temperature.

Solutions of each of the oil-soluble dyes were prepared by dissolving 10 mg of the dye in acetone and diluting to 25 ml in a volumetric flask. These solutions were spotted along the second pencil line on the dried, impregnated paper, a single solution being applied to each mark on the starting line. The volume of each dye solution used was 5 μl (*i.e.* 2 μg of dye) delivered from a micro pipette and confined to a spot 0.5 cm in diameter. When the spots had dried, the paper was folded along the first pencil line and placed over a supporting rod in the developing tank with the end of the paper dipping into the solvent in the trough. One of the spots on each paper was 1-phenylazo-2-naphthol which was used as a reference compound for determining relative R_F values. The chromatograms were developed with mixtures of acetone and water using proportions of 40:60, 45:55, and 50:50 by volume. Development of the chromatograms was continued until the 1-phenylazo-2-naphthol spot had travelled about 30 cm down the paper from the starting line. Each of the oil-soluble dyes was chromatographed, with a given solvent, on four different strips of paper to obtain averaged values. After development of the chromatogram had been completed, the paper was removed from the tank, suspended in a vertical position, and dried at room temperature. R_S values relative to the 1-phenylazo-2-naphthol spot were then calculated and averaged for the four papers. The R_F value of 1-phenylazo-2-naphthol in each of the developing solvents was determined in quadruplicate and the averaged value was used to calculate the apparent R_F values of the other dyes.

DISCUSSION

The initial difficulty in this investigation was the procurement of suitable samples of the alkyl-substituted 1-phenylazo-2-naphthols. Since the dyes differed only slightly in R_F values, especially among isomeric dyes, it seemed desirable to obtain these dyes in as pure a state as possible. Only three dyes of this type, Sudan I, Orange SS, and Oil Red XO, are commercially available and although Sudan I and Orange SS are relatively free from isomeric contaminants, Oil Red XO is known to be a mixture of two isomeric dyes in approximately equal amounts. Rather than using any of these commercial products, all of the dyes employed in this investigation were synthesized in an identical manner by diazotization of the corresponding amines and coupling the resulting diazonium salts with 2-naphthol. Many of the amines needed for the syntheses of these dyes were available from chemical suppliers, usually in reagent grade purity, and were used without further purification. Those amines not commercially available were synthesized using methods which, for the most part, already appear in the literature. The crude product from each of the coupling reactions was purified by repeated recrystallization, to constant melting point, from mixtures of benzene and ethanol; these dyes are readily soluble in benzene but only sparingly soluble in ethanol.

Only one of the dyes reported here, 1-(3,5-dimethylphenylazo)-2-naphthol, was previously unknown. The 2-ethyl-derivative has been reported only once[17] and although its infrared spectrum was given, the melting point for this compound was not reported. All of the remaining dyes appear in the literature one or more times and the melting points given here for these compounds generally agree quite well with the reported values, the exception being that of 1-(2,3-dimethylphenylazo)-2-naphthol which is considerably higher than the sole reported value by MAY AND HUNT[18]. At first it was thought that the material obtained as the 2,3-dimethyl-derivative was not the expected compound. The 2,3-dimethylaniline hydrochloride used in the preparation of this dye was only of practical grade and no information was available as to its identification or its purity since this material decomposes on heating and no melting point could be obtained. The amine was identified by distillation of the free amine after first liberating it from the salt. Almost all of the amine distilled within the range 220–221° which is correct for 2,3-dimethylaniline; unlike most of the isomeric dimethylanilines which boil at or about the same temperature, 2,3-dimethylaniline has a unique boiling point. Additional evidence as to the identity of this amine was obtained by conversion of the distilled amine to its acetyl derivative, m.p. 134–135°; reported m.p. 135.5°[10]. A centre-cut fraction of the distilled amine, when coupled with 2-naphthol in the usual manner, gave the same product as was obtained previously from the hydrochloride salt. Finally, the infrared spectrum of the dye was almost identical with that reported by DOLINSKY AND JONES[17]. Thus it seems to be evident that the dye which was obtained was indeed the expected 2,3-dimethyl-derivative. The validity of the melting point reported by MAY AND HUNT[18] for this compound is somewhat in doubt since it spans a 5° range and also because their melting

points for other oil-soluble dyes of this type are consistently much lower than the values reported by other workers.

The method used for development of the chromatograms was a standard reversed-phase technique with descending flow of the developing solvent. Since the chromatograms were of the "Durchlauf" type and were developed for several days, the chromatography tank was fitted with a constant-head device for supplying fresh developing solvent so that the apparatus required little or no attention. Experimentation revealed that equilibration of the tank for several hours or preferably overnight was necessary in order to obtain reproducible R_F values but that it was not necessary to treat the paper in any manner, exclusive of impregnation, prior to development of the chromatogram. Whatman No. 3MM chromatographic paper was chosen as the support after running comparative chromatograms using this paper and Whatman papers numbers 1, 31, 31 extra thick, and 54. The 31 and 54 papers had the apparent advantage of being faster than the 1 and 3MM papers but it was found that the differences in R_F values of the dyes were smaller, under uniform chromatographic conditions, on these faster papers than on the slower ones. Whatman No. 1 paper was found to be roughly equivalent to 3MM paper in its ability to resolve mixtures of the dyes but it suffered from the disadvantage of having considerably less capacity than that of the thicker 3MM paper. These observations may not be directly attributable to the papers themselves but rather to the amounts of oil which were retained by the papers after impregnation. These papers vary greatly in their abilities to absorb liquids and since the oil was applied in ether solution to the paper, different amounts of this solution would be retained by the various papers and thus, on drying, these papers would contain different amounts of the stationary phase. It was found that varying the amount of oil on the paper, by altering the concentration of the impregnating solution, had a marked effect both on the R_F values and on the degree of separation which was achieved with dye mixtures. Only one stationary phase, light mineral oil, was used in this investigation because numerous chromatograms of oil-soluble dyes run in this laboratory have indicated that this material is at least as good as, and is sometimes superior to, the other commonly used stationary phases. It also has the added advantage of being chemically inert. For impregnation of the paper, it was found that a solution of 5 g of the oil in 100 ml of ether provided the best results both from the standpoint of the capacity of the paper and of the resolutions which could be achieved when using dye mixtures. Lower oil concentrations resulted in reduced capacities while higher oil concentrations gave inferior resolutions and required excessively long developing times.

The choice of acetone–water mixtures as developing solvents for the chromatograms was based on a number of considerations. The dyes were fairly soluble in acetone but insoluble in water and thus the R_F values of the dyes could be easily changed by altering the water content of the developing solvent. In addition, since acetone is completely miscible with water, it provided an infinite range of solvent mixtures of readily ascertained compositions, and also, it prevented separation of the solvent nto two phases during development of the chromatograms due to fluctuations in

temperature. The mineral oil employed as the stationary phase in the chromatograms was relatively insoluble in acetone and even less so in acetone–water mixtures and thus this solvent system prevented excessive depletion of the stationary phase during development of the chromatograms. Some of the stationary phase was eluted from the paper during development of the chromatograms and presumably this could have been prevented by saturation of the developing solvent with mineral oil prior to the development, however, when this was tried it was found to have no observable effect on the chromatograms. Finally, acetone–water provided good resolution of mixtures of the dyes. It was thought that ethanol–water mixtures might also be used despite the fact that the dyes were considerably less soluble in ethanol than in acetone but this solvent system gave inferior resolutions to those obtained with acetone–water mixtures.

Acetone was selected as the solvent for the dyes in preparing stock solutions for application to the paper for some of the same reasons that it was chosen as the organic component of the developing solvents. It also had the desirable features of rapid evaporation so that the spots could be dried quickly and, being part of the developing solvent, small traces which remained after drying the spots would not influence the chromatograms. The amount of each dye which was applied to the papers was arrived at by trial and error and was such that the colour intensity of the spots made observations of their positions easy and yet it did not overload the paper. It was found that 2 μg of each of the dyes gave the best results, although several times this quantity could be applied to the paper without serious overloading taking place. The stock solutions were prepared such that 5 μl of solution contained 2 μg of dye, this being done purely for convenience since a 5 μl pipette was used for application of the dye solutions to the paper.

Table I summarizes the relevant data obtained from the chromatograms of individual 1-arylazo-2-naphthols in the three acetone–water developing solvents used in this investigation. Each figure in the table is an averaged value from four separate chromatograms each of which had a given dye spotted at different positions along the starting line so as to account for any localized differences in the paper or in the stationary phase. It was found that, for a given dye, values varied somewhat from one chromatogram to another depending on the position at which the dye was spotted, however, these deviations were never greater than 4 % from the arithmetic mean. Each of the papers was spotted at the extreme left-hand mark on the starting line with 1-phenylazo-2-naphthol which served as a reference compound and thus allowed the calculation of R_S values (*i.e.* relative displacements) for each of the other dyes on the paper; these values appear in Table I under the heading R_S. Since the phenyl derivative was spotted at the same position on each of the papers, data obtained from different papers can be directly compared. This is important since it was not convenient to spot all thirteen of the dyes on a single paper. Under the heading R_F in Table I, the values for the phenyl derivative are averaged values which were obtained experimentally by retaining the solvent front on the paper. Values appearing under this heading for the other compounds were calculated by multiplication of R_S for the compound by R_F for the phenyl derivative.

TABLE I

DATA OBTAINED FROM CHROMATOGRAMS OF 1-ARYLAZO-2-NAPHTHOLS DEVELOPED WITH ACETONE–WATER MIXTURES

Compound (Aryl group)	*40: 60 Acetone–water Running time 144 h*				*45: 55 Acetone–water Running time 90 h*				*50: 50 Acetone–water Running time 5 h*			
	R_S	R_F	R_M	*Length of spot (cm)*	R_S	R_F	R_M	*Length of spot (cm)*	R_S	R_F	R_M	*Length of spot (cm)*
Phenyl	1.00	0.048	1.30	3.9	1.00	0.089	1.01	4.0	1.00	0.16	0.73	3.9
2-Methylphenyl	0.55	0.026	1.57	3.9	0.59	0.052	1.23	3.9	0.65	0.10	0.95	4.0
3-Methylphenyl	0.50	0.024	1.61	3.7	0.55	0.050	1.28	3.6	0.61	0.095	0.98	4.0
4-Methylphenyl	0.45	0.022	1.66	3.2	0.49	0.044	1.34	3.2	0.57	0.088	1.01	3.4
2-Ethylphenyl	0.30	0.015	1.85	2.6	0.35	0.031	1.49	2.9	0.41	0.064	1.16	3.1
2,3-Dimethylphenyl	0.29	0.014	1.85	4.0	0.33	0.029	1.52	4.1	0.39	0.060	1.19	4.6
2,4-Dimethylphenyl	0.25	0.012	1.91	2.8	0.29	0.026	1.57	3.1	0.37	0.056	1.23	3.2
2,5-Dimethylphenyl	0.29	0.014	1.85	2.6	0.34	0.031	1.49	3.1	0.41	0.064	1.17	3.2
2,6-Dimethylphenyl	0.37	0.018	1.74	2.7	0.42	0.037	1.41	3.1	0.46	0.072	1.11	3.2
3,4-Dimethylphenyl	0.27	0.013	1.88	2.5	0.33	0.029	1.52	2.9	0.39	0.061	1.19	2.7
3,5-Dimethylphenyl	0.24	0.012	1.91	2.8	0.28	0.025	1.59	3.3	0.35	0.055	1.24	3.1
2,4,5-Trimethylphenyl	0.16	0.0079	2.10	2.3	0.20	0.018	1.74	2.6	0.25	0.039	1.39	2.6
2,4,6-Trimethylphenyl	0.17	0.0081	2.09	2.2	0.21	0.019	1.72	2.6	0.26	0.040	1.38	2.6

Several papers have appeared in the literature, dealing with the relation between chemical structure and chromatographic behaviour, which are applicable to the work reported here. In their classical paper, MARTIN AND SYNGE[20] derived a general theoretical equation for the rate of movement of a solute during column partition chromatography. The same equation was later derived more simply by the use of the kinetic theory[21,22]. CONSDEN *et al.*[23] demonstrated that this equation could be applied to paper partition chromatography with some modification and introduced the concept of R_F values which now has achieved general acceptance. For ideal solutions, it was demonstrated mathematically by MARTIN[24] that the partition coefficient, to which the R_F value is related, is dependent on the free energy required to transport one mole of a compound from the stationary phase to the mobile phase. It was also shown by MARTIN that the addition of a given group to a chemical structure will alter the partition coefficient of the compound by a constant factor which is dependent only on the added group and the nature of the two partitioning phases and not on the rest of the molecule. BATE-SMITH AND WESTALL[25] suggested the use of R_M, which has a linear relation to the logarithm of the partition coefficient, in order to facilitate practical application of MARTIN'S rather involved equation. It follows directly from the equation derived by MARTIN that, for compounds comprising a homologous series, a linear relation exists between the R_M values and the number of like substituent groups present in the compounds. The equation also suggests that isomeric compounds should possess identical R_M values, although this conclusion is not strictly valid since, in deriving the equation, no consideration was given to interaction of the groups within a molecule. Indeed, FRANC *et al.* [26–29] found that isomeric substituted benzenes had different R_M values and modified the linear relation for R_M values, derivable from MARTIN'S equation, by a term involving the dipole moments of the compounds. Accounting for polarity differences among isomers in this manner, these workers have obtained good agreement between calculated and observed R_M values.

The chromatographic data obtained in this investigation, which appear in Table I, agree fairly well with the relationships outlined above. Plotting the R_M values obtained with each solvent system against the number of methyl groups present in the dyes gave a straight line in agreement with MARTIN'S relationship although there was some scattering within the groups of isomeric compounds (Fig. 1). Using the method of least squares, a straight line was positioned on each plot and it was found that the slope gradually increased as the concentration of acetone in the solvent system was decreased. This increase in the slope corresponds to an increase in the spread of R_M values and hence to a greater separation of the spots on the chromatogram. The deviations of the R_M values from linearity did not exceed 6 %, 7 %, and 6 % for the 40:60, 45:55, and 50:50 acetone–water solvent systems respectively.

The small but reproducible differences in the R_M (or R_F) values within the three isomeric groups indicate the effect of positional isomerism. Since the separations involve a process of continuous partition between a stationary non-polar phase and a mobile polar phase, any variation in the polarity of the isomers would lead to a corresponding variation in their rates of travel on a chromatogram. A more polar isomer

would favour the mobile phase and hence exhibit a higher R_F value than a less polar isomer. FRANC and co-workers (see above) have used dipole moments with considerable success as a measure of polarity differences between isomers. Unfortunately, this treatment is not directly applicable here because 1-arylazo-2-naphthol compounds

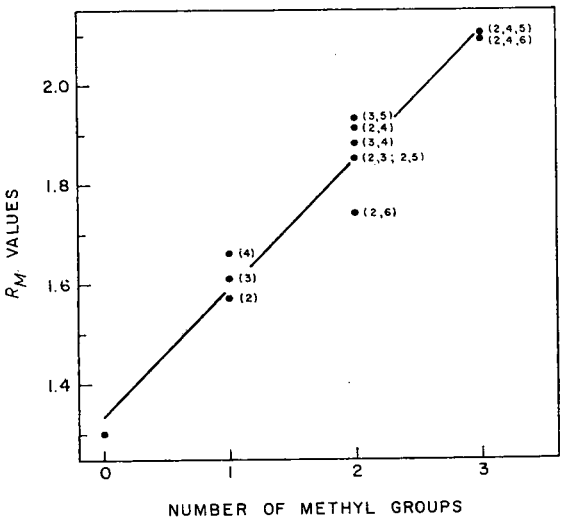

Fig. 1. R_M values of methyl-substituted 1-phenylazo-2-naphthols chromatographed with 40:60 acetone–water plotted against number of methyl groups. The figures in the brackets denote the positions of the methyl groups on the phenyl ring.

do not exist as single species amenable to precise dipole moment determinations but rather as equilibria of azo and hydrazone tautomers[30]. The position of equilibrium in these tautomeric systems is dependent on the nature of the solvent and on the position and character of the substituents.

The relative polarities of the compounds in the three isomeric groups can be determined by considering the known effects of alkyl substituents attached to the phenyl ring on the position of the azo–hydrazone equilibrium. BURAWOY *et al.*[30] have shown that methyl groups favour the more polar hydrazone tautomer in the order 2-methyl > 3-methyl > 4-methyl. Thus, the 2-methylphenyl derivative should be more polar than the 3-methylphenyl derivative and this, in turn, should be more polar than the 4-methylphenyl derivative. Applying the same reasoning to the dimethylphenyl derivatives, the order of polarity should be 2,6-dimethyl > 2,5-dimethyl ≡ 2,3-dimethyl > 2,4-dimethyl > 3,5-dimethyl > 3,4-dimethyl and for the trimethylphenyl derivatives, 2,4,6-trimethyl more polar than 2,4,5-trimethyl. Inspection of Table I and Fig. 1 reveals that the isomers do follow generally the predicted order. A persistent exception is the 3,4-dimethyl isomer which, while it should show the lowest R_F value, shows intermediate behaviour. Bond deformation of the adjacent alkyl groups may be a factor in this instance. It is particularly gratifying to note that even in the highly substituted trimethyl derivatives, the effect of altering the posi-

tion of one methyl group is a small but measurable difference in R_F in the predicted direction.

Almost all the chromatograms run in connection with this work were performed on the individual dyes, however, the data which have been obtained could have practical application in the analysis of aromatic amine mixtures by first coupling the mixed amines with 2-naphthol. This technique was applied to the amine mixtures obtained by reduction of several commercial samples of Ponceau 3R. The azoic component of Ponceau 3R is a mixture of alkyl-substituted anilines including both methylated and ethylated compounds. Chromatography of the dye mixture, thus produced, using 40:60 acetone–water as the developing solvent readily separated the major components. Some of the dyes were not separable even with 40:60 acetone–water, which gave the greatest spread in R_F values. On the basis of the separations which were achieved in this solvent and the R_F values in Table I, two dyes can be separated if their R_F values differ by more than 0.001. Since increased resolution is obtained on decreasing the acetone concentration in the developing solvent, presumably improved separations could be achieved using a smaller concentration of acetone than was used in this work, however, the time required for the development would be rather lengthy. An ethyl derivative, 1-(2-ethylphenylazo)-2-naphthol, was included among the dyes studied here to see whether it would behave like a monomethylated or like a di-methylated compound. Its R_F was similar to those of the dimethylated derivatives and thus the molecular weight of the compound appears to be the dominating factor in the R_F value of these compounds. The averaged length of the spot for each dye after development is included in Table I. There is no significant difference in these values from one solvent to another and it appears that the length of the spots is roughly proportional to the distance travelled down the paper, after an initial rapid lengthening of the spots. Lateral spreading was small except in the lower 25 % of the paper. Longitudinal spreading could be minimized by the use of smaller amounts of the dyes. The size of the spot after development is important when considering the resolution which may be obtained with mixtures of the dyes.

SUMMARY

A number of alkyl-substituted 1-phenylazo-2-naphthols were prepared, of which 1-(3,5-dimethylphenylazo)-2-naphthol is reported for the first time, and these were subjected to reversed-phase paper chromatography using mineral oil-impregnated papers and acetone–water mixtures as developing solvents. R_F values are given for thirteen compounds. R_M values were calculated and these are discussed in the light of present knowledge of the relationship between chemical structure and chromatographic behaviour.

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IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN*

XL. MITTEILUNG. CHROMATOGRAPHISCHE METHODEN ZUR ANALYSE DER GEMISCHE VON ALKYLIERTEN PHENOLEN

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In unserem Laboratorium wurden wir vor das Problem gestellt, komplizierte Gemische, die bei der Alkylierung verschiedener Phenole mit Isobutylen oder Diisobutylen resultieren, qualitativ und quantitativ zu analysieren. Zu diesem Zwecke bedienten wir uns dreier chromatographischer Methoden und zwar der Papierchromatographie, der Säulenchromatographie auf Polyamidpulver und der Gas–Flüssigkeit-Chromatographie.

Alle in dieser Arbeit studierten Phenole wurden in unserem Institut dargestellt, durch Destillation oder Kristallisation gereinigt und standen uns als analysenreine Produkte, deren Identität mittels Elementaranalyse und auf Grund der physikalischen Eigenschaften geprüft wurde[1,2], zur Verfügung. Das Nonyl- und Dodecylphenol waren Produkte der Firma Imperial Chemical Industries, Ltd., Billingham, Durham, England.

PAPIERCHROMATOGRAPHIE

Lösungsmittelsysteme

Zur papierchromatographischen Identifizierung einwertiger Phenole wurden bereits mehrere Methoden vorgeschlagen[3-5]. Wir machten zur Auftrennung alkylierter Phenole von der Chromatographie auf mit Dimethylformamid, Formamid oder Paraffinöl imprägnierten Papieren Gebrauch. Es hat sich gezeigt, dass für die mono- und di-*tert.*-butylierten, bzw. mono-*tert.*-octylierten einwertigen Phenole das Lösungsmittelsystem Dimethylformamid/Hexan geeignet ist, für die höher alkylierten ist es vorteilhafter die Chromatographie auf umgekehrten Phasen im Lösungsmittelsystem Paraffinöl/Methanol, bzw. Methanol–Wasser anzuwenden, wie es aus der Tabelle I ersichtlich ist. Für die Abkömmlinge des Brenzcatechins erwiesen sich als die geeignetsten die Systeme Formamid/Benzol, bzw. Benzol–Chloroform 1:1 (Tabelle II).

In anderen Mitteilungen[6,7] zeigen wir, dass im Falle der Chromatographie auf imprägnierten Papieren die Flecken auf den Chromatogrammen einerseits durch Änderung des Imprägnierungsgrades, andererseits durch Änderung der Zusammensetzung der mobilen Phase beliebig verschoben werden können. In der Tabelle I

* XXXIX. Mitteilung: *Mikrochim. Acta*, (1961) 96.

** Neue Anschrift: Institut für makromolekulare Chemie, Tschechoslowakische Akademie der Wissenschaften, Prag, Tschechoslowakei.

TABELLE I

R_F-WERTE DER EINWERTIGEN ALKYLIERTEN PHENOLE

Phenol	*Lösungsmittelsystem**						*Farbe der Flecken***		
	I	*II*	*III*	*IV*	*V*	*VI*	A_1	A_2	*B*
Phenol	0.06	0.04	0.03	—	—	—	G	Wr	G
4-*tert.*-Butylphenol	0.26	0.17	0.10	—	—	—	OG	RV	RO
2-*tert.*-Butylphenol	0.40	0.29	0.17	—	—	—	OG	BlV	RV
2,4-Di-*tert.*-butylphenol	0.70	0.65	0.45	0.36	0.74	0.81	RO	RO	G
2,4,6-Tri-*tert.*-butylphenol	0.96	0.95	0.90	0.00	0.08	0.45	—	—	—
4-*tert.*-Octylphenol	0.57	0.52	0.29	0.61	0.83	0.81	OkG	OkG	G
2-*tert.*-Octylphenol	0.70	0.65	0.45	—	—	—	G	Bl	RV
Dioctylphenol	0.88	0.93	0.90	0.01	0.19	0.67	RO	RO	G
Nonylphenol	—	0.54	—	0.41	0.77	0.82			
Dodecylphenol	—	—	—	0.13	0.61	0.76	G	RV	G
2-Methylphenol	0.13	0.08	0.05	—	—	—	G	V	O
4-*tert.*-Butyl-2-methylphenol	0.43	0.34	0.18	—	—	—	OG	BlV	RV
6-*tert.*-Butyl-2-methylphenol	0.61	0.56	0.30	0.46	0.71	0.77	OG	Bl	RO
4,6-Di-*tert.*-butyl-2-methylphenol	0.90	0.83	0.70	0.10	0.45	0.73	—	—	—
3-Methylphenol	0.09	0.05	0.04	—	—	—	G	RV	GO
x-*tert.*-Butyl-3-methylphenol	0.51	0.45	0.25	0.66	0.83	0.81	O	V	O
4-Methylphenol	0.09	0.05	0.04	—	—	—	OkG	V	RO
2-*tert.*-Butyl-4-methylphenol	0.52	0.46	0.25	0.68	0.83	0.79	GO	O	G
2,6-Di-*tert.*-butyl-4-methylphenol	0.94	0.91	0.82	0.02	0.13	0.48	—	—	—
3,4-Xylenol	0.11	0.07	0.05	—	—	—	RO	V	O
x-*tert.*-Octyl-3,4-xylenol	0.77	0.76	0.65	0.12	0.54	0.77	RO	RO	G
Cumylphenol	0.14	0.08	0.04	—	—	—	G	Wr	OG
x-*tert.*-Octylcumylphenol	0.77	0.75	0.59	0.04	0.45	0.75	RO	BlV	G
2-Naphthol	0.04	0.02	0.01	—	—	—	O	O	O
1-*tert.*-Octyl-2-naphthol	0.45	0.37	0.20	0.35	0.73	0.77	RO	RO	O
4-Phenylphenol	0.06	0.03	0.02	—	—	—			

* I = 25% Dimethylformamid/Hexan. II = 35% Dimethylformamid/Hexan. III = 50% Dimethylformamid/Hexan. IV = Paraffinöl/Methanol–Wasser (3:2). V = Paraffinöl/Methanol–Wasser (4:1). VI = Paraffinöl/Methanol.

** A = Sichtbarmachung mittels diazotiertes *o*-Chlor-*p*-nitroanilin, A_1 vor und A_2 nach dem Besprühen mit 0.1 *N* NaOH. B = Sichtbarmachung mittels diazotierte Sulfanilsäure.
G = gelb; O = orangenfarben; R = rot; Wr = weinrot; V = violett; Bl = blau; Ok = ocker.

wird am Beispiele des Lösungsmittelsystems Dimethylformamid/Hexan gezeigt, wie durch steigenden Imprägnierungsgrad die R_F-Werte herabgesetzt werden können. Bei Anwendung der mit Formamid imprägnierten Papiere für die Auftrennung der Brenzcatechinderivate können die R_F-Werte durch Steigerung der Polarität (Hexan → Benzol → Chloroform) vergrössert werden (Tabelle II). Macht man von der Chromatographie auf umgekehrten Phasen Gebrauch und wendet das Lösungsmittelsystem Paraffinöl/Methanol–Wasser an, ist es möglich durch Wasserzugabe zum Fliessmittel die R_F-Werte zu vermindern.

Wie wir in einer folgenden Mitteilung[7] eingehender berichten, wird bei Anwendung der Chromatographie auf imprägnierten Papieren eine grössere Schwankung

TABELLE II

R_F-WERTE DER BRENZCATECHINDERIVATE AUF MIT FORMAMID IMPRÄGNIERTEN PAPIEREN

Derivat	*Mobile Phase**		
	I	*II*	*III*
Brenzcatechin	0.02	0.03	0.03
4-*tert.*-Butylbrenzcatechin	0.16	0.29	0.36
4-*tert.*-Octylbrenzcatechin	0.62	0.75	0.79
4,5-Di-*tert.*-butylbrenzcatechin	0.89	0.91	0.92
4,6-Di-*tert.*-butylbrenzcatechin	0.89	0.91	0.92

* I = Hexan–Benzol (2:1). II = Benzol. III = Benzol–Chloroform (1:1).

der R_F-Werte beobachtet. Im Falle des Dimethylformamids wird es u.a. durch bestimmte Flüchtigkeit des Dimethylformamids während des Trocknen der Papierstreifen bei der Imprägnierung verursacht. Es muss also betont werden, dass das Trocknen der Zimmertemperatur angepasst werden soll und im Sommer die Zeit von 10 Minuten nicht überschreiten darf. Die Schwankungen der R_F-Werte beeinflussen jedoch nicht die Qualität der Trennung, da die R_F-Werte aller chromatographierter Verbindungen in einer Richtung verschoben werden.

Sichtbarmachung

Die Sichtbarmachung der Flecken erfolgte durch Besprühen mit einer Eisen(III)-ferricyanidlösung[8], wodurch alle chromatographierten Phenole entdeckt werden konnten. Sie erschienen als intensiv blaue Flecken auf hellgelbem Hintergrund und wurden durch Eintauchen des Chromatogramms in Chlorwasserstoffsäure und nachträgliches Auswaschen in fliessendem Wasser stabilisiert, sodass sie als blaue Flecken auf weissem bis hellblauem Hintergrund erschienen. Für die *o*-substituierten Derivate war eine grünblaue Farbe der Flecken charakteristisch. Für die Derivate der einwertigen Phenole konnten wir noch weitere Sprühreagenzien anwenden: diazotierte Sulfanilsäure[3] und diazotiertes *o*-Chlor-*p*-nitroanilin; auf diese Weise wurden jedoch die Phenole mit allen drei besetzten 2,4,6-Stellungen nicht entdeckt. Die Farben der Flecken sind in der Tabelle I angeführt. Zur Sichtbarmachung der Brenzcatechine war auch ammoniakalische Silbernitratlösung[3] geeignet. Hierbei werden schwarze Flecken auf weissem Hintergrund gebildet, die durch blosses Auswaschen des Chromatogramms in fliessendem Wasser stabilisiert werden konnten.

Quantitative Analyse

Die quantitative Erfassung der einwertigen Phenole führten wir auf die Weise durch, dass wir nach Eluierung die chromatographierten Verbindungen kolorimetrisch mit Hilfe der Reaktion mit diazotiertem *o*-Chlor-*p*-nitroanilin bestimmten. Dieser Methode wird eine separate Mitteilung gewidmet[9].

Bereitung der Chromatogramme

Alle Versuche wurden mit Whatmanpapier Nr. 3 durchgeführt. Streifen von 45 × 13 cm wurden durch eine 50 %, 35 % oder 25 % benzolische Dimethylformamidlösung, oder 20 % äthanolische Formamidlösung oder 10 % Paraffinöllösung in Hexan gezogen und 5–15 Minuten lang durch Aufhängen (das Ende des Streifens mit der Startlinie hängt nach Unten—siehe Lit. [7]) bei Zimmertemperatur getrocknet. Die chromatographierten Verbindungen wurden auf die beschriebene Weise vorbehandelten Papiere in Form 0.5–2 % benzolischer oder äthanolischer Lösungen (2–6 μl) aufgetragen und die Chromatogramme unter Anwendung laufender chromatographischer Ausrüstung absteigend entwickelt. Nach Beendigung der Entwicklung wurden die Chromatogramme an der Luft getrocknet und dann mit Sprühreagenzien besprüht.

Die Sichtbarmachung mittels der Reaktion mit Eisen(III)-ferricyanid erfolgte auf bekannte Weise durch Besprühen mit einem Gemisch von gleichen Teilen einer 15 % Eisen(III)-chloridlösung und 1 % Kaliumferricyanidlösung. Nachdem die Flecken der Phenole erschienen, wurde das Chromatogram durch verdünnte HCl (1 : 1) gezogen und 15 Minuten in fliessendem Wasser gewaschen.

Die Sichtbarmachung mittels diazotiertes *o*-Chlor-*p*-nitroanilin wurde auf folgende Weise durchgeführt: Das Chromatogram wurde mit einer 0.1 % Lösung des stabilisierten Diazosalzes in Wasser, dessen pH durch Zugabe von einigen Tropfen konz. HCl auf 2–3 gebracht wurde, besprüht. Nachträglich wurde noch mit einer 0.1 *N* NaOH-Lösung besprüht. Das stabilisierte Diazosalz wurde nach HEINRICH UND SCHULER[10] hergestellt, nur wurde statt 1-Naphthalinsulfonsäure die 1,5-Disulfonsäure angewendet.

Die Sichtbarmachung mit diazotierter Sulfanilsäure wurde auf bekannte Weise durchgeführt[3].

Chromatographisches Verhalten und Struktur

Den erhaltenen Resultaten konnten folgende Beziehungen zwischen der Konstitution und dem Verhalten bei der Papierchromatographie entnommen werden. Im Falle der polaren stationären Phase (Dimethylformamid, Formamid) ist das Verhalten der Phenole einerseits von der Anzahl der OH-Gruppen, andererseits von der Kohlenstoffatomeanzahl der Alkyle und von ihrer Stellung zu der OH-Gruppe abhängig. Die zweiwertigen Phenole werden, wahrscheinlich auf Grund der grösseren Möglichkeit intermolekulare Wasserstoffbrückenbindungen mit der stationären Phase zu bilden, von der stationären Phase viel mehr festgehalten, als die einwertigen. Deshalb müssen zur Chromatographie der zweiwertigen Phenole Fliessmittel von grösserer Polarität Anwendung finden. Die R_F-Werte der alkylierten Phenole vergrössern sich in der Reihe Phenol, 4-Methylphenol, 3,4-Dimethylphenol, 4-*tert.*-Butylphenol und 4-*tert.*-Octylphenol. Die Substitution in *o*-Stellung verursacht ein grösseres Anwachsen der R_F-Werte, als die *p*-Substitution, sodass die *o*-Isomeren immer höhere R_F-Werte aufweisen. Bei der *o*-Substitution kommt der Einfluss der *tert.*-Butylgruppe deutlicher zum Ausdruck als der Einfluss der Methylgruppe. Deswegen hat das 2-*tert.*-Butyl-4-methylphenol einen grösseren R_F-Wert als das isomere 4-*tert.*-Butyl-2-methylphenol,

und das 2,6-Di-*tert.*-butyl-4-methylphenol einen grösseren R_F-Wert als das isomere 4,6-Di-*tert.*-butyl-2-methylphenol. Wahrscheinlich wird durch sterische Faktoren die intermolekulare Wasserstoffbrückenbindung zwischen der OH-Gruppe und der stationären Phase verhindert. Dementsprechend hat *o-tert.*-Octylphenol denselben R_F-Wert wie das 2,4-Di-*tert.*-butylphenol. Beide Phenole haben dieselbe Anzahl von Kohlenstoffatomen und bei beiden kommen dieselben sterischen Hinderungen in Frage.

Auf umgekehrten Phasen sind die Verhältnisse ganz umgekehrt.

Auf Grund dieser Beziehungen war es möglich dem chromatographischen Verhalten nach bei der bisher als *x-tert.*-Butyl-3-methylphenol bezeichneten Verbindung den Beweis zu erbringen, dass die *tert.*-Butylgruppe in die *o*-Stellung eingetreten ist. Dieses Derivat hat nämlich denselben R_F-Wert wie das isomere 2-*tert.*-Butyl-4-methylphenol. Würde ein 4-*tert.*-Butyl-3-methylphenol vorliegen, würde es einen grösseren R_F-Wert als 4-*tert.*-Butylphenol und einen kleineren als 4-*tert.*-Butyl-2-methylphenol aufweisen. Diese dem papierchromatographischen Verhalten entnommene Tatsache stimmt mit den Erfahrungen bei der Gaschromatographie (siehe weiter) überein.

SÄULENCHROMATOGRAPHIE AUF POLYAMIDPULVER

Zur quantitativen Auftrennung und kolorimetrischen Bestimmung der Brenzcatechinabkömmlinge versuchten wir auch die Chromatographie auf Polyamidpulversäulen[11] anzuwenden, deren Eignung für die Chromatographie der Phenole schon bestätigt wurde[12,13]. Wir benützten zu diesem Zwecke ein Polyamidpulver inländischer Herkunft (Severočeské chemické závody, n.p. Lovosice, Závod Rudník u Vrchlabí), von dem der Anteil mit der Korngrösse 40–100 DIN in die Kolonnen, und zwar in Form einer Suspension in wässrigem Äthanol (1 T. Äthanol: 5 T. Wasser),

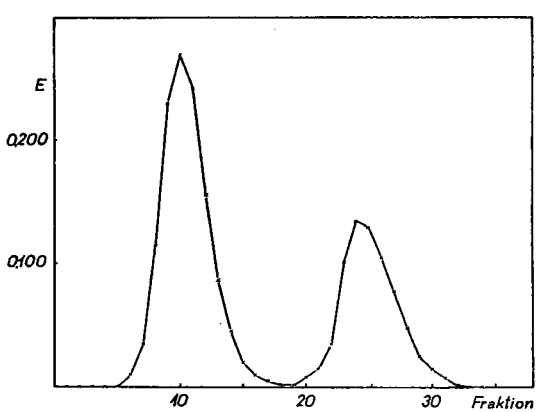

Fig. 1. Graphische Darstellung einer Trennung von 5 mg Brenzcatechin und 5 mg 4-*tert.*-Butylbrenzcatechin auf einer Polyamidpulversäule. 7.5 g Polyamidpulver, Säule 205 cm lang, 16 mm im Durchmesser, Fraktionen à 10 ml, einzelne Fraktionen kolorimetrisch analysiert, E = Extinction. Eluierung: Fraktion Nr. 1–16, Äthanol–Wasser 1:5 (Brenzcatechin); Nr. 17, Äthanol–Wasser 1:4; Nr. 18, Äthanol–Wasser 1:3; Nr. 19–34, Äthanol–Wasser 1:2 (4-*tert.*-Butylbrenzcatechin).

gefüllt wurde. Auf eine Säule von 7.5 g Polyamidpulver, 220 mm lange und 16 mm im Durchmesser, wurden die Brenzcatechine in einer Menge von 5–20 mg, gelöst in 5–10 ml des wässrigen Äthanol, aufgegeben. Die Eluierung wurde mit einem Gemisch Äthanol–Wasser 1:5 begonnen das in weiterem durch an Äthanol reichere Gemische ersetzt wurde. Jede Fraktion wurde—bei qualitativer Trennung—papierchromatographisch analysiert, oder das betreffende Derivat wurde kolorimetrisch oder spektrophotometrisch bestimmt. In der Fig. 1 ist als Beispiel die Auftrennung von 5 mg Brenzcatechin und 5 mg 4-*tert.*-Butylbrenzcatechin graphisch dargestellt. Zur kolorimetrischen Bestimmung des Brenzcatechins sowie des 4-*tert.*-Butylbrenzcatechins machten wir von der Reaktion mit Eisen(II)-Salz und Weinsäure[14] Gebrauch. Die beiden erwähnten Derivate geben bei dieser Reaktion eine violette Färbung, das 4,5-Di-*tert.*-butylderivat reagiert nicht. Es kann dagegen, sowie auch die übrigen Derivate spektrophotometrisch erfasst werden. Über diese quantitative Methode wird gesondert berichtet[9].

Ein grosser Vorteil der Säulenchromatographie auf Polyamidpulver ist, dass die zur Analyse schon benutzte Säule durch blosses Auswaschen mit wässrigem Äthanol zur weiteren Analyse vorbereitet ist und die Analysen auf solche Weise wiederholt werden können.

GAS–FLÜSSIGKEIT-CHROMATOGRAPHIE

Die Gaschromatographie der Phenole, die als Bestandteile des Steinkohlenteers vorkommen können, studierten schon mehrere Autoren (Übersicht siehe Lit.[15]). Die Auftrennung und Identifizierung der Produkte der Alkylierung der Phenole und Kresole mit Isobutylen mittels dieser Methodik wurde bisher noch nicht beschrieben.

Zur Auftrennung dieser Verbindungen machten wir von einer nicht polaren Trennflüssigkeit, Silikonöl, andererseits einer polaren Trennflüssigkeit dem 3,5-Dinitrobenzoat des "Polydiols 400" Gebrauch.

Der Apparat

Die Analysen wurden mit einem Apparat eigener Konstruktion durchgeführt, dessen Einrichtung keine Unterschiede gegenüber den üblichen Apparaten aufwies. Die Detektion der Verbindungen wurde nach Verbrennung und Konversion des gebildeten Wassers auf Wasserstoff durch Bestimmung der Wärmeleitfähigkeit durchgeführt[16]. Die Proben wurden mit einer Kapillarpipette eingebracht[17]. Die Kolonne bildete ein U-förmiges Glasrohr von 4 mm Innendurchmesser und 120 cm Länge. Als fester Träger diente Kieselguhr mit einer Korngrösse von 50–60 Maschen, der mit 20% der betreffenden Trennflüssigkeit getränkt wurde. Die Imprägnierung wurde mittels der acetonischen Lösungen der Trennflüssigkeiten und Abdampfen des Acetons auf gewöhnliche Weise durchgeführt. Als Trägergas wurde reiner Stickstoff angewendet.

Trennflüssigkeiten

Silikonöl. Verwendet wurde die Fraktion des Methylphenylsilikonöls vom Sdp. 245–275°/5·10^{-5} mm Hg.

*Bis-3,5-dinitrobenzoesäureester des "Polydiols 400"** wurde auf folgende Weise dargestellt: 8 g "Polydiol 400" (Chem. Werke Hüls) wurde mit 11 g 3,5-Dinitrobenzoylchlorid auf 70° erwärmt. Nachdem die Chlorwasserstoffentwicklung beendigt war (*ca.* 15 Minuten), wurde das Reaktionsgemisch noch 1 Stunde im Vakuum der Wasserstrahlpumpe erwärmt. Nach Lösung des Reaktionsgemisches in Benzol wurde die benzolische Lösung mit 25 % Natronlauge, verdünnter HCl (1:2) und Wasser ausgeschüttelt, über wasserfreiem Natriumsulfat getrocknet und das Benzol abdestilliert. Auf diese Weise wurde 10.6 g des Produktes, das eine viskose gelbe Flüssigkeit bildete, erhalten.

Trennung der tert.-butylierten Phenole

Die gaschromatographische Auftrennung führten wir in allen Fällen bei 180° und einem Durchfluss von 30 ml Stickstoff/Min. durch. Die Retentionsvolumina der Phenole sind in Tabelle III angeführt. Die typischen Auftrennungen der *tert.*-butylierten Phenole auf beiden Trennflüssigkeiten sind aus den Fig. 2 und 3 ersichtlich.

TABELLE III

RETENTIONSVOLUMINA DER ALKYLPHENOLE

(t = 180°, Durchfl. 30 ml N_2/Min.)

Phenol	*Sdp. in °C*	*Trennflüssigkeit*[a]	
		A	*B*
Phenol	182	1.00	1.00
2-Methylphenol	191	1.49	1.09
4-Methylphenol	202.1	1.54	1.45
3-Methylphenol	202.2	1.54	1.51
2-*tert.*-Butylphenol	221	3.71	1.55
6-*tert.*-Butyl-2-methylphenol	231	4.62	1.09
4-*tert.*-Butylphenol	237	4.27	2.63
2-*tert.*-Butyl-4-methylphenol	237	5.28	1.90
x-*tert.*-Butyl-3-methylphenol	244	5.28	2.14
4-*tert.*-Butyl-2-methylphenol	247	5.66	2.41
2,6-Di-*tert.*-butylphenol	255	8.06	0.62
2,4-Di-*tert.*-butylphenol	263	10.05	2.16
2,6-Di-*tert.*-butyl-4-methylphenol	265	10.7	0.85
4,6-Di-*tert.*-butyl-2-methylphenol	269	11.9	1.36
2,4,6-Tri-*tert.*-butylphenol	278	14.5	0.62
4-*tert.*-Octylphenol	—	16.3	—
V_g Phenol		28.5	163

[a] A = Silikonöl. B = 3,5-Dinitrobenzoesäureester des Polydiols 400.

Es war schon *a priori* offenbar, dass die Trennung dieser Verbindungen einerseits durch ihre Tension (Siedepunkte), andererseits durch sterische Faktoren dirigiert wird. Es konnte erwartet werden, dass die sterischen Einflüsse wegen des grossen Umfangs der *tert.*-Butylgruppe eine bedeutende Rolle spielen werden.

* Ein Polyäthylenglykol vom mittlerem Molekulargewicht 400.

Der Fig. 4a kann entnommen werden, dass bei der Chromatographie auf nicht polarem Silikonöl die Reihenfolge bei der Elution durch die Siedepunkte bestimmt wird. Die Interaktion zwischen den Phenolen und der nicht polaren Trennflüssigkeit

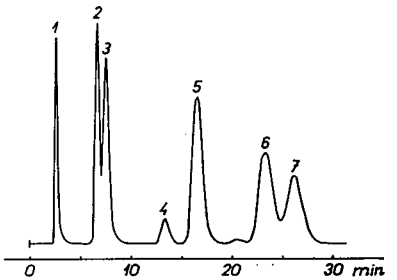

Fig. 2. Trennung eines Gemisches der *tert.*-Butylphenole auf Silikonöl bei 180° und Durchfluss 30 ml N_2/Min. (1) Phenol; (2) 2-*tert.*-Butylphenol; (3) 4-*tert.*-Butylphenol; (4) 2,6-Di-*tert.*-butylphenol; (5) 2,4-Di-*tert.*-butylphenol; (6) 2,4,6-Tri-*tert.*-butylphenol; (7) *tert.*-Octylphenol.

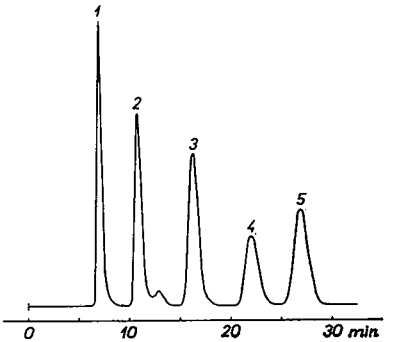

Fig. 3. Trennung der *tert.*-Butylphenole auf 3,5-Dinitrobenzoat des Polydiols 400 bei 180° und Durchfluss 30 ml N_2/Min. (1) 2,6-Di-*tert.*-butyl- und 2,4,6-Tri-*tert.*-butylphenol; (2) Phenol; (3) 2-*tert.*-Butylphenol; (4) 2,4-Di-*tert.*-butylphenol; (5) 4-*tert.*-Butylphenol.

weist nur einen nicht polaren, dispersen Charakter auf und sterische Faktoren kommen nicht zur Geltung.

Bei Anwendung der polaren Trennflüssigkeit — des 3,5-Dinitrobenzoats des "Polydiols 400" — tritt dagegen die Interaktion der Hydroxylgruppe der Phenole mit der Trennflüssigkeit (Bildung der Wasserstoffbrückenbindungen[18]) in Vordergrund und der Einfluss der Tensionen der chromatographierten Verbindungen wird vermindert (Fig. 4b). Eine selektive Auftrennung wird erzielt, wenn durch sterische Faktoren diese Interaktion gehindert wird, was bei den *o*-substituierten Phenolen der Fall ist. Wie aus der Fig. 4b ersichtlich ist, wird schon durch Einführung einer *tert.*-Butylgruppe in die *o*-Stellung eine bemerkenswerte Verminderung der Retentionszeit

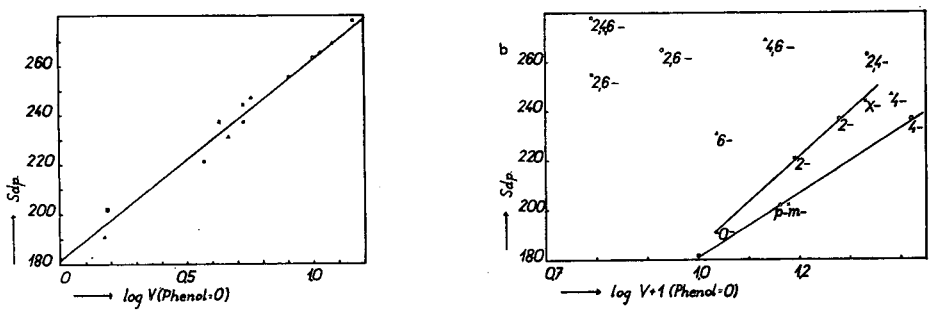

Fig. 4. Logaritmus der relativen Retentionsvolumina der alkylierten Phenole gegen Siedepunkt. (□) Phenole; (△) *o*-Kresole; (○) *p*-Kresole; (×) *m*-Kresole. (a) Stationäre Phase Silikonöl; (b) stationäre Phase 3,5-Dinitrobenzoat des Polydiols 400.

beobachtet, sodass 2-*tert.*-Butylphenol eine um ein weniges grössere Retentionszeit hat wie 3-Methylphenol, das einen um 19° niedrigeren Siedepunkt hat. Sind beide *o*-Stellungen durch *tert.*-Butylgruppen substituiert, so können praktisch keine Wasserstoffbrückenbindungen gebildet werden[19]; diese Verbindungen weisen noch kleinere Retentionszeiten auf als Phenol, das einen um 80° niedrigeren Siedepunkt hat. In Fällen, wo eine Methylgruppe als Substituent auftritt, sind die sterischen Einflüsse kleiner und die Retentionsvolumina wachsen an. Interessant ist der Einfluss der *p*-*tert.*-Butylgruppe. Diese Gruppe allein hat keinen spezifischen Einfluss, sodass das 4-*tert.*-Butylphenol das grösste Retentionsvolumen hat. Sind jedoch zugleich die 2- oder die 2,6-Stellungen substituiert, so wird die Retentionszeit verkürzt und das 2,4-Di-*tert.*-butylphenol wird miteinander mit *x*-*tert.*-Butyl-3-methylphenol eluiert; das 2,4,6-Tri-*tert.*-butylphenol hat dasselbe Retentionsvolumen wie das 2,6-Di-*tert.*-butylphenol, trotzdem es von allen erwähnten Phenolen den höchsten Siedepunkt hat.

Auf Grund dieser Beziehungen und Gesetzmässigkeiten war es möglich die Stellung des Substituenten im Falle des *x*-*tert.*-Butyl-3-methylphenols zu bestimmen. Seinem gaschromatographischen Verhalten nach kann abgeleitet werden, dass die *tert.*-Butylgruppe in *o*-Stellung zur Hydroxylgruppe stehen muss. Wäre sie in der *p*-Stellung, müsste das Retentionsvolumen des *tert.*-Butyl-3-methylphenols auf polarer Phase grösser sein als des 4-*tert.*-Butylphenols. Dieses Resultat steht auch mit dem papierchromatographischen Verhalten dieser Verbindung (siehe oben) im Einklang.

Die Ergebnisse der gaschromatographischen Trennung der alkylierten Phenole können also in dem Sinne zusammengefasst werden, dass zwar auf einzelnen Trennflüssigkeiten nicht alle Phenole getrennt werden, durch Kombination der beiden Methoden ist jedoch die Unterscheidung und Identifizierung aller geprüften Derivate möglich.

DANK

Herrn Dr. J. NOSEK aus unserem Institut und seinen Mitarbeitern sind wir für Zurverfügungstellung der Präparate der Alkylphenole zu Dank verpflichtet. Frau M. BORECKÁ, Frau O. BERANOVÁ und H. J. HAVLÍČEK danken wir für Durchführung der experimentellen Arbeiten.

ZUSAMMENFASSUNG

Zur Trennung und Analyse der Gemische von Alkylphenolen, die aus Alkylierung der Phenole mit Isobutylen, bzw. Diisobutylen resultieren, werden papierchromatographische, säulenchromatographische und gaschromatographische Methoden vorgeschlagen. Die alkylierten einwertigen Phenole werden papierchromatographisch in Lösungsmittelsystemen wie: Dimethylformamid/Hexan oder Paraffinöl/Methanol–Wasser getrennt und identifiziert und für die Alkylderivate des Brenzcatechins mit Formamid imprägnierte Papiere und das Gemisch Benzol–Chloroform als Fliessmittel empfohlen. Die Brenzcatechinderivate können zu analytischen Zwecken auch auf Polyamidpulversäulen bei Anwendung von wässrigem Alkohol als Eluierungsmittel,

abgetrennt werden. Die Alkylderivate des Phenols und der Kresole wurden auch mittels Gas–Flüssigkeit-Chromatographie getrennt und identifiziert. Es wurden Methoden mit einer nicht polaren Trennflüssigkeit (Silikonöl) und mit einer polaren (3,5-Dinitrobenzoesäureester des "Polydiols 400") kombiniert.

SUMMARY

Paper chromatographic, column chromatographic and gas chromatographic methods are proposed for the separation and analysis of mixtures of alkylphenols that have been obtained from phenols by alkylation with isobutylene or di-isobutylene. The monohydric alkylphenols are separated and identified by paper chromatography in solvent systems such as dimethylformamide/hexane or paraffin oil/methanol–water, while formamide-impregnated paper and the solvent system benzene–chloroform are recommended for the separation of the alkyl derivatives of pyrocatechol. For analytical purposes the pyrocatechol derivatives can also be separated on polyamide powder columns with aqueous alcohol as eluant. The alkyl derivatives of phenol and of the cresols are also separated and identified by gas–liquid chromatography. Methods are described in which a non-polar solvent (silicone oil) and a polar one (3,5-dinitrobenzoate of "Polydiol 400") are used.

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AN APPARATUS FOR PREPARATIVE-SCALE GAS–LIQUID CHROMATOGRAPHY

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A number of preparative-scale gas–liquid chromatography units have been described[1–8] which for the most part employ columns of 1 in. or less in diameter and 25 ft. or less in length. Although an increase in column capacity is generally achieved by increasing the diameter of the column, the existing literature is somewhat conflicting with regard to the effect of increased diameter on column efficiency. In some instances, for example, it has been stated[2,9] that an increase in column diameter above 1 in. results in a rapid decline in efficiency. In at least one case[3], however, it was reported that a 3-in. diameter column resulted in little loss in efficiency over that of a 1-in. column. DE WET AND PRETORIUS[10] have studied various factors affecting gas–liquid chromatographic separation of large samples with a 1½-in. diameter column and found that HETP (height equivalent to a theoretical plate) values below 1 cm can be obtained for samples up to 10 ml in volume. A similar study has recently been reported by HUYTEN, VAN BEERSUM, AND RIJNDERS[11]. We describe below a column 1½ in. in diameter with sufficient length so that very efficient separations may be made on reasonably large samples. The unit has been used successfully for a variety of applications, including the separation of catalytically cracked gasoline, kerosene, isobutane–olefin alkylate, and olefin concentrates.

APPARATUS

The column section of the unit is composed of eight vertically mounted 1½-in. × 9-ft. stainless steel tubes connected by 3/8-in. tubing returns at both ends. Column tubes, heaters, and carrier-gas preheater line are cast in aluminum. At the base of the column in separately heated sections are found the sample vaporizer, thermal conductivity cell, and trapping manifold. The thermal conductivity cell is operated on a slip stream from the main flow. Effluent may be trapped from any one of three valves on the manifold.

A view of the entire unit is shown in Fig. 1 and a flow diagram is given in Fig. 2.

Column

The eight tubes of the column are arranged in bundle fashion with the ends of the tubes protruding 1½ in. from the end flanges. The bundle of tubes and the connecting

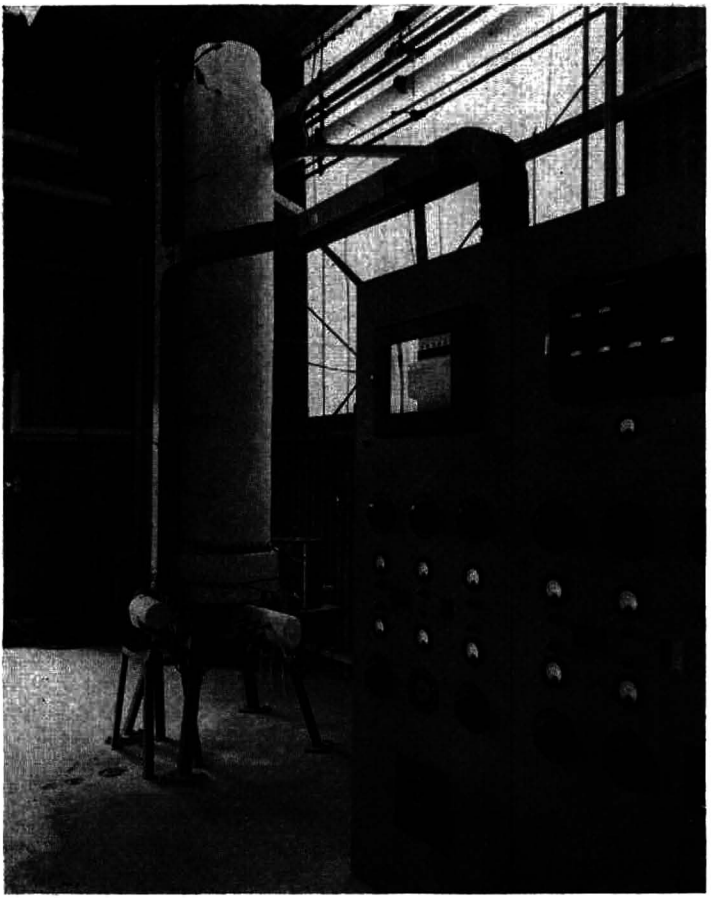

Fig. 1. Preparative-scale gas–liquid chromatograph.

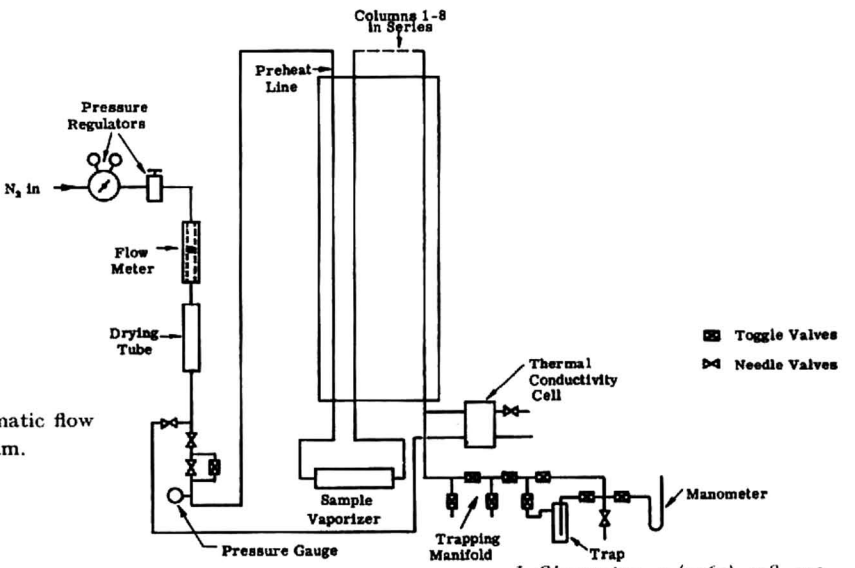

Fig. 2. Schematic flow diagram.

tubing returns are mounted so that the effective column length can be set at 18, 36, 54, or 72 ft., depending upon the number of pairs of individual tubes used. Six ½-in. Chromalox tubular heating elements (No. TS-13645) of about 1000 W each (110 V) are coiled around the eight tubes with 3 ½ turns per heater and a coil height of 1 ¼ ft. These heaters are paired (series) so as to form three circuits. One tubular heater of 3800 W (220 V) passes through the center of the column tube bundle and is used to bring the column rapidly to the desired temperature. The 3/8-in. tubing returns at each end are heated by a Chromalox electric range element (MTC-926MN, 650 W, 110 V) which is located about 1 in. above (or below) the returns.

Four inches of 85 % magnesia insulation covers the body of the column, and insulation caps of 12-in. diameter pipe insulation cover the tubing returns and hold the electric range elements in place.

Sample vaporizer

Carrier gas entering the sample vaporizer is preheated by passage through a 3/8-in. stainless steel tube which extends through the length of the column section. The vaporizer chamber is an 8 × 1 ¼-in. stainless steel bar with a 7/16-in. hole drilled through the center. Heating is provided by two 400-W strip heaters and the tubing making connection with the column is heated with a heating tape. Both the vaporizer and connecting tubing are insulated with asbestos pipe insulation. Samples are injected into the chamber through a serum bottle stopper near the inlet side.

Thermal conductivity cell

A Gow-Mac TE-III, Model No. 9234 thermal conductivity cell is used with a 6-V wet storage battery and a trickle charger. An automatic attenuator is included in the bridge circuit. The thermal conductivity cell is located in a container consisting of a small round can inside a larger round can with 3 in. of insulation between the two. Beaded Chromel A wire which is wound around the inside can heats the cell compartment.

Trapping system

Gas emerging from the last tube of the column is split into two flows; one small stream goes through the thermal conductivity cell, the remainder goes to the trapping manifold. The valves are Hoke 411-series all-metal cam-operated diaphragm valves which can be used up to 250°. Distance between valves was made as small as possible in order to reduce sample holdup and carryover. Final exit of gas from the manifold is through a needle valve used to adjust the outlet pressure. The inlet sides of the traps are connected to the manifold by ball-and-socket joints and the outlet sides are connected to the needle valve with tygon tubing. Heat for the trapping manifold is provided by 12 ft. of No. 25-gage Chromel A resistance wire (500 W), and the manifold is insulated with 1 in. of asbestos.

Instruments

Chromatograms are recorded with a 0–5 mV Brown strip-chart recorder. A six-point,

0–1200° F Weston Indicating Potentiometer controller (Celectray) is used to control the three main heating circuits of the column, as well as those of the sample vaporizer, thermal conductivity cell compartment, and trapping manifold. All other heated points are manually controlled.

High-temperature-limit system

A high-temperature-limit control system was designed to shut off the Celectray in case of failure of a relay. For each of the three main heating circuits of the column, a sensing thermocouple is provided. This high-limit control provides positive shut

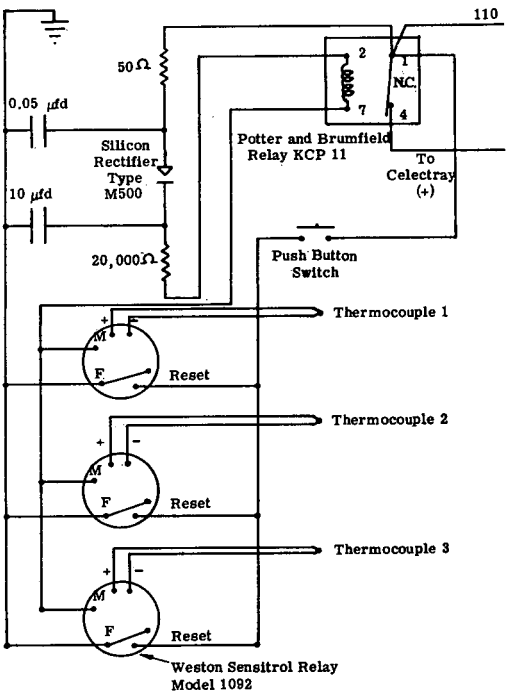

Fig. 3. Schematic wiring diagram for high-temperature-limit circuit

off until the condition has been corrected and the control reset. The system is simple, inexpensive, and can be made to monitor a number of points. A schematic wiring diagram is shown in Fig. 3.

PROCEDURE

Nitrogen carrier gas is dried by passage through a large tube filled with magnesium perchlorate and indicating drierite. A rotameter and a pressure gauge serve as rough indicators of flow rate. More precise flow adjustment is obtained by use of soap film meters of 50- and 350-ml capacity. Due to the large volume of the column, several minutes are required for a steady rate to be reached after each adjustment of the

regulating needle valve. The column is operated with an outlet pressure 10–20 mm above atmospheric pressure so as to assure a flow of 50–100 ml/min through the thermal conductivity cell.

The two silicone liquid phases used, GE-96 and DC-710, were deposited in the amount of 20 wt. % on 20–40 mesh Johns-Manville C-22 firebrick. Changing the column packing of the unit is a relatively simple matter. Removal of the two end insulation caps exposes the ends of the column tubes, and the diameter of the tubes is large enough to allow rapid removal or addition of packing material. It is our experience that uniform packing can be realized without agitation. However, if additional settling is required, a heavy-duty massage-type vibrator produces some vibration of the tubes.

Samples are introduced into the sample vaporizer chamber with hypodermic syringes. For samples larger than 1 ml, a 20-gage needle is used so that injection can be made rapidly. A 5-ml sample can be injected in about 2.5 seconds.

Temperature in the sample vaporization chamber is maintained 50–70° above the column temperature while the trapping manifold is held 20–50° above the column temperature. The thermal conductivity cell is usually operated at the same temperature as the column; however, for some higher-boiling samples the cell is held 10–20° above the column temperature. With its large mass, the body of the column heats up rather slowly; but, once the desired temperature is reached, it maintains a stable temperature to $\pm$ 1°. About 2 hours are required to bring the column to 100°, and 2 additional hours for each 50° increment up to 250°. The recorder baseline does not show appreciable fluctuation due to temperature cycling until 200° is reached.

The phenomenon of peak inversion[12–14] was encountered under certain conditions with the Gow-Mac thermal conductivity cell and with nitrogen as a carrier gas. If gold-plated filaments are used in the cell, rather than tungsten, the temperature at which inversion takes place is raised. Pentane, for example, is inverted below 100° with tungsten filaments, whereas with gold-plated filaments it is not inverted until a temperature between 110° and 150° is reached.

One of the more important and difficult phases of preparative-scale gas–liquid chromatography is recovery of separated material from the carrier gas stream. When one attempts to condense relatively small amounts of vaporized material from a large amount of fast-moving heated carrier gas, the result is usually a fog which is difficult to precipitate. A sophisticated technique for overcoming this difficulty has been described by WEHRLI AND KOVÁTS[15]. We have investigated a number of simpler traps and techniques for collecting fractions.

Two of the most useful designs we have employed feature glass vessels tightly packed with glass wool in one case and with copper turnings in the other. The glass wool trap is 7.6 cm long and 2.5 cm in diameter. Gas enters through a side arm and exits through a central tube, around which the glass wool is wrapped. Gas enters the trap (15 × 1.7-cm Vigreux-type tube) containing copper turnings near the bottom and exits at the top. Recovery with the copper-packed trap is about 97 % and is

somewhat better than the recovery obtained with the glass wool packing. Small amounts of liquid held up by the glass wool can be recovered by centrifuging and withdrawal of the freed liquid with a hypodermic syringe.

For the most part, a dry ice–isopropyl alcohol cooling bath is used. As the carrier gas is nitrogen, the use of liquid nitrogen for cooling relatively small traps results in a freeze-up which restricts the flow of gas.

FACTORS AFFECTING COLUMN EFFICIENCY

Column length

The bundle-type column arrangement permits a simple study of the effect of column length on separating efficiency. A comparison of efficiency at three different column lengths and various sample sizes is shown in Fig. 4. The number of theoretical plates

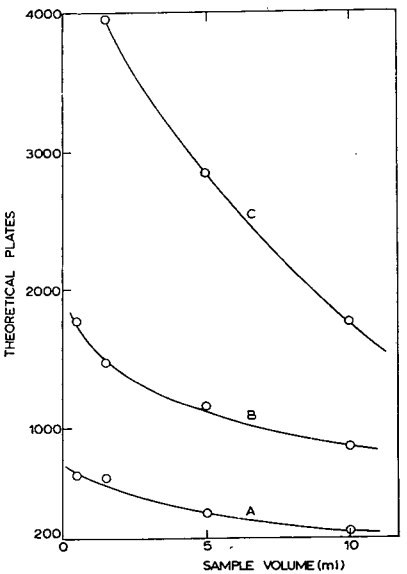

Fig. 4. Effect of sample volume and column length on number of theoretical plates. Sample: benzene, toluene, *m*- and *p*-xylene. Column packing: DC-710 silicone. Column temperature: 110°, Nitrogen flow rate: 1000 ml/min. Measured peak: *m*- and *p*-xylene. Column length: curve A. 18 ft.; curve B, 36 ft.; curve C, 72 ft.

was calculated according to JOHNSON AND STROSS[16]. Throughout the range of sample size indicated, doubling of the column length more than doubles the number of theoretical plates. Although the rate of decrease in efficiency with increasing sample volume is most rapid in the case of the full column length of 72 ft., the point of diminishing returns from an increase in column length has apparently not been reached.

Component concentration

To the extent that the concentration of a given component influences the number of calculated plates, Fig. 4 does not fully exemplify the performance of the apparatus.

In particular, components present in high concentration give rise to peak spreading (column flooding). As an example of the number of plates that can be realized for components present in low concentration, the peak for 2,4-dimethylpentane (2–3 % of sample) in the chromatogram of an 8.7-ml charge of alkylate (cut 4 of Fig. 5 and Table I) shows an efficiency of over 9100 plates.

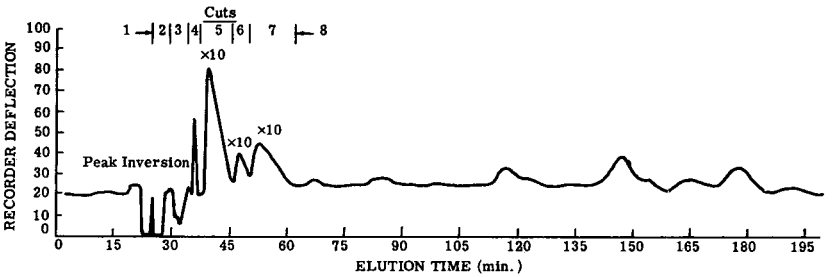

Fig. 5. Chromatogram of alkylate. Sample: 8.7 ml isobutane–butylene alkylate. Column packing: 36 ft. GE-96 silicone + 36 ft. DC-710 silicone. Column temperature: 150°. Nitrogen flow rate: 1000 ml/min.

Flow rate

The effect of carrier gas flow rate on separation efficiency is illustrated in Fig. 6 for 5-ml samples of a five-component mixture. As the flow rate increases, the number

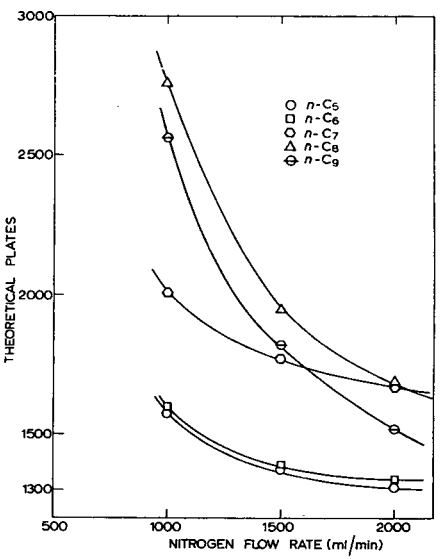

Fig. 6. Effect of flow rate on number of theoretical plates. Column packing: DC-710 silicone. Column temperature: 110°. Column length: 72 ft. Sample volume: 5 ml.

of plates for each component decreases; however, the rate of decrease is much greater for those components which have longer retention times.

Elution time

The relation between theoretical plates and elution time goes through a maximum which is illustrated in Fig. 7. This maximum moves toward shorter retention times with increasing flow rate. Examination of the chromatograms shows that the peaks near maximum efficiency are found to be very nearly symmetrical, while those with shorter retention time are tailed and those beyond the maximum show leading.

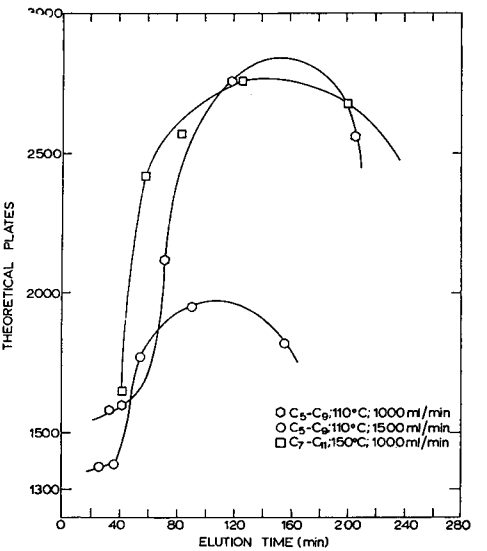

Fig. 7. Relationship between number of theoretical plates and elution time. Column packing: DC-710 silicone. Column length: 72 ft. Sample volume: 5 ml.

This effect may be due to overloading of the column. Nonideal adsorption on the support causes tailing and nonideal solution effects produce leading[17]. Tailing can be avoided by using small samples which do not fall on the strongly curved region of the adsorption isotherm; however, as the samples used here are intentionally large, tailing cannot be avoided in this manner. Leading can also be improved by working at low concentration, where the activity coefficient varies only slightly, or by increasing the temperature in order to produce more nearly ideal solution conditions. It is seen in Fig. 7 that with an increase in temperature of 40°, the relative symmetries of the peaks are changed. Tailing probably occurs for all peaks, with the effect of leading becoming more pronounced as the retention volume increases. Symmetrical peaks occur when there is a balance between tailing and leading. Slow sample vaporization, a frequent difficulty in connection with large sample sizes, is a further factor influencing peak shape.

Stationary liquid

The DC-710 silicone fluid used as one of the column liquid phases produces some separation by hydrocarbon type as may be demonstrated by the separation of 2,4-

dimethylpentane (b.p. 80.51°) and cyclohexane (b.p. 80.74°). On the basis of the formula $2\Delta y/(y_a + y_b)$[18], where Δy is the distance between the two peak maxima and y_a and y_b are the peak widths determined by the intercepts cut on the base line by the tangents to the two peaks, the column resolution for these two peaks with 18 ft. of DC-710 silicone was 0.995, and for 36 ft. of GE-96 silicone plus 36 ft. of DC-710 silicone was 1.99. If the column resolution value is $\geqq 1$ the peaks are completely separated. With the short 18-ft. column of DC-710 silicone, it was possible to prepare a 100 % pure (by mass spectrometric analysis) sample of cyclohexane from the blend.

Sample volume

It has been reported[18,19] that with plug-flow sample introduction of sufficiently small samples, sample volume will not contribute to the peak width. With the apparatus described here, the peak width does depend on sample volume, probably because of several factors. The introduction of large samples by hypodermic syringe cannot be accomplished instantaneously and the charge time increases with increasing sample volume. Sample volumes for which this unit was designed (above 1 ml) are considerably above the limit for nonbroadening allowed by the equation of VAN DEEMTER *et al.*[20]. For samples below about 0.2 ml, the sample volume has only a small effect on the base width, and the number of plates obtained is more uniform. If the plate numbers used here were corrected for the peak widening due to the detector volume[19], the values would be increased by about 10 %.

APPLICATIONS

Light catalytically cracked gasoline

Samples of a 45–120° catalytically cracked gasoline fraction were charged in volumes up to 20 ml with the column operated at 110° and at a nitrogen flow rate of 1000 ml per min. Seven distinct peaks were obtained from a 5-ml charge with 18 ft. of column, 12 peaks with 36 ft., and 16 peaks with 72 ft. (first 36 ft. packed with GE-96 silicone, second 36 ft. with DC-710 silicone). The first three peaks from the run with 18 ft. of column were trapped and rerun on a ¼-in. × 25-ft. GE-96 silicone column. The first peak contained C_6 saturates and olefins, the second contained mainly C_6 olefins plus a small amount of C_7 saturates and olefins (about 1.5 vol. %), and the third cut contained C_6 olefins and C_7 saturates and olefins with the amount of C_7 material increased. With a full column of 72 ft., very narrow concentrates could be obtained from such a sample.

Isobutane–butylene alkylate

A portion of the total liquid product from an experimental alkylation run was charged to the preparative-scale chromatography unit in 5-ml batches with eight cuts being taken (Fig. 5). Further identification was carried out on a ¼-in. × 4-ft. GE-96 silicone column with variable-temperature operation. Results are shown in Table I. In cases where there is a carry-over of a few tenths of a percent, and higher purity

TABLE I

SEPARATION OF ISOBUTANE–BUTYLENE ALKYLATE

Cut	*Components*	*Basis cut, wt. %*	*Basis total liquid product, mole %*
1	Isobutane	47.5	39.3
	n-Butane	51.6	11.4
	Isopentane	0.9	
2	Isopentane	72.4	4.40
	n-Pentane	27.6	1.08
3	*n*-Pentane	0.1	
	2,3-Dimethylpentane	87.7	3.80
	3-Methylpentane	12.2	0.30
4	2,4-Dimethylpentane	100.0	2.51
5	2,3-Dimethylpentane	3.9	0.94
	2,2,4-Trimethylpentane	95.5	14.4
6	2,2,4-Trimethylpentane	0.8	
	Dimethylhexane	99.2	3.66
7	Dimethylhexane	0.7	
	2,3,3- and 2,3,4-Trimethylpentane	83.2	12.6
	Trimethylhexane	15.6	1.88
8	Boiling range, °C*		
	125–140	8.2	0.47
	140–165	17.9	0.83
	165–180	18.5	0.65
	180–200	55.4	1.81

* From elution-time calibration data based on *n*-paraffins.

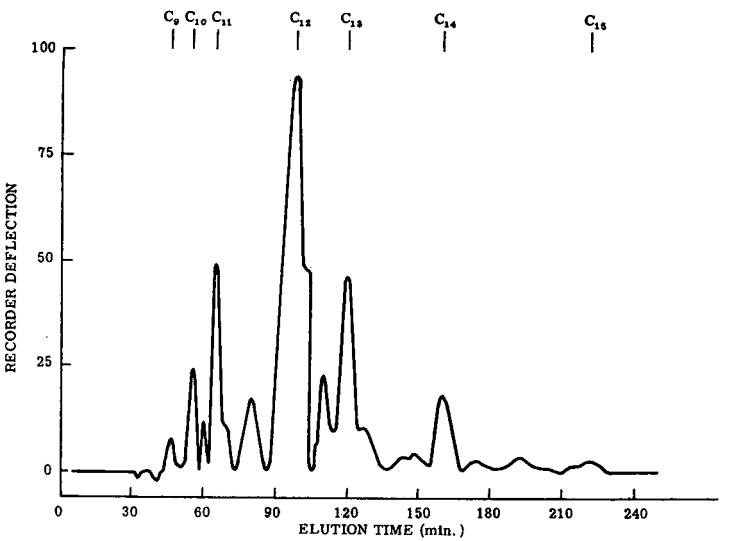

Fig. 8. Chromatogram of kerosene. Sample: 5 ml kerosene. Column packing: 36 ft. GE-96 silicone + 36 ft. DC-710 silicone. Column temperature: 200°C. Nitrogen flow rate: 1000 ml/min.

is desired, the cut points should be made so that the bottom of the valley between peaks is discarded.

Kerosene

Kerosene samples and a number of synthetic blends in the boiling range of 125–270° were run at a column temperature of 200°. A typical chromatogram of kerosene is shown in Fig. 8.

Olefin concentrates

A large number of olefin concentrates in the C_6 to C_{10} range which were prepared from a catalytically cracked gasoline sample by liquid chromatography and distillation were separated in 5-ml portions with 18 ft. of column (DC-710) at a flow rate of 1000 ml/min and column temperatures of 100–200°. The cuts taken from the unit showed that separation by z-number (in the empirical formula C_nH_{2n+z}) with about 90 % selectivity was achieved even though complete resolution was not obtained in all cases.

Purification of 3-methylhexane

The preparative-scale unit was used to prepare a total of 175 ml of naphthene-free 3-methylhexane from Phillips technical-grade material for use in reaction studies. Previous attempts to remove the naphthenes by precision distillation were unsuccessful, but they were completely removed by the preparative-scale unit (5-ml sample, 36 ft. of DC-710 silicone, 110°, 1000 ml/min). Taking into account the sample lost through the thermal conductivity cell side stream, a recovery of 97.5 % was obtained by using two traps in series, the first cooled with dry ice and acetone, and the second with liquid nitrogen. With a narrow-boiling-range sample such as this, it was possible to complete a run every 35 min.

ACKNOWLEDGEMENT

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SUMMARY

An efficient preparative-scale gas–liquid chromatography unit has been eveloped which features a column composed of eight 1 ½-in. × 9-ft. tubes connected in series. The tubes are arranged so that the effective column length can be set at 18, 36, 54 or 72 ft. Operation at temperatures up to 250° and with sample sizes as large as 20 ml is practical. Performance studies indicate that separations equivalent to 9000 or more theoretical stages are possible. The unit has been applied to the preparation of narrow concentrates and compounds of high purity for analytical and reaction studies.

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LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

I. CONDITIONS FOR EXISTENCE

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Of the three basic chromatographic techniques, elution chromatography has long been recognized as uniquely suited for the separation of multicomponent mixtures. The theory of elution chromatography is well developed[1,2] for linear isotherm systems, and considerable progress has been made in the quantitative correlation of solute separabilities with molecular structure in such cases[3]. With few exceptions, linear elution chromatographic (LEC) separations are experimentally noted only in systems with liquid stationary phases. Isotherm linearity, or the validity of Henry's law in stationary and moving phases, is generally recognized as the rule in these chromatographic cases, and the exception in those employing a solid adsorbent for the entire stationary phase. Data have been reported[4–6] (among others) as examples of LEC behavior for solid–liquid and solid–gas chromatographic systems, but the phenomenon has been obscured by the frequent use of deactivated solid stationary phases, where both solid and adsorbed liquid constitute the total stationary phase. SPORER AND TRUEBLOOD[7] have presented the only extensive data for a non-deactivated adsorbent (silica gel) which exhibits isotherm linearity, using frontal analysis. The extent to which isotherm linearity exists in this system is not clear, however, since data are given both from observations on the linear region and extrapolations from non-linear data. Nothing is known about the possible generality of linear elution behavior at practical column loadings for chromatographic systems featuring a solid adsorbent, and this has discouraged systematic studies of the quantitative relationships between solute separability and structure.

Certain physical separations, particularly of petroleum[8,9] and other natural constituent compound classes, appear possible only by elution chromatography from a solid stationary phase. The experimental convenience of adsorption chromatography has also recommended its use where other separation procedures are applicable. The quantitative description of given multicomponent chromatographic separations is only conceivable should linearity in the distribution isotherm exist: Linear adsorption isotherm systems are also potentially of value in obtaining greater insight into the adsorption process, as by examining the variation of solute adsorption energy with systematic change in solute structure. Quantitative comparisons of this type depend on the existence of unique thermodynamic standard states for the adsorbed solutes

compared. The region of isotherm linearity affords such a standard state, comparable to the dilute solution state. Similarly, the adsorbed state can be used to gain information on the intramolecular forces, or configuration, pertinent to the non-adsorbed molecule by forcing it into a co-planar state upon adsorption. Chromatographic separation order has been used in this connection[10] to obtain qualitative interaction forces (or energies) for molecules with sterically hindered internal rotation. Again, use of linear systems would furnish data for quantitative interpretation. Finally, a knowledge of linear elution adsorption chromatographic (LEAC) solute separabilities leads to greater understanding of the more common non-linear chromatographic cases.

Before attempting a thorough investigation of LEAC compound separability, it is desirable to understand the factors which contribute to non-linear separation, to learn how linearity may be attained in practical adsorption systems, and to develop tests for linearity in cases where lack of experimental precision makes it uncertain that linearity exists. The following discussion begins with a theoretical analysis of linearity in the equilibrium adsorption isotherm, and is followed by an experimental study which relates it to linear elution separation.

EQUILIBRIUM ADSORPTION FROM SOLUTION

The Langmuir isotherm offers the simplest and most frequently used model for interpretation of the adsorption process. It is limited by its failure to consider adsorbent heterogeneity, that is, differences in the energy of adsorption sites, and its neglect of lateral interactions between adjacent adsorbed molecules. Furthermore, in most adsorption systems of chromatographic interest, polyatomic molecules are involved which presumably use more than one adsorption site per adsorbed molecule. FOWLER[11] has derived the theoretical isotherm expression for adsorption without lateral interaction of a monatomic gas on a heterogeneous surface. A re-derivation for the adsorption of a polyatomic solute X from solution in a solvent S will be made. With all adsorbent sites occupied, the adsorption equilibrium is

$$X_{(n)} + mS_{(a)} \rightleftharpoons X_{(a)} + mS_{(n)}\,, \tag{1}$$

with the solute requiring m times the number of sites needed by solvent. The present discussion concerns the isotherm region of linearity or near linearity, the adsorbed solute concentration will be small, and therefore lateral interactions involving solute will be almost exclusively those between solute and solvent. These energy terms may be grouped with the site energy since they are independent of solute concentration. The isotherm expression for adsorption of X on a set of homogeneous sites i is then,

$$K_i = \frac{\theta_i}{(X)_n\,(1 - m\theta_i)\,m}\,, \tag{2}$$

the derivation being completely analogous to that for the Langmuir expression. K_i is a thermodynamic equilibrium constant for reaction (1) and sites i, θ_i is the frac-

tion of sites i which are covered by X, and $(X)_n$ is the solution concentration of X. Eqn. (2) reduces to the Langmuir isotherm for m equal one, and is essentially intractable for other cases. By proceeding with the assumption of m equal one we can anticipate a model which will illustrate the qualitative features of near linear adsorption, which is all that is desired at present.

θ, the fraction of the total adsorbent surface covered by X, is given by

$$\theta = \int_{-\infty}^{\infty} \theta_i N_i \, dF_i \; ; \tag{3}$$

here N_i is the fraction of the total number of sites in set i, and F_i is the dimensionless free energy of sites of set i, defined as $-\log K_i$. The distribution coefficient K for solute partitioned between total adsorbed phase and non-sorbed phase is equal to $\theta/(X)_n$. It is desired to solve for K as a function of solute concentration in the case of specific site energy distributions which are illustrative of certain features of isotherm linearity. The combination of eqn. (2) with m equal one and eqn. (3), plus the definitions $K_i = e^{-F_i}$ and $K = \theta/(X)_n$, yields

$$K = \int_{-\infty}^{\infty} \frac{N_i \, dF_i}{e^{F_i} + \theta/K} \tag{4}$$

The limiting value of K as θ approaches zero is the linear distribution coefficient K_0.

The ratio K/K_0 provides a test of isotherm linearity, $1 - K/K_0 \leqq \varepsilon$, with ε arbitrarily small. Corresponding to adsorbent capacity V_a, the specific volume of the adsorbed phase for complete site coverage, there exists a *linear capacity*, θ_ε, at which fractional coverage $(1 - K/K_0)$ equals ε.

In Fig. 1, a hypothetical site energy distribution is shown. We may characterize such distribution functions by their shape or type (*e.g.*, rectangular, triangular, Gaussian), their width or energy spread p, and the average strength of the sites F_0. K/K_0 is thus specified through eqn. (4) by these three loosely defined site distribution

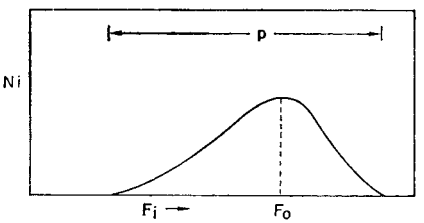

Fig. 1. General site energy distribution curve.

parameters, and by θ. It is interesting and simplifying to note, however, that the ratio of interest K/K_0 is independent of the average strength of the sites, F_0. It may be seen that if F_0 is increased by an amount E, corresponding to the increase of all site free energies by E, e^{F_i} in eqn. (4) is replaced by $[e^{F_i} e^{E}]$, N_i and dF_i remaining unchanged and F_i referring to the original distribution function. But this requires

that the new value of the distribution coefficient K' resulting from this change in F_0 be $K\,e^{-E}$, since substitution of these new values of the changed terms in eqn. (4) results in an identity. Similarly, the new value of the linear distribution coefficient K'_0 is $K_0\,e^{-E}$, and the ratio K/K_0 is thus independent of changes in F_0. Thus, the question of linearity reduces to the value of θ_ε as a function of two site distribution parameters (curve type and energy spread).

Consider, first, the rectangular distribution function, $N_i = 1/p$, for $-p \leqslant F_i \leqslant 0$, shown in Fig. 2. The normalization requirement

$$\int_{-\infty}^{\infty} N_i \, dF_i = 1$$

is observed. Eqn. (4) provides

$$K_0 = (1/p) \int_{-p}^{0} e^{-F_i} \, d\, F_i \tag{5a}$$

and

$$K = (1/p) \int_{-p}^{0} \frac{d\, F_i}{e^{F_i} + \theta/K} . \tag{5b}$$

Combination of integrated eqns. (5a) and (5b) then yields

$$K/K_0 = \theta\, p \left[\frac{e^p}{(e^p - 1)\,(e^{p\theta} - 1)} - \frac{e^{p\theta}}{(e^p - 1)\,(e^{p\theta} - 1)}\right] \tag{6}$$

As p becomes large, eqn. (6) approaches the limit (7) for small values of θ (near the linear region):

$$K/K_0 = \frac{\theta p}{e^{\theta p} - 1} \tag{7}$$

Linear capacity θ_ε is defined by the value of K/K_0, and in eqn. (7) it is seen that for a given value of K/K_0, θ (and linear capacity) is inversely proportional to p. The wide rectangular distribution is illustrative of a general property of all site distributions, linear capacity decreases with increasing site energy spread p.

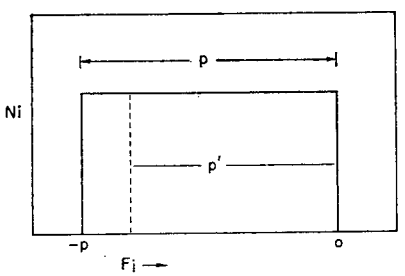

Fig. 2. Rectangular site energy distribution function.

In addition to illustrating one of the general properties of all wide site distributions, the rectangular function of Fig. 2 should prove a good approximation to

natural distributions which have been artificially truncated, as by water deactivation of the adsorbent.

It has been noted by CAHNMAN[12] that partial deactivation of silica by water improves its chromatographic characteristics, and this has proved to be a rather general phenomenon[13]. The effect has been attributed to removal of very strong sites which cause tailing of solute elution bands. The demonstration that K/K_0 is independent of F_0 suggests that strong sites as such do not contribute to non-linearity. It is of interest to consider the effect of adsorbent deactivation on adsorbent linear capacity.

If the rectangular distribution is partially deactivated by a selective adsorbate, so as to approach the truncation indicated in Fig. 2 by the dotted line, the integrations of eqns. (5a) and (5b) can be repeated, changing only the limits of integration to p' and θ, to give finally

$$K/K_0 = \frac{\theta p}{(e^{p'} - 1)} \frac{(e^{p'} - e^{p\theta})}{(e^{p\theta} - 1)} \tag{8}$$

where θ refers to adsorbent loading relative to the original distribution. For large values of p (and hence p'), eqn. (8) reduces exactly to eqn. (7), or *the moderate deactivation of a rectangular site distribution does not change linear capacity when the site energy spread p is large.* That this is so can be seen in terms of the demonstration that K/K_0 is dependent on θ but independent of F_0. When p is large and θ is small, only the sites at small values of F_i are appreciably covered, and the relative loading of these sites determines the relation between K/K_0 and θ. Truncating the distribution curve at the large F_i end does not affect either the latter relationship or any part of the adsorption process. But truncation at the large F_i end of the curve is equivalent, for the dependence of K/K_0 on θ, to truncation at the lower F_i end. The end result is merely a change in value of F_0. Hence, adsorbent deactivation does not change the dependence of K/K_0 on θ until p' becomes small enough for eqn. (8) to fail as an approximation to eqn. (7).

The situation with respect to adsorbent deactivation is different in the case of other site distributions. Where p is small, all site distributions approach the behavior of a homogeneous set, with deactivation causing a proportional *reduction* in linear capacity. As p goes to zero, adsorbent coverage is described by the Langmuir isotherm and

$$K/K_0 = 1 - \theta \tag{9}$$

Here, θ refers to the coverage relative to the sites left after deactivation, so that decline in adsorbent capacity leads to a proportional decline in linear capacity.

Alternately, distributions where N_i declines with F_i, rather than holding constant to some value $-p$, and where p is moderately large show linear capacity *increasing* for moderate deactivation. This is shown for an isosceles triangular distribution in Fig. 3 where $\theta_{.1}$ is plotted *versus* percent truncation or deactivation for various values of p. The values shown in Fig. 3 were obtained by numerical integration of eqn. (4) for K/K_0. The behavior of the triangular distribution is representative of

other declining site energy functions such as exponential or Gaussian distributions. As deactivation proceeds towards 100 %, adsorbent linear capacity must eventually decline.

The experimental observations of CAHNMAN cited above can be attributed to a closer approach to isotherm linearity with increasing adsorbent deactivation. This results, however, not from the removal of strong adsorption sites as strong sites, but as sites on a distribution tail, as in the triangular distribution.

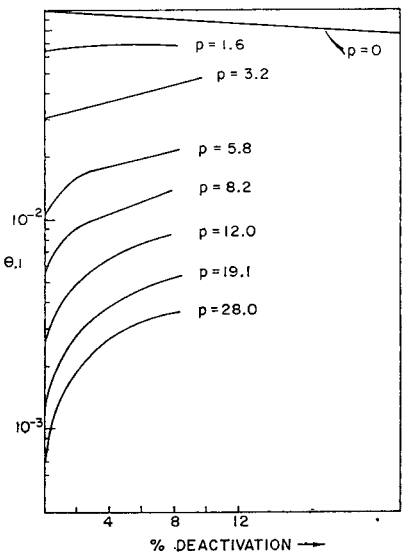

Fig. 3. Linear capacity as a function of site energy distribution width and truncation

Suitable tests for isotherm linearity and the related experimental evaluation of the linear coefficient K_0 for given systems are important. The experimental measurement of K as a function of adsorbent coverage (or solute concentration) over a range of values which includes a region of isotherm linearity (constancy of K) is the surest procedure, but this can be time-consuming. In some cases it is uncertain whether linearity has been achieved at the lowest coverages studied because of the larger experimental error associated with values of K measured at small values of θ. It is desirable to be able to use two values of K at different values of θ close to the region of linearity, and extrapolate to a value of K_0. The Langmuir expression has been used in this connection by SPORER AND TRUEBLOOD[7]. For the homogeneous (Langmuir) distribution

$$K = K_0 (1 - \theta) , \tag{9a}$$

and a linear extrapolation of K as a function of θ to θ equal zero yields K_0. For the wide rectangular distribution with K as a function of θ given by eqn. (7),

$$K/K_0 = 1 - \tfrac{1}{2}\theta p \tag{10}$$

at low values of θ. This may be seen by expansion of eqn. (7) with retention of first order terms. It is observed that linear extrapolation of K *versus* θ for K_0 is again valid. For site distributions characterized by declining values of N_t as site adsorption strength increases, linear extrapolation begins to fail at large values of p, although it is adequate as an empirical relationship for all but the widest distributions. In general, if two K values are measured for solute concentrations (or coverages) differing by at least a factor of 10, and if the difference between these K values is less than 10 %, then the linear extrapolation of K to zero coverage gives K_0 accurate within 5 %. As an extreme example, the test was applied to a very wide exponential distribution, where p was 20 % less than that value sufficient to make K_0 indeterminate and linearity impossible at any value of θ. The extrapolation procedure (within the above conditions) produced a value of K_0 always within 6 % of the theoretical value. The simple condition that K change by no more than 10 % over a 10-fold range in θ requires that even where non-linearity exists, the Freundlich exponent must be equal to or greater than 0.96. Other workers[6] have equated cases this close to linearity with linear systems.

LINEAR ELUTION ADSORPTION CHROMATOGRAPHY (LEAC) AND THE ADSORPTION ISOTHERM

When isotherm linearity exists throughout the elution of a solute from an adsorbent chromatographic column, the equivalent plate treatment of MARTIN AND SYNGE[2] gives solute retention volume R' (eluent volume to elute 50 % of solute from column) as

$$R' = (V_a/V_n)\, K_0 V_0 + V_0 \,. \qquad (11)$$

(V_a/V_n) is the ratio of adsorbed to non-sorbed phases, and V_0 is the total volume of non-sorbed phase within the column. The corrected retention volume, R equal $R' - V_0$, can be defined, as can the equivalent retention volume, $\underline{R}^0$ equal R/W. W is the total adsorbent weight. If V_a is the adsorbent capacity, V_0 equals $V_n\, W$, and

$$\underline{R}^0 = V_a K_0 \,. \qquad (12)$$

If K_0 is calculated from adsorbed phase concentrations in weight of solute per weight of adsorbent, rather than per volume of adsorbed phase, $\underline{R}^0 = K_0$. For experimental cases involving a non-linear isotherm, the non-linear equivalent retention volumes R define an "average" K for the entire elution.

Given the number of equivalent stages for an experimental elution case, and knowing $K/K_0 = f(\theta)$, it is straightforward to formulate the calculation of values of R (and $\underline{R}/\underline{R}^0$) for specified values of the column loading W_s. The actual calculation for most cases of interest strains the capabilities of an intermediate speed computer, however, and is impossible of manual solution. Qualitatively, it is noted that only a fraction of the total adsorbent is accessible to the solute band at any time during elution, and this fraction is proportional to solute elution band width w/R'. The

adsorbent coverage is proportional to the fraction of the total solute which is adsorbed, $V_aK(V_aK + V_n)$. If an intermediate value of w/R' is chosen as a standard of comparison (0.3), then a factor α equal to the fraction of the column "used" by the solute band can be defined, and values of W_s and θ can be associated with equal values of $\underline{R}$ and K, relative to the standard w/R' case:

$$\theta = \frac{0.3W_sR/(R + V_n)}{W_a\,\alpha\,(w/R')} \tag{13}$$

W_a refers to the weight of adsorbed phase per gram of adsorbent.

In Fig. 4, experimental equilibrium (K) and chromatographic ($\underline{R}$) data are plotted against θ for elution of 1,2,3,5-tetramethylbenzene from calcined alumina by pentane. Eqn. (13) was used to calculate values of θ for the chromatographic data from values of W_s. The best superposition of the chromatographic and equilibrium data required a value of α equal to 0.05. This means that 5 % of the chromatographic

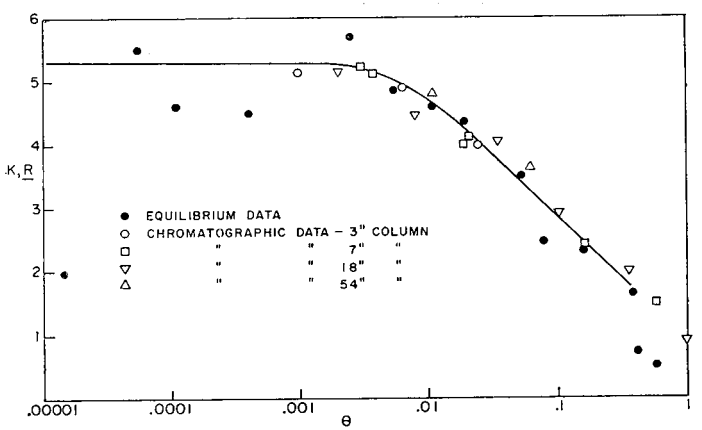

Fig. 4. Superposition of equilibrium and elution K values to show fraction of total column effective in adsorption.

column is effective in containing the solute, or that this fraction of the column, used as an equilibrium system, would have a value of K associated with it equal to $\underline{R}$ for the total column. While the particular value of α must be a function of the shape of the isotherm for each chromatographic system, it will be considered constant for purposes of relating values of W_s and θ in the next section. Returning to the data of Fig. 4, it is noted that the various chromatographic data all fall close to the same curve, even though a wide range in chromatographic conditions are represented (*e.g.*, column lengths from 3 to 54 inches). This means that eqn. (13) is capable of accurately reducing various chromatographic systems to a comparable basis. In addition, the agreement between equilibrium and superimposed chromatographic data is suggestive of a correspondence between $\underline{R}$ and K values.

The maximum column loading permissible for LEAC (linear capacity) with

alumina as adsorbent can be calculated, assuming the most favorable case (a homogeneous site distribution). W_a is equal to about 0.05 for calcined alumina, $\theta_{.1}$ equals 0.1 for a homogeneous site distribution, and taking a value of α equal to 0.05, a maximum value of W_s equal to $2.5 \cdot 10^{-4}$ is calculated for $\underline{R}/\underline{R}^0 \geqslant 0.9$. Since site heterogeneity is not unexpected in the various metal oxide adsorbents (compare next section), it could have been anticipated that LEAC would have been confined to column loadings of the order of 10^{-4} or less for elution from alumina.

EXPERIMENTAL OBSERVATIONS ON SOME ELUTION ADSORPTION CHROMATOGRAPHIC SYSTEMS

Equivalent retention volumes $\underline{R}$ have been measured for a number of chromatographic systems. Fig. 5 shows data for calcined alumina and silica as adsorbents, with a linear isotherm region observable in each case. Since linearity has already been observed by SPORER AND TRUEBLOOD[7] in systems featuring calcined silica, the latter observation is unsurprising. Fig. 6 shows the dependence of adsorbent linear capacity ($\theta_{.1}$) on the water deactivation of alumina, for the elution of naphthalene as solute by carbon tetrachloride as eluent. With from 0 to 2 % water on alumina, $\theta_{.1}$ continues to increase with added water. From this it may be inferred that the effective site distribution is of the "declining" rather than rectangular type for this adsorbent.

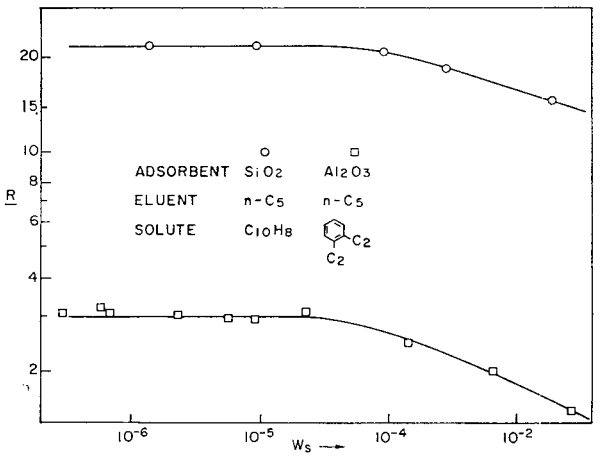

Fig. 5. Examples of linear elution adsorption chromatography.

Since $\theta_{.1}$ is defined in terms of the *original* adsorbent capacity W_a in Fig. 6, the site distribution appears to have become almost homogeneous with the addition of 2 % water. The W_s scale on the right of Fig. 6 gives the maximum column leadings for the maintenance of linear behavior, assuming the standard band width case but not taking into account the fact that the fraction of solute in the adsorbed phase varies. They are useful indications of the relative oil to gel ratios required for linearity in a typical elution system.

A number of $\underline{R}$ *versus* θ (or, what is equivalent at values of $\underline{R}$ close to $\underline{R}^0$, of $\underline{R}$ *versus* W_s) plots have been obtained for different chromatographic systems featuring alumina or silica as adsorbent. Applying the previous criterion for the linear extrapola-

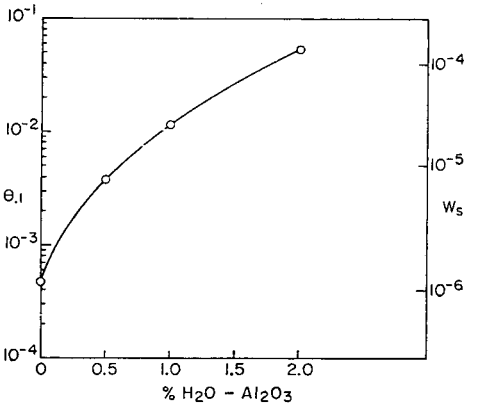

Fig. 6. Linear capacity as a function of adsorbent deactivation; elution of naphthalene by carbon tetrachloride from alumina.

tion of these plots to give values of $\underline{R}^0$, extrapolated values are compared in Table I with those observed in the linear region. This extrapolative procedure is designed to permit extrapolation only of $\underline{R}$ data lying between 0.9 $\underline{R}^0$ and $\underline{R}^0$. For more "extreme" extrapolation, where a ten-fold change in oil to gel ratio causes more than a 10 % change in $\underline{R}$, the linear extrapolation fails. This is also shown in Table I, where the results of extrapolating from $\underline{R}$ equals 0.5 $\underline{R}$ (with the second oil to gel value being 0.1 times the latter) are shown.

TABLE I

THE USE OF LINEAR EXTRAPOLATION FOR VALUES OF $\underline{R}^\circ$

Adsorbent	*Eluent*	*Solute*	$\underline{R}^\circ$*	*Extrapolated values of* $\underline{R}^\circ$	
				*10 %***	*50 %***
1.0 % H_2O–Al_2O_3	*n*-Pentane	Naphthalene	8.4	8.5	6.2
3.9 % H_2O–Al_2O_3	*n*-Pentane	Naphthalene	1.385	1.385	1.27
SiO_2 (calcined)	*n*-Pentane	Naphthalene	22.0	22.5	17.0
1.0 % H_2O–Al_2O_3	CCl_4	Naphthalene	2.33	2.35	
2.0 % H_2O–Al_2O_3	CCl_4	Naphthalene	0.68	0.675	
0.5 % H_2O–Al_2O_3	CCl_4	Methyl ethyl sulfide	6.8	6.75	

* Average value measured in linear region.
** Change in $\underline{R}$ produced by ten-fold change in W_s.

Values of W_s rather than θ are experimentally more accessible, and the two quantities are essentially interchangeable in the extrapolation procedure *when* values

$\underline{R}$ are greater than 1, and V_n for the adsorbent is less than 1. Since θ is proportional to $R/(R + V_n)W_s$, the latter quantity may be substituted exactly for the former in linear extrapolations.

Experimental

The eluents were 99.0 % or reagent-grade commercial chemicals, and were purified prior to use by passage over silica gel for the removal of trace polar constituents. The solutes were American Petroleum Institute standard samples, of higher than 99½ % purity. The adsorbents, Alcoa F-20 grade alumina and Davison 28 mesh silica, were first calcined at 400° for 16 h, following which deactivated samples were made up by addition of water. The percentages of water refer to the grams added per 100 grams of adsorbent. Both equilibrium and chromatographic data were measured at room temperature, 24°. To obtain the equilibrium K data of Fig. 6, the concentration of solute in solution was measured by ultraviolet absorption, and a difference procedure was used to calculate the concentration of solute in the adsorbed phase. The volume of the adsorbed phase, which is required in the calculation of K, was 0.05 ml/g of adsorbent (from the extrapolation of the solute uptake data to infinite solution concentration — Langmuir plot).

DEFINITIONS OF SYMBOLS

a subscript refers to "in adsorbed phase"

F_i dimensionless site free energy, $-\log K_i$

F_0 value of F_i for some particular point (*e.g.*, the maximum of a Gaussian) on a site energy distribution curve

K equilibrium constant defined for total adsorbent–solvent system; $K = \theta/(X)_n$; for the experimental data of Fig. 5 and related discussion, K is defined in terms of concentration of solute per unit weight of total adsorbent rather than volume of adsorbed phase

K_i Langmuir coefficient; (see eqn. (2)); e^{F_i}

K_0 value of K in the limit as solute concentration approaches zero; the linear distribution coefficient

m ratio of solute to solvent molar volumes; see eqn. (1)

n subscript refers to "in non-sorbed phase"

N_i fraction of total adsorbent sites possessing free energy of homogeneous set i

p width of site energy distribution curve in units of F_i; difference in F_i values between two arbitrary points on distribution curve

R' uncorrected retention volume

R retention volume corrected for column dead volume; $R = R' - V_0$

$\underline{R}$ equivalent corrected retention volume; R'/W

$\underline{R}^0$ linear equivalent corrected retention volume; R/W

$S_{(k)}$ solvent or eluent in phase k

V_0 column dead volume; equal to volume of moving phase plus adsorbed phase

V_a volume of adsorbed phase (monolayer) in ml per gram of adsorbent
V_n volume of non-sorbed phase per gram of adsorbent
w band width
W total weight of adsorbent in chromatographic column
W_s oil to gel ratio; weight of solute divided by weight of adsorbent
W_a weight of adsorbed phase per gram of adsorbent
$X_{(k)}$ solute in phase k
$(X)_k$ solute concentration in phase k
α fraction of a chromatographic column which, when used as an equilibrium adsorption system would give a value of K equal to R for the column
θ fraction of adsorbent sites covered by solute
θ_ε linear adsorbent capacity; value of θ at which K/K_0 declines from 1 by amount ε.

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SUMMARY

Adsorbent linear capacity has been shown to be independent of the average strength of adsorption sites, but inversely proportional to site energy distribution width. Water deactivation of an adsorbent normally leads to an increase in adsorbent linear capacity because the site distribution tails toward the strong site end. Linear extrapolation of experimental distribution coefficient values leads to correct linear coefficients if certain precautions are taken.

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THE CHROMATOGRAPHIC SEPARATION OF URANIUM, THORIUM AND RARE EARTHS BY MEANS OF PAPER TREATED WITH A LIQUID ANION EXCHANGER

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Recently, the interest in chemical separation methods based on the use of suitably treated chromatographic papers has increased considerably. Many examples are reported in the literature, and lately, the problem of a quick and dependable method for the preparation of amine-containing papers has been solved in our laboratory[1]. Such papers, which contain an anion exchanger (tri-*n*-octylamine), were successfully applied to the resolution of some mixtures of chemical elements that are very similar. As described, a hydrochloric medium was used as the eluent, because the various elements differ in their ability to form hydrochloric complexes.

The separation of uranium, thorium and rare earths, which is of considerable interest in the field of nuclear chemistry, cannot be adequately performed by conventional paper chromatography since the R_F values of thorium and the rare earths are very similar[2].

Furthermore, thorium and rare earths do not form hydrochloric complexes[3], and hence thorium cannot be separated from rare earths even by the above-mentioned method of elution from aminated paper with hydrochloric acid. Similarly, as is well known, these elements in hydrochloric solution are not retained by anion exchange resins or extracted by liquid anion exchangers. On the other hand, the behaviour of uranium, thorium and the rare earths in nitrate solutions is dissimilar[4–7], their distribution coefficients being very different, so that it is possible to obtain a good separation of the elements from each other.

Since it was shown[1] that paper treated with a liquid anion exchanger (tri-*n*-octylamine) acts very much like a film of an anionic resin, it was decided to investigate the chromatographic separation of uranium, thorium and rare earths by paper chromatography on aminated paper in a nitrate system.

EXPERIMENTAL AND RESULTS

Preparation of the paper

For all the experiments described below, Whatman No. 1 paper was used. The tri-*n*-octylamine (Fluka and Light's) was applied by dipping square or rectangular sheets of paper into a solution of the amine in benzene. This solution was prepared by adding

sufficient amine to benzene to obtain the required molarity, and then shaking for 5 min with three times the volume of nitric acid or nitrate of a given concentration in water. In this way the amine is converted into the nitrate salt; this makes the elution more regular and the solvent front well-defined. Then the organic phase was separated and freed from residual droplets of the aqueous phase by passing it through cotton lint. After soaking the paper, it was allowed to drip and then dried with warm air to remove the excess of benzene. The amount of amine fixed on the paper was determined by weighing a 15 × 15 cm sheet of paper before and after treatment. When using a 0.1 *M* solution of amine in benzene the amount fixed proved to be 0.55 mg/cm^2. Obviously, by using more concentrated solutions, this amount can be considerably increased. After the paper was ready for use it was cut partly into 10 cm diameter discs for circular chromatography, and partly into 5 × 30 cm strips for descending chromatography.

Solutions of the elements

The solutions of the elements were prepared from $Th(NO_3)_4 \cdot 6H_2O$ (B.D.H.), $La(NO_3)_3 \cdot 6H_2O$, $Ce(NO_3)_3 \cdot 6H_2O$ and $UO_2(NO_3)_2 \cdot 6H_2O$(Merck). Since in the first experiments, cerium (III) and lanthanum behaved in the same fashion, only lanthanum was used subsequently.

Circular chromatography

A drop of about 10 μl, containing 25–50 μg of each element, was deposited at the center of the disc and immediately dried. The spot was then developed at room temperature (20°) by slowly dropping 2 or 3 ml of the appropriate eluent at the center of the disc with a micropipette, till the diameter of the wet surface was about 7 cm.

The elution time ranged from 10 to 20 min according to the viscosity and density of the eluent solution and the permeability of the paper. The permeability of the paper in turn depends on the molarity of the amine solution used for the treatment.

After the elution was completed, the paper was dried and the zones developed by means of a 0.1 % solution of morin in alcohol; uranium and thorium are revealed at once, whereas lanthanum (or Ce (III)) appear only after the paper has been exposed to ammonia vapour.

Elution with HNO_3

The R_F values for uranium, thorium and lanthanum were measured as functions of the molarity of the nitric acid used as eluent. In this experiment the paper was treated with an amine solution which had been pre-equilibrated with 10 *M* HNO_3, and the elution was performed with HNO_3 in the range from 1 *M* to 10 *M*. The experimental results reported in Fig. 1 show that in the whole range of HNO_3 molarities, thorium is retained more strongly than uranium, while lanthanum always moves with the solvent front. When the HNO_3 molarity of the eluent increases from 1 to 6, both thorium and uranium become progressively more complexed and hence their R_F values become lower and lower. From 6 *M* to 10 *M*, however, the R_F values rise again.

This fact is probably due to anion exchange between the nitric acid and the anionic complex fixed by the aminated paper. Analogous behaviour was found during the adsorption of thorium and uranium from nitric acid solutions on anionic resins[5-6] or by extraction with tri-*n*-octylamine[7] and tri-iso-octylamine[8].

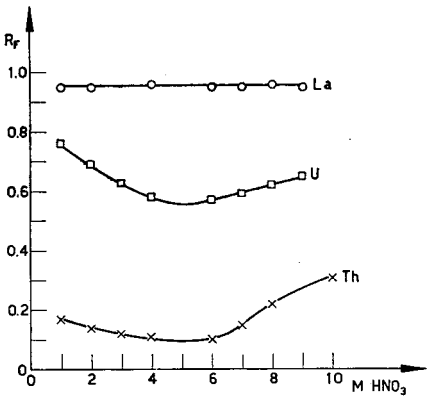

Fig. 1. R_F values on paper treated with 0.1 *M* TNOA in benzene *vs.* molarity of HNO_3 in the eluent.

The R_F values of the three elements (U, Th and La) were compared with the values of the extraction coefficient E°_{a}, by shaking 10 ml of 0.2 *M* tri-*n*-octylamine (TNOA) for 10 min with 10 ml of a 0.01 *M* solution of each element, containing different amounts of HNO_3.

Thorium and lanthanum were determined by complexometry, and uranium by colorimetry. The results are shown in Fig. 2; in Fig. 2a the values of $1/R_F$—1 are given and in Fig. 2b the extraction coefficients E°_{a}. From the plots it is clear that lanthanum is always very poorly extracted (E°_{a} = 0.01 and R_F = 0.96) and that both uranium and thorium show the highest extraction between 6 *M* and 7 *M* HNO_3.

The shape of the curves is very similar for the quantities $1/R_F$—1 and E°_{a}, and under the above conditions the extraction coefficients of uranium are quite close to those of thorium. This fact does not account for the considerable difference in the R_F values that was found in our experiments, but the effect of the amine concentration must also be considered. In fact, as shown in Fig. 3, the extraction of thorium is increased much more than that of uranium when the amine concentration is raised. These experiments were carried out by shaking 10 ml of a 3 *M* HNO_3 solution 0.01 *M* in uranium or thorium, with 2 mmoles of TNOA diluted with varying amounts of benzene. By changing the amine concentration from 0.1 *M* to 2.3 *M*, the extraction coefficient of thorium is increased about 1,000 times, whereas that of uranium is increased only 80 times.

It can be inferred that the amine fixed on the paper behaves as if it were in a concentrated solution, since the solvent is completely removed during the preparation; this would enhance the difference between the R_F of uranium and that of thorium.

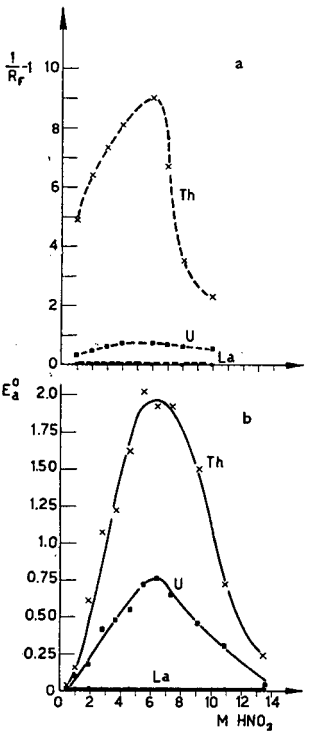

Fig. 2. Comparison of (a) $1/R_F-1$ *vs.* the molarity of HNO_3 in the eluent and (b) the extraction coefficient E_a^0, for 0.2 *M* TNOA in benzene, *vs.* the molarity of HNO_3 in the aqueous phase.

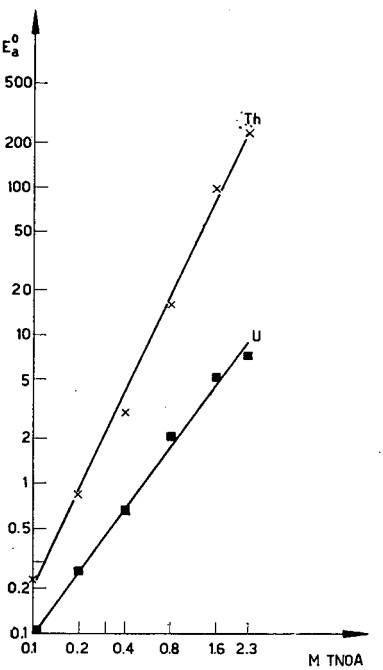

Fig. 3. Effect of the dilution of TNOA (2 mmoles in total) in benzene on the extraction coefficient E_a^0; 3 *M* HNO_3.

Moreover, by treating the paper with solutions of TNOA in benzene, of molarities ranging from 0.01 M to 0.6 M, the R_F values were considerably lowered for uranium and thorium, the decrease being greatest for the latter. These results are shown in Fig. 4.

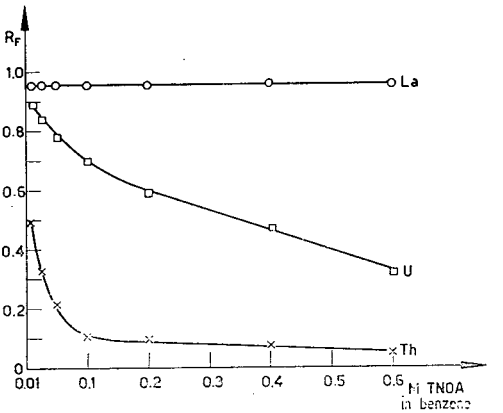

Fig. 4. Effect on R_F of the molarity of the TNOA used for treatment of the paper; eluent 3 M HNO_3.

Therefore the difference in behaviour of the two elements can be influenced both by the molarity of the eluting acid and by that of the amine used to treat the paper.

Elution with nitrate salts

Elsewhere[4], it has been pointed out that the adsorption of uranium on anionic resins can be enhanced by using NH_4NO_3 instead of HNO_3, at the same molarity. In this case thorium behaves approximately as in a HNO_3 solution, whilst the rare earths, which are not adsorbed from HNO_3 solutions, show sufficient adsorption from ammonium nitrate and lithium nitrate solutions[9]. Furthermore, no re-extraction takes place at higher NO_3^- molarities. Therefore, the possibility of using solutions of a nitrate salt as the chromatographic eluent was considered.

Lithium nitrate was investigated first. The 0.1 M TNOA was pre-equilibrated with three times its volume of a solution 6 M in $LiNO_3$ and 0.005 M in HNO_3. The free acid, which was also present in the eluent, was added in order to prevent hydrolysis of the salts of the elements being investigated. The paper was then prepared in the usual way and chromatography was carried out by eluting with lithium nitrate solution, the molarity of which ranged from 1 M to 6 M. The results given in Fig. 5 show that: (a) the R_F values of uranium are lower than those obtained with HNO_3 at the same molarity (see Fig. 1); (b) the R_F values diminish continuously as the molarity of nitrate is increased up to 6 M; (c) for thorium there is no marked difference between HNO_3 and $LiNO_3$; (d) the R_F values of lanthanum are slightly lowered. Generally, the picture of this type of ion exchange chromatography is very similar to that of ion exchange on anionic resins.

Fig. 6 shows the experimental results obtained with 3 M $LiNO_3$ and HNO_3

from 0.025 M to 1 M. These results show that uranium and lanthanum are progressively less retained; in fact, their R_F values increase, whilst those of thorium remain unaffected. In this case, however, the amine solution had not been pre-equilibrated

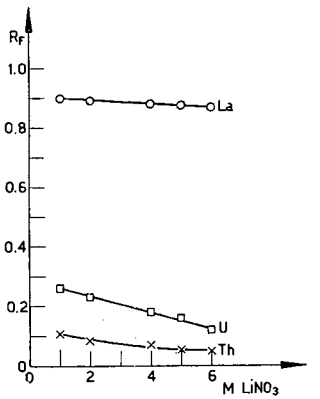

Fig. 5. R_F values on paper treated with 0.1 M TNOA in benzene *vs.* molarity of $LiNO_3$ in the eluent.

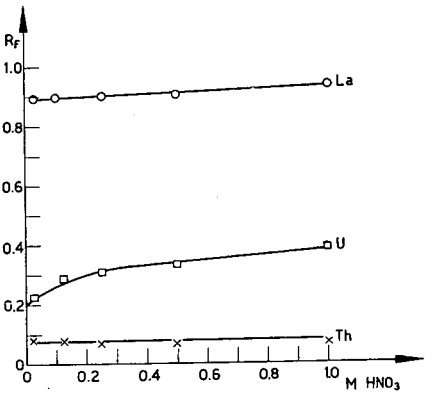

Fig. 6. Effect on R_F of the molarity of the HNO_3 present in the 3 M $LiNO_3$ eluent.

with the nitrate solution. The effect of lithium nitrate is clearly seen in Fig. 7, which shows that by varying the $LiNO_3$–HNO_3 ratio (the total NO_3^- molarity being 6 M), the R_F values of uranium are greatly affected. For these experiments the amine solution had been pre-equilibrated with three times its volume of the respective eluent solution.

Some experiments were carried out with $NaNO_3$, the concentration being varied from 1 M to 8 M (HNO_3 0.005 M). The results plotted in Fig. 8 show that the behaviour of the R_F values is similar to that found with $LiNO_3$; moreover no back-extraction was found up to 8 M, since the R_F values decrease progressively as the molarity increases.

Similar experiments were carried out with NH_4NO_3 of molarities from 1 M to 10 M (HNO_3 0.005 M). The paper was treated with amine pre-equilibrated with 10 M NH_4NO_3 + 0.005 M HNO_3. As shown in Fig. 9, the R_F curve of thorium is very similar to that obtained with the other alkali nitrates, while the R_F values of uranium are lower than those with nitric acid, but higher than with lithium and sodium nitrate. This can be due to the higher acidity arising from free HNO_3, which results from the hydrolysis of NH_4NO_3 solutions. The behaviour of lanthanum with NH_4NO_3 was rather interesting because in this case the R_F values are considerably lower than in all the experiments discussed above.

With NH_4NO_3, as with $LiNO_3$ and $NaNO_3$, no increase of the R_F values with increasing concentration of nitrate ions was found up to 10 M.

The effect of $Ca(NO_3)_2$ and $Al(NO_3)_3$ was also investigated. The results plotted in Fig. 10 show that the effect of calcium nitrate is very similar to that of sodium and lithium nitrate, provided that the concentration of nitrate ion is the same. As

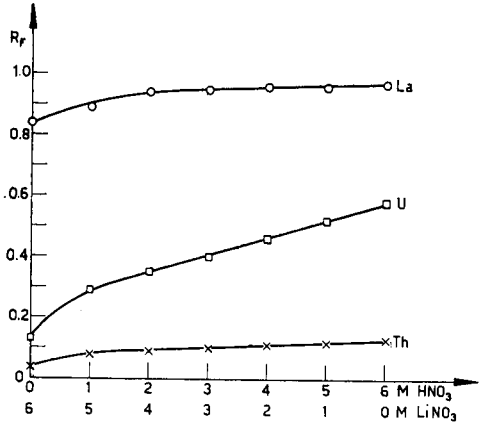

Fig. 7. Effect on R_F of the ratio $HNO_3/LiNO_3$ in the eluent; total nitrate ion molarity 6 M.

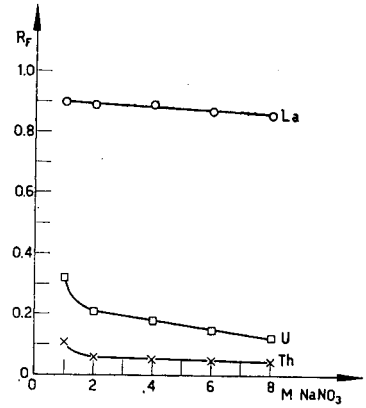

Fig. 8. R_F values on paper treated with 0.1 M TNOA–benzene *vs.* molarity of $NaNO_3$ in the eluent.

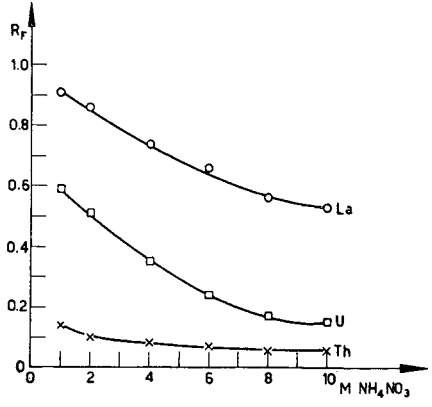

Fig. 9. R_F values on paper treated with 0.1 M TNOA–benzene *vs.* molarity of NH_4NO_3 in the eluent.

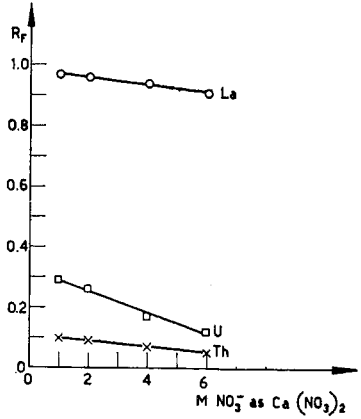

Fig. 10. R_F values on paper treated with 0.1 M TNOA–benzene *vs.* nitrate ion molarity of the $Ca(NO_3)_2$ eluent.

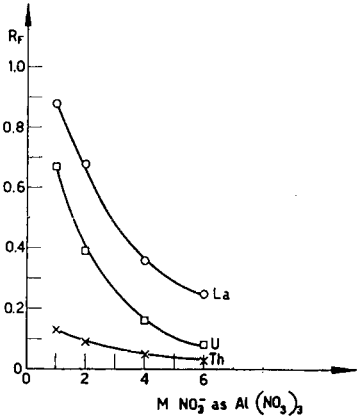

Fig. 11. R_F values on paper treated with 0.1 M TNOA–benzene *vs.* nitrate ion molarity of the $Al(NO_3)_3$ eluent.

shown in Fig. 11, the behaviour of thorium was normal with aluminium nitrate, while the R_F values of uranium were relatively higher at lower molarities of nitrate, but rapidly reached the normal values as the molarity increased. Similar results were obtained also for lanthanum, which, at higher molarities, has lower R_F values than with ammonium nitrate.

For the experiments with the calcium and aluminium salts the paper was treated with an amine solution previously equilibrated with the eluting solution containing the highest concentration of the respective salt (6 *M* and 0.005 *M* HNO_3).

To facilitate comparison of the experimental data, all the results have been collected in Table I.

TABLE I

CIRCULAR CHROMATOGRAPHY
EFFECT OF THE NATURE AND CONCENTRATION OF THE ELUENT ON THE R_F
Paper treated with 0.1 *M* TNOA–benzene.

Molarity NO_3^-		1	2	4	6	8	10
	Th	0.17	0.14	0.11	0.10	0.22	0.31
HNO_3	U	0.76	0.69	0.58	0.57	0.62	0.65
	La	0.95	0.95	0.96	0.95	0.95	0.96
	Th	0.11	0.08	0.07	0.05		
$LiNO_3$	U	0.26	0.23	0.18	0.12		
	La	0.90	0.89	0.88	0.87		
	Th	0.11	0.06	0.05	0.05	0.05	
$NaNO_3$	U	0.32	0.21	0.18	0.15	0.12	
	La	0.90	0.89	0.89	0.87	0.86	
	Th	0.14	0.10	0.08	0.06	0.05	0.05
NH_4NO_3	U	0.59	0.51	0.35	0.24	0.17	0.15
	La	0.91	0.86	0.74	0.66	0.56	0.53
	Th	0.10	0.09	0.07	0.05		
$Ca(NO_3)_2$	U	0.29	0.26	0.17	0.12		
	La	0.97	0.96	0.94	0.92		
	Th	0.13	0.09	0.05	0.03		
$Al(NO_3)_3$	U	0.67	0.39	0.16	0.08		
	La	0.88	0.68	0.36	0.25		

Descending chromatography

Good results in the separation of uranium, thorium and lanthanum were also obtained with descending chromatography.

From the experimental data, which are reported in Table II, the following observations have been made:

(a) Thorium and uranium present well defined oval spots, while lanthanum, which is not retained by the amine, forms a narrow band extending all along the solvent front.

(b) By lowering the amine concentration on the paper, all the other conditions being equal, the R_F values of uranium and thorium increase considerably as in the case of circular chromatography.

(c) The R_F value is unaffected by the distance that the front runs, provided that the other conditions are kept constant.

Some chromatograms were also made by using 0.1 mg and 1 mg of each element. The solution was deposited along a horizontal band at the start; after elution with

TABLE II

DESCENDING CHROMATOGRAPHY

Amount of element deposited: 25 μg; eluent 3 M HNO_3.

Front run cm	*Molarity of pre-equilibrated amine*	R_F		
		Th	*U*	*La*
24	0.20	0.02	0.23	0.98
18	0.10	0.05	0.42	0.97
16	0.05	0.16	0.68	0.97
28	0.05	0.15	0.68	0.97

5 M HNO_3 and development, thorium, uranium and lanthanum showed three distinct bands, which differed in width.

Two experiments were performed with NH_4NO_3 as the eluent, and in this case also lanthanum formed a distinct spot. The experimental data are given in Table III.

TABLE III

DESCENDING CHROMATOGRAPHY

Amount of element deposited: 25 μg; paper treated with 0.05 M TNOA–benzene.

Front run cm	*Molarity of NH_4NO_3 and HNO_3 in the eluent*	R_F		
		Th	*U*	*La*
32	10 M NH_4NO_3 0.02 M HNO_3	0.02	0.20	0.60
29	2 M NH_4NO_3 0.1 M HNO_3	0.05	0.70	0.87

CONCLUSIONS

Good separations of uranium, thorium and rare earths can be obtained by chromatography on paper treated with tri-*n*-octylamine. Nitric acid, or a solution of a nitrate salt, can be used as the eluent. As previously observed for the chloride complexes, in this case also a certain concordance exists between the behaviour of the R_F and that of both the distribution coefficient of anionic resins and the extraction coefficients of liquid anion exchangers. Treatment of the chromatographic paper with various substances that have a certain degree of selectivity for the different elements (amines, phosphines, organo-phosphoric acids, inorganic exchangers[10], etc.) appears to be a useful tool for the analytical chemist, and many separation problems can be solved by

selecting both the appropriate substance for the treatment and a suitable eluent. For instance, the separation of uranium, thorium and lanthanum can be carried out also from hydrochloric solutions by using a paper treated with tri-*n*-octyl-phosphine oxide (TOPO). An experiment was made with a solution of 3 *N* HCl and paper treated with 0.05 *M* TOPO in cyclohexane. In this case, as expected from the extraction curves with TOPO[11], uranium is most strongly retained and presents the smallest R_F (0.06), followed by thorium (R_F = 0.38) and lanthanum (R_F = 0.97).

A new series of experiments is now being performed, in which cellulose powder treated with TNOA is used to carry out column chromatography. Good results have been obtained and will be described in a subsequent paper.

SUMMARY

The chromatographic separation of uranium, thorium and rare earths was carried out by using a paper treated with tri-*n*-octylamine (TNOA).

Solutions containing HNO_3, $LiNO_3$, NH_4NO_3, $NaNO_3$, $Ca(NO_3)_2$ and $Al(NO_3)_3$ were used as eluents. This type of chromatographic separation is analogous to ion exchange on anionic resins or with liquid amines.

The effects of the various parameters on the R_F values are examined.

The separation of thorium, uranium and lanthanum with hydrochloric solutions, on paper treated with tri-*n*-octyl-phosphine oxide was investigated.

The possibility of using columns filled with cellulose powder treated with TNOA, as anionic resin columns is also anticipated.

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Short Communications

A simple technique for the automatic application of solutions to chromatography paper

A technique has been developed for the automatic application of relatively large volumes of dilute aqueous solutions to absorbent paper in such a way as to produce "spots" of a suitable size for chromatography and paper electrophoresis.

The apparatus is shown in Fig. 1. The paper (F), held clear of the bench, is pierced by a needle bearing a length of thin cotton thread (No. 50) with a knot (E) at one end, at the point (D) where application of the solution is to be made. The fibres of the thread and of the paper are then matted together at their point of junction by scratching with the needle, and the thread is cut at a predetermined distance above the paper. The sample to be applied is contained in a pipette (A) supported above the paper and the upper end of the thread is attached by surface tension to the meniscus of the solution in the tip (B).

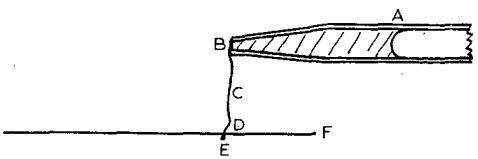

Fig. 1.

The solution soaks down the thread (C) and onto the paper, and when the rate of this process is balanced by evaporation, the area of the "spot" reaches a steady-state diameter and the solute is deposited in a fine ring at the circumference. The ring grows from its inner edge and all the sample is eventually drawn from the pipette. It should be noted that if the initial diameter of the ring on the paper is too small for the sample being used, it will eventually close in giving a uniform disc and subsequent application of solution is extremely slow. An estimate of the amount of solution contained in the thread can be made by observing the levels of the liquid in the pipette before and after the thread is saturated with the solution. This is retained at the end of the process, and if considered significant it can be washed onto the paper using pure solvent from a second pipette. The thread is then removed.

The size of the "spot" was observed to depend on the temperature, humidity and the length of the thread. This length must be determined by experiment under the prevailing conditions. In this laboratory lengths of 1.8 to 2 cm gave spots 0.8 to 1.0 cm in diameter with aqueous solutions, and 0.1 ml was applied in approximately 7 h. More volatile solvents require shorter threads. Mr. J. ASHWORTH has found here that

0.1 ml of a dioxane solution was applied through a 0.8 cm thread in 50 min and yielded a 1.5 cm diameter "spot".

Even though the "spots" produced by this technique were somewhat larger than those obtained on the most favourable occasions using the very much more time-consuming manual technique, equally good, if not better, resolution of urinary phenolic and indolic acids has been obtained[1] on chromatography papers 23 cm square.

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Separation of monosaccharides, disaccharides and trisaccharides on carbon–aluminium oxide columns

A systematic study of the separation of carbohydrates on carbon columns was carried out by TISELIUS[1] in 1940. This method was not widely used until 1950, when WHISTLER AND DURSO[2] found that columns of carbon–Celite proved to be very effective. Thus, the separations of oligosaccharides[3–7] and methylated sugars[8–10] were successively achieved. In most cases carbon Darco G 60 and Celite 535 were used[1,5,7,10]. Celite alone was used for the separation of sugars[11] but it was usually added to carbon in order to increase the rate of flow of the eluate. The use of powdered cellulose instead of Celite was suggested by JERMYN[7]. The separations of sugars were performed on active carbon (40–60 mesh)[12]; columns of cocoanut charcoal (50–200 mesh) were used for separating methylated sugars and uronic acids[13] and the methylated sugars were separated on Al_2O_3 columns as well[14–16].

Since Celite has the tendency to contaminate the fractions[17], while carbon itself decreases the rate of flow of the eluate, we have investigated the separation of glucose, mannose and raffinose on a column containing carbon and aluminium oxide in ratio 3:4. It has been found that the three sugars could be recovered without any loss. The separations were controlled by paper chromatography[18], the results obtained showed that the separations were complete. The purity of the sugars recovered was checked by comparing their electric resistance with that of a mixture of pure sample and 0.5 % sodium chloride. The resistance of the sugar recovered was found to be greater than that of the mixture which meant that the impurities could not surpass 0.5 %. By using an aluminium oxide–carbon column we have succeeded in isolating the α,α-trehalose from baker's yeast[19] and β-gentiobiose from the reaction mixture obtained by the action of emulsin on glucose solution[19].

Experimental

Fractionation was carried out in columns (350 × 12.5 mm) containing active carbon (Degussa) and aluminium oxide (Brockmann) in ratio 3:4. Total weight of adsorbent 21 g. Before chromatographing, the column was washed with distilled water, 15 % ethanol and again with water. The mixture of glucose (52.4 mg), maltose (50.4 mg) and raffinose (52.3 mg) was dissolved in distilled water (5 ml) and chromatographed. The column was eluted with 1 %, 5 % and 15 % aq. ethanol respectively. The elution with ethanol of lower percentage was substituted by ethanol of higher percentage when the eluate ceased to reduce Fehling-solution. The eluates obtained were evaporated to dryness (in vacuum at 45°) to constant weight. Recovered weight: glucose (53.1 mg), maltose (50.9 mg) and raffinose (52.1 mg).

Paper chromatography was carried out by the ascending method, according to JEANES[18]. A mixture of butanol–pyridine–water (3:1:1.5) was used for the development, and the detection of the spots was achieved by means of aniline hydrogen phthalate[20]. The electric resistance of glucose was measured conductometrically:

Substance:	*Conc. in water sol.:*	*Electric resistance:*
Regenerated glucose	1.000 %	3.05 kΩ
Glucose + NaCl	0.995 % 0.005 %	1.70 kΩ

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Paper chromatography of quercetagetin derivatives

In the course of our investigations of *Vitex agnus castus* seeds, it was found that they contain a flavonoid pigment casticin (5,3′-dihydroxy-3,6,7,4′-tetramethoxy-flavone[1]. It became desirable to follow the progress of the purification of casticin and to compare its R_F values with those of other known flavonoids for the purpose of identification. In most solvent systems that are successfully used with flavonoid pigments[2], casticin was found to travel near the front and to show considerable tailing. Therefore, for the paper chromatography of casticin, solvent systems such as are used in the steroid field and many other fields[3], were applied.

Filter paper (Whatman No. 1) was dipped into a solution of formamide or dimethylformamide in acetone (50 % v/v), the excess of solvent removed by blotting between sheets of filter paper and the samples, ranging in quantities from 1 to 30 γ applied in the form of solutions in acetone. The chromatogram was developed by the descending technique, until the mobile phase had travelled about 30 cm. Mixtures of benzene and decalin, saturated with formamide, or decalin saturated with dimethylformamide, were used as mobile phase. Subsequently the chromatogram was dried in a stream of hot air until the mobile phase had evaporated. Visualisation was achieved by conventional methods as for example by examining the paper in U.V. light or by spraying it with a diazotised solution of sulphanilic acid[4].

The results, given in Table I, show that satisfactory chromatograms can be obtained with formamide–benzene–decalin, especially if the ratio of benzene–decalin is adjusted to suit the specific requirements. Artemetin and diethylcasticin cannot be separated since their R_F values are too close together. However, the difference between the R_F values obtained with the dimethylformamide–decalin system is large

TABLE I

R_F VALUES OF QUERCETAGETIN DERIVATIVES

	R_F values in			
	Formamide/ benzene-decalin (1:1)	*Formamide/ benzene-decalin (4:1)*	*Dimethyl-formamide/decalin*	*Colour in U.V. light*
Casticin (5,3′-dihydroxy-3,6,7,4′-tetramethoxy-flavone)	0.26	0.40	0.0	dark brown
Artemetin (5-hydroxy-3,6,7,3′,4′-pentamethoxy-flavone)	0.81	0.83	0.12	dark brown
Diacetylcasticin (5,3′-diacetoxy-3,6,7,4′-tetramethoxy-flavone)	0.65	0.72	0.0	blue fluorescence
Hexamethylquercetagetin (3,5,6,7,3′,4′-hexamethoxy-flavone)	0.52	0.72	0.0	blue fluorescence
Diethylcasticin (5,3′-diethoxy-3,6,7,4′-tetramethoxy-flavone)	0.79	0.83	0.22	blue fluorescence

enough to allow distinct separation. Yet this system has the disadvantage that it causes tailing of the above-mentioned substances.

By substituting petroleum ether for decalin in the solvent system formamide–benzene–decalin, the method can be utilised in large scale work. The separation of artemetin and casticin on a column of cellulose powder was carried out to test the method.

Cellulose powder (200 g, Whatman standard grade) was impregnated with formamide (66 g) in acetone (150 ml). Then the acetone was evaporated at 50° under reduced pressure. The cellulose was pressed into a column 4 × 28 cm. The mixture of artemetin (185 mg) and casticin (198 mg), dissolved in a small amount of the mobile solvent, was dropped on the top of the column and the mobile solvent (benzene and 20 % v/v petroleum ether 30–40°, saturated with formamide) allowed to pass through the column at the rate of 2 ml/1 min. Ten ml portions were collected and examined by paper chromatography. Fractions 1–7 contained artemetin and fractions 10–20 contained casticin. In fractions 8 and 9 neither flavone could be detected. Fractions 1–7, and 10–20, respectively, were combined, and the solvent evaporated. Of artemetin 190 mg and of casticin 200 mg were recovered. After one crystallisation from benzene–petroleum ether both compounds had the original melting points.

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Behaviour of the metals of the 1b group on zirconium phosphate columns and papers

Recent work has revived interest in inorganic ion exchangers and their application[1]. Of these, zirconium phosphate was selected for our purpose because of its properties and high capacity. It was prepared according to the method described in the literature[2].

Columns were made from 6 mm diam. glass tubes constricted at one end and filled to a height of about 40 mm with zirconium phosphate (ZP). Its capacity and distribution coefficients were as previously recorded (capacity = 1.88 mequiv./g).

The zirconium phosphate papers were prepared as follows: Whatman No. 1 paper was impregnated with a solution of 0.2 *M* $ZrOCl_2 \cdot 8H_2O$ in 4 *N* HCl, the excess solution drained off and the paper dried at room temperature. These papers were then

dipped into a solution of 12 % H_3PO_4 in 4 *N* HCl and washed with water until the pH was 4 and again dried at room temperature.

The behaviour of Ag(I), Cu(II) and Au(III) was found to be as follows: Au(III) as $AuCl_4^-$ is not retained on a column of ZP, contrary to what happens on an organic exchanger such as Dowex-50[3]. From a solution of Au(III) and Cu(II) (as $CuCl_2$) only Cu(II) is retained.

Ag(I) and Cu(II) as sulphates are retained on a column and can be readily separated by eluting first with 0.1 *N* HCl (2 ml suffice) to remove the Cu(II) and then with NH_3–NH_4Cl (4 *N*) which removes the silver.

Au(III) has the same R_F value on ZP-impregnated paper as on ordinary Whatman No. 1 paper (R_F = 0.65 – 0.70) when developed with 0.01 *N* HCl or water.

When a mixture of Ag(I) and Cu(II) sulphates are developed with 0.1 *N* HCl, Ag(I) stays on the point of application and Cu(II) has an R_F of 0.7 – 0.8. Thus the behaviour is analogous to that on ZP columns.

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A rapid method for the preparation of packing material for gas chromatographic columns

The generally accepted practice in preparing the stationary phase for gas–liquid chromatography is to dissolve the liquid substrate in a suitable volatile solvent, add this to the support material and then remove the solvent by careful heating. This is often a tedious procedure and is subject to frequent "bumping" of the mixture.

A more satisfactory way of removing the solvent is to carry out the evaporation in a rotating-type vacuum evaporator. This operation takes only a matter of minutes and on completion the packing generally requires very little further conditioning.

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Dünnschicht-Chromatographie

IX. Mitteilung. Schnelltrennung von Digitalis- und Podophyllum-Glycosidgemischen

Zur Chromatographie kleinster Mengen von Herzglycosidgemischen werden zur Zeit vorzugsweise die von SCHINDLER UND REICHSTEIN[1], TSCHESCHE und Mitarb.[2] und KAISER[3] ausgearbeiteten Verfahren auf den mit Formamid oder Octanol imprägnierten Papieren verwendet. Vor zehn Jahren haben STOLL und Mitarb.[4] auf die Leistungsfähigkeit von Silicagel-Säulen zur Trennung derartiger Gemische aufmerksam gemacht. Nach unseren bisherigen Erfahrungen[5] war zu erwarten, dass der Übergang von der "geschlossenen" zur "offenen" Kieselgel-Schicht auch auf dem Gebiet der Steroid-, Triterpen- und Lignanglycoside neben einer Zeitersparnis, eine verbesserte Trennung und eine Spurenanalyse bringen würde.

Unsere Versuche ergaben, dass auf Kieselgel G-Schichten* mit verschiedenen Elutionsgemischen eine Schnelltrennung der genannten Glycosidgruppen möglich ist. So lassen sich z.B. mit einem Methylenchlorid–Methanol–Formamid-Gemisch (80 + 19 + 1; Vol. Teile) auf einer Trennstrecke von 10 bis 12 cm in einer halben Stunde alle 14 uns zur Verfügung stehenden Digitalisglycoside sauber auftrennen. Zur Sichtbarmachung bevorzugten wir neben dem Antimon(III)-Chlorid Reagenz vorallem die von KAISER[3] verwendete Trichloressigsäure–Chloramin-Reaktion. Hiermit lassen sich auf der Kieselgelschicht die Herzglycoside z.T. bis 0.1 μg bei Tageslicht erkennen. Im ultravioletten Licht werden hiermit noch Mengen bis 0.01 μg gut sichtbar. Vergleichsweise sei angeführt, dass bei den genannten papierchromatographischen Methoden die untere Erfassungsgrenze mit 3–5 μg angegeben wird und die Trennzeit zwischen 3 und 14 Stunden liegt[3]. Da nahe beieinanderliegende R_F-Werte keine Aussage über die Trennmöglichkeiten der Substanzen machen, ist in der Fig. 1 ein im Durchlicht nachgezeichnetes Dünnschicht-Chromatogramm wiedergegeben. Auf gleiche Weise lassen sich auch *Strophanthus-*, *Scilla-*, *Convallaria-* und ähnliche Herzglycosidgemische auftrennen. Die Möglichkeiten der quantitativen Auswertung nach dem Abschaben der Zone und Elution der Glycoside ist wie bei den *Rauwolfia*-Alkaloiden[6] gegeben.

Durch die Arbeiten von VON WARTBURG und Mitarb.[7] ist bekannt, dass auch die Lignane der beiden medizinisch verwendeten *Podophyllum*-Arten ursprünglich in glycosidischer Bindung vorliegen. Sie werden vor allem bei der Bereitung des Harzes (Podophyllin) zerlegt. Die harzreiche, nicht im DAB.6. offizinelle, indische Droge (*Podophyllum emodi* Wall.) enthält im Gegensatz zum offizinellen Podophyllin (*P. peltatum* L. aus U.S.A.) kein α- und β-Peltatin, sondern 4'-Demethylpodophyllotoxin[7]. Hierauf lässt sich eine einfache Unterscheidung der morphologisch sehr ähnlichen Wurzeldrogen und auch der Podophylline[8] aufbauen.

Eine Trennung der Lignanglycoside und der Aglukone erreichten wir mit der

* Die Herstellung erfolgte mit Kieselgel G "Merck" und der Desaga-Grundausrüstung zur Dünnschicht-Chromatographie Nr. 600 nach der Arbeitsvorschrift von E. STAHL.

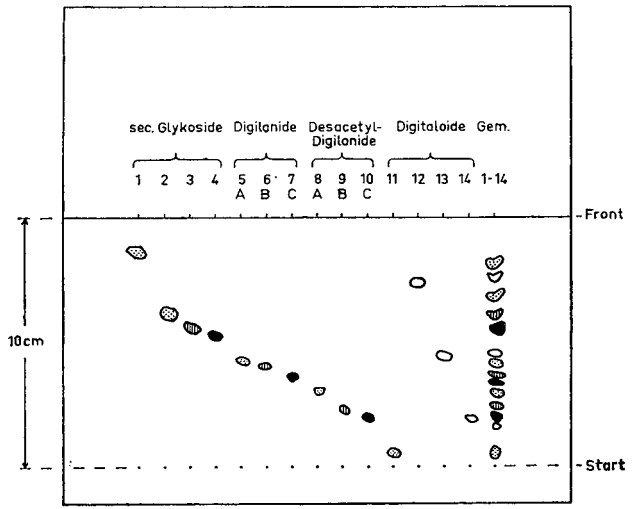

Fig. 1. Dünnschicht-Chromatogramm von Herzglycosiden (je 1 μg) auf einer Kieselgel G-Schicht. (KÜS)[9]. 1. Acetyldigitoxin; 2. Digitoxin; 3. Gitoxin; 4. Digoxin; 5. Digilanid A; 6. Digilanid B; 7. Digilanid C; 8. Desacetyldigilanid A; 9. Desacetyldigilanid B; 10. Desacetyldigilanid C; 11. k-Strophantosid; 12. Cymarin; 13. Proscillaridin A; 14. Scillaren A. G-Gemisch aus gleichen Teilen der Substanzen 1–14. Sichtbarmachung: Trichloressigsäure–Chloramin (15 + 1), 10 Min. 110°. Im langwelligen UV-Licht: Fluoreszenz hellgelb = ▯, braungelb = ⁞⁞, hellblau = ||||, violettblau = ■.

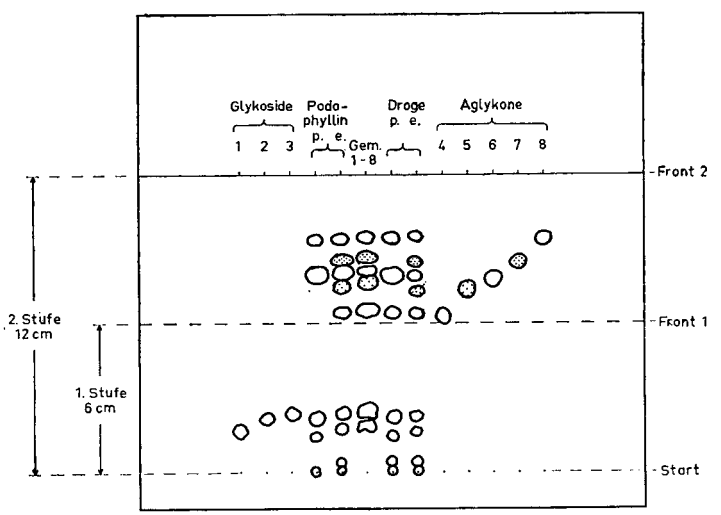

Fig. 2. Dünnschicht-Chromatogramm der *Podophyllum*-Inhaltsstoffe (Kieselgel G; Stufentechnik). 1. α-Peltatin-β-glucosid; 2. Podophyllotoxin-glucosid; 3. β-Peltatin-β-D-glucosid; 4. 4′-Demethylpodophyllotoxin; 5. α-Peltatin; 6. Podophyllotoxin; 7. β-Peltatin; 8. 1-Deshydroxy-podophyllotoxin. Die punktiert gezeichneten Zonen reagieren zusätzlich mit Diazoniumsalzen unter Farbbildung (e. = emodi, p. = peltatum). Von den Reinsubstanzen wurde je 1.0 μg, von den Podophyllinen und der Droge wurde 0.5 mm³ der 10 %igen alkoholischen Auszüge aufgetragen (KÜS).

bereits früher beschriebenen Stufentechnik[9]. Das in der 1. Stufe verwendete Gemisch (Chloroform + 10 % Methanol) trennt die Glycoside. In der 2. Stufe werden mit Chloroform + 35 % Azeton die Aglukone getrennt (Fig. 2). Zwischen beiden Stufen wurde in 5 Minuten mit einem Kaltluftstrom das 1. Elutionsmittel entfernt. Die Trennzeit betrug etwa 15 + 45 Minuten. Zur Sichtbarmachung wurde eine Mischung von konz. Schwefelsäure + Essigsäureanhydrid (1 + 3) aufgesprüht und 15 Minuten auf 100° erhitzt. Die Substanzen traten danach als rote bzw. grauviolette Zonen hervor. Die untere Erfassungsgrenze liegt bei 0.3 μg. Die Fig. 2 lässt erkennen, dass auf diesem Wege eine Unterscheidung der beiden *Podophyllum*-Arten und der hieraus gewonnenen amorphen Harze gut gelingt. Ferner zeigt das Chromatogramm, dass auch im Harz noch kleine Mengen der entsprechenden Lignanglycoside enthalten sind. Abschliessend darf noch erwähnt werden, dass sich die bei der Hydrolyse anfallenden Zucker auf gepufferten Kieselgur G-Schichten in 30 Minuten trennen und bis 0.05 μg nachweisen lassen[10].

Für die grosszügige Überlassung der Reinsubstanzen sind wir der Sandoz A. G. (Basel), insbesondere Herrn Dr. A. HOFMANN, sehr dankbar.

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1-METHYL-5-(2-METHOXYETHYL)-TETRAZOLE—A STATIONARY PHASE FOR GAS–LIQUID PARTITION CHROMATOGRAPHY

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Recents studies in gas–liquid partition chromatographic separations of hydrocarbon pyrolysis and photolysis products[1] have pointed out the versatility of 1-methyl-5-(2-methoxyethyl)-tetrazole as a stationary phase. Columns with 20–25 wt. % of the tetrazole derivative on 30/60 mesh "Chromosorb" (Johns-Manville Products) have been used over the temperature range of —196° to + 80°. Temperature programming of the columns between these limits permits the effective separation of mixtures of hydrogen, nitrogen, carbon monoxide, and many of the C_1 to C_7 acyclic and cyclic alkanes, alkenes, alkadienes and the lower molecular weight aromatic hydrocarbons in one operation. Of special interest in the studies was the gas–liquid partition chromatography(GLPC) of mixtures containing benzene, b.p. 80.1°; 1,3-cyclohexadiene, b.p. 80.5°; cyclohexene, b.p. 83.3°; and cyclohexane, b.p. 80.7°. The chromatogram of a synthetic approximately equimolar mixture of these compounds on the "tetrazole" column is presented in Fig. 1. For comparison, Fig. 2 shows the chromatogram of the

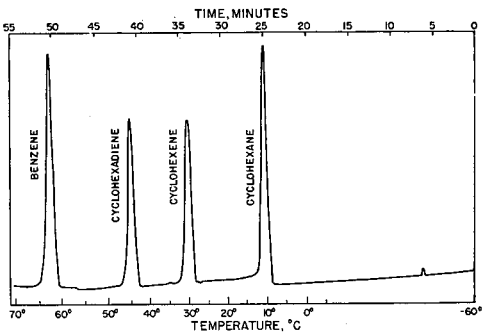

Fig. 1. Gas chromatogram of mixture A: consisting of (a) cyclohexane, (b) cyclohexene, (c) 1,3-cyclohexadiene and (d) benzene. Column T 3: 3/16 in. × 12 ft., 25% 1-methyl-5-(2-methoxyethyl)-tetrazole on 20–40 mesh C 22 brick.

same mixture on a Squalane–"Pelletex" (Godfrey-Cabot Corp., Pampa, Texas) column. No separation occurs on the absorbent type column. The superiority of this new GLPC column for these separations is clearly evident.

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It is noteworthy that, on the tetrazole column, the components of the mixture are separated in order of their increasing unsaturation and essentially in reverse order of their boiling points. Such a behavior indicates complexing of the unsaturated compounds with the stationary phase through weak π bond formation, dipole–dipole interactions or combinations of these. A similar explanation has been advanced by LANGER, ZAHN AND PANTAZOPLOS[2] for the effectiveness of tetrahalophthalate esters

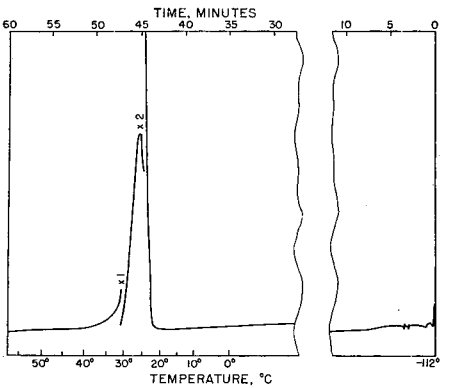

Fig. 2. Gas chromatogram of mixture A on 1.5% squalane on 20–40 mesh Pelletex.

as stationary phases in the separation of aromatic compounds. Supporting evidence for this explanation was found in the infrared spectra of equimolar mixtures of 1-methyl-5-(2-methoxyethyl)-tetrazole with benzene, 1,3-cyclohexadiene and cyclohexene, respectively. Shifts in the absorption maxima averaging 8 cm^{-1} to higher wave numbers for the C–H out of plane bending vibrations in benzene at 675, 1812 and 1950 cm^{-1} are evident. For 1,3-cyclohexadiene and cyclohexene, absorption maxima associated with carbon–carbon double bond stretching frequencies at 1715 and 1695 cm^{-1} and 1650 cm^{-1}, respectively, are shifted 5–10 and 2–5 cm^{-1}, respectively, to lower wave numbers. Shifts of the magnitudes observed correspond roughly to energies of 25 ± 3, 20 ± 1 and 10 ± 1 cal/mole for benzene, 1,3-cyclohexadiene and cyclohexene, respectively, in mixtures with the tetrazole derivative. They are proportional to the net decrease in bond order resulting from dissociation of the olefin–olefin complexes and formation of the olefin–tetrazole complexes.

SHABTAI, HERLING AND GIL-AV[3] have shown that retention volumes using a silver nitrate-saturated glycol stationary phase increase with increasing asymmetry of isomeric olefins and conclude that, for this system, complexing is of major importance but that the polarity of the stationary phase also plays a role in the separation of olefins of similar boiling points. By analogy with other 1,5-disubstituted tetrazoles whose dipole moments range from 5.30 to 7.36 Debye units[4], 1-methyl-5-(2-methoxyethyl)-tetrazole is undoubtedly strongly polar. Confirmatory evidence is shown by the unusually high boiling point (70–80° at 1 μ pressure) for a compound of its low molecular weight (143.17).

The upper temperature limit of this material as a stationary phase is about 80–90°.

Slight decomposition of the tetrazole occurs above this temperature and the chromatographic baseline changes due to the elution of the decomposition products from the column.

Most GLPC columns are quite ineffective when used below the freezing point of the liquid stationary phase. Since the frozen stationary phase does not retain the sample, the column is almost completely inert. The utility of 1-methyl-5-(2-methoxyethyl)-tetrazole as a stationary phase at very low temperature is perhaps due to its ability to supercool markedly without crystallizing. Using pure material and seeding a sample at about —20°, crystallization can occasionally be induced to occur. In general, however, samples will remain liquid although viscous at —50° and merely glass when cooled to liquid nitrogen temperatures.

Elution times or retention volumes are not routinely measured in this laboratory since temperature programming is employed. No effort has been made to make the temperature programming sufficiently linear or reproducible to use elution temperatures since mass spectrometer and infrared identification are the preferred techniques.

Fig. 3 points out the separation for one C_5 and eight C_6 cyclic and acyclic alkanes, alkenes, alkadienes and benzene on an 8 ft. 3/16 in. O.D. column.

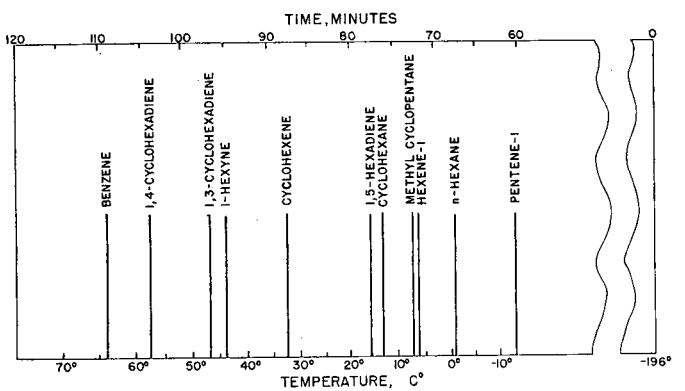

Fig. 3. Separation of one C_5 and eight C_6 cyclic and acyclic alkanes, alkenes, alkadienes and benzene on 1-methyl-5-(2-methoxyethyl)-tetrazole.

A sample of high octane aviation gasoline has been analyzed and the chromatogram is shown in Fig. 4. No attempt was made to identify the individual components.

The initial use of the column was in the study of the pyrolysis of cyclohexene. The pyrolysis was investigated over the temperature range 420–535° to study the mechanism and the energy of activation of the decomposition. The amount of decomposition was determined by the complete product analysis of the sample. The decompositions were limited to a few per cent; therefore, mass spectrometer analysis of the total samples was difficult since the mass spectrum of cyclohexene overlapped the mass spectra of most of the products. Although the absorption columns initially used (Squalane on Pelletex) were able to separate all the products up to the C_4 range, they failed in the separation of the C_5, C_6 and C_7 isomers.

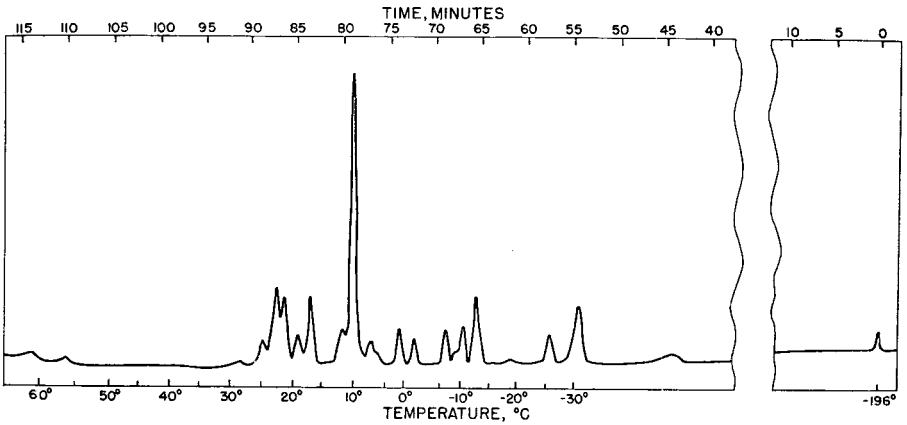

Fig. 4. Gas chromatogram of high octane aviation gasoline. Column T 3: 3/16 in. × 12 ft., 25% 1-methyl-5-(2-methoxyethyl)-tetrazole on 20–40 mesh C 22 brick.

The 8 ft. 3/16 in. column of 20% 1-methyl-5-(2-methoxyethyl)-tetrazole on "Chromosorb" separated all the reaction products completely with the exception of a few of the minor products. A chromatogram that has been run to show the products is shown in Fig. 5.

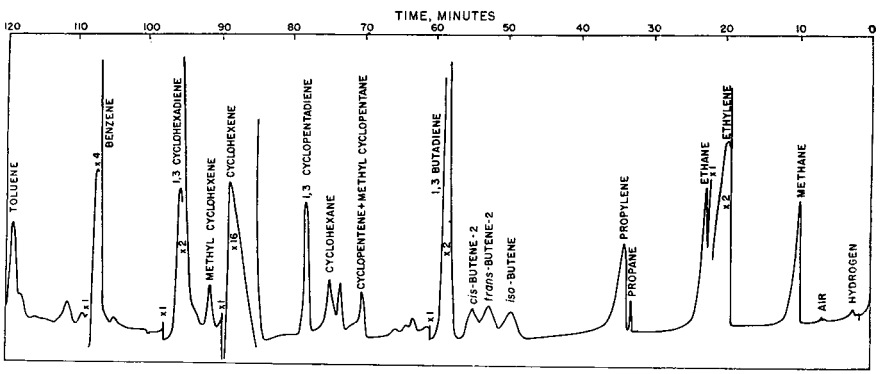

Fig. 5. Gas chromatogram of a partially pyrolyzed sample of cyclohexene on 1-methyl-5-(2-methoxyethyl)-tetrazole.

EXPERIMENTAL

Apparatus and procedure

The columns have been prepared in the standard manner by dissolving the 1-methyl-5-(2-methoxyethyl)-tetrazole in methylene chloride in contact with the "Chromosorb" and evaporating to dryness. The material was then loaded into copper tubing and wound into a 2 in. O.D. coil on a mandril. The apparatus and the temperature programming technique used have been previously outlined[5]. The method of identification of the peaks by capturing the effluent gas of the column and subsequent mass spectrometer analysis have also been discussed[6,7].

Materials

1-Methyl-5-(2-methoxyethyl)-tetrazole. 5-(2-Methoxyethyl)-tetrazole[8] (64 g, 0.5 mole) was methylated in aqueous basic solution with dimethylsulfate at 30–40° following a previously described procedure[9]. After completion of reaction, the solution was cooled to 20° and extracted with four 50-ml portions of methylene chloride. The methylene chloride solution was dried with magnesium sulfate and evaporated on a steam bath leaving an oily product residue. Distillation of this residue gave 29.85 g (42.0 %) of 2-methyl-5-(2-methoxyethyl)-tetrazole, b.p. 60° at 0.2 mm

Anal. calcd. for $C_5H_{10}ON_4$: C, 42.24; H, 7.09; N, 39.41
Found: C, 42.13; H, 7.22; N, 39.48

and 33.70 g (47.5 %) of 1-methyl-5-(2-methoxyethyl)-tetrazole, b.p. 70–80° at 1 μ (Hickman Molecular Still)

Anal. calcd. for $C_5H_{10}ON_4$: C, 42.24; H, 7.09; N, 39.41
Found: C, 41.97; H, 6.89; N, 39.59, 39.77.

ACKNOWLEDGEMENTS

We are indebted to Prof. HANS B. JONASSEN, Tulane University, New Orleans, La. for his encouragement and many valuable discussions. The interest and cooperation of Mr. E. M. BENS in the interpretation of chromatographic data is gratefully acknowledged. Mr. DON PERLICH aided in carrying out some of the early evaluation runs on the column material, and Mr. JOSEPH H. JOHNSON and Mrs. HELEN R. YOUNG carried out the mass spectrometer analyses.

SUMMARY

Data are presented to show the versatility of 1-methyl-5-(2-methoxyethyl)-tetrazole as a stationary phase for the gas–liquid partition chromatographic separations of mixtures of hydrogen, nitrogen, carbon monoxide, C_2 to C_6 cyclic and acyclic alkanes, alkenes, alkadienes and benzene. Its complexing properties and strongly polar nature assist markedly in the separation of a series of cyclic C_6-hydrocarbons of increasing unsaturation having similar boiling points and other C_5 and C_6 straight-chain compounds. Previous clean separation of these compounds by gas chromatography has not been reported.

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IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN*

XLI. MITTEILUNG.

PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG DER ALKOHOLE, GLYKOLE, POLYÄTHYLENGLYKOLE, PHENOLE, MERCAPTANE UND AMINE IN FORM IHRER 3,5-DINITROBENZOYLDERIVATE

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(Eingegangen den 12. September 1960)

In einigen vorangehenden Mitteilungen aus unserem Laboratorium[1-6] zeigten wir, dass die aliphatischen Alkohole, Amine, Glykole und Polyäthylenglykole, sowie ihre Monoäther und Chlorhydrinderivate vorteilhaft in Form ihrer 3,5-Dinitrobenzoylderivate chromatographiert werden können. Die Flüchtigkeit dieser Verbindungen und auch der Mangel an empfindlichen Detektionsmethoden zwingen zur Überführung dieser Verbindungen in geeignete Derivate vor der chromatographischen Behandlung. Wir benützen diese Methoden seit mehreren Jahren zur Identifizierung der Spaltungsprodukte organischer Verbindungen, deren Konstitution wir ermitteln, sowie zur Analyse verschiedener technischer Produkte (z.B. Textilhilfsmittel), die gewöhnlich komplizierte Gemische vorstellen. Da das 3,5-Dinitrobenzoylchlorid auch mit Verbindungen vieler anderen Stoffklassen Derivate bilden kann – deren chromatographisches Verhalten uns unbekannt war, was zu Irrtümern bei der Interpretation der Chromatogramme führen könnte – waren wir gezwungen das Verhalten einer möglichst grossen Anzahl von 3,5-Dinitrobenzoylderivaten zu prüfen. Im Verlauf dieser Arbeit gelang es uns nicht nur die nötigen Informationen über das Verhalten aller in Frage kommenden Derivate zu erhalten, sondern auch eine Reihe von Faktoren, die die papierchromatographische Trennung dieser Verbindungen beeinflussen können, aufzuklären. Die erhaltenen Resultate sind in der vorliegenden Mitteilung zusammengefasst.

EXPERIMENTELLER TEIL

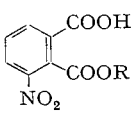
Die Wahl der Derivate

Flüchtige oder unbeständige Verbindungen oder solche Stoffe, bei denen keine empfindliche Detektionsmethode zur Verfügung steht, werden in Form von geeigneten

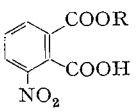
* XL. Mitteilung: *J. Chromatog.*, 5 (1961) 408.

Derivaten chromatographiert[7]. Aus einer grossen Anzahl zu diesem Zweck schon beschriebener Derivate ist es notwendig solche rationell auszuwählen, die die in Folgendem angeführten Eigenschaften besitzen. Vor allem müssen sie solche funktionelle Gruppen enthalten, die eine bequeme und empfindliche Sichtbarmachung der Flecken am Chromatogrammen ermöglichen, bzw. können sie selbst farbig sein oder fluoreszieren. Weiter sollen solche Derivate beständig sein, was auch für ihre Lösungen gelten sollte. Die Reaktion, die zu ihrer Darstellung verwendet wird muss natürlich mit allen in Frage kommenden Verbindungen eindeutig und mit guter Ausbeute verlaufen. Es ist ein grosser Vorteil, wenn die Reaktion eine Isolierung z.B. aus verdünnten wässrigen Lösungen ermöglicht.

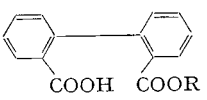
In diesem Sinne wurden für die Papierchromatographie der Alkohole bzw. auch für die Glykole folgende Derivate vorgeschlagen: Xanthogenate (Zusammenfassung siehe[8]), 3,5-Dinitrobenzoate[1,4–6,9–14], 3-Nitrophthalate[15], Diphenate[15], 3,6-Dinitrophthalate[16], oder wurden die Alkohole zu Säuren oxydiert und diese chromatographisch erfasst[17,18]. Auf die Nachteile bei der Anwendung der Xanthogenate zur Papierchromatographie verwiesen wir in der schon zitierten vorangehenden Mitteilung[8]: diese Derivate sind in der Lösung und unter bestimmten Bedingungen auch während der Chromatographie nicht genügend stabil. Die Reaktion mit 3-Nitrophthalanhydrid ist wenig geeignet, da neben den Estern I, die als Hauptprodukt entstehen, auch noch die Ester II gebildet werden, die auf den Chromatogrammen stören. Deshalb wurde die Reaktion mit Diphensäureanhydrid vorgeschlagen, die eindeutig unter Bildung von Estern III verläuft, zur Sichtbarmachung dieser Ester ist man jedoch auf einen Säuren–Basen-Indikator angewiesen. In den letzten Jahren wurden auch farbige Derivate zur Identifizierung und Säulenchromatographie vorgeschlagen



(I)



(II)



(III)

—die Phenylazobenzoate[19] oder *p*-Nitrophenylazobenzoate[20]—die die Frage der Sichtbarmachung überflüssig machen.

Die Phenole können ohne Überführung in Derivate[21] oder in Form der Phenoxyessigsäuren[22] chromatographiert werden. Die aliphatischen Amine werden in Form ihrer Salze[3] oder nach Überführung in 4-Nitrobenzamide[23], bzw. 3,5-Dinitrobenzamide[2,3,24] oder 2,4-Dinitrophenylderivate[25] chromatographiert. Die aromatischen Amine können nur dann als freie Basen chromatographiert werden, wenn sie nicht flüchtig sind; andernfalls werden sie in Bromderivate[26], Azofarbstoffe[27] oder 3,5-Dinitrobenzamide[7] überführt. Für die Mercaptane wurden bisher die 2,4-Dinitrophenylthioäther[28] und weitere Derivate[29–32] vorgeschlagen.

Für unsere Arbeit wählten wir die 3,5-Dinitrobenzoate als vorteilhafteste Derivate aus, da das Reagens, das 3,5-Dinitrobenzoesäurechlorid, nicht nur schnell und eindeutig reagiert, eine gute Sichtbarmachung ermöglicht und als Handels-

produkt zur Verfügung steht, sondern da auch die 3,5-Dinitrobenzoylderivate zur Identifizierung besonders der Alkohole, Glykole und Amine häufig Anwendung finden und eine grosse Anzahl dieser Verbindungen schon als Derivate beschrieben wurden. Für unsere Arbeit war weiter entscheidend, dass das 3,5-Dinitrobenzoylchlorid mit allen erwähnten Verbindungen reagiert, da alle diese Stoffklassen als Bestandteile der von uns analysierten Produkte vorkommen können.

Bei der Reaktion des 3,5-Dinitrobenzoylchlorids werden folgende Typen von Derivaten gebildet (R = Alkyl oder Aryl):

$$R{-}OH + 3,5\text{-}C_6H_3(NO_2)_2CO{-}Cl \longrightarrow 3,5\text{-}C_6H_3(NO_2)_2CO{-}OR$$

$$R{-}NH_2 + 3,5\text{-}C_6H_3(NO_2)_2CO{-}Cl \longrightarrow 3,5\text{-}C_6H_3(NO_2)_2CO{-}NHR$$

$$\begin{matrix} R \\ R \end{matrix}\!\!>\!NH + 3,5\text{-}C_6H_3(NO_2)_2CO{-}Cl \longrightarrow 3,5\text{-}C_6H_3(NO_2)_2CO{-}N\!<\!\begin{matrix} R \\ R \end{matrix}$$

$$R{-}SH + 3,5\text{-}C_6H_3(NO_2)_2CO{-}Cl \longrightarrow 3,5\text{-}C_6H_3(NO_2)_2CO{-}SR$$

Nur im Falle der mehrwertigen Alkohole war der Reaktionsverlauf nicht eindeutig, da sowohl die Monoester IV als auch die Diester V gebildet wurden, was im Kapitel über die Darstellung der Derivate näher diskutiert werden wird.

$$3,5\text{-}C_6H_3(NO_2)_2CO{-}OCH_2{-}CHOH{-}CH_2OH \quad \text{(IV)}$$

$$3,5\text{-}C_6H_3(NO_2)_2CO{-}OCH_2{-}CH(O{-}CO{-}C_6H_3(NO_2)_2\text{-}3,5){-}CH_2OH \quad \text{(V)}$$

Sichtbarmachung der 3,5-Dinitrobenzoylderivate

Zur Sichtbarmachung der farblosen und nicht fluorescierenden Verbindungen auf den Chromatogrammen dient Besprühen mit solchen Reagentien, die Farb- oder Fluorescenzreaktionen hervorrufen, welche empfindlich sein sollen und zu möglichst lange haltbaren Flecken führen sollen. Das Sprühreagens soll leicht darstellbar und lange haltbar sein und die ganze Prozedur soll auf einfachste Weise durchführbar sein.

Zur Sichtbarmachung der 3,5-Dinitrobenzoate wurden bisher folgende Methoden vorgeschlagen:

(1) Das Besprühen mit einer 1-Naphthylamin-[10] oder Rhodamin 6GBN-Lösung[10]; die 3,5-Dinitrobenzoylderivate bewirken das Auslöschen der Fluorescenz dieser Reagentien. Unseren Erfahrungen nach sind diese Methoden nicht sehr empfindlich.

(2) Das Bestrahlen der Chromatogramme, die Formamid oder Dimethylformamid enthalten, mit unfiltriertem Licht der Quarzlampe[1,14], wobei die 3,5-Dinitrobenzoylderivate als violette Flecken erscheinen, die im U.V.-Licht rötlich fluorescieren. Auch diese Methode ist nicht genügend empfindlich und fällt sogar bei manchen Derivaten aus. Ihr grosser Vorteil ist die einfache und schnelle Durchführung der Sichtbarmachung. Die auf solche Weise sichtbar gemachten Chromatogramme können noch mit weiteren Reagentien behandelt werden.

(3) Das Besprühen mit Malonsäurediäthylester und Natronlauge[11] oder mit acetonischer Natronlauge[24]. Diese Methoden haben den Nachteil, dass die Flecken eine begrenzte Zeit haltbar sind.

(4) Das Besprühen mit Zinn(II)-chloridlösung[4], wobei die Dinitroderivate zu den entsprechenden Aminoderivaten reduziert werden, welche durch nachträgliches Besprühen mit einer *p*-Dimethylaminobenzaldehydlösung in die betreffenden Schiffschen Basen übergeführt werden und als gelbe Flecken erscheinen. Diese Methode ist genügend empfindlich; *ca.* 1 μg der Dinitroverbindungen können noch sichtbar gemacht werden und die Flecken sind wochenlang haltbar. Der Nachteil dieser Methode ist, dass sie etwas komplizierter ist, da man nach dem Besprühen mit der Reduktionslösung die Chromatogramme 30–60 Minuten hängen lassen muss, zumal die Reduktion speziell bei manchen Polyäthylenoxyderivaten längere Zeit erfordert. Auch die Zinn(II)-chloridlösung muss nach einigen Tagen frisch bereitet werden. Unseren Erfahrungen nach ist jedoch diese Methode die geeignetste und wir wenden sie schon mehrere Jahre bei Serienarbeiten an.

Die Wahl der Lösungsmittelsysteme

Um das richtige Lösungsmittel zur papierchromatographischen Trennung auszuwählen nehmen wir immer die Löslichkeitsverhältnisse der chromatographierten Verbindungen in Betracht[7]; wir unterscheiden demnach drei Klassen von Lösungsmittelsystemen:

(1) Nicht vorbehandelte Papiere, d.h. Wasser oder besser der Komplex Zellulose–Wasser als stationäre Phase für in Wasser gut lösliche Verbindungen.

(2) Papiere imprägniert mit nicht wässrigen polaren Lösungsmitteln, z.B. Formamid, Dimethylformamid, Propylenglykol, N-Methylformanilid, usw. Als mobile Phase dient ein wenig polares Lösungsmittel, z.B. Hexan, Benzol usw. Diese Systeme sind für semipolare, in Wasser gewöhnlich wenig lösliche oder unlösliche Stoffe geeignet.

(3) Papiere imprägniert mit unpolaren Lösungsmitteln oder wenig polaren, z.B. Paraffinöl, Laurylalkohol, 1-Bromnaphthalin als stationärer Phase. In diesen Systemen werden wenig polare Stoffe chromatographiert.

Es ist notwendig, dass der chromatographierte Stoff in der stationären Phase sehr gut, in der mobilen Phase dagegen mässig oder weniger löslich ist.

Die 3,5-Dinitrobenzoate wurden bisher auf verschiedene Weisen chromatographiert. Wir wollen im Weiteren zeigen, wie den vorangehenden Regeln nach, die Wahl der richtigen und geeignetsten Lösungsmittelsysteme durchgeführt werden soll. Trotzdem die 3,5-Dinitrobenzoate in Wasser praktisch unlöslich sind und sich sehr gut in polaren Lösungsmitteln lösen, versuchten einige Autoren[9] (die Übersicht der bisherigen Methoden siehe Tabelle I) auf nicht vorbehandelten Papieren zu chromatographieren. Selbstverständlich hatte dies wenig Erfolg, da die Derivate sehr hohe R_F-Werte hatten oder Streifen bildeten. Deshalb schlugen weitere Autoren[9,11] das Vorbehandeln der Papiere mit Silikagel vor, d.h. der Verteilungsvorgang wurde mit einer Adsorption kombiniert um die Verminderung der R_F-Werte zu erzielen.

TABELLE I

BISHERIGE METHODEN ZUR PAPIERCHROMATOGRAPHISCHEN TRENNUNG DER 3,5-DINITROBENZOATE DER ALIPHATISCHEN ALKOHOLE

Literatur	RICE, KELLER UND KIRCHNER[9]	MEIGH [10]	ČERNÝ[11]	HUELIN UND KENNETT[12]	MICHEEL UND SCHMINKE[13]	SUNDT UND WINTER[14]	VEČEŘA, GASPARIČ UND SPĚVÁK [1]	BORECKÝ, GASPARIČ UND VEČEŘA[4]
Derivate der	niederen Alkohole	niederen Alkohole	C_1–C_3-Alkohole	C_1–C_{10}-Alkohole	C_1–C_{12}-Alkohole	C_1–C_{12}-Alkohole	C_1–C_4-Alkohole	C_1–C_{18}-Alkohole
Lösungsmittelsysteme	nicht vorbehandeltes Papier, oder Papier imprägn. mit Silikagel. Mobile Phase: wässriges Aceton, Isopropylalkohol oder Pyridin	Methanol/Heptan	Papier imprägniert mit Silikagel. Mobile Phase: Äther + Petroläther	Methanol/Heptan	Acetyliertes Papier. Mobile Phase: Äthylacetat – Dioxan – Wasser 2.0:4.5:4.5	Dimethylformamid/Dekalin	Dimethylformamid/Cyclohexan	Paraffinöl/Dimethylformamid – Methanol – Wasser
Sichtbarmachung	1-Naphthylamin oder 10% KCN	Rhodamin 6GBN	Malonsäurediäthylester + NaOH		1-Naphthylamin oder Benzidin	1-Naphthylamin Rhodamin 6GBN, Bestrahlen mit Quarzlampe	1-Naphthylamin oder Bestrahlen mit Quarzlampe	*p*-Dimethylaminobenzaldehyd nach vorheriger Reduktion mit $SnCl_2$

Eine weitere Verbesserung war das von MEIGH[10] vorgeschlagene Lösungsmittelsystem Methanol–Heptan, in dem schon das Methanol die Rolle einer polaren stationären Phase spielte. Prinzipiell war diese Methode richtig, was auch weitere Autoren[12] bestätigten, nur ist Methanol wegen seiner Flüchtigkeit keine geeignete stationäre Phase, da man die Atmosphäre der Kammer sowie das Papier langwierig sättigen muss. Wir schlugen deshalb Dimethylformamid als stationäre Phase anstatt Methanol vor[1] und erzielten eine gute Auftrennung der Methyl-, Äthyl-, Propyl- und Butylester der 3,5-Dinitrobenzoesäure. Man kann zwar weiter durch Vergrösserung des Imprägnierungsgrades des Papieres die R_F-Werte herabsetzen, doch zeigen die höheren Alkohole in dem System Dimethylformamid/Hexan zu grosse R_F-Werte. Das heisst, dass sie schon in der mobilen Phase (Hexan) zu viel löslich sind. Das ist begreiflich, da durch Verlängerung des Alkylrestes sich die Polarität der Ester um soviel vermindert hat, dass für sie der Kohlenwasserstoff die geeignetere stationäre Phase als Dimethylformamid geworden ist. Deshalb chromatographieren wir die Ester der höheren Alkohole auf mit Paraffinöl imprägnierten Papieren[4] und wenden das Gemisch von Dimethylformamid, Methanol und Wasser als mobile Phase an.

Die 3,5-Dinitrobenzoylderivate der Amine und die betreffenden Ester der mehrwertigen Alkohole werden von Dimethylformamid und Formamid so stark zurückgehalten, dass sie am Start bleiben, wenn Hexan oder Cyclohexan als mobile Phase verwendet wird. Im Falle der mit Formamid imprägnierten Papiere kann man dann die Polarität der mobilen Phase steigern und anstatt Hexan auch von Benzol, Chloroform und Äthylacetat bzw. ihren Gemischen Gebrauch machen. Also durch Änderung der Zusammensetzung des Fliessmittels können wir die R_F-Werte beliebig ändern, um die gewünschten Auftrennungen zu erzielen. Auf diese Weise gelang es uns sukzessiv die homologen Reihen der Alkohole, Glykole, Glykoläther und Chlorhydrine, Polyäthylenglykole, Mercaptane, Amine usw. aufzutrennen. Auch bei Anwendung umgekehrter Phasen, d.h. mit Paraffinöl oder 1-Bromnaphthalin als stationäre Phase war es möglich die R_F-Werte beliebig zu ändern und zwar wieder durch Änderung der Zusammensetzung des Fliessmittels. Die Zugabe von Methanol und Wasser zu Dimethylformamid oder von Wasser zur Essigsäure setzte die R_F-Werte stark herab.

In der Tabelle II sind die Resultate unserer Versuche, sowie alle von uns angewendeten Lösungsmittelsysteme zusammengefasst.

Im Zusammenhang mit dem Problem der besten Auftrennung muss noch einer Tatsache die Aufmerksamkeit gewidmet werden. Von verschiedenen Autoren wird gewöhnlich angenommen, dass zwei Verbindungen aufgetrennt werden, wenn sich ihre R_F-Werte um 0.05 unterscheiden. Im Weiteren wollen wir zeigen, dass diese Ansicht noch einer Ergänzung bedarf. Bei der Papierchromatographie, besonders bei Anwendung der mit organischen Lösungsmitteln vorbehandelten Papiere sind nämlich ganz bemerkenswerte Unterschiede in der Grösse der Flecken in Abhängigkeit von ihrer Lage auf dem Chromatogramm festzustellen. In der Nähe des Startes sind die Flecken klein und scharf abgegrenzt. Je grösser die Strecke die sie aber auf dem Chromatogramm vom Start zurücklegen, umso grösser werden sie durch Diffusion

TABELLE

R_F-WERTE DER 3,5-DINITROBENZOYLDERIVATE IN

Derivate der	*R_F-Werte*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Aliphatischen Alkohole							
Methylalkohol	0.09	0.09	0.19	0.22	0.30	0.33	0.56
Äthylalkohol	0.18	0.16	0.25	0.29	0.58	0.61	0.77
n-Propylalkohol	0.27	0.27	0.47	0.53	0.76	0.78	0.86
Isopropylalkohol	0.31	0.31	0.52	0.58	0.80	0.82	—
n-Butylalkohol	0.37	0.38	0.60	0.64	0.88	0.86	—
Isobutylalkohol	0.41	0.41	0.60	0.65	0.87	0.85	—
sek.-Butylalkohol	0.42	0.42	0.63	0.67	0.90	0.87	—
tert.-Butylalkohol	0.46	0.47	0.68	0.70	0.91	0.88	—
n-Amylalkohol	0.48	0.49	0.75	0.74	0.93	0.90	—
n-Hexylalkohol	0.52	0.57	0.79	0.77	0.94	0.91	—
n-Heptylalkohol	0.61	0.63	0.83	0.81	0.94	0.92	—
n-Octylalkohol	0.70	0.71	0.84	0.85	0.95	0.94	—
2-Octylalkohol	0.75	0.72	0.86	0.87	0.96	0.94	—
n-Nonylalkohol	0.75	0.79	0.87	0.88	0.95	0.94	—
n-Decylalkohol	0.80	0.83	0.90	0.89	0.95	0.94	—
n-Dodecylalkohol	0.90	0.91	0.91	0.90	0.95	0.94	—
n-Tetradecylalkohol	0.92	0.93	0.93	0.92	0.96	0.95	—
n-Hexadecylalkohol	0.93	0.94	0.94	0.93	0.96	0.95	—
n-Octadecylalkohol	0.94	0.95	0.95	0.94	0.96	0.95	—
Cyclcaliphatischen, ungesättigten und heterocyclischen Alkohole							
Cyclopentanol	0.36	0.42	0.60	0.66	0.84	0.85	—
1-Methylcyclopentanol	0.52	0.55	0.70	0.76	0.80	0.90	—
2-Methylcyclopentanol	0.49	0.51	0.69	0.75	0.90	0.90	—
3-Methylcyclopentanol	0.49	0.50	0.69	0.75	0.90	0.90	—
Cyclohexanol	0.51	0.53	0.68	0.74	0.91	0.90	—
Methylcyclohexanol	0.61	0.65	0.77	0.80	0.95	0.92	—
Geraniol	0.70	0.66	0.83	0.84	0.96	0.94	—
akt. Isoborneol	0.64	0.68	0.84	0.85	0.95	0.94	—
l-Menthol	0.77	0.82	0.89	0.90	0.95	0.94	—
Cholesterol	0.95	0.95	0.90	0.91	0.95	0.93	—
Allylalkohol	0.15	0.15	0.30	0.34	0.62	0.65	0.80
Crotylalkohol	0.18	0.18	0.46	0.52	0.80	0.83	—
Undecylenalkohol	0.76	0.82	0.83	0.84	0.95	0.94	—
Oleylalkohol	0.92	0.93	0.90	0.91	0.95	0.94	—
Furfurylalkohol	0.04	0.06	0.12	0.16	0.51	0.57	0.77
Tetrahydrofurfurylalkohol	0.07	0.10	0.15	0.24	0.26	0.29	0.54
Arylalkylalkohole							
Benzylalkohol	0.08	0.12	0.20	0.31	0.72	0.77	0.86
p-Methylbenzylalkohol	0.12	0.16	0.32	0.46	0.80	0.84	—
p-Methoxybenzylalkohol	0.04	0.06	0.13	0.21	0.66	0.71	—
2-Phenyläthylalkohol	0.11	0.13	0.26	0.39	0.76	0.81	—
γ-Phenylpropylalkohol	0.15	0.17	0.35	0.48	0.88	0.87	—

*1 = 50% Dimethylformamid/Hexan.
2 = 50% Dimethylformamid/Cyclohexan.
3 = 25% Dimethylformamid/Hexan.
4 = 25% Dimethylformamid/Cyclohexan.
5 = 20% Formamid/Hexan.
6 = 20% Formamid/Cyclohexan.
7 = 20% Formamid/Hexan–Benzol 4:1.
8 = 20% Formamid/Hexan–Benzol 3:2.
9 = 20% Formamid/Hexan–Benzol 2:3.
10 = 20% Formamid/Hexan–Benzol 1:4.
11 = 20% Formamid/Benzol.
12 = 20% Formamid/Benzol–Chloroform 1:1.
13 = 20% Formamid/Chloroform.

II

VERSCHIEDENEN LÖSUNGSMITTELSYSTEMEN*

R_F-Werte												
8	9	10	11	12	13	14	15	16	17	18	19	20
0.71	—	—	—	—	—	—	—	—	—	0.93	0.17	0.26
0.88	—	—	—	—	—	—	—	—	—	0.92	0.11	0.19
0.92	—	—	—	—	—	—	—	—	—	—	0.06	0.14
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.96	0.91	0.86	0.04	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.94	0.89	0.80	—	0.11
—	—	—	—	—	—	—	—	0.93	0.81	0.69	—	0.07
—	—	—	—	—	—	—	—	—	—	—	—	0.06
—	—	—	—	—	—	—	—	0.82	0.68	0.43	—	0.04
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.75	0.60	0.29	—	—
—	—	—	—	—	—	—	—	0.69	0.51	0.20	—	—
—	—	—	—	—	—	—	—	0.56	0.32	0.07	—	—
—	—	—	—	—	—	—	—	0.41	0.17	0.02	—	—
—	—	—	—	—	—	—	—	0.30	0.07	0.00	—	—
—	—	—	—	—	—	—	—	0.20	0.04	0.00	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.86	0.88	0.77	—	—
—	—	—	—	—	—	—	—	0.95	0.81	0.69	—	—
—	—	—	—	—	—	—	—	0.84	0.73	0.54	—	—
—	—	—	—	—	—	—	—	0.88	0.70	0.47	—	—
—	—	—	—	—	—	—	—	0.77	0.57	S	—	—
—	—	—	—	—	—	—	—	0.09	0.00	0.00	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	0.87	—	—
		—	—	—	—	—	—	0.77	0.67	0.22	—	—
—	—	—	—	—	—	—	—	0.34	0.09	0.01	—	—
—	—	—	—	—	—	—	—	—	—	0.94	0.06	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	0.90	—	0.08
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	0.93	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.97	0.95	0.88	—	—

14 = 20 % Formamid/Chloroform–Äthylacetat 1:1.
15 = 20 % Formamid/Chloroform–Äthylacetat 1:4.
16 = 5 % Paraffinöl/Dimethylformamid–Methanol–Wasser 8:1:1
17 = 5 % Paraffinöl/Dimethylformamid–Methanol–Wasser 4:1:1.
18 = 5 % Paraffinöl/Dimethylformamid–Methanol–Wasser 2:1:1.
19 = 10 % 1-Bromnaphthalin/70 % Essigsäure.
20 = 10 % 1-Bromnaphthalin/90 % Essigsäure.

(*Fortsetzung auf Seite 474*)

TABELLE II

Derivate der	R_F-Werte						
	1	2	3	4	5	6	7
Methylphenylcarbinol	0.14	0.17	0.35	0.48	0.88	0.87	—
Methyläthylphenylcarbinol	0.27	0.32	0.50	0.60	0.90	0.94	—
Cinnamylalkohol	0.07	0.09	0.19	0.29	0.80	0.82	—
Glykole							
Äthylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Äthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	S
1,2-Propylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.02
1,2-Propylenglykol (Diester)	0.00	0.00	0.00	0.01	0.02	0.01	0.27
1,3-Butylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.05
1,3-Butylenglykol (Diester)	0.01	0.01	0.02	0.04	0.03	0.04	0.36
2,3-Butylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.05
2,3-Butylenglykol (Diester)	0.01	0.01	0.03	0.06	0.04	0.07	S
1,4-Butylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.03
1,4-Butylenglykol (Diester)	0.00	0.00	0.01	0.02	0.02	0.03	S
1,6-Hexandiol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.10
1,6-Hexandiol (Diester)	0.01	0.01	0.01	0.02	0.02	S	S
Glyzerin (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glyzerin (Diester)	—	—	—	—	—	—	—
Glyzerin (Triester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Polyäthylenglykole							
Diäthylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.11
Triäthylenglykol (Monoester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Triäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.10
Tetraäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.06
Pentaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.03
Hexaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Heptaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Octaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nonaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dekaäthylenglykol (Diester)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Monoäther der Glykole und Polyäthylenglykole							
Äthylenglykol-mono-methyläther	0.04	0.06	0.14	0.16	0.20	0.26	0.52
Diäthylenglykol-mono-methyläther	0.01	—	0.09	—	0.09	—	0.38
Triäthylenglykol-mono-methyläther	0.00	—	0.07	—	0.03	—	0.22
Äthylenglykol-mono-äthyläther	0.10	0.12	0.26	0.30	0.40	0.41	0.73
Diäthylenglykol-mono-äthyläther	0.05	—	0.15	—	0.20	—	0.56
Triäthylenglykol-mono-äthyläther	0.01	—	0.09	—	0.08	—	0.42
Tetraäthylenglykol-mono-äthyläther	0.00	—	0.07	—	0.02	—	0.24
Äthylenglykol-mono-*n*-butyläther	0.17	0.26	0.49	0.55	0.76	0.80	0.89
Diäthylenglykol-mono-*n*-butyläther	0.13	0.22	0.39	0.49	0.60	0.70	0.82
Triäthylenglykol-mono-*n*-butyläther	0.08	0.17	0.30	0.42	0.35	0.50	0.72
Tetraäthylenglykol-mono-*n*-butyläther	0.04	0.13	0.21	0.32	0.20	0.34	0.57
Pentaäthylenglykol-mono-*n*-butyläther	0.02	0.09	0.13	0.20	0.08	0.16	0.41
Hexaäthylenglykol-mono-*n*-butyläther	0.01	0.06	0.09	0.16	0.04	0.08	0.25
Heptaäthylenglykol-mono-*n*-butyläther	0.01	0.02	0.07	0.11	0.02	0.03	0.14
Äthylenglykol-mono-(2-äthylbutyl)-äther	0.49	0.51	0.67	0.74	0.95	0.92	—
Diäthylenglykol-mono-(2-äthylbutyl)-äther	0.40	0.44	0.61	0.65	0.88	0.87	—
Triäthylenglykol-mono-(2-äthylbutyl)-äther	0.30	0.37	0.53	0.60	0.76	0.78	—
Tetraäthylenglykol-mono-(2-äthylbutyl)-äther	0.18	0.27	0.43	0.55	0.59	0.61	0.85
Pentaäthylenglykol-mono-(2-äthylbutyl)-äther	0.10	0.17	0.32	0.46	0.35	0.45	0.73
Hexaäthylenglykol-mono-(2-äthylbutyl)-äther	0.05	0.10	0.21	0.34	0.18	0.26	0.52

(*Fortsetzung*)

*R*F-Werte												
8	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.96	0.96	0.90	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
0.03	0.07	0.13	0.25	0.33	0.50	0.69	0.79	—	—	—	0.79	0.75
S	0.80	0.93	—	—	—	—	—	—	—	—	0.01	0.08
0.07	0.18	0.32	0.44	0.54	0.71	0.79	—	—	—	—	0.69	0.68
0.68	0.87	0.96	—	—	—	—	—	—	—	—	—	—
0.17	0.38	0.54	0.65	0.74	0.85	0.84	—	—	—	—	—	0.60
0.75	0.90	0.96	—	—	—	—	—	—	—	—	—	—
0.17	0.37	0.52	0.63	0.73	0.84	0.85	—	—	—	—	—	—
0.78	0.95	0.97	—	—	—	—	—	—	—	—	—	—
0.11	0.28	0.44	0.57	0.68	0.80	0.81	—	—	—	—	—	—
0.68	0.80	0.96	—	—	—	—	—	—	—	—	—	—
0.29	0.57	0.72	0.78	0.87	0.89	—	—	—	—	—	—	—
S	0.97	0.98	—	—	—	—	—	—	—	—	—	—
0.00	0.00	0.00	0.00	0.00	0.07	0.33	0.53	—	—	—	—	—
—	0.06	0.24	0.43	0.46	0.66	0.85	0.90	—	—	—	—	—
S	S	0.80	0.89	0.93	0.94	0.94	0.98	—	—	—	—	—
0.03	0.09	0.23	0.37	0.52	0.72	0.70	0.75	—	—	—	0.74	0.76
0.51	0.80	—	—	—	—	—	—	—	—	—	0.01	0.08
0.03	0.11	0.26	0.40	0.64	0.82	0.70	0.72	—	—	—	—	—
0.48	0.79	—	—	—	—	—	—	—	—	—	—	—
0.39	0.76	—	—	—	—	—	—	—	—	—	—	—
0.28	0.68	—	—	—	—	—	—	—	—	—	—	—
0.19	0.58	—	—	—	—	—	—	—	—	—	—	—
0.13	0.47	—	—	—	—	—	—	—	—	—	—	—
0.08	0.35	—	—	—	—	—	—	—	—	—	—	—
0.00	0.24	—	—	—	—	—	—	—	—	—	—	—
0.00	0.14	—	—	—	—	—	—	—	—	—	—	—
0.76	—	—	—	—	—	—	—	—	—	—	—	0.38
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
0.86	—	—	—	—	—	—	—	—	—	—	0.19	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.95	0.90	0.84	0.05	0.19
—	—	—	—	—	—	—	—	—	—	—	0.06	0.24
—	—	—	—	—	—	—	—	—	—	—	0.08	0.30
0.80	—	—	—	—	—	—	—	—	—	—	—	—
0.71	—	—	—	—	—	—	—	—	—	0.95	—	—
0.56	—	—	—	—	—	—	—	—	—	0.95	—	—
0.40	—	—	—	—	—	—	—	—	—	0.95	—	—
—	—	—	—	—	—	—	—	0.95	0.90	0.83	0.02	0.09
—	—	—	—	—	—	—	—	0.95	0.94	0.90	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—

(*Fortsetzung auf Seite 476*)

TABELLE II

Derivate der	*R_F-Werte*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Äthylenglykol-mono-*n*-dodecyläther	0.84	0.95	0.84	0.85	0.95	0.90	—
Diäthylenglykol-mono-*n*-dodecyläther	0.81	0.90	0.70	0.74	0.90	0.82	—
Triäthylenglykol-mono-*n*-dodecyläther	—	—	—	—	—	—	—
Tetraäthylenglykol-mono-*n*-dodecyläther	—	—	—	—	—	—	—
Chlorhydrine							
Äthylenchlorhydrin	0.02	0.04	0.05	0.12	0.24	0.31	0.61
1,2-Propylenchlorhydrin	0.07	0.09	0.21	0.29	0.56	0.61	0.72
α-Dichlorhydrin	0.02	0.05	0.13	0.20	0.43	0.47	0.80
Phenole							
Phenol	0.05	0.06	0.13	0.20	S	S	0.76
o-Kresol	0.09	0.11	0.22	0.31	0.69	0.75	—
m-Kresol	0.09	0.10	0.22	0.30	S	S	—
p-Kresol	0.08	0.09	0.22	0.30	S	S	—
m-Äthylphenol	0.14	0.15	0.32	0.40	0.82	0.84	—
p-tert.-Butylphenol	0.22	0.29	0.48	0.54	0.90	0.92	—
1-Naphthol	0.02	0.04	0.10	0.16	S	S	—
2-Naphthol	0.02	0.04	0.10	0.16	S	0.45	—
Mercaptane, Thiophenole und ihre Derivate							
Äthylmercaptan	0.24	0.22	0.43	0.49	0.75	0.78	—
n-Butylmercaptan	0.46	0.47	0.67	0.69	0.93	0.91	—
n-Hexylmercaptan	0.62	0.66	0.80	0.79	0.95	0.93	—
n-Dodecylmercaptan	0.94	0.93	0.90	0.90	0.97	0.96	—
Äthylenglykol-mono-*n*-dodecylthioäther	—	—	—	—	—	—	—
Diäthylenglykol-mono-*n*-dodecylthioäther	—	—	—	—	—	—	—
Triäthylenglykol-mono-*n*-dodecylthioäther	—	—	—	—	—	—	—
Benzylmercaptan	0.14	0.16	0.28	0.39	0.84	0.87	—
Thiophenol	0.08	0.12	0.17	0.26	0.70	S	—
o-Thiokresol	0.10	0.13	—	—	0.80	0.84	—
p-Thiokresol	0.11	0.15	—	0.39	S	S	—
Primäre aliphatische Amine							
Ammoniak	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methylamin	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Äthylamin	0.01	0.02	0.01	0.04	0.00	0.00	0.00
n-Propylamin	0.02	0.04	0.02	0.07	0.00	0.01	0.00
n-Butylamin	0.04	0.06	0.07	0.13	0.02	0.03	0.03
Isobutylamin	0.04	0.06	0.08	0.15	0.02	0.03	0.03
sek.-Butylamin	0.05	0.07	0.10	0.17	0.02	0.03	0.04
tert.-Butylamin	0.10	0.12	0.20	0.27	0.06	0.07	0.10
n-Amylamin	0.08	0.10	0.15	0.24	0.04	0.06	0.09
Isoamylamin	0.09	0.11	0.17	0.25	0.04	0.06	0.08
n-Hexylamin	0.12	0.14	0.23	0.30	0.07	0.10	0.19
n-Octylamin	0.23	0.28	0.37	0.54	S	S	0.39
n-Decylamin	0.41	0.46	0.54	0.70	S	S	S
n-Dodecylamin	0.60	0.65	0.65	0.78	S	S	S
n-Tetradecylamin	—	—	—	—	S	S	—
n-Hexadecylamin	0.82	0.85	0.73	0.83	S	S	—
n-Octadecylamin	0.89	0.88	0.75	0.84	S	S	—
Sekundäre aliphatische Amine							
Dimethylamin	0.00	0.01	0.01	0.01	0.00	0.00	0.06

(*Fortsetzung*)

R_F-Werte												
8	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
—	—	—	—	—	—	—	—	0.49	0.47	0.07	—	—
—	—	—	—	—	—	—	—	0.65	0.57	0.18	—	—
—	—	—	—	—	—	—	—	—	0.67	0.26	—	—
—	—	—	—	—	—	—	—	—	0.76	—	—	—
0.81	—	—	—	—	—	—	—	—	—	—	0.10	0.29
—	—	—	—	—	—	—	—	—	—	0.95	0.07	—
—	—	—	—	—	—	—	—	—	—	0.95	0.06	0.21
—	—	—	—	—	—	—	—	0.96	—	0.93	0.03	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	0.98	—	—	—	—
—	—	—	—	—	—	—	—	—	—	0.78	—	—
—	—	—	—	—	—	—	—	—	—	—	0.01	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	0.89	—	0.12
—	—	—	—	—	—	—	—	0.94	0.85	0.73	—	0.11
—	—	—	—	—	—	—	—	0.85	0.70	0.51	—	0.07
—	—	—	—	—	—	—	—	0.49	0.18	0.01	—	—
—	—	—	—	—	—	—	—	—	0.38	—	—	—
—	—	—	—	—	—	—	—	—	0.53	—	—	—
—	—	—	—	—	—	—	—	—	0.62	—	—	—
—	—	—	—	—	—	—	—	—	—	0.88	—	—
—	—	—	—	—	—	—	—	—	—	—	—	0.08
—	—	—	—	—	—	—	—	—	—	0.88	—	0.06
—	—	—	—	—	—	—	—	—	—	0.85	—	0.05
0.00	0.00	0.00	0.03	0.04	0.08	0.47	0.66	—	0.91	0.88	—	0.70
0.00	0.04	0.08	0.19	0.28	0.43	0.72	0.79	—	—	—	0.80	0.74
0.04	0.12	0.22	0.35	0.49	0.63	0.83	—	—	—	—	0.77	0.71
0.09	0.22	0.38	0.51	0.65	0.76	—	—	—	—	—	0.69	0.68
0.18	0.37	0.56	0.66	0.77	0.85	—	—	—	—	—	0.60	—
0.17	0.34	0.52	0.63	—	—	—	—	—	—	—	—	0.66
0.15	0.34	0.51	0.65	—	—	—	—	—	—	—	—	—
0.30	0.50	0.67	0.71	—	—	—	—	—	—	—	—	—
0.29	0.50	0.68	0.75	0.85	—	—	—	—	—	—	—	—
0.26	0.46	0.66	—	—	—	—	—	—	—	—	0.45	0.62
0.47	0.68	0.81	0.85	—	—	—	—	0.99	0.98	0.96	0.33	0.56
0.69	0.84	—	—	—	—	—	—	0.98	0.98	0.95	0.17	0.44
—	—	—	—	—	—	—	—	0.95	0.95	0.89	0.10	0.32
—	—	—	—	—	—	—	—	0.93	0.89	S	0.05	0.19
—	—	—	—	—	—	—	—	—	—	—	0.02	0.11
—	—	—	—	—	—	—	—	0.77	0.53	S	—	0.06
—	—	—	—	—	—	—	—	0.62	0.31	S	—	0.03
0.18	0.45	0.60	0.67	0.82	0.88	—	—	—	—	0.92	0.72	0.74

(*Fortsetzung auf Seite 478*)

TABELLE II

Derivate der	R_F-*Werte*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Diäthylamin	0.03	0.06	0.08	0.14	0.08	0.13	0.30
Di-*n*-propylamin	0.08	0.09	0.19	0.22	0.29	0.33	0.58
Di-*n*-butylamin	0.25	0.31	0.47	0.53	0.72	0.77	0.85
Andere Amine							
Anilin	0.00	0.01	0.03	0.07	0.00	0.02	0.02
N-Methylanilin	0.03	0.04	0.07	0.14	0.18	0.24	0.49
N-Äthylanilin	0.05	0.08	0.17	0.23	0.41	0.51	0.70
o-Toluidin	0.02	0.02	0.03	0.05	0.00	0.02	0.00
m-Toluidin	0.02	0.03	0.04	0.08	0.01	0.03	0.00
p-Toluidin	0.02	0.03	0.04	0.08	0.01	0.04	0.00
1-Naphthylamin	0.16	0.18	0.28	0.35	0.55	0.61	—
Diphenylamin	0.03	0.04	0.07	0.14	S	S	S
Benzylamin	0.00	0.01	0.01	0.05	0.00	0.00	0.00
Cyclohexylamin	0.05	0.11	0.15	0.23	0.03	0.04	S
Dibenzyläthanolamin	0.24	0.29	0.52	0.58	0.94	0.94	—
3,5-Dinitrobenzoesäure	0.00	0.00	0.00	0.00	0.00	0.00	0.00

nach allen Richtungen hin, dabei aber auch rundlicher, jedoch nicht mehr so scharf abgegrenzt. Es ist dann selbstverständlich, dass in der Nähe des Starts kleinere Unterschiede der R_F-Werte genügen um eine Auftrennung schon möglich zu machen; dagegen sind in der unteren Hälfte des absteigenden Chromatogramms grössere Unterschiede in den R_F-Werten erforderlich.

Weiter muss in Betracht genommen werden, dass die Wirksamkeit der chromatographischen Auftrennung der ganzen Länge des Chromatogramms entlang wieder nicht die gleiche ist. Am wirksamsten werden zwei Flecken in der Nähe der Mitte des Chromatogramms getrennt; die Trennfähigkeit sinkt, wenn sich die Flecken dem Start oder der Lösungsmittelfront nähren. Diese Gesetzmässigkeit kann folgendem Beispiel entnommen werden: Wir chromatographierten die *n*-Butyl- und *n*-Amyl-3,5-dinitrobenzamide in Systemen mit Formamid als stationäre Phase und änderten die mobile Phase (Hexan, Benzol, Chloroform und ihre Gemische) in der Weise, dass die beiden Verbindungen beliebig hohe R_F-Werte aufwiesen. Die Resultate, die in der Tabelle III zusammengefasst sind, zeigen, dass beide Verbindungen in der Nähe des Starts praktisch nicht getrennt werden, mit wachsenden R_F-Werten jedoch vergrössern sich auch die Unterschiede ihrer R_F-Werte (ΔR_F) bis zu $\Delta R_F = 0.17$ in der Hälfte des Chromatogramms, um sich in der Richtung zur Lösungsmittelfront wieder zu vermindern. Die Resultate aus der Tabelle III sind in der Fig. 1 graphisch dargestellt.

Dass diese Gesetzmässigkeit auch für andere Lösungsmittelsysteme gilt bewiesen wir dadurch, dass wir auf ähnliche Weise die Resultate der Trennung der höheren aliphatischen 3,5-Dinitrobenzoate auf mit Paraffinöl imprägnierten Papieren graphisch verarbeiteten (siehe Fig. 2) und dieselben Ergebnisse erhielten. In diesem Falle handelte es sich sogar um Unterschiede in den R_F-Werten einzelner Glieder

(Fortsetzung)

R_F-Werte												
8	9	10	11	12	13	14	15	16	17	18	19	20
0.57	0.76	0.85	—	—	—	—	—	—	—	—	0.54	0.60
0.77	0.89	0.93	—	—	—	—	—	—	—	0.95	—	0.49
—	—	—	—	—	—	—	—	—	—	0.97	—	—
0.08	0.26	0.45	0.58	0.65	0.71	0.91	—	—	—	—	0.51	0.56
0.76	0.90	—	—	—	—	—	—	—	—	—	—	—
0.86	—	—	—	—	—	—	—	—	—	0.96	—	—
S	S	S	S	S	S	—	—	—	—	—	S	0.55
S	S	S	S	S	S	—	—	—	—	—	S	0.52
S	S	S	S	S	S	—	—	—	—	—	S	S
—	—	—	—	—	—	—	—	—	—	0.94	—	—
—	—	—	—	—	—	—	—	—	—	0.93	0.15	0.32
S	S	S	—	—	—	—	—	—	—	0.91	0.51	0.65
S	S	S	—	—	—	—	—	—	—	0.95	0.46	0.65
—	—	—	—	—	—	—	—	0.96	0.94	0.80	—	—
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	—	0.90	0.89	0.66	0.64

einer homologen Reihe unter verschiedenen Bedingungen (verschiedener Imprägnierungsgrad des Papiers) und auch diesmal zeigte sich, dass am besten diejenigen zwei Verbindungen getrennt werden, die sich gerade in der Hälfte des Chromatogramms befinden.

TABELLE III

R_F-WERTE-UNTERSCHIEDE DES *n*-BUTYL- UND *n*-AMYL-3,5-DINITROBENZAMIDS IN VERSCHIEDENEN LÖSUNGSMITTELSYSTEMEN

Papier imprägniert mit Formamid

Mobile Phase		R_F-*Wert des*		ΔR_F*	R_F'**
		n-Butylamins	*n-Amylamins*		
Hexan		0.01	0.02	0.01	0.01
Hexan–Benzol	9:1	0.02	0.07	0.05	0.045
	8:2	0.11	0.20	0.09	0.155
	7:3	0.19	0.32	0.13	0.255
	6:4	0.28	0.42	0.14	0.345
	5:5	0.31	0.47	0.16	0.39
	4:6	0.31	0.48	0.17	0.395
	3:7	0.42	0.58	0.16	0.50
	2:8	0.48	0.62	0.14	0.545
	1:9	0.57	0.70	0.13	0.635
Benzol		0.70	0.80	0.10	0.75
Benzol–Chloroform	9:1	0.76	0.85	0.09	0.805
	8:2	0.78	0.87	0.09	0.825

* $\Delta R_F = R_F(\mathrm{AmNH_2}) - R_F(\mathrm{BuNH_2})$.

** $R_F' = \dfrac{R_F(\mathrm{BuNH_2}) + R_F(\mathrm{AmNH_2})}{2}$.

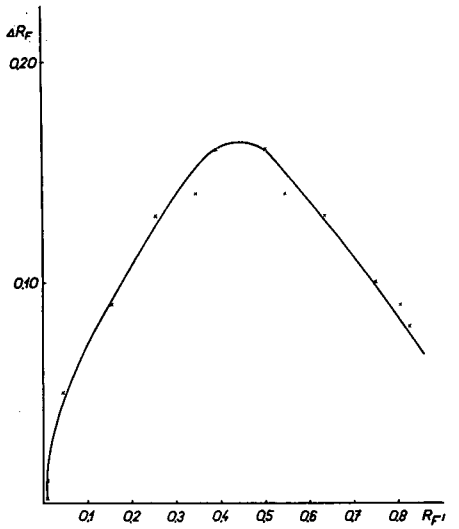


Fig. 1. Graphische Darstellung der Trennfähigkeit entlang der ganzen Laufstrecke. ΔR_F und R_F' wurden der Tabelle III entnomen.

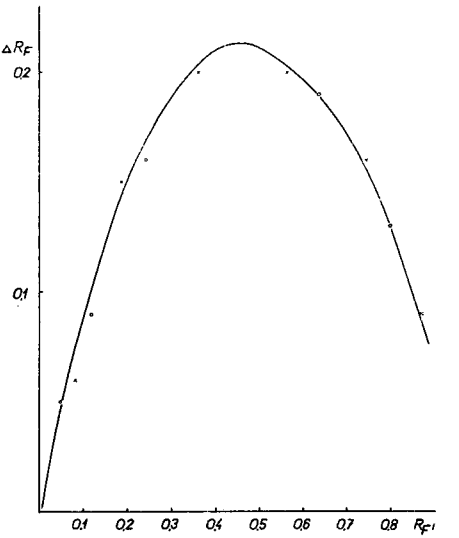


Fig. 2. Graphische Darstellung der Trennfähigkeit entlang der ganzen Laufstrecke. Chromatographiert wurden die höheren Alkyl-3,5-dinitrobenzoate. ΔR_F und R_F' wurden von den Angaben aus der Fig. 5 ausgerechnet. (×) System 5% Paraffinöl/Dimethylformamid–Methanol–Wasser 4:1:1; (○) System 10% Paraffinöl/Dimethylformamid–Methanol–Wasser 4:1:1.

Die Imprägnierung der Papiere

Die Resultate unserer Studien sowie die Erfahrungen anderer Autoren (z.B.[33]) und Privatmitteilungen einer Reihe von Kollegen zwangen uns die Verhältnisse bei der Papierchromatographie auf vorbehandelten Papieren zu untersuchen. Es wurde schon mehrere Male in der Literatur darauf hingewiesen, dass die Schwankungen auf den vorbehandelten Papieren grösser sind, als man gewöhnlich für nicht imprägnierte Papiere angibt, d.h. $\pm$ 0.02. Auch wir machten solche Erfahrungen im Laufe einiger Jahre und fanden, dass dieses Problem etwas komplizierter ist, als es auf ersten Blick zu sein scheint.

Aus den im Folgenden angeführten Ergebnissen ist ersichtlich, dass die absolute Menge der stationären Phase im Papier einen entscheidenden Einfluss auf die Qualität der Chromatogramme und auf die R_F-Werte hat. Man imprägniert die Papiere mit einer Lösung der stationären Phase in einem flüchtigen Lösungsmittel und kann dann den Imprägnierungsgrad z.B. durch Änderung der Konzentration der Imprägnierungslösung einstellen. Aus Fig. 4 und 5 ist ersichtlich, wie die R_F-Werte und die Trennung durch den Imprägnierungsgrad beeinflusst werden: In allen Fällen, die uns bekannt sind, werden die R_F-Werte mit zunehmendem Imprägnierungsgrad vermindert. Enthält das Papier zu wenig stationäre Phase, so werden Streifen gebildet.

Der Imprägnierungsvorgang kann auf verschiedene Weise durchgeführt werden, was auch seine Folgen auf die Qualität der Chromatogramme hat. Prinzipiell werden zweierlei Methoden angewendet:

Methode A[34]. Die Papierstreifen werden in die Imprägnierungslösung eingetaucht oder durch diese durchgezogen und der Überschuss an Imprägnierungslösung durch Abpressen zwischen Filtrierpapieren beseitigt; das Papier wird dann bei Zimmertemperatur an der Luft aufgehängt, damit das flüchtige Lösungsmittel verdampfen kann. Wir imprägnierten auf diese Weise 13 cm breite und 45 cm lange Filtrierpapierstreifen und nach Verdunsten des flüchtigen Lösungsmittels zerschnitten wir das Papier auf 7 cm breite Streifen; durch Wiegen bestimmten wir den Gehalt an stationärer Phase. Die Imprägnierung (siehe Fig. 3a) des Papieres war zwar relativ

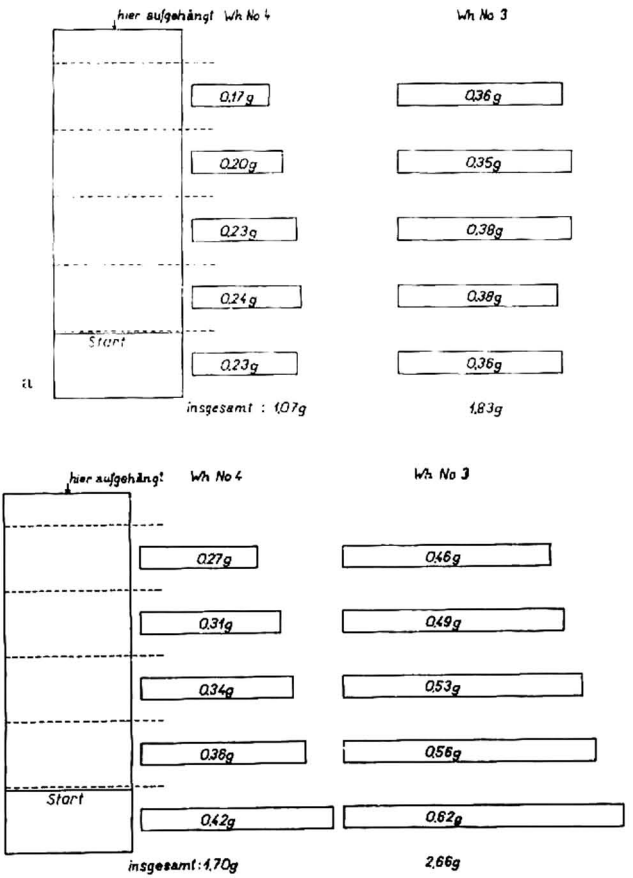


Fig. 3. Die Abhängigkeit der Menge der stationären Phase im Papier von der Art der Imprägnierung. Papier Whatman No. 3 oder 4 (13 × 40 cm) imprägniert mit 20 % alkoholischen Formamidlösung. (a) Nach Durchziehen durch die Imprägnierungslösung und Abpressen zwischen Filtrierpapieren aufgehängt, 10 min auf der Luft getrocknet und nach Zerschneiden (7 cm Streifen) gewogen. (b) Wie oben, nur ohne Abpressen.

gleichmässig, sie war jedoch nicht reproduzierbar. Den grössten Einfluss darauf hatte das Abpressen zwischen Filtrierpapieren. Diese Tatsache ist im Einklang mit den Resultaten von KAUFMANN UND MOHR[35], die dieses Problem eingehend studierten.

Methode B. Die Papierstreifen werden in die Imprägnierungslösung eingetaucht oder durch diese durchgezogen und ohne vorher zwischen den Filtrierpapieren abgepresst zu werden, bei Zimmertemperatur an der Luft aufgehängt. Das Ende des Papierstreifens mit dem Start hängt nach unten. Nach dem Aufhängen fliesst ein Teil der Imprägnierungslösung zum nach unten hängenden Ende des Papierstreifens, tropft zum Teil ab, zum Teil verdampft das flüchtige Lösungsmittel und die stationäre

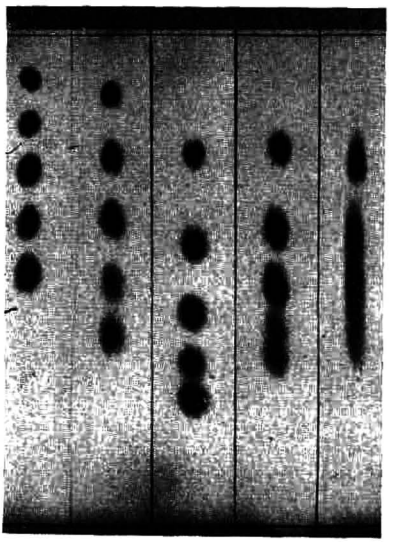


Fig. 4. Chromatogramm der Methyl- bis Amyl-3,5-dinitrobenzoate im System Dimethylformamid/Hexan auf Whatmanpapier No. 3, imprägniert mit acetonischen Dimethylformamidlösungen und getrocknet ohne Abpressen 10 min. Imprägniert mit: (1) 50%, (2) 40%, (3) 30%, (4) 20%, (5) 10% Dimethylformamidlösung.

Phase konzentriert sich auf diesem Ende des Streifens. Wenn jetzt, wie bei der Methode A, die Menge der stationären Phase bestimmt wird, erkennt man (siehe Fig. 3b), dass das Papier viel mehr stationäre Phase enthält als bei der Methode A und dass diese am Ende des Streifens bei der Startlinie konzentriert ist.

Unseren Erfahrungen nach ist die Methode B vorteilhafter; die unregelmässige Imprägnierung ruft keine Störungen hervor. Der grösste Vorteil der Methode ist, dass der Imprägnierungsgrad in gewissen Grenzen beliebig gesteigert werden kann, da durch das Abpressen keine stationäre Phase beseitigt wird und so verloren geht. In der Fig. 6 wird an praktischen Beispielen gezeigt, wie die Imprägnierungsweise die Qualität der Chromatogramme beeinflussen kann. Aus der Figur ist ersichtlich, dass die mittels der Methode A mit einer 50%igen Dimethylformamidlösung vorbehandelten Papiere dieselben Resultate aufweisen wie diejenigen, die mittels Methode B mit einer 25%igen Dimethylformamidlösung imprägniert wurden.

Auch durch das Trocknen der imprägnierten Streifen, welches das Abdampfen des flüchtigen Hilfslösungsmittels zum Ziel hat, können in manchen Fällen Komplikationen eintreten. In Fig. 7 ist graphisch dargestellt, wie aus dem Papier das flüch-

tige Lösungsmittel verdampft und dass im Falle des Formamids, Paraffinöls und 1-Bromnaphthalins nach Abdampfen des flüchtigen Lösungsmittels die Menge der stationären Phase konstant bleibt. Im Falle des Dimethylformamids kann man jedoch nicht unterscheiden, wann alles Benzol verdampft ist, da die stationäre Phase selbst vom Papier verflüchtigt, sodass nach einigen Stunden das Papier praktisch kein Dimethylformamid enthält. Aus den Fig. 8a, b ist ersichtlich, wie das Trocknen der mit Dimethylformamid imprägnierten Papiere die R_F-Werte und die Qualität der Chromatogramme beeinflusst. Bei mit Formamid, Paraffinöl, oder 1-Bromnaphthalin

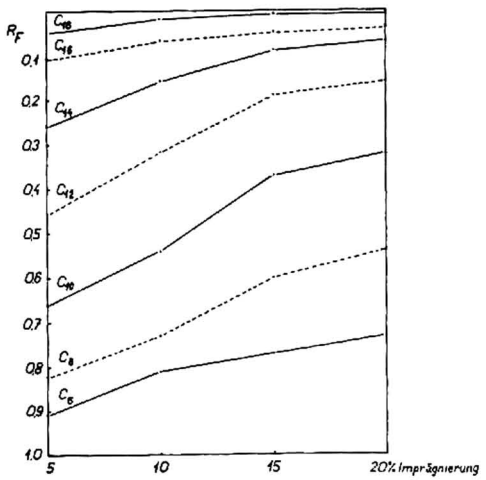


Fig. 5. R_F-Werte der Hexyl- bis Octadecyl-3,5-dinitrobenzoate im System Paraffinöl/Dimethylformamid–Methanol–Wasser 16:4:4 bei verschiedenem Imprägnierungsgrad des Papiers mit Paraffinöl.

vorbehandelten Papieren beobachteten wir diesen Einfluss nicht. Man muss jedoch in Betracht ziehen, dass das Trocknen der Chromatogramme nach der Imprägnierung selbst von einer Reihe von Faktoren beeinflusst wird. Das Verdampfen des Hilfslösungsmittels, sowie der Verlust an Dimethylformamid ist von der Raumtemperatur, der Luftströmung im Digestorium, der Papierdicke und -oberfläche usw. abhängig.

Es ist auch fraglich, wie weit das flüchtige Lösungsmittel aus dem Papier verflüchtigen kann. Wir fanden z.B., dass bei Anwendung von Methanol oder Äthanol als Hilfslösungsmittel zum Vorbehandeln der Papiere mit Formamid oder Dimethylformamid ein Teil des Alkohols in der stationären Phase verbleibt. Als wir mit auf solche Weise vorbehandelten Papieren 3,5-Dinitrobenzoylchlorid chromatographierten, fanden wir stets die Flecken des Methyl-, bzw. Äthyl-3,5-dinitrobenzoats, die durch die Reaktion des Chlorids mit dem Rest des Alkohols gebildet wurden. Das hat zur Folge, dass man die Chromatogramme der Reaktionsgemische nach der Darstellung der 3,5-Dinitrobenzoylderivate, die freies Chlorid enthalten könnten, mit bestimmter Vorsicht interpretieren muss, oder noch besser — man macht von solchen Hilfslösungsmitteln Gebrauch, die keine neue Komplikationen einführen können.

Auch die chemischen Eigenschaften der stationären Phasen und der Lösungsmittel sind in Betracht zu ziehen. So ist bekannt[38], dass z.B. Formamid teilweise zersetzt wird, sodass manche Präparate freies Ammoniak, Ameisensäure oder Ammoniumformiat enthalten.

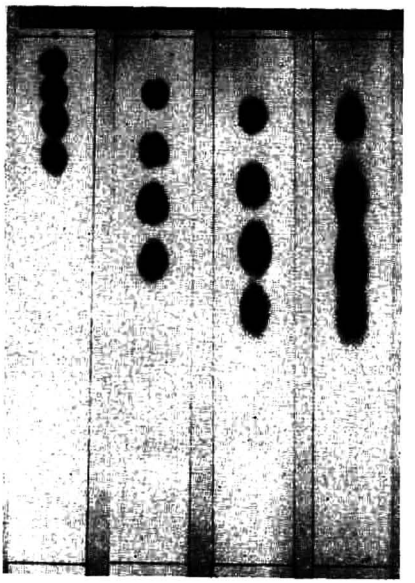


Fig. 6. Chromatogramme der Methyl- bis *n*-Butylester der 3,5-Dinitrobenzoesäure im System Dimethylformamid/Cyclohexan. (1) Imprägniert mit 50% Dimethylformamidlösung und getrocknet durch blosses Aufhängen, 10 min; (2) dasselbe mit 25% Dimethylformamidlösung; (3) imprägniert mit 50% Dimethylformamidlösung, nach Abpressen zwischen Filtrierpapieren getrocknet durch Aufhängen, 10 min; (4) dasselbe mit 25% Dimethylformamidlösung.

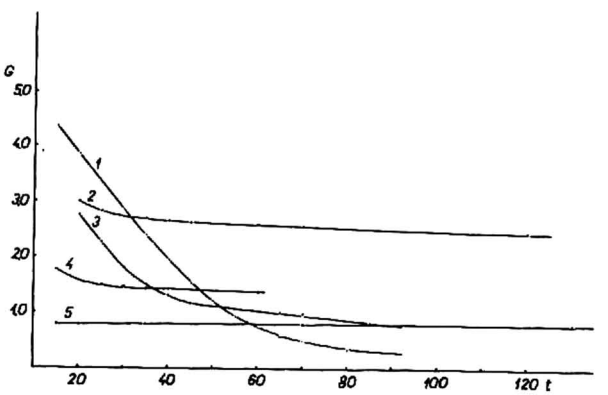


Fig. 7. Verflüchtigung des Hilfslösungsmittels, bzw. der stationären Phase aus dem Papier Whatman No. 4 beim Trocknen nach der Imprägnierung (ohne Abpressen). Temperatur 21.5°, relative Luftfeuchtigkeit 55–60%. G = Menge der stationären Phase im Papier (in g); t = Minuten. (1) Imprägnierung mit 50% benzolischer Dimethylformamidlösung; (2) Imprägnierung mit 20% äthanolischer Formamidlösung; (3) Imprägnierung mit 25% benzolischer Dimethylformamidlösung; (4) Imprägnierung mit 10% 1-Bromnaphthalinlösung in Chloroform; (5) Imprägnierung mit 5% Paraffinöllösung in Hexan.

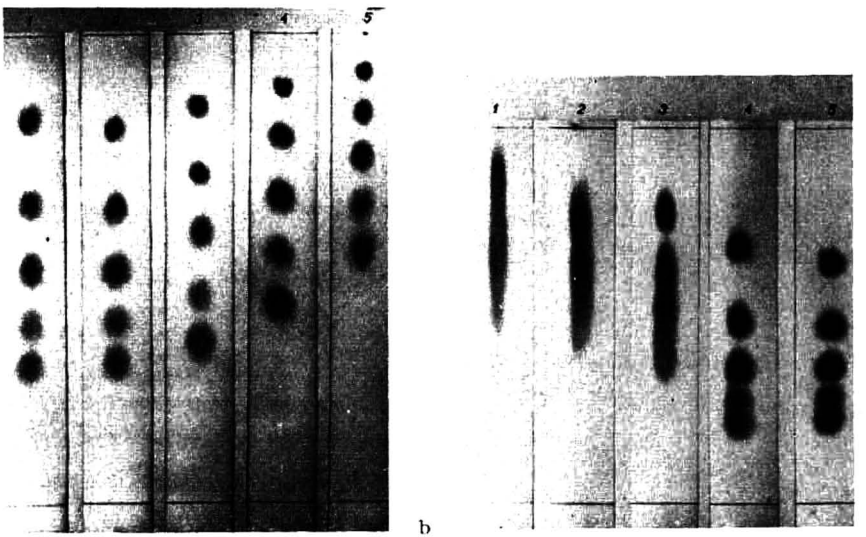


Fig. 8. Chromatogramme der Methyl- bis *n*-Amyl-3,5-dinitrobenzoate im System Dimethylformamid/Hexan. (a) Whatmanpapier No. 3 imprägniert mit 50 % acetonischer Dimethylformamidlösung; (b) dasselbe mit 25 % Dimethylformamidlösung. In beiden Fällen getrocknet ohne Abpressen: (1) 50 min; (2) 40 min; (3) 30 min; (4) 20 min; (5) 10 min.

Ein weiterer Einfluss, den man nicht vernachlässigen darf, ist der Wassergehalt des Papieres, bzw. die Feuchtigkeit der Luft im Laboratorium während des Trocknens der Chromatogramme und der Wassergehalt der Imprägnierungslösungen. Wie schon früher hervorgehoben, spielt die Löslichkeit in der stationären Phase eine entscheidende Rolle für den Verteilungsvorgang. Die Anwesenheit von Wasser würde die Löslichkeit vermindern, was die Vergrösserung der R_F-Werte zur Folge hätte. Diese Ansicht konnten wir durch praktische Versuche bestätigen. Weiter verfolgten wir den Einfluss verschiedener Papiersorten auf die Qualität der Chromatogramme. Unsere Versuche führten wir mit folgenden Papieren durch: Whatman No. 1, 2, 3, 4, Schleicher & Schüll 602, 2043b, 2045a, WF 1 (Papierfabrik Niederschlag) und Binzer Nr. 208. Zugleich wurde die Menge der stationären Phase bestimmt. Unsere Ergebnisse sind in der Tabelle IV angeführt. Wir konnten keine grösseren Unterschiede beobachten, die Qualität der Chromatogramme war in allen Fällen dieselbe.

Durch alle diese beschriebenen Versuche wollten wir erklären, warum im Falle der Chromatographie auf imprägnierten Papieren mit grösseren Streuungen der R_F-Werte gerechnet werden muss als auf den nicht vorbehandelten. Es gibt demnach eine Reihe von Faktoren, die die Resultate beeinflussen können. Unserer Meinung nach genügt es jedoch nur zu wissen, welche von diesen Faktoren störend wirken können und welche Bedingungen eingehalten werden müssen, um die besten Resultate erzielen zu können. Für die Praxis ist es ja gleichgültig ob die R_F-Werte etwas schwanken, solang die Auftrennung reproduzierbar ist.

Wir versuchten die Verlässlichkeit dieser chromatographischen Methoden auf die Weise zu überprüfen, dass wir in einem Zeitabstand von einem Monat 50 Chro-

TABELLE IV

R_F-WERTE DER C_1-C_{14} ALKYL-3,5-DINITROBENZOATE IM SYSTEM 50 % DIMETHYLFORMAMID/HEXAN AUF VERSCHIEDENEN PAPIERSORTEN

	Whatman No. 1	*Whatman No. 2*	*Whatman No. 3*	*Whatman No. 4*	*S & S 602*	*S & S 2043b*	*S & S 2045a*	*Binzer 268*
Menge des Dimethylformamids in g / *3,5-Dinitrobenzoate*	*2.8*	*2.0*	*4.0*	*3.0*	*2.0*	*2.8*	*1.6*	*2.0*
Methyl-	0.08	0.09	0.07	0.08	0.08	0.10	0.17	0.10
Äthyl-	0.18	0.18	0.15	0.15	0.21	0.20	0.32	0.19
n-Propyl-	0.28	0.29	0.23	0.24	0.34	0.33	0.45	0.29
n-Butyl	0.39	0.39	0.33	0.33	0.46	0.46	0.55	0.38
n-Amyl-	0.51	0.49	0.43	0.43	0.56	0.58	0.62	0.46
n-Hexyl-	0.62	0.59	0.51	0.53	0.65	0.70	0.70	0.59
n-Octyl-	0.75	0.71	0.69	0.70	0.75	0.82	0.75	0.71
n-Decyl-	0.83	0.78	0.80	0.82	0.81	0.87	0.78	0.77

matogramme der 3,5-Dinitrobenzoesäuremethyl-, -äthyl-, -*n*-propyl- und -*n*-butylester im System 25 % Dimethylformamid–Hexan darstellten und bei diesen Versuchen die nötigen Parameter soweit wie möglich konstant hielten. Der Imprägnierungsvorgang (Methode B) wurde stets auf die gleiche Weise durchgeführt und die Trocknungszeiten eingehalten; ferner wurde darauf geachtet, dass die Temperatur im Laboratorium sowie in der chromatographischen Kammer den Bereich von 20–23° nicht überschritt. In den Fig. 9a–d sind stets 50 Messungen für einzelne Derivate

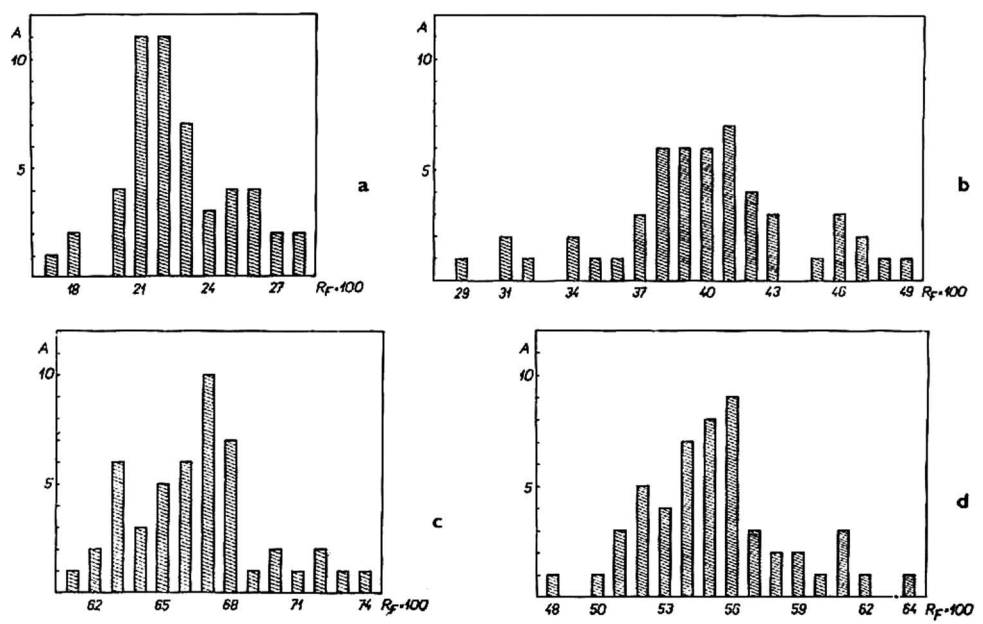


Fig. 9. Streuung der R_F-Werte im System 25 % Dimethylformamid/Hexan (50 Messungen). (a) Methyl-, (b) Äthyl-, (c) *n*-Propyl- und (d) *n*-Butylester der 3,5-Dinitrobenzoesäure. *A* = Anzahl der Messungen.

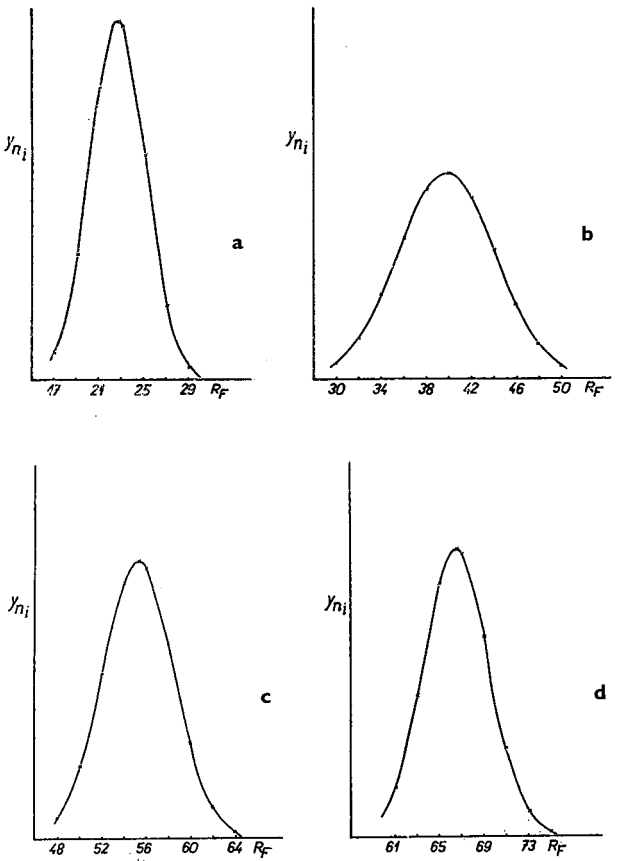


Fig. 10. Graphische Darstellung der Streuung der R_F-Werte. Verarbeitung der Resultate von Fig. 9 (a)–(d) mittels statistischer Mathematik. y_{ni} = Anzahl der beobachteten R_F-Werte in den einzelnen Klassen. (a) Methyl-, (b) Äthyl-, (c) *n*-Propyl- und (d) *n*-Butylester der 3,5-Dinitrobenzoesäure.

TABELLE V

VERARBEITUNG DER RESULTATE MITTELS STATISTISCHER MATHEMATIK

Alkyl-3,5-dinitrobenzoat	*Schwankungen der R_F-Werte*	ΔR_F	$\bar{x}$	s	y_{max}
Methyl-	0.17–0.28	0.11	0.226	0.025	16.44
Äthyl-	0.29–0.49	0.20	0.398	0.043	9.37
n-Propyl-	0.48–0.64	0.16	0.553	0.032	12.61
n-Butyl-	0.61–0.74	0.13	0.665	0.029	13.06

ΔR_F = Schwankungsbreite.
$\bar{x}$ = R_F-Mittelwert.
s = Standardabweichung.
y_{max} = Scheitelordinate der untersuchten Gausschen Verteilung.

graphisch erfasst. Diese Messungen verarbeiteten wir mit Hilfe der statistischen Mathematik[37]; die Resultate sind in der Tabelle V und den Fig. 10a–d zusammengestellt. Es hat sich gezeigt, dass die einzelnen R_F-Werte um einen Mittelwert regelmässig gemäss dem Gausschen Gesetz streuen, dass die Schwankungen am grössten in der Hälfte der Laufstrecke (R_F-Wert *ca.* 0.5) sind und zum Start, sowie zur Lösungsmittelfront sich vermindern.

Darstellung der Derivate

Mit Ausnahme der 3,5-Dinitrobenzoate der Polyäthylenglykolgemische standen uns alle in der Tabelle II angeführten Derivate als authentische Präparate zur Verfügung. Ihre Identität und Reinheit prüften wir durch Schmelzpunktbestimmung und Elementaranalyse.

Die Darstellung von Standardderivaten der Alkohole und Phenole führten wir nach bekannten Methoden durch[38,39]. Die Di- bzw. Triester der mehrwertigen Alkohole stellten wir zum Teil nach BOEHM UND THIEME[40], zum Teil nach der gewöhnlichen Pyridinmethode dar. Über die Darstellung der Monoester der Polyalkohole berichteten wir in einer früheren Mitteilung[5]. Die Mercaptane und Thiophenole überführten wir in die betreffenden Derivate nach WERTHEIM[41] und die Amine nach VEČERA *et al.*[24]. Für die höheren aliphatischen Amine wählten wir die für *p*-Nitrobenzamide empfohlene Darstellungsweise[42]. Wir machten zunächst von der Pyridinmethode Gebrauch bei Serienarbeiten, als wir verschiedene unbekannte Bestandteile der technischen Produkte identifizierten (Vorschrift siehe unten). Es hat sich jedoch gezeigt, dass diese Methode z.B. im Falle des Benzylalkohols unbrauchbar ist; Benzylalkohol gibt unter diesen Bedingungen keinen Ester, dieser kann nur bei gewöhnlicher Temperatur dargestellt werden. Im Laufe weiterer Arbeiten hat sich dann noch gezeigt, dass die geprüften Alkohole, Glykole usw. nicht immer in feste Derivate überführt werden müssen, sondern es genügt kleine Mengen der benzolischen Lösungen der rohen Reaktionsprodukte darzustellen, die direkt und ohne die letzten Spuren der überschüssigen 3,5-Dinitrobenzoesäure zu beseitigen auf die Chromatogramme aufgetragen werden können. Die oben erwähnte Verunreinigung stört nämlich auf den Chromatogrammen überhaupt nicht, da die Derivate von ihr gut abgetrennt werden. Weiter hat es sich gezeigt, dass die betreffenden Derivate durch Reaktion des 3,5-Dinitrobenzoylchlorids mit einem Tropfen der zu prüfenden Substanz in einem Mikroreagensglas durch Erwärmen auf 105–110° gebildet werden können. Das Reaktionsgemisch kann dann nach Auflösen in Benzol direkt auf das Chromatogramm aufgetragen werden. Mit Hilfe dieser Methode ist es uns gelungen von allen bisher geprüften Verbindungen (auch mit Benzylalkohol) die Derivate zur chromatographischen Bestimmung darzustellen. Die Methode ist auch für flüchtige Alkohole geeignet, da diese so schnell mit 3,5-Dinitrobenzoylchlorid reagieren, dass sie nicht Zeit haben zu entweichen.

Es war auch notwendig eine solche Methode zur Verfügung zu haben, die eine Isolierung der Derivate aus verdünnten wässrigen Lösungen ermöglicht. Wir benützten zu diesem Zwecke die Methode nach HOLLEY UND HOLLEY[43].

Mit Ausnahme des Benzylalkohols verliefen die Reaktionen in allen Fällen eindeutig, nur bei den mehrwertigen Alkoholen und Polyäthylenglykolen wurde immer ein Gemisch von Mono- und Diester gebildet, auch wenn ein Überschuss des Chlorids angewendet wurde. Es ist jedoch von grosser Wichtigkeit, dass sich die Mono- und Diester auf den Chromatogrammen ganz verschieden verhalten. Die Monoester werden von der polaren stationären Phase viel stärker festgehalten, sodass in den Systemen, in denen die Diester getrennt werden, die Monoester am Start bleiben. In Systemen, in denen die Monoester geeignet wandern, wandern die Diester mit der Lösungsmittelfront. Es ist auch wichtig zu wissen, dass manche von diesen Verbindungen besser in Form von Monoestern, andere wieder besser in Form der Diester chromatographiert werden. Bei der Darstellung der Derivate kann durch Änderung der Menge des angewandten 3,5-Dinitrobenzoylchlorids die Reaktion zu Gunsten der Bildung der Mono- oder der Diester geführt werden. Wir arbeiten deshalb im Falle der vereinfachten Methode mit unbekannten Proben in der Weise, dass wir zuerst einen Orientierungsversuch mit Reagensüberschuss durchführen und dann dem Resultat des Versuches entsprechend die Reaktion mit geeigneten Mengen des Reagens wiederholen. Bei dieser Darstellungsweise beobachteten wir im Falle der Polyäthylenglykolderivate noch eine Nebenreaktion, die hauptsächlich bei höheren Temperaturen verlief: es wurden als Nebenprodukte die Derivate der betreffenden Chlorhydrine gebildet. Bei den von uns angewandten Reaktionsbedingungen wurden diese Nebenprodukte nur in Spuren gebildet, sodass sie vernachlässigt werden konnten.

Pyridinmethode. 0.1 g Alkohol wurde in 1 ml Benzol gelöst, eine Lösung von 0.5 g, 3,5-Dinitrobenzoesäurechlorid in 3 ml Benzol und 1 ml Pyridin zugefügt und 30 min lang auf kochendem Wasserbade erhitzt. Nach dem Erkalten wurde zweimal mit je 5 ml 50%iger Kalilauge umgeschüttelt, wobei nach jedem Umschütteln ungefähr 50 ml Wasser zur scharfen Abscheidung der Schichten zugegeben wurden. Danach wurde die Benzollösung zweimal mit 5 ml Wasser, zweimal mit 5 ml HCl (1:1) und dann mit Wasser, bzw. einer gesättigten Natriumsulfatlösung bis zur neutralen Reaktion ausgeschüttelt. Die Benzolschicht wurde durch Zugabe von wasserfreiem Natriumsulfat abgeklärt und direkt auf das Papier aufgetragen.

Vereinfachte Methode: 0.05 ml der untersuchten Substanz wurden in einem Mikroreagensglas mit einem Körnchen 3,5-Dinitrobenzoylchlorid versetzt und 10–15 min auf 100–110° erhitzt. Nach dem Erkalten wurden 5 Tropfen einer 5%igen Na_2CO_3-Lösung und 1 ml Benzol zugefügt, das Gemisch mit einem Glasstab tüchtig durchgerührt und die so erhaltene benzolische Schicht auf das Papier aufgetragen.

Methode für wässrige Lösungen: zu ungefähr 10 ml der verdünnten wässrigen Lösung, die *ca.* 1.5 mmol des gelösten Alkohols oder das Aminhydrochlorids enthielt, wurde eine Lösung von 0.5 g 3,5-Dinitrobenzoylchlorid in 30 ml Benzol zugefügt. Unter Schütteln und Kühlung wurden hierauf *ca.* 10 g Kaliumkarbonat zugegeben. Das Gemisch wurde 20 min lang bei normaler Temperatur (bei sekundären Alkoholen und Aminen über Nacht) stehen gelassen und die abgetrennte benzolische Schicht, wie im Falle der Pyridinmethode, mit Kalilauge, Salzsäure, und Wasser gewaschen. Nach dem Trocknen mit wasserfreiem Natriumsulfat wurde das Lösungsmittel abdes-

tilliert, der Rückstand in der nötigen Menge Benzol gelöst und auf das Papier aufgetragen.

Bereitung der Chromatogramme

In diesem Abschnitt wird die Bereitung der Chromatogramme beschrieben, wie wir sie bei täglicher Arbeit durchführen und wie wir sie auch empfehlen durchzuführen.

Der Streifen des chromatographischen Papiers (wir benützten Streifen von 13 × 45 cm) wurde durch die Imprägnierungslösung (25- oder 50%ige benzolische Dimethylformamidlösung, 20%ige alkoholische Formamidlösung, 5%ige Paraffinöllösung in Hexan oder eine 10%ige 1-Bromnaphthalinlösung in Methanol, Chloroform oder Benzol) durchgezogen und so an der Luft im Abzug aufgehängt, dass das Ende des Papierstreifens mit der Startlinie nach unten hing. Die Trocknungszeit betrug 10–30 min; nur bei den mit Dimethylformamid vorbehandelten Papieren war es nötig die Trocknungszeit der Raumtemperatur anzupassen, d.h. im Sommer auf 10 min oder noch weniger zu verkürzen. Die zu chromatographierenden Verbindungen wurden auf die vorbehandelten Papiere in Aceton, Chloroform oder Benzol zu 0.5–1%igen Lösungen gelöst aufgetragen. Alle Chromatogramme wurden unter Benützung normaler chromatographischer Ausrüstung absteigend entwickelt; die Temperatur schwankte dabei im Bereich von 18–23°. Nur im Falle der mit 1-Bromnaphtalin imprägnierten Papiere musste auf genügende Sättigung der beweglichen Phase mit 1-Bromnaphthalin Bedacht genommen werden, um das Herauswaschen der stationären Phase während des Chromatographierens zu vermeiden. Bei den übrigen Lösungsmittelsystemen war dies nicht notwendig. Im Augenblick, in dem das Lösungsmittel die gewünschte Laufstrecke (30–35 cm) zurückgelegt hatte wurden die Chromatogramme aus der Kammer herausgenommen und im Abzug bei Raumtemperatur getrocknet.

Die Sichtbarmachung der Chromatogramme geschah durch Besprühen mit einer Zinn(II)-chloridlösung (0.7 g $SnCl_2 \cdot 2H_2O$, 15 ml konz. HCl und 100 ml Wasser) und nach 30–60 min mit einer 1%igen *p*-Dimethylaminobenzaldehydlösung in alkoholischer Chlorwasserstoffsäure (95 Teile Äthanol und 5 Teile konz. HCl). Die 3,5-Dinitrobenzoylderivate erscheinen als gelbe Flecken auf weissem Hintergrund.

ERGEBNISSE UND DISKUSSION

In der vorliegenden Arbeit werden Methoden zur papierchromatographischen Identifizierung von Alkoholen, Polyalkoholen, Polyäthylenglykolen und ihren Äthern, Phenolen, Aminen und Thiolen nach Überführung in die entsprechenden 3,5-Dinitrobenzoylderivate beschrieben.

Vor allem soll auf dieser Stelle das Prinzip der Methode diskutiert werden. Es hat sich gezeigt, dass das Überführen der erwähnten Verbindungen in die 3,5-Dinitrobenzoylderivate den Vorteil besitzt, dass die ursprünglich flüchtigen oder nicht entdeckbaren Verbindungen in zur Chromatographie geeignete Derivate überführt werden. Bis auf kleine Ausnahmen verläuft die Reaktion eindeutig und es werden

leicht erkennbare (1 μg), stabile Derivate gebildet. Die Darstellungsweise konnte sehr vereinfacht werden, da es nunmehr möglich ist die rohen Reaktionsprodukte zu chromatographieren, ohne die Derivate vorher isolieren zu müssen. Das hat zur Folge, dass auch die kleinsten Mengen von Verunreinigungen erfasst werden können und durch den Reinigungsvorgang nicht beseitigt werden. Auch die Bildung öliger Derivate im Falle von Gemischen, z.B. der Polyäthylenglykole bietet keine Schwierigkeiten. Für die Lösung der in Frage stehenden Probleme ist von Wichtigkeit, dass auf diese Weise das Reagens mit allen oben erwähnten Stoffklassen reagiert, da alle als Bestandteile der von uns analysierten Produkten in Frage kommen.

Es ist begreiflich, dass unter den *ca.* 150 chromatographierten Derivaten einerseits grosse Unterschiede im Verhalten während des Chromatographierens beobachtet werden konnten, andererseits aber wieder eine Reihe von Verbindungen ein sehr ähnliches Verhalten aufwies. Wir überprüften das Verhalten aller Derivate in 20 Lösungsmittelsystemen und fanden für jedes Derivat die geeigneten Bedingungen zu seiner Auftrennung und Identifizierung.

Grundsätzlich machten wir von Lösungsmittelsystemen mit Dimethylformamid, Formamid, Paraffinöl und 1-Bromnaphthalin als stationären Phasen Gebrauch. Der grosse Vorteil dieser Systeme, bei denen mit Dimethylformamid und Formamid imprägnierte Papiere benützt werden, ist ihre Schnelligkeit: das Fliessmittel durchläuft eine 40 cm lange Laufstrecke in 2 Stunden. In den übrigen Systemen legt die mobile Phase dieselbe Laufstrecke erst in 8 oder mehreren Stunden zurück.

Für die mit Dimethylformamid imprägnierten Papiere kann Hexan oder Cyclohexan mit gleichem Erfolg als mobile Phase angewendet werden und die R_F-Werte können in einem bestimmten Bereich durch den Imprägnierungsgrad des Papieres geändert werden.

Für die mit Formamid imprägnierten Papiere kann Hexan oder Cyclohexan, Benzol, Chloroform, Äthylacetat oder ihre Gemische als Fliessmittel Anwendung finden. Mit steigender Polarität der mobilen Phase vergrössern sich auch die R_F-Werte und es ist möglich durch Änderung der Zusammensetzung des Fliessmittels die Flecken auf den Chromatogrammen beliebig zu verschieben.

Im Falle der mit Paraffinöl imprägnierten Papiere überprüften wir in einer vorangehenden Mitteilung[4] die Eignung von 23 mobilen Phasen, die Formamid oder Dimethylformamid, Methanol und Wasser allein oder in Gemischen verschiedener Zusammensetzung enthielten. Durch Erhöhung des Prozentgehaltes im Gemisch an organischem Lösungsmittel tritt eine Erhöhung der R_F-Werte auf. Am wirksamsten in dieser Hinsicht erwies sich das Dimethylformamid, am wenigsten wirksam war Formamid. Die Wasserzugabe setzte die R_F-Werte selbstverständlich herab; sie konnten aber wieder durch Methanolzugabe erhöht werden. Bei höheren Methanolkonzentrationen tritt jedoch die Tendenz zur Streifenbildung in den Vordergrund. Das Formamid hat weiter als Bestandteil der mobilen Phase den Nachteil, dass es vom Papier nur schwierig beseitigt werden kann. Nach allen diesen Erfahrungen können wir als die geeignetsten mobilen Lösungsmittelgemische das System Dimethylformamid–Methanol–Wasser in Verhältnissen 8:1:1, 4:1:1 und 2:1:1 empfehlen,

die alle Forderungen erfüllen. Mit wachsendem Prozentgehalt an Dimethylformamid in diesen Lösungsmittelsystemen wachsen auch die R_F-Werte an.

Für die mit 1-Bromnaphthalin vorbehandelten Papiere wird 70 bis 90%ige Essigsäure als Fliessmittel benützt. Mit wachsendem Wassergehalt werden die R_F-Werte vermindert.

In Folgendem wird angeführt, welche Systeme für bestimmte Stoffklassen empfohlen werden können.

Aliphatische Alkohole. Für die niederen Glieder dieser Reihe, d.h. für Alkohole C_1–C_5 sind die Systeme mit Dimethylformamid oder Formamid als stationäre Phase und Hexan oder Cyclohexan als Fliessmittel geeignet. Macht man von Formamid als stationäre Phase und mehr polaren Lösungsmitteln als mobile Phase Gebrauch, so wandern die Alkoholderivate mit der Lösungsmittelfront mit. Die Qualität der Trennung der niederen Alkohole kann aus den Fig. 4, 6 und 8 beurteilt werden. Alkohole mit höheren Alkylen bilden im System 50% Dimethylformamid/Hexan zwar gut ausgebildete Flecken, diese werden jedoch in der Nähe der Lösungsmittelfront unscharf getrennt (Tabelle II). Vorteilhafter ist hier die Systeme mit Paraffinöl als stationärer Phase und Dimethylformamid–Methanol–Wasser in Verhältnissen

Fig. 11. Chromatogram der 3,5-Dinitrobenzoate der höheren aliphatischen Alkohole. System: Paraffinöl/Dimethylformamid–Methanol–Wasser 16:1.5:1.5. (1) Höhere Fraktion von 3; (2) Myristylalkohol; (3) synthetisches Fettalkoholgemisch; (4) Gemisch von Decyl-, Dodecyl-, Tetradecyl-, Hexadecyl- und Octadecylalkohol; (5) niedere Fraktion von 3.

8:1:1, 4:1:1 oder 2:1:1 als Fliessmittel anzuwenden. In diesen Systemen wandern wieder die niederen aliphatischen Alkohole in der Nähe der Lösungsmittelfront. In der Fig. 11 wird die Auftrennung der höheren Alkohole natürlicher Herkunft mit

gerader Anzahl von Kohlenwasserstoffatome sowie eines Gemisches synthetischer Alkohole gezeigt.

Glykole. Bei der Reaktion des 3,5-Dinitrobenzoylchlorids mit mehrwertigen Alkoholen werden neben den Monoestern auch die Diester, bzw. die Triester der 3,5-Dinitrobenzoesäure gebildet. Mann kan zwar durch Änderung der Menge des zur Reaktion angewandten Chlorids die Reaktion zu Gunsten des einen oder des anderen Ester führen, es entstehen jedoch in jedem Falle auf den Chromatogrammen zwei Flecken, da die Monoester auch bei Anwendung eines Überschusses an 3,5-Dinitrobenzoesäurechlorid gebildet werden. Beide Typen von Estern verhalten sich grundlegend verschieden. Es hat sich gezeigt, dass zur Identifizierung der Glykole vorteilhafter die Monoester herangezogen werden können. Sie bilden in Systemen mit Formamid als stationäre Phase und Gemischen von Hexan und Benzol oder Benzol allein als Fliessmittel gut ausgebildete Flecken und werden gut getrennt. Die Diester stören überhaupt nicht, da sie unter denselben Bedingungen mit der Lösungsmittelfront mitlaufen. Für den Glyzerinmonoester ist Chloroform eventuell im Gemisch mit Äthylacetat die geeignete mobile Phase. Die Diester können zur Identifizierung der Glykole nicht angewendet werden, da sie verwaschene Flecken bilden, die nicht genügend aufgetrennt werden (System Formamid/Hexan).

Monoäther der Glykole und Polyäthylenglykole. Durch die Einführung der Oxyäthylgruppen in die Moleküle der aliphatischen Alkohole werden ihre R_F-Werte auf mit polaren Phasen imprägnierten Papieren sukzessiv herabgesetzt. Man wird also zur Chromatographie dieser Verbindungen solche Systeme wählen, in denen die Alkohole die als Ausgangsprodukte dienten, geeignet grosse R_F-Werte haben, bzw. in der Nähe der Lösungsmittelfront wandern. Für die Methyl- bis Butyl-äther der Glykole, bzw. Polyäthylenglykole sind die Systeme Dimethylformamid/Hexan oder Formamid/Hexan, bzw. Hexan–Benzol 9:1, 8:2 oder 7:3 geeignet. Die Dodecyläther werden demgemäss auf umgekehrten Phasen chromatographiert (Paraffinöl/Dimethylformamid–Methanol–Wasser).

Polyäthylenglykole. Analog wie bei den mehrwertigen Alkoholen werden im Falle der Reaktion der Polyäthylenglykole mit 3,5-Dinitrobenzoylchlorid sowohl die Mono- als auch die Diester gebildet. Die beiden Reihen von Estern verhalten sich auf den Chromatogrammen wieder ganz verschieden. Bei den Monoestern steigen die R_F-Werte zuerst vom Mono- zum Triäthylenglykol, um durch Einführung weiterer Oxyäthylgruppen vermindert zu werden. Die Flecken und Trennung der ersten drei Glieder sind zur Identifizierung geeignet; die höheren Glieder der Reihe weisen kleine Unterschiede im R_F-Wert auf, sodass sich im Gemisch auf den Chromatogrammen nur ein langer Streifen mit einzelnen Maxima bildet. Die Diester der ersten drei Glieder werden voneinander nicht getrennt, die ersten zwei bilden sogar Streifen. Die höheren Glieder bilden dagegen charakteristische und voneinander gut aufgetrennte Flecken.

Zur Auftrennung des Äthylen-, Diäthylen- und Triäthylenglykole eignen sich also die Monoester und das Lösungsmittelsystem Formamid/Benzol, bzw. Benzol–Chloroform 1:1 und zur Identifizierung der höheren Polyäthylenglykole die Diester und das Lösungsmittelsystem Formamid/Hexan–Benzol 4:6.

Chlorhydrine. Für die Trennung des Äthylenchlorhydrins, Propylenchlorhydrins und Dichlorhydrins war das System 25 % Dimethylformamid/Hexan oder Cyclohexan das beste. Bei Anwendung des Formamids als stationäre Phase bildet das Dichlorhydrin einen streifenförmigen Flecken.

Cycloaliphatische Alkohole. Im System 50 % Dimethylformamid/Hexan oder Cyclohexan werden die cycloaliphatischen Alkohole der Anzahl der Kohlenstoffatome nach getrennt, d.h. die Methylcyclopentanole und das Cyclohexanol weisen zwar bestimmte kleine Unterschiede im R_F-Wert auf; diese sind jedoch zu klein, um eine Trennung zu ermöglichen. Das Cyclohexanol verhält sich dabei wie Amylalkohol.

Heterocyclische Alkohole. An dieser Stelle soll nur das interessante Verhalten des Furfurylalkohols und Tetrahydrofurfurylalkohols erwähnt werden. In Systemen mit Dimethylformamid als stationärer Phase hat der Tetrahydrofurfurylalkohol grössere R_F-Werte; auf mit Formamid vorbehandelten Papieren ist die Reihenfolge umgekehrt.

Aromatische Alkohole. Derivate dieser Stoffklasse werden am vorteilhaftesten in Systemen mit Dimethylformamid oder Formamid/Hexan oder Cyclohexan chromatographiert. Für die Praxis ist von gewisser Bedeutung, dass z.B. Benzylalkohol in Systemen mit Dimethylformamid ähnlich wie Methanol wandert; wendet man aber Formamid als stationäre Phase an, dann verhält er sich wie *n*-Propanol.

Phenole. Die Derivate des Phenols und seiner Alkylhomologen bilden in Systemen mit Dimethylformamid als stationärer Phase runde Flecken in der Nähe des Starts; in Systemen mit Formamid bilden sie Streifen. Für diese Klasse von Verbindungen ist est selbstverständlich vorteilhafter sie ohne Überführung in Derivate zu chromatographieren.

Mercaptane. Für die Derivate der Mercaptane sind dieselben Lösungsmittelsysteme geeignet, die zur Auftrennung der Alkoholderivate gute Dienste leisten. Die Mercaptane verhalten sich nämlich wie um einen Kohlenstoffatom reichere Alkohole, also Äthylmercaptan wie *n*-Propanol, Dodecylmercaptan wie C_{13}-Alkohol u.ä.

Thiophenole. Diese Verbindungen sind im System 50 % Dimethylformamid/Hexan als runde Flecken in der Nähe des Starts, im System 25 % Dimethylformamid/Cyclohexan wandern sie etwas schneller als in Hexan und auf mit Formamid imprägnierten Papieren bilden sie streifenförmige Flecken.

Aliphatische Amine. Ein sehr unterschiedliches Verhalten weisen die Derivate der primären und sekundären Amine auf den Chromatogrammen auf. Dabei sei noch bemerkt, das die tertiären Amine mit 3,5-Dinitrobenzoylchlorid nicht reagieren. In Systemen mit Dimethylformamid oder Formamid als stationären Phasen werden die Derivate der primären Amine von der stationären Phase wahrscheinlich wegen der Bildung intermolekularer Wasserstoffbrückenbindungen viel mehr festgehalten als die Derivate entsprechender sekundärer Amine. Die sekundären und primären Amine werden also in Form ihrer 3,5-Dinitrobenzoylderivate bemerkenswert wirksamer getrennt als in Form ihrer Salze. Im System 25 % Dimethylformamid/Hexan oder Cyclohexan ist das Methyl- bis Amylamin am Start, die höheren Amine von Hexyl-

bis Tetradecylamin werden gut aufgetrennt (Fig. 12). Von den Derivaten der sekundären Amine wandern in diesem Lösungsmittelsystem nur die des Di-*n*-propyl- und Di-*n*-butylamins; die Amine mit kleineren Alkylen bleiben am Start. Im System Formamid/Hexan werden vorzüglich die sekundären Amine (Dimethyl- bis Di-*n*-butylamin) aufgetrennt; die niederen primären Alkylamine bis Hexylamin bleiben

Fig. 12. Chromatogramm der höheren Alkyl-3,5-dinitrobenzamide im System 50% Dimethylformamid/Hexan. (1) Myristylamin; (2) Laurylamin; (3) Gemisch von 1, 2, 4 und 5; (4) Decylamin; (5) Octylamin.

am Start, die höheren primären Alkylamine bilden Streifen. In Systemen Formamid/Hexan–Benzol in verschiedenen Verhältnissen werden gut die niederen primären Amine bis Hexylamin aufgetrennt, die übrigen Amine wandern mit der Lösungsmittelfront mit. Eine sehr gute Auftrennung der homologen Reihe der primären Amine von Hexyl- bis Octadecylamin erzielt man im System 1-Bromnaphtalin/90% Essigsäure. Für praktische Analysen ist von Bedeutung, dass auf diese Weise auch das Ammoniak in Form seines Derivats, des 3,5-Dinitrobenzoesäureamids, von den primären und sekundären Alkylaminen abgetrennt wird und in ihrer Anwesenheit verlässlich identifiziert werden kann. Geeignet dazu ist das Lösungsmittelsystem Formamid/Chloroform, bzw. Chloroform–Äthylacetat 1:1.

Andere Amine. Für das Cyclohexylamin und Benzylamin ist das System 25% Dimethylformamid/Cyclohexan geeignet. Die Trennung von Anilin, N-Methylanilin und N-Äthylanilin erfolgt am besten in Systemen Formamid/Cyclohexan oder Hexan–Benzol; für die Identifizierung des Diphenylamins ist das System mit Dimethylformamid als stationärer Phase geeignet. Auf mit Formamid vorbehandelten Papieren bildet sonst das Cyclohexylamin, Benzylamin und Diphenylamin Streifen.

3,5-Dinitrobenzoesäure. Da alle nicht gereinigten Derivate 3,5-Dinitrobenzoesäure enthalten können, die durch die angewandte Detektionsmethode auch sichtbar gemacht wird, ist es notwendig auch über ihr chromatographisches Verhalten zu berichten. In Lösungsmittelsystemen mit Dimethylformamid und Formamid verbleibt die Säure am Start in allen Fliessmitteln, die zur Auftrennung der erwähnten Stoffklassen verwendet werden. Erst im System Formamid/Chloroform–Äthylacetat 2:8 hat sie den R_F-Wert 0.04. In Systemen mit Paraffinöl als stationärer Phase wandert die Säure mit der Lösungsmittelfront mit. Bei Anwendung der mit 1-Bromnaphthalin imprägnierten Papiere und 70–90% Essigsäure als Fliessmittel hat die Säure auch hohe R_F-Werte; manche Derivate der Amine weisen jedoch noch höhere R_F-Werte auf.

Unbekannte Verbindungen. Bei der Identifizierung unbekannter Verbindungen wird dieses Derivat zuerst im System Formamid/Hexan nach Überführung in das betreffende 3,5-Dinitrobenzoylderivat chromatographiert. Hat das untersuchte Derivat einen zu kleinen R_F-Wert, oder ist es sogar am Start, so benützen wir in weiteren Versuchen mehr polare bewegliche Phasen (also Formamid/Hexan → Benzol → Chloroform → Äthylacetat oder ihre Gemische). Hat das untersuchte Derivat im System Formamid/Hexan einen grossen R_F-Wert oder wandert es mit der Lösungsmittelfront mit, so wenden wir in weiteren Versuchen das System Dimethylformamid/Hexan an und wenn dann noch der R_F-Wert zu hoch ist, wiederholen wir das Chromatographieren und arbeiten mit dem System Paraffinöl/Dimethylformamid–Methanol–Wasser.

CHROMATOGRAPHISCHES VERHALTEN UND KONSTITUTION

Den in dieser Arbeit erhaltenen Resultaten können einige Beziehungen zwischen Struktur der chromatographierten Verbindungen und ihrem Verhalten auf den Chromatogrammen entnommen werden (bezogen auf Systeme mit polarer stationärer Phase, also mit Dimethylformamid oder Formamid).

Unter den geprüften Typen von Verbindungen, d.h. den Estern, Thioestern, Alkyl- und Dialkylamiden und der 3,5-Dinitrobenzoesäure werden die Verbindungen folgender Reihe nach von der stationären Phase immer fester zurückgehalten: Ar–CO–SR, Ar–CO–OR, Ar–CO–N(R)$_2$, Ar–CO–NH·R, Ar–CO–NH$_2$ und Ar–COOH, wobei R = Alkyl und Ar = 3,5-$C_6H_3(NO_2)_2$. Dieses Verhalten kann durch die Fähigkeit Wasserstoffbrückenbindungen mit der stationären Phase zu bilden erklärt werden.

In einzelnen Reihen von Verbindungen wird der R_F-Wert hauptsächlich von den Alkylgruppen beeinflusst: den niedrigsten R_F-Wert hat immer die Verbindung mit der Methylgruppe; die R_F-Werte steigen mit wachsender Anzahl der Kohlenstoffatome der Alkylgruppe an. Dasselbe gilt auch für die Anzahl der Methylengruppen bei den mehrwertigen Alkoholen und für die Alkylgruppen der Monoäther der Glykole und Polyäthylenglykole. Eine Doppelbindung verursacht eine bestimmte Erniedrigung der R_F-Werte; z.B. ein Allylderivat verhält sich wie ein Äthylderivat. Die *n*-Alkylderivate und die entsprechenden *iso*-, *sek.*- und *tert.*-Alkylderivate weisen kleine,

jedoch reproduzierbare und regelmässige R_F-Wert Unterschiede auf. Von diesen Isomeren hat das *tert.*-Alkylderivat stets den grössten R_F-Wert. Die Benzylderivate verhalten sich in Systemen mit Dimethylformamid als stationäre Phase wie Methylderivate, in Systemen mit Formamid als stationäre Phase wie die Propylderivate. In der Reihe: Benzylalkohol, Phenyläthylalkohol und Phenylpropylalkohol steigen die R_F-Werte wie zu erwarten. Die Einführung der *p*-Methylgruppe in das Molekül des Benzylalkohols verursacht eine Vergrösserung der R_F-Werte, die *p*-Methoxygruppe verursacht dagegen eine Verminderung dieser Werte. Weitere Beziehungen in der Reihe der aromatisch-aliphatischen Alkohole sind der Tabelle II zu entnehmen.

Cycloaliphatische Verbindungen verhalten sich wie die aliphatischen, um ein Kohlenstoffatom ärmeren Derivate, also Cyclopentanol wie *n*-Butanol, Cyclohexanol wie Amylalkohol und Cyclohexylamin wie Amylamin. Die isomeren Methylcyclopentanole werden voneinander nicht getrennt, was auch bei den isomeren Kresolen und Toluidinen der Fall ist. Phenol selbst hat in Systemen mit Dimethylformamid einen kleineren, mit Formamid einen grösseren R_F-Wert als Methanol; die Einführung einer Alkylgruppe in den aromatischen Ring verursacht Vergrösserung des R_F-Wertes, es steigen also die R_F-Werte in der Reihe: Phenol, Kresole, Äthylphenol, *tert.*-Butylphenol an. Die Naphthole und *p*-Phenylphenol haben kleinere R_F-Werte als Phenol selbst.

Die Diester der Glykole haben kleinere R_F-Werte als die betreffenden aliphatischen Alkohole (Äthylenglykoldiester hat einen kleineren R_F-Wert als Äthylalkoholester), der Triester des Glyzerins hat wieder einen kleineren R_F-Wert als der Diester des Äthylenglykols. Es werden also die R_F-Werte mit steigender Anzahl der Ar–CO–OR-Gruppen vermindert. Die Anwesenheit freier Hydroxylgruppen in den Monoestern der Glykole und Polyäthylenglykole bewirkt ein merkliches Absinken der R_F-Werte. Der Monoester des Äthylenglykols wird also von der stationären polaren Phase stärker zurückgehalten als das Derivat des Äthylalkohols; im Falle des Glyzerins vermindern sich die R_F-Werte in der Reihe: Triester, Diester und Monoester. Die Ätherifizierung einer Hydroxylgruppe im Diol, bzw. seinem Monoester, kommt durch einen bedeutenden Anstieg der R_F-Werte zum Ausdruck. Im Falle der isomeren Butylenglykole kann eine merkliche Abtrennung des 1,4-Isomeren beobachtet werden. Die Chlorsubstitution einer Hydroxylgruppe im Äthylenglykol macht sich grössenordnungsmässig auf die gleiche Art bemerkbar, wie die Ätherifizierung dieser Gruppe durch eine Methylgruppe. Mit zunehmender Chlorsubstitution verringern sich die R_F-Werte. Die Einführung der Oxyäthylgruppen in die Moleküle der Glykole, bzw. Glykolmonoäther, bewirkt eine merkliche Verminderung der R_F-Werte. Eine Ausnahme bilden die ersten drei Glieder der Polyäthylenglykolreihe, das Mono-, Di- und Triäthylenglykol. Bei den Monoestern dieser Reihe steigen die R_F-Werte zuerst vom Mono- zum Tri-Derivat, um durch Einführung weiterer Oxyäthylgruppen wieder vermindert zu werden. Bei den Diestern sind die R_F-Werte der ersten drei Glieder praktisch gleich und erst vom Tetraäthylenglykol an werden mit steigender Oxyäthylgruppenanzahl die R_F-Werte vermindert.

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ZUSAMMENFASSUNG

In der vorliegenden Arbeit wird gezeigt, dass Alkohole, Glykole, Polyäthylenglykole, ihre Monoäther, sowie Chlorhydrinderivate, Phenole, Mercaptane und aliphatische sowie aromatische Amine nach Überführung in die betreffenden 3,5-Dinitrobenzoylderivate auf Grund ihrer Reaktion mit 3,5-Dinitrobenzoylchlorid nebeneinander mit Hilfe der Papierchromatographie identifiziert werden können. Rund 150 Derivate wurden dargestellt und ihr chromatographisches Verhalten in 20 Lösungsmittelsystemen studiert. Es werden die nötigen Vorschriften zur Darstellung der Derivate bei Serienarbeit angegeben und für jede Stoffklasse die besten Bedingungen der Papierchromatographie empfohlen. Es wird von folgenden Lösungsmittelsystemen Gebrauch gemacht: Dimethylformamid/Hexan oder Cyclohexan, Formamid/Hexan oder Cyclohexan, Benzol, Chloroform und Äthylacetat allein oder in Gemischen, Paraffinöl/Dimethylformamid–Methanol–Wasser in verschiedenen Verhältnissen und 1-Bromnaphthalin/70 oder 90% Essigsäure. Die Beziehungen zwischen dem chromatographischen Verhalten und der Struktur der chromatographierten Verbindungen wurden diskutiert.

Im Rahmen dieser Arbeit wurden auch die Verhältnisse der Chromatographie auf imprägnierten Papieren eingehender studiert und eine Reihe von Faktoren, die den chromatographischen Vorgang beeinflussen, aufgeklärt. An praktischen Beispielen der 3,5-Dinitrobenzoylderivate wurde die Wahl der Derivate und der geeigneten Lösungsmittelsysteme diskutiert, sowie der Einfluss der Imprägnierungsart, des Trocknens der Chromatogramme bei der Imprägnierung, usw. demonstriert. Es wurde gezeigt, dass in einem bestimmten Bereich die Verbindungen auf den Chromatogrammen beliebig verschoben werden können.

SUMMARY

The authors show that alcohols, glycols, polyethylene glycols, and their monoethers, as well as chlorohydrins, phenols, mercaptans and aliphatic and aromatic amines, can be identified when present together, by paper chromatography of their 3,5-dinitrobenzoyl derivatives. About 150 derivatives were prepared and their chromatographic behaviour in 20 solvent systems studied. Methods for preparing the derivatives in the case of serial analyses are given, and for each class of compound the best conditions for paper chromatography recommended. The following solvent systems were used: dimethylformamide/hexane or cyclohexane, formamide/hexane or cyclohexane, benzene, chloroform and ethyl acetate alone or in mixtures, paraffin oil/dimethylformamide–methanol–water in various ratios and 1-bromonaphthalene/

70 or 90 % acetic acid. The relations between chromatographic behaviour and structure of the compounds are discussed.

Chromatography on impregnated paper was also closely investigated and a number of factors that affect the chromatographic process were clarified. Taking the 3,5-dinitrobenzoyl derivatives as examples, the choice of derivatives and of suitable solvent systems is discussed, as well as the influence of the method of impregnation, the drying of the chromatograms after impregnation, etc. It is shown that within certain limits the position of the compounds on the chromatograms can be altered.

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THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

II. THE PAPER CHROMATOGRAPHY OF SOME STEROLS, PROVITAMINS, VITAMINS AND PENTACYCLIC TRITERPENOID ALCOHOLS*

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INTRODUCTION

Unlike the paper chromatographic separation of the more polar steroids, the chromatography of highly fat-soluble sterols, vitamins, provitamins and pentacyclic triterpenoid alcohols has not been the subject of many comprehensive studies.

Because of the poor solubility of these compounds in water and polar solvents, the "normal" paper chromatographic methods find little application here. The literature only deals with separations of cholesterol and 7-dehydrocholesterol[1] and of vitamin A and vitamin A acetate[2] in similar "normal" systems.

Impregnation of the paper beforehand is necessary to achieve good chromatographic separations. For this purpose the following agents may be used: phenyl cellosolve[4–6], salts of carboxylic acids[7], aluminium oxide[3] and Quilon (a chromium stearato complex); see Table I. The last-mentioned compound, a water-repellent impregnating agent, has been applied for the separation of some naturally occurring sterols[9–11].

The solvent mixtures used for the paper chromatographic fractionation of sterols, mentioned in Table I, are the following:

(1) Quilon/ethanol–water (8:2)[9].
(2) Quilon/methanol[9].
(3) Quilon/methanol–water–ethylene glycol monomethyl ether (65:20:20)[10].
(4) Quilon/methanol–water (95:5)[10].
(5) Quilon/ethanol–water (8:2)[11].
(6) Sodium stearate, -palmitate/methanol–carbon tetrachloride–water (18:5:2)[7].
(7) Aluminium oxide/hexane–ether (3:1)[3].
(8) Water/phenol–methanol–water (13.5:30:56.5)[1].
(9) Water/isopropanol–water (1:1)[2].
(10) Phenyl cellosolve/heptane[6].
(11) Phenyl cellosolve/heptane[5].
(12) Silicone grease/acetonitril–water (6:4)[8].

* For Part I of this series, see ref.[22].
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TABLE I

DATA CONCERNING THE PAPER CHROMATOGRAPHIC SEPARATION OF SOME STEROLS, VITAMINS AND PROVITAMINS

Compound	*R_F in paper chromatographic system*																
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
Cholesterol	0.52	0.56	0.49	0.73	0.56	0.30	0.77	0.0		0.63	≡0.1		0.29	0.49	0.50	0.35	0.42
Cholesterol acetate						0.11											
β-Sitosterol	0.54	0.65	0.43	0.65	0.47–0.58		0.80				1.0		0.00	0.51	0.42	0.26	0.38 / 0.33
Phytosterol from rapeseed- or olive-oil					0.33–0.41						1.0					0.33 / 0.40	0.42
Stigmasterol	0.53	0.52					0.80			0.30	1.0		0.00				0.38
7-Dehydrocholesterol	0.94	0.88	0.48	0.67				0.9			0.7		0.39	0.57	0.56		
β-Cholestanol	0.56	0.63									1.1						
Ergosterol	0.95	0.84	0.43	0.68		0.47	0.60	0.0		0.37	0.6		0.00	0.56	0.00		0.89
Zymosterol													0.41	0.61	0.48		
Vitamin D_3			0.76	0.91									0.46	0.68	0.68		
Vitamin D_2			0.80	0.91									0.44	0.66	0.69		
Lumisterol													0.27	0.59	0.52		
Suprasterol II													0.36	0.52	0.57		
Tachysterol													0.00	0.00	0.00		
													0.37	0.61	0.58		
													0.98	0.98	0.98		
Vitamin A									about 0.9				0.93 / 0.98				
Vitamin A acetate									about 0.5			0.32					
α-Tocopherol												0.07	0.15	0.58			

(13) Paraffin/ethylene glycol monoethyl ether–*n*-propanol–methanol–water (35:10:30:25)[16].
(14) Paraffin/*n*-propanol–methanol–water (15:82:3)[16].
(15) Paraffin/methanol–water (95:5)[16].
(16) Petroleum b.p. 220–240°/pyridine–water (85:15)[12].
(17) Paraffin/acetic acid–water (84:16)[13–15].

The best separations of these highly fat-soluble sterols and vitamins have been obtained by reversed-phase chromatography, applying as stationary phase: silicone grease[8], petroleums fractions[12] or paraffinum liquidum[13,16].

In the analysis of fats and oils, a good method for the detection of small amounts of animal or vegetable fats in their mixtures is required[22]. Therefore, a paper chromatographic method for the identification of cholesterol, which occurs in animal fats, was badly needed. It is, however, not easy to obtain a complete paper chromatographic separation of cholesterol from the closely related phytosterols occurring in vegetable oils (mainly β-sitosterol and stigmasterol). Of all the systems mentioned in Table I, system No. 17: paraffin/acetic acid–water (84:16) gave the best separation of cholesterol from these phytosterols. The paper chromatographic separations of several sterols, vitamins and provitamins, that are possible with this system were more closely studied. Besides this, an attempt was made to establish more general rules for classifying and "explaining" the paper chromatographic data obtained from the literature and from our own experiments.

PAPER CHROMATOGRAPHIC DATA

Table II presents the R_F values, relative R_S values (S = cholesterol) and the corresponding R_M values of some sterols, fat-soluble vitamins, provitamins and pentacyclic triterpenoid alcohols in the above-mentioned system. The formulae of some of these sterols are given in Fig. 1.

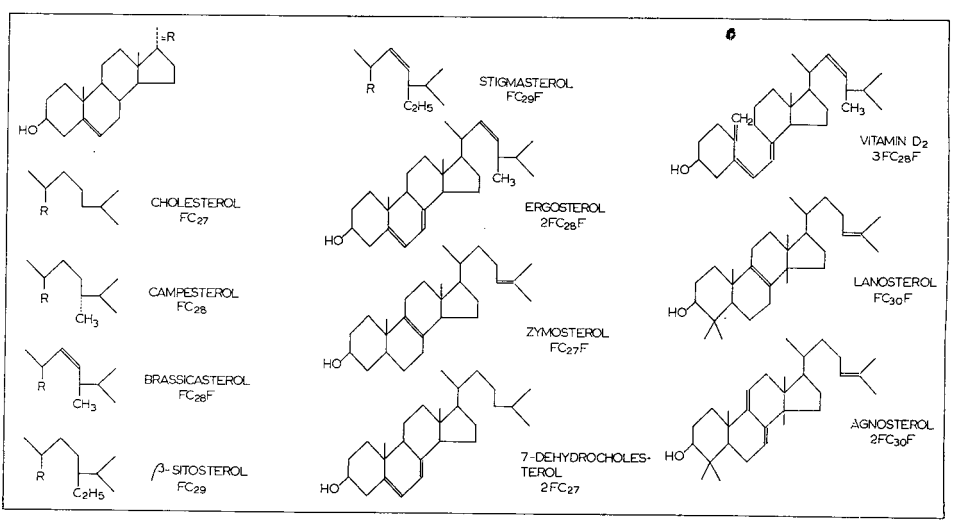


Fig. 1. The formulae of some sterols, mentioned in Table II.

Paper chromatographic reversed phase systems generally present a pure liquid–liquid partition chromatography. The relation between the R_F values or, even better the R_M values, and the number of carbon atoms of substances belonging to a homologous series should be more or less linear. This nearly linear relation is observed, for instance, when separating higher fatty acids in the system undecane/95 % acetic

TABLE II

THE R_F, R_S* (S = CHOLESTEROL) AND R_M** VALUES OF SOME STEROLS, VITAMINS, PROVITAMINS AND PENTACYCLIC TRITERPENOID ALCOHOLS IN THE SYSTEM PARAFFIN/ACETIC ACID–WATER (84:16)

Paper: Schleicher-Schüll 2043 b mgl.; temp. 22–24°.

Compound	*Formula*	*Abbr.*** *form.*	R_F *value*	R_S* *value*	R_M** *value*	R'_M† *calcd.*
Cholesterol	$C_{27}H_{46}O$	FC27	0.33	1.0	0.31	0.28
Δ^7-Cholestenol	$C_{27}H_{46}O$	FC27	0.33	1.0	0.31	0.28
7-Dehydrocholesterol	$C_{27}H_{44}O$	2FC27	0.40	1.22	0.18	0.20
Dihydrocholesterol (β-cholestanol)	$C_{27}H_{48}O$	C27	0.27	0.81	0.43	0.39
Coprostanol	$C_{27}H_{48}O$	C27	0.07	0.22	1.13	—
Epicholesterol	$C_{27}H_{46}O$	FC27	0.26	0.79	0.45	—
			(0.11	0.34	0.91)	
			(0.09	0.30	1.005)	
β-Sitosterol	$C_{29}H_{50}O$	FC29	0.25	0.75	0.48	0.52
Campesterol††	$C_{28}H_{48}O$	FC28	0.29	0.87	0.39	0.40
Stigmasterol	$C_{29}H_{48}O$	FC29F	0.27	0.83	0.43	0.41
Brassicasterol†† (and chalinasterol)	$C_{28}H_{46}O$	FC28F	0.33	1.0	0.31	0.29
γ-Sitosterol	$C_{28}H_{48}O$	FC28?	0.29	0.88	0.39	0.40
22-Dihydroergosterol	$C_{28}H_{46}O$	2FC28	0.34	1.04	0.29	0.32
Zymosterol	$C_{27}H_{44}O$	FC27F	0.43	1.31	0.12	0.17
Ergosterol	$C_{28}H_{44}O$	2FC28F	0.42	1.26	0.14	0.21
Vitamin D_2	$C_{28}H_{44}O$	3FC28F	0.38	1.15	0.21	0.26
Vitamin D_3	$C_{27}H_{44}O$	3FC27	0.36	1.10	0.25	0.25
Dihydrovitamin D_2	$C_{28}H_{46}O$	3FC28	0.29	0.87	0.39	0.37
Pyrocalciferol	$C_{28}H_{44}O$	2FC28F	0.31	0.94	0.35	—
Isopyrocalciferol	$C_{28}H_{44}O$	2FC28F	0.38	1.15	0.21	—
Lumisterol	$C_{28}H_{44}O$	2FC28F	0.33	1.00	0.31	—
Epilumisterol	$C_{28}H_{44}O$	2FC28F	0.37	1.12	0.23	—
Lanosterol	$C_{30}H_{50}O$	FC30F	0.22	0.66	0.55	0.53
Dihydrolanosterol	$C_{30}H_{52}O$	FC30	0.15	0.44	0.75	0.64
Agnosterol	$C_{30}H_{48}O$	2FC30F	0.19	0.59	0.63	0.45
Dihydroagnosterol	$C_{30}H_{50}O$	2FC30	0.18	0.54	0.66	0.56
Cholesterol acetate	$C_{29}H_{48}O_2$	—	0.09	0.25	1.00	—
β-Sitosterol acetate	$C_{31}H_{52}O_2$	—	0.06	0.17	1.20	—
Stigmasterol acetate	$C_{31}H_{50}O_2$	—	0.08	0.24	1.06	—
Ergosterol acetate	$C_{30}H_{46}O_2$	—	0.10	0.29	0.95	—
Cholesterol butyrate	$C_{31}H_{52}O_2$	—	0.04	0.12	1.38	—
7-Hydroxycholesterol	$C_{27}H_{46}O_2$	—	0.74	2.23	−0.46	—
Vitamin A	$C_{20}H_{30}O$	—	0.30	0.91	0.37	—
Vitamin A acetate	$C_{22}H_{32}O_2$	—	0.01	0.03	1.99	—
dl-α-Tocopherol	$C_{29}H_{50}O_2$	—	0.13	0.40	0.83	—

* The R_S value is calculated from R_F sterol/R_F cholesterol with R_S cholesterol = 1.0.

** $R_M = \log (1/R_F - 1)$.

*** The abbreviated formula FC28F means a sterol structure with 28 carbon atoms. The F before and after C_{28} corresponds to the number of double bonds in the sterol nucleus and in the side chain of the molecule respectively.

† These theoretical R'_M values are calculated from R_{M_0} and ΔR_M values.

†† These sterols were not available as pure preparations.

acid[18] and when separating cholesterol esters in the system paraffin/acetic acid–chloroform–paraffin oil (65:25:10)[19].

Analogously, an almost linear relation is obtained when the R_F values of some sterols are plotted against their number of carbon atoms (see Fig. 2).

DISCUSSION

The appearance of critical pairs

The introduction of a double bond produces an increase in the polarity of the molecule (and an increase in R_F value), which is approximately equal to that caused by a decrease in length of the carbon chain by one $-CH_3$ group. Therefore stigmasterol (FC29F), campesterol (FC28) and dihydrocholesterol (C27) have approximately equal R_F values.

Similar to the paper chromatographic separation of higher fatty acids[18], all sterols are arranged in critical pairs (mimic substances according to BUSH[23]). Brassicasterol (FC28F), cholesterol (FC27) and 7-dehydrocholesterol (2FC27), ergosterol (2FC28F), and zymosterol (FC27F) also belong to two different critical pairs.

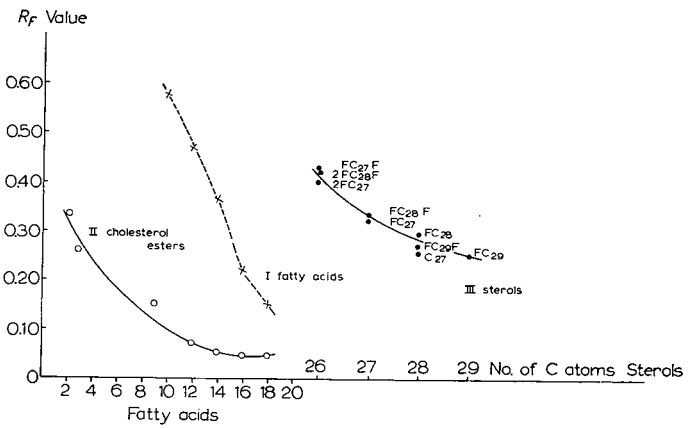


Fig. 2. The R_F values of higher fatty acids, cholesterol esters and sterols plotted against their number of carbon atoms. The higher fatty acids are separated in the system undecane/95% acetic acid[18] (curve I), the cholesterol esters in the system paraffin/acetic acid–chloroform–paraffin oil (65:25:10)[19] (curve II), and the sterols in the system paraffin/84% acetic acid (curve III).

The above-mentioned sterols are separated into four zones on a paper chromatogram, made according to the technique of MATTHIAS[32]. The lowest zone contains β-sitosterol, the second zone campesterol and stigmasterol, the third zone cholesterol and brassicasterol, and the fourth zone 7-dehydrocholesterol, ergosterol and zymosterol (see Fig. 5).

Each critical pair can be characterized by a paper chromatographic value, *pc.V.*, calculated as follows: *pc.V.* = number of carbon atoms minus number of double bonds[21]. In Table III all the sterols with the same *pc.V.* number are arranged horizontally, thus producing the various critical pairs.

The sterols belonging to the same zone may still present a slight difference in R_F

TABLE III

CRITICAL PAIRS OF NON-CONJUGATED STEROLS, CLASSIFIED BY THEIR *pc.V.* NUMBER

pc.V. = $n - M$ (n is the number of carbon atoms and M the number of double bonds in the molecule).

pc.V.	$M = 0$	$M = 1$	$M = 2$	$M = 3$	*Zone*	R_F *values*	R_M *values*
25			Zymosterol, FC27F 7-Dehydrocholesterol, 2FC27	Ergosterol, 2FC28F	4	0.39–0.44	0.10–0.19
26		Cholesterol, FC27	Brassicasterol, FC28F		3	0.32–0.35	0.27–0.32
27	Dihydrocholesterol, C27	Campesterol, FC28	α_1-Sitosterol, FC29F Stigmasterol, FC29F		2	0.26 0.30	0.37–0.45
28	Dihydrocampesterol, C28	β-Sitosterol, FC29	α_2-Sitosterol, FC30F Lanosterol, FC30F		1	0.21 0.26	0.45–0.58
29	Dihydrositosterol, C29	Dihydrolanosterol, FC30				0.14–0.19	0.63–0.79
30	Tetrahydrolanosterol, C30 Tetrahydro-α_2-sitosterol, C30					0.06–0.10	0.95–1.20

value. This difference is too small to permit a separation under normal conditions. We succeeded, however, in separating a mixture of zymosterol and ergosterol, using the circular technique described by SULSER[13].

A complete separation of cholesterol from the most important naturally occurring phytosterols (β-sitosterol and stigmasterol), which is necessary for the analysis of mixtures of animal and vegetable fats[22] and a separation of vitamin A and vitamin A esters can be obtained in the studied system.

The pentacyclic triterpenoid alcohols occurring in wool fat, lanosterol (FC30F) and dihydrolanosterol (FC30), can also be separated. It is, however, impossible to separate agnosterol (2FC30F) and dihydroagnosterol (2FC30), which both have another double bond. A complete separation of vitamin D_2 (3FC28F) and dihydrovitamin D_2 (3FC28), which both have three conjugated double bonds but differ by one non-conjugated double bond, is also impossible. Conjugated double bonds seem to have a strong influence on the R_F value of a sterol molecule.

Calculation of ΔR_M values

The introduction of a non-conjugated double bond into the molecule generally causes a decrease in R_M value. ($R_{M_2} - R_{M_1} = \Delta R_M{}^{C=C} = \ln \Delta\mu^{C=C}/RT$). The $\Delta R_M{}^{C=C}$ value varies between —0.05 and —0.20, with a mean value of —0.11 (see Table IV A and Fig. 4). This value agrees with the difference in the R_M value caused by the introduction of an extra $-CH_3$ group ($\Delta R_M{}^{CH_3} = +0.12$).

TABLE IV

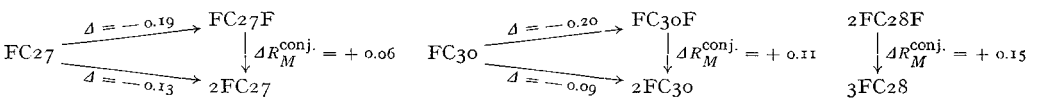
THE EFFECT OF THE INTRODUCTION OF ANOTHER DOUBLE BOND ON THE R_M VALUE

A. The molecule contains no double bond or only one			$\Delta R_M{}^{C=C}$
Dihydrocholesterol (C27)	⟶	cholesterol (FC27)	—0.12
Cholesterol (FC27)	⟶	zymosterol (FC27F)	—0.19
Campesterol (FC28)	⟶	brassicasterol (FC28F)	—0.08
β-Sitosterol (FC29)	⟶	stigmasterol (FC29F)	—0.05
Cholesterol (FC27)	⟶	7-dehydrocholesterol (2FC27)	—0.13
Dihydrolanosterol (FC30)	⟶	lanosterol (FC30F)	—0.20
Dihydrolanosterol (FC30)	⟶	dihydroagnosterol (2FC30)	—0.09
B. The molecule already contains two double bonds			$\Delta R_M{}^{C=C-C=C-C=C}$
1. Ergosterol (2FC28F)	⟶	vitamin D_2 (3FC28F)	+0.07
2. 7-Dehydrocholesterol (2FC27)	⟶	vitamin D_3 (3FC27)	+0.07
3. Lanosterol (FC30F)	⟶	agnosterol (2FC30F)	+0.08
4. Dihydroagnosterol (2FC30)	⟶	agnosterol (2FC30F)	—0.03
5. 22-Dihydroergosterol (2FC28)	⟶	dihydrovitamin D_2 (3FC28)	+0.08

If the molecule contains two (conjugated) double bonds, the introduction of a third one causes only a slight decrease in R_M value (see Table IV B, No. 4) or in most cases when the third double bond is conjugated, even an increase in R_M value.

By irradiation of 7-dehydrocholesterol and ergosterol with U.V. light, vitamins D_3 and D_2, respectively, are formed. When ring B of the cholesterol nucleus opens, producing a system of three conjugated double bonds, an increase in R_M value of about +0.07 is obtained.

In a critical pair, the sterols with several conjugated double bonds generally have higher R_M values than sterols with fewer conjugated double bonds or without any. The R_M values of dihydroagnosterol (2FC30), 7-dehydrocholesterol (2FC27) and dihydrovitamin D_2 (3FC28) are higher than those of lanosterol (FC30F), zymosterol (FC27F) and ergosterol (2FC28F) respectively.



From these examples a mean value of +0.10 caused by the presence of several conjugated double bonds can be calculated.

The agreement between the values: 0.07, 0.10, due to conjugation, and the $\Delta R_M^{CH_3}$ and $\Delta R_M^{C=C}$ values causes the existence of critical pairs. Introduction of a double bond as well as shortening of the carbon chain by removing one $-CH_2$ group results in the sterol being shifted one step to the left in the graph of Fig. 2 (*i.e.* to higher R_F values).

Introduction of another (conjugated) double bond when the molecule already contains two of them, will shift the R_F value one step to the right (*i.e.* to lower R_F values).

The same effect is produced when separated double bonds are "rearranged" into a system with (more) conjugated double bonds. If, for instance, cholesterol (FC27) is first converted into 7-dehydrocholesterol (2FC27) and this into vitamin D_3 (3FC27), the R_F values of cholesterol and vitamin D_3 will be very similar.

Consequently these sterols for the greater part follow the rule of MARTIN regarding the thermodynamic meaning and additivity of the R_M values. The validity of this rule in the field of the more polar adrenocortical steroids has been shown by BUSH[23].

The R_F value of a sterol molecule with a steric configuration related to 3β-cholestanol is predominantly determined by the total number of carbon atoms and the number of non-conjugated and conjugated double bonds in the molecule.

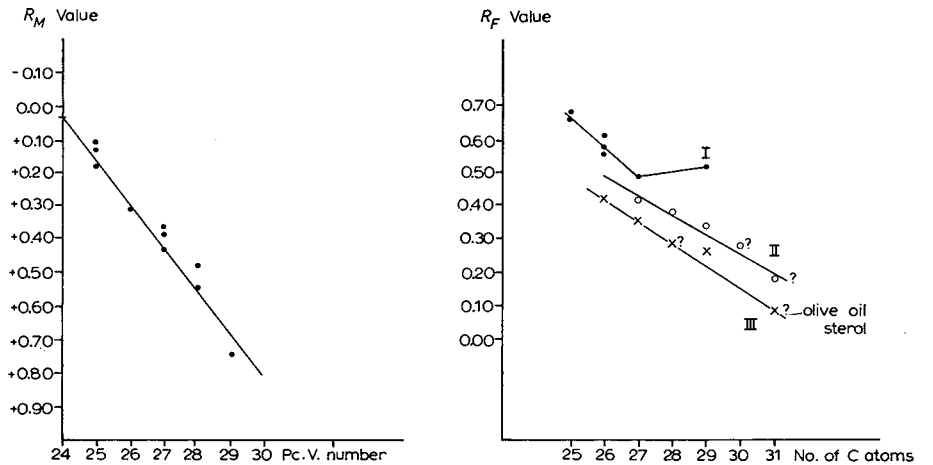


Fig. 3. (a) The R_M values of lanosterol and dihydrolanosterol fit into the linear relation obtained with the other sterols. (b) The linear relation of the R_F values in the systems described by KODICEK[16] (I), SULSER AND HÖGL[13] (II), and DE ZOTTI[12] (III).

The position of the double bonds in the molecule has no influence. Thus cholesterol (Δ^5) and Δ^7-cholestenol have the same R_F values. The place of attachment of the alkyl groups in the molecule has also no influence. Therefore the R_M values of the related pentacyclic triterpenoid alcohols lanosterol (FC30F), dihydrolanosterol (FC30), agnosterol (2FC30F) and dihydroagnosterol (2FC30), which all have two methyl groups at C-4, for the greater part agree with the above-mentioned rules.

Fig. 3a illustrates that the R_M values of the first two non-conjugated alcohols fit into a straight line with the R_M values of the other sterols.

From the graph of Fig. 3a and from the above-mentioned rules, the following ΔR_M values, characteristic for the studied system, can be calculated:

$$R_{M_0} = +0.03 \text{ (}pc.V.\text{ number} = 24\text{)}$$
$$\Delta R_M{}^{CH_3} = +0.12$$
$$\Delta R_M{}^{C=C} = -0.11$$

When two conjugated double bonds are formed:

$$\Delta R_M{}^{C=C-C=C} = -0.11 + \text{exaltation value A of } + 0.03 = -0.08$$

Introduction of another double bond, when the molecule already contains two of them, gives:

$$\Delta R_M{}^{C=C-C=C-C=C} = -0.11 + \text{exaltation value B of } + 0.19 = + 0.08$$

The theoretical R'_M values, mentioned in Table II, are calculated from R_{M_0} and ΔR_M values, according to the formulae:

$$R_{M_2} - R_{M_1} = \Delta R_M{}^x = \ln \Delta\mu^x/RT$$
$$R'_M\text{sterol} = R_{M_0} + n \ln \Delta\mu^{CH_3}/RT + m \ln \Delta\mu^{C=C}/RT + \text{exaltation values}$$

An example of such a calculation gives: R'_M dihydrovitamin D_2, 3FC28 = 0.03 + 4 × 0.12 — 3 × 0.11 + 0.19 = 0.37 (experimental value 0.39).

When the number of double bonds in a sterol molecule is plotted against the R_M values, sigmoid curves are obtained. This is illustrated in Fig. 4.

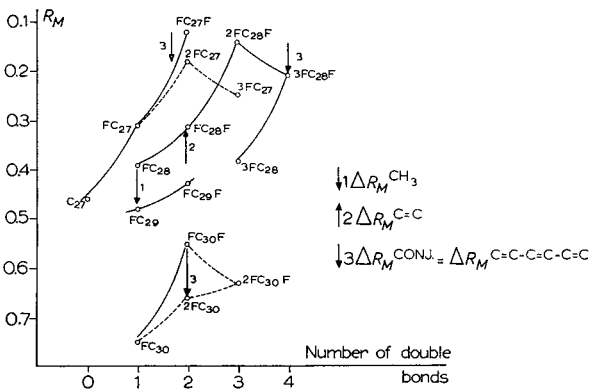


Fig. 4. Relation between the number of double bonds in a sterol molecule and the R_M value. The various ΔR_M values are presented.

APPLICATIONS

By applying these rules regarding the effect of various groups in the sterol molecule on the R_F value, important data concerning not yet identified sterol structures may be obtained.

KABASAKALIAN[24] recently calculated the ΔR_M values of several substituents in the pregnane nucleus. Using these values it is possible to calculate a theoretical R_F value of a steroid of known structure. Good agreement between calculated and experimental values has been achieved. Analogously, the structure of not yet identified steroids or naturally occurring sterols[25] can be determined by using these previously calculated ΔR_M values. Other paper chromatographic systems are useful for this purpose as well. The "R_F–carbon number" graphs of systems Nos. 14, 16 and 17, mentioned in Table I, also show a nearly linear relation (see Fig. 3b).

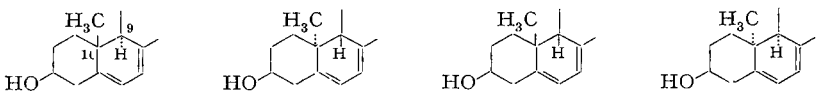
In system 14, the R_F values of the vitamins D_2 and D_3 also fit into the linear relation[16], unlike their behaviour in the system studied by us. The R_F value of β-sitosterol in system 14 is higher than might be expected from the linear relation. In the corresponding systems 13 and 15 of Table I, the deviating R_F values for β-sitosterol and ergosterol (R_F = 0.0) indicate non-ideal liquid–liquid partition chromatography.

In the analogous system: petroleum/pyridine–water (85:15)[12] a similar linear relation can be found (Fig. 3b). Only the R_F value of "fucosterol" obtained by DE ZOTTI[12] does not fit into this relation. This sterol, which has a structure isomeric with that of stigmasterol ($C_{29}H_{48}O$ with Δ^5 and Δ^{28}) should also present an R_F value corresponding with that of stigmasterol. According to curve III of Fig. 3b we should expect an R_F value of 0.37. The R_F value of about 0.41, obtained by DE ZOTTI for "fucosterol", points more in the direction of a sterol belonging to the critical pair: zymosterol–ergosterol.

The R_F values of some unidentified sterols from peanut and whale oil (0.28 and 0.18 respectively) reported by SULSER AND HÖGL[13] fit into the linear relation shown by the other sterols (curve II of Fig. 3b).

Separation of some sterols and steroids that have different steric configurations

Some provitamins such as lumisterol, isopyrocalciferol and pyrocalciferol are isomers of ergosterol. Because of their different steric configurations, they have, however, different R_S values.



	Ergosterol	Lumisterol	Isopyrocalciferol	Pyrocalciferol
	Configuration of the molecule: 3β-OH, juncture A/B: *trans*			
and	10β,9α	10α,9β	10β,9β	10α,9α
R_S value:	1.26	1.00	1.15	0.94

These data show that the R_S values of provitamins belonging to the (allo) series of β-cholestanol and possessing β-configuration at carbon atom 10 are always higher than these of the corresponding provitamins with α configuration at carbon atom 10. The separation of the provitamins with different configurations at carbon atom 10 *i.e.* ergosterol (10β,9α) and lumisterol (10α,9β), or isopyrocalciferol (10β,9β) and pyrocalciferol (10α,9α), is possible under favourable conditions. A separation of provita-

mins with the same configuration at C-10 cannot be obtained. The series of increasing R_S values is as follows:

$$10\alpha,9\alpha < 10\alpha,9\beta < 10\beta,9\beta < 10\beta,9\alpha$$

$$\Delta R_M\ 9\alpha,10\alpha \longrightarrow 9\alpha,10\beta = -0.21$$

$$\Delta R_M\ 9\beta,10\alpha \longrightarrow 9\beta,10\beta = -0.10$$

The lumisterol preparation that we had at our disposal showed also a second zone (R_S = 1.72), only visible by a greenish colour in U.V. light (365 mμ), and of unknown origin.

If the steric configuration of a sterol molecule is different from the (allo) series of β-cholestanol with respect to the configuration at C-5 at the juncture of rings A and B, the R_S values obtained deviate considerably. The R_S value of cholestanol (3β, equatorial OH group, juncture A/B: *trans* or 3β,5α) is considerably higher than that of coprostanol (3β, axial OH, A/B: *cis* or 3β,5β). Thus we can derive a difference in polarity of the molecule given by: 3β,5α > 3β,5β. ΔR_M 3β,5α $\longrightarrow$ 3β,5β = +0.70.

This corresponds with data in the literature[26] concerning C_{19} and C_{21} steroids, which show the polarity sequence: 3β,5α $\geqslant$ 3α,5β > 3β,5β $\geqslant$ 3α,5α.

In general, steroids with an equatorial OH group show a higher polarisy in partition chromatography than those with an axial OH group[26]. Thus cholesterol (3β equatorial) likewise has a higher R_S value than epicholesterol (3α axial). ΔR_M 3β $\longrightarrow$ 3α = + 0.14.

EXPERIMENTAL

Several hexagonal holes are cut out of a sheet of Schleicher and Schüll No. 2043b mgl paper of 20 × 50 cm, according to MATTHIAS[32] (see Fig. 5). The direction of the paper fibres should be parallel to the direction in which the mobile phase moves.

The paper is immersed three times in a 10 % solution of medicinal paraffinum liquidum in petroleum ether (b.p. 60–80°). After drying in the air the degree of impregnation is about 0.15 g/g paper. The stationary and mobile phases are mutually saturated. In the centre of the 1 cm wide bridges 15 μg sterol mixture is spotted. After 16 h accommodation, the chromatogram is developed with the mobile phase: acetic acid–water (84:16) for 40–48 h, by the ascending method. The temperature should be 22–24°; the length of the run 25–30 cm. After drying the chromatogram some sterols are visible in U.V. light (365 mμ) as fluorescent spots (see Table V). After drying for 2 h in the air and 1 h at 80°, the chromatogram is sprayed with a 10 % ethanolic solution of phosphomolybdic acid (Merck) and then heated for about 1–4 min at 80°.

Blue-green spots develop on a quickly darkening light green background.

Dihydrocholesterol and other saturated sterols, if present in quantities of 100 μg, become visible as yellow spots. By spraying with a mixture of ether–concentrated sulphuric acid (2:1) and heating 5–10 min at 80° all saturated sterols become visible as blue-green spots[27].

The R_F values mentioned in Table I were calculated from a great number of

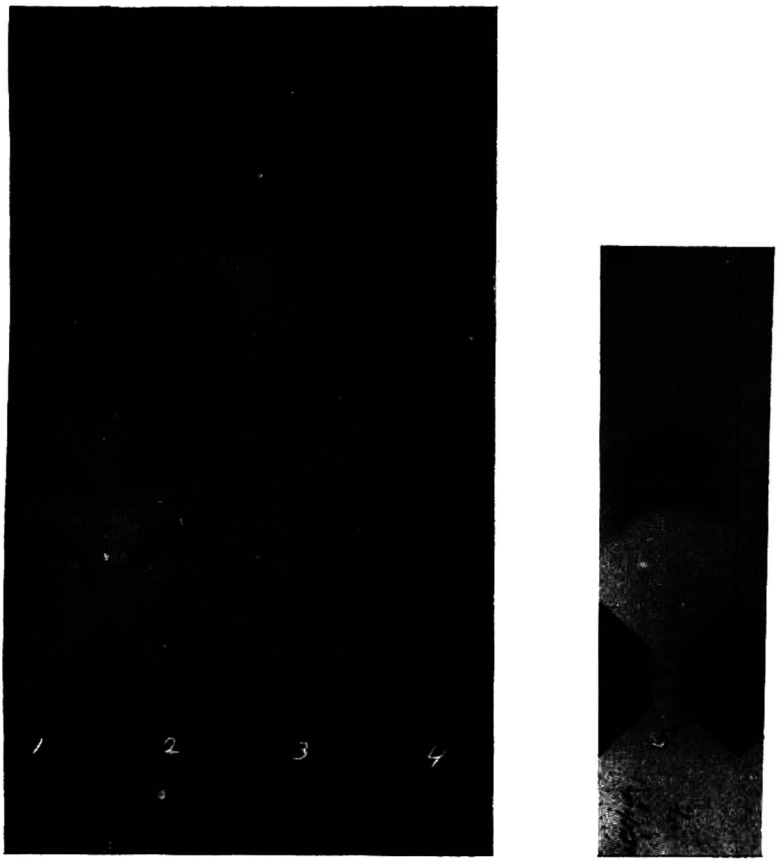


Fig. 5. Separation of some sterols in the system: paraffin/acetic acid–water (84:16). Spot 1: technical wool-fat alcohols, showing cholesterol, lanosterol and dihydrolanosterol. Spot 2: a mixture of ergosterol, cholesterol, stigmasterol, β-sitosterol, dihydrolanosterol and cholesteryl acetate. Spot 3: lumisterol. Spot 4: vitamin D_2. Spot 5: 15 μg of a mixture of cholesterol and phytosterols from soja oil (2:8).

determinations. The precision of the R_F values is approximately ± 0.01. The R_M values were calculated with the formula: $R_M = \log (1/R_F - 1)$.

Near the front some spots produced by oxidation products appear, *e.g.* 7-hydroxycholesterol.

Accompanying sterols in quantities of about 10–30% are demonstrated by spotting 100–400 μg sterol mixture on a sheet of Whatman No. 3 paper, cut in a similar way with bridges of 0.5 cm. Besides the principal spots some weakly coloured zones can be demonstrated.

Colour reactions

Besides the above-mentioned very sensitive detection with phosphomolybdic acid, more specific colour reactions are available for the identification of sterols belonging to the same critical pair.

TABLE V

COLOUR REACTIONS SHOWN BY SOME STEROLS

	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*
Compound	*U.V. light 365 mμ*	*$SbCl_3$*	*Sb_2Cl_5*	*$BiCl_3$*	*$CaCl_2$ in U.V. light*	*Trichloroacetic acid*	*Phosphotungstic acid*	*Silicotungstic acid*	*Dimethyl-p-phenylenediamine*	*Millon's reagent*	*Urea in U.V. light 365 mμ*	*$NaIO_4$-$KMnO_4$*
Cholesterol	—	violet	yellow-brown	violet	—	—	pink-violet	pink-orange	—	very faint yellow	—	—
β-Sitosterol	—	violet	very faint yellow	violet	—	—	pink-violet	pink-orange	—	—	—	—
Stigmasterol	—	faint violet	grey-purple	rose-violet	—	—	violet-brown	faint purple brown	—	—	—	—
Lanosterol	—	orange	yellow	orange	—	—	orange-yellow	orange-yellow	—	—	—(365mμ) +(256mμ)	yellow
Ergosterol	+	very faint purple	pink	—	++	faint purple	faint purple	faint purple-brown	very faint blue	yellow	++	—
Zymosterol	—	pink-brown	orange-yellow	faint yellow	faint	—	yellow-brown	yellow	blue	yellow	faint (365mμ) +(256mμ)	yellow
7-Dehydrocholesterol	+	purple-brown	brown-blue	faint purple-brown	+	green-purple	purple-brown	purple-brown	—	yellow	+	yellow
Vitamin D_2	+	brown	brown-blue	grey-brown	+	—	brown	brown	blue	faint yellow	—	yellow
Lumisterol	+	faint brown	purple	—	+	purple	purple-brown	purple-brown	blue	yellow	+	yellow

In the literature the following colour reactions are mentioned: with antimony (III) chloride[6], antimony (V) chloride[1,27,28], phosphotungstic acid[29], $SnCl_2$ with benzoyl chloride[6] and silicotungstic acid[30,31]. We have found some other colour reactions, *e.g.* with trichloroacetic acid, $CaCl_2$, $BiCl_3$, $NaIO_4$–$KMnO_4$, urea and dimethyl-*p*-phenylenediamine–*m*-tolylenediamine (1:1) also very useful.

The colour reactions are carried out on spots of 100 μg sterol on Whatman No. 3 paper. These more specific reactions, mentioned in Table V, can be used for the identification of not yet identified, naturally occurring sterols[25].

Execution of the colour reactions mentioned in Table V. The dried chromatogram is sprayed with:

II. A solution of antimony (III) chloride (50 % in ethanol). Heat 5–10 min at 70°. The colours produced in daylight (and in U.V. light of 365 mμ) are noted.

III. A solution of antimony (V) chloride (20 % in $CHCl_3$).

IV. A solution of bismuth (III) chloride (33 % in ethanol). Heat some seconds at 60°. Colours are produced in daylight and U.V. light.

V. A solution of $CaCl_2$ (50 % in ethanol–water) and heated 10–15 min at 80–90°. In U.V. light of 365 mμ fluorescent spots are visible.

VI. Moisten the chromatogram with trichloroacetic acid, dissolved in two drops of water.

VII. A solution of phosphotungstic acid (15% in ethanol). Heat some minutes at 60°.

VIII. A solution of silicotungstic acid (25% in ethanol). Heat some minutes at 60°.

IX. A mixture of dimethyl-*p*-phenylenediamine and *m*-tolylenediamine, 1:1 (1 % in water).

X. Millon's reagent (one part of mercury dissolved in two parts of concentrated nitric acid). Heat 2–4 min at 40–50°; spray again and heat.

XI. A solution of urea in water (50 %). Heat 10–50 min at 80°. In U.V. light of 365 mμ and 256 mμ, fluorescent spots appear.

XII. A solution of $NaIO_4$ (1 % in water). Spray after 5 min with 1 % $KMnO_4$.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to Dr. J. G. VAN GINKEL, Director of the Government Dairy Station (Rijkszuivelstation), for his encouragement and permission to publish this paper, and to Miss C. PUNSELIE for her able technical assistance. They thank the Unilever Research Laboratory, Vlaardingen, N.V. van Schuppen, Veenendaal, Koninklijke Nederlandse Gist- en Spiritusfabriek, Delft, N.V. Philips-Duphar, Weesp, and the Laboratories of Organic Chemistry and of Physical Chemistry of the University of Leiden, for their generous gifts of the sterols used in this work.

SUMMARY

The determination of R_F values of several sterols, fat-soluble vitamins, provitamins and pentacyclic triterpenoid alcohols in the system paraffin/acetic acid–water (84:16) is discussed.

There is a nearly linear relation between the R_M (R_F) values and the number of carbon atoms.

Some rules are given regarding the effect on the R_F value of the introduction of one or more double bonds into the molecule.

The ΔR_M values, due to the introduction of a CH_3 group, or of non-conjugated or conjugated double bonds into the molecule are calculated. Sterols with nearly the same R_F values are arranged in so-called critical pairs. Rules concerning these critical pairs are discussed.

In the system mentioned above the separation of the following sterols has been achieved: cholesterol–stigmasterol, β-cholestanol–coprostanol, cholesterol–epicholesterol, lanosterol–dihydrolanesterol and ergosterol–lumisterol, etc.

The system can be used for the identification of not yet identified sterols, isolated from natural sources.

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AUTOMATIC REGISTRATION OF ORGANIC ACIDS IN COLUMN CHROMATOGRAPHY

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(Received October 24th, 1960)

Column chromatographic analysis of organic acids generally requires laborious titration of a large number of fractions. In our laboratories an automatic apparatus has been developed by means of which the different fractions of organic acids are eluted in water immediately after they leave the column, whereby the pH of the solution is lowered below a set value for which the apparatus is adjusted. The lowering of the pH in the solution starts an automatic piston burette which adds NaOH until the pH of the solution again reaches the set value. The movements of the piston burette correspond directly to the amount of NaOH used to neutralize the acids and are recorded automatically.

APPARATUS

The following apparatus was used for the recording and the titration of the organic acids:

pH-stat: Titrator, Model TTT1, Radiometer Co., Copenhagen, the pH was measured using a combined electrode GK 202.

Piston burette: Piston burette, Model E 298, Metrohm, Herisau, equipped with a built-in special resistor.

Recorder: Brown potentiometer, Honeywell, Minneapolis.

Magnetic stirrer: Model E 184, Metrohm, Herisau.

PROCEDURE

Chromatography

The organic acids were placed on a silica gel column and separated according to the method of DONALDSON *et al.*[1]. The acids were eluted with a constantly increasing amount of *n*-butanol in chloroform according to the method of WREN[2].

Elution. In order to make titration of the organic acids possible these had to be eluted in water. A glass cup with an overflow pipe was used as extraction vessel (Fig. 1). When the *n*-butanol–chloroform dropped into the water, a magnetic stirrer kept the liquid surface in the extraction vessel in constant movement; the surface was further disturbed by an air current which passed through the vessel. Without

this air current breaking the surface, chloroform accumulated, markedly impairing the results of the extraction. Distilled water was continuously added to the extraction fluid at a speed twice that by which *n*-butanol–chloroform left the column, thus inhibiting the formation of buffers which might influence the pH changes of the water. A combined glass electrode was also placed in the extraction vessel.

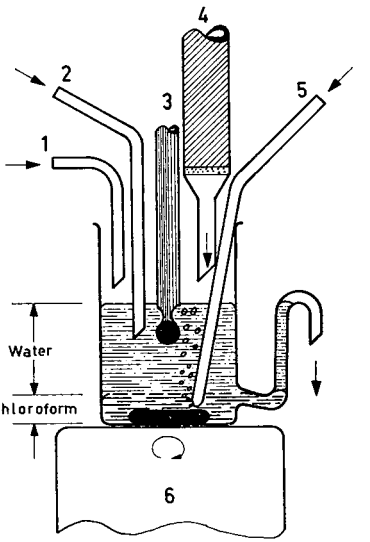

Fig. 1. Extraction vessel used for eluting in water the organic acids leaving the column. The vessel contains a glass electrode (3) for registering the acidity of the solution, one delivery tube for the titration fluid (2), one for the air current (5) and one for the addition of water (1), and a magnetic stirrer (6). The chromatography column (4) also empties into the vessel.

Titration and registration. When the acids are eluted and the pH of the solution has decreased below the threshold value at which the pH-stat is adjusted, an electric circuit is closed which starts the piston burette and 0.005 *N* NaOH is added until the initial pH is reached. The pH-stat was usually adjusted in such a way that the titration started when the pH fell below 6.9. Fig. 2 shows schematically the whole set-up of the apparatus for elution, titration and registration.

A pH meter with adjustable set point, and provided with an integral relay, was employed for pH measurement and automatic titration. As the voltage ratings of controller and piston burette were different, the latter was connected with the control output of the pH meter through another relay, which was added to the pH meter. Thus, manual control of the piston burette also remained possible. For the purpose of recording the position of the piston at a given time (and thus also the added NaOH volume) a 5000-ohm linear precision resistor coil (R_1) was mounted in the piston burette housing, its sliding contact rigidly connected with the piston feed mechanism, so that a well-defined position of this contact on the resistor is obtained for each piston position. This resistor transmitter (R_1) is supplied with direct current from a

1.5-V battery through resistor R_3 and voltage divider R_2. The latter is used to adjust the voltage across resistor R_1 and therefore also serves to position the pen of the connected potentiometer recorder at a predetermined value prior to the experiment. When the piston burette is put into operation by the pH controller and by the intermediate relay, the sliding contact of the transmitter resistor will move and cause a

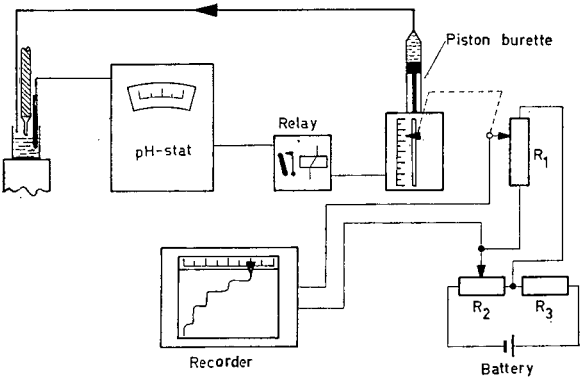

Fig. 2. Schematic outline of the apparatus for automatic registration of organic acids separated by column chromatography.

voltage corresponding to the piston position to appear at the terminals of the recorder. The latter will then continuously record the NaOH consumption in terms of time during the titration process, which lasts about 8 hours.

The recorder was adjusted to give maximum deflection when the piston burette moved from 0 to 10 ml. The burette could be replaced by others with greater or smaller volumes according to the amounts of acids present. This method has advantages over one where the concentration of the NaOH is altered, since a strong base easily causes overtitration.

This apparatus can be applied for the registration of basic or acidic compounds separated by column chromatography. A similar apparatus for purposes other than chromatography has been described by JACOBSEN *et al.*[3].

Organic acids in physiological solutions

In our laboratories the above apparatus has been used for determinations of organic acids in blood and physiological incubation fluids. The material to be analysed was deproteinized with perchloric acid, neutralized with potassium hydroxide and the organic acids eluted from the solution with ether according to SWIM AND UTTER[4]. The acids were then neutralized with NaOH and evaporated to dryness in a vacuum exsiccator at room temperature. The dry residue was acidified with 0.1 *N* H_2SO_4, adsorbed on 1 g silicic acid and placed on a silica gel column[1]. The eluted acids were titrated with 0.005 *N* NaOH. Fig. 3 shows an example of a chromatographic analysis of organic acids after an incubation of rat liver slices for 2 hours in a physiological medium.

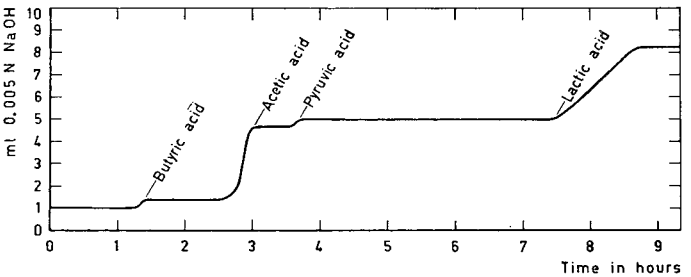

Fig. 3. Example of an automatic recording of organic acids formed in rat liver slices incubated for 2 h in physiological saline solution.

SUMMARY

An automatic apparatus for the registration of organic acids separated by column chromatography is described. An example is given of the determination of organic acids from a physiological incubation fluid. The method can be applied for the registration of other titratable acids and bases.

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RIFOMYCIN

XII.* CHROMATOGRAPHIC STUDIES OF SOME PRODUCTS OF TRANSFORMATION OF RIFOMYCIN B**

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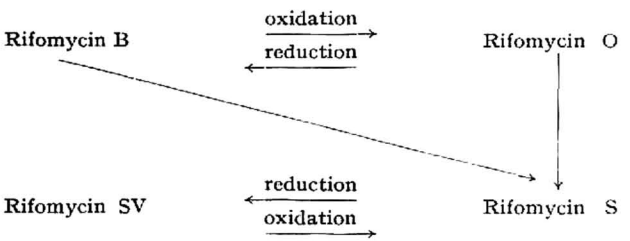
(Received October 14th, 1960)

Rifomycin B is a new antibiotic of clinical interest, which has been isolated by SENSI *et al.*[1,2]. When treated with oxidizing agents, rifomycin B is transformed into rifomycin O, which can be again reduced to rifomycin B[3]. Both rifomycin B and rifomycin O in aqueous solutions show the interesting property of undergoing a transformation into another substance with a higher antibiotic activity, rifomycin S[4]. Rifomycin S can be transformed by mild reduction with ascorbic acid into rifomycin SV[4].

In short, we have already proved that the relationships amongst the above-mentioned antibiotics can be represented by the following scheme:



In the course of chromatographic studies aimed at developing test methods for the antibiotics of this family, we encountered some difficulties due to the ease with which each substance is transformed into the others.

The activation process of rifomycin B and rifomycin O was followed by paper chromatography in order to detect the newly formed compound. *n*-Amyl alcohol–*n*-butyl alcohol (9:1), saturated with an aqueous solution containing ascorbic acid, was currently used as solvent system. The addition of ascorbic acid proved to be necessary in order to make the system reducing. With this method, the only antibiotics separated by paper chromatography are rifomycin B and rifomycin SV.

Figs. 1 and 2 represent the paper chromatography of rifomycin B, rifomycin O, rifomycin S and rifomycin SV, the solvent system being *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6, without and with 0.1 % sodium

* Part. XI: P. MARGALITH AND G. BERETTA, *Mycopathol. et Mycol. Appl.*, 13 (1960) 321.
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ascorbate. In presence of sodium ascorbate (Fig. 2), rifomycin B and rifomycin O give spots with the same R_F value (0.40); these spots are due to rifomycin B. Rifomycin S and rifomycin SV behave in the same fashion, each presenting a spot with the same R_F value (0.87); these spots are due to rifomycin SV.

Using the same solvent system without sodium ascorbate (Fig. 1), rifomycin B develops two spots and this is also the case with rifomycin O. It is assumed that the first spot (R_F 0.25) is rifomycin B, also when rifomycin O is the starting material.

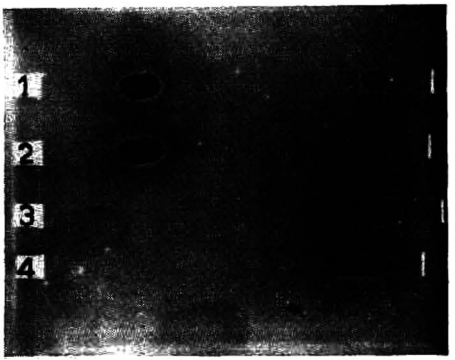

Fig. 1. (1) Rifomycin B; (2) rifomycin O; (3) rifomycin S; (4) rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

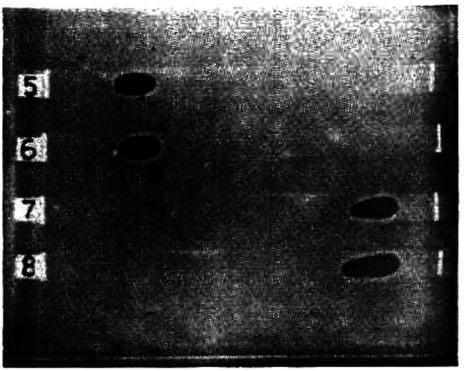

Fig. 2. (5) Rifomycin B; (6) rifomycin O; (7) rifomycin S; (8) rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate.

This assumption is supported by experiments which demonstrate the possibility of rifomycin O being transformed into rifomycin B by the mild reducing power of the chromatographic paper. The second spot (R_F 0.73) is caused by the conversion of rifomycin B or rifomycin O into rifomycin S. Furthermore, we have demonstrated that rifomycin S can be reduced by chromatographic paper to rifomycin SV; no doubt the second spot must be due to rifomycin SV.

The R_F values obtained with the solvent systems with and without sodium ascorbate do not correspond exactly. We suppose that the differences are caused by the presence of sodium ascorbate in the first solvent system. These differences are even more marked if the percentage of sodium ascorbate is increased.

The reduction of rifomycin O to rifomycin B and of rifomycin S to rifomycin SV by the very mild reducing properties of chromatographic paper was demonstrated by the following experiments.

A methanolic solution of rifomycin O was micropipetted onto four Whatman No. 1 paper strips (50 γ on each strip) and chromatographed using as solvent system *n*-amyl alcohol–*n*-butyl alcohol (9:1), saturated with phosphate buffer pH 8.6. The spots corresponding to R_F 0.25 were cut out, collected and eluted with 25 ml methanol. The methanolic solution was concentrated under vacuum to 5 ml. The visible spectrum of this solution clearly shows the characteristics of rifomycin B (Fig. 3).

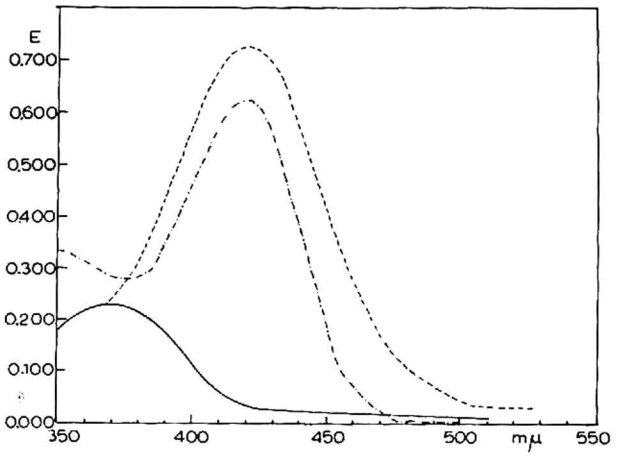

Fig. 3. Reduction of rifomycin O to rifomycin B by chromatographic paper. Visible spectra in methanolic solution at 40 γ/ml concentration of: ——— rifomycin O; - - - - - rifomycin B; —·—·— rifomycin O after paper chromatography.

The same experiment was performed with rifomycin S. The spectrum of the methanolic eluate from four chromatographic strips is identical with that of rifomycin SV (Fig. 4). Elution must be performed very carefully in absence of oxygen, to avoid the possibility of the eluted rifomycin SV being oxidized to rifomycin S. In this case the resulting spectrum of the eluate is a combination of the two forms — one oxidized and one reduced — of the antibiotic.

Various attemps to separate rifomycin O from rifomycin B and rifomycin S from rifomycin SV failed because of the reducing power of the chromatographic paper.

Rifomycin B can be separated from rifomycin SV by means of several solvent

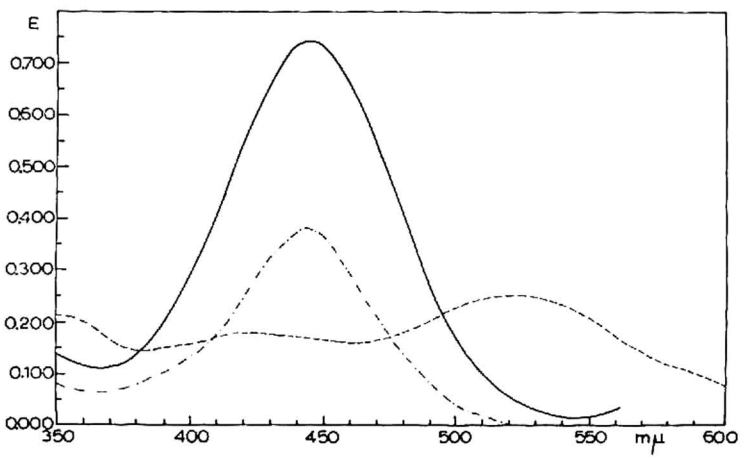

Fig. 4. Reduction of rifomycin S to rifomycin SV by chromatographic paper. Visible spectra in buffer solution pH 7.3 at 40 γ/ml concentration of: ——— rifomycin SV; - - - - - rifomycin S; —·—·— rifomycin S after paper chromatography.

systems. The most suitable systems that we employed for routine analyses are the following:

(1) *n*-Amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate. The ratio amyl alcohol–butyl alcohol is not critical; the small percentage of butyl alcohol markedly increases the mobility of the two antibiotics. The pH value of the aqueous phase must be neutral or slightly alkaline. In fact, both rifomycin B and rifomycin SV are acidic substances of different strength. Rifomycin B is a dibasic acid whilst rifomycin SV is a monobasic acid. Therefore, it is obvious that at a neutral or slightly alkaline pH, rifomycin SV has a higher mobility in organic solvents than rifomycin B (rifomycin B, R_F = 0.40; rifomycin SV, R_F = 0.87) (Fig. 2).

(2) Phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate, saturated with *n*-amyl alcohol–*n*-butyl alcohol (9:1). In this aqueous solvent rifomycin B shows a higher mobility than rifomycin SV (rifomycin B, R_F = 0.67; rifomycin SV, R_F = 0.51) (Fig.5).

(3) *n*-Butyl alcohol saturated with phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate (rifomycin B, R_F = 0.75; rifomycin SV, R_F = 0.95) (Fig. 6).

(4) Phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate saturated with *n*-butanol (rifomycin B, R_F = 0.86; rifomycin SV, R_F = 0.67) (Fig. 7).

All the chromatographic experiments were performed in glass jars, saturated for 6 hours at 22° with the solvent system. Whatman No. 1 paper was dipped in the aqueous buffered phase and dried. About 18 hours were necessary for a chromatographic run of 25 cm if the organic solvents were employed and, only 6 hours if the aqueous solvents were used. The strips were dried at room temperature and developed on agar plates buffered at pH 5.9 using *Sarcina lutea* as the micro-organism test. The optimal quantities of the two antibiotics were about 1 γ of rifomycin SV and 5–10 γ of rifomycin B for each strip.

CENTRIFUGAL CIRCULAR CHROMATOGRAPHY

In order to avoid the activation of rifomycin B in the course of the chromatographic run, centrifugal circular chromatography[5] was employed. With this technique, the time of a chromatographic run is greatly reduced (about 4 min). Rifomycin B was micropipetted at 3 cm from the center of the paper disk (Whatman No. 1). The paper disk was placed on a circular, stainless steel frame in a saturated air-tight chamber and rotated at about 1500 r.p.m. while a thin flow of solvent was aimed at a point about 2 cm from the center. The solvent reached the border of the disk in 4 min. The paper disk was dried and then placed for 15 min on the agar assay plates buffered at pH 5.9 using *Sarcina lutea* as the micro-organism test.

Rifomycin B could be separated from rifomycin SV by using *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

The quantities of rifomycin B and SV were 1 γ and 0.15 γ, respectively. There was no evidence of "activation" of rifomycin B although the solvent system did not contain sodium ascorbate as stabilizer (Fig. 8).

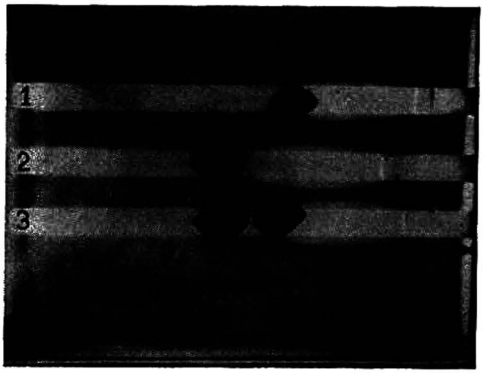

Fig. 5. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate saturated with *n*-amyl alcohol–*n*-butyl alcohol (9:1).

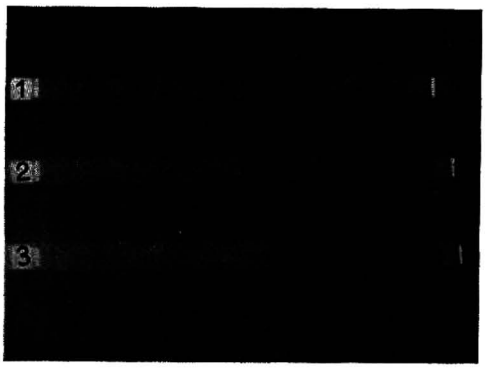

Fig. 6. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: *n*-butyl alcohol saturated with phosphate buffer pH 7.3 containing 0.1 % sodium ascorbate.

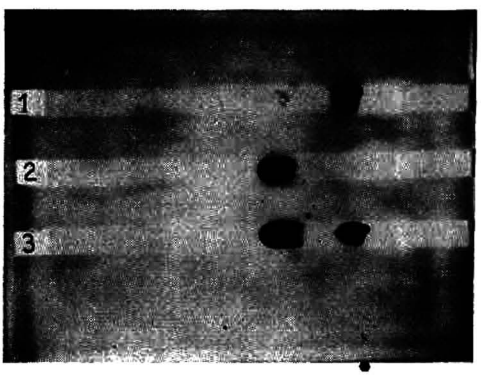

Fig. 7. (1) Rifomycin B; (2) rifomycin SV; (3) rifomycin B and rifomycin SV. Solvent system: phosphate buffer pH 8.6 containing 0.1 % sodium ascorbate saturated with *n*-butanol.

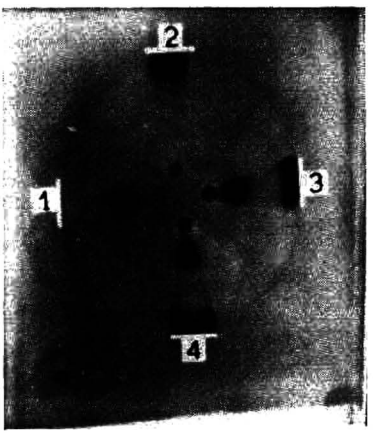

Fig. 8. Centrifugal circular chromatography. (1) Rifomycin B; (2) rifomycin SV; (3, 4) rifomycin B and rifomycin SV. Solvent system: *n*-amyl alcohol–*n*-butyl alcohol (9:1) saturated with phosphate buffer pH 8.6.

THIN-LAYER CHROMATOGRAPHY

The difficulties encountered in the detection of rifomycin O and rifomycin S by paper chromatography mentioned above, can be overcome by using thin-layer chromatography according to STAHL[6–11].

The plates were prepared as described earlier by one of us[12], using 25 g of Silicagel G (a mixture of Silicagel and calcium sulfate) and 50 g of water. A thin layer of Silicagel was obtained on glass plates by a roller machine. The plates were dried at

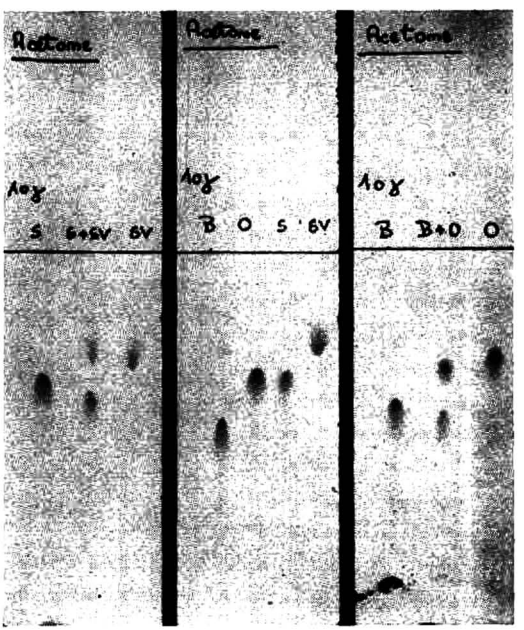

Fig. 9. Thin-layer chromatography of rifomycin B, O, S and SV.

105–110° for 30 min and cooled in a desiccator over Sikkon. The thin layer is fixed on the glass plates by the calcium sulfate. The activity of the Silicagel layer is equivalent to about II–III degrees.

The separation of rifomycin B from rifomycin O and of rifomycin SV from rifomycin S can be achieved by using acetone as solvent (Fig. 9).

10 γ of each of the antibiotics rifomycin B (in methanol solution), O, S and SV (in acetone solution) were applied at 2 cm from one end of the plate at distances of 1 cm. After drying for a short time the end was placed into an air-tight jar (saturated for 6 h with the solvent) containing a 1 cm layer of acetone at the bottom. After a run of 10 cm (15 min) the plate was removed and air-dried. Rifomycin B, O and SV are visualized as clear yellow spots, rifomycin S as a red-violet one.

Fig. 9 shows a good separation of rifomycin B from rifomycin O and of rifomycin S from rifomycin SV. The R_F values of rifomycin O and rifomycin S are about the same and no clear separation occurs with acetone. A mixture of these antibiotics can nevertheless be detected since rifomycin S is red and rifomycin O yellow. In this case a yellow spot appears with a red halo.

SUMMARY

Several chromatographic techniques were used in order to separate rifomycin B, rifomycin O, rifomycin S and rifomycin SV.

Rifomycin B and rifomycin SV can be easily separated by paper chromatography using organic or aqueous systems containing ascorbic acid as stabilizer.

Rifomycin O and rifomycin S are reduced by the paper during the chromatographic run, to rifomycin B and rifomycin SV respectively.

The four antibiotics can be separated by means of thin-layer chromatography on Silicagel.

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THE ANION EXCHANGE BEHAVIOUR OF YTTRIUM, NEODYMIUM AND LANTHANUM IN DILUTE NITRIC ACID SOLUTIONS CONTAINING ETHANOL

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The anion exchange behaviour of the rare earths in nitric acid has been investigated by DANON[1]. These elements were found to be adsorbed to a slight extent by Dowex-1 from concentrated nitric acid solutions, but the small differences between the values of the distribution coefficients did not allow efficient separations. Since replacement of part of the aqueous phase by a non-aqueous component has been shown to enhance anion exchange adsorption and resolution of inorganic ions[2,3], the anion exchange behaviour of some rare earths (Y, Nd and La) in dilute nitric acid solutions containing ethanol has been investigated.

EXPERIMENTAL

Materials and column operation

Resin: Dowex-1, 8X, 50–100 mesh, nitrate form.

Size of resin bed: 22 × 1.9 cm.

Sample: Mixture of specpure rare earth nitrates equivalent to 1 mg each of Y, Nd and La.

Addition of sample to column: Sample was dissolved in 10 ml HNO_3–ethanol solution of the same concentration to be used as eluant and placed in the column head. This solution was allowed to run into the resin bed at a flow rate of 1 ml/min. The resin bed had previously been equilibrated with 300 ml HNO_3–ethanol solution of the same concentration as used for dissolving the rare earth nitrates.

Elution: When the liquid level in the column had almost reached the top of the resin bed, elution was commenced with the appropriate HNO_3–ethanol eluant. Elutions were carried out at room temperature.

The following eluant systems were investigated:

(a) 0.8 *N* HNO_3 and varying concentrations of ethanol (0, 20, 40 and 80 %).

(b) 80 % ethanol and varying concentrations of HNO_3 (0.16, 0.8 and 1.6 *N*).

Solutions of HNO_3 above 1.6 *N* were not employed since they oxidised the ethanol to acetaldehyde.

Eluant solutions were prepared by diluting the proper volume of concentrated HNO_3 to the mark in a standard flask with the appropriate concentration of ethanol.

The rate of flow of eluant through the column was 1 ml/min and the effluent was collected in 20-ml fractions. These fractions were transferred to 80-ml porcelain evaporating basins and taken to dryness on a "low" hot plate.

Spectrographic monitoring of effluent fractions

The following spectrographic conditions were employed for examining the effluent fractions:

Electrodes: The lower electrode (anode) was a flat top NCC regular grade graphite 3/16 in. diameter rod.

The upper electrode was a pointed Champion "ship" carbon 5-mm diameter rod.

Loading electrodes: Flat topped electrodes were rubbed with a circular downward motion around the bottom and sides of the evaporating dishes.

Spectrograph: Hilger (E492) large quartz and glass; glass optics; wavelength range 3,800–5,300 Å; slit width 0.0025 mm; slit height 3 mm; Hilger F958 quartz lens focussed on slit. Kodak 103-0 plate. Samples were arced to completion at 4.5 A DC. Plates were developed for 4½ min in Kodak D19b developer at 20°.

Construction of semiquantitative elution curves

A semiquantitative measure of the concentration of a rare earth in the effluent fractions was obtained by visually estimating the relative intensity of a suitable spectrum line of the element (Y 4374.94; Nd 4303.57; La 4333.73) using an arbitrary 7-stepped spectrum line as a source of reference. Semiquantitative elution curves were constructed by plotting rare earth relative intensities against effluent volume.

RESULTS AND DISCUSSION

Fig. 1 illustrates the results obtained when a series of ethanol concentrations containing 0.8 *N* HNO_3 were employed as eluants. As the ethanol concentration was increased, rare earths showed increased adsorption by the resin. Nd and La were so firmly adsorbed by Dowex-1 from 80% ethanol containing 0.8 *N* HNO_3 that an eluant in which the adsorbed rare earth "complexes" were no longer stable had to be employed for their removal. Water elution was used for this purpose.

From the elution curves obtained, the volume distribution coefficients (D_v) were evaluated from the familiar relationship

$$D_v = V_{max} - i$$

where V_{max} is the number of column volumes of eluant required to obtain an element in maximum concentration in the effluent and i is the fractional interstitial volume (*ca.* 0.4). D_v values (estimated to within $\pm$ 30%) are shown in Table I.

Similar results have been obtained by DANON[4] and MARCUS AND NELSON[5] using acidified nitrate solutions. These workers found the addition of a soluble nitrate such as $LiNO_3$ to a dilute HNO_3 solution resulted in the enhanced adsorption of rare earths

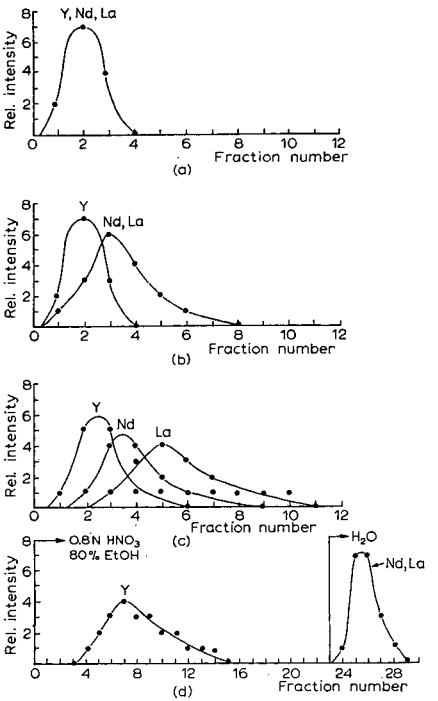

Fig. 1.* The elution of Y, Nd and La from a 22 × 1.9 cm column of Dowex-1,8X, 50–100 mesh resin with 0.8 *N* HNO_3 containing various concentrations of ethanol. Relative intensity expressed in arbitrary units. (a) 0.8 *N* HNO_3–0% EtOH; (b) 0.8 *N* HNO_3–20% EtOH; (c) 0.8 *N* HNO_3–40% EtOH; (d) 0.8 *N* HNO_3–80% EtOH. Flow rate, 1 ml/min. Volume/fraction, 20 ml.

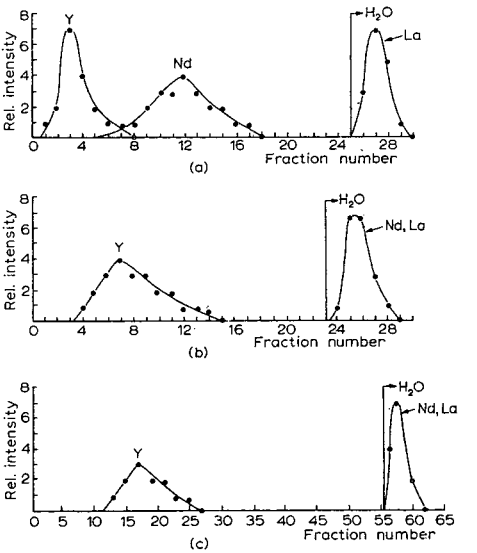

Fig. 2.* The elution of Y, Nd, and La from a 22 × 1.9 cm column of Dowex-1,8X, 50–100 mesh resin with 80% ethanol containing various concentrations of HNO_3. Relative intensity expressed in arbitrary units. (a) 80% EtOH–0.16 *N* HNO_3; (b) 80% EtOH–0.8 *N* HNO_3; (c) 80% EtOH–1.6 *N* HNO_3. Flow rate, 1 ml/min. Volume/fraction, 20 ml. *Note*: In (c) points have been plotted every 40 ml.

by strong-base anion exchange resins. Adsorbabilities increased with increasing $LiNO_3$ concentration, the lighter rare earths being more strongly adsorbed than the heavier earths.

From Fig. 1 it can also be seen that enhanced resolution of Y, Nd and La occurred with increasing ethanol concentration.

The elution curves obtained using 80% ethanol containing various concentrations of HNO_3 are shown in Fig. 2. Rare earths showed increased adsorption by the resin with increasing HNO_3 concentration. La (in 80% ethanol containing 0.16, 0.8 and 1.6 *N* HNO_3) and Nd (in 80% ethanol containing 0.8 and 1.6 *N* HNO_3) were so firmly adsorbed by Dowex-1 that water elution had to be employed for their removal.

D_v values (estimated to within ± 30%) are given in Table II.

MARCUS AND NELSON[5] found the adsorption of rare earths from $LiNO_3$–HNO_3

* Since overlapping rare earths had very similar relative intensities, their elution has been shown by means of single curves in Figs. 1 and 2.

TABLE I

Eluant	D_v		
	Y	*Nd*	*La*
0.8 *N* HNO_3	0.2	0.2	0.2
0.8 *N* HNO_3–20% EtOH	0.2	0.5	0.6
0.8 *N* HNO_3–40% EtOH	0.4	0.7	1.2
0.8 *N* HNO_3–80% EtOH	1.8	$\gg 5$	$\gg 5$

TABLE II

Eluant	D_v		
	Y	*Nd*	*La*
0.16 *N* HNO_3–80% EtOH	0.6	3.4	$\gg 5$
0.8 *N* HNO_3–80% EtOH	1.8	$\gg 5$	$\gg 5$
1.6 *N* HNO_3–80% EtOH	5.0	$\gg 5$	$\gg 5$

to be independent of acidity at low acid concentrations (10^{-4}–10^{-2} *N*). Increasing the acid concentration resulted in decreased adsorption of rare earths.

The rare earth elution sequence obtained with ethanol–HNO_3 systems was according to size

$$Y^{3+} > Nd^{3+} > La^{3+}$$

$$(r = 0.92 \text{ Å}) \; (r = 1.04 \text{ Å}) \; (r = 1.14 \text{ Å})$$

and accorded to that obtained with HNO_3–$LiNO_3$ elution[4,5].

Elution with 0.8 *N* HNO_3–80% ethanol or 1.6 *N* HNO_3–80% ethanol would appear admirably suited for preparing "light" and "heavy" concentrates from mixtures of rare earths.

Although the separation of neighbouring rare earths was not investigated, gradient elution techniques would appear to offer possibilities for effecting such separations.

ACKNOWLEDGEMENTS

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SUMMARY

The adsorption and resolution of Y, Nd and La by a strong-base anion exchange resin from dilute nitric acid–ethanol solutions was examined.

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THE CHROMATOGRAPHY OF PHOSPHATIDES ON SILICIC ACID-IMPREGNATED FILTER PAPER*

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The techniques of paper chromatography have enjoyed wide usage in biochemical research and can be expected to facilitate the solution of many difficult analytical problems. This report is concerned with our analysis of some of the more pertinent variables evident in the chromatography of phosphatides on silicic acid-impregnated paper. Our understanding of the limitations of various solvent combinations and of the characteristics of a variety of silicic acid-impregnated cellulose filter papers has provided us with a versatile system of analysis which we have extensively employed on a number of problems to be reported elsewhere.

The desirability of utilizing several solvent systems has already been discussed[1]; in the present communication we will describe a neutral system (2,6-dimethyl-4-heptanone–methanol–water), an acidic system (2,6-dimethyl-4-heptanone–acetic acid–water), and a basic system (2,6-dimethyl-4-heptanone–pyridine–water) and will demonstrate the necessity of employing all three solvent systems for the thorough analysis of the phosphatides.

MATERIALS AND METHODS

1. *Chromatography jars*

Three sizes of chromatography jars were used:

(a) 35 × 200 mm (Kimble No. 20065) contained 15 ml of solvent mixture. Short runs were made (1–2 ½ h) using 30 × 140 mm paper strips cut at the bottom as shown in Fig. 1a to prevent their clinging to the vessel wall. Trimming in this way resulted in more uniform movement of the solvent. These vessels were capped by polyethylene stoppers, through which a stainless steel wire projected to serve as a hanger for the paper. The principal use of these small jars was to determine the optimal ratio of solvent components necessary for the desired chromatographic separation (*cf.* Figs. 2–5) and were therefore used in batteries of 4–7 as necessary.

* This investigation has been supported by grants HTS5133 and H2564 from the National Institutes of Health, United States Public Health Service.

(b) 150 × 300 mm (Corning No. 6942) contained 200 ml of solvent and employed 140 × 140 mm paper; these jars, capped by a glass plate containing a center hole for the hanger, were used for our normal analytical run of 2–6 h (*cf.* Fig. 1b, right-hand jar).

(c) 150 × 450 mm (Corning No. 6942) contained 200 ml of solvent and employed 140 × 290 mm paper; these jars were used when runs longer than 6 h were necessary for greater separation of the various phosphatides (*cf.* Fig. 1b, left-hand jar).

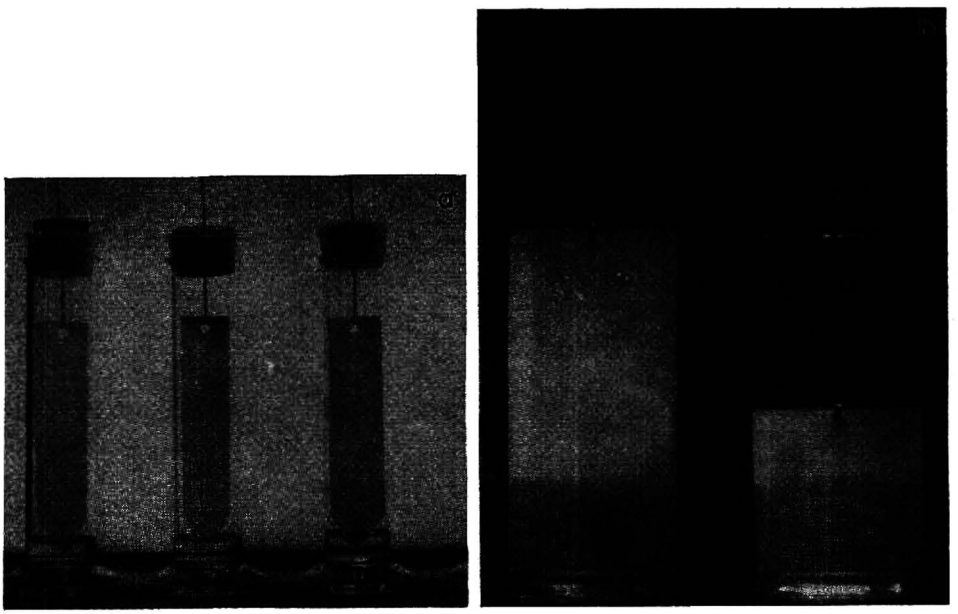

Fig. 1. (a) Chromatogramming in the small jars; normally only one sample was placed on each paper; however, with care to keep the area of sample application small, 2–3 extracts could be quite satisfactorily examined on each paper. (b) 6–8 samples were chromatogrammed on the wide papers shown here; our experience has indicated that an intermediate length paper would probably be the most generally useful.

2. *Impregnation of paper*

Potassium silicate was freshly prepared by slowly (during 5 min) pouring a slurry of 200 g silicic acid (100 mesh powder, Mallinckrodt or Baker Analytical reagent) in 400 ml water into a freshly prepared solution of potassium hydroxide (113 g Baker Analyzed Reagent in 400 ml H_2O) using a 2 l beaker and stirring briskly with a teflon-covered magnetic stirrer. This solution was cooled to room temperature, diluted to 1 l, and filtered through pyrex wool into a 220 × 340 mm pyrex tray. The filter paper (140 × 290 mm) was immersed in this solution for 5 min, let drain vertically for 5 min, then immersed in 6 *N* HCl for 10 min. The HCl was removed by running tap water 30 min and 3 ten-minute changes of distilled water. In this way excessive washing, which tended to leach the silicic acid from the paper, reducing its capacity, was avoided. After drying at room temperature, they were extracted by immersion

in chloroform–methanol (2:1, v/v) and again air dried. Papers impregnated by various dilutions of this 200 g/l solution were also studied; their lower capacity was generally of no advantage. Equivalent impregnations were obtained from a 5:2 dilution of DuPont's Potassium Silicate Solution (Electronics No. 200 Technical).

Clear solutions of a number of commercial sodium silicate preparations (40–42° Be) diluted 1:1, in general, made papers of low capacity, resulting in streaking and with less uniform properties than the two above; these papers tended to tear due to an excess of alkali, making handling difficult and were often full of bubbles due to an excessive carbonate content of the silicate solutions.

The general features to be described were observed with all of the following filter papers impregnated as described, although only Whatman No. 1 and Schleicher & Schüll 2043b were extensively examined: Whatman Nos. 1, 2, 3MM, 4, 5, 7, 11, 20, 31 Double, 40, 41, 41H, 42, 50, 52, 54; Schleicher & Schüll Nos. 2043b, 598. There is no doubt that a careful screening could lead to the selection of one or more of these papers especially useful for specific analyses. As a rule, the thicker papers had greater capacity due to their heavier impregnation.

3. *Solvents*

Our interests in the acid-labile plasmalogens[1,2] stimulated a search for non-acidic solvent systems of equal usefulness in the chromatography of these phosphatides. The simple substitution of pyridine or methanol for the acetic acid in the 2,6-dimethyl-4-heptanone mixtures of MARINETTI AND STOTZ[3] proved to be encouraging. The optimal ratios of the various solvents was ultimately determined by trial resulting in the following three solvent systems: (a) 2,6-dimethyl-4-heptanone–methanol–water 100:25:4; (b) 2,6-dimethyl-4-heptanone–acetic acid–water 100:40:8; (c) 2,6-dimethyl-4-heptanone–pyridine–water 100:75:10. It was found desirable to adjust the ratio of water for each batch of paper to achieve the desired separations (*cf.* Figs. 2–4). This was simply determined in a single run by using the small chromatography jars, the first containing no water, the second 1 part of water, the third two parts, etc., making use of as many jars as necessary. In a similar way the optimal ratio of heptanone:methanol, heptanone:acetic acid, and heptanone:pyridine was determined (*cf.* Fig. 5). The 2,6-dimethyl-4-heptanone (Matheson, technical grade) was purified by passing it through a 30 × 340 mm column containing 25 g Mallinckrodt Silicic Acid 100 mesh, overlaid by 50 g Woelm neutral Aluminium Oxide, activity grade 1; this column effectively removed a yellow contaminant from 500 ml of solvent. Alternately 500 ml of heptanone was shaken with 50 g Al_2O_3 and 2 g Norite and filtered. All other solvents were of analytical reagent grade and were used without further purification. The methanol and the pyridine containing systems were employed at room temperature, while the acetic acid system was used in a cold room at 2° in order to reduce hydrolysis of plasmalogens, although the short runs (1–2 h) at room temperature appeared to be safe and to give better resolution than similar runs in the cold (*cf.* Fig. 4). All three systems were found to be quite stable over many days, provided they were protected from evaporation, and required no equilibration. Chloroform–

Fig. 2. One hour small jar run at room temperature to demonstrate the influence of H_2O concentration in effecting resolution of infarct plasmalogen 8 and cardiolipin 7. Extract was from 24 h infarcted dog heart[1] chromatogrammed on Whatman No. 1 silicic acid-impregnated paper in ketone 100, methanol 25, and water from left to right 0, 1, 2, 4, 6 and 8. Plasmalogen and rhodamin stain.

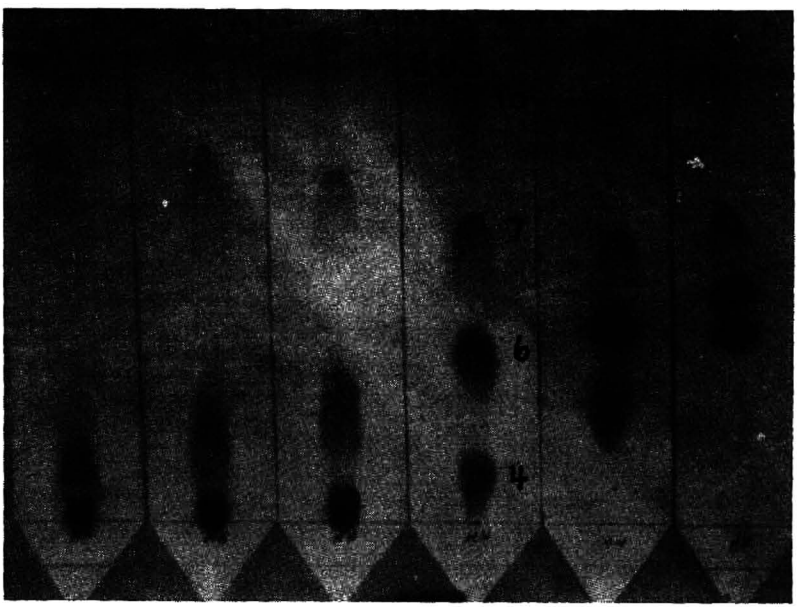

Fig. 3. One hour room temperature run of normal monkey heart extract. Whatman No. 1 impregnated paper in ketone 100, pyridine 75, and water left to right 0, 1, 2, 4, 6 and 8. With some papers 8 parts of water was sufficient to reduce the mobility of cardiolipin to that of phosphatidyl ethanolamine.

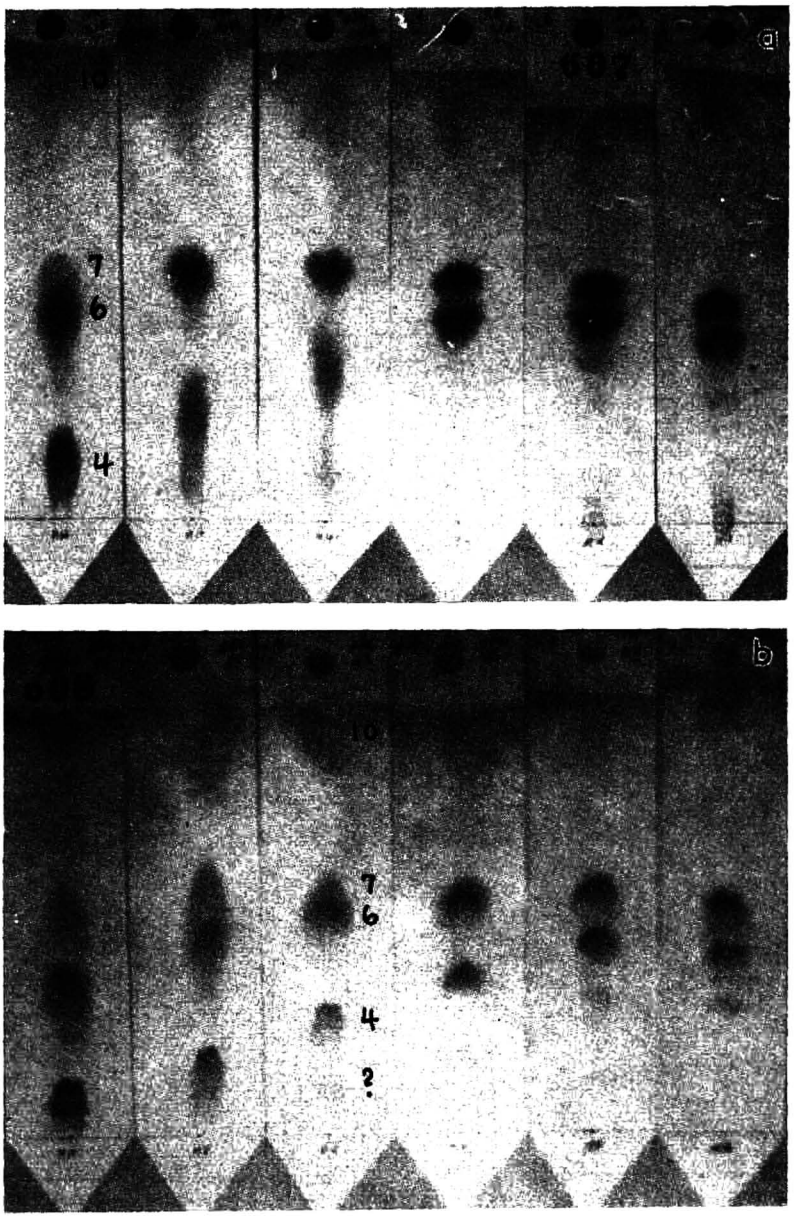

Fig. 4. Same extract and paper as Fig. 3, in ketone 100, acetic acid 40, water from left to right 0, 1, 2, 4, 6 and 8; (a) 3 h at 2°. (b) 1 h at room temperature, plasmalogen and rhodamin stain. The material labeled ? had properties resembling lysolecithin in this solvent system, but not in either of the other solvent systems as was demonstrated by chromatogramming this extract with lysolecithin. Notice that cardiolipin 7 was resolvable only in mixtures of low water content.

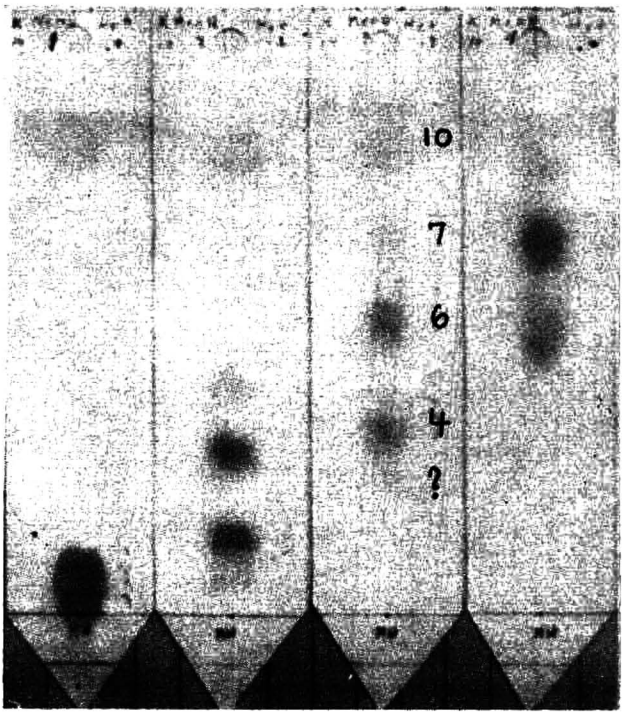

Fig. 5. One hour room temperature run on same extract and paper as Figs. 3 and 4. Ketone 100, methanol left to right 10, 20, 30, 40; water 2; plasmalogen and rhodamin stain. Similar general patterns were obtained on changing the pyridine or acetic acid concentration in their respective solvent systems.

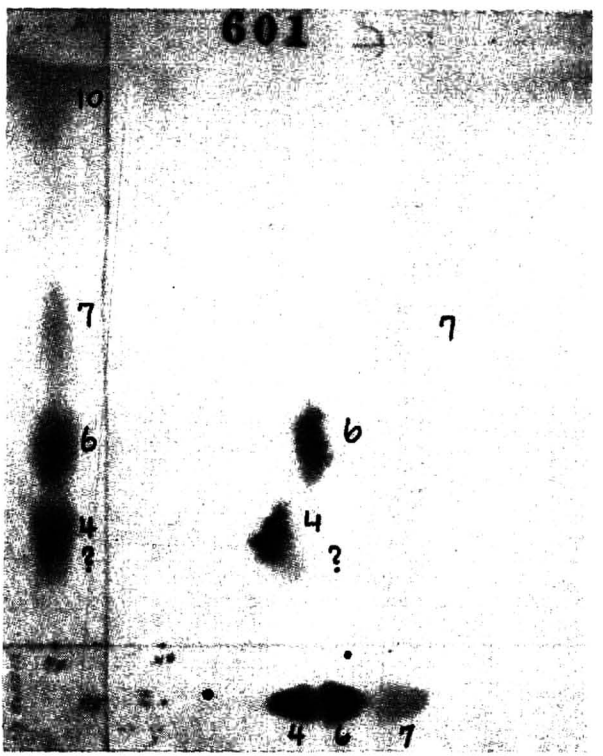

Fig. 6. Two-dimensional chromatogram of same extract and paper as in Figs. 3–5. As oriented here the first dimension was run vertically during 1 h in ketone 100, methanol 40, water 2. After drying, the left-hand strip was cut off, a 25 μl aliquot of extract was added to the bottom strip and the paper again was chromatogrammed (to the right as shown here) during 1 h in ketone 100, pyridine 75, water 6, the left-hand and lower strips representing the separate mobilities. Notice how the material marked ? was resolvable only by the methanol system (see Figs. 2–5); *i.e.*, it had the same mobility as lecithin in the pyridine system. In a similar manner cardiolipin was often shown to be a part of the phosphatidyl ethanolamine spot in the acetic acid system.

methanol 100:10 and chloroform–ethanol 100:15 were similar to the methanol system above, but were much less reproducible even with added water.

A less extensive examination of other ketones (3-methyl-2-butanone, 4-methyl-2-pentanone, 2-pentanone) in various ratios with methanol, pyridine, etc., failed to indicate any general advantage. The higher boiling point of 2,6-dimethyl-4-heptanone was probably responsible for the greater resolving power of this ketone in mixtures as described.

4. *Chromatographic procedure*

Extracts of various animal tissues were prepared according to the method of FOLCH *et al.*[4]; alternately the tissue was freeze-dried and extracted with chloroform–methanol 2:1, 10 ml/100 mg dry weight of tissue; 25 μl of these extracts was generally adequate for analysis. Our extensive experience with cardiac muscle phosphatides[1,2,5] has led us to use extracts of this tissue for control purposes when chromatographing extracts from other sources. Familiarity with the behavior of lecithin and the cephalins in these cardiac muscle extracts as well as their various enzymatic hydrolyses products[2] was far more useful for the interpretation of unknowns than dependence of R_F measurements (which were found to be too variable) and on isolated standard phosphatides which frequently did not keep well even when refrigerated. Similarly the complete battery of spot-tests should be utilized[2] in order to completely characterize the components of each extract. Two-dimensional chromatograms were utilized in order to localize the phosphatides resolved by one solvent system and not by the other; in this way an understanding of the limitations of each solvent system was obtained.

On some occasions, *i.e.*, when the neutral lipid content was high, it was found desirable to prepare the chromatogram in the usual way, but before running in one of the phosphatide solvent systems, the neutral lipids were brought to the top of the paper by a preliminary run in acetone. After drying, the paper was then run in the usual manner. An extension of this technique was to use a long (140 × 290 mm) paper, bring the neutral lipid to the half-way point with acetone, cut the paper in two and chromatogram separately the top half for neutral lipids and the bottom half for phosphatides in the usual manner.

5. *Spot-tests*

Spot-tests useful for characterization of the phosphatides were employed as described earlier[2]. It is essential to emphasize that spot-tests for plasmalogens, after washing briefly in 0.005 M H_2SO_3 to remove residual pyridine, etc., and for unsaturation should be done promptly after completion of the chromatogram in order to avoid the complications arising from air oxidation[6].

RESULTS AND DISCUSSION

The principal results are illustrated by Table I and the figures. In addition it was observed that the mobilities of the lysophosphatides were also markedly influenced by the solvent ratios and were thus not always separable from other phosphatides.

As indicated, it was possible to achieve very adequate resolution of most of the phosphatides providing appropriate adjustments in the solvent ratios were made to meet the situation at hand. In this way we have becomes less dependent on the availability of silicic acid standardized for chromatography or on stable silicate solutions.

TABLE I

DIAGRAM OF THE GENERAL CHROMATOGRAPHIC RELATIONSHIPS OBSERVED IN THE THREE SOLVENT SYSTEMS DESCRIBED

In each case 10 is at the solvent front. The numbers at the right of each column are intended to indicate the variability in the relative mobilities of cardiolipin and phosphatidyl serine, 7 and 5 resp.; as seen from Figs. 2, 3, 4, their mobilities are markedly influenced by the water content of the chromatographic solvent and are thus not always separable from phosphatidyl ethanolamine.

Methanol	*Pyridine*	*Acetic acid*	*Key*	
10	10	10	Neutral lipid	10
		9	Phosphatidic acid	9
8	8	8	Infarct plasmalogen[1]	8
7	7	7	Cardiolipin	7
5	5	6	Phosphatidyl ethanolamine	6
6	6	5		
			Phosphatidyl serine	5
3	4	4	Lecithin	4
4	3	3	Lysophosphatidyl ethanolamine	3
2,1	2	2	Sphingomyelin	2
	1	1	Lysolecithin	1

Although we have examined a large number of extracts freshly prepared from many normal tissues from a variety of animal species, we have never observed free-plasmal (higher fatty aldehyde obtained on hydrolysis of plasmalogen) which, in the solvent systems described, would appear at the solvent front[2] and would react immediately with the leuco-fuchsin of the plasmalogen spot-test. Even prolonged storage in the cold did not result in the production of free-plasmal in most of these extracts.

The substance labeled *cardiolipin* was observed in most tissues examined, with the exception of brain and is the subject of continued investigation.

SUMMARY

Techniques for the uniform impregnation of filter paper with silicic acid for the chromatography of phosphatides under controlled conditions are described.

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NOTE ADDED IN PROOF

The influence of water on the chromatographic mobility of phosphatides has also been discussed in a recent paper by J. E. MULDREY, O. N. MILLER AND J. G. HAMILTON, *J. Lipid Research*, 1 (1959)48.

Short Communications

The adsorption of yttrium, neodymium and lanthanum on a strong base anion exchange resin from dilute hydrochloric acid solutions containing ethanol

In a previous paper[1], the adsorption and resolution of Y, Nd and La by a strong base anion exchange resin from dilute nitric acid solutions containing ethanol was described. The present communication reports the adsorption of Y, Nd and La on a strong base anion exchanger from dilute hydrochloric acid solutions containing ethanol. Rare earths are not adsorbed by strong base anion exchange resins at any concentration of hydrochloric acid solution[2,3]. Hydrochloric acid–ethanol mixtures have been used by LEDERER[4] for the paper chromatographic separation of rare earths.

14 × 1.4 cm columns of Amberlite IRA CG-400, 2-4X, 100–200 mesh, chloride form resin have been used in this work. A mixture of specpure rare earth chlorides equivalent to 1 mg each of Y, Nd and La dissolved in 5 ml HCl–ethanol of the same concentration as to be used as eluant was applied to the top of the resin bed and soaked into the resin at a flow rate of 1 ml/min. The column had previously been equilibrated with 100 ml of the HCl–ethanol mixture to be used as eluant.

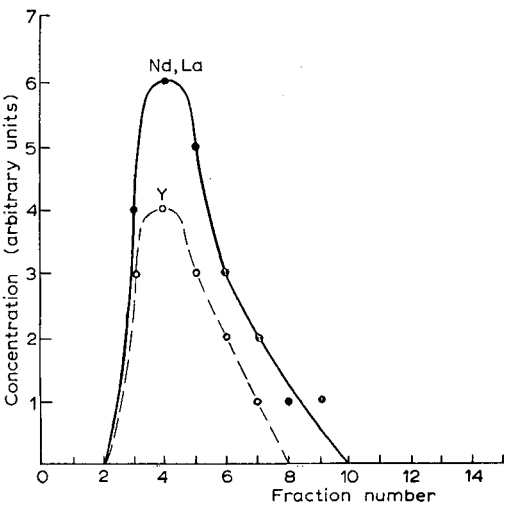

Fig. 1. The elution of Y, Nd and La from a 14 × 1.4 cm column of Amberlite IRA CG-400, 2-4X, 100–200 mesh resin with 1 *N* HCl–80% ethanol. Flow rate, 1 ml/minute. Volume/fraction, 15 ml. *Note:* Since Nd and La had very similar relative intensities, their elution has been shown by means of a single curve.

The following eluant systems were investigated:

(i) 1 *N* HCl and varying concentrations of ethanol (0, 50 and 80 % ethanol).

(ii) 80 % ethanol and varying concentrations of HCl (0.1, 1.0 and 2.0 *N* HCl).

Elution was carried out at room temperature, a flow rate of 1 ml/min being employed. The effluent was collected in 15 ml fractions. Each fraction was taken to dryness and arced to completion at 4.5 A DC. Semiquantitative elution curves were constructed by plotting rare earth relative intensities against the effluent volume. (For details of the spectographic monitoring of the effluent fractions and the construction of semiquantitative elution curves, see EDGE[1]).

A typical semiquantitative elution curve is shown in Fig. 1.

From the elution curves obtained, the volume distribution coefficients (D_v) were evaluated from the familiar relationship

$$D_v = V_{\max} - i$$

where $V_{\max}$ is the number of column volumes of effluent at which an element appeared in maximum concentration and i is the fractional interstitial volume (*ca.* 0.4).

D_v values, estimated to within $\pm$ 30 %, are shown in Table I.

TABLE I

Eluant	D_v *(Y, La, Nd)*
1 *N* HCl	0.3
1 *N* HCl–50 % ethanol	0.6
1 *N* HCl–80 % ethanol	2.4
0.1 *N* HCl–80 % ethanol	1.0
1 *N* HCl–80 % ethanol	2.4
2 *N* HCl–80 % ethanol	3.1

From Table I it is seen that rare earths showed increased adsorption by the resin with increasing ethanol and HCl concentrations.

Y, Nd and La were found to be unresolved by all the HCl–ethanol eluant systems employed.

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Storage of gas chromatographic data using key-punched IBM cards

During the past few years, the use of gas chromatographic techniques has grown at a very rapid rate. As a result, more and more data are being published, but little work has been published on compiling the data in a form convenient for use. SPENCER AND JOHNSON[1] have reported a method of storing data on punched cards; however, their method is limited to hydrocarbons. This paper is a brief description of a system using IBM cards for the storage of gas chromatographic data for a wide variety of compounds.

Two decks of IBM cards are used. One deck consists of name–formula cards (NFC) and the other of gas chromatographic data cards (CDC). The NFC contains the compound name in standard punched card alphabet, molecular formula, and a cross-reference serial number. The original NFC were reproduced from the NFC being used by infrared adsorption spectroscopy groups. Cross-reference serial numbers were assigned to these cards after cards for inorganic materials, polymers, salts, complex compounds, and unstable materials had been removed from the deck. The CDC contains key-punched codes for the liquid phase, solid support or active solid, reference material, relative retentions, column temperature, type of compound (such as ester, aldehyde, ketone, acid, alcohol, amino acid, or steroid), bibliography reference including journal, volume, page, year, and main subject of article, and cross-reference serial number of the compound.

The purpose of the NFC is to identify the compound for which the chromatographic data are listed. A given compound will, therefore, have the same cross-reference serial number, regardless of the number of times data have been coded for this compound. Also, if a given compound can be named in different ways, each name will have the same cross-reference serial number. The NFC are arranged in order of molecular formula; thus, the serial numbers are also in order of molecular formula.

Once the decks of cards have been produced, they can be reproduced, sorted, and rearranged rapidly by means of conventional punched card handling equipment. Thus, the information stored in the system can be rapidly retrieved. The cards can also be used to produce printed lists of all or any part of the information stored, and in almost any order desired. The following printed lists have been useful here:

1. NFC in order of molecular formula.
2. NFC in alphabetical order.
3. CDC arranged as follows:
   a. Major sort; liquid phase.
   b. Intermediate sort; reference material, solid support or active solid, and column temperature.
   c. Minor sort; relative retentions.
4. Compound name or names from merged NFC and CDC in order of molecular formulas. Under the name are all the data for that particular compound, as in 3.
5. A bibliography on gas chromatography in order of the subject of the article.

Lists 1 and 2 are useful for coding new data. Lists 3–5 are used for rapid retrieval of data.

The system can be applied to practical problems by using either the cards and machine sorting or the printed lists. So far, the authors have used the printed lists for:

1. Selection of the liquid phase for separation of a mixture of two or more substances.

2. Tentative identification of unknown materials by comparison of the measured relative retention of the unknown with pertinent relative retentions in the lists. Two or more columns are used for a more positive identification[2].

3. Easy access to literature in which work of interest is described.

4. Comparison and correlation of data from different sources.

Several spaces on the CDC are unused in the present system. It is expected that some of these will later be assigned to data not now being stored in the system. The ratio of liquid phase to solid support and the specific retention volume, for example, are not coded at present; however, since data are being reported more precisely than in the past, it is planned to code this information.

We are grateful to H. W. PATTON and M. V. OTIS for helpful suggestions on setting up this system for storage and retrieval of data; to A. D. ALLEN, C. S. MCCONNELL, Mrs. DORIS W. MOREHEAD, W. E. ROBERTSON, J. N. ROPER, JR., J. L. SANKS, A. L. STONE and M. R. WHITLEY for assistance in preparing the original NFC; to M. H. LEIMKUHLER and A. G. FISH for co-ordinating the work done with the IBM machine card handling equipment; and to Mrs. FRANCES P. HUFFAKER for coding data from the literature.

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Fractionation of sulphosalicylic acid filtrates on diethylaminoethylcellulose

In the course of a study on serum mucoproteins one of the aims of the authors was to investigate the high molecular substances that are not precipitable by sulphosalicylic acid, after the removal of the precipitant.

A method, formerly[1] developed for the separation of urine mucoproteins was used. It consists in the chromatographic separation of the substances on a column of

diethylaminoethylcellulose[2] and their gradient elution with phosphate buffers. The gradient was obtained with the aid of a closed mixer, which contained 0.01 *M* phosphate buffer at the beginning of the experiment. The same buffer, but with an ionic strength that had been increased to 0.6 by adding NaCl, was then added from a supply bottle. As soon as the ionic strength of the eluted solution had reached the value of 0.4, the addition of 0.4 *N* NaOH was started. By this means, a complex gradient of ionic strength and pH was attained. The eluted solution was collected in samples of about 5 ml, using an automatic fraction collector[3]. The samples were measured with a spectrophotometer and the height of their polarographic waves was determined, mixing 0.1 ml of the eluate with 1 ml of the BRDIČKA cobaltic solution[4].

The above-mentioned high-molecular substances were prepared during the so-called "BRDIČKA polarographic filtrate test"[4], by deproteinizing serum with sulphosalicylic acid. As the excess of sulphosalicylic acid interferes with the separation on an ion-exchange column when the final concentration is 0.42 *M*, this acid was removed by repeated dialysis and the dialysate was concentrated by freeze drying. Although BRDIČKA[4] and other authors state that substances causing the polarographic wave are not dialysable, a decrease in polarographic activity was always observed during dialysis. It was found that the most efficient dialysis and thus also the minimum decrease in activity could be achieved by short dialysis without using electric current.

The filtrate obtained from 200 ml of serum was subjected to dialysis in a cellophane sack for 48 hours and after freeze drying it was dissolved in 12 ml of water, re-dialysed in a disk dialyser according to SEEGERS[5] and again lyophilized. During this procedure the polarographic activity decreased to one half of the original value, while the amount of sulphosalicylic acid was reduced to 1/5000–1/10,000 of the original value. Thus, the sulphosalicylic acid constituted about 1/10 by weight of the dry-frozen substances.

In Fig. 1 the results of the separation are shown for the filtrates of normal and pathological sera, as well as for a sample of the urine mucoprotein prepared according to TAMM AND HORSFALL[6] and submitted to the action of sulphosalicylic acid and dialysis in the same way as the filtrates. The figure shows clearly the considerable similarity of the serum mucoproteins that pass into the filtrate and the urine mucoproteins obtained by alcoholic fractionation. In the experiments, carried out with urine mucoproteins, the peaks were called A – E and the zones of their occurrence were plotted on the volume scale. In zone C of the urine mucoproteins that had not been subjected to the action of sulphosalicylic acid several sub-fractions were found, the largest of which was called C_1. The main fraction found in the present experiment corresponds to this sub-fraction C_1. Another striking peak found in the zone of fraction C belongs to sulphosalicylic acid, either bound or free. This is confirmed by the spectra of samples from the peaks of the individual fractions (Fig. 2). The shape of these spectra as well as other properties, leads to the conclusion that fraction E consists of a dye of the urochrome type and fraction C_1 of a component with a rather high content of aromatic amino acids; on the other hand the other fractions have a typical mucoprotein shape.

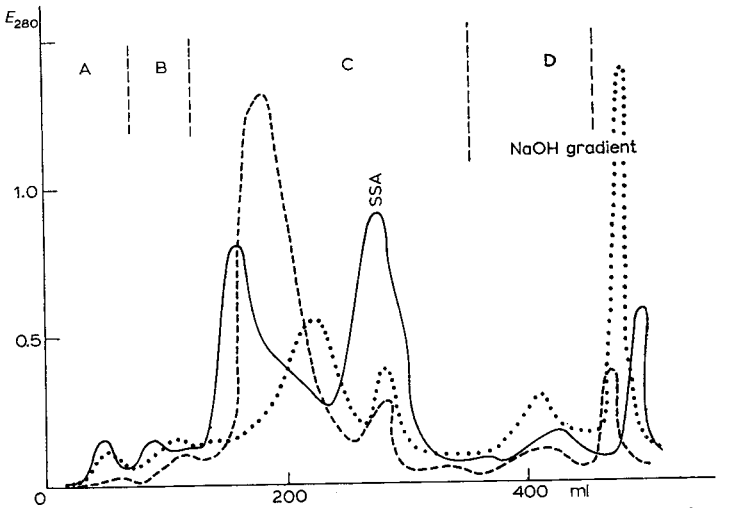

Fig. 1. Absorbances at 280 mμ. —— filtrate from normal serum; – – – – filtrate from pathological serum; ····· urine mucoprotein.

When the polarographic activity of the fractions was investigated (Fig. 3) it was found that all the protein components were active. Urochrome assumed, to be present in fraction E, and sulphosalicylic acid should not yield a catalytic wave with cobalt. The activities found, namely at the peak of sulphosalicylic acid, indicate that these substances are partially bound to proteins. From a comparison of Figs. 1 and 3 it follows that component B possesses the highest polarographic activity, related to the absorbance at 280 mμ. The activity of the fractions decreases in the sequence: A, C, D, E. From the individual areas in Fig. 3 the conclusion can be drawn that component C_1 participates maximally in the polarographic activity of the filtrate. It should, however, be kept in mind that after dialysis only one half of the original

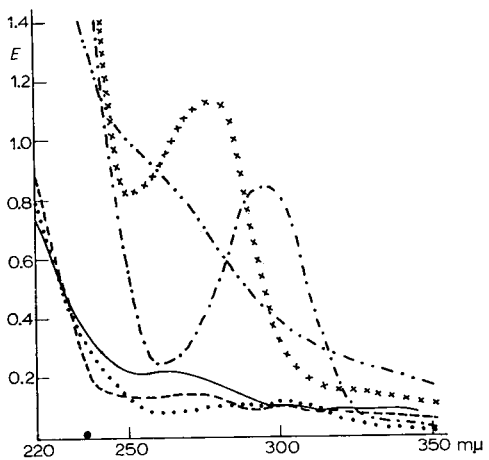

Fig. 2. Spectra of components according to Fig. 1. – – – – A; —— B; × × × C; ····· D; – · · – E; – · – · – sulphosalicylic acid.

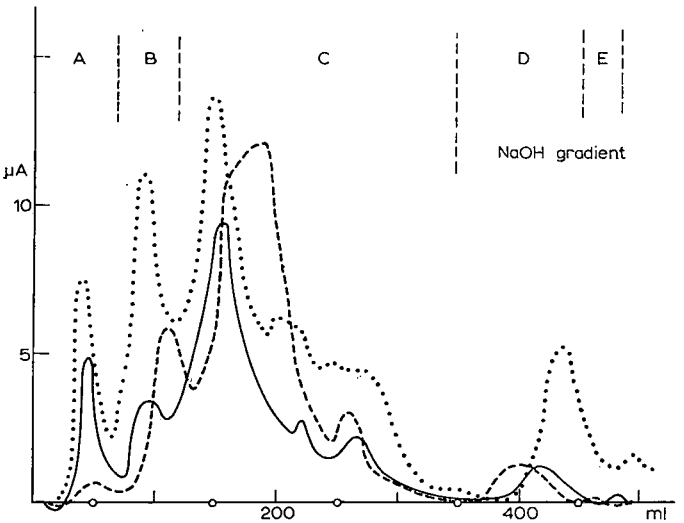

Fig. 3. Polarographic activities; —— filtrate from normal serum; – – – – filtrate from pathological serum; ····· urine mucoprotein.

polarographic activity remains in the filtrate and thus the activity of the original filtrate may be also influenced by other components, which were removed, either partially or completely.

During these experiments, attention was paid to the bond between sulphosalicylic acid and the protein. Attempts to remove all the sulphosalicylic acid from the filtrate by dialysis were unsuccessful, for this operation was accompanied by considerable losses of mucoprotein. A constant ratio between the content of sulphosalicylic acid and the mucoprotein could not be reached, even after a very long period of dialysis. These facts, as well as the presence of a comparatively small quantity of proteins in the fractions of the sulphosalicylic acid peak, support the assumption of a bond, even though it may be a weak one, between the proteins and sulphosalicylic acid. In view of the affinity of the mucoproteins to ions this phenomenon is not unexpected.

Further investigation of the components by analytical and physicochemical methods is being carried out.

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Zirconium selenite as ion-exchanger

According to the literature[1] some zirconium salts have good ion-exchange properties. Since ZrO_2^- is precipitated by H_2SeO_3 to give sparingly soluble compounds, zirconium selenite was prepared from 2 *M* nitric acid solutions of H_2SeO_3 and $ZrOCl_2 \cdot 8H_2O$ (ratio H_2SeO_3:$ZrOCl_2$ 1:1.23). The precipitate was washed with water until the pH was 4 and then dried at room temperature.

The capacity was determined by running a 0.1 *M* solution of NaCl through a column and titrating the acid formed. The capacity was found to be 0.48 mequiv./g.

The behaviour of the IB-group was studied with zirconium selenite as exchanger, in the same way as previously with zirconium phosphate[2].

Paper impregnated with zirconium selenite was prepared as follows:

(1) Solution A: 12.3 g of $ZrOCl_2$ in 150 ml of 2 *M* HNO_3. Whatman No. 1 paper was impregnated with this solution, the excess drained off and the paper dried at room temperature.

(2) Solution B: 10 g of H_2SeO_3 in 150 ml of 2 *M* HNO_3. The paper impregnated with solution A was immersed in solution B. The paper was then washed with water until the pH was 4 and dried at room temperature.

With this impregnated paper the separation of Ag(I) and Cu(II), and of Cu(II) and Au(III) was studied.

(a) With 0.01 *M* HCl as eluent a good separation of Cu(II) (R_F = 0.25) and Au(III) (R_F = 0.66) was obtained. The salts employed were $CuCl_2$ and $AuCl_3$.

(b) With 0.1 *M* HCl as eluent, Ag(I) was precipitated (R_F = 0), while Cu(II) had an R_F of 0.78. The salts employed were Ag_2SO_4 and $CuSO_4$.

The results were similar to those obtained with zirconium phosphate[2].

Instituto de Alta Cultura, M. J. NUNES DA COSTA
Centro de Estudos de Química Nuclear, Porto (Portugal) M. A. S. JERÓNIMO

[1] P. PASCAL, *Nouveau Traité de Chimie Minérale*, Tome 5e, Masson et Cie., p. 726.
[2] M. J. NUNES DA COSTA AND M. A. S. JERÓNIMO, *J. Chromatog.*, 5 (1961) 456.

Received February 6th, 1961

Improved separation of amino acids with a new solvent system for two-dimensional paper chromatography

Some of the major advantages of the paper chromatographic method are its versatility of application to separate any complex group of substances, flexibility of the choice of the moving phase, and the ease and capacity with which information can be obtained. The vast amount of available literature on these subjects has been excellently

reviewed by BLOCK, DURRUM AND ZWEIG[1], LEDERER AND LEDERER[2] and others. Now it is possible to routinely separate most of the common naturally occurring amino acids either unidimensionally using multi-solvent and multi-run systems or two-dimensionally using a number of pairs of solvent systems. The former is comparatively laborious and time-consuming while in the latter the best recommended and the most widely used solvent systems include one or more of phenol, cresol, collidine, lutidine, pyridine, etc., which give offensive and toxic vapors and require special precautions and measures when in constant use. Moreover, in the two-dimensional systems known, which evidently have apparent advantages over the multi-solvent single dimensional technique, satisfactory separation of most of the amino acids is achieved at the cost of resolution of some amino acids, such as leucine and isoleucine, lysine and arginine, glycine and serine or glutamic acid and aspartic acid. The problem of a solvent system which can separate all the common naturally occurring amino acids on a single chromatogram has long remained unresolved.

In the present paper we will describe a new combination of solvent systems for two-dimensional paper chromatography which gives a reasonable resolution of 20 common amino acids as shown in Fig. 1.

The first solvent is *sec.*-butanol–*tert.*-butanol–2-butanone–water in the proportion 4:4:8:5 (v/v) which makes a miscible solvent and was routinely mixed with 0.5% diethylamine (v/v). The use of 0.5% ammonium hydroxide in the solvent instead of diethylamine was also found satisfactory. The second solvent consists of the popular

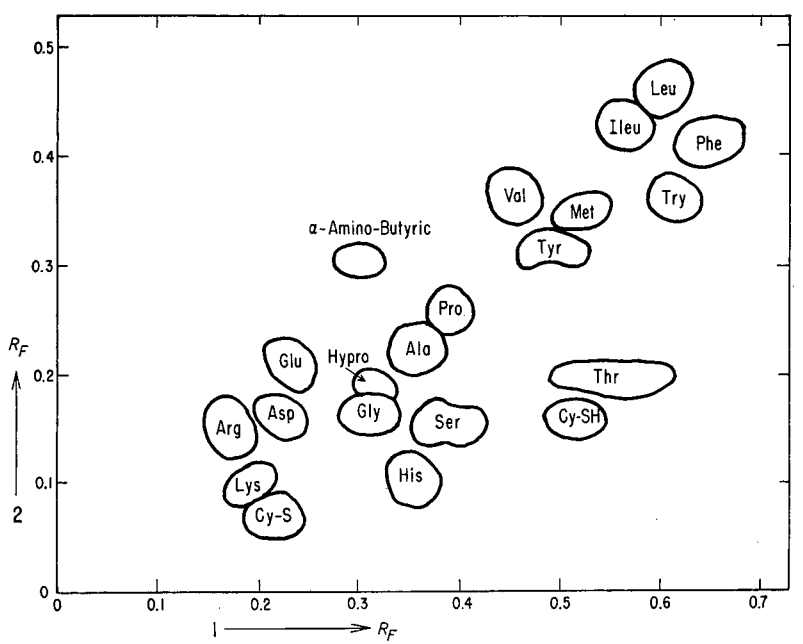

Fig. 1. Two-dimensional chromatogram. Solvent 1 is *sec.*-butanol–*tert.*-butanol–2-butanone–water in the proportion 4:4:8:5 (v/v) + 0.5% diethylamine. Solvent 2 is the upper layer of *n*-butanol–acetic acid–water in the proportion 4:1:5.

solvent mixture containing *n*-butanol–acetic acid–water in the proportion 4:1:5, which separates into two layers. The upper solvent layer was used. Reco chromatographic chambers were found very convenient for descending two-dimensional chromatography, although any general equipment of ascending or descending techniques should be satisfactory. A constant temperature oven of 55–75° was used for heating the chromatograms.

Although hydration in a steam hood gave better definition and more compact spots, this was not found essential. Also, previous saturation of the chamber was not a strict requirement if the chamber is leak proof. About a 15 hour run each way was found quite sufficient to give the necessary separation, and this could be routinely accomplished overnight.

For the best results, a few general precautions recommended in any paper chromatographic procedure were found necessary. The acidity of the hydrolysate, if not neutralized with excess of ammonia, results in streaking and gross distortion. This is best achieved by spotting an equal volume of 6 *N* ammonium hydroxide on the paper followed by aeration by a blower to remove the excess. Diethylamine or ammonia remaining after the fast run interferes with the second acid solvent and results in a blue blackground when sprayed with ninhydrin. This can be avoided by heating the papers for about 2 hours between 55–65°, and this was routinely carried out. After the usual developments, the chromatograms were sprayed with 0.25% ninhydrin in acetone and the color developed at 55–65° for 30 minutes. For quantitative purposes, 0.5% ninhydrin in acetone is recommended.

All the three grade papers gave satisfactory resolution of amino acids with varying definition of the spots. Filter paper, Whatman No. 3, was found to be the most suitable, giving more compact and well-defined spots, its thickness permitting a large concentration of the sample to respond. On No. 1 paper, the spots were somewhat elongated; more so on No. 4. The rate of travel of both the solvents was similar on paper Nos. 1 and 3, but much faster on paper No. 4.

The assistance of Mrs. R. M. Cox is appreciated. This research was supported by the Quartermaster Food and Container Institute for the Armed Forces.

Department of Food Science and Technology, University of California, Davis, Calif. (U.S.A.)

K. S. AMBE
A. L. TAPPEL

[1] R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958.
[2] E. LEDERER AND M. LEDERER, *Chromotagraphy*, 2nd Ed., Elsevier Publ. Co., Amsterdam, 1957.

Received February 8th, 1961

Editor's note. This paper was received before the February issue containing a similar paper (M. MIZELL AND S. B. SIMPSON, JR., *J. Chromatog.*, 5 (1961) 157) had appeared.

BOOK REVIEWS

Electrophoresis with "Oxoid" Cellulose Acetate Strips, a pamphlet of Consolidated Laboratories, Inc., Chicago Heights, Ill.

The use of cellulose acetate as the porous support instead of filter paper offers numerous advantages but requires also modifications in technique. This pamphlet deals with these questions in twenty eight pages and gives some excellent colour reproductions of stained serum patterns. It is actually rather a review article with complete technical details than a commercial pamphlet and should be read by everybody with an interest in electrophoretic techniques. Both the inventor of the technique, Dr. J. KOHN and Consolidated Laboratories should be congratulated on their work.

M. LEDERER (Rome)

Paper Electrophoresis — a review of methods and results, by L. P. RIBEIRO, E. MITIDIERI AND O. R. AFFONSO, Elsevier Publishing Co., Amsterdam, 1961, xii + 464 pages, price Dfl. 37.50.

Several short monographs, as well as various laboratory manuals have already appeared on the subject of paper electrophoresis. *Paper Electrophoresis* by RIBEIRO, MITIDIERI AND AFFONSO, has the great advantage that it provides the largest possible coverage of the whole literature, listing 3226 references. A critical appraisal of the whole subject matter under these conditions is of course impossible, but the research worker will find in this book, united in one volume, much of the literature that has been published in little-known medical journals, which are sometimes difficult to obtain locally.

The main emphasis of the book is on protein separations, this topic being covered in eight chapters dealing with the clinical, analytical and other aspects. The separations of smaller molecules are dealt with in chapters on vitamins, carbohydrates, nucleic acids and their constituents, amino acids and peptides, as well as in a chapter on "other applications". This last chapter, which appears to be somewhat ill-assorted, deals with fourteen topics, such as organic acids, amines, venoms, toxins, porphyrins, carcinogenic substances, dyes, alkaloids, antibiotics, other substances of pharmaceutical interest, indoles, as well as viruses, vegetable proteins and tannins. These topics are covered very capably but some items would perhaps have been more in their place in other chapters. Also in the 12 page chapter on inorganic separations the literature on inorganic acids does not appear to be quite as thoroughly treated as the reviewer would like.

This book should prove invaluable to all chemists, biochemists and medical research workers, as it is so far the only survey available that can be used as a reference book for all aspects of electrophoretic separations on paper. The printing and illustrations are of a high standard and few printer's errors were noted by the reviewer.

Announcement

DAS 4. INTERNATIONALE GASCHROMATOGRAPHISCHE SYMPOSIUM

wird organisiert von der
Fachgruppe "Analytische Chemie"
der
Gesellschaft Deutscher Chemiker
und der
Gas Chromatography Discussion Group
under the auspices of
The Hydrocarbon Research Group
of the Institute of Petroleum
und ist
die 41. Veranstaltung der

EUROPÄISCHEN FÖDERATION
FÜR CHEMIE-INGENIEUR-WESEN

Das Symposium findet vom
13. bis 16. Juni 1962 in Hamburg statt.

Die Vorträge, die auf der Tagung gehalten werden, sollen ähnlich denen sein, die auf den vorhergehenden gaschromatographischen Symposien gehalten wurden. Sie werden in die Hauptgruppen unterteilt:

1. Theorie
2. Apparate und Arbeitstechniken
3. Anwendungen

Diejenigen Vorträge, welche sich mit den Arbeitstechniken und Anwendungen befassen, müssen neue Erkenntnisse zum Inhalt haben, um in das Programm aufgenommen zu werden.

Bei dieser Gelegenheit weisen wir darauf hin, dass die vorläufige Anmeldung zur Teilnahme bis 15. Mai 1961 bei der Gesellschaft Deutscher Chemiker eingegangen sein sollte. Eine frühzeitige Anmeldung ist für die Arbeiten des Organisationskomittees sehr wichtig und für die Versendung weiterer Informationen sowie der Einladung zur endgültigen Anmeldung notwendig.

Interessenten, die Vorträge auf dem Symposium halten bzw. an der Veranstaltung teilnehmen wollen, bitten wir, die notwendigen Anmeldeunterlagen umgehend anzufordern bei:

Gesellschaft Deutscher Chemiker,
Dr. W. FRITSCHE,
Frankfurt (Main), Postfach 9075,
Deutschland.

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CHROMATOGRAPHIC DATA

SUPPLEMENT TO THE

JOURNAL OF CHROMATOGRAPHY

VOL. 5 (1961)

EDITORS

C. B. COULSON
(Inveresk, Scotland)

M. LEDERER
(Rome, Italy)

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

TABLE 1

R_F VALUES OF INORGANIC ANIONS

(I. I. M. ELBEIH AND M. A. ABOU-ELNAGA, *Anal. Chim. Acta*, 23 (1960) 30)

Solvent: Ethanol–pyridine–water–conc. ammonia (60:20:16:4).

Anion	R_F	*Anion*	R_F
Fluoride	0.01	Carbonate	0.10
Ferrocyanide	0.01	Iodate	0.10
Arsenate	0.03	Arsenite	0.12
Chromate	0.03	Sulphite	0.20
Dichromate	0.03	Bromate	0.41
Sulphide	0.03	Nitrite	0.43
Phosphate	0.03	Chloride	0.45
Cyanide	0.06	Bromide	0.50
Sulphate	0.07	Nitrate	0.56
Ferricyanide	0.08	Chlorate	0.60
Borate	0.09	Iodide	0.61
Thiosulphate	0.09	Thiocyanate	0.66

TABLE 2

R_F VALUES (RELATIVE) OF 2-(4-AMINO-4-CARBOXYBUTYL)-THIAZOLE-4-CARBOXYLIC ACID

(J. D'A. JEFFERY, E. P. ABRAHAM AND G. G. F. NEWTON, *Biochem. J.*, 75 (1960) 216)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:4, by vol.).
S_2 = 80% (w/w) phenol in an atmosphere saturated with 50% (v/v) acetic acid.
S_3 = Butan-1-ol saturated with aqueous 0.1 *N* HCl.
S_4 = Butan-2-ol saturated with 3% aqueous NH_3.
S_5 = Propan-1-ol–water (7:3, v/v).
Paper: Whatman No. 1.
Detection: Ninhydrin.

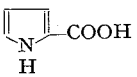
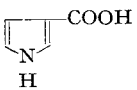
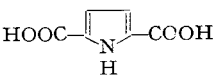
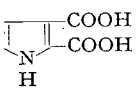
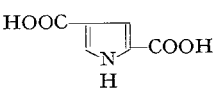
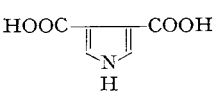
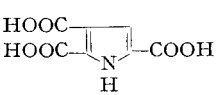
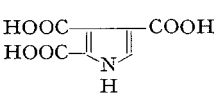
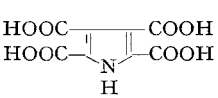
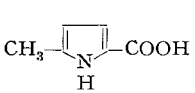
Compound	R_{Gly}*					
	S_1	S_2	S_3	S_4	S_5	
2-(4-Amino-4-carboxybutyl)-thiazole-4-carboxylic acid	1.76	1.37	3.5	0.56	2.12**	0.73***

* R_{Gly} = R_F relative to that of glycine.
** Applied in *N* HCl.
*** Applied in aq. 3% (w/v) NH_3.

TABLE 3

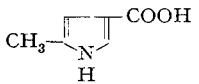
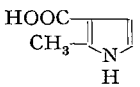
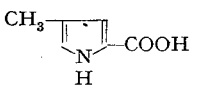
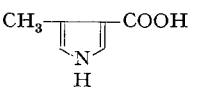
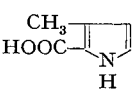
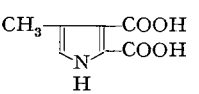
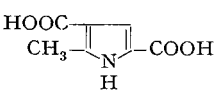
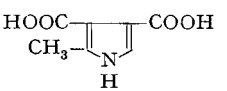
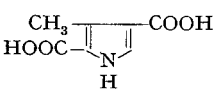
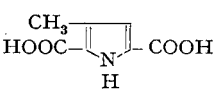
R_F VALUES OF SOME PYRROLIC ACIDS

(R. A. NICOLAUS, *Rass. med. sper.*, 7 (1960) suppl. No. 2)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.12		0.65	0.89	
	0.04	0.29	0.60	0.80	0.48
	0.00		0.47	0.79	
	0.17		0.72	0.43	
	0.00	0.07	0.35	0.72	0.23
	0.11	0.44	0.67	0.52	0.58
	0.00	0.46	0.54	0.36	0.29
	0.00		0.12	0.40	
	0.00	0.02	0.15	0.49	0.06
	0.18		0.64	0.86	

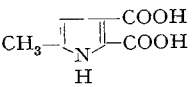
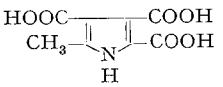
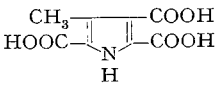
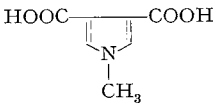
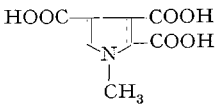
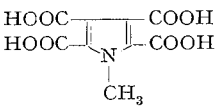
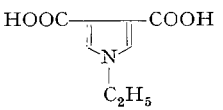
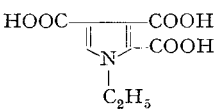
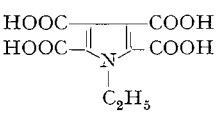
(Continued on p. D3)

TABLE 3 (*continued*)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.09	0.11	0.56	0.84	0.56
	0.08	0.39	0.57	0.85	0.58
	0.18	0.55	0.65	0.85	0.72
	0.08	0.40	0.59	0.84	0.60
	0.19		0.64	0.90	
	0.24	0.60	0.71	0.57	0.40
	0.00	0.01	0.37	0.78	0.30
	0.15	0.52	0.67	0.71	0.69
	0.00		0.33	0.79	
	0.00	0.18	0.44	0.81	0.41

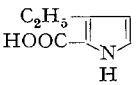
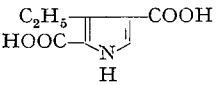
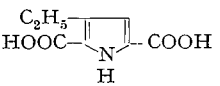
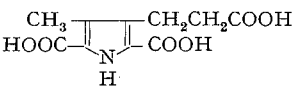
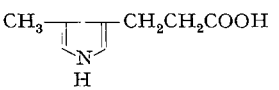
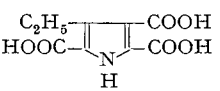
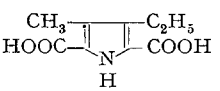
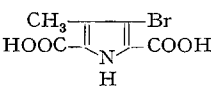
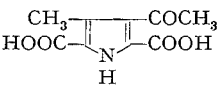
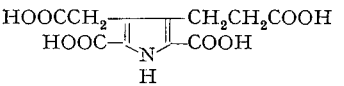
(Continued on p. D4)

TABLE 3 (*continued*)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.00	0.59	0.37	0.78	0.75
	0.00	0.04	0.23	0.34	0.14
	0.00	0.15	0.46	0.30	0.24
	0.12	0.46	0.63	0.50	0.66
	0.00	0.06	0.16	0.44	0.15
	0.00	0.01	0.05	0.15	0.08
	0.22	0.59	0.70	0.64	0.75
	0.00	0.13	0.28	0.54	0.25
	0.00	0.02	0.09	0.23	0.10

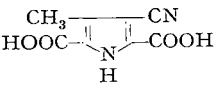
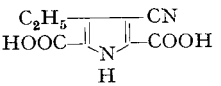
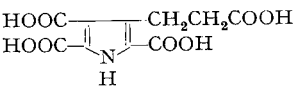
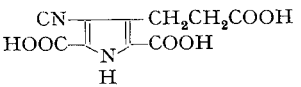
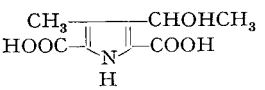
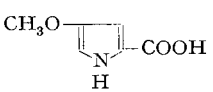
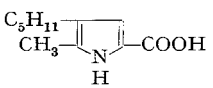
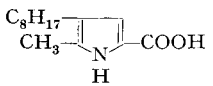
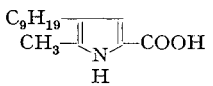
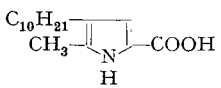
(Continued on p. D5)

TABLE 3 (*continued*)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
		0.69	0.72	0.91	
		0.15	0.39	0.88	
		0.29	0.48	0.87	0.56
	0.00	0.05	0.28	0.86	0.67
			0.73		
	0.00	0.24	0.51	0.54	
	0.05	0.49	0.59	0.94	0.28
	0.02	0.29	0.44	0.84	
	0.02	0.28	0.46	0.79	0.57
	0.00	0.01	0.06	0.58	0.19

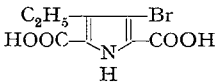
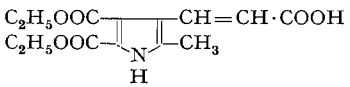
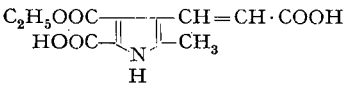
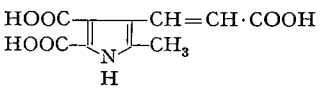
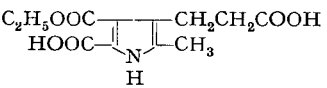
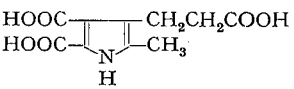
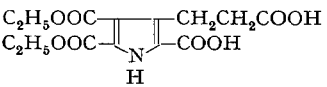
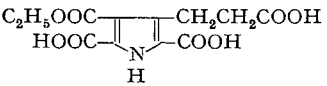
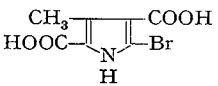
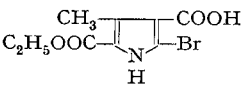
(Continued on p. D6)

TABLE 3 (*continued*)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.01	0.26	0.53	0.74	0.58
	0.05	0.32	0.54	0.82	0.67
	0.04	0.03	0.24	0.46	0.13
	0.00	0.06	0.46	0.70	0.33
		0.35	0.49	0.81	
			0.46	0.70	
	0.50		0.84	0.94	
	0.67	0.82	0.85	0.96	0.92
	0.72	0.83	0.86	0.96	0.92
	0.73	0.82	0.86	0.96	0.92

(Continued on p. D7)

TABLE 3 (*continued*)

Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1:4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.03	0.40	0.55	0.88	0.71
	0.36	0.68	0.82	0.90	0.80
	0.00	0.20	0.54	0.81	0.55
			0.27	0.44	
	0.00	0.23	0.52	0.91	0.55
	0.00	0.41	0.51	0.63	0.44
	0.05	0.20	0.70	0.90	0.74
	0.00	0.09	0.36	0.83	0.36
	0.00	0.11	0.28	0.88	0.35
	0.49	0.65	0.75	0.93	0.82

(Continued on p. D8)

TABLE 3 (*continued*)

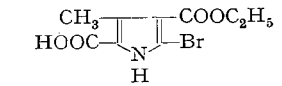
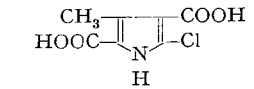
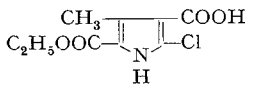
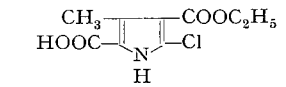
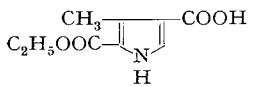
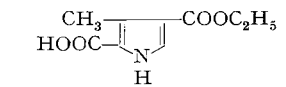
Pyrrolic acid	R_F				
	Butanol–2N NH_4OH (1:1)	*Butanol–ethanol–33% NH_3–H_2O (10:10:1·4)*	*Ethanol–33% NH_3–H_2O (80:4:16)*	*Butanol–acetic acid–H_2O (4:1:5)*	*Propanol–33% NH_3–H_2O (60:30:10)*
	0.65	0.72	0.76	0.93	0.87
	0.00			0.89	
	0.47	0.68	0.76	0.94	0.82
	0.60	0.74	0.76	0.94	0.87
	0.35			0.92	
	0.47	0.69	0.72	0.92	0.85

TABLE 4

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF AROMATIC KETONES

(E. BREUER, H. LEADER AND S. SAREL, *Bull. Research Council Israel*, 9A (1960) 43).

Paper: Whatman No. 1 impregnated with a 25% solution of N,N-dimethylformamide in 96% ethanol.
Solvent: Cyclohexane–carbon tetrachloride–dimethylformamide (20:4:1).
Temperature: 25°.

2,4-Dinitrophenylhydrazone of:	R_F	*2,4-Dinitrophenylhydrazone of:*	R_F
Isobutyrophenone	0.90	1-Acetylnaphthalene	0.53
Butyrophenone	0.86	2-Acetylnaphthalene	0.52
Benzophenone	0.80	Propionaldehyde	0.70
Propiophenone	0.75	Benzaldehyde	0.35
Cyclopropyl phenyl ketone	0.75	Formaldehyde	0.30
Acetophenone	0.58	2,4-Dinitrophenylhydrazine	0

TABLE 5

R_F VALUES OF SOME FATTY AND HYDROXY-FATTY ACIDS

(V. P. SKIPSKI, S. M. ARFIN AND M. M. RAPPORT, *Arch. Biochem. Biophys.*, 87 (1960) 259)

Solvents: S_1 = 95% acetic acid.
S_2 = 65% acetic acid.
Paper: Whatman No. 1 (ascending); washed with 95% acetic acid or glacial acetic acid – 30% H_2O_2 (9:1), depending on the solvent system, then with distilled water.
Temperature of run: 40° (reduce gradually to 35° after 8–10 cm to prevent drying out of front).
Length of run: 17–25 cm.
Time of run: 7–10 h (at elevated temperatures); 15–23 h (at room temperature).
Impregnation: Paraffin oil in benzene; 12%(?) (for S_1); 11%(?) (for S_2).
Detection: Carried out at 60–70°. Distilled water wash (three times; 5 min each); submerged in saturated aqueous bismuth subnitrate solution (30 min); distilled water wash (twice; 5 min each); submerged in 0.01% ammonium sulphide (10 min); rinsed in distilled water (modified from ALIMOVA AND BOLGOVA (1957)). Sensitivity 0.5 μg palmitic acid (for all fatty acids). 1% I_2 in $CHCl_3$ followed by 0.5% starch solution (for unsaturated fatty acids).

Fatty acid	R_F S_1	*S.D.**	R_F S_2	*S.D.**
Myristic	0.80	0.04		
Palmitic	0.72	0.05		
Stearic	0.59	0.05		
Arachidic	0.44	0.05		
Behenic	0.31	0.05		
Lignoceric	0.21	0.04		
Oleic	0.72	0.05		
2-Hydroxymyristic	0.94	0.02	0.89	0.04
2-Hydroxypalmitic	0.93	0.02	0.82	0.04
2-Hydroxystearic	0.93	0.02	0.64	0.04
2-Hydroxylignoceric (cerebronic)	0.92	0.02	0.00	—

* S.D. = Standard deviation.

TABLE 6

R_F VALUES OF SOME HIGHER FATTY ACID METHYL ESTERS

(B. P. SMIRNOV, V. A. POPOVA AND R. A. NISKANEN, *Biokhimiya*, 25 (1960) 368)

Solvent: Acetic acid–acetone (3:1).
Paper: Slow type filter paper (Volodarsky Factory, Leningrad).
Impregnation: 5% petroleum jelly in CCl_4 (to give 8–12% by weight in paper).
Time of run: Up to 36 h.
Detection: By autoradiography (as $R \cdot COO^{14}CH_3$) with Agfa-Röntgen-Duro film.

Acid	R_F
Lignoceric	0.03–0.08
Behenic	0.08–0.18
Arachidonic	0.19
Stearic	0.35
Palmitic	0.48–0.47
Oleic	0.57–0.57
Linoleic	0.65–0.67

TABLE 7

R_F VALUES OF SOME AMINO ACIDS

(R. Y. SHKOL'NIK AND N. G. DOMAN, *Biokhimiya*, 25 (1960) 276)

Solvent: S_1 = Propanol–0.88 ammonia–0.5 % Trilon B soln. (60:30:10, by vol.) (LOUGHMAN AND MARTIN, 1957).
Paper: Leningrad Chromatography Paper No. 2.
Length of run: 24 cm.
Detection: Not given.

Amino acid	R_F
Aspartic acid	0.34
Asparagine	0.37
Glutamic acid	0.41
Arginine	0.47
Histidine	0.54
Glycine	0.55
Lysine	0.55
Tyrosine	0.60
Alanine	0.60
Methionine	0.76
Tryptophan	0.85
Phenylalanine	0.88
Leucine	0.90

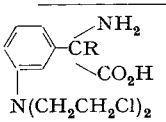
TABLE 8

R_F VALUES OF SOME CHLOROETHYLAMINOARYL-SUBSTITUTED AMINO ACIDS

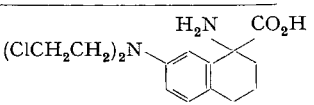
(T. A. CONNORS AND W. C. J. ROSS, *Chem. & Ind. (London)*, (1960) 492)

Solvents: S_1 = *n*-Butanol–ethanol–propionic acid–water (10:5:2:5).
S_2 = *n*-Butanol saturated with water.
Paper: Whatman No. 1.
Detection: Not given.

Compound	R_F	
	S_1	S_2
I	0.74	
II	0.80	
p-Di-(2-chloroethyl)-amino-DL-phenylalanine	0.73	0.51
III	0.82	
o-Di-(2-chloroethyl)-amino-DL-phenylalanine	0.79	0.64



I: R = H
II: R = Me



III

TABLE 9

R_F VALUES OF SOME SUGARS AND RELATED COMPOUNDS

(R. Y. SHKOL'NIK AND N. G. DOMAN, *Biokhimiya*, 25 (1960) 276)

Solvent: Propanol–0.88 ammonia–0.5% Trilon B soln. (60:30:10, by vol.) (LOUGHMAN AND MARTIN, 1957).
Paper: Leningrad Chromatography Paper No. 2 (ascending).
Length of run: 24 cm.
Detection: Not given.

Compound	R_F	*Compound*	R_F
Raffinose	0.35	Fructose	0.55
Maltose	0.43	Galactose	0.56
Sucrose	0.50	Ribose	0.58
Lactose	0.50	Phosphopyruvic acid	0.11
Glucose	0.52	Glucose-1-phosphate	0.13
Sedoheptulose	0.54	Sodium glycerophosphate	0.16

TABLE 10

R_F VALUES (RELATIVE) OF SOME NUCLEOTIDES, CONSTITUENT SUGARS AND RELATED SUGAR PHOSPHATES

(H. G. PONTIS, A. L. JAMES AND J. BADDILEY, *Biochem. J.*, 75 (1960) 428)

Solvents: S_1 = Ethanol–ammonium acetate (pH 7.5) (PALADINI AND LELOIR, 1952).
S_2 = Ethanol–ammonium acetate (pH 3.8) (PALADINI AND LELOIR, 1952).
S_3 = Ammonium sulphate–propan-2-ol–sodium acetate (MARKHAM AND SMITH, 1951).
S_4 = Butanol–pyridine–water (3:2:1.5).
S_5 = Phenol–water (PARTRIDGE, 1948).
Paper: Whatman No. 1.
Detection: U.V. light (Hanovia lamp); alkaline silver reagent; benzidine–trichloroacetic acid; molybdate reagent.

Compound	*R Adenosine**				
	S_1	S_2	S_3	S_4	S_5
Guanosine diphosphate mannose	0.13	0.11	3.47	—	—
Uridine diphosphate glucose	0.27	0.27	4.46	—	—
Uridine diphosphate N-acetyl-glucosamine	0.39	0.36	3.94	—	—
Guanosine diphosphate	0.03	0.05	3.42	—	—
Guanosine-5′-phosphate	0.10	0.32	3.20	—	—
Guanosine-3′-phosphate	0.12	0.41	2.54	—	—
Galactose	—	—	—	0.62	0.54
Glucose	—	—	—	0.70	0.48
Fructose	—	—	—	0.79	0.82
Mannose	—	—	—	0.83	0.61
Acetylglucosamine	—	—	—	0.96	1.34
Glucose-1-phosphate	—	0.60	—	—	—
Glucose-6-phosphate	—	0.62	—	—	—
Fructose-1-phosphate	—	0.68	—	—	—
Fructose-6-phosphate	—	0.73	—	—	—
Fructose-1,6-diphosphate	—	0.29	—	—	—
Sucrose phosphate**	—	0.54	—	—	—

* *R* Adenosine = R_F of substance/R_F adenosine.
** From data of LELOIR AND CARDINI (1955).

TABLE 11

R_F VALUES (RELATIVE) OF SOME MONOSACCHARIDE SULPHATE ESTERS

(A. G. LLOYD, *Biochem. J.*, 75 (1960) 478)

Solvent: S_1 = Butan-1-ol–acetic acid–water (50:12:25, by vol.).
Paper: Whatman No. 3 MM (descending).
Time of run: 48 h.
Temperature of run: 20°.
Detection: Silver nitrate (sugars); aniline hydrogen phthalate (sugars); Elson-Morgan reagent (hexosamines); 10% perchloric acid in ethanol, 5 min at 80–85° (moist atmosphere), $BaCl_2$ solution–sodium rhodizonate (sulphuric acid esters).

Compound	R_G*		
	Parent compound	*Ester sulphates*	
		Di-	*Mono-*
Glucose sulphate	1.0	0.66	0.42
Galactose sulphate	0.95	0.59	0.42
N-Acetylglucosamine sulphate	1.25	0.76	0.54
N-Acetylgalactosamine sulphate	1.15	0.71	0.51

* R_G = R_F of compound/R_F glucose.

TABLE 12

R_F VALUES OF SOME FLAVANONE GLYCOSIDES AND RELATED COMPOUNDS

(W. J. DUNLAP AND S. H. WENDER, *Arch. Biochem. Biophys.*, 87 (1960) 228)

Solvents: S_1 = *n*-Butanol–acetic acid–water (6:1:2).
S_2 = 15% acetic acid.
S_3 = Distilled water.
S_4 = 60% acetic acid.
S_5 = Nitromethane–benzene–water (2:3:5).
Paper: Whatman No. 1 (descending).
Detection: U.V. light.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Isosakuranetin-7-rhamnoglucoside	0.59	0.79	0.51		
Isosakuranetin	0.94			0.83	0.97
Naringin	0.52	0.80	0.63		
Naringenin	0.93			0.76	0.82
Hesperidin	0.45	0.75	0.50		
Hesperetin	0.92		0.78		0.92

TABLE 13

R_F VALUES OF SALICYLIC ACID AND SOME OF ITS METABOLITES

(D. HOSTYNOVÁ, P. PROVAĆ, R. DZURÍC AND T. R. NIEDERLAND, *Farmácia*, 28 (1959) 145)

Solvent: *n*-Butanol–acetic acid–water (40:4:56):0.2 % NH_3 atmosphere.
Paper: P_1 = Schleicher & Schüll 2043b; P_2 = Whatman No. 1.
Time of run: P_1:12–15 h; P_2:6–7 h.
Direction: Ascending.
Temperature of run: 14–18°.
Detection: U.V. light, diazotized sulphanilic acid, 0.2 % $FeCl_3$ solution, diazotized *p*-nitraniline.

Compound	R_F	
	P_1	P_2
Salicylic acid	0.78	0.75
Salicyluric acid	0.66	0.63
Gentisic acid	0.55	0.52
Salicylamide	0.86	0.85

TABLE 14

R_F VALUES OF SOME POSSIBLE METABOLITES OF CHLOROBENZENE

(T. GESSNER AND J. N. SMITH, *Biochem. J.*, 75 (1960) 172)

Solvents: S_1 = Hexane–isopropyl ether (10:1, v/v).
S_2 = Hexane.
S_3 = Butan-1-ol–acetic acid–water (4:1:5, by vol.).
S_4 = Butan-1-ol saturated with water.
S_5 = Benzene–acetic acid–water (1:1:2, by vol.).
S_6 = Pyridine–benzene–acetic acid–water (3:1:5:3, by vol.).
S_7 = Butan-1-ol–benzene–acetic acid–water (1:1:1:5, by vol.).
S_8 = Hexane–isopropyl ether (5:1, v/v).
Paper: Whatman No. 4 (descending).
Impregnation: For S_1, with 0.2*N* Na_2CO_3; for S_2, 20 % (v/v) formamide in methanol; for S_8, 20 % glycerol in methanol.
Times of run: 1.5 h (S_1, S_2, S_8); 3 h (S_5); 7 h (S_3, S_4, S_6, S_7).
Detection: 0.01 % ethanolic dichloroquinonechloroimide, then satd. aq. $NaHCO_3$ spray; 0.1*N* $AgNO_3$ with 1 % (v/v) aq. NH_3 (sp. gr. 0.88)(these first two for phenols); 1:5 (v/v) *Helix pomatia* gastric juice, followed by first reagent after 5 min (for phenolic glucosides); 0.1 *N* HCl spray, then heating (50–60° for 10 min) followed by first reagent (for ethereal sulphates); 0.1 % ninhydrin in butan-1-ol (10 min at 100°); U.V. fluorescence quenching; $Ag_2Cr_2O_7$ reagent. (Last three for the chlorophenyl derivatives.)

Compound	R_F							
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
o-Chlorophenol	0.3	0.5	1.0	0.9	0.8	1.0	1.0	0.9
m-Chlorophenol	0.5	0.7	1.0	0.9	0.8	1.0	1.0	0.9
p-Chlorophenol	0.6	0.6	1.0	0.9	0.8	1.0	1.0	0.9
o,*m* or *p*-Chlorophenyl glucoside	0	—	0.8	0.8	0	0.8	0.6	—
o,*m* or *p*-Chlorophenyl sulphate	0	—	0.6	0.4	0	0.6	0.2	—
o,*m* or *p*-Chlorophenylcysteine	0	—	0.7	0.6	0	0.6	0.5	—
o,*m* or *p*-Chlorophenylmercapturic acid	—	—	0.9	0.5	0.7	0.6	1.0	—
4-Chlorocatechol	0	0.1	1.0	—	0.3	0.9	1.0	0.2
4-Chlororesorcinol	0	—	0.9	—	0.2	0.9	0.9	—
2-Chlororesorcinol	0	—	0.9	—	0.25	0.9	—	0.03
2-Chloroquinol	0	—	0.9	—	0.2	0.9	0.9	0.03
Phenol	0.4	0.3	—	—	0.9	—	—	0.06

TABLE 15

R_F VALUES OF BUTYLATED HYDROXYANISOLE ISOMERS AND THEIR METABOLITES

(B. D. ASTILL, D. W. FASSETT AND R. L. ROUDABUSH, *Biochem. J.*, 75 (1960) 543)

Solvents: S_1 = Butanol–acetic acid–water (4:1:5, by vol.), organic layer of fresh mixture.
S_2 = Benzene–acetic acid–water (2:2:1, by vol.), organic phase.
S_3 = Butanol–formic acid–light petroleum b.p. 66–75° (1:10:10, by vol.). (1) After 10–15 cm development. (2) After 35–40 cm development.

Paper: Whatman No. 1 (descending).

Detection: D_1 = aq. 1% (w/v) $AgNO_3$–aq. 3 *N* NH_3 soln. (1:1).
D_2 = 1% (w/v) sulphanilic acid in 3 *N* HCl–aq. 5% (w/v) $NaNO_2$ (1:1), freshly mixed; dried paper sprayed with aq. 2% (w/v) Na_2CO_3.
D_3 = 0.05% (w/v) ethanolic 2,6-dichloroquinone-chloroimide (solution A); dried paper sprayed with aq. 2% (w/v) sodium borate.
D_4 = Solution A; dried paper sprayed with aq. *N* NH_3 soln.
D_5 = *N* HCl; dried paper heated at 70° for 3 min, then treated as in D_4.

Compound	R_F			*Colour**				
	S_1	S_2	S_3	D_1	D_2	D_3	D_4	D_5
2-*tert.*-Butyl-4-hydroxyanisole (isomer A)	0.96	0.92	(1) 0.90 (2) 0.82	bbn	o	bg	db	—
3-*tert.*-Butyl-4-hydroxyanisole (isomer B)	0.96	0.94	(1) 0.90 (2) 0.90	bbk	or	bp	p	—
2,5-Di-*tert.*-butyl-4-hydroxyanisole	0.95	0.94	(1) 0.94	wbn	wo	pk	—	—
p-Methoxyphenol	0.95	0.80	(1) 0.32	bn	r	b		
Isomer A ethereal sulphate	0.82	0.10	(1) 0.00	—	—	—	—	bg–g
Isomer B ethereal sulphate	0.79	0.07	(1) 0.00	—	—	—	—	btb–pk
Isomer A glucuronide	0.90	0.00	(1) 0.00	—	—	—	—	vwb
Isomer B glucuronide	0.89	0.00	(1) 0.00	—	—	—	—	vwp
Compound H**	0.83	0.00	(1) 0.00	—	o	wp	m	y
tert.-Butylquinol	0.97		(1) 0.50	bk	p	b	bp	

* b = blue; o = orange; g = grey; d = dark; bk = black; r = red; p = purple; w = weak; bt = bright; bn = brown; v = very; m = mauve; y = yellow; pk = pink — = no colour reaction given.

** Tentatively identified as the O-demethylation product of isomer A ethereal sulphate (*i.e.* 2(or 3)-*tert.*-butyl-4-hydroxyphenyl sulphate).

TABLE 16

R_F VALUES OF OESTRIOL AND 2-HYDROXYOESTRIOL

(R. J. B. KING, *Biochem. J.*, 74 (1960) 22P)

Solvent: Acetic acid–water–ethylene dichloride (proportions not given).

Paper: Not given.

Detection: Folin-Ciocalteu (blue without alkali).

Compound	R_F
Oestriol	0.65
2-Hydroxyoestriol	0.05

TABLE 17

R_F VALUES OF SOME STEROLS

(J. W. COPIUS PEEREBOOM AND J. B. ROOS, *Fette, Seifen, Anstrichmittel*, 62 (1960) 91)

Paper: Schleicher & Schüll 2043b mgl, impregnated with liquid paraffin (0.15 g/g of paper).
Solvent: Acetic acid–water (84:16).
Method: Ascending (for 40–45 h).
Temperature: 22–24°.

Sterol	R_S *value* (S = *cholesterol*)
Cholesterol	1.00
γ-Sitosterol	0.75
β-Sitosterol	0.75
Campesterol	0.87
Stigmasterol	0.84
Rapeseed oil phytosterols (brassicasterol?)	1.03
Cholestanol	0.82
7-Dehydrocholesterol	1.17
Ergosterol	1.20

TABLE 18

R_F VALUES OF SOME STEROIDS OF THE CONESSINE SERIES

(A. KASAL, V. ČERNÝ AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 927)

Solvents: S_1 = Methanol–ammonia–water (50:2:48).
S_2 = Methanol–butanol–ammonia–water (80:5:2:13).
Paper: Whatman No. 4 (impregnated with liquid paraffin in light petroleum; 1:10).
Detection: Dragendorff reagent.

Substance	R_F	
	S_1	S_2
Dihydroconessine	0.05	0.50
N-Cyano-dihydroisoconessimine	0.01	0.70
Dihydroisoconessimine	0.23	0.80
N-Chloro-dihydroisoconessimine	0.00	0.15
5α-Conanin-3-one	0.07	0.78
5α-Conanin-3β-ol	0.32	0.86
3β-Acetoxy-5α-conanine	0.00	0.24
18-Dimethylamino-5α-pregn-20-en-3-one	0.00	0.27
18-Dimethylamino-5α-pregn-20-en-3β-ol	0.00	0.63
3β-Acetoxy-18-dimethylamino-5α-pregn-20-ene	0.00	0.06
18-Dimethylamino-5α-pregnan-3β-ol	0.00	0.61
18-Dimethylamino-5α-pregnan-3-one	0.00	0.26
Hexahydroapoconessine	0.00	0.00
18-Dimethylamino-5α-pregnane-20,21-diol-3-one	0.82	0.94
18-Dimethylamino-5α-pregnane-3β,20,21-triol	0.88	0.98
18-Dimethylamino-3-oxo-5α-androstane-17β-carboxylic acid methyl ester	0.01	0.54

TABLE 19

R_F VALUES (RELATIVE) OF DICYANOCOBYRINIC ACID a,b,c,d,e,g-HEXAMIDE f-(DL-2-HYDROXYPROPYL)-AMIDE DIHYDROGEN PHOSPHORIC ACID ESTER (A)

(K. BERNHAUER, F. WAGNER, H. DELLWEG AND P. ZELLER, *Helv. Chim. Acta*, 43 (1960) 700)

Solvents: S_1 = Water-saturated *sec.*-butanol, 0.01 % HCN.
S_2 = S_1 saturated with $KClO_4$.
S_3 = *sec.*-Butanol–water–acetic acid–10 % HCN soln. (100:50:1:0.05).
S_4 = *sec.*-Butanol–water–25 % NH_3 soln.–10 % HCN soln. (100:36:14:0.05).
S_5 = Water-saturated *sec.*-butanol, 0.01 % HCN, 0.5 % sodium tetraphenyl borate.
S_6 = *n*-Butanol–10 % sodium carbonate soln.–10 % HCN soln. (100:100:0.1).
S_7 = Isoamyl alcohol–5 % disodium hydrogen phosphate soln. (100:100), 0.005 % KCN. (Both phases used in the two last-mentioned solvents.)

Paper: Whatman No. 1.
Temperature of run: 22–23°.
Time of run: 18 h.
Detection: Not given.

Compound	*R_F (relative)**						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
A	0.345	0.185–0.22	0.43–0.53	0.29–0.31	0.135–0.15	1.22	1.10

* Factor B = 1 (Factor B = dicyanocobyrinic acid a,b,c,d,e,g-hexamide f-(D-2-hydroxypropyl)-amide.

TABLE 20

R_F VALUES (RELATIVE) OF COBYRINIC ACID a,b,c,d,e,g-HEXAMIDE (VIa) AND ITS AMIDE

(K. BERNHAUER, H. DELLWEG, W. FRIEDRICH, G. GROSS, F. WAGNER AND P. ZELLER, *Helv. Chim. Acta*, 43 (1960) 693)

Solvents: S_1 = Water-saturated *sec.*-butanol, 0.01 % HCN.
S_2 = S_1, saturated with $KClO_4$.
S_3 = *sec.*-Butanol–water–glacial acetic acid–10 % HCN soln. (100:50:1:0.05).
S_4 = *sec.*-Butanol–water–25 % NH_3 soln.–10 % HCN soln. (100:36:14:0.05).
S_5 = Water-saturated *sec.*-butanol, 0.05 % HCN, 0.5 % sodium tetraphenyl borate.

Paper: Whatman No. 1.
Temperature of run: 22–23°.
Time of run: 18 h.
Detection: Not given.

Compound	*R_F (relative)**				
	S_1	S_2	S_3	S_4	S_5
VIa	0.53	0.45	0.97	0.65	0.34
VIa amide	0.90	0.87	0.90	0.91	0.88

* Factor B = 1 (see previous table).

TABLE 21

R_F VALUES OF SUDAN DYES

(J. GASPARIČ AND M. MATRKA, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1969)

Solvents: S_1 = Cyclohexane.
S_2 = Ethanol–water (8:2).
S_3 = Ethanol–ammonia (8:2).
S_4 = Ethanol–water (1:1).
S_5 = Ethanol–ammonia (1:1).
Paper: P_1 = Whatman No. 3 (13 × 40 cm).
P_2 = WF_1.
Impregnation: I_1 = 50% dimethylformamide in ethanol.
I_2 = 10% liquid paraffin in hexane.
I_3 = 50% lauryl alcohol in ethanol.
Detection: Visible light.

Dye	*Name*	*Colour Index No.*	R_F S_1 P_2I_1	S_2 P_1I_2	S_3 P_1I_2	S_4 P_1I_3	S_5 P_1I_3	*Colour**
Aniline→2-Naphthol	Sudan I	24	0.80	0.50	0.55	0.13	0.20	o
2-Toluidine→2-Naphthol			0.86	0.36	0.42	0.09	0.12	ro
3-Toluidine→2-Naphthol			0.86	0.37	0.42	0.09	0.12	ro
4-Toluidine→2-Naphthol			0.86	0.35	0.42	0.09	0.11	ro
4-Amino-1,3-dimethylbenzene→2-Naphthol	Sudan II	92	0.89	0.30	0.38	0.08	0.10	ro
2-Amino-1,4-dimethylbenzene→2-Naphthol			0.90	0.31	0.38	0.06	0.10	ro
o-Anisidine→2-Naphthol	Sudan R	113	0.58	0.68	0.72	0.15	0.25	r
p-Anisidine→2-Naphthol			0.72	0.51	0.59	0.12	0.16	ro
4-Aminoazobenzene→2-Naphthol	Sudan G	248	0.88	0.10	0.14	0.02	0.03	c
4-Amino-3,2′-dimethylazobenzene→2-Naphthol	Sudan IV	258	0.89	0.10	0.14	0.02	0.03	c
4-Amino-3,3′-dimethylazobenzene→2-Naphthol	Sudan Red B		0.88	0.10	0.14	0.02	0.03	c
1-Naphthylamine→1-Naphthol	Sudan Brown	81	0.86**	0.20**	0.40**	0.04**	0.14**	c
2-Naphthylamine→2-Naphthol	Sudan CB	93	0.78	0.30	0.36	0.04	0.09	ro
Aniline→Resorcinol	Sudan G	23	0.45	0.60	0.82	0.13	0.75	y
			0.01	0.82	0.84	0.32	0.42	b
1-Naphthylamine→1,3-Phenylenediamine	Sudan Brown RRN					0.39	0.50	
1-Naphthylamine→1-Naphthylamine	Sudan Brown R		0.06	0.68	0.73	0.07	0.15	
			0.31	0.84	0.85	0.12	0.33	b
4-Amino-1,3-dimethylbenzene→1-Phenyl-3-methyl-5-pyrazolone	Sudan Yellow G		0.90	0.26	0.62	0.04	0.43	y
	Sudan Red 3R		two streaks	0.23	0.40	0.16 0.59	0.02 0.25	c
	Sudan Yellow GRN		0.96	o	o	o	o	y
	Sudan Black B		0.08	0.50	0.62	0.02	0.05	bbl
			0.51	0.84	0.87		0.09	
Dimethylaminoazobenzene	Butter Yellow	19	0.80	0.64	0.73	0.22	0.29	y
4-Aminoazobenzene	Aniline Yellow	15	0.03	0.90	0.90	0.52	0.56	y
Aniline→1-Naphthol *o*-isomer			—	0.90	0.85	—	—	
p-isomer			—	0.10	0.80	—	—	

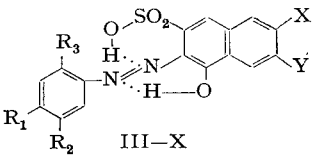
* o = orange; r = red; c = carmine red; y = yellow; b = brown; bbl = blue black.
** Main spot, carmine red.

TABLE 22

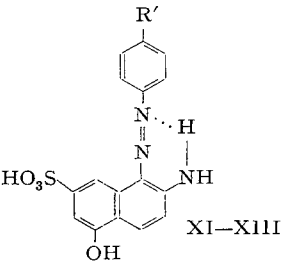
R_F VALUES OF AZO DYES

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 663)

Solvents: S_1 = 80% aqueous ethanol.
S_2 = Ethanol–3 *N* ammonia (8:2).
Paper: Whatman No. 1 (30% liquid paraffin, b.p. 195–275°, in cyclohexane, impregnation; descending).
Temperature of run: 20° ± 1°.
Detection: Visible light.



III–X



XI–XIII

Azo dye						R_F S_1	R_F S_2	*Colour**
I. 2-Amino-5-naphthol-7-sulphonic acid (J-acid)						0.32	0.41	vF
II. 2-Amino-8-naphthol-6-sulphonic acid (γ-acid)						0.32	0.41	vF
III. R_1 = H	R_2 = H	R_3 = H	X = NH_2	Y = H		0.19	0.34	o
IV. R_1 = H	R_2 = H	R_3 = H	X = H	Y = NH_2		0.23	0.36	v
V. R_1 = H	R_2 = SO_3H	R_3 = OH	X = NH_2	Y = H		0.05	0.11	r
VI. R_1 = H	R_2 = SO_3H	R_3 = OH	X = H	Y = NH_2		0.07	0.17	v
VII. R_1 = SO_3H	R_2 = H	R_3 = H	X = NH_2	Y = H		0.07	0.35	o
VIII. R_1 = SO_3H	R_2 = H	R_3 = H	X = H	Y = NH_2		0.09	0.45	v
IX. R_1 = NO_2	R_2 = H	R_3 = H	X = NH_2	Y = H		0.17	0.30**	r
X. R_1 = NO_2	R_2 = H	R_3 = H	X = H	Y = NH_2		0.24	—**	v
XI. R′ = NO_2						0.48	0.49	pv
XII. R′ = SO_3H						0.125	0.38	o
XIII. R′ = H						0.53	0.58	y

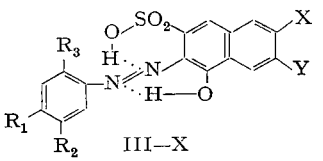
* F = fluorescence; r = red; v = violet; pv = pale violet; o = orange; y = yellow.
** Elongated spot.

TABLE 23

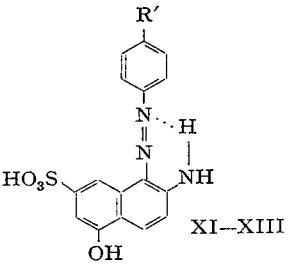
ELECTROPHORETIC MOBILITIES OF AZO DYES

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 663)

Electrolytes: E_1 = 3 *N* ammonia.
E_2 = 1 *N* acetic acid.
Paper: Whatman No. 1.
Mobility units: $u \times 10^5.cm^2.V^{-1}.sec^{-1}$.
Potential: 6–7 V/cm.
Standard: 3-Nitrophthalic acid (u = 20.5).
Detection: Visible light.
Time of run: 3–4 h.



III–X



XI–XIII

Azo dye						*Mobility* E_1	*Mobility* E_2	*Colour**
I. 2-Amino-5-naphthol-7-sulphonic acid (J-acid)						20.5	0.5	vF
II. 2-Amino-8-naphthol-6-sulphonic acid (γ-acid)						20.5	0.5	vF
III. R_1 = H	R_2 = H	R_3 = H	X = NH_2	Y = H		1.5	2.3	o
IV. R_1 = H	R_2 = H	R_3 = H	X = H	Y = NH_2		2.0	0.8	v
V. R_1 = H	R_2 = SO_3H	R_3 = OH	X = NH_2	Y = H		13.4	4.6	r
VI. R_1 = H	R_2 = SO_3H	R_3 = OH	X = H	Y = NH_2		15.6	5.1	v
VII. R_1 = SO_3H	R_2 = H	R_3 = H	X = NH_2	Y = H		10.4	9.0	o
VIII. R_1 = SO_3H	R_2 = H	R_3 = H	X = H	Y = NH_2		13.7	9.0	v
IX. R_1 = NO_2	R_2 = H	R_3 = H	X = NH_2	Y = H		0.3	1.2	r
X. R_1 = NO_2	R_2 = H	R_3 = H	X = H	Y = NH_2		0.3	0.0	v
XI. R′ = NO_2						3.9	1.85	pv
XII. R′ = SO_3H						16.8	11.4	o
XIII. R′ = H						5.6	2.5	y

* F = fluorescence; r = red; v = violet; pv = pale violet; o = orange; y = yellow.

TABLE 24

R_F VALUES OF SOME THIAZINE DYES

(K. B. TAYLOR, *J. Histochem. and Cytochem.*, 8 (1960) 248)

Solvents: S_1 = 2 *N* HCl.
S_2 = Dioxane–2 *N* HCl (92.5:7.5).
Paper: Whatman No. 1 (circular).
Detection: Visible light.
R_{tet} = Radial distance moved by unknown to that moved by tetraethylthionine for a standard run of 5 cm.

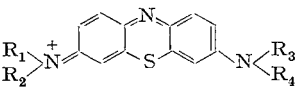
Thiazine dye		R_{tet}	
			
R_1,R_2	R_3,R_4	S_1	S_2
Pr_2	Pr_2	0.65	2.00
Et_2	Et_2	1.00	1.00
Et_2	MeEt	0.94	0.78
Et_2	Me_2	0.81	0.62
EtMe	MeEt	0.82	0.58
EtMe	Me_2	0.70	0.41
Me_2	Me_2	0.54	0.25
Et_2	HEt	0.80	0.90
EtMe	HEt	0.65	0.69
Me_2	HEt	0.51	0.53
Et_2	HMe	0.75	0.78
EtMe	HMe	0.59	0.54
Me_2	HMe	0.46	0.37
Et_2	H_2	0.70	0.78
EtMe	H_2	0.56	0.58
Me_2	H_2	0.39	0.41
EtH	HEt	0.47	0.78
EtH	HMe	0.43	0.65
MeH	HMe	0.38	0.49
EtH	H_2	0.37	0.66
MeH	H_2	0.33	0.53
H_2	H_2	0.27	0.57

TABLE 25

R_F VALUES OF SOME 2-PHENYLNAPHTHO-[1,2]-TRIAZOLE DERIVATIVES

(J. DOBÁŠ AND J. PIRKL, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 912)

Solvent: 5 % aqueous pyridine, after 1 % aqueous sodium chloride elution.
Paper: Whatman No. 4.
Temperature of run: 24–25°.
Detection: U.V. light.

2-Phenylnaphtho-[1,2]-triazole derivative		R_F
I. $R_1 = R_2 = H$	$C_{16}H_{10}N_3NaO_3S \cdot H_2O$	0.354
II. $R_1 = OH$, $R_2 = H$	$C_{16}H_{10}N_3NaO_4S \cdot H_2O$	0.228
III. $R_1 = H$, $R_2 = OH$	$C_{16}H_{10}N_3NaO_4S \cdot H_2O$	0.215
IV. $R_1 = OCH_3$, $R_2 = H$	$C_{17}H_{12}N_3NaO_4S \cdot 2H_2O$	0.211
V. $R_1 = H$, $R_2 = OCH_3$	$C_{17}H_{12}N_3NaO_4S$	0.223
VI. $R_1 = NH_2$, $R_2 = H$		0.197
VII. $R_1 = H$, $R_2 = NH_2$		0.215
VIII. $R_1 = Cl$, $R_2 = H$	$C_{16}H_9ClN_3NaO_3S \cdot H_2O$	0.168
IX. $R_1 = H$, $R_2 = Cl$	$C_{16}H_9ClN_3NaO_3S \cdot H_2O$	0.242
X. $R_1 = SO_3Na$, $R_2 = H$	$C_{16}H_9N_3Na_2O_6S_2 \cdot 2H_2O$	0.575
XI. $R_1 = H$, $R_2 = SO_3Na$	$C_{16}H_9N_3Na_2O_6S_2 \cdot 2H_2O$	0.618
XII. $R_1 = COOH$, $R_2 = H$	$C_{17}H_{10}N_3NaO_5S \cdot 2H_2O$	0.400
XIII. $R_1 = H$, $R_2 = COOH$	$C_{17}H_{10}N_3NaO_5S \cdot 2H_2O$	0.420
XIV. $R_1 = CONH_2$, $R_2 = H$	$C_{17}H_{11}N_3NaO_4S \cdot 1\frac{1}{2}H_2O$	0.181
XV. $R_1 = CN$, $R_2 = H$	$C_{17}H_9N_4NaO_3S \cdot 1\frac{1}{2}H_2O$	0.282
XVI. $R_1 = H$, $R_2 = CN$	$C_{17}H_9N_4NaO_3S \cdot H_2O$	0.228

TABLE 26

R_F VALUES OF SOME AMINOPTERIN DERIVATIVES

(K. SLAVÍK, V. SLAVÍKOVÁ AND Z. KOLMAN, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1929)

Solvents: S_1 = 0.5 % aqueous sodium carbonate.
S_2 = 0.05 *M* acetate (pH 6).
Paper: Whatman No. 3 or No. 4 (ascending).
Detection: U.V. light (280 mμ–313 mμ) and recorded on Foma-Reflex photographic paper (15–30 min exposure).

Compound	R_F	
	S_1	S_2
Tetrahydroaminopterin	0.22	0.17
N^{10}-Formyl-aminopterin	0.77	0.73
N^{10}-Formyl-tetrahydroaminopterin	0.33	0.30
N^{5}-Formyl-tetrahydroaminopterin	0.90	0.86
$N^{5\text{-}10}$-Methylene-tetrahydroaminopterin	0.60	0.47
N^{10}-Hydroxymethyl-aminopterin	0.70	0.65
N^{10}-Hydroxymethyl-tetrahydroaminopterin	0.75	0.75

TABLE 27

R_F VALUES OF SOME COUMARIN DERIVATIVES

(E. STEINEGGER AND A. BRANTSCHEN, *Pharm. Acta. Helv.*, 34 (1959) 334)

Solvent: *n*-Butanol–acetic acid–water (4:1:2).
Paper: Schleicher & Schüll 2045 bm.
Detection: U.V. light ("Chromalite" 2537 Å)—before and after 1 % KOH spray—heating at 100°.

Compound	R_F
Fraxin	0.47
Fraxetin	0.68
Fraxidin	0.81
Isofraxidin	0.81
Fraxinol	0.82
Aesculin	0.51
Aesculetin	0.74
Aesculin-7-monomethyl ether	0.52
Aesculetin-7-monomethyl ether	0.80
Aesculetin-6,7-dimethyl ether	0.82
Cichoriin	0.49
Cichoriin-6-monomethyl ether	0.51
Aesculetin-6-monomethyl ether	0.80

TABLE 28

R_F VALUES OF SOME *Senecio* ALKALOIDS

(C. B. COULSON, P. J. DAVIES AND W. C. EVANS, *J. Comp. Pathol. Therap.*, 70 (1960) 199)

Solvents: S_1 = *n*-Butanol–acetic acid–water (100:30; just saturated).
S_2 = *n*-Butanol–acetic acid–water (100:10; just saturated).
S_3 = *n*-Butanol–acetic acid–water (100:4; just saturated).
S_4 = *n*-Butanol–formic acid–water (100:10; just saturated).
Paper: Whatman No. 4 (ascending; 5 cm × 45 cm).
Detection: Dragendorff.

Compound	R_F			
	S_1	S_2	S_3	S_4
Senecionine*	0.88	0.57	0.54	0.51
Jacodine (jacobine)	0.80	0.73	0.66	0.65

* Tentative identification.

TABLE 29

R_F VALUES OF SOME *Lobelia* ALKALOIDS

(F. KACZMAREK AND E. STEINEGGER, *Pharm. Acta Helv.*, 34 (1959) 330)

Solvent: Benzene (thiophene-free)–chloroform (alcohol-free) (1:1) saturated with formamide.
Paper: P_1 = Schleicher & Schüll 2043 bm.
P_2 = Schleicher & Schüll 2045 bm.
Impregnation: I_1 = Formamide pro analysi (Xenon Co., Lodz, Poland)–acetone (9:20).
I_2 = Formamide purum (Xenon Co., Lodz, Poland)–acetone (9:20).
I_3 = Formamide pro analysi–ammonium formate (9 ml shaken with 0.9 g then added to 20 ml acetone and filtered).
Length of run: 30 cm (descending).
Detection: Dragendorff.

Paper	*Impregnation*	R_F		
		Lobinaline	*Lobelanidine*	*Lobeline*
P_1	I_1	0.00	0.03	0.05
P_1	I_2	0.03	0.11	0.15
P_1	I_3	0.09	0.16	0.22
P_2	I_1	0.00	0.04	0.06
P_2	I_2	0.02	0.09	0.12
P_2	I_3	0.11	0.18	0.25

TABLE 30

R_F VALUES OF SOME *Lobelia* ALKALOIDS

(F. KACZMAREK AND E. STEINEGGER, *Pharm. Acta Helv.*, 34 (1959) 413)

Solvent: Ether–chloroform (1:9).
Paper: Schleicher & Schüll 2045 bm (descending).
Impregnation: Formamide–formic acid–ammonium formate–acetone (9 ml:0.9 g:1 ml:20 ml).
Time of run: 5–6 h.
Detection: U.V. light; Dragendorff.

Compound	R_F
Lobeline	0.41
Lobelanidine	0.50
Lobelanine	0.73

TABLE 31

R_F VALUES OF SOME PAPAVERACEAE ALKALOIDS

(J. SLAVÍK, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1663)

Solvent: S_1 = *n*-Butanol–acetic acid–water (10:1:3).
Paper: Whatman No. 1.
Length of run: 25 cm (approx.).
Detection: U.V. light; Dragendorff reagent.

Alkaloid	R_F
Sanguinarine	0.47
Chelerythrine	0.58
Protopine	0.65
Coptisine	0.44
Berberine	0.61

TABLE 32

R_F VALUES OF SOME PAPAVERACEAE ALKALOIDS

(J. SLAVÍK AND L. SLAVÍKOVÁ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1667)

Solvents: S_1 = *n*-Butanol–acetic acid–water (10:1:3).
S_2 = Water-saturated butanol with varying acetic acid content (see table).
S_3 = Water-saturated ethyl acetate with 2 % acetic acid.
Paper: Whatman No. 1 (descending).
Detection: U.V. light fluorescence.

Alkaloid	R_F									*Fluorescence**
	S_1	S_2, % CH_3COOH							S_3	
		0.5	1	2	3	4	5	10		
Chelirubine	0.53	0.54	0.54	0.50	0.49	0.49	0.48	0.48	0.50	pr
Sanguinarine	0.43	0.37	0.37	0.38	0.37	0.34	0.35	0.40	0.19	o
Sanguirubine	0.39	0.23	0.24	0.28	0.29	0.29	0.31	0.34	0.19	pr
Macarpine	0.48	0.28	0.34	0.37	0.39	0.40	0.42	0.46		cr
Chelilutine	0.67	0.44	0.52	0.54	0.55	0.55	0.55	0.62	0.14	o
Chelerythrine	0.54	0.35	0.38	0.40	0.43	0.43	0.45	0.51	0.08	y
Sanguilutine	0.60	0.30	0.38	0.40	0.43	0.44	0.45	0.53	0.03	o

* p = purple, r = red, o = orange, c = carmine, y = yellow.

TABLE 33

R_F VALUES OF SOME AMINO ACIDS AFTER SINGLE AND MULTI-DEVELOPMENT

(M. S. DUNN AND E. A. MURPHY, *Anal. Chem.*, 32 (1960) 461)

Solvents: S_1 = *tert.*-Butanol–formic acid–water (70:1:29, v/v).
S_2 = *tert.*-Butanol–formic acid–water (70:15:15, v/v).
S_3 = Phenol–water (78:22, v/v)–conc. ammonium hydroxide (94:1, v/v); stabilised by addition of 8-quinolinol (20 mg/lb. phenol).

Paper: Schleicher & Schüll No. 589.

Time of run: S_1, 8 h; S_2, 4 h (single development).

Detection: See table; abbreviations: N = ninhydrin; *p*-D = *p*-dimethylaminobenzaldehyde; D = diazotised sulphanilamide; I = isatin, 0.3% in absolute ethyl alcohol; P-I = platinic iodide; V = vanillin.

Compound	$R_F \times 100$							*Detection*[a]
	S_1		S_2			S_3		
	× 1	× 2	× 1	× 2	× 3	× 1	× 2	
Alanine	48	72	38	68	83	60	77	N*
L-Arginine·HCl (in H_2O)	22	43	(15;26)	(25;40)	(38;54)	79	92	—
L-Arginine·HCl (in 6 *N* HCl)	—	—	—	20	34	72	83	—
Asparagine·H_2O	—	—	13	23	50	44	—	N+[b]
Aspartic acid	34	52	23	37	65	18	25	N*[b]
Citrulline	—	—	21	39	—	67	—	*p*-D*
Cysteine·HCl	—	—	48	(31;48)	(63;71)	(38;72)	—	P-I+
Cystine (in 1 *N* HCl)	7.8	12	5.8	6.9	18	36	49	N*[b]
Glutamic acid	43	63	32	56	70	30	45	N*
Glycine	32	50	23	42	63	39	49	N*[b]
Histidine·HCl·H_2O	16	28	12	16	36	72	84	D*
Hydroxy-L-proline	35	56	30	58	67	70	84	I*
Isoleucine	81	—	77	86	91	86	—	N*
Leucine	81	—	77	86	91	86	—	N*
Lysine·HCl	17	28	12	27	36	78	92	N*[c]
Methionine	65	—	56	76	88	80	—	N*
Methionine sulphone	—	—	—	38	64	—	—	N+
Methionine sulphoxide	33	50	26	61	80	82	92	N+
Norleucine	—	—	82	86	93	86	96	N+
Norvaline	—	—	72	87	92	84	92	N+
Ornithine	—	—	10	20	34	67	—	V+
Phenylalanine	72	—	60	84	89	88	—	N*
Proline	48	—	45	67	80	90	—	I*
Sarcosine·HCl	—	—	47	54	72	77	—	N+
Serine	32	50	23	41	60	39	49	N*[b]
Taurine	—	—	14	34	47	42	88	N+[b]
Threonine	38	60	29	57	70	50	68	N*
Tryptophan	—	—	40	71	81	80	—	N+
Tyrosine (in 1 *N* HCl)	48	72	38	61	76	60	77	N*
Valine	64	—	56	79	85	80	—	N*

Limiting amounts for detecting 0.5 μg^* or 1.0 μg^+ in the presence of 500 μg of L-arginine·HCl.

[a] S_2(× 2), one-dimensional chromatogram.

[b] Two-dimensional chromatogram (S_2 then S_3).

[c] S_3 used but water replaced by pH 6.2 buffer; paper pre-treated with buffer.

TABLE 34

R_F AND RELATIVE R_F VALUES OF SOME AMINO ACID DERIVATIVES

(J. RUDINGER, K. PODUŠKA AND M. ZAORAL, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2022)

Solvent: *n*-Butanol–pyridine–acetic acid–water (15:10:3:12).
Paper: Whatman No. 1.
Detection: VOGES-PROSKAUER reagent (alkaline α-naphthol–diacetyl); ninhydrin.

Compound	R_F
N^{α}-Tosyl-L-α, γ-diaminobutyric acid	0.44
γ-Guanidino-L-α-tosylaminobutyric acid	0.52
β-Guanidino-L-α-tosylaminopropionic acid	0.53
N^{α}-Benzoyl-L-α, γ-diaminobutyric acid	0.41
γ-Guanidino-L-α-benzamidobutyric acid	0.49
β-Ureido-L-α-aminopropionic acid (albizziine)	1.03*
β-Guanidino-L-α-aminopropionic acid	1.38*
γ-Ureido-L-α-aminobutyric acid	1.54*
γ-Guanidino-L-α-aminobutyric acid	1.47*

* R_{Dab} (= R_F of compound/R_F of α, γ-diaminobutyric acid?).

TABLE 35

R_F VALUES OF SOME α-KETO ANALOGUES OF SOME NATURAL DIAMINO ACIDS

(L. MACHOLÁN AND E. SVÁTEK, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2564)

Solvents: S_1 = *n*-Butanol saturated with 1 *N* HCl (1:1).
S_2 = *n*-Butanol–acetic acid–water (4:1:5).
S_3 = *n*-Butanol saturated with water.
S_4 = *n*-Butanol–pyridine–water (5:2:2).
Paper: Whatman No. 4 (descending).
Temperature of run: 20–21°.
Tank size: 85 × 50 × 25 cm.
Detection: 0.1% ninhydrin in acetone; 0.2% 2,4-dinitrophenyl-hydrazine in 1 *N* HCl.

Hydrochloride of	R_F				*Colour with ninhydrin**
	S_1	S_2	S_3	S_4	
α-Keto-γ-aminobutyric acid	0.14	0.19	0.06	0.07	ob
α-Keto-δ-aminovaleric acid	0.19	0.30	0.12	0.22	yo
DL-Pipecolic acid	0.36	0.43	0.23	0.20	v
L-Proline	0.25	0.35	0.14	0.13	y
Δ^1-Piperideine-2-carboxylic acid	0.37	0.42	0.22	0.24	bry

* o = orange; b = brown; y = yellow; br = bright; v = violet.

TABLE 36

ELECTROPHORETIC MOBILITIES (RELATIVE) AT HIGH POTENTIALS OF SOME AMINO ACIDS, PEPTIDES, AND CERTAIN DERIVATIVES

(Z. PRUSÍK AND B. KEIL, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2049)

Electrolyte: Acetic acid–formic acid–water (150:50 ml in 1000 ml), pH 1.9.
Paper: Whatman No. 3 (?).
Potential of run: 85 V/cm (?).
Temperature of run: —5° (?).
Apparatus: According to the authors; horizontal.
Units: $cm^2 . V^{-1} . sec^{-1}$ (U); U/U_{Ala} = mobility relative to that of alanine.
Detection: Ninhydrin.

Compound	U/U_{Ala}	*Compound*	U/U_{Ala}
$CySO_3H$	—0.25	$CySO_3H$. Ala	0.00
ε-DNP-Lys	0.48	$CySO_3H$. Gly	0.00
Try	0.48	$CySO_3H$. Leu	0.00
S-Carboxymethylcysteine	0.46	Leu. $CySO_3H$	0.00
Hypro	0.60	Phe. $CySO_3H$	0.00
$MetSO_2$	0.60	$GluNH_2$. $GluNH_2$	0.62
Phe	0.62	Gly. Tyr	0.64
Cit	0.62	Val. Phe. Lys	0.67
CyS-S	0.67	Ileu. Glu. Lys	0.67
Hexahydrophenylalanine	0.67	Thr. Val	0.68
Met	0.71	Tyr. Gly	0.69
$AspNH_2$	0.73	Leu. Gly. Gly	0.69
Pro	0.75	Leu. Gly	0.81
Thr	0.78	Ser. Ala	0.82
Ala	1.00	Gly. $GluNH_2$	0.82
Gly	1.15	Ileu. Ala. Lys	1.07
Octahydrotryptophan	1.17	Asp. Ser. Lys	1.07
Lys	1.41	Leu. Lys	1.20
Orn	1.41	Ala. Arg.	1.24
$CySO_3H.MetSO_2$	—0.04	Ser. Arg.	1.29
Thr. $CySO_3H$	—0.02	Ala. Hist	1.34

TABLE 37

R_F VALUES OF VARIOUS PEPTIDES

(H. ZAHN AND N. H. LAFRANCE, *Ann.*, 630 (1960) 37)

Solvents: S_1 = *sec.*-Butanol–formic acid–water (75:15:10).
S_2 = *sec.*-Butanol–10% aqueous ammonia (85:15).
S_3 = 80% Phenol.
Paper: Not given (H. ZAHN AND R. KOCKLÄUNER, *Biochem. Z.*, 329 (1953/4) 339).
Detection: Not given (H. ZAHN AND R. KOCKLÄUNER, *Biochem. Z.*, 329 (1953/4) 339).

Compound	R_F		
	S_1	S_2	S_3
L-Ala-L-leu	0.53	0.22	0.84
L-Leu-L-val	0.80	0.30	0.88
L-Tyr-L-leu	0.72	0.30	0.87
L-Tyr-L-leu-L-val	0.90	0.38	—
L-Ala-L-leu-L-tyr-L-leu-L-val	0.94	0.60	0.90

TABLE 38

R_F VALUES OF SOME ISOVALERIC ACID DERIVATIVES

(M. STRASSMAN, J. B. SHATTON AND S. WEINHOUSE, *J. Biol. Chem.*, 235 (1960) 700)

Solvents: S_1 = *n*-Butanol saturated with water.
S_2 = *n*-Butanol saturated with 3% NH_4OH.
S_3 = *n*-Butanol–ethanol–water (5:1:4).
S_4 = *n*-Butanol–formic acid–water (5:1:4).
S_5 = Phenol saturated with water.
S_6 = *sec.*-Butanol–propionic acid–water (19:1:9).
S_7 = *n*-Butanol–pyridine–water (6:4:3).
S_8 = Ethyl acetate–pyridine–water (5:2:5).
S_9 = Ethyl acetate–glacial acetic acid–water (2:1:1).
S_{10} = *n*-Propanol–conc. NH_4OH (6:4).
S_{11} = Phenol (80%).
Paper: Whatman No. 1 (ascending).
Detection: Not given.

Compound	R_F										
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}
2,4-Dinitrophenylhydrazone of α-ketoisovaleric acid	0.66	0.78	0.78	0.95							
Valine					0.71	0.27	0.25				
α,β-Dihydroxyisovaleric acid		0.17	0.68				0.35	0.41	0.79	0.58	0.58

TABLE 39

R_F VALUES OF SOME Δ^3-ISOPENTENYL COMPOUNDS

(H. EGGERER AND F. LYNEN, *Ann.*, 630 (1960) 58)

Solvents: S_1 = Ethanol–conc. ammonia–water (80:4:16).
S_2 = Butanol saturated with 1.5 *N* NH_3.
S_3 = Isoamyl alcohol–collidine–water (10:2:1).
S_4 = Methanol saturated with heptane.
S_5 = Amylene hydrate–glacial acetic acid–water (4:1:2).
Paper: Whatman No. 1 (ascending); for S_4: equilibrated overnight; descending.
Detection: 0.1% $KMnO_4$; U.V. light (3.5-dinitrobenzoates); 0.5% ethanolic α-naphthylamine (3.5-dinitrobenzoates).

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Δ^3-Isopentenoic acid*	0.76	0.27	0.03		
Δ^3-Isopentenol 3,5-dinitrobenzoate				0.7	
Dicyclohexylammonium-Δ^3-isopentenyl phosphate					0.82

* Inseparable from the isomeric dimethylacrylic acid.

TABLE 40

ELECTROPHORETIC MOBILITIES OF Δ^3-ISOPENTENOIC ACID AND DIMETHYLACRYLIC ACID

(H. EGGERER AND F. LYNEN, *Ann.*, 630 (1960) 58)

Electrolyte: Pyridine–acetate (pH 6.2) buffer.
Paper: Whatman No. 1.
Potential of run: 45 V/cm.
Time of run: T_1 = 60 min; T_2 = 65 min (40 mA).
Detection: 0.1 % $KMnO_4$ solution spray of moist paper.
Migration units: cm.

Compound	*Migration*	
	T_1	T_2
Δ^3-Isopentenoic acid	13.1	11.5
Dimethylacrylic acid	10.3	9.7

TABLE 41

ELECTROPHORETIC MOBILITIES OF SOME L-ARABINOSIDO-D-GLUCOSES

(K. WALLENFELS AND D. BECK, *Ann.*, 630 (1960) 46)

Electrolyte: Borate buffer (pH 10).
Paper: Whatman No. 3 (27.5 × 45.5 cm).
Time of run: 60 min.
Potential: 1000 V.
Detection: Silver nitrate reagent.

Compound	M_G
6-(α-L-Arabinosido)-D-glucose (Vicianose)	0.82
4-(α-L-Arabinosido)-D-glucose	0.40
3-(α-L-Arabinosido)-D-glucose	0.76
2-(β-L-Arabinosido)-D-glucose	0.42

TABLE 42

R_G VALUES OF SOME L-ARABINOSIDO-D-GLUCOSES

(K. WALLENFELS AND D. BECK, *Ann.*, 630 (1960) 46)

Solvent: Butanol–pyridine–water (6:4:3).
Paper: Schleicher & Schüll No. 2043b.
Time of run: 75 h.
Detection: Silver nitrate reagent.

Compound	R_G
6-(α-L-Arabinosido)-D-glucose (Vicianose)	0.50
4-(α-L-Arabinosido)-D-glucose	0.55
3-(α-L-Arabinosido)-D-glucose	0.80
2-(β-L-Arabinosido)-D-glucose	0.62

TABLE 43

R_F VALUES OF SOME PHOSPHATIDES AND FATTY ACIDS

(J. E. MULDREY, O. N. MILLER AND J. G. HAMILTON, *J. Lipid Research*, 1 (1959) 48)

Solvent: Benzene–pyridine (100:100, v/v) with water added.
Paper: Glass paper (X-934-AH; H. Reeve Angel & Co., Clifton, N.J., U.S.A.) 10 × 12.5 cm, with prior heating to 600° (30 min).
Impregnation: Dipped in fresh 0.4 % sodium silicate solution; dried over hot-plate.
Treatment: Developed with benzene first; phospholipids remain at start, neutral lipids carried to front.
Detection: Conc. H_2SO_4 spray, then heating to 230° (4 min); (also: ninhydrin; phosphomolybdate; fuchsin–sulphurous acid–mercuric chloride; Dragendorff).

Lipid	R_F								
	Volume of water added								
	0	*2*	*4*	*6*	*8*	*9*	*10*	*11*	*14*
Free fatty acids	0.65	0.60	0.60	0.60	0.70	0.82	0.85	0.85	—
Phosphatidyl choline	0	0	0	0.25	0.50	0.60	0.75	0.80	0.85
Sphingomyelin	0	0	0	0.04	0.25	0.40	0.50	0.65	0.85
Phosphatidyl ethanolamine	0	0	0	0.02	0.10	0.20	0.25	0.50	0.85
Phosphatidyl serine	0	0	0	0	0	0	0	0	0.60

TABLE 44

R_F VALUES OF SOME PHOSPHOLIPIDS

(E. GJONE, J. F. BERRY AND D. A. TURNER, *J. Lipid Research*, 1 (1959) 66)

Solvents: S_1 = Diisobutyl ketone–acetic acid (30:7).
S_2 = *n*-Butyl ether–acetic acid–chloroform–water (40:35:6:5).
S_3 = Ether–acetone–phenol–water (J. W. DIECKERT *et al.*, 1958).
Paper: P_1 = Unimpregnated paper (R. F. WITTER *et al.*, 1957).
P_2 = Silicic acid-impregnated paper (G. V. MARINETTI AND E. STOTZ, 1956).
P_3 = Glass filter paper impregnated with silicic acid (J. W. DIECKERT *et al.*, 1958).
Detection: Rhodamine B; ninhydrin; and iodine vapour.

Lipid	R_F*		
	$S_1 P_1$	$S_2 P_2$	$S_3 P_3$
L-α-Dimyristoyl phosphatidyl ethanolamine	0.55 ± 0.11	0.56 ± 0.12	0.80 ± 0.17
L-α-Dimyristoyl lecithin	0.70 ± 0.07	0.53 ± 0.11	0.75 ± 0.16
Sphingomyelin	0.39 ± 0.10	0.36 ± 0.11	0.67 ± 0.18

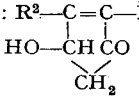
* R_F computed to centre of spot ± half length of spot divided by distance to solvent front.

TABLE 45

R_F VALUES OF SOME HYDROXYDIKETONES AND RETHROLONES

(J. FARKAŠ, H. KOMRSOVÁ, J. KRUPIČKA AND J. J. K. NOVÁK, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1824)

Solvent: Petroleum ether (65–90°)–methanol–water (100:20:1).
Paper: Whatman No. 1.
Detection: 0.1 % $KMnO_4$ spray followed by water wash then by a 0.5 % benzidine in acetic acid spray.
Hydroxydiketone: $R^1CH_2COCH_2CH(OH)COR^2$
Rethrolone:



Compound		R_F	
R^1	R^2	*Hydroxydiketone*	*Rethrolone*
Allyl	Methyl	0.26	0.06
2-Cyclopenten-1-yl	Methyl	0.45	0.11
2-Cyclohexen-1-yl	Methyl	0.79	0.53

TABLE 46

ELECTROPHORETIC MOBILITIES OF SOME PYRIMIDINE DERIVATIVES, AND THE EFFECT OF 5-FLUORO-SUBSTITUTION

(R. W. BROCKMAN, J. M. DAVIES AND P. STUTTS, *Biochim. Biophys. Acta*, 40 (1960) 22)

Electrolyte: Sodium tetraborate (0.05 *M*; pH 9).
Paper: Whatman No. 3MM.
Apparatus: R. MARKHAM (1955).
Time of run: 90 min.
Potential: 750 V.
Standard: Uridine-5′-phosphate (14–16 cm from origin; 750 V, 90 min).
Units: Relative migration distance $= \frac{\text{migration distance (cm) of pyrimidine} \times 100}{\text{migration distance (cm) of uridine-5′-phosphate}}$
Detection: U.V. absorption.

Compound	*Relative migration distance*	
	Unsubstituted compound	*5-Fluoro-substituted compound*
Uracil	30	82
Uridine	66	85
2′-Deoxyuridine	18	60
Cytosine	−5	−4
Cytidine	46	42
2′-Deoxycytidine	−12	−12
Orotic acid	97	113

TABLE 47

ELECTROPHORETIC MOBILITIES OF SOME AZAURIDINE DERIVATIVES

(J. BERÁNEK AND J. SMRT, *Collection Czechoslov. Chem. Commum.*, 25 (1960) 2029)

Electrolyte: Citrate buffer (0.05 *M*, pH 3.7).
Paper: Whatman No. 3.
Units: cm/h.
Potential: 100 V/cm.
Apparatus: Not given (presumably Z. PRUSÍK AND B. KEIL, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2049).
Detection: Not given.

Compound	*Mobility*
6-Azauridine-2′(3′)-phosphate	9
6-Azauridine-2′(3′),5′-diphosphate	13.5
6-Azauridine-5′-phosphate	9

TABLE 48

R_F VALUES OF SOME AZAURIDINE DERIVATIVES

(J. BERÁNEK AND J. SMRT, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2029)

Solvents: S_1 = Isopropanol–ammonia–water (7:1:2).
S_2 = Isopropanol–1 % aqueous $(NH_4)_2SO_4$ (2:1).
S_3 = Saturated $(NH_4)_2SO_4$ solution–0.1 *M* ammonium acetate–isopropanol (79:19:2).
Paper: Whatman No. 1 (S_1, S_2, descending; S_3, ascending).
Detection: Not given.

Compound	R_F		
	S_1	S_2	S_3
6-Azauridine-2′(3′)-phosphate	0.20	0.3	0.77
6-Azauridine-2′,(3′)-cyclic phosphate	0.47	0.4	0.58
6-Azauridine-2′(3′),5′-diphosphate	—	0.12	0.85
6-Azauridine-5′-phosphate	0.20	0.3	0.80

TABLE 49

R_F VALUES OF AZAURACIL, AZAURIDINE AND AZAURIDINE-5′-PHOSPHATE

(R. E. HANDSCHUMACHER, *J. Biol. Chem.*, 235 (1960) 764)

Solvents: S_1 = Butanol–acetic acid–water (10:2:5).
S_2 = Isobutyric acid–0.5 *N* ammonium hydroxide (5:3).
S_3 = Ethyl acetate saturated with 0.05 *M* phosphate buffer, pH 5.0.
S_4 = Isopropanol–6 *N* HCl (17:8).
Paper: Whatman No. 1 (descending).
Detection: U.V. light.

Compound	R_F			
	S_1	S_2	S_3	S_4
Azauracil	0.56	0.60	0.50	0.67
Azauridine	0.38	0.51	0.06	0.67
Azauridine-5′-phosphate	0.11	0.29	0.0	0.72

TABLE 50

R_F VALUES OF SOME AROMATIC ANIONS

(I. JAKUBEC, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1736)

Solvents: S_1 = 1 *N* KCl.
S_2 = 1 *N* K_2SO_4.
Paper: P_1 = Whatman No. 4 (ascending; 17 cm, 24 cm).
P_2 = Schleicher & Schüll 602 hart (ascending; 4 cm, 24 cm).
Impregnation: I_1 = 20% liquid paraffin (d = 0.809) in 50–60° petroleum ether (rolled and exposed to air for 6 h).
I_2 = None.
Detection: Not given.

Compound	R_F			
	$P_1 I_1$		$P_2 I_2$	
	S_1	S_2	S_1	S_2
p-Hydroxybenzoic acid	0.78	0.66	0.82	0.69
Salicylic acid	0.73	0.63	0.78	0.65
p-Aminosalicylic acid	0.61	0.53	0.64	0.54
Phenylcinchoninic acid	0.36	0.23	0.34	0.23
Sulphadimidine	0.81	0.74	0.85	0.77
Sulphanilimide	0.72	0.66	0.71	0.61
Salicylamide	0.60	0.54	0.56	0.49
p-Aminobenzoic acid	0.77	0.69	0.84	0.69
Sulphaguanidine	0.77	0.75	0.71	0.64
Procaine	0.72	0.70	0.83	0.75
Benzocaine	0.49	0.44	0.51	0.40

TABLE 51

R_F VALUES OF SOME AROMATIC ACIDS

(K. REHNELT, *Ber. naturwiss. Ges. Bayreuth*, 10 (1958/60) 232)

Solvents: X-alcohol–ethanol–water–conc. ammonia (5:5:2:1).
Substances chromatographed: (1) Perylene-3,9-dicarboxylic acid.
(2) Pyrenoyl-(3)-β-propionic acid.
(3) Fluoren-9-one-1-carboxylic acid.

X-alcohol	R_F		
	1	*2*	*3*
Methanol	0.51	0.72	0.75
Ethanol	0.49	0.81	0.79
Propanol	0.30	0.74	0.71
Butanol	0.25	0.66	0.64
Pentanol	0.17	0.55	0.52
Hexanol	0.19	0.56	0.50
Heptanol	0.18	0.52	0.47
Octanol	0.13	0.45	0.39

TABLE 52

R_F VALUES OF CAROTENES

(A. JENSEN, *Acta Chem. Scand.*, 14 (1960) 2051)

Solvents: S_1 = Petroleum ether (b.p. 60–80°).
S_2 = Petroleum ether–benzene (80:20, v/v).
Paper: Schleicher & Schüll No. 667 (circular).
Impregnation: 20% Al_2O_3 (activated after impregnation at 150° for 15 min).
Detection: Visible light.

Compound	$R_F \times 100$	
	S_1	S_2
α-Carotene	43	66
β-Carotene	38	62
γ-Carotene	5	15
Phytofluene	77	88
ζ-Carotene	36	60
Neurosporene*	15	25
Lycopene	2	8
Azobenzene**		80

* Second isomer.
** Standard marker.

TABLE 53

R_F VALUES OF SOME *Vinca minor* L. ALKALOIDS

(J. TROJÁNEK, O. ŠTROUF, K. KAVKOVÁ AND Z. ČEKAN, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2045)

Solvents: S_1 = Petroleum ether (50–70°) shaken with 10% I_1.
S_2 = Methanol–1% acetic acid (1:4).
S_3 = Methanol–5% acetic acid (1:1) saturated with octan-2-ol.
Paper: P_1 = Whatman No. 4 (descending).
P_2 = Whatman No. 3 (descending).
Time of run: S_1, 1 h; S_2, 6 h; S_3, 7 h.
Temperature of run: 18–20°.
Impregnation: I_1 = Ammonium formate, anhydrous (50 g/l) dissolved in formamide–ethanol (1:6). Formamide solution initially adjusted to pH 9.9 with 25% ammonia. Resultant pH 7.5.
I_2 = 10% liquid paraffin in petroleum ether.
I_3 = 10% octan-2-ol in acetone.
Detection: Initial heating to 100–110°, then U.V. light or Dragendorff reagent spray.

Compound	F_F			*U.V. fluorescence**
	$S_1P_1I_1$	$S_2P_2I_2$	$S_3P_2I_3$	
Vincaminorine	0.93	0.14	0.48	y(S_2S_3)
Vincaminoreine	0.93	0.66	0.72	wy
Vincamidine	0.39	0.71	0.69	wy

* y = yellow; w = weak.

TABLE 54

R_F VALUES OF SOME LYSERGIC ACID DERIVATIVES

(M. SEMONSKÝ AND V. ZIKÁN, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 2038)

Solvent: Chloroform.
Paper: Not given.
Impregnation: Formamide–5% ammonium formate.
Detection: U.V. light.

Compound	R_F
d-Isolysergic acid (+)-3-cyclopentyl-1-hydroxy-2-propylamide	0.94
d-Lysergic acid (+)-3-cyclopentyl-1-hydroxy-2-propylamide	0.56
d-Isolysergic acid (—)-3-cyclopentyl-1-hydroxy-2-propylamide	0.89
d-Lysergic acid (—)-3-cyclopentyl-1-hydroxy-2-propylamide	0.66

TABLE 55

R_F VALUES OF SOME STEROIDS

(J. R. SWARTWOUT, J. W. DIECKERT, O. N. MILLER AND J. G. HAMILTON, *J. Lipid Research*, 1 (1960) 281)

Solvents: S_1 = Isooctane.
S_2 = Benzene–isooctane (1:1.5).
S_3 = Benzene.
S_4 = Benzene–isooctane (1:1).
S_5 = Benzene–isooctane (2:1.25).
S_6 = Benzene–ethanol (100:1).
S_7 = Isooctane–acetic acid (200:3).
S_8 = Isooctane–acetic acid (200:7).
Paper: Glass paper No. X-934-AH (Reeve Angel & Co., Clifton, N. J.; 15 × 19 cm, ascending).
Impregnation: I_1 = The paper is heated (600° for 30 min), cooled, dipped in aqueous 0.4% sodium silicate, freed of excess with glass rod and dried over hot plate.
I_2 = Silicic acid (J. W. DIECKERT, W. B. CARNEY, R. L. ORY AND N. J. MORRIS, *Anal. Chem.*, 30 (1958) 1442).
Time of run: 7 min.
Detection: Sulphuric acid (conc.) spray, then charred by heating (230°, 4 min).

Compound	R_F							
	S_1I_1	S_2I_1	S_3I_1	S_4I_1	S_5I_1	S_6I_1	S_7I_1	S_8I_2
Coprostanone	0.88							
Cholesterol	0.54							
β-Cholestanol	0.54							
β-Sitosterol	0.54							
Coprostanol	0.70							
Progesterone		0.71						
Pregnane-3α, 20α-diol			0.29					
Allopregnane-3β,20α-diol				0.57				
Androsterone					0.47			
Dehydroepiandrosterone				0.59				
Epiandrosterone				0.66				
Testosterone				0.38				
Oestrone				0.54				
Hydrocortisone						0.50		
Lithocholic acid							0.50	
3,12-Diketocholanic acid								0.64

TABLE 56

R_F VALUES OF SOME STEROIDS

(P. KABASAKALIAN AND A. BASCH, *Anal. Chem.*, 32 (1960) 458)

Solvents: S_1 = Chloroform–formamide.
S_2 = Benzene–formamide.
S_3 = Toluene–propylene glycol.
S_4 = Ligroin–propylene glycol.
S_5 = Heptane–methyl cellosolve.
S_6 = Heptane–phenyl cellosolve.
(*cf.* R. NEHER, *J. Chromatog.*, 1 (1958) 205).

Paper: Whatman No. 1 (descending).

Impregnation: 35 % propylene glycol in methanol; methyl cellosolve, undiluted; phenyl cellosolve, 18 % in acetone (v/v); formamide, stabilised reagent grade (Fisher Scientific Co.).

Temperature of run: 22°.

Detection: Not given.

Compound	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
11α,17α,21-Trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione	0.23					
11α,17α,21-Trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione 21-acetate		0.14				
9α-Fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione						
9α-Bromo-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione 21-acetate	0.17	0.27				
9α-Fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione 21-acetate		0.22				
3β,17α,21-Trihydroxy-16α-methylallopregnan-20-one 21-acetate			0.41			
16α-Methyl-17α,21-dihydroxy-1,4-pregnadiene-3,20-dione 21-acetate			0.71			
16α-Methyl-17α,21-dihydroxy-1,4,9(11)-pregnatriene-3,20-dione 21-acetate			0.65			
9β,11β-Epoxy-16α-methyl-17α,21-dihydroxy-1,4-pregnadiene-3,20-dione 21-acetate			0.62			
3β-Hydroxy-5,16-pregnadien-20-one				0.18	0.18	
3β,17α-Dihydroxy-16α-methyl-21-bromoallopregnan-20-one				0.07		
16α-Methyl-17α,21-dihydroxyallopregnane-3,20-dione 21-acetate				0.15		
2ε,4ε-Dibromo-16β-methyl-17α,21-dihydroxyallopregnane-3,20-dione 21-acetate				0.09		
22α-5-Spirosten-3β-ol(Diosgenin)					0.52	0.24
3β-Hydroxy-5,16-pregnadien-20-one 3-acetate					0.55	
3β-Hydroxy-16α-methyl-5-pregnen-20-one					0.24	0.17
3β-Hydroxy-16α-methylallopregnan-20-one					0.24	0.18
16β-Methyl-17(20)-allopregnene-3β,20β-diol 3,20-diacetate						0.80
16α-Methyl-17α,20α-epoxyallopregnane-3β,20β-diol 3,20-diacetate						0.21

TABLE 57

R_F VALUES OF SOME TRITERPENE ALCOHOLS

(B. PASICH, *Dissertationes Pharm.*, 12 (1960) 201)

Solvents: S_1 = Benzene (purified: B. PASICH, *Dissertationes Pharm.*, 11 (1959) 23).
S_2 = Toluene (agitated with 7% H_2SO_4 (4 h), then washed with NaOH, water and distilled).
S_3 = Cyclohexane (analytical reagent grade).
S_4 = Xylene (refluxed with 90% H_2SO_4 (4:1) for 2 h, then distilled).
Paper: Whatman No. 1 (12 × 40 cm).
Impregnation: Al_2O_3 (B. PASICH, *Dissertationes Pharm.*, 11 (1959) 23); reactivation by heating (110° for 1 h).
Length of run: 30 cm.
Time of run: 3–3.5 h.
Temperature of run: 18°.
Detection: D_1 = $SbCl_5$ in $CHCl_3$.
D_2 = $SbCl_3$ in $CHCl_3$.
D_3 = Phosphotungstic acid in ethanol.
D_4 = Silicotungstic acid in ethanol (25%; spray and heat to 115–118° for 2 min).
D_5 = $ZnCl_2$ in benzoyl chloride.
D_6 = Acetic anhydride and H_2SO_4.
D_7 = Chlorosulphonic acid and Sesolvan NK (Badische Anilin u. Sodafabrik, Ludwigshafen a.Rh.). Dried chromatogram is dipped into Sesolvan NK, transferred to glass plate covered with thin layer of the acid, more of the acid is then rolled on with a glass rod.
D_8 = Red blood cell suspension.
(For D_1 and D_2 see B. PASICH, *Dissertationes Pharm.*, 11 (1959) 23; for D_3–D_6 see B. PASICH, *Dissertationes Pharm.*, 11 (1959) 31.)
Sensitivity: Mean sensitivity in $\mu g/cm^2$.
Light source: V = visible; U.V. = ultraviolet.

Compound	R_F				*Colour**										
					D_1	D_2		D_3	D_4	D_5		D_6		D_7	D_8
	S_1	S_2	S_3	S_4	V	V	U.V.	V	V	V	U.V.	V	U.V.	V	V
Aescigenin	0.03	0.08	0.03	0.04	gn	v	r–v	g–gn	gn–bn	gn	bn	gn–b	gn–bn	pi	h
Primulagenin A	0.04	0.09	0.05	0.06	pi	v	pi	ch	pi–ch	ch–bn	y	ch	v	pi	h
Arnidiol	0.28	0.11	0.23	0.13	bn	ch	r–o	g–pi	y	o	p–y	o	y–bn	pi	h
Betulin	0.31	0.30	0.33	0.16	bn	y	y	pi–bn	p–pi	o	p–b	o	y	pi	h
Lupeol	0.53	0.49	0.45	0.24	bn	o–pi	pi–y	pi–y	bn–pi	o	b	o	y–bn	pi	h
α-Lactucerol	0.76	0.67	0.64	0.42	v	pi	pi–y	pi	p–pi	pi	ch	o	o	pi	h
Sensitivity	—	—	—	—	2–5	20–30		5–8	10–20	5–8		8–10		8–10	5–20

* b = blue; bn = brown; ch = cherry; g = grey; gn = green; h = haemolysis; o = orange; p = pale; pi = pink; r = red; v = violet; y = yellow.

TABLE 58

R_F VALUES OF SOME AZULENES

(V. SÝKORA AND K. VOKÁČ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1702)

Solvents: S_1 = 10.10 % HCl.
S_2 = 13.38 % HCl.
S_3 = 15.37 % HCl.
S_4 = 20.07 % HCl.
S_5 = 25.05 % HCl.
Paper: Whatman No. 1 (ascending).
Impregnation: I_1 = 10 % paraffin oil.
I_2 = 20 % paraffin oil.
I_3 = 30 % paraffin oil.
Impregnation carried out with the paraffin oil in petroleum ether b.p. 40–60°, v/v).
Time of run: 3½ h (I_1); 4 h (I_2); 5 h (I_3).
Detection: Visible light; EM-reagent spray on chromatogram washed to neutrality.

Compound	*I*	R_F				
		S_1	S_2	S_3	S_4	S_5
Vetivazulene	I_1	0.01	0.04	0.08	0.43	0.76
	I_2	0.01	—	0.05	0.35	0.71
	I_3	0.00	—	0.03	0.23	0.57
S-Guiazulene	I_1	0.03	0.11	0.24	0.59	0.83
	I_2	0.02	—	0.17	0.58	0.80
	I_3	0.00	—	0.10	0.48	0.74
Se-Guiazulene	I_1	0.04	0.23	0.42	0.75	0.87
	I_2	0.04	—	0.26	0.69	0.88
	I_3	—	—	0.22	0.57	0.77
Chamazulene	I_1	0.08	0.28	0.42	0.78	0.82
	I_2	0.04	—	0.30	0.68	0.80
	I_3	0.03	—	0.25	0.55	0.83
Se-Chamazulene	I_1	0.13	0.39	0.53	0.84	0.87
	I_2	—	—	—	0.79	0.89
	I_3	—	—	—	—	—
2-Methylchamazulene	I_1	0.08	0.30	0.40	0.76	0.82
	I_2	0.04	—	0.35	0.68	0.82
	I_3	0.03	—	0.28	0.62	0.81
2-Ethylchamazulene	I_1	0.22	0.59	0.67	0.83	0.86
	I_2	0.13	—	0.58	0.81	0.85
	I_3	0.09	—	0.55	0.78	0.85
Artemazulene	I_1	0.54	0.67	0.77	0.82	0.83
	I_2	0.35		0.69	0.83	0.84
	I_3	0.31		0.66	0.82	0.87

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