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A STREAM DIVISION VALVE FOR USE IN AUTOMATIC COLUMN CHROMATOGRAPHY

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Automatic column chromatography is particularly useful when analysis (with automatic monitoring and recording devices) and preparative fractionation of the effluent are achieved simultaneously. With certain monitoring devices such as ultraviolet spectrophotometers and radioactive scanners, no problems arise. With other types of monitoring, such as color reactions which destroy the material under examination, the only practical approach is a constant and accurately controllable division of the effluent stream into analyzed and collected portions. A simple and inexpensive device for this general purpose has been developed in this laboratory and has been used satisfactorily with a Beckman/Spinco Amino Acid Analyzer (Model 120).

The device, designated a stream division valve, consists of a rotary valve which is positioned by a solenoid and spring in opposition, the former being actuated at intervals by a timing device (see Fig. 1).

COMPONENTS

Timing device

The timer used on this stream-division valve consists of a microswitch riding on a split disk which is turned by a synchronous motor. Thus, when the two halves of the split disk are superimposed, the microswitch is open and closed for equal intervals of time. If the two halves of the disk are placed in opposition to each other, the microswitch has a complete disk on which to ride and will be open continuously. Thus, by suitable overlapping of the two halves of the split disk, between 50 % and 100 % of the effluent stream may be diverted to a fraction collector. In practice, it is found that the analyzer will not give satisfactory results with less than 10 % of the effluent. A synchronous motor of 0.5 r.p.s. proved quite satisfactory in this application. This timing device is now commercially available from Western Electro-Mechanical Co., 300 Broadway, Oakland 7, Calif.

Valve

Since most analyzers require that a constant, known amount of effluent be flowing through the reaction coil and colorimeter at all times, this dictates that the actual valve be slightly more complicated than that which is necessary for the mere splitting of fractions (see Fig. 3). A second flow of liquid, equal in flow rate to the effluent stream, must be available so that when the effluent is diverted to the fraction collector, this second liquid can be diverted to the reaction coil, thus maintaining a constant

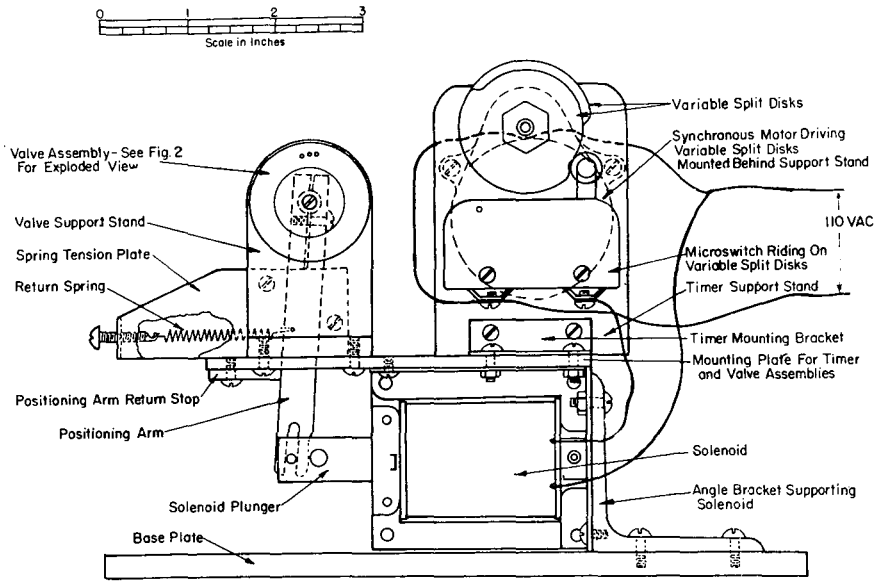

Fig. 1. Assembly detail of the stream division valve. This drawing to scale shows the assembled stream division valve. The timing assembly, constructed as shown for this device, is now commercially available from Western Electro-Mechanical Co., 300 Broadway, Oakland 7, Calif. The solenoid used is a Guardian type 14-Cont-115-A.C. Those structures shown in dashed lines are, as per convention, behind other structures.

flow rate to the reaction coil and colorimeter. This is accomplished by placing three outlets on one face of the valve and two on the other. Outlets A and B of valve face 2 (part No. 4 in Figs. 2 and 3) are connected to a fraction collector and the reaction coil of the analyzer respectively. Inlets 1 and 3 of valve face 1 are connected together and supplied by the auxiliary liquid flow while inlet 2 is supplied by the column effluent stream. Since inlets and outlets are equally spaced in their respective faces, when outlet A is positioned opposite inlet 3, outlet B opposes inlet 2 and auxiliary liquid flows to the fraction collector while column effluent flows to the analyzer reaction coil (see Fig. 3, ON position). When valve face 2 is rotated so that outlet A is opposite inlet 2, outlet B then opposes inlet 1 and column effluent flows to the fraction collector while auxiliary liquid flows to the analyzer reaction coil (OFF position, Fig. 3). If this rotary valve is reciprocated between these two positions, the column effluent will be divided between the fraction collector and the analyzer reaction coil. The accuracy of the division is assured by the precise flow rates available from the metered analyzer pumping system.

The backpressure created by the flow of liquid in the narrow gauge reaction coil tubing in the analyzer is equalized by using an equal length of the same bore teflon tubing leading to the fraction collector. This eliminates the possibility of "rebound" of the liquid column when the valve is rotated, and has an added advantage in that the optical density of an aliquot of the liquid being discharged into the fraction collector is, at the same time, being measured by the colorimeter and recorded on the analyzer chart.

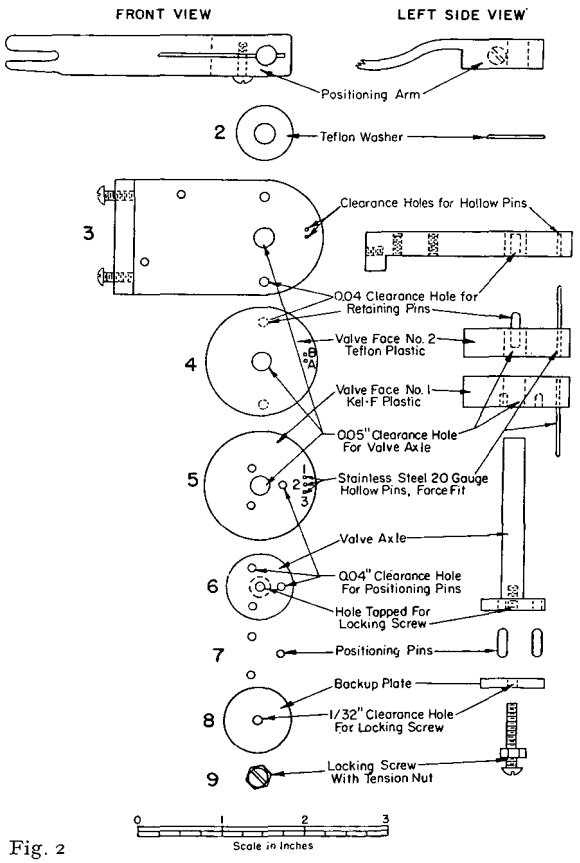

Fig. 2

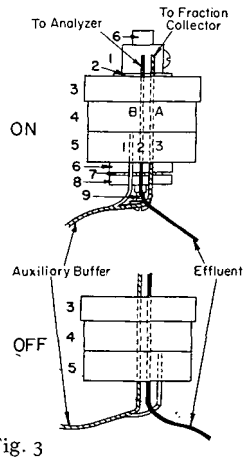

Fig. 3

Fig. 2. Exploded views of valve assembly. This drawing shows an exploded view of the valve assembly in its front view and its left side view as defined by the valve position in Fig. 1. Metal parts 1, 3, 6, 7 and 8 are preferably constructed from stainless steel and coated lightly with silicone grease to decrease corrosion and friction. The tightness of the valve is adjusted by tightening or loosening the tension nut. This adjustment is made less critical by placing $^1/_{16}$ in. rubber pads in the positioning pin holes of valve face 1. An assembled view comparable to the left side view of the valve may be found in the drawing labeled ON in Fig. 3.

Fig. 3. Top view of valve action. The two positions of the rotary valve are shown in this diagram. The numbers next to the components in the diagrams refer to the components so labeled in Fig. 2. The designations ON and OFF refer to the operation of the solenoid.

EFFLUENT DIVISION

The amount of column effluent returning to the analyzer is estimated by determination of the length of time for which the valve occupies each position. The ratio of the time during which the valve returns the column effluent to the analyzer to the time of a complete cycle of the valve is the fraction of the total sample being analyzed and recorded. A more precise determination of this ratio may be obtained by volumetric measurements or by the use of flow meters. The amount of liquid flowing to the fraction collector and through the analyzer should be determined while the valve is operating with only the column effluent liquid flowing.

RESULTS

This stream division valve has been tested by applying standard amino acid mixtures, collecting fractions, and re-applying the effluent fractions to the analyzer. The values for the amounts of the amino acids recorded and collected differed from the calculated values by not more than 5 %, while the fractions collected varied from the peak position on the recorder by not more than 12 sec. The valve is now in routine use for the purification and preparation of peptides.

ACKNOWLEDGEMENTS

The author is indebted to Mr. FRANK POOL, Jr., not only for the actual assembling of the valve, but also for the choice of materials for the final design of the valve.

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SUMMARY

A stream division valve which allows the division of a column effluent for analysis and collection in automatic column chromatography is described. Details of the construction of this valve as well as the results obtained in using it are presented.

THE ADDITIVITY OF RATE AND DIFFUSION PHENOMENA IN CONTINUOUS CHROMATOGRAPHY

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In a recent analysis[1] it has been possible to treat the fairly general problem of pulse-input chromatography with instantaneous equilibrium for linear and nonlinear isotherms, with and without axial dispersion; it had already been demonstrated[2,3] that the partial differential equations of continuous chromatography were very difficult to solve for the case of a finite rate of exchange and that even when analytical solutions are found they are much too complicated for use in correlating experimental data from actual chromatographic columns. While such analytical solutions are of mathematical interest they do not resolve the major problem of the experimental chromatographer who would much rather have a fundamental picture of the way in which finite rate phenomena and axial dispersion can influence elution time and band broadening. To quote GOLDSTEIN[4], who discusses equilibrium theory as a limiting case of kinetic theory, "what is needed, in addition to experimental work to determine the exchange equations, is a method for obtaining a closer approximation to the kinetic theory than is provided by the equilibrium theory without solving the kinetic theory equations in full. It will clearly be useful to do this first for a single solute." It is the purpose of the present communication to develop an approximate model for finite rate phenomena as a perturbation on equilibrium theory such that a useful working knowledge can be evolved of the way in which finite rate phenomena and axial dispersion can influence chromatography. In effect, the present analysis clearly defines the conditions under which dispersion and rate phenomena can be additive such that the final partial differential equation is the continuous rate theory analog of the plate approximation of VAN DEEMTER, ZUIDERWEG AND KLINKENBERG[5].

In the discussion that follows $C(t,z)$ denotes the moles of solute per unit volume of mobile phase, $n(t,z)$ the moles of solute taken up by unit volume of stationary phase, u the constant mobile phase velocity through the interstices, ε the void fraction, D the effective molecular diffusivity of the solute in the mobile phase accounting for the effect of the packing and K is the corresponding virtual coefficient of axial dispersion in the TAYLOR[6,7] sense. The concentration of solute in the mobile phase as a function of time t and axial position z is then given by[1,2]:

$$\frac{\partial C}{\partial t} + u\frac{\partial C}{\partial z} = (D + K)\frac{\partial^2 C}{\partial z^2} - \frac{1}{\varepsilon}\frac{\partial n}{\partial t} \qquad (1)$$

In effect the use of equation (1) presumes that there are no radial concentration gradients within the interstices of the stationary phase so that in the TAYLOR[7,8]

sense this means that dispersion terms of order $(K^2/u)\partial^3C/\partial z^3$ and higher have been neglected. The derivative $(1/\varepsilon)\partial n/\partial t$ represents the exchange of solute between the mobile and stationary phases, so that by assuming a finite rate with a linear isotherm:

$$\frac{\partial n}{\partial t} = k_A C - k_D n \tag{2}$$

where k_A and k_D are the rate constants for absorption and desorption respectively. Equation (2) can then be rearranged into the more convenient form:

$$\frac{1}{k_D}\frac{\partial n}{\partial t} = kC - n \tag{3}$$

where $k = k_A/k_D$ is the equilibrium constant for the linear isotherm. It is clear from eqn. (2) that if $k_D \to \infty$ and $\partial n/\partial t$ is finite as required by eqn. (1), then $n = kC$ and $\partial n/\partial t = k\partial C/\partial t$, corresponding to instantaneous equilibrium at each point in the column. However, small departures from equilibrium can be considered by permitting k_D to have a large, but finite, value when $\partial n/\partial t = k\partial C/\partial t$ may be considered to be the zeroth approximation to $\partial n/\partial t$ in eqn. (3), so that the next higher approximation for n becomes:

$$n = kC - \frac{k}{k_D}\frac{\partial C}{\partial t} \tag{4}$$

By repeating the above perturbation method, it is possible to generate the following infinite series for n:

$$n = k\left(C - \frac{1}{k_D}\frac{\partial C}{\partial t} - \frac{1}{k_D^2}\frac{\partial^2 C}{\partial t^2} - \cdots\right) \tag{5}$$

The most useful approximation for n is that obtained by truncating series (5) at the term of order $1/k_D$, corresponding to expression (4), Thus, substituting eqn. (4) for n into partial differential equation (1) and redefining the coefficients, we obtain

$$\frac{\partial C}{\partial t} + U\frac{\partial C}{\partial z} = (D_c + K_c)\frac{\partial^2 C}{\partial z^2} + R_c\frac{\partial^2 C}{\partial t^2} \tag{6}$$

where:

$$U = u/(1 + k/\varepsilon)$$
$$D_c = D/(1 + k/\varepsilon)$$
$$K_c = K/(1 + k/\varepsilon)$$
$$R_c = k/k_D\,\varepsilon(1 + k/\varepsilon)$$

It is clear from eqn. (6) that the effects of diffusion and a finite rate can be made additive if

$$\frac{\partial^2}{\partial t^2} = U^2\frac{\partial^2}{\partial z^2} \tag{7}$$

when eqn. (6) reduces to the following simple form:

$$\frac{\partial C}{\partial t} + U\frac{\partial C}{\partial z} = (D_c + K_c + U^2R_c)\frac{\partial^2 C}{\partial z^2} \tag{8}$$

Differential eqn. (8) can be further reduced to the form of the parabolic diffusion law by the change of variable $\xi = z - Ut$, when

$$\frac{\partial C}{\partial t} = E_c \frac{\partial^2 C}{\partial \xi^2} \tag{9}$$

where $E_c = D_c + K_c + U^2 R_c$. Equation (9) may now be used to analyze experimental chromatography data in the manner discussed by HOUGHTON[1]. The solution to eqn. (9) for an initial pulse of length L_0 centered around $z = 0$ and containing a solute of concentration C_0 is

$$\frac{C}{C_0} = \tfrac{1}{2} \operatorname{erf}\left(\frac{\xi + \tfrac{1}{2} L_0}{2\sqrt{E_c t}}\right) - \tfrac{1}{2} \operatorname{erf}\left(\frac{\xi - \tfrac{1}{2} L_0}{2\sqrt{E_c t}}\right) \tag{10}$$

It is now appropriate to discuss the conditions under which rate and diffusion phenomena are additive, as in eqns. (8) and (9):

(1) Necessary but not sufficient conditions for an additive law are (a) a linear equilibrium isotherm, (b) the absence of appreciable radial gradients of concentration in the interstices so that dispersion terms of order $(K^2/u)\partial^3 C/\partial z^3$ or higher may be neglected, and (c) the existence of only small deviations from instantaneous equilibrium, corresponding to the neglect of rate terms of order $(1/k_D^2)\partial^3 C/\partial t^3$ and higher. In addition to the above three requirements, the experimental conditions must be such that at least one of the following three conditions is satisfied:

(2) As discussed above, small deviations from equilibrium require R_c to be small, so that if it is also presumed that D_c and K_c are small then the terms on the right of eqn. (6) can be considered negligible compared with those on the left, leading to the Lagrangian subsidiary equation

$$\left(\frac{\partial}{\partial t} + U \frac{\partial}{\partial z}\right) C = 0 \tag{11}$$

Relationship (7) between the distance and time operators follows immediately from degenerate eqn. (11). The assumption of small values of D_c, K_c and R_c corresponds to the case treated by VAN DEEMTER *et al.*[5], who demonstrated additivity for the plate theory using the solution of LAPIDUS AND AMUNDSON[2].

(3) It is not necessary, however, to assume that D_c and K_c are small if the initial pulse width L_0 is small enough, since the solutions for small R_c will still be of the form (10), when it is readily observed that values of $[\xi]$ leading to appreciable ratios C/C_0 must be of order $L_0/2$; thus as $L_0 \to 0$ then $z \to Ut$, leading again to relationship (7) and solution (10).

(4) Since solutions for small R_c will be of the form (10), it is clear that the first time derivative, $\partial C/\partial t$, will decrease as $t^{-\frac{3}{2}}$ while $\partial^2 C/\partial t^2$ will decay as $t^{-\frac{5}{2}}$; similarly $\partial C/\partial z$ will decrease as $t^{-\frac{1}{2}}$ and $\partial^2 C/\partial z^2$ as t^{-1}. Thus as $t \to \infty$ the terms on the right of eqn. (6) will again become negligible in comparison with first derivatives on the left, because the second derivatives decay to zero faster than the first derivatives. Thus the additive eqns. (8) and (9) will become asymptotically valid at large times approaching infinity.

Under the above restrictions the effects of diffusion and rate phenomena are additive in that, to a first approximation, they both cause band broadening without affecting the elution time.

SUMMARY

By considering a finite rate of exchange between the mobile and stationary phases as a perturbation on equilibrium theory, it has been possible to arrive at conditions for the additivity of rate and diffusion phenomena in continuous chromatography. The resulting diffusion equation and its solution for a pulse input provide an alternative method to the plate theory for the treatment of experimental chromatography data.

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A CHROMATOGRAPHIC STUDY OF THE BUFADIENOLIDES ISOLATED FROM THE VENOM OF THE PAROTID GLANDS OF *BUFO PARACNEMIS* LUTZ 1925

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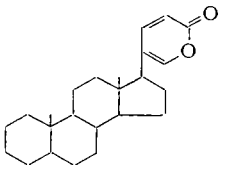
Chromatography is of acknowledged importance in the identification and separation[1] of the bufadienolides (I), the cardiotoxic steroids of toad venoms, which are of increasing pharmacological interest[2–5].

Following on our work on the venoms of *B.ictéricus* Spix 1824 and *B.crucifer* Wied 1821[6], we now report a chromatographic survey of the bufadienolides isolated from the venom of the parotid glands of *B.paracnemis* Lutz 1925.



(I)

B. paracnemis Lutz 1925[7] is a giant toad (13–22 cm) (Fig. 3) common in various states of Brazil (Minas Gerais, Rio de Janeiro, São Paulo, Paraná)[8]. The species possesses voluminous parotid glands averaging 25 % of the length of the individual and is easily distinguished from the northern (Amazonian) *B.marinus* Linnaeus 1758 by the presence of salient *tibial* glands.

A preliminary investigation of the crude venom was based on thin-layer chromatography, the results of which have been briefly reported[9]. In order to achieve separation and identification of the bufadienolides obtained in this first step, repeated column chromatography on aluminium oxide or silicic acid was carried out and the fractionations systematically followed by thin-layer and paper chromatographic analysis.

EXPERIMENTAL

Extraction of the bufadienolides

The viscous mass, collected from 239 individuals by pressure of the parotid glands, was dried in a desiccator (over P_2O_5), yielding 57 g of crude dried venom. The aqueous suspension of this material was repeatedly extracted with chloroform–methanol (9:1)

* This paper was presented in part at the 4th meeting of the Brazilian Chemical Society, Professor FRITZ FEIGL's 70th anniversary, Rio de Janeiro, Nov. 16–23, 1962.

and the concentrated dried organic layers furnished 9.8 g of an amorphous residue containing mainly cardiotoxic steroids.

Column chromatography

Chromatographic fractionations were performed on columns of aluminium oxide (Merck, active neutral) or silicic acid (Mallinckrodt, 100 mesh) by the method of fractional elution[10], using the sequence of solvents benzene, chloroform, methanol (Table I).

TABLE I

COLUMN CHROMATOGRAPHIC FRACTIONATION OF BUFADIENOLIDES

Column 1 (Al_2O_3): 9.5 g of venom extract				*Column 2 (Al_2O_3): 1.72 g from fractions 1–3, column 1*			
Fraction No.	*Solvent system**	*Amount (mg)*	*Bufadienolide***	*Fraction No.*	*Solvent system**	*Amount (mg)*	*Bufadienolide***
1–3	Chf–Bz 1:1	1785	γs, R, M	1–4	Bz	24	γs
4–6	Chf–Bz 3:1	3089	R, M, T	5–9	Chf–Bz 1:1	194	γs, B, M
7	Chf	490	M, T	10–15	Chf–Bz 3:1	102	
8–10	Chf	539		16–20	Chf	419	B, M
11	Chf–Me 50:1	794	M, T, Bt, G,	21–24	Chf–Me 50:1	629	B, M, T, He, G
12–13	Chf Me 50:1	252	He	25–26	Chf–Me 50:2	208	M, T
14	Chf–Me 45:5	289		27–28	Chf–Me 45:5	52	He, G
15–16	Me	627	M, Bt, T, He, Ho, 3 spots				

Column 3 (SiO_2): 0.664 g from fractions 5–20, column 2				*Column 4 (Al_2O_3): 3.2 g from fractions 4–7, column 1*			
Fraction No.	*Solvent system**	*Amount (mg)*	*Bufadienolide***	*Fraction No.*	*Solvent system**	*Amount (mg)*	*Bufadienolide***
1–4	Chf–Bz 2:3	104		1	Chf–Bz 1:1	64	M, R
5–9	Chf–Bz 3:2	11	γs	2–6	Chf–Bz 1:1	42	M, T
10–12	Chf–Bz 4:1	16		7–13	Chf–Bz 3:1	1204	M, (T)
13–15	Chf	5	γs, B	14–19	Chf	1710	
16–23	Chf–Me 50:1	373	B, M	20–21	Chf–Me 50:1	29	M, T
24–25	Me	35		22	Chf–Me 50:2	26	

Column 5 (Al_2O_3): 1.3 g from fractions 8–11, column 1				*Column 6 (Al_2O_3): 1.12 g from fractions 12–16, column 1*			
Fraction No.	*Solvent system**	*Amount (mg)*	*Bufadienolide***	*Fraction No.*	*Solvent system**	*Amount (mg)*	*Bufadienolide***
1–2	Chf–Bz 1:1	44	M, T, Ag	1–4	Chf	280	M, T, Bt, He
3–5	Chf–Bz 1:1	24	M, T	5–9	Chf–Me 50:1	260	M, T, Bt, Ag
6–10	Chf–Bz 3:1	54	M, Bt	10–14	Chf–Me 50:1	184	He, Ho, 2 spots
11–19	Chf	210	T, G	15–19	Chf–Me 50:2	147	M, T, Ag, He
20	Chf–Me 50:1	628	T, (M), (G)	20–23	Chf–Me 45:5	36	Ho, 5 spots
21–23	Chf–Me 50:1	150	T, He, Ho,	24–28	Chf–Me 20:5	41	1 spot (start)
24–26	Chf–Me 50:2	22	1 spot				

* Chf = chloroform; Bz = benzene; Me = methanol.

** Ag = argentinogenin; B = bufalin; Bt = bufotalinin; G = gamabufotalin; He = hellebrigenin; Ho = hellebrigenol; M = marinobufogenin; R = resibufogenin; T = telocinobufogenin; βs = β-sitosterol; γs = γ-sitosterol; parentheses indicate traces.

Thin-layer chromatography (TLC)

Chromatoplates of Silica gel G (Merck) were prepared by procedures already described[9–11] and ascending development was carried out, using one of the solvent systems:

A: Ethyl acetate–cyclohexane (1:2)

B: Ethyl acetate

C: Ethyl acetate–methanol (9:1).

After concentration of the column eluates to a small volume (1–2 ml), aliquots were applied to the plates through capillary tubes.

Paper chromatography

The descending method was selected. The papers (Whatman No. 1) were impregnated with the stationary phase previously diluted with acetone (1:5 v/v) and partially dried (70°) before development. The solvent systems employed were:

A: Formamide–benzene

B: Formamide–chloroform

C: Propylene glycol–water (4:1)/benzene–chloroform (1:1).

Development took from 3 to 96 h. 100 to 150 μg of the steroidal mixture and 30 to 50 μg of the standards were applied along a line 7 cm from one edge of the 50 cm sheets.

Detection

The steroidal compounds were visualized by spraying a saturated chloroform solution of $SbCl_3$ on the chromatoplates or the dried papers (vacuum oven/60°), heating over a hot plate and examining in ultra-violet light. In experiments with preparative TLC, visualization could also be obtained by spraying water[12] on the developed plates, thus showing up the white bands corresponding to the steroidal constituents.

RESULTS AND DISCUSSION

Table I shows the results of the chromatographic fractionations and Table II shows some of the R_F values. Figs. 1 and 2 are examples of thin-layer and paper chromatographic analyses of bufadienolides.

Fractions 1–3 (column 1), 1–9 (column 2) and 1–12 (column 3) (Table I) include

TABLE II

R_F VALUES* (PAPER CHROMATOGRAPHY) OF UNIDENTIFIED POLAR COMPONENTS

Solvent system: formamid/chloroform

	$R_F \times 100$	*Color under U.V. light ($SbCl_3$, after 2 min heating)*
Gamabufotalin	100	Yellow
P_1	80	Pink
P_2	67	Pink
P_3	44	Pink-yellow
P_4	25	Orange
P_5	10	Pink-orange

* Determined with reference to gamabufotalin.

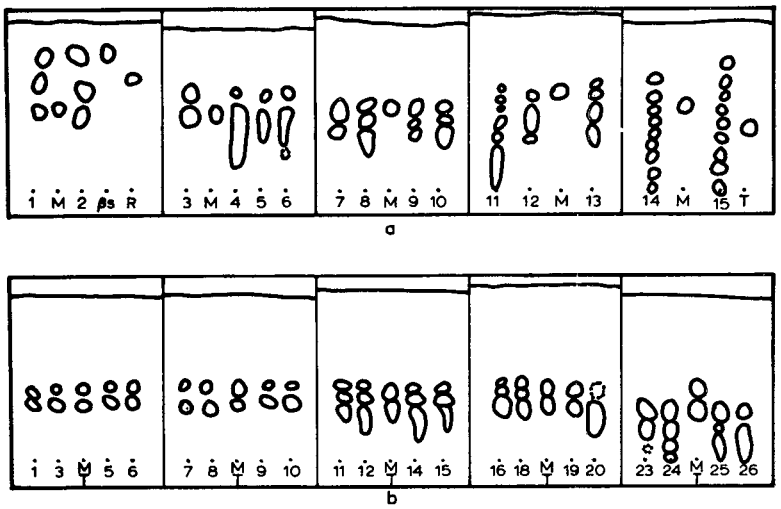


Fig. 1 (a). Thin-layer chromatography of eluates from column 1 (Table I). Solvent: ethyl acetate. (b) Thin-layer chromatography of eluates from column 5 (Table I). Solvent: ethyl acetate.

a substance of the same R_F value as β-sitosterol (TLC, solvent systems: ethyl acetate–cyclohexane (1:2), ethyl acetate). This was considered to be γ-sitosterol. Since the occurrence of this sterol has earlier been recognised in toad venoms[13] and the structure and configuration of β-sitosterol assigned to it[14], a standard of β-sitosterol was used for further identification: (a) column chromatography on silicic acid of fractions 1–9 (column 2) and 1–12 (column 3) followed by elutions from benzene yielded 113 mg of chromatographically (TLC) pure material which was recrystallized in acetone–methanol; yield 51 mg, m.p. 141–143°, $[\alpha]_D^{21°} = -41°$ ($c = 1.03$; chloro-

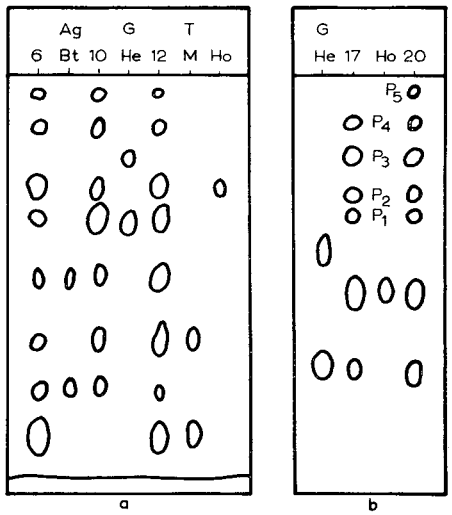


Fig. 2. Paper chromatograms of eluates from column 6 (Table I). Solvent system: formamide–chloroform. Time of development: (a) 3 h; (b) 6 h.

form); (b) infrared spectra of this compound and of β-sitosterol exhibit the same absorption bands in the 1400–650 cm^{-1} region, which agrees with previous findings[15].

Recrystallizations in acetone–ether of: (a) fractions 16–25 (column 3) furnished 45 mg of chromatographically (TLC) pure bufalin, m.p. 235–238°, $[\alpha]_D^{24°} = -11°$ ($c = 1.09$; chloroform); (b) fractions 7–19 (column 4) gave 2.717 mg of chromatographically (TLC) pure marinobufogenin*, m.p. 215–217°, $[\alpha]_D^{24°} = +12°$ ($c = 1.13$; chloroform); and of (c) fraction 20 (column 5) yielded 524 mg of telocinobufogenin, together with traces of marinobufogenin, m.p. 158–162°, $[\alpha]_D^{24°} = +7°$ ($c = 1,07$; chloroform).

In fractions 15–23 (column 6), the presence of 5 spots of lower R_F values than gamabufotalin was demonstrated by paper chromatography (Fig. 2). Further fractionation on a column of silicic acid or by preparative TLC was unsuccessful owing to the complexity of the mixture and its low yield.

Generally speaking, TLC had several advantages, but showed certain limitations when using silica gel G: for instance, in cases in which the resolving power of TLC did not favour the distinct location of the more polar bufadienolides, paper chromatography proved more suitable.

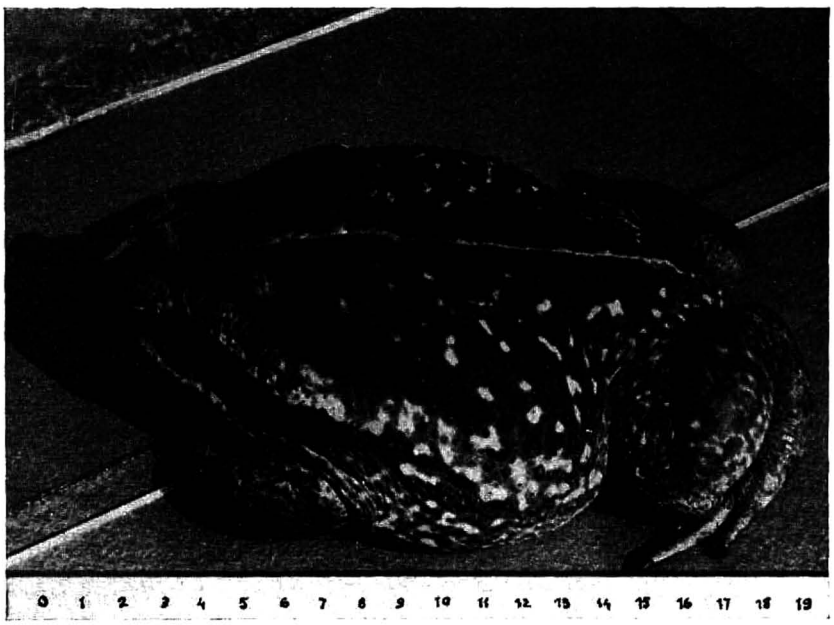


Fig. 3. *Bufo paracnemis* Lutz 1925.

ACKNOWLEDGEMENT

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* Previously isolated from the venom of the same species of toad along with bufotenine and adrenaline[16].

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SUMMARY

A chromatographic study of the bufadienolides isolated from the venom of the parotid glands of *Bufo paracnemis* Lutz 1925 is reported. Column fractionations, followed by thin-layer and paper chromatographic analysis, resulted in the identification of the following compounds: γ-sitosterol, argentinogenin, bufalin, bufotalinin, gamabufotalin, hellebrigenin, hellebrigenol, resibufogenin and telocinobufogenin. Five unidentified compounds of lower R_F values than gamabufotalin were detected by paper chromatography, using the solvent system formamide–chloroform.

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MULTI-DIMENSIONAL CHROMATOGRAPHY USING DIFFERENT DEVELOPING METHODS*

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The efficiency of chromatographic separations of complex mixtures is commonly reduced by the fact that peak or zone overlapping occurs with compounds of different chemical structure. In paper chromatography (PC), thin-layer chromatography (TLC) or electrophoresis the use of two-dimensional chromatograms is known and widely applied[1–4]. In gas chromatography (GC) the application of two or more stationary phases selectively retaining the different components of mixtures is in principle equal to two-dimensional chromatography.

Only few papers[5,6] have been published on the pre-separation of compounds using TLC and the subjection of pre-separated parts of a mixture to gas chromatography. One of these papers used a more detailed combination[6]: pre-separation by means of GLC on one type of stationary phase, followed by TLC separation of the simplified GLC cuts and subsequent gas–liquid chromatographic separation of extracted TLC cuts on another type of stationary phase. In this paper it is shown that the separation on thin layers of adsorbent is a process occurring in the system independent of the vapour pressure of the substances. Under these circumstances the polarity of the adsorbent is much higher than the polarity of all known polar stationary phases used in GLC. Therefore the separation depends in the first place upon the type of functional group and/or their steric hindrances, while the molecular weights play a secondary role. On the other hand, non-polar stationary phases in GLC systems separate to the first approximation according to the increasing C-number in a molecule or according to the increase in molecular weight, as well as the dispersion forces of molecules. The separation is therefore a process in which the vapour pressure of the substances plays a minor role.

If we use GLC as a sampling stage for TLC or PC it is clear that we can open up some new possibilities in the field of isolation and identification of components from complex mixtures of different substances. This type of two-dimensional chromatographic separation exploits to the maximum both separation extremes given by all existing chromatographic methods, *viz.* separation according to the number of C atoms by GLC in the direction of the time axis along the start of the thin layer or the paper and, according to the type of functional group, by TLC or PC in the direction of the capillary flow of the solvent. After this two-dimensional chromatography, it is possible to extract the separated compounds from the thin layer or paper and, if

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necessary, chromatograph microgram quantities by means of GLC on a suitably selected stationary phase.

Other methods of identification, *e.g.* microscopy, crystallography or colorimetry, as described in another paper[7], can be used in connection with the above-mentioned procedure.

THEORY

When different chromatographic methods are applied in sequence, it is necessary to consider the effect of different diffusion processes on zone broadening. If the products of a gas chromatogram are developed on the start of the thin layer or the paper, the condition that neighbouring components are accurately and quantitatively resolved[8] is given by

$$t'_{\max} - t_{\max} = \Delta t_{\max} = 4\sigma \tag{1}$$

As a result of diffusion on the plate, overlapping of both zones may appear, as shown in Fig. 1, when a thin-layer chromatogram is developed. This overlap may be so great that under unsuitable conditions the separatory effect of the gas chromatography is annulled. In order that two different zones may be clearly detected after the TLC separation, the relation between b and σ must also be limited, $b \leqq 2\sigma$.

The width of the zone, b, on the thin layer can be defined in a common form similar to that used in chromatography for defining the degree of separation[9]:

$$n = 16\left(\frac{V_{\max}}{\Delta V}\right)^2 = 16 \cdot \left(\frac{d}{b}\right)^2 \tag{2}$$

where n is the number of theoretical plates, $V_{\max}$ is the volume of mobile phase at the maximum of the concentration of the zone, ΔV is the volume corresponding to the zone width (cut out with tangents at the points of inflexion), and d is the distance of the zone from the start on the layer.

$$b = 4\sqrt{\frac{d^2}{n}} \tag{3}$$

After putting the number of theoretical plates n in terms of the height equivalent of a theoretical plate H, where $n = d/H$, $d = R_F \cdot L$, and L is the length of the layer, equation (3) can be written as

$$b = 4\sqrt{H \cdot d} = 4\sqrt{H \cdot R_F \cdot L} \tag{4}$$

b therefore increases linearly[10] with respect to d and $\Delta b = K_1 \Delta d$ (definition (2)). Since $d^2 = K_2 t$, t being the development time of the chromatogram on the plate, the width of the zone b increases in accordance with $\sqrt{t}$. The spreading from the starting point of the spot to a point of definite width is parabolic. This was the conclusion drawn for paper chromatography[11] and chromatography on plates[9] prepared by drying of a slurry of silica gel G.

The position of the chromatographic zones on the start line is given by $\Delta t_{\max} \cdot v$, where v is the velocity of the plate (when the sample from the gas chromatography column is deposited on it), and along the start line our condition for non-overlapping may be written as

$$b \leqq 2\sigma \leqq \frac{1}{2}\Delta t_{\max} \cdot v \tag{5}$$

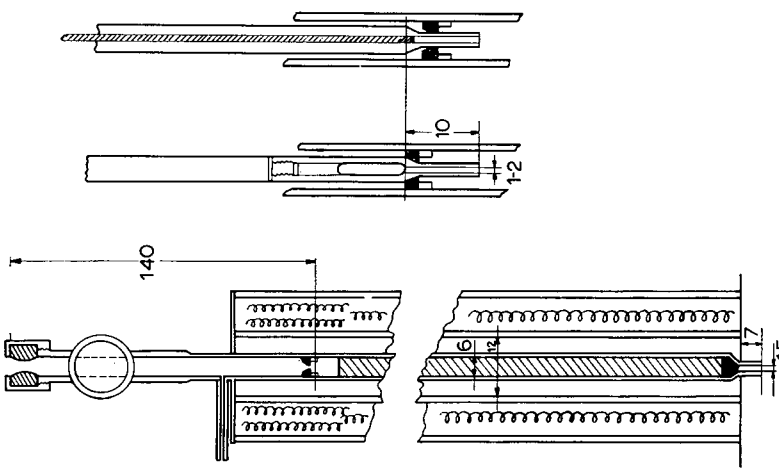

Fig. 2. Scheme of the gas chromatographic column with sampling device in detail (dimensions in mm).

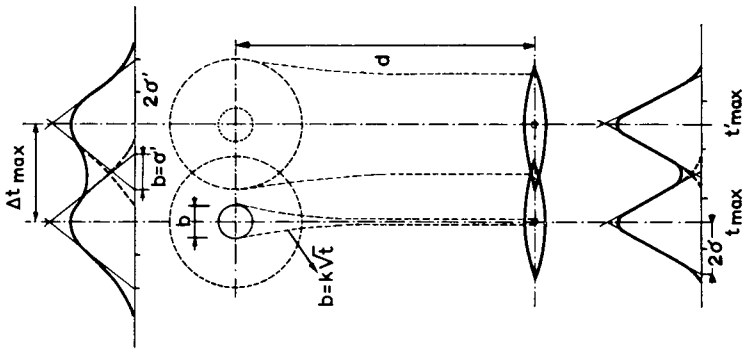

Fig. 1. Band broadening of gas-chromatographic zones on a thin layer.

Combining equations (3) and (5) gives

$$v \geqq \frac{8}{\Delta t_{max}} \sqrt{H \cdot R_F \cdot L} \tag{6}$$

The speed necessary to fulfil this condition will be the lower the larger Δt_{max}, and/or the lower the height equivalent of a theoretical plate, or the ratio of the rate of movement of the solute zone to the solvent front and the length of the layer (that is, the value under the root determining the value of b).

When artificial restriction of the lateral diffusion of the zones has been created by means of dividing the layer into a system of narrow strips of width a, the relation for the minimum speed can be given in the final form:

$$v_{min} = \frac{a}{b} \cdot \frac{8}{\Delta t_{max}} \sqrt{H \cdot R_F \cdot L} \tag{7}$$

METHODS AND MATERIALS

Gas chromatography

The gas chromatography column was a stainless steel tube of 6 mm I.D. and of 100 cm of usual length, as shown diagrammatically in Fig. 2.

The column was placed in a steel jacket of 12 mm I.D. surrounded by 8 semi-circular electric heating elements each of 250 mm length. Current was supplied from the mains supply through an auto-transformer. The injection chamber was heated with a small element to approximately 50° above the temperature of the chromatographic column. The colum outlet was reduced to a capillary of 1.5 mm I.D. and 7 mm long. The sample was introduced into the gas chromatographic column by means of a slightly modified TENNEY–HARRIS pipette[12] or a plunger-operated injection needle[13]. In the first case the sampling pipette was pressed through a silicone gasket which was held tightly in place above the column filling by a metallic O-ring, and the liquid or melted sample was transferred by the carrier gas stream into the heated sampling chamber and thence in vapour form into the column filling. In the second case the sample was forced out from a robust injection tube by a steel wire acting as a plunger. The second method gives better results for low-viscosity liquids and for solids with melting points higher than 100°.

Phenyl methyl silicone elastomer of molecular weight approximately 370,000 (East Bohemian Chemical Works, N.E., Pardubice, ČSSR) was applied as stationary phase as a 10% w/w coating of celite (Johns–Manville Ltd., London, England) of grain size 0.2–0.3 mm. The stationary phase was stable up to 300°. Nitrogen or argon was used as carrier gas.

Thin-layer chromatography

Plates of mirror glass, 100 × 200 mm and 200 × 200 mm, were used and coated with loose powdered silica gel PHH (Spolana N.E., Neratovice, ČSSR; this material is more polar than Merck silica gel G for thin-layer chromatography). An applicator of a common type was used[14] for the silica gel plates of 80 × 190 mm and 180 × 180 mm, grain size 0.05–0.15 mm, thickness of the layer 0.6–0.9 mm. In the case where lateral diffusion is restricted, metal plates made from nickel-coated brass were used. These

were notched at 3-mm intervals with a groove 1 mm in width to provide a thin-layer strip of 1 mm width between the grooves (see Fig. 3).

A glass chromatographic chamber of 150 × 300 × 150 mm or 300 × 300 × 150 mm was used. The slope of the coated plates was 20–30°. The following analytically

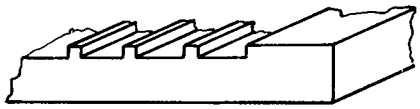

Fig. 3. Schematic illustration of the type of notches.

pure solvents, *n*-hexane, cyclohexane, benzene, chloroform, acetone, ether (Lachema N. E., Brno, ČSSR), were used for measuring R_F values. For some analytical separations, benzene, cyclohexane and a mixture of cyclohexane–benzene (1:1 v/v) were chiefly employed. Developing times of the TLC were usually 8–15 min.

Gas chromatographic sampling technique for TLC

The silica gel plates were placed on desk D on a small flat carriage as shown in Fig. 4.

The movement of the carriage was effected by a continuous screw, S_3, driven by an electric motor, M, which can be operated at different speeds by means of gears S_1 and S_2. The driving velocity is regulated in the range of 2–75 mm/sec. The outlet from the chromatographic column was placed 2 mm above the surface of the silica gel layer and 10–15 mm from the edge. No disturbance of the silica gel was caused by velocities of carrier gas up to 1 ml/sec and under these conditions the starting zone was usually less than 3 mm wide.

Detection and recovery of substances from a TLC plate for further GLC analysis

The spots were detected by forming coloured complexes of the substances under examination with tetracyano-ethylene (TCE)[15]. A saturated solution of TCE in benzene (3.6 g/100 ml) was dropped from a capillary pipette or sprayed on by means of a wetted tooth-brush rubbed on a metal sieve. After evaporation of the benzene in a drying oven at 100°, the yellow-coloured silica gel becomes white and coloured spots of

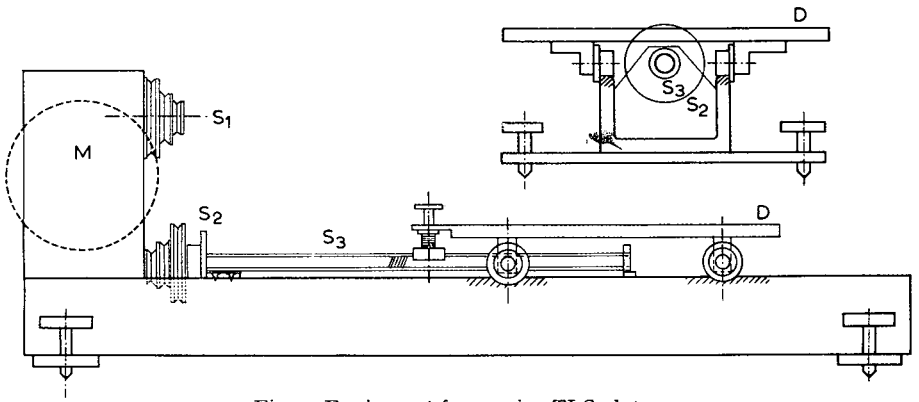

Fig. 4. Equipment for moving TLC plates.

complexes appear. The intensity of the various colours is constant for 1–2 h, but substances of phenolic character deepen in colour during this time. After this time the colours fade according to the type of substance[16], though the effect of the moisture content of the silica gel on the reaction becomes important too. Similar results are observed at higher drying temperatures. In the case of analytical treatment of the chromatograms, TCE solution was dropped on places where spots were expected.

For further gas-chromatographic examination the silica gel corresponding to a zone characterized by a t_{max} value (gas chromatography) and R_F value (thin-layer chromatography) is transferred into a glass capillary shown in Fig. 5.

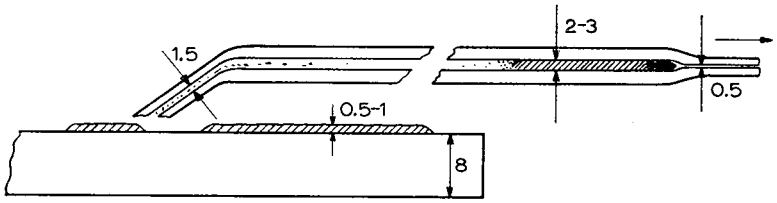

Fig. 5. Transfer capillary for extracting TLC material for GLC analysis (dimensions in mm).

The silica gel is removed by suction into the capillary starting at the centre of the expected zone on the plate and working spirally to the edges. The small silica gel column in the capillary is eluted with methanol or pyridine (0.1 ml at a time). Usually the first two drops from the column contain all the adsorbed material and serve as a storage solution for injection into a sensitive ionisation gas chromatograph. The apparatus used was a commercial high-temperature flame ionisation model (Chrom I, Laboratory Equipments N.E., Prague, ČSSR).

Reference substances

These substances were compounds specially prepared for research purposes (Gesellschaft für Teerverwertung, Duisburg, W.-Germany; The Benzole Producers Ltd., Watford, Herts., England; Coal Tar Research Association, Gomersal, Leeds, England) and purified by GC-sublimation[7], so that they were gas-chromatographically pure.

EXPERIMENTAL AND DISCUSSION OF RESULTS

R_F values of standard compounds and colours of their TCE complexes

The R_F values obtained are summarized in Tables I and II.

The group character of the separation of compounds with different functional groups on silica gel, as described earlier[6], is evident. Aromatic hydrocarbons and heterocyclics without strong electron affinity to silica gel have R_F values of about 0.4–0.5 in non-polar solvents (*n*-hexane, cyclohexane), and heterocyclics with –NH– and –N= functional groups and phenols have R_F values of less than 0.05. In benzene the R_F values of aromatic hydrocarbons increase to 0.90–0.95, those of indoles and carbazoles to 0.60–0.75. Phenols have R_F values of 0.15–0.50, according to the degree of steric hindrance of the hydroxyl group. Heterocyclics with tertiary nitrogen are retained practically on the start. The molecular weight of most substances has little influence on the R_F value.

TABLE I

R_F VALUES OF AROMATIC HYDROCARBONS AND COLOURS OF THEIR COMPLEXES WITH TCE

Compound	*Colour of complex with TCE*	R_F		
		n-Hexane	*Cyclohexane*	*Benzene*
Naphthalene	rose-violet	0.50	0.40	0.94
1-Methylnaphthalene	grey-violet	0.47	0.46	0.94
2-Methylnaphthalene	red-violet	0.46	0.47	0.93
1,2-Dimethylnaphthalene	grey-blue	0.48	0.56	0.94
1,3-Dimethylnaphthalene	grey-violet	0.42	0.53	0.95
1,4-Dimethylnaphthalene	grey-blue	0.55	0.54	0.95
1,5-Dimethylnaphthalene	green-blue	0.44	0.47	0.93
1,6-Dimethylnaphthalene	green-blue	0.47	0.47	0.94
1,7-Dimethylnaphthalene	dark grey	0.50	0.52	0.95
2,3-Dimethylnaphthalene	blue-violet	0.36	0.40	0.95
2,6-Dimethylnaphthalene	blue-violet	0.46	0.44	0.93
2,7-Dimethylnaphthalene	violet	0.45	0.44	0.94
2-Ethylnaphthalene	brown	0.55	0.52	0.93
2,3,5-Trimethylnaphthalene	blue-grey	0.50	0.47	0.93
2-Phenylnaphthalene	red-violet	0.34	0.38	0.92
Biphenyl	orange	0.45	0.45	0.95
3,5-Dimethylbiphenyl	orange	0 40	0 42	0.93
4,4′-Dimethylbiphenyl	light violet	0.39	0.44	0.94
4,5-Benzindane	grey-green	0.50	0.50	0.96
Acenaphthene	coffee green	0.52	0.53	0.95
Fluorene	red-violet	0.40	0.40	0.95
Phenanthrene	violet	0.36	0.40	0.93
1-Methylphenanthrene	violet	0.29	0.32	0.94
Anthracene	blue-green*	0.40	0.42	0.95
2-Methylanthracene	blue-green*	0.40	0.40	0.94
Pyrene	grey-green	0.35	0.38	0.93
Fluoranthene	brown-violet	0.30	0.25	0.92
Chrysene	grey	0.18	0.18	0.94

* Colour disappears within a few seconds.

The colours of TCE complexes are also given in Tables I and II. It is known[16] that the colour formation is connected with the donor–acceptor charge transfer between π electron pairs of aromatic conjugated systems and cyano groups on the unsaturated C skeleton[17]. The colour of the complexes in the visible part of the spectrum has some relation to the structure of the substances under investigation. Hydrocarbons with simple benzene nucleic or substituted nuclei have colours from yellow to red, *e.g.* benzene – yellow, tetramethyl benzene – red, diphenyl – orange, diphenylmethane – orange, 3,5-dimethyldiphenyl – light violet. Tri- or polycyclic aromatic hydrocarbons are grey to violet. A special position is occupied by phenols and some heterocyclics, where, as previously mentioned, the colour change with time indicated that the complexing proceeds by stages. There are also characteristic group colours, *e.g.* red to brown for indoles and blue for carbazoles. Heterocyclic compounds with tertiary nitrogen in the nucleus yield weak yellow to yellow-brown colours.

TABLE II

R_F VALUES OF AROMATIC S, O AND N COMPOUNDS AND COLOURS OF THEIR COMPLEXES WITH TCE

Compound	*Colour of complex*	R_F					
		n-Hexane	*Cyclohexane*	*Benzene*	*Chloroform*	*Ether*	*Acetone*
S compounds							
Thionaphthene	brown	0.47	0.53	0.93			
Diphenylene sulphide	violet	0.46	0.44	0.90			
O compounds							
Cumarone	yellow-orange	0.41	0.41	0.90			
Diphenylene oxide	brown-red	0.37	0.40	0.92			
4-Hydroxyhydrindene	red-brown	0.02	0.01	0.29	0.21	0.81	0.94
5-Hydroxyhydrindene	grey-violet	0.00	0.00	0.18	0.16	0.88	0.95
2,3,5-Trimethylphenol	dark violet	0.00	0.00	0.28	0.33	0.82	0.96
2,4,5-Trimethylphenol	grey	0.00	0.00	0.24	0.26	0.91	0.92
3,4,5-Trimethylphenol	brown-violet	0.00	0.00	0.15	0.17	0.90	0.96
3-Methyl-5-ethylphenol	brown	0.00	0.00	0.19	0.18	0.90	0.94
1-Naphthol	orange	0.01	0.01	0.32	0.26	0.85	0.94
2-Naphthol	brown	0.00	0 00	0.20	0.17	0.86	0.95
2-Hydroxybiphenyl	yellow-brown	0.03	0.02	0.55	0.50	0.90	0.94
4-Hydroxybiphenyl	blue-violet	0.00	0.00	0.23	0.20	0.92	0.95
N compounds							
Indole	brown (green edge)	0.03	0.02	0.65	0.63	0.95	
2-Methylindole	orange	0.03	0.02	0.65	0.60	0.95	
3-Methylindole	brown (violet edge)	0.02	0.03	0.65	0.70	0.92	
5-Methylindole	brown	0.03	0.03	0 65	0.65	0.93	
7-Methylindole	brown	0.04	0.04	0.64	0.65	0.95	
Carbazole	ultramarine blue	0.03	0.02	0.74	0.75	0.92	
2-Methylcarbazole	sky blue	0.03	0.02	0.77	0.70	0.92	
Quinoline	red-orange	0.00	0.00	0.02	—	0.45*	
2-Methylquinoline	red-orange	0.00	0.00	0.04	0.40*	—	
4-Methylquinoline	brown-orange	0.00	0.00	0.04	0.40*	—	
7-Methylquinoline	brown-orange	0.00	0.00	0.03	—	—	
8-Methylquinoline	brown-orange	0.00	0.00	0.03	0.38*	0.70*	
2,6-Dimethylquinoline	brown-yellow	0.00	0.00	0.04	—	—	
7,8-Benzoquinoline	brown-orange	0.00	0.00	0.01	—	—	
Isoquinoline	brown-orange	0.00	0.00	0.03	—	0.44*	
1-Methylisoquinoline	brown	0.00	0.00	0.02	0.29*	—	
3-Methylisoquinoline	grey-brown	0.00	0.00	0.02	—	—	
2-Phenylpyridine	brown-orange	0.00	0.00	0.02	—	—	
Acridine	yellow-orange	0.00	0.00	0.00	—	0.01	

* Tailing from the start to the given R_F value; in the case of small samples remains at the start.

Estimation of minimum plate driving velocity

Zone broadening during the deposition of the substances from the GC column on the TL plate is a diffusion process[18] which can be described by an effective diffusion coefficient:

$$\sigma = \sqrt{2Dt} = \frac{b}{4}$$

$$D = \frac{b^2}{32\,t}$$

$$b = \sqrt{32Dt} = k\sqrt{t}$$

Fig. 6 shows that the relation d and t only approaches the parabolic one described for PC[11] and TLC[9] (from slurry-prepared thin-layer plates). Deviation from the linearity of the relation between d^2 and t is caused by irregular distribution of the solvent. The condition that the driving velocity of the chromatographic front is proportional to the mass flow of solvent[11] is not completely fulfilled.

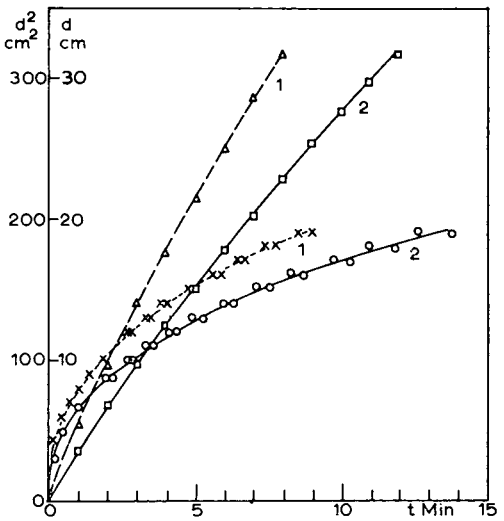

Fig. 6. Relation between distance of the zone from the start and time.
1 = cyclohexane; 2 = benzene.

Data were found for some aromatic hydrocarbons in the system silica gel/benzene, as shown in Table III ($L = 19.0$; $t = 525$ sec; $R_F = 0.94$; $\Delta t_{max} = 1.3$ min).

Values for the minimum driving velocity of the plate estimated from average data:

$$v_{min} = \frac{8}{1.3} \cdot \sqrt{0.00259 \times 18} = 6.15 \times 0.216 = 1.33 \text{ cm/min.}$$

Using notches of $a = 0.30$ cm, the minimum driving velocity is

$$v_{min}\,(\text{diphenyl/acenaphthene}) = 1.33 \cdot \frac{0.30}{0.84} = 0.475 \text{ cm/min.}$$

This is in accordance with experimental results, as may be seen from Fig. 7.

With a driving velocity of 0.22 cm/min (Fig. 7a) the pairs diphenyl and acenaphthene and acenaphthene and fluorene overlap ($b > 2\sigma$), but with a velocity of 0.63 cm/min (Fig. 7b) the separation is better than required by $b = 2\sigma$ and no overlapping can be recorded with a driving velocity of 1.49 cm/min (Fig. 7c) ($b \ll 2\sigma$).

On the other hand, the zones of fluorene and phenanthrene (Fig. 7a) are well separated, even when the driving velocity of the plate is 0.22 cm/min, because Δt_{max} is 3.7 min and

$$v_{min}\ (\text{fluorene/phenanthrene}) = \frac{8}{3.7} \cdot \frac{0.30}{0.84} \cdot 0.216 = 0.163.$$

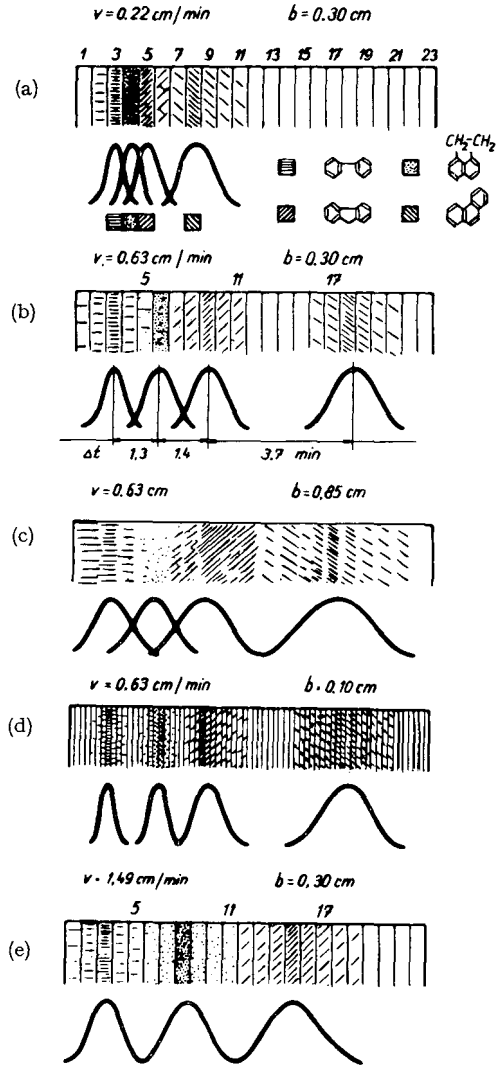

Fig. 7. Examples of zone broadening at different plate driving velocities and different side diffusion hindrances.

TABLE III

MEASURED VALUES OF EFFECTIVE DIFFUSION COEFFICIENT D, PROPORTIONALITY FACTOR k BETWEEN TIME AND ZONE WIDTH, AND HEIGHT OF A THEORETICAL PLATE H FOR SOME AROMATIC HYDROCARBONS

Compound	b *(cm)*	d *(cm)*	$D \times 10^4$ *(cm²/sec)*	$k \times 10^2$ *(cm/sec)*	$H \times 10^3$ *(cm)*
Biphenyl	0.75	18.0	0.335	6.55	2.06
Acenaphthene	0.85	18.0	0.43	7.42	2.65
Fluorene	0.95	18.0	0.56	8.49	3.30
Phenanthrene	0.80	18.0	0.38	7.00	2.35
Mean	0.84	18.0	0.43	7.37	2.59

Finally the example in Fig. 7c shows that the zone broadening on the plate without prevention of lateral diffusion gives the same results with a driving velocity of 0.63 cm/min only as was found in the case of a driving velocity of 0.22 cm/min (Fig. 7a) and notches 0.30 cm wide, because $b > 2\sigma$. A thin-layer chromatogram developed with the same parameters, but with artificial prevention of lateral diffusion at 0.10-cm intervals is practically a copy of the corresponding gas chromatogram, because $b \ll 2\sigma$.

Application to coal tar research

The complexity of coal tar mixtures is extremely great. Gas chromatography alone is not able to yield a complete separation of compounds. It may be shown that the amount of GC-information about the composition of coal tar and similar materials decreases with increasing boiling points of the compounds[19–21]. The new possibilities

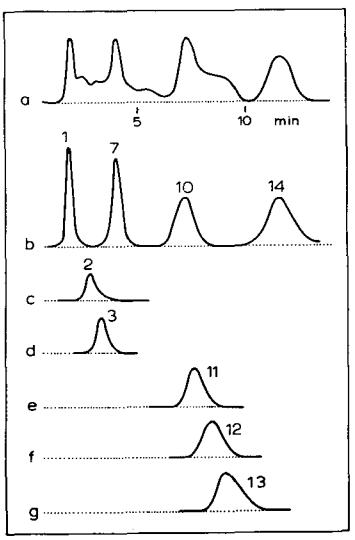

Fig. 8. Gas chromatograms (numbers correspond to those given in Table IV and Fig. 9). (a) original mixture; (b) hydrocarbons extracted from thin layer; (c) extracted zone of quinoline; (d) extracted zone of 4-hydroxyhydrindene; (e) extracted zone of 2-hydroxybiphenyl; (f) extracted zone of 1-naphthol; (g) extracted zone of 2-naphthol.

TABLE IV

PHYSICAL AND CHROMATOGRAPHIC VALUES FOR COMPONENTS OF THE STANDARD MIXTURE I

No.	*Compound*	*Boiling point (°C)*	*Gas-liquid chromatography silicone elastomer (220°) $r_{1,2}$*	*Thin-layer chromatography silica gel/benzene R_F*	*Colour of complex with tetracyano-ethylene*
1	Naphthalene	238	0.46	0.95	rose-violet
2	Quinoline	237	0.67	0.02	yellow-brown
3	4-Hydroxyhydrindene	247	0.77	0.29	brown
4	3-Methylisoquinoline	251	1.0	0.02	yellow-brown
5	Indole	255	0.60	0.60	brown (rose*)
6	5-Hydroxyhydrindene	255	0.94	0.18	violet (light blue-violet*)
7	Biphenyl	255	1.0	0.95	orange
8	3-Methylindole	266	1.1	0.65	purple (rose*)
9	2,4-Dimethylquinoline	273	1.4	0.03	brown-yellow
10	Acenaphthene	277	1.8	0.95	green
11	2-Hydroxybiphenyl	286	1.9	0.50	brown
12	1-Naphthol	288	2.1	0.32	orange
13	2-Naphthol	296	2.2	0.20	brown
14	Fluorene	298	2.9	0.95	violet

* Self-colouring in the presence of air oxygen at 100°.

of the method described appear to be clear from the information in Table IV on the composition of standard mixtures of compounds with boiling points ranging from 200 to 300°.

The chromatographic data are summarised in Fig. 9, which shows the separation on the plate and this is followed by gas chromatograms of the original mixture and separated materials extracted from a group of zones or from individual zones (Fig. 8).

The mixture of compounds with boiling points up to 400° was separated in the same way and is described in Table V and illustrated in Fig. 10.

This method was successfully exploited in research on the composition of coal tars, but it must be mentioned that it may be used without serious changes for the separation of other complex mixtures of industrial nature.

TABLE V

PHYSICAL AND CHROMATOGRAPHIC VALUES FOR COMPONENTS OF THE STANDARD MIXTURE II

No.	*Compound*	*Boiling point (°C)*	*GLC, silicone elastomer (300°) $r_{1,2}$*	*TLC, silica gel/ benzene + n-hexane (1:1 v/v) R_F*	*Colour of complex with tetracyano-ethylene*
1	Acenaphthene	277	0.35	0.93	green
2	1-Naphthol	288	0.40	0.12	orange
3	2-Naphthol	296	0.40	0.07	brown
4	Fluorene	298	0.59	0.93	rose-violet
5	Phenanthrene	340	1.0	0.92	violet
6	Acridine	344	1.1	0.01	yellow-green
7	Carbazole	353	1.2	0.55	ultramarine-blue
8	1-Methylphenanthrene	359	1.4	0.92	violet
9	2-Methylcarbazole	364	1.6	0.58	sky blue
10	Fluoranthene	384	2.0	0.92	violet
11	Pyrene	394	2.60	0.90	grey-green

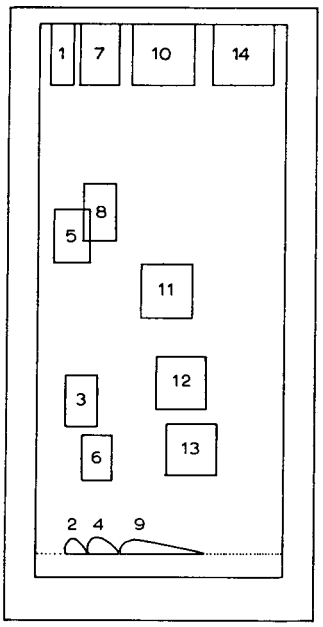

Fig. 9. Position of chromatographic zones after the combined developing (the numbers correspond to those given in Table IV). GLC–220°; TLC–silica gel/benzene.

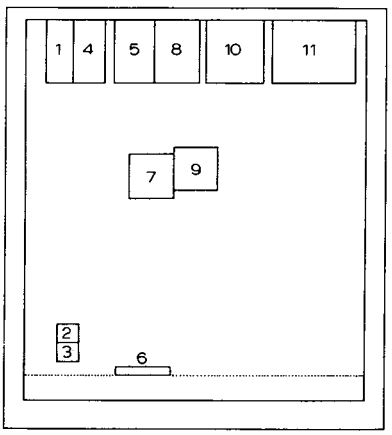

Fig. 10. Situation of chromatographic zones after the combined developing (numbers correspond to those given in Table V). GLC–300°; TLC–silica gel/benzene + *n*-hexane (1 : 1 v/v).

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SUMMARY

A new multi-dimensional chromatographic technique is described. μg to mg quantities from the gas chromatogram are placed on the start-line of a thin layer of adsorbent or of a sheet of chromatographic paper, which is moved past the orifice of the gas chromatograph and then developed in the usual manner. Thus, separation of the materials is obtained by exploiting the two most extreme possibilities given by the existing chromatographic methods, *viz.* gas–liquid chromatography, which separates according to the relative volatility of compounds (in the direction of the time-axis along the start), and thin-layer chromatography or paper chromatography, which separate according to the type of the functional group (in the vertical direction). After the extraction of the separated materials from the thin-layer or paper, GLC, on a suitably selected stationary phase, may be repeated if necessary. The theory for separation and experimental details are given. Further possibilities are obtainable by programming the driving velocity of the plate (paper) which acts in the same manner as temperature programming of a GC column.

The method described was used successfully in the research on the composition of coal tar, but can be applied to other complex mixtures of compounds of different chemical character.

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THE USE OF A WINDOWLESS GAS-FLOW COUNTER FOR DETECTING WEAK β EMITTERS ON PAPER CHROMATOGRAMS

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INTRODUCTION

The windowless gas-flow scanner[1–10] is one of the most sensitive methods for detecting the distribution of weak β emitters on paper chromatogram strips. The fact that no window separates the counting volume from the active sample permits the estimation of the weakest β emitting isotopes, *i.e.* tritium, which has a maximum energy of 18 keV, all of the β particles being absorbed by windows with a superficial density greater than 0.6 mg/cm^2.[11] This type of detector also has the advantage that it produces a continuous trace of the distribution of activity on the chromatogram as opposed to the histogram produced by sub-dividing the paper into sections and counting these individually. These advantages would seem to indicate that a windowless flow counter is the ideal chromatogram scanner. The method does have its limitations, however, and when an apparatus of the type was set up in this laboratory to determine the distribution of tritium and carbon-14 labelled compounds on one-dimensional chromatograms the first results showed a complete lack of reproducibility. It was found that the discrepancies could be attributed to three basic causes, namely: the gas flow rate through the counter, the build up of static charge on the paper, and losses due to volatilisation of the active compound from the paper chromatogram. It was also found that scans from opposite sides of the same chromatogram were different, and that the scanner appears to be approximately eight times more sensitive to spots containing tritiated stearic acid than it is to spots containing tritiated benzoic acid on the same chromatogram. Each of these factors has been investigated separately and a procedure devised whereby it is possible to produce reproducible scans from the same chromatogram. The need to calibrate the sensitivity of the scanner for each individual spot on the chromatogram is discussed and a combustion procedure for this briefly described.

APPARATUS

The active chromatograms were scanned in a gas flow proportional counter, shown schematically in Fig. 1, which is similar in basic design to that described briefly by KISIELESKI AND SMETANA[6]. The paper chromatogram strip is attached to a plate that passes through a hemi-spherical counting chamber. A rack attached to the plate, driven by a pinion connected to an electric motor via a gear box, enables the speed

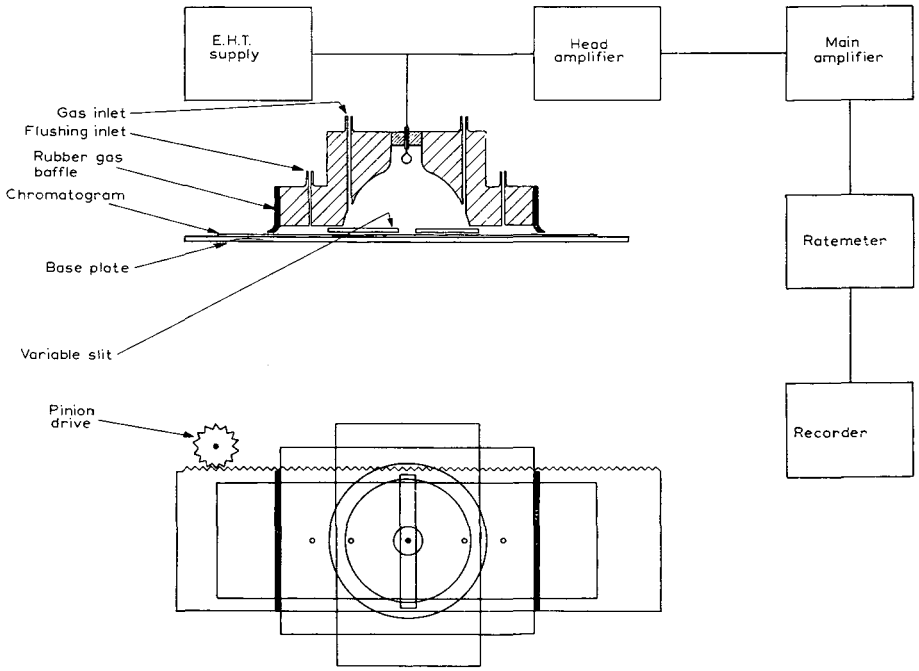

Fig. 1. A diagram showing the essential components of the chromatogram scanner and its associated electronic equipment.

at which the paper traverses the sensitive volume of the counter to be varied. A sliding plate containing rectangular slits of various dimensions is located between the counting chamber and the paper strip. This defines the area of the chromatogram counted at any one instant, and affects the resolution of spots. The counting gas (90 % argon, 10 % methane) is introduced at two points inside the hemi-spherical chamber, and at two points outside the sensitive volume. The gas inlets to the counting chamber are connected to a common supply which is regulated by a needle valve and measured with a Rotameter flow meter. The two flushing inlets are connected to a separate Rotameter. The gas passes out of the counter via the tunnels through which the plate and slit regulator pass.

The E.H.T. (about 2000 V) to the counter is supplied by a power unit (AERE Type 1359A). The pulses are amplified by a head amplifier (AERE Type 1049C), and a main amplifier (AERE Type 1430A), the output of which is connected to a ratemeter (AERE Type 1037C) and a Sunvic recorder.

EXPERIMENTAL

Setting up the equipment

To determine the optimum conditions for running the scanner a piece of filter paper spotted with tritiated stearic acid and carbon-14 labelled palmitic acid was sprayed with a 10 % polyethylene glycol 600 in ethyl alcohol solution, and allowed to dry. The paper was attached to the base plate and the appropriate spot introduced into the counter when necessary.

With the amplifier attenuation settings at 20 and 30 dB for tritium and carbon-14 respectively, and the bias on the ratemeter set at 50 V, the variation of count rate with E.H.T. was measured. The gas flow rates were 0.5 l/min and 0.05 l/min through the counting chamber and the preflushing tubes respectively. A point in the middle of the plateau thus obtained was selected as a suitable working voltage. To determine the optimum bias and attenuation settings the E.H.T. was set at this value and a series of curves of count rate (logarithmic scale) against bias voltage (linear scale) plotted for each attenuation setting of the amplifier. A similar series of curves were plotted for the background. The values of the ratio of count rate to background for the different bias and voltage settings were tabulated and the counter adjusted to the settings where this parameter was a maximum.

Gas flow rate

To investigate the effect of variations of gas flow on a stationary source one of the active spots on the test strip was positioned inside the counter. With the gas flow through the preflushing tubes fixed at 0.05 l/min the total gas flow through the central chamber was varied and the corresponding count rates recorded. Measurements for both carbon-14 and tritium were made for different discriminator and bias settings.

Static build up

The effect of the build up of static charge on the count rate of a stationary source, which has been reported by OSINSKI[8], was demonstrated by recording the variation, with time, of the count rate of spots containing tritium and carbon-14 on two test chromatograms. The electronic equipment was adjusted to the optimum settings for the two isotopes and the gas flow rates set at 0.5 and 0.05 l/min through the chamber and preflushing tubes respectively.

One of the chromatograms was then dipped in a 2 % solution of polyethylene glycol 600 in benzene and allowed to dry. The other chromatogram was sprayed with a suspension of colloidal graphite in aqueous ethyl alcohol until the chromatogram had a uniform dark grey colour.

After this treatment the change in count rate with time of the active areas on both chromatograms was again recorded.

Volatilisation of the sample

To determine the loss of activity during the preparation and storage of chromatograms a number of Whatman No. 1 papers were spotted with known volumes of freshly prepared solutions of tritiated benzoic and stearic acids in alcohols. Some strips were spotted with a single substance and others with a mixture of the two acids. The strips were run for approximately 7 h with a descending solvent consisting of ethyl alcohol (16 parts), 0.880 ammonia solution (1 part) and water (3 parts). The resultant chromatograms were dried and dipped rapidly in 2 % polyethylene glycol 600 in benzene. One chromatogram was rescanned after a second dipping to confirm that no loss of benzoic acid occurred at this stage.

The losses occurring between spotting a chromatogram and scanning it were determined by excising the active areas on chromatograms spotted with a single substance and run for 7 h. The paper was burned in a sealed flask of pure oxygen. A liquid phosphor containing inactive water was injected into the flask and an aliquot

removed after equilibration with the tritiated water formed during combustion[12]. A small piece of paper impregnated with the same volume of tritiated acid solution as that used to spot the chromatograms was assayed in a similar manner.

The chromatograms with two spots were marked with Indian ink containing non-volatile promethium-147[13] to give reference peaks of fixed height on the scans. One chromatogram was stored in the fume hood and scanned at regular intervals. The other was hermetically sealed in a polythene container, and rescanned after standing in the fume hood for 20 days. The polythene container was made from lay-flat tubing one end of which was sealed by placing the tubing between two metal plates and running a very small gas flame over the polythene where it passed through the plates. The chromatogram was inserted in the bag thus formed, and the open end flame sealed when all of the air had been expelled.

Distribution of activity within the chromatogram

A strip of Whatman No. 1 paper 4 cm × 46 cm was spotted with 5 μl of a freshly prepared alcoholic solution of benzoic acid (1.75 μg containing 2.52 μC of tritium) and stearic acid (2.45 μg containing 1.48 μC). Both samples were applied to the same side of the paper by repeatedly touching the surface with a capillary tube containing the active solution. The evaporation of the solvent was accelerated by applying a draught of air over both surfaces of the paper. The paper was equilibrated for 5 h in an atmosphere saturated with the solvent, ethyl alcohol (16 parts) 0.880 ammonia solution (1 part), and water (3 parts), and run for 10 h using the descending solvent technique. The chromatogram was dried, dipped in 2 % polyethylene glycol in benzene, and each face was scanned twice. The areas of paper containing the active spots were then cut from the chromatogram and the activity contained in them determined after combustion[12].

RESULTS AND DISCUSSION

It was found that tritium required a higher gain (regulated by the attenuation setting of the main amplifier) than carbon-14 at optimum counting conditions. This is to be expected because of the difference in their average energies which are 5.5 keV and 50.0 keV for tritium and carbon-14 respectively[11].

Gas flow rate

The effect of gas flow rate on the variation of the count rate of spots containing tritium and carbon-14 on the same chromatogram with high gain and a low bias setting is shown in Fig. 2. Reducing the gain and increasing the bias reduces the counting efficiency for both isotopes, and higher gas flows are required for the count rates to reach a steady value, as shown in Fig. 3.

Back diffusion of air into the counting chamber reduces the gas amplification, this reduces the size of the pulses and results in a loss of counts. This can be averted by increasing the gas flow. However, since a higher gas flow rate increases both the build up of static charge, and the rate of loss of volatile compounds from the paper, it should be kept to a minimum. This can be achieved by working with high gain and low discriminator settings, and by designing the equipment in such a manner that the possibility of back diffusion of air into the counting chamber is reduced to a minimum.

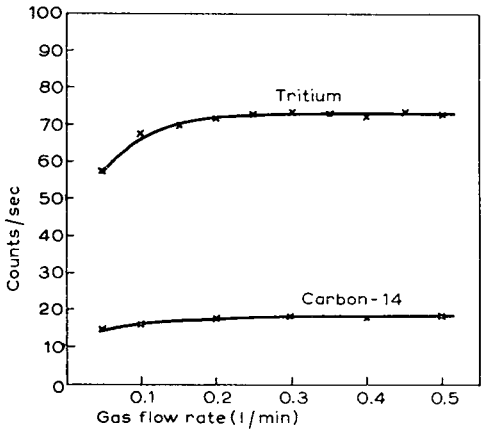

Fig. 2. A graph showing the variation of count rate with the total gas flow through the centra counting chamber with the attenuation on the amplifier set at 8 dB and a bias voltage of 15 V The total gas flow through the flushing tubes was maintained at 0.05 l/min throughout.

Static build up

The rate of loss of counts on a stationary chromatogram due to the build up of static charge is shown in Fig. 4. It can be seen that the losses for tritium are far greater than those for carbon-14. The major factors affecting the rate of decrease of counts were found to be the gas flow rate and the slit width, the losses increasing with increasing gas flow and decreasing when the slit width was decreased.

It was found that when untreated chromatograms containing a number of spots of non-volatile tritiated compounds were scanned on successive days the peak heights varied considerably, reaching a maximum when atmospheric humidity was high. If the same chromatograms were repeatedly scanned, without allowing the paper to

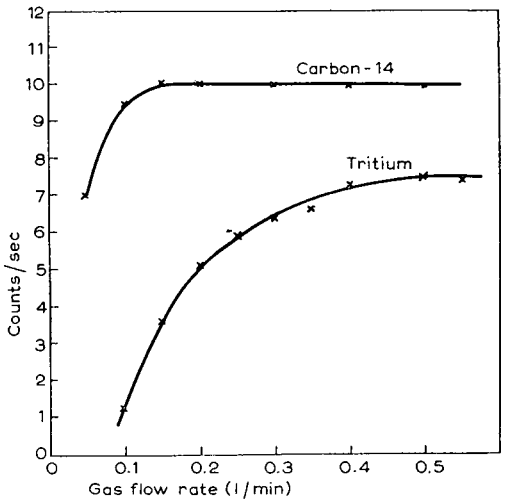

Fig. 3. The variation of the count rate with gas flow rate for the same chromatogram spots used for Fig. 2, but with the attenuation and bias settings adjusted to 16 dB and 50 V respectively.

equilibrate with the atmosphere between scans, the tritium peaks were successively reduced. Successive scans were more reproducible if the counting gas was partially saturated with water vapour but this resulted in an overall lowering of the peak heights.

The problem of the build up of static charge can be completely overcome by rendering the papers more conducting. Two methods have been devised and tested;

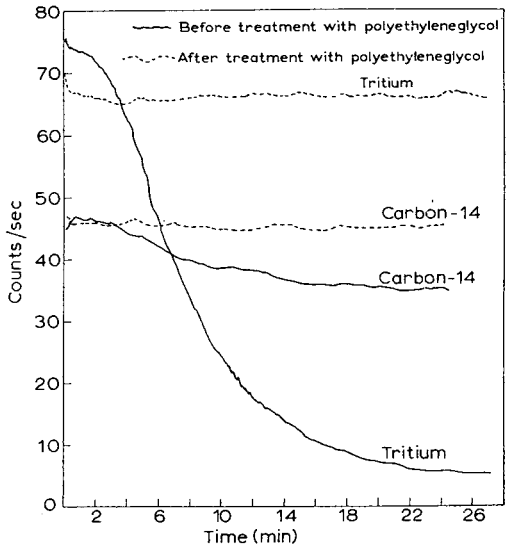

Fig. 4. The variation of the count rate with time of spots containing tritium and carbon-14 on a stationary chromatogram.

one consists of spraying the paper with colloidal graphite, the other consists of dipping or spraying the paper with a solution of a humectant—a substance that keeps the moisture content constant despite environmental changes. Polyethylene glycol 600 was found to be suitable for this purpose. The effect of the latter treatment, which is to be preferred if spots are also to be detected with a colour reagent, is shown in Fig. 4. The peak heights produced by spots containing tritium and carbon-14 on papers treated by either of these methods do not vary by more than 2 % on repeated successive scans.

Loss of activity

The mass of a radioactive material of high specific activity that can be detected by its radiations is very small, usually far less than can be detected by other means. When very small weights of compounds, normally regarded as stable and non volatile, are spread over a relatively large area of paper the fractional losses due to a quite small vapour pressure can be appreciable.

The losses occurring during the preparation of the chromatograms were measured by a combustion method. The count rate of the spots containing approximately 2 μg of benzoic and 2 μg of stearic acid on the final chromatogram was found to be approximately 10 % less than that of the solution applied to the paper for both acids. A

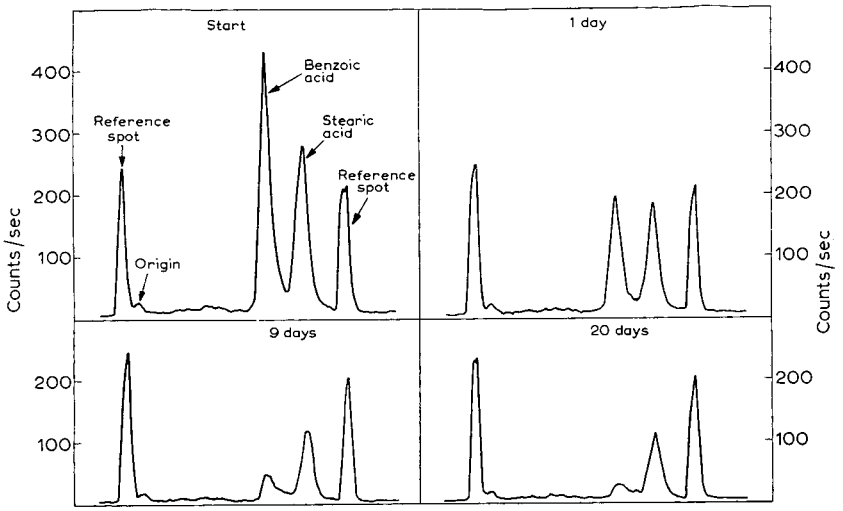

Fig. 5. The variation of scans obtained from the same chromatogram after exposing it to a draught in a fume cupboard for a number of days.

portion of these losses can be attributed to the separation of radiochemical impurities during the chromatographic process.

The change in peak heights that occurs when the chromatogram is exposed to a draught of air over a period of 20 days is shown in Fig. 5. The relatively small reductions in peak height shown in Fig. 6 indicate that these losses can be considerably reduced by encasing the chromatogram in polythene.

Distribution of activity within the chromatogram

The traces obtained when different surfaces of the same chromatogram are scanned are shown in Fig. 7. It can be seen that the peaks on the surface opposite that to which the solutions were applied are greater for both acids. A possible explanation for this effect is that a higher concentration of acids is built up on the side of the paper opposite that to which the capillary is applied during the spotting of the paper. Each time the capillary touches the paper it releases a drop of solution which washes

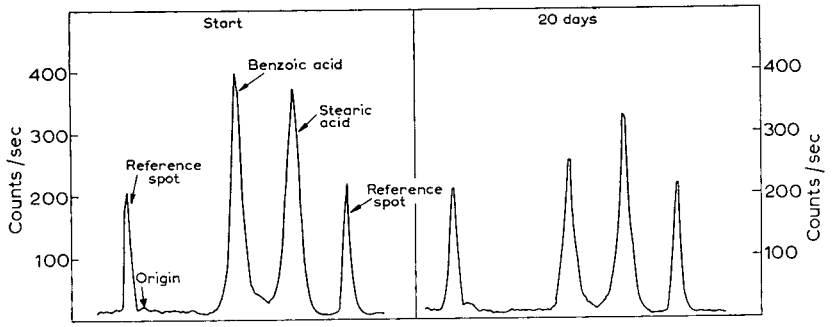

Fig. 6. The variation of scans obtained from a chromatogram containing the same amount of activity as that used for Fig. 5 sealed in a polythene tube from which air was excluded for 20 days.

the acid already in the paper before it as it passes through the paper. What is surprising is that a concentration gradient should be maintained through the relatively small thickness of the paper during the 10 h for which the eluting solvent is passing down the paper. These results, which have been repeatedly reproduced, are in contrast to those of POCCHIARI AND ROSSI[14,15] who observed that different concentrations of activity were detectable on opposite sides of the paper when Whatman No. 1

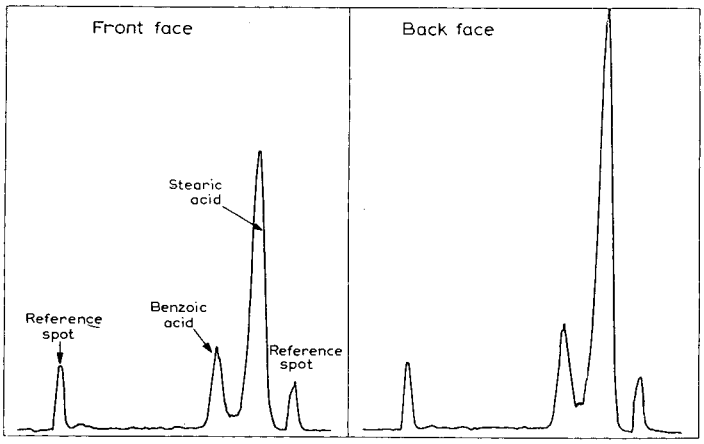

Fig. 7. Scans obtained from different sides of the same chromatograms. The face denoted FRONT was the face to which the spot was applied.

papers were spotted with carbon-14 labelled glucose. They found that chromatographic development led to homogeneous distribution of the activity throughout the paper except for substances left at the origin. WENZEL[10], however, found that the count rate on the side of the paper to which the solution was applied was greater than the reverse side, and that a difference in count rate for the two sides of the paper was maintained after chromatography. HANSEN[16] has observed a small error when scanning both sides of a chromatogram for carbon-14 labelled stearic and palmitic acids, but he does not indicate if the discrepancies are related to the side of the paper to which the original solutions were applied. BIDWELL[17] has observed that there is an erratic increase in surface concentration of carbon-14 labelled compounds when papers are forcibly dried on one face. He used Whatman No. 3 MM paper which is considerably thicker than the Whatman No. 1 papers, used for the experiments described herein, which were usually dried in a gentle draft—the process only taking a few minutes. There was no noticeable difference in the author's results after the papers were forcibly dried on one surface.

A more surprising fact was revealed by combustion of the spots that produced the peaks shown in Fig. 7. The assay showed that the area of the chromatogram corresponding to the benzoic acid peak contained 2.23 μC of the original 2.52 μC of tritium originally applied, and the stearic acid peak contained 1.24 μC of the original 1.48 μC. The area of the stearic acid peak was found to be 4.05 times greater than that of the benzoic acid peak for the front of the chromatogram and 4.55 times for the back. Thus for this pair of acids the normal premise that peak areas are proportional to the activity of the corresponding spots, for the same isotopes on a single chromato-

gram, is invalidated by a factor of eight. A possible explanation is that the benzoic acid is more volatile than the stearic acid and therefore the surface concentration is less than the bulk concentration due to the differences in their vapour pressures. The half-thickness for absorption of tritium β particles is about 0.1 mg/cm^2 and all are absorbed when passing through substances of density greater than 0.6 mg/cm^2. Therefore only tritiated compounds on the outside of the outermost fibres of the paper are detectable. An alternative and more plausible explanation is that the effect is the result of an inherent fundamental process in paper chromatography, the benzoic acid being retained within the cellulose fibres of the paper whereas the stearic acid remains primarily on the surface.

If the second of these explanations is correct the effect could have more far-reaching consequences. For example, the results obtained by the colorimetric determination of concentrations based on surface reactions may be subject to error if the concentration of the substances investigated not only varies from one surface of the paper chromatogram to the other, but also within the cellulose fibres of which the paper is composed.

RECOMMENDED PROCEDURE

In order to obtain reproducible scans from chromatograms containing substances labelled with tritium and carbon-14 the following procedure is recommended: firstly, having found the optimum settings for the electronic equipment for the particular isotope to be measured, the minimum gas flow rate to give a steady count for a sample should be found. Increasing the flow by 50% of this value will normally compensate for additional diffusion when the chromatogram is mobile. Spraying the chromatogram with colloidal graphite to a uniform dark grey colour will prevent the build up of static charge on the paper, which has a pronounced effect when scanning for tritiated substances. Alternatively, the papers can be dipped or sprayed with polyethylene glycol solution. There is a possibility that opposite faces of the same chromatogram will give different scans. It is important therefore that the same side of the chromatogram, with respect to that to which the spot was applied, should always be kept uppermost in the chamber if repeated scans are to be made. If the scans are to be used as the basis for quantitative measurements both faces should be scanned and the results averaged. Chromatograms containing small masses of substances with small vapour pressures should be scanned as soon as possible after preparation. The loss of activity from papers that have to be kept before scanning, or stored for future use, can be considerably reduced if they are hermetically sealed in a polythene tube from which the air has been excluded.

Although adsorption of the above procedure should result in the production of reproducible scans from a single chromatogram containing tritium, it should not be automatically assumed that the same factor can be used to convert peak heights or areas to activity for all compounds. For accurate quantitative analyses the activity of each spot should be checked independently, *i.e.* by combustion.

SUMMARY

A 2π gas flow proportional counter for scanning chromatograms is described. The effects of the gas flow rate, build up of static charge, and the volatilisation of the

sample on the reproducibility of scans are discussed and methods whereby artefacts due to these causes can be minimised are outlined. It is shown that different scans can be obtained from opposite faces of the same chromatogram. It is also shown that when using tritium the peak height, or peak area, on a scan does not necessarily correspond with the activity of the spot on the chromatogram for different substances. Thus a spot containing 1.2 μC of tritiated stearic acid produces a peak on the scan with an area four times greater than that produced by a spot containing 2.2 μC of tritiated benzoic acid on the same chromatogram. An operational procedure based on these findings is outlined.

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PAPIERCHROMATOGRAPHIE ÄTHERISCHER ÖLE

II. DIE DENSITOMETRISCHE BESTIMMUNG UNGESÄTTIGTER TERPEN-ALKOHOLE UND -ESTER IN ÄTHERISCHEN ÖLEN

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München (Deutschland)

(Eingegangen den 28. Oktober 1963)

In zwei vorgegangenen Publikationen berichteten wir über ein von uns entwickeltes neues Verfahren der papierchromatographischen Trennung von ätherischen Ölen[1,2]. Das charakteristische Kennzeichen dieser Methode ist die adsorptive Chromatographie intakter Öle an einem mit Paraformaldehyd imprägnierten Ederol-Papier, ohne dass vorher Derivate hergestellt werden müssen. Das von uns entwickelte Papier ermöglicht damit erstmals auch eine direkte spektrophotometrische Auswertung der Papierchromatogramme von ätherischen Ölen, wie sie auf anderen Stoffgebieten bereits durchgeführt worden ist[3–10]. Zum Unterschied von einzelnen bisher veröffentlichten, sowohl auf Papier[4,5] als auch auf Dünnschichtplatten[12] durchgeführten ähnlichen Bestimmungsmethoden kann dabei auf eine vorangehende Elution[11] oder mit Fehlern[6] behaftete Photoreproduktion der Chromatogrammflecken verzichtet werden. Über Einzelheiten unserer Methode wird im folgenden ausführlich berichtet.

METHODIK

Densitometer

Als Messgerät verwendeten wir das von GRASSMANN UND HANNIG[13,14] in die klinische Serum-Eiweiss-Analyse eingeführte Densitometer (Fig. 1). Dieser sog. "Elphor-Integraph"** stellt ein für die Methode der Transmissionsmessung entwickeltes vollautomatisches, selbst integrierendes Spektrophotometer dar, welches gleichzeitig die Extinktionskurven der gefärbten Chromatogrammflecken aufzeichnet und deren Flächenwerte numerisch anzeigt. Ein Vorteil dieses Gerätes ist ferner, dass nicht ein Nachlaufschreiber die Kurve zeichnet, sondern die Kurvenpunkte jeweils im Augenblick der Messung gestochen werden. Die im Gerät verwendete Null-Messmethode gewährleistet ein besonders hohes Mass an Genauigkeit, da jeder einzelne Messwert erneut auf die Nullinie bezogen wird. Das Gerät arbeitet wie die meisten Densitometer eindimensional. Es ist deshalb erforderlich, dass die Anfärbung des Chromatogrammflecken über die ganze Lichtspaltbreite des Gerätes gleichmässig erfolgt, was durch strichförmiges Auftragen erreicht wird[13].

Papier und Dosierung der Substanzmengen

Das Chromatogrammpapier muss für die Densitometrie optisch möglichst einheitlich sein. Das Ederol-Papier Nr. 208 erschien uns nach zahlreichen Versuchen mit anderen

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** Hersteller: Fa. Dr. Bender & Dr. Hobein, München 15, Deutschland.

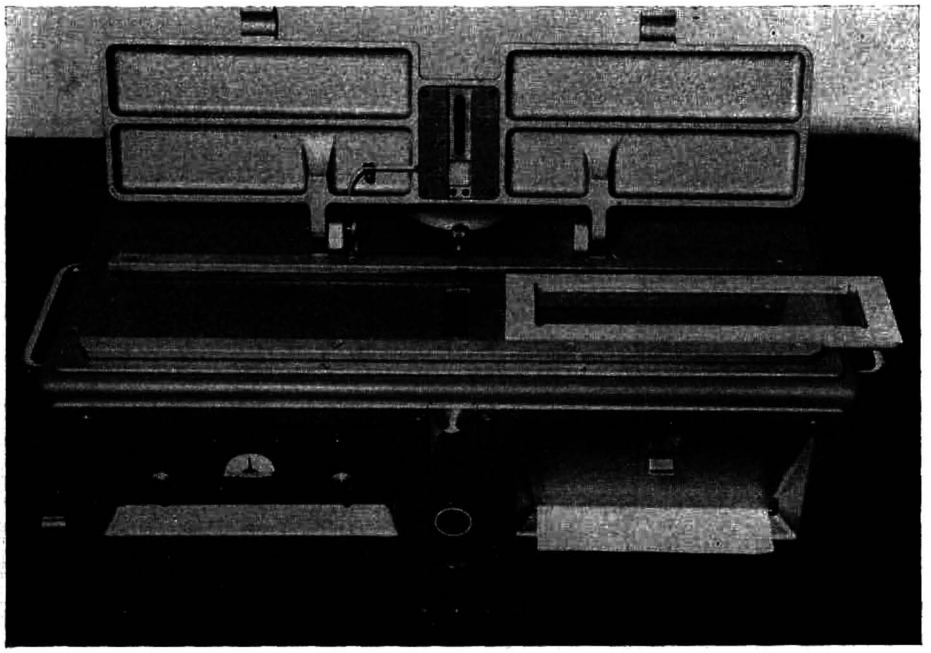

Fig. 1. "Elphor-Integraph", vollautomatisches Transmissionsdensitometer, aufgeklappt, im Deckel oberes Photoelement sichtbar. Links elektronischer Teil, rechts mechanischer Teil.

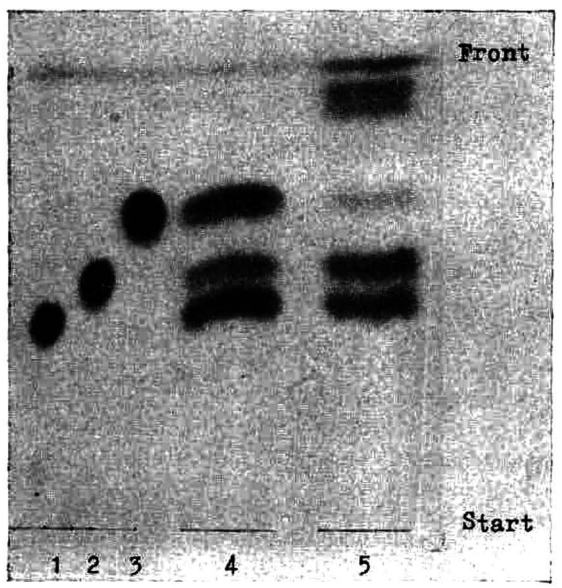

Fig. 2. Aufsteigend auf Ederol 208/P angefertigtes Papierchromatogramm. Fliessmittel: Methylisopropylketon–*n*-Heptan (5:20, V/V); Anfärbung: Osmiumtetroxid. 1 = Geraniol; 2 = Citronellol; 3 = Linalool; 4 = Gemisch der Testsubstanzen; 5 = Geraniumöl für die densitometrische Messung strichförmig aufgetragen.

Papieren auch im Hinblick auf die Trennfähigkeit und Festigkeit nach der Imprägnierung als besonders gut geeignet*. Kleinere Inhomogenitäten fallen nicht ins Gewicht, weil bei der photometrischen Auswertung über die ganze Streifenbreite gemittelt wird[14].

Das Auftragen der Testsubstanzen und ätherischen Öle geschah aus geeigneten Verdünnungen. Die Testsubstanzen wurden als 0.1 %ige Lösungen in *n*-Heptan, *n*-Hexan oder Benzol, die ätherischen Öle in entsprechend stärkerer Konzentration aufgebracht. Zur genauen Dosierung verwendeten wir eine Agla-Spritze**. Die Startflecken wurden dem spaltförmigen Lichtstrahl des Densitometers angemessen als 3 cm breite Striche ausgeführt, wobei zum nächsten Fleck 1.5 cm Abstand gelassen wurden (Fig. 2).

Entwicklung der Chromatogramme

Die Entwicklung der Chromatogramme für die Eichkurven und die zu messenden ätherischen Öle geschah wie früher beschrieben[1,2] und immer unter gleichen Bedingungen nach der aufsteigenden Methode. Dabei wurden je nach der Art des zu untersuchenden Öles verschiedene in Tabelle I zusammengestellte Fliessmittel verwendet.

TABELLE I

GEEIGNETE FLIESSMITTEL

Fliessmittel	*Volumenteile*	*Ätherische Öle*	*Laufzeit (Min.)*
Methylisopropylketon–*n*-Heptan	5:20	Citronellöle Geraniumöle Melissenöle	90
n-Hexan–*n*-Heptan–Eisessig	15:15:2	Lavendelöle Lavandinöle	60

Anfärbung der Chromatogramme

Die nach der Entwicklung 10 Min. an der Luft getrockneten Chromatogramme wurden 120 Min. lang in einer 5-l-Kammer dem Dampf aus etwa 0.25 g Osmiumtetroxid und 50 ml Eisessig (in einem Erlenmeyerkolben) ausgesetzt. Die zwischen den olefinischen Doppelbindungen der reagierenden Substanzen und dem OsO_4 gebildeten Osmium(VI)-säureester liessen sich anschliessend durch Hydrolyse in die Osmium(VI)-säure überführen, wobei eine starke Farbvertiefung und damit verbundene Empfindlichkeitssteigerung resultierte. Dazu erhitzten wir die Chromatogramme nach der Osmiumtetroxidbehandlung 120 Min. im Trockenschrank in einer gesättigten Wasserdampfatmosphäre. Den Dampf entwickelten wir aus einer Schale (ø = 24 cm) mit 500 ml Wasser, die sich im Trockenschrank befand. Danach wurde das Chromatogramm 60 Min. an der Luft getrocknet, in 4 cm breite, für das Densitometer passende Streifen geschnitten und sofort gemessen.

Eine nachträgliche Transparentierung erwies sich als überflüssig, wenn ein

* Das Papier für die Papierchromatographie und Densitometrie ätherischer Öle wird seit einiger Zeit nach unseren Angaben von der Fa. J. C. Binzer, Hatzfeld/Eder, Deutschland, unter der Bezeichnung Ederol 208/P hergestellt.

** Hersteller: Fa. Burroughs Wellcome & Co., London, England.

Stück ungefärbtes Chromatogrammpapier vor das untere vergleichende Photoelement gebracht wurde. Dadurch liessen sich bei beiden Photoelementen etwa gleiche Lichtverhältnisse erzielen. Ausserdem konnten wir, wie bereits MASTNER, FRANEK UND NOVAK[15] feststellten, auf nicht transparentem Papier mit grösserer Empfindlichkeit und auch mit weniger Wiederholungen messen.

Durchführung der densitometrischen Messungen

Für die Messungen verwendeten wir einen Lichtspalt von 1 mm Breite und 36 mm Länge. Die Nullinien-Kompensation, womit auf die Grundlinie der Extinktionskurve eingestellt wurde, nahmen wir zwischen dem Start und dem zu messenden Fleck an der optisch dünnsten Stelle, etwa 2–2.5 cm vor der Mitte des Flecken vor. Dabei zeigte es sich, dass die Ausdehnung der Flecken oft wesentlich grösser war, als das Auge wahrnehmen konnte. Damit liess sich ein Fehler vermeiden, der die Ergebnisse bei Ausschneiden oder Auskratzen von Chromatogrammflecken und deren Bestimmung durch Elution gefährden kann. Gemessen wurde in Richtung zur Front solange, bis das Zählwerk zu addieren aufhörte. Von mindestens 3 Messungen bildeten wir das arithmetische Mittel. Die so erhaltenen Flächenwerte der Extinktionskurven wurden in ein Koordinatensystem eingetragen (Fig. 3). Kehrte bei der

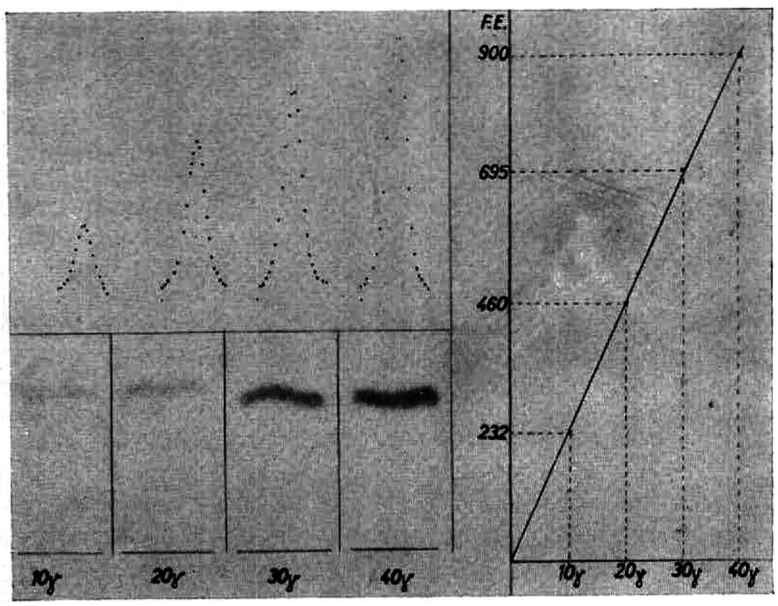

Fig. 3. Chromatogrammstreifen mit steigenden Mengen Geraniol aus einem Chromatogramm, angefärbt mit Osmiumtetroxid, darüber die dazugehörigen, im Densitometer gemessenen Extinktionskurven, rechts die daraus resultierenden Flächenwerte graphisch dargestellt.

Messung mehrerer dicht nebeneinander liegender Flecken die Kurve nicht bis zur Nullinie zurück, so wurde bei geringen Überlappungen der tiefste gestochene Kurvenpunkt des zwischen den beiden Maxima liegenden Tales als Ende der Messung für den davorliegenden Flecken genommen. Dies bedeutet, dass von diesem Punkt

das Lot auf die Grundlinie gefällt wurde. Bei etwa gleicher Form benachbarter Extinktionskurven ist der Fehler so gering, dass er vernachlässigt werden kann.

Eichkurven

Für folgende Reinsubstanzen stellten wir Eichkurven auf: Geraniol, Citronellol, Linalool und Linalylacetat. Zur Ermittelung der Eichkurven wurden jeweils von

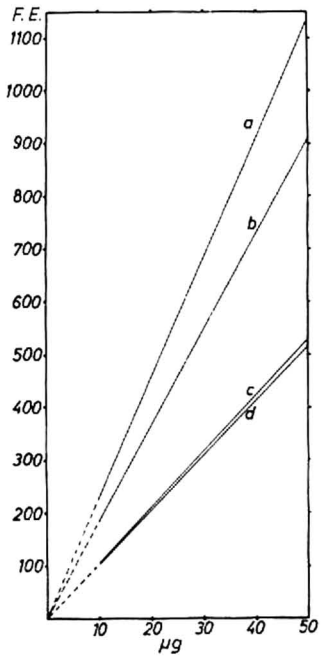

Fig. 4

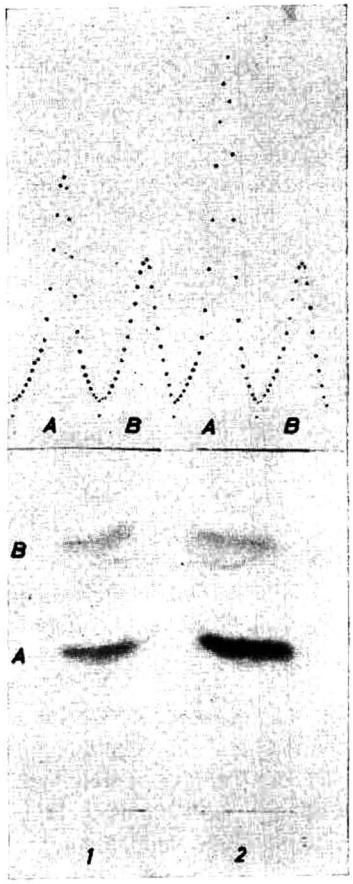

Fig. 5

Fig. 4. Densitometrisch ermittelte Eichkurven für: a = Geraniol; b = Linalool; c = Citronellol; d = Linalylacetat; FE = Flächeneinheiten.

Fig. 5. Chromatogrammstreifen und Extinktionskurven von je 100 µg Lavendelöl Barrême, der zweite Streifen mit Zusatz von 20% Linalool. Fliessmittel: *n*-Hexan–*n*-Heptan–Eisessig (15:15:2, V/V). A = Linalool; B = Linalylacetat.

5 zu 50 µg steigende Mengen reiner Testsubstanz wie oben beschrieben chromatographiert, angefärbt und gemessen. Die arithmetischen Mittelwerte von 10 derartigen Eichchromatogrammen lieferten die Kurvenpunkte für die Eichkurve. Von den vier bisher von uns herangezogenen Substanzen erhielten wir die in Fig. 4 dargestellten Geraden. Der Messbereich ist durch die Konstruktion des Gerätes begrenzt und wird mit zunehmender Zahl olefinischer Doppelbindungen einer Substanz

kleiner, weil die Stärke der Anfärbung zunimmt. Liegt das Maximum der Extinktionskurve eines Wertes ausserhalb des Messbereiches, so ist die Messung nicht mehr exakt.

Überprüfung der Reproduzierbarkeit durch Zusatz reiner Testsubstanz zu Öl mit bekanntem Gehalt

Zu Lavendelöl (Barrême-Qualität) mit 20 % Linalool wurden in verschiedenen Versuchen 10 und 20 μg Linalool auf dem Chromatogramm dazu aufgetragen und überprüft, ob der densitometrisch ermittelte Gehalt der Summe entsprach (Fig. 5). Wie Tabelle II zeigt, wurden in beiden Fällen die hinzugefügten Mengen wiedergefunden.

Reinigung der Testsubstanzen

Linalylacetat reinigten wir von geringen, mit den Terpenen in die Front laufenden Verunreinigungen, indem wir 1 ml auf eine mit aktiviertem (3 Std. bei 150°) Kieselgel

TABELLE II

REPRODUZIERBARKEIT BEI ZUSATZ VON LINALOOL ZU LAVENDELÖL

Aufgetragene Substanzen	*Flächeneinheit*	*Linalool (μg)*	*Mittelwerte (μg)*	*Linalool wiedergefunden (%)*
100 μg Lavendelöl	368	20.8		
	398	22.0	21.6	—
	397	22.0		
Dazu 10 μg Linalool	607	33.3		
	597	32.7	32.2	entspr. 106
	554	30.5		
Dazu 20 μg Linalool	761	41.6		
	745	40.8	40.6	entspr. 100
	716	39.3		

Merck (ø 0.2–0.5 mm) 24 cm hoch beschickte Säule (ø = 1 cm) brachten, zunächst die Verunreinigungen mit Wundbenzin und danach das Linalylacetat mit Benzol eluierten. Nach dem Trocknen mit Natriumsulfat sicc. wurde das Benzol im Vakuum abgedampft und reines Linalylacetat erhalten.

Linalool. Zwei Beimengungen begleiteten das handelsübliche Linalool. Auf der Dünnschichtplatte chromatographiert (Kieselgel G Merck, Benzol–Äthylacetat (95:5, V/V) als Fliessmittel) lag die eine Verunreinigung dicht über, die andere dicht unter dem Linalool. Da beide in einer geschlossenen Säule nicht vom Linalool zu trennen waren, wurden sie über eine mit Kieselgel G Merck angefertigte offene Säule mit Benzol–Äthylacetat (95:5) chromatographiert und, nach Detektion der Zonen an einem Streifen, die Linaloolzone separiert. Nach Elution derselben mit Benzol, Trocknen und Abdampfen des Lösungsmittels (siehe oben) erhielten wir reines Linalool.

Geraniol wurde, da eine Reinigung[16] des handelsüblichen Produktes, das von 5 Verunreinigungen begleitet war, zu keinem befriedigenden Ergebnis führte, durch Verseifen von reinem Geranylacetat (1 Std. mit 5 %iger kalter Natronlauge) erhalten.

Citronellol. Die Reindarstellung von Citronellol aus einem handelsüblichen Produkt gelang durch Entfernung des vorhandenen Geraniols nach TIEMANN UND

SCHMIDT[17], Überführung des Citronellols nach REICHSTEIN[18] in sein 3,5-Dinitrobenzoat und säulenchromatographischer Reinigung desselben. Dazu wurde es zunächst über eine Aluminiumoxydsäule (20 g Al_2O_3 neutral Woelm + 6 % Wasser, ø 21 mm) und anschliessend mit Chloroform über eine Kieselgelsäule (20 g aktiviertes Kieselgel Merck 0.2–0.5 mm, ø 21 mm) chromatographiert. Nach 20 stündiger kalter Verseifung mit 10 %iger methanolischer Kalilauge erhielten wir das in Freiheit gesetzte Citronellol nach Verdünnen mit reichlich Wasser und Ausschütteln mit Äther.

Fehlerrechnung

Zunächst war der Fehlerumfang zu ermitteln, der durch die Flüchtigkeit der ätherischen Öle entsteht. Zu diesem Zweck wurden Versuche mit Linalool angestellt, wobei jeweils 40 µg aufgetragen, 10 Min. lang chromatographiert und dann erst nach wachsenden Zeiten angefärbt und gemessen wurde. Der Vergleich der Extinktionen mit entsprechenden Werten der Eichkurve ergab zu niedrige Mengen bei den Verdunstungsversuchen (siehe Tabelle III). Da die Eichkurven aber unter den gleichen Bedingungen aufgestellt werden, wie sie bei den eigentlichen Messungen unbekannter Werte herrschen, wird dieser Verdunstungsfehler weitgehend kompensiert.

TABELLE III

VERDUNSTUNGSVERLUSTE BEIM LINALOOL DENSITOMETRISCH BESTIMMT

Verdunstungszeit (Min.)	*Substanzverlust* bezogen auf die Werte nach sofortiger Anfärbung (%)*
30	etwa 1
60	2.5– 5.0
120	5.0– 8.0
240	6.0–10.0

* Die Werte bewegen sich in gewissen Spannen, was wohl mit den Bedingungen wie Luftbewegung, Temperatur und Luftfeuchtigkeit zusammenhängt.

Zur Berechnung der Fehler* bei den Messungen wurden die bei der Aufstellung der Eichkurven erhaltenen Werte herangezogen. Aus ihnen war zunächst der Anstieg der Kurven nach der Formel:

$$m = \frac{\text{Extinktionsflächenwert}}{\mu\text{g Testsubstanz}}$$

zu ermitteln.

Für Geraniol erhielten wir z.B. bei 10 Werten mit je 30 µg aufgetragener und normal chromatographierter Substanz folgende Werte:

$$\frac{647}{30} \quad \frac{665}{30} \quad \frac{671}{30} \quad \frac{668}{30} \quad \frac{698}{30} \quad \frac{695}{30} \quad \frac{690}{30} \quad \frac{632}{30} \quad \frac{643}{30} \quad \frac{678}{30}$$

Aus den Quotienten wurden die Fehler errechnet und in der gleichen Weise mit den Eichkurvenwerten der übrigen Testsubstanzen verfahren. Die erhaltenen Fehler sind in Tabelle IV zusammengestellt.

* Nach F. W. KÜSTER UND A. THIEL, *Logarithmische Rechentafeln*, Berlin, 1955, S. 188 und Tafel 12, I.

TABELLE IV

FEHLERGRÖSSEN BEI DER DENSITOMETRISCHEN MESSUNG DER TESTSUBSTANZEN

Substanz	*M*	$10^8 \cdot f^2$	f_m	F_m	E_m	E_w
Geraniol	22.32	536, 840, 000	0.77	0.24	22.32 ± 0.24	± 0.16
Linalool	18.54	2,209, 300, 000	1.57	0.50	18.54 ± 0.50	± 0.33
Linalylacetat	10.25	230, 540, 000	0.51	0.16	10.25 ± 0.16	± 0.11
Citronellol	10.39	808, 170, 000	0.95	0.30	10.39 ± 0.20	± 0.20

In Prozenten ausgedrückt ergibt sich für die einzelnen Stoffe folgender mittlerer Fehler (f_m) für die Einzelmessung:

Geraniol ± 0.7	Linalylacetat ± 1.1
Linalool ± 1.8	Citronellol ± 1.9

ERGEBNISSE

Vergleichende Qualitätsermittelung einiger handelsüblicher ätherischer Öle auf chemisch-analytischem, densitometrischem und gaschromatographischem Wege

Eine Anzahl frischer und gelagerter Lavendel-, Lavandin-, Citronell-, und Melissenöle (vgl. Fig. 6) untersuchten wir mit Hilfe der aufgestellten Eichkurven nach unserem

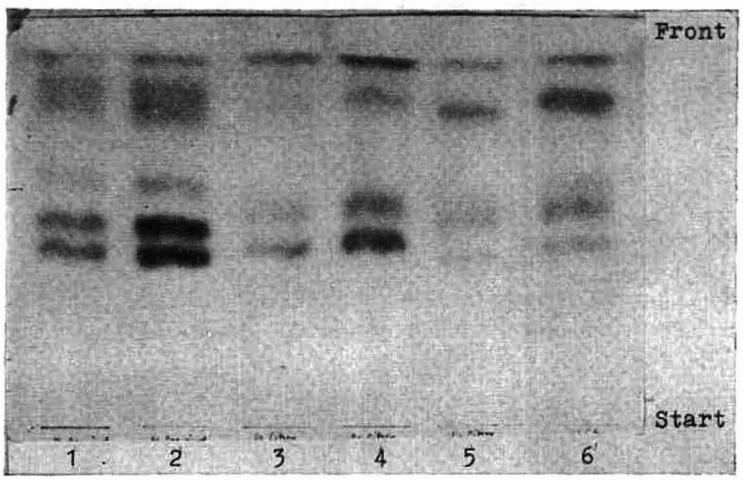

Fig. 6. Für die densitometrische Messung angefertigtes Papierchromatogramm. Fliessmittel: Methylisopropylketon–*n*-Heptan (5:20, V/V); Anfärbung: Osmiumtetroxyd. 1 und 2 = 100 und 200 µg ägyptisches Geraniumöl; 3 und 4 = 100 und 200 µg ceylonesisches Citronellöl; 5 und 6 = 150 und 200 µg javanisches Citronellöl.

densitometrischen Verfahren und nach bisher üblichen chemisch-analytischen Methoden (siehe Tabelle V und VI). Dabei liessen sich oft erhebliche Unterschiede zwischen den Analysenergebnissen feststellen.

Die densitometrisch und gaschromatographisch bestimmten Werte liegen unter den chemisch bestimmten. Die Abweichungen der densitometrischen Ergebnisse von den gaschromatographischen sind einmal dadurch zu erklären, dass es sich bei

TABELLE V

ANALYSENERGEBNISSE VON ÄTHERISCHEN ÖLEN

Ätherische Öle	*Methode*					
	Chem.-analyt.		*Densitometrisch*		*Gaschromatogr.*	
	A	*B*[19]	*C*	*D*	*C*	*D*
Lavendelöl Mt. Blanc 38–40 %	38.8	19.1	33.1	20.7	37.0	37.0
Lavendelöl Mt. Blanc 40–42 %	40.2	19.5	35.4	23.4	23.0	19.0
Lavendelöl Barrême	32.1	21.0	21.5	16.1	22.0	33.0
Lavendelöl Mt. Blanc	42.8	24.8	38.3	21.6	27.0	31.0
Lavandinöl 22–24 %	22.9	22.1	22.1	16.7	21.0	35.0

Erläuterungen: A = Estergehalt (%); B = Alkoholgehalt (%); C = Linalylacetatgehalt (%); D = Linaloolgehalt (%).

TABELLE VI

ANALYSENERGEBNISSE VON ÄTHERISCHEN ÖLEN

Ätherische Öle	*Methode*						
	Densitometrisch			*Gaschromatogr.*			*Chem.-analyt.*
	A	*B*	*C*	*A*	*B*	*C*	*D*
Citronellöl ceylon.	14.2	28.0	—	23.0	22.0	—	60.2
Citronellöl javan.	6.1	22.7	6.8	9.0	18.0	—	75.6
Geraniumöl ostind.	54.5	—	—	68.0	4.0	5.0	—
Melissenöl deutsch	5.5	26.2	8.2	6.0	30.0	5.0	—

Erläuterungen: A = Geraniolgehalt (%); B = Citronellolgehalt (%); C = Linaloolgehalt (%); D = Gesamtgeraniolgehalt (%).

ersteren um Gewichts-, bei letzteren aber um Volumenprozente handelt. Ausserdem beziehen sich die gaschromatographischen Ergebnisse nicht wie bei der Densitometrie auf die gesamte chromatographierte Ölmenge, sondern nur auf den flüchtig gewordenen Anteil. Mit den chemisch-analytischen Methoden andererseits werden bekanntlich nicht einzelne Stoffe erfasst, sondern meist ganze Stoffgruppen.

DISKUSSION

Zwei Faktoren haben hauptsächlich dazu beigetragen, das Problem der direkten chromatographischen Auftrennung von ätherischen Ölen auf Papier und die Frage der densitometrischen Auswertung solcher Papierchromatogramme zu lösen. Es sind die Herstellung eines mit Paraformaldehyd imprägnierten Papieres und die Ausarbeitung einer reproduzierbaren Anfärbemethode für ungesättigte Substanzen* mit Osmiumtetroxid. Neben der Qualität des Papieres[13] ist die Anfärbung der zu messenden Substanzen auf dem Chromatogramm für die Reproduzierbarkeit von ausschlaggebender Bedeutung. Eine farbintensive Anfärbung, die dem Lambert-Beerschen Gesetz folgt und von möglichst vielen Inhaltsstoffen ohne eine Verfärbung des Papierhintergrundes in einem weiten Messbereich gegeben wird, ist die schwer zu erfüllende Voraussetzung. Das von uns verwendete Osmiumtetroxid genügt diesen Anforderungen in idealer Weise. Das mit ungesättigten Substanzen gebildete

* Die densitometrische Bestimmung anderer, auch nicht flüchtiger Naturstoffe soll Gegenstand weiterer Arbeit sein.

Schwarz kommt dem "idealen Grau"[20] sehr nahe, sodass es nicht notwendig ist, monochromatisches Licht zu verwenden. Eine Verfärbung des Hintergrundes tritt nicht ein. Da das Reagens als Dampf zur Anwendung kommt, wird eine 100%ige Durchfärbung der Flecken gewährleistet.

Mit dem von uns entwickelten Verfahren ist es nun in wenig aufwendiger Weise möglich, die Qualität ätherischer Öle in Bezug auf ihre ungesättigten Hauptbestandteile zu bestimmen. Chromatogramme von guten Qualitäten lassen sich als Standardmasstäbe für später produzierte bzw. eingekaufte Öle aufbewahren, da die Färbung unbegrenzt haltbar ist und noch nach Monaten dieselben Messwerte ergibt. Genauso lässt sich das Auftreten von Autoxydationsprodukten während der Lagerung leicht überwachen, da diese am Startpunkt zurückbleiben. Das Verfahren der densitometrischen Bestimmung von ätherischen Ölsubstanzen stellt sich damit neben die Gas- und die Dünnschichtchromatographie. Der Vorteil, dass im Gegensatz zur gaschromatographischen Analyse auch die nicht oder nur sehr schwer flüchtigen Bestandteile in einem Arbeitsgang mit erfasst werden, spielt bei der Beurteilung von alten und schlecht gelagerten Ölen, bei denen sich nicht mehr flüchtige Polymerisationsprodukte gebildet haben, eine Rolle. Während bei der gaschromatographischen Routineanalyse in einem solchen Falle die Summe der Peakflächen aller flüchtigen Stoffe gleich 100% gesetzt und daraus der Prozentgehalt der einzelnen Komponenten berechnet wird, die nicht flüchtigen Anteile also unberücksichtigt bleiben, bezieht man bei der Densitometrie auf eine genau eingewogene Stammlösung. Ausserdem kann man im Gegensatz zur Gaschromatographie sehr verdünnte Lösungen zur Analyse bringen. Verglichen mit der dünnschichtchromatographischen Methode[21–26] besitzt unser Verfahren deren Trennvermögen und den Vorteil kurzer Entwicklungszeiten, ohne ihre Schwierigkeiten bei der quantitativen Auswertung zu teilen.

In einer kürzlich erschienenen Veröffentlichung deutet YORK[27] in einer Arbeit über Harze und Balsame wohl an, dass diese Schwierigkeiten mit Hilfe eines Reflexionsdensitometers überwunden werden können, doch fehlen noch nähere Einzelheiten des Verfahrens. Man würde ausserdem im Falle der Reflexionsmessung eine im Vergleich zur Transmissionsmessung fünf mal geringere Empfindlichkeit in Kauf nehmen müssen[28].

DANK

Unser besonderer Dank gilt Herrn Prof. Dr. L. HÖRHAMMER, Direktor des Institutes für Pharmazeutische Arzneimittellehre der Universität München für die freundliche Förderung unserer Arbeit und Herrn Priv.-Doz. Dr. H. WAGNER für Korrektur und Diskussion.

Ferner sind wir der Fa. Dragoco, Holzminden, die uns die gaschromatographischen Analysen der von uns untersuchten ätherischen Öle anfertigte zu aufrichtigem Dank verbunden. Ausserdem gebührt ihr, wie auch den Firmen Haarmann & Reimer in Holzminden, Frey & Lau in Hamburg-Bahrenfeld und E. Ziegler in Aufsess, unser bester Dank für die freundliche Überlassung von ätherischen Ölen und Testsubstanzen. Ausserdem sind wir der Firma A. Boake, Roberts & Co, London, für die freundliche Überlassung reiner Testsubstanzen sehr verbunden.

ZUSAMMENFASSUNG

Nach papierchromatographischer Trennung auf Ederol-Papier Nr. 208/P und Anfärbung mit Osmiumtetroxid lassen sich ungesättigte Bestandteile ätherischer Öle

reproduzierbar densitometrisch mit Hilfe eines gebräuchlichen Transmissionsdensitometers bestimmen. Es werden die Eichkurven der gut zu trennenden Alkohole Linalool, Geraniol und Citronellol (Fliessmittel: Methylisopropylketon–*n*-Heptan (5:20, V/V)) sowie von Linalylacetat aufgestellt und mit ihnen Gehaltsbestimmungen an Lavendel-, Lavandin-, Geranium-, Citronell- und Melissenölen durchgeführt. Die so erhaltenen Werte werden mit chemisch-analytisch und gaschromatographisch gewonnenen Analysendaten verglichen.

SUMMARY

A method is described with which unsaturated components of essential oils can be determined with good reproducibility. After separation on Ederol paper No. 208/P and coloration by treatment with osmium tetroxide, the components are determined by means of a common transmission densitometer. Using the solvent system methyl isopropyl ketone–*n*-heptane (5:20, v/v), calibration curves were drawn for the alcohols linalool, geraniol and citronellol, which are well-separable, as well as for linalyl acetate. With these curves the contents of these components in oil of lavender, lavandin, geranium, citronella and melissa were determined. The values were compared with those obtained by means of chemical analysis and gas chromatography.

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CONDENSATION OF GLYCINE WITH PHENOL DURING PAPER CHROMATOGRAPHY*

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The identification of metabolic intermediates by two-dimensional paper chromatography has been employed extensively in biochemical investigations. When this method is used to determine the quantitative distribution of intermediates, errors due to the presence of non-enzymically produced products become significant. This problem is considerably enhanced when the precursor or its metabolic products complex with the chromatographic solvent. Apparent concentrations may be significantly lowered with respect to the other intermediates or precursors present. Multiple or irregular spots, variability in R_F values, and poor quantitative recovery are often encountered in paper chromatography, particularly when phenol solvents are employed with amino acids[1–5]. Such formation of multiple spots has been attributed to interaction between amino acids[3,6], to excessive concentrations of the amino acids applied[7], to oxidation of the amino acid[4], and to different ionic forms as related to the pH of the solvent system employed[8]. This report concerns the formation of stable glycine condensation products during paper chromatography with water saturated phenol. The effects of pH, distillation of phenol, and the presence of formaldehyde are considered.

MATERIALS AND METHODS

Whatman No. 1 filter paper (22 × 17 in.) was used either untreated or treated with oxalic acid by immersion for 2 h in a 1 % solution and then washed exhaustively with distilled water. Designated amounts of radioactive glycine-2-^{14}C (1.08 mC/m*M*) from New England Nuclear or Volk were applied to the origin point. The two-dimensional chromatograms were developed first in water-saturated phenol (prepared from Mallinckrodt 88 % phenol, liquified, preservative free) and secondly in *n*-butanol–propionic acid–water[9]. Between solvents, the chromatograms were dried overnight in a hood at room temperature in a slow moving current of air. Timed studies were initiated from the instant of solvent addition to the chromatographic system. Radioactive areas were located on the chromatograms by radioautography employing Dupont Xtra Fast No. 508 X-ray Film. The relative distribution of radio-

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activity in the various areas was determined by counting with an end window Geiger tube[10]. Identification of labeled unknowns was made by co-chromatography with unlabeled compounds and comparison of radioautographs with ninhydrin sensitive areas. Exact coincidence of areas was accepted as indicating identical compounds. Distillation of phenol was performed either in the absence of any additives or by the method of DRAPER AND POLLARD[11]. The water-saturated phenol solvent was prepared by adding the distillate or residue to a limited amount of water until a single phase was obtained. Acid and alkaline hydrolysis of the glycine condensation products was performed in a sealed tube at 20 lb. pressure and 250° F for 2 hours.

DISCUSSION AND RESULTS

A two-dimensional chromatogram of glycine-2-^{14}C on Whatman No. 1 filter paper with water-saturated phenol as the initial solvent and butanol–propionic acid–water as the second solvent is shown in Fig. 1. While a number of active areas were present in addition to glycine, only three gave evidence of being formed during the chromatographic process. These areas, labeled I, II and III, consistently streak in the water-saturated phenol solvent with their tailing end coinciding with the glycine area. This type of streaking suggested that the unknowns were being formed during the development of the chromatogram in phenol. Their R_F values in the second solvent system were considerably larger than that of glycine, thus providing excellent re-

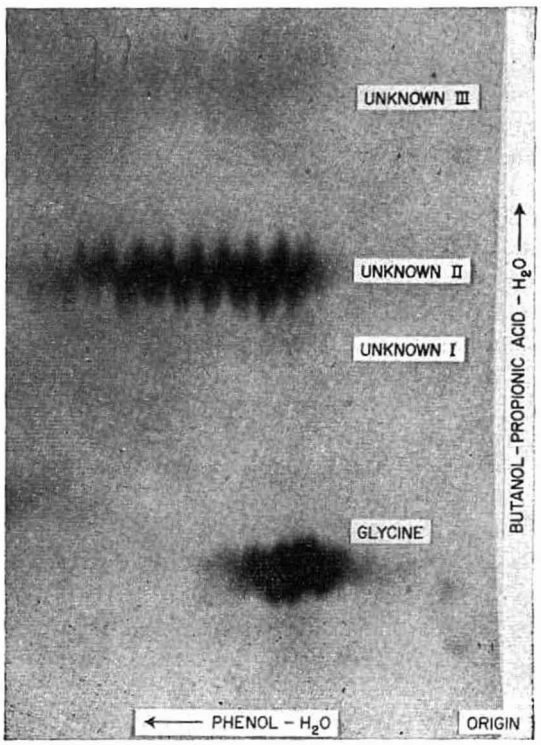

Fig. 1. Radioautogram of chromatogram of glycine-2-^{14}C. Whatman No. 1 filter paper. Solvents: phenol–water (first dimension); *n*-butanol–propionic acid–water (second dimension).

solution. No significant streaking occurred in the second solvent, indicating that no complex formation was taking place. The other areas of activity on the chromatogram were well defined spots with no evidence of streaking and appear to be contaminants of the original glycine preparation or, at least, were formed prior to chromatography.

At adequate levels of material all three unknowns could be detected with standard ninhydrin sprays. Since the ^{14}C-labeled areas and the ninhydrin-sensitive areas exactly coincided, the ninhydrin-sensitive compounds and the ^{14}C-labeled products were the same. Unknown I exhibited the typical purple color of glycine with ninhydrin sprays as soon as sprayed. Unknowns II and III exhibited a yellow color during the five minute heating process following spraying, but two to five days later the color turned to the typical purple color of ninhydrin with glycine. This type of color development (first yellow and then purple) was characteristic of such compounds as glycylglycine and glycylglycylglycine, except that the glycine peptides showed ninhydrin color development from yellow to purple within two to three hours.

Failure by other workers[4] to detect ninhydrin color reaction with these unknown substances from glycine may be due to the low concentration of material used. With commercial liquid phenol and untreated Whatman No. 1 filter paper unknowns I and II were easily detectable with 25 μg applications of glycine at the origin. Unknown III could not be detected at this level. Even applications as low as 10 μg of glycine yielded a detectable ninhydrin sensitive area for unknowns I and II. Chromatography of unlabeled glycine from four different commercial sources with the two-dimensional solvent system yielded, in each case, unknown I and II which could be detected readily with ninhydrin.

Unknowns I and II were stable and could be eluted from the filter paper readily with water. Rechromatography in the same solvent system gave a reproducible R_F with a discrete spot and no evidence of tailing. The approximate R_F values in butanol–propionic acid–water were 0.6 for I, 0.7 for II and 0.9 for III. In phenol–water the R_F values for all three unknowns were nearly the same at about 0.8 to 0.9.

Upon hydrolysis of ^{14}C-labeled unknown II with ammonium hydroxide all the ^{14}C cochromatographed identically with unlabeled glycine (Fig. 2). Partial hydrolysis was obtained with HCl, formic acid, and acetic acid. In each case glycine-^{14}C was released. These results supported the hypothesis that unknown II was a stable glycine condensation product rather than a product of glycine oxidation as has been suggested[4]. However, unknown II did not cochromatograph with α-phenyl-glycine, N-phenylglycine, glycylglycine, glycylglycylglycine, diketopiperazine and phenylglycinate and thus did not appear to be one of these types of possible condensation products.

The glycine condensation was directly related to the phenol employed and to the length of time that the glycine was exposed to the solvent (Table I). When redistilled phenol (redistilled without preservatives present) was used, a four fold increase in condensation products formed during an initial five hour period from glycine-2-^{14}C occurred as compared to the use of nonredistilled commercial phenol or the distillation residue.

Phenol redistilled in the presence of aluminum turnings and sodium bicarbonate yielded only 1.6 % of the total activity as condensation products after twenty hours of chromatography. This suggested that formation of glycine unknowns required the

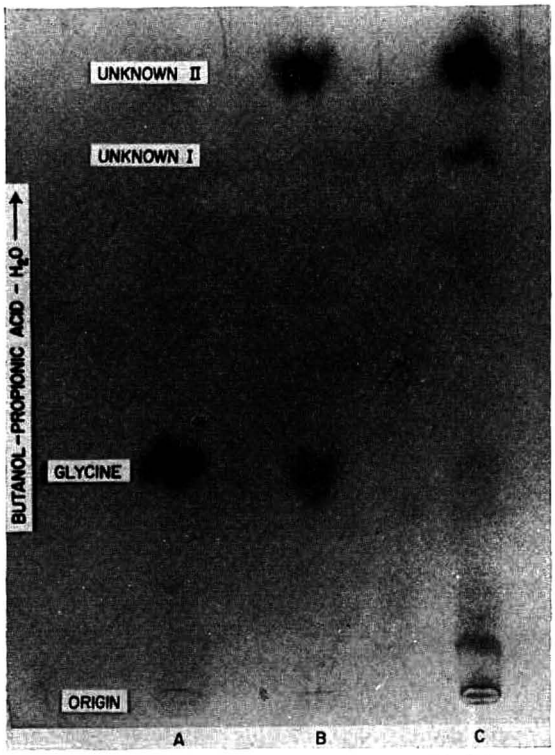

Fig. 2. Radioautogram of the hydrolysis products of unknown II. Whatman No. 1 filter paper. Solvent: *n*-butanol–propionic acid–water. A. Hydrolysis with ammonium hydroxide. B. Hydrolysis with HCl. C. No hydrolysis.

presence of a contaminant which was essentially removed or destroyed by distillation in the presence of aluminum turnings and sodium bicarbonate.

The increase in amount of the glycine condensation products with length of time of chromatography confirmed that their synthesis occurred during the chromatographic process. Direct contact between the glycine and the phenol solvent was required. Suspensions of glycine aliquots spotted on Whatman No. 1 filter paper in a phenol saturated atmosphere for two weeks produced no detectable condensation products.

Adjustment of the pH of the phenol solvent from 3.0 to 9.0 caused no significant decrease in condensation products. However, prewashing Whatman No. 1 filter paper with oxalic acid decreased glycine condensation by about one third. Saturation of the phenol solvent system with 20 % saponin, which has been suggested as a means of preventing amino acid interaction[12], failed to curtail glycine condensation.

Chromatography of glycine in phenol solvent containing 1 % (v/v) formaldehyde for twenty hours resulted in complete conversion of glycine to unknown III. Addition of 1 % (v/v) formaldehyde to the *n*-butanol–propionic acid–water solvent had no effect on glycine chromatography.

One hour incubation of solutions containing glycine-2-^{14}C, formaldehyde, and phenol produced condensation products II and III, while incubation for 24 h caused complete conversion of glycine-2-^{14}C to unknown III (Table II). The formation of

TABLE I

FORMATION OF GLYCINE-2-^{14}C CONDENSATION PRODUCTS IN REDISTILLED PHENOL

Hours in phenol	*Per cent of total radioactivity found in condensation products*		
	Commercial phenol	*Redistilled phenol**	*Phenol residue**
0	0	0	0
5	3.0	13.4	3.5
10	7.4	21.1	6.4
15	10.8	25.5	9.2
20	20.0	36.4	12.5
25	21.4	43.3	19.3

* Distillation of the phenol was performed in the absence of any additives.

TABLE II

EFFECT OF FORMALDEHYDE ON THE FORMATION OF GLYCINE-2-^{14}C CONDENSATION PRODUCTS

Compound	*Phenol* c.p.m.*	*Phenol* + HCHO c.p.m.*
Glycine	16,488	234
Unknown I	170	0
Unknown II	94	3,803
Unknown III	0	17,719

* Phenol was redistilled from aluminum turnings and sodium bicarbonate. Mixtures were incubated at room temperature for 1 h.

unknown II predominated at low formaldehyde concentrations and short incubation times, while unknown III was formed with longer incubation times and high formaldehyde concentrations. Thus unknown II may have been the precursor of unknown III.

By cochromatography it was shown that unknown II was the same material whether formed in commercial nonredistilled phenol or in the presence of added formaldehyde. Radioactive ^{14}C was found in condensation products II and III irrespective of whether formaldehyde-^{14}C or glycine-2-^{14}C were employed (Fig. 3). The differences in distribution of radioactivity between II and III on the chromatogram resulted from differences in formaldehyde concentration. The formaldehyde-^{14}C mixtures contained much less formaldehyde than the glycine-2-^{14}C plus added unlabeled formaldehyde. On chromatograms of biological experiments which utilized formaldehyde-^{14}C, unknown III was a common product. In such experiments unused formaldehyde-^{14}C and a reservoir of glycine from the biological material were usually present.

The structures of unknowns I, II and III have not been established. Our evidence indicates that they are condensation products of glycine, phenol and formaldehyde. Such condensation products have been reported[13]. While such reports have been concerned with formation of glycine–phenol–formaldehyde polymers, polymerization at the low concentrations employed in paper chromatography has not been considered. *Ortho*, *meta* and *para*-hydroxybenzyl alcohols, the postulated initial products of formaldehyde condensation with phenol[14], were considered as intermedi-

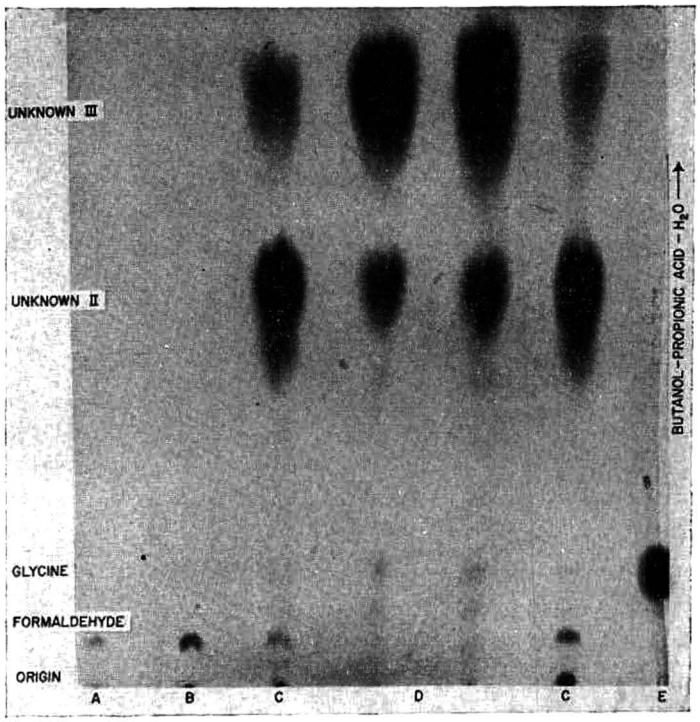

Fig. 3. Radioautogram of a chromatogram of the following mixtures incubated for 1 h prior to spotting on the filter paper. A. Formaldehyde-^{14}C in water-saturated phenol. B. Formaldehyde-^{14}C in water-saturated phenol. C. Formaldehyde-^{14}C and glycine in water-saturated phenol. D. Glycine-2-^{14}C and formaldehyde in water-saturated phenol. E. Glycine-2-^{14}C in water. Solvent for chromatography was *n*-butanol–propionic acid–water.

ates in formation of unknowns I, II and III. However, no increase in the ^{14}C condensation products from glycine-^{14}C was observed when the hydroxybenzyl alcohols were incubated with glycine and phenol mixtures.

Experimental results suggest that commercial phenol contains trace amounts of formaldehyde or formaldehyde condensation products which condense with glycine during paper chromatography. In addition, it appears that ethanolamine forms similar condensation unknowns during paper chromatography with phenol solvents. In chromatography where such condensation should be eliminated, redistillation of the phenol from aluminum turnings and sodium bicarbonate[11] and pretreatment of Whatman No. 1 filter paper with oxalic acid minimize the problem.

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SUMMARY

Three products formed during the paper chromatography of glycine with water-saturated phenol as a solvent have been investigated. These unknowns were stable

condensation products whose formation was dependent upon the presence of glycine and formaldehyde. They were ninhydrin sensitive. Alkaline hydrolysis yielded glycine. For chromatographic practices, the amount of glycine or formaldehyde which may be converted into the condensation products will depend upon the amount of the other component in the mixture. Addition of 1% formaldehyde normally complexes all of the glycine. In usual chromatographic experiments part of the glycine may be complexed by residual formaldehyde present in chromatographic phenol. Glycine condensation may be minimized by redistilling the phenol solvent with aluminum turnings and sodium bicarbonate and by washing the filter paper with 1% oxalic acid.

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PAPER CHROMATOGRAPHY OF DYES

IV. PAPER CHROMATOGRAPHY OF WATER-SOLUBLE DYES

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INTRODUCTION

In previous reports paper chromatography of water-insoluble dyes, namely, disperse dyes[1], vat and sulphur dyes[2], was described, and the subject of the chromatography of indigosols[3] was discussed. Although the latter are water-soluble, they are prepared by solubilizing insoluble vat dyes.

In the present paper, a chromatographic method suitable for water-soluble dyes is described. These dyes represent some 50 % of the total volume of the annual world production of coal tar dyestuffs, amounting to about 200,000 tons. Water-soluble dyes are manufactured practically by all dye producers and the number of trade products at present on the market reaches several thousand. Individual trade products of the various dye manufacturers are frequently identical, a fact easily shown by paper chromatography. The individual dyes, however, differ from each other in purity. The reaction mechanism of dye synthesis is of a complicated and manifold nature. For this reason the impurities occurring in the dye very frequently consist of other dyes or intermediates formed during the course of the reaction. The non-uniformity of the dye mostly depends on the degree of purity of the intermediates and on the tautomerism of the products likely to form isomers. Unreacted intermediates, isomers and other impurities, must be regarded as the main cause of the non-uniformity of dyes. Dyes are either pure homogeneous, *i.e.* they are free from any secondary dye impurities which might have been formed in the course of the dye synthesis itself, or shaded, *i.e.* they contain a small amount of another dye so that the desired shade is obtained, or lastly miscellaneous, these are formed by intentional mixing of several fundamental dyes.

In the selection of dyes for purposes of industrial application, purity of the dye is of great importance, influencing the dyeing technology. For the purity estimation of dyes and of their dyeing behaviour, paper chromatography is of paramount importance.

Water-soluble dyes are represented by a number of organic compounds with typical properties, either derivatives of aromatic hydrocarbons or of heterocyclic compounds of aromatic character. The chromatographic behaviour of these dyes depends generally on the complexity, shape and size of the dye molecule; its functional groups, their nature and positions, the nature of the bonds, and the ionic as well as electrostatic character of the molecules (*e.g.* on polarity and polarizability).

The subject of paper chromatography of water-soluble dyes has already been dealt with by numerous authors[4–12]. A check of the methods described revealed their unsuitability for a comprehensive systematic analysis of dyes of all chemical classes. In most cases, the results of the separation did not confirm the relation between the structure of the dye and its chromatographic behaviour and were not in accordance with the laws of partition chromatography.

In view of the technological importance of these dyes, we have turned our attention to the development of a chromatographic method, by means of which it should be possible, under equal chromatographic conditions, to compare the properties of water-soluble dyes of all chemical classes[13–16].

EXPERIMENTAL

For a systematic analysis of water-soluble dyes of all chemical classes a descending chromatographic technique using Whatman No. 1 paper and a solvent system composed of pyridine–isoamyl alcohol–25% ammonia (1.3:1:1) has proved to be most suitable. This system also gives satisfactory results with a solvent ratio of 1:1:1; its effectiveness remaining the same even when the ratio of pyridine is increased up to 1.9:1:1. Increasing the quantity of pyridine increases the sharpness of the zones, but this is already optimal when the ratio of the components is 1.3:1:1. This solvent system possesses sufficient sensitivity to detect differences in chemical composition and structure of dyes of individual manufacturers and permits chromatographic separation of very complex mixtures of dyes.

The separating properties of the isoamyl alcohol solvent system are also evident with a butan-1-ol–pyridine–water (1:1:1) system. For the determination of the purity of highly substantive azo dyes a butan-1-ol–pyridine–water (0.5:1:1) solvent system was used.

Method

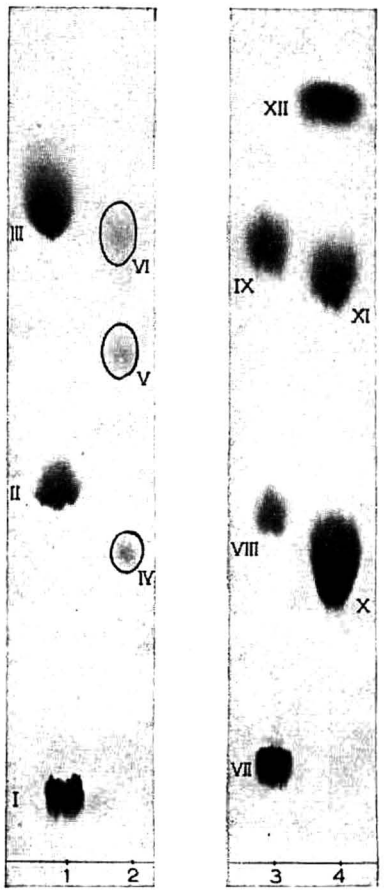
0.1 μl samples of a 1% aqueous solution of water-soluble dye are applied as spots on the start of the chromatogram by means of a micro-pipette. The size of the chromatogram is 160 × 470 mm, the distance of the start from the edge of the paper being 70 mm. The dyes are spotted on the start at intervals of 20 mm from each other. Development of the chromatogram takes place at a temperature of 20–22° for 12 h (preferably overnight) and the length of the run is 400 mm.

RESULTS AND DISCUSSION

Water-soluble dyes of all chemical classes were chromatographed by the method described above, employing an isoamyl alcohol system.

This isoamyl alcohol system is compatible with the conditions of partition chromatography and particularly distinguishes between differences in the partition

Fig. 1. The influence of the flow rate of the isoamyl alcohol solvent system on the quality of separation. A. Descending technique. B. Centrifugal technique. 1. Mixture of nitroso and nitro dyes (I, II, III). 2. Mixture of thiazine dyes (IV, V, VI). 3. Mixture of monoazo dyes (derivatiives of azonaphthalene, VII, VIII, IX). 4. Mixture of monoazo dyes (derivatives of benzeneazonaphthalene, X, XI, XII).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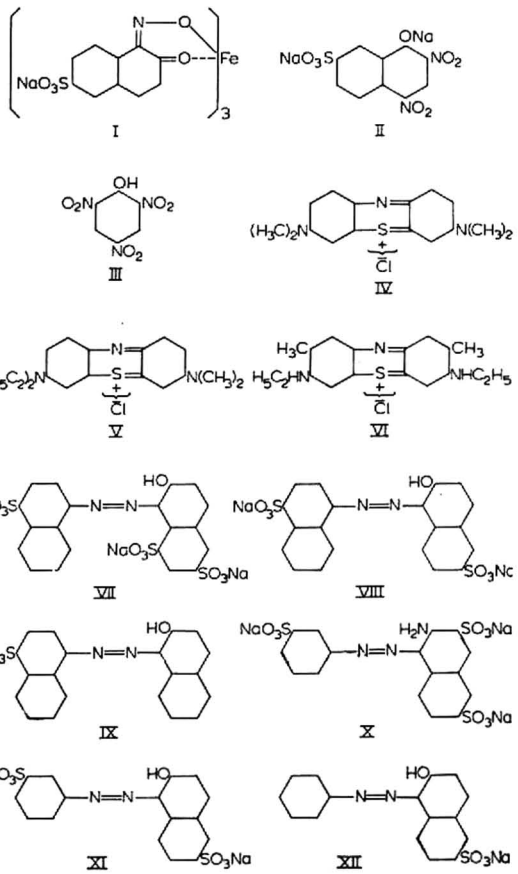
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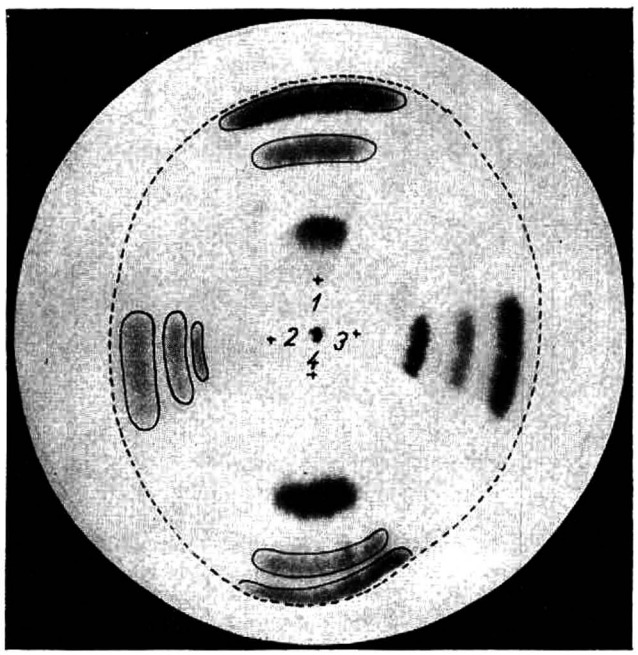
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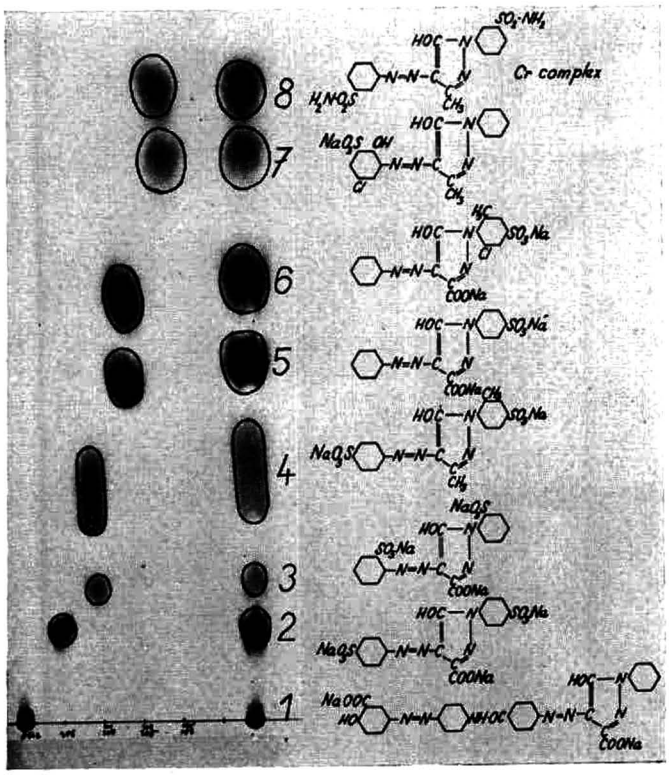

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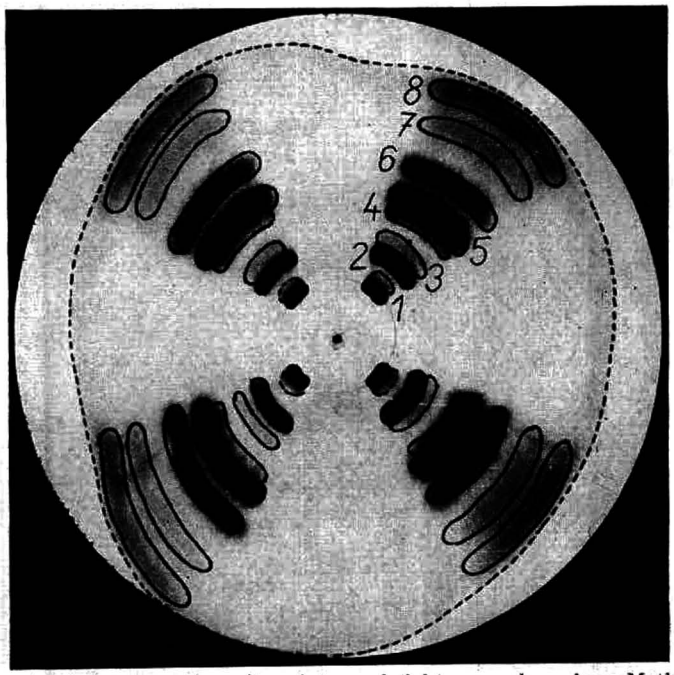


B

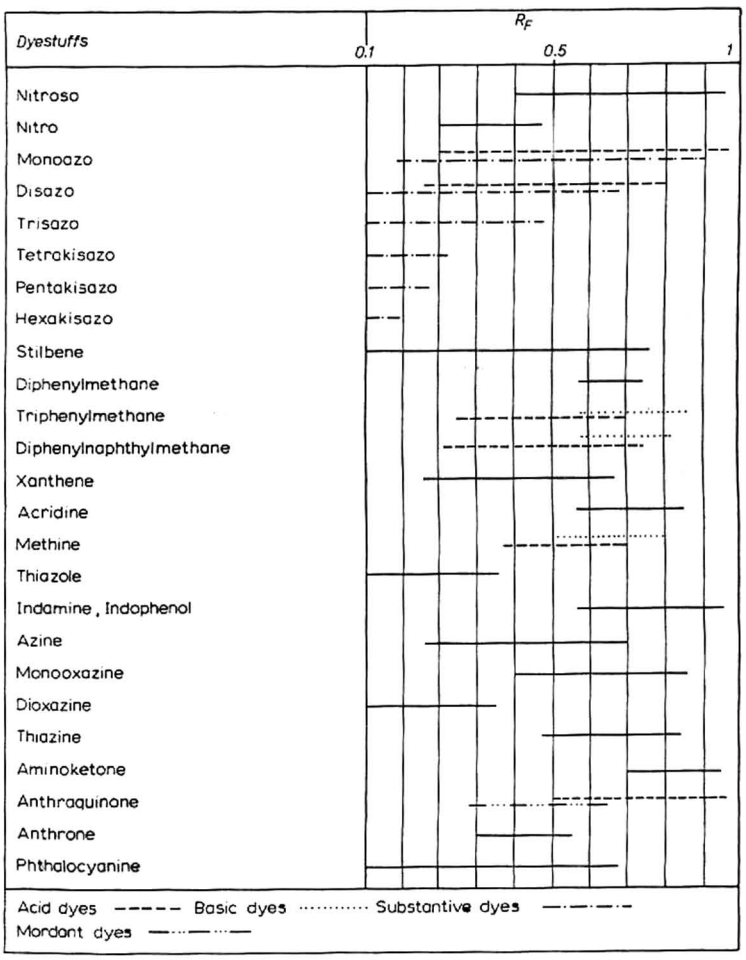
Fig. 2. An example of the separation of a mixture of eight pyrazolone dyes. Methyl derivatives have higher R_F values than carboxy derivatives. A. Descending technique. B. Centrifugal technique.

coefficients and in the solubility of the dyes. For instance, the rate of flow of the solvent system through the paper, which with most systems not compatible with the conditions of partition chromatography, influences the reproducibility of results, fails here to exert any effect whatsoever. This was shown by applying this solvent system in chromatography in a centrifugal field, where the rate of flow of the solvent system through the paper increases many times. Results obtained with chromatograms in a centrifugal field and by the normal descending method were identical. Figs. 1 and 2 show the influence of the flow rate of the isoamyl alcohol solvent system on the quality of separation, the descending and centrifugal chromatographic techniques being compared.

Centrifugal chromatography was carried out with the pressureless apparatus with central spot development described previously by PAVLÍČEK, ROSMUS AND DEYL[17,18]. The chromatograms were cut from Whatman No. 3 paper and the separa-

TABLE I

MEAN R_F VALUES OF WATER-SOLUBLE DYES



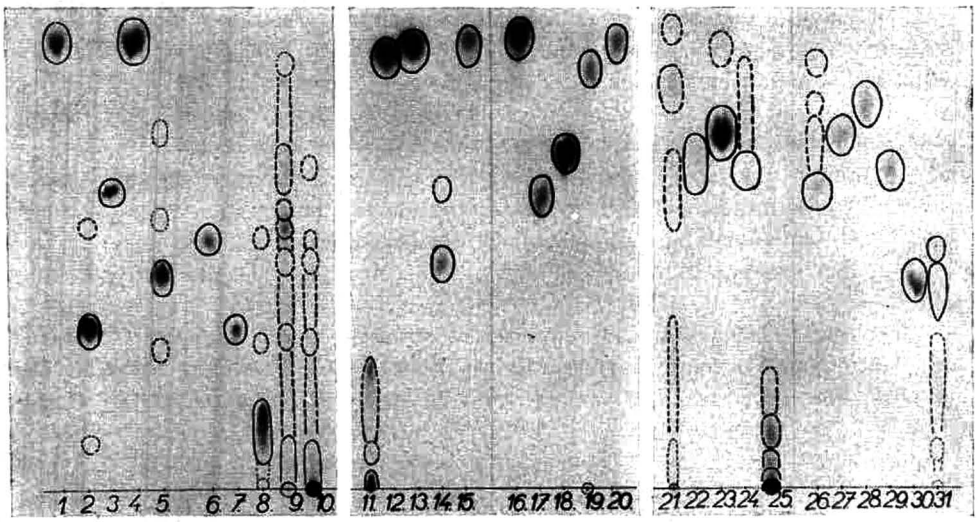


Fig. 3. Example of a chromatographic separation of water-soluble dyes.

1. Picric Acid (nitro), CI 10305
2. Green PLX (nitroso), CI 10020
3. Brilliant Geranine B (monoazo – substantive), CI 15080
4. Cellit Fast Yellow R (monoazo – acid), CI 13145
5. Chloramine Red 8BS (bisazo – substantive), CI 23050
6. Wool Black 6BG (bisazo – acid), CI 20350
7. Sirius Blue 6G (trisazo – substantive), CI 34230
8. Sirius Supra Grey R (tetrakisazo), CI 35870
9. Diamine Catechine G (pentakisazo), CI 36030
10. Diamine Nitrazol Fast Brown BD (hexakisazo), CI 36310
11. Chlorantine Supra Orange T4RLL (stilbene), CI 40265
12. Auramine G (diphenylmethane – basic), CI 41005
13. Malachite Green (triphenylmethane – basic), CI 42000
14. Carbinol Fast Green G (triphenylmethane – acid), CI 42165
15. Victoria Blue R (diphenylnaphthylmethane – basic), CI 44040
16. Victoria Blue 4R (diphenylnaphthylmethane – basic), CI 44045
17. Naphthaline Green V (diphenylnaphthylmethane – acid), CI 44025
18. Methylene Red (xanthene – basic), CI 45006
19. Phosphine E (acridine – basic), CI 46045
20. Astrazone Orange G (methine – basic), CI 48035
21. Thioflavine S (thiazine – substantive), CI 49010
22. Toluylene Blue (indophenol – basic), CI 49410
23. Induline Scarlet (azine – basic), CI 50080
24. Rhoduline Sky Blue 3G (monooxazines – basic), CI 51005
25. Sirius Supra Blue F3GL (dioxazines – substantive), CI 51310
26. Thionine Blue GO (thiazine – basic), CI 52025
27. Brilliant Sulpho Flavine FF (aminoketone–acid), CI 56205
28. Anthralan Blue B (anthraquinone – acid), CI 62130
29. Alizarine Chrome Blue FFG (anthraquinone – mordant), CI 62120
30. Alizarine Rubinol 3 G (anthraquinone – acid), CI 68205
31. Sirius Supra Turquoise Blue GL (phthalocyanine), CI 74180

tion process was carried out for 45 min at 600 r.p.m. The chromatograms were always developed with the same solvent system as that used for the descending technique.

Table I shows mean R_F values of the individual groups of water-soluble dyes obtained by compilation of minimum and maximum R_F values of several hundred dyes of known chemical constitution. The R_F values refer to the isoamyl alcohol solvent system and Whatman No. 1 paper. Fig. 3 shows an example of a chromatographic separation of water-soluble dyes.

Although the establishment of correlation between the structure and chromatographic behaviour of a dye is rather complicated, we have been able to set up some general rules:

(1) Structurally simple dyes, with low physico-chemical activity, have a high R_F, as seen in Table I. With increasing complexity of the dye molecule, its physical and chemical tendency for reaction between the phases and phase-carrier increases proportionally, this being most pronounced with the azo dyes.

The substantive dyes have the lowest R_F values, the acid dyes come next and the basic dyes have the highest R_F values.

(3) The polarizability, substantivity and adsorption affinity increase with increasing number of conjugated double bonds traversing the entire dye molecule, the R_F values decreasing in proportion. If the chain of conjugated double bonds is interrupted, the R_F increases, even though a decrease of the R_F would be expected owing to the number of –N=N– bonds. The greater the number of the azo groups, the lower the R_F. Carbamide and amide conjugated double bond systems replacing the –N=N– bond cause a decrease of the R_F value.

(4) With dyes where the formation of internal hydrogen bonds is possible, the adsorption affinity decreases and the R_F value increases.

(5) With compounds of the same fundamental skeleton the R_F is decreased by $-SO_3H$, –COOH, $-NH_2$ and $-NO_2$ groups. The influence of these groups on the R_F is additive. Halogens and heterocyclic compounds act in the same way. A conductive connection of these groups with a conjugated system of double bonds decreases the R_F value.

(6) With the increase of the total dipole moment of the dye molecule, with compounds of the same fundamental skeleton, the R_F diminishes depending on the position and character of the substituents.

(7) Symmetric dyes have a lower R_F than unsymmetric dyes.

(8) Generally, the R_F value is dependent on the molecular weight. With its increase the R_F value diminishes proportionally with growing capillary adhesion.

Results obtained by the chromatography of various individual industrial important classes of water-soluble dyestuffs will be described in subsequent papers.

ACKNOWLEDGEMENTS

The authors are grateful to Messrs. Ing. Z. DEYL, C. Sc. and Ing. J. ROSMUS of the Central Research Institute of Food Industry, Prague, for kindly permitting them to carry out centrifugal chromatography on their apparatus and for their valuable assistance in the field of centrifugal chromatography.

SUMMARY

A descending paper chromatographic method for water-soluble dyes is described in which a pyridine–isoamyl alcohol–ammonia solvent system is used. The relationship between dye constitution and chromatographic behaviour has been investigated.

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CHROMATOGRAPHIC SEPARATION OF PHENOLS ON ION-EXCHANGE PAPER

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INTRODUCTION

Impregnated papers have been reported to provide improved chromatographic separation of phenols. In addition to partitioning, steric, and other effects by which separations are accomplished on ordinary chromatographic paper, separations on impregnated paper are effected by differences in polarity of individual phenols with respect to the stationary phase, or because of the properties of some phenols to form chelates with the stationary phase that affect the partitioning.

Among the reagents that have been used for these chromatographic separations are sodium tetraborate[1–4], sodium molybdate[5–7], sodium tungstate or vanadate[7], polyamide[8], formamide[9] and dimethylformamide[10].

The separation of some dihydric phenols cannot be conveniently accomplished by the use of some of these inorganic stationary phases, because of the streaking that occurs. A disadvantage of others is the time required to obtain a separation (15–45 h). The method of SUNDT[10], which uses dimethylformamide-impregnated paper with a cyclohexane–ethyl acetate (5:1) solvent, was found to be convenient and useful. However, conditioning of sheets prior to development resulted in "blooming" of some phenols that have low vapor pressures. This prevented the clean separation of guaiacol and alkylguaiacols.

The separation of phenols by a column of cation-exchange resins has been reported[11]. In the present work, paper loaded with ion-exchange resin was used to separate phenols that were studied in connection with those derived from pulping or lignin sources.

EXPERIMENTAL

Reeve Angel Grade SB-2 Amberlite ion-exchange resin-loaded paper was used for this work. The resin, Amberlite IRA-400, a strong base type in the Cl^- form, was used in this form. The use of two solvents (A and B) was studied. Solvent A, which consisted of cyclohexane–ethyl acetate–acetic acid (5:1:1, v/v), provided good separation of monohydric and dihydric phenols, but was less effective for phenolic aldehydes and for moving hydroxybenzoic acids. Solvent B, which consisted of butanol–water–acetic acid (6:2:1), gave better results for the latter two groups, but most simple phenols were not well separated.

* Maintained at Madison, Wisc., in cooperation with the University of Wisconsin.

All chromatographic runs were made at 25° with descending solvent flow in the machine direction of 22-in. strips of the resin-loaded paper. For the separation of monohydric and dihydric phenols with solvent A, the paper strips were preconditioned for several hours in a jar of saturated water vapor before applying the phenols. The moisture content of the paper was found to reduce the rate of solvent front movement and to avoid elongated spots that resulted from rapid solvent travel. No preconditioning was necessary for chromatographic separations that used solvent B. Chromatography jars were well saturated with vapors of the solvent to be used. With solvent A, solvent travel was 40 cm in $2^1/_2$ h. With solvent B, the front moved 38 cm in 4 h.

Sheets were dried in a circulating air oven at 50° for 5 or 10 min. Spots were detected by a light spray of diazotized sulfanilic acid followed by 20% sodium carbonate[12].

RESULTS AND DISCUSSION

R_F values that were determined are given in Table I through IV. These values show slight variations in different runs, apparently depending upon humidity of the paper and flow rate of the solvent. A difference of R_F value of about 0.05 was necessary to obtain resolution of mixtures of compounds with solvent A. In general, *ortho*-isomers could be separated from either *meta* or *para* forms, such as *o*-, *m*- and *p*-cresols, but a mixture of *meta*- and *para*-isomers was not resolved. Excellent separation of a mixture of the four catechols and four guaiacols listed was possible with solvent A. Of the dimethylphenols, the 2,3-, 2,4- and 2,5-isomers could not be separated, and the 3,4-isomer was only partially separated from the 3,5-isomer with solvent A.

The chief advantage of solvent B, butanol–water–acetic acid (6:2:1), was the

TABLE I

MONOHYDRIC PHENOLS

Compound	R_F *value*	
	Solvent A	*Solvent B*
Phenol	0.28	0.73
o-Cresol	0.48	0.81
m-Cresol	0.40	0.81
p-Cresol	0.41	0.81
o-Ethylphenol	0.67	0.87
m-Ethylphenol	0.52	0.87
p-Ethylphenol	0.55	0.86
p-Propylphenol	0.69	0.92
o-Phenylphenol	0.54	0.75
m-Phenylphenol	0.35	0.73
p-Phenylphenol	0.32	0.70
p-Benzylphenol	0.51	0.83
2,3-Dimethylphenol	0.56	0.82
2,4-Dimethylphenol	0.58	0.82
2,5-Dimethylphenol	0.57	0.86
2,6-Dimethylphenol	0.72	0.89
3,4-Dimethylphenol	0.45	0.82
3,5-Dimethylphenol	0.48	0.82
Biphenol	0.02	0.24
2,2′-Dihydroxybiphenyl	0.14	0.53

TABLE II

DIHYDRIC AND TRIHYDRIC PHENOLS

Compound	R_F value	
	Solvent A	Solvent B
Catechol	0.08	0.51
4-Methylcatechol	0.12	0.57
4-Ethylcatechol	0.20	0.69
4-Propylcatechol	0.24	0.73
Guaiacol	0.59	0.79
Creosol	0.70	0.82
4-Ethylguaiacol	0.81	0.87
4-*n*-Propylguaiacol	0.88	0.93
Eugenol	0.08	0.93
Isoeugenol	0.19	0.85
Coniferyl alcohol	0.10	0.65
Resorcinol	0.02	0.31
Orcinol	0.02	0.39
Pyrogallol	0.00	0.18
Pyrogallol 1,3-dimethyl ether	0.10	0.76

greater mobility it afforded the highly polar hydroxybenzoic acids. As with solvent A, solvent B required an R_F difference of about 0.05 to permit separation of individual compounds. It was again impossible to resolve completely a mixture of 2,4-, 2,5- and 3,4-dihydroxy-isomers, but good separations of monohydroxy from dihydroxy types and their derivatives were possible.

SUNDT[10] has pointed out the relationship of pK_a values of some phenols and their R_F values obtained with this chromatographic system. He also suggests that acidity is not the only factor affecting migration of compounds, based upon poor

TABLE III

HYDROXY AROMATIC ACIDS

Compound	R_F value	
	Solvent A	Solvent B
p-Hydroxybenzoic acid	0.04	0.50
m-Hydroxybenzoic acid	0.09	0.47
2,4-Dihydroxybenzoic acid (β-resorcylic)	0.02	0.28
2,5-Dihydroxybenzoic acid (gentisic)	0.02	0.24
3,4-Dihydroxybenzoic acid (protocatechuic)	0.00	0.24
3-Methoxy-4-hydroxybenzoic acid (vanillic)	0.08	0.58
3,5-Dimethoxy-4-hydroxybenzoic acid (syringic)	0.06	0.65
o-Hydroxycinnamic acid	0.11	0.44
p-Hydroxycinnamic acid	0.03	0.46
3,4-Dihydroxycinnamic acid (caffeic)	0.00	0.21
3-Methoxy-4-hydroxycinnamic acid (ferulic)	0.10	0.52
3,4-Dimethoxycinnamic acid	0.08	—
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic)	0.07	0.53
p-Hydroxyphenylacetic acid	0.02	0.46
3,4-Dihydroxyphenylacetic acid	0.00	0.20
3-(*p*-Hydroxyphenyl)-propionic acid	0.08	0.57
3-(3,4-Dihydroxyphenyl)-propionic acid	0.01	0.29

TABLE IV

AROMATIC ALDEHYDES AND KETONES

Compound	R_F value	
	Solvent A	Solvent B
p-Hydroxybenzaldehyde	0.07	0.71
2,4-Dihydroxybenzaldehyde	0.08	0.61
2,5-Dihydroxybenzaldehyde	0.07	0.57
3,4-Dihydroxybenzaldehyde	0.02	0.42
2-Hydroxy-3-methoxybenzaldehyde (*o*-vanillin)	0.64	0.84
Vanillin	0.20	0.75
Veratraldehyde	0.45	0.87
Syringaldehyde	0.10	0.74
Coniferyl aldehyde	0.08	0.71
Cinnamaldehyde	—	0.92
Hydroxymethylfurfural	0.08	0.68
5-Formylvanillin	0.16	0.40
5-Carboxylvanilin	0.03	0.26
2,4-Dihydroxyacetophenone	0.18	0.68
Acetovanillone	0.20	0.78
Acetosyringone	0.34	0.78
2,4-Dihydroxypropiophenone	0.30	0.73
3,4-Dihydroxypropiophenone	0.06	0.55
Maltol	0.42	0.81

correlation between pK_a and R_F values of 2,5- and 2,3-dimethylphenols. R_F values obtained for several phenols in the present work with solvent A are very similar to those of SUNDT. The same relationship of R_F and pK_a values exists, including the non-resolution of 2,5- and 2,3-dimethylphenols, despite their pronounced difference in pK_a values. A comparison of some of the present results with those of SUNDT is given in Table V.

TABLE V

RELATIONSHIP BETWEEN ACID STRENGTH (pK_a) AND R_F VALUES

Compound	pK_a	Reference	R_F*	R_F**
m-Cresol	10.08	13	0.41	0.40
p-Cresol	10.10	14	0.42	0.41
o-Cresol	10.19	14	0.49	0.48
3,5-Dimethylphenol	10.23	15	0.50	0.48
3,4-Dimethylphenol	10.43	15	0.45	0.45
2,5-Dimethylphenol	10.46	15	0.57	0.57
2,3-Dimethylphenol	10.57	15	0.56	0.56
2,4-Dimethylphenol	10.63	15	0.59	0.58
2,5-Dimethylphenol	10.66	15	0.65	0.72

* Dimethylformamide-impregnated paper, SUNDT[10].

** This work: Reeve Angel SB-2 paper, solvent A: cyclohexane–ethyl acetate–acetic acid (5 : 1 : 1).

SUMMARY

Paper loaded with strong base ion-exchange resin in Cl^- form was used to accomplish the chromatographic separation of a number of monohydric and dihydric

phenols and their derivatives by the use of two solvent systems. Data obtained confirmed the results of others who found that there is good correlation between pK_a values of some phenols and their R_F values for separation on paper that contains a highly polar stationary phase. This is not true for other phenols, suggesting that several factors may affect these chromatographic separations.

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SUR LE MARQUAGE DU TRIPHÉNYL-BISMUTH PAR LE RADIO-BISMUTH: ÉTUDE CHROMATOGRAPHIQUE

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INTRODUCTION

Dans un travail précédent[1], l'analyse chromatographique du mélange $Hg(C_6H_5)_2$–$^{203}HgCl_2$ avait permis de mettre en évidence le rôle du chlorure de phényl-mercure dans le marquage du diphényl-mercure par ^{203}Hg. Une analyse similaire intéressant les composés du bismuth s'est avérée nécessaire dans l'étude des conséquences chimiques accompagnant la transmutation d'isotopes du plomb en bismuth, lorsque l'atome de plomb est engagé dans un composé organométallique. Dans ce système, en effet, la présence simultanée de formes organiques, organométalliques et minérales du bismuth n'est pas exclue et il est indispensable de connaître l'importance d'éventuelles réactions d'échange isotopique.

L'échange isotopique du bismuth entre le triphényl-bismuth $Bi(C_6H_5)_3$ (en abrégé $BiPh_3$) et le dichlorure de triphényl-bismuth $Bi(C_6H_5)_3Cl_2$ (ou $BiPh_3Cl_2$) a été examiné par NEFEDOV, SINOTOVA ET TRENIN[2] qui ont déterminé l'énergie d'activation de la réaction d'échange (15.9 kcal/mol) et montré que la vitesse de la réaction était du premier ordre par rapport à chacune des espèces en réaction. Considérant $BiPh_3$ comme un "pseudoatome" dont $BiPh_3Cl_2$ serait le sel, et admettant la règle de Grimm selon laquelle l'addition de n radicaux organiques à un atome d'un élément de numéro atomique Z lui confère les propriétés d'un pseudoatome de propriétés voisines de celles de l'élément Z-n, ces auteurs concluent à un mécanisme d'échange par transfert d'électrons:

$$Bi^*Ph_3 \longrightarrow Bi^*Ph_3^{2+} + 2e^-$$

$$BiPh_3^{2+} + 2e^- \longrightarrow BiPh_3$$

dont le bilan net est:

$$Bi^*Ph_3 + BiPh_3Cl_2 \leftrightharpoons BiPh_3 + Bi^*Ph_3Cl_2$$

Ce raisonnement est étayé par la faible valeur de l'énergie d'activation et par les essais négatifs d'échange isotopique entre l'atome de bismuth d'un composé organométallique et des ions Bi^{3+}. Cette dernière assertion est cependant sujette à caution: à partir d'une solution homogène, dans un solvant organique, de $BiPh_3$ et de $BiCl_3$ marqué par ^{210}Bi, il est possible de séparer après 1 h de contact à la température de 40°, un composé dont le point de fusion et les propriétés sont caractéristiques de $BiPh_3$ et qui retient la presque totalité du bismuth radioactif. L'observation de cet

échange isotopique apparent entre un composé organobismuthique et des ions minéraux du métal, nous a incités à examiner les conditions de ce marquage à l'aide de la chromatographie sur papier dont l'emploi s'était déjà révélé fécond dans le précédent travail[1].

PARTIE EXPÉRIMENTALE

Marquage du triphényl-bismuth par ^{210}Bi ou ^{212}Bi

Le triphényl-bismuth, préparé à partir de chlorure de bismuth et de bromure de phényl-magnésium, est recristallisé dans l'alcool jusqu'à ce que son point de fusion soit constant (78°). $BiPh_3$ est soluble dans l'alcool, le benzène, l'éther, mais décomposé par les acides concentrés ($N > 1$). En solution dans l'éther ou le benzène, il réagit avec le chlorure de bismuth en formant du chlorure de diphényl-bismuth, $Bi(C_6H_5)_2Cl$ (ou $BiPh_2Cl$) peu stable, qui précipite. Sous l'action du chlore, $BiPh_3$ est transformé en $BiPh_3Cl_2$, dérivé organométallique du bismuth pentavalent.

$BiPh_3$ est marqué à partir d'une solution homogène de $BiPh_3$ $2 \cdot 10^{-2}$ M et $BiCl_3$ $2.5 \cdot 10^{-4}$ M marqué par ^{210}Bi (RaE, $T = 5$ j) dans l'alcool ou dans le mélange alcool–benzène (1:1), à la température de 40°. Des parties aliquantes de la solution sont prélevées à intervalles réguliers; les phases organique et minérale sont séparées par addition soit de benzène et d'acide nitrique très dilué (pour la solution dans l'alcool), soit d'acide nitrique très dilué (solution dans le mélange alcool–benzène). Dans tous les cas, $BiPh_3$ est extrait dans le benzène, la solution est lavée avec HNO_3 très dilué, le solvanté est séparé par distillation et $BiPh_3$ recristallisé jusqu'à ce que les critères de pureté radiochimique (activité spécifique constante) et chimique (point de fusion 78°) soient atteints. L'introduction de ^{210}Bi dans $BiPh_3$, en fonction du temps, est représentée par la courbe de la Fig. 1. Après 1 h, 97 % de l'activité est retenue sous

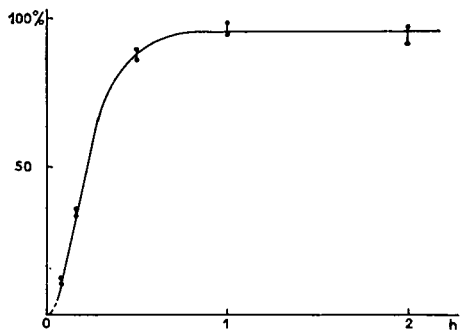

Fig. 1. Fraction de ^{210}Bi incorporée dans $BiPh_3$ en fonction de l'âge d'une solution de $Bi(RaE)Cl_3$ $2.5 \cdot 10^{-4}$ M et de $BiPh_3$ $2 \cdot 10^{-2}$ M dans le mélange alcool–benzène.

forme de $BiPh_3$, dans le mélange alcool–benzène. Le marquage est encore plus rapide en solution alcoolique.

Une expérience similaire a été réalisée avec ^{212}Bi (ThC, $T = 1$ h). A cet effet, $BiCl_3$ est marqué par ^{212}Bi en équilibre de régime avec ^{212}Pb (ThB, $T = 10.6$ h). La présence de ^{212}Pb, générateur de ^{212}Bi, ne gêne en rien la spécificité du marquage de $BiPh_3$, dont l'activité décroît avec la période d' 1 h caractéristique de ^{212}Bi (Fig. 2).

La réalité du *marquage* de $BiPh_3$ (contrairement à un entraînement de bismuth

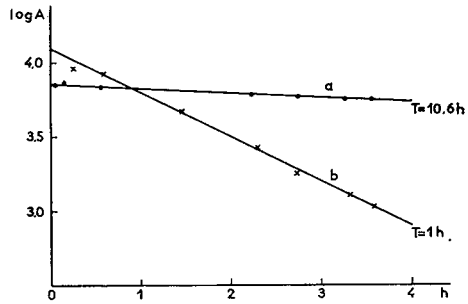

Fig. 2. (a) Évolution de l'activité d'une solution de $BiCl_3$ marqué par ^{212}Bi en équilibre avec ^{212}Pb. (b) Évolution de l'activité de $BiPh_3$ après contact avec la solution précédente.

radioactif par adsorption) prouvée par la constance de l'activité spécifique a encore été vérifiée par la synthèse, à partir de $BiPh_3$ marqué, de $BiPh_3Cl_2$ qui a satisfait aux mêmes critères de pureté chimique et radiochimique, ainsi que par chromatographie sur papier (Fig. 3a et b).

Analyse chromatographique

La séparation d'organobismuthiques par chromatographie ascendante sur papier a été étudiée par NEFEDOV ET GRACHEV[3]; $BiPh_3$ est élué par le benzène et l'éther de pétrole ($R_F = 1$), pour $BiPh_3Cl_2$, les valeurs R_F avec ces solvants sont respectivement 1 et 0. $BiCl_3$ n'est entraîné par aucun solvant ($R_F = 0$).

L'analyse chromatographique ascendante sur papier Whatman No. 1 de la solution âgée d' 1 h, de $BiPh_3$ et Bi^*Cl_3 dans le benzène a été effectuée avec deux solvants: le benzène jusqu'à ce que le déplacement du front de l'éluant ait atteint 7 cm,

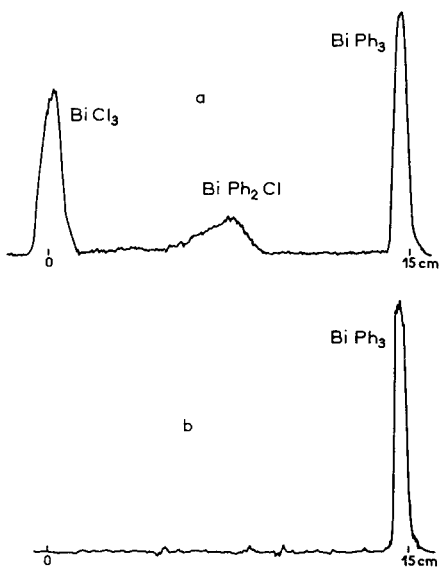

Fig. 3. (a) Analyse radiochromatographique du mélange $BiPh_3$ + $Bi(RaE)Cl_3$. (b) Analyse radiochromatographique après recristallisation de $BiPh_3$ marqué par échange avec $Bi(RaE)Cl_3$.

puis l'éther de pétrole jusqu'à un déplacement total de 15 cm. La mesure de la radioactivité du chromatogramme révèle un pic au point de départ attribué à $BiCl_3$, un pic au front de l'éluant correspondant à $BiPh_3$, ainsi que la présence d'un composé entièrement élué par le benzène, non entraîné par l'éther de pétrole et qui pourrait correspondre à une étape intermédiaire dans le marquage de $BiPh_3$ (Fig. 3a).

Dans les conditions expérimentales du marquage, la formation de $BiPh_2Cl$ est possible:

$$2\ BiPh_3 + BiCl_3 \longrightarrow 3\ BiPh_2Cl \qquad (1)$$

et le composé intermédiaire peut être identifié avec le chlorure de diphényl-bismuth. Effectivement la chromatographie de $BiPh_2Cl$ préparé selon la réaction (1) à partir de $BiPh_3$ ou de $BiCl_3$ marqués, dévoile non seulement que les conditions d'élution sont celles de ce composé intermédiaire, mais révèle aussi l'instabilité de $BiPh_2Cl$ qui est décomposé en cours de chromatographie selon la réaction inverse de (1). Ce n'est qu'en solution suffisamment diluée que le pic de $BiPh_2Cl$ est nettement mis en évidence.

Le radiochromatogramme d'un mélange de $BiPh_3$ et de $BiCl_3$ marqué par ^{36}Cl, obtenu avec le benzène, présente deux pics correspondant à $BiCl_3^*$ ($R_F = 0$) et $BiPh_2Cl^*$ ($R_F = 1$).

Le composé intermédiaire identifié, il est possible de suivre par analyse chromatographique l'évolution de l'activité de $BiCl_3$, $BiPh_3$ et $BiPh_2Cl$ en fonction de l'âge de la solution. La durée relativement longue de la chromatographie au cours de laquelle peuvent se poursuivre des réactions rend le calcul de la cinétique de la réaction aléatoire. Un exemple de la répartition du radio-bismuth entre les trois formes, en fonction de l'âge de la solution, est représenté sur la Fig. 4. Après un marquage initial

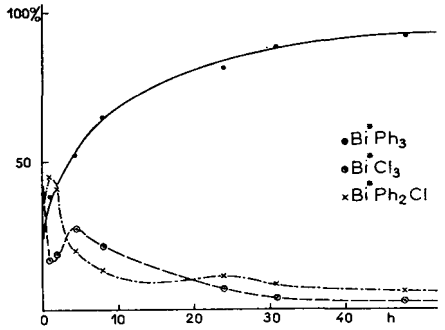

Fig. 4. Répartition du bismuth radioactif en fonction de l'âge d'une solution de $Bi(RaE)Cl_3$ $0.5 \cdot 10^{-3}$ M et $BiPh_3$ $2 \cdot 10^{-2}$ M dans le mélange alcool–benzène.

rapide, l'augmentation de l'activité de $BiPh_3$ est plus lente et tend régulièrement vers un palier, atteint d'autant plus vite que la température est plus élevée. Les activités de $BiCl_3$ et de $BiPh_2Cl$ varient périodiquement et en sens inverse pour atteindre finalement des valeurs très faibles.

A partir de ces résultats, il est possible de proposer le mécanisme de marquage suivant. Dans une première étape, $BiPh_3$ et $BiCl_3$ s'additionnent pour former le chlorure de diphényl-bismuth:

$$2\ BiPh_3 + Bi^*Cl_3 \longrightarrow Bi^*Ph_2Cl + 2\ BiPh_2Cl \qquad (2)$$

C'est la réaction de formation d'un corps instable dans le milieu expérimental, décomposé par l'alcool selon la réaction inverse au cours de laquelle la répartition du bismuth radioactif se fait statistiquement entre $BiPh_3$ et $BiCl_3$:

$$Bi^*Ph_2Cl + 2\ BiPh_2Cl \longrightarrow Bi^*Ph_3 + BiPh_3 + BiCl_3 \quad (3)$$

$$Bi^*Ph_2Cl + 2\ BiPh_2Cl \longrightarrow BiPh_3 + Bi^*Ph_3 + BiCl_3 \quad (4)$$

$$Bi^*Ph_2Cl + 2\ BiPh_2Cl \longrightarrow BiPh_3 + BiPh_3 + Bi^*Cl_3 \quad (5)$$

Les réactions (3), (4) et (5) traduisent la diminution de l'activité du chlorure de diphényl-bismuth formé selon (2) et l'augmentation simultanée de celle du triphényl-bismuth et du chlorure de bismuth. La réaction (6) analogue à (2) regénère le chlorure de diphényl-bismuth:

$$Bi^*Cl_3 + 2\ BiPh_3 \longrightarrow Bi^*Ph_2Cl + 2\ BiPh_2Cl \quad (6)$$

Cependant le radiobismuth passant progressivement dans le triphényl-bismuth, la probabilité de la réaction (7) entre des espèces non marquées s'accroît continuellement au détriment de la réaction (2)

$$2\ BiCl_3 + 4\ BiPh_3 \longrightarrow 6\ BiPh_2Cl \quad (7)$$

Le bilan des réactions (3) à (7) est un échange isotopique apparent selon:

$$2\ Bi^*Ph_2Cl + 2\ BiPh_3 \longrightarrow 2\ Bi^*Ph_3 + 2\ BiPh_2Cl \quad (8)$$

CONCLUSIONS

1. Le triphényl-bismuth est rapidement marqué en système homogène par contact avec du chlorure de radio-bismuth. La réaction est spécifique et permet la séparation de Bi d'autres espèces radioactives (Pb, Hg, Po, Sb).

2. Malgré l'absence de réaction d'échange isotopique entre Bi^{3+} et $BiPh_3$, en présence d'ions Cl^- ou NO_3^- le radio-bismuth se répartit rapidement entre les formes organiques et minérale du bismuth à la suite de réactions chimiques simulant un échange d'atomes de bismuth.

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RÉSUMÉ

Le triphényl-bismuth $Bi(C_6H_5)_3$ a été marqué par contact en milieu homogène avec du chlorure de radio-bismuth. L'analyse chromatographique du mélange $Bi(C_6H_5)_3$–Bi^*Cl_3 a montré que le chlorure de diphényl-bismuth était un composé intermédiaire dans la réaction de marquage.

SUMMARY

In a homogeneous medium, triphenyl-bismuth, $Bi(C_6H_5)_3$, is readily labelled by contact with radioactive bismuth chloride. The labelling reaction has been studied by paper chromatographic analysis of the mixture $Bi(C_6H_5)_3$–Bi^*Cl_3; diphenyl-bismuth chloride was found to be an intermediate species in the reaction.

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Short Communication

Chromogenic activity of carbonyl-substituted resorcinols with Ehrlich reagent

In our previous communication[1] we reported color reactions of 4-alkylresorcinols and some naturally occurring phenols with modified Ehrlich reagent. Study of the former compounds indicated that generally with an increase in alkyl chain a decrease in color reaction was observed. Testing of the latter revealed the usefulness of the reagent for the identification of micro amounts of some natural phenols.

To study possible relationship of color and structure, we have tested* over one hundred phenolic compounds with modified Ehrlich reagent[2]. Herein are reported our results of color reactions of carbonyl-substituted resorcinols.

Among the carbonyl group containing compounds ACHESON *et al.*[3] have examined the three resorcinol carboxylic acids. γ-Resorcylic acid was observed to give pale blue color in an unspecified amount. However, STEELINK[4], MCGEER *et al.*[5] and later ACHESON AND TURNER[6] reported that all of the three carboxylic acids yielded no color reaction. Although no other carbonyl substituted resorcinols were examined, ACHESON AND TURNER[6] concluded that this substituent in general deactivated the benzene ring and thus inhibited the color tests. Phloroglucinolcarboxylic acid and 2,4,6-trihydroxyacetophenone, however, gave a pink color, indicating that additional activation by a phenolic group could mitigate the adverse effect of the carboxyl or other carbonyl group.

Preliminary work performed by us[1] on some carbonyl substituted resorcinols appeared to confirm the finding of the previous workers[4–6] that the presence of a carbonyl group in resorcinol inhibited the color reaction. However, only some 3,5-dihydroxy- and 2,4-dihydroxy-carbonyl group containing compounds were then tested. Subsequent study of 2,6-dihydroxy-carbonyl phenols and comparative tests on all the three types of compounds showed varying and noteworthy results.

Testing technique

For studying color reactions we preferred to use spot tests on paper rather than the paper chromatographic technique employed by the previous workers[3–5]. Generally six different amounts, *viz.* 1,2,5,10, 50 and 100 μg, of each compound were spotted on a Whatman No. 1 filter paper strip. Two λ of the modified Ehrlich reagent (0.5 g *p*-dimethylaminobenzaldehyde in 90 ml absolute ethyl alcohol, 2.8 ml concentrated hydrochloric acid added and volume made to 100 ml with absolute alcohol) was applied on a 3–4 mm diameter spot of each compound. The paper was placed in an oven for one minute at 100°C.

* These results were recently presented to the Annual Meeting of the American Pharmacognosy Society, held at Chapel Hill, North Carolina (U.S.A.), in July 1963. Abstract of the paper is published[2].

In case of some compounds that were colored or found to yield light coloration on heating, a blank spot of each concentration of the test substance was made. Reagent was not applied on this spot. Two λ of the reagent was also separately spotted. After comparing the colors of the separate test compound and reagent controls with the color of these combined, differences were recorded. The color recorded was of the lowest amount of the test compound yielding a noticeable coloration.

TABLE I

COLOR REACTIONS OF CARBONYL SUBSTITUTED RESORCINOLS WITH EHRLICH REAGENT

No.	*Compound*	*Color*	*Amount of test compound* (μg)*
1	2,4-Dihydroxybenzaldehyde	Light pink	50
2	2,4-Dihydroxybenzamide	Light yellow	5
3	2,4-Dihydroxybenzoic acid	Light pink	50
4	2,4-Dihydroxyacetophenone	Light violet	50
5	2,4-Dihydroxypropiophenone	Light pink	100
6	2,4-Dihydroxybenzophenone	Orange-brown	100
7	2,4-Dihydroxybenzaldoxime	Yellow-brown	50
8	2,6-Dihydroxyacetophenone	Pink	1
9	2,6-Dihydroxybenzamide	Pink	1
10	2,6-Dihydroxybenzoic acid	Light violet	1
11	Methyl 2,6-dihydroxybenzoate	Light pink	1
12	3,5-Dihydroxybenzamide	Light yellow	50
13	3,5-Dihydroxybenzoic acid	Light yellow	100
14	Methyl 3,5-dihydroxybenzoate	Light yellow-gray	100
15	3,5-Dihydroxy-4-methylbenzoic acid	Pale yellow**	100

* Spot tests on paper on six different quantities, 1, 2, 5, 10, 50 and 100 μg, of each compound were performed and the lowest amount of the compound that gave noticeable color reaction is recorded.

** This color could not be distinctly differentiated from the reagent blank and might be considered as doubtful.

Color reactions of carbonyl substituted resorcinols

Resorcinol derivatives with electron withdrawing carbonyl substituents such as aldehyde, amide, carbomethoxyl, carboxyl, and ketone functions were examined. The carbonyl substituted phenols with hydroxyl groups in 2,4-, 2,6-, and 3,5- were tested. Color reactions of the spot test are listed in Table I.

The results (Table I) reveal the varying chromogenic activity of the three types of carbonyl substituted resorcinols. 2,6-Dihydroxy-carbonyl containing phenols show the most sensitive color reaction and pink to violet coloration is noticeable even in an amount of 1 μg. The compounds in which hydroxyl groups are in 3 and 5 positions show the least sensitive test and the colored products are yellowish. The variation of chromogenic activity of the three types of carbonyl substituted phenols to yield coloration with modified Ehrlich reagent can be summarized as follows:

2,6-dihydroxy- > 2,4-dihydroxy- > 3,5-dihydroxy-

The usefulness of the reagent in the identification of 2,6-dihydroxy-carbonyl substituted phenols is also indicated from the results (Table I).

Color reactions of carbonyl substituted trihydroxyphenols

To study the compensating effect of an additional hydroxyl group comparative examination of trihydroxy-carbonyl substituted compounds has also been made. Several carbonyl substituted trihydroxyphenols with hydroxyl groups in 2,3,4-, 2,4,5-, 2,4,6-, and 3,4,5- have been tested. It was found that sensitive pink-violet color reactions were observed only in the case of carbonyl containing phloroglucinols. This indicated that the location of the phenolic hydroxyl group was important in mitigating the adverse effect of the carbonyl group. More details on trihydroxy carbonyl substituted compounds and results on many other phenols tested will be published.

Color reactions of 5,7-dihydroxyflavans and related compounds

The difference in chromogenic activity of some carbonyl group containing resorcinol derivatives as against those that do not contain such deactivating function can be used to distinguish natural phenols such as 5,7-dihydroxyflavans and related compounds that have resorcinol moiety in their structure. To determine this we have tested some more such compounds than previously reported[1]. For comparison results of color tests on all these are recorded in Table II.

TABLE II

COLOR REACTIONS OF 5,7-DIHYDROXYFLAVANS AND RELATED COMPOUNDS WITH EHRLICH REAGENT

No.	*Compound*	*Colour*	*Amount of test compound* (μg)*
1	D-Catechin**	Violet	1
2	Chrysin	Light yellow	10
3	Cyanidin chloride**	Pink	1
4	Epicatechin**	Violet	1
5	Fisetin	Yellow-orange	2
6	Genistein	Orange	50
7	Hesperetin	Orange-yellow	5
8	Hesperidin methyl chalcone	Light yellow	10
9	Kaempferol	Light yellow	10
10	Malvidin chloride**	Pink	1
11	Morin	Yellow-green	5
12	Naringenin	Orange-yellow	5
13	Quercetin	Yellow	5
14	Quercitrin	Yellow	5
15	Rhamnetin	Yellow	10
16	Rutin	Yellow	10

* Amount spotted and color observations were made similarly as given in footnote* under Table I.

** The lowest amount that could be detected was 0.1–0.2 μg.

It is indicated from the results (Table II) that the modified Ehrlich reagent is valuable in identifying 5,7-dihydroxyflavans and related compounds which do not have a carbonyl group in 4 position. This reagent gives pink to violet color with such compounds (*e.g.* catechin, epicatechin, cyanidin chloride, and malvidin chloride). Anthocyanidins (such as cyanidin and malvidin) are colored flavan derivatives as compared to catechins which are colorless. The two classes of compounds after detection with our reagent can be additionally distinguished from each other.

In connection with tests on catechin and epicatechin it might be indicated that this type of compounds can also be detected in crude extract of such plants as tea. A spot test on an infusion of tea, which is known to contain several catechins, shows a distinct pink coloration suggesting their presence.

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Separation of saturated and unsaturated fatty acid esters of cholesterol by gas–liquid chromatography

While several procedures for the separation of sterols and their derivatives by gas–liquid chromatography (GLC) have been published[1], the separation of long-chain fatty acid esters of cholesterol has not been specifically reported. This communication deals with the separation of cholesteryl palmitate, stearate and oleate, prepared by trans-esterification of cholesteryl acetate and the appropriate fatty acid ester using sodium methoxide[2].

GLC analysis was carried out on an F & M model 500 temperature-programmed gas chromatographic unit with flame ionization attachment. Of several supports and stationary phases examined under a variety of conditions, the system here described gave the best separation. A 4-ft. spiral stainless steel column (0.3 in. diam.) was packed with 60/70 mesh Anakrome ABS (an acid-washed, base-washed and silicone-treated flux-calcined diatomaceous earth) coated with 2 % SE 30 (silicone rubber gum). The temperatures at the injection port and detector block were 320° and 350° respectively. Flow rates for air, helium and hydrogen were 400, 100 and 30 ml/min respectively. A mixture of cholesteryl palmitate, stearate and oleate (5 mg each) was made up to 0.3 ml with chloroform and a sample of 1–3 μl was injected. Attenuation was kept at 800. The column was programmed from 200–340° at 3°/min.

Cholesteryl palmitate was eluted at 270° and cholesteryl stearate and oleate in a single peak at 290°. The mixture of these three esters (*ca.* 20 mg) was oxidized with

permanganate–periodate[3] at room temperature for 2 h, whereby the ethylenic linkage of oleate is attacked, and the resulting free carboxyl group was esterified with diazomethane. Chromatography of this mixture under the same conditions gave an additional peak at 240° for cholesteryl azelate, while that at 290° diminished in area. Since azelate arises on oxidation of all the common unsaturated esters, oleate, linoleate and linolenate, these will be estimated together as cholesteryl azelate. A method for distinguishing these esters by preliminary separation on a silicic acid–silver nitrate column as described by DE VRIES[4] is being worked out.

Separation by GLC of serum lipids, extracted by BLOOR's method and esterified with diazomethane, was examined. Temperature programming was carried out from 100–330°. The free fatty acid esters, mono- and di-glycerides, cholesterol, cholesteryl palmitate, cholesteryl stearate/oleate and triglycerides separated into distinct peaks. Quantitative aspects will be published elsewhere.

This work was carried out during the tenure of a post-doctorate fellowship of the National Research Council of Canada. I wish to express my sincere thanks to Mr. D. A. MCPHEE for technical assistance.

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Notes

Chromatography on ion-exchange papers

XV. The adsorption of metal ions on cation exchangers from perchloric acid solutions

In a recent paper NELSON *et al.*[1] report the adsorption of metal ions on Dowex-50 from HCl and $HClO_4$ solutions and noted a deviation from "ideal" exchange consisting of a general trend towards increased adsorption at higher concentrations of $HClO_4$. In previous papers[2] we have compared cellulose anion exchangers with anion resin papers and thus could show the extent to which the network influences adsorption. We hence thought that it would be interesting to apply the same comparative technique to cation exchangers and the perchloric acid system as this non-ideal behaviour in concentrated $HClO_4$ has not been adequately explained so far. The

comparison in this case was made between a cellulose sulphonic acid exchanger and a resin sulphonic acid exchanger. The first is marketed now (by Macherey, Nagel & Co.) in the form of paper sheets (capacity 0.4–0.7 mequiv.).

Furthermore no data for inorganic ions have so far been published for the cellulose sulphonate paper. Such data are, of course, of more interest if reported in comparison with the sulphonic resin papers under identical conditions.

Experimental

The following papers were employed:

Amberlite SA-2 paper (containing 45 % of Amberlite IR-120, a nuclear sulphonic resin on a styrene base), washed twice with 2 *N* HCl and distilled water for 30 min and air dried.

MN-Ionenaustauscherpapier (stark sauer), a cellulose sulphonic acid exchanger (capacity 0.4–0.7 mequiv./g) washed twice with 2 *N* HCl and distilled water for 30 min and air dried.

Whatman No. 3MM cellulose paper.

Ascending development of strips about 20 cm long in small volume jars was employed throughout, always chromatographing all three papers simultaneously.

The perchloric acid used was reagent quality (Carlo Erba, Milan) and all dilutions were prepared from 70 % $HClO_4$.

The metal ions were placed on the paper as solutions of the nitrates in 2 *N* $HClO_4$.

Fig. 1 shows the R_F values of 20 metal ions obtained at a room temperature of 20° ± 2°.

Discussion

The lowering of R_F values of some metal ions on the resin paper is identical to that observed by NELSON *et al.*[1] in column experiments. The same decrease of R_F values on the cellulose sulphonic acid at higher $HClO_4$ concentrations occurs also with Th(IV), U(VI), La(III), Y(III) and Sc(III) while these are not adsorbed by pure cellulose (Whatman No. 3MM paper). The possibility of adsorption on the network causing this "non-ideal" behaviour must hence be ruled out for these ions.

On the other hand quite considerable adsorption on pure cellulose may be observed with Pb(II), Bi(III), Ag(I) and Tl(I) and here the decrease of the R_F values on the cellulose sulphonic exchanger is of the same order of magnitude as the adsorption; thus in some instances adsorption also plays a role.

The lowering of R_F values for most of the divalent transition metals seems to be less than that observed by NELSON *et al.*[1]. However, owing to the fragility of the paper we could not work with concentrations higher than 6 *N* $HClO_4$, which is the region where the increased adsorption seems to commence. However, the R_F values of Ga(III) do not decrease at higher $HClO_4$ concentrations, although a minimum around 3 *N* $HClO_4$ was obtained by NELSON *et al.*[1]. It seems that Amberlite IR-120 (in the papers employed here) and Dowex-50 are not strictly comparable for this phenomenon.

Zr(IV) shows some hydrolysis at lower concentrations of $HClO_4$, which causes it to adsorb (or precipitate) on pure cellulose, and should therefore be omitted from any considerations of this mechanism.

Finally we would like to draw attention to the fact that the deviation from

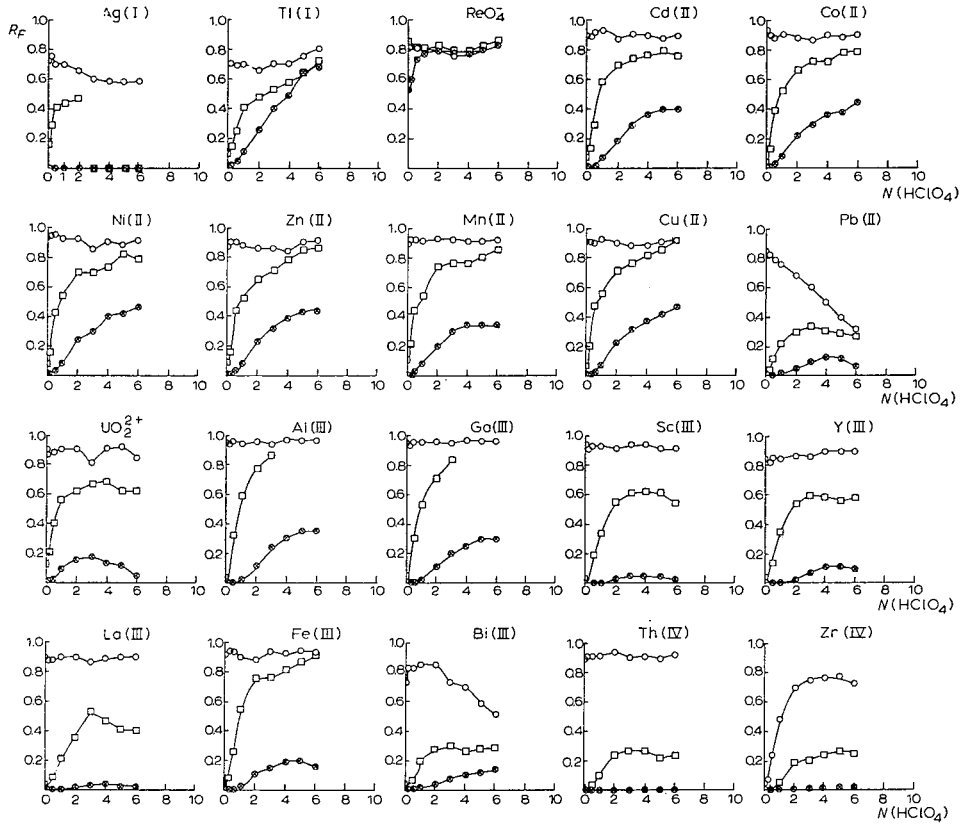

Fig. 1. R_F values of metal ions plotted against the concentration of $HClO_4$ for the following papers: ⊙—⊙ Whatman No. 3MM cellulose paper; ⊡—⊡ Macherey, Nagel & Co. stark saures Ionenaustauscherpapier (containing sulphonic acid groups attached to the cellulose); ⊗—⊗ sulphonic acid resin paper (SA-2).

"ideal" behaviour occurs in the region where the conductivity of $HClO_4$, reaches a maximum. We seem to reach here the gradual dehydration of $HClO_4$, which may first induce the metal ion to dehydrate itself and hence adsorb more strongly on the resin.

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An anomalous chromatographic behaviour of some di- and trinitrophenols

In a previous communication[1] a paper chromatographic method for the separation and identification of variously substituted mono-, di- and trinitrophenols has been described. Recently we have had the opportunity to chromatograph some further derivatives and have found that some di- and trinitro-derivatives of alkylphenols showed an anomalous behaviour.

Experimental

Materials. The preparation of 4,6-dinitro-2,5-xylenol has been described in a previous communication[2]. Its identity was confirmed by melting point determination and elementary analysis. 4,6-Dinitro-3,5-xylenol was prepared by nitration according to ROWE *et al.*[3] and had a m.p. of 114–115° (according to ref. 3, 115–116°). 4,6-Dinitro-2,3-xylenol was prepared by nitration of 2,3-xylenol with nitrous acid[2] and had a m.p. of 83.5–84° (according to ref. 4, 86°). 4,6-Dinitro-2-*tert.*-butyl-5-methylphenol was prepared according to the literature[5] and had a m.p. of 106–107° (according to ref. 5, 107–108°). Other nitrophenols have been described in previous communications[1,2].

Paper chromatography. Paper chromatography was carried out in the same manner as previously[1], *i.e.* use was made of the solvent system 1-bromonaphthalene–80 % acetic acid.

Absorption spectra. Absorption spectra were determined in the same manner as described previously[6].

Results and discussion

In the previous communication[1] we calculated the ΔR_M values for the individual functional groups, *e.g.* for the methyl group *meta* to the hydroxygroup the ΔR_M value was + 0.23, for the methyl group *ortho* to the hydroxygroup + 0.33.

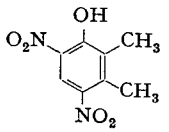
In Table I the calculated and observed R_F values of the newly chromatographed

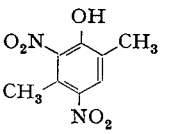
TABLE I

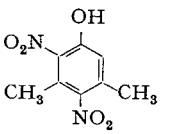
OBSERVED AND CALCULATED R_F VALUES OF SOME NITROPHENOLS

Nitrophenol	R_F *observed*	R_F *calculated*
4,6-Dinitrophenol	0.39	—
4,6-Dinitro-*o*-cresol	0.20	0.23
4,6-Dinitro-*m*-cresol	0.28	0.27
4,6-Dinitro-2,3-xylenol	0.12	0.15
4,6-Dinitro-2,5-xylenol	0.59	0.15!
4,6-Dinitro-3,5-xylenol	0.67	0.12!
4,6-Dinitro-2-*tert.*-butylphenol	0.10	—
4,6-Dinitro-2-*tert.*-butyl-5-methylphenol	0.32	0.06!
4-Nitro-2,5-xylenol	0.76	—
2,4,6-Trinitrophenol	0.55	—
2,4,6-Trinitro-*m*-cresol	0.39	0.42
2,4,6-Trinitro-3,5-xylenol	0.56	0.30!

nitrophenols are summarized. At first sight, in the case of 4,6-dinitrophenols, the calculated R_F values of *o*- and *m*-cresol and 2,3-xylenol derivatives show a satisfactory agreement with the observed values. In the case of the dinitro-derivatives of 2,5- and 3,5-xylenols, however, they differ considerably. As expected, in comparison with 4,6-dinitrophenol the corresponding derivatives of *o*- and *m*-cresol have lower R_F values, the derivative of 2,3-xylenol (I) a still lower value and the derivative of 2-*tert.*-butylphenol the lowest R_F value. Rather surprisingly the derivatives of 2,5- and 3,5-xylenols (II and III) have R_F values greater than the unsubstituted 4,6-dinitrophenol. Their behaviour on the chromatogram is very similar to that of *p*-nitrophenols. The R_F value of one corresponding *p*-nitrophenol is, for comparison, recorded in Table I. The behaviour of 4,6-dinitro-2-*tert.*-butyl-*m*-cresol is analogous. This compound has a higher R_F value than 4,6-dinitro-2-*tert.*-butylphenol, though owing to the presence of the methyl group it should have a lower R_F value.


(I)


(II)


(III)

From the example mentioned it can be deduced that the behaviour of dinitrophenols is anomalous where a methyl group is present in the position between the two nitro groups. When studying the molecular model we found that, in this case, all the three substituents are no longer coplanar with the benzene nucleus and that one of the nitro groups is forced out of coplanarity. 4,6-Dinitro-3,5-xylenol has a greater R_F value than 4,6-dinitro-2,5-xylenol. The difference in their R_F values corresponds to that of the dinitro-derivatives of *o*- and *m*-cresol. Because the anomalous dinitrophenols behave on the chromatogram like *p*-nitrophenols, it may be deduced that the nitro group forced out of coplanarity is that in the *ortho*-position. This, of course, essentially changes the properties and the character of the original molecule, resulting also in changes in chromatographic behaviour. Assuming that displacement of the *o*-nitro group from coplanarity could also affect the hydrogen bonding between the hydroxy and nitro groups, higher R_F values of the anomalously behaving phenols would be obtained. In the case of 2,4,6-trinitrophenols the same anomalous behaviour is shown only in the presence of two methyl groups in the molecules. In 2,4,6-trinitro-*m*-cresol one *o*-nitro group still remains for the hydrogen bonding and therefore this compound does not behave anomalously.

In connection with other work[6] we determined the absorption spectra of those compounds in their yellow alkaline solutions. All 4,6-dinitrophenols have characteristic spectra with 2 maxima at *ca.* 375–380 and 400–420 mμ, whereas *p*-nitrophenols show only 1 maximum at 408–415 mμ (Fig. 1). The spectra of both nitrophenols showing the anomalous chromatographic behaviour are very similar to that of *p*-nitrophenol. In this case, probably, the non-coplanarity has some influence on the electronic states of the molecules. Similar effects of the alkyl groups on the absorption spectra of trinitrobenzenes have been ascribed recently to steric inhibition of resonance and steric enhancement of resonance[7]. Although all these phenomena cannot be directly compared it is evident that in all cases the steric effects play the main role.

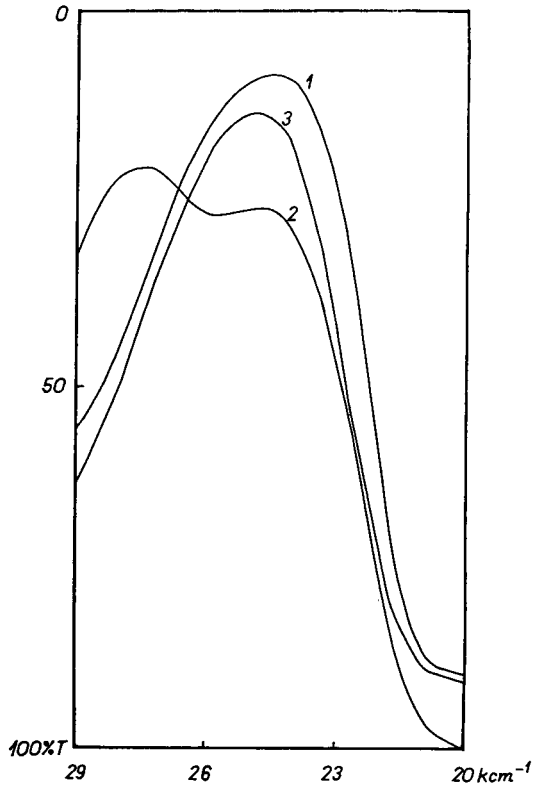


Fig. 1. Absorption spectra of the nitrophenols in ethanolic ammonia. (1) 4-Nitrophenol; (2) 4,6-dinitrophenol; (3) 4,6-dinitro-2,5-xylenol.

These facts illustrate that steric factors may have a considerable effect on the chromatographic behaviour of organic compounds at least if they interfere with the formation of hydrogen bonds. In the case of small molecules with many and/or bulky substituents care must therefore be taken when calculating the theoretical R_F values from the ΔR_M data. Attention should also be paid to possible interactions of those groups and their effect on the character of the whole molecule.

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A simple uni-dimensional separation of nucleosides

In work connected with the analysis of nucleosides in pharmaceutical preparations we required a fast separation of adenosine, cytidine, guanosine, inosine and uridine from each other for purposes of subsequent quantitative determination.

On searching the literature twenty-one different separations were found using mainly buffered or alkaline butanol or isoamyl alcohol as partition systems or aqueous ammonia or buffers for adsorption chromatography on paper. However, none of the published separations gave good R_F differences for all five nucleosides, with the possible exception of when piperidine[1] was used, but no R_F value was quoted for inosine in this case.

As we wanted a rather fast separation, it was decided to try to improve on the adsorption systems already published. The direction in which this search was oriented was given by previous work of this laboratory[2], which had indicated that cellulose ion exchangers can function as adsorbents as well as exchangers and that the adsorbtive properties depended on the type and the polarity of the substituents chosen to confer ion exchange properties to the cellulose.

The R_F values of the five nucleosides on a number of ion exchange papers are given in Table I. Excellent separations with five well-defined spots were obtained with diethylaminoethylcellulose paper (Whatman DE-20) using water as solvent as shown in Fig. 1. The nucleosides are visible as dark spots under ultraviolet light. If placed on the paper in dilute HCl a light spot due to chloride ions is noted near the origin.

Resin-impregnated papers retained all the nucleosides strongly, with the exception of the weak base WB-2 paper which, however, did not separate all five nucleosides.

TABLE I

R_F VALUES OF NUCLEOSIDES ON CELLULOSE ION EXCHANGE PAPERS

Paper	*Solvent*	*Adenosine*	*Cytidine*	*Guanosine*	*Inosine*	*Uridine*
Whatman 3 MM (pure cellulose)	Water	0.48	0.32	0.52	0.70	0.77
Whatman AE 30 (aminoethyl-cellulose)	Water	0.61	0.79	0.18	0.15	0.42
Whatman DE 20 (diethylaminoethyl-cellulose)	Water	0.57	0.80	0.20	0.11	0.37
Macherey-Nagel strongly basic anion exchange paper (with quaternary ammonium groups)	Water	0.90	0.92	0.40	0.74	0.68
Whatman CM 50 paper (carboxymethyl-cellulose)	Water	0.64	0.68	0.67	0.86	0.90
Whatman CT 30 paper (cellulose citrate)	Water	0.13	0.15	0.40	0.70	0.85
Whatman P 20 paper (cellulose phosphate)	Water	0.02	0.02	0.04	0.34	0.84
Macherey-Nagel strongly acidic cation exchange paper (with sulphonic groups)	Water	0.15	0.14	0.22	0.46	0.68
Whatman DE 20	0.5 *N* HCl	all spots on the solvent front				
Whatman DE 20	0.5 *N* NH_4OH	0.52	0.77	0.28	0.35	0.69

We believe that the approach used here to achieve a paper chromatographic separation is novel in one respect, namely that usually the solvent mixture is altered to achieve suitable conditions while here we have altered the nature of the support,

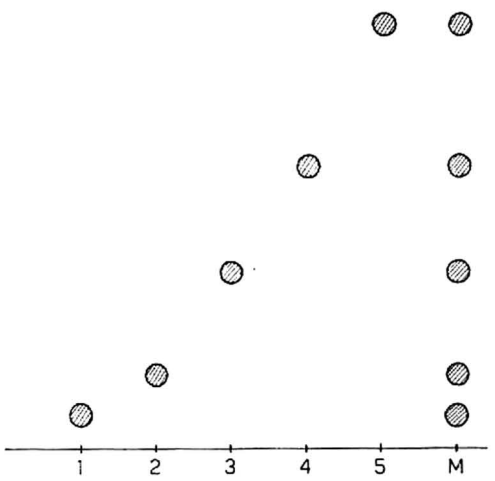

Fig. 1. Chromatogram (schematic) of nucleosides on Whatman DE 20 paper. (1) Inosine; (2) guanosine; (3) uridine; (4) adenosine; (5) cytidine; (M) a mixture of all five compounds.

rather in analogy to gas chromatographic procedures where the stationary phase is usually changed instead of the less important carrier gas.

Work is still in progress for adopting a quantitative procedure after separation and this will be published later.

This work was carried out in the Laboratorio di Cromatografia del C.N.R., Istituto di Chimica, Rome.

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Die Isolierung der 3-Methoxy-4-hydroxy-mandelsäure aus dem Urin unter Verwendung der Keilstreifenmethode

Zur Auftrennung der Phenolsäuren in Urinextrakten wurden bisher neben anderen Trennverfahren[1–5] in der Hauptsache die zweidimensionale Papierchromatographie[6–8] beschrieben. Eine gute Auftrennung der Extrakte zur quantitativen Bestimmung der 3-Methoxy-4-hydroxy-mandelsäure (Vanilmandelic acid, VMA) erzielten wir mit einer eindimensionalen, absteigenden Papierchromatographie unter Verwendung der Keilstreifenmethode nach MATTHIAS[9]. Als Laufmittel diente die organische Phase

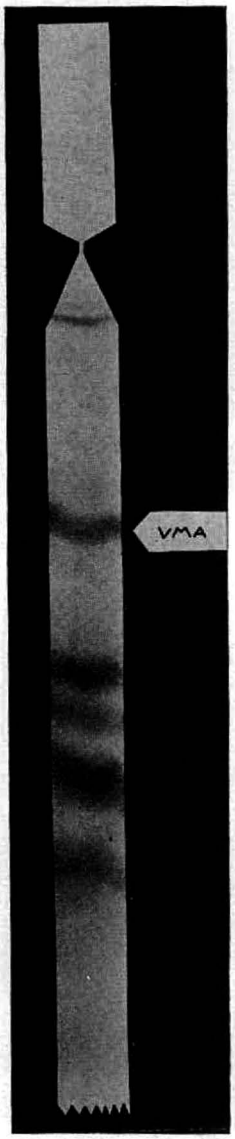

Fig. 1.

des Systems[10] *n*-Butanol-*n*-Butylacetat–10 %ige Essigsäure (2:8:10). Zur Chromatographie wurden die Papiere Nr. 2045 bM (Schleicher und Schüll) und FN6 (VEB Spezialpapierfabrik Niederschlag/Erzgebirge) mit gleichgutem Erfolg verwendet (Fig. 1). Die Laufzeit beträgt 15 Stunden. Beim Besprühen mit 10 %iger Na_2CO_3-Lösung und diazotiertem *p*-Nitranilin[6] erscheint die VMA als blauvioletter, beständiger Diazofarbstoff. Die Lokalisation der Säure erfolgt durch Mitführung eines inneren Standards. Dass die in Frage kommende Zone nur aus dem VMA-Diazofarbstoff besteht, wurde wie folgt gesichert:

Der Urinextrakt wird zuerst im System *n*-Butanol–*n*-Butylacetat–10 %ige Essigsäure (2:8:10) chromatographiert (Keilstreifen, absteigend). Die Lage der VMA-Zone wird an Hand eines Vergleichschromatogramms reiner VMA bestimmt, ausgeschnitten und mit Methanol eluiert. Der Methanolextrakt wird im System[6] Isopropanol–25 % iges Ammoniak–H_2O (8:1:1) absteigend chromatographiert (Keilstreifen). Nach Anfärbung mit diazotiertem *p*-Nitranilin zeigt sich nur eine Zone. Aus einem Parallelstreifen wird die VMA ohne vorangegangene Farbreaktion eluiert und erneut im System Propionsäure–Benzol–H_2O (100:75:5) absteigend chromatographiert (Keilstreifen). Nach der Farbreaktion mit diazotiertem *p*-Nitranilin ist auch hier nur eine Zone zu erkennen.

Die Versuche zeigen, dass bereits die erste Chromatographie zur Abtrennung der VMA von anderen Phenolsäuren des menschlichen Urins ausreicht. Bei einer guten Trennwirkung ist der Zeitaufwand der Methode relativ gering. Über die quantitative Bestimmung weiterer aromatischer Säuren im Urin nach diesem Verfahren hoffen wir später berichten zu können.

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Inexpensive adsorbents for thin-layer chromatography of carbohydrates

The usefulness of thin-layer chromatography (TLC) as a rapid and sensitive analytical technique has been firmly established. However, reports of separations of carbohydrate compounds by TLC have been relatively few compared with other classes of materials. STAHL AND KALTENBACH[1] and PASTUSKA[2] obtained resolution of monosaccharides on Kieselguhr G (Merck) and Silica Gel G (Merck) but oligosaccharides were poorly resolved by the solvent systems employed. Later WEILL AND HANKE[3] demonstrated excellent separations of glucose, maltose, and malto-oligosaccharides on Kieselguhr G (Merck) using combinations of *n*-butanol–pyridine–water as developing solvents. The only disadvantage of this system was that monosaccharide materials followed the solvent front.

Characteristic of the above publications is the use of the adsorbents Kieselguhr G and Silica G for all of the studies. Some of the drawbacks to the use of these materials have been noted above but in addition they are relatively expensive. We have found ordinary celite filter aid materials to be excellent adsorbents for the separation of carbohydrates by TLC and in some instances to give better resolution than that obtained with more expensive adsorbents.

Experimental

Apparatus. The thin-layer chromatography assembly manufactured by Research Specialties Company employing a fixed thickness spreader was used for preparation of the chromatoplates. Borosilicate glass plates (8 in. × 8 in.), were employed for all analyses. Battery jars or large glass cylinders 9 in. in diameter with glass covers were used as development chambers.

Materials. Solutions of anhydrous dextrose (National Bureau of Standards), maltose hydrate (Pfanstiehl Co. C.P.) and xylose, C.P. were prepared by dissolving 100 mg of each compound in distilled water and diluting to 10 ml.

Isomaltose and panose were prepared from maltose by the action of the enzyme, transglucosidase, present in fermentation broths of *Aspergillus niger* cultures according to the procedure of PAZUR AND ANDO[4]. The resulting hydrolysis mixture containing glucose, maltose, isomaltose, panose and minor unknown components was diluted with 2.5 volumes of distilled water prior to spotting the solution on the adsorbent.

n-Butanol, b.p. 116–118° and pyridine, b.p. 115–116° were used in developing solvents.

Adsorbents used were Kieselguhr G (Merck) obtained from Brinkman Instruments, Inc. and Johns-Manville's Hyflo Super-Cel and Filter-Cel.

Silver nitrate solution: 5 g of $AgNO_3$ dissolved in 95 ml of water and 6 ml of concentrated ammonium hydroxide.

Calcium sulfate, $CaSO_4 \cdot 0.5\ H_2O$.

Procedure. Thin-layer chromatoplates of the various Johns-Manville filter aid materials were prepared as follows: 0.8 g of $CaSO_4 \cdot 0.5\ H_2O$ was placed in a mortar, covered with 5 ml of distilled water and ground with a pestle for one minute. Fifteen grams of filter aid and 60 ml of distilled water was added and the mixture ground for an additional 1 to 2 min. The smooth slurry was poured into the spreader and the

prepared immediately. Five 8 in. × 8 in. plates could be obtained in this manner. After air drying 10–15 min the plates were dried at 100° for at least 30 min.

The Kieselguhr G plates were prepared in a similar fashion except no calcium sulfate was added and 20 g of adsorbent to 40 ml of water was employed.

Two μl of the solutions described above were placed 1 in. apart and 1 in. from the bottom of the adsorbents with the aid of a lambda pipette so that the solvent travelled either opposite or perpendicular to the direction of the spreader.

A soft stream of warm air from a hair dryer was directed at the spot to hasten evaporation of sample and keep the size of the spot to $^1/_4$ in.

The solvent front was allowed to proceed 8–10 cm and the development time was 30–80 min depending upon the particular adsorbent. The solvent employed was *n*-butanol–pyridine–water (75:15:10).

After development the plate was air-dried in a hood for 15–20 min and sprayed lightly with ammoniacal silver nitrate. Color development was carried out by heating 5 min at 100°.

Results

The R_F-values of the reference compounds employed and of the products obtained from the enzymatic digest of maltose are shown in Table I.

Discussion

Of the adsorbents employed Filter-Cel and combinations of Filter-Cel with Hyflo Super-Cel provided better resolution of monosaccharides than did Hyflo Super-Cel alone or Kieselguhr G. In the case of the latter two adsorbents glucose and xylose closely followed the solvent front and migrated as a single spot.

The substances detected in the enzymatic digest of maltose were also better resolved on Filter-Cel and formed more compact well defined spots than those obtained on the other adsorbents. However, solvent migration is considerably slower with Filter-Cel. Glucose and maltose in the enzyme digest material were identified by comparison of R_F values of reference compounds and by the rate of disappearance of maltose and the simultaneous formation of glucose due to the glucosidase activity of the enzyme. Isomaltose and panose have been previously identified by PAN, ANDREASEN AND KOLACHOV[5] and PAZUR AND ANDO[4] as the two major synthetic products resulting from the action of transglucosidase activity present in *Aspergillus niger* cultures on maltose. Although isomaltose and panose were not available to use as reference materials, the two major spots migrating below maltose were tentatively designated as isomaltose and panose since these were the only two other major spots detected in addition to glucose and maltose. The other unknown substances detected in trace quantities in the enzyme digest were not characterized but did separate as well defined spots. Their origin appeared to have been impurities in the maltose and enzyme materials employed.

Occasionally, specks of unground calcium sulfate remained in the slurry and tended to cause streaks during preparation of the filter aid plates. Although this was not a serious drawback it could usually be avoided by thorough mixing of the materials.

Thin-layer plates of Filter-Cel and Kieselguhr G could also be conveniently prepared by the spray technique described by BEKERSHY[6] which worked satisfac-

torily in our solvent systems. However, Hyflo Super-Cel due to its larger particle size tended to plug the nozzle of the chromatography spray bottle.

TABLE I

R_F VALUES OF REFERENCE COMPOUNDS AND PRODUCTS FROM ENZYMATIC DIGEST OF MALTOSE

	Adsorbent			
	Filter-Cel	*Hyflo-Super-Cel*	*Hyflo Super-Cel: Filter-Cel (6: 4)*	*Kieselguhr G*
Maltose digest				
Unknown	0.76	—	0.94	—
Glucose	0.67	0.94	0.76	0.96
Maltose	0.48	0.84	0.51	0.83
Isomaltose*	0.25	0.71	0.35	0.71
Panose*	0.15	0.50	0.16	0.55
Unknown	0.06	0.32	—	0.28
Unknown	0.00	0.00	0.00	0.00
Reference compounds				
Xylose	0.85	0.94	0.90	0.95
Glucose	0.67	0.94	0.76	0.95
Maltose	0.48	0.84	0.51	0.83
Development time (min)	80	30	50	50

* Tentative identification.

Conclusions

As a result of our work to date we have found the availability, low cost and high resolving power of ordinary filter aid materials make them excellent adsorbents for thin layer chromatography of carbohydrates. It is hoped similar results can be obtained with other classes of compounds.

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A method for the gas chromatographic separation of estrogens employing a solid injection system*

In recent communications from our laboratory, the gas chromatographic separation of synthetic mixtures of estrogens as well as their isolation from biological material

* This investigation was supported in part by a research grant from The Heart Association of Southeastern Pennsylvania and the American Medical Association Education and Research Foundation.

have been reported[1,2]. In all of the reported investigations injections were carried out with the compounds dissolved in solvent.

A method employed in our laboratory for the gas chromatographic separation of a synthetic mixture of estrone, 17β-estradiol and estriol in the absence of solvent has been developed. The ability to separate the estrogens in the absence of a solvent would be extremely helpful in handling biological samples.

Experimental

An aliquot of redistilled dioxan is drawn up into a 10 μl microsyringe (Hamilton Syringe Co., Whittier, Calif.). Immediately afterwards an equivalent aliquot of a synthetic mixture of estrone, 17β-estradiol and estriol is drawn up into the same microsyringe. The contents of the microsyringe are then placed on a solid injection syringe (Hamilton Syringe Co., Whittier, Calif.). A domestic hair dryer is employed to facilitate the transfer and initially evaporate the solvent. The syringe is then placed in a 101° drying oven for 20 min to ensure the complete evaporation of the solvent.

The sample is introduced, employing the solid injector, into a Barber-Colman, model 10, gas chromatograph equipped with a ^{90}Sr ionization detector.

A 6 ft. long, "U" shaped, pyrex glass column with an internal diameter of 5 mm was packed with a stationary phase of 3% QF-1 (fluorosilicone) on 100/120 Gas Chrom-P (Applied Science Laboratories, Inc., State College, Pa.). The temperature of the column was maintained at 252°, the detector at 274° and the "flash heater" at 319°. Argon was employed as the carrier gas and a flow rate of 88 ml/min was maintained. The column was previously conditioned, while vented to the air at 255° and at the operating pressure of the carrier gas for 24 h.

Results and discussions

In Fig. 1 may be observed the gas chromatographic separation of the estrogens following their introduction into the instrument in solvent. In Fig. 2 may be seen the gas chromatographic separation of the same compounds following their introduction with a solid injection system. It may be readily observed that the peak representing the solvent has been eliminated.

The elimination of the solvent peak will offer three advantages: first, the operating sensitivity can be increased without introducing the interference of a broad solvent peak; secondly, the retention times of the compounds can be decreased, also increasing sensitivity; a third advantage is the ability to introduce into the chromatograph larger volumes of concentrated samples than would be possible employing an injection with a solvent.

Other methods of introducing solids into a gas chromatograph have been suggested by BOWMAN AND KARMEN[3], RENSHAW AND BIRAN[4] and MCCOMAS AND GOLDFIEN[5]. The methods, in each case, as suggested by the other investigators require interruption of the carrier gas flow, a modification of the injection system or difficulty in injecting the samples. None of the above-mentioned objections were observed employing the method suggested in this paper.

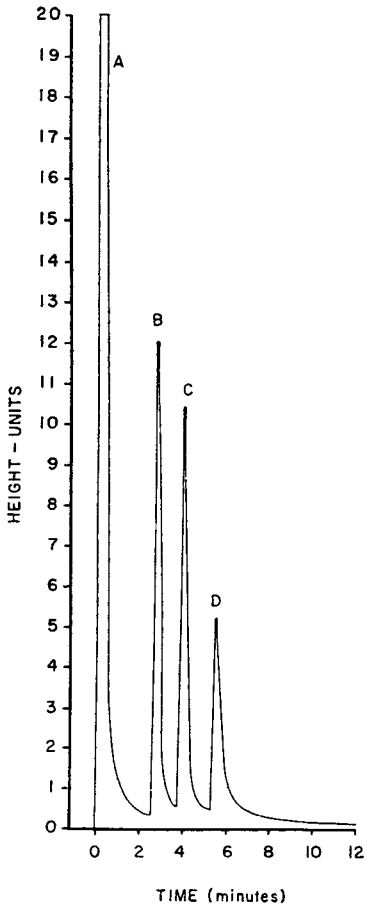

Fig. 1. Estrogens introduced in solvent. Gas chromatographic separation of (A) solvent, (B) 1.0 μg 17β-estradiol, (C) 1.0 μg estrone and (D) 2.0 μg estriol. Conditions: column, 6 ft., 5 mm I.D. pyrex glass column; 3% QF-1 on 100/120 Gas Chrom-P; 252°, 88 ml/min flow rate; detector, 274°; flash heater, 319°.

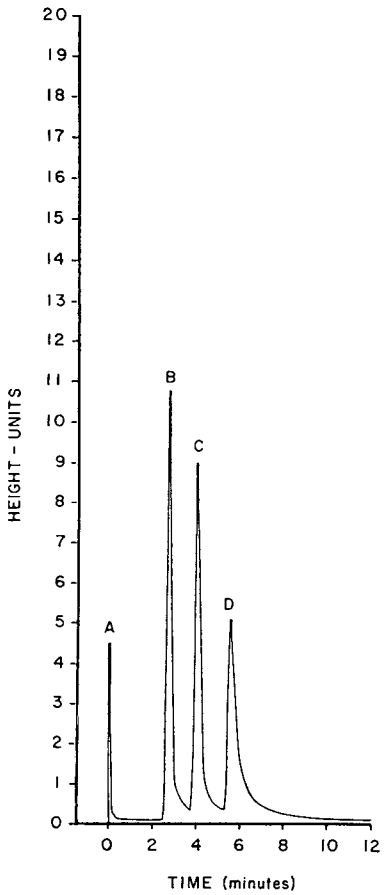

Fig. 2. Estrogens introduced in solid injector. Gas chromatographic separation of (A) air peak, (B) 1.0 μg 17β-estradiol, (C) 1.0 μg estrone and (D) 2.0 μg estriol. Conditions: column, 6 ft., 5 mm I.D. pyrex glass column; 3% QF-1 on 100/120 Gas Chrom-P; 252°, 88 ml/min flow rate; detector, 274°; flash heater, 319°.

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A column and paper chromatographic method for separation of N-dimethylamino-succinamic acid in plant extracts

Recently N-dimethylamino-succinamic acid (B-9) has been used as a size-controlling compound on certain plant species[1-3]. To determine the distribution pattern and the ultimate fate of B-9 in plant tissue, it was necessary to develop a procedure for its chemical analysis. The purpose of this investigation was to devise a method of analysis for the compound by using column and paper chromatographic techniques.

Materials and methods

Initial studies were conducted on standards of ^{14}C-labeled B-9 which had not been subjected to plant metabolism. A water solution containing 0.8 μC of ^{14}C B-9 was passed through ion exchange resins attached in series. The upper column contained IR-120*, a cation exchange resin in the H^+ form, and the lower column contained IR-45*, an anion exchange resin in Cl^- form. The columns measured 8 mm inside diameter and were packed to a height of 15 cm. The resins were prepared essentially as described by ROMBERGER[4].

After passing the ^{14}C B-9 through the resins, 25 ml of distilled water was added as a resin wash. The columns were then disconnected and the IR-120 resin was eluted with 30 ml of 7.5 *N* ammonium hydroxide and concentrated to 1 ml under a warm air stream. The IR-45 resin was eluted with 7.5 *N* formic acid and concentrated to 1 ml under a warm air stream. Aliquots were taken from each fraction including the non-polar material that passed through both resins, and assay of the recovered radioactivity was accomplished in a windowless gas-flow counter.

One gram aliquots of freeze-dried plant material previously treated with non-labeled B-9 and 1 μC of ^{14}C-labeled B-9 were extracted for 4 h at 90° with 50% ethanol. The extract was filtered hot under suction and brought to a constant volume. The total extract was then passed through the ion exchange resins attached in series as previously described.

After elution and concentration, 100 λ of the material from the IR-120 resin was spotted on Whatman 3 MM paper and developed two-dimensionally. The solvent systems employed were as follows: the first dimension in *n*-butanol–methyl ethyl ketone–water (2:4:1, v/v/v) plus 2 ml of ammonium hydroxide per 100 ml of solvent, developed for 15 h; the second dimension was developed in isopropanol–ammonium hydroxide–water (20:1:4, v/v/v) for 15 h. The dried chromatograms were sprayed or dipped in a 1:1 solution of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$, a reagent commonly used in higher concentration for the detection of phenols[5]. The color reagent is sensitive to 0.5 μg of B-9. Autoradiographs were made of the same two-dimensional chromatograms to verify the color test.

Results and discussion

To test the separation efficiency of the ion exchange resins, ^{14}C-labeled B-9 was passed through the columns of resins attached in series. The eluate from the columns was concentrated and aliquots were taken for radioactive assay. The results of this test may be seen in Table I, which shows that of the total radioactivity recovered, 98.1 %

* Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A. Mention of trade names does not imply endorsement by the U.S. Department of Agriculture over similar materials not so named.

TABLE I

DISTRIBUTION OF ^{14}C B-9 STANDARD SOLUTION ON ION EXCHANGE RESINS ATTACHED IN SERIES

Fraction	*Recovered activity*	
	*CCPM**	%
IR-120	55760	98.1
IR-45	80	0.1
Through resins	1000	1.8

* Corrected counts per minute.

was contained on the IR-120 resin, against only 0.1 % on the IR-45 resin, with 1.8 % passing through both resins. The efficiency of recovery of the ^{14}C B-9 on the IR-120 resin was so high that it was selected for use. Since B-9 is a positively charged molecule, one would expect it to adsorb on IR-120 with other positively charged molecules such as amino acids. The efficiency of the IR-120 resin for B-9 was not altered when plant extracts containing the chemical were passed through the column.

In recovering B-9 from a sample plant extract, positively charged molecules other than B-9 are adsorbed and eluted from the IR-120 resin. Therefore, further separation of B-9 after elution was necessary, and this was accomplished by employing two-dimensional paper chromatography. The separation of B-9 from other components of a plant extract eluted from IR-120 such as amino acids by using the two-dimensional paper chromatography is shown in Fig. 1. Positive location and identification of B-9 was done by comparing the standard B-9 solution with a plant extract containing radioactive B-9 (direct counting and autoradiograph) and with a plant extract containing nonradioactive B-9. The amino acids tryptophan and tyrosine, which color blue with the B-9 reagent, do not interfere, but rather aid in the location of B-9.

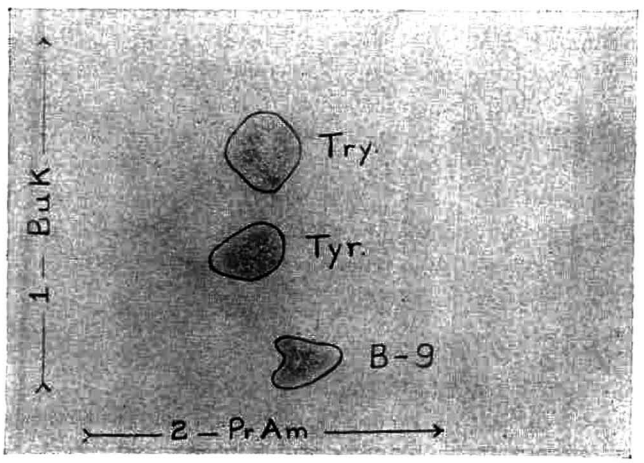

Fig. 1. Separation of B-9 from other positively charged components from IR-120. First dimension (1) developed in *n*-butanol–methyl ethyl ketone–water (2:4:1, v/v/v) plus 2 ml of ammonium hydroxide per 100 ml of solvent for 15 h, R_F 0.06. Second dimension (2) in isopropanol–ammonium hydroxide–water (20:1:4, v/v/v) for 15 h, R_F 0.33. Try = tryptophan; Tyr = tyrosine.

The B-9 extract separated on paper reacts with the reagent and gives a dark blue spot against a yellow background. In a very short time the background turns dark blue, and location of B-9 is difficult or impossible. This problem can be prevented by spraying immediately with 2 *N* ammonium acetate.

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J. Chromatog., 15 (1964) 95–97

Countercurrent distribution of D-lyxose-1-^{14}C*

During the course of countercurrent distribution studies, we observed that D-lyxose-1-^{14}C migrated less rapidly than its unlabeled counterpart during countercurrent distribution. These data are similar to those previously reported[1] for D-arabinose-1-^{14}C and extend to these aldopentoses, during countercurrent distribution in cyclohexane–ethanol, PIEZ AND EAGLE'S[2] caution concerning the use of coincidence of radioactivity and an index of mass as the criterion for identity in studies of labeled amino acids. Implicit in such migration of solutes during countercurrent distribution as well as chromatography is the considerable error that can result in the selection of a single fraction rather than the peak for determinations of specific activity.

Materials

D-Lyxose-1-^{14}C and D-xylose-1-^{14}C with specific activities, respectively, of 0.18 and 0.21 mC per millimole were purchased from Calbiochem. The radiochemical purity of all compounds was found to be higher than 98 % when mass calculated from observed characteristic absorbance and absorbance index[3] was compared with mass computed from radioactivity and sample specific activity. *o*-Aminobiphenyl, purchased from Chemical Procurement Laboratories, College Point, New York, was purified by recrystallization.

Experimental

Countercurrent distribution. Twenty to 30 mg of a mixture of inert pentose and radioactive isomer with a final specific activity of 35–40 μC/mmole were dissolved in 100 ml of lower phase of the cyclohexane–ethanol system described in Fig. 1. The solution, after 24 h to permit anomeric equilibrium, was introduced into the first five tubes of the 100 tube countercurrent train. At the end of the indicated number of transfers, sampled tubes were dried in moving air at 22°. Dried samples were counted for

* Supported by a U.S. Public Health Training Grant.

radioactivity, then each residue was dissolved in 5 ml of water from which 1 ml was taken for the determination of the unlabeled sugar by measuring the absorbance at 370 mμ of the pentosylamine[3] formed in 30 min with *o*-aminobiphenyl.

As in the case of D-arabinose-1-^{14}C and L-arabinose-1-^{14}C, D-lyxose-1-^{14}C migrated less rapidly than unlabeled D-lyxose (Fig. 1) during countercurrent distribution for 600 transfers as well as for 950 transfers. The apparent distribution coefficient (0.11) at 950 transfers, calculated from the position of the tube having the maximum concentration of the pentose, was greater than the corresponding value (0.06) for 600 transfers but such would be expected since no compensation (by calculation or solvent replacement) was made for the gradually decreasing lower phase volumes after 600 transfers. There was no difference between the mobility of D-xylose-1-^{14}C and inert D-xylose in the cyclohexane–ethanol system (Fig. 2). No measurable radioactivity appeared in tubes 0 to 24 or between 60 and 100 when labelled xylose underwent the distribution shown. When the distribution was carried out through 400 transfers, no difference in mobility between the labeled and unlabeled pentose was observed.

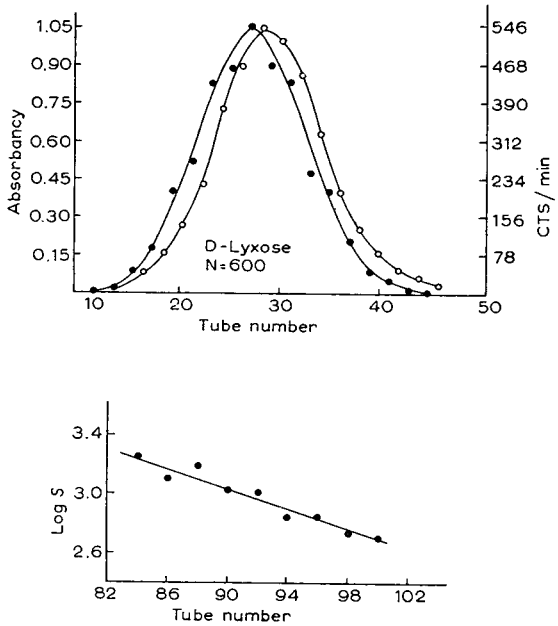

Fig. 1. (Upper) Resolution of D-lyxose-1-^{14}C and unlabeled D-lyxose in the all glass countercurrent apparatus of CRAIG AND POST with each phase volume 10 ml. The solvent system was an equilibrated mixture of 2 parts of cyclohexane with 1 part 95% ethanol at 22°. The upper phase composition, by volume in percent of water, ethanol and cyclohexane, was 0.8, 15.5 and 84.5 as determined by matching spectra of synthetic mixtures of the components using the Perkin-Elmer infrared spectrophotometer, Model 21. The corresponding composition of water, ethanol and cyclohexane in the lower phase was 2.5, 48 and 49: ● = radioactivity, measured with an end window Geiger counter; ○ = absorbance at 370 mμ of the arabinosylamine. Twenty mg of unlabeled D-lyxose mixed with 5 μC of labelled pentose constituted the sample for the 600 transfer distribution shown. (Lower) Plot of log specific activity, log S, against tube number, X, in accordance with $\ln S = [(M_1 - M_2)X/\sigma^2] + [(M_2^2 - M_1^2)/2\sigma^2]$ derived from the ratio of two curves (absorbance, ^{14}C-activity) assuming the normal distribution and that they have the same standard deviation σ, but M_1, the mean of the absorbance curve, differs from M_2, the mean of the ^{14}C activity curve; thus, the slope of the line is the index of resolution for this 950 transfer distribution.

Although the countercurrent distribution of xylose compares a 200 transfer (Fig. 2) extraction with a 600 transfer one (Fig. 1), the greater mobility of xylose (K = 0.26) compared to lyxose (K = 0.06) permits the comparison[4]. Indeed,

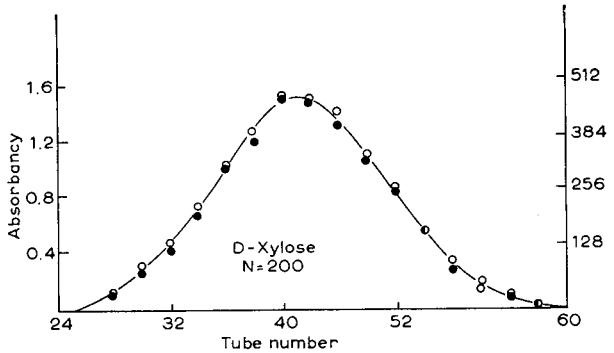

Fig. 2. Absorbancy at 370 millimicrons and radioactivity, a graphical representation of the coincidence of the mobility of D-xylose and D-xylose-1-^{14}C in a distribution similar to that described in Fig. 1 with the exception that the number of transfers was 200. ● = Radioactivity, measured with an end window Geiger counter. O = Absorbance, at 370 millimicrons of the xylosylamine.

if the distribution coefficients of D-xylose-1-^{14}C and inert D-xylose had the same ratio as the corresponding apparent distribution coefficients of D-lyxose-1-^{14}C and unlabeled D-lyxose (Fig. 1), separation of the peaks of the distribution patterns for radioactive and inert xylose would be greater at 200 transfers than that shown for the isotopic partners of D-lyxose (Fig. 1) for 600 transfers.

If our reasoning is correct, ^{14}C on carbon 1 alters the dipole moment of the radioactive lyxose and, thereby, the distribution coefficient of this aldopentose in the cyclohexane system. Possibly several forms (for example, the aldehyde and one or more ring forms) comprise the lyxose sample undergoing such extraction, but lyxose-1-^{14}C contributes to the less rapidly migrating components during countercurrent distribution. Since the isotope effect was not observed with similarly labeled radioactive xylose, it might be that the corresponding equilibria are too one-sided for the effect to be discernible in countercurrent distribution in cyclohexane–ethanol. In these studies, as in the previous one for arabinose, the slightly broader than theoretical[4] distribution for lyxose as well as the slight deviation from linearity of plots of specific activity *versus* fraction number (Fig. 1 — lower) are at least consistent with such polymorphism.

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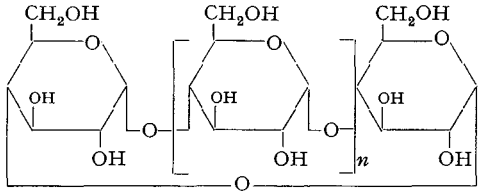
Thin-layer chromatography of cyclodextrins and some other sugars using microchromatoplates

Schardinger dextrins (cyclodextrins) are a homologous group of cyclic saccharides consisting of α 1,4 bound glucose units. They are obtained from the breakdown of starch by the action of *Bacillus macerans* amylase. Their general formula may be expressed by (I):



(I)

in which $n = 4$ for α-cyclodextrin, $n = 5$ for β-cyclodextrin, and $n = 6$ for γ-cyclodextrin.

They are important in that they can form inclusion compounds. During a study of the preparation and properties of cyclodextrins we thought it useful to develop a simple and rapid procedure to distinguish α- from β-cyclodextrin, and the cyclodextrins mentioned from some other sugars, *viz.* glucose and maltose, produced during the preparation of cyclodextrins. γ-Cyclodextrin is not included in our observations, as we have not yet succeeded in isolating it.

CRAMER[1] succeeded in separating α-, β- and γ-cyclodextrin on Schleicher & Schüll 2045b paper, using butanol-2-pyridine–water (1:1:1, v/v/v) as a developing solvent (circular chromatogram). However, we have been unable to separate α- and β-cyclodextrin in this way. On the other hand we did succeed in separating α- and β-cyclodextrin by using a developing solvent described earlier by CRAMER[2] which consisted of *n*-butanol–dimethylformamide (dmf)–water (2:1:1, v/v/v). In our opinion it is not advisable to use Schleicher & Schüll 2045b paper as described by CRAMER, as the time of separation is about 48 h. We obtained more rapid results (18 h approximately) by using Whatman No. 1 paper. In our opinion the best indicator spray is 1% alcoholic iodine solution. This results in the formation of a purple compound with α-cyclodextrin and a yellow one with β-cyclodextrin. The latter is visible only after the brown I_2 background has evaporated sufficiently; in some cases this may take 1 h.

In view of the long separation time of the method described above and of the rather poor identification procedure, we have developed a rapid chromatographic procedure to separate α- and β-cyclodextrins. For this we used the microchromatoplates as described by PEIFER[3].

Preparation of chromatoplates

1. *Silicic acid layers.* We used Kiesel-G "Merck", and followed PEIFER's[3] procedure. The plates ($7^1/_2 \times 2^1/_2$ cm) were steamed and dried to obtain less fragile coatings. The initial activity of the plates was very constant, as was shown by chromatographing the test fluid of the firm of Desaga (60, Hauptstrasse, Heidelberg, Germany)

consisting of butter yellow, sudan red and indophenol blue with benzene as a developing solvent (time of separation: 4 min).

2. *Alusil layers* (silicic acid mixed with alumina). A suspension consisting of 25 g Kieselgel-G and 25 g Aluminiumoxid-G (both "Merck") in 100 ml chloroform–methanol (2:1, v/v) was used. The plates were treated (steaming and drying) by the PEIFER[3] procedure.

Indicator spray

The indicator spray used was a mixture of 10 ml conc. sulphuric acid, 20 ml water and 3 g potassium dichromate. The spray reagent should be used as a fine cloud, and should be blown onto the coating very carefully as there is a chance of loosening the coating. Before spraying, the developing solvent is carefully vaporized by laying the chromatoplate on a hot plate. After spraying, the chromatoplate is put on the hot plate again for a short time until black dots appear. The advantages of the use of microchromatoplates are apparent here as the developing solvent is vaporized very rapidly and the oxidation takes place almost instantaneously.

Developing solvents

After having tried some thirty solvents the following two give the best results:

(1) *n*-butanol–glacial acetic acid–water–pyridine–dmf (6:3:1:2:4), and

(2) *n*-butanol–glacial acetic acid–water (6:3:1).

Separation of α- and β-cyclodextrins

1. *On silicic acid layers.* The difficulty was to find a suitable developing solvent as the solvents used for "common sugars"[4] were found unsuitable for our purpose; however, solvent (1) was found to be most suitable. The R_F of α-cyclodextrin is 0.0 and that of β-cyclodextrin is about 0.5. After evaporating solvent (1) from the plate with a hair-dryer, the plate can be put in, for example, dmf for a short time, in such a way that the dmf front travels $^1/_3$ of the distance of the solvent (1) front ("Stufentechnik"); the R_F of α- and β-cyclodextrin in dmf is about 0.9. It is also possible to make a two-dimensional chromatogram using solvent (1) and dmf; however, the "Stufentechnik" referred to gave the best results.

2. *On alusil layers.* α- and β-cyclodextrins could also be separated on layers of alusil with solvent (1). The R_F of α-cyclodextrin is again 0.0 and that of β-cyclodextrin about 0.5 but the spots were less sharp than when silicic acid layers were used.

Investigations on solvent (1) showed that:

(a) omission of water resulted in tailing of the α-cyclodextrin,

(b) omission of pyridine resulted in tailing of the β-cyclodextrin,

(c) omission of glacial acetic acid resulted in tailing of the α-cyclodextrin,

(d) omission of dmf resulted in an $R_F = 0.0$ for β-cyclodextrin.

It was also observed that cyclodextrins are very soluble in dmf. β-Cyclodextrin has a solubility of 20.0 ± 0.5 g/100 g dmf at 24°, compared with its solubility in water of only 1.5 ± 0.3 g/100 g water.

It is advisable not to put more than 5 μg of each cyclodextrin on the plate.

Separation of α- and β-cyclodextrin, glucose and maltose

The best results were obtained on silicic acid plates (even when separating glucose

and maltose, this in contradiction to STAHL's[4] observations on "macro" plates) using the "Stufentechnik". Development was first with solvent (2), followed by solvent (1). It is advisable to allow solvent (2) to reach the end of the adsorbent and then leave the chromatoplate in this solvent for another 4 min. After this the solvent is vaporised with a hairdryer and the plate is put in solvent (1) (the R_F of α-cyclodextrin in solvent (2) is slightly greater than 0.0). Fig. 1b shows the result. For pur-

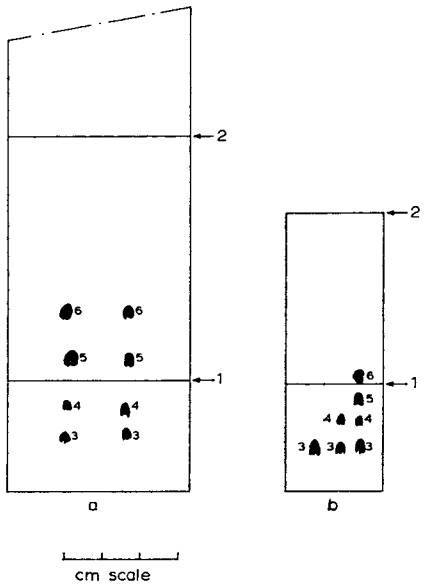


Fig. 1. Separation of α- and β-cyclodextrin, glucose and maltose. 1 = front of solvent (1); 2 = front of solvent (2); 3 = α-cyclodextrin; 4 = β-cyclodextrin; 5 = maltose; 6 = glucose.

pose of comparison (Fig. 1a) we made a chromatogram on a plate prepared by the usual spreading technique with the aid of the Desaga applicator (thickness of the layer 250 μg). When alusil plates were used the spots were not as sharp.

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The author is grateful to Prof. Dr. C. L. VAN PANTHALEON VAN ECK for his interest shown throughout the course of this study and to Mr. W. BREKELMANS for his technical assistance.

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Detection of saccharification products by thin-layer chromatography

In alcoholic fermentations using different kinds of raw materials containing starch as substrate, the saccharification process is generally followed by the disappearance of the colour formed with iodine. More accurate results may be obtained by the determination of the reducing sugars. Such methods only give total results without specifying which sugar is being formed. This paper is concerned with the detection of the saccharification products by thin-layer chromatography, using different saccharifying preparations.

Experimental

Qualitative analysis. Chromatoplates were prepared by coating glass plates (20 cm × 20 cm) with Merck's Kieselgur G according to STAHL[1]. 5 μl spots containing approximately 50 μg of sugar were applied 1.5 cm from the bottom of the plate. A mark was made 10 cm from the point of application so that the migration distance would be the same on all plates. The chromatoplates were irrigated by ascending migration of 5 ml of a solvent mixture. A number of different solvents were tried and a mixture of *n*-butanol–toluene–ethylacetate–water (10:2:5:3) was found to give the best results[2]. The separation was completed within 20 min. The chromatoplates were dried at 60° and then sprayed with anisaldehyde[1] and developed at 100° for 10 min.

Quantitative analysis. Reducing sugars were determined by the method of NELSON[3]. The disappearance of starch was followed by the method of BOWINE *et al.*[4].

Results and discussion

As a basic experiment, the hydrolysis of starch by α- and β-amylase was studied. 160 mg of soluble starch were dissolved in either 10 ml of 0.05 *M* phosphate buffer, pH 7.0, for α-amylase, or 0.05 *M* acetate buffer, pH 4.7, for β-amylase. 1 mg of the enzyme was added in 6 ml of water and the mixture incubated at 37°. Samples were taken at 5 min intervals for the determination of reducing sugar and starch, as well as for chromatography. They were immersed in a boiling bath for 3 min to stop the reaction. All blanks were prepared with an inactivated enzyme preparation.

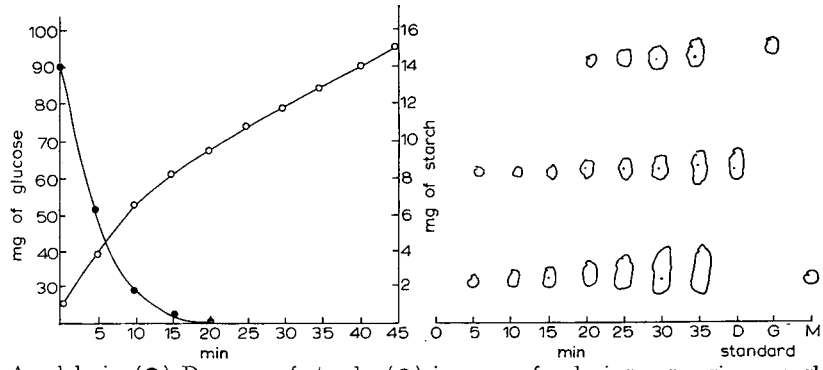

Fig. 1. α-Amylolysis. (●) Decrease of starch; (○) increase of reducing sugar given as glucose. Thin-layer chromatogram in minutes. Solvent system: *n*-butanol–toluene–ethyl acetate–water (10:2:5:3). The standards are: dextrins (D), glucose (G) and maltose (M).

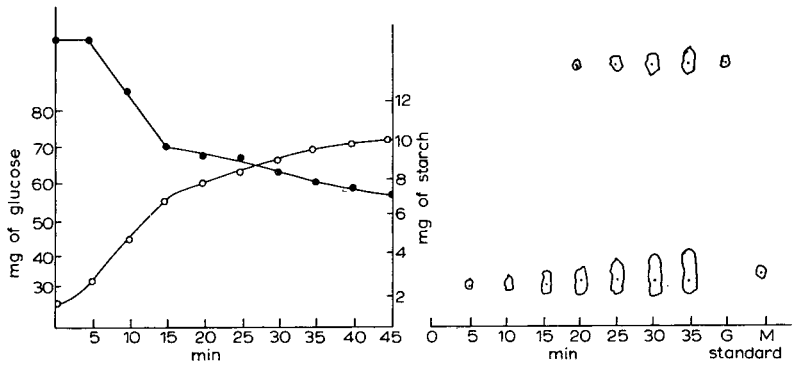

Fig. 2. β-Amylolysis. (●) Decrease of starch; (○) increase of reducing sugar given as glucose. Thin-layer chromatogram in minutes. Solvent system: *n*-butanol–toluene–ethyl acetate–water (10:2:5:3). The standards are: glucose (G) and maltose (M).

The results shown in Figs. 1 and 2 confirm the differences in action of the two amylases, showing the strong formation of dextrins with α-amylase, whereas β-amylase does not form them. On the other hand the hydrolysis of starch is much slower in the latter case. In both experiments we observe that the reducing power is due, at first, mainly to the presence of maltose, glucose being only formed in detectable amounts after 15 min of incubation.

For the saccharification of manioc by barley malt, 50 g of manioc meal were cooked with 1 % barley malt in 250 ml of water for 2 h at 1.7 atm. 10 % of malt were added to the solubilized mash and the pH adjusted to 5.0 with dilute H_2SO_4. The saccharification was followed at 50°, samples being taken for analysis.

As may be seen from Fig. 3, saccharification occurred in 10 min, dextrins, maltose and glucose all being responsible for the appearance of reducing power. Similar results were obtained using mold bran as saccharifying agent.

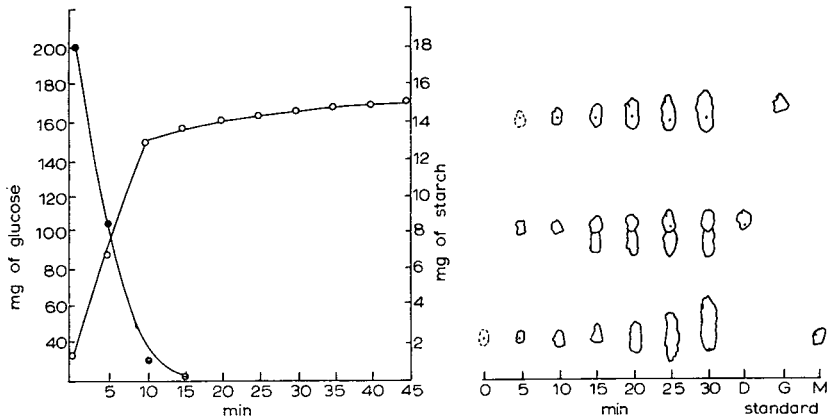

Fig. 3. Saccharification of manioc by barley malt. (●) Decrease of starch; (○) increase of reducing sugar given as glucose. Thin-layer chromatogram in minutes. Solvent system: *n*-butanol–toluene–ethyl acetate–water (10:2:5:3). The standards are: dextrins (D), glucose (G) and maltose (M).

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Sample application in preparative thin-layer chromatography

The successful resolution of mixtures on preparative thin-layer chromatoplates requires uniform application of the sample with respect to both concentration and shape of the starting zone. Uniformity of concentration has been attained by application of a solution of the mixture from a travelling syringe[1]. Recently a much simpler device was described[2] but this suffers from the drawback that a maximum volume of *ca.* 0.3 ml of solution can be added with a single application. Despite careful handling of the applicator, it is not possible to avoid slight irregularities in the shape of the starting zone on repeated application to the same position on a plate. These irregularities become exaggerated as the zones migrate so that separation of compounds with $\Delta R_F < 0.3$ is seldom efficient, especially with quantities greater than 20 mg of mixture per plate (20 × 20 cm). We have found that uniform migrating zones, and efficient separation of compounds with ΔR_F as low as 0.1, are obtained from the narrow, straight-edged bands prepared by the following procedure:

Two parallel cuts (A, Fig. 1), *ca.* 3 mm apart, are made through the adsorbent

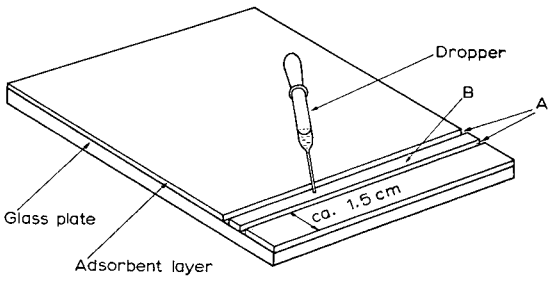

Fig. 1.

layer by means of a thin knife-blade. If these cuts are made by lightly drawing the blade over the adsorbent three or four times, and not by a single stroke, the ridge (B, Fig. 1) will not break. Blow out any loose material in the cuts and apply a solution of the mixture to be resolved, in any convenient solvent, to the ridge with a fine-tipped dropper as uniformly as possible. The ridge should be allowed to dry out thoroughly after each application of solution. The cuts are then filled with *dry* adsorbent, using an aluminium foil mask (M, Fig. 2) with a 1 mm slit (S, Fig. 2) which is

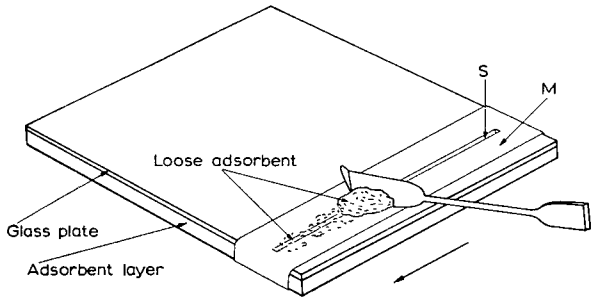

Fig. 2.

placed over each cut in turn. The adsorbent is packed by firmly drawing the back of a large spatula over the slit in the mask (Fig. 2). No special care is needed in handling these layers—the "loose" adsorbent will not fall out when the plates are placed vertically in a chromatographic tank. The chromatograms are run in the usual manner.

By this method, 50 mg crude 7-ketocholesteryl acetate, m.p. 156–158°, $[\alpha]_D$ —98°, prepared by oxidation of cholesteryl acetate with *tert.*-butyl chromate, on a 1 mm layer of silica impregnated[2] with Rhodamine 6 G and developed with benzene, gave, in less than one hour, 48 mg pure 7-ketocholesteryl acetate, m.p. 158–159°, $[\alpha]_F$ —103°, showing a single spot on an analytical thin-layer chromatogram. Material of the same physical constants, but still showing a second weak spot on an analytical thin-layer chromatogram, was obtained from the crude acetate only after six crystallisations from acetone.

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Eine Methode für den direkten Nachweis von Desoxyribonukleasen nach Elektrophorese in Agargel

Zur Prüfung der Eigenschaften von Enzympräparaten wird immer häufiger die Elektrophorese eingesetzt. Besondere Bedeutung gewinnt sie bei der Untersuchung von multiplen Enzymen (Isoenzyme), obgleich die damit erhaltenen Resultate nicht ohne andere Analysendaten bewertet werden dürfen[1].

Unter den Nukleasen sind multiple Formen bei den Streptokokken gefunden worden. Es handelt sich um drei Desoxyribonukleasen, die von Streptokokken der Gruppe A in das Nährmedium abgegeben werden. Zu ihrem Nachweis nach elektrophoretischer Auftrennung in Stärke zerlegte WANNAMAKER[2] den Stärkeblock in Fraktionen, eluierte diese und bestimmte in den Eluaten die Fermentaktivität. Ein einfacherer Nachweis, der die gleichzeitige Untersuchung vieler Proben ohne grossen Aufwand ermöglicht, wird hier beschrieben. Er dürfte sich ganz allgemein nicht nur für den Nachweis von Desoxyribonukleasen sondern darüberhinaus auch für Ribonukleasen eignen.

Prinzip. Die Elektrophorese erfolgt in Agargel. Nach beendeter Trennung wird parallel zur Wanderungsrichtung ein Agarstreifen ausgestanzt und in eine Schale übertragen, wo er mit einem Gemisch aus Agar und Desoxyribonukleinsäure umgeben wird. Nach Bebrütung bei 37° wird der Agar mit Salzsäure überschichtet. An den Stellen, wo im Elektropherogramm eine Desoxyribonuklease vorlag, ist der Streifen von einer Verdauungszone umgeben, während die unverdaute Desoxyribonukleinsäure in fädig-flockiger Form ausfällt.

Die Anwendung der Methode soll am Beispiel des Nachweises der erwähnten extrazellulären Desoxyribonukleasen von *Streptococcus pyogenes* erläutert werden:

Glasplatten von 4.5 × 23 cm werden in einer Schale mit einer ca. 3 mm hohen Schicht von 1.5 % Agar in 0.05 *M* Glycin–NaCl-Puffer, pH 9.0, bedeckt. Aus dem erstarrten Agar wird ein Loch von 2 × 15 mm so ausgestanzt, dass als Wanderungsstrecke in Richtung der Anode zwei Drittel der gesamten Streifenlänge verbleiben. Dieses Loch wird mit einem Gemisch aus gleichen Teilen 3 %igem Agar (abgekühlt auf 45°) und dialysierter Enzymlösung gefüllt. Die Elektrophorese der Proben erfolgt mit gleichem Puffer bei einer Spannung von 150 V im Laufe von ca. 16 Std. Dabei liegen die Glasplatten auf einem mit fliessendem Wasser gekühlten Metallbehälter. Nach beendeter Auftrennung wird aus dem Agar parallel zur Wanderungsrichtung ein Streifen von 5 × 140 mm herausgestanzt und auf eine Glasplatte von 15 × 15 cm übertragen. Bis zu sechs solcher Streifen können auf eine Platte gebracht werden, wobei jedoch zwischen ihnen ein Abstand von etwa 2 cm bleiben muss. Nachdem auf die Platte ein Plexiglasrahmen von 1.5 cm Höhe aufgesetzt wurde, füllt man auf dem nivellierten Giesstisch in die Zwischenräume der Agarstreifen 40 ml eines Gemisches aus gleichen Teilen verflüssigtem 4 %igen Wasseragar und 0.6 % Desoxyribonukleinsäure (hergestellt aus Kalbsthymus[3]) in 0.028 *M* Veronal–Azetatpuffer (Mg^{2+}-Endkonzentration 0.03 *M*) ein. Nach 16–24stündigem Bebrüten der abgedeckten Platten bei 37° überschichtet man den Agar mit 0.5 *N*-Salzsäure und spült nach 15 Min mit Wasser nach. Die Lage der auftretenden Verdauungszonen kann durch Fotografie bei Dunkelfeldbeleuchtung oder auch durch einfaches Durchpausen auf der Rückseite der Glasplatte festgehalten werden.

Fig. 1 zeigt die extrazellulären Desoxyribonukleasen des Streptokokkenstammes

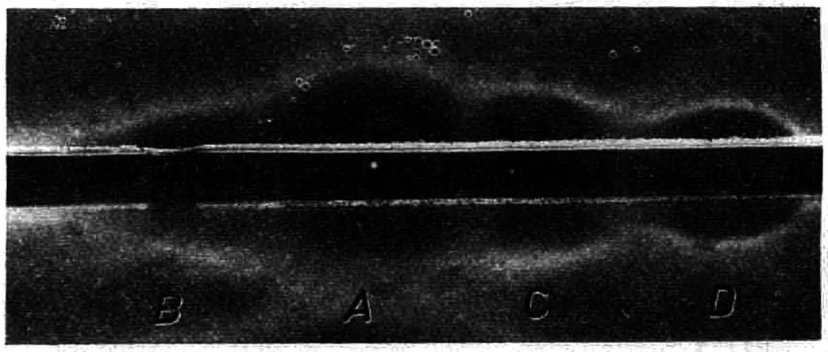

Fig. 1. Die extrazellulären Desoxyribonukleasen des Stammes S 84 von *Streptococcus pyogenes*.

S 84 (Typ 3). Dieser Stamm entlässt in das Nährmedium vier verschiedene Desoxyribonukleasen, welche sich nicht nur elektrophoretisch sondern auch in anderen Eigenschaften unterscheiden. Drei von ihnen können mit den von WANNAMAKER[2] beschriebenen Enzymen A, B und C identifiziert werden. Das vierte entspricht wahrscheinlich dem von AYOUB UND WANNAMAKER[4] erwähnten Enzym und soll deshalb Enzym D genannt werden.

Mit dem Rest des Elektrophoresestreifens kann man zusätzliche Untersuchungen anstellen (z.B. Bestimmung des pH-Optimums und anderer Fermenteigenschaften). Hierzu wird der Streifen in zentimeterbreite Fraktionen zerlegt und diese tiefgefroren. Die nach dem Auftauen abgeschiedene Flüssigkeit (evtl. Zugabe eines kleinen Volumens Puffer) enthält das Ferment.

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The analysis of straight-chain (*n*-C_1–C_9) carboxylic acids by a thin-layer chromatographic method

Straight chain carboxylic acids of low molecular weight are frequently encountered as by-products of biological and chemical processes. The identification of individual acids of this type can be difficult, particularly as they often occur, or are recovered, in dilute aqueous solution. Straight chain carboxylic acids have been analysed by paper chromatography[1–8]. A thin-layer chromatographic method has been described[9] for which it is shown that for the carboxylic acids of even carbon numbers 4 to 18 and

odd carbon numbers 5 to 11 there is a linear relationship between log R_F and the total carbon number of the acids. However, a good separation of the acids of lower molecular weight was not obtained. Another thin-layer chromatographic method is described[10] for the separation of formic, acetic, pyruvic and lactic acids. We have developed a much improved separation by thin-layer chromatography of the straight chain carboxylic acids (n-C_1–C_9) in dilute aqueous solutions.

Experimental and results

Development of a suitable solvent system. The majority of the solvents described in the literature[1–10] for the analysis of low molecular weight fatty acids by paper and thin-layer chromatography are mixtures of a lower alcohol, water and ammonia. Preferably the acids are separated as their salts, *e.g.* salts of the acid with morpholine, ethylamine, ammonia, etc., or as derivatives, *e.g.* hydroxamic acids.

The separation of n-C_1 to C_9 carboxylic acids was examined (both the free acids and the ammonium salts being used) on thin-layer plates of neutral silica gel with various solvent systems including those previously employed by other authors[1–10]. It was found, however, that the best separation was obtained with the solvent system methyl acetate–ammonia 2.5 % vol. aq. (95:5, v/v) and this was adopted for the subsequent investigations.

Spray reagents. Many of the common acid–base indicators were examined as spray reagents for locating the acid zones on the chromatoplate. Alcoholic methyl red was found to be the most suitable.

The separation procedure. The separation of the acids is carried out in the usual way, a 20 cm × 20 cm plate covered with silica gel being used. When the solvent front has reached a line on the chromatoplate line indicating the limit of travel, the plate is removed from the solvent tank and placed in an oven at 105° for 2–3 min to evaporate the solvents. After cooling, the plate is replaced in the tank and again developed with the solvent. Finally the plate is sprayed with alcoholic methyl red

TABLE I

R_F VALUES OF LOW MOLECULAR WEIGHT, STRAIGHT CHAIN CARBOXYLIC ACIDS (AND OF SOME BRANCHED CHAIN ACIDS FOR REFERENCE PURPOSES)

Acid	R_F		
	After double run in fresh solvent	*After double run in solvent aged for 24 h*	*After single run in solvent aged for 24 h*
Formic	0.05	0.07	0.03
Acetic	0.10	0.13	0.06
Propionic	0.15	0.30	0.15
n-Butyric	0.24	0.40	0.22
n-Valeric	0.39	0.50	0.30
n-Hexanoic	0.52	0.57	0.34
n-Heptanoic	0.55	0.60	0.39
n-Octanoic	0.58	0.66	0.43
n-Nonanoic	0.61	0.69	0.45
Trimethylacetic	0.57	0.71	0.47
α-Methylbutyric (*dl*)	0.39	0.65	0.39
β-Methylbutyric	0.34	0.53	0.31
Isobutyric	0.27	0.57	0.32

solution and heated in the oven at 105° until the acids appear as dark red spots on an orange background. The R_F values of the acids are recorded in Table I.

The effect of solvent aging. If the developing solvent was allowed to stand for 24 h in a stoppered flask before use, the R_F values were greatly increased so that only a single development was required in order to obtain a satisfactory separation. Results obtained with the aged solvent are also shown in Table I. Because the R_F values of the acids depend upon the age of the solvent it is clearly necessary with unknown mixtures to run the reference acids at the same time.

Applicability of the method to steam distillates. Since straight chain carboxylic acids of low molecular weight are often isolated from oil products by steam distillation the application of the method to such distillates was investigated. Analysis of steam distillates obtained from a blend of C_1–C_6 acids clearly indicated that as the distillation proceeded the concentrations of the higher molecular weight acids in the distillate became less. Thus, the combination of steam distillation and thin-layer chromatography promises to be a quick and simple way of separating and identifying the straight chain carboxylic acids of low molecular weight encountered in the study of chemical and biological processes.

Discussion

Excellent separation of the *n*-C_1 to C_9 carboxylic acids is obtained either by a single development with the aged solvent mixture methyl acetate–aqueous ammonia or by a double development with fresh solvent. The acids are separated as their ammonium salts, the excess ammonia being driven off by heating the plate after development so that the acids can be located as compact red zones when sprayed with methyl red solution. The separation takes about half an hour for a single development so that the complete analysis including the time taken to prepare the plate requires one hour. The method is very sensitive and it is estimated that 5 μg of an acid can be detected.

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Reversed-phase paper chromatography of some substituted hydrazine derivatives of monosaccharides and related compounds

During the synthesis of some substituted hydrazine derivatives of monosaccharides and related compounds, it was desirable to chromatograph the isolated products and reaction mixtures. The compounds involved migrate in *n*-butanol–ethanol–water (10:1:2) (BEW) and in ethyl acetate–pyridine-water (8:2:1) (EPW), solvent mixtures used in this laboratory for chromatographing sugars and lactones, but either the resolution or the time required is not completely satisfactory. Hydrazones and osazones of monosaccharides are almost immobile in the *n*-heptane–phenoxyethanol reversed-phase paper chromatographic system used for the separation of 2,4-dinitrophenylhydrazones of aldehydes and ketones[1]. In the present study, a reversed-phase Zaffaroni-type solvent system used for the paper chromatography of steroids[2] gave rapid and satisfactory resolution of substituted hydrazine derivatives of monosaccharides and some organic acids and lactones. A number of systems of this general type were screened with typical sugar hydrazones and osazones. This paper reports the separations obtained with the best of these solvent systems—formamide–ethyl acetate–water (1:20:1) and formamide-impregnated paper (FEW).

Experimental

*Compounds and reagents**. Pure D-mannoheptulose was a gift from Dr. NELSON K. RICHTMYER, glucose was National Bureau of Standards sample No. 41, and the remaining commercially available sugars, organic acids, and lactones were used without further purification.

Eastman No. 1866 2,4-dinitrophenylhydrazine, No. 330 phenylhydrazine hydrochloride, and No. 1666 1-benzyl-1-phenylhydrazine hydrochloride were recrystallized before use.

The hydrazide, hydrazone, and osazone derivatives were prepared by the general methods referred to in Table I, column 2. Temperature of reaction, composition of solvent, and molecular ratio of reactants were modified as necessary.

Eastman No. 565 formamide and reagent grade acetone, ethyl acetate, and pyridine were used without further purification. Ethanol, 95 %, was distilled from magnesium turnings.

Preparation of chromatogram. Whatman No. 1 paper "for chromatography", cut 17 × 43 cm across the machine direction, was dipped twice into a mixture of 10 vol. formamide in 90 vol. acetone, drained momentarily, placed between four dry sheets of Whatman No. 1 paper on a smooth, flat surface, and slightly pressed to remove excess solvent. The impregnated paper was hung immediately in still air for 10 to 15 min. Not more than 2 μl of a freshly prepared solution of each compound was applied to the paper with a micropipet in spots 2 cm apart. The paper was suspended in a glass trough in the chromatographic jar, usually within one hour after impregnation, and migration was started immediately by the descending technique.

Benzylphenylhydrazine hydrochloride and phenylhydrazine hydrochloride were

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

TABLE I

R_G[a] VALUES OBTAINED BY REVERSED-PHASE CHROMATOGRAPHY

Solvent: Formamide–ethyl acetate–water (1:20:1, upper phase).
Paper: Whatman No. 1 "for chromatography" (descending).
Impregnation: Formamide–acetone (10% v/v).
Time of run: 2 h.
Temperature of run: 26°.
Detection: Acetone–silver nitrate — alcoholic potassium hydroxide.

Compound	*Method of preparation Ref. No.*	R_G
2,4-Dinitrophenylhydrazine		1.19
2,4-Dinitrophenylhydrazide of:		
Galactonic acid[b]	3	0.06
Gluconic acid[b]	4	0.09
Saccharic acid[b], bis,	4	0.52
Formic acid	5	1.04
Acetic acid	5	1.05
2,4-Dinitrophenylhydrazone of:		
Galacturonic acid	3	0.03[c]
Glucuronic acid	6	0.06[c]
Glucuronolactone	6	0.69
Mannoheptulose[b]	3	0.22
Galactose	3	0.27
Glucose	5	0.25
Mannose	3	0.28
Fructose	3	0.44
Arabinose	7	0.55
Lyxose	7	0.57
Ribose	7	0.63
Fucose	3	0.71
Rhamnose	3	0.85
2,4-Dinitrophenylosazone of:		
Glucose	5	1.02
Sorbose	3	1.05
Arabinose	5	1.16
Xylose	5	1.15
Fucose[b]	5	1.20
Rhamnose[b]	5	1.19
Phenylhydrazine[d]		(0.00, 0.96, 1.09)[e]
Phenylhydrazone of:		
Mannose	8a	0.21
Arabinose	8b	0.46
Phenylosazone of:		
Galactose	9	1.00
Glucose	8c	1.00
Sorbose	9	1.01
Arabinose	10	1.16
Lyxose	10	1.14
Rhamnose	9	1.19

(continued on p. 113)

TABLE I (*continued*)

Compound	*Method of preparation Ref. No.*	R_G
1-Benzyl-1-phenylhydrazine[d]		1.25[e]
1-Benzyl-1-phenylhydrazone of:		
Mannose	8d	0.76
Arabinose	8e	0.96
1-Benzyl-1-phenyl-phenylosazone of:		
Mannoheptulose	11	1.08
Glucose	11	1.16
Fructose	11	1.16
Sorbose	11	1.17
Arabinose	11	1.23
Rhamnose	11	1.23
Free sugar, acid or lactone:		
Uronic acids		0.00
Sugar acids (saccharic acid, and galactonic acid)		0.00–0.05 0.00–0.05
Lactones (galactonolactone, gluconolactone, glucuronolactone)		0.05–0.09
Heptulose		0.00
Hexoses		0.00–0.01
Pentoses		0.01–0.05

[a] R_G = distance migrated by compound/distance migrated by glucose phenylosazone on same chromatogram.
[b] Derivative not reported previously.
[c] Spot not discrete, streaking or bearding.
[d] Prepared from recrystallized hydrochloride derivative, see text.
[e] Principal spots.

dissolved separately in 95 % ethanol containing an equivalent amount of potassium hydroxide and 50 to 100 μg of each free base was spotted on the paper. Twenty-five μg of 2,4-dinitrophenylhydrazine was spotted from pyridine.

The free sugars, acids, and lactones were dissolved in water, and their derivatives in pyridine. One to 5 μg (0.6 μl) of the sugar 2,4-dinitrophenylosazones, about 12.5 μg (2 μl) of the uronic acid 2,4-dinitrophenylhydrazones, and 25 μg (2 μl) of the other derivatives were spotted. Arabinose and mannose phenylhydrazones are somewhat labile in pyridine and were spotted as soon as they dissolved.

Chromatography. The solvent was a mixture of formamide–ethyl acetate–water (1:20:1 v/v/v), shaken and allowed to separate into two phases. Separation is rapid. The upper phase was used as the mobile phase, the lower phase was discarded.

The chromatographic chamber was a cylindrical glass jar 30.3 cm in diameter and 60.5 cm high, sealed with a weighted glass lid. The wall of the jar was lined with Whatman No. 1 paper thoroughly wet with mobile phase, and the bottom of the jar was covered with mobile phase. The chamber was equilibrated at least 16 h before use.

As soon as the papers were spotted, two papers were suspended from each of two glass troughs in the jar and the troughs were filled with solvent. To assure a well-equilibrated atmosphere, the paper liner was rewetted at the beginning of each

chromatographic run. The chromatograms were developed by the descending technique at room temperature (26°) until the solvent front was about 3 cm from the bottom of the paper, usually not more than two hours.

The presence of 2,4-dinitrophenylosazones on the chromatogram was established with certainty by their change from yellow to violet when the still damp chromatogram was exposed to ammonia vapor. After the chromatograms hung overnight in a well ventilated hood, visible spots were outlined, and colorless reducing spots were detected by successive dips in acetone–silver nitrate and alcoholic potassium hydroxide solutions.

Discussion and results

When synthesizing hydrazone and osazone derivatives of sugars and related compounds, the formamide–ethyl acetate–water system is helpful to establish quickly the extent of the reaction, the nature of the derivative formed, and the purity of the isolated compound. It is particularly helpful when preparing hydrazones of sugars because excess reagent and any osazone formed move well ahead of the corresponding hydrazone while unreacted sugar remains near the origin. Microgram quantities of hydrazones and osazones formed by the solvent diffusion technique for microscopic identification of sugars[6,7,10,12,13] may be transferred from the diffusion cell and chromatographed with this system to further confirm the identity of the sugar.

The R_G values of the substituted hydrazine derivatives studied by this reversed-phase system are given in Table I, column 3. The mobilities of the sugar hydrazones or osazones in the FEW system are in about the same order as those of the unreacted sugars obtained by descending chromatography in BEW, that is, in order of decreasing mobility: methyl pentose and glucuronolactone, aldopentose, ketohexose, aldohexose, heptulose, and uronic acid. The data in Table I show that the R_G values are similar for corresponding hydrazine derivatives of stereo-isomers. STOLL AND RÜEGGER[14] also observed this when chromatographing the *p*-nitrophenylhydrazones of sugars in water-saturated solvents.

Hydrazones resolve better than do osazones. Even so, when preparing the 1-benzyl-1-phenyl-phenylosazone derivatives listed in Table I, resolution was adequate to show if the corresponding phenylosazone derivative was also present in the reaction mixture. The 2,4-dinitrophenylhydrazones of mannose, fructose and arabinose resolved better in the FEW system than did the corresponding free sugars in BEW or in EPW; whereas the reverse was true for the 2,4-dinitrophenylhydrazones of mannose, glucose, and galactose.

In the FEW system spots are more discrete if the area of the spot is small, the paper is chromatographed as soon as possible after impregnation and the solvent flow is across the machine direction of the paper. Loading is important—25 μg (2 μl) gave a discrete spot for most of the derivatives but more than 5 μg (0.6 μl) of a 2,4-dinitrophenylosazone may streak excessively.

The R_F values from this system should not be considered absolute because it is difficult to impregnate the paper uniformly. For purposes of comparison or identification, an authentic derivative should be chromatographed adjacent to an unknown.

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Notes on semimicro preparative thin-layer chromatography

Details of preparative thin-layer chromatographic techniques used in this laboratory and mentioned in two recent papers[1,2] are described here since they are of general application. They have been used in the isolation of 2- to 5-mg quantities of material for subsequent identification by ultraviolet and infrared spectral studies and by chromatographic comparison.

Overdeveloping thin-layer plates

For the separation of contiguous spots and bands on thin-layer plates, a continuous, descending method was developed. It gave much better separation at higher loading than BRENNER AND NIEDERWIESER's method[3] and was easier in operation and gave better separation than repeated, ascending development. It resembles the continuous, descending methods by which STANLEY *et al.*[4] washed plates and which MISTRYUKOV recently described[5], as well as the descending method mentioned by BIRKOFER *et al.*[6]. It requires less special equipment than these published methods and makes use of the weight of the plate itself for providing contact with a soft, cloth wick. Although the apparatus is not as simple as that recently reported by BENNETT AND HEFTMANN[7], the method requires less handling of the delicate plates and is easier for routine use.

Plate coatings of activated alumina and unbonded fluorescent silica (silica gel HF_{254}, Brinkmann Instruments, Inc., New York) were employed at thicknesses of 0.25 and 0.50 mm. To minimize side effects during development and to facilitate handling, approximately 1 cm wide bands of surface layer were removed from each vertical edge of the plates. Application of sample and of chromogenic reagents was accomplished by means of a precision streaking device[2,8].

The developing apparatus, whose important details are shown in Fig. 1, consisted of the usual thin-layer plate rack of stainless steel modified so as to support a standard 8.5-in. paper chromatographic trough in its 9-in. metal cradle, bearing the appropriate antisiphon and anchor rods. Leading from each side of the solvent trough and extending over the antisiphon rods are wicks against which thin-layer plates are leaned,

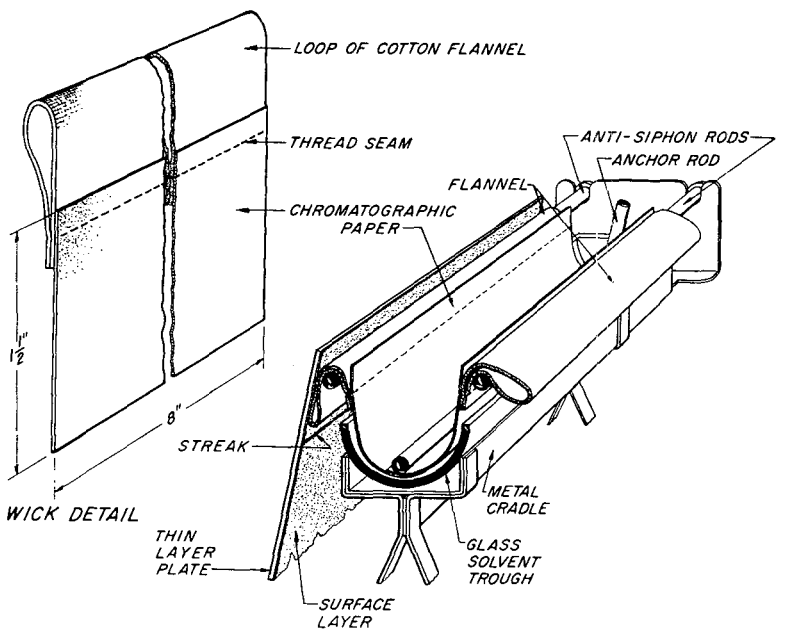

Fig. 1. Apparatus for continuous downward development.

layer side inward, at an approximate angle of 70°. The bottom edges of the plates rest on absorbent mats of heavy filter paper. The entire assembly is contained in a suitably sized chromatographic tank with a well-fitted cover. The wicks consist of a loop of fuzzy cloth (white cotton flannel) sewn to a strip of chromatographic paper (such as Whatman No. 1)—see separate wick detail in Fig. 1. This construction prevents marring of the thin-layer surface while affording good contact, the paper providing a slow solvent feed. The wicks are prewashed with solvent using spare plates.

In operation, two sample-streaked plates are lowered into the solvent saturated tank and pressed firmly against the wicks so that contact is made above the streak. Development begins at once. The time required for the solvent front to migrate to the bottom of the plates is noted and a total time of some multiple of this is tried.

The plates are then removed and dried and band separation observed in ultraviolet light (longwave and shortwave) or by cross streaking at the very edge of the surface layer with a chromogenic reagent. Should band separation be inadequate, the chromogenic-streaked zone is wiped from the plates and development continued until the desired separation is attained (7–18 h has been required with some systems). The

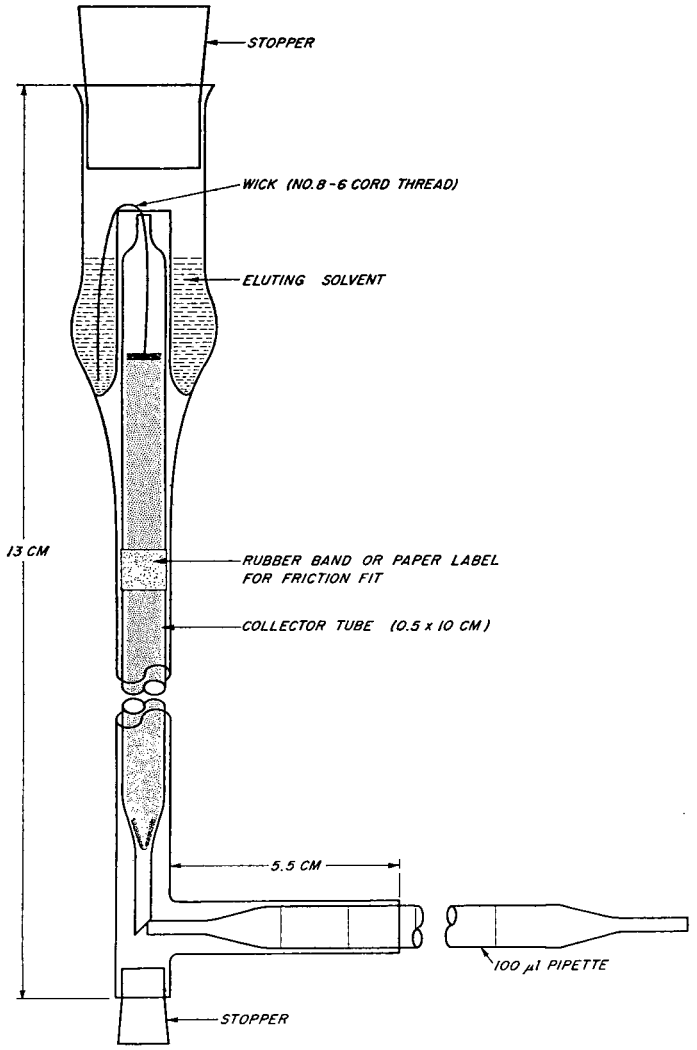

Fig. 2. Apparatus for elution with volatile solvents.

plates are then air dried to remove solvent, and chromogenic reagents if necessary are streaked at intervals in a direction perpendicular to the separated sample bands. The sample bands between the indicating streaks are removed from the plates as described below.

Removal and elution of bands

Bands developed on thin-layer plates in isolation work were removed from the plates and the adsorbed material eluted by the method developed for quantitative thin-layer chromatography[2], with suitable changes in the apparatus to accommodate larger amounts of surface layer and volatile organic solvents. The vacuum-cleaner-type collection tube was made longer and sometimes of greater diameter; a 10 × 0.5 cm (O.D.) tube would hold the absorbent from a 10 × 1.5 to 2 cm band of 0.5 mm thick absorbent layer. The eluting apparatus (see Fig. 2) was extended and fitted with a sidearm for the pipette, and both open ends of the apparatus were loosely stoppered. By removing the lower stopper, micro drops of eluate were taken to spot on thin-layer plates in order to test for completeness of elution. Wicks were washed in the organic solvent and stored dry. More than one thread was used if elution did not start in $^1/_2$–1 h.

Sesquiterpene alcohols on alumina were eluted with methylene chloride; the materials were similarly recovered from potassium bromide pellets which had been used in the determination of their infrared spectra, the pellet being ground to a powder prior to elution. Approximately 1 mg was obtained from each 10 × 0.5 cm tube, 200 μl of solvent being required to elute it from alumina, much less from potassium bromide (compare JANAK's elution from a capillary tube with a few drops[9]). A *p*-coumaroyl ester was similarly eluted from silica with acetone.

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Column chromatography of tryptophan and some related indoles

A chromatographic separation of 5-hydroxytryptophan and tryptophan dissolved in water has been described by CONTRACTOR[1]. This note shows that the procedure can be extended to the quantitative separation of a greater range of indoles in water or in a complex biological system.

Experimental and results

The method was similar to that of CONTRACTOR[1], except that Amberlite CG-50 100 to 200 mesh resin was used instead of 400 mesh.

The results of two typical experiments are shown in Figs. 1 and 2.

Discussion

The preparative separation of indolic metabolites of tryptophan has mainly been achieved by extraction with organic solvents followed by paper chromatography (for a review, see SANDLER[2]). These procedures allow only small amounts of material to be processed at one time, and the recoveries of the final purified products tend to be poor. A group separation of indoles from urine has been described by SCHLOSSBERGER, KUCH AND BUHROW[3]. They used a Sephadex column but were unable to separate 5-hydroxytryptophan from 5-hydroxyindoleacetic acid.

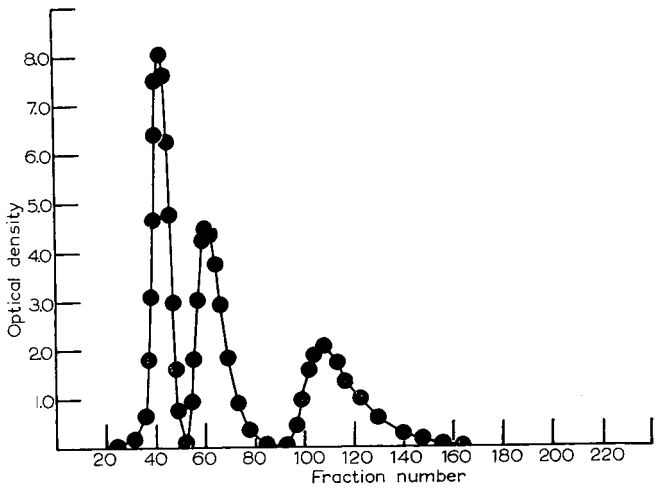

Fig. 1. Chromatographic separation on Amberlite CG-50 resin, 100–200 mesh, H^+ form, pH 7. Column dimensions = 57 × 1 cm. A mixture of 5.05 mg of 5-hydroxyindoleacetic acid, 5.1 mg of 5-hydroxytryptophan and 4.34 mg of tryptophan in 0.001 *N* HCl (0.5 ml) was quantitatively transferred to the column. Flow rate = 2 ml/min. 2-ml fractions were collected. Optical density of each fraction was determined at 275 mμ. Identity of the peaks was established by two-dimensional paper chromatography[4]. Recovery of 5-hydroxyindoleacetic acid (first peak) = 98%, 5-hydroxytryptophan (second peak) = 86% and tryptophan (third peak) = 92%.

Chromatography on CG-50 resin offers many advantages. Indoles can be separated quantitatively from a water solution in a single chromatogram (Fig. 1), or for example in the presence of the contaminants of an enzyme reaction mixture, by successive chromatograms (Fig. 2). Comparatively large amounts of indoles can be obtained in a pure state using this technique.

The 100–200 mesh resin used in the present study is easier to prepare and has a faster flow rate than the 400 mesh resin used previously (CONTRACTOR[1]), whilst possessing similar properties of resolution. The mild conditions provided by using water as an eluant for these labil ecompounds lead to high recoveries.

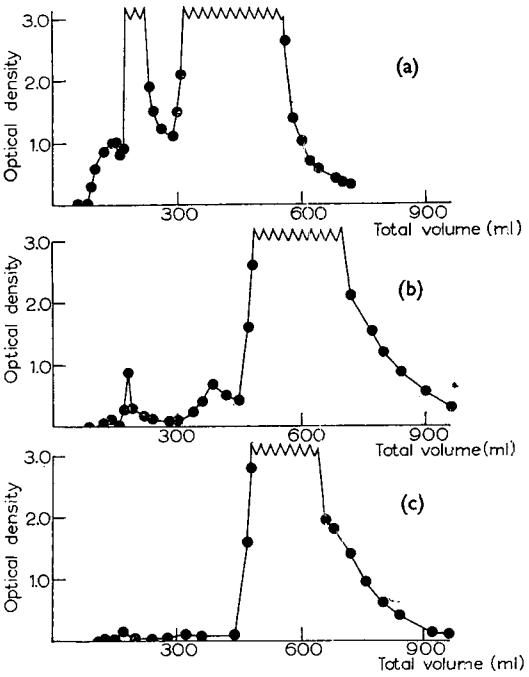

Fig. 2. Successive chromatographic separation of D-5-hydroxytryptophan after incubation of DL-5-hydroxytryptophan (103 mg) with snake venom L-amino acid oxidase[5]. Amberlite CG-50 resin 100–200 mesh, H^+ form, pH 7. Column dimensions = 57 × 2.5 cm. Optical density of each fraction was determined at 275 mμ. Identity of the peaks was established by two-dimensional paper chromatography[4]. (a) Lyophilised reaction mixture was dissolved in a minimal volume of 0.001 *N* HCl and was quantitatively transferred to the column. Flow rate = 2 ml/min. 3-ml fractions were collected. D-5-Hydroxytryptophan and 5-hydroxyindoleacetic acid were present in peak 3 only. (b) Peak 3 (vol. 300–660 ml) of chromatogram (a) was lyophilised and transferred to a fresh column. Flow rate = 2 ml/min. 2-ml fractions were collected. 5-Hydroxyindoleacetic acid and D-5-hydroxytryptophan were present in peaks 2 and 3, respectively. (c) Peak 3 (vol. 460–1000 ml) of chromatogram (b) was lyophilised and transferred to a fresh column. Flow rate = 4 ml/min. 2-ml fractions were collected. Only D-5-hydroxytryptophan was present in the peak. Overall recovery of D-5-hydroxytryptophan at this stage = 68%. For final purification, traces of L-5-hydroxytryptophan were removed by incubating with guinea-pig kidney L-aromatic amino-acid decarboxylase[6], and after lyophilisation, passing through a further column as in (a). Any 5-hydroxytryptamine thus formed is retained on the column.

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GAS CHROMATOGRAPHIC DETERMINATION OF POLYNUCLEAR HYDROCARBONS IN DUST

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The determination of polynuclear hydrocarbons in urban airborne particles can be performed by chromatography on alumina or silica gel, elution with suitable solvents, and determination of the various components either by ultraviolet or fluorescence spectroscopy. Several procedures following this scheme have been suggested[1–3]. Though gas chromatography has been successfully applied to the analysis of aliphatic and aromatic hydrocarbons, no previous work has been carried out on the application of this technique to the determination of polynuclear hydrocarbons in air pollution investigations.

The determination of some polycyclic hydrocarbons by gas chromatography in cigarette smoke[4], coal tar and mineral oil[5,6] has been reported.

The purpose of this work was the study of the operational conditions under which gas chromatography can be applied for the determination of polynuclear hydrocarbons in dust, to develop a method of analysis, and to evaluate the limitations and the advantages of the procedure. Attention has been also paid to the determination of alkanes as they have been found in noticeable amounts in urban dust.

The method has been developed with synthetic mixtures of polynuclear hydrocarbons and checked with air particulate samples obtained by a commercial high volume air sampler.

In order to find a rapid method for the determination of various components in atmospheric dust, some preliminary chromatograms were carried out directly on samples obtained by extraction of dust with cyclohexane and evaporation of the solvent. The large number of components found in dust, however, renders the chromatograms of the samples of little use, so that a preliminary fractionation is considered necessary. An efficient procedure for isolating the hydrocarbons consists in the use of successive extractions of the cyclohexane solution with methanol–water and nitromethane as suggested by HOFFMANN AND WYNDER[7]; in the former operation the cyclohexane phase is freed of hydrophilic compounds and in the latter the hydrocarbons are fractionated, the aromatics being extracted in the nitromethane phase.

EQUIPMENT AND MATERIALS

The gas chromatographic investigation was carried out with a Carlo Erba Fractovap C (mod. PAID/f), equipped with a flame ionisation detector and a linear programmed

temperature system. All the chromatograms reported were carried out with a glass capillary column (35 m long, 0.35 mm I.D.) coated with SE 30 silicone rubber. The working conditions were: column temperature, 200°; injector temperature, 250°; carrier gas, nitrogen at inlet pressure of 0.25 atm; column flow rate, 1 ml/min; splitter 1:100 ratio; sample size 0.5-2 μl of solution containing approximately from 0.5 to 2 μg/μl for each component. The column efficiency measured from the *n*-hexacosane peak is about 10,000 theoretical plates.

The dust sample, kindly supplied by Prof. D'AMBROSIO of the Laboratorio Provinciale Milano, had been previously analysed by paper chromatography and by spectrophotometry.

The solvents employed (cyclohexane (R. P. Carlo Erba), nitromethane (A.S.D.) and methanol (R.P. Carlo Erba)) were purified by fractional distillation, collecting the fractions with a constant boiling point. Their purity was tested periodically by gas chromatography; 50 ml of each solvent was evaporated to a very small volume and injected into the gas chromatograph operating under the usual working condition; no other peaks were observed.

METHOD

Dust samples (0.5–1.0 g) were extracted in a Soxhlet extraction apparatus with 100 ml cyclohexane for about 5 h; after this time the liquid portion, condensed in the thimble, did not show any fluorescence when exposed to ultraviolet light. The thimble was previously extracted with acetone.

The extract was evaporated to a volume of about 5 ml and shaken with a 5 ml mixture of methanol–water (4:1). The methanol extract was shaken three times with 5 ml portions of cyclohexane to recover the hydrocarbons. The separation of the phases was carried out by centrifugation in 25 ml tubes. The combined cyclohexane fractions were extracted 6 times with 5 ml of nitromethane. The cyclohexane and the nitromethane solutions containing respectively the aliphatic and the polycyclic hydrocarbons were evaporated to dryness under reduced pressure. The residues were dissolved with small volumes of ether and concentrated in micro test tubes to about 10 μl.

EXPERIMENTAL

Table I shows the relative retention volumes referred to chrysene ($V_R = 1.0$) of polynuclear hydrocarbons obtained under the above described working conditions. A higher column temperature (230°) was used to elute a certain number of less volatile hydrocarbons. These values show that a fairly good separation is obtained for most hydrocarbons and their identification therefore might be performed by gas chromatography.

The response of the hydrogen detector is proportional to the concentration of the various hydrocarbons but no simple relationship exists between the number of carbon atoms of various molecules and the response of the detector. For example, a mixture of pyrene and fluoranthene with the same molecular weight at the same molar concentration yields elution peaks with different areas, the former being about 1.4 times the latter.

TABLE I

RELATIVE RETENTION VOLUMES AND WEIGHT RESPONSE FACTOR OF POLYNUCLEAR HYDROCARBONS

No.	*Component*	V_R (200°)	A/A_{St}
1	Anthracene	0.13	—
2	Fluoranthene	0.31	1.20
3	Pyrene	0.36	1.61
4	1:2-Benzofluorene	0.48	—
5	1-Methylpyrene + 3-methylpyrene	0.54	0.82
6	Benzo[*ghi*]fluoranthene	0.82	—
7	1:2-Benzanthracene	0.98	—
8	Chrysene	1.00	0.72
9	7,12-Dimethyl-1:2-benzanthracene	2.36	—
10	2:3-Benzofluoranthene	2.63	0.78
11	3:4-Benzopyrene + 1:2-benzopyrene	3.10	0.60
12	Perylene	3.21	0.37
13	20-Methylcholanthrene	4.00	—
14	1,3,5-Triphenylbenzene	4.15	1.00
		V_R (230°)	
15	1:2;5:6-Dibenzanthracene	5.28	
16	1:12-Benzoperylene	5.92	
17	1:2;3:4-Dibenzopyrene	13.6	
18	1:2;4:5-Dibenzopyrene	15.2	
19	Coronene	15.5	

In order to apply gas chromatography to quantitative determinations it has been found necessary to find a suitable internal standard and to evaluate a correction factor for each hydrocarbon.

Several attempts have been made to select a suitable internal standard; 1,3,5-triphenylbenzene (FLUKA), was found to meet the requirements for this purpose. Its retention volume under the conditions described (200°) is 4.15 and is therefore quite different from those of the aromatic hydrocarbons tested.

The correction factors, determined from the ratio of the peak areas of the tested hydrocarbon and the peak area of the internal standard (A/A_{St}), at the same weight concentrations, are reported in Table I.

In artificial mixtures the determination of a hydrocarbon can be performed with an error of $\pm$ 3 %.

Dust samples have been extracted according to the procedure described and a typical gas chromatogram of the nitromethane fraction containing aromatic hydrocarbons is shown in Fig. 1. A large number of peaks is obtained and it seems that in dust, besides the polynuclear hydrocarbons identified by spectrophotometry, other compounds of the same class are present. In Table II are reported the retention volumes of the components eluted from the column. The concentrations of the hydrocarbons identified have been determined by the method described here and the results are compared with the values obtained by spectrophotometry.

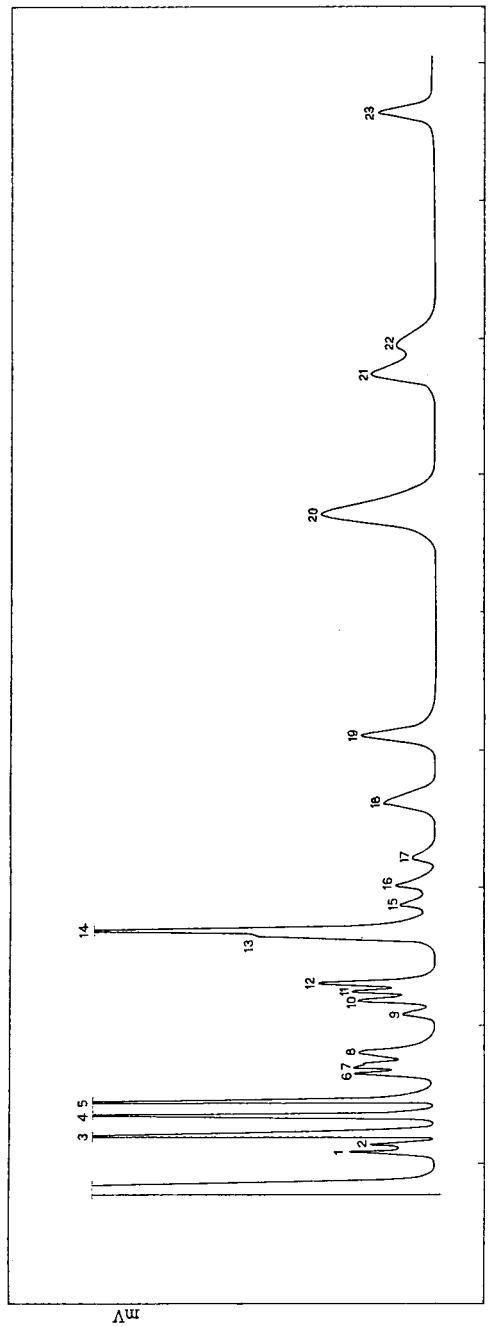

Fig. 1. Gas chromatogram of polynuclear hydrocarbons from a dust sample (nitromethane fraction). The peak numbers correspond to the components reported in Table II.

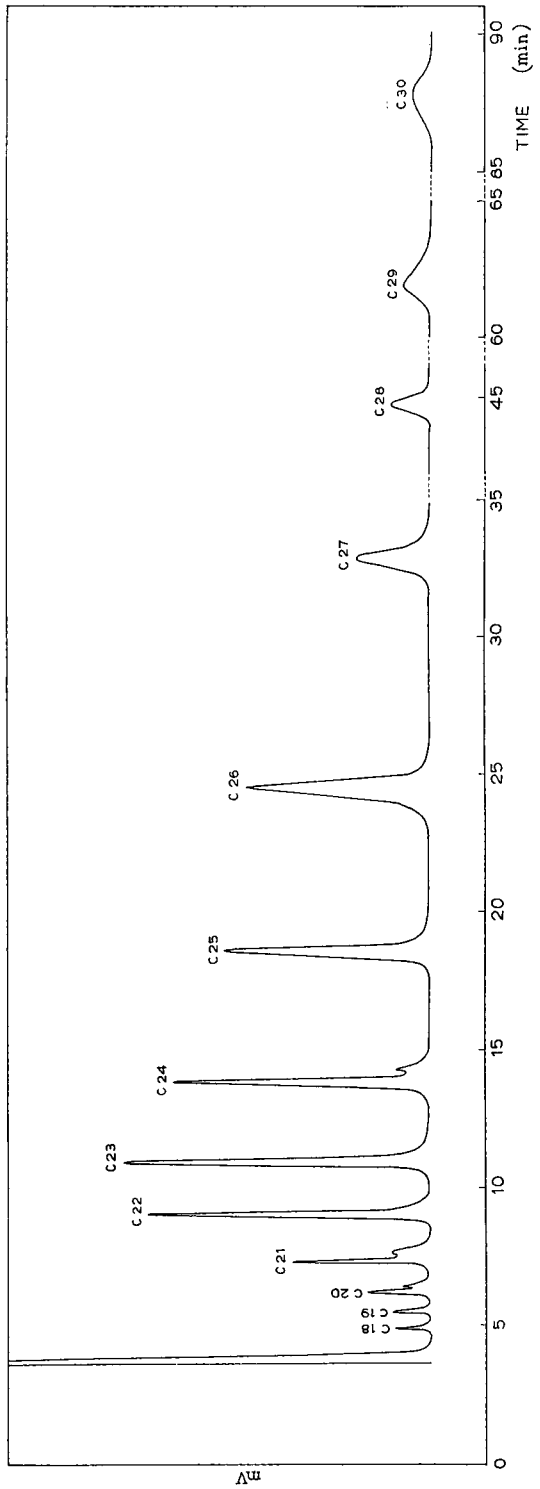

Fig. 2. Gas chromatogram of alkanes from a dust sample (cyclohexane fraction).

TABLE II

SPECTROPHOTOMETRIC AND GAS-CHROMATOGRAPHIC DETERMINATION OF POLYNUCLEAR HYDROCARBONS IN DUST ($\mu/\mu g$)

No.	Components	$V_{R(200°)}$	Sample A		Sample B		Sample C	
			GLC	SPCT	GLC	SPCT	GLC	SPCT
1	—	0.16						
2	—	0.19						
3	—	0.22						
4	Fluoranthene	0.31	115	126	76	60	32	46
5	Pyrene	0.36	114	130.4	78	70.5	37	49.6
6	—	0.45						
7	1:2-Benzofluorene	0.48	Trace	—	Trace	—	Trace	—
8	1-Methylpyrene + 3-methylpyrene	0.54	70	—	80	—	50	—
9	—	0.68						
10	—	0.73						
11	—	0.77						
12	Benzo[*ghi*]fluoranthene	0.82	Trace	—	Trace	—	Trace	—
13	1:2-Benzanthracene	0.98	620	133.2	410	134.9	315	80
14	Chrysene	1.00		—		—		—
15	—	1.09						
16	—	1.18						
17	—	1.29						
18	—	1.50						
19	—	1.76						
20	2:3-Benzofluoranthene	2.63	364	—	277	—	313	—
21	3:4-Benzopyrene + 1:2-benzopyrene	3.10	282	241.5	245	262.3	205	181.2
22	Perylene	3.21	202	—	122	—	135	—
23	1,3,5-Triphenylbenzene	4.15						
24	1:12-Benzoperylene	5.92 (230°)		49.6		58.3		41.3
25	Coronene	15.5 (230°)		10.4		10.8		9.5

Analysis of the cyclohexane fraction

The cyclohexane fraction has been analysed by gas chromatography under the same working conditions, and a typical chromatogram of this fraction is shown in Fig. 2. In order to identify these peaks, a series of alkanes (C_{18},C_{20},C_{24},C_{28}) was chromatographed and the plot of the logarithm of the relative retention volume *versus* number of carbon atoms yielded a straight line. The retention volumes due to peaks of the cyclohexane fractions reported in Table III, fall on this line and correspond to alkanes from C_{18} to C_{30}, with even and uneven number of carbon atoms. These compounds seem to be normal components of the urban dust as have been found in most samples. As alkanes have been identified in cigarette smoke[4] and in coal tar[6] their presence in the dust might be attributed to these sources.

DISCUSSION

The method which has been suggested, consisting of a gas chromatographic determination of a cyclohexane extract preceded by an extraction of hydrophilic compounds, seems to be quite reliable for the determination of polynuclear hydrocarbons in air.

TABLE III

RELATIVE RETENTION VOLUMES OF NORMAL PARAFFINS FROM DUST EXTRACT. CYCLOHEXANE FRACTION

Fraction	V_R	*Fraction*	V_R
C_{18}	0.13	C_{25}	1.50
C_{19}	0.19	C_{26}	2.08
C_{20}	0.26	C_{27}	2.90
C_{21}	0.37	C_{28}	4.00
C_{22}	0.55	C_{29}	5.50
C_{23}	0.73	C_{30}	8.05
C_{24}	1.00		

The recovery of the polycyclic hydrocarbons from the dust is practically quantitative and a complete separation is achieved between the aliphatic and the aromatic fraction. Previous experiments performed using a short alumina column to isolate the polynuclear hydrocarbons from the dust extract did not yield satisfactory results since the high boiling alkanes were eluted together with some polynuclear hydrocarbons; the procedure moreover was time consuming.

The gas chromatographic procedure in comparison with the column adsorption–spectrophotometric method has the limitation that some pairs of hydrocarbons are not separated and yield a single elution peak. They are 1-methylpyrene and 3-methylpyrene, 1:2-benzanthracene and chrysene, 3:4-benzopyrene and 1:2-benzopyrene.

It should be pointed out, however, that by changing the liquid phase (as observed using an Apiezon column) some of these components can be separated.

It seems therefore that by carrying out a gas chromatographic analysis with two liquid phases, such as Silicone rubber S.E. 30 and Apiezon, a complete picture of the composition of polynuclear hydrocarbons in dust should be obtained.

It is convenient to perform the analysis of the nitromethane fraction at two temperatures, 200° and 230°. The use of a higher working temperature is required for the elution of compounds such as coronene; at this temperature the elution of the higher boiling components is rendered easier but a poor resolution for the hydrocarbons which boil at a lower temperature is obtained.

When enough information is available on the retention volumes of various hydrocarbons, chromatograms with programmed temperature can be performed with considerable success.

The agreement between the gas chromatographic and the spectrophotometric methods is fair with some hydrocarbons but not too satisfactory with others. Since both methods present some limitations, an extensive comparative investigation seems desirable.

At present the method which has been developed offers two main advantages: a high speed of analysis and the possibility of realizing a clear separation for various components. The time of analysis of the nitromethane fraction of the dust is less than two hours and might be shortened by carrying out the gas chromatogram at a programmed temperature.

The determination of polynuclear hydrocarbons may become a routine measurement, carried out by non-technical personnel. An interesting feature of the method is

the possibility of detecting and identifying other polycyclic hydrocarbons possibly present in the atmosphere and of following the probable changes. An extensive application of this method might be of a noticeable help in increasing our knowledge of the components of urban airborne particles.

SUMMARY

The gas chromatographic separation of polynuclear and paraffinic hydrocarbons is reported. A procedure for their separation from dust samples is described. The gas chromatographic results are compared with the spectrophotometric data, and the advantages and the limitations of both methods are discussed.

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AMINO ACIDS AND PEPTIDES

II. GAS CHROMATOGRAPHY OF AMINO ALCOHOLS*

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INTRODUCTION

In recent years gas chromatography has become an important analytical tool. Although this technique is best suited to liquids, the method has been applied to the solid amino acids. To date all of the previously reported studies have been carried out on derivatives in which the amino and/or carboxyl group is usually masked or removed by a variety of chemical subterfuges. Modified systems have included methyl esters[1–3], butyl ester hydrochlorides[4], N-acetyl esters[5,6], N-trifluoroacetyl esters (N-TFA)[1,7–12], N-formyl esters[13], N-trimethylsilyl trimethylsilyl esters[14], α-hydroxy esters[15,16], α-chloro esters[17], phenylthiohydantoin and dinitrophenyl methyl ester derivatives[18–20], aldehydes[21–23], amines[24] and nitriles[25].

These various schemes have been utilized only to a very limited extent in current biochemical problems. One practical example involved the use of the dinitrophenyl methyl ester method to identify the individual amino acids formed during the hydrolysis of the polypeptide Gramicidin A[26]. In another study a variety of amino acid intermediates, used in the preparation of the hydroxyprolines, were chromatographed in the form of their acetyl and trifluoroacetyl methyl esters[27]. Recently, the N-trifluoroacetyl ester method was applied to measure the extent of racemization in peptide synthesis[28].

The N-acetyl-amino ester procedure was utilized to determine some protein and polypeptide hydrolysates some time ago[29]. Synthetic di- and polypeptides were analyzed by means of N-TFA esters[8] and O-trimethylsilyl N-TFA methyl esters[30]. Modified determinations were based on polyamino alcohols or polyamines[31] and α-chloroacids[32].

In the belief that removal of the carboxyl function in the amino acids would lead to a volatile series of compounds, the *amino alcohols* were thought to be suitable candidates for gas chromatographic analysis. These materials were previously prepared by hydride reduction of various amino acid esters and were shown to exist as either liquids or low melting solids[33–37]. To date no systematic examination of this class of compounds has been reported in the literature.

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* For the previous paper in this series, see: W. W. LEE, G. T. TONG, A. P. MARTINEZ, B. WEINSTEIN, M. G. M. SCHELSTRAETE, B. R. BAKER AND L. GOODMAN, *J. Med. Chem.*, 6 (1963) 439.

RESULTS AND DISCUSSION

The simplest amino alcohol—2-aminoethanol (*i.e.*, the glycine analog)—was systematically screened against the following stationary phases: apiezon (L), methyl silicone fluid (SF-96), cyano silicone fluid (XF-1150), fluoro silicone (FS-1265 or QF-1), methyl silicone gum (SE-30), methyl phenyl silicone gum (SE-52), cyano silicone gum (XE-60), carbowax (20M), polyglycol (UCON 50 HB-5100 and LB-1715), diethyleneglycol succinate (DEGS), ethyleneglycol succinate (EGS), didecyl phthalate (DDP), bis-2-ethylhexyl tetrachlorophthalate (EHCP), cyclohexyldimethanol succinate (CDMS), phenyldiethanolamine succinate (PDEAS), crosslinked diethyleneglycol adipate (LAC-2-R446), sucrose acetate isobutyrate (SAIB), *m*-polyphenyl ether, hallcomid (M-18), polyamide (Versamid 900), nonylphenoxypolyethylene ethanol (Igepal CO-880), tricresyl phosphate (TCP), 1,2,3-tris-(2-cyanoethoxy)-propane (TCEP) and nonylphenol.

A satisfactory peak was obtained from SF-96, XF-1150, FS-1265, SE-52, XE-60, UCON 5100, UCON 1715, DPP, Igepal and TCP. As a result, these ten substrates were treated with eight additional and different amino alcohols. FS-1265 and Igepal were soon discarded, since they did not discriminate between glycinol and alaninol, while TCP was eliminated because the retention time for glycinol was too long for practical work (11.1 min at 105°). The data for XF-1150, XE-60 and UCON 1715 were in the same category, and do not appear unusually noteworthy at this time.

TABLE I

RETENTION TIMES OF VARIOUS AMINO ALCOHOLS

$$R = -CH\begin{matrix}CH_2OH \\ NH_2\end{matrix}$$

	Column	*SF-96*		*SE-52*		*UCON*	*DDP*
Amino alcohol	*Flow rate helium, ml/min*	*60*	*60*	*60*	*60*	*60*	*75*
	Oven temp., °C	*170*	*200*	*155*	*200*	*178*	*120*
Glycinol	RH	1.0	A	1.6	A	2.6	3.0
Alaninol	RCH_3	1.2	A	1.9	A	2.9	4.0
Valinol	$RCH(CH_3)_2$	2.9	A	4.9	A	4.8	9.4
Leucinol	$RCH(CH_3)CH_2CH_3$	3.7	A	6.6	3.4	11.6	F
Serinol	RCH_2OH	3.9	2.6	8.2	3.3	C	G
Isoleucinol	$RCH_2CH(CH_3)_2$	5.1	2.8	9.8	4.5	5.7	H
Methioninol	$RCH_2CH_2SCH_3$	16.2	7.4	26.1	12.1	39.3	I
Phenylalaninol	$RCH_2C_6H_5$	22.6	9.0	37.1	17.2	D	I
Tryptophanol	$RCH_2C_8H_6N$	A	B	A	B	E	A

A = Compound not injected upon the column.
B = Nothing detected during a run of 150 min.
C = Nothing detected during a run of 30 min.
D = Broad peak centered at 55.3 min.
E = Nothing detected during a run of 70 min.
F = Time of peak maximum varied considerably; usually about 23.2 min.
G = Nothing detected during a run of 45 min.
H = Time of peak maximum varied considerably; usually about 22.1 min.
I = Nothing detected during a run of 90 min.

DDP and UCON 5100 were useful, but were rejected since these columns were operated at temperatures where bleeding of the liquid phase material was a definite problem. The two most outstanding phases were SF-96 and SE-52. The value of these two phases for the various amino alcohols was attributed to a combination of their low polarity and thermal stability properties.

Retention time data in minutes for these last four packings are given in Table I. Tryptophanol was not successfully chromatographed on any of the liquid phases. The separations can no doubt be improved by a temperature programming sequence and moving the analysis to a higher temperature. It is quite possible that chromatography over a lightly loaded column (*i.e.*, 2 % substrate) would produce even better results. We intend to investigate these factors at a later date and also will attempt the mass spectroscopy analysis of the amino alcohols. From a practical viewpoint these early results are encouraging to us, but at this time they do not replace the demonstrated utility of the N-TFA ester systems.

EXPERIMENTAL

Apparatus and procedure

A Wilkens Aerograph Model A-90-P2 with a thermal conductivity detection system was used in this work. Each phase was deposited as a 20 % by weight coating over Chromosorb W, 60–80 mesh; the solid support was previously deactivated with hexamethyldisilazane. The column was 2.0 m long by 6.35 mm outside diameter. The retention times given in Table I were measured for each compound from the moment of injection to the center of each sample peak. Each value represented the average of at least three determinations. Sample size was of the order of 0.2 μl.

Materials

Alaninol, isoleucinol, leucinol, methionol, phenylalaninol, serinol, tryptophanol and *valinol* were purchased from the Research Organic Chemicals Co., 3101 Floye Drive, Los Angeles 46, Calif. *Glycinol* was a Matheson Coleman and Bell product.

ACKNOWLEDGEMENT

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SUMMARY

Amino acids may be reduced by lithium aluminium hydride to afford the corresponding amino alcohols. The amino alcohols are generally high boiling liquids or low melting solids. These compounds may be gas chromatographed over a variety of substrates. Two liquid phases, SF-96 and SE-52, separated in a short time period seven different amino alcohols. This method is an alternative to other analytical schemes for amino acids previously described in the literature.

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SCHWEFEL-HETEROCYCLEN UND VORSTUFEN

32. MITT. DÜNNSCHICHTCHROMATOGRAPHISCH-SPEKTROSKOPISCHE UNTERSUCHUNGEN AN SCHWEFEL-HETEROCYCLEN*

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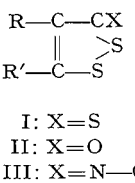
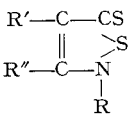
EINLEITUNG

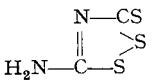
Während schon vereinzelt[1] mit Erfolg die Dünnschichtchromatographie zur Untersuchung und Identifizierung schwefelorganischer Verbindungen herangezogen wurde, ist über eine diesbezügliche Bearbeitung der Schwefelheterocyclen vom Typ der Trithione (I) [1,2-Dithiolthione-(3)], der Dithione (II) [1,2-Dithiol-one-(3)], deren Anile (III), sowie über 1,2-Thiazolinthione-(5) (IV) und Xanthanwasserstoff (V) bisher nicht berichtet worden. Lediglich die Säulenchromatographie fand schon frühzeitig Anwendung[2]. Gelegentlich wurde auch die Papierchromatographie herangezogen, so beispielsweise für die Trennung und den Nachweis in der Natur vorkommender Trithione[3].



I: X=S
II: X=O
III: X=N—C_6H_5



IV



V

Wie wir im folgenden zeigen, erwies sich die Dünnschichtchromatographie in Verbindung mit der Elektronenspektroskopie bei einfachster Handhabung und geringem Zeitaufwand als nützliches und sehr leistungsfähiges Hilfsmittel zur Isolierung und Identifizierung obiger Schwefelheterocyclen, wie auch zur Trennung und Produktanalyse komplizierter Gemische. Die Kombination mit der Elektronenspektroskopie ist insofern bei den genannten Heterocyclen günstig, als diese, wie schon in einer grundlegenden Arbeit für den Typ I festgestellt worden war[4], zwischen 200 und 500 mμ charakteristisch absorbieren. Zudem können die erforderlichen Substanzmengen mit einfachsten Mitteln direkt aus den Dünnschichtchromatogrammen gewonnen werden.

Die Kenntnis des R_F-Wertes und des Elektronenabsorptionsspektrums genügte in allen bisher untersuchten Fällen zur eindeutigen Identifizierung bekannter Systeme.

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** Teilweise Diplomarbeit, Technische Universität Dresden, 1963.

EXPERIMENTELLES

Substanzen

Für die Untersuchungen standen Schwefelheterocyclen aus unserem Arbeitskreis zur Verfügung, deren Synthese bereits hinreichend von anderen Autoren oder von uns (vgl. dazu Zit. 5–9) beschrieben wurde. Die Spektroskopie konnte zu Vergleichszwecken analysenreine und dünnschichtchromatographisch als einheitlich gesicherte oder dünnschichtchromatographisch von Verunreinigungen befreite Verbindungen einsetzen.

Dünnschichtchromatographie

Unter Berücksichtigung allgemeiner Erfahrungen[10] wurden Glasplatten der Abmessung 20 cm × 20 cm mit Kieselgel G nach STAHL (Fa. E. Merck) in einer Schichtdicke von 250 μ präpariert, 15 Min. an der Luft getrocknet und dann etwa 30 Min. bei 120–130° aktiviert. Da sich die Verbindungen I, II und III in Benzol, I und II mit polaren Substituenten speziell in Aceton und IV in Methylenchlorid oder Chloroform hinreichend lösen, trägt man sie vorteilhaft in diesen Solventien in einer Zeitspanne von 10 Min. auf (Luftfeuchtigkeit: 60–65 %). Für V konnten wir kein geeignetes Lösungsmittel finden. Entwickelt wurde grundsätzlich unter Kammersättigung bei einer Laufstrecke von 10 cm und einer Laufzeit von 20–30 Min.

Für die Trennung relativ unpolarer Trithione (I) eigneten sich Petroläther–Benzol (1:1) (Fliessmittel A) oder CS_2 (Fliessmittel B). Andere Gemische ergaben wesentlich schlechtere Trenneffekte (vgl. Fig. 1, aus der die Abhängigkeit des R_F-

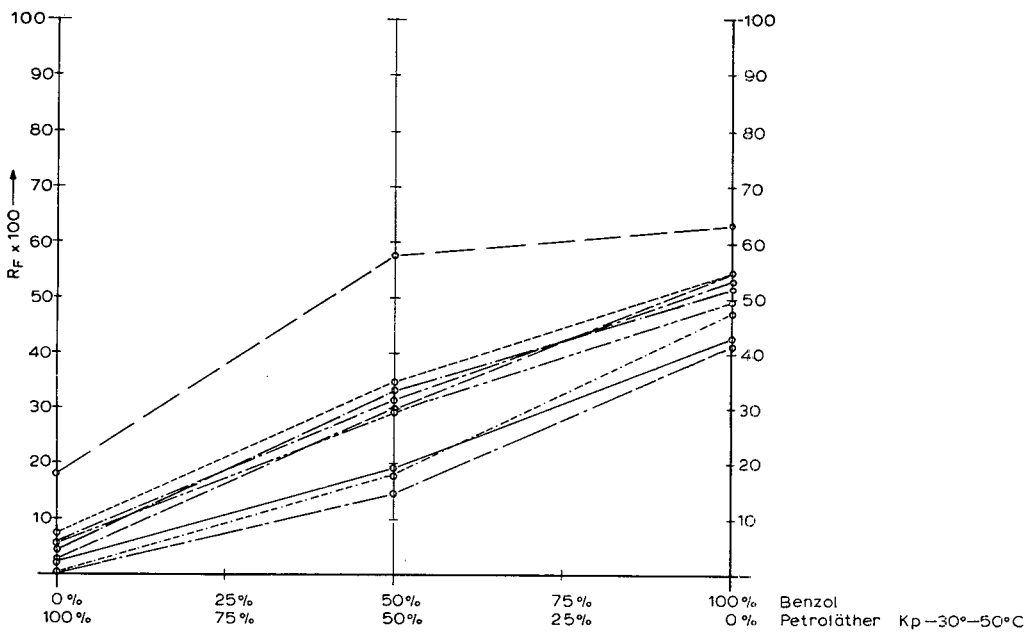


Fig. 1. Abhängigkeit der R_F-Werte von der Zusammensetzung des Fliessmittels A bei Benzotrithion (— —), 4-Äthyltrithion (– – – –), Tetramethylentrithion (—·—), 5-Phenyltrithion (———) 4-*p*-Tolyltrithion (—————), 4-Methyltrithion (———), Trithion (——), Safroltrithion (—··—) und 5-*p*-Methoxyphenyltrithion (————).

Wertes von der Zusammensetzung des Lösungsmittelsystems Petroläther–Benzol hervorgeht). Zur Trennung der Trithione (I) mit polaren Gruppierungen und der Isothiazolinthione (IV) benutzten wir Benzol–Essigester (3:1) (Fliessmittel C). Aceton fand bei mit OH- und COOH-Gruppen substituierten Heterocyclen Anwendung.

Die Einzelkomponenten sind in der Regel farbig und so auf der Platte direkt zu erkennen. Analytische Bedeutung hat aber die Beobachtung, dass Tetracyanoäthylen mit I, II und IV charakteristische Farbreaktionen gibt, die vermutlich auf charge-transfer-Komplexen beruhen (Tabelle I).

TABELLE I

FARBREAKTIONEN MIT TETRACYANOÄTHYLEN

Verbindung	*Farbe*		
	in Methylenchlorid	*auf Kieselgel sofort nach dem Besprühen*	*auf Kieselgel nach 1 min.*
Trithione (I)	rotbraun	rot	braungelb
Dithione (II)	orange	orange	grüngelb
Isothiazolinthione (IV)	intensiv blau	intensiv blau	grüngelb

Im Gegensatz zu I und II bildet IV zudem Farbkomplexe mit Chinon (orange), Chloranil (rot) und Trinitrobenzol (orange).

Diese Farbeffekte sind so auffallend unterschiedlich, dass sie bereits eindeutige Hinweise über den Verbindungstyp geben. Für die synthetisch-präparative Praxis ist dies eine wesentliche Erleichterung.

Stehen nur Quecksilbersalzaddukte der obigen Heterocyclen zur Verfügung, trägt man diese in acetonischer Lösung auf, behandelt den Startfleck mit Schwefelwasserstoffgas und entwickelt die dann in Freiheit gesetzten Heterocyclen wie oben beschrieben.

Elektronenspektroskopie

Zur elektronenabsorptions-spektroskopischen Untersuchung wurden die Einzelkomponenten mitsamt der jeweiligen Kieselgelzone abgetrennt, dann mit spektroskopisch reinem Cyclohexan oder 96 %igem Äthanol extrahiert und das Lösungsmittel durch Zentrifugieren vom Kieselgel befreit. Die Messungen erfolgten mit dem Gitterspektrographen CF 4 der Fa. Optica Milano in 10^{-3}–10^{-4} molarer Lösung. Es wurden nur intensive Banden mit $\log \varepsilon > 3$ angegeben, wobei die Fehler der aufgeführten Wellenlängen und molaren Extinktionskoeffizienten kleiner als 1 mμ bzw. 0.05 $\log \varepsilon$ Einheiten sind.

ERGEBNISSE UND DISKUSSION

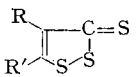
R_F-Werte

Die ermittelten R_F-Werte sind in den Tabellen II und III zusammengestellt. Obwohl nicht in allen Fällen ausgeprägte Unterschiede vorhanden sind, ist doch in Verbindung mit der Spektroskopie eine eindeutige Charakterisierung möglich. 4-Alkylsubstituierte Trithione erfüllen annähernd die MARTINsche Beziehung, nicht dagegen 4,5-Polymethylentrithione.

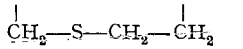
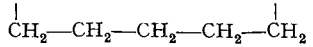
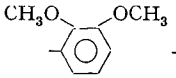
(Fortsetzung des Textes S. 162

TABELLE II

R_F-WERTE UND SPEKTRALE DATEN VON TRITHIONEN UND DEREN ANILE

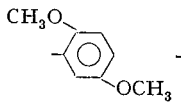
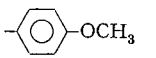
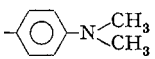
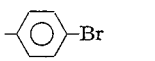
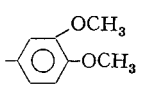
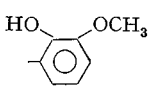
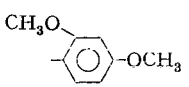
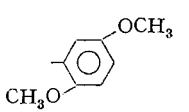
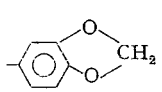


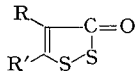
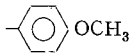
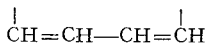
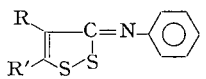
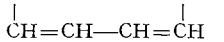
R	R′	$R_F \times 100$ Fliessmittel A	B	C	Lösungsmittel*	Charakteristische Absorptionen $\lambda_{max}(m\mu)$ (log ε)
—H	—H	19	14	63	CH	229 (3.88), 251 (3.92), 336 (3.80), 415 (3.83)[a]
					Ä	225 (3.90); 244 (3.73), 321 (3.61), 410 (3.99)
—H	$-CH_3$	—	—	—	I	223 (4.0), 248 (3.9), 326 (3.8), 413 (3.9)[12]
$-CH_3$	—H	29	23	67	CH	223 (3.92), 249 (3.86), 329 (3.79), 415 (3.89)
					Ä	221 (4.03), 241 sh** (3.75), 270 (3.51), 317 (3.57), 410 (3.95)
$-C_2H_5$	—H	35	33	> 70	CH	226 (4.03), 249 (4.00), 271 sh (3.75), 328 (3.92), 415 (3.98)
					Ä	224 (4.08), 241 sh (3.90), 271 (3.80), 315 (3.81), 410 (4.00)
$-CH_3$	$-CH_3$	27	21	> 70	CH	226 (3.99), 249 (3.96), 270 (3.83), 321 (3.89), 414 (3.96)
					Ä	224 (3.99), 240 sh (3.77), 276 (3.78), 310 (3.67), 409 (3.97)
—*tert.*-C_4H_9	$-CH_3$	—	—	—	I	227 (4.1), 250 (4.1), 269 (3.9), 311 (3.7), 415 (4.1)[12]
—*neo*-C_5H_{11}	—H	—	—	—	I	229 (4.0), 250 (3.9), 326 (3.8), 423 (3.9)[12]
$CH_2—CH_2—CH_2$		26	19	69	CH	221 (3.92), 250 (3.90), 278 (3.74), 340 (3.90), 413 (3.87)
					Ä	221 (3.96), 241 (3.74), 283 (3.74), 330 (3.73), 410 (4.01)
$S—CH_2—CH_2$		32	—	—	CH	430
$CH_2—S—CH_2$		16	—	—	CH	412
$S—CH=CH$		57	—	—	CH	434 sh, 450
$CH_2—CH_2—CH_2—CH_2$		33	24	> 70	CH	229 (3.93), 249 (3.88), 275 (3.76), 327 (3.83), 411 (3.93)
					Ä	225 (3.99), 240 sh (3.76), 279 (3.83), 314 (3.72), 408 (4.02)
$CH=CH—CH=CH$		58	53	> 70	CH	225 (4.01) 245, (4.05), 277 (4.12), 284 (4.13), 309 (3.75), 320 (3.75), 433 (3.85), 450 (3.88)
					Ä	224 (4.03), 244 (3.89), 274 (4.01), 284 (3.99), 290 (3.97), 309 sh (3.74), 431 sh (3.88), 448 (3.93)
$N=CH—CH=CH$		—	—	—	M	245, 290, 456 (3.88)[13]

		35	20	> 70	CH Ä	230 (3.94), 249 (3.85), 268sh (3.69), 323 (3.75), 411 (3.85) 228 (4.09), 244sh (3.73), 274 (3.71), 313 (3.67), 408 (4.01)
		—	—	—	M	290 (3.77), 324 (3.70), 420 (3.99)[4]
		—	—	—	I M	225 (4.01), 248 (3.94), 281 (3.78), 342 (3.87), 417 (3.82)[14] 225 (4.04), 240sh (3.82), 285 (3.79), 330 (3.71), 411 (3.95)
		32	20	> 70	CH Ä	230 (3.98), 250 (3.94), 276 (3.81), 320 (3.84), 418 (3.93) 229 (4.10), 243sh (3.92), 280 (3.93), 310 (3.83), 412 (4.04)
—H	—COOH	0	0	0[b]	CH Ä	245, 271sh, 339, 445 238 (3.97), 270sh (3.90), 313sh (3.41), 320 (3.43), 437 (3.70)
—H	$-CONH_2$	0	0	24	Ä	240 (4.06), 265sh (3.94), 321 (3.31), 440 (3.82)
—H	$-COOC_2H_5$	22	14	> 70	CH Ä	244 (4.15), 272 (3.88), 337 (3.42), 455 (3.75) 240 (4.03), 271 (3.88), 327 (3.32), 450 (3.78)
$-CH_3$	$-COOCH_3$	—	—	—	M	315 (3.35), 445 (3.88)[4]
	$-COOCH_3$	—	—	—	M	322 (3.47), 450 (3.82)[4]
—CN	$-NH_2$	0	0	11	Ä	235 (4.04), 280sh (3.91), 310 (4.15), 367 (3.94)
$-CONH_2$	$-NH_2$	0	0	16	CH Ä	229, 264sh, 282, 309sh, 400 233 (4.07), 284sh (4.00), 313 (4.18), 363 (3.95)
$-COOC_2H_5$	$-NH_2$	0	0	37	CH Ä	248, 266sh, 287, 329, 393 248 (4.11), 289sh (4.17), 311 (4.26), 373 (3.88)
$-COOC_2H_5$	$-NHCOCH_3$	7	0	58	Ä	231 (4.06), 247sh (4.06), 292 (4.03), 342 (3.56), 394 (3.98)
$-COOC_2H_5$					M	305 (4.03), 427 (3.90)[4]
	—H	22	31	> 70	CH Ä	243 (4.09), 283 (3.57), 335 (3.71), 428 (3.71) 238 (4.12), 281 (3.66), 318 (3.63), 420 (3.89)
	—H	30	21	> 70	CH Ä	220sh (4.29), 247 (4.15), 282sh (3.58), 338 (3.77), 430 (3.80) 217sh (4.25), 236sh (4.19), 240 (4.16), 282 (3.62), 320 (3.64), 422 (3.88)
	—H	—	—	—	M	232 (4.27), 315 (3.71), 423 (3.94)[15]
	—H	—	—	—	M	317 (3.67), 418 (3.94)[15]

(Fortsetzung S. 158)

TABELLE II (*Fortsetzung*)

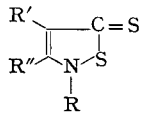
R	*R'*	$R_F \times 100$ *Fliessmittel* A	B	C	*Lösungsmittel**	*Charakteristische Absorptionen* $\lambda_{max}(m\mu)$ (log ε)
	—H	—	—	—	M	315 (3.91), 418 (3.90)[15]
—H		32	17	> 70	CH Ä	230 (4.00), 252 (4.09), 284sh (4.14), 309 (4.25), 437 (3.90) 229 (4.11), 248sh (4.01), 272 (4.03), 316 (4.28), 433 (4.02)
—H		15	5	68	CH Ä	236, 256sh, 278sh, 338, 440 233 (4.08), 267sh (3.89), 349 (4.27), 430 (4.00)
—H		—	—	—	M	320 (3.66), 455 (4.56)[4]
—H		37	22	> 70	CH Ä	232 (4.21), 254 (4.13), 288sh (4.22), 315 (4.34), 439 (3.97) 230 (4.15), 279 (4.03), 321 (4.28), 436 (3.98)
—H		—	—	—	M	230 (4.10), 256 (4.11), 356 (4.22), 432 (4.12)[15]
—H		—	—	—	M	335 (3.94), 473 (4.34)[15]
—H		—	—	—	M	230 (3.89), 258 (3.90), 365 (4.17), 435 (4.06)[15]
—H		—	—	—	M	270 (4.08), 314 (4.08), 440 (410)[15]
—H		18	8	> 70	CH Ä	250, 348, 280sh, 437 230 (4.06), 254 (4.10), 268sh (4.06), 363 (4.19), 431 (4.02)
—CH_3		38	29	> 70	CH Ä	214 (4.16), 250 (4.11), 293 (4.07), 311sh (3.95), 423 (3.96) 215 (4.20), 242sh (4.06), 300 (4.09), 420 (4.02)

		31	14	> 70	CH Ä	234sh (4.36), 260 (4.23), 322 (4.10), 437 (3.99) 244sh (4.44), 257sh (4.11), 310 (4.09), 430 (3.95)
						
—H	—H	—	—	—	CH Ä	226 (3.35), 310 (3.60) 230 (3.33), 310 (3.67)
—H	—COOH	0	0	0[c]	Ä	246 (3.66), 343 (3.60)
—H	—$CONH_2$	0	0	20	Ä	247 (3.80), 348 (3.61)
—H	—$COOC_2H_5$	26	22	66	CH Ä	250 (3.71), 358 (3.53) 250 (3.68), 354 (3.56)
—H		33	27	> 70	CH Ä	277 (4.04), 328sh (3.36) 286 (4.09), 336sh (3.56)
—H		—	—	—	M	225 (3.85), 310 (4.25)[15]
		37	35	> 70	CH Ä	238 (4.19), 254sh (3.75), 266 (3.74), 350 (3.56) 239 (4.18), 254sh (3.79), 354 (3.55)
						
		30	20	> 70	CH Ä	237 (4.20), 365 (3.61) 239 (4.16), 362 (3.59)
—H		33	27	> 70	CH Ä	236 (4.41), 365 (3.84) 240 (4.36), 362 (3.80).

[a] Vgl. Zit. 16. λ_{max} in CH: 230 (2.87), 254 (3.92), 336 (3.81), 415 (3.83).
[b] R_F-Wert in Aceton als Fliessmittel: 18.
[c] R_F-Wert in Aceton als Fliessmittel: 26.
* CH = Cyclohexan; Ä = Äthanol; M = Methanol; I = Isooctan.
** sh = Schulter.

TABELLE III

R_F-WERTE UND SPEKTRALE DATEN VON 1,2-THIAZOLINTHIONEN-(5)



R	R′	R″	$R_F \times 100$ Fliessmittel A	B	C	Lösungsmittel*	Charakteristische Absorptionen λ_{max} (mμ) (log ε)
—CH(CH$_3$)$_2$	—CH$_3$	—CH$_3$	—	—	—	CH Ä	255 (3.78), 303 (3.14), 388 (4.26) 258 (3.75), 366 (4.25)
—CH$_2$CH$_2$OH	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		0	0	4	Ä	260 (3.84), 365 (4.21)
—CH$_2$CH$_2$OCOCH$_3$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	21	CH Ä	256, 305sh**, 385 261 (3.81), 368 (4.23)
—CH$_2$CH$_2$OCO—C$_6$H$_5$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	30	CH Ä	228, 257, 274sh, 282, 384 229 (4.20), 261 (3.88), 280sh (3.26), 367 (4.25)
—CH$_2$CH$_2$CH$_3$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	31	CH Ä	255 (3.80), 304sh (3.10), 385 (4.29) 260 (3.83), 370 (4.28)
—CH$_2$CH(CH$_3$)$_2$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	30	CH Ä	256 (3.80), 303 (3.15), 385 (4.25) 260 (3.74), 367 (4.23)
—CH$_2$CH=CH$_2$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	33	CH Ä	252 (3.78), 306sh (3.10), 383 (4.25) 260 (3.79), 367 (4.21)
—CH$_2$CH$_2$CH$_2$CH$_3$	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—		<5	<5	36	CH Ä	256 (3.85), 307sh (3.18), 386 (4.30) 260 (3.84), 367 (4.31)

—CH_2—	—CH_2—CH_2—CH_2—CH_2—	> 5	< 5	50	CH Ä	254 (3.79), 386 (4.20) 261 (3.82), 369 (4.27)
—CH_2CH_2—	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	44	CH Ä	253, 388 261 (3.81), 368 (4.25)
	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	54	CH Ä	254 (3.83), 310 (3.08), 387 (4.24) 260 (3.78), 369 (4.31)
	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	56	CH Ä	255 (3.86), 386 (4.26) 260 (3.79), 370 (4.28)
	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	54	CH Ä	228sh (4.24), 254 (3.86), 386 (4.26) 221sh (4.23), 260 (3.83), 370 (4.26)
	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	42	CH Ä	254, 305, 386 260 (3.70), 370 (4.25)
	—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	52	CH Ä	258 (4.00), 290sh (3.64), 393(4.31) 262 (3.96), 377 (4.27)
	—CH_2—CH_2—CH_2—CH_2—CH_2—	< 5	< 5	44	CH Ä	250 (3.80), 393 (3.03), 390 (4.22) 260 (3.83), 366 (4.24)

*CH = Cyclohexan; Ä = Äthanol
**sh = Schulter.

Übereinstimmend mit der allgemeinen Erfahrung erniedrigen polare Gruppierungen in I und II, wie NH_2, $CONH_2$, COOH und OH, stark die R_F-Werte.

5-Phenyltrithione mit *para*-ständigen Substituenten am Benzolkern und Trithione mit Elektronenacceptoren zeigen eine Änderung der R_F-Werte in der BROCKMANN-schen Reihe: Br > H > 3,4-OCH_2O > OCH_3; $COOC_2H_5$ > $CONH_2$ > COOH.

Jod- und Quecksilberchlorid-Addukte der Trithione, wie das J_2-Addukt von Trithion und $HgCl_2$-Addukt von 4,5-Dimethyltrithion, werden bei Verwendung der Fliessmittel A oder B gespalten (vgl. Zit. 4) und geben R_F-Werte, die mit denen der reinen Trithione übereinstimmen. Die R_F-Werte der Isothiazolinthione (IV) sind gegenüber denen der Trithione deutlich verschieden, so dass eine Trennung und verlässliche Charakterisierung leicht möglich ist. Phenylsubstituenten am Typ IV bewirken eine Erhöhung des R_F-Wertes.

Aufschlussreich ist in diesem Zusammenhang ein Vergleich isomerer oder isoelektronischer pseudoaromatischer Verbindungen. Es findet sich bei den verschiedensten Typen ein regelmässiger Gang, wobei ein Austausch eines Ringschwefels gegen eine C–C-Doppelbindung eine Erhöhung des R_F-Wertes bewirkt. Die erhöhte Elektronendichte und die leichtere Polarisierbarkeit des Ringschwefels gegenüber der C–C-Doppelbindung erklären diese Unterschiede.

Am besten wird dies an einigen Beispielen deutlich (Zahlenangaben: R_F-Wert × 100, Fliesmittel A):

Thieno[*b*]trithion (57) < Benzotrithion (58) < Dithiochromon (60);
Benzo-1,3-dithiol-2-thion (Benzoisotrithion) (56) < Benzotrithion (58);
Benzoisotrithion (56) < Dithiocumarin (58);
Dithiocumarin (58) < Dithiochromon (60);
Cyclopenta[*c*]thiopyran (Isothialen) (62) < Azulen (68);
Thiophthalsäureanhydrid (10) < Naphthochinon-(1,4) (11).

Diese Parallelen sind sicher weitgehend verallgemeinerungsfähig. Die Kenntnis solcher Gesetzmässigkeiten gestattet eine Abschätzung der R_F-Werte unbekannter oder noch nicht vermessener Systeme und ist eine wesentliche Hilfe in der Laboratoriumspraxis.

Elektronenabsorptionsspektren

Trithione und heteroanaloge Verbindungen zeigen in der Regel eine intensive langwellige Absorption, deren analytischer Wert frühzeitig erkannt wurde[4,11]. In der Praxis scheitert ein spektroskopischer Nachweis aber oftmals an störenden Fremdabsorptionen, die zumindest den kurzwelligeren Bereich umfassen. Nach einer dünnschichtchromatographischen Trennung stehen aber die Spektren jeder Komponente eindeutig im gesamten Messbereich zur Verfügung. Durch Wahl verschiedener Lösungsmittel und damit Kenntnis des Lösungsmitteleffektes wird die Auswertung zudem erleichtert.

In den Tabellen II und III sind charakteristische Absorptionen in unpolaren Kohlenwasserstoffen und in den polaren, protonisierenden Alkoholen angegeben, wobei auch Literaturwerte berücksichtigt wurden.

Die Tabelle IV gibt für einige schon eingehender vermessene Trithione (I) und Isothiazolinthione (IV) charakteristische Absorptionsbereiche sowie die mittlere Verschiebung der Bandenlage beim Wechsel vom Cyclohexan zum 96 %igem Äthanol wieder. Einige weitere allgemeine Regelmässigkeiten seien kurz zusammengefasst.

TABELLE IV

CHARAKTERISTISCHE ABSORPTIONSBEREICHE UND LÖSUNGSMITTELEFFEKT EINIGER TRITHIONE (I) UND 1,2-THIAZOLINTHIONE-5 (IV)

Verbindung	*Lösungsmittel*	*Substitutionstyp*	*Bandenbezeichnung*	*Absorptionsbereich für Maxima (mμ)*	*(Mittlere) Verschiebung beim Wechsel vom Cyclohexan zum Äthanol (cm^{-1})*
Trithion (I)	Äthanol	unsubstituiert	I	410	300 (hypsochrom)
			II	321	1400 (hypsochrom)
			III	244	1150 (hypsochrom)
			IV	225	750 (hypsochrom)
	Cyclohexan, Isooctan	Alkyl-	I	413–423	300 (hypsochrom)
			II	311–329	1200 (hypsochrom)
			III	248–250	1400 (hypsochrom)
			IV	223–229	450 (hypsochrom)
	Cyclohexan, Isooctan	4,5-Polymethylen-	I	411–417	350 (hypsochrom)
			II	320–342	1050 (hypsochrom)
			III	268–278*	550 (bathochrom)
			IV	248–250	1300 (hypsochrom)
			V	221–230	(nicht charakteristisch)
	Äthanol, Methanol	4-Aryl-	I	418–423	450 (hypsochrom)
			II	315–320	1600 (hypsochrom) (bei Phenylsubstitution)
	Äthanol, Methanol	5-Aryl-	I	420–473	200 (hypsochrom)
			II	300–365	700 (bathochrom) (bei Phenylsubstitution)
1,2-Thiazolinthion-(5) (IV)	Äthanol	4,5-Tetramethylen-2-alkyl- oder -aralkyl-	I	366–370	1300 (hypsochrom)
			II	260–261	900 (bathochrom)

* Teilweise als Inflexion.

Trithione. Die Bande I (Bandenbezeichnung siehe Tabelle IV) wird charakteristisch vom Lösungsmittel beeinflusst und ist zum Nachweis der Trithione geeignet. Sie wurde unter 400 mμ lediglich bei den tabellierten 5-Aminotrithionen in polaren Lösungsmitteln beobachtet. Eine Rotverschiebung tritt auf, wenn in 5-Stellung eine Substitution mit Carbonyl-($COOC_2H_5$ > $CONH_2$ > COOH) oder Arylgruppen erfolgt bzw. in 4,5-Stellung aromatische oder pseudoaromatische Ringe ankondensiert sind. Auch bei den isoelektronischen Dithiopyronen-(4) findet man einen ähnlichen Substituenteneinfluss (Lösungsmittel: Äthanol, Angaben in mμ) Trithion: 410, 5-Phenyltrithion: 430, Benzotrithion: 450, Dithiopyron-(4): 381[17], 2-Phenyldithiopyron-(4): 396[17], Benzodithiopyron-(4): 415. Der Bandentyp II ist empfindlich gegen Struktureinflüsse. Alkylsubstitution führt zu einer hypsochromen, zunehmende Spannung eines ankondensierten hydrierten Ringsystems zu einer bathochromen Verschiebung dieser Bande. 5-Aryltrithione zeigen eine auffallend intensive Bande II, deren Lage noch von den Substituenten am Arylrest bestimmt wird[4,15].

Heteroanaloge Trithione. Wie aus Tabelle II ersichtlich ist, tritt bei Austausch von C = S gegen C = N oder C = O eine hypsochrome Verschiebung des langwelligsten Absorptionsmaximums ein (S > $N-C_6H_5$ > O). Auch ringstickstoffhaltige Trithionanaloge geben die erste Bande bei kürzeren Wellenlängen als die Trithione (vgl. Tabelle III; Absorptionsmaxima des Xanthanwasserstoffs (V) in Äthanol: 230 (3.85), 286 (4.28), 310 sh (3.87), 355 (3.80)).

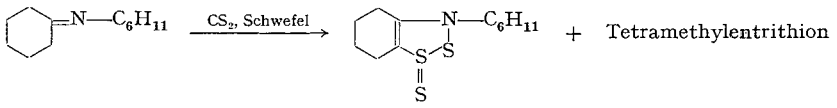
ANWENDUNGSBEISPIELE

Der Wert einer kombinierten dünnschichtchromatographisch-spektroskopischen Untersuchung sei an 3 Beispielen verdeutlicht:

(1) Bei der thermischen Schwefelung von 1-Methylcyclohexen-(1) entstehen Tetramethylentrithion und Benzotrithion[6]. Je nach den Bedingungen ist das Trithiongemisch, das auch bei den Dehydrierversuchen des Tetramethylentrithions mit Schwefel von Interesse ist[6], unterschiedlich zusammengesetzt. Fig. 2 gibt das Spektrum eines aus nahezu gleichen Teilen beider Komponenten bestehenden Gemisches wieder, in dem die spektralen Charakteristika beider Trithione überlagert sind. Eine quantitative Trennung mit Hilfe der Säulenchromatographie versagt. Nach der Dünnschichtchromatographie sind die Einzelspektren eindeutig (Fig. 2) und auch quantitativ auszuwerten. Es sind noch 0.5 % Benzotrithion neben 99.5 % Tetramethylentrithion nachzuweisen und zu erfassen. Der Zeitaufwand für die Trennung und Charakterisierung des Gemisches beträgt etwa 2 Std. bei einer reinen Arbeitszeit von etwa 30 Min.

(2) Bei der Umsetzung des aus Cyclohexanon und Cyclohexylamin erhaltenen Ketimins mit CS_2 und Schwefel[7], enstand ein säulenchromatographisch gereinigtes Isothiazolinthion, das aber, wie aus der dünnschichtchromatographisch-spektroskopischen Untersuchung hervorging, noch Tetramethylentrithion enthielt (Fig. 3).

Die Anwesenheit des Trithions war allein aus den Spektren des Gemisches noch nicht offensichtlich.



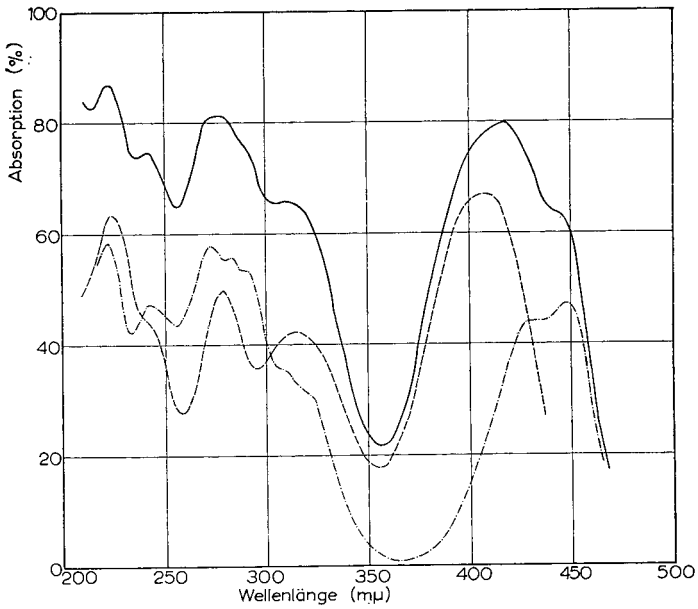


Fig. 2. Absorptionsspektren eines Gemisches von Tetramethylen- und Benzotrithion (1:1) (——) und der chromatographisch getrennten Komponenten Tetramethylentrithion (– – –) und Benzotrithion (–·–·) in Äthanol.

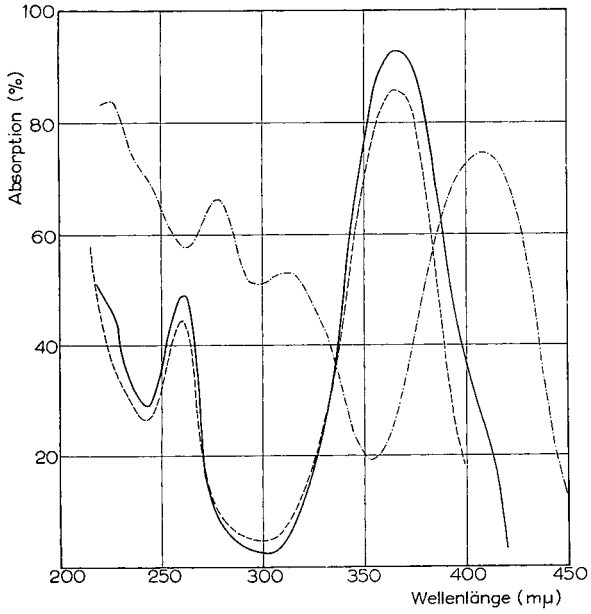


Fig. 3. Absorptionsspektren des Rohproduktes der Umsetzung von N-Cyclohexyl-cyclohexanonimin mit Schwefelkohlenstoff und Schwefel (——) und der chromatographisch abgetrennten Fraktionen Tetramethylentrithion (–·–·–) und 2-Cyclohexyl-3,4-tetramethylenisothiazolinthion-(5) (— — —) in Äthanol.

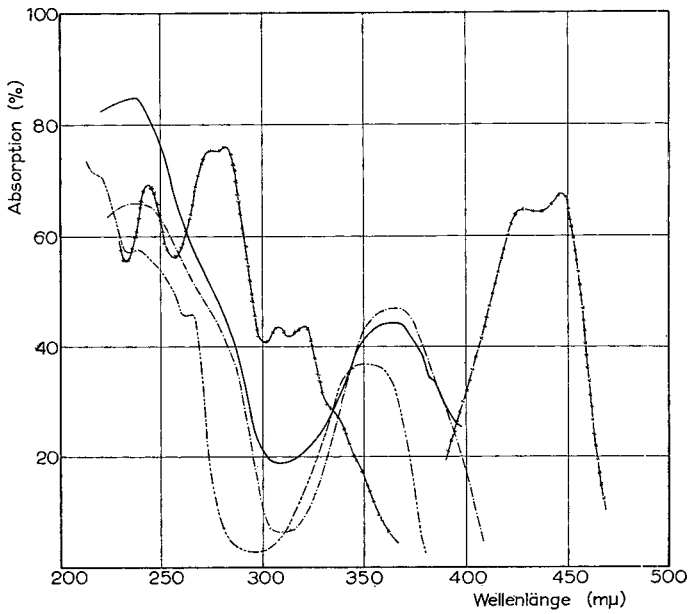
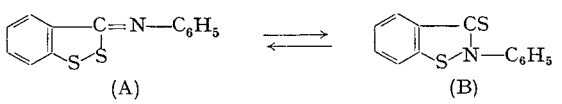


Fig. 4. Absorptionsspektren des Rohproduktes nach CLELLAND UND SALKELD[18] (——), das fol, gende Fraktionen nach der chromatographischen Trennung ergab: Benzodithionanil (–·–·–)- Benzodithion (–· ·–· ·–), und Benzotrithion (–×–×–).

In Fliessmittel B ist sowohl in diesem als auch in vorstehendem Beispiel der Schwefel abtrenn- und nachweisbar ($R_F \times 100$: 95).

(3) Nach der Literatur[18] soll das Anil (A) des Benzotrithions mit dem isomeren Benzoisothiazolinthion (B) in Gleichgewicht stehen ("dynamic equilibrium".).



(A) (B)

Wir haben A nach verschiedenen Methoden (aus Benzotrithion nach CLELLAND UND SALKELD[18], aus S-Methylbenzotrithioniumjodid aus 3-Chlorbenzo-1,2-dithioliumchlorid[8] hergestellt und mit Hilfe der Dünnschichtchromatographie gereinigt. Tatsächlich sind in dem umkristallisierten Produkt noch andere Substanzen vorhanden, die aber nicht B, sondern Benzodithion und Benzotrithion sind (Fig. 4). Wie aus den chromatographisch-spektroskopischen Untersuchungen eindeutig folgt, ist A unter den Bedingungen stabil und nach der Reinigung auch einheitlich. Das Gleichgewicht A $\rightleftharpoons$ B liess sich in keinem Falle bestätigen.

DANK

Für unermüdliche Mithilfe bei der Anfertigung der Spektren möchten wir Frl. I. JOCHEM herzlichst danken. Unser Dank gilt auch staatlichen Stellen der D.D.R. für die grosszügige Unterstützung der Arbeit.

ZUSAMMENFASSUNG

Trithione (I), Dithione (II), deren Anile (III) sowie die heteroanalogen 1,2-Thiazolinthione-(5) (IV) und Xanthanwasserstoff (V) wurden dünnschichtchromatographisch untersucht, spektroskopisch zwischen 210 und 500 mμ vermessen und hinsichtlich ihrer R_F-Werte und spektralen Absorptionen verglichen. Die Brauchbarkeit einer kombinierten dünnschichtchromatographischen und spektroskopischen Arbeitsweise zur Trennung, zum Nachweis und zur Identifizierung wird aufgezeigt.

SUMMARY

1,2-Dithiole-3-thiones, 1,2-dithiol-3-ones and their anils, as well as the hetero-analogues 1,2-thiazoline-5-thiones and xanthanic acid were investigated by thin-layer chromatography, and by spectroscopy in the region 210–500 mμ. The R_F values and the spectral data of these compounds were compared and it was found that correlations with the chemical structure exist. It was shown that thin-layer chromatography in combination with spectroscopy can be used to separate, detect and identify these compounds.

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THE SEPARATION OF HEXOSEPHOSPHATES AND TRIOSEPHOSPHATES BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Thin-layer chromatography has been used for the separation of a wide variety of substances[1-8]. Ease and simplicity of operation, rapidity and versatility are its outstanding characteristics. In the separation of sugars and sugar derivatives, as STAHL AND KALTENBACH[6] have noted, conversion from paper or column chromatography to thin-layer chromatography (TLC) cannot be accomplished without some modification of solvent systems, adsorbents and techniques. STAHL AND KALTENBACH[6] separated free sugars on Kieselgur G. DEFERRARI *et al.*[7] and GEE[8] used silica gel with a starch binder. After a preliminary investigation of silica gel in our laboratory, it was determined that cellulose powder without a binder was a suitable adsorbent for the TLC of sugar phosphates. A two-dimensional development, utilizing an acid with a water-immiscible solvent followed by a water-miscible system at right angles, gave small, concentrated spots with adequate resolution.

MATERIALS

Adsorbent

Cellulose powder MN 300 (particle size $< 10\,\mu$) – Machery, Nagel and Co., Düren, Germany. Obtained from Brinkmann Instruments, Inc., N.Y.

Solvents

Phase I[9]. Water-poor phase from mixture of: 60 ml *tert.*-amyl alcohol, redistilled 101.8°, 30 ml water + 2 g *p*-toluenesulfonic acid. In all solvent systems, the water employed was distilled-demineralized.

Phase II[10]. 66 ml isobutyric acid, redistilled 154.4°, 1 ml concentrated ammonium hydroxide, 33 ml water.

Solvents were obtained from Distillation Products Industries, Rochester, New York. Chemicals were reagent grade, used as received.

Apparatus

Rectangular chromatojars, 20 × 20 cm glass plates, 250 μ fixed-thickness spreader and aligning tray were purchased from Research Specialties Company, Richmond, Calif. Model No. V41 Mineralight (2537 Å) was obtained from Ultra Violet Products, Inc., South Pasadena, Calif.

Standards

Hexosephosphates and triosephosphates, Grade A purity, were purchased from Sigma Chemical Company, St. Louis, Mo., and decationized with the use of Dowex 50-X1, 50–100 mesh, hydrogen form. Standards were freshly prepared in concentrations from 20–300 mg/ml in aqueous solution for application.

Spray reagents

A. 0.2 *M* *m*-phenylenediamine dihydrochloride in 76 % ethanol[11].

B. 5 ml 60 % perchloric acid, 10 ml 1 *N* HCl, 25 ml 4 % ammonium molybdate, 60 ml H_2O[9].

C. 10 % $SnCl_2 \cdot H_2O$ in concentrated HCl freshly diluted 200 fold with 0.5 *M* H_2SO_4[12].

Other spray reagents were used[13–16] in the identification of individual phosphates, but were not incorporated into the routine detection procedure. Sprays were applied with the Universal Aerosol Spray Kit obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

METHODS

Preparation of plates

The plates were coated with a slurry prepared by mixing 15 g of cellulose powder and 90 ml of water with a mortar and pestle. After allowing 10 min at room temperature for the cellulose to set, the plates were activated by heating at 105° for 2 h. Until used, the prepared plates were stored over a desiccant in a closed chamber.

A suitable aliquot (1–10 λ) of each standard or mixture was applied to the lower left corner of a plate, approximately 2 cm from the cellulose edges, with a glass capillary. The spots were permitted to dry between applications to minimize origin size.

Development

Phase I solvent system was allowed a 30 min period to saturate the atmosphere in a chromatojar before chromatography of the plate was initiated. The plates were positioned in the jar for ascending development with the solvent flowing in the direction of slurry application. Six to 8 h were required for the solvent front to move 16–18 cm. The plates were then air dried at room temperature, usually overnight.

Phase II solvent system was given a 15 min saturation period in the chromatojar. The plates were placed at right angles to the first run for ascending development. Two to 4 h were required for the solvent front to move 14–18 cm. After air-drying with a hair drier, the plates were ready for the detection procedure.

Detection and identification

Detection of the hexosephosphates and triosephosphates was accomplished with a series of spray reagents which gave characteristic color responses (see Table I). R_F values and the position of individual standards relative to that of inorganic phosphate further aided in the identification of spots. The sprays were successively applied to each plate according to the following procedure and spots were marked as visualized.

Spray reagent A. After spraying, the plates were heated for 5 min at 110°. They were then viewed under ultraviolet light.

Spray reagent B. After spraying, the plates were dried with the hair drier before

heating for 5–8 min. at 110–120°. Sufficient heating was noted when the edges of the cellulose began to char.

Spray reagent C. This reagent was applied while the plates were still warm from the previous heating. While wet, the spots and colors were readily distinguished. As the plates dried, the background became pale violet. A subsequent spray with concentrated ammonium hydroxide restored the light background, but caused all spots to become dark blue.

RESULTS

The results are presented in Tables I and II and Fig. 1.

TABLE I

COLOR RESPONSES OF HEXOSEPHOSPHATES AND TRIOSEPHOSPHATES FOLLOWING APPLICATION OF INDICATED SPRAY REAGENTS

*Compound**	*Spray A*** (under ultraviolet)*	*Spray B*** (daylight)*	*Spray C*** (daylight)*
PEP	U.V. absorbing spot	Blue	Blue
P_i	Not visible	Yellow-green	Yellow-green
2-PG; 3-PG	Not visible	Blue	Pink
F-6-P	Blue fluorescent	Blue-green	Blue-green
FDP	Not visible	Blue	Grey-green
G-1-P	Not visible	Yellow-green	Yellow-green
G-6-P	Blue fluorescent	Green	Green
Background	Violet	White	Light-blue, violet when dry***

* Abbreviations: PEP = phosphoenolpyruvic acid; P_i = inorganic phosphate; 2-PG = 2-phosphoglyceric acid; 3-PG = 3-phosphoglyceric acid; F-6-P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate.

** See text for details.

*** After plate was completely dried, background was violet and spots were obscured to some extent. A light spray with concentrated NH_4OH restored the pale background and all phosphates were blue.

TABLE II

R_F AND R_{PO_4} VALUES IN PHASE I AND PHASE II SOLVENT SYSTEMS

*Compound**	*Phase I solvent system***		*Phase II solvent system****	
	R_F	R_{PO_4}	R_F	R_{PO_4}
PEP	0.87	1.18	0.23	0.90
P_i	0.74	1.00	0.27	1.00
2-PG; 3-PG	0.68	0.92	0.24	0.94
F-6-P	0.41	0.56	0.20	0.81
FDP	0.34	0.46	0.13	0.52
G-1-P	0.32	0.43	0.27	1.06
G-6-P	0.29	0.39	0.17	0.68

* See Table I for explanation of abbreviations.

** *tert.*-Amyl alcohol–water–*p*-toluenesulfonic acid (see text).

*** Isobutyric acid–ammonium hydroxide–water (see text).

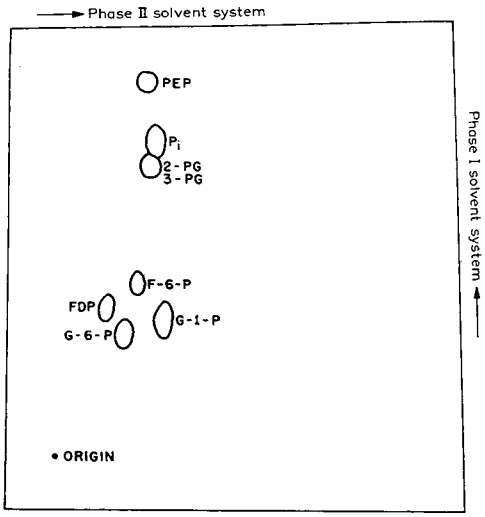

Fig. 1. Tracing of thin-layer chromatoplate following separation of hexosephosphates and triosephosphates. PEP = phosphoenolpyruvic acid; P_i = inorganic phosphate; 2-PG = 2-phosphoglyceric acid; 3-PG = 3-phosphoglyceric acid; F-6-P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate.

DISCUSSION

Experience in the laboratory has shown that methods for the separation of hexosephosphates and triosephosphates, which may be successfully applied in paper chromatography, may not be carried over to TLC with the same degree of success. The work reported is the culmination of many trials with different adsorbents, solvent systems and detection methods. It is pertinent to report these failures as well, since others may be spared the time spent in testing them.

It was found in these studies that the MN 300 cellulose powder gave the best resolution of the adsorbents tested, but was not free of impurities. Thus, in the development of each solvent system a yellow substance was observed which advanced with the front, but appeared not to interfere with the separation of the sugar phosphates. Attempts to remove the impurity by shaking or extracting the cellulose with diethyl ether were not successful. While cellulose for TLC obtained from Research Specialties Corporation, Richmond, Calif., was found to have fewer impurities, the resolutions were not as good as with the MN 300. Similarly, when Avicel (a microcrystalline cellulose sold by American Viscose Company) was tried as an adsorbent, the plates appeared thin, there were no impurities, and the resolution was not as good as was obtained with MN 300.

The solvent systems BANDURSKI AND AXELROD[17] and HANES AND ISHERWOOD[9] used in their paper chromatography methods failed to provide adequate separations on TLC, except for the *tert.*-amyl alcohol–*p*-toluenesulfonic acid–water system suggested by the latter authors. Furthermore, varying the proportions of *tert.*-amyl alcohol, water and *p*-toluenesulfonic acid in the Phase I system as well as the isobutyric acid, ammonia and water in the Phase II system did not improve the results. If anything, the results were not as good as those obtained with the solvent systems described as Phase I and Phase II.

Attempts were made to separate 3-phosphoglyceric and 2-phosphoglyceric acids, but these compounds migrated together while separating from the other sugar phosphates. Glyceraldehyde-3-phosphate appeared to separate, but four spots were obtained with the preparation used in these studies. Further attempts will be made to purify the preparation so that positive identification may be made. However, despite these difficulties, the good resolution of hexosephosphates and some triosephosphates offers a useful tool in the study of these compounds.

SUMMARY

The separation of hexosephosphates and triosephosphates may be obtained by use of TLC, using a cellulose adsorbent without binder. While the hexosephosphates resolved into well-defined spots, 3-phosphoglyceric acid and 2-phosphoglyceric acid did not separate.

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THE QUANTITATIVE SEPARATION AND ESTIMATION BY THIN-LAYER CHROMATOGRAPHY OF LIPIDS IN NERVOUS TISSUE

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During the last four years there has been a great increase in the use of thin-layer chromatography for the separation of lipids but for brain lipids reports have been concerned mainly with the procedure as a qualitative rather than as a quantitative technique.

JATZKEWITZ[1] has determined the eight sphingolipids in brain, quantitatively, by scraping off the silicic acid containing the separated fractions from the plate and estimating the lipids colorimetrically. In 1962, HONEGGER[2] published a semi-quantitative method of estimation of brain lipids by visual comparison of spot densities on thin-layer chromatoplates. DAVISON AND GRAHAM-WOLFAARD[3] have also determined brain lipids quantitatively by thin-layer chromatography, but their method also involves scraping off the silicic acid as well as the lipid and then transfering the "scraped off" material to columns for elution before hydrolysing the lipids for analysis.

The procedure to be described below is considered to be much simpler in that lipids are determined directly on the plate. It is an application of methods by ZÖLLNER, WOLFRAM AND AMIN[4] who estimated cholesterol esters, and PRIVETT, BLANK AND LUNDBERG[5] who estimated mono-, di- and triglycerides by spraying with sulphuric acid and scanning the separated spots with a densitometer.

Two methods for scanning thin-layer chromatograms have recently been published. CSALLANY AND DRAPER[6] coat the silicic acid layer with Neatan and remove it from the plate before scanning, SQUIBB[7] uses a clear plastic onto which the silicic acid is bonded; this can then be cut into strips prior to scanning in standard equipment.

MATERIALS AND EQUIPMENT

Merck's Kieselgel G spread on glass plates 20 cm × 20 cm, in layers about 250 μ thick. Plates are activated 1 h before use at 120° for 30 min. Chloroform, methanol G.P.R., *n*-propanol, distilled once and 12.5 % aqueous ammonia.

The samples were applied to the plate with an Agla micrometer syringe mounted vertically on the coarse adjustment of an old microscope. This enables the sample to be applied to the plate without scratching the surface of the silicic acid.

The chromatoplate is placed on a bed which moves over a scale so that samples are always applied in exactly the same positions.

The scanner is of the reflectance type (Fig. 1) and is manually operated. Rays from the light source strike the plate at right angles and are reflected onto photocells from which the signal passes through an amplifier and is registered on a voltmeter.

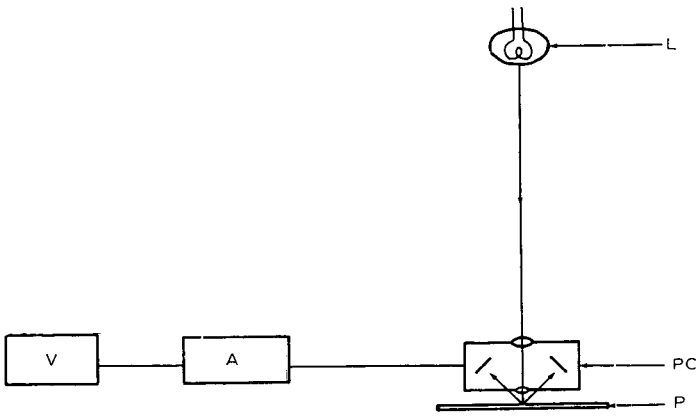

Fig. 1. Block diagram of scanner. A = amplifier; L = light source; P = plate; PC = photocell; V = voltmeter.

METHOD

Total lipids are extracted from freeze-dried brain with $CHCl_3$–MeOH (2:1, v/v) in the ratio of 20 ml of solvent to 1 g of the original wet tissue. An aliquot of the extract (equivalent to 150–200 μg of total lipid) is applied to the silicic acid as a narrow band (0.8 cm × 0.1 cm) under a stream of nitrogen, without prior concentration. Samples are run in duplicate on the same plate in parallel with standard lipids of varying concentration.

The prepared chromatoplates are run in small tanks, previously saturated with the solvents used by JATZKEWITZ[1], except that the volumes of solvents in the second mixture, *n*-PrOH–NH_4OH, are 39:11, which has been found to produce better separations.

After drying, the plates are sprayed with 50 % sulphuric acid to which methyl orange has been added (5 mg %). The plates are then heated at 160° for 20 min when the lipids show up as carbonized spots on a white background. (Fig. 2).

The separated lipids are scanned directly on the plate by the reflectance scanner which measures the optical density at intervals of 0.3 mm along the length of the plate. The trace produced is shown in Fig. 3. A trace is also made along a blank strip of the sprayed, heated silicic acid.

The areas under the curves are measured with a planimeter and the mean value of 3 readings is taken. The area is found to be proportional to the weight of lipid in the sample (see Figs. 4–6). By comparison of the graphs of areas under the curves against weight of standard lipid applied with the area of a particular lipid in the sample, it is possible to calculate the amount of that lipid in the tissue.

Cholesterol is determined colorimetrically by a modified Liebermann-Burchard reaction on an aliquot of the original lipid extract.

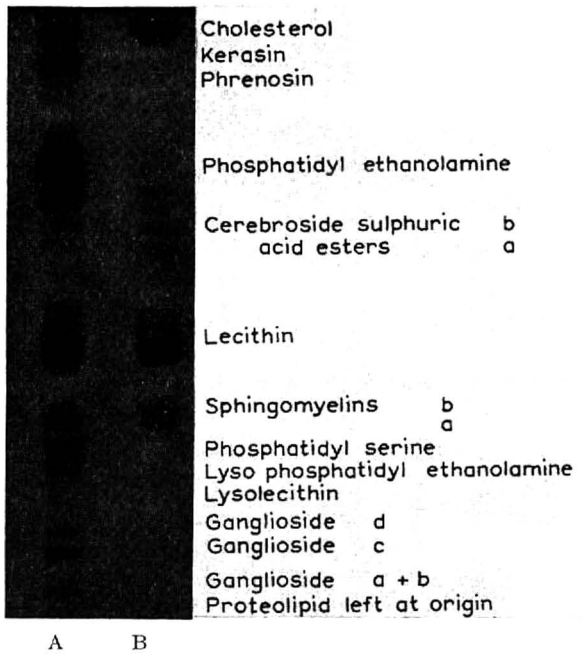

Fig. 2. Lipids in rat brain separated on a thin layer of silicic acid on a glass plate and run first in $CHCl_3$–MeOH–H_2O (14:6:1) to 15 cm and then in *n*-PrOH–NH_4OH (39:11) to 10 cm. Lipids in position A are those found in rat brain. Lipids in position B are "standards": the lecithin is ovolecithin, the cerebroside is synthetic glucocerebroside. The phosphatidyl ethanolamine shows up as two spots when the spot is not overloaded: the faster running is the plasmalogenic form of the phosphatide.

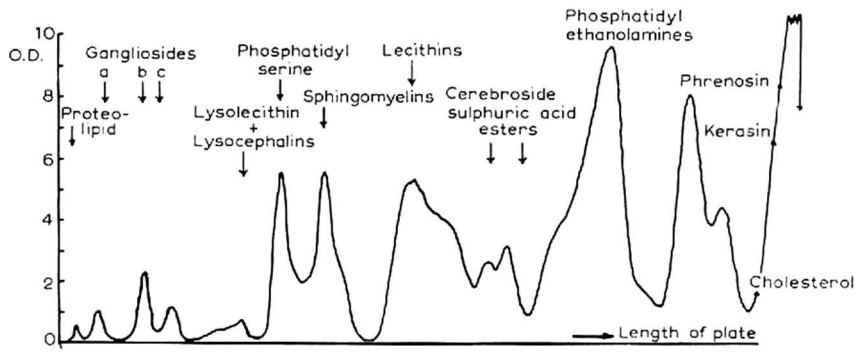

Fig. 3. Separation of lipids. 1.64 mg wet brain tissue ≡ 25 μl lipid extract of brain of stock rat 38 ≡ 151 μg lipid. Total length of plate = 20 cm.

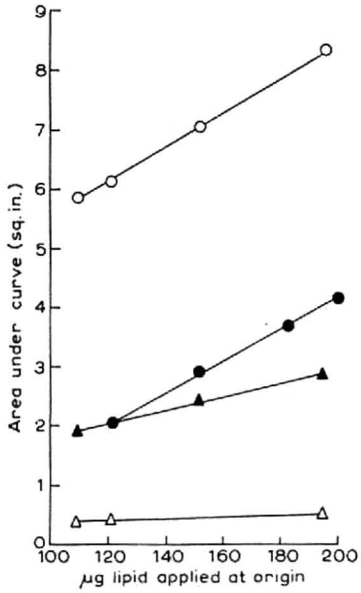

Fig. 4. Graphs showing relationship between area under the curve (sq. in.) and the quantities of different lipids found in varying amounts of total lipid. ● = sphingomyelins; ○ = cerebrosides; ▲ = cerebroside sulphuric acid esters; △ = gangliosides.

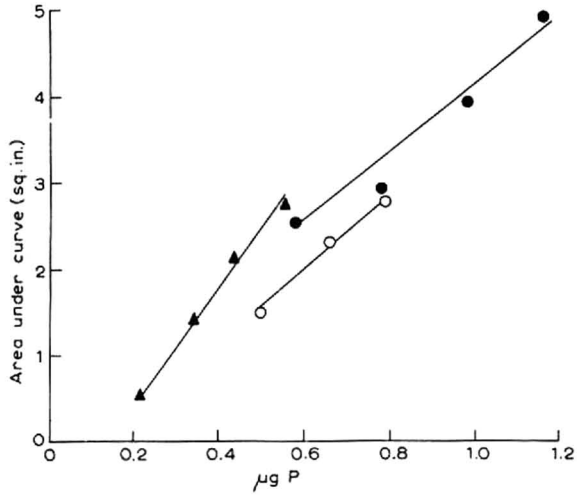

Fig. 5. Graphs showing relationship between areas under curves (sq. in.) and quantities of P of three phospholipids. ▲ = sphingomyelin; ● = phosphatidyl ethanolamine; ○ = ovolecithin.

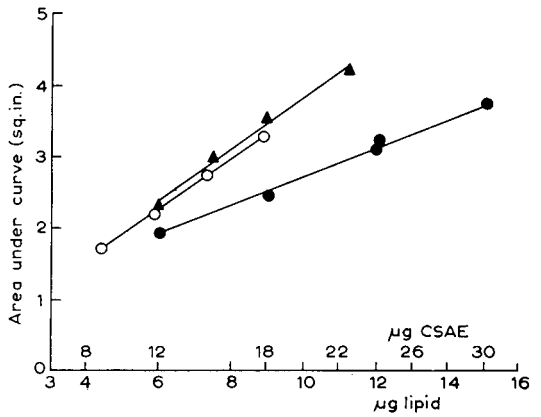

Fig. 6. Graphs showing relationship between area under the curve (sq. in.) and concentration of lipids. ○ = CSAE = cerebroside sulphuric acid esters; ● = sphingomyelins; △ = glucocerebrosides.

RESULTS AND DISCUSSION

Table I shows the reproducibility of the method. As would be expected, the standard errors of the smaller peaks are rather larger than could be desired, but that on the larger peaks is considered sufficiently small to make the method usable.

Methyl orange is added to the sulphuric acid spray so that even coverage of the plate is easier to obtain. Although this produces a slightly darker background due to carbonization of the methyl orange, the advantages greatly outweigh the disadvantages.

TABLE I

REPRODUCIBILITY OF THE METHOD

Lipid	*Amount of lipid applied (μg)*				
	194	*151*	*109**	*85*	*60*
	Area under curve as % of total area				
Ganglioside a + b	0.7	1.0	0.6 + 0.05	0.3	0.5
Ganglioside c	1.3	2.0	1.3 + 0.07	0.8	1.0
Ganglioside d	0.8	1.3	0.7 + 0.09	0.4	0.5
Lysocephalins and lysolecithin	0.8	1.3	1.4 + 0.02	1.4	1.6
Phosphatidyl serine	6.2	9.0	8.4 + 0.78	9.3	10.3
Sphingomyelin	9.0	6.3	7.3 + 0.34	4.1	3.8
Lecithin	19.4	18.9	18.9 + 0.41	18.3	18.9
Cerebroside sulphuric acid esters	7.5	6.6	6.0 + 0.41	4.8	4.8
Phosphatidyl ethanolamine	32.4	31.4	33.2 + 0.59	35.3	35.2
Degradation product of phosphatidyl ethanolamine	2.4	2.8	2.7 + 0.45	2.5	4.2
Cerebrosides	20.4	19.9	20.4 + 0.45	22.6	19.0
Total area under curve (sq. in.)	38.59	36.70	29.7 + 0.7	22.71	16.73
Wet weight of brain tissue (mg)	2.09	1.63	1.18	0.92	0.65

* All values given in this column are the means and standard errors of 5 separate determinations on 3 different days, on 3 different plates.

TABLE II

APPROXIMATE R_F VALUES FOR LIPIDS IN NERVOUS TISSUE RUN IN $CHCl_3$–MeOH–H_2O (14:6:1) TO 15 cm AND THEN IN *n*-PrOH–12.5% aq. NH_4OH (39:11) TO 10 cm

Lipid	R_F
Ganglioside a	0.022
Ganglioside b	0.027
Ganglioside c	0.09
Ganglioside d	0.13
Lysocephalin	0.22
Phosphatidyl serine	0.25
Lysolecithin	0.27
Sphingomyelin a	0.31
Sphingomyelin b	0.33
Lecithin	0.46
Cerebroside sulphuric acid esters a	0.61
Cerebroside sulphuric acid esters b	0.64
Phosphatidyl ethanolamine	0.67–0.80*
Phrenosin	0.93
Kerasin	0.96
Cholesterol	0.99
Free fatty acids	1.00

* The first figure is the R_F value measured from the end of the spot and the second figure is the R_F value measured from the front of the spot.

TABLE III

CONCENTRATION OF LIPIDS IN RAT BRAIN TISSUE AS DETERMINED BY THIN-LAYER CHROMATOGRAPHY (TLC) COMPARED WITH THE RESULTS OF OTHER AUTHORS

Lipid	*Results by TLC Lipid (mg) in total brain of 50 day old rat*	*Other authors' results Lipid (mg) in total brain*	*Reference*
Cerebrosides	19.3*	16.5 (40 day old rat) 14.6 (65 day old rat)	KOCH AND KOCH[8] KISHIMOTO AND RADIN[9]
Cerebroside sulphuric acid esters	11.0	7.2 (40 day old rat)	KOCH AND KOCH[8]
	Lipid P (moles/g) of wet brain tissue		
Sphingomyelin	9.1	8.1 (42-day-old rat) 11.0 (2-month-old rat)	BIETH, FREYSZ AND MANDEL[10] MANDEL AND BIETH[11]
Lecithin	32.2	28.2 (42-day-old rat)	BIETH, FREYSZ AND MANDEL[10]
Total choline phosphatides	41.3	29.4 (3–6 months old)	NIEMIRO AND PRZYJEMSKI[12]

* Using a synthetic glucocerebroside as standard.

Equal quantities of different lipids give varying areas under the densitometric curves (Figs. 5 and 6) so that although the figures given in Table I for the percentages of individual lipids present in the extract can be used for direct comparison of different tissues, they cannot be taken as an absolute indication of the amount of each component present. Therefore it is essential to have pure lipids for use as standards.

The R_F values of the lipid are not reproducible from day to day, but in relation to one another they are constant. Therefore it is advisable to run standards on each plate. The approximate R_F values of the lipids in the solvents used are given in Table II.

The two spots found for sphingomyelin and for the cerebroside sulphuric acid esters are probably due to the difference in fatty acid composition. The gangliosides have differing proportions of hexosamine to neuraminic acid in the molecule.

Not many figures are available in the literature for quantities of lipids in rat brains, especially for cerebrosides, since most of the figures for "cerebroside" given in the literature before 1955 include values for ganglioside as it was the sugar moiety of both which was estimated. Table III shows the results obtained by this method compared with those for rat brains reported in the literature.

ACKNOWLEDGEMENTS

I would like to thank Dr. J. OLLEY for the gift of the sphingomyelin standard; Dr. G. H. SLOANE-STANLEY for the cerebroside sulphuric acid standard; Dr. D. SHAPIRO for the synthetic glucocerebroside; and Dr. R. M. C. DAWSON for gifts of phosphatidyl ethanolamine, phosphatidyl inositol and ovolecithin standards.

All the equipment was designed and made by Mr. P. R. PAYNE and Mr. J. BARKER of this Unit.

SUMMARY

Lipids from brain tissue have been estimated densitometrically after separation on thin-layer chromatograms.

Standard error on five determinations on three different days for lipids present in large amounts is 2%, but on lipids present in smaller amounts the standard error is sometimes as much as 10%.

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A METHOD FOR THE UNIDIMENSIONAL SEPARATION OF PHOSPHOLIPIDS BY THIN-LAYER CHROMATOGRAPHY*

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INTRODUCTION

A number of methods for thin-layer chromatography of phospholipids have been reviewed by MANGOLD[1]. SKIDMORE AND ENTENMAN[2] have employed a two-dimensional thin-layer system which separates seven phospholipids from a rat liver extract. The present communication describes a single solvent system which separates the radioactive phospholipids from pancreas slices into seven discrete spots. The phospholipids may be distinguished from their lyso derivatives by this technique. The lipids are detected by autoradiography and the radioactive spots can be quantitatively cut out from the thin-layer plate.

MATERIALS AND METHODS

Preparation of chromatographic plates

Silica gel G (Merck, Germany) was used directly from the bottle. It was applied to glass plates as a slurry (1/2 w/v), using a modified version of STAHL's[3] original applicator purchased from Research Specialties Co. The thin-layer plates were kept in an oven at a 110° and removed shortly before use.

Developing solvent

A phenol–water solution was prepared by dissolving 5 lbs. of phenol (reagent grade) in 520 ml of distilled water. This solution can be stored at room temperature for several months. The developing solvent was prepared just prior to chromatography by mixing 1.0 ml of concentrated ammonium hydroxide with 99 ml of the phenol stock solution.

Treatment of the chromatographic plates

The plates were developed at room temperature. After approximately 4 h, when the solvent had ascended 12 to 15 cm, the plates were removed and partially dried in an oven at 110°. When it was desired to determine radioactivity in the separated

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phospholipids the plates were sprayed with a water dispersion of polyvinylpropionate (Neatan®, purchased from Brinkmann Instrument Inc.). The plates were then returned to the oven and completely dried. The plates which were used to isolate the radioactive material for alkaline hydrolysis were not sprayed with Neatan®.

Detection of the phospholipids

The reagents and techniques for detecting amino groups, choline, phosphorus, aldehyde, and unsaturation which were used in this study have been described by MANGOLD[1] and SKIDMORE AND ENTENMAN[2]. Most of these reagents can be used after the application of the plastic coating with little loss in sensitivity. The radioactive phospholipids were detected by autoradiography and coincided with the chemically stained regions. The chromatographic plate was placed in a specially constructed holder made by gluing together two 1/8 × 10 × 12 in. sheets of pressed wood, the

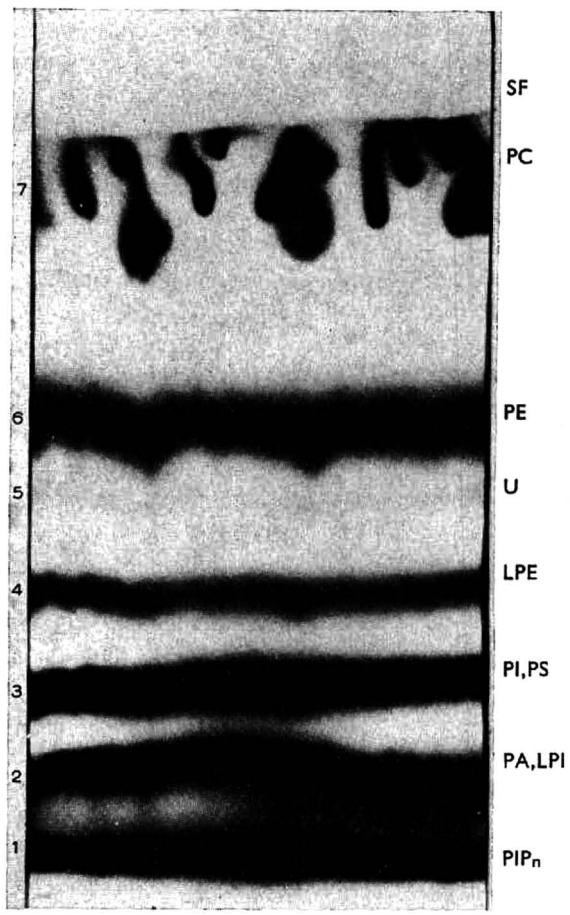

Fig. 1. An autoradiograph showing the separation of the phospholipids of pigeon pancreas tissue. The following abbreviations were used: SF (solvent front); PC (phosphatidyl choline); PE (phosphatidyl ethanolamine); PS (phosphatidyl serine); U (unknown); LPE (lysophosphatidyl ethanolamine); PI (phosphatidyl inositol); PA (phosphatidic acid); LPI (lysophosphatidyl inositol); PIP_n (phosphatidyl inositol polyphosphate).

upper sheet having an 8 × 8 in. square removed to accommodate the plate. An 8 × 10 in. Kodak No-Screen X-ray film was secured over the chromatographic plate by means of spring clamps and the holders were stored in a lightproof cabinet until the film was sufficiently exposed.

Quantitative determination

The radioactive phospholipids spots, detected by autoradiography, were marked with pencil and circled with a razor blade. After moistening with water, the plastic impregnated silica gel patches were lifted off the plate with a spatula and transferred to planchets for counting.

RESULTS AND DISCUSSION

Identification of separated phospholipids

Phospholipids, labelled with radioactive orthophosphate from pigeon pancreas slices[4], were applied to several thin-layer plates. The lipids separated into seven distinct bands (Fig. 1). Several thin-layer chromatograms were sprayed with various chemical reagents, as described in MATERIALS AND METHODS, to aid in the identification of these lipids. The separated phospholipids were further characterized by identifying their radioactive alkaline hydrolysis products (Table I). This was done by scraping the radioactive areas off the plates with a spatula and subjecting the phospholipids adsorbed to the silica gel to mild alkaline hydrolysis by the method of DAWSON[7] and identifying the hydrolysis products by their mobility in two chromatographic systems[7]. The R_F values of the separated phospholipids were also compared to the R_F values of standard phospholipids (Table I). The alkaline hydrolytic products of band 1 (Fig. 1) were not identified. Polyphosphoinositides are labelled with ^{32}P in brain[8] and red blood cells[9]. Chromatography of lipid extracts from these tissues showed that the

TABLE I

IDENTIFICATION OF THE INDIVIDUAL PHOSPHOLIPIDS

Standard compounds were prepared by chromatographing ^{32}P-labelled phospholipids from pancreas slices[4] according to the method of MARINETTI[5] and eluting the separated phospholipids from the paper with 3:1 chloroform–methanol. Lysophosphatidyl inositol was isolated as previously described[6], and sphingomyelin was obtained from the Nutritional Biochemical Corp. The following abbreviations were used: α-GP (α-glycerophosphate); GPI (glycerophosphorylinositol); IMP (inositol monophosphate); GPE (glycerylphosphorylethanolamine); GPC (glycerylphosphorylcholine).

Band No. (Fig. 1)	*R_F value*	*Ninhydrin*	*Dragendorf*	*Fuchsin-sulfurous acid*	*Principal radioactive alkaline hydrolysis products*	*Compound**
1	0.02	—	—	—	unknown	PIP_n**
2	0.12	—	—	—	α-GP, GPI, IMP	PA** and LPI**
3	0.28	—	—	—	GPI and IMP	PI** and PS**
4	0.44	+	—	—	GPE	LPE**
5	0.56	—	—	—	unknown	unknown
6	0.64	+	—	+	GPE	PE**
–	0.74	—	+	—	—	Sphingomyelin**
7	0.92	—	+	+	GPC	PC**

* For abbreviations see the legend to Fig. 1.
** Standard compounds had the same R_F value.

polyphosphoinositides did not move very far from the baseline. Polyphosphoinositides have not, however, been definitely identified in pancreas.

An unknown lipid was detected (Fig. 1, band 5). This lipid may be a diacylglycerophosphatide since it is attacked by bee venom, presumably to yield the lyso derivative (Fig. 2). On alkaline hydrolysis it gave α-glycerophosphate and an unidentified product. There was no positive reaction with the various stains used.

Fuchsin aldehyde tests were positive in bands 6 and 7 (Table I), indicating the presence of ethanolamine and choline plasmalogens.

This chromatographic system also separates the phospholipids from their lyso derivatives (Fig. 2).

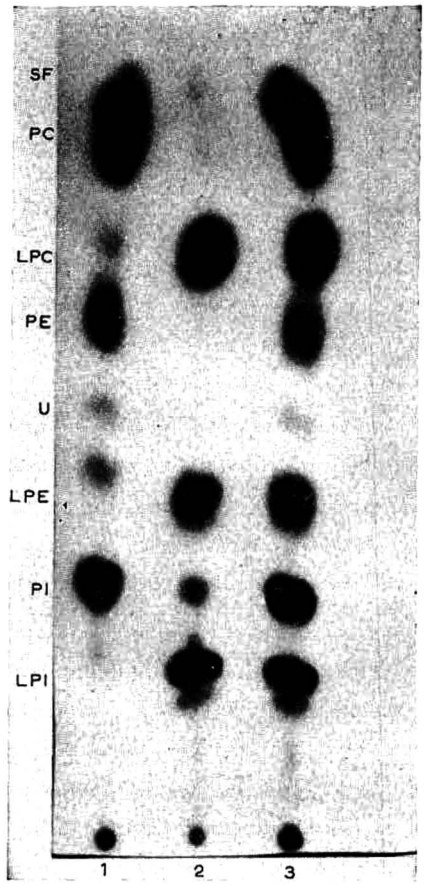

Fig. 2. An autoradiograph showing the separation of the phospholipids and the lysophospholipids. Aliquots of ^{32}P-labelled pancreas phospholipids were incubated with 0.1% bee venom (purchased from Sigma Chemical Company) in an ethereal system similar to that described by HANAHAN[10], except that several mg of alumina were added to promote hydrolysis[11]. The reaction mixture was dried under nitrogen and the lipids were dissolved in ethanol–chloroform–water (5:2:2). Aliquots of this solution were spotted. Phosphatidic acid, polyphosphoinositide and lyso derivatives of these compounds were strongly bound by the alumina and were not present in the lipid extract. Numbers 1 and 2 are duplicate samples of pancreas phospholipids incubated in the absence and presence of bee venom, and 3 is a mixture of the two samples. See Fig. 1 for abbreviations used.

Quantitative estimation of phospholipid radioactivity

The capacity of the plate was determined by spotting varying amounts of pancreas lipid extracts. A range of 1.8 to 18 μg of phospholipid phosphorus were plated and no change in the R_F values of the separated phospholipids was noticed.

Since the spots are relatively small, the autoradiographic method is extremely sensitive, and as little as 50 disintegrations/min of ^{32}P are sufficient to produce a discrete spot in one day. The absence of radioactive streaking allows the separated phospholipids to be counted accurately. The data in Table II show that very precise results can be obtained.

TABLE II

THE QUANTITATIVE ESTIMATION OF RADIOACTIVITY INCORPORATED INTO THE INDIVIDUAL PHOSPHOLIPIDS

Duplicate aliquots of a total lipid fraction (sample 1) and a partially purified phosphatidyl inositol fraction (sample 2) from pigeon pancreas stimulated with carbamylcholine[4] were chromatographed and the radioactivities of the individual phospholipids were determined as described under MATERIALS AND METHODS. Abbreviations are given in the legend to Fig. 1.

Compound	Sample 1 (counts/min)		Sample 2 (counts/min)	
	A	B	A	B
Origin	65	77	—	—
PIP_n	69	51	31	22
LPI + PA	543	519	193	203
PI	1128	1116	1672	1642
LPE	35	35	109	124
PE	130	127	200	213
PC	31	37	—	—
Total recovered counts/min	1992	1960	2205	2204
Total spotted counts/min	2065	2065	2310	2310

Disadvantages of this method are that phosphatidyl inositol and phosphatidyl serine (Band 3) and phosphatidic acid and lysophosphatidyl inositol (Band 2) do not separate. Methods have been reported[2,12,13] which separate phosphatidyl serine and phosphatidyl inositol. One of these methods could be used together with the phenol–NH_3 system to distinguish between them.

ACKNOWLEDGEMENT

The authors wish to thank Dr. L. E. HOKIN, in whose laboratory this work was done, for his advice and encouragement.

SUMMARY

A phenol–water–ammonia solvent system has been utilized to separate most of the known phospholipids and lysophospholipids on thin-layer plates of silica gel G. These compounds were identified by chromatography of their mild alkaline hydrolysis products, various staining reactions, and comparison with known compounds. An unknown radioactive phospholipid has been detected in pigeon pancreas lipids by

means of this chromatographic method. A procedure is described which makes it possible to determine the radioactivity incorporated into the individual phospholipids with a high degree of precision.

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SÉPARATION DES ISOMÈRES GÉOMÉTRIQUES D'ACIDES GRAS MONOÉTHYLÉNIQUES SUR COUCHE MINCE DE TALC. DOSAGE SEMI-QUANTITATIF

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INTRODUCTION

Certains procédés ont été publiés pour séparer les acides gras élaïdisés des isomères dont les liaisons éthyléniques sont de forme *cis*. Il en existe peu, à notre connaissance, qui permettent d'isoler rapidement l'une ou l'autre de ces formes géométriques sans avoir recours à plusieurs opérations intermédiaires.

La méthode de JANTZEN ET ANDREAS[1] fait appel à la préparation de composés d'addition: dérivés acétoxymercuriméthoxy- des esters d'acides gras. Ces dérivés se forment 10 fois plus lentement à partir des acides gras insaturés de forme *trans* qu'avec ceux de forme *cis*. D'après MANGOLD[2], on peut utiliser la chromatographie en couche mince de gel de silice pour séparer le composé d'addition qui s'est formé rapidement à partir des esters d'acides gras de forme *cis* et les esters (de forme *trans*) qui n'ont pas encore réagi avec l'acétate mercurique. En pratique, la résolution de ces deux taches s'effectue très difficilement.

Une autre méthode de séparation est celle décrite par DE VRIES[3,4]. Elle est basée sur la formation de complexes Ag + alcène, qui se forment facilement sur les liaisons éthyléniques *cis*, difficilement sur les *trans*. SCHOLFIELD *et al.*[5,6] se basent sur le même principe pour séparer les esters d'acides *cis* et *trans* par fractionnement à contre-courant. Ces procédés exigent une ou plusieurs opérations supplémentaires pour la récupération des acides gras à partir du complexe formé.

Nous nous proposons de décrire ici une méthode, par simple chromatographie sur couche mince de talc, qui permet de séparer directement et de doser presque quantitativement les acides gras insaturés de forme *cis* et ceux de forme *trans* sans leur faire subir de préparation.

PRINCIPE DE LA MÉTHODE

On applique les mélanges d'acides gras insaturés (de forme *cis* et *trans*) au bas d'une chromatoplaque revêtue de substance peu polaire (silicate de magnésium) et on utilise la différence de vitesse de solubilisation de ces acides gras pour leur élution avec un mélange hydroalcoolique de solvants.

ANALYSE QUALITATIVE

Confection des chromatoplaques

On mélange dans un mortier: 40 g de talc du commerce* et 60 ml de *n*-propanol (ou isopropanol). On verse ce mélange dans un applicateur pour chromatographie en couches minces de type courant après l'avoir réglé à une hauteur de 0.25 mm. On l'étale sur cinq plaques de verre de 20 × 20 cm qu'on fait sécher à l'étuve à 105–110° pendant 20 min.

Application du mélange d'acides gras

On applique le mélange d'acides gras (environ 10 γ) de chaque constituant en solution dans un solvant léger (éther de pétrole par exemple).

Élution

L'élution à la température ambiante de 15–18° est faite à l'aide du mélange hydroalcoolique de 35 ml d'éthanol absolu et 1 goutte d'acide acétique glacial, complété à 50 ml avec de l'eau bi-distillée. Ces volumes et températures doivent être rigoureusement respectés pour obtenir une bonne résolution des différentes taches. L'élution dure de 5–6 h, temps nécessaire pour que le front du solvant arrive à 3 cm du haut de la plaque.

Révélation des plaques

On peut révéler par tous moyens couramment utilisés: vapeurs d'iode ou carbonisation par l'acide sulfurique à 50 % vaporisé sur la plaque, par exemple.

En opérant rigoureusement dans les conditions décrites ci-dessus on obtient les R_F suivants: acide oléique 0.80, acide élaïdique 0.40, l'acide gras saturé correspondant (stéarique) restant fixé sur la ligne de départ.

Influence de la composition du mélange éluant sur la chromatographie

(*a*) *Concentration de l'éthanol: action sur la valeur des R_F et sur la longueur des taches.* Les R_F varient très fortement avec la concentration de l'éthanol dans l'éluant ainsi que l'on peut s'en rendre compte par l'examen du Tableau I et de la Fig. 1.

TABLEAU I

Éthanol pour 50 ml d'éluant (ml)	R_F	
	Acide oléique	*Acide élaïdique*
25	0.50	—
30	0.70	0.10
35	0.80	0.45
40	0.95	0.75

La concentration de l'éthanol dans le milieu hydroalcoolique que nous avons choisie correspond à un système d'élution qui permet d'obtenir les taches les plus ramassées comme on peut le voir par l'examen du Tableau II et de la Fig. 2.

* L'origine du silicate de magnésium importe peu. Cependant nous utilisons de préférence un talc du commerce (Prolabo) débarrassé des débris organiques par lavage et qui adhère mieux aux plaques de verre qu'un produit pur tel que le silicate de magnésium synthétique (Johns Manville).

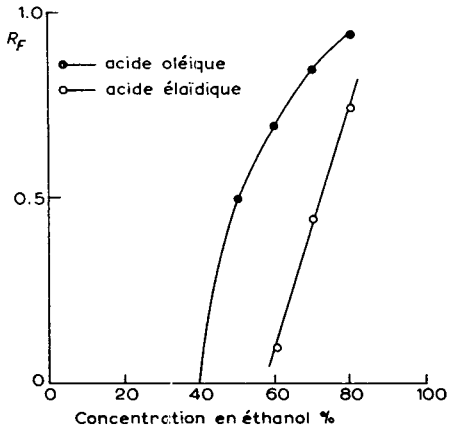

Fig. 1. Influence de la concentration de l'éthanol dans le mélange éluant sur le R_F des taches.

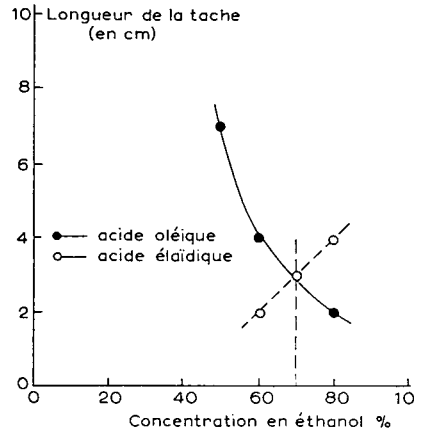

Fig. 2. Influence de la concentration de l'éthanol dans le mélange éluant sur la longueur des taches.

(*b*) *Acidité du mélange hydroalcoolique, action sur les R_F des acides gras élaïdisés.* En augmentant l'acidité du mélange éluant on augmente principalement le R_F des acides gras de forme *trans*.

TABLEAU II

Éthanol pour 50 ml d'éluant (ml)	*Longueur des taches (mm)*	
	Acide oléique	*Acide élaïdique*
25	70	—
30	40	20
35	30	30
40	20	40

ANALYSE QUANTITATIVE

On peut séparer sur des plaques beaucoup plus épaisses des quantités d'acides gras éthyléniques de forme *cis* et de forme *trans* suffisantes pour les titrer et en effectuer une chromatographie en phase gazeuse.

Confection des chromatoplaques préparatives

On mélange dans un mortier: 150 g de talc du commerce et 200 ml de *n*-propanol (ou isopropanol). On verse ce mélange dans un applicateur du type de celui décrit par DAUVILLIER[7] en le règlant à une hauteur de 1 mm. On étale sur 4 plaques de verre de 20 × 20 cm et on fait sécher à l'étuve à 105–110° jusqu'à complète évaporation du propanol.

Application du mélange d'acides gras

On peut appliquer en une seule bande au bas de chaque chromatoplaque jusqu'à 40 mg d'acides gras en solution dans l'éther de pétrole.

Élution

L'élution est faite à l'aide du mélange hydroalcoolique de 105 ml d'éthanol absolu et 2 gouttes d'acide acétique glacial, complété à 150 ml avec de l'eau distillée.

Localisation des taches

Les vapeurs d'iode révèlent instantanément les acides gras de forme *cis* plus lentement les acides gras élaïdisés.

Extraction des acides gras du chromatogramme préparatif

On récupère la poudre de talc imprégnée d'acides gras et l'on procède à leur extraction par un mélange de solvants (méthanol–chloroforme par exemple).

Dosage des acides gras

Le dosage des acides gras peut se faire avec une solution de soude 0.01 *N* à l'aide d'une microburette ou d'une seringue calibrée ou encore au pH-mètre.

Erreur effectuée par dosage titrimétrique

(*a*) *Mélange renfermant uniquement des acides gras monoéthyléniques.* On désire séparer les constituants d'une solution renfermant de l'acide oléique et de l'acide élaïdique à raison de 1 mg (soit 3.5 micro-équivalents) de chaque acide gras par ml. Le Tableau III

TABLEAU III

Volume de la solution d'acides gras (ml)	*Volume théorique de NaOH à verser (ml)*	*Volume pratique de NaOH à verser (ml)*	*Erreur (%)*
5	1.75	1.90	8
3	1.05	1.10	5
1	0.35	0.37	6
0.55	0.19	0.20	5

indique les erreurs que l'on obtient lorsque l'on effectue le dosage à l'aide d'une solution alcoolique de soude 0.01 *N* délivrée par une microburette à pointe plongeant dans la solution d'acides gras.

(*b*) *Mélange renfermant à la fois des acides gras mono- et diéthyléniques et des acides gras saturés.* On fait une solution renfermant (en moles): $0.49 \cdot 10^{-2}$ d'acide oléique, $0.12 \cdot 10^{-2}$ d'acide linoléique, $0.49 \cdot 10^{-2}$ d'acide palmitique, $0.12 \cdot 10^{-2}$ d'acide élaïdique, dont on effectue la séparation sur chromatoplaque de talc.

Comme les R_F des acides gras saturés sont voisins de ceux des acides gras élaïdisés, leurs bandes sont plus ou moins confondues. Pour récupérer les acides gras on procède alors de la façon suivante:

Fraction A: le quart supérieur de la plaque (mesuré entre la ligne de départ et celle du front) qui contient tous les acides gras de forme *cis*.

Fraction B: les trois-quart inférieurs qui renferment tous les acides gras de forme *trans* et les acides gras saturés.

On extrait alors les acides gras de ces fractions et on en effectue le dosage. Théoriquement, le rapport de la somme des acides gras de forme *cis* sur la somme des

acides gras *trans* et saturé est égal à 1. Le rapport des volumes de soude 0.01 *N* versée réellement:

$$\frac{\text{0.65 ml (fraction A)}}{\text{0.66 ml (fraction B)}}$$

est également voisin de l'unité.

L'erreur est plus sensible que pour l'exemple précédent puisqu'elle atteint 7 % (acides gras "*cis*") et même 9 % (acides gras "*trans*"). On pourrait la réduire en utilisant une méthode de titration après drainage du CO_2 par barbotage d'azote telle que celle décrite par SALAMAN ET ROBINSON[8].

En associant la chromatographie gaz–liquide à la chromatographie sur couche mince de talc on détermine le pourcentage d'acides gras saturés extraits avec les acides gras de forme *trans*.

RÉSUMÉ

La séparation des isomères géométriques de ces acides gras insaturés est effectuée sur couche mince de talc du commerce par élution hydroalcoolique. Une méthode titrimétrique permet ensuite d'obtenir leur détermination quantitative. Cette technique, utilisée ou non en association avec la chromatographie gaz–liquide, fournit un moyen de connaître avec une approximation convenable la concentration en isomères *cis* et *trans* de mélanges d'acides gras complexes.

SUMMARY

This paper describes a method of thin-layer chromatography on commercial talc with which it is possible to separate elaidinized octadecamonoenoic and dienoic fatty acids from mixtures of saturated fatty acids and *cis* and *trans* forms of unsaturated fatty acids. By combining this method with gas–liquid chomatography, elaidic acid can be determined with good accuracy.

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COMPLETE STRUCTURAL ANALYSIS OF FATTY ACID MIXTURES BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Gas–liquid, thin-layer and paper chromatography permit the separation of complex mixtures of fatty acids according to chain length, number of double bonds, configuration, etc. (for reviews, see refs. 1, 2, 3). As yet, however, no satisfactory method has been devised for separating structurally isomeric monoenoic acids containing the same number of carbon atoms, but having the double bonds in different positions. Hence one could not hitherto carry out a complete analysis of fatty acids by purely chromatographic means and the components of fatty acid mixtures regarded in many papers as "oleic", "palmitoleic", etc. acids actually consisted of mixtures of position isomers. In the relatively few cases in which an exhaustive structural analysis was carried out, the composition of the unsaturated acid fractions with equal numbers of C-atoms could be determined only from the results of oxidative cleavage[4–8]. This part of the work had to be done on a preparative scale, required considerable amounts of material for analysis and was very time-consuming[9,10]. In a recent modification the methyl esters of the unsaturated fatty acids are fractionated prior to oxidation by preparative thin-layer and gas–liquid chromatography[11]. The modified method is highly sensitive, but still does not discriminate between stereoisomeric acids and involves a large number of operations (twofold runs each by thin-layer chromatography and gas–liquid chromatography, ozonization and reduction of the ozonides).

The present paper describes a method for carrying out the complete structural analysis of fatty acids using only thin-layer chromatography*. It is based on two-dimensional thin-layer chromatography of fatty acids and allows separation of positional isomers of unsaturated fatty acids having double bonds in any position. The spots can be specifically identified with the aid of reference substances and if these are unavailable, then by oxidation of the components directly in the thin layer of the adsorbent. The method is simple, requires very little material (0.5 mg of a mixture of the methyl esters of the fatty acids) and greatly shortens the time required for the analysis.

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DISCUSSION

With the objective of separating the structural isomers of unsaturated acids we investigated the chromatographic behaviour of their methyl esters on plates covered with silica gel impregnated with silver nitrate. Such plates have been recently employed for separation of *cis-trans* isomers[12]. It was shown that the π-complexes formed by the reaction of silver nitrate with the ethylenic bond of the stereoisomeric unsaturated esters differ, much more than the esters alone, in polarity, and hence in their mobility on the plates. One could therefore expect that such plates would also efficiently separate structurally isomeric mono-unsaturated fatty acid esters.

Our results with the chromatography of the methyl esters of structurally isomeric mono-unsaturated fatty acids in various systems showed that they could in fact be separated on silver nitrate-impregnated silica gel (see Table I and Fig. 1). The best separation was obtained with systems I, II, IV and V (see Table I). The same conditions, however, did not lead to satisfactory separation of unsaturated esters of varying chain lengths. In order therefore to achieve complete separation according to chain length, structure and configuration, we made use of two-dimensional chromatography. The mixture was first subjected to thin-layer partition chromatography on silica gel impregnated with dodecane in an acetonitrile–acetone system[13]. This led to separation into groups according to the number of C atoms (each double

TABLE I

R_F VALUES OF ISOMERIC MONO-UNSATURATED METHYL ESTERS ON SILICA GEL IMPREGNATED WITH $AgNO_3$

Compounds	R_F					
	I	*II*	*III*	*IV*	*V*	*VI*
C_{16}						
$CH_3(CH_2)_5CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.62	0.40	0.32	0.35	0.26	0.22
$CH_3(CH_2)_3CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.68	0.46	0.38	0.44	0.34	0.28
C_{18}						
$CH_3(CH_2)_9CH=CH(CH_2)_5COOCH_3$ (*cis*)	0.56	0.34	0.29	0.28	0.23	0.18
$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.64	0.42	0.35	0.39	0.29	0.24
$CH_3(CH_2)_5CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.73	0.49	0.42	0.49	0.41	0.34
$CH_3(CH_2)_7CH=CH(CH_2)_7COOCH_3$ (*trans*)	0.84	0.64	0.59	0.64	0.54	0.44
C_{20}						
$CH_3(CH_2)_7CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.70	0.47	0.37	0.47	0.39	0.29
C_{22}						
$CH_3(CH_2)_{15}CH=CH(CH_2)_3COOCH_3$ (*cis*)	0.58	0.39	0.30	0.31	0.26	0.19
$CH_3(CH_2)_9CH=CH(CH_2)_9COOCH_3$ (*cis*)	0.72	0.51	0.40	0.50	0.41	0.32
C_{26}						
$CH_3(CH_2)_{15}CH=CH(CH_2)_7COOCH_3$ (*cis*)	0.65	0.46	0.34	0.42	0.30	0.23

Solvents: I = Diethyl ether–petroleum ether (b.p. 28–40°) (3:7)
II = Diethyl ether–petroleum ether (b.p. 28–40°) (9:41)
III = Diethyl ether–petroleum ether (b.p. 40–60°) (9:41)
IV = Dipropyl ether–petroleum ether (b.p. 28–40°) (2:3)
V = Dipropyl ether–petroleum ether (b.p. 28–40°) (1:3)
VI = Dipropyl ether–petroleum ether (b.p. 40–60°) (1:3)

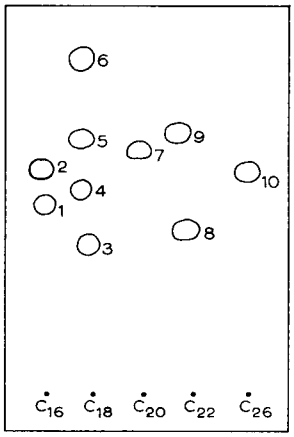

Fig. 1. Separation of isomeric mono-unsaturated methyl esters on $AgNO_3$-impregnated silica gel plates. Solvent: Dipropyl ether–hexane (2:3). Time for saturation of chamber: 40 min. Time of development: 90 min. Length of run: 14 cm. Detection: 50% H_2SO_4. Methyl esters of the acids (50 γ each): 1 = palmitoleic; 2 = palmitvaccenic; 3 = *cis*-octadecen-7-oic; 4 = oleic; 5 = *cis*-vaccenic; 6 = elaidic; 7 = *cis*-eicosen-11-oic; 8 = *cis*-docosen-5-oic; 9 = *cis*-docosen-11-oic; 10 = *cis*-hexacosen-9-oic.

bond being equivalent to shortening of the chain by two CH_2 units)[13,14]. The plates were then impregnated with a solution of silver nitrate and were developed in the second direction with a dipropyl ether–hexane (2:3) system.

In this way, complete separation of the groups into individual components was achieved, the components appearing as clearly defined spots on spraying with an alkaline or ammoniacal solution of bromothymol blue. The R_F values of the methyl

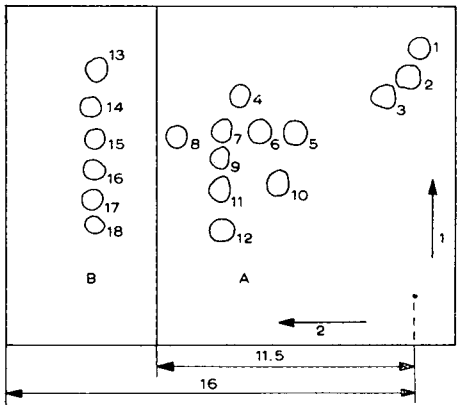

Fig. 2. Two-dimensional chromatography of a mixture of methyl esters of saturated and unsaturated fatty acids (dimensions in cm). Adsorbent: silica gel. First direction: impregnation with a 10% solution of dodecane in hexane. Solvent: acetonitrile–acetone (1:1). Developing time: 90 min. Second direction: impregnation with a 20% $AgNO_3$ solution (zone A only). Solvent: dipropyl ether–hexane (2:3). Developing time: 60 min. Methyl esters of acids (50 γ each): 1 = arachidonic; 2 = linolenic; 3 = linoleic; 4 = palmitvaccenic; 5 = *cis*-octadecen-7-oic; 6 = oleic; 7 = *cis*-vaccenic; 8 = elaidic, 9 = *cis*-eicosen-11-oic, 10 = *cis*-docosen-5-oic; 11 = *cis*-docosen-11-oic; 12 = *cis*-hexacosen-9-oic; 13 = lauric; 14 = myristic; 15 = palmitic; 16 = stearic; 17 = arachidic; 18 = behenic.

esters are given in Table II. Fig. 2 shows the separation of an artificial mixture of the methyl esters of six saturated, nine monoethylenic and three polyenic esters.

It is particularly noteworthy that under the above conditions esters of different chain length, but with the same distance of the double bond from the carboxyl group possess R_F values that are close together, which values increase as the double bond is further removed from the carboxyl group; *trans* isomers move faster than *cis* isomers (see Table II). This circumstance allows the method to be used, not only for complete separation of the mixtures, but also for identification and study of the structure of the components.

TABLE II

R_F VALUES IN TWO-DIMENSIONAL CHROMATOGRAPHY OF METHYL ESTERS OF SATURATED AND UNSATURATED FATTY ACIDS ON SILICA GEL

Compounds	*1st Direction*	*2nd Direction*
Methyl esters of saturated acids		
Dodecanoic	0.78	0.80
Tetradecanoic	0.67	0.80
Hexadecanoic	0.56	0.80
Octadecanoic	0.46	0.80
Eicosanoic	0.36	0.80
Docosanoic	0.28	0.80
Methyl esters of unsaturated acids:		
C_{16}		
cis-Hexadecen-9-oic	0.71	0.39
cis-Hexadecen-11-oic	0.71	0.46
C_{18}		
cis-Octadecen-7-oic	0.60	0.34
cis-Octadecen-9-oic	0.60	0.42
cis-Octadecen-11-oic	0.60	0.51
trans-Octadecen-9-oic	0.60	0.64
C_{20}		
cis-Eicosen-11-oic	0.50	0.52
C_{22}		
cis-Docosen-5-oic	0.40	0.39
cis-Docosen-11-oic	0.40	0.52
C_{26}		
cis- Hexacosen-9-oic	0.25	0.47
Octadeca-9,12-dienoic	0.71	0.08
Octadeca-9,12,15-trienoic	0.80	0.03
Eicosa-5,8,11,14-tetraenoic	0.89	0.00

If reference substances are available, the method permits a complete structural analysis of fatty acids to be carried out without any supplementary procedure. If they are not available, the unsaturated acids can be identified by oxidative cleavage directly in the thin layer of the adsorbent. For this purpose the individual substances were scraped off together with the adsorbent and were applied in the form of an ethereal extract to plates covered with a thin layer of cellulose impregnated with a benzene

solution of dimethylformamide. Oxidative fission of the compounds by the periodate–permanganate reagent[15] was carried out directly on the plates. Development of the resultant mixture of the monocarboxylic acid and the monomethyl ester of the dicarboxylic acid was carried out in the system hexane–diethyl ether–dimethylformamide (40:20:1), the compounds being detected with the aid of a sensitive acid–base indicator[16].

Higher polyenic (*e.g.* arachidonic) acids are difficult to elute from silica gel impregnated with silver nitrate. They were therefore oxidized directly on the silica gel taken from the plate. The mixture was then extracted with ether and the extract applied to the plate covered with dimethylsulphoxide or dimethylformamide-impregnated cellulose (Fig. 4).

The spots obtained for the *n*-alkanoic acid and the monomethyl ester of the dicarboxylic acid were identified with the aid of reference substances (malonic acid derived from divinylmethane patterned esters does not give spots). The results of the oxidation of ten monoethylenic and polyenic acids are shown in Figs. 3 and 4.

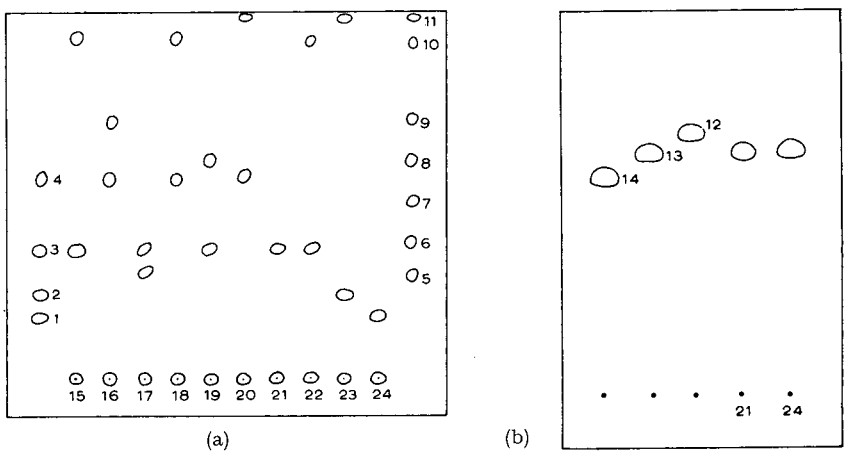

Fig. 3. Oxidative cleavage of unsaturated fatty acids. (a) Adsorbent: cellulose impregnated with a 25% solution of dimethylformamide in benzene. Solvent: hexane–diethyl ether–dimethylformamide (40:20:1). Developing time: 20 min. (b) Adsorbent: silica gel impregnated with a 10% solution of dodecane in hexane. Solvent: acetic acid–acetonitrile (1:4) (saturated with dodecane). Developing time: 60 min. Reference substances: (I) Monomethyl esters of the acids: 1 = glutaric; 2 = pimelic; 3 = azelaic; 4 = nonanedicarboxylic-1,9. (II) Acids: 5 = propionic; 6 = butyric; 7 = valeric; 8 = capronic; 9 = enanthic; 10 = pelargonic; 11 = hendecanoic; 12 = palmitic; 13 = margaric; 14 = stearic. Methyl esters of unsaturated acids subjected to oxidation: 15 = oleic; 16 = *cis*-vaccenic; 17 = linolenic; 18 = *cis*-eicosen-11-oic; 19 = linoleic; 20 = *cis*-docosen-11-oic; 21 = *cis*-hexacosen-9-oic; 22 = elaidic; 23 = *cis*-octadecen-7-oic; 24 = *cis*-docosen-5-oic.

In this system, *n*-alkanoic acids with more than 11 C atoms move along with the front. Oxidation of the higher unsaturated fatty acids yielding such alkanoic acids must therefore be carried out on two plates. In order to develop and identify the monomethyl ester of the dicarboxylic acid, oxidation is carried out on cellulose plates as described above. To develop and detect the second fraction, *i.e.* the monocarboxylic acid with more than 11 carbon atoms, oxidation is carried out on a thin-layer of silica gel impregnated with dodecane and developed with dodecane-saturated acetonitrile–acetic acid (4:1) (Fig. 3b).

It can be seen from Table III that the R_F values of some monocarboxylic acids and monomethyl esters of dicarboxylic acids coincide. However, the combined data of two-dimensional chromatography and oxidative fission always allow accurate determination to be made of the structure of the original unsaturated acids together with a complete structural analysis of any straight chain fatty acid mixture. Quan-

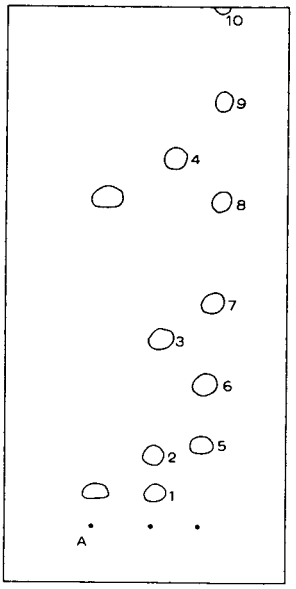

Fig. 4. Chromatography of the oxidation products of methyl arachidonate. Adsorbent: cellulose impregnated with a 25 % solution of dimethylsulfoxide in toluene. Solvent: diethyl ether–hexane (1:2) (chamber saturated with dimethylsulfoxide vapor). Developing time: 35 min. A: Oxidation products of methyl arachidonate. For enumeration of reference substances, see Fig. 3.

titative determination of the components can be achieved either by one of the methods usually employed in thin-layer chromatography[17,18] or by a combination of thin-layer and gas–liquid chromatography[19].

The proposed method thus makes possible the complete structural analysis of mixtures of fatty acids differing not only in the number of C atoms and in the degree of unsaturation, but also in the position of the double bonds, and may therefore become a convenient tool in lipid chemistry.

EXPERIMENTAL

Chromatography was carried out on plates coated with a thin layer of silica gel as follows: A paste of 6.5 g KSK silica gel (150–200 mesh), 0.4 g gypsum and 17 ml 12 % $AgNO_3$ solution is applied to a 9 × 24 cm glass plate and after drying at room temperature for 5–8 h is dried in an oven, the temperature of which is slowly (40–60 min) raised to 104–106° at which temperature the plate is kept for another hour. The plates gradually darken on prolonged storage, but this does not affect their separating efficiency. The grey background disappears when detecting the substances

TABLE III

R_F VALUES OF MONOCARBOXYLIC ACIDS AND MONOESTERS OF DICARBOXYLIC ACIDS ON DIMETHYLFORMAMIDE-IMPREGNATED CELLULOSE

Compounds	R_F
Monocarboxylic acid	
Propionic	0.20
Butyric	0.31
Valeric	0.43
Caproic	0.54
Enantic	0.65
Pelargonic	0.84
Hendecanoic	0.94
Monomethyl esters of dicarboxylic acids	
Glutaric	0.11
Pimelic	0.17
Azelaic	0.31
Nonanedicarboxylic	0.54

by spraying with 50 % H_2SO_4 followed by heating for 5–10 min with a 500 W I.R.-lamp. The substances are revealed as brown, rapidly vanishing spots. It should be mentioned that the separation of the substances and the reproducibility of the results depend greatly upon the degree of saturation of the chamber. Best results were obtained by preliminary saturation for 40–50 min [for the system I and II (see note to Table I) 5–10 min are sufficient]. According to reported data[20] supersaturation of the chamber by attaching filter paper to the walls cuts the developing time by one third. Our observations have shown that despite this, poorer separation of the substances is obtained.

Two-dimensional chromatography was carried out on plates (18 × 18 cm) covered with a paste of 10 g silica gel, 0.6 g gypsum and 25 ml water. After the usual drying procedure, the plates were impregnated by gradual immersion in 10 % (v/v) of dodecane solution in hexane. The extent of impregnation (weight ratio of impregnating substance to adsorbent) can vary within the limits of 0.1–0.2 without any noticeable effect on the separating power. For lower degrees of impregnation the capacity of the layer becomes inadequate and part of the substance moves with the front; for higher degrees, a second frontal line appears.

In the first direction, the mobile phase consisted of a 1:1 acetonitrile–acetone mixture, of which 90 % had been previously saturated with dodecane (6–6.4 ml dodecane per 90 ml mixture at 18–20°). Development took 70–90 min. Mean R_F values for the runs in the first direction are given in Table II.

After development in the first direction the plates were dried for 30 min at room temperature and for 30–40 min at 90–95°, and were then left overnight in air. Part of the plate (zone A, Fig. 2) was impregnated with 20 % $AgNO_3$ solution and the plate was activated by slow heating (1 h) up to 100° and holding at 100–102° for one h*.

* The reagent is much less sensitive for the detection of saturated acids on $AgNO_3$-impregnated silica gel than on the non-impregnated adsorbent. The part of the plate where the saturated methyl esters should be located after the run in the second direction (zone B, Fig. 2) was therefore not sprayed with $AgNO_3$ solution.

A dipropyl ether–hexane mixture (2:3) served as mobile phase for the second direction. The mean R_F values for this direction are also shown in Table II.

After developing in the second direction the plates were dried at 70–80° for 20 min, zone B was sprayed with an alkaline solution of bromothymol blue* (40 mg indicator in 100 ml 0.01 *N* NaOH solution) and the plates were again heated for 10–15 min at the same temperature. The saturated esters were detected as yellow spots on a blue background. The sensitivity was 15–20 γ.

After detection of the saturated esters the plates were sprayed with an ammoniacal solution of bromothymol blue (40 ml of bromothymol blue in 100 ml 20% NH_4OH)**. The methyl esters of unsaturated acids were detected as quickly fading, light or dark blue spots on a gray background. The sensitivity was 40–50 γ.

Oxidative cleavage of the methyl esters of unsaturated acids

(*a*) *Preparation of the plates*. A plate (18 × 18 cm) covered by a thin layer of cellulose as described earlier[21] (9 g cellulose powder, 0.7 g gypsum, 25 ml water) was impregnated by immersion for 10–15 sec in a 25% solution of dimethylformamide in benzene. The plate was then dried for 20 min at room temperature and 2–3 min at 60–70°, following which it was immediately covered by glass (to prevent further evaporation of the impregnated composition). A 1.5–2 cm band was left uncovered for application of the oxidation products.

(*b*) *Oxidation and chromatographic analysis of the oxidation products*. After detecting the methyl esters on silica gel the spots were scraped off together with the adsorbent and the substance extracted with ether (4 × 10 ml). The combined ether extracts were evaporated *in vacuo* to 0.3 ml and were spotted on a cellulose plate.

Each methyl ester spot was oxidized by applying by means of a capillary tube a small amount of oxidizing reagent (0.001 mole K_2CO_3 and 0.001 mole $KMnO_4$ dissolved in 10 ml of water and mixed with a solution of 0.001 mole $NaIO_4$ in 10 ml of water) and heating the plate in a drying oven at 55–60° until the pink potassium permanganate color disappeared (3–5 min). This operation was repeated 2 or 3 times (over-all oxidation time 30–40 min), after which the brown spots formed were moistened with 2 *N* HCl solution. After applying the reference substances the chromatogram was developed in the system hexane–diethyl ether–dimethylformamide (40:20:1). The developing time was 20–30 min. The plate was then placed in an ammonia-saturated glass chamber and sprayed with indicator (200 mg methyl red, 200 mg bromothymol blue, 100 ml formalin, 400 ml ethanol, 3 ml 1 *N* NaOH)[16]. The monocarboxylic acids and monomethyl esters of dicarboxylic acids formed as the result of the oxidation process were detected as yellow spots on a green background (Fig. 3a). At the starting line, a pink 1.5–2 cm band that passes into yellow is observed, whose appearance is apparently due to the presence of hydrochloric acid and of dimethylformamide degradation products. On this band the monomethyl ester of glutaric acid can be detected as an orange colored spot. The sensitivity is 5 γ.

Methyl arachidonate was oxidized directly on silica gel, the reaction mixture then being extracted with ether (3 × 10 ml). The ether extract was then evaporated

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** The reagent should be kept from zone A in order to avoid hydrolysis of the unsaturated esters.

*** A more sensitivitive reagent (10–15 γ) is a solution of 40 mg bromothymol blue in 100 ml water and 2 ml 2 *N* HCl. However, it gives poorer results on further oxidation.

to 0.1 ml and the mixture applied on a cellulose-coated plate, impregnated with dimethylformamide or a 25 % solution of dimethylsulfoxide in toluene (Fig. 4).

In order to identify the compounds which on oxidation yield an *n*-alkanoic acid with more than 11 carbon atoms, the resultant monomethyl ester of the dicarboxylic acid is detected on cellulose plates as described above. To identify the monocarboxylic acid fraction the oxidation is carried out on a thin silica gel layer, impregnated with dodecane*, by applying to the methyl ester spot the oxidizing reagent (0.001 mole $KMnO_4$, 0.001 mole $NaIO_4$, 20 ml water) as described above. The chromatogram is developed in the system acetonitrile–acetic acid (4:1), saturated with dodecane. The developing time was 50–60 min. The plates are then dried at 110–120° for 1 h, sprayed with 10 % solution of phosphomolybdic acid in ethanol and heated by an I.R. lamp (Fig. 3b).

SUMMARY

A method for the complete structural analysis of complex mixtures of fatty acids has been developed based on two-dimensional thin-layer chromatography of their methyl esters on silica gel and identification of the unsaturated acids by oxidative cleavage directly in the adsorbent layer. The method permits the determination both of positional isomers and of stereoisomers of unsaturated fatty acids.

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* Optimal results were obtained with silica gel prepared from liquid glass[22].

THE SEPARATION OF FATTY ACID METHYL ESTERS (INCLUDING "CRITICAL PAIRS") BY THIN-LAYER PARTITION CHROMATOGRAPHY

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Mixtures of saturated higher fatty acids and of unsaturated higher fatty acids, or their methyl esters, may be separated by partition chromatography, but it has been observed that the introduction of a double bond into a molecule gives a change in partition coefficient roughly equivalent to that produced by a reduction in chain length of two methylene groups[1,2]. Thus in the partition chromatography of fatty acids or their methyl esters using a hydrocarbon[3,4] or silicone oil[5] stationary phase, palmitic and oleic acids or their esters, for example, run together and appear as a single spot on the developed chromatogram. Such acids, or their esters, have been termed "critical pairs"[3,6,7]. Numerous methods have been suggested in order to decide whether a spot is composed of saturated or unsaturated material, or both, but these usually require the running of a second chromatogram (or the original chromatogram in the second dimension) after hydrogenation[2,4], oxidation[2,7], or complexing[6] of any unsaturated species present. MICHALEC[8], however, has separated palmitic and oleic acids by two-dimensional paper partition chromatography, development in the second direction being carried out at —8°. APARICIO[9] has shown that lauric acid may be separated from linolenic acid and that myristic may be separated from linoleic acid by paper chromatography, using undecane as stationary phase and aqueous acetic acid as mobile phase.

We now wish to report the separation of critical pairs by thin-layer partition chromatography of fatty acid methyl esters. The identification of the components is facilitated by the use of a detecting agent which gives different coloured spots for saturated and unsaturated methyl esters.

EXPERIMENTAL

Thin layers of Kieselgur G (Merck) (300 μ) were prepared and dried at 100°. After cooling, the layers were impregnated by immersion in 10% v/v liquid paraffin B.P. in petroleum spirit (b.p. 60–80°); the solvent was allowed to evaporate at room temperature for 0.5–24 h.

Methyl esters were prepared from fatty acids (L. Light & Co.) using 12% BF_3 in methanol[10]. Aliquots of solutions of the esters in petroleum spirit (b.p. 60–80°) were applied to the layer surfaces using a Hamilton Microliter Syringe and chromatograms were developed at ambient temperature using a nitromethane–acetonitrile–

acetic acid (75:10:10) mixture over a 10 cm run. It was not found necessary to equilibrate the mobile with the stationary phase. The developed chromatogram was dried and the resolved materials were detected by spraying with a saturated aqueous solution of ferric chloride followed immediately by a 0.1 *M* aqueous solution of sodium molybdate[11], and heating at 140° for about 3–5 min. A very fine spray is required for this operation and the spray jar described by KIRCHNER *et al*[12] was found to be suitable. The detection of unsaturated material with iodine vapour was occasionally found to be advantageous; this in no way affected the result obtained by subsequent spraying with the ferric chloride–sodium molybdate reagent.

RESULTS AND DISCUSSION

Various loadings of liquid paraffin, vaseline and silicone oil (MS200) were used as stationary phase in conjunction with different combinations of acetonitrile, acetic acid, nitromethene, ethanol, acetone, dioxan and water as mobile phase. Best results were obtained using the solvent system specified above together with layers impregnated with 10% liquid paraffin in petroleum spirit (giving a loading of 30 g/100 g Kieselgur G) but small variations in the acetonitrile and acetic acid contents of the mobile phase had no appreciable effect on the result. The chromatograms took 20–25 min to develop in this solvent system.

After treatment of the chromatograms with the ferric chloride–sodium molybdate detecting agent the saturated methyl esters gave orange spots and the unsaturated methyl esters blue-purple spots on a brown background. It was found desirable to

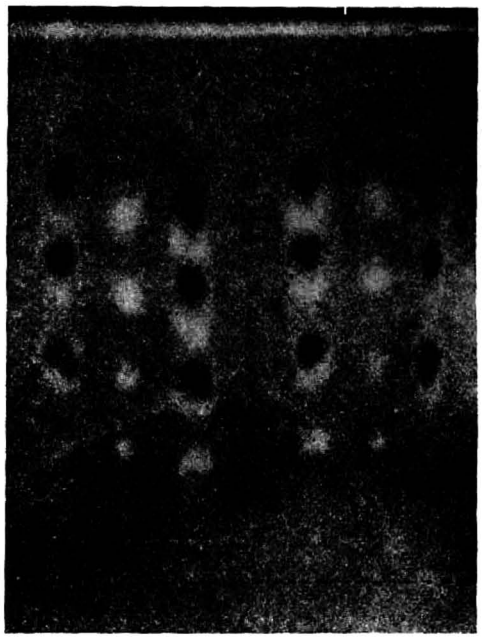

Fig. 1. Separation of fatty acid methyl ester mixtures. Mobile phase CH_3NO_2–CH_3CN–CH_3COOH (75:10:10). Detection: $FeCl_3$–Na_2MoO_4. Dark spots: unsaturated. Light spots: saturated. From top to bottom: (1) C_{18}''', C_{18}'', C_{18}'. (2) C_{12}, C_{14}, C_{16}, C_{18}. (3) C_{18}''', C_{12}, C_{18}'', C_{14}, C_{18}', C_{16}, C_{18}.

watch the chromatogram during heating and sometimes to ring the unsaturated spots when they appeared, as they tended to fade while the saturated spots were being intensified. Excess heating is to be avoided once all the spots are at maximum intensity. The blue-purple spot of methyl oleate was often found to be surrounded by a pale, light coloured halo; experience enables this to be distinguished from slight palmitate contamination.

The R_F values and detection limits for several fatty acid methyl esters are given in Table I, and Fig. 1 shows the separation obtainable for mixtures of unsaturated,

TABLE I

R_F VALUES AND DETECTION LIMITS FOR FATTY ACID METHYL ESTERS

Mobile phase CH_3NO_2–CH_3CN–CH_3COOH (75:10:10)

Methyl esters	R_F *(average of eight determinations)* ± 0.03	$FeCl_3$–Na_2MoO_4		I_2
		μg	*μg/cm²*	*μg*
C_{18}'''	0.69	11	100	1.0
C_{12}	0.62	6	40	
C_{18}''	0.56	11	110	1.0
C_{14}	0.49	4	30	
C_{18}'	0.39	21	270	1.5
C_{16}	0.35	2	20	
C_{18}	0.24	2	30	

C_{12} = methyl laurate
C_{14} = methyl myristate
C_{16} = methyl palmitate
C_{18} = methyl stearate
C_{18}' = methyl oleate
C_{18}'' = methyl linoleate
C_{18}''' = methyl linolenate

saturated, and saturated + unsaturated methyl esters. Methyl linolenate is completely separated from laurate and linoleate from myristate but we were unable to separate completely oleate from palmitate, though such separation was almost achieved using this solvent system.

The minimum quantity of one member of a critical pair which is definitely detectable in a mixture of both members is given in Table II. From this table it will be seen that it is often desirable to use both iodine and ferric chloride–sodium molybdate as detecting agents.

TABLE II

LIMITS OF DETECTION OF ONE MEMBER OF A MIXTURE OF CRITICAL PAIRS (%)

Critical pair mixture	*Detection method*		
	$FeCl_3/Na_2MoO_4$		I_2
	Saturated	*Unsaturated*	*Unsaturated*
C_{18}'''–C_{12}	25	6	1.5
C_{18}''–C_{14}	6	12	1.5
C_{18}'–C_{16}	1.5	50	3

ACKNOWLEDGEMENT

We wish to express our thanks to Mr. J. BARRON of this Institute for photographing the results of this work.

SUMMARY

The separation of critical pairs of fatty acid methyl esters has been carried out by thin-layer partition chromatography, complete separation of linolenate and laurate, linoleate and myristate, and almost complete separation of oleate and palmitate being achieved. The chromatograms took about twenty minutes to develop. A detecting agent was used which gives different coloured spots for saturated and unsaturated methyl esters.

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THIN-LAYER CHROMATOGRAPHY OF β-SITOSTERYL ESTERS

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Sterols are often present in plant tissues not only as the free sterols, but also as their esters. In connection with their studies on corn oil constituents, KUKSIS AND BEVERIDGE[1,2,2a] prepared a number of steryl esters of high purity which could be used as reference compounds for the sterol derivatives present in plants, and studied their separation by reversed-phase chromatography on impregnated paper with three solvent systems.

In studies on the sterol fraction of mulberry (*Morus alba*) leaves, which are believed to contain a factor, or factors, essential for the feeding of the silkworm *Bombyx mori*[3,4] NAYAR AND FRAENKEL[5] have obtained strong evidence that the active material is very similar to β-sitosterol and occurs in the plant in the form of esters which, however, have not been isolated in a crystalline form. Like these authors, we have used the technique of thin-layer chromatography and have studied the R_F values, separation and identification of fifteen sitosteryl esters in six solvent systems. With this method, we have been able to isolate from mulberry leaves a major sterol which, indeed, was identical or at least very similar to β-sitosterol, as well as sitosteryl caprate; it appears that at least one more ester is present which is either sitosteryl palmitate or stearate.

EXPERIMENTAL

Preparation of esters

Most of the esters had been described before[1,2]; the new compounds have been prepared by the following method: a mixture of 0.2 mole each of β-sitosterol and the acid with 0.015 mole of *p*-toluenesulphonic acid in 150 ml of dry benzene was refluxed for 4 h in an oil bath. The filtered solution was concentrated *in vacuo* and the solid residue triturated with acetone and recrystallized from methanol.

β-Sitosteryl pelargonate, m.p. 82–83°. Calculated for $C_{38}H_{66}O_2$: C, 82.3; H, 11.9. Found: C, 82.3; H, 12.0 %.

β-Sitosteryl 10-undecenoate, m.p. 72–73°. Calculated for $C_{40}H_{68}O_2$: C, 82.8; H, 11.7. Found: C, 83.0; H, 11.9 %.

β-Sitosteryl arachidonate, m.p. 70–71°. Calculated for $C_{49}H_{80}O_2$: C, 84.0; H, 11.4. Found: C, 83.4; H, 11.8 %.

Preparation of plates

The chromatography was carried out on glass plates (20 × 20 cm), coated with a layer (250 μ thick) of silica gel G (E. Merck).

The slurry for five plates was prepared by shaking 30 g of silica gel and 60 ml of water in a stoppered flask for 30 sec; it was then transferred to a thin-layer applicator

(Desaga, Heidelberg) which was drawn across the plates. The plates were allowed to dry for 15 min at room temperature and then activated in an oven at 120–130° for 30 min. After cooling they were kept in a vacuum desiccator.

Development

To ensure equilibrium conditions inside the chromatography chamber, the walls were lined with a strip of filter paper dipped into the solvent system (150 ml).

The following solvent systems were used as mobile phases (v/v):

(1) Cyclohexane–benzene (1:1),
(2) Cyclohexane–benzene (2:1),
(3) Cyclohexane–benzene (4:1),
(4) Carbon tetrachloride–chloroform (19:1),
(5) *n*-Heptane–ethyl acetate (19:1),
(6) Chloroform–acetone (19:1).

The starting line was drawn at a distance of 2 cm from the base line. The esters were dissolved in chloroform (0.5 mg/ml chloroform), and 1 μl of each solution was applied with a micropipette.

The glass plates were placed inside a Desaga rectangular glass chamber (21 × 22 × 10 cm) and developed by the ascending technique. The experiments were performed at room temperature (27–30°); 30–90 min. were usually required for the solvent front to reach a distance of 13–15 cm from the starting line.

The plates were then taken out of the chamber, and after marking the solvent front, dried in air for a few minutes and at 120° for 3 min.

Detection

The spots of the esters were detected by spraying the plates in a horizontal position with two reagents, (a) a saturated solution of antimony trichloride in chloroform[6,7] and (b) phosphomolybdic acid (10% in ethanol)[8] and heating at 120° for 5 min.

TABLE I

No.	Ester of β-sitosterol	Empirical formula	R_F values in solvent system					
			1	2	3	4	5	6
1.	Acetate	$C_{31}H_{52}O_2$	0.40	0.22	0.19	0.18	0.48	0.83
2.	Propionate	$C_{32}H_{54}O_2$	0.53	0.33	0.26	0.25	0.53	0.84
3.	Butyrate	$C_{33}H_{56}O_2$	0.55	0.36	0.29	0.26	0.56	0.85
4.	Caproate	$C_{35}H_{60}O_2$	0.70	0.41	0.33	0.31	0.59	0.86
5.	Caprylate	$C_{37}H_{64}O_2$	0.75	0.46	0.39	0.37	0.60	0.88
6.	Pelargonate	$C_{38}H_{66}O_2$	0.74	0.48	0.40	0.36	0.59	0.87
7.	Caprate	$C_{39}H_{68}O_2$	0.76	0.50	0.42	0.38	0.62	0.88
8.	10-Undecenoate	$C_{40}H_{68}O_2$	0.70	0.46	0.35	0.34	0.61	0.87
9.	Laurate	$C_{41}H_{72}O_2$	0.80	0.53	0.43	0.40	0.63	0.90
10.	Myristate	$C_{43}H_{76}O_2$	0.82	0.54	0.44	0.41	0.64	0.90
11.	Palmitate	$C_{45}H_{80}O_2$	0.85	0.54	0.47	0.43	0.65	0.91
12.	Stearate	$C_{47}H_{84}O_2$	0.88	0.55	0.50	0.44	0.66	0.92
13.	Oleate	$C_{47}H_{82}O_2$	0.86	0.53	0.45	0.42	0.65	0.91
14.	Linoleate	$C_{47}H_{80}O_2$	0.89	0.56	0.52	0.45	0.67	0.92
15.	Arachidonate	$C_{49}H_{80}O_2$	0.92	0.58	0.54	0.49	0.69	0.93

Reagent (a) gave violet spots, except for β-sitosteryl pelargonate which gave an orange spot. Reagent (b) gave blue spots against a yellow background.

The R_F values of the β-sitosteryl esters are summarized in Table I.

In order to separate mixtures of β-sitosteryl esters, two-dimensional chromatography was employed, using the solvent systems No. 2 and 4. The starting line was drawn at a distance of 3 cm from the base line and the mixture was developed by ascending chromatography. After the solvent had traveled 14 cm, the plate was removed, dried and developed in a second direction.

The following three mixtures of esters have been studied (Table II):

A: 1, 2, 7, 13, 14, 15
B: 1, 6, 8, 9, 11, 12
C: 1, 2, 3, 4, 5, 7, 10, 14, 15.

TABLE II

No.	Ester of β-sitosterol	R_F in solvent system			
		2		4	
		A	B	B	C
1.	Acetate	0.09	0.10	0.10	0.10
2.	Propionate	0.25			0.13
3.	Butyrate				0.15
4.	Caproate				0.20
5.	Caprylate				0.22
6.	Pelargonate		0.27	0.24	
7.	Caprate	0.28			0.24
8.	10-Undecenoate		0.30	0.28	
9.	Laurate		0.37	0.34	
10.	Myristate				0.26
11.	Palmitate		0.38	0.35	
12.	Stearate		0.34	0.32	
13.	Oleate	0.32			
14.	Linoleate	0.34			0.28
15.	Arachidonate	0.35			0.30

SUMMARY

Fifteen esters of β-sitosterol have been separated by thin-layer chromatography on silica gel G plates, using six solvent systems.

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COMPLEXES OF ORGANOMETALLIC COMPOUNDS

VII. PAPER ELECTROPHORESIS OF $(C_2H_5)_2Pb^{2+}$ AND $(C_2H_5)_3Pb^+$ IN CHLORIDE SOLUTIONS

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The present work deals with the paper electrophoresis of the organometallic cations $(C_2H_5)_3Pb^+$ and $(C_2H_5)_2Pb^{2+}$, in aqueous LiCl solutions of varying molarity. It has been undertaken in order to investigate the applicability of paper electrophoresis to these organo-lead cations, and in particular to study, by this technique, the possibility of the cations forming reversible chloride complexes.

It is well known that the study of reversible complexes in aqueous solutions, and at high ligand concentration, can be carried out by means of ion exchange resins[1]. We have demonstrated that it is possible to study the reversible chloride complexes of organo-lead cations by anion exchange[2], and that useful results can be obtained in this field by the employment of anion exchange papers[3]. The results of the research given in refs. 2 and 3 strongly support the hypothesis of the formation of the complexes $(C_2H_5)_2PbCl_n^{2-n}$ (n ranging from 1 to 4) and $(C_2H_5)_3PbCl_n^{1-n}$ (n ranging from 0 to 3); the values of n are functions of the ligand activity in solution.

On the other hand, it must be kept in mind that the experimental distribution curves of $(C_2H_5)_2PbCl_2$ and $(C_2H_5)_3PbCl$ between resin and aqueous solutions containing the ligand Cl^-, at pH $\simeq$ 7, are continuously increasing with the increase of ligand concentration in solution[2]. Distributions of this kind are often not attributed to anion exchange of negatively charged complexes (see ref. 3).

We have carried out the paper electrophoretic studies reported in this paper, in order to throw some light on this subject. If an eventual agreement of results were obtained with anion exchange and paper electrophoresis, *viz.* with two substantially different techniques, any reasonable doubt as to the existence of reversible chloride complexes of organo-lead cations in neutral solutions would be removed.

In conclusion our work showed that with the electrophoretic technique it is possible to obtain a rapid and accurate separation of $(C_2H_5)_2PbCl_2$ from $(C_2H_5)_3PbCl$. That is why we have also studied the electrophoretic behaviour of Pb^{2+}, since it is the principal decomposition product of organo-lead compounds.

EXPERIMENTAL

The diethyl-lead dichloride and the triethyl-lead chloride used were prepared, stored, checked for purity, and purified as described in earlier papers[2].

Paper electrophoresis was carried out using the technique and apparatus as described by LEDERER AND WARD[4].

Strips of Whatman No. 1 paper (5.5 × 40 cm) were employed. In order to avoid photochemical decomposition during the trials with organo-lead compounds, the sheets of glass containing each strip were enveloped in black paper.

Each electrophoresis was carried out simultaneously on amounts of about 50 γ of each organo-lead chloride (or $PbCl_2$) and on the same quantity of H_2O_2. These compounds were dissolved in aqueous LiCl solutions which were also employed in the electrophoresis. H_2O_2 was used to measure the electro-osmotic flow.

Previous experiments showed that the best results are obtained by employing a potential difference of 135 V for 2 h. The highest current intensity was about 60 mA for concentrated solutions, but in general it was 20–30 mA.

The location of organo-lead cations, after electrophoresis, was revealed by oxidation to Pb^{2+} with Br_2, followed by spraying with potassium rhodizonate. The H_2O_2 was located with KI.

Experiments on electrophoretic separations of solutions of $PbCl_2 + (C_2H_5)_2PbCl_2 + (C_2H_5)_3PbCl$ mixture were also carried out.

RESULTS

The data obtained are reported in Table I, which indicates in mm the displacement (corrected for electro-osmotic flow) of the spot centre from the application point towards the cathode or towards the anode. It must be remembered that $(C_2H_5)_2PbCl_2$ and $PbCl_2$ are adsorbed on Whatman No. 1 paper in LiCl solutions of concentrations $< 0.5\ M$, while $(C_2H_5)_3PbCl$ is adsorbed in [LiCl] $> 4\ M$. A tail formation can be observed for these molarities (see Table I).

Partial decomposition, probably thermal, of the compound $(C_2H_5)_2PbCl_2$ occurred sometimes, during electrophoresis in concentrated LiCl solutions.

The best separation of the components in $PbCl_2 + (C_2H_5)_2PbCl_2 + (C_2H_5)_3PbCl$ mixtures was obtained using LiCl concentrations between 2 and 3 M. (see Table I).

DISCUSSION

The results obtained and reported in Table I show that paper electrophoresis may be used to study electrolytic solutions of organo-lead chlorides. The displacement observed is in fact very clear and reproducible.

From the data given in Table I, it is possible to deduce the formation of reversible complexes between Cl^- and organo-lead cations. The mean number of ligands, determining the charge of the complex, seems to be a function of the concentration of the supporting electrolyte which supplies the ligand. Similar results were obtained for Pb^{2+} (see Table I), which forms the well-known reversible chloride complexes $PbCl_n^{2-n}$.

The isoelectric points for $(C_2H_5)_2PbCl_2$, $(C_2H_5)_3PbCl$ and $PbCl_2$ are at LiCl $\simeq$

TABLE I

PAPER ELECTROPHORESIS OF $(C_2H_5)_2PbCl_2$, $(C_2H_5)_3PbCl$ AND $PbCl_2$

Supporting electrolyte: aqueous LiCl. Applied potential: 135 V for 2 h. The distances travelled are corrected for electro-osmotic flow.

Molarity of LiCl	*Distances travelled in mm**		
	Et_2PbCl_2	Et_3PbCl	$PbCl_2$
3.24	—22	— 9	—34
2.14	—24	— 9	—30
1.47	—20	— 5	—20
1.00	—20	— 4	— 8
0.94	—16	— 3	— 5
0.825	—16	— 1	— 1.5
0.75	—11	0	+ 2
0.69	— 9	0	+ 4
0.54	— 9	+ 3	+ 8
0.45	— 8	+ 3	+ 9**
0.38	0	+ 7	+16**
0.276	+ 4	+ 8	+22**
0.14	+15**	+16	+22**

* + indicates movement towards the cathode, — towards the anode.
** Tail produced by adsorption on cellulose.

0.4 M; LiCl $\simeq$ 0.75 M; LiCl $\simeq$ 0.8 M, respectively. If the isoelectric point corresponds to the presence of an electrically neutral complex, it follows that the complexes $PbCl_n^{2-n}$ (with the average number of ligands $\bar{n} = 2$), $(C_2H_5)_3PbCl_n^{1-n}$ (with $\bar{n} = 1$) and $(C_2H_5)_2PbCl_n^{2-n}$ (with $\bar{n} = 2$) are present at the LiCl concentrations corresponding to the isoelectric point.

In LiCl solutions with a lower concentration than that corresponding to the isoelectric point, cationic complexes or the respective cations (migrating towards the negative pole, *cf.* Table I) would exist, while in more concentrated LiCl solutions, there are anionic complexes (migrating to the positive pole, *cf.* Table I).

These deductions are in accordance with the data reported in ref. 2 for the distribution between aqueous LiCl and an anion exchange resin, corrected according to MARCUS AND CORYELL[5] for the "invasion" of the resin by the electrolyte supplying the ligand.

It is worth while pointing out that the agreement between the electrophoresis data and corrected anion exchange data pertaining to each organo-lead cation, is better for some corrected distribution curves than for others, which in theory are equally probable.

From the investigations reported in ref. 2, two deductions can be made.

(a) In the case of $(C_2H_5)_3PbCl_n^{1-n}$, the neutral complex $(C_2H_5)_3PbCl$ is preponderantly present (that is, $\bar{n} = 1$) in about 2.7 M LiCl. This holds only if we asssume $n = 2$ for the complex which exchanges with the resin sites, applying a correction for experimental distribution according to MARCUS AND CORYELL. On the other hand, if we assume $n = 3$ in the correction, then $\bar{n} = 1$ for *ca.* 1 M LiCl. Values of 2 and 3 for n are both probable on the basis of the usual coordination numbers of lead, and according to anion exchange data neither can be preferred.

(b) In the case of $(C_2H_5)_2PbCl_n^{2-n}$, assuming $n = 3$ for the complex which exchanges with the resin sites, $\bar{n}$ becomes 2 for LiCl $\simeq$ 1 M; if, however, a value of 4

for n is assumed, $\bar{n}$ becomes 2 for LiCl $\simeq$ 0.65 M. The choice of the actual value of n between these two probable values cannot be made by anion exchange data.

By comparing these results with the isoelectric points obtained from electrophoresis, it can be concluded that the corrected anion exchange distribution curves that are in better accordance with the electrophoretic findings, are those obtained by assuming $(C_2H_5)_2PbCl_4^{2-}$ and $(C_2H_5)_3PbCl_3^{2-}$ as the complexes exchanging with the resin sites (see ref. 2). Following the MARCUS AND CORYELL theory for anion exchange distributions, these assumptions lead to the conclusion that the complexes that are fully coordinated are $(C_2H_5)_2PbCl_4^{2-}$ and $(C_2H_5)_3PbCl_3^{2-}$ (see ref. 2).

From these considerations it seems possible that paper electrophoresis will provide qualitative evidence of the formation of organo-lead chloride complexes. This evidence together with corrected anion exchange measurements, should lead to the actual determination of the fully coordinated complexes. Paper electrophoresis is thus useful also in determining the complexes exchanging with the resin sites.

It must be remembered that it is often impossible to obtain the data regarding these complexes necessary for the application of the MARCUS AND CORYELL theory.

In the case of $PbCl_n^{2-n}$, it was not possible to obtain any information about the type of fully coordinated complex by comparing distribution data between resin and solution[6] with the electrophoretic experiments reported above. In fact, if this last complex is either $PbCl_5^{3-}$ or $PbCl_4^{2-}$, corrected anion exchange data show that the respective isoelectric points are at LiCl $\simeq$ 0.7 M and LiCl $\simeq$ 0.95 M, while the isoelectric point obtained by the electrophoretic method is at LiCl $\simeq$ 0.8 M.

A further conclusion is that the electrophoretic method is also useful for the analytical separation of mixtures containing $(C_2H_5)_2PbCl_2$, $(C_2H_5)_3PbCl$ and $PbCl_2$.

SUMMARY

The compounds $(C_2H_5)_2PbCl_2$, $(C_2H_5)_3PbCl$ and $PbCl_2$ were studied by paper electrophoresis in aqueous LiCl solutions of various molarities. Determination was made of the formation of chloride complexes in which the electric charge was a function of ligand concentration in the solution. From the results of these experiments, and from a comparative examination of anion exchange data obtained in previous researches, useful information is obtained for determining the ligand concentrations relative to the preponderance of cationic, neutral and anionic complexes in the aqueous solutions. From this information it is possible to assume $(C_2H_5)_2PbCl_4^{2-}$ and $(C_2H_5)_3PbCl_3^{2-}$ as the fully coordinated complexes in aqueous solutions of LiCl. The electrophoretic method is also useful for separating the components of mixtures of $(C_2H_5)_2PbCl_2$, $(C_2H_5)_3PbCl$ and $PbCl_2$.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF CARBIDES

PART I. THE PREPARATION AND HYDROLYSIS OF RARE EARTH DICARBIDES

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INTRODUCTION

PETTERSON[1] first prepared the dicarbides of lanthanum and yttrium in an electric arc-furnace from the oxides by reduction with carbon. ($M_2O_3 + 5C \rightarrow MC_2 + 3CO$) MOISSAN's pioneering observations[2–5] in this field have stimulated many examinations (some detailed), of the preparation of the carbides of the rare-earth elements.

By 1900 MOISSAN had prepared the following:

(i) yttrium dicarbide by reducing yttria with carbon[2],
(ii) lanthanum dicarbide by reducing the oxide with sugar charcoal[3],
(iii) cerium dicarbide by reducing the dioxide with sugar charcoal[4],
(iv) praseodymium, neodymium, and samarium dicarbides by reducing their oxides with graphite[5,6] in an electric arc furnace.

In a similar electrolytic process, using carbon rods immersed in cerium dioxide in a copper crucible, cerium dicarbide was prepared[7]. STERBA[8] suggested that the reduction of cerium dioxide by carbon at high temperatures proceeds via the intermediate $CeC_2 \cdot 2CeO_2$, which was claimed to be produced after one minute of heating. DAMIENS[9] later denied the existence of this compound, stating it to be a mixture of carbides and oxides of cerium. The existence of a carbide CeC_3 has more recently been reported by WARF[10], but again denied by SPEDDING, GSCHNEIDER AND DAANE[11]. It seems most likely that the reaction proceeds with a step involving such a carbide. WARF's compound was probably a mixture of dicarbide in solid solution with excess carbon. A more likely intermediate is the free metal which combines with carbon to form the dicarbide in the final stage of the reaction[12], and preparation must be carried out under vacuum, or in an inert or reducing atmosphere, but not nitrogen[13] (due to formation of a nitride).

Reaction of the metal with graphite can also form the sesquicarbides M_2C_3, a carbide M_3C, as well as the dicarbide[11]. Indeed, ATOJI[14] reports that cerium dicarbide must always contain at least 5 % w/v sesquicarbide. The dicarbide and sesquicarbide of lanthanum have been prepared by reacting the hydride with graphite[15].

The structures of the dicarbides have been found by neutron-diffraction[12] and X-ray methods[11] to be body-centred tetragonal. The carbon–carbon bond lengths are about 1.3 Å, which is longer than in the acetylide ion. The metal atom is also known

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TABLE I

ANALYSIS OF LIGHT FRACTION OF HYDROLYSIS GASES

	H_2	CH_4	C_2H_2	C_2H_4	CO	CO_2	N_2O	NO	*High fraction*
LaC_2/H_2SO_4	17.9	0.2	68.3	8.9	—	0.4	—	—	4.2
LaC_2/HNO_3	3.4	—	59.0	3.1	—	0.5	19.2	13.2	1.7

to exist in the trivalent state in the carbide, and the odd electron is assigned to a 5d conduction bond.

MOISSAN's hydrolysis results[2–6] indicate a large percentage (*ca.* 70 %) of acetylene produced from each dicarbide, and the work of DAMIENS[9] confirms this. More recent observations of DE VILLELUME[16] are not in general agreement with these results, although the discrepancy is probably due to catalysis or polymerisation effects at the high temperature (200°C). GREENWOOD AND OSBORN[15] hydrolysed lanthanum dicarbide and sesquicarbides using 4 *N* nitric and sulphuric acids. The major component found was acetylene and results are given in Tables I and II. Gas chromatography was used to analyse the gases produced.

Lanthanum and cerium dicarbides have been hydrolysed by PALENIK AND WARF[17] (for results see Table III), who reported ethane as well as the other products of GREENWOOD AND OSBORN[15].

TABLE II

ANALYSIS OF HIGH FRACTION OF HYDROLYSIS GASES

	C_3 *hydrocarbons*	$n\text{-}C_4H_{10}$	*1-Butene*	*2-Butene*		*1,2-Butadiene*	*1,3-Butadiene*	*1-Butene*
				cis	*trans*			
LaC_2/H_2SO_4	3.0	7.9	1.3	13.1	9.8	4.6	55.8	4.6
LaC_2/HNO_3	2.6	2.6	8.0	5.5	8.0	2.6	70.8	—

No figures were reported for non-condensable gases except that they probably amounted to 5 % at room temperature and 15 % at 200°C. Such gases must consist mainly of hydrogen and methane. No solid carbon is reported, and the similarity of results from LaC_2 and CeC_2 lead to the conclusion that the metals in these carbides are in the same oxidation state (III). The solid product of hydrolysis was reported to be amorphous hydroxides showing no X-ray pattern.

In this work, four rare-earth dicarbides have been prepared, and hydrolysed by water and mineral acids at 100°C.

EXPERIMENTAL

Materials

Lanthanum, cerium and yttrium oxides (99.9 % pure); gadolinium and dysprosium oxides (96 % pure) were purchased (New Metals and Chemicals Ltd., London). Neodymium and samarium oxides at least 99 % pure, were isolated from 100 g batches didymium oxide (Thorium Limited, London) using cation-exchange techniques[18,19] and analysed spectrophotometrically[30].

TABLE III

ANALYSIS OF HYDROLYSIS GASES[17]

	LaC_2 25°C	CeC_2 25°C	CeC_2 100°C	CeC_2 200°C
C_2H_6	24.3	19.7	16.3	11.2
C_2H_4	8.45	6.56	4.96	39.6
C_3H_8	0.21	6.03	1.03	13.5
C_3H_6	0.03	0.05	0.53	12.4
n-C_4H_{10}	0.51	6.83	2.32	1.96
C_2H_2	61.5	67.3	63.9	0
1-C_4H_8	1.13	1.39	1.20	1.76
iso-C_4H_8	0	0	0	0.58
Unknown I	0	0	0.37	0.89
trans-2-C_4H_8	0.66	0.84	1.49	3.64
cis-2-C_4H_8	0.72	0.87	1.84	3.74
n-C_5H_{12}	0.05	0	0	3.17
Unknown II	0.03	0.03	0.37	0
1,3-Butadiene	0.05	0.07	2.70	3.56
Unknown III	0	0	0	2.38
1,2-Butadiene	0.26	0.22	0.42	1.48
Unknown IV	2.14	2.10	2.51	0

The carbon was coconut charcoal, and was used throughout the preparations. 1.0000 g of this carbon when heated in a muffle furnace for 10 h at 700°C, gave 0.005 g unburnable residue. Charcoal was finely ground in a pestle and mortar, suitable for mixing with the powdered oxides.

Preparation

15 g of the rare-earth oxide was mixed with the stoichiometric weight of carbon, very finely ground together, and pelletted at 3 tons per sq. inch. The pellets were placed in a carbon crucible, in a Wild Barfield/NRC vacuum resistance furnace Model 2904B. After the pressure had dropped to below 0.01 μ the heating cycle was started, and initiation of the reaction noted by the sudden rise in pressure due to the evolution of carbon monoxide. Reaction occurred at temperatures ranging from 1260°C for lanthanum to 1600°C for gadolinium. Samarium oxide would not react at temperatures obtained in the furnace (1900°C), and dysprosium oxide reacted only very slowly.

TABLE IV

ANALYSIS DATA ON YTTRIUM CARBIDE AND FOUR RARE-EARTH DICARBIDES

	% *Yield*	% *Metal*		*X-ray analysis*		
		Found	*Theoretical*	a_0 (Å)	c_0 (Å)	*Structure*
YC_2	72.4	82.2	78.8	3.66	6.15	b.c.t.§ *
LaC_2	80.6	85.5	85.3	3.93	6.57	b.c.t.
CeC_2	88.2	84.9	85.4	3.87	6.48	b.c.t.**
NdC_2	80.3	86.0	85.7	3.82	6.40	b.c.t.
GdC_2	79.3	87.2	86.7	3.71	6.24	b.c.t.

§ b.c.t. = body-centred tetragonal (*cf.* ref. 14).

* Metal rich. Several lines not indexed, showed presence of other phases, possibly Y_2C_3, YC, and Y_3C.

** Two stray lines, most probably due to Ce_2C_3.

Four rare earth dicarbides and an yttrium carbide were prepared, however, and Table IV shows the yields, chemical analysis by oxalate precipitation and the structures as found by X-ray powder analysis. Deposits formed on cooler parts of the furnace, dissolved in dilute hydrochloric acid and were checked by precipitation with oxalate solution. Such deposits accounted for the low yields of products.

Hydrolysis

A simple all-glass apparatus allowed hydrolytic agents (2.5 ml) to be dropped on to 0.5 g carbide in a 5 ml flask at 100°C. The gases so released were passed over a small calcium chloride drying tube, and through a gas sampling valve where 1.4 ml samples were extracted and swept on to a gas-chromatographic column. The column was 25 ft. of 20 % w/w tetra-isobutylene on Silocel 36–60 mesh at 19°C. The detector was a flame-ionization type which could detect components comprising 0.001 % v/v of the mixture[21]. The flow rates of the gases used in the analysis were 40 ml/min for nitrogen, 20 ml/min for hydrogen and 400 ml/min for air. Fig. 1 shows a schematic diagram of the chromatographic unit.

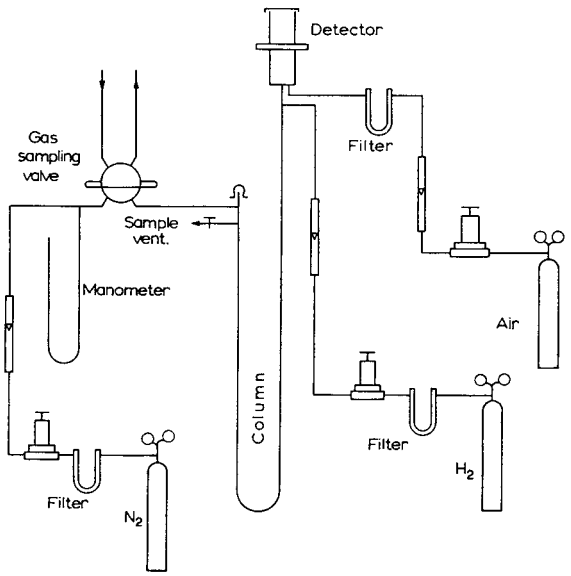

Fig. 1. Schematic diagram of chromatographic analyser unit.

RESULTS

Tables V, VI, and VII show the quantitative breakdown of the hydrolysis products from water, 4 *N* sulphuric acid, and 4 *N* nitric acid, respectively, at 100°C, in volume %. Fig. 2 shows the type of elution patterns obtained from lanthanum dicarbide and water, under differing sensitivities.

The data in Table VIII were obtained under the following conditions:

Temperature 19°C; flow rate, hydrogen 21 ml/min, nitrogen 42 ml/min, and air 400 ml/min; inlet pressure 13 p.s.i.; dead time (hydrogen) 3.7 min; column 25 ft. 20 % w/w tetra-isobutylene on Silocel 36–60 mesh.

TABLE V

HYDROLYSIS PRODUCTS FROM WATER AT 100°

Products	LaC_2	CeC_2	NdC_2	GdC_2	$YC_{1.6}$
Hydrogen	5.64	1.79	2.21	5.07	2.50
Methane	0	0.01	0.28	0.14	4.50
Acetylene	67.72	69.72	66.45	68.23	67.30
Ethylene	7.42	8.67	9.16	8.48	7.15
Ethane	11.13	13.85	12.71	12.35	8.45
C_3H_4	0	0	0	0	0.71
Propylene	0.01	0.01	0.01	0.02	0.93
Propane	0.03	0.06	0.72	0.06	5.07
Isobutane	0.01	0.01	0.03	0.02	0
1-Butene	1.67	0.45	2.08	1.33	1.35
1,3-Butadiene	2.41	1.74	2.69	2.03	0.83
n-Butane	0.36	0.69	0.42	0.51	0.32
trans-2-Butene	0.50	0.87	0.64	0.65	0.34
cis-2-butene	0.55	0.92	0.60	0.65	0.01
1-Butyne	0.22	0.32	0.02	0.03	0.01
1,2-Butadiene	0	0.01	0.03	0.02	0
2-Butyne	0	0.06	0.01	0	0
Others	2.33	0.82	1.94	0.41	0.82

Fig. 3 shows the expected straight-line relationship between log retention time and carbon number for the alkanes and alkenes. Methane is not on the line, since as its short retention time indicates, ideal chromatographic conditions are not realised with this compound. It is also apparent that the true homologue of ethylene and propylene is *cis*-2-butene and the *trans*-2-butene, or 1-butene.

It will be noticed in Fig. 4 that retention data are related to boiling points, *i.e.* the phase separates the gases according to volatility and not structure. Acetylene appears as an exception, having no true boiling point, but subliming at —83.6°C.

TABLE VI

HYDROLYSIS PRODUCTS FROM 4 *N* H_2SO_4 AT 100°C

Products	LaC_2	CeC_2	NdC_2	GdC_2	$YC_{1.6}$
Hydrogen	8.52	6.17	2.22	4.76	4.63
Methane	0	0.01	0.64	0.32	4.63
Acetylene	70.10	70.23	63.84	63.04	69.42
Ethylene	7.42	8.11	10.06	10.47	7.36
Ethane	10.92	11.62	13.86	13.46	8.17
C_3H_4	0	0	0	0	0.36
Propylene	0	0.01	0.01	0.02	0.57
Propane	0.03	0.07	0.57	0.04	2.57
Isobutane	0.01	0.01	0.08	0.01	0.01
1-Butene	0.82	1.03	1.64	1.93	0.77
1,3-Butadiene	0.79	1.18	2.16	2.22	0.55
n-Butane	0.27	0.48	0.35	0.31	0.26
trans-2-Butene	0.41	0.44	0.43	0.42	0.16
cis-2-Butene	0.42	0.48	0.49	0.51	0.34
1-Butyne	0.17	0.01	0.05	0.05	0.05
1,2-Butadiene	0	0	0	0.02	0
2-Butyne	0	0	0.02	0	0
Others	0.12	0.15	3.58	2.42	0.15

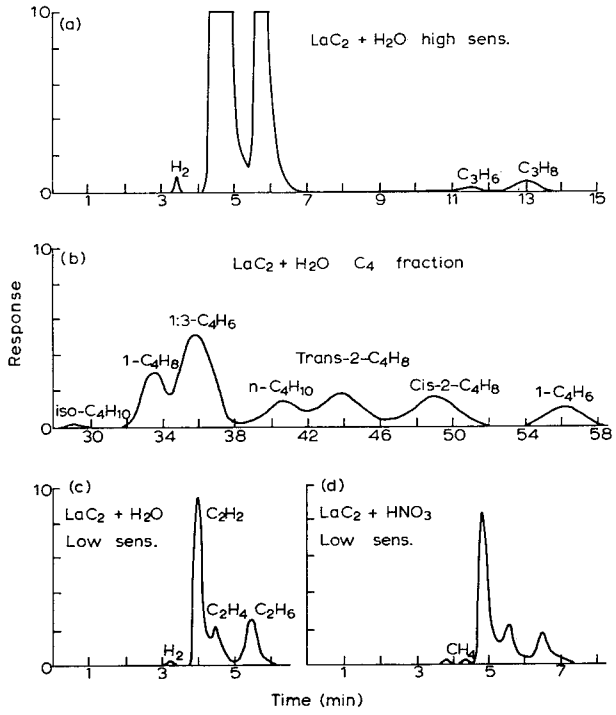

Fig. 2. Elution patterns for the hydrolysis of lanthanum dicarbide with various sensitivities and reagents.

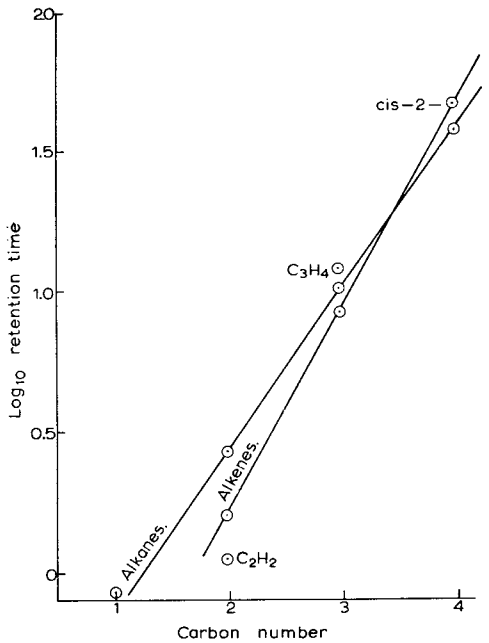

Fig. 3. Relationship between log retention time and carbon number for (i) alkanes and (ii) alkenes.

TABLE VII

HYDROLYSIS PRODUCTS FROM 4 *N* HNO_3 AT 100°C

Products	LaC_2	CeC_2	NdC_2	GdC_2	$YC_{1.6}$
Hydrogen	4.64	5.78	2.72	2.76	5.86
Methane	1.50	1.79	0.36	0.72	7.53
Acetylene	60.87	58.45	58.80	58.70	50.53
Ethylene	9.81	10.86	10.35	10.45	10.71
Ethane	10.82	9.53	8.67	7.86	7.72
C_3H_4	0	0	0	0	0.36
Propylene	0	0.01	0.01	0.02	0.57
Propane	0.05	0.21	1.14	0.05	4.36
Iso-butane	0.34	0.25	0.53	0.07	0.01
1-Butene	0.28	0.42	1.13	1.15	0.49
1,3-Butadiene	0.72	1.10	1.53	0.78	0.43
n-Butane	0.09	0.04	0.29	0.45	0.17
trans-2-Butene	0.13	0.05	0.42	0.58	0.12
cis-2-Butene	0.20	0.25	0.46	0.54	0.25
1-Butyne	0.14	0.04	0	0.02	0.03
1,2-Butadiene	0	0	0.04	0	0
2-Butyne	0.01	0	0.03	0.01	0.01
Nitrogen oxides	10.40	11.26	13.52	15.84	11.26

Association between acetylene molecules has been postulated. Fig. 4 shows the relationship used to identify the unknown hydrocarbons. The diagrams shows the retention times of the three unknown hydrocarbons coupled with the boiling points of three unsaturated C_4 hydrocarbons. Their intersections are either on or very close to the line, identifying the three as 1-butyne, 1,2-butadiene, and 2-butyne in order of elution.

1,2-Butadiyne has the same boiling-point as 1,2-butadiene (10.3°C) but it was not expected to be present in hydrolysis products which contain some hydrogen initially in a highly active state.

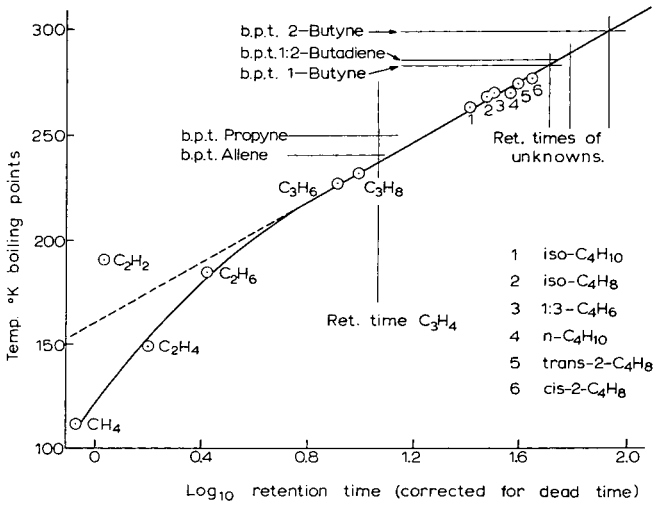

Fig. 4. Relationship between log retention time and boiling point (°K) for the alkanes and alkenes.

TABLE VIII

RETENTION TIMES OF HYDROCARBONS

Hydrocarbon	*B.p. (°K)*	*Corrected retention time (R.T) (min)*	*$Log_{10}RT$*
CH_4	111.7	0.25	0.70
C_2H_2	189.6	1.1	0.04
C_2H_4	149.3	1.6	0.20
C_2H_6	184.9	2.6	0.415
C_3H_6	226.2	8.25	0.92
C_3H_8	231	10.0	1.00
C_3H_4 (?)	—	11.85	1.074
iso-C_4H_{10}	263	26	1.415
1-Butene	26.8	30.3	1.48
1,3-Butadiene	270	32.5	1.51
n-Butane	270	37.1	1.57
trans-2-Butane	274.2	40.0	1.60
cis-2-Butene	276.9	45.1	1.65
1st unknown	—	52.6	1.72
2nd unknown	—	62.0	1.79
3rd unknown	—	87.0	1.94
Isopentane	301	91.0	1.96
n-Pentane	309	123.5	2.09

Another hydrocarbon, probably allene or methyl acetylene was found in the hydrolysis of yttrium carbide. The compound was eluted after propane but the intersections of the retention time with the boiling-points of allene (—32°C) and methyl acetylene (—23.3°C) did not lie on the line; although that due to allene was very close.

DISCUSSION

The results show general agreement with previous observations in this field[15], with close agreement in many instances, and discrepancies in others. Some general comments precede more detailed discussion on the individual carbides.

General comments

Composition of "others". Where water and sulphuric acid were used, this probably consisted of carbon monoxide, carbon dioxide, nitrogen and oxygen. With nitric acid, large quantities of nitric oxide and nitrogen dioxide were released. This is consistent with GREENWOOD AND OSBORN's results on lanthanum carbides[15], although there is a discrepancy over quantities.

Nitric acid. The percentage of C_4 hydrocarbons in the hydrolysis mixture was always less when nitric acid was used. For free radical type of reactions involving collisions of $C_2H\cdot$ radicals with subsequent hydrogenation to form C_4 hydrocarbons, a smaller percentage would be expected since NO is a chain-stopper. Reactions such as:

$$CH_3\cdot + NO \longrightarrow CH_3NO$$

are well known in free radical reactions[22], and the analogous reaction with ethyl radicals is also known. Thus products such as nitro-compounds might be expected from the hydrolysis of carbides with nitric acid, but the conditions of operation of the

gas chromatography apparatus used for these results would not detect such products. EVERED AND POLLARD[23] have shown that alkyl nitrates and nitroalkanes can be satisfactorily eluted at about 110°C using dinonyl phthalate or squalane as solvents.

Nature of reaction. The initial stage of this highly exothermic process is possibly the chemisorption of water molecules on the surface of the carbide. The metal atom probably has a higher affinity for oxygen than hydrogen causing fission of the water molecule to give atomic or ionised hydrogen. Such an entity attacks the carbon in the crystal leading initially to C_2H radicals from acetylides, or CH_2 radicals from methanides, with the corresponding metal hydroxide or hydrated metal oxide. With water and sulphuric acid, the products found are very similar, hence the presence of hydrogen ions does not affect the nature of the reaction, which must therefore be concerned with reaction by the water molecule rather than the hydrogen ion. The carbide is eventually dissolved by acid, but with water there always remains a solid deposit which appears to contain little or no carbon. Uranium carbides showed an oily film on the water, indicating the presence of higher hydrocarbons[24].

If the formation of radicals is assumed, and the undoubted presence of free or active hydrogen (which does not all combine to form hydrocarbons—since hydrogen gas is present in the hydrolysis products in every case), the course of the further reactions may have a wide variety of interpretations.

Rare earth carbides

The first observation that can be made is that there appears to be no trend along the rare earth series. From lanthanum and gadolinium the results are similar under the same conditions although there is a distinct variation in the ease of preparation of these carbides. Even cerium^{III} dicarbide with the possibility of the formation of cerium^{IV} during hydrolysis, does not give different results. Cerium is known to exist in the trivalent state in the dicarbide[14], and has been found to remain so in that valency state when hydrolysed with water or 4 *N* sulphuric acid, but to be oxidised to Ce^{IV} when hydrolysed with 4 *N* nitric acid.

The main difference (apart from the smaller C_4 hydrocarbons produced) between the reactions is the lower percentage of acetylene (58–60 %), and different percentages of ethylene (10–11 %) and ethane (8–11 %), formed with nitric acid when compared to the other two hydrolytic reagents. The results for water are acetylene (66–70 %), ethylene (7–9 %), and ethane (11–14 %) respectively. The higher ethylene figure, and lower ethane figure, is probably also a feature of the unusual reaction of nitric acid where the degree of hydrogenation is apparently curtailed.

Ethylene is probably produced by reactions such as:[25]

$$C_2H\cdot + H \rightarrow C_2H_2$$

$$C_2H_2 + H \rightarrow C_2H_3\cdot$$

$$H + C_2H_3\cdot \rightarrow C_2H_2 + H_2 \rightarrow C_2H_4$$

Ethylene can then combine with hydrogen atoms readily to form the ethyl radical, a reaction which may be surface-catalysed, leading to ethane[26]. The reduction in acetylene is only apparent since, if the percentage of "others" is excluded from the results (*i.e.* contain little or no carbon), the acetylene figure is brought up to a similar value

as those with water, the ethane and ethylene figures also increase. Thus with nitric acid, more carbon is released from the carbide as C_2 hydrocarbons than with water or sulphuric acid.

Very small percentages of methane and C_3 hydrocarbons are present, their complete absence is to be expected from carbides containing C_2 groups in the crystal. The appearance of such species may be due to the presence of a few single carbon atoms in the crystal which react to form methylene radicals. Such radicals could be further hydrogenated to form methane or react with acetylene or $C_2H\cdot$ radicals to form C_3 species[27].

$$CH_3\cdot + C_2H_2 \rightarrow C_3H_5\cdot$$

$$CH_3\cdot + C_2H_4 \rightarrow C_3H_7\cdot$$

The major C_4 hydrocarbon present is 1,3-butadiene which can be formed by the reaction of two C_2 entities followed by hydrogenation.

$$C_2H\cdot + C_2H_2 \rightarrow C_4H_3\cdot^{28}$$

Other C_4 hydrocarbons might be formed by further reaction of this radical with hydrogen, or further free radical reactions, possibly

$$C_2H_5\cdot + C_2H_2 \rightarrow C_4H_7\cdot^{29}$$

$$C_2H_5\cdot + C_2H_4 \rightarrow C_4H_9\cdot^{30}$$

Polymerisation (probably catalysed) appears as a major factor in the hydrolyses at higher temperatures, forming high percentages of C_1 and C_3 hydrocarbons as reported by DE VILLELUME[30,31] and PALENIK AND WARF[17] when reacting cerium dicarbide at 200°C. Little or no acetylene is reported. Thus it is possible that the C_1 and C_3 species are formed through free radical mechanisms involving bond splitting which becomes prominent at higher temperatures. The exothermic nature of the reaction may produce local "hot zones" even though the general temperature is 100°C, and hence cause such reactions to proceed to a minor extent and give only minor yields of C_1 and C_3 species. Further work is necessary to resolve these problems.

Yttrium carbide

Chemical analysis of this carbide showed 82.2 % metal; YC_2 requires 78.8 %. Crystallographic examination showed that the carbide was mainly YC_2 but contained a number of unidentified X-ray lines probably due to another carbide phase. SAMSONOV, KOSOLAPOVA, AND MAKARENKA[32] have described the preparation of three yttrium-carbon phases, YC, Y_2C_3 and YC_2 (from the oxide with carbon *in vacuo*) at temperatures of 1800–1900°C, 1700–1800°C, and 1900°C, respectively. The structure of Y_2C_3 is known to be complex[34], explaining why the unidentified lines on the powdergram from the carbide prepared here could not be indexed. Y_2C is known to be cubic[11], while the structure of YC is not known. Assuming only YC_2 and Y_2C_3 were present, would demand the proportions 75 % Y_2C_3 and 25 % YC_2—this is not possible in view of the powdergram. A more reasonable proposition is that the carbide was mainly YC_2, containing metal, and Y_2C_3. The sesquicarbide (M_2C_3) contains C_2 groups in the

crystal which are therefore acetylides, but produce less acetylene and more methane and C_3 hydrocarbons than the dicarbides!

The same general remarks made for the rare-earth dicarbides can be made for this carbide, but the hydrolysis products were different, containing more methane, propane, propylene and showing the presence of another hydrocarbon, probably C_3H_4–allene or methyl acetylene.

The mechanism of the hydrolysis of the carbides which produce a mixture of hydrocarbons is still not certain, but accurate analyses of the products will certainly help to elucidate the problem. To prove that there is no trend down the rare-earth series, the remaining dicarbides should be hydrolysed, *i.e.* praesodymium, samarium, and dysprosium to lutetium. Europium dicarbide has to date defied preparation. Pure yttrium dicarbide must be prepared and hydrolysed to observe if the products are analogous to the rare-earth carbides. If a trend exists down the series, then yttrium should be compared to dysprosium and holmium—the position predicted from a consideration of atomic radii. More detailed studies on the hydrolysis at higher temperatures, *e.g.* passage of super-heated steam over the heated carbide, to study the effects of temperature on the products are necessary, in order to elucidate whether catalytic or free radical processes are involved. Studies on calcium carbide have already been initiated.

Relative studies of the rare-earth dicarbides and sesquicarbides will be stimulating, since in the sesquicarbides there are 1.5 unpaired electrons, and the carbon is present as C_2 groups. Apparently less acetylene is produced[11] but the reason is not clear.

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SUMMARY

Four rare-earth dicarbides and a yttrium carbide (average composition $YC_{1.6}$) have been prepared and hydrolysed using water or mineral acids. The hydrolysis products, gaseous hydrocarbons, were determined using gas-liquid chromatography. The preparation from the oxide and carbon appears to proceed via the metal, and the results of hydrolysis are discussed from the nature of the carbide, the primary and subsequent free-radical reaction steps. In particular, the unusual results with nitric acid, due to the chain-terminating properties of nitric acid are mentioned.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF CARBIDES

PART II. THE HYDROLYSIS OF URANIUM CARBIDES

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INTRODUCTION

Both uranium mono- and di-carbides are well known, particularly because of the interest of the atomic energy authorities of the world[1]. The dicarbide UC_2 was first prepared by MOISSAN[2] by heating the metal and carbon. Later the compound was prepared by heating an oxide with graphite, with evolution of carbon monoxide[3-5]; usually uranium dioxide was preferred to U_3O_8 as the source of uranium. LITZ, GARRETT AND CROXTON[4] prepared uranium monocarbide UC by passing methane over the finely divided metal, and it has also been prepared from the metal and graphite at 2100°[4]. It is reported that the dicarbide is formed at higher temperatures from both types of reaction[6].

Uranium monocarbide is a face-centred cubic lattice structure[3,4], $a_0 = 4.955$ Å, whilst the dicarbide is a body-centred tetragonal system[4,7]. $a_0 = 3.517$ Å, and $c_0 = 5.987$ Å with a carbon–carbon bond distance of 1.34 Å.

The results of hydrolysis of UC as described by MOISSAN[2,8] were 0.5 % acetylene, 6 % ethylene, 79 % methane, 14 % hydrogen, the gaseous products representing one-third of the combined carbon, and the two-thirds remaining were liquid and solid hydrocarbons. The observations of LEBEAU AND DAMIENS[9] differ considerably from this, when 29 % hydrogen, 20 % methane, 23 % ethane and other gaseous hydrocarbons were reported after 23 days reaction. Recently BRADLEY AND FERRIS[10] and KEMPTER[11] have reported observations on these systems. The results for uranium monocarbide at 25° agree; the approximate percentages being 10 % hydrogen, 87 % methane, 2 % ethane. KEMPTER reports for UC_2, 14 % hydrogen, 17 % methane, 5 % ethylene, 39 % ethane, 2 % propane, 16 % C_4's (mainly *n*-butane), with smaller amounts of C_5- and C_6-hydrocarbons.

The work described here, was nearing completion when the latter two papers were published.

EXPERIMENTAL

Preparation

Uranium monocarbide. U_3O_8 (21.66 g) was mixed with carbon (3.40 g) finely ground and pelleted at 3 tons/sq. in. The pellets were placed in a previously outgassed graphite crucible and heated in a Wild Barfield/NRC vacuum resistance furnace (Model No. 2904B). The reaction,

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$$U_3O_8 + 11C \rightarrow 3UC + 8CO$$

observed by the evolution of carbon monoxide, commenced at 1460° and was complete at 1690°. The temperature was taken up to 1760° for 90 min, when the pressure dropped to below 1 μ. The pressure was never allowed to rise above 100 μ. A grey crystalline, highly pyrophoric product was obtained. Yields *ca.* 92 %. Analysis of a sample showed 95.3 % uranium (UC requires 95.2 %). An X-ray powder photograph showed the product to have face-centred cubic lattice with $a_0 = 4.958$ Å.

Uranium dicarbide. 10 g of uranium dicarbide were a gift from the U.K.A.E., R.E., Harwell. Analysis showed the carbide to contain 90.2 % uranium (UC_2 requires 90.8 %), and an X-ray powder photograph showed a body-centred tetragonal lattice with $a_0 = 3.52$ Å, and $c_0 = 6.00$ Å.

Hydrolysis

A simple all-glass apparatus was constructed which allowed the hydrolytic reagents used (2.5 ml) to be dropped on to the heated carbides (0.5 g) maintained at 100° by an oil bath. The resultant gases passed over a calcium chloride drying tube. The gases were then passed through a gas-sampling valve on to a gas–liquid chromatography column (25 ft. of 20 % tetraisobutylene on 36–60 Silocel ¼ in. O.D. copper tubing) and detected using a flame ionisation detector, after the design of ONGKIEHONG[12]. Temperature of the column was 20°, carrier gas nitrogen, 40 ml/min, hydrogen 20 ml/min, and air 400 ml/min. Fuller details of the apparatus were described previously[13].

RESULTS

Tables I and II show the nature and composition (%) of the hydrolysis products of both carbides at 100° with 3 reagents, water, 4 *N* sulphuric acid and 4 *N* nitric acid

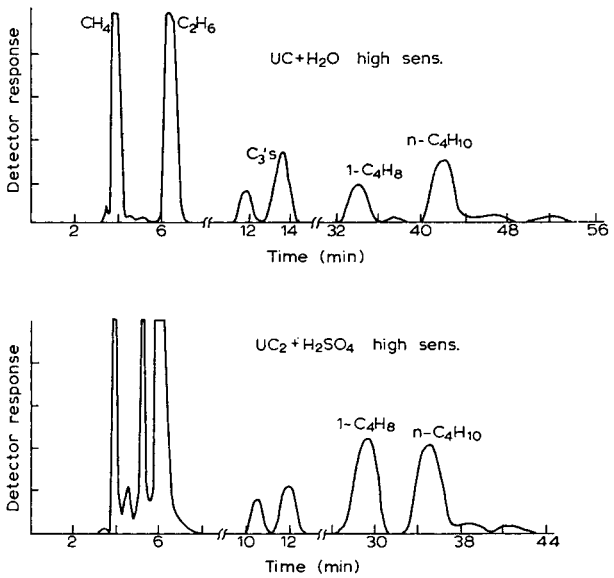

Fig. 1. Elution patterns for the hydrolysis of uranium mono- and dicarbides.

TABLE I

HYDROLYSIS OF URANIUM MONOCARBIDE

	Hydrolysing agent		
	H_2O	$4N\ H_2SO_4$	$4N\ HNO_3$
Hydrogen	9.16	7.47	8.09
Methane	79.05	78.55	64.64
Acetylene	0.57	0.64	0.72
Ethylene	0.43	0.86	0.43
Ethane	5.49	5.86	5.57
Propylene	0.57	0.79	1.32
Propane	1.36	0.27	2.00
1-Butene	0.86	0.91	0.58
1,3-Butadiene	0.42	0.83	0.36
n-Butane	1.40	1.44	1.06
trans-2-Butene	0.31	0.57	0.15
cis-2-Butene	0.31	0.57	0.18
Others	0.07	1.24	14.91
Nitrogen oxides	0	0	

respectively. Fig. 1 shows the elution patterns obtained with the monocarbide and dicarbide respectively. The nature of the products was established using the techniques which have been described previously[13].

TABLE II

HYDROLYSIS OF URANIUM DICARBIDE

	Hydrolysing agent	
	Water	$4N\ H_2SO_4$
Hydrogen	0.54	0.64
Methane	27.71	26.75
Acetylene	0.57	0.64
Ethylene	9.23	8.87
Ethane	44.31	45.62
Propylene	2.00	3.22
Propane	6.00	4.57
1-Butene	4.02	3.92
n-Butane	4.93	4.08
trans-2-Butene	0	0.25
cis-2-Butene	0	0.26
Others	0.69	1.28

With both carbides a small waxy deposit was observed, probably higher hydrocarbons, but insufficient material was available for further study. Work is in progress to elucidate the nature of these products.

DISCUSSION

The results for the uranium monocarbide hydrolysis at 100° agree quite well with those of BRADLEY AND FERRIS[10] at 99°; although the methane content is lower, and the ethane figure is higher. There is agreement with KEMPTER'S[11] results, although

traces of acetylene, propylene and unsaturated C_4 hydrocarbons were also observed. However, the results from uranium dicarbide do not agree with those of KEMPTER at 25°, less hydrogen and methane were found, whilst the work of LITZ[14] (with 47 % hydrogen at 100°) is in even more disagreement. Metal-rich samples would hydrolyse giving more hydrogen than expected, as would samples prepared from the hydride, where hydrogen remained in the lattice.

Tables I and II both show that the hydrolysis products from water and sulphuric acid are almost identical, which indicates that the reaction involves the water molecule, probably in a chemisorption process, rather than as an ionic species. Oxygen has a greater affinity for uranium than hydrogen has, leading to the formation of oxide or hydrated oxide, and the reaction of atomic hydrogen with the carbon in the lattice then follows. In the case of the monocarbide, this clearly results in the methylene radical, with an excess of hydrogen forming methane and ethane as the main products. The lack of ethylene is probably due to its ease of hydrogenation. Over 96 % of the gaseous products are saturated hydrocarbons.

With uranium dicarbide, the carbon is present as carbon–carbon bonded groups in the lattice, with a bond length of 1.34 Å. However, a large percentage of methane (26 %) points to an easy cleavage of this bond (indeed 83 % of the products are saturated hydrocarbons) and can be compared with the rare-earth dicarbides. In these cases, less than 1 % methane is formed, and over 65 % acetylene. The C_3 hydrocarbons are probably formed by reaction of a methylene or methyl radical, with a C_2 entity, and the C_4 hydrocarbons by dimerisation of C_2 radicals.

The results in Table I using nitric acid are different from those using water, and are worthy of separate discussion. Some 15 % of oxides of nitrogen are formed, but even allowing for this, the methane and C_4 hydrocarbon figures are lower, again due to the chain terminating activity of nitric oxide. It is most probable that such compounds as nitromethane, or formaldoxime are formed by the reactions of NO and hydrocarbon radicals in this system, since the formation of oxalic acid has been communicated to us[15]. Work is in progress to elucidate the solutions to these problems.

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SUMMARY

The gaseous products of the hydrolysis of uranium mono- and dicarbides using water, nitric acid and sulphuric acid at 100° is reported. Gas–liquid chromatography was used to elucidate the products. The results are discussed with a view to justifying the number of hydrocarbon products with respect to the nature of the carbide.

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GASCHROMATOGRAPHISCHE ANALYSE VON GEMISCHEN ANORGANISCHER FLUORIDE

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Bei der Elektrolyse von Lösungen anorganischer Verbindungen in wasserfreiem Fluorwasserstoff entsteht an der Anode in den meisten Fällen ein relativ komplexes Gasgemisch. Die vollständige und quantitative Analyse eines solchen Gemisches physikalisch oft sehr ähnlicher, manchmal jedoch in extrem unterschiedlicher Menge vorhandener Verbindungen, erschien rationell nur mit Hilfe der Gaschromatographie durchführbar. Zwei Faktoren erwiesen sich jedoch hierbei besonders erschwerend:

1. Die chemische Agressivität in Form eines hohen Oxydationsvermögens, oft verbunden mit extremer Hydrolyseempfindlichkeit.

2. Die physikalische Ähnlichkeit gewisser Verbindungen, ausgedrückt in nahe beieinander liegenden Siedepunkten und vergleichbarer Löslichkeit in flüssigen Phasen.

Bei der Wahl der stationären Phase musste also sowohl der chemischen Aggresivität der zu analysierenden Verbindung Rechnung getragen, als auch auf ein genügendes Trennvermögen geachtet werden.

Eine Durchsicht der vorhandenen Literatur ergab, dass beide Probleme für sich getrennt gelöst worden waren, die Kombinations-Lösung aber noch nicht befriedigend gelungen war.

So gelang ELLIS UND IVESON[1] 1958 erstmals die Trennung von Fluor, Chlor, Chlortrifluorid und Fluorwasserstoff. Dabei handelt es sich wohl um äusserst aggressive Gase, aber die physikalischen Konstanten sind genügend unterscheidlich. Der hierbei verwendete Gaschromatograph wurde von IVESON UND HAMLIN[2] für Routine-Analysen weiterentwickelt. Für den Bau des Gaschromatographen konnten ausschliesslich Nickel, Monel, Teflon und Hostaflon verwendet werden, da sämtliche andere Werkstoffe von Fluor und einigen seiner Verbindungen angegriffen werden.

Als Trennsäule wurde in beiden Fällen eine, mit Teflonpulver gefüllte Säule benützt, die mit "Halocarbon-Oil" (Polytrifluorchloräthylen) in Mengen bis zu 50 % belegt war. Da Teflon-Pulver nur eine sehr geringe Oberfläche hat und sämtliche Versuche, ein Trennsäulenmaterial mit grosser Oberfläche zu erzeugen scheiterten, wird derzeit die Anwendbarkeit der gaschromatographischen Methode auf die Fluorchemie durch das Fehlen einer Säule mit entsprechender Trennwirkung erschwert. ELLIS, FORREST UND ALLEN[3] geben bei einer, mit 50 % Halocarbon-Oil belegten Teflonsäule 70–100 Trennstufen pro Meter Säulenlänge an. Im Vergleich dazu werden bei Chromsorb 3,000–5,000 Trennstufen pro Meter Säulenlänge erhalten.

PHILLIPPS UND OWENS[4] trennten Chlortrifluorid, Chlor und Fluorwasserstoff an,

mit Halocarbon-Oil belegten Kapillarsäulen aus Teflonschläuchen. Aber auch die Trennleistung dieser Säulen war bei weitem nicht zufriedenstellend.

Andererseits zeigen unzählige Beispiele des überragenden Trennvermögens gewisser stationärer Phasen gegenüber *organischen* Verbindungen (Isomere, Homologe), dass das Problem der Trennung chemisch relativ inerter Substanzen weitgehend gelöst ist.

Um die Methode der Gaschromatographie auf die Identifizierung von Elektrolysegasen bei Elektro-fluorierungen anorganischer Verbindungen anwenden zu können, musste also im Hinblick auf die Trennsäule ein Kompromiss zwischen chemischer Unempfindlichkeit und doch noch ausreichender Trennwirkung angestrebt werden. Die Natur der zu erwartenden Elektrolysengase fordert dementsprechend, dass sich die Trennsäule inert verhält gegen oxydierende und fluorierende Gase wie Sauerstoffdifluorid oder Perchlorylfluorid, weiters, dass sie von geringsten Spuren Wasser befreit werden kann um Hydrolyse von Gasen wie SF_4 oder SiF_4 auszuschliessen und sie andererseits noch eine befriedigende Trennleistung gegenüber Gasen mit Siedepunkten bis —160° aufweist.

Da die uns bekannten gebräuchlichen Verteilerflüssigkeiten mit einigen der dargestellten Fluoride reagieren würden, lag die Verwendung von Adsorbersäulen nahe. Auch eine Trennung von permanenten Gasen lässt sich am besten durch Adsorption durchführen, da die Löslichkeit von Gasen mit tiefen Siedepunkten in den stationären, flüssigen Phasen von Verteilersäulen sehr gering ist.

EXPERIMENTELLES

Die von uns untersuchten Säulen wurden mit Siliciumtetrafluorid auf ihre Verwendbarkeit geprüft. Obwohl diesem Gas in der Literatur[5] träge Reaktion mit Wasserdampf zugeschrieben wird, wurde es anfangs von sämtlichen untersuchten Trennsäulen sogar nach Ausheizen der Säulen auf 250° quantitativ zurückgehalten. Dieser etwas überraschende Befund lässt sich durch die katalytische Beeinflussung der Reaktion mit Wasser durch das Adsorbens erklären. Da andererseits die Energie einer Wasserstoff-Brückenbindung an Fluorid, der Energie einer normalen chemischen Bindung nahe kommt, würde auch ein Bindungsmechanismus dieser Art die quantitative Absorption von Siliciumtetrafluorid erklären.

In der Tabelle I sind die mit Siliciumtetrafluorid getesteten Adsorbentien zusammengestellt, die Vorbehandlung des Füllmaterials und die Konzentration der Belegung mit Halocarbon-Oil (Nr. 13–21, Halocarbon Corp. Hackensack, N.J. U.S.A.) angegeben. Die Versuche mit Sterchamol, Aluminiumoxyd, Aktivkohle und Molekularsieb verliefen erfolglos, das Testgas wurde quantitativ absorbiert. Teflonpulver liess auf Grund seiner hydrophilen Oberfläche wohl alle Gase durch, hatte aber völlig ungenügende Trennwirkung.

Durch entsprechende Vorbehandlung von Kieselgel gelang es ein Adsorbens zu finden, das allen oben gegebenen Anforderungen zufriedenstellend entsprach. Fig. 1 zeigt, wie die Bandenform von Siliciumtetrafluorid von der Ausheiztemperatur des Kieselgels abhängt. Erhitzt man Kieselgel nur auf 250°, so wird Siliciumtetrafluorid vollständig zurückgehalten. Durch Ausheizen des Absorbens auf 600° gelingt es, das Testgas in Form eines langgezogenen Hügels nachzuweisen. Diese Bande ist jedoch weder qualitativ noch quantitativ auswertbar. Wird jedoch Kieselgel eine Stunde bei

TABELLE I

Adsorbens	*Vorbehandlung*	*Belegung*	*Säulenlänge (m)*	*Säulendurchmesser (mm)*	*Säulen-Temp. (°C)*	*Mengenstrom (ml/Min.)*	*Bemerkungen*
Sterchamol (0.3–0.4 mm)	—	15% Halocarbon-Oil	2	4	0	30	SiF_4 wird quantitativ absorbiert
Aktivkohle (Merck, 0.5–0.7 mm)	20 Std., 250°, mit 40% HF vorbehandelt	0–20% Halocarbon-Oil	0.5	4	0 +150	30	SiF_4 wird quantitativ absorbiert
Aluminiumoxyd	20 Std., 250°, mit 40% HF vorbehandelt	0–20% Halocarbon-Oil	0.5	4	0 +100	30	SiF_4 wird quantitativ absorbiert
Molekularsieb (5Å)	10 Std., 250°	—	0.3	4	0 +100	30	SiF_4 wird quantitativ absorbiert
Teflon (Perkin-Elmer Nr. 098–150H)	—	0–50% Halocarbon-Oil	1	4	—20 +50	30	SiF_4 passiert die Säule ohne Trennung von Luft
Kieselgel (Merck, 0.2–0.5 mm)	5 Std., 600°	—	1	4.5	30	100	SiF_4 passiert die Säule teilweise, Bande nicht auswertbar (Fig. 1a)
Kieselgel (Merck, 0.2–0.5 mm)	1 Std., 900°	0% Halocarbon-Oil 30% Halocarbon-Oil	1	4.5	30	100	SiF_4 passiert die Säule mit Tailing (Fig. 1b) Tailing reduziert (Fig. 1c)

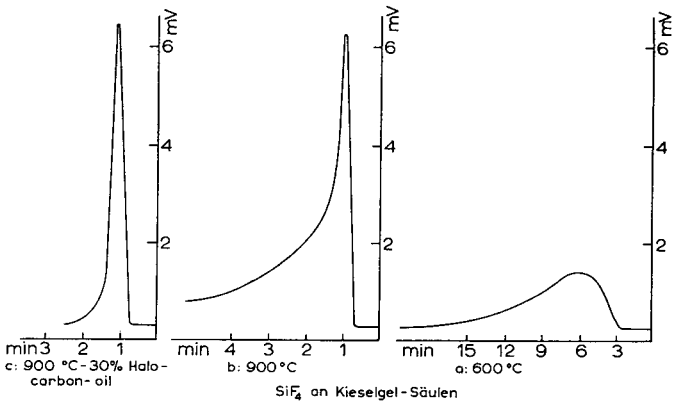

Fig. 1. Abhängigkeit der Bandenform von Vorbehandlungstemperatur und Belegung des Kieselgels.

900° geglüht, so findet anscheinend eine irreversible Veränderung der Oberfläche statt. Das chemisch in Form von Si–O–H Gruppen gebundene Wasser wird unter Vernetzung zu Si–O–Si abgegeben. Siliciumtetrafluorid geht in Form einer reproduzierbaren, quantitativ auswertbaren Bande mit Tailing durch, das durch Belegen der Säule mit Halocarbon-Oil wesentlich reduziert werden kann. (Fig. 1)

Diese Ergebnisse stehen in gewissem Widerspruch zu Angaben von MARVILLET UND TRANCHANT[6], welche berichten, dass Kieselgel höchstens bis zu 600° erhitzt werden dürfe, weil bei höheren Ausheiztemperaturen durch Zusammenbruch der Struktur das Kieselgel als Adsorbens unbrauchbar würde.

Wir konnten jedoch nach einer Methode von CREMER[7] durch Messung des Rückhaltevolumens von Siliciumtetrafluorid bei stationären Bedingungen feststellen, dass die Oberfläche von Kieselgel durch vierstündiges Ausheizen bei 960° kaum

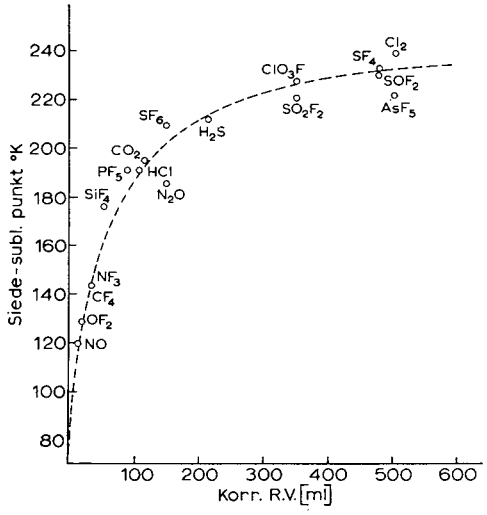

Fig. 2. Siede- und Sublimations-Punkte (°K) gegen Retentionsvolumina.

reduziert wird. Glüht man dagegen Kieselgel 50 Std. bei 960°, so wird die Struktur zerstört, das mittlere spezifische Gewicht steigt auf einen Wert, der um 70 % höher liegt, als jener, der nach vierstündigem Glühen bei derselben Temperatur erhalten wird und die Trennleistung der damit gefüllten Säule fällt extrem ab. Zwischen diesen beiden Ausheizzeiten lag der zur Vorbehandlung von Kieselgel für unsere Aufgaben günstigste Bereich.

Präparierung der Säule

Zur Präparierung einer universellen Standardsäule wurde Kieselgel 1 Std. auf 900° erhitzt, anschliessend mit 30 % Halocarbon-Oil belegt und in eine 4 m lange Säule gefüllt. Die Trennleistung dieser Säule blieb ein halbes Jahr lang trotz sehr häufiger Verwendung konstant.

Eichkurven

Zur Vereinfachung der Auswertung unserer Chromatogramme wurden mit Testgasen Eichkurven der uns interessierenden Verbindungen aufgenommen. Fig. 2 zeigt die Auftragung der nach JAMES UND MARTIN[8] korrigierten Rückhaltevolumina (Tabelle II) der Eichsubstanzen gegen ihre Siedepunkte in °K. Das Trennvermögen der Säule

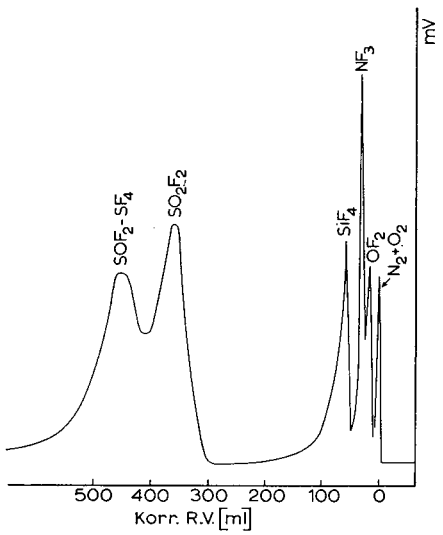

Fig. 3. Gaschromatogramm bei "Standardbedingungen".

wird aus Fig. 3, dem Chromatogramm eines willkürlich zusammengestellten Gasgemisches sichtbar.

Chromatographie

Die von uns gewählten "Standardbedingungen" für die Chromatographie waren folgende:

Trägergas: Wasserstoff, 150 ml/Min.

Säule: Länge 4 m, Durchmesser 6 mm, 30°.

TABELLE II

Gas	*Korrigierte Rückhaltevolumina (ml)*
NO	12.5
OF_2	21.0
NF_3	32.5
SiF_4	57.4
PF_5	96.0
HCl	110
CO_2	121
N_2O	155
SF_6	155
H_2S	220
SO_2F_2	356
ClO_3F	357
SF_4, SOF_2	484
Cl_2	509
SO_2	1050

Stationäre Phase: Silikagel plus 30 % Halocarbon-Oil, 15.5 g/m Säule.
Säulen Druck: Eingang: 1356 Torr; Ausgang: 710 Torr.
Detector: Wärmeleitfähigkeitszelle mit Thermistoren.
Schreibstreifengeschwindigkeit: 2 cm/Min.

Analyse

Zur Analyse wurde ein Fraktometer der Firma Perkin Elmer (Type 116 E) verwendet. Die Registrierung erfolgte mit einem automatischen Kompensationsschreiber (Firma Philips, Type PR 2210 A/21). Die Gasproben wurde mit Injektionsspritzen oder über eine Gasschleife aufgegeben. Die quantitative Auswertung erfolgte nach der von CREMER UND HAUPT[9] ausgearbeiteten "Maxima Methode".

ERGEBNISSE

Unter diesen "Standardbedingungen" wurden durch diese Säule die in Tabelle III angegebenen Verbindungspaare nicht getrennt.

Um diese Verbindungen ebenfalls zu trennen, müsste die Säule auf 6 bis 8 m verlängert werden. Da hiermit die Rückhaltezeiten ebenfalls stark ansteigen würden, wurde in unserem Falle auf die gaschromatographische Trennung dieser Verbindungen verzichtet, und jeweils durch Aufnahme eines Infrarot-Spektrums entschieden, welche der Verbindungen vorlag.

TABELLE III

Verbindung	*Siedepunkt (°C)*	*Verbindung*	*Siedepunkt (°C)*
CF_4	—128	NF_3	—128.4
N_2O	—88.49	SF_6	—63.8 (Subl.)
SO_2F_2	—55.2	ClO_3F	—46.8
SF_4	—40.4	SOF_2	—43.7
AsF_5	—53	Cl_2	—34.6

Zur Charakterisierung unserer Trennsäule sind in Tabelle IV die aus den Chromatogrammen berechneten Trennstufenhöhen (HETP) und die entsprechenden Trennleistungen (n^1) für eine Anzahl von uns untersuchter Gase angegeben.

TABELLE IV

Gas	*HETP (mm)*	n^1
N_2, O_2	7.22	139
OF_2	5.3	189
NF_3	10.05	99.5
SiF_4	6.72	149
PF_5	3.25	308
HCl	16	62.5
CO_2	20.0	50
N_2O	3.6	278
SF_6	13.75	72.5
H_2S	17.1	58.5
SO_2F_2	18.7	53.5
ClO_3F	23.9	42
SF_4, SOF_2	19.46	51.5
SO_2	14.4	69

Wie aus Tabelle III zu ersehen ist, entspricht die Trennleistung unserer Säule bei den von uns gewählten Bedingungen nur einer relativ schlechten Säule, dafür zeigte sie die von uns verlangte Stabilität und allgemeine Anwendbarkeit auch gegenüber sehr aggressiven Verbindungen und war für unsere Aufgabe: eine möglichst schnelle qualitative und quantitative Analyse von komplexen Gemischen dieser Gase mit kleinsten Probemengen (0.1–1 ml Gas) vollauf ausreichend.

DANK

Diese Arbeit wurde im Rahmen des "European Research Contracts Programm" des "Office of Naval Research", Washington, U.S.A., unterstützt, wofür wir sehr zu Dank verpflichtet sind.

Ausserdem danken wir dem Institutsvorstand Prof. Dr. E. HAYEK für seine immer bereitwilligst gewährte Unterstützung und Beratung.

ZUSAMMENFASSUNG

Die Gaschromatographie anorganischer Fluoride wird im Hinblick auf die Analyse der Anodengase von Elektro-Fluorierungen anorganischer Substanzen untersucht. Eine, bei 900° ausgeheizte Kieselgelsäule, die zu 30 % mit Halocarbon-Oil belegt ist, erwies sich genügend stabil gegen die grossteils sehr reaktionsfähigen Gase und zeigte noch genügend Trennvermögen um sie mit Erfolg anwenden zu können. Die Rückhaltevolumina einer Reihe von anorganischen Gasen an dieser Säule werden mitgeteilt und die Trennstufenhöhen berechnet.

SUMMARY

The gas chromatography of inorganic fluorides is investigated in connection with the analysis of the anode gases in electrofluorinations of inorganic substances. A silica gel column, which was preheated to 900° and coated with 30 % Halocarbon Oil, proved to be sufficiently resistant to the mostly very aggressive gases, while still giving good separations. The retention volumes of a series of inorganic gases on this column are reported and the HETP values calculated.

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REVERSED-PHASE CHROMATOGRAPHY OF ALKALINE EARTHS ON PAPER TREATED WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID IN CHLORIDE MEDIUM

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INTRODUCTION

In a previous paper[1] the chromatographic behaviour of alkali metals and alkaline earths was investigated, using papers treated with organic solutions of di-(2-ethylhexyl) orthophosphoric acid (HDEHP), the eluent being acetic acid.

As in the case of the chromatographic separation of rare earths[2], similarity was found between results of liquid–liquid experiments reported in the literature and data obtained in chromatographic investigation.

In the present work the behaviour of alkaline earths in reversed-phase paper chromatography has been further investigated, using hydrochloric acid solutions as the eluents. The R_F values of calcium, strontium, barium, magnesium and beryllium were determined as functions of the HDEHP concentration and of the molarity of the hydrochloric acid used as eluent.

EXPERIMENTAL

Reagents, equipment and treatment of paper

The reagents and equipment used in this work have already been described[1].

Chromatographic paper was treated with cyclohexane solutions of HDEHP previously equilibrated with 2.5 *M* HCl solutions according to the usual procedure[2]. Paper sheets were carefully washed with a 1 *M* HCl solution and rinsed with distilled water before treatment with HDEHP.

Chromatographic procedure

Application of spots, chromatographic procedure and development of spots were generally the same as described previously[1]. For the detection of Ba^{2+} spots, however, tetrahydroxyquinone[3] was preferred to sodium alizarinsulphonate. The spots became immediately visible on spraying a 0.1 % solution in ethanol of this reagent on paper previously exposed to ammonia vapours.

The main work was performed with five different concentrations of HDEHP in cyclohexane, namely 0.010, 0.025, 0.050, 0.075 and 0.100 *M*; experiments were also performed with intermediate concentrations.

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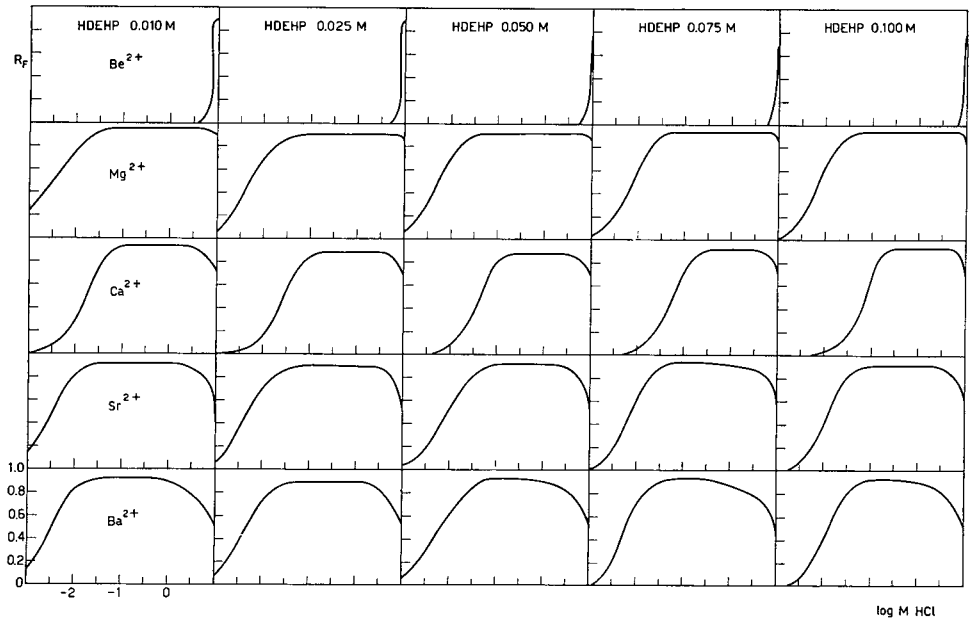

Fig. 1. R_F values of alkaline earth ions plotted *vs.* log M HCl. Paper treated with HDEHP.

Each result was checked at least once under the same conditions, except for a few cases in which the relative position of results at various concentrations left no doubt as to their reliability. Reproducibility of the R_F values within $\pm$ 3 % was considered satisfactory, because of the shortness of the chromatograms and of the difficulty often encountered in obtaining an exact view of the real shape of the eluted and developed spots.

RESULTS AND DISCUSSION

Typical experimental R_F values for alkaline earth ions are collected in Table I as functions of the molarity of the hydrochloric acid used as eluent for papers treated with 0.010 M, 0.025 M, 0.050 M, 0.075 M and 0.100 M solutions of HDEHP in cyclohexane.

In Fig. 1, the results are plotted against the log of the hydrochloric acid concentration. Although experiments were carried out in a range of acidity from 1 M to $1 \cdot 10^{-4}$ M HCl, concentrations lower than $1 \cdot 10^{-3}$ M were omitted in plotting Fig. 1 since the results were of little interest.

The results collected in Fig. 1 agree qualitatively with those reported by PEPPARD *et al.* for the liquid–liquid extraction of calcium, strontium and barium[4].

The decrease in R_F values at very high hydrochloric acid concentrations, particularly noticeable in the case of strontium and barium, was also verified when acetic acid was used as eluent; this problem was also discussed in the previous paper[1].

By applying the equation

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

TABLE I

R_F VALUES FOR ALKALINE EARTH IONS AS FUNCTIONS OF THE MOLARITY OF THE HCl ELUENT. PAPER TREATED WITH HDEHP.

Operating temperature 23° ± 1°

HDEHP molarity	Cations	R_F									
		HCl molarity									
		10	7	2.5	1	0.1	0.04	0.01	0.004	0.001	0.0002
0.010	Be^{2+}	0.85	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.87	0.85	0.94	0.94	0.92	0.92	0 73	0.49	0.20	0.20
	Ca^{2+}	0.79	0.81	0.89	0.95	0.93	0.80	0.25	0.10	0.03	0.00
	Sr^{2+}	0.53	0.78	0.85	0.90	0.89	0.92	0.80	0.48	0.12	0.10
	Ba^{2+}	0.56	0.67	0.81	0.90	0.89	0.87	0.80	0.45	0.12	0.10
		HCl molarity									
		10	3.5	1	0.1	0.035	0.02	0.01	0.006	0.002	0.001
0.025	Be^{2+}	0.84	—	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.88	0.90	0.93	0.89	0.85	0.88	0.71	0.60	0.16	0.10
	Ca^{2+}	0.75	0.90	0.91	0.84	0.59	0.35	0.13	0.06	0.00	0.00
	Sr^{2+}	0.59	0.87	0.93	0.88	0.83	0.84	0.69	0.58	0.15	0.10
	Ba^{2+}	0.54	0.83	0.92	0.88	0.84	0.84	0.69	0.62	0.19	0.10
		HCl molarity									
		10	5	1	0.5	0.1	0.05	0.02	0.01	0.003	0.001
0.050	Be^{2+}	0.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.88	0.88	0.93	0.93	0.91	0.89	0.82	0.66	0.31	0.00
	Ca^{2+}	0.71	0.85	0.87	0.88	0.80	0.54	0.19	0.06	0.00	0.00
	Sr^{2+}	0.49	0.82	0.91	0.90	0.92	0.86	0.70	0.50	0.24	0.07
	Ba^{2+}	0.50	0.75	0.90	0.90	0.95	0.86	0.69	0.58	0.33	0.06
		HCl molarity									
		10	7	2.5	1	0.1	0.035	0.015	0.004	0.002	0.001
0.075	Be^{2+}	0.70	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.95	0.95	0.92	0.92	0.91	0.91	0.75	0.27	0.13	0.04
	Ca^{2+}	0.85	0.92	0.92	0.92	0.71	0.32	0.08	0.00	0.00	0.00
	Sr^{2+}	0.59	0.80	0.87	0.90	0.95	0.93	0.78	0.24	0.06	0.00
	Ba^{2+}	0.55	0.67	0.81	0.84	0.92	0.91	0.78	0.30	0.10	0.00
		HCl molarity									
		10	7	2.5	1	0.1	0.05	0.025	0.01	0.005	0.001
0.100	Be^{2+}	0.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Mg^{2+}	0.88	0.92	0.93	0.93	0.96	0.93	0.83	0.65	0.32	0.02
	Ca^{2+}	0.69	0.89	0.93	0.93	0.67	0.35	0.16	0.04	0.02	0.00
	Sr^{2+}	0.57	0.77	0.88	0.95	0.95	—	0.77	0.44	0.17	0.01
	Ba^{2+}	0.59	0.67	0.84	0.90	0.95	—	0.77	0.43	0.17	0.01

where E_a° is the extraction coefficient of a given element in the liquid–liquid system, and k is a constant that depends on the experimental conditions, the chromatographic R_F value can be used to elucidate some aspects of the extraction mechanism[2].

The equilibrium generally accepted as representing the extraction mechanism of metallic cations by dialkyl esters of orthophosphoric acid, may be written as

$$M^{b+} + c\,HY \rightleftharpoons MY_b \cdot (c - b)\,HY + bH^+ \qquad (2)$$

where M^{b+} is the cation having the valency b, and HY is the extractant molecule disregarding its degree of polymerization.

PEPPARD *et al.*[5,6] have proved HDEHP to be dimeric in solvents such as cyclohexane, which is used in this work. Thus eqn. (2) may be written as

$$M^{b+} + c(HDEHP)_2 \rightleftharpoons M(DEHP)_b \cdot b(HDEHP) \cdot (c - b)\,(HDEHP)_2 + bH^+ \qquad (3)$$

where the extracted species has been indicated without giving any information as to how the extractant is bound to the metallic atom. According to PEPPARD *et al.*[7] single ionization of the dimer $(HDEHP)_2$ occurs, followed by chelation with the metal. More recently MCDOWELL AND COLEMAN[8] have suggested double ionization of the dimer together with coordination of the other molecules of the acid, in the monomeric form, around the metal, to satisfy the metal coordination number. As in the case of the acetate system[1], the latter hypothesis has been adopted here.

From eqns. (1) and (3) the following relationship can be written

$$\log\left(\frac{1}{R_F} - 1\right) = c \log [HDEHP]_{eff} - b \log [H^+] + K \qquad (4)$$

where $[HDEHP]_{eff}$ is the effective HDEHP concentration on paper and K is a term containing the logarithm of the equilibrium constant of reaction (3).

In the case of liquid–liquid systems the analogous equation, $\log E_a^\circ = c \log [(HDEHP)_2] - b \log [H^+] +$ constant, was shown to hold in the case of the HDEHP extraction of lanthanides (III) and actinides (III) as summarized by PEPPARD AND MASON[9]. The expected value 3 for b, and the same value for c was found in that case. The same results were obtained in the paper chromatography of rare earth elements provided that the HDEHP concentration was expressed as the effective concentration, $[HDEHP]_{eff}$, of the extractant in the stationary phase and not as the nominal concentration of the solution used to treat the paper[1,2].

Liquid–liquid extraction of alkaline earth ions with HDEHP solutions[4,8] from inorganic acid solutions gave the expected value of 2 for b and the value of c was 3.

To investigate the analogous chromatographic behaviour, in Figs. 2, 3, 4 and 5 the plots have been reported of $\log (1/R_F - 1)$ *vs.* $\log [H^+]$ for Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} respectively. Beryllium was not included because, as shown in Fig. 1, it is firmly held by the stationary phase throughout a wide range of HCl and HDEHP concentrations. Hydrogen ion concentration, in the range considered, may be assumed to be equal to the hydrochloric acid concentration. In these figures straight lines with different slopes were drawn and the experimental results compared to them. It can be noted for later discussion that very few of these lines have the expected slope of -2.

On the other hand in the plot shown in Fig. 6 of $\log (1/R_F - 1)$ against the

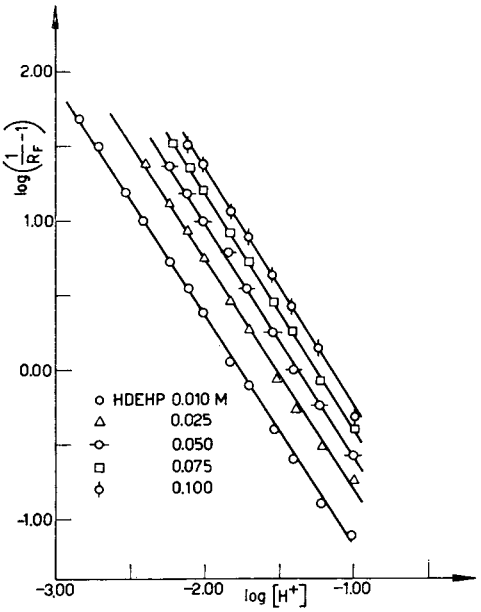

Fig. 2. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for calcium. Paper treated with HDEHP at various concentrations. Slope = —1.5.

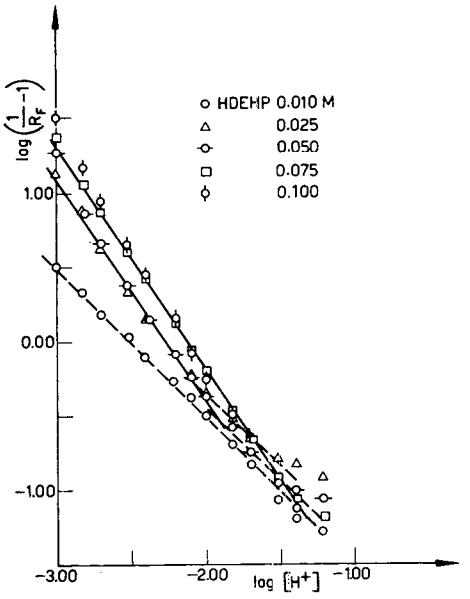

Fig. 3. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for magnesium. Paper treated with HDEHP at various concentrations. Slopes = —1.5 (solid lines); —1.0 (dashed lines).

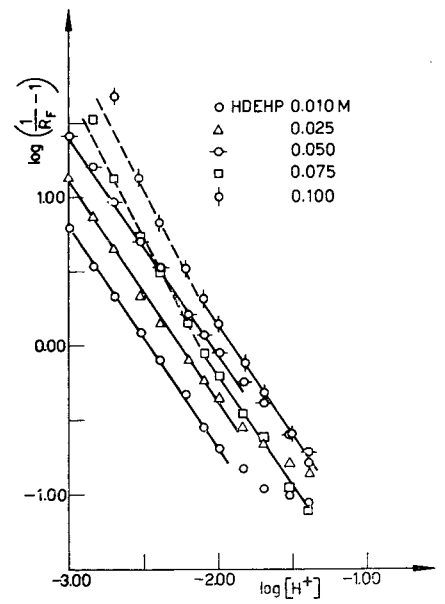

Fig. 4. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for strontium. Paper treated with HDEHP at various concentrations. Slopes = —1.5 (solid lines); —2.0 (dashed lines).

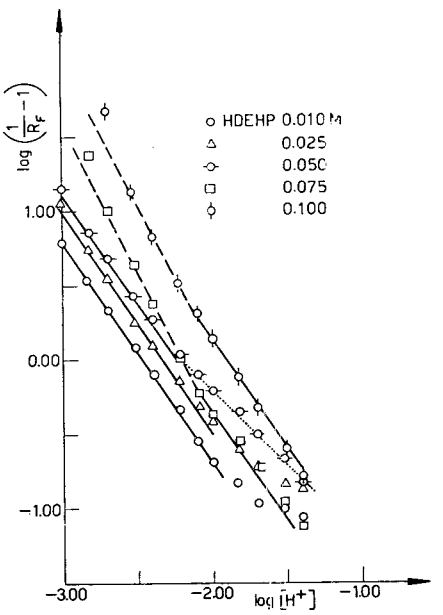

Fig. 5. Plot of log $(1/R_F - 1)$ *vs.* log $[H^+]$ for barium. Paper treated with HDEHP at various concentrations. Slopes = —1.5 (solid lines); —2.0 (dashed lines); —1.0 (dotted line).

logarithm of the effective HDEHP concentration on paper, straight lines having the expected slope of + 3 can be drawn. The effective HDEHP concentration was evaluated following the procedure reported in the previous paper[1]. The two curves refer to Ca^{2+} and Sr^{2+}; Ba^{2+} and Mg^{2+}, when eluted with HCl under the same conditions as Sr^{2+}, give analogous curves which lie very close to that of strontium. In order to have more information on this behaviour, simultaneous elutions were carried out with

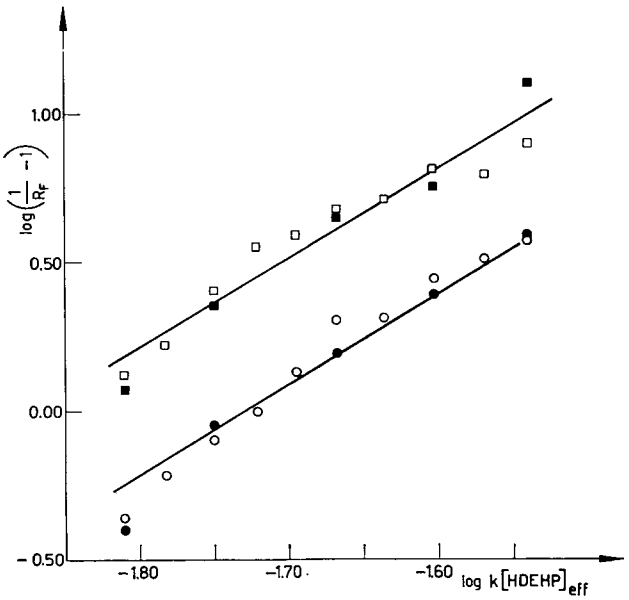

Fig. 6. Plot of log $(1/R_F - 1)$ *vs.* log k $[HDEHP]_{eff}$ for calcium eluted with 0.03 *M* HCl (circles), and strontium eluted with 0.003 *M* HCl (squares). Data from Table II (open symbols) and data from plots in Figs. 2 and 4 (closed symbols).

paper treated with HDEHP solutions at ten different concentrations. Whatman CRL/1 paper strips were used, cut from complete sheets each treated with a different HDEHP concentration. The results are collected in Table II. From this table, data referring to calcium and strontium are shown in Fig. 6 as open symbols. The closed symbols refer to data derived from plots given in Figs. 2 and 4.

From the results of Figs. 2 to 6 it appears that although a relationship such as that shown in equation (4) holds, the values obtained for b are often lower than the stoichiometric value 2 characteristic of the ion involved and confirmed in the liquid–liquid experiments referred to above.

The strontium and barium lines showed the expected — 2 slope, giving a value of 2 for b, only when paper treated with 0.075 and 0.100 *M* HDEHP was used and eluted with HCl at a concentration less than 0.01 *M*.

In some cases a value of b smaller than the cation valency was also reported in the literature for liquid–liquid extraction with HDEHP. PEPPARD *et al.*[10] have shown that in the extraction of thorium(IV) by HDEHP in toluene from aqueous nitrate, perchlorate and chloride solutions, an inverse third (instead of fourth) power dependence of E_a° on H^+ concentration occurs when the acidity is high. This dependence

TABLE II

R_F VALUES FOR ALKALINE EARTH IONS ELUTED WITH HCl, AS FUNCTIONS OF HDEHP CONCENTRATION OF THE SOLUTION USED TO TREAT THE PAPER.

Operating temperature 23° ± 1°

HCl molarity	*Cations*	R_F									
		HDEHP molarity									
		0.010	*0.017*	*0.025*	*0.033*	*0.041*	*0.050*	*0.061*	*0.075*	*0.087*	*0.100*
0.03	Ca^{2+}	0.67	0.60	0.53	0.48	0.40	0.31	0.31	0.25	0.22	0.20
0.01	Mg^{2+}	0.81	0.83	—	0.71	0.68	—	0.62	0.65	0.66	—
	Ca^{2+}	0.28	—	—	0.11	0.09	0.08	0.04	—	0.02	—
	Sr^{2+}	0.77	0.77	0.71	0.70	0.74	0.66	0.59	0.59	0.56	0.47
	Ba^{2+}	0.79	0.75	0.70	0.68	0.71	0.65	0.66	0.62	0.65	0.62
0.003	Mg^{2+}	0.51	0.46	0.34	0.29	0.26	0.25	0.19	0.21	0.20	0.15
	Sr^{2+}	0.42	0.36	0.28	0.21	0.20	0.17	0.15	0.13	0.14	0.11
	Ba^{2+}	0.46	0.41	0.30	0.30	0.22	0.25	0.18	0.21	0.21	0.14

extends to the low acidity with nitrate systems. Such results were explained by postulating that thorium is extracted as both metallic cation Th^{4+} and ThX^{3+} complex with the acid anion X^-. This hypothesis was experimentally confirmed by means of infrared absorption measurements, which revealed the presence of NO_3^- in the extracted species for thorium, cerium, hafnium, and zirconium[11]. Further examples are given for neptunium extracted as $Np(NO_3)^{3+}$ in the HDEHP (toluene) *versus* perchlorate + nitrate system[9] and for beryllium with HDEHP (toluene) in nitrate, sulphate and chloride systems[12,13]. In this case, plots of $\log E_a^\circ$ *vs.* $\log[H^+]$ show E_a° values at high acid concentration greater than expected from the —2 slope that is obtained with more dilute solutions.

Though only the behaviour reported for beryllium concerns a chloride system in the field of alkaline earths, and thus directly refers to the present investigation, the great number of examples reported already suggest that in this case also, as in that of an acetate system, the formation of a compound where the cation is surrounded by both the inorganic anion and HDEHP is responsible for the slopes found in Figs. 2 to 5. It therefore follows that, together with the equilibrium represented by equation (2), the following equilibrium should also hold

$$MX^{b-1} + c(HDEHP)_2 \rightleftharpoons MX(DEHP)_{b-1} \cdot (b - 1)HDEHP \cdot (c - b + 1)(HDEHP) + (b - 1)H^+ \quad (5)$$

The combination of the two equilibria gives a value of b in eqn. (4) lying between 2 and 1, as actually results from this investigation.

As reported above, the value 3 of the coefficient c was expected on the basis of liquid–liquid extraction data. There is, however, a tendency to a minor slope at high HDEHP concentration. At very high HDEHP concentrations a second-power dependence was found to exist between E_a° and the extractant concentration for alkaline earths[14,15] in chloride systems.

In conclusion, from eqns. (3) and (5) the equilibria can be written as:

$$M^{2+} + 3(HDEHP)_2 \rightleftharpoons M(DEHP)_2 \cdot 4\ HDEHP + 2\ H^+$$

$$MCl^+ + 3(HDEHP)_2 \rightleftharpoons MCl(DEHP \cdot HDEHP) \cdot 4\ HDEHP + H^+$$

These equations would explain the mechanism of the retention of alkaline earth ions on paper treated with HDEHP and eluted with hydrochloric acid. High concentrations of acid, as well as low amounts of HDEHP on paper, seem to favour the second equilibrium. Moreover, the value 3 of the $(HDEHP)_2$ coefficient is affected at a high extractant concentration. Probably at still higher concentrations, a value between 3 and 2 should be postulated (and hence some different kind of equilibrium) to justify the experimental results.

Application to chromatographic separations

From the results described above, it appears that many interesting chromatographic separations are feasible.

Various chromatograms were obtained by the ascending technique on 3 × 40 cm strips cut from Whatman No. 1 paper sheets perpendicularly to the machine direction. The paper was treated with HDEHP in the same way as the CRL/1 sheets used in the basic work. Conditions were chosen on the basis of the curves reported in Fig. 1. The chromatographic behaviour of elements included in the present experiments other than alkaline earths was known from work carried out in our laboratory, part of which has been published elsewhere[2].

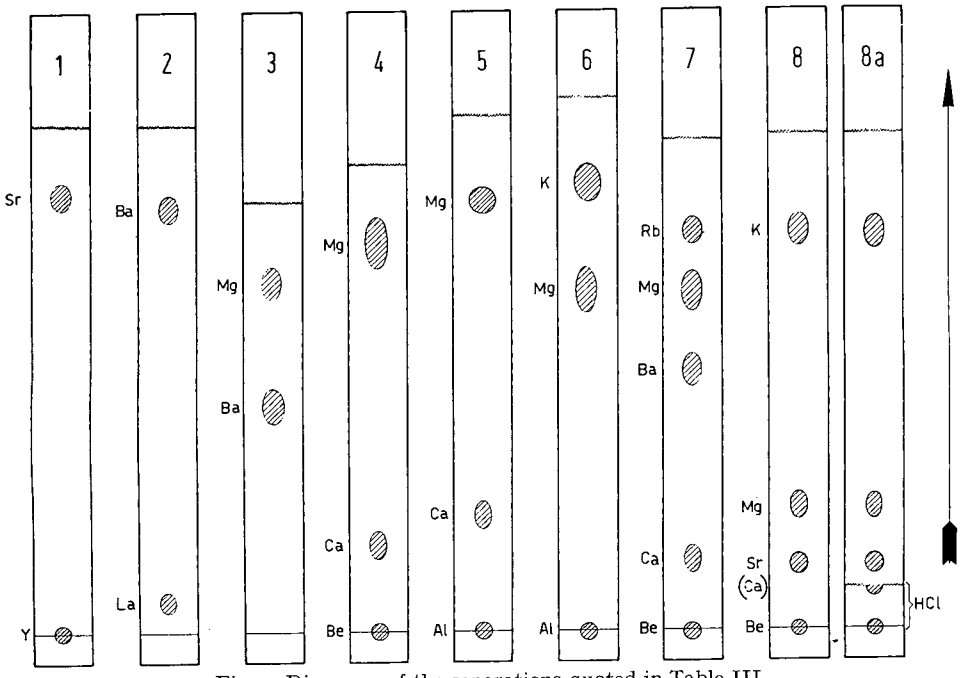

Fig. 7. Diagrams of the separations quoted in Table III.

TABLE III

ASCENDING CHROMATOGRAMS OBTAINED WITH 3 × 40 CM PAPER STRIPS TREATED WITH HDEHP

Operating temperature 23° ± 1°

No.	Separations	Run (cm)	HDEHP (M)	Eluent	R_F
1	Y–Sr	34.5	0.100	0.10 *M* HCl	Y = 0.00; Sr = 0.85
2	La–Ba	34.5	0.100	0.10 *M* HCl	La = 0.06; Ba = 0.83
3	Ba–Mg	29.2	0.075	9 *M* HCl	Ba = 0.52; Mg = 0.80
4	Be–Ca–Mg	31.8	0.100	0.05 *M* HCl	Be = 0.00; Ca = 0.18; Mg = 0.82
5	Al–Ca–Mg	35.0	0.100	0.05 *M* HCl	Al = 0.00; Ca = 0.22; Mg = 0.83
6	Al–Mg–K	36.3	0.100	0.01 *M* HCl	Al = 0.00; Mg = 0.63; K = 0.83
7	Be–Ca–Ba–Mg–Rb	33.2	0.010	3.2 *M* CH_3COOH	Be = 0.00; Ca = 0.14; Ba = 0.53; Mg = 0.69; Rb = 0.82
8	Be(Ca)–Sr–Mg–K	33.7	0.100	2.5 *M* CH_3COOH	Be(Ca) = 0.00; Sr = 0.13; Mg = 0.25; K = 0.80
8a	Be–Ca–Sr–Mg–K	33.7	0.100	2.5 *M* CH_3COOH 0.25 *M* HCl*	Be = 0.00; Ca = (0.08); Sr = 0.13; Mg = 0.25; K = 0.80

* Elution 8a is the same as elution 8 in which an additional HCl elution for a distance of 2.7 cm has been performed to resolve the Be–Ca pair.

Table III collects data of some of the separations obtained, and their diagram is shown in Fig. 7.

In order to compare the acetate system[1] with the chloride system two separations with acetic acid as eluent were also included. They show how the HDEHP–acetic acid system offers even more possibilities of separation than the HDEHP–hydrochloric acid system.

Since the separation obtained by a single elution with acetic acid on paper treated with 0.010 *M* HDEHP (No. 8 in Fig. 7) has the disadvantage of the small retention capacity of paper, the chromatogram was repeated with 0.100 *M* HDEHP treated paper. A double elution was performed first with more concentrated acetic acid, and then, to resolve the pair Ca^{2+}–Ba^{2+}, with hydrochloric acid.

In conclusion, in spite of a somewhat greater versatility of the HDEHP–acetate system with respect to the separation of alkaline earths, the chloride systems are of certain interest, since in most cases a chloride solution of the elements is available.

SUMMARY

Reversed-phase chromatography of alkaline earth metals on paper treated with di-(2-ethylhexyl) orthophosphoric acid (HDEHP) has been investigated, using hydrochloric acid as eluent in a range of concentrations from 10 *M* to $1 \cdot 10^{-4}$ *M*.

The quantity $(1/R_F - 1)$ has been related to the hydrogen ion concentration in the mobile phase, and to the effective HDEHP concentration on the paper.

The chromatographic behaviour has been correlated with that of liquid–liquid extraction systems.

Chromatographic separations of alkaline earths from each other, and from other cations, were performed. Two of them were obtained with acetic acid elutions.

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Notes

Column chromatography of polysaccharides in the presence of urea

The separation of polysaccharides by ion exchange chromatography has been used successfully in certain cases[1] but has lacked any general applicability. This has been because ionic interactions are largely obscured by molecular sieving effects and by association due to hydrogen bonding. A recent paper[2] has reported that these secondary binding forces may be eliminated by the addition of urea to the eluting system. We now wish to report that the use of such urea solutions offers considerable promise in the chromatographic separation of polysaccharides.

Black spruce (*Picea mariana*) is known to contain 3-O-methyl-L-rhamnose[3] in small amounts and, in common with other coniferous woods, alkaline extraction of a chlorite holocellulose yields a mixture of polysaccharides containing predominantly arabinomethoxyglucuronoxylans and galactoglucomannans. Attempted resolutions of such a mixture on a DEAE-cellulose column (OH^- form) using water and aqueous ammonium acetate (2 *N*) did not result in any clear-cut separation but gave approximately equal amounts of the xylan and mannan polymers in the aqueous eluate. When the eluting solvents used were 7 *M* urea and ammonium acetate in 7 *M* urea the fraction eluted with 7 *M* urea was free of xylose and on hydrolysis gave a galactose:glucose:mannose:3-O-methyl-rhamnose ratio of 1.0:2.2:2.5:3.8. The remaining fractions eluted with 2 *N* ammonium acetate in 7 *M* urea contained mainly xylose polymers. The 3-O-methyl-rhamnose was readily identified by its chromatographic behaviour[3] in ethyl acetate–acetic acid–formic acid–water (18:3:1:4) and ethyl acetate–pyridine–water (9:2:2).

These results illustrate the application of this method to polysaccharides and show that the elution pattern is markedly changed in the presence of urea. They also show that the 3-O-methyl-rhamnose is associated with a mannan rather than a xylan polymer. It is likely that the polysaccharide containing the 3-O-methyl-rhamnose has a low degree of polymerization resulting in its separation from the other polymers[2]. It is hoped to study this point later.

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Effects of buffer cations on chromatography of proteins on hydroxylapatite

Three effects were noted when potassium and ammonium phosphates were used as eluting buffers instead of sodium phosphate in hydroxylapatite chromatography[1] of water-extractable soybean proteins: (a) relative areas under the chromatographic peaks varied; (b) degree of resolution of chromatographic fractions differed; and (c) similar fractions eluted at dissimilar phosphate buffer concentrations.

Fig. 1 shows gradient elution diagrams for water-extractable soybean proteins in which identical gradients of sodium, ammonium, and potassium phosphate buffers were used. A 1:10 (meal:water extraction ratio) extract of defatted meal was dialyzed against phosphate buffer, pH 7.6, ionic strength 0.5, which contained 0.01 *M*

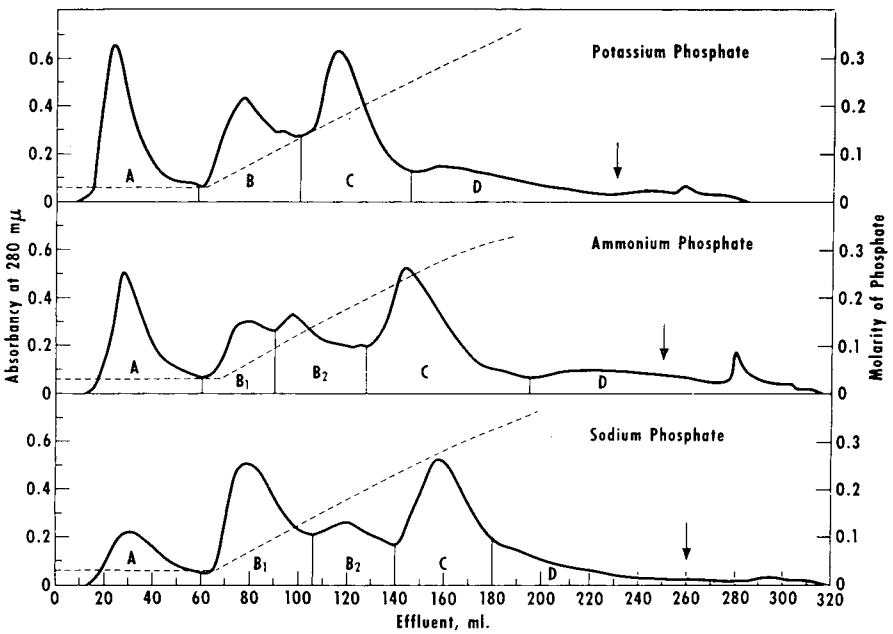

Fig. 1. Gradient elution of water-extractable soybean proteins on hydroxylapatite columns with potassium, ammonium, and sodium phosphate buffers at 25°. Vertical arrow indicates point where *M* phosphate buffer was added to complete elution. Solid curve is absorbancy at 280 mμ in 1-cm cell after 1:4 dilution. Dashed curve is elution gradient determined by conductance measurements. Respective column loads for potassium, ammonium, and sodium buffers were 193, 219, and 180 mg of protein.

mercaptoethanol[1]. The dialyzed solution was made 0.011 *M* with N-ethyl-maleimide to block protein sulfhydryl groups and to react with excess mercaptoethanol; the solution was again dialyzed against 0.03 *M* phosphate buffer, pH 7.6. After dialysis 10 ml of protein solution were placed on a 1 × 25 cm column of hydroxylapatite prepared by the method of ANACKER AND STOY[2] and pre-equilibrated with 0.03 *M* phosphate buffer, pH 7.6. Elution was begun with 320 ml of 0.03 *M* phosphate (pH 7.6) in a mixing chamber. After collecting 20 ml of effluent, a molar solution of

the corresponding buffer at pH 7.6 was added to the mixing chamber at the same rate that the buffer left it (the chamber).

Potassium phosphate eluted the proteins in four major fractions, designated A, B, C and D[1] (Fig. 1) and having the ultracentrifugal compositions shown in Table I. With ammonium phosphate, fraction A is smaller, fraction B is resolved into two parts (B_1 and B_2), and elution of C and D occurs at phosphate concentrations higher than with potassium phosphate. Sodium phosphate elution causes further reduction in the size of fraction A, shows greater resolution of B_1 and B_2, and elutes B_2 and C at phosphate concentrations even higher than with ammonium phosphate.

TABLE I

ULTRACENTRIFUGAL ANALYSIS[a] OF CHROMATOGRAPHIC FRACTIONS OBTAINED WITH DIFFERENT PHOSPHATE BUFFERS

Buffer cation	*Chromatographic fraction*	*Molarity of eluting buffer*[b]	*Total,*[c] %	*Ultracentrifugal composition, %*			
				2S	*7S*	*11S*	*15S + > 15S*
——	Water-extractable proteins	—	100	25	39	25	11
Potassium	A	0.03	24	100[d]	—	—	—
	B	0.07	26	42	35	14	9
	C	0.17	33	7	11	68	14
	D	0.28	17	—	69	22	9
Ammonium	A	0.03	19	100[d]	—	—	—
	B_1	0.07	13	54	39	7	—
	B_2	0.12	18	40	45	15	—
	C	0.23	34	4	14	73	9
	D	0.40	16	—	76	17	7
Sodium	A	0.03	12	100[d]	—	—	—
	B_1	0.07	30	61	26	5	8
	B_2	0.18	16	49	36	15	—
	C	0.27	30	3	24	64	9
	D	~0.36	12	—	65	11	24

[a] Conditions as in ref. 1.
[b] Phosphate concentration at which fractions peaked on elution diagram.
[c] Estimated from areas under elution diagram.
[d] A mixture of two or more slow sedimentating components[1].

However, fraction D is not resolved from fraction C and elutes at a lower phosphate concentration than with ammonium phosphate. Although phosphate concentrations required for elution depend upon the buffer cation, the order of elution does not appear altered and the composition of the fractions does not vary greatly (Table I).

Similar results were obtained upon chromatographing bovine plasma albumin (Armour Lot P67502)* with sodium and potassium phosphates (Fig. 2). Albumin

* Reference to commercial products is for identification only and does not imply endorsement by USDA.

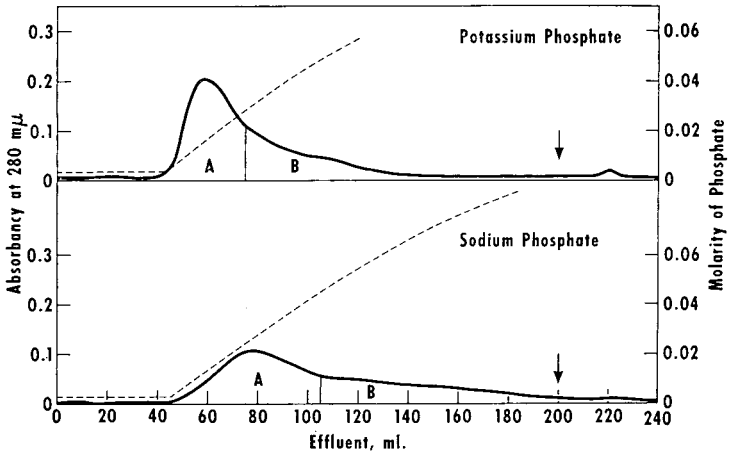

Fig. 2. Gradient elution of bovine plasma albumin from hydroxylapatite columns (1 × 25 cm) with sodium and potassium phosphate buffers, pH 7.6, at 25°. Vertical arrow indicates where 0.25 *M* phosphate at pH 7.6 was added. Solid curve is absorbancy at 280 mμ in 1-cm cell after 1:4 dilution. Dashed curve is elution gradient determined colorimetrically[3]. Column loads were 41 mg.

samples were adsorbed to the columns from 0.003 *M* phosphate, pH 7.6, and eluted by adding 0.25 *M* phosphate, pH 7.6, to 320 ml of 0.003 *M* phosphate in the mixing chamber. Separation into two fractions, A (60 %) and B (40 %), is indicated. Respective concentrations of phosphate at the maxima of the A fractions were 0.015 and 0.025 *M* for potassium and sodium, respectively. After chromatographing albumin with sodium phosphate, the column was treated with *M* potassium phosphate and equilibrated with 0.003 *M* potassium phosphate. Chromatography of another albumin sample with potassium phosphate gave an elution pattern similar to the upper diagram of Fig. 2, and fraction A peaked at 0.014 *M* phosphate. The effect of buffer cations on the chromatographic behavior of plasma albumin therefore appears reversible.

Ultracentrifugal analysis of the original albumin sample indicated 84 % monomer and 16 % dimer. After chromatography with potassium phosphate buffer, fractions A and B (Fig. 2) were dialyzed until free of salts and freeze-dried. Ultracentrifugal analysis of fraction A indicated 83 % monomer and 17 % dimer, whereas fraction B contained 71 % monomer, 16 % dimer, and 12 % of a faster material (possibly a trimer). Clear-cut separation into monomer and dimer[4] did not occur, but a separation tendency is indicated. Because the presence of trimer in fraction B indicates that aggregation occurred, probably the amount of monomer may have been higher in the samples before freeze-drying.

To our knowledge, effects of buffer cations on chromatography of proteins on hydroxylapatite as described here have not been reported previously. However, such effects were anticipated[5,6]. Hydroxylapatite exchanges calcium ions in the crystal lattice for sodium and potassium ions to different extents[7]. This exchange reaction suggests that different buffer cations form crystal surfaces having varying binding strengths for proteins, thereby accounting for the effects reported. A survey of the literature shows that sodium, potassium, and ammonium buffers have been used

individually or in combinations with one another in chromatography of proteins on hydroxylapatite; often it is difficult to ascertain which buffer cation was used. Our results show the importance of knowing exactly which buffer cation is used.

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Gas chromatographic analysis of ethylene and some fluoroethylenes

It was of interest in a recent investigation[1] to separate the C_2-olefins ethylene, 1,1-difluoroethylene and tetrafluoroethylene. Silica gel and silver nitrate–ethylene glycol packings show excellent separational properties for hydrocarbons and olefins[2,3], and in the present work these materials have been used to separate the C_2-olefins.

Using silica gel alone it is found that 1,1-difluoroethylene is separated from the ethylene and tetrafluoroethylene peak, while silver nitrate–ethylene glycol separates ethylene from the fluoroolefins. The quantitative separation of all three olefins is achieved by using both column materials in series.

Experimental and results

Silver nitrate in diethylene glycol on a firebrick support was purchased from the Perkin-Elmer Co., as was the silica gel. A Perkin-Elmer gas chromatograph (154-C) was used.

The silver nitrate phase was packed into a 12 ft. length of $^1/_4$ in. O.D. aluminium tubing and coiled, while the silica gel was contained in a 3.3 ft. Pyrex glass column;

TABLE I

Column used	*Retention times (min)*		
	C_2H_4	CF_2CH_2	C_2F_4
Silver nitrate–ethylene glycol	12.5	7.0	7.0
Silica gel	14.2	19.0	14.2
Silver nitrate–ethylene glycol plus silica gel	30.8	25.6	21.6

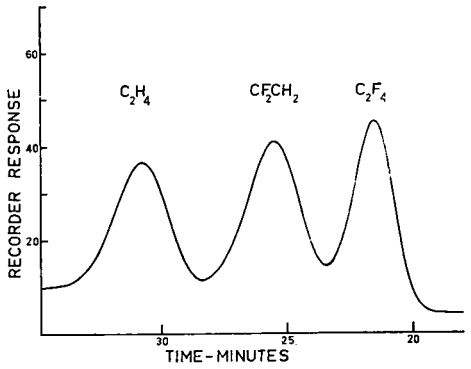

Fig. 1. Chromatogram of olefin mixture (25°, flow rate 36 ml/min).

the latter column was pretreated with nitrogen at 100° before use. Hydrogen was used as a carrier gas at a flow rate of 36 ml/min column temperature was 25°. The olefin mixtures were approximately equimolar and sample size was 0.06 ml.

The rasults of the separation are given in terms of retention times and are recorded in Table I; the recorded chromatograph using the combined columns is shown in Fig. 1.

Retention times may be decreased with some loss in separation by increasing the carrier flow and column temperature. A chromatogram at 45° and flow rate of 70 ml/min is shown in Fig. 2.

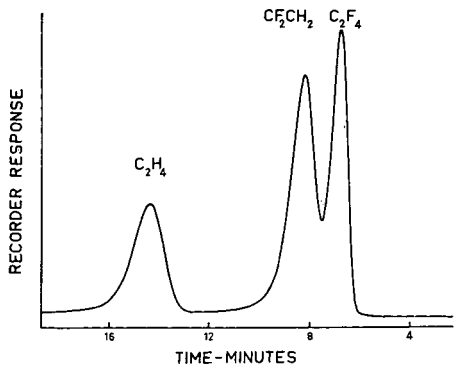

Fig. 2. Chromatogram of olefin mixture (45°, flow rate 70 ml/min).

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A microchromatograph for quantitative estimation of sugars using a paper strip as partition support

Some fifteen years ago FLOOD, HIRST AND JONES[1,2] developed a paper chromatographic method for the quantitative assay of single monosaccharides. In later methods columns of powdered cellulose[3], ion exchange resins[5], or starch[4] were used to obtain separation of the monosaccharides.

This paper describes a convenient paper chromatographic procedure for separation and quantitative colorimetric estimation of single sugars.

Experimental

Munktell filter paper OB was used with *n*-butanol–water–acetic acid (4:1:1) as the mobile phase for the separation of the individual sugars. The paper was cut into 2 to 3 cm wide strips, the length of the paper strip depending on the R_F of the sugars present in the test sample.

Fig. 1 shows the separation of five different sugars: fucose, mannose, glucose, galactose and glucosamine. The paper strip used was 3 cm wide, the temperature was 22° and a *n*-butanol–water–acetic acid mixture served as effluent yielding an average of 5 drops per hour. The values obtained from Fig. 1 serve as a standard which allows us to predict the time when a spot representing a certain sugar will leave the paper strip with the mobile phase. If the paper strip is 25 cm long it can be inferred from Fig. 1 that the individual sugars will leave the strip after the following number of hours: fucose 16, mannose 25, glucose 30, galactose 34 and glucosamine 45–50 h. The time interval between the centers of two subsequent sugar spots eluted from the strip will be at least 4 h. During this interval 20 drops of the effluent will have left the strip (64 drops = 1 ml). The procedure therefore even allows the separation of sugars with rather close R_F values as, for example, galactose and glucose.

The separation of single sugars from mixtures or biological hydrolysates was carried out by one-dimensional descending chromatography in a microchromatograph (see Fig. 2). The microchromatograph consists of a glass airtight chamber in which an atmosphere saturated with chromatographic solvent can be maintained. With a view to better saturation of the atmosphere, the chromatographic solvent was poured not only into the trough but also on the bottom of the chamber. However, the best results were attained when the inside of the chromatographic chamber was lined with filter paper.

Before use the strip of filter paper was washed carefully with chromatographic solvent and dried. A measured amount of the hydrolysate or the mixture of the sugars to be tested is applied at the central portion of the start line by means of micropipettes containing 10–25 μml. The spot of solution containing the sugars was dried and the chromatographic strip placed in the microchromatograph. When the effluent reached the end of the strip and began to form drops, the microchromatograph was connected to the fraction collector of the type described by CARLANDER AND GARDELL[6]. The required number of drops of effluent were collected in each test-tube. The effluent was then evaporated to dryness in a desiccator under reduced pressure. The residue was taken up in 0.5 ml destilled water and 0.5 ml 5 % phenol–water solution was added, followed by 3.0 ml concentrated sulfuric acid. The further steps in the assay procedure were the same as those described by DUBOIS *et al.*[7].

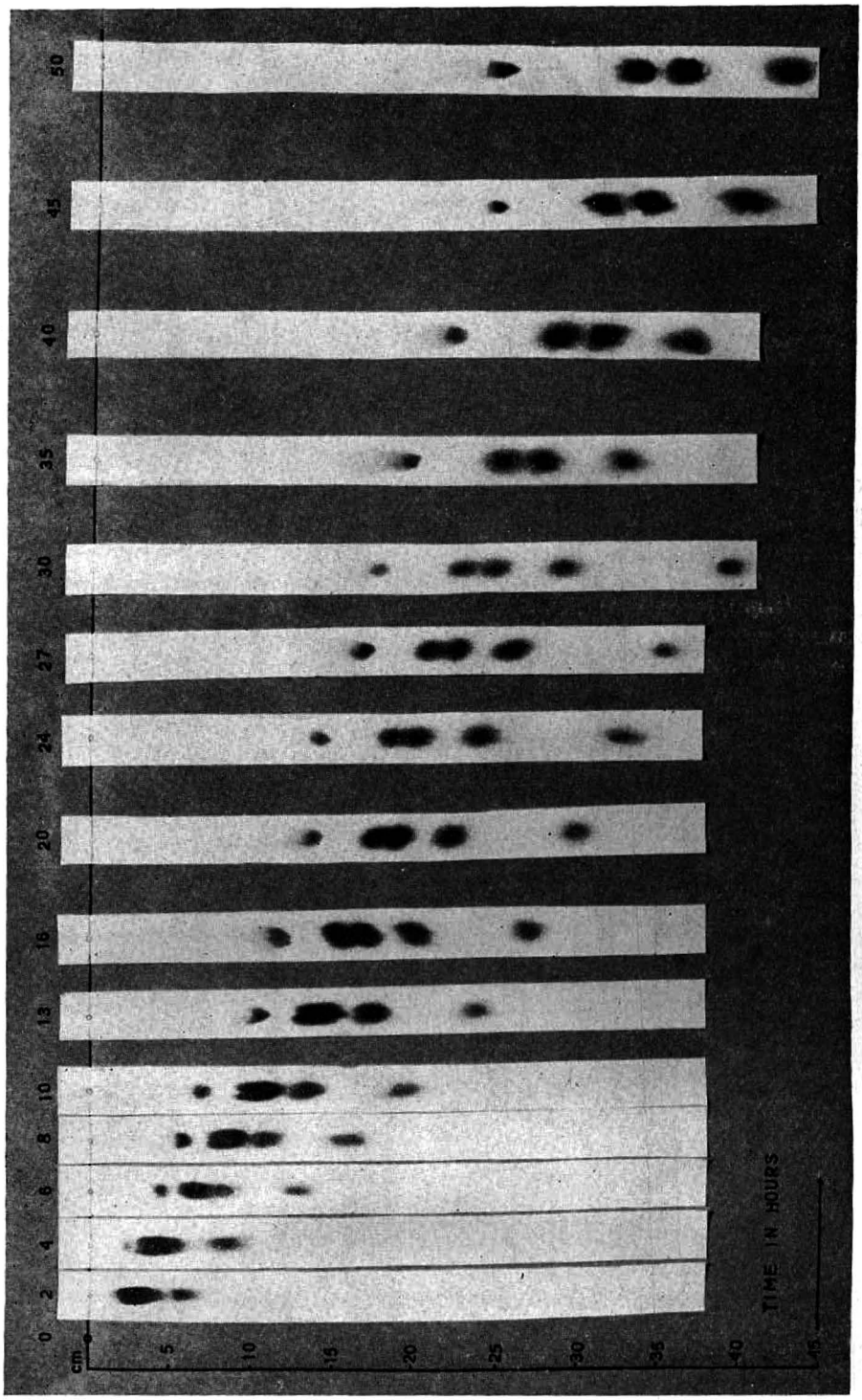

Fig. 1. The position of five sugars on the filter paper of 3 cm width and 45 cm length after different times of flow of the effluent. Further explanation in text.

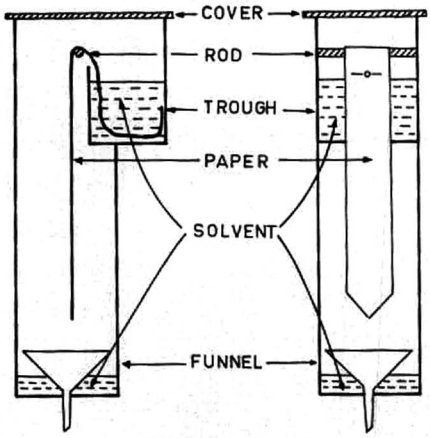

Fig. 2. Diagrammatic drawing representing the microchromatograph.

Glucosamine did not give a positive phenol–sulfuric acid color reaction, therefore the ELSON AND MORGAN[8] procedure as modified by IMMERS AND VASSEUR[9,10] and by IMMERS[11] was applied. The absorbancy was measured at 490 mμ for hexoses and at 480 mμ for pentoses or methyl pentoses and at 530 mμ for glucosamine. A Beckman spectrophotometer model DU with a 10 mm Cortex cell was used for these measurements.

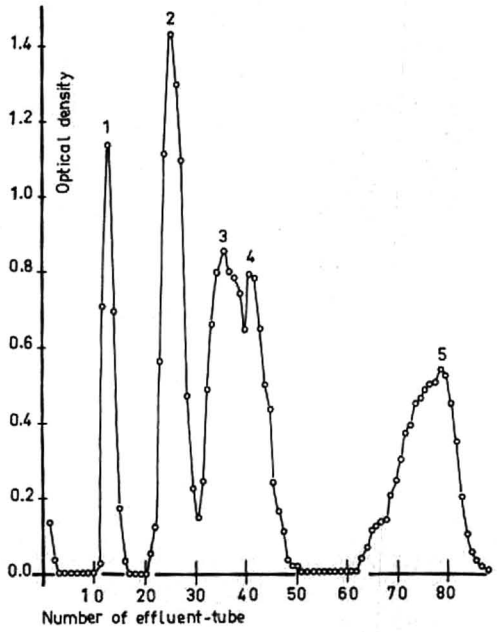

Fig. 3. The concentration (optical densities) of 445 μg fucose (1), 400 μg mannose (2), 290 μg glucose (3), 410 μg galactose (4) and 480 μg glucosamine (5) mixed in 25 μml water as function of effluent tube numbers. The width of filter paper is 2 cm, the length 25 cm. In each test-tube 4 drops of effluent. Absorbancy for fucose at 480 mμ, for mannose, glucose, galactose at 490 mμ and for glucosamine at 530 mμ.

TABLE I

RECOVERY OF ADDED AMOUNTS OF DIFFERENT SUGARS AFTER CHROMATOGRAPHIC PROCESSING

	Fucose			*Mannose*			*Glucose*			*Galactose*			*Glucosamine*		
Run. No.	*Added* μg	*Found* μg	*Recovery* %	*Added* μg	*Found* μg	*Recovery* %	*Added* μg	*Found* μg	*Recovery* %	*Added* μg	*Found* μg	*Recovery* %	*Added* μg	*Found* μg	*Recovery* %
1	445	451	101.3	400	450	112.5	290	253	87.2	410	423	103.1	480	443	92.2
2	190	187	98.3	204	199	97.5	—	—	—	210	202	96.1	156	148	94.2
3	54	53	98.0	55	46	83.6	60	62	103.3	—	—	—	—	—	—
4	116	120	103.4	202	—	—	154	139	90.2	136	130	95.5	166	157	94.6
5	380	361	95.0	408	462	113.2	360	328	91.1	420	397	94.5	259	232	89.7
6*	150	151	100.6	—	—	—	200	198	99.0	—	—	—	—	—	—

* The strip of filter paper was 2.5 cm wide and 5.0 cm long.

The quantity of an individual sugar in a sample can be estimated from a standard curve previously constructed for the particular sugars of the test samples (see GARDELL[4]).

The possibility of separating five single sugars may be illustrated by plotting the concentrations (optical densities) as a function of tube number, see Fig. 3. The total amount of the individual sugars could be calculated by summing the values found in the tubes corresponding to a complete fraction (1, 2, 3, 4 or 5).

The recovery from six series of chromatograms in which varying amounts of the sugars were tested is summarized in Table I.

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Entfernung der Untergrund-Färbung nach Disc-Elektrophorese

Bei der Disc-Elektrophorese wird das Protein-Gemisch nach der Trennung im Polyacrylamid-Gel mit Amido-Schwarz gefärbt. Gemäss der Originalvorschrift von ORNSTEIN UND DAVIS erfolgt die Entfernung des überschüssigen, nicht gebundenen Farbstoffes mit der Trenn-Apparatur in vertikaler Stellung der Gel-Stäbchen durch Anlegen eines Stromes von 10–20 mA je Röhrchen. Dabei kommt es nach unseren Erfahrungen, je nach Dauer der elektrophoretischen Entfärbung des Untergrundes, zu einer Veränderung des Fraktionsmusters: durch Diffusion werden die Bänder nachträglich unscharf und/oder entfärbt; infolge des Auftretens von Gasblasen und der dadurch hervorgerufenen Widerstandserhöhung tritt Erwärmung auf. Diese Störungen können vermieden werden, wenn die kataphoretische Entfärbung *senkrecht* zur Trennrichtung erfolgt. Die zu diesem Zweck konstruierte Apparatur ist in Fig. 1 dargestellt.

In einer runden Plastik-Schale (B) werden in den Boden 12 Plastik-Stücke eingeschweisst, die längliche Schlitze (F) enthalten. Der Boden der Schlitze, die genau der Standard-Grösse der Polyacryl-Säulchen entsprechen, besteht aus einer Glasfritte P4. Nach dem Einlegen der zu entfärbenden Gel-Stäbchen in die Schlitze, werden diese mit Entfärbungsflüssigkeit aufgefüllt. Die Schalen A und B werden mit 7%iger Essigsäure beschickt und zusammengesetzt. Die Schale A enthält in der Mitte den

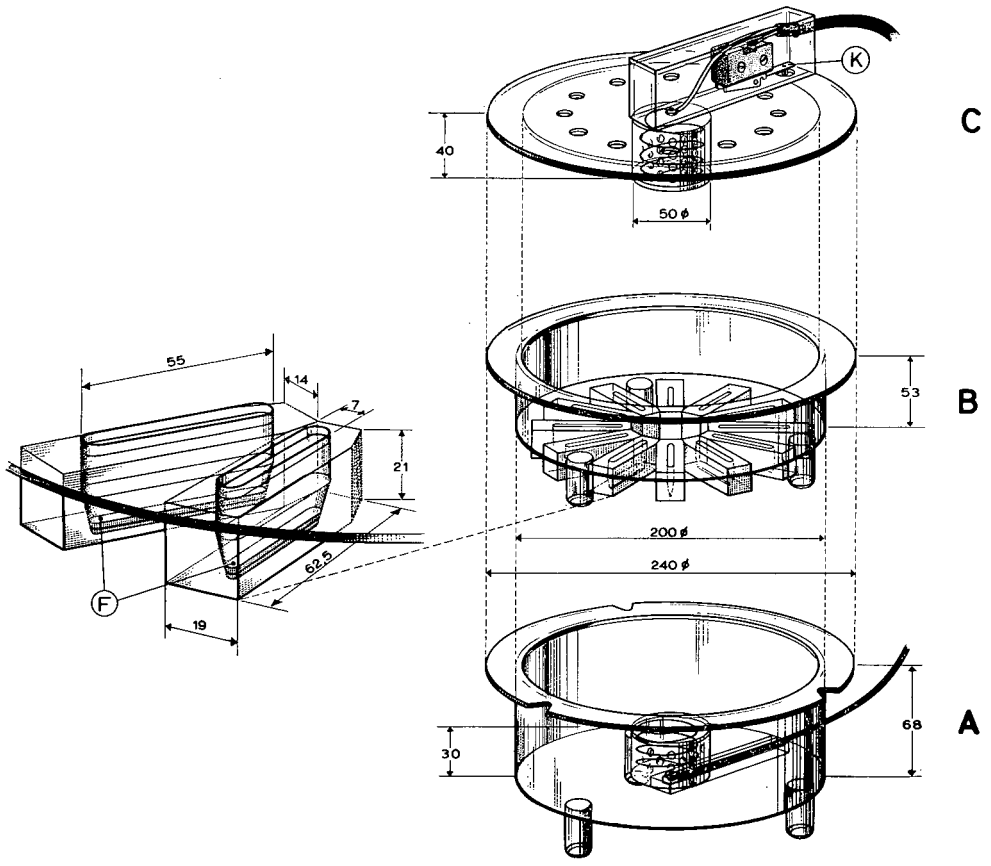

Fig. 1. Apparat zur Untergrund-Entfärbung von Disc-Elektrophorese-Säulchen in horizontaler Lage. (A) Untere Plastik-Schale mit Stromzuführung (Anode). (B) Einsatzschale aus Plastik, in der sich die Schlitze (F) für das Einlegen der zu entfärbenden Gel-Säulchen befinden. (C) Deckel mit Stromzuführung Kathode und Sicherheitsschalter (K).

positiven Pol der Stromzuführung; der andere Pol wird durch Einsetzen des Deckels C in die Lösung getaucht. Die leitende Verbindung erfolgt durch die Glasfilter hindurch. Der Sicherheitskontakt K sorgt dafür, dass der Stromkreis nur geschlossen wird, wenn die Apparatur zusammengesetzt ist. Die Entfärbung erfolgt nach Anlegen der Spannung, die nicht stabilisiert sein muss, sodass auch einfache Gleichrichter benutzt werden können.

Bei einer Stromstärke von 20 mA je Gel-Säulchen findet eine einwandfreie Untergrund-Entfärbung in ca. 30 Min. statt; eine solche ist für eine scharfe Trennung von pflanzlichen Protein-Extrakten entscheidend, wenn ein Verwischen der Bänder nicht mehr auftreten soll.

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Eingegangen den 28. Januar 1964

Quantitative analysis of tyramine by ion exchange chromatography

In the course of basic biochemical studies on banana plant tissue in this laboratory, it became desirable to have a method for the automatic quantitative determination of tyramine together with ammonia, lysine, histidine and arginine. The method of MOORE, SPACKMAN AND STEIN[1,2] has been widely used for the quantitative analysis of basic amino acids on Amberlite IR-120 resin (a strong-acid cation exchanger), but this method has not been satisfactory for the quantitative elution of tyramine. KIRSHNER AND GOODALL[3] have reported the use of Amberlite IRC-50 resin (a weak-acid cation exchanger) for the separation of certain phenolic amines and their subsequent quantitative determination. By modifying and combining these two procedures, a convenient 6-h method has been developed on the Beckman/Spinco amino acid analyzer for the quantitative measurement of tyramine as well as the basic compounds normally determined on this instrument. The very recent paper of PERRY AND SCHROEDER[4] deserves particular attention since they demonstrated the separation of many amines and unidentified bases in urine by chromatography on Amberlite CG-50 resin (chromatographic grade of IRC-50). Their technique is necessarily more complex and time-consuming than the one reported here which is only for five basic constituents commonly found in plants.

Resin and column preparation

One pound of Amberlite CG-50 type 2 resin was cycled once through the sodium and acid forms before use, according to the method of HIRS, MOORE AND STEIN[5]. After the final washing, fines were removed by repeated suspension in distilled water. The resin was then suspended in 3 l of pH 5.28 sodium citrate buffer 0.35 *N* in Na^+. Concentrated NaOH (50 % w/v) was added to the suspension frequently during the next few hours to maintain the pH at 5.28. A final pH correction was made after overnight stirring. The resin was then stored in the pH 5.28 buffer under refrigeration.

The resin was suspended in de-aerated (*i.e.* boiled) pH 5.28 buffer and poured into a water-jacketed chromatographic column (0.9 cm diameter) to a height of 40 cm. A separate circulating water bath maintained the column temperature at 63° ± 2°. Since the column was run at a relatively high temperature, it was also necessary to boil the eluting buffer (pH 5.28) before use to prevent the formation of air bubbles in the resin.

Experimental

Temperatures ranging from 23° to 85° and buffers between pH 3 and 6 were explored using a flow rate of 30 ml/h, which is standard for our instrument. Optimum resolution and separation of ammonia, lysine, histidine, arginine and tyramine was achieved with pH 5.28 buffer and a column temperature of 63°. These conditions resulted in a column pressure of 12 p.s.i.g.

Standard calibration mixtures were made up in pH 5.28 sodium citrate buffer. A four-point standard curve for tyramine can be obtained in one 9-h run on the Beckman/Spinco amino acid analyzer by stopping the elution every hour for the first three hours in order to load the column with an additional tyramine standard.

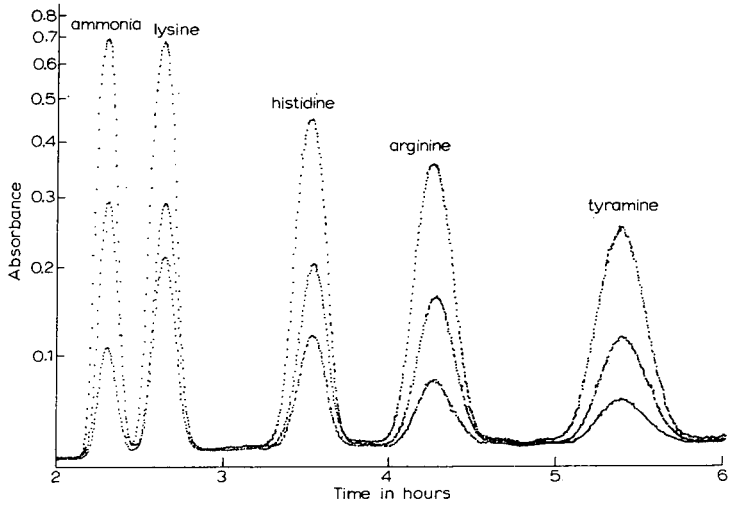

Fig. 1. Chromatographic separation of ammonia, lysine, histidine, arginine and tyramine on an Amberlite CG-50 (type 2) column and the colorimetric estimation as recorded at 570 mμ, suppressed 570 mμ and 440 mμ (lower line) by a Beckman/Spinco amino acid analyzer. Column 0.9 × 40 cm. Flow rate 30 ml/h. Temperature 63°. Pressure 12 p.s.i.g. Elution with Na citrate buffer, pH 5.28, 0.35 N in Na^+.

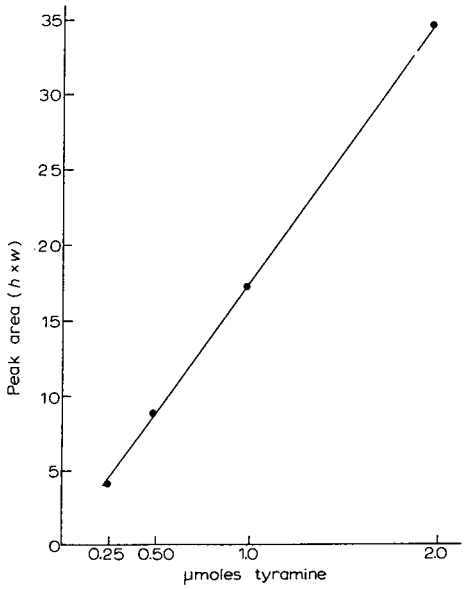

Fig. 2. Standard curve for tyramine when separated on Amberlite CG-50 (type 2) resin and recorded on a Beckman/Spinco amino acid analyzer. Tyramine constant 17.0 ± 0.3.

Results and discussion

Fig. 1 shows the chromatogram of a mixture of standard amino acids and tyramine, 1 μmole of each. Constants calculated (for 1 μmole) by the method of SPACKMAN *et al.*[2] are as follows: ammonia 19.2, lysine 22.1, histidine 18.6, arginine 18.5, tyramine 17.0. Excluding tyramine which cannot be determined on IR-120 resin, these compounds are determined as accurately (*i.e.* equal linearity and sensitivity) off CG-50 columns as off the regular IR-120 resin. The linearity of tyramine in the range 0.25 to 2.0 μmoles is shown in Fig. 2. Analyses of 10 standard samples on the same column resulted in a tyramine constant of 17.0 ± 0.3, indicating a reproducibility of better than 2 %.

Our early efforts to modify the procedure of MOORE, SPACKMAN AND STEIN[1] by changing the temperature, column length, flow rate and pH of the buffer did not improve the results for tyramine. Combining certain features of the method of KIRSHNER AND GOODALL[3] with those of the above method, however, has resulted in a relatively rapid technique for the quantitative determination of tyramine together with ammonia, lysine, histidine and arginine. Application of this procedure required only two changes in the normal operation of the Beckman/Spinco amino acid analyzer: (a) the use of CG-50 resin in place of IR-120 resin and (b) a column temperature of 63° instead of 50°. We have used this technique for the analysis of acidified (0.1 *N* HCl) 80 % ethanol extracts of banana plant tissues which were concentrated to remove ethanol, adjusted between pH 3 and 5.5, and brought to volume with water. When standard amounts of tyramine were added during the homogenization of the tissue, recoveries of tyramine were 96 ± 3 %. No regeneration of the column was necessary. However, the top few centimeters of resin were replaced after each analysis if the plant extracts contained appreciable amounts of tannins and pigments. When an analysis of tyramine alone was desired, changes in column length to 10 cm and temperature to 25° permitted a complete quantitative determination in less than 2 h.

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Relative detector response in gas chromatography

V. Halogenoalkanes, aliphatic aldehydes, pyridines

The investigation of the relative detector response of a thermal conductivity detector to organic compounds of various types, when nitrogen is the carrier gas, is continued with a study of relative responses to members of the homologous series halogenoalkanes, aliphatic aldehydes and pyridines.

TABLE I

RELATIVE DETECTOR RESPONSES TO SOME HALOGENOALKANES, ALIPHATIC ALDEHYDES AND PYRIDINES

Compound	*Mol. wt.*	*Response per mole relative to benzene (= 100)*
Halogenoalkanes		
1-Chloropropane	78.7	90
1-Chlorobutane	92.7	114
2-Chlorobutane		103
1-Chloropentane	106.7	138
1-Chloro-3-methylbutane		129
2-Chloro-3-methylbutane		111
1-Chlorohexane	120.7	162
1-Chloroheptane	134.7	185
1-Bromopropane	123	132
2-Bromopropane		121
1-Bromobutane	137	162
2-Bromobutane		149
2-Bromo-2-methylpropane		131
1-Bromopentane	151	186
1-Bromo-3-methylbutane		173
2-Bromopentane		166
1-Bromohexane	165	210
Iodoethane	156	162
1-Iodopropane	170	187
2-Iodopropane		173
1-Iodobutane	184	211
1-Iodo-2-methylpropane		197
2-Iodobutane		194
1-Iodopentane	198	236
1-Iodohexane	212	259
Aliphatic aldehydes		
Butanal	72	66
Pentanal	86	88
Hexanal	100	108
Heptanal	114	128
Octanal	128	151
Decanal	156	189
Pyridines		
Pyridine	79	100
2-Methylpyridine	93	126
3-Methylpyridine		125
4-Methylpyridine		127
2-Ethylpyridine	107	148
4-Ethylpyridine		146
2,5-Dimethylpyridine		147
2,6-Dimethylpyridine		144
3,4-Dimethylpyridine		131
3,5-Dimethylpyridine		144
4-(*n*-Propyl)-pyridine	121	166
2,4,6-Trimethylpyridine		169
Piperidine	85	95

Experimental

Gas–liquid chromatography was carried out using the apparatus described previously[1]. Nitrogen was used as the carrier gas at a flow rate of approximately 33 ml/min, and the bridge current was 100 mA. Under the conditions used no reversal of peaks was observed for any of the compounds studied.

The compounds were obtained from commercial suppliers and most of them could be obtained chromatographically pure by fractional distillation. In those instances when more than one peak was obtained from a compound even after repeated fractionation, that compound was purified by using a preparative chromatographic apparatus.

Results and discussion

The relative detector responses to a number of halogenoalkanes, aliphatic aldehydes and pyridines are shown in Table I.

An examination of these results shows that there is, in each homologous series, an increase in relative detector response with an increase in molecular weight. The increment for each CH_2 group is approximately 24 units of response for chloroalkanes, 26 units for bromoalkanes, 24 units for iodoalkanes, 20 units for aliphatic aldehydes and 23 units for pyridines. These increments are similar to those found for other homologous series[2,3].

It is also found that, in the halogenoalkanes, there is a decrease in relative response to isomeric compounds with an increase in chain branching. This decrease in relative response is similar to that found previously for isomeric alkyl benzenes[4], aliphatic esters[5] and aliphatic ethers[2].

In the pyridine series it is found that, *ortho*-effects and chain-branching effects being absent, a compound with a group forming part of a side-chain has a similar relative detector response to the isomeric compound which has the group attached to the pyridine ring. In this respect the pyridine series resembles the benzene series[4].

No *ortho*-effect is observed for the monomethylpyridines but the relative response to 3,4-dimethylpyridine is lower than the responses to 2,5-, 2,6-, and 3,5-dimethylpyridines, which are similar.

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Quantitative gas chromatography

We refer to "The Standardization of Gas–Liquid Chromatography for the Analysis of Simple Hydrocarbon Mixtures" in the name of the International Conference of Benzole Producers, which appeared in the November 1963 issue of this journal.

Nine European laboratories took part in an experiment in which the two samples tested had the composition shown in Table I.

Duplicate determinations were performed and repeatability and reproducibility calculated for each sample. The authors concluded "For the first test sample... both the precision and accuracy were reasonably satisfactory. For the second sample however... the precision was appreciably worse. The reasons for the poor repeatability are not apparent".

This effect was predicted by us in a paper entitled "A Statistical Evaluation of Gas-Liquid Partition Chromatography as a Method of Quantitative Analysis" presented at the Fourth International Symposium on Gas Chromatography held under the auspices of Der Deutschen Akademie für Wissenschaften zu Berlin at Leuna, 28th–31st May, 1963, the proceedings of which will be published in due course.

We also showed that it was not meaningful to average repeatabilities over different concentrations. This is because repeatability is concentration-dependent.

Our investigation involved a $9 \times 3 \times 2$ factorial experiment covering 2, 4 and 6 component systems in which one component was varied over the range 0.25–64 % by nine logarithmic steps, while, in the case of the 4 and 6 component systems the ratios of the other 3 or 5 components were maintained constant. Duplicate determinations were made and the whole 54 runs were performed in random order, over a period of two weeks by one operator.

The apparatus and conditions were as follows:

Instrument	Griffin & George D.6 (Prototype)
Column material	Stainless steel
Solid support	JJ's acid washed Celite 545 100/120 BSS mesh
Liquid phase	10 % Poly(ethyleneglycol adipate)
Detector	Martin Gas Density Balance
Carrier gas	Nitrogen
Column temperature	100°, 149°, 177°
Method of injection	Griffin sampler (2 μl).

Analysis of the ranges of duplicate results led to the derivation of the following equation which accounted for 86 % of the variability of the ranges:

$$\text{Range} \times 10^2 = 0.9635\,|C| - 0.009635\,|C|^2 + 0.1386N^2 - 0.7506;$$

where $|C|$ represents the concentration level in % w/w and N represents the total number of components.

Application of this equation to the results quoted by BENZOLE PRODUCERS for the preferred method A gives the values listed in Table I.

Thus 70 % of the variability of the ranges (as measured by sum of squares) has been accounted for.

This demonstrates that the differences observed between the two samples are

TABLE I

Sample number	*Component*	*Concentration (%)*	*Average range found from all Laboratories* ($\times 10^{-2}$)	*Range predicted by equation* ($\times 10^{-2}$)
1	Benzene	98.04	6.67	3.28
	Toluene	0.73	2.11	2.17
	Ethylbenzene	0.80	2.88	2.23
	Cyclohexane	0.43	1.66	1.88
2	Toluene	90.64	25.00	10.89
	Benzene	1.82	4.00	4.44
	Ethylbenzene	2.69	8.62	5.24
	p-Xylene	3.72	14.00	6.17
	n-Nonane	1.13	2.78	3.79

due to the change in number of components and the variation in the concentration of these components.

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Paper chromatography of short chain aliphatic amides

During studies on the use of aliphatic amides by the bacterium *Pseudomonas aeruginosa* as sole source of carbon and nitrogen for growth[1–3], it became necessary to check the purity of the amides and identify very small amounts of amide remaining in the culture media after growth. The use of direct paper chromatography of amides was investigated since, although it is possible to convert amides to their hydroxamates[4] and there exist a number of suitable solvents for chromatography of hydroxamates[5,6], the procedure does not allow distinction to be made between amides such as acetamide, N-methylacetamide and N-acetylacetamide, as these amides give the same hydroxamate. A few methods for direct chromatography of amides have been published but these are for long chain amides[7] or involve treatment of the paper, *e.g.* with 5 % polycaprolactam–formic acid[8]. In the search for a suitable solvent, it was found that many of the usual chromatography solvents were unsatisfactory because the amides travelled with the solvent front, did not move at all, or in the case of *n*-butanol–acetic acid–water did not give adequate separation of the amides. The solvent finally selected was toluene–ethanol (75:25 v/v); amides were detected by conversion to hydroxamates and the hydroxamates visualised with ferric chloride[9,10].

Experimental procedure

1.0 *M* aqueous solutions of amides were used with the exception of glutamine, asparagine and malonamide; saturated solutions of these three amides were used (approx. 0.3, 0.2 and 0.8 *M*, respectively). Approximately 0.002 ml of each amide was applied to Whatman No. 1 chromatography paper, 18 × 22 in., and after the spots had dried the paper was equilibrated for 30 min in a Shandon all glass chromatography tank arranged for descending chromatography and containing a beaker of toluene–ethanol (75:25). At the end of the equilibration period, the solvent was introduced to the edge of the paper and the chromatogram was developed for $2^1/_2$ h in the machine direction of the paper; it was then removed from the tank, the solvent front marked, and dried in an oven at 100° for 3 min. The chromatogram was then sprayed with reagent of the following composition: 80 % methanol, 20 % water (v/v) containing 3 g of sodium hydroxide/100 ml and saturated with hydroxylamine hydrochloride. Although alkaline hydroxylamine is unstable, satisfactory results were obtained with reagent that had been stored for 1 week at 5°. The sprayed chromatogram was placed in an oven at 100° for 10 min and then lightly sprayed with 1 % ferric chloride hexahydrate in 90 % methanol–10 % conc. HCl (v/v); the hydroxamates of each amide appeared as pink spots against a yellow background. Although these pink spots faded slowly, they were still clearly visible after 72 h and since the yellow background due to ferric chloride faded more rapidly, examination of the chromatograms for very faint spots was carried out after 3–6 h when the background was practically white. Using the above procedure, it was possible to detect 5 μg of acetamide; the amount of the other amides detectable varied with their rate of reaction with alkaline hydroxylamine[9].

Some amides could be distinguished on the basis of their hydroxamate colour; thus the hydroxamate of iodoacetamide gave a brown colour with ferric chloride; the pink coloured hydroxamate of cyanoacetamide darkened to become almost black after 24 h and that of thioacetamide became blue around its edge. In addition, thioacetamide can be detected directly by spraying with 5 volumes hydrogen peroxide containing 1 % ferric chloride; the reagent gives an ephemeral red colour with μg amounts of thioacetamide but it destroys the ability of thioacetamide to form a hydroxamate with alkaline hydroxylamine. None of the other amides tested gave any colour with ferric chloride–hydrogen peroxide.

Results

The rates of migration of a number of amides relative to acetamide (R_A) were determined using toluene–ethanol solvent; these are listed in Table I together with their actual R_F's.

Discussion

The above procedure is a rapid way of separating short chain aliphatic amides and locating their position on the chromatogram. The time chosen for heating amides with alkaline hydroxylamine to convert them to hydroxamates is a compromise since there is considerable variation in the times necessary for complete conversion of amide to hydroxamate; *e.g.* formamide requires 60 min at 26° whereas N-methylacetamide requires 420 min at 60°. Therefore if substituted amides are known to be present, it might be necessary to prolong the heating period with alkaline hydroxyl-

TABLE I

R_F AND R_A VALUES (RATE OF MIGRATION RELATIVE TO ACETAMIDE) FOR SOME SHORT CHAIN ALIPHATIC AMIDES

Amide	R_F	R_A
Formamide	0.08	0.6
N-Methylformamide	0.18	1.4
Acetamide	0.13	1.0
Glycolamide	0.07	0.54
N-Methylacetamide	0.32	2.5
N-Acetylacetamide	0.53	4.1
Thioacetamide	0.32	2.5
Cyanoacetamide	0.06	0.45
Fluoroacetamide	0.19	1.5
Iodoacetamide	0.27	2.1
Propionamide	0.26	2.0
Acrylamide	0.21	1.6
Lactamide	0.10	0.8
β-Hydroxypropionamide	0.07	0.54
Butyramide	0.4	3.2
Isobutyramide	0.51	3.9
Malonamide	0.01	0.1
Glutamine	0.0	0.0
Asparagine	0.0	0.0
Glycine amide	0.0	0.0

amine in order to obtain sufficient conversion of the amide to hydroxamate for a detectable colour with ferric chloride to be observed. The procedure can be made quantitative by running duplicate spots and after locating each amide by the above method on one strip, eluting it from the corresponding portion of the second strip and applying the quantitative method involving the correct temperature and time for that particular amide[9,10].

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Frosted glass plates as an aid to thin-layer chromatography

Although STAHL *et al.*[1] were not the first to separate organic substances on thin-layer adsorbents[2], they were responsible for standardizing and popularizing the application of the method. Currently, the potential of thin-layer chromatography is rapidly being explored and extended. However, the present form of the technique is not without its limitations.

The procedure of spreading and drying an adsorbent, such as silica gel, onto a clear glass plate, results in a thin, extremely fragile film (100–500 μ). In an attempt to strengthen the applied film, various kinds of binders have been added to the silica gel. The use of binders, however, does not entirely solve the problem of fragility; solvents containing water tend to increase the delicacy of the silicic acid thin layer[3], during and after chromatography. Also, the use of such binders presents other problems. For example, in the case of phospholipids[4] a load effect is evident.

In order to circumvent these difficulties, peeling and load effect, we sought to overcome the problem in another manner. The peeling and cracking of the silica gel layer is due partly to the fact that smooth glass plates do not offer an adhesive surface. During the course of our investigation, it was found that a more adhesive surface could be provided by etching the clear glass plates.

By sand-blasting clear glass with a variety of substances, it was possible to produce surfaces with varying degrees of roughness. The coarseness of the surface grain had an effect on the R_F values of compounds as well as providing a better adhesive surface. Very coarse surfaces resulted in R_F values lower than that of plain glass. A finer etched surface increased the R_F values. After experimenting and comparing a variety of different frosted plates, it was determined that the ideal surface was the one formed by blasting with a light medium grit of sand (U.S. Patent pending)*.

When these finely etched plates were used for routine thin-layer chromatography, the cracking and peeling of the silica layer, normally exhibited on clear plates, especially in aqueous solvent systems, was not noticed. A comparison of clear and frosted plates may be seen in Fig. 1.

For the same solvent, it was demonstrated that R_F values were slightly higher when mixtures of compounds were chromatographed on etched plates in comparison to clear glass plates (Fig. 2). The increase mobility was most noticeable for compounds having a small R_F value. The effect was due to the capillary action of the frosted surface, itself.

Etched plates were particularly useful in the separation and quantitation of phospholipids. Previously, it has been shown that binders were undesirable, since load effects were evident[4]. Also, quantitating thin-layer chromatographic plates in the customary way presents some inherent problems. Recently, these were summarized by DOIZAKI AND ZIEVE[5]: loss of specimen in attempting to scrape sample area prior to quantitation, inhibiting effect of silica gel on the color development of phosphorus.

The problem of quantitating phospholipids on thin-layer plates was solved by scanning 1 × 8 in. plates directly with a double beam reflecting densitometer (Chromo-

* Frosted glass plates are available from National Instrument Company, Rockville, Md., through Med-Chem. Laboratories, Detroit.

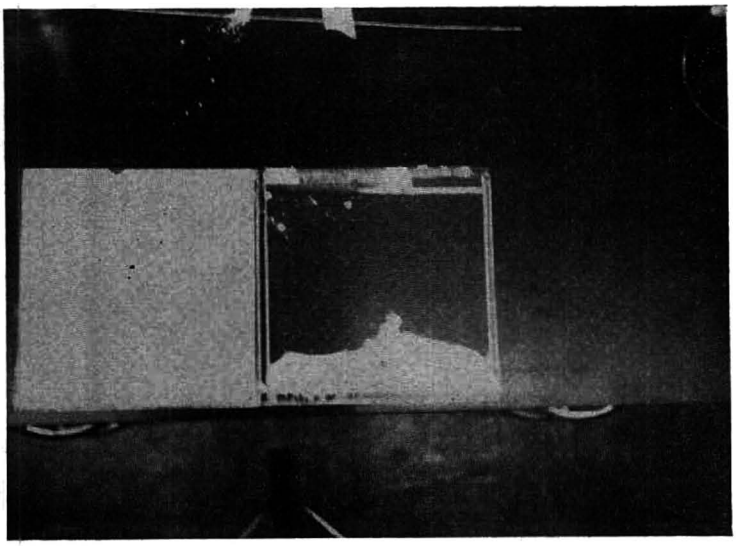

Fig. 1. On the right is an example of peeling and cracking of silica gel thin layer, without binder, covering a smooth glass surface. On the left, is silica gel thin layer, without binder, coated on an etched glass plate. The etched glass plate shows no peeling or cracking.

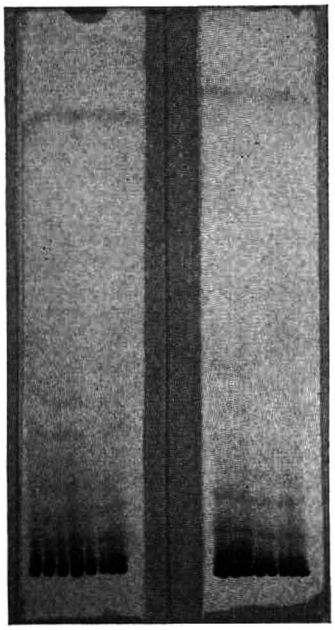

Fig. 2. Comparison of synthetic porphine samples (kindly supplied by A. SZUTKA, University of Detroit), separated on clear and etched glass. The silica gel on an etched glass surface (right) showed a greater number of component substances, especially those with small R_F values.

scan, National Instrument Company, Rockville, Md.). In a previous report, we demonstrated the usefulness of this instrument for agar-gel pherograms, as well as paper chromatograms[6]. Scanning of the thin-layer silica gels, in a similar manner, was now possible because of the greater adhesion between the gel and the etched glass plate. The per cent of each phospholipid fraction could be determined through scanning, and total phosphorus content could be measured on an aliquot of the same sample[7]. By this method, the amount of each phospholipid in a mixture could be calculated. Complete details of this method will be the subject of another report.

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Beitrag zum Problem der Sichtbarmachung von chlorierten Insektiziden am Dünnschichtchromatogramm

Die Papier- und Dünnschichtchromatographie (DC) wurde in den letzten Jahren immer mehr zur Abtrennung geringer Mengen von Pestiziden herangezogen. In Arbeiten auf dem Gebiete der Papierchromatographie (ausführliche Literaturübersichten[1–6]) wurden die Bedingungen der Abtrennung festgelegt und Vorschriften für die quantitative und semiquantitative Bestimmung gegeben.

Die Sichtbarmachung der Phosphor- und Thiophosphorsäureester am Papierchromatogramm ist verhältnismässig einfach da die besondere Beschaffenheit dieser Verbindungen verschiedene Reaktionen ermöglicht. Schwieriger gestaltet sich die Anfärbung und Unterscheidung der chlorierten Kohlenwasserstoffe.

MITCHELL UND PATTERSON[7] und MITCHELL[8] behandeln zu diesem Zweck das Chromatogramm, nach Auftrennung, mit Monoäthanolamin um dann mit Silbernitrat anzufärben. WINTERINGHAM und Mitarb.[9] verwenden Monoäthanolamin, Silbernitrat und ultraviolettes Licht. In späteren Arbeiten verwenden MITCHELL[10,11] wie auch MILLS[12,13] Silbernitrat (in einer 2-Phenoxyäthanollösung). Ein neuer Vorschlag[14]

besteht in der Verwendung von Methylgelb zum Besprühen der Papierchromatogramme und U.V.-Bestrahlung. Die chlorierten Pestizide erscheinen als rote Flecken welche nach einer Stunde verblassen.

Auf dem verhältnismässig jungem Gebiet der Dünnschichtchromatographie verwenden BÄUMLER UND RIPPSTEIN[15] eine Lösung von N,N-Dimethyl-*p*-phenylendiaminhydrochlorid in Natriumäthylatlösung und setzen die Platten der Bestrahlung einer U.V.-Lampe aus.

PETROWITZ UND PASTUSCHKA[16] befassen sich eingehend mit der DC-Trennung chlorierter Kohlenwasserstoffe und verwenden die in der Papierchromatographie erprobte Anfärbung mit Monoäthanolamin, Silbernitrat und Bestrahlung.

WALKER UND BEROZA[17] erzielen die Sichtbarmachung am Dünnschichtchromatogramm indem sie die Platten mit Bromfluoreszein oder Silbernitrat behandeln und mit U.V.-Licht bestrahlen.

BONDI UND GUZMAN[18] und BONDI[19] beschreiben die Anwendung von Tüpfelreaktionen und untersuchen, nebst andern Fremdstoffen, auch chlorierte Insektizide in Lebensmitteln. Sie benützen in geänderter Form den von FEIGL[20] für Amine beschriebenen Tüpfelnachweis. Fluoreszeinchlorid und wasserfreies Zinkchlorid reagieren in diesem Nachweis mit dem Amin unter Freilegung von HCl und Bildung eines Rhodaminfarbstoffes. In der abgeänderten Form ersetzen die chlorierten Kohlenwasserstoffe das Fluoreszeinchlorid und als Reagenz dienen Diphenylamin und Zinkchlorid. Dieser Tüpfeltest ist positiv für Aldrin, Dieldrin, Toxaphen und DDT, während Lindan negative Resultate gibt.

Diese Farbreaktion wurde schon von CUETO[21] zur colorimetrischen Bestimmung von Dieldrin, von GRAUPNER UND DUNN[22] zur Bestimmung von Toxaphen-Rückständen und von MCKINLEY UND GRAHAM[23] zum Nachweis von Captan am Papierchromatogramm (nach Bestrahlung mit U.V.-Licht) angewandt.

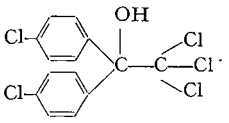
Auf der Suche nach einem geeigneten Anfärbemittel nach Auftrennung der chlorierten Insektizide am Dünnschichtchromatogramm konnten wir mit Diphenylamin–Zinkchlorid ausgezeichnete Resultate erzielen. Die Färbungen sind für eine ganze Reihe von chlorierten Insektiziden ausgesprochen charakteristisch und dadurch ist diese Arbeitsweise vorteilhafter als die oben beschriebenen. Es sind auch keine Vor-oder Nachbehandlungen nötig.

Nach Auftrennung werden die Platten mit dem Diphenyl–Zinkchlorid Reagenz besprüht und direkt in den auf 200° aufgeheizten Trockenschrank gebracht. Bereits nach einigen Minuten erscheinen die chlorierten Insektizide in charakteristischen Färbungen:

DDT	= rosa-rot;
Methoxychlor	= dunkelblau-schwarz;
CPCA (Kelthan)	= blattgrün;
Chlor-DDT	= rostbraun;
Captan	= grün bei 160°, braunlich bei 200°;
Toxaphen	= grau-grün.

Die unterschiedliche Anfärbung und die einfache Arbeitsweise ermöglichen eine rasche und einwandfreie Identifizierung der Pestizide. Sie können auch zur Überwachung eines technologischen Verfahrens, zum Auffinden von interferierenden oder sonstigen chlorierten Begleitstoffen herangezogen werden.

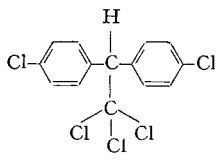
An Hand einer Beispiels soll die Anwendung der beschriebenen Arbeitsweise zur Sichtbarmachung von Begleitstoffen eines Schädlingsbekämpfungsmittels gezeigt werden. In einer frühern Arbeit[24] wurde die Analyse des 1,1-Bis-(*p*-chlorphenyl)-2,2,2-trichloräthanols (CPCA, Acarin, Kelthan) (I), behandelt, ohne Angabe einer



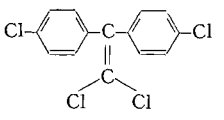
(I)

Methode zur Ermittlung von Begleitstoffen. Die eventuellen Begleitstoffe dieses Acarizids waren DDT (II), DDE (III), Chlor-DDT (IV) und *p,p'*-Dichlordibenzoyl (V). Sämtliche Verbindungen können am Chromatogramm sichtbar gemacht werden.

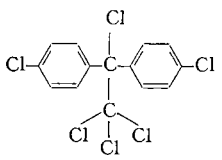
Das CPCA (I), DDT (II) und Chlor-DDT (IV) geben charakteristische Färbungen



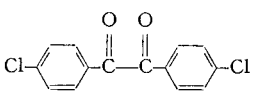
(II)



(III)



(IV)



(V)

(siehe oben) mit dem Diphenylamin–$ZnCl_2$ Reagenz, das DDE (III) und *p,p'*-Dichlordibenzoyl (V) erscheinen nach besprühen mit Jod als hellbraune Flecken.

Ein von uns dargestelltes Gemisch der fünf genannten Verbindungen wurde nach Auftrennung (siehe R_F Werte Tabelle I) zunächst mit Jodlösung besprüht um das DDE und Dichlordibenzoyl zu identifizieren. Die anderen Stoffe sind mit Jod nicht anfärbbar.

Nach Entfernung des Jods mittels Erwärmung auf 80° werden die Platten mit dem Diphenylamin–$ZnCl_2$ Reagenz besprüht und auf 200° erhitzt um die restlichen Verbindungen (CPCA, DDT, Chlor-DDT) in ihren charakteristischen Anfärbungen sichtbar zu machen.

Das DDE und das *p,p'*-Dichlorbenzoyl können ebenfalls mit dem Diphenylamin–$ZnCl_2$ Reagenz sichtbar gemacht werden indem man die Platten, nach Besprühung und Erhitzung auf 100° dem U.V.-licht (Desaga UV-Strahler 700) aussetzt. Sie erscheinen als rosa, beziehungsweise gelbe Flecken.

Die untere Nachweisgrenze ist 3–4 γ für CPCA und Captan, 1 γ für Methoxychlor und Chlor-DDT und 2 γ für DDT und Toxaphen.

TABELLE I

R_F WERTE

CPCA (I)	0.16
Chlor-DDT (IV)	0.96
p,p'-Dichlordibenzoyl (V)	0.50
DDE (III)	0.95

Praktische Durchführung

Reagenzien: 0.5 g Diphenylamin und 0.5 g $ZnCl_2$ werden in 100 ml Aceton gelöst, 0.5 g Jod in 100 ml Chloroform.

Fliessmittel: Petroläther (40–60°)–CCl_4 (1:1).

Desaga Standardausrüstung. Mit Kieselgel G nach Stahl beschichtete 200 × 200 mm Platten; Schichtdicke 0.25 mm.

Nach Auftrennung (Kammersättigung mit Filterpapierstreifen) werden die Platten bei 70° 10 Minuten getrocknet, mit Diphenylamin–$ZnCl_2$ Reagenz besprüht und 5 Minuten bei 200° erhitzt (DDT, CPCA, Chlor-DDT, Captan, Methoxychlor und Toxaphen).

Zur Auffindung von DDE und Dichlordibenzoyl werden die Platten nach Auftrennung und Trocknung mit Jodlösung behandelt.

Der Autor dankt dem Generaldirektor der Makhteshim Chemical Works, Beer-Shewa für die erteilte Unterstützung und die Erlaubnis zur Veröffentlichung und Herrn Prof. A. BONDI für die zur Verfügung gestellten Arbeiten aus seinem Laboratorium.

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Thin-layer chromatography of *Dioscorea* sapogenins

Paper chromatographic separations of steroidal sapogenins have been described by HEFTMANN AND HAYDEN[1], SANNIÉ AND LAPIN[2], MC.ALEER AND KOZLOWSKI[3] and WALL *et al*[4]. The procedures of HEFTMANN AND HAYDEN and SANNIÉ AND LAPIN are complicated by frequent lack of resolution due to tailing of spots at concentrations greater than 5 μg and by difficulties in maintaining stable chromatographic conditions[3]. The non-aqueous systems of MC. ALEER AND KOZLOWSKI and WALL *et al.* are technically more complicated and require several hours for development.

Thin-layer chromatography has been employed by several workers for the separation of steroidal sapogenins. SANDER[5–8] employed the technique for the separation of unknown sapogenins and in the identification of neotigogenin, tigogenin, diosgenin and gitogenin. TLC has been used by BENNETT AND HEFTMANN[9] for the separation of steroidal sapogenins using systems other than those described in this paper and with 50 % sulphuric acid as the detecting agent. SMITH AND FOELL[10] have given R_F values for C_{27} sapogenins with starch bound silica gel thin-layers, using hexane–ethyl acetate (4:1), hexane–ethyl acetate (1:1) and ethyl acetate as solvent systems and with phosphomolybdic acid among their detecting reagents. Spread-layer chromatography has also been applied to some sapogenins[11–13].

The chromatographic methods described in this paper were devised for the rapid separation of the components of the crude sapogenin mixtures isolated from *Dioscorea* tubers[14].

Experimental

A slurry was prepared by mixing silica gel G, 30 g (Research Specialties Company, Richmond, Calif.) with distilled water, 60 ml. This was spread in a layer 0.5 mm thick on glass plates, 20 cm × 20 cm and after standing for 5 min the plates were dried at 100° for 30 min. The sapogenins were applied to the prepared plates as benzene solutions and after waiting for about 1 min for the spots to dry the plates were placed in one of three solvent systems: (1) chloroform-ethanol 95 % (95:5); (2) chloroform–acetone (3:1); and (3) ethyl acetate. Elution was by the supersaturated method of STAHL[15] The procedure of BLUNDEN AND HARDMAN[14] afforded the test solutions: the tubers of *Dioscorea belizensis* Lundell, *D. sylvatica* Ecklon, and *D. villosa* Linn. were disintegrated, fermented, acid hydrolysed and the insoluble material was extracted with petroleum ether. Removal of the solvent gave a residue of sapogenin, 40 μg of which in benzene solution was used to spot the plate from a micropipette. Development of the plate occupied 60–90 min for both the chloroform–ethanol 95 % and the chloroform–acetone systems and 40–60 min for the ethyl acetate system. After development the chromatograms were dried for 2 min, sprayed with antimony trichloride in concentrated hydrochloric acid and heated at 90° for 10 min to enable the full colour of the spots to develop[16].

Results

The chromatographic pictures obtained from the sapogenin test solutions from all three *Dioscorea* species were very similar, irrespective of which solvent system was used. The pictures from *D. sylvatica* and *D. villosa* were identical and all these fractions were detected in the extract from *D. belizensis*. However, in addition to these

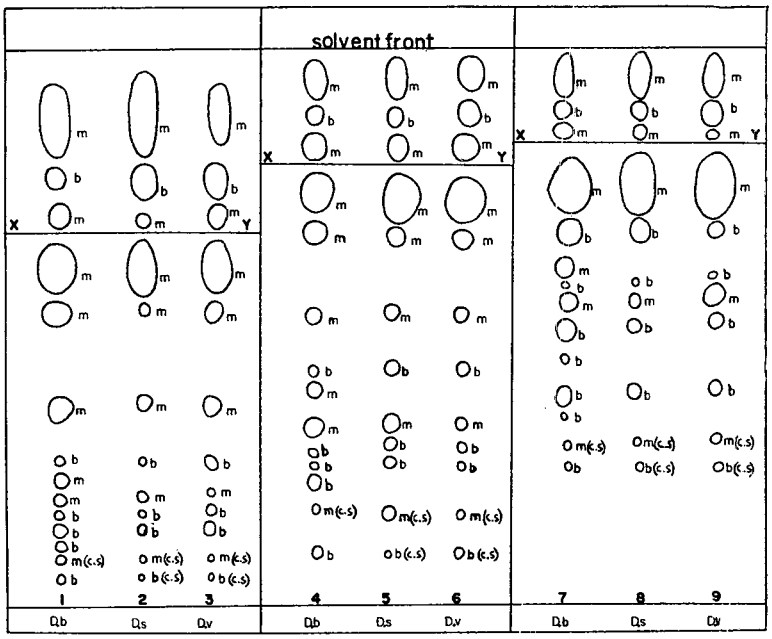

Fig. 1. Separation of steroidal sapogenins from *Dioscorea* species. D.b. = *D. belizensis*; D.s. = *D. sylvatica*; D.v. = *D. villosa*. Solvent systems: for 1, 2 and 3, chloroform–ethanol 95% (95:5); for 4, 5 and 6, chloroform–acetone (3:1); for 7, 8 and 9, ethyl acetate. Colour produced after spraying plates with antimony trichloride in concentrated hydrochloric acid and heating: m = mauve-purple, b = blue. Spots above the line XY were those detected from the first fractions of chloroform eluate from the sapogenin mixture after passage through an alumina column. Spots detected from concentrated solutions only = c.s.

compounds three others were detected from *D. belizensis*, but were not noticed in the chromatograms from the other two species (Fig. 1).

The spots with the highest R_F values were not clear using this concentration. These sapogenins were separated from the others by passage through an alumina column using chloroform as the eluting solvent and collecting the first fractions. Three spots were detected from these fractions from all three *Dioscorea* species (Fig. 1).

Samples of pure sapogenins were run alongside the plant extracts (Fig. 2). The reference compounds used were diosgenin (22α-spirost-5-en-3β-ol), kryptogenin (25α-cholest-5-en-3β,26-diol-16,22-dione), yamogenin (22β-spirost-5-en-3β-ol), botogenin (22α-spirost-5-en-3β-ol-12-one) and pennogenin (22α-spirost-5-en-3β,17α-diol). Of these the botogenin sample when chromatographed separated out into five different spots and could not be used as a reference. The reference compounds were in addition mixed with the tuber extracts from the three species of *Dioscorea* and chromatographed. The kryptogenin separated out from the spots produced by the plant extracts showing it to be absent from these extracts, but the diosgenin and pennogenin did not, indicating strongly their presence in the plant extracts. Diosgenin and its isomer yamogenin, were not clearly separated by any of the solvent systems. Diosgenin was the predominant sapogenin from all three plants when assayed by the standard procedure[14], and in tubers over two years old the following percentages

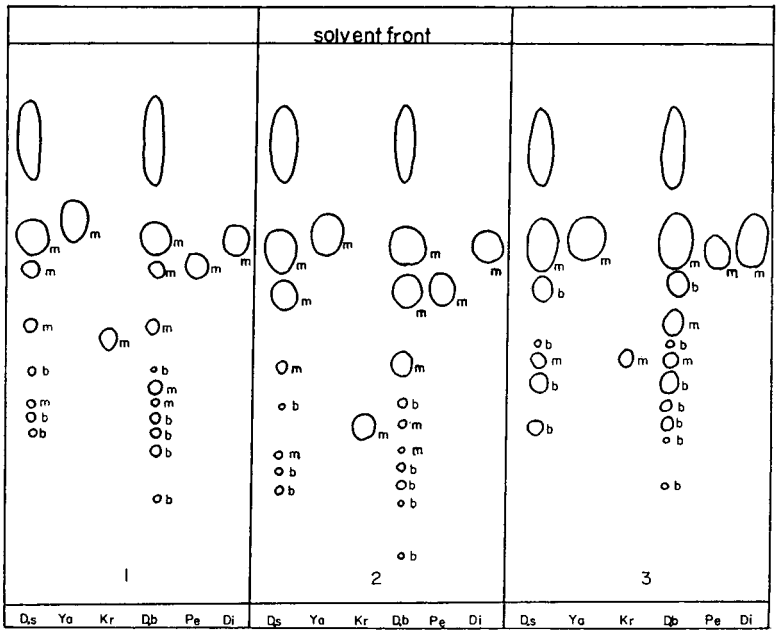

Fig. 2. Separation of steroidal sapogenins from *Dioscorea* species. Ya = yamogenin; Kr = kryptogenin; Pe = pennogenin; Di = diosgenin; D.b. = *D. belizensis* sapogenin mixture; D.s. = *D. sylvatica* sapogenin mixture. (Same spots also produced by *D. villosa*). Solvent systems: for 1, chloroform–ethanol 95% (95:5); for 2, chloroform–acetone (3:1); for 3, ethyl acetate. Colour produced after spraying plates with antimony trichloride in concentrated hydrochloric acid and heating: m = mauve-purple, b = blue.

were obtained, calculated on a moisture free basis: *D. villosa* 0.7% to 1.1%, *D. belizensis* 0.6% to 2.6%, *D. sylvatica* 4% to 6%.

Discussion

The saponin-containing *Dioscorea* tubers used in this study were selected both for their widely different geographical sources and for their representation of three different morphological types: the underground dwarf, rhizome-like organ of *D. villosa* Linn., indigenous to the eastern and central United States[17]; the underground large elongated tuber of *D. belizensis* Lundell, indigenous to British Honduras[18], and thirdly the large circular and plate-like tuber of *D. sylvatica* Ecklon, found in the surface of the soil of subtropical South Africa[19].

The use of all three developing systems proved worthwhile. For example, with the two chloroform systems the mauve-purple spots produced by diosgenin and pennogenin were separated from the extracts of all three *Dioscorea* species, but these two compounds were not separated using the ethyl acetate solvent system. However, with the latter system a blue spot was seen below the combined diosgenin–pennogenin spot; using the two other systems this blue spot was not separated from that of pennogenin. The presence of the two compounds was noticed after spraying, as the blue colour developed first and was then masked by the mauve-purple colour of the pennogenin on heating. Furthermore, the ethyl acetate system separated out more completely the blue spots of low R_F present only in the *D. belizensis* extract.

MARKER AND LOPEZ[20] demonstrated that the ethanolic acid or alkali treatment of the saponins from *D. mexicana* resulted in the formation of several sapogenins which were not naturally occurring in the plant as the glycoside. The large number of spots detected on the chromatograms in this present study probably include these products as well as any arising from disintegration and fermentation of the tuber. Diosgenin is the predominant sapogenin obtained from all three plants. Pennogenin was detected in all three plant extracts. It was shown by MARKER[20] AND LOPEZ that it could arise as a breakdown product. Kryptogenin was readily isolated from the Mexican species of *Dioscorea* studied by MARKER *et al.*[21], but they considered it to be a breakdown product formed during the isolation of the sapogenins[20]. Apparently it was not formed during our assay procedure, which avoids the use of boiling ethanolic hydrochloric acid, for kryptogenin was not detected in the sapogenin mixture from the Central American species *D. belizensis* nor from the other two species.

Detection of the other compounds present was impossible due to lack of reference compounds.

Acknowledgements

We wish to thank the Canadian Foundation for the Advancement of Pharmacy for their generous financial assistance without which this work would have been impossible. Our thanks are also offered to Syntex S.A. for supplying us with the samples of yamogenin, pennogenin and botogenin, and to Mrs. D. MITCHELL for her technical assistance.

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Separation of sugar phosphates and sugar nucleotides by thin-layer chromatography

Several papers describing the application of thin-layer chromatography to the separation of purines, pyrimidines, nucleosides and nucleotides have appeared[1–7], but this technique has not been applied to the resolution of mixtures of sugar phosphates and sugar nucleotides. Using the procedure described below, the separation of these substances can be accomplished within two hours using ECTEOLA layers.

The plates were prepared by suspending 2 g of sieved ECTEOLA-cellulose powder (Serva-Entwicklungslabor, Heidelberg, Germany) in 18 ml of 0.004 *M* ethylenediaminetetraacetic acid pH 7.0, and shaking it vigorously for 5 min. The resulting slurry was poured onto a 20 × 20 cm glass plate and spread in a uniform layer with a glass rod. The plates were dried overnight at room temperature, giving a layer with a thickness of about 200 μ. Afterwards, they were sprayed with 0.1 *M* ammonium tetraborate[8] pH 9.0 (preparation of the ECTEOLA slurry in ammonium tetraborate did not give the same results). After spraying, the layers were dried at 50° for 30 min, and cooled to room temperature before use. No difference was found if the plates were used immediately or within three days.

Solutions of phosphoric esters and nucleotides were spotted 3 cm from the edge of the plate. The layers were placed in closed tanks and the chromatograms were developed with ethanol (95 %)–0.1 *M* ammonium tetraborate, pH 9.0, (3:2). For phosphoric esters only, a solvent prepared with a pH 10 buffer was also used. In this case, the plates had been previously sprayed with buffer of the same pH.

An improvement in the shape of the spots was observed if some cellulose was erased from the layer so as to leave a pattern similar to the one described by MATTHIAS[9] for paper chromatography.

TABLE I

R_P* VALUES OF PHOSPHORIC ESTERS AND NUCLEOTIDES

Substance	*pH of the solvent* 9.0	*Substance*	*pH of the solvent*	
			9.0	10
TDP-glucose**	1.25	N-Acetylglucosamine 1-P	1.29	1.37
UDP-acetylglucosamine	0.82	N-Acetylgalactosamine 1-P	1.12	1.16
UDP-glucose	0.70	Glucose 1-P	1.20	1.15
UDP-galactose	0.47	Mannose 1-P	0.90	0.88
ADP-glucose**	0.61	Galactose 1-P	0.77	0.80
ADP-mannose**	0.50	Mannose 6-P	0.70	0.57
ADP-galactose**	0.40	Fructose 6-P	0.68	0.68
ADP-glyceric acid**	0.43	Fructose 1-P	0.59	0.54
UTP	0.37	Glucose 6-P	0.56	0.39
UDP	0.42	Fructose 1,6-P_2	0.33	0.20
UMP	0.53	3-P-Glyceric acid	0.97	0.95
ATP	0.32	2,3-Diphosphoglyceric acid	0.78	0.80
ADP	0.36			
AMP	0.42			
TMP	1.10			

* Inorganic phosphate moves about 7.2 cm from the point of application. The results are expressed as the ratio of the distance travelled by the phosphate derivative to the distance travelled by inorganic phosphate.

** These nucleotides were generously provided by Dr. E. RECONDO.

The chromatography was stopped when the solvent front had reached the top edge (after about 2 h). After examination for ultraviolet absorbing spots with a Mineralight lamp, the chromatograms were sprayed successively with benzidine–trichloroacetic acid[10] to ascertain the position of the hexose-6-phosphates, and with the molybdate reagent of BURROWS *et al.*[11] for phosphoric esters.

The results obtained for some phosphoric esters and sugar nucleotides are shown in Table I. Satisfactory separations could be obtained also for sugar nucleotides and aldose-1-phosphates by developing the chromatograms with a different solvent mixture ethanol (95 %)–0.1 *M* ammonium tetraborate, pH 9.0, (1:1).

The limits of detection of the substance with the different reagents is as follows in mμmoles: benzidine 10, molybdate 5, and ultraviolet light 1.

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Announcements

THE SOCIETY FOR ANALYTICAL CHEMISTRY

Notice of Joint Meeting

A Joint Meeting of the Midlands Section, the Physical Methods Group and the Thin Layer Chromatography Discussion Panel of the Society for Analytical Chemistry and the Birmingham and Midlands Section of the Royal Institute of Chemistry will be held on Wednesday, 14th October, 1964, at the Medical School, The University, Edgbaston, Birmingham, 15.

The Meeting will take the form of a one-day Symposium on

THIN LAYER CHROMATOGRAPHY

Programme

1.30 p.m. Chairman: Dr. E. V. TRUTER, B.Sc., A.R.C.S., D.I.C., in the Arthur Thompson Hall.
"Thin Layer Chromatography in Clinical Biochemistry" by Dr. J. G. LINES, Ph.D., M.Sc. (Children's Hospital, Birmingham) and "Exploitation of Thin Layer Chromatography in Plant Chemistry" by Dr. E. J. SHELLARD, F.P.S., F.L.S., F.R.I.C. (Chelsea College of Science and Technology).

2.45 p.m. Tea

3.15 p.m. Chairman: Prof. J. R. SQUIRE, M.A., M.D., F.R.C.P., in the Arthur Thompson Hall.

Plenary lecture

"Some New Techniques and Apparatus in the Field of Thin Layer Chromatography" by Prof. Dr. EGON STAHL (University of Saarland, Saarbrücken, West Germany).

4.45 p.m. Tea.

5.30 p.m. Chairman: Prof. J. C. ROBB, B.Sc., Ph.D., D.Sc., F.R.I.C. in the Large Anatomy Theatre.
"The Use of Thin Layer Chromatography in Biosynthetic Studies" by Dr. B. H. DAVIES, B.Sc., A.R.I.C. (University College of Wales, Aberystwyth).

6.30 p.m. Chairman: Mr. W. H. STEPHENSON, F.P.S., D.B.A., F.R.I.C. in the Large Anatomy Theatre.
"Recent Developments in Thin Layer Chromatography Equipment" by Mr. R. P. HIRSCH (Camlab (Glass) Ltd., Cambridge) and "Thin Layer Chromatography on Ion-Exchange Media" by Dr. C. S. KNIGHT, M.Sc., A.R.I.C. (W. and R. Balston Ltd., Maidstone, Kent).

There will be ample time allowed for discussion in each session.

A Trades Exhibition on Thin Layer Chromatographic Equipment and Materials has been arranged in the Chamberlain Museum (Medical School) and will be open from 11 a.m. until 8 p.m.

All enquiries in connection with the Trades Exhibition should be made to Mr. D. M. PEAKE, B.Sc., A.R.I.C., c/o Imperial Metal Industries (Kynoch) Ltd., P.O. Box 216, Kynoch Works, Witton, Birmingham, 6.

Further details can be obtained from MERVYN L. RICHARDSON, A.R.I.C., A.C.T. (Birm), Hon. Secretary, Midlands Section, The Society for Analytical Chemistry, c/o John and E. Sturge Ltd., Lifford Lane, Kings Norton, Birmingham, 30, England.

INFORMAL SYMPOSIUM
ON
BIOLOGICAL APPLICATIONS OF GAS CHROMATOGRAPHY

September 14th, 1964

École Polytechnique, 17, rue Descartes, Paris 5e, France

Programme

9.00 Opening Address—Prof. L. JACQUÉ (École Polytechnique, Paris)
9.10 Biological Applications of Gas Chromatography—E. C. HORNING
10.10 Gas Chromatography of Steroids—W. J. A. VANDENHEUVEL
11.10 Gas–Liquid Chromatography of Carbohydrates—C. C. SWEELEY

14.30 Analysis of Bile Acids by Gas Chromatography and Mass Spectrometry—J. SJÖVALL
15.30 Gas Chromatography of Amino Acids and Amino Acid Derivatives.

Three communications of which the title is not yet fixed will also be presented.

Authors who wish to present a communication on this subject are requested to send a summary before August 1st.

For registration write to G. GUIOCHON, Laboratoire de Chimie, École Polytechnique, 17, rue Descartes, Paris, 5e, France.

(Registration fee: Fr. 20).

THIRD INTERNATIONAL SYMPOSIUM ON CHROMATOGRAPHY

Brussels, September 14th and 15th, 1964

The Belgian Society of Pharmaceutical Sciences wishes to inform you that it is organizing on Monday 14th and Tuesday 15th of September 1964, a third Symposium on Chromatography, in which theoretical aspects and most diversified practical applications will be considered.

All persons interested in chromatography are invited to participate at this Symposium. Registrations will be received until August 1st, 1964. Participants who want to give a lecture are invited to register before July 1st, 1964.

The official languages at the Symposium will be French, Dutch, English and German.

The registration fee has been fixed at 300 Belgian francs, and may be paid to the Postal Account 1090.00 of the "Banque de Bruxelles", on behalf of "Mme HANS—Melle RENOZ—Symposium de Chromatographie", No. A 52/33173.

A scientific exhibition will be held during the Symposium.

The Secretary's office of the Belgian Society of Pharmaceutical Sciences—11, rue Archimède, Brussels 4—will forward registration forms upon request.

THE BOARD OF DIRECTORS

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Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

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PROGRAMMED FLOW GAS CHROMATOGRAPHY

PART I

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The objective of research in gas chromatography in recent years has been the resolution of more complex mixtures. One of the many ways devised to solve this problem is the programmed temperature technique. By means of this technique it is possible to separate not only groups of substances with wide boiling point ranges but also the constituents of each group.

This paper describes the utilization of a programmed flow of the mobile phase[1] in isothermic or temperature programmed runs, in order to obtain the separation of complex mixtures.

In his recent book PURNELL[2] also suggested the possibility of a programmed flow technique; more recently SCOTT[3] proposed the use of programmed flow in preparative scale chromatography with analytical columns.

RELATIONSHIP BETWEEN CHROMATOGRAM PARAMETERS AND THE MOBILE PHASE FLOW RATE IN ISORHEIC** RUNS

The effect will be discussed of the gas flow rate on the parameters that can be directly taken from the chromatogram, *i.e.*, retention time, peak width, peak area and peak height. The system chosen to study these effects consisted of Nujol (as the stationary phase) and various alkanes, in order to minimize secondary effects in the partition process***.

(1) Peak migration and flow rate

Peak migration can be related to flow rate by means of the equation:

$$t_N = KV_L/F_c \qquad (1)$$

The validity of this equation for the system used in this paper is shown in Figs. 1, 2 and 3.

In programmed flow chromatography V_N rather than t_N should be used as the

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** An isorheic process is defined as a process that occurs at a constant flow rate (from the Greek, iso = same and rheo = flow).

*** It should be emphasized here that all the results given and discussed in this paper refer to experimental procedures, equipment and phase systems described in the experimental part. It is not our intention to say that the results presented are general relationships nor that they are particular cases of a more general theory. Further work is in progress to verify this.

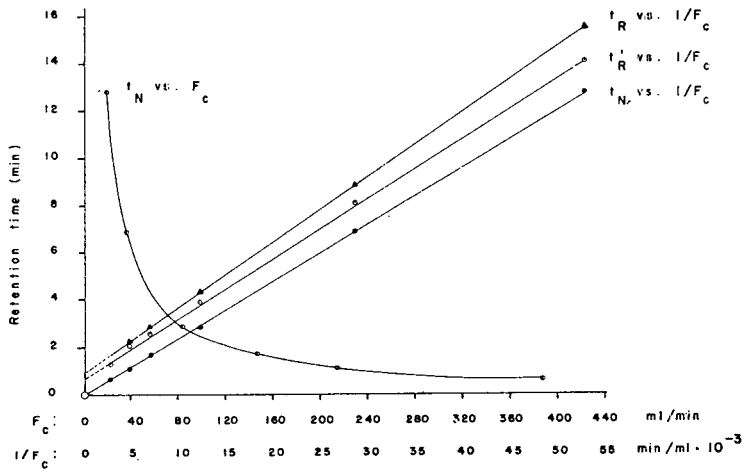

Fig. 1. Plot of retention (t_R) time (min), corrected retention time (t_R') (min) and net retention time ($t_N = jt_R'$) (min) *vs.* $1/F_c$ (min/ml) for 2,3-dimethylpentane; stationary phase: Nujol 20% on CG-SORB TB.

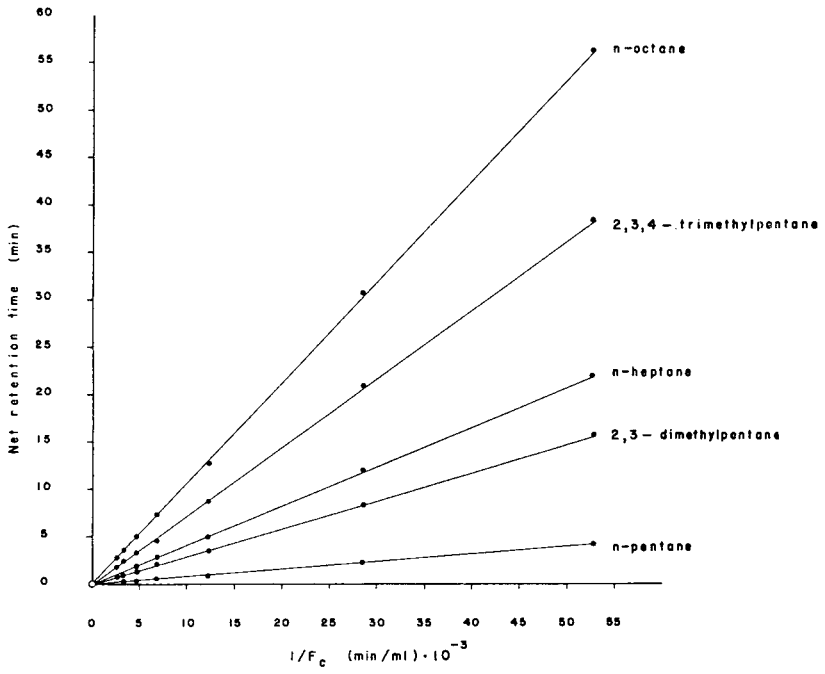

Fig. 2. Plot of net retention time (min) *vs.* $1/F_c$ (min/ml) for various hydrocarbons on Nujol.

retention parameter for the characterization of the peaks since V_N is constant and independent of flow rate. An integral volume measuring device would be highly desirable to accompany any equipment using a programmed flow technique.

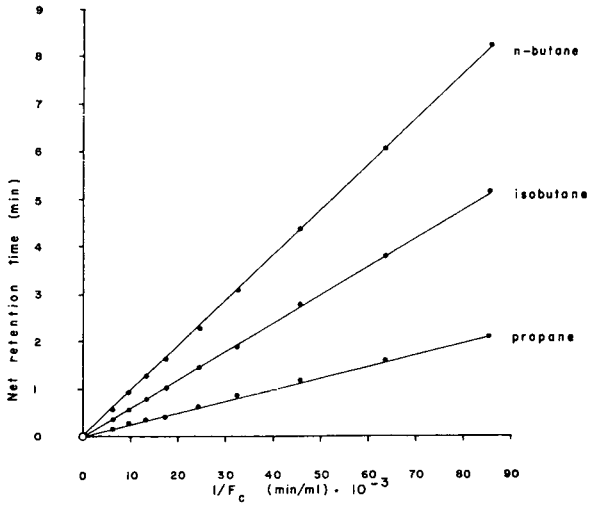

Fig. 3. Plot of net retention time (min) *vs.* $1/F_c$ (min/ml) for propane, isobutane and *n*-butane

(2) *Peak width (w) and flow rate*

Peak width can be related to flow rate by means of the equation:

$$w = A + B\left(\frac{1}{F_c} + \frac{C}{F_c^2}\right) \qquad (2)$$

Fig. 4 shows the plot of *w versus* $(1/F_c + C/F_c^2)$.

It should be noted in the curves of Fig. 4 for propane, isobutane and *n*-butane, that all the coefficients of eqn. (2) are different (Table I). It is possible that the values

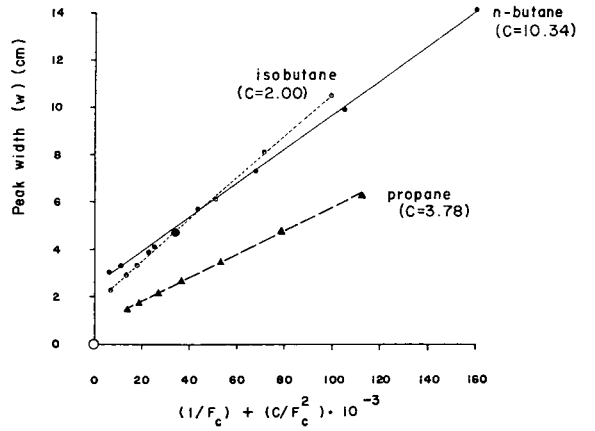

Fig. 4. Plot of peak width (*w*) (cm) *vs.* $(1/F_c) + (C/F_c^2)$ for propane, *n*-butane and isobutane. The values of *C* are shown in the corresponding curves.

TABLE I

COEFFICIENTS OF EQUATION (2)

$$w = A + B\left(\frac{1}{F_c} + \frac{C}{F_c^2}\right)$$

w in cm; F_c in ml/min

Compound	*% in the mixture*	*A*	*B*	*C*
Propane	59.2	0.8	50	3.78
Isobutane	23.9	1.8	88	2.00
n-Butane	16.9	2.4	72	10.34

of these coefficients depend on the amount and the nature of the substance, but it is not likely that the detector-response dependence on flow rate makes any significant contribution. Further work is in process to clarify this.

The peak width is a basic parameter for the calculation of the efficiency of the column and resolution of peaks. The results using eqn. (2) for these calculations are shown in the next section.

The plot of Fig. 4 shows the total peak width. A peak width asymmetry based on the ratio of the front half-peak width to the total peak width was fairly constant, at 0.43 ± 0.03, for all the flow rates.

(3) Peak area and flow rate

The peak area is a major characteristic in quantitative analysis and a knowledge of the behaviour of the peak area with the flow rate is of fundamental importance when using a programmed flow technique for quantitative purposes. The literature contains some references[2,4] to the behaviour of the area with flow rate.

The peak area is certainly dependent on the detector response, which, in the case of the thermoconductivity detectors is not simply a function of the gas flow rate. Nevertheless we found that the peak area behaved as a linear function of $1/F_c$ in our experimental system (as can be seen in Fig. 5) as indicated by eqn. (3),

$$A_{i,F_c} = q_i \cdot \frac{1}{F_c} \tag{3}$$

where q_i, the slope of the line, is dependent on the amount of substance (% i) and probably on its nature (i). The absolute value of q depends also on the nature of the detector response (Y).

As can be seen in Fig. 5, the ratio of the areas is constant at any flow rate.

It is certain that our data depend somewhat on the equipment used in our experiments, but if these results can be generalized, at least for the particular type of detector used, it would be an important feature for quantitative analysis in a programmed flow process.

To a first approximation, valid for very similar substances, the influence of the nature of the substance on the slope could be neglected, and for the same equipment, relative values of q are all that are needed. In this way, q can be directly related to the relative amounts of the constituents and the following equation can be derived to

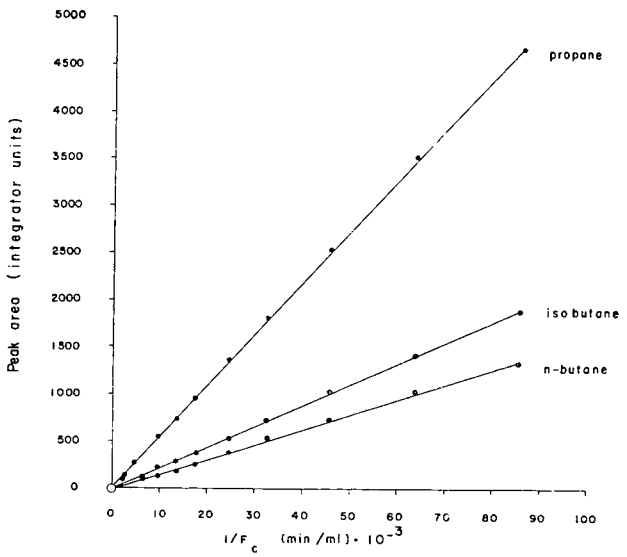

Fig. 5. Plot of peak area *vs.* $1/F_c$ (min/ml) for propane, isobutane and *n*-butane; stationary phase: Nujol 20% on CG-SORB TB 80–100 mesh.

calculate the relative amount of the constituents of a mixture submitted to a programmed flow run.

In order to calculate the amount of any substance by internal normalization, all the areas in the same flow must be related, so that

$$\%\, i = A_{i,\, F_c} / \sum_{1}^{m} A_{m,\, F_{c1}} \tag{4}$$

The area of any component i at a flow rate F_{cn} can be related to the area at a flow rate F_{c1} by means of eqn. (3).

Eqn. (4) can be written as

$$\%\, i = A_{i,\, F_{cn}} \cdot F_{cn} / \sum_{1}^{m} A_{m,\, F_{cn}} \cdot F_{cn} \tag{5}$$

From this equation the various components in a step-programmed flow analysis can be determined. As the amount of substance is constant for all points on the same line (Fig. 5), the slope of the line reflects the influence of the flow rate on the detector response. Thus, in order to calculate a certain area it is necessary to know at what flow rate the area is being measured and in a programmed flow chromatogram eqn. (5) may be used to calculate the amount of substances, provided that the whole peak area is measured at the same flow rate.

(4) Peak height (h) and flow rate

The peak height is also a suitable parameter for quantitative analysis because it is dependent on the amount of substance. Nevertheless it is also very much dependent on the detector response Y. In thermal conductivity detectors, Y is very critical, especially in its dependence on the flow rate.

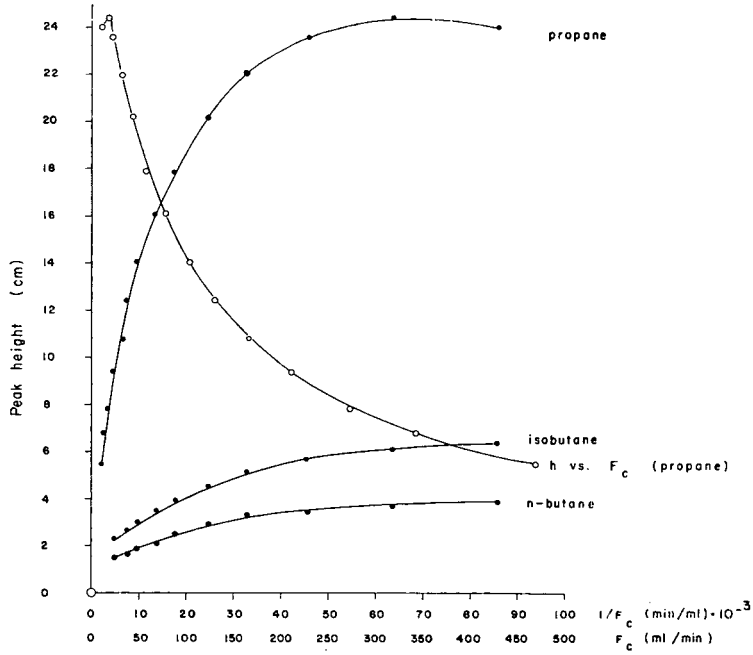

Fig. 6. Plot of peak height (cm) *vs.* $1/F_c$ (min/ml) and F_c (ml/min) for propane, isobutane and butane.

Fig. 6 shows the behaviour of peak height with flow rate for the systems studied. No general function could be obtained to correlate the peak height with flow rate in the detector used.

EFFICIENCY AND FLOW RATE

The efficiency of a chromatographic column can be given as the number of theoretical plates (n) it has, or the height equivalent to a theoretical plate (H). The latter can be expressed as a function of flow by means of the well-known Van Deemter equation.

The number of theoretical plates of a column can be calculated by means of the expression: $n = 16\,(t/w)^2$.

Combining this expression with eqns. (1) and (2) we may write

$$n = \left(\frac{4\,KV_L}{AF_c + B + \dfrac{BC}{F_c}} \right)^2 \tag{6}$$

and

$$\sqrt{H^*} = \frac{L^{1/2}}{4\,KV_L} \left(B + \frac{BC}{F_c} + AF_c \right) \tag{7}$$

This equation is similar to that of Van Deemter in its dependence on flow rate.

Eqn. (7) can be written as

$$H^* = a + \frac{b}{F_c} + cF_c + \frac{d}{F_c^2} + eF_c^2 \tag{8}$$

as compared with $H = a' + b'/F_c + c'F_c$ in Van Deemter's equation.

Again we must emphasize that the validity of eqn. (7) depends on eqn. (2) and this has not yet been shown to be generally applicable. The experimental evidence of this relationship involves a knowledge of the coefficients which can be easily calculated from the experimental parameters of eqn. (2).

Fig. 7 shows the plot of H^*, $\sqrt{H^*}$ and H *versus* F_c.

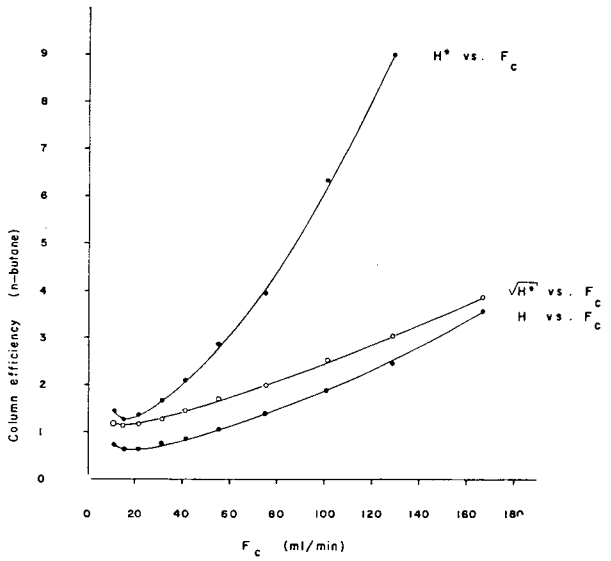

Fig. 7. Plot of column efficiency for *n*-butane as a function of F_c (ml/min). H^* is calculated with net retention parameters. Stationary phase: Nujol 20% on CG-SORB TB, 80–100 mesh.

RESOLUTION AND FLOW RATE

Resolution between two peaks, defined as $R^* = 2\ t_N/W$, may be written as

$$R^* = 2\ V_L\ \Delta K\ F_c/(\sum_i B_i C_i + \sum_i B_i F_c + \sum_i A_i F_c^2) \qquad (9)$$

or in a simplified way:

$$R^* = F_c/(a'' + b''\ F_c + c''\ F_c^2) \qquad (10)$$

This function has a maximum (R_{max}) at $F_c = (a''/(2c''-1))^{1/2}$ and $R = 0$ both at $F_c = 0$ or $F_c = \infty$.

In Fig. 8 the resolution of butane–isobutane peaks is presented as a function of the flow rate, in accordance with eqn. (10).

AN EXAMPLE OF PROGRAMMED FLOW

As an example of the use of the programmed flow technique, a mixture of 13 aliphatic hydrocarbons and benzene was studied.

The first four chromatograms are isorheic (and isothermic) runs in order to show the various resolutions among the peaks.

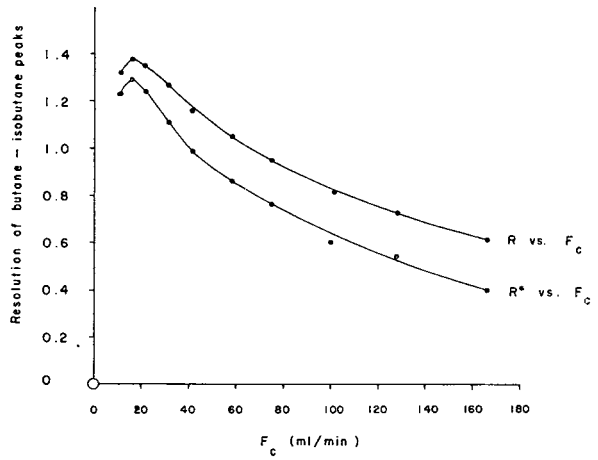

Fig. 8. Plot of the resolution of the mixture *n*-butane–isobutane *vs.* F_c (ml/min). R^* involves net retention parameters.

In chromatogram No. 1 (Fig. 9) at 35 ml/min all the peaks are resolved, except those of isooctane and *n*-heptane (peaks 11 and 12).

The retention times of the last constituents, the octanes, are very large and the peaks are well separated. At the highest flow rate these last peaks are still resolved, and are eluted in 6 min. Nevertheless a very great number of constituents could not be resolved. The other chromatograms (Figs. 10–13), with intermediate flow rates, show intermediate situations.

Chromatogram No. 5 (Fig. 13) shows the results of a programmed flow (discontinuous) run. The resolution obtained at the lowest flow rate is still present although less than half the time was needed for this chromatogram.

It is interesting to note that in a normal chromatographic process an inherent continuous programmed flow process takes place owing to the pressure gradient in the column.

CHARACTERISTICS OF THE PROGRAMMED FLOW PROCESS

1. Equilibrium is reached in the system almost instantaneously. Although the literature recommends times of 10–20 min in order to establish equilibrium conditions,

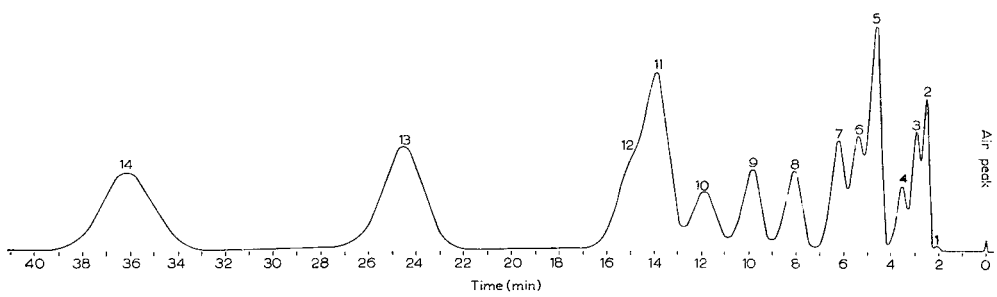

Fig. 9. Chromatogram No. 1. Helium flow rate: 35.1 ml/min. Inlet pressure: 5.0 p.s.i.g. Pressure gradient correction factor (*j*): 0.85. Detector sensitivity: ¼.

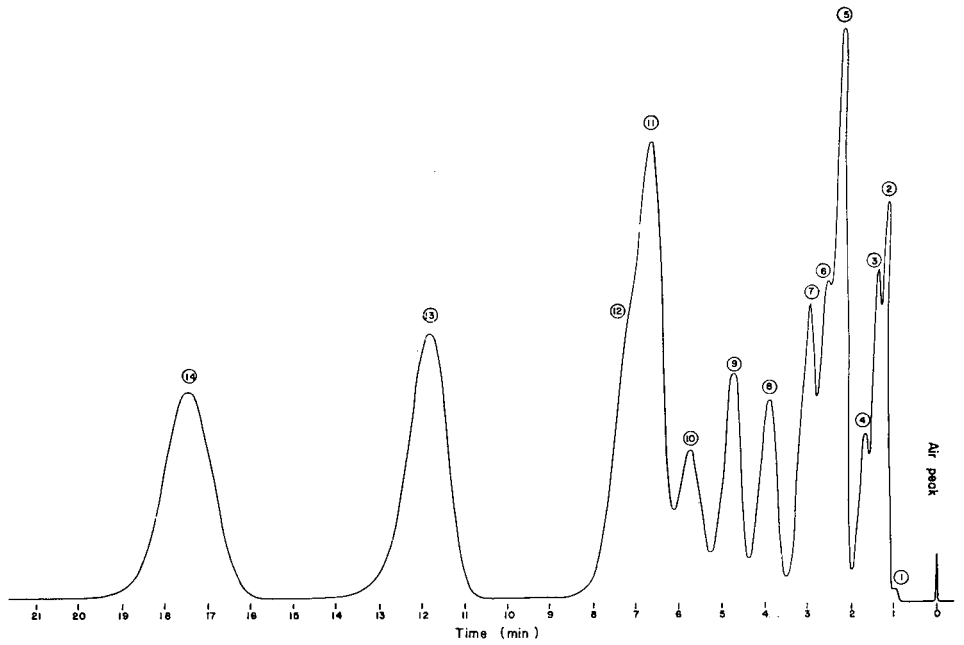

Fig. 10. Chromatogram No. 2. Helium flow rate: 82.9 ml/min. Inlet pressure: 10.0 p.s.i.g. Pressure gradient correction factor (j): 0.73. Detector sensitivity: $\frac{1}{4}$.

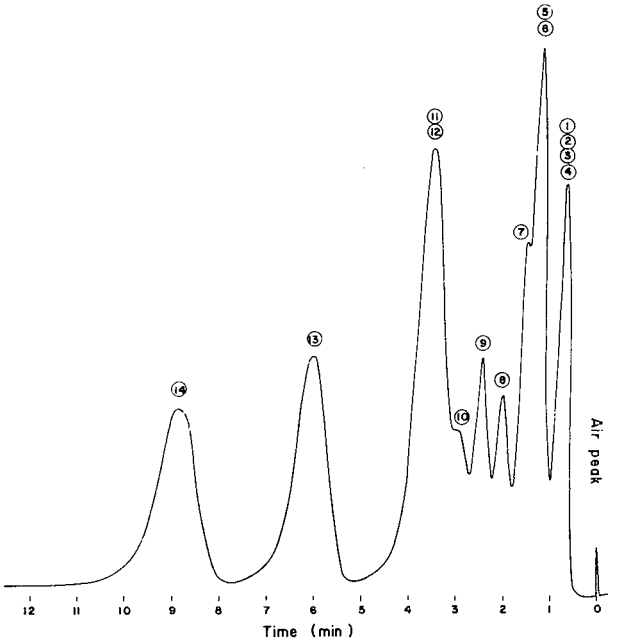

Fig. 11. Chromatogram No. 3. Helium flow rate: 212.9 ml/min. Inlet pressure: 20.0 p.s.i.g. Pressure gradient correction factor (j): 0.55. Detector sensitivity: $\frac{1}{8}$.

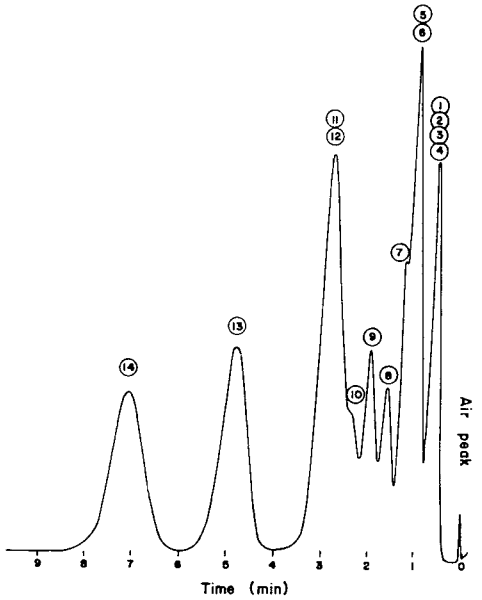

Fig. 12. Chromatogram No. 4. Helium flow rate: 293.4 ml/min. Inlet pressure: 25.0 p.s.i.g. Pressure gradient correction factor (j): 0.50. Detector sensitivity: 1/16.

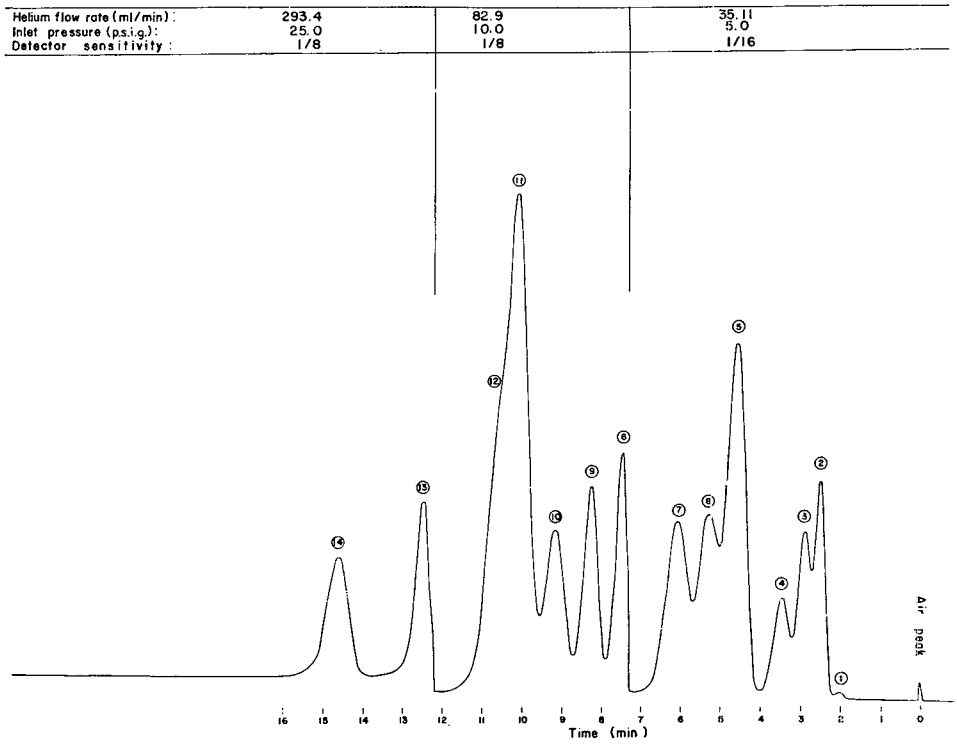

Fig. 13. Chromatogram No. 5. Programmed flow.

we found that very good reproducibility of results could be obtained almost instantly.

2. There is no increase in the volatility of the stationary phase; this allows a wider use of phases.

3. Considerable and almost instantaneous variations in the flow rates are possible, and, as we have already mentioned before, equilibrium is reached in a very short time.

We have used a discontinuous (sequence of isorheic processes) programmation. This is very easy to perform manually and the cost of a step valve for the program is very small.

A continuous process could be used but it is experimentally much more complicated and did not offer any real advantage over the discontinuous process.

4. The programmed flow technique allows the resolution of complex mixtures in isothermic runs or runs with temperature programming. In addition, it has all the inherent advantages of a final temperature much lower than that of an isorheic run.

5. It improves the "quality" of the chromatogram. Sharper peaks (w decreases as F_c increases) with a great reduction in the retention time of the less volatile constituents are obtained, while complete resolution of the more volatile products is still possible.

EXPERIMENTAL

The chromatograms were performed under the following general conditions (the particulars are shown for each chromatogram):

(*1*) *Chromatograph.* Perkin-Elmer "Vapor Fractometer" model 154C with thermistor-detector (Fenwall Electronics Inc., Mass., G 112 Assembly, R_0 at 25°: 6710 Ω. Bridge operated with 8 V).

(*2*) *Column.* ¼ in. copper tubing, 2 m long, containing 3.21 g of Nujol ($d_{26} = 0.88$) on 12.86 g of CG-SORB TB 80–100 m. Effective cross section area of the column: 0.12 cm² (determined from the slope of the straight line obtained from the plot of t_M^0 *vs.* $1/F_c$, according to the expression $t_M^0 = sl \cdot 1/F_c$ (Fig. 14).

The behaviour of the alkanes in the Nujol column can be further characterized by their K value determined from eqn. (1) and the graphs of Figs. 2 and 3.

The values given in Table II were determined.

TABLE II

K VALUES OF ALKANES

(V_L = 3.64 ml Nujol)

Substance	*Boiling point (°C) (1 atm)*	*K*	*Temperature (°C)*
Propane	—42.2	6.67	26
Isobutane	—10.2	16.5	26
n-Butane	—0.6	26.2	26
n-Pentane	36.2	21.1	75
2,4-Dimethylpentane	80.5	66.6	75
2,3-Dimethylpentane	89.8	81.5	75
2,2,4-Trimethylpentane	99.3	114	75
2,3,4-Trimethylpentane	113.4	119	75
n-Octane	125.8	293	75

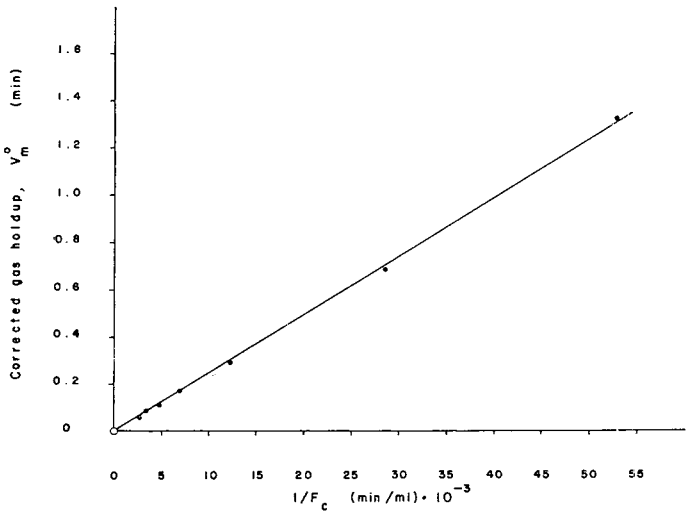

Fig. 14. Plot of corrected gas holdup V_M^0 (min) *vs.* $1/F_c$ (min/ml).

(3) *Recorder.* Speedomax G, Leeds & Northrup, 5 mV, equipped with a Perkin-Elmer Printing Integrator model 194. The recorder speed for the chromatograms of C_5–C_8 was 1 in./min and for the chromatograms of C_3–C_4 was 4 in./min.

(4) *Carrier gas.* Helium.

(5) *Temperature.* For the chromatograms of C_5–C_8: 75°. For the chromatograms of C_3–C_4: 25°.

(6) *Sample* C_5–C_8. A synthetic mixture, containing the following constituents, was made:

1. 2-Methylbutane
2. *n*-Pentane
3. 2,2-Dimethylbutane
4. 2,3-Dimethylbutane
5. 2-Methylpentane
6. 3-Methylpentane
7. *n*-Hexane
8. 2,4-Dimethylpentane
9. 2,3-Dimethylpentane
10. Benzene
11. Iso-octane
12. *n*-Heptane
13. 2,3,4-Trimethylpentane
14. *n*-Octane

(The numbers correspond to those in the chromatograms, Figs. 9–13.)

Sample size: approximately 10 λ were used in each injection (Hamilton syringe).

(7) *Sample* C_3–C_4. Petroleum gas was used as the sample for C_3–C_4 hydrocarbons and contained 59.2 % of propane, 23.9 % of isobutane and 16.9 % of *n*-butane.

Sample size: gas cell ¼ c.c.

NOMENCLATURE

A_{i,F_c} – area of peak i at flow rate F_c
F_c – flow rate of the mobile phase (ml/min)
h – peak height
H – height equivalent to a theoretical plate (calculated from uncorrected retention time)

H^* – height equivalent to a theoretical plate (calculated from the net retention time)
K – partition coefficient
L – length of the column (cm)
R – resolution of peaks (calculated from uncorrected retention time)
R^* – resolution of peaks (calculated from net retention time)
s – effective cross section area of the column (cm^2)
t_N – net retention time
$t_M{}^0$ – corrected retention time of air peak
u – velocity of the mobile phase (cm/min)
V_R – retention volume (uncorrected) (ml)
$V_R{}^0$ – corrected retention volume (ml)
$V_M{}^0$ – corrected gas hold up (ml)
V_L – liquid phase volume (ml)
V_N – net retention volume (ml)
w – peak width

ACKNOWLEDGEMENTS

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SUMMARY

The results obtained on programmed flow gas chromatography of a mixture of hydrocarbons are presented. The following relationships between various parameters and flow rate are discussed:

(1) Peak migration and flow rate of the mobile phase.
(2) Effect of flow rate on peak width, height and area.
(3) Effect of flow rate on efficiency and resolution.

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DIE DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG STEREOISOMERER CYCLOHEXYLAMINE

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Bei Arbeiten, die sich mit der Trennung von einander sehr ähnlichen Substanzen befassen, ist das Vorhandensein einer geeigneten Analysenmethode, die die Erkennung auch kleiner Mengen an Verunreinigungen gestattet, von ausschlaggebender Wichtigkeit.

So ergab sich im Rahmen von Untersuchungen über die Konstellation von substituierten Cyclohexylaminen die Notwendigkeit, eine brauchbare analytische Methode auszuarbeiten, die auch kleine Mengen eines verunreinigenden stereoisomeren Amins neben der Hauptmenge des zugehörigen Stereoisomeren zu erfassen vermochte. Hierzu wurde zunächst die Gaschromatographie verwendet[1]. Diese Methode hat den ausserordentlichen Vorteil, zugleich qualitative und quantitative Ergebnisse zu liefern. Sie war aber nicht auf alle Amine anwendbar. So konnten bisher z.B. die Dekalylamine nicht getrennt werden. Als Ergänzung zu diesem Verfahren wurde jetzt eine dünnschichtchromatographische Methode ausgearbeitet. Sie ist besonders für die Trennung der Amine geeignet, die gaschromatographisch bisher nicht oder nur schlecht getrennt werden konnten. Aber auch die meisten der anderen untersuchten Amine lassen sich glatt trennen. Gegenüber der gaschromatographischen Methode hat die Analyse mit Hilfe der Dünnschichtchromatographie den Nachteil, keine oder doch nur ungefähre quantitative Werte zu liefern. Die Ausführung ist aber ungleich viel einfacher und weniger aufwendig, es kann in wesentlich höherer Verdünnung gearbeitet werden und der Zeitaufwand ist sehr viel kleiner. Aus diesen Gründen ist die dünnschichtchromatographische Methode besonders für die laufende Überwachung von Trennungen, Synthesen usw. geeignet. Sie hat in Verbindung mit Destillationen, Verteilungen und Säulenchromatogrammen Anwendung gefunden. Dabei wurde jede einzelne Fraktion untersucht. Das konnte im Fall der Verteilungen und der Säulenchromatographie durch direktes Auftragen der anfallenden Fraktionen geschehen, ein Konzentrieren erübrigte sich.

Neben der reinen Trennung kann man durch die Dünnschichtchromatographie der primären Alkylcyclohexylamine auch Hinweise auf ihre räumliche Struktur erhalten (vergl. Tabelle I).

Zum besseren Verständnis der Ergebnisse sollen hier noch einmal ganz kurz einige grundlegende Tatsachen über den räumlichen Bau von Cyclohexanderivaten dargestellt werden. Der Cyclohexanring liegt aus energetischen Gründen in der Regel in der Sesselform vor. An ihr kann man zwei verschiedene Stellungen der Wasserstoffatome unterscheiden. Die Äquatorialstellung (e) – die Valenzen stehen in der aus vier Ringatomen gebildeten Ebene – und die Axialstellung (a) – die Valenzen

stehen senkrecht zu dieser Ebene. Das gleiche gilt für etwaige Substituenten. Durch Umklappen kann die eine Sesselform in eine zweite übergehen. Sind für den unsubstituierten Cyclohexanring beide Sesselformen, also beide Konstellationen des Moleküls, gleich, so unterscheiden sie sich bei substituierten Cyclohexanen. Beim Umklappen gerät nämlich jeder Substituent, der sich zunächst in Äquatorialstellung befunden hatte, in Axialstellung und umgekehrt.

Würde man einen an verschiedenen C-Atomen disubstituierten Cyclohexanring flach in einer Ebene ausbreiten, so trüge das *cis*-Isomere beide Substituenten auf derselben Seite, das *trans*-Isomere je einen Substituenten über und unter der Ebene. Man spricht daher von *cis-trans* Isomeren. Diese Bezeichnung ist allgemein üblich. Für das Verhalten und die Reaktionsweise ist aber die Konstellation der Isomeren verantwortlich.

Bei 1,2- und 1,4-disubstituierten Cyclohexanen stehen in dem *trans*-Isomeren beide Substituenten äquatorial bzw. nach dem Umklappen axial, im *cis*-Isomeren steht der eine Substituent äquatorial, der andere axial, beim Umklappen vertauschen die Substituenten ihre Lage. Bei den 1,3-disubstituierten Cyclohexanen kehren sich die Verhältnisse um. Hier stehen die Substituenten am *cis*-Isomeren diäquatorial bzw. diaxial und am *trans*-Isomeren axial-äquatorial bzw. äquatorial-axial.

Es hat sich bei den primären Alkylcyclohexylaminen in jedem der untersuchten Fälle, soweit eine Trennung überhaupt möglich war, gezeigt, dass von zwei Isomeren das mit der am stärksten abgeschirmten Aminogruppe den grösseren R_F-Wert besitzt (Fig. 1, 2 und 3).

Für ein solches Verhalten bietet sich zunächst folgende Erklärung an. Die polare Aminogruppe mit dem einsamen Elektronenpaar wird bei den Adsorptionsvorgängen, die zur Trennung führen, eine ausschlaggebende Rolle spielen. Eine Abschirmung der Aminogruppe führt zu einer verminderten Haftfestigkeit des Moleküls am Adsorbens und damit zu einer höheren Wanderungsgeschwindigkeit, der R_F-Wert wird höher. Am ausgeprägtesten muss dieser Unterschied bei solchen Molekülen sein, die sich wirklich nur durch die Abschirmung der Aminogruppe unterscheiden. Hierher gehören z.B. die *tert.*-Butylcyclohexylamine und die *trans*-Dekalylamine. Beim *tert.*-Butylcyclohexylamin verhindert der raumerfüllende Substituent, beim *trans*-Dekalylamin der starre Bau des Moleküls ein Umklappen des Sechsringes.

Die jeweils zusammengehörigen Stereoisomeren können sich daher nur durch die Stellung der Aminogruppe unterscheiden. Da, wie schon lange bekannt, die Abschirmung eines axialen Substituenten am Sechsring durch die benachbarten 3-ständigen, ebenfalls axialen Wasserstoffe grösser ist, als beim äquatorialen Substituenten durch die benachbarten Wasserstoffe, müssten die Isomeren der oben genannten Cyclohexylamine mit axialer Aminogruppe den grösseren R_F-Wert besitzen. Das ist auch der Fall (siehe Tabelle I).

Schwieriger lässt sich das Verhalten solcher Cyclohexylamine voraussehen, die weder ein starres Ringsystem aufweisen noch durch einen sperrigen Substituenten am Umklappen in die andere Sesselform gehindert werden. Sie werden in einem Konstellationsgleichgewicht vorliegen. Als Beispiel sei das 4-Methylcyclohexylamin angeführt (Fig. 4).

Die *trans*-Form, die es beiden Substituenten gestattet, die äquatoriale Lage einzunehmen, muss aus energetischen Gründen fast ausschliesslich in dieser Form vorliegen. Für das *cis*-Isomere ist dagegen ein Konstellationsgleichgewicht zu erwarten,

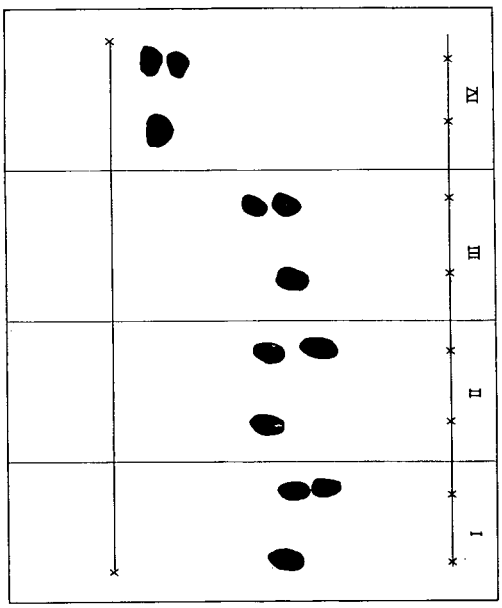

Fig. 1.

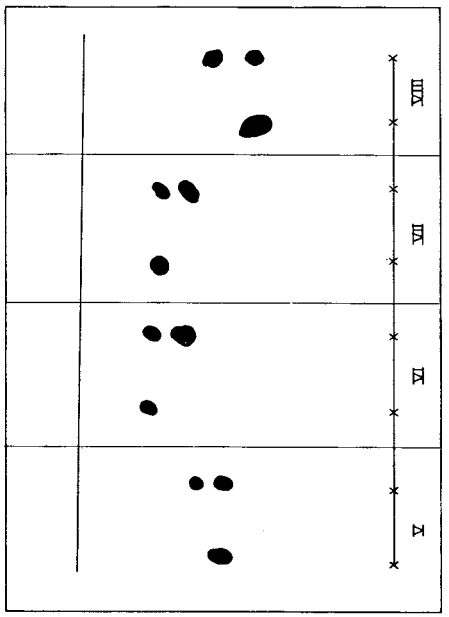

Fig. 2.

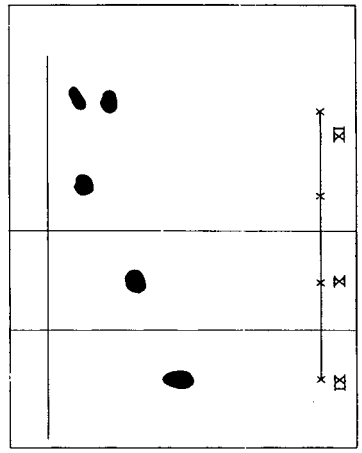

Fig. 3.

Fig. 1, 2 und 3. Dünnschichtchromatogramme einiger alkylsubstituierter Cycloalkylamine. I = 4-Methylcyclohexylamin; II = 4-*tert.*-Butylcyclohexylamin; III = 3-Isopropylcyclohexylamin; IV = Bornyl- und Isobornylamin; V = 3-*tert.*-Butylcyclohexylamin; VI = *trans*-α-Dekalylamin; VII = *cis*-α-Dekalylamin; VIII = *trans*-β-Dekalylamin; IX = *cis*-β-Dekalylamin; X = 2-Methylcyclohexylamin; XI = 2-Isopropylcyclopentylamin. Die Einzelflecke auf den Chromatogrammen stellen mit Ausnahme von IV, IX und X die jeweiligen *cis*-Isomeren dar. Der Einzelfleck bei IV ist Isobornylamin, IX und X sind ungetrennte Gemische beider Isomeren.

TABELLE I

KONSTELLATION UND DÜNNSCHICHTCHROMATOGRAPHISCHES VERHALTEN EINIGER ALKYLSUBSTITUIERTER CYCLOALKYLAMINE

Substanz	*Konstellation**	*Trennung***	*R_F-Wert höher (+) bzw. niedriger (—) als der des zugehörigen Isomeren*
trans-2-Methylcyclohexylamin	ee (aa)	—	
cis-2-Methylcyclohexylamin	ea		
cis-3-Methylcyclohexylamin	ee (aa)	+	—
trans-3-Methylcyclohexylamin	ea		+
trans-4-Methylcyclohexylamin	ee (aa)	+	—
cis-4-Methylcyclohexylamin	ea		+
trans-2-Isopropylcyclohexylamin	ee (aa)	—	
cis-2-Isopropylcyclohexylamin	ea		
cis-3-Isopropylcyclohexylamin	ee (aa)	+	—
trans-3-Isopropylcyclohexylamin	ea		+
cis-3-*tert.*-Butylcyclohexylamin	ee	++	—
trans-3-*tert.*-Butylcyclohexylamin	ea		+
trans-4-*tert.*-Butylcyclohexylamin	ee	++	—
cis-4-*tert.*-Butylcyclohexylamin	ea		+
trans-1-Amino-*trans*-dekalin	e ($-NH_2$)	++	—
cis-1-Amino-*trans*-dekalin	a ($-NH_2$)		+
trans-1-Amino-*cis*-dekalin	e ?($-NH_2$)	++	—
cis-1-Amino-*cis*-dekalin	e ?($-NH_2$)		+
cis-2-Amino-*trans*-dekalin	e ($-NH_2$)	++	—
trans-2-Amino-*trans*-dekalin	a ($-NH_2$)		+
cis-2-Amino-*cis*-dekalin	e ?($-NH_2$)	—	
trans-2-Amino-*cis*-dekalin	e ?($-NH_2$)		
trans-2-Isopropylcyclopentylamin		++	—
cis-2-Isopropylcyclopentylamin			+
Bornylamin		++	—
Isobornylamin			+

* e = äquatorial; a = axial. Steht ($-NH_2$) hinter der Konstellationsangabe, so bezieht sich diese ausschliesslich auf die Konstellation der Aminogruppe.

** Die Trennungen werden nach ihrer Güte mit +, ++ usw. gekennzeichnet. Ein — bezeichnet ein Gemisch, das sich nicht trennen lässt.

in dem jeweils der eine oder andere Substituent axial bzw. äquatorial steht. Betrachtet man von den beiden Konstellationen diejenige mit äquatorialer Aminogruppe, so unterscheidet sie sich von dem *trans*-Isomeren durch die Axialstellung der Methylgruppe. Die andere mögliche Konstellation des *cis*-Isomeren unterscheidet sich von dem *trans*-Isomeren durch die axiale Aminogruppe, also in der gleichen Weise, wie die oben genannten starren Amine. Da einmal die Lage des Gleichgewichtes nicht genau bekannt und für verschieden substituierte aber bewegliche Cyclohexylamine verschieden ist, zudem auch das Verhalten der Konstellation des *cis*-Isomeren des 4-Methyl-

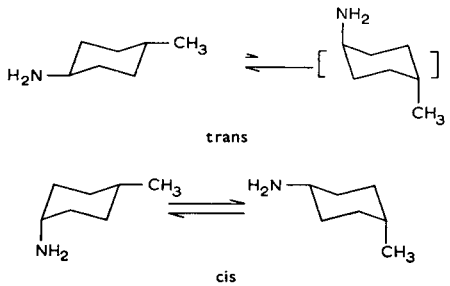

Fig. 4. Die den Sesselformen des Cyclohexans entsprechenden Konstellationen der 4-Methylcyclohexylamine.

cyclohexylamins mit axialer Methylgruppe nicht vorauszusehen ist, so wird der Rückschluss von dünnschichtchromatographischem Verhalten auf die Struktur bei den zum Umklappen befähigten Cyclohexylaminen unsicherer. Das bisher vorliegende Versuchsmaterial zeigt aber, dass, sofern die Trennung überhaupt gelingt, stets das Isomere den höheren R_F-Wert besitzt, das auch den höheren Anteil an axialständiger Aminogruppe im Konstellationsgleichgewicht aufweist. Der Einfluss des axialen Anteiles der Aminogruppe scheint also die anderen sterischen Einflüsse, die durch die Alkylsubstituenten bewirkt werden, zu überwiegen. Man kann daher das dünnschichtchromatographische Verhalten von substituierten primären Cyclohexylaminen als sehr einfache und brauchbare Methode benutzen, um erste Anhaltspunkte für die zunächst unbekannte Konfiguration der Amine zu erhalten, wenn man sich auch darüber klar sein muss, dass es sich zunächst nur um eine Arbeitshypothese handelt, die der Bestätigung durch ein sehr viel umfangreicheres Versuchsmaterial bedarf. Wie berechtigt solche Einwände sein können, zeigt sich bereits bei den N,N-Dimethylierungsprodukten der oben besprochenen primären Amine (Tabelle II).

Die bei den primären Aminen zunächst gültigen Überlegungen versagen bei ihren

TABELLE II

DAS DÜNNSCHICHTCHROMATOGRAPHISCHE VERHALTEN EINIGER N,N-DIMETHYLIERTER ALKYLSUBSTITUIERTER CYCLOALKYLAMINE

Substanz	*Trennung**	*R_F-Wert höher (+) bzw. niedriger (−) als der des zugehörigen Isomeren*
N,N-Dimethyl-*trans*-2-methylcyclohexylamin	+++	+
N,N-Dimethyl-*cis*-2-methylcyclohexylamin		−
N,N-Dimethyl-*cis*-3-methylcyclohexylamin	+++	−
N,N-Dimethyl-*trans*-3-methylcyclohexylamin		±
N,N-Dimethyl-*trans*-4-methylcyclohexylamin	+++	+
N,N-Dimethyl-*cis*-4-methylcyclohexylamin		−
N,N-Dimethyl-*trans*-4-*tert.*-butylcyclohexylamin	++++	−
N,N-Dimethyl-*cis*-4-*tert.*-butylcyclohexylamin		+
N,N-Dimethyl-*trans*-2-methylcyclopentylamin	++	−
N,N-Dimethyl-*cis*-2-methylcyclopentylamin		+

* Siehe Fussnote ** der Tabelle I.

N,N-Dimethylierungsprodukten völlig. Bei diesen sollte die Abschirmung des einsamen Elektronenpaares am Stickstoff wesentlich grösser werden und damit die R_F-Werte steigen und die Trennung sich verschlechtern. Die Reihenfolge der R_F-Werte sollte die gleiche bleiben, da sich an den Konstellationen nichts Grundsätzliches ändert. Nichts von alledem tritt ein (siehe Fig. 5). Die R_F-Werte nehmen keineswegs

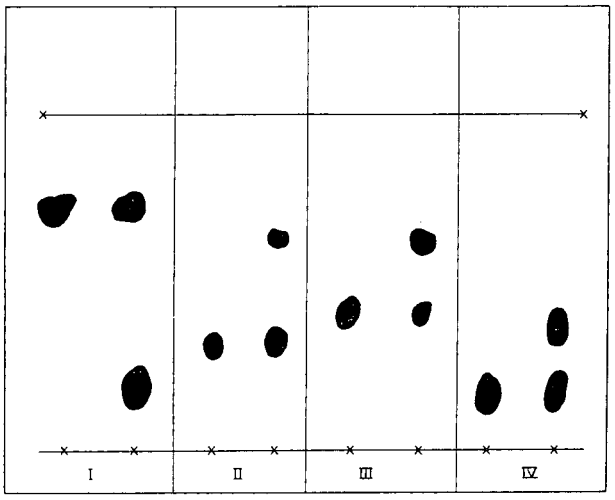

Fig. 5. Dünnschichtchromatogramme einiger N,N-dimethylierter alkylsubstituierter Cyclohexylamine. I = N,N-Dimethyl-4-*tert.*-butylcyclohexylamin; II = N,N-Dimethyl-4-methylcyclohexylamin; III = N,N-Dimethyl-2-methylcyclohexylamin; IV = N,N-Dimethyl-3-methylcyclohexylamin. Die Einzelflecken stellen jeweils die *cis*-Isomeren dar.

zu, die Trennungen werden durchweg besser, die Reihenfolgen kehren sich teilweise um. Daraus wird ersichtlich, wie komplex diese Adsorptionsvorgänge sind und dass im Grunde wenig gesagt ist, wenn man Trennungen mit einem Phänomen erklären will, das sicherlich vorhanden ist, in seiner Bedeutung für den Gesamtvorgang aber nicht abgeschätzt werden kann.

Innerhalb eng begrenzter Stoffklassen können bei der Chromatographie gewisse Zusammenhänge festgestellt werden und innerhalb dieser auch angewendet werden, bei einer Verallgemeinerung ist aber äusserste Vorsicht geboten.

1,2-Disubstituierte Cyclohexylamine weisen im Gegensatz zu ihren 3- oder 4-substituierten Stellungsisomeren einen etwas höheren R_F-Wert auf. Das könnte durch eine sterische Hinderung der Aminogruppe durch den benachbarten Alkylrest gedeutet werden. Eine Trennung der einzelnen *cis-trans* Isomeren war in diesem Fall bei der verwendeten Methode nicht möglich (vergl. Fig. 3 und Tabelle I). Das gelang jedoch im Fall des 2-substituierten Isopropylcyclopentylamines. Hier macht sich wohl die annähernd ebene Form des Cyclopentanringes bemerkbar. Dadurch wird die Abschirmung der Aminogruppe im *cis*-Isomeren sehr viel grösser sein als im *trans*-Isomeren.

Die Nachweisgrenze eines Isomeren, das in kleinen Mengen neben dem hauptsächlich vertretenen zugehörigen anderen Isomeren vorhanden ist, liegt für die relativ schwierig zu trennenden 3- bzw. 4-Methylcyclohexylamine bei 1 % (Fig. 6).

Diese Empfindlichkeit wird auch mit der Gaschromatographie nicht erreicht, da diese Amine nicht ganz getrennt werden[1]; geringe Mengen des einen Isomeren verschwinden damit in der Hauptbande des anderen.

Die Nachweisgrenze für Amine, die sich besser trennen lassen, z.B. die 4-*tert.*-Butylcyclohexylamine oder sogar die N,N-Dimethylamine liegen noch erheblich darunter.

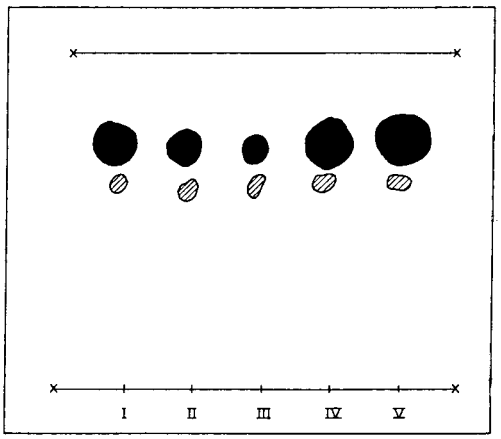

Fig. 6. Der Nachweis von kleinen Mengen *trans*-4-Methylcyclohexylamin neben viel *cis*-4-Methylcyclohexylamin. Die Platte wurde zur Erzielung einer besseren Trennung zweimal entwickelt. I = 2.5% *trans*; II = 5% *trans*; III = 10% *trans*; IV = 2% *trans*; V = 1.3% *trans*.

EXPERIMENTELLER TEIL

Die Darstellung der Amine erfolgte nach den üblichen Methoden. Meist wurden als Ausgangsstoffe die entsprechenden Phenole verwendet. Diese wurden katalytisch hydriert, die entstehenden Alkohole zu den Ketonen oxydiert und über die Reduktion ihrer Oxime oder durch katalytische Hydrierung in Gegenwart von NH_3 in die Amine überführt. Die Trennung der Isomeren gelang durch fraktionierte Kristallisation ihrer Salze (siehe z.B. [2]), durch Gegenstromverteilung[3] oder durch Destillation[4]. Die Konfigurationszuordnung einiger noch nicht bekannter stereoisomerer Cyclohexylamine wurde mit Hilfe der Kernresonanz, der I.R.-Spektroskopie, ihres Verhaltens bei Verteilung und Chromatographie und durch die Bestimmung von Dichte und Brechungsindex getroffen[4].

Die dünnschichtchromatographische Technik

Zur Ausführung der Reihenuntersuchungen wurden die allgemein üblichen 20 × 20 cm grossen Glasplatten, die mit dem Desaga-Streichgerät nach Stahl beschichtet wurden, verwendet.

Die Dünnschichtchromatographie am Glasstab[5] fand bei der Ausarbeitung des Trennverfahrens, insbesondere bei der Suche nach dem geeignetsten Fliessmittel und bei allen Einzelanalysen Anwendung. Die Besonderheit der Dünnschichtchromatographie am Glasstab liegt darin, dass als Träger der Schicht Glasstäbe dienen, die in einem durchbohrten Stopfen stecken. Mit Hilfe dieser Stopfen werden sie auf Reagenzgläser gesetzt, die einige ml des Fliessmittels enthalten und als Trennkammern dienen.

Man kann auf jeden Stab eine Substanzprobe und ein Vergleichsgemisch auftragen und zusammen chromatographieren.

Das Beschichten der Stäbe geschieht durch Eintauchen in eine Anschüttelung des Sorptionsmittels, die sich in einem besonders geformten Standzylinder befindet. Zum Trocknen werden die Stäbe in ein kleines Gestell gehängt.

Der Vorteil dieser Methode liegt in der Ersparnis von Material, Geräten, Fliessmittel und Arbeitsplatz. Sie ist vor allem für die Ausführung von Einzelanalysen gedacht und für die Entwicklung neuer Trennverfahren, bei denen viele verschiedene Lösungsmittelsysteme durchprobiert werden müssen.

Es wurde aufsteigend eindimensional chromatographiert. Bei den in der Tabelle I mit + bezeichneten Trennungen konstellativ ähnlicher Verbindungen wurde zuweilen zur Erzielung besserer Ergebnisse dasselbe Chromatogramm zweimal entwickelt. Hierzu wird Stab oder Platte, wenn das Fliessmittel zum ersten Mal die markierte Höhe erreicht hat, aus dem Fliessmittel genommen, kurz an der Luft getrocknet und sofort noch einmal chromatographiert. Man erreicht damit praktisch eine Vergrösserung der Laufstrecke ohne einen längeren Stab verwenden zu müssen. Ausserdem ist der Zeitaufwand geringer und die Flecken bleiben schärfer.

Schicht

Als Sorptionsmittel wurde Kieselgel G "Merck" verwendet. Für die Dünnschichtchromatographie am Glasstab muss die übliche Zusammensetzung von Kieselgel–Wasser = 1:2 etwas geändert werden, um gute Schichten zu erhalten. Da die Bindefähigkeit des in den einzelnen Kieselgel-Chargen enthaltenen Gipses schwankt, müssen von Lieferung zu Lieferung oft etwas andere Zusammensetzungen gewählt werden. Bisher lagen die Werte zwischen 10 und 12 g Kieselgel auf 22 ml Wasser. Diese Menge reicht dann für die Beschichtung von ungefähr 20 Stäben. Es empfiehlt sich, bei jeder neuen Charge in einem Vorversuch die Mischungsverhältnisse zur Erzielung einer guten Schicht auszuprobieren. Ist die erhaltene Schicht zu dick, wird der Wasseranteil um je 1 ml erhöht, fällt die Schicht zu dünn aus, erhöht man den Kieselgelanteil um jeweils 0.5 g. Die auf diese Weise ermittelte optimale Zusammensetzung wird auf der Kieselgelflasche vermerkt. Die Schicht wird am besten in einem 50 ml Erlenmeyer-Kölbchen angeschüttelt, wobei man die benötigte Menge Kieselgel in das Wasser gibt.

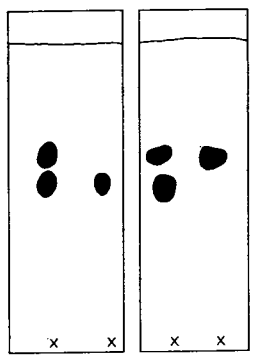

Fig. 7. Am Glasstab gewonnene Dünnschichtchromatogramme. Die Stäbe wurden zur Erzielung einer besseren Trennung zweimal entwickelt. Links: 3-Methylcyclohexylamine. Rechts: 4-Methylcyclohexylamine. Die Einzelflecken stellen jeweils das *cis*-Isomere dar.

Die Steighöhe der Fliessmittelfront betrug auf der Platte 10 cm, auf dem Stab 8 cm.

Zur Dokumentation der Plattenchromatogramme wurde ein Durchschlagpapier auf die Platte gelegt und die Startpunkte, Substanzflecke und Lösungsmittelfront durchgezeichnet.

Vom Stab wurden die Schichten nach Umzeichnung der Flecken mit Hilfe einer Klebefolie in der früher beschriebenen Weise[5] abgehoben und auf ein Blatt Papier geklebt (Fig. 7).

Fliessmittel

Unter den zahlreichen Fliessmitteln, die auf ihre Brauchbarkeit hin untersucht wurden, erwies sich das folgende als am besten geeignet: 17 Teile einer Mischung aus 2 Teilen konzentriertem Ammoniak und 98 Teilen Aceton werden mit 8 Teilen Petroläther 50–70° gemischt. Die Mischungsverhältnisse der 2 % Ammoniaklösung in Aceton mit dem Petroläther können geändert werden. So wurden zur Erniedrigung von zu hohen R_F-Werten diese beiden Bestandteile zu gleichen Teilen gemischt und mit gutem Erfolg verwendet.

Das Fliessmittel ist, wenn wirklich gute Ergebnisse erzielt werden sollen, jeweils nur einmal zu verwenden, muss also bei jedem neuen Chromatogramm ersetzt werden. Das fertig gemischte Fliessmittel ist in einer gut verschlossenen Flasche aufzubewahren und täglich neu herzustellen.

Sprühmittel

1. Eine Mischung aus gleichen Teilen 0.1 *N* Jodlösung und ungefähr 10 % Schwefelsäure.

2. Dragendorff-Reagenz modifiziert nach MUNIER[6]. Stammlösung: 1.7 g basisches Wismutnitrat und 20 g Weinsäure werden in 80 ml Wasser gelöst. 16 g Kaliumjodid werden in 40 ml Wasser gelöst. Beide Lösungen werden gemischt.

Das Sprühreagenz wird aus 5 ml Stammlösung und aus einer Lösung von 10 g Weinsäure in 50 ml Wasser gemischt.

Die Amine wurden in 0.1–0.01 *M* Chloroformlösung aufgetragen.

ZUSAMMENFASSUNG

Es wird eine dünnschichtchromatographische Methode zur Trennung von stereoisomeren alkylsubstituierten Cycloalkylaminen beschrieben. Es besteht ein für Konfigurationsbestimmungen verwertbarer Zusammenhang zwischen dem räumlichen Bau und dem dünnschichtchromatographischen Verhalten der primären Cycloalkylamine. Die Methode gestattet die Erkennung von ungefähr 1 % eines Isomeren im Gemisch mit dem zugehörigen anderen Isomeren.

SUMMARY

A method is described for the separation of stereoisomeric alkyl derivatives of cycloalkylamines by thin-layer chromatography. In the case of primary cycloalkylamines there is a relation between the spatial configuration and the behaviour on thin-layer chromatography, which can be useful for the determination of the configuration. With

this method about 1 % of an isomer can be detected in mixtures with the corresponding isomer.

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STUDIES ON THE CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON DIETHYLAMINOETHYL(DEAE)-CELLULOSE

I. THE EFFECT OF THE CHEMICAL AND PHYSICAL NATURE OF THE EXCHANGER

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INTRODUCTION

Although ion-exchange chromatography on substituted cellulose derivatives[1], particularly diethylaminoethyl-cellulose, has found wide application in the fractionation of serum proteins[2,3], surprisingly few detailed investigations have been undertaken to establish the factors influencing the separation of proteins accomplished by this procedure. Recently the need for such investigations have been emphasized (PORTER[4] and PETERSON AND CHIAZZE[5]).

The work to be described here was undertaken with the object of improving the effectiveness of the DEAE-cellulose chromatographic procedure as a means of fractionating serum proteins. In addition to column chromatography, a rapid batch procedure (STANWORTH[6]) has been used to study a number of the factors influencing the ion-exchange process.

Results of studies of the effect of the degree of substitution and physical form of the exchangers, and also the temperature of elution, are reported in this paper (Part 1). The standard procedure thus evolved has been employed in further studies (reported in Part 2), involving the detailed investigation of the chromatographic properties of individual purified serum proteins and artificial mixtures of these.

METHODS

Preparation of samples

Three different samples of human serum were used. Sample 1 (serum separated from a normal human donor) was used in all the column experiments with laboratory prepared exchanger.

Sample 2, used in the column chromatographic experiments on the commercial exchangers, was prepared by the defibrination of pooled acid citrate dextrose (ACD) plasma which had been stored at 4°.

Sample 3 comprised serum separated from a single donation of blood from a rheumatoid arthritic patient and was used in all the batch chromatographic experiments, providing, in addition, data on the chromatographic behaviour of rheumatoid factor.

Prior to chromatography, all the samples were dialysed against 200 volumes of the starting buffer (pH 7.6, 0.01 *M* phosphate) for 20–24 h. The samples were then centrifuged at 3,000 r.p.m. for 10 min, before application to the chromatographic columns.

Adsorbents

The DEAE-cellulose used was obtained from two sources. One sample ("W") was prepared in the laboratory from wood cellulose, according to the method of PETERSON AND SOBER[1]. This exchanger was powder-like in form and had a degree of substitution of 0.87 mequiv./g, as revealed by titration (with *N*/100 hydrochloric acid) of a suspension of the exchanger in 0.15 *M* saline. The other samples were obtained from commercial sources. These exchangers were floc-like in form, having been prepared from fibrous cotton linters. Their exchange capacities ranged between 0.05 and 1.2 mequiv./g. In this series of experiments the exchangers are referred to as C 5, C 25, C 65 and C 120, where the letter "C" indicates their cotton source and the number their degree of substitution in mequiv./g × 100.

CHROMATOGRAPHIC PROCEDURE

The exchanger "W" (2 g) was washed successively with two 50 ml volumes of 0.05 *M* NaH_2PO_4 solution and phosphate buffer (pH 7.6, 0.01 *M*) before being suspended in 30 ml of the latter buffer and poured into a column (diameter 1 cm). The column was then washed overnight with at least 100 ml of phosphate buffer (pH 7.6, 0.01 *M*), the final length of the column being about 16 cm.

Due to the floc-like nature of the "C" type exchangers, even packing of the columns by means of pouring a slurry of the exchanger into the column and applying positive pressure proved difficult. In addition, the low capacity of these exchangers necessitated the use of larger weights of material (see Discussion). The dry exchanger (3 g) was, therefore, packed a little at a time into the column (diameter 1 cm). After packing, the column was equilibrated by washing with 300–500 ml of the pH 7.6, 0.01 *M* phosphate buffer, giving a column of length between 13 and 15 cm.

The batch chromatographic experiments to be described were performed using 0.5 g quantities of exchanger "W", and 1.0 g samples of the "C" type exchangers. These samples were washed initially with 25 ml volumes of 0.05 *M* NaH_2PO_4 solution and finally equilibrated with phosphate buffer (pH 7.6, 0.01 *M*).

Throughout all the investigations, fresh exchanger was used in each experiment. On no occasion was regenerated material used. In addition all equilibrations were accomplished at room temperature, whilst chromatography was performed at 4° (except where otherwise stated).

ELUTION PROCEDURE

Column experiments

The dialyzed protein samples (volume 5 ml) were carefully applied to the top of the column by means of a Pasteur pipette and then allowed to run in under gravity. Elution was performed by a stepwise procedure using the following series of solvents:

1. Phosphate buffer: pH 7.6, 0.01 *M*.
2. Phosphate buffer: pH 6.3, 0.02 *M*.
3. 0.05 *M* NaH_2PO_4 solution.

The flow-rates for the columns prepared from the "W" and "C" type exchangers were 3-5 ml and 60 ml per hour, respectively.

The use of an additional solvent, 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl, was found to result in the elution of only a small amount of protein of very heterogeneous composition and so was not used in these column chromatographic studies. The effluent was collected in 3 ml fractions using an automatic fraction collector, incorporating a weight-balance syphon.

Examination of the effluent

The protein distribution in the effluent was determined by measuring the $E^{1\,cm}_{280\,m\mu}$ value in silica cells, in a Unicam SP 500 spectrophotometer. Effluent pH was determined by means of a micro electrode and direct reading pH meter, whilst the electrical conductivity was measured with a Mullard conductivity bridge incorporating a cathode ray (magic eye) indicator.

A standard system of pooling effluent fractions was adopted which was based upon the distribution of the main protein peaks in the chromatographic pattern of the whole serum (see Fig. 2).

Batch experiments

After equilibration, as described above for the column procedure, the exchanger ("W") was filtered through a sintered glass disc and the resultant damp exchanger was then intimately mixed with the equilibrated serum sample 3 (volume 1 ml). Following equilibration for a further 30 min, 20 ml of phosphate buffer (pH 7.6, 0.01 *M*) were added and the contents stirred to give a suspension. The sample was then allowed to stand for 30 min at 4° with frequent stirring. Exchanger and supernatant were then separated either by centrifugation, as in the case of the exchanger "W" or by filtration (see above), as in the case of the "C" type derivatives. This process was repeated with 20 ml volumes of the solvents described previously for the column procedure, but with the addition of a fourth solvent, namely 0.05 *M* NaH_2PO_4 solution containing 0.25 *M* NaCl.

In experiments investigating the effect of temperature, equilibration was performed in a thermostatically controlled centrifuge (for temperatures 0°, 10° and 22°) or in a thermostatically controlled water bath (for 37° and 50°). The equilibrated exchanger and solvent were then separated by centrifugation with minimum temperature change.

Concentration of fractions

Concentration of samples was performed at 4° by ultra-filtration through visking tubing ($^8/_{32}$ in. in diameter) using a negative pressure of 50–60 cm of Hg. After ultrafiltration adherent protein was carefully massaged from the sides of the sac and the concentrated fractions stored at —20° in polythene containers.

Analysis of fractions

Protein determinations were carried out on the concentrated fractions by the LOWRY modification[7] of the FOLIN phenol procedure, and their compositions were determined by the immunoelectrophoretic technique using a rabbit antiserum raised against human serum.

RESULTS

Column separations

Four column chromatographic fractionations of defibrinated plasma (serum sample 2) were performed on each of the "C" type exchangers. An analysis of variance indicated that there was no significant difference between the total product recovered from these exchangers at varying degrees of substitution (ranging from 0.05–1.20 mequiv./g).

For comparison, results obtained from the analysis of normal human serum (sample 1) on exchanger "W" are also included in Table I. A more direct comparison

TABLE I

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY IN THE COLUMN CHROMATOGRAPHIC PROCEDURE

The values are given as means of four sets of results together with their standard deviations ($6\bar{x}$)

Sample	*Exchanger*	*Degree of substitution (mequiv./g)*	*Amount of protein applied (mg)*	*Protein recovery (percentage of total applied)*			
				Solvent 1	*Solvent 2*	*Solvent 3*	*Total*
				Phosphate buffer			
				pH 7.6, 0.01 M	*pH 6.3, 0.02 M*	*0.05 M NaH_2PO_4*	
Defibrinated	C5	0.05	240.6 ± 17.6	80.4 ± 6.4	3.0 ± 0.6	9.0 ± 2.1	92.8 ± 4.4
Human	C25	0.25	210.0 ± 15.4	56.7 ± 5.5	2.8 ± 1.1	11.1 ± 2.3	70.5 ± 6.5
A.C.D.	C65	0.65	265 ± 15.8	39.5 ± 4.7	9.7 ± 0.9	32.5 ± 3.8	81.6 ± 7.6
Plasma	C120	1.2	227.6 ± 6.8	13.6 ± 3.9	7.6 ± 1.5	48.6 ± 7.5	68.9 ± 2.0
Normal human serum	Laboratory prepared W	0.87	365.0	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8

of the properties of the two types of exchangers was made, however, by employing the batch procedure (see later). In general, the total recoveries of protein from the "C" type exchanger were not significantly different from those observed with exchanger "W", although significantly greater recoveries were obtained with exchanger C5 (*i.e.* 0.05 mequiv./g).

The elution patterns varied markedly with the chemical nature of the exchanger (see Fig. 1). For instance, the amount of protein eluted with the initial solvent (phosphate buffer; pH 7.6, 0.01 *M*) was inversely related to the degree of substitution of the exchanger. Conversely, there was a direct relationship between the amount of protein recovered with the final solvent (0.05 *M* NaH_2PO_4 solution) and the degree of substitution of the exchanger.

As shown in Fig. 2 a characteristic serum elution pattern was obtained using exchanger "W", pure 7S-γ-globulin being eluted in the initial fractions. On the other hand, comparative immunoelectrophoretic analyses revealed the breakthrough of other proteins in the corresponding fractions from the "C" type exchangers. For instance, proteins such as the β- and α-globulins and the albumin, which are normally firmly bound to the exchanger at pH 7.6 (0.01 *M* phosphate), were eluted along with the 7S-γ-globulin. Consequently, the amount of protein eluted with further solvents was much reduced. Nevertheless, the protein fractions eluted with solvent 3 from these exchangers showed the heterogeneity characteristic of this part of the chromatogram (compare with Fig. 2), indicating that even ex-

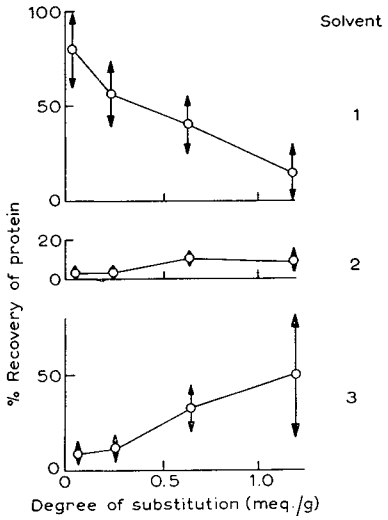


Fig. 1. The effect of the degree of substitution of "C" type exchangers on protein recoveries. The chromatographic technique employed is described in the text, whilst the amounts of protein (serum sample 2) fractionated are recorded in Table I. Each point on the graph represents the mean of four observations. The deviations illustrated by the arrows are derived by multiplying the standard deviations of the mean by the value of t at the 5 % significance (0.05 probability) level for the respective number of degrees of freedom.

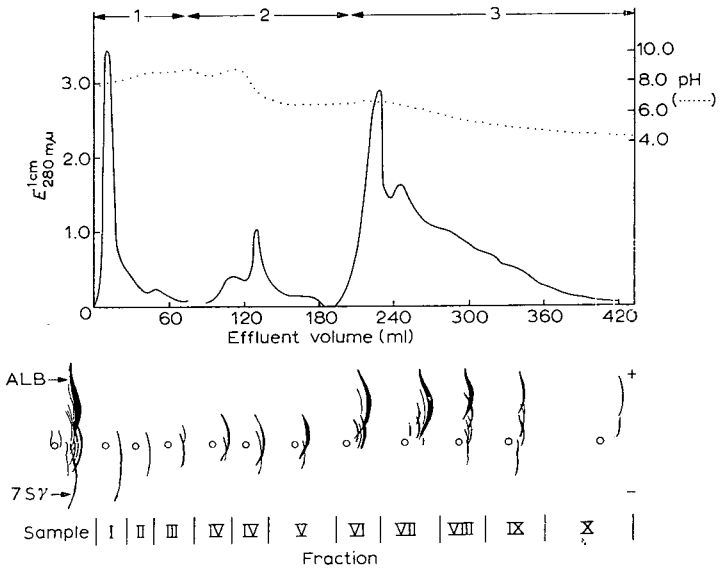


Fig. 2. A typical human serum chromatographic pattern based on the results of four different column separations of 5 ml of serum, sample 1, on type "W" exchanger. The dotted line indicates the pH of the effluent whilst the heavy line represents the protein concentration as measured by absorbancy at 280 mμ. Tracings of immunoelectrophoretic patterns obtained by testing the fractions with rabbit anti-whole human serum are also included. For chromatographic conditions see text.

TABLE II

THE EFFECT OF THE DEGREE OF SUBSTITUTION OF DEAE-CELLULOSE EXCHANGERS ON PROTEIN RECOVERY BY THE BATCH PROCEDURE

The results given are those obtained in experiments using 1 ml samples of serum 3, 1 g amounts of the "C" type exchangers and 0.5 g amounts of exchanger "W"

Exchanger	*Degree of substitution (mequiv./g)*	*Protein recovery (percentage of total applied)*				
		Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Total*
		Phosphate buffer		*0.05 M NaH_2PO_4*	*0.05 M NaH_2PO_4 containing 0.25 M NaCl*	
		pH 7.6, 0.01 M	*pH 6.3, 0.02 M*			
C5	0.05	57.5	13.2	9.9	15.8	96.4
C25	0.25	39.3	12.9	11.9	22.5	86.6
C65	0.65	22.0	8.8	15.0	36.2	82.0
C120	1.2	28.3	6.3	13.5	46.9	95.0
W	0.87	13.0	6.0	9.3	54.5	82.8

changers of a very low degree of substitution (*i.e.* 0.05 mequiv./g) have a retentive capacity for a certain number of serum proteins.

Batch separations

In all the batch experiments performed (see Table II), there were no significant differences in the recovery of total serum protein (sample 3) from the various exchangers ("C" and "W").

These recoveries were greater than those achieved by the corresponding column procedures described above. However, the relative amounts of protein eluted with the various solvents were similar to those observed with the column technique (see Fig. 3).

As in the case of the column separations, breakthrough of proteins other than 7 S-γ-globulin occurred only in the fractions eluted from "C" type exchangers with solvent 1.

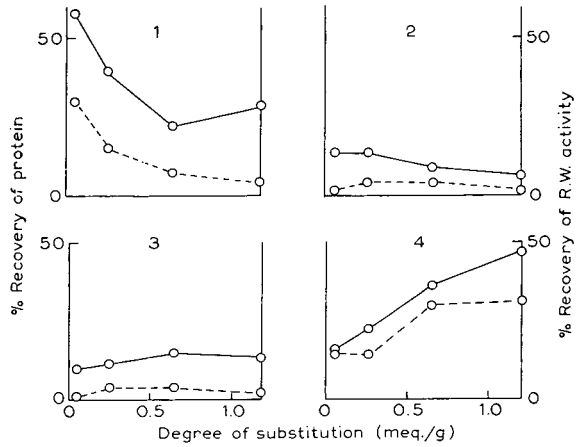


Fig. 3. Effect of the degree of substitution of "C" type exchangers on mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –), during batch experiments using serum sample 3. For chromatographic conditions see text.

As the degree of substitution of the exchangers increased, the α_2-macroglobulin and albumin content in the initial effluent decreased. The major components of fractions eluted from all the different exchangers by the final solvent, however, were the α-globulins (haptoglobin and ceruloplasmin), 19S-γ-globulin and albumin, suggesting that at least these proteins were retained.

By employing rheumatoid serum (sample 3), it was also possible to study the chromatographic behaviour of a human 19S-γ-globulin, the "rheumatoid factor". This protein was detected in the effluent by means of its capacity to agglutinate sensitised sheep erythrocytes, (*i.e.* by the ROSE-WAALER technique, as modified by BALL[8]). It was hoped that the strong affinity shown by this protein for DEAE-cellulose exchangers of about 1 mequiv./g substitution, a factor which complicates its isolation on these derivatives, would be overcome by employing less highly substituted materials. In this way, it might have been possible to first selectively adsorb the rheumatoid factor and subsequently to recover it readily. Unfortunately, however, the widespread distribution of serological activity amongst the various chromatographic fractions (see Fig. 3) indicated no preferential adsorption on "C" type exchangers of relatively low degrees of substitution, thus limiting their use in the isolation of rheumatoid factor.

The effect of temperature on the chromatographic separation of human serum proteins on DEAE-cellulose was also investigated. Changes of temperature between 0–50° had a negligible effect on the specific adsorption capacity of the laboratory prepared exchangers for human serum proteins (see Fig. 4).

The amounts of protein remaining adsorbed at pH 7.6 (0.01 *M* phosphate), at

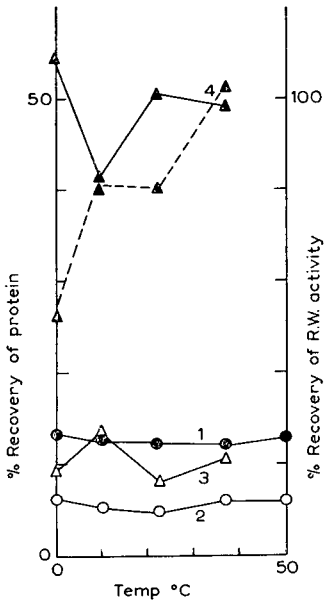


Fig. 4. Effect of temperature on the mean protein recovery (———) and recovery of ROSE-WAALER activity (– – –) during the batch chromatography of serum sample 3 on exchanger "W". Recoveries of activity with solvents 1, 2 and 3 were negligible and so have been omitted from the diagram. For chromatographic conditions see text.

the various temperatures tested, were almost identical and the effluent compositions were very similar. The quantitative and qualitative composition of the protein eluted with the other solvents were comparable, although there was some variation in the recovery of rheumatoid factor; this could, however, be partially attributed to the limitations of a doubling dilution technique of estimation of rheumatoid factor activity.

From the mean protein recoveries obtained in the above experiments, the specific adsorption capacities of the various exchangers in both the column and batch procedures have been determined and these are plotted in Fig. 5. In these experiments the "specific adsorption capacity" is defined as the total amount (mg) of serum protein adsorbed per 100 mg of exchanger, after both have been equilibrated with phosphate buffer (pH 7.6, 0.01 *M*). This permits a comparison of values obtained in these studies with the results reported by PETERSON AND SOBER[1]. As will be seen, the amount of protein adsorbed was related to the degree of substitution of the exchanger. The adsorption curves reached a plateau, above which the substitution of further reactive groupings had little effect on protein adsorption.

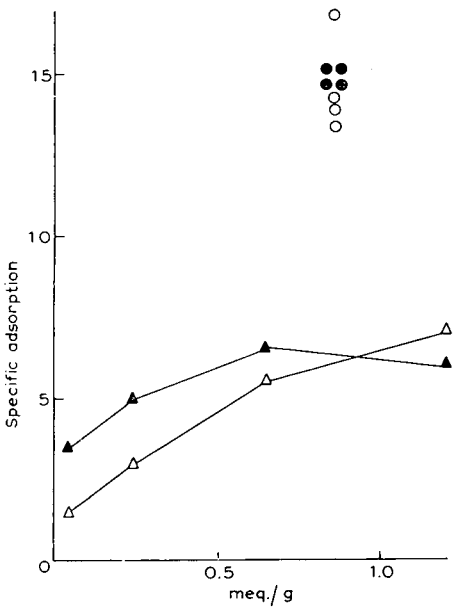


Fig. 5. A comparison of the specific adsorption capacities of "C", (–△–△–, –▲–▲–) and "W" (○, ●) type exchangers for total human serum proteins, by both column (–△–△–, ○) and batch (–▲–▲–, ●) techniques. The batch experiments on "W" type exchangers were performed between the temperatures of 0 and 50°.

DISCUSSION

Although the present study has not led to the ready development of an improved procedure for the chromatographic fractionation of human serum proteins on DEAE-cellulose, the data presented offer an indication of how various factors influence the ion-exchange process.

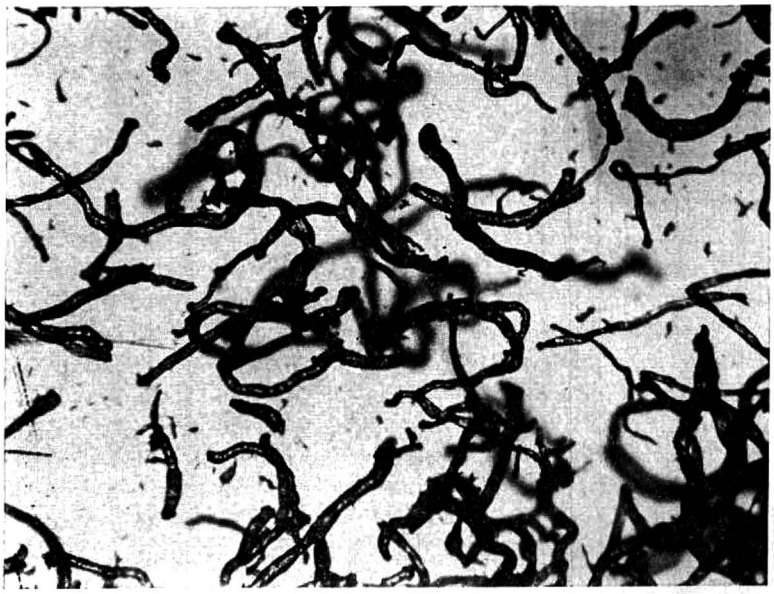
By employing exchangers of varying degrees of substitution it was hoped to

achieve the selective adsorption of certain proteins. Furthermore, by using exchangers of low degrees of substitution it should have been possible theoretically to overcome the irreversible binding which seemed to accompany the chromatography of some of the serum proteins, notably the 19S-γ-globulins. It was found, however, that there was no selective retention of the 19S-γ-globulin (rheumatoid factor) molecule on exchangers of low degrees of substitution (see Fig. 3). On the other hand, smaller molecules such as ceruloplasmin and haptoglobin were preferentially adsorbed under similar conditions in both the column and batch procedures. Hence it would appear that some proteins compete for the available sites on the surface of the exchanger, molecules with a relatively high affinity for the exchanger displacing those with a smaller affinity. It is possible that this property could be applied to the partial purification of specific serum proteins. For example α_2-macroglobulin can be separated from the bulk of the other serum proteins (including 19S-γ-globulin and the other α-globulins) by the batch chromatography of serum on ion exchangers of 0.25 mequiv./g substitution or less, which had been equilibrated with phosphate buffer (pH 7.6, 0.01 M). Theoretically, it should then be possible to adsorb selectively the α_2-macroglobulin on an exchanger of high substitution (0.7–1.0 mequiv./g). In practice, however, it was found that recoveries by such a two-stage process were small.

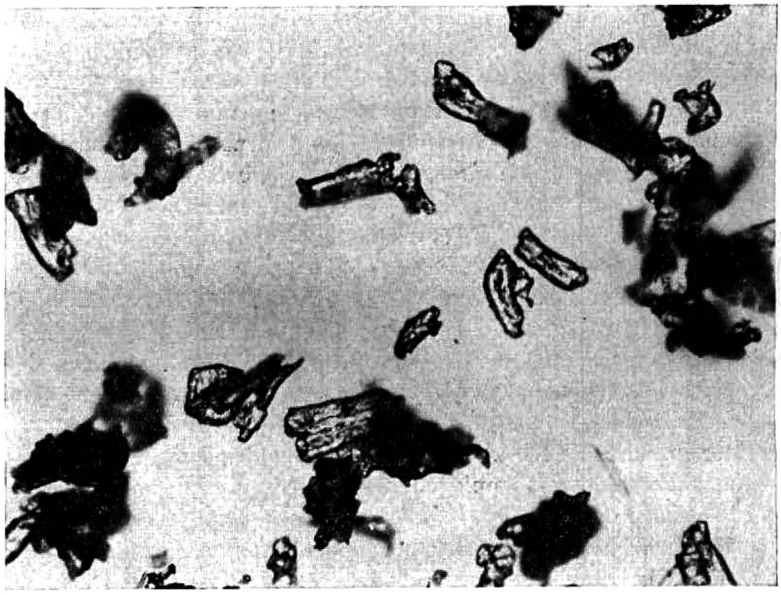
Another useful fraction rich in ceruloplasmin and other α-globulins could also be obtained by a selective adsorption procedure, *i.e.* by equilibrating a mixture of exchanger and serum with 0.05 M NaH_2PO_4 solution and then eluting the exchanger with a solvent of high ionic strength (such as 0.05 M NaH_2PO_4 solution containing 0.25 M NaCl). It should be mentioned, in this respect, that such selective adsorption techniques have already been applied by other investigators (*e.g.* CONNEL AND SHAW[9], STEINBUCH AND QUENTIN[10] and STEINBUCH AND LOEB[11]) to the isolation of serum α-globulins. In order to obtain further information about the relative selectivity of exchangers of differing degrees of substitution it will be necessary to study the adsorption characteristics of individual isolated serum proteins.

It is significant that the specific adsorption capacities obtained in the present series of experiments are approximately one tenth of those observed by PETERSON AND SOBER[1]. Although those investigators employed bovine serum albumin, in elutions with pH 7.0, 0.01 M phosphate buffers, it is difficult to explain the observed differences. Nevertheless, the two sets of results showed the same trend, the amount of protein adsorbed being proportional to the degree of substitution of the exchanger. In contrast, however, the adsorption curves shown in Fig. 5 reached a plateau above which the substitution of further reactive groupings had little effect upon protein adsorption. This is not apparent from the results of PETERSON AND SOBER, where marked increases in adsorption capacity were observed between the ranges 0.45 to 1.34 mequiv./g substitution. The higher adsorption capacity observed during the batch (as opposed to the column chromatography) procedure with "C" type exchangers of less than 0.65 mequiv./g substitution can probably be attributed to the fact that the elution with the first solvent is less efficient than in the column procedure.

As previously mentioned, some protein is adsorbed by exchangers of low substitution. For example, the exchanger C 5 adsorbs 20 % of the total amount of protein adsorbed by exchanger C 120, although its degree of substitution is only 4 % of that of the latter exchanger. This would suggest that either C 5 exhibits considerable non-ionic adsorption, or else a high proportion of the ionizing groups in the exchanger of



(a)



(b)

Fig. 6. Photomicrographs of "C" and "W" type exchangers. A. Exchanger C120, magnification × 100. B. Exchanger W, magnification × 250.

high substitution are not available for combination with the protein. It is assumed here, that the washing procedure has been efficient, and that no protein is trapped within the exchanger.

The differences in the chromatographic properties of the "W" type and "C" type exchangers (of similar substitution) can readily be explained in terms of differences in their physical form, as will be seen from the photomicrographs shown in Fig. 6. The "C" type exchangers are of a coarse physical form being prepared from fibrous cotton linters, whereas the "W" type product was crystalline-like. The effect of the physical form of DEAE-cellulose on its chromatographic behaviour has previously been discussed by PETERSON AND SOBER[1], who showed that gelatinous adsorbents (of fine physical form) possess higher capacities than non-gelatinous adsorbents. As well as affecting the capacity of the exchanger, the physical form also influences the resolution which was not so pronounced on the "C" type exchangers as in the laboratory product. A consideration of the factors which affect chromatographic phenomena on columns could explain these discrepancies (GLUECKAUF[12]). Disturbances due to non-equilibrium between protein solution and exchanger are liable to be more serious in exchangers of coarse form, for there is a greater tendency for longitudinal diffusion and channelling effects as well as less time for equilibration. All these factors tend to reduce the efficiency of the chromatographic process, although considerably higher rates of elution are obtained.

The results of the investigations of the effect of temperature are consistent with the observations of HJERTÉN[13], using calcium phosphate adsorbents. This factor was found to be of limited importance in the DEAE-cellulose chromatography of serum proteins. However, the effect of temperature may prove to be more marked in column procedures employing floc-like exchangers, where rises in temperature will increase diffusion and channelling of protein and solvent thus affecting resolution. The limitations of chromatography at temperatures above 4° are not inherent in the technique itself. Nevertheless, the susceptibility of proteins to denaturation and the risk of increased bacterial activity at these elevated temperatures necessitate fractionation at low temperatures.

The findings discussed here suggest that DEAE-cellulose exchangers prepared from cotton cellulose are inferior to those prepared from wood cellulose, at least as far as the separation of serum proteins is concerned. Ideally, the ion exchangers should be substituted to a degree of about 1 mequiv./g and should be of a physical form which permits ready packing into columns without showing a marked resistance to flow. Nevertheless, exchangers prepared from cotton cellulose have application in batch procedures where packing is not a problem, in spite of the necessity of using relatively greater amounts of such exchangers (owing to their low adsorption capacities).

With the solvent systems employed, the batch procedure was found to effect a resolution of serum proteins comparable to that achieved by the column technique. This means that when a large number of chromatographic fractionations are to be undertaken, the more rapid batch procedure provides a satisfactory alternative.

ACKNOWLEDGEMENTS

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SUMMARY

Studies have been undertaken to establish the influence of various factors on the fractionation of human serum proteins by DEAE-cellulose chromatography. The following points emerged:

1. In both column and batch procedures, the specific adsorption capacities of the various exchangers tested were related to their degree of substitution.

2. Exchangers of low degrees of substitution selectively adsorbed a number of serum proteins, especially α-globulins such as ceruloplasmin and haptoglobin, although they failed to retain preferentially rheumatoid factor.

3. The adsorption capacity of exchanger prepared from wood cellulose (of fine physical form) was twice the capacity of exchangers of similar substitution prepared from cotton cellulose.

4. Variation in the temperature of elution over a range from 0–50° had no effect on the properties of DEAE-cellulose exchanger (prepared from wood cellulose) as revealed by batch chromatographic experiments.

5. The batch technique would appear to provide a rapid alternative method to the column procedure for the chromatographic separation of serum proteins.

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STUDIES ON THE CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON DIETHYLAMINOETHYL(DEAE)-CELLULOSE

II. THE CHROMATOGRAPHIC CHARACTERISTICS OF PURIFIED HUMAN SERUM PROTEINS

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INTRODUCTION

Although, as already mentioned[1], DEAE-cellulose chromatography has been used extensively in the fractionation of serum proteins, there have been no previous reports of the investigations of well characterized individual proteins on this exchangers.

In the work to be described the chromatographic properties of several purified serum proteins have been studied. The group of proteins studied comprised macromolecules with a wide range of molecular size and charge density.

The effect of protein–protein interaction has also been determined by the chromatography of artificial protein mixtures, some of which included isotopically labelled proteins.

Studies of this type are essential if the resolution of serum protein mixtures affected by DEAE-cellulose chromatography is to be improved and if new selective isolation procedures are to be developed.

METHODS

Purified proteins

The human serum proteins used in these investigations are listed in Table I, together with their mode of preparation. The 7 S-γ-globulin, β-lipoprotein and α_2-macroglobulin preparations were shown to be free of other serum proteins by ultracentrifugal analysis and immunoelectrophoretic analysis employing a rabbit antiserum to whole human serum. By similar techniques the siderophilin preparations were found to contain small amounts of contaminating 7 S-γ-globulin (less than 5 %) and the albumin, a β-globulin impurity (2–5 %). In the case of the Lister albumin preparation the β-globulin contaminant was siderophilin.

Isotopic labelling of proteins

Various protein preparations were labelled with ^{131}I by the iodine monochloride technique of McFARLANE[6], as modified by DAVIES *et al.*[7], whilst siderophilin was labelled with ^{59}Fe by an exchange procedure (VEAL AND VETTER[8]). The activity of

TABLE I

METHODS OF PREPARATION OF THE PROTEINS INVESTIGATED

Protein	*Sample numbers*	*Method of separation*
7S-γ-Globulin	1	DEAE-cellulose column chromatographic separation from normal human serum
	2	DEAE-cellulose batch chromatographic separation from outdated acid citrate dextrose plasma (STANWORTH[2])
Siderophilin	1 and 3	DEAE-cellulose column chromatographic separation of F IV–4 obtained by the low temperature ethanol procedure of COHN *et al.*[3]
	2	DEAE-cellulose chromatographic separation of outdated acid citrate dextrose plasma followed by further DEAE-cellulose chromatography of the siderophilin-rich fraction
β-Lipoprotein	1	Subfractionation of Fractions II and III, obtained by COHN method 6[3], by method 9 (ONCLEY *et al.*[4]) to give fraction III-O; zone centrifugation of this fraction in saline density gradient
α_2-Macroglobulin	1	Zone centrifugation in sucrose density gradient of the pellet obtained in the above centrifugation procedure
Albumin	1	Fraction V obtained by COHN method 6[3]
	2	Commercial preparation (LISTER) obtained by the ether fractionation procedure of KEKWICK AND MACKAY[5]

labelled fractions was determined by counting measured aliquots in a thallium activated, well shaped, sodium iodide crystal.

Column chromatographic procedure

With one exception (discussed later) all chromatographic separations were carried out in columns containing 2 g of DEAE-cellulose exchanger prepared in the laboratory from wood cellulose (Solka Floc Grade BW 100) according to the method of PETERSON AND SOBER[9] (column dimensions 16.0 × 1 cm). This material had a degree of substitution of 0.87 mequiv./g. Elution was effected by a stepwise procedure using the following series of solvents:

1. Phosphate buffer: pH 7.6, 0.01 *M*.
2. Phosphate buffer: pH 6.3, 0.02 *M*.
3. 0.05 *M* NaH_2PO_4 solution.

Other practical details are discussed in the previous paper[1].

RESULTS

(a) Studies of single proteins

The amounts of protein eluted with the various solvents are recorded in Table II in which the mean recoveries and the standard deviation of the mean are given. Statistical analysis revealed that the total recoveries of 7S-γ-globulin and albumin were significantly greater than those observed with total serum protein, whilst the recovery of siderophilin was comparable. On the other hand, the recoveries of β-lipoprotein and α_2-macroglobulin were significantly less than that shown by total serum protein.

TABLE II

THE AMOUNTS OF THE VARIOUS SERUM PROTEINS CHROMATOGRAPHED AND THEIR RECOVERIES

Mean recoveries expressed together with their standard deviations ($6\bar{x}$).

Sample	*Number of experiments*	*Amount protein applied (mg)*	*Protein recovery (percentage of total applied)*			
			Phosphate buffer		*0.05 M NaH_2PO_4*	*Total*
			pH 7.6, 0.01 M	*pH 6.3, 0.02 M*		
Normal human serum	4	365	8.2 ± 2.3	7.5 ± 1.1	57.4 ± 6.8	73.0 ± 8.8
7S-γ-globulin	4	20.5 ± 4.2	72.4 ± 4.6	11.9 ± 4.4	6.1 ± 2.6	90.5 ± 3.9
Siderophilin	5	119.4 ± 25.6	52.7 ± 8.0	10.1 ± 3.7	2.9 ± 0.8	65.7 ± 7.3
β-Lipoprotein	2	13.5	0.65 ± 0.65	7.2 ± 3.2	15.4 ± 3.7	23.1 ± 6.4
α_2-Macroglobulin	4	70.1 ± 4.6	2.7 ± 0.9	0.9 ± 0.2	29.4 ± 3.0	33.4 ± 3.1
Albumin	6	157.0 ± 47.8	0.8 ± 0.2	1.1 ± 0.2	82.1 ± 1.3	84.1 ± 1.2
7S-Globulin and siderophilin	3	51.7 ± 15.3	61.0 ± 6.7	13.1 ± 3.9	1.3 ± 0.4	75.2 ± 5.0
7S-Globulin and albumin	2	125 ± 15.0	32.8 ± 4.6	2.4 ± 0.2	52.9 ± 4.9	88.2 ± 0.3
α_2-Macroglobulin and albumin	1	114	0.3	0.8	37.0	38.1
7S-Globulin, siderophilin, α_2-macroglobulin and albumin	1	168	18.9	15.7	46.9	81.5

As was to be expected most of the 7S-γ-globulin failed to bind to the exchanger and so was recovered in the initial solvent front. Nevertheless, this protein exhibited the "trailing" effect also observed by other investigators[10,11].

The elution characteristics of isolated siderophilin were found to differ markedly from those exhibited by this protein during the DEAE-cellulose chromatography of whole serum[12]. For instance, the major part of the isolated siderophilin was eluted by the first solvent (0.01 *M* phosphate, pH 7.6) whereas in separation of whole serum it was completely eluted by the second solvent (0.02 *M* phosphate, pH 6.3). This effect does not appear to have been observed by TOMBS *et al.*[13], in their studies on the chromatographic behaviour of electrophoretically prepared β-globulin fraction.

Recoveries of β-lipoprotein were extremely low (23.1 % ± 6.4), the eluted material being dispersed throughout the greater part of the chromatogram. Similarly the recovery of α_2-macroglobulin was disappointingly low (33.4 % ± 3.1). In contrast to the β-lipoprotein, however, almost all of this protein was recovered with one solvent (the 0.05 *M* NaH_2PO_4 solution). It was eluted ahead of the normal 19S-γ-globulin position[11], and also prior to the albumin (see Fig. 1).

As mentioned earlier, the total recoveries of albumin were constantly high (84.1 % ± 1.2). Again the major portion of this protein (82.1 % ± 1.3) was recovered with the final solvent (0.05 *M* NaH_2PO_4 solution). The chromatographic patterns obtained by duplicate analysis of the same albumin sample showed considerable variability, however. Compare for instance patterns A and B in Fig. 2 which were obtained by parallel analyses on the same albumin preparation (a COHN Fraction V). Comparison with patterns (E and F) given by larger amounts of a different albumin preparation (LISTER) revealed even greater variation. It should be mentioned, however, that the leading minor peaks in these patterns can be attributed to contaminating siderophilin.

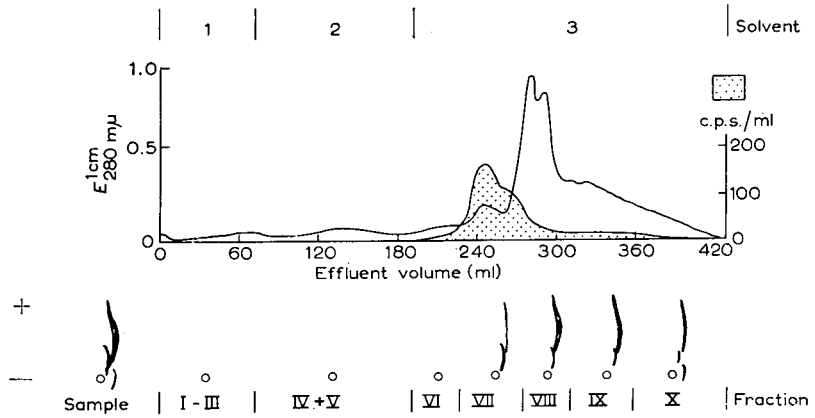

Fig. 1. Chromatography of a mixture of ^{131}I labelled α_2-macroglobulin (12.6 mg) and COHN F V albumin (101.4 mg of preparation 1) on DEAE-cellulose exchanger W. For chromatographic details see text.

In general, the recoveries of the various proteins studied were found to be independent of the amount of material applied to the DEAE-cellulose column. For instance, although the amounts of albumin chromatographed varied between 84–320 mg, the total recovery was remarkably constant, *e.g.* 84.1 % ± 1.2. This is supported by the results of statistical analysis.

(b) Studies of protein mixtures

The mixtures of serum proteins investigated are tabulated in Table II where it can be seen, that with the exception of the ^{131}I labelled α_2-macroglobulin–albumin mixture, the overall protein recoveries were comparable with those shown by the individual

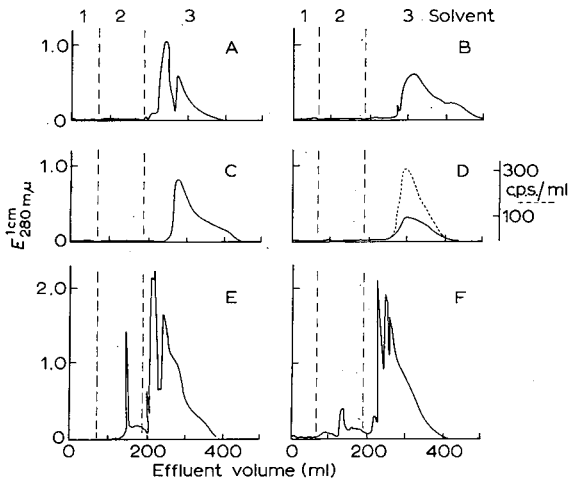

Fig. 2. The chromatographic distribution of albumin on DEAE-cellulose exchanger W. A, B and C are 82.0, 82.0 and 106.0 mg respectively of COHN F V albumin (preparation 1); D is 43.0 mg of ^{131}I labelled COHN F V (preparation 1); and E and F are 320.0 and 293.0 mg of LISTER albumin (preparation 2). For chromatographic details see text.

proteins. Moreover, the positions of elution of various components in the protein mixtures were similar to those exhibited by these proteins in isolated form. Recoveries of labelled siderophilin, and α_2-macroglobulin, which had been added to whole serum were comparable with those shown by the isolated proteins. On the other hand, the recoveries of ^{131}I labelled 7 S-γ-globulin, β-lipoprotein and albumin from whole serum were lower than the corresponding recoveries observed with isolated proteins and simple protein mixtures.

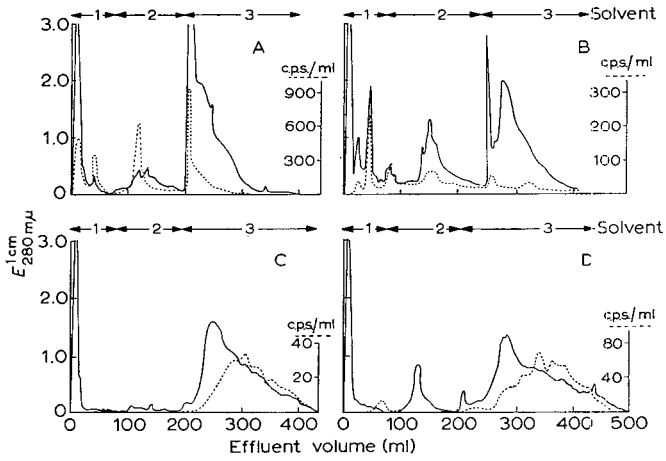

Fig. 3. The chromatography of mixtures of isotopically labelled serum proteins and whole serum on DEAE-cellulose exchanger W. The following isotopically labelled serum proteins were added to 5 ml of normal human serum: A = 6.3 mg of ^{131}I labelled 7 S-γ-globulin preparation 2; B = 141.8 mg of ^{59}Fe labelled siderophilin preparation 3; C = 12.6 mg of ^{131}I labelled α_2-macroglobulin; D = 45.7 mg of ^{131}I labelled albumin preparation 1. Distribution of radioactivity indicated thus: For further chromatographic details see text.

In these experiments, involving the addition of labelled proteins to whole serum, the distribution of protein-bound isotope was often found to differ from that observed during the chromatography of simple mixtures containing the labelled protein. This was particularly noticeable in experiments employing ^{131}I labelled 7 S-γ-globulin (see Fig. 3), where the major portion of the labelled protein was eluted with solvents other than the 0.01 *M* phosphate buffer (pH 7.6). It should also be noted that the total recoveries of protein from isotopically labelled protein–serum mixtures were less than those obtained with serum alone.

DISCUSSION

On the whole, the chromatographic behaviours of isolated serum proteins proved similar to those exhibited during the fractionation of total serum.

As suggested from earlier studies on whole serum, the position of elution of an individual component depends largely on its charge density at the pH employed. Other factors, however, appear to influence the chromatographic separation of certain serum proteins. For example, although both α_2-macroglobulin and ceruloplasmin fall into the α_2-globulin electrophoretic class these proteins exhibit quite

distinct chromatographic properties. Similarly, there are marked differences in the chromatographic behaviour of siderophilin (a β-globulin) and 19S-γ-globulin, in spite of their relatively small differences in electrophoretic mobility.

The results of the studies on artificial protein mixtures failed to demonstrate that protein–protein interaction was responsible for the differing behaviour of these pairs of proteins. It is more likely, however, that molecular size plays a critical role in the ion-exchange chromatography of proteins on substituted cellulose as suggested by PETERSON AND SOBER[14], resulting in the phenomenon known as "size compensation". This could explain the difference in chromatographic behaviour of 7S-γ- and 19S-γ-globulin. Another complication results from variations in the distribution, availability and degree of ionization of the charged groups within a protein molecule. This renders difficult the precise duplication of chromatographic separations of serum proteins. In addition, it is also possible that certain charged groups are not revealed until the protein molecule becomes unfolded on binding to the ion exchanger. Such groupings might be expected to play an active part in irreversible binding, which is assumed to be responsible for the low recoveries of proteins such as α_2-macroglobulin and β-lipoprotein.

The weak affinity of 7S-γ-globulin for exchanger, probably due to its relatively high isoelectric point, is reflected by both its position of elution and its good recovery. Nevertheless, as already mentioned, a certain degree of trailing of the 7S-γ-globulin was observed, successive fractions showing progressively greater electrophoretic mobility (see refs. 10 and 11). The rather abnormal distribution of ^{131}I labelled 7S-γ-globulin (see Fig. 3A) was probably due to configurational changes produced in this molecule due to excessive iodination. Immunoelectrophoresis failed to reveal any such changes, however.

The alterations in the chromatographic behaviour of siderophilin following its isolation were puzzling. This could have been due to irreversible changes in its molecular configuration resulting from the disruption of its combination with other kinds of protein molecules. Such an irreversible change is suggested by the results of experiments involving the addition of ^{59}Fe labelled siderophilin to whole serum (see Fig. 3B), which showed that a large proportion of the added protein was still eluted prematurely. However, this effect could have been due to the use of a large excess of added siderophilin. Alternatively, structural changes not attributable directly to any protein–protein interaction effect could have played a part in the observed anomalous behaviour. The elution of the siderophilin preparations in a number of distinct peaks could have been due to the presence in the mixture of a range of siderophilin molecules saturated to varying extents with iron, or due to the existence of several distinct siderophilins[15].

The poor recoveries and "trailing" of both the β-lipoprotein and α_2-macroglobulin can be attributed to the molecular size of these proteins. Having a large partial specific volume they are susceptible to entrapment between the cellulose particles. In addition, it seems reasonable to assume that the simultaneous disruption of all the groups involved in the adsorption processes is never realised. In the case of the β-lipoprotein there is the additional complication of the high lipid content, which may render the molecule surface active, thereby causing increased affinity between exchanger and protein leading to denaturation. Moreover, this protein is rather insoluble in the aqueous eluting solvents. The elution of the α_2-macroglobulin

prior to albumin would appear to be a reversal of the size compensation phenomenon observed with 7S-γ- and 19S-γ-globulin. The most plausible explanation of this effect is a difference in the availability of the ionizable groups of the two proteins, for their total dicarboxylic and sialic acid contents are similar.

Because of the high dicarboxylic acid content (and hence low isoelectric point), the albumin molecule is adsorbed strongly onto DEAE-cellulose at alkaline pHs. However, this adsorption process must be readily reversible because the albumin is efficiently recovered. This could be due to the marked configurational adaptability of the albumin molecule. The appearance of albumin in a number of peaks and over a wide area of the chromatogram (see Fig. 2) has been attributed to a number of factors including the binding of small ions, especially fatty acids[16], dimer formation[17,18] facilitated by the presence of fatty acid[19,20] and to the concentration dependence of the adsorption isotherms[21]. It has also been suggested that this phenomenon might result from successive stepwise decreases in the capacity of the column under the influence of the eluting buffer[22,23]. Experiments involving the chromatography of COHN F V on DEAE-cellulose prepared from cotton linters (illustrated in Fig. 4),

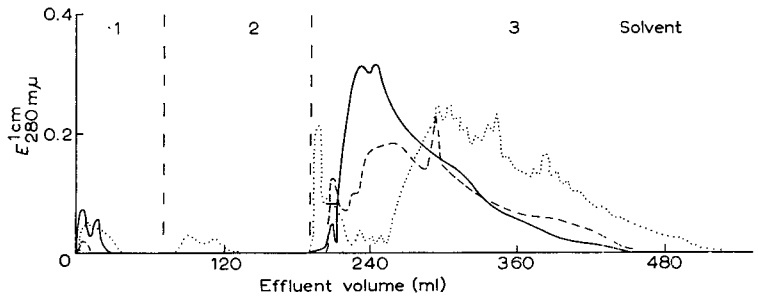

Fig. 4. The effect of oleic acid on the chromatographic behaviour of COHN F V serum albumin (preparation 1) on DEAE-cellulose (commercial exchanger 1.2 mequiv./g substitution). Chromatographic distribution represented as follows: albumin ———, ether extracted albumin – – – and oleic acid saturated albumin · · ·. For additional chromatographic details see text.

indicate that oleic acid saturated protein is more firmly bound to the column and less readily recovered than the other proteins. These results support the observations of previous workers[14,16]. Hence the binding of small, immunologically undetectable molecules, is most probably a factor of paramount importance in the observed chromatographic anomalies of human serum albumin.

As the investigation of isolated serum proteins has confirmed a wide variation in affinity for DEAE-cellulose, and has also failed to demonstrate the occurrence of any obvious protein–protein interactions, it seems probable that further studies of a similar nature could lead to the development of selective adsorption techniques for the isolation of specific serum components. Preliminary studies have demonstrated that batch chromatographic procedures, involving selective adsorption under strictly controlled conditions, could be used for such purposes.

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SUMMARY

1. The chromatographic behaviour of several purified human serum proteins on columns of diethylaminoethyl-cellulose has been established. The group of proteins investigated included 7 S-γ-globulin, siderophilin, β-lipoprotein, α_2-macroglobulin and albumin, *i.c.* molecules with a wide range of physico-chemical properties.

2. Individual proteins were found to differ widely in their recoveries. For example, 7 S-γ-globulin and albumin were recovered in high yield in spite of their widely different affinities for DEAE-cellulose. On the other hand, a major portion of the β-lipoprotein and α_2-macroglobulin investigated (proteins of large molecular size) could not be recovered from the exchange cellulose.

3. The chromatographic characteristics of the isolated proteins closely paralleled those shown by these proteins during the fractionation of simple mixtures, though such a close relationship was not observed in whole serum. Purified siderophilin, however, proved an exception in showing a much reduced affinity for exchanger whether in isolated form or even when added to more complex mixtures.

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LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

VIII. GRADIENT ELUTION PRACTICE. THE EFFECT OF ALKYL SUBSTITUENTS ON RETENTION VOLUME

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INTRODUCTION

The technique of gradient elution has been used frequently in the adsorption chromatographic separation of complex organic mixtures, with the intention of optimizing sample resolution, maximizing sensitivity in the detection of trace components, and minimizing separation time. The theory of gradient elution adsorption chromatography (GEAC) has been treated in the preceding paper[1] of the present series. In this theoretical study it was found that eluent gradients of the so-called *linear strength* form, eqn. (1), are generally optimum in GEAC separation:

$$\alpha\varepsilon^\circ = a + bV \tag{1}$$

Here, $\alpha\varepsilon^\circ$ is the *effective* eluent strength (adsorbent activity function, α, times eluent strength, ε°) of the GEAC binary eluent after the passage of V ml of eluate through the adsorbent column, and a and b are constants for a particular gradient. In GEAC separations with gradients obeying eqn. (1), and for sample sizes within the linear capacity[2] of the column (linear elution adsorption chromatography, LEAC), a simple relationship between solute retention volume R_g, solute structure, and experimental separation conditions is predicted:

$$R_g = \frac{\log\left(2.31\, A_s b W \underline{R}_p\, 10^{-a A_s} + 1\right)}{bA_s} \tag{2}$$

The constants a and b are defined by eqn. (1), A_s refers to the effective solute surface volume, W to the adsorbent weight, and $\underline{R}_p$ to the linear equivalent retention volume of the solute for elution from the same adsorbent by pentane. The calculation of values of A_s and the prediction of $\underline{R}_p$ for a variety of separation systems (varying solute, adsorbent, adsorbent activity) have been detailed in preceding papers of this series and summarized in Part VI[3].

Having demonstrated the theoretical advantages of a linear or near-linear strength gradient in GEAC separation, it remains to consider how such gradients may be attained in practical separations. Additionally, the theory we have derived

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for GEAC separation[1], and particularly eqn. (2), needs to be verified experimentally. Finally, in the course of other studies of the adsorption chromatographic process, the role previously assigned[4] to solute alkyl groups in determining retention volume in strong eluent systems (and in GEAC separation) has been revised. These various items form the basis of the present communication.

EXPERIMENTAL

The GEAC separations described in the present paper were carried out using glass-teflon units similar to that of Fig. 1. Solid glass rods of varying diameter were placed in the strong eluent (B) reservoir to permit variation of its effective cross sectional

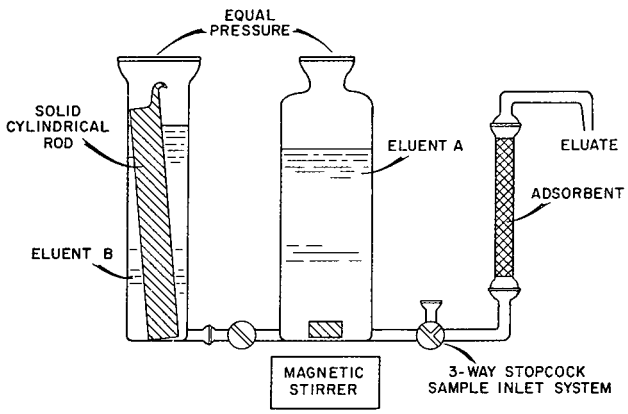

Fig. 1. Gradient elution apparatus used in present study.

area. A predetermined amount of weak eluent (A) was added to its reservoir, and the adsorbent column prewet or left dry, as desired. Sufficient strong eluent (B) was added to the second eluent reservoir to give hydrostatic equilibrium between the two reservoirs:

$$h_A/h_B = d_B/d_A$$

h_A and h_B refer to the height of each liquid head, and d_A and d_B to the respective eluent densities. Sample was introduced through the three-way teflon bore stopcock, all stopcocks opened for flow into the adsorbent column, and elution begun with stirring of the weak eluent reservoir. Measurement of sample retention volume R_g was carried out as in the case of fixed eluent separation[5], being equal to the measured retention volume in ml with a correction for the dead volume of the column in the case of prewet colums. Linear capacity in the systems studied had been previously evaluated[6,7], and the sample sizes used ($< 5 \cdot 10^{-5}$ g/g) insured isotherm linearity.

The use of equipment of the type in Fig. 1 for the GEAC separation of samples of interest is facilitated by the following design considerations. First, the overall size of the apparatus required is roughly proportional to the sample sizes that must be dealt with. However, provision should be made for varying the ratio of cross sectional areas (A_A/A_B) between 0.5 and 16. As the cross sectional area of the rod in vessel B approaches that of vessel B, capillary entrainment of solvent becomes a problem,

so that more than one diameter vessel B is desirable. If the two vessels of Fig. 1 are detachable, as shown, only the strong eluent vessel requires duplication. It is recommended that four sizes of the vessel B be available, giving relative cross sectional areas (A_A/A_B) equal 0.5, 1, 5 and 10.

To prevent non-equilibrium in the solvent mixer, the bore of the stopcock between vessels A and B should be large (at least 2 mm). For ease in introducing the control rod of vessel B, the inside diameter of the top spherical joint of that vessel should be approximately as large as the diameter of vessel B. The stirring bar used in vessel A should be no longer than $^2/_3$ of the diameter of the vessel, to restrict the backflow of eluent A into vessel B. Finally, standard glassware sizes restrict the possible values of (A_A/A_B) to certain discrete values. For most work, the values of this ratio thus available will approximate desired values sufficiently closely. An optimum arrangement might use machined stainless steel or teflon rods, in order to vary the cross sectional area ratio precisely as desired.

LINEAR STRENGTH GEAC SEPARATION

The specific advantages of linear strength gradient elution (*i.e.*, using gradients which obey eqn. (1)) have been derived previously[1]. They include (i) optimum peak spacing (weakly adsorbing components sufficiently separated to be resolved, strongly adsorbing components eluted within convenient separation times), (ii) elution bands of approximate equal width (for maximum sensitivity of detection), and (iii) no band splitting with development of spurious peaks. Eluent strength gradients which are mildly convex, rather than linear, are generally acceptable in GEAC separation, and in many cases are predicted to give a slightly better separation system. The major disadvantage of convex eluent strength gradients is the difficulty of accurately predicting R_g values, due to the inapplicability of eqn. (2) in non-linear separations. For many GEAC systems, particularly in the separation of samples whose constituents are initially unknown and for which R_g values cannot therefore be predicted, this consideration will be unimportant. *Concave gradients are generally undesirable in every respect.*

To determine the form (linear, concave, etc.) of the eluent strength gradient in a given GEAC separation, we must be able to relate the composition of the eluent to the eluate volume V, and the effective eluent strength $\alpha\varepsilon^\circ$ to eluent composition. The latter relationship for a simple binary eluent gradient (pure solvents A and B) has been derived[4]:

$$\alpha\varepsilon^\circ = \alpha\varepsilon^\circ_A + \frac{\log\left(X_B\, 10^{an_b(\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B\right)}{n_b} \qquad (3)$$

Here, α is the adsorbent activity function, ε°_A and ε°_B are eluant strengths, respectively, of A and B, n_b is the value of A_s for B, and X_B is the mole fraction of B in the binary eluent. The values of these various parameters have been reported in previous papers of this series and indexed in Part VI[3].

X_B in eqn. (3) may be related to the corresponding volume fraction V_B through the molecular weights M_A and M_B of A and B,

$$X_B = \frac{V_B}{(1 - V_B)\,(d_A/M_A)\,(M_B/d_B) + V_B} \qquad (3a)$$

so that the necessary relationship between V_B and V for a linear (or other) strength gradient can be specified. A number of experimental devices have been described recently[8–13] which are capable of producing any predetermined eluent gradient or V *versus* V_B relationship (primarily for application to ion exchange gradient elution). These devices are moderately complex and were not investigated in the present study, although they should prove useful where the number of separations contemplated justifies the fabrication of the necessary equipment and the time to become familiar with its use. The application of these devices to GEAC separation appears straightforward in principle, and requires no further comment. Alternately, it is possible to carry out linear strength GEAC separation in simpler devices, particularly of the kind shown in Fig. 1. The derivation of the relationship between V_B and V for gradient devices of this type has been given by at least two authors[14,15], for the case of equal eluent densities d_A and d_B. For the general case of unequal densities an approximate extension of prior derivations for the device of Fig. 1 gives[16]:

$$V_A = [1 - (a^* V/V^\circ_A)]^{(1 - a^*)/a^*} \quad (4)$$

where:

$$a^* = (A_A/A_B)/[(A_A/A_B) + (d_A/d_B)] \quad (4a)$$

V°_A is the volume of solvent in reservoir A prior to the beginning of elution. The variation of V_A with the fractional depletion of *both* solvent reservoirs V/V° (V equal V° when both reservoirs empty) is illustrated in Table I for several values of the parameter a^*.

Figs. 2 and 3 illustrate the dependence of $\alpha\varepsilon^\circ$ on V for several hypothetical chromatographic systems using the apparatus of Fig. 1. In Fig. 2, the eluent parameters n_b and $\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$ are assumed equal to 2 and 0.30, respectively, while in Fig. 3 these eluent parameters are given values of 6 and 0.30 (*i.e.*, the strong eluent B in Fig. 2 is a small molecule and in Fig. 3 a large molecule). The different curves

TABLE I

COMPOSITION OF ELUENT FOR GRADIENT DEVICE OF FIG. 1 *versus* TOTAL ELUATE VOLUME AND a^*

V/V°	V_B *for various values of* a^*								
	0.50	*0.60*	*0.65*	*0.70*	*0.75*	*0.80*	*0.85*	*0.90*	*0.95*
0.05	0.05	0.034	0.027	0.022	0.017	0.013	0.009	0.006	0.003
0.10	0.10	0.068	0.055	0.044	0.035	0.026	0.018	0.012	0.006
0.20	0.20	0.138	0.112	0.091	0.072	0.054	0.038	0.024	0.012
0.30	0.30	0.212	0.174	0.142	0.112	0.085	0.061	0.039	0.019
0.40	0.40	0.289	0.239	0.196	0.156	0.120	0.086	0.055	0.027
0.50	0.50	0.370	0.311	0.257	0.206	0.159	0.115	0.074	0.036
0.60	0.60	0.457	0.389	0.324	0.263	0.205	0.149	0.097	0.047
0.70	0.70	0.552	0.477	0.403	0.330	0.260	0.191	0.125	0.062
0.80	0.80	0.658	0.579	0.498	0.415	0.331	0.247	0.164	0.082
0.90	0.90	0.785	0.710	0.627	0.536	0.438	0.333	0.226	0.115
0.95	0.95	0.864	0.800	0.728	0.632	0.527	0.410	0.283	0.146

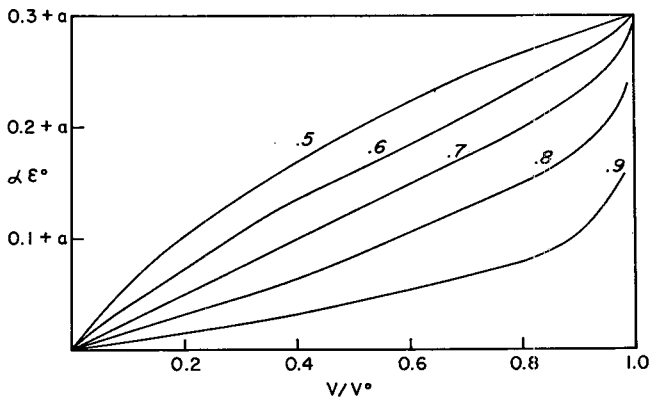

Fig. 2. Eluent strength *versus* eluate volume curves for device of Fig. 1. $\alpha\varepsilon^\circ_A = a$; $\alpha\varepsilon^\circ_B = 0.3 + a$; $n_b = 2$; $(d_A/M_A)/(d_B/M_B) = 1.0$. Values of a^* on curves.

in Figs. 2 and 3 correspond to different values of the gradient apparatus parameter a^*. In Fig. 2, for B small, values of a^* between 0.6 and 0.7 give a reasonably linear gradient for values of V/V° less than 0.9 (*i.e.*, for the first 90 % of the separation). In Fig. 3, where the strong eluent is a large molecule, reasonably linear gradients occur for larger values of a^* (0.90–0.95). For values of a^* less than the linear gradient value, the gradient is convex, and for larger values, concave. As a general observation, *gradient devices of the present type should not be used for V greater than 0.9 V°*, because the gradient frequently becomes severely concave during the last 10 % of the separation. It should also be noted that the larger the strong eluent (value of n_b), the smaller the effective range in eluent strength covered during a single GEAC separation ($0 \leqslant V \leqslant V^\circ$), and the smaller the number of sample components conveniently separable.

As the difference in strength between the two eluents ($\varepsilon^\circ_B - \varepsilon^\circ_A$) is increased, the family of $\alpha\varepsilon^\circ$ *versus* V curves tends to resemble the case of larger n_b. Increasing $\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$ in Fig. 2 would tend to give curves of shape similar to those in Fig. 3, with higher values of a^* required for gradient linearity. The curves of Figs. 2 and 3

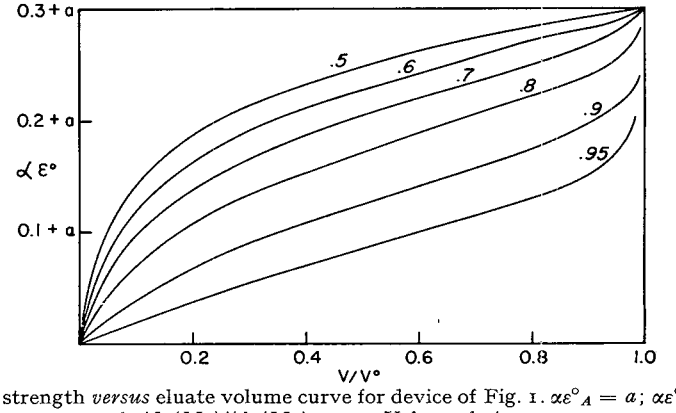

Fig. 3. Eluent strength *versus* eluate volume curve for device of Fig. 1. $\alpha\varepsilon^\circ_A = a$; $\alpha\varepsilon^\circ_B = 0.3 + a$; $n_b = 6$; $(d_A/M_A)/(d_B/M_B) = 1.0$. Values of a^* on curves.

assume $(d_A/M_A)/(d_B/M_B) = 1$. When this ratio is increased, the effect on the $\alpha\varepsilon^\circ$ *versus* V curves is similar to decreasing n_b, but less pronounced.

The curves of Figs. 2 and 3 in conjunction with the data of Table II offer a qualitative guide to the selection of optimum a^* values in practical GEAC separation work. The calculation of similar curves for specific situations is not difficult, using

TABLE II

PROPERTIES OF SOME COMMON ELUENTS FOR USE IN DESIGNING GEAC SEPARATIONS

Eluent	d_A/M_A	d_A	n_b	ε°	
				Al_2O_3	SiO_2
Pentane	0.00861	0.626	5	0.00	0.00
Isooctane	0.00606	0.692	8	0.01	
Cyclohexane	0.00926	0.780	6	0.04	
Carbon tetrachloride	0.01037	1.595	4	0.18	0.14
Benzene	0.01127	0.879	6	0.32	0.25
Methylene chloride	0.01574	1.336	3	0.42	0.32
Acetone*	0.01363	0.792	4	0.56	
Methyl acetate*	0.01253	0.927	5	0.60	
Ethyl acetate	0.01023	0.901	5	0.60	
Dioxan	0.01176	1.035	6	0.63	
Pyridine*	0.01242	0.982	6	0.71	

* ε° values and supporting data to be described in a following communication.

eqn. (3) and Tables I and II. A number of specific linear strength systems are itemized in Table III for the three adsorbents so far studied. These systems are restricted to weak or moderately strong eluents, since very strong eluents such as the alcohols have not yet been studied in the present series of investigations.

At first glance, it might appear particularly useful in GEAC separation to have the weak and strong eluents as widely different as possible (*e.g.*, pentane A, isopropanol B). From Figs. 2 and 3 and the related discussion, however, this would require values of a^* quite close to 1.00, and at V equal 0.9 V° the effective eluent strength would be only a fraction of that of pure B. Consequently, substances eluted readily by pure B will not necessarily be eluted from the column in the linear gradient region ($V \leqslant 0.9\ V^\circ$), although this would generally be true of GEAC systems where the eluent strengths of the two constituent solvents are closer in value. Consequently, much of the expected advantage of a very strong second eluent will be lost inasmuch as many strongly adsorbing solutes will not be eluted in the course of the separation. An additional objection to gradient eluents of widely different strengths exists by virtue of the displacement effect[1], which becomes especially marked in such cases. Displacement causes poor separation of weakly adsorbing solutes, because the initial stages of separation serve only to saturate the column adsorbent with the strong eluent B, all weakly adsorbing compounds being displaced as a single band by the advancing strong eluent front. Gradient systems based on eluents of moderately different strengths (as in Table III) appear optimum from the standpoint of all but very weakly and very strongly held solutes, and it seems likely that most samples of interest can be adequately separated by GEAC systems of this type.

TABLE III

SUMMARY OF SEVERAL BINARY LINEAR STRENGTH GRADIENT ELUTION SYSTEMS

Weak eluent A	*Strong eluent B*	*Adsorbent*		A_A/A_B	$h_A h_B = d_B/d_A$	$\alpha\varepsilon^\circ_A$	bV°
		Type	% H_2O				
n-Pentane	Carbon tetrachloride	Al_2O_3	1	1.1	2.55	0.000	0.130
			2	1.1		0.000	0.111
			4	0.9		0.000	0.098
n-Pentane	Benzene	Al_2O_3	1	13	1.41	0.000	0.161
			2	2.8		0.000	0.157
			4	2.1		0.000	0.145
		SiO_2*	1	2.8		0.000	0.156
			4	1.6		0.000	0.161
			16	1.3		0.000	0.130
		Flor.**	1	13		0.000	0.110
n-Pentane	Methylene chloride	Al_2O_3	1	4.2	2.13	0.000	0.250
			2	3.5		0.000	0.242
			4	2.7		0.000	0.204
		SiO_2*	1	3.5		0.000	0.233
			4	2.3		0.000	0.189
			16	1.9		0.000	0.155
		Flor.**	1	4.2		0.000	0.168
			4	1.9		0.000	0.165
Carbon tetrachloride	Benzene	Al_2O_3	1	4.3	0.55	0.151	0.108
			2	4.3		0.135	0.092
			4	3.4		0.113	0.084
Carbon tetrachloride	Methylene chloride	Al_2O_3	1	3.4	0.84	0.151	0.176
			2	3.1		0.135	0.163
			4	2.6		0.113	0.140
Benzene	Dioxan	Al_2O_3	1	16	1.08	0.269	0.148
			2	10		0.240	0.157
			4	7.7		0.202	0.110
Methylene chloride	Dioxan	Al_2O_3	1	5.2	0.77	0.353	0.142
			2	4.2		0.315	0.122
			4	3.0		0.265	0.102

* Davison Code 12 or equivalent.
** See ref. 3.

In the case of unusually complex samples which are suspected to contain a number of components, some easily eluted and some strongly adsorbed, no simple gradient system of the type so far described will prove wholly satisfactory. Two alternative procedures are useful in this connection, however. Thus, the sample may be separated first by conventional elution chromatography into several fractions by successive elution with a series of eluents j, k, m... of increasing strength ($\varepsilon^\circ_j < \varepsilon^\circ_k < \varepsilon^\circ_m$...). Each of the resulting fractions may then be further separated in an *optimum* GEAC separation as above, since the range of compounds in each of

these fractions should be properly spaced in the right GEAC system. As a general rule, if a sample is eluted by 2–3 column volumes of some eluent j, it will be readily eluted by a GEAC system using j as strong eluent (B). Similarly, all compounds eluted after 2–3 column volumes of the eluent j will tend to be separated in a GEAC separation using j as the weak eluent (A). Consequently, an optimum *overall* separation might be accomplished by elution with 2–3 column volumes of each eluent j, k, etc., in the initial preparation of the various fractions, with the second fraction being reseparated in a GEAC system using eluents j (weak) and k (strong). The third fraction would be reseparated in a GEAC system based on eluents k and m, and so on for the rest of the initial fractions. The various eluents j, k, etc., need not be pure solvents, but can be binary eluents if this is required or convenient. The only difference is the use of eqn. (5) in the calculation of eluent strength in the resulting *ternary* solvent systems[1], rather than eqn. (3) for binary solvents:

$$\alpha\varepsilon^\circ = \alpha\varepsilon^\circ_B + \frac{\log X_C \, 10^{an_c(\varepsilon^\circ_C - \varepsilon^\circ_B)} + X_B}{n_c} \qquad (5)$$

X_C is the mole fraction of C in the eluent, n_c is the value of A_s for the eluent C (same as n_b for eluent B), and ε°_C is the eluent strength of C.

A second solution to the problem of separating complex samples by GEAC is through the use of additional eluent after the initial emptying of the eluent reservoirs. Simple elution with the strong eluent B at this point will serve to further elute sample from the column, but the advantages of GEAC separation in separating additional sample components are rapidly lost; separation time for the most strongly adsorbing components mounts, solute bands broaden with loss in sensitivity of detection, and quite possibly a spurious peak would appear soon after change to pure eluent.

A third and more reasonable approach is the use of a new eluent pair which will continue the original eluent gradient described by eqn. (1), thus extending the range of compounds which may be separated under the original separation conditions, and incidentally permitting the continued prediction of solute R_g values through eqn. (2). As we have seen, the first step of the GEAC separation will normally terminate around $V = 0.9\ V^\circ$, at which point the eluent entering the column will be of some composition x % A–B. Now if the latter *binary* eluent (x % A–B) is used in place of the original weak eluent A in the *second* step of the overall GEAC separation, and a stronger eluent C ($\varepsilon^\circ_C > \varepsilon^\circ_B$) is used as the strong eluent, a value of a^* for the second part of the separation can be calculated for linear strength elution, and a value of V° determined so as to make the slope b in the second stage of the separation equal to that in the first stage. Equation (5) must of course be substituted for eqn. (3) in the resulting calculations. One such ternary eluent GEAC separation of this type is described in the following section. Other ternary eluent separations can be readily formulated.

In Table III are listed values of bV°, which corresponds approximately to the effective eluent strength range available during separation. If the eluent strength gradient were precisely linear throughout the whole separation, bV° would equal $\alpha(\varepsilon^\circ_B - \varepsilon^\circ_A)$. By varying V°, b can be varied at will, and the question of an optimum value of b must be considered. The analysis of such a question is reasonably complex, and dependent upon factors (column efficiency) which are not yet fully

understood. However, as a rough guide, W/V° should be small (less than 0.1), and the size of W will be dictated by the sample size required to be separated, the linear capacity of the adsorbent, etc. The longer the column and the smaller the eluent flow rate (mm/min), the better will be the resulting separation.

THE EXPERIMENTAL VERIFICATION OF EQUATION (2) AND SOME COROLLARY RELATIONSHIPS

The validity of the theoretical analysis of GEAC presented in the previous paper[1] of this series, and of the discussion in the preceding section of this communication, ultimately rests on the accuracy of eqn. (2). For these reasons, as well as to test the applicability of eqn. (2) *per se* in the prediction of GEAC separability, it is important to compare experimental and calculated values of R_g in some representative separations. Experimental R_g values are tabulated in Table IV for four different linear strength GEAC systems (linear isotherm loadings). These four separations include data for two adsorbents and three binary eluent pairs. The 39 solutes include examples from most of the compound types previously studied in fixed eluent separation: hydrocarbons, substituted hydrocarbons, and heterocyclic compounds of various types. With experimental values of R_g ranging from 3 to 96 ml, the average deviation between these 51 experimental and calculated R_g values is only $\pm$ 2.5 ml, fully confirming the accuracy of eqn. (2) in binary eluent linear strength GEAC separation, and verifying our analysis of the experimental characteristics of gradient devices of the type of Fig. 1 (which were used in measuring the experimental values). A few comments on the calculation of these R_g values are in order. Systems II, III and IV of Table IV are linear in strength over the range $V \leqslant 0.9$, as recommended in the preceding section. System I of Table IV is approximately linear over the entire range, deviating only at the lower end ($V < 10$ ml). For this reason, the best approximation to eqn. (1) for system I has a equal to 0.03, rather than to the value of $\alpha\varepsilon^\circ_A$ for the weaker eluent (0.00).

Values of $\underline{R}_p$ used in eqn. (2) may be calculated either from fundamental molecular parameters as reviewed in Part VI[3], or interpolated from $\underline{R}^\circ$ values for other eluents, and even other adsorbent activities. Where the adsorbent is the same or closely similar, the latter procedure is potentially the more accurate, particularly where $\underline{R}_p$ is extrapolated from a retention volume $\underline{R}_A$ for elution by the weak eluent A. The latter procedure, where values of $\underline{R}_A$ had previously been measured, underlies the calculation of R_g values in Table IV. The alternative of calculating $\underline{R}_p$ from tabulated parameters has been illustrated for system I in the previous paper of this series[1].

Table V presents some data for an extended GEAC separation. Following the termination of linearity in the eluent strength gradient in an initial binary eluent system, the gradient was extended by ternary eluent elution using the gradient eluents (A–B), C. The initial binary eluent system was that described for system III of Table IV, for which eluent strength linearity terminates at V equal 0.91 V°. At that point, the composition of eluent entering the column is 65.4 mole % benzene–carbon tetrachloride. A continuation of the original eluent gradient using the new system, 65.4 mole % benzene–carbon tetrachloride (weak), dioxan (strong), was then considered. For a^* equal 0.86 and V° equal 196 ml, it was calculated that b would have

TABLE IV

EXPERIMENTAL TEST OF EQUATION 2; BINARY ELUENT SYSTEMS

Solute	R_g (ml) in system							
	I		*II*		*III*		*IV*	
	Exptl.	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*
Naphthalene	12	8						
Acenaphthylene	19	16						
Phenanthrene	24	27					8	7
Anthracene	27	27						
Fluoranthene	35	40						
Triphenylene	51	53						
Chrysene	48	53						
Benzanthracene	51	53	3	4				
Perylene	63	65						
3:4-Benzpyrene	63	65	6	7				
Benzperylene	76	76						
Picene			15	13			16	22
1:2;4:5-Dibenzpyrene	96	84						
Coronene	86	84						
1:2-Benzcoronene			42	43	41	41		
1,2-Diphenylethane							8	10
Phenyl ethyl sulfide							9	9
Nitrobenzene							30	37
Methyl benzoate							52	53
2-Methoxynaphthalene							22	25
1-Nitronaphthalene			7	5				
1-Cyanonaphthalene			8	7				
1-Acetonaphthalene			23	20	18	18		
2-Acetonaphthalene			20	22				
p-Diethoxybenzene			6	4			65	67
o-Nitroanisole					18	16		
m-Nitroanisole			6	7				
p-Nitroanisole							71	71
m-Dinitrobenzene							82	84
o-Nitroaniline			75	69				
3:4-Benzacridine			8	8	7	8		
7:8-Benzquinoline					9	8		
Quinoline			30	29				
β-Naphthoquinoline			36	38	34	35		
Phenanthridine			38	37	37	35		
1-Azapyrene			40	40				
6-Nitroquinoline			53	58	51	53		
Isoquinoline			67	60	61	56		
Carbazole							50	55

System	*Adsorbent*	*Eluent A*	*Eluent B*	*a**	*a*	*b*	*V°* (ml)	*W* (g)
I	3.7% H_2O–Al_2O_3	Isooctane	Ethyl ether	0.75	0.03	0.00190	133	10.0
II	4.0% H_2O–Al_2O_3	Carbon tetrachloride	Benzene	0.69	0.113	0.00070	109	2.0
III	4.0% H_2O–Al_2O_3	Carbon tetrachloride	Benzene	0.69	0.113	0.00078	95	2.0
IV	17 % H_2O–SiO_2	Pentane	Methylene chloride	0.80	0.000	0.00125	124	1.7

TABLE V

EXPERIMENTAL TEST OF EQUATION 2; EXTENDED TERNARY ELUENT SYSTEM*

Solute	R_g (ml)	
	Exptl.	Calc.
1,2,4-Tricarbomethoxybenzene	105	109
2,4-Dinitroaniline	137	130
1-Azacarbazole	175	181
7-Azaindole	177	179
2-Aminoanthraquinone	204	206

* First stage conditions. System III of Table IV to $V = 87$ ml. Second stage conditions. Weak eluent: 65.4 mole % benzene–carbon tetrachloride. Strong eluent: dioxan. $a^* = 0.86$; $V^\circ = 196$ ml.

the same value (0.00078) as in the initial GEAC separation using carbon tetrachloride and benzene. R_g values for five solutes eluting in the second stage of this extended gradient (ternary eluent) separation are given in Table V, and these data show good agreement with calculated values from eqn. (2) (average deviation $\pm$ 4 ml).

For the four gradient elution systems of Table IV, the importance of displacement was estimated by means of Fig. 2 of the previous paper[1]. In all cases, V_s/V° (the initial fraction of the separation not at equilibrium) was estimated at less than 0.01, or column equilibrium is predicted for V greater than 1 ml. In agreement with this calculation, there is no tendency for the first eluted solutes of Table IV to have larger than calculated R_g values, with the apparent exception of system I. In the latter case, the deviation in the experimental R_g values in the first eluted solutes may actually be shown to be due to the modest failure of eqn. (1) to approximate the true eluent strength *versus* eluate volume relationship at small values of V. In agreement with theory[1], which predicts an absence of band splitting in linear strength GEAC separation, none of the solutes described in Table IV showed any tendency toward giving double peaks.

TABLE VI

BAND WIDTH IN GEAC SEPARATION; DATA FOR SYSTEM II OF TABLE IV

Solute	R_g (ml)	Band width (ml)	w/R_t
1:2-Benzanthracene	2.5	6	1.5
3:4-Benzpyrene	5.7	11	1.3
p-Diethoxybenzene	6.1	22	6.1
1-Nitronaphthalene	7.3	30	7.2
3:4-Benzacridine	7.5	11	1.4
1-Cyanonaphthalene	8.3	17	2.8
Picene	15	37	3.4
2-Acetonaphthalene	20	14	0.7
Quinoline	30	29	1.0
β-Naphthoquinoline	36	34	1.2
Phenanthridine	38	22	0.6
1-Azapyrene	41	49	1.2
6-Nitroquinoline	52	31	0.7
Isoquinoline	67	44	1.1
o-Nitroaniline	75	28	0.7

It is desirable that the widths of solute bands remain approximately constant throughout GEAC separation. While no GEAC system will meet this requirement exactly, linear strength separation is theoretically predicted[1] to give reasonably constant band widths under certain conditions: (i) solutes whose sizes (A_s) do not differ greatly; (ii) column separation efficiency constant throughout separation; (iii) solute R_g values reasonably large. Table VI presents a number of data on solute band width from system II of Table IV. Column separation efficiency (measured by band width in fixed eluent separation) normally varies with eluent flow rate and the viscosity of the eluent; the viscosities of the two eluents of system II (carbon tetrachloride and benzene) are approximately equal, and eluent velocity was held constant during the acquisition of the band width data of Table VI, so column separation efficiency was presumed near constant for these data. The solute sizes are not widely different ($8 \leqslant A_s \leqslant 14$). While the individual band widths vary from 6 to 49 ml in Table VI, band width *is* relatively independent of R_g for R_g greater than 10:

R_g range (ml)	*No. of solutes*	*Average band width (ml)*
0–10	6	16 ± 7
10–30	3	28 ± 8
30–50	3	34 ± 10
50–80	3	34 ± 6

Theoretically, the ratio of band width w to "instantaneous retention volume" R should be constant for R_g large[1]. As seen in Table VI this is only very approximately true, with several striking exceptions. This suggests that column separation efficiency may be a function of the solute.

ALKYL SUBSTITUENTS AND SOLUTE RETENTION VOLUME

The role played by alkyl substituents in determining the retention volume of the solute has been examined briefly in earlier papers[4,5]. For solutes eluted by weak eluents such as pentane, the solute alkyl substituent will be adsorbed along with the remainder of the solute. For elution by stronger eluents, theoretical considerations suggest that most of the alkyl group will lie in the solution phase when the solute adsorbs, since the weakly adsorbing alkyl carbons cannot effectively compete with the more strongly adsorbing eluent for a place on the adsorbent surface. Limited data[6] for elution of some alkyl-substituted solutes from alumina suggest that in strong eluent systems only one of the alkyl carbons of each alkyl substituent lies in the adsorbed phase, and that the area required for adsorption of the solute (A_s) is independent of the length of its alkyl substituents. Because the calculation of A_s for a solute enters into the prediction of R_g values in GEAC separation, some recently acquired data relevant to this point are summarized in Tables VII and VIII.

The data of Table VII (for alumina) and VIII (for silica) illustrate the variation of retention volume with eluent strength for several solute types possessing alkyl substituents of varying length. The general relationship between retention volume $\underline{R}^\circ$ (ml/g) and eluent strength is given by eqn. (6):

TABLE VII

EFFECT OF ALKYL SUBSTITUENTS ON SOLUTE RETENTION VOLUME FOR ELUTION BY STRONG ELUENTS FROM ALUMINA

Solute	n[a]	% H_2O-Al_2O_3	*Eluent*[b]	$\log \underline{R}_p$	$\alpha\varepsilon$[c]	$\log \underline{R}^\circ$ *Exptl.*	$\log \underline{R}^\circ$ *Calc. eqn. (6)*
Naphthalene	0	1.3	P	0.83	0.000	0.83	—
		1.3	10% C–P		0.029	0.60	—
		1.3	50% C–P		0.084	0.16	—
Methyl benzoate	1	1.3	B	2.53[d]	0.259	0.20	—
		3.7	P	1.58	0.000	1.58	—
		3.7	1.5% E–P		0.056	1.08	—
		3.7	5% E–P		0.100	0.68	—
		3.7	15% E–P		0.148	0.24	—
Methyl 3,5-dinitrobenzoate	1	1.3	B	4.46[d]	0.259	0.84	—
		1.3	M	4.72[d]	0.340	−0.04	—
		3.7	25% M–P		0.149	1.22	—
		3.7	B	3.30[d]	0.208	0.30	—
Dimethyl phthalate	1	3.7	25% M–P		0.157	1.48	—
		3.7	B	3.36[d]	0.208	0.86	—
		3.7	70% M–P		0.253	0.32	—
1-Ethylnaphthalene	2	1.3	10% C–P	0.92	0.029	0.66	0.65
		1.3	50% C–P		0.084	0.13	0.13
1-*n*-Butylnaphthalene	4	1.3	10% C–P	0.92	0.029	0.64	0.63
		1.3	50% C–P		0.084	0.07	0.07
1-*n*-Hexylnaphthalene	6	1.3	10% C–P	0.97	0.029	0.66	0.66
		1.3	50% C–P		0.084	0.10	0.08
n-Butyl benzoate	4	1.3	B	2.58[e]	0.259	−0.04	−0.04
		3.7	1.5% E–P	1.65	0.056	1.06	1.08
		3.7	5% E–P		0.100	0.61	0.64
		3.7	15% E–P		0.148	0.09	0.15
n-Decyl benzoate	10	1.3	B	2.68[e]	0.259	−0.22	−0.32
		3.7	1.5% E–P	1.72	0.056	1.05	1.07
		3.7	5% E–P		0.100	0.56	0.56
		3.7	15% E–P		0.148	−0.07	0.00
n-Hexadecyl benzoate	16	1.3	B	2.78[e]	0.259	−0.55	−0.41
		3.7	1.5% E–P	1.72	0.056	1.03	1.03
		3.7	5% E–P		0.100	0.49	0.49
		3.7	15% E–P		0.148	−0.14	−0.10
n-Butyl 3,5-dinitrobenzoate	4	1.3	B	4.64[e]	0.259	0.69	0.73
		1.3	M		0.340	−0.38	−0.49
		3.7	25% M–P	3.34[e]	0.153	1.09	1.03
		3.7	B		0.208	0.20	0.20
n-Hexadecyl 3,5-dinitro-benzoate	16	1.3	B	4.83[e]	0.259	0.55	0.35
		3.7	25% M–P	3.49[e]	0.153	0.80	0.84
		3.7	B		0.208	−0.17	−0.11
Di-*n*-butyl phthalate	4	3.7	25% M–P	3.44[e]	0.153	1.22	1.27
		3.7	B		0.208	0.54	0.49
		3.7	70% M–P		0.253	0.07	−0.15
Di-*n*-hexadecyl phthalate	16	3.7	25% M–P	3.75[e]	0.153	0.89	0.90
		3.7	B		0.208	0.21	−0.12

[a] Number of alkyl carbon atoms in substituent.

[b] % refers to per cent by volume; P = pentane; C = carbon tetrachloride; B = benzene; M = methylene chloride; E = ethyl ether.

[c] Measured through eqn. (6) from $\underline{R}^\circ$ for unsubstituted or methyl-substituted solutes for binary eluents; pure eluents calculated as usual.

[d] Calculated from eqn. (6).

[e] Calculated from value of $\underline{R}_p$ for unsubstituted or methyl-substituted solute as described in text.

$$\log \underline{R}^\circ = \log \underline{R}_p - \alpha\varepsilon^\circ A_s \quad (6)$$

The solute surface volume A_s can generally be expressed as the sum of individual contributions a_i from each solute group i:

$$A_s = \sum^{i} a_i \quad (7)$$

The data of Tables VII and VIII permit the calculation of A_s for each solute type by means of eqn. (6). Comparison of values of A_s for a solute substituted by an alkyl group C_nH_{2n+1} with the A_s value of the unsubstituted or methyl-substituted solute then permits the calculation of values of a_i for alkyl substituents of varying length. Some of the $\underline{R}_p$ values of Tables VII and VIII are experimental values for elution by pentane. Other $\underline{R}_p$ values for unsubstituted or methyl-substituted solutes are extrapolated from $\underline{R}^\circ$ values in strong eluent systems, by means of eqn. (6). Still other $\underline{R}_p$ values for alkyl-substituted solutes related to the latter unsubstituted or methyl-substituted solutes were calculated from these $\underline{R}_p$ values by adding 0.02 α for each additional methylene group (alumina[5]), or subtracting 0.05 α for each such group (silica[7]).

TABLE VIII

EFFECT OF ALKYL SUBSTITUENTS ON SOLUTE RETENTION VOLUME FOR ELUTION BY STRONG ELUENTS FROM DAVISON CODE 12 SILICA

Solute	n[a]	% H_2O–SiO_2	*Eluent*[b]	$\log \underline{R}_p$	$\alpha\varepsilon^\circ$[c]	$\log \underline{R}^\circ$ *Expll.*	$\log \underline{R}^\circ$ *Calc. eqn. (6)*
Methyl benzoate	1	16.0	P	2.13	0.000	2.13	—
		16.0	5% M–P		0.029	1.69	—
		16.0	15% M–P		0.065	1.14	—
		16.0	25% M–P		0.088	0.78	—
n-Butyl benzoate	4	16.0	5% M–P	2.04[d]	0.029	1.59	1.56
		16.0	15% M–P		0.065	0.92	0.98
		16.0	25% M–P		0.088	0.59	0.60
n-Decyl benzoate	10	16.0	5% M–P	1.87[d]	0.029	1.41	1.35
		16.0	15% M–P		0.065	0.68	0.71
		16.0	25% M–P		0.088	0.23	0.29

[a] Number of alkyl carbon atoms in substituent.
[b] % refers to per cent by volume; P = pentane; M = methylene chloride.
[c] Measured through eqn. (6) from $\underline{R}^\circ$ for methyl-substituted solute.
[d] Calculated from value of $\underline{R}_p$ for methyl-substituted solute as described in text.

Table IX summarizes values of a_i calculated for alkyl substituents of varying lengths from the data of Tables VII and VIII. These a_i values are seen to be constant for substituents of a given length, even though derived from a variety of solutes eluted by several eluents from two adsorbents of varying activity (α). A complete tabulation of a_i values for alkyl substituents containing from 1 to 20 carbon atoms is shown in Table X. As a final check on the derived values of a_i, Tables VII and VIII compare calculated values of $\log \underline{R}^\circ$ for the alkyl-substituted solutes with experimental values. The average deviation between calculated and experimental data is only ± 0.05 log units, which represents quite satisfactory agreement.

TABLE IX

EXPERIMENTAL VALUES OF a_i FOR ALKYL SUBSTITUENTS

Group	*Solute*	*Adsorbent*	A_s	a_i	*Av.* a_i
$-C_2H_5$	1-Ethylnaphthalene	Al_2O_3	9.3	1.3	1.3
$-C_4H_9$	1-*n*-Butylnaphthalene	Al_2O_3	10.1	2.1	2.1 ± 0.1
	n-Butyl benzoate	Al_2O_3	10.3	2.3	
	n-Butyl 3,5-dinitrobenzoate	Al_2O_3	15.0	2.0	
	Di-*n*-butyl phthalate	Al_2O_3	13.8	1.9	
	n-Butyl benzoate	SiO_2	14.8*	2.3	
$-C_6H_{13}$	1-*n*-Hexylnaphthalene	Al_2O_3	10.4	2.4	2.4
$-C_{10}H_{21}$	*n*-Decyl benzoate	Al_2O_3	11.6	3.6	3.7 ± 0.1
	n-Decyl benzoate	SiO_2	16.3*	3.8	
$-C_{16}H_{33}$	*n*-Hexadecyl benzoate	Al_2O_3	12.6	4.6	4.3 ± 0.3
	n-Hexadecyl 3,5-dinitrobenzoate	Al_2O_3	17.5	4.5	
	Di-*n*-hexadecyl phthalate	Al_2O_3	17.7	3.9	

* A_s values for some solutes are different on SiO_2 relative to Al_2O_3 (ref. 7).

The data originally used[4] in drawing the conclusion that only one alkyl carbon of a substituent is adsorbed (and that a_i for all alkyl groups equal one, regardless of size) *are included in Table VII*. The present modification in this original conclusion

TABLE X

VALUES OF a_i FOR ALKYL SUBSTITUENTS OF VARYING LENGTH

No. of alkyl carbons	a_i	*No. of alkyl carbons*	a_i
1	1.0	11	3.8
2	1.4	12	3.9
3	1.7	13	4.0
4	2.1	14	4.1
5	2.3	15	4.2
6	2.6	16	4.3
7	2.9	17	4.4
8	3.1	18	4.4
9	3.4	19	4.5
10	3.6	20	4.5

reflects certain limitations on the use of eqn. (6) in the experimental determination of A_s values. Unless the range of eluent strengths covered in such a determination is large (as was not the case in the original study[4]), inaccurate values of A_s can result.

GLOSSARY OF TERMS

a, b	Constants in eluent strength *versus* eluate volume relationship, eqn. (1).
a_i	Contribution of group i to solute surface volume A_s.
a^*	GEAC separation parameter, for device of Fig. 1, defined by eqn. (4a).
A, B, C	Refer to solvents used as eluents in GEAC separation.
A_A, A_B	Cross sectional areas of gradient device of Fig. 1.
A_s	Solute surface volume, see eqn. (6).

d_A, d_B	Densities of eluents A and B.
h_A, h_B	Initial heights of eluent in two chambers of device of Fig. 1.
M_A, M_B	Molecular weights of solvents A and B.
n	Number of alkyl carbons in alkyl substituent.
n_b, n_c	Value of A_s for eluents B and C.
$\underline{R}^\circ$	Solute linear equivalent retention volume (ml/g).
$\underline{R}_p$	Value of $\underline{R}^\circ$ for elution by pentane.
$\underline{R}_A$	Value of $\underline{R}^\circ$ for elution by eluent A.
R_g	Retention volume (ml) of a solute in GEAC separation.
R_t	Instantaneous retention volume (see Part VII[1]).
V	Eluate volume (ml).
V_s	Initial GEAC eluent volume during which displacement occurs (see Part VII[1]).
V°	Total volume of eluent (A and B) stored in gradient device of Fig. 1 prior to beginning of separation.
V°_A	Total volume of eluent A stored in its chamber prior to beginning of separation.
V_A, V_B	Volume fraction of eluents A or B in binary eluent mixture.
W	Weight of adsorbent (g) in column.
w	Solute band width (ml), measured from 4 to 96 % solute elution from column.
X_B, X_C	Mole fraction of eluents B and C in a binary or ternary solvent mixture.
α	Adsorbent activity function.
$\varepsilon^\circ, \varepsilon^\circ_A, \varepsilon^\circ_B, \varepsilon^\circ_C$	Eluent strength parameters for indicated solvents.

SUMMARY

The technique of *linear strength* gradient elution has been studied experimentally under linear isotherm conditions. A previously derived theoretical relationship between solute retention volume (in linear strength gradient elution), solute molecular structure, and experimental separation conditions has been verified. The unique advantages of linear strength separation have been confirmed. Simple experimental devices for carrying out linear strength gradient elution separation are described, and a number of specific linear strength separations cataloged for easy duplication. The effect of solute alkyl groups on retention volume in strong eluent systems has been re-examined on the basis of new experimental data, and previous conclusions with respect to the retention volumes of these solutes have been modified.

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A CONTRIBUTION TO THE THEORY OF THE PAPER CHROMATOGRAPHY OF ACIDS

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INTRODUCTION

Paper chromatography is normally considered as a partition phenomenon. The R_F values of the separated compounds are a function of their partition coefficient (α) between the mobile phase and the stationary phase. For neutral substances, α will be directly related to the difference between the thermodynamic standard potentials in the two phases. These standard potentials will depend upon the composition of the solvent system, which usually consists of a mixture of water and one or more organic liquids. Changes in the relative proportions of the components or variations in the organic liquids will affect the partition coefficients. When acids are separated, the addition of a buffer or a base to the solvent may affect the R_F values by modifying the electrolytic dissociation of the acids, by forming complexes between the cation of the electrolyte added and the anions of these acids and by causing an electric potential to develop between the two phases.

The most simple case is that in which no dissociation of the acids occurs in the mobile phase. This situation has been dealt with by WAKSMUNDZKI AND SOCZEWIŃSKI[1] (see also ref. 2). Only the effect of the buffer or base upon the dissociation of the acid in the stationary phase must be considered in this case. The equilibrium between the two phases is represented by:

$$\begin{array}{ll} \text{stationary phase } (s) & HA_s \rightleftharpoons H_s^+ + A_s^- \\ & \quad \Updownarrow \\ \text{mobile phase } (m) & HA_m \end{array}$$

The equilibrium involved will be determined by the partition coefficient of HA, by the dissociation constant of the acid in the stationary phase and by the pH of that phase.

The assumption that dissociation of the acids does not occur in the mobile phase does not apply to rather polar solvents. Here the presence of ions in that phase should also be considered. The equilibrium between the two phases is now represented by:

$$\begin{array}{lccccc} \text{stationary phase} & HA_s & \rightleftharpoons & H_s^+ & + & A_s^- \\ & \Updownarrow & & \Updownarrow & & \Updownarrow \\ \text{mobile phase} & HA_m & \rightleftharpoons & H_m^+ & + & A_m^- \end{array}$$

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Since ions are now directly involved in the partition of the acid between the two phases, possible differences in electric potential will affect the equilibria. Such potential can be expected to occur generally when electrolytes are added to the solvent system. This can be illustrated for the case where a base BOH is present in the solvent. Then the following equations will hold for the thermodynamic equilibrium:

$$\mu_{0,B^+,s} + RT\ln c_{B^+,s} + FE_s = \mu_{0,B^+,m} + RT\ln c_{B^+,m} + FE_m \quad (1)$$

$$\mu_{0,OH^-,s} + RT\ln c_{OH^-,s} - FE_s = \mu_{0,OH^-,m} + RT\ln c_{OH^-,m} - FE_m \quad (2)$$

Since electroneutrality requires that $c_{B^+,s} = c_{OH^-,s}$ and $c_{B^+,m} = c_{OH^-,m}$ when $c_{H^+} \ll c_{OH^-}$, the relation for $E_s - E_m = \Delta E$ will become on subtracting eqn. (2) from eqn. (1):

$$2F\Delta E = \Delta\mu_{0,OH^-} - \Delta\mu_{0,B^+} \quad (3)$$

where Δ represents the difference of the quantity involved between the stationary phase and the mobile phase.

The values of the thermodynamic standard potentials of the base added to the solvent will therefore influence the partition of the acid between the two phases. Consequently, alteration of a buffer or base in a solvent system will influence the R_F values, even if no change of the pH in the stationary phase has occurred.

In addition, there is the possibility of ion association. This is most likely when the dielectric constant of the solvent is rather low and when the concentration of the complexing ion is large. An effect of the electrolytes added to the solvent system may also be expected.

In the present publication two models are discussed. In the first case, total dissociation of the acids to be separated is assumed to occur in both phases. In the second, ion association is assumed to occur to some extent in the mobile phase, while the acids are almost completely dissociated in the stationary phase.

THEORY

Model 1

A theory is developed first for the case in which a polybasic acid H_kA is completely dissociated in both phases into kH$^+$ and A^{-k}. The thermodynamic equilibrium equation describing the partition of the ion A^{-k} between the two phases is given by eqn. (4), neglecting activity coefficients:

$$\mu_{0,A^{-k},s} - kFE_s + RT\ln c_{A^{-k},s} = \mu_{0,A^{-k},m} - kFE_m + RT\ln c_{A^{-k},m} \quad (4)$$

The R_F value of the acid is a function of the partition coefficient (α) of the acid between the two phases[3]. Since the amounts of the undissociated acid and of the other ions are assumed to be negligible relative to the concentration of the ion A^{-k}, α will by approximation be equal to the partition coefficient of that ion:

$$\alpha_k = \frac{q_m}{q_s}\left(\frac{1}{R_{F,k}} - 1\right) = \frac{c_{A^{-k},s}}{c_{A^{-k},m}} = e^{(-\Delta\mu_{0,A^{-k}} + kF\Delta E)/RT} \quad (5)$$

The index k means that an acid with k acidic groups is considered; q_s and q_m are the cross sections of the two phases. The symbol Δ is explained in the introduction.

Making use of the R_M value as defined by BATE-SMITH AND WESTALL[4], we are able to eliminate the e powers from eqn. (5):

$$R_{M,k} = \log\left(\frac{1}{R_{F,k}} - 1\right) = \log\frac{q_s}{q_m}\alpha_k = \log\frac{q_s}{q_m} - \frac{\Delta\mu_{0,A^{-k}}}{2.3\,RT} + \frac{kF\Delta E}{2.3\,RT} \qquad (6)$$

Assuming that the separated ions do not influence the electric potential significantly, *i.e.* when $kc_{A^{-k}} \ll c_{B^+}$ holds in both phases, we can apply eqn. (3) to eliminate ΔE from eqn. (6):

$$R_{M,k} = \log\frac{q_s}{q_m} - \frac{\Delta\mu_{0,A^{-k}}}{2.3\,RT} + k\,\frac{\Delta\mu_{0,OH^-} - \Delta\mu_{0,B^+}}{4.6\,RT} \qquad (7)$$

The term $\log q_s/q_m$ can be eliminated by using the R_M value of the undissociated acid: $R_{M,k,0}$. The thermodynamic equilibrium equation for the acid is:

$$\mu_{0,H_kA,s} + RT\ln c_{H_kA,s} = \mu_{0,H_kA,m} + RT\ln c_{H_kA,m} \qquad (8)$$

From eqn. (8) the equation for the $R_{M,k,0}$ value can be derived:

$$R_{M,k,0} = \log\frac{q_s\,c_{H_kA,s}}{q_m\,c_{H_kA,m}} = \log\frac{q_s}{q_m} - \frac{\Delta\mu_{0,H_kA}}{2.3\,RT} \qquad (9)$$

The difference of the R_M value of the ion A^{-k} (eqn. 7) and the R_M value of the undissociated acid (eqn. 9) is given by:

$$R_{M,k} - R_{M,k,0} = \frac{\Delta\mu_{0,H_kA} - \Delta\mu_{0,A^{-k}}}{2.3\,RT} + k\,\frac{\Delta\mu_{0,OH^-} - \Delta\mu_{0,B^+}}{4.6\,RT} \qquad (10)$$

It is evident that the first term of the right-hand side of eqn. (10) is related to the differences in the pK's of the acid involved in the two phases. This relation is expressed by the following equation:

$$\frac{\mu_{0,H_kA} - \mu_{0,A^{-k}} - k\mu_{0,H^+}}{2.3\,RT} = -\sum_{1\to k}^{i} pK_i \qquad (11)$$

Equation (10) can be converted by means of eqn. (11) into:

$$R_{M,k} - R_{M,k,0} = -\sum_{1\to k}^{i}\Delta pK_i + k\,\frac{2\,\Delta\mu_{0,H^+} + \Delta\mu_{0,OH^-} - \Delta\mu_{0,B^+}}{4.6\,RT} \qquad (12)$$

It follows from eqn. (12) that, when our model holds, differences of $R_M - R_{M,0}$ for anions of the same valency can only be due to differences of the sums of the ΔpK_i values. ΔpK for monobasic acids is related to the reciprocal value of the dielectric constant D. This relation is expressed by:

$$\Delta pK = a\left(\frac{1}{D}\right) \qquad (13)$$

The factor a depends upon the number, the volume and the position of substituents present in the acid involved. Generally a increases, when steric inhibition of solvation occurs[5]. Acids having no substituents or substituents that are analogous may be expected to have the same value of a and also the same value of $R_M - R_{M,0}$, if the model developed by us holds.

The effect of a change of the base upon the R_M values is shown by eqn. (14):

$$R_{M,k,\mathrm{I}} - R_{M,k,\mathrm{II}} = k \frac{\Delta\mu_{0,\mathrm{B}^+,\mathrm{II}} - \Delta\mu_{0,\mathrm{B}^+,\mathrm{I}}}{4.6\,RT} \qquad (14)$$

I and II denote the two different bases.

It follows from eqn. (14) that in our model the difference between the R_M values is independent of the acid involved, provided acids containing the same number of acidic groups are considered.

Model 2

Having considered almost complete dissociation of the acids in the mobile phase, possible ion association in that phase must now be considered. When the ion association is very small in the stationary phase, the R_M value of the acid is represented by:

$$R'_{M,k} = \log \frac{q_s}{q_m}\alpha'_k = \log \frac{q_s}{q_m} \frac{c_{\mathrm{A}^{-k},s}}{(c_{\mathrm{A}^{-k},m} + c_{\mathrm{BA}^{-k+1},m} + c_{\mathrm{B_2A}^{-k+2},m} + \cdots + c_{\mathrm{B}_k\mathrm{A},m})}$$

$$= \log \frac{q_s}{q_m} \frac{c_{\mathrm{A}^{-k},s}}{c_{\mathrm{A}^{-k},m}\left(1 + \frac{c_{\mathrm{B}^+,m}}{K'_{1,m}} + \frac{c^2{}_{\mathrm{B}^+,m}}{K'_{1,m}\,K'_{2,m}} + \cdots + \frac{c^k{}_{\mathrm{B}^+,m}}{K'_{1,m}\,K'_{2,m}\cdots K'_{k,m}}\right)} \qquad (15)$$

The accent is used to distinguish the quantities involved from those concerned in the first model. Activity coefficients are not considered in this case, either. K'_i is the dissociation constant of the complex $\mathrm{B}_{k-i+1}\mathrm{A}^{-i+1}(i = 1\cdots k)$. The quotient of the concentrations of the completely dissociated ions in the two phases is equal to α_k and can be eliminated from eqn. (15) by means of the relation for $R_{M,k}$ (see eqn. 6). The quotient q_s/q_m is then also eliminated:

$$R'_{M,k} = R_{M,k} - \log\left(1 + \frac{c_{\mathrm{B}^+,m}}{K'_{1,m}} + \frac{c^2{}_{\mathrm{B}^+,m}}{K'_{1,m}\,K'_{2,m}} + \cdots + \frac{c^k{}_{\mathrm{B}^+,m}}{K'_{1,m}\,K'_{2,m}\cdots K'_{k,m}}\right)^{*} \qquad (16)$$

It is evident that where the dissociation constants of the complexes greatly exceed the concentration of the base cation, the logarithmic term of eqn. (16) approaches zero. On the other hand when the dissociation constants $K'_{i,m}$ are much smaller than the concentration of the cation $c_{\mathrm{B}^+,m}$, eqn. (16) approaches:

$$R'_{M,k} = R_{M,k} - \log \frac{c^k{}_{\mathrm{B}^+,m}}{K'_{1,m}\,K'_{2,m}\cdots K'_{k,m}} = R_{M,k} - \sum_{1 \to k}^{i} \mathrm{p}K'_{i,m} - k \log c_{\mathrm{B}^+,m} \qquad (17)$$

* Making use of the mathematical symbol Π indicating multiplication, eqn. (16) becomes:

$$R'_{M,k} = R_{M,k} - \log\left(1 + \sum_{1 \to k}^{j} \prod_{1 \to j}^{i} \frac{c_{\mathrm{B}^+,m}}{K'_{i,m}}\right)$$

The difference $R'_{M,k} - R_{M,k,0}$ is given by the following equation obtained by eliminating $R_{M,k}$ from eqn. (17) by means of eqn. (12):

$$R'_{M,k} - R_{M,k,0} = - \sum_{\substack{i \\ I \to k}} \Delta pK_i + k \frac{2\,\Delta\mu_{0,H^+} + \Delta\mu_{0,OH^-} - \Delta\mu_{0,B^+}}{4.6\,RT} - \sum_{\substack{i \\ I \to k}} pK'_{i,m} - k \log c_{B^+,m} \qquad (18)$$

This is the equation for the R_M value of an acid, which is almost completely associated in the mobile phase and almost completely dissociated in the stationary phase. The R_M values for incomplete association in the mobile phase lie in the range defined by eqns. (18) and (12).

It follows from eqn. (16) that, contrary to what is expected in the first model, the concentration of the cation of the bases also influences the R_M value in the second case considered. This concentration will depend upon the dissociation constant of the base in the solvent and upon the concentration of the base.

The effect of replacing the base by another base is expressed by eqn. (19), which is derived from eqn. (16) and (14):

$$R'_{M,k,I} - R'_{M,k,II} = k \frac{\Delta\mu_{0,B^+,II} - \Delta\mu_{0,B^+,I}}{4.6\,RT} + \log \frac{\left(1 + \frac{c_{B^+,m,II}}{K'_{1,m,II}} + \frac{c^2{}_{B^+,m,II}}{K'_{1,m,II}\,K'_{2,m,II}} + \cdots + \frac{c^k{}_{B^+,m,II}}{K'_{1,m,II}\,K'_{2,m,II}\cdots K'_{k,m,II}}\right)}{\left(1 + \frac{c_{B^+,m,I}}{K'_{1,m,I}} + \frac{c^2{}_{B^+,m,I}}{K'_{1,m,I}\,K'_{2,m,I}} + \cdots + \frac{c^k{}_{B^+,m,I}}{K'_{1,m,I}\,K'_{2,m,I}\cdots K'_{k,m,I}}\right)} \qquad (19)$$

It is evident from eqn. (19) that proportionality of $R'_{M,I} - R'_{M,II}$ to k may not be generally expected. Deviations from such a proportionality, therefore, may indicate complex formation.

DISCUSSION

We are aware of the incompleteness of the proposed models. The approximations are rather rough: activity coefficients are neglected, possible effects of the ions separated upon the partition of charges between the two phases are not considered, nor is the influence of the ions upon the dissociation of the base. When the dielectric constant of the solvent is low and the acids have a large pK, incomplete dissociation of the acids in the mobile phase should also be taken into account. Furthermore, association of the acids or ions may also be possible. On the other hand, consideration of all these possibilities leads to very complex equations.

We have checked one of the most important consequences of the theory developed, namely that the R_M values should depend upon the base applied to the solvent. The solvent system is composed of *n*-propanol–2 *N* methylamine (70:30) or of *n*-propanol–2 *N* isopropylamine (70:30). These solvent systems are denoted by I and II respectively. The acids used by us are assumed to be almost completely dissociated in these solvents, since the bases applied are rather strong and the dielectric constant of the medium is not very low. Addition of a solution of a "Merck" universal indi-

cator showed that acids with a pK_{water} of about 9 are about 50% dissociated in the solvent. Most of the acids used by us for checking the effect of the base have no higher pK values than about 6. It seems rather improbable that the dissociation of these acids in the moving phase should not be nearly complete. Even acids having a pK of about 7, such as the second pK of maleic acid or the third pK of mellitic acid may be dissociated to a large extent, unless the increase of the pK values of these acids on changing from water to the propanol–water mixture is much larger than for the acids present in the universal indicator.

On the other hand extensive formation of ion complexes between the cations of the base and the anions is uncertain. The results obtained must therefore always be considered from two points of view, the presence of single ions in the mobile phase and the occurrence of ion complexes in addition to these ions.

The experiments were carried out as follows:

The acids are spotted once on a sheet of Whatman No. 541 paper within a circle of about 30 mm² as 0.1 *N* solutions in 2 *N* methylamine or in 2 *N* isopropylamine respectively. The chromatograms are then equilibrated for 90 min at 25° and are developed in the horizontal direction. The solvent level is held 4.5 cm from the start-

TABLE I

MEAN R_F VALUES, R_M VALUES AND DIFFERENCES IN R_M VALUES FOUND WITH THE SOLVENTS PROPANOL–2 *N* METHYLAMINE (70:30) (I) AND PROPANOL–2 *N* ISOPROPYLAMINE (70:30) (II) RESPECTIVELY

The mean R_F values have been calculated from the mean R_M values. The chromatograms are made on Whatman No. 541 paper at 25° by the horizontal technique. σ = standard deviation.

Compounds	$R_{F,I}$	$R_{F,II}$	$R_{M,I}$	σ_I	$R_{M,II}$	σ_{II}	$R_{M,I}-R_{M,II}$
Neutral compounds							
Glycerol	0.55	0.56	—0.09	0.05	—0.10	0.02	0.01
Sorbitol	0.35	0.36	0.26	0.05	0.25	0.05	0.01
Average				0.05		0.04	0.01
Monobasic acids							
Nitric acid	0.51	0.64	—0.02	0.02	—0.25	0.02	0.23
Hydrochloric acid	0.41	0.54	0.15	0.03	—0.07	0.02	0.22
Perchloric acid	0.63	0.76	—0.23	0.04	—0.50	0.04	0.27
Formic acid	0.45	0.55	0.09	0.05	—0.09	0.04	0.18
Acetic acid	0.47	0.57	0.06	0.05	—0.12	0.05	0.18
Propionic acid	0.57	0.68	—0.12	0.06	—0.33	0.07	0.21
Butyric acid	0.66	0.75	—0.28	0.03	—0.48	0.07	0.20
Palmitic acid	0.86	0.91	—0.79	0.12	—1.01	0.14	0.22
Isobutyric acid	0.64	0.76	—0.25	0.07	—0.50	0.04	0.25
Iodoacetic acid	0.57	0.71	—0.13	0.03	—0.38	0.04	0.25
Chloroacetic acid	0.53	0.65	—0.06	0.03	—0.26	0.01	0.20
Dichloroacetic acid	0.68	0.78	—0.33	0.03	—0.55	0.06	0.22
Trichloroacetic acid	0.78	0.85	—0.54	0.04	—0.76	0.10	0.22
Benzoic acid	0.68	0.76	—0.32	0.02	—0.51	0.06	0.19
Picric acid	0.82	0.89	—0.66	0.08	—0.91	0.07	0.25
Nicotinic acid	0.56	0.67	—0.10	0.04	—0.31	0.05	0.21
Indolylacetic acid	0.60	0.68	—0.17	0.04	—0.32	0.03	0.15
Average				0.05		0.05	0.215 ±0.007

(continued on p. 367)

TABLE I (*continued*)

Compounds	$R_{F,I}$	$R_{F,II}$	$R_{M,I}$	σ_I	$R_{M,II}$	σ_{II}	$R_{M,I}-R_{M,II}$
Dibasic acids							
Sulphuric acid	0.14	0.30	0.78	0.05	0.37	0.01	0.41
Oxalic acid	0.15	0.32	0.75	0.04	0.33	0.01	0.42
Malonic acid	0.17	0.30	0.68	0.05	0.36	0.05	0.32
Succinic acid	0.19	0.34	0.62	0.04	0.28	0.03	0.34
Fumaric acid	0.20	0.34	0.60	0.09	0.29	0.06	0.31
Maleic acid	0.21	0.38	0.58	0.07	0.22	0.03	0.36
Malic acid	0.17	0.32	0.69	0.02	0.33	0.03	0.36
Tartaric acid	0.15	0.29	0.75	0.04	0.38	0.03	0.37
Phthalic acid	0.33	0.51	0.30	0.10	−0.02	0.04	0.32
Glucose-6-phosphoric acid	0.11	0.20	0.92	0.09	0.61	0.07	0.31
Fructose-6-phosphoric acid	0.12	0.19	0.88	0.07	0.63	0.06	0.25
Phenyl phosphoric acid	0.31	0.49	0.35	0.01	0.01	0.02	0.34
Average				0.06		0.04	0.343 ±0.015
Tribasic acids							
Citric acid	0.10	0.27	0.95	0.02	0.44	0.05	0.51
Aconitic acid	0.10	0.26	0.95	0.03	0.45	0.02	0.50
Tricarballylic acid	0.11	0.24	0.93	0.05	0.49	0.02	0.44
Trimethylcarballylic acid	0.13	0.30	0.83	0.03	0.37	0.02	0.46
Average				0.03		0.03	0.478 ±0.017
Tetrabasic acids							
Pentanetetracarbonic acid	0.04	0.16	1.33	0.03	0.71	0.02	0.62
Ethylenediaminotetraacetic acid	0.10	0.23	0.94	0.03	0.53	0.14	0.41
Fructose-1,6-diphosphoric acid	0.03	0.09	1.49	0.13	1.01	0.11	0.48
Phenolphthalein diphosphoric acid	0.09	0.26	1.00	0.14	0.46	0.06	0.54
Average				0.08		0.08	0.513 ±0.048
Hexabasic acid							
Mellitic acid	0.02	0.22	1.66	0.09	0.54	0.03	1.12

ing line. The duration of development is 7 h; after that time the front is at a distance of about 30 cm from the starting line. The acids were detected according to HOWE[6]. The neutral compounds are oxidized to acids by spraying the chromatograms with a 1 % solution of $NaIO_3$. All determinations of the R_F values are repeated 3–7 times. Table I gives the mean R_F values and the mean R_M values for the acids, and also the differences $R_{M,I} - R_{M,II}$.

It is clearly seen from our results that a significant decrease of the R_M values occurs on changing the base from methylamine to isopropylamine. This can be explained by the fact that the isopropylamine has a larger "affinity" for the mobile phase than the methylamine. On the other hand the more polar methylamine will have a lower thermodynamic standard potential in the stationary phase than isopropylamine. The difference in electric potential between the two phases ΔE will therefore be different for the two solvents. The effect of the change of the bases increases with increasing number of acidic groups as expected from eqns. (14) and (19). This increase is mathematically significant for the differences between monobasic,

dibasic and tribasic acids. The relation of $R_{M,k,I} - R_{M,k,II}$ and k, however, is not strictly linear. The results can be expressed by one of the following two relations, in which A, B, C and D are constants:

$$R_{M,k,I} - R_{M,k,II} = A + kB \quad (20)$$

$$R_{M,k,I} - R_{M,k,II} = kC - k^2D \quad (21)$$

This is illustrated in Fig. 1 and Fig. 2 respectively. The point for mellitic acid has been omitted in these figures, since only one observation was made for the hexabasic acids.

Possibly the additional term in eqn. (19) accounting for complex formation in the mobile phase gives rise to the deviations from linearity. The last term of eqn. (19)

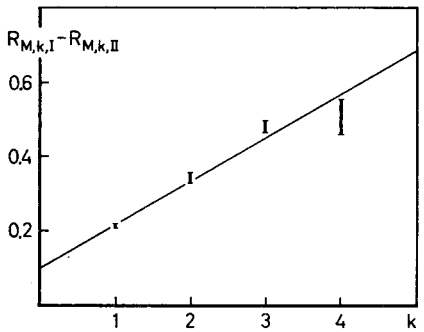

Fig. 1. The effect of changing the base present in the solvent system propanol–2 N base (70:30) from methylamine (I) to isopropylamine (II) upon the R_M values of acids bearing different numbers of acidic groups (k). Average data from Table I. The point for mellitic acid has been omitted. The lengths of the vertical stripes indicate the standard deviations of the values concerned.

is not, however, expected to be very great. The concentrations of the cation in the mobile phase will be equal to the concentrations of the ions in the solvent system before contact with the stationary phase, since the papers are equilibrated before chromatography. The amines have approximately the same pK values, thus the concentration of the methylamine cation will be nearly equal to the concentration of the isopropylamine cation. Furthermore the dissociation constants $K'_{1,m} \cdots K'_{k,m}$

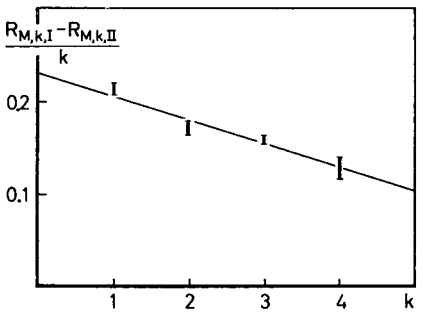

Fig. 2. A plot of $(R_{M,k,I} - R_{M,k,II})/k$ for the same data as given in Fig. 1.

TABLE II

AVERAGE VALUES OF $R_M - R_{M,0}$ FOR UNSUBSTITUTED UNBRANCHED ALIPHATIC ACIDS (A) AND FOR AROMATIC ACIDS (B) AS FOUND BY HOWE[6]

Solvent system propanol–2 N NH_4OH (70:30) at 20° on Whatman No. 1 paper by the ascending technique.

k	A	n	B	n
1	0.49 ± 0.005	7	0.69 ± 0.028	6
2	1.06 ± 0.037	9	1.04 ± 0.06	2
3	1.43	1	1.69	1
4			1.90 ± 0.01	2

are not likely to differ very much for the two bases, unless the ionic volume of the cation plays an important role. At any rate it is evident that the main effect expected theoretically, namely a difference in R_M on changing the base and an increase of this difference with increasing number of acidic groups, is confirmed.

A check of the equations for $R_{M,k} - R_{M,k,0}$, eqns. (12) or (18), has not been carried out by us. HOWE[6], however, recently published a large number of these values for the solvent system propanol–2 N NH_4OH (70:30). He found an increase in $R_M - R_{M,0}$ with increasing number of acidic groups (*cf.* Table II). This increase is roughly proportional to k. It should mean, according to our models, that $\Sigma \Delta$ pK_i or possibly also Σ p$K'_{i,m}$, is approximately linearly related to k.

The differences between monobasic acids belonging to different groups may possibly be explained by differences in Δ pK values. These differences may be due to the presence of aromatic substituents in the acids of group B, Table II, with consequent increase in a. Data for a (eqn. 13), however, are rather scarce, so that we are not able to check this possibility. Differences in pK'_m may also play a role when ion association occurs to some extent in the mobile phase. Values of pK'_m, however, are not available.

It may be concluded, that the observations of HOWE, too, are mainly in accordance with the theories developed. A calculation of $R_{M,k} - R_{M,k,0}$ or $R_{M,k,I} - R_{M,k,II}$ based upon values of ΔpK, pK'_m and thermodynamic standard potentials, however, may give definite evidence for or against the validity of the equations derived.

LIST OF SYMBOLS EMPLOYED

α = partition coefficient between stationary phase and mobile phase
μ = chemical potential
c = concentration
K_i = i^{th} dissociation constant of the acid H_kA
K'_i = i^{th} dissociation constant of the ion complex B_kA
k = number of acidic groups
R = gas constant
T = absolute temperature
q = cross section
D = dielectric constant
F = Faraday constant
E = electric potential

R_F = distance of spot from origin divided by distance of solvent front from origin
R_M = $\log (1/R_F - 1)$
$R_{M,k,0}$ = R_M value of the undissociated acid H_kA
$R_{M,k}$ = R_M value of the acid when partial or complete dissociation occurs
$R'_{M,k}$ = R_M value of the acid when ion association occurs

Operator signs

Π = product
Σ = summation
ln = natural logarithm
log = logarithm to base 10
Δ = difference of the quantity involved between stationary phase and mobile phase

Indices

s = stationary phase
m = mobile phase

ACKNOWLEDGEMENT

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SUMMARY

An extended theory is given for the R_M values of acids separated by paper chromatography in alkaline solvents. Two models are developed. One model deals with the case in which the acids are almost completely dissociated in both the stationary phase and the mobile phase. The second model is based on the assumption that the ions associate to some extent in the mobile phase with the cations of the base from the solvent. It can be predicted that replacing the base in the solvent system by another base will lead to a change in the R_M value and correspondingly to a change in the R_F value in both cases. This effect is also shown experimentally. In addition the influence on this change of the number of acidic groups in the acids is discussed.

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IDENTIFIZIERUNG VON GLYCIDYLÄTHERN

PAPIERCHROMATOGRAPHISCHE IDENTIFIZIERUNG UND TRENNUNG VON 2,4-DINITROPHENYLHYDRAZONEN SUBSTITUIERTER ACETALDEHYDDERIVATE

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In den vorhergehenden Arbeiten[1–3] befassten wir uns mit dem Studium der Trennung und Identifizierung von Glycidyläthern. Eine dieser Arbeiten[2] beschreibt die katalytische Hydratation von Glycidyläthern, die auf diese Weise in α-Alkyl(Aryl)Äther des Glycerins umgewandelt werden können. Die letztgenannten Verbindungen enthalten an die vizinalen Kohlenstoffatome gebundene primäre und sekundäre Hydroxylgruppen und unterliegen sehr leicht der oxydierenden Wirkung der Überjodsäure, wobei sie in die betreffenden Substitutionsderivate des Acetaldehyds überführt werden.

Aus den Arbeiten bezüglich der Identifizierung von organischen Stoffen ist das Bestreben ersichtlich, Derivate mit farbigen Reagentien[4,5] mit den geeigneten funktionellen Gruppen herzustellen, die eine empfindliche Detektion und somit eine weitere Möglichkeit der Identifizierung mittels der chromatographischen Methoden[6,7] ermöglichen. Aus den Beispielen der Identifizierung von Alkoholen als Ester verschiedenartig substituierter Nitrobenzoesäuren und Aldehyde oder Ketone als Nitro- und Dinitrophenylhydrazone, resultieren gute Eigenschaften von Reagentien mit Nitrogruppen[8]. Diese Tatsachen führten uns dazu, dass wir die selektive Oxydation mit der Überjodsäure[9] ausnützten, die 1,2-Diole unter gleichzeitiger Bildung der zugehörigen Aldehyde oxydiert, wobei sie selbst zu Jodsäure reduziert wird. Diese Reaktion gilt im allgemeinen für alle Verbindungen, die an die vizinalen Kohlenstoffatome gebundenen Hydroxylgruppen enthalten und wird für die massanalytische Bestimmung dieser Stoffe[10,11] benutzt. Die Verbindungen, die vizinale primäre und sekundäre Hydroxylgruppen enthalten, unterliegen der Oxydation, wobei z.B. 1,2-Propandiol Acetaldehyd und Formaldehyd gewährt, wogegen 2,3-Butandiol zu Acetaldehyd[12] übergeführt wird:

$$CH_3\text{–}\underset{\displaystyle OH}{\underset{|}{CH}}\text{–}CH_2OH \xrightarrow{HJO_4} CH_3CHO + CH_2O$$

$$CH_3\text{–}\underset{\displaystyle OH}{\underset{|}{CH}}\text{–}\underset{\displaystyle OH}{\underset{|}{CH}}\text{–}CH_3 \xrightarrow{HJO_4} 2\ CH_3CHO$$

Im Sinne der angeführten Oxydationsreaktionen bildet das Molekül von α-Alkyl(Aryl)-

Äthern des Glycerins durch Einwirkung von Überjodsäure ein Gemisch von Aldehyden:

$$R\text{-}O\text{-}CH_2\text{-}\underset{\displaystyle OH}{\underset{|}{CH}}\text{-}CH_2OH \xrightarrow{HJO_4} R\text{-}O\text{-}CH_2\text{-}CHO + CH_2O$$

im gegebenen Fall ein Gemisch von Alkoxyacetaldehyd und Formaldehyd. Diese Verbindungen enthalten eine Carbonylgruppe, die dann mit den geeigneten Reagentien unter gleichzeitiger Bildung von kristallinischer Derivate in die Reaktion eintritt.

In dieser Arbeit haben wir die Möglichkeit der Verwendung von Papierchromatographie für die Verfolgung der Reinheit, Trennung und Identifizierung von Dinitrophenylhydrazonen der durch die Oxydation der Glycerinäther mit Überjodsäure gewonnenen Alkoxy- und Aryloxyderivate studiert. In der Literatur wird eine Reihe von Verfahren für die Trennung verschiedener Aldehyde und Ketone durch die Papierchromatographie beschrieben. Infolge ihrer Flüchtigkeit können diese Stoffe nicht in freiem Zustand sondern nach deren Überführung auf geeignete Derivate chromatographiert werden. Am meisten wurden 2,4-Dinitrophenylhydrazone[14-18] chromatographiert.

Die Oxydation von Glycerinäthern wurde im Medium der verdünnten Essigsäure (50 %ige) durchgeführt, wo unter gleichzeitigem Überschuss der Überjodsäure die Oxydation von Formaldehyd auf Ameisensäure, bzw. auf Kohlendioxyd und Wasser[13] stattfindet, wobei die Carbonylgruppe von Alkoxy-Acetaldehyd unverändert bleibt. Nach durchgeführter Oxydation haben wir den Überschuss der Überjodsäure, sowie die durch die Reduktion gebildete Jodsäure durch Zugabe von festem Kaliumjodid zerstört, das ausgeschiedene Jod mit Natriumthiosulfat in farbloses Tetrathionat übergeführt und die klare Lösung mit Lösung von 2,4-Dinitrophenylhydrazin in Eisessigsäure gefällt. Das ausgeschiedene 2,4-Dinitrophenylhydrazon wurde abfiltriert, mit destilliertem Wasser gewaschen, getrocknet und aus dem Gemisch von Chloroform–Äthanol umkristallisiert. Mit dem im experimentellen Teil angeführten Standardverfahren haben wir 2,4-Dinitrophenylhydrazone der zugehörigen Acetaldehydderivate hergestellt. Die Derivatausbeuten betrugen 55–65 %.

EXPERIMENTELLER TEIL

Die präparative Herstellung von Glycidyläthern und deren Überführung durch katalytische Hydratation in α-Alkyl(Aryl)Äther des Glycerins wurde bereits auf anderer Stelle[2] beschrieben.

Die Oxydation von α-Alkyl(Aryl)Äthern des Glycerins

Die Herstellung von 2,4-Dinitrophenylhydrazon des Cyclohexylacetaldehyds. 1 g Cyclohexyläther des Glycerins wurde in 40 ml 50 %iger Essigsäure in einem 500 ml Erlenmeyerkolben aufgelöst. In diese Lösung wurde bei normaler Temperatur binnen 2 Std. durch Scheidetrichter eine Lösung von 2 g Überjodsäure in 40 ml 50 %iger Essigsäure dosiert. Das Gemisch wurde mit elektromagnetischem Rührer 3 Std. lang gemischt und der Überschuss von Überjodsäure durch sukzessive Zugabe von 3.2 g festem Kaliumjodid zerstört. Das ausgeschiedene Jod wurde durch kristallinisches

Natriumthiosulfat in Tetrathionat übergeführt und die klare, vollständig farblose Lösung mit der Lösung von 2,4-Dinitrophenylhydrazin (1.9 g in 50 ml Eisessigsäure) gefällt. Das gewonnene 2,4-Dinitrophenylhydrazon wurde abfiltriert, mit Wasser gewaschen, getrocknet und aus dem Gemisch von Chloroform–Äthanol umkristallisiert. Die Ausbeute betrug 1.2 g (63 %), Schmelzpunkt 114°. Für $C_{14}H_{18}N_4O_5$ (322.31) berechnet 52.16 % C; 5.63 % H; 17.38 % N, gefunden 52.30 % C; 5.75 % H; 17.67 % N. Auf ähnliche Weise wurden die übrigen 2,4-Dinitrophenylhydrazone (siehe Tabelle I) hergestellt.

TABELLE I

2,4-DINITROPHENYLHYDRAZONE SUBSTITUIERTER ACETALDEHYDDERIVATE

2,4-Dinitrophenylhydrazon	*Summenformel (Molekulargewicht)*	*Schmelzpunkt (°C)*	*Elementaranalyse Berechnet/Gefunden*	
			% C	*% H*
Methoxy-acetaldehyd	$C_9H_{10}N_4O_5$ (254.20)	—	42.52	3.96
Äthoxy-acetaldehyd	$C_{10}H_{12}N_4O_5$ (268.22)	—	44.77	4.51
Propoxy-acetaldehyd	$C_{11}H_{14}N_4O_5$ (282.25)	85	46.81 46.34	5.00 5.34
Isopropoxy-acetaldehyd	$C_{11}H_{14}N_4O_5$ (282.25)	122	46.81 46.72	5.00 5.09
Butoxy-acetaldehyd	$C_{12}H_{16}N_4O_5$ (296.28)	77	48.64 48.41	5.44 5.39
Isobutoxy-acetaldehyd	$C_{12}H_{16}N_4O_5$ (296.28)	61	48.64 48.66	5.44 5.65
Amyloxy-acetaldehyd	$C_{13}H_{18}N_4O_5$ (310.30)	62	50.31 50.20	5.85* 5.99*
Hexyloxy-acetaldehyd	$C_{14}H_{20}N_4O_5$ (324.33)	56	51.84 51.54	6.21 6.47
Heptyloxy–acetaldehyd	$C_{15}H_{22}N_4O_5$ (338.36)	59	53.24 53.00	6.55 6.72
Octyloxy-acetaldehyd	$C_{16}H_{24}N_4O_5$ (352.38)	66	54.53 54.51	6.87 6.95
Nonyloxy-acetaldehyd	$C_{17}H_{26}N_4O_5$ (366.41)	65	55.72 55.36	7.15 7.18
Phenyloxy-acetaldehyd	$C_{14}H_{12}N_4O_5$ (316.26)	122	53.16 53.19	3.82 4.04
Tolyloxy-acetaldehyd	$C_{15}H_{14}N_4O_5$ (330.29)	145	54.54 54.48	4.27 4.39**
p-tert.-Butylphenyloxy-acetaldehyd	$C_{18}H_{20}N_4O_5$ (372.37)	142	58.05 58.33	5.41 5.65
Benzyloxy-acetaldehyd	$C_{15}H_{14}N_4O_5$ (330.29)	114	54.54 54.39	4.27 4.24
Cyclohexyloxy-acetaldehyd	$C_{14}H_{18}N_4O_5$ (322.31)	114	52.16 52.26	5.63 5.75***

* % N: ber. 18.05, gef. 17.91.
** % N: ber. 16.96, gef. 16.85.
*** % N: ber. 17.38, gef. 17.67.

Chromatographie

Die Chemikalien — verwendete Lösungsmittel, Petroläther (Siedepunkt 90–110°), Benzol, Hexan, Aceton und Dimethylformamid — waren analysenreine Produkte. Sie wurden vor der Verwendung überdestilliert und die Fraktion im Bereich des theoretischen Siedepunktes aufgefangen. Die 2,4-Dinitrophenylhydrazone wurden durch

Oxydation von Glycerinäthern mit Überjodsäure hergestellt und mit der Lösung von 2,4-Dinitrophenylhydrazin gefällt. Nach der Isolierung und Kristallisation aus dem Gemisch Chloroform–Äthanol wurden 1 %ige Lösungen von 2,4-Dinitrophenylhydrazonen in Chloroform hergestellt.

Das Papier Whatman Nr. 4 wurde zwecks Imprägnation durch eine 50 %ige Lösung von Dimethylformamid in Benzol gezogen (im Falle des Paraffinöles durch 10 %ige Lösung in Benzol) und in der Luft bei normaler Temperatur für die Dauer von 10–15 Min. aufgehängt, damit Benzol verdunsten kann. Auf das imprägnierte Papier wurden 7 cm vom oberen Rand und 3 cm voneinander mit der Mikropipette die Lösungen von 2,4-Dinitrophenylhydrazonen in Chloroform in der Menge von 5–50 μg aufgetragen. Es wurde absteigend mit dem durch Dimethylformamid gesättigtem Gemisch von Petroläther–Hexan (3:1) entwickelt. Für die mit Paraffinöl imprägnierten Papiere hat sich als durchfliessende Phase das Gemisch von Aceton–Wasser–Dimethylformamid (5:2:3) bewährt. Der chromatographische Zylinder wurde mit den Dämpfen des zugehörigen Lösungsmittelsystems gesättigt, welches bei der Temperatur von 20° ± 1° die Laufstrecke von 35 cm binnen 1.5–2 Std. unter Verwendung der mit Dimethylformamid vorbehandelten Papiere und binnen 6–8 Std. mit Paraffinöl vorbehandelten Papiere zurückgelegt hat.

Detektion

Nach Herausnehmen aus dem Zylinder wurden die Papiere bei normaler Temperatur aufgehängt, damit die zugehörigen Lösungsmittel verdunsten können. Nach dem Verdunsten des Lösungsmittels aus dem Papier wurde das Chromatogramm mit Zinnchloridlösung besprüht, die durch Auflösung von 0.7 g $SnCl_2 \cdot 2\ H_2O$ in 100 ml 15 %iger Chlorwasserstoffsäure hergestellt wurde und bei normaler Temperatur 30 Min. lang hängen gelassen. Nach Ablauf dieser Zeit wurden die Flecke mit 1 %iger Lösung von *p*-Dimethylaminobenzaldehyd in Äthanol mit 5 % Chlorwasserstoffsäure zur Sichtbarmachung besprüht. Die Zinnchloridlösung war jeden Tag frisch zu bereiten. Die geeignetste Art der Sichtbarmachung bestand jedoch in der Einzeichnung der Chromatogrammflecke im ultravioletten Licht der Lampe Philora. Auf angeführte Weise wurden verhältnismässig gut reproduzierbare Chromatogramme mit scharfen und runden Flecken gewonnen, die im Falle der mit Paraffinöl vorbehandelten Papiere eine ein wenig längliche Form (Fig. 2) annahmen.

ERGEBNISSE UND DISKUSSION

Zur chromatographischen Trennung von 2,4-Dinitrophenylhydrazonen der Alkoxy- und Aryloxyderivate des Acetaldehyds haben sich die mit Dimethylformamid und Paraffinöl imprägnierten Papiere Whatman Nr. 4 bewährt. Die Sichtbarmachung haben wir mittels der ultravioletten Lampe bzw. nach Reduktion der Nitrogruppe durch Zinnchloridlösung durch Besprühen mit Ehrlich-Reagens durchgeführt. Die Sichtbarmachung der Flecke auf dem Papier gestattete die Identifizierung bis zu 5 μg 2,4-Dinitrophenylhydrazonen.

Als durchfliessende Phase hat sich im Falle von Dimethylformamid das Gemisch von Petroläther–Hexan bewährt, das bei normaler Temperatur mit Dimethylformamid gesättigt wurde. Für die mit Paraffinöl vorbehandelten Papiere hat sich das System Aceton–Wasser–Dimethylformamid bewährt. Diese Lösungsmittel durch-

fliessen das Papier verhältnismässig schnell, sodass das Chromatographieren bei den mit Dimethylformamid vorbehandelten Papieren ca. 1.5–2 Std. und bei den mit Paraffinöl vorbehandelten Papieren 6–8 Std. in Anspruch nimmt.

In der Tabelle II sind die R_F-Werte und diesen entsprechende R_M-Werte in den Systemen angegeben, die sich bestens bewährt haben. Die beschriebene Methodik

TABELLE II

R_F- UND R_M-WERTE VON 2,4-DINITROPHENYLHYDRAZONEN DER ALKOXY- UND ARYLOXY-DERIVATE DES ACETALDEHYDS

Papier: Whatman Nr. 4.
Imprägnierung: I_1 = 50% Dimethylformamid in Benzol; I_2 = 10% Paraffinöl in Benzol.
System: S_1 = Petroläther–Hexan (3:1), gesättigt mit Dimethylformamid; S_2 = Aceton–Wasser–Dimethylformamid (5:2:3).
Laufzeit: S_1I_1, 1.5–2 Std.; S_2I_2, 6–8 Std.
Länge des Chromatogrammes: 35 cm.
Temperatur: 20° ± 1°.
Detektion: U.V.-Licht der Lampe Philora.

2,4-Dinitrophenylhydrazon	S_1I_1		S_2I_2	
	R_F	R_M	R_F	R_M
Methoxy-acetaldehyd	—	—	—	—
Äthoxy-acetaldehyd	—	—	—	—
Propoxy-acetaldehyd	0.32	0.33	0.88	−0.85
Isopropoxy-acetaldehyd	0.27	0.43	0.85	−0.74
Butoxy-acetaldehyd	0.37	0.23	0.87	−0.82
Isobutoxy-acetaldehyd	0.35	0.27	0.88	−0.85
Amyloxy-acetaldehyd	0.46	0.07	0.85	−0.74
Hexyloxy-acetaldehyd	0.56	−0.10	0.76	−0.49
Heptyloxy-acetaldehyd	0.66	−0.28	0.67	−0.31
Octyloxy-acetaldehyd	0.74	−0.46	0.55	−0.09
Nonyloxy-acetaldehyd	0.82	−0.66	0.41	0.16
Phenyloxy-acetaldehyd	0.07	1.12	0.93	−1.10
Tolyloxy-acetaldehyd	0.11	0.91	0.93	−1.10
p-tert.-Butylphenyloxy-acetaldehyd	0.27	0.43	0.75	−0.48
Benzyloxy-acetaldehyd	0.09	1.06	0.93	−1.10
Cyclohexyloxy-acetaldehyd	0.40	0.18	0.76	−0.49

gewährt eine gute Trennung von 2,4-Dinitrophenylhydrazonen der Alkoxy- und Aryloxyderivate des Acetaldehyds. Bezüglich der Beziehung zwischen den R_F-Werten und der Struktur der chromatographierten Verbindungen, verursacht die Verlängerung der Kohlenstoffkette die Vergrösserung der R_F-Werte, wobei 2,4-Dinitrophenylhydrazone der Aldehyde mit der geraden und verzweigten Kette und derselben Anzahl der Kohlenstoffatome praktisch untrennbar sind. Ähnlich wie bei anderen Homologreihen, kann die Regelmässigkeit in der Homologreihe von 2,4-Dinitrophenylhydrazonen in R_M-Werten ausgedrückt werden, wobei die Abhängigkeit dieser Werte im Bereich von R_F 0.1–0.7 von der Anzahl der Kohlenstoffatome der chromatographierten Stoffe geradlinig ist (Fig. 3). Wie aus der Tabelle II und Fig. 1 und 2 hervorgeht, wurde eine gute Trennung von 2,4-Dinitrophenylhydrazonen auf den mit Dimethylformamid und Paraffinöl vorbehandelten Papieren erreicht. Das Paraffinöl ist jedoch für die Trennung von Dinitrophenylhydrazonen der Aryloxyderivate des Acetaldehyds weniger geeignet, denn die angeführten Verbindungen kommen im gegebenen System fast an das Ende des Chromatogramms und weisen daher dieselben R_F-Werte

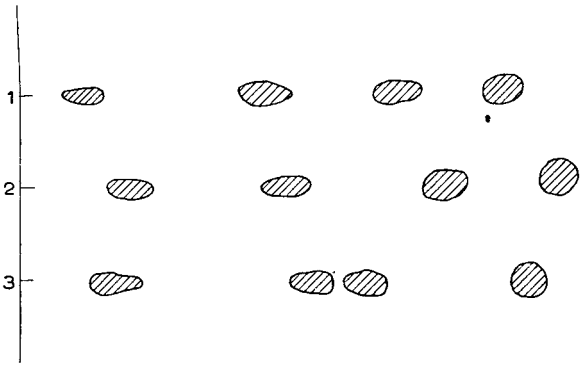

Fig. 1. Chromatogramm von 2,4-Dinitrophenylhydrazonen substituierter Derivate des Acetaldehyds im System Dimethylformamid/Hexan–Petroläther. 1 = Phenyloxy-, Propoxy-, Amyloxy- und Heptyloxy-Acetaldehyd. 2 = Tolyloxy-, *p-tert.*-Butylphenyloxy-, Hexyloxy- und Nonyloxy-Acetaldehyd. 3 = Benzyloxy-, Butoxy-, Cyclohexyloxy- und Octyloxy-Acetaldehyd.

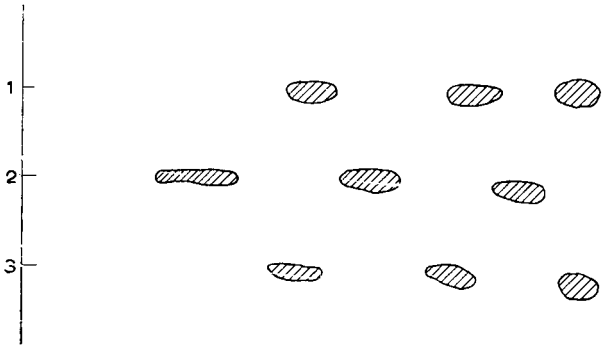

Fig. 2. Chromatogramm von 2,4-Dinitrophenylhydrazonen substituierter Derivate des Acetaldehyds im System Paraffinöl/Aceton–Wasser–Dimethylformamid. 1 = Octyloxy-, Hexyloxy- und Propoxy-Acetaldehyd. 2 = Nonyloxy-, Heptyloxy- und Amyloxy-Acetaldehyd. 3 = Octyloxy-, Cyclohexyloxy- und Butoxy-Acetaldehyd.

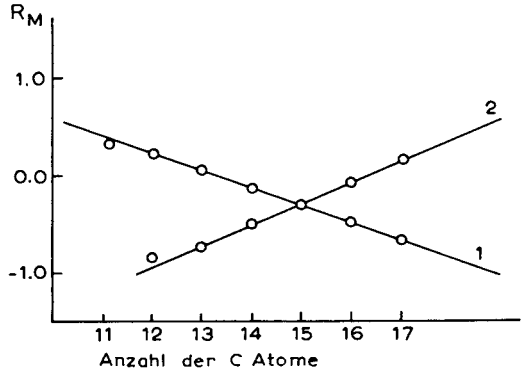

Fig. 3. R_M-Werte von 2,4-Dinitrophenylhydrazonen der Alkoxy-Acetaldehyde. 1 = System Dimethylformamid/Hexan–Petroläther. 2 = System Paraffinöl/Aceton–Wasser–Dimethylformamid.

auf, wobei 2,4-Dinitrophenylhydrazon des *p-tert.*-Butylphenyloxyacetaldehyds unter den gegebenen experimentellen Bedingungen Streifen bildet.

Zu den angeführten Beziehungen ist jedoch zu bemerken, dass die R_F-Werte bei der Chromatographie auf den imprägnierten Papieren eine grössere Streuung aufweisen, weil sie von der Art der Imprägnierung, der Zeit der Papiertrocknung, der Luftfeuchtigkeit und der vollkommenen Sättigung der Kammer mit beiden Phasen[18,19] abhängig sind. Es ist daher nötig, die angeführten Faktoren womöglich konstant zu halten und bei der Identifizierung des unbekannten Glycerinäthers erscheint es als vorteilhaft, gleichzeitig auch die Referenzstoffe zu chromatographieren.

ZUSAMMENFASSUNG

Durch katalytische Hydratation von Glycidyläthern wurden α-Alkyl(Aryl)Glycerinäther gewonnen, die nach erfolgter Isolierung und Reinigung unter Einwirkung von Überjodsäure in die zugehörigen Alkoxy-(Aryloxy-)Derivate des Acetaldehyds übergeführt wurden. Die letztgenannten Stoffe enthalten eine reaktive Carbonylgruppe, die mit 2,4-Dinitrophenylhydrazin kristallinische 2,4-Dinitrophenylhydrazone bildet. Die Papierchromatographie wurde zur Trennung und Identifizierung von 2,4-Dinitrophenylhydrazonen der substituierten Acetaldehydderivate benutzt. Auf den mit Dimethylformamid und Paraffinöl vorbehandelten Papieren Whatman No. 4 wurde in den Systemen Petroläther–Hexan und Aceton–Wasser–Dimethylformamid eine gute Trennung der angeführten Stoffe erzielt. Die Abhängigkeit des R_M-Wertes von der Anzahl der Kohlenstoffatome weist in den verwendeten Lösungsmittelsystemen in der Homologreihe von 2,4-Dinitrophenylhydrazonen einen linearen Verlauf auf.

SUMMARY

Glycide ethers are converted to glycerol α-alkyl(aryl) ethers by catalytic hydration. After isolation and purification the glycerol ethers are treated with periodic acid, whereby the corresponding alkoxy(aryloxy) derivatives of acetaldehyde are obtained. These derivatives possess a reactive carbonyl group which reacts with 2,4-dinitrophenylhydrazine to give crystalline 2,4-dinitrophenylhydrazones. These are separated and identified by paper chromatography. Using Whatman No. 4 paper impregnated with dimethylformamide or paraffin, and the solvent systems petroleum ether–hexane or acetone–water–dimethylformamide, good separations of the compounds investigated were obtained. In these solvent systems the relationship between the R_M values and the number of carbon atoms of compounds belonging to these homologous series proved to be linear.

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A SYSTEMATIC ANALYSIS OF ANTIBIOTICS USING PAPER CHROMATOGRAPHY

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INTRODUCTION

Paper chromatographic studies of compounds in several solvent systems for the purpose of their classification and identification have proved useful in systematic analysis[1]. At a recent symposium[2] papers were presented on the systematic analysis of alkaloids[3], antibiotics[4], synthetic dyes[5], inorganic cations and anions[6,7], aromatic compounds[8] and steroids[9].

Several methods for the systematic analysis of antibiotics are known. The oldest of them is the so-called "summarized paper chromatogram"[10], where the antibiotics are analysed in eight solvent systems and the R_F values of an antibiotic are presented graphically as a "summarized chromatogram". This principle has been used with various modifications by many authors[11–17].

MIYAZAKI *et al.*[18] classified antibiotics using a salting-out paper chromatography technique, in which nine solvent systems with different concentrations of ammonium chloride in water were used. According to their R_F values in these systems antibiotics can be divided into six groups[18,19]. NEMEC *et al.*[20] have also applied it to antibiotics from fungi.

BETINA[21] and BETINA AND NEMEC[22] determined the ionic character of unknown antibiotics and also the possibility of their isolation and purification from the "pH chromatograms".

From the chemical point of view antibiotics are a very heterogeneous group of biologically active compounds, which causes some difficulty in working out a systematic chromatographic analysis. Bearing this fact in mind a systematic analysis was developed along the following lines. The antibiotics are first analysed in four solvent systems, and then are divided into five classes with fourteen subclasses which are further analysed in additional solvent systems for each class.

MATERIALS AND METHODS

Principal solvent systems

The antibiotics were first analysed in the following four solvent systems:

1. Distilled water,
2. *n*-Butanol saturated with water,
3. Ethyl acetate saturated with water,
4. Benzene saturated with water.

The antibiotics were divided into five classes with fourteen subclasses, according to their R_F values in the above solvent systems.

*Additional solvent systems**

The following additional solvent systems were used for individual classes of antibiotics:

Class I:

A. Methanol–water (40:60).
B. *n*-Propanol–water (40:60).
C. Methanol–3 % ammonium chloride in water (70:30).
D. Methyl ethyl ketone–*n*-butanol–water (30:5:65).

Classes II and III**:

E. 3 % Ammonium chloride in water.
F. Isoamyl acetate–methanol–99 % formic acid–water (65:20:5:10).
G. *n*-Butyl acetate–methyl ethyl ketone–0.15 *M* phosphate buffer, pH 7.4 (50:25:5).
H. Ethyl acetate–*n*-hexane–0.15 *M* phosphate buffer, pH 6.0 (65:15:20).

Classes IV and V***:

I. Isoamyl acetate–methanol–99 % formic acid–water (40:20:10:30).
J. *n*-Butanol–methanol–water (40:10:50).
K. Methanol–*n*-hexane (60:40).
L. Benzene–cyclohexanone–0.15 *M* phosphate buffer, pH 7.4 (5:35:60).

Development and detection of chromatograms

Strips of the Whatman No. 1 paper 1 × 35 cm were used, the origin being 3 cm from the lower end of the strips.

For the development of the chromatograms 500 ml glass cylinders were used with 25 ml of the solvents. After applying the antibiotics to the origin and drying in air, the chromatographic strips were immersed to a depth of 1 cm in the solvent. Ascending development at 20 ± 1°, without preliminary saturation of the chromatograms with the vapours of the solvents, was used. The development was stopped when the solvent front reached a distance of 15 cm from the origin.

After drying the chromatograms in air, detection was carried out either bioautographically, using sensitive test microorganisms[23], or, in some special cases, chemically (see Table I).

Antibiotics

Sixty-two antibiotics from actinomycetes, fungi and lichens were studied. Data about the amount of each antibiotic used, the solvent for its solution before application on to paper strips, and the detection technique are given in Table I.

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* All proportions are given by volume.
** Upper layers of systems F, G, and H were used.
*** Bottom layers of systems I, J, K, and L were used.

TABLE I

SOLVENTS AND DETECTION METHODS FOR THE ANTIBIOTICS ANALYZED

Solvents used for the application of antibiotics on to chromatograms: A = acetone; AW = 50% acetone in water; C = chloroform; E = ethyl acetate; M = methanol; W = water.
Detection methods: a = bioautographically with *Bacillus subtilis*; b = bioautographically with *Escherichia coli*; c = bioautographically with *Candida pseudotropicalis*; d = 3% $FeCl_3$ in methanol; e = 3% NH_4Cl in water; f = acidic $KMnO_4$ (according to ref. 25, p. 737); g = conc. H_2SO_4; h = 3% NH_4OH in water; i = original colour.

Antibiotic	*Application*		*Detection*	*Colour reaction*
	Amount (μg)	*Solvent*		
Aburamycin	20	M	a	—
Actinomycin C·HCl	20	A	a	—
Alternariol	60	A	d	dark green
Amphomycin	40	W	a	—
Amphotericin B	250	W	g	blue
Aspergillic acid	60	A	d	red
Azalomycin B	80	M	a	—
Azalomycin F	60	M	c	—
Bryamycin	40	C	a	—
Chloramphenicol	10	M	a	—
Chlorotetracycline·HCl	30	M	d	light brown
Citrinin	80	A	a	—
Congocidin·HCl	40	W	a	—
Cyanein	80	A	c	—
Cyclopaldic acid	40	A	d	dark green
Cycloserine	40	W	a	—
Dihydrostreptomycin (sulfate)	10	W	b	—
Eniantin B	90	A	g	light red
Erythromycin (lactobionate)	20	W	a	—
Etamycin	5	A	a	—
Flavipin	50	M	d	dark green
Flavofungin	80	M	f	decolorized
Fomecin A	30	A	d	blue-green
Frequentin	50	C	d	brown-red
Fuscin	40	A	h	violet
Geodin	30	A	a	—
Gladiolic acid	50	A	e	dark green
Gliotoxin	30	A	a	—
Griseofulvin	100	C	g	yellow
Illudin M	40	A	a	—
Illudin S	20	A	a	—
Javanicin	20	A	i	red-violet
Kanamycin (sulfate)	80	W	b	—
Kojic acid	40	A	d	brown-red
5-Methoxy-*p*-toluquinone	40	A	a	—
Mycophenolic acid	80	A	d	dark green
Neomycin B (base)	250	W	b	—
Novobiocin (Na salt)	10	E	a	—

(Table continued on p. 382)

TABLE I (*continued*)

Antibiotic	*Application*		*Detection*	*Colour reaction*
	Amount (μg)	*Solvent*		
Oleandomycin (base)	30	M	a	—
Oxytetracycline·HCl	60	W	d	light brown
Patulin	100	A	a	—
Penicillic acid	160	M	a	—
Penicillin G (K salt)	0.5*	W	a	—
Penicillin V (K salt)	0.5*	W	a	—
Pleuromutilin	120	A	a	—
Puromycin	120	W	a	—
Quadrilineatin	40	A	e	dark green
Ristocetin	40	W	a	—
Rugulosin	20	A	d	yellow-brown
Sclerotiorin	20	A	g	orange-red
Spinulosin	50	A	d	brown
Spiramycin	80	M	a	—
Streptomycin (sulfate)	10	W	b	—
Synnematin B (cephalosporin N)	10	W	a	—
Telomycin	40	AW	a	—
Terreic acid	80	M	d	brown-red
Tetracycline·HCl	60	W	d	light brown
Trichothecin	20	A	c	—
Usnic acid	80	A	g	brown
Vancomycin	20	W	a	—
Viomycin (sulphate)	160	W	b	—
Viridin	80	A	a	—

* Units

RESULTS

The principles whereby the antibiotics were divided into five classes with fourteen subclasses, according to their R_F values in four solvent systems, are given schematically in Table II. Each subclass is represented by a typical antibiotic in Fig. 1.

The distribution of the antibiotics studied into the classes and subclasses is given in Tables III to VII. R_F values in the four principal and in four of the additional solvent systems are presented graphically as "summarized chromatograms" (or "R_F spectra"[7]) in Figs. 2 to 6.

Class I of antibiotics is characterized as follows: R_F values are equal to zero in the principal solvent systems 3 and 4, and 0.00–0.30 in system 2. There are greater differences in system 1, these were used for the division of this class into three subclasses: 1a, 1b, and 1c (see Table II and Fig. 1).

Class I includes antibiotics of the streptomycin–neomycin group, tetracyclines, cycloserine, synnematin B (cephalosporin N), vancomycin, ristocetin and other antibiotics (Table III). The "summarized chromatograms" of tetracyclines are very similar. There are also similarities between the "summarized chromatograms" of

TABLE II

CLASSIFICATION OF ANTIBIOTICS INTO CLASSES (PART A) AND INTO SUBCLASSES (PARTS B, C, D, E AND F)

Part A	*Classes*				
R_F values in principal systems	*I*	*II*	*III*	*IV*	*V*
R_{F_1}	$>$ 0.00	$>$ 0.60	0.31–0.60	0.00–0.30	0.00–1.00
R_{F_2}	0.00–0.30	$>$ 0.30	$> R_{F_1}$	$> R_{F_1}$	$>$ 0.60
R_{F_3}	0.00	$>$ 0.00	$> R_{F_4}$	$\geqq R_{F_4}$	$>$ 0.60
R_{F_4}	0.00	0.00	0.00–0.60	0.00–0.60	$>$ 0.60

Part B	*Class I*		
R_F values	*Subclasses*		
	Ia	*Ib*	*Ic*
R_{F_1}	$>$ 0.60	0.31–0.60	$\leqslant$ 0.30

Part C	*Class II*	
Subclasses		
IIa	*IIb*	*IIc*
$R_{F_1} > R_{F_2} > R_{F_3}$	$R_{F_1} > R_{F_2} < R_{F_3}$	$R_{F_1} < R_{F_2} < R_{F_3}$

Part D	*Class III*	
R_F values	*Subclasses*	
	IIIa	*IIIb*
R_{F_4}	0.00	0.05–0.60

Part E	*Class IV*		
R_F values	*Subclasses*		
	IVa	*IVb*	*IVc*
R_{F_2}	$> R_{F_1}$	$> R_{F_1}$	$> R_{F_1}$
R_{F_3}	$> R_{F_1}$	0.00	$> R_{F_4}$
R_{F_4}	0.00	0.00	0.05–0.60

Part F	*Class V*		
R_F values	*Subclasses*		
	Va	*Vb*	*Vc*
R_{F_1}	$>$ 0.60	0.31–0.60	0.00–0.30

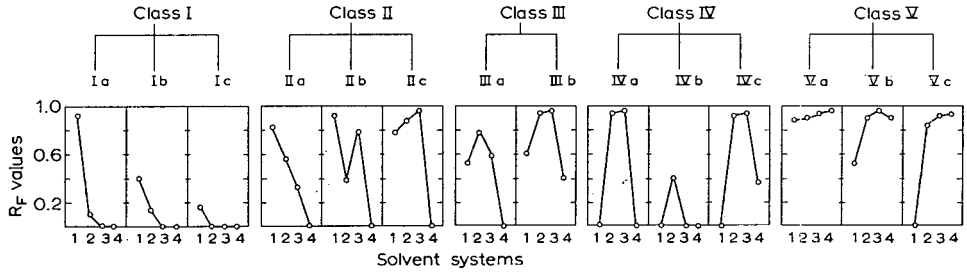

Fig. 1. Typical antibiotics belonging to five classes according to their R_F values in principal solvent systems 1, 2, 3 and 4. From left to right: cycloserine (subclass Ia), chlorotetracycline (Ib), streptomycin (Ic), kojic acid (IIa), penicillin G (IIb), chloramphenicol (IIc), oleandomycin (IIIa), etamycin (IIIb), cyanein (IVa), azalomycin F (IVb), azalomycin B (IVc), illudin M (Va), pleuromutilin (Vb) and viridin (Vc).

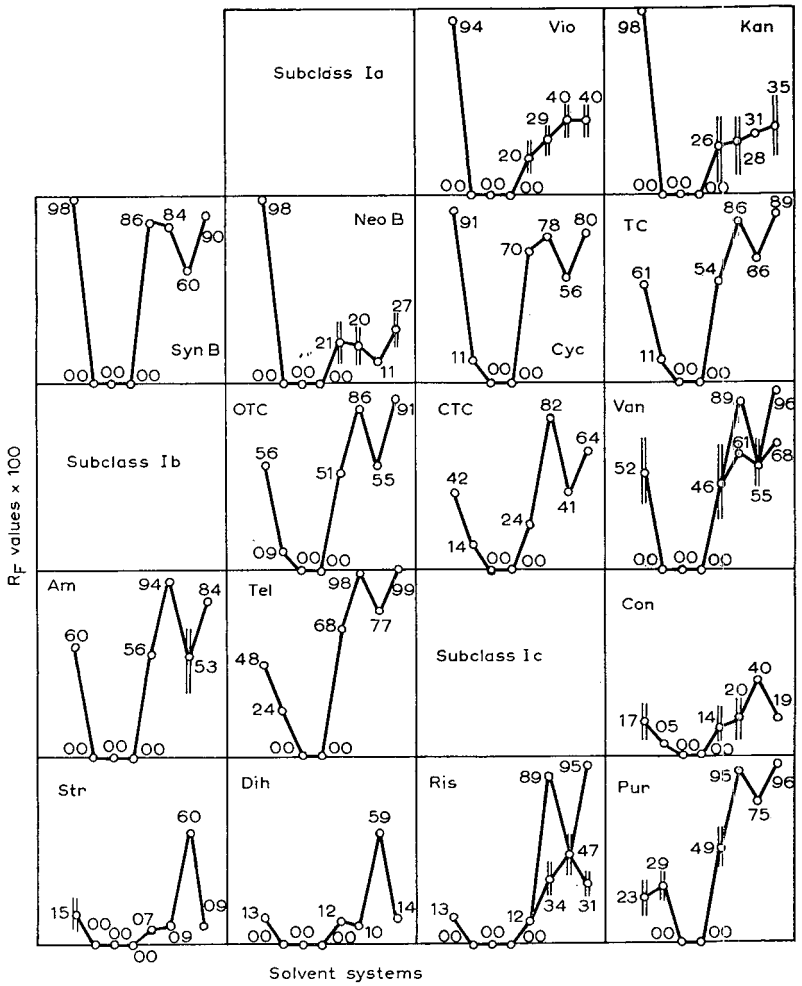

Fig. 2. "Summarized chromatograms" of antibiotics of class I. Subclass Ia: viomycin (Vio), kanamycin (Kan), synnematin B (Syn B), neomycin B (Neo B), cycloserine (Cyc) and tetracycline (TC). Subclass Ib: oxytetracycline (OTC), chlorotetracycline (CTC), vancomycin (Van), amphomycin (Am) and telomycin (Tel). Subclass Ic: congocidin (Con), streptomycin (Str), dihydrostreptomycin (Dih), ristocetin (Ris) and puromycin (Pur). Solvent systems: 1, 2, 3, 4, A, B, C. and D.

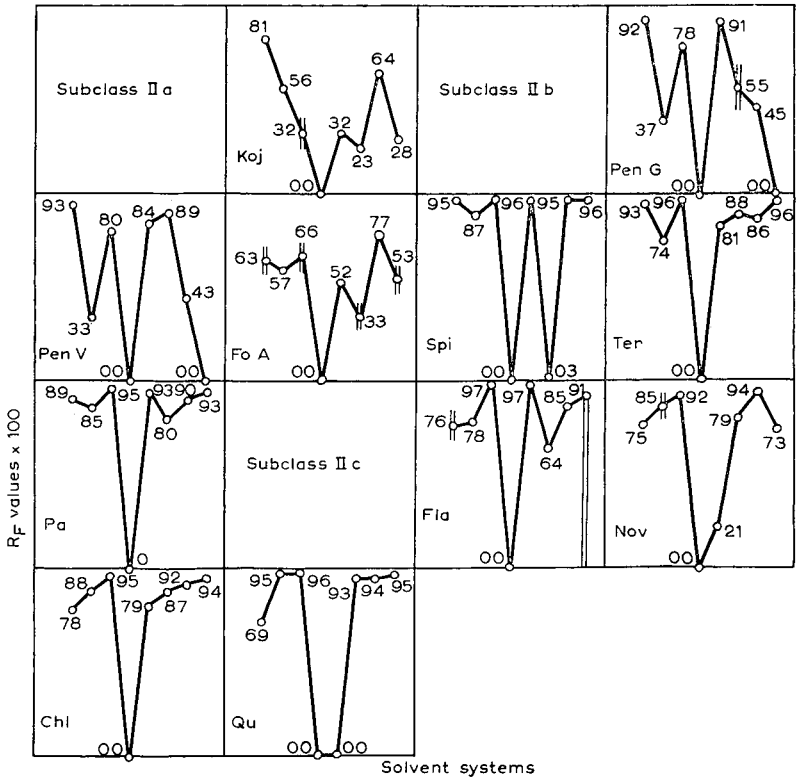

Fig. 3. "Summarized chromatograms" of antibiotics of class II. Subclass IIa: kojic acid (Koj). Subclass IIb: penicillin G (Pen G), penicillin V (Pen V), fomecin A (Fo A), spiramycin (Spi), terreic acid (Ter) and penicillic acid (Pa). Subclass IIc: flavipin (Fla), novobiocin (Nov), chloramphenicol (Chl) and quadrilineatin (Qu). Solvent systems: 1, 2, 3, 4, E, F, G, and H.

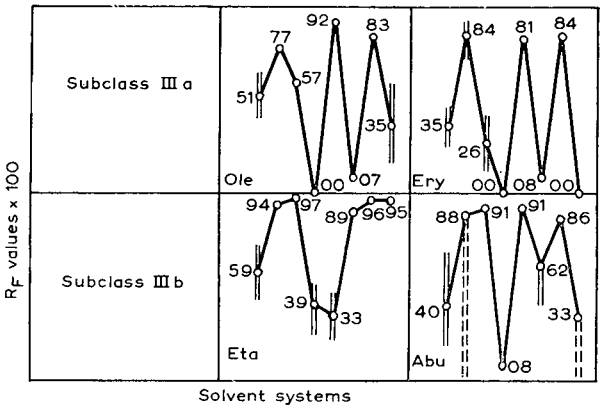

Fig. 4. "Summarized chromatograms" of antibiotics of class III. Subclass IIIa: oleandomycin (Ole) and erythromycin (Ery). Subclass IIIb: etamycin (Eta) and aburamycin (Abu). Solvent systems: 1, 2, 3, 4, E, F, G, and H.

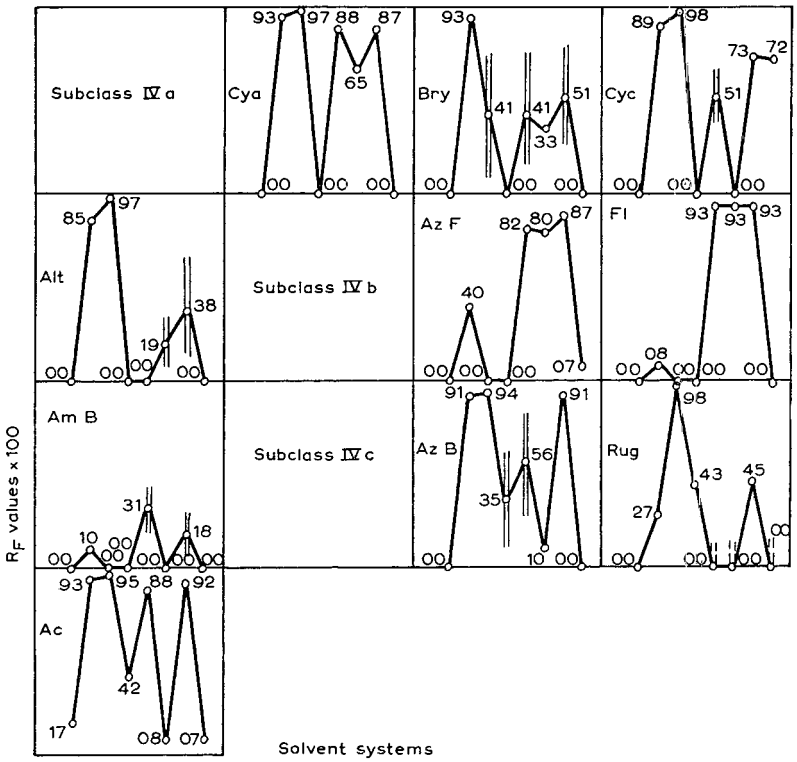

Fig. 5. "Summarized chromatograms" of antibiotics of class IV. Subclass IVa: cyanein (Cya), bryamycin (Bry), cyclopaldic acid (Cyc) and alternariol (Alt). Subclass IVb: azalomycin F (Az F), flavofungin (Fl) and amphotericin B (Am B). Subclass IVc: azalomycin B (Az B), rugulosin (Rug) and actinomycin C (Ac). Solvent systems: 1, 2, 3, 4, I, J, K, and L.

TABLE III

ANTIBIOTICS BELONGING TO CLASS I

Subclass I a	*Subclass I b*	*Subclass I c*
Viomycin	Oxytetracycline	Streptomycin
Kanamycin	Chlorotetracycline	Dihydrostreptomycin
Synnematin B	Vancomycin	Congocidin
Neomycin B	Amphomycin	Ristocetin
Cycloserine	Telomycin	Puromycin
Tetracycline.		

TABLE IV

ANTIBIOTICS BELONGING TO CLASS II

Subclass II a	*Subclass II b*	*Subclass II c*
Kojic acid	Penicillin G	Flavipin
	Penicillin V	Novobiocin
	Fomecin A	Chloramphenicol
	Spiramycin	Quadrilineatin
	Terreic acid	
	Penicillic acid	

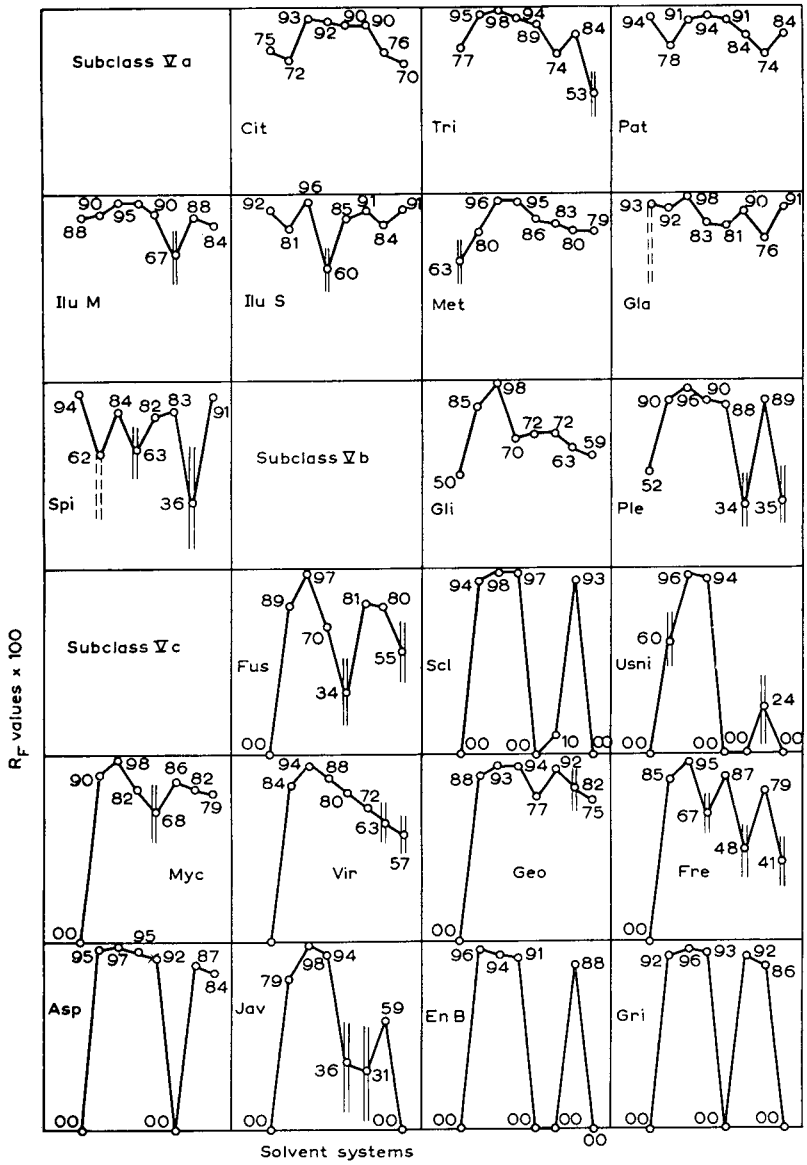

Fig. 6. "Summarized chromatograms" of antibiotics of class V. Subclass Va: citrinin (Cit), trichothecin (Tri), patulin (Pat), illudin M (Ilu M), illudin S (Ilu S), 5-methoxy-*p*-toluquinone (Met), gladiolic acid (Gla) and spinulosin (Spi). Subclass Vb: gliotoxin (Gli) and pleuromutilin (Ple). Subclass Vc: fuscin (Fus), sclerotiorin (Scl), usnic acid (Usni), mycophenolic acid (Myc), viridin (Vir), geodin (Geo), frequentin (Fre), aspergillic acid (Asp), javanicin (Jav), eniantin B (En B) and griseofulvin (Gri). Solvent systems: 1, 2, 3, 4, I, J, K, and L.

streptomycin and dihydrostreptomycin. Commercial samples of vancomycin (Vancocin) and ristocetin (Spontin) were used in our studies. These preparations contain mixtures of vancomycins and of ristocetins, respectively. Double spots in systems

TABLE V

ANTIBIOTICS BELONGING TO CLASS III

Subclass III a	*Subclass III b*
Oleandomycin	Etamycin
Erythromycin	Aburamycin

TABLE VI

ANTIBIOTICS BELONGING TO CLASS IV

Subclass IV a	*Subclass IV b*	*Subclass IV c*
Cyanein	Azalomycin F	Azalomycin B
Bryamycin	Flavofungin	Rugulosin
Cyclopaldic acid	Amphotericin B	Actinomycin C
Alternariol		

TABLE VII

ANTIBIOTICS BELONGING TO CLASS V

Subclass V a	*Subclass V b*	*Subclass V c*
Citrinin	Gliotoxin	Fuscin
Trichothecin	Pleuromutilin	Sclerotiorin
Patulin		Usnic acid
Illudin M		Mycophenolic acid
Illudin S		Viridin
5-Methoxy-*p*-toluquinone		Geodin
		Frequentin
Gladiolic acid		Aspergillic acid
Spinulosin		Javanicin
		Eniantin B
		Griseofulvin

B and D were observed in both cases; these solvent systems could be used for the paper chromatographic separation of the antibiotics mentioned.

Class II of antibiotics differ from class I with respect to their R_F values in the solvent systems 2, 3 and partially in system 1 (see Table II and Fig. 1). Differences in the relationships of the R_F values in systems 1, 2, and 3 served as limits for the subclasses IIa, IIb and IIc.

Class III of antibiotics differ from class II by the R_F values in solvent system 1 and partially (subclass IIIb) in solvent system 4. Differences of R_F values in system 4 were used for the characterization of subclasses IIIa and IIIb.

Both class II and class III have the same additional solvent systems because of some similarities in antibiotics belonging to these two classes. Such antibiotics as penicillins G and V, chloramphenicol, novobiocin and macrolide antibiotics can be shown in these classes. Against this, synnematin B (cephalosporin N), a penicillin with a more hydrophilic side chain in its molecule, does not belong to class II like

penicillins G and V, but to class I which includes antibiotics of a more hydrophilic character.

Antibiotics of class IV have some similarities with classes I, II, and III, when their R_F values in solvent systems 2, 3 and 4 are considered. However, they are akin to the Vc subclass by virtue of their R_F values in solvent system 1. They form a transitional class in our systematic analysis.

Class V is characterized by the R_F values in systems 2, 3 and 4, which are always higher than 0.60, showing a maximum in system 3 in most cases. The characterizations of subclasses Va, Vb, and Vc were established according to differences of the R_F values in solvent system 1.

DISCUSSION

DROZEN[24] discussed the classification and identification procedures of systematic chromatographic analysis in the light of information theory. He mentioned two main different methods of analysis, *viz.* the so-called sequential (step-wise) method and the simultaneous method.

In the chromatographic analysis of antibiotics hitherto known, both principles have been used. SNELL *et al.*[14] classified and identified several peptide antibiotics according to the principles of sequential analysis. ISHIDA *et al.*[10] and most other authors used the principles of simultaneous analysis in several solvent systems. Both methods have their advantages and disadvantages[24].

The advantage of our method of systematic analysis of antibiotics is the use of a combination of both principles. In the first step, antibiotics are analysed simultaneously in the principal solvent systems and are classified into classes and subclasses. The classification is then completed by the second step using the additional solvent systems. Using our combination of sequential and simultaneous analysis, it is not necessary to analyse a compound in many solvent systems as is done in simultaneous analysis. On the other hand, it is possible to compare the "summarized chromatogram" of an antibiotic with others belonging to the same class or subclass, respectively. The chromatographic identification of an unknown antibiotic, of course, must be confirmed by other data (I. R. spectrum, U. V. spectrum and other physical, chemical and biological properties).

MACEK AND PROCHÁZKA[25] mention two possibilities for the evaluation of solvent systems for paper chromatography. Solvent systems are classified according to their decreasing polarity in the eluotropic scale. Another classification of solvent systems is based on their possibility (or impossibility) to be hydrogen donors or acceptors, forming hydrogen bridges.

Both criteria were taken into consideration in our attempt to establish a systematic analysis of antibiotics. The polarity of the principal solvent systems decreases from system 1 to system 4. Solvent system 1 consists of water, which can accept or donate hydrogen, forming hydrogen bridges with other molecules of water. *n*-Butanol, the main component of system 2, also possesses the property of accepting or donating hydrogen and forms hydrogen bridges with molecules of other compounds. Ethyl acetate in solvent system 3 can only accept hydrogen, and benzene in system 4 has the least possibility (compared to the three solvents mentioned) of forming hydrogen bridges.

According to our classification, the hydrophilic character of the antibiotics analysed generally diminishes from subclass Ia to subclass Vc. Structurally related antibiotics (such as tetracyclines, streptomycins, penicillins G and V) have very similar "summarized chromatograms". In some cases the additional solvent systems may help to separate closely related antibiotics (see vancomycin and ristocetin in systems B and D, and penicillins G and V in system F).

In searches for new antibiotics from fungi our systematic chromatographic analysis can be applied when only crude concentrates of the antibiotics are available. An antibiotically active strain is cultivated in liquid medium up to the maximum of the antibiotic activity. Then the mycelium of the fungus is separated from the medium by filtration. Both the filtrate of the cultivation medium and the mycelium of the producing strain are used separately for the preparation of crude concentrates of antibiotics. The crude concentrates are prepared as follows[26]:

(1) 50 ml of acetone are added to 50 ml of the filtrate to precipitate proteins and other compounds that might interfere in the chromatographic analysis of the antibiotics in the filtrate. The mixture is heated to 50° for 10 min and then cooled to laboratory temperature. After precipitation and filtration, the filtrate is evaporated *in vacuo* to dryness. The dry residue is dissolved in 5 ml of 80 % aqueous acetone, filtered and used for the chromatographic studies.

(2) The mycelium of the producing strain is extracted twice with ethyl acetate, filtered and the filtrate is evaporated *in vacuo* to dryness. The dry residue is dissolved in 5 ml of 80 % aqueous acetone, filtered and used for the chromatographic studies of antibiotics from the mycelium.

Results of such chromatographic studies[26] of antibiotics from filtrates of media and from mycelia of 62 strains of antibiotically active fungi are given in Table VIII.

DOSKOČILOVÁ AND VONDRÁČEK[17] in their studies of antibiotics from actinomycetes prepared only concentrates from media and used them for chromatographic analysis of antibiotics. Other authors[27] prepared mixed concentrates of antibiotics

TABLE VIII

CLASSIFICATION OF ANTIBIOTICS FROM 62 STRAINS OF FUNGI

Subclasses	*Antibiotics found in*		
	Media	*Mycelia*	*Both media and mycelia*
Ia	10	—	—
Ib	1	—	—
Ic	—	—	—
IIa	5	—	—
IIb	1	—	—
IIc	5	—	—
IIIa	—	—	—
IIIb	—	1	—
IVa	5	5	5
IVb	7	—	—
IVc	—	1	—
Va	1	6	3
Vb	—	—	—
Vc	—	28	1
	35	41	9

from both the cultivation media and the mycelia of fungi and used them for chromatographic studies.

We found that it is necessary to prepare concentrates from both the cultivation media and the mycelia of strains studied, and that a separate chromatographic analysis of both concentrates of the same strain provides more precise results. We found in many cases that antibiotics in the concentrate from the medium and in the concentrate from the mycelium of the same strain belonged to different classes or subclasses of our systematic analysis. In some cases it was also possible to identify chromatographically antibiotics in concentrates from the medium and from the mycelium of the same strain. Such findings are also very useful for isolation procedures of unknown antibiotics.

In our laboratory data of these chromatographic studies are used not only for the classification but also for the tentative identification of unknown antibiotics. In our screening programme of new antibiotics from fungi it was possible to identify three antibiotics which had been isolated as strictly identical with citrinin[28], gliotoxin[29] and kojic acid[29]. Chromatographic identifications (the "summarized chromatograms", the "pH chromatograms" and the salting-out paper chromatograms) were confirmed by the I. R. spectra and by other physical, chemical and biological properties of these antibiotics.

Data about antibiotics in crude concentrates from filtrates of cultivation media and from mycelia of fungi that are obtained from the "summarized chromatograms" and from the "pH chromatograms"[21,22] help us to determine their ionic character and general possibilities of the isolation procedures[21,22,29]. This principle was applied during isolation and purification procedures of the three antibiotics mentioned and of the new antibiotic cyanein[30].

Of the known antibiotics studied, antibacterial compounds belong mainly to classes I, II and III, whereas antifungal antibiotics or antibiotics with simultaneous antibacterial and antifungal activity belong mostly to classes IV and V, respectively. It is well known that solubility, distribution coefficients and other physico-chemical properties of antibiotics have some relationship to their spectra of antimicrobial activity. This aspect of our systematic chromatographic analysis of antibiotics, of course, needs more detailed studies.

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SUMMARY

An attempt to establish a systematic chromatographic analysis of antibiotics has been made. Sixty-two known antibiotics were distributed into five classes and into fourteen subclasses, according to their R_F values in four principal solvent systems. Principles of the classification of antibiotics are given. For more detailed comparison and characterization of antibiotics further additional solvent systems were used. Additional solvent systems A, B, C, and D were used for class I, systems E, F, G, and H for classes II and III, systems I, J, K, and L were used for classes IV and V.

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CHROMATOGRAPHIC STUDIES ON SULPHUR COMPOUNDS

PART II. CONSIDERATIONS FOR QUANTITATIVE STUDIES OF THE FORMATION AND REACTION OF POLYTHIONATES*

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Before commencing with a description of the methods used in dealing with these problems, it is necessary to consider what advantages are gained and what limitations are set by performing a chromatographic separation prior to analysis**.

The advantage of an initial separation of a complex and labile thionate mixture before analysis is evident when viewed in the light of the classical methods for the determination of these compounds. The polythionates themselves are so similar in their chemical properties that their analysis by classical methods is lengthy, involved, and is liable to give erroneous results except in the hands of experienced research workers[1]. A recent scheme allows the determination of tri-, tetra- and hexa-thionate, in the presence of each other, and has been further extended to include other sulphur oxy-acids at the same time[2]. However, other work[3] suggests the presence of polythionates with more than six sulphur atoms per molecule in many of the reaction mixtures previously studied. Owing to the analytical methods, the presence of these acids would merely augment the amounts of the lower polythionates in the result.

In order that the true value of previous results may be appreciated, the methods of analysis will now be briefly reviewed. The following reactions were made use of by KURTENACKER AND GOLDBACH[4] for the estimation of tri-, tetra- and penta-thionates:

(1) Reaction with alkali sulphites. This causes degradation of higher thionates to trithionate and thiosulphate[5–7].

$$S_{x+3}O_6^{2-} + xSO_3^{2-} \longrightarrow S_3O_6^{2-} + xS_2O_3^{2-} \qquad (1)$$

The excess sulphite is complexed with formaldehyde, and the thiosulphate titrated with iodine solution[8–11].

(2) Treatment with alkali cyanide solution converts higher thionates to sulphate, thiocyanate and thiosulphate[12–15].

$$S_{x+3}O_6^{2-} + (x+2)CN^- + H_2O \longrightarrow SO_4^{2-} + xCNS^- + 2\,HCN + S_2O_3^{2-} \qquad (2)$$

* For Part I see ref. 29.

** The material in this paper is of such a form that for reasons of continuity the experimental details will be set out under separate headings in the following papers of this series, whilst the main reactions are considered in this paper.

Again the liberated thiosulphate is titrated with iodine.

(3) Reduction with alkali sulphide[16–19].

$$S_nO_6^{2-} + S^{2-} \longrightarrow (n - 3)S + 2\ S_2O_3^{2-} \quad (3)$$

As in cases (1) and (2), the liberated thiosulphate is estimated.

From the values of the iodine consumption for these three reactions, simultaneous equations may be set up and solved, by which the concentration of tri-, tetra- and penta-thionate may be determined.

Hexathionate will react with all the above reagents according to eqns. (1), (2), and (3), and another distinguishing reaction is required for its estimation. GOEHRING, FELDMANN AND HELBING[1] utilized the hydrolysis of thionates under controlled conditions; the thiosulphate liberated is titrated with iodine. With excess dilute alkali the overall reactions are represented by[20]:

$$S_6O_6^{2-} \longrightarrow S_5O_6^{2-} + S \quad (4)$$

$$2\ S_5O_6^{2-} + 6\ OH^- \longrightarrow 5\ S_2O_3^{2-} + 3\ H_2O \quad (5)$$

$$4\ S_4O_6^{2-} + 6\ OH^- \longrightarrow 5\ S_2O_3^{2-} + 2\ S_3O_6^{2-} + 3\ H_2O \quad (6)$$

Trithionate is unaffected by this treatment, but is decomposed by strong alkali[21]

$$2\ S_3O_6^{2-} + 6\ OH^- \longrightarrow S_2O_3^{2-} + 4\ SO_3^{2-} + 3\ H_2O \quad (7)$$

With such a scheme, analysis of mixture of tri- to hexa-thionates can be made; however, if higher polythionates are present, the only reliable value is trithionate, which is obtained from reactions (2) and (3). Because of the problems inherent in such schemes, some workers[22,23] have refrained from estimating the concentration of each thionate, but from the ratio of the iodine consumption for reactions (1) and (2) have calculated the average number n, the number of sulphur atoms per molecule. While this method is reliable when higher polythionates are present, the information is of limited use.

Both methods suffer from a major drawback when applied to solutions containing colloidal sulphur, a commonly occurring component of polythionate reaction mixtures. Colloidal sulphur reacts readily with sulphite to form thiosulphates, thus increasing the iodine titration figures for reaction (1) and hence affecting the calculated values of n.

Evidently separation prior to analysis offers a sound method of analysing such compounds; WEITZ AND SPOHN[3] suggested the fractional precipitation of the insoluble benzidine salts for this purpose, and the method has been used to advantage by several Russian workers[24–26]. The benzidine polythionate fractions are analysed for sulphur content by oxidation to sulphate (followed by barium precipitation). Such methods give a semi-quantitative estimation of the amount of each polythionate in the mixture.

Only scant attention has been paid to the application of partition chromatography to this problem. WOOD[27] successfully separated thionates by means of paper electrophoresis, and applied the separation to the detection of thionates in gelatines

as used in photography. Using this technique, WOOD verified KURTENACKER AND CZERNOTSKY'S[28] observations on the production of higher thionates in the arsenic-catalysed decomposition of acidified hydrochloric acid; also a solvent is cited for the paper chromatographic separation of the thionates, using methyl cellosolve as the major organic component.

Further qualitative separations of the polythionates by POLLARD, MCOMIE AND JONES[29], BIGHI, TRABANELLI AND PANCALDI[30] and SCOFFONE AND CARINI[31] have been described. None of these workers has applied these separations to the quantitative analysis of these compounds, although applications of the Pollard, McOmie and Jones' solvent have been made by BLASIUS *et al.*[32–35] in studying Wackenroder's solution and the stability of polythionates, using ^{35}S. Careful examination of reproducibility of each of these methods convinced us that the method of POLLARD, MCOMIE AND JONES[29] was most suited for adaptability as a quantitative micro-method.

Once separated, the analysis of any thionate species is limited to determining the amount or concentration of a solution* of a single characterized substance. Only one reaction or one physical property of the "solution" need be applied to the measurement of this amount instead of the many required by the older methods. There can be no chance of interference due to colloidal sulphur of higher thionates, for dealing with which the classical methods were inadequate. In the case of higher polythionates (those with more than six sulphur atoms), the method must at least be as good as benzidine fractionation. If these compounds do not form discreet zones on the chromatogram (*i.e.* no quantitative separation), it is nevertheless true to say that portions of the zone of higher thionates which have travelled furthest on the chromatogram will contain the components with the highest sulphur number. So that a fractionation can be performed by separating portions of the zone and subjecting these portions to analysis.

In some respects, another advantage is the small quantity of material required for the separation; this factor commends the method for use in the analysis of small samples from kinetic experiments, with no appreciable reduction of the reaction volume. However, small sample volume becomes a necessary condition of chromatographic methods of analysis rather than a chosen desirability, and in many ways adds difficulties and not advantages to the analyst, and becomes the most severe limitation of the method. The other major limitation is that the concentration of the sample solution should not be too high. The reason for these limitations is that, should they be exceeded, conditions are reached when the size of the zones is such that a quantitative separation is no longer obtained. This condition is termed overloading and is especially important when using the rear-phase technique[29].

Two cases of overloading may be distinguished, although, practically, the effects will combine. These may be called, (i) volume overloading and (ii) column overloading; they will be considered in detail.

VOLUME OVERLOADING

This limitation is not of importance in ordinary paper chromatography, since in this process the initial zone applied to the chromatogram is dried before elution. However,

* By solution is meant the material of a chromatographed thionate zone.

such a procedure is not possible with column or rear-phase chromatography owing to the nature of these techniques. Thus, the discussion which follows applies only to the latter methods.

When the sample solution is applied to a column or rear-phase chromatogram, the mobile and static phases for partition have already been prepared by frontal analysis of the solvent mixture, and the column should be homogeneous and at equilibrium (Fig. 1a). If an aqueous solution is now applied to the column, the equilibrium of the column is disturbed at this point and desorption of the less strongly adsorbed components of the solvent mixture will occur, the vacated adsorption sites being filled with water. Since adsorption is a function of concentration, the sufficient addition of water to the top of the column would result in the complete washing out of static and mobile phases (see Fig. 1b).

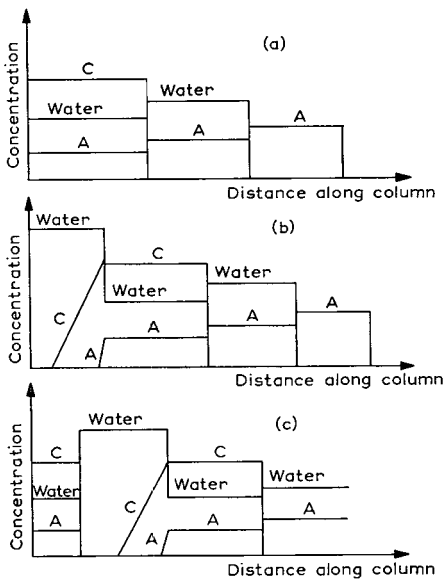

Fig. 1. Assume solvent mixture composed of A, C, and water. (a) Chromatogram showing frontal analysis of solvent mixture when at equilibrium. (b) Application of aqueous solution of solutes. Equilibrium now disturbed. A and C will be replaced by more strongly adsorbed water. Formation of water zone. (c) Fresh solvent mixture added to commence elution of solutes.

Fig. 1c shows the state of affairs when more solvent has been added to elute the mixture of solutes. The solutes themselves, being only of low concentration, cause only slight disturbance of the column equilibrium and their effect is neglected in comparison with that of the water added. This process has caused a "water zone", and zones do not disperse rapidly but travel a considerable distance down the support before completely diffusing into the surrounding solvent. They may be seen when the eluting solvent is coloured with an indicator, as white zones on a coloured background, clearly showing the washing-out processes outlined above. The water zone travels with a velocity similar to that of the solvent front (as would be expected) and a strongly adsorbed solute is soon left behind which may then undergo partition with the eluting

solvent. In this case there is little disturbance. However, for a solute which is only weakly adsorbed on to the cellulose from aqueous solutions, it will travel much further in association with the water in which it was originally dissolved. During this period, only those portions of the zone at the rear of the water zone will be subjected to the partitioning processes. If the water concentration is too great, such an elongated diffuse zone will cause annulment of separation between two adjacent solutes. From the earlier paper of this series[29], the thionates which will be interfered with in this way are those of highest sulphur number, *i.e.* hexa- and higher thionates.

Another and equally important aspect of this problem is that when a solution is applied to dry filter paper it spreads out owing to capillary action until equilibrium is reached and the thickness of the solution impregnating the paper is constant over the zone. Experiments have shown that for Whatman No. 1 filter paper there is an approximately linear relationship between the volume applied to the paper and the area of zone produced, and 5 μl of solution produces an area of 1 cm^2 (see Fig. 2).

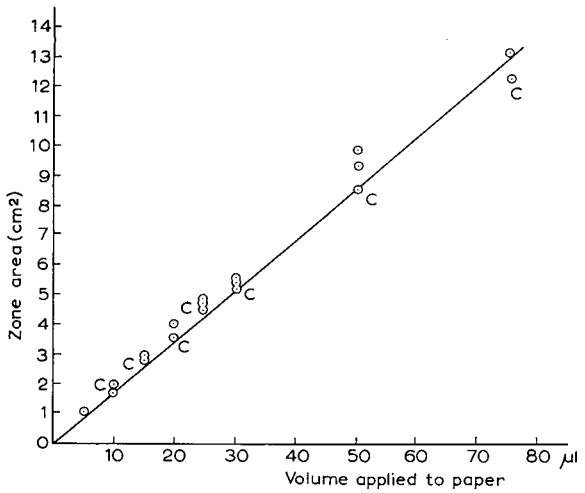

Fig. 2. Area of a zone produced for various volumes of solution applied to the chromatogram. The line is drawn through points corresponding to circular zones (C).

Then for a circular zone, the diameter w (cm) is given in terms of the volume applied v (μl) by

$$w = \sqrt{\frac{0.8v}{\pi}}$$

It is evident from the plate theory of MARTIN AND SYNGE[36] that the larger the area of the initial zone, the greater is the number of theoretical plates necessary for separation of a pair of solutes. Since the height equivalent to a theoretical plate has a constant value for the chromatographic system used, it follows that a longer chromatogram is necessary for separation. However, in practical terms, an extended chromatogram does not necessarily give better resolution, owing to diffusion causing the zones to spread.

A plot of Q_r (concentration of solute in plate r) against plate number r, is usually assumed to be of the Gaussian type, c is the concentration limit of detection, and the points L and L′ where the line $Q = c$ intersects the curve, are the boundaries of the detected zone. Assume the concentration of the initial zone is unit quantity of solute in one plate (Fig. 3).

According to MARTIN AND SYNGE[36], the amount of material in plate $(r + 1)$ is

$$Q_{r+1} = \frac{1}{\sqrt{2\pi r}} \cdot \left(\frac{v}{PV}\right)^r e^{r - v/V} \tag{8}$$

and at the maximum $r = v/V = R$.

Let the plate at L′ be $(n + 1)$

$$Q_{n+1} = \frac{1}{\sqrt{2\pi n}} \cdot \left(\frac{R}{n}\right)^n e^{n-R} = c \tag{9}$$

The diameter of the detected zone, w, is given by LL′, which in terms of number of plates will be,

$$w = 2\,(n - R)$$

Let $n - R = S$, that is $w/2$

$$c = \frac{1}{\sqrt{2\pi(S + R)}} \cdot \left(\frac{R}{S + R}\right)^{S+R} e^S \tag{10}$$

This equation has been solved for $c = 0.01$, and for various values of R the following values of w are obtained,

R	0	50	100	150	200	250
w	1	26	34	40	45	46

These values are all in terms of the unit h. MARTIN AND SYNGE[36] found that for their chromatograms $h = 0.02$ cm and calculations on our chromatograms tend to

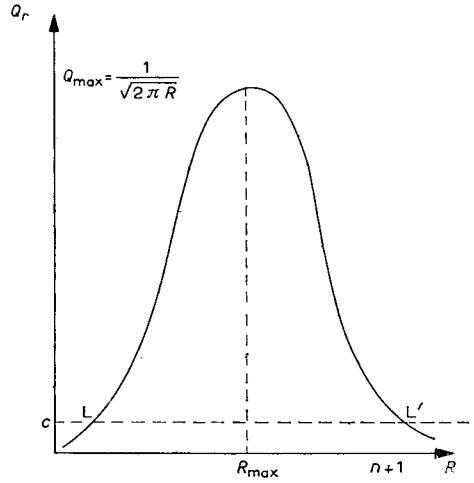

Fig. 3. Graph of Q_r (quantity of solute in plate r) against r (serial number of the plate). Symbols as used in the text.

produce a similar value. Since, in practice, more than one plate is filled with solute to form the inital zone, the values of w given above are merely the increases in the size of a zone. Notice that this process is due to chromatography and not to diffiusion, although since diffusion is always greatest at a steep concentration gradient, its effect would be expected to be greatest at the beginning of the process and become less as the concentration gradients are evened out. At first sight this appears to nullify the statement that diffusion effects make the use of extended chromatograms worthless. However, as the solvent elutes solute down the paper, its progress becomes slower, and hence the longer the chromatogram, the more time there is for diffusion.

The main point we must gather is that the width of the initial zone sets a lower limit to the widths of the zones of the chromatographed materials. If this is too great, then a quantitative separation cannot be attained. This means that an accurate method for applying small volumes of solution to the chromatogram is essential if the separation is to be used in a quantitative method of analysis, especially when using the rear-phase technique.

CONCENTRATION OVERLOADING

The area of a chromatographed zone is strongly dependent upon the concentration of the solution of zone-forming substance applied to the chromatogram.

Equation (10) holds for unit quantity of solute being admitted to the first "plate" of the chromatogram. For a solution of such concentration that an amount x is admitted to the first plate eqn. (10) becomes

$$c = \frac{x}{\sqrt{2\pi(S+R)}} \cdot \left(\frac{R}{S+R}\right)^{S+R} e^{S} \tag{11}$$

or

$$x = \frac{K\,(S+R)^{S+R+0.5}}{R^{S+R} \cdot e^{S}} \tag{12}$$

$$K = c\sqrt{2\pi}$$

The following values of x/c have been obtained for $R = 100$ and various values of S.

S (h units)	0	5	10	15	20	30	50
x/c	$2.4 \cdot 10$	$2.65 \cdot 10$	$3.96 \cdot 10$	$7.34 \cdot 10$	$1.57 \cdot 10^2$	$1.15 \cdot 10^3$	$9.29 \cdot 10^5$

For some time, an experimentally determined linear relationship between the area of a chromatographed zone and the log of the concentration has been used as a basis of analytical methods[37,38]. It appeared likely, therefore, that a plot of S^2 *versus* log x/c should give a straight line, and the result is confirmed (Fig. 4).

Equation of the line is

$$S^2 = 557 \log_{10} \frac{x}{c} - 765$$

S measured in h units.

Now examining eqn. (12), some meaning may be attached to the constants of this equation.

By taking logs and re-arranging

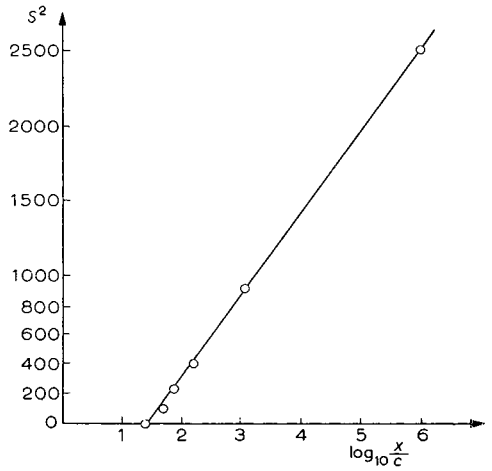

Fig. 4. Graph of S^2 ($S \sim$ area of eluted zone) against log x/c. Intercept on log x/c axis is 1.36.

$$\log_e \frac{x}{c} = \log_e \sqrt{2\pi R} + (S + R + 0.5)\left(\frac{S}{R} - \frac{S^2}{2R^2} + \frac{S^3}{3R^3}\right) - S \qquad (13)$$

Since R is much larger than S for a normal chromatogram, terms of $(S/R)^4$ or higher are neglected.

$$\log_e \frac{x}{c} = \log_e \sqrt{2\pi R} + \frac{S}{2R} + \frac{S^2}{2R}\left(1 - \frac{1}{2R} + \frac{S}{2R^2} + \frac{2S^2}{3R^2}\right)$$

Of the terms containing S, only $S^2/2R$ and $2S^2/3R^2$ contribute, hence other terms are very much smaller. If this is so, then an approximate equation is

$$\log_e \frac{x}{c} = \log_e \sqrt{2\pi R} + \frac{S^2}{2R}\left(1 + \frac{2S^2}{3R^2}\right)$$

Solution for S being

$$S = \pm\sqrt{-1 \pm \frac{2}{3}\sqrt{3\chi R^{3/2}}}$$

$\chi = \log x/c - \log \sqrt{2\pi R} > 1$, but at very low concentrations when S is very small compared with R, sufficiently accurate to use,

$$S^2 = 2R \log_e \frac{x}{c} - 2R \log_e \sqrt{2\pi R}$$

or

$$S^2 = 4.606\, R\,(\log_{10} \frac{x}{c} - \log_{10}\sqrt{2\pi R}) \qquad (14)$$

Interception with the $\log_{10} x/c$ axis will be at log $\sqrt{2\pi R}$. This means that a zone for which

$$\frac{x}{c} = \sqrt{2\pi R}$$

will have no detectable spread and the area of the zone after elution for R plates will be equal to initial area, but unfortunately, no account of diffusion is taken here.

Consider the chromatography of a mixture of two solutes forming an original zone of radius, ρ_z, which, after elution, yields two zones whose centres are at R and R' plates from the origin. Let $x/c = \chi$, and the two values for the two solutes be χ, and χ'.

Now

$$S^2 = 4.606R\ (\log \chi - \log \sqrt{2\pi R}) \tag{14a}$$

$$(S')^2 = 4.606R'\ (\log \chi' - \log \sqrt{2\pi R'}) \tag{15}$$

$$R\rho_z = \rho_z + S$$

$$R'\rho_z = \rho_z + S'$$

$$R' - R = \rho_z + S + \rho_z + S' + d.$$

The two zones will interfere when $d = 0$ and

$$R' - R = 2\rho_z + S + S' \tag{16}$$

This cannot be solved easily, but it may be assumed that since in general $R' - R$ is small compared to R, then $S = S'$.

Thus

$$R' - R = 2(\rho_z + S)$$

$$S = \left(\frac{R' - R}{2}\right) - \rho_z.$$

Substituting

$$\frac{(R' - R - 2\rho_z)^2}{4} = 4.606 \left(\frac{R' + R}{2}\right) \left(\log \chi - \log \sqrt{\frac{2\pi\,(R' + R)}{2}}\right) \tag{17}$$

Thus let $R = 500$, $R' = 600$, $\rho_z = 10$ (h terms), and $x/c = 2 \cdot 10^4$.

In this case, if $h \approx 0.02$ cm, the distance travelled by zone 1 is 10 cm, by zone 2 12 cm, if the diameter of the original zone was 0.2 cm.

Thus eqn. (17) can be used to determine the limiting size of the initial zone for chromatography of solutions of a given concentration, such that overloading does not occur.

It must be pointed out, however, that no account of diffusion has been allowed for these calculations. Diffusion will act towards a lessened resolution of the zones such that the actual limiting concentrations which may be employed in a quantitative separation will be less than those calculated, and the degree by which these are lower will be greater the longer the substances are chromatographed. In a qualitative nature, the effects of spreading of a zone by diffusion are embodied in the equation

$$S_D = \phi\left(\frac{dx}{dR}, t\right)$$

where S_D is spreading due to diffusion; t is period during which zone is chromatographed.

The radius (ρ_R) of a chromatographed zone after travelling R plates can thus be expressed as

$$\rho_R = \rho_0 + S_C + S_D$$

(S_C = spread due to chromatography)

$$\rho_R = \rho_0 + \sqrt{4.606R \log_{10} \frac{\psi}{\sqrt{2\pi R}}} + \phi\left(\frac{dx}{dR}, t\right) \qquad (18)$$

METHODS OF ANALYSIS

In general two procedures are available for the analysis of solutes which have been previously chromatographed. The substances may be extracted from the chromatogram after drying, and determined by classical micro-analytical techniques, or the analysis may be accomplished without extraction by an *in situ* method. Such methods have been adequately described elsewhere[39]. On considering these methods with reference to the thionates, it was seen that the major difficulty with hot extraction methods is that there will be considerable decomposition of the species with the formation of a mixture of products. Such methods are not, therefore, of any value unless a controlled reaction leading to a definite product is instigated during the extraction. However, it appeared that elution of the thionates from the chromatogram would not be followed by any subsequent decomposition, since the species are relatively stable in the solvent used for chromatography[35]. It was therefore necessary to find an analytical procedure for the estimation of micro-quantities of thionate present in a solution containing organic solvent and potassium acetate. All the reactions of thionates used by GOEHRING lead to the production of thiosulphate, so that, if one such reaction was chosen, the method involves the determination of thiosulphate.

Experiments were carried out on the disappearance of colour of an iodine–potassium iodide solution when a solution of sodium thiosulphate is added to it. Some difficulty was found in preparing a reproducible calibration curve for optical density *versus* concentration of iodine solution. This was found to be due to variations in the concentration of potassium iodide; however, when a standardised technique was employed, estimations of standard sodium thiosulphate solutions were possible to less than 2 % relative error.

For the conversion of thionates to thiosulphate, the sulphide degradation

$$S_nO_6^{2-} + S^{2-} \longrightarrow (n - 3)S + 2\ S_2O_3^{2-} \qquad (3)$$

was chosen, since for each mole of thionate, two moles of thiosulphates are produced. After boiling the eluant sample with alkaline sodium sulphide solution, the excess sulphide was removed by addition of freshly precipitated zinc carbonate slurry. The mixture was centrifuged, and made up to a standard volume with standard iodine solution. The optical density was then measured.

Results, however, were most unreliable, even when the utmost care was taken. It is thought that the fault is the solubility of zinc sulphide $7 \cdot 10^{-3}$ g/l at 18°[39], and although being negligible for macro-analysis, introduces considerable error when such small amounts of precipitate are considered.

Several other methods were then tried. They included:

(i) The extraction and oxidation of the thionate-containing section of the paper to sulphate, using potassium chlorate solution. The sulphate was estimated spectrophotometrically using 4-chloro-4'-aminodiphenyl[40]. Unfortunately, this method failed to yield consistent results, probably because of the bisulphite content of the filter paper.

(ii) The alkaline hydrolysis of the polythionate species on the paper to thiosulphate according to the equations[20]

$$4\ S_4O_6^{2-} + 6\ OH^- \longrightarrow 5\ S_2O_3^{2-} + 2\ S_3O_6^{2-} + 3\ H_2O \quad (6)$$

$$2\ S_5O_6^{2-} + 6\ OH^- \longrightarrow 5\ S_2O_3^{2-} + 3\ H_2O \quad (5)$$

$$S_6O_6^{2-} \longrightarrow S_5O_6^{2-} + S \quad (4)$$

while trithionate is decomposed only in concentrated alkaline solution[21]

$$2\ S_3O_6^{2-} + 6\ OH^- \longrightarrow S_2O_3^{2-} + 4\ SO_3^{2-} + 3\ H_2O \quad (7)$$

and titration of the resultant thiosulphate with formaldehyde being used to find the sulphite from the paper. Recovery experiments on chromatographed samples of potassium thiosulphate gave reproducible results with an average recovery of 98 % ± 4 %.

The hydrolysis reactions given above were tested by hydrolysing samples of each thionate with alkali under varying conditions. After adding formaldehyde and neutralising, the solutions were titrated with iodine solution to obtain values of $S_2O_3^{2-}$ produced. Also samples of the hydrolysates were chromatographed to identify the thionate species present.

Trithionate was found to be especially stable to hydrolysis. Boiling with 0.5 *M* caustic soda solution for 2 h only produced about 16 % hydrolysis, while 1 *N* caustic soda was required for quantitative conversion to thiosulphate, the only species observed on chromatographing the hydrolysate. With tetrathionate, standing with 0.5 *N* caustic soda solution was sufficient to cause hydrolysis, and from the alkali consumption, and thiosulphate produced, excellent agreement with equation (6) was obtained, a chromatogram showing only $S_2O_3^{2-}$ and $S_3O_6^{2-}$ to be present. However, on using a large excess of 0.5 *N* alkali, eqn. (6) was no longer obeyed. The results corresponded to $2\,S_4O_6^{2-}$ consuming $4\,OH^-$ with the production of $3\,S_2O_3^{2-}$ to within 2 %. Experiment showed that $S_3O_6^{2-}$ was not hydrolysed under these conditions, and neither did the presence of thiosulphate catalyse the decomposition of $S_3O_6^{2-}$ as it does with higher thionates[34]. Chromatograms showed that $S_3O_6^{2-}$ was present, and it is possible to formulate a stoichiometric equation which represents the alkali consumption correctly,

$$4\ S_4O_6^{2-} + 8\ OH^- \longrightarrow 4\ S_2O_3^{2-} + 2\ S_3O_6^{2-} + SO_4^{2-} + S^{2-} + 4\ H_2O.$$

Since the oxidation of S^{2-} requires twice the concentration of I_2 per mole required for the oxidation of thiosulphate, it can be seen that the iodine consumption would also be correct on this basis, and therefore the reaction seems to be a likely one.

Both 0.5 *N* and 1.0 *N* caustic soda solution caused rapid hydrolysis of penta-

thionate to thiosulphate, the only species detected chromatographically. The results agreed with equation (5).

With regard to hexathionate, the results could not be interpreted on the basis of any stoichiometric eqn. (4), such as that quoted by GOEHRING *et al.*[20] and from the chromatograms it appeared that numerous side reactions were occurring. The hydrolysis could not therefore be used to estimate hexathionate.

It would seem then that although the method would be of use in the analysis of $S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$ and $S_5O_6^{2-}$, it is likely to fail with higher thionates. These results tend to agree with the results obtained by BLASIUS *et al.*[34,35] concerning the stoichiometry of such degradation reactions. It is just these higher thionates which are the main interest in the investigations for which the analytical method was required.

All these proposed methods having been found inadequate for ready and accurate analysis of thionates from chromatograms, attention was focused upon *in situ* methods. Such methods involve measuring the area, optical density, or some other property of the developed zone which bears a simple relationship to the concentration of solute present.

Area measurement requires that the zone should be sharply defined. Preliminary experiments were carried out on trithionate zones, which were developed with ammoniacal silver nitrate solution, the excess silver nitrate being removed with sodium thiosulphate solution. Measurement of the area of the zone was found to be only semi-quantitative. This was mainly due to the difficulty of delimiting the zone boundaries. A major factor in such systems is the sensitivity of the spray reagent[41]. Thus, whereas it is possible that area measurements of zones may afford a method of estimation of solutes, in most cases it is only semi-quantitative.

Measurement of the absorbance of zones using scanning techniques proved much more promising. The measurement of the total absorbance of a small zone was rejected as this would severely limit the volume of solution (not more than 5 μl). The other method was to measure the absorbance of a section of a uniform band. This necessitates the application of a uniform band to the chromatogram, and a suitable machine was designed for this purpose. In designing the machine, care was taken in its specification to adhere to the ideas reported in this paper concerning (a) volume and (b) concentration overloading.

Because of the space needed for these considerations and a description of the machine, details and developments are described in the following paper.

SYMBOLS

A = area of cross-section of column $= A_I + A_L + A_S$.
A_I = area of cross-section of inert support.
A_L = area of cross-section of mobile (liquid) phase.
A_S = area of cross-section of static phase.
α = partition coefficient $= \dfrac{\text{g solute/ml of stationary phase}}{\text{g solute/ml of mobile phase}}$ at equilibrium.
c = concentration limit of detection.
d = distance between centres of gravities of two spots.
h = H.E.T.P. (height equivalent of a theoretical plate).

Q_r = concentration of solute in plate r.
r = serial number of "plate", measured from top of chromatogram.
R = $\frac{\text{displacement of position of maximum concentration of solute}}{\text{simultaneous displacement of liquid surface of developing fluid}}$.
ρ_0 = radius of zone at commencement of elution.
ρ_R = radius of chromatographed zone after travelling R plates (diffusion).
S = radius of zone.
S_C = spreading due to chromatography.
S_D = spreading due to diffusion.
t = time period of chromatographic development.
v = volume of solvent used in development of chromatogram.
V = $h(A_L + A_S)$.
w = diameter of zone.

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SUMMARY

This paper begins with a short review of previous analytical methods for polythionates, and goes on to consider the limitations of the chromatographic method which must be taken into account when the method is to be used for quantitative measurements. A discussion on overloading follows in which two types of overloading are defined due to high volume and high concentration, respectively. In this case of concentration overloading, the treatment leads to an equation which may be used to determine the conditions for interference of two zones whose centres have a given separation.

The actual analytical methods applied are then described. Many macro-analytical methods for the analysis of thionates were adopted to semi-micro scales in the search for a method of estimation which could be coupled with the chromatographic separation. The final method adopted for the estimation of the species was an *in situ* method.

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CHROMATOGRAPHIC STUDIES ON SULPHUR COMPOUNDS

PART III. QUANTITATIVE ANALYSIS OF A MIXTURE CONTAINING THIOSULPHATE AND POLYTHIONATES BY PAPER CHROMATOGRAPHY*

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When using a scanning technique for the determination of peak area measurements, in elution chromatography the absorbances of small segments of a zone are recorded and plotted against position as illustrated in Fig. 1. For the following simple treatment, the zone may be considered to be divided into segments, the smaller each segment the closer will be the approximation to reality.

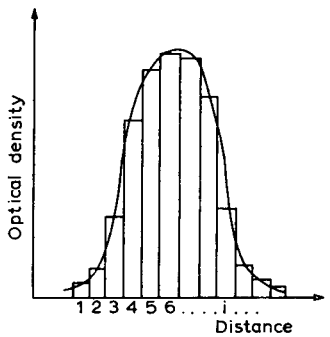


Fig. 1. Area beneath an absorbance section of a zone.

The total absorbance of the zone, I_T, is given by

$$I_T = \Sigma I_i = I_1 + I_2 + \cdots I_n.$$

Subscripts 1, 2 . . . n refer to the numbers of the segments from the start. If the weight of solute in each segment is symbolised by W_i, and if Beer's law is assumed to hold,

$$I_T = k \Sigma W_i = kW_T$$

$$= kW_1 + kW_2 + kW_3 \cdots kW_n,$$

where W_T = total weight of solute in the zone.

If Beer's law does not hold for the regions of higher concentration, then the errors of assuming proportionality between the absorbance of the total zone and

* For Part II of this series, see ref. 2.

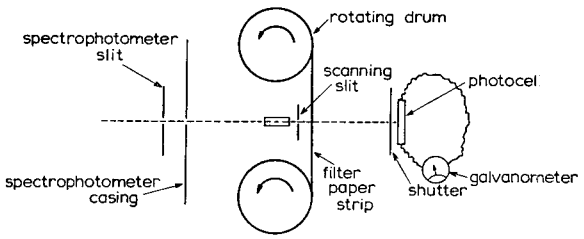
weight of solute therein can only be estimated from an absorbance section. Even with a calibration graph of concentration of solute solution *versus* total absorbance, large errors might be obtained if the zone is distorted owing to interfering substances in the sample solution. Obviously the scanning method offers the best solution of these problems.

To summarise, the method consists in measuring the absorbance of a uniform band of silver sulphide and sulphur on a paper strip by means of a scanning device, the silver sulphide and the sulphur being produced by the decomposition of silver thionates[1] obtained by spraying the chromatogram with silver nitrate solution.

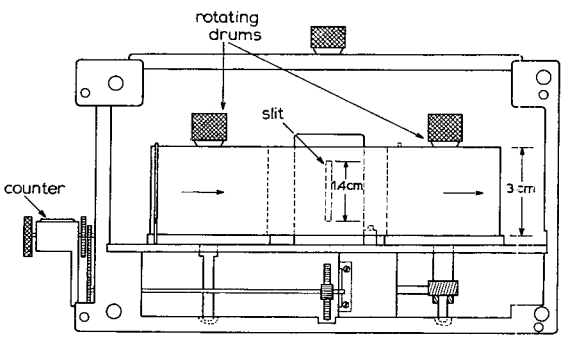
Obviously for the success of such a method, a uniform band of liquid should be applied to the chromatogram, this being done by using a zoning machine, designed to the specification of Part II of this series[2].

THE SCANNING INSTRUMENT

The scanning instrument used consisted of an attachment to the Unicam SP. 500 spectrophotometer. Fig. 2a shows the principle of the attachment, while Fig. 2b shows the attachment diagrammatically. The light beam passes through a slit between two rotatable drums which carry the chromatographic strip. The movement of the strip was hand-operated from outside the cell, and was measured by a geared counter of the cyclometer type. The movement of the strip for one unit on the counter was 0.55 mm,



(a)



(b)

Fig. 2. (a) Principle of the scanning attachment. (b) Diagram of the scanning attachment.

the width of the slit in the scanning instrument was 2 mm, and the height 1.15 cm.

There are two possible ways of using such an instrument, either the total absorbance of a small zone is measured, or an absorbance section of a large band may be determined (Fig. 3). With the first method, a primary requisite is that the diameter of the zone should be less than the height of the slit, *i.e.*, with the apparatus at hand,

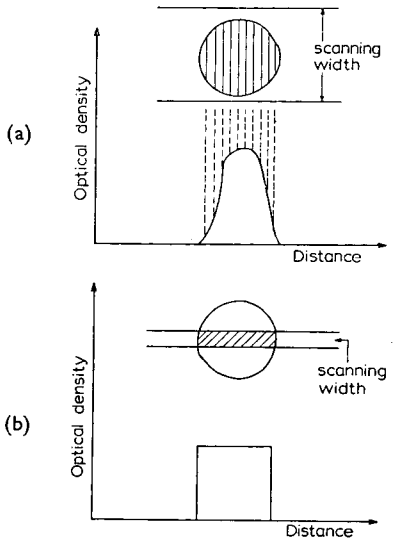


Fig. 3. Scanning methods. (a) Total absorbance of a zone is measured. (b) Absorbance of a section (partial area of a zone) is measured.

less than 1.15 cm in diameter. This means that the volume of solute solution applied to the chromatogram may not exceed 5 μl, if the initial zone is to have such a diameter; owing to spreading of the zones during chromatography[2], it would be necessary to use much smaller volumes. The accurate measurement and delivery of such small volumes is difficult. The Agla-micrometer syringe, an instrument widely used for such purposes, allows the measurement of volumes with an error of $\pm$ 0.05 μl. Thus, while the relative error is only 0.5 % for 10 μl, for 1 μl it is 5 %.

The imposition of a small volume must simultaneously restrict the concentration of solute which may be chromatographed, if previous concentration of the solute solution is not possible or undesirable. This introduces further errors. Owing to variations in the chromatographic paper, its absorbance is not constant; the variations are small but appreciable—this may be referred to as base-line instability. Variations of $\pm$ 0.01 absorbance units were found to be normal in the system studied; for a substance having a peak height of one absorbance unit, this error requires least consideration. However, it comes into prominence for dilute zones and, coupled with volume errors, is sufficient to ensure that this method of working gives results which are only semi-quantitative.

The absorbance *versus* distance plot obtained by this method gives a curve which is partially influenced by the shape of the zone and is thus not a true absorbance cross-section. (Fig. 3 shows the absorbance cross-section and the type of curve likely

to be found on scanning a circular zone of uniform cross-section). The area under the curve will be proportional to the concentration of the zone-forming solute if Beer's law is obeyed, whereas if the absorbance cross-section is taken, this will only be true if the zone has uniform concentration or, in the case of a band, has identical absorbance cross-section throughout its length.

Since the utilization of such small zones of diameter in order that the absorbance of the entire zone may be measured is liable to give inaccurate results, the problem is to produce a uniform band of solute on the chromatogram. The procedure in this case is shown in Fig. 4a, which depicts part of a developed paper chromatogram including a zone which has been applied to the paper as a band. The ends of the band (shaded) will not have uniform cross-sections, and may not be used for scanning. Any portion of the middle section of the band of suitable width, *i.e.*, greater than the height of the slit in the scanning attachment, may be chosen for absorbance measurement. The width normally used is 3 cm; this is cut out and scanned as in Fig. 4b. It gives an absorbance cross-section as shown in Fig. 4c.

If V is the volume per cm of solution applied to the chromatogram on the starting line, and c is the concentration of the solute, then the weight, W, of the solute in the section of the zone scanned is given by

$$W = cV\alpha,$$

where α = the width of zone scanned = slit height.

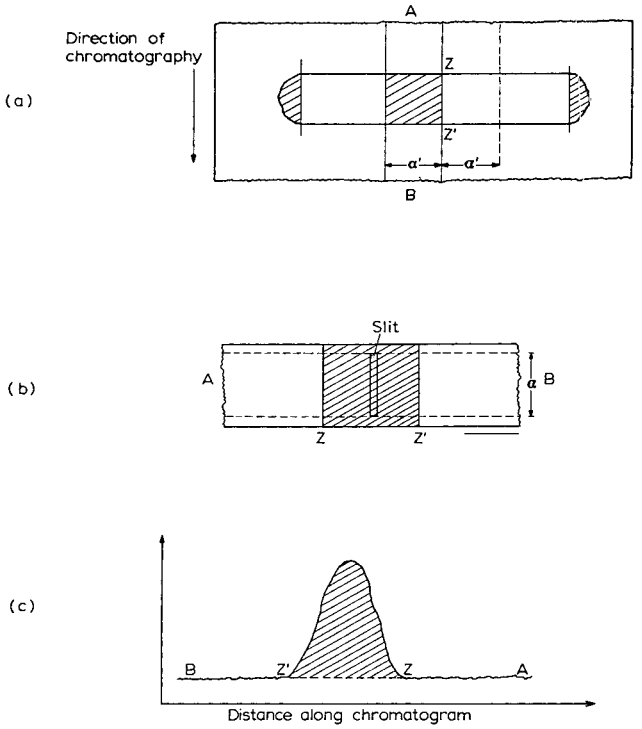


Fig. 4. (a) Part of a chromatogram showing a band. (b) Section of a chromatogram cut for scanning. (c) Optical density section.

If the shaded area in Fig. 4c is A sq. cm, and if Beer's law is obeyed,

$$W = k_1 A \alpha,$$

where k_1 is a constant.

Thus, the concentration is given by

$$c = \frac{k_1 A \alpha}{V \alpha} = \frac{k_1 A}{V}.$$

If V is kept constant,

$$c = k_2 A, \text{ where } k_2 = k_1/V.$$

Thus, it is seen that the criterion for success is that V, the volume of solution applied per cm, should be constant. This was found impossible to achieve manually, and therefore the zoning machine was used.

Several zoning machines have been described in the literature[3-5], but none have been used for applying to chromatograms in the stage of elution; hence it was necessary to design such a machine. The machine used is shown in Figs. 5 and 6 and, diagrammatically, in Fig. 7b. The block B supports an Agla micrometer syringe A, which was filled with the solution to be applied to the chromatogram. A rubber band was fastened round the syringe plunger and looped over the block B so that the plunger was kept in contact with the metal micrometer piston D. B was mounted on a

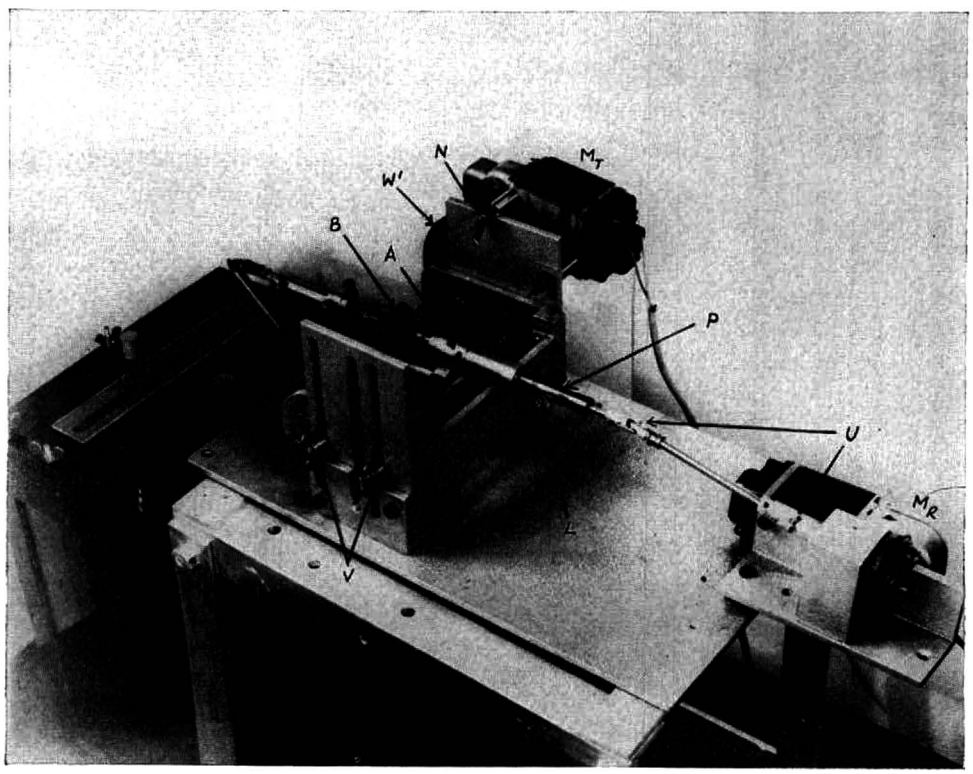


Fig. 5. The zoning apparatus (rear view).

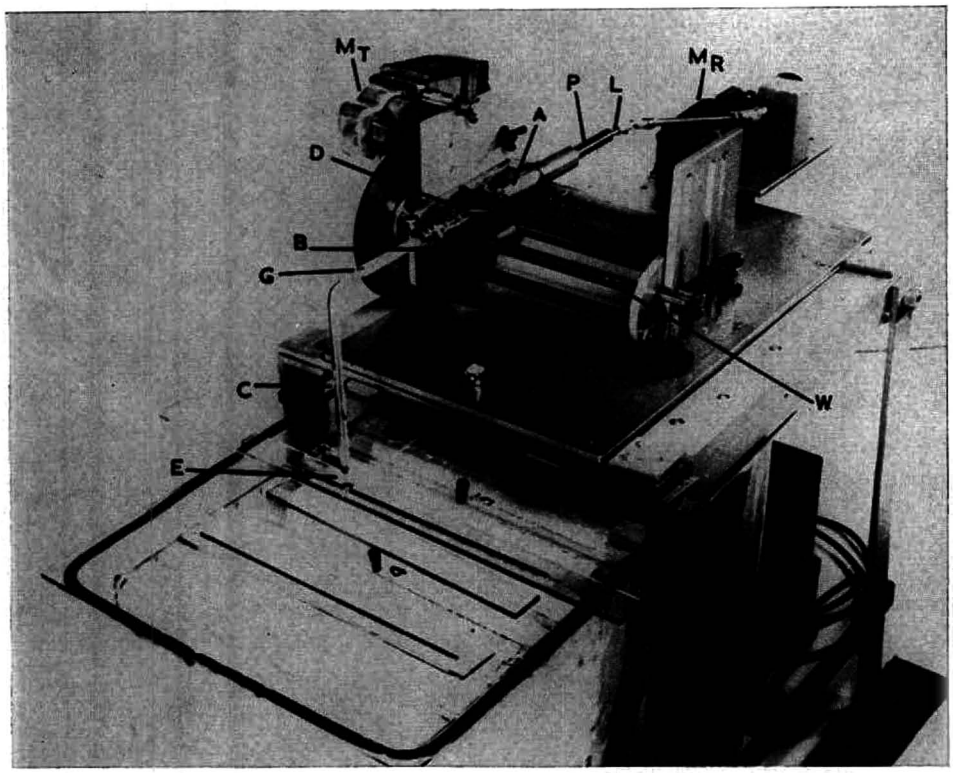


Fig. 6. The zoning apparatus (front view).

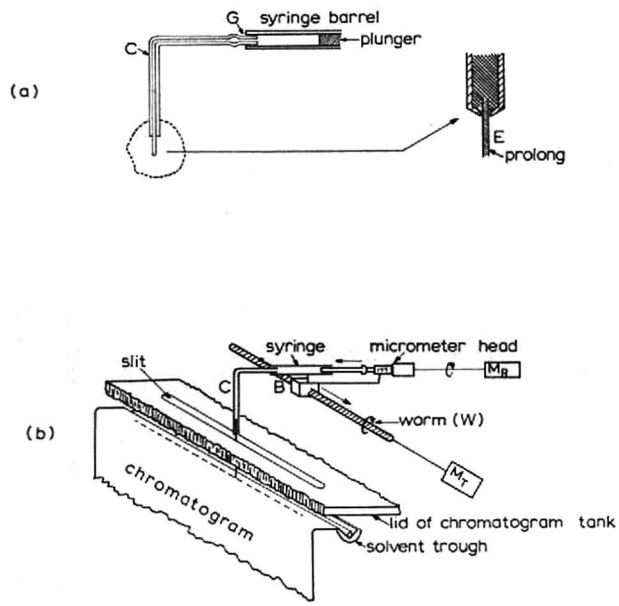


Fig. 7. (a) Syringe adaptor. (b) Operation of the zoning apparatus.

worm W. This worm was rotated by a D.C. motor M_T, which caused horizontal traverse of the syringe, while at the same time a second motor M_R caused rotation of the micrometer head and piston of the syringe, thus ejecting the solution through the adaptor C on to the paper chromatogram. After investigating different power units to drive the motors, the syringe delivered 6.13 μl in 107 sec; the maximum possible traverse used was 12 cm.

The motor M_R is coupled to the syringe head in such a way that the transverse motion of the syringe does not interfere with the rotation or place a torque upon the syringe. This is allowed by the combined action of the universal joints U. Since, as the micrometer head turns, the distance between it and M_R increases, it was necessary to incorporate the Teflon piston P, lubricated with oil, which maintained a rotational torque on the brass sleeving by means of the locating pin L, which is free to slide in a slit. The micrometer head is detached from the coupling by means of the Allen screw A_1 (see Fig. 5).

In order that the machine could be used with different chromatogram tanks, the slots V were cut to allow vertical displacement of the syringe. The motor M_T may be disconnected from the gear wheel W, by loosening the nut N. This is necessary at the beginning and end of a zoning operation. The syringe fittings are clamped into place by means of the brass block B, fitted with Allen screws.

Some difficulty was encountered initially in maintaining a liquid contact between the end of the tube C and the chromatographic paper. Since the chromatogram cannot be supported in a rigid manner and stretches during elution, no fixed alignment can be made between the height of the tip of tube C and the paper. Because of this, the delivery tube occasionally lost contact with the paper with consequent formation of a liquid drop and loss of uniformity in application of the solution.

To obviate this drawback, it was found necessary to introduce the thin glass canulus E (Fig. 7a). At the start of a run the canulus was pushed into C so that there is no contact with the paper. The motor M_R was set going and, as the solution slowly expelled from the syringe, so the canulus slowly emerged from its sheathing until it approximated to the surface of the paper. At this moment M_T was switched on and the end of the canulus touched the paper surface while undergoing vertical transverse. During the continued journey of the syringe the canulus rests upon the paper, delivering with a uniform flow and no drop formation. The tube C was fixed at an acute angle to the paper in the direction of motion to avoid any puncturing of the paper, which was sometimes found to occur when the tube was held in an upright position. When it was desired to conclude the application, the joint G was turned to raise the "stylus" from the paper, and both motors switched off. The slit in the lid of the chromatography tank was then covered with a glass plate, and the solutions allowed to chromatograph[6].

It was soon found that the opening of this slit for the time necessary to carry out the application of samples resulted in a substantial loss of equilibration inside the tank marring the chromatographic separation. In further experiments, the slit cover was replaced by another cover bearing a small hole in it, 0.5 cm diameter, through which the adaptor could pass. This was then removed over the slit at the same speed as the syringe; in this way only a very small outlet for the vapours in the tank was permitted, and the equilibrium was not seriously disturbed.

The further procedure was as illustrated in Fig. 4. Calibration graphs for $S_2O_3^{2-}$,

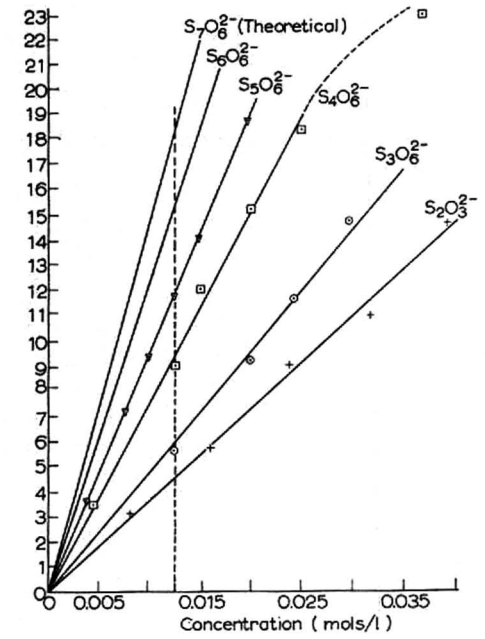


Fig. 8. Variation of area beneath absorbance section with concentration of applied solution

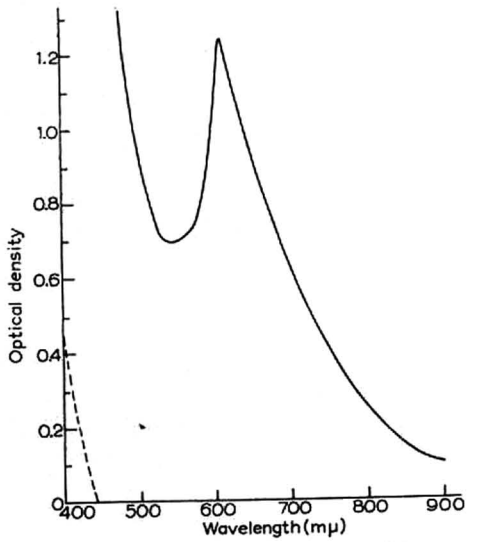


Fig. 9. Absorption spectra of silver sulphide and sulphur on filter paper. —— AgS + S stain from tetrathionate; – – – blank filter paper. Spectrophotometer slit width = 0.07 cm.

$S_3O_6^{2-}$, $S_4O_6^{2-}$, $S_5O_6^{2-}$, and $S_6O_6^{2-}$ were obtained (Fig. 8) using scans made at 600 mμ, since a maximum in the absorption spectra occurs at this wavelength (Fig. 9). The spectra of silver sulphide alone, as obtained by scanning a zone from thiosulphate, and the spectra of silver sulphide plus sulphur, obtained from tetrathionate, show no differences either in shape or position of the maximum. A typical separation of $S_3O_6^{2-}$, $S_4O_6^{2-}$ and $S_5O_6^{2-}$ is shown in Fig. 10. The results show that the method will allow estimation of thionates, the values being subject to an error of about 4%. This large error stems from two causes:

(i) the impossibility of standardising the developing technique to more than a qualitative degree;

(ii) there seems to be no possibility of obtaining absorbance measurements with respect to a comparative standard during the scanning;

(iii) there are variations in absorbance due to irregularities in the filter paper.

The development of the chromatograms involves spraying them with silver nitrate solution, heating before an electric fire to decompose the silver thionates

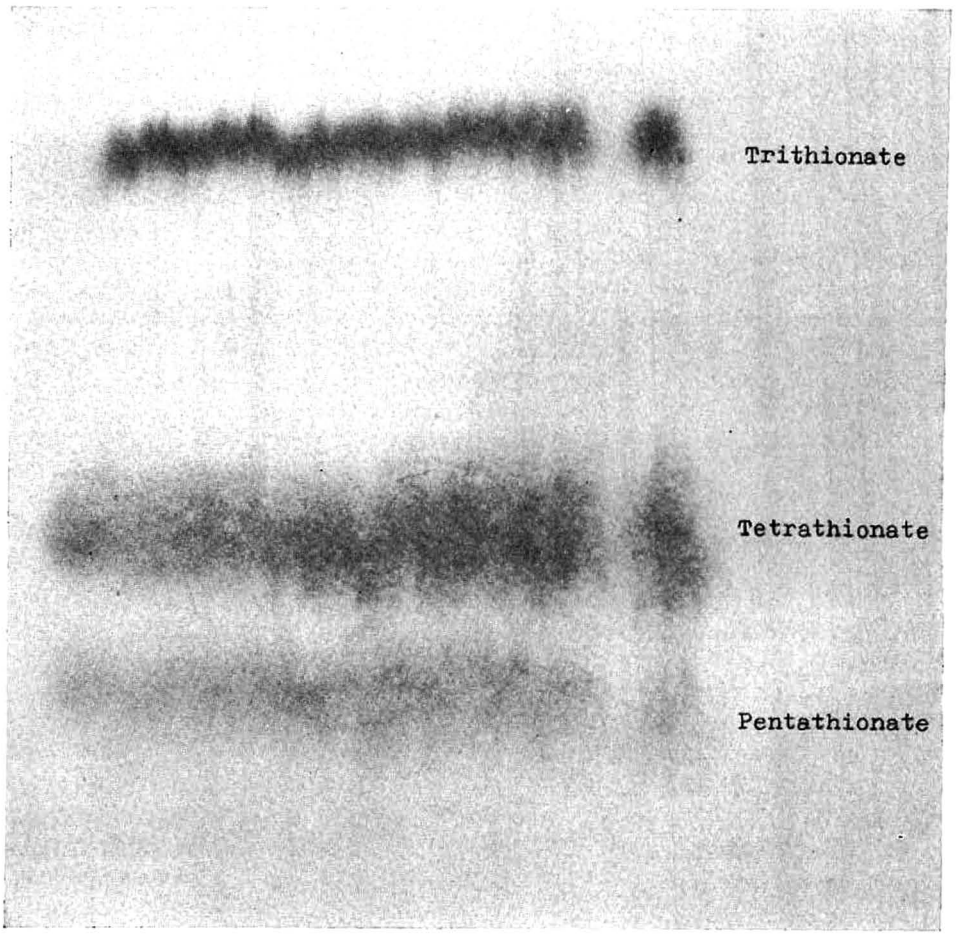


Fig. 10. Separation of tri-, tetra- and penta-thionate zones.

to sulphide and sulphur, washing in concentrated thiosulphate solution to remove excess silver, then in distilled water, and finally drying. Of these processes, that which is least readily standardised is spraying. The results of spraying vary considerably with the experience of the operator. The most even spraying was obtained by using a fine, light spray directed from a distance of a few feet on to the paper. As saturation is reached, a sheen may be seen on holding the chromatogram obliquely, all chromatograms were sprayed until this sheen appeared evenly all over the paper. By this means, gross variations were ruled out.

When using the spectrophotometer for measuring the absorbance of solutions, it is usual to use two cells which may be placed alternatively in the light beam. One of these contains pure solvent and the other is the solution whose absorbance it is desired to measure. For any given measurement, the blank cell is put into the beam first, and the width of the slit in the spectrophotometer adjusted until a certain voltage is produced by the detector. With the same setting of the slit width, the blank cell is replaced by that containing the solution, and the absorbance is measured, which is thus relative to that of the solvent. Not only does this comparison method nullify any effects due to light absorption by the solvent at certain wavelengths, it also ensures that any variations in the light source do not influence the measured absorbance. When scanning a chromatogram, it is not possible to do this, however, since once the scan is commenced, it may not be interrupted and the construction of the instrument does not permit simultaneous measurement of two absorbance sections. The time taken to complete a scan of a single zone is not less than 15 min, and it was found that quite considerable variation in intensity of the light source sometimes occurred over such periods. In order to reduce the extent of these effects, a blank strip was inserted in the scanning attachment such that a marked position interrupted the light beam. The slit width was then adjusted to bring the voltage to a certain value and the actual scan of the zone was then carried out using this slit width. After a scan, the blank was again inserted to check the variation in light intensity and, if necessary, the slit width readjusted. With a blank standardised at absorbance 0.153, the slit width varied between 0.15 and 0.20 mm over a period of months. No assessment can be made of the variations encountered during an actual scan, although it was sometimes observed that the baseline on one of the sides of the zone was higher than the other. However, it is thought that a large part of the error in the results obtained is due to this cause.

In an attempt to eliminate the variation in absorbance due to irregularities in the filter paper, translucing agents were used. Paraffin oil[7], Nujol[8], methyl salicylate[9], and a mixture of Nujol and *n*-amyl alcohol[9] have all been reported as reducing absorption and these were investigated. The strips of filter paper were soaked in the various organic solvents, and dried between filter paper. Using a constant slit width of 0.035 cm, the strips were scanned at 600 mμ. The maximum deviations in absorbance were calculated. In Table I these results are shown and can be compared.

From these results it was seen that a strip soaked in Nujol alone for 5 min and then dried between filter papers reduced the absorption of the paper to the greatest degree. Next a tetrathionate scan on plain filter paper with the scan of the same strip was obtained after immersion in Nujol. In both cases the absorbance of the filter paper background was adjusted to 0.180 by variation of the slit width. The third scan was carried out on a different background absorbance (see Table II).

TABLE I

COMPARISON OF VARIOUS TRANSLUCING AGENTS AT A CONSTANT SLIT WIDTH OF 0.035 CM

Agent	*Method of application*	*Optical density*
Plain		0.78 ± 0.015
Nujol	Soak for 5 min and dry between filter paper	0.04 ± 0.015
Nujol 60 amyl alcohol 40	Soak for 5 min and dry between filter paper	0.180 ± 0.08
Methyl salicylate	Soak for 5 min and dry between filter paper	0.112 ± 0.02
Amyl alcohol	Soak for 5 min and dry between filter paper	0.82 ± 0.017
Nujol*	Soak for 5 min, hang for 1 h, and dry between filter paper	0.096 ± 0.018
		0.083 ± 0.031
Nujol*	Soak for 5 min, hang for 3 h, and dry between filter paper	0.065 ± 0.032
Nujol*	Soak for 5 min, hang for 8 h, and dry between filter paper	0.095 ± 0.031
Nujol*	Soak for 5 min, leave wet for 24 h, and then dry	0.136 ± 0.024

* Slit width = 0.067 cm.

From these results it was seen that although Nujol did reduce the background, it also reduced the size of peak obtained by a factor of 4. Thus it is preferable to scan the untreated strip.

On referring to Fig. 8, it is seen that the method is most sensitive for higher thionates and least sensitive for thiosulphate. Consideration of why this is also allows

TABLE II

COMPARISON OF TRANSLUCING AGENTS FOR RELATIVE SENSITIVITY

	Area (sq. in.)	*Slit width (cm)*	*Background absorbance*
Plain	20.14	0.138	0.180
Nujol	5.13	0.066	0.180
Nujol	4.99	0.077	0.050

some conclusions regarding the slopes of the straight lines in the figure to be drawn, and the theoretical prediction of the curve for $S_7O_6^{2-}$ and so on can be made.

BASSETT AND DURRANT[1] showed that the silver thionates decompose according to:

$$Ag_2S_3O_6 \longrightarrow Ag_2S + 2\ SO_3 \quad (1)$$

$$Ag_2S_4O_6 \longrightarrow Ag_2S + 2\ SO_3 + S \quad (2)$$

$$Ag_2S_5O_6 \longrightarrow Ag_2S + 2\ SO_3 + 2\ S \quad (3)$$

while silver thiosulphate decomposes as follows:

$$2\ S_2O_3^{2-} \longrightarrow S^{2-} + 2\ SO_3^{2-} + S \quad (4)$$

It is evident at once that the stain produced for tetrathionate should be twice as dense as that for thiosulphate. Also, if the absorptions due specifically to Ag_2S, M_2SO_4, and S are a, b and c, respectively, then the absorbances of the thionates should be in the ratio:

$$S_3O_6^{2-}:S_4O_6:S_5O_6 = 1:1 + \frac{c}{a+2b}:1 + \frac{2c}{a+2b}.$$

It may be shown that if, for an absorbance such as those measured, the absorbance is increased in a certain ratio at all points on the curve, then the area under that curve will be increased in the same ratio.

It is therefore to be expected that the areas obtained from thionate zones corresponding to a given concentration should bear a simple ratio to one another. This is in fact the case. In Fig. 8 the ordinate at concentration 0.0125 moles/l intersects the curves at the A values given in Table III.

TABLE III

AREAS OF THIONATE ZONES

	$S_2O_3^{2-}$	$S_3O_6^{2-}$	$S_4O_6^{2-}$	$S_5O_6^{2-}$
A	4.5	6	9.4 (9)	11.8 (12)
Ratio to $S_2O_3^{2-}$	1	1.33	2	2.66
Ratio to $S_3O_6^{2-}$		1	1.5	2

This would suggest that $c/(a + 2b) = 0.5$, and these ratios are thus directly correlated to eqns. (1), (2), (3), and (4). Evidently, for any given higher thionates (assuming the decomposition of the silver salt is similar) the appropriate ratio may be found by adding on the requisite number of contributions due to sulphur atoms to the value of 1 for $S_3O_6^{2-}$. In this way the theoretical curves for $S_6O_6^{2-}$ and $S_7O_6^{2-}$ have been drawn. The theoretical curve and experimental curve are coincident. This procedure has considerable advantages when dealing with substances such as higher thionates, in which case it is usually difficult to obtain specimens suitable for preparation of calibration curves.

An intriguing application of this analysis method so far neither tested nor exploited is the possibility of recording a continuous "picture chromatogram" of the

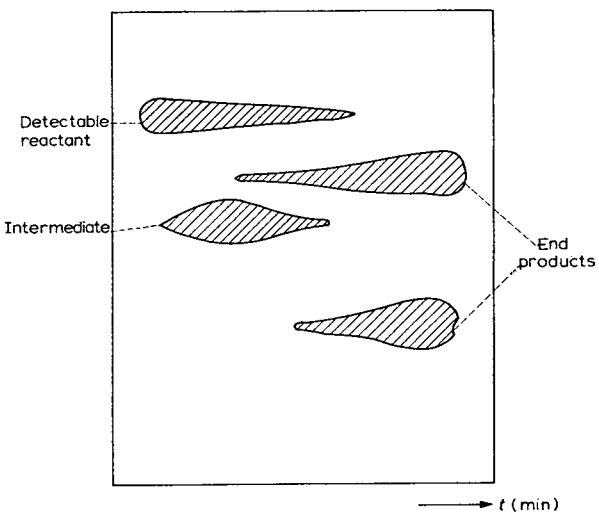


Fig. 11. Possible application of the zoning instrument to kinetic studies.

progress of a slow reaction. The automatic zoning machine takes about 15 min to traverse 10 cm and continuously applies solution to the chromatogram during this time. Given a homogeneous reaction in solution with a velocity such that it would be useful to study a 15 min period of the reaction, then by setting the reaction mixture in the syringe and zoning as described previously, the chromatogram produced would give a complete record of the kinetics of the reaction during this time. It has already been noted that for reactions involving thionates, reaction ceases as soon as the solution is applied to the paper[6]. A diagram of a chromatogram illustrating this application in the case of a hypothetical reaction is shown in Fig. 11. The exact times at which certain products are formed may be calculated, and by the method outlined above, the concentration of any component at any desired time may be found.

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SUMMARY

The "band" analysis method for the quantitative determination of thiosulphate, tri-, tetra-; penta-, and hexa-thionates has now reached a stage of development where it can be used to estimate the concentration of these ions in unknown mixtures.

The method should be very useful in following the course of reactions involving the species, as by using it their appearance and disappearance can be followed.

The main advantage of this method over classical methods is that each species is identified unequivocally and analysed separately.

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SEPARATION OF INORGANIC IONS IN FUSED SALTS BY MEANS OF CHROMATOGRAPHY AND ELECTROPHORESIS ON GLASS FIBER PAPER

III. EFFECT OF WATER, OXYGEN AND SUPPORT ON THE MIGRATION OF INORGANIC IONS DISSOLVED IN THE LiCl–KCl EUTECTIC AT 450°

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Electromigration on glass fiber paper can be employed to obtain simply and rapidly useful information concerning the nature of ions present in fused salts, and also to perform some analytical separations of inorganic ions[1–3].

Before determining the electrophoretic behaviour of an ion, it is necessary to ascertain whether the ion interacts with the support and also whether precipitation due to the ions present in the fused electrolyte could occur. This can be studied by chromatographic experiments and only when the ion travels freely with the front of the fused eutectic, can it be assumed that neither precipitation nor interaction occurs.

In this paper, the causes of precipitation of different metallic ions dissolved in the LiCl–KCl eutectic are discussed. Suitable experimental conditions for avoiding such precipitation were determined by chromatography.

EXPERIMENTAL

Preparation of dehydrated eutectic

Finely powdered potassium chloride (Erba RP) and lithium chloride (Erba RP) were oven dried and then mixed in molar proportions of LiCl:KCl = 59:41. This mixture was dried again at 94°[4] and then dehydrated with anhydrous HCl in an assembly similar to that described by GRUEN AND MCBETH[5]. The anhydrous pellets of eutectic were then stored under CCl_4 previously distilled over P_2O_5. The solutions of ions being examined were obtained by dissolving the respective anhydrous chlorides in the eutectic. Anhydrous chlorides of Th(IV), Cr(III), Co(II), Mn(II) are not readily available commercially, therefore the corresponding hydrated chlorides were dehydrated by heating in a stream of anhydrous hydrogen chloride. The chlorides were then dissolved in the fused eutectic and anhydrous hydrogen chloride was bubbled through, for approximately $^1/_2$ h. In this way, the chlorides of all of the elements studied were perfectly dissolved in the molten eutectic.

Apparatus

The electrophoretic experiments with non-dehydrated LiCl–KCl eutectic were performed in a furnace similar to that described in a previous paper[2].

The experiments with dehydrated eutectics were performed in the stainless steel apparatus shown in Fig. 1. This apparatus consists of a cylindrical furnace, which contains an electrophoretic chamber of slightly smaller diameter than the furnace that can be tightly closed. The chamber can be easily withdrawn and transferred to a

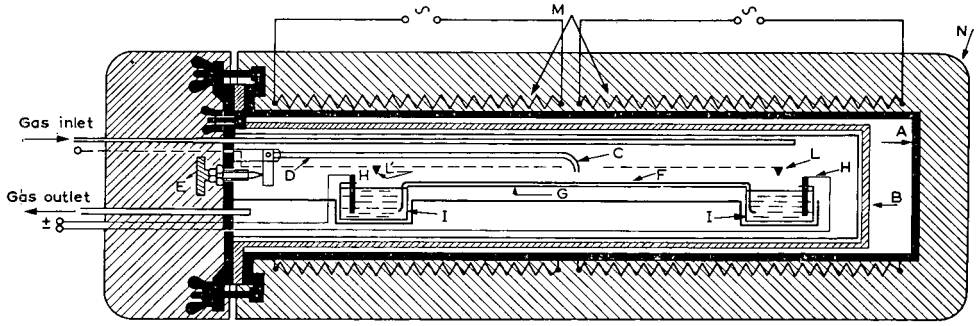

Fig. 1. Cross-sectional view of the apparatus: A = furnace; B = electrophoretic chamber; C = capillary; D = tube supporting the capillary; E = screw to rise or lower tube D; F = glass fiber paper; G = pyrex glass plate; H,H′ = graphite electrodes; I,I′ = pyrex vessels; L,L′ = thermocouples; M = Ni–Cr heating wire; N = insulating jacket.

dry-box in order to prepare the experiment. The furnace is electrically heated by two electric resistances controlled by two thermocouples.

The chromatographic experiments were performed in an assembly similar to that described in ref. 1 (Fig. 3).

Techniques for electrophoretic and chromatographic experiments in anhydrous conditions

In order to perform chromatographic and electrophoretic experiments under perfectly anhydrous conditions, all the preliminary operations must be done in a dry-box.

The dehydrated salts, prepared as described above, are dissolved in the anhydrous eutectic contained in the apparatus shown in Fig. 2. Anhydrous hydrogen chloride is

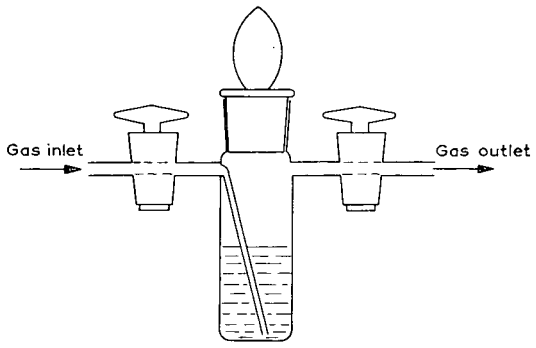

Fig. 2. Apparatus used to dissolve chlorides of metal ions in molten LiCl–KCl eutectic.

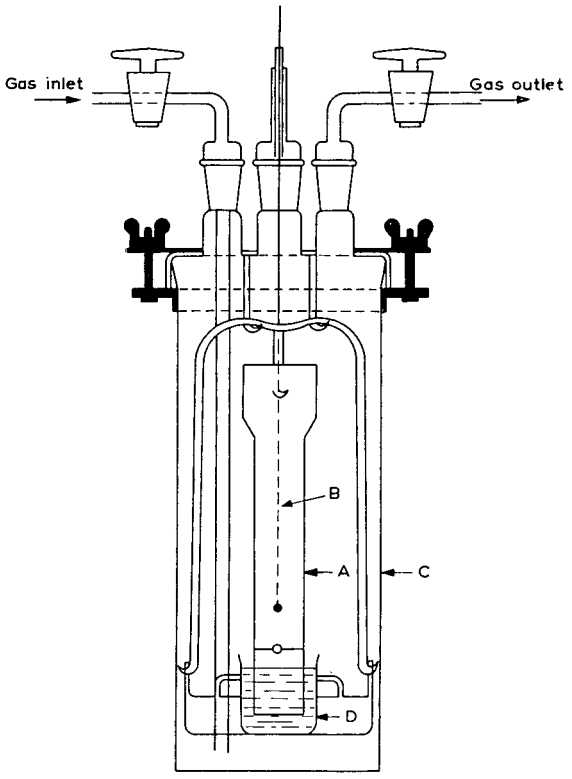

Fig. 3. Apparatus for chromatographic experiments. A = glass fiber paper; B = thermocouple; C = pyrex cylinder; D = pyrex vessel.

passed for approximately $^1/_2$ h, followed by anhydrous nitrogen for about 10 min. In the chromatographic experiments the ion to be examined is placed, by means of a glass capillary, directly on the fiber glass paper in the dry-box. To perform electrophoresis under anhydrous conditions, the capillary C (Fig. 1) is filled with the melt containing the ion being examined. The strip of glass fiber paper is placed on the support G, and the vessels I–I′ are filled with the dehydrated eutectic. The electrophoretic chamber is then removed from the dry-box and placed in the furnace. At this stage, only one section of the furnace was heated, in order to melt only the eutectic contained in one vessel. The glass fiber strip is then chromatographically impregnated by the fused chlorides. After about 2 h, the other section of the furnace is heated in order to melt the eutectic contained in the second vessel. In this way, the junction of the two chromatographic fronts is no longer located at the center of the strip, but is displaced towards one of the vessels. It is thus possible to avoid any concentration of the impurities of the paper at the center of the strip where the spot is placed.

The current between the two vessels is then switched on and allowed to become constant, and is then turned off. The tube D is then lowered by means of an external device E, so that the end of the capillary C lies directly on the glass fiber strip. In order to make sure that most of the salt flows from the capillary to the strip, this operation is repeated several times. An electric field (3.3 V/cm) is then again applied for 1–3 h

according to the electrophoretic mobility of the ions being examined. During the whole experiment, a slow stream of anhydrous nitrogen is passed through the electrophoretic chamber.

RESULTS AND DISCUSSION

(*a*) *Chromatography of metal ions*

The chromatographic experiments, under air and using the hydrated LiCl–KCl eutectic, showed that while some metallic ions travel freely with the front of the fused eutectic, others remain at the point of application (see Table I, column a).

To determine whether these ions stayed at the point of application as a result of a reaction with the support or because they were precipitated as oxides, the possible sources of O^{2-} ions in the melt were eliminated. The main cause was found to be the appreciable solubility of water in the fused eutectic even at temperatures of 450° to 500°[4]. The eutectic should therefore be carefully dehydrated in order to avoid the hydrolysis reactions in this temperature range.

The O^{2-} ion could also be present in the melt as a result of the oxidation of the Cl^- ion by the oxygen present in the chromatographic chamber, according to the reaction:

$$2\ Cl^- + \tfrac{1}{2}\ O_2 = Cl_2 + O^{2-} \qquad (1)$$

The equilibrium constant for this reaction is not known. Its numerical value should be very small as the potential of the Cl_2/Cl^- system, referred to the Pt/Pt^{2+} electrode, is 0.322 V[7], while that for the O_2/O^{2-} system, according to DELARUE[8,9], is in the range 0 to 0.06 V.

The small concentration of O^{2-} ions from reaction (1), when air is used as filling gas, can be high enough to precipitate those ions forming highly insoluble oxides. Moreover, the presence of oxygen in the filling gas, may cause the oxidation of some ions such as Fe(II) or U(IV), modifying their chromatographic and electrophoretic behaviour. Therefore, in experiments with ions forming highly insoluble oxides in LiCl–KCl melts and ions that could be oxidized, it is necessary to use pure nitrogen as filling gas for the chromatographic chamber.

A third cause of precipitation of very slightly soluble oxides could be the glass fiber paper. Glass can contain an appreciable quantity of water which dissolves in the melt[6]. Therefore, the strips were dehydrated at 480° and stored in a dry-box. Furthermore alkali oxides contained in the glass could dissolve in the fused eutectic giving rise to O^{2-} ions. A powdered quartz support could possibly give better results than the one used in these experiments.

Drastic conditions would be required, *i.e.* a stream of anhydrous hydrogen chloride, in order to achieve the complete elimination of O^{2-} ions. Chromatographic experiments in anhydrous hydrogen chloride atmosphere were unsuccessful, as in this case the glass fiber strip is no longer wetted by the fused eutectic. Therefore, the wettability of the glass fiber paper by the LiCl–KCl eutectic, might be related to the presence of traces of water or O^{2-} ions. As it is not possible to operate under such drastic conditions, a different acceptor of O^{2-} ions of medium strength ($S_2O_7^{2-}$) was used in some further experiments.

The results obtained by chromatography under various conditions are reported in Table I. In column (a) the results obtained in experiments performed without special

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF INORGANIC IONS IN MOLTEN LiCl–KCl EUTECTIC AT 450° UNDER VARIOUS CONDITIONS

Ions	*(a) Non-dehydrated eutectic*	*(b) Anhydrous eutectic, anhydrous air*	*(c) Anhydrous eutectic, anhydrous nitrogen (99.998%)*	*(d) Anhydrous eutectic with 10% $Na_2S_2O_7$, anhydrous nitrogen (99.998%)*
Na (I)	Front	Front	Front	
Rb (I)	Front	Front	Front	
Cs (I)	Front	Front	Front	
Ag (I)	Front	Front	Front	
Tl (I)	Front	Front	Front	
Cd (II)	Front	Front	Front	
Pb (II)	Front	Front	Front	
Zn (II)	Front	Front	Front	
Co (II)	Front	Front	Front	
Mn (II)	Application point	Front	Front	
Ni (II)	Application point	Front	Front	
UO_2 (II)	Application point	Front	Front	
Cr (III)	Application point	Application point	Front	
Fe (III)	Application point	Application point	Application point	Front
Th (IV)	Application point	Application point	Application point	Front with tail
CrO_4 (II)	Front	Front	Front	
CrO_3	Front	Frout	Front	

precautions (hydrated eutectic and presence of air) are listed. In columns (b), (c) and (d) the results obtained with dehydrated eutectic, using respectively air (b), nitrogen (99.998 % purity) (c), and $Na_2S_2O_7$ 10 % (d) are reported.

These results show that, once the causes for precipitation of oxides are eliminated, the ions, except Th(IV), move freely with the front of fused eluant. Therefore, as expected, adsorption on glass is negligible, taking into account the high ionic concentration, *i.e.* the strong eluting power of the eutectic LiCl–KCl.

From these experiments, the ions examined can be arranged according to the solubility of the oxides in LiCl–KCl eutectic and these solubilities correspond to those found by DELARUE[9] by dissolving the oxides in the LiCl–KCl eutectic.

(b) Electrophoresis of inorganic ions

The electrophoretic behaviour was determined in anhydrous eutectic under anhydrous nitrogen (99.998 % purity) for those ions moving freely in the chromatographic experiments. All the preliminary operations took place in a dry-box as already described. The results reported in Table II show that UO_2(II) gives an anionic complex and Cr(III), Ni(II), Tl(I) and Mn(II) behave as cations.

TABLE II

MOVEMENT OF INORGANIC IONS IN FUSED ANHYDROUS LiCl–KCl EUTECTIC AT 450° AND 3.3 V/cm

Ions	*Distance (cm) moved in 2 h*
Mn(II)	4(+)
Ni(II)	4(+)
Tl(I)	12(+)
Cr(III)	13(+)
Fe(III)	0 *
Th(IV)	0 *
CrO_4(II)	1(—)
CrO_3	1(—)
UO_2(II)	2(—)

(+) Movement towards cathode; (—) movement towards anode. Values are from center of initial spot to center of zone after moving.

* Insoluble precipitate formed.

In order to study the electrophoretic behaviour of Fe(III) and Th(IV) in molten LiCl–KCl eutectic, some experiments using a stream of anhydrous hydrogen chloride, were carried out. As shown earlier, however, in an anhydrous hydrogen chloride atmosphere, the glass fiber paper cannot be wetted by the eutectic. Therefore, the glass fiber strip was first impregnated in a nitrogen atmosphere, then the dehydration with a stream of anhydrous hydrogen chloride was performed. Under these conditions, diffusion of the ions along the whole strip was observed in a very short time ($\sim$ 1 h) without any applied e.m.f.

Therefore, we were not able to obtain any information about the electrophoretic behaviour of Fe(III) and Th(IV) in molten LiCl–KCl eutectic.

The electrophoretic behaviour of some ions giving soluble oxides has been reported elsewhere[2]. It has to be pointed out that ions precipitating as oxides at the application

point, showed rather high mobilities once the O^{2-} ion concentration in the melt was lowered (see conditions in Table II).

The CrO_4^{2-} ion shows a very slight mobility similar to that shown by CrO_3. This could be due to a decomposition of the CrO_4^{2-} into CrO_3 and O^{2-}

$$CrO_4^{2-} \rightleftharpoons CrO_3 + O^{2-} \qquad (2)$$

The chromic anhydride formed, is soluble in the molten eutectic in its molecular state[8], so that elution to the front in chromatography and no movement in electrophoresis would be expected. This has been confirmed experimentally. The CrO_4^{2-} ion can only exist in the melt in appreciable concentration in the presence of a relatively high O^{2-} ion concentration. Therefore, the electrophoretic mobility must be related to the equilibrium constant of reaction (2) and to the O^{2-} ion concentration. Only when complete elimination of O^{2-} ions has been achieved, could chromic anhydride be used for the determination of the electroendosmotic effect in fused salts. Owing to the electroendosmotic effect, the data reported in Table II for chromic anhydride are not necessarily conclusive as it is difficult to avoid completely traces of O^{2-} ions, using glass fiber paper as support.

CONCLUSIONS

The results obtained indicate that electrophoresis of inorganic ions in molten LiCl–KCl eutectic is only possible, without special precautions, for ions forming soluble oxides in this melt. For ions forming insoluble oxides a dehydrated eutectic and an atmosphere of an inert anhydrous gas, *viz.* pure nitrogen, is necessary.

Chromatography in fused salts proved to be a very simple method for obtaining information concerning the relative solubility of various oxides. Electrophoresis in fused salts proved to be useful in providing information about the nature of the ions in fused salts. The different electrophoretic mobilities and solubilities of the oxides in a particular eutectic can be used to achieve separation of inorganic ions.

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The authors wish to express their thanks to Prof. V. CAGLIOTI and Dr. M. LEDERER for helpful discussions.

SUMMARY

The chromatographic and electrophoretic behaviour of different metal ions dissolved in LiCl–KCl eutectic was studied. It was found that:

(a) Traces of water in the eutectic precipitate metal ions forming sparingly soluble oxides in fused alkali chlorides.

(b) The ions examined did not react with the glass fiber paper employed as support.

(c) When the ions to be examined are easily oxidized or give sparingly soluble oxides in molten chlorides the experiments must be performed in an inert gas atmosphere (water and oxygen free).

The chromatographic and electrophoretic behaviour of some inorganic ions is reported and the electroendosmotic effect in fused chlorides, employing glass fiber paper as support, is discussed.

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Notes

The separation of oestrogens by horizontal thin-layer chromatography

The technique of separating several oestrogens by thin-layer ascending chromatography has been described by LISBOA AND DICZFALUSY[1] and LUISI *et al.*[2], and has since found extensive application.

The authors found that horizontal chromatography on silica gel layers improved the separation of oestrogens, both in mixtures of pure substances and in extracts of biological fluids.

The advantages of this technique are speedy development with high resolving power, and better preparative purification with continuous development. REISERT AND SCHUMACHER[3] have used a similar technique for the fractionation of urinary 17-ketosteroids.

Table I lists both R_F values and the R_{Oe} values relative to oestrone, of the several oestrogens investigated. The solvent system is cyclohexane–ethyl acetate (35:65, v/v).

TABLE I

Compound	$R_F \pm$ S.D.		$R_{Oe} \pm$ S.D.	
Oestrone	0.87	0.03	1.00	—
17β-Oestradiol	0.73	0.03	0.83	0.03
16-*epi*-Oestriol	0.45	0.02	0.52	0.02
Oestriol	0.17	0.01	0.20	0.01

Experimental

A suspension of silica gel G (7731, Merck) was applied on glass plates (20 cm × 20 cm) using a Desaga thin-layer applicator, calibrated to give a 250 μ layer according to LUISI *et al.*[2]. The resulting plates were dried in air and activated at 110° in an oven. Small amounts of steroids (0.5 to 5 μg) in absolute ethanol (0.5–5 μl) were spotted on the starting line, approximately 1.5 cm from the bottom edge of the plate. Care was taken not to apply steroids within 2 cm of the side edges because the reproducibility of chromatographic mobilities was considerably poorer in this portion of the plates.

The plates were developed, at room temperature, by the horizontal technique using the B.N.-Kammer apparatus (Desaga, Heidelberg); development time is about 20 min. The solvent front was marked and the plates were air-dried and then heated, for 10 min, in an oven at 60°.

The chromatoplates were then sprayed with a freshly-prepared solution of BARTON'S[4] reagent or with 2,4-dinitrophenylhydrazine 0.5 % in alcoholic sulphuric acid 10 % v/v.

Different proportions of the present solvent system and other solvent systems were investigated and no appreciable improvements noted.

The purification of oestrogens contained in urinary extracts has also been attempted and is still being studied.

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A method for automatic packing of Sephadex columns

Various methods have been investigated and used for packing Sephadex columns. One, described by FLODIN[1], gave good results with Sephadex G-25 and G-50. Another packing method, useful for all Sephadex types, was later developed by the same author[2]. In this latter method, the extension tube with the Sephadex suspension, connected to the column to be packed, has to be emptied of the supernatant as soon as it is depleted of gel and refilled with new suspension. This must be repeated until the desired bed length has been obtained. The packing of large columns with this method would be very time consuming. Furthermore, after several fractionations, the flow in such large columns may cease altogether so that frequently repacking is necessary.

In this note an automatic packing method is described which does not require continuous service during the packing process. This method can also be used for column materials other than Sephadex, although it is especially suitable for this material.

Method

A schematic diagram of the packing setup is shown in Fig. 1. The column is carefully mounted vertically and the outlet is closed. It is filled with the desired liquid and a small amount of Sephadex suspension is added. When a layer of 2–5 cm has formed[2] the system is closed, as shown in Fig. 1, and the outlet is opened. From the flask with the Sephadex suspension under stirring, the gel grains are entrained by the liquid which is siphoned through the PVC tubing into the column.

The packing rate depends upon the siphoning head between the level of the Sephadex suspension in the flask and the bottom of the column and also upon the

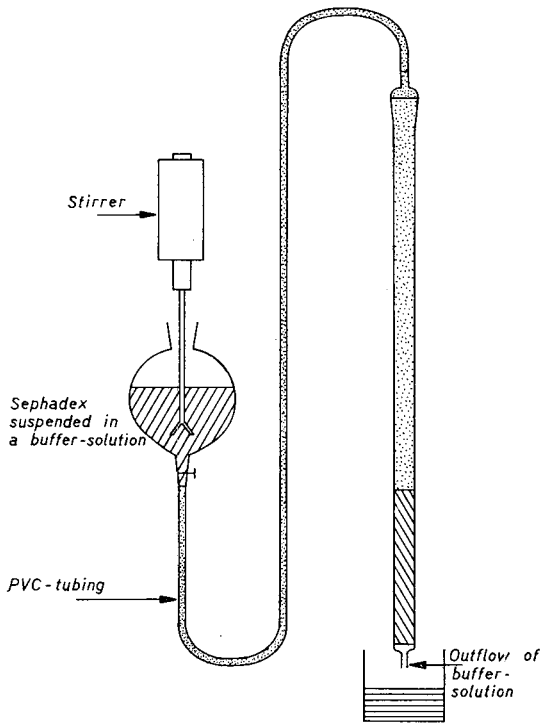

Fig. 1.

speed of the stirrer and the concentration and the type of Sephadex. Good packing can be obtained with a Sephadex concentration in the flask of about 2 %. The compactness of the Sephadex column formed is controlled by the siphoning head. By connecting a T-tube to the flask, two columns can be packed at the same time under identical conditions.

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An adaptable air stream evaporator for multiple samples

A versatile apparatus, of high capacity, for the evaporation of solvent from multiple samples has been designed from simple components. A portable *air-feeder rack*, Fig. 1, serves to hold the vessels containing the solvents to be evaporated and to distribute an air supply over the surface of the solvents. Chamber A, for the delivery of air, fits over the rack, B, and is adjustable in height above the rack with wing nuts and bolts projecting from the sides of the rack. Removable shelves, C, with varied size holes can accommodate a large variety of tubes or flasks. Projecting from Chamber A are 4 in., 16 gauge, stainless steel tubes whose centers coincide with the mouths of the flasks or tubes in rack B. Chamber A contains a central nipple to receive an air hose located in the *warm air cabinet*, Fig. 2. The warm air cabinet serves to hold the air-feeder racks at temperatures above ambient during the evaporation process. Its size is a function of the size and number of air-feeder racks, which it is desired to contain. When mounted in a hood, filters at top and bottom allow a continual flow of clean air to remove solvent vapors. A low temperature heating tape is suspended from the lower, stationary, perforated sheet-metal shelf. A thermoregulator on an upper side wall joins heating tape and input current. Filtered air is distributed within the cabinet with glass and plastic tubing and can be quickly connected to, or disconnected from the nipple on Chamber A. Sliding front doors of clear plastic serve to maintain temperature and keep out dust. Both heater and air pump can be controlled with an automatic timing device.

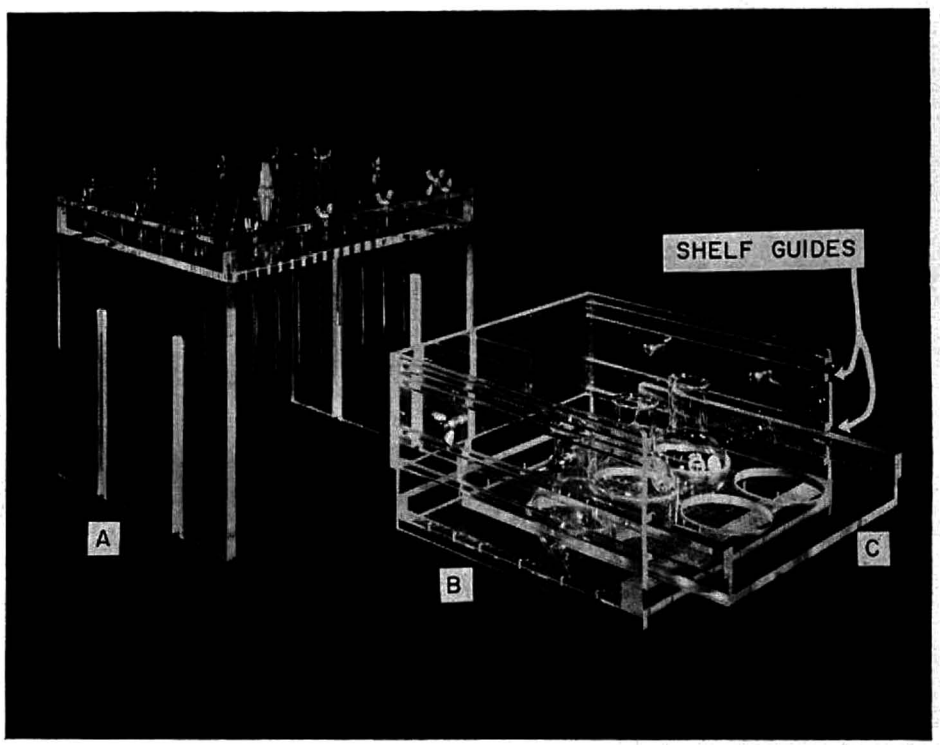

Fig. 1.

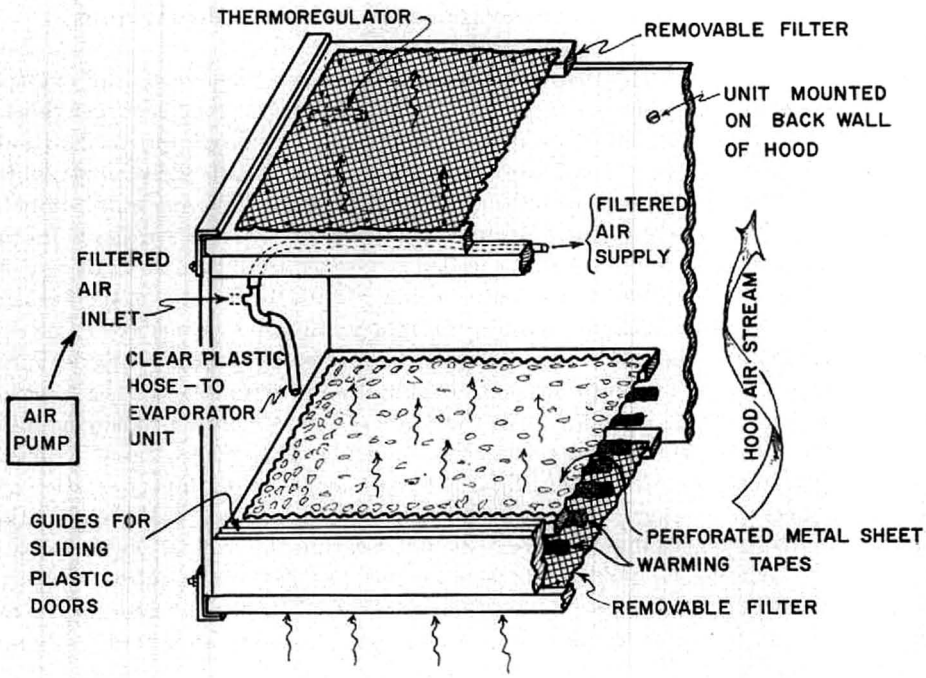

Fig. 2.

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Über das Auftreten von Fehlsubstanz-Zacken bei der Gaschromatographie

In der russischen Zeitschrift *Zavodskaja Laboratorija* wurde kürzlich über eine neue Methode der Gaschromatographie, die Vakanz-Chromatographie, berichtet, bei der die zu untersuchende Probe dem Trägergas dauernd beigemischt und durch einen Einlass reinen Trägergases ein Vakanz-Chromatogramm der Probe erzeugt wird[1].

Ohne diese Arbeit zu kennen, haben wir Untersuchungen über das gleiche Phänomen angestellt, über die im folgenden berichtet sei.

In der Literatur über Gaschromatographie[2,3] und von den Herstellern der

Gaschromatographen und Trägergase wird allgemein kleinen Verunreinigungen des Trägergases — abgesehen von deren unter Umständen schädlichem Einfluss auf Säulenfüllungen, der hier nicht beachtet werden soll — keine grosse Bedeutung beigemessen. Da der Hitzdrahtdetektor als Differentialanzeiger zwischen einen vor Probengeber und Säule abgezweigten und den von der Säule kommenden Gasstrom geschaltet ist, seien kleine Verunreinigungen für die Genauigkeit der Anzeige unerheblich. Wesentlich sei lediglich eine sehr genaue zeitliche Konstanz der Trägergaszusammensetzung.

Bei Experimenten zum Nachweis kleiner Wasserstoffmengen in katalytisch erzeugtem Sauerstoff stiessen wir auf zunächst unerklärliche Zacken scheinbar unbekannter Verunreinigungen. Sie können jetzt als Loch- oder Fehlsubstanz-Zacken erklärt und zugeordnet werden.

Es wurde ein 116E von Perkin-Elmer mit 1 mV-Sargent-Schreiber verwandt. Trennsäule: Molekularsieb 5 Å, 2 m lang, Hitzdrahtdetektor, 0.5 Atm Tärgergasvordruck, Säule bei Zimmertemperatur, Gaseinlass 1 ml 10^{-2} Torr können noch sicher nachgewiesen werden.

Mit normalem Bombenstickstoff als Trägergas zeigte der katalytisch entwickelte Sauerstoff eine kleine, deutlich getrennte Vorzacke mit der Retentionszeit von Wasserstoff oder Helium, die bei der verwendeten Anordnung schlecht getrennt werden. Eine Gegenprobe mit normalem Bombensauerstoff ergab jedoch die gleiche Zacke, wodurch die Aussage über den Wasserstoffgehalt des katalytisch erzeugten Sauerstoffs unsicher geworden war. Diese kleine Vorzacke blieb völlig unverändert, wenn man Bombensauerstoff mit BTS-Katalysator nachreinigte (Entfernung eventuell vorhandenen Wasserstoffs), verflüssigte und wieder verdampfte (Verminderung des Helium- oder Wasserstoffgehaltes) oder den katalytisch erzeugten Sauerstoff, der ausser Wasserstoff nichts enthalten kann, vor allem kein Helium, mit BTS-Katalysator behandelte. Aufschluss über die Natur dieser merkwürdigen "Verunreinigung" gaben Experimente mit Bombensauerstoff als Trägergas. Dann dürften nur die Verunreinigungen des katalytisch erzeugten Sauerstoffs Zacken ergeben. Eichung mit Wasserstoff ergab eine Nachweisgrenze von 10^{-2} Torr, das sind $0.3^0/_{00}$ bei 720 Torr (mittlerer Atmosphärendruck in München) Einlass. Einlass von Bombensauerstoff ergab erwartungsgemäss keine Zacke, Einlass des katalytisch erzeugten Sauerstoffs ergab zwei Zacken.

Die Nachprüfung unserer Vermutung, dass es sich um Loch-Zacken handele, ergab folgendes: Zumischen von 99.99%-igem Argon zum katalytisch erzeugten Sauerstoff ergab an der Stelle der einen Zacke eine grosse Zacke nach der andern Seite — die Argon-Zacke. Die zweite der beiden zuerst gefundenen kleinen Zacken blieb unverändert. Damit war eine der kleinen Zacken als Argon-Loch identifiziert. Zumischen von Bombenstickstoff zum katalytisch erzeugten Sauerstoff ergab an der Stelle der zweiten kleinen Zacke eine grosse Zacke nach der anderen Seite — die Stickstoff-Zacke; gleichzeitig wurde die Zacke des Argon-Loches auf etwa $^1/_{10}$ verkleinert.

Die Erklärung der Loch-Zacken ist folgende: Bombensauerstoff enthält Stickstoff und Argon als Verunreinigungen. Dieses "Gasgemisch" strömt durch Säule und Vergleichszelle, die Brücke ist abgeglichen, wie wenn reines Trägergas verwendet würde. Beim Einlass des reinen, katalytisch erzeugten Sauerstoffs entsteht eine Zone reinen Sauerstoffs, nach vorne und hinten durch eine Grenze vom stickstoff- und

argonverunreinigten Trägergassauerstoff abgesondert. Sauerstoff wandert mit der ihm eigenen Geschwindigkeit durch die Säule. Etwas langsamer wandert die vordere Argongrenze. Ebensoschnell wie diese wandert hinter der eingelassenen Zone katalytisch erzeugten Sauerstoffs die neue Argongrenze des unreinen Trägersauerstoffs. Beide Grenzen schliessen ein Argon-Loch ein, das sich mit Argongeschwindigkeit fortbewegt und bei der Retentionszeit des Argon die Hitzdrahtbrücke stört, mithin eine Argonloch-Zacke, entgegengesetzt der Argon-Zacke, erzeugt. Das gleiche gilt mit Stickstoffwanderungsgeschwindigkeit für das Stickstoff-Loch. Die Sauerstoffmoleküle der eingelassenen Zone reinen Sauerstoffs überholen die vorderen Verunreingungen und ergeben natürlich keine Zacke.

Auf die gleiche Weise ist die zuerst beobachtete Begleitzacke des Sauerstoffs mit Bombenstickstoff als Trägergas zu deuten. Bombenstickstoff enthält eine Verunreinigung, die Bombensauerstoff nicht in dem Masse enthält, und die natürlich bei allen Reinigungsmethoden des Sauerstoffs drin bleibt. Diese Loch-Zacke hatte die Retentionszeit des Heliums oder Wasserstoffs. In der Tat enthält Bombenstickstoff, ebenso nachgereinigter Stickstoff, mit dem die gleiche Loch-Zacke erschien, nach Angaben der Firma Linde je 200 bis 300 p.p.m. Helium und Neon. Eine Überschlagsrechnung ergab, dass die von uns beobachtete Zackenhöhe etwa diesem Wert entspricht.

Es ist anzunehmen, dass in vielen Fällen nicht identifizierbare Zacken von "Verunreinigungen" oder "Nebenprodukten" in Wirklichkeit derartige Loch-Zacken sind. Deshalb wird bei empfindlichen Nachweisen auf grösste Reinheit des Trägergases zu achten sein.

Wie erwähnt, wurde beim Zumischen von Stickstoff auch die Argonloch-Zacke stark vermindert, weil Bombenstickstoff auch Argon als Verunreinigung enthält. Dies bedeutet, dass beim Prüfen auf Verunreinigungen bei Abwesenheit einer Zacke nicht unbedingt auf Abwesenheit der Verunreinigung geschlossen werden darf, da es durchaus möglich ist, dass ein Verunreinigungs-Loch und eine Verunreinigung sich gerade kompensieren und dann nicht bemerkt werden.

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The separation of phenylhydrazones of volatile carbonyl compounds by vapour phase chromatography

The fractionation of carbonyl compounds and their identification generally involve very complex operations. We are particularly referring to the analysis of odoriferous constituents, where carbonyl compounds are present in very limited quantities, and to the study of ozonolysis fragments of complex organic substances.

The problem is even more complex when the compounds in the mixtures have different volatilities. In this case separation involves the formation of non-volatile derivatives and their resolution.

The problem is therefore now shifted to the separation of the derivatives. Among these, the 2,4-dinitrophenylhydrazones (2,4-DNPh) are generally preferred for known reasons. Chromatographic techniques are very useful in the fractionation of 2,4-DNPh mixtures into single components but the use of gas–liquid chromatography (GLC) for identification purposes is impossible, unless the carbonyl compounds can be regenerated and subsequently analysed by gas chromatography[1,2]. Obviously, a direct method of GLC-analysis for carbonyl compound derivatives would greatly assist in the study of such complex mixtures in small amounts.

The literature on this subject, as far as we know, is very scarce: CASON AND HARRIS[3] have examined mixtures of dioximes of volatile aldehydes on celite–di-2-ethylhexyl phthalate columns, and LOHR AND WARREN[4] have examined the oximes of benzaldehyde and salicylaldehyde on a celite–silicone column.

We have examined some aldehyde phenylhydrazones by GLC and here some preliminary data are presented for the gas chromatography of phenylhydrazones of normal aldehydes from C_1 to C_{12}.

Columns of SE 30 (2 %) on chromosorb W were used with a flame ionization detector. Nitrogen (flow rate 25 c.c./min) was used as carrier gas, and the column temperatures were between 120° and 190°. The peaks obtained were well shaped and no decomposition products were noted. A plot of the log of retention times of the *n*-aldehyde phenylhydrazones against the number of carbon atoms indicates a straight line relationship.

We intend to investigate this further and to expand these initial data.

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[1] J. W. RALLS, *J. Agr. Food Chem.*, 8 (1960) 141.
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News

SECOND INTERNATIONAL SYMPOSIUM

THE STATIONARY PHASE IN PAPER AND THIN-LAYER CHROMATOGRAPHY

The Symposium organized by the Chromatography Group of the Czechoslovak Chemical Society was held on June 10th–12th, 1964 at Liblice castle near Prague. The programme was divided into five sections:

I. Chromatography papers

Introductory reviews

A. GRÜNE, Dassel (Deutschland): Cellulose-Papier.
F. MICHEEL, Münster (Deutschland): Papiere aus chemisch modifizierter Cellulose.
C. S. KNIGHT, Maidstone (Great Britain): Ion-exchange paper chromatography.
Č. MICHALEC, Praha (Czechoslovakia): Non-cellulosic materials.

Discussion contributions

J. FELLEGI, Bratislava (Czechoslovakia): Optical properties of chromatographic papers.
J. ŠRÁMEK, Dvůr Králové (Czechoslovakia): Einfluss der chemischen Eigenschaften der Cellulose und des Charakters der stationären Phase auf das chromatographische Verhalten von Farbstoffen.
K. MACEK, Praha (Czechoslovakia): Anwendung von Glasfaserpapier zur Verankerung von Formamid.

II. Thin-layer materials

Introductory reviews

E. STAHL, Saarbrücken (Deutschland): Kieselgel.
L. LÁBLER, Praha (Czechoslovakia): Other inorganic materials.
P. WOLLENWEBER, Düren (Deutschland): Cellulosepulver. Chemisch modifizierte Cellulose.
B. GELOTTE, Uppsala (Sweden): Thin-layer gel filtration on Sephadex.
H. ENDRES, München (Deutschland): Polyamide.

Discussion contributions

J. GASPARIČ AND A. CEE, Pardubice-Rybitví (Czechoslovakia): Dünnschicht-Chromatographie substantiver Farbstoffe auf Kieselgel mit wässriger stationärer Phase.
P. ŠIPOŠ AND J. POLČIN, Bratislava (Czechoslovakia): Separation of the products of the oxidative degradation of glycides.
J. FOHL, Ostrava (Czechoslovakia): Der Einfluss des Adsorptionsmittels auf die Trennung aromatischer Kohlenwasserstoffe und heterocyklischer Verbindungen.
Z. PECHAN, Brno (Czechoslovakia): Chemically modified cellulose in thin layers.
V. V. RACHINSKIĬ, Moscow (S.S.S.R.): Sorption properties of ion-exchange celluloses.
E. SIMONIANOVÁ AND M. RYBÁK, Praha (Czechoslovakia): Dünnschicht-Chromatographie von Eiweisstoffen auf Phosphocellulose.
J. COPIUS-PEEREBOOM, Leiden (The Netherlands): Some properties of polyamide layers.
J. FELLEGI, Bratislava (Czechoslovakia): Chromatography on polyamide.

III. Stationary liquids and adsorbents in paper chromatography

Introductory reviews

R. CONSDEN, Maidenhead (Great Britain): The cellulose–water complex.
Ž. PROCHÁZKA, Praha (Czechoslovakia): Polar organic solvents.
E. CERRAI, Milano (Italy): The use of liquid ion-exchangers in paper chromatography.
Č. MICHALEC, Praha (Czechoslovakia): Adsorbents.

Discussion contributions

K. MACEK, Praha (Czechoslovakia): Einige Bemerkungen zu der Zusammensetzung von wässrigen Zweiphasensystemen.

J. CHURÁČEK, Pardubice (Czechoslovakia): Einige Bemerkungen zur Arbeit auf imprägnierten Papieren.

J. GASPARIČ, Pardubice-Rybitví (Czechoslovakia): Papierchromatographie von Phenolen und aromatischen Aminen unter Anwendung verschiedener polarer stationärer Phasen.

A. WAKSMUNDZKI AND S. PRZESLAKOWSKI, Lublin (Poland): Chromatography of some metal ions on paper impregnated with high-molecular weight amines and developed with thiocyanate solutions.

J. BORECKÝ, Pardubice-Rybitví (Czechoslovakia): Die Gegenüberstellung der Chromatographie auf Kieselgeldünnschicht und auf mit Kieselgel imprägniertem Papier bei der Trennung der Äthylenoxydaddukten.

IV. Stationary liquids and impregnations for thin layers

Introductory reviews

J. PITRA, Praha (Czechoslovakia): Zur Frage des Charakters und der Funktion des an Adsorbentien verankerten Wassers.

Ž. PROCHÁZKA, Praha (Czechoslovakia): Polare organische Lösungsmittel.

J. W. COPIUS-PEEREBOOM, Leiden (The Netherlands): Lipophilic liquids as stationary phase in thin-layer chromatography.

Discussion contributions

A. WAKSMUNDZKI AND R. MAŃKO, Lublin (Poland): Separation of phenols on thin layers of formamide-impregnated silicagel.

V. General problems of the stationary phase

Introductory reviews

E. STAHL, Saarbrücken (Deutschland): Standardisierung von Adsorbenzien und Trägermitteln.

S. HEŘMÁNEK, Praha (Czechoslovakia): Interaktionen zwischen der zu trennenden Substanz und der stationären Phase.

M. BRENNER, Basel (Schweiz): Die stationäre Phase als Ursache für die Ausbildung von Fliessmittel-Gradienten. Einfluss der Gradienten auf den Trenneffekt und die Gültigkeit des Gruppenadditivitäts-Prinzips. Förderung der Gradientenbildung in der BN-Kammer und Ausnützung im polyzonalen Trennverfahren.

I. M. HAIS, Hradec Králové (Czechoslovakia): The shape of spots as influenced by the stationary phase.

A. WAKSMUNDZKI AND E. SOCZEWIŃSKI, Lublin (Poland): The influence of the pH of the stationary phase on the chromatographic behaviour.

K. MACEK, Praha (Czechoslovakia): Verankern von Reagenzien.

C. G. HONEGGER, Basel (Schweiz): Schichtdicke in der Dünnschichtchromatographie.

Discussion contributions

J. FRANC, Pardubice-Rybitví (Czechoslovakia): Die Rolle intermolekularer Wasserstoffbrücken in der papierchromatographischen Trennung gewisser aromatischer Verbindungen.

M. ŠARŠÚNOVÁ AND V. SCHWARZ, Bratislava (Czechoslovakia): Wechselwirkung zwischen einigen zu trennenden Arzneimitteln, der mobilen Phase und dem Adsorbenten.

K. MACEK, Praha (Czechoslovakia): Querschnitt der stationären und mobilen Phase in der Papierchromatographie.

E. SOCZEWIŃSKI, A. WAKSMUNDZKI AND R. MAŃKO, Lublin (Poland): The volume ratio of the mobile and stationary phases in impregnation methods of paper chromatography.

V. V. RACHINSKIĬ, Moscow (S.S.S.R.): Theory of the longitudinal distribution of the mobile phase in paper and thin layer chromatography.

Z. VACEK, Neratovice (Czechoslovakia): Über einige Anomalien bei der pH-Chromatographie substituierter Phenole.

J. W. COPIUS-PEEREBOOM, Leiden (The Netherlands): Separation of sterols on silver nitrate impregnated adsorbent layers.

Announcements

THE 16TH PITTSBURGH CONFERENCE
ON
ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY, INC.,
EXPOSITION OF MODERN LABORATORY EQUIPMENT

Preliminary Announcement of 1965 Pittsburgh Conference

The Sixteenth Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Inc. will be held at the Penn-Sheraton Hotel in Pittsburgh, Pa., U.S.A., March 1–5, 1965. Approximately 200 papers on all phases of analytical chemistry and spectroscopy will be presented. Symposia on the following subjects are proposed for the 1965 Conference:

1. Coblentz Society Symposium in honor of Prof. HARRISON RANDALL
2. Steel Symposium, Modern Analytical Techniques in Today's Steel Mills
3. N.M.R. Symposium
4. Infrared Symposium on Group Frequency Intensities
5. Chemical Separations
6. The Management Viewpoints of Analytical Chemistry
7. On Stream X-ray Analyses Symposium
8. Gas Chromatography Symposium on (a) Process Monitoring and Control, (b) Flavors and Aroma, (c) Biomedical Investigations, and (d) Accuracy of Generalized Method
9. Universal Emission Spectrochemical Techniques.

Original papers on all phases of analytical chemistry and spectroscopy are invited. A brief abstract (150 words) of each paper will be printed in the program. Three copies of this abstract, with a letter listing the names of the authors, the laboratory in which the work was done, and the current addresses of the authors, should be addressed to:

Dr. WILLIAM G. FATELEY, Program Chairman,
The Sixteenth Pittsburgh Conference, Inc.,
Mellon Institute,
4400 Fifth Avenue,
Pittsburgh, Pa., 15213, U.S.A.

The final date for receipt of abstracts is *October 15, 1964*.

In addition to the program of technical papers, there will be an exhibition of the newest analytical instrumentation. More than 125 companies will display instru-

ments, chemicals, and equipment. A complete program of activities for wives and lady attendees at the Conference is also being planned for the 1965 Pittsburgh Conference. Finally, the sixth ENC (Experimental Nuclear Magnetic Resonance Conference) will be held at Mellon Institute Pittsburgh. For the convenience of those wishing to attend both meetings, the NMR sessions of the Pittsburgh Conference will be scheduled for Monday, March 1.

INTERNATIONAL SYMPOSIUM ON MICROCHEMICAL TECHNIQUES—1965

August 22–27, 1965

The Pennsylvania State University will conduct this 1965 Symposium at University Park, Pennsylvania, U.S.A. The program is being organized by the American Microchemical Society (formerly the Metropolitan Microchemical Society) with the sponsorship of the International Union of Pure and Applied Chemistry. The technical sessions and social events will parallel in organization and conduct those of the corresponding 1961 Symposium held under similar auspices and sponsorship. Details on registration and local arrangements will become available early in 1965. If you attended the 1961 Symposium, such information will be sent to you routinely; otherwise, the Organizing Committee should be informed of your possible interest.

The Pennsylvania State University, located in the heart of Nittany Valley, is an ideal location for the 1965 Symposium. It is located near the geographical center of the State of Pennsylvania, about 275 miles west of New York City, 190 miles from both Philadelphia and Washington, D.C. and 140 miles east of Pittsburgh. The University will provide adequate accommodations and facilities for individuals or families. The lectures will be held in buildings conveniently located on the beautiful campus. There are many attractions in the social program for families and those wishing to renew acquaintanceships.

A call is now being made for research papers directed toward small-scale operations, techniques, and methods in all phases of chemistry, including clinical chemistry and biochemistry. Papers centered on history, speculation, or review will not be considered. Although the technical sessions eventually organized will depend on the papers offered, it is hoped to have sessions on the following subjects: (1) micro methods in structural elucidation; (2) micro techniques in peptide studies; (3) clinical and forensic analysis; (4) novel micro and ultramicro approaches in organic elemental analysis; (5) micro techniques with high-energy materials; (6) novel micro separation methods; (7) inorganic microanalysis and trace analysis; (8) micro methods in air and water pollution studies; (9) determination of physical properties with small samples; (10) education for instrumentation; (11) general papers.

Regulations governing papers

1. All correspondence regarding papers (or requests for addition to the Symposium mailing list) should be directed to: Mr. HOWARD FRANCIS, Jr., Vice-Chairman,

Intern. Sym. Microchem. Techniques—1965, c/o Pennsalt Chemicals Corp., 900 First Ave., King of Prussia, Pennsylvania, U.S.A.

2. A descriptive title and an abstract must be received by January 31, 1965, and the text of the paper by April 30, 1965. Papers will not be considered for presentation "by title" or in absentia.

3. The abstract of not more than 300 words, preferably in English, should provide a more definite description of the nature and scope of the paper than conveyed by the title and should make clear to an expert in the relevant field the novelty of the research findings or results being presented. Unusual symbols and complex mathematical formulae should not be used. An original and two carbon copies should be submitted. The edited abstract will appear in the Symposium program. The names of the author or authors should be given along with the name and address of the company or institution at which the work was performed. If the present address of any author is different, both addresses should be given.

4. The letter of transmittal of the abstract should contain the following information: (a) the name of the person who will present the paper in the case of multiple authors; (g) a statement that the paper in substance or essence has not or will not be submitted for publication elsewhere; (c) the language of the oral presentation (English being preferred even if a manuscript must be closely followed); (d) the estimated presentation time (not to exceed 25 minutes with 5 additional minutes for discussion); (e) the size of lantern slides to be shown, either 3.5 by 4.0 in. (8.9 by 10.2 cm) or 2.0 by 2.0 in. used with 35-mm transparencies; (f) any special facilities that may be required.

5. The right of original publication of all research papers contributed to the Symposium is reserved by the *Microchemical Journal*, published by Academic Press under the auspices of the American Microchemical Society. A release to publish elsewhere cannot be entertained. The exception to this rule is that the plenary lectures will be published in the International Union of Pure and Applied Chemistry's journal, *Pure and Applied Chemistry*.

6. The full text of the paper with illustrations must be submitted in duplicate. Each manuscript will be acknowledged on receipt and will be placed in type for appearance in issues of the *Microchemical Journal* to be mailed directly following the Symposium. Papers should conform in organization to the practices of that Journal as described in recent issues on the page entitled "Information to Authors"; copies of that page are available on request.

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

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Gas Chromatography

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4. TECHNIQUES II

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4b. Programmed temperature GC

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THE FLAME IONIZATION DETECTOR

A SIMPLE ELECTROMETER FOR LINEAR, LOGARITHMIC AND INTEGRAL RESPONSES

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INTRODUCTION

This paper discusses a relatively simple and inexpensive electrometer circuit for use with the flame ionization detector, providing wide-range linear, logarithmic or integral responses.

At the high sensitivity end of the linear range, the responses are adequate for most practical purposes, and at the low sensitivity end macro concentrations can be handled. Provision is made so that sensitivity can be varied during a run over the whole range without moving the baseline. The logarithmic mode automatically provides for stepless sensitivity changes across the available range so that no peaks go off-scale, while small traces still show up: this is a feature very useful for preliminary scouting work. The integral mode provides a simple coulometric device peculiarly adapted to the flame detector, and requires little additional expense.

The construction of the circuit is fairly straightforward, and practical hints are given, based on long experience of battery-operated high impedance DC electrometers. While no criticism of highly sensitive mains-operated AC stabilized electrometers is intended (apart from expense and sometimes temperamental behaviour), their sensitivity is largely wasted on the flame detector, which generates relatively large currents, so large indeed that the drift problem with simple DC circuits can be eliminated for practical purposes by operating at very low gain. For the special requirements of the logarithmic circuit, which requires a voltage swing of about 0.5 V at the input, the low gain is an advantage. It is possible that some of the more complicated circuits would be overloaded with such a voltage. In any case, the installation of the logarithmic or integrating circuits would require internal modifications to commercial electrometers and would need expert advice.

In the discussion, a good deal of attention is given to input circuit background compensation, since this is not only convenient for normal linear response but essential for integration, and we are not aware of any published analysis of the theory of compensation in the input circuit.

DISCUSSION

Background compensator

Compensation for background current in the input circuit is advantageous for two reasons. Firstly, it allows the sensitivity to be varied over the whole available range

without shifting the baseline, and secondly it avoids overloading the electrometer with useless input voltage from high backgrounds.

In its simplest form, the compensator consists of a very high resistance in series with a variable voltage source, connected across the detector between collector and ground. The polarity is such that ion current is drawn from the collector and returned to the jet electrode, by-passing the range resistor. The variable voltage is adjusted until the background ion current is exactly neutralized and there is then no current through the range resistor in the absence of a signal, and hence no voltage across it. A different range resistor can thus be inserted without shifting the baseline. In this simple form, the compensator has been described by DESTY, GOLDUP AND WHYMAN[1].

This compensator causes a reduction in the response when a signal appears, because the signal current divides between the compensator and range resistors in inverse proportion to their resistances: thus, if the compensator resistance is $10^{12}\ \Omega$ and the range resistance $10^{10}\ \Omega$, then 1 % of the signal response is lost. The errors are thus easily calculable, and in many cases negligible.

If the background current is high, as from column bleeding, then the compensator voltage required may become inconveniently large if the high value compensator resistance is retained. The alternative is to reduce the compensator resistance to allow for the greater compensation required. Generally, this procedure is convenient since high sensitivity is in any case impracticable at high background because of drift. The principle can be extended to cope with very high background currents, the available sensitivity being restricted then to low levels, in the ranges in which thermal conductivity detectors are usually employed, where linear response is still obtainable from the flame detector if electrode geometry and voltage are adequate[2].

The compensator can be modified so that there is no error arising from division of signal current. This is done by feeding back from the output a voltage equal to the input signal voltage and subtracting this feedback from the compensator voltage. The net voltage across the compensator resistor is thus unaffected by the signal, and the current drawn through it remains equal to the background current only. The feedback is most conveniently and reliably derived from a re-transmitting slidewire on the recorder. For simple linear operation, this refinement is not essential since the error introduced by the compensator is easily calculable. The situation is different for integration at high sensitivity (*vide infra*) because the error is cumulative with time and not easily calculable, although large signal currents can still be handled without substantial error.

In fairness to AC stabilized electrometers, it should be said that the simple forms of the compensator are probably always adequate with them since their dynamic input resistance is reduced by a large factor, due to high negative feedback, so that the effective ratio of the compensator resistance to the range resistance is much larger than that calculated from the static resistances.

Linear circuit and basic design (Fig. 1)

The linear ranges are conventional and arranged in decades in the input, with a single step output attenuator. The bias on the cathode is set to correspond with the low end of the straightest portion of the characteristic curve, and for the designed maximum positive input voltage swing of 0.5 V (with output attenuator at X1) the linearity of response across the chart does not err by more than about 0.5 %. Using

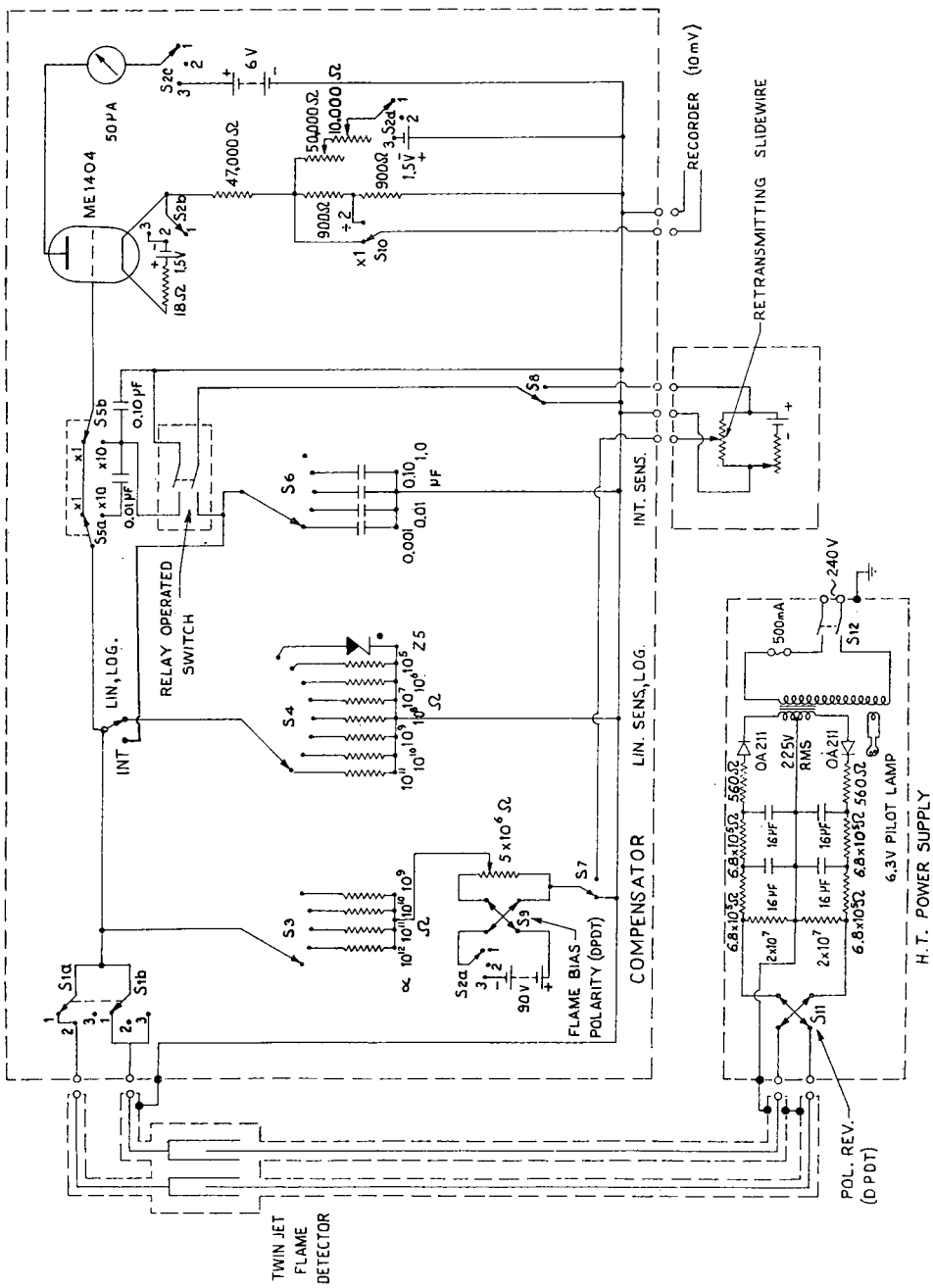

Fig. 1. Electrometer and power supply for twin jet flame detector.

the output attenuator (voltage swing 1 V) the non-linearity does not exceed 1%. Linearity is assisted by the cathode resistance, which biases the cathode (and does not affect the dynamic resistances of the range resistors). It is important to connect the filament battery and associated current-limiting resistance as shown, for positive-going signals (*i.e.* for positive jet, negative collector); the whole filament and cathode circuit would need rearranging for negative-going signals.

The output load resistances shown are calculated for a 10 mV recorder. They may be changed appropriately for other sensitivities within reason, without interfering appreciably with the bias. Where a variety of recorder sensitivities may be used from time to time, it is convenient to arrange plug-in change-over sets of matched resistors. The coarse recorder-balancing potentiometer may be placed inside the cabinet.

The microammeter is very useful for fault-finding, and occasionally for following the progress of very large off-scale peaks: sometimes it is possible to detect the existence of several peaks. The normal base current is about 28 μA. The ganged switch S2 (two decks, three pole, three position) switches the filament on before the anode, in line with generally recommended procedures with electrometer tubes, to reduce circuit drift: the middle position also serves for stand-by, and the associated gangs switch the other batteries on and off.

The filament source is a 1.5 V heavy duty dry cell, which will last at least a year with normal daily operation. It is important to insulate this cell from the electrometer case, since leakage through the cardboard shell by-passes the cathode resistor and causes serious noise. Insulation is simply and reliably provided by wrapping in polythene. Current sources except the flame high tension are placed within the cabinet and wedged to prevent movement.

The valve is a Mullard ME 1404 sub-miniature electrometer triode, mounted vertically to reduce filament sag, and upside down for convenience in wiring. The gain of the circuit is about $^1/_{50}$, wired as shown for a 10 mV recorder, with the output attenuator X1. The low gain reduces circuit drift to negligible amounts and is compensated for by using higher value input resistors: the main disadvantage is that on the highest sensitivity (10^{11} Ω input) the time constant is appreciable (about 5 sec), but in any case at this sensitivity some damping is required for flame noise.

All the high impedance circuitry must be well insulated. Ceramic decks are used on the wafer switches. Wiring is done with heavy gauge rigid tinned wire without spaghetti. Including all auxiliary circuit switches, the insulation resistance should be about $5 \cdot 10^{12}$ Ω, with desiccation. Desiccation is not essential, at around 50% r.h., except for the highest sensitivities.

All ground leads should be brought to a common point within the cabinet, before connection to frame: this avoids transient pick-up which can arise if any portion of the cabinet forms part of the circuit.

The flame high tension should be at least 320 V, and is provided in the circuit shown by a simple two-polarity source using half-wave rectification, unstabilised but heavily smoothed. Batteries can of course be used instead. Twin jets are shown for use with a reference column when desired: when so used the reference jet is made negative. This arrangement is preferable to symmetrical balanced circuits because the background currents mutually cancel and do not tend to saturate the input. In theory opposed twin jets dispense with the need for a compensator, balance being obtainable by regulating the gas rates.

The circuit is designed for use with jets insulated from ground. We use 23 gauge hypodermic tube with glass press-shrunk insulators. For moderately high temperatures sealing with Araldite is feasible. Some designers go to considerable lengths to keep the jets at ground potential, but we are inclined to think that this practice leads to as many difficulties as it avoids.

We do not recommend placing ignition or temperature measuring devices in the vicinity of the jet tip since they distort the field and lead to non-linear response at high loadings.

Logarithmic response

In our experience of service work in a fairly large chemical firm, the need was often felt for some kind of logarithmic response when doing qualitative work involving all sorts of samples in which trace impurities might be important, and in which perhaps even the major components and their relative amounts were unknown or doubtful. With the normal linear presentation, often either several runs at various sensitivities and sample sizes are required, or considerable agility and close attention must be exercised in range changing during the run. At fixed sensitivities some peaks are likely to be off-scale and the peak maximum position indeterminate, while on the other hand small traces may be missed because no tests are made with large injections at high sensitivity. With range changing, although the compensation of the steady background is a big help, the tails of large peaks are inevitably blown up on switching to higher sensitivity, and the record is correspondingly messy. Automatic attenuation has its virtues but again the record is not very easy to read and the apparatus is an additional expense.

A logarithmic response covering five or six decades would provide for most sensitivity requirements and give an unbroken trace.

Early attempts to obtain logarithmic response were based on the known principle that in a triode taking positive grid current the logarithm of grid current may be proportional, or roughly proportional, to plate current. These attempts were reasonably successful using a Mullard ME 1404 operated, as indicated by the makers, for log response. However, it was not found easy to get a good log/linear effect, and the sensitivity in the vicinity of the baseline was governed by the magnitude of the background current, all of which flowed through the grid. Circuit modifications were required which made switching from the linear to the logarithmic mode rather complicated.

We have now found that the resistance *versus* current characteristic of a silicon diode in the forward direction gives the required log/linear relationship over many decades (nine or more if desired) if the diode is placed in the position of a normal range resistor, while the resistance near 0 V can in selected cases be high enough for reasonably high sensitivity, *e.g.* equivalent to a linear resistance of $10^{10}\ \Omega$. Used with the compensator circuitry already described, no modifications to the normal circuit are required (except an additional position on the linear range switch) provided the voltage swing at the electrometer input for full scale on the recorder is of the order of 0.5 V. The measured slope of the diode characteristic is about 78.5 mV for each decade in current flow, so that 0.5 V will allow for over six decades. In other words, 1 % of full scale near the maximum on the recorder represents about a million times more sample concentration at the flame than the same deflection near the minimum.

Three diodes were examined experimentally to determine the slope (all nearly parallel, 78.5 mV per decade) but the resistances at a given current differed considerably. Since the highest possible resistance is desirable at the low current end, to give high sensitivity while still in the log/linear region, it is necessary to select the particular diode to be used. Of the three examined, two were zeners and one was an ordinary diode. The forward bias voltage required on each to reach the log/linear region was about 120 mV and at this voltage the measured currents corresponded to resistances as follows:

Phillips OA 200: $1.2 \cdot 10^8\ \Omega$.
Intermetall Z5: $10^{10}\ \Omega$.
Intermetall Z10: $4 \cdot 10^9\ \Omega$.

Thus, the Z5 zener was the best, and this was used since it gave quite adequate sensitivity for practical purposes. At lower forward bias, the sensitivity is higher but not quite log/linear, and near 0 V, the capacitance effect of the diode itself makes response very sluggish. It seems very probable that higher resistances and/or lower capacitances could be found among the many silicon diodes available, if one was prepared to examine a number of classes and a number within each class. A quick selection without the need for a very sensitive electrometer could be made by measuring the current at a fixed voltage above about 0.5 V. Thus, the Z5 passed 4.2 μA at 0.55 V, and the other two gave larger currents than this at the same voltage. Manufacturers' data sheets do not usually give adequate information at low currents, and the variability within a class appears to be high. Subsequent tests over a number of diodes showed that about one in eight Intermetall zeners were suitable, while none could be found among ordinary diodes.

To calibrate the recorder for logarithmic presentation on an arbitrary scale, it is first necessary to establish the pen movement corresponding to one decade. This can be done by applying sufficient current to the input through a resistance high enough to allow neglect of the diode resistance. For example, with the Z5 two pen positions are established using a 10^9 resistor with two voltages, 30 and 300, in the ratio of $^1/_{10}$. The difference between the pen positions then gives the length of one decade to within an approximation error of 1 %. This gives the initial basis for laying off the logarithmic scale. However, since it is desirable to have the low end starting with zero for zero signal, and as this cannot be done on a true logarithmic scale, the initially plotted log scale is further modified. If the initial plot is numbered 1, 10, 100, etc. on the main index lines, subtraction of unity from all values gives a new scale with the index lines now labelled, 0, 9, 99, etc. (It will be seen that the function has been changed from $\log x$ to $\log (x + 1)$. When $x = 0$, $\log x$ is minus infinity, but $\log (x + 1)$ is zero.) The higher decades on this scale are practically identical with the original scale, and the infinity of decades between 0 and 1 on the original scale have been compressed into the space between 0 and 1 (originally 1 and 2) on the new scale.

To provide the necessary forward bias of 100–120 mV when using the flame detector it is only necessary to undercompensate for the baseline current. For example, with the circuit shown, having a gain of $^1/_{50}$, the compensator is adjusted until the pen is about 2 mV upscale from true input zero. The modified log scale is then moved along to place the zero over the pen position, and relative signal strengths can then be read off directly. The log presentation does not look very handsome at first sight,

because the peak bases are very broad because of adsorption tails. This appearance, however, merely indicates the enormously greater sensitivity at the low end as compared with the other. There is no actual loss of resolution or of available information, indeed the clear indication of long adsorption tails is quite a striking demonstration of how persistent such effects can be.

We have not ourselves attempted to use the logarithmic presentation for accurate quantitative work but there is no reason why it should not be used for quite reasonable accuracy, and it could be used for example, for monitoring plant gas streams for impurity tolerances without elaborate automatic sensitivity switching. For the highest accuracy the diode should be thermostated, since for a given current the voltage developed is a rather complicated function of temperature.

Integrating circuit

A circuit for the integration of the output of ionization detectors has been published by VAN DER GRINTEN AND DIJKSTRA[3]. In this circuit the driving voltage for the detector is provided by a charged condenser, and the integral is obtained by measuring the decay in the voltage of the condenser as current is drawn from it.

A compensator is provided for background current, but this compensator is not applicable for ordinary linear response.

We have now devised an integrating circuit which makes use of the same compensator as for linear response, and which can be incorporated with the linear and logarithmic circuitry discussed above with comparatively little extra trouble.

In our integrator the range resistors are disconnected and replaced by a range of condensers. Signal current is then accumulated on an integrating condenser instead of being dissipated through a resistor. Since the instantaneous signal is essentially a current, the integrated signal is essentially coulombic, and the voltage developed is directly proportioned to coulombs accumulated. The maximum voltage is limited to that corresponding to full scale deflection on the recorder, the integration being then continued by automatically and momentarily shorting the condenser.

Normally, condenser integration is not feasible in simple DC systems because the back voltage affects the signal, and also when the signal decreases the charge leaks away through the source. In the special case of the flame ionization detector neither of these effects applies. In the first place the signal current is a saturation current, driven by a large excess voltage, and is not affected in the least by small back voltages; while in the second place the detector is effectively equivalent to a perfect diode and cannot leak back. Leakage does take place through the compensator resistor, and for good results at high sensitivities (*i.e.* when using small integrating condensers) the compensator feedback refinement is advisable. For low sensitivity work the leakage may be tolerable without feedback. At the lowest sensitivity the size of the condenser required becomes embarrassingly large (10 μF), and for convenience the circuit can be arranged to divide the voltage developed across a smaller condenser, by use of a supplementary condenser network, thus multiplying the effective value of the integrating condenser. In the circuit shown a nominal 10 to 1 multiplication is effected, extending the effective maximum capacity from 1 μF to approximately 10 μF. If the capacitor values are precisely as shown, *viz.* 1.0, 0.1 and 0.01 μF for the integrating condensers and division network, the actual multiplication factor would be 11.1. The condenser shorting mechanism not only shorts the integrating condenser,

but when the multiplier is used a second contact is closed as a precaution to ensure that the grid is kept to reference potential, although in fact drift caused by grid current is negligible at this low sensitivity.

The condensers used must have a high quality dielectric (*e.g.* polystyrene) since lossy dielectrics retain charge on shorting.

The shorting mechanism consists of a microswitch (closed by the pen carriage when it reaches full scale) which actuates a relay inside the electrometer cabinet. This indirect circuit is used to avoid high impedance lines to the recorder. The relay is an ordinary two contact normally-open type, but modified by removing the spring and relying on gravity (by tilting) to effect opening, and also modified by remounting the fixed contacts on high insulation material (Perspex). The coil is bridged by a 100 μF condenser to eliminate transients, which have already been minimised by the low power requirement.

When the relay closes the pen returns towards baseline, but signal is not lost since, except during the moment of shorting, the charge is still being accumulated. The pen recommences its upscale traverse at the reading corresponding to the charge accumulated during its downscale movement, and up to a point the pen speed does not affect the result. This point is reached when the rate of charge is so large that the pen cannot follow it, and the indication of this condition is failure of the pen to return more than halfway towards the baseline. If this happens a larger condenser is required. Chart speed is only significant with respect to readability. The total integral is measured from the baseline reference, not from the lower points of the trace (which incidentally give an envelope roughly indicative of the peak shape).

If the compensator feedback refinement is used, it is sufficient for ordinary purposes to adjust the feedback voltage until the full end to end voltage across the retransmitting slidewire, applied across the electrometer input, is just sufficient to drive the recorder full scale. This is done by closing switch 8, holding down the microswitch on the recorder, and adjusting the rheostat of the slidewire. The feedback provided—by setting the slidewire current on the more sensitive ranges—is virtually inoperative when using the condenser multiplier, as the feedback then also needs increasing tenfold. This of course could be done if thought worthwhile. For the most accurate and sensitive work a further fine adjustment can be made such that no detectable drift occurs with the pen at any position on the scale: this adjustment allows for constant ohmic leakage other than through the compensator and also for the constant component of grid current.

(Condenser integration on the above lines could be applied to any one-way current generating device substantially unaffected by small back voltage, *e.g.* photoemissive cells, ion chambers.)

A rough correlation between an integrator reading and sample size can be calculated by allowing 0.1 C per gram atom of carbon in oxidation states not higher than alcohols or ethers. Thus for example a 10 mV excursion on the recorder for the circuit shown (output X1, gain $^1/_{50}$) using the 1.0 μF condenser is equivalent to 0.5 V at the condenser or $5 \cdot 10^{-7}$ C, or $5 \cdot 10^{-6}$ gram atoms of carbon (as above defined), or 60 μg. With the equivalent of 10 μF the range thus extends up to milligrams, the maximum possible depending on the speed characteristics of the recorder and the rapidity of elution.

At the other end of the scale, with the 0.001 μF condenser and a reading of

1 % of full scale on the recorder, the smallest readable amount would be 0.0006 μg. For accuracy in absolute terms at high sensitivity the stray capacitance of the input, including the appreciable capacitance of the cable to the detector would need to be measured independently. The stray capacitance can be as much as 50 pF, and this could be used alone as the integration capacitance if the ultimate realisable sensitivity was required. The usefulness of extremely sensitive integration is perhaps limited more by chemical drift than by instrumental possibilities.

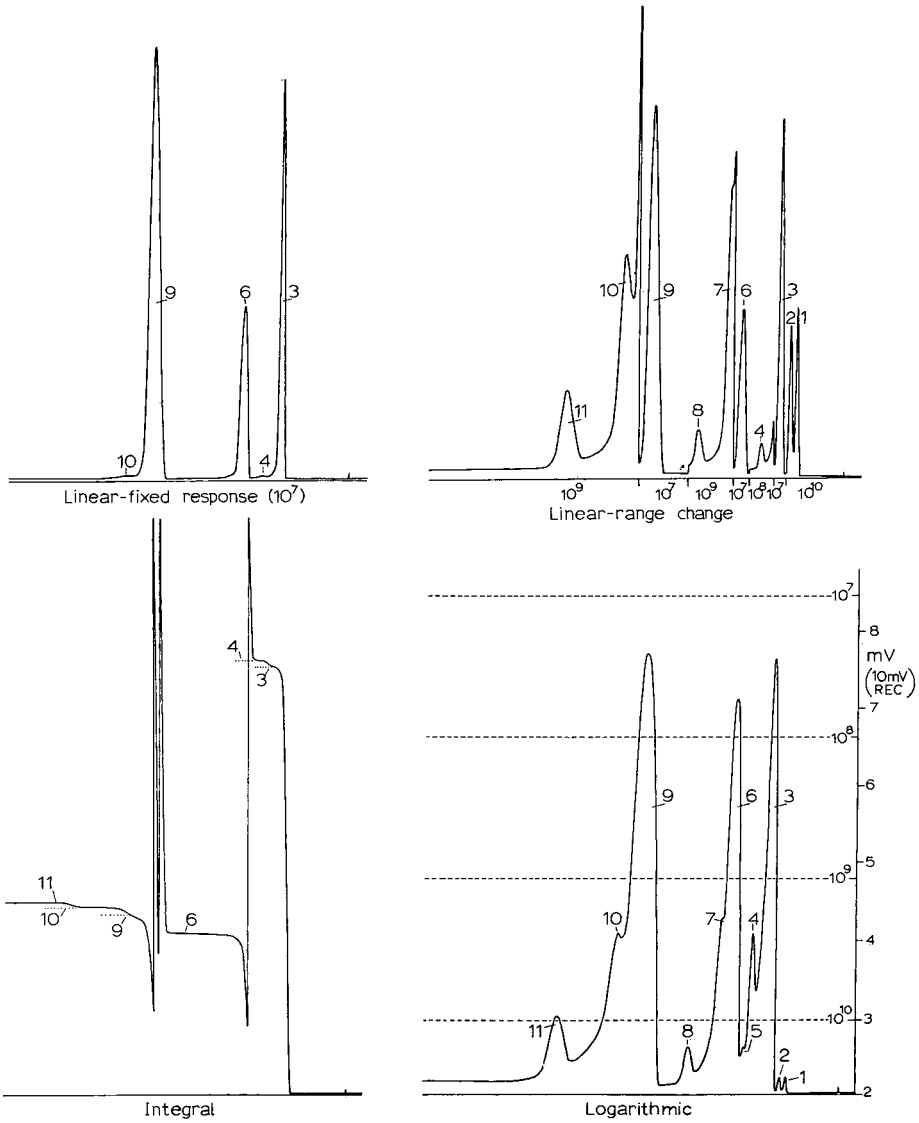

Fig. 2. Comparison of four types of presentation of the same signals. Vertical scale on log trace indicates linear resistors which must not be exceeded for on-scale linear traces of peaks up to the corresponding heights. Integrator set on 1.0 μF (without feedback).

Traces illustrative of the three functions of the circuit are shown in Fig. 2.

In conclusion we would like to suggest more use of the flame ionization detector at low sensitivities, where linearity can still be attained with adequate detector design, and where drift and noise problems are almost eliminated. There is still a large area of use for low sensitivity work because one is then not restricted to low volatility partition liquids. For these reasons we have designed the circuit to be operable under conditions permitting the use of volatile packings, and the injection of undiluted microlitre amounts of sample without splitting, assuming of course that an adequately designed detector is used in conjunction.

SUMMARY

A detailed description of a versatile DC electrometer for use with the flame ionization detector is given. The circuit provides for baseline compensation, and for logarithmic and integral responses, obtained by relatively simple electrical devices, all in the input circuit, as well as wide-range linear presentation in the usual way.

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THE CRITICAL EXAMINATION OF COMMERCIALLY AVAILABLE DETECTORS FOR USE IN GAS CHROMATOGRAPHY

PART I. THE MACRO ARGON AND FLAME IONISATION DETECTORS

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INTRODUCTION

Due to the increasing interest in the quantitative accuracy that can be obtained in gas chromatographic analyses, a precise knowledge of the characteristics of commercially available detectors is essential. This paper, which is the second of a series concerned with the investigation of these characteristics, gives the results from the detailed study of two detectors, the Macro Argon Detector, Pye Cat. No. 12216, and the Flame Ionisation Detector, Pye Cat. No. 12210 (manufactured to a design by Ionics Inc.). The response of each detector was examined over a wide range of concentration levels using a method of assessment previously described in this journal[1]. Where possible, operating conditions are given for each detector, over which satisfactory quantitative results can be obtained. The experimental work was confined to conditions normally employed in the practice of gas chromatography.

EXPERIMENTAL

The apparatus used, together with the theory of the dilution system employed, has been described by FOWLIS AND SCOTT[1]. The response of each detector was examined using diisopropyl ether, *n*-heptane, toluene and chlorobenzene at various flow-rates and operating temperatures. However, each detector has its own specific operating parameters other than those of the chromatographic equipment with which it is associated, so prior to each test the voltage, temperatures and flow-rates were adjusted to the value required and known masses of the solutes placed in the dilution vessel. The peak height, equivalent to a current or voltage signal produced by the detector, was measured for each successive operation of the automatic sampling valve. This procedure was carried out altering each pertinent variable as required. So that the results could be presented in the form of a graph relating signal to mass of solute entering the detector, it was necessary to calculate the concentration of solute in grams per second leaving the dilution vessel and to express this in terms of mass of solute entering the detector.

A worked example for a particular set of operating conditions is given to illustrate the method employed. The concentration of solute in the exit gas is dependent on the properties of the dilution system, *viz.* partition coefficient of solute, gas and

liquid volumes of the vessel, gas flow-rate, temperature and pressure. The gas pressure in the dilution vessel was measured with a static mercury manometer, the temperature with a thermocouple immersed in the liquid and the partition coefficients of the substances concerned obtained from their retention ratios to *n*-heptane. The partition coefficients of *n*-heptane, which were taken from the results of DESTY AND GOLDUP[2] and EVERED AND POLLARD[3], were plotted as their logarithms against the reciprocal of the absolute temperature. The partition coefficient of *n*-heptane at any temperature was taken from the regression line through these points. The temperature in the dilution vessel tended to fluctuate due to the thermal inertia in the system. The fluctuation had an amplitude of approximately $\pm$ 0.4° and so the temperature was measured over a period of time and the mean value taken as the temperature of the dilution vessel. The flow-rate was measured by means of a calibrated soap-film meter, exterior to the apparatus and therefore at room temperature. This flow-rate was corrected to the temperature and pressure existing in the dilution vessel. An example of the calculation carried out for a test of the flame-ionisation detector using diisopropyl ether is given below.

Conditions

Carrier gas flow-rate: 60.0 ml/min
Hydrogen flow-rate: 60.0 ml/min
Oxygen flow-rate: 60.0 ml/min
Detector voltage: 100 V
Solute: Diisopropyl ether
Volume of liquid in the dilution (V_L): 4.93 ml.
Volume of gas in the dilution vessel (V_g): 23.07 ml

Determination of flow-rate through the dilution vessel

The mean flow-rate through the dilution vessel (5 observations) = 15.38 ml/min at 21° and 760 mm pressure.
The pressure in the dilution vessel = 769 mm Hg.
The maximum temperature of the dilution vessel = 70.10°.
The minimum temperature of the dilution vessel = 68.30°.
The mean temperature of the dilution vessel (20 observations) = 69.40°.
Thermocouple correction obtained by comparison with N.P.L. mercury in glass thermometer = + 0.5°.
The corrected temperature of the liquid in the dilution vessel = 69.9°.
Thus, the gas flow-rate through the dilution vessel at 69.9° and 769 mm pressure = 17.72 ml/min.

The partition coefficient (K_1) of *n*-heptane at 69.9° = 200.
Time for elution of air = 41.4 sec.
Time for elution of diisopropyl ether = 161 sec.
Time for elution of *n*-heptane = 445 sec.

Thus, the retention ratio of diisopropyl ether with respect to *n*-heptane = 0.296.
Hence, the partition coefficient of diisopropyl ether (K_2) at 69.9° = 59.3.
Thus

$$V_g + K_2 V_L = 315.3 \text{ ml}$$

Hence

$$\frac{Q}{V_g + K_2V_L} = 5.62 \cdot 10^{-2} \text{ min}^{-1}$$

The weight of diisopropyl ether placed in the dilution vessel was $5.4 \cdot 10^{-3}$ g. Thus, the initial concentration of diisopropyl ether in dilution vessel exit gas is

$$\frac{\text{mass}}{V_g + K_2V_L} = 1.71 \cdot 10^{-5} \text{ g/ml}$$

Gas volume equivalent to peak width at base = 20.8 ml.

Assuming the concentration of solute in the carrier gas at the peak maximum is twice the average concentration in the peak and 1-ml samples of exit gas from the dilution vessel are placed on the column, then the concentration of solute in the detector at the peak maximum, X°_{gn},

$$X^{\circ}_{gn} = \frac{1.71 \times 10^{-5} \times 2}{20.8} = 1.65 \cdot 10^{-6} \text{ g/ml}$$

(assuming the first sample is taken at $t = 0$).
Thus,

$$\text{since } X_{gt} = X_{g0} \exp\left[-\left(\frac{Qt}{V_g + K_2V_L}\right)\right],$$

a graph of log X_{gt} against time (t) can be constructed. From this graph values of solute concentration in g/ml at the peak maximum for each sample were calculated and results presented as a graph of $\log_{10}$ solute concentration against $\log_{10}$ detector current. If the slope of this graph is unity, then the detector has a linear response.

Since the column flow-rate was 60.0 ml/min, the mass of solute entering the detector in unit time is numerically equal to the concentration in g/ml. The ionisation efficiency was calculated from the current produced when a mass of 10^{-9} g of solute entered the detector in one second.

Flame ionisation detector

A diagram of the detector is shown in Fig. 1. It is constructed of stainless steel and contains a cylindrical electrode insulated from the body by means of a Teflon sleeve. After continuous operation for about 8–10 weeks there were distinct signs of corrosion on the walls of the detector itself and on the tubular electrode. The corrosion on the electrode caused some instability and had to be removed. A more corrosion-resistant stainless steel or a good quality gold film on the inside of the detector body and over the electrode should eliminate this effect. The quality of the plating should be such that the temperature changes which occur in the detector do not cause flaking.

If concentrations near the limit of the detector sensitivity are used, good electrical shielding is required or the baseline will be unstable. The values of the noise level given were for the detector together with its ancillary equipment. The amplifier characteristics contributed a large proportion of the total noise.

Results. The presentation of the results in the form of $\log_{10}$ ionisation current plotted against $\log_{10}$ of the concentration simplifies the assessment of the linearity of the detector. The detector has a linear response if the slope of this curve is unity.

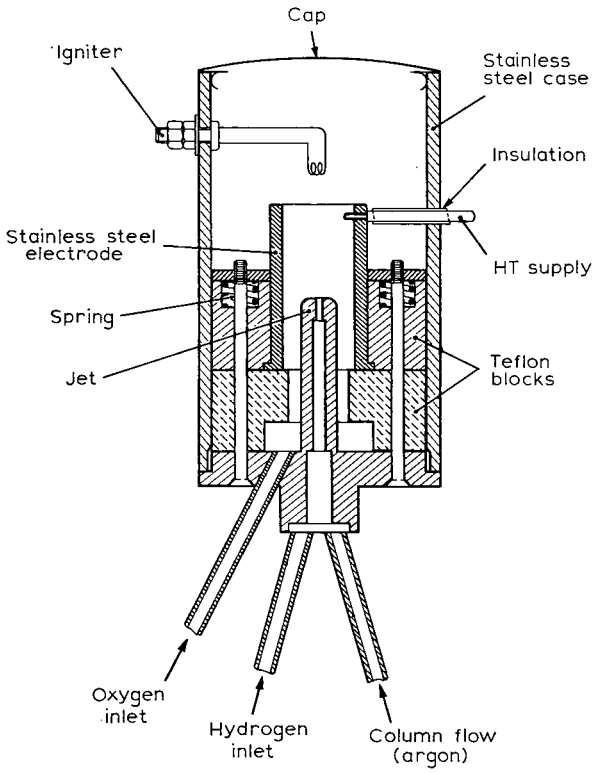

Fig. 1. Cross section of the flame ionisation detector.

If the detector response is non-linear then the slope may neither be unity nor the curve linear. The results obtained with different substances are shown in Fig. 2 (a, b, c, d) and for different operating conditions in Fig. 3 (a, b, c, d). The tables attached to Fig. 2 and 3 give detector characteristics and the experimental conditions under which these were obtained. The absolute sensitivity is taken as that concentration which will give a current equivalent to twice the noise level.

Discussion of results. The flame-ionisation detector has a linear response for the four compounds examined over a wide concentration range (10^{-6} to 10^{-10}) g/ml, and is relatively insensitive to the applied voltage. The voltage range examined was 25 to 150 V, and it can be seen from Fig. 3 (d) that all the points for the various voltages lie on the same straight line, over a concentration range of four orders. Fig. 3(a) and 3(b) show that the detector is relatively insensitive to changes in column flow-rate, but if the oxygen flow is reduced below the 1:1 ratio of hydrogen to oxygen there is a tendency for the detector response to become non-linear (as shown by Fig. 3(c), where the response index is 1.13). The optimum operating conditions were found to be:

column flow-rates up to 60 ml/min
hydrogen flow-rate 60 ml/min
oxygen flow-rate 60 ml/min
applied voltage 25–150 V.

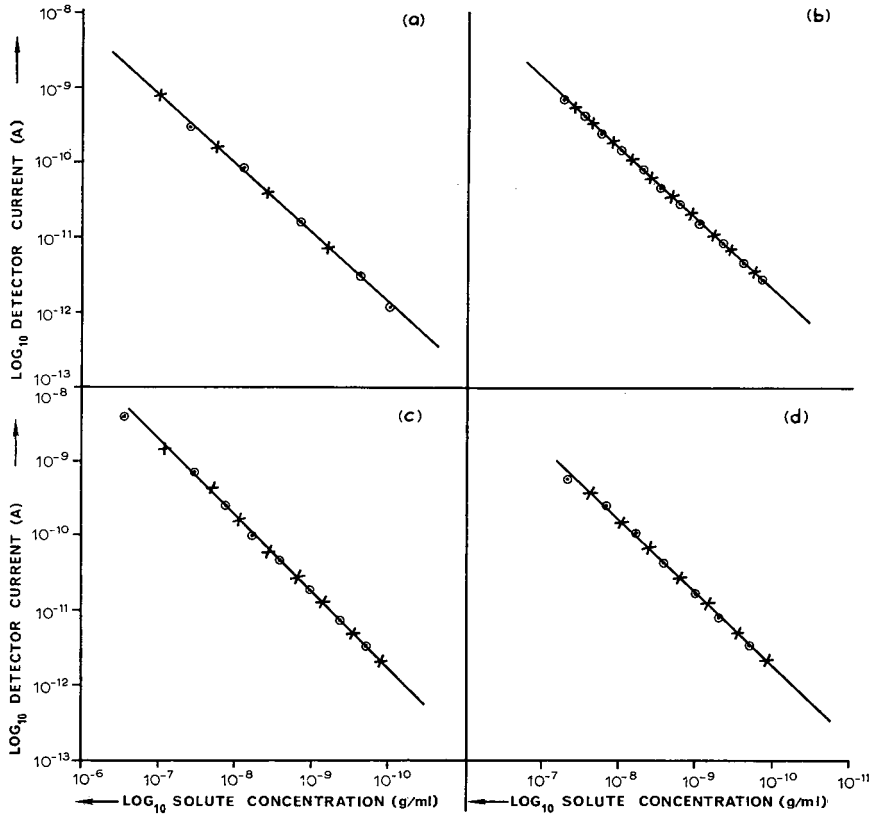

Fig. 2. Response curves for the flame ionisation detector for different solutes. Detector temperature: 23°. Solute: (a) diisopropyl ether; (b) *n*-heptane; (c) toluene; (d) chlorobenzene. Detector flow-rate: 60 ml/min. Hydrogen flow-rate: 60 ml/min. Oxygen flow-rate: 60 ml/min.

	Voltage	*Minimum detectable signal (estimate) g/ml*	*Ionization efficiency %*	*Response index*	*Range g/ml*	*Noise level A*
(a)	50	$6.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	0.96	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	100	$6.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	0.96	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
(b)	50	$3.0 \cdot 10^{-11}$	$1.5 \cdot 10^{-3}$	1.05	10^{-7} — 10^{-10}	$8 \cdot 10^{-13}$
	100	$3.0 \cdot 10^{-11}$	$1.5 \cdot 10^{-3}$	1.05	10^{-7} — 10^{-10}	$8 \cdot 10^{-13}$
(c)	50	$5.0 \cdot 10^{-11}$	$2.0 \cdot 10^{-3}$	0.97	$5 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	100	$5.0 \cdot 10^{-11}$	$2.0 \cdot 10^{-3}$	0.97	$5 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
(d)	50	$3.8 \cdot 10^{-11}$	$1.8 \cdot 10^{-3}$	0.98	$5 \cdot 10^{-8}$ — 10^{-10}	$8 \cdot 10^{-13}$
	100	$3.8 \cdot 10^{-11}$	$1.8 \cdot 10^{-3}$	0.98	$5 \cdot 10^{-8}$ — 10^{-10}	$8 \cdot 10^{-13}$

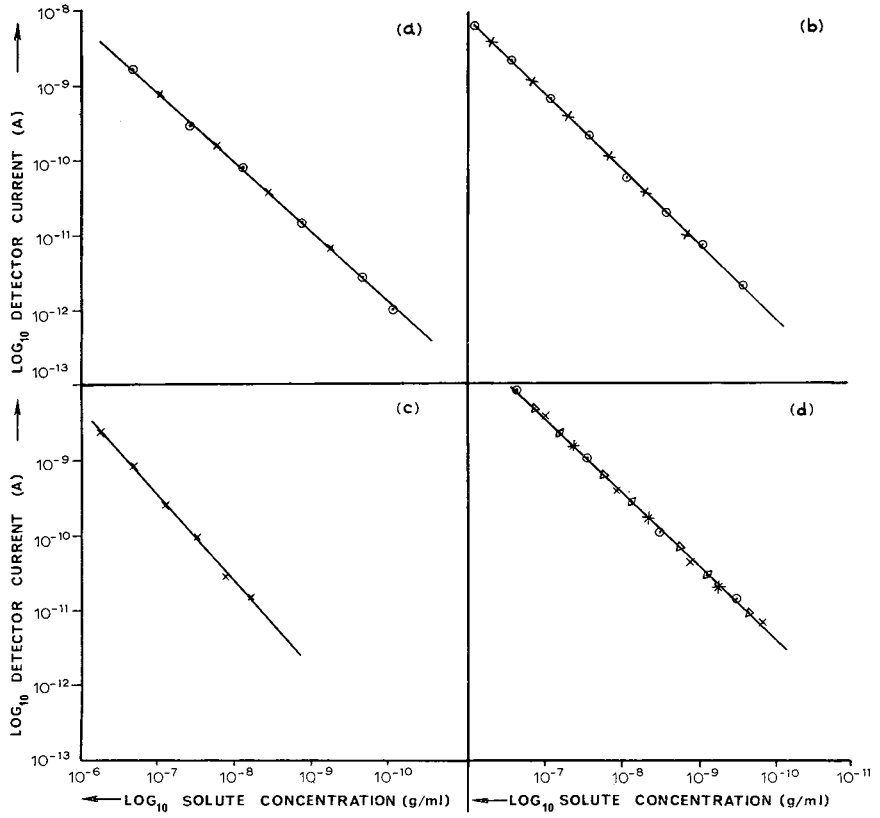

Fig. 3. Response curves for the flame ionisation detector for various flow-parameters and voltages. Detector temperature: 23°. Solute: (a), (b) and (c) diisopropyl ether; (d) *n*-heptane. Detector flow-rate: (a), (c) and (d) 60 ml/min; (b) 30 ml/min. Hydrogen flow-rate: (a), (b) and (d) 60 ml/min; (c) 90 ml/min. Oxygen flow-rate: 60 ml/min.

	Voltage	*Minimum detectable signal (estimate) g/ml*	*Ionization efficiency %*	*Response index*	*Range g/ml*	*Noise level A*
(a)	50	$6.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	0.96	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	100	$6.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	0.96	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
(b)	50	$9.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	1.01	10^{-6} — $5 \cdot 10^{-10}$	$8 \cdot 10^{-13}$
	100	$9.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-3}$	1.01	10^{-6} — $5 \cdot 10^{-10}$	$8 \cdot 10^{-13}$
(c)	100	$3.5 \cdot 10^{-10}$	$2.5 \cdot 10^{-4}$	1.13	10^{-6} — 10^{-8}	$8 \cdot 10^{-13}$
(d)	25	$1.8 \cdot 10^{-11}$	$4.0 \cdot 10^{-3}$	0.98	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	50	$1.8 \cdot 10^{-11}$	$4.0 \cdot 10^{-3}$	0.98	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	75	$1.8 \cdot 10^{-11}$	$4.0 \cdot 10^{-3}$	0.98	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	100	$1.8 \cdot 10^{-11}$	$4.0 \cdot 10^{-3}$	0.98	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$
	150	$1.8 \cdot 10^{-11}$	$4.0 \cdot 10^{-3}$	0.98	$2 \cdot 10^{-7}$ — 10^{-10}	$8 \cdot 10^{-13}$

The ionisation efficiency calculated for this detector compares favourably with that obtained by ONGKIEHONG[4] and DESTY *et al.*[2], although the limit of its sensitivity is not as high as the values quoted by these authors. The lower sensitivity is probably due to the higher noise level of the ancillary equipment and not to the flame-ionisation detector itself. Results show that the flame ionisation detector is very satisfactory for quantitative gas chromatographic analysis. It is possible, however, to obtain higher sensitivity if one is prepared to accept a detector with a non-linear response.

Macro argon ionisation detector

A diagram of the detector is shown in Fig. 4. The detector is constructed of brass and contains a central cylindrical electrode that terminates in a small sphere. The electrode,

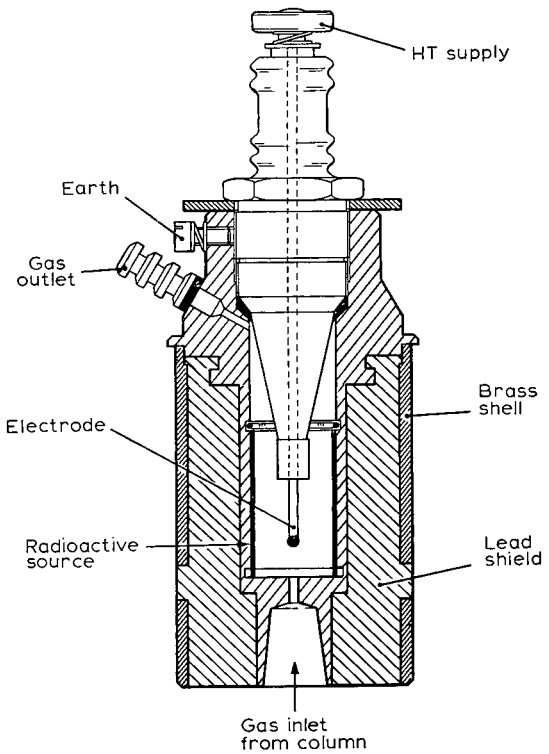

Fig. 4. Cross section of argon ionisation detector.

which is a modified sparking plug, is insulated from the body of the detector by means of a ceramic sleeve. A radioactive source (strontium-90) in the form of a foil is fitted concentric with the electrode.

The response of the detector was examined at various voltages and flow-rates with, and without, a linearizing resistance incorporated in the anode lead and at temperatures of 50° and 70°.

Results. In Figs. 5 and 6 the data obtained are presented as a graph of $\log_{10}$ ionisation current against $\log_{10}$ concentration. The absolute sensitivity, ionisation

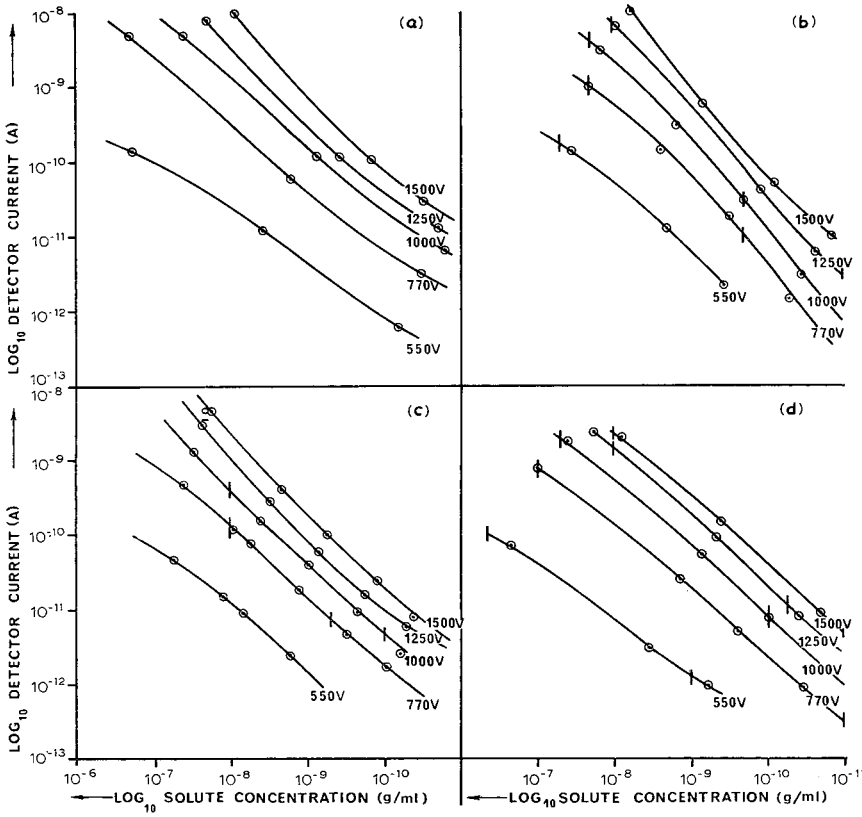

Fig. 5. Response curves for the argon ionisation detector for different solutes. With linearising resistance. Column flow-rate: 60 ml/min. Detector temperature: 75°. Solute: (a) toluene; (b) chlorobenzene; (c) *n*-heptane; (d) diisopropyl ether.

	Voltage	*Minimum detectable signal (estimate) g/ml*	*Ionization effiiciency %*	*Response index*	*Range g/ml*	*Noise level A*
(a)	550	$6.5 \cdot 10^{-11}$	$4.7 \cdot 10^{-3}$			$6.0 \cdot 10^{-12}$
	770	$<10^{-13}$	$4.2 \cdot 10^{-2}$			$3.0 \cdot 10^{-12}$
	1000	$<10^{-13}$	$1.5 \cdot 10^{-1}$			$3.0 \cdot 10^{-12}$
	1250	$<10^{-13}$	$3.4 \cdot 10^{-1}$			$3.6 \cdot 10^{-12}$
	1500	$<10^{-13}$	$7.5 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(b)	550	$2.4 \cdot 10^{-11}$	$2.2 \cdot 10^{-2}$	0.91	$5 \cdot 10^{-8}$ — $5 \cdot 10^{-10}$	$6.0 \cdot 10^{-12}$
	770	$2.4 \cdot 10^{-12}$	$6.4 \cdot 10^{-2}$	0.95	$2 \cdot 10^{-8}$ — $2 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1000	$8.5 \cdot 10^{-12}$	$2.3 \cdot 10^{-1}$	0.99	$2 \cdot 10^{-8}$ — $2 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1250	$1.5 \cdot 10^{-13}$	$5.0 \cdot 10^{-1}$	1.51	10^{-8} — 10^{-11}	$3.6 \cdot 10^{-12}$
	1500	$2.0 \cdot 10^{-13}$	$9.4 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(c)	550	$4.2 \cdot 10^{-10}$	$1.1 \cdot 10^{-3}$			$6.0 \cdot 10^{-12}$
	770	$1.0 \cdot 10^{-11}$	$1.6 \cdot 10^{-2}$	0.95	10^{-8} — $5 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1000	$1.0 \cdot 10^{-12}$	$3.9 \cdot 10^{-2}$	0.93	10^{-8} — 10^{-10}	$3.0 \cdot 10^{-12}$
	1250	$1.1 \cdot 10^{-13}$	$8.7 \cdot 10^{-2}$			$3.6 \cdot 10^{-12}$
	1500	$<10^{-13}$	$1.8 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(d)	550	$3.2 \cdot 10^{-11}$	$1.4 \cdot 10^{-3}$	0.87	$5 \cdot 10^{-7}$ — 10^{-9}	$6.0 \cdot 10^{-12}$
	770	$9.0 \cdot 10^{-12}$	$1.8 \cdot 10^{-2}$	0.92	10^{-7} — $5 \cdot 10^{-11}$	$3.0 \cdot 10^{-12}$
	1000	$1.0 \cdot 10^{-13}$	$4.6 \cdot 10^{-2}$	0.89	$5 \cdot 10^{-8}$ — 10^{-10}	$3.0 \cdot 10^{-12}$
	1250	10^{-13}	$8.3 \cdot 10^{-2}$	0.86	10^{-8} — $5 \cdot 10^{-11}$	$3.6 \cdot 10^{-12}$
	1500	10^{-13}	$1.5 \cdot 10^{-1}$	0.88	10^{-8} — 10^{-11}	$6.0 \cdot 10^{-12}$

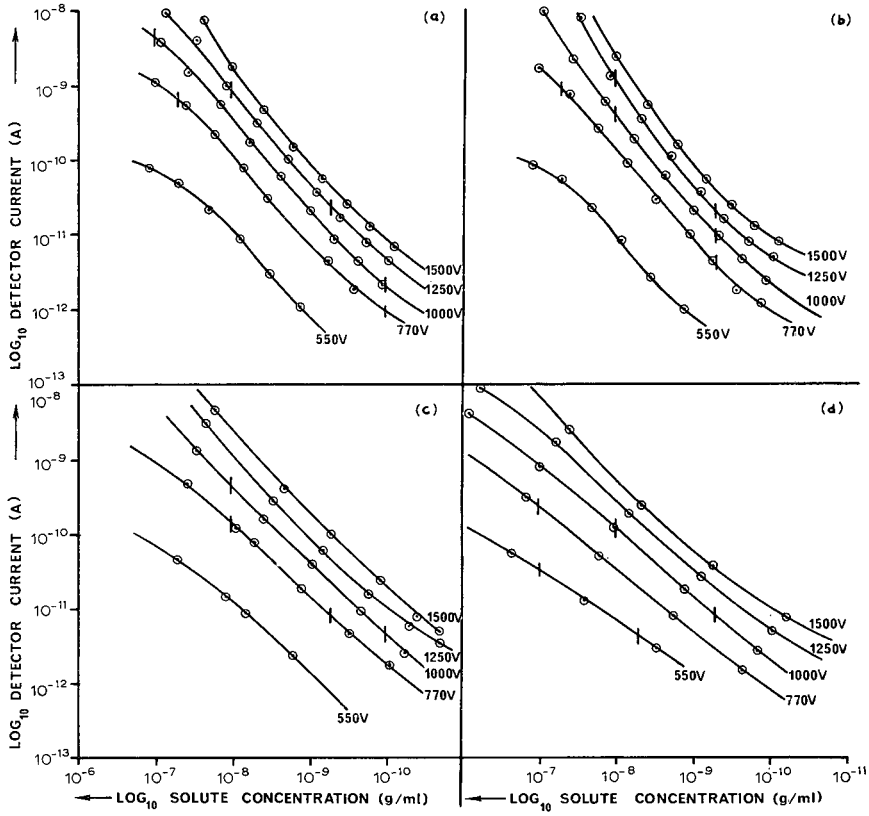

Fig. 6. Response curves for the argon ionisation detector at different flow-rates and temperatures. (a), (c) and (d) with linearising resistance; (b) without linearising resistance. Column flow-rate: (a) and (b) 30 ml/min; (c) and (d) 60 ml/min. Detector temperature: (a), (b) and (c) 75°; (d) 46°. Solute: *n*-heptane.

	Voltage	*Minimum detectable signal (estimate) g/ml*	*Ionization effiiciency %*	*Response index*	*Range g/ml*	*Noise level A*
(a)	550	$6.0 \cdot 10^{-10}$	$2.1 \cdot 10^{-3}$	1.11	10^{-8} — 10^{-9}	$6.0 \cdot 10^{-12}$
	770	$3.0 \cdot 10^{-11}$	$2.7 \cdot 10^{-2}$	1.14	$5 \cdot 10^{-8}$ — 10^{-10}	$3.0 \cdot 10^{-12}$
	1000	$9.0 \cdot 10^{-12}$	$6.9 \cdot 10^{-2}$	1.08	10^{-7} — 10^{-10}	$3.0 \cdot 10^{-12}$
	1250	$1.0 \cdot 10^{-12}$	$1.7 \cdot 10^{-1}$	1.60	10^{-8} — $5 \cdot 10^{-10}$	$3.6 \cdot 10^{-12}$
	1500	$1.0 \cdot 10^{-12}$	$3.7 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(b)	550	$7.5 \cdot 10^{-10}$	$2.3 \cdot 10^{-3}$			$6.0 \cdot 10^{-12}$
	770	$1.5 \cdot 10^{-11}$	$2.9 \cdot 10^{-2}$	1.26	$5 \cdot 10^{-7}$ — $5 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1000	$5 \cdot 10^{-12}$	$8.3 \cdot 10^{-2}$	1.19	10^{-8} — $5 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1250	$1 \cdot 10^{-12}$	$2.0 \cdot 10^{-1}$	1.30	10^{-8} — $5 \cdot 10^{-10}$	$3.6 \cdot 10^{-12}$
	1500	$<10^{-13}$	$5.0 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(c)	550	$4.2 \cdot 10^{-10}$	$1.1 \cdot 10^{-3}$			$6.0 \cdot 10^{-12}$
	770	$1.0 \cdot 10^{-11}$	$1.6 \cdot 10^{-2}$	0.95	10^{-8} — $5 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1000	$1.0 \cdot 10^{-12}$	$3.9 \cdot 10^{-2}$	0.93	10^{-8} — 10^{-10}	$3.0 \cdot 10^{-12}$
	1250	$1.1 \cdot 10^{-13}$	$8.7 \cdot 10^{-2}$			$3.6 \cdot 10^{-12}$
	1500	$<10^{-13}$	$1.8 \cdot 10^{-1}$			$6.0 \cdot 10^{-12}$
(d)	550	$4.0 \cdot 10^{-10}$	$6.8 \cdot 10^{-4}$	0.81	$5 \cdot 10^{-7}$ — $5 \cdot 10^{-9}$	$6.0 \cdot 10^{-12}$
	770	$1.0 \cdot 10^{-11}$	$3.3 \cdot 10^{-3}$	0.89	10^{-7} — 10^{-10}	$3.0 \cdot 10^{-12}$
	1000	$2.0 \cdot 10^{-12}$	$1.1 \cdot 10^{-2}$	0.92	10^{-8} — $5 \cdot 10^{-10}$	$3.0 \cdot 10^{-12}$
	1250	$4.0 \cdot 10^{-13}$	$2.8 \cdot 10^{-2}$			$3.6 \cdot 10^{-12}$
	1500	$4.1 \cdot 10^{-13}$	$5.7 \cdot 10^{-2}$			$6.0 \cdot 10^{-12}$

efficiencies and response indices for the four substances are also included in Fig. 5. The argon detector has a linear response for any given substance only over a small concentration range and at a particular voltage. Throughout the investigation a flame-ionisation detector was operated in series with the macro-argon detector and at all times it showed a linear response. Thus, any deviation from a linear response of the argon-ionisation detector was due solely to the detector and not to any part of the calibration system.

In Figs. 6(a) and 6(b) a comparison of response indices and concentration ranges for different voltages indicates that the use of a linearising resistance does not significantly improve the linearity of the detector. This fact has been substantiated by plotting detector current against voltage for different concentrations of *n*-heptane, with and without a linearising resistance (see Fig. 7).

In view of the capacity for chlorinated hydrocarbons to undergo electron capture, it is surprising to see, from Fig. 5(b), that the argon detector has an approximately linear response over the concentration range 10^{-8} to 10^{-10} g/ml for chlorobenzene when operated at 770 and 1000 V.

Since the response indices for the four substances are generally outside those

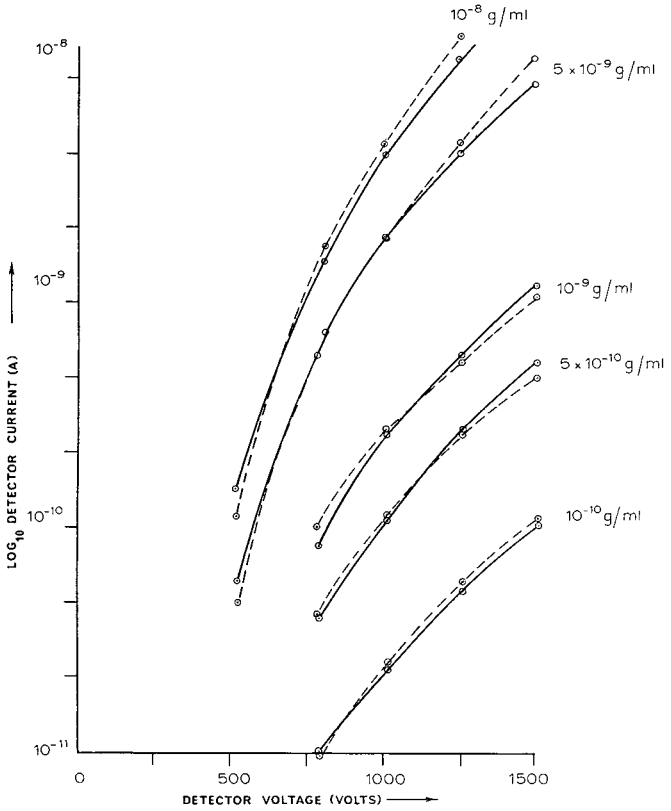

Fig. 7. Variation of ionisation current with voltage at different solute concentrations for the argon ionisation detector. With linearising resistance: ———. Without linearising resistance: – – –. Column flow-rate: 30 ml/min. Detector temperature: 75°. Solute: *n*-heptane.

acceptable for any detector in quantitative analysis, calibration of the macro-argon detector is therefore necessary for each compound at each particular voltage.

Figs. 6a, 6c and 6d show that increases in temperature and flow-rate improve the response index of the detector for *n*-heptane.

The maximum sensitivity for all four components studied was of the order of 10^{-13} g/ml. Where maximum sensitivity is required the argon detector can be advantageously used, but when quantitative results are necessary with the minimum of calibration then the flame-ionization detector should be employed.

ACKNOWLEDGEMENT

The authors would like to thank Mr. T. E. YOUNG for his valuable assistance in this work.

SUMMARY

The flame-ionisation detector has a linear response over a range of four orders of concentration, *i.e.* 10^{-6} to 10^{-10} g/ml, and this range could probably be extended to six orders by employing suitable experimental conditions. The detector response is independent of the applied voltage, over the range of 25–150 V, and has a sensitivity of approximately 10^{-11} g/ml.

The macro-argon ionisation detector has a linear response over a small concentration range at one particular voltage, the value of which may vary from one substance to another. Inclusion of a linearising resistance does not result in a linear response. Calibration of the macro-argon detector is essential for accurate quantitative analysis due to the variability of the response index. The sensitivity of the detector is about 10^{-13} g/ml.

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THE CHOICE OF CARRIER GAS IN PREPARATIVE GAS CHROMATOGRAPHY

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In analytical gas chromatography with katharometer detection, hydrogen or helium are the best carrier gases. The use of nitrogen results in a considerable loss of sensitivity, and the non-linearity of the detection together with possible peak reversals are additional drawbacks with this gas. In preparative scale gas chromatography, however, gas consumption is an important factor. Helium is ideal, but the price is prohibitive, at least for the majority of institutions. Hydrogen is of course dangerous, although it may be pointed out here that an air–hydrogen mixture only becomes inflammable at hydrogen concentrations of more than 4%. This means that in a fairly large room ($5 \times 6 \times 4$ m) an entire cylinder (± 5 m^3) may be emptied without danger of explosion. Undoubtedly, however, in preparative gas chromatography the use of nitrogen should be reconsidered.

Because of the increased sample loads of preparative gas chromatography the sensitivity of detection is no longer critical. With helium and hydrogen no special problems are encountered.

With nitrogen and katharometer detection, however, the troublesome effect of peak inversion can occur. BOHEMEN AND PURNELL[1] have shown that the signal of a katharometer detector is influenced by several factors. The thermal conductivity factor heats the katharometer when a substance passes and this gives a "positive" deflection. The molar heat capacity of the substances, however, also plays a part and has a cooling effect. If the heat capacity factor is the more important, peak inversion occurs. High gas flow rates, spiral filaments and high temperatures of filament and detector oven favour peak inversion.

With hydrogen and helium, thermal conductivity far outweighs the other factors, but with nitrogen this is not the case and peak inversion can occur easily with this gas. Commercial katharometer blocks are usually designed for analytical purposes and have as small a volume as possible. The use of such a detector block for preparative purposes with nitrogen at high flow rates produces just the conditions where peak inversion is most easily observed. This is for example the case for the Wilkens Autoprep 700 preparative gas chromatographic unit. The chromatograph has also analytical possibilities and in fact the detector block is the same as in analytical chromatographs produced by this firm. Inversion then occurs easily. This seemingly very annoying phenomenon can, however, be guided in such a way that inversion is the normal state of affairs. This is achieved by setting the detector oven at a high temperature (250°) and by using high flow rates and high detector currents. Most

substances then show completely reversed peaks with a strong signal over the whole possible concentration range. All that is necessary then is to switch the polarity of the recorder.

This is for example the procedure to be followed using the instrument mentioned above for all concentrations of acetone, ethyl acetate and benzene, substances for which badly shaped peaks and inversion trouble have been reported frequently in the literature. The highest signal for a reversed peak can be obtained with a nitrogen gas flow of 250 ml and a detector current of 200 mA. The efficiency of the columns is, however, impaired by such a high gas rate using nitrogen and the high katharometer currents considerably shorten the detector block life time. The same detector current will heat a katharometer wire to a much higher temperature in a nitrogen stream than in a hydrogen or helium stream. With more normal conditions *e.g.* gas rate 200 ml and a detector current of 150 mA, the response is only slightly less and the separation is good. This is shown in the chromatogram of Fig. 1 where 500 μl of a mixture of acetone, ethyl acetate and benzene are separated under these conditions on a 6 m glass column of 9 mm diameter filled with chromosorb W with 30 % SE30. The recorder terminals are reversed.

An important point with all detectors is the linearity of the response to sample load. With helium and hydrogen the response of a katharometer detector is linear for very small sample loads and shows decreasing signals for the large samples used in preparative chromatography. The reverse is true for the inverted peaks obtained with nitrogen. For small samples there is no linearity and the response first falls off with increasing sample, while remaining linear for larger sample loads. With a flow rate of 300 ml and 150 mA as bridge current the sensitivity of the detection in hydrogen and nitrogen for large samples is nearly the same.

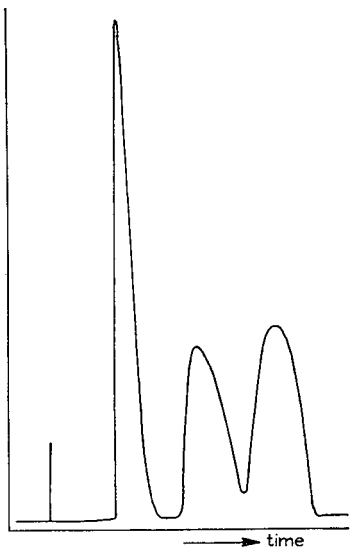

Fig. 1. Separation of acetone, ethyl acetate and benzene (0.5 ml) on 6 m column, diameter 9 mm, 30 % SE30 on Chromosorb W with nitrogen as carrier gas, showing peak inversion over whole concentration range as explained in text.

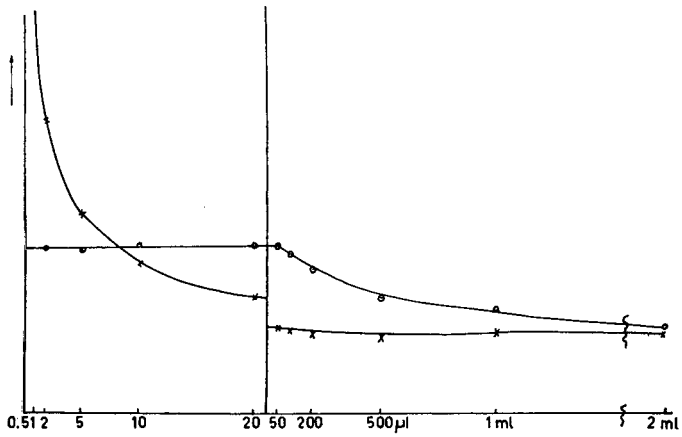

Fig. 2. Response of katharometer detection in hydrogen (⊙ normal peaks) and nitrogen (× inverted peaks) as further explained in text.

The linearity discussed above is shown in Fig. 2. To obtain easily readable diagrams, all measured peak surfaces were recalculated as peaks for 2 ml and the sample scale ordinate was compressed for larger sample loads.

The absolute value of the surfaces cannot be compared in Fig. 2, because measurements were obtained on different recorders and with a different flow rate and detector current. The scales were adapted to show that for really large samples the surface area is about the same for the normal peaks in hydrogen and the inverted peaks in nitrogen. For small samples then the inverted peak surface with nitrogen is larger, but the peaks are lower and broader than for the positive peaks obtained with hydrogen. This is a normal result with regard to the higher non-linear response in nitrogen (response is lower at peak maximum) and this is also due to the difference in column efficiency with the two gases for small samples, as will be explained later.

Column efficiency and carrier gas

Column efficiency is defined here as the number of plates calculated from the equation $n = C(V'_R \cdot V_R/W^2)$ where V_R and V'_R are the uncorrected and corrected retention times and W is the band-width. C is a constant and is 18.5 when the band is measured at 10 % of its maximum height[2]. Comparisons of the carrier gases were obtained by chromatographing the same substance under exactly the same conditions. The optimum gas rates for nitrogen and for hydrogen and helium differ slightly, being somewhat lower for nitrogen. Varying the gas rates between 50 and 500 ml makes no difference to the general line of results and conclusions as given below.

With analytical quantities, the efficiency of larger bore columns is much better with hydrogen and helium than with nitrogen. The difference is between 20 and 100 % and varies with column length and grain size of the support material.

With increased sample loads for preparative purposes the efficiency drops sharply as has indeed been long known. It is important that this efficiency decrease is less marked with nitrogen than with hydrogen and helium. This can be concluded from the following diagram showing the plate number *versus* the sample volume at a gas rate of 200 ml/min for the different carrier gases (Fig. 3).

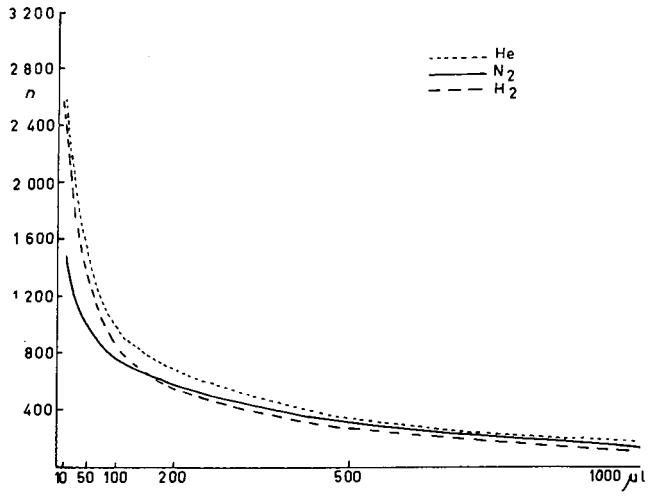

Fig. 3. Plate number of 6 m coiled glass column, diameter 9 mm, as function of sample load of iso-octane with helium, hydrogen and nitrogen as carrier gas.

The same pattern was observed for shorter or longer columns and for a number of different grain sizes of the support.

The conclusion is that for analytical purposes the superiority of helium and hydrogen over nitrogen on large bore columns is unquestionable, but that for preparative quantities there is no very great difference between the three gases from the all-important point of efficiency. It is of course not easy to measure retention times, peak heights and band-widths on the distorted peaks of preparative gas chromatography, and the value of the plate number as a measure of efficiency is questionable in this case. However, in as far as the plate number is an expression of relative band-width, it is useful in this respect. The measured peak heights and band-widths are only proportional to the actual values in the column for linear detection over the whole concentration range of the peak. This in only so for small concentrations and in hydrogen; for all other cases the calculated plate number values must be too low. It is believed, however, that this detection effect is only of minor importance and that the general line of results about the plate number is not influenced by it.

Example of a separation with hydrogen and nitrogen

A separation with hydrogen and nitrogen is shown in the diagrams obtained with decalin (Figs. 4 and 5). The concentration difference at the base and top of the peaks is much greater than could be inferred from the chromatograms. The height of the minimum between the peaks in the separation with nitrogen is reached experimentally with only 1 % of the sample load of the preparative separation, although this height is 12 % of the peak height of *trans*-decalin. This is caused by the sensitivity for small samples in nitrogen as explained in the discussion of Fig. 2. As a result, even when the minimum between two peaks does not reach the base line the recovered substances are purer than would be expected. The substances recovered from the preparative separations of Fig. 4 and 5 by simply switching the collection bottles at the minimum between the two peaks (this means that no waste period was observed)

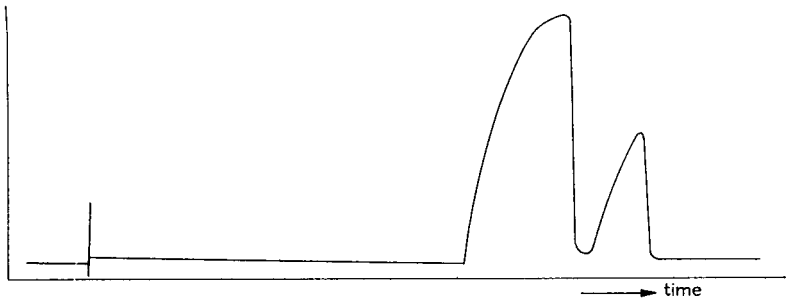

Fig. 4. Separation of 0.5 ml decalin (*trans* and *cis*) on 6 m coiled glass column, diameter 9 mm, filled with 30 % SE30 on Chromosorb P with hydrogen as carrier gas.

were analysed by ordinary gas chromatography. No trace of *cis*-contamination of *trans*-decalin or *vice versa* could be detected with katharometer detectors. On a flame ionisation instrument with a capillary column, less than 0.5 % *cis*-decalin was found in the *trans*-decalin from the separation with nitrogen; the other substances were pure. This is indeed surprising considering the tracing shown in Fig. 5 obtained with nitrogen.

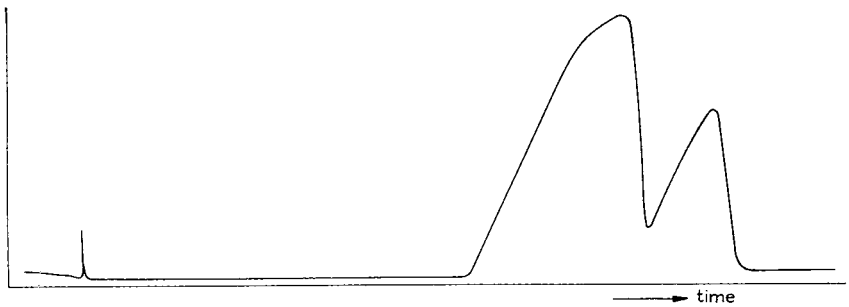

Fig. 5. The same as in Fig. 4 but with nitrogen as carrier gas. The inverted peaks become normal after switching the polarity of the recorder terminals.

ACKNOWLEDGEMENT

The "Fonds voor het collectief fundamenteel wetenschappelijk onderzoek" is thanked for financial help to the laboratory. Thanks are also due to M. VERSTAPPE for technical assistance.

SUMMARY

Helium and hydrogen as carrier gases are more suitable than nitrogen for preparative gas chromatography. Having regard, however, to the high price of helium and the danger of hydrogen, nitrogen may be preferred. For small analytical sample loads on large bore columns helium and hydrogen are far superior to nitrogen, but for larger sample loads this difference is not so great. The separations obtained with nitrogen are much better than indicated by the recorded chromatograms because of the non-linearity of the detection.

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THEORY OF PARTITION CHROMATOGRAPHY

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The theory of chromatographic processes has been dealt with in numerous recent articles and some excellent reviews of the subject are available[1–5]. The treatments vary from a phenomenological approach in the equilibrium theories, which are based on the "effective plate" concept, to rate theories, where the approach is kinetic. The object of the latter is to elucidate the actual mechanism of the chromatographic process and to determine the concentration profile of the elution curve as a function of fundamental parameters such as flow rate, feed concentration, partition coefficient, solute diffusion coefficient, etc. The treatment along these lines requires the specification of a model and the introduction of some simplifying assumptions such as linear sorption isotherms, plug flow of the moving phase, neglect of longitudinal diffusion, etc. Some recent contributions in this field are listed in refs. 6–9.

In the present article a kinetic approach based on a simple physical model of a chromatographic column is presented. The model is fairly general and may be applied to most of the different chromatographic procedures in use. A detailed treatment will, however, be given here only for the case in which the different solutes do not interact with each other. It applies best therefore to different types of partition chromatography, such as gel filtration and some forms of gas–liquid chromatography.

We will start by specifying the model. It is based on a model treatment of diffusion processes advanced in a recent article by the author[10]. It was shown that diffusion problems can be handled by means of a model consisting of a subdivision of space into compartments, separated by membranes. In this model all the resistance to diffusion is concentrated in the membranes, which thus constitute the resistance elements of the model, whereas the compartments constitute the capacity elements. The diffusion coefficient for a solute in a medium can be reproduced by choosing the proper value for the permeation coefficient of the membranes. This principle can be applied to a chromatographic column by representing the moving and stationary phases of the column by compartments, separated by a membrane and visualizing them as constituted of two long grooves separated by the membrane. A cross section is shown in Fig. 1. Owing to the basic assumptions of the model, the concentrations in a cross section are uniform within each compartment. The expression for concentration equilibration for a single solute within a narrow strip of the grooves may then be readily derived. Taking also into account the possibility of an unequal partition of the solute between the two phases, the following expression is obtained:

$$\frac{1}{\gamma} c_2 - c_1 = \left(\frac{1}{\gamma} c_2^\circ - c_1^\circ\right) e^{-mt} \qquad (1)$$

with

$$m = a\left(\frac{1}{V_1} + \frac{1}{\gamma V_2}\right) \tag{2}$$

where

V_1, V_2 = volumes per unit membrane area of respective compartments
c_1, c_2 = concentrations of solute in respective compartments
a = permeation constant for solute
γ = solute partition coefficient.

In addition the following mass conservation relation holds for the solute:

$$c_1V_1 + c_2V_2 = c_1^\circ V_1 + c_2^\circ V_2 \tag{3}$$

From eqns. (1) and (3) it follows that

$$c_1 = c_1^\circ + \left(\frac{c_2^\circ}{\gamma} - c_1^\circ\right)\frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 - e^{-mt}\right) \tag{4}$$

$$\frac{1}{\gamma}c_2 = c_1^\circ + \left(\frac{c_2^\circ}{\gamma} - c_1^\circ\right)\frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 + \frac{V_1}{\gamma V_2}e^{-mt}\right) \tag{5}$$

These relations are now applied to the model of the chromatographic column. The column is divided into cells of equal width l and the operation of the column is assumed to take place in discontinuous steps of duration τ. The procedure of the operation is as follows. During the time interval τ the solutions on the two sides of the membrane in every cell are equilibrated according to eqn. (1). At the end of the step the solutions in the moving phase of each cell are instantaneously shifted to the next

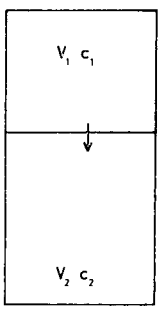

Fig. 1. Cross section of the column model.

cell and the equilibration procedure is repeated. If the velocity of the moving phase in the longitudinal direction of the column is v then obviously $l = v\tau$. A schematic representation of the column operation is shown in Fig. 2. The cells are numbered from left to right and the solution is assumed to enter the column from the left side. The concentrations in the ith cell at time t are denoted by f_{it} and γg_{it} for the moving and stationary phase respectively. Neglecting for the moment longitudinal diffusion and using eqns. (4) and (5) the material balance equations may be written down:

$$f_{i+1,\,t+\tau} = f_{it} + (g_{it} - f_{it})\frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 - e^{-m\tau}\right) \tag{6}$$

$$g_{i,\,t+\tau} = f_{it} + (g_{it} - f_{it})\frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 + \frac{V_1}{\gamma V_2}e^{-m\tau}\right) \tag{7}$$

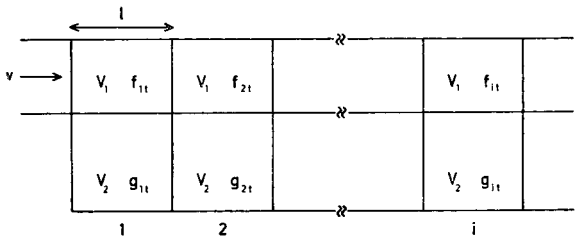

Fig. 2. Schematic representation of the column operation.

From these relations the differential equations for the column operation may readily be derived. The spatial variable x is introduced, and since the cell width is $v\tau$, where v is the velocity of the moving phase, eqns. (6) and (7) may be written

$$f(x + v\tau, t + \tau) = f(x,t) + [g(x,t) - f(x,t)] \frac{\gamma V_2}{V_1 + \gamma V_2} (1 - e^{-m\tau}) \tag{8}$$

$$g(x,t + \tau) = f(x,t) + [g(x,t) - f(x,t)] \frac{\gamma V_2}{V_1 + \gamma V_2} \left(1 + \frac{V_1}{\gamma V_2} e^{-m\tau}\right) \tag{9}$$

Expanding the left members and the exponential terms in the right members into power series and rearranging, we get

$$\frac{\partial f}{\partial x} v\tau + \frac{\partial f}{\partial t} \tau = (g - f) \frac{\gamma V_2}{V_1 + \gamma V_2} m\tau + \text{higher terms in } \tau \tag{10}$$

$$\frac{\partial g}{\partial t} \tau = -(g - f) \frac{V_1}{V_1 + \gamma V_2} m\tau + \text{higher terms in } \tau \tag{11}$$

Substituting for m from eqn. (2) and dividing by τ, we get for the limit $\tau = 0$

$$\frac{\partial f}{\partial t} + v \frac{\partial f}{\partial x} = \frac{a}{V_1} (g - f) \tag{12}$$

$$\frac{\partial g}{\partial t} = -\frac{a}{\gamma V_2} (g - f) \tag{13}$$

Here the parameter a determines the rate of the lateral diffusion in the column. It may be shown[10] that, if diffusion in the stationary phase is the rate determining step,

$$a = 2 \frac{D_2}{V_2} \tag{14}$$

where D_2 is the solute diffusion coefficient in the stationary phase. At this stage longitudinal diffusion may also be taken into account by superposing on eqns. (12) and (13) the concentration changes obtained from the diffusion equation (15)

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \tag{15}$$

Thus, the final equations take the form

$$\frac{\partial f}{\partial t} + v\frac{\partial f}{\partial x} = \frac{D_2}{V_1 V_2}(g - f) + D_1\frac{\partial^2 f}{\partial x^2} \tag{16}$$

$$\frac{\partial g}{\partial t} = -\frac{D_2}{\gamma V_2^2}(g - f) + D_2\frac{\partial^2 g}{\partial x^2} \tag{17}$$

The same equations may of course also be derived from a continuous-flow model. Equations (16) and (17) contain the following fundamental parameters:

v = velocity of the moving phase

γ = solute partition coefficient

D_1, D_2 = diffusion coefficients in the moving and stationary phases respectively

V_1, V_2 = volumes per (interphase) area of the moving and stationary phases respectively.

All these parameters are in principle determinable. The solution of the differential equations thus gives the solute distribution in the column as a function of time and position. In the case of a mixture of non-interacting solutes, different values have only to be assigned to the parameters γ, D_1 and D_2 to obtain the distributions of the different solutes, and hence the separation efficiency of the column. However, as analytical solution of the differential equations seems impossible, a procedure is presented for numerical solution of the problem using the original eqns. (6) and (7). The effect of longitudinal diffusion may be considered by adding to the right members of these equations the concentration changes due to longitudinal diffusion. Using the diffusion-model treatment in the longitudinal direction (see Fig. 2) and assuming constant diffusion coefficients, the following concentration increments according to eqn. (9) in ref. 10 are obtained

$$\Delta f_{it} = \tfrac{1}{2}\alpha_1(f_{i-1,t} - 2f_{it} + f_{i+1,t}) \tag{18}$$

$$\Delta g_{it} = \tfrac{1}{2}\alpha_2(g_{i-1,t} - 2g_{it} + g_{i+1,t}) \tag{19}$$

where α_1 and α_2 are determined by the diffusion coefficients in the respective phase. Noting that the volumes per area of the compartments in the model for longitudinal diffusion are $v\tau$, eqn. (15) in ref. 10, gives

$$D_i = \tfrac{1}{2}v^2\tau\alpha_i \tag{20}$$

$$i = 1,2$$

As $\alpha_i \leqslant 1$, this equation obviously imposes a lower limit on τ for longitudinal diffusion. The final equations are now obtained from eqns. (6) and (7) and eqns. (18) and (19). With the substitutions

$$\eta = \frac{\gamma V_2}{V_1 + \gamma V_2}(1 - e^{-m\tau}) \tag{21}$$

$$\xi = \frac{\gamma V_2}{V_1 + \gamma V_2}\left(1 + \frac{V_1}{\gamma V_2}e^{-m\tau}\right) \tag{22}$$

and with a new time unit having the length τ, we get

$$f_{i+1,\,t+1} = (1 - \eta)\, f_{it} + \eta g_{it} + \tfrac{1}{2}\,\alpha_1(f_{i-1,t} - 2\, f_{it} + f_{i+1,t}) \tag{23}$$

$$g_{i,t+1} = (1 - \xi) f_{it} + \xi\, g_{it} + \tfrac{1}{2}\,\alpha_2(g_{i-1,t} - 2\, g_{it} + g_{i+1,t}) \tag{24}$$

From these equations the distribution of solute in the chromatographic column may be obtained. Here, a simplified treatment is considered in which longitudinal diffusion is neglected. Thus instead of eqns. (23) and (24) the following are used:

$$f_{i+1,\,t+1} = (1 - \eta)\, f_{it} + \eta\, g_{it} \tag{25}$$

$$g_{i,\,t+1} = (1 - \xi)\, f_{it} + \xi\, g_{it} \tag{26}$$

From eqn. (26) we get by recursion

$$\begin{aligned} g_{i,\,t+1} &= (1 - \xi)\, f_{it} + \xi\{(1 - \xi)\, f_{i,\,t-1} + \xi[(1 - \xi)\, f_{i,\,t-2} + \cdots\} = \\ &= (1 - \xi)\, f_{it} + (1 - \xi)\xi f_{i,\,t-1} + (1 - \xi)\xi^2 f_{i,\,t-2} + \cdots (1 - \xi)\xi^t f_{i0} \end{aligned} \tag{27}$$

Insertion of this into eqn. (25) gives

$$\begin{aligned} f_{it} = (1 - \eta)\, f_{i-1,\,t-1} &+ \eta(1 - \xi)\, f_{i-1,\,t-2} + \eta(1 - \xi)\xi\, f_{i-1,\,t-3} + \\ &+ \eta(1 - \xi)\, \xi^{t-2} f_{i-1,0} \end{aligned} \tag{28}$$

A matrix (f_{ij}) of order n is now defined, having its elements determined by eqn. (28) with $j = t + 1$ and $i, j = 1, 2 \cdots n$. With reference to Fig. 2 the following interpretation of the element f_{ij} is obtained. It specifies the concentration in the moving phase in the ith cell at the time $(j - 1)\tau$. The time will always be given as the time at the beginning of an equilibration period. Thus, a row of the matrix represents the concentration in a particular cell at different times from $t = 0$ to $t = (n - 1)\tau$ and a column of the matrix represents the concentration distribution in the chromatographic column at a particular instance of time. The initial conditions are specified by the values of f_{1j} for $j = 1, 2, \cdots n$ and f_{i1} for $i = 1, 2, \cdots n$. The value of f_{1j} may be interpreted as the concentration in the solution that enters the column at the time

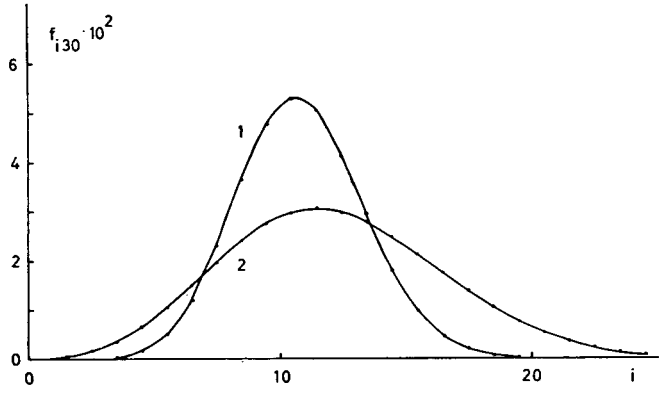

Fig. 3. Solute distribution in the moving phase of the column. For curve 1, $\tau \gg \tau^1/_2$ (*i.e.* equilibrium is established); for curve 2, $\tau = \tau^1/_2$ ($\tau^1/_2$ = half-time for attainment of equilibrium in lateral diffusion).

$(j - 1)\tau$ and that of f_{i1} as the concentration in the ith cell at $t = 0$. Specifying the coefficients in eqn. (28) and the initial values f_{1j} and f_{i1} and assigning to all f_{it} with zero or negative indices the value zero, all the elements f_{ij} in the matrix may be calculated from eqn. (28).

The calculations may conveniently be carried out with digital computers. Then matrixes of very high orders may be obtained, in which the conditions of continuous operation of the column are approached. In the present work a few calculations were carried out with hand-operated calculators giving matrixes of the 30th order. The results are shown in the form of the last columns, representing the solute distribution in the moving phase at the end of the time period considered ($t = 30\tau$). They are presented diagrammatically in Fig. 3. In the calculations the following initial conditions and values on the parameters determining the coefficients in eqn. (28) were used:

$f_{11} = 1, f_{1j} = f_{i1} = 0$ for $i, j = 2, 3, \cdots n$.
$\gamma = 1$
$V_1/V_2 = 0.5$
$\eta = {}^2/_3$ and ${}^1/_3$ for curve 1 and 2 respectively
$\zeta = {}^2/_3$ and ${}^5/_6$ for curve 1 and 2 respectively.

It should be noted that owing to the basic assumptions in the diffusion model (uniform concentrations in the compartments separated by the membrane) the geometry of the column filling does not enter into the treatment explicitly, but is taken into account by the ratio V_1/V_2. It is easy to show that where the stationary phase consists of a filling of tight-packed spherical beads the ratio

$$\frac{V_1}{V_2} = \frac{3\sqrt{2}}{\pi} - 1 \approx 0.350.$$

Thus, in the present calculations a rather loosely packed column is considered.

The results shown in Fig. 3 demonstrate the influence of lateral diffusion (local non-equilibrium) on the chromatographic process. It has a negative effect on the separation efficiency, it causes the broadening of a peak but affects only slightly its translational velocity. Thus, in general it is not possible to separate substances in a column on the basis of differences in diffusion coefficients. Only if one of the substances has a very small diffusion coefficient may it be separated from other substances with considerably higher diffusion coefficients. Thus it may be concluded that in partition chromatography separation is mainly due to differences in partition coefficients. The present calculations can of course only give a superficial picture of the possibilities of the method and further work, including more detailed computations with the aid of digital computers is in progress.

Finally some of the approximations made in the present treatment are considered. In the first place longitudinal diffusion has been neglected. The latter is most pronounced in the moving phase and causes the broadening of a chromatographic peak, but leaves its translational velocity unaffected. Therefore from the present calculations the optimal performance of an ideal column is obtained.

The diffusion model used in this treatment may be considered to give a first order approximation of the diffusion process (*e.g.* diffusion into a spherical particle (radius = r) is represented by an exponential function with the half-time $\tau_{1/2} =$

0.039 r^2/D, in close agreement with VERMEULEN's[11] approximate formula $\tau_{1/2} = 0.030\ r^2/D$). Higher order approximations could be obtained by a repeated application of the diffusion model, *e.g.* a spherical particle could be considered to consist of several concentric zones, each representing an element of the diffusion model. However, this would considerably complicate the numerical calculations and it is felt that the first order approximation is sufficient to bring out the essential features of the lateral diffusion effect.

The discreet operation of the column also introduced an approximation. However, by decreasing the length of the time period τ, conditions for the continuous operation of a column are approached. The magnitude of the deviation from the conditions for continuous operation may be estimated from calculations with different τ values.

SUMMARY

A rate theory for partition chromatography, based on a simple physical model, is presented. It has the object of determining the concentration profile of an elution curve from fundamental parameters, characteristic of the solute and the column operation. A method for a numerical solution of the problem with the aid of digital computers is also given.

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ANION-EXCHANGE CHROMATOGRAPHY OF SUGAR PHOSPHATES WITH TRIETHYLAMMONIUM BORATE

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Several methods of separation of sugar phosphates and other phosphoric esters by ion exchange chromatography have been described[1–6]. The recovery of the sugar phosphates after most of these separations is troublesome and time consuming, which diminishes the effectiveness of the technique and its application to microscale use.

This paper describes a method which allows separation of most of the sugar phosphates on an anion-exchange resin column and permits their almost quantitative recovery. It is based on the use of linear gradient elution with ammonium or triethylammonium borate and on the removal of these salts after freeze-drying by distillation with methanol.

EXPERIMENTAL

Materials

Fructose-1-P was synthesized according to RAYMOND AND LEVENE[7]. Fructofuranose-2-P and fructopyranose-2-P were prepared according to PONTIS AND FISCHER[8]. Mannose-1-P was kindly given by Dr. A. MORENO and galactose-1-P, N-acetylglucosamine-1-P and N-acetylgalactosamine-1-P were generously provided by Dr. C. E. CARDINI. All the other sugar phosphates were commercial samples.

Analytical procedures

The following analytical methods were used: BARTLETT's[9] method for phosphate, the anthrone method[10] for reducing power, ROE's[11] method for fructose and REISSIG *et al.*'s[12] method for acetylhexosamines.

Column chromatography

Dowex-1 X4 resin (200–400 mesh) was used for the chromatography of the hexose phosphates. Dowex-1 (Cl^-) resin was converted into the borate form by passing 0.8 *M* potassium tetraborate until all the chloride had been displaced. It was then washed with water until the effluent gave no precipitate on addition of silver nitrate.

The columns used were 60 cm long, 0.5 cm in diameter and were prepared by allowing a suspension of resin to settle until a bed 45 cm high was obtained. Consider-

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able shrinkage occurred during the chromatography. Therefore, the columns were disassembled after each run, and the resin washed again with 0.8 *M* potassium tetraborate and water as above. The resin could be reused many times, except for a layer at the top which became dark during the run and which was replaced every time with fresh resin.

The ammonium[8] or triethylammonium tetraborate used for elutions was prepared by mixing a freshly made boric acid solution with ammonia or triethylamine. Thus, a 0.4 *M* triethylammonium tetraborate was prepared by dissolving 99.2 g of boric acid and 112 ml of triethylamine in water and making the volume up to one liter.

The mixture of sugar phosphates to be separated was adjusted to pH 8 with ammonium hydroxide and applied to the column. After washing the column with water the sugar phosphates were eluted with a linear gradient of ammonium or triethylammonium borate. Fractions of 1.4 ml were collected at a flow rate of 1.0–1.5 ml/min. Aliquots of every second fraction were taken for analysis.

Columns of the size indicated above could be used for separation of 2 to 100 μmoles of a complex mixture of sugar phosphates.

Recovery

The fractions under each peak were pooled and freeze dried. When ammonium borate was used as eluant, a very light powder remained which was easily removed by two or three evaporations to dryness with methanol. With triethylammonium borate a residue that stuck to the wall of the flask was left and made removal with methanol slower. Freeze drying can be replaced by evaporation to dryness in a rotary evaporator when ammonium borate has been used as eluant.

The sugar phosphates thus freed from salts were dissolved in water and adjusted to pH 7.

Radioactivity measurements

When radioactive samples were under investigation, suitable aliquots from the column fractions were plated on aluminum disks and evaporated to dryness on a water bath. Methanol was then carefully added taking care not to overflow the planchet. Depending on the aliquot, two or three methanol additions were needed to eliminate all the salt. With aliquots of 0.5–1.0 ml it was found convenient to treat the planchet perimeter with silicon grease, in order to keep the liquid centered.

Counting was carried out with a gas flow counter (Tracerlab, Inc.).

RESULTS AND DISCUSSION

The commonly occurring monophosphorylated sugars have nearly identical dissociation constants[13]. This makes their complete separation by simple ion-exchange unlikely.

The use of borate, introduced by KHYM AND COHN[1] permitted the separation of glucose-1-P, glucose-6-P, fructose-6-P and ribose-5-P from each other and from fructose-1,6-P_2. KHYM AND ZILL[14] had used relatively concentrated solutions of borate for the separation of neutral sugars, but for phosphoric esters, the use of dilute borate on the basis of the following reasoning[1] was decided upon. "Since borate ion exists only in alkaline solutions where phosphate esters are doubly ionized and

hence more strongly bound to anion exchangers, the use of borate as the replacing ion as well as the complex-forming ion involves higher concentrations than are compatible with easy recovery of the separated esters". Hence, KHYM *et al.*[2] developed methods by which borate complexes of phosphate esters could be separated in chloride or sulfate systems containing only sufficient borate to allow complexing. However, this did not simplify the recovery procedure, as the removal of the eluting salts required the use of cation and anion exchangers[2] or solvent extraction[6].

The problem of recovery can be overcome by replacing potassium or sodium borate as eluants with ammonium or triethylammonium borate. These salts are easily removed by freeze-drying and distillation with methanol. In this way sugar phosphates can be freed from borate in concentrations up to 0.5 *M*. Ammonium borate has already been used as an eluant by PONTIS AND FISCHER[8] for the separation of fructose 2-phosphates from fructose-1-P. It has now been found that the use of triethylammonium borate as eluant gives more reproducible results for complex separations. Hence, this salt is generally used, particularly for analytical runs. Ammonium borate is employed when substances are easily separated, especially for preparative runs on account of its easier removal (see Experimental).

The application of this technique permits almost quantitative recovery of the sugar phosphates (Table I). Moreover, if BARTLETT's[9] procedure for phosphate de-

TABLE I

AMOUNT OF SUGAR PHOSPHATES RECOVERED

Sugar phosphate	*μmoles added to column*	*Percent recovery*	*μmoles before freeze-drying**	*Percent recovery*
N-Acetylglucosamine-1-P	2.3	93	1.65	86
N-Acetylgalactosamine-1-P	7.0	95	5.7	93
Glucose-1-P	2.7	97	2.2	95
Galactose-1-P	12.0	105	6.0	92
Mannose-1-P	9.0	98	7.2	94
Fructose-6-P	7.3	98	6.7	91
Mannose-6-P	15.0	94	3.0	93
Fructose-1-P	8.0	94	7.2	84
Glucose-6-P	9.6	91	5.0	100
Galactose-6-P	9.0	97	8.0	96
Fructose-1,6-P_2	9.0	94	6.4	81

* Only part of the ester recovered after the column was submitted to freeze-drying.

termination is employed, the method can be used with as little as 0.2 μmole of aldose-1-phosphates and fructose-2-phosphates. A further advantage is that these acid labile esters are not exposed to strong acid conditions during their isolation.

The results obtained with the different sugar phosphates are shown in Figs. 1 and 2. Similar elution patterns have been obtained by GOODMAN *et al.*[3] and DIEDRICH AND ANDERSON[6]. However, the triethylammonium borate system offers the advantage that the procedure is simpler and that the substances do not become so dilute.

The separation between fructose-6-P and glucose-6-P is complete (Fig. 1A). A simple procedure is thus available for the purification of fructose-6-P. The triethylammonium borate gradient used for the more common sugar phosphates also resolves mixtures of fructose-2-phosphates and fructose-1-P (Fig. 1C). On the other hand, the

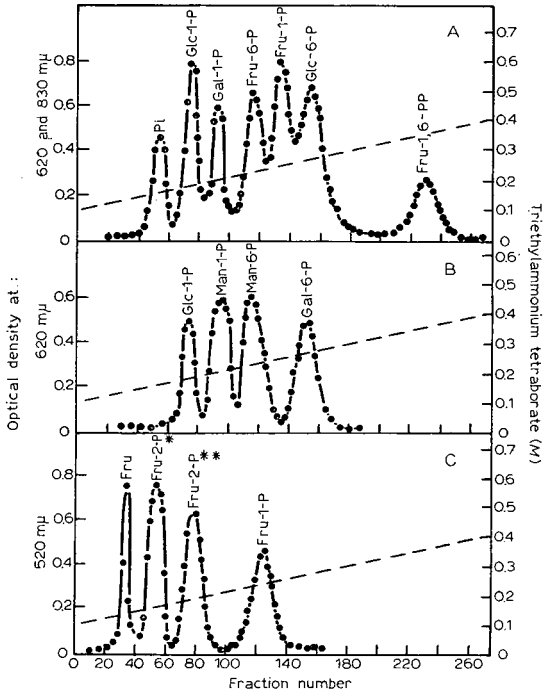

Fig. 1. Ion-exchange chromatography of sugar phosphates. The broken line represents the gradient from 0.1 *M* to 0.4 *M* triethylammonium tetraborate (360 ml total volume). The mixtures applied to the column were as follows, in μmoles: [A] P_i (2); glucose-1-P (9); galactose-1-P (7); fructose-1-P (10); fructose-6-P (10); glucose-6-P (17); fructose-1,6-P_2 (9). [B] Glucose-1-P (5); mannose-1-P (18); mannose-6-P (17); galactose-6-P (20). [C] Fructose (1); fructofuranose-2-P (Fru-2-P*, 3); fructopyranose-2-P (Fru-2-P**, 4); fructose-1-P (6). Assays were as follows: anthrone for sugar phosphates in A and B, ROE's[11] method for fructose compounds in C, and BARTLETT's[9] method for inorganic phosphate.

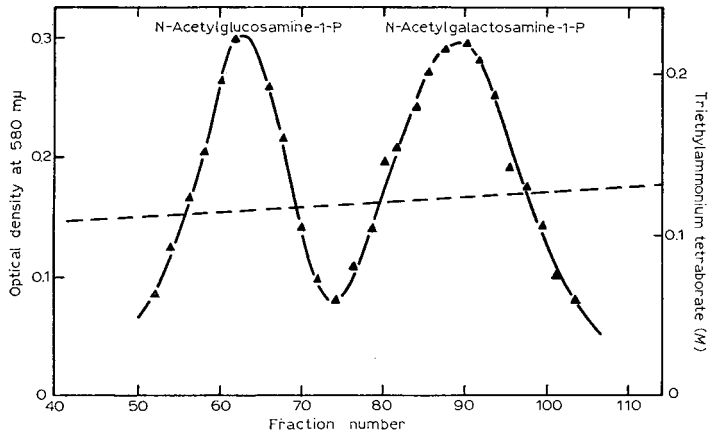

Fig. 2. Ion-exchange chromatography of N-acetylhexosamine 1-phosphates. The broken line represents the gradient from 0.1 *M* to 0.14 *M* triethylammonium tetraborate (200 ml total volume). The mixture applied to the column contained N-acetylglucosamine-1-P (2 μmoles) and N-acetylgalactosamine-1-P (5 μmoles). Assay according to REISSIG *et al.*[12] after acid hydrolysis.

separation of N-acetylglucosamine-1-P and N-acetylgalactosamine-1-P can be achieved as shown in Fig. 2, by employing a less steep gradient than that used for the other esters.

The use of ammonium or triethylammonium borate as eluant is also advantageous when dealing with radioactive samples. In this case, suitable aliquots from the column fraction can be plated directly on aluminum disks, evaporated to dryness on a water bath, and treated with methanol until no salt residue is left. Self-absorption owing to the presence of salts is completely eliminated. In the chromatogram shown in Fig. 3, fructose-1-P-^{14}C, 36,000 counts/min, was applied together with the esters indicated. After plating and counting, 37,000 counts/min were recovered.

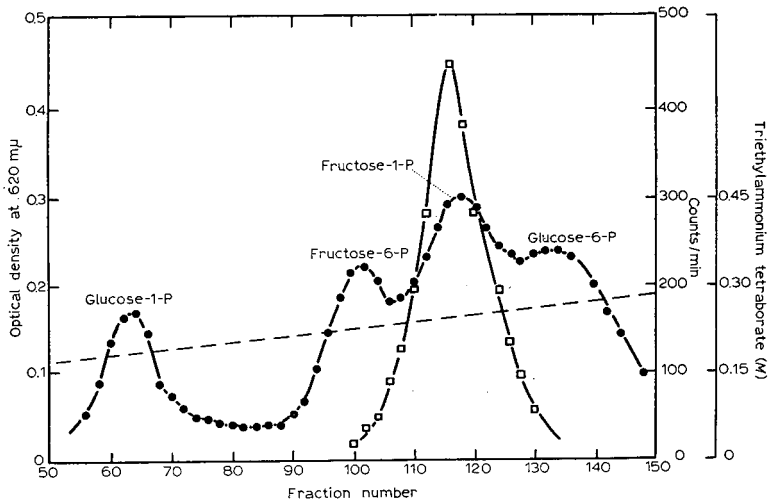

Fig. 3. Ion-exchange chromatography of fructose-1-P-^{14}C. The broken line represents the gradient from 0.1 *M* to 0.35 *M* triethylammonium tetraborate (300 ml total volume). Fructose-1-P-^{14}C 36,000 counts/min was mixed with a solution containing approximately 10 μmoles of each sugar phosphate and applied to the column. Counting of radioactive aliquots as described in text. , anthrone assay; □, counts/min.

As the free sugars are eluted at the beginning of the gradient (Fig. 1C), this method is very suitable for the purification of labelled sugar phosphates obtained through enzymatic reactions where adenosine triphosphate is the usual phosphorylating agent. This nucleotide, as well as adenosine mono- and diphosphate, is retained on the column.

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SUMMARY

A method is presented which allows the separation of most of the sugar phosphates on an anion-exchange resin column and permits their almost quantitative recovery. It is based on the use of linear gradient elution by ammonium or triethylammonium tetraborate and on the removal of these salts, after freeze-drying, by distillation with methanol.

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THE DETERMINATION BY REFLECTANCE SPECTROPHOTOMETRY OF AMINO ACIDS RESOLVED ON THIN-LAYER PLATES

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INTRODUCTION

The obvious advantages afforded by paper chromatography in the analysis of amino acids has resulted in an extensive literature in the field[1]. A considerable number of these publications deal with problems involving the quantitative estimation of the acids[2] following their resolution. In this connection, much interest has been shown in the *in situ* determination of the acids as a means of eliminating tedious elution procedures and thus expediting the analyses. POLSON *et al.*[3] made measurements by visually comparing the colors resulting from the addition of ninhydrin to standard and unknown amino acid concentrations. BULL *et al.*[4] devised a procedure for the estimation of amino acids separated by paper chromatography which involved direct photometry of the spots. The accuracy attained by this and some of the other direct photometric methods[5–7] developed subsequently was of the order of $\pm$ 5 % to $\pm$ 12 %. LUGG AND MCEVOY-BOWE[8,9] achieved increased precision by employing a specially designed densitometer having a small aperture and by taking account of texture irregularities in the chromatographic paper.

The promise of reduced tailing, increased sensitivity, and greater speed and resolution[10] has induced many investigators to resort to the thin-layer technique in the analysis of amino acids. Unfortunately, since methods developed for the *in situ* estimation of acids separated on paper are not applicable to chromatoplates, there is need for a procedure whereby this can be accomplished in separations involving thin plates. The possibility of using reflectance measurements for this purpose was suggested by a study of the application of spectral reflectance to thin-layer chromatography[11]. Results of this research demonstrated that the components of dye mixtures resolved on thin-layer plates can be determined with a precision of approximately $\pm$ 5 % by direct examination of the plates. If the reflectance measurements are carried out on spots removed from the plates and packed in an appropriate cell, the degree of precision attained is identical to that afforded by transmittance.

EXPERIMENTAL

The amino acids studied (DL-alanine, L-arginine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-norvaline, DL-phenylalanine, DL-serine, DL-threonine and DL-valine) were of Calbiochem A Grade purity. Stock solutions containing 500 mg of the acids per 50 ml of solution were used in making up

the dilution series employed in this research. Distilled water served as the solvent throughout and the solutions were applied as spots by means of a Hamilton microsyringe in 5 μl increments. The 20 × 5 × 0.35 cm plates were coated with adsorbent by distributing a Merck silica gel G–water (4:10) mixture with a glass rod which rested on one thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating 0.2–0.3 mm thick. The plates were dried at 180° for 2 h and stored in a desiccator. The amino acids were chromatographed in *n*-propanol–water (64:30) by the one-dimensional ascending technique described by BRENNER AND NIEDERWIESER[10], and the plates were then dried at 60° for 30 min in a mechanical convection oven.

The spray reagent, consisting of 90 g of *n*-butanol, 10 g of phenol and 0.4 g of ninhydrin, was one described by BULL *et al.*[4]. An even dispersion of the spray was achieved by using an atomizer in conjunction with compressed air at a distance of 30 to 40 cm from the plate. After the gas pressure was adjusted to forestall impairment of the adsorbent surface, the plates were sprayed until they first appeared translucent. An excess of spray was avoided to preclude leaching out of the amino acids. The plates were next exposed to a stream of cold air for 5 min, dried in a mechanical convection oven at 60° for 15 min, and then stored in the dark at about 10° in a refrigerator until required for the determination. These drying conditions were selected after a consideration of the results of various investigations[6,12,13] which indicated that drying temperatures exceeding 60° substantially reduced recoveries of amino acids from paper chromatograms.

Direct spectral examination of these plates was accomplished with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment. A Beckman Model DU Spectrophotometer, likewise equipped for the measurement of diffuse reflectance, was employed to examine spots scraped off the chromatoplates. The cells used to hold the sample and reference material as well as the technique utilized in preparing material for examination have been described elsewhere by the authors[11]. With these cells 40 mg of the material being studied sufficed to give a thin layer, of an approximate thickness of 0.3 mm and an approximate diameter of 1.8 cm, dimensions which have been found to be optimum for the analysis. The reference standard in all cases consisted of adsorbent from the plate under investigation.

For the analysis of spots removed from chromatoplates, the 40 mg comprising the sample were weighed to $\pm$ 0.3 mg and then ground in a small agate mortar for two periods of 1 min each to insure homogeneity and uniform particle size. Samples were weighed and worked up in a low humidity, air-conditioned room to avoid the condensation of moisture. With the exception of this period, the samples were stored in a large desiccator from the time they were removed from the refrigerator until they were introduced into the reflectance attachments of the spectrophotometers. In addition to the calcium chloride desiccant, the desiccator contained dry ice as a coolant to ensure color stability.

RESULTS AND DISCUSSION

Color development

The necessity for storing the samples at reduced temperatures was pointed up by the results of a study of the variation of the color developed using the ninhydrin spray described above with time and temperature. A dilution series of leucine ranging from 5.0 to 50 μg was employed for this study with readings being taken at 520 mμ. Spots

were removed from the chromatoplates and prepared for analysis by the procedure outlined above. As may be seen in Fig. 1, the color intensity of the ninhydrin complex of leucine decreased by as much as five reflectance units within 24 h when the samples were kept at room temperature (28°). To attain the degree of precision inherent in the reflectance technique[11], the storage temperature had to be maintained below 10°. When

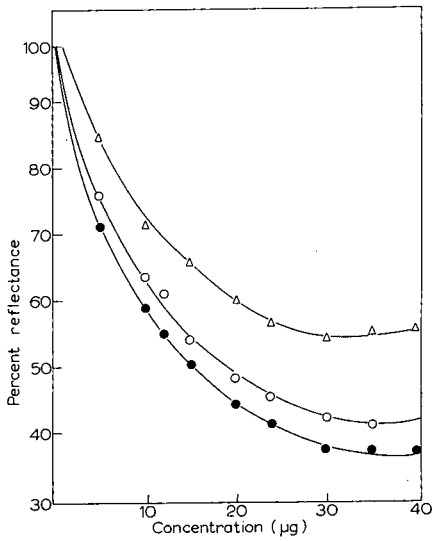

Fig. 1. % reflectance at 520 mμ of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration and time. ●–● after 2 h; ○–○ after 24 h; △–△ after 3 days at room temperature (28°).

this precaution was observed with a similar dilution series, the average change over a 24 h period was found to be 1.0 reflectance unit and the greatest decrease noted was of the order of 2.0 reflectance units. The data for this study are presented in Table I. Similar results were obtained with all of the amino acids listed above except phenylalanine and glutamic acid. In these two instances changes ranging up to three and five reflectance units, respectively, were noted.

The ninhydrin spray reagent used initially was a modification of one described by BRENNER AND NIEDERWIESER[10]. It was prepared by mixing 50 parts by volume of a 0.2 % solution of ninhydrin in absolute alcohol with 3 parts by volume of a 1 % solution of $Cu(NO_3)_2 \cdot 3H_2O$ in absolute alcohol and then adding 20 ml of glacial acetic acid to 100 ml of the resulting mixture. The spray described by BULL *et al.*[4] was found to provide greater sensitivity, however, and was employed in the acquisition of the data presented in this report. Fig. 2, which depicts % reflectance and absorbance at 520 mμ of the ninhydrin complex of leucine adsorbed on silica gel as a function of concentration, shows clearly the increased color intensity resulting from the use of the latter spray. Both sprays yield analytically useful data which take the form of a straight line up to concentrations of 30 μg when absorbance is plotted *versus* the square root of the concentration. Sensitivities found for leucine—0.5 μg for the BULL spray and 2 μg for the modified BRENNER-NIEDERWIESER spray — were consistent with the 0.5 μg value reported by PRATT AND AUCLAIR[14] for a 0.1 % solution of ninhydrin in

TABLE I

% REFLECTANCE AT 520 mμ OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF CONCENTRATION AND TIME FOR SAMPLES STORED BELOW 10°

Concentration of dilution series (μg leucine)	*Time*			*Range in reflectance units*
	2 h (% R)	*6 h (% R)*	*24 h (% R)*	
0.5 × 10	71.7	71.9	70.9	1.0
1.0	59.3	59.3	58.7	0.6
1.2	56.0	55.9	55.2	0.8
1.4	52.4	51.4	51.2	1.2
1.6	50.0	49.2	48.5	1.5
2.0	46.4	45.8	44.4	2.0
2.2	45.0	44.6	44.0	1.0
2.4	43.1	42.2	41.7	1.4
2.6	41.8	41.8	42.2	0.4
2.8	40.1	39.5	38.9	1.2
3.0	39.2	38.9	37.8	1.4
3.2	38.5	38.5	38.1	0.4
3.4	38.0	37.8	37.4	0.6
3.6	37.8	37.2	37.2	0.6
4.0	37.2	36.3	36.0	1.2
5.0	36.8	36.3	36.3	0.5
Average range in reflectance units:				1.0

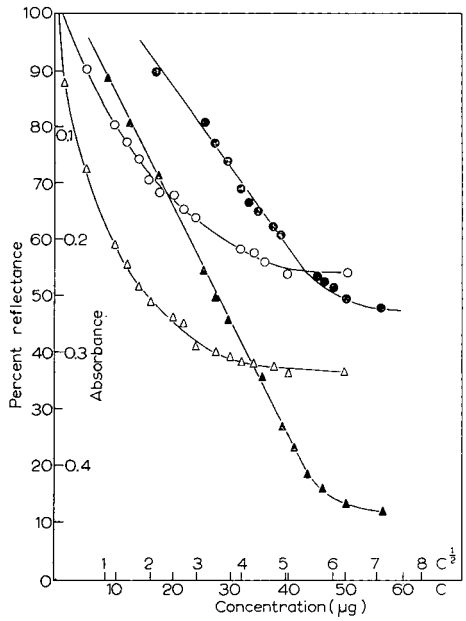

Fig. 2. % reflectance and absorbance at 520 mμ of ninhydrin complex of leucine adsorbed on silica gel as a function of concentration. Modified BRENNER–NIEDERWIESER spray: ○–○ % reflectance *vs.* C; ●–● absorbance *vs.* $C^{1/2}$, BULL *et al.* spray, △–△ % reflectance *vs.* C; ▲–▲ absorbance *vs.* $C^{1/2}$.

n-butanol used in conjunction with paper chromatograms. The difference can be ascribed to the three- or four-fold dilution of the ninhydrin complex with silica gel adsorbent that occurs during the preparation of the sample for analysis.

The procedure outlined above for the development and measurement of the color due to the ninhydrin complex was suggested by the results of a study of the effects of temperature and time of development, and of post-development storage time, upon color intensity. Chromatoplates, spotted with identical amounts of leucine (30 μg) and sprayed with the BULL reagent, were developed at different temperatures (27°, 60° and 90°) for varying periods of time (10, 15 and 20 min) and then read at 1, 4 and 24 h intervals following their storage in the dark at room temperature. The results of the study are summarized in Table II as mean values of % reflectance readings

TABLE II

% REFLECTANCE AT 530 mμ OF NINHYDRIN COMPLEX OF LEUCINE ADSORBED ON SILICA GEL AS A FUNCTION OF TEMPERATURE AND TIME OF COLOR DEVELOPMENT, AND OF POST-DEVELOPMENT STORAGE TIME (AT 27°)

	Development temperature						
	90° ± 2°			*60° ± 1°*			*27° (room temperature)*
Development time (min)	*10*	*15*	*20*	*10*	*15*	*20*	
Storage time (h)	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*	*(% R)*
1	47.6	46.6	46.9	44.9	43.9	45.7	
4	54.2	52.7	53.2	49.4	48.2	51.3	41.3
24	81.6	79.0	80.2	72.5	69.1	72.3	53.8

obtained from three replicates for each set of experimental conditions. Although plates read an hour after the ninhydrin color had been developed yielded the lowest reflectance readings regardless of the other variables, a longer interval was utilized since it lends itself more to serial analyses. Because it afforded greater precision, development at 60° for 15 min was preferred to development at room temperature despite the fact that the latter method produced a more intense color. Indeed, of the readings taken at the 4-h interval, only those obtained at room temperature exhibited a range in excess of 1.5 reflectance units. There the ranges found corresponded to 3.0 reflectance units. These results agree substantially with those obtained by MCFARREN *et al.*[6] with paper chromatograms. Such was not the case with an attempt to enhance color intensity by treating the chromatoplates with steam for 5 min following development at 60° for 10 min. Whereas BULL *et al.*[4] employed this technique successfully with paper chromatograms, similar treatment of thin-layer plates had the reverse effect. In one case the % reflectance was found to increase from 43.9 to 55.2.

Direct examination of chromatoplates

The quantitative potential of the direct examination technique is indicated by Fig. 3, which shows the reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Since it has already been shown[11] that the precision provided by this procedure is of the same order as that afforded by direct

transmission methods applied to paper chromatography[4–7], no further work was done on this aspect of the research.

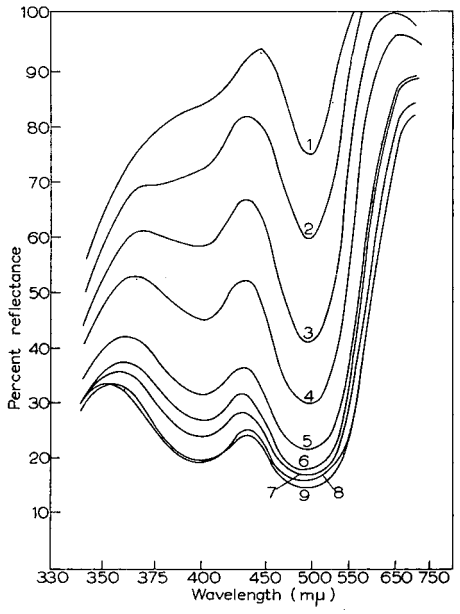

Fig. 3. Reflectance spectra of various concentrations of leucine adsorbed on silica gel and sprayed with ninhydrin reagent. Concentrations in mg: (1) 2.00; (2) 5.00; (3) 10.0; (4) 15.0; (5) 20.0; (6) 25.0; (7) 30.0; (8) 35.0; (9) 40.0.

Examination of spots removed from chromatoplates

A considerable increase in precision was achieved by analyzing spots removed from chromatoplates. The reproducibility one can anticipate for readings obtained for different spots of the same concentration of various amino acids adsorbed on silica gel and sprayed with ninhydrin reagent is indicated in Table III. In the case of each acid three 30 μg replicates were chromatographed over a distance of 15 cm in one dimension and prepared for analysis according to the procedure outlined in the experimental section. For this study the size of the analytical samples was increased to 60 mg and the reflectance at 515 mμ was determined 12 h after the ninhydrin color had been developed. An average standard deviation of 1.42 % was obtained for the fourteen sets; the largest standard deviation found for any one set was the 2.32 % value observed with alanine. This degree of precision was attainable, however, only with sets subjected to the procedure at one and the same time. Particularly large deviations resulted when plates were sprayed at times differing by an hour or more. In fact the precision of the determinations was limited by elements associated with the generation of the ninhydrin color and not by deviations arising from the packing of the sample cell, which were of secondary importance. This was in contrast to results obtained for a stable system whose analyses involved no chromogenesis[11]. On the other hand, when standards were processed along with samples under investigation, it was possible to achieve this order of precision with spots containing as little as 2 μg

of an amino acid. The best possible results were obtained when sets containing 3 to 4 replicates were chromatographed in the same chamber.

The possibility of using a single standard curve in the determination of several amino acids was suggested by a consideration of the color densities and standard deviations recorded in Table III. Accordingly the relationship between reflectance and

TABLE III

REPRODUCIBILITY OF REFLECTANCE READINGS OBTAINED AT 515 mμ FOR DIFFERENT SPOTS OF THE SAME CONCENTRATION OF AMINO ACIDS ADSORBED ON SILICA GEL AND SPRAYED WITH NINHYDRIN REAGENT

Compound	*Range (% R)*	*Mean (% R)*	*Std. dev. (% R)*
Alanine	45.4–47.0	45.8	2.32
Arginine·HCl	60.4–62.2	61.2	1.37
Glutamic acid	60.9–63.0	62.3	1.94
Glycine	68.5–71.0	69.6	1.83
Isoleucine	47.4–49.0	48.0	1.80
Leucine	48.8–49.6	49.3	0.88
Lysine·HCl	59.8–60.2	60.0	0.33
Methionine	54.2–55.7	55.0	1.38
Norleucine	48.4–49.2	49.0	0.34
Norvaline	46.8–47.9	47.6	1.47
Phenylalanine	69.5–71.2	70.5	1.26
Serine	53.2–54.9	54.3	1.76
Threonine	60.0–61.8	61.3	1.85
Valine	48.4–49.8	49.2	1.47
		Av. std. dev.:	1.42

the concentration of the ninhydrin complexes of various amino acids adsorbed on silica gel was determined by means of the procedure employed for the precision study. Reflectance readings were taken 6 h after the ninhydrin color had been developed for dilution series covering the 0.5 to 50 μg range. Some of the standard curves obtained during the course of this study are shown in Fig. 4. It was found that one standard curve could indeed be used for the determination of norvaline, valine, norleucine, isoleucine and leucine without affecting the precision significantly. As may be seen in Fig. 4, the largest spread found between the curves for norvaline and leucine, which bracketed those for the other group members, only amounted to two reflectance units. Although standard curves for the other nine acids in general resembled those shown in Fig. 4, no other grouping analogous to the norvaline–leucine set was found. This was not surprising since the acids differed not only in their color development characteristics but also in their sensitivities. Sensitivities (in μg) found for the acids were as follows: alanine, glutamic acid, isoleucine, leucine, norleucine, norvaline, serine and valine —0.5; glycine —0.8; methionine —2.5; threonine —5; lysine —8; arginine —10; and phenylalanine —12. The interchange that occurred in the relative positions of the curves for glycine and threonine, which are included in Fig. 4, with increased concentration is indicative of some of the complexities encountered. A more useful form of these data is obtained when absorbance is plotted *versus* the square root of the concentration, as is done in Fig. 5, since this results in a linear relationship in the concentration range of analytical interest.

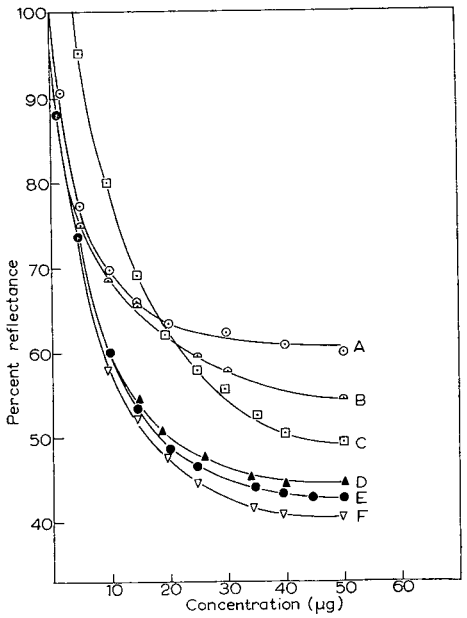

Fig. 4. % reflectance at 515 mμ of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of concentration. (A) Glycine. (B) serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.

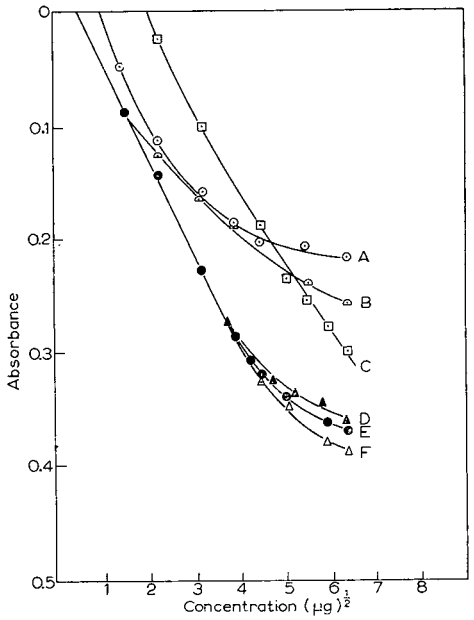

Fig. 5. Absorbance at 515 mμ of ninhydrin complexes of various amino acids adsorbed on silica gel as a function of the square root of concentration. (A) Glycine. (B) Serine. (C) Threonine. (D) Leucine. (E) Norvaline. (F) Alanine.

CONCLUSIONS

When specified precautions are observed in the generation of the ninhydrin color, amino acids resolved on chromatoplates can be determined by reflectance spectrophotometry. Direct examination of the plates yields results having the same range of precision, 5 to 12 %, as is reported for direct photometric methods developed for paper chromatograms. A degree of precision approaching that afforded by transmittance is achieved if the reflectance measurements are carried out on spots removed from the chromatoplates. Unlike analyses dealing with stable systems and involving no chromogenesis, the precision attained is limited by elements associated with the color development process and not by deviations arising from the preparation of the analytical sample. The dilution of the ninhydrin complex with silica gel adsorbent that occurs during the sample preparation reduces the sensitivity of the reflectance method to approximately half that reported for transmission measurements carried out with paper chromatograms, but the advantages afforded by the thin-plate technique more than compensate for this reduced sensitivity. These include more rapid resolution, increased precision and no requirement that the substrate be treated to compensate for textural irregularities. Over the concentration range best suited for analysis, 2 to 30 μg, a linear relationship is observed with most of the acids studied when absorbance is plotted against the square root of the concentration. Although the present study was restricted to one-dimensional thin-film chromatography, there appears to be no reason why spectral reflectance cannot be applied to the two-dimensional process with equal success.

SUMMARY

A procedure was devised whereby amino acids resolved on chromatoplates can be determined by spectral reflectance. Direct examination of plates yielded a degree of precision comparable to that afforded by direct transmission methods applied to paper chromatography. Precision approaching that afforded by transmittance is attained if the reflectance measurements are carried out on spots removed from the plates and specified precautions are observed in the generation of the ninhydrin color.

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QUANTITATIVE DETERMINATION OF THE EQUILIBRIA OF COPPER, COBALT, NICKEL AND CADMIUM IONS IN CHLORIDE SOLUTIONS BY MEANS OF ION-EXCHANGE PAPERS

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In a previous investigation[1] it was shown that the equilibria established in a solution containing a cation and a ligand may be determined quantitatively by means of chromatography with ion-exchange papers.

The rapidity and simplicity of operation with ion-exchange papers are, indeed, attractive factors which recommend their use for quantitative measurements, but since the results of the calculation depend upon the accuracy with which the R_F values are measured, caution should be applied before a general application of this method.

The purpose of this study was the application of the procedure, developed previously[1], to solutions of copper, cobalt, nickel and cadmium ions in the presence of chloride in order to evaluate the possibilities and the limitations of this method.

EXPERIMENTAL

Ion-exchange paper SA-2 (Rohm and Haas) containing about 45 % Amberlite IR-120 (a sulphonic resin) was used. The sodium ion of the resin was replaced by hydrogen ion by equilibrating the ion-exchange papers with 3 *M* hydrochloric acid for 30 min; the papers were washed several times by allowing them to equilibrate with distilled water for 30 min after each wash.

Copper, cobalt, nickel and cadmium perchlorate solutions were prepared by adding 3 *M* perchloric acid to an excess of the solid carbonates; the solutions were filtered and then boiled to eliminate carbon dioxide. The copper content was determined iodometrically and the other cations were determined by means of titrations with ethylenediaminetetraacetic acid (EDTA). The working solutions were 0.1 *M* Cu^{2+}, 0.01 *M* Co^{2+}, 0.4 *M* Ni^{2+} and 0.4 *M* Cd^{2+} at these concentrations well defined, round spots were obtained when samples of about 10 μl were eluted with the solutions described below.

The experimental procedure was the same as outlined in the previous paper[1]. The chromatograms were run at 25° by the ascending technique.

The various cations were detected by spraying the ion-exchange papers with a solution of hexacyanoferrate, $K_4Fe(CN)_6$, for copper, with H_2S for cadmium, dimethylglyoxime for nickel and α-nitroso-β-naphthol for cobalt.

All the systems were investigated at constant ionic strength (3000 mM, except for the Cd^{2+}–Cl^- system which was 1000 mM) and at a constant hydrogen ion concentration. The ligand concentration was made to vary by adding hydrochloric acid, $H^+(Cl^- + ClO_4^-) = 3000$ mM, except for the Cd^{2+} system where $H^+(Cl^- + ClO_4^-) = 1000$ mM. A different ligand range was chosen for the latter system as the cadmium–chloride complexes are more stable than the others; with a high ligand concentration the spots travel with the front.

RESULTS

The experimental results obtained for the investigated cations are collected in Table I. $[A^-]$ is the concentration by analysis of the ligand of the eluting solution, which is assumed to be equal to the equilibrium concentration; A_L/A_S is the ratio of the amount of solvent to the amount of resin in a cross section of the paper. The partition coefficient (α) is related to the chromatographic R_F value by the equation:

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

In Fig. 1 the partition coefficient α is plotted against the ligand concentration for the systems investigated.

By assuming complex formation between an ion and a chloride ligand ($M^{n+} + j\ Cl^- \rightleftharpoons MCl^{(n-j)+}$) and considering the partition on the resin of the various chemica

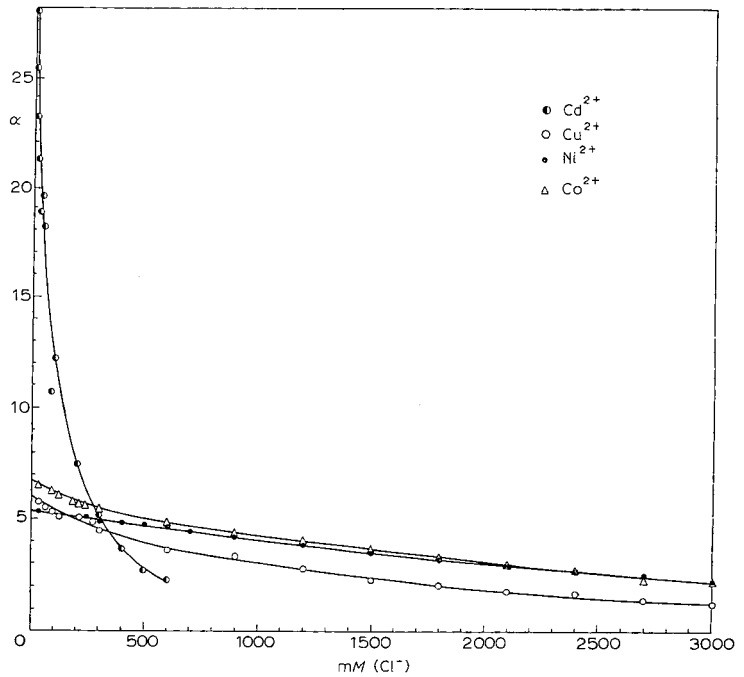

Fig. 1. Values of α plotted against chloride concentration for various cations.

TABLE I

R_F AND A_L/A_S VALUES AT VARIOUS LIGAND CONCENTRATIONS FOR THE INVESTIGATED CATIONS

$[A^-]mM$	A_L/A_S	Cu^{2+}		Co^{2+}		Ni^{2+}		$[A^-]mM$	A_L/A_S	Cd^{2+}	
		R_F	α	R_F	α	R_F	α			R_F	α
0								0			30.8
30	3.52	0.38	5.74	0.35	6.54	0.40	5.28	5	3.367	0.11	27.24
60	3.52	0.39	5.50					10	3.365	0.12	24.68
90	3.51	0.40	5.27	0.36	6.25			15	3.363	0.13	22.51
120	3.51			0.37	5.98			20	3.361	0.14	20.65
150	3.50	0.41	5.04					30	3.357	0.15	18.32
180	3.50							40	3.353	0.16	17.60
210	3.50			0.38	5.71			50	3.349	0.17	16.35
240	3.50					0.41	5.03	100	3.328	0.22	11.80
270	3.49					0.41	5.02	200	3.286	0.32	7.15
300	3.49	0.44	4.45	0.39	5.46	0.42	4.82	300	3.244	0.40	4.97
600	3.45	0.49	3.60	0.42	4.96	0.43	4.57	400	3.203	0.48	3.47
900	3.43	0.51	3.29	0.44	4.37	0.45	4.19	500	3.162	0.56	2.48
1200	3.40	0.55	2.78	0.46	3.99	0.47	3.83	600	3.120	0.60	2.08
1500	3.36	0.60	2.24	0.48	3.64	0.49	3.50				
1800	3.33	0.62	2.04	0.50	3.33	0.51	3.20				
2100	3.30	0.65	1.78	0.53	2.95	0.53	2.93				
2400	3.27	0.66	1.68	0.54	2.79	0.55	2.68				
2700	3.24	0.70	1.39	0.58	2.35	0.57	2.44				
3000	3.21	0.73	1.19	0.59	2.23	0.59	2.23				

TABLE II

FORMATION CONSTANTS FOR CHLORIDE COMPLEXES WITH VARIOUS CATIONS

Ion-exchange papers ($t = 25°$)	*Other investigations*	
	Cu^{2+}	
$\beta_1 = 0.94 \pm 0.05\ M^{-1}$ $\beta_2 = 0.16 \pm 0.07\ M^{-2}$ $\beta_3 = 0.05 \pm 0.08\ M^{-3}$	$\beta_1 \cong 1\ M^{-1}$ $\beta_2 = 0.1 - 0.4\ M^{-2}$ $\beta_3 = 0.02 - 0.06\ M^{-3}$ $\beta_4 = 0.003 - 0.01\ M^{-4}$	Spectrophotometry[2] ($t = 22°$)
	Ni^{2+}	
$\beta_1 = 0.23 \pm 0.03\ M^{-1}$ $\beta_2 = 0.06 \pm 0.03\ M^{-2}$ $\beta_3 = 0.001 \pm 0.006\ M^{-3}$	No evidence of anionic complex even in 12 M HCl	Anion exchange[3]
	Co^{2+}	
$\beta_1 = 0.61 \pm 0.05\ M^{-1}$ $\beta_2 = 0.11 \pm 0.04\ M^{-2}$	$K_1 = 4 \cdot 10^{-3}\ M^{-1}$ $K_2K_3 = 3 \cdot 10^{-4} M^{-2}$	Spectrophotometry[4] ($t = 18°$)
	Cd^{2+}	
$\beta_1 = 18 \pm 3\ M^{-1}$ $\beta_2 = 90 \pm 55\ M^{-2}$	$\beta_1 = 38.9\ M^{-1}$ $\beta_2 = 170\ M^{-2}$ $\beta_3 = 257\ M^{-3}$	Cd–Hg/3 M $NaClO_4$[5] ($t = 25°$)

species, the data have been treated, according to the procedure of the previous paper[1], to determine the unknown values of the equation:

$$\alpha = l_0 \frac{1 + l\,[Cl^-]}{1 + \sum_{j=1}^{N} \beta_j\,[Cl^-]^j}$$

Table II shows the values obtained in this investigation and compares them with the values available from the literature.

DISCUSSION

The data reported in Table II show that equilibria which establish in solution can be determined by means of ion-exchange papers by applying the FRÖNAEUS method. Satisfactory results are obtained for the determination of equilibrium constants for the first or second step of weak complexes. When a system has a higher degree of complexity and the complexes are very stable, the inaccuracy connected with the R_F determination affects the calculations much more and renders the graphic extrapolations unreliable. For example, an error corresponding to ± 0.01 in the determination of R_F, affects the calculation of α by about ± 0.2.

In general therefore, the results are less accurate than ones obtained by means of ion-exchange resins and other standard methods. The procedure, however, offers the advantage of a greater rapidity so that it can be satisfactorily applied, especially on a qualitative basis, to obtain information on the strength of various complexes. The behaviour of α, which is obtained by simple calculations and the trend of the graph α *versus* ligand concentration, is particularly indicative. The degree of steepness of the curve is an indication of the stability of the complexes. When complexes are also formed at a very low ligand concentration, as in the case of the Cd^{2+}–Cl system, the curve is quite steep.

ACKNOWLEDGEMENT

One of us (M.G.) is indebted to the Ministero della Pubblica Istruzione for a grant given to contribute to this investigation.

SUMMARY

Equilibria which establish in a solution containing a cation (Cu^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+}) and chloride have been studied by means of chromatography on ion-exchange papers; complex formation constants have been determined and the possibilities of this method are discussed and evaluated.

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CHROMATOGRAPHY ON ION EXCHANGE PAPERS

XIII. THE ADSORPTION OF INORGANIC IONS FROM SULPHATE SOLUTIONS

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INTRODUCTION

Comparison of the adsorption of ions on various cellulose and resin anion exchangers from HCl and HBr solutions has led us to conclude that not only ion exchange but also adsorption plays a considerable role in the retention of ions on anion exchangers[1].

We now extended these studies to include nitrate and sulphate solutions and encountered several technical difficulties. Nitric acid seemed to attack the cellulose ion exchangers and in neutral lithium nitrate solution hydrolysis occurred with most of the complex ions of interest. In sulphuric acid solutions two fronts were formed, presumably due to the preferential adsorption of SO_4^{2-} over HSO_4^- ions and some ions move with this front making comparisons with for example HCl solutions difficult. However, in sulphate solutions a salting-out effect was observed which adsorbed ions otherwise not adsorbed on resins or paper. It was found that by choosing a suitable salting-out agent, anions such as Cl^-–Br^-–I^- could be separated on cation exchange resins.

EXPERIMENTAL

(i) The adsorption of ions from sulphuric acid solutions on anion exchangers

Fig. 1 shows the R_F values of some ions on Whatman No. 1 paper, amino-ethyl cellulose paper (AE 30), DEAE cellulose paper (DE 20) and strong anion exchange resin paper (SB-2). Fe(III) forms a very narrow zone on the "second front" up to which SO_4^{2-} appears to saturate the paper.

The behaviour of ReO_4^- requires some comment. In Fig. 2 we have plotted the R_F values of ReO_4^- in HCl, HBr and H_2SO_4. If this adsorption were governed by ion exchange, sulphuric acid, being a divalent acid, should desorb perrhenate more than HCl or HBr. We shall show below that this strong adsorption is due to the rather strong salting-out effect of polyvalent acids and salts.

(ii) The adsorption of anions from sulphate solutions on cellulose and cation exchange resin papers

The rather considerable salting-out effect of sulphates was already employed by SARGENT AND RIEMAN[2] in the salting-out chromatography of neutral organic substances on both cation and anion exchange resins.

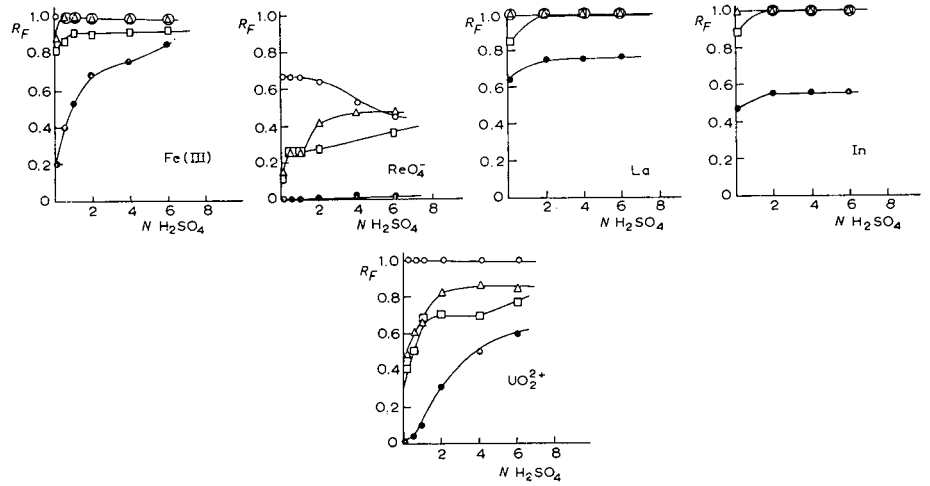

Fig. 1. R_F values of some metal ions plotted against the concentration of H_2SO_4 for various ion exchange papers. ●—● SB-2 (strong base resin)paper; △—△ DEAE (Whatman DE-20) paper; □—□ Aminoethyl (Whatman AE-30) paper; ⊙—⊙ Whatman No. 1 cellulose paper.

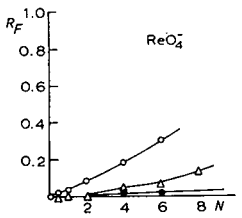

Fig. 2. R_F values of perrhenate plotted against the concentration of the eluant acid on strong base resin (SB-2) paper. ●—● H_2SO_4; ⊙—⊙ HBr; △—△ HCl.

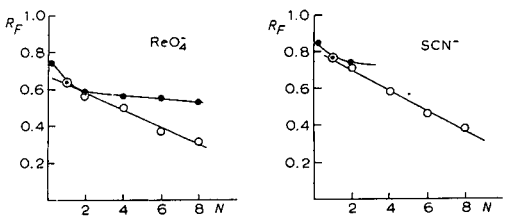

Fig. 3. R_F values of perrhenate (left) and thiocyanate (right) plotted against the normality of H_2SO_4 and $(NH_4)_2SO_4$ on cellulose paper (Whatman No. 3MM). ●—● H_2SO_4; ⊙—⊙ $(NH_4)_2SO_4$.

In order to obtain some indications of salting-out in absence of anion exchange, we examined a range of inorganic anions on pure cellulose paper (the thicker Whatman 3MM paper), on sulphonic resin paper (SA-2) and on carboxylic resin paper (WA-2) with both sulphuric acid and ammonium sulphate solutions.

Numerous anions such as CrO_4^{2-}, MoO_4^{2-}, SeO_4^{2-}, TeO_3^{2-} give comets and double spots and hence we report in Table I only those ions which led to the formation of sharp spots.

As may be expected from the incomplete ionisation of H_2SO_4, its salting-out effect is lower than that of ammonium sulphate (see Fig. 3).

While oxy-anions are adsorbed to a relatively small extent, remarkably good adsorption and separations can be obtained with halides on strong cation exchangers (see for example Fig. 4).

With this kind of adsorption chromatography on ion exchange papers we seem to have arrived at the extreme of adsorption mechanisms on ion exchange resins which do

TABLE I

R_F VALUES OF INORGANIC ANIONS ON CELLULOSE PAPER AND CATION EXCHANGE RESIN PAPERS WITH AMMONIUM SULPHATE AND SULPHURIC SOLUTIONS AS SOLVENTS

W 3MM = Whatman 3MM cellulose paper; SA-2 = sulphonic resin (SA-2) paper; WA-2 = carboxylic resin (WA-2) paper.

Anion	*Paper*	*$(NH_4)_2SO_4$ solution as solvent*				
		1 N	*2 N*	*4 N*	*6 N*	*8 N*
I^-	W 3MM	0.76	0.74	0.67	0.54	0.46
	SA-2	0.87–0.62	0.85–0.59	0.71–0.46	0.59–0.29	0.47–0.27
	WA-2	0.84	0.79	0.71	0.63	0.51
Br^-	W 3MM	0.84	0.81	0.73	0.70	0.64
	SA-2	0.86	0.83	0.75	0.70	0.62
	WA-2	0.89	0.85	0.77	0.74	0.70
Cl^-	W 3MM	0.87	0.80	0.79	0.77	0.74
	SA-2	0.93	0.90	0.87	0.77	0.75
	WA-2	0.92	0.88	0.81	0.80	0.76
IO_3^-	W 3MM	0.86	0.85	0.83	0.82	0.81
	SA-2	0.91	0.86	0.88	0.86	0.85
	WA-2	0.83	0.81	0.79	0.81	0.81
BrO_3^-	W 3MM	0.89	0.85	0.82	0.82	0.81
	SA-2	0.91	0.93	0.80	0.81	0.75
	WA-2	0.85	0.84	0.81	0.82	0.79
ClO_3^-	W 3MM	0.83	0.78	0.77	0.81	0.80
	SA-2	0.83	0.79	0.74	0.75	0.65
	WA-2	0.87	0.85	0.82	0.82	0.81
NO_2^-	W 3MM	0.86	0.87	0.80	0.79	0.74
	SA-2	0.70–0.54	0.59	0.55	0.71–0.46	0.70–0.51
	WA-2	0.02	0.03	0.04	0.07	
NO_3^-	W 3MM	0.85	0.83	0.81	0.74	0.72
	SA-2	0.88	0.83	0.81	0.75	0.64
	WA-2	0.85	0.86	0.82	0.72	0.70
ReO_4^-	W 3MM	0.62	0.57	0.50	0.37	0.32
	SA-2	0–0.10	0–0.09	0–0.08	0–0.06	0–0.05
	WA-2	0.57	0.53	0.43	0.32	0.25
SCN^-	W 3MM	0.76	0.71	0.59	0.47	0.39
	SA-2	0.12–0.40	0.09–0.31	0.06–0.26	0.04–0.22	0–0.18
	WA-2	0.76	0.72	0.63	0.51	0.43

Anion	*Paper*	*H_2SO_4 solution as solvent*					
		0.1 N	*1 N*	*2 N*	*4 N*	*6 N*	*8 N*
ReO_4^-	W 3MM	0.74	0.63	0.59	0.56	0.55	0.53
SCN^-	W 3MM	0.84	0.77	0.75	—	—	—

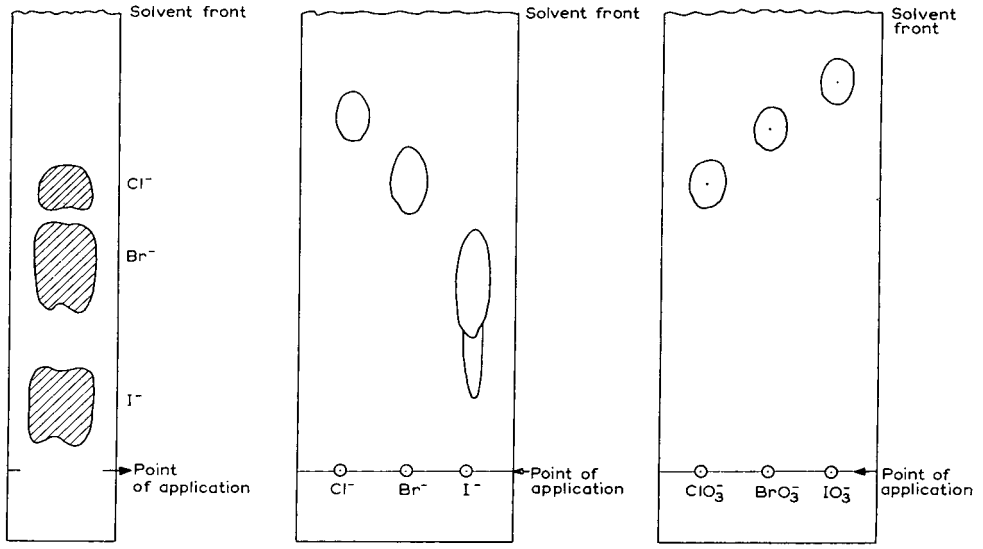

Fig. 4. Some chromatograms of inorganic anions on sulphonic resin (SA-2) paper with 8 *N* $(NH_4)_2SO_4$ as solvent. Left: separation of Cl^-–Br^-–I^-. Centre: movement of Cl^-, Br^- and I^- when run side by side. Right: movement of ClO_3^-, BrO_3^- and IO_3^- when run side by side on one sheet.

not involve the functional groups of the resin. It is not likely that there will be any analytical advantages in a halide separation on cation exchangers. We believe, however, that it is an excellent illustration of the possible factors involved in adsorption on resins and cellulose exchangers.

SUMMARY

The adsorption of inorganic anions on cation exchange resins from sulphate solutions was studied and could be shown to be governed by a salting-out mechanism.

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CHROMATOGRAPHIC STUDIES ON SULPHUR COMPOUNDS

PART IV. THE DECOMPOSITION OF ACIDIFIED THIOSULPHATE AND POLYTHIONATE SOLUTIONS

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The products formed when an alkaline thiosulphate solution is acidified with mineral acid are very dependent on the conditions, *e.g.* concentration of thiosulphate and acid, rate of mixing and temperature.

With dilute acid, the usual products are sulphur, and sulphur dioxide:

$$H_2S_2O_3 \longrightarrow S + SO_2 + H_2O \qquad (1)$$

although at higher concentrations of acid, hydrogen sulphide has been detected and the solution is found to contain sulphuric acid and polythionic acids[1–5], while at medium acidities, sulphur oils are formed. There is often a time lag between the mixing of the solutions and the precipitation of sulphur which was found by LANDOLT[6] to be directly proportional to the dilution of thiosulphate.

There are numerous postulated mechanisms with the primary intermediate considered to be thiosulphurous acid and sulphoxylic acid[7,8]. While many mechanisms have been put forward to account for the observed facts, they all include unlikely intermediates or neglect the condition of acidity in the solution[9].

SCHMIDT[10] has shown that in non-aqueous solution anhydrous thiosulphuric acid decomposes into sulphur trioxide and hydrogen sulphide:

$$H_2S_2O_3 \longrightarrow H_2S + SO_3 \qquad (2)$$

He assumes this is the primary step in the aqueous solution also:

$$H^+ + S_2O_3^{2-} \longrightarrow HSO_3^- + S \qquad (3)$$

while eqn. (3) explains the formation of sulphite and sulphur.

The polythionates are prepared by the reaction of the products of reaction (2):

$$H_2S + 2\ SO_3 \longrightarrow H_2S_3O_6 \qquad (4)$$

Now in acid solution, trithionate would react with excess thiosulphate to form higher thionates, *viz.*:

$$S_3O_6^{2-} + H^+ + S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + HSO_3^- \qquad (5)$$

$$S_4O_6^{2-} + H^+ + S_2O_3^{2-} \longrightarrow S_5O_6^{2-} + HSO_3^- \qquad (6)$$

$$S_5O_6^{2-} + H^+ + S_2O_3^{2-} \longrightarrow S_6O_6^{2-} + HSO_3^- \qquad (7)$$

Thus in essence, the production of trithionate as the primary thionate, followed by build-up of higher thionates.

GOEHRING[7] suggests the formation of thiosulphurous acid as an unstable intermediate:

$$H_2S_2O_3 \xrightarrow{H_2O} H_2S + H_2SO_4 \quad (8)$$

$$H_2S_2O_3 \longrightarrow H_2SO_3 + S \quad (9)$$

which is then followed by:

$$H_2S + H_2SO_3 \longrightarrow H_2S_2O_2 + H_2O \quad (10)$$

This intermediate immediately reacts with excess thiosulphate to yield $S_6O_6^{2-}$ which is then degraded by sulphite to produce the lower polythionates:

$$H_2S_2O_2 + 2\ H_2S_2O_3 \longrightarrow H_2S_6O_6 + 2\ H_2O \quad (11)$$

also:

$$H_2S_2O_2 + 2\ H_2SO_3 \longrightarrow H_2S_4O_6 + 2\ H_2O \quad (12)$$

then:

$$H_2S_6O_6 + HSO_3^- \longrightarrow H_2S_5O_6 + S_2O_3^{2-} + H^+ \quad (7a)$$

The serious objection to reactions such as (7a), is that it would be expected to move in reverse direction because of the large excess of thiosulphate and acid present.

Thus in this mechanism hexathionate is first formed, which is in contradiction to that of SCHMIDT[10], where trithionate is formed. Thus it can be seen that positive identification of the primary thionate would help to decide which mechanism is most likely.

DAVIS[9] contributes yet another explanation:

$$S_2O_3^{2-} + H^+ \rightleftarrows HS_2O_3^- \quad (13)$$

followed by:

$$HS_2O_3^- + S_2O_3^{2-} \longrightarrow HS_3O_3^- + SO_3^{2-} \quad (14)$$

thus the sulphane-monosulphonic acids[10] are built up to:

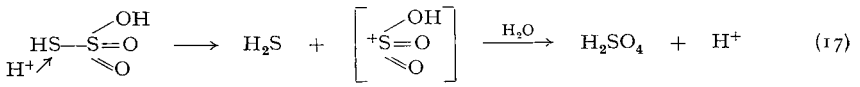
$$HS_9O_3^- \longrightarrow S_8 + HSO_3^- \quad (15)$$

Polythionates are formed by:

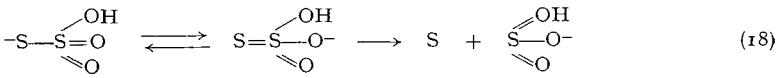
$$\begin{array}{l} \mathrm{SH} \\ \mathrm{(S)}_x \\ \mathrm{SO_3^-} \end{array} \rightleftarrows \begin{array}{l} \mathrm{S^-} \\ \mathrm{(S)}_x \\ \mathrm{SO_3H} \end{array} + \begin{array}{l} \\ \mathrm{(S)}_y\mathrm{—SH} \\ \mathrm{SO_3^-} \end{array} \longrightarrow \begin{array}{l} \\ \mathrm{(S)}_x\mathrm{—(S)}_y\mathrm{—SO_3^-} \\ \mathrm{SO_3H} \end{array} + \mathrm{SH^-} + \mathrm{S} \quad (16)$$

On this theory, polythionates from $H_2S_3O_6$ to $H_2S_{17}O_6$ should be formed, while the sulphur is depositing. DAVIS[9] based his postulates and kinetics on the results of LA MER and co-workers[11–14] who studied the system spectrophotometrically in dilute aqueous solution at constant ionic strength. Chemical differentiation of the species formed was not made.

POLLARD AND JONES[15] suggest a further possibility, and consider that thiosulphate can degrade in two ways depending on the acidity of the solution:

(a)  (17)

which occurs in strongly acid solution only, and:

(b)  (18)

This breakdown yields sulphur and bisulphite which occur in weakly acid solutions.

Now considering three conditions of acidity.

(*i*) *Weakly acid solution.* The reaction is entirely represented by (18).

(*ii*) *Strong acid solution.* Reaction (17) is the major breakdown but (18) also contributes $H_2S_2O_3$.

This is followed by reaction (10) and then by reaction of thiosulphate and sulphite with the formed thiosulphurous acid, polythionates up to 6 sulphur atoms are produced.

(*iii*) *Medium acidity.* In a strict range of acidities, sulphur hydrides (sulphanes) of high molecular weight are formed as oils[16,17]. Their formation may be explained by assuming reaction (19) to occur to a slight extent, while (18) is the major reaction.

The formation of oils occurs by:

$$H^+ + HS^- + x\,S \longrightarrow H_2S_{x+1} \qquad (19)$$

The absence of these oils at low acidities is explained by non-occurrence of reaction (17), whilst at high acidities it is postulated they occur only transiently, but react with thiosulphuric acid to produce sulphane-monosulphonic acids which have increased stability in concentrated acid solution:

$$H_2S_x + H_2S_2O_3 \rightleftarrows H_2S_{x+1}O_3 + H_2S \qquad (20)$$

Polythionate formation is accounted for by the reaction, which shows

$$H_2S_2O_2 + 2\,H_2S_{x+1}O_3 \longrightarrow H_2S_{2x+4}\,O_6 + 2\,H_2O$$

the formation of higher thionates than $S_6O_6^{2-}$ since x cannot be less than 1.

Breakdown of the higher polythionates into free radicals follows, with recombination to smaller units:

$$HO_3S\text{–}S_y\text{–}SO_3H \longrightarrow HO_3S_a + S_bO_3H \qquad (21)$$

$$2\,HO_3S_a \longrightarrow H_2S_{2a}O_6 \qquad (22)$$

$$2\,HO_3S_b \longrightarrow H_2S_{2b}O_6 \qquad (23)$$

Thus a detailed chromatographic study of the acid decomposition of thiosulphate, to determine the order of formation of the thionates and the stability of these species in such acid solutions might well assist to elucidate the mechanisms involved.

According to YANITSKII AND VALANCHUNAS[18,19] when sulphide was present with the ratio of $S_2O_3^{2-}:S^{2-} \geqslant 3.1$, no free hydrogen sulphide remained, and 87–92 % of the original sulphur was in the form of polythionates. The stoichiometric equation is:

$$3\ H_2S_2O_3 + H_2S \longrightarrow H_2S_6O_6 + S + 3\ H_2O \qquad (24)$$

When hypophosporous acid was substituted for H_2S, 95 % of total sulphur was obtained as pure hexathionate. The equations to represent the formation of this acid were given as:

$$H_2S_2O_3 + H_2O \rightleftarrows 2\ H_2SO_2 \qquad (25)$$

$$2\ H_2SO_2 + H_2S \longrightarrow H_2S_2O_2 + S + 2\ H_2O \qquad (26)$$

$$H_2S_2O_2 + 2\ H_2S_2O_3 \longrightarrow H_2S_6O_6 + 2\ H_2O \qquad (11)$$

Fractionation of the benzidine salts showed the presence of octathionate. These workers further report that when the available sulphur was increased by the addition of sulphite, the major product was octathionate with some hexathionate, and when a ratio of thiosulphate:sulphide:sulphite of 6:7:5 was used, 98 % of the thiosulphate sulphur occurred as octathionate.

In this case too, it seems that a qualitative investigation by paper chromatographic techniques would yield interesting confirmation of the presence of octathionate, since the Russian workers analysed their products by fractional precipitation of the benzidine salts.

EXPERIMENTAL

Rear-phase chromatography[20]

Solvent: Tertiary butanol, 15 ml; acetone 65 ml; water 25 ml; potassium acetate 0.5 g; pH value of solution 9.6 (glass electrode).

When a paper chromatogram is eluted with this solvent, two phases are formed on the paper by frontal analysis. The leading phase contains free acetic acid, whilst the rear phase contains a high concentration of potassium ions. If the thionates are adsorbed on to the chromatogram whilst dry, and then eluted, the leading phase reaches the thionates and hydrolysis occurs by means of the unadsorbed hydrogen ions in the solvent, giving free polythionic acids. This process stops when all the free hydrogen ions in the area covered by the original spot are used up, and thus the thionates are present both as potassium and free acid salts. The acid salts travel faster than the salts, separation occurs, and on developing the chromatograms, two spots are obtained from a single thionate.

The "rear-phase" technique essentially consists of ensuring that the thionates remain as single spots by eluting them as potassium salts only. This is achieved by allowing the phase boundary to pass the starting line before the solution of the thionates is applied to the paper.

Even if the thionate is applied in concentrated hydrochloric acid, only one spot is obtained, the thionate being converted to the potassium salt by the buffering action of the high concentration of the potassium ions in the rear phase. The only effect noticed with such acid is slight distortion of the trithionate and tetrathionate spots due to displacement by the chloride ion interposed between them.

Apparatus

The chromatograms were run in a large glass tank (46 cm × 30 cm × 41 cm) having a ground glass edge, and plate glass lids ground against each other, rendering the tank vapour-tight. The tank contained four solvent troughs, 2.5 cm wide, 25 cm long, supported 1 cm from the top of the tank by a glass frame. At either end of each trough was a supporting glass bar, and at either side a glass bar to prevent the trough sliding along. A further 4 cm away was another horizontal bar which acted as a support for the filter paper, and provided the horizontal portion of the chromatogram for *in situ* adsorption of the solutes. The lids were arranged so that a hole, 0.6 cm diameter, was directly over the centre of each trough, and a long slit over the starting line of the chromatogram (situated approximately half-way between the trough and filter paper supporting bar). The slits, covered by a thin plate glass strip during the equilibration and elution, were only uncovered to admit the samples of solute. The circular holes were likewise plugged with rubber bungs except during the admission of the solvent.

Procedure

Whatman No. 1 paper was used for all "rear-phase" chromatography. The strips were cut out so that the solvent flowed parallel to the machine direction of the paper, with the starting line 6.5 cm from one end. Thus when this end of the paper was folded, and placed in the solvent trough, the solvent feed was 2.5 cm from the starting line and located directly beneath a slit in the tank lid. The bottom of the chromatogram was folded, forming a pad, and secured with paper clips. The length of the paper from the top to the pad was 46 cm. In this manner two objects were achieved:

(a) the paper did not trail on the bottom of the tank, and (b) there was less decrease in velocity of the solvent due to it being allowed to run off the end of the paper.

Equilibration was carried out by placing four Petri dishes in the bottom of the tank, each containing about 20 ml of solvent. The chromatogram strips were in place, and the tank sealed during this period.

After a 12 h period, the solvent was poured into the troughs and allowed to elute the paper, this process being known as pre-elution. Normally the solvent contained 1 % w/v Phenol Red to indicate the position of the phase boundary. This was omitted in certain cases where it would interfere with the detecting spray used after eluting the solutes. When the phase boundary was marked in this way, it was used as an indication of the length of pre-elution needed, *i.e.* the samples were admitted when the distance between the phase boundary and the starting line was sufficient to ensure that no double spotting occurred. The samples were taken into thin drawn-out glass tubes by capillary action and these were held in contact with the paper for about 3 sec. Thus the sample was transferred to the paper. The slit was closed again and elution allowed to take place. After about 10 h, the chromatograms were removed and sprayed with developing agent, washed and dried.

The actual length of the equilibration, pre-elution and elution periods depended on the temperature at which these processes were carried out, and it was best to elute chromatograms at lower temperatures, *ca.* 10°.

Detection of thionates

The chromatograms were sprayed with 0.5 *N* silver nitrate solution, and warmed in

front of an electric fire to decompose the unstable silver thionates to silver sulphide and sulphur. Unless the chromatograms were washed free of the excess silver nitrate, they darkened on exposure to the light. The only reliable method was to wash them in distilled water, then in a 10% w/v solution of sodium thiosulphate, and finally in clean distilled water. The chromatograms were then dried. The sensitivity of the method is increased by viewing the dried chromatograms under ultra violet light when $7 \cdot 10^{-6}$ g of thionate over 0.5 cm^2 of developed zone is clearly visible.

Detection of other species

(*i*) *Dithionate.* 1% Benzidine in absolute alcohol + 0.001 *M* potassium permanganate gives a blue spot on a pale brown background.

(*ii*) *Sulphite.* 5% w/v zinc sulphate solution plus 5% sodium nitroprusside solution (freshly prepared) gives a red colour.

(*iii*) *Rongalite* (sodium formaldehyde sulphoxylate). 0.1 *N* silver nitrate solution gives silver sulphide and metallic silver.

(*iv*) *Sulphide.* 0.1 *N* silver nitrate solution gives brown-black silver sulphide.

(*v*) *Sulphate.* 0.1 *M* barium chloride solution plus sodium rhodizonate solution produces a white spot on a pink background.

R_F values

The R_F values given in Table I were calculated taking $S_4O_6^{2-} = 1.0$ as the standard, this was because of the difficulty of calculating true R_F values due to the spots being applied to the chromatograms after the solvent front had passed the starting line.

TABLE I

R_F VALUES OF SULPHUR COMPOUNDS

Anion	R_F	*Anion*	R_F
SO_4^{2-}	0.02	SO_3^{2-}	0.77
S^{2-}	0.04	$S_2O_8^{2-}$	0.89
$S_2O_3^{2-}$	0.11	$S_4O_6^{2-}$	1.00
$S_2O_6^{2-}$	0.38	$S_5O_6^{2-}$	1.25
$S_3O_6^{2-}$	0.67	$S_6O_6^{2-}$	1.43
Rongalite	0.71		

Thus by combining a knowledge of R_F values and sprays, the species may be identified.

RESULTS

A preliminary study of the stability of polythionates and thiosulphate in hydrochloric acid

The aims of this investigation were to determine:

(a) The strength of hydrochloric acid in which the three lower polythionates were stable.

(b) The effects of thiosulphate on these stabilities.

(c) The effect of formaldehyde solution in these stabilities.

(d) The products from the decomposition of thiosulphate.

Many workers[21–25] have suggested that in reaction mixtures, higher polythionates are built up from lower polythionates by the reaction with thiosulphate:

$$H^+ + S_xO_6^{2-} + S_2O_3^{2-} \rightleftarrows S_{x+1}O_6^{2-} + HSO_3^- \qquad (27)$$

If this is so, addition of thiosulphate to the solution should increase the higher polythionates present. Formaldehyde should remove the bisulphite ion from the equilibrium, and also assist the formation of the higher polythionates.

In this series of experiments, the thionate solution was placed in the reaction vessel, followed by hydrochloric acid. To the mixture so formed, thiosulphate and then 30 % formaldehyde solution were added where indicated, and left for 12 h at room temperature.

Stability of trithionate in hydrochloric acid

In experiments Nos. 1–8, 0.110 *M* potassium trithionate solution, and in experiments Nos. 9–13, 0.283 *M* potassium trithionate were used. In each reaction the HCl required was added to 1 ml of thionate solution. Each acidity was duplicated, and 1 ml of water was added to the first sample, whilst 1 ml of 30 % formaldehyde solution was added where indicated. This procedure was used in order that both the solution with the formaldehyde and that in which it was absent, would have the same total acidity. Results are given in Table II.

TABLE II

STABILITY OF POTASSIUM TRITHIONATE IN HCl

Expt. No.	*Molarity of trithionate*	*Molarity of HCl*	*Analyses after 12 h**	
			Formalin absent	*Formalin present*
1	0.055	—	$S_3O_6^{2-}$	$S_3O_6^{2-}$
2	0.037	0.33	$S_3O_6^{2-}$	$S_2O_3^{2-}$
3	0.028	1.5	$S_3O_6^{2-}$	—
4	0.037	2	$S_3O_6^{2-}$ + slight S	—
5	0.028	3	$S_3O_6^{2-}$ + slight S	—
6	0.028	4.5	$S_3O_6^{2-}$ + slight S	—
7	0.0185	6	$S_3O_6^{2-}$ + slight S	—
8	0.0185	7	$S_3O_6^{2-}$ + slight S	—
9	0.047	6	$S_3O_6^{2-}$ + slight S	—
10	0.031	7	$S_3O_6^{2-}$ + slight S	—
11	0.031	8	$S_3O_6^{2-}$ + slight S	—
12	0.031	9	$S_3O_6^{2-} + S_4O_6^{2-}$ + S	—
13	0.013	10	$S_3O_6^{2-} + S_4O_6^{2-}$ + S + t $S_6O_6^{2-}$	—

* t = trace.

Trithionate is quite stable for up to 12 h in the range 0–1.5 *M* hydrochloric acid, above this acidity decomposition occurs to precipitate sulphur, while in strong acid conditions (> 8 *M* HCl) some tetra- and hexathionates are formed.

Formalin catalyses the decomposition of trithionate so quickly that no thionates are present in acidities greater than about 0.3 *M* acid after 12 h.

Stability of tetrathionate in hydrochloric acid

In experiments Nos. 14–21, 0.085 *M* potassium tetrathionate solution was used, while in experiments Nos. 22–26, a solution of 0.292 *M* potassium tetrathionate was employed. The reaction conditions were obtained as described for trithionate, and the results are given in Table III.

TABLE III

STABILITY OF POTASSIUM TETRATHIONATE IN HCl

Expt. No.	*Molarity of tetrathionate*	*Molarity of HCl*	*Analysis after 12 h**	
			Formalin absent	*Formalin present*
14	0.047	Nil	$S_4O_6^{2-}$	$S_4O_6^{2-}$, $S_3O_6^{2-}$, $S_5O_6^{2-}$ + S
15	0.032	0.33	$S_4O_6^{2-}$	$S_4O_6^{2-}$, t $S_3O_6^{2-}$, $S_5O_6^{2-}$ + S
16	0.024	1.5	$S_4O_6^{2-}$	$S_4O_6^{2-}$ + S
17	0.032	2	$S_4O_6^{2-}$	S
18	0.024	3	$S_4O_6^{2-}$	S
19	0.024	4.5	$S_4O_6^{2-}$	S
20	0.016	6	$S_4O_6^{2-}$	S
21	0.016	7	$S_4O_6^{2-}$	S
22	0.049	6	$S_4O_6^{2-}$	S
23	0.033	7	$S_4O_6^{2-}$	S
24	0.033	8	$S_4O_6^{2-}$	S
25	0.036	7.9	$S_4O_6^{2-}$	S
26	0.013	10.0	$S_4O_6^{2-}$ + S	S

* t = trace.

Tetrathionate is stable for up to 12 h even in 8 *M* hydrochloric acid. Only in the strongest acid media, *ca.* 10 *M*, did the degradation of $S_4O_6^{2-}$ occur, to deposit sulphur.

Formaldehyde assists the decomposition of tetrathionate, and at low acidities up to 1.5 *M* HCl, tri- and penta-thionates are formed. At greater acidities, degradation is complete, and only S is observed.

Stability of pentathionate in hydrochloric acid

In experiments Nos. 27–34, 1 ml of 0.106 *M* potassium pentathionate, and in experiments Nos. 35–39, 1 ml of 0.302 *M* potassium pentathionate were employed. Other conditions were analogous to those for trithionate. The results given in Table IV.

Pentathionate is unstable in hydrochloric acid solutions of low acidity, 0–2 *M*, but is stable for up to 12 h in the range 3.0–6.9 *M*. At greater acidities tetrathionate, hexathionate and sulphur appear due to disproportionation of pentathionate.

$$2\ S_5O_6^{2-} \longrightarrow S_4O_6^{2-} + S_6O_6^{2-}$$

The results obtained in the presence of formalin are interesting as evidence for the production of higher thionates than hexathionate was obtained. Thus hepta- and octa-thionates are observed in the range 4.5 to 6.9 *M* hydrochloric acid. Above this range the only product found was sulphur. This phenomenon is worthy of detailed re-examination, especially in a quantitative manner.

The influence of sodium thiosulphate

Trithionate. The experimental details were as before, except for the addition of 1 ml

TABLE IV

STABILITY OF PENTATHIONATE IN HCl

Expt. No.	Molarity of pentathionate	Molarity of HCl	Analyses after 12 h*	
			Formalin absent	Formalin present
27	0.053	—	$S_5O_6^{2-}$, t $S_4O_6^{2-}$	$S_5O_6^{2-}$, t $S_4O_6^{2-}$
28	0.035	0.33	$S_5O_6^{2-}$, t $S_4O_6^{2-}$	$S_5O_6^{2-}$, t $S_4O_6^{2-}$
29	0.0265	1.5	$S_5O_6^{2-}$, t $S_4O_6^{2-}$	$S_5O_6^{2-}$, t $S_4O_6^{2-}$
30	0.035	2.0	$S_5O_6^{2-}$, t $S_4O_6^{2-}$	$S_5O_6^{2-}$, t $S_4O_6^{2-}$
31	0.265	3.0	$S_5O_6^{2-}$	$S_5O_6^{2-}$, $S_6O_6^{2-}$
32	0.265	4.5	$S_5O_6^{2-}$	$S_8O_6^{2-}$ + S
33	0.0176	6.0	$S_5O_6^{2-}$	$S_8O_6^{2-}$, $S_7O_6^{2-}$ + S
34	0.0176	6.9	$S_5O_6^{2-}$	$S_8O_6^{2-}$, $S_7O_6^{2-}$ + S
35	0.05	6	$S_5O_6^{2-}$	S
36	0.034	7	$S_5O_6^{2-}$, t $S_4O_6^{2-}$, t $S_6O_6^{2-}$	S
37	0.036	8	$S_5O_6^{2-}$ + t S	S
38	0.038	7.9	$S_5O_6^{2-}$ + t S	S
39	0.014	10	$S_5O_6^{2-}$ + t S	S

* t = trace.

of 0.01 *M* sodium thiosulphate to each solution. Experiments Nos. 40–47 contained 1 ml of 0.1021 *M* potassium trithionate. Experiments Nos. 48–52 contained 1 ml of 0.2829 *M* potassium trithionate. The results are given in Table V.

Trithionate is stable in the presence of thiosulphate in the range 0 to 1.2 *M* HCl, at greater acidities sulphur is formed. As the acidity rises above 7 *M* tetra-, penta- and hexathionate are observed to be present.

Formaldehyde again assists the degradation of trithionate so that no thionates were present in acidities greater than 2.4 *M* HCl. Comparing these results with those obtained in Table II, it is seen that the occurrence of tetra-, penta- and hexa-thionate is increased at the high acidities, and the decomposition in formalin is retarded. Table V must also be compared with Table IX in the next section, since much of these effects will come from the degradation of thiosulphate with acid.

TABLE V

STABILITY OF TRITHIONATE IN THE PRESENCE OF THIOSULPHATE

Expt. No.	Molarity of trithionate	Molarity of HCl	Analyses after 12 h*	
			Formalin absent	Formalin present
40	0.034	—	$S_2O_3^{2-}$, $S_3O_6^{2-}$	$S_2O_3^{2-}$, $S_3O_6^{2-}$
41	0.0256	0.25	$S_3O_6^{2-}$	$S_2O_3^{2-}$, $S_3O_6^{2-}$
42	0.02	1.2	$S_3O_6^{2-}$	$S_2O_3^{2-}$, $S_3O_6^{2-}$
43	0.0256	1.33	$S_3O_6^{2-}$ + S	$S_2O_3^{2-}$, $S_3O_6^{2-}$
44	0.02	2.4	$S_3O_6^{2-}$ + S	$S_2O_3^{2-}$
45	0.02	3.6	$S_3O_6^{2-}$ + S	—
46	0.015	5.0	$S_3O_6^{2-}$ + S	—
47	0.015	6.0	$S_3O_6^{2-}$ + S	—
48	0.0314	6.7	$S_3O_6^{2-}$ + S	—
49	0.021	7.38	$S_3O_6^{2-}$, t $S_4O_6^{2-}$, $S_5O_6^{2-}$, $S_6O_6^{2-}$ + S	—
50	0.022	8.47	$S_3O_6^{2-}$, t $S_4O_6^{2-}$, $S_5O_6^{2-}$, $S_6O_6^{2-}$ + S	—
51	0.022	8.53	$S_3O_6^{2-}$, t $S_4O_6^{2-}$, S_5O_6, $S_6O_6^{2-}$ + S	—
52	0.010	9.37	S	—

* t = trace.

TABLE VI

STABILITY OF TETRATHIONATE IN THE PRESENCE OF THIOSULPHATE

Expt. No.	*Molarity of tetrathionate*	*Molarity of HCl*	*Analyses after 12 h**	
			Formalin absent	*Formalin present*
53	0.032	—	$S_2O_3^{2-}$, $S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
54	0.024	0.25	$S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
55	0.019	1.2	$S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
56	0.024	1.33	$S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
57	0.019	2.4	$S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
58	0.019	3.6	$S_4O_6^{2-}$	$S_2O_3^{2-}$, $S_4O_6^{2-}$
59	0.0135	5.0	$S_4O_6^{2-}$	$S_4O_6^{2-}$ + S
60	0.0135	6.0	$S_4O_6^{2-}$	S
61	0.036	6.74	$S_4O_6^{2-}$	S
62	0.022	7.38	$S_4O_6^{2-}$	S
63	0.023	8.47	$S_4O_6^{2-}$, t S	S
64	0.023	8.53	$S_4O_6^{2-}$, t $S_5O_6^{2-}$, $S_6O_6^{2-}$ + S	S
65	0.010	9.37	$S_4O_6^{2-}$, t $S_5O_6^{2-}$, $S_6O_6^{2-}$ + S	S

* t = trace.

Tetrathionate. The conditions were as for trithionate (under this heading). In experiments Nos. 53–60 0.0948 *M* potassium tetrathionate, in experiments Nos. 61–65 0.2923 *M* potassium tetrathionate was used. The results are given in Table VI.

Tetrathionate is stable in the presence of thiosulphate in the range 0 to 7.4 *M* HCl. At greater acidities penta- and hexa-thionates are formed while S is deposited. Formalin again assists the breakdown of $S_4O_6^{2-}$, so that no thionates are present at acidities greater than 5 *M* hydrochloric acid.

Comparing the results of Table VI with those of Tables III and IX, it is seen that penta- and hexa-thionates occur at high acidities in the presence of thiosulphate, but not when it is absent. Thiosulphate once again retards the decomposition of formalin.

Pentathionate. The conditions were as for the experiments with trithionate

TABLE VII

STABILITY OF PENTATHIONATE IN THE PRESENCE OF THIOSULPHATE

Expt. No.	*Molarity of pentathionate*	*Molarity of HCl*	*Analyses after 12 h**	
			Formalin absent	*Formalin present*
66	0.038	—	$S_4O_6^{-}$, $S_5O_6^{2-}$ + S	$S_4O_6^{2-}$, $S_5O_6^{2-}$ + S
67	0.028	0.25	$S_4O_6^{2-}$, $S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_4O_6^{2-}$, $S_5O_6^{2-}$, $S_6O_6^{2-}$
68	0.023	1.20	$S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_5O_6^{2-}$, $S_6O_6^{2-}$
69	0.028	1.33	$S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_5O_6^{2-}$, $S_6O_6^{2-}$
70	0.023	2.4	$S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_5O_6^{2-}$, $S_6O_6^{2-}$
71	0.023	3.6	$S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_8O_6^{2-}$ + S
72	0.016	5.0	$S_5O_6^{2-}$, $S_6O_6^{2-}$	$S_8O_6^{2-}$ + S
73	0.016	6.0	$S_5O_6^{2-}$, $S_6O_6^{2-}$	S
74	0.036	6.74	$S_5O_6^{2-}$, $S_6O_6^{2-}$	S
75	0.0235	7.38	$S_5O_6^{2-}$, $S_6O_6^{2-}$ + t S	S
76	0.025	8.47	$S_5O_6^{2-}$, $S_6O_6^{2-}$ + t S	S
77	0.025	8.53	$S_5O_6^{2-}$, $S_6O_6^{2-}$ + t S	S
78	0.011	9.37	$S_5O_6^{2-}$, $S_6O_6^{2-}$ + t S	S

* t = trace.

solution. In experiments Nos. 66–73 0.126 *M* potassium pentathionate was used. In experiments Nos. 74–78 0.3025 *M* potassium pentathionate was used. The results are given in Table VII.

Pentathionate is unstable in the presence of thiosulphate in neutral solution, and at low acidities (0–2.5 *M* HCl), $S_4O_6^{2-}$ is formed, whilst at any acidity greater than this $S_6O_6^{2-}$ also occurs. Formalin again assists the production of octathionate in the range 3.6 to 6 *M* HCl, while at higher acidities there is complete degradation to sulphur. Comparing these results with those of Table IV, it is seen that hexathionate occurs at much lower acidities when the thiosulphate ion is present, both in the presence and absence of formalin.

GENERAL CONCLUSION AND DISCUSSION

The region of stability of the thionates under investigation in hydrochloric acid, have been tabulated in Table VIII.

TABLE VIII

STABILITY OF THIONATES

Anion	*Range of stability*			
	Without thiosulphate		*With thiosulphate*	
	Formalin absent	*Formalin present*	*Formalin absent*	*Formalin present*
Trithionate	Neutral to 1.5 *M*	Neutral	Neutral to 1.2 *M*	Neutral to 1.3 *M*
Tetrathionate	Neutral to 5 *M*	—	Neutral to 7.4 *M*	Neutral to 3.6 *M*
Pentathionate	3–6 *M*	1.5 *M*	—	—

Whilst these results are only qualitative, and a further detailed quantitative investigation is needed, experiments being repeated at intermediate acidities, and also at different time intervals, some general principles are shown.

(i) As the number of sulphur atoms in the polythionic acids increases, the region of stability is formed at higher acidities.

(ii) Thiosulphate undergoes exchange reactions with the thionates, the products depending on the acidities of the media, but also prolongs the existence of tri- and tetrathionate in formalin.

(iii) Formalin also accelerates reaction, probably by the removal of any sulphite ions from the solutions as formaldehyde bisulphite.

The absence of higher thionates in solutions of trithionate may be explained by the instability of these substances at low acidities, while at higher acidities trithionate itself is unstable. Each time the SO_3^{2-} fraction is hydrolysed off, it is complexed by the formalin, and thus the total thionate sulphur content of the solution is diminished. Eventually all the sulphur is converted into the formaldehyde bisulphite form and free sulphur, which is formed by complete degradation of a high polythionate:

$$H^+ + S_2O_3^{2-} + S_xO_6^{2-} \longrightarrow S_{x+1}O_6^{2-} + HSO_3^- \qquad (27)$$

When $x = 6$ or 7, it appears such thionates have very limited stability ranges of acidity; outside these ranges complete degradation rapidly occurs.

Since this work was undertaken, Blasius and co-workers[25,26] have extended these studies to alkaline pH's, and the results are confirmatory. All the reactions are slow, which allows a correct analysis of the polythionates present by the paper-chromatographic method.

Decomposition of sodium thiosulphate

30.0 mmol of sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$) in 25 ml of water was added to 40 mmol of hydrochloric acid (20 ml of 2 *M* acid). The solution was analysed for thionate at time intervals. Sulphur precipitated immediately. The results are given in Table IX.

TABLE IX

DECOMPOSITION OF THIOSULPHATE

Time (min)	*Thionates present*
0	$S_2O_3^{2-}$
0.5	$S_2O_3^{2-}$, $S_3O_6^{2-}$
1.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$
2.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$
4.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$
8.5	$S_2O_3^{2-}$, $S_3O_6^{2-}$
11.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$
13.5	$S_2O_3^{2-}$, $S_3O_6^{2-}$
20.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$
26.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$
32.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$
39.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$
43.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$
67.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$, t $S_5O_6^{2-}$
80.0	$S_2O_3^{2-}$, $S_3O_6^{2-}$, $S_4O_6^{2-}$, $S_5O_6^{2-}$

These results confirm BLASIUS AND BURMEISTER's results[28] and show that the first polythionate formed in the decomposition of thiosulphate in 1 *M* hydrochloric acid is trithionate. The remaining species are built up from this as the decomposition continues. This supports SCHMIDT's[10] mechanism which regards the primary reaction as the decomposition of thiosulphuric acid as depicted by eqn. (2), followed by eqn. (4), and reactions with excess thiosulphate to yield higher thionates.

Another possible mechanism is the formation of thiosulphurous acid[15] which is hydrolysed to monosulphurhydrate[27,28] and sulphoxylic acid:

$$H_2S_2O_2 + H_2O \rightleftarrows HSOH \text{ and } S(OH)_2 \qquad (28)$$

The sulphoxylic acid reacts with sulphite to give trithionate:

$$S(OH)_2 + 2\ HSO_3^- \rightleftarrows S_3O_6^{2} + 2\ H_2O \qquad (29)$$

or more probably:

$$HSOH + SO_3H^- \longrightarrow HSSO_3H + OH^- \qquad (30)$$

$$HSSO_3H + SO_3H^+ \longrightarrow H_2S_3O_6 + HS^- \qquad (31)$$

As the concentration of trithionate increases, so formation of higher thionates proceeds.

Because $S_4O_6^{2-}$, $S_5O_6^{2-}$ and $S_6O_6^{2-}$ are not found in the early stages of the reaction, it must be assumed that:

(a) the hydrolysis according to eqn. (28) lies to the right hand side,

(b) that reaction (29) is fast compared to the reaction:

$$S(OH)_2 + 2\ HS_2O_3^- \longrightarrow S_5O_6^{2-} + 2\ H_2O \qquad (32)$$

cf. ref. 29 and 30.

A comparison of the rates of reaction of sulphite, and thiosulphate with sulphur dichloride in aqueous solution, is being undertaken to help to substantiate the above mechanism.

Investigations on the effect of the addition of sulphide, hypophosphite and sulphite on the acid decomposition of sodium thiosulphate[18,19]

50 mmol of sodium thiosulphate and 12 mmol of sodium sulphide or hypophosphite were dissolved separately in 35 ml of water. This was added to 35 ml of concentrated hydrochloric acid at −5°. Sulphur deposited immediately in the sulphide experiment and in 45 min with the hypophosphite. The mixture was analysed chromatographically and the results are given in Table X.

TABLE X

REACTION OF THIOSULPHATE WITH SULPHIDE OR HYPOPHOSPHITE

Time (min)	*Polythionates present (F) $S_nO_6^{2-}$*	
	Sulphide	*Hypophosphite**
1	4, 5, 6, 7, 8	8
5	4, 5, 6, 7, 8	8
10	4, 5, 6, 7, 8	t 4, 5, 6, 8
15	4, 5, 6, 7, 8	4, 5, 6, 8
20	4, 5, 6, 7, 8	4, 5, 6, 7, 8
30	4, 5, 6, 7, 8	4, 5, 6, 7, 8
40	4, 5, 6, 7, 8	4, 5, 6, 7, 8
50	4, 5, 6, 7, 8	4, 5, 6, 7, 8
120	t 4, 5, 6, 7, 8	4. 5, 6, 8
210	t 4, t 5, 6, 7, 8	

* Underlined species denotes the predominant species; t = trace.

Hexathionate is the predominant species, which is in agreement with YANITSKII AND VALANCHUNAS'S[18,19] results, for the sulphide addition, whilst octathionate is formed initially in the hypophosphite case which breaks down to lower thionates, the main product after 100 min being pentathionate. In this latter case, the Russian workers[18,19] found 95 % pure hexathionate, which they precipitated by the addition of benzidine hydrochloride after removal of the sulphur. Benzidine octathionate was obtained first, then the hexathionate salt, and finally the mother liquor contained pentathionate.

Thus in general outline the two series of experiments agree.

The reactions involved in the sulphide addition seem to be pretty clear, in that it involves a large concentration of H_2S being present to drive the reaction:

$$H_2S + SO_2 \rightleftarrows H_2S_2O_2 \qquad (10)$$

to completion, then follow two reactions:

$$H_2S_2O_2 + 2\ H_2S_2O_3 \rightleftarrows H_2S_6O_6 + 2\ H_2O \qquad (11)$$

and

$$H_2S_2O_2 + 2\ H_2SO_3 \rightleftarrows H_2S_4O_6 + 2\ H_2O \qquad (12)$$

followed by build-up and degradation of these two species to give S_5, S_7 and S_8 species.

However, the hypophosphite case is not so clear, because only one product was initially found, *i.e.* octathionate, and we feel the only explanation somehow involves the reducing power of hypophosphorous acid.

If this could force an extra-sulphur atom into $H_2S_2O_3$ to form the disulphanemonosulphonic acid $H_2S_3O_3$, then with thiosulphurous acid:

$$H_2S_2O_2 + 2\ H_2S_3O_3 \longrightarrow H_2S_8O_6 + 2\ H_2O$$

but even under such circumstances, the initial appearance of other thionates would be expected.

When studies of the decomposition of thiosulphate in the presence of both sulphide and sulphite are carried out, a complex series of thionates are produced from traces of $S_4O_6^{2-}$ to $S_7O_6^{2-}$, whilst hexathionate is the predominant species. This is in contrast to the results found by YANITSKII AND VALANCHUNAS[19], who found pentathionate the predominant species; however, it must be remembered that the precipitation by benzidine as means of analysis was not complete until after 3–4 days. Thus it is possible that hexathionate was their primary product, but pentathionate being more stable in the acidity range studied, was formed, but one would have thought that indications of other thionates besides pentathionate would have been observed.

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SUMMARY

Since many of the reactions involving thionic acids occur in acid media, it was felt that a qualitative study of the stability of these substances in various concentrations of hydrochloric acid was desirable. One of the main reactions in acid media is said to be:

$$H^- + S_2O_3^{2-} + S_x\ O_6^{2-} \rightleftarrows S_{x+1}\ O_6^{2-} + HSO_3^-$$

The effect of thiosulphate and formaldehyde (which removes bisulphite) was therefore observed.

Results obtained agreed with the above equation and also showed that as the number of sulphur atoms in the thionates increases, the concentration of acid in which the thionate is stable increases. Experiments conducted to ascertain the primary thionate produced in the decomposition of acidified thiosulphate solution are described, this being trithionate; a possible mechanism is suggested for this reaction.

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CHROMATOGRAPHIC STUDIES ON SULPHUR COMPOUNDS

PART V. A STUDY TO SEPARATE THIOSULPHATE, SULPHITE AND THE LOWER POLYTHIONATES BY ANION-EXCHANGE CHROMATOGRAPHY

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Anion-exchange chromatography has proved such a powerful technique for the separation of homologous series or closely-related inorganic anions, *e.g.* polyphosphates[1,2], lower phosphorous acids[3], and the imido-ring and chain phosphates[4–6], that it was decided to attempt to separate the polythionates using this technique. Work had already been carried out in these laboratories[7] using both strongly and weakly basic anion-exchangers, but of unspecified cross-linkages, and assumed to be about 10% from swelling characteristics. Using the former resin, thiosulphate was eluted with both N and $3N$ sodium chloride solution. Trithionate and tetrathionate could not be eluted with such solvents, buffered at, pH 4 or pH 2, or unbuffered, or with $2N$ sulphuric acid. With the weakly basic resin, tetrathionate was eluted very slowly with $3N$ sodium chloride, not at all with citric acid, and very slowly with $0.5N$ and $2N$ sulphuric acid. $6N$ hydrochloric acid eluted tetrathionate but slowly from both resins, and concentrated hydrochloric acid eluted both these thionates, but too rapidly for a separation to occur.

While the present research was in progress, IGUCHI[8] published results showing a separation of the thionic acids using an anion-exchange column of Dowex-1, 2% cross-linked D.V.B.* IGUCHI[8] also reported a separation of sulphite, sulphate, sulphide and thiosulphate, using different solvents. Since polythionates are usually found together with thiosulphate and sulphite in mixtures, these separations are of limited use. A separation of polythionates, sulphite and thiosulphite is desirable.

Anion-exchange chromatography has the advantage over paper chromatography, in that relatively large volumes of solution at high concentrations can be used.

EXPERIMENTAL

Salts

(1) AnalaR sodium thiosulphate $Na_2S_2O_3 \cdot 5H_2O$.
(2) AnalaR sodium sulphite $Na_2SO_3 \cdot 7H_2O$.
(3) Potassium trithionate, for preparation see ref. 9.
(4) Potassium tetrathionate, for preparation see ref. 10.
(5) Potassium pentathionate, for preparation see ref. 11.

* Divinylbenzene.

Determination

The thiosulphate and sulphite were estimated in the collected 5 ml fractions, using the conventional iodine titration method. When thiosulphate and sulphite occurred together, the sulphite was complexed with formaldehyde, and the thiosulphate determined alone; the sulphite figure being obtained by difference.

The method used for the determination of the polythionates[12] was titration under Andrew conditions, *i.e.* direct titration with standard potassium iodate solution, in the presence of 6*M* hydrochloric acid and carbon tetrachloride.

Anion-exchange chromatography

Resin: De-Acidite FF, Water regain 1.6–2.00 (cross-linkage D.V.B. 2 %), mesh size 52–100 B.S.S.

Column: 20 cm long × 0.75 cm^2 cross-section area. The column after packing was washed well with 9*M* hydrochloric acid and finally with water.

TABLE I

Solvent (M HCl)	*Volume used (ml)*	*Trithionate*		*Tetrathionate*		*Pentathionate*	
		S.V.	*F.V.*	*S.V.*	*F.V.*	*S.V.*	*F.V.*
3	75	35	70	—	—	—	—
6	65	—	—	95	110	—	—
9	50	—	—	—	—	145	180
Concentration of thionate applied (mmol)		0.04		0.04		0.04	
% Recovery		100 ± 1		101 ± 2		98 ± 1.5	

S.V. = Break through or appearance volume of species.
F.V. = Final or disappearance volume of species.

Conditions of elution

The conditions for elution (see Table I) are by a batch method, since optimum elution conditions for each species are so different that gradient elution proved inefficient. A typical separation is shown in Fig. 1, while Fig. 2 shows the elution of trithionate under identical conditions, but using an 8 % D.V.B. cross-linked resin.

Since the separation of the polythionates was satisfactory, a separation of sulphite and thiosulphate was sought. The final solvent used was a solution of 2*M*

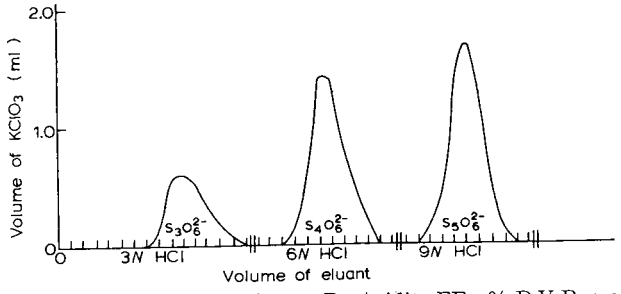

Fig. 1. Elution of the polythionates from a De-Acidite FF 2 % D.V.B. cross-linked resin.

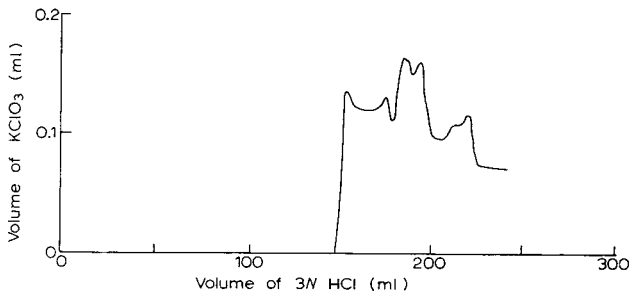

Fig. 2. Elution of trithionate from an 8% D.V.B. cross-linked resin.

potassium hydrogen phthalate (pH = 4). Carefully controlled experiments showed that the polythionates and thiosulphate were stable in this media for much longer periods of time than that taken for the anion-exchange separation. However, when the separation of thiosulphate and trithionate was attempted, a complication arose. On changing the solvent after elution of the thiosulphate, and passing 3*M* hydrochloric acid through the column, free phthalic acid was produced and the column was blocked. Thus after passage of the phthalate buffer, a further buffer, pH4, made up of sodium acetate–hydrochloric acid was also passed down the column, to remove the phthalate buffer.

By combination of these two methods, the separation shown in Figs. 3 and 4 was achieved, with the conditions given in Table II.

Fig. 3 shows the complete separation, while Fig. 4 gives the total graph of SO_3^{2-} + $S_2O_3^{2-}$, and also the $S_2O_3^{2-}$ only, whilst Fig. 4b gives the resultant elution patterns of SO_3^{2-} and $S_2O_3^{2-}$ when the $S_2O_3^{2-}$ contribution is subtracted from the $S_2O_3^{2-}$ + SO_3^{2-} graph.

The behaviour of hexathionate in 9*M* HCl was studied, and although it is sepa-

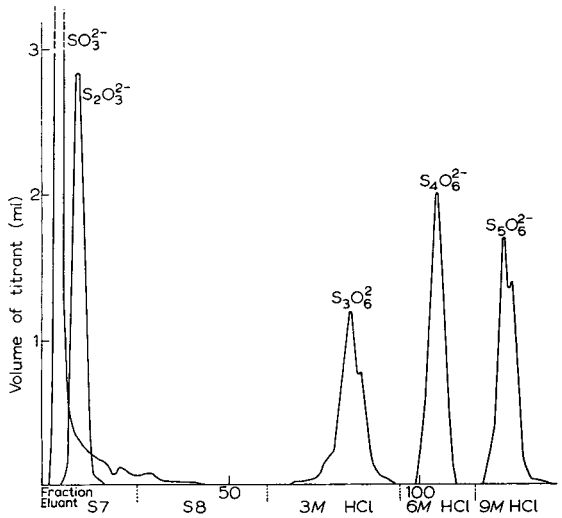

Fig. 3. Elution of polythionates, sulphite and thiosulphate from a 2% cross-linked resin.

TABLE II

Eluants	*Ion eluted*
(1) 250 ml of 2 *M* potassium hydrogen phthalate (S7)	SO_3^{2-}, $S_2O_3^{2-}$
(2) 250 ml of sodium acetate–hydrochloric acid buffer, pH 4 (S8)	—
(3) 250 ml of 3 *M* HCl	$S_3O_6^{2-}$
(4) 250 ml of 6 *M* HCl	$S_4O_6^{2-}$
(5) 250 ml of 9 *M* HCl	$S_5O_6^{2-}$

rated from pentathionate, tailing of the peak was noted. Work is in progress to correct this phenomenon, then it is hoped to apply this method to study reactions where these ions are found.

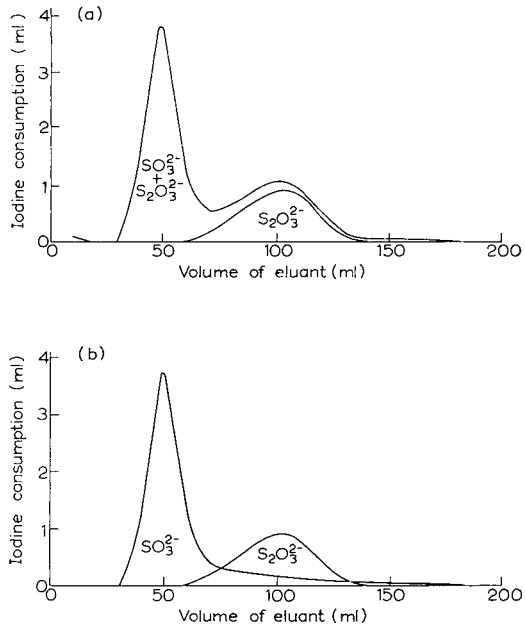

Fig. 4. (a) Elution of sulphite and thiosulphate from a 2 % cross-linked resin. (6) Elution of sulphite and thiosulphate from a 2 % cross-linked resin, calculated from (a).

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SUMMARY

A method has been devised for the analysis of sulphite, thiosulphate, trithionate, tetrathionate and pentathionate by anion-exchange chromatography.

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Notes

Eine einfache Vorrichtung zum Aufbrechen von abgeschmolzenen Glasampullen im Gaschromatographen

Bei Versuchen über die Radiolyse kleiner Substanzmengen im hiesigen Laboratorium begegneten wir dem Problem, abgeschmolzene Glasampullen mit dem zu analysierenden Inhalt (1–100 mg) an festen oder flüssigen Verbindungen im Verdampferteil eines Gaschromatographen zu öffnen. Über Vorrichtungen zur Zertrümmerung abgeschmolzener Ampullen ist bereits berichtet worden[1,2]. Die nachstehend beschriebene Anordnung besitzt gegenüber den bisher verwendeten den Vorteil, dass die Abdichtung nur an einer einzigen Stelle geschieht und dass nur ein bewegliches Teil vorhanden ist. Sie erwies sich als sicher im Betrieb und gestattete es, die Reste der zertrümmerten Ampullen einfach und rasch zu entfernen. Die erhaltenen Gaschromatogramme besassen einwandfreie Auflösung und gewährleisteten die quantitative Analyse der Substanzgemische.

Die Vorrichtung besteht aus den in der Fig. 1 gezeigten metallischen Teilen, nämlich aus dem Kolonnenaufsatz A und dem Zertrümmerer B. Der Aufsatz wird durch eine Metallverschraubung* an die Säule des Gaschromatographen angeschlossen. Er dient zur Aufnahme des Zertrümmerers und besitzt hierzu eine Bohrung, deren

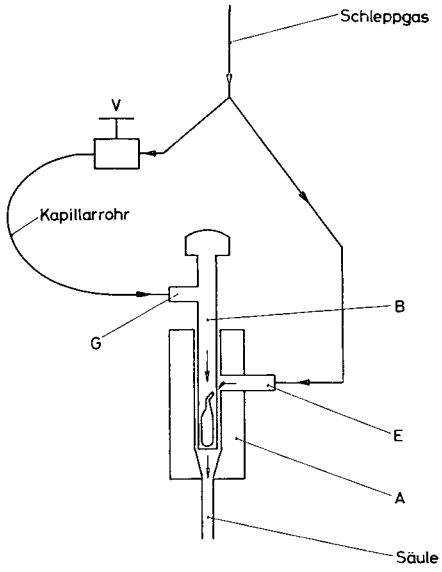

Fig. 1. Schema der Zertrümmerungsapparatur und des Gasstroms.

* Hersteller: J. Walterscheid KG, Siegburg-Lohmar.

Durchmesser knapp den äusseren Durchmesser des Zertrümmerers übertrifft. Wie Fig. 2 zeigt, sorgt beim Einschieben des Teils B die stopfbuchsenartige Kombination aus Gummiring O, Metallring M und Lochschraube LS für die Dichtung zwischen den beiden Teilen. Eine Heizwicklung W hält den unteren Teil des Aufsatzes auf hoher Temperatur, um das sofortige Verdampfen des Ampulleninhalts zu gewährleisten. Die Dichtung am oberen Teil wird durch die Wasserkühlung K geschützt. Der Aufsatz besitzt einen seitlichen Ansatz E, durch den das Schleppgas in die Bohrung Z einströmt.

Das Prinzip der Zertrümmerung lässt Fig. 2 erkennen: Die Ampulle U wird zunächst in die Metallhülse H gesteckt und diese anschliessend über das Gewinde SG mit dem Metallstab S verbunden. Dieser besitzt eine Bohrung, durch die das bei G eintretende Schleppgas die Ampulle umspült. Der Gasstrom tritt durch die Bohrungen

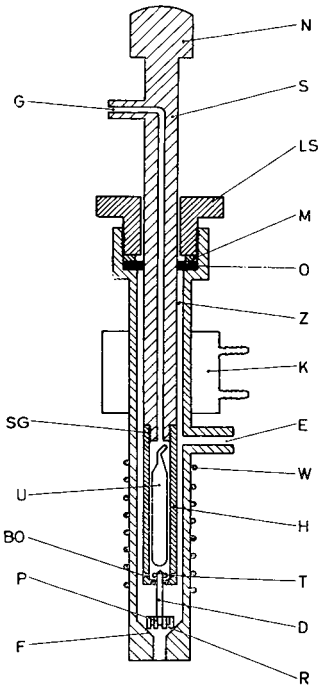

Fig. 2. Querschnitt durch die Zertrümmerungsapparatur in schematischer Darstellung.

BO am unteren Boden der Hülse aus. Durch die zentrale Bohrung der Hülse ragt der Dorn D, der durch den Stift T in der Hülse gehalten wird. Am unteren Ende des Dorns sitzt die Platte P, die eine Reihe senkrecht und radial verlaufender Rillen R besitzt.

Der Zertrümmerer wird zunächst in den Aufsatz geschoben, bis die Platte am Konus F aufsitzt, was sich durch den eintretenden Widerstand bemerkbar macht. Nun wird durch Anziehen der Lochschraube LS für eine gute Dichtung gesorgt. Dann wird der Hahn V in Fig. 1 geöffnet, das Schleppgas strömt durch die flexibele Kapillare, tritt bei G in den Stab S ein und umströmt die Ampulle U in der Hülse. Nach

kurzer Zeit hat sich konstanter Druck eingestellt, und die Ampulle ist gleichzeitig erwärmt worden. Durch Druck oder leichten Schlag auf den Kopf N des Stabes S wird die Ampulle zertrümmert. Der verdampfte Inhalt wird von dem von oben eintretenden Gasstrom erfasst und durch die Bohrungen der Hülse und die Rillen auf die Säule gespült. Eine Rückdiffusion nach oben wird durch den bei E eintretenden Gasstrom verhindert. Nach Beendigung des Versuchs wird der Hahn V geschlossen und dann der Stab herausgezogen. Um ein Ausströmen des bei E eintretenden Schleppgases zu vermeiden, wird die Vorrichtung mit einem Blindstab verschlossen.

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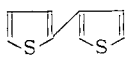
Gas chromatography in the separation and identification of isomeric bithienyls

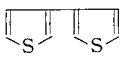
The reported methods for the separation and identification of isomeric bithienyls and polythienyls are lengthy and are not sensitive to low concentrations. SEASE AND ZECHMEISTER[1] separated bithienyl from terthienyl and other polythienyls by chromatographing on alumina from solvent solutions. WYNBERG AND BANTJES[2] used chromatography on basic alumina together with U.V. and I.R. absorption bands and mass spectral analysis for the separation and identification of the isomeric bithienyls.

In the radiation chemistry of thiophene a rapid, direct and highly sensitive method for the detection and analysis of radiation induced isomeric bithienyls was needed. Preliminary work with gas chromatography using thermistor and thermal conductivity detectors did not separate the three isomeric bithienyls (I, II, III).

2,2′-Bithienyl
(I)



2,3′-Bithienyl
(II)



3,3′-Bithienyl
(III)

A flame ionization detector employing a 6 ft. ¼ in. O.D. stainless steel column packed with 15 % silicone gum rubber (SE-30) coated on Chromosorb W (60–80 mesh) also did not separate the three isomers. However, a Carbowax 20 M column described below gave good separation of the three bithienyls.

Experimental

The bithienyls were separated using an FM Model 720 Chromatograph with a Model 1609 flame ionization detector. The 6 ft. ¼ in. O.D. stainless steel column was packed with 15 % by weight of Carbowax 20 M on Chromosorb W (80–100 mesh).

The experimental conditions were:

Carrier and gas flow: helium 130 ml/min; hydrogen 58 ml/min; air 640 ml/min.

Injection port temperature: 260°.

Detector temperature: 205°.

Column temperature: 220°.

Injections of 1 μl of a mixture of thiophene and isopropanol solutions of the isomeric bithienyls were made. The concentration of the three bithienyls present in 1 μl of isopropanol–thiophene solvent mixture are shown in Table I. The isomeric bithienyls

TABLE I

GAS CHROMATOGRAPHY OF ISOMERIC BITHIENYLS ON CARBOWAX 20 M

Bithienyl	*Retention time (min)*	*Concentration (μg in 1 μl solvent)*	
		used in Fig. 1	*lowest amount detectable*
2,2′-	6.6	0.96	0.028
2,3′-	7.6	1.0	0.029
3,3′-	9.2	0.92	0.027
(*Solvents*)			
Isopropanol	0.5		
Thiophene	0.6		

used were purified and characterized by Dr. H. WYNBERG, as described in ref. 2. Trace amounts of the isomers were present in each of the bithienyls. In addition, solvent dilutions of the isomeric bithienyls were made containing approximately 3 and $8 \cdot 10^{-8}$ g of each compound per μl.

Results and discussion

Fig. 1 shows the excellent separation of the three isomeric bithienyls obtained with the Carbowax 20 M column. The lowest concentration of bithienyls detectable was approximately $3 \cdot 10^{-8}$ g with an attenuator setting of 10 × (Table I). There was no improvement in the resolution of the three isomers by using programmed temperature

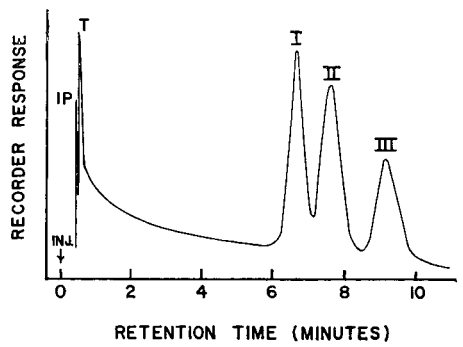


Fig. 1. Gas chromatogram of solvent solution of isomeric bithienyls. Column = 15% Carbowax 20 M on Chromosorb W (80–100 mesh). Column temperature = 220°. Injection port temperature = 260°. Helium flow = 130 ml/min. Sample size 1.0 μl. Attenuator setting = 200 ×. Peak I = 2,2′-bithienyl. Peak II = 2,3′-bithienyl. Peak III = 3,3′-bithienyl. IP = isopropanol. T = thiophene.

chromatography (120° start and 10° per min). Isothermal chromatography at 188°, 220° and 240° gave good separation of the isomers. The sharpest peaks were obtained at a temperature of 240°.

A flame ionization detector with the Carbowax 20 M column gave the best separation of isomeric bithienyls with a number of substrates studied (tricresyl phosphate, silicone gum rubber SE30 and Apiezon L grease). The Carbowax column gave excellent separation and identification of low concentrations of the isomeric bithienyls produced in the radiation chemistry of thiophene. The column material should be useful in the separation and identification of other isomeric heterocyclic compounds.

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SIGMUND BERK

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Chromatography of fat-soluble food dyes on thin starch layers with stationary non-polar phases

Of the fat-soluble food dyes only the natural ones are permitted in moist countries. The use of synthetic fat-soluble dyes became restricted since Japanese research workers found them to be carcinogenic, especially Butter Yellow[1,2]. For this reason colouring of foods with these synthetics was prohibited in several countries, including Czechoslovakia[3]. The strict restriction, or even prohibition of these substances calls for a suitable, and above all, rapid analytical method of detection, or as the case may be, even for determination, of these substances in food inspection and research work.

Chromatographic methods based on adsorption[4] or on partition[5–7] are mostly used for the detection of fat-soluble food dyes. A classical system of identification of lipophilic food dyes has been described by THALLER AND SCHELLER[8] and JAX AND AUST[9]. These methods are based on pre-separation into groups by adsorption column chromatography; the components are then identified by means of partition paper chromatography. As the adsorption material alumina, various clays, etc., were employed. The use of partition paper chromatography which a stationary non-polar phase (paraffin oil) was first described by LINDBERG[5]. For the stationary phase other substances such as *n*-lauryl alcohol, oleic acid, diacetylethylene glycol monostearate[10],

and the like were also used; paraffin oil, however, found the widest application. The disadvantage of paper partition chromatography is that it is rather time consuming.

This drawback has been obviated to a certain extent by using thin-layer chromatography. For the partition of synthetic dyes this method was used first by MOTTIER AND POTTERAT[11,12], LAGONI AND WORTMANN[13], and by DAVÍDEK, POKORNÝ AND JANÍČEK[14,15]. All these methods make use of the adsorption ability of alumina or other adsorbents. Therefore the usefulness of the method is somewhat limited as it can only be applied to substances showing substantially different adsorption affinities. In some mixtures it was impossible to identify individual dyes by this method. In such cases a combination of the method with partition paper chromatography appeared necessary.

In the present paper the chromatographic separation of fat-soluble food dyes using partition chromatography on thin layers of starch with a stationary non-polar phase is described. This method allows the principle of partition chromatography to be used even with the thin layer modification and so perfect separations of the said dyes could be obtained.

Experimental

The following dyes were studied: Yellow AB, Yellow OB, Orange SS, Sudan I, II, III, IV, and GN, Sudan Red G, and Butter Yellow. For the chromatographic partition 0.05–0.5 % dye solutions in ethanol, according to their solubility and coloring intensity, were used (see Table I). The chromatograms were developed with the following chromatographic solvent systems: aqueous methanol of various concentrations (50–70 %); a mixture of methanol 80 %, glacial acetic acid 5 % and water 15 %; a mixture of methanol 80 %, glacial acetic acid 10 % and water 10 %; mixtures of dioxan–water (3:2), and dioxan–water–ammonia (15:4:3).

Preparation of chromatographic plates. The chromatographic plates are prepared in the usual manner in wet state. To a 10 % solution of paraffin oil in petroleum ether, starch is added so as to obtain a fairly spreadable suspension (with about 10 g starch). This suspension is used for the preparation of the plate. After application on the glass plate and evaporation of the solvent, the plate is ready for use for chromatographic partition.

TABLE I

CONCENTRATION OF DYES USED

Dye	*Concentration mg/100 ml*
Yellow OB	2.00
Yellow AB	2.00
Orange SS	0.62
Oil Red OS	0.69
Sudan I	1.22
Sudan II	0.67
Sudan III	0.56
Sudan IV	0.65
Sudan Red G	0.64
Sudan Yellow 3G	1.13
Butter Yellow	1.19
Sudan GN	3.00

It appeared necessary first to establish the time required for evaporation of the solvent after the layer had been applied. The plates were prepared according to the procedure described above and dried in air for various time intervals; thereupon standards of dyes were applied and subjected to partition by means of the solvent system: methanol 80 %, water 15 %, and glacial acetic acid 5 %. The results are given in Table II.

TABLE II

INFLUENCE OF DRYING TIME OF THE PLATE UPON R_F VALUES

Drying time	R_F		
	Yellow OB	*Yellow AB*	*Orange SS*
20 min	0.63	0.73	0.33
30 min	0.60	0.73	0.32
90 min	0.58	0.68	0.30
150 min	0.59	0.71	0.28
180 min	0.58	0.72	0.30
210 min	0.57	0.70	0.27
270 min	0.58	0.72	0.28
7 h	0.56	0.70	0.26
24 h	0.53	0.68	0.28
48 h	0.50	0.61	0.19

As shown in the table, for perfect removal of the solvent a drying time of 20 min at room temperature is sufficient. Longer drying does not substantially influence the partition properties of the plate.

It was then necessary to establish the optimum concentration of paraffin oil for impregnating the thin layer. The preparation procedure was the same as before. For impregnation solutions of 1–25 % paraffin oil in petroleum ether were used. The plates were dried for 30 min at room temperature, and it was found that the optimum concentration of paraffin oil is about 5–10 % (Table III). After the optimum conditions had thus been established, the partition of lipophilic synthetic dyes was examined first in model mixtures. The results of these experiments are shown in Table IV. The solvent systems methanol 80 %, water 15 % and glacial acetic acid 5 %, and methanol 80 %, water 10 % and glacial acetic acid 10 % proved best. In these solvent systems even multi-component mixtures, such as are encountered in foodstuffs, can be successfully separated.

The dyes were usually isolated from the fat by acid extraction, adsorption on alumina, or extraction after saponification. As we have found before[14], for quantitative

TABLE III

INFLUENCE OF PARAFFIN OIL CONCENTRATION UPON R_F VALUES

Oil concentration %	R_F		
	Yellow OB	*Yellow AB*	*Orange SS*
1	0.88	0.91	0.67
5	0.70	0.74	0.34
10	0.57	0.71	0.29
20	0.44	0.58	0.14
25	0.32	0.42	0.10

TABLE IV

R_F VALUES OF THE INDIVIDUAL DYES

Dye	R_F in the solvent system				
	1	2	3	4	5
Yellow OB	0.04	0.29	0.59	0.57	0.61
Yellow AB	0.06	0.43	0.61	0.71	0.71
Orange SS	0.01	0.08	0.41	0.29	0.38
Oil red OS	0.00	0.00	0.10	0.03	0.04
Sudan I	0.02	0.11	0.40	0.36	0.41
Sudan II	0.01	0.04	0.32	0.19	0.28
Sudan III	0.00	0.03	0.27	0.10	0.24
Sudan IV	0.00	0.02	0.18	0.04	0.10
Sudan red G	0.04	0.26	0.54	0.53	0.58
Sudan yellow 3G	0.02	0.14	0.48	0.33	0.48
Butter yellow	0.04	0.23	0.57	0.59	0.66
Sudan GN		0.48	0.63	0.64	0.74
		0.75	0.95	0.94	0.98

* Solvent systems: (1) 50% methanol.
(2) 70% methanol.
(3) 100% methanol.
(4) methanol–water–glacial acetic acid (16:3:1).
(5) methanol–water–glacial acetic acid (8:1:1).

isolation of the dye only extraction after saponification may be applied, and it is this method that we employed to isolate the dyes from model fat samples.

Method. 25 g of the coloured fat was weighed into a flask, and after addition of 200 ml of 50% alcoholic potassium hydroxide saponified by refluxing on a boiling water-bath for 30 min. Thereupon 160 ml water was added to the reaction mixture, and it was extracted with three separate portions of 50 ml of pentane. The extract was washed and dried and the solvent distilled off *in vacuo*; the residue was then dissolved in 2 ml ethanol. This solution was used for the chromatographic procedure.

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Selenoamino acid identification on paper chromatograms

The selenium analogues of methionine, S-methylcysteine and cystine have been identified in plant and animal protein[1–3]. Radioautography of hydrolysates of proteins from plants, microorganisms and animals grown in the presence of selenium-75 has been used almost exclusively in the identification of these amino acids by paper chromatography[1–3]. Isotope techniques are necessary as the selenium amino acids occur in concentrations so low as to render detection by ninhydrin almost impossible. The problem is further complicated by the similarity of the R_F values of the selenium amino acids to those of the naturally occurring sulfur amino acids.

In order to overcome the above complications, a method has been developed which can be used to identify specifically selenomethionine and selenocystine on paper chromatograms in quantities as low as 0.5 mμ moles. This method is based on the reaction of hydriodic acid with sulfoxides as described by TOENNIS[4]. This reaction of hydriodic acid with sulfoxides was later incorporated into a starch–hydriodic acid spray for paper chromatograms by THOMPSON[5].

The amino acid (protein hydrolysate) is spotted on paper in the normal manner. Selenomethionine and selenocystine are then selectively oxidized prior to chromatography by holding the spot over 15 % hydrogen peroxide for 45 to 60 sec. A petri dish containing enough hydrogen peroxide to allow 1 cm from the peroxide to the paper is excellent for the purpose.

After chromatography, the chromatogram is dried at room temperature. The starch–hydriodic acid spray is made fresh daily as follows: (1) 5 % soluble starch; (2) 0.035 *M* sodium iodide; (3) 1.0 *M* hydrochloric acid. Stock starch solution is prepared and stored; the sodium iodide and hydrochloric acid are added immediately before use. Care must be taken to spray the paper very lightly. Selenomethionine selenoxide and selenocystine diselenoxide react in less that 45 sec to give a purplish color which fades to brown on drying at 50° in a forced draft oven. Selenocystine gives more color per mole than selenomethionine. This is probably due to the formation of the diselenoxide, or possibly two selenic acids, by peroxide oxidation.

Preservation of the chromatograms is accomplished by placing them in a manila envelope in which they are pressed against a paper which has been similarly sprayed and dried. In this manner 0.5 mμmole spots have been clearly visible after two weeks.

The sulfur amino acids are not oxidized by the described conditions and will therefore not interfere with the identification of the selenoamino acids. With this technique 0.5 mμmoles of selenomethionine was detected in the presence of 200 mμmoles of methionine. The two amino acids were mixed, applied to the paper, and then the selenomethionine oxidized as described, prior to placing the spotted paper in the chromatography tank. Similar mixtures of selenocystine and cystine have given analogous results.

The sulfur amino acids can be identified by the ninhydrin reaction. Before spraying with ninhydrin, it is necessary to immerse the chromatogram in an ammonia atmosphere for 1 h. This process decolorizes the paper and clear ninhydrin spots are obtained.

Alternatively, the chromatogram can be immersed in a peroxide atmosphere for 30 min. This oxidizes the sulfur amino acids and they give positive starch hydriodic acid spots. Also, the sulfur amino acids are frequently oxidized when the paper is

dried following development by certain solvents as described by THOMPSON[5]. The rate of color formation for the sulfoxides is markedly slower than for the selenoxides and, therefore, provides a very good method of differentiation.

Table I shows some R_F values obtained for selenomethionine and selenocystine treated as described above. Methionine and cystine were identified by ninhydrin. The

TABLE I

R_F VALUES (× 100) OF OXIDIZED SELENOMETHIONINE, SELENOCYSTINE, METHIONINE AND CYSTINE

The selenoamino acids were oxidized prior to chromatography and were identified by the starch–hydriodic acid spray described in the text. All chromatography was done on Whatman No. 1 paper.

Solvent	R_F × 100			
	Oxidized Se-methionine	*Oxidized Se-cystine*	*Methionine*	*Cystine*
Butanol–acetic acid–H_2O (4:1:1)	22	8	55	4
Ethanol–1 *M* NH_4OH–H_2O (90:5:5)	20	5	38	6
Phenol–H_2O–NH_4OH (100:20:0.03)	95	26	76	21

differences in R_F values between the selenoxides and their unoxidized sulfur analogues illustrate the usefulness of this technique. It is anticipated that selenomethylcysteine can also be identified by this method.

A previous publication from this laboratory described the identification of selenomethionine by radioautography from hydrolysates of protein taken from *E. coli* grown in ^{75}Se-selenite[6]. The method described in this report was applied to similar protein hydrolysates and selenomethionine was clearly observed. Other spots were observed, which correspond to selenocystine, but which have not yet been unequivocally identified[6,7].

We are grateful to Dr. J. MCCONNELL for his gift of samples of selenomethionine and selenocystine. JAMES SCALA is a Predoctoral Fellow, Division of General Medical Sciences, U.S. Public Health Service.

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Thin-layer Chromatography

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Gas Chromatography

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JUVET, JR., R. S. AND DAL NOGARE, S.: Gas chromatography. *Anal. Chem.*, 36 (1964) 36R–51R — a review of 639 papers mostly from 1962/1963.

KEENAN, R. G.: Recent developments in analytical methods for occupational health. *J. Occupational Med.*, 5 (1963) 301–306 — brief review of GC on pp. 303–304.

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2c. Thermodynamics and theoretical relationships

EVANS, M. B. AND HIGGINS, G. M. C.: Use of retention index in qualitative gas–liquid chromatography. *Nature*, 202 (1964) 83 — $\log R_{xN} = (I/100 - N)b$ (R_{xN} = retention of an unknown relative to that of a *n*-alkane, I = retention index, N = number of carbon atoms of *n*-alkane, b = increment in the log of the retention per methylene group).

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RACKOW, B.: Die thermodynamische Bedeutung der chromatographischen Information und deren Eigenschaften. *Z. Chem.*, 4 (1964) 109–110 — probability instead of peak area is incorporated in the information equation.

3. TECHNIQUES I

3a. Detectors

BAITINGER, W. E. AND AMY, J. W.: Correlations between electron capturing ability and structure. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 28, p. 54 — relative sensitivities are given.

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KING, JR., W. H.: Piezoelectric sorption detector. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 4, p. 50 — quartz crystal is coated with GC stationary phases and vibrated at 9 Mc; response speed is 0.05 sec, output signal is frequency, sensitivity (selective) about 10^{-9} g.

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PIRINGER, O., TATARU, E. AND PASCALAU, M.: Construction and operation of the electrolytic conductivity detector. *J. Gas Chromatog.*, 2 (1963) 104–106 — CO_2 formed by combustion is detected by differential electrolytic conductivity method; full electric scheme is given.

PRÖSCH, U., VORMUM, G. AND ZÖPFL, H. J.: Vergleichende Untersuchungen mit verschiedenen geschlossenen radioaktiven Präparaten für einen Argonionisationsdetector der Gaschromatographie. VI. Mitt. Geschlossene Präparate. *Kernenergie*, 7 (1964) 26–33 — of 10 mC ^{90}Sr, 1 mC ^{90}Sr, 500 mC ^{3}H and 5 mC ^{85}Kr sources, the last was found the best.

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ROESLER, J. F.: A preliminary study of the characteristics of a photoionization detector for gas chromatography. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 7, p. 51 — about 5 ml sample of 2.8 p.p.m. C_3H_8 in N_2 gives a response of 0.66 μA.

SEIBEL, A. C.: A high temperature hydrogen flame ionization detector. A critical evaluation of design and operating characteristics. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 5, p. 50.

SMITH, V. N. AND FIDIAM, J. F.: Electron drift-velocity detector for gas chromatography. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 3, p. 50 — an argon detector with negative voltage pulse duration; sensitivity for $N_2 \sim 10^{-10}$ g/sec.

3b. Column performance and filling studies

CHOVIN, P.: Study of polar phases for use in gas chromatography. *J. Gas Chromatog.*, 2 (1964) 83–88 — β-cyanoethyl derivatives of polyols, thiols, phenols and thiophenols were studied.

HARUKI, T., MORII, T. AND SATO, K.: The effect of injection port temperature on peak broadening in gas chromatography. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 62, pp. 59–60.

PORTER, R. S., HINKINS, R. L., TORNHEIM, L. AND JOHNSON, J. F.: Computer optimization of mixed liquid phases for gas chromatography. *Anal. Chem.*, 36 (1964) 260–262 — a machine computation programme (IBM 7090) selects the best ratio of stationary phases for up to 50 compounds; the influence of mixed phases on retention of polar substances is not considered.

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SHAPRAS, P. AND CLAVER, G. C.: The use of polymers as liquid phase in the gas chromatographic analysis of high boiling point compounds. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 25, p. 53 — polystyrene, styrene–acrylonitrile copolymer and polycarbonate are used and advocated for temperatures up to 300° as stationary phases in glass columns.

TAKEUCHI, T. AND SETSUDA, T.: Support materials for use with lightly-loaded column for gas chromatography. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 1648–1651 (English summary A104) — celite 545, C-22 firebrick, α-alumina, carbon black, NaCl, glass beads and graphite were examined; C-22 with PEG-400 was the best.

3c. Apparatus, accessories and materials for GC

ANDREEN, B. H. AND KNIEBES, D. V.: Design and performance of a portable ionization chromatograph. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 80, p. 63 — for field estimation powered by rechargeable batteries.

ASHMEAD, H. L., COUCH, B. C. AND SCHMIT, J. A.: A continuous extractor for the analysis of trace gases in liquids. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 83, pp. 63–64.

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HOFFMANN, R. L. AND SILVEIRA, JR., A.: Multiple-port fraction collector for gas chromatography. *Anal. Chem.*, 36 (1964) 447–448 — detailed photos are given; recoveries for C_3–C_{11} alcohols are 95%.

JACOBSEN, J. K.: An ultra pure hydrogen generator for gas chromatography. *15th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 2–6, 1964*, Abstr. No. 78, p. 63 — hydrogen at a flow rate of 0–200 ml/min under 60 p.s.i.g. pressure is obtained by electrolysis of water and purified by diffusion through a hollow Pd–Ag tube as cathode; no N_2, water vapour and other impurities.

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Announcement

3. Internationales Fluorsymposium, München 1965

Das 3. Internationale Fluorsymposium findet vom 30. August bis 2. September 1965 in München statt und wird von der Gesellschaft Deutscher Chemiker organisiert. Der wissenschaftliche Teil wird von Herrn Prof. Dr. F. Weygand, Technische Hochschule München, bearbeitet.

Anmeldeformulare für die Teilnahme und zur Vorankündigung von Diskussionsvorträgen können bei der:

GDCh-Geschäftsstelle,

6000 Frankfurt/Main, Postfach 9075, Deutschland

angefordert werden. Dabei ist zu bemerken, dass diese Voranmeldungen bis zum 30. September 1964 bei der GDCh-Geschäftsstelle eingegangen sein müssen.

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TABLE 1

R_F VALUES OF SOME METAL IONS

(J. HALMEKOSKI AND F. SUNDHOLM, *Suomen Kemistilehti*, B 36 (1963) 63).

Solvents: Mixtures of aqueous HCl and ketones (10:90).
Paper: Whatman No. 1 and No. 42; descending.
Time of run: *ca.* 18 h with cyclohexanone; 4–7 h with methyl ethyl ketone.
The R_F values were calculated with respect to the liquid (ketone) front.

Ketone in solvent mixture and paper used	*Cation*	$R_F \times 100$									
		ml conc. HCl in 100 ml of the solvent mixture									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Methyl ethyl ketone, Whatman No. 1	Hg^{2+}	74	75		88		90		89		91
	Cu^{2+}	6	13		63		72		73		77
	Cd^{2+}	22	25		77		86		90		95
	Bi^{3+}	30	46		74		84		75		65
	As^{3+}	13	29		70		84		94		95
	Sn^{2+}	*	*		86		93		95		95
	Sb^{3+}	21	37		77		93		94		95
	Fe^{3+}	38	41		95		> 95		> 95		> 95
	UO_2^{2+}	10	22		77		87		90		90
Methyl ethyl ketone, Whatman No. 42	Hg^{2+}	84	87	*	*	83					
	Cu^{2+}	4	6	31	52	60					
	Cd^{2+}	16	28	38	66	70					
	Bi^{3+}	56	60	62	65	74					
	As^{2+}	5	10	27	59	70					
	Sn^{3+}	79	84	88	89	92					
	Sb^{3+}	26	49	48	80	83					
	Fe^{3+}	78	80	81	87	92					
	UO_2^{2+}	7	10	25	56	65					
Cyclohexanone, Whatman No. 1	Hg^{2+}			36		*	51	56	67	83	
	Cu^{2+}			20		28	37	40	54	60	
	Cd^{2+}			32		40	46	59	72	77	
	Bi^{3+}			*		46	53	60	68	79	
	As^{3+}			27		41	51	56	74	86	
	Sn^{2+}			83		86	88	85	87	88	
	Sb^{3+}			68		88	88	*	87	91	
	Fe^{3+}			80		75	80	92	90	89	
	UO_2^{2+}			30		38	42	49	70	80	

* Elongated spot.

TABLE 2

R_F VALUES OF SOME INORGANIC IONS

(R. P. BHATNAGAR AND N. S. POONIA, *Anal. Chim. Acta*, 30 (1964) 211)

Paper: Whatman No. 1.
Development: radial.
Solvent: Chloroform–acetone–amyl alcohol–conc. HCl (47.6:23.8:23.8:4.5).

Ion	R_F	*Ion*	R_F
WO_4^{2-}	0.00	Au^{3+}	1.00
MoO_4^{2-}	0.69	Pt^{4+}	0.77
UO_2^{2+}	0.28	Pd^{2+}	0.57
Zr^{4+}	0.00	Fe^{3+}	0.92
Th^{4+}	0.11	Cu^{2+}	0.74
VO^{2+}	0.13	Cd^{2+}	1.00
		Ag^{+}	0.10

TABLE 3

R_F VALUES OF INORGANIC IONS ON STYRENATED PAPER

(J. R. A. ANDERSON, S. DILLI, J. L. GARNETT AND E. C. MARTIN, *Nature*, 201 (1964) 772)

Solvent: *n*-Butanol saturated with 1 *N* HCl.
Paper: Whatman No. 41 with 18% (styrene) graft.

Ion	R_F	*Ion*	R_F
As^{3+}	0.70	Ni^{2+}	0.19
Sb^{3+}	0.72	Cd^{2+}	0.57
Sn^{2+}	0.82	Bi^{3+}	0.72
Fe^{3+}	0.26	Cu^{2+}	0.20
Al^{3+}	0.18	MoO_4^{2-}	0.42
Zn^{2+}	0.66	Be^{2+}	0.27
Au^{3+}	0.86	Co^{2+}	0.17
Hg^{2+}	0.81	Mn^{2+}	0.20

TABLE 4

R_F VALUES OF IODIDE, IODATE AND TELLURITE

(A. MOGHISSI, Kernforschungszentrum Karlsruhe, unpublished results)

Solvents: S_1 = Acetone–water–conc. ammonia (6:1:1).
S_2 = Acetone–water–conc. ammonia (6:2:1).
S_3 = Ethanol–water–conc. ammonia (6:2:1).
Paper: Whatman No. 1.

Compound	R_F		
	S_1	S_2	S_3
Iodide	0.95	0.95	0.71
Iodate	0.35	0.55	0.31–0.37
Tellurite	0.05	0.15	0.16

TABLE 5

HIGH VOLTAGE ELECTROPHORESIS OF INORGANIC ANIONS ON THIN LAYERS

(A. MOGHISSI, *Anal. Chim. Acta*, 30 (1964) 91)

Electrolyte: 0.1 *N* NaOH.
Thin layer: Kieselgel (Woelm or Merck).
Voltage: 45 V/cm.
Time: 2 min.

Ion	*Distance moved (mm)*	*Ion*	*Distance moved (mm)*
SCN^-	35	Br^-	60
SeO_3^{2-}	30 T	BrO_3^-	45
TeO_3^{2-}	22 T	NO_3^-	58
I^-	60	NO_2^-	55
IO_3^-	51	SO_4^{2-}	56
Cl^-	55	PO_4^{3-}	0
ClO_3^-	53		

T = Tailing.

TABLE 6

R_F VALUES OF SOME CHLOROAMMINEPLATINUM(II) COMPLEXES

F. BASOLO, M. LEDERER, L. OSSICINI AND K. H. STEPHEN, *Ric. Sci., Suppl.*, 32 (II-A) (1962) 485

Solvents: S_1 = *N*/10 HCl.
S_2 = 2 *N* HCl.
S_3 = Butanol–2 *N* HCl.
Paper: P_1 = Whatman No. 3 MM.
P_2 = Whatman No. 1.
Detection: D_1 = $SnCl_2$ + KI in HCl.
D_2 = Iodine vapours.

Compound	R_F			*Colour*		
				D_1		D_2
	S_1P_1	S_2P_1	S_3P_2	*Cold*	*Heating*	
$Pt(NH_3)_4^{2+}$	0.92	0.79	0.06	—	green (slowly)	red
$Pt(NH_3)_3Cl^+$	0.69	0.66	0.10	—		brown
cis-$Pt(NH_3)_2Cl_2^0$	0.70	0.67	0.23	red	—	brown
trans-$Pt(NH_3)_2Cl_2^0$	0.45–0.50	0.48	0.17	—	red	brown
$PtCl_4^{2-}$	0.91	—	0.58	red	—	brown
$PtCl_6^{2-}$	0.91	—	0.64	red	—	brown

TABLE 7

R_F VALUES OF SOME CHLOROPYRIDINEPLATINUM(II) COMPLEXES

(F. BASOLO, M. LEDERER, L. OSSICINI AND K. H. STEPHEN, *Ric. Sci.*, 32 (II-A) (1962) 485)

Solvents: S_1 = 2 *N* HCl.
S_2 = Butanol–*N* HCl.
S_3 = Benzene–hexane (9:1).
Paper: P_1 = Whatman No. 3 MM.
P_2 = Whatman No. 1.
P_3 = Whatman No. 1 impregnated with 10% dimethylformamide in ethanol.

Compound	R_F		
	S_1P_1	S_2P_2	S_3P_3
$[PtPy_4]Cl_2$	0.96	0.38	0
cis-$PtPy_2Cl_2$	0.87	0.61	0.13
trans-$PtPy_2Cl_2$	—	trail	0.81
cis-$[Pt(Py)_2(NH_3)_2]Cl_2$	—	0.02	0
trans-$[Pt(Py)_2(NH_3)_2]Cl_2$	—	0.03	0
cis-$Pt(NH_3)_2Cl_2$	0.67	—	0
trans-$Pt(NH_3)_2Cl_2$	0.48	0, 0.10	0

TABLE 8

R_F VALUES OF THE $[Pt^{II}Py_{4-x}(NH_3)_x]Cl_2$ SERIES

(F. BASOLO, M. LEDERER, L. OSSICINI AND K. H. STEPHEN, *Ric. Sci.*, 32 (II-A) (1962) 485.

Solvents: S_1 = Butanol–2 *N* HCl.
S_2 = 1.2 *N* HCl.
S_3 = 3.6 *N* HCl.
S_4 = 5 *N* HCl.
S_5 = 7.2 *N* HCl.
Paper: P_1 = Whatman No. 1.
P_2 = Amberlite SA-2 paper H^+ form.

Compound	R_F				
	P_1S_1	P_2			
		S_2	S_3	S_4	S_5
$[Pt(NH_3)_4]Cl_2$	0.06	0.03	0.29	0.49	0.66
trans-$[PtPy_2(NH_3)_2]Cl_2$	0.09	0.01	0.14	0.27	0.47
cis-$[PtPy_2(NH_3)_2]Cl_2$	0.06	0	0.16	0.32	0.57
$[PtPy_4]Cl_2$	0.41	0	0.06	0.11	0.25

TABLE 9

R_F VALUES (THIN LAYER) OF ORGANIC ACIDS

(H. GOEBELL AND M. KLINGENBERG, *Chromatographie Symposium II, 1962*, published by the Société Belge des Sciences Pharmaceutiques, Brussels, 1963, p. 153)

Solvents: S_1 = Ethanol–ammonia–water (8:2:1).
S_2 = Isobutanol–5 *M* formic acid (2:3).
Thin layer: Pure cellulose.

Compound	R_F	
	S_1	S_2
Citrate	0.05	0.31
cis-Aconitate	0.07, 0.10	0.29, 0.82
dl-Isocitrate	0.08, 0.39	0.28, 0.82
Glyoxalate	0.14	0.70
Oxalacetate	0.15	0.20
Malonate	0.17	0.64
l-Aspartate	0.18	0.08
l-Malate	0.21	0.46
l-Glutamate	0.24	0.14
Succinate	0.27	0.80
α-Ketoglutarate	0.31	0.51
Fumarate	0.32	0.89
l-Lactate	0.56	0.80
Pyruvate	0.60	0.78
Fluoroacetate	0.60	0.86
β-Hydroxybutyrate	0.66	0.88

TABLE 10

R_F VALUES (THIN LAYER) OF ISOMERIC $C_{12}H_8O_4$ COMPOUNDS

(M. J. D. VAN DAM, *Rec. Trav. Chim.*, 83 (1964) 31)

Technique: Thin-layer chromatography on silica gel.
Solvent: Toluene–95% acetic acid (1:1).
Detection: Iodine vapours or viewing under U.V. light.

Compound	R_F
3-Carboxy-6-phenyl-2-pyrone	0.71
Phenylpropargylidenemalonic acid	0.40
2-Carboxy-5-phenyl-2,4-pentadien-(1,4)-olide	0.63
6-Carboxy-3-phenyl-2-pyrone	0.58

TABLE 11

R_F VALUES (THIN LAYER) OF HOMOLOGOUS PHENOLS

(J. HALMEKOSKI AND H. HANNIKAINEN, *Suomen Kemistilehti*, B 36 (1963) 24)

Solvents: S_1 = Chloroform.
S_2 = Ethyl acetate.
S_3 = *n*-Propanol.
S_4 = Chloroform–methanol (99:1).
S_5 = Chloroform–methanol (90:10).
S_6 = Benzene–methanol (95:5).
S_7 = Benzene–ethyl acetate (3:7).
S_8 = Benzene–methanol–acetic acid (45:8:4).
S_9 = Benzene–dioxane–acetic acid (90:25:4).
S_{10} = *n*-Butyl ether (saturated with water)–acetic acid (10:1).
S_{11} = *n*-Butanol–benzene–water (1:40:40); organic phase.
S_{12} = Xylene–methyl ethyl ketone–formamide (25:25:1).
Thin layer: Kieselgel G (E. Merck AG), activated at 100–105° for 30 min.
Developing agent: Folin–Denis reagent, followed by 25% ammonia.

Homologous series	*Compound*	$R_F \times 100$											
		S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
I	Phenol	28	66	68	32	63	35	56	56	54	53	17	56
	4-Methylphenol	30			33	64	38	56	58	56	54	18	58
	4-Ethylphenol	32			34	65	40	57	58	57	55	18	59
	4-Propylphenol	24	69	71	35	66	42	58	59	57	56	19	61
II	Guaiacol	63	68	68	53	62	49	48	57	57	52	27	60
	4-Methylguaiacol	60				63	49	49	59	58	50	24	63
	4-Ethylguaiacol	59				63	50	50	60	60	50	23	67
	4-Propylguaiacol	57	68	66	53	64	52	51	62	62	54	26	73
III	Catechol	07	62	64	08	40	17	41	45	40	46		63
	4-Methylcatechol				09	42	19	42	47	41	46		64
	4-Ethylcatechol				10	44	20	43	49	42	48		64
	4-Propylcatechol	09	64	59	13	46	22	45	52	44	49		64
IV	Vanillin	37	58	53		66	36	34	55	49	26	13	44
	4-Acetoguaiacone	33	56	53		69	34	35	51	49	21	10	41
	4-Propioguaiacone	38	61	59		71	40	39	58	54	31	15	46
V	Protocatechuic aldehyde		51	54	03	39	07	30	42	28	23		31
	4-Acetocatechol		50	56	05	42	07	33	41	28	18		30
	4-Propiocatechol		52	57	06	45	10	35	46	35	26		34

TABLE 12

R_F VALUES (THIN LAYER) OF HOMOLOGOUS PHENOLS

(J. HALMEKOSKI AND H. HANNIKAINEN, *Suomen Kemistilehti*, B 36 (1963) 24)

Solvents: S_1 = Chloroform.
S_2 = Ethyl acetate.
S_3 = *n*-Propanol.
S_4 = Chloroform–methanol (99:1).
S_5 = Chloroform–methanol (90:10).
S_6 = Benzene–methanol (95:5).
S_7 = Benzene–ethyl acetate (3:7).
S_8 = Benzene–methanol–acetic acid (45:8:4).
S_9 = Benzene–dioxane–acetic acid (90:25:4).
S_{10} = *n*-Butyl ether (saturated with water)–acetic acid (10:1).
S_{11} = *n*-Butanol–benzene–water (1:40:40); organic phase.
S_{12} = Xylene–methyl ethyl ketone–formamide (25:25:1).
Thin layer: Polyamide (Woelm AG).
Developing agent: Folin–Denis reagent, followed by 25% ammonia.

Homologous series	*Compound*	$R_F \times 100$											
		S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}
I	Phenol	32			36	66	37	64	72	66	64	22	68
	4-Methylphenol	36			41	74	40	68	74	71	70	26	71
	4-Ethylphenol	42			48	78	45	71	77	76	76	31	74
	4-Propylphenol	46			53	84	51	75	79	81	83	37	77
II	Guaiacol	89				88	77		83	86	78	75	85
	4-Methylguaiacol	90				90	79		86		80	81	87
	4-Ethylguaiacol	92				91	81		88		82	82	88
	4-Propylguaiacol	93				91	83		89	88	85	84	90
III	Catechol	05	52	64		37	11	35	42	37	37	04	41
	4-Methylcatechol		47	66		49	14	36	47	42	42	05	45
	4-Ethylcatechol		44	70		54	17	38	54	47	49	06	49
	4-Propylcatechol	10	43	73		58	22	42	59	53	57	07	54
IV	Vanillin	76			72	88	52	66	81		53	47	67
	4-Acetoguaiacone	75			76	89	56	68	84		54	50	69
	4-Propioguaiacone	79			78	91	63	70	87		63	61	74
V	Protocatechuic aldehyde		24	57		34	02	14	40	14	14		27
	4-Acetocatechol		29	62		43	03	18	46	18	15		28
	4-Propiocatechol		32	66		47	05	22	50	22	21		32

TABLE 13

R_F VALUES OF HOMOLOGOUS PHENOLS

(J. HALMEKOSKI AND H. HANNIKAINEN, *Suomen Kemistilehti*, B 36 (1963) 25)

Solvents: S_1 = Xylene–methyl ethyl ketone–formamide (25:25:1); paper treated with formamide.
S_2 = Xylene; paper treated with formamide.
S_3 = Chloroform; paper treated with formamide.
S_4 = Petroleum ether (boiling range 100–140°)–xylene–formamide (96:2:2); paper treated with formamide.
S_5 = *n*-Butyl ether; paper treated with formamide.
S_6 = Cyclohexane; paper treated with formamide.
S_7 = Cyclohexane-pyridine (25:1); paper treated with formamide.
S_8 = Cyclohexane; paper treated with dimethylformamide.
S_9 = Cyclohexane–pyridine (25:1); paper treated with dimethylformamide.
S_{10} = Xylene–dimethylformamide (9:2); paper treated with dimethylformamide.
S_{11} = *n*-Butanol–benzene–water (1:40:40); organic phase.
S_{12} = *n*-Butanol saturated with 2 per cent ammonia.
S_{13} = Methyl ethyl ketone–water–diethylamine (921:77:2).

Paper: Whatman No. 1.

Homologous series	*Compound*	$R_F \times 100$												
		S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}
I	Phenol	69		45			05	26	08	19	79			
	4-Methylphenol	77		62			08	37	11	24	81			
	4-Ethylphenol	84		76			14	53	17	32	83			
	4-Propylphenol	90		85			27	70	23	41	87			
II	Guaiacol	56	58		31	65		40						
	4-Methylguaiacol	72	71		47	75		53						
	4-Ethylguaiacol	83	82		68	84		65						
	4-Prophylguaiacol	92	89		80	89		78						
III	Catechol	12		05		13		03				24		
	4-Methylcatecol	21		12		21		04				39		
	4-Ethylcatechol	36		22		36		11				61		
	4-Prophylcatechol	54		38		55		23				77		
IV	Vanillin	44	21	69		15						81	40	85
	4-Acetoguaiacone	44	24	80		16						80	44	89
	4-Propioguaiacone	65	48	89		37						91	59	91
V	Protocatechuic aldehyde	10		02								07	30	80
	4-Acetocatechol	09										10	43	88
	4-Propiocatechol	17		07								27	34	93

TABLE 14

R_F VALUES OF CATECHOLS

(J. HALMEKOSKI AND A. NISSEMA, *Suomen Kemistilehti*, B 35 (1962) 188)

Solvents: S_1 = Water saturated ethyl ether.
S_2 = Water saturated isopropyl ether.
S_3 = Water saturated *n*-butyl ether.
S_4 = Water saturated *n*-amyl ether.
Paper: Whatman No. 1.

Compound	R_F			
	S_1	S_2	S_3	S_4
Catechol	0.96	0.75	0.68	0.50
4-Methylcatechol	0.96	0.81	0.77	0.67
4-Ethylcatechol	0.96	0.86	0.87	0.83
4-Propylcatechol	0.97	0.88	0.92	0.89

TABLE 15

R_F VALUES (THIN LAYER) OF ADRENALINE DERIVATIVES

(J. HALMEKOSKI, *Suomen Kemistilehti*, B 36 (1963) 58)

Solvents: S_1 = *n*-Butanol satured with aqueous sulphur dioxide solution (H_2SO_3).
S_2 = *n*-Butanol–acetic acid–H_2SO_3 (4:1:5).
S_3 = *n*-Amyl alcohol–acetic acid–ethanol–H_2SO_3 (4:1:1:5).
S_4 = *n*-Butanol–*n*-propanol–acetic acid–H_2SO_3 (4:1:1:5).
Thin-layer: Kieselgel G, buffered to pH 4 or with additions of sodium salts of chelate-forming anions.
Temperature: 19–21°.
Developing agent: Folin–Denis reagent.

Solvent system	*Layer treated with*	$R_F \times 100$								
		Adrenaline	*Oxedrine*	*Noradrenaline*	*Adrenone*	*Corbadrine*	*Oxamphetamine*	*Isoprenaline*	*Metaoxedrine*	*3-Hydroxytyramine*
S_1	—	27	35	41	38	57	69	45	44	53
	Na_2MoO_4	06	40	12	06	14	71	11	47	17
	Na_2WO_4	05	48	06	05	12	72	14	53	12
	Borax	15	38	14	07	18	51	20	40	28
S_2	—	33	42	52	41	56	64	48	48	54
	Na_2MoO_4	10	40	13	10	21	66	21	50	25
	Na_2WO_4	18	45	17	08	27	71	34	53	29
	Borax	27	56	30	17	37	73	40	61	46
S_3	—	27	30	34	27	38	48	31	34	37
	Na_2MoO_4	05	35	14	06	19	56	17	39	19
	Na_2WO_4	09	36	17	08	22	60	20	40	21
	Borax	13	34	20	16	22	60	20	36	23
S_4	—	42	45	52	45	60	67	53	51	56
	Na_2MoO_4	22	47	25	16	31	64	24	47	24
	Na_2WO_4	24	50	30	19	36	64	31	52	31
	Borax	28	55	36	24	42	73	46	61	42

TABLE 16

R_F VALUES (ALSO THIN LAYER) OF SULPHAMIDES

(M. TH. VAN DER VENNE AND J. B. T'SIOBBEL, *Chromatographie Symposium II*, 1962, published by the Société Belge des Sciences Pharmaceutiques, Brussels, 1963, p. 196)

Solvents: A = Isopropanol–25% ammonia (10:2.5). B = Methyl acetate–methanol–25% ammonia (80:8:15). C = Methyl ethyl ketone–methanol–25% ammonia (80:16:19). D = Chloroform–methanol–25% ammonia (80:45:15). E = Methyl isobutyl ketone–acetone–25% ammonia (25:100:25). F = Isopropanol–amyl acetate–25% ammonia (5:2:2). G = Isoamyl acetate–pyridine–25% ammonia (70:75:20). H = Isoamyl acetate–pyridine–1% ammonia (80:15:60). DB′ = Chloroform–ethyl acetate–methanol–25% ammonia (40:40:40:16). B′ = Ethyl acetate–methanol–25% ammonia (80:18:15). C′ = Methyl isobutyl ketone–methanol–25% ammonia (80:30:17). E′ = Chloroform–acetone–25% ammonia (18:100:20).

Paper: Whatman No. 1.

Thin-layer systems: KB′ = Ethyl acetate–methanol–25% ammonia (85:30:25); Kieselgel G. AlB′ = Ethyl acetate–methanol–25% ammonia (85:15:15); Alumina G. KE = Methyl isobutyl ketone–acetone–25% ammonia (25:100:25); Kieselgel G. PE = Methyl isobutyl ketone–acetone–25% ammonia (25:100:5); Polyamide. PB′ = Ethyl acetate–methanol–25% ammonia (85:15:5); Polyamide.

Compound	$R_F \times 100$																
	A	B	C	D	E	F	G	H	DB′	B′	C′	E′	KB′	AlB′	KE	PE	PB′
Sulfacetamide	35	16	45	35	35	29	16	9	29	12	15	20	—	19	38	12	7
Sulfanilamide	59	97	98	71	95	72	72	60	76	95	83	95	97	96	79	84	73
Sulfathiazol	42	44	64	48	63	45	25	20	44	27	32	50	80	42	57	18	12
Sulfapyridine	43	65	68	65	63	44	57	67	58	53	50	54	77	28	55	84	69
Sulfathiourea	24	18	40	20	42	20	17	15	19	11	11	29	60	28	49	10	5
Sulfaguanidine	48	77	83	54	73	55	46	32	54	64	50	76	50	78	64	47	33
N,N-Dimethyl-acroyl-sulfanilamide	52	39	58	66	56	49	32	27	50	31	36	40	74	38	54	22	12
Sulfadiazine	27	18	33	39	34	25	17	22	29	12	12	18	49	19	38	30	13
Sulfamerazine	31	28	43	52	42	33	26	36	38	18	18	27	59	25	45	49	25
Sulfamethazine	41	44	53	67	53	43	41	53	51	34	32	35	69	35	51	67	46
Cinnamein sulfanilamide	60	97	97	72	95	72	72	60	77	95	82	95	97	96	79	85	74
Sulfaethylthiodiazole	50	47	70	67	64	48	26	13	55	34	33	48	80	41	61	14	13
Maleylsulfanilamide	44	37	56	36	52	36	25	14	34	24	22	35	81	34	61	13	8
Succinylsulfanilamide (sodium salt)	25	14	24	21	29	21	11	8	18	8	11	12	6	12	39	4	6
N^1-Benzoyl-sulfanilamide	53	55	73	68	68	50	30	20	60	46	46	55	85	50	59	10	7
Phthalylsulfacetamide	19	3	10	18	14	15	4	3	13	2	5	4	26	6	33	0	0
Phthalylsulfathiazole	23	10	20	25	28	24	7	5	18	6	9	11	50	6	44	1	0
4-Homosulfanilamide salt of 1-sulfanilyl-2-thiourea	23	18	39	21	44	20	19	12	20	10	14	30	60	28	49	10	5

sulfanilamide																	
Sulfisomidine	47	36	49	64	46	42	20	16	45	31	28	33	74	39	48	20	17
N^4-Carbethoxysulfoethyl thiodiazole	76	77	92	94	73	73	35	34	72	59	63	81	88	63	61	24	23
Sulfisoxazole	53	46	66	63	58	49	23	18	46	25	30	45	77	34	53	8	7
Sulfanilamido-2-phenyl-pyrazole	63	59	84	79	70	59	36	22	57	43	52	72	86	56	57	13	9
N^1-Isopropoxysulfanilamide	65	77	86	92	71	63	37	25	69	53	55	74	92	58	61	18	14
Sulfanilamido-4,5-dimethyloxazole	47 13	48 10	63 13	69	55 17	46 15	22 6	22 6	49 13	28 3	37 7	44	47 +t	—	58 +t	25 6	17 5
Sulfathiazole-formaldehyde	57 27	71 9	79 15	90 26	69 14	63 23	36 6	25 4	67 22	30 6	56 15	67 9	—	—	—	0	6 23
2-Sulfanilamido-3-methoxypyrazine	34	36	49	57	44	36	25	26	42	22	25	36	63	34	50	25	17
N^1-Acetyl-2-sulfanilamido-3-methoxypyrazine	35	t	t	t	t	38	t	t	t	t	t	t	76	t	54	97	t
Sulfa-4,6-dimethoxy-pyrimidine	45	64	69	73	63	48	45	62	62	46	49	35	85	46	57	58	45
2,4-Dimethyl-6-sulfanil-amido-1,3-diazine	45	54	68	58	62	42	28	34	60	44	37	69	81	45	57	57	25
2-Sulfanilamido-5-methylpyrimidine	29	30	41	53	39	30	27	32	39	14	22	27	59	26	45	50	26
2-Sulfanilamido-5-methoxydiazine	26	29	38	50	39	25	28	37	36	15	26	27	60	25	45	35	35
3-Sulfanilamido-6-methoxypyridazine	37	40	48	68	47	46	30	34	47	22	34	32	73	36	50	47	30
N^1-Acetyl-3-sulfanilamido-6-methoxypyridazine	38	t	t	t	t	40	t	t	t	t	t	t	70	t	54	97	t

t = tailing.

TABLE 17

R_F VALUES (ALSO THIN LAYER) OF PYRONES AND THIOPYRONES

(C. PÁRKÁNYI AND R. ZAHRADNÍK, *Collection Czech. Chem. Commun.*, 27 (1962) 1355)

Solvents: S_1 = Carbon tetrachloride–acetic acid (4:1).
S_2 = Diethyl ether.
A: Paper Whatman No. 1, descending (except where otherwise stated).
B: Thin-layer chromatography on Al_2O_3 (neutral, activity III).
Detection: 0.1 *N* $KMnO_4$ acidified with H_3PO_4.

Compound	R_F	
	A, S_1	*B*, S_2
γ-Pyrone	0.55 0.41*	0.15
γ-Thiapyrone	0.53	0.09
γ-Thiopyrone	0.74	0.67
γ-Thiothiapyrone	0.72	0.61
α-Thiothiapyrone	0.81	0.71
3-Nitro-γ-pyrone	0.08**	---

* Ascending.
** Ascending; detection: visual.

TABLE 18

R_F VALUES OF SOME MONOSUBSTITUTED PURINES

(F. G. STANFORD, The Radiochemical Centre, Amersham, Bucks., England, unpublished results, July, 1963)

Solvents: S_1 = *n*-Butanol–water–conc. ammonia (172:18:10).
S_2 = *n*-Butanol–*N* sodium acetate–*N* HCl (7:120:60).
S_3 = Ethyl acetate satd. pH 6 phosphate buffer (lower phase).
Paper: Whatman No. 1 (ascending for *ca.* 16 hours).
Temperature of run: Room (*ca.* 20°).
Detection: U.V. light (250–280 mμ).
Samples: 25 μg per spot in dilute ammonia.

Compound	R_F			*U.V. colour*
	S_1	S_2	S_3	
6-(Furfurylamino)-purine	0.90	Streaks	Streaks	White
6-Chloropurine	0.50	0.70	0.75	Yellow
6-Mercaptopurine	0.07	0.47	0.50	Yellow-green
6-Hydroxypurine (hypoxanthine)	0.07	0.66	0.70	Grey
6-Aminopurine (adenine)	0.25	0.54	0.53	Grey

TABLE 19

R_F VALUES (THIN LAYER) OF VARIOUS AMINO ACIDS

(M. BRENNER AND A. NIEDERWIESER, *Experientia*, 16 (1960) 378)

Solvents: S_1 = 96% Ethanol–water (63:37).
S_2 = Propan-1-ol–water (64:36).
S_3 = Butan-1-ol–acetic acid–water (60:20:20).
S_4 = Phenol–water (75:25).
S_5 = Propan-1-ol–34% NH_3 (67:33).
S_6 = 96% Ethanol–34% NH_3 (77:23).
Thin layer adsorbent: Silicic acid (Kieselgel G, Merck; air dried overnight).
Detection: Ninhydrin (E. D. MOFFAT AND R. I. LYTLE, *Anal. Chem.*, 31 (1959) 926).

Compound	$R_F \times 100$					
	S_1	S_2	S_3	S_4	S_5	S_6
α-Aminocaproic acid	66	65	60	69	58	60
Tryptophan	65	62	56	63	55	58
Phenylalanine	63	58	49	55	54	60
Norleucine	61	57	49	52	53	59
Isoleucine	60	53	46	49	52	58
Leucine	61	55	47	48	53	58
Tyrosine	65	57	47	47	42	51
Dihydroxyphenylalanine	—	—	45	34	—	—
Methionine	59	51	40	49	51	60
Norvaline	56	50	38	42	49	57
Valine	55	45	35	40	48	56
Alanine	47	37	27	29	39	40
β-Alanine	33	26	27	30	30	29
Threonine	50	37	25	26	37	40
Serine	48	35	22	20	27	31
Glycine	43	32	22	24	29	34
Hydroxyproline	44	34	20	38	28	31
Proline	35	26	19	50	37	30
Sarcosine	31	22	17	37	34	31
Cysteic acid	69	50	14	04	17	21
Glutamic acid	63	35	27	10	14	15
Aspartic acid	55	33	21	06	09	07
Histidine HCl	33	20	06	32	38	42
Lysine HCl	03	02	05	09	18	11
Arginine HCl	04	02	08	19	10	06
Cystine	39	32	16	12	27	22

TABLE 20

R_F VALUES OF N^{ε}-(1-HYDROXY-2-ACETAMIDO-4-FLUORENYL)-DL-LYSINE AND RELATED COMPOUNDS

(C. C. IRVING AND H. R. GUTMANN, *J. Org. Chem.*, 26 (1961) 1859)

Solvents: S_1 = Cyclohexane–*tert.*-butanol–pyridine–water (16:2:2:1) (J. H. WEISBURGER, E. K. WEISBURGER, H. P. MORRIS AND H. A. SOBER, *J. Natl. Cancer Inst.*, 17 (1956) 363).
S_2 = 70% methanol.
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
S_4 = *tert.*-Butanol–formic acid–water (70:15:15).
S_5 = Methyl ethyl ketone–propionic acid–water (75:25:30).
S_6 = Cyclohexane–*tert.*-butanol–pyridine–water (16:2:2:1).
Paper: P_1 = Whatman No. 1 (ascending).
P_2 = Whatman No. 1 (descending).
Time of run: T_1 = 7–8 h.
T_2 = 15–18 h.
Detection: D_1 = Folin–Ciocalteau reagent followed by 20% aq. Na_2CO_3 spray.
D_2 = 0.3% ninhydrin in ethanol.

Compound	R_F							
	P_1T_1							P_2T_2
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
N^{α}-Tosyl-N^{ε}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine benzyl ester	0.82	0.87	0.96					
N^{ε}-(1-Hydroxy-2-acetamido-4-fluorenyl)-DL-lysine				0.55	0.51	0.00	0.98	0.53
DL-Lysine					0.10			0.20

TABLE 21

ELECTROPHORETIC MOBILITIES OF N^{ε}-(1-HYDROXY-2-ACETAMIDO-4-FLUORENYL)-DL-LYSINE AND LYSINE

(C. C. IRVING AND H. R. GUTMANN, *J. Org. Chem.*, 26 (1961) 1859)

Electrolyte: 0.25 *N* acetic acid.
Paper: Whatman No. 3 MM.
Apparatus: Beckman Spinco Model R (Duostat power supply).
Potential applied: 200 V (about 2.2 mA).
Time of run: 2.5 h.
Mobility: *M* = cm/sec per V/cm.
Detection: D_1 = Folin–Ciocalteau reagent followed by 20% aq. Na_2CO_3 spray.
D_2 = 0.3% ninhydrin in ethanol.

Compound	*M*
N^{ε}-(1-Hydroxy-2-acetamido-4-fluorenyl)-DL-lysine	6.7
DL-Lysine	15

TABLE 22

R_F VALUES OF FLUORENYLCYSTEINE DERIVATIVES

(E. K. WEISBURGER AND R. E. BOYD, *J. Chem. Soc.*, (1964) 515)

Solvents: S_1 = Butan-2-ol–3 % aq. ammonia (3:1).
S_2 = Butan-1-ol–acetic acid–water (4:1:5).
S_3 = Propan-1-ol–2 *N* aq. ammonia (1:9).
Paper: Whatman No. 3 MM.
Developing agent: Ninhydrin (for cysteines), dichromate–silver nitrate (for cysteines and mercapturic acids).

Compound	R_F*		
	S_1	S_2	S_3
S-9-Fluorenylcysteine	0.62–0.73	0.82–0.88	0.80–0.87
S-(4-Methylenefluorenyl)-cysteine	0.61–0.72	0.82–0.84	0.87–0.90
S-2-Biphenylylcysteine		0.84–0.89	0.88–0.93
S-9-Fluorenylmercapturic acid	0.64–0.79	0.83–0.92	0.90–0.92
S-(4-Methylenefluorenyl)-mercapturic acid	0.64–0.80	0.81–0.92	0.85–0.92
S-2-Biphenylylmercapturic acid		0.83–0.91	0.90–0.94

* Measured from back to front of spot.

TABLE 23

R_F VALUES (THIN LAYER) OF FLUORENYLCYSTEINE DERIVATIVES

(E. K. WEISBURGER AND R. E. BOYD, *J. Chem. Soc.*, (1964) 515)

Solvents: S_1 = *m*-Cresol saturated with 0.1 *M* borate buffer, pH 8.2.
S_2 = Chloroform–butan-2-ol–0.05 *M* sodium benzoate (60:36:4).
S_3 = Propan-1-ol–light petroleum (b.p. 30–60°) (1:1) saturated with 0.3 *M* acetate buffer, pH 6.
Thin layer: Silica gel G.

Compound	R_F*		
	S_1	S_2	S_3
S-9-Fluorenylcysteine	0–0.10	0	0
S-(4-Methylenefluorenyl)-cysteine	0–0.10	0	0
S-2-Biphenylylcysteine	0–0.10	0	0
S-9-Fluorenylmercapturic acid	0.14–0.37	0–0.08	0.11–0.19
S-(4-Methylenefluorenyl)-mercapturic acid	0.14–0.37	0–0.08	0.10–0.18
S-2-Biphenylylmercapturic acid	0.14–0.37	0–0.08	0.10–0.16

* R_F values were not reproducible from one run to another, but the compounds showed the same movement relative to each other.

TABLE 24

R_F VALUES OF SYNTHETIC SULPHUR-CONTAINING AMINO ACIDS

(S. OOMORI AND S. MIZUHARA, *Arch. Biochem. Biophys.*, 96 (1962) 179)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:4, v/v).
S_2 = Phenol–water (4:1, v/v; 0.2% NH_3).
Paper: Not specified.
Detection: Not specified.

Compound	R_F*	
	S_1	S_2
(+) and (—)-Isovalthines [S-(isopropyl-carboxylmethyl)-cysteine]	0.61, 0.605	0.46, 0.465
S-(1,1-Dimethyl-2-carboxyethyl)-L-cysteine	0.55	0.53
S-(1,2-Dimethyl-2-carboxyethyl)-L-cysteine	0.58	0.52
S-(Ethyl-2-carboxyethyl)-L-cysteine	0.59	0.47
S-(Propylcarboxymethyl)-L-cysteine	0.62	0.49
S-(2-Methyl-3-carboxypropyl)-L-cysteine	0.57	0.45

* Somewhat variable according to conditions but the relative positions of the spots are constant.

TABLE 25

R_F VALUES OF SOME PEPTIDES

(E. WALTON, J. O. RODIN, C. H. STAMMER AND F. W. HOLLY, *J. Org. Chem.*, 26 (1961) 1657)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, upper phase).
S_2 = Butan-1-ol–1.5 *N* NH_4OH (1:1, upper phase).
S_3 = Methyl ethyl ketone–pyridine–water (4:1:1.6).
Paper: Whatman No. 1, 32 cm diameter (circular).
Detection: D_1 = Ninhydrin.
D_2 = Diazotised sulphanilic acid.

Compound	R_F			*Detection*	
	S_1	S_2	S_3	D_1	D_2
L-Val-L-Tyr-L-Tyr-L-Isoleu-L-His-L-Pro-L-Phe-OMe·diHCl	0.80		1.0	+	
L-Val-L-Tyr-L-Tyr	0.70		0.80	+	
L-Isoleu-L-His-L-Pro-L-Phe	0.70		0.90	+	
N-Cbz-L-Val-L-Tyr-L-Tyr		0.5–0.7			+

TABLE 26

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF KETONES AND KETO ACIDS

(C.-C. LIANG, *Biochem. J.*, 82 (1962) 429)

Solvent: Butan-1-ol–ethanol–aq. 2 *N* NH_3 soln. (7:1:2, v/v).
Paper: Not specified.
Time of run: 20 h.
Detection: Alkali reagent (air-dried paper dipped in 2% NaOH in 90% ethanol).

2,4-Dinitrophenylhydrazone of	R_F*			*Colour***		
	I	*II*	*III*	*I*	*II*	*III*
Dihydroxyacetone	0.92	—	—	B	—	—
Acetone	0.97	—	—	B	—	—
Glyoxal	0.31	0.50	—	S	O	—
Methylglyoxal	0.00	0.80	0.87	B-V	O	P-V
α-Oxoglutaric acid***	0.50	—	—	Ol-B	—	—
Oxaloacetic acid***	0.13	0.38	0.55	dB	C	Ol-Y
Glyoxylic acid***	0.24	0.45	—	S	Y-B	—
Pyruvic acid***	0.33	0.55	—	C	Ol-Y	—
Acetoacetic acid***	0.36	—	—	Y-C	—	—
Phenylpyruvic acid***	0.70	—	—	B	—	—
α-Oxoisovaleric acid***	0.72	—	—	Y-B	—	—
α-Oxo-β-methylvaleric acid***	0.75	—	—	B	—	—
α-Oxoisocaproic acid***	0.75	—	—	B	—	—
Free 2,4-dinitrophenylhydrazine	0.88	0.95	—	Y-B	Y-B	—

* I, II and III denote isomeric forms of the hydrazones of the same keto compound.
** B = brown; C = chocolate; O = orange; Ol = olive; V = violet; P = pinkish; S = salmon; Y = yellow; d = dark.
*** Found in blood.

TABLE 27

R_F VALUES OF TRIMETHYLHEXANOIC AND TRIMETHYLHEXANEDIOIC ACIDS

(R. H. MAZUR, *J. Org. Chem.*, 26 (1961) 1289)

Solvents: S_1 = Butan-1-ol–acetic acid–water (5:2:3).
S_2 = Methyl ethyl ketone–pyridine–water (60:15:25).
S_3 = Propan-2-ol–conc. NH_4OH–water (7:1:2).
Paper: Not specified.
Detection: Ninhydrin for 6-amino-derivatives.

Compound	R_F		
	S_1	S_2	S_3
6-Amino-3,3,5-trimethylhexanoic acid	0.79	0.55	
2,4,4-Trimethylhexanedioic acid			0.41
6-Amino-3,5,5-trimethylhexanoic acid	0.75	0.58	
2,2,4-Trimethylhexanedioic acid			0.41

TABLE 28

R_F VALUES (THIN LAYER) OF LIPIDS AND RELATED SUBSTANCES PRESENT IN BRAIN

(H. JATZKEWITZ AND E. MEHL, *Z. Physiol. Chem.*, 320 (1960) 251)

Solvents	*Length of run (cm at 20°)*	*Time of run*
S_1 = *n*-Heptane (pre-saturated chamber)	10	0.5 h
S_2 = Carbon tetrachloride	18	3.5 h
S_3 = Ethylene dichloride (pre-saturated chamber)	15	50 min
S_4 = Chloroform (pre-saturated chamber)	15	1 h
S_5 = Ethylene dichloride–methanol (98:2)	15	1.5 h
S_6 = Petrol ether (b.p. 40–70°)–ether (70:30)	15	1 h
S_7 = Chloroform–glacial acetic acid (96:4)	10	1.5 h
S_8 = Chloroform–methanol (95:5)	10	50 min
S_9 = Ether	10	80 min
S_{10}* = (a) Propan-1-ol–12.5% NH_3 (80:20) with at least 12 h prior chamber saturation	8	1.75 h
(b) S_5 with at least 12 h prior chamber saturation	17	1.75 h
(c) Chloroform–96% acetic acid (95:5) (separation occurs immediately following solvent addition; R_F is calculated for a 17 cm run)	10.5	1.25 h
(a), (b) and (c) used sequentially.		

* A run prior to spotting carried out with S_5 to remove background.

Thin-layer adsorbent: Silicic acid (Kieselgel G, Merck).

Detection: D_1 = Bromothymol blue reagent (50 mg bromothymol blue + 1.25 g boric acid + 8 ml *N* NaOH + 112 ml H_2O), or

D_2 = Modified bromothymol blue reagent (40 mg bromothymol blue + 100 ml 0.01 *N* NaOH).

*Compound***	$R_F \times 100$***									
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}
Squalene	64									
Cholesterol lignocerate		57								
Cholesterol stearate	0	50	75	79	94	—	—	—	—	94
Cholesterol oleate		43								
Cholesterol linolenate		32								
Palmitic acid	0	27	53	70	91	92	—	—	—	
Lignoceric acid methyl ester	0	27	58	70	91	92	—	—	—	
Tripalmitin–triolein	0	0	41	75	94	91	—	—	—	80
Cerebronic acid methyl ester	0	0	18	38	80	47	—	—	—	78
α-Hydroxystearic acid methyl ester	0	0	15		77					
Distearin				27	75	29	92	95	—	
Diolein	0	0	7	(15)	(59)	(21)	(89)	(91)	—	73
Cholesterol	0	0	12	21	50	25	88	91	90	67
C_{16}–C_{24} fatty acids	0	0	0–9	0–9	0–18	0–22	90	15–60	10–77	56
Glycerol monostearate	0	0	0	2	9	0	29	47	54	47
Ceramide	0	0	0	0	0	0	10	28	10	43
Cerebronic acid							31			
α-Hydroxystearic acid	0	0	0	0	0	0	27	0	(4)	41

** About 10 μg used.

*** R_F values are reproducible under the same conditions.

TABLE 29

R_F VALUES (THIN LAYER) OF BRAIN PHOSPHOLIPIDS

(H. JATZKEWITZ AND E. MEHL, *Z. Physiol. Chem.*, 320 (1960) 251)

Solvents: S_1 = Propan-1-ol–12.5 % NH_3 (80:20) with at least 12 h prior saturation of chamber.
S_2 = Propan-1-ol–17 % NH_3 (70:30) with at least 12 h prior saturation of chamber.
S_3* = (a) S_1.
(b) Ethylene dichloride–methanol (98:2) with at least 12 h prior saturation of chamber.
(c) Chloroform–96 % acetic acid (95:5) (immediate separation occurs on addition of solvent; R_F calculation to a 17 cm front).
(a), (b) and (c) used sequentially.
Thin-layer adsorbent: Silicic acid (Kieselgel G).
Time of run: 3.5 h (S_1); 3.75 h (S_2); 1.75 h ($S_{3(a)}$).
Length of run: 10 cm (S_1, S_2); 8 cm ($S_{3(a)}$).
Detection: D_1 = Bromothymol blue reagent.
D_2 = Ammonium molybdate–vanadium chloride reagent.
D_3 = Diphenylamine reagent.

*Compound***	$R_F \times 100$		
	S_1	S_2	S_3
Ceramide	85		43
Cerasin sulphate	69	66	29
Cerebrone sulphate	61	66	25
Cerasin (nervone?)	47	20–60	20
Cerebrone (hydroxynervone?)	40	20–60	20
Lecithin	25	53	15
Sphingomyelin	13	40	11
Ganglioside fraction	0	11	
		(18)?	0–3
		24	
Cephalin	0	0	0

* A run with ethylene dichloride–methanol (98:2) carried out before spotting so as to remove background.

** *cf.* H. JATZKEWITZ, *Z. Physiol. Chem.*, 320 (1960) 134, for the classification of certain fractions. About 10 μg sample used.

TABLE 30

R_F VALUES (THIN LAYER) OF VARIOUS SUGARS

(G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427)

Solvents: S_1 = Benzene–methanol–acetic acid (1:3:1).
S_2 = Methyl ethyl ketone–methanol–acetic acid (3:1:1).
Thin layer adsorbent: Silicic acid (Kieselgel G, Merck).
Detection: Naphthoresorcinol reagent.

Compound	R_F		*Colour**	
	S_1	S_2	S_1	S_2
D(+)-Glucose	0.63	0.42	bv	v
D(+)-Mannose	0.58	0.32	lb	bgn
D(+)-Galactose	0.55	0.32	bv	ggn
L(+)-Arabinose	0.62	0.42	bgn	bgn
D(+)-Xylose	0.59	0.39	lb	b
L(+)-Rhamnose	0.67	0.52	gn	bgn
D(—)-Fructose	0.52	0.31	blr	rbn
L(—)-Sorbose	0.51	0.24	r	rbn
Sucrose	0.63	0.29	r	rbn
Lactose	0.56	0.25	rv	gb
D(+)-Glucuronic acid	0.48	0.34	b	b
D(+)-Galacturonic acid	0.36	0.10	b	bgn

* b = blue; bl = black; bn = brown; g = grey; gn = green; l = light; r = red; v = violet.

TABLE 31

R_F VALUES OF CEREAL PHENOLIC COMPOUNDS

(M. S. BARDINSKAYA AND T. A. SHUBERT, *Biokhimiya*, 27 (1962) 58)

Solvent: Butan-1-ol–acetic acid–water (3:2:95).
Paper: Not specified.
Detection: D_1 = U.V. light (fluorescence).
D_2 = Diazotised sulphanilic acid.
D_3 = Phloroglucinol in conc. HCl.

Compound	R_F	*Colour**		
		D_1	D_2	D_3
trans-Ferulic acid	0.31	B	VB	n
cis-Ferulic acid	0.58	B	VB	n
trans-*p*-Coumaric acid	0.46	wBL	R	n
cis-*p*-Coumaric acid	0.72	wBL	R	n
Caffeic acid	0.28	bB	GBr	n
Vanillic acid	0.51	n	O	n
p-Hydroxybenzoic acid	0.61	n	Y	n
Synapaldehyde	0.39	wYG	Re	L
Coniferylaldehyde	0.44	BG	Y	RL
Vanillin	0.62	n	n	O
p-Hydroxybenzaldehyde	0.73	n	n	YBr

* B = blue; Br = brown; G = green; L = lilac; O = orange; R = red; Re = rose; Y = yellow; b = brilliant; w = weak; n = negative.

TABLE 32

R_F VALUES (THIN LAYER) OF CERTAIN PHENOLS AND PHENOLIC ACIDS

(G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 355)

Solvents: S_1 = Benzene–dioxane–acetic acid (90:25:4).
S_2 = Benzene–methanol–acetic acid (45:8:4).
Thin layer adsorbent: Kieselgel G.
Detection: Diazotised sulphanilic acid and other diazotised reagents.

Compound	R_F	
	S_1	S_2
Phenol	0.76	0.60
Catechol	0.58	0.54
Resorcinol	0.56	0.52
Hydroquinone	0.54	0.46
Pyrogallol	0.32	0.45
Phloroglucinol	0.34	0.32
Guaiacol	0.83	0.72
Vanillin	0.70	0.64
m-Hydroxybenzoic acid	0.49	0.51
m-Hydroxybenzoic acid ethyl ester	0.76	0.61
Gentisic acid	0.30	0.40
Gentisic acid methyl ester	0.71	0.61
Protocatechuic acid	0.32	0.39
Protocatechuic acid ethyl ester	0.50	0.62
Gallic acid	0.18	0.23
Gallic acid ethyl ester	0.36	0.38
Syringaldehyde	0.60	0.57
Syringic acid	0.48	0.60
β-Resorcylic acid	0.54	0.52
p-Coumaric acid	0.49	0.52
Vanillic acid	0.54	0.61
Ferulic acid	0.50	0.58

TABLE 33

R_F VALUES OF CATECHOLAMINES

(J. DALY, L. HORNER AND B. WITKOP, *J. Am. Chem. Soc.*, 83 (1961) 4787)

Solvents: S_1 = Methanol–butan-1-ol–benzene–water (2:1:1:1).
S_2 = Methyl ethyl ketone–propionic acid–water (15:5:6).
S_3 = *sec.*-Butanol–formic acid–water (75:15:10).
S_4 = Butan-1-ol–acetic acid–water (4:1:1).
Paper: Whatman No. 1.

Compound	R_F			
	S_1	S_2	S_3	S_4
2,4,5-Trihydroxyphenethylamine	0.56	0.47	0.17	0.08
4,5-Dihydroxy-2-methoxyphenethylamine	0.68	0.50	0.31	0.15
2,5-Dihydroxy-4-methoxyphenethylamine	0.76	0.54	0.33	0.18

TABLE 34

R_F VALUES OF SOME ANALOGUES OF CHLORAMBUCIL RING ISOMERS AND COMPOUNDS INVOLVED IN THEIR SYNTHESIS

(W. A. SKINNER, M. G. M. SCHELSTRAETE AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 1554)

Solvents: S_1 = Benzene–methanol–water (2:6:1).
S_2 = Water-saturated butanol.
S_3 = Butanol–acetic acid–water (5:2:3).
Paper: P_1 = Schleicher & Schüll 2495, acetylated (descending).
P_2 = Whatman No. 1 (descending).
Detection: U.V. light.

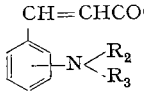
Compound	R_F		
	S_1P_1	S_2P_2	S_3P_2
Methyl *o*-aminocinnamate	0.92	0.78	
Methyl *o*-[bis-(2-hydroxyethyl)-amino]-cinnamate·HCl	0.75		
Methyl *o*-(2-hydroxyethylamino)-cinnamate	0.58		
Methyl *o*-[bis-(2-hydroxyethyl)-amino]-hydrocinnamate·HCl	0.33	0.79	
o-[Bis-(2-chloroethyl)-amino]-hydrocinnamic acid (*o*-Norchlorambucil)	0.48		
m-Aminohydrocinnamic acid·HCl			0.68
Methyl *m*-aminohydrocinnamate·HCl	0.70	0.34	0.66
Methyl *m*-[bis-(2-hydroxyethyl)-amino]-hydrocinnamate	0.57	0.82	
m-[Bis-(2-chloroethyl)-amino]-hydrocinnamic acid (*m*-Norchlorambucil)	0.71		
Methyl 4-{*o*-[bis-(2-hydroxyethyl)-amino]-phenyl}-butyrate·HCl	0.73		
Methyl 4-(*o*-aminophenyl)-butyrate	0.88		
Methyl 4-(*o*-aminophenyl)-butyrate *p*-tolylsulphonate		0.03	
4-{*o*-[Bis-(2-chloroethyl)-amino]-phenyl}-butyric acid	0.34		
4-(*m*-Aminophenyl)-butyric acid·HCl	0.69		

TABLE 35

R_F VALUES OF SOME NITROGEN MUSTARDS DERIVED FROM CINNAMIC ACID (CHLORAMBUCIL ANALOGUES)

(W. A. SKINNER, M. G. M. SCHELSTRAETE AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 1674)

Solvent: Benzene–methanol–water (2:6:1).
Paper: Schleicher & Schüll 2495, acetylated (descending).
Detection: U.V. light.



Compound			R_F
R_1	$R_2 = R_3$	*Isomer*	
C_2H_5	H	*m*	0.41
C_2H_5*	$-CH_2CH_2OH$	*m*	0.76
C_2H_5	$-CH_2CH_2OH$	*p*	0.71
H	$-CH_2CH_2Cl$	*o*	0.44
H	$-CH_2CH_2Cl$	*m*	0.68
H	$-CH_2CH_2Cl$	*p*	0.62
C_2H_5	$-CH_2CH_2Cl$	*p*	0.28

* Hydrochloride. Both hydrochloride and free base have the same R_F value, indicating ease of dissociation of the salt to the base.

TABLE 36

R_F VALUES (THIN LAYER) OF CERTAIN STEROIDS AND DERIVATIVES OF ETIANIC ACID METHYL ESTERS

(M. BARBIER, H. JÄGER, H. TOBIAS AND E. WYSS, *Helv. Chim. Acta*, 42 (1959) 2440)

Solvents: See table; E = ethyl acetate; C = cyclohexane; H = *n*-hexane.
Thin-layer adsorbent: Silicic acid (Kieselgel G, Merck).
Detection: $SbCl_3$ in $CHCl_3$ (*cf.* D. F. LAWDAY, *Nature*, 170 (1952) 415).

Compound	*Solvent*	R_F
3β-Acetoxy-5β-etienic acid (14, 15) methyl ester	EC (15:85)	0.47 ± 0.1
3β-Acetoxy-5β-etienic acid (8,14) methyl ester	EC (15:85)	0.47 ± 0.1
3β-Acetoxy-5β-etianic acid methyl ester	EC (15:85)	0.47 ± 0.1
3β-Acetoxy-5β-14-hydroxyetianic acid methyl ester	EC (15:85)	0.22 ± 0.05
3β-Acetoxy-14,15-oxido-5β-etianic acid methyl ester	EC (15:85)	0.23 ± 0.05
3β-Acetoxy-8,14α-oxido-5β-etianic acid methyl ester	EC (15:85)	0.18 ± 0.05
3β-Acetoxy-15α-hydroxy-5β-etianic acid methyl ester	EC (15:85)	0.16 ± 0.05
3β,12β-Dihydroxy-5β-etianic acid methyl ester	EC (30:70)	0.08 ± 0.05
	EH (75:25)	0.54 ± 0.1
3β-Acetoxy-12β-hydroxy-5β-etianic acid methyl ester	EC (30:70)	0.40 ± 0.07
3β,12β-Diacetoxy-14β-hydroxy-5β-etianic acid methyl ester	EC (30:70)	0.42 ± 0.07
3β,12β-Diacetoxy-5β-etianic acid methyl ester	EC (30:70)	0.52 ± 0.1
3β-Acetoxy-12-keto-14β-hydroxy-5β-etianic acid methyl ester	EC (25:75)	0.41 ± 0.07
3β-Acetoxy-12-keto-5β-etianic acid (14,15)methyl ester	EC (30:70)	0.48 ± 0.1
3β,12β-Diacetoxy-11-keto-5β-etienic acid methyl ester	EC (30:70)	0.32 ± 0.05
3α-Acetoxy-11-keto-5β-etienic acid methyl ester	EC (30:75)	0.58 ± 0.1
3α-Acetoxy-11β-hydroxy-5β-etianic acid methyl ester	EC (30:70)	0.52 ± 0.1
5β-Cholestan-3-one	EC (15:85)	0.71 ± 0.05
5α-Cholestan-3-one	EC (15:85)	0.86 ± 0.05
Cholest-4-en-3-one	EC (15:85)	0.63 ± 0.05
Cholesterol	EC (30:70)	0.16 ± 0.05
3,4-Oxidocholestane	EC (30:70)	0.85 ± 0.1

TABLE 37

R_F VALUES OF CYTIDINE DERIVATIVES

(J. P. H. VERHEYDEN AND J. G. MOFFATT, *J. Am. Chem. Soc.*, 86 (1964) 1236)

Solvents: S_1 = Ethanol–*M* ammonium acetate, pH 7.5 (5:2).
S_2 = *n*-Propanol–ammonium hydroxide–water (6:3:1).
S_3 = Isopropanol–ammonium hydroxide–water (7:1:2).
Paper: Schleicher & Schüll 589, orange ribbon.

Compound	R_F		
	S_1	S_2	S_3
Cytidine	0.73	0.52	0.72
Cytidine 2′(3′)-phosphate	0.37	0.25	0.30
Cytidine 5′-phosphate	0.30	0.17	0.20
Cytidine 2′(3′),5′-diphosphate	0.13	0.07	0.05
Cytidine 2′,3′-cyclic phosphate 5′-phosphoromorpholidate	0.57	0.39	0.56
Cytidine 2′,3′-cyclic phosphate 5′-phosphoroanisidate	0.51	0.44	0.56
Cytidine 2′,3′-cyclic phosphate 5′-diphosphate	0.18	0.11	0.10
Cytidine 2′-phosphate 5′-diphosphate	0.11	0.05	0.03
Cytidine 2′(3′)-phosphate 5′-phosphoromorpholidate	0.35	0.21	0.24
Cytidine 2′,3′-cyclic phosphate 5′-phosphate	0.22	0.18	0.18
Cytidine 5′-phosphoroanisidate	0.69	0.59	0.72

TABLE 38

R_F VALUES OF 8-BROMOPURINES AND PURINE DERIVATIVES

(R. E. HOLMES AND R. K. ROBINS, *J. Am. Chem. Soc.*, 86 (1964) 1242)

Solvents: S_1 = 5% aqueous ammonium hydrogen carbonate.
S_2 = Ethanol–water (7:3, v/v).
S_3 = Dimethylformamide–ammonium hydroxide–isopropanol (25:10:65, v/v).

Compound	R_F		
	S_1	S_2	S_3
8-Bromoguanosine	0.69	0.69	0.19
8-Bromoadenosine	0.64	0.73	0.51
8-Bromo-2′-deoxyadenosine	0.65	0.78	0.26
8-Bromoguanine	0.38	0.67	0.12
8-Bromoadenine	0.28	0.62	0.24

TABLE 39

R_F VALUES OF METHYL PHOSPHATES AND 2′,3′-CYCLIC PHOSPHATES OF NUCLEOSIDES

(D. LIPKIN, J. S. DIXON AND P. T. TALBERT, *J. Am. Chem. Soc.*, 83 (1961) 4772)

Solvents: S_1 = Satd. aq. $(NH_4)_2SO_4$–propan-2-ol–water (79:2:19) (R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 49 (1951) 401).
S_2 = Propan-2-ol–water (70:30)/NH_3 atmosphere (R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552).
S_3 = Isoamyl alcohol layered on 5% aq. Na_2HPO_4 (C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 1466).
Paper: Whatman No. 1 (ascending).
Detection: D_1 = U.V. light (254 mμ).
D_2 = Sodium metaperiodate spray for vicinal glycol groups (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162).
D_3 = Molybdate spray for phosphorus-containing compounds (C. A. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).

Compound	R_F		
	S_1	S_2	S_3
C-2′-P methyl ester	0.63	0.45	
C-3′-P methyl ester	0.56	0.45	
C-2′,3′-P	0.48	0.38	0.78
A-2′-P methyl ester	0.25	0.44	
A-3′-P methyl ester	0.16	0.43	
A-2′,3′-P	0.12	0.41	0.52
U-2′-P methyl ester	0.57	0.40	
U-3′-P methyl ester	0.57	0.40	
U-2′,3′-P	0.51	0.34	0.76
G-2′-P methyl ester	0.43	0.26	
G-3′-P methyl ester	0.30	0.21	
G-2′,3′-P	0.28	0.18	0.67

* A = adenosine; C = cytidine; G = guanosine; P = phosphoric acid; U = uridine.

TABLE 40

R_F VALUES OF SOME 5-FLUOROPYRIMIDINE NUCLEOSIDES AND RELATED COMPOUNDS

(I. WEMPEN, R. DUSCHINSKY, L. KAPLAN AND J. J. FOX, *J. Am. Chem. Soc.*, 83 (1961) 4755)

Solvents: S_1 = Butan-1-ol–water (86:14).
S_2 = Butan-1-ol–acetic acid–water (5:2:3).
S_3 = Butan-1-ol–1*N* NH_4OH (86:14).
S_4 = Propan-2-ol–HCl (170:41) diluted to 250 ml with H_2O.
S_5 = Ethanol–water (18:15).
Paper: Schleicher & Schüll 597 (ascending).
Detection: U.V. light.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Uracil	0.33	0.59	0.27	0.73	0.63
5-Fluorouracil	0.42	0.61	0.16	0.75	0.63
Cytosine	0.20	0.55	0.20	0.52	0.52
N,N-Dimethyl-	0.47	0.63	0.50	0.45	0.74
5-Fluoro-	0.27	0.59	0.27	0.52	0.49
N-Methyl-5-fluoro-	0.48	0.71	0.47	0.60	0.72
Uridine	0.15	0.52	0.11	0.68	0.53
5-Fluoro-	0.33	0.58	0.04	0.75	0.67
4-Thio-5-fluoro-	0.65	0.73	0.30	0.83	0.77
Cytidine	0.08	0.52	0.10	0.50	0.43
N-Methyl-	0.25	0.60	0.28	0.59	0.66
N,N-Dimethyl-	0.32	0.61	0.31	0.59	0.69
5-Fluoro-	0.17	0.56	0.19	0.56	0.48
N-Methiyl-5-fluoro-	0.36	0.64	0.39	0.52	0.76
2′-Deoxyuridine	0.36	0.63	0.24	0.79	0.71
5-Fluoro-	0.46	0.64	0.07	0.80	0.75
4-Thio-5-fluoro-	0.75	0.80	0.33	0.92	0.85
2′-Deoxycytidine	0.17	0.61	0.23	0.61	0.63
N-Methyl-	0.41	0.66	0.50	0.73	0.79
N,N-Dimethyl-	0.49	0.69	0.58	0.67	0.79
5-Fluoro-	0.29	0.63	0.32	0.66	0.63
N-Methyl-5-fluoro-	0.53	0.70	0.50	0.64	0.83
N-Ethyl-5-fluoro-	0.67	0.79	0.67	0.73	0.89
N-Propyl-5-fluoro-	0.85	0.87	0.85	0.84	0.90
N-*n*-Butyl-5-fluoro-	0.85	0.88	0.87	0.92	0.96
N,N-Dimethyl-5-fluoro-	0.65	0.80	0.57	0.67	0.88

TABLE 41

R_F VALUES OF SOME NUCLEOSIDE-5′-PHOSPHATE METHYL ESTERS

(W. SZER AND D. SHUGAR, *Biokhimya*, 26 (1961) 840)

Solvents: S_1 = Propan-2-ol–NH_4OH (sp. gr. 0.88)–water (70:10:20, v/v).
S_2 = Ethanol–1 *M* ammonium acetate (70:30, v/v).
S_3 = Butan-1-ol saturated with saturated aqueous boric acid.
Paper: Whatman No. 1 (descending).
Time of run: 20–24 h (S_1, S_2).
Detection: U. V. light (2535 Å).

Compound	R_F		
	S_1	S_2	S_3
Uridine	0.49	0.73	0
3-N-Methyluridine	0.78	0.90	0
3-N-Methyl-2′-O-methyluridine	0.83	0.94	0.72
Uridine-5′-phosphate	0.18	0.26	0
3-N-Methyluridine-5′-phosphate	0.25	0.48	0
Dimethyl ester of 3-N-methyluridine-5′-phosphate	0.72	0.84	0
Monomethyl ester of 3-N-methyluridine-5′-phosphate	0.55	0.76	0
Dimethyl ester of 3-N-methyl-2′-O-methyluridine-5′-phosphate	0.78	0.93	0.46
Monomethyl ester of 3-N-methyl-2′-O-methyluridine-5′-phosphate	0.62	0.85	0.29
Dimethyl ester of uridine-5′-phosphate	0.65	0.80	0
Monomethyl ester of uridine-5′-phosphate	0.41	0.64	0

TABLE 42

R_F VALUES (RELATIVE) OF 2-AMINO-9-(5′-DEOXY-β-D-RIBOFURANOSYL)-9-H-PURINE-6-THIOL AND RELATED COMPOUNDS

(E. J. REIST, P. A. HART, L. GOODMAN AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 1557)

Solvents: S_1 = Water-saturated butan-1-ol.
S_2 = 5% aq. Na_2HPO_4.
Paper: Whatman No. 1 (descending).
Detection: D_1 = U.V. light.
D_2 = Bromine spray for thioethers (E. J. REIST, P. A. HART, L. GOODMAN AND B. R. BAKER, *J. Am. Chem. Soc.*, 81 (1959) 5176).

Compound	R_{Ad}*	
	S_1	S_2
2-Amino-9-(5′-deoxy-β-D-ribofuranosyl)-9-H-purine-6-thiol	0.61, 0.68	1.55, 1.47
5′-S-Ethyl-2′,3′-O-isopropylidene-5′-thioguanosine**	1.71, 1.84	
5′-Deoxy-2′,3′-O-isopropylideneguanosine**	1.54, 1.70	
5′-S-Ethyl-5′-thioguanosine	1.2	1.6
5′-Deoxyguanosine	0.34	1.84
2′,3′-Di-O-acetyl-5′-deoxyguanosine	1.42	
2′,3′-Di-O-acetyl-5′-deoxythioguanosine	1.62	
Adenine	1.00	1.00

* R_{Ad} = R_F compound/R_F adenine.
** R_{Ad} varied from chromatogram to chromatogram but could be used to follow sulphur removal.

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