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CHANGES OCCURRING WITH THE IMMOBILE LIQUID PHASE IN GAS–LIQUID CHROMATOGRAPHY

II. THE EFFECT ON RETENTION VOLUMES

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KELLER, BATE, COSTA AND FORMAN[1] reviewed the literature dealing with transformations which occur with the immobile liquid phase during gas–liquid chromatography and experimentally examined changes in retention volumes as columns were used either for prolonged periods of time or at conditions outside of those recommended for the partitioning liquids concerned. The actual distribution of liquid was determined by cutting the column into short sections and extracting the partitioner in each section. Rates of loss of partitioner from the support were determined by heating in air. Two kinds of discontinuities in the column were recognized. One was a discontinuity of the nature of the partitioner which could arise from complete evaporation of the immobile liquid leaving exposed support at the inlet of the column. Solutes might experience adsorption on the support in this region followed by solution in the liquid partitioner farther along the column. With the alcohols, two peaks could be produced from a single species if the solute had been preceded by another alcohol. This was explained by adsorption of the first alcohol followed by its displacement by the second alcohol. The second discontinuity was one of concentration of the immobile phase on the support. This was explained by assuming that the partitioner partially evaporated from the support at the inlet end on encountering unsaturated carrier. As soon as the carrier became saturated and evaporation ceased the concentration of the immobile phase rose from a low value to that of the original packing employed. There was no change in the mechanism by which solutes were retained but instead there was a very sharp change in partitioner concentration. Experimentally it was found that the semi-logarithmic plots of retention volumes *vs.* the number of carbon atoms for the homologous series of primary alkyl alcohols, as determined on a column subjected to periods of heating at elevated temperature between chromatograms, showed a change in the intercept of the line but no change in slope when tested at the 5 % significance level. It is the purpose of this communication to investigate this result more fully from a theoretical point of view. The problem is relevant to column conditioning prior to use, *e.g.*, heating while carrier is passed through the column, which seems to improve its performance, and the lifetime of a column where it seems that the performance deteriorates. Presumably some changes within the column are completed in a short period of time while others occur continuously.

Direct observation of such changes is destructive, *e.g.*, cutting the column into sections and examining the packing in each section. Hence indirect observations, *e.g.*, changes in chromatographic behavior or column bleeding, are preferred if the column is to be preserved. Here we examine the kinds of changes which may occur and attempt to relate these to changes in chromatographic behavior through an appeal to theory. We also raise the question as to whether it is meaningful to describe the packing material in terms of percentage liquid placed on the support if during the conditioning process or use of the column both of these change to something new and unknown.

CLASSIFICATION OF CHANGES

Changes occurring with the immobile phase can be physical or chemical. Physical changes may be classified as: (1) Microscopic. There is a redistribution of the liquid among the capillary spaces of the support and at the contact points of the particles which minimizes the surface free energy and eventually gives a uniform free energy over the entire liquid surface. This is probably the primary process which takes place during column conditioning and can only be achieved with the column packed. This initial microscopic equilibration necessitates a preconditioning period. Once accomplished, it is complete unless the packing is physically disturbed. (2) Macroscopic. Unless the carrier gas is presaturated with partitioner before entering the column, partitioner will evaporate from the packing to alter the total quantity of liquid present and its distribution on the column. Experimentally this loss and redistribution occurs in the early sections of the column[1]. Such changes will depend upon the nature of the partitioner, its homogeneity, the operating temperature, and the volume and flowrate of the carrier passed through the column. If the liquid is heterogeneous, the rate of loss of each component will depend upon the temperature and the ideality of the solution. If the column is operated at several temperatures several discontinuities may be introduced into the column as has been shown with polyethylene glycol[1].

We classify as chemical changes any transformation which alters the specific interactions of the solutes with the immobile liquid or partitioner–support combination by virtue of a change in properties of the liquid or its distribution. Such changes may be classified as: (1) Evaporation of the partitioner to expose the support or to enhance its participation in retention. (2) Changes due to the heterogeneity of the liquid applied to the support. A loss of a component or a contaminant of the liquid may leave behind a partitioner of properties different from the original liquid. (3) Chemical reaction with contaminants present in the liquid and/or carrier gas, *e.g.*, oxidation, catalyzed decomposition, esterification, etc. (4) Chemical changes inherent with the liquid, *e.g.*, polymerization, pyrolysis, dehydration, etc.

It is conceivable that a chemical change might not appreciably affect solute–partitioner interactions but so change the physical properties of the immobile liquid as to seriously alter the kinetic steps involved and change the range of carrier velocities in which the near-equilibrium approximation is valid. An example would be an increase in viscosity of the liquid phase. If, indeed, diffusion through the partitioning liquid is the rate controlling step, then this change is particularly important with regard to column efficiency.

THEORY

As our model, we assume that the chromatographic tube is packed with an impermeable solid which is coated with a liquid of cross-sectional area A_L. The solute molecules partition themselves between this immobile liquid and the flowing gas phase of cross-sectional area A_M. There is no adsorption of solute by the support nor at the gas–liquid interface, the chemical properties of the immobile liquid are independent of the amount applied, and the surface free energy of the liquid has reached its equilibrium value and is uniform over the entire liquid surface. This model is most closely approximated by columns packed with spherical glass beads covered with a thin liquid film. The definition of R as the fraction of solute molecules in the mobile phase leads to the expression:

$$R = \frac{1}{1 + \alpha(A_L/A_M)} \tag{1}$$

where α is the ratio of the concentration of solute in the stationary phase to that in the mobile phase, *i.e.*, the distribution coefficient. R is also the distance moved by the point of maximum concentration of a particular solute relative to that of the front of the mobile fluid phase (R_F) subject to the limitations discussed by GIDDINGS AND KELLER[2]. Physical changes would bring about a change in (A_L/A_M) leaving α constant while strictly chemical changes not involving column bleeding would bring about a change in α leaving (A_L/A_M) constant.

The retention time of a solute in a column[3] is:

$$t = \int_0^L \mathrm{d}x/u \tag{2}$$

where L is the length of the column, $\mathrm{d}x$ the element of length, and u the average velocity of the vapor. Since $u = Rv$ where v is the average velocity of the mobile carrier gas, then:

$$t_i = \int_0^L \mathrm{d}x/R_i v \tag{3}$$

where the subscript refers to the ith component of the mixture being chromatographed. This expression neglects extra-column contributions to t_i, *e.g.*, sample injector and detector designs, etc. Here we assume R_i to be independent of the column length and flowrate. This is not generally true since changes in composition of the liquid, non-linearity in the retention isotherm, and a non-equilibrium situation associated with high flow velocities may cause R_i to depend strongly on the distance of the solute along the column. It is expedient to make this assumption which is in part justified by the fact that the data reported in the earlier publication were collected at moderate operating conditions. Then, using the flowrate to convert to retention volumes:

$$V_i = \frac{2A_M L}{3R_i} \frac{(P_i/P_o)^3 - 1}{(P_i/P_o)^2 - 1} \tag{4}$$

where P_o is the pressure of the carrier gas at the column outlet and P_i is the pressure at the inlet. Substituting for R_i and letting:

$$K_p = \frac{2}{3}\frac{(P_i/P_o)^3 - 1}{(P_i/P_o)^2 - 1}$$

$$V_i = LK_pA_M + \alpha_i LK_pA_L \tag{5}$$

The partition coefficient, α, is zero for an unretained solute. Assuming this to be true of air, the first term is the retention volume of the air peak. Two practices are common to the literature. Eqn. (5) is valid where retention times are measured from the instant of sample introduction into the column. If one measures retention times from the air peak, then:

$$V_i^a = \alpha_i LK_pA_L \tag{6}$$

as was done by KELLER and coworkers[1]. These volumes may be further reduced to standard temperature and pressure.

It will become increasingly more apparent that eqn. (4) and all subsequent expressions involve average values. This is permissible only in the absence of large velocity gradients and is particularly important here where the column is non-uniform with regard to the distribution of partitioner. For example, with a large velocity gradient each solute would spend a far longer period of time at the inlet end of the column in contact with the partitioner distribution particular to this region than at the outlet end, where the flow velocity is great. The solutes would encounter a partitioner distribution in time which differs from the measurable distribution in distance along the column. Average values based on the spatial distribution would be inappropriate with large P_i/P_o ratios. As Table I shows, K_p values did not differ greatly from unity and the velocity gradient should not have been very large.

The partition coefficient, α, is an equilibrium constant related thermodynamically to the difference in chemical potential, $\Delta\mu^\circ$, of solute in the stationary phase and solute in the mobile phase, both at some standard concentration[2]. For ideal behavior:

$$\alpha = e^{-\Delta\mu^\circ/RT} \tag{7}$$

Substituting in eqn. (6) and expressing the result in logarithmic form:

$$\ln V_i^a = \ln A_L K_p L - \frac{\Delta\mu^\circ}{RT} \tag{8}$$

The difference in chemical potential can be expressed as a linear combination of terms descriptive of the groups present in the molecule. For a homologous series:

$$\Delta\mu^\circ = n_{CH_2}\Delta\mu^\circ_{CH_2} + \sum\Delta\mu_j^\circ \tag{9}$$

where $\Delta\mu^\circ_{CH_2}$ is characteristic of the methylene group while $\Delta\mu_j^\circ$ refers to other kinds of groups (methyl, hydroxyl, etc.). Eqn. (8) becomes:

$$\ln V_i^a = \ln A_L K_p L - \frac{\sum\Delta\mu_j^\circ}{RT} - n_{CH_2}\frac{\Delta\mu^\circ_{CH_2}}{RT} \tag{10}$$

On the basis of the simple picture presented here, if there is only a change in the cross-sectional area of the partitioning liquid, A_L, due to a loss or redistribution, then the

intercept of the semi-logarithmic plot of the retention volume *vs.* the number of carbon atoms will change but not the slope. If the column loses material to the flowing gas equally throughout its entire length then A_L represents the real cross-sectional area of the liquid. This is not the case. Since material is lost predominantly from the inlet end, A_L is an effective value which does not represent the actual distribution of partitioner. If the difference in chemical potential changes by virtue of an alteration of the chemical nature of the partitioner, then the slope of the plot will be altered, if such chemical change affects $\Delta\mu^\circ_{CH_2}$ for the methylene groups. We, however, envision that retention of polar compounds, *e.g.*, alcohols, on a polar partitioner, *e.g.*, glycol, is primarily due to the interaction of the polar groups of the materials which is largely independent of the methylene groups, *i.e.*, the interaction is described by a term in $\Sigma\Delta\mu_j^\circ$ which in turn affects the intercept. Since interaction of this kind is probably the predominant one in most chromatographic separations and since it cannot be separated from A_L without some independent means of determining this latter quantity, it is not likely that one can deduce the kind of change which occurs in the column from an examination of chromatographic behavior alone. Eqn. (10) is valid only when retention volumes are measured relative to the air peak. Eqn. (5) is not amendable to as simple a separation of physical and chemical effects. Rearrangement and expansion in an infinite series gives:

$$\ln V_i = \ln \frac{K_p L}{A_M} + \frac{\alpha A_L}{A_M} - \frac{1}{2}\alpha^2 \left(\frac{A_L}{A_M}\right)^2 + \frac{1}{3}\alpha^3 \left(\frac{A_L}{A_M}\right)^3 + \ldots \tag{11}$$

which is valid for $-1 < \alpha A_L/A_M < 1$. If one neglects higher ordered terms and expands the exponential form of α, then, again neglecting higher ordered terms:

$$\ln V_i = \ln \frac{K_p L}{A_M} + \frac{A_L}{A_M}\left[1 - \frac{\Delta\mu^\circ}{RT} + \frac{1}{2!}\left(\frac{\Delta\mu^\circ}{RT}\right)^2 - \ldots\right] \tag{12}$$

$$= \ln \frac{K_p L}{A_M} + \frac{A_L}{A_M} - \frac{A_L}{A_M}\frac{\Delta\mu^\circ}{RT} \tag{13}$$

Substituting eqn. (9) for $\Delta\mu^\circ$:

$$\ln V_i = \ln \frac{K_p L}{A_M} + \frac{A_L}{A_M} - \frac{\Sigma\Delta\mu_j^\circ}{RT}\frac{A_L}{A_M} - n_{CH_2}\frac{\Delta\mu^\circ_{CH_2}}{RT}\frac{A_L}{A_M} \tag{14}$$

The effect of physical changes with the partitioner cannot be separated from chemical changes. For diagnostic purposes, retention volumes relative to the air peak are to be preferred.

HETP has been reported to vary directly with the effective thickness squared of the liquid layer[4]. This thickness behaves like A_L on redistribution and its effective value will decrease with the kind of redistribution reported by KELLER *et al.*

If one has two columns, (1) and (2), which differ only in the amount of liquid phase present, the difference of the intercepts is given by:

$$\ln V_i^a(1) - \ln V_i^a(2) = \ln \frac{A_L(1)}{A_L(2)} + \ln \frac{K_p(1)L(1)}{K_p(2)L(2)} \tag{15}$$

The volume of liquid adhering to each particle is the volume of the coated particle of radius r_2 minus the volume of the support particle of radius r_1 or:

$$\frac{4}{3}\pi(r_2^3 - r_1^3) = W_L/n\rho_L \tag{16}$$

where W_L is the total weight of liquid on the column, ρ_L its density, and n the number of particles in the column. In a similar fashion, the cross-sectional area of the liquid phase is the cross-section of the coated particle minus the cross-section of the support or:

$$A_L = \pi(r_2^2 - r_1^2) \tag{17}$$

If $(r_2 - r_1)$ is factored from each polynomial, its value from eqn. (16) substituted in eqn. (17), and the result simplified by completion of the square, then:

$$A_L = \frac{3\pi W_L}{4n\rho_L\left[r_2 + r_1 - \frac{r_2 r_1}{r_2 + r_1}\right]} \tag{18}$$

Assuming that $r_1 = r_2 = r$ or that the thickness of the liquid is negligible compared to the radius of the particle:

$$A_L = \frac{\pi W_L}{2n\rho_L r} \tag{19}$$

We may write:

$$\frac{A_L(1)}{A_L(2)} = \frac{W_L(1)n(2)}{W_L(2)n(1)} \tag{20}$$

since ρ_L and r are the same for both columns. Also:

$$n = W_S d_S \tag{21}$$

where W_S is the weight of the bare support material in the column and d_S is the number of support particles per gram. Using this, eqn. (15)

$$\ln V_i^a(1) - \ln V_i^a(2) = \ln \frac{W_L(1)W_S(2)}{W_L(2)W_S(1)} + \ln \frac{K_p(1)L(1)}{K_p(2)L(2)} \tag{22}$$

If there is more than one immobile phase involved in the retention of the solute, *e.g.*, adsorption by the solid support or retention at the liquid–gas interface as suggested by MARTIN[5] such that α as measured chromatographically by a treatment based on eqn. (1) does not compare with values found by other independent means, then we may attempt to separate these effects by defining as many arbitrary partition coefficients, α, β, γ,... as necessary to describe these other active interfaces. For example, let β equal the concentration of solute at the support–liquid interface (moles/unit surface)/concentration of solute in the mobile gas phase. Following the development of GIDDINGS AND KELLER[2], then for two active immobile phases:

$$R = \frac{1}{1 + \alpha(A_L/A_M) + \beta(A_S/A_M)} \tag{23}$$

Here one may try to assign values to the cross-sectional area of the solid support from measurements performed on the support particles. If β can be obtained from non-chromatographic results, R may be calculated. Otherwise β must be determined from R for appropriate values of α, A_L, A_S, and A_M. Extension of eqn. (23) to other active sites such as the liquid–gas interface or an attempt to distinguish between the partitioning in the adsorbed partitioner and the liquid held in capillary puddles assuming the $\Delta\mu^\circ$ of the solute to be different in these two regions, leads to speculation as to the values of both the partition coefficient and the effective cross-section. We make further remarks about this distribution of liquid regions later.

For a liquid phase held on a support which contributes to retention, eqn. (6) becomes:

$$V_i^a = \alpha_i L K_p A_L + \beta_i L K_p A_S \tag{24}$$

We presume that some sort of functional relationship exists between A_L and A_S such that $A_S = \mathrm{f}(A_L)$, *i.e.*, the participation of the support in retention depends upon the quantity and perhaps the distribution of the liquid in the column. In order to characterize the variation of $\ln V_i^a$ with the number of methylene groups in a homologous series when both liquid partition and solid adsorption occur, we take the total derivative of the logarithmic form of eqn. (24) with respect to α_i and β_i to obtain:

$$\mathrm{d}(\ln V_i^a) = \frac{A_L \mathrm{d}\alpha_i + \mathrm{f}(A_L)\mathrm{d}\beta_i}{\alpha_i A_L + \beta_i \mathrm{f}(A_L)} \tag{25}$$

Substitution of eqn. (9) in (7) and differentiation with respect to n_{CH_2} yields:

$$\frac{\mathrm{d}\alpha_i}{\mathrm{d}n_{CH_2}} = -\frac{\alpha_i}{RT}\Delta\mu^\circ_{CH_2(L)} \tag{26}$$

and an analogous expression for $\mathrm{d}\beta_i/\mathrm{d}n_{CH_2}$. Here $\Delta\mu^\circ_{CH_2(L)}$ is the difference in chemical potential for distribution of methylene groups between the liquid partitioner and the vapor state while $\Delta\mu^\circ_{CH_2(S)}$ pertains to solute distributed between the active solid and the vapor. With these substitutions, eqn. (25) becomes:

$$\frac{\mathrm{d}(\ln V_i^a)}{\mathrm{d}n_{CH_2}} = -\frac{\alpha_i A_L \Delta\mu^\circ_{CH_2(L)} + \beta_i \mathrm{f}(A_L)\Delta\mu^\circ_{CH_2(S)}}{RT[\alpha_i A_L + \beta_i \mathrm{f}(A_L)]} \tag{27}$$

If we assume that the nature of the chemical interaction between the liquid and the solid as it affects the solute activity is independent of the quantity of partitioner or its distribution then $\Delta\mu^\circ_{CH(L)}$ and $\Delta\mu^\circ_{CH_2(S)}$ are constants. Deviation of this plot from linearity suggests that the solid support participates in retention through the non-linearity of $\mathrm{f}(A_L)$. MARTIN[5] suggested that for his particular case the solid surface area is proportional to the bulk liquid above a 3 % liquid load. With linearity of A_S in A_L, eqn. (27) becomes:

$$\frac{\mathrm{d}(\ln V_i^a)}{\mathrm{d}n_{CH_2}} = -\frac{k_1\alpha_i + k_2\beta_i}{\alpha_i + k_3\beta_i} \tag{28}$$

That eqn. (28) equals a constant requires that $\alpha_i/\beta_i = k$ which implies that:

$$\Delta\mu^\circ_{CH_2(L)} = K + \Delta\mu^\circ_{CH_2(S} \tag{29}$$

where K is a constant. This is always true if, as we have supposed, both chemical potentials are constant.

The opportunity to write:

$$A_S = kA_L \tag{30}$$

represents a considerable algebraic simplification since eqn. (24) then becomes:

$$V_i^a = (\alpha_i + k\beta_i)LK_pA_L \tag{31}$$

and the remainder of the equations follow as before with $\alpha_i = \alpha_i'$ and $\Delta\mu_i^\circ = \Delta\mu_i^{\circ\prime}$ where α_i' and $\Delta\mu_i'$ are measures of the combined influence of the partitioning liquid and active solid support in retention. Thus participation of the solid support is equivalent in effect to chemical change of the partitioner under the assumptions made here and there is no non-linearity introduced into $\ln V_i^a$ *vs.* n_{CH_2}.

It is to be clearly understood that eqn. (30) states that the average value of A_S is a linear function of the average value of A_L. The effect of particular distributions as a function of the distance along the column has not been explored experimentally.

EXPERIMENTAL

KELLER *et al.*[1] prepared two 1.5 m columns with packings of polyethylene glycol on firebrick. Column 4 contained 16.45 g of packing holding 31.6 % liquid phase. From this $W_S(1) = 11.25$ g. Column 5 contained 11.80 g of packing holding 2.80 % liquid which gives $W_S(2) = 11.47$ g and $W_L = 0.33$ g. These columns were conditioned, a series of chromatograms performed, the column heated at a higher temperature, another series run, and this procedure repeated once again. After the third series of chromatograms the columns were cut into sections and the immobile phase determined in each. Column 4 lost a great deal of volatile material during conditioning so that the liquid load for the first or *a*-series chromatograms was doubtful. Column 5 was

TABLE I

LIQUID LOAD CALCULATED FROM CHROMATOGRAPHIC RESULTS

Column	*Series*	*Intercept*	K_p *(av.)*	W_S *(g)*	*Calculated amount of liquid*	
					W_L	%
4	*a*	1.916	0.9025	11.25	3.01	21.0
	b	1.756	0.9119	11.25	2.06	15.4
	c	1.624	0.9231	11.25	1.54	12.0
5	*d**	0.964	0.9378	11.47	—	—
	e	0.564	0.9387	11.47	0.131	1.13
	f	0.446	0.9495	11.47	0.097	0.84

* Standard for comparison with $W_L = 0.33$ g and per cent loading of 2.80.

prepared from packing which had been heated in a drying oven prior to preparation of the column. As evidenced by negligible column bleeding, it lost its volatiles during this process and we have confidence that its liquid load did not change during conditioning. This justifies its use as a standard for the comparison of the series. Table I

lists the observed intercepts and the average value of K_p for the columns. From these data we can calculate the weight of liquid on each column. Polyethylene glycol is a poor substance to use because of the pronounced bleeding during conditioning and the redistribution on the support. It is not surprising, therefore, that the calculated per cent liquid load (21.0%) for the *a*-series is much lower than the amount found on the original packing (31.6%). After the *c*-series, the column was cut and the liquid distribution determined. This is shown in Fig. 9 of the earlier communication[1]. The maximum liquid in any one section was 14%. The average per cent liquid in each section as computed from the actual distribution was 12.5 which is remarkably close to the 12.0 % reported in Table I. The same thing was done for column 5 after the *f*-series to give an average liquid load of 1.23 % whereas the table shows 0.84%, a difference of 0.4%. These results are probably within experimental error.

DISCUSSION

With regard to retention volumes only, we conclude that if the immobile liquid phase undergoes a macroscopic redistribution of partitioner without chemical change, the column behaves as if it were uniformly coated with the liquid remaining. It also appears that the change in intercept but constancy of slope reported in the earlier paper can be explained satisfactorily by physical macroscopic redistribution alone. These conclusions are only valid in the region of small velocity gradients within the column and where the amount of retention at other interfaces is proportional to the liquid load. The problem of the effect of redistribution on peak shapes and plate height may well require a different approach. This will be the subject of a subsequent paper.

The majority of support materials used in gas chromatographic columns are not impermeable but are porous and permeated by capillary cavities which may be interconnecting. Capillary columns do not have smooth bores nor are they perfectly circular in cross section. These non-uniformities lead to non-uniform distributions of liquid. Even glass beads may deviate severely from the model because the bulk of the liquid is located at the contact points between the beads. Thus A_L or the thickness of the liquid film is an effective value or a kind of average value which is not likely to describe reality at any location in the column. The problem is to calculate this effective value. This is not unique to gas chromatography nor is it particularly new to chromatographic literature[2]. GIDDINGS[6] has been much concerned with the effective thickness of this film since HETP is intimately dependent upon it. Using a model of adjacent conical cavities ("saw-tooth" profile) in a solid he has derived theoretical results important to further extension of our knowledge. If the liquid wets the solid then adsorption forces and capillary forces compete for the liquid. For a loading of 15 % liquid on a granular porous support, GIDDINGS calculates 2 to 4 % to be adsorbed liquid and 12 to 13 % to be in the capillary cavities. This conclusion has implications for those using columns of low liquid loading in an effort to realize greater column efficiency. If the chemical potential of a solute is different for surface adsorption on the support, solution in the capillary liquid, and solution in the adsorbed liquid then chromatographic behavior will be strongly dependent on column bleeding and partitioner redistribution because the extent of participation of these three sites depends on the liquid load. At low loads even a small loss is likely to alter their relative involvement. We predict that if the extent of participation of each kind of site can be

represented by an average cross-sectional area and if these are in turn linear functions of the cross-sectional area of the liquid partitioner then $\ln V_i^a$ *vs.* n_{CH_2} is linear and chromatographic behavior can be described by an effective partition coefficient characteristic of all three mechanisms. Any change in value of this coefficient is reflected by a change in slope of the plot. If these average cross-sections are related non-linearly then the plot is non-linear. Adsorption at the gas–liquid interface may also be included in this treatment. Attempts to separate these individual contributions involve the assessment of a partition coefficient and an effective cross-section for each active phase. For some of these phases, the concept of a cross-section is very unrealistic and it is unlikely that such values can be assigned from other than chromatographic data.

Although the chemical potential of the partitioning liquid held by adsorption is assumed to be the same as that held in capillary puddles, it is not necessarily true that the chemical potential of a solute dissolved in these two regions will be the same. For an inactive support holding a non-polar partitioner we do not expect much organization of the molecules of the partitioner in the adsorbed regions and there should be little difference between the liquid in the two regions and there should be equivalent solute behavior in each. However, for an active support holding a polar partitioner we might well expect such a difference. This distinction is probably only important at a few tenths of a per cent liquid load. Users of low load columns can expect that chromatographic sequences will be altered in an unpredictable manner as they reduce the loading and that $\ln V_i^a$ *vs.* n_{CH_2} plots may be non-linear.

For normal loadings we expect support participation to be minimal and that partitioning will predominantly occur in the liquid held by capillaries. The problem is merely the calculation of the effective A_L by an averaging process. Here we conclude that a change in the intercept of the semi-logarithmic plot indicates a change in A_L and/or $\Sigma \Delta\mu_j^\circ/RT$. Our work indicates that one may determine which of these may bring about this variation if one knows the weight of the liquid remaining on the column since A_L as a function of W_L is independent of both the microscopic and macroscopic distribution. Moreover, it is meaningful to report specific retention volumes even if there has been a macroscopic redistribution if the actual weight, not the preconditioning weight, of the partitioner on the column is used. Direct weighing of the column would furnish W_L and not involve its destruction.

An additional remark is that if the liquid is deposited on the support in such a manner that the surface free energy is not uniform over the entire surface of liquid, microscopic redistribution can occur either by fluid flow over the surface or by vaporization and recondensation. GIDDINGS[6], on the basis of some assumed parameters, concludes that the two mechanisms are about equally involved, that the time required to reach an equilibrium distribution depends upon the average pore volume of the support, and that for a diatomaceous earth it is about thirty minutes. In general, this adjustment is apparently a small fraction of the lifetime of the column.

SYMBOLS

A_L cross-sectional area of the immobile liquid phase.
A_M cross-sectional area of the mobile phase.
A_S cross-sectional area of the immobile support.

α_i distribution coefficient of the ith solute for liquid partition.
α_i' effective distribution coefficient of the ith solute where retention is at several active interfaces.
β_i distribution coefficient for retention of the ith solute on a solid surface.
γ_i, δ_i... distribution coefficients for retention of the ith solute at active interfaces.
d_S number of particles of support per gram.
k, k_1 k_2, k_3, K numerical constants.
K_p $\frac{2}{3}\frac{(P_i/P_o)^3 - 1}{(P_i/P_o)^2 - 1}$.
L column length.
$\Delta\mu^\circ$ difference in chemical potential of a solute in the stationary phase and solute in the mobile phase, both at some standard concentration.
$\Delta\mu^\circ_{CH_2}$ $\Delta\mu^\circ$ for the methylene group.
$\Delta\mu^\circ_j$ $\Delta\mu^\circ$ for functional groups other than methylene.
$\Delta\mu^\circ_{CH_2(L)}$ $\Delta\mu^\circ_{CH_2}$ for distribution between immobile liquid and vapor.
$\Delta\mu^\circ_{CH_2(S)}$ $\Delta\mu^\circ_{CH_2}$ for distribution between immobile solid and vapor.
$\Delta\mu_i^{\circ\prime}$ effective $\Delta\mu_i^\circ$ of the ith solute where retention is at several active interfaces.
n number of particles in the column.
n_{CH_2} number of methylene groups in a molecule.
P_i pressure at the column inlet.
P_o pressure at the column outlet.
R_i fraction of the ith solute molecules in the mobile phase.
R universal gas constant.
r_1 radius of the support particle.
r_2 radius of the liquid coated particle.
ρ_L density of the partitioning liquid.
t_i retention time of the ith solute.
T absolute temperature.
u average velocity of solute vapor.
v average velocity of carrier gas.
V_i retention volume of the ith solute measured from the instant of sample entrance into the column.
V_i^a retention volume of the ith solute measured from the air peak.
W_L total weight of liquid on the column.
W_S total weight of uncoated support in the column.
dx element of column length.

ACKNOWLEDGEMENTS

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SUMMARY

The immobile liquid phase of a gas chromatographic column may undergo physical and chemical changes during conditioning and use. Chemical changes are those which affect the difference in chemical potential of a solute between the solution in the partitioning liquid and the vapor state. Macroscopic physical changes are those which change the total amount of liquid and/or its distribution on the support. Microscopic physical redistribution of partitioner occurs until the surface free energy of the liquid is minimal and uniform over the entire liquid surface. This latter redistribution is probably complete within a small fraction of the lifetime of the column. The assumption that the packing consists of impermeable solid particles coated with a uniform liquid film leads to the conclusion that a chemical change which affects $\Delta\mu^\circ$ for the methylene groups of a homologous series changes the slope of the semi-logarithmic plot of retention volume *vs.* the number of carbon atoms in the molecule whereas the intercept is altered by either a change in $\Delta\mu^\circ$ for the functional groups of the molecule or by a change in the effective cross-sectional area of the partitioner. Evidence is given that the change due to the latter can be calculated by finding the average amount of liquid on the column irrespective of its distribution. The effect of retention by more than one kind of immobile phase is discussed.

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COMPARISON OF HELIUM AND ARGON IN IONIZATION DETECTORS

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The action of argon ionization detectors for gas chromatography has been ascribed by LOVELOCK[1,2] to the occurrence of inelastic collisions between metastable excited argon atoms and molecules of other gases. As a result of these collisions, the excited argon imparts its energy to the second species of molecule and itself returns to the ground state. If the second molecular species has an ionization potential below 11.6 eV, the energy of the lowest excited state of argon, the collision results in ionization and a consequent increase in the number of charge carriers. The magnitude of the resulting increase in electrical conductivity may be taken as a measure of the concentration of the second molecular species.

On the basis of this hypothesis, an increase in sensitivity was predicted if helium were substituted for the argon since the excited states of helium are more energetic (more than 20 eV) than those of argon. When helium was used experimentally, however, "high baseline currents" and low sensitivity were observed[3,4], and gases with high ionization potentials such as oxygen or nitrogen caused diminution of the current flow in these detectors. This behavior was attributed to the presence of impurities in the helium. Upon collision with excited helium, the impurity molecules were ionized, which caused high baseline current. The excited helium returned to the ground state, so that the addition of more nitrogen or oxygen to the gas stream only interfered with the process of creating excited states and so reduced the total current.

When purified helium was used[5] oxygen and nitrogen caused the predicted increases in current and highly sensitive detection of these gases was found possible. This was reported to require exhaustive purification of the helium.

We have previously reported finding that helium supplied commercially is sufficiently pure for this last effect if the design of the detector cell is modified somewhat and if reasonable care is exercised in making gas connections[6]. This observation permitted us to evaluate the use of helium in an ionization detector, to determine the response to atmospheric gases and to organic vapors containing a variety of functional groups, and to compare these responses to those obtained when argon was used.

MATERIALS AND METHODS

A detector cell composed of a coaxial rod and cylinder was used (Fig. 1). Polytetrafluoroethylene ("Teflon") was used as insulator and mechanical mounting of the center electrode. Care was taken in the construction of this cell so that no leakage of

gas occurred between the insulator and the central electrode, or at the junction of the insulator and the cylinder. A self sustained, direct current electric discharge was excited between the central electrode and the cylinder[7].

A high negative potential from a North Hills Electronics Co. Model CS 120 Constant Current Source was put on the cylinder, while the central anode was

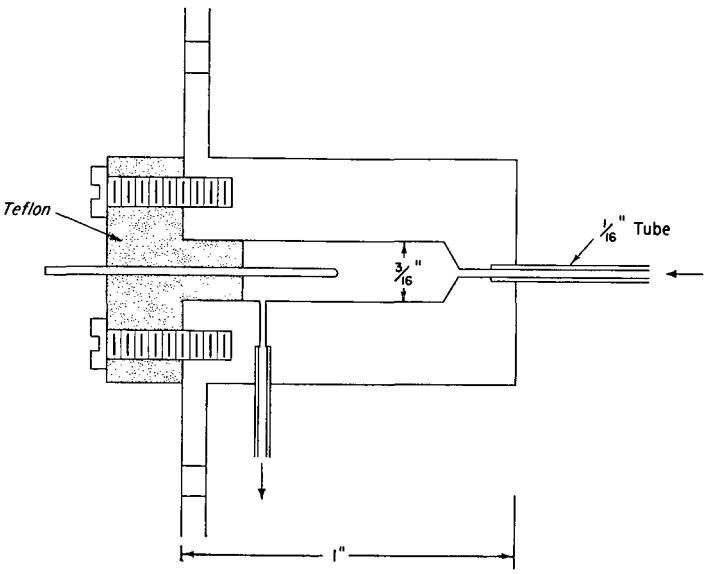

Fig. 1. Longitudinal section of a detector cell (schematic).

grounded. One μA was put through the gas in the detector. A signal proportional to the voltage necessary to cause this current to flow was recorded, using appropriate zero suppression.

When studying the effect of addition of atmospheric gases, the inlet tube of the detector was connected to the outlet of a glass gas chromatography column, 3 ft. long, containing molecular sieve 5 A, 30–50 mesh. A silicone rubber seal was used to make the glass-to-metal connection. A 10 μl syringe (Hamilton Co.) was used to inject samples of air into this column through a silicone rubber septum. To evaluate the response of the detector to concentrations of oxygen and nitrogen smaller than those obtainable by injecting 1 μl of air, additional carrier gas was supplied to the detector cell using a "T" connection. To evaluate the response of the detector to organic vapor, a capillary column, stainless-steel, 0.010 in. inside diameter, 100 ft. long coated with Apiezon L was used to supply graded quantities of vapor. Despite using a stream divider to reduce the time constant of the inlet, whereby more than 99 % of the gas was vented and less than 1 % entered the column, and despite brisk (100 cc/min) dilution flow at the detector, the detector was overloaded when 1 μl liquid samples of organic materials were injected into the inlet. A series of solutions were prepared containing 1 % test material in either toluene or isooctane, and a second series of 2 % solutions of the same materials in these solvents. Each of the materials tested was well separated from either isooctane or toluene by the column so that the sensitivity of

the detector to it could be determined by injecting 3 μl samples of their solutions in these solvents. The response of the detector to each test substance was determined by measuring the area of the peaks after establishing that peak area was linearly related to the quantity injected.

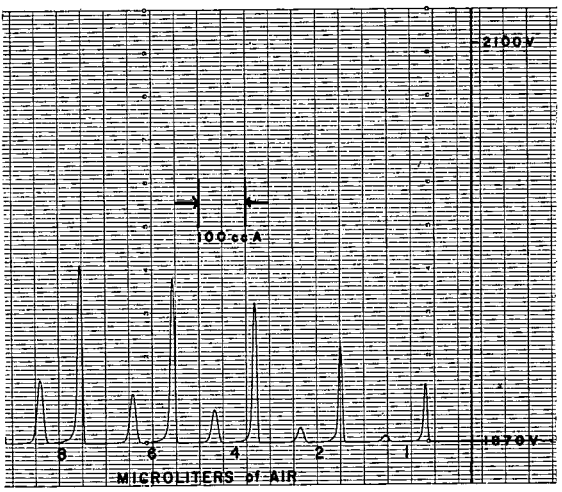

Fig. 2. A series of air samples, analyzed by a molecular sieve column, detected by an argon discharge.

RESULTS

Atmospheric gases

The response of an argon discharge to a series of air samples by a molecular sieve column is shown in Fig. 2. The baseline voltage is 1870 V. The presence of either oxygen or nitrogen caused an increase in this voltage. The effect of oxygen was comparatively much greater.

The response of a helium discharge to a similar series of samples is shown in Fig. 3. This series of analyses was done using a freshly installed, incompletely activated column so that the gas reaching the detector cell may be assumed to have contained contaminating gases. The baseline voltage was 500 V. Both oxygen and nitrogen raised the voltage (decreased the conductivity) of the discharge. The sensitivity to nitrogen and oxygen was more nearly comparable.

The sensitivity of the argon discharge or the impure helium discharge to oxygen or nitrogen decreased with increasing concentration of these gases. The decrease in sensitivity was somewhat specific. When, for example, the helium discharge was deliberately contaminated with nitrogen, the sensitivity of the discharge to both oxygen and nitrogen was decreased, but the decrease was relatively greater with respect to nitrogen. When the gas was deliberately contaminated with oxygen, the sensitivity to both gases again decreased. Under this circumstance, the sensitivity to oxygen decreased more. Contamination of the gas in the detector with air gave the expected intermediate result.

Fig. 4 shows the responses obtained with the same experimental helium dis-

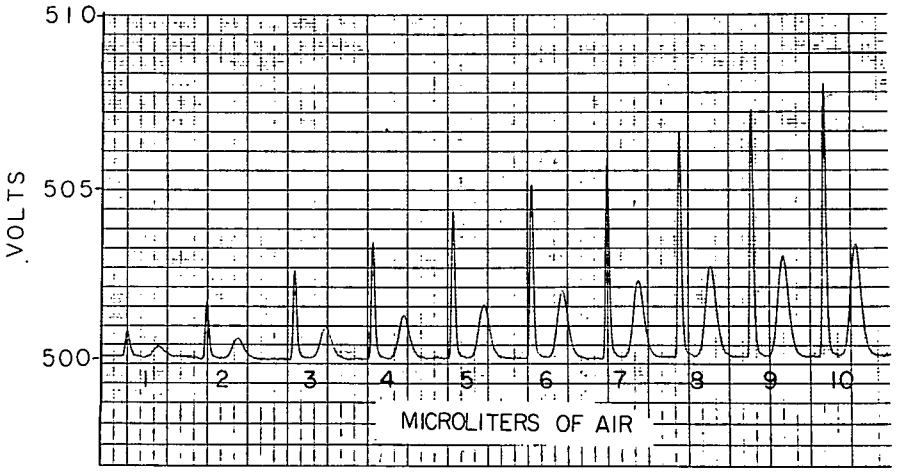

Fig. 3. A series of air samples, analyzed by a molecular sieve column, detected by an "impure" helium discharge.

charge setup as above except that the column had been "activated" by heating at 200° overnight with helium flowing through it. The baseline voltage increased from 500 to 800 V. Addition of either nitrogen or oxygen now increased the conductivity of the discharge (lowered the voltage). The sensitivity to both was greatly increased. It was necessary to add a dilution flow of 1000 cc of helium per minute to the detector to avoid overloading. Despite this dilution, the response to oxygen showed overloading at concentrations that resulted when 10 μl samples of oxygen were analyzed. More than twice this concentration of nitrogen in the helium was required to overload the detector. As may also be seen, the minimum voltage of the discharge reached when

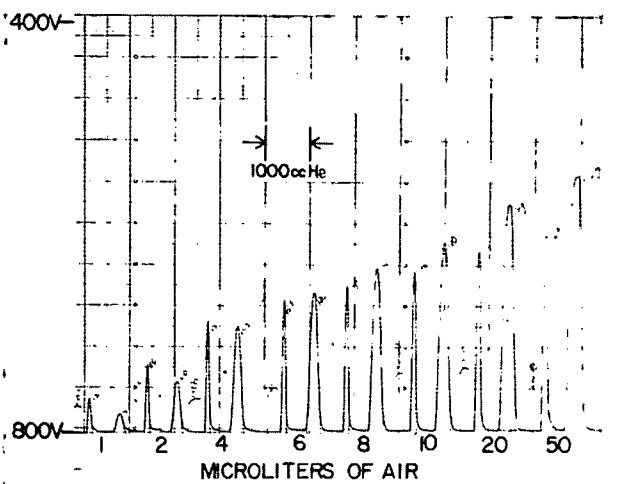

Fig. 4. A series of air samples, analyzed by a molecular sieve column, detected by a "pure" helium discharge.

oxygen was added to the helium was greater than the minimum reached when nitrogen was added.

It was possible to monitor the process of outgassing the molecular sieve column by observing the voltage and the response of the helium detector. When the column was freshly installed the voltage across the helium discharge ranged from 600 to 700 V, and oxygen and nitrogen both caused increases in voltage. The sensitivity was comparatively low, but within several hours, the voltage dropped to 550 V, and the sensitivity to both oxygen and nitrogen increased. Both continued to cause increases in voltage. With still further outgassing, but without a marked change in voltage, the sensitivity to low concentrations of nitrogen decreased. At this point low concentrations of nitrogen decreased the voltage while oxygen continued to increase the voltage. The response to oxygen then also changed toward increasing the conductivity of the gas.

The sensitivity, when the helium was sufficiently pure so that addition of oxygen and nitrogen caused decreases in the voltages (increased conductivity) was at least two orders of magnitude greater than that of the "impure helium" system in which these gases decreased the conductivity.

By repeatedly heating the molecular sieve column to 200° with helium flowing through it and then allowing it to cool, and by repeatedly heating the detector cell to 200°, we were able to raise the voltage across the helium discharge to a maximum of 1100 V. This was accomplished with considerable experimental difficulty. Sensitivity to oxygen and nitrogen increased with the increase in voltage. Disconnecting the detector from the column for less than ten seconds returned the voltage to 600 V and required repeated baking of the detector cell before the voltage could be raised again. We postulated that contamination of the electrode surfaces with water was responsible for a major part of this effect.

TABLE I

RESPONSE OF ARGON AND HELIUM DISCHARGES TO ORGANIC COMPOUNDS

	Argon			*Helium*		
	Response per mole	*Response per unit wt.*	*Response per gram C*	*Response per mole*	*Response per unit wt.*	*Response per gram C*
Chlorobenzene	2.28	1.49	1.95	0.92	0.58	0.74
Toluene	2.14	1.72	1.57	1.62	1.28	1.15
Fluorobenzene	1.47	1.11	1.24	0.71	0.55	0.60
Benzene	1.38	1.29	1.17	1.15	1.06	0.95
Iso-octane	1.16	0.74	0.74	1.43	0.81	0.89
Cyclohexane	1.09	0.93	0.91	1.43	1.23	1.19
Heptane	1.04	0.75	0.74	1.34	0.97	0.95
Pentane	1.00	1.00	1.00	1.00	1.00	1.00
Acetone	0.57	0.71	0.95	0.42	0.59	0.78
Methylene chloride	0.52	0.43	2.59	0.81	0.70	4.06
Chloroform	0.42	0.26	2.22	0.97	0.59	4.85
Ether	0.42	0.66	0.53	0.44	0.69	0.54
Methyl ethyl ketone	0.38	0.40	0.50	1.13	1.14	1.41
Carbon tetrachloride	0.38	0.19	2.04	1.14	0.54	5.66

Organic vapors

The response of both argon and helium discharges to organic compounds with different functional groups was variable (Table I). Within a series of closely related compounds, such as the aliphatic hydrocarbons, pentane, cyclohexane, heptane, and isooctane, or the halogenated hydrocarbons, the response appeared to be related to the number of molecules of these substances introduced, rather than to the weight.

The responses of the argon discharge to compounds within this limited group may be classified into the responses to aliphatic hydrocarbons, which were all similar, the responses to aromatic hydrocarbons, including toluene, benzene, chlorobenzene, and fluorobenzene which were greater and the responses to compounds containing oxygen such as acetone and those containing halogens, which were less.

The responses of the helium discharge to the same group of compounds were grossly similar. The discharge was most sensitive to toluene. The sensitivities per mole to aliphatic hydrocarbons, halogenated aromatics, and halogenated aliphatics were all equal. In the group of compounds tested, only diethyl ether and acetone were detected with lower sensitivity. The helium discharge showed somewhat less variability of response than the argon discharge. This was only a matter of degree rather than an easily discernible qualitative difference.

DISCUSSION

A stable electric discharge in a gas requires that free electrons be released at a rate equal to the rate at which they are lost. This requires that a population of ions and excited gas molecules exist in just sufficient concentration to cause the release of these electrons. If the discharge is self-sustained, this population of electrons, ions and excited molecules must be maintained without dependence on any source of initiating electrons. A stable gas discharge thus implies a rather unique condition of the gas.

In the usual operation of an ionization detector, a given voltage is impressed across the electrodes of the detector cell and the current is measured. The voltage chosen may, however, be less than optimal for maximum sensitivity when one gas is used, while yet large enough to result in destructive currents when other gases are used. Since the experiments reported here were designed specifically to compare the effects of using different gases, an attempt was made to use comparable conditions, and the use of a constant current, self-sustained, gas discharge was selected. This defined the conditions of excitation of the gas necessary for maintaining a self-sustained gas discharge as the conditions at which the effects of using different gases were compared.

Since the electrical characteristics of a self-sustained discharge are by definition independent of the level of radioactivity present, no radioactive source was required.

The voltage across the discharge depends primarily upon the composition of the gas. Because of this and because it is independent of the current in the same manner that the voltage across a voltage regulator tube is independent of the current, it is a better variable to follow than the current. Although the effect of small changes in current is small, the current was held constant electronically. A constant current system tends to be more stable especially when the test materials reduce the electrical conductivity of the gas.

Each of the discharges tested was found to be sensitive to each of the atmospheric gases and organic vapors added. LOVELOCK has reported[1] that the argon ionization detector is insensitive to nitrogen. We have noted that the sensitivities of the "impure" helium discharge and the argon discharge to a given atmospheric gas decreased with increasing concentration of that gas and postulated that different levels of impurities in the argon may explain the differences in the results reported from different laboratories.

By painstaking attention to the details of leaktight construction of the detector cell and by prolonged outgassing of the molecular sieve column, the connecting tubing, and the detector cell itself, we were able to cause the voltage across a "pure" helium system to rise to 1100 V when the effluent of a molecular sieve column alone was put through the detector. When the effluent of a capillary column was added, however, we were not able to obtain a voltage higher than 700 V. Since this same voltage could have indicated either moderate contamination of the helium or rather pure helium, we have determined which of the two it did actually indicate by injecting air into the capillary column. The responses to organic vapors described were each obtained using helium that responded to the addition of air with an increase in electrical conductivity, or what we described as "pure" helium.

This same test was not useful when the argon discharge was used, since the response of the argon discharge to air was always in the direction of decreased conductivity. Since the presence of relatively large concentrations of air in the argon reduces the increase of its conductivity when organic vapors are added, the ability of organic vapors to drop the voltage to 500 V was taken as evidence that the argon was not grossly contaminated with air.

The sensitivity of the pure helium system to oxygen, nitrogen and argon was greater than that of any of the other discharges described. The sensitivity of the same discharge to organic vapors was comparable to the sensitivity of the argon discharge to these vapors and was greater, approximately in proportion to the purity of the helium, than the sensitivity of the impure or contaminated helium system. Since this increase in sensitivity was obtained concomitantly with increase in sensitivity to oxygen and nitrogen, any real increase in sensitivity in terms of increased signal to noise ratio is achieved only through a great deal of effort.

Despite increased sensitivity to oxygen, the response of the pure helium discharge to organic compounds containing oxygen was still less than to similar compounds containing only carbon and hydrogen. Failure to obtain a marked improvement in nonspecificity of response over that obtained using the argon discharge, combined with the increase in experimental difficulty make its comparative usefulness for detection of organic materials, except under unusual circumstances, somewhat doubtful.

SUMMARY

Sensitive detection of atmospheric gases and organic vapors using ionization detectors has been accomplished with either helium or argon carrier gas. Addition of permanent gases in low concentration increased the electrical conductivity of an electric discharge excited in helium, while addition of higher concentrations of the same gases decreased it. Addition of permanent gases to an argon discharge reduced its conductivity.

Addition of small concentrations of organic vapors to either an argon or a helium discharge increased its conductivity. The sensitivity of both helium and argon discharges to compounds with different functional groups varied. The variation in sensitivity of the helium discharge, although somewhat less than that of the argon discharge, was found to be still pronounced.

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SCALE-UP OF GAS CHROMATOGRAPHY COLUMNS

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It is known that retention time usually passes through a slight minimum and then increases linearly with increasing sample size for compounds which produce ideal symmetrical peaks[1]. This is caused primarily by the increased band width at larger sample sizes. This effect can be overcome by using larger diameter columns which allow proportionally greater amounts of sample to be contained in the same band width obtained with the smaller columns. It has been observed that sample size may be quadrupled as column diameter is doubled if the length is increased by 450 %[2]. Increasing column length by a factor of three, all other conditions remaining the same, has resulted[3] in tripling the amount of sample that could be satisfactorily separated.

BEROES[4] observed that increasing the column diameter at constant linear carrier gas velocity resulted in increased efficiency, or lower values of HETP, the height equivalent to a theoretical plate. He proposed that the effect of channeling along the walls decreases with increasing internal column diameter. This indicates that the column flow patterns, and especially the wall effect, are significantly altered in large columns. He also observed that as column diameter increases, the retention time decreases sharply if the volumetric carrier gas flow rate is kept constant. GRANT AND VAUGHAN[5] found that the carrier gas flow rate must be increased in proportion to the increased cross-sectional area if the retention times were to remain the same.

The amount of a sample whose components boil approximately 1° apart that can be completely resolved has been shown to be approximately one-fourth the amount that can be separated if the components differ in boiling points by 10 to 20°[3,6]. In both cases, the columns were deliberately overloaded to leave only a small time interval between each component.

DIMBAT, PORTER AND STROSS[7] observed that the slight loss in efficiency caused by increasing the column diameter from 7 to 42 mm was almost completely offset by an increase in column length of less than 50 %.

KIRKLAND observed that for a limited number of systems, there was no difference in efficiency when the column diameter was increased from 4.75 to 31 mm if comparable operating temperatures were used. He adjusted the operating temperature of the larger column to a value which gave the same retention times as obtained with the smaller column.

For small samples, 2 μl or less, KIRKLAND used a sample vaporizer 50–75° above the boiling point of the highest boiler in the sample. For 10 μl samples, it was necessary to increase the vaporizer temperature to a level 100–150° above the boiling

temperature of the highest boiler to ensure that instantaneous volatilization during the 5–7 sec required for injection. POLLARD AND HARDY[8] have found that the effect of a ten-second sample injection period causes a change in HETP of only 1% as compared to plug injection.

These differences in experimental results indicate that the efficiency of separation cannot be attributed to particle size and weight ratio of substrate to support, but must also be some function of the column diameter and length and other factors associated with them. With columns of a given length, efficiency can be radically affected by changes in the porosity of the packing, velocity and temperature gradients across the column, and by channeling caused by poor sample injection and distribution techniques.

It is known that chromatographic columns operate most efficiently when the sample is distributed in a very narrow band as it enters the column, *i.e.*, efficiency increases as the injection approaches plug flow. As the column diameter is increased, the problem of maintaining plug injection becomes increasingly difficult. GOLAY[9] has shown that proper design and location of "distribution plates" for large diameter column can, to some extent, prevent the loss in efficiency usually encountered when scaling up analytical columns to preparative size. These plates tend to greatly reduce channeling and to flatten the velocity profile and so reduce leading and tailing.

The temperature gradients in an analytical column of small diameter operated in a constant temperature bath are quite small at low flow rates, and increase with flow rate. With small samples in such columns operated with a low carrier gas flow rate, changes in the temperature gradients caused by the heats of adsorption and desorption of the sample components should be small. With large samples in columns of large diameter, the adsorption and desorption heat effects may become large because of the wide solute band widths usually encountered in such systems. If this is the case, the temperature profiles will be distorted as the rate of heat exchange between the column and the surrounding constant temperature medium is slower than that for small columns at the same linear carrier gas flow rate. The result is that the solute is distributed in a non-isothermal band which becomes progressively more distorted as the band moves down the column. In the design of the Beckman "Megachrome" unit, an attempt has been made to overcome this problem by the use of several small columns operated in parallel for the separation of large (10–50 g) samples. An alternative to multiple column operation might be to pack the annular space between two large, concentric tubes or pipes, *i.e.*, pack the annulus between a 2-in. and a 4-in. tube and have the constant temperature medium circulating on both sides of the column. Another alternative might be the use of heat conducting rods inserted axially in the column to break up the temperature profiles in large diameter columns by acting as heat sinks.

The modified Reynolds number, based on average particle diameter and superficial vapor velocity,

$$N'_{Re} = \frac{d_p V_o \rho}{\mu}$$

was used in this work as it has been found to be an excellent method for correlating data to show the physical effects of varying, either independently or collectively, the velocity, viscosity, density, and packing particle size on fluid flow. Its usefulness as a

design factor in the construction of preparative-scale gas chromatography columns is a natural extension from common chemical engineering design methods for packed absorption towers, fixed-bed catalytic reactors, etc. The study reported here was designed to determine the effect of varying the modified Reynolds number and the ratio of column diameter to particle diameter, Z, on retention time for samples of constant size at moderate carrier gas flow rates. For this work, the density and viscosity of the carrier gas were used at average column absolute pressure as being representative of the bulk gas phase.

EXPERIMENTAL

Apparatus

The columns used consisted of a 129.4 cm packed length of borosilicate glass tubing surrounded by a vapor jacket composed of a 135.0 cm section of 5.1 cm internal diameter glass tubing. The column assembly has already been described[1]. The column diameters tested were 3.1, 4.6, 7.0, 9.0 and 18.1 mm. The thermal conductivity cell, recorder, and flow control system were as previously described.

Test mixtures

Test mixtures were prepared as follows. No. 1: 2-butanone, ethoxyethane, 2-propanone, and 2-(1-methylethoxy)-propane. No. 2: methanol, ethanol, 2-propanol, 1-propanol, 2-methyl-2-butanol, and 2-butanol. No. 3: ethyl formate, vinyl acetate, ethyl acetate, and methylethyl acetate. The choice of components for the test mixture was governed by the desire to have short total analysis times. The purity of the sample components was the highest normally available. Each mixture consisted of an equivolume mixture of the listed components.

Procedure

The column packing was 35 g of dibutyl phthalate per 100 g of $-48 + 65$ Tyler standard mesh type C–22 Johns-Manville firebrick. The method of packing preparation and system operation were as previously described. The columns were maintained at 98.6° by refluxing water in the vapor jacket. Samples were injected into the system through a self-sealing rubber serum cap. The sample size used in this work was 10 μl. Sample injection and vaporization was complete within 2 sec or less.

RESULTS AND DISCUSSION

Flow rate changes

The 3.1, 4.6, 7.0, and 9.0 mm inside diameter columns were tested at carrier gas flow rates at 5.0, 10.0, 19.6, 36.5, 58.5, and 78.0 ml of helium per min. The 18.1 mm inside diameter column was tested at flow rate of 5.4, 32.5, and 78.0 ml/min. Inlet pressure to all the columns except the 18.1 mm I.D. column was 20 p.s.i.g. For the latter column, the inlet pressure required was 30 p.s.i.g.

For these runs the ratios:

$$Z = D_T/d_p$$

of column diameter to average particle diameter were 12.3, 18.3, 27.8, 35.7, and 71.8

in order of increasing column diameter. The average particle diameter was 0.25 mm which corresponds to the —48 + 65 mesh Tyler standard screen fraction. The retention time is greatly dependent on the Reynolds number (or at least velocity) for the work done at the two lowest values of Z. At the two highest values of Z, the retention time is only slightly effected by changes in the Reynolds number. Under these conditions a plot of corrected retention time ($T_R°$) *vs.* N'_{Re} gave straight, nearly horizontal

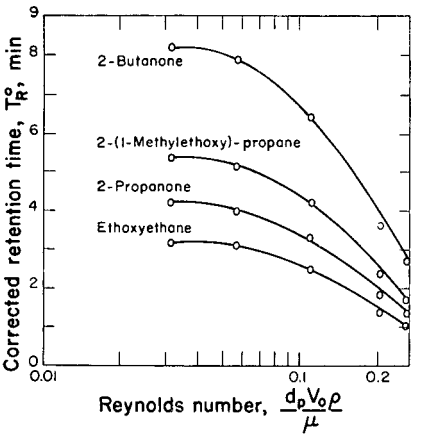

Fig. 1. Effect of modified Reynolds number on corrected retention time. Particle diameter: 0.25 mm (—48 + 65 Tyler standard mesh). Column diameter: 3.1 mm.

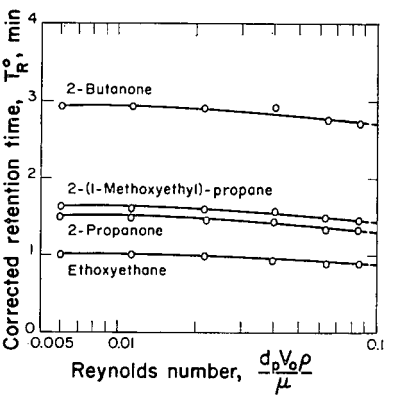

Fig. 2. Effect of modified Reynolds number on corrected retention time. Particle diameter: 0.25 mm (—48 + 65 Tyler standard mesh). Column diameter: 7.0 mm.

lines which were approximately parallel. Representative data obtained with the ether–ketone test mixture (No. 1) under the above conditions is presented in Fig. 1 for $Z = 12.3$. The data points in this figure are numerical averages of triplicate observations. Deviations between observations were less than 0.8 % in all cases. As

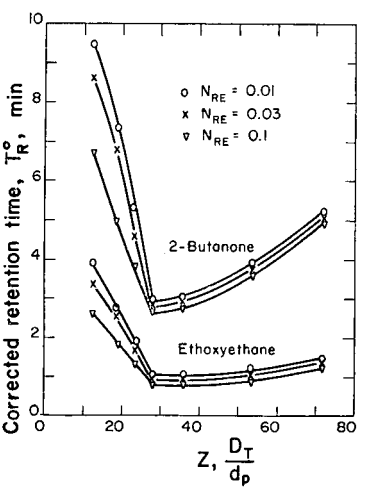

Fig. 3. Effect of column to particle diameter ratio on corrected retention time. Particle diameter: 0.25 mm. Column diameter: variable.

the average column pressure increased with increasing flow rate the retention times plotted have been corrected for the compressibility of the vapor phase by the method of JAMES AND MARTIN[10]. The case where $Z = 27.8$ seems to be a transition point (Fig. 2). The curvature, unlike that exhibited in Fig. 1, is very slight. The transition produces a definite minimum value of $T_R°$ when plotted against Z for different Reynolds numbers. This is shown in Fig. 3 for 2-butanone and ethoxyethane. The only explanation that presented itself for these minima was that the wall effect becomes appreciable at the lower Z values, resulting in a widening of the solute band. This increase in band width may be caused by the fact that the velocity at a point approximately one particle diameter from the column wall may be as much as 100 % greater than the velocity of the column[4,11,13] because of radical porosity changes. The result is increased channeling of the flow as the column diameter is decreased as is indicated by the change in the curvature on the left. As N'_{Re} increases, the minimum value of $T_R°$ decreases as would be expected.

Particle size effects

In order to be sure that the curves in Fig. 3 were not peculiar to the system used (test mixture 1 on the —48 + 65 dibutyl phthalate column) additional work was done using test mixtures 2 and 3. For this work, the column internal diameter was held constant at 4.6 mm and the —28 + 48, —48 + 65, —65 + 100, —100 + 150, and —150 + 200 mesh Tyler standard fractions of crushed C–22 firebrick were used to prepare a series of packings, each having 35 g dibutyl phthalate per 100 g brick. Similar packings using dibutyl sebacate were also prepared in these size fractions. When the corrected retention times were plotted *vs.* Z for $N'_{Re} = 0.01$ and 0.1, the curves were found to have minimum values of $T_R°$ at $Z = 26$ to 29 for the components of test mixtures 2 and 3 in the dibutyl phthalate packings. This work was duplicated for the same test mixtures in the dibutyl sebacate columns. Minimum values of $T_R°$ were found at $Z = 24$ to 27. For both these two series of tests, the column temperature was 98.6°. The same flow rates as used in the ether–ketone phase of the work were used for the 4.6 mm internal diameter column just described.

Further work was done using the —28 + 48 Tyler mesh dibutyl phthalate packing in each of the 5 columns. For these runs the column inlet pressure was 30 p.s.i.g. in all cases. The flow rates tested were the same as those used for the initial work using test mixture No. 1. Minimum values of $T_R°$ were found at $Z = 20$ to 25.

The left branch of the curves in Fig. 3 may be explained by the fact that the porosity increases near the column wall. As the column diameter was increased, the proportions of the relatively porous area to the total column cross-sectional area decreased, causing a decrease in the average column porosity. The limiting retention time $T_R°$ can be calculated[12] from:

$$V_R° = V_o + KV_s = FT_R° \tag{1}$$

or:

$$T_R° = V_o/F + KV_s/F \tag{2}$$

As the temperature is constant the partition coefficient K should not change. Decreasing in the average column porosity by increasing the column inside diameter caused a decrease in the area available for the gas phase in any cross-section. Although

the total amount of liquid phase present is increased by decreasing the porosity in this manner this should not have much influence on the retention time for work done using a given particle size since the solubility of the sample in the substrate is not generally a limiting factor in the case of high substrate to brick weight ratios such as used in this work.

With increasing average column pressure the volume of gas in the column at standard conditions increases, while the volumetric flow rate remains constant. This would cause an increase in the retention time. It is suggested that above Z values of approximately 25 to 30 the effect of increased average column pressure will overcome the effect of changes in the average column porosity. The result is an increase in retention time as seen in the right branch of the curves in Figs. 3 and 4.

The shift in the location of the minima illustrated in Fig. 4 when using different

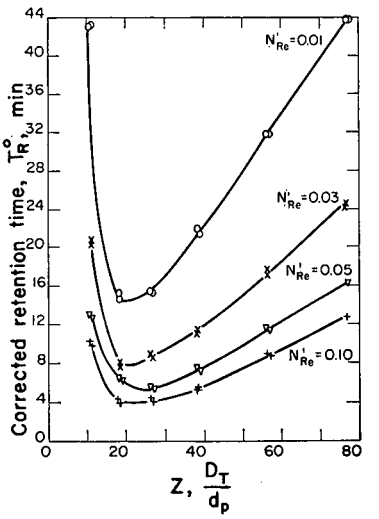

Fig. 4. Effect of column to particle diameter ratio on corrected retention time for ethanol. Particle diameter: variable. Column diameter: 4.6 mm.

particle diameters in the 4.6 mm column compared with those obtained using a constant particle size in columns of different diameter is undoubtedly due to the change of the thickness of the substrate film caused by changing the particle diameter while holding the ratio of substrate to brick constant. These changes in liquid film thickness are not too large as the surface areas of the packings, as determined by the Johns–Manville Corp., Mannville, N.J., by the Brunauer–Emmett–Teller method were: 4.0, 3.2, 3.0, 3.0, and 2.3 m^2/g for the — 28 + 48, — 48 + 65, — 65 + 100, — 100 + 150, and — 150 + 200 Tyler standard screen fraction, respectively.

Temperature effects

The effect of changing the Reynolds number by changing the operating temperature has been investigated using room temperature, 65°, 98.6°, and 127.5° for the — 48 + 65 mesh dibutyl phthalate and dibutyl sebacate packing in the 4.6 mm diameter column at flow rates of 5.0, 27.5, 50.6, 61.8 and 76.5 ml helium per min. In

all cases when corrected retention time was plotted *vs.* log Reynolds number, straight lines were obtained as would be expected.

CONCLUSIONS

From the data collected it appears that the contribution of the wall effect to retention time is a general phenomenon. It is also concluded that large scale chromatography columns can be prepared which will operate at maximum efficiency and minimum retention time if the ratio of column diameter to particle diameter is maintained at approximately 25. This is in close agreement with a Z value of 30 obtained by SCHWARTZ AND SMITH[11] using various packing sizes and shapes in 2 to 4 inch pipes. Further work is in progress and will be reported later.

ACKNOWLEDGEMENT

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SUMMARY

The effect of the modified Reynolds number, N'_{Re}, and the ratio of column diameter to support particle diameter, Z, have been studied in 5 column sizes and 5 particle sizes at 6 flow rates, for several test mixtures of oxygenated aliphatics. The results indicate that minimum values of the pressure-drop-corrected retention time, $T_R°$, will be obtained at Z values of 20–29, with an average value close to $Z = 25$.

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POSSIBLE RADIATION HAZARDS ARISING FROM THE USE OF RADIOACTIVE DETECTORS IN GAS CHROMATOGRAPHY

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INTRODUCTION

Radioactive detectors for use in gas chromatography were first described by LOVELOCK[1]; these detectors consist essentially of an ionisation chamber containing a source of radiation in the form of a radioactive foil. Commonly used sources of radiation are 10 to 20 mC of strontium-90 or promethium-147, 200 mC of tritium, and 100 μC radium-226 or radium-D.

The hazards which may arise from handling radioactive materials fall into two categories; namely, external radiation, that is radiation originating from sources outside the body, and internal radiation, which results from the entry of radioactive material into the body. In the case of external radiation, the magnitude of the hazard depends on the nature of the radiation (whether α-, β-, or γ-rays), on the isotope concerned and its activity, on the distance from the source, on the presence of shielding and on the time of exposure. Some exposure to external radiation is almost inevitable whenever radioactive materials are used, but with proper precautions this should be very slight in the use under consideration. In the case of internal radiation, the magnitude of the hazard depends not only on such physical aspects as radioactive half life and the nature and quantity of the radiations emitted, but also on the uptake and localisation of the element in question in the body, and on the method and rate of its elimination from the body. In the use of radioactive materials in gas chromatography, the internal hazard can be almost entirely eliminated if proper attention is paid to laboratory technique, cleanliness and good ventilation.

In general, the radioactive detectors in gas chromatography are handled by persons who would not otherwise be concerned with radioactive materials and it is therefore desirable, unless there are very strong reasons to the contrary, not to have to classify them as radiation workers. This means that the annual dose received by the whole body and individual organs (except the thyroid gland) from both external and internal radiation should not exceed 1.5 rem, the corresponding dose to the skin and the thyroid gland being 3 rems[2]. The question of a higher figure for the hands and forearms, feet and ankles is at present under review and an annual maximum permissible dose of 7.5 rems may be agreed.

CONSIDERATION OF THE POSSIBLE HAZARDS

The various possibilities of external and internal hazard are considered separately below. Internal hazards will only arise in the event of the liberation of radioactive

material, which may occur as a result of (a) mechanical damage to the foil during assembly and cleaning, (b) emission of radioactive gas such as radon (from ^{226}Ra) and tritium, (c) chemical attack of the foil by the vapour under investigation, and (d) the effect of heat.

External radiation

In the case of tritium and promethium-147 foils the detector body itself is sufficient to absorb all the primary β radiation. The same may, in general, be true of Ra-D foils but, if the apparatus is constructed entirely of glass, some additional shielding may be necessary to stop the more energetic β particles. However, in stopping β-particles bremsstrahlung* is produced and, with the exception of the β-radiation from tritium, shielding is usually necessary against it. For the ^{90}Sr and ^{226}Ra sources in current use, external shielding must be provided which is sufficiently thick to reduce to acceptable levels the β-radiation and any associated bremsstrahlung, as well as the γ-radiation in the case of ^{226}Ra. The protection of β-ray sources by various materials is discussed by HAYBITTLE[3], and WYARD[4] gives a formula for calculating the dose rate due to bremsstrahlung. Although lead is very often used as a shielding material, some other material such as copper should be used if it is intended to use the gas chromatography apparatus at temperatures in excess of 250° on account of the low melting point of lead (327°).

As an example of the importance of bremsstrahlung, one detector containing a 20 mC ^{90}Sr source was submitted for test here and found to give a dose rate due to bremsstrahlung of about 100 mrad/h at its surface despite the fact that the body of this particular detector was constructed of brass about 1 cm thick and was shielded with 1 cm lead. The dose rate at an accessible point can be reduced to an acceptable level by enclosing the detector in a screen of suitable dimensions; a screen placed 20 cm from the surface of the assembled detector would limit the dose rate to 0.75 mrad/h, which would permit continuous handling, but handling the shielded detector without its screen would be limited to about one and a half hours per week for a person not classified as a radiation worker (assuming 7.5 rems/year is accepted as the limit for the hands). This is in accordance with I.C.R.P. concepts; in the United Kingdom the Factory Sealed Sources Regulations 1961[5] may impose more severe limitations if persons are not "classified workers".

So far only handling and protection of the assembled detector have been discussed but, in certain instances, operators may wish to build or clean their own detector. In these circumstances the use of forceps is necessary in order to reduce the very high radiation dose which would otherwise be received by the hands in close proximity to the unshielded active area of the radioactive source. Some radiations, namely the α-rays from ^{226}Ra and Ra-D, as well as the β-rays from tritium are so readily absorbed that they do not penetrate to the sensitive parts of the skin and hence are considered to produce no external radiation hazard. More energetic β-rays do penetrate deeper, and the dose rate at the surface of the skin due to β-radiation can be roughly estimated if we assume that at a distance of 10 cm from an unfiltered point source of 1 μC, of ^{226}Ra and daughters, Ra-D and daughters, $^{90}Sr + {}^{90}Y$, and ^{147}Pm, the dose rate will be approximately 25, 9, 15, and 2.5 mrad/h, respectively.

* Bremsstrahlung is a form of secondary electro-magnetic radiation which is produced during the absorption of β-rays.

For example, 100 μC ^{226}Ra will give a β-ray dose rate of approximately 2.5 rad/h and 20 mC ^{90}Sr a β-ray dose rate of approximately 300 rad/h when handled with forceps 10 cm long. In addition to the β-radiation, there will also be a γ dose of approximately 9 mrad/h in the case of the ^{226}Ra source. As will be seen from the above figures, the handling of unshielded sources will, with the exception of tritium sources lead to dose rates far in excess of that which can be accepted continuously by persons not classified as radiation workers and should therefore be restricted to the persons classified as radiation workers and the appropriate handling techniques for such sources must be used.

Internal radiation

(*a*) *Mechanical damage.* The tritium sources consist of a copper strip covered with a thin evaporated layer of titanium in which the tritium is absorbed; the other sources consist of silver or gold foil strips in which the isotope is firmly bonded. In the case of the ^{226}Ra and Ra-D foils, the isotope is completely enclosed by the gold foil except at the cut ends of the strip. On either side of the active area there is an inactive area, 2.5 to 7.5 mm wide, depending on the source, which may be used for handling the source or for fixing it in the detector. In order that the α-radiation may penetrate the foil, the front face of the foil has a very thin "window" 4 to 5 μ in thickness. The ^{147}Pm foils are similar except that there are no active ends, and the isotope is completely enclosed within the foil. In the case of ^{90}Sr, the isotope is completely enclosed but the thickness of the active face is 50 μ.

To prevent mechanical damage to the extremely fragile "window" over the active area the sources should only be manipulated by their inactive edges and again the use of forceps is indicated.

In cases where it is necessary to clean the detector it is recommended that the detector be flushed out with a non-corrosive solvent which is suitable for the deposit to be dissolved. If the source has suffered any mechanical damage or damage due to chemical action, the wash liquor will be radioactive and it is advisable that workers should wear rubber gloves when handling the wash liquor and should decontaminate the apparatus used after completion of the washing. The liquor itself should be regarded as radioactive waste and treated accordingly with respect to disposal. Under no circumstances should the foils be swabbed, or damage will result.

(*b*) *Emission of radioactive gas.* Experiments to determine the leakage of radon from radium-226 sources were carried out at the Radiological Protection Service (R.P.S.) with a foil containing 100 μC radium and have shown that about 10^{-4} μC of radon escape from the foil each minute when the apparatus is operating at a temperature of 200°. There was also evidence that when the foil was cut into two pieces the rate of emission increased possibly because the length of cut edge was increased.

Assuming that the source is in a room of 1000 ft.^{3} having three air changes per hour, the concentration of radon in air would then be about 10^{-10} μC/cm^3. This is 30 times less than the value, $3 \cdot 10^{-9}$ μC/cm^3, recommended by the International Commission on Radiological Protection for uncontrolled areas, assuming a 40 hour week. Under conditions of a fairly large room and normal ventilation the leakage rate of radon does not present a hazard, but where space is restricted and ventilation poor it is advisable to lead the outlet of the column outside the building or into a fume extractor.

Data appertaining to leakage from tritium sources can be obtained on application to the Radiochemical Centre[6] who state that molecular tritium is slowly evolved from a 200–300 mC source at room temperature to the extent of 5 μC/day. There is a further reduction in activity of about 40 μC/day as a result of radioactive decay. The leakage rate has been found to increase with elevation of temperature; for example, it increases ten fold between 200° and 250°, 1 % of the tritium in the source being lost in 24 hours at 250°. It would therefore be advisable to limit the use of these sources to a maximum temperature of 200° and to lead the effluent gas outside the building or into a fume extractor. In any event, the ventilation of the room in which the detector is used should be sufficient to ensure that the average concentration of tritium in the form of tritium oxide does not exceed the maximum permissible level of $5 \cdot 10^{-7}$ μC/cm^3 for non-occupational exposure for a 40 hour week or $2 \cdot 10^{-4}$ μC/cm^3 of molecular tritium (T_2 or HT)[2].

(*c*) *Chemical attack.* It is difficult to predict what the effect of chemical attack on these foils will be. The tritium sources are reported[6] as being affected by acidic and basic vapours to varying degrees dependent on temperature. The strontium foils, by virtue of their sealed ends and of their thicker "window", will be less subject to attack than the ^{147}Pm, Ra-D and ^{226}Ra foils, with their thinner "windows", but the ultimate effect of chemical corrosion on the "window" will be the same. The Ra-D and ^{226}Ra foils are also more vulnerable to chemical attack owing to their open ends.

An 80 μC Ra-D foil was tested by the author by passing a number of corrosive vapours through the detector containing it. Various temperature conditions and rates of gas flow were used. The effluent gas was passed into a vapour trap instead of being allowed to discharge into the air as usual and the foil itself was washed with ether after each run. The amount of activity found in the trap and the washings was found to depend to a marked degree upon the temperature of the device and the previous treatment of the foil. When hydrogen chloride was used it was found that no damage to the Ra-D foil occurred at temperatures below 100° but that damage occurred above this temperature.

Once damage had occurred to the foil, up to 10^{-4} μC/h of Ra-D and daughters were identified in the vapour trap when the chromatograph was run with organic substances unlikely to give corrosive breakdown products. However, up to ten times as much Ra-D and daughters were found in the trap when further samples of compounds having breakdown products such as hydrogen chloride, chlorine and thionyl chloride were introduced into the chromatography column. Assuming that the effluent gas discharges into a 1000 ft.^{3} room having three air changes per hour the concentration of Ra-D in the atmosphere would be about 10^{-12} μC/cm^3 when the apparatus is used for non-corrosive vapours and up to 10^{-11} μC/cm^3 in the case of corrosive vapours. These are respectively one tenth and equal to the maximum concentration in air which is recommended by the I.C.R.P. for non-classified workers, assuming a 40 hour week. However damage once started is progressive, and the amount of damage depends on the concentration of the breakdown products in the carrier gas and also on the presence of water vapour which aggravates the damage. In exceptional cases the compound under investigation can itself produce a volatile compound with the radioactive material in the foil, in which case the activity in the effluent gas would certainly exceed the quantities stated above. For example, the R.P.S. was asked to examine a number of organic phosphohalogen compounds one of

which, phenyl-dichlorophosphine, removed as much as 0.02 μC of Ra-D from the source when a single sample was introduced into the chromatography column. If this 0.02 μC of Ra-D had been allowed to enter a 1000 ft.^{3} laboratory it would have produced an average concentration of twenty times the maximum permissible level.

(*d*) *Effect of heat.* The effect of heat on tritium and ^{226}Ra sources and the production of radioactive gas has already been referred to.

In the case of the α-emitting foils it is known that, if the temperature is allowed to rise above 450°, damage to the "window" occurs with resulting leakage of activity[7]. There is also some evidence to suggest that, over prolonged periods of heating, damage may occur below this temperature. Tests on sources up to temperatures of 250° appear to result in little or no damage to the source as will be seen below, but it would seem that breakdown of the foil takes place progressively in the temperature range 250–450°. No tests were carried out on ^{147}Pm foils but it would be expected that their behaviour would be similar to other foils.

Tests were carried out at the R.P.S. on a detector containing a 20 mC ^{90}Sr source. The temperature was maintained at 200° and argon was continuously passed through the detector for about a month. The effluent gas from the detector was passed into a trap and contents of the trap were assayed daily for ^{90}Sr. Amounts of the order of 10^{-6} μC/day were detected; this is, of course, very small in comparison with the 20 mC of ^{90}Sr actually enclosed within the foil and, if escaping into a well-ventilated room, would give air concentrations well below the maximum permissible concentration for non-classified workers of $3 \cdot 10^{-11}$ μC/cm^3.

CONCLUSIONS

Provided that this extremely useful type of detector is treated with due regard to the fact that it contains radioactive material, there is very little hazard involved. Handling of the sources should be kept to a minimum for the reasons mentioned above, and overheating of the detectors should be avoided; the temperature should preferably be below 250°. As a safety measure, it would be advisable to pass the effluent gas from the apparatus into a fume cupboard, in order to minimise the hazard which would arise as a result of overheating or chemical attack. It is possible to avoid some of these problems by enclosing the foil in, for example, a thin glass envelope. This would protect the foil from chemical attack and would contain any isotope released as a result of overheating. This form of protection, however, is only suitable in the case of fairly energetic β-emitters such as ^{90}Sr and would, of course, exclude those with insufficient energy to penetrate the glass. It must not be forgotten, however, that external shielding of the detector as a whole is still necessary.

ACKNOWLEDGEMENTS

The author wishes to thank her colleagues at the Radiological Protection Service for the many helpful discussions during the preparation of this paper, and for the calculation of the dose rates due to β-radiation and measurement of the bremsstrahlung from β-emitting foils.

SUMMARY

The hazards which may arise due to the use of radioactive foils in the gas chromatography detectors are reviewed; they are external radiation, effect of mechanical damage emission of radioactive gases, the effect of chemical attack and the effect of heat. Overheating of the foils is the principle source of danger to the majority of users, but the other hazards particularly effect those persons who build or service their own apparatus.

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RELATIVE RETENTION TIMES OF C_1–C_8 HYDROCARBONS OVER DIFFERENT COLUMNS AND AT DIFFERENT TEMPERATURES*

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INTRODUCTION

An extensive investigation of catalytic dehydrogenation and isomerization of hydrocarbons carried out in our laboratory[1,2] required a reliable method to analyze the numerous reaction products. Gas–liquid chromatography gave satisfactory quantitative and qualitative analysis for the C_1–C_8 hydrocarbons involved in our studies. Due to the complexity of most samples, a number of different columns were used in the identification of each unknown reaction product. This study presents the relative retention times of the hydrocarbons studied over seven different columns at different temperatures.

EXPERIMENTAL

Two Podbielniak Chromacon Vapor Phase Chromatographic Analytical Apparatus (Models No. 9475 3A and 9580) and an F and M Model 300 Gas Chromatograph were used throughout this study. Liquid samples were injected from Hamilton microliter syringes. Sample sizes varied from 0.2 μl to 1 μl. Gaseous samples were introduced by medical gas-tight syringes. Sample sizes varied from 0.1 to 2 ml. All determinations were done at constant temperatures. Helium was used as carrier gas. The different columns used are listed in Table I. All columns were made from 1/4 in. O.D. copper tubes, bent in spiral forms. Flow rates were measured at the column outlet using a bubble flowmeter. Theoretical plate numbers for the different columns were calculated using the formula:

$$\text{Number of theoretical plates} = 5.54 \left[\frac{t_r}{W_{1/2}}\right]^2$$

where t_r was the time between introduction of the sample and the peak emergence, and $W_{1/2}$ was the peak width at half height.

The number of theoretical plates was determined using 0.2 μl samples of *n*-hexane on columns A and B, and similar quantities of *n*-octane or toluene on columns C and D. For column F an 0.1 ml ethane sample was used.

n-Hexane was selected as reference.

* Taken in part from a dissertation submitted by S. M. CSICSERY to the Graduate School in partial fulfillment of the requirements for the PH. D. degree, December 1961.

TABLE I

DESCRIPTION OF GAS–LIQUID PHASE CHROMATOGRAPHIC COLUMNS USED IN HYDROCARBON RELATIVE RETENTION TIME STUDIES

Column	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Liquid phase	35% Dimethylsulfolane–dipropylsulfone (ratio 27:73)	33% Dimethylsulfolane	5% 7,8-Benzoquinoline	20% Dinonyl phthalate ester	7% Di-*n*-propyl tetrachlorophthalate ester		15% Carbowax "600"
Solid support	Firebrick	Firebrick	Firebrick	Celite	Celite	Davison's 950 Silica Gel	Gas–Chrom P
Mesh	30/60	100/120	100/120	30/60	30/60	60/200	80/100
Length of column (ft.)	35	15	26	10	30	8	14
Theoretical plates	2200	5800	6900	1000		1290	
Flow rate (ml He/min)	70–130	50–75	28–35	40–80	50–100	20	60
Inlet pressure (p.s.i.)	13–17	25–32	40	10–20	18–30	30	30
Column temperature (°C)	28	30–75	86–108	86–124	96–124	28	125

Relative retention time, α_x, was defined as:

$$\alpha_x = \frac{r_x - r_{air}}{r_{nHA} - r_{air}} = \frac{K_x}{K_{nHA}}$$

where r_{air} was the retention of air, which was not retarded in the columns; r_x and r_{nHA} were the retentions of compound X and *n*-hexane; and K_x and K_{nHA} were the corresponding partition coefficients, taken as ratios of the solute concentrations in the liquid phase to those in the gas phase. For column F, the only gas–solid adsorption chromatographic column used in this study, ethane was selected for a reference.

All measurements were done at the highest sensitivities available on each in-

TABLE

RELATIVE RETENTION TIMES OF C_1–C_8 HYDROCARBONS

		B.P. (°C)	A	B			
	Column temp. (°C)		28	30	43	52	75
1	Carbon monoxide	−192					
2	Methane	−161.49	0.002				
3	Ethane	−88.63	0.016	0.017	0.023		
4	Ethylene	−103.71	0.019	0.02	0.028	0.03	
5	Carbon dioxide	−78.5	0.038	0.044	0.06	0.059	
6	Propane	−42.07	0.047	0.050	0.061	0.066	
7	Propylene	−47.70	0.077	0.086	0.102	0.125	
8	Isobutane	−11.73	0.091	0.093	0.11	0.12	
9	Cyclopropane	−33		0.121			
10	*n*-Butane	−0.50	0.138	0.142	0.164	0.176	
11	Neopentane	9.503	0.147	0.15	0.17	0.188	
12	Acetylene	−84		0.19			
13	1-Butene	−6.26	0.210	0.233	0.256	0.28	
14	Isobutylene	−6.900	0.223	0.247	0.271	0.29	
15	Allene	−34.5		0.267			
16	*trans*-2-Butene	0.88	0.270	0.305	0.325	0.36	
17	Isopentane	27.852	0.290	0.295	0.32	0.36	
18	*cis*-2-Butene	3.72	0.317	0.358	0.381	0.405	
19	*n*-Pentane	36.074	0.378	0.388	0.41	0.455	
20	1,1-Dimethylcyclopropane	21	0.366	0.39		0.476	
21	3-Methyl-1-butene	20.061	0.375	0.40	0.426	0.48	
22	2,2-Dimethylbutane	49.741	0.52	0.52	0.54	0.593	
23	1,3-Butadiene	−4.413	0.45	0.54	0.555	0.595	
24	Methylacetylene	−23.22		0.58			
25	1-Pentene	29.968	0.545	0.595	0.616	0.655	0.77
26	3,3-Dimethyl-1-butene	41.24	0.615		0.66	0.69	
27	2-Methyl-1-butene	31.163	0.635	0.69	0.705	0.760	
28	2-Methylpentane	60.271	0.740	0.725	0.74	0.776	
29	*trans*-2-Pentene	36.353	0.660	0.735	0.748	0.79	
30	2,3-Dimethylbutane	57.988	0.74	0.74	0.758	0.794	

strument, and special care was taken to avoid overloading the columns by using the smallest possible samples. The temperature control and the accuracy of the temperature measurements varied among the instruments used.

Many of the hydrocarbons and other compounds were commercial products or API samples. Some of the less common hydrocarbons were prepared in our laboratory.

The relative retention times of the hydrocarbons are presented in their increasing order (over column B) in Table II. A few alcohols, ketones, and other compounds are listed at the end of Table II. The relative retention times presented here are the averages of a large number of measurements. The number of significant figures indicates the accuracy of each relative retention time value.

II

OVER DIFFERENT COLUMNS AND AT DIFFERENT TEMPERATURES

C		*D*				*E*		*F*	*G*	
86	*108*	*86*	*95*	*114*	*124*	*96*	*124*	*28*	*125*	
								0.003		1
								0.095		2
		0.024						1.000		3
								2.10		4
								1.85		5
0.06		0.07	0.07	0.09	0.10	0.07		5.0		6
0.075		0.08	0.09		0.12	0.09				7
		0.13		0.16	0.18					8
										9
0.19		0.19	0.20	0.215	0.247	0.19				10
										11
										12
		0.19	0.20	0.21						13
										14
										15
0.28		0.234	0.25	0.26	0.306	0.26				16
0.33		0.35	0.38	0.40	0.43					17
		0.255	0.27	0.28	0.31	0.27				18
0.43	0.46	0.44	0.46	0.47	0.506	0.45	0.55		0.55	19
0.35		0.347								20
0.35		0.335								21
0.557		0.594		0.65					0.56	22
										23
				0.485						24
										25
										26
0.48		0.48								27
0.773		0.768	0.787	0.79	0.825	0.80				28
0.58	0.60			0.57	0.575	0.58				29
0.726		0.68							0.63	30

(continued on p. 38)

TABLE II

		B.P. (°C)	A	B			
	Column temp. (C°)		28	30	43	52	75
31	*cis*-2-Pentene	36.942	0.725	0.812	0.813	0.825	
32	3-Methylpentane	63.282	0.875	0.873	0.876	0.910	
33	1,4-Pentadiene	25.967		0.895	0.90	0.936	
34	2-Methyl-2-butene	38.568	0.850	0.935	0.92	0.942	0.97
35	1,1,2-Trimethylcyclopropane	57		0.94		0.98	
36	*n*-Hexane	68.740	1.00	1.00	1.00	1.00	1.00
37	3-Methyl-1-pentene	54.14		1.06	1.04		
38	4-Methyl-1-pentene	53.88	1.01	1.06	1.04		
39	Cyclopentane	49.262	1.02	1.14	1.14	1.15	
40	4-Methyl-*cis*-2-pentene	56.30		1.14	1.14		
41	4-Methyl-*trans*-2-pentene	58.55	1.08	1.18	1.16		
42	2,2-Dimethylpentane	79.197				1.19	
43	Isopropylcyclopropane	70		1.21			
44	2,3-Dimethyl-1-butene	55.67	1.17	1.28	1.24	1.20	
45	2,4-Dimethylpentane	80.500	1.30	1.32	1.21	1.20	
46	Isoprene	34.067	1.29	1.51	1.46	1.41	
47	2,2,3-Trimethylbutane	80.882				1.43	
48	Cyclopentene	44.242		1.55	1.53	1.47	1.46
49	1-Hexene	63.485	1.44	1.57	1.51	1.47	
50	2-Methyl-1-pentene	60.7	1.485	1.62	1.55	1.54	
51	*trans*-3-Hexene	67.08	1.47	1.65	1.575	1.54	1.53
52	4,4-Dimethyl-*trans*-2-pentene	76.75		1.67	1.58	1.57	
53	*trans*-2-Hexene	67.87	1.61	1.76	1.67	1.66	1.57
54	*cis*-3-Hexene	66.44	1.61	1.76	1.67	1.66	
55	3,3-Dimethylpentane	86.064		2.0	1.7		
56	2-Ethyl-1-butene	64.66	1.69	1.84	1.80	1.75	
57	Methylcyclopentane	71.812	1.74	1.78	1.74	1.71	1.67
58	2-Methyl-2-pentene	67.29	1.73	1.93	1.85		
59	*cis*-2-Hexene	68.84	1.83	2.01	1.9	1.82	
60	3-Methylhexane	91.850				1.84	
61	1-*trans*-3-Pentadiene	42.032	1.73	2.06	1.97	1.92	1.84
62	3,3-Dimethylpentene	77.54		2.25	2.02		
63	2,2,4-Trimethylpentane	99.238	2.47		2.02	1.95	
64	3-Methyl-*trans*-2-pentene	67.63	1.92	2.14	2.1	2.00	
65	2,3-Dimethylpentane	89.784		2.2	1.92		
66	1-*cis*-3-Pentadiene	44.068	1.9	2.31	2.19	2.1	2.02
67	4,4-Dimethyl-*cis*-2-pentene	80.42				2.08	
68	3-Ethylpentane	93.475				2.09	
69	3-Methylcyclopentene	65.0		2.32		2.12	2.07
70	3-Methyl-*cis*-2-pentene	70.45	2.12	2.35	2.2	2.12	
71	2,3,3-Trimethyl-1-butene	77.87		2.54	2.22	2.18	
72	1-Ethyl-1-*cis*-2-dimethylcyclopropane			2.5	2.20		
73	1-Ethyl-1-*trans*-2-dimethylcyclopropane			2.6	2.30		
74	2,4-Dimethyl-1-pentene	81.64			2.26	2.16	
75	Cyclohexane	80.738		2.6	2.46	2.36	2.27

(continued)

C		*D*				*E*		*F*	*G*	
86	*108*	*86*	*95*	*114*	*124*	*96*	*124*	*28*	*125*	
						0.65				31
0.84		0.87	0.886		0.91				0.91	32
										33
0.60		0.60								34
										35
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		1.00	36
										37
										38
										39
										40
										41
1.2										42
										43
										44
1.21		1.25								45
0.88										46
1.28										47
1.08										48
							1.15			49
1.07		1.02								50
1.13			1.13	1.1	1.07	1.2				51
										52
1.2			1.23	1.2	1.19	1.4				53
1.2			1.23	1.2	1.19	1.4				54
1.46										55
1.23										56
1.315	1.34	1.39	1.39	1.37	1.38	1.39				57
1.24		1.17								58
1.23			1.23	1.2	1.19	1.43				59
1.85		1.88			1.69				1.47	60
										61
										62
2.08		2.08								63
		1.25								64
1.67		1.81								65
										66
										67
1.91		2.06			1.82				1.49	68
1.31										69
1.35		1.34								70
1.45										71
2.1										72
2.1										73
										74
1.66	1.77	1.86	1.85		1.80	1.85	1.27			75

(continued on p. 40)

TABLE II

		B.P.(°C)	A	B			
	Column temp. (°C)		28	30	43	52	75
76	*n*-Heptane	98.427		2.5	2.38	2.25	2.1
77	2,3-Dimethyl-2-butene	73.21	2.42	2.70	2.59	2.47	
78	Cyclopentadiene	42		2.92	2.82	2.68	2.42
79	2,5-Dimethylhexane	109.103			2.81	2.70	
80	2,3-Dimethyl-1-pentene	84.26		2.95	2.60		
81	3-Methyl-2-ethyl-1-butene	89		3.25	2.81		
82	2,4-Dimethylhexane	109.429				2.82	
83	2,4,4-Trimethyl-1-pentene	101.44			3.1		
84	2,2,3-Trimethylpentane	109.841				3.24	
85	1-Methylcyclopentene	75.8		3.76		3.3	
86	2,3-Dimethylbutadiene	68.78			3.8	3.6	
87	1-Heptene	93.643		3.6	3.48		
88	3,4-Dimethyl-*cis*-2-pentene	87		3.77	3.34		
89	3,4-Dimethyl-*trans*-2-pentene	87		3.94	3.43		
90	*cis*-3-Heptene	95.75		3.9	3.60		
91	2-Methylheptane	117.647				3.70	
92	2,4,4-Trimethyl-2-pentene	104.91			3.8		
93	*trans*-3-Heptene	95.67		4.25	3.84		
94	2,3,4-Trimethylpentane	113.467	4.7		3 90	3.75	
95	Methylcyclohexane	100.934		4.2	3.88		
96	*trans*-2-Heptene	97.95		4.6	4.10		
97	Ethylcyclopentane	103.466		4.76	4.38	4.0	
98	2,3,3-Trimethylpentane	114.760				4.06	
99	3-Ethylhexane	118.534				4.08	
100	*cis*-2-Heptene	98.5		5.0	4.53		
101	3,4,4-Trimethyl-1-pentene	104				4.53	
102	Cyclohexene	82.979		5.40	4.9	4.63	
103	2,3-Dimethyl-2-pentene	97.46		5.54	4.83		
104	2-Methylpentadiene			5.6	5.0	4.73	
105	3,3-Dimethyl-2-ethyl-1-butene	110				4.84	
106	2,5-Dimethyl-1-hexene	111.6			5.9	5.5	
107	2-Ethyl-1,3-butadiene	75		6.1		5.1	
108	2,3,3-Trimethyl-1-pentene	108.31				5.15	
109	*n*-Octane	125.665		6.3	5.62	5.05	4.0
110	2-Methylpentadiene			6.5	5.8	5.4	
111	3,4,4-Trimethyl-2-pentene	112				5.63	
112	1-Methyl-*trans*-2-ethylcyclopentane	121.2			6.55	5.85	
113	3-Methyl-1,3-pentadiene	77		6.64		5.5	
114	2-Methylcyclopentadiene	71		7.4		5.7	
115	2,3,4-Trimethyl-2-pentene	116.26			7.28		
116	1-Methyl-2-ethylcyclopentene	127.4			7.4		
117	2,3,4-Trimethyl-1-pentene	108			7.50		
118	3-Ethyl-3-hexene	116			7.6	6.3	
119	1-*trans*-2-Dimethylcyclohexane	123.419			7.32	6.56	
120	1-Methylcyclopentadiene	71		8.3		6.35	

(continued)

C		*D*				*E*		*F*	*G*	
86	*108*	*86*	*95*	*114*	*124*	*96*	*124*	*28*	*125*	
2.23	2.10	2.22	2.14	2.02	1.97	2.15	1.43			76
		1.47							1.71	77
										78
2.62		2.30								79
										80
										81
2.73										82
2.62										83
2.82									2.0	84
1.99		1.84								85
1.6									2.25	86
										87
2.2										88
2.2										89
										90
3.57										91
2.84										92
										93
3.28		3.55								94
2.83		3.2	3.0		2.72	3.1	2.10			95
2.77						2.5				96
3.10		3.2	3.1		2.77	3.2				97
3.42										98
4.03		4.20			3.29				2.34	99
3.0						2.7				100
										101
2.9										102
2.2		1.89							2.8	103
3.26									2.6	104
										105
4.00		3.88								
2.3		1.96								106
										107
5.0	4.4	5.1	4.99	4.17	3.8	4.68	2.8			108
2.4		2.02								109
										110
3.66									2.7	111
4.9		4.87			4.08					112
		2.12								113
2.44		2.0								114
4.41										115
					6.7					116
4.02										117
5.18					3.62				3.19	118
5.34			5.42		4.56	6.5	3.9			119
2.50		2.0								120

(continued on p. 42)

TABLE II

		B.P. (°C)	A	B			
	Column temp. (°C)		28	30	43	52	75
121	1-*trans*-4-Dimethylcyclohexane	119.351			5.82	5.34	
122	1-*cis*-3-Dimethylcyclohexane	120.088			6.07	5.49	
123	Cycloheptane	117					
124	1-*cis*-4-Dimethylcyclohexane	124.321			7.85	7.03	
125	1-*trans*-3-Dimethylcyclohexane	124.450			8.0	7.13	
126	3-Ethyl-2-hexene	121			8.6	7.1	
127	1-Octene	121.280			8.47	7.55	
128	2,2,3,4-Tetramethylpentane	133.016					
129	1-Methyl-*cis*-2-ethylcyclopentane	128.050			9.0		
130	1-*cis*-2-Dimethylcyclohexane	129.728			10.3	9.11	
131	Ethylcyclohexane	131.783			10.4	9.2	
132	5-Methylcyclopentadiene			9.7			
133	2,5-Dimethyl-1,5-hexadiene	114.3			12.8	11.2	
134	2,2,3,4-Tetramethylpentene						
135	Methylcycloheptane	134			13.7	12.0	
136	Cyclooctane	147					
137	Benzene	80.100		19		14.2	10.2
138	Toluene	110.625				30.7	
139	Ethylbenzene	136.186					37.6
140	*p*-Xylene	138.351					39.3
141	*m*-Xylene	139.103					39.7
142	*o*-Xylene	144.411					53.0
143	Styrene	145.2					
144	Ethyl ether	34.5		1.47		1.30	
145	Acetone	56.5				7.4	5.7
146	2-Methyl-2-propanol	82.8					
147	1-Bromopropane	70.9					
148	3-Pentanone	102.7					
149	3,3-Dimethyl-2-butanone	106.2					
150	2,2-Dimethyl-3-pentanone	124.5					
151	2,2,3-Trimethyl-3-pentanone	135					
152	2,2,3-Trimethyl-3-pentanol	154					
153	3-Ethyl-3-hexanol	160					
154	Water	100.000					

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(continued)

C		*D*				*E*		*F*	*G*	
86	*108*	*86*	*95*	*114*	*124*	*96*	*124*	*28*	*125*	
4.5										121
4.9		4.87			3.97					122
5.2			5.2			5.5				123
5.28										124
5.48		5.80			4.63					125
5.56					3.90				3.53	126
5.22			4.8			5.6				127
5.60										128
6.2					5.1					129
6.5		7.15	6.78	5.9	5.63	7.1	4.5			130
6.9		7.15	6.78	5.9	5.63	7.12	4.5			131
										132
5.8		4.9								133
6.95										134
8.6		9.1	8.52	7.4	6.9		5.6			135
14.1					10.2	14.9	8.5			136
3.67	3.6	2.80	2.70	2.56	2.50	4.0	2.55		7.12	137
8.66	7.9	6.45	6.10	5.48	5.10	9.2	5.37		11.8	138
18.1	15.1	13.5	12.2	10.2	9.2	16.8	9.08		18.0	139
19.3	16.2	14.5	13.6			21.0	11.0			140
20.5	17.1	15.0	13.8	11.3	10.2	20.2	10.6		19.2	141
25.9	21.2	18.6	16.7	13.65	12.2	27.0	13.6		24.2	142
30.6					13.2	29.0			34.0	143
0.85										144
6.0		0.95			0.9				4.0	145
									4.5	146
					1.93				4.57	147
									9.1	148
10.0									8.4	149
13.7									11.0	150
16.3										151
									30.5	152
									39.6	153
									2.2	154

SUMMARY

Relative retention times over different gas–liquid chromatographic columns of 154 C_1–C_8 hydrocarbons and other compounds are presented. The liquid substrates used were: dimethylsulfolane, dimethylsulfolane–dipropylsulfone, 7,8-benzoquinoline, dinonyl phthalate, di-*n*-propyl tetrachlorophthalate, and Carbowax 600. Relative retention times of several gases over a solid adsorption column with Davison's 950 Silica Gel are also presented. Column characteristics and conditions of operation are described.

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RAPID PAPER CHROMATOGRAPHIC FRACTIONATION OF COMPLEX MIXTURES OF WATER-SOLUBLE SUBSTANCES

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In research on the constituents of biological extracts our laboratories have often required rapid procedures for rough fractionation and tentative characterization of unknown constituents in small amounts of crude extracts. Paper chromatography on a fast-running paper such as Whatman No. 4 has proved to be the easiest and fastest first stage, and several solvent systems have gradually been evolved for the fractionation of relatively water-soluble substances. A large number of known reference substances have been run in these solvents, and the purpose of this paper is to make available to other workers this accumulation of R_F values.

The first solvent in the series is an 8:8:4:1 mixture of isopropanol, pyridine, water, and glacial acetic acid, for which we commonly use the name IPWA. The use of this solvent for separation of common inorganic ions[1] and of many carbohydrates and polyols[2] has been described previously. A second solvent, BuPWA, a 12:6:4:1 mixture of isobutanol, pyridine, water, and glacial acetic acid, was devised to give lower R_F values and better separation of substances having high R_F values in IPWA[3]. A third solvent, MePWA, a 6:6:4:1 mixture of methanol, pyridine, water, and glacial acetic acid, has not yet been described but is useful for separation of substances having low R_F values in IPWA.

These solvents, used with Whatman No. 4 paper, give complete chromatograms within 2 h and can be sufficiently air-dried even at room temperature in 15 min to allow use of reagents to detect spots. The use of these solvents requires no preliminary equilibration, since their water content is high, and R_F values are not altered by small variations in temperature or solvent composition. The capacity (for substances at least moderately water-soluble) is high, and interference caused by the presence of salts is minimal, since all anions move as the pyridinium salts, and all cations as the acetate salts. Any spray reagent can be used to detect spots, although with certain reagents the chromatograms may require a 24 h drying period or prior extraction with acetone or exposure to steam to remove residual pyridine. We have found that detection of spots by many reagents is improved if the Whatman No. 4 paper is pre-washed before use. The method of GORDON AND HEWEL[1], which uses 80:15:5 distilled water, pyridine, and glacial acetic acid, is satisfactory.

The major disadvantage of these solvents is low resolving power, since relatively

elongated spots are formed, as an unavoidable consequence of the high speed of travel through relatively coarse paper. Since all the solvents are weakly acidic, they do not effect some separations (*e.g.*, arginine from lysine) that are possible in strongly acidic or alkaline solvents in which differential ionization of strongly acidic or basic groups occurs. In fact, our solvents are general-purpose systems and will often be, for any specific separation, inferior to the specialized solvents devised for specific classes of substances. We have used them primarily for the first-stage purification of unknown biologically-active or radioactive compounds; the relatively broad bands so obtained are easily eluted or transferred to a second paper strip for more detailed characterization in a second solvent system or by paper ionophoresis. The original extract is simplified by the preliminary chromatography, and subsequent operations are usually much more informative than they would be if carried out on the crude extracts.

LIMITATIONS OF R_F VALUES IN CHARACTERIZATION OF UNKNOWNS

The compounds for which reference R_F data have been accumulated in our laboratories are mostly of biochemical interest and include many amines and amino acids, peptides, carbohydrates, polyols, carboxylic acids, some dinitrophenyl derivatives, vitamins, antibiotics, nucleosides and inorganic ions. For many of these substances we have previously published ionophoretic mobility data[4,5]. Paper ionophoresis is usually our second stage in identifying unknown substances, because it can yield information about ionizing groups and molecular weight even when the unknown does not match any of our reference compounds.

Table I lists compounds in order of increasing 100 R_F value in BuPWA, IPWA and MePWA. Each listed value is the mean of at least two observed values, usually not differing by more than 3 units, obtained on separate 2-h ascending chromatograms with test spots of 10–20 μg. In our earlier detailed studies using IPWA, however, we have shown that the R_F value and spot size of a substance are affected by the quantity of substance in the initial spot, by its state (*e.g.*, free tartaric acid, potassium tartrate, and calcium tartrate give somewhat different values), and by the kind and amount of other substances present. These effects can be lessened by using pyridine and acetic acid in the original extraction solvent or on the spotted chromatogram to pre-equilibrate the mixture before running, by adding pyridinium sulfate to reduce interference by polyvalent cations or barium acetate to reduce interference by polyvalent anions, and in other ways. Nevertheless, the R_F of a substance in a complex mixture may be 5 or more units higher or lower than that of the pure substance. A substance in a crude extract having a 100 R_F value of 30 may therefore be any one of the reference substances falling in the range from 25 to 35 in the Table; and even larger deviations are sometimes found.

Table I gives values for most of the reference compounds in IPWA and at least one other solvent. The best purification and characterization is achieved in the range from 20 to 50. For example, the best separation of glycine, alanine and α-aminobutyric acid is obtained in IPWA, where the 100 R_F sequence is 20, 36 and 46, while for valine, isoleucine and leucine, BuPWA gives the best sequence (29, 40, 46). Nothing is gained by running a substance with a high IPWA value in MePWA, or a low IPWA value in BuPWA. When values in other solvents are also in the table, these

TABLE I

$100R_F$	*BuPWA*	*IPWA*	*MePWA*
5	DL-Methionine sulfoximine (20, 50)	Adenosine-5′-triphosphate (Na_2) (51) *Djenkolic acid (26) *Lysyl-glycine (42)	
6	Fructose-6-phosphate (Ba) (31, 74) *meso*-Inositol (31, 63) 2-Ketogluconic acid (40, 69) D-Melezitose (54, 78) Scyllo-inosose (38, 68)	Thiamine pyrophosphate (58)	
7	*1,4-Diaminobutane (34, 70) *Glutamic acid (24, 55) *Lactose (46, 73) *Melibiose (45, 74)	*Glycyl-L-histidine (37) *O-Phosphoserine (38) Phosphocholine	
8	DL-Methionine sulfoxide (24, 56) *Potassium ion (15, 45) *Tartaric acid (29, 65)	Ethylene-dinitrilo-tetra-acetic acid (EDTA) (52) *Histidyl-histidine (35)	
9	Gluconic acid (38, 67) Homoserine (31, 60) N-Methyl-glycine (32, 62)	Barium ion (50) Iodate ion (33)	
10	Alanyl-glycine (27, 62) *Alanyl-glycyl-glycine (27, 61) *N-Aminoethyl-piperazine (31, 70) *Calcium ion (35, 75) *Ethylenediamine (28, 61) Glycerophosphoric acid (37, 76) Hydroxyproline (33, 57) Methionine sulfone (37, 63) *Oxalic acid (32, 69) Sarcosine (32, 62) Taurine (38, 61) *Uric acid (45, 70)	Adenosine-5′-diphosphate (Na) (59) *Carnosine (39) Glycyl-asparagine (36) *Homocystine *allo*-Hydroxylysine (44) *Ornithine (46)	
11	*β-Alanine (28, 60) Barbital (diethyl-barbituric acid) (25, 63) *D-Galactosamine (47, 72) *Deoxyadenylic acid (31, 60) *Glycinamide (30, 58)	Ferrocyanide ion *Glycyl-aspartic acid (47)	
12	*Cellobiose (53, 74) Diethylenediamine (32, 66) Gulonic acid (42, 72) *D-Maltose (57, 78) *Phosphate ion (42, 71) Threonine (35, 64)	Asparagine (35) Cysteic acid (39) *Fructose-1,6-diphosphate (Ba) (62)	

TABLE I (*continued*)

$100 R_F$	*BuPWA*	*IPWA*	*MePWA*
13	5-Ketogluconic acid (53, 75) *Piperazine (31, 65)	Aminomethylenesulfonic acid (67) *Aspartic acid (40) 2,4-Diaminobutyric acid (41) Ferricyanide ion (72) *Glycyl-glycyl-glycyl-glycine (47) Methionine methylsulfonium ion (47) *O-Phosphoethanolamine (51)	
14	α-Methyl-glutamic acid (38, 73) *Sodium ion (26, 60) *Uridylic acid (49, 75)	*Lysine (49) D-Penicillamine (53) *Spermidine (61)	
15	*α-Alanine (36, 64) *D-Glucosamine (53, 76) *Magnesium ion (57)	*Arginine (51) *Glycyl-glycyl-glycine (52) *Potassium ion (8, 45) Rubidium ion (40) *Tetraethylenepentamine (59)	
16	*Arcaine (41, 78) *Diglycolic acid (54, 74) *Proline (36, 65)	Cesium ion (38) *Histidine (45) Sulfate ion (64)	
17	Arsenate ion (47, 80) *L-Quinic acid (50, 75) *D-Turanose (64, 80)	*Alanyl-asparagine (51) Glycyl-serine (54) Mucic acid (60) Saccharic acid	
18	*3,4-Dihydroxyphenylalanine (42, 66)	Glutamine (45) *Glycyl-L-glutamic acid (55) Glycyl-glycine (47) *2-Thiolhistidine (43)	
19	α-Amino-*n*-butyric acid (46, 74) *FD & C Blue No. 1 (63) *3-Dimethylamino-*n*-propyl-amine (36, 74) Lithium ion (61) *Sucrose (62)	Strontium ion (66)	**meso*-Lanthionine
20	*D-Galactose (62, 78) *Glycyl-L-tyrosine (43, 77) D-Trehalose (52, 75)	*Glycine (51) DL-Methionine sulfoximine (5, 50)	
21	*Calcium ion (35, 75) *Citric acid (62) *Guanine (52, 77) *N-Hydroxyethyl-piperazine (53, 74) Tetramethyl-ammonium ion (47, 81)	*Glucose-6-phosphate (Ba) (69) *Guanylic acid (63)	Glutathione (oxidized)

(continued on p. 48)

TABLE I (*continued*)

$100 R_F$	*BuPWA*	*IPWA*	*MePWA*
22	*D-Glucoheptose (63, 77)		α,ε-Diamino-pimelic acid
23	*L-Cysteinesulfinic acid (48, 71) Glyceric acid (59) Hydantoic acid (49, 72) α-Hydroxyglutaric acid (52, 77)	Glutathione (reduced) (57) *Glycyl-L-proline (58)	
24	Choline (52, 86) *DNP-α,ε-diaminopimelic acid (48, 73) *Dulcitol (galactitol) (64) *Ethanolamine (50, 76) Gluconolactone (62, 90) Glycyl-L-tryptophan (46, 77) *Methylamine (50, 74) Pipecolic acid (48, 75) Quebrachitol (63)	*5′-Adenylic acid (60) Deoxyguanylic acid (62) *Glutamic acid (7, 55) DL-Methionine sulfoxide (8, 56) *Riboflavin-5-phosphoric acid (72)	
25	α-Amino-isobutyric acid (52, 79) Bromate ion (57, 69) *Mannitol (66) *Mucochloric acid (55, 74) Pyridoxamine (58) *D-Sorbitol (64)	Barbital (diethylbarbituric acid) (11, 63) *Sodium ion (14, 60)	
26	Floridoside (65) *D-Glucose (65) *Sedoheptulose (62, 76) Thiamine (70) Tyrosine (56, 77)	*Cytidylic acid (64) *Serine (55)	*Djenkolic acid (5)
27	Acetylcholine (56, 89) Allyl-glycine (49, 74) *2-Amino-2-(hydroxymethyl)-1,3-propanediol (62, 84) *Orotic acid (55)	Alanyl-glycine (10, 62) *Alanyl-glycyl-glycine (10, 61) *Deoxycytidylic acid (65) Lactobionate (68) Vitamin B_{12} (65)	
28	*3-Amino-1-propanol (57) α-Aminovaleric acid (58, 80) Chloride ion (48, 78) 3-Dimethylamino-1,2-propanediol (86) Guanosine (60) Pinitol (67) *2-Pyrrolidone carboxylic acid (56, 75)	*β-Alanine (11, 60) *Ethylenediamine (10, 61) Glycyl-alanine (50)	**allo*-Cystathionine
29	Glycyl-leucine (55, 77) *α-Ketoglutaric acid (66) *Riboflavin (65) Valine (51, 79)	*3′-Adenylic acid (62) *Tartaric acid (8, 65)	

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
30	Dimethylamine (54, 79) *L-Malic acid (63, 81) *D-Mannose (70) Methionine (54, 79) Xanthopterin (48, 68) *Xanthosine (66)	*Glycinamide (11, 58)	
31	*D-Arabinose (65, 79) 2-Dimethylaminoethanol (65) *Fructose (69) Leucyl-glycine (62)	*N-Aminoethylpiperazine (10, 70) *Deoxyadenylic acid (11, 60) Fructose-6-phosphate (Ba) (6, 74) Homoserine (9, 60) *meso*-Inositol (6, 63) *Piperazine (13, 65)	
32	*3-Hydroxy-piperidine (63, 83) *L-Sorbose (69)	*Agmatine (66) Diethylenediamine (13, 66) α-D-Galacturonic acid (67) N-Methyl-glycine (9, 62) *Oxalic acid (10, 69) Sarcosine (10, 62)	Cytidine-diphospho-choline
33	*L-Arabitol (73) Cytidine (60) Guanidine (56, 75) Tryptophan (53, 71)	*Cadaverine (67) Hydroxyproline (10, 57)	Diphosphopyridine-nucleotide Iodate ion (9)
34	*3-Amino-2-propanol (59, 86) *Cytosine (53) Glycyl-phenylalanine (49, 79) *Inosine (67)	*N-Acetyl-L-histidine (62) *1,4-Diaminobutane (7, 70) *Glucose-1-phosphate (Na) (70)	
35	4-Amino-5-imidazolecarboxamide (59, 72) L-Arabinose (70) Bromide ion (66) Deoxyguanosine (79) Isopropylamine (62, 85) Phenylalanine (57, 81) *D-Tagatose (70, 83) *D-Xylose (71)	*Calcium ion (10, 75) *L-Ergothionine (67) Threonine (12, 64)	Asparagine (12) *Histidyl-histidine (8)
36	*Xanthine (64, 76) L-Xylose (73)	*α-Alanine (15, 64) *3-Dimethylamino-*n*-propylamine (19, 74) *Proline (16, 65) Sulfamate ion (60)	Glycyl-asparagine (10)
37	N-Acetylglucosamine (75) *Adonitol (D-Ribitol) (73) Ethionine (61, 81)	*Barbituric acid (55) Cysteine (63) Glycerophosphoric acid (10, 76) *Glycyl-DL-methionine (70) Methionine sulfone (10, 63)	*Glycyl-L-histidine (7) *Glycyl-lysine

(continued on p. 50)

TABLE I (*continued*)

$100 R_F$	*BuPWA*	*IPWA*	*MePWA*
38	Cyanoacetic acid (68)	Gluconic acid (9, 67) α-Methyl-glutamic acid (14, 73) Scyllo-inosose (6, 68) Taurine (10, 61)	Cesium ion (16) *O-Phosphoserine (7)
39	ε-Amino-*n*-caproic acid (68, 86) *DNP-*allo*-δ-hydroxylysine (66) *Pyrrolidine (62) *Pyruvic acid (72) *Isoriboflavin (58)		*Carnosine (10) Cysteic acid (12)
40	*5-Hydroxy-2,4-dichloro-phenoxy-acetic acid (75) Isoleucine (63, 82) *D-Lyxose (75) *Malonic acid (71) *Uracil-5-carboxylic acid (61) Urea (62, 75)	5-Hydroxytryptophan (59) 2-Ketogluconic acid (6, 69)	*Aspartic acid (13) Rubidium ion (15)
41	*Acetylglutamic acid (78, 89) **cis*-Aconitic acid (78) Aluminum ion (69) *L-Fucose (75) *p*-Hydroxyphenyl-pyruvic acid (76) Hypoxanthine (62)	*Arcaine (16, 78)	2,4-Diaminobutyric acid (13)
42	**trans*-Aconitic acid (75) Deoxycytidine (69) *Glycolic acid (71) Gulonic lactone (78, 84) Nitro-ferricyanide ion (84) *D-Ribose (76) Tetraethylammonium ion (68, 93)	*3,4-Dihydroxyphenyl-alanine (18, 66) Gulonic acid (12, 72) L-Kynurenine (61) *Phosphate ion (12, 71)	*Lysyl-glycine (5)
43	Adenosine (68)	*Glycyl-L-tyrosine (20, 77)	*allo*-Hydroxylysine (10) *2-Thiolhistidine (18)
44	*Epinephrine (81) *Isocytosine (66, 79)		
45	*Erythritol (77, 85) *Kynurenic acid (63) Nitrate ion (75, 89)	*Melibiose (7, 74) *Raffinose (77) *Uric acid (10, 70)	Glutamine (18) *Histidine (16) *Potassium ion (8, 15)
46	L-Ascorbic acid (85) Chromate ion (70) *3-Hydroxytyramine (76) Leucine (65, 83)	α-Amino-*n*-butyric acid (19, 74) Glycyl-L-tryptophan (24, 77) *Lactose (7, 73) Malto-tetraose	*Ornithine (10)
47	*DNP-djenkolic acid Lead ion (71)	Arsenate ion (17, 80) *D-Galactosamine (11, 72) Isomaltose Tetramethylammonium ion (21, 81)	*Glycyl-aspartic acid (11) Glycyl-glycine (18) Methionine methyl sulfonium ion (13)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
48	*Adenine (60) Chloromycetin (69, 91)	Chloride ion (28, 78) *L-Cysteinesulfinic acid (23, 71) DNP-α,ε-diaminopimelic acid (24, 73) Pipecolic acid (24, 75) Xanthopterin (30, 68)	
49	*DNP-histidine Uridine (84)	Allyl-glycine (27, 74) Glycyl-phenylalanine (34, 79) Hydantoic acid (23, 72) *Uridylic acid (14, 75)	*Lysine (14)
50	*p*-Aminobenzoyl-glutamic acid (80, 89) 2-Deoxy-D-glucose (80) DNP-L-asparagine *Shikimic acid (78) *Xanthurenic acid (74)	*Ethanolamine (24, 76) *Methylamine (24, 74) *L-Quinic acid (17, 75)	Barium ion (9) Glycyl-alanine (28) DL-Methionine sulfoximine (5, 20)
51	Chlorate ion (81) Fumaric acid (80) *Manganous ion (84)	Maltotriose	Adenosine-5′-triphosphate (Na_2) (5) *Arginine (15) *Alanyl-asparagine (17) *Glycine (20) *O-Phosphoethanolamine (13)
52	Deoxyadenosine (70) *L-Rhamnose (82)	α-Aminoisobutyric acid (25, 79) Choline (24, 86) *Guanine (21, 77) α-Hydroxyglutaric acid (23, 77) D-Trehalose (20, 75)	Ethylene-dinitrilo-tetraacetic acid (EDTA) (8) *Glycyl-glycyl-glycine (15)
53		*Cellobiose (12, 74) *Cytosine (34) *D-Glucosamine (15, 76) *N-Hydroxyethyl-piperazine (21, 74) 5-Ketogluconic acid (13, 75) Tryptophan (33, 71)	D-Penicillamine (14) *Glycyl-glycyl-glycyl-glycine (13)
54	DNP-glutamine	*Diglycolic acid (16, 74) Dimethylamine (30, 79) D-Melezitose (6, 78) Methionine (30, 79)	Glycyl-serine (17)
55	Aminotriazole (70) *DNP-glycyl-aspartic acid Iodide ion (78) Thiourea (72, 80) *Tyramine (80)	α-Aminovaleric acid (28, 80) Glycyl-leucine (29, 77) *Mucochloric acid (25, 74) *Orotic acid (27)	*Barbituric acid (37) *Glutamic acid (7, 24) *Glycyl-L-glutamic acid (18) Phosphocholine *Serine (26)
56	Aureomycin (78) Glucuronic lactone (84)	Acetylcholine (27, 89) Guanidine (33, 75) *2-Pyrrolidone-carboxylic acid (28, 75) Tyrosine (26, 77)	DL-Methionine sulfoxide (8, 24)

(continued on p. 52)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
57	Cyclamate ion (83) *Maleic acid (89) Uracil (73)	*3-Amino-1-propanol (28) Bromate ion (25, 69) *Magnesium ion (15) *D-Maltose (12, 78) Phenylalanine (35, 81) Valine (29, 79)	Glutathione (reduced) (23) Hydroxyproline (10, 33)
58	DL-Glyceraldehyde (70) *Leucyl-tyrosine (86, 95)	*Isoriboflavin (39) Pyridoxamine (25)	*Glycinamide (11, 30) *Glycyl-L-proline (23) Thiamine pyrophosphate (6)
59	*Tryptamine (81)	4-Amino-5-imidazolecarboxamide (35, 72) *3-Amino-2-propanol (34, 86) Glyceric acid (23)	Adenosine-5′-diphosphate (Na) (10) 5-Hydroxytryptophan (40) *Tetraethylenepentamine (15)
60	*Lactic acid (82) Picolinic acid (75)	*Adenine (48) Cytidine (33) Guanosine (28)	*5′-Adenylic acid (24) *β-Alanine (11, 28) *Deoxyadenylic acid (11, 31) Homoserine (9, 31) Mucic acid (17) *Sodium ion (14, 25) Sulfamate ion (36)
61	Dihydroxyacetone (86) *DNP-alanyl-glycyl-glycine Glycerol (82) Urocanic acid (80, 86)	Ethionine (37, 81) Lithium ion (19) *Uracil-5-carboxylic acid (40)	*Alanyl-glycyl-glycine (10, 27) *Ethylenediamine (10, 28) L-Kynurenine (42) *Spermidine (14) Taurine (10, 38)
62	*Tricarballylic acid	*2-Amino-2-(hydroxymethyl)-1,3-propanediol (27, 84) *Citric acid (21) *D-Galactose (20, 78) Gluconolactone (24, 90) Hypoxanthine (41) Isopropylamine (35, 85) Leucyl-glycine (31) *Pyrrolidine (39) *Sedoheptulose (26, 76) *Sucrose (19) Urea (40, 75)	*N-Acetyl-L-histidine (34) *3′-Adenylic acid (29) Alanyl-glycine (10, 27) Deoxyguanylic acid (24) *Fructose-1,6-diphosphate (Ba) (12) N-Methyl-glycine (9, 32) Sarcosine (10, 32)
63	*Aesculin (89) *Endophthalic acid (89) Thiocyanate ion (88)	*FD & C Blue No. 1 (19) *D-Glucoheptose (22, 77) *3-Hydroxypiperidine (32, 83) Isoleucine (40, 82) *Kynurenic acid (45) *L-Malic acid (30, 81) Quebrachitol (24)	Barbital (diethyl-barbituric acid) (11, 25) Cysteine (37) *Guanylic acid (21) *meso*-Inositol (6, 31) Methionine sulfone (10, 37)
64		*Dulcitol (galactitol) (24) *D-Sorbitol (25) *D-Turanose (17, 80) *Xanthine (36, 76)	*α-Alanine (15, 36) *Cytidylic acid (26) Sulfate ion (16) Threonine (12, 35)

TABLE I (*continued*)

$100\,R_F$	*BuPWA*	*IPWA*	*MePWA*
65	*DNP-taurine	*D-Arabinose (31, 79) 2-Dimethylaminoethanol (31) Floridoside (26) *D-Glucose (26) Leucine (46, 83) *Riboflavin (29)	*Deoxycytidylic acid (27) *Piperazine (13, 31) *Proline (16, 36) *Tartaric acid (8, 29) Vitamin B_{12} (27)
66	Strychnine (90)	Bromide ion (35) *DNP-*allo*-δ-hydroxylysine (39) *Isocytosine (44, 79) *α-Ketoglutaric acid (29) *Mannitol (25) *Xanthosine (30)	*Agmatine (32) Diethylenediamine (13, 32) *3,4-Dihydroxyphenylalanine (18, 42) Strontium ion (19)
67	*DNP-glycine *2-Phenylethylamine (83)	*Inosine (34) Pinitol (28)	Aminomethylenesulfonic acid (13) *Cadaverine (33) *Ergothionine (35) α-D-Galacturonic acid (32) Gluconic acid (9, 38)
68	*DNP-serine Thymidine (90) Thymine (79)	Adenosine (43) ε-Amino-*n*-caproic acid (39, 86) Cyanoacetic acid (38) Tetraethylammonium ion (42, 93)	Lactobionate (27) Scyllo-inosose (6, 38) Xanthopterin (30, 48)
69	*Flavine-adenine dinucleotide (84)	Aluminum ion (41) Chloromycetin (48, 91) Deoxycytidine (42) *D-Fructose (31) *L-Sorbose (32)	Bromate ion (25, 57) *Glucose-6-phosphate (Ba) (21) 2-Ketogluconic acid (6, 40) *Oxalic acid (10, 32)
70	*DNP-hydroxyproline Hippuric acid (88) α-Ketobutyric acid (92)	Aminotriazole (55) L-Arabinose (35) Chromate ion (46) Deoxyadenosine (52) DL-Glyceraldehyde (58) *D-Mannose (30) *D-Tagatose (35, 83) Thiamine (26)	*N-Aminoethylpiperazine (10, 31) *1,4-Diaminobutane (7, 34) *Glucose-1-phosphate (Na) (34) *Glycyl-DL-methionine (37) *Uric acid (10, 45)
71	6-Chloropicolinic acid (81) Nicotinic acid (83)	*Glycolic acid (42) Lead ion (47) *Malonic acid (40) *D-Xylose (35)	*L-Cysteinesulfinic acid (23, 48) *Phosphate ion (12, 42) Tryptophan (33, 53)
72	Ethylene glycol (86) Nicotinamide (85)	*Pyruvic acid (39) Thiourea (55, 80)	4-Amino-5-imidazolecarboxamide (35, 59) Ferricyanide ion (13) *D-Galactosamine (11, 47) Gulonic acid (12, 42) Hydantoic acid (23, 49) *Riboflavin-5-phosphoric acid (24)

(continued on p. 54)

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
73	*Cobalt ion (92)	*Adonitol (D-Ribitol) (37)	DNP-α,ε-diaminopimelic acid (24, 48)
	*Nickel ion (92)	*L-Arabitol (33)	*Lactose (7, 46)
		Uracil (57)	α-Methylglutamic acid (14, 38)
		L-Xylose (36)	
74	Phenoxymethyl-penicillin (89)	*Xanthurenic acid (50)	Allyl-glycine (27, 49)
	Tetra-*n*-propylammonium ion (82, 94)		α-Amino-*n*-butyric acid (19, 46)
			*Cellobiose (12, 53)
			*Diglycolic acid (16, 54)
			*3-Dimethylaminopropylamine (19, 36)
			Fructose-6-phosphate (Ba) (6, 31)
			*N-Hydroxyethyl-piperazine (21, 53)
			*Melibiose (7, 45)
			*Methylamine (24, 50)
			*Mucochloric acid (25, 55)
75	Beryllium ion (91)	N-Acetylglucosamine (37)	*Calcium ion (10, 35)
	Copper ion (88)	*trans*-Aconitic acid (42)	Guanidine (33, 56)
		*L-Fucose (41)	5-Ketogluconic acid (13, 53)
		*5-Hydroxy-2,4-dichloro-phenoxy-acetic acid (75)	Pipecolic acid (24, 48)
			*2-Pyrrolidone-carboxylic acid (28, 56)
		*D-Lyxose (40)	*L-Quinic acid (17, 50)
		Nitrate ion (45, 89)	D-Trehalose (20, 52)
		Picolinic acid (60)	Urea (40, 62)
			*Uridylic acid (14, 49)
76	Gentisic acid (89)	*p*-Hydroxyphenylpyruvic acid (41)	*Ethanolamine (24, 50)
		*3-Hydroxytyramine (46)	*D-Glucosamine (15, 53)
		*D-Ribose (42)	Glycerophosphoric acid (10, 37)
			*Sedoheptulose (26, 62)
			*Xanthine (36, 64)
77	*Amphetamine (90)	*Erythritol (45, 85)	*D-Glucoheptose (22, 63)
	*DNP-threonine		Glycyl-leucine (29, 55)
			Glycyl-L-tryptophan (24, 46)
			*Glycyl-L-tyrosine (20, 43)
			Guanine (21, 52)
			α-Hydroxyglutaric acid (23, 52)
			*Raffinose (45)
			Tyrosine (26, 56)
78	Cyclamycin	Acetyl glutamic acid (41, 89)	*Arcaine (16, 41)
	*DNP-glycyl-proline	*cis*-Aconitic acid (41)	Chloride ion (28, 48)
		Aureomycin (56)	*D-Galactose (20, 62)
		Gulonic lactone (42, 84)	*D-Maltose (12, 57)
		Iodide ion (55)	D-Melizitose (6,54)
		*Shikimic acid (50)	

TABLE I (*continued*)

100 R_F	*BuPWA*	*IPWA*	*MePWA*
79	Pyridoxine (88)	Deoxyguanosine (35) Thymine (68)	α-Amino-isobutyric acid (25, 52) *D-Arabinose (31, 65) Dimethylamine (30, 54) Glycyl-phenylalanine (34, 49) *Isocytosine (44, 66) Methionine (30, 54) Valine (29, 51)
80	*Itaconic acid (92) Propylene glycol (90) *Succinic acid (93)	*p*-Aminobenzoyl-glutamic acid (50, 89) 2-Deoxy-D-glucose (50) Fumaric acid (51) *Tyramine (55) Urocanic acid (61, 86)	α-Aminovaleric acid (28, 58) Arsenate ion (17, 47) Thiourea (55, 72) *D-Turanose (17, 64)
81	*DNP-α-alanine (95) *DNP-β-alanine	Chlorate ion (51) 6-Chloropicolinic acid (71) *Epinephrine (44) *Tryptamine (57)	Ethionine (37, 61) *L-Malic acid (30, 63) Phenylalanine (35, 57) Tetramethylammonium ion (21, 47)
82	*DNP-glycyl-tyrosine *DNP-proline	Glycerol (61) *Lactic acid (60) *L-Rhamnose (52) Tetra-*n*-propylammonium ion (74, 94)	Isoleucine (40, 63)
83	*2-Chlorophenoxyacetic acid (92)	Cyclamate ion (57) Nicotinic acid (71) *2-Phenylethylamine (67)	*3-Hydroxypiperidine (32, 63) Leucine (46, 65) *D-Tagatose (35, 70)
84		*Flavine-adenine dinucleotide (69) Glucuronic lactone (56) *Manganous ion (51) Nitro-ferricyanide ion (42) Uridine (49)	*2-Amino-2-(hydroxymethyl)-1,3-propanediol (27, 62) Gulonic lactone (42, 78)
85	Cadmium ion (97) *3,4-Dihydroxyphenylacetic acid *DNP-allyl-glycine *DNP-galactosamine *DNP-glycyl-phenylalanine *DNP-methionine *β-Methylglutaconic acid	L-Ascorbic acid (46) Nicotinamide (72)	*Erythritol (45, 77) Isopropylamine (35, 62)
86	*DNP-α-Aminoisobutyric acid *DNP-glucosamine *DNP-phenylalanine Zinc ion (91)	Dihydroxyacetone (61) 3-Dimethylamino-1,2-propanediol (28) Ethylene glycol (72) *Leucyl-tyrosine (58, 95)	ε-Amino-*n*-caproic acid (39, 68) *3-Amino-2-propanol (34, 59) Choline (24, 52) Urocanic acid (61, 80)
87	4-Chloro-3-methyl-phenoxy-acetic acid *DNP-2,4-diaminobutyric acid *DNP-tryptophan		

(continued on p. 56)

TABLE I (*continued*)

100 R_F	BuPWA	IPWA	MePWA
88	*Glutaric acid	Copper ion (75) Hippuric acid (70) Pyridoxine (79) Thiocyanate ion (63)	
89	*4-Chloro-2-methyl-phenoxy-acetic acid Hydroxyacetone (97) *2,4,5-Trichlorophenoxyacetic acid	*Aesculine (63) *Endophthalic acid (63) Gentisic acid (76) *Maleic acid (57) Phenoxymethyl-penicillin (74)	Acetyl choline (27, 56) Acetyl-glutamic acid (41, 78) *p*-Aminobenzoylglutamic acid (50, 80) Nitrate ion (45, 75)
90	*p*-Aminobenzoic acid	*Amphetamine (77) Dehydroascorbic acid Propylene glycol (80) Strychnine (66) Thymidine (68)	Gluconolactone (24, 62)
91	*Gibberellic acid Quinine	Beryllium ion (75) Zinc ion (86)	Chloromycetin (48, 69)
92	*DNP-alanyl-leucine *DNP-α-aminobutyric acid *DNP-methionine sulfoximine *2-Hydroxyphenylacetic acid	*2-Chlorophenoxyacetic acid (83) *Cobalt ion (73) *Itaconic acid (80) α-Ketobutyric acid (70) *Nickel ion (73)	
93	Caffeic acid	*Succinic acid (80)	Tetraethylammonium ion (42, 68)
94			Tetra-*n*-propylammonium ion (74, 82)
95	*DNP-isoleucine DNP-leucine *DNP-methionine sulfoxide *Indole-3-acetic acid	*DNP-α-alanine (81)	*Leucyl-tyrosine (58, 86)
96-97	*Apolon *DNP-α-aminobutyric acid *2-Hydroxycinnamic acid *4-Hydroxycinnamic acid *2-Hydroxy-4-methoxycinnamic acid *Umbelliferone (7-hydroxy-coumarin)	Cadmium ion (85) Hydroxyacetone (89)	

are given in parentheses following the name of the compound at each place where it is listed; when ionophoretic mobility values have been published for the compound, its name is preceded by an asterisk. Thus, in the BuPWA 15 column, one finds *alpha-alanine (36, 64) to indicate that this compound is listed (together with compounds that have the same value) in the IPWA 36 and MePWA 64 columns and also in the ionophoretic tables of THORNBURG *et al.*[5]. Values in a second and third solvent

may support a tentative identification but since these solvents differ in "moving power" rather than in resolving power, they are not sufficient for final characterization of an unknown.

USE OF THE CHROMATOGRAPHIC SOLVENTS

The extract of an unknown material may be made in water, but we normally use 10% or 20% methanol containing 1% of pyridine and 1% of acetic acid to effect a pre-equilibration, and also to permit storage without decomposition by microorganisms. If the unknowns are thermo-stable, briefly heating in boiling water is desirable to coagulate proteins, which may otherwise ruin the chromatographic separation. In many instances 50% methanol can be used to give an extract relatively free of proteins and of excessive amounts of salts, sugars, or other major constituents. After spotting and drying, brief extraction of the spot with acetone can sometimes eliminate acetone-soluble undesirable impurities. Extracts or spotted strips can be stored in a freezer below 0°C, but oxidative or hydrolytic decomposition can be a serious problem, which can only be solved by lyophilizing in glass ampoules, vacuum-sealing, and storing at low temperature.

Our simple apparatus for one-dimensional ascending chromatography has been described[1] and is commercially available in somewhat modified form from Microchemical Specialities Company, Berkeley, Calif.

All extracts are first run in IPWA, with several test spots to allow use of different reagents to locate acidic or basic spots, polyols, amines, fluorescent substances, or other classes of compounds. With extracts containing radioactive substances, one strip is scanned with a thin mica window Geiger counter to locate spots of high activity. When biological activity (vitamins, antibiotics) is being investigated, strips are cut into regions (usually 0–30, 30–70, and 70–100) which are eluted and tested. A second test strip is subdivided to test the boundaries (*e.g.*, 20–40, or 60–80). The results usually make possible localization within a band not more than 20 units in width.

When a substance has a value less than 40 in IPWA, the extract is also run in MePWA; when the IPWA value is higher than 60, the extract is also run in BuPWA.

When an unknown has been located, a large quantity of extract is banded on a relatively wide strip of washed paper and run in the solvent in which its 100 R_F value is nearest to 40. The region containing the desired band is cut out of this preparative strip. Usually the cut is narrow and designed to remove the diffuse upper and lower regions of the band. While this causes some loss, the bulk of the material is obtained in purer form by such trimming. The cut-out strip can be eluted with several ml of water or 10% methanol, but the solution so obtained may be too dilute for subsequent work. Such elution is usually done only when a degradation or derivatization (such as reaction with dinitrofluorobenzene[5]) is to be attempted, or passage through an ion-exchange column is planned. For direct transfer to a second paper strip, we usually insert one edge of the strip cut from the chromatogram in water and let the capillary flow move the solutes to the other edge. When this edge is touched briefly to a point or line on a strip of paper to be used for further chromatography or for paper ionophoresis, the solutes are transferred as a relatively concentrated solution. To check the efficiency of transfer, a spot of a dilute dye solution can be applied to the center of

the strip before it is dipped in water. When paper ionophoresis by the method of WERUM *et al.*[4] is to be used, a spot of the reference dye solution ABA will serve both as an indicator of transfer and a mobility standard in the ionophoresis.

USE OF OTHER PAPERS AND DEVELOPMENT INTERVALS

When Whatman No. 4 paper is used, development intervals longer than 2 h do not much improve separation of spots. Shorter periods (as little as 10 min) are occasionally useful when a desired substance has an R_F much higher or lower than the substances from which it is to be separated. For example, serine (IPWA 26) and glycerol (IPWA 82) are well separated in 10 min. Since spots in such short chromatograms are more compact, smaller quantities can be detected. Similar compact chromatograms can be produced by using slower papers (such as the medium-speed S & S 2043-B, or the very slow S & S 589) for 2- or 3-h runs. We use 2-h runs on S & S 2043-B when minor spots are difficult to detect in the Whatman No. 4 strips, since spot area on slow papers is usually not more than twice that of the initial spot (while on Whatman No. 4 strips the area may be 10 times larger, largely because of outward diffusion in all directions). Longer runs (4–8 h) on S & S 2043-B are sometimes useful when maximal resolving power is required, since the compactness of spots often allows complete separation of substances with small R_F differences. For small-scale isolation, as described in the preceding section, we still prefer full-length chromatograms on Whatman No. 4 paper because the desired bands can be cut out with less attention to the precise location of the band. On small compact strips an error of a millimeter or two in the location of the cuts can greatly decrease the yield or the purity of the isolated band. Thicker papers (such as Whatman 3 MM or S & S 470) increase the capacity of chromatograms, but usually with a loss either in speed or in resolution of spots.

COMPARISON OF REFERENCE R_F VALUES WITH THOSE IN OTHER SOLVENTS

For carbohydrates, it has been shown that the sequence of R_F values is the same in most solvents, except phenol–water[6]. This makes it possible to estimate values for carbohydrates not included in Table I, by comparison with published data of other workers. For example, KAISER[7] has presented data for rhamnose (BuPWA 52) and several exotic sugars found in cardiac glycosides, from which it can be estimated that digitalose would fall near BuPWA 60 and digitoxose near BuPWA 75.

Unfortunately many other compounds are much more sensitive to the composition of the chromatographic solvent. For example, the R_F sequence for alanine, valine, pyroglutamic acid, and leucine in collidine–lutidine solvent[8,9] is similar to that in IPWA, but taurine has an R_F near that of valine while in IPWA it is near alanine. In 9:1 *n*-butanol–acetic acid/water, the R_F sequence of taurine, alanine, valine and leucine[9] is similar to that in BuPWA, but pyroglutamic is near leucine while in BuPWA it is near valine. R_F values even in solvents somewhat similar in composition to ours are not useful for prediction of R_F values in the IPWA group of solvents, except possibly when two or more reference R_F values in a homologous series in another solvent can be fairly closely matched with values in one of the IPWA group.

The IPWA group is the result of selection from several hundred experimental solvent systems. Many of the systems tried were variations in proportions of the same

components, which gave slightly different R_F values but fundamentally similar chromatograms. Other components (such as 2-butanone, acetonitrile, propionic acid) were occasionally tried but never produced clearly superior chromatograms. BuPWA, IPWA and MePWA were finally adopted in order to have a minimal number of standard systems, different primarily in "moving power". Many attempts to attain a general-purpose one-phase system with much lower "moving power" than BuPWA have been unsuccessful. Many specialized systems containing relatively hydrophobic liquids such as benzene or benzyl alcohol are described in the literature, but in our experience some of these have proved to be of low capacity and very sensitive to interference, to give erratic R_F values, or to be incompatible with many spray reagents.

Of the many solvents described in the literature that differ considerably in resolving power from the IPWA group, we have found an 8:8:4:1 mixture of *tert.*-butanol, methyl ethyl ketone, water and diethylamine most useful for rapid paper chromatography. This is essentially the alkaline system used by REDFIELD[10] in small-scale two-dimensional paper chromatography. It is more sensitive to salts and other interfering factors than the IPWA group and not as useful as a first solvent for crude extracts.

SUMMARY

Three alcohol–pyridine–water–acetic acid solvent systems have proved useful for preliminary fractionation of crude biological extracts by paper chromatography in 2 h or less. High solute concentrations and the presence of salts are usually tolerated but proteins may interfere. The primary system contains isopropanol; this is replaced by isobutanol if R_F values of substances to be isolated are too high for optimal resolution, and by methanol if R_F values are too low. R_F data for many organic and inorganic compounds in the three systems are tabulated. Unknown substances are recovered from the chromatograms in partially purified form for further characterization by other chromatographic solvents or paper ionophoresis.

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PAPIERCHROMATOGRAPHIE VON γ-ARYL-α,β-DIHALOGEN-$\Delta^{\alpha,\beta}$-CROTONLACTONEN UND ÄHNLICHEN SUBSTANZEN*

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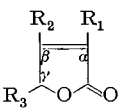
(Eingegangen den 8. März 1962)

Bei der Synthese von γ-Aryl-α,β-dihalogen-$\Delta^{\alpha,\beta}$-crotonlactonen (I), beim Studium einiger ihrer Solvolyseprodukte und ebenso beim Studium einer Reihe weiterer in dieses Arbeitsgebiet[1,2] gehöriger Substanzen leistete die Papierchromatographie wertvolle Hilfe sowohl bei der Reinheitskontrolle der einzelnen Präparate als auch namentlich bei der semiquantitativen Analyse der verschiedenen unter bestimmten Arbeitsbedingungen gewonnenen Reaktionsprodukte.



(I)

In der Literatur liegt keine Erwähnung über die Papierchromatographie derartiger Stoffe vor mit Ausnahme von einigen am Anfang der Tabelle II angeführten einfacheren aromatischen Säuren, die jedoch nicht das Wesentliche der vorliegenden Arbeit bildeten. Ähnlich wie bei einem anderen Typ von Substanzen aus der Gruppe der Kanzerostatika — und zwar der substituierten Purine[3] — befassten wir uns mit dem systematischen Studium dieser Substanzen, suchten die optimalen Bedingungen für Trennung und Nachweis am Papier und waren bestrebt, aus den gewonnenen Ergebnissen einige allgemeine Schlussfolgerungen über die Beziehung zwischen Struktur und chromatographischem Verhalten abzuleiten.

EXPERIMENTELLER TEIL

Papier

Alle Nachweisreaktionen sowie Entwicklung in wasserhaltigen Systemen wurden auf Papier Whatman No. 1, die Chromatographie in Systemen mit verankertem Formamid auf Papier Whatman No. 4 durchgeführt.

Nachweisreaktionen

Der Nachweis der einzelnen Substanzen erfolgte auf Papieren, wo die Proben in Mengen von 2, 5, 15 und 50 μg aufgetragen wurden, nach kurzfristigem Entwickeln

* Teilweise vorgetragen an der Konferenz über Papierchromatographie, Prag, den 21. Juni 1961.

in einem System mit verankertem Formamid mittels der weiter unten angeführten Nachweisreagenzien. Die Substanzen 32 und 43 wurden im System S 2 entwickelt, und die Crotonlactone (mit Ausnahme einiger stärker polarer) wurden ausser in Systemen mit Formamid noch in Systemen mit verankertem Dimethylformamid und Petroleum entwickelt. In allen Fällen wurde das Chromatogramm vor dem Nachweis bei 100° getrocknet. In den Tabellen wird sehr starke Reaktion (Empfindlichkeit 1–3 μg) mit + + +, mittelstarke Reaktion (4–10 μg) mit + +, schwache Reaktion (11–30 μg) mit + und sehr schwache Reaktion (30–300 μg) mit — bezeichnet.

D 1. Beobachtung von Verlöschen im Licht der Niederdruck-Quecksilberlampe (Chromatolite).

D 2. Beobachtung des Verlöschens im Licht der Niederdruck-Quecksilberlampe nach Besprühen des Chromatogramms mit 0.0025 %iger Fluoresceinlösung in 0.5 *N* Ammoniak.

D 3. Das Chromatogramm wird mit 25 %iger wässriger Methylaminlösung besprüht, und nach Trocknen bei Laboratorium-Temperatur wird es 1 Min auf 100° erhitzt, sodann auf eine mit konz. Schwefelsäure bestrichene Glasplatte gelegt.

D 4. Das Chromatogramm wird mit dem Gemisch gleicher Volumina 0.1 *N* $AgNO_3$ und 5 *N* Ammoniak besprüht und dem Sonnenlicht ausgesetzt.

D 5. Das Chromatogramm wird mit 0.2 %iger Lösung von 2,4-Dinitrophenylhydrazin in 0.1 *N* methanolischer HCl besprüht.

D 6. Das Chromatogramm wird mit dem Gemisch gleicher Volumteile 1 % $KMnO_4$ und 2 % Na_2CO_3 besprüht.

Lösungsmittelsysteme

Die Chromatogramme wurden absteigend bei konstanter Temperatur von 20° in den folgenden Systemen entwickelt:

S 1. *n*-Butanol–Essigsäure–Wasser (4:1:5).

S 2. Isopropanol–Ammoniak–Wasser (10:1:1).

S 3. Papier imprägniert mit 50 %iger äthanolischer Formamidlösung; Chloroform fliesst durch.

S 4. Papier wird mit 50 %iger äthanolischer Formamidlösung mit einem Gehalt von 5 % Ammoniumformiat (auf Formamid berechnet) imprägniert; Chloroform fliesst durch.

S 5. Formamid/Benzol.

S 6. Formamid + Ammoniumformiat/Benzol.

S 7. Formamid/Benzin.

S 8. Dimethylformamid/Cyclohexan.

S 9. Papier wird mit 20 % Petroleum in Cyclohexan imprägniert; 60 %iges Isopropanol fliesst durch.

ERGEBNISSE

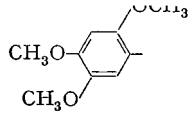
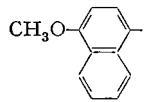
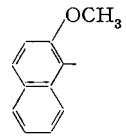
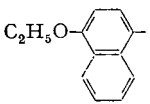
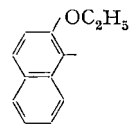
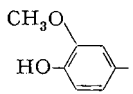
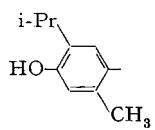
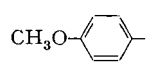
Die Ergebnisse des chromatographischen Verhaltens der Crotonlactone gehen am besten aus der Tabelle I hervor.

Zum Nachweis bewährte sich als Universalreagens Methylamin mit Schwefelsäure (D 3), womit bei den substituierten Crotonlactonen intensiv gefärbte Flecken entstanden. Die Farbe der Flecken wurde ausser durch die Struktur der Sub-

TABELLE I

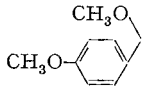
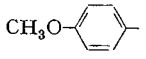
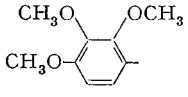
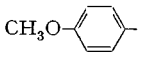
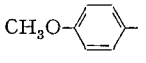
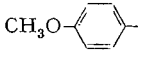
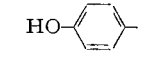
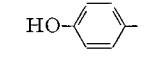
R_F-WERTE EINIGER CROTONLACTONE

No.	R_3	R_2	R_1	Nachweisreaktion* D1	D2	D3** a	D3** b	D3** c	D4	D5	D6	Lösungsmittelsystem ($R_F \times 100$) S3	S5	S7	S8	S9
1	CH_3O-	Cl	OH	+++	+++	G++			+	+++	++	65	32	00	00	97
2	CH_3O-	Cl	OCH_3	++	+++	O+++	O+++	O++	Ø	+	+		95	42	26	86
3	CH_3O, CH_3O-	Cl	H	—	+	R++	R+++	O++	Ø	Ø	—		96	67	41	84
4	CH_3O-	Cl	Cl	Z+++	+++	G++	O++	O++	+	+	+		95	55	36	83
5	C_2H_5O-	Cl	Cl	Z+++	+++	G++	O++	O++	+	+	+		97	77	58	74
6	C_3H_7O-	Cl	Cl	+++	+++	G—	V+	Ø	+	+	+		97	89	72	64
7	C_4H_9O-	Cl	Cl	Z+++	+++	G++	O++	O+++	+	+	+		97	92	82	53
8	CH_3O, CH_3O-	Cl	Cl	Z+++	+++	H+++	V+++	RH+++	+	+	+		94	21	14	89
9	CH_3O, OCH_3, CH_3O-	Cl	Cl	+++	+++	GH++	R++	RH+++	+	+	+		96	60	39	85
10	OCH_3, CH_3O-, OCH	Cl	Cl	+++	+++	G++	G++	GH++	+	Ø	+		96	57	20	89

11		Cl	Cl	+++	+++	Gr+++	Gr+++	Gr+++	+	+	+		97	25	12	91
12		Cl	Cl	+	+++	O+++	S+++	Gr++	+	+	+		97	79L	40	68
13		Cl	Cl	+	+++	Gr++	Gr++	Gr++	+	+	+		96	63L	27	72
14		Cl	Cl	+	+++	O+++	SH++	V+++	+	+	+		96	93	68	54
15		Cl	Cl	+	+++	Gr++	Gr++	G++	+	+	+		96	79	37	67
16		Cl	Cl	+	+++	H+++	H++	H++	+	Z	+	86	68	02	01	95
17		Cl	Cl	++	+++	GH+++	GH++	H++	+	+	+	96	90	06	09	94
18		Br	OCH_3	++	+++	O+++	O+++	GH++	Ø	+	+		95	44	28	87

(Fortsetzung S. 64)

TABELLE I (*Fortsetzung*)

No.	R_2 R_1 / R_3 O O	R_3	R_2	R_1	*Nachweisreaktion** D1	D2	D3** a	D3** b	D3** c	D4	D5	D6	*Lösungsmittelsystem (R_F × 100)* S3	S5	S7	S8	S9
19			Br	H	++	+++	R+++	R+++	R++	Ø	Ø	—		96	59	36	83
20			Br	Br	Z+++	+++	G++	O+++	RH+++	+++	+	+		97	47	21	85
21			Br	Br	+++	+++	Ø	G—	G+	+++	Ø	+		97	60	37	79
22			Br	Br	+++	+++	GH++	RH++	RH++	+++	+	+		97	47	24	88
23			Br	OH	+++	+++	GH+			++	+++	++	72	38	00	02	97
24			Br	Cl	Z+++	+++	G++	O++	O++	+++	+	+		94	50	28	82
25			Cl	Br	Z+++	+++	G++	O++	O++	+++	+	+		94	53	30	84
26			Cl	Cl	Z++	+++	GH+++			Ø	Z	++	49	25	00	02	97
27			Br	Br	Z++	+++	GH+++	GH++		+++	Z	++	48	25	00	02	97
28			Cl	Cl	++	+++	G—		G+	+	+	+		96	64	52	78

* G = Gelbfärbung; H = Braunfärbung; O = Orangefärbung; R = Rotfärbung; S = Graufärbung; V = Violettfärbung; Gr = Grünfärbung; Z = Zersetzung; L = langgezogene Flecken; Ø = keine Reaktion.

** Papier imprägniert mit a = Formamid, b = Dimethylformamid, c = Petroleum.

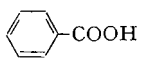
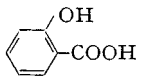
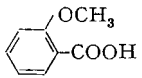
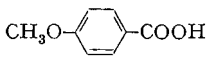
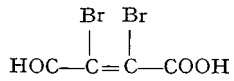
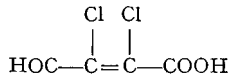
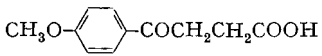
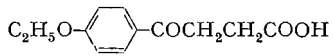
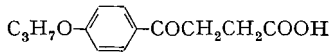
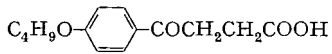
stanzen auch sehr wesentlich durch die benützte verankerte Phase beeinflusst. Als Grundlage wählten wir die Färbung auf Formamid-Chromatogrammen. Crotonlactone, die in γ-Stellung durch den p-Alkoxyphenylrest und in α- oder β-Stellung durch Halogen substituiert sind, reagierten mit diesem Reagens in der Regel gelb (z.B. 4–7, 20, 24, 25). Ersatz des Halogens in α-Stellung durch Hydroxyl hatte zumeist keinen Einfluss auf die Verfärbung des Flecks (1, 23). Hingegen führte Substitution mit Methoxyl in Stellung α oder γ zu Orange- oder Rotfärbung (2, 3, 18, 19). Bei Erwägung des aromatischen Substituenten in Stellung γ ersieht man die Bedeutung des Substituenten am Benzolkern: Abwesenheit der Substituenten (21, 28) setzt die Empfindlichkeit des Nachweises sehr wesentlich herab, und in manchen Fällen braucht überhaupt keine Reaktion einzutreten (21). Methoxyl in p-Stellung verursacht Gelbfärbung, Einbringen einer weiteren Methoxylgruppe in m-Stellung bewirkt Braunfärbung (8, 9). Bei Ersatz des Formamids als verankerter Phase durch Dimethylformamid oder Petroleum verschiebt sich die Gelbfärbung zumeist nach Rot, sodass die feinen Strukturunterschiede nicht so deutlich sind. Anscheinend ist zur Reaktion die Anordnung RO–C_6H_4–C–C=C– erforderlich, die mit Schwefelsäure gelb reagiert. Der Einfluss von Amin (bzw. einer anderen stickstoffhaltiger Komponente in der verankerten Phase) beruht offenbar nicht nur auf der Öffnung des entsprechenden Crotonlactons, sondern auch auf der Reaktion mit dieser Verbindung.

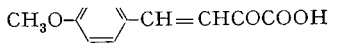
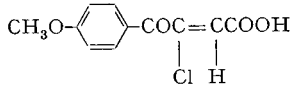
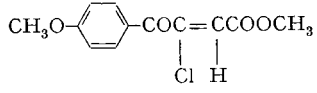
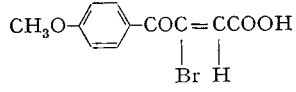
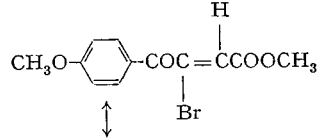
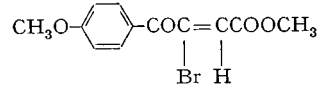
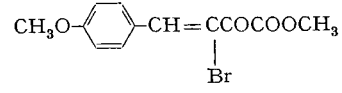
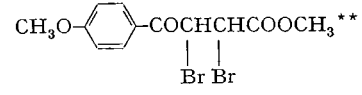
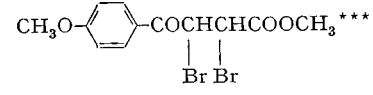
Verhältnismässig allgemein gültig für diese Stoffgruppe ist auch das Verlöschen im Licht der Niederdruck-Quecksilberlampe Chromatolite, namentlich nach Besprühen des Chromatogramms mit Fluorescein (D 2): In der Reihe der untersuchten Substanzen wurden nur die Verbindungen mit Methoxyl in γ-Stellung (3, 19) und ferner Naphthalinderivate (12–15) schwächer nachgewiesen. Verhältnismässig schwach ist der Nachweis mit 2,4-Dinitrophenylhydrazin, mit dem die meisten Crotonlactone reagieren. Es reagieren z.B. die nicht, die in α-Stellung nichtsubstituiert sind (3, 19). Hingegen reagieren in α-Stellung durch die Hydroxylgruppe substituierte Crotonlactone sehr stark (Ketolanordnung – 1, 23). Dieselbe Schlussfolgerung gilt auch für die Verwendung von Kaliumpermanganat (D 6). Mit Silbernitrat reagieren praktisch alle Crotonlactone, die in α-Stellung durch Halogen oder Hydroxyl substituiert sind. Hierbei reagieren die chlorsubstituierten Crotonlactone schwach, die bromsubstituierten Crotonlactone hingegen sehr stark (20–22, 24, 25, 27).

Mit Ausnahme der mit Hydroxyl substituierten Crotonlactone handelt es sich um Verbindungen hydrophoben Charakters, für die sich folgende Lösungsmittelsysteme am besten bewährten: Systeme mit verankertem Formamid und Benzin als mobiler Phase, mit verankertem Dimethylformamid und Cyclohexan als mobiler Phase, oder umgekehrte Phasen mit verankertem Petroleum und 60 %igem Isopropanol als mobiler Phase. Aus dem Zusammenhang zwischen chromatographischem Verhalten und der Struktur der Substanzen ergaben sich geläufige Gesetzmässigkeiten[4]: Substitution durch Brom erhöht die R_F-Werte weniger als Substitution mittels Chlor, Hydroxyl setzt die R_F-Werte herab, die Methylengruppe erhöht das R_F, Methoxyl erniedrigt die R_F-Werte (gilt nur für die stärker hydrophile stationäre Phase). Beim Methoxyl kommt es allerdings noch auf Zahl und Anordnung dieser Gruppen am Benzolkern an: Die disubstituierten Derivate mit den Methoxylgruppen in o-Stellung (8) wandern am Papier langsamer als das unsubstituierte Derivat oder Derivate mit den Methoxylgruppen in p-Stellung. Bei trisubstituierten vicinalen Methoxylderivaten (9, 22) tritt jedoch starke Erhöhung der R_F-Werte ein,

TABELLE II

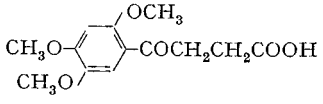
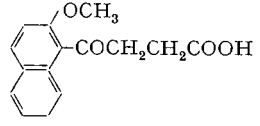
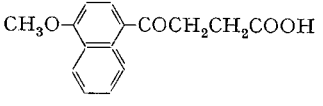
R_F-WERTE EINIGER AROMATISCHEN SÄUREN

No.	Säure	Nachweisreaktionen						Lösungsmittelsystem ($R_F \times 100$)									
		D1	D2	D3	D4	D5	D6	S1	S2	S3	S4	S4*	S5	S6	S7	S8	S9
31		++	++					92	56		34						
32		+++	+++	Ø	Ø	Ø	—	91	66		02	2.7		00			
33		—	+	Ø	Ø	Ø	Ø	89	51		44			09			
34		++	+++	Ø	Ø	Ø	Ø	92	55		44			22			
35		+	+	Ø	+++	++	Ø	89	Z48		03	6.5	00	00			
36		+	+	Ø	Ø	—	Ø	91	Z66			3.7					
37		++	+++	Ø	Ø	+++	Ø	89	56		43			07			
38		++	+++	Ø	Ø	+++	Ø	90	63		66			19			
39		++	+++	Ø	Ø	+++	Ø	91	68		82			38	00	02	
40		++	+++	Ø	Ø	+++	Ø	93	69		89			54	00	04	91
41		++	++	Ø	Ø	+++	Ø	93	90					95	22	23	92
42		+++	+++	G++	Ø	+++	+	91	38		11	30					

43		++	+++	G++	Ø	+++	++	74	59	00	0				
44		+++	+++	G+++	Ø	Ø	+	91	67	07	22				
45		++	++	R++	Ø	Ø	—	92	87			96	53	34	88
46		+++	+++	G++	+++	+	+	90	65	05	19.5				
47		++	++	R+	+	Ø	—	92	87			95	51	32	90
48		++	++	O+	++	—	—	92	87			96	50	39	88
49		+++	+++	O+	++	++	+	92	Z			96	58	29	85
50		++	++	O++	+++	—	—	93	Z87				81	51	81
51		+	++	G++	+++	+	—	93	Z87				83	49L	77

(Fortsetzung S. 68)

TABELLE II (*Fortsetzung*)

No.	*Säure*	*Nachweisreaktionen*						*Lösungsmittelsystem ($R_F \times 100$)*									
		D1	*D2*	*D3*	*D4*	*D5*	*D6*	*S1*	*S2*	*S3*	*S4*	*S4**	*S5*	*S6*	*S7*	*S8*	*S9*
52		+++	+++	G++	Ø	++	Ø	88	43		69			06			87
53		++	++	G++	Ø	Ø	Ø	93	62		73			31	00		92
54		++	+++	G++	Ø	Ø	Ø	89	63		85			51L	00		89L

* Durchlaufchromatogramm, 15 Stunden, Entfernung in cm.
** Isomer mit niedrigerem Schmelzpunkt.
*** Isomer mit höherem Schmelzpunkt.
Weitere Erläuterungen siehe in Tabelle I.

während bei den asymmetrischen Derivaten (11) die Beweglichkeit annähernd zwischen den disubstituierten *ortho*-Derivaten und den trisubstituierten vicinalen Derivaten liegt.

Die Ergebnisse des chromatographischen Verhaltens der aromatischen Säuren sind in der Tabelle II zusammengestellt.

Zum Nachweis bewährte sich als Universalmethode die Beobachtung des Verlöschens im Licht der Niederdruck-Quecksilberlampe (D 1). Dieses Verlöschen ist auf 5–10 μg der zu analysierenden Säuren empfindlich, und nach Besprühen mit Fluorescein (D 2) erhöht sich die Empfindlichkeit auf 2–3 μg. Von den übrigen zum Nachweis angewandten Verfahren kann für Säuren mit einer Ketogruppe die Reaktionen mit 2,4-Dinitrophenylhydrazin (D 5) benützt werden, die bei jenen Säuren sehr empfindlich ist, welche in benachbarter Stellung nicht durch Halogen substituiert sind. Substitution mittels Halogen setzt die Empfindlichkeit ungefähr auf das Zehnfache herab (44, 45, 47, 48). Substanzen mit Doppelbindung in der Seitenkette lassen sich durch alkalische Kaliumpermanganatlösung (D 6) nachweisen, diese Reaktion ist aber nicht sehr empfindlich. Zum spezifischen Nachweis der in der Seitenkette durch Brom substituierten Substanzen verwendeten wir Silbernitrat (D 4): Bei dieser Reaktion können durch zwei Bromatome substituierte Substanzen (35, 50, 51) sehr empfindlich nachgewiesen werden, bei Verbindungen mit einem Bromatom war die Reaktion bei freien Säuren (46) empfindlicher als bei Methylestern (47–49).

Sehr interessant ist der Nachweis mancher aromatischer monoalkoxylierter Säuren mit C_4-Seitenkette mittels Methylamin und Schwefelsäure (D 3): Substanzen, die in dieser Seitenkette eine Doppelbindung aufweisen (42, 43) oder durch Halogen substituiert sind (44, 46), reagieren im Falle freier Säuren mit Gelbfärbung. Die entsprechenden Methylester reagieren orange bis rot (45, 47–49). Eine Ausnahme bilden ferner jene aromatischen Säuren, die am aromatischen Kern entweder mehr Alkoxygruppen besitzen (52) oder die anstelle der Benzoylgruppe eine Naphthoylgruppe aufweisen (53, 54): Diese Säuren reagieren dann mit dem Reagens D 3 gelb, auch wenn in der Seitenkette keine Doppelbindung besteht oder wenn sie nicht durch Halogen substituiert sind.

Von den Lösungsmittelsystemen bewährte sich für freie Säuren stärker hydrophilen Charakters das System Isopropanol–Ammoniak (S 2), für weniger hydrophile Säuren das System Formamid/Chloroform und für Säuremethylester das System Dimethylformamid/Cyclohexan. Bei Systemen mit Formamid war es vorteilhaft, zur verankerten Phase Ammoniumformiat zuzusetzen, da sonst die Flecken langgezogen waren. Beim Studium der Beziehung zwischen chromatographischem Verhalten und Struktur stellten wir ausser den üblichen Gesetzmässigkeiten verschiedene Abweichungen vom üblichen Verhalten fest. So wanderte die Salicylsäure im System S 2 rascher als die Benzoesäure, die *o*-Methoxybenzoesäure wanderte langsamer als die *p*-Methoxybenzoesäure und Bromierung setzte in Formamid-Systemen die R_F-Werte bei freien Säuren (42, 46) herab: Dies wird wahrscheinlich durch die stärkere Acidität der bromierten Derivate verursacht. Bei den analogen Methylestern werden nämlich durch Bromierung die R_F-Werte erhöht.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde das chromatographische Verhalten von 28 substituierten Crotonlactonen und 24 substituierten aromatischen Säuren untersucht.

Es wurden optimale Bedingungen für den Nachweis und Systeme zur Trennung und Charakterisierung dieser Substanzen gefunden und die Beziehungen zwischen dem chromatographischen Verhalten und der Struktur dieser Verbindungen untersucht.

SUMMARY

The chromatographic behaviour of 28 substituted crotonlactones and 24 substituted aromatic acids was investigated. The best conditions for identification of these substances, as well as systems for their separation and characterization are described. The relationships between chromatographic behaviour and structure of these compounds was investigated.

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A CONVENIENT SEPARATION OF ALKALOID MIXTURES BY PARTITION CHROMATOGRAPHY, USING AN INDICATOR IN THE STATIONARY PHASE*

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Partition chromatography is a versatile and precise tool, which has been of great use in the separation of mixtures of closely related organic substances whose resolution by classical chemical methods would be impractical or impossible. When the two immiscible phases employed are neutral (*i.e.*, when there are no acidic or basic solvents, and no buffer in the polar phase), the advantage is offered of permitting visual detection of bands of acidic and basic materials on the column through the use of an indicator in the stationary phase. Sulfonphthalein indicators, which are highly polar in both forms, cover a wide range of pK_a values, and are readily available commercially, are quite ideal for this purpose.

Such a method has been extensively used for the separation of organic acids, both on silica gel and on Kieselguhr, using Bromcresol Green as the indicator on the column; some early papers describing this are listed at the end of this paper[1–5]. However, application of the method to resolution of alkaloid mixtures is apparently without precedent. Two related experiments have been reported: CLAYTON AND STRONG in 1954[6] separated volatile aliphatic amines on Celite, with phenolphthalein as an indicator in the polar phase; and TRAUTNER AND ROBERTS in 1948[7] made use of an indicator to follow the separation of hyoscine and hyoscyamine on silica gel, but the indicator was not on the column during the separation; it was added at various stages in a non-polar solvent, the band positions were marked, and the indicator solution was removed.

EXPERIMENTAL

A. Selection of a partition system

A mathematical treatment of partition chromatography indicates that the best resolution of bands on a column (*i.e.* the greatest ratio between the difference of R_F's and the difference of partition coefficients) occurs in the area where the partition coefficient in the column is equal to 1 (at about $R_F = 0.5$). As the average pressure-packed Kieselguhr column such as those described below has a retention volume of

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parghly three times the volume of the stationary phase, the actual ideal average routition coefficient of a mixture (mobile phase: stationary phase) is about 0.3.

The partition coefficient for the mixture to be separated can be determined in a 10-ml Erlenmeyer flask, using a variety of solvent mixtures, and (for the case of most alkaloids) Mayer's test on the upper and lower phases as a qualitative estimate of the amount of material in each. If the mixture contains a large amount of alkaloidal tar (which would probably have an R_F of near 0 in the system useful for separating the pure bases), this should be removed first by adsorption chromatography, precipitation of solid fractions, or simple extraction of the mixture with the mobile phase of the system to be used.

In choosing a solvent mixture, a number of factors must be considered. Primary among these is the solubility of the alkaloids of the mixture to be separated in various solvents. Most complex organic molecules, such as many alkaloids, contain both polar and non-polar portions and thus are ideally suited to separation by partition chromatography. It should be possible to find, for such compounds, a good polar and non-polar solvent which can be rendered essentially immiscible by addition of one or two indifferent solvents; for example, many alkaloids are relatively soluble in ethylene dichloride and in methanol, and these solvents may be separated by addition of hexane and water, in which many alkaloids are relatively insoluble. These four components are the ones which have proved most useful in this Laboratory, but many other variations are possible, depending upon the nature of the compounds to be separated. If possible, one should avoid highly volatile solvents (such as pentane or chloroform), and non-polar solvents which have a high solvation action on polar solvents (such as ethyl acetate), for a stable equilibrium will be difficult to obtain if these are used. If the non-polar phase draws in a large amount of the polar solvent, not only will the equilibrium of the solvent system be unstable but, furthermore, the indicator will be eluted from the column. Other important considerations are: (1) the polar phase should not be buffered (as the indicator will work poorly); (2) the mixture to be separated should be reasonably soluble (at least 10 %) in the mobile phase; (3) no component of the system should be highly toxic, acidic or basic, highly reactive, or corrosive; (4) no component of the mobile phase should be excessively high-boiling, for this would make necessary tedious post-extractions of the fractions to recover the material; (5) the system need not normally contain over four components; and (6) the polar phase should not be more than 50 % of the system, as much would be wasted.

The systems which have been found most applicable in this Laboratory are those containing ethylene dichloride and Skellysolve B in various ratios less than 2:3 (over which point the mobile phase takes up too much of the polar solvents), methanol, and enough water to effect separation (see below for specific systems). A system containing benzene, methanol, and water was also used successfully in one case, but was not very stable. In another Laboratory, hexane, heptane, and cyclohexane were found to be interchangeable with Skellysolve B[8]. Systems containing carbon tetrachloride and ethanol were also satisfactory, though less so than the ones eventually used most. A system employing Methyl Cellosolve and butanol gave a satisfactory coefficient in one case, but the alkaloid mixture to be separated was very sparingly soluble in the mobile phase.

It must be emphasized that this procedure for selection of a solvent system is

only applicable to mixtures of individual compounds which are fairly close in chemical properties. Although this method is of exceptional utility in separation of mixtures which would be unaffected by ordinary chemical resolutions, it is by no means a substitute for chemical separations of crude and complex mixtures. Thus, a mixture of crude *Buxus* alkaloids will give a large number of overlapping bands from R_F 0.0 to R_F 1.0 when added to a column such as described in the section below. However, after the mixture has been chemically separated into various fractions by salt precipitation, pH fractionation, and extraction with various solvents, each of the resulting fractions is then readily separable by this method—with somewhat different systems being used for different fractions. Similarly, a mixture of the bisbenzylisoquinoline bases isochondrodendrine (which is insoluble in chloroform) and tetrandrine (which is soluble in chloroform) is easily separated by a simple extraction, but behaves in a confusing fashion under the above treatment (because the two components differ markedly in an important chemical property).

B. Selection of an indicator

Sulfonphthalein indicators are highly polar in both forms, and normally will be retained in the stationary phase on a column, unless the mobile phase contains moderate amounts of a polar solvent. Fortunately, they also cover a very wide range of pK_a values. Those most likely to be applicable to alkaloid work are listed in Table I; most of the latter are readily available commercially.

TABLE I

COMMON SULFONPHTHALEIN INDICATORS

No.	*Name*	*pH range*	*Color*	
			acid	*base*
1.	Bromcresol Green	3.8–5.4	yellow	blue
2.	Chlorphenol Red	5.0–6.6	yellow	red
3.	Bromcresol Purple	5.2–6.8	yellow	purple
4.	Bromphenol Red	5.4–7.0	yellow	red
5.	Dibromophenol-tetrabromophenol sulfonphthalein	5.6–7.2	yellow	purple
6.	Bromthymol Blue	6.0–7.6	yellow	blue
7.	Phenol Red	6.8–8.4	yellow	red
8.	Cresol Red	7.2–8.8	yellow	red
9.	Metacresol Purple	7.4–9.0	yellow	purple
10.	Thymol Blue (basic range)	8.0–9.6	yellow	blue

That indicator should be chosen which is farthest down the table, and will clearly turn to the basic color when a solution of about 0.1 mg of its acid form in one ml of the stationary phase of the system to be used is treated with one mg of alkaloid mixture. The use of indicators farther down the list (with a higher pH range) will result in minor bands remaining invisible on the column; if those higher up the list (with a lower pH range) are used, the areas between bands will not return to a yellow color, separation will be observed poorly if at all, and the whole column will soon become discolored.

C. Testing on a column

After a partition system and an indicator have been selected for a given mixture, they should be incorporated into a small column to check the separation; the procedure may then be extrapolated to preparative scale (see below).

A convenient size for the test column is 1.3 cm i.d., with a Teflon stopcock on the bottom and a 100 ml bulb on the top (many variations are possible, of course). About 250 ml of the system is made up and equilibrated, 6 ml of the polar phase and 100 ml of the non-polar phase are withdrawn and combined with 1 mg of the indicator, and the whole mixture is swirled and poured rapidly onto 5 g of Johns-Manville "Celite" 503 (Hyflo "Supercel" is equally satisfactory). The resulting suspension is shaken vigorously; the stationary phase becomes uniformly distributed on the Celite. If the indicator chosen has a pK_a of 6 or less, the Celite will change it partially or wholly to the basic form; in such a case, the flask containing the mobile phase and support is waved briefly over a bottle of conc. hydrochloric acid until the indicator on the support just regains its yellow color. The mixture is then slurried in portions into the open column, the latter having been checked for verticality and protected at the bottom by glass wool, sea sand, and a filter paper disk. When all of the support has been transferred to the column, it is packed tightly with about 10 lbs. air pressure (from a line or rubber bulb); a snug-fitting filter paper disk is dropped onto the top of the packing, and compressed with a tamp until the top of the column is about the same density as the bottom. The column is then ready for use; after the excess solvent has been passed through or withdrawn, 10–50 mg of alkaloid mixture may be added in 0.2 ml of mobile phase, including a non-polar dye to mark the front (American Cyanamid Calco Oil Red or Blue). Elution then may proceed at a rate not exceeding one drop per five seconds. If the column and system are satisfactory, separation should be observed immediately; the bands may be collected as they leave the bottom of the column.

D. Large-scale separations

Large columns (2.0 to 25.0 cm in diam. or even larger) are best constructed by dry-packing; a good dry-packed column about 5 cm in diam. will, in general, give far better comparative resolution than a good slurry-packed one even of small size. The column should be perfectly cylindrical (to prevent extensive wall effects, which are particularly serious in this method of visual band detection) and have no constrictions above the base; a close-fitting solid wood or metal tamp should be constructed for the packing operation.

A 4.5 cm column may be packed with 200 ml of stationary phase and 80 mg indicator on 330 g of Celite 545. The stationary phase and indicator are added to the dry Celite, and the mixture is equilibrated by shaking or rolling it until all lumps are gone. It is then packed into the column with the aid of a powder funnel in at least twenty increments, tamping each one down very thoroughly before adding the next. A layer of fine-grain sea sand and a snug-fitting thick filter paper disk (such as Whatman 3 mm) are placed on top of the completed column (which will be about 5 cm in length), the tamp is placed on the top disk, the lower stopcock is opened, and about 1000 ml of mobile phase is added. The mobile phase running from the bottom is recycled until the entire column is fully wet (*i.e.*, no pressure ridges are visible; this will take about 48 h of running).

The total packing time should not exceed one hour. The final column should be tested with a narrow dye band before use (for channeling, irregularities, or undue diffusion). The alkaloid mixture (1–3 g) may be added in 10–15 ml of mobile phase, and passed through at a rate of 5 ml/min.

The R_F value of a given alkaloid in a given system will be slightly higher in this column than in the smaller slurry-packed one above; although the dry-packing gives a far tighter column, not as much stationary phase can be placed on the carrier.

Such a large column in our Laboratory has been used over thirty times with no obvious decrease in efficiency, and has been used successfully after standing idle under a variety of temperature and humidity conditions for six months.

RESULTS

The following specific applications of this method (summarized in Table II) have been investigated to date. It is highly recommended that an experimenter new to partition chromatography and/or this method try one of these, or another known example, before attempting investigation of an unknown mixture, as a certain degree of technique is required which comes best with experience.

A. Separation of the alkaloids of the Buxaceae[9,10] (see Fig. 1)

This general method was first developed to separate the various complex fractions of the alkaloids of *Buxus sempervirens* L. The alkaloids of this and related plants can be considered as representative of compounds having a highly non-polar saturated nucleus to which are attached a few polar functional groups.

Two solvent systems, (A) ethylene dichloride–Skellysolve B–methanol–water (5:10:2:0.3) and (B) ethylene dichloride–Skellysolve B–methanol–water (1:10:2:0.16) have served to separate all fractions so far investigated into their component individual alkaloids, which in many cases crystallize directly from the column fractions. The compounds are sufficiently strong bases that Phenol Red may be used for an indicator. Bromthymol Blue, Thymol Blue, and Cresol Red have also been employed in another Laboratory[8]; and substitution of hexane, heptane, or cyclohexane in system A does not affect the resolution[8]. Two examples of separations are described here, as typical of the success of this method.

The "acetone-insoluble" fraction gave, in system A, four major bands of R_F's 0.76, 0.68, 0.59 and 0.48; the first three of these were readily crystallizable from acetone or methanol, the last crystallizable with difficulty. A, minor band at R_F 0.18 was also crystallized from methanol with difficulty.

The "Skellysolve B-soluble" fraction gave, in system B, five major bands at R_F's 0.90, 0.78, 0.68, 0.55 and 0.45, of which the first one and last two only have been crystallized at this time; three minor bands appear at R_F's 0.31, 0.21 and 0.10, of which only the first has been crystallized. To save time and solvent, the last of these bands is best removed by a preliminary pass of the mixture through a large column of system A. The material running near the front is then added to the columns of system B.

Although a column 4.5 cm in diam. will handle only 2 g of mixture per run, no other convenient method has been found in this Laboratory to give any crystalline alkaloids from these mixtures. Preliminary work on other fractions has been prom-

TABLE

APPLICATIONS TO VARIOUS

Mixture		*Buxus sempervirens (acetone-insoluble)*	*Buxus sempervirens (Skellysolve B-soluble)*	*Rauwolfia vomitoria (crude)*	*Zygadenus paniculatus (refined)*
System used		*A*	*B*	*F*	*E*
Indicator used		*Phenol Red*	*Phenol Red*	*Bromcresol Purple*	*Bromthymol Blue*
Band I	R_F	0.76	0.90	0.75	0.36
	%	22 %	20 %	12 %	23 %
	identity by	new alkaloid a b c	new alkaloid a c	"*a*" c	neogermitrine b c
Band II	R_F	0.68	0.78	0.60	0.19
	%	35 %	8 %	25 %	20 %
	identity by	new alkaloid a b c	new alkaloid c	"*b*" + trace "*a*" + one minor base c	neogermidine + germidine etc. b c
Band III	R_F	0.59	0.68	0.36	0.16
	%	14 %	26 %	25 %	50 %
	identity by	new alkaloid a b c	new alkaloid c	ajmaline + trace "*b*" c	zygacine b c
Band IV	R_F	0.48	0.55	0.25	
	%	8 %	16 %	22 %	
	identity by	new alkaloid a c	new alkaloid a b c	Three minor bases + trace ajmaline c	
Band V	R_F	0.18	0.45	0.12	
	%	1 %	8 %	6 %	
	identity by	new alkaloid a c	new alkaloid a b c	Two minor bases c	
Other bands			Three minor bands R_F's 0.31, 0.21, 0.10		

ising, and two more crystalline alkaloids have been obtained from one of these fractions by use of system A.

This method also gave five crystalline alkaloids when used for the mixture of bases of genus *Pachysandra*, employing the system ethylene dichloride–Skellysolve B–methanol–water (2.5:15:2:0.3) (C)[8].

The systems Skellysolve B–methyl cellosolve–*n*-butanol–water (8:1.5:0.5:0.4); Skellysolve B–benzene–ethanol–water (8:2:5:0.9) and Skellysolve B–carbon tetrachloride–methanol–water (5:5:5:0.6), were all satisfactory for the "acetone-insoluble" fraction in terms of partition coefficient, but were inferior to system A above for various reasons (possession of a poor solvent action, a poor equilibrium, and a toxic component, respectively).

Large dry-packed columns containing the above system A were very stable, could be used over wide variations in temperature, gave no loss of efficiency after

II

ALKALOID MIXTURES

Commercial "Veratrine" (refined)	*Commercial "Veratrine" (front-running)*	*Cissampelos pareira (crude)*	*Cyclea peltata (crude)*	*Cyclea peltata (front-running)*
D	*E*	*D*	*D*	*E*
Bromthymol Blue	*Bromthymol Blue*	*Bromcresol Purple*	*Bromcresol Purple*	*Bromcresol Purple*
above 0.50 80 % cevadine + veratridine see next column	0.33 62 % cevadine a b c	0.65 10 % *l*-curine b c	above 0.70 70 % tetrandrine + fangchinoline see next column	0.45 57 % tetrandrine b c
0.31 9 % sabadine + minor bases b c	0.15 38 % veratridine b c	0.32 3 % hayatin + iso- chondrodendrine b c	below 0.70 10 % isochondroden- drine + minor bases b c	0.25 43 % fangchinoline b c
0.11 2 % various minor constituents b c				

Code: Systems: A, ethylene dichloride 5 : Skellysolve B 10 : methanol 2 : water 0.3.
B, ethylene dichloride 1 : Skellysolve B 10 : methanol 2 : water 0.16.
D, ethylene dichloride 8 : Skellysolve B 12 : methanol 3 : water 0.6.
E, ethylene dichloride 3 : Skellysolve B 12 : methanol 2 : water 0.24.
F, ethylene dichloride 6 : Skellysolve B 10 : methanol 2.5 : water 0.5.

Methods of identification: a = by crystallization; b = by infrared spectrum; c = by paper chromatography, or rarely by a single sharp narrow band on the partition column being used for the separation.

repeated use or prolonged disuse, and gave excellent separations of very closely related compounds. An occasional large slurry-packed column gave equally good results, but most columns packed by that method were loose and gave much diffusion of bands.

B. Separation of the alkaloids of the Menispermaceae[11]

Good results were obtained in the separation of the bisbenzylisoquinoline alkaloids of *Cyclea peltata* Diels and *Cissampelos pareira* L., although the presence of the chloroform-insoluble isochondrodendrine in both mixtures created some difficulty.

The systems used were ethylene dichloride–Skellysolve B–methanol–water (8:12:3:0.6) (D) and ethylene dichloride–Skellysolve B–methanol–water (3:12:2:0.24) (E). Bromcresol Purple was used as an indicator, and the tests were conducted in a slurry-packed column of 1.8 cm i.d., with 15 ml of stationary phase on 15 g of Celite.

The mixed *Cyclea* alkaloids were first chromatographed in the stronger system (D); all running at R_F greater than 0.7 was collected, and run in the weaker system (E). That running behind R_F 0.7 had an infrared spectrum similar to isochondrodendrine; paper chromatography showed it to be a mixture of several bases, possibly including isochondrodendrine. The separation in the weaker system gave two bands of R_F's 0.45 and 0.25, which were shown by infrared spectra and paper chromatography to be pure tetrandrine and fangchinoline, respectively.

The mixed *Cissampelos* alkaloids were separated in the stronger system (D), and gave only two bands; a large amount of the mixture (isochondrodendrine and related bases, and tars) remained insoluble in the mobile phase. The front band (R_F 0.65) had

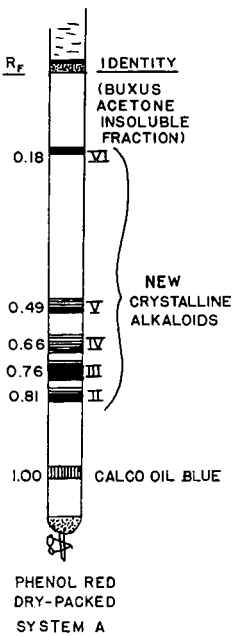

Fig. 1. Diagram of a column separation of the *Buxus* alkaloids.

an infrared spectrum and paper-chromatographic behavior identical with those of *l*-curine. The second band (R_F 0.32) was a mixture, probably containing hayatin and isochondrodendrine.

Thus, although neither of these alkaloid mixtures has been fully separated into individual components by this method, both have yielded some pure alkaloids with a minimal amount of effort and loss of material.

C. Separation of indole-type alkaloids

A complex mixture of alkaloids from *Rauwolfia vomitoria* Afzel., containing at least eight alkaloids (but no reserpine), was separated visually into fractions of R_F's 0.75 (1), 0.60 (2), 0.36 (3), and 0.25 (4) by the system ethylene dichloride–Skellysolve B–methanol–water (6:10:2.5:0.5) (F), using Bromcresol Purple as an indicator. System A with the same indicator could also be used. Fraction 1 contained only one alkaloid (*a*); fraction 2 contained a small amount of *a* and of another base, but was

mostly a third alkaloid (*b*); fraction 3 contained a small amount of *b*, but was mostly ajmaline (identified by paper chromatography); and fraction 4 contained a small amount of ajmaline and three minor bases. Some of the mixture (about 5 %) remained insoluble in the mobile phase. Bases *a*, *b*, and ajmaline were the major components of the total mixture; further work on these, including cleaner separations on better columns and crystallization of the major bases, is in progress.

In a co-operating Laboratory, a variety of other mixtures of indole alkaloids (including ajmaline and ibogaine) have been separated into crystalline components by the use of systems A and C. It was noted that some of these mixtures could be better resolved by alumina chromatography[8].

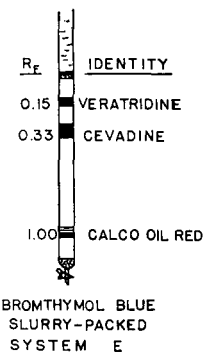

Fig. 2. Separation of a natural mixture of cevadine and veratridine.

D. Separation of the alkaloids of the tribe Veratreae (see Fig. 2)

This general method, which has been shown to be applicable to non-polar highly saturated alkaloids and fairly polar aromatic bases, required surprisingly little modification to be adapted to the highly polar Veratrum-type polyhydroxy ester alkaloids.

A sample of total alkaloids from *Zygadenus paniculatus* Nutt., chromatographed in system E with Bromthymol Blue as indicator, gave three bands only. The germine tri-esters—almost totally neogermitrine—ran at R_F 0.36; the germine diesters (mostly neogermidine and germidine) ran at R_F 0.19; and almost pure zygacine appeared at R_F 0.16. Only small amounts of the aromatic zygadenine-3-esters isolated from another sample[12] were present.

Striking success was encountered in the separation of a natural mixture of veratridine (veracevine-3-veratroate) and cevadine (veracevine-3-angelate) from commercial "Veratrine" (an extract of *Schoenocaulon officinale* Gray). Using system E, cevadine ran at R_F 0.33 and veratridine at R_F 0.15; there was a wide area between the bands which contained no solid, and each band was completely homogeneous. The cevadine band crystallized readily from acetone–water (m.p., becomes anhydrous at 140°, then melts at 204–207°). Veratridine has never been crystallized; efforts on this sample were equally without success. When compared with previous methods[13], this represents a highly efficient resolution of these two compounds.

Slower-running bands in Veratrine could be collected by the use of system D. Two bands, at R_F's 0.31 and 0.11, were visible; the first of these contained three bases, but was probably mostly sabadine; it represented about 10 % of the total Veratrine mixture.

A system, benzene–methanol–water (10:4:0.6), was also used for these bases; although it gave fair results, its equilibrium was highly unstable, probably because the benzene took much methanol into the non-polar phase.

Further work on all of these separations, and more applications of this general method, will be reported in future papers.

SUMMARY

A convenient method has been developed for the separation of the alkaloids of *Buxus sempervirens* L. This method involves the use of partition chromatography on Kieselguhr, with an indicator in the stationary phase; the separate bands are detected visually, collected as a whole and in many cases crystallized directly. General considerations are presented concerned with the application of this method to other alkaloidal mixtures. The method is shown to give rapid and convenient separations of mixtures of bisbenzylisoquinoline alkaloids; of complex mixtures of indole alkaloids; and of mixtures of veratrum ester alkaloids, including an efficient and complete separation of a natural mixture of cevadine and veratridine.

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DEMONSTRATION OF STEROIDAL FUNCTIONAL GROUPS ON PAPER CHROMATOGRAMS

II. α-KETOLS AND GLYCOLS

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As a continuation of a previous communication[1], reporting the search for spot test methods to demonstrate certain functional groups of steroids on paper chromatograms, the present paper will deal with methods for detecting an α-ketol or a *vic*-glycol grouping.

Periodate has been widely used for the detection of polyols and non-reducing sugars[2–5], deoxysugars[6–8] and hydroxy-amino acids[9] on paper chromatograms. These methods are developed either on the basis that periodate is consumed by the polyols or that characteristic products—formaldehyde, acetaldehyde or malonaldehyde—are formed on oxidation. Periodate oxidation of steroidal α-ketols and 1,2-diols yields similar products. In fact, the identification of steroidal α-ketol and 1,2-diol structures by virtue of the characteristic oxidation products has been thoroughly studied and widely applied[10,11]. However, besides the demonstration of the formation of a 17-ketone through periodate oxidation of a 17,20-diol[12,13], no methods have been reported to identify other oxidation products directly on a paper chromatogram. In the present paper, four tests, namely, those for formaldehydogenic, acetaldehydogenic, aldehydogenic (non-volatile) and acidogenic steroids, which can be directly applied to papergrams will be described.

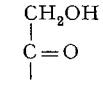
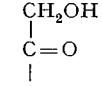
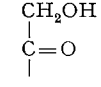
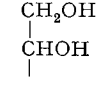
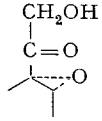
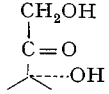
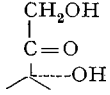
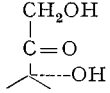
TEST FOR FORMALDEHYDOGENIC STEROIDS

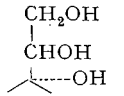
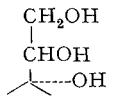
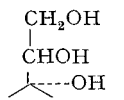
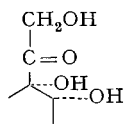
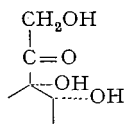
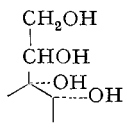
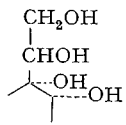
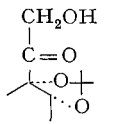
Steroids (C_{21}) with a 21-ol-20-one or a 20,21-diol structure produce formaldehyde on periodate oxidation[10,11]. The procedure described below is adapted from the method of SCHWARTZ for the detection of serine on papergrams[9].

The dried papergram is sprayed with a reagent consisting of 1 vol. of a saturated aqueous potassium periodate solution plus 3 vol. of 95 % ethanol. Four to eight minutes later, the partially dried paper is sprayed with a freshly prepared reagent containing 15 g of ammonium acetate, 0.3 ml of glacial acetic acid and 1 ml of 2,4-pentanedione in 100 ml of methanol. Ten minutes after the second spray, a formaldehydogenic steroid appears as a yellow-green fluorescent spot on a dark background under U.V. ("Mineral Light"). The spot becomes yellow under ordinary light on a colorless background in 30–60 min. The background gradually turns yellow on longer standing. The development of the yellow color at the end of 30 min is regarded as a more reliable

TABLE I

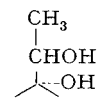
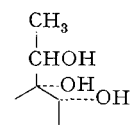
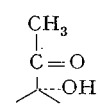
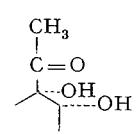
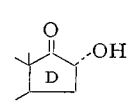
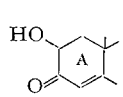
RESULTS OF TESTS FOR STEROIDAL α-KETOLS AND GLYCOLS

Compound	*Structure under test*	*Solvent system*	R_F	*Test for formaldehydogenic steroids*	*Test for acetaldehydogenic steroids*	*Test for aldehydogenic steroids*	*Test for acidogenic steroids*	*Phenol red borate test*	*Tetrazolium reduction test*
Cortexone[a]		HTMW	0.81	+	—	—	+	—	+
3β,21-Dihydroxy-5α-pregnan-20-one[b]		HTMW	0.74	+		—	+	—	+
Corticosterone[a]		HTMW	0.34	+			+	—	+
20β-Dihydrocortexone[c]		HTMW	0.39	+		+	—	—	—
16α,17α-Oxidocortexone[a]		HTMW	0.76	+			+		—
Cortexolone[a]		BMW	0.70	+	—	—	+	—	+
3β,17α,21-Trihydroxy-Δ⁵-pregnen-20-one[b]		BMW	0.59	+			+	—	+
Cortisol[a]		BMW	0.12	+		—	+	—	+

20β-Dihydrocortexolone[c]		BMW	0.35	+	±	—	—	—
20β-Dihydrocortisol[c]		CMW	0.52	+	+	—	—	—
3α,17α,20α,21-Tetrahydroxy-5α-pregnan-11-one[b]		CMW	0.56	+	±	—	—	—
16α-Hydroxy-cortexolone[a]		CMW	0.88	+	—	+	+	+
Triamcinolone[a]		CMW	0.22	+	—	+	+	+
16α,17α,20β,21-Tetrahydroxy-Δ^4-pregnen-3-one[c]		CMW	0.66	+	+	—	+	—
9α-Fluoro-11β,16α,17α,20β,21-pentahydroxy-Δ^4-pregnen-3-one[c]		CMW	0.15	+	+	—	+	—
Triamcinolone-16α,17α-acetonide[a]		HTMW	0.21	—		—	—	+

(continued on p. 84)

TABLE I (*continued*)

Compound	*Structure under test*	*Solvent system*	R_F	*Test for formaldehydogenic steroids*	*Test for acetaldehydogenic steroids*	*Test for aldehydogenic steroids*	*Test for acidogenic steroids*	*Phenol red borate test*	*Tetrazolium reduction test*
3-Keto-Δ^4-pregnen-20β-ol[a]		HTMW	0.88	—	—			—	—
3-Keto-Δ^4-pregnene-17α,20β-diol[c]		HTMW	0.53	—	+			—	—
3-Keto-Δ^4-pregnene-16α,17α,20β-triol[c]		HTMW	0.22	—	+			+	—
17αHydroxy-progesterone[a]		HTMW	0.72	—	—	—		—	—
16α,17α-Dihydroxyprogesterone[a]		HTMW	0.59	—	—	+	—	+	—
16α-Hydroxyandrostenedione[a]		HTMW	0.50	—	—	+	+	—	+
2β-Hydroxytestosterone[a]		HTMW	0.45	—		+	—	—	+

Progesterone[a] (tested by direct spotting)			—	—	—	—	—	—
Androstenedione[a] (tested by direct spotting)			—	—	—	—	—	—
Testosterone[a]	HTMW	0.70	—	—	—	—	—	—
Δ^1-Testotolactone[a]	HTMW	0.40	—	—	—	—	—	—

HTMW = BUSH B-1 system, *n*-hexane being used in place of petroleum ether, run at 37°; BMW = Benzene–methanol–water (1:1:1), by TUZSON's technique[14]; CMW = Chloroform–methanol–water (2:1:2) by TUZSON's technique[14].

[a] From Squibb steroid collection including those obtained commercially and those produced by microbial conversions.
[b] From USP reference standards.
[c] Prepared by sodium borohydride reduction of the corresponding 20-ones. See text under the section of acetaldehydogenic steroids.

criterion than the yellow fluorescence produced in 10 min after the second spray. Certain non-formaldehydogenic steroids, *e.g.* 16α-hydroxyandrostenedione and 16α, 17α-dihydroxyprogesterone, produce a doubtful trace of fluorescence but no characteristic bright yellow color.

The compounds used in studying this test are listed in Table I. All steroids with a 21-ol-20-one or 20,21-diol structure respond well to this test while those without either of these two structures give negative results. Steroids in which an α-ketol side-chain is joined to an isopropylidene-dioxy-group (a 16,17-acetonide) form exceptions to this test. A separate experiment, carried out in test tubes, showed that such a "hindered" α-ketol resists oxidation in a dilute potassium periodate solution; more drastic conditions, *e.g.* treatment with a 2 % periodic acid solution at 37° overnight, is required for oxidation. It is not known whether a hindered 20,21-diol behaves similarly.

A 10 γ cortexone spot on an area of 1 sq. cm can be readily demonstrated on a papergram. The method is not applicable to ZAFFARONI type papergrams because traces of non-volatile solvent or impurities contained therein are often also formaldehydogenic.

TEST FOR ACETALDEHYDOGENIC STEROIDS

Only the 21-unsubstituted C_{21} steroids with a 17,20-diol structure yield acetaldehyde on periodate oxidation. The procedure developed, which is a modification of SCHWARTZ's[9] method for threonine is as follows.

The dried papergram is sprayed with a reagent prepared by dissolving 2.5 g of periodic acid in 10 ml of water and diluting the resulting solution with 90 ml of *tert.*-butanol. Three to seven minutes later, the partially dried paper is sprayed with a freshly prepared reagent containing 2 g of sodium nitroprusside, 15 ml of piperidine in 100 ml of methanol. Ten to fifteen minutes after the second spray, an acetaldehydogenic steroid appears as a blue spot on a straw colored background. The whole background turns blue after 20 minutes. Exposing the sprayed paper strip momentarily to ammonia vapor intensifies the blue color.

As shown by the data given in Table I, only 3-keto-Δ^4-pregnene-17α,20β-diol and 3-keto-Δ^4-pregnene-16α,17α,20β-triol, among all the compounds tested showed a positive reaction. These two compounds were prepared on a microscale[15-17] by reducing 17α-hydroxyprogesterone and 16α,17α-dihydroxyprogesterone respectively with sodium borohydride using the method of NORYMBERSKI AND WOODS[18] as described below. The fact that no steroids tested besides the two mentioned showed a positive reaction conclusively demonstrated that the method is specific for acetaldehydogenic steroids.

To a solution containing 500 γ of 17α-hydroxyprogesterone in 0.1 ml of methanol which had been cooled to 4° in a cold room, 0.1 ml of a freshly prepared 0.1 % solution of $NaBH_4$ in methanol which had also been cooled to 4° was added. The test tube was closed with a polyethylene stopper and kept at 4° for one hour. The reaction was stopped by the addition of 0.1 ml of 3 *M* acetic acid in water and the steroids were extracted with 2.5 ml of chloroform after the addition of 0.7 ml of a saturated solution of ammonium sulfate. The chloroform extract was evaporated to dryness with the aid of a gentle current of air (*cf.* ref. 19). The residue was taken up in a small volume of

1:1 methanol–chloroform and an aliquot was chromatographed. The major spot which was detectable on a HAINES' U.V.-scanner[20] and moved with an R_F value considerably lower than that of 17α-hydroxyprogesterone was taken to represent the expected product—3-keto-Δ^4-pregnene-17α,20β-diol. The compound itself was not isolated in pure form. Other steroidal 20β-ols, as listed in Table I, were prepared by exactly the same procedure.

The nitroprusside reaction for acetaldehyde and secondary amines is well known[21]. It has been widely used for the determination of acetaldehydogenic glycols[22], including steroids[23]. As a spray reagent for paper chromatography, HULME AND ARTHINGTON used it for the detection of proline[24]; WALDRON and EDWARD used it for 6-deoxysugars[6,7] and SCHWARTZ used it for threonine[9]. The method reported here is just another example for its adaptation as a spray reagent. The use of *tert.*-butanol as the solvent for periodic acid is probably the only unique feature of the present technique. In fact, other lower alkanols can also be used; *tert.*-butanol seems to give the most satisfactory result.

A 10γ papergram spot of 3-keto-Δ^4-pregnene-17α,20β-diol occupying an area of 1 sq. cm. is readily detectable. The method is again not applicable to ZAFFARONI type papergrams.

TEST FOR ALDEHYDOGENIC STEROIDS

A steroid containing a secondary α-ketol or a *vic*-glycol structure, except a 17,20-diol, generates a non-volatile aldehyde on periodate oxidation. It is obvious that such an aldehyde cannot be detected by double spraying technique as used in the tests described above for formaldehydo- or acetaldehydogenic steroids because the cellulose of the paper also produces aldehydes on oxidation. This problem can obviously be solved by using the same technique developed for demonstrating a steroid alcohol by direct chromic acid oxidation on a papergram[1].

The part of the papergram bearing the spot to be tested is sprayed with a reagent prepared by dissolving 2.5 g of periodic acid in 10 ml of water and diluting the resulting solution with 90 ml 95 % ethanol. The other part of the papergram is covered with a piece of cardboard at the time of spraying. (See the drawings in a previous paper[1].) Immediately after spraying, the paper strip is rolled on the form of a hollow cylinder and kept for four hours in an atmosphere saturated with 95 % ethanol. Upon removal, it is air dried and then transferred, still in the form of a cylinder, to another jar which contains a shallow layer of 1:1 methanol–chloroform mixture on the bottom. An ascending type chromatogram is developed until the solvent front moves at least 2 in. beyond the area which has been wetted by the periodic acid solution. The strip is again air dried, the part which has been wetted by the periodic acid solution is cut off and the rest is sprayed with Schiff's reagent[25]. One hour later the air dried strip is placed in a closed container to prevent the evaporation of sulfur dioxide and left at room temperature overnight. The aldehyde spot now situated in the front becomes deep blue on an almost colorless background. Traces of red or purple areas along the front are not considered as a positive result.

This test is applicable to papergrams developed with both ZAFFARONI* and BUSH systems. The compounds used in this study are also listed in Table I. Both side-chain or ring *vic*-glycols and secondary α-ketols respond well to this test. The 16α,17α-diol

* ZAFFARONI type papergrams are dried in a 100° oven equipped with a forced air draft system.

structure of triamcinolone and related compounds constitutes an exception. A separate experiment carried out in test tubes showed that the 16α,17α-diol structure of triamcinolone is destroyed after the treatment with periodic acid but the product gives a negative Schiff's test. Probably rearrangement similar to that reported by SMITH *et al.*[26] could have taken place during the oxidation, yielding a non-aldehydic product.

Repeated tests with steroids with a 17α,20,21-triol structure showed that the results were variable (see Table I). Apparently the factors influencing the rate of oxidation which in turn determines whether a 17-ketone or a 17-aldehydo structure would be formed as the end product are not easily controlled. In view of this variation together with the failure of the 16α,17α-diol structure of triamcinolone to give a positive test, it is to be emphasized that in using this test, although a positive result indicates unequivocally the presence of a 1,2-diol or a *sec.*-α-ketol structure, a negative result does not necessarily mean the absence of such groupings.

Although Schiff's reagent has been used for detecting certain steroidal aldehydes[27], is was found in the present study that the reaction between Schiff's reagent and a water-insoluble steroidal aldehyde is very slow. It is therefore essential to keep the sprayed and dried paper strip inside a closed container to keep the background from turning deep red due to the loss of sulfur dioxide. Many new reagents are known for detecting aldehydes[28–30]. Schiff's reagent has been regarded as a rather standard reagent for aldehydes, and has been used in the present study; it is entirely possible that the newer reagents might be more advantageous.

TEST FOR ACIDOGENIC STEROIDS

Periodate oxidation of a steroidal α-ketol generates a non-volatile acid as one of the products. It is considered feasible to detect the acid formation from readily oxidizable neutral steroids directly on a papergram. The following procedure proves satisfactory for such a test.

The dried papergram is sprayed with a reagent containing 1 vol. of a saturated aqueous KIO_4 solution and 3 vol. of 95 % ethanol. One hour later, the paper strip is sprayed with the phenol red–Tris buffer reagent developed for steroid acids[31]. The acidogenic steroids appear immediately as yellow spots on a light pink background. The spots become more conspicuous on drying.

From the results given in Table I, it can be seen that all primary steroid α-ketols respond well to this test. Secondary α-ketols, *e.g.* 2β-hydroxytestosterone, give negative results. Separate microchemical experiments carried out in test tubes showed that these α-ketols are not readily oxidized by a brief treatment with a dilute KIO_4 solution. It is also to be noted that a ring D α-ketol, namely 16α-hydroxyandrostenedione, gives a strong positive test and as discussed under the test for formaldehydogenic steroids, a side chain α-ketol joined to a 16,17-acetonide, namely triamcinolone-16,17-acetonide, is not expected to respond to this test.

This test is also not applicable to ZAFFARONI type paper chromatographs.

PHENOL RED BORATE BUFFER SPRAY FOR CERTAIN STEROID GLYCOLS

This test is a direct adaptation of the methods reported by BRADFIELD AND FLOOD[32] and HOCKENHULL[33] for polyols. The reagent is prepared by mixing 2 ml of a 0.1 %

solution of phenol red in 95% ethanol, 1 ml of an aqueous 0.15 *M* H_3BO_3 and 20 ml of methanol. To this mixture, 0.1 *N* NaOH is added dropwise until the solution becomes pink and does not produce any more perceptible change in color on further addition of 1 to 2 drops. Immediately after spraying, steroidal ring *cis*-1,2-diols appear as yellow spots on a light pink background. The spots become more conspicuous on drying. To exclude the possibility that the spot could be an acid, another identical strip should be sprayed with phenol red–Tris buffer reagent[31] where a glycol would not change the color of the indicator.

As shown by data listed in Table I, all steroid 16α,17α-diols responded to this test without exception, while steroids with a 20β,21-diol, a 17α,20α,21-triol, or a 17α,21-diol-20-one structure gave negative results. These results indicate that a ring *cis*-1,2-diol structure is required for a positive test, agreeing with the information known for carbohydrates[34]. A 1,2- or a 1,3-diol in the side chain apparently does not lower the pH far enough to turn the color of phenol red, although it is known that such glycols also form "chelated orthoborates"[35]. No steroidal diaxial 1,3-diols were tested. It is therefore not known if such a structure would respond to the borate test. In view of the fact that a steroidal diaxial 1,3-diol forms an acetonide[36] readily, such a possibility must be taken into consideration in interpreting a positive result obtained with this test.

This test is applicable to papergrams of both BUSH and ZAFFARONI* types. A 10 γ papergram spot of 16α,17α-dihydroxyprogesterone on an area of 1 sq. cm can be readily detected.

TETRAZOLIUM REDUCTION TEST FOR α-KETOLS

To complete the picture concerning tests characteristic for α-ketols and glycols, the results obtained with tetrazolium reduction tests are also included in Table I. The reagent used was a solution of 1 mg of tetrazolium chloride per ml of 1 *N* KOH in 90% aqueous methanol (v/v), which was essentially the same as that reported by NOWACZYNSKI *et al.*[37]. While primary α-ketols, *e.g.* cortexone, develop a highly intense color within 5 minutes after the reagent is applied by spraying or dipping, the secondary α-ketols, *e.g.* 2β-hydroxytestosterone, produce only a weak color, indistinguishable from that produced by many non-reducing Δ^4-3-ketones, *e.g.* androstenedione or 7α-hydroxyprogesterone (*cf.* ref. 38). However, when the sprayed paper strip is allowed to air dry thoroughly (40 min or longer) and then heated at 80°–90° for 5 min, the color produced by 2β-hydroxytestosterone becomes as intense as that of cortexone, while that produced by non-reducing Δ^4-3-ketones undergoes only slight changes. On the basis of these results, it is recommended that to determine if an unknown is a secondary α-ketol by using the tetrazolium reduction test, known compounds, *e.g.* those mentioned above, should be used as positive and negative controls and the increase in the color intensity on heating should be used as the criterion. Commercial samples of both 2,3,5-triphenyltetrazolium chloride or blue tetrazolium—3,3-dianisole-bis-4,4-(3,5-diphenyl)-tetrazolium chloride—were found to give virtually identical results, the latter giving more intense coloration. Since only the qualitative aspect of the reduction was emphasized in the present study, the impure

* ZAFFARONI type papergrams are dried in a 100° oven equipped with a forced air draft system.

nature of the commercial blue tetrazolium samples as pointed out by BUSH AND GALE[39] was not seriously considered.

DISCUSSION

Since all the tests reported here are based on well established reactions, the results should mean an indication of the presence or absence of the structural elements tested. However, in view of the exceptions observed, namely, the negative result obtained with triamcinolone acetonide in the test for formaldehydogenic steroids and non-aldehydogenic nature of the structure of 16α,17α,21-triol-20-one, a negative result should be interpreted with caution. The principal theme of the present study is really a development of shortcut methods whereby a well established reaction can be carried out directly on a paper chromatogram.

Although the different tests reported here can be used for the detection of spots on a papergram, they are not as sensitive as many other methods, such as U.V.-scanning or phosphomolybdic acid[31]. They are rather recommended for the demonstration of certain structural elements after it is ascertained that a paper gram spot contains a sufficient quantity of the compound to be tested. Furthermore, it is always advisable that in testing an unknown, a compound known to give a positive test and another known to give a negative test should be used at the same time as controls.

SUMMARY

Simple and convenient methods are described whereby a steroid which forms formaldehyde, acetaldehyde, a non-volatile aldehyde or a non-volatile acid on periodate oxidation can be demonstrated directly on a paper chromatogram. A method for demonstrating certain steroidal glycols by using a borate–phenol red spray and a modified procedure for applying the tetrazolium reduction test on a paper chromatogram are also presented.

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CENTRIFUGAL CHROMATOGRAPHY

IV. A SIMPLE DISTRIBUTOR FOR CENTRIFUGAL CHROMATOGRAPHY*

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The majority of hitherto published papers dealing with centrifugal chromatography show[1–4] that McDONALD'S apparatus has found applications in chromatographic separation. We have previously[5] pointed out some disadvantages of this system. Both McDONALD'S method of excentric overpressure feeding of the mobile phase and the central spot distributor of PAVLÍČEK *et al.*[5] have the same drawback, *viz.* the time-consuming and complicated manner in which feeding of the mobile phase from the reservoir takes place. With the arrangements used it is difficult to change the mobile phase rapidly (using the same distributor), which is essential for multi-purpose application of the centrifugal chromatograph.

The mobile phase distributor constructed by TATA AND HEMMINGS[6] is simple, but does not permit changing of the mobile phase during the chromatographic process (*e.g.* gradient elution).

The aim of this paper is to describe a simple central-type distributor for the

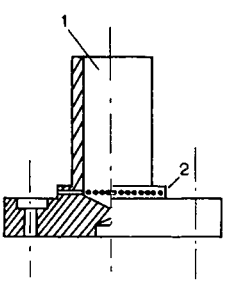


Fig. 1. Mobile phase distributor – sectional view. 1 = hollow space of the distributor – for mobile phase and porous material (glass wool); 2 = system of capillary hollows.

mobile phase, which, in contrast with previous types, permits the mobile phase to be changed rapidly. At the same time, it requires no regulation of flow and is very suitable for papers of low capacity (Whatman No. 1, 2 and 4; Ederol 202, Schleicher & Schüll 2043b). The solvent flow is regulated merely by the centrifugal force and the sorption capacity of the paper.

* For Part III, see Z. DEYL AND J. ROSMUS, *J. Chromatog.*, 8 (1962) 537.
** Director: Ing. FRANTIŠEK VONEŠ.

The mobile phase distributor consists of a polyamide or, preferably, of a stainless-steel hollow cylinder, the base of which is provided with 50 holes 0.2 mm in diameter. The cavity of the cylinder is filled with suitable porous material, glass wool being recommended (Fig. 1). Chromatographic paper, with a circle of the same diameter as the outer dimension of the distributor cut out from the center, is fixed by means of a

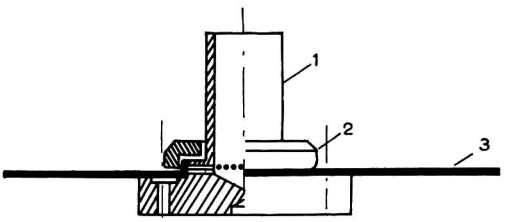


Fig. 2. Chromatographic paper – fixation technique. 1 = hollow cylinder; 2 = fastening ring; 3 = chromatographic paper.

fastening ring. The inner side of the latter is recessed to conform with the recessing of the base of the distributor. By pressing the ring the chromatographic paper starts to swell up until it tightly covers the apertures of the distributor (Fig. 2). The whole distributor unit is firmly coupled with both the motor axle and the three arms, which clasp the paper at its edge (Fig. 3).

When changing the mobile phase the wool must be removed from the distributor with forceps, the distributor rinsed with a suitable solvent (ethanol) and the chromato-

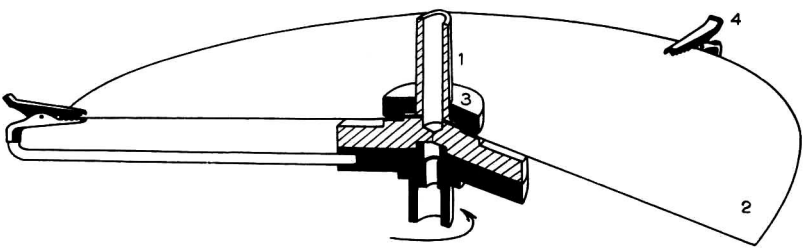
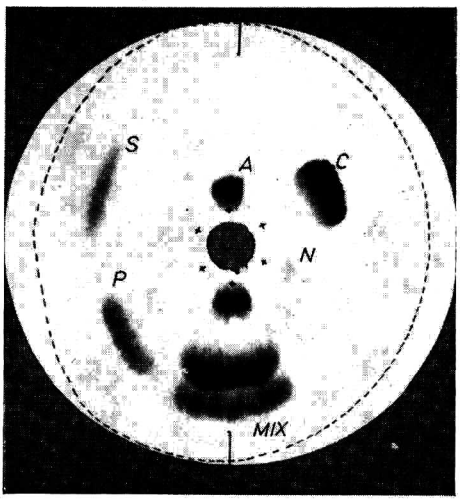


Fig. 3. Arrangement of the apparatus. 1 = distributor; 2 = chromatographic paper; 3 = fastening ring; 4 = crocodile clips.

graph left for 2 min. Then the distributor is filled with new material, chromatographic paper inserted, and the mobile phase pipetted onto the bottom of the chromatographic chamber. The chromatograph is then run for another 2 min to permit saturation of the chamber. Finally the mobile phase is pipetted into the distributor to be developed as usual.

The properties of this distributor were tested by chromatography of standard Schleicher & Schüll dyes using 2 % sodium citrate in 2.5 % aqueous ammonia. Fig. 4 shows the differences between the results of separation on using different types of chromatographic paper. The separation is generally faster than with the central spot distributor described by PAVLÍČEK *et al.*[5] but slower than with MCDONALD'S apparatus. The quality of separation, however, was found to be just the reverse. With the above described distributor it is approximately the same or a little worse



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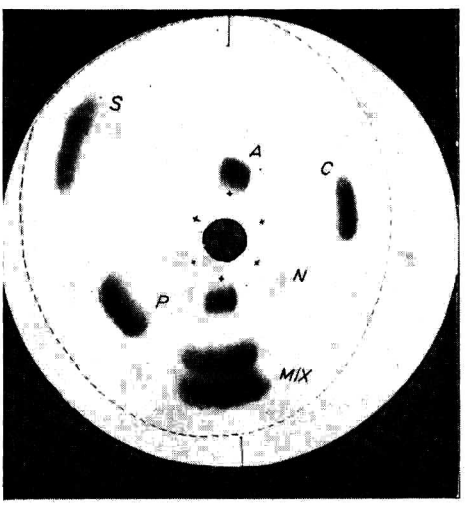

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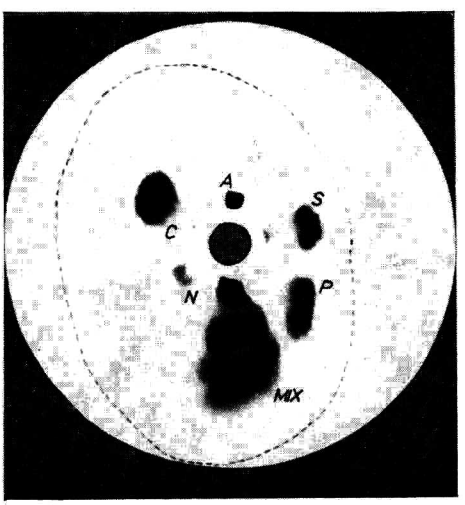

C

Fig. 4. Comparison of different chromatographic papers, using standard dyes of Schleicher & Schüll for testing chromatographic papers. A = Whatman No. 1; B = Whatman No. 3; C = Ederol 225.

than with the central-ball distributor, but much better than with McDONALD's excentric distributor.

For a comparison of the separation time for different types of chromatographic papers see Table I.

TABLE I

DEVELOPING TIME FOR VARIOUS KINDS OF CHROMATOGRAPHIC PAPERS

750 r.p.m., 2 % sodium citrate in 2.5 % aqueous ammonia, diameter of the chromatogram 25 cm

Kind of paper	*Developing time (min)*	*Quality of separation*
Whatman No. 1	20	good
Whatman No. 2	18	good
Whatman No. 3	15	good
Whatman No. 4	12	good
Ederol 202	18	good
Ederol 225	10	sufficient
Schleicher & Schüll 2043 b	20	excellent

ACKNOWLEDGEMENT

Thanks are due to Messrs. Schleicher & Schüll for kindly providing some samples of standard dyes.

SUMMARY

In this paper a simple distributor of the mobile phase for centrifugal paper chromatography is described. This distributor has some advantages over other systems described previously, *viz.* the more rapid and simple manner in which the mobile phase is fed from the reservoir. Since this device enables the mobile phase to be changed rapidly, it can be used for such types of chromatography where this is necessary, as well as for gradient elution paper chromatography.

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UN MESUREUR AUTOMATIQUE DE CHROMATOGRAMMES RADIOACTIFS SUR BANDES DE PAPIER

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INTRODUCTION

Au cours d'une étude sur les séparations de produits de fission et de terres rares radioactives par chromatographie sur bandes de papier, la nécessité d'un enregistrement automatique de l'activité s'est rapidement fait sentir.

Il importait de pouvoir mesurer plusieurs chromatogrammes les uns à la suite des autres, de préférence au cours de la nuit, la journée étant normalement consacrée aux élutions.

Le principe du déroulement des bandes étant un peu différent de ceux habituellement rencontrés dans le commerce, nous avons pensé qu'il pouvait être utile de publier quelques détails sur cette réalisation qui nous a donné satisfaction.

PRINCIPE

La caractéristique principale de notre réalisation réside dans l'emploi, comme mécanisme dérouleur de bandes, du même "bloc moteur-magazin" que celui de l'enregistreur graphique; celui-ci étant, notamment par raison d'économie, du type galvanomètre d'Arsonval (Rectigraphe Chauvin et Arnoux).

Ce dérouleur "bloc magazin" légèrement modifié par adjonction d'un blindage et de contacts de commande et de repérage, entraîne une bande sans fin, en acétate de cellulose, de 90 cm de long aux maximum, ayant la même largeur (15 cm) et les mêmes perforations que le papier d'enregistrement.

En outre, cette bande sans fin porte d'un côté des perforations latérales tous les 10 cm comme repères de longueurs et, de l'autre côté, une perforation unique commandant le déplacement latéral du château de plomb avec son détecteur.

Les chromatogrammes sont tendus sur la bande sans fin à l'aide de ruban adhésif et, suivant les cas, 3 ou 5 pistes sont prévues.

De cette manière nous avons pu, de façon tout à fait routinière, mesurer soit 15 bandes de 20–25 cm de longueur utile et de 1–1.5 cm de large, soit 3 bandes de 50–80 cm de longueur utile et de 3 cm de large.

Le reste du système est classique (Fig. 1); le tube compteur surplombant une "fenêtre" ajustable par bonds (0.5 à 20 mm) est relié à un débimètre (ratemeter)

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possédant de préférence une gamme logarithmique et dont les indications sont enregistrées sur un papier se déroulant à vitesse proportionnelle.

Un des avantages de notre réalisation est précisément qu'il est aisé d'enregistrer à une vitesse sous multiple (1/2, 1/4, 1/8) du déroulement des chromatogrammes et d'obtenir directement un graphique de format commode, par exemple 12.5 cm pour un chromatogramme développé sur 50 cm.

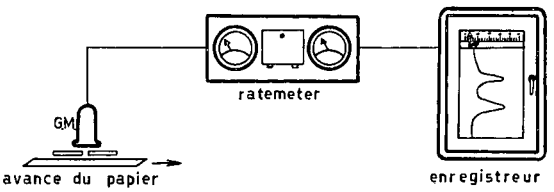

Fig. 1. Principe de l'appareil.

En outre, les repères de position espacés de 10 en 10 cm et l'origine du chromatogramme s'inscrivent automatiquement sous la forme de petits traits verticaux se trouvant un peu sous la ligne du zéro.

La Fig. 2 nous montre la reproduction d'un tel enregistrement. Il s'agit d'une

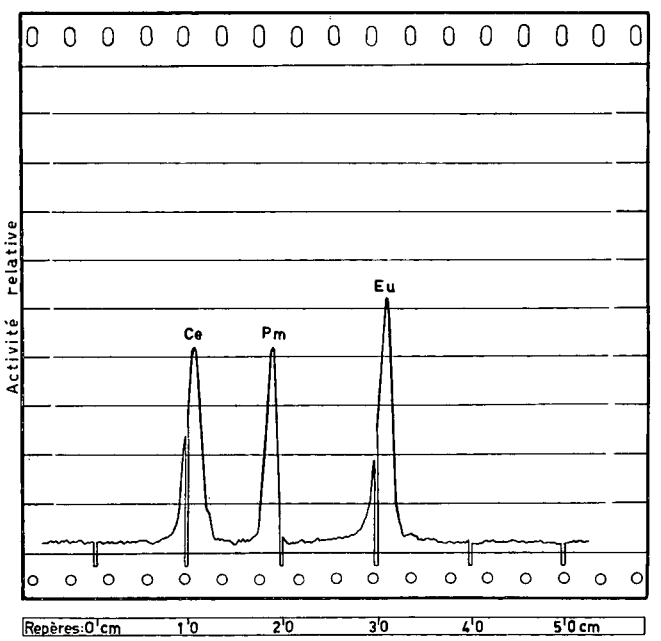

Fig. 2. Enregistrement obtenu sur l'appareil après la mesure d'une bande de 50 cm de longueur.

séparation de terres rares obtenue sur une bande de 50 cm de longueur utile, le défilement étant fait 240 mm à l'heure et l'enregistrement 60 mm à l'heure (réduction au quart).

DESCRIPTION DE L'APPAREIL

La disposition générale des organes est conforme au croquis de la Fig. 3.

Nous examinerons successivement:

A. Le système d'entraînement des chromatogrammes.
B. Le château de plomb mobile.
C. Le panneau général de commandes.
D. Le "ratemeter".
E. L'enregistreur graphique.
F. Le compteur auxiliaire à traction directe.
G. La réserve à chromatogrammes.

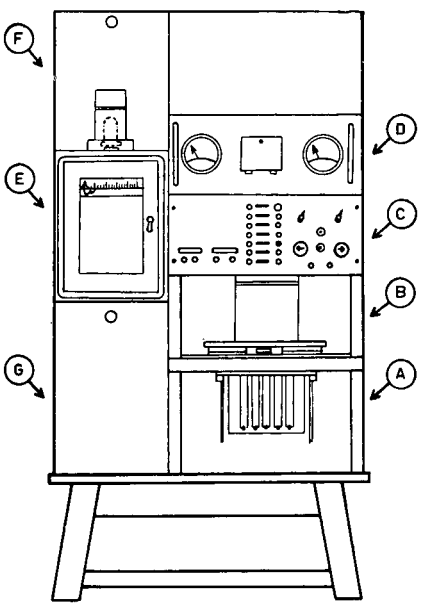

Fig. 3. Disposition générale des organes. A = Mécanisme de déroulement de la bande sans fin. B = Château de plomb mobile avec tube G.M. incorporé. C = Tableau de commande pour l'ensemble de l'appareil. D = Ratemeter linéaire et logarithmique "ECKO". E = Appareil enregistreur "Chauvin & A.". F = Château de plomb auxiliaire pour examen rapide des bandes. G = Réserve de chromatogrammes.

A. Le système d'entraînement des chromatogrammes

Tout comme le bloc "moteur magazin" du "Rectigraphe", celui qui entraîne la bande sans fin isolante est fourni avec 3 paires de roues dentées permettant, dans le modèle "normal" les vitesses 240, 120, 60, 30 et 15 mm à l'heure; celles-ci conviennent parfaitement et le changement d'une paire d'engrenages est simple et rapide.

Cet ensemble aisément amovible (Fig. 4) se suspend horizontalement sous l'ouverture d'une plaque d'acier de 8 mm d'épaisseur qui est solidaire du bâti et supporte le château de plomb à glissières.

A cet effet, la plaque de suspension est pourvue à l'arrière de deux ergots simi-

laires à ceux de l'enregistreur et, à l'avant, de deux pattes agrippant deux autres ergots ajoutés sur les flancs du bloc magazin.

Sur la platine du bloc magazin, une réglette transversale en "plexiglass" surélève la bande transporteuse juste sous la fente de mesure, elle fait office de tendeur pour les chromatogrammes en vue d'assurer une géométrie de comptage reproductible.

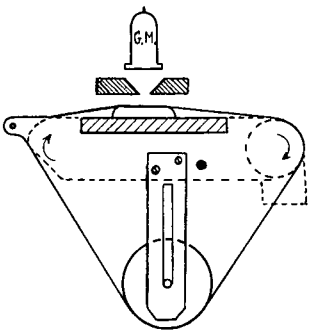

Fig. 4. Coupe du dispositif entraîneur.

Le cas échéant, un rouleau de "plexiglass' de 10 cm de diamètre, suspendu à l'intérieur de la bande accentue encore, par son poids, la tension; il est guidé par deux pattes enfichables sur les flancs du bloc magazin.

La réglette en "plexiglass", également amarrée sur les flancs du "bloc magazin", porte, incrustées de part et d'autre à petite distance des bords, deux lamelles ressort qui, en l'absence de bande transporteuse, sont chacune en contact électrique franc avec une patte d'acier inoxydable maintenue à plat, sur les côtés de la réglette, par une lame en acier à ressort instantanément amovible (Fig. 5).

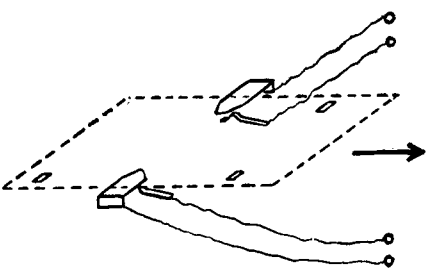

Fig. 5. Réalisation des déplacements et du repérage.

En fonctionnement, les deux côtés de la bandé transporteuse glissent entre les deux parties du contact qui, de ce fait, demeurent ouvertes excepté au passage des perforations.

La bande sans fin dont on prépare à l'avance quelques exemplaires de longueur optimum (65–85 cm) est utilisable un grand nombre de fois; c'est une feuille d'acétate de cellulose de 2/10 mm d'épaisseur, découpée à la largeur et poinçonnée aux cotes du papier enregistreur. La jonction, après un léger amincissement des extrémités, est soigneusement collée à l'aide du minimum de liquide spécial pour film photographique en respectant évidemment l'espace entre les perforations.

Enfin, sous la platine, à l'intérieur du bloc, une plaque de plomb de plus de 1 cm d'épaisseur sert à améliorer le blindage vis-à-vis du rayonnement général des autres chromatogrammes.

B. Le château de plomb mobile (Figs. 6–9)

Le blindage mobile du tube compteur consiste essentiellement en un cylindre de plomb avec parois de 5 cm d'épaisseur, coulé entre deux tubes de fer sertis sur une plaque d'acier (250 × 370 × 18 mm) supportant l'ensemble; il est coiffé d'un couvercle de plomb de même épaisseur.

Au-dessus de la plaque, derrière le château proprement dit, se trouve l'écrou de bronze qui entraîne l'ensemble dans son déplacement latéral par la rotation de la vis mère (diam. 14 mm, pas 1 mm) (Fig. 6).

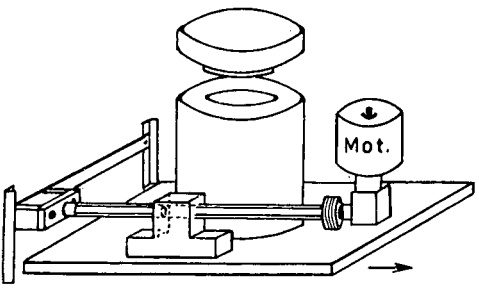

Fig. 6. Mécanisme du château de plomb.

Un côté de celle-ci est solidement amarré au bâti et l'autre est terminé par un accouplement caoutchouté relié directement à la démultiplication du moteur à renversement de marche; celui-ci est fixé au bâti et entraîne la vis à une vitesse de 1 à 2 tours à la seconde.

Sous la plaque d'acier se trouvent les parties femelles des quatre glissières (bronze) et un plateau circulaire en acier de 2 cm d'épaisseur percé en son centre d'un trou de 3 cm de diamètre et, dans le sens avant-arrière d'un tunnel où coulissent les blocs de bronze qui forment la fenêtre ajustable (Fig. 7), un ressort interne situé au fond du tunnel assure le contact permanent entre les deux blocs formant la fenêtre, le tout étant retenu par une goupille traversant la plaque d'acier sur l'avant du château. Nous pouvons ainsi obtenir les largeurs de fenêtres de 0.5, 1, 2, 5, 10 et 20 mm, la longueur étant toujours de 2.5 cm.

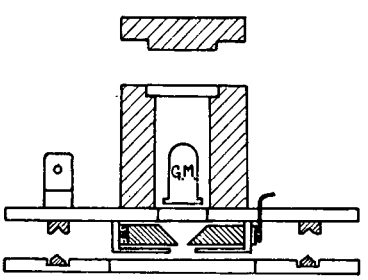

Fig. 7. Coupe dans le château de plomb.

A l'arrière de la plaque mobile se trouvent encore deux réglettes isolantes pourvues l'une de trois, l'autre de cinq encoches, correspondant aux positions d'arrêt du tube compteur au-dessus des différentes pistes (Fig. 8); leurs rôles est d'actionner momentanément les contacts de maintien de l'alimentation du moteur après cessation de l'impulsion de commande du déplacement.

Le tube compteur actuellement utilisé est du type à fenêtre en bout, à vie illimitée (EW3H de 20th Century); il est suspendu de manière réglable par son socket au centre d'un cylindre d'aluminimum assurant sa protection mécanique et son centrage.

Il s'avère cependant que certains isotopes émetteurs K tels ^{153}Gd et ^{169}Yb sont pratiquement indétectables avec le compteur de Geiger; son remplacement par un détecteur à cristal scintillant s'impose.

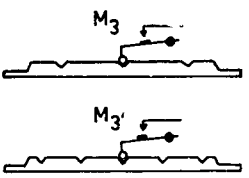

Fig. 8. Réglettes des contacts de positions.

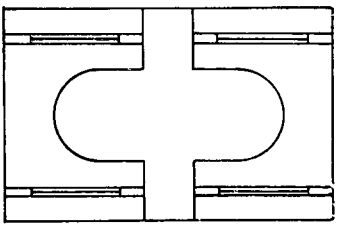

Fig. 9. Plaque support avec glissières.

Enfin, tout cet assemblage peut se déplacer latéralement sur la partie mâle des glissières en fonte, ajustées sur une plaque d'acier solidaire du bâti; celle-ci possède l'ouverture nécessaire à la course du plateau circulaire (Fig. 9).

C. Le panneau général de commandes

Sur le devant du panneau sont réunies les commandes suivantes, le schéma des raccordements étant sur la Fig. 10.

1. Interrupteur général I_1
2. Avance des chromatogrammes I_4
3. Alimentation du "ratemeter" I_2
4. Alimentation de l'enregistreur I_3
5. Index marqueur des repères I_6
6. Alimentation du moteur I_5
7. Commande manuelle du déplacement B_1
8. Sens de marche du château I_7
9. Réglage de la vitesse du déplacement R_1
10. Choix du programme I_8
    - (a) pas de déplacement
    - (b) position centrale ou extrémités
    - (c) 3 pistes espacées de 4 cm
    - (d) 5 pistes espacées de 2.5 cm

Les particularités du fonctionnement électrique sont détaillées ci-dessous:

(a) Les positions de repos correspondant aux différentes pistes ou aux fins de course sont obtenues par l'ouverture des contacts de maintien M_3 (3 pistes) et M_3'

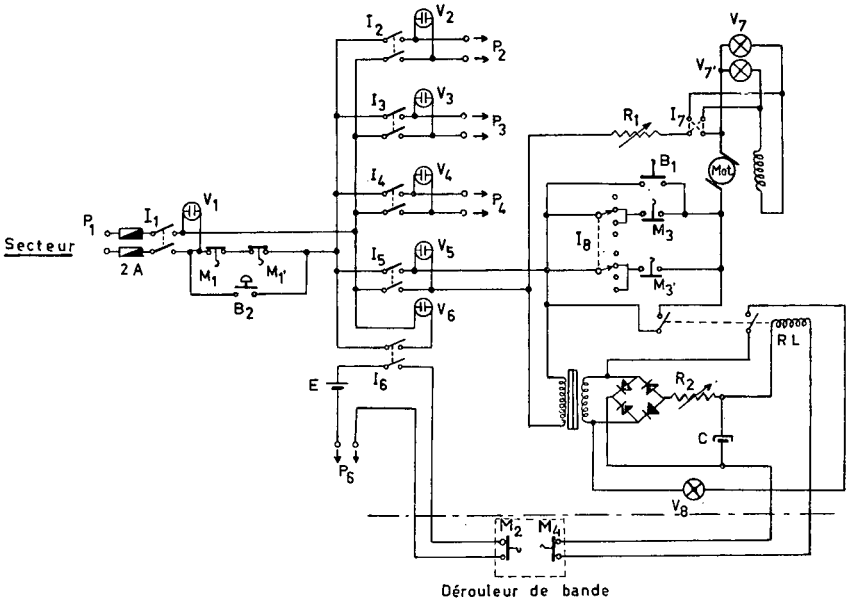

Fig. 10. Alimentation générale et déplacement du château de plomb.

$I_1.P_1.V_1$	Alimentation générale
$I_2.P_2.V_2$	Alimentation "ratemeter"
$I_3.P_3.V_3$	Alimentation enregistreur
$I_4.P_4.V_4$	Avance de la bande
$I_5.V_5$	Déplacement du château de Pb
$I_6.P_6.V_6$	Marqueur vers entrée enregistreur
$I_7.V_7.V_7'$	Sens de déplacement du château
I_8	Choix du programme (0, 1, 3, 5 pistes)
$M_1.M_1'$	Micro-switch de fin de programme
$M_2.E$	Contact des repères—pile sèche
$M_3.M_3'$	Micro-switch de positionnement du château
M_4V_8	Contact de changement de piste-voyant
Mot.	Moteur de déplacement du château pour changer de piste
R_1	Ajustage de la vitesse du moteur-voyant
$R_2.C$	Ajustage de la durée d'enclenchement du relais
B_1	Changement manuel de piste
B_2	Réarmement du programme
RL	Relais de changement automatique de piste

(5 pistes) par l'intermédiaire des encoches prévues dans les deux réglettes isolantes solidaires du chariot.

Lorsque les parties plates des réglettes poussent sur les contacts M_3 ou M_3' (Fig. 8) ceux-ci sont maintenus fermés et le déplacement se poursuit jusqu'au cran adjacent.

(b) Le changement de piste s'amorce par le pontage momentané du contact M_3 ou M_3' durant 2 à 3 sec, soit à la main par un bouton poussoir, soit automatiquement par la fermeture du relai (RL); à chaque tour de piste celui-ci se ferme une seule fois.

Dès que le déplacement a fermé M_3 ou M_3' le déplacement se poursuit jusqu'à l'encoche adjacente.

(c) Comme le déroulement des bandes chromatographiques est relativement lent (15 à 240 mm/h), on ne peut obtenir directement la durée adéquate (2–3 sec) de fermeture du contact d'amorçage M_4 par le simple fait du passage de la perforation de la bande tractrice entre ses pôles.

Afin que le relai (6 V) n'enclenche qu'une seule fois par tour de piste, le temps

juste nécessaire à l'amorçage du déplacement, malgré la persistance beaucoup plus longue de la fermeture de M_4, nous avons eu recours à l'artifice suivant:

Un condensateur C (2000 μF, 15 V) est chargé, en permanence, à travers une résistance variable R_2, ajustée à une valeur telle que, en régime d'équilibre, le courant passant dans la bobine du relai soit insuffisant pour le maintenir fermé. Comme en fonctionnement, le contact M_4 reste normalement ouvert, isolé par la bande tractrice, le condensateur demeure chargé à la tension de pointe fournie par le redresseur (12 V environ).

Lors du passage de la perforation, le contact M_4 est brusquement établi, le relai branché directement sur le condensateur chargé ferme énergiquement et se maintient ainsi quelques secondes seulement en vidant la charge du condensateur qui lui ne peut recharger à la tension de pointe tant que le contact M_4 reste fermé.

(d) Les contacts M_1 et $M_{1'}$ sont des "micro-switchs" de fin de course; en fin de programme, ils coupent l'alimentation générale.

D. Le "ratemeter"

Nous utilisons le "Ratemeter" N522 A de EKCO; son courant de sortie, relativement important (5 mA pour la déviation totale), permet en effet l'utilisation d'un enregistreur galvanométrique de prix très raisonnable.

Ses gammes de mesure vont de 3 à 10,000 coups par seconde pour la déviation totale avec 0.2, 1, 5, 20 ou 80 sec de constante de temps; ce choix est fixé d'une part par la vitesse de déroulement et la largeur de la fente et, d'autre part, par l'activité mesurée.

Récemment, nous avons complété cette installation avec une gamme logarithmique à 3–4 modules (0.1 à 1000 ou 1 à 10,000 à la sec) dont le principe est basé sur la caractéristique quasi-logarithmique de la tension (0.1 à 0.4 V) aux bornes d'une diode à jonction au silicium, en fonction du courant direct qui la traverse.

E. L'enregistreur graphique

Nous utilisons le "Rectigraphe" (Chauvin et Arnoux) (sensibilité: 5 mA pour la déviation totale); c'est essentiellement un galvanomètre à cadre mobile dont le tracé est rendu rectiligne par un dispositif mécanique particulier de l'articulation du bras de l'aiguille. L'élongation maximum est de 13 cm (Fig. 2).

Un micromoteur synchrone entraîne le papier enregistreur à vitesse constante; le modèle normal utilisé possède trois jeux d'engrenages aisément amovibles donnant les vitesses suivantes: 15, 30, 60 et 120 mm/h.

Il est à remarquer que nos chromatogrammes étant actuellement développés sur 50 cm et plus, nous enregistrons toujours à une vitesse sous-multiple du déroulement, par exemple, déroulement à 240 mm/h et enregistrement à 60 mm/h avec constante de temps de 20 sec ou, si les activités sont trop voisines du fond continu, déroulement à 60 mm/h et enregistrement à 15 mm/h avec constante de temps de 80 sec.

De cette manière, nous avons directement un enregistrement de format commode et suggestif (15 × 20 cm).

On notera sur la Fig. 2 que la trace est interrompue périodiquement par des retours brusques de la plume sous la ligne du zéro à chaque passage d'une perforation repère de la bande entraîneuse. L'enregistrement en est fort peu affecté et les mesures de R_F sont ainsi rendues aisées et précises.

Le dépassement inverse du zéro est nécessaire pour distinguer nettement le marqueur sur un faible fond continu; on l'obtient sans peine en insérant, avec la polarité convenable, une pile de 1.5 V en série dans le circuit cadre mobile-contact marqueur-enregistreur (E, Fig. 10).

F. Le compteur auxiliaire à traction directe

Dans les cas où l'on désire enregistrer l'activité d'une seule bande dont la longueur ne dépasse pas 20 cm, nous avons prévu d'utiliser directement la traction du papier enregistreur pour déplacer le chromatogramme.

Ce dernier est fixé, à l'aide de ruban adhésif, sur une réglette de verre qui coulisse horizontalement sous un autre tube compteur monté au-dessus et un peu en retrait de l'enregistreur proprement dit.

L'extrémité avant de la réglette de verre est munie d'un ruban de toile dont l'autre bout, passant par une ouverture pratiquée dans le dessus du caisson du rectigraphe juste au-dessus du papier enregistreur, est fixé au milieu de ce dernier au moyen de ruban adhésif. Un petit rouleau facilite le glissement du ruban de toile au coude et un "microswitch" arrête le déroulement à l'arrivée de la latte de verre dans l'étrier de fin de course.

G. La réserve à chromatogrammes

Une douzaine de plaques de verre (40 × 25 cm) glissant dans des rainures pratiquées dans des flancs en bois servent à stocker momentanément un grand nombre de bandelettes nécessitant éventuellement d'autres examens (décroissance, absorption, etc.).

REMERCIEMENT

L'auteur tient à remercier l'Institut des Sciences Nucléaires pour les crédits qui lui ont permis cette réalisation ainsi que Messieurs E. JEUNEHOMME et R. CONTRARDY, qui l'ont aidé efficacement dans la réalisation mécanique et le câblage.

RÉSUMÉ

Dans l'appareil décrit, un bloc "moteur-magazin" identique à celui de l'enregistreur graphique, est utilisé pour obtenir le défilement successif des chromatogrammes sous le tube compteur; l'enregistrement est d'un format réduit commode.

Une bande sans fin, en acétate de cellulose, porte jusqu'à 15 bandes de papier de 20–25 cm utile, réparties sur 5 pistes espacées de 2.5 cm ou 3 bandes de 50–80 cm utile, réparties sur 3 pistes espacées de 4 cm.

Après exploration d'une piste, le tube compteur et son château de plomb se déplacent latéralement jusqu'à la suivante.

Le reste de l'installation est classique; il comporte un "ratemeter" suivi d'un enregistreur à plume rectilinéaire de prix modique, du type à cadre mobile.

SUMMARY

An apparatus is described in which a unit identical to that of the graphic recorder is used to move the chromatograms successively under the counter tube; the record is of a conveniently reduced size.

An endless strip made of cellulose acetate carries up to 15 paper bands of 20–25 cm effective length, arranged on 5 tracks at a distance of 2.5 cm from each other, or 3 bands of 50–80 cm effective length, arranged on 3 tracks spaced at 4 cm.

After scanning one track, the counter tube with its lead casing moves sideways to the next track.

The rest of the set-up is classical; it consists of a ratemeter connected to a not too expensive rectilinear pen recorder of the moving coil type.

CONTINUOUS ELECTROPHORETIC SEPARATIONS OF RADIOACTIVE RARE EARTH MIXTURES

III. SURVEY OF EXPERIMENTS IN 0.05 *M* LACTIC ACID

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INTRODUCTION

In our previous reports[1,2] some continuous electrophoretic separations of three-component and five-component rare earth mixtures in 0.05 *M* lactic acid were described. These results gave us some indications that in the series of the rare earths there might be a relationship between the cathodic mobilities of the rare earths and their atomic numbers, or their ionic radii respectively. The experiments reported here confirm this presumption and at the same time give complete information about the separability in 0.05 *M* lactic acid of mixtures of rare earths with atomic numbers from 57 to 71.

EXPERIMENTAL AND RESULTS

The experiments here described were carried out in successive runs without changing the filter paper curtain, and without switching off the electric current. The first solution, containing a particular rare earth, was pumped onto the starting point of the curtain during 10 min. Electrophoresis was then continued for a period of 2 h without applying the rare earth solution to the curtain. At the end of this period a set of collecting glasses was inserted, and the next rare earth solution was pumped onto the starting point of the curtain for 10 min. Then during a period of 2 h the first rare earth applied was collected and its distribution in the collecting glasses was estimated by an appropriate method. Afterwards the solution of the third rare earth was applied to the curtain during 10 min, and at the same time the previously applied rare earth was collected during 2 h in a new set of glasses, and so on.

In this way all the rare earths were applied to the curtain in successive runs at intervals of 2 h, each particular rare earth solution being pumped separately onto the starting point of the curtain during a period of 10 min, and the distribution of each of the rare earths was estimated in the corresponding set of collecting glasses. The apparatus[3,5] and general experimental conditions[1] have been described previously.

The diagram shown in Fig. 1 represents the distribution curves of the rare earths at the outlet of the filter paper curtain as estimated by G.M. counting of the effluent in the collecting glasses. In accordance with the experimental procedure, the activities correspond to a 10 min continuous run. Because of the short half lives of Pr, Sm, Dy and Ho, these rare earths were not applied in a radioactive form, and so only

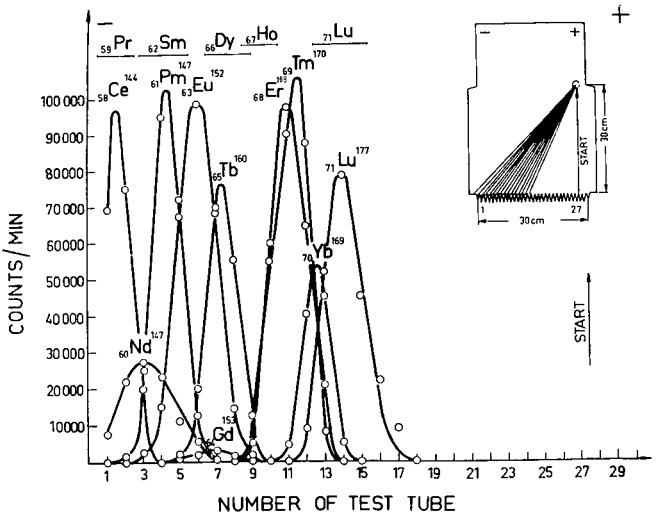

Fig. 1. Distribution curves of the rare earths at the outlet of the filter paper curtain as estimated by G.M. counting of the contents of the collecting glasses. For inactive rare earths only the width of the bands is indicated in the diagram. The activities correspond to a continuous run of 10 min. The dimensions of the filter paper curtain and the position of the starting point is sketched. Electrolyte, 0.05 *M* lactic acid; paper, Munktell No. 20/250; voltage drop, 300 V; mean electric field strength, 10 V/cm; electric current, 20 mA; pumping rate, 0.2 ml/h; time in which each sample is pumped on the curtain, 10 min.

the width of their bands in the collecting glasses could be estimated. In this case it was supposed that the peaks of their distribution curves were in the middle of the respective bands.

Fig. 2 represents a diagram in which the atomic numbers of the rare earths are plotted *vs.* the appearance of the peaks of the distribution curves of the rare earths

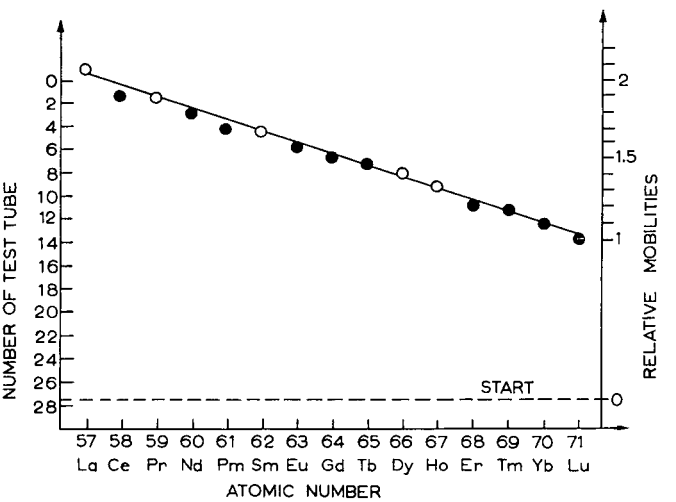

Fig. 2. Plot of atomic numbers of rare earths *vs.* their relative electrophoretic mobilities in 0.05 *M* lactic acid. ● = radioactive rare earths; ○ = inactive rare earths. The positions of the peaks of the distribution curves at the outlet of the apparatus were taken from Fig. 1.

at the outlet of the apparatus. The position of the peaks was estimated from the experimental data given in Fig. 1. At the same time the atomic numbers of the rare earths were plotted against the cathodic electrophoretic mobilities relative to that of lutetium. These relationships give a straight line in the diagram.

Using the data for the ionic radii of the rare earths reported by SEABORG[6], which give an approximately linear relationship between the atomic numbers of these elements and their ionic radii, the diagram in Fig. 3 was constructed. Like the

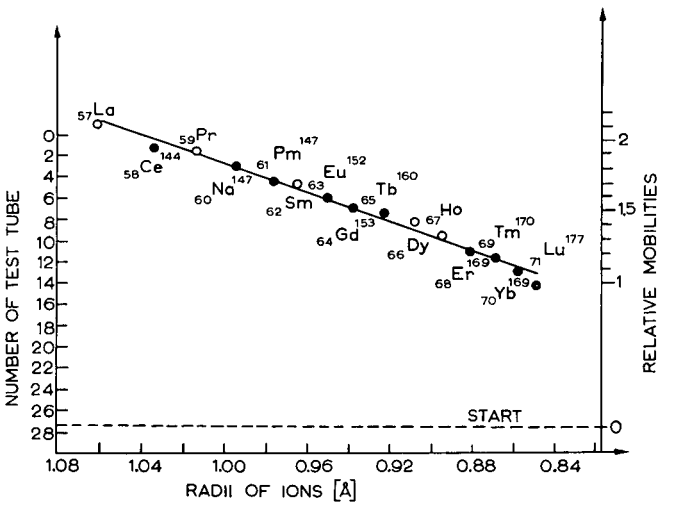

Fig. 3. Plot of ionic radii of rare earths *vs.* their relative electrophoretic mobilities in 0.05 *M* lactic acid. The values of the ionic radii were taken from SEABORG[6]. ● = radioactive rare earths; ○ = inactive rare earths.

previous diagram, this figure shows that a straight line relationship exists between the radii of ions of rare earths and their cathodic electrophoretic mobilities. From the experimental results presented here, it is obvious that the cathodic electrophoretic mobilities of the rare earths in 0.05 *M* lactic acid are directly proportional to their ionic radii, respectively indirectly proportional to their atomic numbers.

DISCUSSION

In 0.05 *M* lactic acid it was not possible to separate rare earth mixtures containing more than five components, even with an apparatus that was considerably enlarged in the horizontal direction[2]. Nevertheless, in order to obtain relative mobilities by the continuous electrophoretic method for as many as 15 rare earths in successive experiments, it was necessary to maintain the experimental conditions as constant as possible from experiment to experiment.

With a given voltage drop at the electrodes it is essential that the electric current remain constant from experiment to experiment, because this seems to be the most convenient indication of the constancy of the temperature in the filter paper curtain and of evaporation and consequently of the constancy of the vertical speed of the background electrolyte, provided the filter paper curtain is not changed during the whole set of experiments. The variation of the vertical speed of flow of the back-

ground electrolyte from sheet to sheet of filter paper, even if these were of the same quality and of the same batch, appeared in our experiments to be too considerable to give comparable results in successive experiments.

We tried to ascertain relative electrophoretic mobilities of rare earths using continuous electrophoresis in a moist chamber with a membrane blocking the continuously rinsed vertical electrodes, despite the fact that the paths travelled by the substances in this technique show smaller or greater curvatures in the sense of an apparent acceleration depending on experimental conditions[4,5]. Because of the relatively great electrophoretic mobilities of the rare earths in 0.05 *M* lactic acid, the small conductivity of the background electrolyte, the relatively small electric field strength used, and a relatively great vertical speed of flow of the background electrolyte, the paths travelled were very close to straight lines[1,2]. In this particular case the relative distances travelled in the horizontal direction are directly proportional to the relative electrophoretic mobilities.

In our experiments the use of radioactive rare earths proved very useful because it enabled us to work with very dilute solutions, which give minimum distortion of the electric field strength in the area of the travelling spot and allowed relatively accurate estimations of the peaks of the distribution curves determined in the collecting glasses. In a few cases, where radioactive rare earths were unsuitable because of their short half lives, the width of the bands in the collecting glasses could only be estimated; in these cases it was assumed that the peaks of the distribution curves were in the middle of the respective bands.

Although the experiments described here were actually carried out discontinuously in successive runs in intervals of 2 h, every rare earth solution being pumped separately onto the starting point of the curtain during 10 min, the results correspond to continuous runs. The angles of inclination of the paths travelled are, according to the theory[3,5], only a function of the mobilities, if the electric field strength and the vertical speed of the background electrolyte are constant. The quantity of the material which drips out of the apparatus corresponds in this particular case to only a 10 min continuous run, but the time between input and output of the substance depends on the vertical speed of the background electrolyte and on the sorbability of the curtain for the particular substance under given experimental conditions.

Our results show that in the direction of increasing atomic number, or decreasing ionic radius respectively, the cathodic mobility decreases linearly. This decrease of the cathodic mobility indicates that the capacity of the rare earths to form complexes with lactic acid increases.

This result seems to be in accordance with various reports in the literature concerning other complexes and different techniques.

LEDERER[7] reported on one-dimensional electrochromatographic experiments with all the rare earths using 1% citric acid as electrolyte. He obtained the same sequence of rare earths but no linearity. SHVEDOV AND STEPANOV described some continuous electrophoretic separations of mixtures of some rare earths that appear among fission products. From their results it is obvious that the sequences, so far reported, both in citric acid[8] and in trilon B[9,10] are in accordance with our results. The same refers to ion exchange using as eluent lactic acid or lactates[11–14], citrate[11] or EDTA[11] solutions, and to extraction of nitrates with tributyl phosphate[15], or extraction of acetylacetonates with acetylacetone[16].

SUMMARY

Using continuous electrophoresis the relative mobilities of rare earths in 0.05 *M* lactic acid were estimated. Within the series of rare earths with atomic numbers ranging from 57 to 71 a relationship was found between the cathodic mobilities of these rare earths and their atomic numbers, or their ionic radii respectively. This relationship is presented in diagrams which give straight lines. In the direction of increasing atomic number, or decreasing ionic radius respectively, the cathodic mobility decreases, indicating that the capacity of these rare earths to form complexes with lactic acid increases. At the same time information about the continuous electrophoretic separability of rare earth mixtures in 0.05 *M* lactic acid for preparative purposes is given. Various problems concerning the determination of relative mobilities using the continuous electrophoretic method are discussed in detail.

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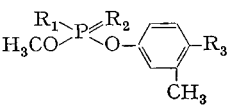
Notes

Papierchromatographische Trennung aromatischer Phosphorsäureester-Insektizide

Das Insektizid LEBAYCID® enthält als Wirkstoff O,O-Dimethyl-O-(3-methyl-4-methylmercaptophenyl)-thiophosphat = Mercaptophos (I) (siehe Tabelle I). Nach Applikation an Pflanzen ist eine Oxydation des Esters durch Einwirkung von Licht, Luftsauerstoff und pflanzeneigenen Enzymen zu den Verbindungen II–VI möglich. Darüberhinaus ist eine Isomerisierung von I zur S-Methylverbindung VII denkbar, die wiederum zu Sulfoxyd (VIII) und Sulfon (IX) oxydiert werden kann.

TABELLE I



Verbindung	R_1	R_2	R_3
I	$-OCH_3$	$=S$	$-SCH_3$
II	$-OCH_3$	$=S$	$-SO\cdot CH_3$
III	$-OCH_3$	$=S$	$-SO_2\cdot CH_3$
IV	$-OCH_3$	$=O$	$-SCH_3$
V	$-OCH_3$	$=O$	$-SO\cdot CH_3$
VI	$-OCH_3$	$=O$	$-SO_2\cdot CH_3$
VII	$-SCH_3$	$=O$	$-SCH_3$
VIII	$-SCH_3$	$=O$	$-SO\cdot CH_3$
IX	$-SCH_3$	$=O$	$-SO_2\cdot CH_3$

Eine vollständige papierchromatographische Auftrennung eines Gemisches der Verbindungen I–IX, die sich in ihren physikalischen Eigenschaften nur wenig unterscheiden, gelingt durch Kombination zweier verschiedener Systeme.

Papierchromatographische Auftrennung

System 1. In diesem System verwendeten wir die acetylierten "Ederol"-Papiere* No. 202/11.5/100, 202/12.8/100 und 202/14.8/100.

In der Literatur werden mehrere Bezeichnungsweisen für den Acetylierungsgrad verwendet. Wir folgen hier den Ausführungen der Herstellerfirma der "Ederol"-Papiere[1]:

"Gebräuchlich ist die Angabe in Prozent Acetylgruppe; ebenso findet man auch die Angabe in Prozent Essigsäure. Häufig wird der vorliegende Acetylgehalt auf den

* Hersteller: J. C. Binzer, Hatzfeld/Eder, Deutschland.

maximal erreichbaren (= 61.7 %) bezogen. Die Angabe erfolgt dann in Prozent der möglichen Acetylierung. Berücksichtigt man, dass für den Gebrauch in der Praxis nicht so sehr der Grad der Acetylierung als vielmehr das erreichte Ausmass der Hydrophobierung interessiert, so erscheint es am besten, den Acetylierungsgrad in Prozent der maximal möglichen Aufnahme von drei Acetylgruppen pro Zuckerbaustein (= 61.7 % Acetyl bzw. 62.5 % Essigsäure oder 44.8 % CH_3CO–) auszudrücken."

Die Angabe 202/11.5/100 bedeutet, dass es sich um die Papiersorte 202 mit 11.5 % des möglichen Acetylgehaltes handelt. Da, wie in Tabelle III dargestellt, der Acetylierungsgrad in unseren Versuchen für die Auftrennung der verschiedenen Metaboliten von entscheidender Bedeutung ist, geben wir in Tabelle II eine Gegenüberstellung der verschiedenen Bezeichnungsweisen für die drei von uns benutzten Papiersorten.

TABELLE II

GEGENÜBERSTELLUNG VERSCHIEDENER MÖGLICHKEITEN ZUR ANGABE DES "ACETYLIERUNGSGRADES"

Acetylierungsgrad	*Entsprechende Angabe in*		
	% Acetyl	*% Essigsäure*	*% CH_3CO– = Gew. zunahme*
11.5/100	7.1	7.2	5.15
12.8/100	7.9	8.0	5.7
14.8/100	9.1	9.25	6.6

Wir arbeiteten in aufsteigender Technik mit der Fliessmittelkombination Aceton–Acetonitril–Wasser (1:1:3). Die Substanzen I, II, III, IV, VII und IX werden in diesem System 1 aufgetrennt. Die Verbindungen V, VI und VIII hingegen werden nicht mehr getrennt, sondern bilden gemeinsam einen Fleck.

System 2. Das von uns entwickelte System 1 wird in idealer Weise ergänzt durch Chromatographie an propylenglykolimprägniertem Papier (Schleicher & Schüll 2043 b) mit Toluol–*n*-Hexan (7:3) als Fliessmittel. Dieses System wurde von BENJAMINI *et al.*[2]

TABELLE III

R_F-WERTE DES "LEBAYCID"-WIRKSTOFFS MERCAPTOPHOS UND SEINER UMWANDLUNGSPRODUKTE

Verbindung	*System 1 Acetylierungsgrad des Papiers*			*System 2*	*Farbe der Flecke nach Besprühen mit $PdCl_2$-Lösung*
	11.5/100	*12.8/100*	*14.8/100*		
I	0.20	0.07	0.04	0.92	ocker
III	0.39	0.22	0.15	0.88	braun
VII	0.49	0.33	0.27	0.82	gelb
IV	0.59	—	—	0.75	hellgelb
II	0.66	0.54	0.49	0.60	braun
IX	0.74	—	—	0.37	goldgelb
VI	0.86	0.78	0.79	0.24	—
VIII				0.15	goldgelb
V				0.07	—

zur Trennung von Derivaten des Insektizids O,O-Diäthyl-O-*p*-methylsulfinylphenylthionophosphat angewendet. Die R_F-Werte der einzelnen von uns untersuchten Substanzen sind ebenfalls in Tabelle III wiedergegeben.

Die Substanzen V, VI, VIII und IX werden mit System 2 sehr gut aufgetrennt. Demgegenüber gelingt eine Trennung der Substanzen I, III und VII nicht mehr, da die Fleckengrösse mit steigenden R_F-Werten zunimmt und die Unterschiede der R_F-Werte relativ gering sind.

In beiden Systemen beträgt die Laufzeit bei Zimmertemperatur und einer Steighöhe der Lösungsmittelfront von 30–35 cm etwa 15 Stunden.

Soll eine quantitative Analyse aller Komponenten eines Gemisches vorgenommen werden, so bestimmt man nach Chromatographie im System 1 die Verbindungen I, II, III, IV, VII und IX sowie die Summe der Substanzen V, VI und VIII. Auf einem zweiten Chromatogramm im System 2 braucht man dann nur noch das Verhältnis der Verbindungen V, VI und VIII untereinander zu bestimmen.

Steht nur eine sehr geringe Substanzmenge zur Verfügung, so kann man nach Chromatographie im System 1 die Verbindungen V, VI und VIII mit Chloroform oder Aceton eluieren und anschliessend im System 2 chromatographieren.

Sichtbarmachen der Flecke

Die Verbindungen I–IV und VII–IX geben gelbe bis braune Flecke nach Besprühen mit einer Lösung von 0.5 g $PdCl_2$ und 2 ml konz. HCl in 100 ml Wasser. 10–20 μg der Verbindungen können auf diese Weise noch gut sichtbar gemacht werden. Die Farben der einzelnen Flecke sind in Tabelle III wiedergegeben.

Es ist bisher nicht gelungen, die Substanzen V und VI nach Chromatographie im System 2 unmittelbar auf dem Papier anzufärben. Ein Nachweis ist jedoch möglich durch Ausschneiden der den Verbindungen entsprechenden Zonen aus dem Chromatogramm, Nassveraschung und Mikrophosphorbestimmung[3]. Diese Methode bietet zugleich die Möglichkeit einer quantitativen Analyse.

Über die Applikation von ^{32}P-markiertem LEBAYCID an Pflanzen, die quantitative Analyse des Metabolitengemisches nach Auftrennung in den beschriebenen Systemen und die daraus sich ergebenden Folgerungen auf den Oxydationsmechanismus wird an anderer Stelle ausführlich berichtet werden[4].

Das System 1 eignet sich auch zur Auftrennung anderer Organophosphorsäureester ähnlicher Struktur. Z.B. lassen sich Parathion und Paraoxon damit vorzüglich trennen (R_F 0.26 bezw. R_F 0.69 bei Acetylierungsgrad 10.3/100).

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The behaviour of inorganic anions on papers loaded with anion-exchange resins

In a previous paper we have recorded the R_F values of numerous inorganic anions on Whatman weak anion exchange resin paper using N KCl and N KNO_3 as developing solvents[1]. Since many separations are possible we decided to extend this work to other papers loaded with anion-exchange resins which had become commercially

TABLE I

R_F VALUES OF INORGANIC ANIONS ON SEVERAL PAPERS LOADED WITH ANION-EXCHANGE RESINS USING N KNO_3 AS SOLVENT

Anion	*Whatman weak anion-exchange resin paper*	*Amberlite WB-2 paper*	*Amberlite SB-2 paper*
Chloride	0.57	0.52	0.66
Bromide	0.35	0.43	0.43
Iodide	0.10	0.24	0.12
Thiocyanate	0.07	0.16	0.10
Chlorate	0.48	0.45	0.49
Bromate	0.74	0.49	0.75
Iodate	0.95	0.33	0.88
Periodate	0.06	0	0.06
Nitrite	0.49	0.41	0.63
Selenite	0.90	0.39	0.89
Selenate	0.82–1	0.36	0.90
Tellurite	0.03–0.40	0	0.03–0.27
Thiosulphate	0.75	0.10	0.83
Chromate	0.18–0.57	0	0–0.17
Molybdate	0.63	0	0.18
Vanadate	0.08	0	0.05
Arsenite	0.84	0.79	0.79
Arsenate	0.89–1	0.48	0.88
Orthophosphate	0.90–1	0.42	0.92
Ferrocyanide	0.50	0	0.43
Ferricyanide	0	0	0

available in the meantime, namely the Amberlite resin papers WB-2 and SB-2 containing about 45 % of Amberlite IRC-50 and Amberlite IRA-400 respectively.

The Amberlite WB-2 paper was first neutralised with HCl, washed and then converted to the nitrate form. All papers were converted to the nitrate form by immersing the papers in 2 N KNO_3 for 30 min, washing with distilled water, repeating this process and drying at room temperature.

The solutions of the anions to be chromatographed were approximately 1.5 % to 2 % solutions of the alkali or ammonium salts in distilled water.

As shown in Table I there is little difference between the ion-exchange papers. However, the Amberlite WB-2 paper had a greater tendency to decomposition than the other two papers and yielded dark coloured spots with chromate, vanadate and molybdate.

Table II shows the R_F values with 0.1, 0.5 and 1 N KNO_3 on Amberlite SB-2 paper (strong base) and on ordinary cellulose paper. The latter was used to confirm that adsorption on cellulose is small for most ions. As was shown by KERTES AND

TABLE II

R_F VALUES OF INORGANIC ANIONS ON AMBERLITE SB-2 PAPER WITH 0.1 N, 0.5 N AND N KNO_3 SOLVENT

Anion	*0.1 N KNO_3*	*0.5 N KNO_3*	*1.0 N KNO_3*	*R_F values with N KNO_3 on cellulose paper (Whatman No. 1)*
Chloride	0.22	0.51	0.66	1
Bromide	0.10	0.33	0.43	1
Iodide	0.03	0.07	0.12	0.84
Thiocyanate	0.02	0.06	0.10	0.81
Chlorate	0.12	0.34	0.49	1
Bromate	0.28	0.60	0.75	1
Iodate	0.60	0.87	0.88	1
Periodate	0	0.05	0.07	1
Nitrite	0.21	0.49	0.63	1
Selenite	0.64	0.86	0.89	0.86
Selenate	0.19	0.80	0.90	1
Tellurite	0–0.25	0–0.25	0.03–0.27	0.34–0.86
Thiosulphate	0.08	0.43	0.83	1
Chromate	0.03	0.06	0–0.17	1
Molybdate	0–0.11	0–0.22	0.18	0–0.72–1
Vanadate	0	0	0.05	0.4–1
Arsenite	0.75	0.78	0.79	0.80
Arsenate	0.61	0.84	0.88	0.91
Orthophosphate	0.66	0.88	0.92	1
Ferrocyanide	0	0.03	0.43	1
Ferricyanide	0	0	0	1

LEDERER[2] the change of R_F value with the change of the normality of the eluting ion should obey an equation of the type:

$$n \log[NO_3] = \log\left(\frac{1}{R_F} - 1\right) + \text{const.}$$

providing that the anion-exchange equilibrium obeys the law of mass action.

When the results of Table II are plotted graphically (R_M [$= \log (1/R_F - 1)$] values *versus* the logarithm of the nitrate concentration) straight lines are obtained; however, the tangent of the line, which should indicate the valency of the anion, is practically the same for monovalent anions such as Cl^-, Br^-, I^-, SCN^-, ClO_3^-, BrO_3^-, IO_4^- and NO_2^- and for phosphate, arsenate, and arsenite, the values for the tangent being between 0.8 and 1.1. A lower tangent (0.57) for chromate and a higher one (1.76) for thiosulphate seems to indicate that in such solutions the charge on an anion cannot be determined from anion-exchange equilibria.

Thanks are due to Miss E. FARULLA for technical assistance.

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A note on preparative scale gas chromatography

The obvious way to attain preparative scale separation by gas chromatography is to increase the sample size and the column diameter. This results, however, invariably in a decrease of the resolving power of the column. A number of reports on this all important resolution factor suggesting ways of improvement have appeared in the literature. In this journal for example FRISONE[1] claims that a series of column restrictions with diminishing opening has a beneficial effect. Although we experimented with a large number of possible forms and combinations of restricting devices, such as those depicted schematically in Fig. 1, we could not confirm this statement.

Fig. 1. Restricting devices. (a) Gradually diminishing openings spaced 25 cm apart; also tested with identical openings of 10 mm spaced 7 cm apart; (b) conical and (c) and (d) semiconical shapes; openings of 10 mm, tried under several spacing conditions.

SICILIO AND KNIGHT[2] claim to have obtained an improvement in the results for methane, ethane and propane by placing a short piece of tubing with a larger bore (3/8 in.) at the beginning of the column (2/8 in.). Experiments in this laboratory with a larger bore section (1 3/4 in.) on a 200 and 400 cm 1-in. column with mixtures of iso-octane, benzene and toluene revealed no appreciable effect.

A definite improvement can be obtained, however, by temperature programming a preparative separation. The chromatograms in Fig. 2 show this clearly.

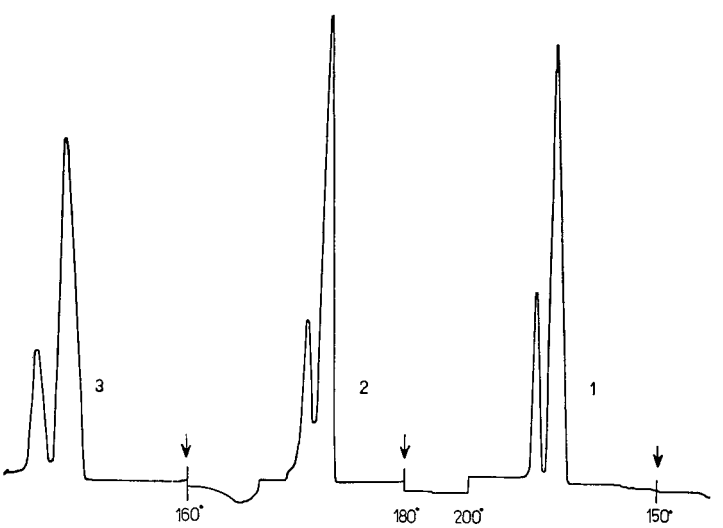

Fig. 2. Wilkens Aerograph A90, 3 mV recorder, filament current 170 mA, carrier gas H_2, 100 ml/min. Attenuator 1/32. 0.5 ml of decalin in each run on a 260 cm column, ø 10 mm, filled with silicone oil 550 (20%) on celite. (1) Programmed run; (2) at 180°; (3) at 160°.

0.5 ml of a mixture of *cis*- and *trans*-decalin was placed on a column of 260 cm length and 10 mm internal diameter, filled with silicone oil 550 (20%) on celite. Run 1 was temperature programmed from 150 to 200° in 30 min by simply setting the oven power of the instrument at full power. The separation is complete. This is not the case at a constant temperature of 180° or even 160°. Still lower constant temperatures could result in a complete separation, but this required more time. There is an additional point in favor of the programmed run in that the bands are compressed and the concentration of the substances in the outflowing carrier gas is thus much higher, resulting in easier recovery of the eluted substances.

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Zur Trennung von Betain und Cholin an Ionenaustauschern

Für die quantitative Bestimmung von Betain in Pflanzenextrakten ist die Fällung mit Reineckesalz (Ammonium-tetrarhodanato-diamminchromiat) nach CROMWELL UND RENNIE[1] gut geeignet. Die Methode erfordert jedoch für Serienbestimmungen einen grossen Zeitaufwand. Soll neben Betain auch Cholin bestimmt werden, so muss man nach BANDELIN UND PANKRATZ[2] das Cholin als Reineckat in stark alkalischer Lösung fällen und abfiltrieren. Dann säuert man das Filtrat an und lässt das Betainreineckat auskristallisieren. Nach STREET *et al.*[3] sind jedoch die aus Pflanzenextrakten mit Reineckesalz erhaltenen Cholinfällungen häufig schwer zu filtrieren, wobei ausserdem die Gefahr der Zersetzung in alkalischer Lösung besteht.

Eine eindeutige Trennung von Cholin, Betain und anderen quarternären N-Verbindungen ist nach CHRISTIANSON *et al.*[4] durch Säulenchromatographie an Dowex 50 mit Salzsäure steigender Konzentration möglich. Für eine vereinfachte Trennung von Cholin und Betain, die auch für Serienbestimmungen geeignet ist, absorbieren HRDÝ UND LOCHMANOVÁ[5] die in der Lösung enthaltenen Anionen an einer Säule von Amberlite IRA 400 (OH-Form). Der Durchlauf passiert danach den schwach sauren Kationenaustauscher Amberlite IRC 50 (H-Form), welcher nur Cholin absorbiert. Der Durchlauf dieser Säule wird eingedampft, der Rückstand in Eisessig aufgenommen und darin Betain mit Perchlorsäure titriert. Das auf der Säule fixierte Cholin wird mit 1 *N* Salzsäure eluiert und mit Reineckesalz gefällt. CARRUTHERS *et al.*[6] arbeiten zur Bestimmung von Betain in Zuckerrübensäften nach dem gleichen Prinzip, geben aber die Säfte auf eine Säule mit einer Mischung aus dem stark basischen Austauscher De-Acidite FF und dem schwach sauren Amberlite IRC 50. Betain wird nicht absorbiert und im Durchlauf mit Reineckesalz nach WALKER UND ERLANDSEN[7] bestimmt.

Wir haben versucht, die Trennung von Betain und Cholin mit nur *einem* Austauscher zu erreichen. Für die Isolierung der in Zuckerrübensäften vorkommenden freien Aminosäuren verwenden wir eine Säule mit dem stark sauren Kationenaustauscher Lewatit S 100 (H-Form) (NIEMANN[8]), der die anorganischen Kationen, die Aminosäuren, Betain und Cholin absorbiert (SCHNEIDER *et al.*[9]). Aminosäuren und Betain werden mit 2 *N* Ammoniak eluiert. Nach dem Abdampfen des Ammoniaks wird Betain mit Reineckesalz in saurer Lösung gefällt und direkt gravimetrisch oder nach Auflösung in Aceton colorimetrisch[2] bestimmt. Der Austauscher wird nach Spülung mit Wasser mit 2 *N* Salzsäure gewaschen und so für den nächsten Versuch regeneriert. Dabei wird gleichzeitig das Cholin eluiert. Das Eluat wird eingeengt und darin das Cholin mit Reineckesalz in saurer Lösung gefällt und gravimetrisch oder colorimetrisch[2] bestimmt.

Unsere Modellversuche mit Standardlösungen und Zuckerrübensäften ergaben eine quantitative Elution und eine eindeutige Trennung von Betain und Cholin. Wir konnten durch papierchromatische Untersuchungen der Reineckatfällungen die Befunde von BREGOFF *et al.*[10] und CARRUTHERS *et al.*[6] bestätigen, dass in Zuckerrüben neben Glycinbetain keine anderen Betaine vorkommen.

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Wick systems in circular paper chromatography

Many techniques for the controlled transport of solvent from the reservoir to the paper have been studied in order to improve the reliability, simplicity, convenience, and speed of circular paper chromatography. These techniques have ranged from the introduction of solvent by means of a self-regulating pipette[1] to the use of various wick systems.

In one group of wick systems, direct contact between solvent and paper is effected by a small, cut-out portion of the chromatogram dipping into a solvent reservoir. RUTTER[2] used a single cut-out strip for the entire chromatogram, whereas PHILIPPU[3] used an individual wedge for each segment of the chromatogram. In a second group of wick systems, indirect contact between solvent and paper is effected by means of

some connecting agency. Among the devices that have been used are capillary tubes[4], single or multiple cotton threads[5], and detachable filter paper wicks of various shapes[6], such as a cylinder of rolled paper, a small paper cone, or simply a paper strip.

A new indirect contacting technique, based on the use of an inert, porous cylinder as the wick, has been found in practice to be somewhat simpler and more convenient than the above techniques. The desired properties of this type of wick are satisfactorily evinced by an unglazed ceramic filtering crucible (style No. 528–30, Laboratory Equipment Corp., St. Joseph, Michigan). The crucible, as a rigid unit, is also ideally suited as a center support for the paper in large-diameter (11 inch), circular chromatographic chambers (Pyrex pie plates, Corning Glass Works, Corning, New York, edges ground flat). The same unit may be used repeatedly, facilitating reproducibility among runs, although its low cost permits it to be disposed of after use. A typical system is shown in Fig. 1. Similar application of other devices made from porous or sintered glass or metal, foamed plastic, etc., in hollow or full shapes is readily apparent.

The porous cylinder wick technique appears equivalent in performance to several widely used wick systems when judged by the ability to separate a mixture of amino acids. The data listed in Table I indicate that the wick system does not affect the individual amino acid R_F values within experimental error. A similar lack of dependence was shown by the zone widths.

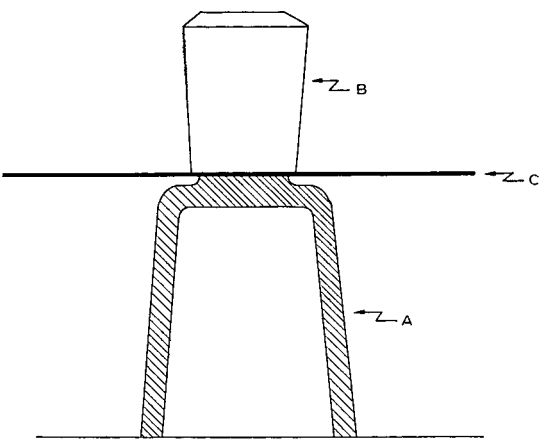

Fig. 1. Porous cylinder wick system. A = unglazed ceramic crucible; B = glass weight; C = chromatogram.

All four wick systems gave symmetrical, mildly eccentric, elliptical development patterns, with an average ratio of major to minor axis of 1.11 ± 0.01.

The rate of solvent flow for each wick technique can be influenced by the geometry and characteristics of the wick and by the distance between paper and solvent. It has been shown[5] for the cotton thread technique that the square root of the solvent flow is proportional to the length of the wick and that the rate depends on the number and the thickness of the strands, and on their position with respect to the center of the paper disc. The rate of flow in the capillary technique similarly depends on the

TABLE I

OBSERVED R_F VALUES[a] OF INDIVIDUAL AMINO ACIDS

Solvent system: organic phase of the mixture *n*-butanol–glacial acetic acid–water in the ratio 40:10:50

	Wick system			
	Capillary tube	*Cotton plug*	*Porous cylinder*	*Philippu*
DL-Aspartic acid	0.37 ± 0.01	0.34 ± 0.02	0.36 ± 0.02	0.34 ± 0.01
β-Alanine	0.44 ± 0.02	0.42 ± 0.01	0.44 ± 0.01	0.41 ± 0.01
DL-Valine	0.58 ± 0.01	0.56 ± 0.01	0.57 ± 0.01	0.56 ± 0.01
DL-Isoleucine	0.68 ± 0.01	0.67 ± 0.01	0.69 ± 0.01	0.67 ± 0.01

[a] Values represent the average of four runs and the average deviation.

diameter and height of the capillary[4]. We have found that the rate may also be varied by the use of either a bundle of thin-walled capillaries (*e.g.*, melting point tubes) or a series of concentric tubes with capillary spacing. The solvent flow rate may be modified in the porous cylinder technique by use of a device of different porosity, size or shape, and in the Philippu technique, by use of a different effective wick length and width. The average rate of chromatographic development observed for each wick system, in units of time (h) for the solvent front to move 12.3 cm, was as follows: capillary 5.1, cotton plug 4.4, porous cylinder 4.5, and Philippu, 2.5.

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Prevention of excessive foaming

Excessive foaming during the concentration or distillation of aqueous solutions is a common problem, which is generally solved by the addition of silicone oil to the solution. In many cases, however, such as when the concentrate is to be analysed by means of paper chromatography, this should be avoided, since it might affect the results.

It was found that in such cases the inconvenience of excessive foaming could be prevented by coating a partly straightened paper clip with a silicone grease, such as Dow Corning Stopcock Grease, and hanging it on the mouth of the vessel. Sometimes it is desirable to replace the paper clip by a glass capillary similarly bent. In the case of distillations the silicone grease can be applied as a thin layer on the tip of the condenser.

Thus when the foam rises it touches the silicone coated paper clip (or bent capillary, or condenser tip) and then breaks down.

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Nachweis schwer hydrolysierbarer Phosphatester mit Hilfe von H_2O_2 und $FeSO_4$

Der Nachweis mancher Phosphatester stösst auf Schwierigkeiten, da sich die Phosphatgruppe unter den üblichen Nachweisbedingungen nicht abspalten lässt. Bei dem Versuch, Phenyl- bzw. Naphthylphosphat nach dem Verfahren von HANES UND ISHERWOOD[1] auf dem Chromatogramm nachzuweisen, erhielten wir keine Phosphatflecke. Wir haben daher für derartige Phosphatester eine Methode entwickelt, die die Abspaltung der Phosphatgruppe gestattet. Der Nachweis des freien Phosphates kann dann nach einem der üblichen Verfahren erfolgen.

Die Methode beruht auf der Tatsache, dass Aromaten durch H_2O_2 und $FeSO_4$ leicht hydroxyliert bzw. unter Öffnung des aromatischen Ringes oxydiert werden können. Die oxydierende Wirkung von H_2O_2 und $FeSO_4$ wurde bereits 1894 von FENTON[2] entdeckt. Später haben HABER UND WEISS[3] die Reaktion des Eisensalzes mit dem H_2O_2 näher untersucht und kamen zu dem Schluss, dass hierbei OH-Radikale entstehen, die als Oxydationsmittel auf verschiedene Verbindungen einwirken können. Bei der Behandlung von Benzol mit FENTON'S Reagenz erhielten HABER UND WEISS[4] Phenol und Polyphenole.

Bei der Einwirkung von FENTON'S Reagenz auf Naphthylphosphat stellten wir

fest, dass der Aromat unter Aufspaltung des Ringes bis zu Phthalsäure oxydiert wird. Gleichzeitig wird hierbei die Phosphatgruppe abgespalten. Die Reaktion verläuft über mehrere Zwischenstufen (eine eingehendere Beschreibung unserer Untersuchung hinsichtlich dieser Reaktion erfolgt demnächst in einem anderen Zusammenhang). Möglicherweise kann als eine der ersten Stufen der Reaktion eine Hydroxylierung angenommen werden. Die aromatischen *p*- und *o*-Hydroxyphosphatester werden relativ leicht unter Abspaltung der Phosphatgruppe weiteroxydiert, wie PATTERMANN UND WIELAND[5] am Beispiel des Naphthohydrochinonphosphates zeigen konnten. Unsere Versuche ergaben, dass sich die Reaktion der Phosphatester mit FENTON's Reagenz auch auf dem Papier durchführen lässt. Wir haben zu diesem Zweck die Chromatogramme zunächst mit einer 5 %igen $FeSO_4$-Lösung und anschliessend mit einer 5 *M* H_2O_2-Lösung besprüht. Bei dieser Behandlung wird das Phosphat abgespalten, kann aber nicht unmittelbar darauf nach einem der üblichen Verfahren nachgewiesen werden, da H_2O_2 oder eines der Reaktionsprodukte, möglicherweise Peroxyde, die Molybdänreaktion stören. Diese Produkte werden völlig beseitigt, wenn man die trockenen Chromatogramme über Nacht in einer Ammoniakatmosphäre hängen lässt.

Zum Vergleich unserer Entwicklungsmethode mit der von HANES UND ISHERWOOD haben wir je zwei Chromatogramme von Äthanolamin-N-phosphatmonobenzylester, Naphthylphosphat und Phenylphosphat laufen lassen und das eine Mal das Phosphat nach Vorbehandlung mit FENTON's Reagenz, das andere Mal ohne dieses nach HANES UND ISHERWOOD nachzuweisen versucht. Nur im ersten Falle erhielten wir positive Ergebnisse.

Das Verfahren wurde mit Erfolg bei einer Reihe von Monobenzylphosphorylestern und -amiden angewendet, bei denen die üblichen Nachweismethoden versagten. Die Methode versagte jedoch bisher bei den aromatischen Triestern der Phosphorsäure.

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Gas chromatography of the amino acid esters in ammonia

The voltages and temperatures used with the hydrogen flame ionization detector[1] are such that the water vapor formed in the combustion and introduced with the sample contributes little to the signal of the detector. Similarly, ammonia gas (commercial anhydrous) added to nitrogen as the carrier gas in gas chromatography increases the de-

tector current of the hydrogen flame by an amount small enough for the maintenance of a satisfactory baseline. This addition of ammonia makes possible the analysis of amino acid esters injected directly into the column as solutions of their hydrochloride salts.

The hydrochloride salt of an amino acid ester (dissolved in alcohol) when injected onto a short (1/4 in. × 6 in.) polyethylene glycol adipate column (22 % on Chromosorb W, 50 to 100 mesh*) with nitrogen alone (50 cc/min) as a carrier gas, yielded

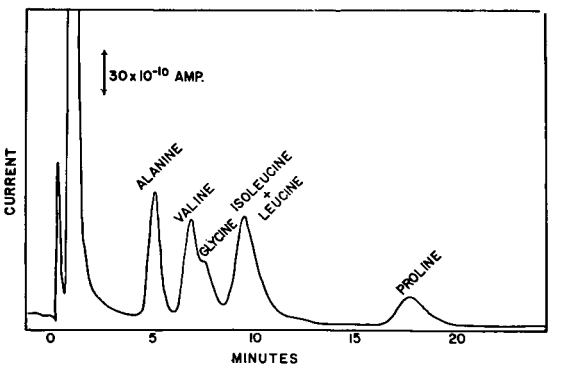

Fig. 1. Gas chromatogram of a mixture of alanine, valine, glycine, isoleucine, leucine and proline converted into the *n*-butyl ester hydrochlorides with butyl alcohol and dry HCl. Temp. of the column was 131°; N_2 carrier gas flow was 50 cc/min; NH_3 gas flow was 8 cc/min; detector was H_2 flame ionization*. The amount of each amino acid injected onto the column was $1.1 \cdot 10^{-4}$ g.

only the alcohol peak and a steady base line of $50 \cdot 10^{-10}$ A for a period of one hour. Upon the addition of ammonia (6 cc/min) to the carrier gas stream, the base line rose to $100 \cdot 10^{-10}$ A and a well defined peak of the amino acid ester emerged. The pure ester hydrochlorides (some ethyl and some *n*-butyl) of the following amino acids have been successfully chromatographed in the ammonia–nitrogen carrier gas system on a short (1/4 in. × 6 in.) polyethyleneglycol adipate column: alanine, valine, glycine, isoleucine, leucine, proline, aspartic acid, threonine, methionine, serine, glutamic acid, phenylalanine, lysine and hydroxyproline. A long column (1/4 in. × 6 ft.) of the adipate polyester proved satisfactory only for the analysis of mixtures of the lower boiling esters. The extent of amide formation (if any) and its relationship to column length, temperature and the column packing has not yet been determined.

Fig. 1 illustrates the gas chromatogram of a mixture of the hydrochlorides of the *n*-butyl esters of alanine, valine, glycine, leucine, isoleucine and proline obtained from a long (1/4 in. × 6 ft.) polyethyleneglycol adipate column at 131°.

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BOOK REVIEWS

Protides of the Biological Fluids, Proceedings of the 9th Colloquium, Bruges, 1961, edited by H. PEETERS, Elsevier Publ. Co., Amsterdam, 1962, x + 373 pages, price Dfl. 45.—.

The ninth colloquium follows the pattern adopted in the previous colloquia. There are five general discussions: on the purity of proteins (by R. LONTIE), on automation (by W. H. MARSH), on polypeptide hormones (by R. SCHWYZER), on genetic aspects of protein metabolism (by P. S. CHEN) and on the transport function of serum proteins (by H. BENNHOLD).

Sixty three original contributions are grouped under the headings analytical methods, protein structure, protein turnover and metabolism, carrier function of proteins, proteins and genetics, and finally, isolated fractions. The problems of automation in clinical analysis receive considerable attention in the plenary lectures, the original papers and the round table conference.

J. Chromatog., 9 (1962) 124

Gas Chromatography Abstracting Service, Preston Technical Abstracts Co., 1718 Sherman Avenue, Evanston, Ill., U.S.A.

The abstracting service under review was started several years ago; however, it was not mentioned in a recent discussion of the literature of gas chromatography (*Nature*, 194 (1962) 822), nor did the reviewer learn of its existence till very recently, and thus we believe that a description is justified.

The Service abstracts all journal articles and books on gas chromatography immediately after publication and presents them on standard 5 in. × 8 in. Unisort punched cards. In 1960 the number of cards prepared was 1300 and in 1961 about 1600. The cards are supplied monthly to subscribers to the service at a rate of $ 10 a month. This is further supplemented by newsletters mainly announcing courses in gas chromatography and new books.

The card system has the advantage that abstracts can be furnished much faster than is the case with a yearly volume such as *Gas Chromatography Abstracts*, but this is achieved at an incomparably higher price. The reviewer feels nevertheless that it should be subscribed to by all university libraries, as well as all industrial laboratories interested in this technique.

MICHAEL LEDERER (Rome)

J. Chromatog., 9 (1962) 124

News

THE PITTSBURGH CONFERENCE ON ANALYTICAL CHEMISTRY AND APPLIED SPECTROSCOPY

EXPOSITION OF MODERN LABORATORY EQUIPMENT

Preliminary Announcement of 1963 Pittsburgh Conference

The Fourteenth Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy will be held at the Penn-Sheraton Hotel in Pittsburgh, Pa., U.S.A., March 4–8, 1963. Approximately 190 papers on all phases of analytical chemistry and spectroscopy will be presented. A symposium entitled *Solution Techniques in X-Ray and Emission Spectroscopy* will be cosponsored with the Society for Applied Spectroscopy. The Coblentz Society is coarranging a symposium on *Techniques Related to Infrared Spectroscopy*. Additional symposia with the titles *Nuclear Magnetic Resonance–Nuclei Other Than Hydrogen, Gas Chromatographic Analysis of Metallo-Organics and Related Compounds, Uses of Reaction Rates in Analytical Chemistry*, and *The Analysis of Refractory Metals*, will also be held.

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 A. STRAUB, Program Chairman,
The Fourteenth Pittsburgh Conference,
Applied Research Laboratory,
United States Steel Corporation,
Monroeville, Pa., U.S.A.

The final date for receipt of abstracts is October 15, 1962. One copy of the complete paper must be submitted by January 1, 1963.

In addition to the program of technical papers, there will be an exhibition of the newest analytical instrumentation. More than 130 companies will display instruments, chemicals, and equipment. A complete program of activities for wives and lady attendees at the Conference is also being planned for the 1963 Pittsburgh Conference. Finally, the fourth OCEANS (Omnibus Conference on Experimental Aspects of Nmr Spectroscopy) will be held at Mellon Institute, Pittsburgh, from Thursday, Feb. 28 through Saturday, March 2, the week preceding this Conference. The first day will be devoted primarily to broad-line work, the second to experimental developments of general interest and the third to high resolution techniques. For the convenience of those wishing to attend both meetings the Nmr sessions of the Pittsburgh Conference will be scheduled for Monday, March 4.

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ÉTUDE DE L'UTILISATION DU TÉFLON COMME SUPPORT EN CHROMATOGRAPHIE GAZ-LIQUIDE

APPLICATION À LA SÉPARATION DE CORPS TRÈS POLAIRES

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INTRODUCTION

L'application de la chromatographie gaz-liquide aux mélanges contenant des constituants très polaires se heurte le plus souvent à de grosses difficultés dues à l'apparition sur l'enregistrement d'importantes traînées derrière les pics, voire à des adsorptions quasi irréversibles[1,2]; ceci est généralement attribué à l'effet des sites d'adsorption plus ou moins actifs qui restent sur le support après son traitement[3]; en effet les isothermes d'adsorption sont généralement non linéaires et la cinétique des phénomènes d'adsorption est sensiblement plus lente que celle des phénomènes de dissolution et d'évaporation qui interviennent en chromatographie gaz-liquide. De plus le volume de rétention des corps fortement adsorbés dépend de la masse de l'échantillon utilisée[4].

De nombreuses publications décrivent diverses méthodes visant à supprimer cet effet, surtout par modification des propriétés de supports usuels tels la brique réfractaire broyée et tamisée. Ainsi KNIGHT[5], propose, pour la séparation de corps possédant les groupes OH ou NH_2, de saturer le gaz vecteur par de la vapeur d'alcool ou d'amine. JOHNS[6] diminue la granulométrie; WEHRLI ET KOVATS[7], empoisonnent les sites actifs d'adsorption en utilisant une grande quantité de phase stationnaire. ORMEROD ET SCOTT[8] déposent sur la brique de l'argent en quantité importante.

Il est bien connu[9] que le lavage de la brique par l'eau régale dissout le fer (environ 1 % de la brique), ce qui diminuerait considérablement les traînées, mais pour la séparation de mono et diamines on peut préférer un traitement par une base forte saturant les sites actifs par des groupes OH et incorporant au support au moins 1.8 % de KOH[10].

D'autres méthodes ont été proposées pour diminuer les propriétés adsorbantes des supports, propriétés étudiées par BENS[4]. On a en particulier étudié divers procédés de traitement par des dérivés du silicium, le diméthyldichlorosilane[4,11-13] et l'hexaméthyldisilazane[14], visant à estérifier les groupes Si–OH libres à la surface du support et à les remplacer par des groupes $Si–CH_3$. Cette réaction et la proportion de groupes OH estérifiés a été étudiée par LOWEN ET BROGE[15]. On peut, dans certaines conditions, obtenir par cette méthode une surface entièrement revêtue de groupes CH_3. Il est cependant curieux de constater que, malgré un tel traitement, les volumes de rétention semblent dépendre du support[13].

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L'idéal, évidemment, serait de disposer d'un support réellement inactif. S'il est inaccessible il se trouve cependant que l'on dispose de quelques supports peu actifs, comme les billes de verre[16] avec lesquelles on ne peut faire de colonnes chargées en phases stationnaires, les hélices d'acier inoxydable, qui donnent des efficacités très faibles, et, surtout, la poudre de téflon.

En effet, le téflon est connu pour être chimiquement inerte: il résiste bien aux attaques par les réactifs (sauf le fluor et les métaux alcalins) et aux solvants. Il a une légère tendance à adsorber les amines, les alcools et l'eau. Il est thermiquement stable, jusque vers 290°. Toutes ces caractéristiques rendent intéressante l'étude de son emploi comme support.

Nous avons utilisé principalement la poudre de téflon vendue sous le nom de Haloport F*, qui est une dispersion de polytétrafluoroéthylène; c'est une poudre légère, blanche, ayant une forte tendance à s'agglomérer. Les dimensions des grains constituant la poudre sont principalement comprises entre 500 et 400 μ. Le Tableau I donne les caractéristiques comparées de la Célite, du Chromosorb et de la poudre de téflon.

TABLEAU I

CARACTÉRISTIQUES PHYSIQUES DE DIFFÉRENTS SUPPORTS SOLIDES

Type	*Surface spécifique*[a] *(m^2/g)*	*Volume des pores (cm^3/g)*	*Densité absolue (g/cm^3)*	*Densité apparente (g/cm^3)*
Chromosorb[b] R	4.8	1.1	2.26	0.40
Chromosorb W	1.2	2.78	2.20	0.20
Célite C 29 924	0.45	0.80	2.30	0.40
Téflon	0.64	0.40	2.3	0.73

[a] Déterminée par la méthode B.E.T., par BAKER, LEE ET WALL[9].
[b] JOHN MANVILLE, 22 East 40th Street, New York 16, N.Y. (U.S.A.).

PRÉPARATION DES COLONNES

Les performances d'une colonne de chromatographie gaz–liquide dépendent essentiellement de la façon dont elle a été réalisée. La préparation comprend le choix du support, son traitement physique (broyage, élutriation, tamisage) et chimique (lavage acide, basique, par l'eau régale), puis l'imprégnation du support par la phase stationnaire choisie et enfin le remplissage de la colonne.

Le traitement chimique est ici sans objet. Le traitement physique se ramène à un tamisage. Étant donné les difficultés rencontrées cette opération est supprimée et la poudre de téflon imprégnée de phase liquide telle quelle. Un tamisage rudimentaire est alors effectué, avant le remplissage de la colonne. On rencontre à chacune des étapes de la préparation de multiples difficultés dues aux propriétés de la poudre de téflon:

1. Collage des grains par la phase stationnaire qui se dépose sur la surface des grains. Ce phénomène se rencontre dans la préparation de toutes les phases et c'est pour éliminer les agglomérats ainsi formés qu'on tamise la phase stationnaire après

* F. & M. Scientific Corporation, 1202 Arnold Avenue Air Base, New Castle, Del. (U.S.A.).

imprégnation. Malheureusement, il est très difficile de tamiser la poudre de téflon sans la faire prendre en masse. On y arrive, cependant, en la versant doucement sur un tamis de 0.5 mm qui ne doit pas être agité. En recommençant sur le tamis 0.4 mm, on parvient à isoler une fraction dont la granulométrie est à peu près homogène, mais dont les dimensions moyennes sont assez élevées, ce qui constitue une limite intrinsèque à l'efficacité des colonnes que l'on pourra préparer avec ce produit[2].

2. La poudre de téflon utilisée est, en fait, de la poudre à mouler, si bien qu'il est normal de voir les grains adhérer facilement les uns aux autres et prendre en masse à la moindre pression, tout en perdant leur porosité.

3. Au cours du remplissage, ce phénomène peut provoquer des dégâts irrémédiables car il facilite la formation de bouchons dans l'intérieur de la colonne et l'apparition de zones trop tassées précédant des sections vides de remplissage. On ne peut donc, dans ces conditions, s'étonner de trouver de faibles efficacités.

Nous avons utilisé deux méthodes d'imprégnation de la poudre de téflon:

La première (colonne TP_1) consiste à remplir d'abord la colonne par de la poudre de téflon non imprégnée (suivant la méthode citée ci-dessous), puis à effectuer l'imprégnation du support en faisant percoler au travers de la colonne une solution de la phase stationnaire choisie dans un solvant volatil; cette solution est introduite sous pression de 2 à 3 kg/cm^2 d'azote. Quand une quantité suffisante de solution a traversé la colonne, on effectue un balayage par un gaz inerte durant 24 h sous une pression de 1 kg/cm^2, à froid, puis on chauffe pour éliminer les traces de solvant (réf. [1], discussion et réf. [17]).

La deuxième méthode de remplissage (colonnes TP_2, TP_3, TP_4, TN_1) est celle utilisée habituellement pour remplir les colonnes où le support est de la brique réfractaire: le support est imprégné par une solution à 5 % environ contenant la quantité désirée de phase stationnaire dissoute dans le chlorure de méthylène qui est chassé par évaporation à l'ambiante puis à 40°, pendant que le produit est continuellement brassé.

Nous avons utilisé des colonnes en cuivre rouge recuit et en acier inoxydable de longueurs définitives comprises entre 150 et 200 cm, de diamètre intérieur 4 mm, de diamètre extérieur 6 mm. Ces tubes sont préalablement dégraissés par un solvant volatil (trichloroéthylène ou chlorure de méthylène), puis séchés dans un courant de gaz inerte (argon ou azote) (*cf.* Fig. 1). La colonne (C) qui mesure environ 30 à 40 cm

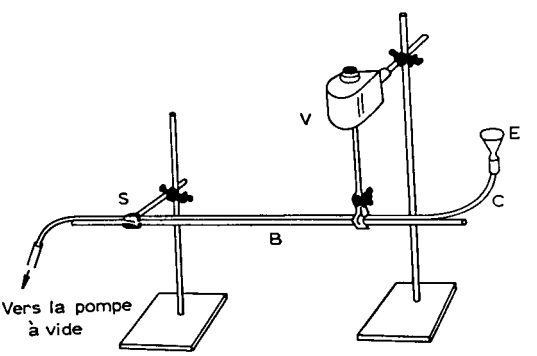

Fig. 1. Schéma de l'installation de remplissage des colonnes: B = barre métallique; C = colonne; E = entonnoir; S = support; V = vibreur.

de plus que sa longueur définitive, est rigidement fixée sur une barre métallique rectiligne (B). On bouche provisoirement avec un tampon de coton hydrophile l'extrémité reliée à la pompe à vide. On fixe à l'autre extrémité un petit entonnoir (E). La barre est, ensuite, prise avec la colonne dans le bras d'un vibreur magnétique (type Vibromixer) (V) et soutenue en un autre point (S). Un opérateur introduit le téflon *grain par grain* dans la colonne tandis qu'un autre bat la colonne pour éviter la formation de bouchons.

APPAREILLAGE

Nous avons utilisé un chromatographe Perkin-Elmer type 116.E, muni d'un détecteur à thermistances. Les colonnes ont été étudiées à des températures constantes comprises entre 50 et 150°; la grande majorité des mesures ont été faites à 70° et à 130°. Le système d'injection est chauffé à 50° environ audessus de la température de la colonne. Les échantillons injectés sont faibles: 0.4 μl pour les solutés purs, 0.5 à 1 μl pour les mélanges (seringue Hamilton de 10 μl).

Nous avons également utilisé un appareil construit au laboratoire à l'aide d'un bloc détecteur Perkin-Elmer et d'une électronique analogue à celle décrite par KIESELBACH[18]. La sensibilité obtenue est environ 10 fois plus forte que celle du 116 E.

RÉSULTATS

Nous décrirons successivement les caractéristiques de ces colonnes: efficacité et vieillissement, puis leur application à la séparation des corps très polaires.

Nous avons étudié sept colonnes différentes dont deux comme références: la première est à 20 % en poids de Polyglycol 1500 (Naphtachimie 60-UK-35) sur brique C22 broyée (tamis 22 et 23 Afnor, soit 0.125 à 0.160 mm) traitée à l'eau régale (colonne CP_1), la deuxième en téflon non imprégné nous a permis d'étudier la rétention et les propriétés du téflon (colonne T_1). Les autres caractéristiques des colonnes sont données dans les Tableaux II et III.

TABLEAU II

CARACTÉRISTIQUES DES COLONNES UTILISÉES

Désignation	CP_1	T_1	TP_1	TP_2	TP_3	TP_4	TN_1
Longueur (cm)	200	200	200	150	200	227	
Support	Chromosorb	téflon	téflon	téflon	téflon	téflon	téflon
Masse de support (g)	12.06	14.1	13.8	13.96	13.2	13.8	
Phase liquide	Polyglycol 1500* (P.G.)	sans	P.G.	P.G.	P.G.	P.G.	P.S.N.G.**
Masse de phase liquide (g)	3.01	0	?	1.56	3.3	3.44	
Teneur en phase (%)	20	0	~5	10	20	20	10
Volume gazeux de la colonne (cm^3)	19.2	19.7	19.6	14.1	14.2	16.3	
Section de la veine gazeuse (cm^2)	0.096	0.098	0.098	0.094	0.071	0.072	

* Naphtachimie (203 Fg Saint-Honoré, Paris 8e) 60-UK-35.
** Polysébaçate de néopentyl glycol[17].

TABLEAU III

PARAMÈTRES D'EFFICACITÉ DES COLONNES

Désignation	*A*	*B*	*C*	$HETP_{min}$ *(cm)*
CP_1	0.07	0.8	0.0007	0.10
T_1	0.25	1.15	0.063	0.60
TP_1	0.25	1.1	0.040	0.52
TP_2	0.16	1.0	0.030	0.35
TP_3	0.10	0.7	0.012	0.23
TP_4	0.12	0.75	0.012	0.24

(*a*) *Efficacité des colonnes*

Nous avons tracé les courbes de VAN DEEMTER[19] (Figs. 2 et 3) pour chaque colonne à 70°, en utilisant le méthanol comme soluté. Nous avons injecté des échantillons de 0.4 μl afin d'éviter la surcharge des colonnes qui pourrait fausser les résultats en augmentant le HETP.

Nous avons calculé pour chaque colonne les constantes A, B et C à partir de l'équation des hyperboles obtenues:

$$H = A + B/u + Cu$$

où u est la vitesse linéaire du gaz vecteur dans la colonne (*cf.* Tableau III).

On constate ainsi que:

$H_{CP_1} < H_{TP_3} \simeq H_{TP_4} < H_{TP_2} < H_{TP_1} < H_{T_1}$ (H valeur minimale de HETP)

$A_{CP_1} < A_{TP_3} \simeq A_{TP_4} < A_{TP_2} < A_{TP_1} \simeq A_{T_1}$

$B_{CP_1} \simeq B_{TP_3} \simeq B_{TP_4} < B_{TP_2} < B_{T_1} \simeq B_{TP_1}$

$C_{CP_1} \ll C_{TP_3} \simeq C_{TP_4} < C_{TP_2} < C_{TP_1} < C_{T_1}$

On sait que A est la constante de diffusion apparente[20], diffusion entre filets gazeux ayant effectué des trajets de longueur différente et que B est la constante de diffusion longitudinale du soluté dans la phase gazeuse, ou diffusion moléculaire.

On observe de grandes valeurs de A et B lorsque le remplissage des colonnes a été irrégulièrement fait et que s'y succèdent des zones très tassées suivies ou précédées de zones vides[3]. A cet égard les colonnes TP_3 et TP_4 sont satisfaisantes puisqu'elles donnent des valeurs de B du même ordre que celle obtenue avec CP_1. Si la valeur de A est sensiblement plus grande cela est dû à la taille moyenne des grains de téflon sensiblement plus gros que ceux de la brique C 22 broyée (125 à 160 μ pour CP_1 contre 400 à 500 μ pour le téflon).

La constante C représente les résistances aux transferts de masse (au travers des phases liquides et gazeuses ou à leur interface): son interprétation est encore très discutée; elle est la somme de plusieurs facteurs dont la valeur et le nombre même dépendent des auteurs[3,16,20–27].

On sait, cependant, que la valeur de C dépend de la granulométrie moyenne du support, de la dispersion de cette granulométrie, de la porosité des grains, de la surface

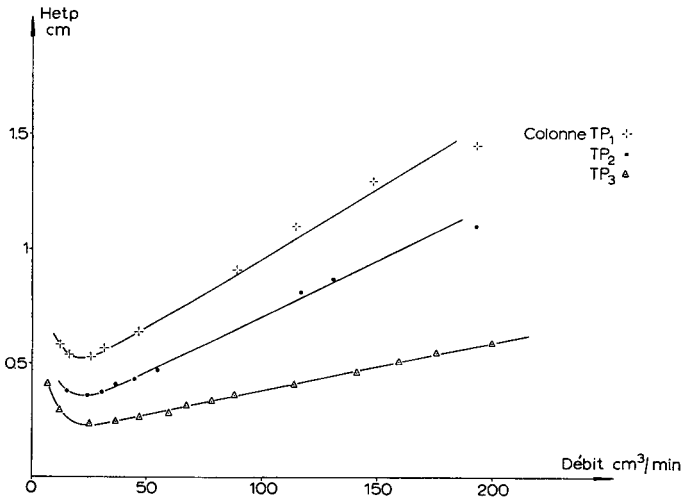

Fig. 2. Variation de HETP en fonction du débit pour diverses colonnes ayant la poudre à mouler de téflon comme support.

spécifique du support, de la nature et de la quantité de phase stationnaire, de sa dispersion sur le support, de la nature du soluté.

On constate que les colonnes faites avec le téflon comme support ont un terme C 20 à 100 fois plus grand que CP_1. Les termes de transfert de masse en phase gazeuse étant sensiblement les mêmes dans les deux cas, nous pensons que cette différence pourrait s'expliquer par l'action de l'épaisseur moyenne de phase stationnaire, beaucoup plus grande dans le cas du téflon, un peu à cause de la surface spécifique assez faible, beaucoup parce que le téflon se laisse mal mouiller par les liquides, surtout s'ils sont polaires[28]. Les valeurs de C très élevées observées pour TP_1 et TP_2, malgré

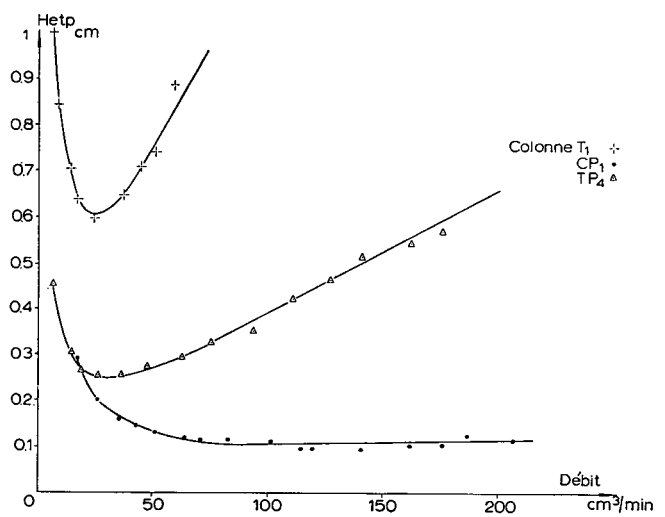

Fig. 3. Comparaison de l'efficacité de diverses colonnes.

des taux d'imprégnation plus faibles, peuvent s'interpréter par l'adsorption sur le téflon (la valeur de C pour T_1 est assez élevée) et par le mode de remplissage défectueux de TP_1.

La méthode de remplissage des colonnes est très délicate si l'on veut obtenir des efficacités convenables; c'est pourquoi nous avons décrit en détail le mode opératoire auquel nous avons abouti, après plusieurs essais peu fructueux: les premières colonnes que nous avons faites, ainsi que de nombreuses colonnes dont l'emploi a été décrit[1], ne donnaient que 70 à 100 plateaux théoriques au mètre.

La colonne utilisée par BORFITZ[29], qui n'en donne pas l'efficacité mais un chromatogramme à partir duquel on peut la calculer, fait 370 plateaux théoriques, mais sa longueur n'est pas connue. Le chromatogramme publié par Perkin-Elmer pour sa colonne W correspond à une efficacité de 400 plateaux.

Pour chercher la cause de cette piètre efficacité, nous avons découpé ces colonnes en sections successives et avons alors constaté la présence de zones successivement trop tassées et très molles, et parfois même de zones vides de longueur importante.

En améliorant la méthode de fabrication nous avons pu obtenir des colonnes telles que TP_3 (20 % de polyglycol) qui a 430 plateaux théoriques par mètre et un facteur de résolution voisin de 1.7 pour le couple méthanol–éthanol, contre 2.3 pour la colonne de polyglycol sur chromosorb. Les colonnes TP_3 et TP_4 exécutées avec le même lot de téflon imprégné de polyglycol par les mêmes opérateurs sont quasi identiques, ce qui prouve la bonne reproductibilité que l'on peut obtenir dans la fabrication de ces colonnes.

Le gain d'efficacité ainsi obtenu est dû en partie à ce que l'on coupe les deux extrémités de la colonne après remplissage. Par exemple la colonne TP_4, initialement de 300 cm environ, gagnait 200 plateaux après raccourcissement des extrémités de 35 et 32 cm: on élimine ainsi les zones non uniformément remplies, l'une trop tassée du côté de la pompe à vide, l'autre pas assez.

A partir des enregistrements effectués pour le tracé des courbes de VAN DEEMTER, nous avons calculé pour chaque colonne le taux de dissymétrie des pics de méthanol obtenus[30]. On trouve (Tableau IV) la valeur de ce taux pour un débit de 100 cm³/min, on observe une valeur nulle pour le débit correspondant à l'efficacité maximale. Le taux de dissymétrie apparemment modeste observé pour le méthanol sur CP_1 ne doit pas faire illusion. Il correspond déjà à une traînée accentuée. Par ailleurs le support utilisé pour CP_1, traité à l'eau régale, est relativement satisfaisant.

En conclusion, si l'on procède avec soin il est possible d'obtenir d'une manière reproductible des colonnes à support téflon ayant une efficacité environ moitié de celle des colonnes classiques.

TABLEAU IV

TAUX DE DISSYMÉTRIE DES PICS À 100 cm³/min (70°)

Colonne	T_1	CP_1	TP_1	TP_2	TP_3	TP_4
Taux de dissymétrie %	20	14	6.5	6	7	9

(b) Vieillisement des colonnes au téflon

Nous avons constaté qu'après une série prolongée d'expériences: 500 à 600 injections

successives de produits polaires à une température élevée (H_2O, alcools, amines, esters, xylénols, etc.) certaines colonnes n'avaient plus la même efficacité.

La Fig. 4 donne les courbes de VAN DEEMTER pour la colonne TN_1 (téflon im-

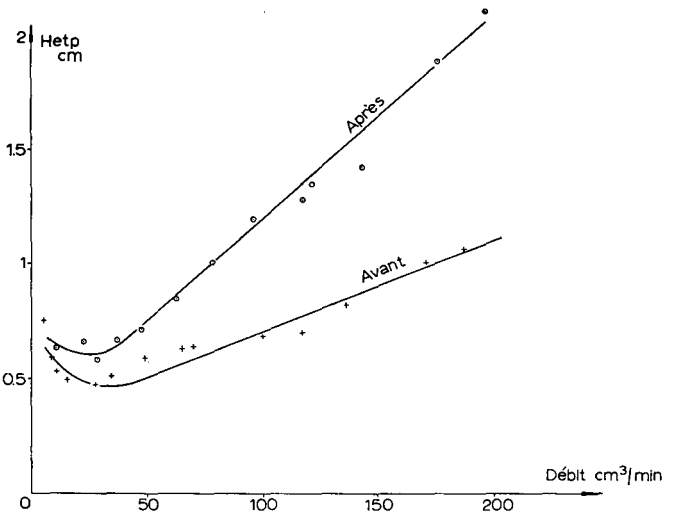

Fig. 4. Efficacité de la colonne TN_1 avant et après vieillissement.

prégné de 10 % de sébaçate de néopentylglycol, longueur 200 cm) avant et après un demi-millier d'injections correspondant à autant d'heures de séjour, à des températures comprises entre 70 et 180°, réparties comme indiqué Tableau V. On constate une diminution de l'efficacité de 25 % environ. Le Tableau VI donne les valeurs des constantes expérimentales de l'équation de VAN DEEMTER pour cette colonne.

TABLEAU V

VIEILLISSEMENT DES COLONNES
NATURE ET QUANTITÉ DES ÉCHANTILLONS INJECTÉS

	Injections	*µl (environ)*
H_2O	60	70
Alcools	120	50
Amines	120	50
Hydrocarbures	60	30
Esters	100	50
Xylénols (180°C)	20	10
Divers	40	20
Méthanol pour courbes d'efficacité	40	20
	560	300

Comme on pouvait s'y attendre les valeurs de A et B n'ont pas sensiblement changé, tandis que C a augmenté de 70 %, entraînant l'augmentation de H de 25 % (Fig. 4).

La constatation de ce phénomène nous a suggéré de faire une étude systématique sur le vieillissement des colonnes. Nous avons choisi la colonne TP_4 (20 % en poids de

polyglycol). La colonne a été purgée pendant une nuit à 100° avec un débit de 100 cm³/min d'hydrogène, puis nous avons tracé une première courbe de VAN DEEMTER (*cf.* Tableau VI).

Nous avons ensuite injecté d'une façon systématique un mélange de composition connue. Nous avons calculé pour chaque injection (de 0.4 μl) le volume de rétention

TABLEAU VI

VIEILLISSEMENT DES COLONNES · PARAMÈTRES D'EFFICACITÉ

Colonne	*A*	*B*	*C*	$HETP_{min}$
TN_1 avant	0.3	1.54	0.034	0.46
TN_1 après	0.3	1.47	0.057	0.58
TP_4 après 25 injections	0.12	0.75	0.012	0.24
TP_4 après 100 injections	0.12	0.74	0.012	0.23
TP_4 après 300 injections	0.12	0.74	0.012	0.23

absolu de l'éthanol et les volumes de rétentions relatifs des autres solutés par rapport à l'éthanol (Tableau VII). On constate nettement une première phase d'équilibrage, accompagnée d'une légère diminution (environ 3 %) des volumes de rétentions absolus jusqu'à la vingtième injection environ, qui s'explique par le départ des fractions de la phase stationnaire de bas poids moléculaire. A partir de là les volumes de rétentions absolus restent constants. Nous avons déterminé l'efficacité de la colonne après 25, 50, 100, 200, 300 injections. Nous n'avons pas constaté de changement ni dans le $HETP_{min}$, ni dans les constantes *A*, *B*, *C* (*cf.* Tableaux III et VI).

TABLEAU VII

VIEILLISSEMENT DE LA COLONNE TP_4 À 70°. ÉVOLUTION DES VOLUMES DE RÉTENTION

Injection No.	$V_R^{0'}$ *éthanol*	V_R^0 *relatifs (à l' éthanol)*				
		*1**	*2*	*3*	*4*	*5*
7	644.3	0.254	0.421	0.633	0.771	2.37
9	622.3	0.254	0.405	0.627	0.755	2.43
20	621.3	0.251	0.420	0.633	0.769	2.45
40	625.9	0.251	0.419	0.633	0.763	2.42
78	619.7	0.254	0.422	0.633	0.765	2.46
200	617	0.254	0.423	0.632	0.765	2.46
300	618	0.255	0.424	0.632	0.765	2.47

* 1 = formiate de méthyle; 2 = acétate de méthyle; 3 = acétate d'éthyle; 4 = méthanol; 5 = eau.

Les propriétés plastiques du téflon auraient pu provoquer un tassement progressif du support avec fermeture de pores ou expulsion de phase liquide. A 100°, un tel processus de vieillissement semble lent et l'on peut escompter un service satisfaisant pendant une durée dépassant sensiblement plusieurs centaines d'heures. A des températures supérieures, la durée de vie peut cependant être nettement raccourcie.

(c) *Application*

(1) *Volumes de rétention.* Nous avons déterminé sur ces colonnes, à 70°, les volumes de rétention relatifs à l'alcool éthylique d'un certain nombre de composés (Tableau VIII). Nous avons rappelé sur ce tableau la quantité de polyglycol contenue dans chaque colonne, ce qui permettrait de calculer les volumes de rétentions spécifiques à 70°, connaissant le volume de rétention spécifique de l'éthanol, qui figure également au Tableau VIII.

TABLEAU VIII

VOLUMES DE RÉTENTION RELATIFS À L'ÉTHANOL (70°)

Solutés	*Colonnes*					
	CP_1	T_1	TP_1	TP_2	TP_3	TP_4
Eau	2.773	2.466	2.521	2.431	2.445	2.475
Méthanol	0.7803	1	0.655	0.759	0.765	0.771
Propanol	2.012	1.949	1.860	1.938	1.963	2.013
Butanol	4.165	—	3.509	4.071	4.039	4.029
Monométhylamine	—	—	0.155	0.156	0.177	0.181
Monoéthylamine	—	—	0.333	0.331	0.326	0.328
Diméthylamine	—	—	0.140	0.144	0.149	0.152
Diéthylamine	—	—	0.331	0.332	0.329	0.331
NH_3 (ammoniac)	—	—	0.094	0.087	0.085	0.090
Formiate de méthyle	—	—	0.300	0.273	0.251	0.257
Acétate de méthyle	—	—	0.431	0.431	0.432	0.437
Acétate d'éthyle	—	—	0.614	0.712	0.654	0.657
Quantité de polyglycol (g)	3	0	?	1.6	3.4	3.44
V_g éthanol	139	—	?	151	140	143

(2) *Indices de rétention.* Nous avons mesuré les indices de KOVATS[7] de trois séries homologues: alcools normaux, aldéhydes normaux et acétates d'alcools normaux sur les colonnes CP_1, TP_1 et TP_4; ces indices sont réunis au Tableau IX où nous avons également reproduit les valeurs fournies par KOVATS[7] sur Emulphor O, phase qui s'apparente au Polylgycol 1500 quoique moins polaire, puisqu'il s'agit d'un polyglycol de masse moléculaire 500 dont les chaînes sont "terminées" par éthérification avec l'alcool octadécylique[7], tandis que le polyglycol 1500 utilisé n'est pas terminé et présente donc des fonctions alcools libres. Cette différence de polarité a déjà été signalée par CHOVIN ET LEBBE[31] qui ont donné une méthode d'estimation des polarités des phases stationnaires: attribuant les polarités 1 et 0 respectivement au β,β'-oxydipropionitrile et au Squalane, ils calculent une polarité de 0.50 pour l'Emulphor O et de 0.76 pour le polyéthylène glycol 1500, analogue à notre phase stationnaire.

Il est normal dans ces conditions que nous observions des indices sensiblement supérieurs à ceux donnés par KOVATS. Le taux d'imprégnation très élevé (40 %) utilisé par cet auteur, au détriment de l'efficacité de ses colonnes, lui permet de rendre très faible l'action du support. C'est cette action qui permet d'expliquer les différences entre les indices mesurés sur nos trois colonnes: il subsiste, sur le support de CP_1, des sites actifs d'adsorption et cette colonne apparaît la plus polaire. Quant aux différences constatées entre TP_1 et TP_4 elles nous paraissent liées à la faible teneur de TP_1 en une phase dont la composition n'est peut-être pas exactement la même que celle de TP_4, en raison de son mode de remplissage.

(3) *Exemples d'analyses.* Bien que la colonne CP_1 soit plus efficace pour le méthanol que la colonne TP_4 (2000 plateaux théoriques contre 1000) lorsqu'on veut séparer des produits très polaires (amines, eau) l'utilisation de CP_1 reste limitée.

TABLEAU IX

INDICES DE RÉTENTION SUR COLONNES CP_1, TP_1, TP_4, À 100°
(Perkin-Elmer 116E)

Soluté	*Indices mesurés par Kovats*	*Indices sur Chromosorb CP_1*	TP_1	TP_4
Eau		1112	1035	1054
Méthanol	731	923	860	892
Éthanol	775	961	896	934
Propanol	878	1064	1000	1034
Butanol	978	1168	1102	1140
Pentanol	1095	1267	1200	1245
Hexanol	1195	1371	1300	1342
Heptanol		1474	1400	1437
Acétaldéhyde	598	701	658	697
Propionaldéhyde	694	799	766	791
Butyraldéhyde	783	889	854	883
Aldéhyde valérianique	885	987	957	978
Hexaldéhyde	978	1082	1056	1079
Acétate de méthyle	718	819	786	820
Acétate d'éthyle	781	875	839	877
Acétate de propyle	870	983	936	971
Acétate de butyle	966	1061	1028	1067
Acétate d'amyle	1066	1151	1121	1169

Les Figs. 5, 6 et 7 reproduisent les enregistrements correspondant à trois analyses faites sur deux colonnes CP_1 et TP_4 dans les mêmes conditions opératoires. Le premier enregistrement (Fig. 5) montre la séparation d'une solution aqueuse d'ammoniac, de méthanol, éthanol, propanol et isopentanol. Sur CP_1 on ne trouve pas le pic de l'ammoniac (adsorption trop forte) et l'isopentanol se trouve complètement masqué dans la traînée du pic de l'eau; au contraire sur TP_4 on distingue facilement les pics de l'ammoniac et d'isopentanol. Le deuxième enregistrement (Fig. 6) correspond à un mélange de trois amines, d'éthanol et d'eau. On voit que la séparation fournie par la colonne CP_1 est médiocre, les pics de la diéthylamine et de l'isobutylamine sont presque complètement masqués par la traînée du pic de diméthylamine, traînée qui dépasse nettement le pic de l'éthanol. Sur la colonne TP_4 les traîneées sont très faibles et on voit même apparaître des pics représentant des impuretés contenues par les amines. Le troisième enregistrement (Fig. 7) montre qu'il est impossible d'analyser sur CP_1 un mélange de diéthylamine, eau, éthanol. La disparition du pic de la diéthylamine a déjà été observée auparavant, lorsqu'on a déterminé les volumes de rétentions relatifs sur la colonne CP_1 (Tableau VIII). Cependant sur l'enregistrement on voit aprés l'éthanol une bosse mais qui ne peut même pas servir à déterminer avec précision un temps de rétention.

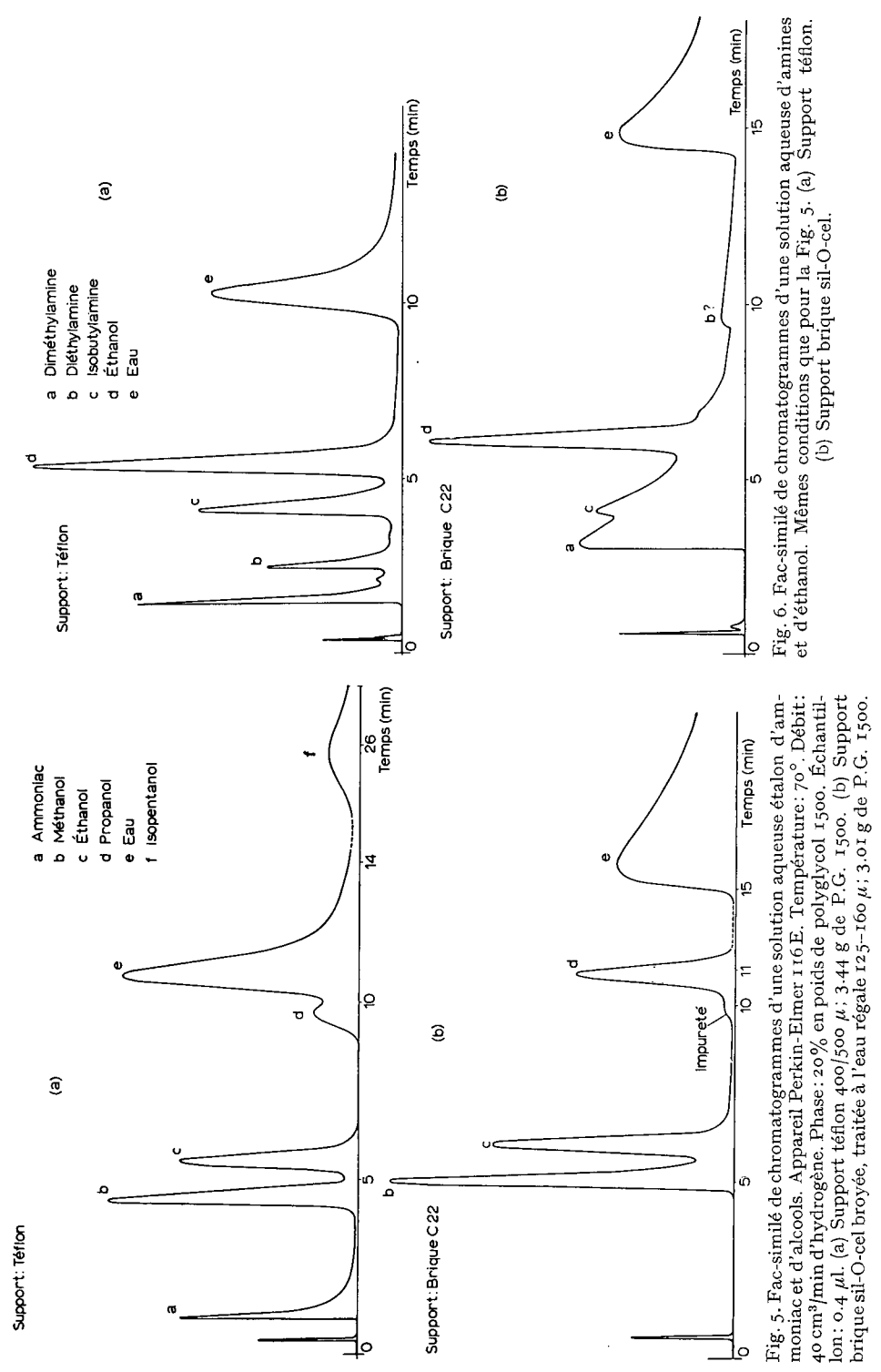

Fig. 5. Fac-similé de chromatogrammes d'une solution aqueuse étalon d'ammoniac et d'alcools. Appareil Perkin-Elmer 116 E. Température: 70°. Débit: 40 cm³/min d'hydrogène. Phase: 20% en poids de polyglycol 1500. Échantillon: 0.4 µl. (a) Support téflon 400/500 µ; 3.44 g de P.G. 1500. (b) Support brique sil-O-cel broyée, traitée à l'eau régale 125–160 µ; 3.01 g de P.G. 1500.

Fig. 6. Fac-similé de chromatogrammes d'une solution aqueuse d'amines et d'éthanol. Mêmes conditions que pour la Fig. 5. (a) Support téflon. (b) Support brique sil-O-cel.

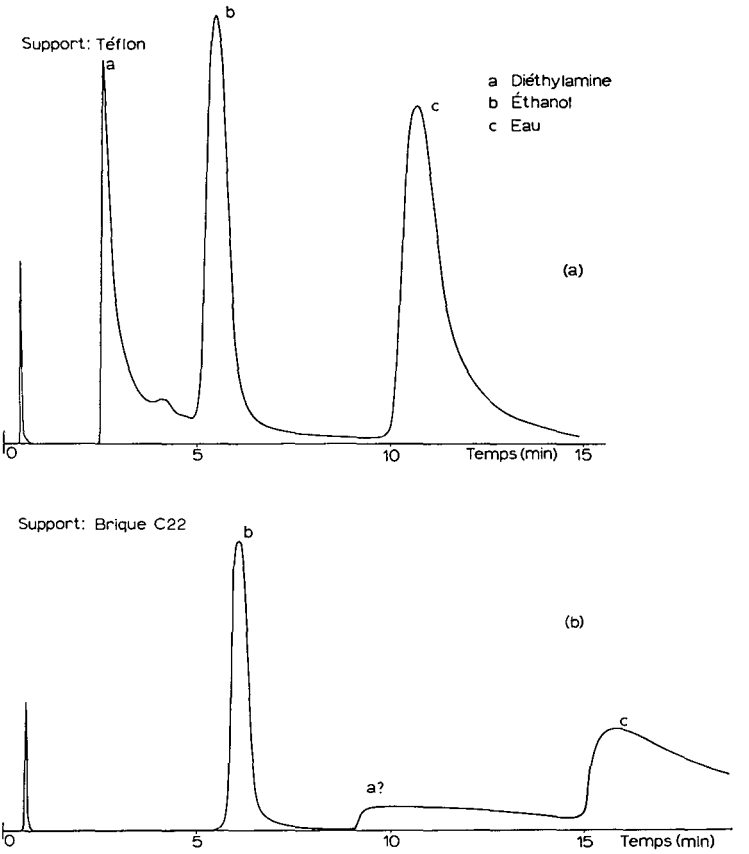

Fig. 7. Fac-similé de chromatogrammes d'un mélange de diéthylamine d'éthanol et d'eau. Mêmes conditions que pour la Fig. 5. (a) Support téflon. (b) Support brique sil-O-cel.

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RÉSUMÉ

Nous avons montré qu'il était possible d'obtenir, en utilisant la poudre à mouler de téflon comme support, des colonnes de chromatographie gaz–liquide dont l'efficacité, bien reproductible, est, pour des solutés peu polaires, au moins la moitié de celle des colonnes courantes, préparées avec des supports traditionnels. Ces nouvelles colonnes permettent par contre de réaliser des séparations qu'il était jusqu'à présent très difficile, voire impossible, d'obtenir: leur utilisation est à recommander pour l'analyse de mélanges contenant des composés très polaires.

SUMMARY

By using polytetrafluoroethylene molding powder it is possible to obtain reproducible

gas–liquid chromatographic columns. The efficiency for strongly polar solutes is about the same as for non-polar ones, and at least half, or more, of that obtained with conventional supports. With these columns it is now possible to obtain separations which were hitherto very difficult or impossible: these columns are very useful in the analysis of mixtures of strongly polar compounds.

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GAS–LIQUID CHROMATOGRAPHY IN QUALITATIVE ANALYSIS

PART IV. A SIMPLE METHOD OF CALCULATING R_{x9} VALUES AT ELEVATED COLUMN TEMPERATURES

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INTRODUCTION

The R_{x9} method[1] of presenting relative retention ratios requires a knowledge of the retention of the *n*-alkane used as standard relative to that of *n*-nonane. At moderate column temperatures this can be obtained graphically from the plot of the logarithm of retention against carbon number (the "log plot") for the calibration series of *n*-alkanes[2]. At high column temperatures a graphical technique is impracticable and as "least squares" procedures are too time-consuming for routine application a simple method of calculating R_{N9} values (*i.e.* the retention of *n*-alkanes relative to *n*-nonane) from the slope of the "log plot" has been evolved with virtually no loss of precision.

PROCEDURE AND RESULTS

The method for obtaining R_{x9} values requires the following steps:

(1) Calculation of the column dynamic dead volume.

(2) Evaluation of a precise value of b (the slope of the "log plot") which is required to calculate R_{N9} values of the *n*-alkane standards from $\log R_{N9} = b(N - 9)$.

(3) Use of this data to calculate R_{x9} from $R_{x9} = R_{N9} \times R_{xN}$.

Calculation of column dead volume

The column dead volume must be calculated since it has been shown that even with detectors which do give an air peak, the volume to the air peak is not coincident with the dynamic column dead volume[3]. After recording the chromatogram of a suitable calibration series of *n*-alkanes[2] the column dead volume can be calculated using either of three methods.

(1) The static column interstitial volume can be calculated using the expression:

$$d_0 = \frac{2.54\,SV}{60\,F}\left\{1 - \frac{B_p}{D_p} - \frac{PB_p}{D_L\,(100 - P)}\right\}$$

where

d_0 = distance from injection point to dead volume point on the recorder chart in cm,
S = the recorder chart speed in in./h,
V = volume of the column packed,

F = carrier gas flow rate in cm³/min at the average column pressure,
B_p = bulk density of the support,
D_p = true density of the support,
P = percentage (w/w) of stationary phase,
D_L = density of stationary phase.

This is subtracted from the observed retention distances of the *n*-alkanes measured between the point of injection and the peak maximum on the recorder chart. The adjusted retention distances are then plotted against carbon number using 2 or 3 cycle log-linear graph paper. Owing to the fact that the static and dynamic column dead volumes may not be identical (due to stream-lining of the gas flow for instance) a further correction is applied computed from the expression:

$$\delta a = \frac{2.303 \sum \delta \log R}{\sum 1/R_{\text{ext.}}}$$

where

$\delta \log R = \log R - \log R_{\text{ext.}}$,
R = retention distance from the assumed dead volume,
$R_{\text{ext.}}$ = value obtained from the extrapolation of the linear part of the graph,
δa = the further correction to the retention distance to be subtracted or added to the first set of adjusted distances depending upon the sign of the deviation from the linear "log plot"[4].

The above expression is more convenient than that previously reported[2] and is more precise in the presence of random scatter. When the further adjusted retention distances are replotted they are generally found to give a straight line, however occasionally a second correction may be necessary to linearise the "log plot" (for example in the unlikely event of the calculated static dead volume being seriously in error).

(2) Alternatively the "log plot" may be linearised by graphical trial and error.

(3) Finally, the method reported by GOLD[5], which uses the retentions of three compounds of the same homologous series, which according to the author need not necessarily be *n*-alkanes, may be used. Care must obviously be exercised when using this method since in the presence of random deviations serious errors will result. Whereas GOLD states that his method can be used with any 3 members of a homologous series we have found that it is necessary to take the retentions of compounds near the beginning, middle and end of the calibration series in order to get consistently reliable results, by comparison with the other linearisation methods. For instance, with the calibration series[2] of *n*-alkanes C_{14} to C_{26} we would recommend the use of C_{16}, C_{20} and C_{24}. Since the lower polar homologues deviate systematically from linearity[6] dead volumes calculated by GOLD's method using non-hydrocarbon homologous series will be far less reliable.

Though the three methods yield comparable results the first is to be preferred on grounds of higher precision.

Calculation of R_{N9} values

The retentions of *n*-alkanes are given by the expression:

$$\log R = a + bN \qquad (1)$$

where R is the retention measured in any convenient units, N the carbon number, and a and b are constants. When the standard is n-nonane, as with the R_{x9} method, $\log R$ for n-nonane is zero by definition, so that:

$$\log R_{N9} = b(N - 9) \tag{2}$$

Thus knowledge of the slope of the "log plot" (the b factor) enables one to calculate all R_{N9} values by means of the simple equation (2) so obviating the necessity of using very large graph paper at high temperatures.

The procedure for calculating b, which is illustrated by determinations on neopentyl glycol succinate polyester stationary phase at 160° and 183°, is as follows:

(1) The retentions of an even number of consecutive n-alkanes are listed in order of increasing retention; if the calibration series involved an odd number of n-alkanes the first member is neglected as the errors in measuring retention are generally greater with the shorter retention components.

(2) The list is divided into two and the n-alkanes designated Rm_1, $Rm_2 \ldots Rm_n$, Rn_1, $Rn_2 \ldots Rn_n$ in order of increasing retention.

(3) The logarithms of the adjusted or corrected retentions are then recorded (using 5 figure tables) and the slope b calculated from:

$$\frac{\log Rn_1 - \log Rm_1}{\frac{\mathcal{N}}{2}}, \frac{\log Rn_2 - \log Rm_2}{\frac{\mathcal{N}}{2}}, \cdots \text{etc.},$$

where $\mathcal{N}$ is the even number of n-alkanes in the calibration series. The range of n-alkanes needed for precise results is given in Part II of this series[2].

A consideration of the propagation of random errors reveals that the ratio of the probable error in b using the "simple mean" (as derived above) and the least squares method is defined by:

$$\frac{Pb^{\wedge}}{Pb^{\square}} = \frac{2}{\sqrt{3}} \frac{\sqrt{(n-m)(n-m+2)}}{(n-m+1)}$$

where

$Pb^{\wedge}$ = the probable error in b by the simple mean method,
$Pb^{\square}$ = the probable error in b by the least squares method,
n = the carbon number of the last member of the calibration series,
m = the carbon number of the first member used in the calculation of b.

This ratio lies between 1.0 when $n - m$ is small and 1.15 when $n - m$ is large. Thus, the insignificant improvement in the accuracy of b by use of the least squares method instead of the "simple mean" does not justify the use of this tedious technique.

The "weighting" of observations is frequently somewhat arbitrary, thus in order to calculate the best value for b we propose to weight directly according to retention. The weighting for the shorter retentions should more rigorously be replaced by retention squared whilst the higher retentions should have unit weighting as experience has shown that the reproducibility of highly retained materials comes down to a fairly constant level. Similarly, in correcting for column dead volume the expression:

$$\delta a = \frac{2.303 \sum \delta \log R}{\sum 1/R_{\text{ext.}}}$$

TABLE I

R_{N_9} VALUES FOR *n*-ALKANES ON NEOPENTYL GLYCOL SUCCINATE POLYESTER AT 160°

Carbon number	*Observed retention (cm)*	*Corrected retention (cm)*	*Logarithm of corrected retention*	*b*	*Rm*	*b* × *Rm*	R_{N_9} *b* = 0.1704 ± 0.0001*	R_{N_9}** *b* = 0.1702 ± 0.0001*
12	1.92	1.60	0.20412				3.25	3.24
13	2.67	2.35	0.37107				4.80	4.79
14	3.82	3.50	0.54407				7.11	7.10
15	5.48	5.16	0.71265	0.16968	1.60	0.27150	10.53	10.51
16	7.93	7.61	0.88138	0.17042	2.35	0.40049	15.59	15.53
17	11.62	11.30	1.05308	0.17036	3.50	0.59626	23.08	22.99
18	17.00	16.68	1.22220	0.17016	5.16	0.87802	34.17	34.02
19	25.07	24.75	1.39358	0.17069	7.61	1.29895	50.58	50.35
20	37.15	36.83	1.56620	0.17053	11.30	1.92699	74.9	74.5
21	54.47	54.15	1.73360				110.9	110.3
22	80.77	80.45	1.90553		$\Sigma = 31.52$	$\Sigma = 5.37221$	164.2	163.1
23	119.50	119.18	2.07622				243.0	241.4

$$\bar{b} = \frac{5.3722}{31.52} = 0.17044 \pm 0.00009^{*}$$

* ± refers to the probable standard deviation of $\bar{b}$ (*i.e.* P_b) calculated from the expression $P_b^2 = \frac{\sum W^2 \delta b^2}{[\sum W]^2}$ where W = weighting factor for each observation and δb = deviation of each observation from the weighted mean.

** Calibration carried out on the same column 4 h later. The difference between the two values of *b* thus determined is consistent with the calculated probable standard deviation of $\bar{b}$ in each case thus implying a satisfactory constancy of experimental conditions.

TABLE II

R_{N9} VALUES FOR *n*-ALKANES ON NEOPENTYL GLYCOL SUCCINATE POLYESTER AT 183°

Carbon number	*Observed retention (cm)*	*Corrected retention (cm)*	*Logarithm of corrected retention*	*b*	*Rm*	*b* × *Rm*	R_{N9} $b = 0.1498 \pm 0.0001$	R_{N9}* $b = 0.1495 \pm 0.0001$
14	2.15	1.85	—					
15	2.90	2.60	0.41497				5.61	5.59
16	4.00	3.70	0.56820				7.92	7.89
17	5.50	5.20	0.71600				11.19	11.13
18	7.65	7.35	0.86629	0.14999	2.60	0.38998	15.79	15.70
19	10.62	10.32	1.01368	0.14946	3.70	0.55300	22.29	22.16
20	14.95	14.65	1.16584	0.14969	5.20	0.77840	31.48	31.26
21	20.95	20.65	1.31492	0.14977	7.35	1.10082	44.44	44.11
22	29.47	29.17	1.46494	0.15011	10.32	1.54914	62.75	62.23
23	41.43	41.13	1.61416	0.14977	14.65	2.19413	88.6	87.8
24	58.50	58.20	1.76492				125.1	123.9
25	82.40	82.10	1.91434		$\Sigma = 43.82$	$\Sigma = 6.56547$	176.6	174.8
26	116.30	116.00	2.06446				249.4	246.6
							352.1	347.9

$$\bar{b} = \frac{6.56547}{43.82} = 0.14983 \pm 0.00008$$

* Calibration carried out 4 h later; again the difference between the two values of *b* is consistent with the calculated probable standard deviation of $\bar{b}$ in each case.

which implicitly weights as the reciprocal of retention has been chosen rather than the more rigorous:

$$\delta a = \frac{2.303 \sum \frac{\delta \log R}{R_{ext.}}}{\sum 1/R^2_{ext.}}$$

on the grounds of computational convenience.

The "weighted mean" of b is given by the expression:

$$\bar{b} = \frac{\sum b \frac{Rm \cdot Rn}{Rm + Rn}}{\sum \frac{Rm \cdot Rn}{Rm + Rn}} \tag{3}$$

where Rm and Rn are the retentions of the two n-alkanes used in each case. This simplifies to:

$$\bar{b} = \frac{\sum b \cdot Rm}{\sum Rm} \tag{4}$$

since the ratio of Rm/Rn is effectively constant.

(4) The "weighted" mean value of b obtained from eqn. (4) is then used to calculate R_{N9} values using eqn. (2). The results are shown in Tables I and II.

(5) R_{x9} values for any unknown can be calculated using the n-alkanes as standards from:

$$R_{x9} = R_{xN} \times R_{N9} = R_{xN} \times 10^{b(N-9)}$$

where

R_{x9} = retention of the material being measured relative to n-nonane,
R_{xN} = retention of material being measured relative to the internal standard,
R_{N9} = retention of the standard relative to n-nonane.

The accuracy of retention values obtained using this technique are clearly dependent upon the precision with which b can be measured. The data in Tables I and II indicate that when suitable calibration series of n-alkanes are used with sensibly constant operating conditions R_{x9} values can be obtained with a reproducibility of within 1 % even at these elevated temperatures.

The factors which influence the long term reproducibility of b are to be investigated shortly.

EXPERIMENTAL

Chromatograms were obtained using an apparatus consisting of glass columns (5 ft. long and 4 mm i.d.) with a modified flame ionisation detector[7]. The carrier gas was a 3:1 (by volume) mixture of hydrogen and nitrogen. The column was packed with a 20% w/w mixture of neopentyl glycol succinate polyester (Applied Science Laboratories) and acid-washed (1 % phosphoric acid) 60–72 mesh celite. The column was heated by means of a vapour jacket; cyclohexanol (160°) and 2-ethylhexanol (183°). In order to minimise the acid catalysed dehydration of the alcohol used in the vapour jacket ~ 2 % of triethanolamine was added along with ~ 0.01% of topanol O.C as antioxidant. Samples (~ 10 γ) were added as ~ 5 % w/v solutions in n-heptane by

means of stainless steel capillary pipettes. The *n*-alkanes were obtained commercially except for certain members between C_{20} and C_{26} which were kindly supplied by Mr. C. G. SCOTT of Lobitos Oilfields Ltd.

SUMMARY

A method of calculating R_{N9} values for the higher *n*-alkanes using the slope of the "log plot" for the *n*-alkanes is described.

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COUNTERCURRENT ELECTROPHORESIS ON PAPER

VII. MEASUREMENT OF ELECTROPHORETIC MOBILITIES ON PAPER BY MEANS OF THE FRONTAL METHOD*

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INTRODUCTION

In a number of studies attempts have been made to work out an exact method for measuring electrophoretic mobilities on paper (for reviews of these methods see the various monographs, *e.g.* ref. 1). The mobilities are measured by the rate of migration of the zones of the given substances. We can call this technique the "elution technique", because we consider that the main difference between chromatography and electrophoresis on paper lies only in the nature of the force which causes the motion of the substance being studied: in chromatography it is the flow of the solvent, in paper electrophoresis the electric field. The authors of the papers mentioned above try to diminish the flow of the electrolyte in the paper. Under conditions where elimination of the electrolyte flow is impossible they introduce appropriate corrections. Some authors consider that if the migration rate of the zone moving along the strip remains constant this proves that the disturbing flow has been eliminated. In the preceding paper[2], however, we have shown that the path can be a linear function of time only when the expression for the distribution equilibrium which results from the electrophoretic process on the paper, is a linear equation. We shall call this expression the "electrophoretic distribution isotherm". A constant migration rate can occur in spite of a nonlinear isotherm, but only by the fortuitous compensation of various factors.

When the distribution isotherm is nonlinear the movement of the zone depends on the concentration. From a single experiment it is quite impossible to conclude whether the change of velocity is caused by the nonlinearity of the distribution isotherm or by other factors. The dependence of the mobility on the concentration is another reason for considering the measurement of the zone migration rate unsuitable for the required purpose. To give values of mobilities without the corresponding values of the concentration is therefore meaningless. It is not easy to determine the concentration when the "elution" method is used, and it is also difficult to determine theoretically which point on the concentration profile of the zone is most suitable for measurement.

The purpose of this paper is to show that the frontal technique applied to the measurement of electrophoretic mobilities is able to give correct values at any concentration.

* For Part VI see ref. 2.

The frontal method has been theoretically worked out for free electrophoresis (see *e.g.* ref. 3). The free mobility of a substance U_0 ($cm^2 \cdot sec^{-1} \cdot V^{-1}$) can be calculated from the relation

$$u_0 = U_0 \kappa / i \tag{1}$$

where u_0 ($cm \cdot sec^{-1}$) is the measured linear velocity of the moving boundary, κ ($\Omega^{-1} \cdot cm^{-1}$) the specific conductivity and i ($A \cdot cm^{-2}$) the current density. κ corresponds to the solution on that side of the boundary where the substance being investigated is present.

Similarly the theory of frontal chromatography has been thoroughly worked out. For the equilibrium case the following equation is valid

$$(\delta x/\delta t)_c = u_s/[1 + q'(c)] \tag{2}$$

where x (cm) is the distance from the upper end of the column, t the time (sec), u_s the linear velocity of the solvent ($cm \cdot sec^{-1}$) and c ($g \cdot cm^{-3}$) the concentration of the substance under consideration in the mobile phase. The distribution isotherm $q(c)$ expresses the amount of the substance in the stationary phase ($g \cdot cm^{-3}$); $q'(c) = dq(c)/dc$.

For the nonequilibrium stationary case and for a distribution isotherm with $q''(c) < 0$ the equilibrium equation is valid in the point $\bar{x}$ defined by the relation (see ref. 4; x and $\bar{x}$ are distances from the end of the column):

$$\int_0^{q(c^0)} x \cdot dq(c) = \bar{x} \cdot q(c^0) \tag{3}$$

When the path travelled is expressed as a distance between two points in both of which the front was already in the stationary state, any point on the concentration profile of the front (any concentration) can be used for this expression. The distance must then be determined always for this point. The integration may be carried out graphically, the concentration profile of the front being known. The value of c° is the value corresponding to the values (*e.g.* of R_F) determined by the frontal method. It is the concentration of the solution entering the chromatographic column.

The velocity u_s is a constant for a homogeneous chromatographic column and the value of $q'(c)$ depends on the concentration only. Therefore the velocity $(\partial x/\partial t)$ is also a function of the concentration only. The distance x_f travelled by the front of the solute in the equilibrium case and the distance $\bar{x}$ in the nonequilibrium stationary case, are then linear functions of time.

The frontal method makes it possible to determine the distribution isotherm of the given substance from measurements at different concentrations c°. In this case the equation of the mass balance takes the following form: $c^\circ(x_s - x_f) = x_f q(c^\circ)$. Here the indices f and s refer to the solute and the solvent respectively. The distance x is expressed in ml of the stationary phase corresponding to the length unit of the column. The following equation is evidently valid

$$q(c^0) = \alpha c^0 \left(\frac{u_s}{u_f} - 1\right) \tag{4}$$

Here α is the porosity. (For calculation of the term α the swelling of the supporting medium must be considered. This question will be treated in another paper.)

By means of the equation given in the previous paper we can apply the equations mentioned above to electrophoresis on paper. We have shown that $U_s = u_s/E = U_0/f$, where E is the potential gradient ($V \cdot cm^{-1}$) and f the tortuosity factor. When all kinds of liquid flow, except the electro-osmotic flow, are eliminated and the observed macroscopic velocity of the front is corrected for electro-osmosis the following equation is valid

$$q_e(c^0) = \alpha c^0 \left(\frac{U_0}{U_{cor.} f} - 1\right) \tag{5}$$

Here q_e is the electrophoretic distribution isotherm, $U_{cor.}$ the mobility of the front corrected for electro-osmosis. The values $U_{cor.}$, q_e and U_0 correspond to the concentration c°.

EXPERIMENTAL

Materials

Whatman No. 4 chromatographic paper was used; its structural parameters were taken from ref. 5.

Acetic acid solutions prepared from the concentrated acid (analytical reagent purity) were used as electrolyte.

The acidic azodye Kashmir Blue T.G. Extra (from Farbenfabriken F. Beyer, Elberfeld, Germany) was used as a sample for the study. The solubility of this dye is 0.092 % in 1 N acetic acid. The contribution of the dye to the conductivity is negligible in comparison with the conductivity of 1 N acetic acid.

Apparatus

The apparatus for measuring the concentration profile of the front was essentially the same as the apparatus described in the previous paper[2], but with the following differences: the vessels were connected by a glass tube with taps to bring the levels to the same height. This permitted exchange of the whole volume from one vessel without opening the wet chamber. The ends of the paper strip were dipped directly into the solution contained in the vessels, and were not wrapped in cellophane membranes.

The detector was moved along the strip and the galvanometer deflections were read. The temperature of the paper strip was measured by a thermistor thermometer. The potential gradient was determined as the voltage between two movable contacts 1 cm apart.

In this method calibration of the detecting arrangement is not necessary. Under the conditions employed the electro-osmotic flow was negligible, as was established by experiments with glucose. The constant velocity of the dye front (see Fig. 3) was evidence of the absence of all other liquid flow. Fig. 1 shows that the zones of the dye in different sections of the strip remained at their original positions after the alternating current was switched on. They broaden only by diffusion (symmetrically).

Experimental technique

After the apparatus and surrounding space had been thermostated the vessels were filled with acetic acid solution and leveled. When leveling was accomplished the

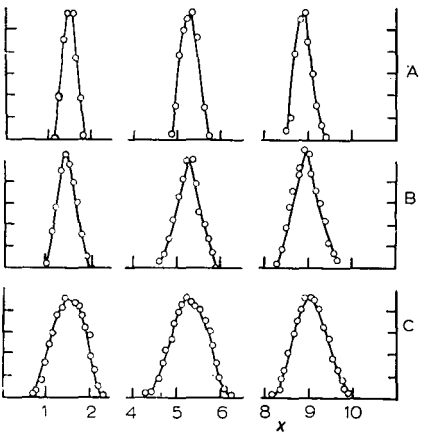

Fig. 1. Change of zone shape with time (alternating current). A = original shapes; B = after 4 h; C = after 8 h.

connecting tube was disconnected. The surface of the defatted glass plate (in the horizontal position) was moistened with 3 ml of acetic acid solution.

The dry chromatographic paper strip from which the impurities had been eluted by 25 ml acetic acid was moistened by dipping it into the solution. Then it was put onto the glass plate.

After the wet chamber had been closed direct current of about 2.40 mA was switched on for 12 hours to establish equilibrium conditions. After this period the acetic acid solution in one vessel was replaced by the same volume of a solution of the dye of known concentration. During the experiment the galvanometer deflection and the potential gradient were measured. An example of the concentration profile thus obtained is shown in Fig. 2. It is evident that this is not the equilibrium but the stationary non-equilibrium case. The shape of the profile does not alter during the movements of the front.

The dependence of the distance travelled on the time is linear as can be seen in Fig. 3. The potential gradient reached a constant value along the strip with an ac-

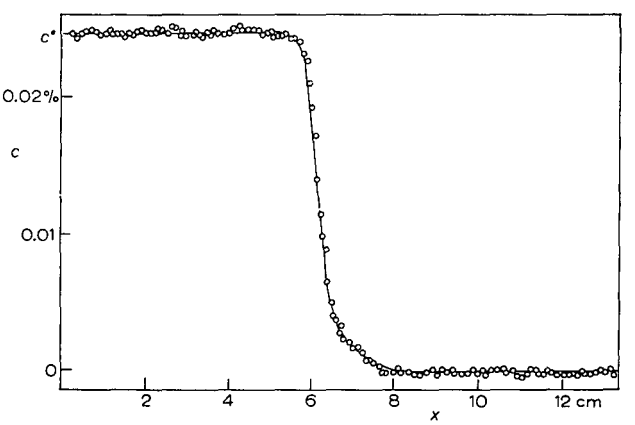

Fig. 2. Example of concentration profile of the front.

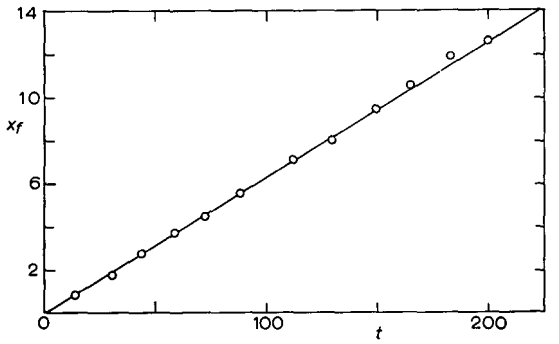

Fig. 3. Relationship between the distance x_f (cm) travelled by the front of the dye and the time t (min) for 0.025 % Kashmir Blue in 1.0 N acetic acid.

curacy of ± 2 %. Under the conditions employed the chromatographic paper contained 2.52 g of water per 1 g dry paper. A value of 0.65 was taken for swelling water. The tortuosity factor was taken equal to 1.30. The temperature of the paper strip was 20°, the temperature of the surrounding space of the apparatus was kept at about 27°.

All measurements were carried out at least three times. Even for the lowest concentrations the experimental error was not greater than 5 %.

All measurements were performed in 1.0 N acetic acid. As has already been stated the contribution of the dye to the overall conductivity is negligible. Several experiments were performed with a dye that is a better conductor, *viz.* Guinea Red 4B, in 0.1 N acetic acid. In this case the potential gradient changes simultaneously with the concentration (see Fig. 4). These changes could be used to indicate the position of the front for colourless substances.

The mobility in free solution was measured in the same way as in the previous paper[2]. The experimental error was ± 5 %.

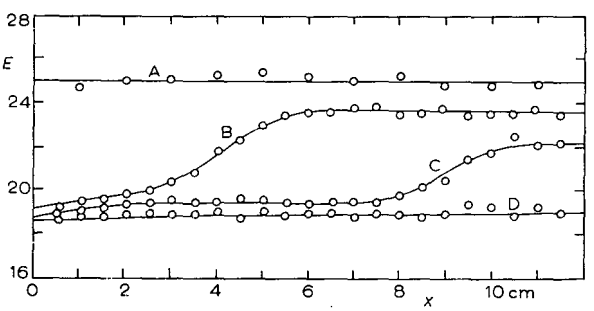

Fig. 4. Potential gradient E (V/cm) along the strip for 0.025 % Guinea Red in 0.1 N acetic acid. The curves A, B, C and D were measured after 0, 2, 4 and 8 h respectively.

RESULTS

With the arrangement described above the dependence of the linear velocity of the front U on the potential gradient E give a straight line going through the origin (Fig. 5). The electrophoretic mobility of the dye on paper, $U = u/E$ (under the conditions employed no correction for any flow in the paper need be made and $U = U_{cor.}$),

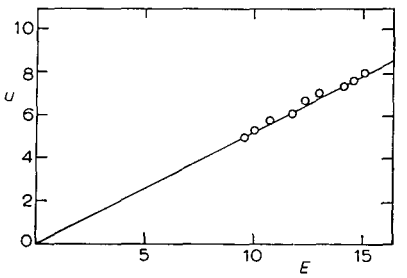

Fig. 5. Relationship between the front velocity u (cm/min) and the potential gradient E (V/cm) for 0.025 % Kashmir Blue in 1.0 N acetic acid.

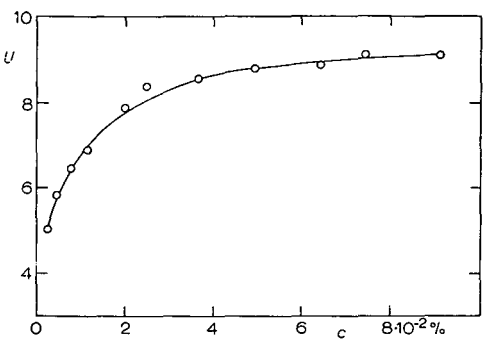

Fig. 6. Relationship between the mobility U (10^{-5} cm^2/V. sec) of Kashmir Blue and its concentration c in 1.0 N acetic acid.

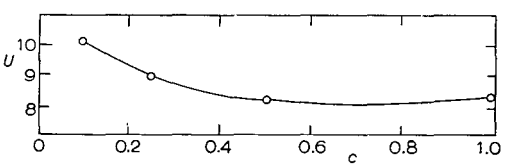

Fig. 7. Relationship between the mobility U (10^{-5} cm^2/V. sec) of the dye and the concentration c (mole/liter) of acetic acid for 0.025 % Kashmir Blue.

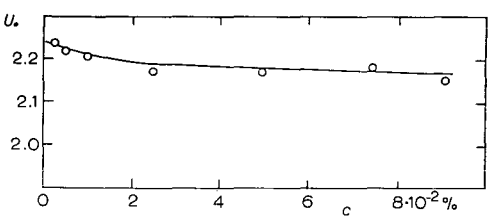

Fig. 8. Relationship between the mobility in free solution U_0 (10^{-5} cm^2/V. sec) of Kashmir Blue and its concentration c (g/liter) in 1.0 N acetic acid.

depends as we expected on the concentration of the dye (Fig. 6). This mobility also depends on the concentration of acetic acid (Fig. 7). The free mobility U_0 is slightly dependent on the concentration (Fig. 8).

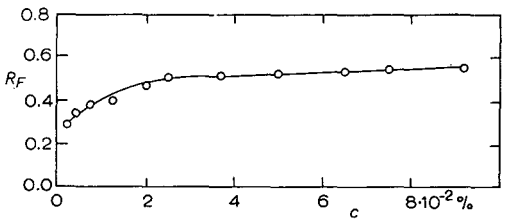

Fig. 9. Relationship between the R_F of Kashmir Blue and its concentration c (g/l)in 1.0 N acetic acid.

It is possible to calculate the factor R_F from the measured values of U and U_0 by means of the relation $R_F = Uf/U_0$. The dependence of R_F on the concentration is plotted in Fig. 9. By means of eqn. (5) the electrophoretic distribution isotherm, $q_e(c)$, can be determined. The function obtained is plotted in Fig. 10.

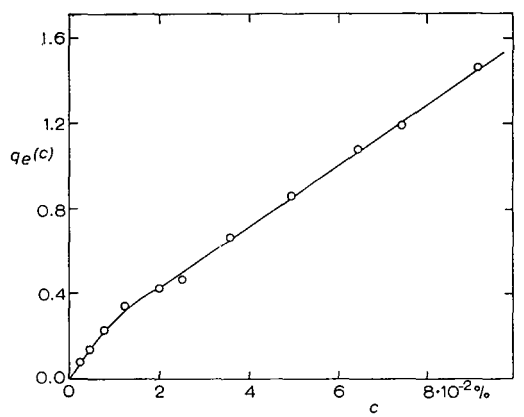

Fig. 10. Electrophoretic distribution isotherm q_e (c) of Kashmir Blue in 1.0 N acetic acid.

CONCLUSIONS

The results obtained show that the frontal method has the expected advantages for the measurement of electrophoretic mobilities on paper. The time dependence of the distance travelled by the front is linear even in the case of a nonlinear distribution isotherm and a nonequilibrium process. Therefore the changes of the migration rate of the front can serve as a sensitive indicator of errors in the experimental arrangement. This method permits the determination of the dependence of the mobility on concentration.

The value of R_F may be calculated from the results obtained. It is possible to compare the values obtained in this way with the values that were determined directly. Agreement of these values would be a further proof of the concepts given in the preceding communication of this series[2]. The dependence of the chromatographic

coefficient R_F on the concentration causes complications similar to those occurring in the case of the electrophoretic mobility and therefore this problem will be studied in the next paper in this series.

The mobility measurements give also the values of the electrophoretic distribution isotherms. Application of the GLUECKAUF method[6] to electrophoresis[2] also gives the values of this function. Thus these methods supplement each other. The GLUECKAUF method is suitable for smaller concentrations, while the frontal method is better for higher concentrations. The fact that the mobilities are independent of the potential gradient provides evidence that the electrophoretic distribution isotherm does not depend on the potential gradient in the range of concentrations and potential gradients employed.

Therefore the frontal method is important for the precise measurement of the true mobilities and their dependence on the concentration and for the verification of various opinions about the nature of the separation processes in electrophoresis on paper.

SUMMARY

The frontal method of measuring chromatographic distribution isotherms was applied to paper electrophoresis. Measurements were performed for a model coloured substance. Concentration dependence of free electrophoretic mobility, of electrophoretic mobility on paper, and of R_F were obtained.

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A NEW ARGON IONIZATION DETECTOR FOR GAS CHROMATOGRAPHY

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INTRODUCTION

During recent years continuing efforts have been directed towards the development of various types of ionization detectors for gas chromatography. Two of these, namely the argon β-ray and the flame ionization detector, are extensively used because of their extremely high sensitivity to the component gases.

The argon β-ray ionization detector, which was developed by LOVELOCK in 1958[1], is based on the principle that organic molecules are ionized by collision with metastable argon atoms. It has been shown that the reactions taking place within the ionization chamber may be represented as follows:

$$\text{Argon} + \alpha\text{- or } \beta\text{-rays} \longrightarrow \text{Argon}^+ + \text{e (primary)}$$
$$\text{Argon} + \text{e} + \text{high voltage} \longrightarrow \text{Argon}^* \text{ (metastable)} + \text{e}$$
$$\text{Organic vapor} + \text{Argon}^* \longrightarrow \text{Organic vapor}^+ + \text{e} + \text{Argon.}$$

The emergence of an organic vapor from the chromatographic column can be detected and measured by an increase in the ionization current. In the reactions shown above the role of the radioactive source is principally to provide primary free electrons for securing the stability of the detector.

There are, however, several possible methods of producing primary free electrons, instead of using a radioactive source. For instance, LOVELOCK[2] suggested the possibility of utilizing a subsidiary discharge, such as corona discharge, silent low pressure discharge or radio-frequency discharge. Along these lines he proceeded to devise successfully a spark gap detector, with which he measured the breakdown potential as a function of the vapor concentration. But good stability of the ionization current could not be achieved.

More recently LOVELOCK[3] devised another type of detector, the photo-ionization detector. The details of its performance, however, have not yet been reported.

This paper is concerned with a new argon ionization detector. Its features are the following:

1. Primary electrons are generated by a self-sustained subsidiary discharge.

2. The subsidiary discharge is excited in the scavenging gas stream, ahead of the sensing chamber. A small fraction of electrons generated by this discharge passes into the sensing chamber with the scavenging gas.

3. The field-intensified ionization current is measured in the sensing chamber.

EXPERIMENTAL ARRANGEMENT

General design

It is well known that the ionization properties of a corona discharge are very sensitive to impurities. However, as LOVELOCK[2] has shown, the use of a corona discharge by itself for the detection of impurities has the disadvantage that it is difficult to obtain a reliable and reproducible operation, inasmuch as the emission of electrons from the corona electrode is disturbed by the presence of impurities. Alternatively, it may be possible to utilize a corona discharge subsidiarily for generating free electrons and directing a fraction of them into an ionization chamber, where the field-intensified ionization current is to be measured. However, even when using a corona discharge as such, instability could not be avoided as long as the corona discharge was maintained in the atmosphere of the effluent gas. In this case, the signal current of the sensing electrode depends on the number of primary electrons, that suffers fluctuation as mentioned above.

With this fact in mind, we have attempted to design a suitable instrument and this is shown schematically in Fig. 1. With argon as carrier gas, it has two flow paths in parallel: (1) through a gas regulating valve, the sample-introducing device, column and detector (carrier gas flow), and (2) through a gas regulating valve, subsidiary discharge electrodes and detector (scavenging gas flow). The main feature of this system is the incorporation of the electrodes for the subsidiary discharge in the scavenging gas stream in front of the sensing chamber of the detector. This makes it possible to keep the subsidiary electrodes separated from the effluent gas and thus set up a stable discharge. A fraction of electrons generated by this discharge falls

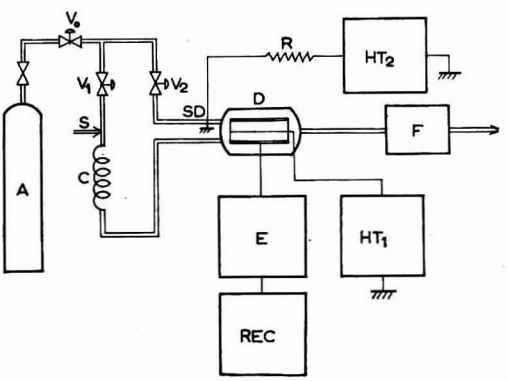

Fig. 1. Schematic diagram of apparatus. A = Argon cylinder; V_0, V_1, V_2 = Gas regulating valves; S = Sample introducing device; C = Column; SD = Subsidiary discharge electrodes; D = Detector; F = Flow meter; HT_1 = High voltage supply for detector; E = Electrometer; REC = Recorder; HT_2 = High voltage supply for subsidiary discharge; R = Current limiting resistance.

into the sensing chamber with the flow of scavenging gas. The confluence of the carrier and the scavenging gas within the sensing chamber yields an ionization current with a steady level, and hence an increase in ionization occurs when an organic vapor emerging from the chromatographic column enters the chamber.

The detector

The detector used in this preliminary experiment is shown in Fig. 2 and the details

of its construction are given in Fig. 3. For easy construction, the detector housing is made of Kovar glass tube with an outer diameter of 30 mm and a length of 65 mm. The cathode, which also acts as an ion-collector, is a stainless steel cylinder of 20 mm outer diameter and 40 mm length. The anode, which is mounted along the axis of the cylinder, is made of 0.2 mm diameter tungsten wire.

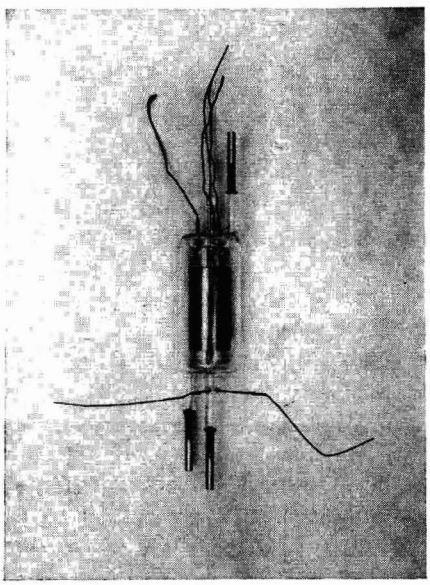

Fig. 2. Photograph of the detector.

The inlet and outlet tubes are sealed to each end of the envelope. They are made of Kovar glass tube, 5 mm o.d., and can be connected to the gas flow system by means of Kovar-to-glass joints.

The electrodes of the subsidiary discharge are incorporated in the tube for the scavenging gas inlet, located at a distance of about 10 mm from the envelope. The electrode construction found to be most convenient consists of two wires inserted opposite each other perpendicular to the gas flow; one of them is a tungsten wire 0.1 mm in diameter and the other a Kovar wire 0.5 mm in diameter.

In order to obtain a stable subsidiary discharge it is important to clean the surface of the electrodes thoroughly. Furthermore, it was found in our experiments that the stability depends critically upon the space between the electrodes, and that

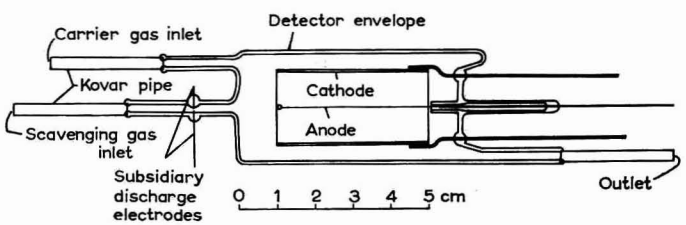

Fig. 3. Cross section of the detector.

fluctuation is more likely to occur with somewhat larger spaces. Therefore it is necessary to mount the electrodes close to each other (*ca.* 0.1 mm). This is probably because a short gap discharge is in essence not very different from low pressure discharge, and thus a stable discharge may be maintained easily at relatively low applied potentials.

RESULTS

Subsidiary discharge

Experiments were first carried out on the characteristics of the subsidiary discharge in order to prove that it is stable enough to serve as a source of primary electrons. Fig. 4 shows current–potential curves at a scavenging gas flow rate of 12 ml/min. The solid curves represent the ionization current as a function of the potential of the high voltage supply, measured with circuits of current-limiting resistance 100 MΩ

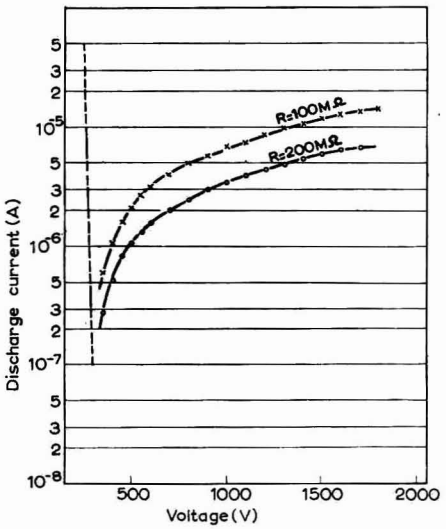

Fig. 4. Current–voltage curves for subsidiary discharge. Solid curves show the discharge current as a function of the potential of the high voltage supply, while the dashed curve shows the discharge current as a function of the inter-electrode potential.

and 200 MΩ, respectively. Until the potential reaches about 300 V, the starting potential of the discharge, there is no appreciable current (10^{-13} A). At a potential slightly higher than 300 V, pulses of discharge start suddenly, accompanied by a faint emission of light. Thereafter, with increasing potentials, the frequency of the intermittent discharge rises very rapidly at first, while later it gradually goes over to a continuous and steady discharge with a current of several micro-amperes.

The variation of the current as a function of the potential is shown more clearly in the current recordings given in Fig. 5, in which it can be seen that stable discharge is established at all potentials above 500 V. Thus in this region of potential the subsidiary discharge may be used as the source of primary electrons.

The manner in which this discharge was maintained is easily understood from the dashed curve in Fig. 4, which shows the relation between current and interelectrode

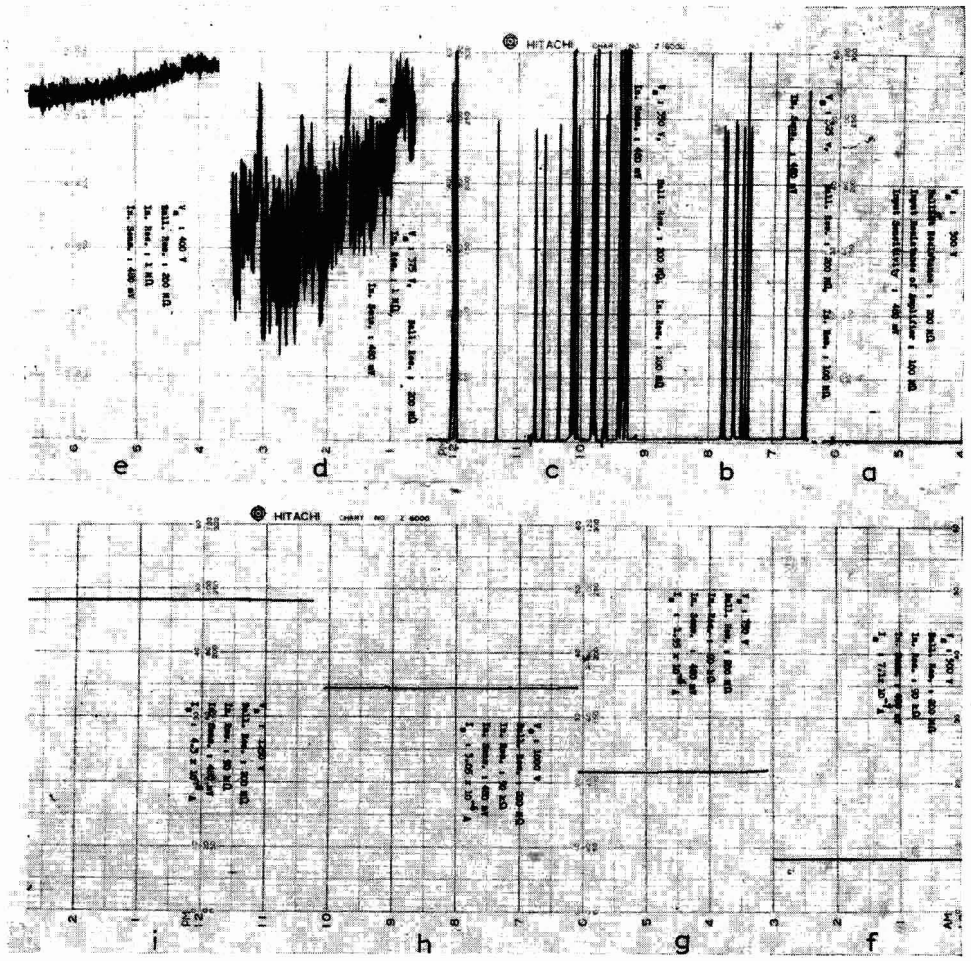

Fig. 5. Recorded curves for subsidiary discharge current. Note stable discharge being established for potentials above 500 V. (a) V = 300 V; (b) V = 325 V; (c) V = 350 V; (d) V = 375 V; (e) V = 400 V; (f) V = 500 V; (g) V = 750 V; (h) V = 1000 V; (i) V = 1250 V.

potential. The vertical rise of the current at a critical voltage proves clearly that at that voltage the immediate breakdown into spark occurred, after which a glow discharge of a small current was maintained without the appearance of corona. Since the glow discharge is less affected by external disturbances and is thus more stable than the corona discharge, it is more suitable as a source of primary electrons. This immediate breakdown into spark occurs only when the gap is less than a certain critical value, called the corona point distance, as KIP[4] had found when investigating corona discharge.

Despite the results of the experiments presented here, it must be borne in mind that, as mentioned in the preceding section, the characteristics of the discharge depend critically upon the dimensions of the electrodes and the space between them. This disadvantage may be overcome by exciting the discharge in an atmosphere of

helium, in which it is more easily and more stably sustained than in argon[5]. A gas chromatographic method using argon as carrier gas and helium as scavenging gas, with a subsidiary discharge excited in the latter, is now under investigation.

The background current

In Fig. 6 the background current flowing between the detecting electrodes is shown. The curves were made at different values of the subsidiary discharge current, the gas flow rate being kept constant (carrier gas flow of 23 ml/min and scavenging gas flow of 12 ml/min).

It can be seen that the background current is about 10^5 times less than the subsidiary discharge current, which indicates that only a very small fraction of electrons, generated by the discharge, can survive long enough to reach the sensing chamber.

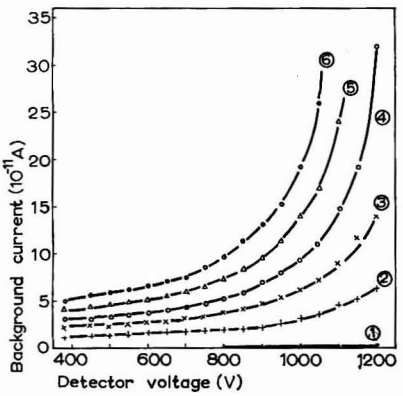

Fig. 6. Curves for background current plotted against detector voltage at various values of the subsidiary discharge current. Carrier gas flow 23 ml/min. Scavenging gas flow 12 ml/min. Subsidiary discharge current: (1) 0; (2) 2.10^{-6}A; (3) 4.10^{-6}A; (4) 6.10^{-6}A; (5) 8.10^{-6}A; (6) 10.10^{-6}A

The much smaller amount of background current than that of the conventional argon ionization detector has an advantage in that even with a high input resistor of the electrometer it can be easily suppressed to zero base line. Moreover, it should be noted that since the ionization efficiency depends on the intensity of the background current, the sensitivity can only be controlled by varying the current of the subsidiary discharge.

In Fig. 7 the background current plotted against the flow rate of the scavenging gas is shown. The linear relationship indicates clearly that primary electrons are not generated in the sensing chamber by photo-ionization, but are conveyed by the scavenging gas. Although the scavenging gas dilutes the concentration of the component gas it is to be expected that the increase in the background current with the gas flow will compensate the reduction of the ionization efficiency.

The stability of the background current depends, of course, mainly on the stability of the subsidiary discharge. It was found that preliminary ageing by running the discharge at a relatively large current is necessary to obtain a good base line stability. Under optimum operating conditions, the background noise can be reduced to about $1 \cdot 10^{-13}$A.

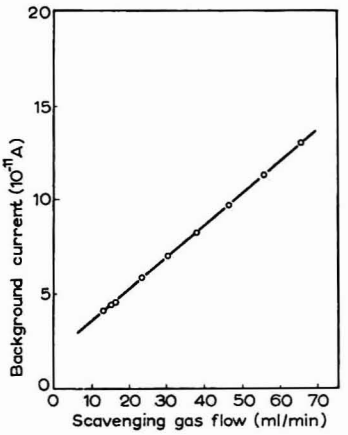

Fig. 7. Background current as a function of the flow rate of the scavenging gas. Detector voltage 600 V. Subsidiary discharge current 6.10^{-6}A. Note the linear increase in the background current with the flow of scavenging gas.

Performance

In order to estimate the sensitivity of the detector, a three-way stopcock was employed to introduce small quantities of test gases. The stopcock shown in Fig. 8 was so designed that one of the paths is connected to a vacuum–gas filling system and, on turning the stopcock, the confined test gases are entrained by the carrier gas. In practice, the test gases were introduced as argon–sample gas mixtures at various concentrations and pressures.

In Fig 9 is shown the sensitivity, in terms of coulombs/g, to propane over a range from 10^{-8} to 10^{-5} g at various values of detector voltage and a subsidiary discharge current of $5 \cdot 10^{-6}$A. It can be seen that the sensitivity is nearly constant over a range up to about 10^{-6} g of propane and that at large quantities it begins to fall. Phenomenologically, as the quantity of propane increases above 10^{-6} g, the peak height tends to saturate gradually, and ultimately peak inversion occurs showing

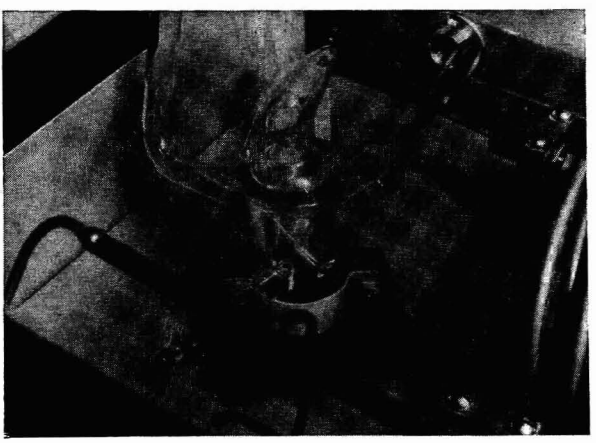

Fig. 8. Three-way stopcock for sample introduction.

double peaking. These quenching effects, as was observed by CONDON *et al.*[6], seem to be characteristics of all types of argon ionization detectors.

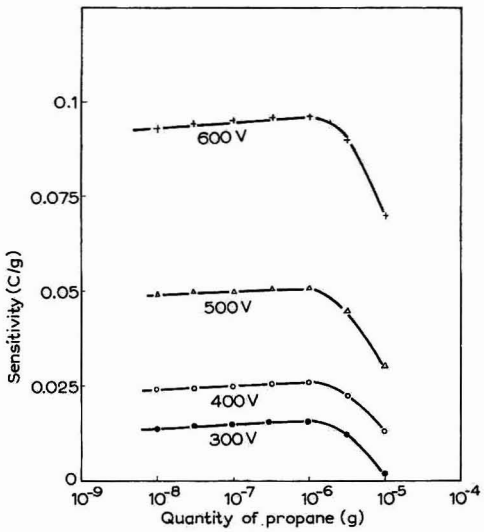

Fig. 9. Sensitivity to propane at various values of detector voltage indicated by the figures on the curves. The subsidiary discharge current is $5 \cdot 10^{-6}$ A.

Fig. 10 shows the sensitivities to C_2–C_5 paraffin hydrocarbons as a function of detector voltage. Each curve was obtained with a quantity of $5 \cdot 10^{-7}$ g at a subsidiary discharge current of $5 \cdot 10^{-6}$A. The results show a progressive increase in sensitivity with increasing length of the carbon chain. Furthermore it should be noted that there are appreciable differences in sensitivity between those gases for which the ionization potential lies below or above the metastable potential of argon.

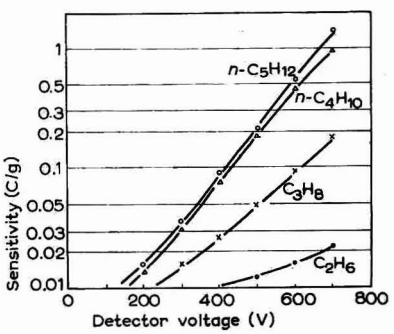

Fig. 10. Sensitivities to $5 \cdot 10^{-7}$ g of ethane, propane, *n*-butane and *n*-pentane plotted against detector voltage. The subsidiary discharge current is $5 \cdot 10^{-6}$ A.

As an example of analyses carried out with the ionization detector, three chromatograms are given in Fig. 11. They were obtained on 0.13 ml samples (gas volume) of

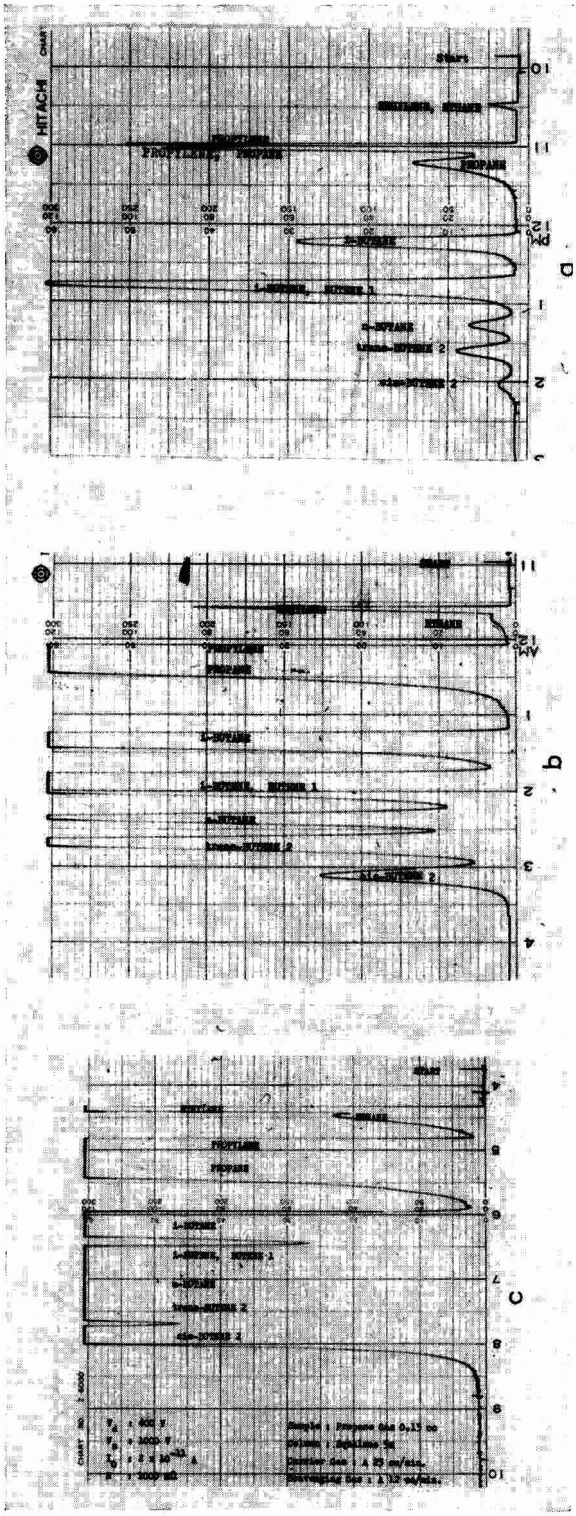

Fig. 11. Chromatograms of 0.13 ml C_2–C_4 hydrocarbon mixtures. (a) Full scale: $5 \cdot 10^{-9}$ A. (b) Full scale: $5 \cdot 10^{-10}$ A. (c) Full scale: $5 \cdot 10^{-11}$ A.

C_2–C_4 hydrocarbon mixtures under the following conditions:

Argon carrier gas	23 ml/min
Argon scavenging gas	12 ml/min
Column	Squalane 5 m
Temperature	20°
Subsidiary discharge current	$4 \cdot 10^{-6}$ A
Background current	$2 \cdot 10^{-11}$ A
Detector voltage	400 V
Input resistance of electrometer	$1 \cdot 10^{9} \Omega$.

The whole appearance of the chromatogram is shown in Fig. 11a. But it must be noted that when the sample contained 50.5% of propane ($1.28 \cdot 10^{-4}$ g) and 22% of propane ($5.35 \cdot 10^{-5}$ g), double peaking occurred. This is an example of overloading of the sample.

For the minimum quantity that can be detected by the detector, CONDON *et al.*[6] gave the following relation:

$$\text{Min. det. (g/sec)} = \frac{2 \times \text{noise (A)}}{\text{signal (A)/quantity (g)/band width (sec)}}$$

As can be seen in Fig. 11b, $6.5 \cdot 10^{-7}$ g *cis*-butene (0.2% of the sample) gives a peak of $2.1 \cdot 10^{-10}$ A with a band width of 70 sec, whereas in Fig. 11c it can be seen that the noise current is $1 \cdot 10^{-12}$A or less. Thus the minimum amount of *cis*-butene that can be detected is $8.9 \cdot 10^{-11}$g/sec. Though this value is slightly inferior to that of LOVELOCK's simple detector[7], we may expect that a great improvement will be obtained with a micro version of our detector.

Effect of the background current

It was found that the detector operated stably even when the background current was reduced to $5 \cdot 10^{-12}$A, the minimum value in our experiments, as long as a stable subsidiary discharge was maintained. But if the high voltage applied to the discharge electrode was reduced to zero, the operation was not satisfactory, because no primary electrons could exist in the chamber. Under these circumstances no peaks were observed at a detector voltage below 1000 V. If the voltage was increased further, peaks corresponding to easily ionizable components appeared sharply. As an example, a chromatogram obtained on the same sample is shown in Fig. 12.

The results of this analysis seem to contradict to those obtained by HAAHTI *et al.*[8] who reported that their detector operated successfully even without a radioactive source. It should, however, not be overlooked that in the absence of a radioactive source they observed an appreciable background current, though it is not clear where the primary electrons came from.

It is clear from what has been said above that the existence of primary electrons is essential. As regards the optimum intensity and the minimum safe operating intensity of the background current, not enough data are at hand at present to permit of discussion.

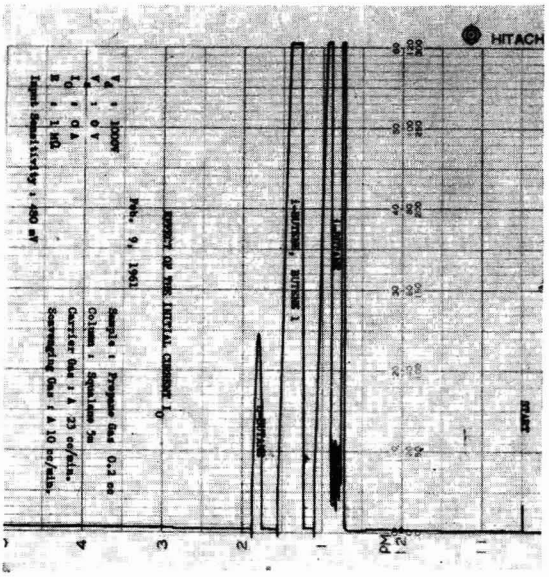

Fig. 12. Chromatogram of 0.2 ml C_2–C_4 hydrocarbon mixtures. Background current is 0.

ACKNOWLEDGEMENT

The author wishes to express his appreciation to Dr. M. SUGAWARA and Dr. S. TAKEI for their encouraging support and guidance throughout these experiments, and to Mr. M. YAMAMOTO for his valuable discussions and his examination of the manuscript.

SUMMARY

This paper is concerned with a new argon ionization detector. Its features are the following:

1. Primary electrons are generated by a self-sustained subsidiary discharge, instead of by means of a radioactive substance.

2. The subsidiary discharge is excited in the scavenging stream, ahead of the sensing chamber. A small fraction of electrons generated by this discharge passes into the sensing chamber with the flow of the scavenging gas.

3. Field-intensified ionization current is measured in the sensing chamber.

Details of the construction of the detector, the characteristics of the subsidiary discharge and performance of the detector are described.

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PAPER ELECTROPHORESIS THROUGH DIALYSING BARRIERS

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INTRODUCTION

Paper eletrophoresis has been successfully used for the separation of low-molecular weight organic compounds, suchs as vitamins, sugars, polyalcohols, purine and pyrimidine bases, amino acids, etc., and of various inorganic compounds[1].

In many instances, however, such as when these compounds are being studied in blood specimens, the samples must be previously deproteinized. The usual deproteinization procedures must frequently be avoided, since they can lead to the loss of the compounds mentioned. Hence the technique of centrifugal–ultrafiltration was devised to separate unstable, low-molecular weight substances from blood proteins[2].

A further complication arises when the specimens to be examined are very small such as blood samples drawn from mice. In these cases deproteinization and electrophoretic separation of low-molecular weight compounds were achieved simultaneously by means of the intercalation of dialysing barriers in the electrophoresis paper strip.

MATERIALS AND METHODS

Paper electrophoresis was carried out by the hanging strip method, using a Shandon equipment. The conditions (buffer, voltage and time) were chosen according to the case.

The dialysing barriers are prepared as follows: a starting line is drawn with a soft pencil at the middle of a 4 × 37 cm filter paper (Macherey-Nagel No. 261) strip. Two other lines are drawn parallel to the starting line, at a distance of 0.5 cm from each side of it and similarly spaced from the borders of the strip. Along them a 1 mm slit is cut by means of a razor blade. Two drops of collodion (4% w/v in ethyl ether–ethanol, 3:1) are then left to run over the slits and the solvent is evaporated at room temperature for a few minutes; the apertures are thus covered with a semi-permeable membrane.

The paper strip so prepared is soaked in the chosen buffer for about half an hour, excess buffer is blotted out with filter paper and the sample to be studied is applied over the starting line, as usual. Four V-shaped segments are then cut from the paper

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at the end of the slits, leaving two 1 mm wide collodion bridges as the sole connections between the three segments of the strip, as can be seen in Fig. 1.

This set-up is placed in the electrophoretic chamber, and a chosen potential is

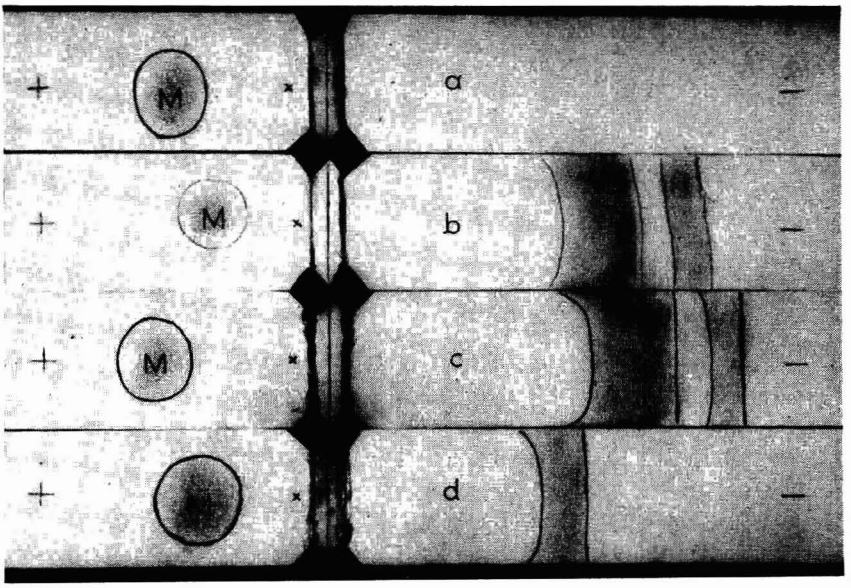

Fig. 1. Paper electrophoresis (veronal–acetate buffer, pH 9.0, 330 V/4 h) through dialysing barriers of (a) mouse serum, (b) streptomycin, (c) mouse serum plus streptomycin and (d) serum from a mouse previously injected with streptomycin. M = marker (bromothymol blue).

applied during a suitable time, which is usually double that needed for the same separation by routine methods. The collodion bridges act as dialysing barriers, through which only the electrically charged, low-molecular weight compounds will pass.

RESULTS AND DISCUSSION

The above method was tried successfully for the separation of sugars, amino acids and antibiotics from blood samples. Fig. 1 shows the result of the barrier-electrophoresis (veronal–acetate buffer pH 9: $u = 0.06/330$ Volts; 4 h) of mouse blood serum drawn 5 min after the intravenous injection of 4 mg streptomycin sulphate and prepared as described previously[3], comparing it with those found for a standard solution of streptomycin, a sample of serum from an untreated mouse and an identical sample with streptomycin added. Guanidine compounds were localized by the Sakaguchi reaction, as described by Wu[4], and proteins were stained by bromophenol blue–zinc sulphate[5].

As the electrical conductivity of the dialysing barrier is not constant, a suitable marker (M) must be spotted on the paper strip, in order to evaluate the displacement of the compound being studied. In the present case a drop of an alcoholic solution of bromothymol blue (0.1% w/v) was spotted in the middle of the paper strip, 0.5 cm to the anode side of the barrier. Streptomycin and the dye migrate in opposite directions, and thus the antibiotic can be assayed colorimetrically free from interfering substances, after elution from the paper.

ACKNOWLEDGEMENTS

The present work was carried out at the Central Laboratory of Tuberculosis in collaboration with the Institute of Phthisiology and Pneumology of the University of Brazil. One of the authors (R.C.R.B.) received a grant from the National Research Council of Brazil.

SUMMARY

The technique of paper electrophoresis through dialysing barriers was devised to separate low-molecular weight, electrically charged compounds from proteins. It is based on the intercalation of collodion bridges in the electrophoresis paper strip, one at each side of the starting line, the displacement of proteins being thus barred while smaller molecules move freely.

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DETECTION OF TUMOUR-INHIBITING MANNITOL DERIVATIVES BY MEANS OF PAPER CHROMATOGRAPHY

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Research work concerning the mechanism of the action by which anti-tumour agents produce their inhibitory effect has made it necessary to separate and analyse both the original substance and its decomposition products. Owing to its sensitivity, paper chromatography seems to be suitable for the identification of those agents that give a colour reaction. Degranol (1,6-bis(β-chloroethylamino)-1,6-dideoxy-D-mannitol dihydrochloride)[1] and Mannitol-Myleran (1,6-dimethanesulphonyl-D-mannitol)[2,3] both mannitol derivatives, were justifiably expected to lend themselves for such experiments. Runs were performed on Schleicher & Schüll paper No. 2043/b with ascending development. The test substances were dissolved in distilled water and transferred to the paper as samples containing 50 to 200 μg. Mannitol was run at the same time as model substance. Various solvent mixtures and spraying reagents were compared in the course of our experiments. It was found that both Degranol and Mannitol-Myleran were detectable by means of the procedures employed (Fig. 1). The R_F values obtained with various solvent systems are listed in Table I. With *n*-propanol–ethyl acetate–water (7:1:2) the highest R_F values were obtained. While the R_F values of Degranol and mannitol were of approximately the same order of magnitude, the R_F of Mannitol-Myleran proved to be significantly higher.

TABLE I

R_F VALUES OF MANNITOL, DEGRANOL AND MANNITOL-MYLERAN (MM) IN VARIOUS SOLVENT SYSTEMS

	R_F			
	1	*2*	*3*	
Degranol	0.12	0.15	0.25	0.06
MM	0.45	0.43	0.62	0.57
MM-decomposition product	0.19	0.16	0.28	0.18
Mannitol	0.18	0.17	0.28	0.20

1 = *n*-butanol–acetic acid–water (5:2:1)[4]; 2 = *n*-butanol–ethanol–water (4:1:5)[5]; 3 = *n*-propanol–ethyl acetate–water (7:1:2)[6]; 4 = *tert.*-isoamyl alcohol–*n*-propanol–water (8:2:3)[7].

Preliminary investigations had proved that spraying reagents containing an indicator were best suited for the demonstration of tumour-inhibiting mannitol derivatives. The results obtained with different reagents are assembled in Table II, in

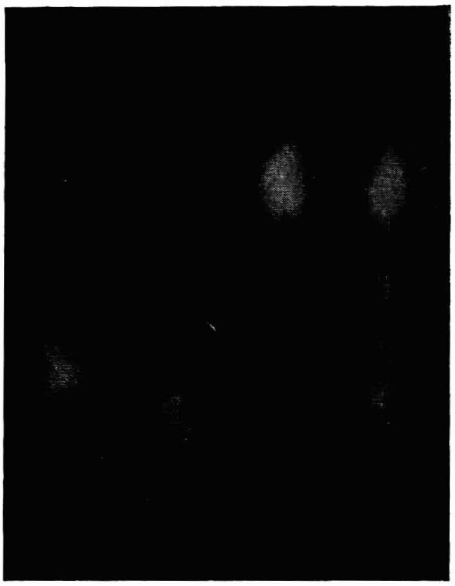

Fig. 1. Chromatogram of anti-tumour mannitol derivatives. Solvent system: *n*-butanol–ethanol–water (4:1:5). Spraying reagent: phenol red. Ascending development. (1) Mannitol; (2) Degranol; (3) Mannitol-Myleran; (4) Mannitol-Myleran, 6 day old solution. Amount applied 200 μg in each case.

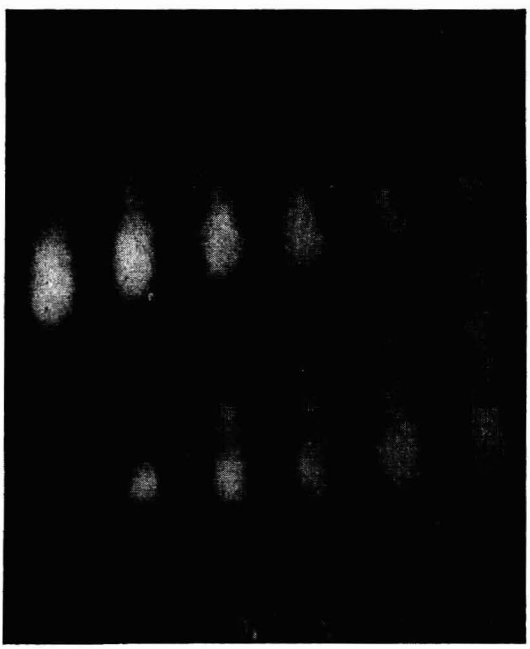

Fig. 2. Chromatograms of solutions of Mannitol-Myleran which were allowed to age. Solvent system: *n*-butanol–ethanol–water (4:1:5). Spraying reagent: phenol red. Ascending development. A = Mannitol-Myleran; B = decomposition product of Mannitol-Myleran. 1 = fresh solution; 2–6 = 1, 2, 4, 7 and 9 day old solutions, respectively. Amount applied 200 μg in each case.

which the colour reactions as well as the comparative colour intensity of the compounds examined are shown. Of the four indicator-containing reagents, methyl red gave a particularly weak reaction. Degranol gave a moderate reaction, mannitol a less marked one, while with Mannitol-Myleran, no colour reaction at all was obtained.

TABLE II

COLOUR RESPONSE OF MANNITOL, DEGRANOL AND MANNITOL-MYLERAN (MM) TO VARIOUS SPRAYING REAGENTS

	Mannitol	*Degranol*	*MM*
Bromophenol blue[8]	+++ yellow	++ brownish red	+++ yellow
Bromocresol green[8]	+++ yellow	+++ yellow	+++ yellow
Phenol red[9]	+++ yellow	+++ yellow	+++ yellow
Methyl red[10]	+ violet	++ violet	— —
Permanganate[11]	+++ yellow	++ brown	— —

The number of crosses indicates the intensity of the reaction; — = no reaction.

Reagents containing phenol red appeared to be the most sensitive of all the reagents examined. The lower limit of sensitivity was about 2 μg for all the reagents examined.

The reagent $KMnO_4$ yielded a yellow spot with mannitol, a brown one with Degranol, and failed to react with Mannitol-Myleran.

Experiments with tissue cultures showed that if solutions of Mannitol-Myleran were left to stand they gradually lost their cytotoxicity[12,13]. This process ran parallel with a decrease in the value of the pH, a phenomenon that was significant only during the first 24 hours (Table III).

TABLE III

CHANGES IN THE pH VALUE OF MANNITOL-MYLERAN SOLUTION AT DIFFERENT INTERVALS (DISSOLUTION IN DISTILLED WATER)

	Fresh	*24 h*	*48 h*	*72 h*
pH values	3.0	1.8	1.7	1.65

Paper chromatographic analyses of samples collected at different intervals revealed a gradual decrease in the intensity of the Mannitol-Myleran spot, and the simultaneous emergence of a new spot of growing intensity in the zone of mannitol (Fig. 2). In view of the fact that the diminution of the pH value practically terminates after the first 24 hours, while the disappearance of the Mannitol-Myleran spot is a slow process which lasts some 3 to 4 weeks, it seems safe to assume that the two phenomena are not directly connected. If the solution of Mannitol-Myleran is left standing for a

few hours, methanesulphonic acid is split off and this renders the solution strongly acid[3]. The new spot that arises is, therefore, presumably that of mannitol, a supposition which is confirmed by the observed R_F value (Table I, Fig. 1). The comet formation seen in connection with the new spot was probably due to the decomposition products of acid character (Fig. 2), since the spraying reagent was extremely sensitive in respect to pH. No similar phenomenon was observed in connection with Degranol: time produced neither a weakening of its spot nor the appearance of a new spot.

SUMMARY

Paper chromatography has proved to be suitable for the separation of both Degranol and Mannitol-Myleran. Experiments made in this connection showed that Degranol was more stable than the other agent.

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FURTHER STUDIES ON THE ISOLATION AND IDENTIFICATION OF ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC PRODUCTS

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INTRODUCTION

In a former paper[1] we described the chromatographic methods that we used for the isolation and identification of isonicotinic acid hydrazide (INH) and its metabolic products. On another occasion[2] paper electrophoresis was used for the isolation of the isonicotinyl-hydrazone of pyruvic acid.

The possibility of combining the two techniques led us to study the use of buffered papers for the chromatographic separation of INH metabolites after the electrophoretic run.

Furthermore, new and more sensitive reagents were used for the localization of the spots and more derivatives were studied. In the present paper we report both the new methods and the results that complete our first paper on this subject.

MATERIALS AND METHODS

Deproteinization

The technique of centrifugal ultrafiltration[1], slightly modified[3,4], was always used for the separation of water-soluble compounds from proteins. The residue left after the evaporation of the filtrate (80°/vacuum), however, was now taken up in 1 % (w/v) ammonium hydroxide[5]. This permitted a better recuperation of the derivatives after the chromatographic purification.

Chromatographic purification

Descending paper chromatography, as described previously[1], was used for the purification of INH metabolites. The use of ammonia solutions prevented the loss of derivatives such as isonicotinic acid, which have low R_F values when in the form of sodium salts[1]. The absorption region, localized under the U.V. lamp, was cut out and eluted with 1 % ammonium hydroxide. Concentration of the eluted material, prior to paper electrophoresis or chromatography, was carried out at 80° under vacuum, and the dry residue was dissolved again in a few microliters of 1 % ammonia.

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Paper electrophoresis

The use of paper electrophoresis permits the separation of the acid metabolites of INH, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid, from the other metabolites. Our first studies[2] were carried out using *M*/15 phosphate buffer, pH 7.0, but more recent experiments have shown that 0.1 *M* TRIS (tris-hydroxymethyl-aminomethane) buffer, pH 7.0, is superior. The samples were spotted on the paper (Macherey-Nagel No. 261), which was then sprayed with the buffer solution, excess buffer being removed by blotting with filter paper. Electrophoresis was then carried out as usual (in our case in a Shandon apparatus), applying a potential of 300 V for a period of 3 hours.

Paper chromatography

Two-dimensional paper chromatography was carried on as described previously[1], with the exception that, for the acid metabolites, ammonium salts were now employed. On the other hand, the use of papers impregnated with 0.1 *M* TRIS buffer, pH 7.0, gave good results and opened up the possibility of combining paper chromatography and paper electrophoresis in two-dimensional separations. In this case the best results were obtained with isoamyl alcohol and with *n*-butyl alcohol, both saturated with 0.1 *M* TRIS buffer, pH 7.0.

Paper electrophoresis combined with paper chromatography

On a sheet of Macherey-Nagel No. 261 filter paper, measuring 20 × 37 cm, a line was drawn at 2.5 cm from the longer edge; another line was drawn in the middle of this line and perpendicular to it, the intersection being the starting point. The samples were spotted and the paper was sprayed with TRIS buffer as described. Paper electrophoresis was then carried out along the longer axis of the paper under the same conditions as before. After the electrophoretic run the paper was dried in the oven (80°) and ascending paper chromatography was carried out along the shorter axis, using one of the buffered solvents quoted above.

Localization of the spots

As was seen before[1], certain compounds, such as the isonicotinyl-hydrazone of pyruvic acid, give only discrete absorption spots under the U.V. lamp and a negative reaction with cyanogen bromide. We found that spraying with 0.1 *N* HCl and drying in the oven at 80° before a second exposure to BrCN made them appear as bluish spots that were easily discernible.

Very good results were also obtained with the reagent recently devised by GREULACH AND HAESLOOP[6] for the identification of hydrazide derivatives (1 % w/v aqueous ferric chloride plus an equal volume of 1 % w/v aqueous potassium ferricyanide). This reagent has the advantage that it can be used after the reaction with cyanogen bromide. Isonicotinic acid and isonicotinamide do not react.

The complete procedure for the localization of INH metabolites is then as follows: (1) observation under the U.V. lamp; (2) treatment with cyanogen bromide for 1 h followed by observation under the U.V. lamp; (3) exposure to ammonia vapours and observation in daylight and under the U.V. lamp; (4) spraying with 0.1 *N* HCl and drying in the oven at 80°, followed by treatment with BrCN and

observation under the U.V. lamp (in some cases the second exposure to BrCN is not necessary, depending on the degree of impregnation after the first one); (5) spraying with the GREULACH-HAESLOOP reagent.

RESULTS AND DISCUSSION

Great improvements on our former techniques for the chromatographic purification of INH metabolites were obtained by using 1 % ammonium hydroxide as solvent. In Table I the R_F values are given for the metabolites that were most affected by the change—isonicotinic acid (INAcid), the isonicotinyl-hydrazone of pyruvic acid (Py.INHzone) and the isonicotinyl-hydrazone of acetaldehyde (Ac.INHzone)—besides those obtained for the derivatives that have not been studied before—isonicotinamide (INAmide) and di-isonicotinic acid hydrazide (di-INH).

TABLE I

R_F-VALUES OF SOME INH-METABOLITES ON MACHEREY-NAGEL NO. 261 FILTER PAPER

Sample	*R_F value in solvent**		
	1	*2*	*3*
Py-INHzone**	0.41–0.22	0.30–0.17	0.56
Ac-INHzone	0.40	0.29	0.70
INAcid**	0.29	0.27	0.61
INAmide	0.66	0.93	0.72
Di-INH	0.37	0.85	0.67

* 1 = Isopropanol–1 % NH_4OH (20:3) (ascending); 2 = Butanol saturated with 1 % NH_4OH (ascending); 3 = Propanol–NH_4OH (70:30) (descending).
** As ammonium salts.

As can be seen, the use of 1 % ammonia as solvent prevents the loss of isonicotinic acid, which occurred when the metabolites were purified as their sodium salts[1], since now the R_F values in propanol-ammonia are all over 0.5.

On the other hand, the isonicotinyl-hydrazone of pyruvic acid, as its ammonium salt, gave double spots in isopropanol–ammonia and in ammonia-saturated butanol. The spots were eluted and the amount of pyruvic acid in each one of them was calculated as described previously[2]. 56.2 % of the hydrazone was found in the spot with the higher R_F value and 43.8 % of it was found in the slowest spot. It was assumed that the spots were due to isomers of the same derivatives.

The values given in Table I, together with the ones already found for INH and its metabolites[1] were plotted in the map shown in Fig. 1, which represents a two-dimensional chromatogram run by the ascending technique with (a) isopropanol–1 % ammonia (20:3) and (b) ammonia (1 %)–saturated butanol.

In Fig. 1 we can see that one of the spots of the ammonium salt of the isonicotinyl-hydrazone of pyruvic acid is partially superimposed by the spot of the isonicotinyl-hydrazone of acetaldehyde. In this respect the sodium salt is superior, since it has a lower R_F value[1] and superposition is avoided.

The results obtained by paper chromatography of INH metabolites on TRIS-buffered papers are shown in Table II. As can be seen, *n*-butanol saturated with

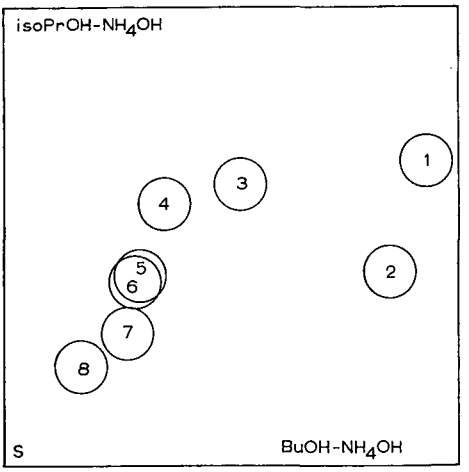

Fig. 1. Map of a two-dimensional paper chromatogram of INH metabolites (Macherey-Nagel No. 261 filter paper, unbuffered) run with (a) isopropanol–NH_4OH and (b) butanol–NH_4OH, by the ascending technique. (1) INAmide; (2) di-INH; (3) INH; (4) acetyl-INH; (5) Py-INHzone (56.2% of the ammonium salt); (6) Ac-INHzone; (7) INAcid (ammonium salt); (8) Py-INHzone (47.7% of the ammonium salt).

TRIS separates the two isomers of the isonicotinyl-hydrazone of pyruvic acid, but this does not happen with isoamyl alcohol saturated with TRIS.

The use of 1% ammonia as solvent is essential when buffered papers, such as those described are used. We found that sodium carbonate affects the displacement of the metabolites by interfering with the buffer. This does not happen when they are

TABLE II

R_F-VALUES OF INH AND SOME OF ITS METABOLITES ON MACHEREY-NAGEL NO. 261 FILTER PAPER IMPREGNATED WITH 0.1 *M* TRIS BUFFER, pH 7.0

Sample	*R_F value in solvent**	
	1	*2*
INH	0.40	0.62
Acetyl-INH	0.39	0.57
Py-INHzone**	0.05	0.06–0.33
Ac-INHzone	0.47	0.63
INAcid**	0.08	0.23
INAmide	0.51	0.62
Di-INH	0.45	0.63

* 1 = Isoamyl alcohol saturated with TRIS buffer (ascending); 2 = Butanol saturated with TRIS buffer (ascending).

** As ammonium salts.

dissolved in ammonia (Table III), since no residue is left after drying the spotted samples.

Attention must be paid to the fact that the ammonium salt of isonicotinic acid sublimes at rather low temperatures, and that some loss is bound to occur when the papers are dried in the oven at 110°.

TABLE III

EFFECT OF THE SOLVENT UPON THE R_F-VALUE ON BUFFERED PAPERS

Macherey-Nagel No. 261 filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0. Solvent system: isoamyl alcohol saturated with 0.1 *M* TRIS buffer, pH 7.0. Sample: isonicotinyl-hydrazone of acetaldehyde, 0.1 % (w/v).

Sample (μg)	*Solvent*	R_F	*Interfering substance (μg Na_2CO_3)*
2	1 % NH_4OH	0.69	—
4	1 % NH_4OH	0.68	—
6	1 % NH_4OH	0.68	—
2	10 % Na_2CO_3	0.64	20
4	10 % Na_2CO_3	0.58	40
6	10 % Na_2CO_3	0.42	60

The electrophoretic mobilities found for the various metabolites of INH on Macherey-Nagel filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0, after application of a potential of 300 V during 3 hours, are shown in Table IV.

As was noticed before, paper electrophoresis is specially useful for the separation of the acid metabolites, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid, especially when combined with ascending paper chromatography, as shown in Fig. 2.

Table V summarises the results obtained with the sequential procedure for the localization of INH and its metabolites. It must be noted that the colours produced by the reactions involving cyanogen bromide are frequently affected by traces of

TABLE IV

ELECTROPHORETIC MOBILITY OF INH AND ITS METABOLITES

Macherey-Nagel No. 261 filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0; potential applied 300 V for 3 hours

Sample	*Displacement*	
	pole	*cm*
INH	—	1.4
Acetyl-INH	—	1.4
Py-INHzone	+	5.8
Ac-INHzone	+	1.6
INAmide	—	1.4
INAcid	+	8.5
Di-INH	+	1.6

ammonia in the paper, by the concentration of BrCN within the developing chamber and by the time of contact. A simpler procedure giving more consistent results, consists in observing the chromatogram under the U.V. lamp and placing it in an atmosphere of BrCN plus ammonia, thus omitting steps 2 and 3.

TABLE V

RESULTS OBTAINED WITH THE SEQUENTIAL PROCEDURE FOR THE LOCALIZATION OF INH AND SOME OF ITS METABOLITES ON PAPER CHROMATOGRAMS

(The values given are the amounts in μg)

Step	*Description*	*INH*	*Acetyl-INH*	*Py-INHzone*	*Ac-INHzone*	*Di-INH*	*INAcid*	*INAmide*
1	U.V.	brownish	brownish	brownish	slate	slate	brownish	brownish
		5	15	2	1	2	15	10
2	BrCN/visible	—	brownish	brownish	brownish	brownish	—	—
		—	15	4	1	2	—	—
3	BrCN/U.V.	brownish	yellow	brownish	brownish	brownish	brownish	brownish
		5	1	2	0.6	0.6	10	10
4	NH_4OH/visible	yellow	brownish	brownish	brownish	brownish	yellow	yellow
		0.4	1	4	0.8	0.6	0.5	0.5
5	NH_4OH/U.V.	yellow	yellow	brownish	brownish	brownish	yellow	yellow
		1	0.5	2	0.2	0.2	0.5	0.5
6	HCl/BrCN/U.V.	—	yellow	bluish	brownish	bluish	—	—
		—	1	2	0.6	2	—	—
7	GREULACH-HAESLOOP	deep blue	deep blue	deep blue	deep blue	deep blue	—	—
		1	0.2	0.6	0.2	0.2	—	—

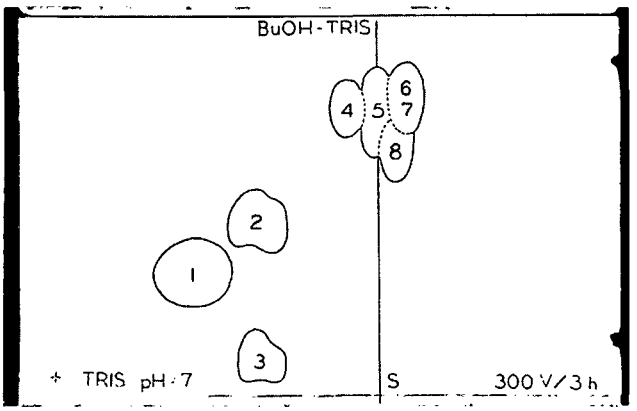

Fig. 2. Two-dimensional paper electrophoresis (0.1 *M* TRIS buffer, pH 7.0, 300 V, 3 h) and ascending paper chromatography (*n*-butanol satd. with TRIS buffer) of INH and some of its metabolic products: (1) INAcid; (2) and (3) Py-INHzone; (4) di-INH; (5) Ac-INH-zone; (6) INH; (7) INAmide; (8) acetyl-INH.

ACKNOWLEDGEMENTS

The present work was carried out at the Central Laboratory of Tuberculosis, in collaboration with the Institute of Phthisiology and Pneumology of the University of Brazil. The authors wish to acknowledge the assistance of the National Research Council of Brazil, from which one of them (R.C.R.B.) received a grant, and to thank Dr. B. P. LISBOA, from the Karolinska Institute (Sweden), for kindly supplying some samples of INH derivatives.

SUMMARY

Proceeding with former studies[1] on the isolation and identification of some of the metabolic products of isonicotinic acid hydrazide, the authors combined paper electrophoresis and paper chromatography in two-dimensional separations, which they found to be specially useful for the study of acid metabolites, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid. Better separations of the non-acid metabolites were obtained as before[1], by means of two-dimensional paper chromatography on unbuffered papers.

Dissolution of the metabolites in 1% ammonium hydroxide instead of in 10% sodium carbonate as usual, gave better results in the sense that isonicotinic acid was not lost during the chromatographic purification and that the R_F values on the buffered papers were not affected by the amount of solution spotted.

The technique for the localization of the spots was greatly improved by the use of a new and very sensitive reaction for hydrazide derivatives, carried out at the end of a sequential procedure devised to reveal all the metabolic derivatives in the range of 1 to 0.2 μg/sq. cm.

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SEPARATION OF HOMOLOGOUS SERIES OF 2,4-DINITROPHENYLOSAZONES BY COLUMN PARTITION CHROMATOGRAPHY

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This manuscript describes chromatographic systems for separating homologous series of the 2,4-dinitrophenylosazone* derivatives of α-keto-aldehydes and 2,3-diketones. To the author's knowledge, only CORBIN[1] has successfully resolved the members of these two classes.

In the work reported below, column partition systems are described in which the stationary phase is alkaline. It is well known that both 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones change from a yellow or orange hue in neutral solvents to various shades of red, purple or blue under the proper alkaline conditions. This phenomenon, which is also shown by other *meta*-dinitro compounds[2], has been studied extensively and has been discussed by JONES AND HANCOCK[3], JONES *et al.*[4], BRAUDE AND JONES[5], TIMMONS[6], BOHLMANN[7], NEUBERG AND STRAUSS[8], and in references cited by these investigators. Since the wavelength of maximum absorption of the 2,4-dinitrophenylhydrazones or osazones going from neutral to alkaline conditions is shifted toward the visible region of the spectrum, visual detection of a spot or zone is enhanced. Paper chromatograms of 2,4-dinitrophenylhydrazones are usually sprayed with alcoholic base for this reason.

TURBA AND SCHRADER-BEIELSTEIN[9], VAN DUIN[10,11] and SCHWARTZ *et al.*[12,13] have employed alkaline adsorbents for fractionation of 2,4-dinitrophenylhydrazones. However, no partition system employing a strongly alkaline phase has been described for separating an homologous series of 2,4-dinitrophenylhydrazones. The stability of 2,4-dinitrophenylhydrazones and osazones adsorbed on a strong anion-exchange resin has been demonstrated by SCHWARTZ *et al.*[14]. It, therefore, seemed feasible to attempt to introduce partition systems employing a strong base.

EXPERIMENTAL

*Reagents and apparatus***

Ethanolamine. Fisher's highest purity grade is redistilled under reduced pressure. The fraction boiling at 56–58° at 4 mm pressure is collected and stored at 4° in a tightly

* The term 2,4-dinitrophenylosazone or merely osazone in this manuscript is used synonymously with bis-(2,4-dinitrophenylhydrazone). It should also be understood that only derivatives were investigated even though the derivative term has sometimes been omitted in the text.

** The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U.S. Department of Agriculture.

stoppered, brown glass bottle. Ethanolamine is hygroscopic and should not be unduly exposed to moist air.

Benzene. ACS grade benzene is redistilled and then dried over anhydrous sodium sulfate for 24 h before use.

Benzene saturated with ethanolamine. Redistilled, dried benzene is saturated by shaking, or more conveniently by stirring (preferably magnetically with a Teflon covered bar) 50 ml of redistilled ethanolamine per gallon of benzene for at least 2 h. Complete phase separation takes at least 16 h. However, a faint turbidity in the benzene phase is not detrimental and in the prescribed method will develop anyhow.

Celite. Johns-Manville Analytical Grade Celite is dried at 140–150° for 96 h. The dried product should be protected against moisture.

Chromatographic column. A borosilicate glass column 2.6 cm × 29 cm with a coarse sintered glass disc is suitable.

Siphon. A siphon to deliver approximately 5 ml is used. If necessary fractions smaller than 5 ml may be collected in order to obtain purer fractions.

Cation-exchange resin. Amberlite IR 120 (H^+), 16–50 mesh, 8–10 % cross linked, is washed with distilled water until a colorless effluent is obtained. It is then washed with 6 column volumes of ACS grade methanol and then with 4 column volumes of redistilled benzene.

Column preparation

The same mechanical procedure is used in the preparation of all columns. Equipment should be clean and dry.

α-Keto-aldehydes. Seven ml ethanolamine and 0.5 ml distilled water are mixed in a 6-in. mortar and 15 g celite is added. The contents of the mortar are ground with a pestle until a homogeneous preparation is obtained. This is best accomplished by grinding, scraping the sides of the mortar completely with a stainless steel spoon and regrinding. The scraping and regrinding procedure is repeated 4 or 5 times. The impregnated celite is transferred to a 500 ml Erlenmeyer flask and 200 ml of mobile phase (benzene saturated with ethanolamine) is added. A 1.5-in. Teflon-coated stirring bar is dropped in, the mouth of the flask covered with aluminum foil and the contents stirred for 15 min maintaining a speed sufficient to produce a visible vortex in the slurry. A layer of glass wool should be placed between the flask and magnetic stirrer before the onset of stirring. This will prevent the temperature of the solution from rising significantly. At the end of this period the bar is removed and the slurry poured through a wide stemmed (so-called powder) funnel to within 2 in. from the top of a chromatographic tube clamped at the outlet. Air bubbles are removed by rotating a stainless steel rod through the slurry in the tube. The column is then packed under nitrogen pressure (2–3 p.s.i.) until the height of mobile phase above the packing is about 1 in. The remainder of the slurry is then added, the flask rinsed clean with mobile phase and the rinsings added to the column. Air bubbles are removed from the remainder of the slurry by stirring above the packed portion of the column. Nitrogen pressure is applied again until the last of the mobile phase has entered the column. The top of the column is then carefully leveled by applying light pressure from a stainless steel tamping rod similar to that described by CORBIN *et al.*[15].

It is important for best results to obtain an even, level column surface. A small amount of celite may resist firm packing and may obscure observation of the true

column surface. If this occurs, it is best to rinse the sides of the tube down carefully with small (about 10 ml) portions of mobile phase, pour out the suspended celite and then repeat this until the surface is free of unpacked celite. The column surface may then be retamped until smooth and perpendicular at all points to the walls of the tube.

Applications of sample. Collection of effluent is begun upon application of the sample. The sample of α-keto-aldehydes in a 10 ml beaker is dissolved in 5 ml of mobile phase. It is important that complete solution be obtained. With the amounts of dicarbonyls chromatographed in this study, 5 ml is sufficient to effect solution. Solution may be hastened by covering the beaker securely with aluminum foil and heating gently for a few minutes on the steam bath and then allowing the solution to cool to room temperature. The solution is applied to the column by allowing it to run from a pipette rotated rapidly around the upper inner wall of the tube. In quantitative work the beaker should be rinsed with the minimum volume of mobile phase until all color is removed and the rinsings applied to the column in the manner described above. After the last of the colored solution has just entered the column, the sides of the tube are rinsed down with a few milliliters of mobile phase. The latter is allowed to percolate into the column and 10 ml of mobile phase is added. A ball of glass wool (about 2 g) previously saturated with ethanolamine by soaking and squeezing out the excess, is then pushed into the column to within approximately 0.5 in. of the column surface and the tube filled with mobile phase. The glass wool impregnated with stationary phase is necessary to compensate for changes in the composition of mobile phase if a constant temperature room is not used. If the chromatogram is not developed at constant temperature, solution of some stationary phase will occur if the temperature rises. The use of glass wool impregnated with ethanolamine insures that the mobile phase remains saturated with stationary phase throughout the development.

Removal of ethanolamine from effluent. The effluent from the column contains a fine suspension of ethanolamine which renders the fractions unsuitable for spectrophotometric evaluation. It is, of course, also necessary to remove the ethanolamine from the osazones at some point if further analysis of the fractions is desired. Both of these problems are overcome by adsorbing the base onto a cation-exchange resin. The use of a cation-exchange resin in the microanalysis of 2,4-dinitrophenylhydrazones and osazones has been described by SCHWARTZ *et al.*[14]. Their results showed that all types of commonly occurring 2,4-dinitrophenylhydrazones and osazones pass through the resin quantitatively and without change.

For the purpose of removing ethanolamine the exchanger is packed into a coarse sintered glass funnel (approximately 2.2 cm i.d. × 5 cm from top to sintered disc). Five grams of resin (dry basis) will quantitatively take up the ethanolamine from about 250 ml of mobile phase. The conditioned exchanger is placed just below the siphon. The exchanger is changed at a suitable time, that is, when there is a minimum of 3 blank fractions emerging from the column (see Figs. 3 and 4). Regeneration of the exchanger is accomplished by washing with 5 column volumes of *N* HCl, followed by distilled water until a neutral effluent is obtained and then with methanol and benzene as described under reagents and apparatus.

2,3-Diketones. The procedure for preparation of the column for separating 2,3-diketones is exactly the same as that for the α-keto-aldehydes except that 8 ml ethanolamine is used and the water is omitted.

Glyoxal. Glyoxal, the only vicinal dialdehyde, requires a special column for chromatography. It is much too slow moving in the systems described above to be eluted in a reasonable length of time. It can be separated from all other saturated dicarbonyls on a column composed of 7.5 g celite, 1.5 ml water, and 3.5 ml ethanolamine. The column is constructed as described for the other classes.

RESULTS AND DISCUSSION

Figs. 1 and 2 are photographs, and Figs. 3 and 4 plots of absorbancy *vs.* fraction number of the α-keto-aldehyde and diketone chromatograms, respectively.

Coloring

There is a sufficient contrast in hue (see Table I) between the 2 classes so that visual differentiation can be made with confidence*. This color difference has always been manifested provided that coloring occurs. Coloring (and separation) will not occur if (a) insufficient stationary phase is used, (b) too much water is present, or (c) excessively high flow rates are employed.

TABLE I

COLOR, R-VALUES AND RECOVERY OF 2,4-DINITROPHENYLOSAZONES

2,4-Dinitrophenyl-osazone of	*Color*	R_{Bu}*	R_{Mg}*	R_{Khex}*	*Recovery %*
α-Ketononanal	blue	—	0.09	0.42	98
α-Ketooctanal	blue	—	0.11	0.54	95
α-Ketoheptanal	blue	—	0.15	0.70	102
α-Ketohexanal	blue	—	0.22	1.00	98
α-Ketopentanal	blue	—	0.31	1.44	97
α-Ketobutanal	blue	—	0.57	2.65	97
α-Ketopropanal (methylglyoxal)	blue	—	1.00	4.58	96
2,3-Octanedione	grey	0.24	—	—	102
2,3-Heptanedione	grey-violet	0.30	—	—	100
2,3-Hexanedione	—	0.43**	—	—	—
2,3-Pentanedione	violet	0.62	—	—	102
2,3-Butanedione (diacetyl)	violet	1.00	—	—	101
Glyoxal	blue	—	3.90	—	99

* Abbreviations: Bu = 2,3-butanedione; Mg = methylglyoxal; Khex = α-ketohexanal.

$$R = \frac{\text{volume of effluent to peak of compound}}{\text{volume of effluent to peak of standard}}.$$

** Value calculated from graph of $\log_{10}$ of effluent volume *vs.* number of carbons in parent compound.

With approximately equimolar concentrations of members of a given class, the intensity of the color increases on the chromatogram as the chain length of the member decreases. 0.05 μm of methylglyoxal or diacetyl show up as intensely colored bands under the prescribed conditions, even after having moved the full length of the column.

* Separation of the various classes of 2,4-dinitrophenylosazones (*e.g.*, non-vicinal diketones and dialdehydes, vicinal diketones, α-keto-aldehydes and vicinal dialdehyde) has been accomplished in this laboratory. The method greatly simplifies the analysis of complex mixtures of dicarbonyls and makes color differences between classes less critical, but still desirable from a confirmatory standpoint.

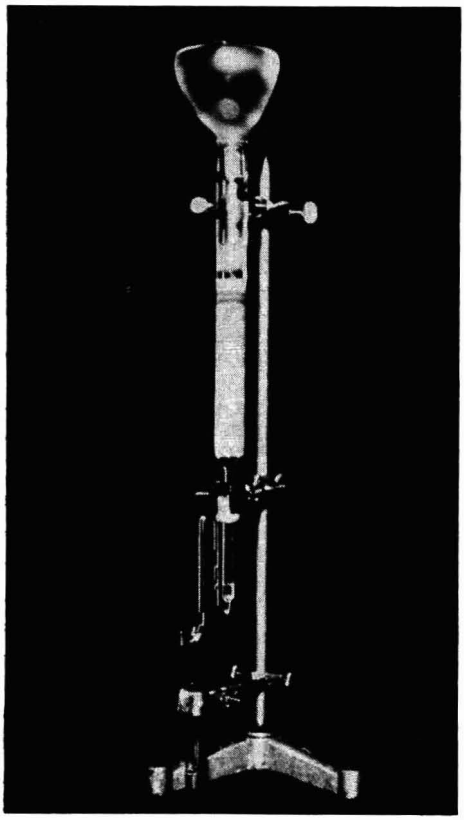

Fig. 1. Chromatogram of α-keto-aldehyde 2,4-dinitrophenylosazones. 1 h development. Approximately 0.05 μm each.

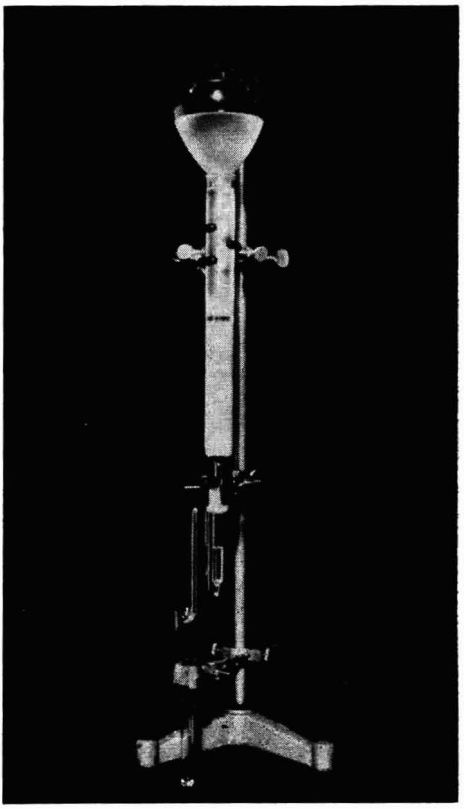

Fig. 2. Chromatogram of 2,3-diketone 2,4-dinitrophenylosazones. 45 min development. Approximately 0.05 μm each.

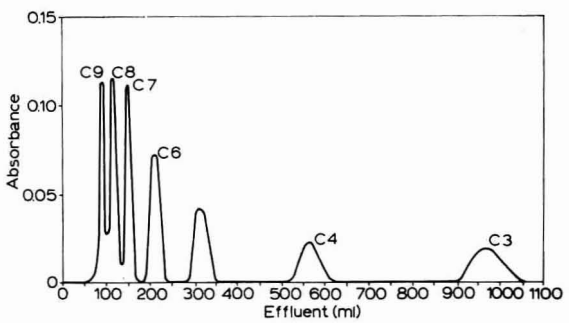

Fig. 3. Plot of absorptivity *vs.* volume of effluent for chromatogram of α-keto-aldehyde 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 133 ml/h; fractions read at 390 mμ.

The colors of the bands can be intensified by using more ethanolamine in the case of the diketones. This effect can be produced with the α-keto-aldehydes by using more ethanolamine and/or decreasing the water content of the stationary phase. A markedly superior separation between members is achieved for both classes when

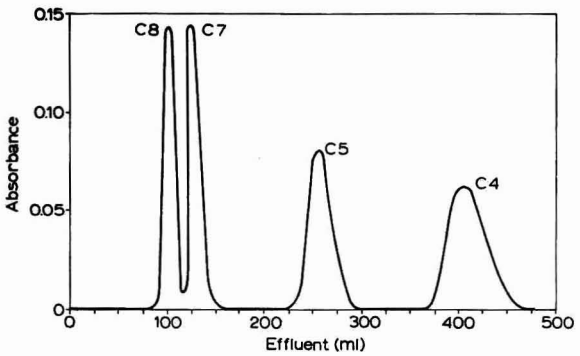

Fig. 4. Plot of absorptivity *vs.* volume of effluent for chromatogram of 2,3-diketone 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 92 ml/h; fractions read at 390 mμ.

the amount of stationary phase is increased. However, this is obtained only at a considerable increase in the time needed for elution of a given band. Also, the long-chain diketones assume the color of the shorter members. The chromatograms presented in this paper, therefore, represent a compromise between better resolution and completion of the chromatogram in a reasonable length of time. Nevertheless, the investigator can vary his conditions to suit the nature of the constituents in the unknown sample.

Reproducibility

The volume of mobile phase needed to elute a given zone was found to vary considerably from one chromatogram to the next. This was due largely to variations in flow rate. However, the volume of mobile phase needed to elute a given compound relative to an internal standard was found to be quite constant. It is felt that the *R* values (Table I) can be used with greater confidence than trying to control all of the variables involved, especially in light of the critical role played by moisture.

Stability and recovery studies

Although ample evidence attesting to the stability of 2,4-dinitrophenylhydrazones and osazones, under alkaline conditions is accumulating, it was felt that the long exposure of some of the osazones to ethanolamine warranted investigation of additional stability and recovery data. For this purpose, pairs of diketones and α-keto-aldehydes, which are well separated from each other (*e.g.*, C_8 and C_5 diketones, C_9 and C_6 α-keto-aldehydes, etc.) were chromatographed in the usual manner. The bands were collected and recoveries determined spectrophotometrically in chloroform. Each recovered compound was then cochromatographed with the same concentration of authentic unchromatographed derivative in the proper system recommended by CORBIN[1]. A continuous flow analyzer (Canal Industrial Corp., Bethesda, Md.) with a 390 mμ filter was used to monitor the chromatogram.

Recoveries are given in Table I. All compounds chromatographed as a single peak with no other bands being detectable either visually or by the instrument. These data indicate that dinitrophenylosazones are quite stable in alkali, a fact which might find considerable application in view of the good solubility of these compounds in non-aqueous bases.

Miscellaneous

The chromatogram of glyoxal is shown in Fig. 5. All other dicarbonyls investigated in this solvent system move with the front and do not change color in the system. It is not known whether other classes of aliphatic dicarbonyls (*i.e.*, unsaturated) would form osazones which would be blue on the chromatogram under the conditions for preparing the glyoxal column.

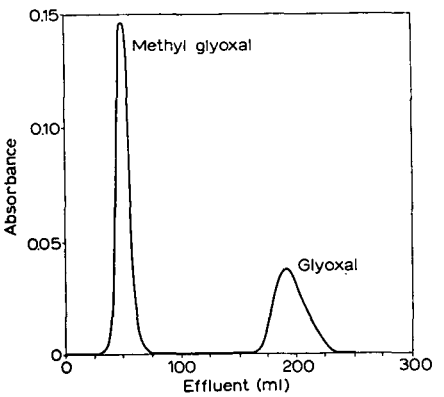

Fig. 5. Plot of absorptivity *vs.* volume of effluent for chromatogram of glyoxal and methylglyoxal 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 150 ml/h; fractions read at 390 mμ.

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The author wishes to express his gratitude to Dr. EARL HAMMOND, Iowa State University for a gift of the α-keto-aldehydes.

SUMMARY

Ethanolamine–benzene systems are described for the partition chromatography of the 2,4-dinitrophenylosazone derivatives of homologous series of α-keto-aldehydes and 2,3-diketones. Each class produces a different vivid color on the chromatogram facilitating differentiation and permitting very small amounts of derivative to be followed visually down the column.

A special system is also described for the chromatography of the 2,4-dinitrophenylosazone of glyoxal, the only saturated vicinal dialdehyde.

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STEROIDS

CCXI. CENTRIFUGALLY ACCELERATED PAPER PARTITION CHROMATOGRAPHY OF STEROIDS*

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The analysis of steroids by paper partition chromatography[2] has made possible the study of a variety of problems related to their biosynthesis and metabolism. This has been true not only in biochemical studies but also in organic synthesis[3]. Normally the development of a chromatogram in which the solvent travels 30 cm from the line of sample application takes 2 h or more. In reaction rate studies which require many consecutive analyses the time factor can be important. Recently, the advent of centrifugally accelerated paper chromatography[4] presented a possibility for making this technique even more useful by reducing the time for analysis from hours to minutes. The following is a study of the variables in the centrifugally accelerated partition chromatography of steroids on paper.

EXPERIMENTAL AND RESULTS

Apparatus

The apparatus (Fig. 1) consists of two 10 in. aluminium pie plates, B and G, between which is held the impregnated chromatographic paper C. The lower plate, G, is

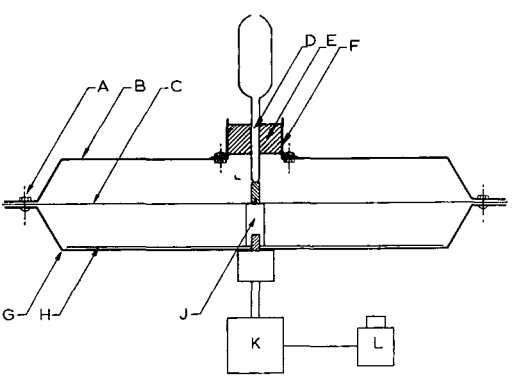

Fig. 1. A: Screw to hold covers together. B: Upper plate. C: Paper. D: Solvent applicator. E: Cork liner to hold applicator. F: Flange to hold cork liner. G: Lower plate. H: Paper liner. J: Brass rod support. K: Motor and shaft assembly. L: Variac to control motor velocity.

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attached to the motor shaft and centered by means of a threaded ½ in. brass rod J, which supports the center of the paper. This support has a raised point which pierces, the center of the paper disc. A Whatman No. 3 MM liner, covers the inside bottom of the lower plate and is held in place by J. The liner is wet with the mobile phase before the start of a run. The upper plate, B, has a 1 in. hole in the center. A flange, F, 1.25 in. high is centered over the hole, and has a cork, E, with a hole for the solvent applicator, D. The whole assembly is held together by means of four screws, A, evenly spaced around the perimeter of the plates. The speed of the motor is controlled by variac, L.

The volatile solvents used in steroid chromatography require that a closed system be used. In addition, paper wet with the stationary phase is not strong enough to be supported only at the center while it is being accelerated centrifugally. Even if supported with pins[5] it does not suspend at the velocity used in this work and therefore it must be held around the edge. The apparatus described meets both of these requirements.

Solvent application

The solvent is applied to the center of the paper disc by means of a paper wick inside a 6 mm internal diameter glass tube 9 cm high with a 4 cm high × 13 mm internal diameter reservoir at the top. An indented ridge about an inch above the lower end prevents the wick from receding completely into the glass tube. The tube is long enough that the wick can be centered on the point of support J before the upper lid is lowered so that the solvent is applied exactly to the center of the paper disc. The wick is made by tightly rolling a one inch width of washed Whatman No. 1 paper around a wooden cotton applicator stick. The stick is recessed to allow for the point on support J.

The velocity of the solvent, passing through the wick is regulated by the amount of paper that is used for the wick. Flow rates of 1 ml/min were used in this work. The wick is calibrated by placing 6 ml of hexane saturated with formamide in the applicator and measuring the time required for a given volume to pass through. Five to six ml of solvent was applied to the paper disc. Due to the effect of centrifugal velocity most determinations required 10 to 15 min to apply this amount.

This method of solvent application was adopted because of the difficulties encountered in using capillaries. After each run, the wick is cleaned of stationary phase by applying vacuum at the upper end of the tube and placing the wick in some methanol, then drying by pulling the wick out of the solvent and allowing air to pass through with vacuum applied. Once a wick is prepared it is good for a large number of determinations.

Paper preparation and sample application

The Whatman No. 1 paper discs were 27 cm in diameter with the center marked and a circle inscribed of 2 cm radius for sample applications. Four holes were punched which corresponded to the screws, A. The discs were impregnated with the stationary phase in the normal manner[2] and the samples applied to the inscribed circle as spots or arcs. It was found that the mode of sample application was very important. If samples were applied dissolved in methanol–chloroform 1:1 there was extensive streaking and tailing in the resulting chromatograms. It was necessary to dissolve the

steroids in chloroform saturated with formamide* if formamide was the stationary phase or benzene if propylene glycol was used. In general 10 to 20 μg of each steroid was applied. No deleterious effect was found in using quantities up to 100 μg.

Centrifugal acceleration

Contrary to what has been found for centrifugally accelerated adsorption chromatography[6] R_F values in accelerated partition chromatography of steroids are not independent of the velocity. At velocities above 200–250 r.p.m. the steroids did not move from the line of application. This is probably because at high velocities the solvent travels too fast to allow for partitioning to occur. The effect of centrifugal acceleration is shown in Table I.

In all subsequent work a velocity of 150 r.p.m. was used.

TABLE I

EFFECT OF CENTRIFUGAL FORCE ON R_F-VALUES OF A MIXTURE OF HYDROCORTISONE, CORTISONE AND 11-DESOXY-17-HYDROXYCORTICOSTERONE[a]

Velocity (r.p.m.)	*Hydrocortisone*	*Cortisone*	*11-Desoxy-17-hydroxycorticosterone*	*Time (min)*
0	0.43	0.81	1.0	40
150	0.47	0.79	0.97	10
300	0	0	0	105

[a] Capillary flow 1 ml/min, Whatman No. 1 impregnated with formamide–methanol, 1:3, chloroform satd. formamide.

Stationary phase content of paper

The amount of stationary phase in the paper affected the appearance of the steroid spots. When formamide–methanol or propylene glycol–methanol 1:1 was used the steroid spots had some tendency to streak and in some cases tailing occurred and no separation was apparent. If too little stationary phase was used (15%) the same effect occurred. Therefore the paper discs were impregnated with methanol solution containing 25% stationary phase. When run under these conditions, the steroid spots elongated laterally but there was no tailing apparent.

R_F values of steroids

To eliminate the possibility of variations in R_F values due to any number of factors, it is best to run standards on the same disc of paper. Although the solvent front assumes an oval shape rather than a circle[6] R_F measurements are still reliable. Thus, when 4 samples of cortisone were run on the same disc, the R_F values obtained were 0.73, 0.74, 0.75 and 0.75 even though the solvent front travelled anywhere from 70 to 77 mm from the line of sample application. In Table II are listed the results of some steroids run separately and in mixtures.

Excess mobile phase can be applied to the paper to give the same effect as in regular descending partition chromatography where the mobile phase is allowed to run off the paper. The advantage in centrifugal chromatography is that the steroids

* Personal communication from Dr. J. DE FLINES, Koninklijke Nederlandsche Gist- en Spiritusfabriek N.V., Delft, The Netherlands, to whom we offer our thanks.

TABLE II

R_F VALUES OF STEROIDS SEPARATELY AND IN MIXTURES

Conditions: 150 r.p.m.; stationary phase applied as 25% in methanol; wick velocity 1 ml/min; 5 to 6 ml applied.

Stationary phase	*Mobile phase*	*Time of run (min)*	*Mixture*	R_F	
				Separately	*Mixture*
Formamide	Chloroform	10	Estriol	0.10	0.08
			Estradiol	0.80	0.74
			Estrone	0.97	0.95
Formamide	Chloroform	10	Hydrocortisone	0.49	0.47
			Cortisone	0.78	0.79
			11-Desoxy-17-hydroxycorticosterone	0.97	0.97

do not leave the paper because the solvent evaporates where the paper extends beyond the plates and the steroids travel only that far.

ACKNOWLEDGEMENTS

We wish to thank Dr. ALEJANDRO ZAFFARONI for helpful discussions and Dr. OTTO HALPERN for assistance in the design and construction of the apparatus.

SUMMARY

The apparatus and method for centrifugally accelerated partition chromatography of steroids is described. It is shown that the centrifugal velocity must be relatively low for separations to occur. The other factors studied were the mode of sample application and the stationary phase content of the paper. Using the described procedure, separations occur in 10–15 min. This makes it particularly suited to analyses which require short development times such as in chemical or enzymic kinetic studies or to determine the homogeneity of fractions in column chromatography.

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PAPIERCHROMATOGRAPHISCHE TRENNUNG VON CYCLISCHEN PEPTIDEN-OLIGOMEREN DES CAPROLACTAMS

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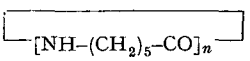
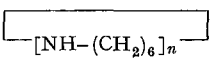
Bei der Hitzepolymerisation von Caprolactam entsteht ein Gleichgewichtsgemisch, das neben Caprolactam auch dessen cyclische Oligomere enthält.

Die papierchromatographischen Untersuchungen dieser Ringmoleküle wurden erst von ZAHN UND REXROTH durchgeführt[1]. Die Cycloamide (I, n = 1 bis 4) wurden in Pyridin–Wasser (70:30) 20 Stunden chromatographiert, ohne gute Ergebnisse zu erhalten. Die Unterschiede zwischen den R_F-Werten der einzelnen cyclischen Oligomeren waren sehr niedrig (0.02 bis 0.04). Im Phenol–Wasser (80:20) trennten sich die untersuchten Substanzen nicht und wanderten zusammen mit der Lösungsmittelfront. Um bessere Trennungsmöglichkeiten zu erhalten hatten ZAHN UND SPOOR in ihren späteren Arbeiten[2–4] die Cycloamide mit Lithiumaluminiumhydrid zu den entsprechenden cyclischen Aminen (II) reduziert und diese in *sek.*-Butanol–Ameisen-



(I)



(II)

säure–Wasser (75:15:10) über 4 Tage chromatographiert. Dieses Verfahren erlaubte die Identifizierung und quantitative Analyse[4] der cycl. Oligomeren (I, n = 2 bis 6) in Polycaprolactam Extrakten. Das benutzte Lösungsmittel trennte nicht die cycl. Di- und Trimere Amine voneinander.

Eine ausgezeichnete papierchromatographische Trennung von Ringamiden wurde von ROTHE mit einem neuen Lösungsmittel: Tetrahydrofuran–Petroläther–Wasser (186:14:10) erzielt[5–7]. Anschliessend wurde von ihm festgestellt, dass das Polycaprolactam auch die grösseren Ringamide (I, n = 5 bis 9) enthält.

In der vorliegenden Arbeit wurde eine neue Methode zur Trennung der cycl. Oligomeren beschrieben, die auf einer spezifischen Affinität der phenolischen Substanzen zu den Peptidbindungen[8] beruht. In dieser Methode ist Wasser, das mit Thymol gesättigt ist, die mobile Phase und das Thymol, mit welchem die Papierstreifen imprägniert wurden, die stationäre Phase.

Die Thymollösungen wurden bisher in der Papierchromatographie nur selten angewandt. GRASSMANN UND RIEDEL[9] benutzten die Thymollösungen zur Identifizierung der DNP-Aminoalkohole, PAWEŁKIEWICZ UND WALERYCH[10] zur Trennung der Corphyrine und BARTOSIŃSKI[11] zur Trennung der Riboflavine von ihren Nucleotiden.

EXPERIMENTELLER TEIL

Zur Ausführung unserer Versuche diente das Whatman Papier No. 1. Die Streifen (35 × 17.5 cm) wurden frisch mit 0.75 %-iger methanolischen Thymollösung imprägniert und an der Luft getrocknet. Man hatte die Papierstreifen erst in der Entfernung von 2 cm von der Startlinie imprägniert (Fig. 2). Das uns zur Verfügung stehende Caprolactam und die reinen cycl. Oligomeren lösten wir 1 %ig in einem Gemisch von Isopropanol–Methanol–Wasser (1:1:1)[1] und brachten auf die Papierstreifen auf. Als Lösungsmittel verwendeten wir Wasser mit Thymol gesättigt. Es wurde aufsteigend chromatographiert. Die Papierstreifen blieben ca. 4 Stunden in den Glaskammern bei 20 ± 2°. Die Chromatogramme wurden bei Zimmertemperatur getrocknet. Die Anfärbung der Caprolactam- und cycl. Peptidenflecke erfolgte durch Besprühen mit methanolischer Kaliumwismutjodidlösung[12]. Papierchromatogramme, die mit diesem Reagens entwickelt wurden gaben rote Flecke der komplexen Verbindungen des Caprolactams[12] und seiner cycl. Oligomeren[13]; der Hintergrund der Papierstreifen blieb gelb.

RESULTATE UND DISKUSSION

Zur Imprägnierung der Papierstreifen hatten wir die methanolischen Thymollösungen von verschiedenen Konzentrationen angewandt. Die besten Ergebnisse wurden auf mit 0.75 %iger Thymollösung imprägnierten Papierstreifen erzielt. Mit der Steigerung des Gehalts an Thymol im Papier, nahmen die R_F-Werte des Caprolactams und cycl. Oligomeren bedeutend ab (Fig. 1). Die Anwendung niedriger Thymolkonzentrationen führte zur Schweifbildung bei niederen Cycloamiden (I, n = 1 bis 3).

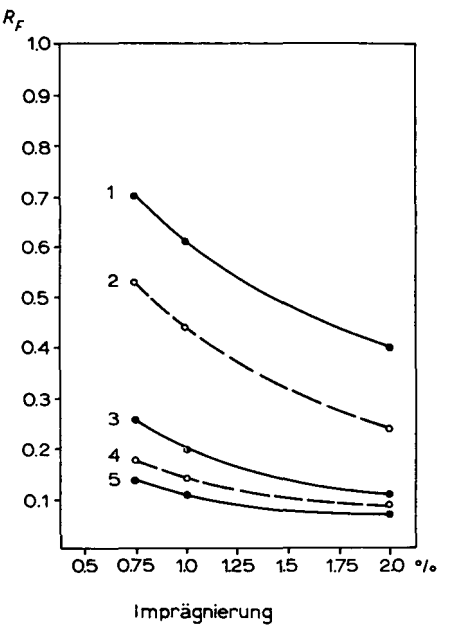


Fig. 1. R_F-Werte von Cycloamiden bei verschiedenem Imprägnierungsgrad des Papiers mit Thymol. (1) Monomer; (2) Dimer; (3) Trimer; (4) Tetramer; (5) Pentamer.

Es wurde festgestellt, dass man besser die höheren cycl. Peptiden trennen kann, wenn die Papierstreifen nicht auf ihrer ganzen Oberfläche, sondern erst in der Entfernung von 2 cm von der Startlinie imprägniert werden. Die Ergebnisse sind auf der Fig. 2 und in der Tabelle I dargestellt.

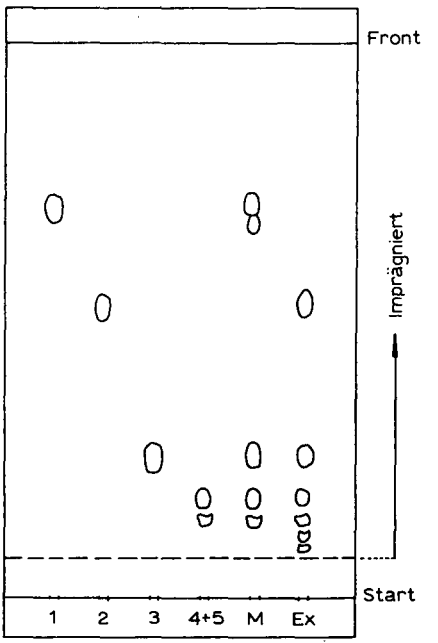


Fig. 2. Chromatogram der Cycloamide (I) auf Whatman Papier No. 1 imprägniert mit 0.75 % methanolischer Thymollösung. Mobile Phase: Wasser mit Thymol gesättigt. (1) Caprolactam; (2) Dimer; (3) Trimer; (4) Tetramer; (5) Pentamer; (M) Gemisch von (1), (2), (3), (4), und (5); Ex = Polycaprolactam Extrakt.

Die Cycloamide wanderten auf den Papierchromatogrammen gemäss ihrem steigenden Molekulargewicht. Am weitesten wandert das Monomer, dann folgen Dimer, Trimer und die höheren Homologen in der Reihenfolge ihrer Ringgrösse. Diesen Trennungseffekt kann man auf die Bildung der zwischen Peptidbindungen –NH·CO– der cycl. Oligomeren und Hydroxylgruppen des Thymols entstehenden Wasserstoffbrücken zuschreiben.

Die beschriebene Methode wurde zur Analyse eines von Caprolactam befreiten Methanolextraktes des Polymeren angewandt. Auf den Papierchromatogrammen konnten wir gleichfalls die Flecke der höheren cycl. Oligomeren (Hexa- und Heptameren) nachweisen.

ZAHN und Mittarbeiter, sowie ROTHE hatten zur Identifizierung des Caprolactams und der Cycloamide das sogenannte Chlorverfahren[1] angewandt. Wir fanden dazu die Kaliumwismutjodidlösung besser geeignet. Dieses Reagens gibt mit Caprolactam und cycl. Oligomeren rote Komplexverbindungen, die leicht auf dem gelben Untergrund der Papiere nachweisbar sind.

Die Nachweisempfindlichkeit dieser Reaktion gegen cycl. Oligomeren (auf dem unbehandelten Papier) ist derselben Grösse wie bei Caprolactam und beträgt 5–10 μg.

TABELLE I

R_F-WERTE VON CAPROLACTAM UND CYCL. OLIGOMEREN AUF MIT THYMOL IMPRÄGNIERTEM PAPIER

$\left[\text{NH-(CH}_2\text{)}_5\text{-CO}\right]_n$		R_F-Werte		
		Imprägniert mit Thymollösung		
		0.75%	1.0%	2.0%
$n = 1$	Monomer	0.70	0.61	0.40
$n = 2$	Dimer	0.53*	0.44*	0.24
		0.68**	0.59**	
$n = 3$	Trimer	0.26	0.20	0.11
$n = 4$	Tetramer	0.18	0.14	0.09
$n = 5$	Pentamer	0.14	0.11	0.07
$n = 6$	Hexamer	0.11		
$n = 7$	Heptamer	0.09		

* Einzeln.
** In Mischung.

Die Imprägnierung der Papierstreifen mit Thymol erniedrigt etwas die Empfindlichkeit der Reaktion (10–20 μg).

Diese papierchromatographischen Untersuchungen wurden auch auf andere Lactame und cycl. Peptide ausgedehnt.

DANK

Für die Überlassung synthetischer Oligomeren (Di- bis Pentameren) möchte ich an dieser Stelle Herrn Dr. MANFRED ROTHE aus dem Institut für Faserstoff-Forschung der Deutschen Akademie der Wissenschaften zu Berlin bestens danken. Fräulein LUDMIŁA ZIÓŁKOWSKA danke ich auch für ihre zuverlässige Hilfe.

ZUSAMMENFASSUNG

Es wurde eine einfache papierchromatographische Methode zur Trennung cyclischer Oligomeren des Caprolactams auf mit 0.75 %iger Thymollösung getränktem Papier als stationäre Phase und mit Thymol gesättigtem Wasser als mobile Phase beschrieben.

Die Flecke des Caprolactams und der Ringamide wurden mit Kaliumwismutjodidreagens nachgewiesen. Die Empfindlichkeit dieser Reaktion liegt unter 10 μg.

SUMMARY

A simple method for separating cyclic oligomers of caprolactam by paper chromatography is described. The separation is carried out on filter paper strips impregnated with thymol (stationary phase). A saturated aqueous solution of thymol is used as the mobile phase.

Caprolactam and the cycloamides were identified by means of potassium bismuth iodide. The sensitivity of this reaction is below 10 μg.

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DISCREET SEPARATION OF POLYCYCLIC HYDROCARBONS IN AIR BORNE PARTICULATES USING VERY LONG ALUMINA COLUMNS

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INTRODUCTION

Polycyclic hydrocarbons in recent years have become very important in air pollution studies; particularly so as several members of this family of compounds have demonstrable carcinogenic properties[1,2]. The correlation between lung cancer mortality and the air borne concentrations of specific polycyclic hydrocarbons, 1:12-benzoperylene and 3:4-benzopyrene, has been statistically established in certain highly polluted areas of the industrial midland counties of Great Britain[3,4].

In work pioneered by KOTIN[5], COOPER[6], WEDGEWOOD AND COOPER[7], LINDSEY *et al.*[8], COMMINS[9], KOTIN *et al.*[10–12] and others column chromatography on activated alumina or silica gel was used for primary division. However, the columns were generally of the order of three to six inches in length so that in many cases poor separation was achieved. This led to some excellent techniques of paper separation being devised, but these methods did not allow separation of the polycyclic compounds without at least some preliminary column separation.

The method outlined of long column separation then ultraviolet assay has proved very satisfactory for the discreet separation and identification of polycyclic compounds. Indeed, in many instances of quantitative estimation no other spectral media or chromatographic techniques are considered necessary.

APPARATUS AND MATERIALS

Activated alumina

Activated aluminium oxide, B.D.H. 100–200 mesh was heated in an air oven at 120°C for 72–96 h then brought to equilibrium over 50 % sulphuric acid for at least 36 h. The moisture content of the alumina was then 13–13.5 %.

Columns

A slurry of the alumina in cyclohexane was added to a 26.5 × 0.5 in. column to a depth of 23 in. with moderate vibration. Care was taken at all time to prevent the column becoming dry.

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Reagents

Where possible spectroscopic grade reagents were used. Because of the large volumes of cyclohexane required it was not economically possible to use this grade reagent so distillation followed by introduction of 5 g/l freshly activated alumina into the reagent bottles was adopted. A blank was then run on the purified reagent. This generally gave acceptable purity but in rare instances fractionation on a 1 in. diameter column packed to 16 in. with 3/16 in. raschig rings was necessary.

For the solid–liquid extraction, acetone, benzene or cyclohexane were used. For developing the column cyclohexane–ether was preferred.

Ultraviolet illumination

A Hanovia 3650 Å hand ultraviolet lamp was used to locate fluorescent zones on the column and to follow the passage of eluted material so that efficiency of separation and rate of elution could be systematised.

Ultraviolet spectrophotometer

A Beckman DK 2 split beam ratio recording spectrophotometer was used for ultraviolet spectral analysis of the eluates, with 3 ml quartz cells of 1 cm light path.

METHOD

Air particulate samples were obtained by passing 20–50 cubic ft./min through tared filter pads using a M.S.A. fixed flow, or Staplex variable flow, High Volume Air Sampler.

The filter pads of glass fibre or thick paper were weighed before and after the test against an equivalent standard pad and adjustments made for differential moisture content. This procedure was adopted because:

(i) the thick paper pads absorb moisture at a fast rate if dried in an air oven to 105°C then brought to equilibrium in a desiccator, so that accurate weighing is impossible (with glass pads this would not be an objection);

(ii) the loss of some polycyclic members such as fluorene is said to be appreciable if air dried at this temperature.

Where possible glass fibre pads dried before and after use in a desiccator are preferred but they have the drawbacks of low mechanical strength and low capacity.

The pads were extracted in a Soxhlet extraction apparatus with acetone solvent for about 5 h, the actual time being determined by a check on the fluorescence of the liquid condensed in the thimble portion. When an ultraviolet lamp showed this condensate to be free from fluorescent material, the extraction was stopped and the extracted liquid evaporated to dryness on a water bath to eliminate the acetone.

The solid residue was dissolved in cyclohexane, then transferred to the top of a 1/2 × 23 in. activated alumina column using a glass capillary tube fitted with rubber suction bulb and the minimum quantity of cyclohexane, certainly not exceeding 3 ml. Two circles cut from glass filter pad and inserted gently onto the top of the column prevented the alumina from being disturbed. Several loose strands of ether-washed "glass wool" fibres were placed on top of these discs.

The main eluting solvent, purified cyclohexane, was then run into the column

from a cylindrical separating funnel suspensed above the column and 25 ml fractions collected with a LKB radirac fraction collector.

It was found that the columns were very sensitive to pressure (possibly due to the increased resistance encouraging pulsing of the bed) hence only gravity percolation was employed. After the first 8–10 samples gradually increasing amounts of ether were added to the cyclohexane up to a maximum of 30 %, the specific quantities being determined by the even elution of the various bands down the column. Passage of these bands was closely followed with a 3650 Å ultraviolet lamp, so that care could be exercised in keeping the zones horizontal and sharply defined, hence systematising the ether additions.

Total samples numbered seventy to one hundred and twenty. These samples were transferred to brown phials then evaporated on a low temperature water bath to eliminate the ether and concentrate the samples.

Each fraction was separately run on a Beckman DK2 Ratio Recording Ultraviolet Spectrophotometer.

The order of elution of the polycyclic compounds is constant and this fact vastly simplifies systematic enquiry of unknown fractions. In Table I the relative elution rate of some of these compounds is given.

TABLE I

ORDER OF ELUTION OF POLYCYCLIC COMPOUNDS ON ACTIVATED ALUMINA WITH CYCLOHEXANE–ETHER SOLVENT

Aliphatics
Olefins
Benzene, toluene and derivatives
Naphthalene and naphthalene derivatives
Acenaphthene
Fluorene
Phenanthrene
Anthracene
Pyrene
Fluoranthene
1:2-Benzofluorene and 2:3-benzofluorene
9:10-Benzophenanthrene
1:2-Benzanthracene
Chrysene
3:4-Benzopyrene
1:2-Benzopyrene
Perylene
2:3-Benzofluoranthene
1:12-Benzoperylene
Anthanthrene
Coronene

A library of standard substances has been compiled allowing standard solutions of known concentrations in cyclohexane to be made. The graphs of these standards besides characterising and identifying polycyclic compounds enable quantitative measurements to be made using the Beer-Bouguer law of linear concentration/absorbance dependence.

The importance of graphing the actual standard substance under identical conditions to the run of unknowns cannot be too highly stressed. For the quantitative

determinations use was made of a local baseline technique, an extension of the work of COOPER[6], particularly where background absorbance interfered. When this effect did occur it was more evident at wave lengths from 280 mμ to the air absorbance at 180 mμ.

Generally the range of wave length values was 440–210 mμ the technique of scale magnification being often adopted particularly in the range 440–360 which precedes a cam change on the Beckman DK2 spectrophotometer.

Paper chromatography may sometimes be used to help resolve badly mixed fractions. The solvent systems acetic acid–ether–water (12:5:20) and *n*-propanol–ether–water (20:9:4) on acetylated paper, Carl Schleicher and Schüll 2043b mgl, proved the most satisfactory. SPOTSWOOD'S system[13] ethanol–toluene–water (17:4:1) and that of BERGMANN AND GRUENWALD[14] methanol–toluene–water (10:1:1) were also used.

R_F values for the acetic acid–ether–water system are given in Table II.

TABLE II

R_F VALUES ON ACETYLATED PAPER S & S 2043b WITH SOLVENT ACETIC ACID–ETHER–WATER (12:5:20)

Compound	*R_F value*	*Colour with 253.7 mμ ultraviolet light*
3:4-Benzopyrene	0.43	purple
1:2-Benzopyrene	0.33	purple
Fluoranthene	0.88	intense white-blue
1:9-Benzanthrone	0.81	intense olive green
1:2-Benzanthracene	0.56	violet
Pyrene	0.74	deep royal blue
Phenanthrene	0.77	faint blue-brown
Chrysene	0.71	ochre
3:4-Benzofluorene	0.66	royal blue
1:2-Benzofluorene	0.57	royal blue
Perylene	0.34	light blue
1:12-Benzoperylene	0.26	purple
1:2;3:4-Dibenzopyrene	0.15	lemon yellow
3:4;9:10-Dibenzopyrene	(0.05), (0.18)	white-blue brown
1:2;3:4-Dibenzanthracene	0.22	purple
3-Methylcholanthrene	0.28	violet
Phenanthraquinone	0.88	light brown (visible light)
Pentacene	0.26	fawn

Some mixing occurs at times between polycyclic compounds adsorbed to about the same degree. This is most prevalent with the compounds benzophenanthrene–benzanthracene–chrysene but only in the rare case where the concentration of benzanthracene is small compared to the other two tetracyclic isomers do all three appear on the one graph. Even when this effect occurs it is only over a small range and can be resolved quantitatively by three-dimensional analysis. Normally the benzophenanthrene–benzanthracene and benzanthracene–chrysene couplets allow ready quantitation by two-dimensional techniques.

Typical analysis of an air sample will emphasise the above arguments.

The sample, on the roof of Sydney Town Hall was collected by passing 422,500 cubic ft. of air at 50 cubic ft./min to give a total solid deposit of 2.011 g. After extraction and column chromatography 87 fractions were obtained.

SYSTEMATIC ANALYSIS

Benzene, toluene and derivatives. Shown in fractions 5,6. Characteristic wave lengths benzene 268 (slight), 261, 255, 248, 244 mμ; toluene 268, 265S, 262, 255, 248, 244 mμ.

Acenaphthene. Fractions 16, 17 (mainly) 18. Wave length maxima 229 (very strong), 280, 289, 301, 307, 321 mμ.

Fluorene. Trace in fraction 21. Wave length maxima 255, 262, 272 mμ.

Phenanthrene. Trace in fraction 22 mixed with anthracene. Wave lengths 244, 251, 258S, 264S mμ. Presence not confirmed by chromatography on acetylated paper with acetic acid–ether–water (12:5:20).

Anthracene. Fractions 22 and 23. Wave length maxima 221, 246S, 252 mμ.

Pyrene. Fractions 24, 25, 26, 27 (mixed). Very characteristic spectra at 231, 238S, 241, 252, 262, 273, 306, 319, 336 mμ. Fig. 1 shows sample 25.

Fluoranthene. Fractions 27, 28, 29. Very characteristic spectra at 236, 243S, 249, 263, 271S, 277, 288 mμ. Fraction 28 is shown in Fig. 2.

1:2-Benzofluorene and 2:3-benzofluorene. Only a trace is evident in fractions 31, 32. The fractions concentrated to 1 ml were chromatographed on acetylated paper with acetic acid–ether–water (12:5:20) and a faint trace of these compounds appeared to be present.

9:10-Benzophenanthrene. Fractions 40, 41, 42 (mixed with benzanthracene). Wave length maxima 250, 260, 275, 286 mμ. Quantitative estimation of fraction 42 requires two-dimensional analysis. Fig. 3 shows sample 40.

1:2-Benzanthracene. Mixed with benzophenanthrene in sample 42, with chrysene in samples 43, 44. Diagnostic wave lengths 226, 256, 277, 298, 316 mμ. See Fig. 4 showing sample 42, Fig. 5, sample 43.

Chrysene. Fractions 43, 44, 45, 46, 47, 48. Pertinent wave lengths 241, 250S, 259, 269, 282, 295, 306, 321. Fig. 6 shows sample 46.

3:4-Benzopyrene and 1:2-benzopyrene. Together in fractions 50, 51, 52. 3:4-Benzopyrene is readily identified by magnifying scale in region 360–450 to give diagnostic wave lengths at 385 mμ, 403 mμ. The 403 peak is used for quantitative estimation of 3:4-benzopyrene. See Figs. 7, 8.

2:3-Benzofluoranthene. Evident in fractions 59, 60. Wave length maxima 257, 263S, 277, 282S, 290, 294S, 303 mμ. Fraction 59 is shown in Fig. 9.

1:12-Benzoperylene. Fractions 61, 62, 63, 64, 65. Characteristic wave lengths 250, 259.5, 275, 286 mμ. Fig. 10 compares pure 1:12-benzoperylene with sample 63.

Anthanthrene. A quantity evident in fractions 69, 70. Fluorescent spectra using a mercury lamp and a Corning filter 5840 to radiate the fluorescent cell at 365 mμ gave wave length maxima at 426, 434, 456 and 488 mμ.

Coronene. The sample of coronene was spread over samples 70 (mixed) 71, 72, 73, 74, 75, 76. A comparison between sample 73 and pure coronene is given in Fig. 11.

Concentration of the polycyclic hydrocarbons on a weight and a volume basis are given in Table III.

TABLE III

CONTENT OF POLYCYCLIC HYDROCARBONS, ATMOSPHERIC SAMPLE, SYDNEY TOWN HALL

Compound	*Hydrocarbons p.p.m. of solid*	*Hydrocarbons μg/100 m³*
Acenaphthene	3.9	0.07
Anthracene	2.8	0.05
Pyrene	15.2	0.26
Fluoranthene	17.9	0.30
9:10-Benzophenanthrene	14.2	0.24
1:2-Benzanthracene	3.5	0.06
Chrysene	20.1	0.34
1:2-Benzopyrene	18.8	0.32
3:4-Benzopyrene	20.7	0.35
2:3-Benzofluoranthene	3.0	0.05
1:12-Benzoperylene	21.8	0.37
Anthanthrene	8.0	0.13
Coronene	15.9	0.27

DISCUSSION

Column adsorption chromatography is undoubtedly the best means of initial separation of the polycyclic mixture. Figs. 1–11 are actual photographs of a run of unknown fractions graphed on the paper supplied with the Beckman spectrophotometer. Spectra of the pure compounds are superimposed on these graphs and are indicated by a broken or dotted line. The clarity of separation and purity of the compounds relative to the standard substances is evident. Although with proper care such good separation is obtained, the method is lengthy and often some compromise must be reached between clear separation and elution time. For more routine investigations where five or six of the more common polycyclic compounds are required the column length could be cut to say 10 in. allowing comparable care of elution.

As air samples are much more complex than the pure polycyclic compounds which give such good results on paper, at least some arbitrary fractionation on alumina is required. If only qualitative data is wanted, paper chromatography from this point may well be used. SPOTSWOOD's method[13] of activated cellulose columns with solvent systems analogous to those used in paper chromatography has advantages over the normal paper methods in the far larger surfaces available for adsorption. For quantitative analysis of such samples, however, the good initial separation obtained by long alumina columns is very necessary.

With very impure samples of a tarry nature the method of CAHNMANN AND KURATSUNE[15], who partitioned polycyclic compounds between cyclohexane and aqueous methanol (1:4) was tried. The partition coefficients cyclohexane phase/water–methanol (1:4) phase for the polycyclic hydrocarbons found in the extracts varied from 14 to 150. A further partition between cyclohexane and nitromethane with coefficients nitromethane phase/cyclohexane phase from 1.5 to 4.4 was suggested by HOFFMANN AND WYNDER[16]. However, with both these methods there was often a carryover into the cyclohexane phase of unknown substances which poisoned the alumina, preventing good separation. Preliminary purification of tarry samples on 12 in. alumina columns with repeated ether washing appeared to be the best solution.

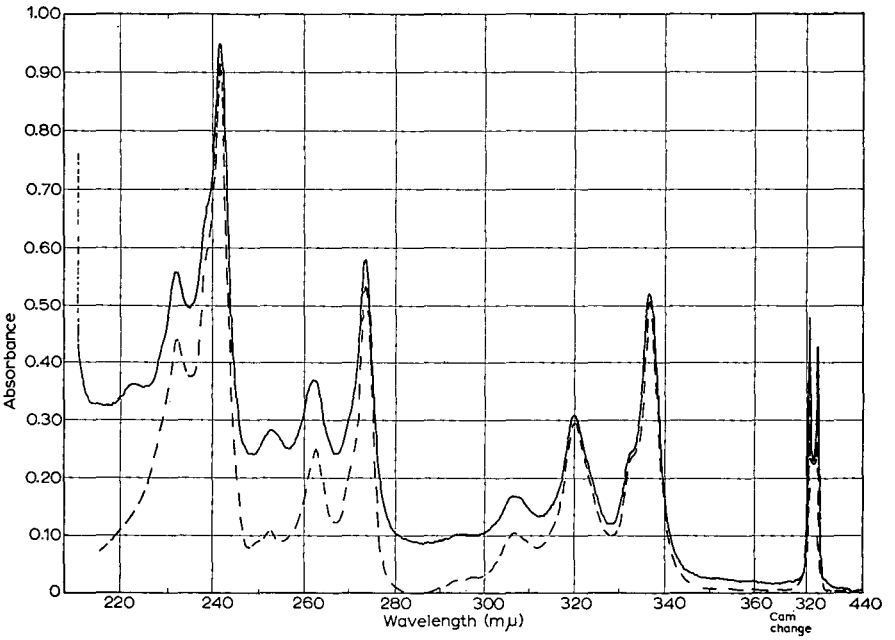

Fig. 1. Sample 25 (——); pyrene (– –).

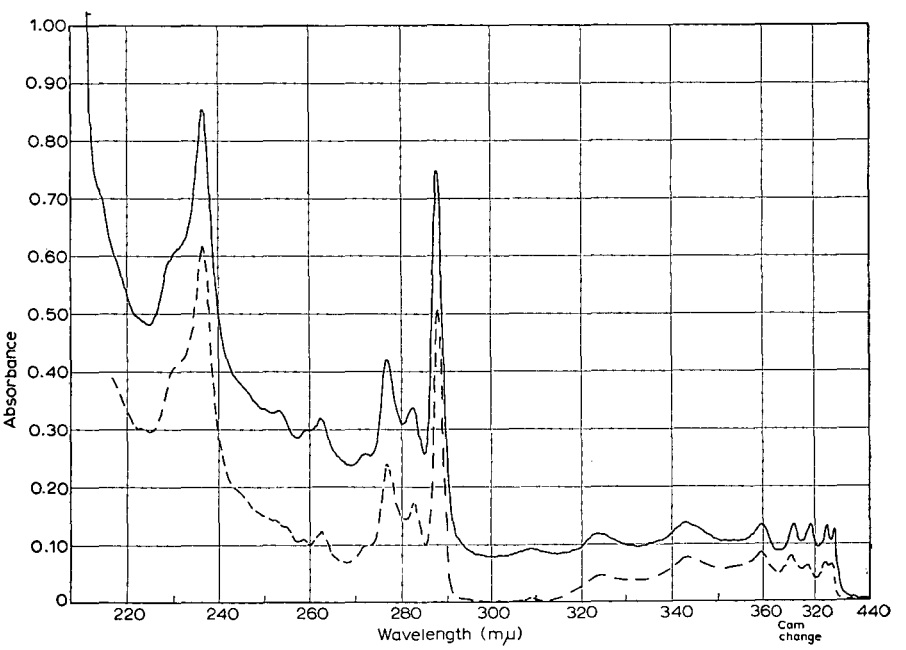

Fig. 2. Sample 28 (——); fluoranthene (– –).

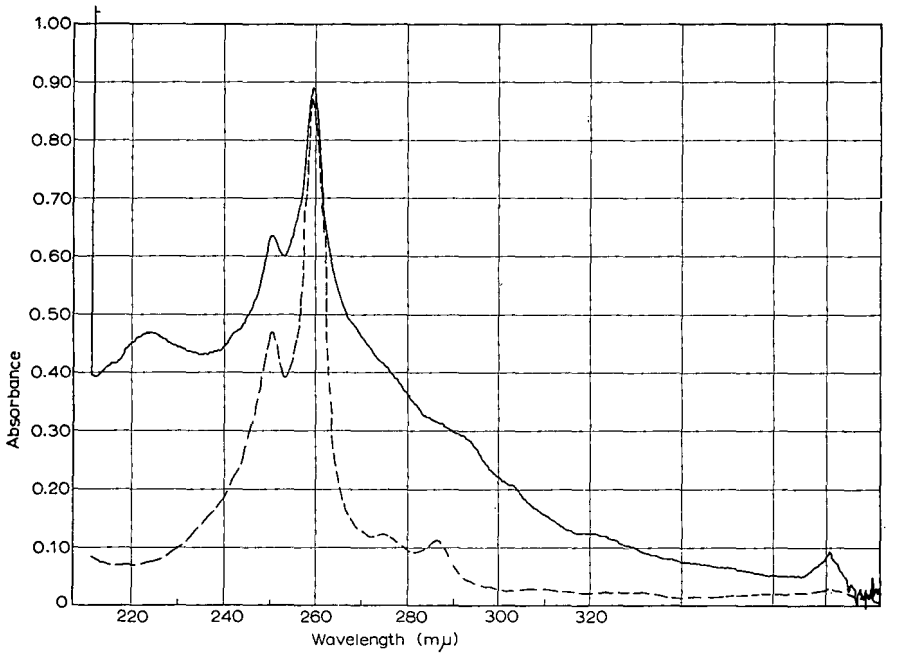

Fig. 3. Sample 40 (——); 9:10-benzophenanthrene (– –).

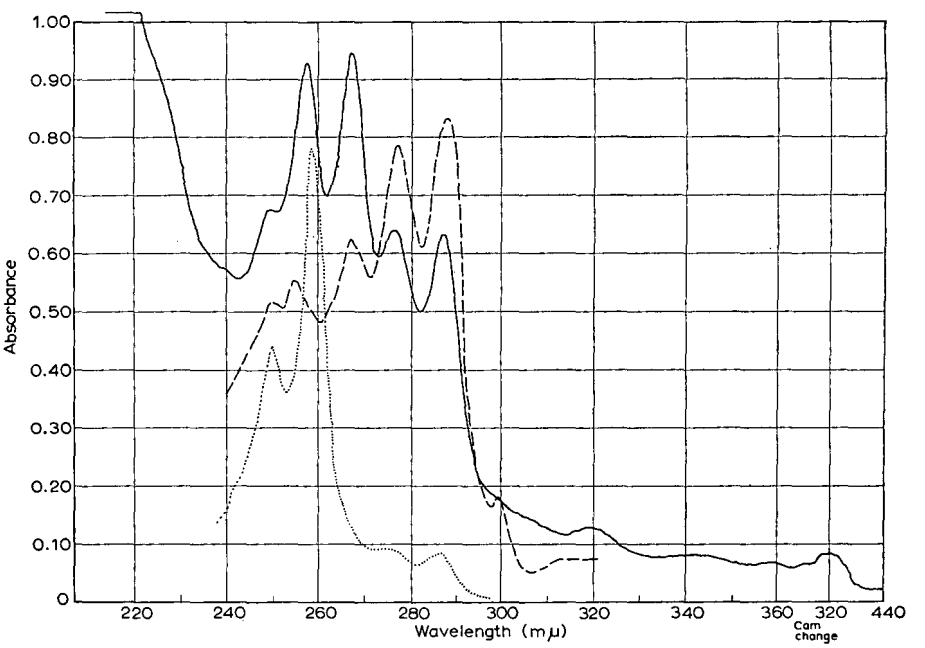

Fig. 4. Sample 42 (——); 9:10-benzophenanthrene (· · ·) and 1:2-benzanthracene (– –).

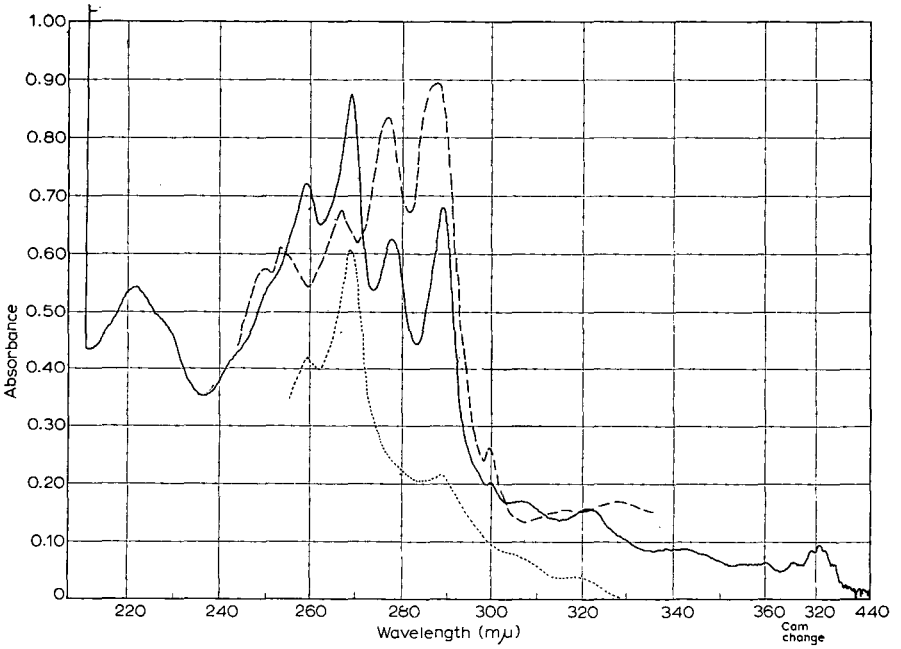

Fig. 5. Sample 43 (——); 1:2-benzanthracene (– –) and chrysene (· · ·).

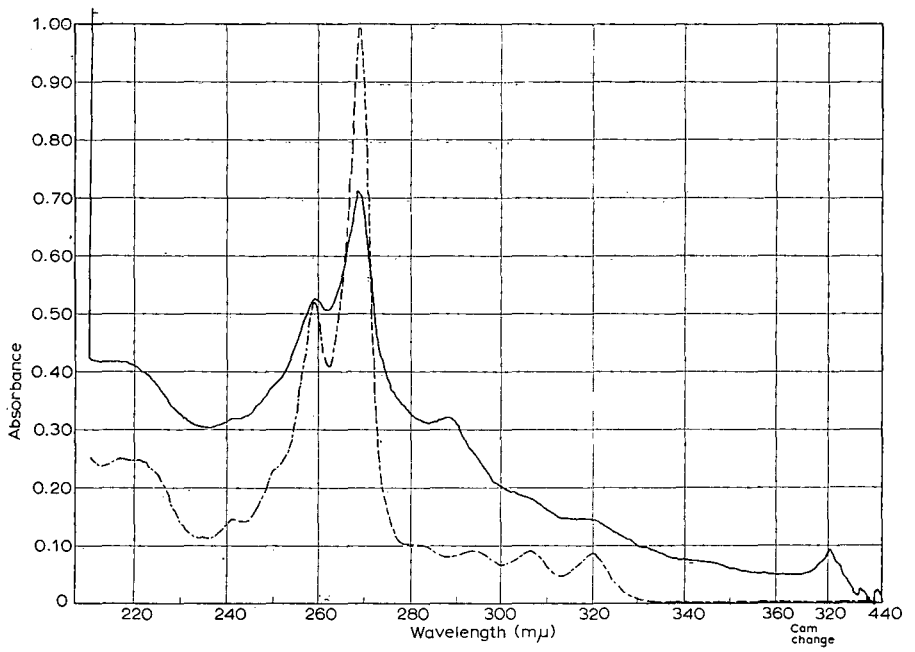

Fig. 6. Sample 46 (——); chrysene (– –).

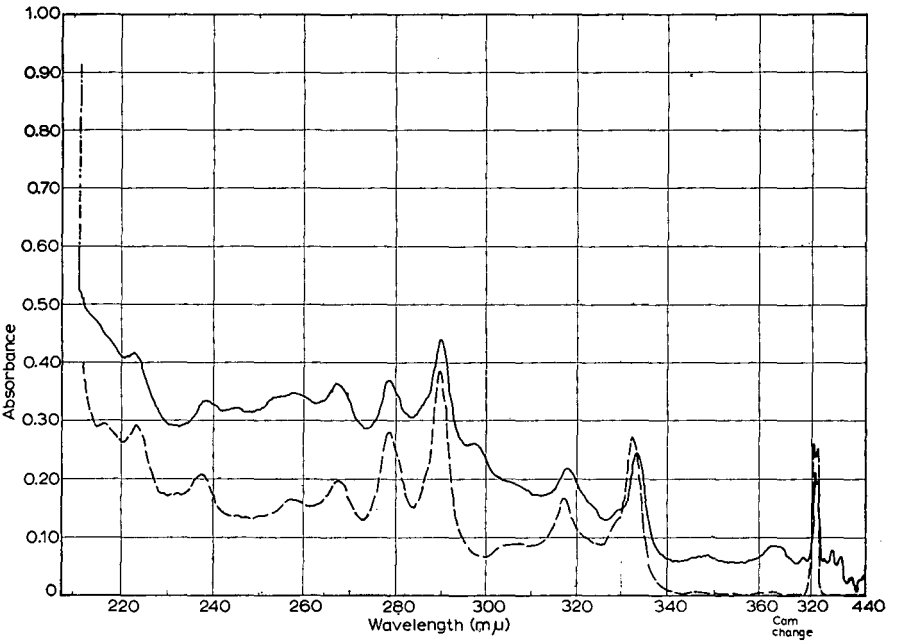

Fig. 7. Sample 51 (——); pure 1:2-benzopyrene (– –).

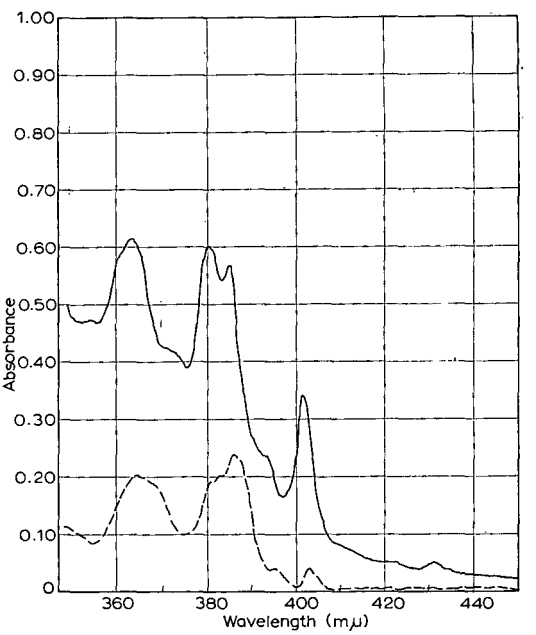

Fig. 8. Sample 52 (——); pure 3:4-benzopyrene (– –).

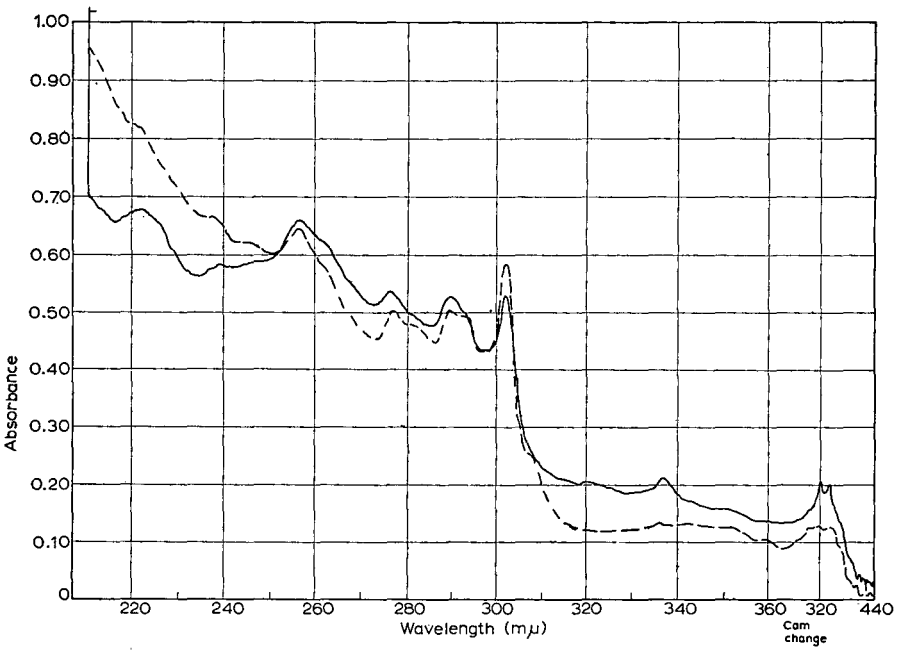

Fig. 9. Sample 59 (——); 2:3-benzofluoranthene (– –).

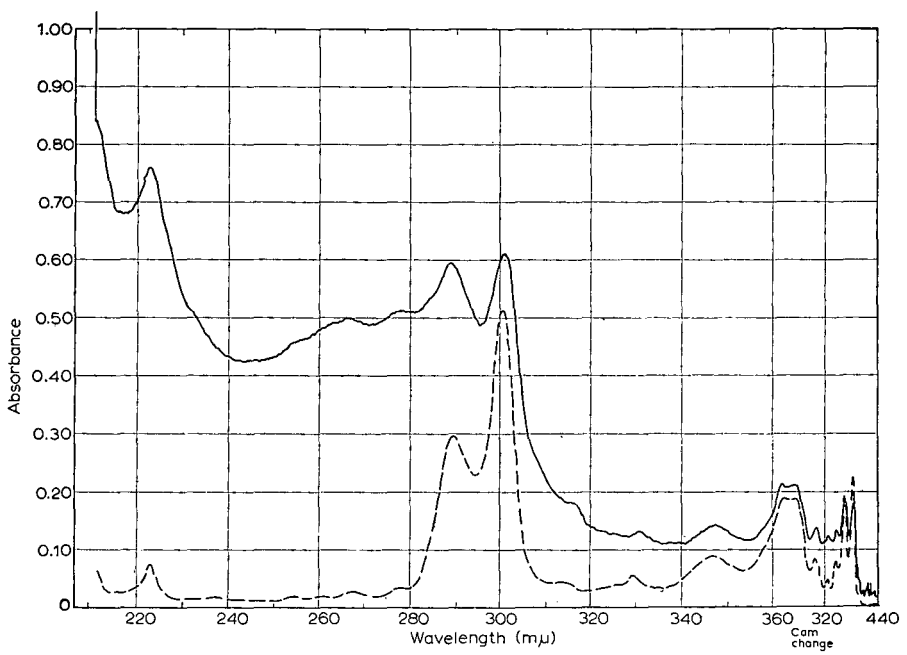

Fig. 10. Sample 63 (——); 1:12-benzoperylene (– –).

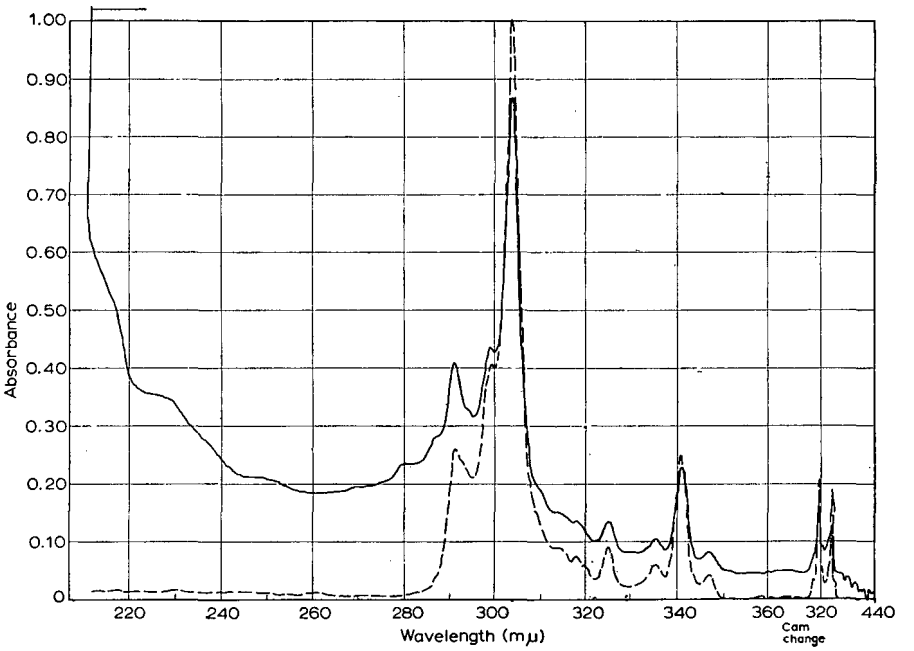

Fig. 11. Sample 73 (——); coronene (– –).

SUMMARY

Use has been made of an unusually long alumina column to obtain good separation of the complex polycyclic fraction of air particulates.

In many instances of quantitative estimation no other spectral media or chromatographic technique other than ultraviolet spectroscopy need be contemplated.

An example has been given of the chromatographic separation and identification of a typical air pollution sample.

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SEPARATIONS BY REVERSED-PHASE COLUMN PARTITION CHROMATOGRAPHY WITH KEL-F SUPPORTING TRI-*n*-OCTYLPHOSPHINE OXIDE

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In recent years reversed-phase partition chromatography has received increasing interest in the field of inorganic chemistry, since it helps to solve problems relating to new analytical procedures or to the preparation of certain specific elements. In this connection, the chemistry of nuclear materials or, in general, nuclear reactor technology has provided the incentive.

Tri-*n*-butyl phosphate (TBP), the well known organic solvent which is widely used in the liquid–liquid extraction of substances such as uranium, plutonium, zirconium, thorium, etc., has been fixed on different types of supports and used as the stationary phase in column chromatography.

FIDELIS AND SIEKIERSKI[1,2] as well as ESCHRICH[3] used suitably treated kieselguhr powder as a support for TBP, while SMALL[4] used styrene–divinylbenzene powder.

TBP is not the only extractant which gives good results. Di-(2-ethylhexyl) orthophosphoric acid on alumina[5,6], on polystyrene–divinylbenzene[6], or on cellulose powder[7], and tri-*n*-octylamine[8] (TNOA) or tri-*n*-octylphosphine oxide[9] (TOPO) on cellulose powder, have also been used with success.

Recently, a new organic support has been proposed by HAMLIN and co-workers[10], who fixed TBP or TNOA on poly-trifluoro-chloro-ethylene (Kel-F) and used this material for the selective retention of uranium and its purification.

Since in our laboratory as mentioned above[9], the system TOPO–cellulose has given good results in the separation of many elements or for the isolation of traces from undesired major components, it was our aim to combine the selectivity of TOPO with the high capacity of Kel-F and to investigate the chromatographic behaviour of this system.

The experiments and results are reported in the present paper.

EXPERIMENTAL

Reagents and equipment

Kel-F moulding powder (chemical composition $[\text{-CClF-CF}_2\text{-}]_x$, low density type, grade 300) was supplied by Minnesota S.p.A. (Milan). It is a halofluorocarbon polymer, which is non-inflammable, exceptionally stable and resistant to temperature as well as chemically highly inert.

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Tri-*n*-octylphosphine oxide (chemical compositon $(C_8H_{17})_3PO$; mol. wt. 386.65; m.p. 54–55°) was supplied by Eastman Organic Chemicals, New York, U.S.A.

All the acids, salts and organic compounds were analytical grade.

The chromatographic columns were 8.6 mm i.d. pyrex tubes fitted with a fritted glass disc at the bottom.

The Kel-F powder was ground in a laboratory hammer-mill and reduced to the required size by means of a series of standard sieves.

Preparation of Kel-F/TOPO

The general procedure was as follows: to 5 g of Kel-F powder of the required grain size, a solution containing 4 g of TOPO in 9 ml of cyclohexane was added and the mixture stirred in a small beaker until it was homogeneous. Then 20 ml of 1:1 HCl was added and the mixture stirred again for a few minutes and finally allowed to stand for one hour. After that, the mixture was poured into the column and the voids were eliminated by gently pressing with a glass rod. A 12 cm high bed was obtained which was washed with 200 ml of 1:1 HCl to eliminate the excess of organic solution.

Three slightly different preparations (A, B and C) were made in order to select relatively good operating conditions of the bed.

In preparation A, Kel-F powder of 100–170 mesh grain size was used, in preparation B, the grain size was 35–50 mesh, and in preparation C a slight variation of preparation A was obtained by volatilizing cyclohexane at 60° before adding the 20 ml of 1:1 HCl.

Types A and B were used for the separation Fe–Co–Ni, and the results given in Fig. 1 show that the smaller grain size has a favourable effect on the separation and definition of the peaks.

A comparison between types A and C, reported in Fig. 2, shows that on elution of iron with 0.5 *M* H_2SO_4 the curve is steeper in the case of type A than in that of

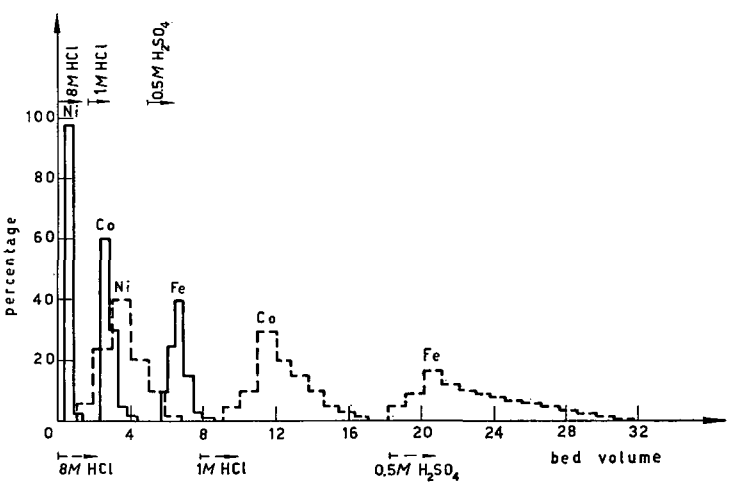

Fig. 1. Effect of grain size of Kel-F on the separation of Ni–Co–Fe. Solid line: 5 g Kel-F 100–170 mesh + 4 g TOPO–cyclohexane (10 ml). Dashed line: 5 g Kel-F 35–50 mesh + 4 g TOPO–cyclohexane (10 ml). Bed volume: 7 cm^3; feed solution: Fe–Co–Ni, 10 mg each in 0.5 ml of 8 *M* HCl; flow-rate 0.5 ml/min.

type C. This probably means that TOPO has a higher reactivity when in the presence of the organic solvent than when it is in the solid state even though adsorbed on the surface of the organic support.

These results led us to use the type A preparation for further investigations and the break-through curve reported in Fig. 3 was determined with a Fe^{3+} solution. The

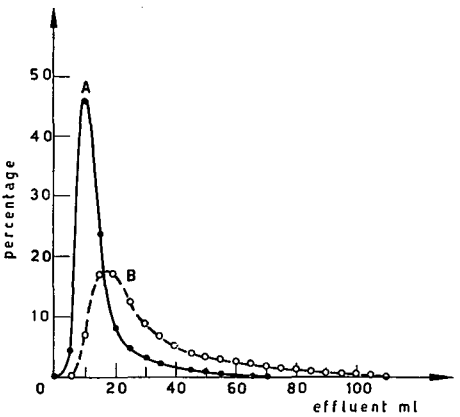

Fig. 2. Elution peak of 10 mg Fe with 0.5 *M* H_2SO_4 at 1 ml/min. Curve A: 100–170 mesh Kel-F; TOPO added in cyclohexane solution without volatilization of the organic solvent. Curve B: the same as curve A, but using a preparation of Kel-F/TOPO from which cyclohexane had been removed by volatilization at 60°.

column was pre-treated with 50 ml of 8 *M* HCl and then a solution containing 1 mg/ml of iron in 8 *M* HCl was percolated through the column at 1 ml/min. Iron was well retained by the bed, and its retention within the column could be followed by the slow downward movement of the yellow band. Iron broke through after 115 ml, and the bed was saturated with about 130 mg of iron.

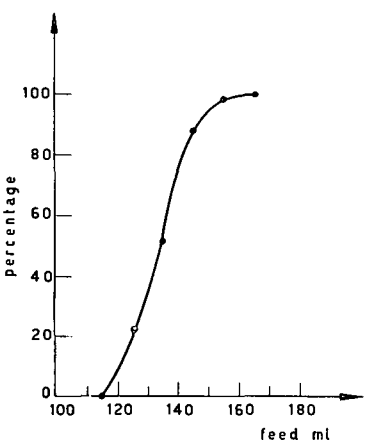

Fig. 3. Break-through curve of iron on a 100–170 mesh Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Feed: 8 *M* HCl with 1 mg/ml Fe at a rate of 1 ml/min.

CHEMICAL SEPARATIONS

On the basis of results obtained in experiments carried out some time ago with TOPO-treated cellulose powder[9] or with paper chromatography[11], the following separations were investigated with Kel-F/TOPO columns. As a general procedure, the hydrochloric solution of the ions to be separated was fed onto the top of the column, and then elution was started with the various solvents at the rates indicated in the legends to the figures.

Separation of Ni–Co–Fe^{3+}–U^{6+}

Nickel is not complexed by hydrochloric acid, and hence it is not retained by the bed. In fact the pale green band of this element moves with the solvent front (8 *M* HCl), followed by the blue band of cobalt which is only weakly fixed. Iron and uranium (yellow bands) are firmly held on the upper part of the column. As shown in Fig. 4, after the break-through of nickel, cobalt is rapidly eluted with 1 *M* HCl, whilst iron and uranium are removed with 0.5 *M* H_2SO_4 and 4 *M* H_3PO_4 respectively.

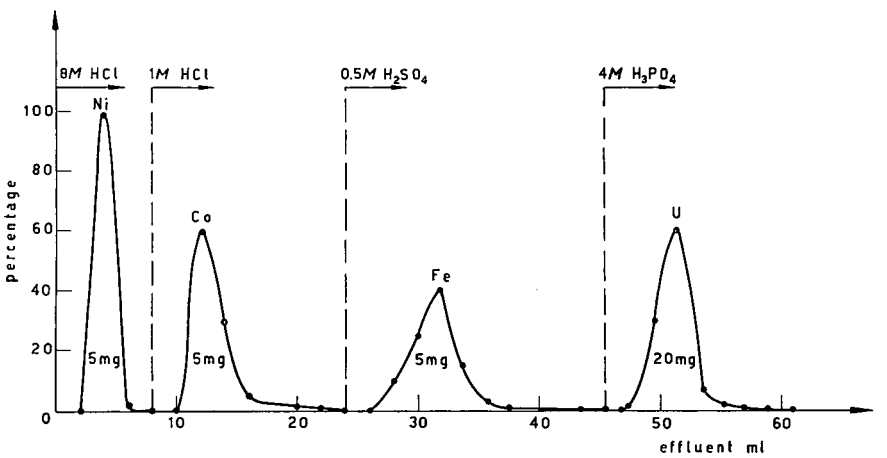

Fig. 4. Separation of Ni–Co–Fe^{3+}–U^{6+} on a Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Flow-rate 0.5 ml/min. Feed solution volume 0.5 ml.

Separation of Al–Cu–Fe^{3+}–U^{6+}

Aluminium travels with the solvent front (8 *M* HCl) and copper (yellow band) moves more slowly. In Fig. 5 are shown the elution peaks obtained by using 1 *M* HCl for copper, 0.5 *M* H_2SO_4 for iron and 4 *M* H_3PO_4 for uranium.

Separation of V^{4+}–Ti^{4+}–U^{6+}

The blue band of vanadium runs along the column with 8 *M* HCl, whilst titanium and uranium are strongly held. Titanium can be eluted, rather slowly, with 2 *M* H_2SO_4, while for uranium 4 *M* H_3PO_4 is used, as usual (Fig. 6).

Separation of Mn–Cu–U^{6+}

This separation is shown in Fig. 7; manganese is not retained at all in 4 *M* HCl and forms a pink band which moves with the front. Copper (yellow band) is eluted with 1 *M* HCl and uranium (yellow band) with 4 *M* H_3PO_4.

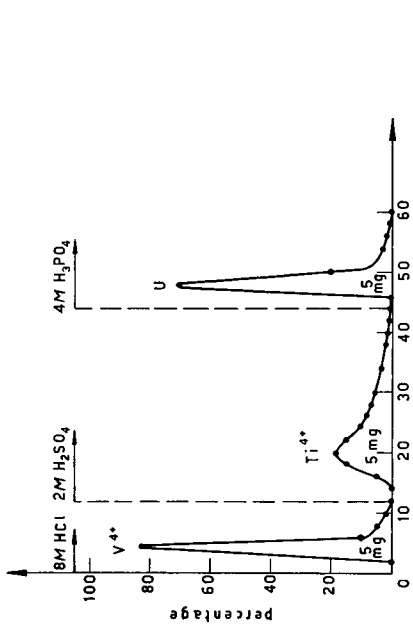

Fig. 6. Separation of V^{4+}–Ti^{4+}–U^{6+} on a Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Flow-rate 1 ml/min. Feed solution volume 1 ml.

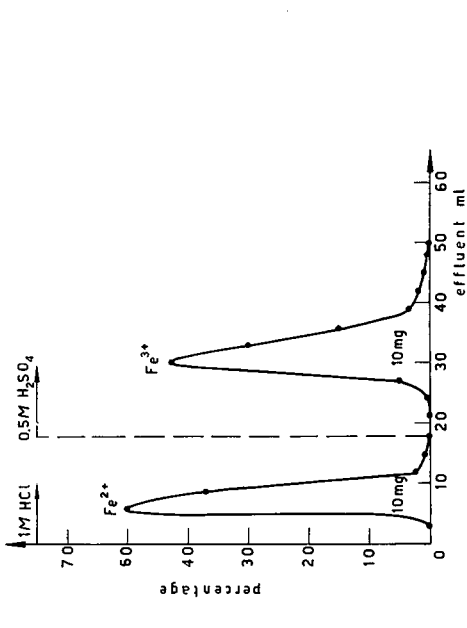

Fig. 8. Separation of Fe^{2+}–Fe^{3+} on a Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Flow-rate 1 ml/min. Feed solution volume 3 ml.

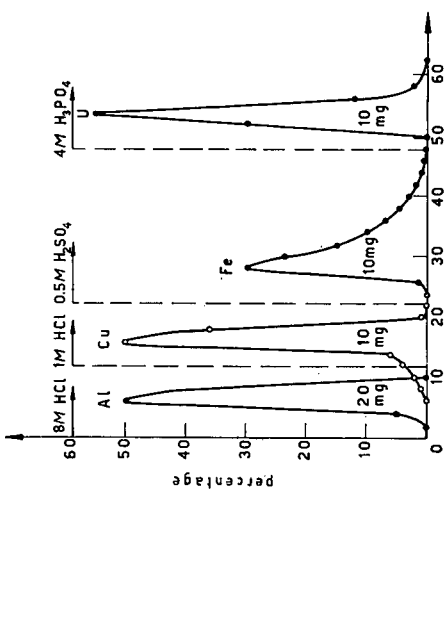

Fig. 5. Separation of Al–Cu–Fe^{3+}–U^{6+} on a Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Flow-rate 0.5 ml/min. Feed solution volume 2 ml.

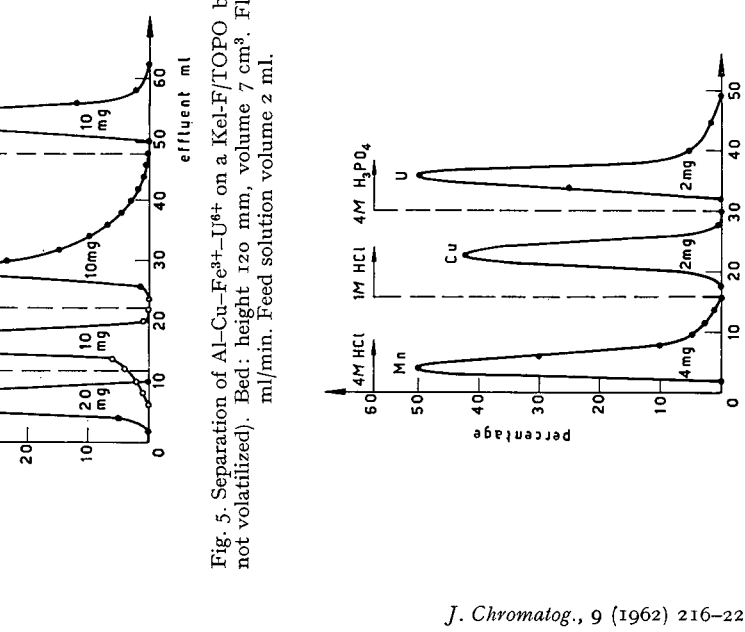

Fig. 7. Separation of Mn–Cu–U^{6+} on a Kel-F/TOPO bed (solvent not volatilized). Bed: height 120 mm, volume 7 cm^3. Flow-rate 1 ml/min. Feed solution volume 1 ml.

Separation of Fe^{2+}–Fe^{3+}

Since divalent iron does not form chloride complexes, it can be separated from trivalent iron. In Fig. 8 the elution of Fe^{2+} with 1 *M* HCl and that of Fe^{3+} with 0.5 *M* H_2SO_4 are shown.

ISOLATION AND QUANTITATIVE DETERMINATION OF SOME ELEMENTS BY MEANS OF KEL-F/TOPO BEDS

Our previous experiments indicated that the exchange rate and selectivity of Kel-F/TOPO columns are adequate for the isolation of some elements when in the presence of large amounts of other elements. To demonstrate the practical usefulness of the method, some typical cases were investigated and the results obtained are reported in the following sections.

Simultaneous quantitative determination of traces of cobalt and iron in nickel

A synthetic solution was prepared by dissolving 1 g of nickel containing 0.2 % w/w of cobalt and iron respectively in 25 ml of 8 *M* HCl. The column used in previous separations, after treatment with 8 *M* HCl, was fed with the synthetic solution at 2 ml/min.

As mentioned above, nickel was not retained, so that after washing with 20 ml 8 *M* HCl to remove the last traces of nickel, cobalt was eluted with 10 ml of 1 *M* HCl at 0.5 ml/min. The cobalt-containing solution was evaporated to dryness and re-dissolved with water. Cobalt was then determined by complexometry, back-titrating with 0.01 *M* $MgSO_4$ and using Eriochrome Black T as indicator[12]. The recovery of cobalt was 102 %.

Iron was finally eluted with 30 ml of 0.5 *M* H_2SO_4 at 0.5 ml/min; complexometric titration with EDTA and salicylic acid[13] showed a recovery of 100.5 %.

If only iron has to be determined in nickel, a shorter bed (3 cm) can be used; after percolation of the solution at 4 ml/min, iron can be eluted directly with 10 ml of 0.5 *M* H_2SO_4.

If the amounts of cobalt and iron are smaller than those considered above, a spectrophotometric method can of course be used instead of the complexometric titration.

Simultaneous determination of uranium and thorium in dilute solutions containing foreign ions

Uranium and thorium are very selectively retained by TOPO from a 1 *M* HNO_3 solution[9,11]; therefore they can easily be separated from alkali metals, alkaline earths, rare earths and from the majority of the chemical elements.

A synthetic solution was prepared consisting of 200 ml of 1 *M* HNO_3 which contained 40 mg of each of the following ions: Na^+, Ca^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Al^{3+}, Y^{3+} and La^{3+}.

The solution was fed at 4 ml/min onto a 3 cm high bed of type A Kel-F/TOPO which had been pre-treated with 20 ml of 1 *M* HNO_3. After washing the feed container and the bed with 100 ml of 1 *M* HNO_3 to eliminate any trace of foreign elements, thorium was removed from the bed with 100 ml of 0.5 *M* HCl. In a 10 ml sample of the solution obtained, thorium was determined by spectrophotometry with thoron, the recovery being 96 %. Uranium was eluted with 25 ml of 1 *M* H_3PO_4, and 5 ml of the solution was checked for uranium with $NaOH$–H_2O_2 colorimetry. The recovery was 101.5 %.

Determination of traces of zirconium in copper

Zirconium is quantitatively held by Kel-F/TOPO in 1:1 HNO_3, whilst copper is not retained at all. A synthetic solution was prepared in the following way: 1 g of copper was dissolved in 10 ml of conc. HNO_3, and then 3 mg of zirconium were added. This solution was reduced to 2 or 3 ml by evaporation and then 20 ml of 1:1 HNO_3 was added.

The liquid obtained was fed onto the bed, which had been pre-treated with 20 ml of 1:1 HNO_3, at a flow-rate of 1 ml/min. The bed was washed with 20 ml of 1:1 HNO_3 at the same flow-rate and finally zirconium was eluted at 0.5 ml/min with 20 ml of 0.5 *M* oxalic acid.

After elimination of oxalic acid by heating the solution with perchloric acid, the liquid was evaporated almost to dryness, then 50 ml of distilled water were added, and zirconium was determined by complexometry, back-titrating with 0.01 *M* $FeCl_3$ and salicylic acid[12]. The error in this analysis was ± 3 %.

When zirconium is present in microgram quantities, colorimetric determination of this element becomes necessary.

Evaluation of the exchange capacity of a type A Kel-F/TOPO bed, after 15 cycles

The exchange capacity of a type A Kel-F/TOPO bed which had been used for 15 consecutive cycles was determined again at the end of the last cycle. The usual solution of iron, 1 mg/ml, in 8 *M* HCl was used. The exchange capacity was found to be about 30 % less than the initial value. In view of the fact that during the numerous cycles the bed comes into contact with hydrochloric, nitric, sulphuric, and phosphoric acid of various concentrations, this result can be considered as quite satisfactory.

CONCLUSIONS

Kel-F was found to be a suitable inert support for solutions of TOPO–cyclohexane. As was to be expected from the general data on halofluorocarbon polymers, this support is practically unaffected by acids, alkalies and, to a large extent, also by temperature. By virtue of its porosity it is able to retain a considerable amount of the organic extractant which is not appreciably stripped by the aqueous solution used in the chromatographic process.

As confirmed also by preceding work[9,11], the great extractive ability of TOPO and its good reaction rate make it suitable for use in a great variety of chemical separations, narrow peaks being obtained. In addition, traces of some elements in dilute solutions and in the presence of foreign ions can be retained by passing the liquid rapidly through short beds of Kel-F/TOPO. The columns can be prepared very quickly and each column can withstand many operative cycles with only a relatively small loss of exchange capacity.

SUMMARY

The use of chromatographic columns filled with Kel-F (poly-trifluorochloro-ethylene) supporting concentrated solutions of tri-*n*-octylphosphine oxide (TOPO) in cyclohexane has been investigated.

After selecting the best way of preparing the columns examples are given of the following separations: Fe^{3+}–Co–Ni–U^{6+}, V^{4+}–Ti^{4+}–U^{6+}, Mn–Cu–U^{6+}; Al–Cu–Fe^{3+}–U^{6+} and Fe^{2+}–Fe^{3+}.

Short columns of Kel-F/TOPO were also used for the isolation and quantitative determination of traces of elements in dilute solutions and in the presence of large amounts of foreign ions. The following examples are discussed: Fe and Co in Ni, U and Th in the presence of 13 foreign ions and Zr in Cu.

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EINE PAPIERCHROMATOGRAPHISCHE TRENNUNG VON FLUOR-THIONO-DIPHOSPHORSÄUREALKYLAMIDEN

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Im Verlaufe unserer Arbeiten über Derivate von Fluor-diphosphorsäuren[1,2] beschäftigten wir uns u.a. auch mit einer Verbindung folgender Struktur:

$$[N(C_2H_5)_2](F)P(S)—O—P(O)[N(C_2H_5)_2](F) \quad (I)$$

Einige Beobachtungen bei der Herstellung und Isolierung der Verbindung liessen vermuten, dass I die entsprechenden Dithiono- (II) und Dioxohomologen (III) als Nebenprodukte enthielt. Dieser Sachverhalt war analytisch nicht prüfbar, da sich II und III aus I infolge thermischer Symmetrifizierungsreaktionen in gleicher Menge bilden; zudem unterscheiden sich die Siedepunkte der drei Verbindungen nur wenig[2].

Der Nachweis der drei Verbindungen im erhaltenen Syntheseprodukt gelang papierchromatographisch auf folgendem Wege: Nach zahlreichen Versuchen erwies sich eine Anordnung mit umgekehrten Phasen als geeignet. Die Substanzen werden auf ein mit Petroleum imprägniertes Papier (stationäre lipophile Phase) aufgetragen und mit 50 % Essigsäure (mobile hydrophile Phase) absteigend chromatographiert[3]. Hierbei blieb die Verbindung II in Startnähe, Verbindung I befand sich mit einem R_F-Wert von etwa 0.4 in der Mitte des Streifens, und Verbindung III war in Frontnähe anzutreffen. Die R_F-Werte der Flecke von II und III konnten mit den auf anderem Wege rein dargestellten Verbindungen[2] überprüft und bestätigt werden.

Die R_F-Werte werden bei dem beschriebenen Verfahren — von geringen Schwankungen abgesehen — ausschliesslich vom Verhältnis der Gruppen P=S/P=O im Molekül bestimmt. So zeigten Chromatogramme der nachstehend aufgeführten, an *anderen* Gerüststellen variierten Moleküle IV und V stets nur einen Fleck in Frontnähe, entsprechend der Abwesenheit von P=S-Gruppen:

$$[N(C_2H_5)_2](F)P(O)—O—P(O)[N(C_2H_5)_2]_2 \quad (IV)$$

$$[N(C_2H_5)_2](F)P(O)—O—P(O)[N(CH_3)_2]_2 \quad (V)$$

Auf ähnliche Weise konnten in einem Produkt der erwarteten Struktur:

$$[N(C_2H_5)_2](F)P(S)—O—P(O)[N(C_2H_5)_2]_2 \quad (VI)$$

* Neue Anschrift: Farbenfabriken Bayer AG, Wuppertal-Elberfeld (Deutschland).
** Verstorben.

wieder (sehr geringe) Anteile des Dithiono- und des Dioxohomologen (IV) neben der Hauptsubstanz VI nachgewiesen werden, wobei die Lage der R_F-Werte wiederum nur vom P=S/P=O-Verhältnis im dargestellten Sinne abhängig war.

Wir glauben, dass sich das Verfahren auch zur Klärung von Problemen aus der Chemie andersartig substituierter Thiono-di- oder -polyphosphorsäurederivate benutzen lässt.

Bemerkenswert ist noch, dass die Chromatogramme der Verbindungen I und VI anstelle *eines* Fleckes mit mittlerem R_F-Wert deren *zwei* zeigten. Diese waren jedoch so eng benachbart, dass sie häufig kaum getrennt werden konnten. Es liegen also Produkte von praktisch gleichem P=S/P=O-Verhältnis vor, die sich möglicherweise nur durch verschiedene räumliche Struktur unterscheiden. Eine exakte Erklärung hierfür steht jedoch noch aus.

EXPERIMENTELLER TEIL

Aus Papier Schleicher & Schüll Nr. 2043A werden Streifen entsprechenden Formats zurechtgeschnitten und nach REINDEL UND HOPPE[4] zugespitzt. Man zieht die Streifen schnell durch ein Gemisch von Petroleum (Sdp. 200–240°)–Petroläther im Verhältnis 4:6, bewegt sie leicht schwenkend einige Male hin und her und presst sie zwischen Filterpapier ab. Mittels spitz ausgezogenen Glasstabes wird ein sehr kleiner Tropfen der (unverdünnten) Substanz auf den Startpunkt gesetzt. Nach 15–20 Min. wird der Streifen absteigend mit 50% Essigsäure chromatographiert. Die Atmosphäre der Kammer wird mit dem Laufmittel und etwas Petroleum gesättigt. Die Laufzeit beträgt 17–21 Stunden bei 18°. Danach wird der Streifen aus der Kammer genommen und auf Filterpapier liegend trocknen gelassen. Vorm Besprühen mit dem üblichen Molybdatreagenz[5] wird der Streifen zur Beseitigung des Petroleums 15 Min. bei 160° im Trockenschrank gehalten. Nach dem Aufbringen des Molybdatreagenzes wird 7 Min. auf 85–95° erwärmt. Danach muss der Streifen 4–7 Tage liegen bleiben. Dies ist zum völligen Abbau der thionoschwefelhaltigen Verbindungen erforderlich. Schwefelfreie Verbindungen werden dagegen sofort zersetzt. Zuletzt werden die Flecke durch Besprühen mit einer sehr verdünnten $SnCl_2$-Lösung sichtbar gemacht: Dunkelblaue Flecke auf blassblauem Grund.

Für die einzelnen Verbindungen wurden Flecke mit den in Tabelle I angegebenen R_F-Werten erhalten.

TABELLE I

Verbindung	*R_F-Werte für die Strukturen*			
	=P(S)–O–P(S)=	*=P(S)–O–P(O)=*		*=P(O)–O–P(O)=*
I	0.06	0.37	0.47	0.84
II	0.07	—	—	—
III	—	—	—	0.85
IV	—	—	—	0.86
V	—	—	—	0.90
VI	0.06	0.52	0.61	0.89

DANK

Der eine von uns (C.St.) ist der Firma ISIS-Chemie, Zwickau/Sachsen, Deutschland, für die Unterstützung der Arbeit zu Dank verplichtet.

ZUSAMMENFASSUNG

Es wird ein Verfahren zur papierchromatographischen Trennung von Fluor-thiono-diphosphorsäurealkylamiden mitgeteilt. Man chromatographiert auf petroleumgetränktem Papier mit 50 % Essigsäure. Die R_F-Werte sind nur vom Verhältnis der P=S/P=O-Gruppen im Molekül abhängig.

SUMMARY

A procedure for the separation of fluoro-thiono-diphosphoric acid alkylamides by means of paper chromatography is reported. Chromatography is carried out on paper impregnated with petroleum and 50 % acetic acid serves as solvent. The R_F values depend only on the P=S/P=O ratio in the molecule.

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SEPARATION OF HYPOPHOSPHITE, PHOSPHITE AND PHOSPHATE BY ANION-EXCHANGE CHROMATOGRAPHY

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Iodometric methods for estimating hypophosphite, phosphite and phosphate in a mixture[1,2] are unsatisfactory when the mixture also contains other lower oxyanions or thioxyanions of phosphorus, since these also react with iodine. A scheme employing gradient elution anion-exchange chromatography similar to that developed by GRANDE AND BEUKENKAMP[3] and other investigators[4–9] is used to obtain a separation of hypophosphite, phosphite and phosphate. Quantitative separations, using two independently developed schemes, one at pH 6.8 and the other at pH 11.4 are described.

EXPERIMENTAL

Materials

B.D.H. Reagent Grade sodium hypophosphite $NaH_2PO_2 \cdot H_2O$; and disodium phosphite, $Na_2HPO_3 \cdot 5H_2O$ were twice recrystallised from aqueous solution.

B.D.H. AnalaR disodium hydrogen orthophosphate $Na_2HPO_4 \cdot 12H_2O$ was used without further purification.

B.D.H. Reagent Grade potassium chloride.

Preparation of chromatographic columns

Dowex-1 X8 anion-exchange resin (mesh size 100–200; batch number 3246) was made into a slurry with distilled water and poured into a glass column fitted with a tap at the lower end, and surrounded by a water-jacket suitable for the circulation of water from an external source. The resin was allowed to settle until the length of the resin bed was 50 cm. It was then washed with 200 ml 4 *N* hydrochloric acid, followed by distilled water until the effluent was free of chloride ions. Finally the column was washed with 200 ml of buffered potassium chloride of the same concentration and pH as the initial eluant solutions.

Chromatographic separation

1.0 ml of a solution of a mixture of the anions to be analysed, containing up to 2000 μg of phosphorus per anion per ml, was pipetted on to the column. When the sample solution had almost been completely absorbed in the resin, it was washed into the resin bed by about 2 ml of initial buffered potassium chloride eluant solution.

The anions were eluted from the column using a gradient of potassium chloride concentration obtained by the method described by GRANDE AND BEUKENKAMP[3].

TABLE I

CONDITIONS OF THE CHROMATOGRAPHIC SEPARATIONS

Figure number	*Column dimensions cm*	*KCl concentration in mixing vessel ml/M*	*KCl concentration in reservoir M*	*Temperatures °C*	*pH**	*Flow rate ml/h*
1	50 × 1.5	750/0.05	0.20	18	6.8	60
2	50 × 1.0	750/0.075	0.10	18	11.4	40
3	50 × 1.0	750/0.075	0.10	~ 2**	11.4	25

* Buffer solutions: pH 6.8–25 ml of 2 *M* ammonium acetate solution per l of potassium chloride solution; pH 11.4–20 ml of 0.880 AnalaR ammonia per l of potassium chloride solution.
** ~ 2° obtained by pumping ice-cold water through the water jacket of the column.

10 ml fractions of effluent were collected by means of a syphon pipette and an automatic fraction collector.

Analysis of the fractions

The phosphorus content of each 10 ml fraction was determined by modification of the phosphovanadomolybdate method[10]. The 10 ml fractions were transferred to conical flasks and boiled for one hour with 10 ml of conc. nitric acid (1.42) and 5 ml of bromine water. After cooling, the contents of the conical flasks were transferred to 100 ml graduated flasks. 5 ml of an ammonium vanadate solution (containing 5 g of ammonium vanadate and 20 ml of conc. nitric acid (1.42) per l) were added, followed by 10 ml of 10 % w/v ammonium molybdate solution. The solutions were diluted to 100 ml with distilled water.

The absorbance of the solutions was measured in 1 cm cells at 400 mμ employing a Unicam SP 500 spectrophotometer, after allowing not less than 15 min for full development of the coloured complex.

RESULTS AND DISCUSSION

An examination of Figs. 1, 2 and 3, shows that excellent separations were obtained using both schemes. The order of elution of phosphite and phosphate is reversed on changing the pH of the eluant solution from 6.8 to 11.4. This is presumably because

TABLE II

RETENTION VOLUMES AND PHOSPHORUS RECOVERY

	Hypophospite		*Phosphite*		*Phosphate*	
Figure number	*Retention volume ml*	*Recovery %*	*Retention volume ml*	*Recovery %*	*Retention volume ml*	*Recovery %*
1	150	99	330	98	260	97
2	85	—	285	—	410	—
3	70	98	215	99	370	103

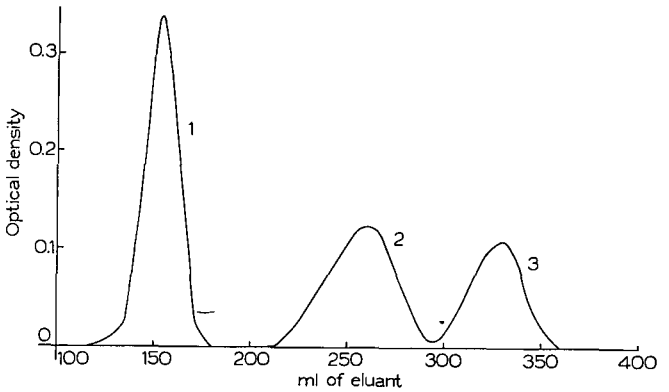

Fig. 1. Separation at a pH of 6.8 at 18°. (1) Hypophosphite; (2) phosphate; (3) phosphite.

the predominant phosphate ion is singly charged in the former solution and doubly charged in the latter case, whilst the predominant phosphite ion is the same in both cases.

$$\left.\begin{aligned} H_3PO_4 &\rightleftharpoons H_2PO_4^- + H^+ \quad K_1 = 1.1 \cdot 10^{-2} \\ H_2PO_4^- &\rightleftharpoons HPO_4^{2-} + H^+ \quad K_2 = 7.5 \cdot 10^{-8} \\ HPO_4^{2-} &\rightleftharpoons PO_4^{3-} + H^+ \quad K_3 = 4.8 \cdot 10^{-3} \end{aligned}\right\} \text{ at } 18°$$

$$\left.\begin{aligned} H_3PO_3 &\rightleftharpoons H_2PO_3^- + H^+ \quad K_1 = 7 \cdot 10^{-3} \\ H_2PO_3^- &\rightleftharpoons HPO_3^{2-} + H^+ \quad K_2 = 2 \cdot 10^{-5} \end{aligned}\right\} \text{ at } 25°$$

Comparison of the separation at room temperature and at 2° indicates that the retention volumes of the three anions are decreased at the lower temperature, and the effect is greatest for phosphite. As a result the separation of phosphate and phosphite is increased at pH 11.4 and decreased at pH 6.8.

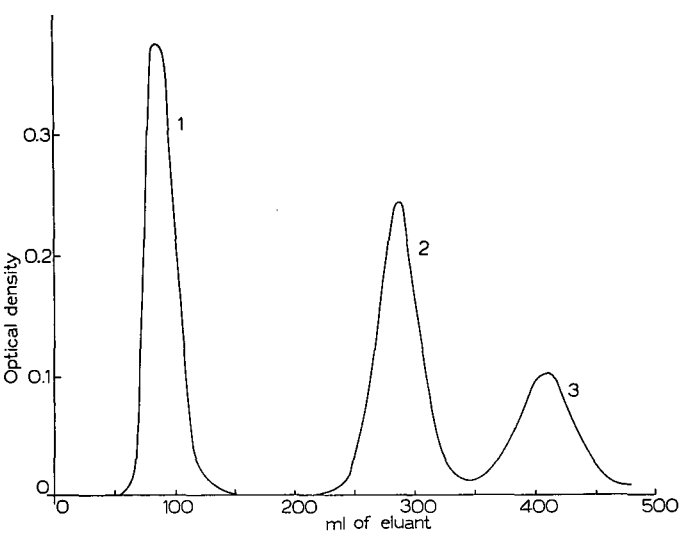

Fig. 2. Separation at a pH of 11.4 at 18°. (1) Hypophosphite; (2) phosphite; (3) phosphate.

Further work in these laboratories has shown that at pH 6.8 the condensed lower oxyanions of phosphorus[11], and at pH 11.4 the thioxyanions both of low and high phosphorus oxidation number[12] have greater retention volumes than the three anions separated by this scheme. As a result, these anions do not interfere with the method of analysis outlined in this paper.

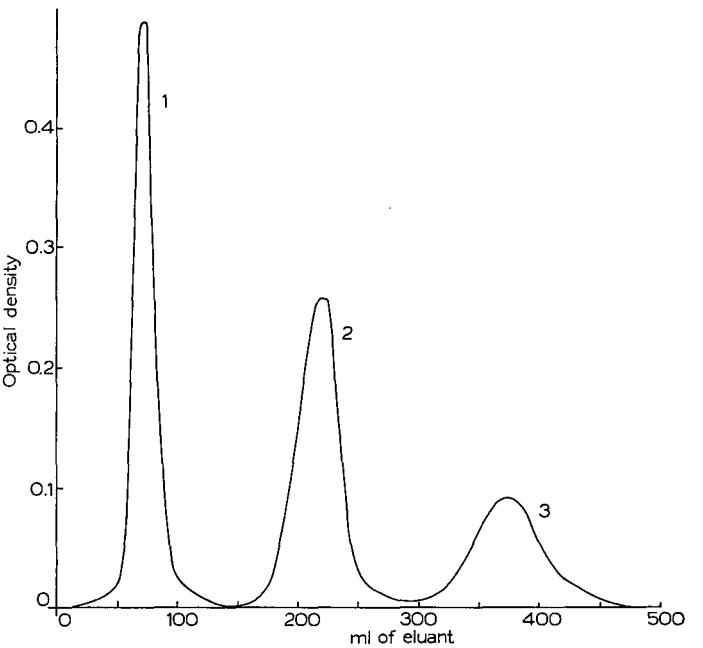

Fig. 3. Separation at a pH of 11.4 at 2°. (1) Hypophosphite; (2) phosphite; (3) phosphate.

SUMMARY

Separations of hypophosphite, phosphite and phosphate have been developed using gradient elution anion-exchange chromatography at pH 6.8 and 11.4. The separation at pH 11.4 is improved by reducing the column temperature to ~2°.

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Short Communications

Séparation des isomères de Te 127 et 129 par électrophorèse sur papier

La technique d'électrophorèse sur papier a été utilisée avec succès pour mettre en évidence les différentes formes chimiques des atomes "chauds" radioactifs formés au cours d'une réaction nucléaire induite par les neutrons[1-3]. Dans le cadre de travaux sur l'étude des conséquences chimiques de transformations radioactives spontanées, nous avons été amenés à employer cette même technique pour séparer les isomères nucléaires du tellure.

La séparation d'isomères nucléaires en filiation n'est possible que dans la mesure où le rayonnement γ de la transition isomérique subit une conversion interne. L'éjection d'un électron d'une couche profonde peut être suivie d'une série de transitions avec effet Auger, conduisant à une ionisation multiple de l'isomère à l'état fondamental. Ce processus est accompagné d'effets chimiques pouvant entraîner une modification profonde de la molécule initiale, et en particulier (si la nature chimique de l'atome et le milieu le permettent), une forme chimique de l'isomère fondamental différente de celle de l'isomère excité. Ces conditions sont remplies pour les isomères du ^{127}Te et du ^{129}Te pour lesquels les rayonnements de la transition isomérique sont fortement convertis (à 100% pour l'isomère de masse 127)[4]. En milieu non organique, les modifications chimiques se traduiront par des réactions d'oxydation ou de réduction, et la séparation des isomères se ramène à la séparation des états d'oxydation du tellure.

C'est ainsi que divers auteurs[5,6] mettant à profit des réactions d'oxydo-réduction, ont coprécipité sélectivement les ions tellurite et tellurate en présence d'entraîneurs. Cette addition d'entraîneurs n'est pas toujours souhaitable et la multiplicité des réactions chimiques risque de modifier la distribution initiale des états

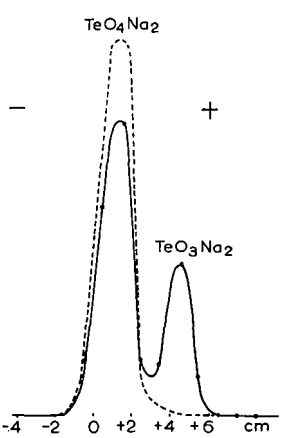

Fig. 1. Électrophorégrammes immédiatement après l'électrophorèse; (——) et 3 jours plus tard (– – –).

d'oxydation du tellure résultant de la transition isomérique. La technique d'électrophorèse sur papier ne présente pas ces inconvénients.

La solution de tellurite de sodium ($^{127}Te + {}^{129}Te$), pratiquement exempte d'entraîneurs, est oxydée en tellurate par l'eau oxygénée à chaud, et le pH ajusté à la valeur désirée. Lorsque le système est en équilibre radioactif (après trois jours environ), la séparation du tellurite formé au cours de la transition isomérique est effectuée selon la technique de GRASSINI ET LEDERER[7] dans les conditions suivantes: électrolyte NaOH 0.5 N; papier Arches 304, 40 × 2.7 cm; durée 45 min; tension 300 V.

L'électrophorèse achevée, la bande de papier est découpée cm par cm et en raison de la faible activité, les échantillons sont mesurés à l'aide d'un ensemble de

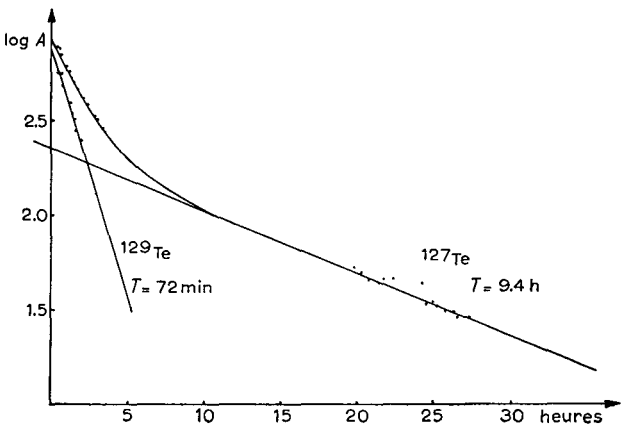

Fig. 2. Évolution de l'activité du pic "tellurite".

comptage à faible bruit de fond (1 C/min) et les pics d'activité du tellurite et du tellurate sont ainsi localisés (Fig. 1). Il est intéressant de connaître la proportion des isomères fondamentaux de chaque isotope, qui ont été réduits lors de la transition isomérique. A cet effet, on suit l'évolution de l'activité du pic du tellurite (Fig. 2) dont la courbe révèle les deux périodes des isomères fondamentaux:

$$^{127m}Te \xrightarrow{105\ j} {}^{127}Te \xrightarrow{9.4\ h}$$

$$^{129m}Te \xrightarrow{33.5\ j} {}^{129}Te \xrightarrow{72\ min}$$

A titre d'exemple une solution de tellurate de sodium à pH 3 est réduite en tellurite dans la proportion de 28% pour ^{127}Te et de 35% pour ^{129}Te.

L'importance du phénomène de réduction varie un peu avec le pH de la solution de tellurate de sodium. Ce fait a déjà été signalé par WILLIAMS[5], mais les valeurs trouvées par cet auteur sont en général supérieures à celles obtenues par électrophorèse.

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The chromatography of polylysine

In the chromatography of partial hydrolysates of polylysine on columns of carboxymethyl-cellulose[1], conditions were found that would resolve the first 20 members of a homologous series of lysine polypeptides. The resolution of a partial acid hydrolysate of polylysine is shown in the upper chromatogram of Fig. 1. The first 5 peaks in order of their emergence from the column were identified as lysine, di-, tri-, tetra-, and penta-lysine respectively by comparative chromatography on paper[2] and carboxymethyl-cellulose columns using lysine, di-lysine[3], and the products of trypsin hydrolysis of poly-L-lysine[2] as reference compounds. In this way, the peak number was

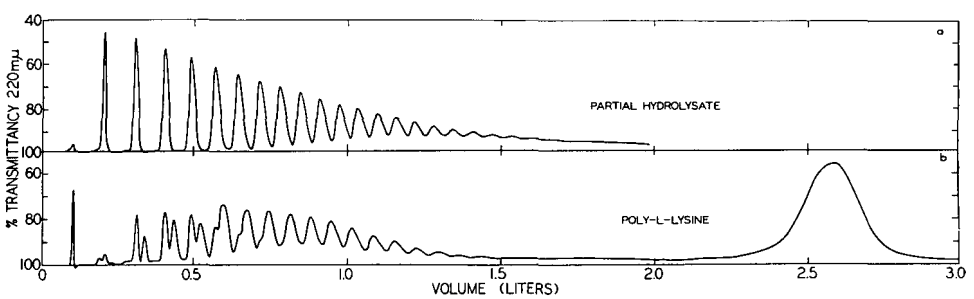

Fig. 1. Chromatograms of polylysine developed on a 40 × 0.9 cm column of 4 g carboxymethyl-cellulose (0.7 mequiv./g capacity, Na^+ form) with an exponential gradient of NaCl. The 1 l constant volume mixing flask, initially filled with water, was replenished with 0.82 *M* NaCl (after 2 l of flow, with 1.73 *M* NaCl) at 2.0 ml/min. Upper chromatogram is of a partial acid hydrolysate of 30 mg high molecular weight poly-L-lysine. Lower chromatogram is of 40 mg poly-L-lysine·HCl synthesized by initiating polymerization of ε-carbobenzoxy-L-lysine N-carboxy anhydride in dioxane (4% w/v) with NH_3 at a molar ratio of 12 and after 48 h reacting the polymerization mixture with anhydrous HCl at 90°.

shown to be identical with the number of lysine residues per molecule in each fraction.

A plot of the logarithm of the peak number or the number of lysine residues per molecule against the molarity of sodium chloride in the column at elution with a linear gradient disclosed a simple correlation between the molecular weight and the eluent concentration. This relationship is shown in the straight line labeled polylysine in Fig. 2. That this relationship may be a general one for the chromatography of other polyelectrolytes on substituted cellulose ion-exchange columns was suggested by plotting the published data on the chromatography of polythymidylic acid[4] and

polyglutamic acid[5] on DEAE-cellulose (Fig. 2). This linear relationship between the logarithm of the number of monomer residues per molecule larger than the trimer and the molarity of salt required for elution suggests a new method for the simultaneous separation and determination of both the molecular weight and the size distribution of the lower members of such homologous series of polyelectrolytes.

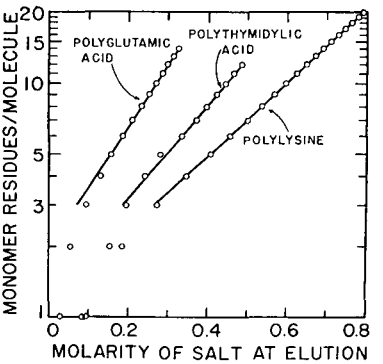

Fig. 2. Relationships of molecular weight to salt concentration in linear gradient elution analysis. Polylysine was developed on the column of Fig. 1 with a linear gradient (1 mM/min) of NaCl. Polythymidylic acid data are from KHORANA AND VIZSOLYI[4]. Polyglutamic acid data are from MILLER[5].

This linear correlation between the logarithm of the degree of polymerization and the molarity required for elution underwent nearly parallel translation towards higher molarities as the length of the carboxymethyl-cellulose column was changed from 16 to 150 cm or as the gradient rate was increased from 1 to 3 mM/min. Hence, in such chromatographic analyses, reproducible results are obtained only if conditions are carefully controlled.

The literature indicates that in the ion-exchange chromatography of polyelectrolyte molecules, the R_F would tend to be either 0 or 1, and would change abruptly from one extreme to the other when the composition of the eluent is changed within a very narrow range[6,7]. PORTER[8] has pointed out that the principal disadvantage of ion-exchange cellulose chromatography lies in the difficulty of finding conditions in which there is a finite partition coefficient of polyelectrolyte molecules between the ion-exchange cellulose and the eluent. These conditions were realized with polylysine by the use of an exponential gradient produced by a constant volume mixer. Selected members of the polylysine series from the dimer to the 15-mer moved through a 40 cm column of carboxymethyl-cellulose during gradient elution analysis (Fig. 1) with R_F values between 0 and 0.31. Thus, when the 15-mer was developed with 0.55 M sodium chloride (the composition of eluate in which it emerged during exponential gradient development) it was eluted at 4.8 liquid hold-up volumes or with an R_F value of 0.21.

These techniques have been used for the analysis of polylysine preparations, one of which is shown in the lower chromatogram of Fig. 1. Ninety-seven per cent of the 220 mμ absorbancy applied to the column was recovered in two widely separated regions. We conclude that this ammonia initiated polylysine preparation[9] had a bimodal molecular weight distribution. The members of the lower series emerged

in double peaks located near the position of each of the smaller peptides in the partial acid hydrolysate. At least two homologous polylysine series were present in the lower molecular weight fractions which probably differ in their amino or carboxyl terminal end. The lower molecular weight series contained peptides with from one to about twenty lysine residues per molecule. The degree of polymerization of the higher series was estimated at 250 from the intrinsic viscosity in dimethyl formamide of the recarbobenzoxylated fraction[10,11]. Such analyses have shown a bimodal molecular weight distribution in the products of polymerization of ε-carbobenzoxy-L-lysine N-carboxy anhydride initiated in dioxane solution with ammonia at molar ratios of monomer to initiator of from 6 to 50[12]. We believe that the new principles illustrated in this study will be of value in the isolation and analysis of polyamino acids, polynucleotides, proteins, and other polyelectrolytes.

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Detection of ketosteroids on chromatograms

The detection of ketosteroids may be carried out on untreated strips by means of contact photography in U.V.-light at about 253 mμ. This method, however, is specific for Δ^4-3-ketosteroids and therefore saturated ketosteroids are not detectable in this way. The strips may also be treated with various, more or less specific reagents; in this case fluorescence in U.V. light or colouring of the spots occurs. However, treated chromatograms cannot be used for further quantitative evaluation, owing to the

stained background. Using fluorescence methods the strips usually undergo complete destruction. Moreover, the nonsteroid substances, such as neutral lipids and phospholipids, which are still present in the chromatographed extracts, give similar reactions with some reagents[1] and can therefore cause misinterpretation of the results.

For the detection of ketosteroids 2,4-dinitrophenylhydrazine (DNPH) was chosen as one of the most general reagents for ketones; an aqueous solution was used, since an alcoholic one may elute steroids from the chromatogram before the reaction with DNPH takes place.

In order to eliminate most, if not all, of the sources of error due to staining, the possibility was investigated of removing the unspecific chromogens and an excess of DNPH, without destroying the steroid hydrazones formed. Since it is well known, that various organic hydrazines are easily decomposed by mild oxidants, such as the Benedict reagent[2], several inorganic salts with oxidative properties were tested.

The best results were obtained with a solution of $KMnO_4$ made slightly alkaline with Na_2CO_3. It was proved by densitometry at 380 mμ, that almost all the colour due to phospholipids and any excess of the reagent disappeared after alkaline permanganate treatment during 10–30 min at room temperature in the pH range 7.5–10, while the steroid hydrazone spots remained unchanged after a similar treatment during 6 hours. A solution of ascorbic acid was used to remove the MnO_2 formed.

Pure steroids and corpus luteum extracts alone, or with steroids added, were chromatographed in the Bush system A (heptane–80% methanol) at 34° on Whatman No. 1 paper, using the "Keilstreifen" method of MATTHIAS[3] and the ascending technique. The results are given in Table I.

TABLE I

	PGS	*TSN*	*ADN*	*PGN*	*DHA*	*17-OH-PGS*	*PDN*
Final colour of the spot	orange	red-orange	purple-orange	lemon yellow	light lemon yellow	red-orange	yellow
Amount visually detectable $\mu g/cm^2$ (*ca.*)	0.5	0.5	0.5	1	5	0.5	1

Abbreviations: PGS = progesterone; TSN = testosterone; ADN = Δ^4-androstene-3,17-dione; PGN = Δ^5-pregnen-3β-ol-20-one; DHA = dehydroepiandrosterone; 17-OH-PGS = 17α-hydroxyprogesterone; PDN = pregnane-3,20-dione.

Reagents

(1) 0.1% solution of DNPH in 2 *N* HCl; stable for 2–3 weeks if stored in a cool and dark place.
(2) 1% $KMnO_4$.
(3) 10% $Na_2CO_3 \cdot 10H_2O$.
(4) Neutralizing fluid: dilute 10 ml of (3) to 100 ml with water.
(5) Decolorizing fluid: mix 2 ml of (2) and 1 ml of (3); dilute to 100 ml with water. Stable for one day.
(6) Reducing fluid: 0.2% solution of ascorbic acid; add one drop of concentrated hydrochloric or sulphuric acid per 100 ml of the fluid. Stable for one day.

All operations should be carried out at room temperature.

Procedure

The chromatograms are drawn through the DNPH solution and held in the air for 10 min. The wet strips are then washed for 1 min in the neutralizing fluid, 10 min in the decolorizing fluid, 5 to 10 min in the reducing fluid and finally in tap water.

If the decolorization is not complete, decolorizing with subsequent washings repeated once or twice will be helpful. After the last washing in water the strips are air-dried and can be stored for further use.

The procedure described increases the sensitivity of the DNPH qualitative test about 5 times, as compared with the data of NEHER[4], when non-impregnated papers are used.

Owing to its simplicity and satisfactory results, the method described seems to be a convenient basis for quantitative analysis of ketosteroid hydrazones. These applications are now under detailed investigation and will be published later.

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Two-dimensional paper chromatography of cholesteryl esters

A new method for the separation of critical pairs

During recent years many authors have studied various chromatographic systems for the separation of cholesteryl esters[1–4]. Although in several cases good results were obtained no useful resolution of critical pairs of these substances by paper chromatography was described.

A two-dimensional technique in which adsorption and reversed-phase partition systems are combined enabled us to solve this problem.

Experimental

Cholesteryl esters. Esters of saturated fatty acids were synthetized by the method of KAUFMANN, MAKUS AND DEICKE[5]. Unsaturated esters were prepared enzymically according to MAHADEVAN AND GANGULY[6].

Impregnation of paper with silica gel. Whatman No. 3 paper (18 × 46 cm) was immersed in a mixture of about 2.5 % (v/v) solution of sodium silicate (prepared by dilution of a stock solution 36–38° Bé) and 5 % ammonium chloride in the ratio 100:30 (v/v). The excess of impregnating agent was removed by rubbing both sides of the paper with a glass rod. After drying in the air overnight the papers were washed three times in distilled water (*e.g.* using 1000 ml for six sheets of 18 × 46 cm) for

10 min. The chromatograms were then dried for 30 min at 80° and stored in a box. This procedure is a minor modification of HAMILTON's method[7].

Impregnation of paper with paraffin oil. The impregnation of silica gel papers with paraffin oil was performed according to MICHALEC AND STRAŠEK[1]. The scheme of this technique is given in Fig. 1.

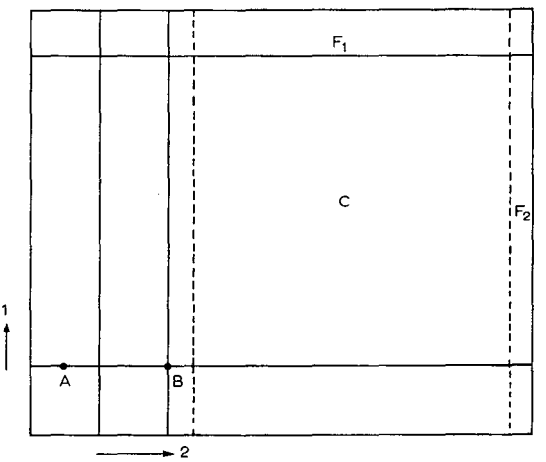

Fig. 1. Scheme of the two-dimensional technique. (A) Start for control chromatogram; (B) start; (F_1) front line for first run; (F_2) front line for second run; (C) part of chromatogram impregnated with paraffin oil (between the two dotted lines).

The mixture of esters (2–5 μg of each substance) was spotted on the start A and B, 2 cm from the lower end of the paper. The chromatogram was then developed with petroleum hydrocarbon (b.p. 60–90°) twice to the front line F 1,1 cm from the upper end. After drying in air, part A was cut off and detection was carried out in order to check the separation in the first run. Then part C was impregnated up to the dotted line (about 0.5–1 cm from B) by immersing it in a 0.5 % solution of paraffin oil in ether. In the second run the mobile phase was acetic acid–chloroform–paraffin oil (80:15:5, v/v/v), development being carried out for 4–5 h to the second front line F 2.

All the chromatographic runs were performed at room temperature (20 ± 2°).

Detection. After drying at 80–90° the chromatogram was sprayed with phosphomolybdic acid (10 % in ethanol) and heated for about 5 min at the same temperature. Blue-green spots developed on a quickly darkening yellow-green background.

Besides this very sensitive detection, the reaction with 50 % antimony trichloride in acetic acid was also used. The mobilities of the cholesteryl esters are summarized in Table I.

Results and discussion

By applying this method we were able to separate some critical pairs of cholesteryl esters. In the first dimension on the paper impregnated with silica gel (adsorption chromatography) it is possible to separate the esters according to the degree of unsaturation. The mobilities (R_F values) decrease in the order: saturated → monoethenoic → di-ethenoic → tri-ethenoic and tetra-ethenoic → more unsaturated esters.

In the second dimension on the paper impregnated with paraffin oil (reversed-phase partition chromatography) the esters were separated into the members of the homo-

TABLE I

R_F VALUES OF CHOLESTERYL ESTERS

Compound	R_F	
	silica gel	*paraffin oil*
Cholesterol	0.10	0.96
Cholesteryl formate	0.76	0.83
Cholesteryl acetate	0.52	0.84
Cholesteryl butyrate	0.63	0.77
Cholesteryl caproate	0.70	0.67
Cholesteryl caprylate	0.77	0.59
Cholesteryl caprinate	0.80	0.52
Cholesteryl laurate	0.82	0.45
Cholesteryl myristate	0.83	0.38
Cholesteryl palmitate	0.85	0.31
Cholesteryl stearate	0.87	0.24
Cholesteryl arachidate	0.87	0.19
Cholesteryl behenate	0.87	0.15
Cholesteryl oleate	0.72	0.31
Cholesteryl palmitoleate	0.71	0.37
Cholesteryl linoleate	0.58	0.38
Cholesteryl linolenate	0.40	0.44
Cholesteryl arachidonate	0.39	0.43
Cholesteryl eicosenate	0.72	0.23
Cholesteryl erucate	0.72	0.19
Cholesteryl $C_{20:5}$ / Cholesteryl $C_{22:6}$	0.25	0.50

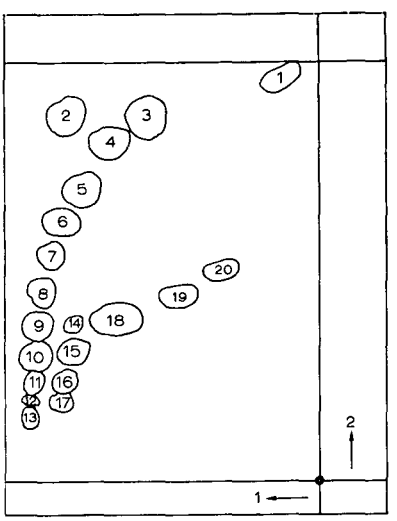

Fig. 2. Mixture of synthetic cholesteryl esters. (1) Cholesterol; (2) cholesteryl formate; (3) cholesteryl acetate; (4) cholesteryl butyrate; (5) cholesteryl caproate; (6) cholesteryl caprylate; (7) cholesteryl caprinate; (8) cholesteryl laurate; (9) cholesteryl myristate; (10) cholesteryl palmitate; (11) cholesteryl stearate; (12) cholesteryl arachidate; (13) cholesteryl behenate; (14) cholesteryl palmitoleate; (15) cholesteryl oleate; (16) cholesteryl eicosenate; (17) cholesteryl erucate; (18) cholesteryl linoleate; (19) cholesteryl linolenate and arachidonate; (20) cholesteryl $C_{20:5}$ and $C_{22:6}$.

logous series. It is well known that the problem of separating critical pairs of, for instance fatty acids, sterol esters, etc., by reversed-phase paper chromatography is difficult to solve. Chromatography at low temperatures imposes certain restrictions and with the other techniques, which include hydrogenation of unsaturated substances on the paper, oxidation with peracids and the addition of mercuri-methoxy

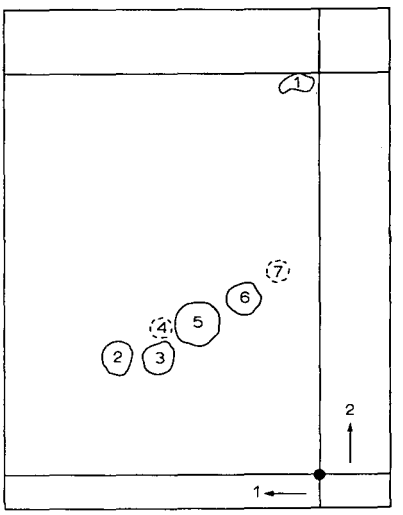

Fig. 3. Cholesteryl esters of human blood serum. (1) Cholesterol; (2) cholesteryl palmitate; (3) cholesteryl oleate; (4) cholesteryl palmitoleate; (5) cholesteryl linoleate; (6) cholesteryl arachidonate; (7) cholesteryl esters of more unsaturated fatty acids.

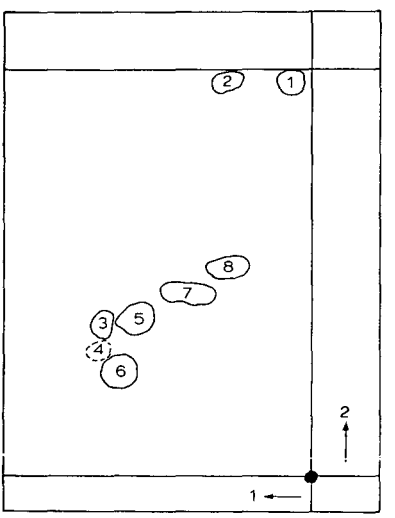

Fig. 4. Cholesteryl esters of fatty acids from rapeseed oil prepared enzymically. (1) Cholesterol; (2) unknown; (3) cholesteryl palmitate; (4) cholesteryl stearate; (5) cholesteryl oleate; (6) cholesteryl erucate; (7) cholesteryl linoleate; (8) cholesteryl linolenate.

groups to the double bounds, it is not possible to obtain on one chromatogram the whole spectrum of all the substances present in the material being analysed.

The two-dimensional system is very simple and, combined with a sensitive detection method, it constitutes a relatively quick and convenient method suitable for the separation of many critical pairs of cholesteryl esters.

This technique was used for the qualitative characterization of cholesteryl esters in various biological materials and for the identification of the spectrum of higher fatty acids in oils and fats after enzymic esterification with cholesterol.

These results will be discussed in another paper.

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Notes

The decomposition of nine amino acids during chromatography on paper

It has recently been shown[1] that some decomposition of glycine takes place during chromatography on Whatman No. 4 paper using phenol–water and *n*-butanol–propionic acid–water as developing solvents. Losses of up to 38% were obtained in this system; these losses could be minimized by distillation of the phenol before use, and by pre-treatment of the paper with oxalic acid. Filter paper pre-treated in this way had a pH of 4.9, compared with 6.7 for untreated paper. This study has now been extended to include eight other amino acids.

Before use, the following amino acids were purified chromatographically on oxalic acid-treated Whatman No. 4 paper, using distilled phenol[2]–water followed by *n*-butanol–propionic acid–water[3]: DL-[2-^{14}C]alanine (8.2 μC/mg); DL-[4-^{14}C]aspartic acid (10.7 μC/mg); DL-[1-^{14}C]glutamic acid (9.0 μC/mg); [2-^{14}C]glycine (8.9 μC/mg); L-[^{14}C]leucine (8.0 μC/mg); DL-[3-^{14}C]phenylalanine (21.1 μC/mg); DL-[3-^{14}C]serine (1.7 μC/mg); DL-[2-^{14}C]tyrosine (2.3 μC/mg); and D-[4,4'-^{14}C]valine (3.1 μC/mg).

Each amino acid was eluted with water, evaporated to dryness under reduced

TABLE I

BREAKDOWN OF NINE AMINO ACIDS DURING CHROMATOGRAPHY

Pretreatment of paper	*Dimensions*	*1st solvent**	*2nd solvent**	*Extent of breakdown of amino acid (%)*									
				Alanine	*Aspartic acid*	*Glutamic acid*	*Glycine*	*Leucine*	*Phenyl-alanine*	*Serine*	*Tyrosine*	*Valine*	*Average for all amino acids*
None	1	Ph.	—	2.0	3.4	4.8	10.4	5.7	3.0	2.4	0.6	1.6	3.8
None	1	Redist. Ph.	—	4.8	4.4	5.9	10.6	6.4	3.7	2.0	1.3	1.8	4.6
None	1	But.–Prop.	—	3.5	3.2	3.7	9.2	10.7	7.7	1.0	2.5	3.3	5.0
None	2	Ph.	But.–Prop.	3.0	1.9	3.8	11.1	14.2	9.0	2.7	4.0	6.8	6.3
None	2	Redist. Ph.	But.–Prop.	3.4	2.3	4.9	12.6	12.9	11.2	2.2	4.6	6.3	6.7
None	2	But.–Prop.	Ph.	7.1	3.8	3.6	6.4	10.0	7.1	1.9	0.0	0.4	4.5
None	2	But.–Prop.	Redist. Ph.	3.9	3.7	4.3	8.9	9.8	5.5	0.8	0.5	1.6	4.3
Oxalic acid	1	Ph.	—	2.4	3.7	5.7	8.7	4.0	1.5	2.1	0.5	1.0	3.3
Oxalic acid	1	Redist. Ph.	—	1.9	3.2	5.2	8.0	3.2	2.8	1.3	0.4	0.7	3.0
Oxalic acid	1	But.–Prop.	—	2.8	2.3	3.7	6.7	8.4	7.6	1.2	3.3	2.2	4.3
Oxalic acid	2	Ph.	But.–Prop.	2.5	3.5	4.8	6.7	11.2	10.5	1.3	1.7	3.0	5.0
Oxalic acid	2	Redist. Ph.	But.–Prop.	1.5	2.2	2.4	6.6	8.4	10.4	1.4	3.3	2.8	4.3
Oxalic acid	2	But.–Prop.	Ph.	0.6	0.7	5.2	8.1	5.4	2.8	0.9	0.6	0.2	2.7
Oxalic acid	2	But.–Prop.	Redist. Ph.	0.9	7.3	1.9	9.0	2.8	2.3	1.1	0.0	0.8	2.9

* Abbreviations: Ph. = phenol–water; Redist. Ph. = redistilled phenol–water; But.–Prop. = *n*-butanol–propionic acid–water.

pressure at 40°, and redissolved in water (0.1 *N* HCl in the case of tyrosine). Aliquots of each amino acid solution containing 0.1 μC of ^{14}C were spotted onto oxalic-acid treated, or untreated, Whatman No. 4 paper, and developed either in one or in two dimensions as shown in Table I. The positions of radioactive substances were determined by radioautography (Kodak Single-Coated Blue-Sensitive Medical X-Ray Film, exposed for 36 days). The radioactive areas were excised from the chromatograms and the radioactivity in each spot was counted automatically in the apparatus described by MOSES AND LONBERG-HOLM[4].

The values reported in Table I are percentages of the total ^{14}C on each chromatogram present in substances other than the corresponding amino acid being investigated; the chemical nature of none of these other substances has been determined. Each of the amino acids investigated showed some breakdown during chromatography; in some cases as many as twenty decomposition products were detected. The extent of decomposition varied from zero with tyrosine in two instances, to over 14% with leucine in one system. Generally, glycine, leucine and phenylalanine showed the greatest degree of breakdown, and tyrosine the least; the other five substances were intermediate between these extremes.

Unlike the earlier findings with glycine[1], distillation of the phenol before use had no detectable effect on amino acid breakdown. Even glycine, the breakdown of which was earlier found to be much less extensive after distillation of the phenol, was unaffected. Presumably this is related to the quality of the undistilled phenol used on the two occasions: HUGGINS AND MOSES[1] employed British Drug Houses (Poole, England) Phenol Detached Crystals B.P.C., while in the present study the source was Mallinckrodt (St. Louis, Mo., U.S.A.) Phenol Liquefied Analytical Reagent.

Pretreatment of the chromatography paper with oxalic acid reduced the amount of decomposition by an average of 30% compared with untreated paper. The greater acidity of the oxalic acid-treated paper was probably responsible for much of this effect. However, oxalic acid specifically might also be of importance, by virtue of its binding of calcium and magnesium ions in the paper; there was some reduction in the extent of breakdown with oxalic acid-treated paper even when butanol–propionic acid–water (pH 2.5) alone was used as a solvent. The beneficial effect of oxalic acid treatment agrees with the earlier studies on the chromatographic breakdown of glycine[1].

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Volman collector for gas chromatography

The problem of aerosol formation, or "fogging", is a normal complication of gas chromatographic purification of liquids. It is particularly troublesome with high molecular weight compounds, and results in low trapping efficiency. If traps are packed with defatted cotton, glass wool, glass beads, silica, or other materials, their efficiency for small samples can be increased, but it becomes necessary to rinse the packing with a suitable solvent to recover the sample. An additional disadvantage is that packed traps are prone to clog with large samples. An electrical precipitator has

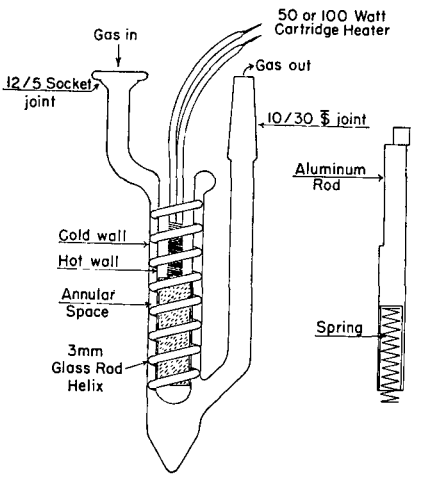

Fig. 1. Trap with cartridge or aluminum rod heaters.

been suggested[1,2] but the dangers of such a trap are obvious. The Volman trap, which can be made by any competent glass blower, is very effective in stopping the fogging phenomenon and, therefore, is practical for preparative work in gas chromatography. A large temperature gradient in the trap prevents fog formation[3].

Our variation of the Volman trap (Fig. 1) permits its use for collecting fractions from gas chromatographic runs. The gases emerging from the apparatus are forced in a spiral path down the annular space between the center well wall, heated by the cartridge, and the outer wall, cooled by ice, dry ice, or liquid nitrogen. The glass helix was found necessary in order to force the gas to take a longer path than without the helix; *i.e.*, some aerosol was observed in the effluent gas stream from the traps without the helix. If the cartridge heater is used, a thermocouple can be inserted with the heater in order to adjust the thermal gradient. Temperatures of 150–200° have been found to be sufficient for most cases.

For chromatographs that suspend traps from a rotating heated plate (*e.g.*, the Megachrom*), a spring-loaded aluminum rod held in contact with the plate can be used to conduct the heat to the center well (Fig. 1, inset). This eliminates the need for electrical wiring and permits assembly to rotate freely with the fraction cutter.

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

If the sample size is in the milligram region, then the material must be vacuum transferred. If the sample size is in the gram region, a Teflon tube can be inserted down the gas outlet tube of the collector, and the sample removed with a syringe or bulb. The collector can then be rinsed clean with appropriate solvents, dried, and thus be ready for another collection.

Trapping yield data have been obtained with ethyl caproate and limonene. In the 1-gram range, yields of 90–95 % were obtained with ice as the coolant. Yields slightly higher than 95 % were obtained with dry ice as the coolant.

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The isolation of DDT, parathion and lindane from biological fatty materials by liquid–liquid partition chromatography

The isolation of organic insecticides from biological fatty matter constitutes a fundamental step for their ultimate quantitative estimation. While studying the applicability of some of the methods described in the literature, the work of JONES AND RIDDICK[1] and that of BURCKFIELD AND STORRS[2] stimulated us to develop liquid–liquid partition chromatographic columns for the isolation of the above-mentioned insecticides.

JONES AND RIDDICK isolated several insecticides by partitioning them between acetonitrile and *n*-hexane. BURCKFIELD AND STORRS replaced acetonitrile by N,N-dimethyl-formamide. By using these solvent pairs in chromatographic columns we succeeded in recovering milligram quantities of DDT, parathion and lindane for more than 90 % out of their solutions in concentrated insect extractives or in peanut oil. One of the advantages of these columns is the possibility of using them more than once.

Application of this method to microgram quantities, often occurring in biological material, could not at the moment be checked owing to the lack of a detection method of adequate sensitivity.

Methods for isolating the insecticides

(1) *Isolation of DDT.* Celite 545 (JOHNS MANVILLE), from which the fines have been slibbed off, is washed with concentrated hydrochloric acid and then with distilled water and dried. Of this material 5 g are mixed thoroughly with 2 g of dimethyl-

formamide by shaking in a stoppered flask until a homogeneous slightly damp powder is obtained. *n*-Hexane saturated with dimethylformamide is added until a smooth slurry is formed. Then the slurry is brought in small portions into a chromatographic tube (length 20 cm, inner diameter 1 cm) which is plugged with glasswool at the constricted end and which contains about 5 ml *n*-hexane saturated with dimethylformamide.

The contents of the tube are packed down slightly with a glass tamping rod after each addition. A loose packing of the column is of crucial importance for obtaining a good separation. After preparation of the column the excess of liquid is drained off until a small volume remains above the kieselguhr.

The concentrated insect extractives are dissolved in 5 ml of *n*-hexane saturated with dimethylformamide. This solution is poured quantitatively into the column, taking care not to disturb the surface of the kieselguhr, whereupon elution is started.

The elution rate should not be more than 5 ml in about 2 min. The fatty materials are eluted with the first 20 ml of hexane. Then about 10 ml almost pure solvent are collected, after which the insecticide appears with the following 20 ml of eluents.

(2) *Isolation of lindane.* The column used for the isolation of lindane is prepared, in the same way as described for DDT, from 3 g of kieselguhr and 1.2 g of dimethylformamide. A lower boiling solvent, *viz.*, *n*-pentane may be used instead of *n*-hexane in order to minimize losses during evaporation of the solvent. The lindane appears in the eluate when 30 ml of the solvent have percolated through the column.

(3) *Isolation of parathion.* The column employed for the isolation of parathion differs from those used for DDT and lindane. It consists of 3 g of cellulose powder (Whatman No. 1)[3], and 1.2 g of acetonitrile as the stationary phase; *n*-hexane saturated with acetonitrile is used as the mobile phase. Packing and elution are carried out as described above.

The retention volume of parathion is also 30 ml.

Recovery tests

These were carried out by evaporating the solvent of the eluate fractions, after which the residues obtained were weighed and then analyzed quantitatively by means of gas–liquid chromatography. A J. H. BECKER Gaschromatograph (Delft, the Netherlands) was used with katharometer detection. The column employed was a coiled copper tube (length 50 cm, inner diameter 0.4 cm) filled with 1.6 g of Embacel (May and Baker) impregnated with Apiezon L (10:1 weight ratio).

Hydrogen was used as the carrier gas. The working conditions were as follows:

	Parathion	*Lindane*	*DDT*
Column temperature	220°	220°	230°
Gas flow rate	60 ml/min	60 ml/min	120 ml/min
Retention volume	174 ml	123 ml	744 ml

Under these conditions the smallest amount of the insecticides that could be detected was about 50 γ. Aiming at a greater sensitivity we applied the combustion method of BEERTHUIS *et al.*[4] with helium as the carrier gas.

In this method the components leaving the G.L.C. column are burned over copper oxide at 1000°, after which the carbon dioxide formed is detected by the katharometer. In this way the smallest detectable amount was lowered to 2 γ.

This sensitivity, however, does not yet allow the determination of the minute amounts of the insecticides often present in biological material. This analysis requires application of ionisation detectors as developed by LOVELOCK AND MCWILLIAM[5,6]. Then quantities as small as 0.01 γ can be estimated.

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Bestimmung des Methylbromids und Äthylbromids nebeneinander

Äthylbromid wird weitgehend in der organischen Industrie verwendet. Es wurde von uns bei einer Gelegenheit verlangt es zu prüfen, ob und wieviel Methylbromid darin enthalten ist.

Obwohl in der Literatur viele und die verschiedensten Methoden zur Äthylbromid- bzw. Methylbromidbestimmung vorliegen[1–12], fanden wir keine Beschreibung einer Methode, welche die Bestimmung dieser Verbindungen nebeneinander behandelt. Inbetracht der physikalischen Eigenschaften dieser Verbindungen (Tabelle I) nahmen

TABELLE I

Formel	*Molgewicht*	*Schmelzpunkt °C*	*Siedepunkt °C*	*Dampfdruck Torr (20°C)*
CH_3Br	94.95	— 93.66	+ 3.56	1250.0
CH_3CH_2Br	108.98	—119	+38.0	386.0

wir es vor, ihre Trennung bzw. Bestimmung mittels Gaschromatographie zu versuchen.

In der Literatur konnten in erster Linie für das Äthylbromid gaschromatographische Angaben gefunden werden[13–16]. Die Angaben beziehen sich aber auf die verschiedensten Versuchsbedingungen. Wir begannen deshalb unserer Versuche mit der Bestimmung der Retentionszeiten und der relativen Retentionsfaktoren der reinen Substanzen, bei einheitlichen Versuchsumständen. Da das chromatographische Verhalten von halogenierten Kohlenwasserstoffverbindungen häufig auf Tetrachlorkohlenstoff bezogen wird[16], wurden auch Bestimmungen mit Tetrachlorkohlenstoff weiterhin mit Azeton unternommen, das bei der industriellen Verwendung oft als Lösungsmittel dient. Bei den Versuchen wurde ein mit Wärmeleitfähigkeitszelle versehener, ungarischer Gaschromatograph, Typ Labor, benützt. Die für geeignetst gefundenen Versuchsbedingungen sind in der Tabelle II beschrieben.

TABELLE II

Temperier-temperatur	52°
Trägergas	gereinigter Wasserstoff
Trägergasdruck	1.1–0.95 kg/cm^2
Trägergasgeschwindigkeit	75 ml/min
Kolonnenlänge	3 m
Innerer Durchmesser der Kolonne	5 mm
Füllung der Kolonne	20% β,β'-Oxydipropionitril auf Schamotteziegelpulver von 30/60 mesh Korngrösse
Einwage	20 μl
Detektor	Wärmeleitfähigkeitszelle
Detektortemperatur	52°
Brückenstromintensität	110 mA
Empfindlichkeit des Kompensographen	200 mm/mV
Empfindlichkeitsstufe	20%
Geschwindigkeit des Kompensographpapiers	1 cm/min

Die Kolonnenfüllung wurde nach WEST *et al.*[16] gewählt, doch mit dem Unterschied, dass als Trägersubstanz anstatt Chromosorb hitzebeständiges Ziegelpulver diente. Als Bezugsverbindung gebrauchten wir ebenfalls Tetrachlorkohlenstoff. Die Ergebnisse von unter angegebenen Versuchsbedingungen unternommenen Messungen sind in Tabelle III zusammengestellt.

TABELLE III

Verbindung	*Relativer Retentionsfaktor*
Methylbromid	0.496
Äthylbromid	0.783
Tetrachlorkohlenstoff	1.000
Azeton	2.342

Aus den Daten lässt es sich feststellen, dass Methylbromid neben Äthylbromid auch in Gegenwart von Azeton und Tetrachlorkohlenstoff bestimmt werden kann. Letzterer Bestimmung kommt besonders in den organischen Betriebsanalysen eine Bedeutung zu. Fig. 1. zeigt das Chromatogramm eines quaternären Gemisches unter den angegebenen Versuchsbedingungen.

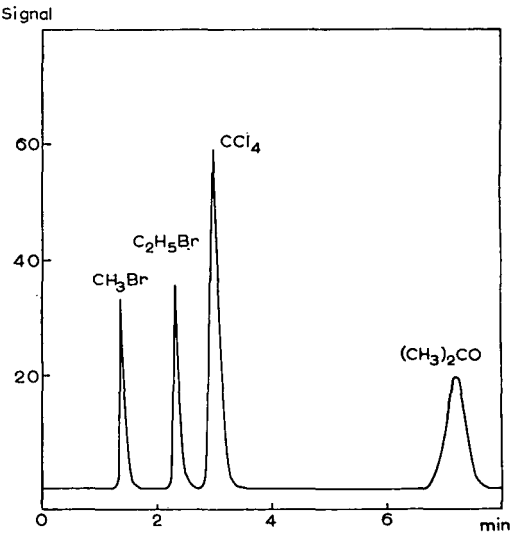

Fig. 1. Chromatogramm eines quaternären Gemisches (10% Methylbromid, 10% Äthylbromid, 40% Tetrachlorkohlenstoff und 40% Azeton).

Es wurden auch Versuche ausgeführt um die geringste Methylbromidmenge zu bestimmen, die sich neben bestimmbarer Äthylbromidmenge mit genügender Genauigkeit noch erkennen lässt. Bei Einwagen von 20 μl konnte, vorausgesetzt, dass während der Elution des Methylbromids volle Empfindlichkeit eingeschaltet wurde, 0.1% Methylbromid in Äthylbromid noch nachgewiesen, 1% mit befriedigender Genauigkeit bestimmt werden. Diese untere Grenze kann durch höhere Apparatsempfindlichkeit und grössere Einwagen noch weiter herabgesetzt werden, wobei jedenfalls die Bestimmung des Äthylbromids dieser Bestrebung Grenzen setzt.

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Separation of the valency states of some elements on paper impregnated with zirconium phosphate

The application of paper chromatography has been extended during recent years by using papers impregnated with both organic and inorganic exchangers. ALBERTI AND GRASSINI[1] have shown that filter papers impregnated with zirconium phosphate can be successfully employed for the chromatographic separation of numerous cations. The advantage of chromatography with such papers lies in the fact that pure aqueous solutions containing different inorganic solutes can replace the organic mixtures normally used in conventional chromatography.

The paper chromatographic separations of elements in different valency states using organic solvent systems have been reported by various workers[2–6]. We have now found that strips impregnated with zirconium phosphate can be successfully used with aqueous solutions as eluents or for the rapid separation and detection of several elements in different valency states when present in admixture with each other.

TABLE I

Valency states of the elements	*Composition of the eluent*	*R_F values*
Fe(II) and Fe(III)	0.5 *N* H_2SO_4	Fe(II) = 0.84 Fe(III) = 0.0
U(IV) and U(VI)	3.0 *N* HCl	U(IV) = 0.0 U(VI) = 0.72
U(IV) and U(VI)	3.0 *N* H_2SO_4	U(IV) = 0.22 U(VI) = 0.68
U(IV) and U(VI)	3.0 *N* HNO_3	U(IV) = 0.03 U(VI) = 0.54
Ce(III) and Ce(IV)	1.0 *N* H_2SO_4	Ce(III) = 0.64 Ce(IV) = 0.06
Ce(III) and Ce(IV)	1.0 *N* HCl	Ce(III) = 0.68 Ce(IV) = 0.0
Cr(III) and Cr(VI)	Saturated solution of Na_2SO_4	Cr(III) = 0.84 Cr(VI) = 0.3
As(III) and As(V)	1.0 *N* HCl	As(III) = 0.66 As(V) = 0.14
As(III) and As(V)	1.0 *N* HNO_3	As(III) = 0.70 As(V) = 0.06
V(IV) and V(V)	Na_2HPO_4–citric acid buffer pH 7	V(IV) = 0.06 V(V) = 0.83 (slight tailing)
Mo(V) and Mo(VI)	4 *N* HCl	Mo(V) = 0.63 Mo(VI) = 0.35
Hg(I) and Hg(II)	0.1 *N* HNO_3	Hg(I) = 0.0 Hg(II) = 0.69

Experimental

Strips (4 cm × 25 cm) of Whatman No. 1 chromatography grade filter papers were drawn at a uniform rate through a 10 % solution of zirconium oxychloride in 4 *N* HCl

and then hung up to dry. The strips were then passed through a solution of 12 % phosphoric acid in 4 *N* HCl and dried at room temperature. Then they were washed with distilled water until free from acid (pH = 4). For further activation, the strips were slowly passed through a 1:3 phosphoric acid solution (containing HCl) at 50°. After drying at room temperature they were again washed with distilled water until free from acid (pH = 4). The strips prepared in this way were used for the separations. Usual methods were employed for the detection of the spots.

In Table I the R_F values of various elements in different valency states are given.

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Structure and chromatographic properties of carbohydrates

II. The liquid–liquid partition mobilities of aldono-γ-lactones*

It is a simple matter to resolve isomeric carbohydrate substances by partition chromatographic means and so the mobilities of compounds depend not only upon fundamental features of the molecules, *e.g.* molecular weight and "hydrophilic nature" (as determined by the number of unsubstituted hydroxyl groupings present), but upon subtler factors related to configuration and conformation. Little attention has been paid to the correlation of structure with chromatographic properties or to the study of the detailed physical phenomena upon which the mobilities depend. Some interrelationships between the disposition of the hydroxyl groupings on pyranose sugars and their chromatographic behaviour have, however, been noted[2], and these have lately been reinterpreted in conformational terms[3]. Thus, free sugars which can assume chair conformations having few axial hydroxyls are less mobile than those which have several. We now note some correlations in the γ-lactone series.

All the measurements (see Table I) were made on Whatman No. 1 papers developed with butan-1-ol–ethanol–water (4:1:5). The members of each group were run together at 25° on one paper and the mobilities quoted in the table are relative to the fastest in each series.

From erythrono- and threono-γ-lactones it is seen that a C_2–C_3 *cis*-diol reduces mobility relative to a *trans*-diol. The pentono-γ-lactones show that the isomer (*lyxo*-) with the configuration which has three hydrophilic groupings on one side of the ring

* For Part I, see ref. [1].

TABLE I

MOBILITIES OF γ-LACTONES
(RELATIVE TO FASTEST ISOMER IN EACH SERIES)

Tetrono-		*Pentono-*		*2-Hydroxymethylpentono-*		*Hexono-*	
Erythrono-	0.78	Ribono-	0.92	Ribono-	0.85	Allono-	0.93
		Arabono-	1.00	Arabono-	0.86	Altrono-	0.92
Threono-	1.00	Xylono-	0.99	Xylono-	1.00	Glucono-	(0.98)
		Lyxono-	0.76	Lyxono-	0.74	Mannono-	0.77
						Gulono-	0.60
						Idono-	1.00
						Galactono-	0.89
						Talono-	(0.89)

travels slowest and that a C_2–C_3 *cis*-diol (*ribo*-) confers lower mobility than a C_3–C_4 *cis*-hydroxy-hydroxymethyl system (*xylo*-).

In the 2-C-hydroxymethyl-pentono-γ-lactones[4] where the situation is complicated by the introduction of another hydrophilic group on the ring, again it is evident that a C_2-C_3 *cis*-diol has the effect of reducing the rate of travel and that the isomer with the *lyxo*-configuration has lowest mobility.

With the hexono-γ-lactones in which simple relationships between structural features and chromatographic properties could be expected to be masked by complexities introduced by the flexible 1,2-dihydroxyethyl grouping at C_4, it is still noticeable that when the three oxygenated substituents are on one side of the five-membered ring (mannono- and gulono-), marked reductions in the rates of travel occur. We were not able to determine the mobilities of the gluco- and talo-isomers directly. The figure given for the former is calculated from the relative mobilities of the *gluco*- and *manno*-isomers in this solvent quoted by ABDEL-AKHER AND SMITH[5]. The talono-γ-lactone figure is quoted as 0.89 as it was unresolvable from the *galacto*-isomer. In an acidic solvent it travels 1.06 times faster than its epimer[6].

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A method for the improved resolution of basic amino acids on the automatic amino acid analyzer

In the course of performing analyses of basic amino acids on the 15 cm column developed with pH 5.28, 0.35 *N* sodium citrate buffer at 50° by the method of SPACKMAN, STEIN AND MOORE[1], difficulties are frequently encountered. These include skewing and spreading of peaks resulting from surface irregularities or poor resins, and marked build-up of pressure due to disintegration of the fine resin particles. In addition, traces of unusual amino acids are sometimes present in protein hydrolyzates which cannot be resolved from the four main constituents.

Because of these problems, a new procedure has been developed which gives greater resolution and greater sensitivity. The sample is loaded on the 50 cm (instead of 15 cm) column and developed with pH 5.28, 0.70 *N* (instead of 0.35 *N*) sodium citrate buffer at 50°. As in the original method, the ninhydrin is not started for 30 min to allow the acidic and most of the neutral amino acids to pass through. Tyrosine and phenylalanine emerge as discrete peaks preceding lysine. Fig. 1 illustrates the appearance of a protein hydrolyzate which has been analyzed by this procedure. Six hours are required for the determination.

The degree of resolution obtained with the 50 cm column at 30°–50° using pH 4.26, 0.38 *N* sodium citrate buffer[1] would not be expected under the present conditions. Fig. 2 illustrates the resolution obtainable with a synthetic mixture of basic amino acids and other slow-moving compounds. It demonstrates that a quick preliminary screening of physiological fluids is possible, and that in some case this 6 h procedure may be used in place of the 22 h 30°–50° pH 4.26 method. It also demonstrates that

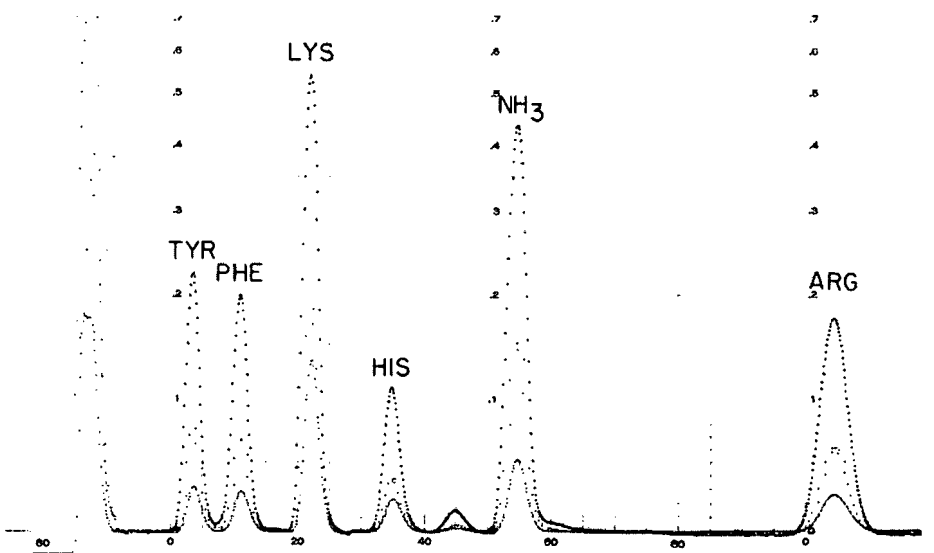

Fig. 1. Chromatographic analysis of hydrolyzate of adult *Phormia* actin, on 50 cm column developed with pH 5.28, 0.70 *N* sodium citrate at 50°. A small peak is present between histidine and ammonia which is probably 1-methylhistidine, resulting from contamination of the protein with a small amount of anserine.

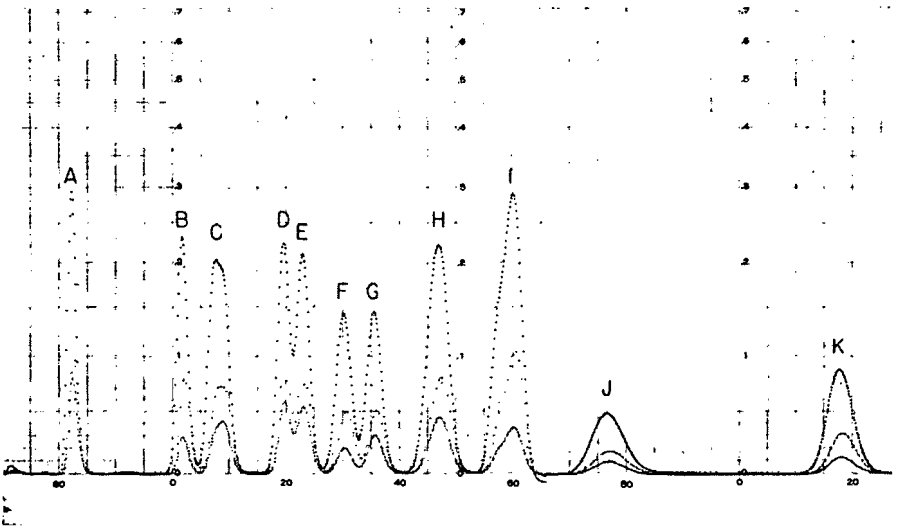

Fig. 2. Chromatographic analysis of mixture of basic amino acids and other slow-moving compounds, on 50 cm column developed with pH 5.28, 0.70 *N* sodium citrate at 50°. (A) α-aminobutyric acid; (B) glucosamine; (C) galactosamine and hydroxylysine; (D) ornithine; (E) lysine; (F) isomer of galactosamine*; (G) histidine; (H) 1- and 3-methyl-histidines; (I) ammonia, ethanolamine and carnosine; (J) tryptophan; (K) arginine.

some of the rarer compounds that may contaminate a protein hydrolyzate can be clearly resolved, such as the 1-methylhistidine present in the hydrolyzate of Fig. 1.

In addition to its usefulness with the automatic amino acid analyzer, the pH 5.28, 0.70 *N* sodium citrate buffer has been used successfully for a number of years[2] on 15 cm columns of 200–400 mesh Dowex 50 X 12 at 50° with 6–8 ml/h flow rate. The resolution obtainable by this manual procedure is very much inferior to that obtained by the automatic procedure described here.

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* Present in D(+)-galactosamine HCl, C-grade, lot No. 104403 supplied by California Corporation for Biochemical Research, and obtained by hydrolysis of chondroitin.

Paper electrophoretic separation of some fission products

Ruthenium-106 has always been found to be one of the most difficult fission products to deal with in fission mixtures[1–3], because of its wide range of stable valency states (0 to 8) and also because of its great tendency to form a great variety of covalent and coordination compounds. Even in solutions of nitric acid which is well known for its reluctance to enter into covalent linkage with other elements, fission product ruthenium has been shown[3–5] to occur as the nitrosylruthenium radical (RuNO)(III), complexed with nitrato, nitro, hydroxo and aquo ligands. Thus this element forms a series of mononuclear complexes from highly charged anions to the aquo complex with the maximum positive charge, to which stable polymers still add themselves and thus complicate the picture in the separation of fission products. It has been shown[1], for example, that even the alkali element ^{137}Cs is contaminated with ^{106}Ru when it is separated from fission products.

For some time we have been making attempts to find a reaction that would yield a single ionic species of ruthenium, irrespective of its valency and complex state, which could then serve as starting point for a paper chromatographic or paper electrophoretic separation of this element. It was found[6] that the reaction of sodium or potassium nitrite with ruthenium salts is most suitable for this purpose. Simple heating on the water-bath for about half an hour with $N/2$ solution of sodium or potassium nitrite in excess (about twice the amount of nitrite necessary to convert all the ruthenium into the hexanitrocomplex) was sufficient to transform all the nitrato- or chlorocomplexes of ruthenium into a single, highly charged (fast moving species in paper electrophoresis) anionic complex.

In the work presented in this paper we have examined the paper electrophoretic behaviour of some principal fission products in $N/2$ $NaNO_2$ solution as electrolyte, in order to investigate the possibility of separating ^{106}Ru from these products by paper electrophoresis in this electrolyte.

Experimental

The usual glass plate technique of paper electrophoresis described by LEDERER AND WARD[7] was employed. All electropherograms were carried out at 250 V, with $N/2$ $NaNO_2$ as electrolyte on 2.7 × 40 cm paper strips (Papier Arches No. 302), which were sandwiched between two glass plates (8 × 31 cm).

The solutions of the fission products were dried on the water-bath and taken up again with $N/2$ $NaNO_2$ solution. This solution was heated on the water-bath for half an hour and then applied, after cooling, to the paper strip with a micropipette along a previously marked line. After the electrophoresis the paper was dried and scanned, using a 2 mm wide slit. The position of the spots on the electropherograms was also located by autoradiography. Identification of the different elements on the electropherogram of the mixture was carried out by comparing the migration of each element on a separate electropherogram obtained under the same conditions.

Results

Fig. 1 shows a typical electropherogram of ^{137}Cs, ^{90}Sr, ^{90}Y, ^{95}Zr, ^{95}Nb, ^{147}Pm, ^{144}Ce, and ^{106}Ru, applied as a mixture on the paper. Since zirconium, promethium, and cerium gave slightly soluble species on heating with $N/2$ $NaNO_2$ solution, these elements were

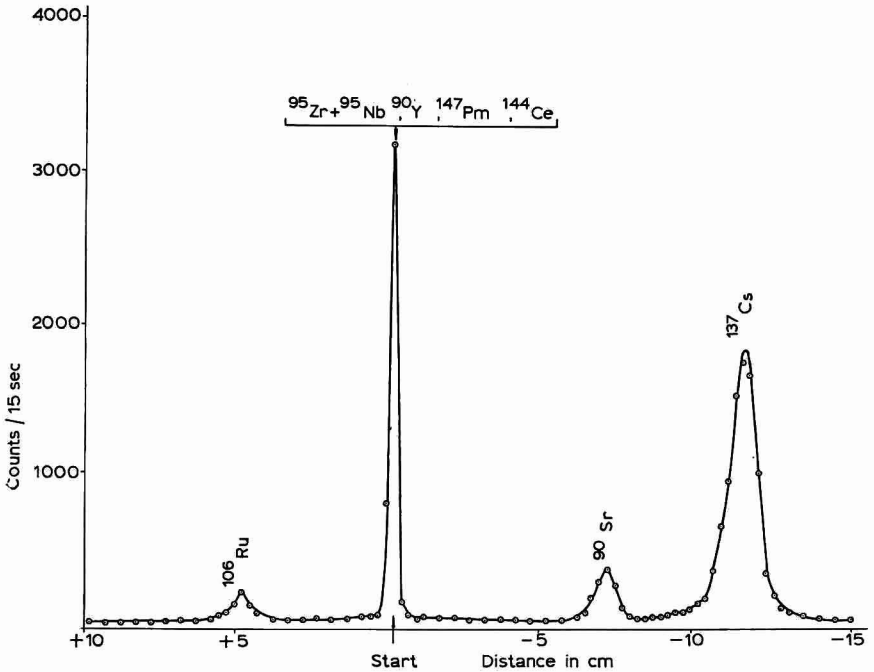

Fig. 1. Electropherogram (250 V; 50 min; electrolyte: $N/2$ $NaNO_2$) of a mixture of ^{106}Ru, ^{95}Zr, ^{95}Nb, ^{90}Sr, ^{90}Y, ^{147}Pm, ^{144}Ce, and ^{137}Cs.

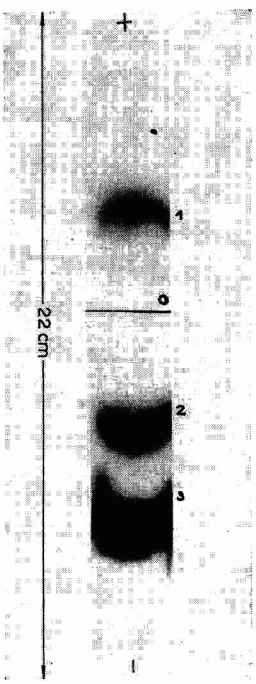

Fig. 2. Autoradiogram of the electropherogram (250 V; 30 min; electrolyte: $N/2$ $NaNO_2$) of a mixture of ^{106}Ru, ^{90}Sr, and ^{137}Cs. (1) ^{106}Ru; (2) ^{90}Sr; (3) ^{137}Cs.

added to the above mixture without previous heating with sodium nitrite solution. Technetium moves also as an anion but is slower than ruthenium. Fig. 2 gives the autoradiogram of the electropherogram of a mixture of ^{137}Cs, ^{90}Sr (+ ^{90}Y), and ^{106}Ru. The autoradiogram was taken after about one month when ^{90}Y, which stays at the point of application, had already decayed (its half-life is 62 h). A complete separation of ^{106}Ru, ^{90}Sr, and ^{137}Cs from ^{95}Zr, ^{95}Nb, ^{90}Y, ^{147}Pm, ^{99}Tc and ^{144}Ce is thus possible by paper electrophoresis using $N/2$ $NaNO_2$ solution as electrolyte.

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Système simple pour création d'un gradient de concentration de la phase mobile

Le système de deux réservoirs superposés explicité par la Fig. 1 ne nécessite aucun système d'agitation et aucune surveillance en cours de chromatographie. L'installation de ce système sur une colonne à chromatographie ne présente aucune difficulté particulière.

Dans le réservoir supérieur, on place un volume variable de la phase qui induit le gradient. Dans le reservoir inférieur, on place un volume donné de la phase de départ.

L'ordre le plus rationnel des manipulations est le suivant: Remplissage du réservoir supérieur. Création d'un vide partiel à la bouche par l'intermédiaire du bec. Remplissage du reservoir inférieur. Solidarisation hermétique des deux réservoirs par le rodage de jonction. Création d'un vide partiel dans le reservoir inférieur après avoir retourné l'ensemble du système.

Le système est alors placé sur la colonne, le bec du réservoir inférieur affleurant le niveau liquide de la colonne, et les robinets des réservoirs sont ouvert doucement.

Une baisse du niveau liquide dans la colonne provoque une entrée d'air par le bec du réservoir inférieur et il s'écoule un certain volume de liquide de celui-ci, ce qui a pour effet de diminuer le niveau liquide de ce réservoir inférieur. Par un mécanisme identique un même volume de liquide s'écoule du réservoir supérieur au réservoir inférieur. De la conservation de l'équilibre résulte un double niveau constant, l'un dans la colonne, l'autre dans le réservoir inférieur. Le passage de l'air créé dans le réservoir inférieur, qui agit comme chambre de mélange, un régime turbulant suffisant pour assurer un brassage efficace du mélange des phases.

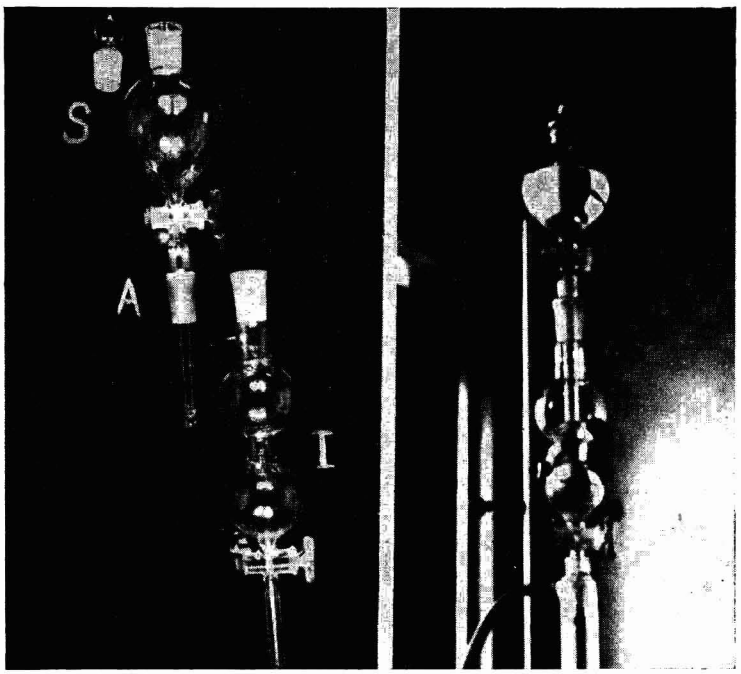

Fig. 1. A gauche, les divers éléments du système décrit. S = réservoir supérieur; I = réservoir inférieur; A = rodage de jonction des deux réservoirs. A droite, un système en cours de fonctionnement, installé sur une colonne à chromatographie.

Il faut observer certaines règles dans la construction de ce système afin d'évite. que ne se produise un bloccage de l'écoulement par des bulles d'air. Le diamètrr minimum du canal des robinets est de 3 mm tandis que celui des becs est de 10 mme La capacité totale d'un reservoir ne doit pas descendre au dessous de 200 ml.

Analyse des gradients obtenus

Soit on veut installer un gradient de concentration de la phase mobile à partir de la concentration C_1 et tendant vers la concentration C_2. Soit dans le réservoir inférieur le volume V juste nécessaire de la phase de concentration C_1 pour que le bec du réservoir supérieur y plonge. Supposons un grand volume de phase de concentration C_2 dans le réservoir supérieur.

Quand il s'écoule un volume dv de liquide du réservoir inférieur dans la colonne, il s'écoule un même volume dv du réservoir supérieur au réservoir inférieur. La variation de concentration dC qui se produit dans le réservoir inférieur est alors:

$$dC = \frac{dv\ (C_2 - C)}{V}$$

C étant la concentration dans le reservoir inférieur avant l'écoulement du volume dv.

Intégrons entre les limites C_1 et C_2, et o et v:

$$\int_{C_1}^{C_2} \frac{V dC}{C_2 - C} = \int_o^v dv$$

On arrive à l'expression:

$$C = C_2 - (C_2 - C_1)\ e^{-\frac{v}{V}}$$

qui donne la concentration de la phase du réservoir inférieur en fonction du volume de liquide écoulé du système.

Quand le volume écoulé est égal au volume de la phase placée dans le réservoir supérieur, on obtient un pallier de concentration.

Par action sur les facteurs C_1, C_2, V et sur le volume de phase placé dans le réservoir supérieur, on obtient un grand nombre de combinaisons possibles que l'on peut déterminer par le calcul.

Si le volume de phase de concentration C_1 dans le réservoir inférieur est plus grand que V, on obtient un pallier préalable de concentration C_1, fonction du volume en excès de V.

Selon la valeur de V, la pente du gradient entre les concentrations limites est plus ou moins grande.

Selon le volume de phase de concentration C_2 placé dans le réservoir supérieur, on obtient plus ou moins vite un pallier limite de concentration.

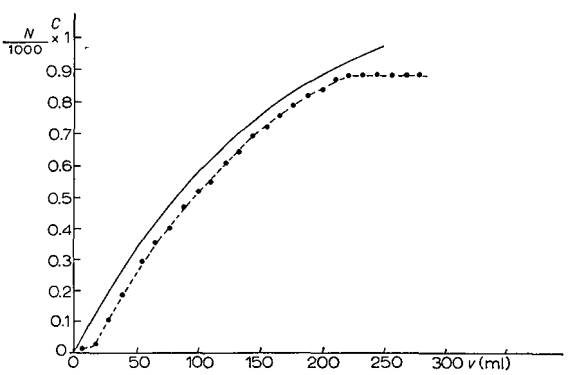

Fig. 2. Parallélisme entre une courbe théorique et une courbe expérimentale représentant la concentration en SO_4H_2 de l'écoulement d'un système en fonction du volume écoulé; — courbe théorique ($C = C_2 - (C_2 - C_1)\ e^{-v/V}$); – – – courbe expérimentale.

La Fig. 2 montre le parallélisme entre une courbe expérimentale et la courbe théorique. Les valeurs des variables étaient:

$$C_2 = SO_4H_2 \frac{N}{1000} 1.23 \text{ (200 ml)}; \quad C_1 = 0 \text{ (Eau distillée)}; \quad V = 160 \text{ ml}.$$

L'écoulement du réservoir inférieur se faisait dans un petit entonnoir à robinet (volume mort de 20 ml) à raison d'une goutte par seconde. Le dosage a été fait par NaOH dans des fractions collectées de 11.2 ml.

Le volume mort et le volume des fractions collectées expliquent le décalage de la courbe expérimentale par rapport à la courbe théorique. Le parallélisme des deux courbes démontre le mélange correct des phases dans le réservoir inférieur.

Ce travail a été executé dans le cadre de la Fondation de Diététique expérimentale et appliquée (Pr. Ag. H. SARLES), subventionnée par l'Institut National d'Hygiène et le Centre National de la Recherche Scientifique.

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Reçu le 19 mars 1962

A frame for photographic processing of the autoradiograms of paper chromatograms or electrophoregrams*

The development, washing and fixation of large sized X-ray films in shallow dishes is time consuming when larger series have to be worked up. The usual stainless steel frames with clips which are suspended in high earthenware tanks have the disadvantage that adjacent films can come into contact with each other which might lead to scratching of the emulsion. A frame (Fig. 1, A, B) has therefore been constructed to hold the films and enable them to be transferred from one tank to another as a compact block. It is based on the well-known principle of DATTA's frame[1]. A template (Fig. 1, C) consisting of two plates equipped with aligning pins for a precise location of the film even in complete darkness serves for punching the films, which are then slid onto the rods of the frame.

The main device consists of two frames made of the "Fatradur" hard polyvinyl thermoplastic resin or similar material. The supporting frame is equipped at each of the shorter edges with Fatradur rods with male thread at the top; holes are drilled in the corresponding positions in the top frame. After loading the supporting frame with the films (f), which are separated by one or more spacers to prevent contact, the top frame is placed in position and secured by a nut (N).

The punching device consists of two plates connected by hinges (b). The lower plate (P1) is equipped with three or more aligning pins (R) for the film and holes for the punch. The upper plate (P2) has similar holes as well as pits above the pins. After

* Demonstrated by O. HOREŠOVSKÝ, Z. FRANC AND I. M. HAIS at the Conference on Paper Chromatography in Prague, June 21, 1961.

the film (f) has been placed on the lower plate and aligned to touch the pins, it is covered by the upper plate and the punch (K) is driven in all the holes in succession.

This equipment has been used successfully for a couple of years. Special experiments have shown that there is no difference in the intensity of the blackening of

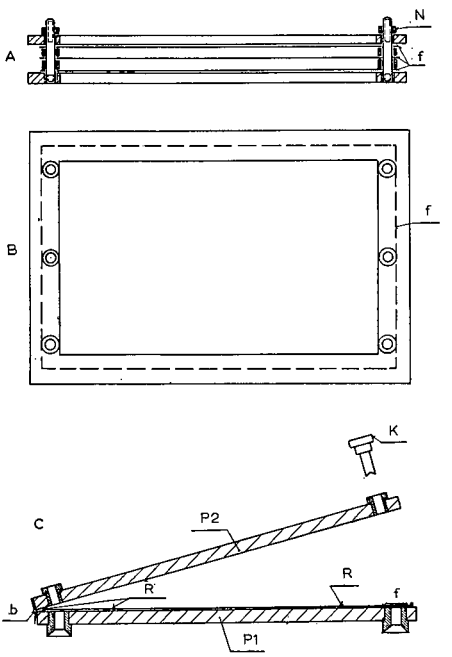

Fig. 1. (A, B) Carrier frame; the actual length of the six rods and number of films depends on the dimensions of the tank. (C) Punching device.

the spots or background, depending on the number and relative position of the films on the frame. There is no obvious reason why the same equipment should not be used for the development and fixation of diagnostic radiographs, but most radiologists prefer individual processing under visual control. On the other hand, in the case of autoradiograms, identical treatment of the whole series is critical for comparative purposes.

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Sur une nouvelle technique chromatographique sur papier des amino-acides soufrés

Parmi les nombreuses techniques chromatographiques proposées pour les amino-acides, peu sont applicables aux dérivés soufrés et plus précisément à la séparation des premiers stades de l'oxydation de la cystéïne, de la cystéïnamine, et de la méthionine.

Seules ont été proposées, par CAVALLINI, MONDOVI ET DE MARCO[1] une technique utilisant le mélange collidine–lutidine, et par WINEGARD, TOENNIES ET BLOCK[2] le mélange classique phénol–eau. Encore, dans ce dernier cas le disulfoxyde de cystine ne se sépare-t-il pas de l'acide cystéïnesulfinique et de tous façons s'agit-il de solvants peu volatils, difficiles à eliminer.

Nous intéressant aux premiers termes du métabolisme oxydatif de la cystéïne, nous avons été amenés à mettre au point une méthode de séparation de ces différents composés, qui joint à une bonne sensibilité une grande simplicité et qui permet de surcroit la récupération des produits par élution.

Après divers essais, nous nous sommes fixés au solvant suivant: éthanol (à 95°)–chloroforme pur–eau (60:30:10).

Les chromatogrammes présentent l'aspect selon la Fig. 1.

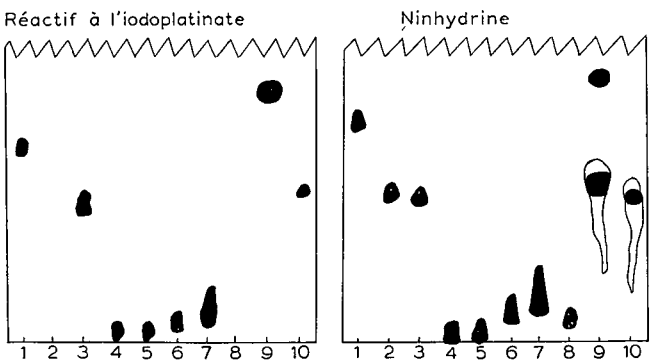

Fig. 1. Chromatogrammes des amino-acides soufrés. 1 = Méthionine; 2 = methionine sulfone; 3 = méthionine sulfoxyde; 4 = cystéïne; 5 = cystine; 6 = disulfoxyde de cystine; 7 = acide cystéïne sulfinique; 8 = acide cystéïque; 9 = cystéïnamine; 10 = disulfoxyde de cystamine.

Nous avons mesuré les déplacements, non plus par rapport au front, car dans certains cas, pour obtenir des déplacements aussi grands que possible nous avons laissé couler le solvant au delà du bord dentelé, mais par rapport à la méthionine, acide aminé facile à obtenir dans un bon état de pureté et donnant une tache ronde parfaitement définie.

Les $R_{méthionine}$ sont donc, dans ces conditions à 20°, présentés dans le Tableau I.

On peut améliorer la séparation des taches de faibles $R_{méthionine}$ d'une part en laissant couler jusqu'à ce que les taches aient migré aussi loin que possible, d'autre part en substituant le méthanol absolu à l'éthanol à 95°.

Les chromatogrammes sont révélés:

(a) D'une part à la ninhydrine, selon les procédés classiques.

TABLEAU I

	Rméthionine	
	Avec la ninhydrine	*Avec le réactif à l'iodoplatine*
Cystéïne	0.13	0.12
Cystine	0.14	0.12
Disulfoxyde de cystine[3]	0.24	0.21
Acide cystéïne sulfinique[4]	0.38	0.30
Acide cystéïque[5]	0.25	n'apparaît pas
Méthionine	1	1
Méthionine sulfone[6]	0.51	0.48 apparaît très difficilement
Méthionine sulfoxyde[7]	0.51	0.47
Cystéïnamine	1.25	1.42 valeur du sommet d'une tache nette
Disulfoxyde de cystamine[8]	0.67	0.63 dans une traînée

(b) D'autre part avec le réactif à l'iodoplatinate préconisé par WINEGARD *et al.*[2]. Il faut remarquer que la méthionine sulfone et l'acide cystéïque ne réagissent pas avec ce dernier réactif et que la cystine ne réagit que fort lentement.

(c) Enfin l'acide cystéïne sulfinique est révélé au moyen d'un mélange HCl–IK, ainsi qu'à l'aide du réactif au chlorure ferrique indiqué par MONDOVI *et al.*[9], la cystéinamine et la méthionine réagissant, quant à elles avec le réactif au nitroprussiate.

On voit que, compte tenu des différentes techniques de révélation, on se trouve en présence d'une méthode permettant la séparation et l'identification faciles des premiers termes d'oxydation des différents composés soufrés essayés, et qui, par la volatilité du solvant permet l'élution des composés, sans entraîner de parasite provenant du solvant.

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Gas chromatography fraction collector and transfer system

This note describes a method for the collection of fractions from a gas chromatograph and a unique method for their subsequent introduction into other systems without contamination.

Fig. 1 depicts an array of Hoke TY 440 valves which constitutes one collection station of a manifold attached to the effluent stream of a chromatograph. In order to minimize absorption of materials on the inside surfaces of the manifold the valve

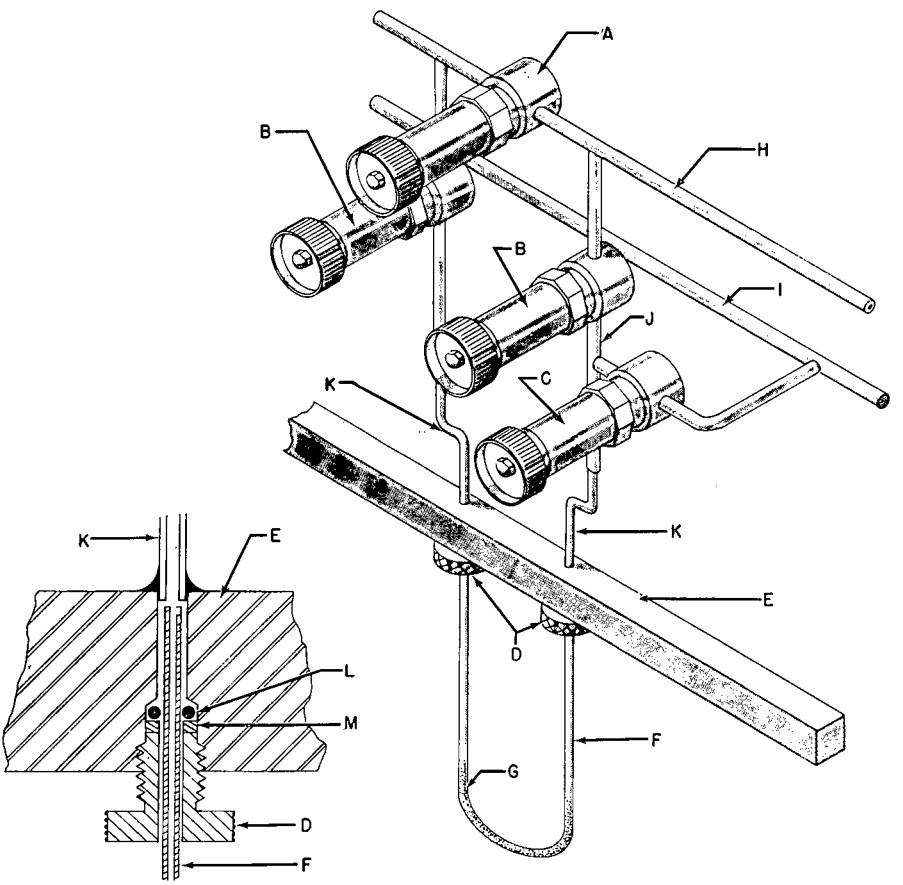

Fig. 1. Manifold assembly for the recovery of gas chromatography fractions: A = effluent bypass valve; B = sample loop valves; C = vacuum exhaust valve; D = knurled compression nuts; E = stainless steel 1/2 in. × 1/2 in. bar; F = glass U tube 4 mm o.d. nominal wall pyrex; G = silica sand; H = effluent line 1/4 in o.d. × 1/16 in. i.d.; I = vacuum line 1/4 in. o.d. nominal wall; J = loop return line 1/4 in. o.d. nominal wall; K = 1/8 in. o.d. nominal wall; L = "O" ring; M = thrust washer.

and tubing assembly is maintained at a temperature of about 100°C by a heating tape. The direction of flow is from left to right via line H. By means of valves A, B, the

carrier gas stream can be diverted through the glass sample loop F. Valve C is opened to remove the carrier gas via vacuum line I.

The traps consist of glass U tubes constructed of standard wall 4 mm o.d. pyrex; they are attached to the manifold by compression "O" ring seals illustrated in the cross sectioned detail part of Fig. 1. These seals have proven quite reliable and have been in constant use in this laboratory for over two years. The traps have a section

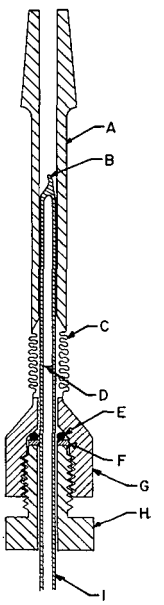

Fig. 2. Device for vacuum transfer of recovered fractions. A = stainless steel male inner connector ground joint; B = seal off tip of glass U tube arm; C = stainless steel sylphon bellows; D = file scratch on glass tubing; E = "O" ring; F = thrust washer; G = knurled body; H = knurled jamb nut; I = glass U tube.

of silica sand in the bottom of the loop retained by plugs of quartz wool. This provides the necessary entrapment for efficient sample recovery. For permanent gases such as methane and carbon monoxide, activated charcoal is substituted for the sand. The trap is held in liquid nitrogen during the collection of a fraction, and while still cold the carrier gas is removed by evacuation through exhaust valve C to a pressure of about 10^{-4} mm Hg. While evacuated and still cold (liquid N_2 temperature), the trap is sealed off at points about 1 inch below the jamb nuts D. A splicing torch has been found highly satisfactory for this operation. The samples are thus sealed off in glass and may be stored indefinitely.

Transfer of the sample to another system by vacuum handling is accomplished by means of the device illustrated in Fig. 2. A small file scratch is made on one of the glass arms about 2 inches below the sealed off end. This is inserted through the "O" ring compression seal E until the scratch is positioned well inside a section of flexible metal sylphon bellows C. The assembly is attached to the system and evacuated. Introduction of the sample is accomplished by flexing the assembly enough to snap the tubing inside at the scratch point.

The above system has been used for collecting several thousand fractions from a chromatograph with subsequent introduction into a mass spectrometer. It has proven to be efficient and trouble-free.

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Improved sampling valve for gas chromatography

For the gas chromatographic analysis of engine exhaust and air for trace amounts of hydrocarbons, a gas sampling valve with the following features was required: (1) no detectable gas leaks, (2) no contamination or adsorption of the sample, (3) fast switching speed for instantaneous sample injection, (4) interchangeable sample volumes with the smallest about 0.1 ml, and (5) good sampling precision. Five different valves available for laboratory gas chromatographs have been tried, but all failed to meet these requirements.

KARASEK AND AYERS[1] have described a unique gas sampling valve for use with industrial gas chromatographs. One of these pneumatically operated valves, obtained from the Greenbrier Instrument Company, Ronceverte, West Virginia, is shown in Fig. 1 with a four-way solenoid valve (PAL type, Ross Operating Valve Company, Detroit, Michigan). Either 1/16- or 1/8-in. Swagelok fittings may be used and a 1/16-in. tube delivering a sample volume of 0.14 ml is shown.

As received from the manufacturer the valve leaked seriously, but otherwise showed promise of meeting the above requirements. It therefore appeared worthwhile to develop a method for making the valve gastight. This method and an example of the use of the valve in the analysis of highly diluted hydrocarbon mixtures are discussed in this paper.

The disassembled valve is shown in Fig. 2. The stainless steel part (left) is separated from the brass base by a Teflon diaphragm, which opens and closes the valve ports by means of air pressure. In initial attempts to eliminate leaks, other diaphragm materials were evaluated, including Mylar, polyethylene, polyvinyl chloride, and polyurethane. Only with the last material was the valve gastight at 20 p.s.i.g. nitrogen. However, in trials with nitrogen samples containing 0.1 % benzene, the polyurethane adsorbed 10 times as much benzene as did the Teflon diaphragm, so that it was discarded as unsatisfactory for quantitative work.

The surfaces separated by the diaphragm bore the marks (mainly long scratches) of the grinding operation in the manufacture of the valve. It was found that the valve could be made gastight with Teflon diaphragms by polishing both surfaces to a mirror-like finish. The stainless steel part was first polished with 2/0, 3/0, then 4/0 Carborundum emery paper taped to a glass plate. Finer polishing was done with No. 9, No. 6, and No. 1 diamond pastes (Elgin National Watch Company, Elgin,

Illinois) on Gamal cloth-covered wheels. Linde B alumina, then Burrell C-RO chromic oxide powders on similar wheels were used to obtain a mirror-like finish.

The brass base, being softer than stainless steel, did not require as elaborate a polishing procedure. After removing the alignment prongs, it was polished with Linde A alumina on a wax wheel, then Linde B alumina and Burrell C-RO chromic oxide on cloth-covered wheels.

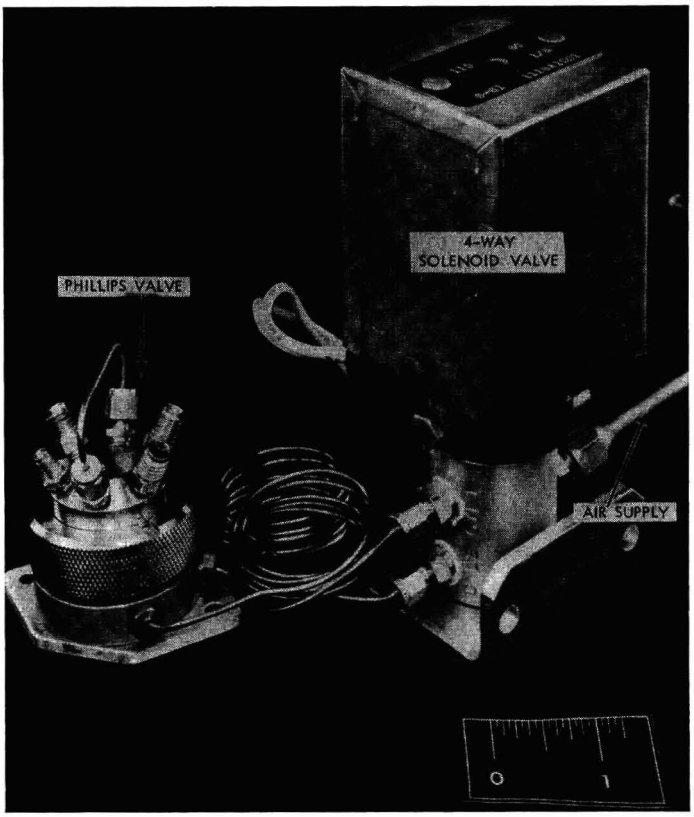

Fig. 1. Phillips gas sampling valve with air pilot valve.

The polishing materials that coat the walls of the channels were removed with triple solvent (equal portions of acetone, chloroform and toluene) in an ultrasonic cleaner. The valve was reassembled with a new Teflon diaphragm and tested under water with 70 p.s.i.g. hydrogen (95 p.s.i.g. air pilot pressure). No gas leaks could be detected.

The valve has been routinely used with a laboratory gas chromatograph for about a year, much of the time with 50 p.s.i.g. hydrogen carrier gas. Leaks, particularly at the sample gas ports, have occurred 3 times; but in all cases the trouble was due to minute metal and dirt particles getting into the valve because of inadequate filtering of the gas streams. After cleaning the valve and installing a new Teflon diaphragm, gastight service was again obtained. All streams are now filtered with metal screens and, in some cases, with porous metal discs.

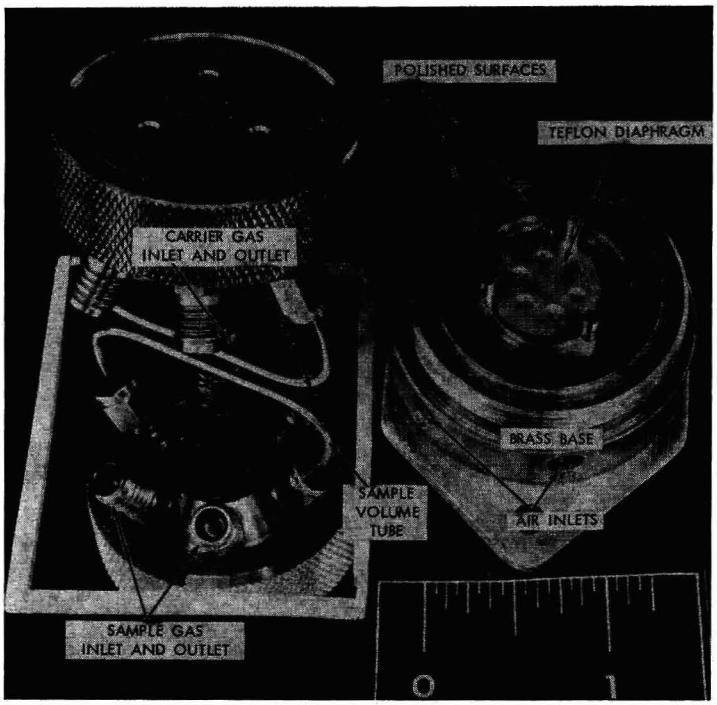

Fig. 2. Disassembled Phillips valve.

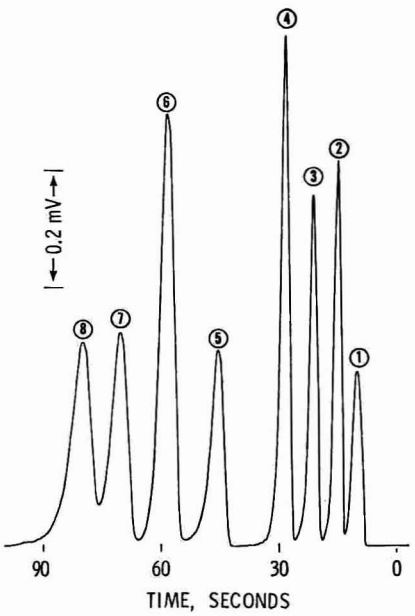

Fig. 3. Chromatogram showing rapid separation using a packed capillary column. Conditions: Perkin-Elmer flame ionization detector, range 1, attenuation × 64. Sample volume: 0.14 ml. Column: 1 m × 0.020 in. i.d. (1/16 in. o.d.) stainless steel tubing packed with 75 mg of 120–140 mesh Burrell silica gel coated with 5 % Apiezon C. Temperature: 26°. Gas flows (23°, 760 mm Hg): 220 ml/min air, 7.4 ml/min H_2 carrier gas (50 p.s.i.g. inlet pressure), 65 ml/min 60/40 nitrogen/hydrogen mixture. (1) 215 p.p.m. methane; (2) 195 p.p.m. ethane; (3) 205 p.p.m. ethylene; (4) 215 p.p.m. propane; (5) 195 p.p.m. acetylene; (6) 210 p.p.m. isobutane; (7) 140 p.p.m. *n*-butane; (8) 200 p.p.m. propylene. Balance: nitrogen.

One of the notable features of the valve is the small volume of the internal channels. The volumes of the sample tube shown in Fig. 2 and internal channels are 57 and 83 μl, respectively, for a total sample volume of 140 μl. The smallest sample that has been measured with the valve is 86 μl. By virtue of these small volumes, the valve has been advantageously used with both Golay and packed types of narrow bore columns. Fig. 3 shows an example of the separation of a nitrogen-diluted mixture of eight light hydrocarbons obtained with a 1/16-in. o.d. packed column. The column was connected directly to the valve and stream splitting was neither used nor required. However, stream splitting has been tried and by this means 33 μl of the same mixture has been separated with an alumina-packed column in one minute.

The precision of the valve has not been extensively investigated; however, the average deviation of peak heights for repeat analyses of dilute hydrocarbon mixtures is usually better than $\pm$ 0.5 %.

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EDITORIAL

This issue has been compiled because over the last few months an unusually large number of papers dealing with techniques and applications of thin-layer chromatography have been received by this office.

In one case several laboratories had worked out techniques for the separation of steroids and related compounds and had reported their results within several days of each other. Hence it was decided to collect all recent papers on thin-layer chromatography into one issue so as to facilitate a comprehensive view of the techniques involved.

It has been the editor's opinion for some time that interesting results could be obtained by applying the R_M value concept to structure–R_F value relationships for adsorption systems. Thin-layer chromatography has produced much more data for such work than adsorption column chromatography. It is hoped that the information collected in this issue will help to stimulate work in this direction.

Rome, August 1962 MICHAEL LEDERER

THIN-LAYER CHROMATOGRAPHY OF SUCROSE ESTERS AND MIXTURES OF RAFFINOSE AND SUCROSE*

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Widespread interest in the development of sucrose as a raw material for industrial chemicals has been stimulated by the development of sucrose esters as surface-active agents. These esters are synthesized by an alkaline transesterification procedure, whereby the reaction is terminated at a time favoring mono- or di-esterification of sucrose. Methods have been lacking to determine the composition of these esterification products, except by an average saponification equivalent value. A simple isolation procedure for determination of specific products has not been successfully developed for the reaction mixture, which usually contains a mixture of monoester, diester and unreacted sucrose. The average saponification equivalent value gives only a rough approximation of ester distribution.

Partially substituted sugar derivatives can be separated on silica columns[1,2]. With proper choice of solvents, the separation results in fractions of different degrees of substitution. As a routine technique for product evaluation, this procedure is time consuming and tedious. We have reported the composition of sucrose monostearate by gas chromatography[3]. This method is useful for structural composition data, but requires time to prepare sugar derivatives which have gas chromatographic mobility. The development of thin-layer chromatography with silica gel[4] suggested a rapid method to study product composition of these sugar esters and sucrose. During the period of our investigation, PASTUSKA[5] reported the successful use of thin-layer chromatography for carbohydrate separations. The technique of chromatography on chromatostrips as developed by MILLER AND KIRCHNER[4] provides a rapid and simple method for thin-layer chromatographic separation without elaborate equipment. Thin-layer chromatography on chromatostrips was extended to include the separation of sucrose and raffinose as part of a general study of separation of sugars.

EXPERIMENTAL

Qualitative analysis

Chromatostrips were prepared by coating pieces of single-weight window-glass, 1/2 in. × 5 1/2 in. in size, with silica gel*** Merck, No. 7729, Germany, containing 5%

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

*** Obtained from E. Merck A.G., Darmstadt, Germany. Reference to a company or product by name does not imply approval or recommendation of the product by the Department of Agriculture to the exclusion of others that may also be suitable.

starch binder as described by MILLER AND KIRCHNER[4] and modified by APPLEWHITE *et al.*[6]. Fluorescent zinc salts were not added to the coating materials because the esters could be located with a spray of a 0.2 % solution of dichlorofluorescein in 95 % ethanol.

Two microliter spots containing 10–200 μg of sucrose esters dissolved in chloroform were applied at a distance of 1.5 cm from the bottom of the strip. A mark was made at a distance of 10 cm from the point of sample application so that replicate strips would migrate the same distance for R_F comparisons. The chromatostrips were irrigated by ascending migration of 1 ml of solvent mixture contained in a stoppered test tube. A number of different solvents were tried and a solvent mixture of toluene, ethyl acetate, and 95 % ethyl alcohol (10:5:5, by vol.) gave the best separations of sucrose esters (Fig. 1, strips 1–5). The chromatostrips were dried in air and

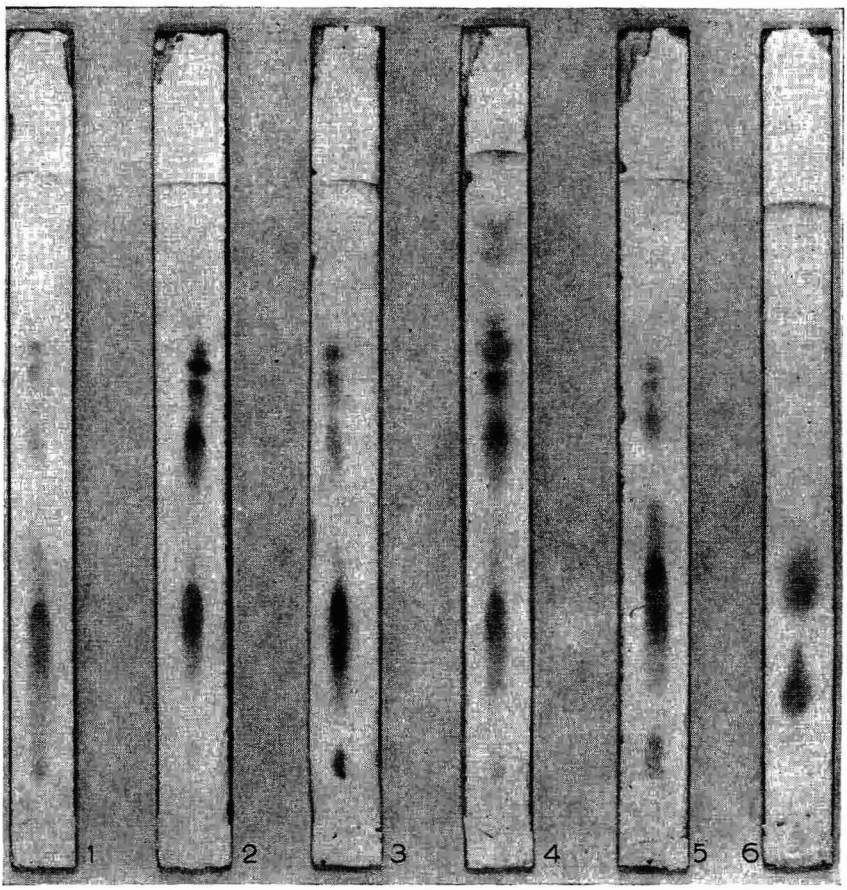

Fig. 1. Developed chromatostrips showing separations of sucrose esters, and sucrose and raffinose. The strips were prepared from silica gel G (for thin-layer chromatography, Merck), sprayed with dilute sulfuric acid, and heated at 110°. Sucrose esters chromatographed were: 1. Sucrose monopalmitate (200 μg); 2. Sucrose dipalmitate (200 μg); 3. Sucrose monolaurate (200 μg); 4. Sucrose lardate (200 μg); and 5. Sucrose tallowate (200 μg). Chromatostrip 6 shows the separation of 35 μg sucrose from 35 μg raffinose. See text for identification of components.

sprayed with the dichlorofluorescein indicator solution. The sucrose esters appeared as yellow spots on an orange background, when illuminated with a long-wave U.V. lamp. The time required for analysis was about 30 min.

Aqueous solutions of sucrose and raffinose mixtures were resolved on chromatostrips using a solvent composed of isopropyl alcohol–toluene–ethyl acetate–water (10:2:5:2.5, by vol.). The highly polar solvents migrate slowly on silica gel, and a period of 1–2 h was necessary for solvent migration of 10 cm. For sucrose and raffinose, the irrigated chromatostrips were sprayed after drying with a naphthoresorcinol–phosphoric acid indicator (5 vols. of 0.2 % naphthoresorcinol in acetone plus 1 vol. of 9 % phosphoric acid in water). The sugar-containing spots were revealed after a 5–10 min heating period at 110°. A typical separation is shown in Fig. 1, chromatostrip 6.

Quantitative analysis

The speed and ease of separation of sucrose-containing components on silica gel chromatostrips suggested the further application of this technique to obtain quantitative data.

Replicate chromatostrips were prepared as described for qualitative analysis so that each strip contained 100–200 μg of sample. After solvent irrigation, one strip was sprayed with the appropriate indicator to locate the fractions present. The R_F values of the various separated materials were noted on the sprayed strips and corresponding areas were scraped from the unsprayed chromatostrips. The silica gel sugar fractions were transferred to 10 mm filter tubes containing coarse fritted discs and each was eluted with 5 1-ml portions of dimethylformamide. The dimethylformamide solutions were evaporated to dryness *in vacuo* at 50–60° in a rotary evaporator. Blanks for unknowns were prepared from blank areas of irrigated chromatostrips and were treated in the same way as the sugar-containing areas. The evaporated residues were analyzed directly for ketose by the colorimetric method of ROE[7] and measured at 490 mμ on a Bausch and Lomb Spectronic 20 colorimeter. Standard samples of sucrose gave recoveries within 10 % of the applied amount and are shown in Table I.

TABLE I

EFFICIENCY OF RECOVERY OF SUCROSE FROM DEVELOPED SILICA GEL CHROMATOSTRIPS PREPARED WITH STARCH BINDER

Sucrose applied (μg)	*Sucrose recovered (μg)*
100	101
80	74
60, 60	60, 63
40	37
20	19

Analyses for sugar esters were made in a like manner except the residues were saponified with 1 ml of 0.2 *N* sodium hydroxide in 90 % ethanol by heating for 5 min at 60° in a water bath followed by addition of the resorcinol–hydrochloric acid reagents for ketose color development. Some typical results for commercial sucrose esters are

shown in Table II. All samples were measured against sucrose as a standard. The precision of the sucrose ester analysis was considered satisfactory for the purpose of this investigation. Seven replicate analyses were made on commercial samples of sucrose monopalmitate and on sucrose dipalmitate. The major constituent, monoester, in the sucrose monopalmitate averaged 75%, with a standard error of 2.3%. The diester content of sucrose dipalmitate averaged 66% with a standard error of 1.9%.

TABLE II

COMPOSITION OF SOME SUCROSE ESTER SAMPLES ANALYZED BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY WITH SILICA GEL

Ester	% *Sucrose*	% *Monoester*	% *Diester*
Sucrose monolaurate	8	81	11
Sucrose monopalmitate	2	84	14
Sucrose dipalmitate	–	36	64

DISCUSSION

The resolution of the sugar esters was better on silica gel than on paper. The silica gel acts as a strong adsorbent for hydroxyls, thereby adding to the separation power achieved by liquid partition. In Fig. 1 the chromatostrips 1, 2, 3, 4, 5 show separations of several sucrose ester samples. The solvent mixture used gave separations as follows: unsubstituted sucrose remained at the origin; sucrose monoester appeared as a long spot; and the sucrose diesters used here resolved into a series of four to five spots. Chromatostrip 4 shows two other fast moving spots which may represent substitution of greater degree than diesterification. If desired, adjustment of solvent composition could be made to study the more highly esterified products. Chromatostrip 6 shows the separation of sucrose and raffinose with raffinose as the slower migrating sugar.

For qualitative examination the same type of resolution resulted when either starch or calcium sulfate was used as a binder for the silica gel. Chromatostrips prepared with starch are recommended as they are easy to handle and can be marked with a pencil. However, chromatostrips prepared with silica gel G (Merck, Germany)* containing calcium sulfate as a binder have the advantage that they can be sprayed with dilute sulfuric acid and heated at 110° for 5–10 min to reveal spots. For the sucrose esters, the dichlorofluorescein indicator was preferred over the corrosive acid spray. However, the heated sulfuric acid spray reagent is a much more general indicator than dichlorofluorescein.

Ten to 200 μg amounts of sample can be detected by this technique, depending upon the number of components present. Samples exceeding 200 μg form diffuse spots which tail, due to overloading of the strip; samples of about 100 μg are useful for both qualitative and quantitative analysis.

The distinct separation of sucrose from raffinose and sucrose monoesters and diesters, made it possible to remove fractions from the chromatostrips for quantitative colorimetric measurement. The quantitative measurements were made for

* Obtained from Brinkmann Instruments, Inc., Great Neck, L. I., N. Y.

monoesters or diesters collectively, as no attempt was made to measure each of the resolved diester spots individually. A yellow color is eluted from the silica gel which contributes to color absorbance at 490 mμ. A blank determination was, therefore, included in the procedure to correct for this impurity, but the preparation of the chromatostrips is not uniform enough to prevent some variation in absolute measured sucrose values.

The resorcinol–hydrochloric acid reagents of ROE[7] were used to develop the characteristic ketose color. A specific ketose reagent was considered necessary under the conditions described to avoid difficulties from glucose-like impurities in starch. Although an aqueous solution of the starch binder used gave a ketose reaction with these reagents, the ketose color precursor from the starch was not eluted with dimethylformamide, and did not interfere in the optical density measurement. Silica gel containing sugar gave low values from known samples when eluted with water. Samples eluted with dimethylformamide gave better recoveries. The silica gel G strips gave poor recovery of sucrose materials even after dimethylformamide elution. Values were consistently low, due either to mechanical losses (silica gel G chromatostrips are weakly held together and tend to form loose powders) or to the fact that calcium sulfate binder may contribute strong adsorption forces.

ACKNOWLEDGEMENT

I wish to thank Dr. T. H. APPLEWHITE for his helpful suggestions on the technique of silica gel chromatostrips.

SUMMARY

The technique of qualitative and quantitative silica gel chromatography on glass strips has been adapted to separate mixtures of sucrose esters, and sucrose and raffinose. Solvent mixtures are described which give rapid, definitive separations of these compounds. The separated components can be eluted from the silica gel and measured for sucrose content by the colorimetric procedure of ROE.

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THIN-LAYER CHROMATOGRAPHY OF ACYL DERIVATIVES OF SUGARS

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In connection with our work[1] on acyl derivatives of aldoses we adapted thin-layer chromatography for the separation and characterisation of anomers.

On acylation of sugars a mixture of anomers is generally obtained. Thin-layer chromatography is a rapid, advantageous method for the investigation of the purity of the acyl derivative isolated. This is very important, because mixtures of anomers have been described as pure compounds in the literature. On the other hand, these acyl derivatives of sugars cannot be characterised by ordinary paper chromatography, unless the paper is specially treated. MICHEEL AND SCHWEPPE[2,3] reported the separation of sugar acetates by reversed-phase chromatography on acetylated paper, and WICKBERG[4] has described the separation of acetylated sugars by chromatography on papers impregnated with polar solvents. We consider thin-layer chromatography superior to paper chromatography in simplicity and speed, for the analytical separation of these sugar derivatives.

STAHL[5], who applied thin-layer chromatography to a wide range of compounds, has recently described the separation of free sugars on Kieselgur-G layers.

We separated acetyl and benzoyl derivatives of sugars by using glass plates coated with a mixture of silicic acid with 10% starch as binder. Development by the ascending method was employed with benzene, alone, or mixed with more polar solvents. A solvent system of 30% v/v ethyl acetate in benzene was one of the best for the resolution of fully acetylated sugars; the benzoyl derivatives were separated using benzene alone or a mixture of benzene with 0.5% methanol. Mixtures of chloroform and benzene were also used. It generally took 15–20 min to reach a height of about 13 cm.

In all cases we detected the spots with the silver nitrate–ammonia–sodium methylate reagent recommended by CADENAS AND DEFERRARI[6]. After spraying, the plates were heated for 10 min at 110°.

All acetates and benzoates showed up as light brown spots which were fluorescent when examined under ultraviolet light. Acetates reacted more quickly than benzoates, and acetylated disaccharides much more slowly than acetylated monosaccharides.

Acetates were resolved with more polar solvents than the corresponding benzoates.

EXPERIMENTAL

Smooth glass plates (15 × 18 cm) were used. They were coated with an even layer, 0.5 mm thick, of an adsorbent mixture of silicic acid Mallinckrodt, chromatographic

grade, with 10% starch as binder. The coating mixture for nine plates was prepared by a method similar to that reported by KIRCHNER, MILLER AND KELLER[7]. Thirty grams of silicic acid were thoroughly mixed with 3 g of starch; both had been sifted through a 200 mesh sieve. The mixture was stirred with 69 ml of distilled water, while heating on a water bath at 80–85° until it thickened. It was then cooled with stirring to room temperature and spread on the glass plates. These plates were activated by heating for 2 h at 110°. The chromatoplates were kept in a desiccator over potassium hydroxide. The starting points were marked on the plates at 15 mm from the base.

Ten milligrams of the sample were dissolved in 1 ml of chloroform and two drops of the solution were applied with a micropipette on the marked points. After evaporation of the solvent, the plates were placed in the chromatographic chamber, containing sufficient solvent to wet *ca.* 0.5 cm of the plates. The jar had to be saturated with the solvent vapours for 2 hours before development. The time of development for a height of about 13 cm was 15 min. After development, the plates were removed and the solvent was allowed to evaporate. The dried plates were sprayed with the silver nitrate–ammonia–sodium methylate reagent[6], and heated for 10 min at 110°.

All acetates and benzoates were detected as brown spots which were fluorescent when examined under ultraviolet light.

The acetyl and benzoyl derivatives investigated were of our own making.

RESULTS

The separation of fully benzoylated monosaccharides is shown in the chromatograms of Figs. 1, 2 and 3, and the R_F values are given in Tables I and II.

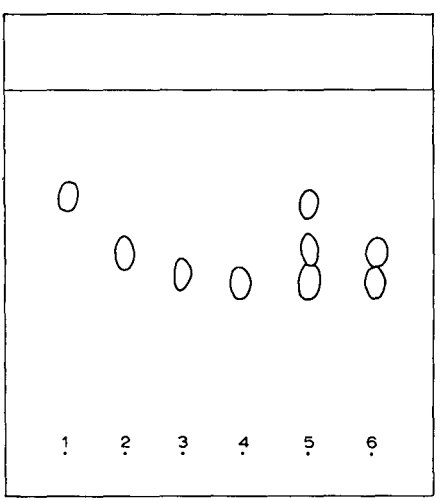

Fig. 1. Thin-layer chromatogram of benzoylated sugars. Solvent system: chloroform–benzene, 3:7 (v/v). (1) Tetra-O-benzoyl-α-D-lyxopyranose; (2) penta-O-benzoyl-α-D-glucopyranose; (3) penta-O-benzoyl-β-D-glucopyranose; (4) hexa-O-benzoyl-D-*glycero*-α-D-*galacto*-heptose; (5) mixture of 1, 2 and 3; (6) mixture of 2 and 3.

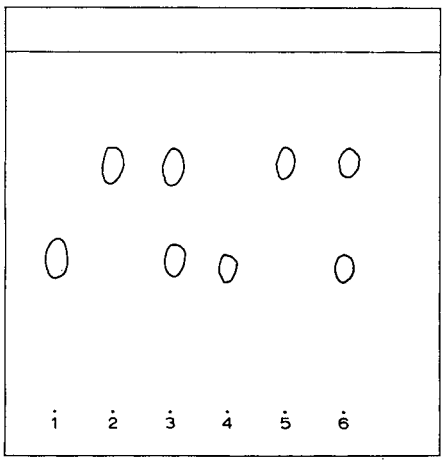

Fig. 2. Thin-layer chromatogram of anomeric benzoylated sugars. Solvent system: 0.5% methanol in benzene. (1) Hexa-O-benzoyl-D-*glycero*-α-L-*manno*-heptose; (2) hexa-O-benzoyl-D-*glycero*-β-L-*manno*-heptose; (3) mixture of 1 and 2; (4) hexa-O-benzoyl-D-*glycero*-α-D-*gulo*-heptose; (5) hexa-O-benzoyl-D-*glycero*-β-D-*gulo*-heptose; (6) mixture of 4 and 5.

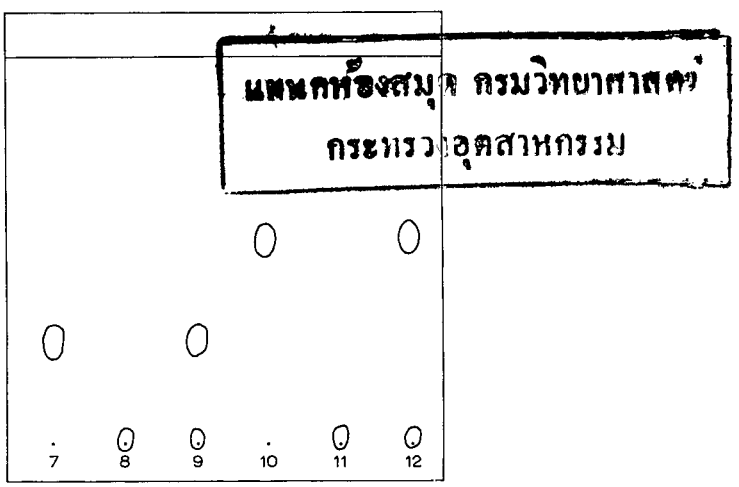

Fig. 3. Thin-layer chromatogram of anomeric benzoylated sugars. Solvent: benzene. (7) Hexa-O-benzoyl-D-*glycero*-α-D-*galacto*-heptose; (8) hexa-O-benzoyl-D-*glycero*-β-D-*galacto*-heptose; (9) mixture of 7 and 8; (10) penta-O-benzoyl-α-D-galactopyranose; (11) penta-O-benzoyl-β-D-galactopyranose; (12) mixture of 10 and 11.

TABLE I

R_F VALUES OF BENZOYLATED SUGARS

Fig. 1 No.	*Compound*	R_F [a]
1	Tetra-O-benzoyl-α-D-lyxopyranose	0.70
2	Penta-O-benzoyl-α-D-glucopyranose	0.56
3	Penta-O-benzoyl-β-D-glucopyranose	0.49
4	Hexa-O-benzoyl-D-*glycero*-α-D-*galacto*-heptose	0.39

[a] Solvent system: chloroform–benzene, 3:7 (v/v).

TABLE II

R_F VALUES OF ANOMERIC BENZOYLATED SUGARS

Figs. 2 and 3 No.	*Compound*	R_F
1	Hexa-O-benzoyl-D-*glycero*-α-L-*manno*-heptose	0.42[a]
2	Hexa-O-benzoyl-D-*glycero*-β-L-*manno*-heptose	0.68[a]
4	Hexa-O-benzoyl-D-*glycero*-α-D-*gulo*-heptose	0.40[a]
5	Hexa-O-benzoyl-D-*glycero*-β-D-*gulo*-heptose	0.69[a]
7	Hexa-O-benzoyl-D-*glycero*-α-D-*galacto*-heptose	0.26[b]
8	Hexa-O-benzoyl-D-*glycero*-β-D-*galacto*-heptose	0.0[b]
10	Penta-O-benzoyl-α-D-galactopyranose	0.52[b]
11	Penta-O-benzoyl-β-D-galactopyranose	0.0[b]

[a] Solvent system: 0.5% methanol in benzene.
[b] Solvent: benzene.

As is readily seen, the mobilities of the anomeric benzoates show considerable differences, while, for the anomeric acetates investigated, the R_F values of a pair of anomers differ only slightly (Fig. 4, Table III).

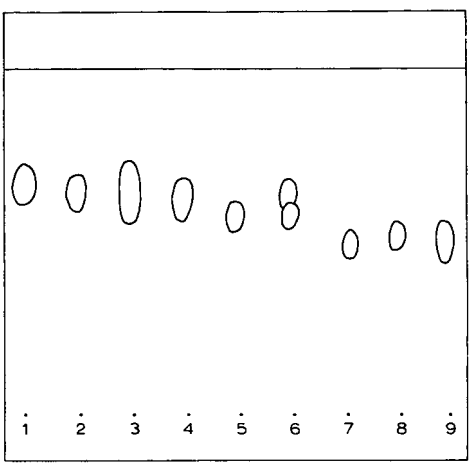

Fig. 4. Thin-layer chromatogram of anomeric acetylated sugars. Solvent system: ethyl acetate–benzene, 3:7 (v/v). (1) Penta-O-acetyl-α-D-glucopyranose; (2) penta-O-acetyl-β-D-glucopyranose; (3) mixture of 1 and 2; (4) penta-O-acetyl-α-D-galactopyranose; (5) penta-O-acetyl-β-D-galactopyranose; (6) mixture of 4 and 5; (7) hexa-O-acetyl-D-*glycero*-α-L-*manno*-heptose; (8) hexa-O-acetyl-D-*glycero*-β-L-*manno*-heptose; (9) mixture of 7 and 8.

TABLE III

R_F VALUES OF ANOMERIC ACETYLATED SUGARS

Fig. 4 No.	*Compound*	R_F [a]
1	Penta-O-acetyl-α-D-glucopyranose	0.66
2	Penta-O-acetyl-β-D-glucopyranose	0.65
4	Penta-O-acetyl-α-D-galactopyranose	0.64
5	Penta-O-acetyl-β-D-galactopyranose	0.57
7	Hexa-O-acetyl-D-*glycero*-α-L-*manno*-heptose	0.49
8	Hexa-O-acetyl-D-*glycero*-β-L-*manno*-heptose	0.52

[a] Solvent system: ethyl acetate–benzene, 3:7 (v/v).

MICHEEL AND SCHWEPPE could not separate the anomeric penta-acetyl-glucoses on acetylated paper but WICKBERG resolved anomeric acetates on papers impregnated with polar solvents.

It is interesting to note that in all cases the benzoyl or acetyl derivative with a 1,5-*trans* configuration had a higher R_F value than the respective anomer, with the exception of D-*glycero*-D-*gulo*-heptose. On the other hand, R_F values were found to be very dependent on the molecular weight for the fully acetylated and benzoylated sugars (Tables IV and I).

TABLE IV

R_F VALUES OF ACETYLATED SUGARS

Figs. 5 and 6 No.	*Compound*	R_F	
		a	*b*
1	Tetra-O-acetyl-α-D-lyxopyranose	0.78	0.74
2	Tetra-O-acetyl-β-D-xylopyranose	0.71	0.69
3	Penta-O-acetyl-β-D-glucopyranose	0.68	0.63
4	Penta-O-acetyl-β-D-mannopyranose	0.57	0.52
5	Octa-O-acetyl-6-β-D-glucopyranosyl-α-D-mannose	0.28	0.27
6	Octa-O-acetyl-gentiobiose	0.27	0.22

[a] Solvent system: ethyl acetate–benzene, 3:7 (v/v).
[b] Solvent system: methanol–benzene, 2:98 (v/v).

The acetylated sugars were resolved with two solvent systems, 30% v/v ethyl acetate in benzene (Fig. 5) or 2% v/v methanol in benzene (Fig. 6).

Table V lists the R_F values of benzoyl-pentoses. The mobilities of these isomeric compounds are rather similar (Fig. 7).

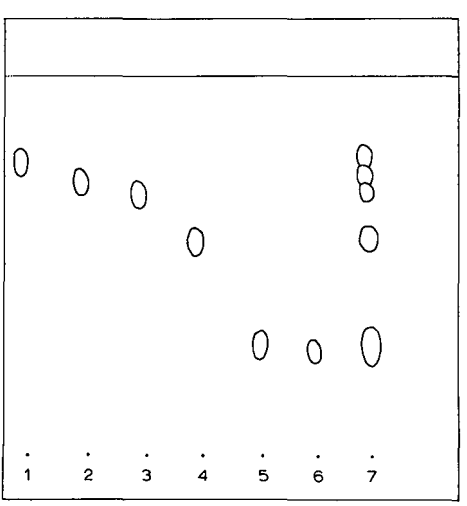

Fig. 5. Thin-layer chromatogram of acetylated sugars. Solvent system: ethyl acetate–benzene, 3:7 (v/v). (1) Tetra-O-acetyl-α-D-lyxopyranose; (2) tetra-O-acetyl-β-D-xylopyranose; (3) penta-O-acetyl-β-D-glucopyranose; (4) penta-O-acetyl-β-D-mannopyranose; (5) octa-O-acetyl-6-β-D-glucopyranosyl-α-D-mannose; (6) octa-O-acetyl-gentiobiose; (7) mixture of 1, 2, 3, 4, 5 and 6.

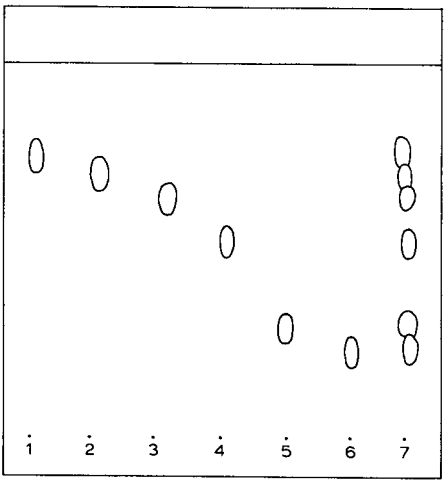

Fig. 6. Thin-layer chromatogram of acetylated sugars. Solvent system: methanol–benzene, 2:98 (v/v). (1) Tetra-O-acetyl-α-D-lyxopyranose; (2) tetra-O-acetyl-β-D-xylopyranose; (3) penta-O-acetyl-β-D-glucopyranose; (4) penta-O-acetyl-β-D-mannopyranose; (5) octa-O-acetyl-6-β-D-glucopyranosyl-α-D-mannose; (6) octa-O-acetyl-gentiobiose; (7) mixture of 1, 2, 3, 4, 5 and 6.

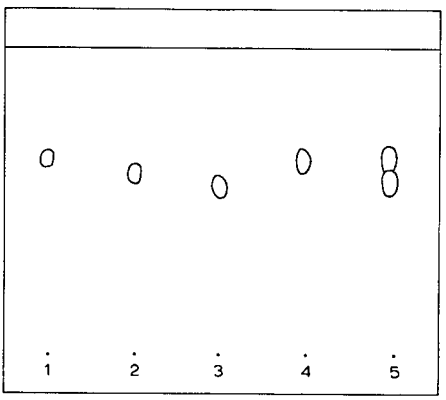

Fig. 7. Thin-layer chromatogram of tetra-O-benzoyl-pentoses. Solvent system: ethyl acetate–benzene, 3:97 (v/v). (1) Tetra-O-benzoyl-α-D-xylopyranose; (2) tetra-O-benzoyl-α-D-lyxopyranose; (3) tetra-O-benzoyl-α-D-ribopyranose; (4) tetra-O-benzoyl-β-L-arabinopyranose; (5) mixture of 1, 2, 3 and 4.

TABLE V

R_F VALUES OF TETRA-O-BENZOYL-PENTOSES

Fig. 7 No.	*Compound*	R_F[a]
1	Tetra-O-benzoyl-α-D-xylopyranose	0.64
2	Tetra-O-benzoyl-α-D-lyxopyranose	0.59
3	Tetra-O-benzoyl-α-D-ribopyranose	0.54
4	Tetra-O-benzoyl-β-L-arabinopyranose	0.62

[a] Solvent system: ethyl acetate–benzene, 3:97 (v/v).

We could distinguish the furanoid from the pyranoid form of penta-O-acetyl-galactoses. The penta-O-acetyl-α-D-galactopyranose travelled faster than the corresponding furanoid derivative, and the penta-O-acetyl-β-D-galactopyranose had a higher R_F value than the penta-O-acetyl-β-D-galactofuranose (Table VI, Fig. 8).

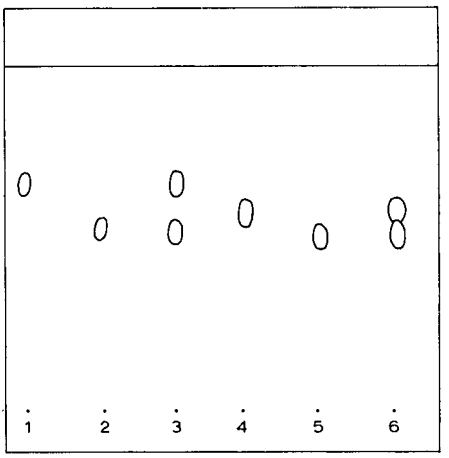

Fig. 8. Thin-layer chromatogram of penta-O-acetyl-galactoses. Solvent system: ethyl acetate–benzene, 3:7 (v/v). (1) Penta-O-acetyl-α-D-galactopyranose; (2) penta-O-acetyl-α-D-galactofuranose; (3) mixture of 1 and 2; (4) penta-O-acetyl-β-D-galactopyranose; (5) penta-O-acetyl-β-D-galactofuranose; (6) mixture of 4 and 5.

We also succeeded in separating a mixture of partially benzoylated glucoses (Fig. 9). As was to be expected, the R_F values increased with acylation (Table VII).

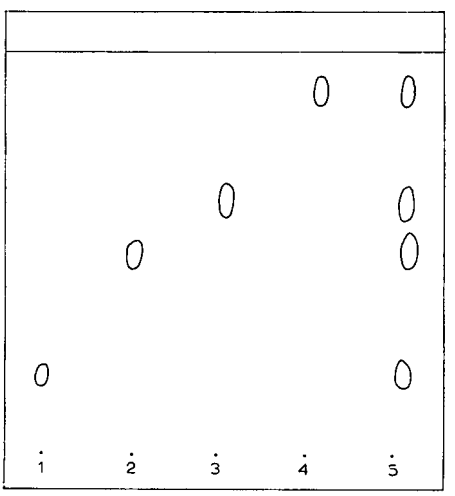

Fig. 9. Thin-layer chromatogram of partially benzoylated glucoses. Solvent system: ethyl acetate–benzene, 4:6 (v/v). (1) 5,6-Di-O-benzoyl-D-glucofuranose; (2) 1,2,3-tri-O-benzoyl-D-glucopyranose; (3) 3,5,6-tri-O-benzoyl-D-glucofuranose; (4) 3,4,5,6-tetra-O-benzoyl-aldehydo-D-glucose; (5) mixture of 1, 2, 3 and 4.

TABLE VI

R_F VALUES OF PENTA-O-ACETYL-GALACTOSES

Fig. 8 No.	*Compound*	R_F[a]
1	Penta-O-acetyl-α-D-galactopyranose	0.65
2	Penta-O-acetyl-α-D-galactofuranose	0.52
4	Penta-O-acetyl-β-D-galactopyranose	0.56
5	Penta-O-acetyl-β-D-galactofuranose	0.49

[a] Solvent system: ethyl acetate–benzene, 3:7 (v/v).

TABLE VII

R_F VALUES OF PARTIALLY BENZOYLATED GLUCOSES

Fig. 9 No.	*Compound*	R_F[a]
1	5,6-Di-O-benzoyl-D-glucofuranose	0.18
2	1,2,3-Tri-O-benzoyl-D-glucopyranose	0.47
3	3,5,6-Tri-O-benzoyl-D-glucofuranose	0.61
4	3,4,5,6-Tetra-O-benzoyl-aldehydo-D-glucose	0.88

[a] Solvent system: ethyl acetate–benzene, 4:6 (v/v).

SUMMARY

Acyl derivatives of sugars have been separated and characterised by thin-layer chromatography. We consider this method of great value for the resolution and identification of a mixture of anomers.

Separation was completed within 20 minutes, with benzene alone or mixed with more polar solvents.

In all cases the acyl derivative with a 1,5-*trans* configuration had a higher R_F value than the respective anomer, with the exception of D-*glycero*-D-*gulo*-heptose.

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THIN-LAYER CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZINE DERIVATIVES OF HYDROXYCARBONYL COMPOUNDS

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In connection with our study of the degradation of carbohydrates it was necessary to separate complex mixtures of mono- and bis-2,4-dinitrophenylhydrazones derived from hydroxycarbonyl compounds. Although the chromatography of 2,4-dinitrophenylhydrazones has been studied extensively, very little has been done with those listed in Table I. WOLFROM AND ARSENAULT[1,2] reported the separation of some of these compounds on columns of deactivated silica gel, whilst REICH AND SAMUELS[3] use an aluminium oxide column to separate the derivatives of pyruvaldehyde and hydroxypyruvaldehyde. Thin-layer chromatography has now been found to be a rapid method of determining the purity of these hydrazones and of examining complex mixtures of them.

METHOD

Adsorbent

Aluminium oxide G* (20 g) mixed with water (40 ml) and silica gel G* (20 g) with water (40 ml) were used as adsorbents. They were applied as slurries, 1½ minutes after mixing, with an applicator[4] giving a film 0.25 mm thick on glass plates (20 × 5 or 20 × 20 cm). The plates were air dried and could be activated further by drying at 100° for 2 hours. For some separations the plates were deactivated by holding them for a few hours at 20° in an atmosphere of 70% relative humidity. To obtain reproducible R_F values, the water content of the adsorbents must be constant since water lowers their activity. Activated plates were stored in a desiccator, but in hot humid weather some loss of activity took place during handling. However if standard reference substances are used on each plate, rigidly controlled conditions are not necessary.

Solvent

Mixtures of toluene and ethyl acetate were found to be the most useful solvent systems; increasing the proportion of ethyl acetate increased their eluting power. The best mixture depended on the compounds to be separated, the adsorbent, and its activity (see Table I).

Chromatographic procedure

Solutions of the hydrazones (0.01–0.1%) in acetone or tetrahydrofuran were applied to the plates by means of 1 μl capillary pipettes. After a few minutes the plates were

* E. Merck, A.G., Darmstadt.

placed in glass jars of appropriate size containing solvent at the bottom. The sides of the jars were lined with filter paper moistened with the solvent. The adsorbent side of each plate was close to and facing the paper.

The progress of the separation could be seen since all the hydrazones gave yellow spots. Usually when the solvent had travelled 15 cm the plate was removed and allowed to dry for 15 min. The spots were then sprayed with a solution of sodium hydroxide (2%) in ethanol (90%) to give intense characteristic colours (Table I), some of which took a few minutes to develop fully on the silica gel plates. The colours soon faded but a permanent record was obtained by colour photography.

Two-dimensional separations were carried out similarly on the square plates. With aluminium oxide useful results were obtained in our work by running the chromatogram first in solvent (c) on partially deactivated adsorbent, then allowing the plates to dry in air for 20 min and running them at right angles in toluene. Silica gel plates were run first in solvent (b) on activated adsorbent, air dried and run in toluene. The R_F values in the second solvent depended on the humidity of the atmosphere in which the plates were dried, and were higher than those in Table I, but in the same order.

Two-dimensional separations were also carried out with two different adsorbents (Fig. 1). The chromatograms were run as usual on the first adsorbent. After drying for 20 min excess adsorbent was scraped off the plate leaving a strip on one side with the spots of the first separation. The plate was placed in the applicator with the strip of the first adsorbent protruding from the applicator tank. The rest of the plate was coated in the normal way, air dried, and run in the second solvent. Plates precoated with both adsorbents gave unsatisfactory results since the solvents ascended the two adsorbents at different rates, causing distortion of the front and of the spots.

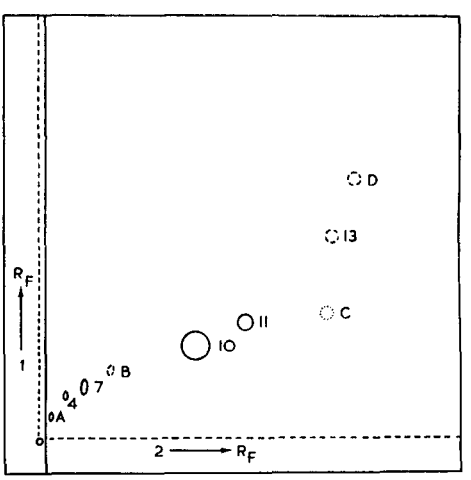

Fig. 1. Two-dimensional chromatogram on two adsorbents. The first adsorbent was silica gel (air dried) and the second aluminium oxide (deactivated). The same solvent system toluene–ethyl acetate 3:1 (v/v) ascended the full length of the plate (20 cm) in the two directions. The sample was the mixture of 2,4-dinitrophenylhydrazine derivatives obtained from the degradation products of fructose heated at 100° in 0.03 *N* oxalic acid for 2 h. A large proportion of compound 4 had been removed by crystallization from acetone. The colours given by the alkaline spray were: A, 4, B, and C purple; 10, 11, 13, and D brown; and 7 blue. The code for the numbers is as in Table I; A, B, C, and D are unidentified.

Preparation of the hydrazones

Most of the hydrazones (mono and bis) could be prepared from the parent carbonyl compounds with 2,4-dinitrophenylhydrazine (2%) in perchloric acid (30%) by the general procedure of NEUBERG, GRAUER, AND PISHA[5]. To prevent osazone formation, derivatives 1, 5, 6 and 9 (Table I) were prepared in the absence of acid, by refluxing a solution of the carbonyl compound and the hydrazine in ethanol[3,6]. Refluxing solutions of α-hydroxymonocarbonyl compounds with 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid yielded the osazones[1,3,7,8]. This reagent gave with glycerose and dihydroxyacetone a mixture[3] of two bishydrazones, 12 and 15, which were separated on an aluminium oxide column.

All the derivatives were obtained pure by recrystallization. Their melting points agreed with those in the literature, except for derivatives 7, 11, and 13 which are new and will be described elsewhere.

RESULTS AND DISCUSSION

Thin-layer chromatography of 2,4-dinitrophenylhydrazine derivatives of hydroxycarbonyl compounds achieved good separation as indicated in Table I. The method is quick, taking about 30 min, and requires extremely small samples. Spots of 0.1 μg are

TABLE I

THIN-LAYER CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES

2,4-Dinitrophenylhydrazone / Parent carbonyl compound	Colour with NaOH	R_F value × 100						
		Al_2O_3 plates				SiO_2 plates		
		a	b	c	d	a	b	c
1 D-Glucose	brown	0	0	0	0	0	0	0
2 D-Glucosone*	purple	0	0	0	3	0	0	10
3 3-Deoxy-D-*threo*-hexosone*	purple	0	0	0	15	0	2.5	23
4 3-Deoxy-D-*erythro*-hexosone (3-deoxyglucosone)*	purple	0	0	0	20	0	2.5	26
5 DL-Glycerose	brown	0	0	0	8	0	1.8	12
6 Dihydroxyacetone	brown	0	0	3	25	0	4	17
7 3,4-Dideoxy-*trans*-Δ^3-D-hexosone*	blue	0	0	3	46	0	9	43
8 3,4-Dideoxy-D-hexosone*	purple	0	0	7	49	0	9	41
9 Glycolaldehyde	brown	0	13	38	67	0	16	39
10 5-(Hydroxymethyl)-furfural	brown	0	20	50	63	0	21	48
11 2-(Hydroxyacetyl)-furan	brown	1.5	32	60	70	2	26	50
12 Hydroxypyruvaldehyde*	purple	0	25	65	85	0	48	74
13 Bis-(5-methylenefurfural) ether	brown	1	59	85	85	1.5	45	66
14 Glyoxal*	purple	2	73	87	86	9	67	80
15 Pyruvaldehyde*	purple	5	77	89	87	13	70	80
16 Furfural	brown	42	83	89	88	27	62	74

(a) Toluene; (b) toluene–ethyl acetate 3:1 (v/v); (c) and (d) toluene–ethyl acetate 1:1 (v/v); (a) (b) and (c) adsorbent activated at 100°, (d) adsorbent deactivated, see text.

* These are all αβ-dicarbonyl compounds, their bishydrazones could also be derived by osazone formation from any of the corresponding α-hydroxy-monocarbonyl compounds. Number 2 was also prepared from D-glucose, D-mannose, and D-fructose, 4 from 3-deoxy-D-glucose, 14 from glycolaldehyde, and both 12 and 15 from either glycerose or dihydroxyacetone.

easily seen and their intensity is increased by the alkaline spray, specially for those giving blue or purple colours. The blue or purple colours seem to be specific for the 1,2-bis-derivatives.

Hydroxyl groups account for most of the adsorption and largely determine the R_F value of these hydrazones as can be seen from Table I, where they are listed in order of decreasing number of hydroxyl groups.

The R_F values also depend on the adsorbent, its activity, and on the solvent system as shown in Table I. It should be noted that reversal of order frequently takes place on changing either the adsorbent or the solvent system. To increase R_F values of polyhydroxy derivatives it is better to deactivate the adsorbent by increasing its water content than to increase the proportion of ethyl acetate beyond 50% since the spots of low R_F values become increasingly elongated. In the solvent systems in Table I all the compounds shown gave single round spots.*

Two-dimensional separations with one or two adsorbents (Fig. 1) were found most useful for the examination of complex mixtures, and this is undoubtedly due to the flexibility in the choice of experimental conditions as the adsorbent, its activity, or the solvent system may be varied for each direction.

ACKNOWLEDGEMENT

The author is indebted to Mr. D. GALLIMORE for technical assistance.

SUMMARY

A simple and rapid method is described for chromatographing mono- and bis-2,4-dinitrophenylhydrazones derived from hydroxycarbonyl compounds. The derivatives were separated by the thin-layer technique on aluminium oxide or silica gel plates with toluene–ethyl acetate mixtures as solvents. Spraying with sodium hydroxide intensified the spots and gave blue or purple colours only with the 1,2-bishydrazones. Complex mixtures were resolved by two-dimensional methods using the same or a different adsorbent for each direction.

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* The present work confirms the preparation of pure glycerose hydrazone by the method of REICH AND SAMUELS[3]. This hydrazone gave a single spot in all the systems in Table I and also on deactivated silica gel using benzene–ether as solvent (*cf.* WOLFROM AND ARSENAULT)[1,2].

SEPARATION OF OXYGENATED FATTY COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

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Since thin-layer chromatography (TLC) was evolved by KIRCHNER *et al.*[1] in 1951, its simplicity, quickness and efficiency have led to widespread use of this technique. In the field of fats, MANGOLD AND MALINS[2] applied TLC for the separation and identification of natural lipid components such as hydrocarbons, triglycerides, sterols, fatty acids, etc. MORRIS, HOLMAN AND FONTELL[3] used TLC in conjunction with other methods of separation for the identification of naturally occurring epoxy acids. Mono-, di-, and triglycerides in a mixture were resolved by TLC using various mixtures of ether and petroleum ether for development[4]. MANGOLD[5] has recently reviewed comprehensively the applications of TLC to the separation of lipids.

A number of mono- and polyhydroxy compounds based on ricinoleic and undecylenic acids and the corresponding alcohols, have been prepared by SUBBARAO AND ACHAYA[6,7] for utilization purposes. ROOMI AND SUBBARAM[8] have prepared a series of epoxy, dihydroxy and halohydroxy derivatives, which occur as intermediates in stereochemical transformations, starting from erucic acid and its *trans* isomer brassidic acid. The application of TLC to the separation of these well-defined and pure compounds is described in this paper. These studies have enabled us to make some useful generalizations regarding the effect on the mobility during TLC of the number and position of hydroxy groups, chain length, presence of epoxy and halohydroxy group, stereochemical configuration and degree of saturation.

MATERIALS AND METHODS

Table I lists the compounds used, their starting materials and method of preparation. The reduction of esters with sodium was carried out according to HANSLEY'S procedure[9]. Hydroxylation to yield *threo* products, and epoxidation with peracetic acid, were performed according to the methods described by SWERN and co-workers[10,11]. Alkaline permanganate oxidation[12] was used to prepare the *erythro* dihydroxy isomers used. Hypohalogenation was conducted according to KING[13].

Using a thin-layer applicator (Desaga, Heidelberg), glass plates (20 × 20 cm) were coated with a well-stirred suspension of silica gel G (E. Merck, Darmstadt; 30 g in 60 ml water) to give a layer approximately 270 μ in thickness. The plates were dried at 105–110° for 30 min and preserved in a desiccator until required. The compounds were dissolved in methanol and 10 μg was applied with a micropipette on starting points 2–3 cm from the end of the plate. The plate was then placed inside the chamber

TABLE I

COMPOUNDS USED IN THE EXPERIMENTS, THEIR STARTING MATERIALS AND METHOD OF PREPARATION

No.	*Compound used and method of preparation*	*Prepared from*
	A. Reduction with sodium	
1.	Undecenyl alcohol	Methyl undecylenate
2.	Oleyl alcohol	Methyl oleate
3.	Ricinoleyl alcohol	Methyl ricinoleate
	B. Hydroxylation	
	(i) with peracetic acid	
4.	*threo*-9,10-Dihydroxystearic acid	Oleic acid
5.	*threo*-13,14-Dihydroxydocosanoic acid	Erucic acid
6.	1,10,11-Trihydroxyundecane	Undecenyl alcohol
7.	1,9,10-Trihydroxyoctadecane	Oleyl alcohol
8.	1,9,10,12-Tetrahydroxyoctadecane	Ricinoleyl alcohol
	(ii) with cold alkaline permanganate	
9.	*erythro*-9,10-Dihydroxystearic acid	Oleic acid
10.	*erythro*-13,14-Dihydroxydocosanoic acid	Erucic acid
	C. Formoxylation	
11.	1,10-Dihydroxyundecane	Undecenyl alcohol
12.	1,9(10)-Dihydroxyoctadecane	Oleyl alcohol
	D. Esterification	
13.	Methyl *threo*-9,10-dihydroxystearate	*threo*-9,10-Dihydroxystearic acid
	E. Hydrogenation	
14.	Stearyl alcohol	Oleyl alcohol
15.	1,12-Dihydroxyoctadecane	Ricinoleyl alcohol
16.	Methyl 12-hydroxystearate	Methyl ricinoleate
	F. Epoxidation	
17.	*cis*-9,10-Epoxystearic acid	Oleic acid
18.	Methyl *cis*-9,10-epoxystearate	Methyl oleate
19.	*cis*-9,10-Epoxystearyl alcohol	Oleyl alcohol
20.	*cis*-13,14-Epoxydocosanoic acid	Erucic acid
21.	*trans*-13,14-Epoxydocosanoic acid	Brassidic acid
	G. Hydrohalogenation	
22.	*threo*-13,14-Chlorohydroxydocosanoic acid	*cis*-13,14-Epoxydocosanoic acid
23.	*erythro*-13,14-Chlorohydroxydocosanoic acid	*trans*-13,14-Epoxydocosanoic acid

for development. The solvent systems consisted of mixtures of ether and petroleum ether (40–60°) in varying proportions (by volume); for the development of fatty acids, the solvent system contained in addition 1 % of glacial acetic acid to avoid smearing. Only the proportion of ether in the system is mentioned subsequently. Usually 40–60 min were required for the solvent front to cover a distance of 15 cm. The plates were then sprayed with 50 % (v/v) sulphuric acid and kept for 15 min at 105–110°. The organic compounds showed up as brown spots on a white background. The separations were reproducible without difficulty.

RESULTS AND DISCUSSION

The three sections in Figs. 1 and 2 each depict a single run. For purposes of comparison, the same compound was sometimes included in more than one TLC run.

Fig. 1B shows that a clear separation of mono-, di-, tri- and tetrahydroxyoctadecanes could be achieved by means of 80% ether in petroleum ether. This system was selected after trying a 10% ether system, which caused only the monohydroxy compounds to move, and a 30% ether system with which only dihydroxy compounds moved from the starting point. In a later trial (item *q*, Fig. 1C), tetrahydroxyoctadecane separated into two discrete spots when developed with a 90% ether system. Our subsequent work suggests that this may be caused by the presence of both *erythro* and *threo* components.

Separation of a C_{11} series containing one, two and three hydroxy groups could also be achieved with the 80% ether system. The R_F value of an undecane was lower

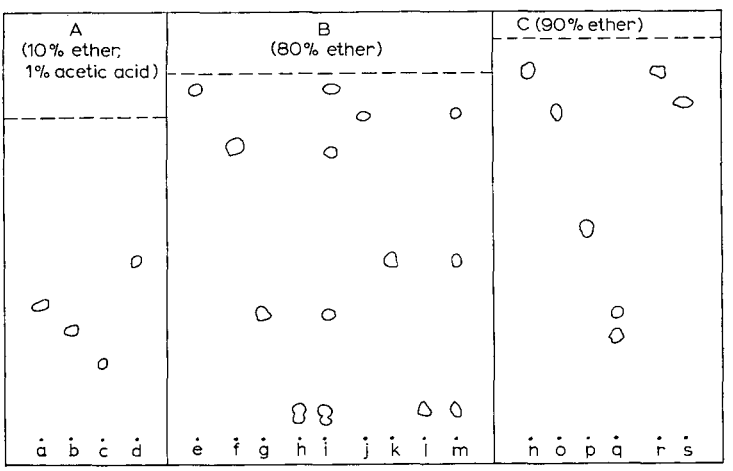

Fig. 1. Separation by thin-layer chromatography of oxygenated derivatives of fatty acids, esters and alcohols, using 10%, 80% and 90% ether–petroleum ether systems (– – – solvent front).

		$R_F \times 100$
a	*threo*-13,14-Chlorohydroxydocosanoic acid	42
b	*erythro*-13,14-Chlorohydroxydocosanoic acid	34
c	Methyl 12-hydroxystearate	24
d	*cis*-13,14-Epoxydocosanoic acid	56
e	Stearyl alcohol	96
f	1,12-Dihydroxyoctadecane	81
g	1,9,10-Trihydroxyoctadecane	35
h	1,9,10,12-Tetrahydroxyoctadecane	07
i	Mixture of *e*, *f*, *g* and *h*	96, 81, 35, 07
j	Undecenyl alcohol	89
k	1,10-Dihydroxyundecane	49
l	1,10,11-Trihydroxyundecane	08
m	Mixture of *f*, *k* and *l*	89, 49, 08
n	Stearyl alcohol	93
o	1,12-Dihydroxyoctadecane	82
p	1,9,10-Trihydroxyoctadecane	53
q	1,9,10,12-Tetrahydroxyoctadecane	34, 26
r	Oleyl alcohol	93
s	Ricinoleyl alcohol	83

than that of an octadecane containing the same number of hydroxy groups; trihydroxyundecane happened to have an R_F value similar to that of tetrahydroxyoctadecane (Fig. 1B). Small differences in chain length were not distinguishable, *e.g.* cetyl alcohol was inseparable from stearyl alcohol (item *c*, Fig. 2A).

Unsaturation had no perceptible effect on the mobility of hydroxy compounds; oleyl alcohol and stearyl alcohol had the same R_F value (items *r* and *n*, Fig. 1C).

The presence of the hydroxy group in the middle or at the end of a chain, *i.e.* whether the alcohol is secondary or primary, made little difference to the R_F value, as shown by the similar mobilities of (a) methyl 12-hydroxystearate and stearyl alco-

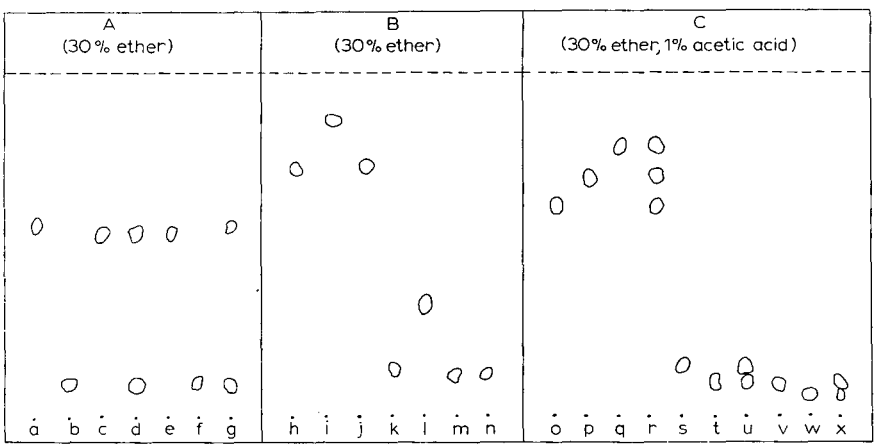

Fig. 2. Separation by thin-layer chromatography of oxygenated derivatives of fatty acids, esters and alcohols, using a 30% ether–petroleum ether system (– – – solvent front).

		$R_F \times 100$
a	Oleyl alcohol	55
b	Ricinoleyl alcohol	10
c	Mixture of 35% cetyl and 65% stearyl alcohols	52
d	Mixture of *a*, *b* and *c*	52, 10
e	Methyl 12-hydroxystearate	52
f	Methyl *threo*-9,10-dihydroxystearate	10
g	Mixture of *e* and *f*	52, 10
h	Stearyl alcohol	72
i	Methyl *cis*-9,10-epoxystearate	86
j	Methyl 12-hydroxystearate	72
k	1,12-Dihydroxyoctadecane	12
l	*cis*-9,10-Epoxystearylalcohol	32
m	Methyl *threo*-9,10-dihydroxystearate	11
n	1,9(10)-Dihydroxyoctadecane	11
o	*cis*-9,10-Epoxystearic acid	61
p	*cis*-13,14-Epoxydocosanoic acid	69
q	*trans*-13,14-Epoxydocosanoic acid	79
r	Mixture of *o*, *p* and *q*	61, 69, 79
s	*threo*-13,14-Dihydroxydocosanoic acid	14
t	*erythro*-13,14-Dihydroxydocosanoic acid	09
u	Mixture of *s* and *t*	14, 09
v	*threo*-9,10-Dihydroxystearic acid	09
w	*erythro*-9,10-Dihydroxystearic acid	06
x	Mixture of *v* and *w*	09, 06

hol (items *j* and *h*, Fig. 2B), (b) ricinoleyl alcohol and methyl 9,10-dihydroxystearate (items *b* and *f*, Fig. 2A), and (c) 1,9 (10)-dihydroxyoctadecane and methyl *threo*-9,10-dihydroxystearate (items *n* and *m*, Fig. 2B).

One epoxy group conferred greater mobility, and hence a higher R_F value, than one hydroxy group. Thus *cis*-9,10-epoxystearyl alcohol, carrying one epoxy and one hydroxyl group, had a higher R_F value than either 1,12-dihydroxyoctadecane or methyl *threo*-9,10-dihydroxystearate, which both contain two hydroxyl groups (items *l*, *k* and *m*, Fig. 2B).

The separation of *cis*- from *trans*-13,14-epoxydocosanoic acid was achieved with 30 % ether in petroleum ether containing 1 % acetic acid. A mixture of *cis*-9,10-epoxystearic acid, *cis*-13,14-epoxydocosanoic acid and *trans*-13,14-epoxydocosanoic acid was clearly resolved into the three components (item *r*, Fig. 2C). *threo*-13,14-Dihydroxydocosanoic acid was separable from the corresponding *erythro* acid, which had a lower R_F value. Similarly, resolution was achieved of *threo*- and *erythro*-9,10-dihydroxystearic acids (items *s*, *t* and *v*, *w*, Fig. 2C). 13,14-Dihydroxydocosanoic acid had an R_F value sufficiently different from that of 9,10-dihydroxystearic acid to distinguish the two compounds; in analogy to other separations mentioned in this paper, a solvent system containing a higher proportion of ether will undoubtedly result in further movement and sharper resolution.

Epoxy compounds moved faster than the halohydrins derived from them, and in turn, these halohydrins had a higher R_F value than the monohydroxy compounds (Fig. 1A). Among the halohydrins, the *threo* derivatives had a slightly higher R_F value than the corresponding *erythro* derivatives. 13,14-Chlorohydroxy-, 13,14-bromohydroxy- and 13,14-iodohydroxydocosanoic acids, of which the chromatograms are not shown, could not be distinguished from each other by TLC.

TLC therefore offers an excellent method for the rapid identification and separation of compounds of the types described above.

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It is a pleasure to thank Dr. G. S. SIDHU and his colleagues, Miss A. BHRAMARAMBA and Mr. MOHD. SWALEH, for help in using the TLC technique.

SUMMARY

Thin-layer chromatography was applied to the separation of various types of fatty compounds, using mixtures of ether and petroleum ether, of varying proportions as solvent systems. The following separations were obtained (the values in parentheses indicate the proportion of ether in the solvent system): (a) compounds of the same chain length differing in the number of hydroxy groups (80 %); (b) compounds differing in chain length by about four or more carbon atoms (80 %); (c) compounds with an epoxy group from the corresponding compound with a hydroxy group or a halohydroxy group; and (d) *cis* isomers from *trans* isomers (30 %).

Thin-layer chromatography could not be used to separate (a) compounds differing only in degree of saturation; (b) compounds with primary hydroxyl groups from similar compounds with secondary hydroxyl groups; (c) chloro-, bromo- and iodohydrins from each other.

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CHROMATOGRAPHY OF SERUM LIPID FRACTIONS ON A THIN LAYER OF Al_2O_3

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Chromatography on a thin layer of silica gel with a binder introduced by STAHL[1–6], was used with satisfactory results for the separation of lipids from human blood serum[7], brain[8] and various oils and fats[9]. Silica gel layers impregnated with silicon permitted the separation of homologues of higher fatty acids and their esters respectively[10]. Recently MOTTIER's method[11] has been employed more extensively, where for the separation of different substances a thin layer of Al_2O_3 without any binder is used (so-called spread-layer chromatography).

In the present work the method of spread-layer chromatography with Al_2O_3 as adsorbent is used for the quantitative separation of lipids from human serum.

EXPERIMENTAL

Materials

Aluminium oxide, analytical grade (Lachema, n.e., ČSSR), washed with distilled water and dried, was used. Its pH was adjusted with hydrochloric acid to 4 and the activity (according to BROCKMANN[12]) to IV.

As standards the following preparations were used: cholesterol (Chemofarma, n.e., ČSSR), cholesterol palmitate (Merck, GFR), palmitic acid (Lachema, n.e., ČSSR), crude lecithin purified ten times by repeated precipitation according to JOHNSON *et al.*[13]. The above substances were dissolved in chloroform and applied to the chromatoplates in amounts of 1 mg (in palmitic acid 1 mmole). Further BLOOR's[14] extract from 50 ml human serum was used. The extract was evaporated and re-extracted with 50 ml petroleum ether (b.p. 60°). For estimations 1 ml of this extract was always used. The other chemical substances used were analytical grade with the exception of heptane which was purified by distillation.

Preparation of the spread layer. Aluminium oxide was poured on a glass plate 16 cm × 23 cm and was smoothed with a rubber-tipped rod to form a homogeneous layer 1–1.5 mm thick. The chromatograms were developed in glass chambers by the ascending method at an angle of 30°.

Method

Two cm from the edge of the chromatogram a mixture of standards and samples to be analysed was applied in the form of a strip. The chromatograms were developed in a system of petroleum ether–ether (95:5). Afterwards the chromatoplates were removed and dried at room temperature. The detection was carried out either under

ultraviolet light at 365 mμ or by spraying the chromatogram with a solution of bromthymol blue[8]. In this system cholesterol esters and triglycerides were separated. Phospholipids, fatty acids and cholesterol remained at the start. Cholesterol ester fractions and triglyceride fractions were removed from the layer by a vacuum pump and eluted from the adsorbent[15]. The cholesterol esters were eluted with 4 ml chloroform and the absorbent rinsed with 3 × 2 ml chloroform. The combined eluates were evaporated to dryness and cholesterol was estimated by ABELL's method[16]. Triglycerides were similarly eluted with ethanol–ether (1:1). In the residue after evaporation triglycerides were estimated using STERN AND SHAPIRO's method[17] or they were first subjected to hydrolysis with 50% KOH and after neutralisation the liberated fatty acids were estimated by TROUT's method[18]. (It cannot be ruled out that in the triglyceride fraction mono- and diglycerides were also present.)

From the remaining chromatoplate part of the Al_2O_3 layer was removed from the site of the triglyceride fraction to the front. The surface of the glass plate was supplemented by new aluminium oxide and smoothed to form a homogeneous layer.

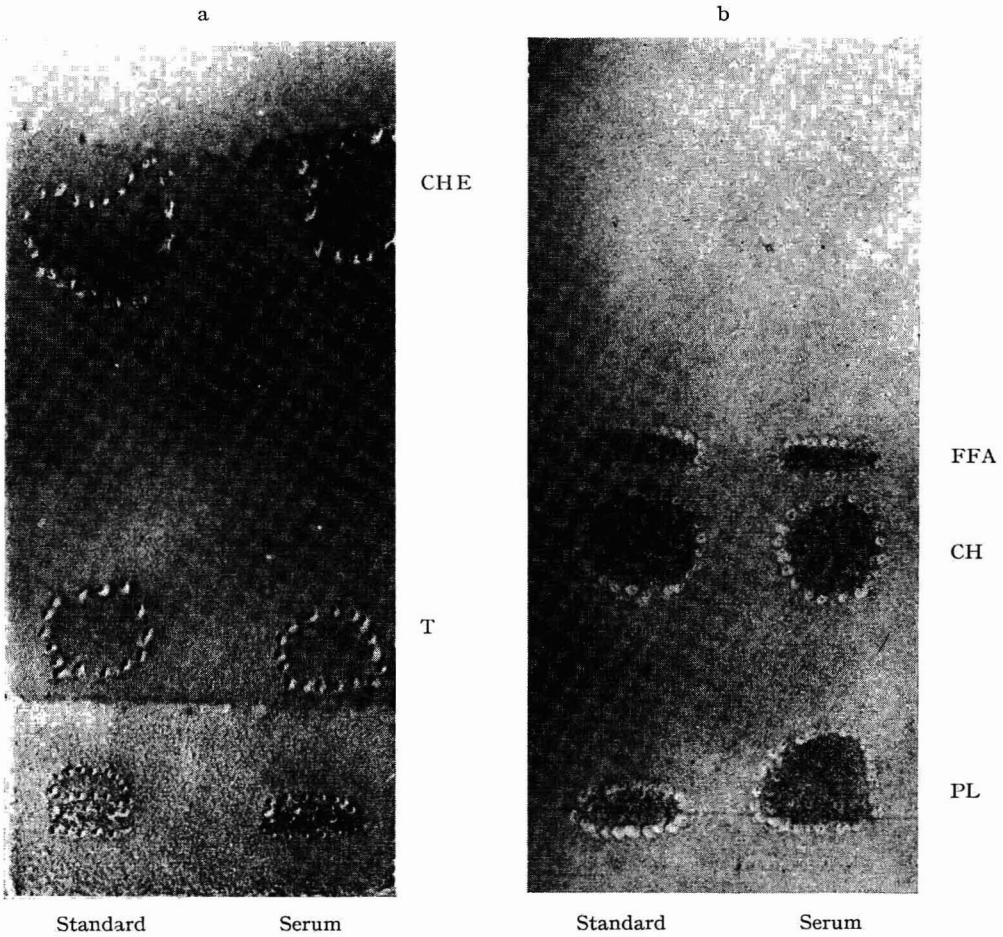

Fig. 1. Samples of chromatograms. (a) System 1: petroleum ether–ether (95:5). (b) System 2: petroleum ether–ether–acetic acid (94.5:5:0.5).

The chromatoplate prepared in this manner was developed in a system of petroleum ether–ether–acetic acid (94.5:5:0.5)*. The subsequent procedure was the same as above. In this system fatty acids, cholesterol and phospholipids were separated. The fractions were removed by a vacuum pump from the layer and eluted. Cholesterol was eluted and estimated as for the cholesterol esters. Fatty acids were eluted by the extraction mixture according to TROUT *et al.*, with sulphuric acid and water and estimated by titration. Phospholipids were subjected to hydrolysis (as in the case of the triglycerides) and the liberated fatty acids were estimated also by TROUT's method.

The separation of lipid fractions in the above systems is apparent from the photographs (Fig. 1).

RESULTS

In the tables individual lipid fractions are given. The following abbreviations are used:

CH = cholesterol	FFA = free fatty acids
CHE = cholesterol esters	PL = phospholipids
T = triglycerides	FA = total fatty acids

The R_F values of lipid fractions in individual systems are summarized in Table I. In the above method the recovery estimated is given in Table II.

TABLE I

COMPOSITION OF SOLVENT SYSTEMS AND R_F VALUES OF LIPID FRACTIONS

(Activity of Al_2O_3 IV, pH 4)

Solvent system	*Composition*	R_F				
		CH	*CHE*	*T*	*FFA*	*PL*
Petroleum ether–ether	95:5	0	0.91	0.30	0	0
Petroleum ether–ethanol	98:2	0.10	0.87	0.38	0.06	0
Petroleum ether–ether–acetic acid*	94.5:5:0.5	0.35	—	—	0.47	0
Heptane–acetic acid	98:2	0.29	—	—	0.37	0

TABLE II

RECOVERY OF LIPID FRACTIONS AFTER CHROMATOGRAPHY ON A SPREAD LAYER OF Al_2O_3

Sample	*Lipid fractions in percentage by weight*				
	CH	*CHE*	*T*	*FFA*	*PL*
1	96	97	99	93	105
2	96	98	102	113	102
3	97	95	105	81	102
4	93	103	101	91	94
5	94	89	101	103	105
6				111	
7				101	
8				101	
9				101	
Average	95	96	102	98	102

* The composition of this system must be modified for some commercial types of Al_2O_3. It proved useful to raise the ether content and the acetic acid content.

In Table III the values of lipid fractions from BLOOR's extract corresponding to 1 ml of serum are given.

The values of fatty acids in individual lipid components after fractionation were compared with the total value before fractionation (Table IV).

The method presented gives results which are in satisfactory agreement with the data of MAN AND ALBRINK[19] (Fig. 2).

TABLE III

CONTENT OF LIPID FRACTIONS IN EXTRACT FROM SERUM AFTER SEPARATION ON A SPREAD LAYER OF Al_2O_3

Sample	*Serum lipid fractions*				
	CH (mg)	*CHE (mg)*	*T (mg)*	*FFA (mequiv.)*	*PL (mequiv.)*
1	0.64	1.54	0.68	0.63	6.41
2	0.62	1.46	0.96	0.61	8.10
3	0.69	1.47	1.22	0.59	7.30
4	0.86	1.41	0.87	0.69	7.68
5	0.66	1.48		0.86	5.84
6	0.65	1.32		0.67	6.00
7	0.70	1.35		0.75	5.84
8	0.59	1.35		0.55	5.75
9	0.69	1.49		0.76	5.63
10		1.46			5.22
Average	0.66	1.43	0.93	0.68	6.38
Standard deviation	0.03	0.07	0.19	0.09	0.92

TABLE IV

COMPARISON OF LIPID COMPONENTS AFTER FRACTIONATION AND THEIR SUM WITHOUT FRACTIONATION (FA = 100%)

Lipid fraction	*Content of fraction*	
	mequiv.	*Percentage*
CHE	2.31	17.0
T	3.61	26.5
FFA	0.68	5.0
PL	6.38	46.9
Sum	12.98	95.4
FA	13.60	100.0

DISCUSSION

A method of chromatographic separation of lipid fractions which is rapid, simple and reliable and has sufficient separating capacity is still the object of research. In the paper presented, the authors describe optimal conditions for the use of Al_2O_3 for the separation of lipid fractions. Under the conditions described above (pH, activity of Al_2O_3) aluminium oxide is well suited for this purpose. Spread layers without a binder have some advantages as compared with thin layers with a binder. These include the easy and rapid preparation of chromatoplates, rapid development, rapid drying after

development and easy removal of the adsorbent with the separated fractions for elution. The easy preparation of the homogeneous layer makes it possible to remove the part of the layer containing the components separated after first development and to substitute a new layer of adsorbent. During development with a second system on a chromatoplate thus renewed the remaining components are separated.

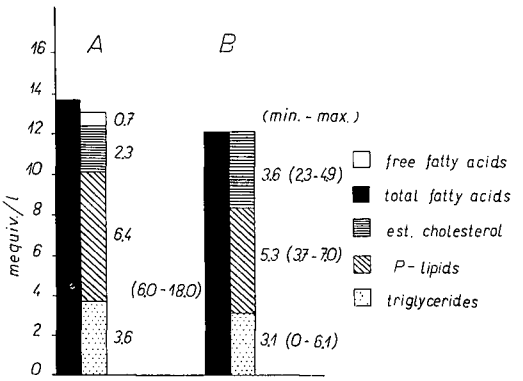

Fig. 2. Comparison of values of lipid components in human serum obtained (A) by the present method, (B) with values obtained by MAN AND ALBRINK.

The results obtained showed that by this method it is possible to achieve rapid quantitative separation of all lipid components of human serum. The recovery of this method is within the same range as that of other chromatographic methods. From Table IV it is apparent that the difference between the sum of fatty acids in individual lipid fractions and the total fatty acid content of the original extract is 4.6%. Comparison of the content of lipid fractions after separation on a spread layer with their estimation directly in the extract is usually not possible in view of the non-specificness of common analytical methods. Results obtained by the method submitted are in good agreement with the results obtained by a specific analytical method, where such a method exists (*e.g.* determination of lipid phosphorus).

By using chromatography on a spread layer the interference of phospholipid in estimation of FFA (free fatty acids) is excluded.

SUMMARY

Spread-layer chromatography on Al_2O_3 was modified for the separation of lipid fractions from human blood serum. The procedure recommended can be used for the separation and quantitative estimation of lipid fractions.

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CHROMATOGRAPHISCHE TRENNUNG EINFACHER ALIPHATISCHER THIOPHOSPHORSÄUREESTER MITTELS DÜNNSCHICHTCHROMATOGRAPHIE

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Bei Untersuchungen zur Bildungsweise von Thiophosphorsäureestern benötigten wir eine schonende, rasche und wirksame Trennmethode für diese relativ leicht flüchtigen Substanzen. Die ausgewählten Modellester erwiesen sich ausser der sauren Verbindung $(C_2H_5O)_2PSSH$ wie viele Phosphatinsektizide als stark hydrophob und wanderten selbst auf mit Dimethylformamid imprägniertem Papier in die Nähe der Laufmittelfront. Alkohole und Wasser im Laufmittel wollten wir möglichst vermeiden, deshalb kam eine Umkehrung der Phasen nicht in Frage[1]. Bei der Gaschromatographie, wie sie für derartige Zwecke bereits von CADOGAN und Mitarbeitern[2] benutzt wird, werden die Substanzen thermisch belastet.

Die Dünnschichtchromatographie ist inzwischen zur Trennung von Phosphatinsektiziden[3] und für deren Abbauprodukte[4] verwendet worden. Das Verfahren schien im Hinblick auf die Flüchtigkeit der Substanzen besonders vorteilhaft durch die wesentliche kürzere Laufzeit und durch die grössere Kapazität der Schicht gegenüber normalen Chromatographiepapieren. Nach Vorversuchen über das Schichtmaterial benutzten wir Aluminiumoxid als Adsorbens und das schon von MEINHARD UND HALL[5] verwendete Bindemittel Stärke. Diese Autoren verkleisterten die Stärke vor dem Aufstreichen. KIRCHNER, MILLER UND KELLER[6] beschrieben derartige Schichten als unterschiedlich aktiv je nach Trocknungsart und Verweildauer an atmosphärischer Luft; STAHL[7] dagegen fand sie bei sehr unterschiedlicher Trocknungsart mit Kieselsäure als Adsorbens als gleich aktiv wie dasselbe SiO_2 ohne jedes Bindemittel.

Wir mischten Stärke und Adsorbens wie SEILER[8] trocken miteinander, rührten mit Wasser an, liessen aber die aufgetragene Schicht nicht vor dem Aktivieren antrocknen, sondern brachten sie nass in einen vorher auf 120° geheizten Trockenschrank. Auf diese Weise verkleistert anscheinend die Stärke noch, ehe das Wasser vollständig verdampft ist, und man erhält prächtige harte Schichten. Sie lassen sich mit Bleistift beschriften und verlieren nur wenig an Aktivität selbst bei längerem ungeschützten Liegen an der Luft. Beim Sichtbarmachen der Ester in der von uns[9] beschriebenen Weise mit Perchlor- und Perjodsäure braucht die besprühte Platte nur in Wasser gebracht zu werden, und es erscheinen blaue Flecke (anstatt der wesentlich schlechter sichtbaren Jodabscheidungen). Anschliessend kann man die Platten an der Luft trocknen lassen. Sie bleichen etwas aus, kräftige Färbungen bleiben jedoch noch wochenlang erkennbar.

Die Leistungsfähigkeit der Methode sei an drei Beispielen demonstriert.

(1) Die Trennung der von KABATSCHNIK UND MASTRJUKOWA[10] als Einwirkungsprodukte des Äthylalkohols auf niedere Phosphorsulfide festgestellten drei Ester $(C_2H_5O)_2PSSH$, $(C_2H_5O)_2PS(SC_2H_5)$ und $(C_2H_5O)_2PSH$ ist leicht möglich (Fig. 1).

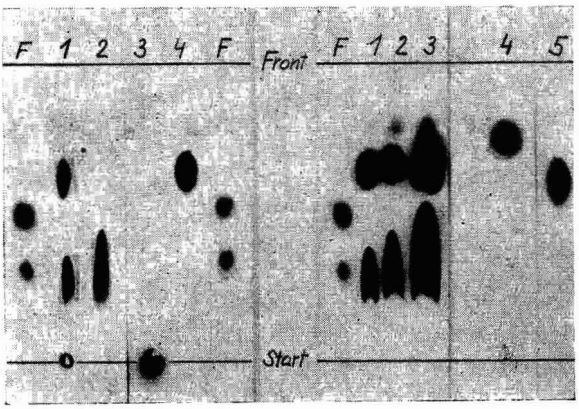

Fig. 1. Fig. 2.

Fig. 1. Laufmittel *n*-Heptan–Aceton (10:1, v/v); Strecke Start–Front 14 cm; Laufzeit 12 Min. F = Testfarbstoffe; unterer Fleck Sudanrot G, oberer Fleck Buttergelb. 1 = Gemisch; 2 = $(C_2H_5O)_2PSH$; 3 = $(C_2H_5O)_2PSSH$; 4 = $(C_2H_5O)_2PS(SC_2H_5)$.

Fig. 2. Laufmittel *n*-Heptan–Aceton (10:1, v/v); Strecke Start–Front 14 cm; Laufzeit 12 Min. F = Testfarbstoffe; unterer Fleck Sudanrot G, oberer Fleck Buttergelb. 1, 2, 3 = das fragliche $(C_2H_5O)_2PO(SC_2H_5)$ in verschiedenen Mengen aufgetragen; 4 = $(C_2H_5)_2S$; 5 = $(C_2H_5O)_3PS$.

(2) Das Chromatogramm eines zweifelhaften Präparates, bezeichnet als Triäthylthiolphosphat, Kp. bei 115°/18–20 mm Hg (Fig. 2), gewonnen analog zur Vorschrift von HILGETAG UND TEICHMANN[11] für den entsprechenden Methylester aus äthanolischer Natronlauge, Phosphorthiochlorid und Diäthylsulfat, zeigt ausser dem

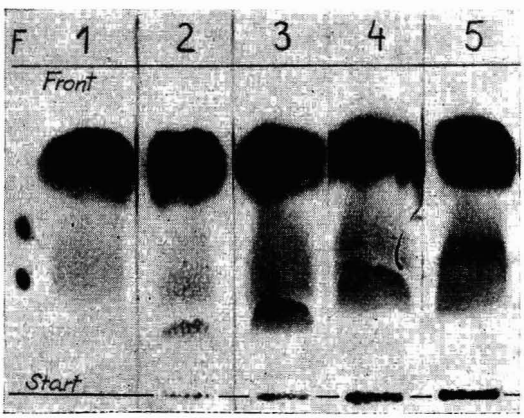

Fig. 3. Laufmittel *n*-Heptan–Aceton (10:1, v/v); Strecke Start–Front 14 cm; Laufzeit 12 Min. F = Testfarbstoffe; unterer Fleck Sudanrot G, oberer Fleck Buttergelb. 1 = $C_2H_5PS(OC_2H_5)_2$ vor dem Erhitzen; 2, 3, 4, 5 = Proben, die nach 1, 2, 3, 4 Stunden Kochen der Substanz mit dem gleichen Volumen C_2H_5I entnommen wurden.

langgezogenen Hauptfleck zwei Verunreinigungen an. Die am weitesten gewanderte Substanz dürfte Diäthylsulfid sein, ihr folgt mit kurzem Abstand Triäthylthionophosphat. Bei der Beurteilung der Flecke muss man beachten, dass der Thiolester bedeutend schlechter auf das Sprühreagenz anspricht als der entsprechende Thionoester[9].

(3) Die Isomerisierung des Diäthylesters der Äthylthionophosphonsäure kann leicht sichtbar gemacht werden (Fig. 3). KABATSCHNIK UND MASTRJUKOWA[12] erhielten den entsprechenden Thiolester in 33 % Ausbeute nach dreistündigem Erhitzen der Thionoverbindung mit Äthyljodid im geschlossenen Rohr auf 140–150°. Wir kochten ein Gemisch gleicher Volumina Thionoester und Äthyljodid am Rückfluss und entnahmen stündlich eine Probe. Man erkennt deutlich die Zunahme der Flecke am Start (Triäthylsulfoniumjodid) und darüber (Thiolester). Bei diesem Versuch wurde (im Hinblick auf quantitative Auswertung) viel mehr Substanz als sonst aufgetragen: pro Platte 20 μl Gemisch, entsprechend etwa 10 mg Ester.

Über weitere Versuche und unsere Folgerungen berichten wir an anderer Stelle.

EXPERIMENTELLES

Herstellung der Dünnschicht

Glasplatten gleicher Dicke (20 cm lang, 4 bzw. 6 cm breit) werden auf eine etwa 18 cm breite Gummiplatte aufgelegt und mit einem nach MACHATA[13] hergestellten Gerät bestrichen (Schichtdicke 1/4 mm). Nach kurzer Übung erhält man damit durchaus zufriedenstellende Schichten.

1.5 g Weizenstärke und 28.5 g Aluminiumoxid werden im Mörser gut verrieben und danach mit destilliertem Wasser versetzt, bis ein dünner Brei entsteht. Die gut durchgerührte Masse wird in den Streicher gefüllt und sofort verstrichen. Die nötige Wassermenge schwankt etwas je nach Herkunft des Al_2O_3. Die Platten für die Abbildungen wurden mit einem Al_2O_3 der Firma Merck, Darmstadt hergestellt, dessen wässriger Auszug schwach alkalisch reagierte. Für 30 g Gemisch benötigten wir 35 ml Wasser und erhielten daraus etwa 900 cm^2 bestrichene Fläche.

Unmittelbar nach dem Streichen legt man die Platten für eine Stunde in den auf 120° vorgeheizten Trockenschrank. Bei nur 100° Trockentemperatur können die Schichten bereits bis zur Unverwendbarkeit lose sein.

Aktivität der Dünnschicht

Zum Vergleich mit anderen Schichten trennten wir nach dem Vorschlag von STAHL[14] ein Testfarbstoffgemisch mit Benzol als Laufmittel. Bei einer Strecke Start–Front von 10 cm (durchlaufen in 8 Min) zeigten die Flecke folgende R_F-Werte: 0.38 (Indophenol), 0.51 (Sudanrot G) und 0.70 (Buttergelb).

Die verhältnismässig geringe Aktivitätsabnahme der Schicht bei längerem Lagern an Luft ohne Feuchtigkeitsabschluss wird deutlich, wenn man die Lage der Farbflecke auf den Abbildungen vergleicht. Die Schicht für Fig. 3 lag vor Gebrauch 20 Stunden lang ungeschützt im Zimmer, für die Fig. 1 und 2 dienten frisch hergestellte und eben ausgekühlte Platten.

Laufmittel

Nach zahlreichen Vorversuchen fanden wir ein Gemisch von *n*-Heptan + Aceton 10 + 1 (Volumteile) für unsere Bedürfnisse gut geeignet. Dieses Laufmittel trennt Indophenol und Sudanrot G nur schlecht (blauer Fleck sitzt unmittelbar oberhalb des roten), deshalb verwendeten wir nur noch Buttergelb und Sudanrot G als Testfarbstoffe. Der Acetongehalt des Laufmittels sinkt bei Gebrauch ab, die Farbstoffflecke nähern sich dem Start. Filtrierpapierstreifen an der Wandung beschleunigen die Sättigung der Kammeratmosphäre mit den Dämpfen der Laufmittel.

Bei allen Versuchen bewegte sich das Laufmittel aufsteigend.

Sichtbarmachen der Flecke

Sprühreagenz: 10 g H_5IO_6 p. a. in 100 ml 70%iger $HClO_4$ p. a. lösen, dazu einige mg V_2O_5. Vor Gebrauch kurz aufschütteln.

Das Gemisch ist äusserst aggressiv und hat für flüchtige Substanzen den grossen Vorteil, dass man die Chromatogramme noch laufmittelfeucht damit ansprühen kann. Gleichzeitig mit dem Sichtbarmachen beginnt der Abbau der Ester zum Phosphat. Die weitere Einwirkung des Gemisches im Sinne von SMITH UND DIEHL[15] zur Nassveraschung bereitet unmittelbar die quantitative Bestimmung des Phosphors in den Flecken vor.

ZUSAMMENFASSUNG

Die Trennung organischer Phosphorschwefelverbindungen durch Dünnschichtchromatographie an Aluminiumoxid mit etwas Stärke als Bindemittel wird beschrieben. Die angegebenen Beispiele sind von Reaktionsgemischen genommen, die bei der Herstellung der Ester von Thiosäuren des Phosphors auftreten.

SUMMARY

The separation of organic phosphorus sulphur compounds by thin-layer chromatography on alumina with some starch is described. Examples are taken from reaction mixtures occurring in the preparation of phosphorus thioesters.

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THIN-LAYER CHROMATOGRAPHY USING THE DESCENDING TECHNIQUE WITH NON-BOUND ALUMINA PLATES

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Thin-layer chromatography on non-bound alumina has recently been proved as a very useful method for rapid analytical and quantitative separation of various mixtures[1–4]. But as far as separation for preparative purposes is concerned, this method suffers one disadvantage, namely the necessity to apply too great an excess of alumina to make it practicable for chromatography of intermediate quantities of mixtures. The reason is that, with solvent systems of great elution power, alumina displays lower adsorption capacity (as is obvious from the higher R_F values) and with the less polar solvents the R_F values are too low to permit effective separation of zones in a single run. This disadvantage is overcome as shown below by applying a flow of solvent (as in column chromatography) which permits effective separation and more effective use of adsorbent. Moreover, when the thin layer (0.4–0.5 mm) is applied, this descending technique gives good analytical separation of compounds otherwise inseparable by the usual one-run method[4]. The idea is somewhat similar to that of BRENNER AND NIEDERWIESER[5], except that the chromatograms are run in a closed tank and a solvent flow is secured not by evaporation but by gravity forces.

EXPERIMENTAL

The simple device for descending plate chromatography consists of a development chamber, usually a shallow round dish (a, Fig. 1) 34 cm in diameter and 10 cm in height with a ground cover, a solvent tank (b) 24 × 3.5 × 3 cm in size which is placed as high as the cover of the chamber permits (for the given size of dish, 4 cm from the bottom), and a frame (c). The frame (c) serves as a support for the glass plate (d) (24 × 24 cm) when alumina is being spread and samples placed, and for securing the solvent flow from the solvent tank into the chromatographic chamber. The frame (c) can be made of any sort of material of sufficient corrosive resistance; aluminium proved quite satisfactory for all solvent systems using alumina. Two modifications of a "pumping" system were tried, the first with a strip of filter paper inserted into the upper and lower slits (e), the second with the width of the slits (e) being carefully regulated to ensure the rise of the solvent by capillary forces up to the alumina layer and down by gravity into the chamber (0.1–0.2 mm). Both systems are equally satisfactory.

The chromatography is carried out as follows. The glass plate (d) is placed on the frame (c). The upper and lower planks (f and g) are fixed at a height which will give the desired thickness of alumina layer (the play between the edges of the glass plate and

the side-pieces (f) and (g) should not exceed 0.2–0.5 mm), and a uniform layer of alumina is spread with the help of the simple device (h) (or by a glass rod thickened at the ends) 10–15 mm from the upper edge of the plate. The margin so formed is filled with alumina (3–4 ml) on which the sample is first adsorbed (by evaporation of the mixture of compounds, alumina and appropriate solvent on the film evaporator).

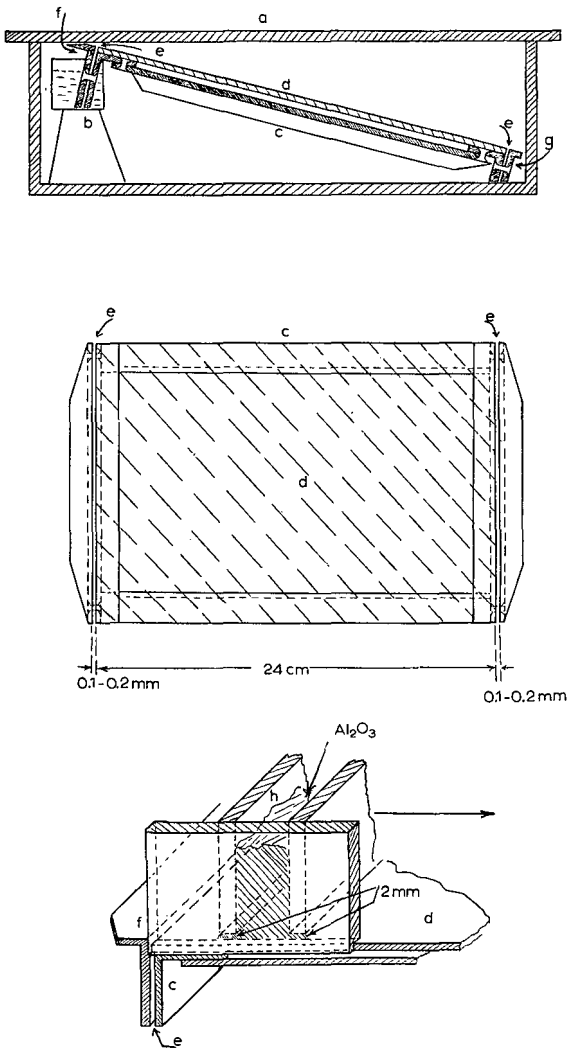

Fig. 1. Chromatographic chamber and its arrangement.

To secure compact zones on the chromatogram, the Al_2O_3 with adsorbed compound should be spread uniformly (spreading by spatula is usually satisfactory). The chromatoplate so prepared is placed with the frame in the chamber (a) with the side-piece (f) dipping into the solvent tank, and the lower end of the frame (g) resting on the bottom of the chamber.

The quantity of sample and the solvent system are determined by preliminary runs on the analytical plates[4], and it is quite obvious that for compounds of close R_F values the charge should be lower and the solvent system less polar than for great R_F differences. For separation of compounds of close R_F values better results are obtained by the method of placing the sample adopted in column chromatography, *i.e.* a solution of the mixture in solvent of the lowest possible polarity is poured into the solvent tank (b) and when all solvent has been drained out, the tank is filled with the developing mixture. For good separation it is sometimes necessary to recirculate the solvent, or to wash out of the plate some components of the mixture. A combination of data from analytical chromatograms and flow-rate determinations for the given system should be carefully considered, especially for compounds invisible under U.V. light. For such compounds detection of the zone position is made either by spraying with an appropriate reagent[2,3] or by iodine vapour. In the latter case, a slow flow of moist air saturated with iodine vapours is used and a narrow band so "painted" along the plate is then examined under U.V. light*.

RESULTS

A typical example of separation is given below. A mixture of two isomeric alcohols (R_F 0.25 and 0.48 on alumina, activity II, in chloroform–acetone, 3:1), prepared by $NaBH_4$ reduction of 0.71 g N-benzoyl-4-keto-*trans*-decahydroquinoline (R_F 0.85), and 4 ml of alumina was spread in a uniform layer at the upper end of the plate with a layer of alumina 2 mm thick (activity II, ~ 80 c.c.) Development of the chromatogram with chloroform (200 ml) was watched under U.V. light and stopped when the zone of one isomeric alcohol reached the end of the plate (traces of the starting material at this moment were washed out). After drying, the zones were collected on a sintered glass suction filter (closed by a cork with an inlet tube of 1–2 mm diameter), using a water pump. The adsorbed material was extracted with methanol and 0.59 g of one isomer (m.p. 104°) and 0.14 g of the other isomer (m.p. 140°) of N-benzoyl-4-hydroxydecahydroquinoline were obtained.

SUMMARY

A convenient procedure for a descending method of analytical and preparative scale thin-layer chromatography on non-bound alumina is described.

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* Good results were obtained by detecting the zones with filter paper "prints"—narrow filter paper strips (5–6 mm) which are gently pressed against the wet chromatograms and then treated with an appropriate reagent.

THIN-LAYER CHROMATOGRAPHY OF SOME STRONGLY ADSORBED AMINES ON NON-BOUND ALUMINA PLATES

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Thin-layer (or column) chromatography of basic compounds such as secondary or primary amines on alumina presents great difficulties because of the strong adsorption affinity of the N-H group. Even solvents of great elution power do not give compact spots or zones on chromatograms. Moreover, it was noticed that these solvents (alcohols and lower ketones) were somewhat inferior to less polar mixtures, since solvents miscible with water enhance adsorption through the increase of adsorbent activity by dehydration. The obvious solution is the introduction of water into such solvent systems; in some instances, in fact (see below), addition of water does improve the results considerably and produces very compact spots. Another approach is to try to use competitive adsorption of some lower amines which are components of the elution system. Generally speaking, any amine may be used for the purpose but the solvent systems containing ammonia proved most effective. The effect of lower aliphatic amines is similar but their application is not so practicable for thin-layer chromatography because of too slow desorption. This slow desorption produces a coloured background on detection, especially by iodine vapour–U.V. technique,

TABLE I

No.	Compound	R_F values in					
		S_1	S_2	S_3	S_4	S_5	S_6
1	*trans*-Decahydroquinoline (I)	0.75 (e)	—	—	0.63 (c)	0.74 (c)	0.25 (et)
2	*cis*-Decahydroquinoline (II)	0.64 (e)	—	—	0.63 (e)	0.74 (c)	0.27 (e)
3	4-Hydroxy-I (m.p. 130°)	0.50 (c)	0.17 (e)	—	—	0.34 (c)	—
4	4-Hydroxy-I (m.p. 170°)	0.45 (c)	0.14 (e)	—	—	0.31 (c)	—
5	4-Hydroxy-II (m.p. of HCl salt 261°)	0.55 (c)	0.26 (e)	—	—	0.39 (c)	—
6	4-Hydroxy-II (m.p. of HCl salt 201°)	0.30 (c)	0.14 (e)	—	—	0.22 (c)	—
7	4-Chloro-I (m.p. 92°)	—	—	—	0.71 (c)	—	0.40 (e)
8	4-Keto-I	—	—	0.61 (c)	—	—	—
9	4-Benzoyloxy-I (m.p. of HCl salt 276°)	—	0.82 (c)	—	—	0.78 (c)	0.33 (e)
10	4-Acetoxy-I (m.p. 86°)	0.76 (c)	0.54 (c)	—	—	0.70 (c)	—
11	4-Acetoxy-I (m.p. of HCl salt 249°)	0.64 (c)	0.16 (e)	—	—	0.62 (c)	—
12	4-Acetoxy-II (m.p. of HCl salt 193°)	0.79 (c)	0.13 (e)	—	—	0.72 (c)	—
13	4-Acetoxy-II (m.p. of HCl salt 258°)	0.59 (c)	0.14 (e)	—	—	0.64 (c)	—
14	7-Hydroxy-I (?) (m.p. of HCl salt 282°)	0.48 (e)	0.2 (et)	—	—	0.23 (c)	—
15	7-Hydroxy-I (?)	0.32 (e)	—	—	—	—	—
16	O-Acetate of No. 14	—	0.67 (c)	—	—	0.67 (c)	0.20 (e)
17	N-Acetate of No. 14	—	0.58 (c)	—	—	—	—

(continued on p. 315)

TABLE I (*continued*)

No.	Compound	R_F values in					
		S_1	S_2	S_3	S_4	S_5	S_6
18	$\Delta^{8,8a}$-Octahydroquinolone-4	0.81 (c)	0.63 (c)	—	0.11 (e)	—	—
19	(1)-*cis*-Perhydropyrindine (III)	0.66 (e)	—	—	—	0.52 (c)	—
20	4-Keto-III	—	0.73 (c)	0.56 (c)	—	0.70 (c)	—
21	4-Hydroxy-III (m.p. 117°)	—	0.35 (c)	—	0.30 (c)	0.42 (c)	—
22	4-Hydroxy-III	—	0.21 (e)	—	0.20 (e)	0.35 (c)	—
23	N-Acetyl-4-acetoxy-III	—	—	0.53 (c)	—	0.69 (c)	—
24	N-Acetyl-4-hydroxy-III (m.p. 117°)	—	—	0.40 (c)	0.15 (c)	0.46 (c)	—
25	N-Benzoyl-4-hydroxy-III (m.p. 193°)	—	—	0.51 (c)	—	0.52 (c)	—
26	N-Benzoyl-4-hydroxy-III (m.p. 153°)	—	—	0.41 (c)	—	0.49 (c)	—
27	4-Hydroxy-3-methylpiperidine	0.42 (e)	0.1 (et)	—	—	—	—
28	Piperidine	—	—	0.2 (et)	—	0.55 (c)	—
29	Pyrrolidine	—	—	0.2 (et)	—	0.53 (c)	—
30	Morpholine	—	—	0.27 (et)	—	0.57 (c)	—
31	Cyclohexylamine	—	—	0.42 (et)	—	0.59 (c)	—
32	Benzylamine	—	—	0.67 (c)	—	0.64 (c)	—
33	Ethanolamine	—	—	0.2 (et)	—	0.14 (et)	—
34	Ethylenediamine	—	—	0.2 (et)	—	0.17 (et)	—

and masks the spots. Table I gives the R_F values of some nitrogen compounds on chromatograms with non-bound alumina[1], activity III, in the following solvent systems: S_1 = acetone–methanol–water (8:2:1); S_2 = methyl ethyl ketone–water (15:1); S_3 = acetone–heptane (1:1); S_4 = chloroform–NH_3 (saturated at 22°); S_5 = chloroform/NH_3–96% ethyl alcohol (30:1); S_6 = chloroform/NH_3–benzene (1:1). The type of spot is marked in Table I as follows: c = compact (round or almost so); e = elongated; et = elongated with a tail.

For reference purposes, R_F values of some N-acyl derivatives are given. All R_F values for S_3 not marked in the table are of the order 0.1–0.2 (et). Exclusion of water from S_2 lowers the R_F values and leads to tail formation, *e.g.*, for compound No. 18, the R_F value drops from 0.63 for S_2 to 0.1–0.2 (et) for dry methyl ethyl ketone. The content of alcohol in the NH_3-type systems is of great importance for the separation of geometrical isomers, *i.e.* compounds Nos. 21–22 can be distinguished in S_4 but they give practically the same R_F values in chloroform/NH_3–methanol (15:2, R_F 0.79), or isopropanol/NH_3 (satd.)–heptane (1:1, R_F 0.53), or *n*-butanol/NH_3 (satd.) (R_F 0.9).

SUMMARY

Solvent systems for the thin-layer chromatography of some strongly adsorbed amines on alumina plates are described.

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1 E. A. MISTRYUKOV, *Collection Czech. Chem. Commun.*, 26 (1961) 2071.

THE ANALYSIS OF MIXTURES OF ANIMAL AND VEGETABLE FATS

III. SEPARATION OF SOME STEROLS AND STEROL ACETATES BY THIN-LAYER CHROMATOGRAPHY*

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Small amounts of animal and vegetable fats in mutual mixtures can be detected by chromatographic analysis of their sterols. A separation of cholesterol (abbreviated formula FC27) and phytosterols, such as β-sitosterol (FC29), stigmasterol (FC29F), and campesterol (FC28), has been accomplished by reversed-phase paper chromatography in the system paraffin oil/acetic acid–water (84:16)[1]. The migration rates of these sterols as well as of several others and the relationship between structure and R_F value were studied[2]. However, a more general application of this sterol analysis to the routine procedures of biochemistry and fat chemistry is hampered by the time-consuming character of reversed-phase paper chromatography, the times of accommodation and of elution being 16 h and 44 h respectively.

At present thin-layer chromatography (TLC) is being applied to the analysis of several groups of lipoid substances (for reviews see, *e.g.*, ref.[3]). Separations in the group of sterols and related triterpenoid alcohols by TLC on layers of silicic acid were already mentioned by JANECKE[4] (cholesterol–vitamin D_3), and TSCHESCHE[5] (*e.g.* lanosterol–β-amyrin).

We have found that separations within this group of sterols and triterpenoid alcohols can also be achieved on layers of "kieselgur G" (Merck). The relative R_S values of some sterols are given in Table I.

Although some sterols that differ in the type and number of double bonds are clearly separated (*e.g.* cholesterol–ergosterol, and lanosterol–cholesterol), the separation of cholesterol from the phytosterols could not be achieved. The structural differences between these related sterols, which only consist in the presence of one or two methyl groups or a double bond in the side chain, are too small to make separations by methods based on adsorption chromatography possible.

Recently, the technique of reversed-phase TLC has been applied by KAUFMANN *et al.*[6] to the analysis of several lipoid substances, such as fatty acids, cholesterol esters, fatty alcohols, diglycerides, and even of closely related mixed triglycerides, such as palmitodiolein and tripalmitin. For the greater part the principles and results of this technique are analogous to those of reversed-phase paper chromatography, but the advantages are a tenfold reduction in the time of analysis, smaller, less diffuse spots, which permit better separations, and the possibility of spraying with

* For Parts I and II of this series, see refs. [1] and [2].

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TABLE I

R_S[a] VALUES OF SOME STEROLS ON KIESELGUR G

Mobile phase: cyclohexane–ethyl acetate (99.5:0.5). Temperature: 23°. Detection: phosphomolybdic acid. Spotted amounts: 0.6 μg. Time of run: 2–3 h.

Compound	R_S	*Compound*	R_S
Cholesterol	≡ 1.0	7-Dehydrocholesterol	0.93
β-Sitosterol	1.00	Zymosterol	1.02
Stigmasterol	1.00	Dihydrolanosterol	1.38
Dihydrocholesterol	0.93	Lanosterol	1.37
Ergosterol	0.89	Agnosterol	1.35
Vitamin D_2	1.11	Dihydroagnosterol	1.34

[a] S = cholesterol.

more agressive colour reagents. We have applied the techniques described by KAUFMANN *et al.* to the analysis of some sterols and sterol acetates.

Chromatoplates of kieselgur G (Merck) are impregnated with undecane* by dipping them into a 10% solution of this hydrocarbon in petroleum ether. After drying in the air, hexagonal holes are brushed out of the kieselgur layer, leaving four chromatograms modelled on the MATTHIAS technique (Fig. 1). Quantities of about

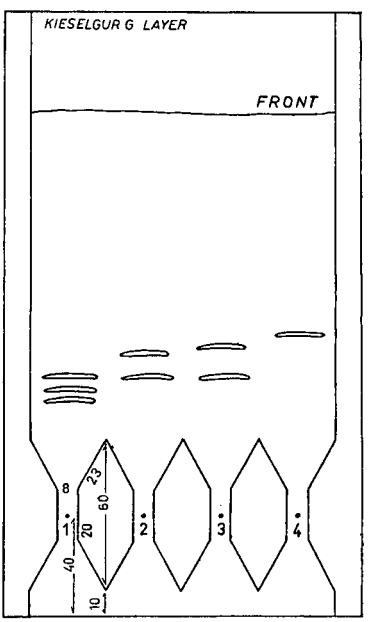

Fig. 1. Separation of some sterol acetates by reversed-phase TLC. Adsorbent: kieselgur G (Merck). Stationary phase: undecane stand. Mobile phase: acetic acid–water (92:8). Time of run: 5 h. Length of run: 20 cm. Detection: phosphomolybdic acid. Temperature: 23°. Measurements in mm. Spot 1 = 5 μg of a cholesterol acetate–soya bean oil phytosterol acetate 4:6 mixture. Spot 2 = Cholesterol acetate–epicholesterol acetate 1:1 mixture. Spot 3 = Ergosterol acetate–cholesterol acetate 1:1 mixture. Spot 4 = 7-Dehydrocholesterol acetate.

* Available from J. HALTERMANN, Hamburg.

5 μg of the sterols and sterol acetates are spotted, and finally the chromatoplate is developed for 5–6 h with acetic acid–water, 90:10 or 92:8 mixtures respectively. After drying, the plate is sprayed with a phosphomolybdic acid solution or with other sensitive colour reagents. The small, clearly discernible blue-green bands of ergosterol, cholesterol, stigmasterol, β-sitosterol, agnosterol and their respective acetates are completely separated, the results being for the greater part analogous to those obtained in the separation of sterols by reversed-phase paper chromatography (Fig. 1).

Preliminary experiments stressed the importance of mutual saturation of the mobile and stationary phases at a temperature of 22–24°. If this condition has not been fulfilled, dissolutions occur caused by solving of the stationary phase, or by variations in temperature. In that case a second front line above the starting points appears on the chromatoplate. The reproducibility of the degree of impregnation is far less than with reversed-phase paper chromatography; consequently there is a greater variation in the R_F values. The relative R_S values (S = cholesterol) in both systems, which prove to be fairly reproducible, are given in Table II.

TABLE II

R_S[a] VALUES OF SOME STEROLS AND STEROL ACETATES OBTAINED IN REVERSED-PHASE TLC
Systems: (A) undecane/acetic acid–water (90:10); (B) undecane/acetic acid–water (92:8).

Compound	*Abbr. formula*	*R_S values of sterols in system A*	*R_S values of sterol acetates in system B*
Cholesterol	FC27	≡1.0	≡1.0
Stigmasterol	FC29F	0.93	0.91
β-Sitosterol	FC29	0.86	0.83
Brassicasterol	FC28F	1.00	1.00
Ergosterol	2FC28F	1.16	1.22
7-Dehydrocholesterol	2FC27	1.12	1.26
Lanosterol	FC30F	0.84	0.97
Dihydrocholesterol	C27	0.90	0.89
Epicholesterol	FC27	0.90	1.16
Agnosterol	2FC30F	0.76	0.86

[a] S = cholesterol.

By applying other stationary and mobile phases, *e.g.* silicone oil, paraffin oil, etc., with acetic acid, propionic acid, monochloroacetic acid etc. water mixtures, similar results can be obtained; an example is the separation of sterol acetates in the system tetradecane* (b.p. 240–250°)/acetic acid–water (96:4).

The results obtained in the separation of sterols and sterol acetates are, on the whole, analogous. A difference in behaviour is shown, for instance, by the pair cholesterol–epicholesterol; the former has the highest R_F value in system A, whereas the migration rate of epicholesterol acetate in system B appears to be higher than that of cholesterol acetate. In the analysis of mixtures of vegetable and animal fats, reversed-phase TLC of the sterol acetates offers some practical advantages; the melting point of these sterol acetate mixtures is used in the phytosterol acetate test of BÖMER and gives already some indication about the composition of the fat mixture.

* Available from J. HALTERMANN, Hamburg.

The critical pair cholesterol–brassicasterol, both of which have the same R_F values in these reversed-phase systems, was separated by adding 0.5 % Br_2 to the mobile phase according to the technique of KAUFMANN *et al.*[6]. The band of brominated brassicasterol acetate then moves ahead of the cholesterol acetate dibromide with a relative R_S value of about 1.19. The detection was accomplished by means of a chlorosulphonic acid–acetic acid, 1:2 mixture[5], blue to purple bands being obtained.

Because of this differentiation of cholesterol and all the phytosterols, reversed-phase TLC was applied with success to the analysis of mixtures of animal and vegetable fats.

Further experiments are in progress.

Experimental procedure

Glass plates of 14 × 24 cm were coated with a mixture of kieselgur G (Merck)–water (1:2) according to the technique of STAHL. After heating for 1/2 h at 115° the resulting 0.2 mm layer was impregnated with undecane* by dipping the chromatoplate carefully into a 10% solution of undecane (b.p. 190–220°) in petroleum ether (b.p. 40–60°). Care must be taken that the layer is not damaged by unnecessary handling. The chromatoplate is held 1 min with the bottom side upwards and is then stored 1 h at room temperature to evaporate the petroleum ether. An amount of approx. 0.50 or 0.30 g undecane is left on the plate (about 0.13 g or about 0.09 g undecane per g kieselgur respectively). After placing an appropriate mould on the chromatoplate hexagonal holes are scratched out of the layer with a brush. The sterol or sterol acetate solution (5 mm^3 of a 0.1 % ethereal solution) is spotted in the centre of the 8 mm wide "bridges". The chromatoplate is developed with the acetic acid–water, 90:10 or 92:8 mixture by the ascending technique at a temperature of 23°. The 10 % undecane solution and the mobile phase are mutually saturated at the same temperature the day before. The saturated acetic acid–water layer is introduced into a chromatographic vessel of 19 × 7 × 30 cm, supplied with filter paper at the sides to ensure complete saturation. When the solvent front has travelled 20 cm in 5–6 h at a temperature of 23–25° the development is interrupted, and the plate is dried about 3 h in the air and 45 min at 90°. After spraying with a 20 % ethanolic solution of phosphomolybdic acid (Merck), the chromatoplate is heated about 5–10 min at 90°. Blue-green bands appear on a light green background.

ACKNOWLEDGEMENTS

The authors express their thanks to Ir. J. B. ROOS and to Dr. J. G. VAN GINKEL, Director of the Government Dairy Station.

SUMMARY

The behaviour of several sterols and related compounds on chromatoplates prepared from kieselgur G has been studied. Using a cyclohexane–ethyl acetate mixture (99.5:0.5), the separation of some sterols, *e.g.* ergosterol–cholesterol, cholesterol–lanosterol, cholesterol–vitamin D_2 has been achieved. In the reversed-phase system: undecane/acetic acid–water several sterols or their corresponding acetates can be

* Availabe from J. HALTERMANN, Hamburg.

fractionated. In this way cholesterol acetate and the acetates of the major phytosterols, *viz.* β-sitosterol and stigmasterol, are clearly separated, thus enabling the analysis of mixtures of vegetable and animal fats. By adding bromine to the mobile phase a differentiation of the critical pair of sterols cholesterol–brassicasterol has been accomplished.

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NOTE ADDED IN PROOF

Further experiments showed that in addition to the acetic acid–water mixtures the same separation of sterols etc. can be accomplished applying an acetic acid - acetonitrile 1:3 solvent mixture. The time of development with this undecane/acetic acid–acetonitrile 1:3 system is considerably less than with the acetic acid–water systems, *viz.* only 1 1/2–2 h.

The R_S values given in Table II indicate that a fractionation of cholesterol and dihydrocholesterol in these systems is possible. Spotting 50–80 μg of sterol acetates we were able to detect even small amounts of dihydrocholesterol acetate (down to 5%) in an excess of cholesterol acetate.

THE SEPARATION OF 19-NOR-STEROIDS BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL*

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(Received March 16th, 1962)

The application of thin-layer chromatography by several workers to the separation and identification of steroids and sterols has been reviewed by DEMOLE[1]. BARBIER *et al.*[2] have obtained good separations of less polar steroids on silica gel plates using different proportions of ethyl acetate in cyclohexane as developing solvent. We have studied the chromatographic properties of thirty-eight 19-nor-steroids by this technique during an investigation of the metabolites of 17α-ethynyl-19-nor-steroids in body fluids and tissues. Steroid spots on the chromatograms were made visible by spraying with antimony trichloride in chloroform[3]. This reagent gave specific colors with the various compounds in daylight and under ultraviolet light. These colors, together with the R_S values with reference to 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one, can be used for preliminary identification of the individual steroids.

EXPERIMENTAL

Glass plates 20 cm × 20 cm × 0.2 cm were used. Distilled water (70 ml) was added to a flask containing 30 g of silica gel G (Merck) and the flask was shaken vigorously for 30 sec. A layer 0.3 mm thick of the resulting suspension was applied to 5 glass plates using the Desaga applicator obtained from Brinkmann Instruments, Inc., Long Neck, N. Y. The plates were allowed to stand at room temperature for 30 min, and were then heated in an air oven at 110–120° for one hour. The plates were cooled in a desiccator until required for use.

Steroids were applied in quantities of 50–100 γ at points 2 cm from the lower edge of the plates. Application was made in chloroform–methanol solution. Development was carried out in ethyl acetate–cyclohexane in the proportions of either (1:1) or (3:7). In either case the solvent was placed on the bottom of a rectangular tank (Brinkmann Instruments Inc.) to a height of 1 cm. The plates were placed in the tank and were removed when the solvent had ascended to a distance of 1 cm from the upper edge of the plate. The time of development was 90–105 min.

The developed plates were heated to 100–110° and immediately placed in a fume hood and sprayed with a saturated solution of antimony trichloride in chloroform. The color of the spots was observed immediately after spraying, and after a period of 24 h at room temperature. The plates were examined under a long wave

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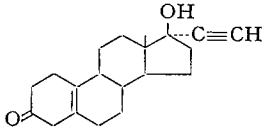
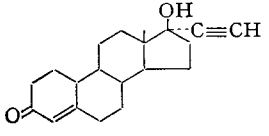
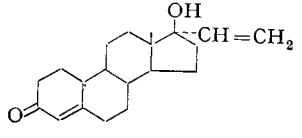
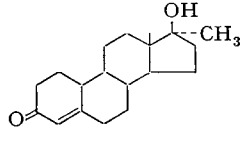
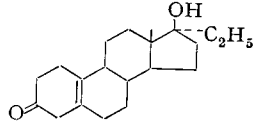
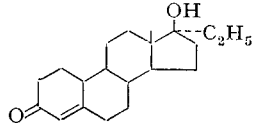
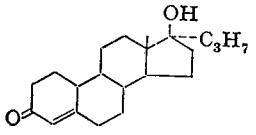
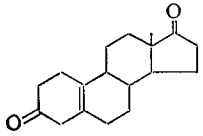
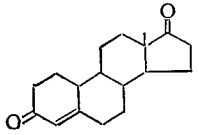
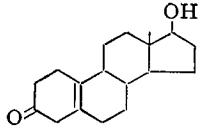
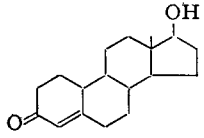
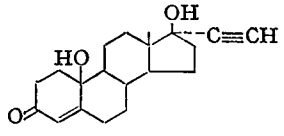
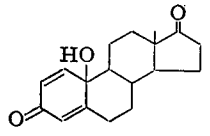
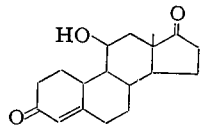
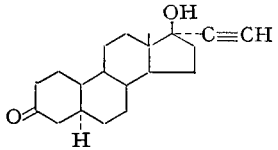
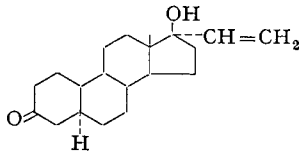
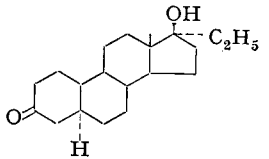
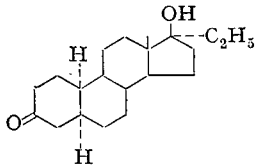
TABLE I

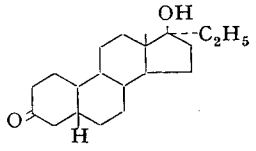
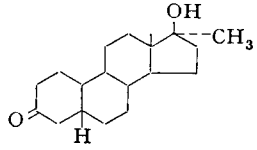
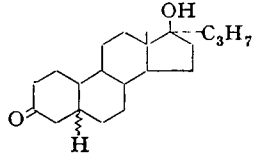
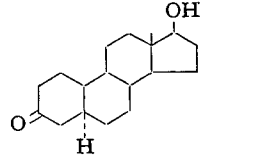
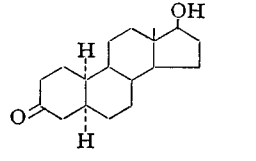
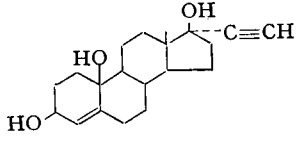
No.	Compound	Ethyl acetate/cyclohexane in mobile phase	R_F	R_S*	Standard error of R_F	Daylight color		Color under U.V. light after 10 min
						After 10 min	After 24 h	
1		1:1 3:7	0.74 0.51	1 1	±0.02 ±0.02	purple	gray-violet	purple-violet absorption
2		1:1 3:7	0.55 0.30	0.74 0.59	±0.01 ±0.01	gray-violet	gray-violet	red absorption
3		1:1 3:7	0.56 0.31	0.76 0.60	±0.03 ±0.03	red-purple-brown	red-brown	strong pink-red absorption
4		1:1 3:7	0.40 0.21	0.54 0.41	±0.05 ±0.03	orange-red	red-violet	pink absorption
5		1:1 3:7	0.68 0.46	0.92 0.88	±0.01 ±0.01	orange	pink-orange	bright yellow absorption with blue fluorescent border
6		1:1 3:7	0.47 0.27	0.63 0.52	±0.03 ±0.01	purple-red	pink with violet border	pink to purple-red absorption

7		1:1 3:7	0.41 0.19	0.55 0.37	±0.02 ±0.01	pink-orange	pink-orange	orange yellow absorption
8		1:1 3:7	0.71 0.50	0.96 0.96	±0.03 ±0.01		bright yellow	strong sky-blue absorption
9		1:1 3:7	0.25 0.13	0.33 0.26	±0.03 ±0.03		bright yellow to blue	strong sky-blue absorption
10		1:1 3:7	0.51 0.29	0.70 0.57	±0.03 ±0.01	yellow	gray-brown	green-blue fluorescence
11		1:1 3:7	0.36 0.14	0.49 0.27	±0.04 ±0.02		bright blue, or by heating bright sky blue	bright blue absorption
12		1:1 3:7	0.28 0.10	0.37 0.13	±0.01 ±0.01	pink to brown-violet	pink-brown	pink-brown absorption with strong blue border
13		1:1 3:7	0.36 0.21	0.48 0.41	±0.05 ±0.03	brown-yellow	orange-brown	dark purple absorption

(continued on p. 324)

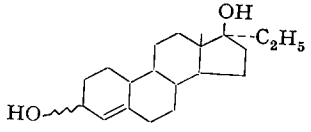
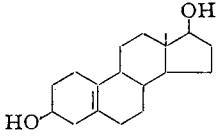
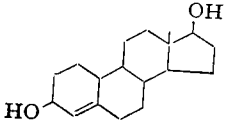
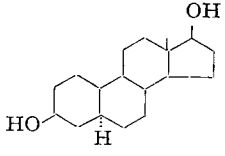
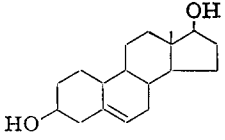
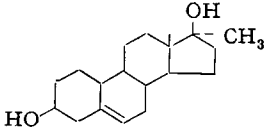
TABLE I (*continued*)

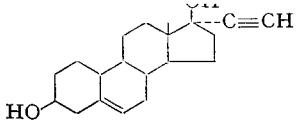
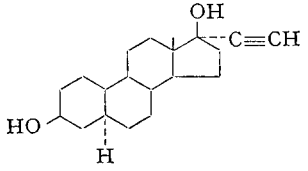
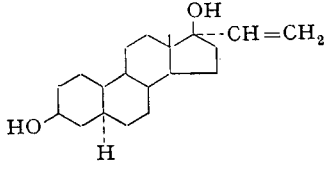
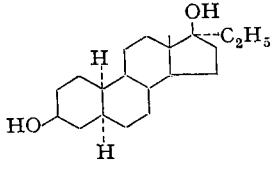
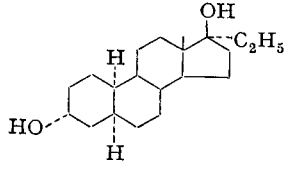
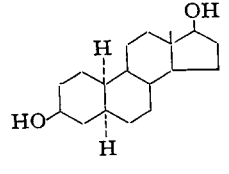
No.	*Compound*	*Ethyl acetate/cyclohexane in mobile phase*	R_F	R_S*	*Standard error of* R_F	*Daylight color*		*Color under U.V. light after 10 min*
						After 10 min	*After 24 h*	
14		1:1 3:7	0.25 0.11	0.33 0.21	±0.01 ±0.01		bright green-yellow	strong sky-blue fluorescence
15		1:1 3:7	0.72 0.42	0.97 0.82	±0.05 ±0.05	brown-yellow	gray-violet	brown absorption
16		1:1 3:7	0.70 0.41	0.94 0.79	±0.03 ±0.02	orange-yellow-brown	orange-brown	orange-brown absorption
17		1:1 3:7	0.64 0.39	0.86 0.75	±0.03 ±0.03	orange-brown	orange-brown	bright brown absorption
18		1:1 3:7	0.63 0.38	0.85 0.74	±0.03 ±0.03	brown-pink	red-brown	bright brown absorption

19		1:1 3:7	0.65 0.39	0.87 0.76	±0.04 ±0.03	brown-orange	orange-brown	bright brown-red absorption
20		1:1 3:7	0.59 0.36	0.79 0.69	±0.02 ±0.01	pink-brown with violet border	orange-brown	blue-pink fluorescence
21		1:1 3:7	0.76 0.53	1.03 1.03	±0.05 ±0.02	pink-brown	red-brown	blue-pink fluorescence
22		1:1 3:7	0.59 0.36	0.79 0.70	±0.02 ±0.02		bright orange-yellow	weak blue absorption
23		1:1 3:7	0.63 0.40	0.84 0.77	±0.03 ±0.02		yellow	weak blue absorption
24		1:1 3:7	0.16 0.05	0.22 0.10	±0.01 ±0.01	0′ grass green 5′ sky blue 10′ ink blue	dark blue-violet	blue absorption

(continued on p. 326)

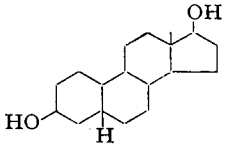
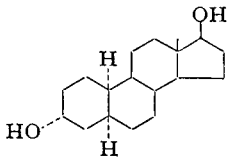
TABLE I (*continued*)

No.	Compound	Ethyl acetate/cyclohexane in mobile phase	R_F	R_S*	Standard error of R_F	Daylight color		Color under U.V. light after 10 min
						After 10 min	After 24 h	
25		1:1 3:7	0.53 0.27	0.71 0.52	±0.04 ±0.04	grey-green violet to purple	grey-green violet	grey-green-blue absorption
26		1:1 3:7	0.36 0.21	0.48 0.41	±0.04 ±0.02	pink-purple	grey-violet	dark brown red absorption
27		1:1 3:7	0.33 0.16	0.44 0.33	±0.04 ±0.01	pink-red to purple-red	red-purple	dark red absorption
28		1:1 3:7	0.39 0.25	0.52 0.45	±0.04 ±0.02	purple-red	red-purple	dark red absorption
29		1:1 3:7	0.38 0.23	0.51 0.45	±0.03 ±0.02	purple-violet	purple	purple absorption
30		1:1 3:7	0.43 0.26	0.59 0.51	±0.02 ±0.02	grey-green	grey-brown	grey-green absorption

31		1:1 3:7	0.54 0.29	0.73 0.57	±0.02 ±0.02	purple	grey-violet	dark purple absorption
32		1:1 3:7	0.53 0.31	0.71 0.60	±0.02 ±0.01	canary yellow	yellow-green-brown	grey-yellow brown absorption
33		1:1 3:7	0.53 0.30	0.71 0.59	±0.02 ±0.01	orange	orange-brown	orange absorption
34		1:1 3:7	0.65 0.41	0.88 0.79	±0.02 ±0.01	orange	orange-yellow	orange-yellow absorption
35		1:1 3:7	0.54 0.32	0.72 0.61	±0.02 ±0.01	orange	orange-brown	orange absorption
36		1:1 3:7	0.48 0.25	0.65 0.49	±0.01 ±0.01		bright brown	weak gray-blue absorption

(continued on p. 328)

TABLE I (*continued*)

No.	*Compound*	*Ethyl acetate/ cyclohexane in mobile phase*	R_F	R_S*	*Standard error of* R_F	*Daylight color*		*Color under U.V. light after 10 min*
						After 10 min	*After 24 h*	
37		1:1 3:7	0.54 0.28	0.71 0.54	±0.03 ±0.02		bright brown	weak grey-blue absorption
38		1:1 3:7	0.42 0.24	0.57 0.47	±0.03 ±0.01		bright brown	weak grey-blue absorption

* R_S values determined with reference to 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one (compound No. 1).

ultraviolet light 10–20 min after spraying. The lamp used was a "Blak-Ray", obtained from Ultra-Violet Products Inc., San Gabriel, Calif., and emitted mainly at about 3660 Å.

RESULTS

The steroids examined were 19-nor-ketones and alcohols many of which possessed 2-carbon side chains at position 17. The R_F and R_S values, together with the colors given with the antimony trichloride reagent are listed in Table I for the individual compounds. Typical chromatograms obtained with most of these compounds in the two solvent systems are shown in Figs. 1 and 2.

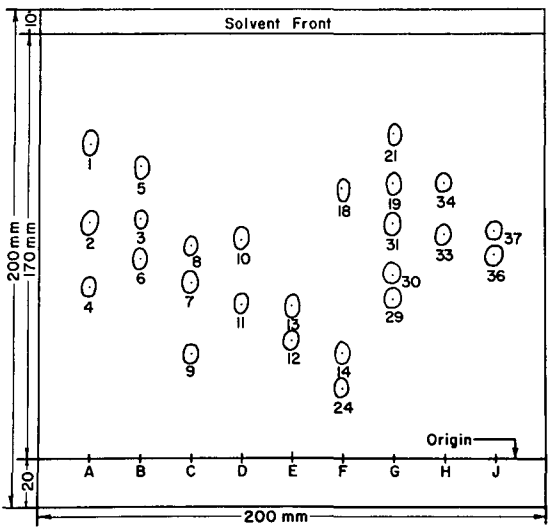


Fig. 1. Example of chromatogram obtained on 0.3 mm silica gel plate in the system ethyl acetate–cyclohexane (1:1). Numbers refer to steroids in Table I. (A) 0.15 mg of mixture of 1,2 and 4; (B) 0.15 mg of mixture of 3, 5 and 6; (C) 0.15 mg of mixture of 7, 8 and 9; (D) 0.10 mg of mixture of 10 and 11; (E) 0.10 mg of mixture of 12 and 13; (F) 0.15 mg of mixture of 14, 18 and 24; (G) 0.25 mg of mixture of 19, 21, 29, 30 and 31; (H) 0.10 mg of mixture of 33 and 34; (J) 0.10 mg of mixture of 36 and 37.

DISCUSSION

The chromatographic data and color reactions described above have proved useful in our laboratory in the detection and preliminary identification of 19-nor-steroids and their metabolites in body fluids and tissues following the administration of these compounds to humans and animals. The color given with antimony trichloride, while specific for each steroid, varied in shade and intensity with the concentration of the steroid and the time of heating the chromatogram before spraying. Care must be taken to compare the color of unknowns with standard spots of approximately the same intensity on the same chromatogram. The R_F values of the steroids varied somewhat on different chromatograms as shown in the standard errors in Table I. In all cases, the R_S values, based on the running speed relative to that of 17α-ethynyl-17β- hydroxy-5(10)-estren-3-one were much less variable than were the R_F values.

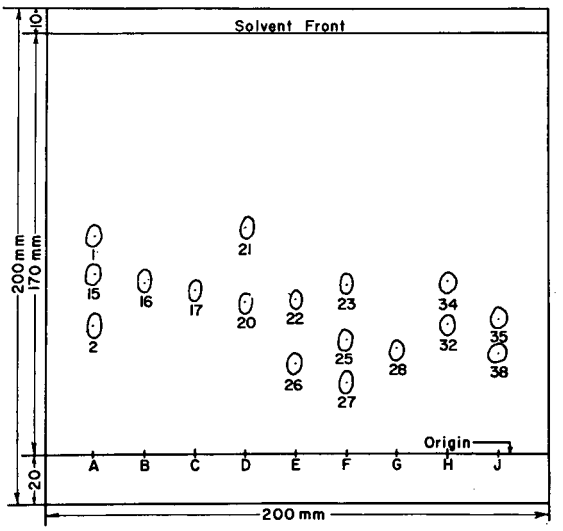


Fig. 2. Example of chromatogram obtained on 0.3 mm silica gel plate in the system ethyl acetate–cyclohexane (3:7). Numbers refer to steroids in Table I. (A) 0.15 mg of mixture of 1, 2 and 15; (B) 0.05 mg of 16; (C) 0.05 mg of 17; (D) 0.10 mg of mixture of 20 and 21; (E) 0.10 mg of mixture of 22 and 26; (F) 0.15 mg of mixture of 23, 25 and 27; (G) 0.05 mg of 28; (H) 0.10 mg of mixture of 32 and 34; (J) 0.10 mg of mixture of 35 and 38.

ACKNOWLEDGEMENTS

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SUMMARY

The separation of 19-nor-steroids by thin-layer chromatography on silica gel, and subsequent identification of the individual compounds by spraying the chromatograms with antimony trichloride in chloroform is described. Chromatographic mobilities and colors developed with the antimony trichloride reagent are listed for thirty-eight 19-nor-steroids.

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STEROIDS

CCXV. THE QUANTITATIVE ANALYSIS OF STEROIDS BY THIN-LAYER CHROMATOGRAPHY*

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(Received May 8th, 1962)

The development of thin-layer chromatography (TLC) by KIRCHNER *et al.* in 1951[2] and 1952[3,4] was not fully appreciated until its extension by STAHL[5] in Europe beginning in 1953. Since then TLC has spread to every field involving separations and its usefulness is attested to by the literature (for reviews see ref. 6). Often, the resolution by TLC surpasses that of paper partition chromatography and in some cases it is as good as gas chromatography[7,8]. The first application of TLC to the separation of steroids[9] was rapidly followed by many others (see ref. 10) showing that this method is applicable to many different types of steroids.

The excellent resolution and rapid development times with TLC offers many advantages for quantitative applications. Methods of quantification reported in the literature have included quantitative analysis by gas chromatography after separation by TLC[11], densitometry of sprayed plates[12] and comparison techniques[13]. In some of his original work, KIRCHNER[2,14] detected U.V. absorbing steroids by adding an inorganic phosphor[15] to the coating. Under ultra violet irradiation, U.V. absorbing compounds stand out as dark spots against a bright background. The spots were then eluted and quantitative measurements made with a spectrophotometer. GÄNSHIRT AND MORIANZ[16] also used this technique for the separation and quantitative analysis of methyl and propyl *p*-hydroxybenzoates with excellent results.

The quantitative analysis of non-U.V. absorbing steroids has usually presented a more difficult problem. They must first be detected in a non-destructive way so that they may be extracted and determined quantitatively by colorimetric or physical methods. In general, non-U.V. absorbing steroid zones are detected by colorimetric reactions. Of the several general reagents used for detecting steroids, iodine vapor[17] appears to be the mildest. It was found during our investigations that detection of steroids by iodine vapor does not affect them markedly for quantitative analysis by other methods afterwards. The effect seems to be one of adsorption since the iodine spots generally disappear shortly after the plates have been removed from the iodine vapor. The use of a phosphor in the thin layer did not affect their detection with iodine vapor.

These techniques have been adapted to the quantitative analysis of steroids by TLC on a semi micro scale. The procedures, and our results with this method are described.

* This work was supported by National Institutes of Health contract No. SA-43-PH-2448. For Part CCXIV see ref. 1.

EXPERIMENTAL

Preparation of coating material

The phosphor used in this work was GS-115 green emission phosphor of U.S. Radium Corp. With 100 mg of phosphor/30 g of coating material (silica gel G or aluminum oxide G with $CaSO_4$ binder)* the green fluorescence was adequate when viewed under a lamp peaking at 254 mμ. The contrast is seen best in a U.V. viewing box (Chromato-Vue) although a hand lamp will serve. In order to remove solvent extractable interferences, the 30 g of adsorbent with 100 mg of phosphor added was extracted three times with 75 ml of boiling methanol (redistilled) with stirring. Fines were effectively removed by filtering with suction through a coarse porosity fritted Pyrex glass filter funnel. After washing and filtering three times, the powder was dried in an oven before it was applied to the plates in the usual manner.

In extracting steroids from zones, erratic or high results were obtained if the coating material was not extracted before preparation of the plates. Tests with methanol and ethanol extraction showed that the lowest blanks were obtained after extraction with methanol. In all quantitative determinations the extracted steroids were read against a blank.

Steroid extraction from zones

After solvent development, the plate is viewed under U.V. light and the U.V. absorbing zones are marked with an ample margin around the zone. The powder in the zone is extracted from the plate by means of the zone extractor shown in Fig. 1. The

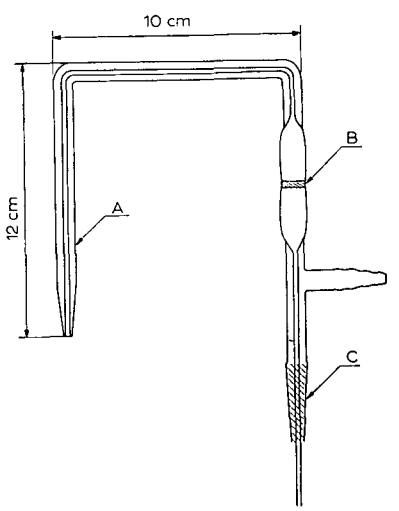

Fig. 1. Zone extractor.

T/S 10/30 joint can fit 5, 10, 25 or 50 ml Kimble or Pyrex volumetric flasks. Throughout this work, 10 ml volumetric flasks were used. With a volumetric flask attached, suction is applied at the hose connection and the steroid zone can be sucked off the

* Obtained from Brinkmann Instruments Inc., 115 Cutter Mill Road, Great Neck, N.Y. (U.S.A.)

plate by rubbing it loose with the inlet tube, A. The powder is carried by the rushing air and stops on top of medium porosity disc, B. After the powder in the zone has been completely removed it is extracted *in situ*. With the vacuum still applied, ethanol is sucked up through A and the steroid is extracted directly into the volumetric flask. The zone extractor can be cleaned by reverse flushing with water and methanol. Occasionally it is necessary to clean the extractor with concentrated sulfuric acid.

After the U.V. absorbing zones have been removed the plate is inserted into a clear jar containing iodine crystals. In a few seconds brown spots appear wherever there are non-U.V. absorbing steroids. The plate is removed immediately and the zones marked quickly. After the spots disappear, the zones are extracted as above. Any trace of adsorbed iodine left is probably removed under the conditions (air and partial vacuum) of zone removal.

Extraction of the steroid from the adsorbent was complete with the first ml of ethanol when quantities of 50 μg of progesterone were used. Using quantities of 1–8 ml of ethanol, recoveries of 94–100% were obtained with an average recovery of 96.4% from silica gel and 97.8% from alumina (Table I). In general practice 8 ml of ethanol was used for extraction.

TABLE I

% RECOVERY OF 50 μg PROGESTERONE FROM ALUMINA AND SILICA GEL

Solvent vol. (ml)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Average*
Alumina	99.8	98.1	94.4	96	98.1	96	100	100	97.8
Silica gel	96.3	94.1	94.8	96	96	99.8	96	98.1	96.4

Application of steroids

Since the quantities of steroid which are used are small there would be an appreciable error in weighing the amounts applied. Furthermore, the coating on the plate is too fragile to stand repeated applications at the same spot such as is done in paper chromatography in order to quantitatively transfer a weighed sample. In this work the solution was applied to the starting line from a 100 μl Kirk type transfer micro pipet. The use of the 100 μl pipet led to much greater reproducibility than could be obtained with smaller micro pipets or the microliter syringes commonly used in gas chromatography.

The concentration of the steroid solution was such that the steroid was applied to the plate in one application from a 100 μl pipet. In any given experiment the solutions were applied with the same micro pipet to eliminate errors due to differences between pipets. For strongly U.V. absorbing steroids such as Δ^4-3-ketones, 100 μg of steroid or 1 mg/ml concentrations were used. For weak U.V. absorbers such as estrogens, 300 μg of steroid were applied. In order to reduce the errors in weighing and dilution, the appropriate amount of sample was weighed in a 5 or 10 ml volumetric flask and dissolved in distilled chloroform. Other solvents did not prove as suitable as chloroform for sample application. The more polar solvents such as methanol tended to leave the steroid as a ring rather than a spot. It was found convenient to warm the plate on an electric hot plate at about 50° while applying the solution in order to increase the rate of evaporation, thus speeding up sample application and keeping the spot small.

RESULTS

The results of multiple determinations are shown in Table II. In these experiments pure testosterone and 4-chloro-17α-hydroxyprogesterone standards were used. The spots containing approximately 100 μg each of steroid were placed on a silica gel plate without development and extracted with 8 ml of ethanol into 10 ml volumetric flasks which were then made to the mark. The exact amount of steroid applied to the plate with the 100 μl pipet was determined by placing the same volume in a 10 ml volumetric flask (in triplicate), making to volume and determining the absorbance in the ultraviolet. The results listed for testosterone and 4-chloro-17α-hydroxyprogesterone thus represent the percentage recovery from the plate. In these cases the $E^{1\%}_{1\,cm}$ calculated for the standard was taken as 100%. The percentage in any case was calculated as follows:

$$\frac{E^{1\%}_{1\,cm}\ \text{problem}}{E^{1\%}_{1\,cm}\ \text{reference}} \times 100 = \% \qquad (1)$$

TABLE II

STATISTICAL ANALYSIS OF REPETITIVE DETERMINATIONS

Steroid	*Results*	$\bar{X}$	*d*	*s*
Testosterone (std.)	95.6, 94.0, 95.6, 94.0, 95.6	94.96	0.77	± 0.87
4-Chloro-17α-hydroxy progesterone (std.)	96.1, 94.8, 95.1, 94.8, 95.9, 96.1	95.47	0.58	± 0.63

$\bar{X}$ = mean.

d = average deviation = $\frac{\Sigma X_i}{n}$ where X_i is deviation of individual values from the mean $\bar{X}$ and n is the number of determinations.

s = standard deviation = $\sqrt{\frac{\Sigma(X_i)^2}{n-1}}$.

The analysis of different steroids of different degrees of purity are listed in Table III. In each case the determination was carried out in triplicate and compared against standards run under the same conditions. The results obtained by TLC are compared with those obtained by other methods. Many analyses were made by more than one operator and are designated by the letters A, B or C. All these analyses were carried out on silica gel G with phosphor. The results listed for 6α-fluoro-16α-hydroxy-dihydrocortisone-16,17-acetonide-21-acetate are for two different lots as indicated by the brackets.

In addition to the analysis of impure lots of steroids, a synthetic blend of testosterone and isotestosterone was separated and determined quantitatively (Table IV). In this experiment a mixture of 130.8 μg testosterone and 80 μg isotestosterone was run on aluminum oxide G with phosphor for a distance of 15 cm using benzene–ether (1:1). The determination was performed in triplicate against a standard run at the same time.

TABLE III

ANALYSIS OF IMPURE STEROIDS

	Steroid	*Solvent system**	*% Purity found*	
			TLC	*Other***
1	11-Desoxycortisone	C:E 3:2	95.5 (A) 95.6 (B)	97.4 (p)
2	19-Nor-progesterone	B:E 1:1	93	95.5 (p)
3	4-Chloro-17α-hydroxyprogesterone	C:E 3:1	95	96 (p)
4	3-Methyl ether of 17α-ethynylestradiol	B:E 9:1	97.5 (B) 98.6 (C)	97 (g)
5	6α-Fluoro-16α-hydroxydihydrocortisone-16,17-acetonide-21-acetate	C:E 3:2	92.9 (A) 92.2 (B) 95.2 (C)	95.8 (p)
			82.8 (A) 82.0 (C)	83.5 (p) 81.5 (cp)

* C = chloroform; E = ethyl acetate; B = benzene.

** p = paper partition chromatography; cp = column partition; g = gas–liquid chromatography.

TABLE IV

SEPARATION AND ANALYSIS OF TESTOSTERONE AND ISOTESTOSTERONE

Steroid	R_F	*% Found*	*Average*	*% (Theory)*
Testosterone	0.37	62.3, 63.0, 63.2	62.8	62.1
Isotestosterone	0.47	39.1, 39.1, 38.8	39.0	37.9

TABLE V

DETECTION OF PURE STEROIDS WITH IODINE VAPORS

Steroid (standards)	$E^{1\%}_{1cm}$				*% Recovery after iodine*	*Thin layer**
	U.V.		*Iodine*			
	Duplicate	*Average*	*Duplicate*	*Average*		
Testosterone acetate	507 509	508	482 491	487	96	S
	493 493	493	477 483	480	97	A
3-Methyl ether of 17α-ethynylestradiol	63.2 64.4	63.8	62.2 60.8	61.5	96	S
	63.7 64.3	64.0	60.8 60.8	60.8	95	A
Cortisone	403 413	408	421 431	426	104	S
	400 395	398	390 385	388	98	A
Corticosterone	409 409	409	410 430	420	103	S
	416 426	421	416 408	412	98	A
6-Dehydro-testosterone acetate	759 771	765	750 769	760	99.3	S
	768 750	759	750 750	750	98.8	A

* S = Silica gel G with phosphor. A = Aluminum oxide G (neutral) with phosphor.

As mentioned in the introduction, the analysis of non-U.V. absorbing steroids was carried out by first detecting the zone with iodine vapors. In order to establish that this method of detection did not interfere with the subsequent colorimetric reaction for quantification, the effect was first studied on U.V. absorbing steroids containing in aggregate the most common steroid groups or groupings.

The experiments were performed in duplicate both on silica gel G and aluminum oxide G (neutral). The procedure followed was to place four equal spots of the steroid on a plate containing phosphor. Two of the spots were detected and marked under U.V. light and determined as described above. After these were removed, the plate was placed in a clear jar with iodine vapors and the steroid spots marked and extracted in the normal manner. The values obtained with the steroids detected by U.V. light were taken as 100% and the values obtained after iodine vapor detection were related to this. The results are given in Table V.

This method of detecting steroids was then applied to the separation and quantification of a synthetic mixture of androsterone and isoandrosterone. The method of quantification chosen was the Zimmermann reaction. For each steroid a calibration curve was prepared as follows: duplicate samples of 10, 50 and 100 μg were placed in 10 ml volumetric flasks and the solvent evaporated. To each flask was added 0.2 ml of a 2% ethanol solution of *m*-dinitrobenzene and 0.2 ml of an ethanolic 2.5 *N* potassium hydroxide solution. The flasks were stoppered, shaken and left in the dark at 25° for one hour. Immediately before being read against a blank on a Beckman DK-2, each flask was made to the mark with ethanol (the ethanol used was 96% spectroscopic grade).

After the steroid mixture had been separated and detected, each zone was eluted with 2 ml of ethanol into a 10 ml volumetric flask. This was evaporated on a steam bath under a stream of nitrogen and treated as above. Table VI lists the results.

TABLE VI

ANALYSIS OF ANDROSTERONE AND ISOANDROSTERONE IN MIXTURE

System: neutral alumina; 15 cm; hexane–ethyl acetate (1:1)

Steroid	R_F	*Found (μg)*	*Average*	*Theory*
Isoandrosterone	0.95	48, 53.5, 56.5	52.7	51.5
Androsterone	0.76	48.5, 51.5, 52.5	50.8	52

Analysis of an impure lot of isoandrosterone by this method gave 92% purity while analysis by gas chromatography gave 89%.

DISCUSSION

As mentioned earlier the use of thin-layer chromatography has distinct advantages over paper partition chromatography. Not the least of these is the fact that there is less diffusion in TLC than in partition chromatography. This results in smaller more compact zones with less tendency to streak and tail. Thus greater resolution is possible and more reproducible quantitative results are obtained. Due to variations in layer

thickness and activity as well as variables in solvent composition, the R_F values in TLC are not as reproducible as in paper partition chromatography. However, this is not a problem when analyzing steroids for purity since obviously the biggest zone is the steroid whose purity is being determined. Quantitative analysis of steroids by gas–liquid chromatography has the disadvantage that all steroids do not give the same molar response[18]. Unless the impurities are known, and calibration curves prepared, this may lead to considerable error when using the method of internal normalization. This problem does not arise in quantitative analysis by TLC.

The limitless variations in solvent composition that can be used in TLC allows one to make experiments with high concentrations of the impure steroid so that even minor impurities may be resolved and detected by iodine once the best solvent mixture is found. It also permits one to observe whether any impurities are travelling close to the major component. Allowances can then be made for this when marking the major zones after detection, so that these impurities are not included in the zone being extracted. Again, the application of samples as small spots aids in this respect since resolution is enhanced. The formation of small spots is aided by warming the plate to about 50° while the 100 μl of solution is applied. The flow rate of solution onto the thin layer must also be controlled to keep the spot small.

All the above procedures for quantitative analysis can probably be scaled down by using microliter syringes and smaller volumes and sample weights but with probable attendant increases in error and decreases in reproducibility. If it is desired to detect much lower quantities of U.V. absorbing steroids it would be advisable to increase the phosphor content in the layer so that the contrast becomes greater under U.V. light.

The detection of steroids by iodine vapor was investigated on compounds containing in aggregate the following functional groups or groupings: aromatic ring A; methyl ether of phenolic ring A; tertiary hydroxyl; primary hydroxyl; ketone; Δ^4-3-ketone; $\Delta^{4,6}$-3-ketone; 17α,21-diol-20-ketone; 21-ol-20-ketone; ethynyl; and ester (Tables V and VI). Although in some cases there is a discrepancy of as much as 4 or 5% after detection by iodine as compared with U.V. detection, the net results indicate that there is very little reaction of iodine with the U.V. chromophore, *i.e.* the Δ^4-3-ketone, $\Delta^{4,6}$-3-ketone or aromatic ring A groups. The addition of iodine to double bonds is known to be very slow even in solution and furthermore is easily reversible. Under the conditions of detections used here, there should be little or no reaction since it is in the dry state where there is likely to be less reaction than in solution. To determine if any reaction occurred when testosterone acetate was detected by this method, about 0.5 mg was placed on a silica gel plate and exposed to iodine vapor. When the spot became visible the plate was removed and the color of iodine allowed to disappear. Another equal amount of testosterone acetate was then placed beside it and the chromatogram developed with chloroform. No new spots were visible in iodine vapor after solvent development indicating that no new products had been formed by this method of detection. The same procedure was followed for each steroid in Table V using solvent systems which had been shown to resolve the largest number of impurities. The only steroid which gave a new compound was cortisone, although the amount was very small compared to the total.

The functional grouping in cortisone which might be sensitive to iodine vapor is the α-ketolic side chain. It has been reported that cortisone gives a blue color when

detected by an iodine solution[19] indicating that there may be some reaction taking place. However, when cortisone is detected by iodine vapor in TLC the appearance of the spots is the same as for any other steroid at the concentrations used in this method. Only when high concentrations were used (500 $\mu g/cm^2$) was a blue color visible. In order to check that there is no appreciable reaction with the α-ketolic group when detected by iodine vapor, cortisone was tested with blue tetrazolium reagent. For this experiment six equal spots of pure cortisone were placed on a silica gel G plate containing phosphor. Three were detected by U.V. light, extracted and the blue tetrazolium reaction run. The other three spots were detected first by iodine vapor, then treated in exactly the same manner as the previous three spots. The absorbances were measured on a DK-2. The absorbances obtained with the cortisone detected by U.V. gave values of 0.318, 0.333 and 0.365 for an average value of 0.339. The cortisone detected by iodine vapor gave values of 0.311, 0.335 and 0.354 for an average value of 0.333. The difference is less than 2% indicating that there is little if any reaction of iodine vapor with the α-ketolic group of cortisone.

The foregoing indicates that the detection of steroids in TLC by iodine vapor is primarily an adsorption phenomenon and there is very little if any chemical reaction with most functional groups usually present in steroids. Detection by this method does not interfere with subsequent colorimetric reactions.

SUMMARY

A method has been developed for the quantitative analysis of U.V. and non-U.V. absorbing steroids utilizing thin-layer chromatography. U.V. absorbing steroids are detected by means of a phosphor in the thin layer, removed and determined quantitatively in a spectrophotometer. Non-U.V. absorbing steroids are detected by iodine vapor, then removed before being determined quantitatively by colorimetric reactions.

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THIN-LAYER CHROMATOGRAPHY OF STEROIDS ON STARCH-BOUND SILICA GEL CHROMATOPLATES

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Early work associated with the thin-layer chromatographic technique was done on chromatoplates prepared using various starches as the binder for the adsorbent[1–4]. Little attention[5–7] has been given to this means of operation since the reports of STAHL[8,9], who employed gypsum as binder in his technique. To our knowledge only one instance has been reported where steroids have been chromatographed on starch-bound thin-layers[6]. We wish to report our experience of the past two years using thin-layer silica gel chromatoplates prepared with rice starch for the analysis of several classes of steroids.

The gypsum-bound thin-layer chromatoplates prepared according to STAHL[8] are fragile and do not withstand handling, transportation, storage, display, etc. without flaking. They cannot be marked with pencil satisfactorily[9]. These disadvantages offset the advantages of use of corrosive reagents and destructive methods of visualization. We find that for routine analysis the durable thin-layers obtained using starch as binder are the preparation of choice. Starch-bound thin-layers resist flaking, may be stored and transported freely, and can be marked with pencil both before and after chromatographic irrigation.

The methods for preparation of the thin-layers using rice starch (other starches may also be used) as binder are modifications of the procedures of REITSEMA[4], and commercially available spreading equipment is used. The technique is otherwise that of STAHL. The solvent systems useful with gypsum-bound plates are of use on starch-bound plates also. In our experience, with a given solvent system the mobility of a given steroid on starch-bound plates may be greater than, less than, or equal to its mobility on gypsum-bound plates (using "Kieselgel G" of E. Merck, Darmstadt). Relative mobility data for several steroids are presented in Table I.

The silica gel–water–rice starch mixture before spreading has a pH of 2.7, and the thin-layers so produced (Procedure A) have an acid reaction to indicator paper. These acid thin-layers have been acceptable for most routine purposes.

Thin-layer chromatography of sensitive steroids is not without artifact formation, however. Sensitive steroid 16β-esters are altered on alumina chromatoplates[10], and we have observed cases where acid-sensitive ethylene ketals have been hydrolyzed on the acidic rice starch-bound silica gel plates.

Neutralization of the silica gel–water–rice starch mixture to pH 6.4 (range 6.1–6.7) affords a neutral chromatoplate (Procedure B) with the durability and chromatographic properties of the acidic plates. The neutral thin-layers offer a more suitable means of analysis where sensitivity to acid is encountered. Thin-layers prepared with

TABLE I

RELATIVE MOBILITY DATA OF SEVERAL CLASSES OF STEROIDS

Steroid	R_F			
	Hexane–ethyl acetate (4 : 1)	*Hexane–ethyl acetate (1 : 1)*	*Ethyl acetate*	*Benzene–2-propanol (4 : 1)*
C_{18}-steroids				
Estrone	0.07	0.57	—	—
Estrone 3-acetate	0.06	0.55	—	—
Estrone 3-methyl ether	0.24	0.68	—	—
Estradiol-17α	0.03	0.42	—	—
Estradiol-17α 3-methyl ether	—	0.52	—	—
Estradiol-17β	0.02	0.37	—	—
Estradiol-17β 3-methyl ether	0.07	0.47	—	—
Ethinylestradiol-17β	0.04	0.56	—	—
Estriol	0.00	0.02	—	—
Equilin	0.04	0.50	—	—
Equilenin	0.04	0.47	—	—
C_{19}-steroids				
Testosterone	0.01	0.15	0.47	—
19-Nortestosterone	0.01	0.12	0.42	—
17α-Methyltestosterone	0.01	0.17	0.48	—
4,5α-Dihydrotestosterone	0.04	0.32	0.63	—
17α-Methyl-4,5α-dihydrotestosterone	0.03	0.32	0.63	—
17α-Ethinyltestosterone	0.03	0.28	0.70	—
4-Androstene-3,17-dione	0.02	0.20	0.55	—
5α-1-Androstene-3,17-dione	—	0.32	0.69	—
5α-Androstane-3,17-dione	—	0.42	0.72	—
1,4-Androstadiene-3,17-dione	0.00	0.14	0.44	—
Dehydroisoandrosterone	0.02	0.25	0.59	—
C_{21}-steroids				
Progesterone	0.05	0.36	—	—
3β-Hydroxy-5-pregnen-20-one	0.06	0.38	—	—
3β-Hydroxy-5,16-pregnadien-20-one	0.05	0.37	—	—
3β-Acetoxy-5,16-pregnadien-20-one	0.34	0.75	—	—
3β-Hydroxy-5α-16-pregnen-20-one	0.09	0.44	—	—
Cortisone	—	0.07	0.38	0.75
Cortisone 21-acetate	—	0.18	0.64	—
Hydrocortisone	—	0.06	0.38	0.55
Hydrocortisone 21-acetate	—	0.17	0.62	—
Prednisone	—	0.06	0.38	0.69
Prednisone 21-acetate	—	0.17	0.61	—
Prednisolone	—	0.05	0.35	0.46
Prednisolone 21-acetate	—	0.13	0.57	—
Cortexone	—	0.22	0.56	0.96
Cortexone 21-acetate	—	0.37	0.71	—
Reichstein's Substance S	—	0.15	0.51	—
11-*epi*-Hydrocortisone	—	0.02	0.20	0.33

(continued on p. 341)

TABLE I *(continued)*

Steroid	R_F			
	Hexane–ethyl acetate (4:1)	*Hexane–ethyl acetate (1:1)*	*Ethyl acetate*	*Benzene–2-propanol (4:1)*
C_{27}-steroids				
Diosgenin	0.18	0.67	—	—
Diosgenin 3-acetate	0.73	—	—	—
Tigogenin	0.18	0.67	—	—
Tigogenin 3-acetate	0.74	—	—	—
Smilagenin	0.26	0.72	—	—
Smilagenin 3-acetate	0.73	—	—	—
Hecogenin	0.02	0.31	0.58	—
Hecogenin 3-acetate	0.26	0.89	—	—
Gentrogenin 3-acetate	0.25	0.89	—	—
Sarsapogenin 3-acetate	0.68	—	—	—
Chlorogenin	0.00	0.04	0.18	—
Kryptogenin	0.00	0.22	0.25	—
Pennogenin	0.04	0.48	0.68	—
Tomatidine	0.00	0.02	0.03	—
Cholesterol	0.36	0.73	—	—
5α-Cholestan-3-one	0.77	0.91	—	—

buffers or at high pH did not have the durability properties of favor, and we have not studied these preparations further. STAHL has reported on such thin-layer chromatoplates, however[11,12].

Detection of steroids on the rice starch-bound plates is accomplished by the same means as would normally be used on paper chromatograms or on gypsum-bound thin-layers. Although the starch-bound plates contain organic matter and thus cannot be subjected to the extremes of heat and chemical exposure possible for gypsum-bound plates, we have encountered relatively few interferences from the rice starch. The greater durability of the plates in day-to-day operations more than accommodates for these limitations.

We have applied many visualization procedures successfully without modification. Thus antimony trichloride, phosphoric acid, trichloracetic acid, 2,4-dinitrophenylhydrazine, the Zimmermann reagent, etc. work well, as does isonicotinic acid hydrazide for detection of Δ^4-3-ketones and $\Delta^{1,4}$-3-ketones[13]. Quenching of ultraviolet light fluorescence permits ready detection of Δ^4-3-ketones, $\Delta^{1,4}$-3-ketones, and other unsaturated steroids. The soda fluorescence procedure of BUSH[14] specific for Δ^4-3-ketones cannot be applied.

Phosphomolybdic acid (10% in ethanol)[15] is the most widely useful visualization technique at our disposal. Whereas phosphomolybdic acid frequently does not give good sensitivity on paper chromatograms and severe background coloration occurs, on the rice starch-bound thin-layer chromatograms (Procedure A) excellent sensitivity and contrast has been achieved with a number of different unsaturated steroids. By heating the sprayed chromatoplate until the solvent front appears as an intense blue line (usually not more than ten minutes at 100°) the steroid zones appear as well

contrasted blue spots against a lemon yellow background which does not deteriorate even after several days.

Sensitivity measurements indicate that less than 0.06 μg of estrone, 0.025 μg of 3β-hydroxy-5-pregnen-20-one, 0.125 μg of progesterone or testosterone can be detected on chromatographically irrigated plates (prepared by Procedure A). The sensitivity of phosphomolybdic acid for progesterone on chromatoplates of different composition is: Procedure A, 0.125 μg; Procedure B, 0.25 μg; Silica gel G according to STAHL, 1 μg.

The sensitivity of the phosphomolybdic acid reagent is definitely a function of acidity, and on neutral plates (Procedure B) the sensitivity is less. A modified reagent incorporating hydrochloric acid must be used in order to obtain comparable sensitivities. Very little color formation occurs on alkaline plates. Neutral or acid plates previously visualized with phosphomolybdic acid lose the lemon yellow background color on spraying with 10% ethanolic alkali, and blue spots on a colorless background result.

With many phenolic steroids, their methyl ethers and acetate esters, phosphomolybdic acid gives a red color which turns blue on heating. This intermediate red color appears immediately after spraying for 1,3,5(10),16-estratetraen-3-ol methyl ether and for certain other unsaturated phenolic steroids. The phenolic steroids may also be detected on thin-layers with the Turnbull blue reagent (1% Fe Cl_3–1% $K_3Fe(CN)_6$)[16].

Reducing steroids cannot be detected as such on rice starch-bound plates using alkaline tetrazolium salts or with alkaline silver nitrate for the background coloration is too intense. Tetrazolium salts can be used for this purpose on gypsum-bound plates, however[17], and where such reducing steroids must be detected, the gypsum-bound plates must be used.

Concentrated sulfuric acid can be used on the rice starch plates, this finding being reported quite early in the use of such thin-layers for chromatography[2,18]. We have detected 5α-cholestan-3-one (which does not respond to phosphomolybdic acid) as a rose violet coloration using concentrated sulfuric acid spread over the irrigated chromatoplate. Deterioration will take place in a few hours. The Liebermann-Burchard reagent (acetic anhydride–concentrated sulfuric acid, 4:1) can be used satisfactorily for unsaturated sterols on the rice starch-bound chromatoplates.

Reproducible mobility data of the same quality as obtained on gypsum-bound thin-layers can be obtained with the starch-bound plates. Relative mobility of steroids in the several solvent systems studied is influenced by the amount of steroid applied to the plate. For routine analysis a 1–5 μg sample is used. For 10 μg samples the resolved spots are still only about 1.2–1.5 cm^2 in size. Heavily loaded plates (25–50 μg) can be run; however, comparison of R_F values on such analyses with R_F data obtained with less sample is not appropriate.

Preparative work can be done with the rice starch-bound chromatoplates without interference from the starch. A 10 mg sample of testosterone was applied to an acid plate, run, located under ultraviolet light, and eluted with methanol, yielding 8.8 mg of crystalline testosterone identified by infrared spectra.

EXPERIMENTAL

The apparatus available commercially from C. Desaga GmbH, Heidelberg, was used throughout. The 20 × 20 cm plates were prepared five at a time, with a 275 μm thin-layer of the silica gel preparation.

Silica gel preparation

Procedure A. Thirty grams of finely divided silica gel (Fisher No. S-158, No. 1 impalpable powder) and 1.5 g of powdered rice starch (Matheson, Coleman, and Bell) were placed in a 250 ml erlenmeyer flask and 50 ml of water was added. The mixture was stirred gently to wet all the material, then heated on a steam bath for twenty minutes, until the preparation thickened. An additional amount of water was then added (usually 20 ml but possibly as little as 15 ml depending on the batch of silica gel), the mixture was stirred well to break up any lumps, and the preparation was again heated on a steam bath for twenty minutes. The mixture was cooled to room temperature on the desk, mixed well, and spread with the Desaga apparatus. The amounts used are sufficient to fill the apparatus and will coat five 20 × 20 cm glass plates.

Procedure B. The exact same proportions of silica gel, water, and rice starch were used, except that the 50 ml of water initially added is composed of 34 ml of water plus 16 ml of 0.1 *N* sodium hydroxide solution. The procedure is otherwise the same as Procedure A.

The thin-layers are made by spreading either preparation with a uniform motion. After air drying in place for 10–20 min (loss of appearance of moisture) the plates are stacked in a metal frame and dried in an oven at 100° for three hours. Whereas the plates are ready for use after drying for as little as two hours, they are conveniently left in the oven at 100° until just prior to use, at which time they are removed, cooled, and spotted with the samples in the usual way. Samples of 1 mg/ml concentration in methanol are applied with suitable microliter pipettes on the start line, etc., and developed with the selected solvent. Desaga rectangular glass chambers, 10 × 22 × 21 cm, were used, with a filter paper liner to assure saturation of the chamber with the solvent. Solvent rise of about 15 cm in 60 min is usual with the solvent systems described.

Detection

After air drying for a few minutes the chromatoplates (Procedure A) were sprayed in a horizontal position with a 10% solution of reagent phosphomolybdic acid in 95% ethanol. The sprayed plate was dried with a hand-held electric hot air drier until the odor of ethanol was lost, then dried at 100° in an electric oven until the solvent front appeared as a clearly visible blue line or zone (not over ten minutes). Overheating past this point will darken the lemon yellow background and faint spots will not be observed. After cooling the blue spots are outlined in pencil and their positions recorded with the Haloid Xerox 914 copy machine[19].

For neutral chromatoplates prepared according to Procedure B, 4 ml of concentrated hydrochloric acid was added to each 100 ml of the 10% phosphomolybdic acid reagent solution.

SUMMARY

A thin-layer chromatographic procedure for steroids is described wherein the thin-layer is prepared from silica gel with rice starch as binder.

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THIN-LAYER PARTITION CHROMATOGRAPHY
A QUICK METHOD OF CHROMATOGRAPHY FOR STEROIDS

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Work on thin-layer chromatography of steroid compounds has up to now been chiefly based on adsorption chromatography similar to the classical process of TSWETT[1–9]. Though some of the papers[5–11] have dealt with the separation of more polar steroids, the best results have been obtained with mixtures of lipophilic steroids.

In our work we have based thin-layer chromatography on a pure partition process and have obtained very good results with steroids of widely differing polarities.

A practically inert medium spread on a glass plate was saturated with a liquid stationary phase, after which the steroid mixtures were applied. A mobile phase ascending the plate separated the steroids according to their partition coefficients. The separation of steroids of widely differing polarities, ranging from sterol esters to corticosteroids, was effected with solvent systems commonly used in paper chromatography. Particularly good results were obtained with Zaffaroni's solvent systems based on formamide or glycols as stationary phases.

Reversed-phase systems also gave good results in the fractionation of the least polar steroids.

With classical solvent systems based on water as the stationary phase, "tailing" could not be avoided and the results were unsatisfactory.

It should be emphasized that the unusually short time of development is the greatest advantage of this method. Using Celite No. 545* with an admixture of gypsum as binder and formamide as the stationary phase, chromatograms were obtained whereby the solvent front covered a distance of 10 cm in 3 to 7 minutes depending on the mobile phase used. In spite of so short a time of development, the separation of steroids differing in structure by only one double bond was achieved, *e.g.* methyltestosterone from Δ^1-dehydro-methyltestosterone.

For spot detection all the reagents used for steroids in paper chromatography and also the drastic reagents recommended for thin-layer chromatography are suitable. We used concentrated sulphuric acid, concentrated phosphoric acid, antimony trichloride, triphenyltetrazolium chloride (TTC), tetrazolium blue and isonicotinic acid hydrazide (INH). Concentrated phosphoric acid has proved very useful, giving intense fluorescence and different colours with different steroids. The same reagent used in paper chromatography as 20% solution is much less sensitive.

The amounts of the substances used in the analyses were 0.1 to 10 γ.

* Johns Manville, International Corp., New York.

EXPERIMENTAL

Six plates of mirror glass, 5 mm thick, 100 mm wide, 180 mm long, were covered with a slurry prepared by mixing 7 g of Celite No. 545, 0.4 g of gypsum and 40 ml of water. The Celite and gypsum were both sifted through a DIN 1171 sieve (pore size 0.07 mm). The mixture was spread on each plate with a glass rod; afterwards the plates were shaken by hand for a short time to obtain a uniform layer of Celite. The plates were then dried for an hour in an oven, the temperature being gradually raised from 20° to 120°. The dried plates were kept in a desiccator.

The starting points (1.5 cm from the lower edge of the plate and at distances of 1.5 cm from each other) and a line at a distance of 10 cm from these points, were marked on the plates with a needle. The coating of the plate was saturated with the stationary phase by means of a fine sprayer. The amount of stationary phase on the plate was estimated by weighing the plate before and after spraying. (In routine work one can do this by counting the number of sprays applied.) Immediately after spraying, the mixtures to be analysed and, where necessary, the reference standards, were applied to the starting points by means of a micropipette and the plate was placed in a chamber containing the mobile phase to a depth of 1 cm. Development was carried out until the solvent front had reached the marked line (2–7 min). The chromatogram

TABLE I

R_F VALUES OF SOME STEROIDS ON CELITE NO. 545

Solvent system	*Z-1*	*Z-2*	*Z-3*	*Z-4*	*Z-5*	*GP-1*	*N*	*P-1*	*Detection reagent*
Time (min)	*3*	*4*	*5*	*4*	*5*	*5–6*	*15*	*7–9*	
Amount of stationary phase (g)	*1.3*	*1.4*	*0.6*	*0.4*	*0.5*	*0.2*	*0.15*	*0.3*	
Cholesteryl benzoate							0.11	0.12	H_3PO_4,$SbCl_3$
Cholesteryl acetate							0.30	0.24	H_3PO_4,$SbCl_3$
Cholesterol							0.75	0.87	H_3PO_4,$SbCl_3$
Pregnenolone acetate	0.94						0.91	0.93	H_3PO_4,$SbCl_3$
Androstenolone acetate	0.84						0.93	0.94	H_3PO_4,$SbCl_3$
Androstenedione		0.79				0.75			H_3PO_4,INH
Methyltestosterone		0.70				0.43			H_3PO_4,INH
Δ^1-Dehydro-methyltestosterone		0.36				0.15			H_3PO_4,INH
Testosterone		0.46				0.19			H_3PO_4,INH
Substance S acetate			0.89						H_3PO_4,TTC
Cortisone acetate			0.44						H_3PO_4,TTC
Prednisone acetate			0.41						H_3PO_4,TTC
Cortisol acetate			0.30						H_3PO_4,TTC
Prednisolone acetate			0.17						H_3PO_4,TTC
Substance S				0.77	0.94				H_3PO_4,TTC
Cortisone				0.30	0.78				H_3PO_4,TTC
Prednisone				0.26	0.72				H_3PO_4,TTC
Cortisol				0.13	0.40				H_3PO_4,TTC
Prednisolone				0.09	0.26				H_3PO_4,TTC

Solvent systems:

Z-1 = formamide/*n*-hexane
Z-2 = formamide/*n*-hexane–benzene (1:1)
Z-3 = formamide/benzene
Z-4 = formamide/benzene–chloroform (1:1)
Z-5 = formamide/chloroform
GP-1 = propylene glycol/ligroin
N = petroleum/methanol–*n*-butanol–water (40:30:30)
P-1 = paraffin oil/methanol–water (95:5)

was dried in an oven at 90–100°, until the heavy fumes of formamide or glycols had disappeared. After cooling, the appropriate detection reagent was applied and, if necessary, the plate was heated again.

The solvent systems, R_F values and detection reagents are listed in Table I.

SUMMARY

A method of thin-layer partition chromatography using Celite No. 545 and Zaffaroni's solvent systems is described. This method has the advantage that the time of development is very short (3–7 min). The separation of steroids of widely differing polarities was satisfactory.

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THIN-LAYER CHROMATOGRAPHY OF CORTICOSTEROIDS

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In recent years the technique of thin-layer chromatography on glass plates has gained recognition as a highly useful analytical method in organic and biological chemistry (for reviews *cf.* refs.[1-8]). In view of the advantages[3] of this method over paper partition chromatography, its application to the field of corticosteroids, where the latter technique has been widely used[9-11], should be explored.

STÁRKA AND MALÍKOVÁ[12], using spread-layer chromatography (in which adsorbent without binder is spread dry on a glass plate) to separate pregnanediols and pregnanetriols, also reported R_F values for cortisone, corticosterone, 17α-hydroxyprogesterone, and progesterone, and ADAMEC, MATIS AND GALVÁNEK[13] employed the same technique for the separation of 11-desoxycortisol, cortisone, cortisol, tetrahydrocortisone, and tetrahydrocortisol. METZ[14] mentioned the use of thin-layer chromatography for separation of corticosteroids without giving any examples, and other workers[15-17] have reported R_F values for various corticosteroids.

In our opinion, the spread-layer technique offers no important advantages over the conventional thin-layer method, except for preparative purposes, and has the disadvantage that the layers are easily disturbed. Accordingly, we have investigated the application of thin-layer chromatography to the identification and separation of corticosteroids and have made some observations on the relation between structure and relative mobility.

EXPERIMENTAL

The Desaga–Brinkmann apparatus for thin-layer chromatography* was used, with Silica Gel G as adsorbent. The solvents were 99 Mol % pure or Spectranalyzed grades**, if available; otherwise reagent grades were used. Chromatograms were recorded by tracing or by photographing with a Polaroid Model 800 Land Camera*** mounted on a Polaroid Model 208 Copymaker***. The procedures described by BRENNER *et al.*[18-20] were used in preparing the chromatoplates and developing the chromatograms, with the following exceptions.

Preparation of plates

Since it is impossible to prepare uniform layers on 20 × 5 cm plates by the standard technique[20], a row of 20 × 20 cm plates was placed on the template and a length of

* Brinkmann Instruments Inc., Great Neck, N.Y.
** Fisher Scientific Co.
*** Polaroid Corp., Cambridge, Mass.

glass tubing, 5 mm in diameter, was placed between the long retaining ledge and the plates. The smaller plates were then aligned in rows of four, with their longer edges parallel to the long edge of the template, on top of the larger plates, placing a few drops of water under each small plate and sliding it back and forth until it adhered well to the larger plate. It is essential that the thickness of the plates within each row be uniform. Each plate was measured with a micrometer and its thickness marked on the underside with a diamond marking pencil.

Small plates have the advantages that they will not crack when heated strongly and, of course, require much less solvent for development. In the concentration range used in our work, up to eight samples can be placed on one plate.

The plates were allowed to dry overnight in an air-conditioned room at 25° and were then developed once with chloroform[21], ten at a time, in a 30.5 × 9.9 × 27.6 cm chamber. Plates were stored in a metal rack, protected from dust by a plastic cover, and all chromatograms were developed in the same air-conditioned room.

Samples were applied as 0.01% solutions in hexane, dichloromethane, or mixtures of these solvents. Since the lower limit of detection is 0.01 μg (less for some compounds), submicrogram quantities, usually 0.1 μg of each compound, were used in this work. It is advantageous to apply quantities of less then 1 μg, because samples can be spotted closer together and separations are better, but adequate separations can still be obtained in the 5 μg range. Some tailing was observed with 10 μg or higher amounts.

Development

The following solvent systems were used:

A: Chloroform–methanol–water, 188: 12: 1.

B: Chloroform–methanol–water, 485: 15: 1.

C: Chloroform–methanol–water, 90: 10: 1.

D: Ethyl acetate–chloroform–water, 90: 10: 1.

The volume of solvent system used for development was always 15 ml; it is important that this be kept constant[20]. The point of application was marked and a finish line, 10 cm from the starting points, was drawn in the layer with a needle. When the solvent front reached this line, its flow ceased, but the plate was kept in the chamber until the solvent penetration, which is easily observed visually, appeared uniform up to the line[18]. Development took from 25 to 30 min in Systems A, B, and C and 21 min in System D. The plates were then dried in the hood under an infrared lamp*; usually 2–3 min was sufficient.

Chromatoplates prepared on microscope slides, 25 × 75 mm and 50 × 75 mm, by the method of HOFMANN[22] were very convenient for exploratory work. The solvent systems used in this study rose a 50-mm distance in 10 min or less. PEIFER[23] has recently published a different method for preparing such small plates.

Detection

For the detection of spots 50% sulfuric acid was found to be the most useful spray. When working with submicrogram quantities, it is important that the entire surface of the plate be covered by the spray, but not to the extent that it appears wet.

* A. H. Thomas Co.

The John chromatographic spray bottle* is excellent for this purpose. After the spraying, the plates were heated on a hot plate in the hood and the spots were observed under long-wave ultraviolet light with an SL-3660 Mineralight*. A surface temperature of 175°–200° is necessary to reveal all the corticosteroid spots.

Another useful spray reagent is Bromthymol Blue solution[24], which gives white spots on a dark-blue background after exposure of the plate to ammonia vapors. This method does not require heating for the detection of spots, but is less sensitive, the lower limit being about 0.1 μg.

RESULTS AND DISCUSSION

Fig. 1 summarizes the results. Compounds 1–11, all Δ^4-pregnene derivatives having keto groups at positions 3 and 20, were completely separated by System A. While progesterone and pregnane-3,20-dione were not well separated in this system due to their proximity to the solvent front, a good resolution of these compounds was achieved in the less polar System B. Of particular interest is the separation of aldosterone, cortisone, and cortisol, since paper-chromatographic methods for effecting this separation are tedious. These three compounds were even better separated in the more polar System C, as shown in Fig. 1.

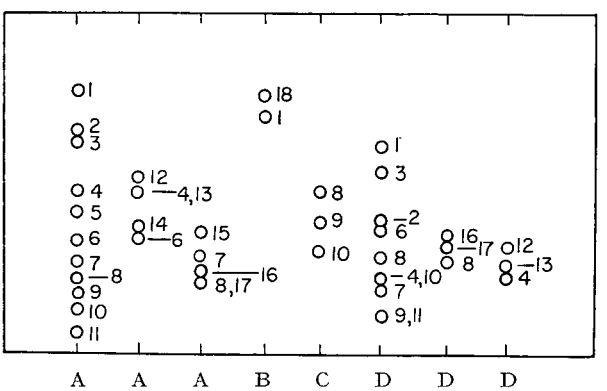

Fig. 1. Separation of corticosteroids in Systems A-D (see text). (1) Progesterone (Δ^4-pregnene-3,20-dione); (2) 17α-Hydroxyprogesterone (Δ^4-pregnen-17α-ol-3,20-dione); (3) 11-Desoxycorticosterone (Δ^4-pregnen-21-ol-3,20-dione); (4) 11-Dehydrocorticosterone (Δ^4-pregnen-21-ol-3,11,20-trione); (5) 11α-Hydroxyprogesterone (Δ^4-pregnen-11α-ol-3,20-dione); (6) 11-Desoxycortisol (Δ^4-pregnene-17α,21-diol-3,20-dione); (7) Corticosterone (Δ^4-pregnene-11β,21-diol-3,20-dione); (8) Cortisone (Δ^4-pregnene-17α,21-diol-3,11,20-trione); (9) Aldosterone (Δ^4-pregnen-18-al-11β,21-diol-3,20-dione); (10) Cortisol (Δ^4-pregnene-11β,17α,21-triol-3,20-dione); (11) 11-Epicortisol (Δ^4-pregnene-11α,17α,21-triol-3,20-dione); (12) Allopregnan-21-ol-3,11,20-trione; (13) Pregnan-21-ol-3,11,20-trione; (14) Pregnane-17α,21-diol-3,20-dione; (15) Allopregnane-11β,21-diol-3,20-dione; (16) Allopregnane-17α,21-diol-3,11,20-trione; (17) Pregnane-17α,21-diol-3,11,20-trione; (18) Pregnane-3,20-dione.

The positional isomers, compounds 2, 3, 5 and 6, 7 and the axial–equatorial 11-hydroxyl epimers 10 and 11 were separated in System A. In the two cases where analogous Δ^4, 5α, and 5β steroids were available for comparison (4, 12, 13 and 8, 16, 17), the Δ^4 and 5β steroids were not separated in System A, although the 5α com-

* A. H. Thomas Co.

pounds were separated from the other two. On the other hand, the Δ^4, 5β pairs 1, 18 and 6, 14 were separated. It is perhaps noteworthy that in the cases where separation did not occur in System A, an 11-keto group was present, while in the other two cases position 11 was unsubstituted. Separation of 4, 12, 13 and 8, 16, 17 was possible in System D; in both cases mobility was in the order $5\alpha > 5\beta > \Delta^4$.

The separation of Δ^4-pregnenes in System A was in line with the accepted concepts of "polarity"[9,11], except that compound 4, in spite of having one more keto group, moved faster than compound 5. In System D, however, the relative mobilities seemed to bear little or no relation to "polarities". Cortisol, for instance, differing from corticosterone only in having an extra OH group (17α), actually ran ahead of the latter. Cortisone and 11-dehydrocorticosterone, also differing by a 17α-OH group, followed the same pattern, but in the case of compounds 3 and 6 the 17α-hydroxy analog was more "polar".

The sequence reversals in Systems A and D should be valuable for confirming identities of compounds. Both solvent systems are suitable for the separation of the adrenocorticosteroids aldosterone, cortisone, and cortisol.

In many cases, chromatoplates on microscope slides gave adequate resolution in a short time. For instance, compounds 1, 2, 4, 5, 6, 7, 8, 10, and 11 were separated in 9 min using System A, and aldosterone was separated from cortisone and cortisol in 7 min in System D.

The time required for spots to appear after spraying with 50 % sulfuric acid may aid in their identification. Cortisol appeared almost as soon as heating began, while cortisone and 11-dehydrocorticosterone, requiring a much higher temperature, were the last to appear. When microgram quantities were used, the spots showed characteristic colors when sprayed with this reagent and heated.

Although R_F values in thin-layer chromatography are reproducible when all variables are controlled[20], they are very likely to vary from one laboratory to another. This is especially true when, as in this work, the chromatoplates have been kept in equilibrium with the atmosphere of the room. Thus, no useful purpose would be served by reporting R_F values. Correlation between results under different conditions can usually be established by including one or two reference compounds per plate.

ADAMEC, MATIS AND GALVÁNEK[13] found that their spread-layer chromatographic method for corticosteroids gave satisfactory results with urinary extracts, and they expressed the opinion that it should be applicable to quantitative analysis. MANGOLD[5] has listed the methods used for quantitative evaluation of thin-layer chromatograms and has commented on the compromise between accuracy and practicality. The speed and economy inherent in thin-layer chromatography certainly warrant further exploration of its applicability to the analysis of biologically important steroids.

SUMMARY

The separation of eighteen corticosteroids and pregnane derivatives in submicrogram quantities by thin-layer chromatography is reported. Positional isomers, axial–equatorial hydroxyl epimers, and A/B *cis–trans* isomers were successfully resolved. Rapid separation of aldosterone, cortisone, and cortisol was achieved. Reversal of some relative mobilities was observed with two solvent systems.

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THIN-LAYER CHROMATOGRAPHY OF STEROIDAL SAPOGENINS

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For the separation of steroidal sapogenins by paper chromatography, SANNIÉ AND LAPIN[1] used mixtures of petroleum ether, chloroform, and acetic acid, HEFTMANN AND HAYDEN[2] petroleum ether–toluene–ethanol–water systems, and MCALEER AND KOZLOWSKI[3] and WALL *et al.*[4] nonaqueous stationary phases, such as formamide, phenylcellosolve, or propylene glycol. The shortcomings of the aqueous systems are failure to attain equilibrium conditions and tailing, whereas the nonaqueous systems are technically more complicated[5] and require several hours for development. Furthermore, the separation of isomers differing in the configuration of the C-25 methyl group by paper chromatography presents difficulties. Although a good separation between smilagenin and sarsapogenin had been reported[2], the failure of OKANISHI *et al.*[6] to reproduce this result has led us to the discovery that our observation was due to an impurity in the sample of sarsapogenin*. SANNIÉ AND LAPIN[1] have also reported the separation of sarsapogenin from smilagenin, but later observations[6–8] showed little or no differences in their R_F values in the same systems.

Recently, VANDENHEUVEL AND HORNING[9] have applied gas–liquid chromatography to the separation of sapogenins. Their method is not capable of resolving C-25 isomers, and the separation of diosgenin from tigogenin is not sharp.

Thin-layer chromatography has been employed in the separation of unknown sapogenins and in the identification of neotigogenin, tigogenin, diosgenin, and gitogenin by SANDER[10–13] and in work on the structure of convallamarogenin by TSCHESCHE, SCHWARZ AND SNATZKE[14]. CARRERAS MATAS[15] impregnated thin-layer plates of silica gel with formamide and used them for partition chromatography of steroidal saponins. HEŘMÁNEK *et al.*[16,17] and ČERNY *et al.*[18] have reported R_F values of some sapogenins for spread-layer chromatography.

In the course of our work on the biosynthesis of diosgenin[19] we have found it necessary to separate small amounts of sapogenins, especially the C-25 isomers. Both partition and adsorption chromatography on thin layers were found eminently suitable to this purpose.

EXPERIMENTAL

Except as described below, chromatograms were prepared and developed as in the preceding paper[20]. Trifluoroacetates of sapogenins were prepared as follows: To 0.2

* Due to a typographical error, Table II, Ref.[2] shows "Yamogenin acetate"; this should read "Yamogenin".

ml of 0.01–0.1% solution of the sapogenins in hexane or dichloromethane, 2 μl of trifluoroacetic anhydride was added. After thorough shaking for 1 min, 1 ml of 2 *N* aqueous sodium carbonate was added and the mixture was again thoroughly shaken. Samples were spotted directly from the organic layer.

Preparation of plates

Unless otherwise specified, Silica Gel G was used as adsorbent. The Kieselguhr G* plates used for partition chromatography were not conditioned by development with chloroform. The silica gel–kieselguhr mixture was prepared by shaking 15 g of Silica Gel G and 15 g of Kieselguhr G in a stoppered flask for a few seconds, adding 60 ml of water, and shaking for 1 min. These plates were conditioned with chloroform.

Development

The following solvent systems were used (minutes required for development in parentheses):

A: Dichloromethane–methanol–formamide, 93:6:1 (28).
B: Toluene–ethyl acetate–formic acid, 57:40:3 (36).
C: Cyclohexane–acetone, 1:1 (26).
D: Cyclohexane–ethyl acetate–water, 600:400:1 (29).
E: Cyclohexane–ethyl acetate–water, 1000:1000:3 (28).
F: Chloroform–methanol–water, 485:15:1 (26).
G: Chloroform–methanol–water, 188:12:1 (28).
H: Chloroform–toluene, 9:1 (34).
I: Hexane–toluene–ethanol–water, 100:50:5:45 (42).
J: Hexane–ethanol–water, 40:3:7 (30).

Detection

Detection of sapogenins with 50% sulfuric acid was possible at a much lower temperature than in the case of corticosteroids[20], 120°–140° being sufficient. The lower limit of detection for diosgenin was 0.005 μg under ultraviolet light, while saturated sapogenins could be detected in 0.01 μg quantities in Systems A-H.

Partition chromatography

To ensure equilibrium conditions inside the chromatography jar, the wall of the cylindrical chamber (229 mm high × 60 mm diameter)* was lined with two strips of filter paper, 15 × 4 cm; one strip dipped into the solvent system (15 ml), the other one was impregnated with water and suspended on the opposite side without touching the solvent. This was accomplished by use of a steel paper clip held in place by an external magnet. Phase equilibrium in the chamber was attained within 6 min.

Usually 0.02 μg of each compound was applied to a Kieselguhr G plate as a 0.002% solution in hexane. Up to four samples may be spotted in the middle 3 cm of the plate, 1 cm apart. A finish line was drawn 15 cm from the starting points.

The plate was impregnated with water by supporting it, adsorbent side down, across the top of a 3-l beaker of boiling water until it was thoroughly wet. It was then placed in the hood, and, as soon as the water began to recede at the corners of the

* Brinkmann Instruments, Inc., Great Neck, N.Y.

layer, the plate was transferred to the developing chamber. It was placed in the chamber with the adsorbent layer facing the solvent-saturated liner.

RESULTS AND DISCUSSION

Preliminary experiments showed that sapogenins differing in number and/or kind of polar groups were easily separable in solvent systems commonly used in thin-layer chromatography. However, the C-25 isomers were not separated, and resolution of diosgenin–tigogenin mixtures was poor. Partition systems of the type used for paper chromatography[2] were then tried in an effort to achieve these separations. When Silica Gel G layers were used with these systems, the monohydroxysapogenins remained near or at the origin. Although impregnation of the chromatoplates with water resulted in greater mobilities, uniform impregnation of the layers could not be achieved and movement of the sapogenins was erratic. However, Kieselguhr G plates, impregnated with water, gave reproducible separations of monohydroxysapogenins. The amount of water in the layer is a critical factor; too much water causes enlargement of the spots and poor separation, while tailing occurs when the plates are too dry. The R_F values are also dependent on the water concentration and therefore exact reproducibility of mobilities is difficult. However, the pattern of separation is not affected by changes in mobilities due to variations in water content. As Fig. 1 shows, smilagenin, tigogenin, and diosgenin were separated by System I, but the C-25 isomers, smilagenin–sarsapogenin and tigogenin–neotigogenin were

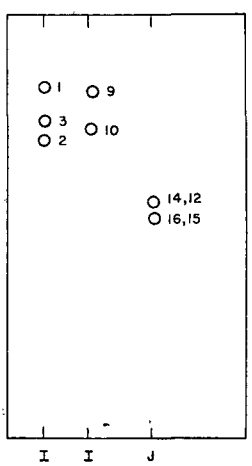

Fig. 1. Separation of sapogenins by partition chromatography on Kieselguhr G (for solvent systems A-J see text). (1) Smilagenin (5β,25α-spirostan-3β-ol); (2) Diosgenin (25α-Δ^5-spirosten-3β-ol); (3) Tigogenin (5α,25α-spirostan-3β-ol); (4) Gentrogenin (25α-Δ^5-spirosten-3β-ol-12-one); (5) Hecogenin (5α,25α-spirostan-3β-ol-12-one); (6) Isochiapagenin (25α-Δ^5-spirostene-3β,12β-diol); (7) Kryptogenin (25α-Δ^5-cholestene-3β,26-diol-16,22-dione); (8) Chlorogenin (5α,25α-spirostane-3β,6α-diol); (9) Sarsapogenin (5β,25β-spirostan-3β-ol); (10) Neotigogenin (5α,25β-spirostan-3β-ol); (11) 3-Episarsapogenin (5β,25β-spirostan-3α-ol); (12) Tigogenin acetate; (13) Diosgenin acetate; (14) Smilagenin acetate; (15) Neotigogenin acetate; (16) Sarsapogenin acetate; (17) Gentrogenin acetate; (18) Hecogenin acetate; (19) Kryptogenin acetate; (20) 3-Episarsapogenin acetate; (21) Tigogenin trifluoroacetate; (22) Diosgenin trifluoroacetate; (23) Smilagenin trifluoroacetate; (24) Sarsapogenin trifluoroacetate; (25) Neotigogenin trifluoroacetate; (26) Gentrogenin trifluoroacetate; (27) Hecogenin trifluoroacetate.

not resolved, although their relative mobilities differed slightly. In the form of their acetates, the C-25 isomers could be separated in System J, but in this system the acetates of the 5α–5β pairs tigogenin–smilagenin and neotigogenin–sarsapogenin were not separated. When the chromatoplates were impregnated with the aqueous phase of System I by the technique of BRENNER, NIEDERWIESER AND PATAKI[21], results were similar to those obtained on plates impregnated with water, except that the C-25 isomers were better separated and the resolution of diosgenin and tigogenin was poorer.

The use of System I seems to be restricted to monohydroxysapogenins, as the hydroxyketo compound gentrogenin remains at the origin, while sterols move with the solvent front. A drawback of Systems I and J is the limited concentration range; the lower limit of detection is 0.02 μg, while amounts greater than 0.2 μg give spots too large for good resolution.

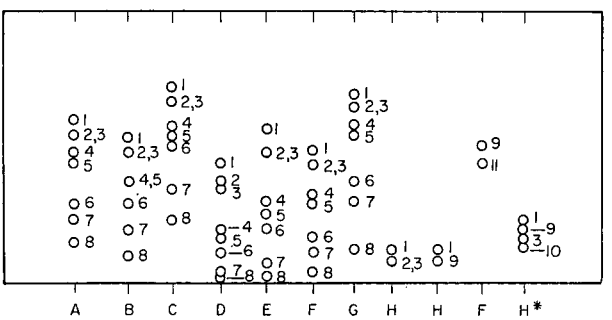

Fig. 2. Separations of sapogenins on Silica Gel G (for legend see Fig. 1). * Kieselguhr G–Silica Gel G plate.

Separations of various sapogenins on Silica Gel G are shown in Fig. 2. The A/B *cis–trans* isomers smilagenin and tigogenin were well separated in Systems A-G, and the axial–equatorial epimers sarsapogenin and 3-episarsapogenin were well resolved in System F. The wide separation of the dihydroxysapogenins isochiapagenin* (3β,12β) and chlorogenin (3β,6α) shows the sensitivity of this method in differentiating positional isomers. It is interesting that gentrogenin and hecogenin were separated by most of these systems, while diosgenin and tigogenin, having the same structural difference, were not. Only in System D was there any significant separation of diosgenin from tigogenin.

The C-25 isomers showed no differences in relative mobility in Systems A-G. In System H, however, separation between smilagenin and sarsapogenin occurred, even though they had moved only a short distance from the origin. When the mobilities were increased by the addition of as little as 1% of methanol to System H, a mixture of the isomers was no longer resolved. This left only two alternatives for increasing mobilities: decreasing the activity of the adsorbent or decreasing the polarity of the sapogenins.

When Silica Gel G was replaced by Kieselguhr G, the relative mobilities of the C-25 isomers increased and showed a considerable difference, but resolution was vitiated by tailing. However, on plates made from a 1:1 mixture of Kieselguhr G

* Prepared by reduction of gentrogenin with sodium borohydride[22].

and Silica Gel G, tailing was eliminated, while mobilities exhibited a satisfactory increase. Fig. 2 (H) shows the separation of two pairs of C-25 isomers.

The other approach to increasing mobilities was also successful. The acetates of the C-25 isomers were even better separated than the free sapogenins on Silica Gel G in System H. Corresponding 5α-, 5β-, and Δ^5-sapogenins were also separated in the form of their acetates, as were the 3α- and 3β-OH epimers. Fig. 3 shows the separation of the acetates of six monohydroxysapogenins in System H. It should be noted that the sequence of the A/B *cis–trans* isomers is reversed in the case of the

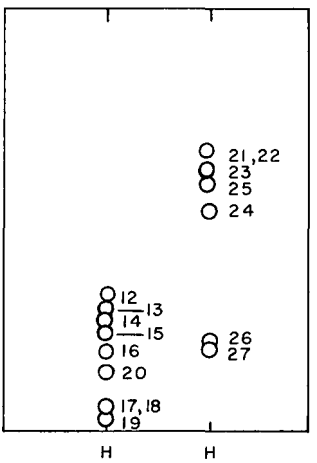

Fig. 3. Separation of sapogenin acetates and trifluoroacetates on Silica Gel G (for legend see Fig. 1).

acetates. Separation of monohydroxysapogenin acetates occurred only in System H or other solvent mixtures of low polarity, *e.g.*, chloroform–carbon tetrachloride and cyclohexane–benzene. In more polar systems, containing ethyl acetate or methanol, these acetates had equal relative mobilities.

For the rapid analysis of sapogenin mixtures it would be helpful if they could be esterified directly on the plate. This is not possible in the case of acetylation, but it was found that trifluoroacetic anhydride reacts almost instantaneously with 3-hydroxy steroids. The trifluoroacetates can be prepared by spotting the anhydride directly over compounds on the plate, but drying in the hood for several minutes is necessary to remove the trifluoroacetic acid formed as a by-product. It is therefore usually preferable to prepare this derivative as indicated in the Experimental Part. Fig. 3 shows the separation of a mixture of sapogenin trifluoroacetates in System H. The lesser polarity of these derivatives in comparison with the acetates is an added advantage.

There is a considerable difference in the reactivity of the Δ^5- and the saturated sapogenins toward the 50% sulfuric acid spray. The former quickly show a bright fluorescence, while the latter take much longer and never appear as intense. When microgram quantities are applied, the Δ^5-compounds are bright red or orange and become purple, while the saturated compounds are pale yellow and become brown.

The useful concentration range of the sapogenin method is greater than that for corticosteroids[20]. Except for the partition systems I and J, no tailing was observed with 50 μg quantities, and some separations were still possible at this level.

In the application of this method to unknown sapogenin mixtures, one of the polar Systems A-G should be used first, to give a separation into classes according to the number and kind of polar groups. This may be followed by a nonpolar system such as H to show differences at C-25, preferably after acylation. The partition system I is the most suitable for distinguishing between diosgenin and tigogenin. The combined use of several of the systems given will permit the identification of any of the monohydroxysapogenins, even if available only in microgram quantities.

SUMMARY

The application of thin-layer chromatography to the separation of mixtures of steroidal sapogenins and their esters has been investigated. Not only sapogenins differing in the number and kind of polar groups, but also those differing in A/B ring junction and in configuration of hydroxyl and C-25 methyl groups have been resolved. A rapid method for partition chromatography on thin layers of Kieselguhr G was developed. Mixed silica gel–kieselguhr layers were also found useful, as was a method for acylating hydroxyl groups directly on the thin-layer plate.

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THIN-LAYER CHROMATOGRAPHY OF STEROLS

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Although thin-layer chromatography has only come into widespread use in the past four years, it has already gained recognition as a valuable tool in the analysis of steroids. Among the classes of steroids to which thin-layer and spread-layer chromatography have been applied are: estrogens[1–6], androgens[4–10] and other C_{19}-steroids[8,9,11,12], corticosteroids[4,9,12–16] and other C_{21}-steroids[4,5,7,9,11,12,14,16,17], cardenolides[18,19] and cardiac glycosides[19–21], etianic acid derivatives[4,13], bile acids and their esters[5,22–24], sterols[4,5,7,9,11,13,17,25,26] and cholesterol esters[4,5,7,27–31], cholestanones[13,32], sapogenins[4,5,9,33–38] and saponins[20], alkaloids[4,5,9,33,35,39], and aromatized steroids[40].

An examination of the above references reveals that separation by thin-layer chromatography is relatively easy where differences exist in the kind, number, position, or configuration of polar groups, but difficult in the absence of such differences. In certain cases a difference in substitution on a carbon atom adjacent to a polar group is sufficient to make separation possible (*e.g.*, progesterone and pregnane-3,20-dione[16]), and the resolution of A/B *cis–trans* isomers having a polar group in position 3 can be accomplished.

The scarcity of data concerning the influence on separability of structural differences remote from polar groups has led us to make a study of such effects. The eight 3β-sterols selected for this investigation differ only in Ring B and/or in the side chain and are of considerable biological interest.

EXPERIMENTAL

Except as described below, chromatograms were prepared and developed as in our previous papers[16,38].

Silica Gel G plates were used for all solvent systems except B, where Silica Gel G–Kieselguhr G (1:1)[38] was the adsorbent.

The composition of the solvent systems was as follows (minutes required for development in parentheses):

A: Cyclohexane–ethyl acetate–water, 600:400:1 (29).

B: Cyclohexane–heptane, 1:1 (25).

C: Cyclohexane–ethyl acetate–water, 1560:440:1 (34).

D: Isooctane–carbon tetrachloride, 19:1 (29).

Sterols were applied in 0.1 μg quantities.

RESULTS AND DISCUSSION

As previously reported[4,5,9,17], the mixtures of hydrocarbons with more polar solvents, commonly used for thin-layer chromatography, failed to separate 3β-sterols differing only by degree of unsaturation or number of carbon atoms in the side chain. Thus, a mixture of cholesterol, stigmasterol, β-sitosterol and desmosterol moved as a single spot in System A (see Fig. 1), and ergosterol and 7-dehydrocholesterol likewise failed to separate.

In view of our previous finding that isomers of sapogenins differing only in the configuration of a C-25 methyl group are resolved by mixtures of nonpolar solvents[38], solvent systems of this type were tested for the thin-layer chromatography of sterols. Mixtures of cyclohexane or isooctane with a series of solvents in descending order of polarity were examined, using cholestane and Δ^{16}-cholestene* as model compounds. While no separation was achieved with chloroform, toluene, or benzene, these hydrocarbons were resolved when carbon tetrachloride was used (System D, Fig. 1).

However, the four sterols differing in the side chain were not separated in systems containing carbon tetrachloride. A resolution of the pairs with saturated and unsaturated side chains β-sitosterol–stigmasterol and cholesterol–desmosterol was finally effected by a mixture of saturated hydrocarbons (System B, Fig. 1). Because

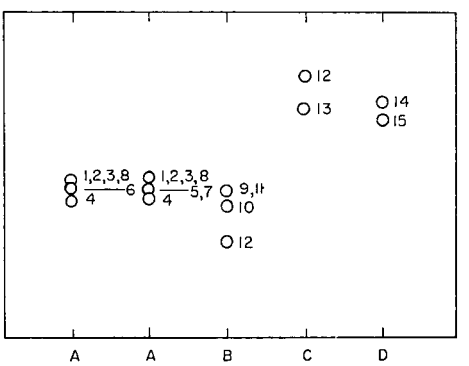

Fig. 1. Separation of sterols and sterol esters (for solvent systems A-D see text). (1) Cholesterol (Δ^5-cholesten-3β-ol); (2) Stigmasterol (24α-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol); (3) β-Sitosterol (24α-ethyl-Δ^5-cholesten-3β-ol); (4) Δ^7-Cholesten-3β-ol; (5) Ergosterol (24β-methyl-$\Delta^{5,7,22}$-cholestatrien-3β-ol); (6) Cholestan-3β-ol; (7) 7-Dehydrocholesterol ($\Delta^{5,7}$-cholestadien-3β-ol); (8) Desmosterol ($\Delta^{5,24}$-cholestadien-3β-ol); (9) Cholesterol trifluoroacetate; (10) Stigmasterol trifluoroacetate; (11) β-Sitosterol trifluoroacetate; (12) Desmosterol trifluoroacetate; (13) Desmosterol acetate; (14) Cholestane; (15) Δ^{16}-Cholestene.

of the very low polarity of this system, appreciable mobilities were only obtained by chromatographing the sterols in the form of their trifluoroacetates on Kieselguhr G–Silica Gel G (1:1)[38]. A separation of cholesterol acetate and desmosterol acetate under similar conditions has recently been reported by MILLER, HAMILTON AND GOLDSMITH[41], who used glass paper impregnated with silicic acid as the adsorbent and isooctane as the developing solvent.

Even in System B an alkyl substituent in the side chain has no influence on mobility, as is shown by the failure of cholesterol trifluoroacetate and β-sitosterol

* Generously supplied by Dr. G. V. NAIR.

trifluoroacetate to separate. Thus, the large difference in mobilities between stigmasterol trifluoroacetate and demosterol trifluoroacetate can only be due to the difference in position of the double bond.

In contrast to sterols with different side chains, compounds differing in the degree and/or position of unsaturation in ring B were separable in polar systems (System A, Fig. 1). The greatest separation occurred between cholesterol and Δ^7-cholesten-3β-ol, but the latter sterol and cholestan-3β-ol, differing only by a double bond four carbon atoms removed from the hydroxyl group, were also separated, as were cholesterol and 7-dehydrocholesterol. It is interesting that 7-dehydrocholesterol, in spite of having one more double bond than Δ^7-cholesten-3β-ol, shows greater mobility. The difficulty of separating 5α- from corresponding Δ^5-steroids is again evident here, in the case of cholesterol and cholestan-3β-ol.

The trifluoroacetates of sterols 1–8 were not separable in polar systems. This is in agreement with our observations on sapogenin acetates[38]. However, as Fig. 1 shows, resolution of desmosterol acetate from its trifluoroacetate was possible in the polar system C. This suggests that while separations based on differences in the acidic portion of the ester are possible in both polar and nonpolar systems, only the latter are suitable for resolution on the basis of differences in the alcohol portion. The literature on the separation of cholesterol esters[4,5,7,27,28,31] also indicates that separations on the basis of differences in the acid portions are usually feasible.

The general applicability of the 50 % sulfuric acid spray was demonstrated by its ability to reveal even a saturated hydrocarbon, cholestane, in a concentration of 0.1 μg. A temperature of about 200° was necessary in this case, but for the sterols about 120° was usually sufficient.

Further work is needed before a systematic correlation of structural differences with separability is possible, but our results show that thin-layer chromatography is capable of rather subtle discriminations when the proper conditions are chosen. Our failure to separate sterols differing only by alkyl substituents in a saturated side chain may reflect a limitation of the method. Undoubtedly further improvements in the separation of the biologically important sterols differing in degree of unsaturation in ring B will be possible by experimenting with other systems, although no solvents of the type of System B could be found to give better resolution.

SUMMARY

3β-Sterols differing in unsaturation in ring B and in the side chain were separated by thin-layer chromatography. Differences in resolving power between polar and nonpolar systems were observed.

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SEPARATION OF GALLIC ACID AND ITS ESTERS ON THIN LAYERS OF POLYAMIDE POWDER

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In recent years gallic acid and its esters have found increasing application as stabilizers of oils and fats, especially those of animal origin. These substances are preferred in cases when a prolonged storage period of the prepared fat before its consumption is anticipated. In most countries only certain specified antioxidants in very low concentrations (0.01–0.05%) are permitted. Consequently reliable methods for detection and determination of these substances appeared necessary.

In the methods generally used, the isolation of antioxidants from oil or fat is effected by extraction with dilute alcohol or another suitable organic solvent, and the extracts thus obtained separated by chromatography. A series of paper chromatographic methods have been described, *e.g.* ZIJP[1], GANDER[2], DEHORITY[3], and others. Acetylated paper, or a paper impregnated with a fixed, less polar phase, usually paraffin or olive oil is employed. One disadvantage of these paper chromatographic methods lies in the prolonged development time of the chromatogram, usually about 3–9 hours, according to the complexity of the sample to be separated. Antioxidants can be detected much more quickly using thin-layer chromatography. The method was first used by SEHER[4,5], who succeeded in separating some antioxidant mixtures on a thin layer of silica gel. The same author separated mixtures of even 10 antioxidants using two-dimensional chromatography.

In our laboratory a method using chromatography on thin layers of polyamide powder has recently been elaborated[6]. Polyamide had already been used as a chromatographic adsorbent, especially for the isolation of phenolic substances. The advantages of polyamide chromatography lie not only in the high adsorption capacity, but also in the reversibility of the sorption process which allows the use of this adsorbent for analytical purposes. We used thin-layer chromatography on polyamide powder some time ago for separation of some flavonoids and antioxidants[7,8], and in the present paper the chromatographic separation of gallic acid and its esters is described.

EXPERIMENTAL

Solutions of gallic acid and its methyl, ethyl, *n*-propyl, lauryl, and *n*-octyl esters were prepared in a concentration of about 1% in pure methanol.

As the solvent system the following solvents or their mixtures were used: methanol, anhydrous ethanol, *n*-butanol, acetone, petroleum ether (b.p. 60–70°), ether, benzene, chloroform and carbon tetrachloride.

TABLE I

R_F VALUES OF GALLIC ACID AND ITS ESTERS

Chromatographic system	R_F*					
	GA	*MG*	*EG*	*PG*	*OG*	*LG*
Methanol	0.32	0.60	0.70	0.62	0.71	0.62
Ethanol	0.31	0.53	0.67	0.55	0.80	0.80
Chloroform	0.0	0.0	0.0	0.0	0.09	0.12
Carbon tetrachloride	0.0	0.0	0.0	0.0	0.0	0.08
Ether	0.04	0.07	0.13	0.28	0.45	0.67
Butanol–acetic acid–water (4:1:5)	0.28	0.60	0.70	0.68	0.89	0.89
Carbon tetrachloride–methanol (7:3)	0.06	0.29	0.39	0.46	0.63	0.80
Carbon tetrachloride–ethanol (7:3)	0.05	0.19	0.26	0.44	0.62	0.80
Carbon tetrachloride–methanol (3:2)	0.10	0.39	0.52	0.52	0.70	0.77
Carbon tetrachloride–ethanol (3:2)	0.08	0.33	0.44	0.47	0.73	0.85

* GA = gallic acid; MG = methyl gallate; EG = ethyl gallate; PG = propyl gallate; OG = octyl gallate; LG = lauryl gallate.

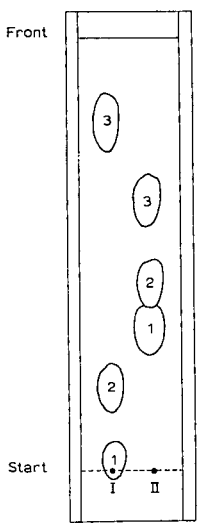

Fig. 1. Chromatogram of a mixture of gallic acid and its esters. Solvent system: carbon tetrachloride–ethanol (7:3). Development time: 45 min. Temperature: 20°. Polyamide powder graining: 0.15 mm. Layer thickness: 1 mm. (I) Spot No. 1 = gallic acid; 2 = methyl gallate; 3 = lauryl gallate. (II) Spot No. 1 = ethyl gallate; 2 = propyl gallate; 3 = octyl gallate.

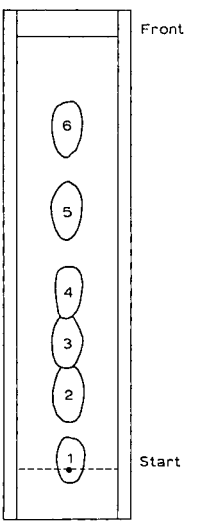

Fig. 2. Chromatogram of a mixture of gallic acid and its esters. Solvent system: carbon tetrachloride–ethanol (7:3). Development time: 50 min. Temperature 20°. Polyamide powder graining: 0.15 mm. Layer thickness: 1 mm. Spot No. 1 = gallic acid; 2 = methyl gallate; 3 = ethyl gallate; 4 = propyl gallate; 5 = lauryl gallate; 6 = octyl gallate.

The polyamide powder used was the commercial product manufactured by the firm Severočeské chemické závody Lovosice, n.p., factory "Rudník". Before use the product was sieved through standard sieves. The optimal grain size for the preparation of the plates is 0.1–0.2 mm.

The chromatographic plates were prepared according to DAVÍDEK AND PROCHÁZKA[6] by applying the polyamide powder to plates of sheet glass 60 × 350 mm, and spreading it roughly with a spatula. Perfect smoothness was achieved by carefully rolling the layer with a simple roller consisting of a glass rod fitted at both ends with a piece of rubber tubing. The breadth of the prepared plate was controlled by the distance between the two pieces of tubing, and the thickness of the chromatographic layer depended on the wall thickness of the tubing.

The sample was applied, taking care not to damage the layer (holding the micropipette at a distance of about 1–2 mm). After the sample had dried the plate was placed into the chamber at an angle of 20–30°, and was developed by the ascending technique until the solvent front had penetrated for some 30 cm (or less, according to the complexity of the mixture to be separated). The wet plate was sprayed in the usual manner with the reagent; this operation, however, required great care in order to avoid disturbing the thin layer by the stream of spray mist.

DISCUSSION

In preliminary experiments we found this method suitable for the separation of gallic acid and its esters. It was first necessary to establish the simplest solvent systems which would give separation of the greatest possible number of the gallates examined. The results of these experiments are shown in Table I and Figs. 1 and 2.

The most advantageous solvent systems for the separation of gallic acid and its esters were mixtures of methanol or ethanol with carbon tetrachloride, in various ratios. Best results were obtained with the mixtures ethanol–carbon tetrachloride, 3:7. Using this system, all the substances studied could be separated.

CONCLUSION

The newly elaborated method for the separation of gallic acid and its esters on a thin layer of polyamide powder shows several advantages, particularly in speed, in which it surpasses all previous methods. The preparation of the chromatographic plates is very rapid and simple, and the time required for development even of a very complex mixture (6 components) does not in any case exceed 60 minutes. The method is feasible in any routine laboratory without special and expensive equipment.

SUMMARY

Chromatographic separation of gallic acid and its esters on a thin layer of polyamide powder has been described. The method is rapid, convenient and feasible even in laboratories with minor equipment. The method was successfully tested in the separation of substances isolated from stabilized fats.

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THIN-LAYER CHROMATOGRAPHY OF THIOPHENE DERIVATIVES

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While there are several accounts[1-4] of the application of normal column chromatographic techniques to the separation of thiophene derivatives, there has been no extensive use[5] of qualitative techniques such as paper chromatography. This is probably due to the lack of suitable methods for the detection of these compounds. Most of the sensitive tests[6,7] for the presence of thiophenes require the use of conc. sulphuric acid to catalyse a condensation with a carbonyl component. The reaction with isatin to give "indophenines" is typical.

The presence of sulphuric acid in these tests has made the use of paper chromatography difficult but this does not apply to thin-layer chromatography[8,9] for qualitative separations. The use of aggressive reagents for detection in this method is well known.

In connection with other work in progress, qualitative separations were required and a range of standard thiophenes and derivatives have, therefore, been tested by this technique.

GENERAL PROCEDURE

Thin-layer chromatographic apparatus from Desaga G.m.b.H. was used with Alumina G and Silica G (Merck) as adsorbents in films of 275 μ thickness. The plates were activated by heating at 120° for one hour immediately before use and the thiophenes (ten on each plate) were then added with a micro-pipette in the usual way as solutions in chloroform (50 γ of thiophene derivative in 25 μl of solvent). After equilibrating in the tank for 30 min the plates were irrigated with petroleum ether (b.p. 40–60°), benzene–chloroform (9:1) or methanol over a period of approximately 30 min at room temperature. Some of the thiophenes tested were volatile under these conditions but this difficulty was overcome by carrying out the complete operation in a cold room at 4°.

When the chromatogram was complete the spots were detected by fluorescence in U.V. light (2450 Å) followed by spraying with a solution of isatin (0.4 %) in conc. sulphuric acid. Other carbonyl reagents (ninhydrin, benzil and phenanthraquinone) were tested and also gave positive results but showed no advantages. After examination the plates were heated for a few minutes at 120° when colour reactions which had not occurred at room temperature were observed and spots already visible at room temperature often showed characteristic colour changes.

RESULTS AND DISCUSSION

We have found that non-polar thiophenes can be separated on alumina by elution with petroleum ether, thiophenes of moderate polarity on silica by elution with benzene–chloroform (9:1) and the very polar thiophene derivatives by methanol on silica. There does not seem to be any characteristic colour produced by the thiophenes tested but there is a predominance of blue and violet colours. Only two thiophenes failed to react with the reagent but this effect was only observed on silica plates; positive reactions were obtained on alumina. There were small variations in the R_F values recorded but variation is often encountered with this technique and was overcome by running a standard compound on all chromatograms. α-Terthienyl was suitable for this purpose.

TABLE I

R_F VALUES AND COLOUR REACTIONS

Compound	*Concentration (γ)*	*Colour under U.V.*	*Colour with isatin*	R_F × 100
Adsorbent: Alumina G		*Solvent: Petroleum ether (b.p. 40–60°)*		
2-Methylthiophene*	50	—	Yellow → green blue	76
3-Methylthiophene*	300	—	Blue	82
2-Ethylthiophene*	300	—	Deep yellow	92
2,5-Dimethylthiophene*	300	—	Pink	95
2,3,5-Trimethylthiophene*	50	—	Violet → pink	87
2,3-Dimethyl-4-ethylthiophene*	50	—	Brown	84
Tetramethylthiophene*	50	—	Violet → pink	89
Methyl 3-thienyl sulphide*	50	—	Orange	92
n-Decyl 3-thienyl sulphide*	50	—	Pale violet	96
Bithienyl[1]	50	Violet	Blue	80
α-Terthienyl[1]	50	Yellow	Wine red → blue green	57
α-Quaterthienyl[1]	50	Lime green	Pale green	26
5,5′-Dichlorobithienyl[10]	50	Lime green	Violet	89
5,5′-Dimethylbithienyl[10]	50	Violet	Yellow	76
α-Phenyl-α-bithienyl[10]	50	Yellow	Green blue	50
5,5′-Diphenylbithienyl[10]	50	Blue	Wine red → mauve	16
5,5″-Dimethylterthienyl[10]	50	Yellow	Wine red → mauve	48
Adsorbent: Silicagel G		*Solvent: Benzene–chloroform (9:1)*		
Thiophene-carboxaldehyde	300	Crimson	— (Yellow**)	34 (84)**
2-Nitrothiophene	300	Crimson	— (Pale blue**)	61 (91)**
2-Benzoylthiophene	300	Crimson	Violet (120°)	39
2-Acetylthiophene	300	Crimson	Violet (120°)	25
Adsorbent: Silicagel G		*Solvent: Methanol*		
Thiophene-carboxylic acid	150	Crimson-violet	Grey → blue (120°)	65
β-(α-Thienyl)-acrylic acid	150	Crimson-violet	Violet	57
4-(α-Thienyl)-butyric acid	150	Crimson-violet	Orange	60
Bithienyl-5-carboxylic acid[10]	50	Deep violet	Yellow	63
2,2′-Bithienyl-methylamine hydrochloride	50	Pale violet	Violet	35

* Volatile samples run at 4°.

** When run at the same concentration on alumina and eluted with benzene–chloroform (9:1) these samples gave the results indicated (*e.g.* yellow).

The lowest concentrations of the various thiophenes which could be detected on the plates after development varied between different compounds and also depended on the solvent system and the adsorbent being used. The concentrations recorded are those which produce an intense spot and do not represent the lower limits of detection. Later work[11] indicates that the silica-gel/benzene–chloroform system is of more general application than is represented by the results given in Table I.

ACKNOWLEDGEMENTS

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One of us (G.T.P.) wishes to acknowledge the receipt of a post-graduate D.S.I.R. studentship.

SUMMARY

A method for the qualitative separation of various thiophene derivatives by means of thin-layer chromatography is described.

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Short Communications

Dünnschicht-chromatographische Trennungen von Aminosäuren an Cellulose-Schichten

Die Dünnschicht-Chromatographie (DC) hat sich auf Grund hervorragender und der Papier-Chromatographie (PC) in mancherlei Hinsicht überlegenen Trennungen kleinster Mengen von Substanzgemischen innerhalb sehr kurzer Zeit einen festen Platz in der Chromatographie gesichert. Über Technik und praktische Durchführung der DC ist inzwischen in zahlreichen Arbeiten berichtet worden, so dass der Hinweis auf einige zusammenfassende Übersichten[1–3] genügen dürfte.

Obschon zahlreiche Methoden zur papier-chromatographischen Trennung von Aminosäuren bekannt sind, erschien es sinnvoll festzustellen, ob die bekannten Vorzüge der DC auch bei Aminosäuretrennungen auftreten und zwar besonders hinsichtlich der Trenndauer und der Empfindlichkeit. MUTSCHLER UND ROCHELMEYER[4] trennten Aminosäuren auf gepufferten Kieselgel-Schichten; BRENNER UND NIEDERWIESER[5] führen Aminosäuretrennungen auf normalen, d.h. ungepufferten Kieselgel-Schichten durch und halten eine Pufferung für überflüssig.*

Ziel der vorliegenden Arbeit ist die systematische Untersuchung der Trennmöglichkeiten von Aminosäuren auf Cellulose-Schichten. TEICHERT, MUTSCHLER UND ROCHELMEYER[6] haben schon kurz auf diese Trennmöglichkeiten hingewiesen, jedoch ohne Angabe von Einzelheiten.

Praktische Durchführung

Die Herstellung der Schichten aus Cellulosepulver erfolgte mit dem Streichgerät der Firma C. Desaga GmbH, Heidelberg. Dazu wurden 15 g Cellulosepulver MN 300 (ohne Gipszusatz) zur Dünnschicht-Chromatographie nach E. STAHL** mit 90 ml dest. Wasser in einem Mixgerät angerührt. Diese Menge ist ausreichend zum Beschichten von 5 Glasplatten 200 × 200 mm. Nach 10 Minuten Trocknen bei 105° sind die Platten gebrauchsfertig. Zur Chromatographie wurden etwa 0.5 μl einer 0.1 %igen Lösung der Aminosäuren in 0.1 *N* HCl 1.5 cm vom unteren Plattenrand mit einer Mikropipette aufgetragen. Die schärfsten und saubersten Trennungen erzielte man bei Auftragung von 0.5–1.0 μl einer 0.1 %igen Lösung je Komponente, d.h. also bei Auftragung von etwa 1 γ je Aminosäure. Die verwendeten Laufmittel sind in Tabelle I aufgeführt.

Nach erfolgter Trennung wurden die Platten mit Ninhydrinlösung besprüht (0.2–0.3 g Ninhydrin in 95 ml Methanol + 5 ml 2,4,6-Collidin). Die besprühten Platten wurden 30 Minuten auf etwa 70° erhitzt, wobei verschieden gefärbte Flecken

* Inzwischen wurde eine weitere Arbeit über die Dünnschicht-Chromatographie von Aminosäuren auf Kieselgel G-Schichten bekannt und zwar von FAHMY, NIEDERWIESER, PATAKI UND BRENNER[7].

** Firma Macherey, Nagel & Co., 516 Düren-Rl. (Deutschland).

TABELLE I

ZUSAMMENSTELLUNG DER UNTERSUCHTEN LAUFMITTEL

L_1 = *n*-Butanol–Eisessig–Wasser (4:1:5), obere Phase
L_2 = Pyridin–Methyläthylketon–Wasser (15:70:15)
L_3 = Propanol–Wasser (7:3)
L_4 = Methanol–Wasser–Pyridin (80:20:4)
L_5 = *n*-Butanol–Ameisensäure–Wasser (75:15:10)
L_6 = Propanol–8.8 %iges Ammoniak (8:2)
L_7 = Äthanol–*n*-Butanol–Wasser–Propionsäure (10:10:5:2)
L_8 = Phenol–Wasser (8:2) (nicht geeignet!)
L_9 = Phenol, mit Phosphatpufferlösung pH 12 gesättigt (Pufferlösung: 0.067 *M* NaOH + 0.67 *M* $Na_2HPO_4 \cdot 12 H_2O$ (1:1)) (nicht geeignet!).

langsam sichtbar wurden. Die von BRENNER UND NIEDERWIESER[5] nachstehend beschriebene Beobachtung ist empfehlenswert. Sie markieren die bei Erwärmung der besprühten Platten langsam entstehenden Farbflecken *in statu nascendi*, d.h. die Flecken kommen oft punktförmig zum Vorschein und gehen dann leicht nach vollständiger Entwicklung langsam ineinander über, besonders bei nahe zusammenliegenden R_F-Werten.

TABELLE II

R_F-WERTE EINIGER AMINOSÄUREN

Laufmittel: siehe Tabelle I.
Schicht: Cellulosepulver MN 300 zur Dünnschicht-Chromatographie nach E. STAHL*.
Entwicklungsdauer: 30 min bei L_2 (aufsteigend).
60 min bei L_1, L_4, L_5, L_6, L_7 (aufsteigend).
90 min bei L_3 (aufsteigend).

Aminosäure	*R_F-Werte*						
	L_1	L_2	L_3	L_4	L_5	L_6	L_7
DL-α-Alanin	0.38	0.06	0.48	0.60	—^{xx}	0.16	0.38
L(+)-Argininium-monochlorid	0.46	0	0.09	0.07	0.15	0.09	0.16
L-Asparagin	0.41	0.04	0.18	0.27	0.15	0.09	0.15
L-Asparaginsäure	0.46	0	0.18	0.54	0.26	0.03	0.34 0.20^{n}
L-Cystin	0.14	0	0.13^{s}	0.13^{sx}	0.07	0	0.04
L-Glutaminsäure	0.35	0	0.42	0.66	0.34	0	0.29^{x}
Glykokoll (Glycin)	0.29	0.03	0.35^{x}	0.39	0.29	0.08	0.23^{x}
DL-Isoleucin	0.67	0.24	0.69	0.82	—^{xx}	0.47	0.64
L-Leucin	0.70^{x} 0.50^{n}	0.27 0.24^{n}	0.71 0.59^{n}	0.80 0.63^{n}	—^{xx} —	0.50 —	0.68 —
L(+)-Lysinium-monochlorid	0.42	0	0.08	0.09	0.11	0.07	0.14
DL-Methionin	0.50	0.25	0.58	0.68	—^{xx}	0.30	0.53
DL-Serin	0.46	0.04	0.25	0.45	0.21	0.11	0.22 0.28^{n}
DL-Tryptophan	0.60	0.34	0.48 0.51^{n}	0.46	—^{xx}	0.35	0.52
L(−)-Tyrosin	0.46	0.30	0.56	0.60	—^{xx}	0.18	0.45
DL-Valin	0.54	0.15	0.59	0.77	—^{xx}	0.35	0.55

* Firma Macherey, Nagel & Co., 516 Düren-Rl. (Deutschland).

^{n} = Nebenfleck; ^{s} = schwach angefärbt; ^{x} = gezogen; ^{xx} = sehr lang gezogen.

Laufmittel L_5, L_8 und L_9 eignen sich bei den angewandten Bedingungen nicht zur Trennung von Aminosäuren auf Cellulose-Schichten. Entweder erscheinen die Flecken nach dem Besprühen mit Ninhydrin garnicht oder aber sehr lang gezogen und verformt. Dagegen liefern die übrigen Laufmittel in den meisten Fällen schöne runde Flecken. Die sauren Aminosäuren bleiben bei Anwendung basischer Laufmittel am Start (vgl. R_F-Werte in Tabelle II).

Diskussion der Ergebnisse

Auch mit Hilfe der DC lassen sich auf Cellulose-Schichten nicht alle Aminosäuren bei Einsatz nur eines Laufmittels trennen. Eine Anzahl Laufmittel können aus der PC übernommen werden. Aminosäuretrennungen auf Cellulose-Schichten MN 300* (ohne Gipszusatz) verlaufen wesentlich besser und schärfer als auf Cellulose-Schichten mit Gipszusatz. Weil diese Tendenz bei allen angewendeten Laufmitteln deutlich wurde, wird auf eine Wiedergabe der R_F-Werte bei Schichten mit Gipszusatz verzichtet. Die Laufzeit der Trennungen auf Cellulose-Schichten liegt zwischen 30 und 90 Minuten und damit weit unter dem Zeitbedarf für papier-chromatographische Methoden. Mengen von 0.5 bis 1 γ können eindeutig und leicht nachgewiesen werden. Die Empfindlichkeit der dünnschicht-chromatographischen Methode ist also wesentlich grösser als die bei papier-chromatographischen Methoden.

Wissenschaftliche Abteilung der Firma Macherey, Nagel & Co., Düren-Rl. (Deutschland) PAUL WOLLENWEBER

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* Firma Macherey, Nagel & Co., 516 Düren-Rl. (Deutschland).

Thin-layer chromatography

Chromatoplate analysis of the bufadienolides isolated from toad venoms

Chromatoplate analysis, owing to its versatility, is receiving extensive attention from organic chemists[1]. It has been successfully applied to the analysis of steroids[1] and recently STAHL has extended its use to the rapid resolution of cardiac glycosides[2].

Work on the isolation and identification of the cardiotoxic principles (bufadienolides or "bufogenins") from Brazilian toad venoms (*Bufo ictéricus* Spix, *Bufo crucifer* Wied and *Bufo paracnemis* Lutz) has been undertaken in this laboratory[3], and in connection with these studies, we wish to report the results of chromatoplate analysis of this group of substances.

Paper chromatography has been used for identification and preparative isolation of bufogenins, with propylene glycol–water or formamide as stationary phases[4]. Detection of the steroidal compounds is accomplished by spraying a saturated chloroform solution of $SbCl_3$, then heating and reading in natural or ultra-violet light.

Using glass plates coated with silica gel, we have found that a good and rapid resolution of the bufogenin group could be easily achieved with the following solvents: ethyl acetate, ethyl acetate–cyclohexane (80:20), or ethyl acetate–acetone (90:10). Good results were also obtained by using ethyl acetate saturated with water.

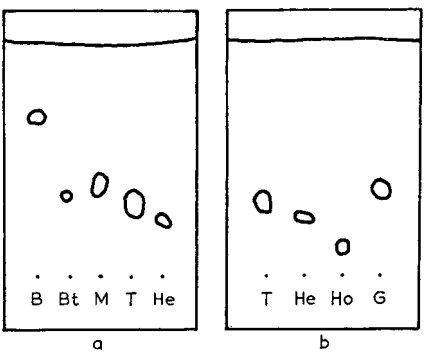

Fig. 1. Solvent: ethyl acetate, time of development (a) 55 min, (b) 60 min. B = Bufalin; Bt = Bufotalinin; M = Marinobufogenin; T = Telocinobufogenin; G = Gamabufotalin; He = Hellebrigenin; Ho = Hellebrigenol; R = Resibufogenin.

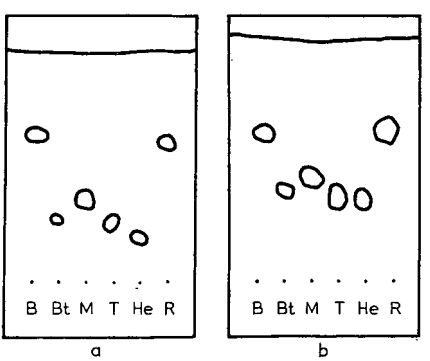

Fig. 2. (a) Solvent: ethyl acetate–cyclohexane (80:20); time 75 min. (b) Solvent: ethyl acetate–acetone (90:10); time 40 min.

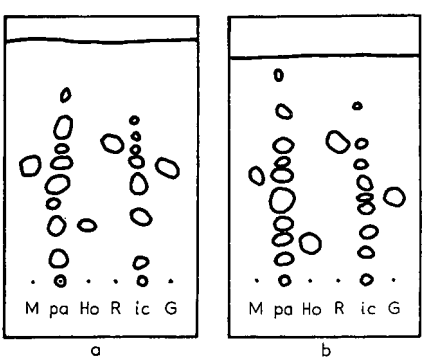

Fig. 3. (a) Solvent: ethyl acetate saturated with water; time 40 min. (b) Solvent: ethyl acetate–acetone (90:10); time 35 min. pa = extract from *B. paracnemis* Lutz; ic = extract from *B. ictéricus* Spix.

Detection of the spots was carried out by spraying with the $SbCl_3$ reagent, heating and reading under ultra-violet light: a bright fluorescence of each of the bufogenins permitted their easy location.

Figs. 1, 2 and 3 show the general pattern obtained in the chromatographic separations.

On investigating the sensitivity of the method, we also found that quantities as low as 1 μg of two pure samples (marinobufogenin and telocinobufogenin) could be distinctly located.

In a few runs, we used the crude extract isolated from the parotid glands of two species of toads (*B. ictéricus* Spix and *B. paracnemis* Lutz) (Fig. 3).

In Table I, the R_F values of eight bufogenins are given. Each vertical column corresponds to one chromatoplate experiment. Slight variations of the R_F values can be observed; these were difficult to avoid owing to differences in the thickness of the coatings and changes in moisture.

TABLE I

R_F VALUES OF SOME BUFOGENINS

Solvents: A = ethyl acetate; B = ethyl acetate–cyclohexane (80:20); C = ethyl acetate–acetone (90:10); D = ethyl acetate saturated with water.

Bufogenin	$R_F \times 100$							
	A		*B*		*C*		*D*	
Resibufogenin	61		60	61	60		66	
Bufalin	62		61			64		62
Bufotalinin	31		22			39		34
Marinobufogenin	42	43	34	32	47		50	
Gamabufotalin		31		26	37		47	
Telocinobufogenin	34		23			28		37
Hellebrigenin	28	27	18			25		30
Hellebrigenol		9		7	17		23	

Experimental

Rectangular glass plates (10 × 18 cm) were coated with silica gel (Merck's Silica Gel G). The plates were activated at 100° before being used. Samples were deposited along a line 3 cm above the bottom of the plate, and from 30 to 75 min were necessary for development, which was in the ascending direction.

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Trennung einfacher Zucker auf Cellulose-Schichten

Auf verschiedenen Gebieten der Naturstoffchemie wurde die Dünnschicht-Chromatographie bereits mit Erfolg angewandt[1]. Eine Spurenanalyse von Zuckern an Schichten von Kieselgel ist kürzlich publiziert worden[2]. Die vorliegende kurze Mitteilung bringt einen Beitrag zur chromatographischen Trennung von Zuckern an der Dünnschicht von Cellulose-Pulver. Das Verfahren wurde ausgearbeitet zur Identifizierung der Hydrolyse-Produkte pflanzlicher Polysaccharide. Über Zielsetzung und Ergebnisse dieser Versuche wird an anderer Stelle berichtet[3].

Beschreibung des Verfahrens

Es wurde im allgemeinen nach den Angaben eines Prospektes der Firma Macherey, Nagel u. Co., Düren gearbeitet. Von dieser Firma stammte auch das verwendete Cellulose-Pulver (Cellulose-Pulver MN 300). 15 g Pulver werden mit 90 ml aqua dest. *ca.* 30 sec im Starmix homogenisiert. Ein Zusatz von Gips oder anderen Bindemitteln erwies sich nicht als notwendig. Der Cellulose-Brei wird in einer Schichtdicke von 0.25 mm aufgetragen. Wir benützten hierbei Platten und Streichgerät der Standardausrüstung für Dünnschicht-Chromatographie der Firma Desaga, Heidelberg. Die feuchte Schicht wird 10 min bei *ca.* 100° im Trockenschrank getrocknet.

Die Substanzen werden zur Chromatographie in Abständen von 2 cm auf einer Startlinie 3 cm vom unteren Rand der Platten aufgetragen.

Als Laufmittelgemische wurden verwendet:

I. Essigsäureäthylester–Pyridin–Wasser (2:1:2), beide Phasen[4].
II. Phenol gesättigt mit Wasser–1% Ammoniak[5].
III. Isopropanol–Pyridin–Eisessig–Wasser (80:80:10:40)[6].

Man chromatographiert in Glaströgen der Firma Desaga bei "Kammerübersättigung"[7]. Die Platte wird aus dem Trog genommen, wenn die Laufmittelfront den oberen Rand erreicht hat und getrocknet. Bei Verwendung von Gemisch I wurde die Platte meist zu einem zweiten Lauf in gleicher Richtung und mit dem gleichen Laufmittel nochmals in den Trog gestellt. Das Verfahren benötigt bei zweimaliger Chromatographie etwa 4 Stunden.

Die Zucker lassen sich mit Anisidinphthalat (0.1 *M* Lösung *p*-Anisidin und Phthalsäure in 96% Äthanol) anfärben[8]. Dabei färben sich Hexosen grün, Pentosen rotviolett, Methylpentosen gelbgrün, Uronsäuren braun.

Ergebnisse

In den untersuchten Polysaccharid-Hydrolysaten kamen hauptsächlich die in der Tabelle I aufgeführten Zucker und Uronsäuren vor.

Wie die Laufwerte, bezogen auf die von der Glucose zurückgelegte Strecke (R_G-Werte) zeigen, lassen sich die Monosaccharide Galactose, Glucose, Mannose, Xylose, Ribose, Rhamnose bei Verwendung von Laufmittel I gut trennen (vergl. Fig. 1). Lediglich die Flecken von Mannose und Arabinose liegen sehr dicht beisammen. Diese Zucker können jedoch durch Rechromatographie mit Laufmittel II völlig getrennt werden.

Für die Papierchromatographie der Uronsäuren schlug SULSER das Laufmittelgemisch III vor[6]. Wir übertrugen es auf die Dünnschicht und erreichten eine befriedigende Trennung von Glucuronsäure, Mannuronsäure und Galacturonsäure. Das

TABELLE I

R_G-WERTE DER ZUCKER NACH ZWEIMALIGER CHROMATOGRAPHIE AUF EINER SCHICHT VON CELLULOSE-PULVER (SYSTEM I)

D(+)-Glucose	1.00
D(+)-Galactose	0.90
D(+)-Mannose	1.09
L(+)-Arabinose	1.11
D(+)-Xylose	1.25
D(—)-Ribose	1.42
L(+)-Rhamnose	1.52
D(+)-Glucuronsäure	—
D(+)-Galacturonsäure	—
D(+)-Mannuronsäure	—

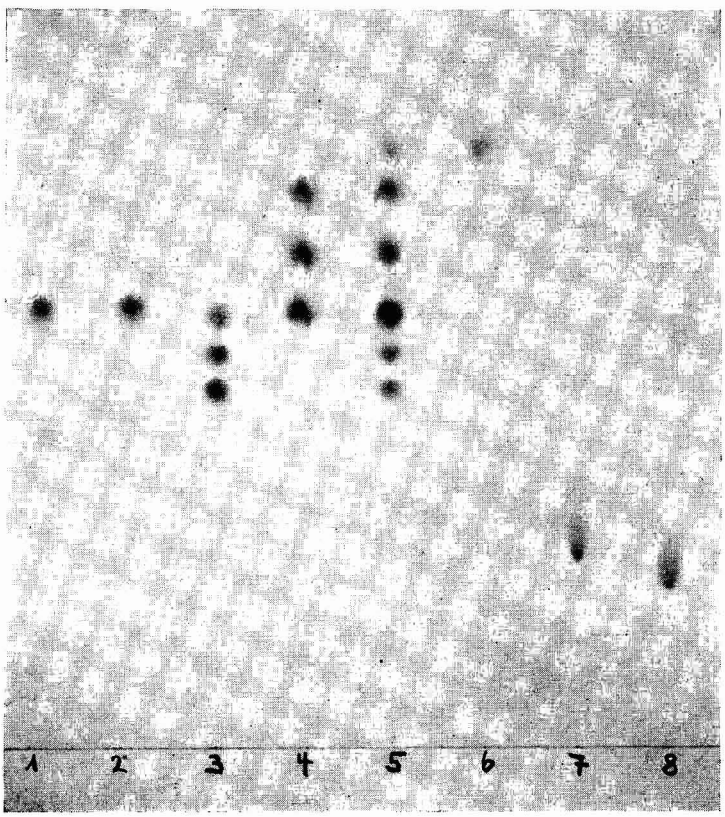

Fig. 1. Dünnschicht-Chromatographie von Zuckern auf einer Schicht von Cellulose-Pulver (2malige Chromatographie im System I). (1) Mannose; (2) Arabinose; (3) Galactose, Glucose, Mannose; (4) Arabinose, Xylose, Ribose; (5) Gemisch von 3,4 und 6; (6) Rhamnose; (7) Glucuronsäure; (8) Galacturonsäure. Von den Pentosen wurden je 1.25 μg aufgetragen, von den übrigen Zuckern je 2.5 μg. Die Anfärbung erfolgte mit Anisidinphthalat.

Cellulose-Pulver kann hierbei mit einer 0.5%igen Lösung von Versen (Äthylendiamintetraessigsäure) vorbehandelt werden (Entfernung von Kationen).

Mit Hilfe des Verfahrens können sehr kleine Substanzmengen identifiziert werden. Die Nachweisgrenze bei Anfärbung mit Anisidinphthalat lag für Hexosen und Methylpentosen bei 0.5 μg, für Pentosen und Uronsäuren bei 0.1–0.2 μg. Optimal sind Konzentrationen von *ca.* 2 μg für Hexosen und *ca.* 1 μg für Pentosen.

Versuche zu einer quantitativen Auswertung der Chromatogramme sind im Gange.

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* Direktor: Prof. Dr. R. GRAU.

Notes

Dünnschicht-Chromatographie von ^{14}C-Dinitrophenyl-aminosäuren

Zur Abtrennung von ^{14}C-Aminosäuren wurden Substanzgemische mit 1-Fluor-2,4-dinitrobenzol (DNFB) umgesetzt. Nach allgemein bekannten, bewährten Verfahren sind die entsprechenden Dinitrophenyl-aminosäuren (DNP-Aminosäuren) in praktisch quantitativen Ausbeuten zu erhalten. Zur Auftrennung der ^{14}C-DNP-Aminosäuren wurde die Dünnschicht-Chromatographie angewandt.

Dieses Vorgehen bringt folgende wesentliche Vorteile für ^{14}C-Aminosäuren:

Zeitsparende, methodisch einfache Abtrennung der DNP-Aminosäuren von anderen Stoffgruppen und damit relativ geringer Verlust an Aktivität;

Erhaltung der C-Zahl der Aminosäuren;

Eigenindikation der farbigen DNP-Aminosäuren auf dem Chromatogramm.

Methoden

Aus Gäransätzen von Traubenmost mit Weinhefe unter Zusatz von L-^{14}C-Glutaminsäure* isolierten wir neben ^{14}C-Alkoholen unter anderen auch ^{14}C-Aminosäuren. Die

* CFB 10 uniformly labelled, Radiochemical Centre, Amersham.

Hefen wurden aus den Versuchs-Ansätzen durch Zentrifugieren entfernt; alle flüchtigen Bestandteile im Vakuum abdestilliert. 0.05–0.1 g Destillations-Rückstand (entsprechend dem von BRENNER, NIEDERWIESER UND PATAKI[1] angegebenen Konzentrationsbereich für Aminosäuren) wurden mit einer Lösung von 0.3 g $NaHCO_3$ in 2 ml Wasser und anschliessend mit einer Lösung von 0.2 ml DNFB* in 4 ml Äthanol versetzt. Der Ansatz wird 2 Stunden bei Zimmertemperatur geschüttelt (verdunkelt). Anschliessend wird in einem Wasserbad von 40° der Alkohol im Vakuum entfernt und der Rückstand mit 2–5 ml Wasser in einen Schütteltrichter überführt. Durch dreifaches Ausziehen mit je 15 ml Äther ist der Überschuss an DNFB sowie Dinitrophenol (DNP-OH) zu entfernen. Nach Ansäuern der wässrigen Unterphase werden die DNP-Aminosäuren mit Äther (3 × 15 ml) oder Essigester extrahiert, die Extrakte getrocknet (Na_2SO_4) und das Lösungsmittel im Vakuum entfernt. Die zurückbleibenden DNP-Aminosäuren werden zur Chromatographie in Aceton bzw. Essigester aufgenommen.

Zur *Chromatographie* der ^{14}C-DNP-Aminosäuren ist die nach STAHL standardisierte Dünnschicht-Chromatographie geeignet:

Dünnschicht-Platten (200 × 200 mm) wurden mit Kieselgel G (Merck) beschichtet**. Vorbehandlung der beschichteten Platten und Chromatographie der DNP-Aminosäuren analog zu BRENNER *et al.*[1].

Fliessmittel 1 (I. Dimension): Toluol–Pyridin–2-Chloräthanol–0.8 *N* Ammoniak (100:30:60:60). Unterphase zum Äquilibrieren der Platten; Oberphase als Fliessmittel.

Fliessmittel 2 (II. Dimension): Chloroform–Benzylalkohol–Eisessig (70:30:3).

Auswertung

Zur Auswertung der Spots auf ihren Gehalt an ^{14}C werden die Chromatogramme folgendermassen vorbereitet:

1. Imprägnieren mit einem Material, das mit Kieselgel einen elastischen Film bildet, der von der Platte abgezogen werden kann.

2. Abheben oder Absaugen einzelner Flecke von der Platte und Elution der DNP-Aminosäuren; gegebenenfalls Rechromatographie mangelhaft getrennter Komponenten.

Ein dünner Film wird erzielt, wenn z.B. nach BAROLLIER[2] mit Collodium–Glycerin oder nach LICHTENBERGER[3] mit Kunststoffdispersionen (Polyacrylsäureester, Polyvinylidenchlorid, Polyvinylpropionat***) imprägniert wird.

Die Impulsgehalte der ^{14}C-DNP-Aminosäuren können nach verschiedenen Methoden mit unterschiedlicher Impulsausbeute bestimmt werden:

(a) Aufsetzen eines Endfenster-Zählrohres auf die Zonen der DNP-Aminosäuren.

(b) Ausstanzen der Zonen, Überführen der Plättchen in einen Probenwechsler mit Methan-Durchflusszähler.

(c) Schneiden des Filmes in Streifen und Auswerten mit einem Radio-Papierchromatographen.

(d) Verbrennen der Proben und Messen der Aktivität des $^{14}CO_2$ in der Gasphase[4].

(e) Autoradiographische Identifizierung der aktiven Zonen[5].

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** Streichgerät und Ausrüstung zur Dünnschicht-Chromatographie nach E. STAHL der Firma Desaga, Heidelberg.

*** "Neatan" der Firma E. Merck, Darmstadt.

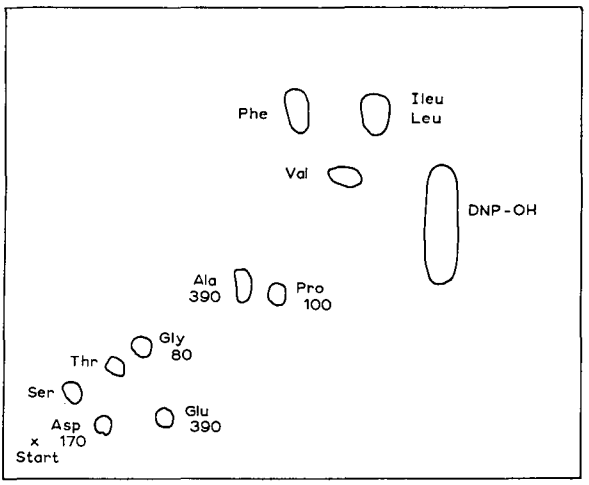

Fig. 1. Dünnschicht-Chromatogramm von ^{14}C-DNP-Aminosäuren.

Aus Fig. 1 wird die Trennung von DNP-Aminosäuren eines Destillations-Rückstandes ersichtlich. Die angegebenen, um den Wert des Untergrundes korrigierten, Impulse wurden nach Imprägnieren des Dünnschicht-Chromatogrammes mit Collodium, Abziehen des Filmes und Ausstanzen der Flecke mit einem Handprobenwechsler* gewonnen.

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[1] M. BRENNER, A. NIEDERWIESER UND G. PATAKI, *Experientia*, 17 (1961) 145.
[2] J. BAROLLIER, *Naturwiss.*, 48 (1961) 404.
[3] W. LICHTENBERGER, *Z. Anal. Chem.*, 185 (1962) 111.
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* Messgerätekombination der Firma Frieseke u. Hoepfner, Erlangen.

A device for simultaneous application of multiple spots on thin-layer chromatographic plates

The application of materials on thin-layer plates[1] with single capillary pipettes is tedious and frequently results in unsatisfactory separations due to inadvertent disturbance of the adsorbent layer at the point of application. These factors become particularly objectionable when mixtures are applied in a series of spots for preparatory separation of components. Preparation of such chromatograms is time consuming and the uneven distribution of sample across the plate and deviation of solvent flow around the attendant holes in the adsorbent layer often results in areas of overlap between adjacent bands on development of the chromatogram. A search for means of obviating these difficulties has resulted in the development of the sample applicator described herein.

Materials and construction

The apparatus as depicted in Fig. 1 consists of a multiple pipette holder made from an aluminum bar (1) with holes drilled near its end which permit it to slide freely

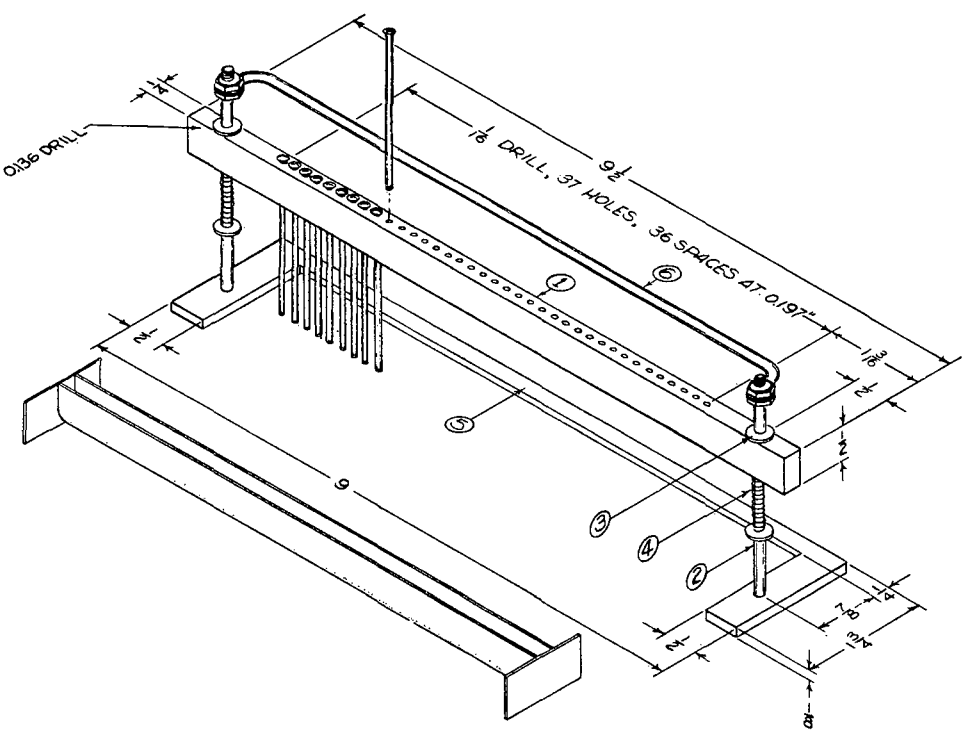

Fig. 1. Construction details for multiple spot applicator.

on two vertical 1/8 in. brass rods (2) and which is held up against adjustable stop washers (3) by partially compressed springs (4). The top and bottom stop washers are 1/4 in. O.D. and 3/32 in. I.D. and are enlarged slightly with a punch so that they may

be forced onto the vertical rods. The bottom washers which support the springs are soldered in place while the top washers remain in place by friction and may be adjusted by forcing them either up or down. The two vertical rods which are threaded, screwed into tapped holes, and soldered in place in the cutout base plate (5) and the upper horizontal tie rod (6) form the rigid support for the apparatus. Thirty-seven precisely drilled 1/16 in. holes spaced 0.197 in. (0.5 cm) on centers in the aluminum crossbar carry the spotting pipettes. These are made from pieces of 1.5 mm O.D. Pyrex melting point capillary tubing (Corning No. 9530) selected so that they will just pass through the holes in the crossbar with no friction. A funnel stop is blown at the upper end of each tube and the tubes cut at a length such that the tips will extend to within 1/16 in. of the surface of the chromatographic plate when placed in the crossbar. The delivery tips are fire polished and squared off by grinding on a flat piece of fine carborundum wetted with water. Progress in the latter operation should be observed under suitable magnification and the grinding continued only until the contact surface of the tip is smooth.

A sample trough of suitable volume and design from which the pipettes may be filled for spotting preparatory plates may be constructed from metal, glass, or plastic depending upon the chemical nature of the samples and solvents employed. A trough of the design shown in Fig. 1 was constructed of No. 23 gauge Monel metal and has proven to be satisfactory for use with non-corrosive solvents.

By removing alternate pipettes from the applicator, the remaining pipettes then may be loaded with individual samples from micro-test tubes. For this purpose a support of suitable dimensions drilled with 19-1/4 in. (1 cm) on centers to accept small test tubes made from 6 mm glass tubing may be constructed of metal, wood, or plastic.

Operation

In replicate spotting of plates for preparatory separation of fractions in bands or spotting a series of individual chromatograms, the device is positioned over the trough or the test tubes in their support and the pipettes lowered into the test solutions by depressing the crossbar on its supporting springs by applying pressure with the index fingers placed at each end of the crossbar. The crossbar is then allowed to return to its normal position and the applicator with its pipettes charged with sample(s) is lifted and positioned over the chromatographic plate using the inside edge of the base plate to index the line of spot application at 7/8 in. from the bottom of the plate. The crossbar is then slowly depressed to slightly beyond the point at which the pipettes make contact with and begin to discharge sample on the adsorbent layer. When the spots have enlarged to the desired diameter, the crossbar is allowed to return to its normal position. The operation may be repeated when the solvent has evaporated from the spots should deposition of more material on the plate be desired.

Applicators of the design described above are in use in several laboratories at the University of Connecticut. These have been used successfully for the deposition of glycerides, phospholipids, hydrocarbons, steroids, substituted cyclohexanols, alkaloids, and 2,4-dinitrophenylhydrazones in bands on thin-layer plates with various appropriate solvents including ether, hexane, benzene, carbon tetrachloride, and chloroform. In addition to the great saving in the time of application, their use has resulted in little or no disturbance of the adsorbent layer and upon development of the

plates the separated bands have been remarkably straight with no areas of overlap between adjacent bands.

Acknowledgement

The author is grateful to R. G. LIGHT for preparation of the drawing and its reproduction.

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Dünnschichtchromatographie von Lactonen, Lactamen und Thiol-lactonen

Für synthetische[1] und biochemische Arbeiten[2] auf dem Gebiet der Lactone, Lactame und Thiol-lactone war es notwendig, ein Nachweisverfahren auszuarbeiten, das es gestattete, Synthese- und Stoffwechselprodukte mit geringem Zeitaufwand zu untersuchen und zu identifizieren. Wegen ihrer einfachen Handhabung bedienten wir uns der Dünnschichtchromatographie nach STAHL[3]. Die Platten für die Chromatographie wurden nach STAHL[4] mit einem Streichgerät (Desaga, Heidelberg) mit Kieselgel G (Merck) beschichtet. Die Schichtdicke beträgt *ca.* 250 μ. Die Substanzen wurden in einem Abstand von $1^1/_2$–2 cm vom unteren Rand entfernt aufgetragen. Die Laufstrecke betrug durchschnittlich 10–13 cm.

Die Lactone lassen sich durch den Eisen–Chelat-Komplex ihrer Hydroxamsäuren nachweisen[5]. Sie werden zuerst durch ein Gemisch von gleichen Teilen 12.5 % NaOH in Methanol und 5 % Hydroxylamin-hydrochlorid-Lösung in Methanol in ihre Hydroxamsäuren überführt, die nach 10 Min. durch Besprühen mit Eisessig und einer 10 % Fe(III)-chlorid-Lösung in Wasser als braune Flecke sichtbar gemacht werden können.

Durch Auftragen verschieden grosser Mengen und anschliessender Chromatographie wird die Erfassungsgrenze bei Butyrolacton bestimmt. 0.07 μMol können noch nachgewiesen werden. Bei zu hoher Konzentration ($> 3\mu$Mol) tritt Schwanzbildung und eine Verschiebung der R_F-Werte zu höheren Werten ein.

An Papierchromatogrammen konnten Lactame mit Hilfe von Ninhydrin und anschliessender Jodbedampfung nachgewiesen werden[6]. Die besten Resultate an Dünnschichtchromatogrammen erzielten wir mit Hilfe des Dragendorff-Reagens. Dieses wird folgendermassen zubereitet[7]. Zu 850 mg Wismutnitrat in 50 ml 20 % Essigsäure werden 8 g KJ in 20 ml Wasser hinzugefügt. Man verdünnt 10 ml dieser Stammlösung mit 20 ml Essigsäure und 100 ml Wasser. Lactame reagieren rotbraun auf gelbem Hintergrund. Die untere Erfassungsgrenze lag für N-Methyl-pyrrolidon

TABELLE I

R_F-WERTE VON LACTONEN

Lösungsmittelsysteme: A. Diisopropyläther; B. Diisopropyläther–Essigester (80:20); C. Diisopropyläther–Isooctan (20:80); D. Diisopropyläther–Isooctan (60:40)

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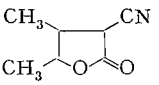
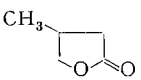
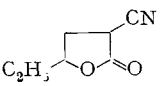
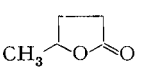
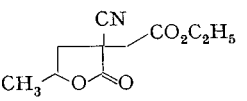
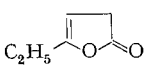
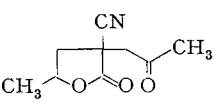
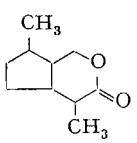
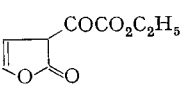
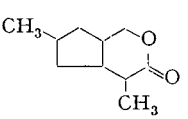
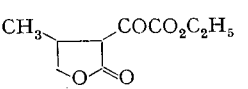
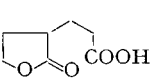
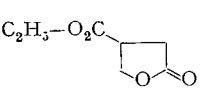
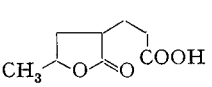
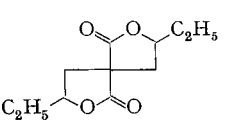
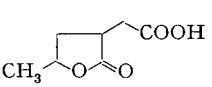
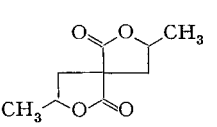
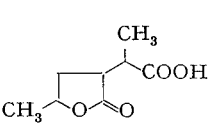
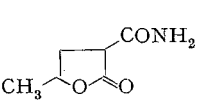
	0.29	0.53		0.42	0.86
	0.43	0.71		0.38	0.87
	0.36	0.65		0.50	0.75
	0.87	Front		0.07	0.66
	0.74	0.93		0.41	0.62
	0.64	0.93		0.49	0.59
	0.21	0.52		0.13	0.46
	0.22	0.32		0.21	0.44
	0.16	0.47		0.08 0.19	0.21 0.36
	0.24	0.52		0.00	0.09

(Fortsetzung S. 383)

TABELLE I (*Fortsetzung*)

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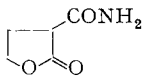
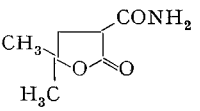
	0.00	0.04				
	0.00	0.16				

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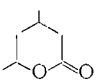
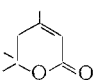
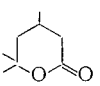
	0.49	0.64	0.14	0.31
	0.51			0.42
		0.88		0.31
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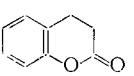
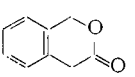
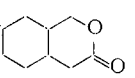
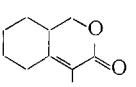
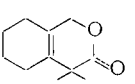
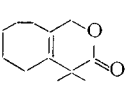
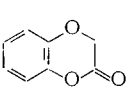
	0.68	0.86	0.12	0.40
	0.82	0.94	0.25	0.39
	0.43	0.66		
	0.70	Front	0.18	0.25
	0.91	Front	0.21	0.37
	0.91	Front	0.21	0.44
			0.49	0.71

TABELLE II

R_F-WERTE VON LACTAMEN

Lösungsmittelsysteme: siehe Tabelle I

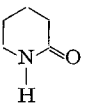
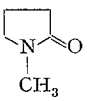
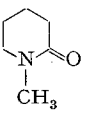
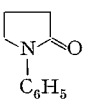
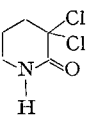
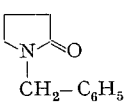
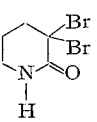
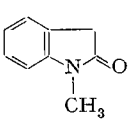
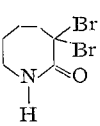
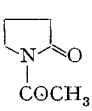
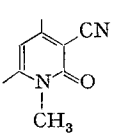
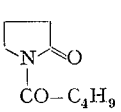
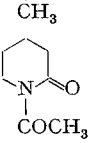
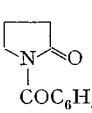
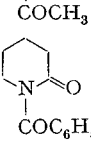
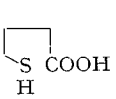
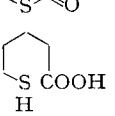
	A	*B*	*D*		*A*	*B*	*D*
	0.11	0.05			0.12	0.08	0.00
	0.26	0.26			0.18	0.15	0.06
	0.34	0.37			0.03	0.22	
	0.19	0.14			0.11	0.21	
	0.44	0.49	0.10		0.22	0.43	
	0.58	0.53	0.19		0.06	0.03	
	0.75	0.87	0.36		0.56	0.62	0.24
	0.32	0.51	0.14		0.51	0.56	0.10

TABELLE III

R_F-WERTE VON THIOL-LACTONEN

Lösungsmittelsysteme: siehe Tabelle I

	A	*C*	*D*		*A*	*C*	*D*
	Front	0.86	0.62		Front	0.95	0.46
	0.80	0.60	0.51		0.71	0.65	0.23

bei 0.1 μMol, Schwanzbildung wird bei Konzentrationen > 5 μMol festgestellt. Thiollactone werden durch Alkali leicht geöffnet. Die so entstehende SH-Gruppe lässt sich mit Nitroprussidnatrium (2 % in 75 % Äthanol[8]) anfärben. Bei Thiol-butyrolacton liegt die untere Erfassungsgrenze bei 0.1 μMol, Schwanzbildung tritt erst bei Konzentrationen $> 10\mu$Mol auf.

Als mobile Phasen untersuchten wir Hexan, Cyclohexan, Isooctan, Chloroform, Aceton, Benzol, Diisopropyläther, Diisoamyläther und Essigester. Die besten Trennergebnisse werden mit Diisopropyläther (Merck) und dessen Gemische mit Isooctan (ASTM Schuchardt) oder Essigester (Merck) erreicht. Es gelingen auf diese Weise auch Trennungen von Stellungsisomeren wie z.B. 4-Methyl-butyrolacton und 5-Methyl-butyrolacton.

Die R_F-Werte sind in Tabelle I–III angegeben.

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[1] F. KORTE UND K. H. BÜCHEL, *Angew. Chem.*, 71 (1959) 709 (Zusammenfassung).
[2] F. KORTE UND H. J. SCHREIBER, *Ann.*, im Druck; F. KORTE UND J. VOGEL, unveröffentlicht.
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[7] I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, Band I, Gustav Fischer Verlag, Jena, 1958, S. 758.
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Eingegangen den 4. April 1962

J. Chromatog., 9 (1962) 381–385

Chromatographie sur couches minces des dérivés de la diphénylamine. Application à l'analyse des poudres

Pour stabiliser les coton-poudres on a utilisé la diphénylamine, qui était en effet introduite par ALFRED NOBEL en 1889. Au cours du vieillissement des poudres il se forme des dérivés nitrosés et nitrés de la diphénylamine. Évidemment la meilleure méthode pour surveiller les poudres à diphénylamine serait de déterminer dans quelle mesure la diphénylamine s'est transformée et en quels dérivés. Ainsi la marine française[1] emploie quelques épreuves colorimétriques qualitatives de diphénylamine et de trinitrodiphénylamine. HAHN[2] a séparé les dérivés formés en poudres par polarographie, mais cette méthode ne convient pas plus à des analyses de routine que la chromatographie sur colonne d'acide silicique effectuée par SCHROEDER et collaborateurs[3]. Plusieurs chercheurs ont aussi essayé la chromatographie sur papier, mais on n'a pas réussi à atteindre une séparation complète des dérivés nitrosés et nitrés de la diphénylamine.

Cherchant une méthode de routine rapide pour la surveillance des poudres au stockage nous avons essayé la technique microchromatographique d'adsorption sur

couches minces, spécialement développée par STAHL[4]. Nous avons travaillé avec plaques de verre 20 × 20 cm couvertes d'une couche d'acide silicique (Merck gel de silice G selon STAHL) d'une épaisseur de 0.25 mm. Les chromatogrammes ascendants sont faits sur un trajet de 10 cm pendant environ 30 minutes avec les solvants suivants: chloroforme, benzène, toluène, cités d'après l'ordre décroissant de leur pouvoir éluant. Nos essais ont été faits sur huit substances commerciales: diphénylamine, triphénylamine et six dérivés nitrosés ou nitrés (voir Tableau I). En outre, nous avons fait des essais comparatifs avec les produits obtenus en traitant les substances mentionnées ci-dessus par les gaz NO et NO_2 et quelques essais préliminaires avec deux poudres à simple base (une à 98.5% nitrocellulose, 1.5% diphénylamine de 1938; une à 98.6% nitrocellulose, 1.0% diphénylamine de 1952). Dans ces derniers essais le stabilisant et les dérivés formés de celui-ci étaient extraits par le chlorure de méthylène. Les substances ont été déposées comme solutions dans l'acétone sur les chromatoplaques.

La plupart des substances sont colorées, et il n'y a pas de difficulté à démontrer leurs taches. Les nitrocomposés donnent des taches d'une couleur jaune à orange, celles de la 4-nitrosodiphénylamine sont bistrées, et les autres sont incolores mais se font graduellement beige grisâtre à l'air. Nous avons révélé les chromatogrammes par pulvérisation d'une solution dans l'alcool de $NaNO_2$ à 0.2% et d'acide sulfurique 1 N, ce qui colore les taches de la diphénylamine et de la N-nitrosodiphénylamine d'une nuance bleu verdâtre. Les taches de la triphénylamine prennent des couleurs différentes suivant les concentrations de l'amine et des réactifs. Pour conserver les résultats nous avons copié les plaques avec réflectographie à transfert.

Nous n'avons pas rencontré de difficultés à séparer les substances examinées, et pour cette raison nous n'avons pas fait une vaste examination d'éluants. Nous avons trouvé que le benzène était le meilleur d'entre les trois solvants essayés. Les valeurs des R_F sont présentées dans le Tableau I. Le traitement par les gaz NO et NO_2 des dérivés de la diphénylamine a donné des suites de dérivés, qui ont été bien résolus sur les chromatogrammes.

TABLEAU I

VALEURS R_F POUR DIPHÉNYLAMINE, TRIPHÉNYLAMINE ET QUELQUES DÉRIVÉS NITROSÉS ET NITRÉS

Substances	*Éluant*		
	Chloroforme	*Benzène*	*Toluène*
Diphénylamine	0.86	0.62	0.61
N-Nitrosodiphénylamine	0.81	0.46	0.41
4-Nitrosodiphénylamine	0.28	0.07	0.05
2-Nitrodiphénylamine	0.86	0.58	0.57
2,4-Dinitrodiphénylamine	0.81	0.43	0.39
2,4'-Dinitrodiphénylamine	0.77	0.37	0.32
sym.-Hexanitrodiphénylamine	0.05	0.05	0.06
Triphénylamine	0.91	0.75	0.78

Nous avons réussi à identifier plusieurs des taches aussi bien par les R_F que par la spectrographie. Dans les Figs. 1–2 nous illustrons les essais avec les deux poudres à simple base, dont une (Fig. 2) a dégagé des vapeurs nitreuses après seulement 25 jours à 65°. Les chromatogrammes n'ont pas été révélés. La différence entre les poudres ne

ressort pas si bien dans les figures comme sur les chromatogrammes originaux, mais il est évident que la séparation est bonne et qu'une substance entremêlée (2,4'-dinitrodiphénylamine) se sépare bien. La quantité de substance nécessaire est illustrée par le

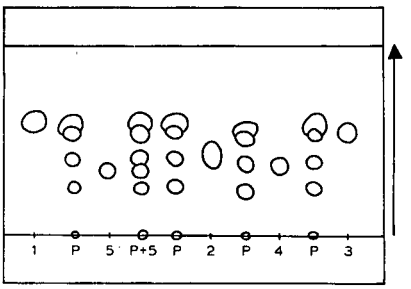

Fig. 1. Chromatogramme comparé de quelques dérivés de diphénylamine et des extraits d'une poudre de 1952 d'un taux en diphénylamine à 1.0%. Éluant: benzène. P = Extraits de poudre; 1 = diphénylamine; 2 = N-nitrosodiphénylamine; 3 = 2-nitrodiphénylamine; 4 = 2,4-dinitrodiphénylamine; 5 = 2,4'-dinitrodiphénylamine.

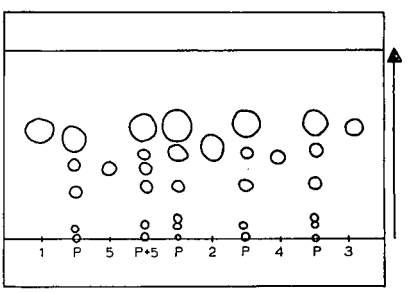

Fig. 2. Chromatogramme comparé de quelques dérivés de diphénylamine et des extraits d'une poudre de 1938 d'un taux en diphénylamine à 1.5%. Éluant: benzène. Notations: voir Fig. 1.

fait que 1 g de poudre a été extrait par 30 ml de chlorure de méthylène, l'extrait a été évaporé et dissous dans 1 ml d'acétone, dont environ 2–5 μl ont été appliqués sur la couche mince. Des substances pures, 1 μg a été suffisant pour produire une tache bien visible.

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[1] G. POINT, communication personnelle.
[2] C. HAHN, communication personnelle.
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Reçu le 20 avril 1962

A method for the more efficient utilization of thin-layer chromatoplates

A method has been devised for doubling the useful area of the Stahl thin-layer chromatoplates. The plates are coated with adsorbent in the usual manner and allowed to dry at room temperature for about 10 minutes. Then they are inverted and a layer of adsorbent is applied to the reverse side. Contrary to what might be expected such manipulation does not damage the underside. The doubly coated plates are cooked and stored as usual. However, care must be taken to prevent damage to the underside when the test material is spotted. For this purpose the plates may be placed on a frame which holds them slightly elevated from the work bench. The frame which touches the plate only along a thin outer border resembles a picture frame and is easily constructed from wood. The detection of the chromatographed material and the making of permanent records with a common letter copying machine are not hindered by the presence of adsorbent on both sides of a plate. This addition to the thin-layer chromatography technique proved very satisfactory while reducing the space and manipulations customarily required in thin-layer chromatography.

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Received May 4th, 1962

Thin-layer chromatography of urinary metabolites of chlorpromazine and related psychotropic drugs

Thin-layer chromatography, in spite of manifold successful applications, is not currently applied to metabolic studies of psychotropic drugs.

In the course of further researches on the urinary excretion of chlorpromazine (CPZ) and chemically related compounds (chlorprotixene (CPX), imipramine (IP) and amitriptyline (ATL)), this technique has given better results than the techniques used in our previous, analogous investigations[1,2].

Thin-layer chromatography on Kieselgel-G layers was employed; chromatoplates (18 × 23 cm) were prepared and activated according to STAHL[3,4]. The most suitable solvent was the system *n*-butanol–acetic acid–water (88:5:7 for CPZ and CPX; 65:15:20 for IP and ATL). Ascending chromatographic runs of 12–13 cm were performed at room temperature (26–27°C), in closed vessels. The same procedure was carried out with normal rabbit urine to which the four compounds in appropriate concentrations had been added. Detection was performed by spraying the plates with concentrated H_2SO_4. CPZ and its metabolites exhibit spots of various colours, from red to violet; IP, ATL and related metabolites show green (IP) and brick-red (ATL) fluorescence under short wave U.V. light (250 mμ) after heating (95–100°C); CPX and its metabolites exhibit a clear orange-red fluorescence under Wood's light.

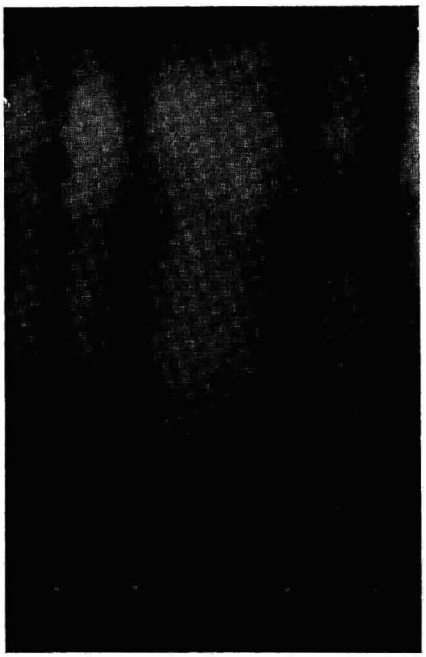

Fig. 1.

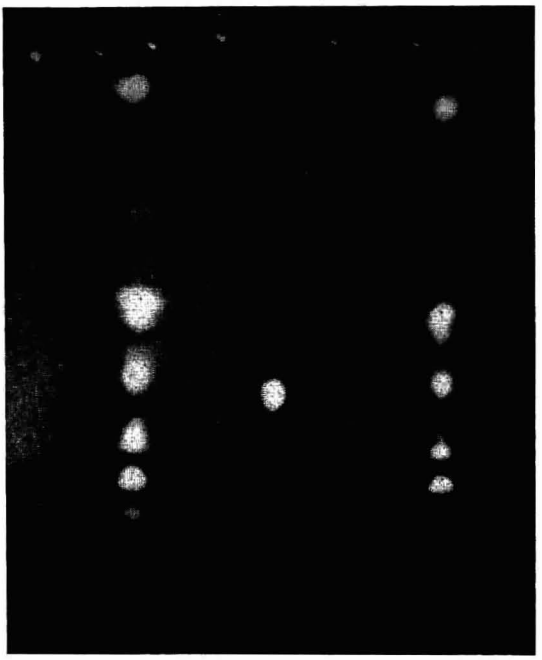

Fig. 2.

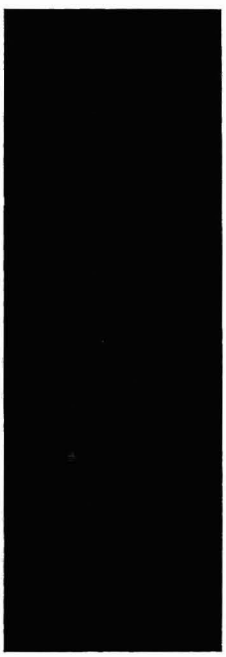

Fig. 3.

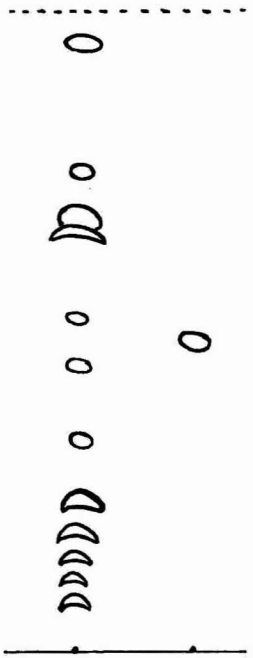

Fig. 4.

Figs. 1–4. Chromatograms of rabbit urine after treatment with chlorpromazine (Fig. 1–I), chlorprotixene (Fig. 2–I and III), imipramine (Fig. 3–I) and amitriptyline (Fig. 4–I). Figs. 1–II, 2–II, 3–II, 4–II show the results obtained with normal rabbit urine to which chlorpromazine (Fig. 1–II), chlorprotixene (Fig. 2–II), imipramine (Fig. 3–II) and amitriptyline (Fig. 4–II) respectively were added.

In the urine of rabbits treated with CPZ, CPX, IP and ATL (100 mg/kg p.o.), 9, 12, 12 and 12 fractions respectively, were detected (Figs. 1–4).

Preliminary experiments indicated the feasibility of performing on the plates some particular chemical reactions designed to establish the nature of the main biotransformation products. This technique can also be applied to other biological fluids or tissue extracts, as well as to other chemically related psychotropic drugs.

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[1] T. BERTI AND M. FERRARI, *Minerva med.*, 50 (1959) 1729.
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[3] E. STAHL, *Chemiker-Ztg.*, 82 (1958) 10.
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Received April 10th, 1962

Rapid method for permanent recording of thin-layer chromatograms

One of the most important recent advances in microanalytical technique has been the development of thin-layer chromatography[1]. The inconvenience of handling and storing finished thin-layer chromatograms, however, has made desirable a means of permanently recording the information obtained. Manual tracing on paper, the simplest method in use, yields at best only an approximate reproduction. Shadowgraphing on photographic paper produces an exact negative replica but requires darkroom facilities unavailable in many laboratories. Moreover, photographic processing is time consuming. In this communication a method of duplication is described which eliminates the undesirable features of wet processing through the use of dry process Diazo* paper. By this method positive replicas of thin-layer chromatograms can be made quickly and cheaply under ordinary laboratory conditions with no special equipment or chemicals.

In Fig. 1 are shown a thin-layer chromatogram and its DRIPRINT replica prepared in the following manner. The chromatogram was placed horizontally over a desk lamp equipped with two cylindrical 15 W fluorescent bulbs, the inverted shade serving as a support for the glass plate. A piece of cellophane laid over the coated surface of the chromatogram protected the surface from abrasion by the paper, the paper from chemical attack by residual spray, and functioned as a negative (Fig. 1) for recording such information as the location of the origin and labels identifying the substances applied. A sheet of DRIPRINT paper was then placed over the cellophane followed by a glass plate to hold the various layers stationary. After exposure of the paper to the light for 10 min it was placed in a covered glass jar containing an open

* Diazo paper is a direct positive blueprinting paper. The brand used was DRIPRINT HC 241B (F speed) supplied by Eugene Dietzgen Co., 407 10th St., N. W., Washington, D. C. A sheet 8 in. × 10 in. costs about 1/2 cent. The paper can be handled freely in ordinary laboratory light.

beaker of conc. NH_4OH. In the presence of ammonia gas unexposed areas, corresponding to spots on the chromatogram, appeared immediately as bright blue spots on a white background. After 10 to 15 sec the permanent mirror image of the chromatogram was removed from the jar. (The mirror image is superior in definition to the real image, produced when the paper is placed on the uncoated side of the plate and illuminated from the coated side.).

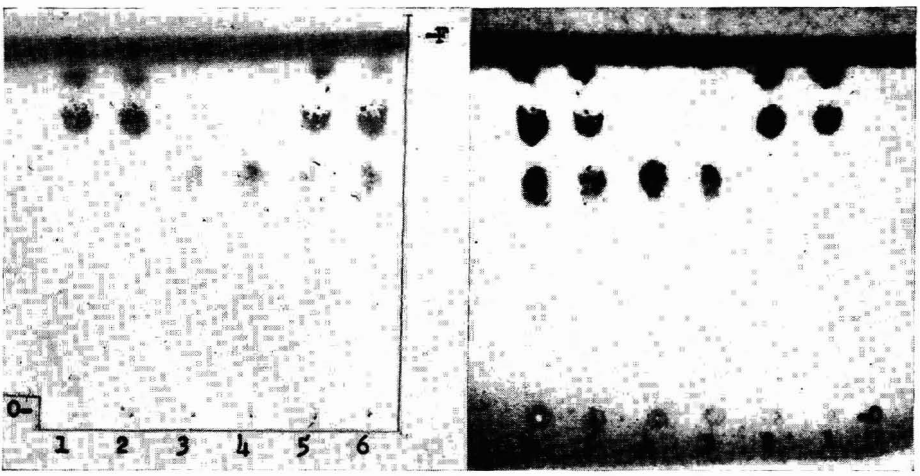

Fig. 1. Left. Thin-layer chromatogram. Adsorbent Silica Gel-G (STAHL). Fifty micrograms of crude dibenzal derivatives of D- and L-idonic acid and D- and L-idosaccharic acid were spotted at 1 cm intervals on line O at 1, 2, 3, and 4 resp.; 5 and 6 are mixtures of 1 + 3 and 2 + 4 resp. Solvent abs. methanol, 6.5 cm ascent to line F. Sprayed with conc. H_2SO_4 and heated 15 min at 150°. Characters typed on paper mask and superimposed at time photograph was taken. Right. DRIPRINT replica. Characters typed in descending numerical order on cellophane sheet, reversed at time of exposure.

It can be seen from Fig. 1 that contrast in the replica is actually better than in the original; this effect is especially noticeable in spots remaining at the origin which are only faintly discernible in the chromatogram but clearly visible in the copy.

GORDON has described the recording of U.V. absorbing spots from paper chromatograms on ferric ferricyanide blueprint paper[2]. Although in this application Diazo paper was inferior to iron-treated paper, a dry process because of its simplicity is in general to be preferred to a wet one.

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Non-destructive zone developing in thin-layer chromatography

The separation of compounds by column chromatography fails at times; however, thin-layer chromatography[1] has been found to fail only rarely*. When thin-layer chromatography is used to separate reaction mixtures to isolate the products, the location of the various compounds is a problem, for there is neither a foolproof nor a convenient non-destructive way to locate the developed zones.

We have overcome this problem in these laboratories by employing the simple technique of *spraying the developed thin-layer chromatoplates with water*. When the water-moistened plate is held against a dark background, it is found that most of the chromatoplate is translucent; however, there are bands which are not translucent, but white. These regions have been found to contain the components of the mixture. Clearer zones are sometimes obtained by first saturating the chromatoplate with water and then letting the plate dry until the zones are distinctly visible. The process may be repeated if it is necessary.

This technique has proved to be very valuable when larger amounts of compounds were to be separated by spotting the entire edge of a large (20 × 20 cm) chromatoplate. Up to 40 mg of a mixture has been separated on a plate covered with silica gel G, followed by evaporation of the solvent, location of the bands with the water spray, circumscription of the areas with a stylus, evaporation of the water by drying in an oven, removal of the absorbant and compounds by scraping the appropriate bands from the chromatoplate with a spatula, and extraction of the absorbant with an appropriate solvent.

The following mixtures were successfully separated and are illustrative:

(1) 3 mg of a cholestanone and α-cholestanol mixture, developed by a 20:20:60 pentane–ether–benzene solution.

(2) 40 mg of a 3-octyl-α-cholestan-3-ol, 3-octyl-β-cholestan-3-ol, α-cholestanol, and β-cholestanol mixture, developed by benzene first and then 30:70 ether–benzene solution.

(3) 25 mg of a *cis*-1-octyl-4-*tert*.-butylcyclohexanol, *trans*-1-octyl-4-*tert*.-butylcyclohexanol, *trans*-4-*tert*.-butylcyclohexanol, and *cis*-4-*tert*.-butylcyclohexanol mixture, developed by benzene first, and then 80:20 benzene–ether solution.

It has been found that the compounds can be water soluble and still give a white spot; however, they must not be water wetting.

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* We have not been able to separate α- and β-cholestanol from cholesterol.

A photographic method for recording chromatograms

Various methods have been used for recording thin-layer chromatograms of substances which are visible under ordinary light[1,2], and procedures involving photographic contact paper have been utilized for recording paper chromatograms of compounds which absorb in the ultraviolet region. A plastic film transfer technique for permanent preservation of the adsorbent layer of thin-layer chromatograms has recently been reported[3], and reagents and equipment are commercially available[4].

With compounds which do not yield visible spots on chromatograms without chemical development (*e.g.*, nucleotides and their derivatives), the spots can be outlined under ultraviolet illumination and, in the case of thin-layer chromatograms, their location can be transferred to tracing paper or cellophane sheets[5] to form a type of permanent record. This procedure, requiring relatively long exposure to ultraviolet light, is tedious and also precarious because of the fragility of the layer.

A photographic method has been devised using Polaroid film. The procedure is simple and rapid, does not require elaborate equipment, gives a permanent record, decreases exposure of personnel to short wavelength ultraviolet radiation, and produces excellent results.

Method

A 4 × 5 view camera is attached to a vertical enlarger stand, the baseboard of which supports the chromatogram on a nonfluorescing background. The camera is fitted

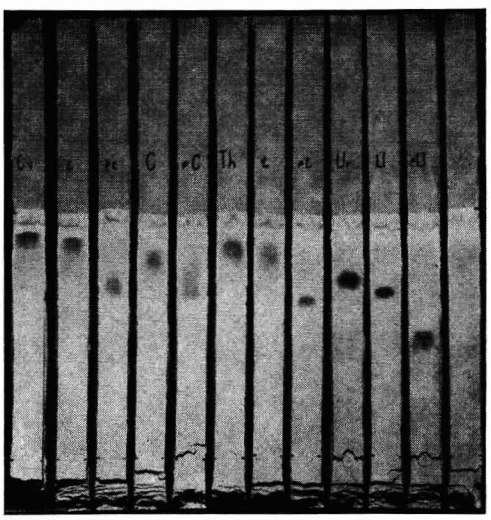

Fig. 1. Photograph from Polaroid negative of a thin-layer chromatogram (20 × 20 cm grooved-glass plate).

with a Polaroid Land Film Holder for film packets, and a Wratten G filter is used over the lens. The chromatograms are evenly illuminated by two ultraviolet lamps[6] set at each side of the copyboard at a 45° angle, as in a standard photographic setup[7]. A photolamp or an incandescent lamp is used for illumination during critical focusing on the ground-glass screen. The room is then darkened, the ultraviolet lamps are

turned on, and an exposure is made on Polaroid film Type 55 P/N using a time of approximately 2 min at f 5.6 for a lens-to-subject distance of 18 in. The film yields a positive 4 × 5-in. print in 20 sec and is usually satisfactory for determination of R_F values. In addition, a negative is obtained which can be processed without darkroom facilities. The negative can then be used for photographic enlargements to aid further in making desired determinations or filed for future reference. It was found that the Polaroid prints resulted in much better visualization, particularly of very weak spots, than those obtained by former methods.

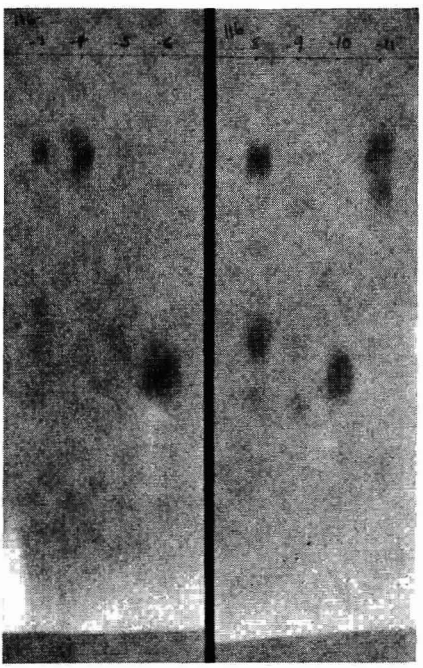

Fig. 2. Photograph from Polaroid negative of paper chromatograms (9 cm wide strips, Whatman No. 31).

Considerable modification could be made, depending on available equipment; for instance, satisfactory results were attained using only one ultraviolet lamp, although uniform illumination was more difficult.

Typical results obtained are illustrated in Fig. 1 for a 20 × 20-cm grooved-glass TLC plate[8] with cellulose as adsorbent and in Fig. 2 for 9-cm wide Whatman No. 31 paper strips.

This work was performed under the auspices of the U.S. Atomic Energy Commission.

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[5] K. RANDERATH, *Biochem. Biophys. Res. Commun.*, 6 (1962) 452.
[6] Model C-81, Ultra-Violet Products, Inc., San Gabriel, California.
[7] Kodak Data Book, *Copying*, 6th Ed., Eastman Kodak Company, 1958.
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Separation and identification of methylol derivatives of cardanol

Various investigators have used different methods and techniques to separate and identify the reaction products of various phenols with aldehydes under alkaline conditions[1]. With a substituted phenol, such as cardanol (the chief component of commercial cashewnut shell liquid), the identification of the intermediate products is even more difficult, and available methods do not enable easy separation for identification of these products.

Preliminary results indicate that it is possible to separate the intermediate products formed in the cardanol–formaldehyde reaction under alkaline conditions by using the technique of thin-layer chromatography[2].

Materials and method

(1) Cardanol (1 mol.) was treated with formaldehyde (1 mol.) in presence of 40% KOH solution (1 mol.) and the reaction mixture was kept at room temperature (25°) in a stoppered bottle. After a period of 24 hours, 1 g of the reaction mixture was removed from the bottle and diluted ten times with ethanol. The reaction mixture was kept for 11 days and at intervals of 24 hours, samples were taken out as indicated above.

(2) Finely divided silica gel (200 mesh) was mixed with twice its weight of water and a little gypsum. The paste was then uniformly spread on glass plates, 20 cm in length, and dried in an oven for 2 hours at 100°.

(3) The samples collected at intervals of 24 hours were taken for spotting. After spotting these solutions on the glass plates, the chromatogram was run for 55 minutes in ethyl acetate–toluene (60:40) mixture. Four spots were obtained from the samples taken on the fifth day and there was no change up to the eleventh day. The chromatograms were developed with diazotised sulphanilic acid.

Three methylol derivatives with R_F values of 0.738, 0.586 and 0.448 respectively were detected. The fourth spot was identified and confirmed to be that of unreacted cardanol.

One of the derivatives was crystalline and melted sharply at 60°. It had an R_F value of 0.738. This derivative was separated and identified after hydrogenating the

alkyl side chain. The resulting product melted at 91°, which corresponds to that of the product isolated by JONES AND ROBSON[3] and designated as the 6-methylol of tetrahydro-anacardol. The crystalline methylol derivative obtained would therefore be 6-methylol-cardanol.

Thus, thin-layer chromatography shows signs of usefulness in these investigations. Further work on this subject is in progress.

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[3] E. R. H. JONES AND I. K. M. ROBSON, to British Resin Products Ltd., *Brit. Pat.* 634,960 (1950).

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BOOK REVIEWS

Progress in Industrial Gas Chromatography, edited by H. A. SZYMANSKI, Plenum Press, Inc., New York, 1961, price $ 10.00.

The title of this volume is very misleading, because this book does not deal with the developments and applications of gas chromatography for industrial purposes, but is a collection of the proceedings of the advanced sessions of the annual Gas Chromatography Institute held at Canisius College, Buffalo, N.Y., in 1961, where some aspects of gas chromatography have been discussed from a practical point of view.

The volume consists of twelve reports and a summary of the panel discussion dealing with instrumentation and operating technics of gas chromatography. Most of the contributions are not original, but are reviews on topics that are already available in specialized books. The majority of the authors, who are connected with firms manufacturing gas-chromatography apparatus, treat the subjects with competence and clarity. Some contributions are, however, only short recommendations on certain subjects. For instance, one paper on the use of chromatography for analysis of odors, flavors and air pollution consists of only four pages and does not contain a single reference. Since this book, according to the advertising flap, should keep "a scientist working in this field abreast of the latest developments", it seems that, at least in this field, the aim of the editors has not been reached.

The reviewer is very doubtful about the aims of publishers who issue volumes of the proceedings of a meeting as progress in a certain field. Unless these meetings have been well organized and the papers carefully selected overlapping of subjects usually occurs and the scientific and technical level of the various papers will not be the same; these remarks certainly apply to this volume.

A. LIBERTI (Naples)

Chromatography, edited by E. HEFTMANN, Reinhold Publishing Corporation, New York, 1961, xxv + 753 pages, price $ 17.50.

Dr. HEFTMANN together with 33 outstanding research workers in the field of chromatography has compiled a book which deals equally with adsorption, ion exchange and partition chromatography, gas chromatography and zone electrophoresis. Each chapter is written by a different author, some chapters having been translated by Dr. HEFTMANN.

Part I of the volume deals with the fundamentals of chromatography; there is a historical introduction by L. ZECHMEISTER, a general chapter on differential migration methods by H. H. STRAIN, one on theory by J. C. GIDDINGS, two chapters on adsorption (by C. H. GILES and L. HAGDAHL, resp.), two on partition (by J. C. GIDDINGS AND R. A. KELLER and by K. MACEK, resp.). The techniques of gas chromatography are described by A. I. M. KEULEMANS AND H. M. MCNAIR, the theory of electrophoresis by H. J. MCDONALD and the technique by H. MICHL, the principles of ion exchange by H. F. WALTON and the technique by R. KUNIN. A special chapter is devoted to molecular sieve processes (by P. FLODIN AND J. PORATH) and gives a good survey of the possibilities of sephadex chromatography.

Part II contains a series of chapters on the applications of chromatography: amino acids and peptides, proteins, lipids, terpenes etc., steroids, carbohydrates, alkaloids, nucleic acids and related substances, chlorophylls and various porphyrins, water-soluble vitamins and antibiotics, phenols, inorganic ions, non-hydrocarbon gases, hydrocarbons.

There is some lack of uniformity, which even the best editor cannot avoid when each chapter is written by another author; thus some chapters give extensive bibliographies while others only quote a few papers. It also happens that the general part contains much more information on inorganic separations in its various chapters than the chapter specifically devoted to them. There is also an author and a subject index, the latter curiously containing also some authors' names.

The book will be invaluable for anyone teaching or learning chromatographic methods, as it presents a survey of most of the possibilities of this technique. It can also be recommended as a handbook in certain fields. The text is richly illustrated, well printed and some chapters list good selections of R_F values and electrophoretic mobilities.

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EIN EINFACHES PROGRAMMSTEUERGERÄT FÜR DIE GASCHROMATOGRAPHIE

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(Eingegangen den 24. Mai 1962)

Es gibt heute verschiedene Programmsteuergeräte, die in bestimmten Zeitabständen Impulse z.B. auf einen Schrittschaltwähler geben und damit ein Schaltprogramm ausführen. Oft ist jedoch das Volumen des Programms beschränkt, die Programmaufstellung zeitraubend und der zeitliche Abstand der Impulse ungenau. Wir haben daher einen sehr einfachen Impulsgeber entwickelt, der zeitlich beliebig lange Programme mit grosser Genauigkeit zu steuern erlaubt. Die Aufstellung des Impulsprogramms ist dabei sehr einfach. Das Gerät wird für die automatisch ablaufende Gaschromatographie verwendet. Die Entwicklung eines Gaschromatogramms von einem Stoffgemisch mit mehreren Komponenten in sehr verschiedenen Konzentrationen bedingt eine exakte Bereichsumschaltung am Registrierinstrument von empfindlichen zu weniger empfindlichen Bereichen und umgekehrt, wenn man die "Peaks" für die in hoher Konzentration vorliegenden Komponenten auf dem Registrierpapier halten und die "Peaks" der in geringer Konzentration vorliegenden Komponenten mit grosser Registrierempfindlichkeit aufnehmen will. Es gibt hierfür auf dem Markt Geräte, die mit zwei Begrenzungskontakten am Recorder und einem vor- und rücklaufenden Schrittschaltwerk jeweils bei annähernd Vollausschlag des Recorders den nächst höheren und bei der Verringerung des Ausschlages auf etwa 30 % den nächst niedrigeren Bereich automatisch einschalten. Es hat sich nun gezeigt, dass ein so aufgezeichnetes Chromatogramm praktisch nicht auswertbar ist, da bei mehreren Bereichsumschaltungen die Kurven zwar auf dem Papier bleiben, aber infolge der zahlreichen Umschaltunterbrüche quantitativ fast nicht auswertbar sind.

Wenn der zeitliche Ablauf eines Chromatogramms bekannt ist, kann man nun ein synchron mit dem Chromatogramm laufendes Programmsteuergerät anwenden, das zu den vorgeschriebenen Zeiten die vorher bestimmten Bereiche einschaltet. Ein solches Gerät ist für Serienanalysen sehr vorteilhaft, besonders dann, wenn die zur quantitativen Bestimmung auszuwertenden Flächenintegrale der Peaks automatisch auf dem gleichen Diagrammpapier aufgezeichnet werden können. Bei zeitlich längeren Programmen ist hierzu eine Umschaltung mit einer Zeitgenauigkeit von *ca.* 0.5 % notwendig. Die von uns entwickelte Einrichtung besteht aus zwei Geräten: dem Impulsgeber und dem eigentlichen Schaltgerät.

(1) *Impulsgeber* (Fig. 1 und 2)

Das Impulsprogramm wird auf einem 10 mm breiten, beliebig langen Papier-

streifen in bestimmten Abständen durch kräftige Bleistift-Striche senkrecht zur Länge markiert. Der markierte Papierstreifen wird zu Beginn auf der Scheibe A aufgewickelt und der Anfang zwischen der Antriebswelle E und der Andruckrolle C durchgezogen. Der Papierstreifen liegt dabei auf der festen Rolle B auf. Wie bei einem Magnettonbandgerät wird der Papierstreifen durch die von einem Synchronmotor angetriebene Präzisionsantriebswelle E mit konstanter Geschwindigkeit

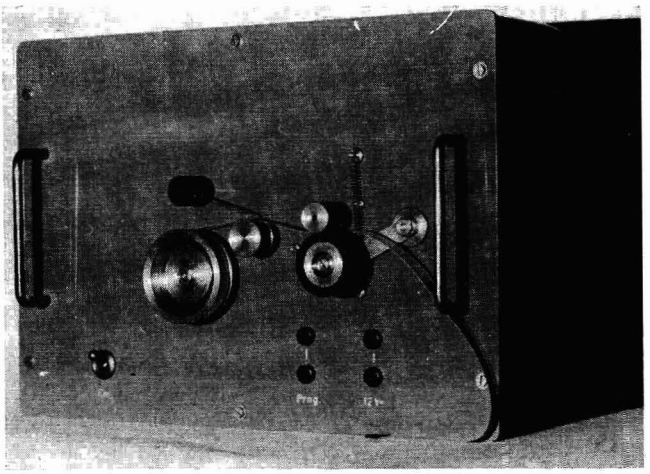

Fig. 1. Impulsgeber.

nach rechts gezogen. Die geschliffene, mit der Feder H belastete Gummiandruckrolle C verhindert jeden Schlupf. Die Antriebswelle E ist zur Einstellung verschiedener Vorschubgeschwindigkeiten auswechselbar. Die Rolle A ist mit einer einstellbaren Rutschkupplung versehen, sodass der Papierstreifen immer gespannt ist. Nach Passieren der Antriebsrolle läuft der Streifen frei ab. Auf dem Scheitel der festen Rolle B wird das Papier gleichzeitig von zwei parallelen, vorne abgerundeten, elektrisch voneinander isolierten Metallfedern unter leichtem Druck abgetastet. Die gedachte Verbindungslinie der Federspitzen verläuft quer zur Papiervorschubrichtung. Während des Papiervorschubs berühren die Federspitzen in bestimmten Zeitabständen gleichzeitig die quer zur Laufrichtung markierten Bleistiftstriche. Dabei wird der elektrische Widerstand zwischen den beiden isolierten Abtastspitzen

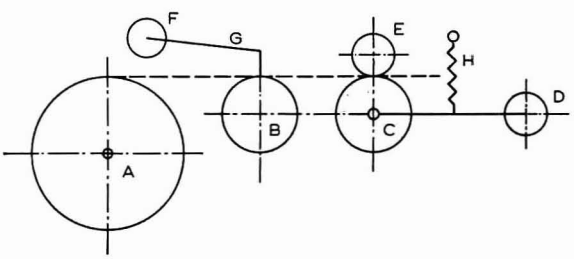

Fig. 2. Impulsgeber. A = Ablaufrolle; B = Kontakttisch; C = Gummiandruckrolle; D = Lagerung des Hebels zur Andruckrolle; E = Antriebswelle; F = Lagerung der Kontaktfedern; G = Kontaktfedern; H = Zugfeder zu Andruckrolle; – – – = Papierstreifen.

von *ca.* 500,000 auf 20,000 Ω herabgesetzt, da Graphit ein guter elektrischer Leiter ist. Die Federn stehen mit einem elektronischen Kaltkathoden-Relais in Verbindung und durch die radikale Widerstandsänderung wird dieses erregt.

(2) *Schaltgerät und automatischer Bereichsumschalter*

Der Recorder wird mit 2 Mikroschaltern ausgerüstet, von denen einer bei 15–30 %, der andere bei 95 % des Skalenendwerts Kontakt gibt. Die Schaltung (Fig. 3) erlaubt 3 Funktionen: 1. Vollautomatische Bereichsumschaltung. 2. Programmgesteuerte Bereichsumschaltung. 3. Handbetrieb. Die auf dem Schaltschema dargestellte Funktion entspricht der vollautomatischen Umschaltung. Der 15–30 % Mikro-

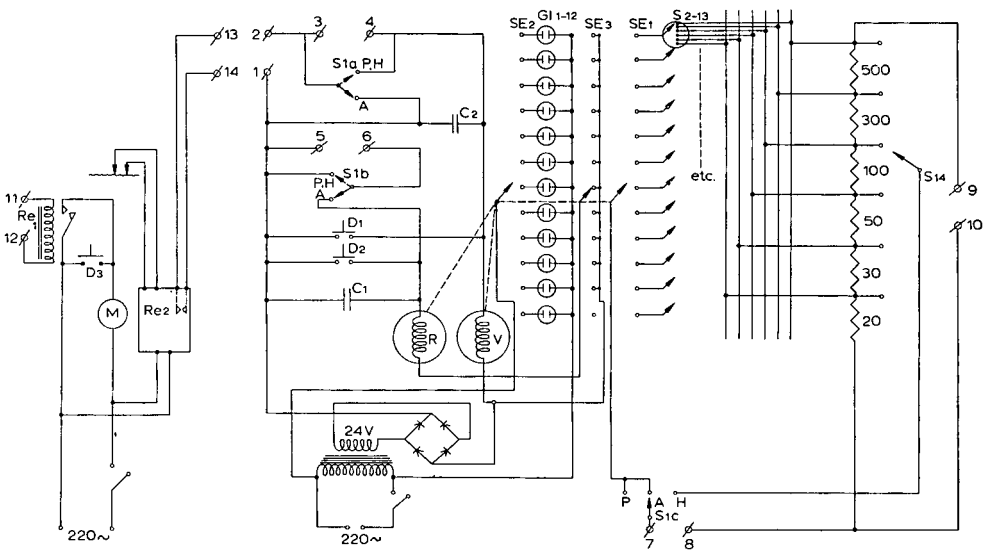

Fig. 3. Schaltung.

V	= Schrittschaltwerk vorwärts	D_{1-3}	= Drucknopf, normal offen
R	= Schrittschaltwerk rückwärts	$C_{1\cdot 2}$	= Papierkondensator 1 μF, 250 V
Re 1	= Relais 12 V =	Gl_{1-12}	= Signalglimmlampen
Re 2	= Kaltkathodenrelais	SE_{1-3}	= Schalterebenen für Schrittschaltwähler 1 × 12 Kontakte
S_1	= Schalter 4 × 3 Kontakte		
S_{2-13}	= Schalter 1 × 6 Kontakte	M	= Antriebmotor
S_{14}	= Schalter 1 × 6 Kontakte	P, A, H	= Programm, Automatik, Hand

Anschlüsse: 1, 2: Verbindung mit Impulsgeber
3, 4: Verbindung mit Mikroschalter 95 %
5, 6: Verbindung mit Mikroschalter 10–30 %
7, 8: Recorder
9, 10: Detektorausgang
11, 12: Startspannung 12 V
13, 14: Verbindung mit Programmgerät

schalter liegt an den Klemmen 5 und 6, der 95 % Mikroschalter an den Klemmen 3 und 4. Bei Betätigung dieser Schalter wird entweder das vorwärtsschaltende Schrittschaltrelais V oder das rückwärtsschaltende Relais R betätigt. Als Schrittschaltrelais nahmen wir 2 Kuhnke Schrittschaltrelais Type Sch B 50–25°; 24 V–51 W–40 % Einschaltdauer, der Firma H. Kuhnke, Malente/Holstein, Bundesrepublik Deutsch-

land*, die aus je einem Drehmagneten mit Ratsche bestehen und auf deren Achse ein 12-Stufen Präzisionsdrehschalter (Contraves, Zürich), montiert wurde. Bei Erregung dreht sich die Achse des Drehmagneten um 30° vorwärts und schaltet dabei den 12-Stufenschalter über die Ratsche um jeweils eine Stufe vorwärts. Am Ende der Schalterachse befindet sich ein zweites Schrittschaltrelais zur Rückwärtsschaltung. Ausser der Schalterebene für die Bereichsumschaltung, befinden sich noch zwei weitere Schalterebenen auf der Achse, von denen die eine die Glimmlampen zur Markierung der Schaltstufe einzuschalten gestattet, während die zweite zur Unterbrechung der Relaiszuleitung des Relais R in der Anfangsstellung dient. Diese Unterbrechung ist notwendig, da der Stufenschalter vor der Anfangsstellung einen Anschlag besitzt und ein Ansprechen des Relais R in der Anfangsstellung zu starke mechanische Kräfte auf die Ratsche der blockierten Schalterachse ausüben würde.

An den Kontakten des 12-Stufenschalters befindet sich je der Abgriff eines kleinen 6-Stufen Radioschalters. Jeder Kontakt des Radioschalters ist mit einem passenden Abgriff des Spannungsteilers zur Bereichsumschaltung verbunden. Damit besteht die Möglichkeit, auf die aufeinanderfolgenden Kontakte des 12-Stufenschalters einen beliebigen der 6 Bereiche zu schalten. In der Stellung Vollautomatik werden die Bereiche 2, 4, 10, 20, 40, 100 mV der Reihe nach auf die Kontakte des 12-Stufenschalters gelegt. Das Chromatogramm beginnt in der Grundstellung 2 mV. Wenn der Recorderausschlag 95 % von 2 mV erreicht hat, schaltet der entsprechende Mikroschalter auf den nächst höheren Bereich um. Geht der Ausschlag wieder auf 15–30 % zurück, wird der entsprechende Mikroschalter den nächst niedrigeren Bereich einschalten, bis schliesslich die Ausgangsstellung wieder erreicht ist.

Die zahlreichen Schaltunterbrüche der vollautomatischen Bereichsumschaltung machen — wie erwähnt — eine quantitative Auswertung des Chromatogramms meistens unmöglich. Man hat es nun bei dem beschriebenen Gerät in der Hand, sich mit weniger als 6 Umschaltstufen zu begnügen, indem man beispielsweise nur zwei oder drei Bereiche auf den Stufenschalter schaltet und damit ein übersichtlicheres Chromatogramm erhält.

In der Stellung "Programm" des Schalters S 1 werden die Klemmen 3 und 4, 5 und 6 und damit die Mikroschalter kurzgeschlossen und die Klemmen 1 und 2 geöffnet. Die Klemmen 1 und 2 werden mit den normal offenen Klemmen 13 und 14 des Impulsgebers verbunden. Wenn diese Kontakte geschlossen sind, wird das Schrittschaltrelais V um eine Stufe vorwärts geschaltet. Das Relais R ist dabei ausser Betrieb. Je nachdem welche Bereiche auf die aufeinanderfolgenden Kontakte des 12-Stufenschalters geschaltet werden, kann für jeden Peak der geeignetste Bereich gewählt werden.

Zur Aufstellung eines Chromatographierprogramms wird zunächst ein vollautomatisches Chromatogramm hergestellt (Fig. 4). Aus diesem Chromatogramm ergeben sich die günstigsten Bereiche und die genauen Umschaltzeiten der Bereiche. Längs des Diagrammstreifens mit dem Chromatogramm wird der 10 mm Papierstreifen gelegt und der Nullpunkt mit Farbstift markiert. An den geeigneten Umschaltpunkten werden die Bleistiftmarkierungen angebracht. Nach dem Einspannen des Papierstreifens wird die Nullmarkierung unter die Abtastspitzen gelegt. Zugleich mit der Injektion der Probe wird ein Fusspedal am Chromatographen betätigt, das

* Die Relais wurden so geändert, dass die Ratschen im Ruhezustand ausgeklinkt sind, jedoch sind auch fertige, vorwärts- und rückwärtsschaltende Relais erhältlich.

über zwei Relais auf dem Diagrammpapier eine Markierung macht (Startpunkt) und den Papiervorschub des Recorders einschaltet. Gleichzeitig wird das Relais Re 1 des Impulsgebers erregt und der Synchronmotor eingeschaltet, wobei der Ablauf des Impulsgeberstreifens beginnt. Durch diese Kombination können die Umschaltungen zeitlich sehr exakt vorgenommen werden (Fig. 5).

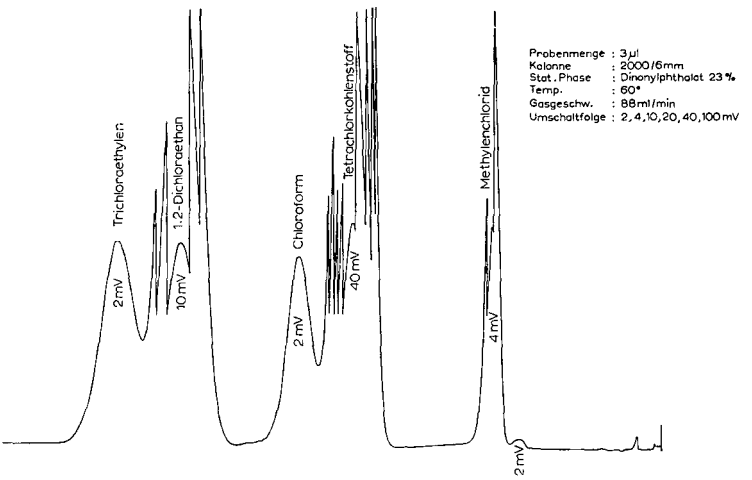

Fig. 4. Vollautomatische Umschaltung der Bereiche.

Ausser für gaschromatographische Serienanalysen eignet sich die beschriebene Programmsteuerung besonders auch für die präparative Gaschromatographie, bei welcher die anfallenden Fraktionen in einem Durchgang oft zu klein sind, sodass mehrfache Probeneingaben nötig werden.

Es versteht sich von selbst, dass das Programmsteuergerät auch für andere Programme als in der Gaschromatographie üblich verwendet werden kann.

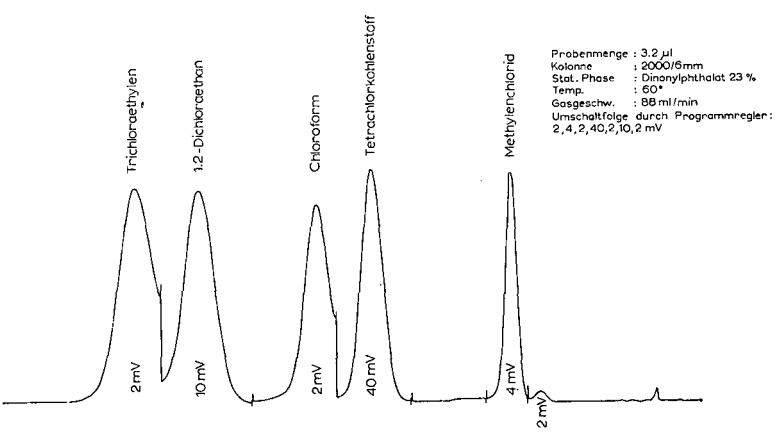

Fig. 5. Umschaltung der Bereiche nach vorbestimmtem Programm.

DANK

Der Direktion der Ciba danke ich für die Erlaubnis zur Veröffentlichung dieser Arbeit. Ebenso sei die Mitarbeit der Herren G. GRASS und P. SPRÜNGLI aus meinem Laboratorium mit Dank erwähnt.

ZUSAMMENFASSUNG

Es wird ein einfaches Gerät für die Gaschromatographie beschrieben, mit welchem die Recorderbereiche bei der Aufnahme von Chromatogrammen entweder vollautomatisch oder nach einem beliebig einstellbaren Programm eingeschaltet werden können.

SUMMARY

A simple apparatus for use in gas chromatography is described. The apparatus can be adjusted so that the recorder range is governed completely automatically or so that it can be programmed as desired.

THE RELATIONSHIP BETWEEN SOLID SUPPORT, COLUMN EFFICIENCY, AND STEROL QUANTITATION BY GAS CHROMATOGRAPHY

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Since the first report of gas chromatographic separation of C-27 sterols there have been several papers describing quantitative methods. Although most authors reporting sterol quantitation have used argon ionization detectors, results and interpretations have varied. SWEELEY AND CHANG[1] first pointed out a need for individual calibration of each sterol in a mixture. Within limits these authors and others[2] found C-27 sterol responses to be linear in an ionization detector. Recently ROSENFELD *et al.*[3] have shown non-linear responses with cholesterol and coprostanol. My own experience has been that while these substances are never truly linear, under certain conditions they approach linearity[4] and because of relationships between response, retention time and oxygenation, quantitation of a variety of sterols may be accomplished using appropriate standards. I have recently observed that when the column efficiency is changed, molar responses and limits of linearity for sterols may be markedly altered. This can be advantageous, but calls for the modified approach to quantitation of sterol mixtures reported below.

METHODS

Apparatus and conditions are similar to those previously used[4]. Briefly they are Barber-Colman Model 10 argon capillary ionization detector, 6 ft., 1/4 in. I.D. column. Column temperature 250°, flash heater 270°, detector 260°, anode voltage 1500. Two column packings were used, Chromosorb-W (120–140 mesh)** and Gas Chrom-P (80–100 mesh)***. The former packing gives a lower efficiency column and the latter packing gives a higher efficiency one. Both packings were presiliconized and coated with 1% SE-30 silicone gum rubber† [5]. Theoretical plates were calculated by the method described by HARDY AND POLLARD[6]. Areas were calculated as previously described[4]. A standard mixture of sterols was used throughout the study except where noted. This contained in each milliliter of methylene chloride, cholestane 0.2 mg, cholestan-3β-ol 0.6 mg, cholestane-3β,7α-diol 1.0 mg, and cholestane-3β,5α,6α-triol 5.0 mg. Cholestan-3β-ol serves as a satisfactory substitute for cholesterol and

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appears to be more stable in solution. Methylene chloride is preferred as a solvent because there is minimal tailing of solvent peaks.

RESULTS AND DISCUSSION

Earlier work[4] was done using Chromosorb-W (120–140 mesh) with a 1% coating of SE-30. When the column support was changed to Gas Chrom-P (80–100 mesh) a large increase in efficiency was obtained so that theoretical plates were about twice those of previous columns. The increased column efficiency markedly changed compound thresholds and linear ranges for those compounds with oxygen functions. The differences became larger as the number of oxygen molecules incorporated into these steroids increased. More highly oxygenated compounds such as cholestanediols and cholestanetriols quantitated well with smaller amounts of material than were previously used. This was very helpful in the study of fecal sterol mixtures in which these compounds were present in relatively small amounts. Paradoxically, the quantitation of simple compounds such as cholesterol, became more complicated since useful linearity decreased as column efficiency increased. When increased column efficiency emphasized the non-linearity of the argon detector to the point that a good linear range for comparison with an internal standard such as cholestane was difficult to determine, changes in my original method of quantitation of complex mixtures of fecal sterols[4] were essential since that method was based on the broad linear ranges afforded by low efficiency columns. Furthermore, as will be shown below, responses and linear ranges can change during continuous use of a column so that repeated calibration during quantitative studies is necessary. The different responses observed with the change of solid support are compared in Table I. Columns labeled A represent

TABLE I

VARIATION OF COLUMN EFFICIENCY AND RESPONSE WITH SOLID SUPPORT*

Compound	*Micrograms injected*		*Peaks specific response* ($cm^2/\mu g$)		*Relative response to cholestane*		*Theoretical plates*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Cholestane	0.6	0.3	4.30	4.33	1.00	1.00	930	2200
Cholestan-3β-ol	1.8	0.9	3.03	3.56	0.701	0.821	900	2700
Cholestane-3β,7α-diol	3.0	1.5	2.17	3.20	0.505	0.740	760	1760
Cholestane-3β,5α,6α-triol	15.0	7.5	0.61	3.18	0.140	0.735	1120	2500

* Figures in columns labeled A are from low efficiency columns (Chromosorb-W, 120–140 mesh); figures in columns labeled B are from high efficiency columns (Gas Chrom-P, 80–100 mesh).

the low efficiency support and B the high efficiency one. While the hydrocarbon cholestane is not affected by the change in support, the responses of more highly oxygenated compounds are moderately increased by the increasing column efficiency. The effect is most marked for cholestane-3β,5α,6α-triol which was in mid-linear range on one support and above linear range on the second support with an injection one half as large. The increased column efficiency made it difficult to define a linear portion of the dose–response curve for the oxygenated compounds. Calibration curves for

cholestan-3β-ol for the two supports are shown in Fig. 1. With the original support (Curve A) the linear range is relatively broad and easily distinguished while with the Gas Chrom-P (Curve B), the linear range is more difficult to define and the dose–response curve is paraboloid in shape. While sensitivity is increased accurate quantitation has become more difficult. These results are due to a combination of factors.

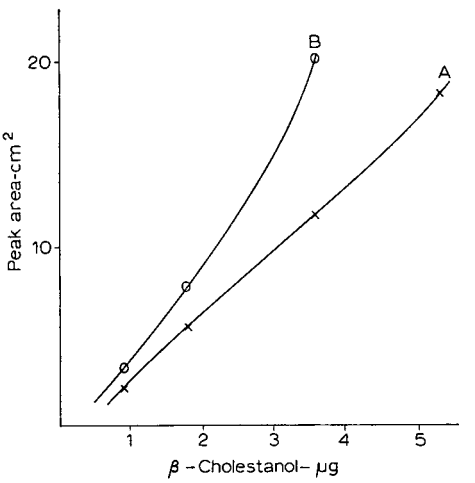

Fig. 1. Calibration curves for β-cholestanol with two different supports. See text.

First of all the basis for linearity of the argon instrument is that response is a function of both the total mass delivered and the rate at which the mass is being presented to the detector[7]. As the number of theoretical plates increases, sharper mass peaks are presented to the detector. Absolute threshold values diminish and detector over-load is experienced with correspondingly smaller amounts. A second factor which is responsible in part for the differences observed in Table I probably relates to the adsorption characteristics of the solid support. It may well be that the finer mesh Chromosorb-W was responsible for more adsorption and tailing than was the Gas Chrom-P although both supports were prepared in identical fashion.

Variation in column efficiency and detector response during continuous use

During a period when the Gas Chrom-P column was in continuous use quantitating fecal non-saponifiable material, it was observed, by comparing standards, that responses were changing gradually and these had to be meticulously taken into account in calculating the fecal sterols. Table II gives the data from two similar injections of the standard sterol mixture made 20 h apart. The changes seen in Table II are not due to any errors in injection technique since three standard injections were made each time. The differences in column detector performance obvious in Table II are two-fold. Each peak specific area (molar response) has increased from 38 to 72 %. The relative response of each peak to cholestane has increased significantly in each instance. Cholestanetriol is beyond linearity with the dose of 7.5 μg whereas in the earlier sample it was linear at the 10 μg level. Of interest is the fact that on the low efficiency column[4] 7.5 μg was barely over threshold. One may account for the differences of Table II by scrutiny of Fig. 2 which contains reproductions of the chro-

TABLE II

COMPARISON OF RESPONSE OF STANDARD STEROL MIXTURE BEFORE AND AFTER 20 HOURS CONTINUOUS USE

Sample number*	*Compound***	*Relative retention time*	*Microgram injected*	*Area (cm^2)*	*Area/unit weight*	*Relative response to cholestane*
2	A	1.00	0.4	1.07	2.67	1.00
	B	1.61	1.2	2.71	2.25	0.842
	C	2.50	2.0	3.76	1.88	0.704
	D	3.62	10.0	17.7	1.77	0.662
36	A	1.00	0.3	1.11	3.70	1.00
	B	1.64	0.9	2.89	3.22	0.870
	C	2.51	1.5	4.03	2.68	0.725
	D	3.62	7.5	22.8	3.04	0.822

* Sample 2 was 2.0 μl injection of standard mixture; sample 36 was 1.5 μl injection.
** A = cholestane; B = cholestan-3β-ol; C = cholestane-3β,7α-diol; D = cholestane-3β,5α,6α-triol.

matograms of the standards at the beginning of the series (A) and at the end (B). While the retention times can be superimposed, the peaks of the later chromatogram are sharper, higher, and narrower. Theoretical plates have been increased from an average of 1900 to 2300, despite the fact that chromatogram A is a smaller dose which should calculate to more theoretical plates, other factors being equal[6]. The effect, not apparent for cholestane, is more obvious as oxygen functions are added to the molecule. Note the gross loss of linearity represented by the two peaks of cholestane-3β,5α,6α-triol. The cholestanetriol peak in curve B is recorded at 35 % sensitivity. In other words, the rate at which molecules are being presented to the detector has increased. Since the machine is not truly linear to begin with, this change in column efficiency

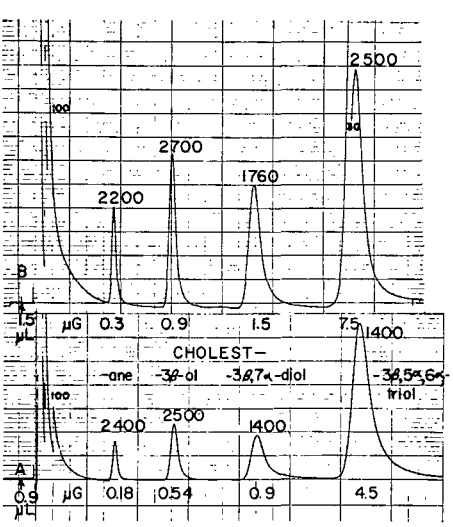

Fig. 2. Chromatograms of standard mixture of sterols at beginning (A), and end (B) of 20 h of fecal sterol quantitation. Theoretical plates for each peak are noted above, and dose size in micrograms is noted below each peak. The small numerals 30 and 100 are relative gains.

profoundly affects results in the same proportion as peak specific area was affected, *i.e.* 38–72 %. A second factor which may be responsible for the changes noted in Table II may be related to gradual filling of binding sites open on the adsorbent. These effects were noted despite routine loading doses[4] prior to quantitation.

Quantitation with high efficiency supports

The principles of quantitation of complex mixtures of fecal sterols are identical to those previously published[4]. Modification of the method is necessary to account for changing responses both with dose and time. Quantitation in this instance is done by running standards at regular intervals throughout the series of determinations and obtaining peak specific areas from one of two interpolations described below. Since the peak specific area is changing regularly as the column efficiency changes this may be plotted as shown in Fig. 3. Fig. 3 is plotted from data made from a series of 44 sample

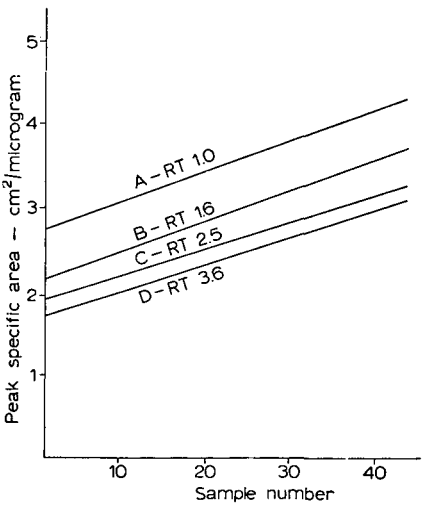

Fig. 3. Change of peak specific area with time during quantitative series. A = cholestane; B = cholestan-3β-ol; C = cholestane-3β,7α-diol; D = cholestane-3β,5α,6α-triol. RT = relative retention time to cholestane.

runs, each lasting about 30 min. Standards were run at intervals during the study. The responses of all compounds changed gradually with time. The lines are plotted by compound name and retention time so that unknown peaks at any time can be interpolated into the drawing on the basis of retention time[4] and sample number. Fig. 3 is satisfactory for quantitation when peaks are known to be in linear range, but this may be difficult to ascertain under the conditions described above.

The second method for determining peak specific areas of unknown peaks and one which takes the non-linearity and changing column efficiency into account is based on Figs. 3 and 4. Fig. 4 is a plot of relative responses to cholestane and peak height for each standard compound from the same series of determinations described for Fig. 3. It can be seen from Fig. 4 that relative responses increase gradually as larger amounts of standard are injected. This relationship holds well over a broad range of column efficiencies. The lines drawn in Fig. 4 are from an average of 12 points

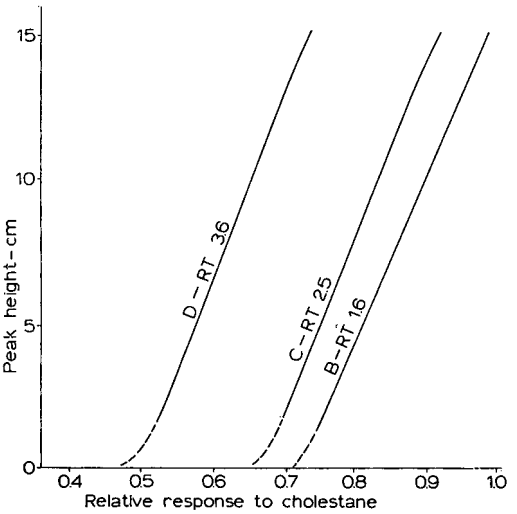

Fig. 4. Variation of relative response with peak height. B, C, D are same as in Fig. 3. RT = relative retention time to cholestane.

for each line. From Fig. 4 a line representing the relative retention time of any unknown may be interpolated visually and from the peak height of the unknown, a relative response is noted. The peak specific area of cholestane can be determined from Fig. 3 and the calculations made according to the formula:

$$m = \frac{A}{Sc \times RR} \tag{1}$$

where: m = the weight of the unknown peak;
A = the area of the unknown peak;
Sc = the peak specific area of cholestane;
RR = the relative response of the unknown peak determined from Fig. 4.

This method of quantitation does not require an internal standard although when used it can be helpful. The internal standard is most useful when the proportion of cholesterol and coprostanol peaks is high with respect to other non-saponifiables, *i.e.* when the animal is fed a cholesterol enriched diet. Under these conditions a relatively large amount of internal standard may be added which will not be influenced significantly by small peaks in fecal non-saponifiable material with retention times equal to cholestane. When the cholesterol and coprostanol content of feces is low with respect to other non-saponifiable materials, small peaks may interfere with internal standardization and must be corrected for by two separate chromatograms, with and without the internal standard. Without the internal standard careful control of volumes is essential.

While the method outlined above seems laborious the advantages are great. A fractional analysis is helpful even though each component cannot always be identified. Plant sterols such as β-sitosterol which appear in many diets are separated from cholesterol and coprostanol so that the latter may be quantitated without interference. Table III is an example of the data obtained from balance studies in two groups of animals on high fat diets supplemented with 1 % cholesterol. In group A the fat is butter in which the sterol is largely cholesterol and the cholesterol–coprostanol peaks

TABLE III

VALUE OF FRACTIONAL STEROL ANALYSIS BY GAS CHROMATOGRAPHY

Animal number	*Diet**	*Sterol per g rat feces measured by:*				
		Gas chromatography				*Digitonin precipitation*
		Coprostanol	*Cholesterol*	*Sitosterol***	*Total*	
1	A	11.2	40.7	< 1	51.9	50.4
2	A	14.8	29.2	< 1	44.0	47.4
3	A	4.4	48.7	< 1	53.1	54.0
4	B	6.7	37.2	6.6	43.9	60.6
5	B	12.8	22.5	6.0	41.3	43.1
6	B	6.0	46.0	6.5	58.5	64.5

* Diet A contains 20% butter, 1% cholesterol; diet B contains 20% unsaturated margarine (Emdee Margarine, Pitman-Moore Co., Indianapolis 6, Ind.).

** Quantitation of sitosterol is only approximate since this method is based upon C-27 sterols and their oxygenated derivatives.

account for more than 95% of total fecal sterol under these conditions, closely agreeing with gravimetric data from digitonin precipitation. In group B, the fat is a highly unsaturated vegetable margarine with 0.7% content of sitosterols. When cholesterol and coprostanol peaks were analyzed, their sum was significantly less than the digitonin data indicated, the difference accounted for by fecal metabolites of the vegetable sterols. This information could not be appreciated in any study of fecal sterols by the Liebermann-Burchard or digitonin precipitation methods. Qualitative and quantitative differences such as cholesterol–coprostanol ratios[2] are also readily apparent. With improved column efficiency the more highly oxygenated compounds have better responses and can be more easily and accurately measured. The price for these results is meticulous control of quantitation with appropriate standards. It has not been my experience that "recalibration is not a serious problem" as claimed by ROSENFELD *et al.*[3] and repeated recalibration appears essential. Simple reliance upon linearity or "almost linear" can lead to errors as much as 100% in quantitative values. Since gas chromatography has the capacity for superior analysis of the complex sterols of feces it is important that the problems encountered with this method be clarified early before issues are clouded by conflicting results due to varied methodologies rather than varied biologies.

ACKNOWLEDGEMENTS

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SUMMARY

The argon ionization detector is not a truly linear instrument for the quantitative analysis of sterols. Because of this large changes in the molar response will occur when column efficiency is changed. These effects are described and methods are outlined for the quantitation of complex sterol mixtures under varying conditions. The necessity for careful control and standardization is emphasized.

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THE SEPARATION OF C_6F_{14} ISOMERS BY GAS CHROMATOGRAPHY AND THE EFFECT OF STATIONARY PHASE CONCENTRATION

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This paper describes the resolution of some of the isomers of C_6F_{14}, perfluorohexane, by gas–liquid partition chromatography.

An earlier report[1] discussed the partition chromatography of fluorocarbons on various stationary-phase liquids at temperatures near the boiling points of the compounds. The results showed that fluorocarbons may best be separated into molecular weight classes by partitioning on stationary media in which they form as nearly ideal solutions as possible. Such liquids as Kel-F oils and $(C_4F_9)_3N$ were used at concentrations of 0.1 to 0.2 c.c. per c.c. of total column volume. It has subsequently been found that at such high concentrations in the column fluorocarbon stationary phases do not separate the close-boiling isomers of fluorocarbon alkanes as well as do hydrocarbon stationary phases. Furthermore great improvement in the resolution of fluorocarbons by the hydrocarbon stationary phase is obtained by reducing the column temperature to room temperature. Since the activity coefficients of fluorocarbons in a hydrocarbon solvent are large, the capacity of the hydrocarbon partitioning column and the appearance times are unusually low, compared to that obtained on a stationary phase with which ideal solutions are formed. Thus, nonideal solutions possess some advantage in a GLPC of nonpolar molecules. At a column temperature of 30° it is possible to elute fluorocarbons up to C_6F_{14} from 2 meters of *n*-hexadecane (0.2 c.c. $C_{16}H_{34}$/c.c. total column volume) in a few minutes at ordinary carrier gas flow rates (*ca.* 20 c.c./min in 0.2-in. i.d. tubes). However, a 2-m length is not sufficient for separating the isomers of C_6F_{14}.

EXPERIMENTAL

In the work with C_6F_{14} partitioned on *n*-hexadecane, a series of columns was investigated in which the thickness of the stationary phase on the supporting particles and the volume of stationary phase in the column were varied. Table I lists the information on these columns. In all columns, except 3A, the particles were acid-washed Chromosorb-P, 35 to 80 mesh. From these tests the ratio of *n*-hexadecane-to-solid support was selected that best resolved a mixture of the isomers of C_6F_{14}. This mixture was obtained in this laboratory as a fraction of the total material produced by the electrolysis of hexyl sulfide in anhydrous HF[2]. The best column was selected by noting the resolution of the C_6F_{14} material at room temperature. Various quantities ranging from 1 μl to 30 μl on the 0.197-in. i.d. tubes were charged. Carrier gas flow rates from 1 to 35 c.c./min were used.

TABLE I

COLUMNS WITH *n*-HEXADECANE SUBSTRATE

Column No.	*Length (m)*	*Total packing (g)*	*g $C_{16}H_{34}$ per g solid*	*c.c. packing per c.c. total at R.T.*	*c.c. $C_{16}H_{34}$ per c.c. total at R.T.*	*i.d. tube (in.)*
38[a]	2	18.7	0.19	0.32	0.10	0.197
36[a]	2	19.9	0.25	0.35	0.13	0.197
37[a]	2	19.8	0.31	0.36	0.16	0.197
3A[b]	2	23.6	0.40	0.51	0.22	0.197
3C[a]	17	2047	0.40	0.72	0.352	0.50
40[c]	8	104.3	0.40	0.49	0.248	0.197
37B[a]	2	22.8	0.51	0.46	0.26	0.197

[a] Particle density of uncoated solid support = 1.85 g/c.c.
[b] Particle density of uncoated solid support = 1.95 g/c.c.
[c] Particle density of coated solid = 1.36 g/c.c.

It was found (a) that the resolution was best with 0.4 g of *n*-hexadecane per g of supporting solid; (b) that a maximum charge of about 5 μl was possible without destroying the resolution; (c) that 12 to 16 m total length of column was sufficient to produce adequate resolution, and (d) that a carrier flow rate of 80 cm/min at the exit was near the optimum for this separation.

On the basis of these tests column 3C was prepared in 0.5-in. i.d. steel tubing for the preparative scale work. This column was arranged in the vertical plane. The resolution in this column was at least as good as that obtained in the smaller diameter tubes at the same total length. The use of nitrogen as carrier, rather than hydrogen used in the 0.197-in. i.d. tube, contributed to the better resolution in the 0.5-in. tube. The volume of *n*-hexadecane per unit total column was greatest in the larger tube even though the ratio of *n*-hexadecane to solid support was 0.40 g/g in both diameters.

In Table II are given the conditions of operation and appearance times on the preparative column and of two other hydrocarbon-coated columns of the smaller

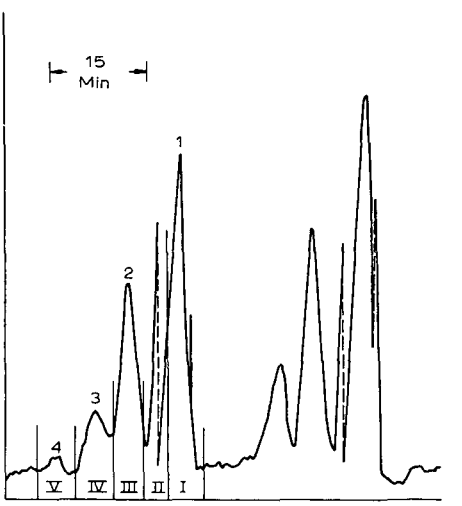

Fig. 1. C_6F_{14} mixture on column 3C; 17 m of *n*-hexadecane. Roman numerals indicate cuts in preparative scale runs. Arabic numerals designate isomers.

TABLE II

CHROMATOGRAMS OF C_6F_{14} MIXTURE ON *n*-HEXADECANE

Column 40: total length = 8 m; i.d. = 0.197 in.; T = 28°; P_i = 1 atm; P_o = 1 atm. Void vol. = 85 c.c.; $C_{16}H_{34}$ vol. = 38.8 c.c. Hydrogen flow rate at exit = 16 c.c./min = 82 cm/min.

Compound	*Peak app. time (min)*	*Corrected retention vol. (c.c.)*	*Partition coeff. K*	$K_{n\text{-}C_6}/K$
Air	8.3	117	1.21	
n-C_6F_{14}	22.0	314	0.170	1.0
2-CF_3-C_5F_{11}	24.3	346	0.149	1.14
3-CF_3-C_5F_{11}	25.5	364	0.139	1.22
$(CF_3)_2C_4F_8$	28.7	410	0.119	1.43

Column 40 + 3A + 37B in series: total length = 12 m; i.d. = 0.197 in.; T = 28°; P_i = 19.2 p.s.i.a.; P_o = 14.7 p.s.i.a. Void vol. = 125 c.c.; $C_{16}H_{34}$ vol. = 57.5 c.c. Hydrogen flow rate at exit = 16 c.c./min = 82 cm/min.

Air	14	193	0.85	
n-C_6F_{14}	37.5	520	0.145	1.0
2-CF_3-C_5F_{11}	40.5	563	0.131	1.11
3-CF_3-C_5F_{11}	42.5	590	0.124	1.17
$(CF_3)_2C_4F_8$	46	640	0.112	1.29

Column 3C: total length = 17 m; i.d. = 0.50 in.; T = 28°; P_i = 29.7 p.s.i.a.; P_o = 14.7 p.s.i.a. Void vol. = 490 c.c.; $C_{16}H_{34}$ vol. = 760 c.c. Nitrogen flow rate at exit = 108 c.c./min = 85 cm/min.

n-C_6F_{14}	77	5300	0.162	1.0
2-CF_3-C_5F_{11}	84.5	5820	0.146	1.11
3-CF_3-C_5F_{11}	90	6200	0.136	1.19
$(CF_3)_2C_4F_8$	96	6610	0.127	1.28

diameter. The auxiliary equipment used in the preparative separation has been described previously[3]. A typical chromatogram obtained during the quantity separation of the C_6F_{14} mixture on column 3C is shown in Fig. 1. The vertical lines on the middle group of peaks show the cut points. The volume of each charge was approximately 0.05 c.c. and they were injected at 30-min intervals. Since complete resolution was not obtained in one pass, each cut was reprocessed on the 0.5-in i.d. column to obtain the pure isomers.

Each isomer was identified by nuclear magnetic resonance spectroscopy[4] as follows:

Peak	*Isomer*
1	*n*-C_6F_{14}
2	Perfluoro-2-methylpentane
3	Perfluoro-3-methylpentane
4	Perfluoro-dimethylbutane isomers

Because of the small quantity of dimethylbutane isomers, these two molecular species were not isolated separately. Pure perfluoro-2,3-dimethylbutane from another source[5] established the appearance time of the fourth peak as that of this compound.

Analysis of the pure fractions on columns 40 + 3A + 37B in series, using hydrogen as carrier gas in a Perkin-Elmer Vapor Fractometer with thermistor detectors at 28°, showed no trace of peaks other than that of the particular isomer.

Perfluoropentane from the electrolysis of pyridine in HF has also been resolved into perfluoro-*n*-pentane and perfluoro-2-methylbutane by the same procedure.

WHY HYDROCARBON SUBSTRATES ARE BETTER

The hydrocarbon possesses the advantage over the more compatible fluorine-containing stationary phases primarily because the retention time of fluorocarbons in hydrocarbon is low at low temperatures. The greater relative partition coefficients found at lower temperatures, compared to higher temperatures, produces better resolution on thermodynamic grounds. Furthermore, the spreading of the peaks by diffusional processes is minimized by operation at low temperatures and low retention time.

Another effect contributing to the advantage of the hydrocarbon substrates for fluorocarbon separation is found in the work of GOLAY[6] leading to the use of capillary columns. This work has shown that the spreading of peaks is low when the ratio of capacity in the gas phase to capacity in the stationary phase is high. Hydrocarbon stationary phases possess lower solubility of fluorocarbons and thus lower capacities at a given fluorocarbon partial pressure than do the same amounts of fluorocarbon liquid substrates. That is, for a given capacity (or partial pressure) in the gas phase the solubility (or capacity) in the liquid phase at a given temperature is inversely proportional to the activity coefficient of the gaseous component in that liquid phase. The relative partition coefficient of isomeric fluorocarbons are very similar, no matter what the nature of the solutions formed in the stationary phase, since the activity coefficients of isomeric fluorocarbons in a given solvent are essentially equal to one another. If this statement is true, merely reducing the quantity of fluorine-containing substrate per total column volume to a value which gives a ratio of capacities in the liquid and gas phases equal to that prevailing in the successful hydrocarbon substrate columns, should, by the GOLAY theory, produce resolutions comparable to those found in the hydrocarbon substrates. The following experiment demonstrates that this expectation is confirmed.

EFFECT OF SUBSTRATE CONCENTRATION ON RESOLUTION OF C_6F_{14} ISOMERS

The chromatogram of the original mixtures of the C_6F_{14} isomers on column 16 (Table III), 2 m long, (0.7 g of $Cl(CF_2CFCl)_3CF_2COOC_2H_5$ per g of Chromosorb) was one broad peak with no resolution of the isomers. Column 50 (Table III) was prepared with approximately one-fourth of this concentration of the ester on the solid support (0.22 g of ester per g of Chromosorb). The column length packed with this preparation was 7.35 m, approximately four times the length of column 16, so that approximately the same total quantity of stationary liquid phase was present in both columns 16 and 50. Operation of the 7.35-m column 50 at the same exit hydrogen gas velocity as in the

TABLE III

COLUMNS WITH FLUOROCARBON STATIONARY PHASES*

i.d. = 0.197 in.; particle density of uncoated support = 1.85 g/c.c.

Column No.	*Length (m)*	*Total packing (g)*	*g stat. phase per g solid*	*c.c. packing per c.c. total at R.T.*	*c.c. stat. phase per c.c. total at R.T.*
		**Kel-F acid 8114 ethyl ester*			
16	2.0	31	0.70	0.44	0.19
50	7.35	75.9	0.222	0.29	0.0565
		**Perfluorotributylamine*			
8A	2.0	33.6	1.71	0.46	0.288

2-m column 16 produced the chromatogram of 4 μl of charge shown in Fig. 2A and Table IV. Fairly good resolution of isomers was thus obtained merely by spreading the liquid substrate over a greater solid supporting area. The substrate concentration ratio of approximately 4 to 1 was chosen on the basis of the known ratio of approximately 1 to 4 for the activity coefficients of C_6F_{14} in the Kel-F acid ester and in *n*-hexadecane, respectively[1].

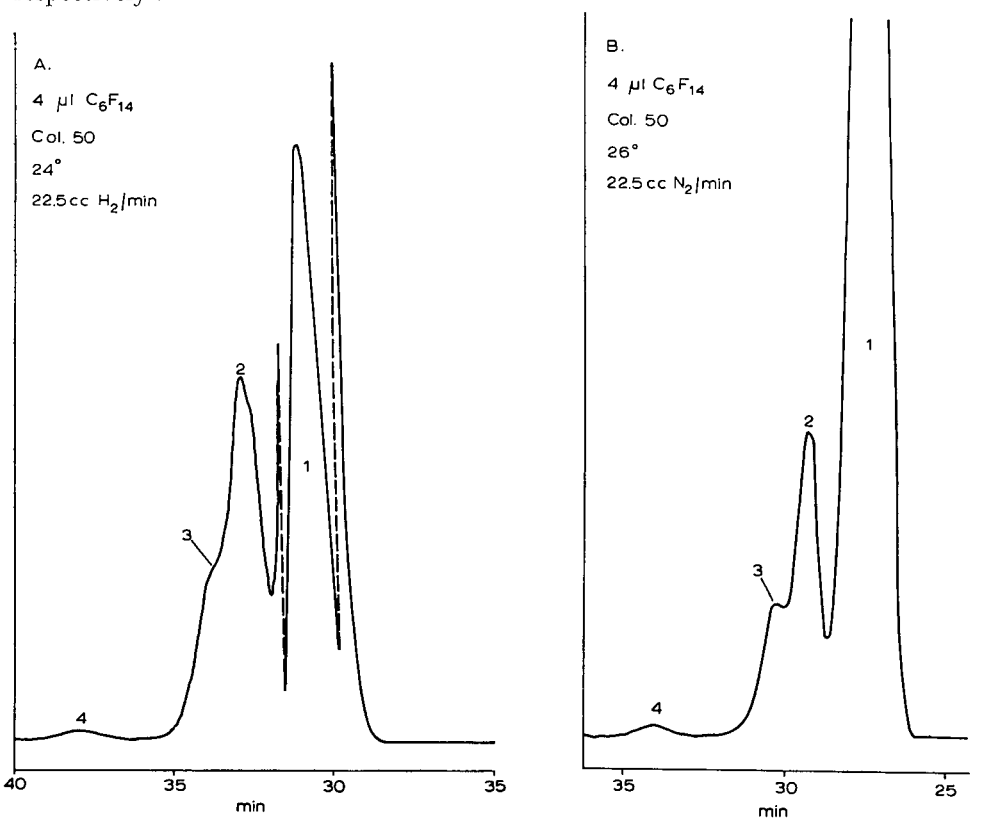

Fig. 2. C_6F_{14} mixture on 7.35 m of $Cl(CF_2CFCl)_3CF_2COOC_2H_5$. (A) Hydrogen carrier gas. (B) Nitrogen carrier gas.

On the basis of the GOLAY theory similar resolutions of C_6F_{14} isomers can be obtained on column 50 and column 40 (8 m of 0.4 g *n*-hexadecane per g of Chromosorb). The chromatogram of 4 μl of the same C_6F_{14} mixture on *n*-hexadecane column 40 is shown in Fig. 3 and Table II. The resolution here is somewhat better than that

TABLE IV

CHROMATOGRAM OF C_6F_{14} MIXTURE ON KEL-F ACID ESTER

Column 50: total length = 7.35 m; i.d. = 0.197 in.; T = 24°; P_i = 1.2 atm; P_o = 1 atm. Void vol. = 102 c.c.; $Cl(CF_2CFCl)_3CF_2COOC_2H_5$ vol. = 8.15 c.c. Hydrogen flow rate at exit = 22.5 c.c./min = 115 cm/min.

Compound	*Peak app. time (min)*	*Corrected retention vol. (c.c.)*	*Partition coeff. K*	K_{n-C_6}/K
Air	6.7	139	0.22	—
n-C_6F_{14}	30.8	630	0.0154	1.00
2-CF_3-C_5F_{11}	32.7	666	0.0145	1.06
3-CF_3-C_5F_{11}	34.2	700	0.0136	1.13
$(CF_3)_2C_4F_8$	37.8	770	0.0122	1.26

obtained on Kel-F ester column 50 using hydrogen carrier gas, but the approximate equivalence of the two columns is evident. The spreading of the peaks is greater on the Kel-F ester substrate even though the appearance times are lower. If the equivalence of the columns were strictly in accordance with GOLAY's theory, the Kel-F ester should be better than the hexadecane since the total volume of substrate per unit quantity of support is greater in the hexadecane column. GOLAY's theory indicates that the thinner substrate layer should give the better resolution. There must be other effects which counteract the deleterious effect of thicker liquid films in the hydrocarbon partitioning column.

An additional parameter that should be minimized according to the GOLAY theory is the void volume in the column. The hexadecane column has the advantage in this respect because of the greater liquid volume per unit solid support allowed by low liquid phase solution capacity.

A further consideration is the magnitude of gas phase diffusion coefficient. Fig. 2B shows the chromatogram of the C_6F_{14} mixture on column 50 under conditions identical with that of Fig. 2A except that nitrogen was used in Fig. 2B in place of hydrogen as carrier gas. The resolution is better with nitrogen. GOLAY's theory gives the spread per unit column length as:

$$2DS/F + [0.01 + 0.03\,(C/C_1 + 1)]\,(F/D)$$

where S is the cross-section of the moving phase, F is the carrier volumetric flow rate, C is the capacity of the moving phase, C_1 is the capacity of the stationary phase, and D is the diffusion coefficient in the moving phase. The first term is spreading due to longitudinal diffusion in the gas phase. The second term, $0.01\,F/D$, is the spreading due to finite time for transfer between moving and stationary regions of the carrier gas, and the third term is due to the finite time for transfer between phases. The first term is proportional to D; the last two are inversely proportional to D. Depending upon which terms are the larger, the resolution can increase or decrease with decrease in

D. Because of the large molecular weight fluorocarbons, D is smaller with a given carrier gas than in the case of hydrocarbon molecules of the same number of carbon atoms. D with nitrogen is less than D with hydrogen, so that it appears for these fluorocarbons that the first term is more important than the others, since nitrogen gives better resolution than does hydrogen.

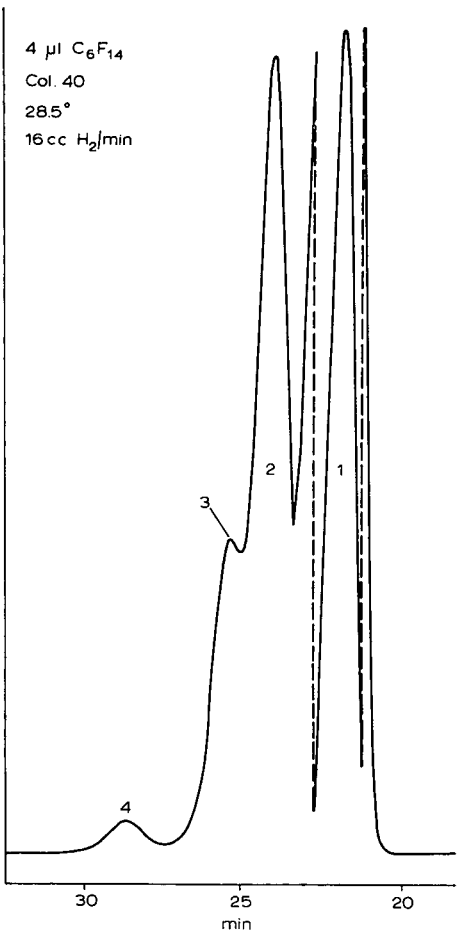

Fig. 3. C_6F_{14} mixture on 8 m of *n*-hexadecane.

Comparison of the data for the various C_6F_{14} isomers on *n*-hexadecane (Table II) with those on the Kel-F ester (Table IV) confirms the expectation that the relative partition coefficients of these fluorocarbons are essentially independent of the nature of the stationary phase, even though the partition coefficients themselves are ten times larger in the hexadecane than in the Kel-F ester.

REMARKS ON HYDROCARBON RESOLUTION IN NONIDEAL SOLUTION SUBSTRATES

For analysis of hydrocarbon mixtures the use of fluorocarbon stationary phases was studied to determine if the nonideal solution possesses any advantages over ideal solu-

tions. Experimental explorations in this direction are described below which indicate that nonideal solution chromatography of C_6H_{14} isomers is not quite as attractive as that for fluorocarbons. It is to be expected at the start from thermodynamic studies of solution that each hydrocarbon isomer will have a different activity coefficient in solution with a given solvent. Even in the absence of such evidence, the fact that the boiling points of hydrocarbon isomers differ considerably from one another would lead one to expect different solution behavior of the isomers one from the other. The boiling points of fluorocarbon isomers all lie within a small temperature range of a few degrees or tenths of a degree, and thus, are essentially equivalent in solution behavior. The condition holding for fluorocarbon isomers demonstrated above, namely that the relative partition coefficients are essentially independent of the type of solution behavior encountered, does not hold for hydrocarbon isomers in fluorocarbon solvents. Nonideal solution behavior may thus destroy or enhance the ideal solution resolution, depending upon the relative magnitudes of the individual activity coefficients of each hydrocarbon isomer when dissolved in a particular nonideal solution substrate. It is desirable that in a given solvent the activity coefficients of isomeric hydrocarbons should decrease with increase in normal boiling point. Actually, they probably increase with increase in boiling point when the substrate is a fluorocarbon. A typical behavior is illustrated by Figs. 4 through 7, which are chromatograms of a mixture containing predominantly three hexane isomers and one heptane isomer.

Fig. 4 illustrates a chromatogram of the C_6H_{14} mixtures in the conventional

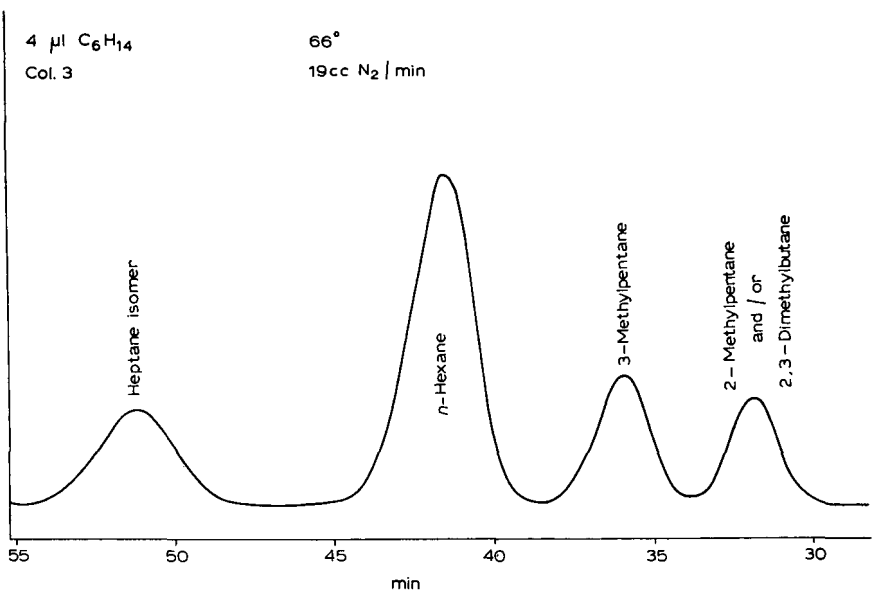

Fig. 4. C_6H_{14} mixture on 2 m of *n*-hexadecane.

manner on *n*-hexadecane (column 3, ref. [1]) at 66° with a 4-µl charge. Fig. 5 is the same material at 20° on column 16, the 2-m Kel-F ester packing of high substrate concentration, using hydrogen carrier gas. The resolution is relatively poor in the latter case.

As shown in Fig. 6A, when the C_6H_{14} mixture is run with hydrogen carrier gas on the 7.35-m column 50 containing essentially the same total amount of Kel-F ester as column 16, the resolution is greatly improved over that obtained in the two preceding columns. When nitrogen is substituted for hydrogen as carrier at otherwise identical conditions (Fig. 6B), the hydrocarbon peaks are more widely spread out. This behavior

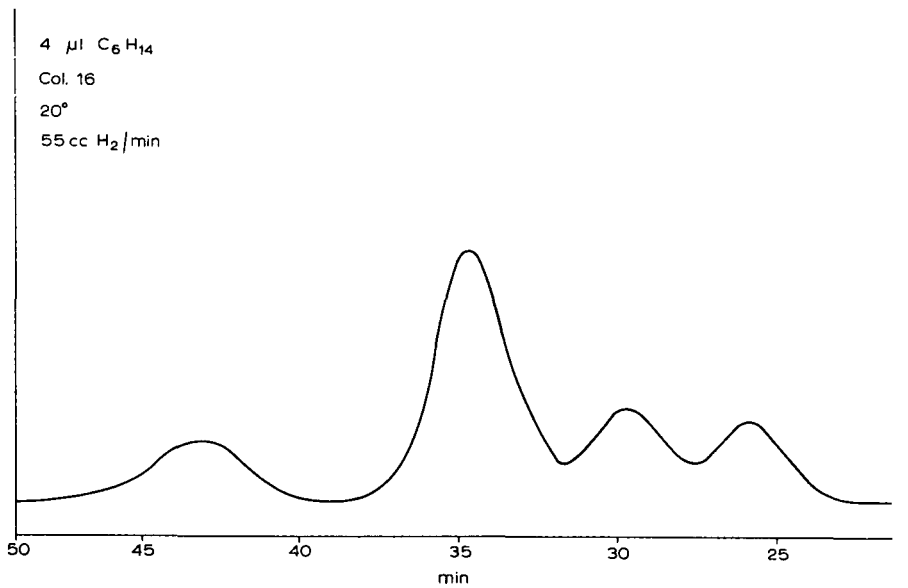

Fig. 5. C_6H_{14} mixture on 2 m containing 12.8 g $Cl(CF_2CFCl)_3CF_2COOC_2H_5$.

with respect to carrier gas molecular weight is the opposite to that found for the fluorocarbon mixture above. The resolution of the hydrocarbon is decreased by decreasing the diffusion coefficient, D, indicating that the second and third terms of the GOLAY theory are the more important for the hydrocarbon C_6H_{14} mixture.

Although the hydrocarbons form rather nonideal solutions in the Kel-F ester substrates they are not sufficiently different in their behavior to alter the relative appearance times appreciably from those observed using a hydrocarbon substrate (*e.g.*, *n*-hexadecane). This is probably due to the fact that a large part of the nonideal behavior in the Kel-F ester substrate arises from dipole–induced-dipole effects which are not as dependent upon the structure of the hydrocarbon isomer as would be purely induced-dipole–induced-dipole effects encountered in a purely nonpolar substrate.

When a nonpolar substrate (perfluorotributylamine) is used to resolve the C_6H_{14} mixture, the result is the chromatogram of Fig. 7. Here the very different activity coefficients of the C_6H_{14} isomers in this solvent cause the isomers to appear very close together. The activity coefficient of the *n*-C_6H_{14} is larger than that of the isomeric structures[7], and since the vapor pressure of the *n*-C_6H_{14} is lower than that of the other isomers, it appears in a relatively shorter time than that corresponding to its vapor pressure. In this instance all the isomers appear close together.

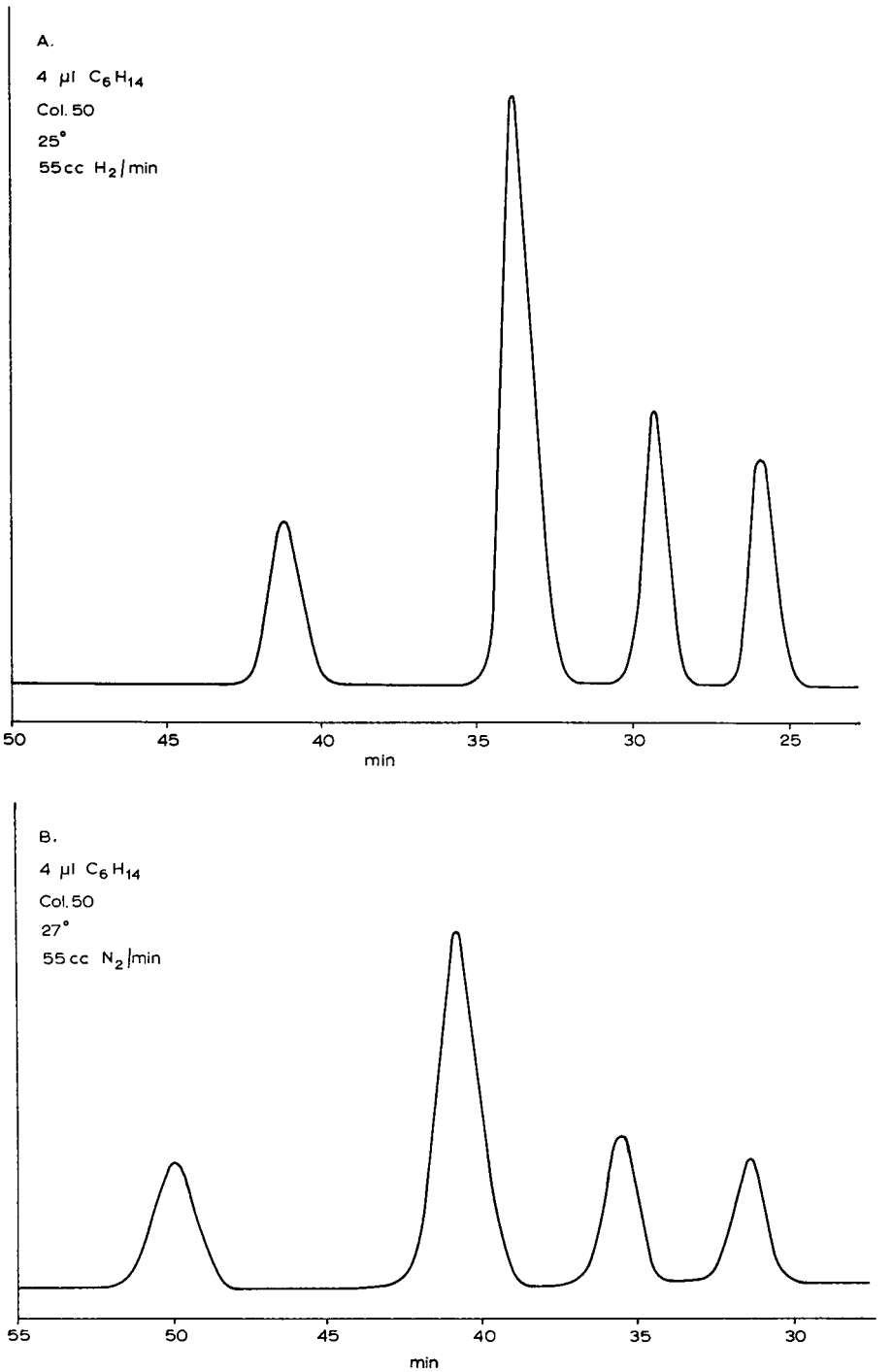

Fig. 6. C_6H_{14} mixture on 7.35 m containing 13.8 g of $Cl(CF_2CFCl)_3CF_2COOC_2H_5$. (A) Hydrogen carrier gas. (B) Nitrogen carrier gas.

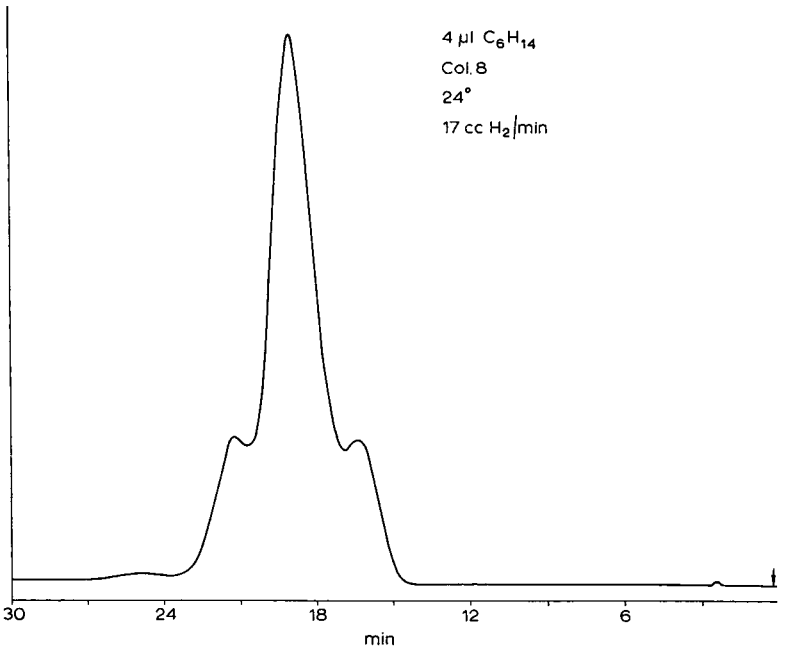

Fig. 7. C_6H_{14} mixture on 2 m of perfluorotributylamine.

CONCLUSIONS

(1) The Kel-F ester column 50, in which the substrate concentration is 0.0565 c.c./c.c. total column volume on 35 to 80 mesh Chromosorb is rather versatile. It allows good resolutions of both hydrocarbon and of fluorocarbon mixtures in column lengths of several meters. To obtain equivalent resolutions longer columns are required for fluorocarbon isomers than for hydrocarbon isomers.

(2) The *n*-hexadecane substrate at ordinary concentration in the column (0.2 to 0.4 c.c./c.c. total) on 35 to 80 mesh Chromosorb-P provides fairly good resolution of C_6F_{14} fluorocarbon isomers at room temperature when the column length is 8 to 16 m. This is the best type of column obtained so far for fluorocarbon isomer mixtures in that it produces the sharpest peaks and adequate resolution.

ACKNOWLEDGEMENTS

Messrs. T. E. TAYLOR and G. W. VINING carried out most of the separations in this work, and were supported in part by the Minnesota Mining and Manufacturing Company and in part by the National Science Foundation, Grant G14591.

SUMMARY

The separation of the isomers of perfluorohexane by gas–liquid chromatography is difficult for two reasons: (1) the isomers have similar vapor pressures in that they all boil within a range of one or two degrees, and (2) they all show essentially identical

thermodynamic behavior in any given solvent. The latter behavior precludes the existence of selective solvent effects, so useful in hydrocarbon systems, to aid in the separation. Satisfactory stationary phases for this isomer separation are hydrocarbons at concentrations of 0.3 c.c. per c.c. of column or chlorofluorocarbons at 0.05 c.c. per c.c. of column volume. Approximate retention volumes for n-C_6F_{14} are 8 c.c. per c.c. of n-hexadecane at 28°, and 77 c.c. per c.c. of $Cl(CF_2CFCl)_3CF_2COOC_2H_5$ at 24°. The relative behaviors of the partitioning media are explained on the basis of the GOLAY theory. The resolution of the hydrocarbon hexanes is poor on perfluorocarbon substrates because of large differences in the thermodynamics among the hydrocarbon isomers in these solvents.

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ANALYSIS OF LIQUID ODORANTS BY GAS CHROMATOGRAPHY

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INTRODUCTION

Before the introduction of gas chromatography, the analysis of odorants was a tedious undertaking. Usually, the odorant was carefully fractionated by distillation and each cut analyzed by physical and chemical means. However, with the aid of gas chromatography, commercial odorants and odorant mixtures may now be analyzed quickly and accurately. This paper compares an isothermal method used successfully for several years in analyzing commercial odorants with a recently developed programmed temperature method which is particularly helpful in analyzing blends with wide boiling ranges. Area response factors were determined for the compounds encountered in odorants, and the accuracy of the isothermal and programmed temperature methods were evaluated. Previous methods have not reported response factors for these compounds[1–7].

ISOTHERMAL METHOD

Experimental procedure

A Perkin-Elmer Model 154B Vapor Fractometer with a thermistor detector is used. The column is 7 ft. by 1/4-in. O.D. aluminum tubing containing 3.5 ± 0.1 g packing per ft. of 42–60 mesh Johns-Manville GC-22 insulating firebrick impregnated with 28.6 wt. % didecyl phthalate. Helium is used as the carrier gas at a flow rate of 60 ml/min measured at 25° and atmospheric pressure. Column inlet pressure is 6.5 p.s.i.g., column temperature is 50°, and sample size is approximately 0.01 ml. In order to make sure that the whole sample is accounted for, the carrier gas flow is reversed at the end of the time allotted for forward flow, and the detector is switched to the column inlet. The time of this backflush is usually 10 min longer than that allowed for forward flow. The backflush should be negligible for a satisfactory analysis.

Retention data

Fig. 1 is a typical chromatogram of a mixture of mercaptans. The *n*-amyl mercaptan is accounted for as a backflush peak in this analysis to illustrate the backflush technique. As expected, this peak is quite wide. In the analysis of the sulfide blend shown in Fig. 2, the forward flow was continued until the highest boiling compound (thiophan) present in the blend was eluted.

Fig. 3 shows the retention time as a function of boiling point for both mercaptans and sulfides. Although didecyl phthalate is somewhat polar, a single straight line correlation is obtained. More polar columns would yield two lines, one for mercaptans and one for sulfides. The single correlation allows easy identification of unknown

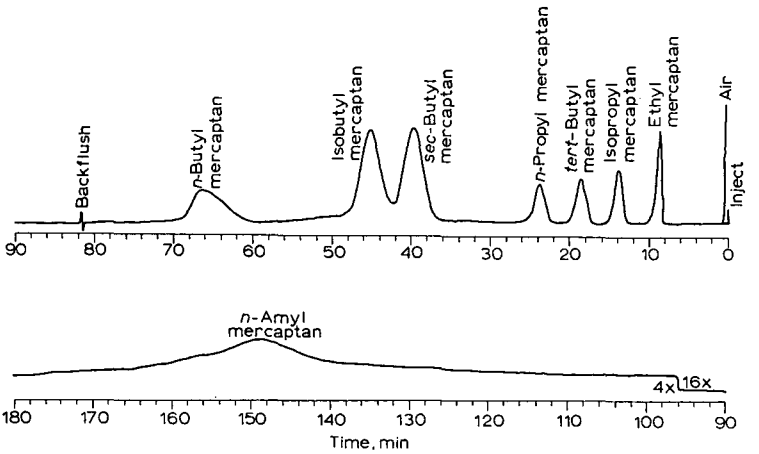

Fig. 1. Isothermal chromatogram of mercaptan blend.

mercaptans and sulfides. The separation of *n*-propyl mercaptan and methyl ethyl sulfide is not complete. However, this is not serious because methyl ethyl sulfide is not normally present in odorants.

Response factors

Relative weight correction factors are normally used in gas chromatographic calculations to convert area per cent to weight per cent. This corrects for variation in detector response of the individual components. The correction is made by multiplying the area of each peak by the respective correction factor. The factors for mercaptans and sulfides were determined by analyzing blends containing known amounts of mercaptans and sulfides with a known amount of *n*-heptane. The weight correction factor for a component relative to that of benzene is calculated by:

$$f_C = \frac{A_H}{A_C} \times \frac{W_C}{W_H} \times f_H$$

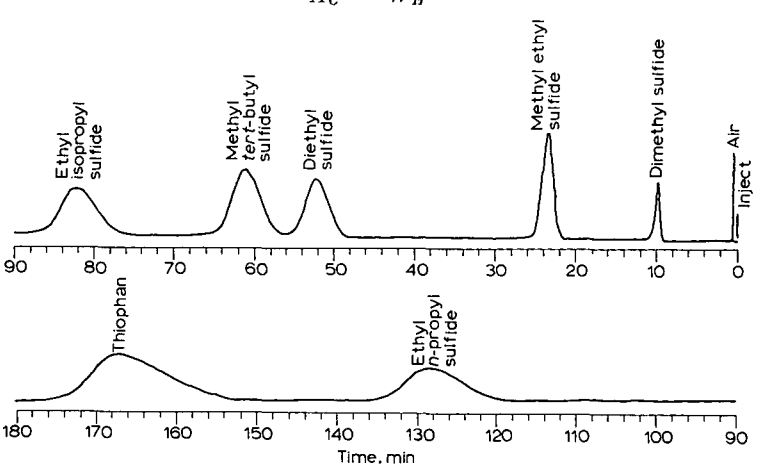

Fig. 2. Isothermal chromatogram of sulfide blend.

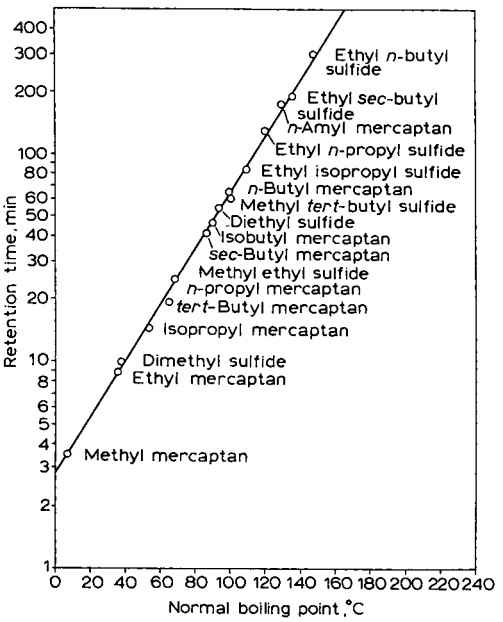

Fig. 3. Correlation of retention time with boiling point for mercaptans and sulfides.

where A_H and A_C are the areas of the *n*-heptane and the component, W_H and W_C are the weights of the *n*-heptane and the component, and f_H (= 0.898) is the weight correction factor of *n*-heptane relative to benzene ($f = 1.00$). Weight correction factors for mercaptans and sulfides are shown in Table I. The values tend to increase with molecular weight and are quite different for the mercaptans and sulfides. Failure to use these correction factors in the analysis of odorants would lead to error.

TABLE I

WEIGHT CORRECTION FACTORS

Compound	*Isothermal*	*Programmed temperature*	*Average*
Ethyl mercaptan	1.42	1.42	1.42
Isopropyl mercaptan	1.60	1.59	1.59
tert.-Butyl mercaptan	1.64	1.67	1.65
n-Propyl mercaptan	1.48	1.50	1.49
sec.-Butyl mercaptan	1.64	1.58	1.61
Isobutyl mercaptan	1.58	1.63	1.61
n-Butyl mercaptan	1.70	1.62	1.66
n-Amyl mercaptan	2.02	2.03	2.03
Dimethyl sulfide	0.91	0.94	0.92
Methyl ethyl sulfide	0.97	0.97	0.97
Diethyl sulfide	0.96	0.96	0.96
Methyl *tert.*-butyl sulfide	1.10	1.10	1.10
Ethyl isopropyl sulfide	1.05	1.07	1.06
Ethyl *n*-propyl sulfide	1.22	1.21	1.22
Thiophan	1.08	1.04	1.06

Molar response factors

Molar response factors relative to benzene (= 100) are related to weight correction factors by:

$$\text{Molar response factor} = \frac{\text{molecular weight}}{\text{weight correction factor}} \times \frac{100}{78}$$

(78 = molecular weight of benzene).

MESSNER *et al.*, obtained a linear relationship between molar response and molecular weight for several homologous series[8]. However, molar response factors calculated from the average relative weight correction factors in Table I and plotted *vs.* boiling point in Fig. 4 show a decrease in response factors at high molecular weights. This

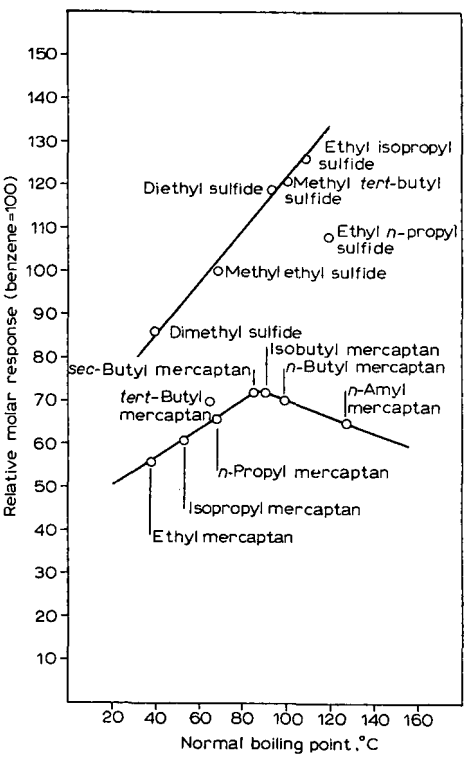

Fig. 4. Correlation of molar response factors with boiling point for mercaptans and sulfides at 50°.

decrease was observed in repeated measurements. Although the reason for this decrease in molar response factor is not known, it has been observed with other substances and is always associated with high boiling and/or high molecular weight substances.

Quantitative results

Three runs were made on each of the blends of mercaptans and sulfides shown in Figs. 1 and 2, and the experimental compositions were calculated using the area correction factors in Table I. Good agreements were obtained with actual compositions as is seen in Tables II and III.

TABLE II

ANALYSIS OF MERCAPTAN BLEND BY ISOTHERMAL METHOD

Component	Composition, wt. %				Standard deviation
	Actual	Run 1	Run 2	Run 3	
Ethyl mercaptan	10.7	11.0	10.8	10.7	0.2
Isopropyl mercaptan	10.8	10.8	10.8	11.0	0.1
n-Propyl mercaptan	11.2	11.7	11.3	11.4	0.3
tert.-Butyl mercaptan	12.2	12.5	12.6	12.3	0.3
sec.-Butyl mercaptan	11.3	11.6	11.2	11.0	0.3
Isobutyl mercaptan	11.8	11.9	12.4	12.1	0.4
n-Butyl mercaptan	29.5	29.4	28.7	29.3	0.5
n-Amyl mercaptan	2.5	1.1	2.2	2.2	0.8

TABLE III

ANALYSIS OF SULFIDE BLEND BY ISOTHERMAL METHOD

Component	Composition, wt. %				Standard deviation
	Actual	Run 4	Run 5	Run 6	
Dimethyl sulfide	8.6	8.6	9.0	8.6	0.2
Methyl ethyl sulfide	8.6	8.6	8.8	8.6	0.1
Diethyl sulfide	10.0	10.0	10.2	9.9	0.1
Methyl *tert.*-butyl sulfide	15.4	15.4	15.7	15.2	0.2
Ethyl isopropyl sulfide	12.6	12.7	12.8	12.8	0.2
Ethyl *n*-propyl sulfide	16.4	16.7	16.4	16.3	0.2
Thiophan	28.3	27.9	27.2	28.5	0.7

PROGRAMMED TEMPERATURE METHOD

Experimental procedure

Although isothermal operation gives a satisfactory analysis for most odorants, programmed temperature operation decreases the analysis time and eliminates backflushing of the higher boiling compounds. A 24-ft. by 1/4-in. O.D. stainless steel column containing 2.3 $\pm$ 0.2 g packing per ft. of 60–80 mesh Johns-Manville Silicone Treated Chromosorb W impregnated with 28.6% didecyl phthalate is used in the programmed temperature method. The column is programmed from 35–180° at 2°/min. The retention temperature is measured with a thermocouple placed inside the column at the exit. The helium flow rate is 75 ml/min measured at 25° and atmospheric pressure, and the column inlet pressure varies from 31–53 p.s.i.g. over the temperature range. A high resistance tungsten thermal conductivity detector is used and operated at 150°. Sample size is approximately 0.04 ml. A single-column unit constructed at Calresearch was used for the analysis reported here.

Retention time data

Chromatograms of mercaptans and sulfides analyzed by the programmed temperature procedure are shown in Figs. 5 and 6. Comparison with the isothermal chromatograms of the identical samples in Figs. 1 and 2 illustrates the savings in time which can be realized by programmed temperature operation. The resolution is greater due to the

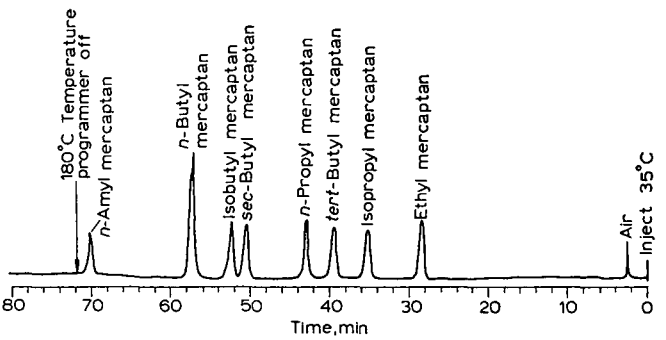

Fig. 5. Programmed temperature chromatogram of mercaptan blend.

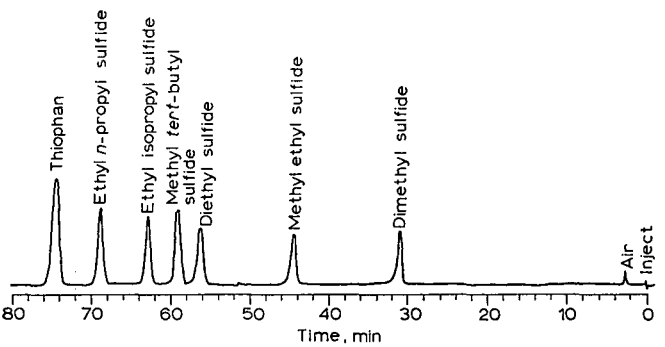

Fig. 6. Programmed temperature chromatogram of sulfide blend.

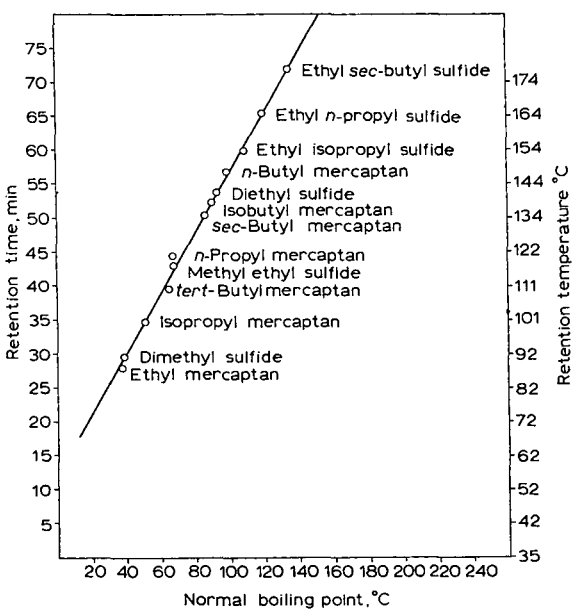

Fig. 7. Correlation of retention time and retention temperature with boiling point for mercaptans and sulfides under programmed temperature operation.

increased column length. In addition, the peaks are all evenly spaced which avoids crowding in the front and large voids toward the end of the chromatogram. In linear programmed temperature gas chromatography, the retention time is directly proportional to the boiling point for homologous series, whereas, in isothermal gas chromatography the logarithm of the retention time is proportional to the boiling point. As with the logarithmic plot of retention times shown in Fig. 3, a single boiling point correlation of all the mercaptans and sulfides is obtained in Fig. 7 regardless of the branching. The right-hand ordinate shows the retention temperature at which the compounds elute from the column. The departure from linearity of the temperature scale is due to a slight lag in the column temperature during programming.

Response factors

Weight correction factors were measured for the programmed method in the same manner as in the isothermal method. Within the limits of measurement, identical values are obtained as seen in Table I. This illustrates that relative correction factors are independent of flow rate, temperature, concentration, and type of thermal conductivity detector.

Quantitative results

Three analyses were made on each of the mercaptan and sulfide blends analyzed by the isothermal method. Standard deviations from actual compositions are, in general, slightly higher for the programmed method. However, the agreements are still quite good as seen in Tables IV and V.

TABLE IV

ANALYSIS OF MERCAPTAN BLEND BY PROGRAMMED TEMPERATURE METHOD

Component	*Composition, wt. %*				*Standard deviation*
	Actual	*Run 1*	*Run 2*	*Run 3*	
Ethyl mercaptan	10.7	11.1	10.7	12.5	1.1
Isopropyl mercaptan	10.8	10.8	10.5	9.9	0.5
n-Propyl mercaptan	11.2	11.5	10.7	10.6	0.5
tert.-Butyl mercaptan	12.2	12.1	11.5	11.5	0.6
sec.-Butyl mercaptan	11.3	11.8	12.2	11.3	0.6
Isobutyl mercaptan	11.8	11.3	11.9	12.1	0.3
n-Butyl mercaptan	29.5	29.0	30.1	29.8	0.5
n-Amyl mercaptan	2.5	2.4	2.4	2.3	0.1

TABLE V

ANALYSIS OF SULFIDE BLEND BY PROGRAMMED TEMPERATURE METHOD

Component	*Composition, wt. %*				*Standard deviation*
	Actual	*Run 4*	*Run 5*	*Run 6*	
Dimethyl sulfide	8.6	8.3	8.5	9.3	0.4
Methyl ethyl sulfide	8.6	8.6	8.2	8.8	0.3
Diethyl sulfide	10.0	10.2	9.9	9.7	0.2
Methyl *tert.*-butyl sulfide	15.4	15.7	15.0	15.4	0.3
Ethyl isopropyl sulfide	12.6	12.3	12.6	12.3	0.2
Ethyl *n*-propyl sulfide	16.4	17.0	16.2	16.0	0.4
Thiophan	28.3	28.0	29.6	28.6	0.8

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SUMMARY

Isothermal and programmed temperature methods for gas chromatographic analysis of mercaptans and sulfides used in odorants are discussed. Relative retention times and compound response factors are summarized. Chromatograms of known mixtures are shown. Average standard deviations from actual concentrations are 0.3% for the isothermal method and 0.5% for the programmed method.

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GRADIENT DE POLARITÉ EN CHROMATOGRAPHIE DE PARTAGE EN PHASE INVERSÉE

APPLICATION À LA SÉPARATION DES ACIDES MONO-, DI- ET TRI-HYDROXYCHOLANIQUES

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Ce travail préliminaire a été fait en nous inspirant des techniques de séparation des acides biliaires par chromatographie de partage de BERGSTRÖM ET SJÖVALL[1,2] et NORMAN[3]. La séparation des acides biliaires se fait sur colonne de kieselguhr siliconé en phase inversée, selon la méthode décrite par HOWARD ET MARTIN en 1950[4]. La phase mobile consiste en de l'alcool méthylique aqueux saturé de la phase fixe. Plus la phase mobile est riche en alcool méthylique, plus vite apparaissent les acides biliaires dans le liquide d'élution.

L'ordre de sortie des acides cholaniques avec des substituants oxygénés en position 3, 7 et 12 est le suivant:

Acide 3α,7α,12α-trihydroxycholanique = acide cholique,
Acide 3α,7α-dihydroxy-12-cétocholanique,
Acide 3α-hydroxy-7,12-dicétocholanique,
Acide 3α,12α-dihydroxy = 3α,7α-dihydroxycholanique = acides désoxycholique et chénodésoxycholique,
Acide 3,7,12-tricéto = 3α-hydroxy-12-céto = 3α-hydroxy-7-cétocholanique,
Acide 3α-hydroxycholanique = acide lithocholique,
Acide 3,12-dicéto = 3,7-dicétocholanique,
Acide 3-cétocholanique.

En physiologie humaine, les acides biliaires les plus intéressants sont les acides cholique (Tri-OH), désoxycholique et chénodésoxycholique (Di-OH) et accessoirement l'acide lithocholique (Mono-OH).

Nous avons pensé qu'il serait intéressant d'obtenir en une seule opération chromatographique les acides mono-, di- et tri-hydroxycholaniques:

(1) sous forme de trois pics suffisamment étroits pour permettre un dosage titrimétrique valable;

(2) en évitant que l'acide cholique ne soit trop rapidement élué.

Cela est possible à condition d'établir un gradient de polarité de la phase mobile. En diminuant progressivement la polarité de la phase mobile par enrichissement en alcool méthylique, on accélère progressivement l'élution des acides hydroxycholaniques.

PARTIE EXPÉRIMENTALE

Solvants

On a utilisé les trois systèmes de solvants suivant:

	Phase mobile (ml)	*Phase fixe (ml)*
Système 50%	méthanol–eau (150:150)	Chloroforme–octanol *sec.* (15:15)
Système 55%	méthanol–eau (165:135)	Chloroforme–octanol *sec.* (30 :30)
Système 60%	méthanol–eau (180:120)	Chloroforme–octanol *sec.* (40:40)

L'alcool octylique secondaire peut être remplacé par de l'alcool isooctylique secondaire, les élutions étant alors retardées.

Support

Le kieselguhr a été préparé de la façon suivante.

Le kieselguhr commercial (Touzart et Matignon, France) est mis en suspension dans un large excès d'acide chlorhydrique 2 *N* durant 24 h. Ensuite il est abondamment lavé à l'eau distillée jusqu'à neutralité, puis séché 24 h à l'étuve à 120°. Ensuite il est mis en suspension 24 h dans de l'heptane dans lequel on ajoute approximativement 5 à 10 % de diméthyl-dichlorosilane. Après le kieselguhr est lavé abondamment à l'alcool éthylique à 98° jusqu'à ce que l'alcool coule absolument limpide. Puis après 24 h à l'étuve à 120° le kieselguhr est prêt à l'usage. Chaque lot de kieselguhr ainsi préparé a été contrôlé par chromatographie de produits purs afin de vérifier l'absence de rétention, rétention qui en cas de préparation defectueuse se manifeste surtout pour le premier produit élué.

Produits utilisés

Acide cholique et acide désoxycholique, Hoffmann-La Roche (France). Acide lithocholique, BLB (France).

Appareillage

Le gradient de polarité de la phase mobile s'obtient par le système de deux réservoirs explicité par la Fig. 1 (voir aussi réf.[5]). Dans le réservoir inférieur se place un volume déterminé V de la phase mobile de plus forte polarité (concentration C_1 en méthanol) et dans le réservoir supérieur un volume variable de la phase mobile de polarité plus faible (concentration C_2 en méthanol) qui induit le gradient de polarité. Ce système placé sur la colonne fonctionne à double niveau constant, un dans la colonne, l'autre dans le réservoir inférieur. Une baisse de niveau dans la colonne determine l'écoulement d'un certain volume de liquide du réservoir inférieur à la colonne, ceci provoquant l'écoulement d'un même volume de liquide du réservoir supérieur au réservoir inférieur. La concentration en méthanol de l'écoulement du réservoir inférieur est donné en fonction du volume v écoulé par la formule:

$$c = C_2 - (C_2 - C_1)e^{-\frac{v}{V}}$$

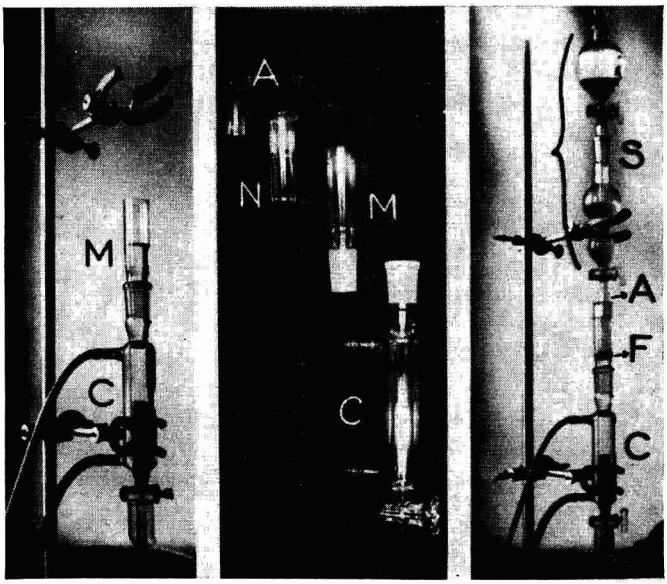

Fig. 1. Au milieu, les divers éléments de la colonne à chromatographie. A = Cylindre creux échancré à une base, se plaçant dans la nacelle perforée N. Cette nacelle contenant la couche filtrante est placée dans le manchon M qui s'adapte à la colonne à jaquette C destinée à recevoir le support imprégné de phase fixe. A gauche, aspect présenté avant la mise en place de la nacelle N contenant la couche filtrante et le cylindre A. A droite ensemble en cours de marche. Le bec du réservoir inférieur du système à gradient S plonge dans le cylindre A. Le liquide qui s'écoule du réservoir inférieur traverse obligatoirement la couche filtrante F avant de parvenir dans le manchon M par les orifices situés à mi-hauteur de la nacelle N.

Le passage de l'air de la colonne au réservoir supérieur créé un régime turbulent suffisant pour assurer un brassage efficace dans le réservoir inférieur qui joue le rôle de chambre de mélange. Quand le volume écoulé du système est égal au volume de phase placé dans le réservoir supérieur, on obtient un pallier de concentration. L'allure du gradient est déterminé par V, C_2, C_1 et par le volume placé dans le réservoir supérieur.

Dans les résultats présentés,

C_1 = phase mobile du système 50 %,

C_2 = phase mobile du système 55 % ou 60 %,

V = 75 ml,

et volume de phase dans le réservoir supérieur = 210 ml.

On a utilisé le type spécial de colonne explicité par la Fig. 1. En effet, il se produit lors du contact des phases mobiles de polarité différente dans le réservoir inférieur une sursaturation en phase fixe et une émulsion plus lourde que la phase mobile apparaît. On se débarasse de cette émulsion en faisant passer la phase mobile au travers d'une couche filtrante. Dans le fond de la nacelle N (Fig. 1) on place 0.250 g de kieselguhr siliconé non imprégné de phase fixe. Ce kieselguhr est mis en suspension dans de la phase mobile du système 50 %. (Pour d'autres systèmes de solvants, il est parfois nécessaire d'imprégner partiellement la couche filtrante de phase fixe afin qu'elle se mélange correctement à la phase mobile.) En cours de chromatographie, la phase trouble qui s'écoule du réservoir inférieur traverse obligatoirement la couche fil-

trante et ensuite on constate qu'elle est limpide à la sortie de la nacelle. Ce montage particulier est nécessaire, car si la couche filtrante est placée au sommet de la colonne, il se produit souvent après un certain volume écoulé une couche de phase fixe qui coiffant le sommet de la colonne gène le déroulement normal de la chromatographie.

Technique opératoire

A 4.5 g de kieselguhr siliconé on mélange intimement 4 ml de la phase fixe du système 50 %. Le kieselguhr ainsi imprégné de phase fixe est mis en suspension dans un petit volume de phase mobile du système 50 % puis est placé dans la colonne. Après homogénéisation et tassement par écoulement de la phase mobile, la substance à analyser est déposée; quand elle a pénétrée dans la colonne, on ajoute une couche de sable lavé puis de la phase mobile du système 50 %. On place la nacelle contenant la couche filtrante avec le cylindre de verre intérieur et ensuite le système à gradient.

Les quantités d'acides biliaires purs utilisés pour une colonne de 4.5 g de kieselguhr ont été de l'ordre de 5 à 10 mg pour l'acide cholique et les acides dihydroxycholaniques et de 1.5 à 4 mg pour l'acide lithocholique.

La température était de 22.5°.

Le volume des fractions collectées était de 4 ml.

L'écoulement était réglé à raison d'une goutte toute les 4 sec.

Dosage des fractions

Il a été fait par titrimétrie avec une solution méthylique de NaOH $N/100$ avec comme indicateur une solution à 0.05 % de bleu de bromothymol dans de l'alcool méthylique.

La récupération des produits purs se situe invariablement au alentour de 97 % (97 % $\pm$ 2).

La caractérisation des pics élués a été faite par chromatographie sur papier entre plaques[6], avec les systèmes de solvants décrit par SJÖVALL[7].

DISCUSSION DES RÉSULTATS OBTENUS

La Fig. 2 schématise les résultats de chromatographie d'un mélange d'acides cholique (Tri-OH), désoxycholique (Di-OH) et lithocholique (Mono-OH) avec et sans gradient de polarité.

Sans gradient de polarité, avec la phase mobile du système 50 %, l'acide cholique s'élue sous forme d'un pic étroit avec un maximum vers 70 ml d'éluat. L'établissement d'un gradient de polarité entre la phase mobile du système 50 % et la phase mobile du système 55 % ou 60 % (gradient 50–55 % ou gradient 50–60 %) ne modifie pas de façon appréciable l'élution de l'acide cholique. Il existe un temps de latence avant que ne se manifeste l'effet accélérateur du gradient, dû en particulier au volume mort de la colonne du point de vue effet du gradient (volume de phase mobile du système 50 % compris entre la nacelle et le sommet de la colonne de support).

L'acide désoxycholique avec la phase mobile du système 50 % sort en traînant de 180 à 300 ml d'éluat et ne peut être dosé valablement. Par contre sous l'effet d'un gradient de polarité, son élution accélérée se fait sous forme d'un pic correct, dosable. Le maximum du pic se trouve à 190 ml pour un gradient 50–55 % et à 160 ml pour un gradient 50–60 %.

L'acide lithocholique sans gradient et avec un gradient 50–55 % reste dans la

phase fixe. Par contre avec un gradient 50–60 %, il s'élue entre 240 et 280 ml et peut être l'objet d'un dosage titrimétrique.

Cette méthode avec un gradient de polarité dit "saturé" (les deux phases mobiles utilisées étant saturées en phase fixe) est plus satisfaisante que la méthode avec gradient de polarité dit "non saturé" où la phase de plus faible polarité qui induit le

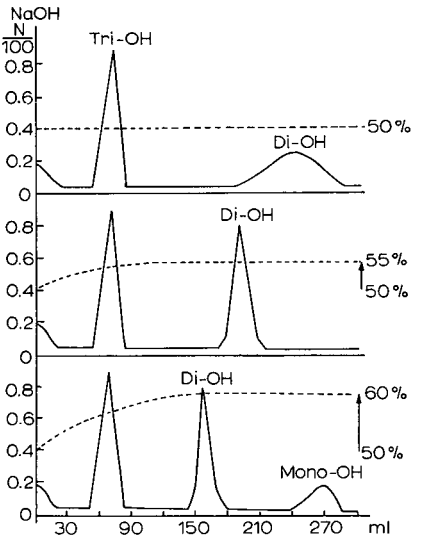

Fig. 2. En pointillé les courbes théoriques donnant la concentration en méthanol de l'écoulement du système à gradient en fonction du volume d'élution. Tri-OH = acide cholique; Di-OH = acide désoxycholique; Mono-OH = acide lithocholique. En haut, phase mobile du système 50 %. Au milieu, gradient 50–55 %. En bas, gradient 50–60 %. (Pour explication, voir texte).

gradient n'est pas saturée en phase fixe. (Dans ce cas une couche filtrante est inutile.) Nous avons essayé des gradients 50–60 %, 50–55 % et 50–52.5 % en plaçant dans le réservoir supérieur de la phase mobile du système 50 % auquelle on ajoutait une quantité determinée d'alcool méthylique de façon à obtenir une concentration de 60 %, 55 % ou 52.5 % en méthanol. Avec un gradient non saturé 50–60 %, l'acide lithocholique s'élue en même temps que l'acide désoxycholique sous forme d'un pic aigu presque symétrique. Avec un gradient non saturé 50–55 %, ces deux acides sortent ensembles mais sous forme d'un pic asymétrique. Par contre avec un gradient non saturé 50–52.5 % ces deux acides sortent séparés, mais il y a élargissement des pics d'élution avec interpénétration de la base des pics. Avec un gradient non saturé, la colonne de phase fixe se déplace par rapport au support par désaturation progressive de la colonne de haut en bas au profit de la phase mobile. L'emploi de la méthode de gradient "saturé" est préférable parce que à priori le support ne se désature pas en phase fixe, la phase mobile restant saturée quoique sa polarité varie.

REMERCIEMENTS

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RÉSUMÉ

Description d'une méthode de chromatographie de partage en phase inversée avec utilisation d'un gradient de polarité de la phase mobile.

Exemple de séparation des acides mono-, di- et tri-hydroxycholaniques en une seule chromatographie dans des conditions permettant un dosage titrimétrique valable.

SUMMARY

A method of reversed phase partition chromatography is described, in which a gradient in the polarity of the mobile phase is utilized.

An example is given of the separation of mono-, di- and trihydroxycholanic acids in a single chromatographic operation under such conditions that a satisfactory titrimetric estimation is possible.

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SEPARATION OF PHENOL AND DEOXYRIBONUCLEIC ACID BY SEPHADEX GEL FILTRATION*

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INTRODUCTION

KIRBY has reported on the use of certain salt solutions and phenol in liberating deoxyribonucleic acid (DNA) from mammalian cells[1]. This report has been confirmed and enlarged upon by COLTER *et al.*[2], who showed that DNA preparations isolated by this method are remarkably free of contamination by polypeptides, polysaccharides, and ribonucleic acid (RNA). Most recently, MANDELL AND HERSHEY[3] have demonstrated the utility of phenol for extracting phage DNA of extremely high molecular weight with virtually no shear degradation. Present methods for the removal of phenol from aqueous DNA preparations involve repeated extractions with diethyl ether. The authors suggest the use of Sephadex gel filtration as a method of removing phenol residues from aqueous DNA preparations.

MATERIALS AND METHODS

DNA from two sources was used in these experiments. Labeled DNA was isolated, by mercaptoethanol–tryptic lysis[4], from HeLa S-3 cells grown in culture media containing tritiated thymidine. Purified and highly polymerized salmon sperm DNA was purchased from the California Corporation for Biochemical Research. Sephadex G-25 and G-50 were purchased from Pharmacia Laboratories, Inc. Redistilled phenol was used for extraction purposes, and all chromatographic techniques were carried out at room temperature.

Ultraviolet optical density measurements were carried out in a Beckman DU spectrophotometer. The distribution of tritium activity was followed using a Packard Model 314EX Tri-Carb liquid scintillation counting system.

The presence of deoxyribose in fractions from the Sephadex columns was assessed by the diphenylamine reaction of DISCHE, as described by SCHNEIDER[5] and modified in our laboratory. The diphenylamine reagent consisted of 1 g of diphenylamine recrystallized from hexane, 100 ml of glacial acetic acid, and 2.75 ml of concentrated H_2SO_4. The reagent was prepared immediately before use. To 1 ml of column effluent was added 1 ml of 10% trichloracetic acid and 2 ml of diphenylamine reagent. The mixture was heated for 10 min at 90°, cooled to room temperature, and absorbance at 600 mμ was measured. Preliminary simultaneous deoxyribose and

* Work performed under the auspices of the U.S. Atomic Energy Commission.

phosphorus determinations on salmon sperm DNA samples then permitted quantitative evaluation of the amount of DNA added and recovered in the effluent from the Sephadex columns, expressed as micrograms of DNA phosphorus. Recoveries in each case were essentially quantitative, and phenol did not interfere in the determination.

The Lowry protein determination, as described by CHOU AND GOLDSTEIN[6], was carried out on 1-ml aliquots of samples. The optical density at 750 mμ was determined using a Bausch and Lomb Spectronic 20 spectrophotometer. This method was used to determine the distribution of phenol in column effluents.

EXPERIMENTAL

One hundred mg of salmon sperm DNA were stirred with 100 ml of 0.01 *M* phosphate buffer, pH 7.2, for 48 h at 5°. The resulting suspension was centrifuged at 85,000 × *g*, for 30 min. The supernatant was removed and assayed for deoxyribose, Lowry coloration, and its optical density at 260 mμ. A 20-ml aliquot of this solution was stirred with an equal volume of water-saturated phenol for 30 min at room temperature. The two phases were separated by low-speed centrifugation, and the aqueous layer was removed and assayed for deoxyribose. A 5-ml aliquot was allowed to percolate into a 2 × 30 cm column of Sephadex G-25 which had been equilibrated with 0.01 *M* phosphate buffer, pH 7.2. The column was eluted with 0.01 *M* phosphate buffer, and 4-ml fractions were collected at 5-min intervals. Aliquots of each fraction were analyzed for deoxyribose, phenol, and absorbance at 260 mμ (Fig. 1).

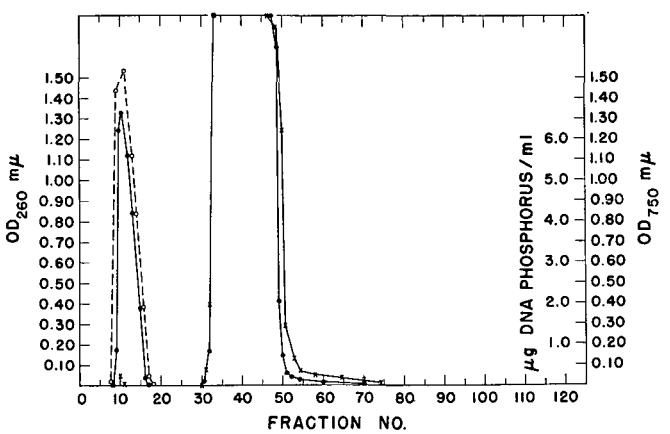

Fig. 1. Gel filtration of an aqueous DNA–phenol mixture. Column, 2.0 × 30 cm; Sephadex G-25; sample volume, 5 ml; eluant, H_2O; fractions collected, 4 ml every 5 min. ●—●—● = O.D. 260 mμ; ×—×—× = O.D. 750 mμ (Lowry determination); O—O—O = μg DNA phosphorus/ml.

The experiment was repeated using a freshly prepared phenol extract of DNA. Two ml of this extract were placed on a 2 × 30 cm column of Sephadex G-25 which had been equilibrated with distilled water. The column was eluted with distilled water, and 5.5-ml fractions were collected at 5-min intervals. Aliquots of each fraction were analyzed for deoxyribose, phenol, and absorbance at 260 mμ (Fig. 2).

The experiment was repeated a third time, the only difference being that the Sephadex column was equilibrated and eluted with 0.015 *M* NaCl. The results obtained were identical to those exemplified in Figs. 1 and 2.

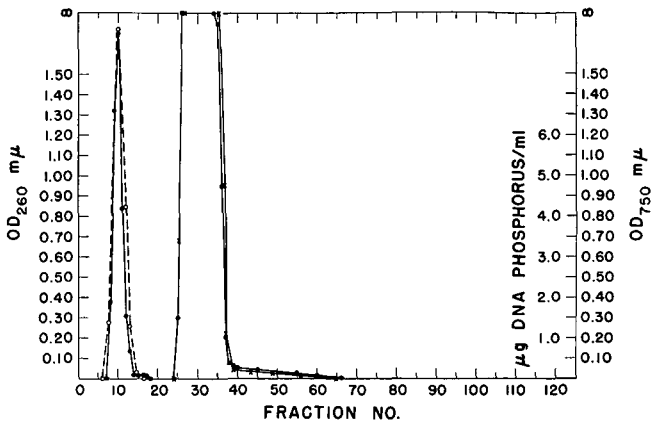

Fig. 2. Gel filtration of an aqueous DNA–phenol mixture. Column, 2.0 × 30 cm; Sephadex G-25; sample volume, 2 ml; eluant, 0.01 *M* phosphate buffer, pH 7.2; fractions collected, 5 ml every 5 min. ●—●—● = O.D. 260 mμ; ×—×—× = O.D. 750 mμ (Lowry determination); ○—○—○ = μg DNA phosphorus/ml.

Finally, an aqueous phenol extract was obtained from HeLa S-3 cell lysate. This extract contained less than 1 μg of DNA per ml and possessed an activity of 16,500 counts/min/0.1 ml, quantity corrected for phenol quenching. A 1-ml aliquot of this extract was percolated into a 1 × 15 cm Sephadex G-50 column which had been equilibrated with distilled water. The column was eluted with distilled water, and 2-ml fractions were collected at 5-min intervals. Aliquots of each fraction were analyzed for optical density at 260 mμ and tritium activity (Fig. 3).

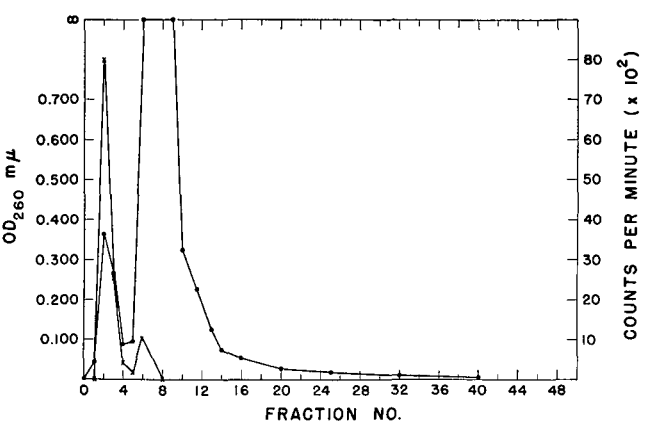

Fig. 3. Gel filtration of an aqueous mixture of phenol and tritium-labeled HeLa S-3 DNA. Column, 1 × 15 cm; Sephadex G-50; sample volume, 1 ml; eluant, H_2O; fractions collected, 2 ml every 5 min. ●—●—● = O.D. 260 mμ; ×—×—× = counts/min.

DISCUSSION

It may be seen that gel filtration on a Sephadex G-25 or G-50 column effects a separation of the components of a phenol–water extract containing DNA. The DNA peak was characterized by its absorbance at 260 mμ and its deoxyribose content. The phenol peak was characterized by its absorbance at 260 mμ and its color formation with the Lowry reagent. Pilot studies carried out on different samples of DNA suggested that purified, protein-free DNA gave a negligible coloration with this reagent.

A separation of DNA and phenol on Sephadex occurred when the column was eluted with water, 0.01 *M* phosphate buffer, or 0.015 *M* NaCl. Duplicate determinations of deoxyribose by the DISCHE method indicated 100 % recovery of the applied DNA in the effluent. While it was impossible to carry out parallel assays of the HeLa S-3 DNA eluted from the Sephadex G-50 column, data based on the total counts of each fraction suggest quantitative recovery in this case as well.

The method offers the significant advantage of virtually quantitative recovery of DNA from phenol-extracts of cell lysates in the absence of denaturation by organic solvents and with a minimum of mechanical shear.

SUMMARY

Mixtures of deoxyribonucleic acid (DNA) and aqueous phenol have been separated into their components, phenol and DNA, by Sephadex gel filtration. Recovery of DNA is quantitative. This procedure is suggested as an adjunct to the phenol extraction method for DNA isolation.

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THE CHROMATOGRAPHY OF FLAVONOID AGLYCONES IN THE SOLVENT SYSTEM BENZENE–ACETIC ACID–WATER

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INTRODUCTION

Although a large number of solvent systems have been used for the paper chromatography of flavonoid compounds[1–3] few are capable of separating flavone, isoflavone and flavanone aglycones over a wide range of R_F values. The most commonly used solvent system *n*-butanol–acetic acid–water (4:1:5 by vol., or its many single phase variations) clearly separates flavonoid glycosides with good definition of spots, but tends to concentrate most aglycones over a narrow range of high R_F values. Aqueous solvents containing various amounts of acetic acid likewise give narrow spreads of R_F values for non-glycosidic flavonoids (see Tables in refs. [1,3,4]).

In work on ether-soluble polyphenols[5] and hydrolysates of plant extracts we have found the solvent system benzene–acetic acid–water (125:72:3 by vol.) to be most useful for the separation of these compounds. This single phase benzene solvent was devised by SMITH[6], as an improvement on the organic phase of benzene–acetic acid–water (2:2:1 by vol.) commonly used for the chromatography of phenolic acids[6,7]. Excellent separations were obtained with this as the first solvent in two-dimensional chromatography, followed by 2 *N* ammonia[5] or aqueous acetic acid as the second solvent. In the present study the chromatographic behaviour in this solvent system of 43 natural or synthetic flavones, isoflavones, flavanones, flavonols and flavanonols is reported.

EXPERIMENTAL

Solutions of the flavonoid compounds in ethanol (10–20 μg of material) were applied as narrow bands (*ca.* 0.5 × 2 cm) on Whatman No. 1 paper and chromatographed in benzene–acetic acid–water (125:72:3 by vol.) by the descending technique. All compounds were chromatographed under comparable conditions at temperatures in the range 20° ± 2°. One hour or longer was allowed for the preliminary equilibration of the papers. Under these conditions the solvent took 3.5–4 h to descend 40 cm. Because of its volatility, the present solvent system is very sensitive to temperature effects, and phasic separation in the paper has sometimes been observed at higher temperatures, particularly with thicker grades of paper.

Location of compounds was by the usual methods (*viz.* U.V. ± NH_3, U.V. + $AlCl_3$)[1]. In the case of the flavanones and flavanonols the identity of the spots was confirmed by spraying with the specific $NaBH_4$ reagent[8]. Diazotised sulphanilic acid (Pauly's reagent) was used as a general spray reagent. This reagent was prepared as described by SMITH[6] with the following slight modifications. A 0.69 % solution of $NaNO_2$ was used instead of the 5 % solution and the reagent was applied by spraying instead of dipping.

RESULTS

R_F values for the compounds studied are given in Table I. These values were obtained under closely comparable conditions and in most cases represent average values of

TABLE I

R_F VALUES OF FLAVONOID AGLYCONES IN BENZENE–ACETIC ACID–WATER (125:72:3) AND THEIR COLOUR REACTIONS WITH SULPHANILIC ACID

*Compound**	R_F	*Colour with diazotised sulphanilic acid***
Flavanone		
1 5,7,3′,4′-OH (Eriodictyol)	0.29	Br-O
2 7,4′-OH	0.45	R-Br
3 5,7,4′-OH (Naringenin)	0.49	O-Br
4 7,4′-OH; 3′-OMe	0.55	R-Br
5 5,7,3′-OH; 4′-OMe (Hesperetin)	0.60	O
6 5,7,4′-OH; 3′-OMe (Homoeriodictyol)	0.61	O
7 7-OH	0.79	P
8 5,7-OH (Pinocembrin)	0.80	Br-O
9 4′-OH; 8,3′-OMe	0.85	P
10 6,7,3′,4′-OMe	0.87	—
11 5-OH; 7,4′-OMe	0.93	O
Flavanonol		
12 5,7,3′,4′-OH (Taxifolin)	0.09	Br-O
13 5,7-OH (Pinobanksin)	0.66	Y-Br
Isoflavone		
14 5,7,3′,4′-OH (Orobol)	0.20	Pu
15 7,4′-OH (Daidzein)	0.35	R-Br
16 5,7,4′-OH (Genistein)	0.41	Br
17 5,7,3′-OH; 4′-OMe (Pratensein)	0.54	Br-O
18 7-OH; 4′-OMe (Formononetin)	0.72	—
19 5,7-OH; 4′-OMe (Biochanin A)	0.77	Br
20 5,7,2′-OH; 6,5′-OMe (Podospicatin)	0.76	Br-O
21 5,2′-OH; 6,7,5′-OMe	0.86	Br-O
Flavone		
22 5,7,3′,4′-OH (Luteolin)	0.17	Br-O
23 5,7,4′-OH (Apigenin)	0.33	Br-O
24 5,7,3′-OH; 4′-OMe (Diosmetin)	0.47	R-Br
25 5,7,4′-OH; 3′-OMe (Chrysoeriol)	0.47	Br-O
26 5,7,8-OH (Norwogonin)	0.51	—
27 5,6,7-OH (Baicalein)	0.61	—
28 5,7-OH; 4′-OMe (Acacetin)	0.75	Br-O
29 5,7-OH (Chrysin)	0.78	Br-O
30 5-OH; 7-OMe (Tectochrysin)	0.93	Br-O

(continued on p. 451)

TABLE I *(continued)*

*Compound**	R_F	*Colour with diazotised sulphanilic acid***
Flavonol		
31 5,6,7,3′,4′-OH (Quercetagetin)	0.00	—
32 7,8,3′,4′-OH	0.02	—
33 6,7,3′,4′-OH	0.02	—
34 7,3′,4′-OH (Fisetin)	0.09	Br-O
35 5,7,5′,6′-OH	0.09	Br-O
36 5,7,3′,4′-OH (Quercetin)	0.10	Br-O
37 5,7,4′-OH (Kaempferol)	0.30	Br-O
38 7,3′,4′-OMe	0.89	—
39 Flavonol	0.91	—
Flavonol 3-methyl ether		
40 5,7-OH; 3′,4′-OMe	0.77	Br-O
41 5,7-OH; 4′-OMe	0.79	Br-O
42 5,7,3′,4′-OMe	0.86	—
43 5-OH; 7,3′,4′-OMe	0.93	O-Y

* Sources of compounds: compounds 3, 5, 23, 31, 34, 36, 37 were from commercial sources; 17, 28 were isolated from plant materials[9,10]; 15, 16, 18, 19 were previously synthesised[11]; the rest were donated by other workers (see ACKNOWLEDGEMENTS).

** Br = brown; O = orange; Pu = purple; R = red; P = pink; Y = yellow; — = no colour.

5 or 6 individual determinations. They are reproducible to within ± 0.02. Good discreet spots were obtained from all compounds studied. The colours given with the diazotised sulphanilic acid reagent are also listed in Table I. Glycosides and anthocyanidins move very slowly in this solvent and were not included in the study.

DISCUSSION

It can be seen from the R_F values given that the benzene–acetic acid–water solvent system is capable of separating aglycones of all the classes of flavonoid compounds tested giving a good range of R_F values for a large variety of structural patterns. This solvent system, being much less polar, complements the polar aliphatic solvents typified by butanol–acetic acid–water. Corresponding R_F values in benzene–acetic acid–water are in general much lower than in the butanol solvent, with the result that substances such as the flavonoid aglycones which are very mobile in the latter are well distributed in the former. Glycosides and the more highly hydroxylated aglycones are not very mobile in the present solvent system however, and are best separated in butanol–acetic acid–water.

With the exception of bisdiazotised benzidine[12], diazotised aromatic amines do not appear to have been used extensively as spray reagents for flavonoid compounds although they are commonly used for other phenolic substances[6,13]. We have found diazotised sulphanilic acid to be an excellent reagent, usually giving shades of brown but often more distinctive colours which are useful as further aids to identification. It gives less intense backgrounds than other diazonium salts and the colours of the spots keep for long periods without appreciable change.

The well known general principles governing the behaviour of phenolic compounds

in partition chromatography[3,14,15] are well borne out in the present study. In Fig. 1 the R_M values [14][R_M = log $(1/R_F - 1)$] of some selected structures are presented. It is readily apparent that the order of R_F values is the same in each class indicating that the effects of structural variations upon R_F values are similar in all classes of flavonoids studied. Comparison of compounds having the same substitution patterns (graphs A, B, C, etc. in Fig. 1) show that in all cases R_F values vary regularly in the order: flavanone > isoflavone > flavone. ROUX and co-workers[15–17] have noted the greater mobility of flavan derivatives in both adsorption and partition systems, and attribute this to the non-planar ring structure in these compounds.

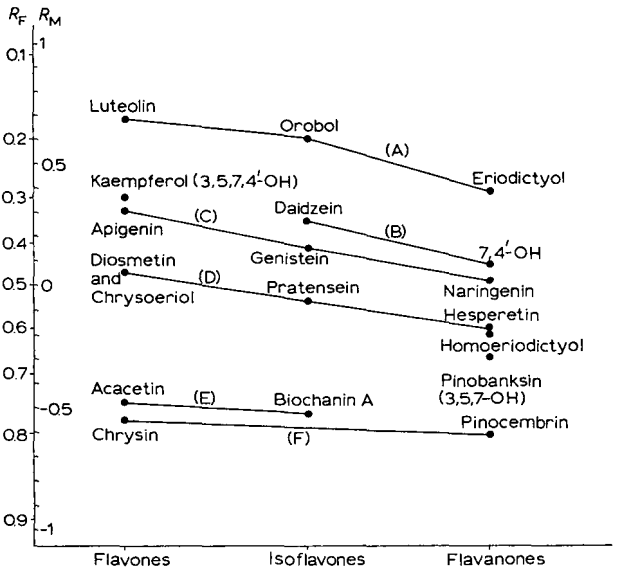

Fig. 1. Relation of R_M to structure in different classes of flavonoids when chromatographed in benzene–acetic acid–water. Graph (A) 5,7,3',4'-OH; (B) 7,4'-OH; (C) 5,7,4'-OH; (D) 5,7,3'-OH; 4'-OMe and 5,7,4'-OH; 3'-OMe; (E) 5,7-OH; 4'-OMe; (F) 5,7-OH.

The principle that hydroxyl groups decrease and methoxyl groups increase the R_F value is also well demonstrated. The positive ΔR_M effect of a hydroxyl group (other than a 5-OH, to be discussed below) is clearly illustrated in Fig. 1 (*cf.* graphs A and C, C and F, and D and E). In contrast to the observation of BATE-SMITH AND WESTALL[14] that in butanol–acetic acid–water the rise in R_F for the methylation of a hydroxyl group is much less than the rise in R_F caused by the complete removal of a hydroxyl group, the lyophilic effect of a methoxyl group is much greater in benzene–acetic acid–water. Methylation almost completely reverses the effect of hydroxylation (*cf.* graphs C, E, and F, Fig. 1) or actually causes a greater R_F rise than dehydroxylation (*cf.* graphs A, C, and D, Fig. 1). The greater lyophilic effect of the methoxyl group in benzene solvents is also apparent in the previous results of SIMPSON AND GARDEN[18].

The abnormal effect of 5-OH and 3-OH groups in partition chromatography, due to their formation of hydrophobic chelate ring systems with the 4-carbonyl group has been clearly demonstrated by SIMPSON and co-workers[18,19]. In the present work the

increase in R_F due to the introduction of a 5-OH group is again illustrated in Fig. 1 (*cf.* graphs B and C, and B and F). Numerous other examples can be found in results listed in Table I. The suppression of the normal positive ΔR_M contribution of a hydroxyl, due to hydrogen bonding of the 3-OH group, can be demonstrated by comparisons of the R_M values for kaempferol, luteolin and apigenin, and for those of pinobanksin, naringenin, and pinocembrin (Fig. 1). The second example suggests that simultaneous hydrogen bonding of both 5- and 3-OH groups[18] is also possible in flavanonols.

The benzene–acetic acid–water solvent system, because of excellent separations it gives with many classes of phenolic compounds, is recommended as the solvent of choice for the first direction in the two-dimensional paper chromatography of less water-soluble polyphenols in plant extracts. Also because of the regular manner in which mobility varies with structure in the classes of flavonoids studied, comparisons of R_M values in this solvent would be useful for the prediction of structures of unknown substances belonging to these classes of natural products.

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SUMMARY

The R_F values of 43 natural and synthetic flavonoid aglycones in the solvent system benzene–acetic acid–water (125:72:3 by vol.) are recorded. Diazotised sulphanilic acid has been found to be a useful general spray reagent for these compounds.

Excellent separations are obtained for all the classes of compounds studied. The effect of structural variation upon R_F is similar for all classes. All the known effects of hydroxylation, methylation and chelation on the chromatographic behaviour of flavonoids were observed in this solvent system. R_F values for compounds having the same substitution pattern vary in the order flavanone > isoflavone > flavone.

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PAPER-ELECTROPHORETIC STUDIES OF THE SOLUTION CHEMISTRY OF RaD (^{210}Pb), RaE (^{210}Bi) AND RaF (^{210}Po)

II. IN NITRIC ACID AND PERCHLORIC ACID

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In a recent communication[1] we described the behaviour of RaD, RaE and RaF on paper electrophoresis in hydrochloric acid. The present paper concerns the study by the same technique of the separability and of the nature of the ionic species of these radio-elements in acids with which complexes are less likely to be formed, namely nitric and perchloric acids.

EXPERIMENTAL

The general experimental procedure and the apparatus used in this study remain the same as those described in Part I. For experiments with nitric acid, the solution of RaD, RaE, and RaF (in radioactive equilibrium) in N HNO_3 was applied on the paper while the solution of these elements in N $HClO_4$ formed the test solution when perchloric acid was used as electrolyte. Electrophoresis was carried out on Arches 302 paper strips (2.7 × 40 cm) sandwiched between two glass plates (8 × 30 cm) by applying a potential difference of 300 V D.C. for 45 min. The position of the elements on the electropherogram was located using the Frieseke-Hoepfner FH 452 automatic scanner by the method already described.

RESULTS AND DISCUSSION

(1) *In nitric acid*

In Table I, we present the electrophoretic displacement on paper of RaD, RaE and RaF in nitric acid in the concentration range 0.0005 N to 3.6 N. Fig. 2 shows some of the typical electropherograms obtained when the concentration of nitric acid was varied. As the NO_3^- ion has little tendency to complex formation, the hydrolysis of the ions in low concentrations of this acid is very marked. The three elements in question, being variously charged, undergo hydrolysis to different extent; RaD with two positive charges is less hydrolysed, the great tendency of RaE to give a number of complex hydrolysis products in dilute nitric acid is well known[2], while RaF with four positive charges has the greatest tendency to complex formation and consequently also to hydrolysis[3]. In very dilute nitric acid, *e.g.* 0.0005 N, all three elements are hydrolysed and lie as a single band at the point of application (Fig. 1, a).

TABLE I

ELECTROPHORETIC DISPLACEMENT (300 V, 45 min) OF RaD, RaE AND RaF IN NITRIC ACID MEDIUM

Normality of the acid	*Displacement (mm)*		
	RaD	*RaE*	*RaF*
0.0005	The three elements lie in a single band (from +4.8 to —12.0, maximum at 0).		
0.001	RaD and RaE lie in a single band (from +3.0 to —24.0, maximum at —4.8).		0
0.01	RaD and RaE lie in a single band (from +2.4 to —24.6, maximum at —14.4).		0
0.025	RaD and RaE lie in a single band (from +3.0 to —24.0, maximum at —10.2).		0
0.05	RaD and RaE lie in a single band (from +3.0 to —24.0, maximum at —12.6).		— 1.8
0.1	RaE still trails from +1.8 while some of RaD begins to move faster. The two elements thus give a large diffused band from +1.8 to —72.0 with a maximum at —24.0.		— 2.4
0.2	—43.2 (trailing from —13.2 to —72.0)	—15.3 (trailing from 0 to —24.0)	— 2.4
0.25	—58.8	—31.2 (trailing from — 7.8 to —39.0)	— 4.8
0.3	—60.0	—33.0 (trailing from — 6.0 to —39.0)	— 7.2
0.35	—65.1	—36.6 (trailing from —15.0 to —45.0)	— 7.8
0.4	—72.6	—51.6 (trailing from —30.0 to —54.0)	—14.0
0.45	—77.4	—52.8 (trailing from —30.0 to —60.0)	—16.2
0.5	—76.2	—56.4 (trailing from —39.0 to —63.6)	—22.8
0.6	—68.7	—52.2 (trailing from —36.0 to —58.8)	—21.9

(continued on p. 457)

TABLE I (*continued*)

Normality of the acid	*Displacement (mm)*		
	RaD	*RaE*	*RaF*
0.75	—71.0	—56.4	—25.2
0.8	—65.4	—52.8	—24.0
0.9	—58.2	—46.8	—20.7
1.0	—50.4	—39.9	—15.6
1.2	—49.2	—42.2	—19.2
1.4	—44.4	—38.4	—17.4
1.6	—39.5	—31.2	—13.8
1.8	—36.0	—33.0	—16.8
1.9	—34.8	—34.8	—14.4
2.0	—33.0	—33.0	—13.8
2.25	—24.0	—24.0	— 8.7
2.5	—22.8	—22.8	— 7.5
3.0	—15.0	—15.0	— 4.2
3.25	—13.8	—13.8	— 3.2
3.5	—18.0	—18.0	— 3.3
3.6	— 2.7	— 2.7	— 2.7

In 0.001 N HNO_3, there is already a visible tendency for RaD to move cationically. As the concentration of the acid in the electrolyte is increased, the hydrolytic effect on the ions becomes less and less and they begin to move faster. The sequence of

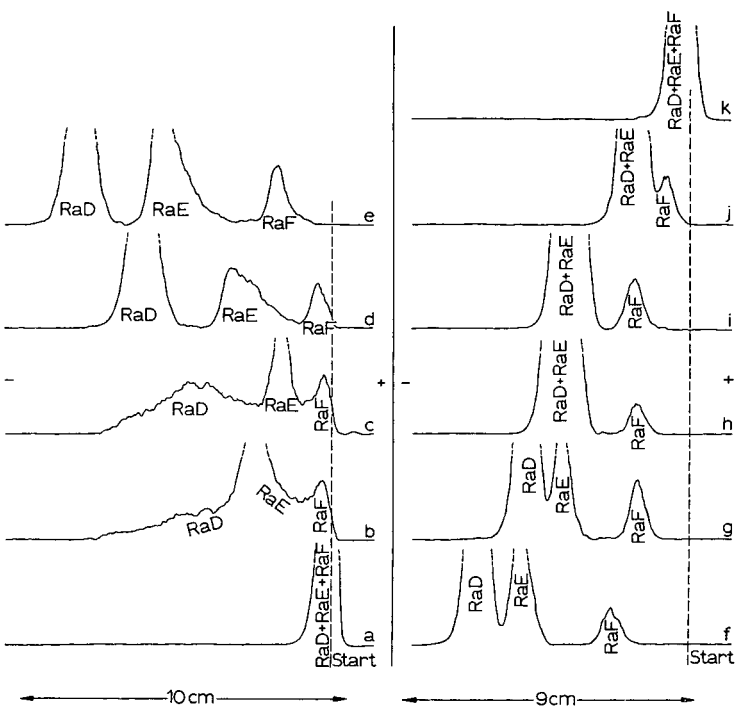

Fig. 1. Electropherograms (300 V, 45 min) of RaD, RaE and RaF in nitric acid as electrolyte. (a) 0.0005 N; (b) 0.1 N; (c) 0.2 N; (d) 0.25 N; (e) 0.45 N; (f) 0.8 N; (g) 1.0 N; (h) 1.5 N; (i) 1.8 N; (j) 2.5 N; (k) 3.6 N.

their migration rate is RaD > RaE > RaF. Although the difference in the migration rate of the three ions is already noticeable on the electropherogram in the electrolyte 0.1 *N* HNO_3 (Fig. 1, b), RaE moves as a wide diffused band which overlaps with RaF at its anionic end, and with RaD at the cationic one. It is only from 0.25 *N* HNO_3 (Fig. 1, d) that the separation of the three elements is rendered possible where the RaE band still trails to touch the cationic extremity of the RaF band but is completely separated from the RaD band. The separation of the three elements is complete in the concentration range 0.35 to 0.6 *N* of nitric acid, 0.45 *N* HNO_3 giving the best results (Fig. 1, e). In electrolyte more concentrated than 0.6 *N* HNO_3, RaE is not hydrolysed and moves as a rapid compact band whose velocity approaches that of RaD. Overlapping between the extremities of the bands of RaD and RaE is therefore obtained (Fig. 1, f, g). Separation of the still slower RaF from the two other elements becomes more and more easy and distinct under these conditions. In 1.5 *N* HNO_3 the electrophoretic bands of RaD and RaE are almost superimposed (Fig. 1, h). No distinction between the RaD and RaE bands is possible in concentrations higher than 1.8 *N* HNO_3, but the separation of polonium is still feasible (Fig. 1, i). On the electropherograms in electrolytes of concentrations higher than 2.5 *N* HNO_3 the migration rate of RaD and RaE approaches that of RaF (Fig. 1, j), due presumably to complexation with NO_3^- ions at high concentrations of nitric acid so that overlapping between the bands is obtained, but one can still separate most of the RaF from the RaD and RaE by electrophoresis on paper in nitric acid of concentration as high as 3.5 *N*. In 3.6 *N* HNO_3 (Fig. 1, k), the highest concentration that could be tried, RaD, RaE and RaF lie in a single band at —2.7 mm. Unfortunately, experiments using acid of higher concentrations could not be carried out because the acid attacked the paper. We can only conclude that the concentrations at which the change over from cationic to anionic nature takes place for RaD, RaE and RaF in nitric acid lie beyond 3.6 *N* HNO_3. Paper electrophoresis of polonium in nitric acid showed similar behaviour to that shown in mixtures with RaD and RaE.

TABLE II

ELECTROPHORETIC DISPLACEMENT (300 V, 45 min) OF RaD, RaE AND RaF USING DIFFERENT MIXTURES OF NITRIC ACID AND POTASSIUM NITRATE SOLUTION AS ELECTROLYTE

Composition of the electrolyte	*Displacement (mm)*		
	RaD	*RaE*	*RaF*
N HNO_3	—50.4	—39.6	—16.2
N HNO_3 95% + *N* KNO_3 5%	—48.0	—30.6 (trailing from —15.0 to —37.8)	— 5.75
N HNO_3 50% + *N* KNO_3 50%	—50.4	—26.4 (trailing from — 3.0 to —33.0)	— 4.5
N HNO_3 5% + *N* KNO_3 95%	—41.4	—15.6 (trailing from 0 to —22.0)	— 2.4
N KNO_3	—29.1	— 0.9	— 0.9

(2) *In mixtures of nitric acid and potassium nitrate solution*

Table II summarises the electrophoretic migration on paper of RaD, RaE and RaF in different electrolytes where the concentration of NO_3^- is kept constant at 1 *N* but the pH of the solution is varied by replacing progressively the nitric acid by potassium nitrate. Even 5 % KNO_3 in the electrolyte in place of nitric acid produces a considerable change in the electrophoretic mobility of the highly charged ions RaE and RaF, while the mobility of the relatively less charged RaD undergoes little change (Fig. 2, a, b). The sharp electrophoretic band of RaE in *N* HNO_3 is replaced by a band which trails, while the migration of RaF is reduced from —16.2 mm to —5.75 mm; thus RaF seems to be more sensitive to changes of pH than RaE. This effect is more and more pronounced as the proportion of potassium nitrate in the electrolyte is increased (Fig. 2 c, d). In *N* KNO_3, RaE and RaF are completely hydrolysed and remain in a single band at the point of application while RaD still preserves its cationic nature (Fig. 2 e). Similar migration is observed for polonium when it is subjected to electrophoresis alone in the above media.

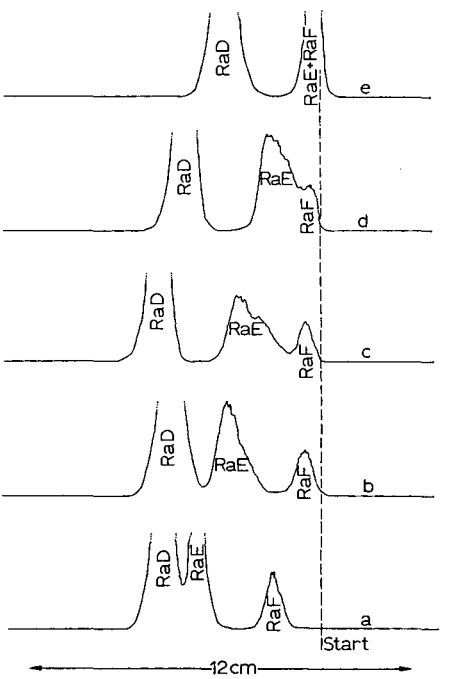

Fig. 2. Electropherograms (300 V, 45 min) of RaD, RaE and RaF in the electrolytes (a) *N* HNO_3; (b) *N* KNO_3 (5 %) + *N* HNO_3 (95 %); (c) *N* KNO_3 (50 %) + *N* HNO_3 (50 %); (d) *N* KNO_3 (95 %) + *N* HNO_3 (5 %); (e) *N* KNO_3.

(3) *In perchloric acid*

Perchloric acid, being a very strong acid, attacks the paper even at a concentration of 1.0 *N*. Studies in this medium could therefore be made only in the concentration range 0.01 *N* to 1.0 *N*. Fig. 3 shows the typical electropherograms obtained. The elements are easily hydrolysed in perchloric acid; thus in 0.1 *N* $HClO_4$ even bivalent RaD is hydrolysed and gives a diffused cationic band while RaE and RaF undergo complete hydrolysis and lie in a single band at the point of application. It is only in concentrations higher than 0.25 *N* that RaD moves as a compact cationic band while

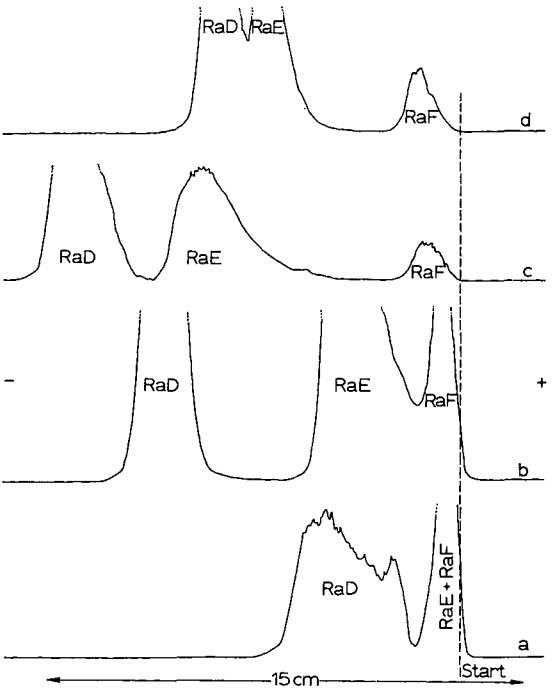

Fig. 3. Electropherograms (300 V, 45 min) of RaD, RaE and RaF in perchloric acid as electrolyte. (a) 0.1 N; (b) 0.25 N; (c) 0.6 N; (d) 1.0 N.

RaE, which is completely separated from RaD at this concentration, still trails in perchloric acid of concentrations as high as 0.85 N. One can, however, obtain the separation of RaD, RaE and RaF in concentrations of 0.25 N to 0.75 N $HClO_4$. With increase in the concentration of the acid, the distance between the RaE and RaF bands increases while RaD and RaE are less and less widely separated. Thus, in 1 N $HClO_4$ the bands of RaD and RaE overlap while the separation of RaF from the other two elements is very distinct. Trailing in the band of RaF is observed even in 1.0 N $HClO_4$ showing that even at this concentration this element is hydrolysed. Similar behaviour in paper electrophoresis for polonium alone applied to the paper was observed in this acid. This is presumably due to the high charge of the ion.

SUMMARY

An attempt has been made to define the concentration range of nitric and perchloric acids which give separations of RaD, RaE and RaF in electrophoresis on paper. The effect on the ionic nature of these elements of varying the concentration of these acids and also of the progressive replacement of nitric acid by potassium nitrate, keeping the NO_3^- ion concentration constant, has been examined.

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DISTRIBUTION OF SPECIES ALONG A BAND IN SEPARATIONS BY DISPLACEMENT CHROMATOGRAPHY

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INTRODUCTION

Of the different theoretical treatments to explain the chromatographic phenomena in columns, the plate theory originally developed by MARTIN AND SYNGE[1] and later extended by MAYER AND TOMPKINS[2] and by GLUECKAUF[3] has been most successful. These authors have applied the plate theory mainly to elution chromatography where bell-shaped elution curves are obtained. In recent years, displacement chromatography, characterised by flat-topped elution curves has acquired some prominence, especially in the field of rare earth separations[4,5] and in the separation of the isotopes of nitrogen[6] and those of lithium[7], using cation exchange resins. In the course of investigations on the separation of the isotopes of nitrogen, SPEDDING, POWELL AND SVEC[6] observed a plateau region (where the composition was uniform and equal to the initial composition) in the middle of the band separating the two ends where the separation of the species occurred. However, no successful attempts appear to have been made to predict quantitatively the concentration distribution of the species in the region following and preceding this plateau. In the present paper detailed calculations are given to determine the concentration distribution of the species along the length of the band during the course of separation and the calculated values are experimentally verified.

THEORETICAL

Consider two species M_1 and M_2 distributed uniformly (initially) along the length of the band of length L. For the sake of simplicity let us assume that the species are ions of equal charge. If the mole fraction of M_1 is N_0 then that of M_2 is $1 - N_0$. According to the concepts of the plate theory this band may be assumed to consist of a number of plates, say, n, of equal height h, so that $n \cdot h = L$. In the chromatographic separation process the eluant will gradually displace the adsorbed species at the rear boundary and the solution will come to equilibrium with the rest of the species during its movement along the band and redeposit the species on the resin at the front boundary. Two special features of displacement chromatography are: (1) the formation of sharp rear and front boundaries, and (2) the constancy of the length of the band under equilibrium conditions irrespective of the distance of elution. Separation is possible only if there is equilibrium between the species according to the exchange reaction:

$$M_1^r + M_2^s \rightleftharpoons M_1^s + M_2^r \qquad (1)$$

where the superscripts r and s refer to the resin and solution phases respectively. The distribution of species between the solution and resin phases at any point on the band, at equilibrium, is governed by the relation:

$$\frac{N^s}{1 - N^s} = \alpha \frac{N^r}{1 - N^r} \tag{2}$$

in which N^s and N^r denote the mole fractions of M_1 in the solution and resin phases respectively, and α is the equilibrium constant of reaction (1). α is sometimes also called the separation factor. If $\alpha > 1$, M_1 will concentrate at the front edge and if $\alpha < 1$, it will concentrate at the rear edge. There will be no separation when $\alpha = 1$. When one of the species tends to concentrate at one end of the band, the other species tends to concentrate at the other end and in the middle section the composition remains the same as in the original mixture. This plateau region will disappear when the separation is complete.

Rear section

Let AB (Fig. 1) represent the resin phase, divided into a number of theoretical plates, R_1, R_2... R_n, of equal height, h, and CD be the solution phase, the corresponding plates in equilibrium with the resin phase being S_1, S_2,... S_n. Initially let the resin phase contain a mixture of M_1 and M_2 in the mole fraction ratio of N_0 to $1 - N_0$, distributed uniformly throughout the length of the band. As a result of elution allow the con-

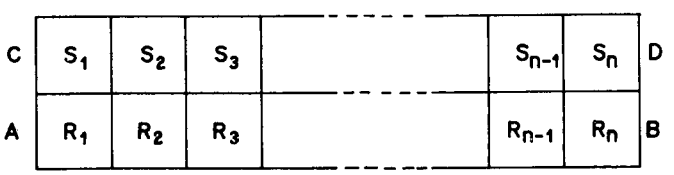

Fig. 1. "Plates" along the resin column.

tents of plate R_1 to go to the solution phase plate S_1. Since the whole of the contents of R_1 is transferred to S_1 the composition of the two plates will remain the same. Transfer the solution to S_2 and now $N_{R_1} = N_{S_1} = N_{S_2}$, which symbols represent the mole fractions of M_1 in R_1, S_1 and S_2 respectively. Allow this solution to come to equilibrium with the resin phase R_2. Let the composition of the resin and solution phases after equilibration be $N_{R_2}{}^E$ and $N_{S_2}{}^E$ respectively. Then from material balance considerations the following relation holds good:

$$N_{S_2} + N_{R_2} = N_{R_2}{}^E + N_{S_2}{}^E \tag{3}$$

and since there is equilibrium between the two phases:

$$\frac{N_{S_2}{}^E}{1 - N_{S_2}{}^E} = \alpha \frac{N_{R_2}{}^E}{1 - N_{R_2}{}^E} \tag{4}$$

Transfer the solution from S_2 to S_3 and allow it to come to equilibrium with R_3, then again:

$$\frac{N_{S_3}{}^E}{1 - N_{S_3}{}^E} = \alpha \frac{N_{R_3}{}^E}{1 - N_{R_3}{}^E} \tag{5}$$

This process of equilibration can be continued throughout the band. From eqns. (4) and (5):

$$N_{R_2}{}^E = \frac{N_{S_2}{}^E}{\alpha + (1 - \alpha)N_{S_2}{}^E} \tag{6}$$

$$N_{S_2}{}^E = \frac{\alpha N_{R_2}{}^E}{1 + (\alpha - 1)N_{R_2}{}^E} \tag{7}$$

$$N_{R_3}{}^E = \frac{N_{S_3}{}^E}{\alpha + (1 - \alpha)N_{S_3}{}^E} \tag{8}$$

$$N_{S_3}{}^E = \frac{\alpha N_{R_3}{}^E}{1 + (\alpha - 1)N_{R_3}{}^E} \tag{9}$$

and from material balance:

$$N_{S_3}{}^E + N_{R_3}{}^E = N_{R_3} + N_{S_2}{}^E \tag{10}$$

Rearranging eqn. (10) and substituting the value of $N_{S_2}{}^E$ from eqn. (7) we have:

$$N_{S_3}{}^E = \frac{\alpha N_{R_2}{}^E}{1 + (\alpha - 1)N_{R_2}{}^E} + \Delta N_{R_3}{}^E \tag{11}$$

where:

$$\Delta N_{R_3}{}^E = N_{R_3} - N_{R_3}{}^E$$

Substituting the value of $N_{S_3}{}^E$ in eqn. (8) one obtains:

$$N_{R_3}{}^E = \frac{[\alpha N_{R_2}{}^E + \Delta N_{R_3}{}^E \{1 + (\alpha - 1)N_{R_2}{}^E\}]}{\alpha\{1 + (\alpha - 1)N_{R_2}{}^E\} + (1 - \alpha)[\alpha N_{R_2}{}^E + \Delta N_{R_3}{}^E \{1 + (\alpha - 1)N_{R_2}{}^E\}]} \tag{12}$$

or in general, the equilibrium composition of the resin phase of any particular plate, R_n, is related to that of the immediate next plate, R_{n+1}, by the relation:

$$N_{R_{n+1}}{}^E = \frac{[\alpha N_{R_n}{}^E + \Delta N_{R_{n+1}}{}^E\{1 + (\alpha - 1)N_{R_n}{}^E\}]}{\alpha\{1 + (\alpha - 1)N_{R_n}{}^E\} + (1 - \alpha)[\alpha N_{R_n}{}^E + \Delta N_{R_{n+1}}{}^E \{1 + (\alpha - 1)N_{R_n}{}^E\}]} \tag{13}$$

where:

$$\Delta N_{R_{n+1}}{}^E = N_{R_{n+1}} - N_{R_{n+1}}{}^E$$

If $\alpha > 1$, $\Delta N_{R_{n+1}}{}^E \geqslant 0$, and if $\alpha < 1$, $\Delta N_{R_{n+1}}{}^E \leqslant 0$. Both at the rear and front sections of the band there is overall mass transfer between the solution and resin phases and therefore $\Delta N_{R_n}{}^E >$ or < 0, depending on the value of α. At the plateau region $\Delta N_{Rn}{}^E = 0$, and eqn. (13) reduces to $N_{R_{n+1}}{}^E = N_{Rn}{}^E$.

At the rear section of the band the value of $\Delta N_{Rn}{}^E$ varies continuously until it becomes 0 at the plateau region. If we repeat the cycle of plate equilibration starting with the contents of the second plate, R_2, a similar type of relation will be obtained

for the composition of any two successive plates. For a given and particular value of N_0 different curves can be plotted representing the actual concentration distribution of the species on the band during the separation process. A typical set of curves is given in Fig. 2, calculated for $\alpha = 2$ and $N_0 = 0.5$. Each curve represents the

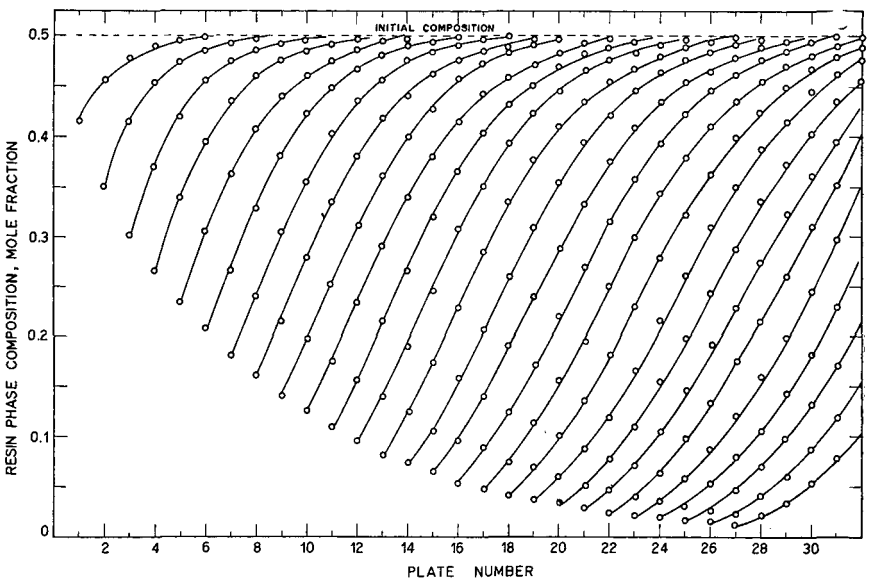

Fig. 2. Theoretical curves representing the concentration distribution of species in the rear section of the band during the course of separation for $\alpha = 2$ and $N_0 = 0.5$.

concentration distribution in different plates for a particular cycle of equilibration. If the height of a theoretical plate (HETP) is known the actual concentration variation along the length of the band, as a result of elution through a known distance, can be computed.

Front section

The general equation (13) will hold good also for the front section of the band. However, the following points have to be considered before applying the equation:

(1) The composition of the first plate, which is formed as a result of the transfer of species from the rear edge of the band through the plateau, will be the same as the composition of the solution at the plateau, *i.e.* equal to:

$$\frac{\alpha N_0}{1 + (\alpha - 1)N_0}$$

(2) The net mass transfer between the solution and resin phases during further development of the band starts from this plate.

(3) The composition of the resin phase in the last plate of the band is equal to the composition of the equilibrium solution in the preceding plate.

Bearing these points in mind, curves representing $N_{Rn}{}^{E}$ at different values of "n"

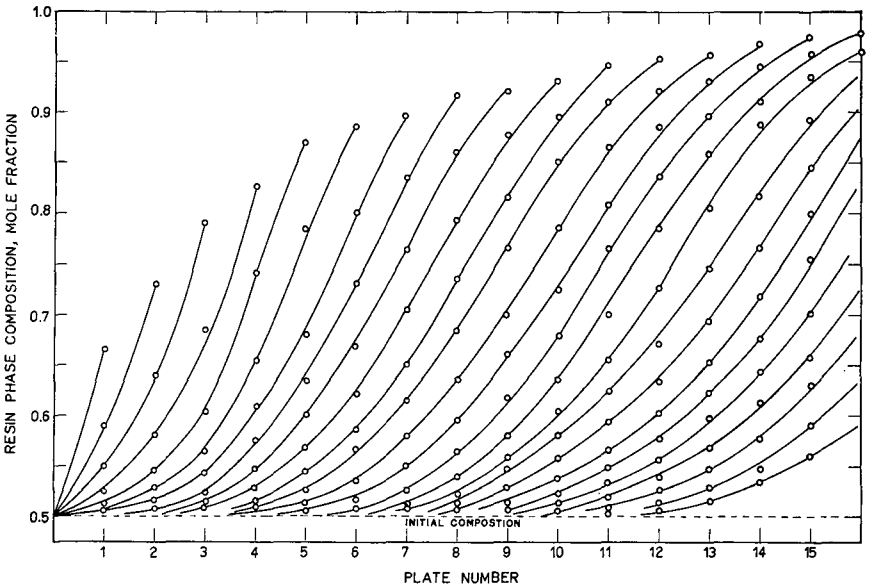

Fig. 3. Theoretical curves for the concentration distribution in the front section of the band, $\alpha = 2$, and $N_0 = 0.5$.

can be plotted for any given value of α and N_0. A typical set of curves is given in Fig. 3, for $N_0 = 0.5$ and $\alpha = 2$, each curve representing a particular set of equilibration.

Plateau region

It has been pointed out earlier that in the plateau region the composition of the resin phase remains constant. A general overall picture of the composition of the rear, the plateau and the front regions of the band, and those of the corresponding solution phases in equilibrium with the resin phase, for $\alpha = 2$ and $N_0 = 0.5$, is given in Fig. 4. The fraction of the band occupied by the plateau region depends on α and the number of band displacements. For an ideal case, the length l_1 of the rear section and l_2 of the front section, where separations have taken place, can be calculated for any band displacements. The length of the plateau will then be equal to $L - (l_1 + l_2)$. So long as the plateau region exists, the composition, $N_P{}^S$, of the solution which is going to the front region through the plateau is related to N_0 by the equation:

$$N_P{}^S = \frac{\alpha N_0}{1 + (\alpha - 1)N_0} \tag{14}$$

The net amount of M_1 transferred through this region (since $\alpha > 1$) when the band moves v band lengths is $v \cdot L(N_P{}^S - N_0) \cdot C$, where C is the capacity of the resin per unit length of the column. Suppose that as a result of this mass transfer there develop bands of length l_1 at the rear and of length l_2 at the front section, then from material balance considerations we have*:

$$(N_P{}^S - N_0) \cdot v \cdot L \cdot C = N_0 \cdot l_1 \cdot C = (1 - N_0) \cdot l_2 \cdot C \tag{15}$$

* This is true only if the length of the band is large compared to the mixing zone.

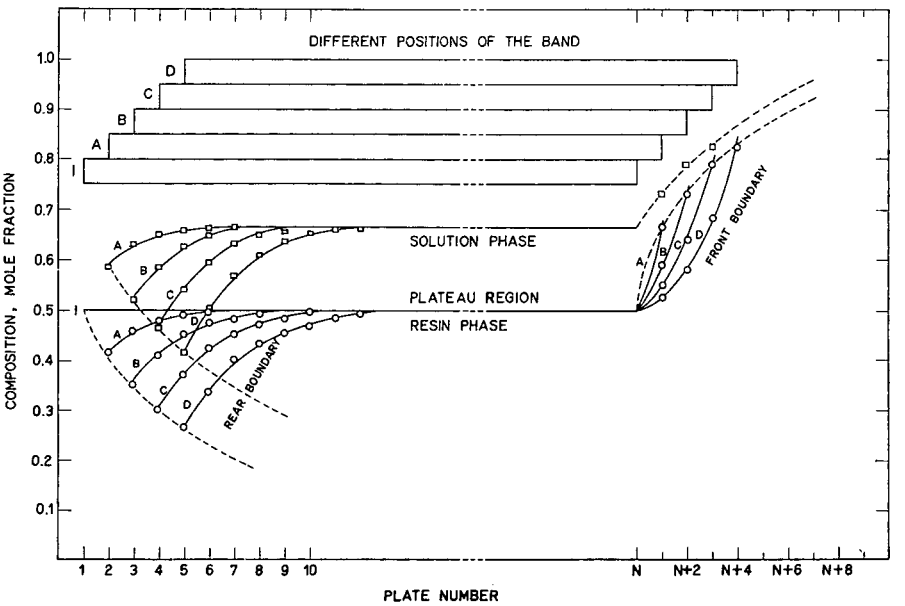

Fig. 4. Theoretical curves for the concentration distribution in the solution and resin phases along the length of the band.

Substituting the value of $N_P^S - N_0$ from eqn. (14) into eqn. (15) and rearranging gives:

$$l_1 = \frac{(1 - N_0)\,(\alpha - 1)\cdot v\cdot L}{1 + (\alpha - 1)N_0} \tag{16}$$

$$l_2 = \frac{N_0(\alpha - 1)\cdot v\cdot L}{1 + (\alpha - 1)N_0} \tag{17}$$

and the length of the plateau region:

$$L - (l_1 + l_2) = L\left[1 - \frac{v\cdot(\alpha - 1)}{1 + (\alpha - 1)N_0}\right] \tag{18}$$

When the plateau disappears, $L = l_1 + l_2$, and then it follows from eqn. (18) that:

$$v = \frac{1 + (\alpha - 1)N_0}{(\alpha - 1)} \tag{19}$$

This value of v also represents the minimum number of band displacements required for the separation of two species. A similar equation was obtained by SPEDDING *et al.*[6] by a different approach, but expressions for l_1 and l_2 were not derived.

EXPERIMENTAL

Height equivalent to a theoretical plate (HETP)

For the experimental verification of the theoretical predictions made in the preceding

section one should know the height of a theoretical plate. SPEDDING, POWELL AND SVEC[6] have shown that HETP (h) can be calculated using the relation:

$$h = \log \alpha \cdot \frac{\Delta L}{\Delta \log (r)} \qquad (20)$$

where $\Delta L/\Delta \log (r)$ is the slope of the L–log (r) line, L and r being the length and the corresponding ratio of the two species in the band at the steady state. In the present case the value of α is equal (for all practical purpose) to the ratio of the stability constants of the complexes involved. From the results of SCHWARZENBACH, GUT AND ANDEREGG[8], α is 3.38 for the Nd–Sm–EDTA system and 1.62 for the Pr–Nd–EDTA system.

An equimolar mixture of samarium and neodymium was absorbed on a cation exchange resin, Dowex-50W, X-8, 200 mesh, contained in a 2 cm diameter glass column to a height of about 5 cm. After saturation the resin was thoroughly washed with deionised water. This rare earth mixture was then eluted with EDTA solution, 0.01 M, pH adjusted to 8.3, at the rate of 0.2 ml/min using 40 cm length of copper retaining bed. As the rare earth band came out of the column the eluate was collected in different fractions and the corresponding lengths of the band were noted. The rare earths in the eluate were precipitated as oxalates, ignited to oxide, and analysed

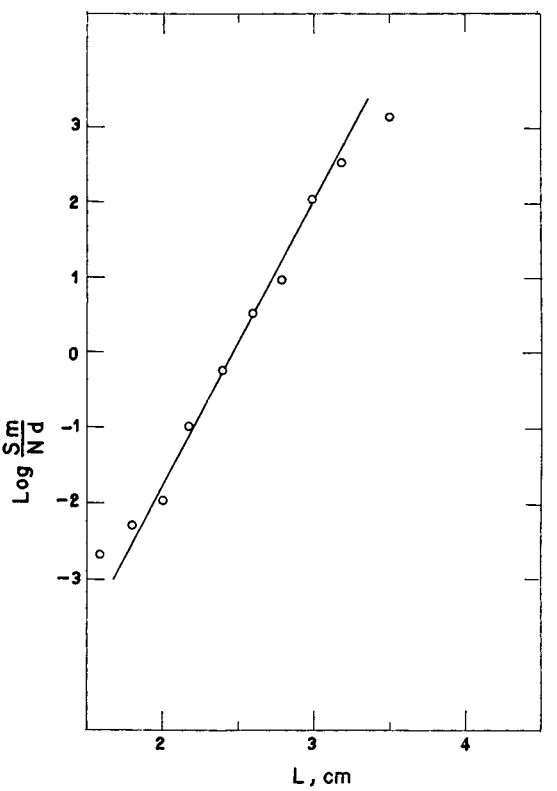

Fig. 5. Height equivalent to a theoretical plate.

for their Nd and Sm contents by the method described by BANKS AND KLINGMAN[9]. The results are plotted in Fig. 5. The HETP, calculated from these plots using eqn. (20) gave a value of 0.14 cm.

Distribution of species along the band during separation

The actual concentration distribution of the species on the resin phase before the steady state is reached cannot be determined correctly by analysing the eluate, because the composition of the resin phase is continuously changing while the solution is passing through the column. Therefore, a static approach was made to determine the resin phase composition during the separation.

A glass column, about 30 cm long, was fitted with a sintered glass disc (with a rim of thin rubber band) at the bottom in such a way that it can be moved smoothly. The bottom end of the column was closed with a rubber stopper containing a glass tube which served as outlet for the solution (Fig. 6). The column was filled to a height

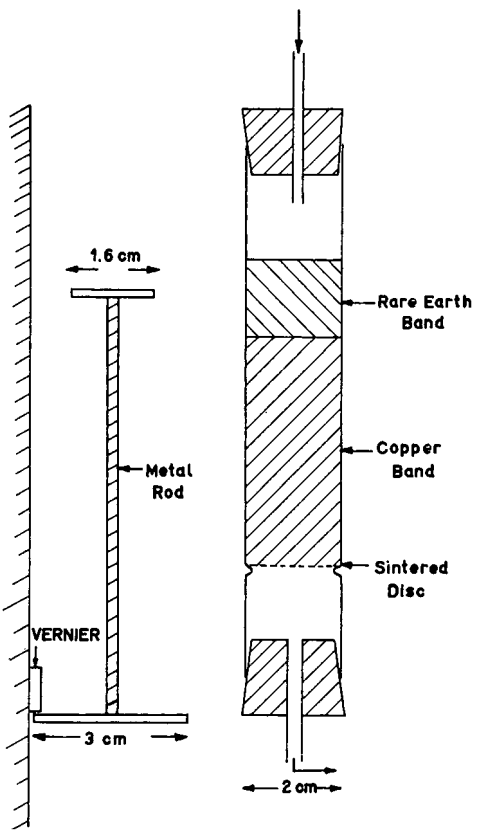

Fig. 6. Experimental set-up for the determination of the concentration distribution during the separation of neodymium and samarium.

of 20 cm with the copper form of the resin and then the rare earth form of the resin containing equal amounts of neodymium and samarium was poured in to a height of

5 cm and pressed gently. Special care was taken to avoid mechanical mixing at the copper–rare earth boundary. EDTA solution, 0.01 *M*, adjusted to pH 8.3 with ammonia, was passed through the column at a rate of 0.2 ml/min until the required amount of NH_4^+ band was formed at the top of the rare earth band. The displacement was 0.45 cm in expt. I, 1.4 cm in expt. II, and 20 cm in expt. III where the steady state was reached. After the required amount of displacement of the band, the solution was drained off by suction. The rubber stopper at the bottom of the column was removed and the sintered glass disc was raised carefully by means of a metal rod, with two discs attached to it, one at the top and another at the bottom (Fig. 6). The movement of this rod was measured using the bottom disc with a vernier scale correct to 0.05 cm. As the resin bed moved up the column the NH_4^+ band, which could be recognised by its white colour, was cut off from the top with a sharp blade. Afterwards the rare earth band was raised and was cut off every 0.2 cm length, and each time the length of the band cut was determined from the movement of the disc at the bottom. The rare earth from each portion of the band was extracted from the resin by repeated treatment with EDTA solution, precipitated as oxalate, ignited to oxide and analysed as before. The results of the three experiments along with the theoretical predictions are given in Fig. 7. It can be seen that within the limits of

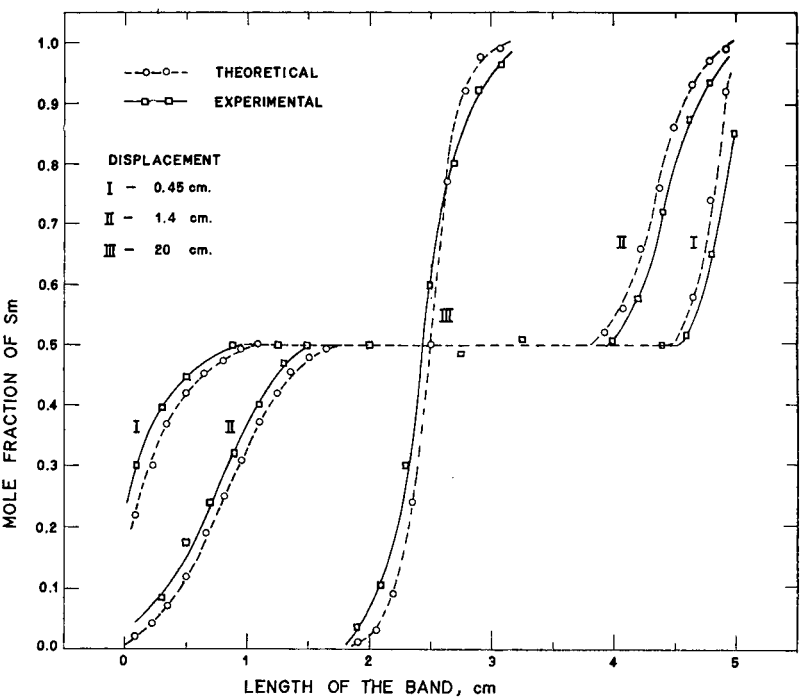

Fig. 7. Comparison of theoretical and experimental results for the separation of neodymium and samarium.

experimental error the agreement between theoretical and experimental values are reasonably good.

Verification of equations (16) and (17)

For verifying eqns. (16) and (17) it is preferable to take coloured rare earths so that the lengths of the rear and front sections of the band can be measured during the course of separation. A mixture of Pr and Nd was used for verifying eqn. (16) and a mixture of Nd and Sm for eqn. (17).

An equimolar mixture of praseodymium and neodymium was absorbed on the cation exchange resin in a 1 in. diameter column to a height of about 40 cm. It was then eluted with EDTA, 0.01 *M* adjusted to pH 8.3, at a rate of 0.5 ml/min using copper retaining bed. When separation occurred the praseodymium band could be identified by its green colour. The distance of elution was measured from the movement of the center of the rare earth band and at the same time the length of the praseodymium band was also measured. The length of the Pr band expected theoretically from eqn. (16) was calculated. When separation is complete the length of the Pr band will remain constant and will be equal to half the total length of the rare earth band. The experimental and theoretical results are given in Fig. 8.

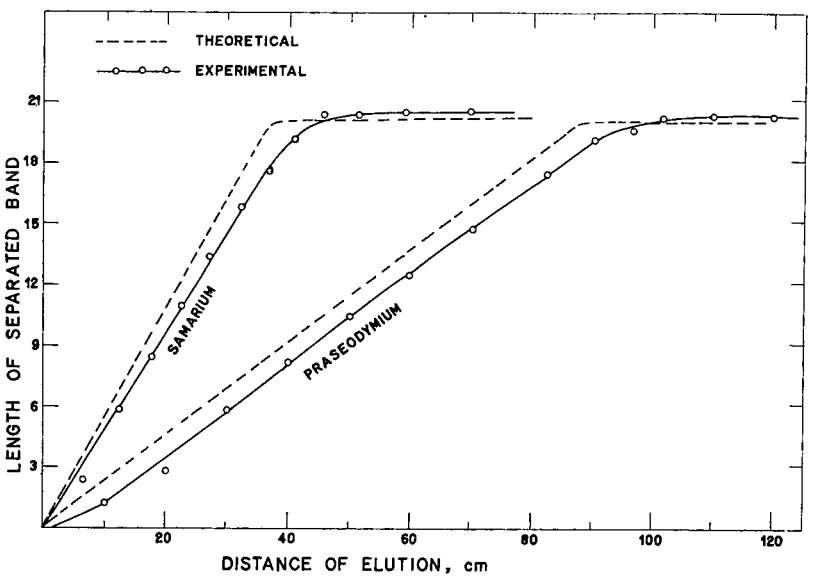

Fig. 8. Verification of equations (17) and (18).

Similar experiments were also carried out with a mixture of Nd and Sm in order to verify eqn. (17). The results are given in Fig. 8. In both cases good agreement is observed between theory and experiment.

SUMMARY

The distribution of species along the band during separation by displacement chromatography is discussed from a theoretical view point, using the concepts of the plate theory. Detailed calculations are presented for the determination of the concentration distribution in the rear and the front regions of the band, for the separation

of two species originally present in equal amount and having a separation factor of 2. An experimental procedure is described for verifying the theoretical results. Data obtained for the separation of neodymium and samarium and of praseodymium and neodymium, using EDTA as eluant and copper as retaining ion confirmed the validity of the theoretical treatment.

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IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN*

LII. MITTEILUNG. PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG EINIGER ALKYLIERTEN AMINOBENZOLSULFONSÄUREN

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Die alkylierten Aminobenzolsulfonsäuren sind wichtige Hilfsmittel in einigen Handelstypen der Farbstoffe. Für diesen Zweck benutzt man besonders die alkylierten Metanil- und Sulfanilsäuren mit Methyl-, Äthyl- oder Benzyl- als Alkyl. So ist z.B. Benzylsulfanilsaures Natrium als Solutionsalz B (I.G. Farbenindustrie), Solutionsalz G (Geigy), Liovatin S (Sandoz), Solution Salt BN (I.C.I.), Dibenzylsulfanilsaures Natrium als Solution Salt SV (I.C.I.), Dimethylmetanilsaures Natrium als Dinaton (I.G. Farbenindustrie) usw. bekannt[1].

In dieser Mitteilung wollen wir eine Methode beschreiben, die wir in unserem Laboratorium zur Trennung und Identifizierung dieser Stoffe benützen. Wir chromatographieren sie unter Anwendung der Mischungen von *n*-Propylalkohol oder *n*-Butylalkohol mit Ammoniak. Für die Dibenzylsulfanilsäure kann man auch von den mit Laurylalkohol imprägnierten Papieren und dem Gemisch Ammoniak–Ameisensäure (85 %) 9:1 als mobile Phase Gebrauch machen.

EXPERIMENTELLER TEIL

Alle Versuche führten wir mit dem Whatmanpapier No. 3 durch. Die chromatographierten Verbindungen wurden als wässrige Lösungen auf die Chromatogramme aufgetragen und in meisten Fällen wurde bei Raumtemperatur entwickelt. Als bewegliche Phase diente ein Gemisch von *n*-Propylalkohol–Ammoniak (2:1) oder *n*-Butylalkohol gesättigt mit Ammoniak. Bei der Umkehrphasenchromatographie wurde ein Papierstreifen durch eine 5 %ige benzolische Lösung von Laurylalkohol gezogen und zwecks Verdunsten des Benzols 10 Minuten an der Luft aufgehängt gelassen. Als bewegliche Phase wurde da ein Gemisch von Ammoniak–85 % Ameisensäure (9:1) benutzt. In diesem Falle ist es vorteilhaft bei höherer Temperatur (30–35°) zu arbeiten. Die Alkohole waren handelsübliche Produkte und ebenso Ammoniak, dass ungefähr 25 %ig war.

Nachdem das Lösungsmittel bis zu einer Entfernung von ungefähr 30 cm vom Startpunkt gewandert war, wurden die Chromatogramme der Kammer entnommen und durch aufhängen an der Luft getrocknet.

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Die Sichtbarmachung der Flecken wurde durch besprühen mit folgenden Sprühreagenzien durchgeführt:

(A) mit einem frisch bereiteten Gemisch von 1 % Kaliumhexacyanoferrat(III)-lösung und 15 % Eisen(III)-chloridlösung (1:1)[2] unter Bildung intensiv blauer Flecken auf hellgelbem Hintergrund. So gewonnene Chromatogramme können durch Eintauchen in verdünnte Chlorwasserstoffsäure und nachträgliches Auswaschen in fliessendem Wasser stabilisiert werden, sodass sie als blaue Flecken auf weissem bis hellblauem Hintergrund erscheinen;

(B) mit 0.1 %iger wässriger 1-Diazo-2-chlor-4-nitrobenzol–1,5-Naphthalindisulfonatlösung[3] (B1) mit nachträglichem Besprühen mit 10 %iger Kalilauge (B2), wobei einzelne Verbindungen charakteristische Färbungen zeigen;

(C) mit 0.05 % wässriger Pinakryptolgelblösung[4], wobei verschiedene intensive, im U.V.-Licht erkennbare blaugraue Flecken erscheinen;

(D) mit einer *p*-Dimethylaminobenzaldehydelösung (1 g *p*-Dimethylaminobenzaldehyd wird in 95 ml Äthanol und 5 ml konzentrierter Chlorwasserstoffsäure gelöst); in einigen Fällen entstehen gelbe Flecken.

Die R_F-Werte und die Färbung bei der Sichtbarmachung sind in der Tabelle I zusammengefasst.

ERGEBNISSE UND DISKUSSION

Die Resultate unserer Versuche zeigten, dass das Verhalten der geprüften Stoffe den laufenden Gewohnheiten entspricht. Die R_F-Werte werden mit steigender C-Atomzahl um zum einwandigen Verteilung dieser Verbindungen ausreichende Werte höher. Einigermassen kleiner ist der Unterschied der R_F-Werte der Verbindungen mit gleicher C-Atomzahl, die nur strukturell verschieden sind. In diesen Fällen kann man der verschiedenen Färbungen bei der Sichtbarmachung (besonders B1 und B2) ausnützen, oder die Unterschiede der R_F-Werte durch die Entwicklung auf Durchlauf grösser zu machen. So z.B. für die Verteilung der Sulfanil- und Metanilsäure kann man vom System *n*-Butylalkohol gesättigt mit Ammoniak auf Durchlaufchromatogramm (Laufzeit 20–30 Stunden) Gebrauch machen.

Um einzelne Verbindungen eindeutiger zu identifizieren wählten wir mehrere Detektionsmethoden, von denen jede gewisse Vorteile hat. Das Besprühen mit Eisen (III)-ferricyanidlösung hat den Vorteil grosser Empfindlichkeit und Universalität, sodass man alle geprüften Verbindungen gleichzeitig sichtbarmachen kann. Die Sichtbarmachung mittels diazotiertem 2-Chlor-4-nitroanilin ist in einigen Fällen nicht so empfindlich (besonders bei höher alkylierten Derivaten, z.B. Dibenzylsulfanilsäure), aber der Vorteil dieser Methode ist, dass einzelne Verbindungen charakteristische Färbungen, die schon beim Besprühen mit wässriger Lösung der Diazoverbindung entstehen, zeigen. In einigen Fällen entstehen charakteristische Veränderungen durch nachträgliches Besprühen mit Alkalilauge. Beim Besprühen mit Pinakryptolgelb entsteht im U.V.-Licht erkennbare blaugraue Färbung, die desto intensiver ist, je höher die ursprüngliche Aminobenzolsulfonsäure alkyliert ist. Diese Färbung ist so charakteristisch, dass man gegebene Verbindungen schon durch diese Detektion von anderen Sulfonsäuren, die oft gleichzeitig anwesend sind (z.B. Tamol NNO, was ein Produkt der Kondensation der Naphthalin-2-sulfonsäure mit Formaldehyd ist) und mit Pinakryptolgelb auch unter Entstehung charakteristischer gelben bis braunen Färbungen reagieren[5], unterscheiden kann. Die Sichtbarmachung

TABELLE I

R_F-WERTE UND FÄRBUNG BEI SICHTBARMACHUNG VON ALKYLAMINOBENZOLSULFONSÄUREN

Säure	*R_F-Wert im Lösungsmittelsystem*		*Färbung bei Sichtbarmachung*				
	S 1	*S 2*	*A*	*B 1*	*B 2*	*C*	*D*
Orthanilsäure	0.24	0.59	dunkelblau	braungelb	karminrot bis rotviolett	blaugrau	gelb
Metanilsäure	0.17	0.51	dunkelblau	braungelb	karminrot bis rotviolett	schwach blaugrau	gelb
N,N-Dimethylmetanilsäure	0.35	0.70	dunkelblau	blauviolett	violettblau	blaugrau	
N,N-Diethylmetanilsäure	0.61	0.80	dunkelblau	purpurviolett	violett	blaugrau	
Sulfanilsäure	0.14	0.47	dunkelblau	gelb	karminrot bis rotviolett	schwach blaugrau	gelb
N-Monomethylsulfanilsäure	0.24	0.59	dunkelblau	gelb	intensiv gelb	schwach blaugrau	schwach gelb
N,N-Dimethylsulfanilsäure	0.31	0.65	dunkelblau	braunviolett	violettbraun	blaugrau	schwach gelb
N-Monobenzylsulfanilsäure	0.39	0.73	dunkelblau	braungelb	gelbbraun	intensiv blaugrau	schwach gelb
N,N-Dibenzylsulfanilsäure	0.76	0.84	dunkelblau	rosa	schwach rosa	blauschwarz	—

* S 1 = *n*-Butylalkohol mit Ammoniak gesättigt; S 2 = *n*-Propylalkohol–Ammoniak (2:1); A = Eisen (III)-ferricyanidlösung; B 1 = 1-Diazo-2-chlor-4-nitrobenzol–1,5-Naphthalindisulfonatlösung; B 2 = 10% KOH nach vorherigem B 1; C = Pinakryptolgelblösung; D = *p*-Dimethylaminobenzaldehydlösung.

mit *p*-Dimethylaminobenzaldehydlösung ist besonders für nicht alkylierte Sulfonsäuren, die intensiv gelbe Flecken bieten, geeignet. Die alkylierte Säuren reagieren in einigen Fällen auch positiv, aber nur schwach.

Die Flecken der einzelnen Verbindungen sind insgesamt fast rund, nur bei höher alkylierten Derivaten (z.B. Dibenzylsulfanilsäure) tritt bei Anwendung vom System *n*-Butylalkohol gesättigt mit Ammoniak gewisse Streifenbildung ein. Aus diesem Grunde wählten wir für die Identifizierung dieser Verbindung noch ein weiteres Lösungsmittelsystem und zwar unter Benutzung umgekehrter Phasen. In diesem Falle bildet diese Verbindung einen fast runden Fleck, der einen R_F-Wert von 0.36 hat, während Benzylsulfanilsäure einen R_F-Wert von 0.88 hat und die anderen geprüften Verbindungen mit der Lösungsmittelfront wandern. Für die Sichtbarmachung ist in diesem Falle das Besprühen mit Pinakryptolgelb am besten geeignet.

Zusammenfassend kann man sagen, dass man die angegebenen Methoden vorteilhaft zur Identifizierung der Alkylaminobenzolsulfonsäuren benutzen kann, in vielen Fällen auch in komplizierten Industrieprodukten, ohne vorher die einzelne Komponente isolieren zu müssen.

ZUSAMMENFASSUNG

Es wurde eine Methode zur Trennung und Identifizierung einiger alkylierten Aminobenzolsulfonsäuren mittels Papierchromatographie auf unvorbehandelten Papieren mit *n*-Propylalkohol oder *n*-Butylalkohol–Ammoniak Gemisch oder auf mit Laurylalkohol imprägniertem Papier mit Ammoniak–Ameisensäure Gemisch als durchlaufende Phase ausgearbeitet.

SUMMARY

A method is described for the separation and identification of some alkylaminobenzenesulphonic acids. Paper chromatography was applied, using either untreated paper with *n*-propanol–ammonia or *n*-butanol–ammonia as solvent systems, or paper impregnated with lauryl alcohol and ammonia–formic acid as solvent system.

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PAPER CHROMATOGRAPHY OF DYES
I. PAPER CHROMATOGRAPHY OF DISPERSE DYES*

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The establishment of a suitable chromatographic method for the determination of the purity and identity of disperse dyes, has aroused interest because of the importance of these dyes for the treatment of synthetic fibres.

INTRODUCTION

Paper chromatography of disperse dyes was described by a number of authors[1–14]. We have studied these methods and found that they are generally not sufficiently sensitive for the separation of more complex mixtures of disperse dyes, and, furthermore, that with the majority of methods there is a lack of relation between the chemical constitution of the dyes and their chromatographic behaviour, this being an indispensable requirement for exact analysis. Very satisfactory results were obtained by the method of GASPARIČ AND TÁBORSKÁ[12,13]. The only drawbacks are the necessity of impregnating the chromatography paper with 1-bromonaphthalene, a rather time-consuming procedure, and the unpleasant odour of this substance.

While developing our method we found very effective chromatographic separation of disperse dyes could be obtained on unmodified chromatography paper by employing aqueous pyridine solvent systems. Of thirty brands of chromatography papers, Schleicher & Schüll 589[3] (blue ribbon) quantitative filter paper proved the only one suitable[11,14].

CHROMATOGRAPHIC TECHNIQUE

In the developing of chromatograms, the ascending technique was employed. The distance from the start of the chromatogram to the edge of the paper was 30 mm and the separation track was 400 mm long. Solutions of dyes were applied as spots of 1% acetone solution by means of a micro pipette in 0.05 mg quantities. Chromatograms were developed for 12 hours at 20–22°.

For disperse azo dyes, the solvent system pyridine–water (1:3), and for anthraquinone disperse dyes the solution pyridine–water (1:5) were found suitable.

The R_F values of disperse dyes in pyridine eluent systems depend on the chemical structure of the dyes and on the functional groups and substituents.

Figs. 1 and 2 show the chromatographic separation of a mixture of disperse azo dyes and Fig. 3 shows that of a mixture of anthraquinone disperse dyes.

* This topic was briefly dealt with in the report entitled "Systematic method of paper chromatography of water-soluble and insoluble dyes" presented at the Conference and Symposium on Paper Chromatography (Prague, 1961).

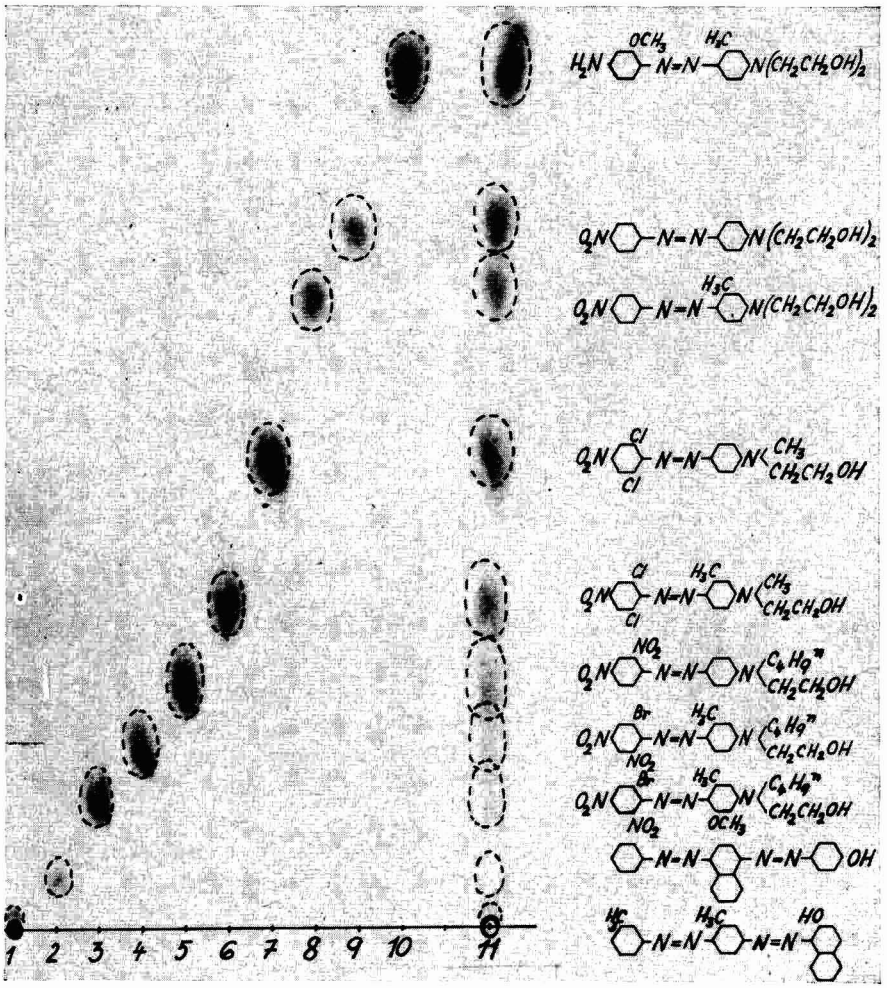

Fig. 1. Example of chromatographic separation of a mixture of disperse azo dyes.

RESULTS

We have analysed by the method described some six hundred disperse dyes of various commercial names. The results obtained have enabled us to complete some data given in the Colour Index[15] on analogous disperse dyes of varying trade names. In Table I a compilation is made of the results of separation of the more important disperse dyes of known constitution. In this compilation, the system used in the Colour Index was followed.

From the results shown in Table I, it is evident that in many cases the dyes contain secondary components (often isomers) originating in the reaction by which

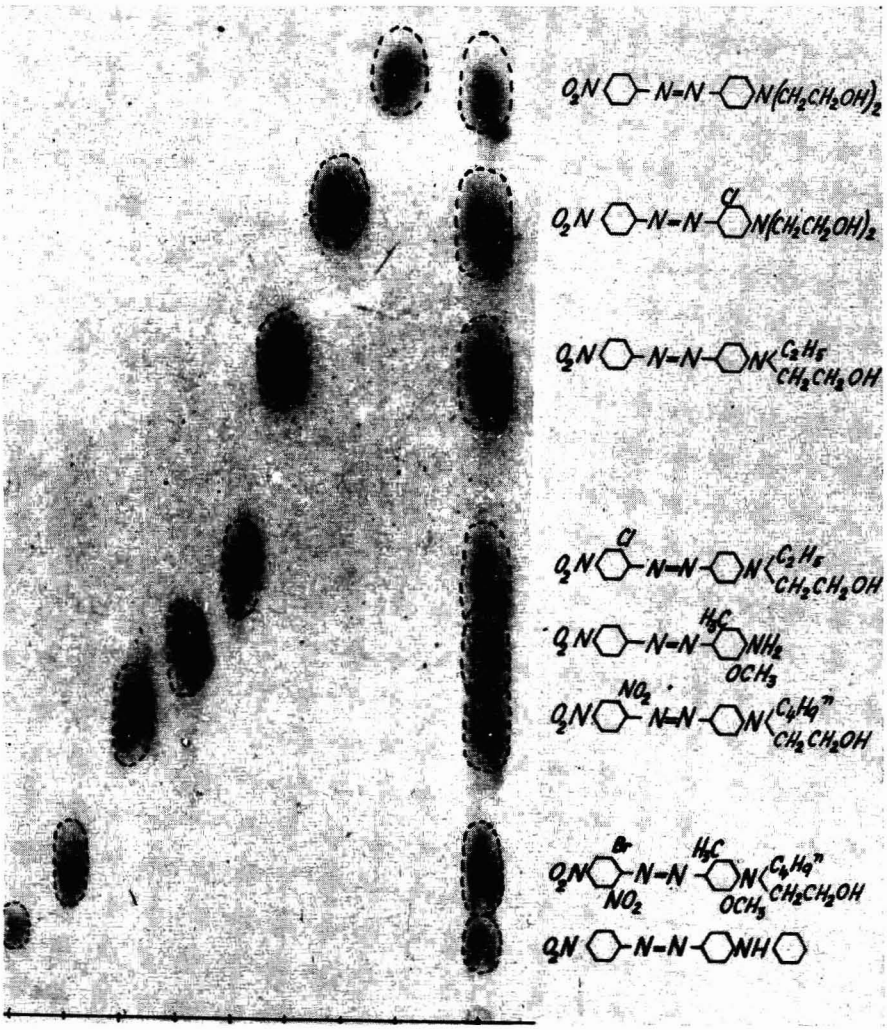

Fig. 2. Example of chromatographic separation of a mixture of disperse azo dyes.

they are produced. Anthraquinone blue dyes of more complex chemical constitution (more complex substitution) contain the most secondary components.

The number and character of the secondary components (by-products) in analogous dyes is not always the same. This may be explained by the varying degree of purity of intermediate products and the perfection of the method of manufacture.

CONCLUSIONS

From the chromatographic behaviour of these dyes the following conclusions may be made:

Fig. 3. Example of chromatographic separation of a mixture of anthraquinone disperse dyes.

(a) Disazo dyes possess a lower R_F value than monoazo dyes.

(b) Substitution of azo- and anthraquinone derivatives entailing a substantial influence on molecular weight tends to lower the R_F value.

(c) Anthraquinone dyes are adsorbed to a lesser degree, therefore possess a higher R_F value than azo dyes, nitro dyes, methine derivatives and dyes of the arylaminoquinone and aminonaphthalimide group.

(d) The presence of $-OCH_3$ and $-CH_3$ groups and of halogens decreases the R_F value of azo dyes as their number increases.

(e) With anthraquinone dyes, considerable decrease of R_F value is caused by alkylation and arylation of the amino group in the nucleus. With aminoanthraquinones

TABLE I

RESULTS OF CHROMATOGRAPHIC SEPARATION OF DISPERSE DYES

Colour index	*Commercial name*	*Chemical constitution*	*Colour of band*	R_F
10350	Celliton Fast Yellow RR	*p*-Amidophenol → 1-chloro-2,4-dinitrobenzene	Yellow	0.55
11005	Celliton Fast Orange GR	*p*-Nitroaniline → anilinomethanesulphonic acid	Dull orange	0.00
			Orange	0.51
			Orange*	0.87
11025	Diazo Nero Microsetile G	*p*-Nitroaniline → N,N-dimethylaniline	Brown	0.00
			Golden yellow	0.72
11035	Cellitazol AZN	2-Chloro-4-nitroaniline → N,N-dimethylaniline	Orange	0.00
			Brown	0.46
11080	Dispersol Fast Orange A	*p*-Nitroaniline → diphenylamine	Orange	0.07
11100	Celliton Fast Brown 3R	2,6-Dichloro-4-nitroaniline → 2-(N-methylanilino)-ethanol	Reddish orange	0.56
11110	Dispersol Fast Scarlet B	*p*-Nitroaniline → 2-(N-ethylanilino)-ethanol	Pink	0.23
			Pink*	0.28
			Red	0.59
			Pink*	0.68
11115	Celliton Fast Rubine B	2-Chloro-4-nitroaniline → 2-(N-ethylanilino)-ethanol	Yellow*	0.00
			Pink*	0.12
			Yellow*	0.15
			Pink*	0.21
			Bordeaux	0.40
			Pink*	0.53
11118	Artisil Fast Scarlet GP	*p*-Nitroaniline → 3-(N-ethylanilino)-1,2-propanediol	Pink	0.65
11120	Celliton Red Violet RR	2,4-Dinitroaniline → 2-(N-butylanilino)-ethanol	Violet	0.03
			Pink*	0.16
			Violet	0.30
			Yellow	0.81
11130	Dispersol Fast Red R	*p*-Nitroaniline → 2,2′-(phenylimino)-diethanol	Red	0.68
			Pink	0.80
			Yellow	0.82
11150	Celliton Fast Scarlet R	2-Nitroaniline → 2,2′-(*m*-chlorophenylimino)-diethanol	Pink*	0.56
			Scarlet	0.76
			Pink	0.82
11180	Celliton Red R	*p*-Nitroaniline → 2-(*m*-toluidino)-ethanol	Orange*	0.02
			Pink	0.45
			Pink	0.69
11190	Celliton Fast Brown 5R	2,6-Dichloro-4-nitroaniline → 2-(N-methyl-*m*-toluidino)-ethanol	Dull yellow	0.00
			Pink	0.16
			Dull yellow	0.23
			Reddish brown	0.41
			Pink	0.53
11195	Celliton Violet R	2,4-Dinitroaniline → 2-(N-butyl-*m*-toluidino)-ethanol	Violet	0.24
11200	Celliton Discharge Violet B	2-Bromo-4,6-dinitroaniline → 2-(N-butyl-*m*-toluidino)-ethanol	Violet*	0.11

11205	Celliton Discharge Blue 3R	2-Bromo-4,6-dinitroaniline → 2-(N-butyl-5-methyl-*o*-anisidino)-ethanol	Blue	0.07
11210	Celliton Fast Red GG	*p*-Nitroaniline → 2,2′-(*m*-tolylimino)-diethanol	Pink	0.58
			Red	0.75
			Orange	0.86
11215	Celliton Fast Rubine 3B	2-Chloro-4-nitroaniline → 2,2′-(*m*-tolylimino)-diethanol	Crimson	0.62
11220	Cellitazol GG	4-Nitro-*o*-anisidine → 2,2′-(*m*-tolylimino)-diethanol	Brown	0.78
			Brown	0.85
11225	Celliton Discharge Rubine BBF	2-Amino-5-nitrophenol → 2,2′-(*m*-tolylimino)-diethanol	Pink	0.63
11230	Celliton Fast Scarlet RN	*p*-Nitroaniline → (N-2-hydroxyethyl-*m*-toluidino)-propionitrile	Pink	0.07
			Pink	0.37
			Scarlet	0.50
			Pink	0.70
			Scarlet	0.75
			Pink	0.86
11250	Cibacet Scarlet G	*p*-Nitroaniline → cresidine	Pink	0.31
11255	Cibacet Diazo Black B	Reduce the nitro group of C.I. 11250	Brown	0.00
			Dull yellow*	0.67
			Dull yellow	0.79
11365	Cibacet Diazo Black GN	*p*-Nitroaniline → 1-naphthylamine	Reddish brown	0.00
			Reddish brown	0.52
			Brown	0.77
			Dull brown*	0.88
			Bluish green	0.94
11410	Celliton Discharge Blue RRF	2-Bromo-4,6-dinitroaniline → 1-*sec.*-butyl-1,2,3,4-tetrahydro-7-methyl-3-quinolinol	Blue	0.20
11430	Celliton Discharge Blue 3G	2-Chloro-4-nitroaniline → 1,2,3,4-tetrahydrobenzo-(*h*)quinoline-3,7-diol	Blue	0.25
			Pink	0.77
11435	Celliton Discharge Blue 5G	2-Amino-6-methoxybenzothiazole → 1,2,3,4-tetrahydro-benzo(*h*)quinoline-3,7-diol	Blue	0.27
			Blue*	0.46
			Blue	0.78
			Pink	0.80
11835	Celliton Fast Yellow G	*p*-Aminoacetanilide → *p*-cresol	Yellow	0.62
12700	Sudan Yellow 3G	Aniline → 3-methyl-1-phenyl-5-pyrazolone	Yellow	0.20
12790	Celliton Yellow 5G	*m*-Nitroaniline → 4-hydroxy-1-methylcarbostyril	Yellow	0.63
			Dull yellow*	0.86
12795	Celliton Yellow 3GN	*o*- and *p*-Nitroaniline → 4-hydroxy-1-methylcarbostyril	Yellow	0.82
26080	Dispersol Fast Orange B	Aniline → 1-naphthylamine → phenol	Orange	0.00
			Orange	0.09
26090	Celliton Fast Yellow 5R	*p*-Phenylazoaniline → *o*-cresol	Yellow	0.09
37235	Cellitazol BN	*o*-Dianisidine	Brown	0.00
			Brown*	0.18
			Brown	0.82
48000	Celliton Fast Yellow 7G "F"	Condense ethyl cyanoacetate with *p*-(N-butyl-2-chloroethyl-amino)-benzaldehyde	Yellow	0.26

(continued on p. 482)

TABLE I (*continued*)

Colour index	*Commercial name*	*Chemical constitution*	*Colour of band*	R_F
48005	Celliton Fast Yellow 3G "F"	Condense ethyl cyanoacetate with *p*-(N-methyl-*p*-phenetidino)-benzaldehyde	Yellow	0.38
			Yellow	0.95
56060	Celliton Fast Green 3B	Condense the leuco form of 8-amino-5-hydroxy-1,4-naphthoquinone imine with *p*-benzoyloxy-aniline, then oxidise with air	Bluish green	0.69
			Green	0.87
56200	Celliton Brilliant Yellow FFA-CF	Condense 4-aminonaphthalic anhydride with 2,4-xylidine	Yellow	0.86
58900	Duranol Brilliant Yellow 6 G	Benzanthrone, 3-methoxy-	Yellow	0.25
60505	Celliton Pink R	Anthraquinone, 1-methylamino-	Pink	0.68
60700	Celliton Orange R	Anthraquinone, 1-amino-2-methyl-	Orange	0.42
60710	Duranol Red 2B	Anthraquinone, 1-amino-4-hydroxy-	Red	0.72
60755	Celliton Fast Pink RF	Anthraquinone, 1-amino-2-methoxy-4-hydroxy-	Pink	0.33
			Pink	0.75
61100	Cibacet Violet 2R	Anthraquinone, 1,4-diamino-	Violet	0.00
			Violet	0.73
			Violet	0.81
61105	Celliton Fast Violet 6B	Anthraquinone, 1-amino-4-methylamino-	Pink	0.74
			Violet	0.79
			Blue	0.82
61110	Artisil Direct Blue 2RP	Anthraquinone, 1-amino-4-anilino-	Blue	0.00
			Blue	0.18
			Blue*	0.86
61115	Celliton Fast Blue FR	Anthraquinone, 1-amino-4-anilinomethoxy-	Grey	0.00
			Blue	0.27
			Violet	0.61
			Blue	0.68
			Blue	0.78
			Violet	0.85
61500	Celliton Fast Blue B	Anthraquinone, 1,4-dimethylamino-	Bluish violet	0.50
			Violet	0.66
61505	Celliton Fast Blue FFR	Anthraquinone, 1-methylamino-4-(2-aminoethanol)	Blue	0.68
			Blue	0.76
			Blue	0.81
61510	Celliton Fast Blue FW	Anthraquinone, 1-methylamino-4-[2-(2-aminoethoxy)-[ethanol]	Blue	0.34
			Blue	0.54
			Blue	0.64
			Blue	0.80
61540	Celliton Fast Green 5	Anthraquinone, 1-methylamino-4-(1-*p*-aminoanilino-2 hydroxyethyl)-	Bluish green	0.71
			Bluish green	0.82
61545	Celliton Fast Blue BF	Anthraquinone, 1,4-di-(2-aminoethanol)	Violet	0.47
			Blue	0.59
			Blue	0.66
62015	Duranol Red X3B	Anthraquinone, 1,4-diamino-2-methoxy-	Red	0.77
62030	Celliton Fast Violet B	Anthraquinone, 1,4-diamino-5-nitro-	Bluish violet	0.82

62035	Celliton Fast Blue FFB	Anthraquinone, 1-amino-2-carbamyl-4-methylamino-	Blue	0.61
			Blue	0.70
			Dull violet	0.83
62050	Celliton Fast Blue FFG	Anthraquinone, 1-amino-2-carbamyl-4-cyclohexylamino-	Blue	0.08
			Blue	0.46
			Dull blue*	0.58
			Dull blue	0.65
			Dull violet	0.82
62500	Artisil Blue Green GP	Anthraquinone, 1,4-di-(2-hydroxyethyl)-2-amino-5,8-dihydroxy-	Blue	0.40
			Blue	0.51
			Blue	0.62
			Dull blue	0.83
63305	Duranol Blue G	Anthraquinone, 1,5-bis-methylamino-4,8-dihydroxy-	Blue	0.25
			Blue	0.71
			Blue	0.81
64500	Celliton Blue ex.	Anthraquinone, 1,4,5,8-tetraamino-	Grey	0.00
			Blue	0.29
			Violet	0.58
			Blue	0.83
64505	Celliton Blue 3G	Anthraquinone, 1,4,5,8-tetraamino-, methylated	Bluish violet	0.00
			Blue	0.07

* Spot hardly visible.

the R_F value is dependent on the number of amino groups in the nucleus and decreases proportionally with their number. Hydroxyethanolamino derivatives possess a higher R_F value than the amino derivatives.

(f) Methine derivatives possess a lower R_F value than nitro dyes and dyes of the arylaminoquinone and aminonaphthalimide groups.

(g) The position and character of the substituents in aromatic nuclei considerably influence the R_F value. With compounds of the same fundamental skeleton and of a different character of substituents, dyes with smaller dipole moments show a higher R_F value.

SUMMARY

Disperse dyes have been separated, using two aqueous pyridine eluents. The relation between dye constitution and chromatographic behaviour has been examined.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART I. THE HYDROLYSIS OF TRIPHOSPHONITRILIC CHLORIDE

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The hydrolysis of triphosphonitrilic chloride $(PNCl_2)_3$ can be conveniently separated into two distinct stages:

(1) The hydrolysis of triphosphonitrilic chloride in neutral or alkaline solution to trimetaphosphimic acid $[PN(OH)_2]_3$[1–5].

(2) The hydrolysis of trimetaphosphimic acid in acid solution through a variety of phosphorus-nitrogen containing acids to orthophosphate and ammonia[6–8].

The reaction (1) is slow, and stepwise substitution of chlorine atoms by hydroxyl groups occurs. The partially substituted intermediates, of which five are theoretically possible, are called chlorohydrines. STOKES[9,10] prepared a mixture of chlorohydrines containing largely the dihydroxy-tetrachlorohydrine, but was unable to determine whether or not substitution occurred in pairs on the same phosphorus atoms. The solid triphosphonitrilic chloride is unreactive with water, and this lack of reactivity is probably due to their insolubility in water. By use of ethereal solutions of triphosphonitrilic chloride it can be brought into intimate contact with water.

A mixture of chlorohydrines was prepared by interrupting the hydrolysis of triphosphonitrilic chloride after a definite time. STOKES[9] reported the preparation of the tetrachlorohydrine $[P_3N_3Cl_4(OH)_2]$ by stopping the hydrolysis after 6–8 h, but was unable to separate it from small quantities of other chlorohydrines.

This communication is the first of a series of investigations concerning the hydrolysis of the phosphonitrilic halides. The aims of the research were:

(1) To study the hydrolysis of triphosphonitrilic chloride under neutral conditions, when trimetaphosphimic acid is the final product, and postulate the order in which the chlorine atoms are replaced by hydroxyl groups (Part I).

(2) To determine the course of the acid hydrolysis of the trimetaphosphimate ion, propose mechanisms for all stages, and determine the rates of the hydrolysis of the ring compounds (Part II).

(3) To study aspects of the hydrolysis of higher chlorides in view of the results obtained for the trimer (Parts III, IV, V).

EXPERIMENTAL

Preparation of intermediate chlorohydrines

Triphosphonitrilic chloride (30 g) was dissolved in diethyl ether (300 ml) and the

solution agitated with water (100 ml) for 6 h. The aqueous layer then contained hydrochloric acid and trimetaphosphimic acid, while the diethyl ether contained unchanged triphosphonitrilic chloride and a mixture of chlorohydrines.

The diethyl ether was separated and dried over calcium chloride, overnight. The greatest part of the diethyl ether was then distilled off on a water bath, and the final residues by blowing a current of hot dry air over the product. The unreacted phosphonitrilic chloride was removed by washing with a small volume of benzene in which the chlorohydrines are relatively insoluble, followed by a washing with carbon disulphide. The product which was obtained in about 5 % yield, due to continuous conversion of the chlorohydrine to the metaphosphimic acid, was dried over silica gel in a vacuum desiccator.

Preparation A. Found: N, 13.6; P, 29.8; Cl, 42.0. Calculated for $P_3N_3Cl_4(OH)_2$: N, 13.5; P, 29.9; Cl, 45.6.

A similar preparation was carried out but the hydrolysis was allowed to proceed for 50 h before isolation of the chlorohydrine.

Preparation B. Found: N, 14.6; P, 30.8; Cl, 30.4. Calculated for $P_3N_3Cl_2(OH)_4$: N, 15.3; P, 33.9; Cl, 25.9.

Both products were unstable in a moist atmosphere, first absorbing water and becoming sticky solids (releasing hydrogen chloride), and later hardening to brittle solids.

Preparation of sodium trimetaphosphimate[6]

Trimeric phosphonitrilic chloride (15 g) was dissolved in diethyl ether (75 ml), and this solution was shaken with a solution of sodium acetate (55 g) in water (100 ml) for 94 h.

Crystals of sodium trimetaphosphimate began to crystallise from the aqueous layer after about 15 h, but at least 80 h were required for complete decomposition of any chlorohydrines formed as intermediate products.

The sodium salt of the acid was filtered off in almost quantitative yield (the product being insoluble in the relatively concentrated sodium chloride solution), washed with 50 % v/v aqueous ethanol and finally with 96 % ethanol, followed by drying in a vacuum desiccator.

Found: N, 10.9; P, 24.6; H_2O, 19.6. Calculated for $Na_3P_3(NH)_3O_6 \cdot 4H_2O$: N, 11.0; P, 24.8; H_2O, 20.0.

Paper chromatography of the chlorohydrines

Paper chromatographic separations of the chlorohydrine reaction products were largely unsuccessful because of a large amount of tailing; probably resulting from the hydrolysis of the species during the elution of the chromatogram.

The technique used was descending elution, with BIBERACHER's basic solvent[11] for 13 h at 18° using Whatman No. 541 filter paper in the conventional all-glass apparatus[12].

The position of a particular species on a paper chromatogram is usually recorded by its R_F value, which is defined as the ratio of distance moved by the species to the distance moved by the solvent front. But in cases where the solvent is eluted off the lower edge of the chromatogram, we recorded the R_x value which we define as the ratio of the distance moved by the species to the distance moved by orthophosphate.

Under the normal standard conditions[13] of elution, the R_x values are subject to deviate from the values given below by $\pm$ 5 %. When large quantities of some cation or anion other than a phosphate species occur in a position close to a phosphate species, the spot is "pushed" from its original position and may have an R_x value outside the $\pm$ 5 % limit. This is easily recognised, however, since the spot usually assumes a crescent shape around the spot of the interfering ion.

BIBERACHER's basic solvent gave chromatograms of the chlorohydrines which showed three quite distinct spots with R_x values 2.45, 1.20 and 0.54. The spot at R_x 1.20 is due to trimetaphosphimate, but with no very pure samples of chlorohydrines available, the identity of the other spots can only be suggested.

Preparation A gave a large spot at R_x value 2.45 and a smaller spot at R_x 1.20 with tailing in between. The analysis shows the composition of the chlorohydrine to approximate to $P_3N_3Cl_4(OH)_2$ and this makes it highly probable that the spot with R_x value 2.45 is due to the tetrachlorohydrine.

Preparation B showed only a trace with R_x value 2.45, but spots at R_x values 1.20 and 0.54. By similar reasoning to the above, it was highly probable that the spot at R_x 0.54 was due to the dichlorohydrine $P_3N_3Cl_2(OH)_4$.

The trimetaphosphimate found in each case is formed by hydrolysis of the chlorohydrines, as it is unlikely to be obtained in preparations where the chlorohydrines are recovered from the ethereal layer. Sodium trimetaphosphimate is insoluble in diethyl ether.

Ion-exchange chromatography of the chlorohydrines

Further evidence to support the postulations as to the identity of the spots on the paper chromatograms, was added when preparations A and B were subjected to ion-exchange separations. Ion-exchange chromatographic separations of phosphate mixtures were effected by a modification of the gradient elution method of GRANDE AND BEUKENKAMP[14], and the technique will be described fully in Part II of this series[15].

The elution patterns for Preparation A and Preparation B are shown in Fig. 1.

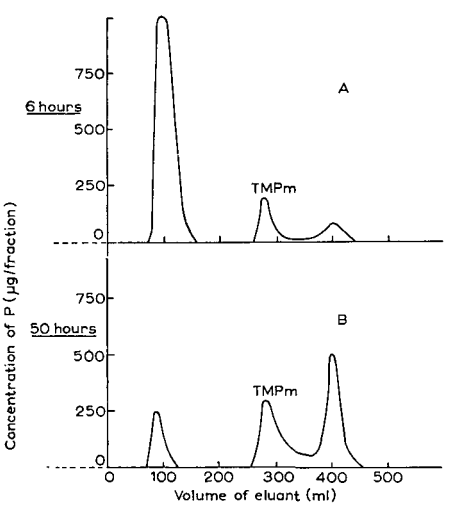


Fig. 1. Elution behaviour of the chlorohydrine preparations. (A) 6 h preparation; (B) 50 h preparation.

For Preparation A, about 85 % of the total phosphorus occurred in the species whose peak was fraction 9, and was assigned to the tetrachlorohydrine $P_3N_3Cl_4(OH)_2$. The small peak with maximum at fraction 27 was the trimetaphosphimate peak, whilst the very small peak at fraction 40 was assigned to the dichlorohydrine.

Preparation B also gave 3 peaks, but the concentration of phosphorus in the peak at fraction 9 was very much smaller amounting to only about 10 % of the total phosphorus. The size of the fractions with peaks at fractions 27 and 40 had increased, and contained about 30 % and 60 % of the total phosphorus respectively. Notice also the tail forward of the species with peak at fraction 40 towards the trimetaphospimate peak. Fraction 40 was assigned to the dichlorohydrine.

No absolute proof as to the identities of the peaks at fractions 9 and 40 was obtained since all attempted methods of isolation yielded products contaminated with large amounts of trimetaphosphimate. From the difference in elution patterns of Preparations A and B coupled with the analysis figures for the products, and for the tendency of hydroxyl groups to substitution in pairs (similarly for ammonia and fluorine), we are confident that the peaks have been correctly assigned.

The patterns are definitely not due to any condensed phosphate or imidophosphate species, as paper chromatography in a number of solvents has shown such species to be absent and they are not formed from chlorohydrines under the conditions of elution (see Part II).

Infra-red spectra of the chlorohydrines

The infra-red spectra of the two preparations were recorded (in potassium bromide discs) and whilst showing certain differences, they were not diagnostic. They are listed in Table I, together with those of triphosphonitrilic chloride and sodium trimetaphosphimate tetrahydrate. The position of the peak is given by the frequency in cm^{-1}, and the intensity of the absorption is indicated by s = strong, m = medium, w = weak, while the shape of the peak is indicated by b = broad, n = narrow, sh = shoulder. Spurious peaks, due to moisture absorbed by potassium bromide are indicated by an asterisk.

The P–Cl vibrations in the region 400–700 cm^{-1} did not give any well-defined narrow peaks, but the absorption in decreasing intensity was $(PNCl_2)_3$, Preparation A, Preparation B. This can be seen in the 550 cm^{-1} line for $(PNCl_2)_3$ which was strong, until it disappeared completely in sodium trimetaphosphimate. A showed strongest absorption at 1232 cm^{-1}, rather close to the P–N stretching vibration of the parent chloride at 1256 and 1222 cm^{-1}, whilst B showed only medium absorption in the region 1210–1240 cm^{-1}. The other noticeable trend is the much larger peak at 1410 cm^{-1} in B than in A, its complete absence in $(PNCl_2)_3$, and its appearance at 1360 cm^{-1} in sodium trimetaphosphimate, and these may be the asymmetric stretching frequency of:

whilst the 950 cm^{-1} peak in A, 960 cm^{-1} in B, and 935 cm^{-1} in sodium trimetaphosphimate may be the symmetrical stretching frequency of the above grouping.

A paper chromatographic study of the hydrolysis

Triphosphonitrilic chloride (2 g) was dissolved in diethyl ether (20 ml) and the solution

TABLE I

INFRA-RED ABSORPTION FREQUENCIES OF THE CHLOROHYDRINES

Triphosphonitrilic chloride	*Preparation A*	*Sodium trimetaphosphimate*	*Preparation B*
*3450 m	*3440 s	*3420 s	*3420 s
2960 w	2950 wsh	3180 msh	3220 ssh
2370 vw	—	—	2350 w
2110 vw	—	—	—
*2030 wn	—	—	—
1973 vw	—	—	—
1876 vw	—	—	—
1745 vwsh	—	1690 msh	—
*1631 vb	*1640 mb	*1642 mb	*1640 mb
1372 wsh	1410 w	1360 m	1410 m
1317 msh	1287 ssh	1330 m	—
—	—	1286 m	—
1256 ssh	1232 s	—	1242 m
1222 vsb	—	1200 s	1210 m
1000 wsh	950 mb	1121 m	1018 mb
—	—	965 s	960 mb
—	—	935 s	—
875 mn	870 vw	860 w	—
—	—	820 m	—
786 vw	—	805 m	—
—	—	738 m	732 m
675 wsh	640 w	—	—
625 s	595 m	—	—
550 s	532 m	—	535 w
—	—	—	505 w
410 s	470 m	—	445 vw
—	382 w	—	385 w

agitated with water (7 ml). Samples were removed from both the aqueous and ethereal layers at intervals and chromatographed using BIBERACHER's basic solvent[11]. Samples from the aqueous layer gave well defined spots, but a great deal of streaking and tailing occurred with the samples from the ethereal layers for the reasons discussed above. However, the course of the reaction is given in Table II.

Interpretation of the chromatograms

The chlorohydrines formed as intermediates are extremely soluble in ether and were found with the phosphonitrilic chloride. The aqueous layer contained trimetaphosphimic acid and hydrochloric acid formed during the hydrolysis. In the presence of such acid, the trimetaphosphimic acid was further hydrolysed, accounting for the presence of ortho-, trimeta-, diimido-trimetaphosphate, and chain phosphates. (This decomposition is discussed in Part II.)

The identification of species present in the ethereal layer was made difficult by the tailing of the triphosphonitrilic chloride.

DISCUSSION

The exact location of the substituted hydroxyl groups in the chlorohydrines has not been discovered, but the formation of large amounts of the di-substituted and tetra-substituted compounds generally, with very little, if any, of the mono-, tri- and penta-sub-

TABLE II

PHOSPHATE SPECIES DETECTED DURING THE HETEROGENEOUS HYDROLYSIS OF TRIPHOSPHONITRILIC CHLORIDE

Time(h)	*Aqueous layer*		*Ether layer*	
	Species	R_x *value*	*Species*	R_x *value*
0	None	—	$(PNCl_2)_3$	1.5–3.5 (streak)
3	Ortho (trace)	1.00	TMPm (trace)	1.20
	TMPm	1.20	$(PNCl_2)_3$	1.5–3.5 (streak)
9	Chain phosphates	0.45	TMPm (trace)	1.20
	Ortho	1.00	$P_3N_3Cl_4(OH)_2$ (trace)	2.50
	TMPm	1.20	$(PNCl_2)_3$	3.10 (tailing)
24	Chain phosphates	0.45	$P_3N_3Cl_2(OH)_4$ (trace)	0.55
	Ortho	1.00	TMPm (trace)	1.20
	TMPm	1.20	$P_3N_3Cl_4(OH)_2$	2.50
	DITMP (trace)	1.45	$(PNCl_2)_3$	3.10
48	Same species as 24 h			
72	Same species as 48 h			
100	Chain phosphates	0.45	$P_3N_3Cl_2(OH)_4$	0.55
	Ortho	1.00	TMPm (trace)	1.20
	TMPm	1.20	$P_3N_3Cl_4(OH)_2$(trace)	2.50
	DITMP	1.45	$(PNCl_2)_3$ (trace)	3.10
	TMP (trace)	2.40		

Abbreviations: TMPm = trimetaphosphimate; DITMP = diimidotrimetaphosphate; TMP = trimetaphosphate.

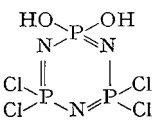
stituted compounds indicates that substitution probably occurs in pairs. Whether substitution of a pair of chlorine atoms attached to the same phosphorus atom, or two chlorine atoms attached to different phosphorus atoms occurs, has been argued for substitution of numerous types of groups[16–18], and it is now obvious that the order of substitution depends upon the nature of the group being substituted. BECKE-GOEHRING *et al.*[17] suggested that strongly nucleophilic reagents attacked the trimer in the 2- or 4-position, with further substitution in the 6-position, but with weak electron donors, substitution occurred in another sequence. Proton magnetic resonance[19] has recently shown that with dimethylamine, substitution occurs in the order 2-mono; 2,4-di; 2,4,6-tri; 2,2,4,6-tetra; 2,2,4,4,6-penta and 2,2,4,4,6,6-hexa.

However, HEFFERNAN AND WHITE[20] have shown from nuclear magnetic resonance data, that when chlorine atoms are replaced by fluorine atoms, substitution of first two atoms occurs on the same phosphorus atom. They also showed that in the more rare trisubstituted compound, two of the substituted fluorine atoms reside on one phosphorus atom and the third fluorine atom on one of the neighbouring phosphorus atoms, the third phosphorus still retaining two chlorine atoms. Whilst it is also possible that steric effects must be taken into account, and that pairwise substitution of dimethylamine on the same phosphorus atom does not occur because of the steric strain which would be caused by substituting two large groups on one atom.

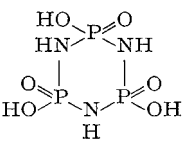
Some relationship usually exists between structure and R_x value when a series of related compounds are considered, and in the same way some relationship usually

exists between structure and retention volume in ion-exchange chromatography. In both these separational techniques, trimetaphosphimate appeared between the spots or peaks assigned to $P_3N_3Cl_4(OH)_2$ and $P_3N_3Cl_2(OH)_4$. At first sight it appears that no correlation exists, but if we consider the number of replaceable hydrogen atoms, we see that it rises from two for $P_3N_3Cl_4(OH)_2$, to three for $P_3(NH)_3O_3(OH)_3$ to four for $P_3N_3Cl_2(OH)_4$. (Trimetaphosphimic acid is tribasic, only 3 of its 6 hydrogen atoms being replaceable by sodium atoms.) Triphosphonitrilic chloride, whilst not chromatographing satisfactorily shows a centre of gravity of the spot at an R_x value of 3.1, which fits satisfactorily with above correlation.

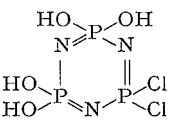
Water is a nucleophilic reagent because of its unshared electron pair, but only a weak one, and because of this, substitution of the hydroxyl groups in pairs takes place. However, nuclear magnetic resonance measurements are needed to prove whether this is possible, and the following structures are proposed:



Tetrachlorohydrine
2 replaceable H's



Trimetaphosphimate
3 replaceable H's



Dichlorohydrine
4 replaceable H's

This work has shown that paper chromatography and ion-exchange chromatography may be used to study the chlorohydrines, but other techniques are needed to confirm these results. Raman spectroscopy should be a useful technique in following the formation of intermediates during the hydrolysis of the polymeric chlorides. A study of the hydrolysis of the phosphonitrilic fluorides and bromides to the corresponding metaphosphimic acids through the analogous intermediate fluoro- or bromohydrines may prove more successful. Both of these suggestions are under active study in these laboratories, and the results will be published later.

ACKNOWLEDGEMENTS

One of the authors (R.W.W.) wishes to acknowledge financial help from the Albright & Wilson (Mfg.) Co. Ltd., for the tenure of a Maintenance Grant. We are indebted to the Albright & Wilson (Mfg.) Co. Ltd., for generous supplies of the polymeric phosphonitrilic chlorides and to the Oldbury Research Staff of that company for valuable advice and discussions, and our thanks are extended throughout this series of papers.

SUMMARY

The paper describes a paper- and anion-exchange chromatographic investigation of the heterogeneous hydrolysis of triphosphonitrilic chloride under neutral conditions, when trimetaphosphimic acid is the final product. Evidence concerning the order in which the chlorine atoms are replaced by hydroxyl groups is also given.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART II. THE HYDROLYSIS OF SODIUM TRIMETAPHOSPHIMATE

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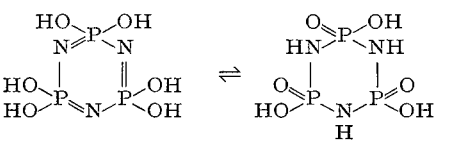
(Received May 3rd, 1962)

GLADSTONE[1–5] obtained an acid which analysed to $P_2N_2O_5H_6$, by the action of water on an ethereal solution of trimeric phosphonitrilic chloride, and which he regarded as the diamide of pyrophosphoric acid. STOKES[6] interpreted GLADSTONE'S results in another way, and pointed out that two molecules of trimetaphosphimic acid (TMPm) with three molecules of water has some empirical formula of $P_2N_2O_5H_6$. The trimetaphosphimic acid is rapidly hydrolysed by the hydrochloric acid formed in the reaction, but alkali metal salts of the metaphosphimic acid are easily prepared by agitation of an ethereal solution of the chloride with an alkali metal acetate or base.

Three atoms of hydrogen are replaceable by alkali metals, while silver is able to replace either 3 or 6 hydrogen atoms. Two tautomeric forms of TMPm are possible:



(a) (b)

STOKES[6] favoured formula (b), and this has been confirmed by potentiometric titration and infra-red spectroscopy[7].

Trimetaphosphimic acid is unstable, but its salts are stable in neutral or alkaline conditions. Treatment in strong acid, however, causes hydrolysis to proceed, the final products being orthophosphate and ammonia[6].

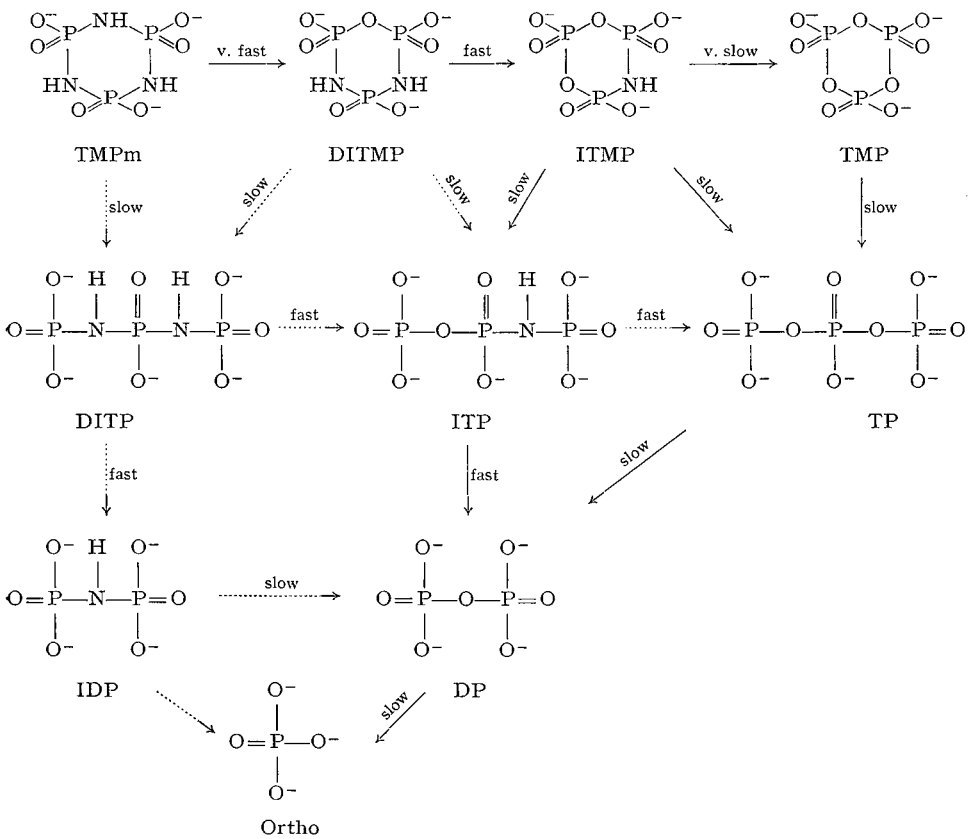
When the reaction is limited, by the choice of suitable conditions, STOKES' analysis[6] of the mixture indicated the presence of:

(i) Unchanged trimetaphosphimic acid $P_3N_3O_6H_6$ (TMPm)
(ii) Diimidotriphosphoric acid $P_3N_2O_8H_7$ (DITP)
(iii) Imidodiphosphoric acid $P_2NO_6H_5$ (IDP)
(iv) Pyrophosphoric acid $P_2O_7H_4$ (DP)
(v) Orthophosphoric acid PO_4H_3 (Ortho)
(vi) Ammonium ions (NH_4^+).

Little further work was carried out on the hydrolysis until NARATH, LOHMAN AND QUIMBY[8,9] found that acid hydrolysis proceeds largely through intermediate

ring compounds with one, two and three oxygen atoms successively replacing the original imide linkages. Possible paths of the decomposition suggested by these workers are outlined below, the main sequence following the solid arrows.

It is interesting to note that the mechanism proposed by STOKES[6] involved the scheme concerned with the broken arrow sequence of reactions. STOKES[6] isolated the first intermediate and DE FICQUELMONT[10] prepared it by a different method, both reporting it to be the chain diimidotriphosphate (DITP) instead of DITMP.



Abbreviation	*Name of acid species*
TMPm	Trimetaphosphimate
DITMP	Diimidotrimetaphosphate
ITMP	Imidotrimetaphosphate
TMP	Trimetaphosphate
DITP	Diimidotriphosphate
ITP	Monimidotriphosphate
TP	Triphosphate
IDP	Imidodiphosphate
DP	Diphosphate
Ortho	Orthophosphate

NARATH *et al.*[9], however, found on repetition of either preparation, only ring products, predominantly DITMP.

This communication describes the hydrolysis of trimetaphosphimate in acid solution through a variety of phosphorus-nitrogen containing acids, finally to orthophosphate and ammonia. QUIMBY, NARATH AND LOHMAN[9] have described the hydrolysis of each of the species included in their scheme for the decomposition of TMPm, and suggested mechanisms for the hydrolysis. The results of this study confirm in detail their results. This work provided a "proving ground" for the paper chromatographic and ion-exchange procedures which were used to study the hydrolysis of the higher polymeric phosphonitrilic chlorides of which we have observations up to the octamer.

A paper chromatographic study of the hydrolysis of sodium trimetaphosphimate

The sodium trimetaphosphimate was prepared by hydrolysis of triphosphonitrilic chloride[6].

The hydrolysis was carried out under exactly the same conditions as those of QUIMBY, NARATH AND LOHMAN[9], namely 60° and in hydrochloric acid, sodium acetate buffered solution at a pH 3.6. The hydrolysis was allowed to proceed for about three weeks and samples were chromatographed in BIBERACHER'S[11] basic, QUIMBY'S neutral[9] and GASSNER'S[12] acid solvents. Although this was carried out as a qualitative study, but using standardised techniques[13], the approximate percentage of total phosphorus present as each species is estimated from the relative size and intensity of the spots (especially with chromatograms eluted with BIBERACHER'S[11] solvent) and shown in Table I.

TABLE I

Time (h)	*TMP* $R_x = 2.40$	*ITMP* $R_x = 1.80$	*DITMP* $R_x = 1.45$	*TMPm* $R_x = 1.20$	*Ortho* $R_x = 1.00$	*Chain phosphates* $R_x = 0.45$
0	—	—	—	100	—	—
0.25	—	—	25	75	—	—
1	—	—	50	50	—	—
2	—	2	64	34	—	—
4	—	8	80	10	2	—
20	—	32	65	—	3	—
30	—	45	47	—	4	4
48	—	60	28	—	6	6
72	3	62	15	—	10	10
91	5	63	12	—	10	10
117	8	60	8	—	15	10
163	8	58	4	—	16	12
192	9	58	2	—	18	13
235	10	56	—	—	20	14
285	10	52	—	—	23	15
331	10	50	—	—	25	15
380	10	45	—	—	28	17
450	10	40	—	—	30	20

Samples of the hydrolysates were chromatographed in the acid and neutral solvents, and in each case they confirmed the results of the BIBERACHER'S chromatograms. The neutral chromatograms showed the development of the ring imidophosphates which were detected. These species, together with their R_x values are shown in

Table II, but of course, species reported as orthophosphate could be mixtures of orthophosphate and chain phosphates.

Samples chromatographed in GASSNER's acid solvent showed increasing amounts of orthophosphate from 4 h to 30 h, and a trace of pyrophosphate after 20 h.

TABLE II

Time(h)	*Species present**
0	TMPm (0.21) only
2	TMPm (0.21) [30] + DITMP (0.30) [60] + trace Ortho (1.00)
4	TMPm (0.21) [10] + DITMP (0.30) [80] + ITMP (0.50) [5] + Ortho (1.0) [5]
20	DITMP (0.30) [60] + ITMP (0.50) [30] + Ortho (1.00) [10]
48	DITMP (0.30) [50] + ITMP (0.50) [25] + Ortho (1.00) [25]

* R_x values are given in parentheses; relative amounts in square brackets.

The hydrolysis of TMPm at 60° at pH 10.0

Sodium trimetaphosphimate was dissolved in a sodium bicarbonate–sodium hydroxide buffer pH 10.0 previously heated to 60°. Samples were removed at intervals to 170 h, eluted in the three chromatographic solvents already mentioned.

No hydrolysis products were obtained during the study, showing the inert nature of the P–N–P bonds in alkaline solution[9].

A paper chromatographic study of the hydrolysis of sodium diimidotrimetaphosphate

Sodium diimidotrimetaphosphate was prepared by two methods[6,10] both of which had previously, but erroneously been described as preparations of sodium diimidotriphosphate.

Found: P, 28.8; N, 8.6. Calculated for $Na_3P_3(NH)_2O_7 \cdot H_2O$: P, 28.9; N, 8.7.

Sodium diimidotrimetaphosphate was dissolved in sodium acetate–hydrochloric acid buffer pH 3.6 at 60°. Samples were removed at intervals and chromatographed in BIBERACHER's basic solvent. An estimation of the approximate percentage of total phosphorus present as each species is given in Table III.

TABLE III

Time (h)	*TMP* $R_x = 2.40$	*ITMP* $R_x = 1.80$	*DITMP* $R_x = 1.45$	*Ortho* $R_x = 1.00$	*Chain species* $R_x = 0.45$
0	—	—	100	—	—
0.25	—	10	90	—	—
1	—	20	80	—	—
2	—	30	70	—	—
4	—	35	65	—	—
20	—	45	50	trace	5
48	—	50	35	5	10
72	5	55	20	10	10
91	5	60	15	10	10
117	7	60	10	12	12
163	9	55	5	15	15
235	10	55	3	17	15
331	12	48	—	25	15
432	13	40	—	30	17

The initial stage of this hydrolysis, DITMP to ITMP was much slower than the stage TMPm to DITMP, the half life of DITMP being about 25 h. Orthophosphate again appeared before TMP in the hydrolysis products.

The hydrolysis of DITMP at 60° and pH 10.0

Sodium diimidotrimetaphosphate was dissolved in a sodium bicarbonate–sodium hydroxide buffer pH 10.0 previously heated to 60°. Samples were removed at intervals and eluted in all the three chromatographic solvents.

The species on all the chromatograms had low R_x values caused by the excess of sodium ions in the buffer solution, making identification of most species impossible. However, from the basic and neutral solvents it was possible to say that no ITMP or TMP were formed, but that chain phosphates, the identities of which could not definitely be determined, were formed. Orthophosphate was detected after about 120 h.

It was concluded that alkaline hydrolysis of DITMP occurs by a different path to the acid hydrolysis, and probably yields chain imidophosphates very slowly through fission of the P–O–P linkage; further work is to be published[14].

An ion-exchange study of the hydrolysis of TMPm at 60° and pH 3.6

BEUKENKAMP, RIEMAN AND LINDENBAUM[15] showed the possibilities of anion exchange chromatography in the separation and quantitative analysis of phosphate mixtures, together with theoretical equations to describe elution characteristics. Later[16], the theory was developed to permit calculation of the positions of elution maxima when eluants of different concentrations were passed successively through the column. A number of advantages, especially those of time and accuracy were introduced by GRANDE AND BEUKENKAMP[17] when they published details of gradient elution technique for the separation of the lower condensed phosphates. The chromatographic separations of the imidophosphates formed in these hydrolyses were effected by a modification of the gradient elution method.

The apparatus used was exactly similar to that described by GRANDE AND BEUKENKAMP. An ion-exchange column, 19 cm long and 2 cm diameter contained in a glass tube fitted with a tap, was used. The column contained 25 g of Amberlite CG-400 resin (100–200 B.S.S. mesh) in the chloride form. It was found that resins from different production batches may differ in the sharpness of peaks obtained, probably due to minor changes in cross-linkage or particle size. The eluant solution for the mixing bottle was 1 l of 0.10 *M* potassium chloride solution buffered to pH 5.0 with a potassium acetate–acetic acid buffer, and the eluant solution for the reservoir was 1.00 *M* potassium chloride solution, also buffered to pH 5.0 with acetate buffer. After elution, the phosphorus was determined by the phosphovanadomolybdate method[18], because of the lack of interference of other ions and the stability of the complex when compared to the more sensitive molybdenum blue methods[19,20].

The retention volumes of the lower condensed phosphates and the imidophosphates were found, and are given in Table IV. In each case a 5 ml solution of the phosphate containing 200–400 μg of P/ml was used.

All the trimeric ring imidophosphate preparations gave single sharp elution peaks, and showed the presence of little or no impurities, but the chain imidophosphates partially decomposed under the conditions of hydrolysis.

TABLE IV

Species	Retention volumes on a 19 cm column	
	Preparation	Retention volume (ml)
Orthophosphate	A. & W.*	100
Pyrophosphate	A. & W.*	250
Triphosphate	A. & W.*	400
Trimetaphosphate	A. & W.*	640
Imidotrimetaphosphate	Ref. 22	450
Diimidotrimetaphosphate	Refs. 6, 10	360
Trimetaphosphimate	Ref. 6	270
Imidodiphosphate	Ref. 23	190**
Diimidotriphosphate	Ref. 24	280**

* Albright & Wilson.
** Partially decomposed under conditions of elution.

From Table III, a quantitative separation of the phosphate species formed in the reaction is possible.

Sodium trimetaphosphimate was hydrolysed at 60° and pH 3.6 in sodium acetate–hydrochloric acid buffer. 5 ml samples were removed at intervals and subjected to ion-exchange separations. The types of separations obtained are given in Fig. 1.

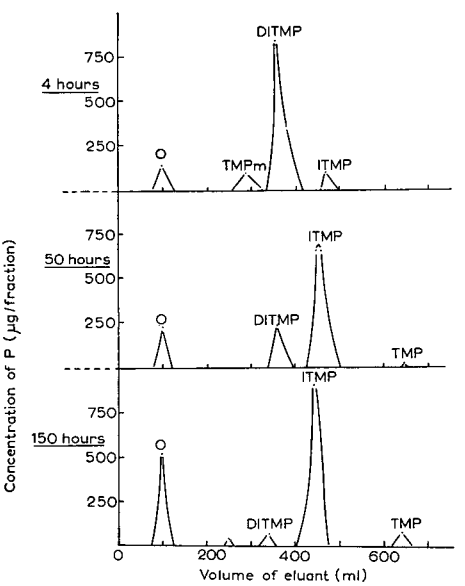
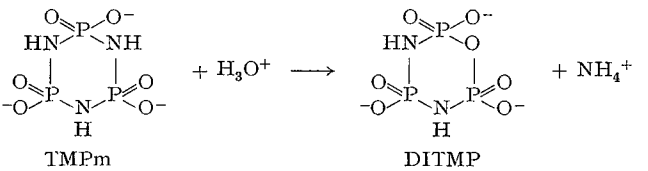


Fig. 1. Elution patterns for the hydrolysis of trimetaphosphimate at pH 3.6 and 60°.

The elution curves confirm the results of paper chromatographic study, except their failure to find any chain phosphate species except pyrophosphate. These quantitative results emphasise the stability of ITMP under these conditions.

Comparison of the rates of hydrolysis of TMPm, DITMP and ITMP

The first stages in the acid hydrolysis of TMPm, DITMP and ITMP all involve the elimination of one molecule of ammonia, whether the mechanism involves a ring or chain intermediate, *e.g.*



TMPm DITMP

The hydrolyses studied by paper and ion-exchange chromatography, were carried out in buffer solutions where the liberated ammonia is absorbed by the buffer without affecting the pH of the solution. However, in unbuffered solutions the liberated ammonia causes the pH to rise, and initially this concentration of ammonia is proportional to the concentration of the imidophosphate species which has been hydrolysed.

Two methods were employed to detect the ammonia being liberated:

(1) A solution of the imidophosphate at 60° was adjusted to pH 3.6 by addition of hydrochloric acid. The hydrolysis was allowed to continue while the pH of the solution was recorded by a potentiometric recorder connected to a conventional recording pH meter. The velocity constants were not calculated from the results due to the non-linear relationship between pH and the amount of ammonia liberated. The relative rates of the hydrolyses are shown in Fig. 2, but the other difficulty is that as the pH rises, the slower the rate of hydrolysis becomes.

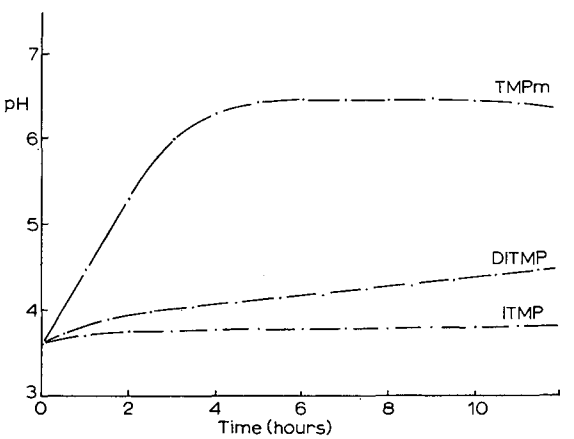


Fig. 2. Rate of change of pH for the hydrolysis of trimeric ring imidophosphates at pH 3.6 and 60°.

(2) A solution of the imidophosphate at 60° was adjusted to pH 3.6 by addition of dilute hydrochloric acid from an E.I.L. Model 24, automatic titration unit. The instrument was then set to adjust the pH to 3.6 by addition of more dilute acid, immediately the pH rose above the detection limits of the apparatus, about $\pm$ 0.20 pH

units. The volume of acid added was proportional to the amount of ammonia liberated in the hydrolysis, especially over the initial stages of the reaction (see Fig. 3). Difficulty was encountered with the hydrolysis of ITMP because the formation of

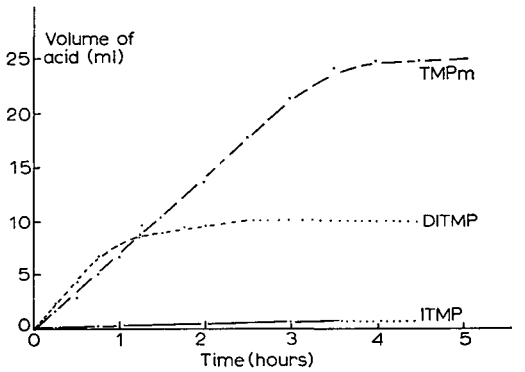


Fig. 3. Rates of hydrolysis of trimeric ring imidophosphates at pH 3.6 and 60°.

orthophosphate (from the decomposition of the imido chain phosphates) caused the pH to drop. Approximate hydrolysis rates were calculated for the initial stages of each reaction, assuming first order reaction kinetics, because of constant pH.

Species	*Reaction rate* (min^{-1})
TMPm	$8 \cdot 10^{-3}$
DITMP	$6 \cdot 10^{-3}$
ITMP	$7 \cdot 10^{-4}$

This work was not meant to provide an accurate study, but to determine approximate reaction rates. The assumption made was that little orthophosphate is formed over the initial stages of the reaction, and hence does not affect the pH of the solution. All these rates are now being carefully checked by quantitative ion-exchange procedures[14].

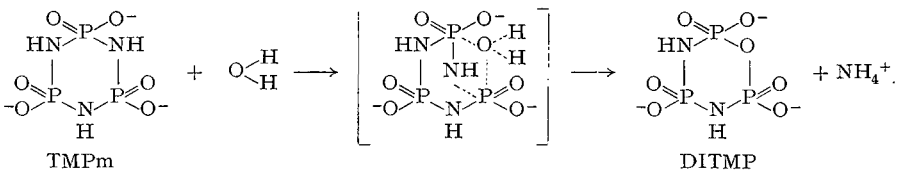
The mechanism for the hydrolysis of trimetaphosphimate

Paper chromatography and ion-exchange chromatography, together with elemental analysis and pH titrations of isolated intermediates, showed that the hydrolysis of TMPm proceeded through ring imidophosphates as reported by QUIMBY, NARATH AND LOHMAN[9]. No chain imidophosphates were detected, but it is possible that small amounts were formed which were hydrolysed very rapidly to orthophosphate. However, failure to detect orthophosphate over the initial stages of the reaction make this unlikely. Two mechanisms can be proposed for the hydrolysis[9]: one involves a chain amidoimidophosphate intermediate, and the other a complex ring intermediate; the former is the one favoured.

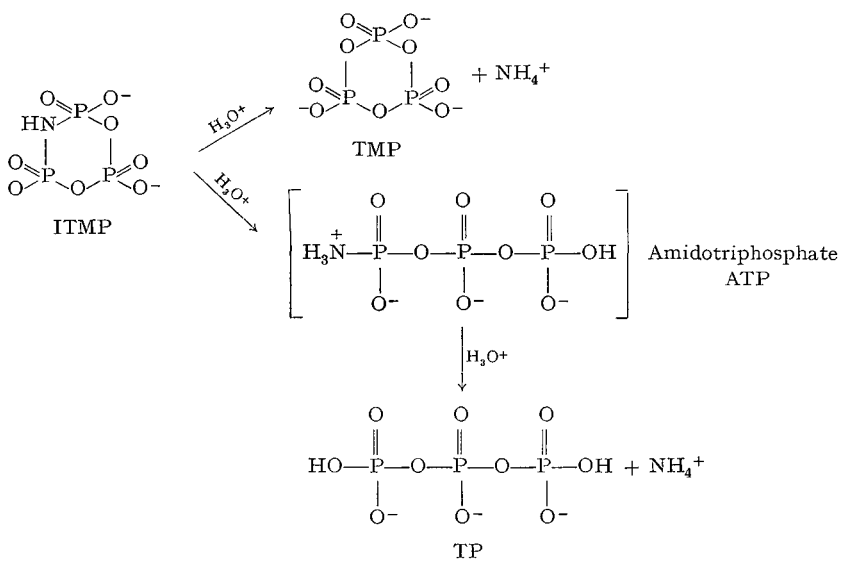
Both these mechanisms have been reconsidered in view of the results obtained from the hydrolysis of TMPm, higher metaphosphimic acids, and a study of molecular

models of the acids. It has been concluded that the actual mechanism probably lies somewhere between the two models, and that it involves the simultaneous breaking and reformation of bonds.

If we consider that a complex ring intermediate is formed, then the first stage of the hydrolysis can be represented as follows:

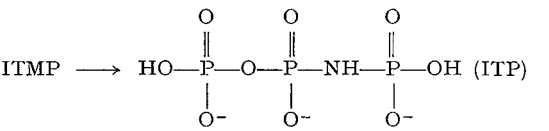


The ring intermediate which, momentarily, contains a four membered ring, could be formed by donation of electron pairs of the oxygen atom of an approaching water molecule into the empty $3d$-orbitals of the phosphorus atoms. DYATKINA AND SYRKIN[21] have discussed the interaction of electron pairs of one atom with vacant d-orbitals of another which occurs in phosphorus compounds and results in the formation of supplementary bonds and increased bond strength. In phosphonitrilic halides, such interaction can occur between an unshared $2s$-pair of nitrogen and an empty $3d$-orbital of phosphorus, in addition to the sp^3d-electrons engaged in the formation of five bonds, and this situation also arises in the trimetaphosphimate anion. When we consider the approach of a water molecule competition arises between the $2s$- and $2p$-electron pairs of the oxygen atom of the water molecule and the $2s$-electron pair of the nitrogen for donation into the emty $3d$-phosphorus orbitals. If this tendency is greater for oxygen, the four membered ring complex will be formed. This would not be stable, it would decompose at the P–N bonds with the resultant formation of DITMP.



ITMP would be formed from DITMP and TMP from ITMP by an exactly similar mechanism. However, TMP is only formed in very small amounts from ITMP, and orthophosphate appears before TMP is detected. Thus ITMP must hydrolyse by two paths, one of which must involve a chain.

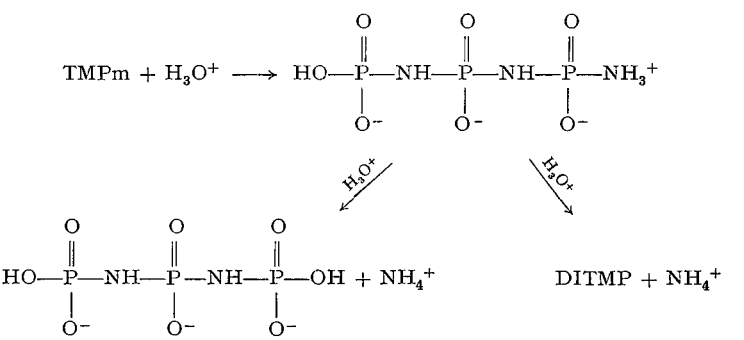
ITMP can either break at a P–N link, or at a P–O linkage, which in a chain form would give:



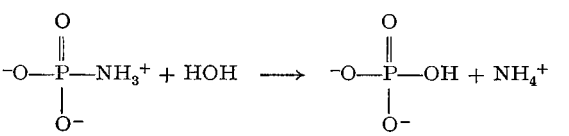
This would easily decompose[14] into pyrophosphate and orthophosphate at pH 3.6, and is most likely in view of the failure to detect triphosphate in the hydrolysis products.

But the strong argument against the complex ring mechanism is that the phosphorus atoms are too far apart for a P–O–P bond to be formed at the same time as a P–N–P bond in a ring compound, even taking into account the large size of the $3d$-orbitals of the phosphorus atom.

The principal argument against the chain mechanism is that chain imidophos phates are known to degrade rapidly to orthophosphate in acid solution. Only one chain intermediate can be formed from TMPm, monamidodiimidotriphosphate:

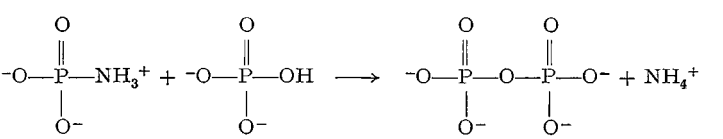


QUIMBY also assumes, and we confirm, that as no orthophosphate is formed immediately, only DITMP is formed. However, it has been shown that the hydrolysis of monamidophosphate which is very fast, yields about 95 % orthophosphate by the reaction:



(1)

whilst a condensation reaction (2) occurs to only 5 %:



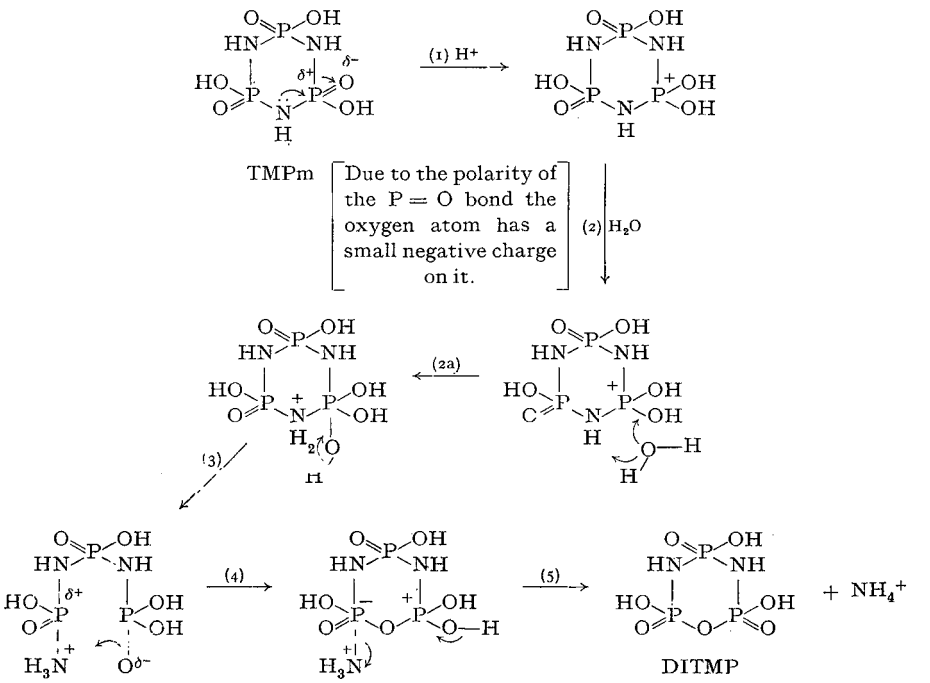
(2)

There appears no reason why the hydrolysis reaction (1) should be any less important than the condensation reaction (2), especially when long chain amidophosphates are considered, and which makes the chain mechanism seem improbable.

Having indicated the advantages and disadvantages of both mechanisms, we suggest a "ruptured ring" mechanism, incorporating ideas from both, in which bond rupture and reformation simultaneously occur.

The major steps in the hydrolysis involve: (1) protonation of a P atom, (2) approach of a water molecule, and (2a) bonding to the P atom, (3) rupture of the P–N bond, (4) formation of a P–O–P bond by electron transfer from the P–O bond on the adjacent P atom, (5) splitting out of the ammonium ion.

A detailed mechanism of the hydrolysis of TMPm to DITMP is given below.



The steps of the mechanism are thought to occur almost simultaneously so that immediately a P–O–N bond is broken, a P–O bond is formed. The mechanism proposes that a P–O bond is first formed by donation of a lone pair of the oxygen of the water molecule (into the empty *d*-orbital of the P atom) before a P–N bond breaks, another P–O bond is then formed, completing the ring before the second P–N bond breaks.

It is proposed that it is the P–O group on the P atom which is active in the reformation of the ring step due to its polarity, electrons being transferred from this bond to form a second P–O bond, with the adjacent P atom which has a small positive charge induced on it by the attacked $-NH_3^+$ group. This mechanism explains the stepwise substitution of oxygen linkages for imide linkages. Similar mechanisms are proposed for the hydrolysis of DITMP and ITMP. The rate of hydrolysis decreases going from TMPm to DITMP to ITMP as expected if the number of imide linkages

available for substitution are considered. ITMP must be hydrolysed by two paths, one yielding TMP by a mechanism similar to that for TMPm to DITMP, and the other yielding ortho- and pyrophosphates. The mechanism of this second path is thought to be attacked at one of the oxygen linkages of the ITMP resulting in cleavage of the ring and formation of ITP, which is immediately hydrolysed to ortho- and pyrophosphates.

Cleavage of a P–O–P linkage is more difficult than a P–N–P linkage. In TMPm, only P–N–P linkages are present and hydrolysis is rapid, DITMP has two imide and one oxygen linkage with the result that attack at the strong oxygen linkage is slight. However, when we come to ITMP, the number of oxygen to imide linkages is 2:1, and attack at oxygen linkages becomes important. The hydrolysis of ITMP to TMP is much slower than DITMP to ITMP due to reduction in imide linkages available for substitution. The fission to ortho- and pyrophosphate is also slow because of stability of P–O–P linkages.

As a general rule in this series, attack at a P–N–P bridge yields a ring compound, but attack at a P–O–P bridge yields a chain compound. Thus acid hydrolysis of DITMP yields ITMP whilst alkaline hydrolysis yields the chain DITP.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. F. W. McOmie for valuable discussion, and Drs. Quimby, Narath and Lohman for manuscripts of their papers before publication.

SUMMARY

A mechanism is proposed for the course of the limited hydrolysis of the trimetaphosphimate ion, using the techniques of paper and anion-exchange chromatography as methods of separating the complex series of products which is obtained. As a general rule, attack at a P–N–P linkage in a ring compound yields a ring compound containing a P–O–P linkage instead, whilst attack at a ring P–O–P linkage gives a chain polyphosphate. Thus acid hydrolysis of diimidotrimetaphosphate gives imidotrimetaphosphate, whereas alkaline hydrolysis produces diimidotripolyphosphate.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART III. THE HYDROLYSIS OF TETRAMETAPHOSPHIMIC ACID

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(Received May 19th, 1962)

The only work reported concerning the hydrolysis of the higher cyclic phosphonitrilic polymers and the metaphosphimic acids is that of STOKES[1-3]. The tetramer $(PNCl_2)_4$ is hydrolysed much more readily than the trimer to the very stable tetrametaphosphimic acid. No intermediate chlorohydrines, of which seven are theoretically possible have been isolated.

Tetrametaphosphimic acid is so stable[3] that long and vigorous action with acid is necessary for its hydrolysis, when orthophosphoric acid and ammonia are formed, and no intermediate hydrolysis products were found. STOKES[4] found that the higher the chloride in the series, the less stable it was to water when in ethereal solution. In the light of work by QUIMBY, NARATH AND LOHMAN[5] and POLLARD, NICKLESS AND WARRENDER[6] on trimetaphosphimate, it was decided that the hydrolysis of tetrametaphosphimate would be worthy of reinvestigation.

PREPARATION OF TETRAMETAPHOSPHIMIC ACID[3]

Tetrametaphosphimic acid is not attacked by cold dilute hydrochloric acid, and hence may be prepared by the action of water on ethereal solution of the chloride.

Tetrameric phosphonitrilic chloride (5 g) was dissolved in diethyl ether (75 ml) and agitated with water (25 ml) for two days. Crystals of the free acid began to separate out after about half an hour and after a few hours, a thick mass of crystals suspended in dilute hydrochloric acid was obtained.

When all the chloride had reacted, agitation was continued for a further few hours to decompose any intermediate chlorohydrines, before filtering off the acid and washing with ethanol. The acid was recrystallised from hot water.

Found: N, 15.9; P, 34.9; H_2O, 9.9.

Calc. for $[P{\cdot}N(OH)_2]_4{\cdot}2H_2O$: N, 15.9; P, 35.1; H_2O, 10.2.

PREPARATION OF AMMONIUM TETRAMETAPHOSPHIMATE[3]

Ammonium tetrametaphosphimate has been prepared by decomposing the tetrameric chloride with either ammonium hydroxide or ammonium acetate. Unlike the trimer, tetrameric phosphonitrilic chloride does not give a chloramide with ammonium

hydroxide. However, repetition of STOKES[3] work did not yield a pure ammonium salt, but a very viscous liquid containing a large proportion of orthophosphate.

Ammonium tetrametaphosphimate, however, was readily prepared by using ammonium acetate.

Tetrameric phosphonitrilic chloride (10 g) was dissolved in diethyl ether (200 ml) and the solution was agitated with a solution of ammonium acetate (40 g) in water (80 ml) for 3 days. Crystals of the ammonium salt began to separate out after a few hours. The product was filtered off, washed with ethanol, dissolved in dilute ammonium hydroxide and reprecipitated with ethanol.

Found: N, 24.1; P, 26.0; H_2O, 15.8.

Calc. for $(NH_4)_4P_4(NH)_4O_8 \cdot 4H_2O$; N, 23.9; P, 26.2; H_2O, 15.8.

Sodium tetrametaphosphimate can be prepared by using sodium acetate in place of ammonium acetate, but the sodium salt does not precipitate well with ethanol and tends to form an oil.

Tetrametaphosphimic acid (abbreviated TeMPm) is a tetrabasic acid, showing one inflection point (all four hydrogens are equivalent) at a pK value of 3.25.

The retention volume of TeMPm on an ion-exchange column under the conditions described in Part II of this series[6] was 300 ml.

DETERMINATION OF THE CONDITIONS SUITABLE FOR A STUDY OF THE HYDROLYSIS

Paper chromatographic study

Tetrametaphosphimic acid was chosen for the initial investigation of the hydrolysis, but due to difficulties encountered because of insolubility, and all subsequent hydrolyses were carried out on the neutral ammonium salt. However, even when this salt was used, some precipitation of the acid ammonium salt $(NH_4)_2P_4N_4O_8H_6$ occurred after the hydrolysis had commenced.

Suspensions of TeMPm (0.1 g) in 15 ml of buffer solution, or of acid, were kept at 60°, and samples were removed at intervals and chromatographed in BIBERACHER's basic solvent[7].

The acids were hydrolysed in sodium acetate–hydrochloric acid buffers of 3.6 and 1.0, and also in 1.0 N and 4.0 N hydrochloric acid and the results are shown in Table I.

TABLE I

Time (h)	*pH 3.6*	*pH 1.0*	*N HCl*	*4 N HCl*
2	Only TeMPm (R_x = 0.90) present			
4	Only TeMPm (R_x = 0.90) present			
20	Only TeMPm (R_x = 0.90) present			
40	Trace ortho	Trace ortho	Trace ortho	TeMPm
70	Ortho + spots at R_x's 1.80 and 2.40	Ortho only	Ortho only	TeMPm
122	Ortho + spots at R_x's 1.80 and 2.40	Ortho only	Ortho only	Ortho and TeMPm

Only orthophosphate was detected in the solutions treated with acid or the buffer solution of pH 1.0, but the solution at pH 3.6 was found to contain species with R_x values corresponding to the trimeric ring phosphates, imidotrimetaphosphate (ITMP) and trimetaphosphate (TMP).

A 5 ml sample was buffered to pH 3.6 and after 200 h also subjected to ion-exchange separation, and the following species were detected: (i) orthophosphate, (ii) trace pyrophosphate, (iii) TeMPm, (iv) ITMP, and (v) TMP.

It appeared that after long hydrolysis at pH 3.6 and 60°, trimeric ring imidophosphates and oxyphosphates were formed together with orthophosphate. As the ring phosphates only appeared at pH 3.6, there seemed to be a limit of acidity which could be employed, if TeMPm was to be prevented from decomposing directly to orthophosphate, and it was decided to study the reaction over the pH range 2–5, and the results are given in Table II.

TABLE II

Time (h)	*pH 5.0*		*pH 3.6*		*pH 2.85*		*pH 2.0*	
0.25			Tetrametaphosphimate only (R_x = 0.90)					
3.0			Tetrametaphosphimate only (R_x = 0.90)					
6.0	TeMPm only		TeMPm only		TeMPm Ortho (trace) + spots	(0.90) (1.0) (1.45)	TeMPm Ortho (trace) + spots	(0.90) (1.0) (1.45, 1.80)
24	TeMPm	(0.90)	TeMPm Ortho Spot	(0.90) (1.0) (1.45)	TeMPm Ortho Spots	(0.90) (1.0) (1.45, 1.80, 2.40)		
48	TeMPm	(0.90)	TeMPm Ortho Spots	(0.90) (1.0) (1.45, 1.80)	Ortho Spots	(1.0) (0.45) (1.45) (1.80) (2.40)	Ortho Spots	(1.0) (0.45) (1.45) (1.80) (2.40)
72	TeMPm Ortho Spot	(0.90) (1.0) (1.45)	TeMPm Ortho Spots	(0.90) (1.0) (1.45) (1.80) (2.40) trace	Ortho Spots	(1.0) (0.45) (1.80) (2.40)	Ortho Spots	(1.0) (0.45) (1.80) (2.40)

The same intermediates were formed over the whole range of pH 2.0–5.0, but the more acid the buffer solution, the faster the rate of hydrolysis.

Although at this stage, no intermediate had been isolated, all evidence indicated the fission of the tetrameric ring to a trimeric ring, as the initial step. The evidence may be summarised as,

(1) R_x values of intermediates in BIBERACHER's solvent were identical with those of diimidotrimetaphosphate (DITMP) (1.45), ITMP (1.80), and TMP (2.40).

(2) The order in which the spots occur was in agreement with that expected if trimetaphosphimate (TMPm) or DITMP were formed as first intermediates.

(3) Orthophosphate was formed at the same time as first ring intermediate, and is to be compared with the hydrolysis of TMPm in which orthophosphate is not formed until after ITMP is present.

(4) Retention volumes on ion-exchange columns were identical for those of ITMP and TMP.

Ion-exchange study

Ammonium tetrametaphosphimate was dissolved in a buffer pH 2.0 previously heated to 60°. The solution was maintained at 60°, and 5 ml samples were removed at intervals and subjected to ion-exchange separations. The results can be seen in Fig. 1.

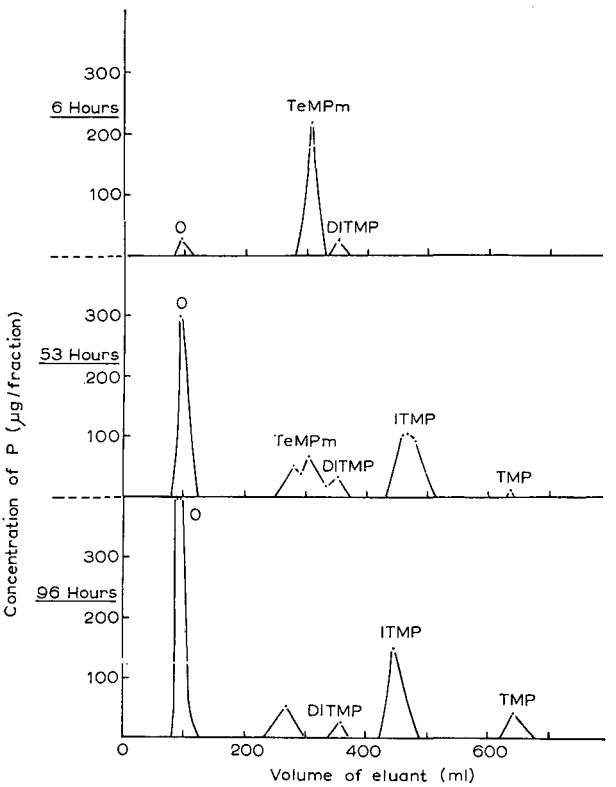


Fig. 1. Elution patterns for the hydrolysis of tetrametaphosphimate at pH 2.0 and 60°.

The ion-exchange study showed the formation of large amounts of orthophosphate as the trimeric rings were formed, providing excellent evidence that the products obtained did contain three phosphorus atoms. Although the quantity of phosphorus present as orthophosphate was always slightly greater than the sum of the phosphorus present as trimeric rings (possibly due to hydrolysis of the trimeric rings themselves to orthophosphate at pH 2.0), there was fairly good agreement that one orthophosphate and one trimeric phosphorus ring was formed from one tetrameric ring.

The first stage of the hydrolysis of TeMPm was very slow when compared with

that of TMPm. The lower pH (namely 2.0) caused the trimeric rings to hydrolyse more quickly when they were formed, than at pH 3.6, this being noticed in the build-up of TMP.

Although DITMP was the first product of the hydrolysis of TeMPm, it is possible, considering the relative rates of the reaction concerned, that TMPm is actually the first product. Since any TMPm formed would be very rapidly hydrolysed to DITMP, with the result that it may not be detected. The possible mechanisms involved in the formation of DITMP and TMPm as initial products of the hydrolysis are discussed later.

ISOLATION OF A SODIUM DIIMIDOTRIMETAPHOSPHATE FROM THE HYDROLYSIS OF TeMPm

To the present, the evidence for the presence of trimeric rings was chromatographic data, and it was decided to make an attempt to isolate a trimeric ring imidophosphate. The path of the hydrolysis was followed by paper chromatography of the reaction mixture in BIBERACHER's basic solvent at frequent intervals, and interrupting the hydrolysis when the required species was present in large concentration. It was then isolated by fractional precipitation with magnesium nitrate mixture prepared as follows: magnesium nitrate (50 g) and ammonium nitrate (17.5 g) were dissolved in water, sufficient ammonium hydroxide added to give a faint odour, and diluting to 500 ml with water.

Method

Ammonium tetrametaphosphimate was dissolved in *p*-toluenesulphonate buffer pH 1.70, and the solution heated at 100° for 9 h. After cooling to room temperature, ammonium hydroxide solution was added until the solution was just alkaline, and excess magnesium nitrate reagent was added which precipitated all the chain phosphates or chain imidophosphates. The magnesium precipitate was centrifuged off, and the centrifugate neutralised with dilute nitric acid before addition of excess silver nitrate which precipitated all the remaining phosphate species. The silver phosphate precipitate was centrifuged off, washed well with water and dissolved in the minimum of 2 *N* ammonium hydroxide solution. A little magnesium nitrate reagent was added to this ammoniacal solution to precipitate any remaining chain species. This precipitate was centrifuged off, and the solution neutralised with nitric acid. The silver salts which were precipitated on neutralisation were centrifuged off, and formed the first fraction containing mainly trimeric phosphates but also TeMPm. A second, and purer fraction was obtained by adding slowly some more silver nitrate solution. This precipitate was centrifuged off and thoroughly washed with water, finally being treated with 2 *N* sodium chloride solution acidified with acetic acid, when silver chloride was precipitated, leaving the sodium imidophosphate in solution. The sodium salt was precipitated from solution by addition of absolute alcohol.

Found: N, 8.5; P, 28.7.

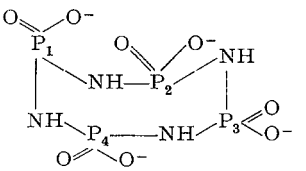
Calc. for $Na_3P_3(NH)_2O_7 \cdot H_2O$; N, 8.7; P. 28.9.

The product gave a spot with R_x value 1.45 when chromatographed in BIBERACHER's basic solvent, and was identical in every way to sodium diimidotrimetaphosphate prepared by the method of DE FICQUELMONT[8] (see Part II of this series[6]) *e.g.* infra-red spectra, potentiometric titration.

THE MECHANISM OF THE HYDROLYSIS OF TeMPm

The only product of the hydrolysis of the tetrametaphosphimate ion previously reported is orthophosphate[3], but from the observations of QUIMBY *et al.*[5] and POLLARD *et al.*[6] on the hydrolysis of TMPm, it was decided to investigate the hydrolysis of TeMPm. It was expected that any intermediates isolated from the hydrolysis would be eight-membered ring compounds, in which the imide linkages had been partially replaced by oxygen linkages, analogous to DITMP and ITMP.

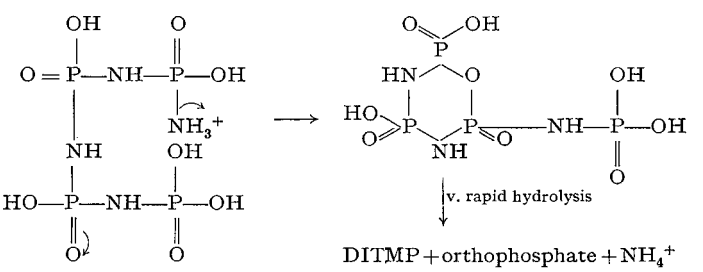
But the products detected were orthophosphate, and the trimeric ring compound DITMP in approximately 1:1 ratio. The DITMP then proceeded to hydrolyse further to ITMP, TMP and orthophosphate.



TeMPm

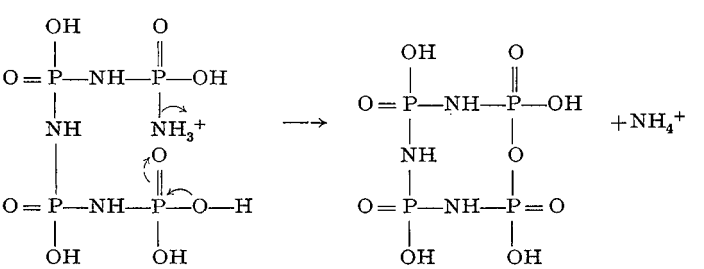
Again the complex ring intermediate (using the boat configuration of TeMPm across the phosphorus atoms P_1 and P_3), or open chain mechanisms can be postulated as for the trimeric ring system[5] but it is believed the ruptured ring system is a better explanation.

The first three major steps of the reaction, (1) protonation of a P atom, (2) approach of a water molecule and (2a) bonding to the P atom, (3) rupture of the P–N bond are as for the trimer[6] but when we consider the formation of the P–O–P bond in step (4) we find there are two P =O bonds in suitable positions for electron transfer to take place to the adjacent P atom. Transfer from one of these would form the six-membered ring DITMP, whilst the second would form an eight-membered ring. In practice we find the six-membered ring being formed, probably again by linkage through P atoms P_1 and P_3 instead of P_2. The two possible ring reformation steps of TeMPm may be represented as follows:



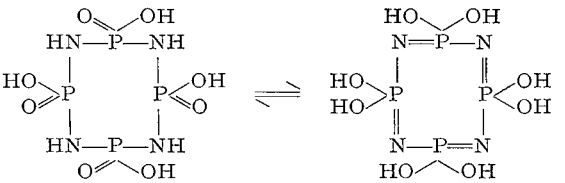
(1)

DITMP + orthophosphate + NH_4^+

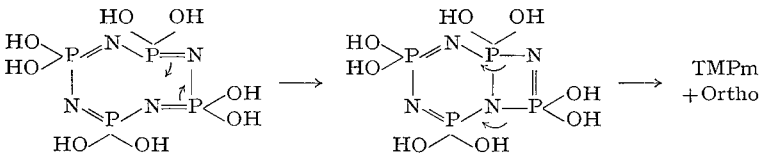


(2)

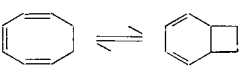
A mechanism can also be proposed for the formation of small amounts of TMPm and depends on the existence of a small amount of the tautomeric form TeMPm containing alternate single and double bonds.



With a boat conformation of the ring, P atoms on opposite sides of the ring are in a position of close proximity, but also a P atom is also in close proximity to a N atom on the opposite side of the ring, so that bonding may occur as shown.



Such an intermediate, involving a four-membered ring, would not be very readily formed, and is probably the reason why no TMPm is found in the hydrolysis of TeMPm (*cf.* Part IV of this series[9]). Once formed, the intermediate would be expected to form TMPm by rupture of the two P-N bonds of the unstable four-membered ring. An analogous situation in organic chemistry, is where a 6:4 system exists and is well known, *e.g.* cyclooctatriene (I) and the bridged compound biscyclo-octa-2,4-diene (II)[10].



(I) (II)

SUMMARY

A kinetic investigation of the hydrolysis of tetrametaphosphimic acid is described. The products of hydrolysis are diimidotrimetaphosphate and orthophosphate; the ruptured ring mechanism is used to provide an explanation of this behaviour.

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CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS
PART IV. THE HYDROLYSIS OF PENTAMETAPHOSPHIMIC ACID

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The only work reported on the hydrolysis of the higher cyclic phosphonitrilic halide polymers is that due to STOKES[1-3]. Metaphosphimate salts above the tetramer cannot be prepared by the action of acetates due to the formation of secondary products. A smooth saponification can be carried out by the action of a concentrated solution of sodium hydroxide on an ethereal solution of the halide[4]. The salts of the pentamer and higher acids are not crystallisable, the alkaline salts being precipitated from aqueous solution by alcohol as syrups. The syrups can be converted into amorphous solids by dehydrating under alcohol.

STOKES[4] detected the following acids in the hydrolysis of pentametaphosphimic acid with hot dilute acetic acid:

(i) Tetrametaphosphimic acid $P_4N_4O_8H_8$.
(ii) Triimidotetraphosphoric acid $P_4N_3O_{18}H_9$.
(iii) Diimidotriphosphoric acid $P_3N_2O_8H_7$.
(iv) Orthophosphoric acid PO_4H_3.

In view of the results of the hydrolysis of trimetaphosphimic acid (TMPm)[5,6] and tetrametaphosphimic acid (TeMPm)[7], it was decided to reinvestigate the hydrolysis of pentametaphosphimic acid (PMPm). STOKES[8] had erroneously concluded that TMPm gave chain imidophosphate intermediates when hydrolysed in acid solution, and it seemed unlikely that such products would be formed in the hydrolysis of PMPm.

PREPARATION OF SODIUM PENTAMETAPHOSPHIMATE

Pentameric phosphonitrilic chloride (Albright & Wilson Ltd.) (4 g) was dissolved in diethyl ether (20 g) and agitated with a solution of sodium hydroxide (5 g) in 20 g of water for about two days. The alkaline solution was separated off and the sodium pentametaphosphimate precipitated as a thick syrup by the addition of 2–3 volumes of ethanol. The syrup was washed by stirring with small volumes of 60 % v/v aqueous ethanol, then dissolved in a minimum of water, reprecipitated by addition of alcohol and washed with 60 % v/v aqueous ethanol until free from sodium chloride. Stirring under renewed samples of ethanol, removed water from the sodium salt which was obtained as a white amorphous powder. After allowing to stand overnight under ethanol, the sodium salt was filtered off and dried in vacuo over sulphuric acid. Found: P, 26.8; N, 11.7. Calc. for $[PN(ONa)_2]_5$: P, 26.9, N, 11.9. The R_x values of PMPm are given in Table I.

TABLE I

R_x values of PMPm	Chromatographic solvent
c.13	Acid[9]
0.08	Neutral[5]
0.70	Alkaline[10]

The retention volume of pentametaphosphimate under the conditions described previously[6] was 300 ml and a minor peak at 230 ml. A potentiometric titration of pentametaphosphimic acid (prepared from the sodium salt by a cation-exchange procedure) gave two inflection points (see Fig. 1) corresponding to four and five replaceable hydrogen ions respectively. This indicated that one unit of the molecule

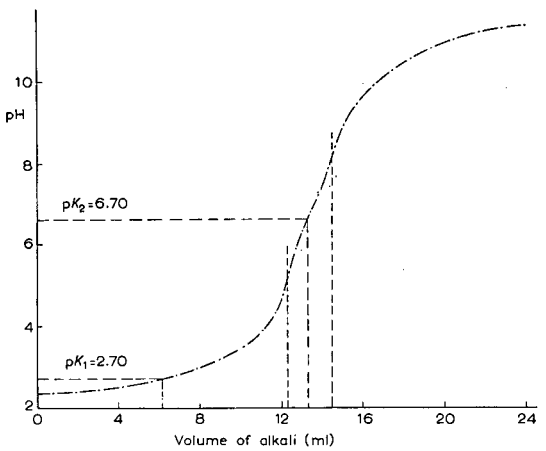


Fig. 1. Potentiometric titration of pentametaphosphimic acid

was probably in a different environment to the other four units. From a molecular model of the pentametaphosphimic acid it was thought that an acid hydrogen atom may point inside the ring instead of away from it as do those of the trimer and tetramer, and consequently is more difficult to replace. STOKES[4] reported the preparation of two sodium salts of the pentamer, one containing four sodium atoms, and the other, five.

A PAPER CHROMATOGRAPHIC STUDY OF THE HYDROLYSIS OF SODIUM PENTAPHOSPHIMATE AT 60° AT pH 2.0 AND 3.6

Samples of the sodium pentametaphosphimate were removed and eluted in BIBERACHER's basic solvent[10], and the neutral solvent of QUIMBY *et al.*[5]. The results for the basic solvent are shown in Table II (figures in brackets refer to the R_x value of species).

The neutral solvent chromatograms confirmed these results. From the R_x values of the species obtained and from the order in which they appear, it is almost

certain that the hydrolysis of the pentamer occurs by the initial formation of a trimer ring imidophosphate, with simultaneous production of large quantities of orthophosphate.

TABLE II

Time (h)	*pH 3.6*	*pH 2.0*
0	PMPm (0.70)	PMPm (0.70)
2	PMPm (0.70) True ortho (1.0)	PMPm (0.70) Trace ortho (1.0)
6	PMPm (0.70) Ortho (1.00) Trace TMPm (1.20)	PMPm (0.70) Ortho (1.0) Trace TMPm (1.20) DITMP (1.45) ITMP (1.80)
24	PMPm (0.70) Ortho (1.00) TMPm (1.20) DITMP (1.45) Trace ITMP (1.80)	PMPm (0.70) Ortho (1.00) Trace TMPm (1.20) DITMP (1.45) ITMP (1.80)
50	Trace PMPm (0.70) Ortho (1.00) TMPm (1.20) DITMP (1.45) ITMP (1.80)	Trace PMPm (0.70) Ortho (1.00) Trace TMPm (1.20) DITMP (1.45) ITMP (1.80) Trace TMP (2.40)

Abbreviations: PMPm = pentametaphosphimate; TMPm = trimetaphosphimate; DITMP = diimidotrimetaphosphate; ITMP = imidotrimetaphosphate; Ortho = orthophosphate.

The most interesting point about the hydrolysis of the pentamer, when compared with that of the tetramer, was the appearance of trimetaphosphimate. As more trimetaphosphimate was found at pH 3.6, than at pH 2.0, it is likely that the presence of TMPm depends on the initial rate of the first step. In the very slow hydrolysis of TeMPm, any TMPm which was formed would immediately be hydrolysed to DITMP. DITMP would then in turn be hydrolysed to ITMP.

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

Sodium pentametaphosphimate was dissolved in a sodium acetate–hydrochloric acid buffer pH 3.6 and kept at 60° for about 10 days. 5 ml samples were removed at intervals for ion-exchange separations. The results are shown in Fig. 2.

The paper chromatograms eluted in BIBERACHER's basic solvent showed orthophosphate and a trace of trimetaphosphimate after 2 1/2 h and orthophosphate, TMPm, DITMP, and trace ITMP after 20 h. Although TMPm decreased, a trace was still present after 280 h. This showed that TMPm was being formed continually, and could not be due to TMPm impurity in the sample, as TMPm is completely hydrolysed in about 20 h under these conditions[6].

The ion-exchange elution patterns are very complicated over the volume range from 220 ml to 380 ml, due to the two PMPm peaks and DITMP occurring in this region. It is impossible to determine from these patterns if any TMPm or TeMPm were present. But there is an approximate relation that 2 orthophosphate ions are produced for every trimeric ring phosphate.

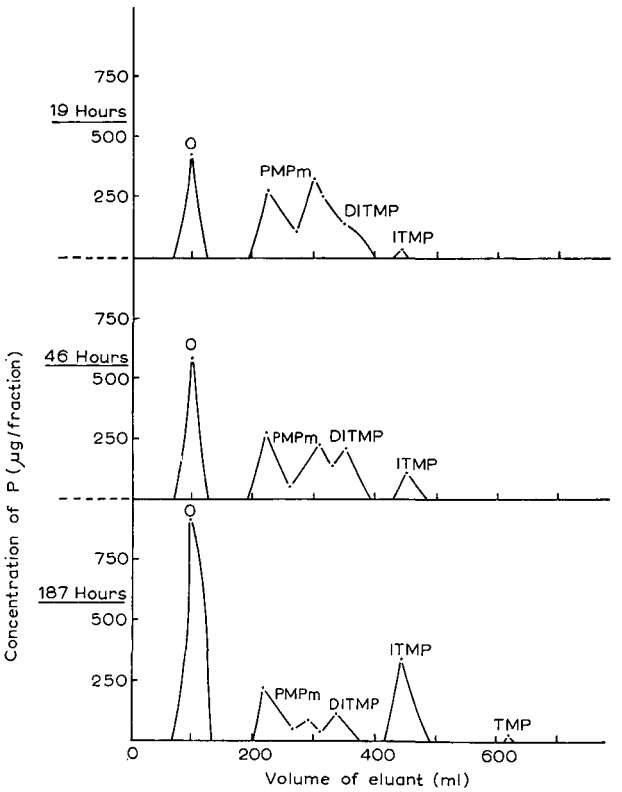


Fig. 2. Elution patterns for the hydrolysis of pentametaphosphimate at pH 3.6 and 60°.

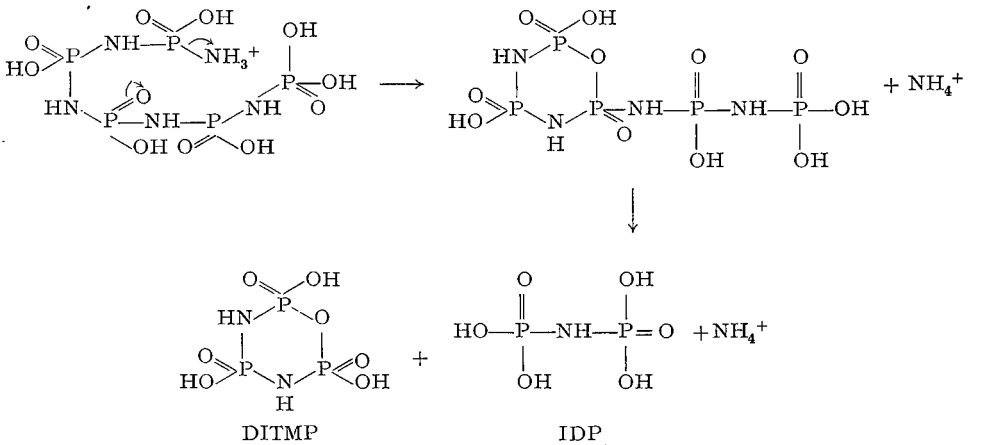
The ratio of 2:1 is expected if the pentamer decomposes to a trimeric ring eliminating two molecules of orthophosphate.

No attempt was made to isolate a trimeric ring compound from the hydrolysis products of PMPm (because of lack of sufficient starting material). There can be little doubt however, that the products detected by both paper and ion-exchange chromatography were trimeric ring imidophosphates. No evidence was obtained for the production of TeMPm as an intermediate, as reported by STOKES[4] but evidence for TMPm was found. We would like to suggest that STOKES[4] mistakenly identified this product TMPm for TeMPm.

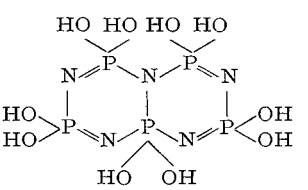
THE HYDROLYSIS OF PENTAMETAPHOSPHIMATE

The mechanisms suggest for the formation of TMPm and DITMP in the hydrolysis of TeMPm[7] and are also thought to occur in the hydrolysis of PMPm.

In the ruptured ring mechanism it is again likely that the chain will reform into a six-membered ring, DITMP being formed with imidodiphosphate (IDP) being eliminated. Imidodiphosphate would immediately hydrolyse to ortho- and pyrophosphates under the reaction conditions[11].

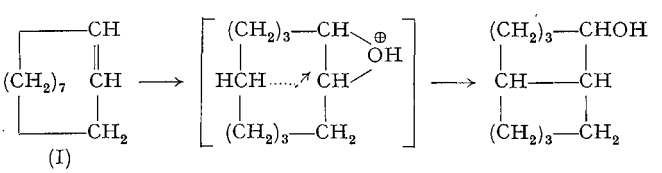


For the formation of the TMPm, bonding could again take place between a nitrogen atom and a phosphorus atom on opposite sides of the ring due to their close proximity in such large ring systems. The intermediate obtained would have a 6:6 structure.



This intermediate would be expected to be formed more readily than the analogous intermediate in the TeMPm hydrolysis from a consideration of strain in the molecule. This intermediate would give one molecule of TMPm and one molecule of imidodiphosphate on rupture of the two P–N bonds of one ring.

An analogous situation exists in organic chemistry when the ten-membered ring compound *cis*-cyclodecene (I) is oxidised, some bonding taking place across the ring[12].


(I)

In this case, however, the product is stable and no further breakdown occurs as in the case of PMPm.

SUMMARY

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

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Notes

Eine Vorrichtung zur Einführung kleinster Probenmengen in gaschromatographische Kolonnen

Bei der gaschromatographischen Bearbeitung von schwer flüchtigen Stoffen wie Steroiden stiessen wir auf die bekannte Schwierigkeit, Probemengen von weniger als 1 μg in die Kolonne einzuführen. Zur reproduzierbaren Injektion von Lösungen mit 10 μl-Injektionsspritzen müssen mindestens 1 μl angewendet werden. Durch den mehr als 1000 fachen Überschuss an Lösungsmittel entstehen im Chromatogramm so lange Schwänze, dass relativ früh erscheinende Peaks nicht mit grösster Empfindlichkeit aufgezeichnet werden können. Ausserdem ist die reproduzierbare Injektion gegen hohe Kolonnendrücke nur mit einer erstklassigen Injektionsspritze möglich. Sogenannte "Splitter" sind meistens zu ungenau und verlangen sehr konzentrierte Lösungen. Wir haben daher eine Einrichtung entwickelt, zu welcher zwar Lösungen angewendet werden, bei der aber die Probe lösungsmittelfrei in die Kolonne eingebracht werden kann.

Die Grundlage der Vorrichtung bildet das Hochdruckinjektionssystem nach SCOTT[1] in seiner kommerziellen Ausführung von W.G. Pye & Co. Ltd., Cambridge. Diese Einschleusvorrichtung (Fig. 1) wird normalerweise mit sogenannten Dipperkapillaren benutzt: Die flüssigen Proben werden in eine kurze geeichte Glaskapillare

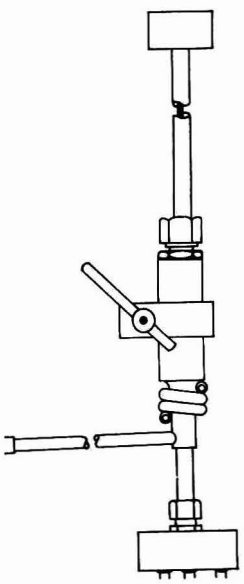

Fig. 1. Injektionssystem (W.G. Pye & Co. Ltd.).

eingesaugt, die sich an einem Ende eines *ca.* 21 cm langen Drahts befindet. Am anderen Ende des Drahts ist ein Eisenkern befestigt. Das Ganze wird mit dem Eisenkern voran in ein *ca.* 21 cm langes, einseitig geschlossenes Messingrohr geschoben. Das geschlossene Ende des Rohrs besteht aus Eisen, um welches ein Ringmagnet gelegt wird, der sich und den Eisenkern in der Schwebe hält. Das Rohr wird auf die Einschleusvorrichtung geschraubt, welche mit der Kolonne verbunden ist. Die Schleuse enthält ein Stück Gummischlauch, der zum dichten Abschluss des Kolonneneingangs gegen aussen durch einen Exzenter zusammengedrückt werden kann. Nach dem Aufschrauben des Rohrs mit der Probe wird die Schleuse geöffnet und der Ringmagnet entfernt. Die Kapillare fällt dann direkt in den Kolonneneingang.

Zur lösungsmittelfreien Eingabe in die Kolonne wurde das beschriebene System folgendermassen abgeändert: an Stelle der Kapillare wird am Ende des 205 mm langen, 1 mm starken Stahldrahts eine 15 mm lange aus einem Schmelzpunktröhrchen hergestellte Glasspitze mit Wasserglaskitt aufgekittet. Unmittelbar oberhalb der Glasspitze sind an den Stahldraht zwei aus Draht hergestellte Führungsbügel hart angelötet. Auf dem Stahldraht befindet sich zwischen Glasspitze und Eisenkern eine frei bewegliche zentrierte Messingführungsscheibe, deren Durchmesser der Innenseite des aufschraubbaren Messingrohrs entspricht. Sie dient der genauen Zentrierung der Glasspitze im aufschraubbaren Rohr. Ihr Durchmesser ist grösser als der Schleusendurchgang, so dass sie beim Fallen des Drahts immer an der gleichen Stelle hängen bleibt. Der Stahldraht mit Eisenkern und Glasspitze — kurz Einsatz genannt — wird in eine Führung (Fig. 2) eingeklemmt. Im rechten Winkel dazu befindet sich die Führung der Injektionsspritze. Unter Beobachtung mit einer starken Lupe bringt

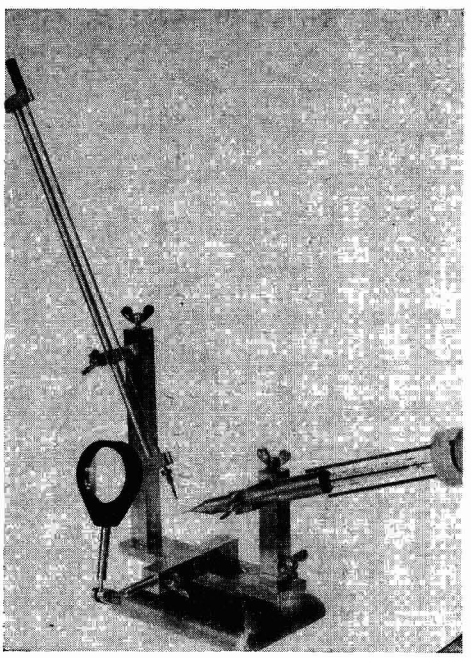

Fig. 2. Justierbares Gestell zum Aufbringen der gelösten Probe auf die Glasnadel.

man nun die mit *ca.* 1 μl Lösung der Probe gefüllte Injektionsspritze mit der angeschliffenen Seite der Nadel an die Glasspitze und drückt den Spritzenkolben bis zum Anschlag. Es können mit Sicherheit 1 μl Lösung als ein Tropfen an die Spitze gebracht werden. Für grössere Mengen spritzt man zusätzlich 1 μl Portionen, nachdem der vorher aufgebrachte Tropfen annähernd angetrocknet ist. Verwendet man Chloroform als Lösungsmittel, so hat sich das Lösungsmittel in längstens 3 min vollständig verflüchtigt. Der Einsatz wird nun in das Messingrohr geschoben und mit dem Ringmagneten befestigt. Das Rohr wird auf die Schleuse geschraubt und diese langsam zum Druckausgleich geöffnet. Zur Entfernung der Luft aus dem Messingrohr wird für 3 min ein am Ende des Rohrs befindliches Nadelventil wenig geöffnet. Danach wird das Nadelventil geschlossen und während *ca.* 4 min die vollständige Wiederherstellung des ursprünglichen Kolonnendrucks abgewartet. Zur Aufgabe der Probe in die Kolonne wird der Haltemagnet nach oben geschoben. Der Einsatz fällt durch die Schleuse und die Glasnadel bohrt sich in die oberste Schicht der Kolonne hinein. Nach *ca.* 10 sec wird der Einsatz mit Hilfe des Ringmagneten wieder in das obere Rohr gebracht und die Schleuse geschlossen.

Bei den Anfangsversuchen bestand die oberste Schicht der Kolonne aus unbedeckter Trägersubstanz (Gaschrom P der Applied Science Laboratories, Inc., State College, Pa., U.S.A.). Als Testsubstanz wurde Cholestan verwendet. Um scharfe Peaks zu erhalten, musste diese Schicht durch einen um die Kolonne gewickelten Heizdraht auf *ca.* 60° über die Kolonnentemperatur gebracht werden. Da vermutet wurde, dass eine Aufheizung nur zur Verminderung der Restadsorptionskräfte von Gaschrom P notwendig war, wurde reinster Quarzsand (Korngrösse 72–96 mesh) als oberste Schicht eingesetzt. Mit Quarzsand waren die Peaks ohne Vorheizung schärfer als bei Gaschrom P mit Vorheizer. Die Reproduzierbarkeit entsprach der Genauigkeit, mit welcher eine Dosierung von 1 μl mit einer Injektionsspritze (Hamilton 10 μl) ohne Gegendruck möglich ist. Ausser einigen Recorderausschlägen in der ersten Minute, hervorgerufen durch Gasdruckschwankungen während der Probenaufgabe, waren keine Nullpunktsabweichungen feststelbar. Die auf die Glasnadel aufgebrachten Mengen betrugen bei unseren Versuchen 0.01–5 μg. Natürlich ist das Verfahren nur bei sehr schwer flüchtigen Proben anwendbar.

Der Direktion der CIBA A.G., Basel danke ich für die Erlaubnis zur Veröffentlichung dieser Arbeit. Ebenso sei die Mitarbeit der Herren G. GRASS und P. SPRÜNGLI aus meinem Laboratorium mit Dank erwähnt.

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Eingegangen den 14. Mai 1962

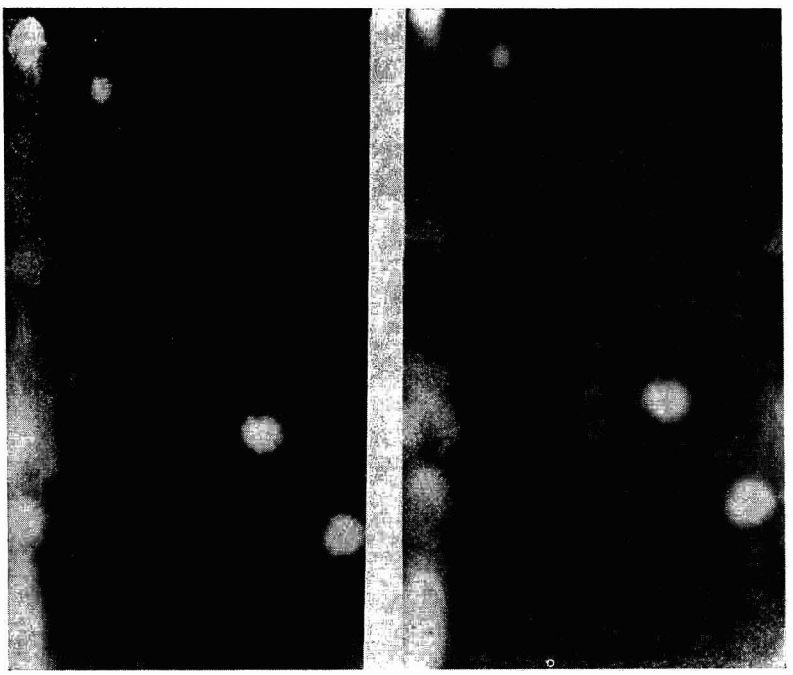
A simplified technique for ultraviolet photography of paper chromatograms

Contact photography provides a useful permanent record of ultraviolet-absorbing[1] and fluorescing[2,3] spots on paper chromatograms. It does involve, however, the separate steps of exposure, development, rinsing, fixing, washing and drying and is therefore somewhat time-consuming. In addition, when Kodabromide F-5 paper is used to photograph fluorescent spots, we have found it necessary to follow under the safelight the darkening of the spots in the developing solution, removing the paper before the background starts to darken also.

With the recent introduction of the French "Polymicro" materials and processing apparatus*, designed primarily for contact and enlarging purposes, a study was carried out of possible applications to paper chromatography.

Method

Contact photographs are taken in the usual way, using the 254 mμ handlamp** for absorbing spots, and the 360 mμ handlamp** with Kodak 2A filter[3] for fluorescent spots. The latter procedure must be carried out in the dark-room. The film is then fed



(a) (b)

Fig. 1. Contact photographs of ultraviolet-absorbing spots on a paper chromatogram using (a) Polymicro film, (b) Kodabromide F-5.

* Distributed in U.S.A. by Federal Manufacturing & Engineering Corporation, Garden City, New York.

** Mineralight Products Inc.

into the processing machine, from which it emerges developed, fixed, and almost dry, with very little tendency to curl. A variety of translucent and opaque (matt or glossy) papers of different sensitivities is available. For absorbing spots, we preferred high contrast "Glossy" paper (slow), using an exposure of 4½ seconds at 1½ feet. For fluorescing spots, good results were obtained using "Document" paper (fast), with an exposure time of 25 seconds at 6 inches.

The technique has proved to be much simpler than conventional film (Fig. 1). Photography of ultraviolet-absorbing spots can be carried out without a dark-room, under conditions of subdued daylight. Fluorescent spots can be photographed without special attention during development. The entire procedure takes less than a minute, which compares very favorably with conventional methods.

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Paper chromatography of quinoline-N-oxide derivatives

The biological behavior of 4-nitroquinoline-N-oxide and related compounds has received wide attention in the past few years. However, the experimental difficulties encountered when dealing with small amounts of reaction products of 4-nitroquinoline-N-oxide in the case of biological materials constitute a serious problem. This work concerns the paper chromatographic separation of quinoline-N-oxides and some related quinolines.

The results are summarized in Table I. The solvent systems employed in these experiments were the following:

(1) *sec.*-Butanol saturated with water, ascending development for 16 h.

(2) Methyl ethyl ketone–*sec.*-butanol–water (2:2:1), ascending development for 14 h.

(3) Ethyl acetate–*sec.*-butanol–water (1:1:1). The upper phase was used in the ascending direction for 14 h.

(4) Methyl ethyl ketone–*sec.*-butanol–water–28% ammonia (40:40:20:1), ascending development for 16 h.

(5) Isoamyl alcohol–acetone–water (4:2:1), ascending development for 16 h.

(6) Methanol–*n*-heptane[1], descending development for 20 h. The solvent front was allowed to run off in this case.

All materials were samples prepared in our laboratory. The filter paper was

TABLE I

MOBILITIES OF QUINOLINE-N-OXIDES

	Rp values in					*Distance moved (cm)*	*Colors* in*			
	sec.-Butanol–water	*Methyl ethyl ketone–sec.-butanol–water*	*Ethyl acetate–sec.-butanol–water*	*Methyl ethyl ketone–sec.-butanol–water–28% ammonia*	*Isoamyl alcohol–acetone–water*	*Methanol–n-heptane*	*U.V. light*	*Dragendorff's reagent*	*Na pentacyanoammineferroate*[2]	*Diazobenzenesulfonic acid*[3]
4-Nitroquinoline-N-oxide	0.91	0.95	0.97	0.94	0.82	24.4	g			
4-Hydroxyquinoline-N-oxide	0.94	0.93	0.83**	0.38	0.73**	—	wb			
5-Nitroquinoline-N-oxide	0.86	0.90	0.91	0.80	0.74	11.1	rbn			
3-Nitroquinoline-N-oxide	0.75	0.94	0.91	0.78***	0.74	13.3	rbn			
2-Aminoquinoline-N-oxide	0.76	0.73	0.67**	0.78	0.60**	4.3	bv	rbn		y
4-Hydroxyaminoquinoline-N-oxide	0.73	0.70	0.60	0.66***	0.54	—	wb		bn	ybn
4-Aminoquinoline-N-oxide	0.63	0.59	0.48**	0.66	0.50	0.9	wb	p	grb	o
4-Carboxymethylthioquinoline-N-oxide	0.31	0.26	0.12	0.35	0.10	0.6	v		p †	y
S-(1-Oxido-4-quinolyl)-L-cysteine	0.32	0.13	0.06	0.23	0.07	—	v		p †	y
4-Nitroquinoline	0.92	0.86	0.54**	0.88	0.72**	6.1	bv	p	bn †	
2-Aminoquinoline	0.73	0.56	0.43**	0.94	0.65**	4.7	v	p	pb	
4-Aminoquinoline	0.49	0.39	0.17	0.94	0.30	0.3	v	o	py	py †

* bn = brown; bv = blue violet; g = green; grb = greyish blue; o = orange; p = pink; pb = pale blue; py = pale yellow; rbn = red brown; v = violet; wb = white blue; y = yellow; ybn = yellowish brown.

** Tailing.

*** Partly decomposed.

† Faint.

Tōyō No. 53 paper, except in the case of solvent 6 where Tōyō No. 51A paper was used.

From the R_F values and the mobilities listed in Table I it can be seen that, although it was impossible to separate all the compounds listed with any one of the solvent systems, combination of two or three solvent systems yielded a separation of all the compounds. The spots were easily detected by irradiation with U.V. light (2537 Å), in which the tested compounds give a characteristic fluorescence. Some compounds were also detected by spraying with suitable reagents, but the coloration was not so noticeable as that obtained by U.V. irradiation. Solvents 3 and 5 caused tailing of some spots, but these solvents gave favorable results in particular cases.

Thanks are due to Dr. KEN'ICHI TAKEDA, director of our laboratory and to Dr. EIJI OCHIAI, emeritus Professor of the University of Tokyo, for their encouragement. The work described here was aided by a grant from the Ministry of Education, Tōkyō, Japan.

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Quantitative determination of succinic dehydrogenase activity after histochemical reaction by means of chromatography

The quantitative determination of histochemical reactions constitutes one of the most important problems of histochemical research. This also refers to one of the most widely used histochemical enzyme reactions, the determination of succinic dehydrogenase by means of neotetrazolium. In the presence of a suitable substratum and dehydrogenases neotetrazolium, tetrazolium-blue and other ditetrazolium salts[1,2] are converted not only to the completely reduced blue formazan, but also to semi-reduced red coloured monoformazan of which considerable quantities are formed. The separation of the two formazan types is important, because the data of FARBER *et al.*[3] seem to indicate that with low enzyme activity the red, and with high activity the blue diformazan is formed.

The authors decided to develop a method for the separation of the different formazan types, and thus render possible the study of their behaviour and their quantitative determination.

The succinic dehydrogenase activity was investigated by means of the histochemical method of NACHLAS *et al.*[4] using sections of the kidney, liver and heart of albino-rats, and neotetrazolium (KETI, Budapest). One part of the section was

examined microscopically, while the other part was applied—from the 0.85% NaCl solution used for washing the sections after the histochemical reaction—to Whatman No. 1 chromatographic paper. After drying at room temperature, the sections adhered perfectly to the surface of the paper and were practically inseparable. After equilibration for half an hour chromatography was carried out for about 1 hour in the Bush B 5[5] solvent system (benzene–methanol–water; 100:55:45). Under the effect of the chromatography the formazans moved out from the sections and the dye fractions separated from each other. The blue-coloured diformazan moved close to the front, while the R_F value of the semireduced red-coloured monoformazan was smaller and less definite (Fig. 1).

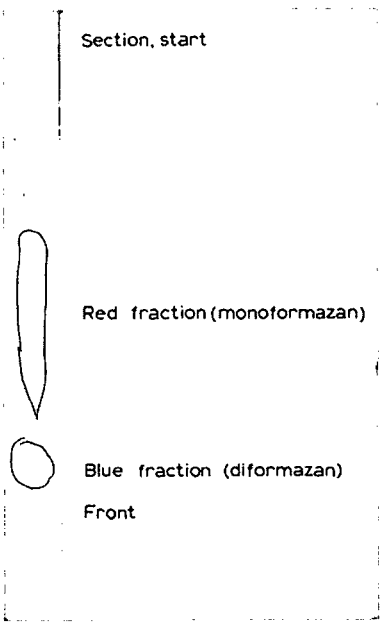

Fig. 1. Formazans after chromatography. The section does not contain any formazan. The separation of the red and the blue formazan is conspicuous.

After separation the fractions were subjected to further examination. The authors observed that the red-coloured formazan can be reduced further to blue diformazan by dipping the strips into an alkaline ascorbic acid solution (10 mg ascorbic acid in 1 ml 2 *N* NaOH).

The separated red- and blue-coloured fractions were eluted with a mixture of ethyl acetate–methanol (7:3). Quantitative determinations were made by means of the Unicam SP 500 type spectrophotometer. The red-coloured fraction was measured at the wavelength of maximum absorption, 480 mμ, the blue one at 530 mμ. The extinction values were referred to the unit weight of the sections applied to the paper.

In Table I the quotients of the amounts of the red (monoformazan) and blue (diformazan) fractions are given. The data show an interesting regularity: the quotients are characteristic for the different organs. (The values of the liver sections are considerably greater than those of the heart sections.) The quotients for the kidney

are less uniform. This is due—at least partly—to the fact that the heart and the liver are histologically relatively homogeneous, whereas in the kidney regions of different succino-dehydrogenase activity can be observed.

TABLE I

QUOTIENTS OF THE QUANTITIES OF RED-COLOURED MONOFORMAZAN AND BLUE-COLOURED DIFORMAZAN MEASURED SEPARATELY IN SEVERAL ORGANS OF TEN RATS

Rat No.	*Liver*	*Heart*	*Kidney*
1.	1.83	1.78	2.44
2.	3.68	1.57	1.00
3.	2.36	1.64	1.33
4.	7.75	2.71	1.95
5.	4.06	2.21	3.50
6.	4.16	1.40	3.90
7.	4.39		2.32
8.	6.58	3.23	4.46
9.	4.36	2.74	3.46
10.	4.60	1.42	1.66
Average	4.18	2.10	2.60

The present paper only deals with the quantitative evaluation of the histochemical reaction of the succinic dehydrogenase activity. The authors believe that this method can also be applied to the quantitative evaluation of various other histochemical reactions. Further work in this direction is in progress.

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Modified multichamber gradient mixer and mechanical column packing in ion exchange chromatography

The most common methods for gradient mixing have been reviewed by BOCK AND LING[1], PARR[2], TUNG AND TAYLOR[3] and SVENSON[4]. A series coupled open vessel gradient mixer was described by PETERSON AND SOBER[5] for use in ion exchange chromatography which can produce almost any type of gradient. PETERSON[6] later described a modified multichamber gradient mixer. The multichamber gradient apparatus described in this note is more flexible and less expensive than the apparatus previously described. It can be operated as one unit or as several interconnected subunits, each one in turn can produce gradients of any selected volume.

A modified gradient mixing device

The gradient mixers were constructed from inexpensive polypropylene graduated cylinders and stirrers, as shown in Fig. 1. Each cylinder was provided with a motor mounted on a machined collar. The cylinders were mounted in groups of three on aluminum stands and interconnected via teflon stopcocks. This was accomplished

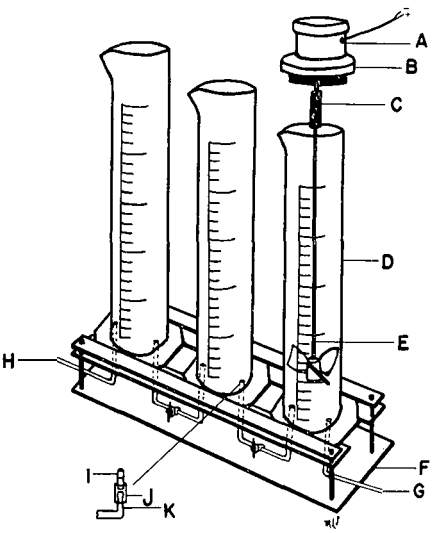

Fig. 1. A modified gradient mixing device. A = Synchronous timing motor (150 r.p.m.) (Hansen Mfg. Co., No. K29RA). B = Machined motor collar, centers stirrer. C = Aluminum driveshaft-stirrer coupler. D = Polyethylene graduated cylinders (Scientific Plastics Co. No. 3666). E = Polyethylene stirrer (Scientific Plastics Co. No. 6160). F = Aluminum stand. G = Gradient outlet (leads to additional gradient device, glass float check valve or column). H = Gradient inlet (plug if not used in combination with other cylinders). I = Nylon hyperdermic needle adapter (Clay-Adams Inc. No. A-1001). J = Tygon tubing (1/8 in. I.D.). K = Pyrex tubing of glass and teflon stopcock.

by drilling two 5/32 in. holes into the bottom of each cylinder and press fitting into them nylon hyperdermic needle adapters, cut to the proper length. A butt joint was made between the adapter and the stopcock with tygon tubing.

When three subunits were linked in series, a multichambered gradient mixing device was obtained which was comparable to that already described[5,6]. However, it was assembled for a fraction of the cost which included nine graduated cylinders, stirrers, and motors (about $ 110). In addition, very little machine work was required in making motor collars, stirrer couplers and stands to which the cylinders were mounted.

The distinct advantage of this multichamber gradient mixing apparatus was the ease and inexpensiveness of assembly and its ability to be broken down into subunits. These subunits could be used independently or connected in sequence. This enabled one to introduce a gradient change of any desired volume at any position of the chromatogram. By elevating the subunits one above the other and interconnecting them with glass float check valves, as shown in Fig. 2, gradients would flow in sequence starting with the gradient from the uppermost subunit. The subunits and valves were arranged to permit one to vary the volume and/or change the eluant at will. The change from one gradient to the next could be made gradually, if the first solution in the second gradient was identical to the last solution in the first gradient, or in a step if they were not the same.

Glass float check valve

The glass float check valve (D) in Fig. 2 permitted various gradients or stepwise elutions to flow in sequence automatically. This was particularly useful when such changes occurred during the night. The valve was constructed from a lapped Pyrex 12/5 mm ball and socket joint which would close when the float chamber was filled with liquid. By elevating the subunits and linking them to the valves in the manner shown in Fig. 2, the hydrostatic pressure from the first unit was greater than that from the second. Therefore, the valve remained closed and liquid flowed from the

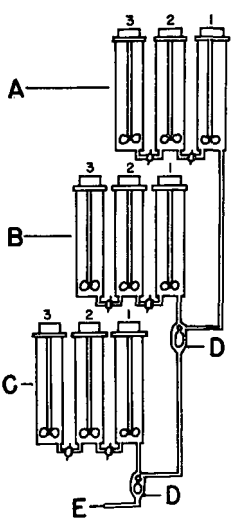

Fig. 2. Assembly of multiple gradient mixing devices on three levels. A = First gradient mixer at highest level. B = Second gradient mixer below level of first. C = Third gradient mixer at lowest level. D = Glass float check valve. E = Gradient outlet (to pump and/or to column).

first unit. As soon as the first unit was emptied, the float valve automatically opened and liquid flowed from the second unit. By inverting the valves during the initial filling, entrapped air was easily displaced. This was important as trapped air sometimes caused difficulties.

Pump packing cellulose columns

A peristaltic pump proved very useful for packing cellulose ion exchange columns. By pumping a slurry, consisting of one part of cellulose to sixty parts of buffer, from a magnetically stirred reservoir to a column filled with the same slurry, reproducible and uniform packing was assured. Flow rates of 10 and 3 ml/min were used for 2.5 × 30 cm columns. The tubing (1/8 in. I.D.) from the reservoir to the column via the peristaltic pump, was small enough to keep the slurry moving in suspension yet not so small that cellulose plugged the lines. Slurries which were too thick produced plugging.

Tygon tubing was used because it was durable in the peristaltic pump. The tubing was connected to the column by passing it directly through a rubber stopper. The rate of flow from the column during packing was constant and controlled by the pump. The pressure during packing increased from zero to about 10 lbs., depending on the height and rate that the column was packed. Columns packed by this method were almost without exception reproducible and evenly packed. This was demonstrated by passage of a narrow blue band through the column, when it was equilibrated with 0.01 *M* glycine[7]. Poorly packed columns usually resulted from packing the column too fast or from using a slurry which was too thick. Such columns gave uneven and/or diffuse blue bands.

Pump packing was also applied with success to larger Sephadex columns. When anaerobic conditions were necessary[8], pump packing was very convenient. Pump packing does not require constant attention. One only needs to be present to terminate the packing before the column is pumped dry.

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Carbonaceous fouling of catherometer filaments

The decomposition of organic materials on gas–liquid chromatography catherometer filaments may arise from either the materials being analysed or from bleeding of the column substrate. The latter effect is particularly pronounced with the more polar materials such as polyesters operated at high temperature. In this laboratory polyester substrates were used in one apparatus almost exclusively for over a year in the analysis of the long-chain highly-unsaturated fatty acid esters derived from marine oils, requiring operating temperatures of 200–230° (helium carrier gas). The directions for the use of the commercial apparatus* employed recommended regular flushing of the detecting block with both polar and non-polar solvents. This was carried out routinely on a weekly basis, additionally whenever the column was changed, and also necessarily when the recorder baseline became noisy.

The latter operating hindrance was usually overcome by such flushing, and discolouration of the rinse liquid indicated that some deposited material was being removed from the interior of the detector block. During the period in question the output bridge twice became unbalanced to the point of requiring a shunt resistor of the order of first 3000 Ω and subsequently 1200 Ω being placed in parallel with the reference catherometers in order to obtain a satisfactorily balanced bridge output. Ultimately the recorder baseline became too noisy to be acceptable and the detecting catherometers were replaced. The opportunity was then taken of examining the condition of this pair of catherometers. These had been mounted horizontally in the detection block, each in a diffusion compartment, with catherometer A being below the input and outlet orifices of the block, and catherometer B above. Under these conditions catherometer A was, as expected, the more seriously affected. The primary fouling agent was most noticeable at the more exposed end of the filament coil (Fig. 1) and as can be seen consisted of a furry black deposit joining numerous helices. The

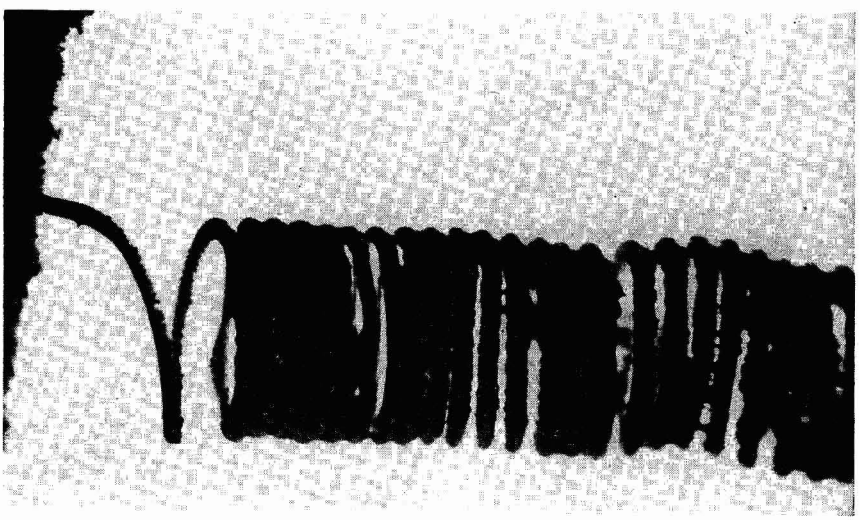

Fig. 1. Exposed end of catherometer A, showing crowding of helices and nodular deposits.

* Aerograph A-110-C, Wilkins Inst. Co., Walnut Creek, Calif., U.S.A.

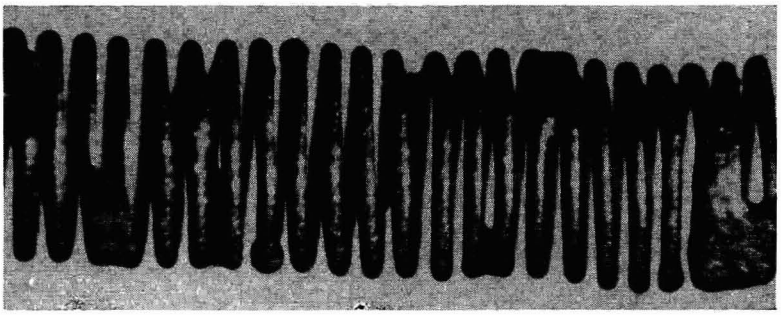

Fig. 2. Catherometer A nearer center, showing smoother carbonaceous deposits between helices.

same effect occurred to a lesser extent at the mounting end of the catherometer filament and intermittently, but more smoothly, throughout the length (Fig. 2). Nodules of the same material were noted on many helices, although not necessarily joining the latter. Other materials found on both catherometers (Fig. 3) included glass wool fibres from the packing of the exit end of the columns, and a metal spiral, possibly turned off the metal column connecting nut (brass) by the stainless steel block connection. Filament B was fouled to a lesser extent than A, although again chiefly at the ends, and also more particularly at the end remote from the mounting head. There were fewer glass wool fibres, suggesting that these may have been washed into the lower filament by the solvent flushing operations.

Attempts were made to remove the deposit with numerous solvents, both hot and cold, with little success. Alcoholic potassium hydroxide was then tried for several days, also without effect. No oxidizing solutions were tested, but it was thought that

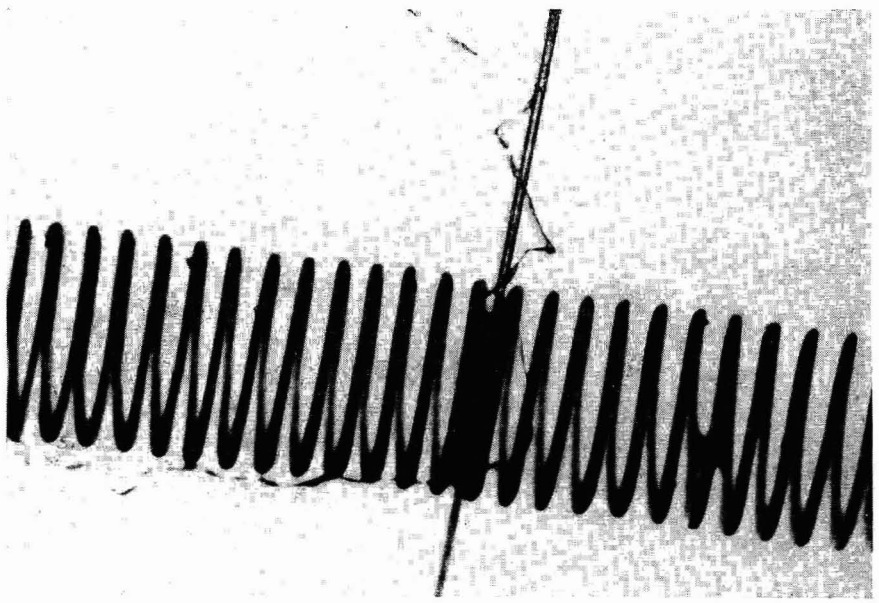

Fig. 3. Catherometer B near center, showing a glass wool fibre, and a metal turning. The fouling is less than on catherometer A.

possibly the residual deposit might be burned off by gently heating the filament in an atmosphere of oxygen. Accordingly catherometer A was mounted in a glass tube with the leads in series with a variable resistance and a source of 12 V direct current. Oxygen was passed slowly through the tube and the current gradually increased. No effect was observed up to 150 mA (compare 250 mA current for entire bridge with four catherometers). At a slightly higher current the center portion of the filament glowed a dull red. Examination of the hot catherometer with a lens indicated no immediate effect on the nodules of deposit, or the deposits linking individual helices, although these were all at dull red heat. On increasing the current slightly, catherometer A broke in the center. Subsequent microscopical examination failed to show any alteration in the fouled ends which had not been visibly heated, presumably due to conduction of heat to the mounting rods. Catherometer B was similarly treated and in this case the catherometer curled near the center into a larger helix (Fig. 4). This occurred at a slightly higher temperature and was accompanied by formation of a gray deposit

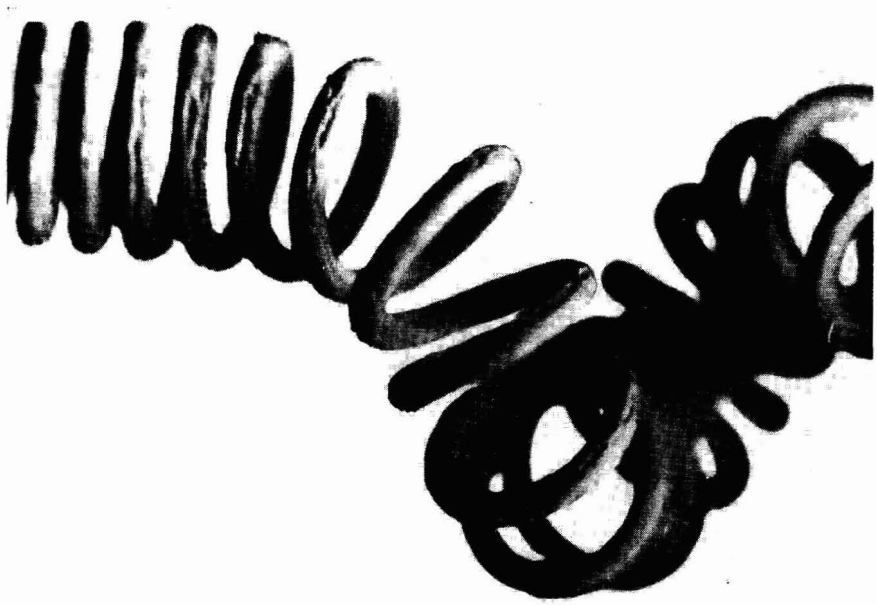

Fig. 4. Catherometer B, near center, after heating.

on the surface of the filament, as well as by apparent cracking of the metal. The visibly heated portion of the catherometer extended nearly to the ends, although again without appreciable effect on the fouling deposits in the end helices.

Comparison of the used filaments with a replacement filament* (Fig. 5) indicates that the helices at both ends of the filament in the former are pushed closer together. Since the center portion of the catherometer apparently operates at a higher temperature than the ends, this portion may stretch in time, allowing the end helices to approach each other. At the same time the lower temperature of the ends would encourage condensation of relatively non-volatile materials leading to the formation of

* Gow-Mac Instrument Company, Madison, N.J., U.S.A.

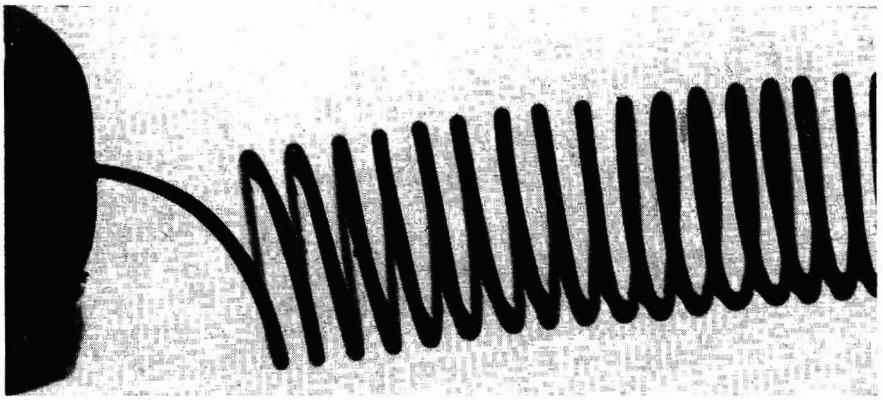

Fig. 5. Unused catherometer at exposed end.

tarry materials which are probably in part responsible for the noisy recorder baseline, but which may be removed by solvent flushing. In the course of time, however, these tarry deposits would carbonize leading to the permanent changes actually observed in catherometer resistance. Partial contacts of carbon nodules or deposits between helices (Fig. 1) are probably responsible for the eventual permanent recorder baseline noise, and also for the eventual partial collapse of the filament helix at the ends, since the reference catherometers, although in service for the same period, were clean and physically similar in all respects to the new catherometers. It may be concluded that if solvent treatment fails to reduce this noise, and all other causes have been eliminated, the replacement of the appropriate detecting catherometers may well be obligatory.

Photographs were taken through a Leitz Ortholux microscope fitted with an Olympus 4 × objective, using a Leitz 35 mm photomicrographic attachment. Total enlargement to film 20.5 ×.

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The separation of amino acids on paper impregnated with zirconium phosphate

The separation of amino acids on cellulose phosphate and resin-loaded papers has recently been discussed by KNIGHT[1]. Zirconium phosphate, either in columns or when loaded on paper has so far only been applied to the separation of inorganic substances. Since paper loaded with zirconium phosphate can easily be prepared in the laboratory[2] and since it should have properties similar to cellulose phosphate paper we thought it of interest to study the movement of amino acids on zirconium phosphate paper under

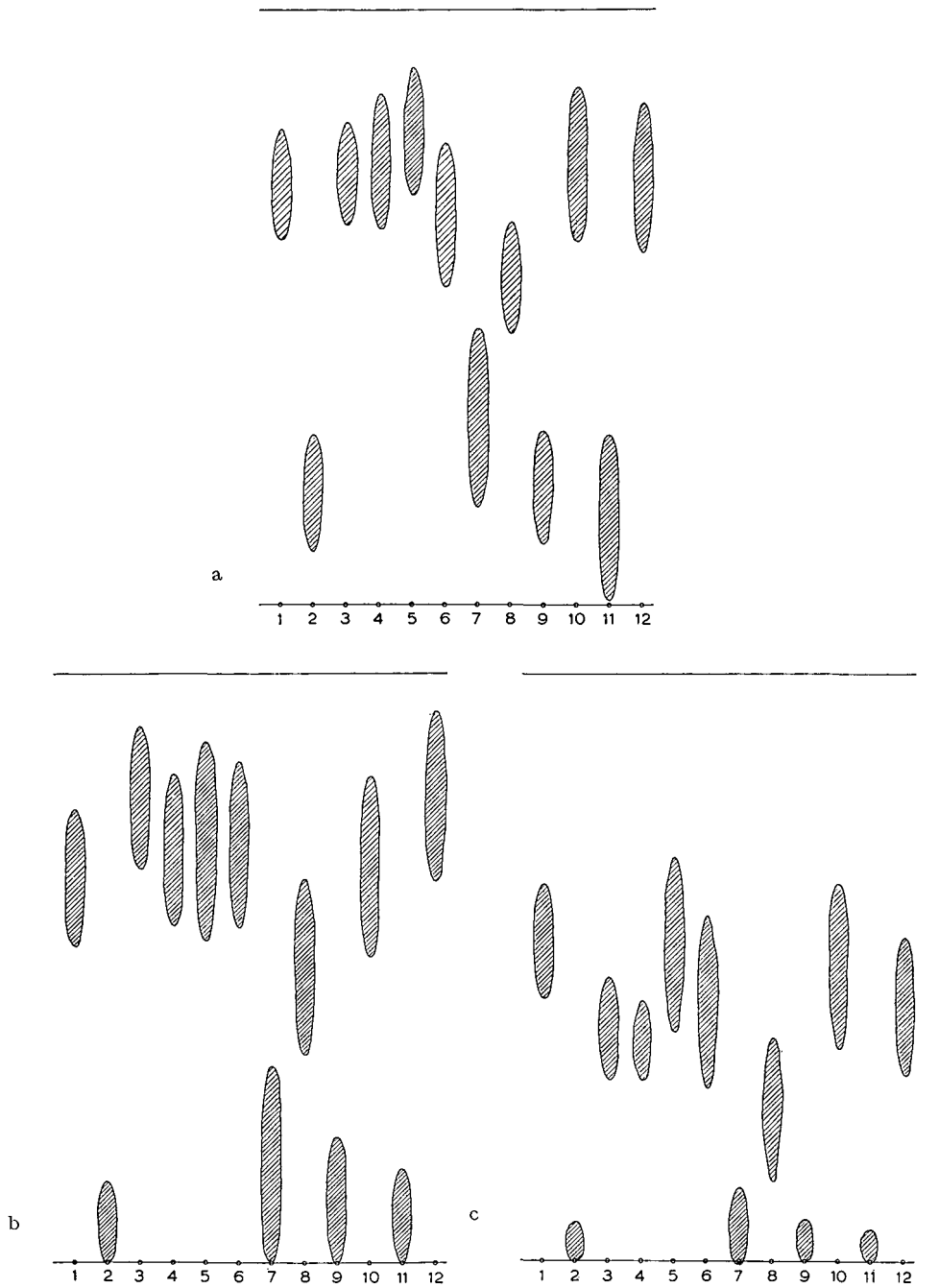

Fig. 1. Chromatograms of amino acids on filter paper loaded with zirconium phosphate. Point of application: below; liquid front: top limit. (1) Tyrosine; (2) cystine; (3) alanine; (4) proline; (5) threonine; (6) glutamic acid; (7) lysine; (8) tryptophan; (9) arginine; (10) serine; (11) histidine and (12) valine. (a) Solvent: HCl–sodium acetate buffer, 0.2 *M*, pH = 2.1. (b) Solvent: HCl–sodium acetate buffer, 0.2 *M*, pH = 3.1. (c) Solvent: HCl–sodium acetate buffer, 0.2 *M*, pH = 4.5.

the same conditions as applied by KNIGHT[1] for the other ion-exchange papers.

Figs. 1 a-c show the movement of 12 amino acids using acetate buffers of various pH values. The results are essentially the same as those obtained by KNIGHT[1] with other ion-exchange papers.

We think that these observations may be of interest for amino acid separation since it would be possible to prepare zirconium phosphate columns completely free of organic impurities (unlike cellulose phosphate), yet with similar properties.

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Détermination par chromatographie en phase gazeuse de petites quantités d'azote et d'oxygène dans le protoxyde d'azote

Au lieu des méthodes communes d'analyse par absorption de gaz, plutôt incertaines et laborieuses, on a pensé obtenir un dosage rapide et suffisamment précis de O_2 et N_2 dans le protoxyde d'azote moyennant la technique de la chromatographie en phase gazeuse.

Conditions expérimentales

(1) *Colonnes d'absorption.* Pour la séparation on a eu recours à un système de deux colonnes, l'une sur tamis moléculaire et l'autre avec l'huile de vaseline à forte viscosité déposée sur brique pilée (60–80 mesh) (covo Erba).

L'emploi de deux colonnes est déterminé par le fait que la première sert à séparer l'O_2 et le N_2, la seconde le N_2O de la somme oxygène azote.

Le tamis moléculaire 5A Linde, adsorbe en effet définitivement le N_2O en laissant passer nettement séparés le N_2 et l'O_2[1].

Le tamis moléculaire est fort sensible à l'humidité et il est donc opportun d'introduire dans la colonne des échantillons complètement anhydres; dans ce but on a inséré, entre la chambre d'injection et la colonne, un tube court en forme de U contenant du perchlorate de magnésium[2].

Pour ne pas commettre de fautes dans le prélèvement de l'échantillon il est opportun de poser un tube analogue entre la bouteille contenant le gaz à analyser et la seringue. La tendance à la formation de queues indique que la substance dessiccative doit nécessairement être remplacée.

(2) *Introduction des gaz.* Au lieu d'enrichir l'échantillon en condensant le protoxyde avec de l'air liquide, on a préféré injecter une plus grande quantité, 5 cm^3 environ.

On a adopté cette dernière solution après avoir contrôlé, par expérience, l'influen-

ce de la variation de pression à l'intérieur de la colonne au moment de l'introduction de l'échantillon[3,4] en injectant des quantités de mélanges aux doses connues.

D'autres auteurs[5,6] se sont servis aussi de cette technique en injectant, pour d'autres séparations, jusq'à 3 cm³ de gaz.

L'appareil employé est le Gasofract 300 B avec cellule de détection par thermoconductivité. Un robinet à deux voies permet la déviation du gaz porteur d'une colonne à l'autre.

Le prélèvement de l'échantillon est exécuté au moyen d'une seringue parfaitement calibrée dont l'aiguille a la pointe fermée par une membrane de caoutchouc que l'on perce au moment de l'injection. Il est nécessaire d'effectuer de nombreux lavages de la seringue avec le gaz à analyser.

Le seul inconvénient c'est que le tamis dure peu parce qu'il se sature de N_2O après une quarantaine d'injections; toutefois on peut fort bien le régénérer à 350° pendant quatre heures environ, lorsqu'on commence à remarquer dans le chromatogramme une séparation défectueuse de O_2–N_2.

Résultats

Grâce à cette technique on a pu analyser rapidement des mélanges de N_2O contenant des quantités d'oxygène jusqu'à 5 mm³ et d'azote jusqu'à 10 mm³ avec une erreur

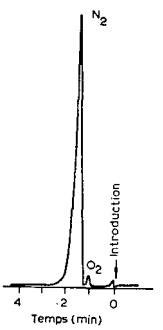

Fig. 1.

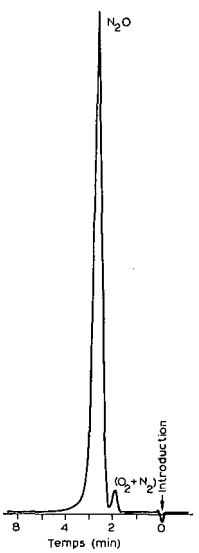

Fig. 2.

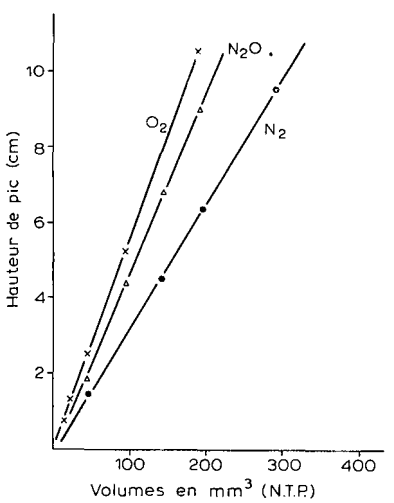

Fig. 3.

± 5%. La Fig. 1 montre la séparation N_2O–O_2–N_2 sur tamis moléculaire à 20°. La colonne a 90 cm de long et 6 mm de diamètre intérieur. Gaz porteur: hydrogène 50 cm³/min. Intensité 160 mA, volume du mélange introduit 4.962 cm³ (N.T.P.). Composition: 4.9 mm³ O_2 (0.11%), 225 mm³ N_2 (4.56%). La Fig. 2 montre la séparation N_2O–(N_2 + O_2) sur brique pilée (60–80 mesh) avec 25% d'huile de vaseline à forte viscosité. La colonne a une longueur de 6 m et 6 mm de diamètre intérieur. Gaz porteur: hydrogène 50 cm³/min. Intensité 160 mA. Volume du mélange introduit: 272.2 mm³ (N.T.P.). Composition: 259.4 mm³ N_2O (95.40%), 22.74 mm³ O_2 + N_2 (4.60%).

On peut voir sur le diagramme de la Fig. 3 le rapport constant entre la hauteur des pics et la quantité du gaz introduit. On peut ainsi contrôler, avec les deux colonnes, la correspondance des valeurs quant à la composition du mélange analysé.

Laboratorio di Igiene e Profilassi, Reparto chimico, Arezzo (Italy) MARCELLO MORGANTINI

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Announcement

IV. SYMPOSIUM ÜBER GAS-CHROMATOGRAPHIE IN DER DEUTSCHEN DEMOKRATISCHEN REPUBLIK

VEB LEUNA-WERKE "WALTER ULBRICHT" IN VERBINDUNG MIT DER DEUTSCHEN AKADEMIE DER WISSENSCHAFTEN ZU BERLIN, UNTERKOMMISSION FÜR GAS-CHROMATOGRAPHIE

Der VEB Leuna-Werke "Walter Ulbricht" beabsichtigt in Zusammenarbeit mit der Unterkommission für Gas-Chromatographie bei der Sektion Chemie der Deutschen Akademie der Wissenschaften zu Berlin

vom 28. bis 31. Mai 1963
das IV. Symposium über Gas-Chromatographie

im Klubhaus des VEB Leuna-Werke "Walter Ulbricht", Leuna (Kr. Merseburg) zu veranstalten.

Wir gestatten uns, Sie zu diesem Symposium einzuladen. Vortragsanmeldungen und Beteiligung an einer am Tagungsort vorgesehenen Geräteausstellung wollen Sie bitte bis zum 1. November 1962 ankündigen an die Adresse:

Unterkommission für Gas-Chromatographie,
Leipzig O5, Permoserstrasse 15.

Es ist vorgesehen, das Vortragsmaterial des Symposiums noch vor der Tagung den Teilnehmern in Buchform als Manuskript gedruckt zur Verfügung zu stellen, um eine angeregte wissenschaftliche Diskussion zu fördern. Wir erlauben uns daher, die Manuskripte der Vorträge bis zum 15. Januar 1963 anzufordern.

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TABLE 1

R_F VALUES OF SOME TRYPTAMINE DERIVATIVES

(K. MACEK AND S. VANĚČEK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 2705)

Solvents: S_1 = *n*-Butanol–acetic acid–water (4:1:5).
S_2 = *n*-Amyl alcohol–acetic acid–water (4:1:5).
Paper: Whatman No. 1.
Detection: D_1 = U.V. light (high pressure mercury vapour lamp: Philora).
D_2 = U.V. light (low pressure mercury vapour lamp: Chromatolite).
D_3 = U.V. light (Chromatolite) after spraying with 0.0025% fluorescein in 0.5 *N* NH_4OH.
D_4 = *p*-Dimethylaminobenzaldehyde (1 g) in acetone–HCl (95 ml + 5 ml), spray.
D_5 = D_4 spray, then heated (100°; short period) followed by 3% nitroprusside in 50% trichloroacetic acid spray, further heating (100°; 3–5 min) and U.V. light viewing.
D_6 = 0.2% ethanolic ninhydrin soln.
D_7 = Reagent spray (1 g iodine azide in 100 ml 0.005 *N* iodine soln.) followed by starch soln. spray.
D_8 = Formaldehyde (35–40%)–conc.HCl–water (1:1:2), spray.

Substance	$R_F \times 100$		*Colour*[a,b]							
	S_1	S_2	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8[c]
Tryptamine	65	35		+b	++b	++f	++bf	++		++g (++o)
5-Methyltryptamine	70	44		+b	++b	++f	++b	++		++o (++o)
5-Fluorotryptamine	68	41	+b	+b	++b	++f	++bf	+a		++g (++g)
5-Methoxytryptamine	59	24			++b	++bf	++b	+az		+r (++v)
5-Ethoxytryptamine	66	35			++b	++bf	++b	+az		+r (++v)
5-Methylmercaptotryptamine	70	39			++	++bf	++bf	++a	++	++r (++v)
6-Fluorotryptamine	72	42	+a	+a	++b	+f	+f	++hf		S (++g)
6-Methoxytryptamine	61	28	+b	++b	++b	++b	++z	+a		++gh (++o)
6-Ethoxytryptamine	67	36	+b	++b	?	++b	++z	?		?
5,6-Methylenedioxytryptamine	56	23	+a	++a	++b	++b	++z	+a		++f (++v)
7-Methylmercaptotryptamine	70	41			++	++b	++b	++a	++	+g (+v)

[a] If no result given for detection agent reaction negative.
[b] Abbreviations: a = gray; b = blue; g = yellow; h = brown; n = negative; o = orange; r = rose; v = quenched; f = violet; z = green; S = weak reaction.
[c] Assessment in parenthesis is for U.V. fluorescence.

TABLE 2

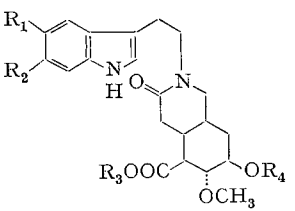
R_F VALUES OF SOME LACTAMS

(K. MACEK AND S. VANĚČEK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 2705)

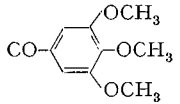
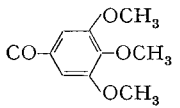
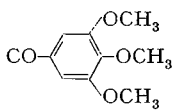
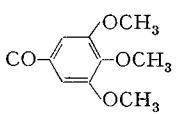
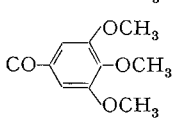
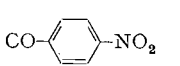
Solvents: S_1 = Paper impregnated with 50 % ethanolic formamide solution; solvent: chloroform.
S_2 = Paper impregnated with 50 % ethanolic formamide solution (containing 5 % ammonium formate); solvent: chloroform.
S_3 = Formamide/benzene–chloroform (1:1).
S_4 = Formamide + ammonium formate/benzene–chloroform (1:1).
S_5 = Formamide/benzene.
S_6 = Formamide + ammonium formate/benzene.
S_7 = Formamide/benzene–cyclohexane (1:1).
S_8 = Formamide + ammonium formate/benzene–cyclohexane (1:1).

Paper: Whatman No. 4.

Detection: D_1 = U.V. light (high pressure mercury vapour lamp (Philora)).
D_2 = U.V. light (low pressure mercury vapour lamp (Chromatolite)).
D_3 = U.V. light observation following spray with 0.0025 % fluorescein in 0.5 *N* ammonia (Chromatolite).
D_4 = 3 % sodium nitroprusside in 50 % trichloroacetic acid, spray, then U.V. light viewing.
D_5 = *p*-Dimethylaminobenzaldehyde (1 g) in ethanol–HCl (95 ml : 5 ml).
D_6 = D_5 spray followed by heating (100°, short period), then D_4 spray followed by further heating (100°, 3–5 min).



				$R_F \times 100$								Colour[a,b]					
R_1	R_2	R_3	R_4	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4[c]	D_5	D_6
F	H	CH_3	H	48	54	09	13	02	02					+b	Sa (Sf)	Sf	+f
CH_3O	H	CH_3	H	63	71	10	16	02	02			Sb	Sb	+b	+a (++f)	++bf	++b
C_2H_5O	H	CH_3	H	78	85	23	29	04	05			Sb	Sb	+b	+a (++f)	++bf	++b
CH_3S	H	CH_3	H	74	83	19	26	04	07			Sz		++	++h (+fh)	++f	++b
H	CH_3O	Lactone		90	93	51	58	16	20			Sb		+b	+o (++zb)	++b	++zb
H	H	CH_3	$COCH_3$	97	98	79	87	50	54	05	09	+b	+b	+b	+o (+fh)	++f	++b
CH_3	H	CH_3	$COCH_3$	97	98	85	91	66	72	09	15			+b	+ah (++f)	++f	++b

F	H	CH_3	$COCH_3$	96	98	79	83	48	54	04	07			+b	Sah (Sah)	Sf	+f
H	CH_3O	CH_3	$COCH_3$	97	98	81	86	47	55	03	06			+b	+h (++zb)	++f	++zb
CH_3	H	CH_3						90	93	40[d]	52			?	?	+f	++b
F	H	CH_3						88	87	17	29			++b	+a	Sh	+f
C_2H_5O	H	CH_3						92	92	29	44			++b	+a (Sf)	++bf	++b
CH_3S	H	CH_3						91	91	26	42	Sa	Sa	++b	+a	Sf	+b
H	CH_3O	CH_3						92	87	53[d]	51[d]			+	+h (++zb)	++b	++zb
H	H	CH_3				93	93	80	81	28	27	+v	++	++	Sah (Shf)	++f	++b

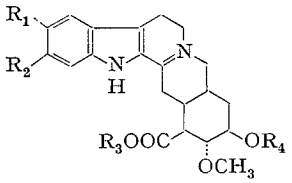
[a] If no result is given then the substance does not react with the detection agent.
[b] Abbreviations: a = gray; b = blue; g = yellow; h = brown; n = negative; o = orange; r = rose; v = quenched; f = violet; z = green; S = weak reaction.
[c] Assessments in parentheses refer to reactions on formamide-impregnated papers; notes in parentheses indicate colours assessed in daylight.
[d] Elongated spots.

TABLE 3

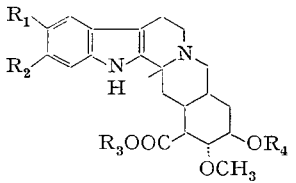
R_F VALUES OF SOME DEHYDRORESERPENOID AND RESERPENOID COMPOUNDS

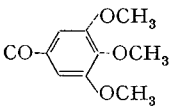
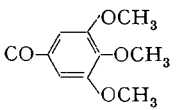
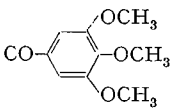
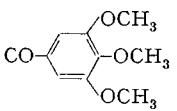
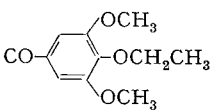
(K. MACEK AND S. VANĚČEK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 2705)

Solvents: S_1 = Paper impregnated with 50 % ethanolic formamide solution; solvent: chloroform.
S_2 = Paper impregnated with 50 % ethanolic formamide solution (containing 5 % ammonium formate); solvent: chloroform.
S_3 = Formamide/benzene–chloroform (1:1).
S_4 = Formamide + ammonium formate/benzene–chloroform (1:1).
S_5 = Formamide/benzene.
S_6 = Formamide + ammonium formate/benzene.
S_7 = Formamide/benzene–cyclohexane (1:1).
S_8 = Formamide + ammonium formate/benzene–cyclohexane (1:1).
S_9 = Formamide + ammonium formate/cyclohexane.
Paper: Whatman No. 4.
Detection: D_1 = U.V. light (high pressure mercury vapour lamp (Philora)).
D_2 = U.V. light (low pressure mercury vapour lamp (Chromatolite)).
D_3 = U.V. light observation (Chromatolite) following spray with 0.0025 % fluorescein in 0.5 *N* ammonia.
D_4 = 3 % nitroprusside in 50 % trichloroacetic acid spray, then viewing in U.V. light.
D_5 = *p*-Dimethylaminobenzaldehyde (1 g) in ethanol–HCl (95 ml:5 ml).
D_6 = D_5 spray followed by heating (100°; short period) then D_4 spray followed by further heating (100°; 3–5 min).



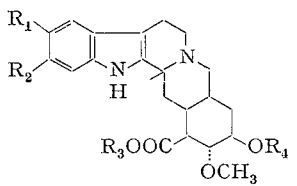
				$R_F \times 100$									Colour[a,b]					
R_1	R_2	R_3	R_4	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	D_1	D_2	D_3	D_4[c]	D_5	D_6
H	CH_3O	Lactone		01	045	00	00						++z	++z	++z	++z (+z)		
CH_3O	H	CH_3	$COCH_3$	97	75	78	22	28[d]	10				++gh	++gh	++h	++gh (+zh)		
H	CH_3O	CH_3	$COCH_3$	89	49	54	08	09	03				++z	++z	++z	++z (+gh)		

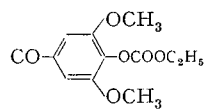
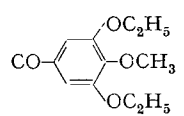
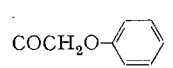
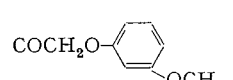
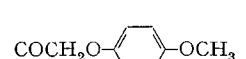
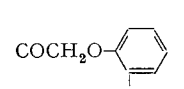


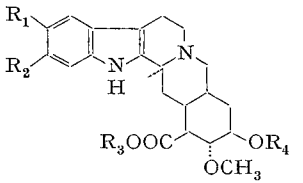
R_1	R_2	R_3	R_4	$R_F \times 100$									Colour[a,b]					
				S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	D_1	D_2	D_3	D_4[c]	D_5	D_6
H	H	CH_3	H	64	16	15	015						+a	+b	+b	++z		
H	CH_3O	CH_3	H	63	15	13	01						++z	++z	++z	++		Shf
H	CH_3O	CH_3						96	75	61	20	00	++z	++z	++z	++z (Sz)		Sh
H	H	CH_3						95	83	75	34	00	++a	++a	+	++z		
F	H	CH_3						97	85	74	36	00	Sa	+a	+	+z		
C_2H_5O	H	CH_3						97	91	76	41	00	+zh	+zh	+h	++gh (Sz)		Sb
H	CH_3O	CH_3						96	85	76	36	00	++z	++z	+z	++z (Sz)		Shf

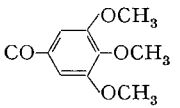
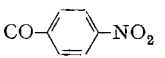
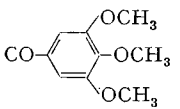
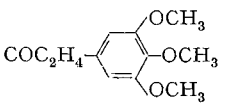
(continued on p. D6)

TABLE 3 (*continued*)



R_1	R_2	R_3	R_4	$R_F \times 100$									Colour[a,b]					
				S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	D_1	D_2	D_3	D_4[c]	D_5	D_6
H	CH_3O	CH_3						96	89	74	31	00	++z	++z	+z	++z		Shf
H	CH_3O	CH_3						97	92	88	57	01	++z	++z	+	++z		Shf
H	CH_3O	CH_3						94	64	55	12		++z	++z	S	++z (+z)		Shf
H	CH_3O	CH_3						91	65	50	12		++z	++z	S	++z (+z)		Shf
H	CH_3O	CH_3				97	81	91	61	46	10		++z	++	S	++z (+z)		
H	CH_3O	CH_3						81	45	29	06		++z	++	S	++		



				$R_F \times 100$									Colour[a,b]					
R_1	R_2	R_3	R_4	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	D_1	D_2	D_3	D_4[c]	D_5	D_6
CH_3O	H	CH_3	H	88	57	50	11	19	04				++gh	++gh	Sr	++gh (Sz)		Sb
CH_3O	H	CH_3	$COCH_3$					96	85	69	42	01	++gh	++a	Sh	++gh (Sz)		Sb
CH_3	H	CH_3								97	92	10	++ah	++a	+	++z (Sz)		
H	H	CH_3								94	86	05[d]	++z	+a	+	++z		
H	H	CH_3								95	88	055	++a	++a	+	++z		
H	H	CH_3								92	81	02	++z	++a	+a	++z		
H	H	H	H	85	52	52	13	30	06				++z	++z	Sb	++z		

[a] If no result is given then the substance does not react with the detection agent.
[b] Abbreviations: a = gray; b = blue; g = yellow; h = brown; n = negative; o = orange; r = rose; v = quenched; f = violet; z = green; S = weak reaction.
[c] Assessments in parentheses refer to reactions on formamide-impregnated papers; notes in parentheses indicate colours assessed in daylight.
[d] Elongated spot.

TABLE 4

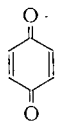
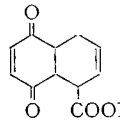
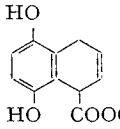
R_F VALUES OF HYDRONAPHTHALENE AND DECALIN DERIVATIVES

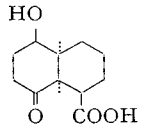
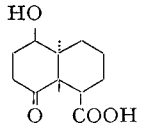
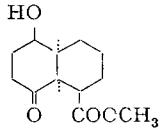
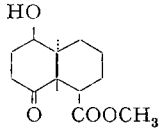
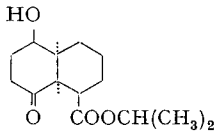
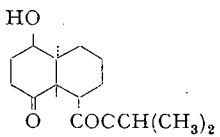
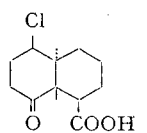
(K. MACEK AND S. VANĚČEK, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 2705)

Solvents: S_1 = Isopropanol–ammonia–water (10:1:1).
S_2 = *n*-Amylalcohol–acetic acid–water (4:1:5).
S_3 = Paper impregnated with 50% ethanolic formamide solution; solvent: chloroform.
S_4 = Paper impregnated with 50% ethanolic formamide solution (containing 5% ammonium formate); solvent: chloroform.
S_5 = Formamide/benzene–chloroform (1:1).
S_6 = Formamide/benzene.
S_7 = Formamide/benzene–cyclohexane (1:1).
S_8 = Dimethyl formamide/cyclohexane.

Paper: P_1 = Whatman No. 1 for all aqueous based systems.
P_2 = Whatman No. 4 for all formamide based systems.

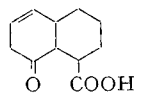
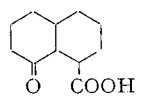
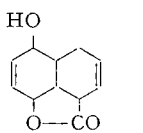
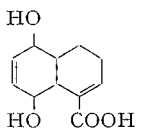
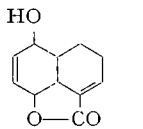
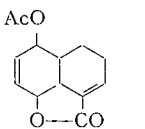
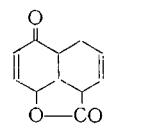
Detection: D_1 = U.V. light (high pressure mercury vapour lamp: Philora).
D_2 = U.V. light (low pressure mercury vapour lamp: Chromatolite).
D_3 = U.V. light (Chromatolite) after spraying with 0.0025% fluorescein in 0.5 *N* ammonia.
D_4 = 1% $KMnO_4$ + 2% Na_2CO_3 (1:1), spray.
D_5 = 0.1 *N* $AgNO_3$ + 5 *N* NH_4OH (1:1), spray (chromatogram heated to 100°).
D_6 = 0.2% 2,4-dinitrophenylhydrazine in 0.1 *N* HCl (methanolic), spray.
D_7 = Potassium periodate (satd. aq.) spray followed after 6 min with 0.1 *M* benzidine in 50% methanol–acetone–0.2 *N* HCl (10:2:1).
D_8 = 0.5% ethanolic Bromophenol Blue (formamide systems incompatible).
D_9 = U.V. light observation of chromatogram laid on layer of conc. H_2SO_4 on glass plate.

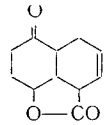
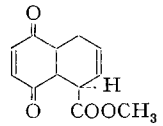
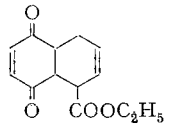
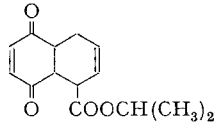
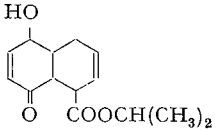
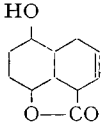
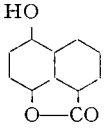
Substance	$R_F \times 100$								*Colour*[a,b]								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
						74	45	20	++v (o)	++ (o)	++ (o)	++ (o)	++[c] (+)	++ (o)			+bv
		75	10[d]	12					+b (++g)	+ (++g)	++ (++)	++ (++)	++[c] (+)	++ (++)	+ (o)	++	+o
		87	055	05					o (++g)	++ (+)	++ (++)	++ (++)	++[c] (++)		+ (+)		+o

	38	72		03					o (S)	S (+)	++n (++n)	+
	42	77		04					o (S)	+ (++)	++n (++n)	+
		?	?	?								
		88	80	85	54	31				+ (+)		
		?	?	?								
		92	95	95	83	66	34	++b (+b)	+b (+b)	+ (+)		
				57	38	22[d]				(S)	+ (+)	++n (+n)	+

(continued on p. D10)

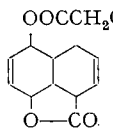
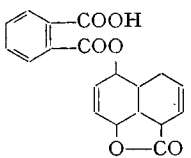
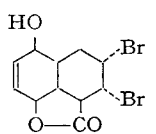
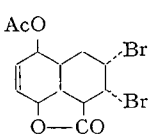
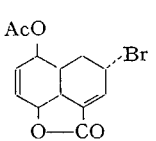
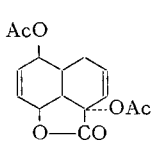
TABLE 4 (*continued*)

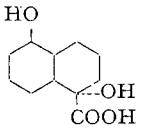
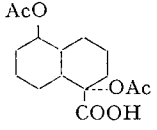
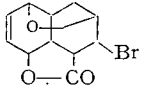
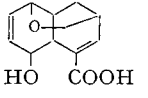
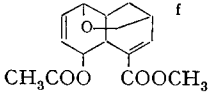
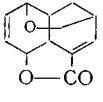
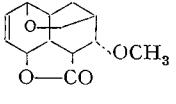
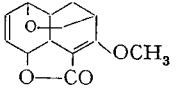
Substance	$R_F \times 100$								Colour[a,b]								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
		83	53[d]	51	19[d]					++ (++)	++ (++)	++ (++)	(S)	+ (++)	++n (++n)	++	
	57	88		72	38	22[d]							(S)	(S)	++n (+n)	+	
	80	79	37	40	13	05						++ (++)					++z
	34	71		01								++ (++)	(S)		++n	+	
		79	58	59	22	10				+ (+)	+ (+)	++ (++)					++z
					90	74	38					++					
			87	89	66	46	14		(+z)	(++z)	S (++)	++ (++)	+ (++)	+ (+)			+go

		81	84	45	25	05				++ (++)		+ (S)		
				84	74	39	08	(++v)	++ (++v)	++ (++)	++ (++)	++[c] (++)	++ (++)	(S)	+o
				92	85	63	18	(++v)	++	++	++ (++)	++[c] (++)	+ (++)	(S)	+o
				97	92	82	36	(++v)	++ (++v)	++ (++)	++ (++)	++[c] (++)	+ (++)	(S)	+o
		82	84	50	28	06			+ (+)	+ (+)	++ (++)				
	75	57	60	17	06						++ (++)				+r[e]
	80	57	59	23	09										+z (+r)[e]

(continued on p. D12)

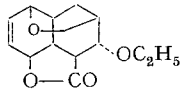
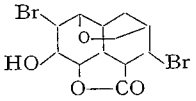
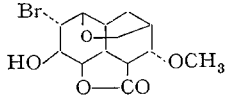
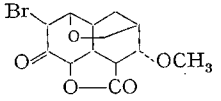
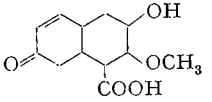
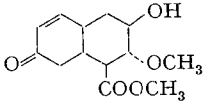
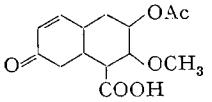
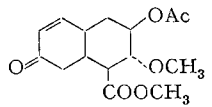
TABLE 4 (*continued*)

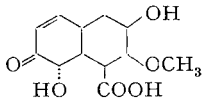
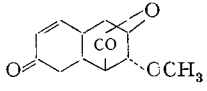
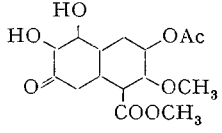
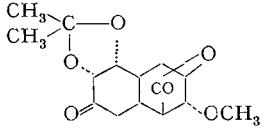
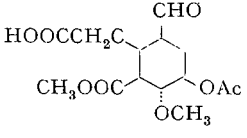
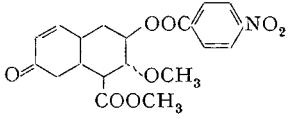
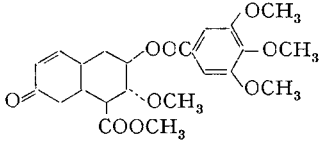
Substance	$R_F \times 100$								Colour[a,b]								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
	79	25[d]	36	09[d]					+b			++ (++)	(S)		++n	+	+z
	85	12[d]	16							(+)	+ (+)	++ (++)	(S)		+n	++	
	88	54	54	29	20	04					S	++ (++)	++ (++)		(Sn)		+z (++z)
					94	79	14					++ (++)	S (++)				(++z)
					89	63	07			++	+ (+)	+ (++)	+ (++)				++z
					91	71	12					++ (++)					++z

	48	77	00	00										+n (+n)	++	
	70	92													++	
					80	66	28	045		+	+ (++)	++ (++)	++ (++)			++z
	47	69	03	02							+ (+)	++ (++)		+n (+n)	++	++z
 [f]						89	71	30				++ (++)				
					78	54	20			++ (+)	++ (++)	++ (++)				++z
					81	58	24					++ (++)				+z
			88	88	57	27	06		++b (++b)	+b (+b)		++ (++)				

(continued on p. D14)

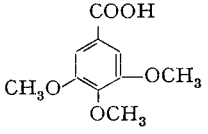
TABLE 4 (*continued*)

Substance	$R_F \times 100$								Colour[a,b]								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
					91	77	50	18	+b (+b)	+b (+b)	++ (++)						+z
	48	80	41		17	10	02			+	+ (+)	+ (+)	++ (++)				
		82	44		17	07			+b	+b			(+)				
		70	31	55[d]	12[d]					++	+ (+)	++ (++)	+ (++)	+ (++)			
	28	63		02					+b	+	+ (+)	++ (++)		++ (++)	+n (+n)	++	
		78	70	71	27	085				+	+ (+)	++ (++)		++ (++)			
	45	84	17	34	07[d]						+ (+)	++ (++)		+ (++)	+n (+n)	++	
					96	84	53	11	+b (+b)		+ (+)	++ (++)		+ (+)			

		54	00	00						++	++ (+)	•++ (++)	(+)	++ (+)	+n (+n)	++	Sg
			82	85	47	17	025	00		++ (+)	++ (++)	++ (++)		++ (+)			
		65	48	53	11				++b (+az)	+b (+a)	(+)	+ (+)	(S)	++ (++)	+ (+)		
					93	74	37				(S)		(S)	+ (+)			+r
		51	92	32[d]	37	07[d]								+ (+)			
					97	94	79	05	+v (+v)	++ (++)	++ (++)	++ (++)		+ (+)			
					97	96	86	04		++ (+)	++ (+)	+ (+)		+ (+)			

(continued on p. D16)

TABLE 4 (*continued*)

Substance	$R_F \times 100$								Colour[a,b]								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9
$CH_2=CH-CH=CHCOOH$	52	92	00						(+az)	++ (+a)	++ (S)	++ (++)			+n	++	
	52	88	21	58	18[d]					++ (++)	++ (++)					+	

[a] If no result given for detection agent reaction negative.
[b] Abbreviations: a = gray; b = blue; g = yellow; h = brown; n = negative; o = orange; r = rose; v = quenched; f = violet; z = green; S = weak reaction.
Assessment in parenthesis refers to results for formamide-impregnated paper.
[c] Immediate reaction.
[d] Elongated spot.
[e] Daylight colour.
[f] Detection not attempted where amount insufficient.

TABLE 5

R_F VALUES OF DIMETHYL-, TRIMETHYL- AND TETRAMETHYL-MANNOSES

(O. K. ORLOVA AND E. P. ROMENSKAYA, *Biokhimiya*, 26 (1961) 646)

Solvent: *n*-Butanol–ethanol–water (40:11:19).
Paper: Not specified (18–20 cm diam. for circular method).
Time of run: 4 h.
Detection: Aniline hydrogen phthalate.

Compound	R_F			
	Circular		*Descending**	
	A	*B*	*A*	*B*
Tetramethyl-mannose	0.96	0.98	0.97	0.97
Trimethyl-mannose	0.89	0.91	0.89	0.88
Dimethyl-mannose	0.74	0.76	0.75	0.73

A = separate sugars; B = hydrolysate.
* See O. K. ORLOVA, *Biokhimiya*, 23 (1958) 4.

TABLE 6

R_F VALUES (RELATIVE) OF METHYL ETHERS OF 6-DEOXYHEXOSES

(A. P. MACLENNAN, H. M. RANDALL AND D. W. SMITH, *Biochem. J.*, 80 (1961) 309)

Solvents: S_1 = Butan-1-ol–ethanol–water (4:1:5, by vol., upper phase).
S_2 = Butan-1-ol–pyridine–water (6:4:3, by vol.).
Paper: Whatman No. 1 (descending).
Length of run: 50 cm.
Detection: *p*-Anisidine–HCl spray (110°, 3–5 min).

Compound	R_{Rh}*		R_{TMG}*		*Colour***
	S_1	S_2	S_1	S_2	
2-O-Methylfucose	1.32	1.18	0.57	0.79	r-b
3-O-Methylfucose	1.18	—	0.53	—	g-b
3,4-Di-O-methylfucose	1.60	—	0.70	—	g-b
2-O-Methylrhamnose	1.48	1.28	0.65	0.85	r-b
3-O-Methylrhamnose	1.36	—	0.60	—	g-b
2,4-Di-O-methylrhamnose	1.96	1.46	0.86	0.96	g-b
2,3-Di-O-methylrhamnose	1.92	—	0.83	—	r-b
3,4-Di-O-methylrhamnose	1.98	—	0.88	—	g-b
2,3,4-Tri-O-methylrhamnose	2.32	—	0.99	—	g-b

* R_{Rh} = R_F compound/R_F rhamnose; R_{TMG} = R_F compound/R_F tetramethylglucopyranose.
** r = red; b = brown; g = green.

TABLE 7

R_F VALUES (RELATIVE) OF FIVE SUGARS

(N. L. BLUMSON AND J. BADDILEY, *Biochem. J.*, 81 (1961) 114)

Solvents: S_1 = Butan-1-ol–pyridine–water (6:4:3) (A. JEANES, C. S. WISE AND R. J. DIMLER, *Anal. Chem.*, 23 (1951) 415).
S_2 = Phenol–water (9:1) (S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
Paper: Whatman No. 1 (2 *N* CH_3COOH wash, then water wash).
Detection: D_1 = Alkaline $AgNO_3$ reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444).
D_2 = Phloroglucinol–HCl reagent (E. BORENFREUND AND Z. DISCHE, *Arch. Biochem. Biophys.*, 67 (1957) 239).

Compound	R_{Ri}*	
	S_1	S_2
Fructose	0.85	0.65
Glucose	0.77	0.47
Mannose	0.85	0.55
Galactose	0.70	0.56
Rhamnose	1.09	1.05

* R_{Ri} = R_F compound/R_F ribose.

TABLE 8

ELECTROPHORETIC MOBILITIES OF FIVE SUGARS

(N. L. BLUMSON AND J. BADDILEY, *Biochem. J.*, 81 (1961) 114)

Electrolyte: 0.05 *M* sodium borate solution (pH 9.2) (R. CONSDEN AND W. M. STANIER, *Nature*, 169 (1952) 783).
Paper: Whatman No. 1.
Apparatus: R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
Potential applied: 7.5 V/cm.
Time of run: 6 h.
Migration units: *M* (cm).
Detection: Modified alkaline $AgNO_3$ reagent (J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65).

Compound	*M*
Fructose	1.15
Glucose	1.30
Mannose	0.89
Galactose	1.18
Rhamnose	0.66

TABLE 9

R_F VALUES (RELATIVE) OF SOME NUCLEOTIDES AND SUGARS

(J. BADDILEY, N. A. HUGHES AND A. L. JAMES, *J. Chem. Soc.*, (1961) 2574)

Solvents: S_1 = Ethanol–*M* ammonium acetate (pH 3.8) (70:35, v/v).
S_2 = Butan-1-ol–pyridine–water (6:4:3, v/v).
Paper: Whatman No. 1 (descending).
Detection: Not specified.

Compound	R_A*	R_R**
	S_1	S_2
α-D-Glucose-1-phosphate	0.60	
Fructose-1-phosphate	0.68	
Guanosine-5′-phosphate	0.32	
Guanosine-5′-pyrophosphate	0.05	
Diguanosine-5′-pyrophosphate	0.27	
Guanosine-5′-N-cyclohexylphosphoramidate	0.55	
Guanosine diphosphate glucose	0.12	
Guanosine diphosphate fructose	0.12	
Guanosine diphosphate mannose	0.13	
Glucose		0.70
Mannose		0.83
Fructose		0.79

* R_A = R_F compound/R_F adenosine.
** R_R = R_F compound/R_F ribose.

TABLE 10

R_F VALUES OF THYMIDINE AND URIDINE DERIVATIVES AS WELL AS CERTAIN SUGARS

(J. H. PAZUR AND E. W. SHUEY, *J. Am. Chem. Soc.*, 82 (1960) 5009)

Solvent: Ethyl alcohol–*M* ammonium acetate, pH 7.5 (7:3, by vol.).
Paper: Not specified.
Detection: U.V. light.

Compound	R_F
Thymidine triphosphate	0.11
Thymidine diphosphate	0.20
Thymidine monophosphate	0.26
Thymidine	0.76
Thymine	0.70
Uridine diphosphate glucose	0.22
Uridine triphosphate	0.07
α-D-Glucose-1-phosphate	0.18
D-Glucose	0.60
L-Rhamnose	0.68

TABLE 11

R_F VALUES (RELATIVE) OF SOME 3-AMINO-2,3-DIDEOXY-β-D-RIBOFURANOSIDES AND RELATED COMPOUNDS

(C. H. ANDERSON, W. W. LEE, L. GOODMAN AND B. R. BAKER, *J. Am. Chem. Soc.*, 83 (1961) 1900)

Solvents: S_1 = Benzene–water–methanol (2:1:6) (T. WIELAND AND W. KRACHT, *Angew. Chem.*, 69 (1957) 172).
S_2 = Water.
S_3 = Butanol–acetic acid–water (5:2:3) (D. M. BROWN, A. TODD AND S. VARADARAJAN, *J. Chem. Soc.*, (1956) 2388).
S_4 = Water saturated butanol (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162).
S_5 = Ethyl acetate–pyridine–water (2:1:2) (M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402).

Paper: P_1 = Schleicher & Schüll No. 2043B, acetylated (descending).
P_2 = Whatman No. 1 (descending).

Detection: D_1 = U.V. light.
D_2 = Ninhydrin reagent.
D_3 = Bromine reagent (F. WEYGAND, H. J. BESTMANN AND H. ZIEMANN, *Chem. Ber.*, 91 (1958) 1040).
D_4 = Periodate–permanganate reagent (R. U. LEMIEUX AND H. F. BAUER, *Anal. Chem.*, 26 (1954) 920).

Compound	R_{Ad}*				
	S_1P_1	S_2P_2	S_3P_2	S_4P_2	S_5P_2
Methyl-3(2)-chloro-2,3-dideoxy-2(3)-(ethylthio)-β-D-arabino(xylo)furanoside			1.56	3.10	
Methyl-3-deoxy-3-(ethylthio)-5-O-trityl-β-D-xylofuranoside	0.68 to 0.85				
Methyl-3(2)-amino-2,3-dideoxy-2(3)-(ethylthio)-5-O-trityl-β-D-arabino(xylo)furanoside	0.59				
Methyl-3-acetamido-2,3-dideoxy-2-(ethylthio)-5-O-trityl-β-D-arabinofuranoside	1.00				
Methyl-2-acetamido-2,3-dideoxy-3-(ethylthio)-5-O-trityl-β-D-xylofuranoside	1.00				
Methyl-3-acetamido-2,3-dideoxy-5-O-trityl-β-D-ribofuranoside	1.20				
Methyl-2-acetamido-2,3-dideoxy-5-O-trityl-β-D-ribofuranoside	1.20				
Methyl-3-deoxy-5-O-trityl-β-D-ribofuranoside	0.63				
Methyl-2,3-dideoxy-2-(ethylthio)-3-(tritylamino)-5-O-trityl-β-D-arabinofuranoside	0.19				
3-Acetamido-2,3-dideoxy-D-ribose		2.60	0.89		1.17
2-Amino-2,3-dideoxy-D-ribose·HCl			0.99		0.71

* R_{Ad} = R_F compound/R_F adenine.

TABLE 12

R_F VALUES (RELATIVE) OF COMPOUNDS IN THE 3′-AMINO-2′,3′-DIDEOXYADENOSINE CLASS AND RELATED ANALOGUES

(W. W. LEE, A. BENITEZ, C. D. ANDERSON, L. GOODMAN AND B. R. BAKER, *J. Am. Chem. Soc.*, 83 (1961) 1906)

Solvents: S_1 = Benzene–water–methanol (2:1:6) (T. WIELAND AND W. KRACHT, *Angew. Chem.*, 69 (1957) 172).
S_2 = Butan-1-ol–acetic acid–water (5:2:3) (D. M. BROWN, A. TODD AND S. VARADARAYAN, *J. Chem. Soc.*, (1956) 2388).
S_3 = Butan-1-ol–methyl ethyl ketone–water (5:3:2).
S_4 = Chloroform–ethanol–water (10:10:6; lower phase) (T. H. KRITCHEVSKY AND A. TISELIUS, *Science*, 114 (1951) 299).
S_5 = Butan-1-ol–water-saturated (J. G. BUCHANAN, *Nature*, 168 (1951) 1091).
S_6 = $(NH_4)_2SO_4$–isopropanol–water (2:28:70) (*cf.* R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 49 (1951) 401).
S_7 = Ethyl acetate–pyridine–water (2:1:2) (M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402).
S_8 = 5% Na_2HPO_4 pH 8.9 (C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 1466).
Paper: P_1 = Schleicher & Schüll No. 2496 (acetylated) or Ederol (acetylated) (descending). (For S_1)
P_2 = Whatman No. 1. (S_2-S_8)
Detection: U.V. light.

Compound	R_{Ad}*							
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
9-[3′-Azido-2′,3′-dideoxy-2′-(ethylthio)-β-D-arabinofuranosyl]-adenine	1.43		1.90	1.58				
9-[2′-Azido-2′,3′-dideoxy-3′-(ethylthio)-β-D-xylofuranosyl]-adenine	1.33		1.90	1.58				
9-[3′-Amino-2′,3′-dideoxy-2′-(ethylthio)-β-D-arabinofuranosyl]-adenine		1.08	1.38					
9-(3′-Amino-2′,3′-dideoxy-β-D-ribofuranosyl)-adenine		0.83	(1.13**)					1.34
9-(2′-Amino-2′,3′-dideoxy-β-D-ribofuranosyl)-adenine		0.76						1.28
3′-Deoxyadenosine***					0.80	1.28	1.14	
2′-Deoxyadenosine					0.69			

* R_{Ad} = R_F compound/R_F adenine.
** Trace constituent.
*** R_{Ad} for water: 1.51.

TABLE 13

R_F VALUES OF NUCLEOSIDE PHOSPHITES AND SIMILAR COMPOUNDS

(J. A. SCHOFIELD AND A. TODD, *J. Chem. Soc.*, (1961) 2316)

Solvents: S_1 = Propan-2-ol–ammonia (sp. gr. 0.88)–water (7:1:2).
S_2 = Butan-1-ol–acetic acid–water (5:2:3).
S_3 = Propan-2-ol–ammonia (sp. gr. 0.88)–acetic acid–water (4:1:2:2).
Paper: Whatman No. 1 (ascending).
Detection: Not specified.

Compound	R_F		
	S_1	S_2	S_3
2′,3′-O-Isopropylideneadenosine	0.93		
Phosphorous acid	0.13		
2′,3′-O-Isopropylideneadenosine-5′-phosphite	0.72		
2′,3′-O-Isopropylideneadenosine-5′-phosphate	0.29		
2′,3′-O-Isopropylideneuridine-5′-phosphite	0.87		
2′,3′-O-Isopropylideneuridine	0.58		
Thymidine-3′-phosphite	0.62		
Thymidine-5′-phosphite	0.53		
Deoxyadenosine-5′-phosphite	0.59		
Deoxyadenosine-3′-phosphite	0.46		
Deoxyadenosine-5′-phosphate	0.16	0.23	0.63
Deoxyadenosine	0.70		

TABLE 14

ELECTROPHORETIC MOBILITIES OF FOUR 1-β-D-ALDOPENTOFURANOSYL-URACILS AND THEIR THYMINE HOMOLOGUES

(J. F. CODINGTON, R. FECHER AND J. J. FOX, *J. Am. Chem. Soc.*, 82 (1960) 2794)

Electrolytes: E_1 = Borate buffer pH 6.0–6.05 (225 g boric acid in 4 l water, dissolved with warming), adjusted to given pH with 10 *N* NaOH).
E_2 = Borate buffer, pH 6.0.
Paper: P_1 = Whatman No. 3MM.
P_2 = *cf.* M. P. GORDON, O. M. INTRIERI AND G. B. BROWN, *J. Am. Chem. Soc.*, 80 (1958) 5161.
Apparatus: A_1 = E. C. model (E. C. Apparatus Co., Swarthmore, Pa., U.S.A.).
A_2 = *cf.* ref. for P_2.
Units: Anodic migration in cm.
Time of run: T_1 = 240 min.
T_2 = 180 min.
Potential drop: Pd_1 = 700 V at 17–19 mA.
Pd_2 = 600–700 V at 50 mA.
Detection: U.V. light after drying at 100–120°.

Aldopentofuranosyl group	*M*	
	$E_1P_1A_1T_1Pd_1$ *Uracil derivative*	$E_2P_2A_2T_2Pd_2$ *Thymine derivative**
Arabino	−2.7	−5.0
Xylo	+6.0	+2.9
Ribo	+9.9	+7.3
Lyxo	+14.3	+12.5

* Reported by M. P. GORDON, O. M. INTRIERI AND G. B. BROWN, *J. Am. Chem. Soc.*, 80 (1958) 5161.

TABLE 15

R_F VALUES OF VARIOUS PURINES, PURINE PHOSPHATES AND RELATED COMPOUNDS

(A. HAMPTON AND M. H. MAGUIRE, *J. Am. Chem. Soc.*, 83 (1961) 150)

Solvents: S_1 = Isopropanol–1% aq. $(NH_4)_2SO_4$ (2:1) (N. ANAND, V. M. CLARK, R. H. HALL AND A. R. TODD, *J. Chem. Soc.*, (1950) 3665).
S_2 = 0.3 *M* potassium phosphate buffer pH 6.9–isoamyl alcohol (two layers).
S_3 = Butan-1-ol–water (4:1:5).
Paper: Schleicher & Schüll No. 597 (ascending).
Detection: D_1 = U.V. photography (Corning Filter No. 9863).
D_2 = Periodate spray (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162), for *cis*-glycols.
D_3 = Molybdate–perchloric acid (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107), for phosphates.

Compound	R_F		
	S_1	S_2	S_3
6-Chloropurine	0.84	—	0.79
Hypoxanthine	0.57	0.61	0.38
6-Chloro-9-β-D-ribofuranosyl-purine	0.76	0.70	0.65
Inosine	0.46	0.72	0.28
6-Chloro-9-(2′,3′-O-isopropylidene-β-D-ribofuranosyl)-purine	0.90	0.60	0.90
Methyl di-*p*-nitrophenyl phosphate	0.90	0.00	0.90
Methyl *p*-nitrophenyl hydrogen phosphate	—	0.75	0.53
Di-*p*-nitrophenyl hydrogen phosphate	0.88	0.49	0.73
p-Nitrophenol	0.90	0.33	0.92
6-Chloro-9-(2′,3′-O-isopropylidene-β-D-ribofuranosyl)-purine-5′-di-*p*-nitrophenyl phosphate	0.90	0.00	0.90
6-Chloro-9-(2′,3′-O-isopropylidene-β-D-ribofuranosyl)-purine-5′-*p*-nitrophenyl hydrogen phosphate	0.78	0.65	0.73
D-*p*-Tolylurea	0.90	0.00	0.95
6-Chloro-9-β-D-ribofuranosyl-purine-5′-phosphate	0.54	0.82	0.24
Inosine-5′-phosphate	0.23	0.81	0.10

TABLE 16

R_F VALUES (RELATIVE) OF CERTAIN FURANOSYLURACIL DERIVATIVES AND RELATED COMPOUNDS

(R. FECHER, J. F. CODINGTON AND J. J. FOX, *J. Am. Chem. Soc.*, 83 (1961) 1889)

Solvent: Isopropanol–water (7:3).
Paper: Schleicher & Schüll No. 597.
Temperature of run: 22–23°.
Time of run: 18 h.
Detection: Not given.

Compound	R_F
2,2′-Anhydro-1-(3′,5′-di-O-mesylarabinosyl)-uracil	0.66
1-(3′,5′-Di-O-mesylarabinofuranosyl)-uracil	0.80
2,3′-Anhydro-1-(5′-O-mesyllyxofuranosyl)-uracil	0.64
1-(5′-O-Mesyllyxofuranosyl)-uracil	0.71
1-β-D-Lyxofuranosyluracil	0.64

TABLE 17

R_F VALUES (RELATIVE) OF SOME NUCLEOTIDES

(N. L. BLUMSON AND J. BADDILEY, *Biochem. J.*, 81 (1961) 114)

Solvents: S_1 = pH 3.8 ethanol–ammonium acetate–water.
S_2 = pH 7.5 ethanol–ammonium acetate–water.
S_1 and S_2: A. C. PALADINI AND L. F. LELOIR, *Biochem. J.*, 51 (1952) 426.
Paper: Whatman No. 1 (washed with 2 *N* CH_3COOH, then water).
Detection: D_1 = U.V. light.
D_2 = Perchloric acid–molybdate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).

Compound	R_{Ad}*	
	S_1	S_2
Uridine-5′-phosphate	0.72	0.33
Uridine-5′-pyrophosphate	0.38	0.18
Guanosine diphosphate mannose	0.23	0.30
Uridine diphosphate glucose	0.40	0.41
Thymidine-5′-phosphate	0.77	0.50
Uridine diphosphate N-acetylglucosamine	0.54	0.53

* R_{Ad} = R_F compound/R_F adenosine.

TABLE 18

R_F VALUES (RELATIVE) OF SOME PYRIMIDINES AND NUCLEOSIDES

(N. L. BLUMSON AND J. BADDILEY, *Biochem. J.*, 81 (1961) 114)

Solvents: S_1 = Butan-1-ol–water (R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294).
S_2 = Propan-2-ol–NH_4OH (sp.gr. 0.88)–water (85:1.3:15) (A. D. HERSHEY, J. DIXON AND M. CHASE, *J. Gen. Physiol.*, 36 (1953) 777).
S_3 = Propan-2-ol–conc. HCl (171:41) (G. R. WYATT, *Biochem. J.*, 48 (1951) 584).
Paper: Whatman No. 1 (2 *N* CH_3COOH wash, then water wash).
Detection: D_1 = U.V. light.
D_2 = Perchloric acid–molybdate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).

Compound	R_{Ad}*		
	S_1	S_2	S_3
Guanine	0.33	0.41	0.63
Uracil	0.78	0.97	1.88
Thymine	1.19	1.33	2.17
Uridine	0.38	0.79	1.83
Thymidine	1.12	1.46	2.31

* R_{Ad} = R_F compound/R_F adenine.

TABLE 19

R_F VALUES OF ASPARTIC ACID β-METHYL ESTER

(J. F. BIERNAT, R. RZESZOTARSKA AND E. TASCHNER, *Ann.*, 646 (1961) 125)

Solvents: S_1 = Pyridine–acetic acid–ethyl acetate–water (5:1:5:3).
S_2 = Pyridine–*sec.*-amyl alcohol–water (7:7:6).
Paper: Whatman No. 1 (descending).
Detection: Not specified.

Compound	R_F	
	S_1	S_2
L-Aspartic acid β-methyl ester	0.33	0.16

TABLE 20

R_F VALUES OF α-AMINO ACID *tert.*-BUTYL ESTERS

(E. TASCHNER, A. CHIMIAK, B. BATOR AND T. SOKOLOWSKA, *Ann.*, 646 (1961) 134)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Butan-1-ol–ethanol–water (5:1:4).
Paper: Whatman No. 1 (ascending).
Detection: Not specified.

tert.-Butyl ester of	R_F	
	S_1	S_2
Glycine	0.63	0.54
DL-Alanine	0.65	0.64
DL-α-Aminobutyric acid	0.71	0.69
DL-Valine	0.69	0.74
DL-Norvaline	0.75	0.70
L-Leucine	0.81	0.81
DL-Isoleucine	0.80	0.79
DL-Norleucine	0.81	0.80
DL-Phenylalanine	0.81	0.83
DL-Proline	0.66	0.62
L-Aspartic acid (diester)	0.81	0.86
L-Glutamic acid (diester)	0.83	0.82
L-Glutamic acid (γ-methyl ester)	0.71	0.70
S-Benzyl-L-cysteine	0.82	0.83
ε-Cbz-L-lysine	0.83	0.81

TABLE 21

R_F VALUES OF SOME N-(2-NITRO-4-SULPHOPHENYL)-AMINO ACIDS

(H. ZAHN AND K. H. LEBKÜCHER, *Biochem. Z.*, 334 (1961) 133)

Solvents: S_1 = Butan-2-ol–formic acid–water (75:15:10).
S_2 = Aqueous 80% phenol.
Paper: Not specified.

*Compound**	R_F	
	S_1	S_2
NSP-Glycine	0.13	0.20
NSP-Alanine	0.30	0.25
NSP-Valine	0.56	0.32
NSP-Leucine	0.62	0.35
NSP-Phenylalanine	0.54	0.37
NSP-Serine	0.08	0.14
NSP-Aspartic acid	0.13	0.10
NSP-Glutamic acid	0.19	0.12
N^{ε}-NSP-Lysine	0.10	0.46
N^{ε}-NSP-Hydroxylysine	0.14	0.58
O-NSP-Tyrosine	0.16	0.48
2-Nitrophenolsulphonic acid	0.24	0.52

* Abbreviation: NSP-amino acid = N-(2-nitro-4-sulphophenyl)-amino acid.

TABLE 22

R_F VALUES OF SOME GLYCINE PEPTIDES

(A. SCHMITT AND G. SIEBERT, *Biochem. Z.*, 334 (1961) 96)

Solvent: Butan-1-ol–acetic acid–water (8:2:2).
Paper: Not specified.
Detection: Not specified.

Peptide	R_F
L-Leucylglycine	0.60
Glycyl-L-leucine	0.61
L-Prolylglycine	0.29
Glycyl-L-proline	0.37
Glycyl-L-valine	0.48
Glycyl-L-serine	0.07
L-Serylglycine	0.16
Glycyl-L-threonine	0.19
Carnosine	0.10
Glycylglycine	0.17
Glycylglycylglycine	0.13

TABLE 23

R_F VALUES OF SOME γ-GLUTAMYL PEPTIDES AND RELATED COMPOUNDS

(E. TASCHNER, C. WASIELEWSKI, T. SOKOLOWSKA AND J. F. BIERNAT, *Ann.*, 646 (1961) 127)

Solvents: S_1 = Pyridine–acetic acid–ethyl acetate–water (5:1:5:3).
S_2 = Butan-1-ol–acetic acid–water (4:1:5).
S_3 = Butan-1-ol–acetic acid–water (62:12:26).
S_4 = Butan-1-ol–ethanol–water (5:1:4).
S_5 = Isoamyl alcohol–pyridine–water (7:7:6).
(S_2 organic phase ≡ S_3 (Editors))
Paper: Whatman No. 1 (descending).
Detection: Not specified.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
L-Glutamic acid α-*tert.*-butyl γ-methyl diester	0.89	0.78			
L-Glutamic acid α-*tert.*-butyl ester	0.78	0.71			
γ-L-Glutamylglycine	0.19	0.13			
L-Aspartic acid di-*tert.*-butyl ester·HCl			0.86		0.90
γ-L-Glutamyl (α-*tert.*-butyl ester)-L-aspartic acid di-*tert.*-butyl ester·HCl			0.93	0.94	0.93
γ-L-Glutamyl-L-aspartic acid	0.17		0.17	0.05	0.14

TABLE 24

R_F VALUES OF S-BENZYLCYSTEINE PEPTIDES AND PEPTIDE AMIDES

(K. JOŠT AND J. RUDINGER, *Collection Czech. Chem. Commun.*, 26 (1961) 2345)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5; S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
S_2 = Butan-1-ol–pyridine–acetic acid–water (S. G. WALEY AND J. WATSON, *J. Chem. Soc.*, (1953) 475).
Paper: Whatman No. 1 (descending).
Detection: Not specified.

*Compound**	R_F	
	S_1	S_2
H·Cys(Bz)·Phe·OH·H_2O	0.84	0.82
H·Cys(Bz)·Leu·OH	0.86	0.87
H·Cys(Bz)·Tyr·OH·0.5H_2O	—	0.75
H·Cys(Bz)·Tyr(Me)OH·1.5H_2O	—	0.75
H·Cys(Bz)·Phe·NH_2	0.82	0.86
H·Cys(Bz)·Gly·NH_2	0.40	0.57
H·Cys(Bz)·Leu·NH_2·HCl	—	0.90
H·Cys(Bz)·Tyr·Gly·OH·0.5H_2O	—	0.69
H·Gly·Cys(Bz)·Tyr·NH_2·0.5H_2O	—	0.66
O-Methyltyrosine	0.41	—

* All optically active amino acids have L-configuration.

TABLE 25

R_F VALUES OF GLYCYL-L-PHENYLALANYL-L-PHENYLALANYL-L-TYROSYL-L-THREONYL-L-PROLYL-L-LYSINE ACETATE

(J. E. SHIELDS AND F. H. CARPENTER, *J. Am. Chem. Soc.*, 83 (1961) 3066)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1) (L. J. REED, *J. Biol. Chem.*, 183 (1950) 451).
S_2 = Butan-1-ol–acetic acid–water–pyridine (30:6:24:50) (S. G. WALEY AND J. WATSON, *Biochem. J.*, 55 (1953) 328).
S_3 = Phenol–water (160:40) (I. SMITH, *Chromatographic Techniques*, Interscience, New York, 1958, p. 60).
S_4 = Ethanol–water–0.9 *N* NH_4OH (180:10:10) (I. SMITH, *loc. cit.*).
S_5 = *tert.*-Butanol–water–methyl ethyl ketone–diethylamine (80:80:40:8) (R. R. REDFIELD, *Biochim. Biophys. Acta*, 10 (1953) 344).
Paper: Whatman No. 1.
Detection: Not given.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Gly-Phe-Phe-Tyr-Thr-Pro-Lys acetate	0.33	0.61	0.91	0.40	0.55

TABLE 26

R_F VALUES OF SOME CARBOBENZOXY AND TOSYL DERIVATIVES OF PHENYLALANINE AND LYSINE PEPTIDES RELATED TO THE INSULIN B CHAIN

(J. KUNDE AND H. KAHN, *Ann.*, 646 (1961) 137)

Solvents: S_1 = Butan-2-ol–90% formic acid–water (75:15:10).
S_2 = Butan-2-ol–10% NH_4OH (85:15).
Paper: Not specified.
Detection: Not specified.

Compound	R_F	
	S_1	S_2
Cbz-Gly-L-Phe	0.94	0.34
Cbz-L-Phe-L-Tyr Et ester	0.96	0.89
L-Phe-L-Tyr Et ester·HBr	0.74	0.89
Cbz-Gly-L-Phe-L-Phe-L-Tyr	0.95	0.55
N^{α}-Cbz-N^{ε}-Tos-L-Lys-L-Ala Me ester	0.95	0.95
N^{ε}-Tos-L-Lys-L-Ala Me ester·HBr	0,70	0.95
Cbz-L-Pro-N^{ε}-Tos-L-Lys-L-Ala Me ester	0.95	0.95
L-Thr-L-Pro-N^{ε}-Tos-L-Lys-L-Ala Me ester	0.65	0.76
Cbz-L-Gly-L-Phe-L-Phe-L-Tyr-L-Thr-L-Pro-N^{ε}-Tos-L-Lys-L-Ala Me ester	0.95	0.95
Cbz-L-Phe-L-Phe-L-Tyr Et ester	0.96	0.89
Cbz-L-Phe-L-Phe-L-Tyr	0.94	0.51
L-Phe-L-Phe-L-Tyr	0.78	0.35
Gly-L-Phe-L-Phe-L-Tyr	0.65	0.15

TABLE 27

R_F VALUES OF SOME PHOSPHOLIPIDS

(H. KALLER, *Biochem. Z.*, 334 (1961) 451)

Solvents: S_1 = *tert.*-Butanol–butan-1-ol–0.001 *N* HCl (3:1:1) (S. HAJDU, H. WEISS AND E. TITUS, *J. Pharmacol. Exptl. Therap.*, 120 (1957) 99).
S_2 = Butan-1-ol–ethanol–acetic acid–water (8:2:1:3) (K. B. AUGUSTINSSON AND M. GRAHAM, *Acta Chem. Scand.*, 7 (1953) 906).
S_3 = Propan-1-ol–acetic acid–water (8:1:1) (F. M. HUENNEKENS, D. J. HANAHAN AND M. UZIEL, *J. Biol. Chem.*, 206 (1954) 443).
S_4 = Ethyl methyl ketone–acetic acid–water (9:1:2).
Paper: Schleicher & Schüll 2043b.
Detection: D_1 = Phosphorus detection reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107, or H. E. WADE AND D. M. MORGAN, *Nature*, 171 (1953) 529).
D_2 = Phosphomolybdic acid reagent (T. H. BEVAN, G. I. GREGORY, T. I. MALKIN AND A. G. POOLE, *J. Chem. Soc.*, (1951) 841).
D_3 = Ninhydrin reagent (U. BEISS AND O. ARMBRUSTER, *Z. Naturforsch.*, 13b (1958) 79).
D_4 = Dinitrophenyl reagent (for sphingosinephosphorylcholine), spray; 1–2% 2,4-dinitrofluorobenzene in ethanol; then 5% aq. Na_2CO_3 solution; paper still moist washed in a tank with running tap water for 30 min; best viewed in short wave U.V. light with background of light blue fluorescent paper.

Compound	R_F			
	S_1	S_2	S_3	S_4
Sphingosinephosphorylcholine	0.34	0.58	0.71	0.29
Sphingosine	0.71	0.78	0.95	0.98
Choline	0.17	0.27	0.27	0.12
Sphingomyelin	0.79	0.83	0.97	Streak
Lysolecithin	0.66	0.77	0.88	0.70

TABLE 28

R_F VALUES OF SOME PHOSPHATIDES AND SPHINGOLIPIDS

(H. WAGNER AND L. HÖRHAMMER, *Biochem. Z.*, 333 (1960/61) 511)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5).
S_2 = Pyridine–butan-1-ol–water (7:25:25) + dibutyl ether (20:5).
Paper: Schleicher & Schüll paper (30 cm × 20 cm) treated with formaldehyde soln. DAB6 (30–40%) + glacial acetic acid (p.a.) + ammonium thiocyanate (100:5:0.2) at 10 atm for 3 h; formaldehyde paper (ascending) (L. HÖRHAMMER, H. WAGNER AND G. RICHTER, *Biochem. Z.*, 331 (1959) 155).
Detection: D_1 = Ninhydrin reagent.
D_2 = Phosphomolybdic acid reagent.
D_3 = Rhodamine B reagent.
(D_1, D_2, D_3 used sequentially.)

Compound	R_F*	
	S_1	S_2
Cerebroside I	0.85	0.80
Cerebroside II (SO_4 ester)	0.60	0.25
Sphingomyelin	0.78	0.33
Ganglioside I	0.25–0.33	0.0–0.10
Ganglioside II	0.18–0.24	

* Average values for a number of determinations.

TABLE 29

R_F VALUES (THIN LAYER) OF PHOSPHATIDES AND GLYCOLIPIDS

(H. WAGNER, L. HÖRHAMMER AND P. WOLFF, *Biochem. Z.*, 334 (1961) 175)

Solvents: S_1 = Chloroform–methanol–water (65:25:4).
S_2 = Chloroform–methanol–water (60:35:8).
(S_1 and S_2: monophasic, freshly prepared).

Thin-layer carrier: Silica gel (Kieselgel G Merck nach E. STAHL); water suspension (25 g/50 ml for 5 plates, 12 × 20 cm); spread and then activated (110°; 20 min).

Temperature of run: 20–23°.

Time of run: 1.75–2 h.

Length of run: 15 cm.

Detection: D_1 = Ninhydrin sprayed (0.2% in 95 ml butan-1-ol and 5 ml 10% acetic acid); then heated (105°; 20 min), for aminophosphatides (red-violet spots).
D_2 = Dragendorff reagent spray (MUNIER AND MACHEBOEUF modification): solution I = basic bismuth nitrate (1.7 g) in 20% acetic acid (100 ml); solution II = potassium iodide (40 g) in water (100 ml). Spray reagent prepared by mixing solution I (20 ml) with solution II (5 ml) and water (70 ml), for choline phosphatides (orange to red-orange spots).
D_3 = Ammonium molybdate–perchloric acid spray (ammonium molybdate (3 g), water (25 ml), *N* HCl (30 ml), 60% perchloric acid (15 ml)); then heated (105°; 20 min), blue-black spots on weak grey background, for other lipids.
D_4 = Diphenylamine reagent (10% alc. diphenylamine (20 ml), conc. HCl (100 ml), acetic acid (80 ml)); spray with diphenylamine soln.; leave in HCl–acetic acid solution atmosphere for 30 min at 105°; then follow with D_2.

Compound	R_F		*Detection**			
	S_1	S_2	D_1	D_2	D_3	D_4
Amino acids	0–0.10		+	—	—	
Lysolecithin	0.21 ± 0.037		—	+	+	
Lecithin	0.39 ± 0.055		—	+	+	
Sphingomyelin	0.29 ± 0.055	0.46	—	+	+	+
Colaminecephalin	0.57 ± 0.075		+	—	+	
Cerebroside unesterified	0.78 ± 0.075	0.70–0.80	—	—	+	+
Cerebroside H_2SO_4-ester		0.50–0.54				+
Cardiolipin	0.92 ± 0.015		—	—	+	
Ganglioside a		0.25				+
Ganglioside b		0.30				+

* Positive reaction: + ; negative reaction: —.

TABLE 30

R_F VALUES (INCLUDING THIN LAYER) OF FOUR BOVINE BRAIN GANGLIOSIDES

(R. KUHN, H. WIEGANDT AND H. EGGE, *Angew. Chem.*, 73 (1961) 580)

Solvents: S_1 = Butan-1-ol–pyridine–water (6:5:4).
S_2 = Propan-1-ol–water (7:3).
Paper: P_1 = Schleicher & Schüll 2043b Mgl (descending).
Time of run: 18 h (S_1P_1).
Thin-layer adsorbent: T_1 = Silica gel G (plate activated: heated to 140° for 3 h).
Detection: D_1 = Not specified (for paper chromatography).
D_2 = Bromothymol blue.
D_3 = Ehrlich aldehyde reagent.

Compound	*Composition (mol/mol glucose)*						R_F	
	Fatty acid (stearic acid)	*Sphingosine*	*Glucose*	*Galactose*	*N-Acetyl-galactosamine*	*Lactaminic acid (NANA)*	S_1P_1	S_2T_1
G I	1	1	1	2	1	1	0.66	0.55
G II	1	1	1	2	1	2	0.47	0.35
G III	1	1	1	2	1	2	0.40	0.23
G IV	1	1	1	2	1	3	0.28	0.18

TABLE 31

R_F VALUES OF METHYLATED URACIL DERIVATIVES

(K. L. WIERZCHOWSKI AND D. SHUGAR, *Acta Biochim. Polon.*, 7 (1960) 63)

Solvent: Benzene–ethanol–water (169:45:15, v/v/v) (J. F. W. MCOMIE, E. R. SAYER AND J CHESTERFIELD, *J. Chem. Soc.*, (1957) 1830).
Paper: Whatman No. 1 (ascending).
Detection: Not specified.

Compound	R_F
Uracil	0.03
5-Methyluracil (thymine)	0.05 (tailing)
6-Methyluracil	0.07
5,6-Dimethyluracil	0.15
1,6-Dimethyluracil	0.32
3,6-Dimethyluracil	0.48
1,3,5-Trimethyluracil	0.72
1,5,6-Trimethyluracil	0.93
1,3,5,6-Tetramethyluracil	0.97

TABLE 32

R_F VALUES OF SOME PYRIMIDINES AND DIHYDROPYRIMIDINES

(C. JANION AND D. SHUGAR, *Acta Biochim. Polon.*, 7 (1960) 309)

Solvent: Butanol–acetic acid–water (2:1:1) (R. E. CLINE AND R. M. FINK, *Anal. Chem.*, 28 (1956) 47; R. M. FINK, R. E. CLINE, C. MCGAUGHEY AND K. FINK, *Anal. Chem.*, 28 (1956) 4).
Paper: Whatman No. 1 (ascending).
Detection: D_1 = 1% *p*-Dimethylaminobenzaldehyde spray.
D_2 = 0.5 *N* NaOH spray following D_1.

Compound	R_F	*Colour**	
		D_1	D_2
Dihydro-uracil	0.57	n	y
Dihydro-1-methyluracil	0.89	p	p
Dihydro-3-methyluracil	0.88	p	p
Dihydro-1,3-dimethyluracil	0.90	p	p
Dihydro-1-acetyluracil	0.70	n	y**
Dihydro-orotic acid	0.43	n	y
Dihydro-thymine	0.68	n	y
Dihydro-1,3-dimethylthymine	0.66	n	y
Dihydro-6-methyluracil	0.70	n	y
Dihydro-1,3,6-trimethyluracil	0.80	n	y
Dihydro-uridine	0.45	n	y
Dihydro-3-methyluridine	0.65	n	y
Dihydro-uridylic acid	0.25	n	y
Dihydro-thymidine	0.62	n	y
Dihydro-3-methylthymidine	—	n	n
Dihydro-thymidylic acid	0.37	n	y
1,3-Dimethyluracil	—	n	y–p***
1,3-Dimethylbarbituric acid	—	o	o
1,3-Dimethylbarbital	—	n	n

* n = none; p = pink; y = yellow; o = orange.
** Ring opening is probably preceded by deacetylation.
*** 1 *N* NaOH exposure as decomposition is too slow in 0.5 *N* NaOH.

TABLE 33

R_F VALUES OF SOME ETHOXYPYRIMIDINE DERIVATIVES

(C. JANION AND D. SHUGAR, *Acta Biochim. Polon.*, 7 (1960) 309)

Solvent: Methanol–acetic acid–water (2:1:1).
Paper: Whatman No. 1 (?).
Detection: *p*-Dimethylaminobenzaldehyde.

Compound	R_F		*Colour* reaction after hydrogenation*
	Before hydrogenation	*After hydrogenation*	
1-Methyl-4-ethoxypyrimidine	1.0	1.0	r
2,4-Diethoxypyrimidine	0.87	0.65, 1.0	y, r
2-Hydroxy-4-ethoxypyrimidine	0.85	0.85	r

* r = red; y = yellow.

TABLE 34

R_F VALUES OF ADENOSINE TRIPHOSPHATE AND ITS ANALOGUES

(M. IKEHARA, E. OHTSUKA, S. KITAGAWA, K. YAGI AND Y. TONOMURA, *J. Am. Chem. Soc.*, 83 (1961) 2679)

Solvents: S_1 = Propan-2-ol–1 % aq. $(NH_4)_2SO_4$ (2:1).
S_2 = Propan-2-ol–1 % aq. $(NH_4)_2SO_4$ (3:2).
S_3 = Butan-1-ol–H_2O (86:14).
S_4 = Saturated $(NH_4)_2SO_4$–H_2O–propan-2-ol (79:19:2).
S_5 = Saturated $(NH_4)_2SO_4$–0.1 *M* ammonium acetate–propan-2-ol (79:19:2).
S_6 = Propan-1-ol–NH_4OH–H_2O (6:3:3).
(Descending except S_3.)
Paper: Toyo Filter Paper No. 51-A.
Detection: U.V. light, phosphate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107); $BaCl_2$–rhodizonate reagent (for sulphate; J. BADDILEY, J. G. BUCHANAN AND R. LETTERS, *Proc. Chem. Soc.*, (1957) 147).

Compound	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
9-(3′-Hydroxypropyl)-6-aminopurine			0.32			0.74
9-(3′-Hydroxypropyl)-6-aminopurine-3′-monophosphate		0.49			0.19	
9-(3′-Hydroxypropyl)-6-aminopurine-3′-triphosphate		0.43			0.33	
9-(4′-Hydroxybutyl)-6-aminopurine			0.36			
9-(4′-Hydroxybutyl)-6-aminopurine-4′-monophosphate		0.64			0.30	
9-(4′-Hydroxybutyl)-6-aminopurine-4′-triphosphate		0.26		0.59		
6-Methylamino-9-β-D-ribofuranosyl-purine-5′-triphosphate	0.57			0.65		
6-Dimethylamino-9-β-D-ribofuranosyl-purine-5′-triphosphate	0.15					
Adenosine-5′-sulphatopyrophosphate					0.37	0.34
Adenosine monophosphate					0.27	0.21
Adenosine diphosphate					0.40	0.19
Adenosine triphosphate					0.45	0.15
Inorganic sulphate						0.28
Inorganic phosphate						0.22

TABLE 35

R_F VALUES OF ADENINE AND ADENOSINE

(K. BERNHAUER, P. GAISER, O. MÜLLER AND O. WAGNER, *Biochem. Z.*, 333 (1960/61) 106)

Solvents: S_1 = Butan-1-ol–ethanol–water (50:15:35, v/v) (F. CRAMER, *Papierchromatographie*, 4th Ed., Verlag Chemie, Weinheim/Bergstr., 1958).
S_2 = Propan-1-ol–1 % NH_4OH (2:1) (W. PFLEIDERER, unpubl.).
S_3 = Butan-1-ol–5 *N* acetic acid (2:1) (W. PFLEIDERER, unpubl.).
Paper: Schleicher & Schüll 2043 A (presumed).
Detection: U.V. light.

Compound	R_F		
	S_1	S_2	S_3
Adenine	0.46	0.56	0.46
Adenosine	0.37	0.54	0.35

TABLE 36

R_F VALUES OF ADENINE NUCLEOSIDE AND ADENOSINE

(K. BERNHAUER AND O. MÜLLER, *Biochem. Z.*, 334 (1961) 199)

Solvents: S_1 = Butan-1-ol–ethanol–water (50:15:35, by vol.).
S_2 = Propan-1-ol–water (2:1).
S_3 = Propan-1-ol–5 *N* acetic acid (2:1).
S_4 = Propan-1-ol–1% NH_4OH (2:1).
S_5 = Butan-1-ol–5 *N* acetic acid (2:1).
Paper: Not specified.
Detection: Not specified.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Adenine nucleoside	0.35	0.58	0.42	0.53	0.18
Adenosine	0.38	0.63	0.48	0.59	0.27

TABLE 37

R_F VALUES (THIN LAYER) OF SOME NUCLEOTIDES

(K. RANDERATH, *Angew. Chem.*, 73 (1961) 674)

Solvents: S_1 = 0.15 *M* NaCl.
S_2 = 0.01 *N* HCl.
Thin-layer adsorbent: Ecteola (0.41 mequiv. N/g; Serva Co., Entwicklungslabor, Heidelberg).
Time of run: 15 min.
Detection: U.V. light.

Compound	R_F	
	S_1	S_2
Adenosine-5′-monophosphate	0.57	0.26
Adenosine diphosphate	0.36	0.08
P^1-[Adenosyl-(5′)-]-P^2-methylpyrophosphate	0.46	
P^1-[Adenosyl-(5′)-]-P^2-phenylpyrophosphate	0.32	
Adenosine triphosphate	0.21	
Guanosine-5′-monophosphate	0.55	0.14
Guanosine diphosphate	0.37	0.03
Guanosine triphosphate	0.17	
Uridine-5′-monophosphate	0.80	0.13
Uridine diphosphate	0.63	0.00
Uridine triphosphate	0.44	
Cytidine-5′-monophosphate	0.74	0.31
Cytidine diphosphate	0.51	0.11
Cytidine triphosphate	0.34	
Inosine-5′-monophosphate	0.74	
Adenosine-3′-monophosphate	0.48	
Guanosine-3′-monophosphate	0.44	
Uridine-3′-monophosphate	0.75	
Cytidine-3′-monophosphate	0.71	
Ribonucleic acid (high mol. wt.)*	0.00	

* Spinach leaf isolate.

TABLE 38

R_F VALUES (INCLUDING THIN LAYER) OF SOME NUCLEOSIDES AND RELATED COMPOUNDS

(K. RANDERATH, *Angew. Chem.*, 73 (1961) 674)

Solvent: Water (distilled).
Paper: P_1 = Ederol 202 (Binzer Co., Hatzfeld/Eder).
Thin-layer adsorbents: T_1 = Ecteola (0.26 mequiv. N/g; Serva Co., Entwicklungslabor, Heidelberg).
T_2 = Cellulose (K. RANDERATH AND H. STRUCK, *J. Chromatog.*, 6 (1961) 365).
T_3 = Kieselgel G.
Detection: U.V. light.

Compound	R_F			
	T_1	T_2*	P_1	T_3
Adenine	0.29	0.30	0.38	0.57
Adenosine	0.56	0.53	0.56	0.75
Guanine	0.33	0.37	0.38	0.66
Guanosine	0.50	0.58	0.57	0.80
Hypoxanthine	0.46	0.55	0.57	0.74
Inosine	0.61	0.70	0.73	0.82
Uracil	0.73	0.72	0.75	0.78
Uridine	0.84	0.81	0.84	0.85
Cytidine	0.82	0.80	0.77	0.76

* From K. RANDERATH AND H. STRUCK, *J. Chromatog.*, 6 (1961) 365.

TABLE 39

R_F VALUES OF DIPHOSPHOPYRIDINE NUCLEOTIDE AND METABOLICALLY RELATED PRODUCTS

(H. KRÖGER, H. W. ROTTHAUWE, B. ULRICH AND H. HOLZER, *Biochem. Z.*, 333 (1960/61) 155)

Solvents: S_1 = Pyridine–H_2O (2:1).
S_2 = 95% Ethanol–0.1 *N* acetic acid (1:1).
S_3 = Propan-1-ol–H_2O (4:1).
Paper: Whatman No. 4.
Temperature of run: 25°.
Time of run: 8 h.
Detection: D_1 = U.V. light (fluorescence).
D_2 = KCN solution (dip); then D_1 (fluorescence), for compounds containing quaternary base residues.
D_3 = BrCN–benzidine spray (reddish), for compounds containing nicotinic acid or nicotinamide residues.

Compound	R_F		
	S_1	S_2	S_3
Diphosphopyridine nucleotide	0.60	0.56	0.00
Nicotinamide riboside	0.81	0.84	0.34
Nicotinamide mononucleotide	0.33	0.69	0.01
Adenosine diphosphate ribose	0.75	0.67	0.01
Nicotinamide	0.89	0.84	0.85
Nicotinic acid	0.81	0.82	0.54

TABLE 40

R_F VALUES OF NUCLEOTIDES AND RELATED COMPOUNDS

(W. SZER AND D. SHUGAR, *Acta Biochim. Polon.*, 8 (1961) 235)

Solvents: S_1 = Propan-2-ol–NH_4OH (d = 0.88)–H_2O (70:10:20, v/v/v).
S_2 = Ethanol–*M* ammonium acetate (70:30, v/v).
Paper: Whatman No. 1 (ascending).
Detection: Not specified.

Compound	R_F	
	S_1	S_2
Uridine	0.49	0.73
N-Methyluridine	0.78	0.91
N-Methyl-2′,3′-O-isopropylidene-uridine	0.86	—
Uridine-5′-phosphate	0.18	0.26
N-Methyluridine-5′-phosphate	0.25	0.48
Uridine-5′-pyrophosphate	—	0.10
N-Methyluridine-5′-pyrophosphate	—	0.16
Dihydrouridine-5′-pyrophosphate	—	0.10
Dinucleotide MeUpUp	0.90	0.18
Trinucleotide MeUpMeUpUp	0.40	0.12
Poly-(MeU)	0.00	0.00

TABLE 41

R_F VALUES OF NUCLEOSIDE DERIVATIVES

(A. HAMPTON, *J. Am. Chem. Soc.*, 83 (1961) 3640)

Solvents: S_1 = Ethanol–water (7:3).
S_2 = Propan-2-ol–water–NH_4OH (70:25:5).
S_3 = Butan-1-ol–acetic acid–water (5:2:3).
S_4 = Propan-2-ol–water (7:3).
Paper: Schleicher & Schüll 597 (ascending).
Detection: U.V. light (Corning filter No. 9863).

Compound	R_F			
	S_1	S_2	S_3	S_4
Uridine	0.53	0.47	0.55	0.63
2′,3′-O-Isopropylidene-uridine	0.67	0.70	0.79	0.82
Cytidine	0.50	0.55	0.51	0.55
2′,3′-O-Isopropylidene-cytidine	0.66	0.75	0.77	0.80
Adenosine	0.47	0.57	0.60	0.58
2′,3′-O-Isopropylidene-adenosine	0.59	0.75	0.80	0.78
Guanosine	—	0.37	0.46	0.49
2′,3′-O-Isopropylidene-guanosine	—	0.68	0.78	0.75
Inosine	0.49	0.50	0.46	0.52
2′,3′-O-Isopropylidene-inosine	0.62	0.70	0.77	0.78
6-Thioinosine	—	0.45	0.48	0.57
2′,3′-O-Isopropylidene-6-thioinosine	—	0.70	0.79	0.79
6-Thioguanosine	—	0.36	0.46	—
2′,3′-O-Isopropylidene-6-thioguanosine	—	0.61	0.78	—
8-Azaguanosine	—	0.40	0.47	—
2′,3′-Isopropylidene-8-azaguanosine	—	0.67	0.81	—
Di-*p*-nitrophenyl hydrogen phosphate	0.72	0.83	0.84	0.85

TABLE 42

R_F VALUES OF FOUR AUXIN–PHENOL COMPLEXES AND PARENT COMPOUNDS

(A. C. LEOPOLD AND T. H. PLUMMER, *Plant Physiol.*, 36 (1961) 589)

Solvent: Isopropanol–water (8:2).
Paper: Not specified.
Detection: D_1 = Visible light.
D_2 = U.V. fluorescence.
D_3 = Ehrlich colour.

Compound	R_F	*Colour**		
		D_1	D_2	D_3
Indoleacetic acid (IAA)	0.75	—	b	pu
Catechol	0.88	—	(q)	—
IAA–catechol	0.45	c	(q)	o
Chlorogenic acid	0.60	—	y	—
IAA–chlorogenic acid	0.45	r	(q)	br
Caffeic acid	0.65	—	y	—
IAA–caffeic acid (1st pigment)	0.55	p	(q)	y
IAA–caffeic acid (2nd pigment)	0.45	br	(q)	o

* c = chestnut; r = rose; pu = purple; b = blue; y = yellow; br = brown; o = olive; p = pink; (q) = quench; — = negative.

TABLE 43

R_F VALUES (THIN LAYER) OF KHELLIN AND RELATED COMPOUNDS, AS WELL AS VULPININC, USNIC AND EVERNIC ACIDS

(E. STAHL AND P. J. SCHORN, *Z. Physiol. Chem.*, 325 (1961) 263)

Solvents: S_1 = Toluene–ethyl formate–formic acid (5:4:1).
S_2 = Benzene–chloroform (1:1).
Solvent components: E. Merck Co., Darmstadt (p.a.), "for chromatography"
Thin-layer carrier: Silica gel (Kieselgel G for thin-layer chromatography No. 121241, E. Merck Co., Darmstadt; mixed with twice its weight of distilled water*; applied at 250 μ layer to 200 × 200 mm glass plates; dried at 105° for 30 min).
Apparatus: E. STAHL, *Chem.-Ztg.*, 82 (1958) 323; (manufactured by C. Desaga Co., Ltd., Heidelberg).
Detection: D_1 = U.V. light; for khellin and related compounds.
D_2 = Anisaldehyde–H_2SO_4 reagent (8.5 ml methanol + 0.5 ml anisaldehyde + 1.0 ml conc. H_2SO_4; mixed in this order, sprayed and heated: 100–110°; 10–15 min), for the lichen acids.

Compound	R_F		*Colour***	
	S_1	S_2	D_1	D_2
Khellin	0.47		B	
Khellol	0.30		Y–G	
Khellol glycoside	0.00		Bl	
Visnagin	0.45		Y–G	
Vulpinic acid		0.80		Y
Usnic acid		0.65		BlV
Evernic acid		0.11		R
Orcinol		0.03		R

* Water is replaced by 0.3 *M* aq. sodium acetate for flavones and 0.5 *N* oxalic acid for lichen acids.

** B = brown; Bl = blue; G = gray; R = red; V = violet; Y = yellow.

TABLE 44

R_F VALUES (THIN LAYER) OF SOME OPIUM ALKALOIDS AND THEIR DERIVATIVES

(E. STAHL, *Angew. Chem.*, 73 (1961) 646*)

Solvents: S_1 = Chloroform–ethanol (9:1; K. TEICHERT, E. MUTSCHLER AND H. ROCHELMEYER, *Deut. Apotheker Ztg.*, 100 (1960) 283, 477).
S_2 = Chloroform–ethanol (9:2; see S_1).
S_3 = Dimethylformamide–diethylamine–ethanol–ethyl acetate (5:2:20:75; see S_1).
S_4 = Benzene–heptane–chloroform–diethylamine (6:5:1:0.02; see S_1).
S_5 = Methanol (G. MACHATA, *Mikrochim. Acta*, 47 (1960) 79).
S_6 = Chloroform–acetone–diethylamine (5:4:1; D. WALDI, K. SCHNACKERZ AND F. MUNTER, *J. Chromatog.*, 6 (1961) 61).

Thin-layer adsorbent: T_1 = Kieselgel G (E. Merck A.G., Darmstadt), normal.
T_2 = Kieselgel G (E. Merck A.G., Darmstadt), alkalinized.
T_3 = Cellulose powder.
T_4 = Cellulose powder, formamide impregnated.

Detection: See *.

Compound	R_F					
	S_1T_1	S_2T_2	S_3T_3	S_4T_4	S_5T_1	S_6T_1
Morphine	0.02	0.02	0.27	0.00	0.24	0.10
Dihydromorphinone (= Dilaudid ®)	0.05	0.13	0.27	0.06	0.14	0.24
Dihydrocodeine (= Paracodin ®)	0.06	0.22	0.34	—	—	0.38
Dihydrocodeinone (= Dicodid ®)	0.10	0.28	0.34	0.57	—	0.51
Codeine (= Morphine methyl ether)	0.12	0.33	0.41	0.37	0.26	0.38
Morphine ethyl ether (= Dionin ®)	0.14	0.37	0.44	0.57	—	—
Acetyldihydrocodeinone (= Acedicon ®)	0.24	0.59	—	0.90	—	—
Dihydrohydroxycodeinone (= Eucodal ®)	0.47	0.70	0.79	0.75	—	—
Papaverine	0.74	0.78	0.86	0.89	0.74	0.67
Narcotine	0.78	0.81	0.92	0.94	0.69	0.72

* *Cf.* other references given.

TABLE 45

R_F VALUES OF CORRINOID CONJUGATES

(K. BERNHAUER, P. GAISER, O. MÜLLER AND O. WAGNER, *Biochem. Z.*, 333 (1960/61) 106)

Solvents: S_1 = Water satd. butan-2-ol with 1% acetic acid.
S_2 = Water satd. butan-2-ol.
S_3 = Aq. butan-2-ol–NH_3.
S_4 = Water satd. butan-2-ol with 0.5% sodium tetraphenyl borate.
(W. FRIEDRICH AND K. BERNHAUER, in K. F. BAUER, *Medizinische Grundlagenforschung*, Vol. 2, Georg Thieme, Stuttgart, 1959, p. 661.)

Paper: Schleicher & Schüll 2043A (ascending; in dark).
Time of run: 20 h.
Detection: U.V. light

Compound	R_F			
	S_1	S_2	S_3	S_4
Cobalamine conjugate	0.20	0.15	0.10	0
Cobinamide conjugate	0.22	0.19	0.13	0.06

TABLE 46

R_F VALUES (THIN LAYER) OF SINGLE AZO DYES ON ALUMINAS WITH DIFFERENT ACTIVITIES

(S. HEŘMÁNEK, V. SCHWARZ AND Z. ČEKAN, *Collection Czech. Chem. Commun.*, 26 (1961) 3170)

Solvent: Carbon tetrachloride.
Thin-layer adsorbent: Alumina (Lachema); activities II–V (see table) (H. BROCKMANN AND H. SCHODDER, *Ber.*, 74 (1941) 73) (7 cm × 24 cm × 0.6 mm).
Binder: None.
Length of run: 17–20 cm.
Time of run: 20–24 min.
Detection: U.V. light.

Azo dye	R_F*			
	II	*III*	*IV*	*V*
Azobenzene	0.59	0.74	0.85	0.95
p-Methoxyazobenzene	0.16	0.49	0.69	0.89
Sudan Yellow	0.01	0.25	0.57	0.78
Sudan Red	0.00	0.10	0.33	0.56
p-Aminoazobenzene	0.00	0.03	0.08	0.19

* R_F values ± 0.04.

TABLE 47

R_F VALUES (THIN LAYER) OF SINGLE AZO DYES ON DIFFERENT TYPES OF ALUMINA

(S. HEŘMÁNEK, V. SCHWARZ AND Z. ČEKAN, *Collection Czech. Chem. Commun.*, 26 (1961) 3170)

Solvent: Carbon tetrachloride.
Thin-layer adsorbent: Alumina of different types (see table) (7 cm × 24 cm × 0.2 mm).
Binder: None.
Length of run: 17–20 cm.
Time of run: 20–24 min.
Detection: U.V. light.

Alumina	R_F				*Activity**	
	Azo-benzene	*p-Methoxy-azobenzene*	*Sudan Yellow*	*Sudan Red*	*Method 1*	*Method 2*
Kaznějov, commercial grade	0.75	0.51	0.28	0.10	III	III
Kaznějov, neutral	0.66	0.33	0.08	—	II	II$^{\frac{1}{2}}$
Kaznějov, neutral according to Reichstein	0.80	0.59	0.39	0.20	III$^{\frac{1}{2}}$	III$^{\frac{1}{2}}$
Lachema, commercial grade	0.79	0.53	0.25	0.06	III	III
Merck, anhydr., standardised	0.78	0.56	0.40	0.18	III$^{\frac{1}{2}}$	III$^{\frac{1}{2}}$
Woelm, anionotropic	0.72	0.45	0.26	0.10	III	III

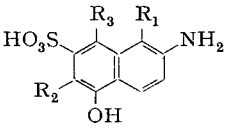
* Method 1: classical method (H. BROCKMANN AND H. SCHODDER, *Ber.*, 74 (1941) 73). Method 2: by thin-layer chromatography.

TABLE 48

R_F VALUES OF SOME DIAZO- AND AZONAPHTHALENE DERIVATIVES AND RELATED COMPOUNDS

(Z. J. ALLAN AND J. PODSTATA, *Collection Czech. Chem. Commun.*, 26 (1961) 1862)

Solvent: 20% NH_3–isoamyl alcohol–pyridine (1:1:1).
Paper: Whatman No. 4 (descending).
Detection: D_1 = Visible light, dry paper alone.
D_2 = After NH_3 fumes.
D_3 = After drop of 2.5 *N* NaOH.
D_4 = After HCl fumes.



I, R_1=Y, R_2=R_3=H
II, R_1=R_3=H, R_2=Y
III, R_1=R_2=H, R_3=Y
IV, R_1=R_2=Y, R_3=H
V, R_1=H, R_2=R_3=Y
VI, R_1=R_3=Y, R_2=H

Y = $-N_2C_6H_4NO_2(p)$

Compound	*Position of azo-group*	R_F		*Colour**			
		1st values reported	*2nd values reported*	D_1	D_2	D_3	D_4
I	1	0.55	0.65	wr	bv	rb	wr
II	6	0.5	0.65	crho	crho	rhb	r
III	8	0.7	0.75	o	bg	b	yho
IV	1,6	0.8	0.85	wr	wr	bhv	v
V	6,8	0.8	0.85	yhr	yhr	yhbn	wr
VI	1,8	—	0.95	o	bshy	b	o

* b = blue; bh = bluish; bn = brown; bsh = brownish; c = clear; g = gray; o = orange; r = red; rh = reddish; v = violet; w = wine; y = yellow; yh = yellowish.

TABLE 49

R_F VALUES OF AMINOANTHRAQUINONES

(J. GASPARIČ AND J. MARHAN, *Collection Czech. Chem. Commun.*, 27 (1962) 46)

Solvent: Pyridine–water (1:1) saturated with 1-bromonaphthalene.
Paper: Whatman No. 3.
Impregnation: 1-Bromonaphthalene.
Detection: Visible light.

Compound	R_F
1-Aminoanthraquinone	0.38
1-Amino-2-chloroanthraquinone	0.14
1-Amino-4-chloroanthraquinone	0.25
1-Amino-2-bromoanthraquinone	0.10
1-Amino-4-bromoanthraquinone	0.21
1-Amino-2-chloro-4-bromoanthraquinone	0.07
1-Amino-2,4-dibromoanthraquinone	0.06
1-Amino-2-chloroanthraquinone-4-sulphonic acid	0.93
1-Aminoanthraquinone-2-carboxylic acid	0.93
1-Amino-4-bromoanthraquinone-2-sulphonic acid	0.93

TABLE 50

R_F VALUES OF CERTAIN INORGANIC CATIONS

(M. ŠTEFFEK, *Chem. Listy*, 55 (1961) 1221)

Solvents: S_1 = Allyl alcohol–water–ethyl acetate–acetic acid (25:15:15:15).
S_2 = Allyl alcohol–water–ethyl alcohol–acetic acid (25:10:10:10).
S_3 = Trichloroethylene–water–methanol–acetic acid (17:8:9:30).
S_4 = Chloroform–methanol–water–acetic acid (35:30:10:40).
S_5 = Chloroform–methanol–water–acetic acid (40:15:9:35).
S_6 = Chloroform–methanol–water–acetic acid (35:7:8:25).
Paper: Niederschlag 388-h or Schleicher & Schüll 589, blue band (not more precisely specified).
Circular: S_1–S_6; ascending: S_1, S_2.
Detection: Group reagents.

Cation	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
Ag^+	0.75	0.78	0.48	0.42	0.50	0.41
Pb^{2+}	0.48	0.52	0.59	0.51	0.62	0.54
Hg^{2+}	0.87	0.79	0.86	0.75	0.80	0.78
Cu^{2+}*	0.62	0.65	0.68	0.61	0.67	0.63
Bi^{3+}	0.74	0.72	0.81	0.76	0.82	0.80
Fe^{3+}	0.94	0.85	0.75	0.80	0.86	0.88

*Cu^{2+} and Cd^{2+} R_F values are the same.

TABLE 51

R_F VALUES OF CERTAIN INORGANIC CATIONS

(M. ŠTEFFEK, *Chem. Listy*, 55 (1961) 1221)

Solvents: S_1 = Butyl acetate–amyl alcohol–methanol–water–HCl (30:20:3:2:15).
S_2 = Butyl acetate–amyl alcohol–water–HCl (50:10:1:20).
Paper: Niederschlag 388-h or Schleicher & Schüll 589, blue band (not more precisely specified).
Circular: S_1, S_2; ascending: S_2.
Detection: Group reagents.

Cation	R_F	
	S_1	S_2
Pb^{2+}	0.43	0.35
Cu^{2+}	0.60	0.59
As^{5+}, As^{3+}	0.82	0.85
Sb^{3+}	0.98	1.00
Au^{3+}	1.00	1.00
Cr^{3+}	0.27	0.30
V^{5+}, V^{3+}	0.38	0.32
Ni^{2+}	0.25	0.31
Co^{2+}	0.52	0.50
UO_2^{2+}	0.70	0.61
Fe^{3+}	1.00	1.00

TABLE 52

R_F VALUES OF PHOSPHATES AND CONDENSED PHOSPHATES

(R. H. KOLLOFF, *Anal. Chem.*, 33 (1961) 373)

Solvents: S_1 = Dioxan–H_2O–trichloroacetic acid–NH_4OH (60 ml:35 ml:5 g:0.25 ml).
S_2 = As S_1 (70 ml:27.5 ml:2.5 g:0.25 ml).
S_3 = As S_1 (70 ml:27.5 ml:5 g:0.25 ml).
S_4 = As S_1 (72.5 ml:25 ml:2.5 g:0.25 ml).
S_5 = As S_1 (80 ml:15 ml:5 g:0.25 ml).
S_6 = Acetone–H_2O–trichloroacetic acid–NH_4OH (28–30%) (65 ml:30 ml:5 g:0.35 ml) (D. N. BERNHARDT AND W. B. CHESS, *Anal. Chem.*, 31 (1959) 1026).
S_7 = As S_6 (80 ml:15 ml:5 g:0.35 ml).
S_8 = Propan-2-ol–H_2O–trichloroacetic acid–NH_4OH (28–30%) (70 ml:25 ml:5 g:0.3 ml) (J. P. EBEL, *Bull. Soc. Chim. France*, 20 (1953) 991, 998, 1089, 1096; E. KARL-KROUPA, *Anal. Chem.*, 28 (1956) 1091).
(Order of addition in S_1–S_5: trichloroacetic acid in water, NH_4OH, then *p*-dioxan).

Paper: Whatman No. 31 DT (7 in. × 9 in.; ascending).

Chromatographic chamber: Borosilicate jar (6 in. o.d. × 12 in. height) lined with Whatman No. 31 DT dipping in 150–200 ml of solvent and covered with polyethylene sheet.

Time of run: 40 min (for S_2).

Length of run: 5 3/4 in. to 5 7/8 in. (for S_2).

Detection: D_1 = Ammonium molybdate spray: $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g) in water (450 ml); conc. H_2SO_4 (5 ml) added, then conc. HNO_3 (20 ml) added with vigorous stirring; mix well. Store in polyethylene container away from light. Stable for 5–10 days.

D_2 = Ammoniacal reducing chromatographic spray stock soln.: 1-amino-2-naphthol-4-sulphonic acid (1.5 g) in water (75 ml) containing Na_2SO_3 (7 g); $NaHSO_3$ (90 g) dissolved in water (700 ml). Solutions combined and diluted to 1 l. Stable for 6 months in dark away from U.V. light.
Spray reagent: stock soln. (25 ml) mixed with water (50 ml) and conc. NH_4OH (25 ml). Prepared daily.

Procedure: Dry chromatograms (air: 5–10 min, oven: 70°). Hydrolyse phosphates on paper in steam–HCl atmosphere (100°, 30 min) in hydrolysis chamber, dry. Spray D_1, then dry (air: 10 min, oven: 70°), then spray D_2 and dry (air: 20–25°).

Phosphate	R_F *(approx.)*[a]							
	S_1[b]	S_2[b]	S_3[c]	S_4[d]	S_5[e]	S_6[d]	S_7[e]	S_8[d]
Length of run (cm)	*23.3*	*15*	*15*	*15*	*15*	*14*	*14*	*13*
Ortho	0.91	0.90 (0.88)	0.95 (0.90)	0.90	0.85	0.89 (0.90)**	0.77***	0.83 (0.80)**
Pyro	0.82	0.65 (0.63)	0.80 (0.65)	0.65	0.26	0.67	0.21***	0.49
Tripoly	0.73	0.45 (0.43)	0.64 (0.48)	0.39	—	0.51		0.33*
Tetrapoly	0.64	—	(0.29)	—	—	—*		—*
Trimeta	0.55	0.15 (0.15)	0.26 (0.18)*	0.13	—	0.31*		0.21*
Tetrameta	0.44	—	(0.10)*	—	—	0.16*		0.10*
Long chain	0.00	—	(0.00)	—	—			0.00*

[a] 10–25 µl of sample solution applied. [b] Sample solution strength: 1% (2%). [c] Sample solution strength 2% (1%). Note: at 1% level about equal distribution of components; at 2% level, distribution: ortho (0.5), pyro (8.2), tripoly (87.4), trimeta (4.0). [d] Sample solution strength: 1% (20%). Distribution: ortho (0.5), pyro (13.0), tripoly (82.0), trimeta (4.4). [e] Sample solution strength: 20%. Distribution: ortho (99.5), pyro (0.5).

* Incomplete resolution. ** No resolution. *** Pyro band spread and diffused.

TABLE 53

R_F VALUES OF CARBOHYDRATE O-ISOPROPYLIDENE DERIVATIVES

(J. E. G. BARNETT AND P. W. KENT, *Nature*, 192 (1961) 556)

Solvent: Methanol–H_2O (6:4, v/v).
Paper: Cellulose acetate ("Oxoid" strips for electrophoresis; 20 × 4.5 cm; Messrs. Oxo Ltd., London; washed thoroughly in methanol, dried; ascending).
Time of run: 2 h.
Temperature of run: 2–5°.
Detection: D_1 = Immerse in solution of 2,4-dinitrophenylhydrazine (0.4% w/v) in 2 *N* HCl (D. E. BLAND, *Nature*, 164 (1949) 1093); carbonyl, O-isopropylidene and O-benzylidene give deep yellow zones, intensified by brief 10% (w/v) NaOH wash.
or D_2 = Float on surface of 1% (w/v) $KMnO_4$ on 1 *N* H_2SO_4 for 2 min, immerse for 1 min, then wash in running water; purple-brown spots (turn brown on standing) (Ž. PROCHÁZKA, *Chem. Listy*, 44 (1950) 43).

Compound	R_F	*No. of free hydroxyl groups*
1,2; 4,5-Di-O-isopropylidene-3-mesyl-D-fructose	0.15	0
1,2-O-Benzylideneglycerol	0.23	1
Methyl O-isopropylidene DL-glycerate	0.28	0
1,2; 3,4-Di-O-isopropylidene-6-tosyl-D-galactose	0.32	0
Methyl 2,3-O-isopropylidene-α,β-D-ribofuranoside	0.48	1
1,2; 5,6-Di-O-isopropylidene-D-glucose	0.52	1
1,2; 3,4-Di-O-isopropylidene-D-galactose	0.55	1
1,2-O-Isopropylideneglycerol	0.65	1
Methyl DL-glycerate	0.66	2
(±)-Butan-1,3-diol	0.73	2
1,2-O-Isopropylidene-D-glucofuranose	0.80	3
2,3,4,6-Tetra-O-methyl-D-glucose	0.90	1
Methyl 2,3,6-tri-O-methyl-α,β-D-glucoside	0.98	2
3-O-Methyl-D-glucose	0.98	4

TABLE 54

R_F VALUES OF OXALURIC ACID AND ALLANTOIC ACID

(G. D. VOGELS, *Biochem. Z.*, 334 (1961) 457)

Solvents: S_1 = Phenol–water (80:20, w/v).
S_2 = Butan-1-ol–acetic acid–water (12:3:5, v/v).
S_3 = Butan-1-ol–pyridine–water (6:4:3, v/v).
S_4 = 96% ethanol–25% ammonia (100:7, v/v).
Paper: Whatman No. 1 (descending).
Detection: Not specified.

Compound	R_F			
	S_1	S_2	S_3	S_4
Oxaluric acid	0.25	0.12	0.05	0.30
Allantoic acid	0.18	0.14	0.00	0.04

TABLE 55

ELECTROPHORETIC MOBILITIES OF DINITROPHENYL DERIVATIVES OF KANAMYCIN, MYCERIN AND COLIMYCIN

(A. B. SILAEV, G. S. KATRUCHA AND V. M. STEPANOV, *Biokhimiya*, 26 (1961) 10)

Electrolyte: 85 % formic acid–glacial acetic acid–water (28:20:52).
Paper: Not specified (36 cm long).
Potential applied: 220 V.
Time of run: 2.5 h.
Mobility units (M): cm towards cathode.
Detection: Not specified.

Compound	*M*
Kanamycin	7.2
Mono-DNP-kanamycin	5.4
Di-DNP-kanamycin	3.6
Tri-DNP-kanamycin	1.8
Tetra-DNP-kanamycin	0.0
Mycerin	7.9
Mono-DNP-mycerin	6.9
Di-DNP-mycerin	5.7
Tri-DNP-mycerin	4.3
Tetra-DNP-mycerin	2.9
Penta-DNP-mycerin	1.5
Hexa-DNP-mycerin	0.0
Colimycin	7.8
Mono-DNP-colimycin	6.8
Di-DNP-colimycin	5.6
Tri-DNP-colimycin	4.3
Tetra-DNP-colimycin	2.8
Penta-DNP-colimycin	1.5
Hexa-DNP-colimycin	0.0

TABLE 56

R_F VALUES OF SOME NEUTRAL AND PHENOLIC STEROIDS

(H. A. KETZ, H. WITT AND M. MITZNER, *Biochem. Z.*, 334 (1961) 73)

Solvent: Chloroform saturated with formamide.
Paper: Schleicher & Schüll 2043 b.
Impregnation: 45 % formamide in acetone.
Time of run: 4–6 h.
Detection: 1 % ferric chloride + 1 % potassium ferrocyanide (R. NEHER, *J. Chromatog.*, 1 (1958) 122, 205).

Steroid	R_F*
Neutral	
Androsterone	0.56
Dehydroepiandrosterone	0.28
Testosterone	0.20
Phenolic	
Oestrone	0.80
Oestradiol-17α	0.60
Oestradiol-17β	0.50

* Average values.

TABLE 57

R_F VALUES OF BILE ACIDS ON DIFFERENT PAPERS

(Y. I. KARBACH, *Biokhimiya*, 26 (1961) 305)

Solvents: S_1 = Toluene–85 % formic acid–glacial acetic acid–methanol (6:0.1:0.25:0.3).
S_2 = Dichloroethane–85 % formic acid–glacial acetic acid–methanol (5:6.8:0.5:0.4).
Paper: P_1 = Leningrad type B.
P_2 = Leningrad type 4-M.
P_3 = Whatman No. 1.
P_4 = Whatman No. 4.
P_5 = Whatman No. 42.
P_6 = Schleicher & Schüll No. 595.
P_7 = Schleicher & Schüll No. 597.
Length of run: 18.5 cm.
Detection: $SbCl_3$ in $CHCl_3$ dip, then heating at 90–95° for 5 min and U.V. light.

Chromatography paper	R_F			
	S_1		S_2	
	A	*B*	*C*	*D*
P_1	0.35	0.69	0.12	0.58
P_2	0.43	0.73	0.14	0.70
P_3	0.36	0.64	0.12	0.58
P_4	0.40	0.64	0.14	0.64
P_5	0.42	0.70	0.15	0.81
P_6	0.59	0.79	0.17	0.75
P_7	0.50	0.67	0.25	0.81

A = cholic acid; B = deoxycholic acid; C = taurocholic acid; D = glycocholic acid.

TABLE 58

R_F VALUES OF VARIOUS Δ^5-ANDROSTENE AND Δ^5-PREGNENE DERIVATIVES

(L. STÁRKA, *Collection Czech. Chem. Commun.*, 26 (1961) 2452)

Solvents: S_1 = Petrol ether.
S_2 = Carbon tetrachloride.
S_3 = Benzene.
Paper: Whatman No. 1.
Impregnation: 30 % Methanolic triethylene glycol.
Detection: D_1 = Alkaline *m*-dinitrobenzene reagent.
D_2 = Saturated chloroform–acetic anhydride (4:1) solution of $SbCl_3$.

Compound	R_F		
	S_1	S_2	S_3
Δ^5-Androsten-3β-ol-17-one	0.22	0.60	0.68
3β-Acetoxy-Δ^5-androsten-17-one	0.92	—	—
Δ^5-Androstene-3β,7α-diol-17-one	0.00	0.04	0.08
Δ^5-Androstene-3β,7α-diol-17-one monoacetate	0.47	0.55	0.80
3β,7α-Diacetoxy-Δ^5-androsten-17-one	0.78	0.87	0.92
Δ^5-Pregnene-3β,7α-diol-20-one			0.20
7α-Acetoxy-Δ^5-pregnen-3β-ol-20-one			0.83
3β,7α-Diacetoxy-Δ^5-pregnen-20-one			0.98

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