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EXPERIMENTAL STUDY OF THE EFFECT OF COLUMN LENGTH AND PRESSURE ON THE HETP IN GAS CHROMATOGRAPHY

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SUMMARY

HETP *vs.* carrier gas velocity curves were measured on columns of different lengths operated under laminar flow conditions at either a constant outlet or a constant inlet column pressure, as well as on columns of a given length operated at various levels of the outlet or the inlet column pressure, respectively. Both high and low amounts of liquid stationary phase on the support were employed. With columns operated at a constant outlet pressure, the minima on the curves were shifted towards lower HETP and flow velocity values and the ascending branches of the curves acquired a more dished curvature upon increase of column length. The reverse situation was encountered with columns operated at a constant inlet pressure. An increase in the mean absolute column pressure brought about by an increase of either the outlet or the inlet column pressure always resulted in a pronounced decrease of the minimum HETP and optimum velocity, as well as in a steeper and straighter ascending branch of the HETP *vs.* velocity curve when working with the high liquid load packing; with a low loading of liquid stationary phase an inappreciable decrease of the minimum HETP occurred under the above conditions. Disagreements between the present results and those obtained by other authors are discussed.

In a previous paper[1], the effects of varying the parameters determining the absolute column pressure on the HETP in gas chromatography have been discussed. It was shown that the above effects may be described by the equations

$$\bar{H} = A + \frac{B_s}{\bar{u}} + \frac{B_m}{\bar{u}P_o + (\eta L/2K)\bar{u}^2} + C_m P_o \bar{u} + C_m(\eta L/2K)\bar{u}^2 + C_s\bar{u} \qquad (1)$$

and

$$\bar{H} = A + \frac{B_s}{\bar{u}} + \frac{B_m}{\bar{u}P_i - (\eta L/2K)\bar{u}^2} + C_m P_i \bar{u} - C_m(\eta L/2K)\bar{u}^2 + C_s\bar{u} \qquad (2)$$

for columns operated under laminar flow conditions and at moderate pressures and

pressure drops. Eqns. 1 and 2 refer to the cases of work at either a constant column outlet pressure (P_o) or a constant column inlet pressure (P_i); A, B_s, B_m, C_m, and C_s are the constants of the extended Van Deemter equation, L and K are the column length and the column permeability constant, η is the carrier gas viscosity coefficient, and $\bar{H}$ and $\bar{u}$ stand for the apparent HETP and the average carrier gas forward velocity determined from the column length and the gas hold-up time, respectively. The present work is an experimental version of the investigation to the above problems.

EXPERIMENTAL

The experiments were designed in such a way that they covered all the aspects indicated by eqns. 1 and 2. Packings with both a high and a low loading of liquid phase were used in columns of different lengths, operated at a constant outlet pressure and variable inlet pressure and *vice versa*, as well as in columns of a given length, operated at different constant levels of either the column outlet pressure or column inlet pressure, respectively.

The column lengths were 0.75 and 3 m, the longer one being made up of four 0.75 m segments. The column segments were stainless steel tubes of 3 and 5 mm inner and outer diameters, respectively, filled, in the straight state, by pouring the packing in while gently tapping the tube for about 2 h and making sure that the weights of packing in the individual segments did not vary more than 0.05 g. The spaces, necessary for plugging the packing, did not exceed 20 μl at each end of the segment; sleeve couplings with teflon sealings, providing for a leakproof face-to-face connection, were used. 25 and 3 wt.% dinonyl phthalate on Chromosorb P 60/80 mesh were employed as the column packings. They were prepared by the slurry technique using dichloromethane as the solvent. The support (Carlo Erba, Italy) had previously been dried at 200° for 3 h prior to coating it with the liquid phase (Griffin & George Ltd., Great Britain). The weights per 0.75 m segment of the 25 and 3% packings were 3.43 and 2.70 g, respectively. Pentane and hexane were used as the solutes with the high and the low load of liquid phases, respectively, the column temperature being kept at 40° in all cases.

The measurements were carried out on a Becker Multigraph 409 (Becker Delft, Holland) equipped with a Servogor RE 512 recorder (Goerz Electro G.m.b.H., Austria); thermal conductivity detection was used and hydrogen was the carrier gas. The injection port and detector temperatures were maintained at the column temperature (40°). The built-in gas flow control unit was disconnected and substituted by an external one in order to be able to extend the inlet pressure range beyond the conventional limits. Further, the measuring cell outlet was connected to a unit comprising a pressure gauge, damping bottle, and needle valve, thus providing control for the column outlet pressure. The flow diagram of the whole pneumatic arrangement is shown in Fig. 1. The use of the narrow-bore sample port insert liner, supplied with the apparatus, as well as the small dead volume of the path between the column outlet and the detector measuring cell are supposed to result in minimum zone broadening effects outside the column.

The sample itself consisted of the saturated vapours of either pentane or hexane at 25° in hydrogen containing traces of air. About 40 μl charges of this sample were injected by a Zimmermann syringe (Zimmermann, Leipzig). This method of sample

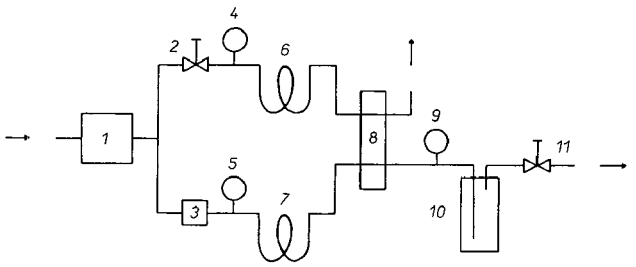

Fig. 1. Flow diagram of the pneumatic arrangement of the gas chromatograph used. 1 = tank cylinder pressure controller; 2 and 11 = needle valves; 3 = Brooks flow controller; 4, 5 and 9 = pressure gauges; 6 = reference column; 7 = measuring column; 8 = measuring cell; 10 = damping bottle.

introduction resulted in optimal sizes of both the pentane (hexane) and air peaks while preventing the possible broadening effects incidental to the volatilization of a liquid sample charge. Full detector sensitivity was employed at bridge currents varying between 250–300 mA.

The mean carrier gas velocity was determined by means of the air peak maximum. The $\bar{H}$ values were calculated from the formula $\bar{H} = L/8(\ln 2)(b/\Delta b_{1/2})^2$ where b and $\Delta b_{1/2}$ designate the peak maximum distance from the start line and the peak width at the half peak height, respectively[2].

The laminar flow of the carrier gas was controlled by checking the linearity of the dependence of the directly measured carrier gas volume flow rate, at the given column outlet pressure, on the parameter $(P_i^2 - P_o^2)/P_o$.

RESULTS AND DISCUSSION

The curves in Fig. 2 refer to measurements with the high loading of liquid phase on the support and a constant column outlet pressure. Curves 1 and 2 were obtained with the 3 and 0.75 m long columns, respectively, the column outlet being kept at the atmospheric pressure in both cases. Curve 3 was obtained with the 0.75 m column and an outlet pressure of 3 atm. The shift of the minimum towards lower $\bar{u}$ and $\bar{H}$ values upon elongating the column is perceptible but not very significant; this is due to the fact that the minimum occurs in a region of relatively low flow velocities and, accordingly, low flow-induced excess pressures with high liquid phase loads, *i.e.*, the ratio of the mean absolute column pressures corresponding to the minima on the curves for the longer and the shorter column, respectively, approaches unity.

On the other hand, with the ascending parts of the above curves, in the region of the highest flow velocities employed, the slope of the tangent to curve 1 (longer column) is about twice as large as that to curve 2 (shorter column). This is obviously due to the considerably greater curvature of the branch concerned of curve 1. In the above flow velocity region, the ratio of the slopes of the tangents to curves 1 and 2 should obey the expression[1] $\{C_s + C_m[(\eta L_1 \bar{u}/K) + P_o]\}/\{C_s + C_m[(\eta L_2 \bar{u}/K) + P_o]\}$, where L_1 and L_2 stand for the lengths of the longer and the shorter column, respectively.

Comparison of curves 3 and 2 shows that the effects of the increase of the column outlet pressure, while maintaining constant column length are similar to the above

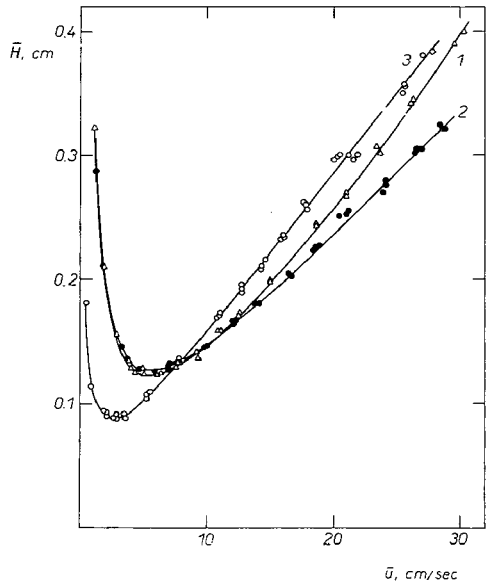

Fig. 2. Measurements at a constant column outlet pressure, high loading of liquid phase on the support. 1 = L 3 m, P_o 1 atm., P_i 1.1–3.91 atm; 2 = L 0.75 m, P_o 1 atm, P_i 1.02–1.67 atm; 3 = L 0.75 m, P_o 3 atm, P_i 3.06–3.75 atm.

column length effects in so far as the trends in the changes of the shape and location of the curve are concerned. The shift of the minimum upon the increase in the column outlet pressure is much more significant than that caused by the increase in the column length. This state of affairs reported earlier by SCOTT[3], is obviously brought about by the fact that the mean absolute column pressure corresponding to the minimum on curve 3 is much higher than that incidental to the minima on both curve 2 and even curve 1; owing to low flow velocities corresponding to the minima on all the curves in Fig. 2, an increase in the column outlet pressure is more effective than an increase in the column length in shifting the minimum towards the region of lower $\bar{u}$ and $\bar{H}$ values.

As for the effect of the outlet pressure on the ascending branch of the $\bar{H}$ *vs.* $\bar{u}$ curve, the ratio of the slopes of the tangents to curves 3 and 2, in the region of high velocities, should be given by $\{C_s + C_m[(\eta L_2\bar{u}/K) + P_{o3}]\}/\{C_s + C_m[(\eta L_2\bar{u}/K) + P_{o2}]\}$ where P_{o3} and P_{o2} are the higher and the lower column outlet pressure, respectively, which is in conformity with the courses of the respective curves in Fig. 2.

Another typical effect incidental to the increase in the column outlet pressure is the straightening of the ascending branch of the curve (*cf.* curve 3). This effect may be explained by referring to the relation for the respective slopes, $d\bar{H}/d\bar{u} = C_s + C_m[(\eta L\bar{u}/K) + P_o]$. It is apparent from this relation that the significance of the $\bar{u}$-containing term, which is responsible for the dished curvature of the ascending branch, decreases with increasing values of P_o. On the whole, the situation depicted in Fig. 2 is in agreement with eqn. 1.

It is worth mentioning that the results of our measurements are at variance with the results obtained by HALÁSZ *et al.*[4] for a similar system. The above authors

found quite opposite trends in the changes of the slope of the ascending branch of the curves and the position of the minimum upon increasing the column outlet pressure. On the other hand, our results are in agreement with those found by LOCKE AND BRANDT[5]. It is interesting to notice that HALÁSZ *et al.*'s measurements were carried out at absolute column pressures varying within fairly wide limits (column outlet pressures of within 0.5–6.0 atm) with N_2 carrier gas, whereas LOCKE AND BRANDT performed their measurements at subatmospheric column outlet pressures using He and CO_2 as carrier gases, which implies substantially lower absolute column pressures in LOCKE AND BRANDT's case. Our measurements were carried out at absolute column pressures comparable with those employed by HALÁSZ *et al.*, but with H_2 as the carrier gas, so that the carrier gas density approached more or less that in LOCKE AND BRANDT's measurements. Hence, it seems that it is the carrier gas density that decides whether the effects of the mean absolute column pressure on the $\bar{H}$ *vs.* $\bar{u}$ curve are predictable, in virtue of the simple mean pressure and stream-line flow concepts[1]. The above situation indicates the contingency of pressure induced turbulence occurring in the column.

Fig. 3 illustrates the situation in the case of measurements with the high loading of liquid phase on the support and a constant column inlet pressure. Again, the shifts in the slope and location of the curves upon changing either the column length or the column inlet pressure are in good agreement with eqn. 2. Curves 1 and 2 were obtained by measurements with the longer and the shorter column, respectively, at a column inlet pressure of 5.5 atm. Curve 3 was obtained with the shorter column at a column inlet pressure of 3.0 atm. The gentler slope of the ascending branch and the higher minimum HETP with curve 1, as compared to curve 2, is caused by the necessity of

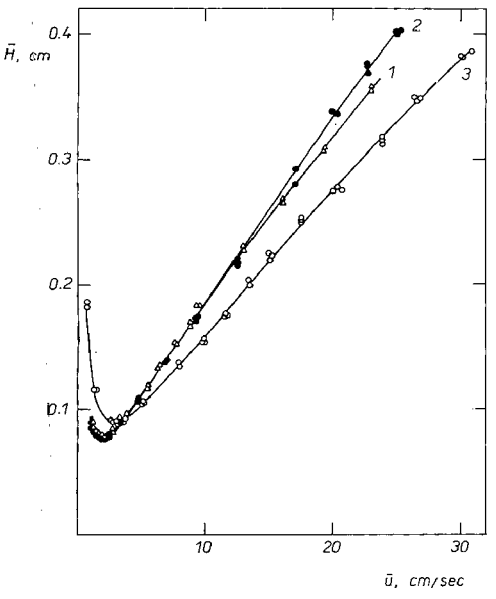

Fig. 3. Measurements at a constant column inlet pressure, high loading of liquid phase on the support. 1 = L 3 m, P_i 5.5 atm, P_o 5.4–3.52 atm; 2 = L 0.75 m, P_i 5.5 atm, P_o 5.48–4.83 atm; 3 = L 0.75 m, P_i 3.0 atm, P_o 2.92–2.16 atm.

having to apply a lower column outlet pressure with the longer column to get a given flow velocity than in the case of the shorter column. This obviously implies a lower mean absolute column pressure in the longer column than in the shorter one for the given flow velocity; the ratio of the slopes of the tangents to curves 1 and 2 should correspond to: $\{C_s + C_m[P_i - (\eta L_1 \bar{u}/K)]\}/\{C_s + C_m[P_i - (\eta L_2 \bar{u}/K)]\}$ in a region of high flow velocities. Variations in the column inlet pressure parameter have the same consequences here as in the work with constant column outlet pressures. The decrease of the mean absolute column pressure by decreasing the column inlet pressure from 5.5 to 3.0 atm (curve 3) results in a pronounced rise of the minimum HETP and in a gentler slope of the ascending branch of the curve; the ratio of the slopes of curves 3 and 2 is characterised by $\{C_s + C_m[P_{i3} - (\eta L_2 \bar{u}/K)\}/\{C_s + C_m[P_{i2} - (\eta L_2 \bar{u}/K)]\}$. All curves in Fig. 3 display perceptibly domed ascending branches, the curvature again being most notable in the case of the lowest mean absolute column pressure (curve 3). The domed curvature is due to the negative sign of the factor $\eta L\bar{u}/K$ in the relation $d\bar{H}/d\bar{u} = C_s + C_m[P_i - (\eta L\bar{u}/K)]$, applicable at higher flow velocities.

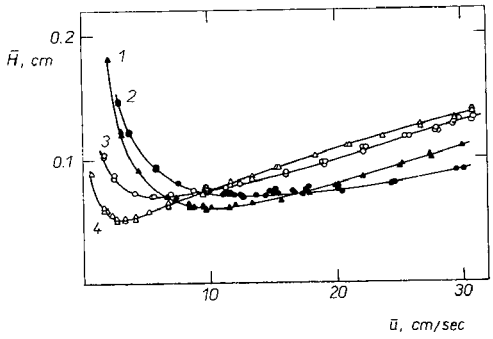

Fig. 4. Measurements with low loading of liquid phase on the support, 1,2,3 = constant column outlet pressure, 4 = constant column inlet pressure. 1 = L 3 m, P_o 1 atm, P_i 1.24–4.5 atm; 2 = L 0.75 m, P_o 1 atm, P_i 1.08–1.91 atm; 3 = L 0.75 m, P_o 3 atm, P_i 3.02–3.94 atm; 4 = L 3 m, P_i 6.5 atm, P_o 6.45–3.18 atm.

Fig. 4 illustrates the situation in the case of the low loading of liquid phase. Curves 1 and 2 correspond to measurements with an atmospheric column outlet pressure with the longer and the shorter column, respectively. Curve 3 represents the case of the shorter column operated at an outlet pressure of 3 atm. Curve 4 was obtained with the longer column operated at a constant inlet pressure of 6.5 atm. It is apparent from the comparison of curves 1 and 2 that the shifts of the minima towards lower flow velocities upon elongating the column are much more significant than in the corresponding case with a high concentration of liquid phase. However, the ratio of the slopes of the tangents to the curves at high velocities is similar to that for the corresponding curves obtained with the high concentrations of liquid phase, though the absolute values of the slopes are much lower with the lower concentration of liquid phase. This confirms the concept that the enhancement of the steepness of the ascending branch upon elongating the column or raising the column outlet pressure is, in practice, associated only with the situation in the gaseous phase and, conse-

quently, that the absolute change in the slope is only slightly dependent on the stationary liquid loading.

The decrease of the minimum HETP with the increase of the column length is larger than that expected in terms of eqn. 1. This is very likely due to longitudinal diffusion within extracolumn spaces; the significance of the latter rises on decreasing the column length and the retentive capacity of the packing. The above extracolumn contribution to the HETP, $\bar{H}_e$, can be derived from the expression $\bar{H}_e = 2RDV_e/Lv$ where R is the retardation factor, D is the diffusion coefficient of the solute in the mobile phase, V_e is the extracolumn volume, v is the mobile phase volume flow-velocity within the volume V_e, and L is the column length. This explanation is supported by the fact that an increase in the column outlet pressure with the same column (*cf.* curve 3) resulted in practically no change in the minimum HETP. The slight drop in the minimum HETP with curve 4, compared to curve 1, may be regarded as incidental to the appreciably higher mean absolute column pressure corresponding to the minimum on curve 4.

CONCLUSIONS

Under laminar carrier gas flow conditions and at moderate pressures and pressure drops, the variations in the course of the HETP *vs.* flow velocity curves, brought about by varying the column length and by changing the parameters of either the column outlet or inlet pressures, are in good agreement with the respective variations predictable by virtue of simple concepts of the role of the mean absolute column pressure, for both high and low concentrations of liquid phase on the support.

Under the above conditions, column elongation results in a slightly lower or unchanged minimum HETP, lower optimum carrier gas flow velocity, and a slight increase in the dished curvature of the ascending branch of the $\bar{H}$ *vs.* $\bar{u}$ curve when working at a constant column outlet pressure. In the case of constant column inlet pressure, an increase in the column length leads to a higher or unchanged minimum HETP, higher optimum flow velocity, and enhanced domed curvature of the ascending branch of the curve.

In both the above-mentioned cases, an increase of the mean absolute column pressure by elevating either the outlet or the inlet column pressure results in a remarkable shift of the minimum on the $\bar{H}$ *vs.* $\bar{u}$ curve towards lower values of both $\bar{H}$ and $\bar{u}$ when working with high concentrations of liquid phase and to little or no decrease in the minimum $\bar{H}$ and a significant decrease of the optimum $\bar{u}$ in case of low loads of liquid phase. The ascending branch of the $\bar{H}$ *vs.* $\bar{u}$ curve grows steeper and straighter upon elevating the absolute column pressure at a constant column length.

The conflicting situations arrived at by other authors seem to stem partly from pressure induced turbulent flow of the carrier gas in the column.

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CHROM. 4883

ÉTUDE DES PARAMÈTRES DE LA CHROMATOGRAPHIE EN PHASE GAZEUSE REALISÉE EN COUPLANT UN GRADIENT LONGITUDINAL ET UNE PROGRAMMATION DE TEMPÉRATURE

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SUMMARY

Study of gas chromatographic parameters obtained by coupling a longitudinal temperature gradient with a linear temperature programmation

Gas chromatography with a longitudinal temperature gradient can be coupled with a linear temperature programmation. By this method retention times may be calculated by means of graphical determination, using the following four parameters: value of temperature gradient, rate of temperature elevation, column length, and inlet temperature at the time of injection. The role of these four parameters in the determination of retention time is defined. By introducing several parameters the method provides more versatility and reduces the time of analysis when compared with other methods, *viz.* isothermal, linear temperature programmation, and longitudinal temperature gradient used alone.

INTRODUCTION

Comme nous l'avions indiqué précédemment[1,2], la chromatographie à température programmée, bien qu'elle permette un gain de temps par rapport à la chromatographie isotherme, présente cependant un inconvénient majeur. En effet on constate aisément que les solutés les moins volatils sont animés d'une trés faible vitesse d'élution en début de programme, lorsque la température choisie est faible afin d'éluer les solutés volatils de façon convenable, et ce fait est responsable d'un allongement du temps de rétention des solutés les derniers élués.

Par ailleurs nous avons proposé une méthode de chromatographie réalisée avec un gradient longitudinal établi de température le long de la colonne. Ainsi la température varie d'une façon linéaire avec l'abscisse de la colonne, et elle ne dépend pas du temps.

Pour pallier cet inconvénient de la chromatographie à température programmée, nous avons donc imaginé une méthode de chromatographie nouvelle, dans laquelle

sont réalisés simultanément une programmation de température et un gradient longitudinal établi de température le long de la colonne[2].

Nous nous proposons dans ce travail de relier la valeur du temps de rétention obtenue avec cette méthode aux différents paramètres qui interviennent lors de l'élution: la longueur de la colonne, la température de l'entrée de la colonne au moment de l'injection du soluté, la valeur de la vitesse de la programmation de la température, et la valeur du gradient longitudinal établi de température. Il sera possible ainsi de définir le rôle joué par chacun de ces paramètres vis à vis de l'élution du soluté, dans le cas de cette chromatographie couplée. En ayant soin de réaliser ce même travail en employant les autres méthodes, soit chromatographie isotherme, chromatographie avec programmation de température et chromatographie avec gradient longitudinal établi de température, il pourra être fructueux de comparer les valeurs obtenues et de préciser le gain de temps permis par cette nouvelle méthode.

PARTIE EXPÉRIMENTALE

Les équations établies dans la partie théorique permettent de calculer pour chaque valeur du paramètre choisi le temps de rétention des solutés élués avec notre méthode. Il suffit pour cela d'avoir préalablement déterminé de façon expérimentale la loi de variation du temps de rétention isotherme t_{rT} avec la température. Les calculs assez nombreux ont été résolus à l'aide d'une machine IBM (Ordinateur 1620 Modèle 2).

Nous avons jugé prudent cependant de vérifier expérimentalement un certain nombre de valeurs ainsi calculées. Les conditions opératoires et l'appareillage utilisé peuvent être rapidement décrites.

Chromatographe: Perkin-Elmer F7 avec catharomètre
Colonne: longueur 2 m; diamètre intérieur 3 mm
Matière: acier inoxydable
Liquide stationnaire: 2.5% caoutchouc Silicone SE-52
Gaz vecteur: hélium; débit 45 cm^3/min

La programmation de température est celle de l'appareil et nous avons quelque peu perfectionné son système pour rendre la vitesse linéaire[2]. Le gradient longitudinal de température est réalisé avec une résistance électrique isolée que l'on a enroulée autour de la colonne de telle sorte que le nombre de spires par unité de longueur varie selon une progression arithmétique[1].

ÉTUDE THÉORIQUE

Détermination du temps de rétention obtenu avec la chromatographie couplant la programmation de température et le gradient longitudinal établi de température

Nous formulerons deux hypothèses: tout d'abord l'hypothèse classique, que l'intéraction soluté–solvant obéit à la loi de Henry. Ensuite une hypothèse primordiale pour notre étude, à savoir que le gradient de pression du gaz vecteur est suffisamment faible pour que la vitesse linéaire du gaz vecteur soit constante le long de la colonne. Grâce à la seconde hypothèse, en tout point d'une colonne isotherme, la vitesse du soluté est reliée à la vitesse linéaire moyenne du gaz vecteur $\bar{V}$ et au facteur de rétention R_F par la relation:

$$\frac{dx}{dt} = \bar{V} \cdot R_F \tag{1}$$

De même le facteur de rétention est égal au rapport du temps de rétention du gaz vecteur t_g par celui du soluté t_{rT}.

$$\frac{dx}{dt} = \bar{V} \cdot \frac{t_g}{t_{rT}} \tag{2}$$

En pensant que évidemment la longueur L de la colonne est égale au produit de la vitesse moyenne du gaz vecteur par son temps de rétention, l'éqn. 2 s'écrit:

$$\frac{dx}{dt} = \frac{L}{t_{rTL}} \tag{3}$$

Cette relation (3), formulée grâce à la seconde hypothèse, vérifie la relation linéaire du temps de rétention isotherme du soluté t_{rTL} avec la longueur de la colonne L.

Avec cette méthode nouvelle, la température en un point de la colonne est une fonction des deux variables indépendantes que sont l'abscisse de ce point et le temps. On peut donc écrire la différentielle totale:

$$dT = \left(\frac{\partial T}{\partial x}\right)_t \cdot dx + \left(\frac{\partial T}{\partial t}\right)_x \cdot dt \tag{4}$$

dans laquelle

$$\left(\frac{\partial T}{\partial x}\right)_t = a \tag{5}$$

et

$$\left(\frac{\partial T}{\partial t}\right)_x = b \tag{5a}$$

a étant la valeur constante du gradient longitudinal de température et b étant la valeur de la vitesse de l'élévation de la température, constante en tout point de la colonne.

L'éqn. 4 s'écrit alors simplement:

$$dT = a \cdot dx + b \cdot dt \tag{6}$$

En remplaçant dans l'éqn. 6, dx par sa valeur tirée de la relation (3), on obtient l'équation différentielle

$$dT = \left(\frac{a \cdot L}{t_{rTL}} + b\right) \cdot dt \tag{7}$$

dont l'intégration permet de calculer le temps de rétention t_r obtenu avec la méthode décrite:

$$t_r = \int_{T_i}^{T_r} \frac{t_{rTL}}{a \cdot L + b \cdot t_{rTL}} \cdot dT \tag{8}$$

T_i étant la température à l'entrée de la colonne au moment de l'injection et T_r étant la température de rétention du soluté, c'est à dire la température à la sortie de la colonne au moment de la sortie du soluté.

De plus, les temps de rétention t_r et la température de rétention T_r sont reliés par la relation obtenue en intégrant l'éqn. 6.

$$t_r = \frac{T_r}{b} - \frac{T_i + a \cdot L}{b} \tag{9}$$

Pour déterminer la valeur du temps de rétention t_r, on réalise l'intégration graphique de l'intégrale (8), en définissant seulement la borne inférieure T_i.

$$t_r = \int_{T_i}^{T} \frac{t_{rTL}}{a \cdot L + b \cdot t_{rTL}} \cdot dT \tag{10}$$

On trace sur le même graphique, la courbe intégrale de l'éqn. 10, et la droite représentative de l'éqn. 11

$$t = \frac{T}{b} - \frac{T_i + a \cdot L}{b} \tag{11}$$

L'intersection de ces courbes permet de définir le temps de rétention t_r et la température de rétention T_r (Fig. 1). Remarquons que le terme $t_{rTL}/a \cdot L + b \cdot t_{rTL}$, situé dans l'éqn. 10 est indépendant de la longueur L, d'après la seconde hypothèse. Ainsi, lorsque l'on fait varier la longueur de la colonne, on constate les faits suivants: l'intégrale indéfinie (10) conserve la même forme; la droite de l'éqn. 11 subit une translation égale à $(a \cdot L)/b$.

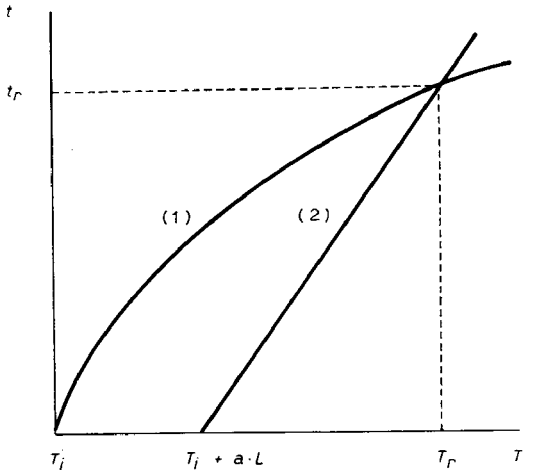

Fig. 1. Détermination graphique du temps de rétention pour la méthode couplée.

Courbes: (1) $t = \int_{T_i}^{T} \frac{t_{rTL}}{a \cdot L + b \cdot t_{rTL}} \cdot dT$;

(2) $t = \frac{T}{b} - \frac{T_i + a \cdot L}{b}$.

Détermination du temps de rétention obtenu avec la chromatographie avec gradient longitudinal établi

Cette chromatographie a été décrite précédemment[1] et nous présenterons içi une méthode différente pour la détermination du temps de rétention.

Le temps de rétention obtenu avec la chromatographie avec gradient longitudinal de température établi peut être déterminé avec l'éqn. 8 dans laquelle on choisit une valeur nulle pour b.

$$t_r = \frac{1}{a \cdot L} \int_{T_i}^{T_r} t_{rTL} \cdot dT \tag{12}$$

et la limite d'intégration T_r est constante et définie par la relation

$$T_r = T_i + a \cdot L \tag{13}$$

L'équation peut être intégrée graphiquement lorsque l'on connait la loi de variation du temps de rétention isotherme avec la température, et l'on obtient aisément le temps de rétention obtenu avec la méthode.

Détermination du temps de rétention obtenu avec la chromatographie avec programmation de température

L'éqn. 10 ne peut être utilisée dans ce cas. Le calcul doit être refait, selon la méthode décrite par HABGOOD ET HARRIS[4]. L'élimination du terme dt entre les relations (3) et (5a) permet d'obtenir l'équation différentielle

$$b \cdot dx = L \cdot \frac{dT}{t_{rTL}} \tag{14}$$

qui revient après l'intégration entre o et L, et entre T_i et T_r

$$b = \int_{T_i}^{T_r} \frac{dT}{t_{rTL}} \tag{15}$$

L'intégration graphique de cette éqn. 15 permet de déterminer la valeur de la température de rétention T_r, et le temps de rétention est alors calculé aisément avec la relation (16):

$$t_r = \frac{T_r - T_i}{b} \tag{16}$$

VARIATION DU TEMPS DE RÉTENTION AVEC LES DIFFÉRENTS PARAMÈTRES

Il existe quatre paramètres indépendants en chromatographie avec programmation de température et gradient longitudinal positif de température: le gradient longitudinal positif de température établi a, la vitesse linéaire d'élévation de la température b, la longueur de la colonne utilisée L et la température T_i à l'entrée de la colonne au moment de l'injection.

Nous ferons varier successivement chacun de ces quatre paramètres en maintenant les trois autres constants.

TABLEAU I

COMPARAISON DES TEMPS DE RÉTENTION DU *n*-DÉCANE EN FONCTION DE *a*

Chromatographie avec	*a (°C/m)*				
	0	*5*	*10*	*15*	*25*
Gradient seul T_r	80	90	100	110	130
t_r	446	345	291	250	198
Gradient plus programmation T_r	126.5	133	140	147	163
t_r	185.5	172	160	149.5	131

Variation du temps de rétention avec la valeur du gradient longitudinal

Nous avons calculé la valeur des temps de rétention obtenus avec différentes valeurs de a, en maintenant les valeurs des trois autres paramètres constants: $L = 2$ m, $T_i = 80°$ et $b = 0.25°$/sec. Les valeurs de a sont exprimées en °C/m.

Tout d'abord, il nous a paru intéressant de comparer les valeurs des temps de rétention obtenues en utilisant les mêmes valeurs de a, avec la chromatographie avec gradient longitudinal, et avec la chromatographie couplant le gradient et la programmation (Tableau I). Nous avons choisi comme soluté le *n*-décane, et les temps de rétention sont exprimés en seconde.

L'examen du Tableau I nous permet d'apprécier le gain de temps réalisé avec la seconde méthode par rapport à la première, et celà bienqu'il diminue lorsque croît la valeur donnée au gradient.

Nous avons ensuite étudié la loi de variation du temps de rétention de divers

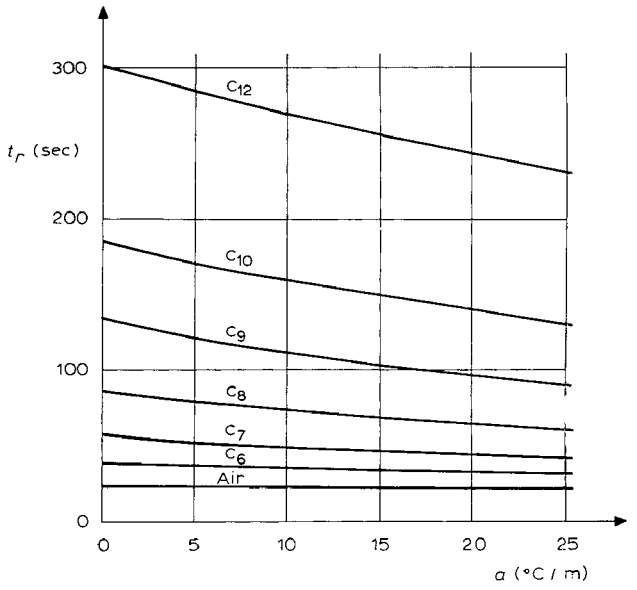

Fig. 2. Variation du temps de rétention avec la méthode couplée pour différentes valeurs du gradient a en maintenant les valeurs des autres paramètres constants: $b = 0.25°$/sec, $L = 2$ m et $T_i = 80°$.

solutés avec la valeur donné au gradient a. À la valeur nulle de a correspond la chromatographie à programmation de température. Les temps de rétention ont été déterminés expérimentalement et par le calcul en utilisant les éqns. 15 et 16. Pour chaque valeur non nulle de a, les temps de rétention ont été mesurés et calculés avec les éqns. 10 et 11. Nous avons choisi, en plus de l'air, comme solutés les alcanes normaux compris entre l'hexane et le dodécane.

Nous pouvons ainsi apprécier sur la Fig. 2 la diminution des valeurs des temps de rétention des alcanes élués avec la méthode couplée, par rapport au temps obtenus avec la chromatographie avec programmation de température, et fait intéressant, nous constatons que ce gain de temps et d'autant plus sensible que l'alcane a une condensation en atomes de carbone plus grande. Ainsi la méthode couplée provoque un resserrement des pics des solutés sans pour autant diminuer de façon excessive le temps de rétention des solutés les plus volatils.

Variation du t_r avec la valeur de la vitesse de montée en température b

Tout d'abord il nous parait intéressant de comparer les temps de rétention d'un soluté (le *n*-décane) obtenus d'une part avec la chromatographie à programmation

TABLEAU II

COMPARAISON DES TEMPS DE RÉTENTION DU *n*-DÉCANE EN FONCTION DE b

Chromatographie avec		b (°C/sec)		
		0	0.1	0.25
Programmation de temperature	T_r	80	107	126.6
	t_r	446	270	185
Methode couplée	T_r	110	129.5	147.5
	t_r	250	196	150

de température et d'autre part avec la méthode couplée. Les temps de rétention du Tableau II ont été déterminés pour différentes valeurs de b, et avec des valeurs constantes pour les trois autres paramètres: $L = 2$ m, $T_i = 80°$ et $a = 15°$/m (pour la méthode couplée).

Le gain de temps permis par la méthode couplée, sur la chromatographie avec programmation de t °C, décroit évidemment lorsque l'on choisit une valeur de b plus élevée, mais il demeure toujours fort important.

Nous avons représenté sur la Fig. 3, la variation en fonction de la valeur de la vitesse b du temps de rétention de plusieurs alcanes élués, avec la méthode couplée. Les trois autres paramètres sont maintenus constants: $a = 15°$/m, $L = 2$ m et $T_i = 80°$. À la valeur nulle de b, il correspond la chromatographie avec gradient longitudinal de température établi.

On constate ainsi une décroissance importante du temps de rétention lorsque b croit, et cette décroissance est d'autant plus importante que le point d'ébullition de l'alcane est élevé. Ainsi la conclusion ressemble quelque peu à la conclusion corres-

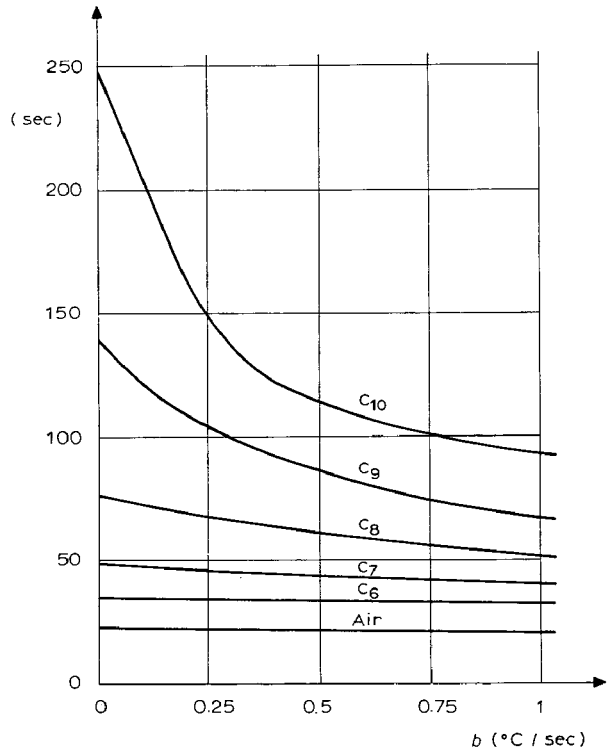

Fig. 3. Variation du temps de rétention avec la méthode couplée en fonction de la vitesse d'élévation de la température b en maintenant les valeurs des autres paramètres constants: $a = 15°/m$, $L = 2$ m et $T_i = 80°$.

pondant à l'influence de la valeur du gradient a, à savoir que la méthode couplée provoque un resserrement des pics des solutés en modifiant très peu le temps de rétention des solutés les plus volatils.

Variation du temps de rétention avec la longueur de la colonne

Le temps de rétention d'un soluté élué avec une colonne de longueur déterminée, peut être calculé de façons différentes selon les cas: en chromatographie couplée, en définissant l'intersection des courbes représentatives des éqns. 10 et 11; en chromatographie avec gradient longitudinal de température, en intégrant graphiquement l'éqn. 12 dans laquelle la borne T_i est définie par la relation 13; en chromatographie avec programmation de température, en utilisant les éqns. 15 et 16.

Nous avons représenté sur la Fig. 4, la variation du temps de rétention des alcanes normaux cités, en fonction de la longueur de la colonne, en maintenant chaque fois constantes les valeurs des autres paramètres: $a = 15°/m$, $b = 0.25°/sec$ et $T_i = 80°$.

Nous constatons ainsi que si le temps de rétention est proportionnel à la longueur de la colonne dans le cas de l'air, fait prévisible selon la seconde hypothèse, il n'en est pas de même pour les alcanes. De plus nous voyons que la concavité des courbes

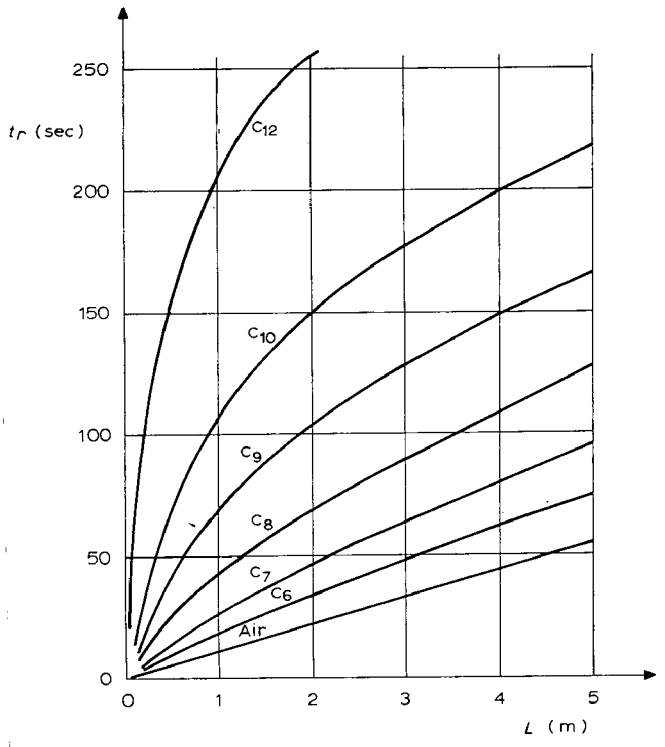

Fig. 4. Variation du temps de rétention avec la méthode couplée en fonction de la longueur de la colonne L, en maintenant les valeurs des autres paramètres constants, soit $a = 15°/m$, $b = 25°/sec$ et $T_i = 80°$.

s'accentue lorsque croit le nombre d'atômes de carbone des solutés. Ainsi lorsque la longueur de la colonne croit, le temps de rétention des derniers solutés croit bien moins vite que le temps des premiers.

Il nous a paru utile de comparer la croissance du temps de rétention avec la longueur de la colonne, obtenue avec les quatre cas de chromatographie, sur la Fig. 5 où l'alcane choisi est le *n*-décane. On constate ainsi que l'allongement du temps de rétention avec la longueur de la colonne devient de plus en plus faible lorsque l'on passe de la chromatographie isotherme à la chromatographie avec gradient, puis à la chromatographie avec température programmée, et enfin à la méthode couplée.

Variation du temps de rétention avec la valeur de la température à l'entrée de la colonne T_i

Les études de la variation du temps de rétention des solutés avec la valeur donnée à la température T_i à l'entrée de la colonne au temps o de la programmation, a été réalisée en maintenant constantes les valeurs des trois autres paramètres: $a = 15°/m$, $b = 0.25°/sec$ et $L = 2$ m.

On a représenté cette variation sur la Fig. 6, en choisissant les alcanes cités précédemment. Nous constatons ainsi que l'élévation de la température T_i provoque une diminution considérable du temps de rétention de tous les solutés, surtout si l'on

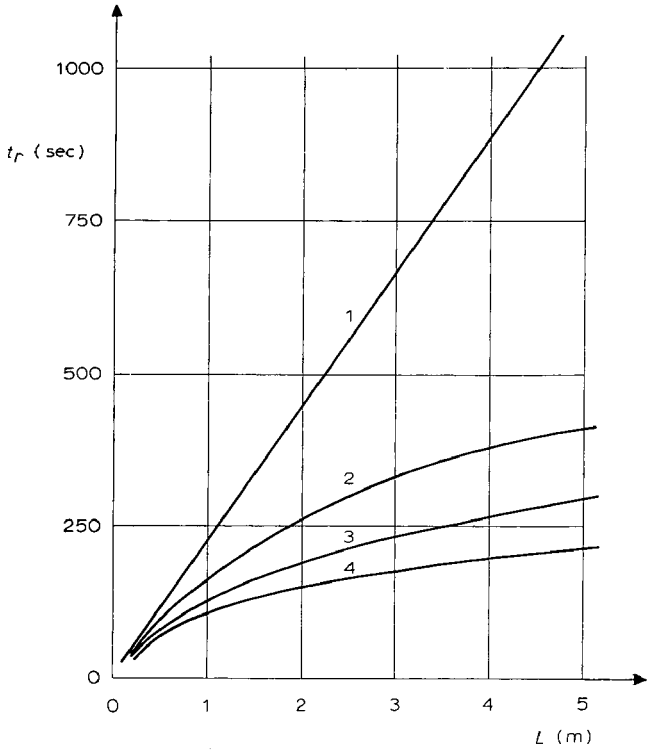

Fig. 5. Comparaison des temps de rétention du *n*-décane obtenus pour les différentes méthodes en fonction de la longueur de la colonne L en maintenant les valeurs des autres paramètres constants: $a = 15°$/m, $b = 25°$/sec et $T_i = 80°$. Courbes: (1) isotherme, (2) gradient seule, (3) programmation seule et (4) programmation plus gradient.

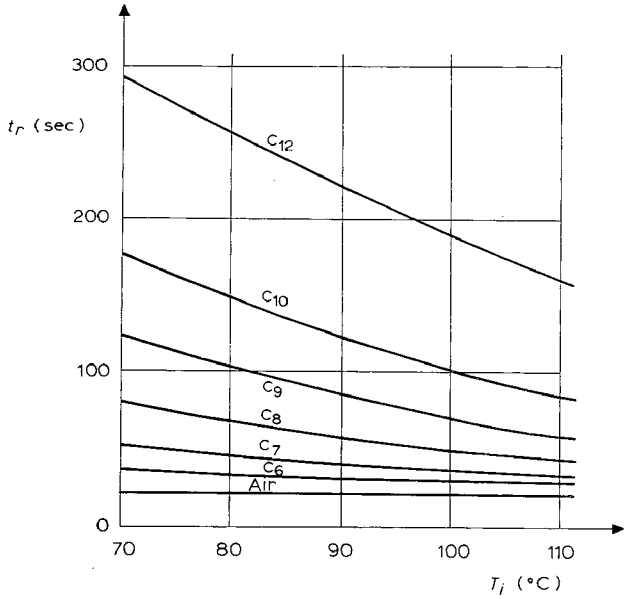

Fig. 6. Variation du temps de rétention avec la méthode couplée en fonction de la température de l'entrée de la colonne au moment de l'injection T_i en maintenant les valeurs des autres paramètres constant, soit $a = 15°$/m, $b = 0.25°$/sec et $L = 2$ m.

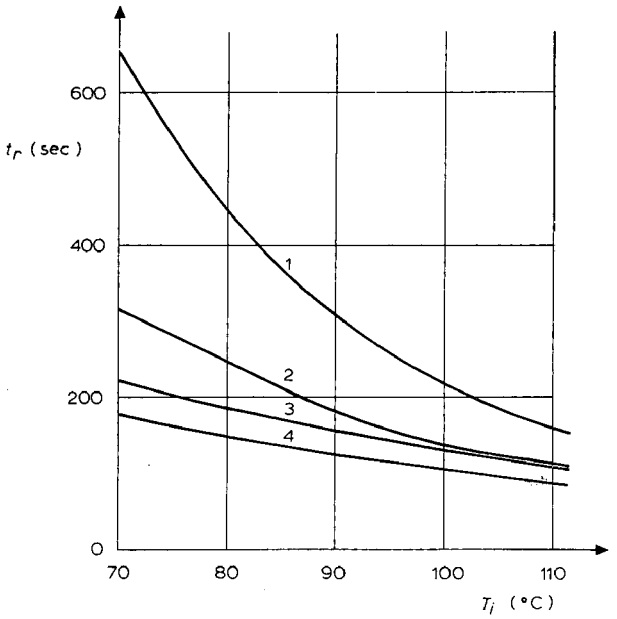

Fig. 7. Comparaison des temps de rétention du *n*-décane pour les différentes méthodes en fonction de la température de la colonne au moment de l'injection T_i en maintenant les valeurs des autres paramètres constant: $a = 15°/m$, $b = 0.25°/sec$ et $L = 2$ m. Courbes: (1) isotherme, (2) gradient seul, (3) programmation seule et (4) programmation plus gradient.

considère les temps de rétention corrigés du temps de rétention de l'air. Ainsi en choisissant T_i égale à 110° plutôt que 70°, on divise par deux les temps de rétention corrigés des divers solutés.

Sur la Fig. 7, nous pouvons comparer la variation du temps de rétention du *n*-décane avec la valeur donnée à T_i, obtenue avec les quatre méthode de chromatographie. En chromatographie isotherme, l'influence de T_i est considérable, puisque le temps de rétention décroit de façon exponentionnelle avec l'inverse de la température absolue. Cette diminution du temps est encore élevée avec la chromatographie avec gradient longitudinal. Par contre elle devient bien moins grande, quoique toujours importante, dans les cas de la chromatographie avec température programmée et dans le cas de la méthode couplée.

CONCLUSIONS

Ce travail a ainsi eu un double but: d'abord de définir le rôle joué, vis à vis du temps de rétention des solutés par chacun des quatre paramètres qui interviennent dans la chromatographie couplant la température programmée et le gradient longitudinal de température: vitesse de l'élévation de la température, gradient longitudinal, longueur de la colonne et température à l'entrée de la colonne au moment de l'injection.

Ensuite l'étude comparée des temps obtenus avec chacune des quatre méthodes décrites, a permis de dégager les avantages essentiels de la méthode couplée sur les trois autres: en effet, fournissant un gain de temps fort appréciable même sur la chro-

matographie à température programmée, la méthode couplée présente l'avantage supplémentaire de provoquer un resserrement des pics des solutés sans pour autant réduire de façon gènante le temps des solutés très volatils.

RÉSUMÉ

La chromatographie en phase gazeuse couplant le gradient longitudinal et la programmation de température, est étudiée tant sur le plan théorique que sur le plan expérimental. Le rôle joué par les différents paramètres sur les temps de rétention des solutés est précisé: gradient longitudinal de température, vitesse d'élévation de la température, longueur de la colonne et température à l'entrée de la colonne à l'injection. Les valeurs des temps de rétention obtenues avec cette méthode ont été comparées aux valeurs correspondantes obtenues en utilisant les autres méthodes de chromatographie: isotherme, à température programmée et avec gradient longitudinal seul. Il est ainsi possible d'apprécier le gain de temps permis par cette nouvelle méthode.

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CHROM. 4887

RELATIONS BETWEEN GAS CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE

III. THE NATURE OF INTERNAL ROTATION IN METHYL DERIVATIVES OF BENZENE*

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SUMMARY

The absence of a large isotope effect in the gas chromatographic behaviour of methyl derivatives of benzene indicates that the methyl rotation is not free in our model compounds, but corresponds to rotation–oscillation.

An incremental value in log α is found whenever one methyl group is in *ortho* position with respect to another. Additional absorption bands, which are the expected overtones of methyl non-symmetric deformations in the infrared spectra of such compounds, suggest that this "*ortho*-shift" effect in gas chromatography is due to perturbation of the methyl rotation–oscillation.

Conversely, it is suggested that the "*ortho*-shift" effect in gas chromatography may be exploited as an additional or a complementary technique in the investigation of configuration and conformation of isomers in organic chemistry.

INTRODUCTION

There is a growing interest in the correlation of chemical structure with behaviour of compounds in gas–liquid chromatography (GLC). The most general statistical mechanical approach, taking into account the effects of the kinetic energy and the configurational potential energy of the molecule in the liquid phase, is, at present, too complicated for practical calculations. No such approach has been reported. The statistical methods, proposed so far, concentrate on the contributions of the configurational potential energy which is only possible however, with the simplifying assumption of hard sphere molecules[3,4]. With the latter methods some fundamental molecular details get lost and it is therefore difficult to account for many of the structural variations of organic chemistry.

In this study the effects of the kinetic energy are investigated and an attempt

* For parts I and II, see refs. 1 and 2.

is made to eliminate the effects of the configurational potential energy by extreme simplification of the experimental conditions with regard to the liquid phase.

The sensitivity of the energy functions to slight variations of chemical structure and the precision of the additivity rules have been demonstrated in a preliminary investigation[10].

A direct relation[1] was shown between the partition coefficient in GLC and the ratio of partition functions of statistical mechanics. Extreme simplifications of the mathematical expressions were obtained when the relative retention α, instead of the absolute retention, were studied. Applying the analysis of variance[1,5] to discriminate between a number of hypotheses postulated, it was found that the separation of benzene and its methyl derivatives on a nonpolar liquid stationary phase corresponds to energy contributions due to translation and internal rotation, the contributions of other types of energy cancel. Under normal GC conditions[1]:

$$\log \alpha = \Delta \log [M^{3/2} \cdot Q_f] \tag{1}$$

where M is the molecular weight, and Q_f is its contribution of the internal rotation.

Analysis of the mathematical expressions for the free and the hindered internal rotational energies suggests that the nature of the methyl rotation can be conveniently determined by introducing hydrogen isotopes at strategic positions* in the model compounds.

THEORY

Two types of internal rotation are theoretically considered in our model compounds. They represent special cases according to the height of the potential barrier hindering internal rotation, for which mathematical expressions have been developed.

Free internal rotation

The height of the potential barrier is small compared to the kinetic energy associated with torsional momentum. For molecules with a number of *light* symmetric tops attached to a *heavy* rigid frame, as described by PITZER AND GWINN[7], as a good approximation each top contributes a factor Q_f to the partition function

$$Q_f = \frac{(8\pi^3 k_B T)^{\frac{1}{2}}}{hn} \cdot I_m{}^{\frac{1}{2}} \tag{2}$$

where

k_B = Boltzmann's constant
h = Planck's constant
T = absolute temperature
n = symmetry factor of top
$I_m = I_m{}^0$ = moment of inertia of a light symmetric top. This simplification is a good approximation in the case of our model compounds.

Substituting a trideuterated methyl in the place of a methyl group in our model compounds will double the value of I_m.

* The mixed trideuteromethyl and methyl derivatives of benzene in this investigation are a type of compound which have not been described in literature before. Their synthesis, purification and identification is described elsewhere[6].

Therefore according to eqns. 1 and 2 a contribution of approximately

$$\Delta \log \alpha = \log \sqrt{2}$$

is to be expected for every trideuterated methyl group in the case of free internal rotation

Hindered internal rotation

The height of the potential barrier is high compared to the kinetic energy associated with torsional momentum, frequency of tunnelling through the barrier is negligible in comparison to the rotation–oscillation frequency.

The total rotational energy of the molecule according to TOWNES AND SCHAWLOW[8] is given by

$$W = \tfrac{1}{2}I_x\omega_x^2 + \tfrac{1}{2}I_y\omega_y^2 + \tfrac{1}{2}I\dot{\chi}^2 + \tfrac{1}{2}\frac{I_1I_2}{I}\dot{\theta}^2 + \tfrac{1}{2}V_0(1-\cos 3\theta) \qquad (3)$$

The first three terms in eqn. 3 correspond to the rotational contributions in the case of "rigid rotation" which drop out of GLC relations[1]. The fourth and fifth terms are the contributions of internal rotation–oscillation, being the kinetic and the potential energy, respectively. For the rotation–oscillation $\dot{\theta}$ should be proportional to the frequency ν, thus

$$\theta^2 \sim \nu^2 = \frac{k}{4\pi^2} \cdot \frac{I}{I_1I_2} \qquad (4)$$

where

k = a force constant, independent of isotope substitution
ω, χ and θ are angular velocities
θ = angle of rotation of substituent relative to the frame
I = moment of inertia
V_0 = potential barrier hindering internal rotation.

Therefore the moments of inertia cancel out for the kinetic energy of internal rotation, which is the fourth term in eqn. 3.

In the last term of eqn. 3 the energy is controlled by the potential field effects, V_0. The gravitational constant being extremely small means that by good approximation mass effects do not contribute to V_0. Substituting a trideuterated methyl in the place of a methyl group in our model compounds therefore has no influence on the last two terms of eqn. 3. In contrast to the case of free internal rotation, a much smaller contribution

$$\Delta \log \alpha \ll \log \sqrt{2}$$

should be expected for every trideuteromethyl group in the case of hindered internal rotation.

Higher order terms of hindered internal rotation

The shape of the potential barrier in eqn. 3 is not given perfectly by the cos 3θ variation, but more generally should be written as a Fourier series[8]

$$V = \sum_p (a_p \cos 3p\theta + b_p \sin 3p\theta) \qquad (5)$$

where

V = potential barrier hindering internal rotation
p = 1,2, ..., p indicates the order of the terms

Eqn. 3 is a good approximation for isolated methyl groups. Studying Stuart-Briegleb molecular models it is seen that the presence of another methyl group in the *ortho* position will interfere with the rotation–oscillation movements of the methyl groups. This will cause higher order terms to appear in eqns. 3 and 5. In GC this means an additional contribution to log α which is the "*ortho*-shift" effect in our model compounds.

At the same time the appearance of additional absorption bands, overtones (higher order terms of the Fourier series) associated with methyl vibrations, should be expected in the IR spectra of such molecules.

RESULTS

Nature of methyl rotation

As is shown in Table I a contribution of the isotope effect was found experimentally for every trideuterated methyl group, amounting to

$$\Delta \log \alpha = -0.0205 \pm 0.0009$$

This value is much less than log $\sqrt{2}$ and clearly points to the case of hindered internal rotation with a high potential barrier (eqn. 3) and not to the case of free internal rotation (eqn. 2).

The experimental values of $\Delta \log \alpha$ are too small to allow the assumption of an intermediate height of the potential barrier. On the other hand, considering the relatively high values of Q_f that were found in an earlier investigation[1], the extreme case of a very high potential barrier corresponding to stiff molecules with methyl

TABLE I

RETENTION OF TRIDEUTEROMETHYL ANALOGUES RELATIVE TO NORMAL METHYL DERIVATIVES OF BENZENE ON APIEZON L AT 95°

The relative retention α of perdeuterobenzene is given for comparison.

Benzene derivative	α	$\Delta \log \alpha$	$\Delta \log \alpha$ *per* CD_3
1-CD_3	0.9810 ± 0.0010	−0.0192	−0.0192
1-CD_3-2-CH_3	0.9805 ± 0.0010	−0.0197	−0.0197
1-CD_3-3-CH_3	0.9792 ± 0.0010	−0.0210	−0.0210
1-CD_3-4-CH_4	0.9808 ± 0.0007	−0.0194	−0.0194
1-CD_3-2,3-di-CH_3	0.9791 ± 0.0004	−0.0211	−0.0211
2-CD_3-1,3-di-CH_3	0.9808 ± 0.0004	−0.0194	−0.0194
1-CD_3-2,4-di-CH_3	0.9801 ± 0.0004	−0.0201	−0.0201
2-CD_3-1,4-di-CH_3	0.9786 ± 0.0004	−0.0216	−0.0216
4-CD_3-1,2-di-CH_3	0.9786 ± 0.0005	−0.0216	−0.0216
2-CD_3-1,3,5-tri-CH_3	0.9793 ± 0.0005	−0.0209	−0.0209
1,2-di-CD_3	0.9602 ± 0.0004	−0.0406	−0.0203
1,3-di-CD_3	0.9573 ± 0.0006	−0.0436	−0.0218
1,4-di-CD_3	0.9605 ± 0.0008	−0.0402	−0.0201
2,4-di-CD_3-1,3,5-tri-CH_3	0.9590 ± 0.0002	−0.0418	−0.0209
1,2,4-tri-CD_3	0.9354 ± 0.0007	−0.0667	−0.0222
C_6D_6	0.9786 ± 0.0010	−0.0216	

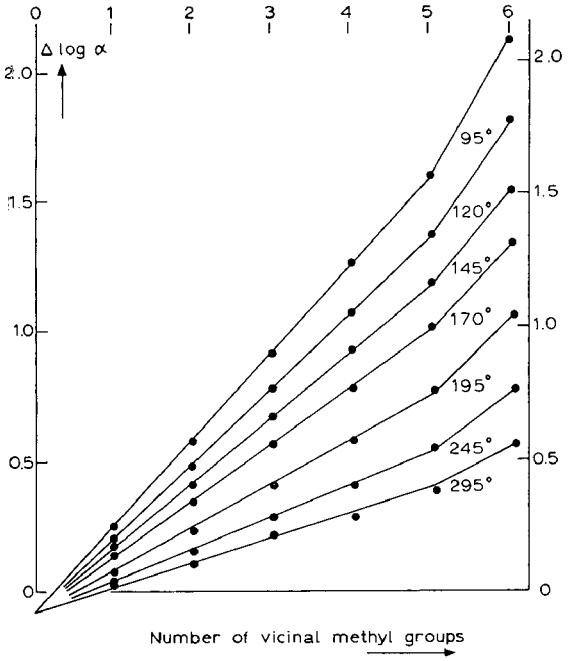

Fig. 1. Contribution of Δlog α of isolated and vicinal methyl groups (M1 through M6) of the methyl derivatives of benzene on Apiezon-L at 95–295°. Data reported in ref. 1.

rotation frozen, can be excluded in our temperature range. It is therefore concluded that methyl rotation in our model compounds corresponds to rotation–oscillation with occasional tunnelling through a high potential barrier.

Higher order terms of hindered internal rotation

Every addition of one methyl group in a position *ortho* to another causes perturbation of rotation–oscillation in both of the methyl groups every time. By approximation the same incremental value for the "*ortho*-shift" in GLC should therefore be expected in the series M1 through M5 (an isolated methyl group is indicated as M1, a string of vicinal methyl groups is treated as one substituent indicated as M2 through M6). In Fig. 1 experimental Δ log α of M1 through M5, by good approximation, indeed show a linear relationship.

Introduction of the sixth methyl group closes the ring of methyl groups causing extra perturbations. The Δlog α of M6 therefore should be higher than would be expected from the linear relationship of M1 through M5 (see Fig. 1).

It can be concluded therefore that, on applying perturbation theory, the GC behaviour of twelve out of thirteen model compounds can be correlated by means of eqns. 1, 3 and 5 and the values of Fig. 1; hexamethyl-benzene is the only exception.

Supporting evidence from IR spectra

All the deuterated model compounds of Table I show strong CD_3 stretching bands at 2232 $\pm$ 5, 2210 $\pm$ 5, 2132 $\pm$ 6 and 2060 $\pm$ 10 cm^{-1} in their IR spectra.

As these bands are sharper and are situated at much larger distance from each other in comparison to those of CH_3, it is convenient to study the appearance of overtones in this region: overtones of CD_3 non-symmetric deformation might be expected here (calculated from the CH_3 vibrations[9] of propene using $\bar{\nu}_D/\bar{\nu}_H = 0.73$). A number of additional absorption bands of medium strength are indeed found in the IR spectra of 1,2-di-CD_3-benzene (2158, 2130, 2118 and 2080 cm^{-1}) and 1,2,4-tri-CD_3-benzene (2170, 2110, 2075 and 2055 cm^{-1}).

Correspondingly, inspection of the IR spectra of all normal methyl derivatives of benzene showed that the absorption bands in the region between 2960 and 2850 cm^{-1} have a more complicated pattern when vicinal methyl groups are present in the molecule. When the vicinal methyl groups are not identical, that is CD_3 *ortho* to CH_3, no additional absorption bands are found in both the regions 2230–2050 and 2960–2850 cm^{-1} or 2600–2450 cm^{-1}. This suggests that combination bands of CD_3 and CH_3 non-symmetric deformations are absent or extremely weak, and the additional bands observed are the overtones expected.

It is suggested therefore that addition of one methyl group in a position *ortho* to another causes perturbation in methyl group non-symmetric deformation vibrations. The resulting overtones are spectroscopically strongly active when the vicinal methyl groups are identical. When the vicinal methyl groups are mixed (CD_3 *ortho* to CH_3) perturbation is still present, causing the same "*ortho*-shifts" in GC, however because of in-phase and out-of-phase coupling of the vibrations ($\bar{\nu}_D/\bar{\nu}_H = 0.73$) these overtones are, by chance, spectroscopically non-active.

There is no reason to believe that perturbation, whenever it occurs, should be limited to one type of methyl vibration only. It is therefore concluded that, applying perturbation theory, the "*ortho*-shift" effect in our model compounds in GC is related to the appearance of additional absorption bands in their IR spectra. Conversely, this suggests that the "*ortho*-shift" effect in GC may be exploited as a new technique in the investigation of configuration and conformation of isomers in organic chemistry. This will be of particular convenience in those cases where internal rotation is spectroscopically not active in IR, Raman and microwave spectrometry.

EXPERIMENTAL

The mixed trideuteromethyl and methyl derivatives* of benzene[6] were obtained by the Wurtz–Fittig synthesis, purified by GLC and identified by IR** and NMR*** spectrometry. The NMR spectra showed a high isotopic purity for the methyl groups; this was confirmed by mass spectrometry. Perdeuterobenzene was commercially available.

IR spectra

Double beam grating IR spectrophotometer, Perkin-Elmer Model 337; 2–10 mg sample in 0.3 ml carbon tetrachloride, 0.5 mm KBr cell.

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* Synthesis and purification were performed with the assistance of Miss M. C. HAZELEGER.
** IR spectra were measured by Mrs. M. A. G. DEN DULK-BARENS.
*** NMR spectra were measured by Miss L. A. MARS.

GC conditions

Chromatograph, Hewlett-Packard/F & M, Model 5750.
Detector, hydrogen flame ionisation detector.
Recorder, Hewlett-Packard, −0.05–1 mV, 1 sec.
Carrier gas, He at 100 ml/min measured at the exit at 20° and 1 atm.
Sample size, 0.01 to 0.10 mg per component.
Analysis temperature, 95°.

Column K47: 9 m × 0.5 cm I.D. coiled copper tube filled with Apiezon L (5:95) on Chromosorb P DMCS (100/120 mesh), efficiency *ca.* 12.000 theoretical plates; analysis time 550 min for pentamethylbenzene.

Column K56: 4 m × 0.5 cm I.D. coiled stainless steel tube filled with Apiezon L (5:95) on Chromosorb P DMCS (100/120 mesh), efficiency *ca.* 6.000 theoretical plates.

Other conditions and correction factors are given in ref. 10.

Evaluation of retention data from partially resolved pairs

Evaluation of partially resolved normal peak pairs has been studied by VANDENBELT AND HENRICH[11].

Peak pairs that have 50% or better separation have their apparent maxima at the same positions as the maxima of the component peaks[11]. No corrections are therefore needed for the peak positions of the di-CD_3 and the tri-CD_3 derivatives relative to their fully protonated analogues. Representative chromatograms are shown in Fig. 2, showing separations on column K56.

On the high resolution column K47 some separations (not shown) were obtained that were even better than 90%.

The mono-CD_3 derivatives, even with the high resolution column K47, have a separation of approximately 10% relative to their fully protonated analogues. In such cases the apparent peak maxima are shifted relative to the maxima of the component peaks. The corrections are most conveniently evaluated[11] when the com-

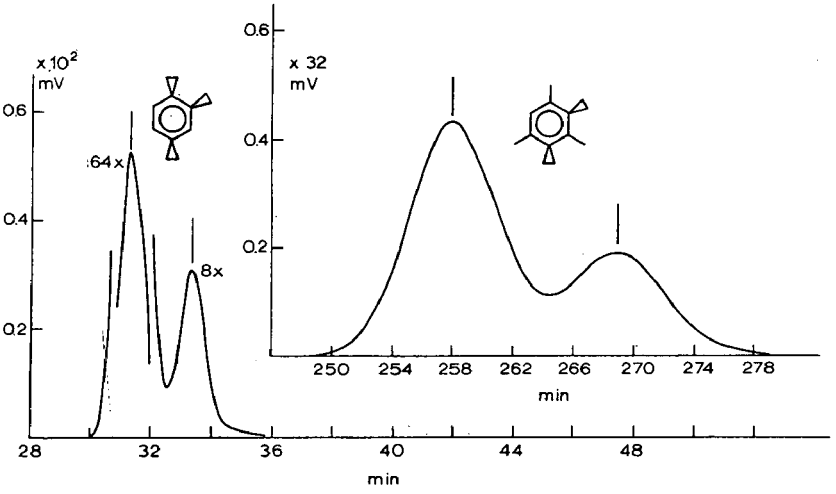

Fig. 2. Separations of 2,4-di-CD_3-1,3,5-tri-CH_3-benzene and 1,2,4-tri-CD_3-benzene from their fully protonated analogues on an Apiezon L GC column K56 at 95°.

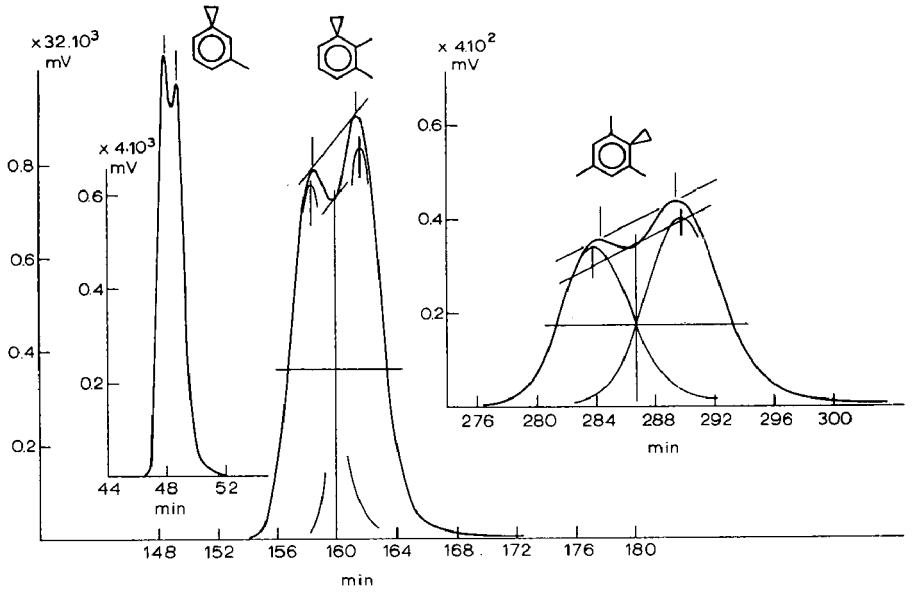

Fig. 3. Separations of 1-CD_3-3-CH_3-benzene, 1-CD_3-2,4-di-CH_3-benzene and 2-CD_3-1,3,5-tri-CH_3-benzene on an Apiezon L GC column K47 at 95°.

ponents have equal peak height. Sample composition* was adjusted with this in mind.

Alternatively, corrections to the position of the peak maxima were obtained by the following approach as demonstrated in Fig. 3. A straight line joining the two apparent peak maxima is drawn. The tangent to the valley is drawn parallel to the first line. Its point of contact is, by approximation, the point where both components contribute equally to the amplitude of the detector signal. The point at half height is a common point of the two component peaks (see Fig. 3). The lower front part of the second component is drawn through the common point parallel to the lower front part of the first component (which coincides with the lower front part of the apparent peak for separation of 10% or better). The amplitudes for the top part of the first component peak are found by computation. The top part of the second component peak is constructed in an analogous way. The two methods produce practically the same corrections for the location of the peak maxima. Representative gas chromatograms are shown in Fig. 3.

Trideuteromethyl benzene and perdeuterobenzene are eluted very rapidly and they are, therefore, more difficult to separate from their fully protonated analogues. They were injected separately with a number of internal standards. Repeat experiments were performed until the required accuracy was attained.

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CHROM. 4896

A SECOND-ORDER REACTION ON A GAS CHROMATOGRAPHIC COLUMN

I. COMPUTATIONAL ANALYSIS BY THE PLATE THEORY

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SUMMARY

Plate theory is used to compute the behaviour of a system in which a reversible bimolecular reaction $A + C \rightleftharpoons 2B$ is occurring during the passage of a mixture of A, B and C through a gas chromatographic column. An apparent equilibrium constant calculated from the elution profile will usually be much less than the true equilibrium constant. Curves are presented showing in detail how the apparent equilibrium constant and centre band width in the elution profile vary with feed pulse volume and with flow rate.

INTRODUCTION

Reaction on a gas chromatographic column has drawn wide attention in theory and in practice. As reactors, chromatographic columns have an entirely different character from ordinary steady flow reactors. Sometimes the conversion becomes much higher than expected from the equilibrium constant, which is understood easily since reactants and products are separated as they flow through a chromatographic reactor. Dehydrogenation reactions are among the best examples of this, since the hydrogen liberated usually has less affinity for a column and flows faster than the reactant and the other product. Indeed patents[1–3] have been awarded for this type of reactor.

Various theoretical treatments[4–10] have been developed, for various types of reactions on chromatographic columns, treating the extent of conversion, the elution curve profile, the effect of the shape of the feed pulse, and so forth. These treatments, however, neglect the band-broadening caused by diffusion and other non-idealities in chromatographic behaviour.

KOCIRIK[11] applied rate theory[12] to take band broadening into account for the case of a first order reaction. If, however, a reaction with a rate law more complicated than first order is to be considered, application of rate theory results in too complicated

a mathematics to be solved analytically, and too many adjustable parameters (such as diffusion constant, mass transfer coefficient, and fractions of volumes of mobile and stationary phases, besides reaction rate constants) when a numerical solution is attempted.

We therefore wish to report the results of applying the plate theory[12–15] to reactions of the type A + C $\rightleftharpoons$ 2B on a chromatographic column. The plate theory has been criticised[16] because of the obscurity of its relationship to physical mechanisms. But it is very useful in a study in which the mechanisms of band broadening are not going to be analysed in detail, because it expresses broadening in terms of a single parameter, the number of theoretical plates. The theory also leads to economical computer programmes, which proved convenient in varying parameters to match the experimental results reported in Part II[17].

METHOD OF ANALYSIS

In taking the material balance at the i-th plate in a conventional manner, the rate of production of components A, B and C is included. If we assume that the reaction A + C $\rightleftharpoons$ 2B occurs in the stationary phase, and that the rates may be expressed as those of a bimolecular reversible process, the material balances become,

$$\begin{aligned}(V_G + K_A V_L)\,(\mathrm{d}A_i/\mathrm{d}t) &= w(A_{i-1} - A_i) + k_0(B_i^2 - K_e A_i C_i)\\ (V_G + K_B V_L)\,(\mathrm{d}B_i/\mathrm{d}t) &= w(B_{i-1} - B_i) - 2k_0(B_i^2 - K_e A_i C_i)\\ (V_G + K_C V_L)\,(\mathrm{d}C_i/\mathrm{d}t) &= w(C_{i-1} - C_i) + k_0(B_i^2 - K_e A_i C_i)\end{aligned} \qquad (1)$$

where:

A_i, B_i, C_i = concentrations of components in stationary phase of i-th plate
V_G, V_L = volumes of mobile (gaseous) and stationary (liquid) phase in a plate
K_A, K_B, K_C = partition coefficients of components between mobile and stationary phases
w = volume rate of flow of carrier gas
K_e = equilibrium constant for the reaction A + C $\rightleftharpoons$ 2B in the mobile phase
$k_0 = V_L k K_B^2$, where k is the second-order rate constant in the stationary phase

For the first plate ($i = 1$), A_0, B_0 and C_0 appear in eqns. 1. These designate concentrations in the feed pulse.

Experimentally, retention volumes V_{RA}, V_{RB} and V_{RC} for the three separate components can be found either by feeding each component separately or, if practicable, by choosing conditions so that k_0 is negligibly small. (The retention volumes are to be understood as those measured when the feed pulse V_0 is small compared to the retention volume.) Then the number of theoretical plates, n, and hence $V_G + K_A V_L$, etc., can be calculated by $n = 16 V_{RA}^2/(\text{band width})_A^2$ and $n(V_G + K_A V_L) = V_{RA}$ and similar equations with subscripts B and C replacing A.

The numerical solution of eqns. 1 was computed with an IBM 7044 computer under the following conditions:

$t \leq 0$: $A_i = B_i = C_i = 0$ for $0 \leq i \leq n$
$0 \leq t \leq t_0$: $A_0 = A_0^0\,\varphi(t)$; $B_0 = B_0^0\,\varphi(t)$; $C_0 = C_0^0\,\varphi(t)$
$t > t_0$: $A_0 = B_0 = C_0 = 0$

where $\varphi(t)$ is a step function, but with infinities at the steps avoided by rounding off in an arbitrary manner, for which purpose a cosine curve was used:

$$0 \leq t \leq zt_0: \quad \varphi = 0.5[1 - \cos(\pi t/zt_0)]$$
$$zt_0 \leq t \leq (1 - z)t_0: \quad \varphi = 1$$
$$(1 - z)t_0 \leq t \leq t_0: \quad \varphi = 0.5[1 + \cos\{\pi(t - t_0 + zt_0)/zt_0\}]$$

The arbitrary width z was taken to be 0.05.

For a finite reaction rate, eqns. 1 were solved by the method of HAMMING[18]. For an infinite reaction rate, a treatment similar to MAGEE's for instantaneous reactions[8] was applied. Instead of eqns. 1, the following equations have to be solved:

$$\begin{aligned}
(V_G + K_A V_L)\,(\mathrm{d}A_i/\mathrm{d}V) &= (A_{i-1} - A_i) + \delta_i \\
(V_G + K_B V_L)\,(\mathrm{d}B_i/\mathrm{d}V) &= (B_{i-1} - B_i) - 2\delta_i \\
(V_G + K_C V_L)\,(\mathrm{d}C_i/\mathrm{d}V) &= (C_{i-1} - C_i) + \delta_i \\
B_i^2/A_i C_i &= K_e
\end{aligned} \tag{2}$$

where $\mathrm{d}V = w\mathrm{d}t$, the volume of carrier gas passing through a plate in time $\mathrm{d}t$, and δ_i is the amount of conversion required to establish the equilibrium instantaneously. Eqns. 2 were also solved numerically with the IBM 7044 computer.

In the numerical work, concentrations can be expressed in terms of an arbitrary concentration unit, provided that k_0 is related to that same unit. The feed pulse was assumed to contain an equilibrium mixture of the components, and the value (100) chosen for the equilibrium constant was close to experimentally attainable values for the reaction $I_2(g) + Br_2(g) \rightleftharpoons 2IBr(g)$.

Values chosen for parameters arose from preliminary work on the Br_2–IBr–I_2 system, but are mostly somewhat different from the values required in Part II. They are:

$A_0^0 = C_0^0 = 1.0$ conc. unit, and $K_e = 100$, hence $B_0^0 = 10.0$ conc. unit
$V_{RA} = 54.0$ ml; $V_{RB} = 74.8$ ml; $V_{RC} = 108.0$ ml

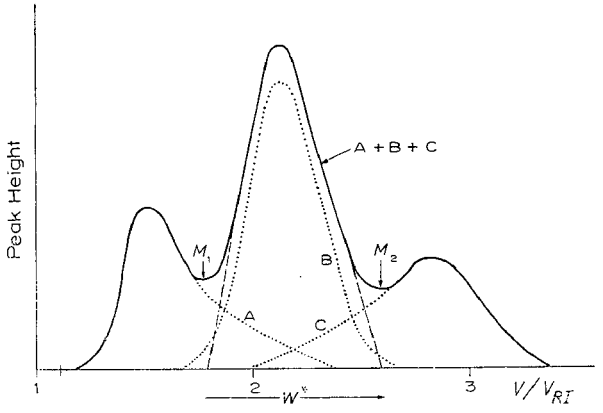

Fig. 1. A typical computed elution profile (A + B + C), with dotted lines showing the contributions from the separate components. M_1 and M_2 are minima used in calculating K_{eff}*. W* is the centre band width from tangents at inflection points. $n = 200$; $K_e = 100$; $V_0 = 0.5$ ml = 0.0136 V_{RI}; $k_0 = 200$ ml min^{-1} (conc. unit)$^{-1}$; $w = 76$ ml/min. From the profile, $K_{eff} = 2.94$ and $K_{eff}^* = 9.01$.

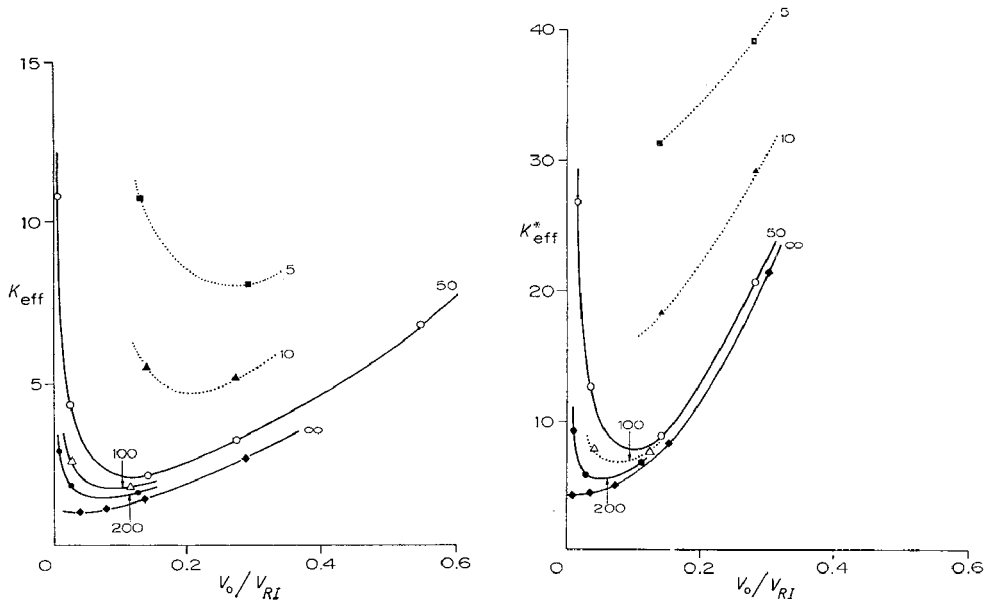

Fig. 2. Variation of apparent equilibrium constant K_{eff} with feed pulse volume V_0. Numbers on curves are values of reaction rate parameter k_0. n, w and K_e are the same as in Fig. 1.

Fig. 3. The same as Fig. 2, for K_{eff}* instead of K_{eff}.

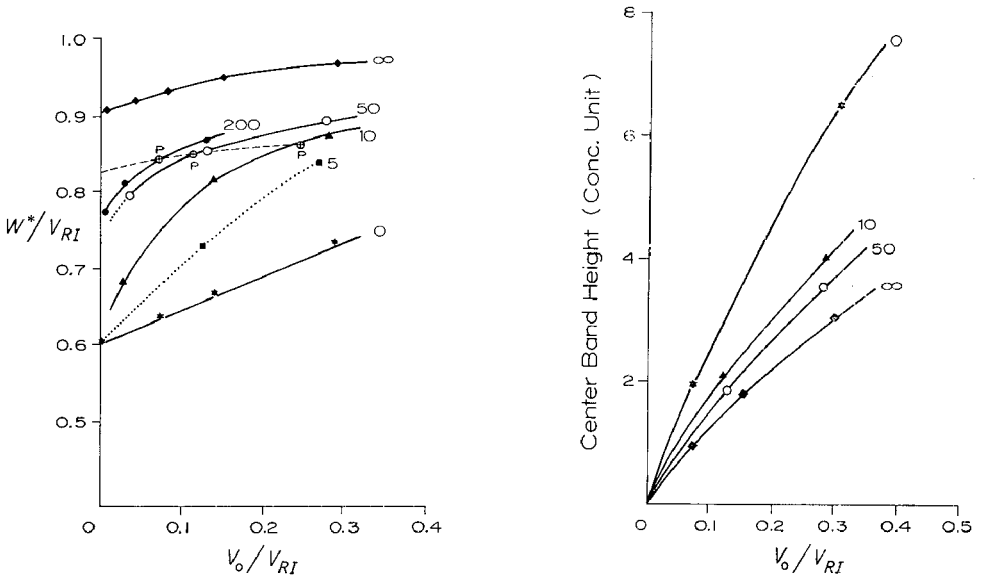

Fig. 4. Centre band width W^* against feed pulse volume V_0. Numbers on curves are values of k_0. Points marked P correspond to minima in Fig. 2. The dotted line is the locus of P (essentially constant W^*). n, w and K_e are the same as in the previous figures.

Fig. 5. Centre band height against feed pulse volume V_0 for various k_0 (numbers on curves). n, w and K_e are the same as in the previous figures.

$V_{RI} = nV_G = 36.8$ ml = retention volume of a component which has no affinity with the stationary phase (zero partition coefficient); in practice, this was regarded as being the same as the retention volume of air, designated V_{RAIR}.

$n = 200$
$w = 30.0, 76.0, 120.0$ ml/min
$V_0 = wt_0 = 0.5$ to 20 ml
$k_0 = 5, 10, 50, 100, 200$ ml min^{-1} (conc. unit)$^{-1}$

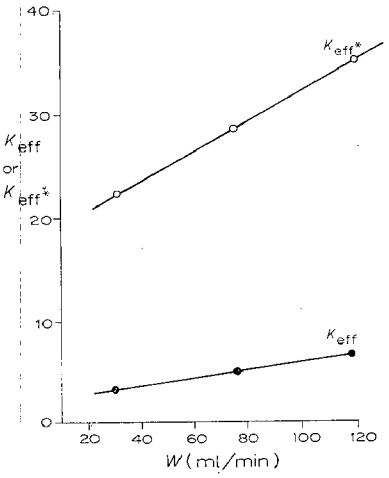

Fig. 6. Apparent equilibrium constants K_{eff} and K_{eff}* against flow rate for a low reaction rate, $k_0 = 10$ ml min^{-1} (conc. unit)$^{-1}$. $V_0/V_{RI} = 0.272$; n and K_e are the same as in the previous figures.

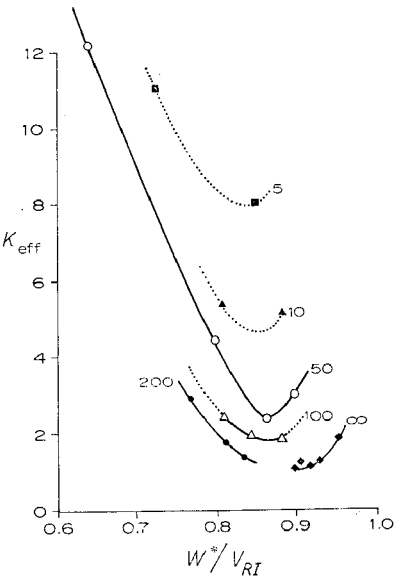

Fig. 7. K_{eff} against centre band width W* for various k_0 (numbers on curves). n, W and K_e are the same as in Figs. 1–5.

A typical computed elution profile is shown in Fig. 1. To describe the behaviour of the system the following quantities were chosen:

$$K_{eff} = [\int_0^\infty B(t)\mathrm{d}t]^2/[\int_0^\infty A(t)\mathrm{d}t \int_0^\infty C(t)\mathrm{d}t]$$

$$K_{eff}^* = [\int_{M_1}^{M_2}(A + B + C)\mathrm{d}t]^2/[\int_0^{M_1}(A + B + C)\mathrm{d}t \int_{M_2}^\infty (A + B + C)\mathrm{d}t]$$

where:

M_1 and M_2 are minima in the elution profile, as marked in Fig. 1;

W* = band width parameter = distance in volume units between intersections of tangents at the inflection points of the centre band of the profile with the horizontal axis;

K_{eff} is an apparent equilibrium constant related to the actual total amounts of A, B and C in the eluted material; but it is not accessible to experimental measurement, because of band overlap in the elution profile;

K_{eff}* is a parameter which can be calculated from an experimental elution

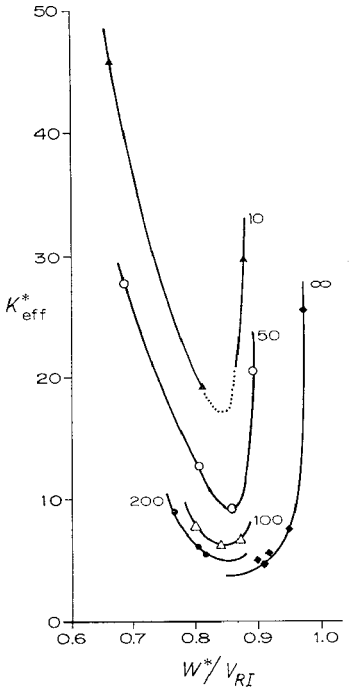

Fig. 8. The same as Fig. 7, for K_{eff}* instead of K_{eff}.

profile; it should normally have a value similar to K_{eff}, but is not precisely related to total amounts of A, B and C.

Results of computations are displayed in Figs. 2–8.

DISCUSSION

Chromatographic separation must slow down the combination of A and C relative to the decomposition of B; thus K_{eff} and K_{eff}* are always less than the true equilibrium constant K_e. The precise value of K_{eff} can be thought of as depending on two factors, which sometimes compete with each other so that, for example, K_{eff} passes through a minimum as V_0 is varied at constant k_0. These two factors are: (a) The reaction rate term, which increases both with the rate parameter k_0 and with the concentration term at any plate $(B_i^2 - K_e A_i C_i)$. A higher reaction rate term tends to decrease K_{eff}. (In Fig. 2, for any constant V_0, K_{eff} becomes smaller as k_0 increases.) (b) The degree of overlapping of the two peaks. Clearly, greater overlapping tends to bring K_{eff} closer to the true K_e.

When the feed pulse volume V_0 is increased, both the concentrations at any plate and the overlapping become larger. At low V_0 the concentration effect is dominant, and at high V_0 the overlapping effect. This occurs because, as discussed by VAN DEEMTER *et al.*[12], the concentration of any single component (*e.g.* A) increases linearly with V_0 up to $V_0 n^{\frac{1}{2}}/V_{RA} \sim 1$, and thereafter increases more slowly (compare Fig. 5, showing centre band height against V_0); whereas the band width increases slowly at the beginning, but linearly above $V_0 n^{\frac{1}{2}}/V_{RA} \sim 2.5$.

Hence each curve of K_{eff} against V_0 in Fig. 2 has a minimum, except that for $k_0 = \infty$. In that case, the reaction is completed at each plate no matter how small the concentrations are. The curve displays the overlapping effect by itself, and K_{eff} increases monotonically with V_0.

The band width parameter W^* (Fig. 4) depends chiefly on the component B, but is larger than the true band width of B because of overlapping (Fig. 1). At any constant V_0, W^* increases with reaction rate parameter k_0. This is because the increasingly rapid production of A and C from B causes the overlapping to become worse; in terms of band shape, the A and C bands (Fig. 1) become asymmetric, with "tails" pointing towards the centre band.

A curve of W^* against V_0 for constant k_0 (Fig. 4) seems to be divisible into two parts: an initial rapid increase, with strong curvature, followed by a slower, more nearly linear increase. This is best seen at $k_0 = 10$, 50 or 200. The transition between the two regions lies roughly at the point marked P on some of the curves in Fig. 4; this is the point at which K_{eff} has its minimum value (Fig. 2). Up to the point P, K_{eff} decreases as V_0 rises, which means that the peaks for A and C are increasing relative to B, so that their tails are contributing increasingly to W^*. Beyond the point P, this effect is reversed; the A and C peaks thereafter contribute successively less and less to the centre peak, and W^* increases less rapidly than in the case of symmetrical bands, $k_0 = 0$.

It is interesting that the points P, for a wide range of k_0 values, lie at roughly the same W^*; thus the importance of extent of overlapping in the dependence of K_{eff} on V_0 can be seen in a surprisingly simple way, in relation to band width.

The flow rate dependence of K_{eff} is shown in Fig. 6. Clearly, a higher flow rate gives less time for reaction to occur, and K_{eff} rises with the flow rate. This effect becomes smaller as reaction rate increases and ultimately, for instantaneous reaction ($k_0 = \infty$), K_{eff} does not depend on flow rate.

In the last two diagrams (Figs. 7 and 8) K_{eff} and K_{eff}^* are plotted against W^*. These diagrams are useful in practice because W^* is easily evaluated from the chromatogram, whereas the feed pulse volume V_0, used as independent variable in Figs. 2 and 3, is rather difficult to evaluate experimentally because, for example, the kinetics of evaporation of a liquid sample in the injector are not known.

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CHROM. 4897

A SECOND-ORDER REACTION ON A GAS CHROMATOGRAPHIC COLUMN

II. DECOMPOSITION OF IBr ON A CHROMATOGRAPHIC COLUMN

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SUMMARY

The computational analysis described in Part I[1] is applied to the behaviour of the system Br_2–IBr–I_2 on a column of Kel-F grease supported on teflon powder. The experimental results are well matched by computations for instantaneous reaction. This implies a rate constant $k \gtrsim 5 \times 10^4$ l mole^{-1}sec^{-1} and an activation energy $E_a \lesssim 10.6$ kcal mole^{-1} for the decomposition of IBr in solution in Kel-F grease at 94°.

EXPERIMENTAL

The apparatus is essentially a gas chromatograph in which the whole flow system is made of pyrex glass, except that the detector block is stainless steel (SS 316). The detector is a thermal conductivity cell with teflon-clad tungsten filaments (from Gow Mac). The attenuator of an Aerograph A90 P3 chromatograph was used.

Column

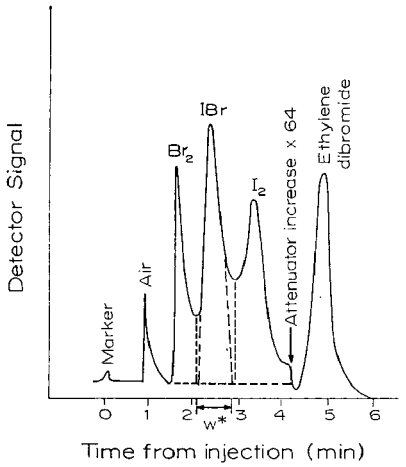
The column consisted of 4.5 m × 5 mm I.D. pyrex tubing, containing 10% Kel-F grease No. 90 (chlorofluorocarbon) coated on Chromosorb T (teflon powder). The coating was done by dissolving the Kel-F grease in chloroform, mixing with the Chromosorb T, and drying over a water bath. Since the dry product is difficult to pack evenly in the column (probably because it easily acquires electrostatic charge), the coated Chromosorb was made into a slurry with a mixture of equal volumes of acetone and water, which does not dissolve the coating. The slurry was introduced into pyrex tubing, previously bent into shape for fitting into the oven of the gas chromatograph, with a glass wool plug at the end of the tubing, and with suction applied by means of an aspirator.

Standard operating conditions

The operating conditions were as follows: Filament current: 200 mA; flow rate: 76.0 ml/min at the exit (room temperature, atmospheric pressure); temperatures: injector 180°, column 93–95° and detector 130°; inlet pressure: 1.5 atm.

Calibration

Solutions of bromine and iodine (both Baker Analyzed Reagent Grade) in ethylene dibromide (B.D.H. Reagent Grade) were injected quantitatively by means of Hamilton microsyringes, and the peak areas were measured by a disc integrator attached to the recorder (Leeds and Northrup Speedomax H). Both lay on the same linear calibration curve of peak area *versus* moles injected. Mixtures of Br_2 and I_2 were injected quantitatively; this time three overlapping peaks appeared on the chromatogram (Fig. 1). The first and third peaks corresponded to Br_2 and I_2, re-



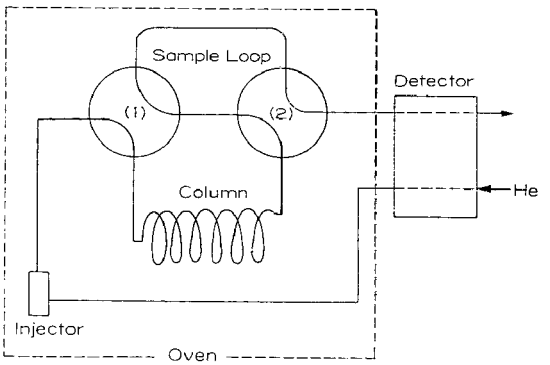


Fig. 1. A typical chromatogram.

Fig. 2. Apparatus for recycling experiment. 1 and 2 are four-way stopcocks.

spectively, and the centre one was assigned to IBr. The total area of the three peaks, plotted against total moles of Br_2 and I_2 injected, fell on the same straight line as the separate Br_2 and I_2 calibrations. Therefore the calibration curve for IBr was taken to be that same straight line.

Procedures

Solutions of equimolar amounts of bromine and iodine in ethylene dibromide were injected quantitatively. All operating conditions were maintained at the standard values given above except where one of them was being varied to test its effect. The ranges of those variations were: volume injected, 1–10 μl; flow rate, 20.7–122.0 ml/min; injector temperature, 150–189°; column temperature, 88–100°. The standard solution for injection contained 0.19 mole/l of total Br_2 and I_2, and solutions from 0.096–0.503 mole/l were used to test the effect of concentration.

To demonstrate unequivocally that reaction was occurring on the column, the apparatus in Fig. 2 was used to recycle a part of the eluted material through the column. 10 μl of a solution was injected. The appearance of the signal was observed on the recorder, and the middle part of a peak was trapped in the sample loop by turning stopcock (2) through 90° at the appropriate moment. In separate runs, each of the three peaks of the elution profile was sampled in this way. After the elution

was over, the sample trapped in the loop was fed into the gas chromatograph again by turning stopcock (1) through 90°. The volume of the sample loop was 5.7 ml, and that of each peak was about 60 ml.

The characteristics of the chromatograms were expressed numerically by calculating the parameters K_{eff}* and W*, as defined in Part I: K_{eff}* is the area of the centre part of the chromatogram divided by the product of the areas of the first and third parts, the lines of division being taken at the minima in the profile; W* is the distance in volume units between the intersections with the horizontal axis of tangents at the inflection points of the centre peak. A typical chromatogram is shown in Fig. 1.

RESULTS AND DISCUSSION

Calibrations

The retention volume of air, V_{RAIR}, was used as an approximation to V_{RI}, the retention volume of a component insoluble in the stationary phase, and was V_{RAIR} = 82.3 ml. Retention volumes of the three components of the reaction mixture were: Br_2, 135.0 ml; IBr, 199.0 ml; I_2, 270.0 ml.

The number of theoretical plates calculated from the retention volume and bandwidth of the I_2 peak was $n = 500 \pm 80$.

Recycling experiment

Fig. 3 shows the results of recycling a cut from each peak in the eluted material.

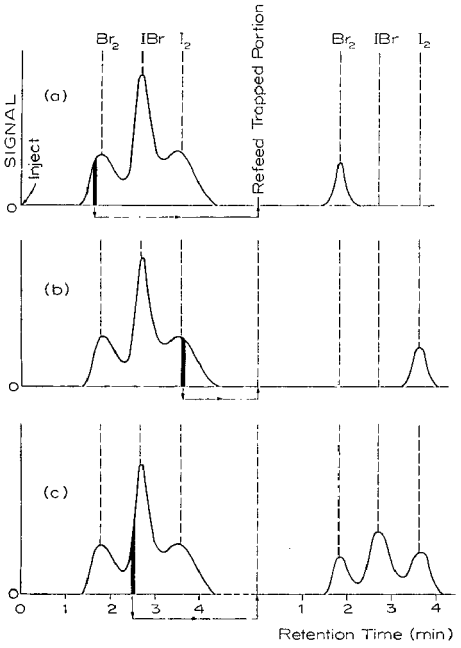


Fig. 3. Results of a recycling experiment. The shaded bands are the trapped portions of eluted material. Peak heights not to scale; attenuation is reduced by a factor usually of 10–20 between original chromatogram and recycled sample. In (c) K_{eff}* = 5.0 and 1.5 in original and recycled material, respectively.

TABLE I

DATA SHOWING THAT CHANGES IN SOME CONDITIONS DO NOT AFFECT K_{eff}*

	Time from preparation of solution to injection (h)							
	2.0	*2.5*	*3.0*	*23*				
K_{eff}*	4.5	4.5	4.7	4.6				
	Injector temperature (°C)							
	150	*160*	*172*	*189*				
K_{eff}*	5.5	5.2	4.7	6.0				
	Flow rate (ml/min)							
	20.7	*28.5*	*37.4*	*40.0*	*53.9*	*76.0*	*96.7*	*122.0*
K_{eff}*	4.2	3.8	4.4	4.6	4.0	4.5	4.3	5.2

The sample from the first peak gave only a bromine peak on recycling, and the sample from the third peak gave only an iodine peak. The sample from the centre peak, however, gave on recycling three peaks again, with a value of K_{eff}* even smaller than that of the original chromatogram (1.5 in place of 5). This is conclusive evidence that reaction is occurring in the column. It indicates also that K_{eff}* depends on the total amount of sample, as is expected from the K_{eff}* — V_0 behaviour predicted by computation (Part I, Fig. 3). Study of the dependence of K_{eff}* on a number of variables, as described below, in fact showed that the total amount of the sample was the most important variable.

Dependence of K_{eff} on various conditions*

Limits of error in calculating K_{eff}* from chromatograms are fairly large, because of the difficulty of locating precisely the minima between the peaks, and are estimated at ± 15%. Limits of error on W* are ± 10%. Within these limits, K_{eff}* was found to be independent of the following:

(a) time from preparation of solution to injection, which might affect the amount of IBr formed in the solution before injection (Table I);

(b) injector temperature (Table I);

(c) concentration of the sample (Fig. 4a);

(d) flow rate (Table I).

The last-mentioned observation is an important one, since it is expected only for $k_0 = \infty$, and therefore suggests very rapid reaction. Further evidence for a high k_0 is provided by the strong dependence of K_{eff}* on the total amount of sample (Fig. 4a), in which K_{eff}* exhibits some very low values, but varies monotonically with amount of sample, showing no minimum. It is not quite clear what variable is best to use in displaying this variation. As mentioned in Part I, the feed pulse volume, which is the convenient variable for computations, is not accessible to direct experimental measurement. It can be calculated from W* on the assumption that $k_0 = \infty$, but there is a large uncertainty in the lowest V_0 values, since V_0 varies very rapidly with W* in

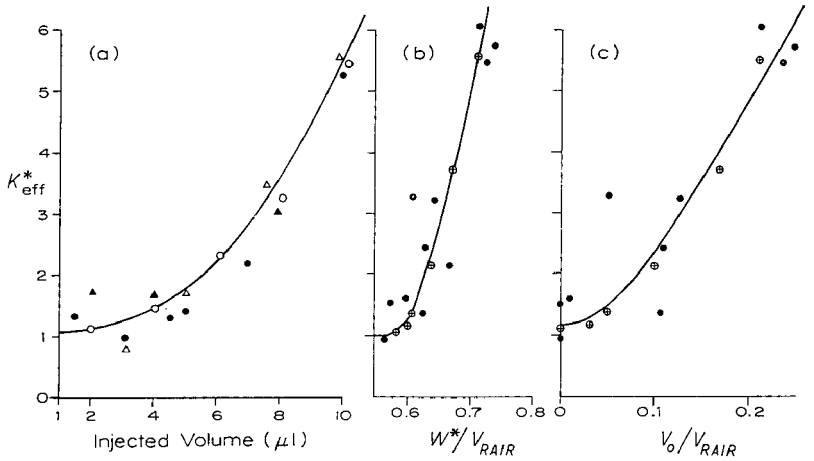


Fig. 4. Apparent equilibrium constant K_{eff}* plotted against (a) injected volume, (b) centre band width W*, and (c) feed pulse volume calculated from W* assuming instantaneous reaction. In (a), symbols specify total concentration in injected solution: ▲, 0.0963 mole/l; ○, 0.1375 mole/l; ●, 0.1925 mole/l; △, 0.503 mole/l. In (b) and (c), solid circles are experimental points and open circles are computed points for instantaneous reaction.

this region. Three variables are used in Figs. 4a, b and c: the total amount injected; the centre bandwith W*; and V_0 calculated from W*. Figs. 4b and c also show some computed values of K_{eff}*, for $k_0 = \infty$, for comparison with the experimental ones. These computations and the computation of V_0 from W* were carried out in the same manner as the computations in Part I, but with the new values $n = 500$ and $K_e = 169$ (at 95°). The latter value is calculated from standard thermochemical data[2,3] for the reaction $I_2(g) + Br_2(g) \rightarrow 2\ IBr(g)$, which yield $\Delta G^\circ = -2720 - 2.80\,T$ cal/mole. The composition of the feed pulse had little effect on the computed value of K_{eff}*, and was taken as the equilibrium composition in most computations (for example, at $V_0/V_{RAIR} = 0.0983$, the equilibrium composition $A_0 = C_0 = 1.00$, $B_0 = 14.75$ and the very much out-of-equilibrium composition $A_0 = B_0 = C_0 = 5.00$ yield, respectively, 2.09 and 1.95 for K_{eff}*).

Computations for the value $k_0 = 200$, the highest finite value used, are close to the limit of reliability of the computer programme at computation times we could afford (some runs took 40 min). The total amount of material in the computed elution curve sometimes differed by as much as a few percent from the amount specified in the input pulse, and computed K_{eff}* and W* should be considered to have error limits of $\pm 5\%$. In this connection it should be recognized that the plate theory was used in computations (as described in Part I) because a more sophisticated programme to solve differential equations allowing explicitly for diffusion, etc., was found incapable of handling high reaction rates in a reasonable time. The programme used for instantaneous reaction (see Part I) was both faster and much more reliable, as indicated by the match of total input to total output, than that for finite rates at its practical limit of $k_0 = 200$ ml min^{-1} (conc. unit)$^{-1}$.

The lower limit of the rate constant k

Figs. 4b and c show good agreement between observed values and those computed

for $k_0 = \infty$. The family of curves in Fig. 3 of Part I indicates that conspicuous changes in the curve from its $k_0 = \infty$ form occur only below $k_0 = 100$, and the indication of high reaction rate is thus, more precisely, $k_0 > 100$ ml min^{-1} (conc. unit)$^{-1}$. The arbitrary concentration unit is to be found, in any comparison with experiment, by equating the total input pulse concentration in the computation to that in the experiment. For all computed points in Figs. 4b and c, concentrations were $A_0 = C_0 = 1$, $B_0 = 14.75$, total 16.75 concentration units. If the experimental concentration is a mole/l, then the inequality becomes $k_0 > (1675/a)$ ml min^{-1} (mole/l)$^{-1}$. Clearly, the lower the value of a to which we find agreement between experiment and the "high k_0" computed curves, the more restrictive this condition becomes. In the present experiments, the lowest a arises from an injected solution of 10 μl with a total I_2 and Br_2

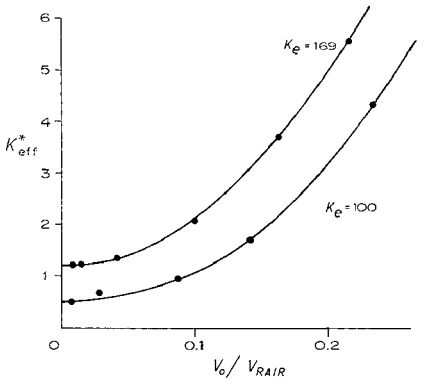


Fig. 5. K_{eff}* against feed pulse volume V_0 for two values of the true equilibrium constant K_e. n = 500, w = 76 ml/min, $k_0 = \infty$ (computed points).

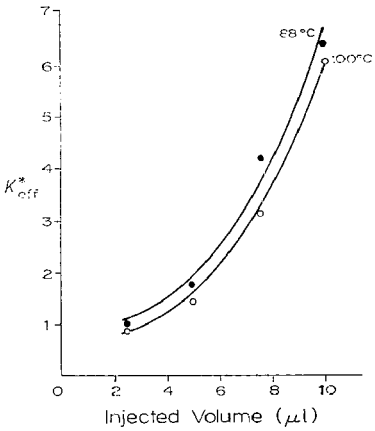


Fig. 6. K_{eff}* against injected volume for two column temperatures; experimental results.

concentration in solution of 0.0963 mole/l. To convert this into a gas phase concentration a after evaporation of the injected solution, the pulse volume V_0 is found from Figs. 4a and 5, which yield, for 10 μl solution, $V_0 = 0.21\ V_{RAIR} = 0.21 \times 82.3$ ml = 17.3 ml. Hence $a = 0.0963$ mole l$^{-1} \times 10^{-5}$ l/17.3 ml $= 5.5 \times 10^{-5}$ mole l^{-1}. Hence the inequality becomes $k_0 \gtrsim 3.0 \times 10^7$ ml min^{-1} (mole/l)$^{-1}$.

k_0 may be converted into the rate constant k by $k = k_0/K_B{}^2V_L$, where $V_L = (V_{RB} - nV_G)/K_B = (V_{RB} - V_{RAIR})/nK_B = (199.0 - 82.3)/nK_B = 116.7/nK_B$. Since nV_L is the total volume of the Kel-F grease coating, which can be calculated from its known weight of 7 g and density of 2.4 g/ml as 3 ml, $K_B = 116.7/nV_L = 116.7/3 = 39$.

Then $k \gtrsim 3.0 \times 10^7/[(39)^2 \times (3/500)]$ l min^{-1}mole^{-1} $= 5 \times 10^4$ l sec^{-1}mole^{-1}.

A very similar estimate can be made from the absence of flow rate dependence of K_{eff}*. An examination of the behaviour of various parameters in the computations showed that, to a good approximation, a β-fold increase of flow rate should have the same effect on K_{eff}* as a β-fold decrease in k_0. We examined the effect of a 6-fold increase in flow rate, and found no change in K_{eff}* (Table I). Since K_{eff}* becomes fairly strongly dependent on k_0 below $k_0 = 50$, this indicates $k_0 \gtrsim 6 \times 50 = 300$.

This is three times the previous estimate, but the flow rate experiments were at twice the concentration of the previous calculation, so that the two estimates of the lower limit of k are only a factor of 3/2 apart.

Since second-order rate constants for reactions in liquid solutions are commonly of the order $k \sim 10^{11} \exp(-E_A/RT)$ l sec^{-1}mole^{-1}, the lower limit given here corresponds to $E_A \lesssim 10.6$ kcal/mole for the decomposition of IBr in solution in Kel-F grease.

The effect of column temperature

Fig. 6 shows that K_{eff}* values at 100° lay consistently below those for 88° column temperature. The ratios for the four pairs of points in Fig. 6 are K_{eff}*(100°)/K_{eff}*(88°) = 0.85, 0.80, 0.75, 0.95, mean 0.84 $\pm$ 0.04. The stated limit of error is better than that which would be expected from the $\pm$ 15% limits given above for individual K_{eff}* values, which would indicate 0.84 as only just significantly different from unity in an average of four pairs of readings.

Computations showed (Fig. 5) that although the numerical values of K_{eff}* are very different from the true equilibrium constant K_e, changes in K_e are nevertheless reflected in roughly proportionate changes in K_{eff}*. The two values of K_e used in the computations of Fig. 5 are in the ratio 100/169 = 0.59. The ratio of K_{eff}* values varies from 0.40 at $V_0/V_{RAIR} = 0$ to 0.60 at $V_0/V_{RAIR} = 0.2$. For the two temperatures used in the experimental work, K_e values from the usual thermochemical data yield $K_e(100°)/K_e(88°) = 160.5/181.3 = 0.885$, to be compared with the K_{eff}* ratio of 0.84.

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CHROM. 4891

THE NEGATIVE ALKALI FLAME DETECTOR RESPONSE*

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SUMMARY

Under certain conditions, the alkali flame detector will give negative response (inverted peaks) for organic compounds containing halogen, nitrogen, or other elements. With a modification of the alkali flame detector particularly suited for such a study, the range and magnitude of negative response was defined in terms of alkali bead bore, hydrogen flow, carrier gas flow, electrode height, and other parameters. Organic compounds containing chlorine, bromine, iodine, and nitrogen, were used as test substances; an organophosphate was studied under the same conditions for purposes of comparison. Cl, Br, I, N, and C can show either positive or negative response and each can be distinguished from the others by proper choice of parameters. Both halides and carbon compounds show stronger response in the negative mode than in the positive mode. The use of two different carrier gases, nitrogen and helium, did not cause significant differences, except in its effect on the response of nitrogen compounds. In general, a large bead bore, a high hydrogen supply and/or a low carrier gas flow favor negative response. These are also the conditions which establish a large area of contact between flame and alkali surface and, consequently, a large background current. In order to obtain reproducible response of adequate magnitude, the Rb_2SO_4 salt surface must be smooth and homogenous. The described detector functions can be used for qualitative microanalysis of hetero-organic compounds by gas–liquid chromatography.

INTRODUCTION

In the short, but turbulent history[1] of the alkali flame detector (AFD), negative responses (inverted peaks) have been observed by many authors[2–4,15]. Such behavior was usually considered an interesting nuisance. Few authors tried to use this effect for analytical purposes. DRESSLER AND JANÁK analyzed sulfur compounds as negative peaks[5]. We reported recently on the use of the negative AFD response for the de-

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termination of chlorinated hydrocarbon insecticides[6]. In this field of residue analysis, the electron capture detector is used almost exclusively, and the AFD does not measure up either in sensitivity or reliability.

Nonetheless, a negative peak is an interesting phenomenon in its own right. Most important, in this respect, is the AFD's capability to distinguish between certain atoms. In the above mentioned study[6], for example, chlorine effected negative, phosphorus positive peaks.

The interest in the negative response appears especially well justified in the area of trace analysis. Chromatographing minute amounts of interesting compounds present in a complex mixture, the best one can hope for is a series of well-separated chromatographic peaks. If these peaks can indicate the presence or absence of a particular heteroelement (by their normal or inverted form) valuable information can be gained. In this study, we have therefore investigated the general behavior of selected halogen, nitrogen, phosphorus, and carbon compounds and tried to define the areas and magnitudes of negative response.

There is a host of parameters influencing the AFD's performance characteristics, which need to be considered in a systematic study. The nature of the salt has been investigated intensively by several authors (ref. 1, pp. 13). Less studied was the surface of these salts, which are in contact with the hydrogen flame. The shape and the position of the electrode in relation to the flame is another critical parameter. Different elements will exhibit response maxima at different electrode heights[7]. Then, the hydrogen, carrier, and air flow are decisive for the performance of the detector, perhaps in this order of relative importance. The magnitude and direction of polarizing voltage is another parameter which has been frequently studied. Most important both in a practical and a theoretical context is the way in which alkali is introduced to the flame (ref. 1, p. 4). Finally, there are several other characteristics of the detector to be considered such as its operating temperature, its geometric and electric design, its pattern of air flow, etc.

Some of the literature on the alkali flame detector is controversial, mainly because different investigators worked with differently designed instruments and the AFD seems to reward every innovation or modification with a wealth of new and unexpected responses. Consequently, the definition of working parameters becomes very important. In this as in other studies, the obtained results depend to a great degree on the particular modification of the detector, the type of alkali source and electrode used, etc., and may not be immediately reproducible by other breeds of alkali flames.

In order to cut down on variables, several AFD features were taken from earlier work and held constant throughout this investigation. For instance, the collector electrode was a platinum loop of 7 mm I.D., which had worked well in a recent study of negative response. Rubidium sulfate was the only salt used, since it had been shown to work well with both halogens and nitrogen. The version of the AFD employed was similar to one reported earlier[6], with a slightly altered detector configuration to permit frequent alkali bead changes. Other constant parameters included the air flow and the potential on the collector electrode.

The distance of the electrode from the alkali salt surface ("electrode height") has been shown to be of considerable importance. Both in positive[7] and negative response mode[6], it can be used to maximize response and discriminate against other

alkali flame-active elements. However, other variables can affect the performance of the AFD to an even greater degree.

The abundant literature on the subject—and our own experience—made it perfectly clear that the shape and temperature of the flame, and the area of its contact with the alkali surface, are of critical importance. This indicates the bore of the alkali bead and the flows of carrier gas and hydrogen as the prime variables. A small bead bore will tend to yield a tall and narrow flame with little contact with the alkali salt, a big bore will produce a low and broad flame which, so to speak, sits on the bead surface. The carrier gas, besides lowering the temperature of the hydrogen flame, will also alter the flame shape. Thus, the carrier flow can become very critical and has to be closely controlled. The dependence of response on the hydrogen flow is perhaps the best documented of the variables in the literature on the AFD (ref. 1, p. 9).

In this work, we have tried to define carrier gas and hydrogen gas flow between the extreme limits, *e.g.*, with hydrogen from the smallest flow which still supports the flame, to the highest flow where the background exceeds the buck-up capability of the electrometer or the noise becomes excessive. In view of several notices on the subject[8–10], two different carrier gases, nitrogen and helium, were used and also varied within a range as wide as possible.

EXPERIMENTAL

The chromatographic system described earlier[6] was used throughout this study. In order to facilitate frequent alkali bead changes, the bead was left in the steel ring used for pressing and held on the detector jet tip with a collar as shown in Fig. 1. It was produced in a screw-type pellet press (similar to the Carle model for IR work) with highly polished metal surfaces. Rubidium sulfate (99.9% pure, K & K) was finely ground in an agate mortar, mixed with a small amount of distilled water,

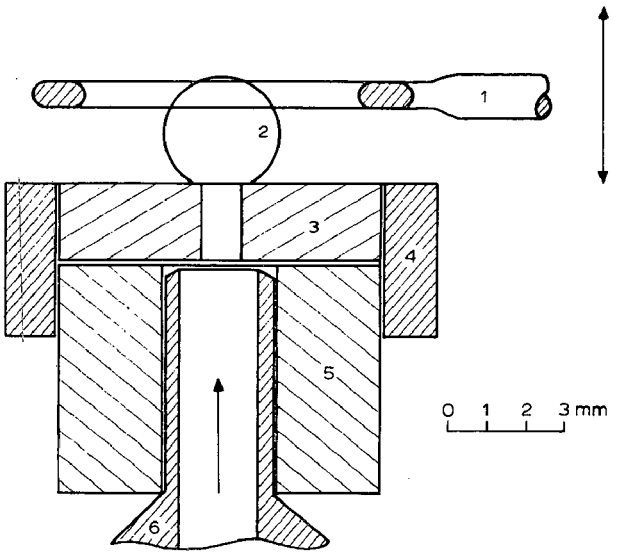

Fig. 1. Modified alkali flame detector. 1 = Adjustable electrode; 2 = hydrogen flame; 3 = rubidium sulfate; 4 = stainless steel; 5 = brass collar; 6 = detector jet tip.

filled into the steel ring, the pellet press closed, and pressure applied in stages with the help of a torque wrench. In intervals of about 5 min, from 5 to 35 ft. lbs. were applied in 5 ft. lb. steps. The final pressure on the bead was estimated in the order of 10^4 to 10^5 p.s.i. The amount of rubidium sulfate filled approximately half of the steel ring. The alkali salt pellet was left in the steel ring and drilled with a high-speed drill press. The salt surface must come out clean and smooth in order to obtain adequate and reproducible negative response.

Chlorobenzene, bromobenzene, iodobenzene; benzylamine, aniline; triethylphosphate, trimethylphosphate; *p*-xylene, *n*-decane, *p*-cymene, 1-octanol, and anisole served as test compounds. The halides were used in concentrations of 1% (w/v) and 0.01% (w/v), the nitrogen and carbon compounds at 1% (v/v), the phosphorus compounds at 0.0001% (w/v) and 0.001% (v/v) in *n*-hexane or acetone.

Unless otherwise indicated, all response profiles were measured with the collector electrode of 7 mm I.D. at +240 V, and an air flow of 250 ml/min. Several Rb_2SO_4 pellets were produced with bores of 0.50, 0.75, 1.0, 1.5, 2.0, and 3.0 mm. In a systematic way, the electrode height, the hydrogen flow, and the carrier gas flow of both nitrogen and helium were varied.

Table I shows the symbols and abbreviations used throughout this paper. The precise conditions of the measurement series are either given in the legends or are evident from the figures.

TABLE I

			C	△ △	*p*-xylene
Cl	—△———△—	chlorobenzene	C	—□———□—	*n*-decane
Br	—□— ——— —□—	bromobenzene	C	○ ○	*p*-cymene
I	—○·· ———·· ○—	iodobenzene	C	▽ ▽	1-octanol
N	—▽— — — —▽—	aniline	C	◇ ◇	anisole
P	—◇·· ——— ·· ◇—	triethylphosphate	N	—▼— — — —▼—	aniline

RESULTS AND DISCUSSION

Any study on the characteristics of the AFD will encounter the problem of reproducibility. As BRAZHNIKOV *et al.* have stated in their recent comprehensive review[1], there are many conflicting data on the AFD to be found in the literature and what holds true for one particular modification of the detector may not necessarily hold true for another.

It seemed in this study that negative response characteristics are somewhat harder to reproduce than positive ones. With several beads, no (or a very small) negative response was obtained. Negative response appears related to the smoothness and evenness of the bead surface. A jagged or cracked surface causes a great decrease if not virtual elimination of negative response.

Once the technique of bead production was perfected, and smooth, hard surfaces

were obtained in every case, the results became reproducible. Results from beads with different diameters fitted very well into series, thereby indicating the validity of the obtained data.

Rubidium sulfate is not the easiest of salts to press and drill, but was chosen for its good performance with different kinds of elements in the AFD. The use of a sulfate demands some skill in the production of the bead. Alkali halides, on the other hand, which are quite easy to press, were not used because the reported depression of halogen response[8].

The picture of the AFD modification (Fig. 1) shows a typical rubidium sulfate bead (in this case with a 1-mm inner bore) which has approximately the same characteristics as the bead which has been reported[6]. The only change from the earlier version was to keep the alkali salt bead in the steel ring used for pressing, and to mount it on the detector jet tip with a brass collar. This allowed frequent exchange of beads which did not get chipped, or scratched on their surface, or lose their firm seat on the detector jet tip.

Size and direction of the collector potential

In our instrument, the collector electrode of the AFD is connected to a battery box which in turn feeds the signal to the electrometer. The detector jet tip and the detector housing are on ground potential. It was interesting to check the dependence of the background current on the magnitude and the direction of the potential on the collector electrode, since this profile is undoubtedly a function of detector design[11]. The results are shown in Fig. 2. While positive response (especially with phosphorus compounds) can be very much greater than the background current, negative response can obviously not exceed this amount. Therefore, the available background current is of considerable interest for a negative response study—a characteristic akin to the electron capture detector.

The background current measured with the positive collector electrode varies with the applied potential and has not yet reached saturation at 290 V. On the other

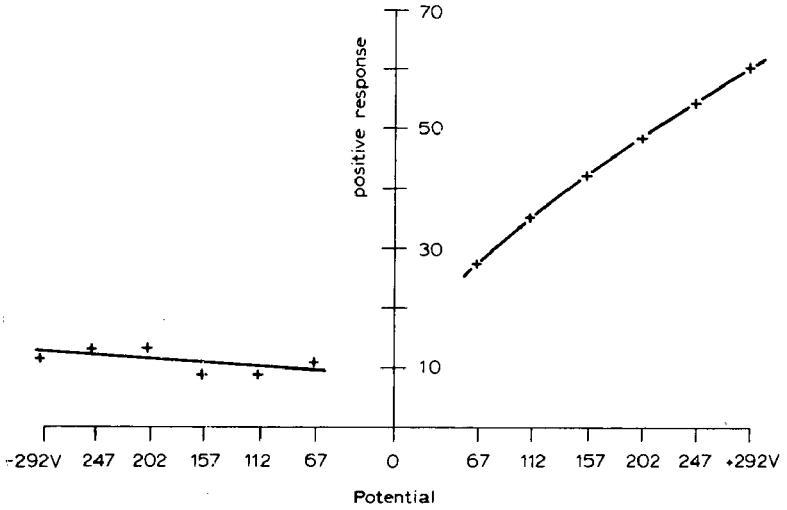

Fig. 2. Background current at various potentials. Electrode height, 2 mm.

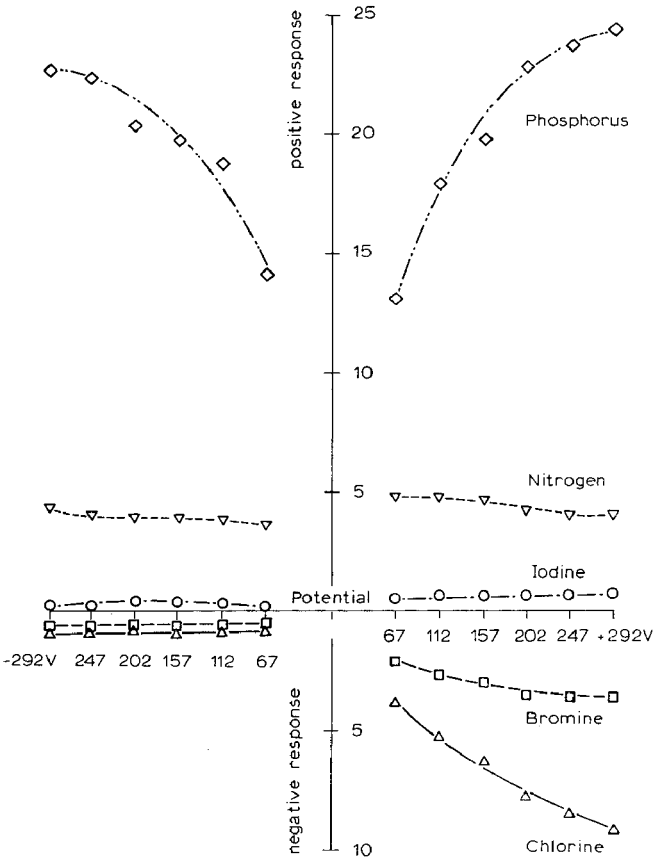

Fig. 3. Response profiles at various potentials. Electrode height, 2 mm. 1-μl triplicate injections of 0.01% solutions of chlorobenzene, bromobenzene, iodobenzene, and benzylamine; and 0.001% solution of triethylphosphate. Column temperatures: 90° (Cl, Br, I), 170° (N, P). Flow rates, in ml/min: H_2, 33; N_2, 50; and air, 250.

hand, the background measured with a negative collector electrode potential is significantly smaller and essentially independent of the applied voltage. This indicates that ions other than Rb^+ and e^- play a major part. Since electrons are easier collected than alkali cations, one would expect their complete collection well within the applied range of potential. On the other hand, the current due to Rb^+ should vary considerably with the potential.

Fig. 3 shows the response of several volatile compounds as affected by the magnitude and the direction of the applied potential. Phosphorus response rises with the applied potential and shows the same type of curve as the background current (at positive potential); this is in accord with other versions of the AFD. The responses at negative and positive potentials are almost exact mirror images of each other. Consequently, phosphorus response at negative potential does not parallel anymore the background current.

Nitrogen shows approximately the same response regardless of the size or direction of the potential.

The halogens, on the other hand, show a clearly different behavior. The negative response of bromine and chlorine appears to parallel the background current (the potential) on both sides. Halide response is thus greatly diminished at negative potentials and does not exhibit the symmetrical curves like phosphorus. This makes some sense since negative response represents a reduction in available background current.

The regular battery box used with the Barber-Colman gas chromatograph contains a 240-V battery. This seemed good enough for all further measurements and those were consequently conducted at a positive potential of 240 V on the collector electrode. A thorough study of the interesting relationship between potential and response was outside the scope of this study, which is mainly concerned with relative response profiles.

The effect of electrode height

The response of the AFD is a function of the shape of the collector electrode[12,13,15] and its height above the alkali salt bead, or in other words, its position in regard to

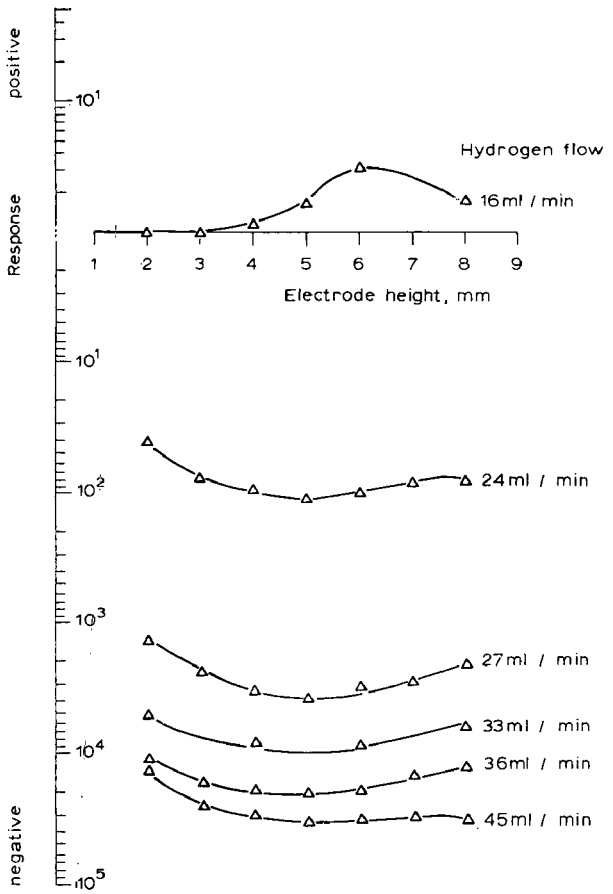

Fig. 4. Chlorine response profiles at various electrode heights and hydrogen flows. 1-μl triplicate injections of 1% chlorobenzene. N_2 flow rate, 50 ml/min.

the flame[7]. Different elements have response maxima at different electrode heights and this phenomenon can be used to determine one particular element at optimum sensitivity and discriminate against other alkali flame-active elements[6,7].

Consequently, the variations of response with different electrode heights were monitored for each particular setting of bead diameter and flow parameters, from 1 to 9 mm above the bead. Typical of such a measurement is the one shown in Fig. 4. The curves were measured with chlorine; bromine behaves similarly but iodine is much more erratic.

As this study progressed, flow parameters were measured over a large range and the effects shown in these studies were of much larger magnitude than the ones brought about by the change in the electrode height at one particular setting. The relatively large diameter of the collector electrode might also tend to diminish the effect of electrode height. While it is very important to find the optimum height for some particular type of analysis, averages, and later the response at a fixed height (2 mm above the bead), were used in the context of this study. These may be considered representative of the general trends observed.

Effect of bead bore

The effect caused by varying inner diameters of the salt bead has never been extensively investigated except in one study[14]. The fact that a narrow bore produces a tall, narrow flame, and a wide bore a low and broad one, underscores its importance for the AFD. A tall and narrow flame will be farther away from the electrode and will contact less alkali surface. Consequently, the AFD will show a lower background current. A broad flame will get closer to the electrode (at a low electrode height), it will touch a greater alkali surface area and produce a higher amount of background

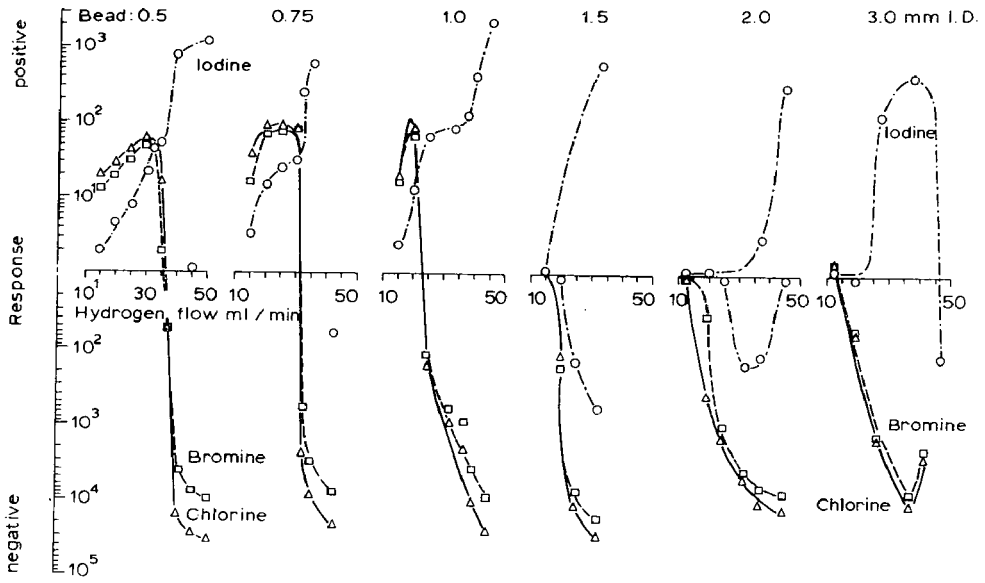

Fig. 5. Halogen response profiles at various hydrogen flows and bead bores. Electrode height, 2 mm. 1-μl triplicate injections of a 1% solution of chlorobenzene, bromobenzene and iodobenzene. N_2 flow rate, 50 ml/min.

current. Regardless of whether a surface mechanism or a mechanism operative in the flame is assumed, the area in contact with the flame and the temperature in the contact region will be decisive factors in determining the response.

Fig. 5 shows the dependence of halogen response on the bead bore (inner diameter), measured for each bead with different hydrogen flows. In this as in the other figures of this paper, the symbols and abbreviations shown in Table I are used. The study shows a consistent behavior of the response profiles as the bead diameter is increased. Bromine and chlorine show both positive and negative response at small bead diameters. The area of positive response, however, decreases until it is virtually nonexistent for bead bores higher than 1.5 mm. This is clearly seen by following the hydrogen flows at which the response profile crosses the abscissa and the response turns from positive to negative. With bead diameters of 0.5, 0.75, 1.0, and 1.5 mm, the crossover points lie at 36, 32, 23, and 15 ml/min of hydrogen, respectively. As the bead diameter increases, the iodine, whose response has been positive up to 1.0 mm, begins to exhibit split peaks., *i.e*, a negative and a positive peak as indicated by a dubble set of data in the figures. This peak shape is similar to that shown for bromine in chromatogram No. 4, Fig. 12; it should not be confused with the familiar rabbit ear

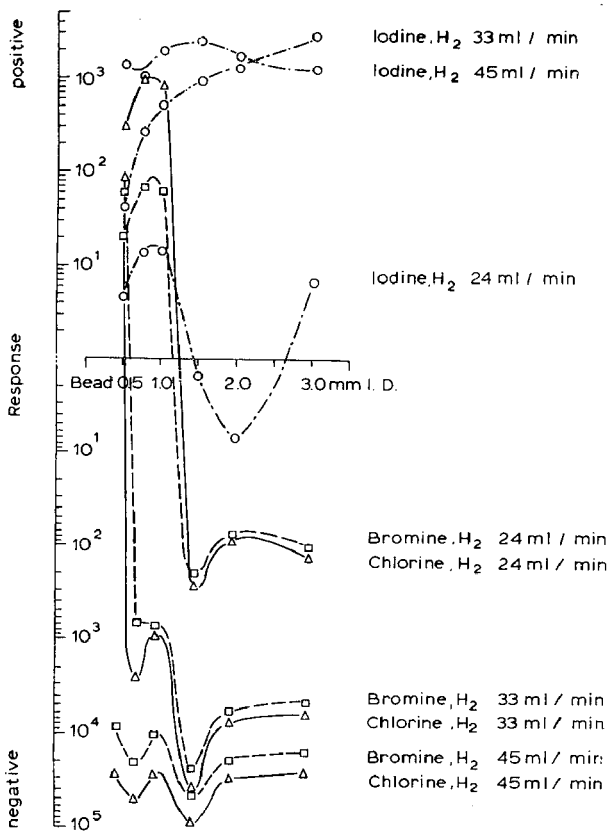

Fig. 6. Halogen response profiles at various bead bores at three selected hydrogen flows. Data are averages from measurements at electrode heights between 1 and 9 mm. 1-μl triplicate injections of 1% chlorobenzene, bromobenzene and iodobenzene. N_2 flow rate, 50 ml/min.

peak which represents a concentration phenomenon. As the large bead bores are approached, iodine finally begins to turn toward negative response.

It appears from Fig. 5 that a larger bead diameter and a higher hydrogen flow rate favor the negative mode of response. The maxima of negative response measured for chlorine and bromine are much larger than those of the positive response range, and they show little variation with bead bore. The recorded data were measured with the electrode 2 mm above the bead.

Fig. 6 shows some of the same data brought into a different form. Response profiles were measured with electrode heights of 1 to 9 mm and the averages values were plotted for chlorine, bromine, and iodine at three different hydrogen flows. Again, larger bead bores favor the negative response, at least for bromine and chlorine. From 1.5 mm I.D. upward, the negative response for bromine and chlorine as well as the positive response for iodine remain in approximately the same order of magnitude.

For the purpose of comparison, Figs. 7 and 8 show the relationship between the response of a phosphorus compound and the hydrogen flow as well as the bead bore diameter. The response is positive throughout and, of course, much larger than that obtained for halogen or nitrogen on an equal weight basis. Fig. 7 shows several somewhat similar response profiles resulting from different bead bores. The response, as expected, varies in an approximately linear fashion with the hydrogen flow and the different bead diameters do not seem to cause any appreciable or noteworthy effect.

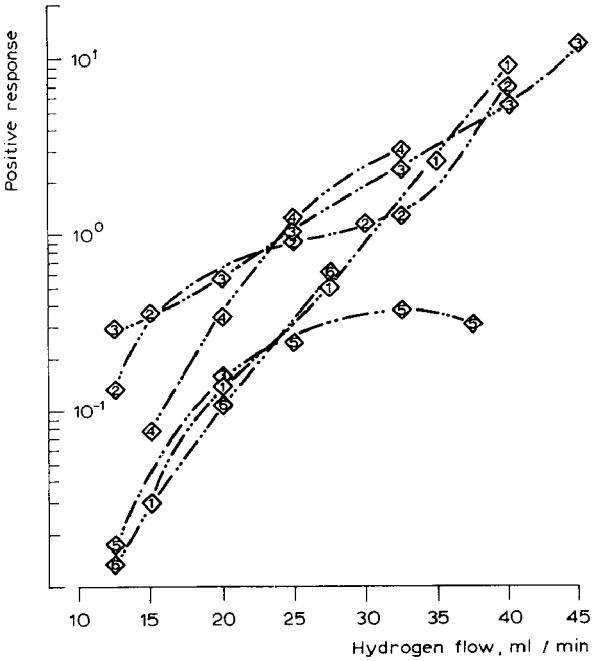

Fig. 7. Phosphorus response profiles for varying hydrogen flow and six bead bores. Electrode height, 2 mm. 1-μl triplicate injections of 0.0001% triethylphosphate. N_2 flow rate, 50 ml/min. Bead bores, in mm: 0.50 (1), 0.75 (2), 1.0 (3), 1.5 (4), 2.0 (5), 3.0 (6).

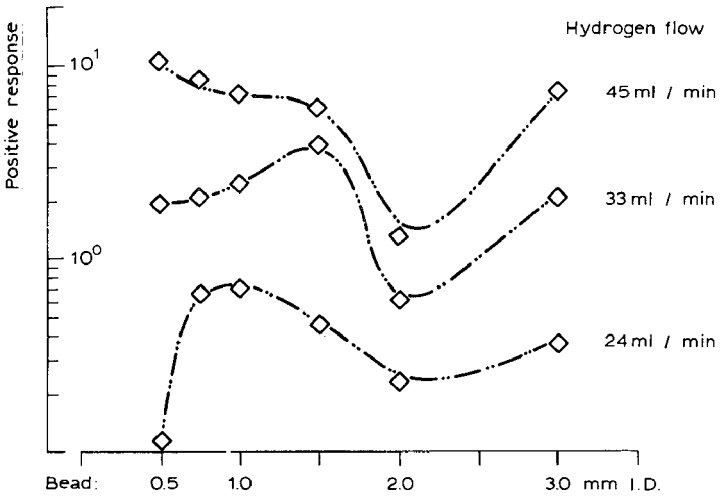

Fig. 8. Phosphorus response profiles at different bead bores and three selected hydrogen flows. Data are averages from measurements at electrode heights between 1 and 9 mm. 1-μl triplicate injections of 0.0001% triethylphosphate. N_2 flow rate, 50 ml/min.

In Fig. 8, three hydrogen flows selected from the same experimental series are used to plot the response profiles for a variation in bead bore. If phosphorus compounds are determined with this particular AFD version, a bore of approximately 1 mm I.D. would probably be the one of choice. The effect introduced by various hydrogen flows is greatest with the very narrow bore; at 45 ml/min, the response is about a hundred times as large as at 24 ml/min of hydrogen. This response ratio decreases and then again increases with increasing bead bore.

It should be noted that it is possible to obtain negative response even for phosphorus, when other electrode dimensions and flow parameters are used. The negative response for phosphorus, however, is dwarfed by the normal positive one and no particular effort was made to characterize these negative ranges for phosphorus.

Effects of carrier gas

We had initially assumed that the influence of the carrier gas flow on the response would be relatively small in comparison with the effects of the hydrogen flow. This, however, was not the case, and the carrier gas supply proved to be a decisive parameter in determining the amount of response. Both nitrogen and helium are commonly used as carrier gases with the AFD and were also used in this study. Of the two, helium is supposed to yield the better response[8–10].

Fig. 9 shows the response profiles for the halogens in dependence on the carrier gas flow. Both helium and nitrogen are used at different levels of hydrogen. Halide response generally increases with decreasing carrier gas flow, that is, under conditions characterized by a flame of higher energy in contact with more alkali salt surface. Only chlorine is negative at the lowest hydrogen flow; bromine and iodine remain positive. As the hydrogen supply is increased, bromine begins to shift and joins chlorine on the negative side. At the very highest hydrogen flow, iodine begins to turn negative also. This is an instructive example for the fact that conditions which favor

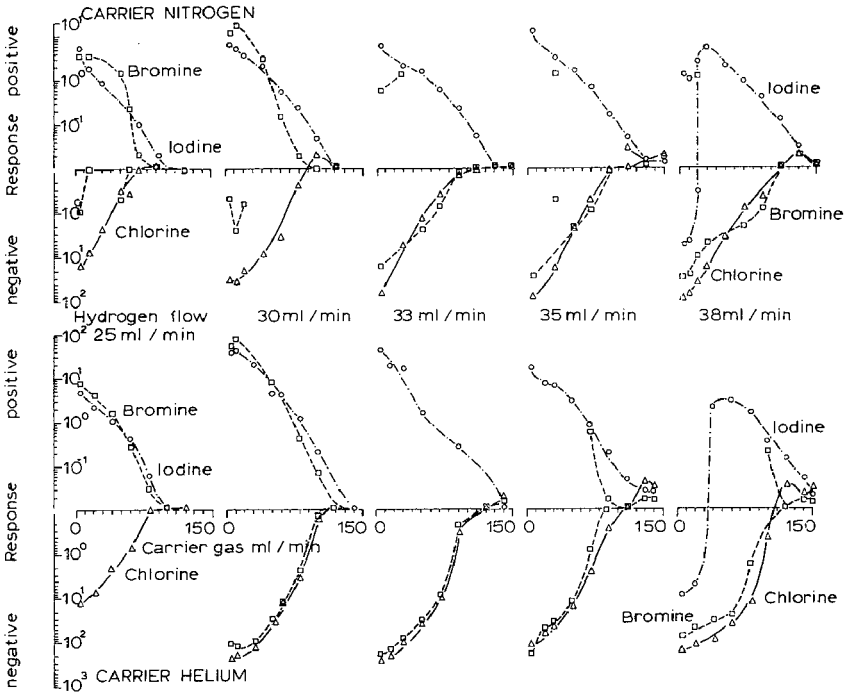

Fig. 9. Halogen response profiles for varying carrier gas flow (nitrogen and helium) and five selected hydrogen flows. Electrode height, 2 mm. Bead bore, 1.0 mm. 1-μl duplicate injections of 0.01 % chlorobenzene, bromobenzene and iodobenzene.

a larger and higher energy contact of the flame with the alkali salt surface tend to favor negative response. These conditions are high hydrogen flow, low carrier gas flow, and a large bore diameter.

While an increase in carrier gas diminishes the negative response, it likewise diminishes, in most cases, the positive response. It should be noted that the data in Fig. 9 were obtained with amounts of halides different from those represented in the previous figures and this fact should be accounted for when response values are compared. Fig. 9 shows that little difference exists between nitrogen and helium in the context of halogen response profiles.

The same conclusion does not hold true for the measurements depicted in Fig. 10. A series of carbon compounds is shown in comparison to a nitrogen compound. Since the response profiles for carbon compounds do not vary much according to their structure, the data points for all of them are recorded, but a curve is drawn only for *n*-decane. The study shows several interesting effects. First, the response curves for nitrogen and carbon compounds are very dissimilar at low hydrogen levels, but become more and more alike at high hydrogen flow rates. Secondly, the response for nitrogen seems decidedly lower with helium as a carrier gas than with nitrogen. This effect becomes especially noticeable with a large hydrogen supply. Thirdly, and in concurrence with other results in this study, the higher hydrogen flow seems to favor the negative response, as can be seen by the crossover points for carbon compounds of 60, 95, and 110; and 80, 100, and 120 for the respective hydrogen flows of 25, 30,

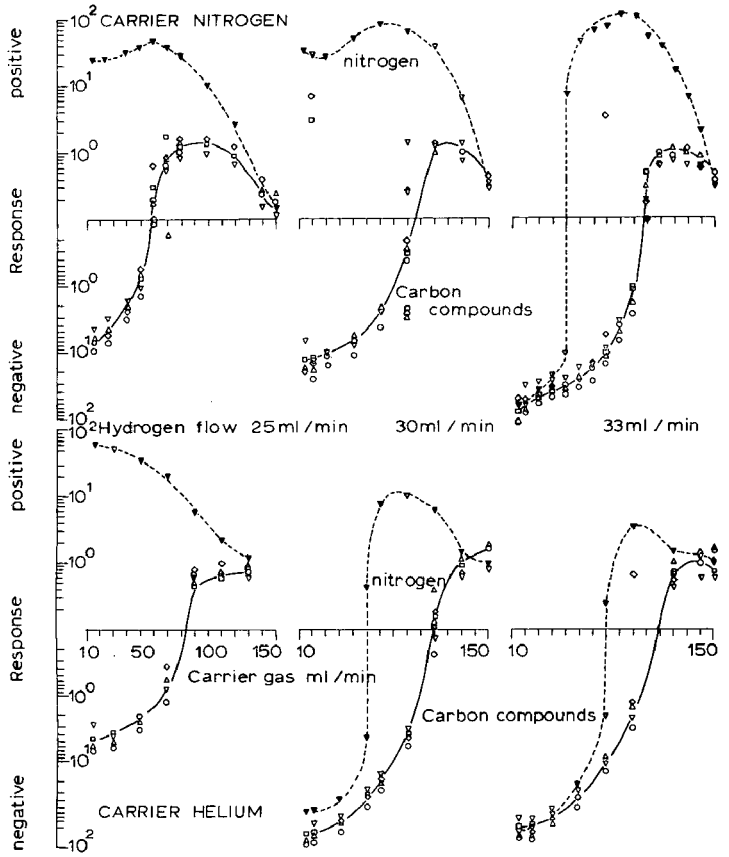

Fig. 10. Nitrogen and carbon response profiles for varying carrier gas flow (nitrogen and helium) and three selected hydrogen flows. Electrode height, 2 mm. Bead bore, 1.0 mm. 1-μl single injections of 1% aniline and 1% each of *p*-xylene, *n*-decane, *p*-cymene, 1-octanol, and anisole.

and 33 ml/min. Fourthly, the negative response for carbon compounds seems to be about one order of magnitude larger than the positive response and it is apparently not dependent on chemical structure. It should be remembered that the nitrogen compound used in the study, aniline, contains six carbon atoms and only one nitrogen atom. Therefore, the response profile for this compound is made up from contributions both of carbon and nitrogen. In areas, however, where the aniline peak is much larger than the carbon compound signal the recorded curves can be considered fairly representative of the true nitrogen response profile.

The greatest importance of response profiles such as these lies in the possibility to distinguish nitrogen-containing compounds from those containing only carbon, hydrogen, and oxygen. This is achieved from chromatograms only. Split peaks were obtained occasionally in our studies. Where this occurred, the data were recorded both for the area of the negative and that of the positive peaks. Several such data can be noticed in Fig. 10; however, most measurements follow a consistent curve.

It is an interesting but unexplained conclusion from Fig. 10 that better response for nitrogen compounds is obtained with nitrogen as carrier gas than with helium.

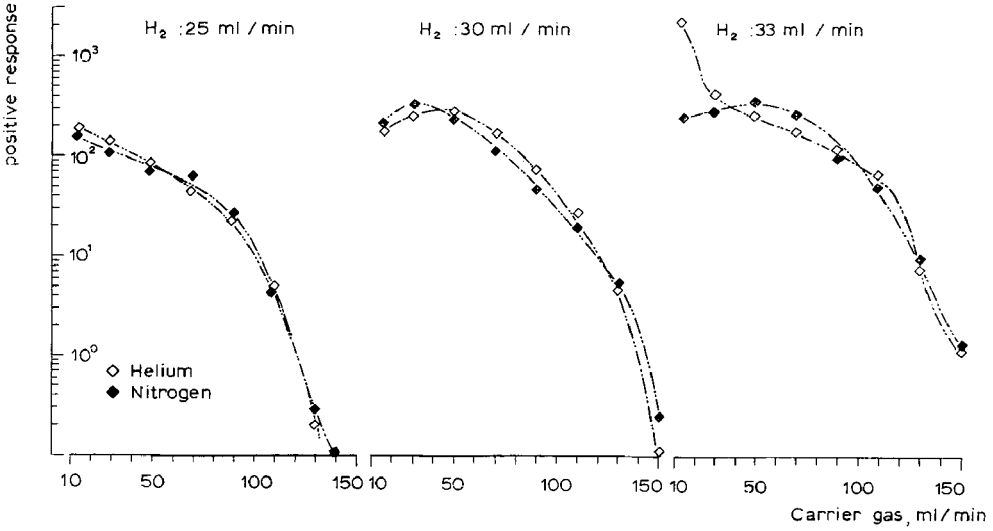

Fig. 11. Phosphorus response profiles for varying carrier gas flow (nitrogen and helium) and three selected hydrogen flows. Electrode height, 2 mm. Bead bore, 1 mm. 1-μl triplicate injections of 0.001% trimethylphosphate.

In Fig. 11, a similar comparison of the effects of nitrogen and helium as carrier gases is recorded for a phosphate. In our modification of the AFD, the difference between helium and nitrogen appears negligible.

Application of the negative AFD response

In an earlier study[6], we have used the negative response of the AFD toward chlorine for the determination of chlorinated hydrocarbon insecticides. No such application is included in the present study. It is obvious, however, that the defined conditions can be used to affirm the suspected presence of a particular hetero-atom in a molecule represented by a peak in the chromatogram. Fig. 12 shows several typical chromatograms obtained in the serial measurements. Halogen, nitrogen, and carbon compounds are represented by both negative and positive peaks, including some chromatograms with "mixed" responses. It is the latter case which allows us to distinguish between the hetero-elements.

These chromatograms were all run after a very short equilibration of the detector. This was necessary for finishing a particular series of measurements in a reasonable time under the same conditions. In an actual analysis, however, the electrode height should be optimized and the detector given adequate time for equilibration. This results in chromatograms which are both more reliable and of greater aesthetic appeal.

Chromatogram No. 4 in Fig. 12 contains a split bromine peak. It is interesting to note that split peaks generally show the same pattern (*e.g.* the negative peak is followed by the positive peak) for one particular element in a series of measurements. In the same series of measurements, however, another hetero-atom might show the opposite peak behavior (*e.g.* the positive peak is followed by the negative one). Such, for instance, was the case with halogen and carbon compounds in one of the described series.

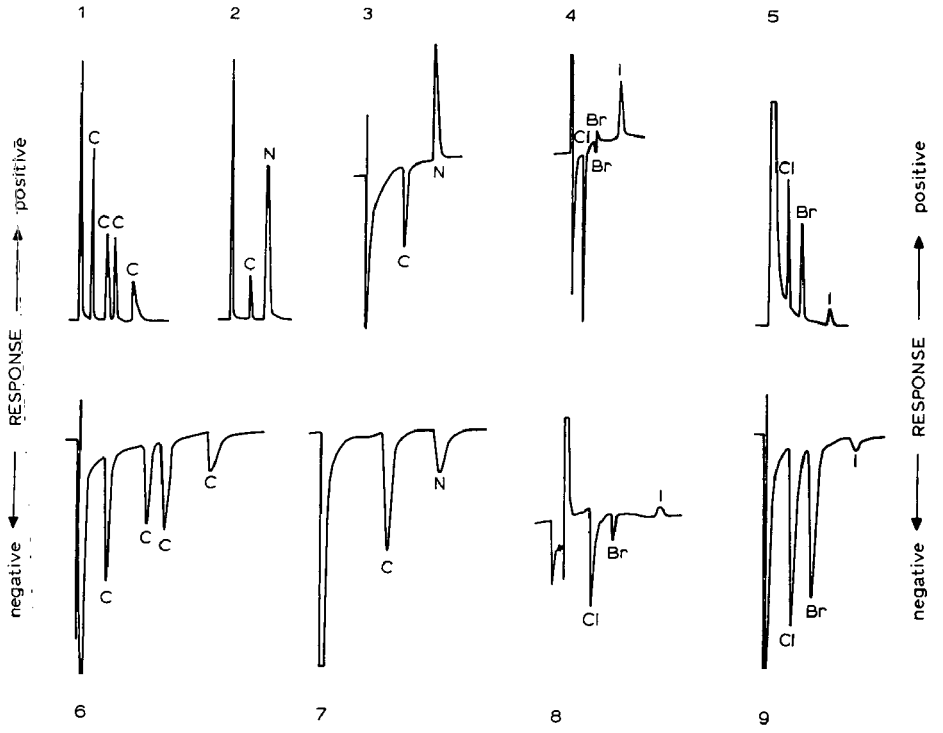

Fig. 12. Typical chromatograms at various flow rates.

	Chromatogram No.								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
N_2(He) flow rate (ml/min)	92	92	46	78	50	37	(27)	42	(43)
H_2 flow rate (ml/min)	26	26	33	30	16	33	30	37	37

Electrode heights, 6 mm (No. 5) and 2 mm (all others). Bead bore, 1.0 mm. Compounds as listed in Table I.

In summary, this study covers some of the more important variables which influence the ranges and magnitudes of negative response for several alkali flame-active elements. It does not—nor could it within the context of one paper—attempt to cover all of the parameters which may affect response. Simple as the AFD appears to the eye, a thorough study of its characteristics can become quite involved; not to mention any attempts directed at elucidating its *modus operandi*.

The negative response of the alkali flame detector still represents somewhat of a curiosity. It can be used to a certain advantage in quantitative measurements of halides or nitrogen compounds. Its main potential, in our opinion, lies in its capability to distinguish between several hetero-elements in organic compounds separated by gas chromatography.

ACKNOWLEDGEMENTS

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GAS CHROMATOGRAPHIC SEPARATION AND DETECTION OF C_1 TO C_3 MONOCARBOXYLIC ACIDS AS THE *p*-SUBSTITUTED BENZYL ESTERS

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SUMMARY

A rapid, sensitive gas chromatographic method for the separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids is presented. The procedure involves reaction of microliter volumes of an ethanolic carboxylic acid solution mixture, down to the 0.1 $\mu g/\mu l$ concentration level, with an appropriate *p*-substituted benzyl halide in a sealed capillary melting point tube at 110°. The esters formed in the reaction mixture are then isothermally eluted from a general-purpose OV-17 glass column.

INTRODUCTION

Separation and/or detection of the lower monocarboxylic acids in foods and in chemical, biological and ecological media has been achieved using spot tests, paper and thin-layer chromatography[1–5], column partition chromatography[6,7] and by gas chromatography as the free acids or after derivatization under a variety of experimental conditions[8–14].

Color tests are often subject to interference and are therefore of limited utility. Column, paper and thin-layer chromatographic techniques are virtually indispensable to the chemist but the lower volatile fatty acids sometimes demand assiduous sample preparation and control of conditions, and may require relatively long development times for optimum separation. Though excellent results are reported in the literature for most of the gas chromatographic methods for the lower carboxylic acids, many require specialized columns which are not always convenient for those control laboratories which routinely process a wide diversity of chemical substances.

Therefore, in this paper we describe a sensitive method for the rapid separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids by isothermal gas chromatography on an all-purpose 5% OV-17 on Gas-Chrom Q column as their *p*-methylbenzyl, *p*-bromobenzyl or *p*-nitrobenzyl esters.

EXPERIMENTAL

Materials

The carboxylic acids used were of reagent grade quality.

The following *p*-substituted benzyl halides were used: *p*-nitrobenzyl iodide, K & K (95–99%); *p*-bromobenzyl bromide, K & K (95–99%); *p*-methylbenzyl bromide, Eastman (highest purity); *p*-nitrobenzyl bromide, Eastman (highest purity).

p-Nitrobenzyl, *p*-methylbenzyl and *p*-bromobenzyl esters of formic, acetic and propionic acids were obtained commercially where available (*p*-nitrobenzyl acetate, K & K, 95–99%; *p*-methylbenzyl acetate, K & K, 95–99%) or synthesized according to the procedures described below.

Synthesis of p-substituted benzyl propionate and acetate reference materials. Propionic acid or acetic acid (3 g) dissolved in water (5 ml) was neutralized to a phenolphthalein end-point with 20% aqueous sodium hydroxide solution. A few drops of dilute hydrochloric acid were added so that the final solution was faintly acid to litmus paper. To this was added the appropriate *p*-substituted benzyl bromide (1 g) dissolved in alcohol (25 ml) and the mixture was heated under reflux for 3 h. The mixture was evaporated to half volume and then diluted with water. After extraction into ether, the organic layer was dried over anhydrous sodium sulfate, the solvent removed and the residual oil distilled *in vacuo*.

Synthesis of p-substituted benzyl formate reference materials. The *p*-substituted benzyl alcohol (1 g) was heated under reflux in formic acid (20 ml) containing concentrated HCl (three drops) for 2 h. The mixture was cooled, diluted with ether and shaken with several portions of 3% sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulfate, the ether removed under a flow of dry nitrogen, leaving an oil which on vacuum distillation yielded the desired product. The halide may also be used, but the yields appeared to be considerably lower.

p-Bromobenzyl formate was synthesized according to the above procedure using *p*-bromobenzyl bromide.

Reference solutions

All reference esters were made up to a concentration of 1 μg/μl in ethanol.

Reagent solutions

The reagent solutions were prepared as follows: Individual carboxylic acid solutions were made up to a concentration of 1 μg/μl in ethanol. An ethanolic mixture of all three carboxylic acids was made up so as to contain 1 μg/μl of each component. Solutions of potassium hydroxide, *p*-nitrobenzyl iodide, *p*-methylbenzyl bromide, and *p*-bromobenzyl bromide each contained 3 μg per μl ethanol.

Micro sample preparation and gas chromatography

The carboxylic acid solution (10 μl) was transferred by means of a Hamilton microsyringe into a capillary melting point tube along with potassium hydroxide in ethanol solution (3 μl) and the appropriate *p*-substituted benzyl halide solution (5 μl) described above (in the case of solution 2, 15 μl were used). The tube was flame sealed and incubated at 110° for 1 h.

After cooling, 2 μl of the reaction mixture were injected into a gas chromato-

graph* equipped with a flame ionization detector unit and fitted with a 5% OV-17 on Gas-Chrom Q (100–120 mesh) glass column (4 ft. × $\frac{1}{8}$ in. I.D.) preconditioned at 280° for 24 h. Column and injection port temperatures were, respectively: for the *p*-methylbenzyl series 100° and 225°, for the *p*-bromobenzyl series 120° and 240°, and for the *p*-nitrobenzyl series 145° and 265°. Nitrogen flow was 80 ml/min, hydrogen flow 50 ml/min, column inlet pressure 8 p.s.i.g., and attenuation × 100.

RESULTS AND DISCUSSION

In order to have an adequate method for the identification of lower molecular weight carboxylic acids by gas chromatography, it was desirable to have at least three different derivatives of each acid. The *p*-substituted benzyl esters offered the most advantages as they are readily prepared and are amenable to gas chromatography.

Reference material preparation

When preparing the reference materials, not all of the ester derivatives were commercially available, hence the appropriate ones were synthesized as described. The feasibility of the synthesis routes was established by gas chromatographic comparison of the pure (95–99%) commercial ester—where available—with the vacuum distillate of the same ester prepared by laboratory synthesis. In addition, IR spectral traces were used to confirm the ester linkage by the presence of the carbonyl stretching frequency band near 1740 cm^{-1}. The correct structure for each of the remaining synthesized esters was then inferred *a posteriori* from these data. The gas chromatograms of the esters synthesized for this investigation did indicate that small quantities of impurities (*e.g.* the corresponding alcohol) were present after a single vacuum distillation but further purification did not appear to be warranted.

Micro sample preparation

In the simulated microanalysis of each carboxylic acid with each *p*-substituted benzyl halide, injection of the reaction mixture was subsequently followed by injection of the ethanolic solution of the reference ester and then finally by injection of a 1:1 mixture of the first two (see Fig. 1, chromatograms a, b and c). In every case, gas chromatography of the reaction mixture afforded a peak of identical retention time to that of the reference ester thus confirming the formation of the expected ester in the capillary tube. In a given series, ester retention times increased with molecular weight of the carboxylic acid such that a mixture of the three acids could be easily resolved after the derivatization step. Fig. 1, chromatogram d, clearly demonstrates a typical separation of a formic, acetic and propionic acid mixture after benzylation, in this instance effected with *p*-bromobenzyl bromide and ethanolic KOH at 110°. Table I gives the retention times, calculated from the point of injection, for each ester and for other relevant compounds in a series. The reaction mixtures gave, in addition to a symmetrical ester peak, a sharp secondary "reaction artifact" peak presumably resulting from isomerization or decomposition of the thermally unstable *p*-substituted benzyl halides but the nature of the product was not investigated. Ethanolic solutions of the halides can, however, be stored at 0° for a week without undergoing appreciable

* Research Specialities Co. "600 series". The FID unit was Model 660.

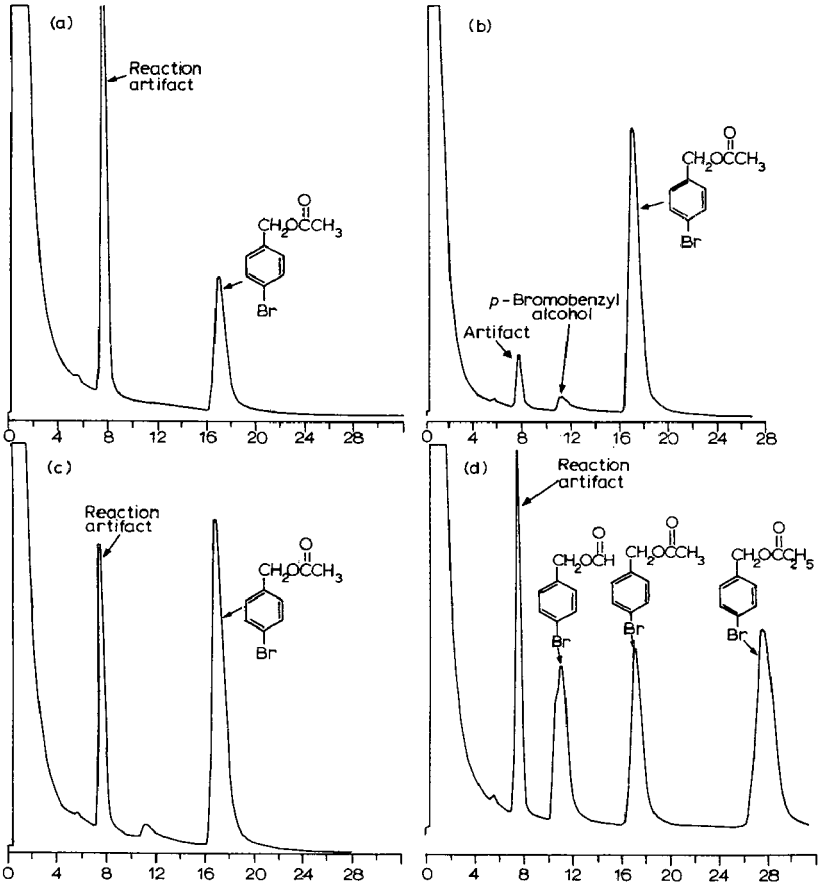

Fig. 1. (a) Reaction mixture (micro preparation using acetic acid solution). (b) Reference standard solution. (c) Reaction mixture of (a) + reference standard of (b) 1:1. (d) Reaction mixture (micro preparation using three-component acid solution).

TABLE I

RETENTION TIMES (min) OF *p*-SUBSTITUTED BENZYL FORMATE, ACETATE AND PROPIONATE ESTERS AND OTHER RELEVANT RELATED COMPOUNDS

	p-Methyl-benzyl (column temp. 100°)	*p-Bromo-benzyl (column temp. 120°)*	*p-Nitro-benzyl (column temp. 145°)*
Formate	8.4	10.9	11.0
Acetate	15.1	17.0	15.3
Propionate	26.3	27.4	22.8
Alcohol	6.6	10.2	12.6
Halide	9.3	12.7	25.5
Artifact	5.7	7.5	7.2

degradation. The presence of some unreacted *p*-nitrobenzyl iodide and its hydrolysis product *p*-nitrobenzyl alcohol was noted in the reaction mixtures of the *p*-nitrobenzyl esters. The iodide was found to be superior to the bromide in this series giving a cleaner reaction mixture and generally enhancing ester formation. In each series, the equilibrium constant for the esterification reaction appeared to be smallest for formic acid, this being suggested by the relative peak heights of the formate, acetate and propionate esters. Optimum results were realised with *p*-bromobenzyl derivatization; peaks were sharp and symmetrical and, aside from the "reaction artifact", the formation of the ester in the capillary tube was attented by no additional observable side reactions except in the case of the formate where, under the stated conditions, a small shoulder peak suggested the presence of some of the corresponding alcohol (see Fig. 1, chromatogram d).

With the appropriate attenuation setting, as little as 10 ng of reference ester were detected. However, in the micro preparation, detection of the carboxylic acid was not usually successful with sample solution concentrations lower than 0.1 $\mu g/\mu l$. The presence of appreciable amounts of water in the reaction mixture promoted virtually complete hydrolysis of the halide to the benzyl alcohol so that aqueous carboxylic acid solutions at the 1 $\mu g/\mu l$ level afforded only a very small ester peak. Nevertheless, detection of the acid was still possible when aqueous solutions of this concentration were diluted tenfold with ethanol and treated as described.

Though a number of chromatographic methods have already been proposed for the detection and separation of C_1 to C_3 monocarboxylic acids in various media such as in foods, in cigarette smoke or as pollutants in river waters, the present method might prove useful for such determinations carried out by control laboratories which, of necessity, employ general-purpose columns in their day to day operation.

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GAS–LIQUID CHROMATOGRAPHIC RETENTION DATA OF SOME ALIPHATIC AND ALICYCLIC SULPHIDES*

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SUMMARY

Gas chromatographic retention indices of some lower aliphatic and alicyclic sulphides on isodecyl phthalate and Apiezon L columns at 110–150° are given and correlated.

INTRODUCTION

The identification and analysis of sulphur compounds in mixtures with hydrocarbons has been a topical analytical problem in the chemical refining and processing of crude petroleum[1]. The aim of the present work is to utilise the empirical rules with respect to the properties of retention indices[2] for substances that have not yet been investigated experimentally in this way[3].

The retention indices of some lower aliphatic and alicyclic sulphides on two stationary phases of different polarity have been measured and the temperature and structural dependences investigated. KOVÁTS' relationship[2], providing for the logarithmic interpolation of the position of the peak of a substance between two neighbouring *n*-paraffins, was used in the calculation. Provided the logarithmic scale of the reference paraffins is linear beyond the interval investigated as well, it is possible to carry out extrapolations. Over a narrow temperature interval, the temperature dependence of the retention index may be considered as approximately linear, though it has been found to be hyperbolic[4] over a wider interval.

EXPERIMENTAL

A Chrom 2 apparatus (Laboratory Instruments, N.E., Prague, Czechoslovakia), partly modified (injection port, gas flow stabilisation) to fit the character of the work, was used.

Measurements were carried out on 1600 mm long columns of 3 mm I.D., wound

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into helices. Apiezon L (ApL) (Edwards High Vacuum Ltd., Crawley, Great Britain) and isodecyl phthalate (IDP) (Carlo Erba S.p.A., Milan, Italy) were used as stationary phases. As a support, Chromosorb W 80–100 mesh (Johns-Manville, Trenton, N.J., U.S.A.) was employed; it was dried at 120° and coated by 20 wt.% of the stationary phase. The Apiezon L and isodecyl phthalate columns contained 4.3 and 4.1 g of the packings, respectively. The Apiezon L column was conditioned at 160° and the column with isodecyl phthalate at 155°. The separation efficiencies of the columns were 2490 (ApL) and 2680 (IDP) theoretical plates, as measured for a decane peak at 120°.

Each member of the homologous series of sulphides was chromatographed at five different temperatures within the interval of 110–150°; except that with the Apiezon L column a temperature interval of 120–160° was used in the measurement of the thiacyclopentanes. The temperature was stabilised to ± 0.2°. For each sulphide a pair of *n*-paraffins was chosen such that the three-component mixture prepared from them was well resolved by chromatography.

About 0.1 μl of the mixture was injected with a Hamilton syringe, each charge being carried out five times on both columns at all the above temperatures. The column hold-up time was measured by injecting 5 μl of town gas. The measurement was

TABLE I

RETENTION INDICES OF MODEL SULPHIDES

Sulphide	*Isodecyl phthalate*			*Apiezon L*		
	110°	*130°*	*150°*	*110°*	*130°*	*150°*
Me-S-Me	579	585	592	516	523	528
Et-S-Me	—	687[a]	—	—	623[a]	—
Pr-S-Me	783	787	792	718	723	727
Bu-S-Me	—	869[a]	—	—	810[a]	—
sec.-Bu-S-Me	843	849	853	784	790	796
Et-S-Et	759	765	772	694	698	704
Pr-S-Et	—	864[a]	—	—	801[a]	—
Bu-S-Et	957	962	965	896	901	906
sec.-Bu-S-Et	—	898[a]	—	—	841[a]	—
Pr-S-Pr	—	958[a]	—	—	894[a]	—
Bu-S-Pr	1047	1052	1057	986	992	995
sec.-Bu-S-Pr	996	999	1005	940	946	951
iso-Pr-S-*sec.*-Bu	941	946	951	886	890	897
tert.-Bu-S-*tert.*-Bu	968	975	982	913	920	927
sec.-Bu-S-*sec.*-Bu	—	999[a]	—	—	952[a]	—
iso-Bu-S-*iso*-Bu	1048	1052	1060	991	997	1003
Bu-S-Bu	1146	1148	1155	1082	1086	1092
2-Me-thiacyclopentane	—	936[a]	—	—	885[a]	—
2-Et-thiacyclopentane	1026[b]	1031	1039	977[b]	981	994
2-Pr-thiacyclopentane	1120[b]	1125	1139	1071[b]	1077	1088
2-Bu-thiacyclopentane	1217[b]	1221	1228	1168[b]	1174	1182

[a] Correlated value.
[b] Temperature 120°.

carried out with 15 different sulphides and 6 *n*-alkanes (C_7–C_{12}), all being the products of Lachema, Brno, Czechoslovakia. The purity (min. 95%) was satisfactory.

The average values calculated of the retention indices are summarised in Table I. The interval of confidence for the retention indices of the sulphides investigated did not exceed $\pm$ 2 units on both columns at 130°.

RESULTS AND DISCUSSION

The fifteen sulphides measured (Table I) were arranged into seven homologous series. In this instance, a series of at least three sulphides is called a homologous series, the retention indices of which are in a mutual relation reflecting a gradual defined change in the structure of the compounds. In each of these series, it is possible to correlate the retention index with the varying structural parameter.

The determination of the retention indices of the sulphides which have not been measured in the individual homologous series are quoted in Fig. 1.

The values of the retention indices of sulphides that were not amenable to direct measurement were determined by interpolation or extrapolation with the aid of the correlation dependence of the retention indices on the carbon numbers of the sulphides measured. The increaments of the symmetrically substituted sulphides represent the

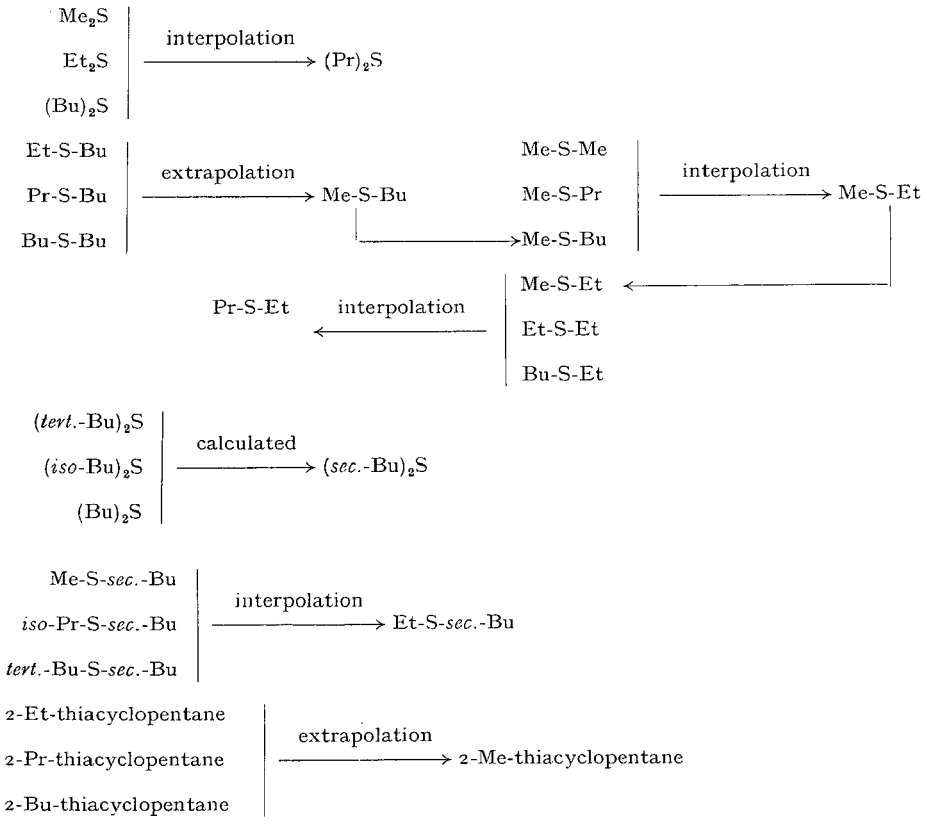

Fig. 1.

structural changes of both substituents in the molecule. The same increaments, from the qualitative viewpoint, with the nonsymmetrical sulphides are due to only one of the substituents while the other one remains unaffected.

The individual homologous series of the sulphides and the values I, ΔI, and I_0 $(\partial I/\partial T)$ for both columns and for three of the originally employed five temperatures are summarised in the tables. In addition, the intervals of confidence $(i = st/\sqrt{n})$ calculated from five replicate injections of each sample for a confidence level of 0.95 have been quoted for all the retention indices of all the experimentally measured sulphides on both columns, at 130°.

TABLE II

RETENTION INDICES OF HOMOLOGOUS DIALKYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me_2S	579	585	592	0.82	516	523	528	1.17	62
$\partial T/10°$	3	3.5			3.5	2.5			
Et_2S	759	765	772	0.99	694	698	704	1.12	67
$\partial T/10°$	3	3.5			2	3			
$(Pr)_2S$	—	958[a]	—		—	894[a]	—		64
$(Bu)_2S$	1146	1148	1155	0.95	1082	1086	1092	0.71	62
$\partial T/10°$	1	3.5			2	2			

[a] Interpolation.

In compliance with the known findings[2,3] on the temperature dependence of retention indices, it can also be seen that the retention index values of sulphides show approximately linear variations with temperature within a narrow temperature interval (50°). With respect to the retention indices of members of homologous series

TABLE III

RETENTION INDICES OF HOMOLOGOUS ALKYL METHYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me-S-Me	579	585	592	0.82	516	523	528	1.17	62
$\partial T/10°$	3	3.5			3.5	2.5			
Et-S-Me	—	687[b]	—		—	623[b]	—		64
Pr-S-Me	783	787	792	1.12	718	723	727	0.24	64
$\partial T/10°$	2	2.5			2.5	2			
Bu-S-Me	—	869[a]	—		—	810[a]	—		59

[a] Extrapolation.
[b] Interpolation.

TABLE IV

RETENTION INDICES OF HOMOLOGOUS ALKYL ETHYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me-S-Et	—	687[a]	—		—	623[a]	—		64
Et-S-Et	759	765	772	0.99	694	698	704	1.12	67
∂T/10°	3	3.5			2	3			
Pr-S-Et	—	864[a]	—		—	801[a]	—		63
Bu-S-Et	957	962	965	0.34	896	901	906	0.53	61
∂T/10°	2.5	1.5			2.5	2.5			

[a] Interpolation.

TABLE V

RETENTION INDICES OF HOMOLOGOUS ALKYL PROPYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me-S-Pr	783	787	792	1.12	718	723	727	0.24	64
∂T/10°	2	2.5			2.5	2			
Et-S-Pr	—	864[a]	—		—	801[a]	—		63
Pr-S-Pr	—	958[a]	—		—	894[a]	—		64
Bu-S-Pr	1047	1052	1057	0.74	986	992	995	0.80	60
∂T/10°	2.5	2.5			3	1.5			

[a] Interpolation.

TABLE VI

RETENTION INDICES OF HOMOLOGOUS ALKYL BUTYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me-S-Bu	—	869[a]	—		—	810[a]	—		59
Et-S-Bu	957	962	965	0.34	896	901	906	0.53	61
∂T/10°	2.5	1.5			2.5	2.5			
Pr-S-Bu	1047	1052	1057	0.74	986	992	995	0.80	60
∂T/10°	2.5	2.5			3	1.5			
Bu-S-Bu	1146	1148	1155	0.95	1082	1086	1092	0.71	62
∂T/10°	1	3.5			2	2			

[a] Extrapolation.

TABLE VII

RETENTION INDICES OF HOMOLOGOUS ALKYL *sec.*-BUTYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
Me-S-*sec.*-Bu	843	849	853	0.49	784	790	796	0.30	59
$\partial T/10°$	3	2			3	3			
Et-S-*sec.*-Bu	—	898[a]			—	841[a]	—		57
Pr-S-*sec.*-Bu	996	999	1005	0.58	940	946	951	0.32	53
$\partial T/10°$	1.5	3			3	2.5			
iso-Pr-S-*sec.*-Bu	941	946	951	0.18	886	890	897	1.32	56
$\partial T/10°$	2.5	2.5			2	3.5			
tert.-Bu-S-*sec.*-Bu	981	987	994	1.16	930	936	942	0.32	51
$\partial T/10°$	2.5	3.5			3	3			

[a] Interpolation.

of nonbranched sulphides, it is notable that, at all the temperatures and on both columns, the retention index values of neighbouring members in a given homologous series differ from each other by about 100 units on a given stationary phase and at a given temperature. Anomalies occur only between lowest members of the homologous series, *i.e.* with those for which the number of carbon atoms does not exceed five.

A different situation occurs with branched sulphides (Tables VII and VIII). The retention indices for the homologous series of the branched nonsymmetrical secondary butyl sulphides are correlated with the number of CH_3 groups on the first carbon of the variable substituent in the molecule. In this case, the increment of the retention index due to the CH_3 group only amounts to about 50 units.

TABLE VIII

RETENTION INDICES OF DIFFERENT DIBUTYL SULPHIDES

Sulphide	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	110°	*130°*	*150°*		*110°*	*130°*	*150°*		
$(Bu)_2S$	1146	1148	1155	0.95	1082	1086	1092	0.71	62
$\partial T/10°$	1	3.5			2	3			
$(iso\text{-}Bu)_2S$	1048	1052	1060	0.93	991	997	1003	1.32	55
$\partial T/10°$	2	4			3	3			
$(sec.\text{-}Bu)_2S$	—	999[a]	—		—	952[a]	—		47
$(tert.\text{-}Bu)_2S$	968	975	982	0.60	913	920	927	0.47	55
$\partial T/10°$	3.5	3.5			3.5	3.5			

[a] Calculated.

TABLE IX

RETENTION INDICES OF HOMOLOGOUS 2-ALKYL THIACYCLOPENTANES

Thiacyclopentane	*Isodecyl phthalate*			± *i*	*Apiezon L*			± *i*	*ΔI*
	120°	*130°*	*150°*		*120°*	*130°*	*150°*		
2-Me	—	936[a]	—		—	885[a]	—		51
2-Et	1026	1031	1039	1.28	977	981	994	1.08	50
$\partial T/10°$	5	4			4	6.5			
2-Pr	1120	1125	1139	1.04	1071	1077	1088	0.49	48
$\partial T/10°$	5	7			6	5.5			
2-Bu	1217	1221	1228	1.02	1168	1174	1182	1.33	47
$\partial T/10°$	4	3.5			6	4			

[a] Extrapolation.

In Table VIII, it is possible to follow the changes of the retention indices of the symmetrical dibutyl sulphides with the gradual shifting of the CH_2 group from the interior of the molecule to its periphery. In this series, (*sec.*-Bu)$_2$S has not been measured. It is possible to estimate from the retention indices of the isomers measured, that the retention index of (*sec.*-Bu)$_2$S will be within an interval confined by the retention index values of (*tert.*-Bu)$_2$S and (*iso*-Bu)$_2$S. The third KOVÁTS' rule[2] may be used to determine the above value. To do this, the retention indices of *tert.*-Bu-S-*sec.*-Bu ($I_{130}^{ApL} = 936$) and (*tert.*-Bu)$_2$S ($I_{130}^{ApL} = 920$) are used. Thus, employing numerical calculation, one obtains for (*sec.*-Bu)$_2$S at 130° on an Apiezon L column a retention index value of $I_{130}^{ApL} = 952$ and on the isodecyl phthalate column, at the same temperature, a value of $I_{130}^{IDP} = 999$.

If the retention index of (*sec.*-Bu)$_2$S is calculated by the second KOVÁTS' rule, *i.e.* from the difference between the boiling points of two isomeric sulphides, one obtains a value of $I_{130}^{ApL} = 966.5$ for the nonpolar column.

The accuracy of calculation is limited by the approximative character of the rules, and the values so obtained may be used for identification as a first approximation only.

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GAS CHROMATOGRAPHIC BEHAVIOUR OF TRICYCLIC SATURATED HYDROCARBONS

THE RELATION BETWEEN THE RETENTION INDICES AND THE STEREOCHEMICAL STRUCTURE OF THE TRICYCLANES

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SUMMARY

The authors determined the Kováts retention indices of stereoisomers of 8 bicyclic and tricyclic saturated hydrocarbons containing five- and six-membered rings in a molecule. The retention data were obtained by means of capillary columns using polar (Carbowax 20M) and non-polar (SE-30) stationary phases. The relationships between the retention indices, the homomorphic factors, ΔI values and the chemical (or stereochemical) structures of the hydrocarbons (or stereoisomers) studied allow one to ascribe an unknown stereoisomer with its hydrocarbon structure (*i.e.* to differentiate between stereoisomeric mixtures of two or more hydrocarbons), to predict the retention values of the individual stereoisomers, and to contribute, on the basis of the retention data, to the determination of configurations of so far unknown stereoisomers.

INTRODUCTION

The comparison of gas chromatographic retention of diverse organic compounds on stationary phases of different polarity has recently become a general method used for identifying these compounds[1–3].

The most common method for the determination and interpretation of the relationships between the chemical structure of various compounds and their retention behaviour is a two-dimensional graphical presentation of the data obtained (most frequently on logarithmic scales). The chromatographic data measured on a non-polar stationary phase are plotted against the data of the same series of compounds, measured on a polar phase. The graph represents a number of dependences, the most important of them (for the given pair of stationary phases of different polarity) being the following:

(1) The points corresponding to compounds of a particular type occur in the graph in a particular characteristic zone.

(2) The points corresponding to members of homologous series lie on parallel straight lines, each straight line being characteristic for a particular homologous series.

(3) The points corresponding to isomers (*i.e.* the points lying on different straight lines of the homologous series) lie on separate segment lines parallel to one another, each of them being characterised by the molecular mass of the isomers lying on the particular segment line.

The above rules, which are valid generally, can be used to predict the chromatographic behaviour of known compounds as well as for attributing a particular chemical structure to unknown compounds on the basis of their chromatographic behaviour.

The stereoisomers of polycyclic naphthenic hydrocarbons containing five- and six-membered rings in a molecule are a specific case of isomerism based only on absolute configuration of the mutual attachment of the individual rings in a molecule. Our previous gas chromatographic analyses of stereoisomeric mixtures of tricyclic saturated hydrocarbons[4], and the relationships found between the structure of adamantane compounds and their chromatographic behaviour[5,6], have given us the idea of trying to solve this complex problem of determining the configuration of the individual stereoisomers of the hydrocarbons mentioned by making use of gas chromatographic data and their interpretation.

Thus the purpose of the present work is, apart from the usual tabulating of gas chromatographic data of compounds so-far undescribed on the usual stationary phases, to determine the relationship between the configurations of the individual stereoisomers and their gas chromatographic behaviour and to evaluate the possibility of utilising the dependences, so found, for the determination of the configurations of stereoisomers so far unknown.

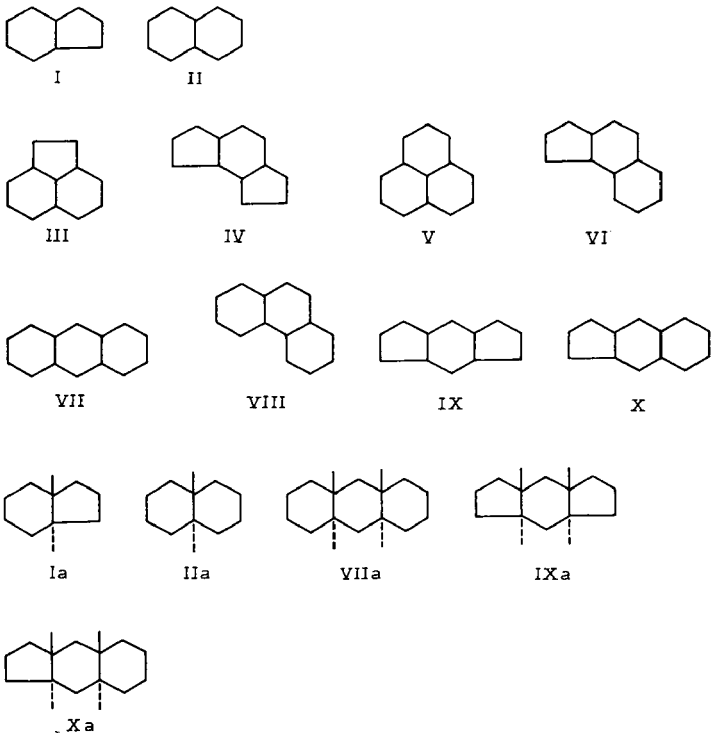
EXPERIMENTAL

The elution data were measured with a Chrom II instrument (Laboratorní přístroje, Prague, Czechoslovakia) fitted with a flame ionisation detector. Nitrogen was used as carrier gas. Stainless steel capillary columns, 0.25–0.30 mm in diameter, 50 m long, were used for the measurements. The elution data of the hydrocarbon series studied were measured on stationary phases of SE-30 and Carbowax 20M.

The following series of stereoisomeric mixtures of bicyclic and tricyclic saturated hydrocarbons, the preparation of which has been described previously[4], were used for the measurements:

Bicyclo [4,3,0] nonane*	(I)
Bicyclo [4,4,0] decane*	(II)
Tricyclo [7,2,1,$0^{5,12}$] dodecane	(III)
Tricyclo [7,3,0,$0^{2,6}$] dodecane	(IV)
Tricyclo [7,3,1,$0^{5,13}$] tridecane	(V)
Tricyclo [8,3,0,$0^{4,9}$] tridecane	(VI)
Tricyclo [8,4,0,$0^{3,8}$] tetradecane	(VII)
Tricyclo [8,4,0,$0^{2,7}$] tetradecane	(VIII)

* Stereoisomeric mixtures of bicyclic hydrocarbons were obtained by the catalytic hydrogenation of the corresponding aromatic hydrocarbon.



The Kováts retention indices were measured by a standard method[7] with the Carbowax 20M phase at 125, 150 and 175° and with the SE-30 at 150, 175 and 200°. In the actual calculations of the individual retention indices the retention distances reduced by those of methane injected simultaneously with the mixture analysed, were used. The values given in Table I are average values of 3–5 analyses. The difference of extreme values of the individual determinations did not exceed two units of the retention index. The average values were rounded-off to whole units.

RESULTS AND DISCUSSION

Influence of stereoisomer configuration on the magnitude of the homomorphic factor

Many studies (especially those of SCHOMBURG[1]) dealing with the structural interpretation of gas chromatographic data of a great number of organic compounds have shown that the values of the retention index are subject to an incremental change. This incremental character will stand out clearly if the relative scale introduced by SCHOMBURG, called the homomorphic factor, is used for structural interpretation.

The *H* factor of the stereoisomers examined, and given in Tables I, II and IV, is the difference between the retention index of a stereoisomer and that of an *n*-paraffin with an equal number of C-atoms (both values being measured on the same phase and at the same column temperature).

TABLE I

THE KOVÁTS INDICES OF STEREOISOMERS OF TRICYCLANES ON SE-30 AND CARBOWAX 20 M

The stereoisomers of perhydroanthracene: number 1 = *trans–syn–trans*; number 3 = *cis–trans*; number 4 = *cis–syn–cis*.
$H = I_{\text{stereoisomer } C_n} - I_{n\text{-alkane } C_n}$; $\Delta I = I^{\text{Carbowax 20M}} - I^{\text{SE-30}}$.

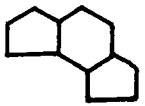
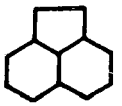
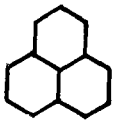
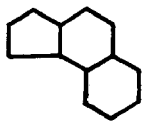
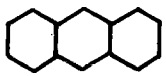
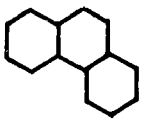
Compound	*Stereo-isomer*	*SE-30*					*Carbowax 20 M*					ΔI_{150}
		I			$\delta I/10°$	H_{150}	*I*			$\delta I/10°$	H_{150}	
		150°	*175°*	*200°*			*125°*	*150°*	*175°*			
	1						1428	1458	1484	11.2	258	
	2						1452	1480	1505	10.6	280	
	3	1307	1326	1343	7.2	107	1477	1508	1535	11.6	308	201
	4	1323	1343	1362	7.8	123	1503	1537	1566	12.6	337	214
	5	1328	1348	1365	7.4	128	1514	1547	1578	12.8	347	219
	6	1352	1373	1391	7.8	152	1550	1585	1619	13.8	385	233
	1	1274	1291	1310	7.2	74	1430	1462	1494	12.8	262	188
	2	1295	1314	1334	7.8	95	1466	1499	1533	13.4	299	204
	3	1317	1338	1359	8.4	117	1504	1539	1574	14.0	339	222
	4	1323	1344	1365	8.4	123	1504	1539	1574	14.0	339	216
	5	1352	1374	1396	8.8	152	1554	1591	1630	15.2	391	239
	1	1377	1400	1424	9.4	77	1553	1590	1628	15.0	290	213
	2	1416	1442	1468	10.4	116	1614	1652	1691	15.4	352	236
	3	1429	1456	1482	10.6	129	1641	1681	1721	16.0	381	252
	1	1409	1433	1456	9.4	109	1592	1628	1660	13.6	328	219
	2	1422	1446	1469	9.4	122	1613	1652	1687	14.8	352	230
	3	1437	1461	1484	9.4	137	1636	1674	1709	14.6	374	237
	4	1444	1468	1491	9.4	144	1647	1685	1720	14.6	385	241
	5	1461	1486	1511	10.0	161	1676	1718	1755	15.8	418	257
	1	1481	1506	1531	10.0	81	1658	1696	1734	15.2	296	215
	2	1494	1519	1545	10.2	94	1680	1718	1755	15.0	318	224
	3	1513	1537	1562	9.8	113	1705	1744	1782	15.4	344	231
	4	1549	1576	1602	10.6	149	1761	1804	1844	16.6	404	255
	5	1559	1587	1616	11.4	159	1777	1819	1860	16.6	419	260
	1	1493	1515	1540	9.4	93	1672	1712	1751	15.8	312	219
	2	1513	1538	1564	10.2	113	1705	1747	1786	16.2	347	234
	3	1537	1563	1589	10.4	137	1741	1786	1826	17.0	386	249
	4	1552	1575	1602	10.0	152	1765	1810	1850	17.0	410	258
	5	1568	1596	1624	11.2	168	1796	1845	1886	18.0	445	277

TABLE II

THE KOVÁTS INDICES OF STEREOISOMERS OF BICYCLANES ON SE-30 AND CARBOWAX 20 M

$H = I_{\text{stereoisomer } C_n} - I_{n\text{-alkane } C_n}$; $\Delta I = I^{\text{Carbowax 20 M}} - I^{\text{SE-30}}$.

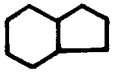
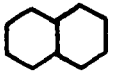
Compound	*Stereo-isomer*	*SE-30*					*Carbowax 20 M*					ΔI_{150}
		I			$\delta I/10°$	H_{150}	*I*			$\delta I/10°$	H_{150}	
		100°	*125°*	*150°*			*100°*	*125°*	*150°*			
	1	963	975	987	4.8	87	1086	1102	1125	7.8	225	138
	2	996	1010	1023	5.4	123	1138	1158	1183	9.0	283	160
	1	1061	1075	1089	5.6	89	1195	1218	1244	9.8	244	155
	2	1101	1116	1132	6.2	132	1260	1285	1314	10.8	314	182

TABLE III

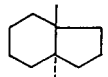
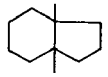
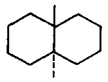
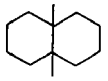
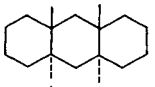
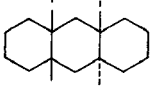
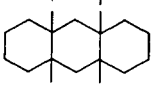
THE KOVÁTS INDICES OF CYCLOHEXANE ON SE-30 AND CARBOWAX 20 M

Compound	*Stereo-isomer*	*SE-30*				*Carbowax 20 M*			
		I			*δI/10°*	*I*			*δI/10°*
		50°	*75°*	*100°*		*50°*	*75°*	*100°*	
		662	667	675	2.6	740	746	751	2.2

In the case of saturated tricyclic hydrocarbons of the type examined, the absolute configurations of only a very limited number of stereoisomers are known; so far, only the absolute configurations of the stereoisomers of perhydroanthracene

TABLE IV

RELATIONSHIP BETWEEN H FACTOR AND CONFIGURATION OF STEREOISOMER

Stereoisomer	H_{150}			
	SE-30		*Carbowax 20 M*	
	Trans	*Cis*	*Trans*	*Cis*
	88[a]		163[a]	
	87		225	
		123		283
	89		244	
		132		314
	81		296	
	113		344	
		149		404

[a] The values were obtained by the extrapolation of the measured values in Table III.

have been ascertained reliably, three of them being employed in the present work (*trans–syn–trans*, *cis–trans* and *cis–syn–cis*). For the purpose of structural interpretation of the *H* factors found, the series of tricyclic stereoisomers was extended by stereoisomers of bicyclo [4,3,0] nonane (hydrindane), bicyclo [4,4,0] decane and cyclohexane (Tables II and III).

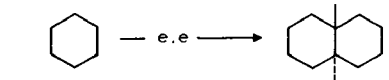
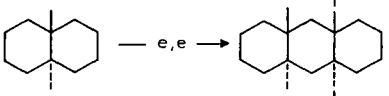
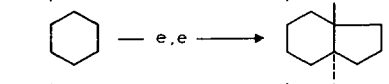
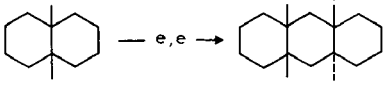
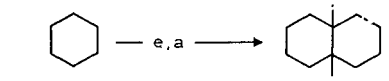
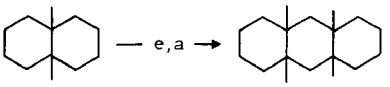
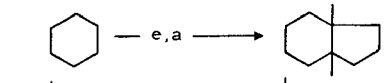
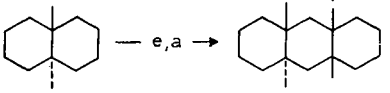
The survey given in Table IV shows that in the series of cyclic hydrocarbons chosen the magnitude of the *H* factors depends on configuration.

The influence of configuration is most pronounced in the values of the *H* factor measured on a non-polar phase (SE-30). With stereoisomers whose molecules contain only *trans* configurations, the value of the *H* factor is nearly constant (81–89), and is independent of the number of rings in the molecule as well as of whether they are six-membered or five-membered rings or a combination of the two. The above results may be compared with the data on monocyclic saturated hydrocarbons measured on a non-polar stationary phase (squalane) by SCHOMBURG[8], who found that the enlargement of a five-membered saturated hydrocarbon ring to a six-membered one does not result in a change in the *H* factor of the compound, which is not true for the

TABLE V

THE INFLUENCE OF CONFIGURATION ON THE VALUE OF THE RETENTION INDEX INCREMENT IN THE CASE OF ENLARGEMENT OF MOLECULES BY OTHER SATURATED RINGS

n_{CA}, n_{CB} = C-number of the compounds C_A and C_B.

Hydrocarbon		$\delta I^{SE\text{-}30}_{150}$	$\delta I^{Ca3b\text{-}20M}_{150}$
C_A	C_B	$n_{CB} - n_{CA}$	$n_{CB} - n_{CA}$
		100	120
		98	113
		100	121
		95	107
		111	138
		104	122
		112	140
		106	125

enlargement of rings with a different number of carbon atoms. The *cis* configurations in the molecule (ranging in the given series from 123 to 149) give rise to an increase in the value of the H factor with the number and size of the rings. The H factor of *cis–trans* perhydroanthracene lies, in the middle, between the values of the H factors of stereoisomers with *trans* configurations only and those of stereoisomers with *cis* configurations only.

With the H factors measured on the polar Carbowax 20M the influence of the configurations was not so pronounced. The H factors corresponding to *trans* configurations are even lower here than those corresponding to *cis* configurations; the H factor, however, greatly increases with the number of rings.

The dependences found suggest that even in the other tricyclic hydrocarbons given in Table I the H factors for *trans* stereoisomers will be lower than those for *cis* stereoisomers.

Structural interpretation of the change in retention index caused by a change in polarity of the stationary phase

It can be assumed that the values of $I^{SE\text{-}30}$ of the series of stereoisomers studied will, in general, be a function of the number of C-atoms in a molecule, the chemical structure of the hydrocarbon (common for all stereoisomers of one hydrocarbon) and the stereochemical structure (absolute configuration). The values of $I^{Carbowax\ 20M}$ will be further influenced by the dipole–dipole interaction of the molecules of the stereoisomers and the molecules of the stationary phase, the carbon rings being known to contribute to the ΔI values in general.

The graphical presentation of the values given in Tables I and II (Fig. 1) provides a system of line segments (corresponding to the respective stereoisomeric mixtures) designated by WALRAVEN[2,9] as an imbricated series*. The stereoisomers of the individual hydrocarbons always lie on one line segment. The line segments of C_9, C_{10}, C_{12}, C_{13}, and C_{14} hydrocarbons are separate and almost parallel. The stereoisomers of C_{14} hydrocarbons all lie together on one line segment, the stereoisomers of C_{13} hydrocarbons lie on two line segments, and those of C_{12} hydrocarbons also lie on two line segments.

The nearly equal values of the H factors of the *trans* stereoisomers on a non-polar stationary phase (SE-30) given in Table IV mean that the points corresponding to stereoisomers with this configuration will be situated in the graph (Fig. 1) on a straight line. Stereoisomers Ia, IIa and VIIa may be considered to be members of a "homologous series", which offers the possibility of estimating the retention values of the missing members. It may be assumed that the points corresponding to stereoisomers IXa and Xa will be found on the straight line of the above mentioned "homologous series".

Fig. 1 also shows that all the stereoisomers of hydrocarbons VII and VIII lie on one segment line of the imbricated series. It may be assumed that the same will hold for tricyclanes with one or two five-membered rings in a molecule, and that stereoisomers of hydrocarbons VI and X will lie on a common segment line of the imbricated series. A common segment line may also be assumed for stereoisomers of hydrocarbons IV and IX.

* The area arrangement of the individual segment lines is similar for any kind of isomeric series and resembles the arrangement of roof tiles (hence called "roof-tile effect").

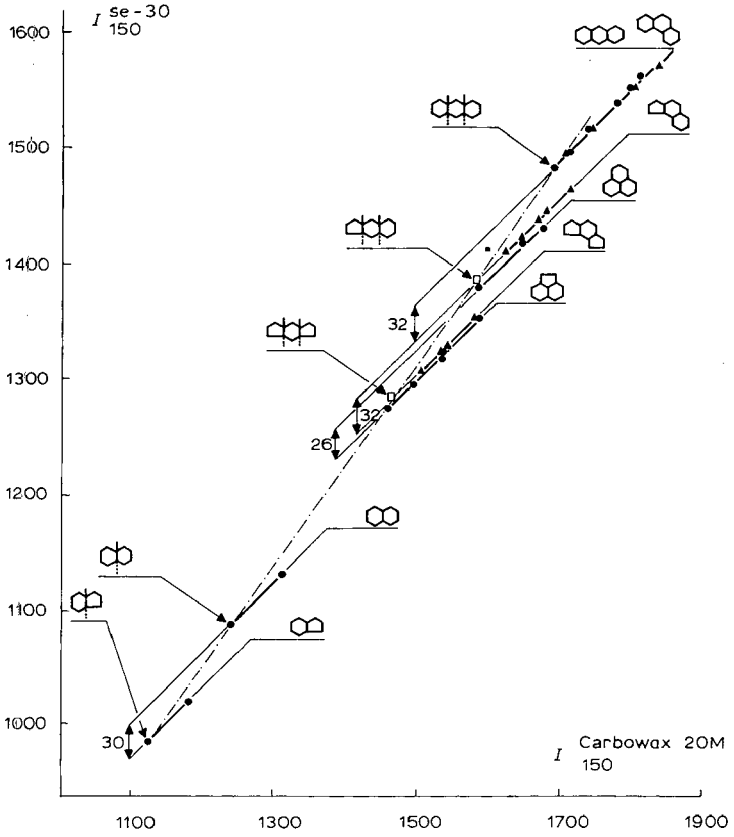
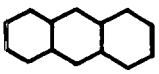
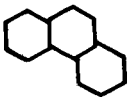


Fig. 1. The "rooftile effect" of the stereoisomers of bicyclic and tricyclic saturated hydrocarbons with five- and six-membered rings. (———) the segment lines of the imbricated series; (— · — · —) the "homologous series" line of *trans*-stereoisomers; (□) estimated retention values of the so far unknown stereoisomers. The segment line of C_{14} hydrocarbons: (●) the stereoisomers of



(▲) the stereoisomers of



The point corresponding to stereoisomer IXa will occur on the graph at the point of intersection of the straight line (joining stereoisomers Ia, IIa and VIIa) with the segment line of the imbricated series common for stereoisomers of hydrocarbons IV and IX. For stereoisomer IXa, the following values were read off the graph:

$I_{150°}^{SE\text{-}30}$: 1,280 and $I_{150°}^{Carbowax\ 20M}$: 1,463

The point corresponding to stereoisomer Xa will likewise be found at the point of intersection of the straight line of the "homologous series" of *trans* stereoisomers with the segment line of the imbricated series common for stereoisomers of hydrocarbons VI and X. The following values were read off the graph (for stereoisomer Xa):

$I_{150°}^{SE\text{-}30}$: 1,378 and $I_{150°}^{Carbowax\ 20M}$: 1,577

It can also be seen from the graph that the segment lines of hydrocarbons IV, VI

and VIII are separated from each other by 32 units of the retention index, and those of hydrocarbons I and II by 30 units. Thus the enlargement of a five-membered saturated ring to a six-membered one in the hydrocarbons whose structure was being studied shifts the segment line of the imbricated series by 30–32 units (on the above mentioned pair of stationary phases). The stereoisomers of hydrocarbons containing one carbon atom common for all three rings (hydrocarbons III and V) lie on their own segment lines of the imbricated series and are separated from each other by 26 units only. They are also distinct from the segment lines of the hydrocarbons described above containing an equal number of carbon atoms in a molecule.

The location of a point corresponding to any stereoisomer of a polycyclic saturated hydrocarbon of the type studied (containing five-membered and six-membered rings in a molecule) will, in general, be determined on the graph by:

(1) The number of carbon atoms in a molecule,

(2) The molecular structure,

(3) The absolute configuration of the stereoisomer.

Points 1 and 2 determine the segment line on which the point corresponding to the given stereoisomer should be situated; point 3 determines the position on this segment line.

Influence of configuration due to a substituent on the change in the retention index, caused by the extension of the molecule by a five- or six-membered saturated carbon ring

When enlarging the basic monocyclic skeleton to a bicyclic or tricyclic molecule, other rings can be attached either by two equatorial bonds or by one axial and one equatorial bond (which has a bearing on the possibility of *cis* and *trans* configurations of the ring attachment).

Table V shows that the change in the retention index caused by the attachment of other rings is affected by the following possibilities:

(1) The attachment of a second ring (to a monocyclic system) by two equatorial bonds results in a change in the retention index of 100 units (related to one $-CH_2-$ group) when measured on SE-30 and by 120 units on Carbowax 20 M. Attachment of a third ring (to a bicyclic system), results in a change, corresponding to one $-CH_2-$ group, which is somewhat lower.

(2) Enlargement of the basic skeleton by attaching another ring by means of one axial and one equatorial bond, causes a change in the retention index which is generally higher when compared with the previous method.

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CHROM 4880

CHROMATOGRAPHY OF BIOGENIC AMINE METABOLITES AND RELATED COMPOUNDS ON LIPOPHILIC SEPHADEX

II. THE CATECHOLAMINES AND THEIR 3-O-METHYLATED DERIVATIVES

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SUMMARY

N-Perfluoroacyl derivatives of adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine and 3-methoxytyramine were prepared and chromatographed on Sephadex LH-20 in solvent systems of 1,2-dichloroethane and methanol. Separations of these compounds were achieved. The systems are highly efficient (about 1200 theoretical plates/m) and fast (2–4 h). The application of the method in studies on biogenic amine metabolism and purification prior to gas chromatography–mass spectrometry is described.

INTRODUCTION

Many useful methods exist for the separation of the catecholamines and their O-methylated metabolites[1,2]. Adsorption chromatography on alumina[3,4] or on thin-layer chromatography systems[5,6] have been widely used. Ion-exchange chromatography on strongly acidic columns of crosslinked polystyrene (*e.g.* Dowex 50-X4 and Amberlite CG-120) have been of great value especially in combination with quantitative fluorimetric measurements[7,8]. Weak cationic exchangers have been useful in the isolation of amines from urine. Also systems using cellulose, including paper chromatography, have been employed[9–13].

Recently SJÖVALL *et al.* have introduced the use of lipophilic Sephadex for highly efficient liquid–gel partition chromatography of steroids, fat-soluble vitamins and other compounds of biological interest[14]. In a previous communication we described the separation of acidic and neutral metabolites of several biogenic amines on Sephadex LH-20 (ref. 16). In the present study we report the preparation of derivatives of the catecholamines and the O-methylated basic metabolites suitable for liquid gel chromatography and their separation on Sephadex LH-20. Applications of the method to studies on catecholamine metabolism are described.

EXPERIMENTAL

Materials

Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. This material was refluxed three times for 2 h in methanol, dried and used to pack the columns as described below. Organic solvents were of analytical purity.

The amines were supplied by the following sources: 3-hydroxytyramine·HCl, L-noradrenaline bitartrate monohydrate and L-epinephrine bitartrate from Sigma Chemical Co., St. Louis, Mo., U.S.A., DL-metanephrine·HCl and DL-normetanephrine from Winthrop Laboratories, New York, U.S.A.; 3-methoxy-4-hydroxy-phenyl ethylamine·HCl from Calbiochem AG, Luzern, Switzerland. ^{3}H-labelled dopamine (spec. act., 2.5 Ci/mmole, generally labelled) and L-[7-^{3}H]noradrenaline (2.34 Ci/mmole) were from the Radio-chemical Centre, Amersham, Bucks., Great Britain and 3-methoxy-4-hydroxy-β-[5-^{3}H]phenylethylamine (12.6 Ci/mmole) and DL-[7-^{3}H]normetanephrine (3.8 Ci/mmole) from New England Nuclear, Boston, Mass., U.S.A.

Preparation of derivatives

Amines or their salts were dissolved in a mixture (1:1) of ethyl acetate and perfluoro acid anhydride. After 30 min at room temperature, the solvent and reagent were evaporated by a stream of nitrogen. Methanol (1–2 ml) was added and the sample left at room temperature for 1 h for the trifluoroacetyl derivatives and overnight for the pentafluoropropionyl and heptafluorobutyryl derivatives. After evaporation to dryness, the material was dissolved in a small volume of the solvent mixture of 1,2-dichloroethane–methanol and applied to the Sephadex LH-20 column.

For characterisation by gas–liquid chromatography (GLC) and mass spectrometry (MS), the N-perfluoroacyl derivatives prepared as described above were trimethylsilylated. The sample was dissolved in 10 μl of dry pyridine and 90 μl of N,O-bis-(trimethylsilyl)acetamide[15]. After 1 h at 40° the reagent was removed by a stream of nitrogen. The sample was redissolved in hexane or ethyl acetate before analysis by GLC–MS.

Preparation of the column

Sephadex LH-20 columns were prepared in solvent mixtures of 1,2-dichloroethane and methanol (9:1, 8:2 and 7:3, v/v) as described previously[16]. For a general discussion on the practical and theoretical aspects of liquid–gel chromatography see SJÖVALL *et al.*[14].

Sephadex LH-20 was equilibrated with an excess of the solvent mixture for 2 h, the slurry poured into the glass columns (750 × 10 mm) and allowed to settle under free flow. The bed volume was about 45 ml, the flow rate was about 0.15 ml/cm^{2}/min. Fractions of 0.8–1.0 ml were collected. A disc of porous Teflon (LKB-Produkter, Bromma, Sweden, Cat. No. 4290-04) was placed on top of the bed to protect the gel surface.

The sample was applied in 0.3–0.5 ml of solvent. After application the top of the column was connected to a reservoir which was filled with about 300 ml of the eluting solvents.

The amine derivatives were detected in the effluent by measuring the absorption at 280 nm in a spectrophotometer (Zeiss Model PMQ II). Radioactivity was

measured in a gas flow counter (Frieseke Hoepfner Model FH 51) operated in the proportional range or by liquid scintillation counting in a Packard Model 3375 or 2002 scintillation spectrometer.

The void volume (V_0) was determined to be about 33% of the bed volume by application of 10 mg of polyvinylpyrrolidone and gravimetric determination of its appearance in the eluting medium. β-Carotene (0.2–0.3 mg) was used as an internal standard. This compound which is easily observed by its orange colour appears at $1.3 \times V_0$ in the solvent system 1,2-dichloroethane–methanol (7:3).

Determination of recovery

The N-trifluoroacetamide of ^{3}H-labelled dopamine, 3-methoxytyramine, and the 7-O-methyl-N-trifluoroacetamide of ^{3}H-noradrenaline, normetanephrine, were prepared to determine the recoveries on Sephadex LH-20 columns (4 × 400 mm). Each compound was put on five replicate columns. The radioactivity was followed in the eluate. The fractions composing the radioactive peak were pooled and evaporated to dryness under nitrogen and counted in a liquid scintillation spectrometer.

The N-trifluoroacetamide of dopamine was also applied on the same type of column in 1 mg and 1 μg amounts. In these experiments the UV absorption at 280 nm and fluorescence at 340 (activation 270) were measured in the eluate.

Preparation of brain extract

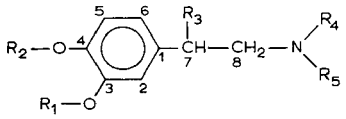
A rabbit was stunned by a blow on the head and bled to death. The brain was rapidly taken out and chilled in Krebs–Henseleit solution containing glucose 0.2%, ascorbic acid 0.02% and disodium EDTA 0.015% (ref. 17). The brain was chopped with scissors into small fragments weighing 10–20 mg each. 2 g (wet weight) of this material were incubated at 37° with 5 ml of Krebs–Henseleit solution and 2 μCi of ^{3}H-dopamine. After 60 min, 2 ml of 2 *M* HCl was added together with carrier amines (0.5 mg) and the sample was homogenised using an Ultraturrax model 45/2 (Jancke & Kunkel AG, G.F.R.) homogeniser. The sample was centrifuged and the supernatant was adjusted to pH 4 and applied to a Dowex 50-X4 column (4 × 60 mm). The effluent and two-ml washes were used for analysis of acids and neutral metabolites. The amines were eluted with 1 *M* HCl in methanol, and the solution was evaporated to dryness.

RESULTS AND DISCUSSION

Preparation of derivatives of amines for liquid–gel chromatography

The structures of the compounds studied are given in Fig. 1. Free amines are known to give tailing in liquid–liquid and gas–liquid chromatography systems due to hydrogen bonding effects. To protect the amino group, while keeping the hydroxyl groups free to interact during the separation, we used the reactions described by GREER *et al.*[18]. These reactions are outlined in Fig. 2 using normetanephrine as an example.

In this procedure the compounds are first fully perfluoroacylated. The trifluoroacetyl, pentafluoropropionyl and heptafluorobutyryl derivatives of dopamine, noradrenaline, adrenaline, metanephrine, normetanephrine and 3-methoxytyramine



R_1 = H, CH_3
R_2 = H, CF_3CO, C_2F_5CO, C_3F_7CO, $(CH_3)_3Si$
R_3 = H, OH, CH_3O, CF_3COO, C_2F_5COO, C_3F_7COO
R_4 = H, CH_3
R_5 = H, CF_3CO, C_2F_5CO, C_3F_7CO

Fig. 1. The structure without the R_1–R_5 substituents is represented as I. The amines used in this paper are metanephrine (I, $R_1 = R_4 = CH_3$; $R_2 = R_5 = H$, $R_3 = OH$), normetanephrine (I, $R_1 = CH_3$; $R_2 = R_4 = R_5 = H$; $R_3 = OH$), 3-methoxytyramine (I, $R_1 = CH_3$; $R_2 = R_3 = R_4 = R_5 = H$), adrenaline (I, $R_1 = R_2 = R_5 = H$; $R_3 = OH$; $R_4 = CH_3$), noradrenaline (I, $R_1 = R_2 = R_4 = R_5 = H$; $R_3 = OH$) and dopamine ($R_1 = R_2 = R_3 = R_4 = R_5 = H$).

gave single peaks on GLC representing the fully acylated derivatives[19,20]. The first step in the reaction sequence outlined in Fig. 2. was fast with all three perfluoro acid anhydrides.

The second step involving methanolysis proceeded more slowly. This was more noticeable for the heptafluorobutyryl derivatives than for the trifluoroacetyl derivatives. Dopamine N-heptafluorobutyramide (I $R_1 = R_2 = R_3 = R_4 = H$, $R_5 = C_3F_7CO$) and 3-methoxytyramine N-pentafluoropropionamide (I $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, $R_5 = C_2F_5CO$) were formed slower than the corresponding derivatives of the β-hydroxylated amines.

In methanol solution the phenolic ester group is preferentially methanolysed whereas the amide group is left intact. Compounds with a hydroxyl group α to the ring (*e.g.* noradrenaline and adrenaline) undergo a nucleophilic substitution reaction to give the methyl ether.

The nucleophilic displacement reaction could be of first (S_N1) or second (S_N2) order. Starting with an optically active compound the S_N1 mechanism gives a racemate, while the S_N2 reaction would lead to inversion of the configuration at the

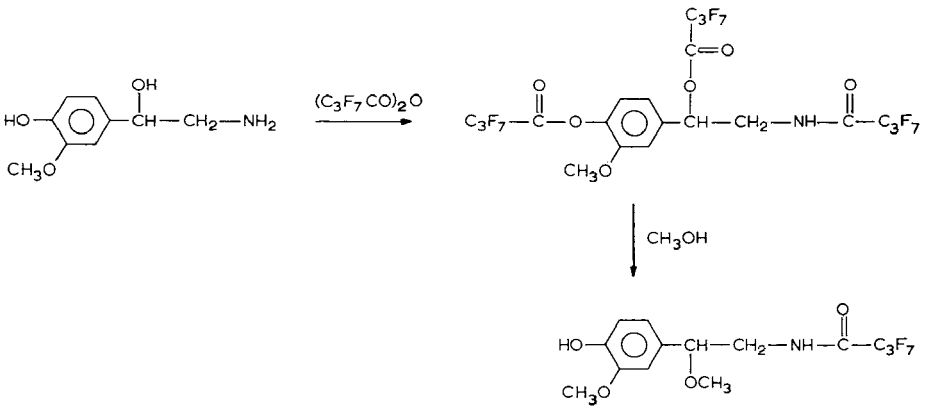
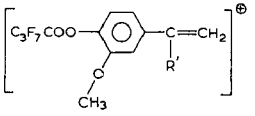
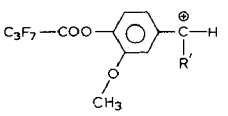
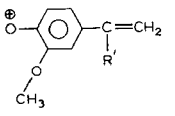
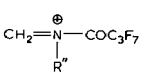
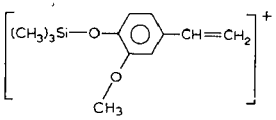
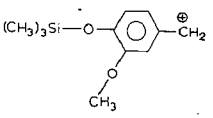
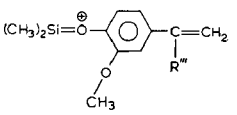
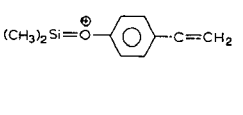


Fig. 2. Normetanephrine is O,O,N-acylated with heptafluorobutyric anhydride (a fast reaction). This derivative dissolved in methanol forms slowly (overnight) a compound with the structure I, $R_1 = CH_3$; $R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = C_3F_7CO$.

TABLE I

MASS SPECTROMETRIC DATA FOR DERIVATIVES OF 3-METHOXYTYRAMINE (3-MT), NORMETANEPHRINE (NM) AND METANEPHRINE (M)
M+ = molecular ion.

4-O,N-Diheptafluorobutyryl derivatives	*M+*				
3-MT; R′ = H, R″ = H	559 (12%)	346 (100%)	333 (12%)	149 (29%)	226 (3%)
NM; R′ = C_3F_7COO, R″ = H	771 (7%)	558 (100%)	545 (3%)	361 (14%)	226 (11%)
M; R′ = C_3F_7COO, R″ = CH_3	785 (1%)	558 (25%)	545 (3%)	361 (2%)	240 (100%)

7-O-Methyl-4-O,N-diheptafluorobutyryl derivatives	$M^{\oplus}$	$M^{\oplus}-15$				
3-MT	435 (45%)	420 (17%)	222 (50%)	209 (100%)	207 (13%)	
NM; R‴ = OCH_3	465 (14%)	450 (18%)			237 (100%)	206 (53%)
M; R‴ = OCH_3	479 (12%)	464 (50%)			237 (100%)	206 (100%)

asymmetric centre, giving an optically active product. To investigate this mechanism L-noradrenaline was heptafluorobutyrylated and then methanolysed. The product was found to have opposite sign of the optical rotation as compared to the parent compound. Thus the reaction seems to be of the S_N2 type.

The UV spectrum of the O-methyl-N-heptafluorobutyryl derivative of normetanephrine showed a $\lambda_{max.}$ at 278 nm in methanol. In 0.01 *M* NaOH in methanol the absorption maximum underwent a bathochromic shift to a $\lambda_{max.}$ of 288 nm, indicating the presence of a phenolic hydroxyl group.

The structures of the derivatives were confirmed by their mass spectra. Table I presents the *m/e* values and probable structures of the main fragments of O,N-heptafluorobutyryl and 7-O-methyl-O,N-heptafluorobutyryl derivatives of 3-methoxytyramine, normetanephrine and metanephrine. The fragmentation α to the nitrogen atom is more frequent than the β cleavage. In the trimethylsilyl derivatives the molecular ion with a loss of a methyl group (M^+-15) is also prominent.

Separation of amine derivatives on Sephadex LH-20

The 7-O-methyl-N-trifluoroacetyl and 7-O-methyl-N-heptafluorobutyryl derivatives of metanephrine, normetanephrine and noradrenaline were prepared. The N-trifluoroacetyl and the N-heptafluorobytyramides of dopamine and 3-methoxytyramine were produced. The 7-O-methyl-N-trifluoroacetyl derivative of adrenaline was readily prepared while the preparation of the 7-O-methyl-N-heptafluorobutyryl derivative unexpectedly was not successful. The compounds were chromatographed on Sephadex LH-20 in solvent systems of 1,2-dichloroethane–methanol (8:1 or 7:3). The elution volumes relative to β-carotene are given in Table II.

The above 7-O-methyl-N-trifluoroacetyl derivatives of metanephrine, normetanephrine and 3-methoxytyramine separated well in systems of 1,2-dichloroethane–methanol (9:1 and 8:2). This is illustrated in Fig. 3. The 7-O-methyl-N-trifluoroacetyl derivatives of the corresponding catecholamines (adrenaline, noradrenaline, dopamine) required the more polar solvent mixture (7:3) for elution within a reasonable volume.

TABLE II

SEPARATION OF CATECHOLAMINE DERIVATIVES ON A SEPHADEX LH-20 COLUMN

Parent compound	*Structure (I)*[a]					*$ClCH_2$-CH_2Cl–CH_3OH (8:2)*	*$ClCH_2$-CH_2Cl–CH_3OH (7:3)*
	R_1	R_2	R_3	R_4	R_5		
Metanephrine	CH_3	H	CH_3O	CH_3	CF_3CO	1.55	1.44
Normetanephrine	CH_3	H	CH_3O	H	CF_3CO	1.90	1.74
3-Methoxytyramine	CH_3	H	H	H	CF_3CO	2.20	1.93
Adrenaline	H	H	CH_3O	CH_3	CF_3CO	—	1.92
Noradrenaline	H	H	CH_3O	H	CF_3CO	—	2.35
Dopamine	H	H	H	H	CF_3CO	—	2.65
Metanephrine	CH_3	H	CH_3O	CH_3	C_3F_7CO	1.53	1.40
Normetanephrine	CH_3	H	CH_3O	H	C_3F_7CO	1.77	1.65
3-Methoxytyramine	CH_3	H	H	H	C_3F_7CO	2.10	1.88
Adrenaline	H	H	CH_3O	CH_3	C_3F_7CO	—	—
Noradrenaline	H	H	CH_3O	H	C_3F_7CO	2.85	2.18
Dopamine	H	H	CH_3	H	C_3F_7CO	3.40	2.61

[a] See Fig. 1.

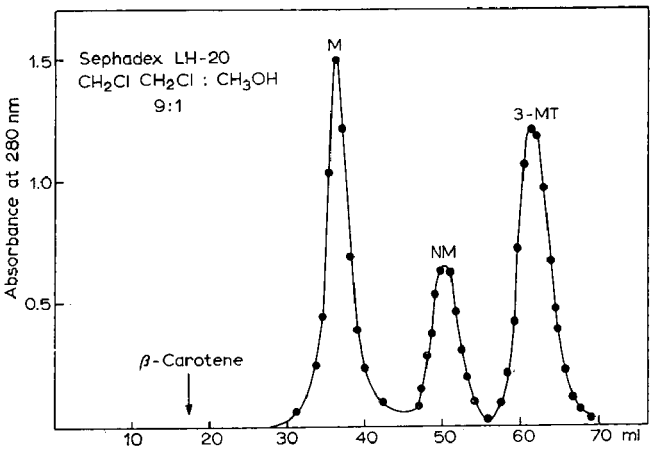


Fig. 3. Separation of 7-O-methyl-N-trifluoroacetyl derivative of metanephrine (= M), normetanephrine (= NM) and 3-methoxytyramine (= 3MT).

In this solvent system the catecholamines were separated from each other (Fig. 4) and also from the corresponding O-methylated metabolites, except for the 3-methoxytyramine and adrenaline derivatives which were not resolved under these conditions.

The separation between the N-trifluoroacetyl derivatives of the amines was better than that of the corresponding N-heptafluorobutyryl compounds (Table II). The peak shapes for the metanephrine, normetanephrine and 3-methoxytyramine derivatives were symmetrical, indicating that adsorption plays a minor role in the

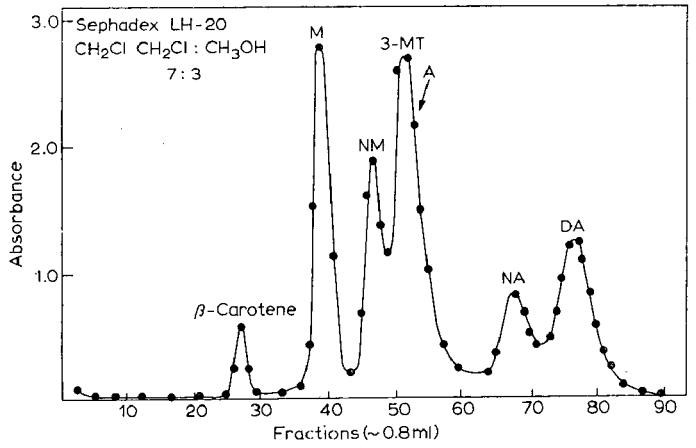


Fig. 4. Separation of metanephrine-O-methyl-N-trifluoroacetamide (M) (I, $R_1 = R_4 = CH_3$; $R_2 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), normetanephrine 7-O-methyl-N-trifluoroacetamide (NM) (I, $R_1 = CH_3$; $R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), 3 MT = 3-methoxytyramine-N-trifluoroacetamide (3-MT) (I, $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$; $R_5 = CF_3CO$) not resolved from A = adrenaline 7-O-methyl-N-trifluoroacetamide (I, $R_1 = R_2$ H; $R_3 = CH_3O$; $R_4 = CH_3$; $R_5 = CF_3CO$), NA = noradrenaline 7-O-methyl-N-trifluoroacetamide (NA) (I, $R_1 = R_2 = R_4 = H$; $R_3 = CH_3O$; $R_5 = CF_3CO$), DA = dopamine N-trifluoroacetamide (DA) (I, $R_1 = R_2 = R_3 = R_4 = H$; $R_5 = CF_3CO$).

separation. The height equivalent to a theoretical plate (HETP) in 1,2-dichloroethane–methanol (7:3) was for the 7-O-methyl-N-trifluoroacetyl derivative of metanephrine (I $R_1 = R_4 = CH_3$, $R_2 = H$, $R_3 = CH_3O$, $R_5 = CF_3CO$) 0.72 mm, for normetanephrine (I $R_1 = CH_3$, $R_2 = R_4 = H$, $R_3 = CH_3O$, $R_5 = CF_3CO$) 0.79 mm and for the N-trifluoroacetyl derivative of 3-methoxytyramine (I $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, $R_5 = CF_3CO$) 0.61 mm. The derivatives of the catecholamines adrenaline, noradrenaline and dopamine gave peaks which were broader and sometimes showed a tendency to tailing (Fig. 4).

Recovery

The recoveries in the submicrogram range of the derivatives of ^3H-labelled 3-methoxytyramine, noradrenaline and normetanephrine were 94.0 ± 15% (SD), 89.8 ± 2.4% (SD) and 105.5 ± 6% (SD), respectively.

The recovery of 1 mg of dopamine was 92.0 ± 5.6% (SD) (four columns). In the microgram range the recovery of dopamine was of the same order, while in the submicrogram range the yield was lower and more variable.

Application in studies on catecholamine metabolism

Chopped rabbit brain was incubated with ^3H-dopamine as described under EXPERIMENTAL. Carrier normetanephrine, 3-methoxytyramine, dopamine and

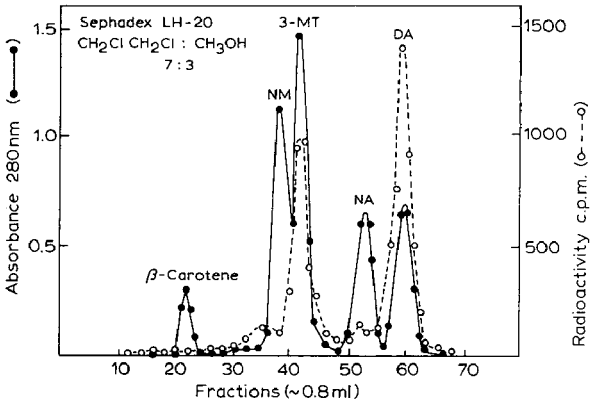


Fig. 5. Separation of brain basic metabolites of [^3H]dopamine after incubation with chopped rabbit brain. The solid line represents absorption at 280 nm due to 7-O-methyl-trifluoroacetyl amides of carrier metabolites and the broken line represents the corresponding radioactive derivatives.

noradrenaline were added and the basic metabolites of dopamine isolated. After preparation of trifluoroacetyl derivatives and methanolysis, the material was subjected to chromatography on Sephadex LH-20 in 1,2-dichloroethane–methanol (7:3). The chromatography is shown in Fig. 5. Two major radioactive peaks appear corresponding to the retention volume of the 3-methoxytyramine and dopamine derivative, respectively. A smaller peak at the position of the noradrenaline derivative was also observed.

Use of derivatives in gas chromatography with electron capture detection

GREER *et al.*[18] used trifluoroacetic anhydride to acylate metanephrine, normetanephrine and 3-methoxytyramine. According to HORNING *et al.*[21], the trimethylsilyl ethers of the N-heptafluorobutyryl derivatives of catecholamines were proposed to have high electron capture responses.

In our experience[22] and according to CLARKE *et al.*[19] the heptafluorobutyryl amides of phenylalkyl amines give considerably lower EC-responses than the corresponding esters. To investigate this matter more closely we prepared the O-trimethylsilyl-N-heptafluorobutyryl and O,N-heptafluorobutyryl derivatives of 7-O-methylmetanephrine and 3-methoxytyramine and compared the EC-responses of these derivatives. The results are shown in Table III. It appeared that the O-trimethylsilyl-N-heptafluorobutyryl derivatives gave 5% or less of the EC-response of the O,N-heptafluorobutyryl compounds. It seems therefore that O-trimethylsilyl-N-heptafluorobutyryl derivatives have only limited use in conjunction with EC-detection. Our results therefore do not support the proposal made by HORNING *et al.*[21] that these derivatives would be suitable for use with EC-detectors. The pentafluoropropionyl or heptafluorobutyryl esters seem to be the best derivatives so far [22].

TABLE III

ELECTRON CAPTURE RESPONSES FOR N-HEPTAFLUOROBUTYRYL AND 7-O,N-DIHEPTAFLUOROBUTYRYL DERIVATIVES OF O-METHYLATED CATECHOLAMINES

Parent compound	*Derivative (I)*[a]					*Electron capture response*[b] *(dieldrin = 100)*
	R_1	R_2	R_3	R_4	R_5	
Metanephrine	CH_3	$(CH_3)_3Si$	CH_3O	CH_3	C_3F_7CO	2.9
Metanephrine	CH_3	C_3F_7CO	CH_3O	CH_3	C_3F_7CO	55
3-Methoxytyramine	CH_3	$(CH_3)_3Si$	H	H	C_3F_7CO	0.7
3-Methoxytyramine	CH_3	C_3F_7CO	H	H	C_3F_7CO	65

[a] See Fig. 1.
[b] Area measurement by triangulation.

The mechanisms of separation in liquid–gel chromatography on lipophilic Sephadex have been discussed in detail by SJÖVALL *et al.*[14]. Depending on the solvent system and the dextran derivative used, "straight-phase" or "reversed-phase" partition effects are achieved in addition to the molecular-sieve mechanism.

Both straight-phase liquid–gel partition and gel filtration mechanisms seem to operate in the separations described here. The compounds with the relatively polar catechol structure were as a group retained as compared to their O-methylated analogues. Within each of these groups there are differences in polarity. The tertiary amides are less polar than the secondary. There are also small differences in molecular size: adrenaline > noradrenaline > dopamine and metanephrine > normetanephrine > 3-methoxytyramine. Both these factors seem to be important in the separation of these closely related compounds. Accordingly, the order of elution is adrenaline, noradrenaline and dopamine derivatives in the catecholic group and metanephrine, normetanephrine and 3-methoxytyramine derivatives among the 3-O-methylated compounds (Table II, Fig. 3). A gel filtration effect is also supported by the finding

that the N-heptafluorobutyryl derivatives were much less well separated from each other as compared to the N-trifluoroacetyl compounds (Table II).

The chromatography system was developed with the aim to produce an efficient and convenient purification step prior to quantitative analysis by gas chromatography. Since the N-heptafluorobutyryl derivatives seem to have no advantages in terms of high EC-response, it would be preferable to use the N-trifluoroacetyl derivatives as these compounds separate better on the Sephadex LH-20 column. To obtain a derivative suitable for analysis with EC-detector, the free phenolic group may be heptafluorobutyrylated (Table III).

In conclusion a simple two-step reaction sequence is used to prepare derivatives of the catecholamines and their O-methylated metabolites for column chromatography on Sephadex LH-20. The separation of these closely related compounds was achieved, probably by a combination of a liquid–gel partition and a molecular-sieve mechanism in an organic solvent mixture.

These chromatography systems incorporate many attractive features such as high efficiency, speed and high recovery[14] in the submicrogram range. The columns can be used repeatedly without repacking. The separated compounds appear in a small volume of volatile organic solvent that is easily removed. This and the fact that the amine group already is protected by an acyl group should make the procedure suitable for use in connection with GLC and MS.

ACKNOWLEDGEMENTS

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CHROM 4890

THE SEPARATION OF THE ULTRAVIOLET-ABSORBING CONSTITUENTS OF URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-resolution liquid chromatograph has been used to separate more than ninety ultraviolet-absorbing constituents from human urine. A 200 μl urine sample was chromatographed on a 0.24 × 100 cm column packed with 12–15 μ anion exchange resin. Over ninety ultraviolet-absorbing constituents were separated in less than 20 h by eluting with a linear acetate gradient at a flow rate of 8 ml/h and a pressure of 1000–1600 p.s.i. The column temperature was maintained at 21° for the first 4 h and then increased to 60° for the final 16 h of the run. The column effluent was monitored with an ultraviolet photometer that permitted absorbance to be recorded linearly at scales ranging from 0.02 to 0.64 absorbance unit full scale. A relative standard deviation of 1–4% was observed between the elution times of similar peaks from three identical chromatographic runs. The advantages of pre-fractionating urine by DEAE chromatography before anion-exchange chromatography was demonstrated.

INTRODUCTION

Body fluids such as urine contain many compounds that have been shown to have a correlation with various pathological states[1]. Recently, PAULING[2] has indicated that the concentration of even normal constituents is important in the maintenance of proper mental health. It is obvious that the qualitative and quantitative knowledge of the normal or pathologic constituents in body fluids would be beneficial in the evaluation of body function. Unfortunately, these constituents are contained in a complex biological mixture and often must be separated before qualitative and quantitative analysis may be performed. In several instances a specific colorimetric or enzymatic method is used for the analysis of a single constituent, but these methods invariably suffer from interference from other constituents contained in the body fluid[3].

SCOTT and co-workers at the Oak Ridge National Laboratory have developed a system, designated the "UV Analyzer", which separates the UV-absorbing constituents of body fluids by high-resolution anion-exchange chromatography[4–8].

With this system they have demonstrated the separation of more than one hundred UV-absorbing constituents from human urine in less than 40 h. Several of these separated constituents have been qualitatively identified[6,9-10] but this has proved difficult due to the small sample quantities involved and their resultant dilution by the eluting solvent.

A different approach is that of using the chromatogram, obtained with the "UV Analyzer", as a profile or fingerprint in evaluating body function. Reference profiles of "normal" subjects are then obtained and compared with profiles from patients having various metabolic or pathologic abnormalities. Using this approach, emphasis must be placed on obtaining accurate and precise reference profiles. We report here, using the chromatographic procedure developed by SCOTT *et al.*[4], that reproducible, urinary profiles may be accurately and precisely obtained with a commercially available liquid chromatograph.

EXPERIMENTAL

Materials

Chemicals. The 0.015 *M*, pH 4.40 and 6.0 *M*, pH 4.40 sodium acetate buffers were prepared from analytical grade sodium hydroxide and acetic acid. The reference compounds, known to be in urine, were purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Resins. The anion-exchange resin Aminex BRX having a nominal 8% divinylbenzene cross-linkage and a particle size distribution of 12–15 μ was a gift from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin, as received from the manufacturer, was swollen for 24 h in 0.015 *M* sodium acetate, pH 4.40 before column packing. The DEAE-Sephadex was purchased from Pharmacia, Piscataway, N.J., U.S.A.

Column preparation. A 0.24 × 100 cm stainless-steel column was dynamically packed with the Aminex BRX anion resin as described by SCOTT AND LEE[11]. An extension (0.54 × 25 cm) was added to the column and both were filled with a 50:50 slurry of the resin and 0.015 *M* sodium acetate, pH 4.40. The column was packed at 1000 p.s.i. using a Milton Roy Minipump and equilibrated for several hours with the acetate buffer. The extension was then removed and the column placed in the chromatographic system.

Chromatographic system. The system used was the Varian Aerograph Model LCS-1010 Liquid Chromatograph, a schematic of which is shown in Fig. 1. The system is capable of gradient elution at pressures up to 3000 p.s.i. The gradient is formed by pumping (Pump 1) a solution of concentrated eluent from Buffer Reservoir 1 into the gradient chamber previously filled with a predetermined volume of dilute eluent from Buffer Reservoir 2. The resultant gradient is pumped through the column by means of a Milton Roy Milroyal D (Pump 2), which is capable of leakfree operations up to 3000 p.s.i. A linear gradient is produced by maintaining the flow rate through the column at twice the rate of flow of the concentrated eluent into the gradient chamber[12]. The initial volume of dilute eluent in the gradient chamber determines the slope of the linear gradient. Both convex and concave gradients in solvent concentration with time can be generated by adjusting the relative flow rates into and out of the gradient mixing chamber. The system has a timing delay which

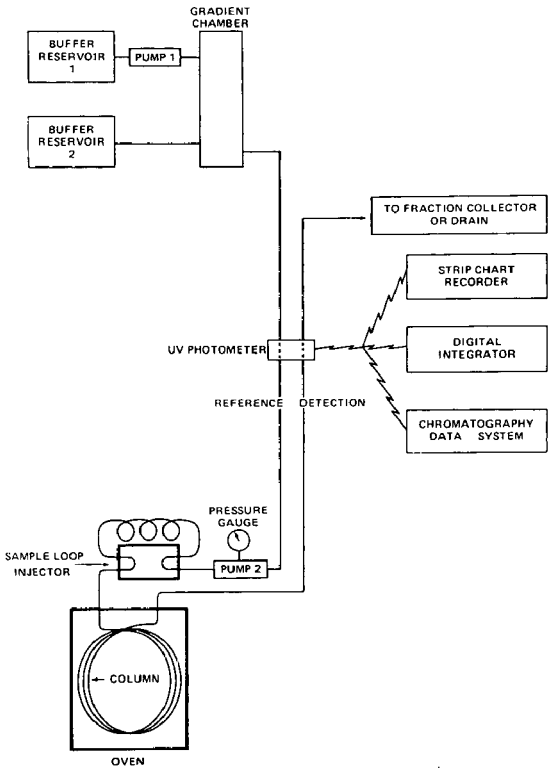

Fig. 1. Schematic of the Varian Aerograph Model LCS-1010 Urine Analyzer.

allows a timed elution with the dilute buffer (*i.e.*, up to 30 min) before the start of the gradient elution. The sample is introduced into the column through a sample loop injector at the top of the instrument. The column is housed in an air oven which is capable of maintaining the column temperature to within $\pm 1°$ of the selected setting. The column effluent is monitored by a sensitive UV photometer, operating at 254 nm, which is equipped with a cylindrical flow cell having a 1 mm diameter and 10 mm path length (*i.e.*, 8 μl cell volume). The detector output is linear in absorbance units and is linear with respect to solute concentration in accordance with Beer's Law. Full scale absorbance ranges from 0.02 up to 0.64 are provided in binary steps. In addition, a nonlinear high absorbance range is provided for qualitative monitoring of highly absorbing samples. The photometer output is displayed on a 10 mV strip chart recorder or fed into a digital integrator or a chromatography data handling system for data acquisition and processing. To minimize baseline drift, due to the UV absorbance of the concentrated acetate buffer, the gradient stream flows through the reference cell in the UV photometer before it enters the 3000 p.s.i. pump.

The column effluent, after passing through the sample cell of the detector, is routed either to a drain or to a fraction collector.

Sample preparation. Urine samples were collected from healthy male subjects and were refrigerated between the time of collection and the time of analysis. A

urine sample, that had been fractionated into an acidic fraction and into a combined basic and neutral fraction by DEAE chromatography, was provided by Dr. LINUS PAULING and Dr. ART ROBINSON of Stanford University.

Anion-exchange chromatography. To introduce a urine sample onto the column, the eluent flow was stopped, the pressure reduced, and the 200 μl sample loop filled with urine. The urine sample was then eluted with a linear acetate gradient at a flow rate of 8 ml/h. The column pressure required to maintain this flow rate varied from 1000 to 1600 p.s.i. depending on the column temperature and the viscosity of the acetate gradient. The linear acetate gradient was formed by placing 90 ml of 0.015 *M* sodium acetate, pH 4.40 in the gradient chamber and pumping into it 6.0 *M* sodium acetate, pH 4.40 at a flow rate of 4 ml/h. As mentioned earlier, the resulting gradient is delivered to the column at a flow rate of 8 ml/h. The optimal column temperature, as determined by SCOTT *et al.*[4] was maintained at 21° for the first 4 h and increased to 60° for the final 16 h of a chromatographic run. Between runs the column was equilibrated with the 0.015 *M* sodium acetate for 4 h. Thus, allowing 20 h for the chromatography and 4 h for equilibration, 1 sample may be chromatographed per 24-h day.

DEAE chromatography. A urine sample was fractionated into an acidic fraction and into a combined basic and neutral fraction by modifying the chromatographic procedure of HORNING[13]. DEAE-Sephadex (diethylaminoethyl-Sephadex), a weakly basic anion-exchange support, was suspended in distilled water, washed with 0.5 *N* sodium hydroxide and rewashed with distilled water to pH 7.0. The water was decanted, the resin resuspended in 0.01 *M* pyridine acetate and the pH adjusted to 5.0 with glacial acetic acid. A 2 × 10 cm glass column was then filled with a slurry of the resin and the resultant column washed with 50 ml of distilled water. A 10 ml urine sample was allowed to percolate into the column and the basic and neutral urinary fraction eluted with 100 ml of distilled water. The acidic fraction was then eluted with 150 ml of 1.5 *M* pyridine acetate. The 100 ml water fraction containing the basic and neutral constituents and the 150 ml pyridine acetate fraction containing the acidic constituents were both lyophilized to dryness. The dry samples were then reconstitued to 10 ml with distilled water and 200 μl aliquots of each and a 200 μl aliquot of the unfractionated urine chromatographed by anion-exchange chromatography.

RESULTS

A typical urine chromatogram of a 100 kg male subject is seen in Fig. 2. The column resolved 99 UV-absorbing peaks from this particular urine. By increasing the column length from 100 to 200 cm we have been able to increase the number of resolved peaks to more than 110, but at the expense of increasing the analysis time to 45 h. Therefore, in the subsequent runs we have utilized the 100 cm column and an analysis time of 20 h and have found these conditions to be the best compromise between resolution and length of analysis time. In addition, the profile of the acetate gradient employed in this (Fig. 2) and subsequent separations is indicated.

Although identification of every individual peak is difficult, a few peaks may be identified by comparing elution times with reference compounds. For example, in Fig. 2, the identity of the uric and hippuric acid peaks eluting at 5.3 and 13.4 h,

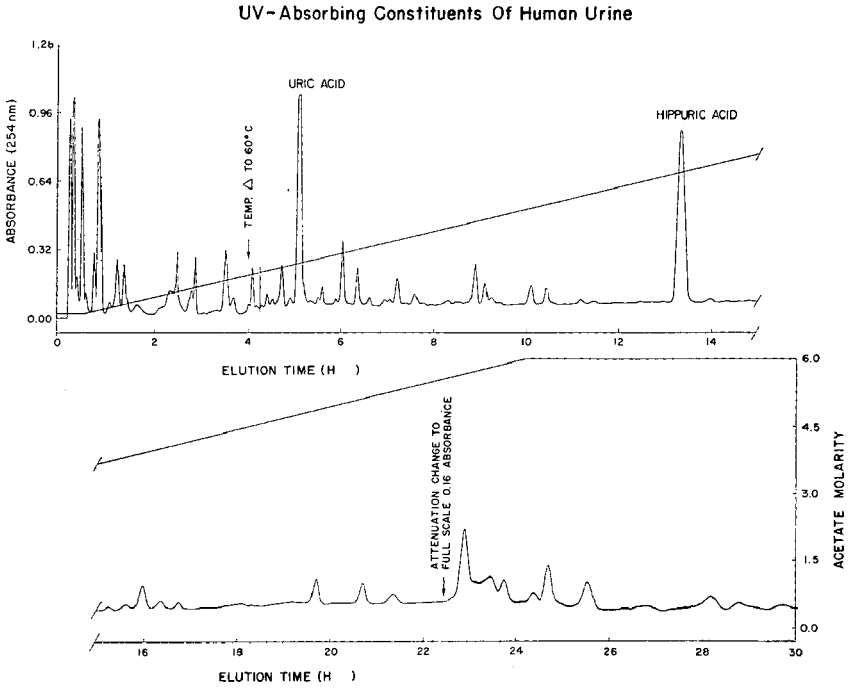

Fig. 2. Separation of the UV-absorbing constituents of human urine by anion-exchange chromatography. Conditions: columns, 0.24 × 100 cm; resin, Aminex BRX (12–15 μ) strongly basic anion exchanger; eluent, linear sodium acetate gradient varying in concentration from 0.015 M, pH 4.4 to 6.0 M, pH 4.4; flow rate, 8 ml/h; pressure, 1000–1600 p.s.i.; column temperature, ambient to 4 h, 60° for remainder of run; sample, 200 μl human urine.

respectively, was determined by comparing their elution times with those of reference uric and hippuric acid. Since this type of identification, and problems associated with it, have been discussed previously[6,9] the following chromatograms will be discussed on the basis of profiles instead of individual peak identities.

TABLE I

PRECISION OF THE ANION EXCHANGE CHROMATOGRAPHIC METHOD USED TO SEPARATE THE UV-ABSORBING CONSTITUENTS OF URINE

Numbers represent the mean ± the relative standard deviation (column 3) for 3 determinations (Fig. 3).

Peak number	*Elution time (min)*	*Relative standard deviation (%)*
1	30.9	3.9
2	53.8	4.2
3	85.8	2.5
4	205.6	2.1
5 (Uric acid)	285.8	1.7
6	486.6	2.6
7 (Hippuric acid)	701.5	1.7
8	822.6	1.9
9	968.7	1.1
0	1003.6	2.5

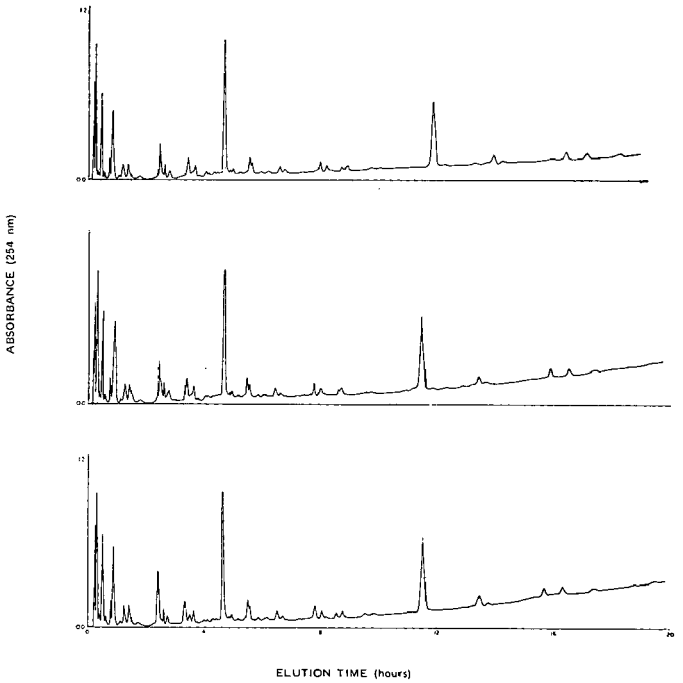

Fig. 3. Reproducibility of the separation of the UV-absorbing constituents of human urine by anion-exchange chromatography. Chromatograms resulted from three identical chromatographic analyses using the same conditions listed under Fig. 2. The 200 μl sample was the same urine in all three cases.

To demonstrate the reproducibility of the chromatographic procedure, three identical chromatographic analyses were performed using aliquots of the same urine sample. The three resultant chromatograms are seen in Fig. 3. The elution times of 10 reference peaks were determined and found to have a relative standard deviation of $\pm$1–4% (Table I). As expected, the greatest deviation between elution times was found in the early region of the chromatograms since separation here is dependent on non-ionic adsorption[9], which is strongly affected by many factors. Compounds that are more strongly retained by the resin (*i.e.*, compounds that elute in the 2–20 h region of the chromatogram) are not markedly influenced by non-ionic factors and therefore the elution times, of similar peaks in this region, have relative standard deviations of only $\pm$1–3%.

To determine the precision of the method on a long term basis, the elution times for uric and hippuric acid from 15 chromatographic runs were obtained and found to have a relative standard deviation of $\pm$2.8 and 3.0%, respectively. Thus, with a precision of $\pm$1–4%, it is obvious that the chromatographic procedure is reproducible, which is mandatory when one attempts to detect and correlate differences in urinary profiles.

It has been previously reported[6,9] that peaks representing a class of compounds will elute at certain times from an anion-exchange column. For example, basic and neutral compounds elute in the first few hours while the acidic compounds elute much later in a chromatographic run. To confirm this, urine was prefractionated

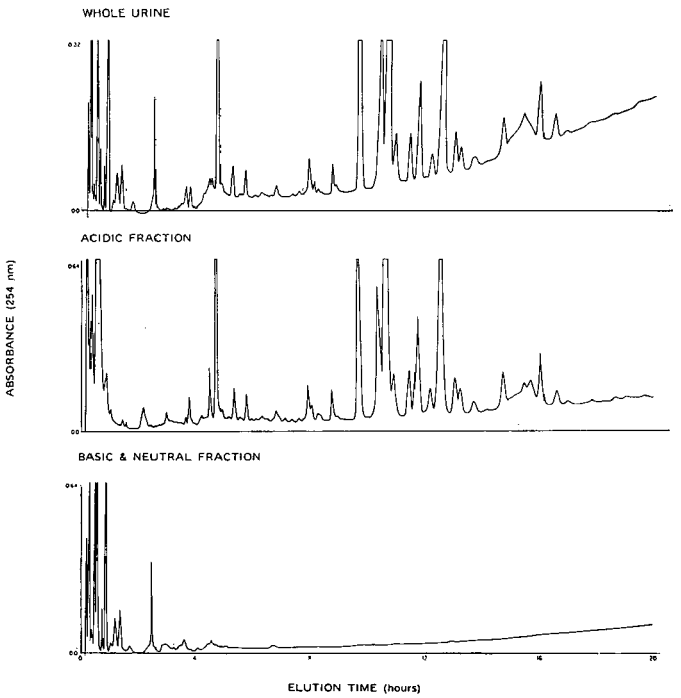

Fig. 4. The separation of the UV-absorbing constituents of DEAE-fractionated urine. The top chromatogram represents the profile of a whole urine sample. The same urine was fractionated into an acidic fraction and into a basic and neutral fraction by DEAE chromatography and then chromatographed by anion-exchange chromatography. The middle and bottom chromatograms represent the acidic fraction and the basic and neutral fraction, respectively. Chromatographic conditions are the same as listed in Fig. 2.

into an acidic fraction and into a combined basic and neutral fraction by DEAE chromatography. Aliquots of these fractions were then chromatographed by anion-exchange chromatography which resulted in the following chromatograms (Fig. 4). The first chromatogram was obtained from the chromatography of an aliquot of the whole urine. The second and third chromatogram represent the acidic and the combined basic and neutral fractions, respectively.

It is evident that the acidic, basic and neutral components of urine elute at discrete intervals from the anion-exchange column. For example, the 4–20 h region of the whole urine chromatogram correlates with the 4–20 h region of the acidic fraction chromatogram. This indicates that the acidic urinary components are eluting in this time interval, an observation which is consistent with previous studies[6,9]. On the other hand, the chromatogram of the basic and neutral fraction correlates with the 0–3 h region of the whole urine chromatogram indicating that the basic and neutral urinary constituents are eluting in this time interval. In addition, there appears to be a region of overlap at 2–4 h where either acidic, basic or neutral components may elute.

In the chromatogram of the acidic fraction, several compounds elute at 1–2 h from the anion-exchange column which do not appear in the chromatogram obtained

from the whole urine. It is probable that these peaks are due to the elution of the neutral or aglycon moieties of conjugated compounds (*i.e.*, glucuronides, sulfates, phosphates, etc.) that arise from the hydrolysis of the conjugates by the chromatographic conditions of the DEAE chromatography. The peaks representing the conjugated compounds could be ascertained by first hydrolyzing the urine, either enzymatically or chemically, prior to the anion-exchange chromatography. Thus, by comparing the resulting anion-exchange chromatogram with that obtained from unhydrolyzed urine, the peaks that correspond to conjugated compounds could be clearly determined. Previously, this approach has been utilized to isolate the aglycon moiety of the conjugated metabolites of phenacetin[9]. In addition, being able to correlate definite peaks with conjugated compounds would be useful since the degree of conjugation is often used as a basis for liver function tests[14,15].

DISCUSSION

The results presented here confirm the data of SCOTT and co-workers who have previously demonstrated the applications and advantages of separating the UV-absorbing constituents of urine by high pressure anion-exchange chromatography. In earlier reports SCOTT and his group have used this technique to evaluate urine from leukemic and schizophrenic patients[4], the diurnal pattern of a normal male human[5], a 2-year old girl who had a neuroblastoma[10] and other pathological states such as hereditary nephritis, Lesch-Nyhan syndrome, gout, alkaptonuria and maple syrup urine disease[16]. In addition, VAVICH AND HOWELL[17] and KELLEY AND WYNGAARDEN[18] have used an Oak Ridge Analyzer in the evaluation of urine for its UV-absorbing compounds from normal newborn and young children and patients with gout, respectively.

Thus, liquid chromatography has been demonstrated to be a particularly useful technique for the separation of complex biological mixtures into their individual components. For example, it allows one to separate a highly complex mixture such as urine, since in liquid chromatography one can easily vary many chromatographic parameters. In addition, we have found the technique to be reproducible to within 1–4%. Assuming a molar absorptivity of 10,000, ng quantities of UV-absorbing urinary constituents can be detected and quantitatively determined. Previously, one was only able to obtain this degree of sensitivity with gas chromatography. However, the gas chromatographic analysis of urinary constituents usually requires first a derivatization procedure consisting of several reaction steps. Using liquid chromatography, a sample can be analyzed directly which eliminates the concern for quantitative derivatization or chemical degradation arising from the derivatization procedure.

To obtain the high resolution necessary in the separations presented here the use of a column filled with an efficient ion-exchange resin of small particle size is required[4]. This small resin, however, requires operation at high pressures to obtain usable flow rates. Thus, a system to perform these separations must be capable of (1) continuous operation at high pressures, (2) must be compatible with high-efficiency columns, and (3) must have a sensitive column monitor to quantitatively detect the minute quantities of solutes that are separated by these high resolution columns. We have found the LCS-1010 Urine Analyzer to meet all of the above

requirements. The pumping system of the LCS-1010 is capable of reliable operation at pressures up to 3000 p.s.i., and we have observed only a 1–4% deviation in the elution times of similar peaks, which indicates a stable and reproducible flow rate.

The UV photometer has been discussed earlier and it was shown to be capable of detecting ng quantities of UV-absorbing compounds[12,19]. An additional advantage, apart from its high sensitivity, is the fact that its output is linear in absorbance, thus linear in concentration. This allows for data acquisition by a digital integrator or computer data system in addition to the readout obtained from a strip chart recorder. In the future, this digital collection of data will prove useful when data from two urinary profiles are compared either manually or, preferably, with a computer.

In regards to the chemical stability of the anion-exchange resin used in this study, we have analyzed, on a single column, more than 60 urine samples over a 9-month period without loss of resolution. This agrees well with the results of SCOTT *et al.*[4] who have analyzed over 100 urine samples on a single column without encountering any evidence of chemical degradation of their resin.

In addition to the chemical stability of the resin, we have found the BRX anion-exchange resin to be mechanically stable when operated at high pressures. We attribute this to the fact that we have used resin of 8% cross-linkage* which contributes to the mechanical stability of the resin. Also, the BRX resin was a narrow cut of small particle size which, when "dynamically packed", has been shown by SCOTT AND LEE[11] to yield a uniformly packed column bed that is operable at high pressures.

In conclusion, we have reconfirmed, using a commercially available liquid chromatograph, that high-pressure anion-exchange chromatography is a useful technique for the separation of the UV-absorbing constituents of human urine. The resultant chromatograms or profiles are an indication of the metabolic state of a subject and should prove useful in the diagnosis of several abnormal metabolic or pathological conditions. However, to use such profiles for diagnostic purposes, dietary effects must be considered as YOUNG[20] has observed different urinary profiles from the same individual when different diets have been ingested. Consequently, in conjunction with Dr. L. PAULING and Dr. A. ROBINSON of Stanford University, we are presently analyzing urine samples obtained from subjects on a chemically defined diet. This should allow us to ascertain or minimize the effect of diet on urine composition which is necessary if urinary profiles are to be utilized for diagnostic purposes.

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* % cross-linking indicates the % of divinylbenzene that is incorporated into the polymer bead prior to attaching ionic groups.

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CHROM. 4886

ASSAY OF COCAINE IN THE PRESENCE OF PROCAINE AND QUININE BY COLUMN CHROMATOGRAPHY

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SUMMARY

A column chromatography method is presented for the separation and quantitation of cocaine in the presence of procaine, quinine and lactose. Separation procedures are described to afford purification of cocaine for infrared absorption spectrum examination.

INTRODUCTION

Illicit cocaine is examined in the forensic laboratories as a fine, white powder, invariably occurring as a hydrochloride salt; occasionally crude-appearing brown samples have been examined. Adulteration and dilution are effected by illicit dealers with procaine and lactose respectively; and less frequently, quinine is used as an adulterant. The dilution has been mostly in the range of 10 to 60%.

The rapid detection tests for cocaine are described in the U.S. Treasury Manual[1]. The preferred methods are in the use of cobalt thiocyanate test and in the formation of the characteristic microcrystals with platinic chloride. These tests afford a rapid and convenient procedure for the presumptive identification of cocaine.

Procaine is detected by the Sanchez color test[1] for cyclic aromatic amines resulting in a vivid red color. Quinine possesses an intense blue fluorescence under UV light, and is readily characterized by placing a sample preferably in an acid solution under fluorescent light.

Thin-layer method for chromatography is recommended if materials other than those mentioned, *i.e.*, procaine, quinine, and lactose are suspected. COMER AND COMER[2] have reviewed a number of papers describing the use of thin-layer chromatography (TLC) for the separation of different kinds of drugs. He has listed some sixteen different TLC systems for separating cocaine from a number of local anesthetics and

from a number of analgesics-antipyretics. In addition, this paper offers a simple TLC system using Eastman Chromatogram sheets for separating cocaine from procaine and quinine. We have not hitherto encountered adulterants other than procaine and quinine in the illicit samples.

YOUNG[3] reported a chemical method for the determination of cocaine in the presence of procaine in which cocaine is hydrolyzed to yield methanol as a product. Methanol is distilled and is measured colorimetrically, using the well-known permanganate oxidation procedure. The method proved time-consuming for forensic work and a number of chloroform shake-out methods[1] were adapted to provide a final residual product suitable for volumetric titration. This procedure proved to be not only laborious but was attended by losses in recovery simply because of its many manipulative steps.

The manual[1] also describes a determination of cocaine, procaine, and tetracaine, employing a direct reading of the three components in alkaline solution and calculating the amounts by simultaneous equation.

The method is beset by the errors introduced by overlapping curves and these errors have been compensated by the introduction of experimental factors. The errors introduced for cocaine determination become more pronounced when proportionally more adulterant is present in the sample than cocaine.

The present method employs the principle of ion pair formation and its extraction by partition chromatography. This principle is discussed by HIGUCHI *et al.*[4] and it has been adapted by LEVINE and co-workers[5–8] for the separation of a large number of pharmaceutical amines.

Among the common anions tested, including chloride, phosphate and sulfate, nitrate proved to be the most efficient for quantitative purposes. Potassium nitrate in HCl solution is used as a stationary phase and chloroform for elution. HCl acts to retain quinine and possibly other basic materials[6] which may be present in the cocaine sample. The eluant from the column is acidified and measured by UV absorption.

PROCEDURE

Mix 4 g of Celite 545 (Johns Mansville) with 2 ml of 1 *M* KNO_3 in 0.1 *N* HCl and transfer to a column, such as used by LEVINE[5]. Tamp the mixture on a pad of fine glass wool. Pipette 1 ml of aliquot of solution containing 50 mg of sample in 1 *M* KNO_3 in 0.1 *N* HCl onto the surface of the column. Irrigate the column with 45 ml of chloroform saturated with water (spectro-grade solvent preferred); collect the eluant in a 50-ml volumetric flask containing 5 ml of methanol and 5 drops of concentrated HCl. Bring the solution to 50 ml mark with chloroform and read its UV spectrum from 340 to 255 nm, max. at 275 nm, using chloroform as a reference.

Prepare a quantitative standard by dissolving 10 mg of cocaine·HCl in 50 ml chloroform containing 5 ml methanol and 5 drops HCl. Absorbance at 1%/1 cm was 32.5 using a Cary 15 spectrophotometer under these conditions. Calculate % cocaine, as hydrochloride, using the following equation:

$$\frac{A_{\text{sample}}}{A_{\text{standard}}} \times \frac{10}{50} \times 100 = \% \text{ cocaine} \cdot \text{HCl}$$

Clean-up for IR examination

Shake the chloroform eluant saved from the assay with 10 ml of water. Discard the chloroform phase. Adjust the pH of the aqueous extract with dilute NH_4OH just to alkalinity (observe white precipitate) and extract with 50 ml chloroform twice. Filter the chloroform extract into a beaker and then treat the filtrate with sodium sulfate to remove water. Filter and evaporate the extract to dryness. Press the resulting crystalline material into KBr for IR spectrum examination for cocaine base.

DISCUSSION

While most forensic laboratories do not quantitate fillers or adulterants present in illicit narcotic samples, procaine and quinine can be eluted from the column using triethylamine in chloroform according to a procedure by LEVINE[5]. 1 *N* HNO_3 or 1 *M* KNO_3 can be used in the column *per se* if procaine is the only adulterant in the illicit cocaine.

In the clean-up procedure, there is the hazard of hydrolyzing cocaine to benzylecgonine and methanol in the presence of water and heat[9]. Therefore, a desiccant such as sodium sulfate is used to dry the chloroform extract prior to evaporation over a hot steam bath.

For the clean-up, 10 mg of cocaine can be recovered by the method. If sufficient amount of material cannot be recovered, *ca.* 100 mg of sample can be passed through the column to recover enough material for IR examination.

On occasions, this laboratory has analyzed brown colored cocaine powder preparations containing procaine as an adulterant. The interference due to the presence of chloroform-soluble colored component was removed by washing the sample in the column, under standard conditions, with 50 ml of water-saturated 1,1-dichloroethane, which has been used in the clean-up of brown heroin[10]. The cocaine which was eluted subsequently with chloroform yielded a UV curve similar to those obtained with standard cocaine; recovery values of cocaine standards under standard conditions are shown in Table I.

TABLE I

RECOVERIES OF COCAINE STANDARDS

mg added	*mg recovered*	*% recovery*
15.00	14.89	99.2
12.00	11.90	99.2
10.00	9.95	99.5
8.00	7.91	99.1
5.00	4.92	98.9

HEAGY[11] reported that cocaine can be purified sufficiently for IR determination by its relative solubility in methylene chloride over procaine which is sparingly soluble in this solvent at room temperature. To a sample of illicit cocaine mixed with procaine, just enough methylene chloride is added to the mixture to dissolve cocaine. The extract is filtered immediately and evaporated to dryness. The residue is recrystallized with petroleum ether and pressed into KBr for IR spectrum reading.

TABLE II

TLC OF COCAINE, PROCAINE AND OTHER COMPOUNDS OF FORENSIC INTEREST

Solvent mixture: 40 ml chloroform, 10 ml ethyl acetate, and 10 drops conc. NH_4OH. Chromatography sheet: Eastman Chromatogram.

Compounds	*Typical* R_F
Cocaine	0.84
Procaine	0.45
Quinine	0.05
Heroin	0.34
Codeine	0.11
Morphine	0.00
LSD	0.34
Mescaline	0.05
STP	0.13
PCP	0.89
Nupercaine	0.45
Benzocaine	0.67
Tetracaine	0.11
dl-Amphetamine	0.00
DMT	0.10

In addition to those TLC solvent systems listed by COMERS AND COMERS[2] the authors suggest a very simple, rapid procedure using a solvent mixture containing 40 ml chloroform, 10 ml ethyl acetate and 10 drops of ammonium hydroxide. Eastman chromatosheets are cut to approximately $1\frac{1}{2}$ in. and 4 in. and irrigated in the solvent mixture in a small bottle accommodating this sized sheet. The spots are revealed by using iodoplatinate reagent. Table II shows the typical R_F values obtained for cocaine, procaine, quinine and other forensically important compounds.

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CHROM. 4903

CHARACTERIZATION OF PHOSPHONOMYCIN BY MICROCHROMATOGRAPHIC AND RELATED TECHNIQUES

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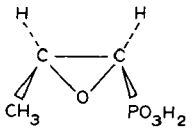
SUMMARY

Phosphonomycin has been characterized by R_F values and color reactions in a variety of chromatographic systems which include thin-layer and paper chromatography. Gas–liquid chromatography was carried out on the di-trimethylsilyl derivative. The gas–liquid chromatography peak was identified by mass spectrometry. Paper chromatography was adapted for purification of a crude concentrate and the purity monitored by gas–liquid chromatography.

INTRODUCTION

Phosphonomycin has been isolated from fermentation sources by ion-exchange and adsorption methods[1]. The antibiotic has been shown to have the following structure[2]:



This report describes microchromatographic and related techniques useful for characterization of the antibiotic. These procedures have been applied in monitoring the purity of concentrates and in establishing the identity of antibiotics present in fermentation broths or in samples of biological origin. Detection of small quantities on paper chromatography (PC) or thin-layer chromatography (TLC) is possible by bioautographic methods. In addition, chemical detection by reagents useful for phosphonates can be carried out using larger quantities of the compound. The procedure of BORST-PAUWELS[3] employing ΔR_m between two paper chromatographic systems was studied for the determination of the number of charged groups in the antibiotic using both crude and highly purified samples. Gas–liquid chromatography (GLC) and combined GLC–mass spectrometry (MS)[4,5] were employed to provide information concerning the composition and purity of samples of antibiotics.

MATERIALS

Reagent grade chemicals were used for the preparation of derivatives or detection reagents. Thin-layer plates were obtained from Anal. Tech. Whatman No. 1 paper was used for PC.

METHODS

PC and TLC

The antibiotic-containing samples were spotted as aqueous solutions containing 1–20 μg of phosphonomycin in up to 25 μl of solution. The paper strips were developed by descending chromatography until the solvent front traveled 20–25 cm. The procedure has been applied to fermentation broths, urine samples from laboratory animals and humans, and partially purified concentrates. For preparative PC crude phosphonomycin (30–50% pure) obtained after primary solvent partition purification[1] was streaked on paper sheets at a loading of about 1 mg/cm. The sheets were developed with system D. The zones located by bioautography were cut out and eluted with anhydrous methanol to obtain purified antibiotic.

Thin-layer plates were loaded with up to 150 μg of phosphonomycin in 2–5 μl and developed by the ascending technique. Mixed solvents were freshly prepared. Bioautographs were carried out by placing the dried plate on filter paper in contact with agar plates seeded with a sensitive organism.

The following solvent systems were used (volume ratios): System A, *n*-propanol–2 *N* methylamine (7:3); System B, *n*-propanol–2 *N* isopropylamine (7:3); System C, *n*-butanol–acetic acid–water (3:1:1); System D, *n*-butanol–acetic acid–water (4:1:1); System E, isopropanol–conc. ammonia–water (7:1:2); System F, methanol–water–triethylamine (80:20:5). The mobility of the antibiotic in the various systems is indicated in Table I.

TABLE I

R_F VALUES OF PHOSPHONOMYCIN IN VARIOUS SYSTEMS

Carrier	*System*					
	A	*B*	*C*	*D*	*E*	*F*
Paper	0.19	0.26	—	0.26	—	—
Cellulose thin layer	—	—	—	0.26	0.18	—
Silica Gel G thin layer	—	—	0.33	—	—	0.75

GLC and combined GLC–MS

The sample (preferably as the ammonium or benzylamine salt) was dissolved in bis-trimethylsilylacetamide (BSA, a powerful trimethylsilylating reagent[5,6]) and heated for 5 min at 60° or allowed to stand at room temperature for 30 min to form the trimethylsilyl (TMSi) derivative. Aliquots (1–2 μl of 0.5% solutions) of the reaction mixtures were used directly for analysis. Chromatography was carried out with a Barber-Colman Model 5000 instrument equipped with a 6 ft. × 4 mm glass U-tube; 4% F-60 (DC-560) coated over 1.5% SE-30 on 100–120 mesh acid-washed and silan-

ized[5] Gas-Chrom P; the temperature was programmed from 65° to 220° at 3°/min, and then held at the upper limit for 10 min; 15 p.s.i.; hydrogen flame ionization detection. Similar column conditions were employed with an LKB Model 9000 combined gas chromatograph–mass spectrometer. Mass spectra were obtained using 70 eV ionizing potential, 250° ion source temperature, 50 μA filament current, and 3.5 kV accelerating potential.

Detection of phosphonomycin

Bioautography

The antibiotic is readily detected at low levels (1–5 μg) by bioautographic techniques using agar seeded with a strain of *Proteus vulgaris* (MB-838). This procedure has been applied to both PC and TLC. Acidic systems must be well dried to eliminate acetic acid which affects the growth of the microorganism.

Phosphonic acid reagents

Molybdate reagent. Although phosphonomycin cannot be detected by molybdate reagents useful for phosphates, reagent containing 3% perchloric acid and 1% ammonium molybdate in 0.01 *N* hydrochloric acid readily reveals 5–30 μg of antibiotic as an intense blue zone after heating for 5 min at 85° (ref. 7).

Iron sequestering. In common with many phosphates, phosphonomycin sequesters iron. TLC or PC zones containing 20 μg or more of antibiotic can be readily detected using this property. The chromatogram is first sprayed with 0.1% ferric chloride ($FeCl_3 \cdot 6H_2O$) in 80% aqueous ethanol. After drying, the antibiotic is revealed as a white or light buff colored zone on a pinkish background by spraying with a 1% solution of sulfosalicylic acid in 80% ethanol.

Non-specific reagents. Positive tests are also obtained with less specific reagents such as dilute alkaline potassium permanganate or sulfuric acid–ammonium sulfate reagents. Impurities were sometimes revealed by ninhydrin spray or UV fluorescence.

RESULTS AND DISCUSSION

In order to ascertain the number of charged groups on phosphonomycin, the R_F values for a number of known compounds were determined by PC in systems A and B. The resulting R_F, R_M and ΔR_M values are tabulated in Table II. The system was originally investigated with crude material prepared by ion-exchange procedures.

TABLE II

MOBILITY OF MODEL COMPOUNDS AND PHOSPHONOMYCIN

Compound	*System A*		*System B*		
	R_F	R_M	R_F	R_M	ΔR_M
Tartaric acid	0.16	0.72	0.31	0.35	0.37
Lactic acid	0.51	−0.02	0.69	−0.23	0.21
Gluconic acid	0.21	0.58	0.25	0.48	0.10
Novobiocin	0.75	−0.48	0.86	−0.71	0.23
Phosphonomycin	0.19	0.63	0.33	0.31	0.32
Crude phosphonomycin	0.19	0.63	0.26	0.45	0.18

TABLE III

MOBILITY OF PHOSPHONOMYCIN IN URINE SAMPLES

Paper chromatography; human urine samples in systems A and B; bioautograph detection. Samples A–D from human source provided by Dr. I. FOLTZ. Sample E from dog (5–24 h).

Phosphonomycin content		*Amount spotted (μl)*		*Observed R_F*		ΔR_M
(μg/ml)		*Unknown*	*Control*	*System A*	*System B*	
Control	1000	—	0.25	0.21	0.31	0.23
A	1050	0.10	—	0.17	0.22	0.14
		0.25	—	0.17	0.23	0.16
		0.5	—	0.17	0.22	0.14
		—	0.5	0.18	0.27	0.23
		0.5	0.5	0.17	0.21	0.11
		—	0.25	0.18	0.27	0.23
B	1550	—	0.25	0.19	0.35	0.36
		0.1	—	0.16	0.25	0.24
		0.25	—	0.16	0.26	0.27
		0.5	—	0.17	0.27	0.23
		0.25	0.5	0.17	0.27	0.23
		—	0.25	0.17	0.32	0.36
C	400	—	0.25	0.19	0.29	0.24
		0.5	—	0.18	0.25	0.18
		1.0	—	0.19	0.24	0.13
		1.0	0.5	0.19	0.25	0.15
		—	0.25	0.23	0.32	0.20
D	90	—	0.25	0.19	0.30	0.26
		0.5	—	0.18	0.23	0.13
		1.0	—	0.18	0.27	0.23
		2.0	—	0.18	0.21	0.12
		2.0	0.5	0.17	0.20	0.23
		—	0.25	0.20	0.33	0.29
E	200	—	0.25	0.18	0.32	0.33
		1.0	—	0.20	0.34	0.31
		1.0	0.25	0.20	0.34	0.31

The ΔR_M value obtained indicated that phosphonomycin contained one PO_3H_2 group. This observation was consistent with the ion-exchange behavior of the material. With pure material the observed ΔR_M is somewhat greater but is still less than that usually obtained with dibasic acids.

It is recognized that chromatographic behavior of a compound may be affected by the presence of other substances. The paper chromatographic behavior of the antibiotic in urine samples was studied using systems A and B, with detection by bioautography. The data obtained from a series of five patients is tabulated in Table III. In these experiments all samples originating from a single individual were run on a single sheet. The data show clearly that extraneous substances influence the mobility of the antibiotic, and co-chromatography was required to show that the antibiotically-active substance present in the urine was indeed unchanged phosphonomycin.

Examination of Table III indicates considerable variation in observed ΔR_M which is primarily due to changes in the observed R_F in system B. Although some variation was obtained with system A, this may be due to the difficulty of reading the center of the bioautograph zone, which extends over 0.1 R_F equivalent or more depending on the sensitivity of the bioautograph and the amount loaded on the chromatogram. The reading was normally taken at the apparent zone center. However,

TABLE IV

SOLVENT DEMIXING IN SOLVENTS A AND B; EFFECT OF ADDED CALCIUM CHLORIDE
Bioautograph detection; 1 μl load sodium phosphonomycin (1 mg/ml).

Day	*Observed* R_F		ΔR_M
	System A	*System B*	
1, normal	0.22	0.35	0.28
2, normal	0.20	0.32	0.27
3, normal	0.20	0.30	0.23
5 cm below origin	0.17	0.26	0.24
+ 1 mg/ml $CaCl_2$	0.18	0.29	0.27
4, normal	0.22	0.34	0.26
5 cm below origin	0.21	0.29	0.19
+ 10 mg/ml CaCl	0.17	0.29	0.30
5, normal	0.25	0.30	0.11
5 cm below origin	0.23	0.28	0.12
+ 10 mg/ml $CaCl_2$	0.23	0.28	0.12

the presence of urine contaminants or extraneous solids affects the mobility in system B. Consequently, the systems were studied over several days to determine day to day variation and the effect of added calcium chloride. Solvent demixing was studied by spotting additional samples 5 cm below the origin. The data obtained (Table IV) indicate that added calcium chloride diminishes the R_F values in both systems and that solvent demixing can be important. The large day to day variation and the solvent demixing effect were indicative of a large effect of minor variation in the moisture content of the paper on the observed R_F value. This was confirmed by chromatography with systems containing 80:20 and 60:40 *n*-propanol–aqueous amine systems. The data tabulated in Table V also show the effect of added calcium chloride and solvent demixing on the R_F and ΔR_M values in the two systems.

It is clear that this chromatographic method of determining the number of charged groups requires careful attention to all experimental details as well as standardization of the reagents with known compounds. A cautious interpretation of the data is indicated.

TABLE V

R_F VALUES OF PHOSPHONOMYCIN (SODIUM SALT)
Solvent A: *n*-propanol–2 *N* methylamine; solvent B: *n*-propanol–2 *N* isopropylamine; both 80:20 and 60:40. Bioautograph detection.

System	*Observed* R_F		ΔR_m
	A	*B*	
System 80:20			
Normal	0.07	0.12	0.25
5 cm below origin	0.06	0.12	0.33
+ 10 mg/ml $CaCl_2$	0.03	0.12	0.64
System 60:40			
Normal	0.43	0.47	0.07
5 cm below origin	0.38	0.42	(−0.07)
+ 10 mg/ml $CaCl_2$	0.41	0.34	0.07

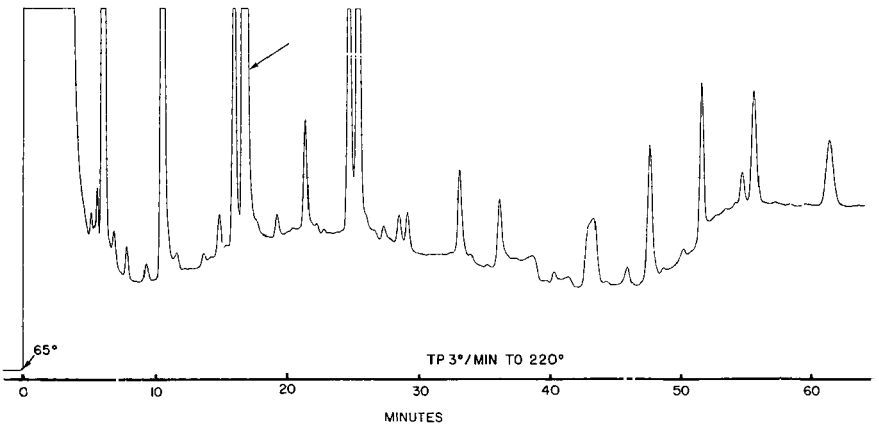


Fig. 1. GLC analysis of a phosphonomycin concentrate (before purification by PC)treated with bis-trimethylsilylacetamide. Conditions given under METHODS.

The value of GLC and combined GLC–MS in following the isolation and purification of a natural product is illustrated in Figs. 1 and 2. Fig. 1 shows the chromatogram resulting from the analysis of a sample of a phosphonomycin concentrate (before purification by PC) treated with BSA; the multi-component nature of the sample is evident. Preparative paper chromatography (System D) of the antibiotic concentrate yielded a product of increased biological activity which, following treatment with BSA, was subjected to GLC. As can be discerned from Fig. 2, this material is largely free from contamination. Note that the predominant peak in Fig. 2 possesses the same retention time as one of the larger peaks found in the non-paper chromatographically purified concentrate (see Fig. 1, arrow); the peaks also gave the same mass spectrum. GLC of the BSA-treated crystalline benzylamine salt of phosphonomycin yielded only two peaks. One corresponded in retention behavior (17 min) to the large peak from the paper chromatographically purified sample; combined GLC–MS of these 17 min

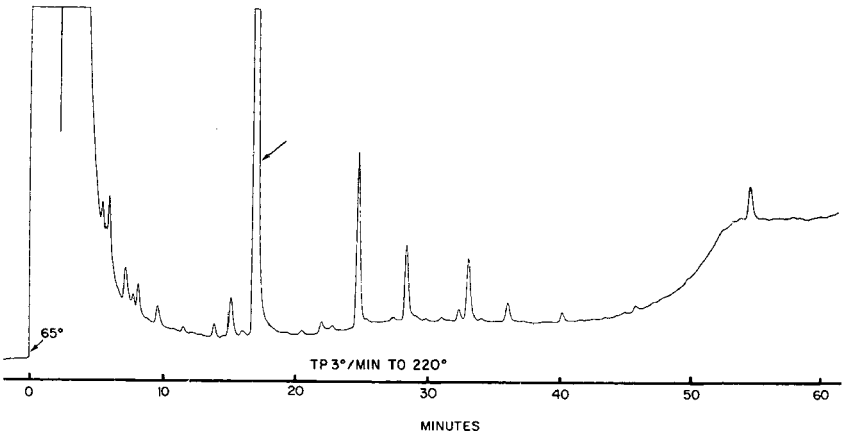


Fig. 2. GLC analysis of a phosphonomycin concentrate (after purification by PC) treated with bis-trimethylsilylacetamide. Conditions given under METHODS.

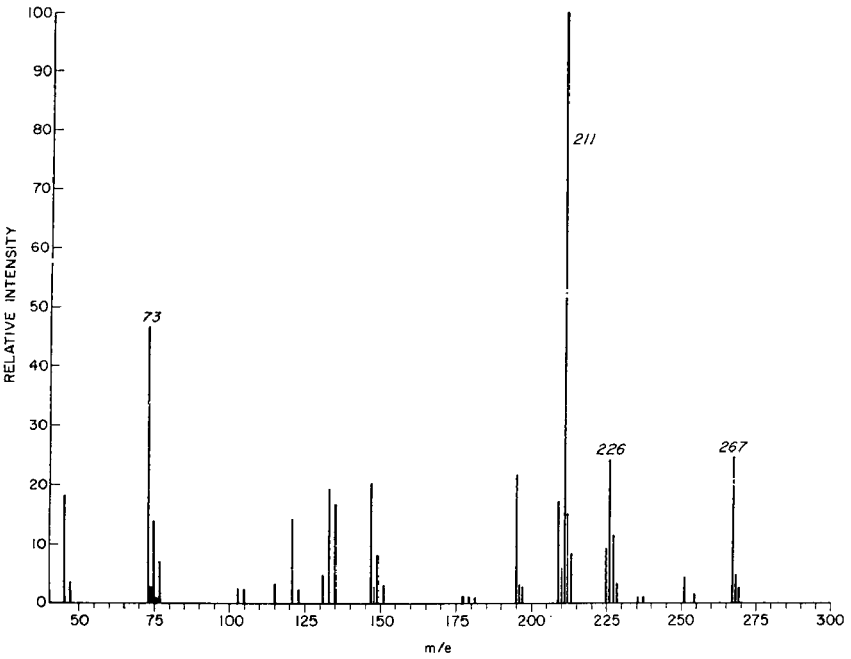


Fig. 3. Mass spectrum of the di-trimethylsilyl derivative of phosphonomycin obtained via combined GLC–MS. Conditions given under METHODS.

peaks confirmed the identical nature of the two compounds. The other peak (of shorter retention time) from the benzylamine salt was shown to be the TMSi derivative of benzylamine.

The mass spectrum of the di-TMSi ester of phosphonomycin is presented in Fig. 3. This compound (molecular weight 282) fails to display a molecular ion (M; *m/e* 282), but does produce a fragment ion of *m/e* 267, corresponding to loss of a methyl group (M-15), not uncommon for TMSi compounds[4,5]. Proposed structures for other major fragment ions are *m/e* 226, formally $(HOP(OSi(CH_3)_3)_2)^+$, resulting from scission of the carbon–phosphorus bond with transfer of a proton from the epoxide moiety to the phosphonate moiety; *m/e* 211, $(226\text{-}CH_3)$; and *m/e* 73, $(Si(CH_3)_3)^+$.

ACKNOWLEDGEMENT

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CHROM. 4873

FRACTIONATION OF DNA ON A METAL ION EQUILIBRATED CATION EXCHANGER

I. CHROMATOGRAPHIC PROFILES OF DNA ON AN IR-120 Al^{3+} COLUMN

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SUMMARY

Amberlite IR-120, a polystyrene sulphonate type of cation exchanger, equilibrated with Al^{3+} has been used for the fractionation of DNA in conjunction with a discontinuous gradient elution procedure. This affords a quantitative and reproducible recovery of DNA in seven distinct fractions each differing in base composition.

Rechromatography can be successfully carried out on the major DNA fractions obtained by fractionation on the IR-120 Al^{3+} column.

INTRODUCTION

Although several procedures exist for the resolution of DNA[1] they are rather unsatisfactory due to experimental limitations like low capacity of the column, high UV positive shedding in the blank, irreversible adsorption, clogging up of the column, poor reproducibility, incomplete recovery, need for drastic eluting agents, slow flow-rates leading to inactivation of marker molecules, etc. In many cases fine resolution is also not possible. The need for the development of an alternate procedure devoid of these drawbacks, therefore, still exists.

Amberlite IR-120 resin equilibrated with Al^{3+} has been employed for the purification of several phosphate metabolising enzymes from agave juice[2]. As a logical extension of this work, studies were initiated on the fractionation of DNA using an IR-120 Al^{3+} column, an account of which follows.

EXPERIMENTAL AND RESULTS

Deoxyribonucleic acid

DNA was isolated from buffalo liver (Mammalia, Ruminantia), the chief milking animal in this country, by SEVAG's procedure[3]. It was a white, fibrous, 85 to 90% pure preparation containing 0.5 to 3.0% protein residue and was free from RNA contamination. Purity of DNA was examined by usual methods[4–7].

The fibrous nature suggested native DNA which was subsequently confirmed

by hyperchromicity data[8], *i.e.*, an increase in the UV absorbance by 40% at 260 mμ and by enzymatic studies[9].

The sodium salt of DNA was also isolated from *Bacillus subtilis* and phage T_2 using SEVAG's procedure[3] and KIRBY's phenol method[10], respectively. Both the preparations were tested for their purity and nativeness by the usual procedures[4–9].

IR-120 Al^{3+} column

10 g of dry regenerated Amberlite IR-120 Na^+ form (BDH, Great Britain), a polystyrene sulphonate, equilibrated with 0.2 *M* aluminium chloride solution was used for the IR-120 Al^{3+} column. Glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was percolated through the column till the pH of the inflow and effluent were the same. This column was then used for fractionation purposes.

Al^{3+} was specifically chosen as a counter-balancing cation as it is nontoxic, noncorrosive and inert in biological systems.

The following environmental factors, likely to influence the chromatographic behaviour of DNA, were studied.

Capacity of the IR-120 Na^+ form of the adsorbent for Al^{3+}

A known amount of 0.2 *M* aluminium chloride solution, judged as in excess from pilot experiments, was percolated through 10 g of the regenerated Na^+ form of the IR-120 resin at a rate of 10–15 ml per h. The column was washed with sufficient amount of glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) to remove any loosely retained Al ions and the amount of Al^{3+} in the effluent and buffer washings was de-

TABLE I

CAPACITY OF IR-120 Na^+ FORM OF THE ADSORBENT FOR Al^{3+}

Description	*Amount of Al^{3+} (mg)*
Loaded on 10 g Na^+ form of resin	563.50
Present in effluent and buffer washings	452.70
Retained on 10 g Na^+ form of resin	110.80
Capacity of 1.0 g resin for Al^{3+}	11.08

termined by GENTRY AND SHERRINGTON's method[11]. The results are cited in Table I which shows that 11.08 mg of Al^{3+} can be maximally retained per g of IR-120 Na^+ form of the resin.

Capacity of the IR-120 Al^{3+} form of the adsorbent for DNA

135 ml of a homogeneous solution of DNA (0.5 mg/ml), in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*), was loaded on to a column containing 10 g IR-120 Al^{3+} resin and allowed to percolate at the rate of 10–15 ml per h, which ensured satisfactory equilibrium conditions. 5 ml fractions were collected till there was no DNA in the effluent. The column was then washed with three bed volumes of the above buffer to remove any loosely retained DNA. DNA adsorbed on IR-120 Al^{3+} column was desorbed by 1.0 *N* sodium hydroxide solution and estimated by BURTON's re-

TABLE II

CAPACITY OF IR-120 Al^{3+} FORM OF THE ADSORBENT FOR DNA

Description	*DNA expressed as O.D. of DPA positive material*
Loaded on 10 g IR-120 Al^{3+} resin	182
Present in effluent and buffer washings	44
Retained on 10 g IR-120 Al^{3+} resin	138
Eluted with 1.0 *N* NaOH from IR-120 Al^{3+}	136
Capacity of 1.0 g IR-120 Al^{3+} resin for DNA	13.6 $\cong$ 5.0 mg

action[4]. The results are given in Table II. It shows that 13.6 O.D. units of DNA are maximally retainable per g of the Al^{3+} form of the IR-120 resin.

Effect of the amount of adsorbent on the profiles of DNA

Two columns containing respectively 2 g and 10 g of IR-120 Al^{3+} resin were employed. The procedure followed for the adsorption and elution is discussed in detail under *Typical chromatographic profiles of DNA*. The fractions collected were assayed for DNA content by BURTON's reaction[4].

It was found that the amount of adsorbent used did not have any effect on the nature of the DNA profiles. COHN[12] found that the amount of adsorbent used affects the profiles if the sample exceeds or approaches the capacity of the column. Similar observations have also been made by TOMPKINS[13]. However, the present studies using an IR-120 Al^{3+} column were carried out below the capacity of the column. Recently however, AYAD *et al.*[14] found that the resolution of the transforming principles of *B. subtilis* could be improved by increasing the amount of adsorbent layer in which fractionation occurs.

Effect of the amount of DNA on the profiles

20 O.D. and 40 O.D. units of DNA was loaded on two separate IR-120 Al^{3+} columns, each containing 10 g of the adsorbent. Adsorption and elution was carried out by a procedure described under typical chromatographic profiles. The fractions collected were assayed as before[4].

It was found that the amount of DNA did not have any effect on the nature of the profiles. The percentage distribution of DNA was practically the same in different fractions in spite of the different amounts of DNA loaded. Furthermore, there was no cumulative effect due to eluting agents as observed by DAVILA *et al.*[15].

Effect of the aging of DNA on the profiles

Freshly prepared DNA, and DNA stored for six months at 25° and 4° were chromatographed on an IR-120 Al^{3+} column under an identical set of experimental conditions keeping other parameters constant, *viz.* source of DNA, method of isolation and deproteinisation etc.

Aging or storage of DNA had no effect on its chromatographic behaviour. Theoretically, the chromatographic profiles need not change unless DNA is associated

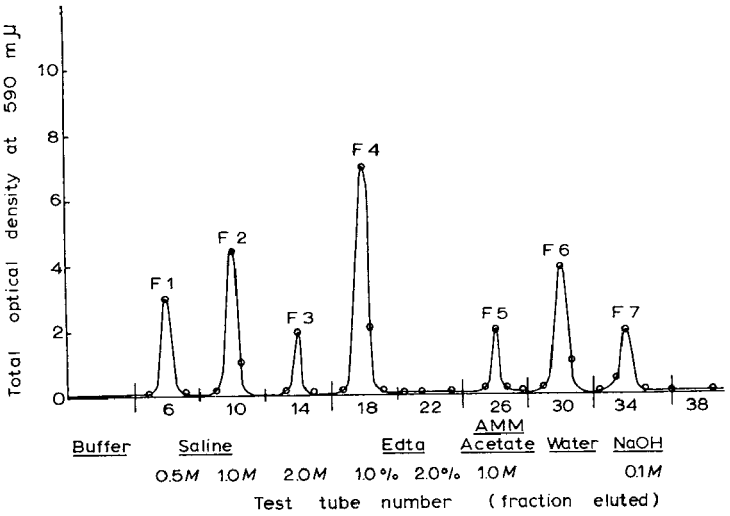

Fig. 1. Typical chromatographic elution profile of buffalo liver DNA (SEVAG's method[3]) on an IR-120 Al^{3+} column.

with deoxyribonuclease activity, which causes depolymerisation and subsequently results in deviation from the typical profiles.

Typical chromatographic profiles of DNA

A homogeneous solution of DNA, in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*), was loaded on to an IR-120 Al^{3+} column, and percolated at the rate of 10–15 ml per h. The effluent was collected, and the column was washed with at least three bed volumes of the above buffer to remove any loosely retained DNA. Adsorbed DNA was then desorbed by 100 ml of each of the following eluting agents in the given sequence: 0.5 *M*, 1.0 *M*, 2.0 *M* saline; 1.0%, 2.0% EDTA; 1.0 *M* ammonium acetate, glass distilled water and finally 0.1 *M* sodium hydroxide solution. The fractions, each of 25 ml, were collected and assayed for DNA content by BURTON's reaction[4]. The same procedure was followed in all the above studies.

The percentage of total eluted DNA by different eluting agents is given in Table III. It shows that DNA is 100% retainable and can be recovered completely by different eluting agents in seven well-defined fractions (F_1–F_7). Fig. 1 shows a

TABLE III

TYPICAL CHROMATOGRAPHIC PROFILES OF BUFFALO LIVER DNA ON AN IR-120 Al^{3+} COLUMN

Percent retention	*Percent elution by*								*Percent elution*
	Saline			*EDTA*		*Ammonium acetate 1.0 M*	*Distilled water*	*Sodium hydroxide 0.1 M*	
	0.5 M	*1.0 M*	*2.0 M*	*1.0%*	*2.0%*				
	F_1	F_2	F_3	F_4	F'_4	F_5	F_6	F_7	
100	10.0	15.0	7.0	33.0	Nil	8.0	17.0	10.0	100

TABLE IV

BASE COMPOSITION OF DNA FRACTIONS OBTAINED ON AN IR-120 Al^{3+} COLUMN

Fraction eluted by	1×10^{-2} *M moles of*				$\frac{A+T}{G+C}$	$\frac{Pu}{Py}$	*Nature of fraction*
	A	*G*	*C*	*T*			
Unfractionated DNA	5.51 ± 0.16	4.62 ± 0.12	4.81 ± 0.08	5.58 ± 0.08	1.17	0.96	A–T rich
0.5 *M* saline	5.25 ± 0.10	5.09 ± 0.12	4.88 ± 0.00	5.25 ± 0.15	1.06	1.02	A–T rich
1.0 *M* saline	4.84 ± 0.09	6.48 ± 0.09	6.82 ± 0.00	5.12 ± 0.12	0.76	0.93	G–C rich
2.0 *M* saline	3.99 ± 0.11	4.28 ± 0.03	4.40 ± 0.12	4.03 ± 0.06	0.93	0.98	G–C rich
1.0% EDTA	15.74 ± 0.10	15.46 ± 0.14	15.50 ± 0.25	15.18 ± 0.14	1.00	1.02	A = G
1.0 *M* ammonium acetate	2.01 ± 0.07	2.86 ± 0.21	2.48 ± 0.00	2.33 ± 0.05	0.85	0.99	G–C rich
Glass distilled water	2.67 ± 0.21	3.32 ± 0.00	3.47 ± 0.13	2.95 ± 0.00	0.85	0.94	G–C rich
0.1 *M* sodium hydroxide	4.17 ± 0.06	4.26 ± 0.00	3.94 ± 0.18	4.12 ± 0.01	1.00	1.02	Almost A = T = G = C

typical chromatographic profile after the total O.D. at 590 mμ has been plotted against the test tube number of the fraction eluted.

Use of 1.0 *M* ammonium acetate first and then of 1.0% EDTA did not alter typical chromatographic behaviour of DNA.

Base composition of different fractions of DNA

The base composition of the DNA fractions was studied to see if DNA fractionated according to the differences in base composition.

Each fraction was concentrated on a rotating evaporator and then precipitated by chilled ethanol. The precipitate was hydrolysed by MARSHAK AND VOGEL'S procedure[16]. Aliquots of each fraction were chromatographed on Whatman No. 1 paper using isopropanol–hydrochloric acid–water, 65:16.6:18.4 as the solvent. Simultaneously, a standard solution of each base was chromatographed. After a 17 h development, the chromatogram was removed from the chamber and dried in air at room temperature. The resolved bases were detected under a Chromatolite lamp, and eluted in 5 ml of 0.1 *N* hydrochloric acid and the O.D. was recorded between 230 to 290 mμ. Blank strips opposite each of these bases were cut from the paper and the O.D. of each of these blanks was subtracted from the O.D. of the corresponding base. The molar extinction coefficients of standard solutions of adenine, guanine, cytosine and thymine agreed closely with the reported values serving as a check on the procedure. The base ratios calculated are presented in Table IV which gives the base composition data of the unfractionated DNA and its seven distinct fractions. The recovery of the bases is greater than 90%. Variations in base composition are considered to be experimentally significant and are also confirmed by OTH'S procedure which was modified by ROGER *et al.*[17]. The base composition data obtained by two different methods, based upon two different approaches, are in good agreement as shown in Table V.

From Tables IV and V it is clear that unfractionated DNA as well as 0.5 *M* saline elutable fraction are A–T rich, while the subsequent two fractions elutable by 1.0 *M* and 2.0 *M* saline are G–C rich. With a change in the nature of eluting agent, *i.e.* with 1.0% EDTA, the fraction coming out has all the bases in almost equal pro-

TABLE V

BASE RATIOS OF DNA FRACTIONS

Fraction eluted by	*A/G ratio by*	
	Paper chromatography	*Modified Oth's procedure*
Unfractionated DNA	1.17	1.15
0.5 *M* saline	1.06	1.10
1.0 *M* saline	0.76	0.90
2.0 *M* saline	0.93	0.98
1.0% EDTA	1.00	1.04
1.0 *M* ammonium acetate	0.85	0.90
Glass distilled water	0.85	0.88
0.1 *M* sodium hydroxide	1.00	—

TABLE VI

RECHROMATOGRAPHY OF DNA FRACTIONS ON AN IR-120 Al^{3+} COLUMN

Fraction loaded	*Percent retention*	*Eluting agent*	*Percent elution*	*Total elution*	*Inference*
0.5 *M* Saline (F_1)	100	0.5 *M* saline alone	100	100	Homogeneous fraction
1.0% EDTA (F_4)	100	Glycine–sodium hydroxide (F 4.1)	52	100	Heterogeneous fraction originally or heterogeneity due to dissociation in absence of Mg^{2+}
		0.5 *M* Saline (F 4.2)	15		
		1.0% EDTA (F 4.3)	34		
Glycine–sodium hydroxide (F 4.1)	100	Glycine–sodium hydroxide alone	100	100	Possibly homogeneous fraction
0.5 *M* Saline (F 4.2)	100	0.5 *M* saline alone	100	100	Possibly homogeneous fraction
1.0% EDTA (F 4.3)	100	Glycine–sodium hydroxide (F 4.3a)	43	100	Heterogeneous originally or as a result of disaggregation in absence of Mg^{2+}
		0.5 *M* saline (F 4.3b)	58		
1.0% EDTA (F_4) + 0.005 *M* Mg^{2+} 4°, 24 h	100	1.0% EDTA alone	100	100	Homogeneous aggregate as a result of Mg^{2+} binding

portions. The fractions eluted by 1.0 *M* ammonium acetate and glass distilled water are again G–C rich. The last fraction eluted by 0.1 *M* sodium hydroxide is slightly A–T rich.

Repeated chromatographic fractionation

Rechromatographic study of two major fractions, *viz.* the 0.5 *M* saline and 1.0% EDTA elutable fractions was carried out. These fractions were concentrated on a rotatory evaporator and precipitated by two volumes of distilled ethanol. The precipitate dissolved easily in the glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) in case of 0.5 *M* saline elutable fraction, but not in case of the 1.0% EDTA elutable fraction. The latter was dissolved in 2.0% sodium carbonate solution and the pH was adjusted back to 8.6.

Rechromatography of the 0.5 M saline elutable fraction. A homogeneous solution of the 0.5 *M* saline elutable fraction (F_1) was loaded on to the IR-120 Al^{3+} column and was found to be 100% retainable. It was also quantitatively eluted with 0.5 *M* saline only. The results are given in Table VI. It should be noted that the 0.5 *M* saline elutable fraction is re-elutable by the same eluting agent indicating its chromatographic homogeneity.

Rechromatography of the 1.0% EDTA elutable fraction. A homogeneous solution of the 1.0% EDTA elutable fraction (F_4) was loaded on to the IR-120 Al^{3+} column and was found to be 100% retainable. When it was eluted by the usual sequence of eluting agents it was found that the adsorbed material is separated into three fractions, a glycine–sodium hydroxide elutable (F 4.1), a 0.5 *M* saline elutable (F 4.2) and a 1.0% EDTA elutable (F 4.3) fraction. No elution was possible with intermediate eluting agents like 1.0 *M* and 2.0 *M* saline.

Further chromatographic procedure. F 4.1 and F 4.2 were adsorbed on a fresh IR-120 Al^{3+} column and eluted at the same position on elution. F 4.3 was found to be 100% retainable on a fresh column and could be resolved into two fractions, a glycine–sodium hydroxide elutable (F 4.3a) and a 0.5 *M* saline elutable (F 4.3b) fraction.

Rechromatography of the 1.0% EDTA elutable fraction (F_4) after Mg^{2+} equilibration. After concentration and precipitation, the precipitate was dissolved and equilibrated with 0.005 *M* Mg^{2+} at 4° for 24 h (0.005 *M* Mg^{2+} ion concentration was expected to be sufficient to nullify the chelating effect of any traces of EDTA remaining). This fraction was then chromatographed on a fresh IR-120 Al^{3+} column and was found to be 100% retainable and could only be eluted at its original location and height by 1.0% EDTA.

The results are given in Table VI and Fig. 2. The latter depicts the chromatographic profiles of the 1.0% EDTA elutable fraction and subfractions, before and after Mg^{2+} equilibration. It is worth noting that the F_4 fraction subfractionated in the absence of Mg^{2+} appears as a single entity after Mg^{2+} equilibration.

Chromatographic behaviour of B. subtilis DNA and phage T_2 DNA

A homogeneous solution of *B. subtilis* DNA and phage T_2 DNA in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was separately loaded on to an IR-120 Al^{3+} column and its behaviour was studied under experimental conditions identical to those used for the buffalo liver DNA fractionation.

It was found that neither of the DNA preparations were retained in absence or

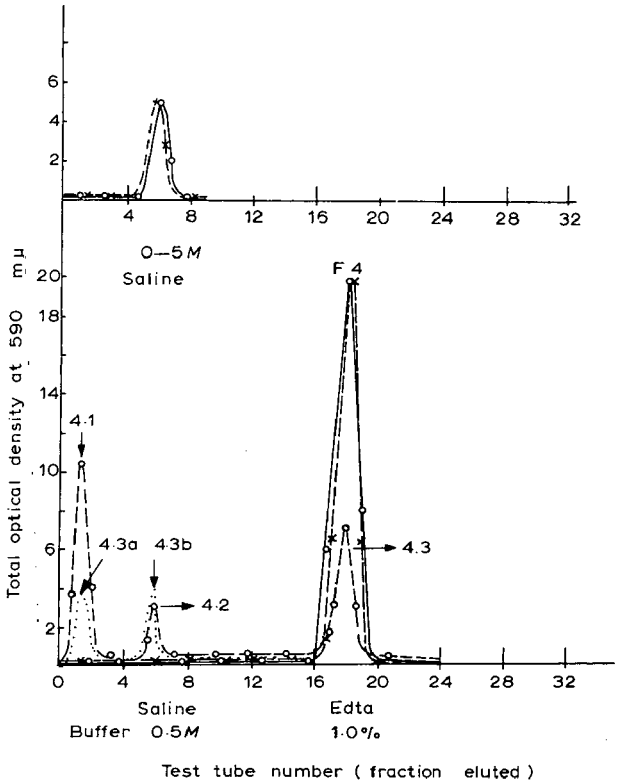

Fig. 2. Rechromatography of DNA fractions on an IR-120 Al^{3+} column. (a) O—O, chromatography of F_1; ×— — —×, rechromatography of F_1. (b) O—O, chromatography of F_4; O— —O, rechromatography of F_4 to give 4.1, 4.2 and 4.3; ×— — —×, rechromatography of F_4 + Mg^{2+}; · · · · ·, rechromatography of $F_{4.3}$ (4.3a and 4.3b).

presence of Mg^{2+}. Mg^{2+} equilibration was tried to see whether it could modify the behaviour. It is difficult to indicate precisely the reason for the failure in retention of these preparations. It could be that rapidly dividing DNAs are not acceptable by the column as could be judged from chromatographic behaviour of human hepatoma DNA[9]. It looks as if the IR-120 Al^{3+} column resolves DNA from rapidly dividing cells and that of somatic tissue.

DISCUSSION

The present fractionation procedure is not conventional ion-exchange chromatography, as in addition to the resin, Al ions are also involved in the adsorption as revealed from equilibrium dialysis data[9]. It could be looked upon as complex-ion-exchange chromatography or ligand-exchange chromatography.

Although a discontinuous gradient elution procedure has certain limitations, for routine explorative purposes it is quick and convenient, particularly for manual

operations. Fractionation can easily be carried out on any scale by adjusting the size of the column; furthermore, the setting up of the column is extremely simple and inexpensive. Discontinuous gradient elution was therefore employed in the present studies and was found to be reasonably satisfactory, when the basic technique had been established, for the general study of the heterogeneity of DNA.

It is known that fractions with high G–C content are eluted with a decreasing salt concentration. The odd elution behaviour of A–T rich fractions with a low salt concentration in the present studies (0.5 *M* saline) has also been noted by CHENG AND SUEOKA[18] in the case of mouse testes DNA profiles on a methylated serum albumin kieselguhr column. This was explained by possible contamination due to an unusual base like methylcytosine. In the present studies however no unusual base was detected and it is unlikely that DNA from a mammalian source will contain any odd base.

In the light of these observations, it was concluded that the IR-120 Al^{3+} column effects the fractionation of DNA extensively and reproducibly, the basis being differences in base composition.

The possibility that the three fractions obtained on rechromatography of the F_4 fraction are genuinely independent fractions, which were eluted together as a single peak by the somewhat drastic and indiscriminate elutability of EDTA in the first chromatographic separation of the DNA is unlikely as rechromatography of the F 4.3 fraction yields two more fractions (F 4.3a, F 4.3b) yet again. Another possibility that 1.0% EDTA elutable fraction (F_4) has undergone structural changes due to its conversion to DNA–Al complex as a result of previous passage through the adsorbent and therefore may have diminished its affinity towards the adsorbent, is not supported by existing circumstantial data[9]. Depolymerisation or denaturation as a result of the passage of the DNA through the adsorbent does not explain the rechromatographic behaviour. It has already been proved that denaturation does not significantly alter the chromatographic behaviour of DNA[19].

Thus, overlapping of the fractions due to the purging action of the eluting agent, structural alterations, depolymerisation, or denaturation as a result of passage through the adsorbent, do not explain the rechromatographic behaviour of the 1.0% EDTA elutable fraction (F_4). The subsequent emergence of two more fractions—F 4.3a and F 4.3b— suggests a labile character for the EDTA elutable fraction and its dissociation into subunits. The quantitative emergence of F_4 after Mg^{2+} equilibration as a single peak due to the coalescence of the F 4.1, F 4.2 and F 4.3 fractions adds to this view. The original EDTA fraction may be dissociated or disaggregated into three subunits by the demineralising action of EDTA giving three fractions and Mg^{2+} addition may cement them back together. Mg^{2+} is known to be a structural ingredient of polyribosomes, a critical concentration of which maintains its integrity as well as functional state[20,21]. OTAKA *et al.*[22] noticed that the chromatographic behaviour of RNA, on DEAE-cellulose, was very much dependent on the molarity of Mg^{2+}; two peaks found at low Mg^{2+} concentrations merged into a single peak at a high Mg^{2+} concentration. It is possible that Mg ions exhibit a unique role in the structural integrity of DNA as well in the present experiments. Mg ions have already been known to bring about conformational changes in nucleic acid molecules[23,24].

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THE USE OF R_C VALUES FOR STRUCTURAL STUDIES IN THE PAPER CHROMATOGRAPHY OF PHENOLS

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SUMMARY

An investigation was carried out of the chromatographic behaviour of some alkyl derivatives of phenol, pyrocatechol, hydroquinone, and 4,4'-isopropylidenebisphenol using papers impregnated with formamide or monomethylformamide. The R_F values of the compounds were related to a homologous series of standards; pyrocatechol and 4-alkylpyrocatechols containing C_1–C_{12} alkyls were used for this purpose and were assigned to R_C values. The ΔR_C contributions of the individual groups were established, and their additivity was verified.

INTRODUCTION

The chromatographic behaviour of the alkyl derivatives of phenols has already aroused much interest. From the point of view of chromatography, the above compounds represent very simple systems, which along with their easy detectability makes them suited for investigations of the relationships between the structure and chromatographic behaviour. These investigations are in most cases based on the well-known MARTIN's relationship, which can be written as follows:

$$R_M = \Sigma \Delta R_M + K \qquad (1)$$

where $R_M = \log (1/R_F - 1)$; the ΔR_M are the contributions of the individual parts of the molecule of the chromatographed compound, and K is a constant which in the first place includes the ratio of the cross section of the stationary to the mobile phase. Several authors[1–6] have succeeded in verifying the above relationship for the alkyl derivatives of phenols. In so far as other relationships[7] which have been found are concerned, the deviations were mainly due to experimental difficulties[1].

Attempts have been made to reduce the experimental errors, on the one hand, by improving the techniques used, *e.g.*, by preventing the evaporation of the phases in the course of chromatography between aluminium foils[8], and on the other, by correcting the R_F or R_M values obtained in the measurements by means of one or

two standards[9]. However, even if the reproducibility is satisfactory, an important complication may arise, namely, that the term K in eqn. 1 is not constant, since both phases exhibit a concentration gradient along the paper strip[10,11]. For instance, COPIUS PEEREBOOM[11] found that the ratio of the cross-sections of both phases on a paper impregnated with paraffin oil decreased from 14.6 (start) to 2.0 (front). These complications can be avoided by using a procedure suggested by DECKER[12]. The compound being analysed is run together with a homologous series of standards chosen in advance. Integers are assigned to the individual members of this series; DECKER suggests the symbol R_C for these values (analogous to the Kováts' indices in GC). The R_C value of the compound being analyzed is determined by interpolation on an experimental curve obtained by plotting the R_F values of the standards against their R_C values. Thus, a value of a hypothetical member of the homologous series is assigned to the compound being analysed, this member having the same position on the chromatogram as the compound. Since the R_M values of both the above compounds are equal, and if relationship (1) is valid, it then follows:

$$\Sigma \Delta R_M + K = n \cdot \Delta R_M(CH_2) + \Delta R_M \text{ (basic compound)} + K$$

where n is the number of the carbon atoms in the chain of the hypothetical standard. Since $R_C = n$, the following holds

$$R_C = \sum \frac{\Delta R_M}{\Delta R_M(CH_2)} - \frac{\Delta R_M \text{ (basic compound)}}{\Delta R_M(CH_2)}$$

By introducing ΔR_C for the ratio $\Delta R_M/\Delta R_M(CH_2)$, we obtain the relationship

$$R_C = \Sigma \Delta R_C - \Delta R_C \text{ (basic compound)} \qquad (2)$$

At constant temperature, the R_C value is independent of the degree of impregnation, any gradient of the phases and their evaporation, as long as their chemical composition remains unchanged.

EXPERIMENTAL

The materials used were prepared according to the literature data or by repurifying commercial products.

4-Alkylpyrocatechols having C_6 to C_{12} alkyls were prepared by acylation of pyrocatechol (1 mole) with the corresponding carboxylic acid (1 mole) and using boron fluoride etherate (1.2 mole) at 80° for 3 h (yield 20–30% of the theoretical); this was followed by reduction of the isolated 4-acylpyrocatechol according to CLEMMENSEN (see ref. 13): 4-hexylpyrocatechol (b.p. 150–160°/1 Torr), 4-heptylpyrocatechol (m.p. 65.5–66.5°, ref. 13, 65–67°, 4-octylpyrocatechol (m.p. 60.5–61.5°, ref. 13, 57–58°), 4-nonylpyrocatechol (72.5–73.5°, ref. 13,68°), 4-decylpyrocatechol (69–70.5°), 4-undecylpyrocatechol (m.p. 81–82°), 4-dodecylpyrocatechol (m.p. 76–77°). 3-*tert.*-Butyl-5-ethylpyrocatechol and 3-*tert.*-butyl-5-propylpyrocatechol were prepared by alkylation of 4-ethylpyrocatechol or 4-propylpyrocatechol (0.01 mole) with *tert.*-butyl alcohol (0.01 mole) in acetic acid, catalysed by sulphuric acid (0.01 mole) at room temperature for 24 h; after the usual treatment of the reaction mixture, the raw alkylation product was used.

Chromatograms were run on Whatman No. 1 paper, impregnated with 20% formamide in ethyl acetate or with 20% monomethylformamide in benzene (dried for 10 min), employing the techniques without removal of the excess of the phase by filtration through the filter paper. In the formamide–chloroform system the descending technique was used; in the other cases, ascending chromatography was employed. The chromatograms were detected by spraying them with a freshly prepared mixture of a 15% solution of ferric chloride and 1% solution of potassium ferricyanide. The R_C values listed in Tables II and IV were obtained as the mean from at least three measurements. The maximum differences between the individual measurements of R_C and the mean value were $\pm$ 0.25 in the formamide systems, and $\pm$ 0.5 in the system with stationary phase of monomethylformamide.

RESULTS AND DISCUSSION

In the present work, alkyl derivatives of the following compounds were chromatographed, and their R_C values determined: phenol, pyrocatechol, hydroquinone, 4,4'-isopropylidenebisphenol (subsequently referred to as bisphenol A). The alkyl groups studied were: methyl, ethyl, isopropyl, *tert.*-butyl, 1,1,3,3-tetramethylbutyl groups (subsequently referred to as *tert.*-octyl) and 1,1,3,3,5,5-hexamethylhexyl (subsequently referred to as *tert.*-dodecyl) groups, as well as some other model compounds. Pyrocatechol and 4-alkylpyrocatechols containing C_1 to C_{12} alkyls were chosen as the homologous series of standards; their polar character covered the whole range of polarity of the compounds studied. The first member in this series, *i.e.* pyrocatechol, was assigned an R_C value equal to zero, 4-methylpyrocatechol was assigned $R_C = 1$ etc., *cf.* Table I.

Deviations from the validity of relationship (1) could be expected with the first and second member of the homologous series of standards[1,12]. To evaluate the

TABLE I

R_C AND R_F VALUES OF 4-ALKYLPYROCATECHOLS

Paper impregnated with 20% formamide (I_1) or monomethylformamide (I_2), developed with chloroform (S_1) or a mixture of isopropyl ether and heptane (1:2) (S_2).

Compound	R_C	R_F *values in the chromatographic system*		
		I_1S_1	I_1S_2	I_2S_2
Pyrocatechol	0	0.05	0.04	—
4-Methylpyrocatechol	1	0.10	0.07	—
4-Ethylpyrocatechol	2	0.18	0.13	—
4-Propylpyrocatechol	3	0.30	0.24	0.05
4-Butylpyrocatechol	4	0.45	0.40	0.08
4-Pentylpyrocatechol	5	0.60	0.55	0.13
4-Hexylpyrocatechol	6	0.71	0.68	0.21
4-Heptylpyrocatechol	7	0.78	0.78	0.31
4-Octylpyrocatechol	8	0.81	—	0.42
4-Nonylpyrocatechol	9	—	—	0.54
4-Decylpyrocatechol	10	—	—	0.64
4-Undecylpyrocatechol	11	—	—	0.71
4-Dodecylpyrocatechol	12	—	—	0.77

magnitude of these deviations the polarity of the first four members of the series of 4-alkylpyrocatechols investigated was lowered by introducing the *tert.*-butyl group into position 6; the derivatives thus obtained were chromatographed in a system with a stationary phase of monomethylformamide. The R_C values (Table V) of the neighbouring members of this new homologous series differ by approximately a unit, *i.e.* the increments are the same as in the case of the higher members of the original series; this means that no great deviations occur.

The pyrocatechol derivatives are extremely sensitive towards detection, which allows the use of small amounts of these compounds and thus chromatographic runs of several standards from one starting line without influencing their R_F values. Chromatographic runs were carried out on paper impregnated with formamide or monomethylformamide, and a mixture of diisopropyl ether–heptane (1:2) was used as the mobile phase; in the case of formamide, chloroform was also used. The R_F values of most of the phenols investigated are very close in both the formamide systems, but differ in compounds having varying abilities of hydrogen bond formation. This is of practical importance, *e.g.* in the analysis of reaction mixtures formed by the alkylation of phenols, when it is possible by chromatographing in both these systems to differentiate between the derivatives formed by alkylation on the oxygen atom and polyalkyl derivatives due to alkylation in the nucleus. The system where monomethyl-

TABLE II

R_C VALUES OF COMPOUNDS USED IN THE CALCULATIONS OF THE ΔR_C INCREMENTS

Paper impregnated with 20% formamide (I_1) or monomethylformamide (I_2), developed with chloroform (S_1) or a mixture of diisopropyl ether–heptane (1:2) (S_2).

Compound	*Chromatographic system*		
	I_1S_1	I_1S_2	I_2S_2
Phenol	3.7	3.7	4.8
2-Methylphenol	5.2	5.1	6.2
2-Ethylphenol	6.3	6.5	7.6
2-Isopropylphenol	—	—	8.7
2-*tert.*-Butylphenol	—	—	10.6
2-Methoxyphenol	—	—	5.3
4-Isopropylphenol	—	—	7.6
4-Benzylphenol	—	6.3	6.6
4-Methoxyphenol	—	—	3.4
2,6-Dimethylphenol	—	6.0	7.7
4-Isopropylpyrocatechol	3.0	3.0	—
4-*tert.*-Butylpyrocatechol	—	—	3.6
2-*tert.*-Butylhydroquinone	1.8	2.9	—
2-*tert.*-Octylhydroquinone	4.7	—	5.2
2-*tert.*-Dodecylhydroquinone	—	—	8.2
2,5-Diisopropylhydroquinone	4.1	5.1	—
2,5-Di-*tert.*-butylhydroquinone	6.3	—	—
2,6-Di-*tert.*-butylhydroquinone	—	—	8.1
4,4′-Methylenebis(2,6-xylenol)	—	—	3.7
4,4′-Isopropylidene-bis(2-methylphenol)	—	4.5	—
2,6-Dimethyl-4,4′-isopropylidene-bisphenol	6.1	—	—
2,6-Diethyl-4,4′-isopropylidene-bisphenol	—	—	4.7
2,6-Diisopropyl-4,4′-isopropylidene-bisphenol	—	—	6.6
2-*tert.*-Butyl-4,4′-isopropylidene-bisphenol	6.8	—	—
2-Methyl-6-*tert.*-butyl-4,4′-isopropylidene-bisphenol	—	—	6.3

TABLE III

ΔR_C INCREMENTS OF INDIVIDUAL GROUPS

Paper impregnated with 20% formamide (I_1) or 20% monomethylformamide (I_2), developed with chloroform (S_1) or a mixture of diisopropyl ether–heptane (1:2) (S_2).

Group	*Chromatographic system*		
	I_1S_1	I_1S_2	I_2S_2
ΔR_C (basic compound)	−8.1	−8.7	−12.7
Phenyl	2.0	1.6	0.8
Phenolic group	−6.4	−6.6	−8.7
p-Isopropyl[a]	3.0	3.0	2.8
p-*tert.*-Butyl	—	—	3.6
p-Methoxy	—	—	−1.4
o-Methyl	1.5	1.4	1.4
o-Ethyl	2.6	2.8	2.8
o-Isopropyl	3.4	4.0	3.9
o-*tert.*-Butyl	4.5	5.8	5.8
o-*tert.*-Octyl	7.4	—	9.1
o-*tert.*-Dodecyl	—	—	12.1
o-Methoxy	—	—	0.5
o,*o*′-Dimethyl	3.8	2.3	2.9
o,*o*′-Diethyl	—	—	5.0
o,*o*′-Diisopropyl	—	—	6.9
o,*o*′-Di-*tert.*-butyl	—	—	12.0
o-Methyl-*o*′-*tert.*-butyl	—	—	6.6

[a] Isopropylidene group was calculated as isopropyl group.

formamide is used as the stationary phase is suitable for less polar phenols. The R_F values of standards are given in Table I, the R_C values of the compounds investigated are presented in Tables II and IV. It was found during this study that the R_C values of some of the compounds analysed were not completely reproducible, especially in the presence of monomethylformamide. The reproducibility observed in the preliminary experiments with dimethylformamide was even less. Such a fluctuation of values is probably due to changes in the water content of the stationary phase. This is also corroborated by a comparison between chromatograms run in winter and in summer, when there are considerable differences in the humidity of the air at the same temperature. In systems containing formamide a small fluctuation in the water content does not lead to any essential changes in the R_C values. The validity of relationship (2) was verified by comparing the measured and calculated values of R_C of the phenolic derivatives studied (Table IV), and the agreement was found to be satisfactory in most cases. The values of the increments ΔR_C (Table III) were calculated from the R_C values of compounds listed in Table II. The isopropylidene bridge in the alkylated bisphenol A's was calculated as an isopropyl group. The comparison of the bisphenols with the analogous mononuclear phenols allows the determination of the constant—ΔR_C (basic compound) in eqn. 2. The ΔR_C values for alkyls in the position *ortho* to the hydroxy group holds only if the other *ortho* position is free. If both the *ortho* positions are occupied with alkyls, the total value of both alkyls was determined. If the other *ortho* position is also occupied by a hydroxy group, *i.e.* in the case of *ortho* alkyl derivatives of pyrocatechol, it is not possible to employ the ΔR_C values from

TABLE IV

R_C VALUES OF PHENOLIC COMPOUNDS

Paper impregnated with 20% formamide (I_1) or monomethylformamide (I_2), developed with chloroform (S_1) or a mixture of diisopropyl ether–heptane (1:2) (S_2).

Compound	*Chromatographic system*					
	I_1S_1		I_1S_2		I_2S_2	
	Found	*Calc.*	*Found*	*Calc.*	*Found*	*Calc.*
2-Methoxyphenol	6.5	—	3.8	—	5.3	—
4-Methoxyphenol	3.8	—	2.4	—	3.4	—
4-Isopropylphenol	6.9	6.7	6.6	6.7	7.6	—
2-Methoxy-4-*tert.*-butylphenol	—	—	—	—	10.3	9.8
4-*tert.*-Butylpyrocatechol	3.6	—	3.7	—	3.6	—
4-*tert.*-Octylpyrocatechol	6.6	—	6.9	—	6.8	—
4-*tert.*-Octyl-5-methylpyrocatechol	—	—	7.0	—	6.9	—
4-*tert.*-Dodecylpyrocatechol	—	—	—	—	9.9	—
2-*tert.*-Butyl-5-methylhydroquinone	3.3	3.3	4.2	4.3	—	—
2-*tert.*-Octyl-5-methylhydroquinone	6.2	6.2	—	—	7.1	6.6
2,5-Diisopropylhydroquinone	4.1	—	5.1	—	4.1	3.9
2,5-Di-*tert.*-butylhydroquinone	6.3	—	—	—	7.8	7.7
2-Methoxy-4-*tert.*-butylphenol	—	—	—	—	9.3	8.9
2-*tert.*-Butyl-4-methoxyphenol	—	—	—	—	9.1	9.2
4,4′-Isopropylidene-bisphenol	2.3	2.3	2.0	1.7	—	—
2-Methyl-4,4′-isopropylidene-bisphenol	3.9	3.8	3.0	3.1	—	—
2,6-Dimethyl-4,4′-isopropylidene-bisphenol	6.1	—	3.9	4.0	—	—
4,4′-Isopropylidene-bis(2-methylphenol)	5.4	5.3	4.5	—	—	—
6-Methyl-4,4′-isopropylidene-bis(2-methylphenol)	—	—	5.3	5.4	4.0	4.0
4,4′-Isopropylidene-bis(2,6-dimethylphenol)	—	—	6.2	6.3	5.3	5.5
2-Ethyl-4,4′-isopropylidene-bisphenol	5.0	4.9	4.4	4.5	—	—
4,4′-Isopropylidene-bis(2-ethylphenol)	7.5	7.5	6.7	7.3	5.2	5.3
6-Ethyl-4,4′-isopropylidene-bis(2-ethylphenol)	—	—	—	—	7.2	7.5
4,4′-Isopropylidene-bis(2,6-diethylphenol)	—	—	—	—	10.2	9.7
2-Isopropyl-4,4′-isopropylidene-bisphenol	5.6	5.7	5.8	5.7	4.0	3.6
4,4′-Isopropylidene-bis(2-isopropylphenol)	—	—	—	—	7.1	7.5
6-Isopropyl-4,4′-isopropylidene-bis(2-isopropylphenol)	—	—	—	—	10.5	10.5
2-*tert.*-Butyl-4,4′-isopropylidene-bisphenol	6.8	—	7.0	7.5	5.3	5.5
2,6-Di-*tert.*-butyl-4,4′-isopropylidene-bisphenol	—	—	—	—	11.5	11.7
4,4′-Isopropylidene-bis(2-*tert.*-butylphenol)	—	—	—	—	10.5	11.3
2-*tert.*-Octyl-4,4′-isopropylidene-bisphenol	—	—	—	—	8.8	8.8
2-*tert.*-Dodecyl-4,4′-isopropylidene-bisphenol	—	—	—	—	11.7	11.8
2-Methyl-2′-*tert.*-butyl-4,4′-isopropylidene-bisphenol	—	—	—	—	6.8	6.9
2,6-Dimethyl-2′-*tert.*-butyl-4,4′-isopropylidene-bisphenol	—	—	—	—	7.9	8.4
6-Methyl-2′-*tert.*-butyl-4,4′-isopropylidene-bis(2-methylphenol)	—	—	—	—	8.8	9.2
2-Methyl-6-*tert.*-octyl-4,4′-isopropylidene-bisphenol	—	—	—	—	9.8	—
2-Methyl-2′-*tert.*-octyl-4,4′-isopropylidene-bisphenol	—	—	—	—	10.4	10.2
4,4′-Methylene-bis(2,6-xylenol)	—	—	4.7	4.3	3.7	—
4,4′-Methylene-bis(2-methyl-6-*tert.*-butylphenol)	—	—	—	—	11.1	11.1
3,3,3′,3′-Tetramethyl-1,1′-spirobiindane-6,6′-diol	6.7	—	6.4	—	5.3	—
3,3,3′,3′-Tetramethyl-1,1′-spirobiindane-6,6′,7-triol	3.4	—	2.6	—	—	—

TABLE V

R_C VALUES OF THE 3-ALKYL DERIVATIVES OF PYROCATECHOL

Paper impregnated with 20% monomethylformamide, developed with a mixture of diisopropyl ether–heptane (1:2).

Compound	R_C
3-*tert.*-Butylpyrocatechol	7.2
3-*tert.*-Butyl-5-methylpyrocatechol	8.1
3-*tert.*-Butyl-5-ethylpyrocatechol	9.2
3-*tert.*-Butyl-5-propylpyrocatechol	10.3
3,5-Di-*tert.*-butylpyrocatechol	11.0
3-Methyl-5-*tert.*-butylpyrocatechol	6.1
3-Isopropylpyrocatechol	4.7

Table III for the calculations. These compounds have higher R_C values than the calculated ones. Another example of the finding that the chromatographic behaviour of phenolic derivatives can be influenced by some other structural feature is illustrated by the behaviour of 4-*tert.*-octylpyrocatechol and 4-*tert.*-octyl-5-methylpyrocatechol, whose R_F values are virtually identical.

It can be concluded, therefore, that in order to determine ΔR_C values which would characterise all the structural features of a substituted phenol molecule, it would be necessary to investigate a much larger set of compounds than we had at our disposal.

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Notes

CHROM. 4882

Gas–liquid chromatography of Caramiphen hydrochloride and its analogues*

Parpanit (Caramiphen hydrochloride, 2'-diethylaminoethyl 1-phenylcyclopentanecarboxylate hydrochloride) and many of its analogues have been prepared and studied in these laboratories as possible antidotes for anticholinesterase poisoning[1,2]. The feasibility of using gas–liquid chromatography both as a criterion of purity for these compounds and as a means for their quantitative analysis has been briefly examined. Hydrochloride salts of this type decompose to the corresponding free bases during GLC and consequently it is possible to determine quantitatively either the free base or hydrochloride by GLC analysis.

Experimental

Except where otherwise specified, all gas–liquid chromatography was done using a Hewlett-Packard, F and M 5750 Research Chromatograph, employing a 6 ft. × $\frac{1}{4}$ in. O.D. glass column filled with 3.8% UCW 98 on Chromosorb W (DMCS), 60–80 mesh. Conditions were standardised at: column temperature 195°, injector 220°, detectors 235° (thermal conductivity detector when compound recovery was desired and flame ionisation detector for all other chromatography), helium flow rate 25 ml/min, hydrogen and air pressure 10 and 36 p.s.i., respectively.

The hydrochloride salts (5 mg) were dissolved in chloroform (20 μl) and 0.1-μl aliquots of these solutions were injected for chromatography. The corresponding free bases were obtained by treating 50 mg of each of the individual salts with 1 ml of 1 *N* sodium hydroxide. The resultant oily suspensions, after shaking for 5 min, were extracted with ether (4 × 1 ml). Samples (1 μl) of the combined ether extracts were injected into the chromatograph. Table I gives a list of the retention times observed for twenty-four compounds of this type.

Discussion

The apparatus and conditions described under *Experimental* gave a lower detectable limit of 1×10^{-11} g for Parpanit when 0.01 μl of a chloroform solution (1×10^{-3} mg/ml) was injected. Other stationary phases and loadings were examined, *e.g.* 6% nitrile gum XE-60 or 10% UCW 98, but a lower detectable limit of only 10^{-9} g was observed. Severe peak broadening was obtained with the XE-60 column when larger injections (0.8 mg) were made for isolation and recovery of samples.

Initial experiments on the chromatography of Parpanit revealed that both forms (salt and base) gave a single peak with identical retention times. Table I indicates that this behaviour is a characteristic common to all the analogues examined. Investigation of this phenomenon by preparative chromatography of the free base (5-μl neat injections) and hydrochloride (1.2 mg in chloroform, 10-μl injections) of Parpanit, coupled with IR examination, showed both recovered materials to be the free base.

* Issued as DREO Report No. 625.

TABLE I

RETENTION TIMES FOR FREE BASES AND HYDROCHLORIDE SALTS OF PARPANIT AND ANALOGUES

No.	*Compound*	*Retention time (min)*	
		Free base	*Hydrochloride salt*
1	2′-Diethylaminoethyl 1-phenylcyclopentanecarboxylate (Parpanit)	6.4	6.3
2	2′-Diethylaminoethyl 1-*m*-methylphenylcyclopentanecarboxylate	7.6	7.6
3	2′-Ethylmethylaminoethyl 1-phenylcyclopentanecarboxylate	5.1	5.1
4	2′-Ethylaminoethyl 1-phenylcyclopentanecarboxylate	4.0	4.1
5	2′-Isopropylethylaminoethyl 1-phenylcyclopentanecarboxylate	8.0	8.0, 5.0[a], 10.0[a]
6	2′-Diisopropylaminoethyl 1-phenylcyclopentanecarboxylate	9.6	9.7, 2.4[a]
7	2′-Isopropylpropylaminoethyl 1-phenylcyclopentanecarboxylate	10.2	10.3
8	2′-Di-*n*-propylaminoethyl 1-phenylcyclopentanecarboxylate	10.6	10.6, 3.0[a]
9	2′-Di-*n*-butylaminoethyl 1-phenylcyclopentanecarboxylate	17.8	17.8
10	2′-Diisobutylaminoethyl 1-phenylcyclopentanecarboxylate	13.9	14.0
11	2′-Di(2″-butyl)aminoethyl 1-phenylcyclopentanecarboxylate	17.5	17.5
12	2′-*tert.*-Butylaminoethyl 1-phenylcyclopentanecarboxylate	5.6	5.6
13	2′-*tert.*-Butylmethylaminoethyl 1-phenylcyclopentanecarboxylate	8.4	8.4
14	2′-*tert.*-Butylethylaminoethyl 1-phenylcyclopentanecarboxylate	10.2	10.2
15	3′-Diethylaminopropyl 1-phenylcyclopentanecarboxylate	8.4	8.4
16	2′-Dicyclohexylaminoethyl 1-phenylcyclopentanecarboxylate	12.7	12.8, 3.6[a]
17	2′-Dibenzylaminoethyl 1-phenylcyclopentanecarboxylate	5.3	5.4
18	2′-Piperidinoethyl 1-phenylcyclopentanecarboxylate	12.0	12.0
19	1′-Methyl-4′-piperidinyl 1-phenylcyclopentanecarboxylate	7.4	7.4
20	1′-Methyl-4′-piperidinyl 1-*o*-methylphenylcyclopentanecarboxylate	10.6	10.6
21	1′-Methyl-4′-piperidinyl 1-*m*-methylphenylcyclopentanecarboxylate	10.2	10.2
22	1′-Methyl-4′-piperidinyl 1-phenylcyclobutanecarboxylate	5.3	5.4
23	1′-Methyl-4′-piperidinyl 1-*p*-methylphenylcyclobutanecarboxylate	7.2	7.1
24	1′-Methyl-4′-piperidinyl 1-*p*-methoxyphenylcyclobutanecarboxylate	12.6	12.6

[a] Minor peaks.

A similar examination of all the compounds listed in Table I was not made, but it is assumed that the same conversion of salt to free base during chromatography is responsible for the correspondence in retention times for salts and bases. The facile interconversions observed for these amino ester hydrochlorides, although known in the literature, were unexpected because of the extensive experimental precautions taken by UMBREIT and co-workers[3] to ensure the conversion of their amine hydrochlorides to the free bases prior to GLC analysis by packing the injection part of the apparatus with 20% KOH coated on Chromosorb 101. It seems probable that the conversion of Parpanit and its analogues to the free bases observed here owes its origin to the fact that the latter are considerably weaker bases (pK_a values range from 7.7–8.1, ref. 4) than the amines examined by UMBREIT and co-workers[3] (pK_a values range from 9.8–10.6, ref. 5) and consequently form less stable salts. Some of the hydrochlorides shown in Table I (compounds 5, 6, 8, and 16) exhibit minor peaks in addition to the major peak attributed to the free base. No effort was made to identify these materials since their presence did not detract from the general usefulness of the method for identification or analysis of the compounds.

Decomposition during gas–liquid chromatography has been observed with amino acids[6], diols[7], unsaturated compounds[8], amine salts[3], corticosteroids[9], sterols[10], and terpene alcohols[11]. The cause of decomposition of some of these compounds has been attributed to the type of column packing[6,7] or to the column material itself[3].

Several column packings were examined in the present work to determine if the column packing was critical for the interconversion. Since all the amine hydrochlorides behaved in the same manner on all the columns examined it seems likely that the interconversion owes its origin to the thermal instability of the compounds themselves rather than to the action of the packing materials. The necessity of treating the solid support, especially diatomaceous earth types, with organic bases such as tetrahydroxyethylethylenediamine (THEED)[12], tetraethylenepentamine (TEP)[12], and polyethyleneimine (PEI)[13], or inorganic bases such as sodium or potassium hydroxide[12–16] to prevent tailing of amines is well known and has been emphasized by UMBREIT and co-workers[3]. In the work reported here, however, Chromosorb W commercially treated with dimethyldichlorosilane performed extremely well and no additional pretreatment was necessary.

An increase in retention time is associated with an increase in the bulk and number of carbon atoms contained in the substituents attached to the nitrogen atom (*cf.* compounds 4 and 1, 1 and 8, 8 and 9 in Table I). Branching of the carbon chain attached to nitrogen lessens the retention time in relation to that of the straight-chain homologue (*cf.* compounds 8 and 6, 9 and 10 in Table I). These observations are probably mainly manifestations of the differences in volatilities of the compounds as reflected by the boiling point behaviour of aliphatic amines, possibly modified by donor and acceptor hydrogen-bonding effects which are known to be very active in the chromatography of amines[16], and which can vary considerably in magnitude with variation in the type and method of pretreatment of the solid support[17].

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Design of a column for the gas chromatographic analysis of chlorinated hydrocarbon pesticides

The mixed silicone–fluorosilicone column of BURKE AND HOLSWADE[1] commonly used for pesticide residue analysis does not sufficiently separate hexachlorobenzene (HCB) and α-benzene hexachloride (α-BHC), which is the isomer normally used in estimation of total benzene hexachloride. Occurrence of peaks for these compounds necessitates re-analysis on another, more polar column, which may not be satisfactory for separation of the other chlorinated hydrocarbons of interest because of peak overlaps or impractically long retention times.

Theory

Separation of pesticides in order of their vapour pressures alone is not readily feasible. The most direct way to an ideal column would be by selection of suitable solute–solvent interactions, using differences in the structure, size or shape of the molecule. Data of this kind is not always easily available, and is rather complex to apply.

Many effective chromatographic systems have been designed by trial and adjustment, but the bewildering array of commercially available stationary phases complicates the choice, and many workers tend to use certain favourite phases already on their shelves[1]. Some guidance can be obtained by using the classification of stationary phases described by BROWN[2], who ascribed to a number of compounds vectors of electron-acceptor, electron-donor, and non-polar properties. From retention data found by experiment on a range of compounds with different characteristics on a BROWN plot, several stationary phases may be chosen as closest in properties required for best separation of the solutes of interest. Intermediate properties closer to ideality may then be obtained from mixtures of these phases, the retention data being predictable by methods such as those used by HILDEBRAND AND REILLEY[3].

Mixed stationary phases are tailored by manipulating retention times directly by changing composition. This is done by standardising or normalising other variables. If liquid volume (V_l) in a column is much smaller than gas volume (V_g), it will be linear with partition ratio (k), thus:

$$k = K \frac{V_l}{V_g}$$

where K is the partition coefficient.

Partition ratio is the relation between solute retention time (t_R) and that of an unsorbed material, usually air (t_A), the solute retention time having been corrected for the air peak.

Partition ratios for experimentally different columns may be normalised for gas volume (V_g) and packing weight (w) as follows:

$$k = k_{(\mathrm{exp})} \times \frac{V_{g(\mathrm{exp})}}{V_{g(\mathrm{norm})}} \times \frac{w_{(\mathrm{norm})}}{w_{(\mathrm{exp})}}$$

where (exp) means experimental and (norm) normalised.

Thus t_R becomes a linear function of V_l.

For two stationary phases (A and B) in one column, $V_l = V_{lA} + V_{lB}$, and $t_R = t_{RA} + t_{RB}$. Then

$$\frac{t_{RA} + t_{RB}}{V_{lA} + V_{lB}} = \text{constant}$$

As the stationary phase composition is altered from all "A" to all "B", the retention time will change in a linear fashion from t_{RA} to t_{RB}. Therefore t_R (and hence k) can be predicted for any solute for any column composition.

The optimum mixture may be found for any pair of solutes from the separation factor (SF), which is the ratio of the larger to the smaller of the two partition ratios. It is a useful guide to separating power, as the lower the SF, the more highly efficient must be the column, to effect resolution. Plotting SF against column composition will show the optimum proportions of the mixture.

In practice, the use of mixed phases may introduce several difficulties, *viz.* (1) Differing temperature stabilities can change column characteristics by preferential bleeding or chemical alteration of one component. (2) Interaction between components can result in anomalous behaviour. Series or mixed-bed packing can minimise this problem. (3) Series packing itself can introduce differences in pressures gradients in different sections of the column, and change partition ratios.

Experimental

The phases selected for study have a variety of positions of the BROWN plot. They are: DC-200 (methyl silicone); QF-1 (trifluoro propyl methyl silicone); XE-60 (cyanoethyl methyl and dimethyl silicone); Zonyl E-7 (fluoroalkyl pyromellitic ester); NPGS (poly(neopentyl glycol succinate), (HI-EFF 3B)).

Retention data were collected for the pesticides on the selected stationary phases under the following conditions: instrument, Packard Model 803; detector, electron capture; column, glass, 6 ft. × 4 mm I.D.; support, Gas-Chrom Q, 80–100 mesh; temperature, 200°; flow rate, 200 ml of nitrogen per min.

Some results are shown in Table I.

TABLE I

GC RETENTION TIMES OF CHLORINATED HYDROCARBON PESTICIDES ON SELECTED PHASES

Retention times are corrected for retention of unsorbed solute by using measurements from the solvent peak.

Phase	*Retention time relative to Dieldrin = 100 (RRTD)*				
	10% DC-200	*10% QF-1*	*10% XE-60*	*10% Zonyl*	*2% NPGS*
HCB	22	19	18	22	11
α-BHC	24	25	30	25	30
Lindane	28	31	41	36	44
DDE	100	69	81	136	92
Dieldrin	100	100	100	100	100
DDD	140	116	192	341	257
DDT	171	127	192	—	222

Discussion

Of those phases which satisfactorily separate HCB, α-BHC and lindane, XE-60 and QF-1 do not distinguish sufficiently between DDD and DDT, and Zonyl appears to cause some breakdown of DDT. NPGS seems more useful, although separation of DDE and Dieldrin is inadequate. As QF-1 selectively retains the oxygenated compound Dieldrin, the possibility of using it to modify a column of NPGS emerged.

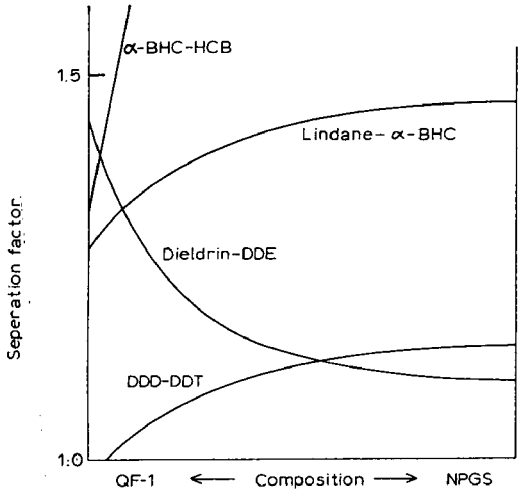

Fig. 1. Plot of separation factors to find optimum substrate composition.

Partition ratios for each solute were calculated for both pure stationary phases from experimental results, then predicted for a range of mixtures from 2% QF-1–0% NPGS to 0% QF-1–2% NPGS. Separation factors were calculated for pairs of pesticides, and plotted against column composition, as shown in Fig. 1. It would appear that optimum separation is available at close to equal proportions of the two constituents.

Confirmatory experiments

A column was made, to contain 2% QF-1 and 2% NPGS. The method of coating

TABLE II

RELATIVE RETENTION TIMES OF CHLORINATED PESTICIDES ON PROPOSED COLUMN

Pesticide	*RRTD*	*Pesticide*	*RRTD*
HCB	13	Heptachlor epoxide	68
Methoxychlor	27, 420	Chlordane	74, 27
Chlordane	27, 74	DDE	88
Heptachlor	28	Dieldrin	100
α-BHC	29	Endrin	113
Aldrin	29	DDT	209
Lindane	41	DDD	235
Telodrin	42	Methoxychlor	420, 27

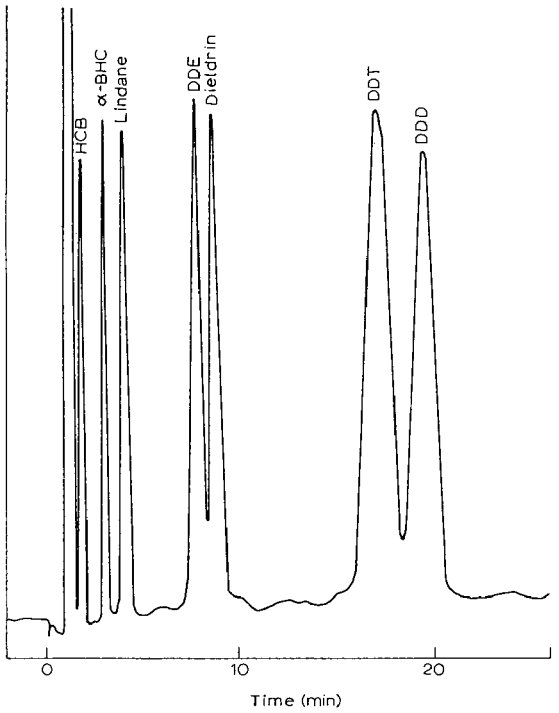

Fig. 2. Chromatogram of chlorinated pesticides on a column of 2% QF-1 and 2% NPGS.

was as follows: Dissolve the required weights of QF-1 and NPGS in acetone and chloroform, respectively. Mix and dilute to approximately 100 ml with acetone in a conical flask. Add 10 g of Gas-Chrom Q (80–100 mesh). Boil for a few minutes until support appears to be degassed. Remove solvent by evaporation and dry by gentle agitation in a stream of dry nitrogen.

This column exhibited retention characteristics close to those predicted. The pesticides are satisfactorily separated under the conditions used. A chromatogram is shown in Fig. 2. It has since been discovered that total column loading may be reduced to 1% without loss of retention characteristics or peak symmetry. Very rapid analyses are possible at this level.

Retention data

Experimentally found retention times, relative to Dieldrin, of a range of chlorinated pesticides, are collected in Table II.

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Die Kombination von Dünnschicht- und Gaschromatographie bei der Identifizierung aliphatischer Hydroxy- und Dicarbonsäuren

Chromatographisch vorgetrennte Substanzen werden häufig durch anschliessende IR-Spektroskopie identifiziert[1]. In der qualitativen Analyse von Fettsäuren lässt sich zu ihrer Identifizierung vorteilhaft die Dünnschicht- mit der Gaschromatographie kombinieren[2,3]. Die Dünnschichtchromatographie auf Cellulose wurde zur Abtrennung der Milchsäure von den aus Muskelgewebe mitextrahierten Säuren eingesetzt[4]. Die sich anschliessende Bestimmung der Milchsäure erfolgte nach ihrer Elution mit Wasser mittels chemischer Methoden[4]. Eine Kombination von Dünnschicht- und Gaschromatographie erschien bei der Identifizierung von aliphatischen, nicht zur Fettsäurereihe gehörigen Carbonsäuren, von Vorteil.

Methode

Die zu untersuchenden Lösungen und Extrakte von Hydroxy- und Dicarbonsäuren wurden auf Schichten, die zu gleichen Teilen aus Kieselgur und Kieselgel G bestanden, aufgetragen und mit dem Laufmittel: Benzol–Äthanol–NH_4OH (25%) (10:20:5) dünnschichtchromatographiert[5]. Sichtbar gemacht wurden die Säuren vor dem Auskratzen mit Bromkresolgrün. Die abgeschabten Säurebanden blieben zur Elution mehrere Stunden (oder über Nacht) mit Methanol im Überschuss stehen. Die methanolische Lösung der Säuren konnte durch Abfiltrieren mittels Porzellantiegel vom silikathaltigen Trägermaterial abgetrennt werden. Die am Rotavapor eingeengten Lösungen wurden nun zum Methylieren mit 0.15–0.3 ml Methanol–BF_3 aufgenommen. Die bei Zimmertemperatur über Nacht gebildeten Methylester konnten ohne weitere Vorarbeiten, in Mengen von 1–4 μl direkt in den Gaschromatographen injiziert werden. Das miteluierte Bromkresolgrün störte die Gaschromatographie nicht. Die Trennung der Säuremethylester erfolgte auf Reoplex (400) (10% auf Chromosorb W)[6] mit einem Perkin-Elmer-Gerät F_{20}, mit FID-Detektor ausgestattet. Wie hierzu früher beschrieben[7], war es vorteilhaft mit Temperaturprogrammierung zwischen 60° und 170° und einer Anstiegsrate von 3°/min zu arbeiten. Die Durchflussgeschwindigkeit des Trägergases Stickstoff betrug 65 ml/min. Der Wasserstoffverbrauch wurde auf eine Strömungsgeschwindigkeit von 33 ml/min und synthetische Luft auf 350 ml/min eingestellt.

Ergebnisse

(a) Untersuchung eines Reinsubstanzgemisches

Aus einer Säurelösung, die jeweils 0.2%ig an Bernsteinsäure und Fumarsäure und 0.4%ig jeweils an Glykolsäure und β-Hydroxybuttersäure sowie 0.4%ig an Na-Lactat war, wurden pro Platte 100–250 μl strichförmig aufgetragen. Unter den in der Methodik beschriebenen Bedingungen konnten folgende R_F-Werte ermittelt werden: Bernstein-, 0.17; Fumar-, 0.32; Glykol-, 0.42; Milch-, 0.55; und β-Hydroxybuttersäure, 0.65 (Hauptbande). Zusammengehörige Fraktionen von mehreren Platten wurden zum Teil gemeinsam eluiert und zur Gaschromatographie vorbereitet. Jede individuelle Bande der Dünnschichtplatte enthielt nach dem Methylieren im Gas-

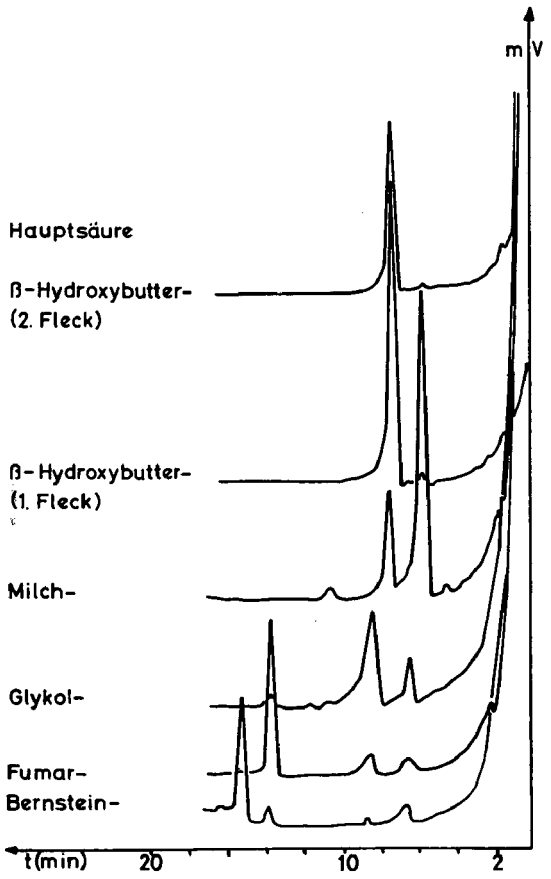

Fig. 1. Gaschromatographie der Methylester der aus Silikatplatten eluierten Säuren eines Reinsubstanzgemisches, auf Reoplex (400). (Anordnung der Gaschromatogramme entsprechend zunehmender R_F-Werte im Dünnschichtchromatogramm.)

chromatogramm, wie Fig. 1 zeigt, eine Hauptfraktion und geringe Mengen der übrigen Lösungsmittelpartner. β-Hydroxybuttersäure ergab mehrere Banden, von welchen in Fig. 1 nur die ersten beiden aufgezeigt wurden.

(b) Chromatographie einzelner Hydroxysäuren

Sowohl β-Hydroxybuttersäure, wie auch 3-Hydroxybuttersäure ergaben bei der Dünnschichtchromatographie neben einer Hauptfraktion, mehrere darüber liegende Nebenfraktionen (vgl. Tabelle I). Nach Elution und Methylieren der letzteren konnte im Gaschromatogramm jedoch für alle nur ein, jeweils an der Stelle der Hauptbande liegender Peak, beobachtet werden.

Es wurde deshalb vermutet, dass es sich bei diesen Nebenfraktionen um kettenförmige Polymere der entsprechenden monomeren Hydroxysäuren handeln könnte. Aus den Estoliden dürften sich beim Eluieren, bzw. Methylieren, die zu Grunde liegenden monomeren Hydroxysäuren zurückgebildet haben.

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHIE VON HYDROXYBUTTERSÄUREN

Laufmittel: benzol–äthanol–NH_4OH (10:20:5); Schicht: Kieselgur–Kieselgel (1:1).

	R_F-Wert	Mengenverhältnis der Fraktionen zueinander[a]
3-Hydroxybuttersäure		
Hauptfraktion	0.55	1.00
Nebenfraktion	0.65	0.39
Nebenfraktion	0.72	0.21
β-Hydroxybuttersäure[b]		
Hauptfraktion	0.55	1.00
Nebenfraktion	0.64	0.15
Nebenfraktion	0.70	0.12
Nebenfraktion (sehr schwach)	0.79	—

[a] Nach Elution der Banden gaschromatographisch ermittelt. Innerer Standard: Maleinsäuredimethylester.

[b] Zur besseren Ermittlung der Nebenbanden wurden höhere Konzentrationen als im Reinsubstanzgemisch aufgetragen. Dadurch wurden die R_F-Werte erniedrigt.

Diese Annahme wird vor allem dadurch gestützt, dass beim Lösen und Methylieren des ringförmigen Lactids, der Milchsäure, im Gaschromatogramm ebenfalls nur ein einzelner Peak an der Stelle des entsprechenden Methylesters beobachtet wird. Bei der Dünnschichtchromatographie des in Wasser gelösten Lactids konnte jedoch auch Lactylmilchsäure, das Zwischenprodukt der hydrolytischen Spaltung, nachgewiesen werden.

Wie aus Tabelle I entnommen werden kann, lassen sich die beiden isomeren Hydroxybuttersäuren dünnschichtchromatographisch unter angegebenen Bedingungen nur schwer von einander trennen. Eine gaschromatographische Differenzierung ihrer Methylester ist jedoch auf Reoplex (400) ohne Schwierigkeiten möglich, da der Methylester von 3-Hydroxybuttersäure wesentlich später, als derjenige von β-Hydroxybuttersäure, aus der Säule austritt (vgl. Fig. 2).

Die Methylester von Glykol- und β-Hydroxybuttersäure liegen dagegen in ihren Retentionsvolumen auf Reoplex (400) so nahe zusammen (vgl. Fig. 2), dass sich bei ihnen für den qualitativen Nachweis, die Dünnschichtchromatographie anbietet (vgl. R_F-Werte, Abschnitt a).

(c) Säureuntersuchungen an Lebensmitteln

Leber. Aus Rinderleber wurden, wie früher für Muskelgewebe und Fleischerzeugnisse[7] beschrieben, die Säuren extrahiert. Der Säureextrakt konnte dünnschichtchromatographisch in die Fraktionen A–G aufgetrennt werden (vgl. Fig. 3). Für die einzelnen Fraktionen wurden folgende R_F-Werte ermittelt: Frakt. B, 0.09; Frakt. C, 0.19; Frakt. D, 0.41; Frakt. E, 0.56; Frakt. F, 0.68; und Frakt. G, 0.75. Bei den Fraktionen B, C, D und F reichte die Konzentration auf dem Dünnschichtchromatogramm für eine anschliessende Gaschromatographie aus (vgl. Fig. 4). Übereinstimmend mit

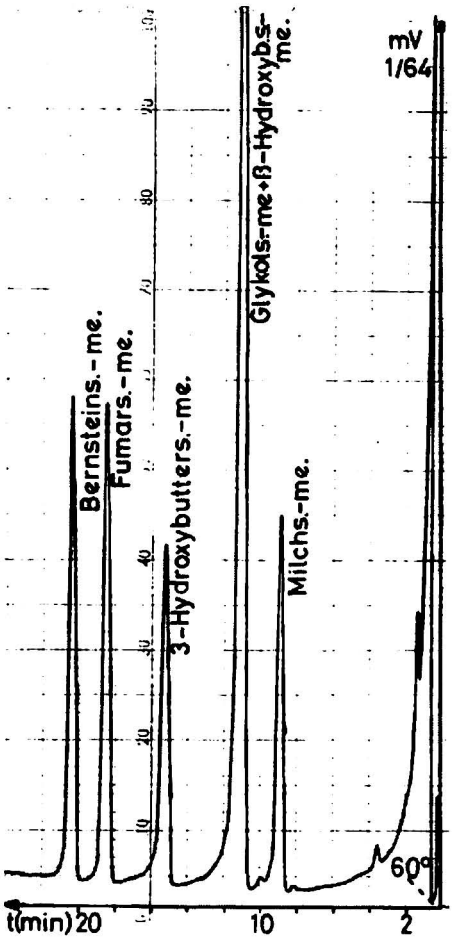

Fig. 2. Gaschromatographie der Methylester eines Reinsubstanzgemisches auf Reoplex (400), temperaturprogrammiert ab 60°, 3°/min. Empfindlichkeit 1/64 der maximalen Geräteempfindlichkeit. Abkürzung: me. = Methylester.

beiden Verfahren wurde damit im Vergleich mit Reinsubstanzchromatogrammen Fraktion B als Bernsteinsäure, Frakt. C als Fumarsäure und Frakt. D als Milchsäure identifiziert. In Fraktion F wurde mittels Gaschromatographie Milchsäure nachgewiesen. Entsprechend den vorhergehenden Ausführungen dürfte es sich hierbei um die Bande von Lactylmilchsäure handeln (vgl. Abschnitt b). In Fraktion G waren höhere Fettsäuren angereichert. Die hier eingesetzten Bedingungen für die Dünnschichtchromatographie eigneten sich jedoch nicht für eine vollständige Abtrennung der Fettsäuren von den übrigen Fraktionen. Die Gaschromatographie (in Fig. 4) wurde deshalb nach Erscheinen der Methylester der aliphatischen Nichtfettsäuren abgebrochen. Bei Fraktion E dürfte es sich, wie Vergleichsuntersuchungen an Hähnchenmuskulatur mit entsprechend höheren Säurekonzentrationen zeigten, um 3-Hydroxybuttersäure handeln. Bei Leber reichte die Konzentration dieser Fraktion nicht zur Aufarbeitung für ein Gaschromatogramm aus.

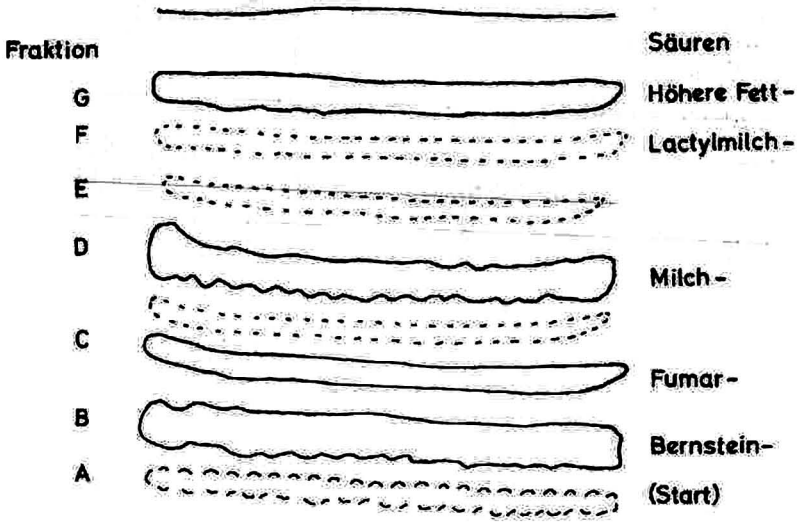

Fig. 3. Dünnschichtchromatographische Auftrennung der aus Leber extrahierbaren Säuren in Fraktionen. Schicht: Kieselgur–Kieselgel (1:1). Laufmittel: Benzol–Äthanol–NH_4OH (10:20:5).

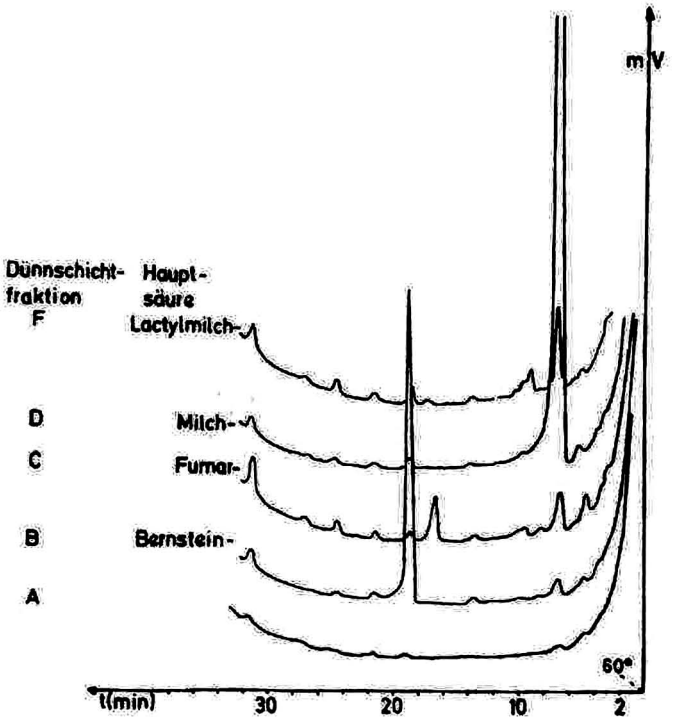

Fig. 4. Gaschromatographie der Methylester von dünnschichtchromatographisch vorgetrennten Säurefraktionen aus Leber. (Anordnung der Gaschromatogramme der einzelnen Banden entsprechend dem Dünnschichtchromatogramm in Fig. 3.)

Fig. 5. Dünnschichtchromatographische Auftrennung der aus Apfelsaft extrahierbaren Säuren in Fraktionen. Schicht: Kieselgur–Kieselgel (1:1). Laufmittel: Benzol–Äthanol NH_4OH (19:20:5).

Apfelsaft. Entsprechend wie bei Leber, konnte bei der Säureuntersuchung eines käuflichen Apfelsaftes verfahren werden. Die Säuren wurden mit Äther ausgeschüttelt und dünnschichtchromatographisch in die Fraktionen A–F aufgetrennt (vgl. Fig. 5). Auf Grund der R_F-Werte in der Dünnschicht und der Retentionszeiten der Methylester auf Reoplex (400), im Vergleich zu Reinsubstanzchromatogrammen, erfolgte die Identifizierung (vgl. Fig. 5 und 6).

In Fraktion A (R_F 0.05) wurde Äpfelsäure und in geringer Menge Citronensäure (in Fig. 6 nicht angezeichnet) nachgewiesen. Fraktion B (R_F 0.12) enthielt Bernsteinsäure; Fraktion C (R_F 0.24) Fumarsäure und Fraktion D (R_F 0.47) die in frischem Kernobst nicht vorhandene Milchsäure. Fraktion E (R_F 0.51) und Fraktion F (R_F 0.60) wurden nicht identifiziert (vgl. Fig. 5).

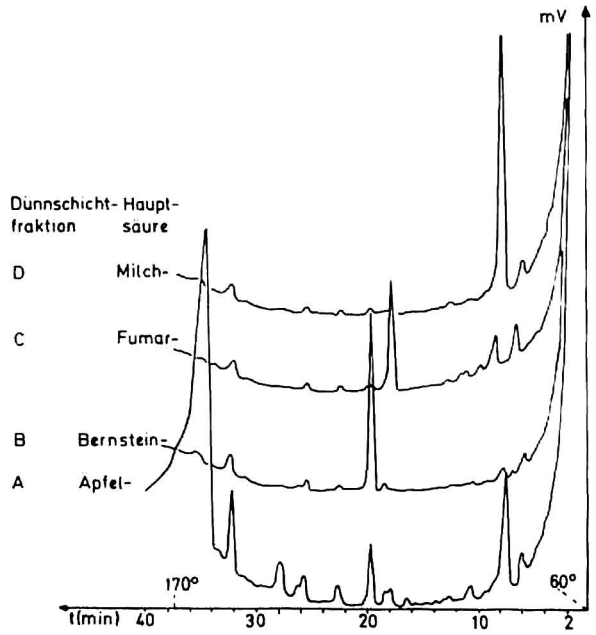

Fig. 6. Gaschromatographie der Methylester von dünnschichtchromatographisch vorgetrennten Säurefraktionen aus Apfelsaft, temperaturprogrammiert 60–170°, 3°/min. (Anordnung der Gaschromatogramme entsprechend dem Dünnschichtchromatogramm in Fig. 5.)

Schlussfolgerungen

Die Empfindlichkeit des Säurenachweises durch Kombination von Dünnschicht- und Gaschromatographie hängt sowohl vom Grad der Eluierbarkeit einer Säure aus dem silikathaltigen Trägermaterial, wie auch von der Intensität des Elektronenstromes beim Ionisieren der Substanz im FID-Detektor ab. Bei Bernstein- und Fumarsäuredimethylester (hiermit vergleichbar ist auch Maleinsäuredimethylester) ist die Empfindlichkeit im FID-Detektor höher, als bei den überprüften Hydroxysäuren. Bei den letztgenannten ist der Nachweis bei Milchsäuremethylester wiederum empfindlicher, als für Glykolsäuremethylester. Die überprüften Dicarbonsäuren lassen sich ausserdem offenbar leichter als die Hydroxysäuren aus dem Silikat-Trägermaterial eluieren.

Um die hochsiedenden Methylester von Äpfelsäure und Citronensäure nachzuweisen, ist es vorteilhafter in der Gaschromatographie bei 170° isotherm zu arbeiten. Citronensäure kann mit der angegebenen Kombination von Dünnschicht- und Gaschromatographie schlecht nachgewiesen werden, da ihre Elution aus der Dünnschichtplatte unzureichend ist, und ihr Methylester ausserdem nur eine geringe Flüchtigkeit hat. Durch die Kombination von Dünnschicht- und Gaschromatographie konnte neben den monomeren Hydroxysäuren auch der Anteil der gaschromatographisch allein nicht bestimmbaren Estolide für β-Hydroxybutter- und 3-Hydroxybuttersäure abgeschätzt werden.

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CHROM. 4885

Buffer preservative failure and modification of in-line buffer filter for automated step-change amino acid analyzers

One of the continuing problems in operation of automated liquid ion-exchange amino acid analyzers is microbial contamination of the buffers. Failure of the usually recommended preservatives, octanoic acid and pentachlorophenol, apparently is much more common than generally recognized or admitted. Moreover, problems attributed to other things, such as entrapped air, careless buffer preparation, or insufficiently "cleaned" sample, may in fact be unrecognized microbial contamination. Troubles have appeared with either sodium or lithium citrate buffers above about pH 4. The higher the pH, the more likely the difficulties.

Because of short column length for basic amino acids and very short running time, difficulties are encountered less frequently in two-column analyses of simple protein hydrolysates. However, serious problems have arisen with extended runs on longer columns, as required for complex mixtures such as physiological fluids or plant or soil extracts. For example, before signs of fungal growth were ever visible in our system, excessive column back-pressure (> 500 p.s.i.) developed in the basics column (0.9 × 27 cm PA-35 resin) which was ultimately traced to blockage of the resin support screen and/or teflon sponge at the bottom of the column. In one instance (unattended night run), pressures exceeded connector clamp strength and the whole bed of expensive spherical bead resin was pumped out and lost. This was baffling since blockage is usually at the top of or within the column, which results in abnormal compression or apparent shrinkage of the resin leading in the extreme to failure of top rather than bottom of column connections. Subsequent staining tests and microscopic examination showed contaminant material was passing the full length of the resin bed only to lodge in the resin support disc. Fortunately, regenerating hydroxide removed enough contaminant to permit a series of runs but resistant residuals did accumulate; this necessitated periodic replacement of the clogged disc. The problems were accentuated by changing to a three-buffer single-column (0.9 × 60 cm) routine for acidic, neutral and basic amino acids.

Increased dosages of octanoic acid and pentachlorophenol were ineffective or interfering. Other preservatives at initially effective concentrations appear either no better, alter sequence of elution, degrade resolution or otherwise are incompatible. MARAVALHAS[1] recently suggested diethylcarbonate as a more useful preservative which acts "...fairly well" under his conditions in South America. It remains to be seen how effective this chemical will prove for others, or how long it will remain effective before tolerant or adaptive organisms develop, as they have for octanoic acid and pentachlorophenol.

Meanwhile, analyzer owners interpose a variety of in-line filters just ahead of or in the column top connector. An effective unit for high-pressure, high-flow rate systems is the "micule filter"* (available through Beckman-Spinco, Palo Alto, Calif., U.S.A.). This is in effect a miniature column (0.9 cm I.D.) containing about 1-cm depth of inert resin beads (of essentially the same particle diameter as used in the analytical

* Mention of trademark or company names is for identification purposes and does not constitute preferential endorsement by the U.S. Department of Agriculture.

column) sandwiched between two teflon sponge disks. This inert resin supposedly is the essential part of the filter for removing all particulates, including fungal contaminants. In our system, the porous teflon sponge supports are actually the effective filters. The special resin is not needed, thereby saving both the cost and the fuss of removal, clean-up and replacement of the resin. In its place, we have simply added additional teflon sponges—as a convenience and to minimize mixing or buffer hold-up volume. When excessive back-pressure develops, the top sponge is removed; a clean disk may then be inserted, preferably at the bottom.

The teflon sponge disks are the same as those regularly used for resin support in the analytical column, and are commercially available. Alternatively, extra disks may be cut to snug-fit size, with an ordinary sharp cork borer, from a $\frac{1}{8}$-in.-thick sheet of 50–55% nominal porosity teflon sponge. New or clogged disks are cooked in refluxing concentrated nitric acid for several hours until pure white (overnight is convenient and insures complete cleaning). We might add that our sheet of stock teflon was variegated gray rather than the typical white. Microscopic examination showed the grayish color was from charcoal-like particulates in the teflon. The HNO_3 treatments removed these. They also disappeared after baking in a muffle-furnace at $> 600°$ (suggestion from Dr. P. B. HAMILTON, Dupont Institute, Wilmington, Del., U.S.A.). Clogged disks from the buffer filter probably could be adequately cleaned by similar "baking", provided the teflon sponge withstands such repeated heat treatments.

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The identification of polymyxin B sulphate

The chemistry and the structure of the polymyxin antibiotics have been elucidated by the studies of VOGLER AND STUDER[1], who published a complete list of the known polymyxins. The list shows that polymyxins are peptides with very similar structure, containing, in addition to amino acids, a fatty acid. They are always found as a mixture of two peptides, differing only in their fatty acid composition. Table I shows the amino acid and fatty acid components of the different polymyxin antibiotics.

TABLE I

AMINO ACID AND FATTY ACID COMPONENTS OF THE POLYMYXIN ANTIBIOTICS

Sample	L-*DAB*	L-*THR*	L-*LEU*	D-*LEU*	L-*ILE*	D-*PHE*	D-*SER*	D-*VAL*	*Fatty acid*
Polymyxin A	+	+	−	+	−	−	−	−	6 MOA
Polymyxin B_1	+	+	+	−	−	+	−	−	6 MOA
Polymyxin B_2	+	+	+	−	−	+	−	−	IOA
Polymyxin C	+	+	−	−	−	+	−	−	6 MOA
Polymyxin D_1	+	+	−	+	−	−	+	−	6 MOA
Polymyxin D_2	+	+	−	+	−	−	+	−	IOA
Polymyxin E_1	+	+	+	+	−	−	−	−	6 MOA
Polymyxin E_2	+	+	+	+	−	−	−	−	IOA
Polymyxin M	+	+	−	+	−	−	−	−	6 MOA
Circulin A	+	+	−	+	+	−	−	−	6 MOA
Circulin B	+	+	−	+	+	−	−	−	IOA
Polypeptin	+	+	+	−	+	+	−	+	?

Polymyxins B and E are the least toxic and at present are the only ones used in medicine. For that reason the pharmaceutical control of these antibiotics requires a specific identification, which is able to distinguish these polymyxins from the others. In this paper, a complete method for the identification of polymyxin B sulphate, specifically, as a pure substance and in pharmaceutical preparations, is given.

Material and methods

All chemicals used, except (+)-6-methyloctanoic acid (MOA) and isooctanoic acid (IOA), are commercially available. The two acids were synthetised from (−)-2-methylbutanol-1 and 2-methylpropanol-1 as described by VOGLER AND CHOPPARD-DIT-JEAN[2]. Cellulose layers for thin-layer chromatography were prepared by coating in the usual way with Cellulose G.

Amberlite IR C50 (sodium form) was prepared by stirring the resin (acid form) for 10 min with 1 *N* sodium hydroxide. The mixture is washed with water by decantation and transferred into a chromatographic column (1 cm in diameter) until a height of 20 cm is obtained.

Amino acid analysis

5 mg polymyxin B sulphate is dissolved in a tube in 0.5 ml of 5.6 *N* hydrochloric acid. The tube is sealed and heated for 22 h at 110°. The liquid is evaporated to dryness

in a warm air stream, the residue dissolved in 0.5 ml water and reevaporated. The amino acids are dissolved in 0.5 ml water. 2 μl of this solution are used for the thin-layer chromatographic analysis on Cellulose G. The following solvent system is used (non-saturated chamber): propanol-2–water–formic acid–pyridine (80:20:4:1). 1 μl of 1 mg/ml solutions of L-leucine, D-phenylalanine, L-threonine and L-α,γ-diaminobutyric acid are used as standards. Two-dimensional development with the above solvent and with methyl ethyl ketone–pyridine–acetic acid–water (70:15:2:15) can be used to check the absence of serine, valine and isoleucine.

After development and drying the plate is sprayed with a solution of 100 mg ninhydrin in a mixture of 70 ml ethanol, 21 ml acetic acid and 2.9 ml collidine. The spots appear after heating for 5 min at 60°. This staining reagent is reported as being the most convenient for the detection of amino acids on cellulose[3].

Fatty acid analysis

30 mg polymyxin B sulphate is hydrolysed in 1 ml 5.6 *N* hydrochloric acid at 120° in a sealed tube for 6 h. The hydrolysate is diluted with water and extracted three times with ether. After drying over anhydrous sodium sulphate the ether is evaporated and the residue dissolved in 1 ml carbon disulphide. 1 μl of this solution is injected into a gas chromatograph on a 20% DEGA–3% phosphoric acid column (on acid washed Chromosorb G, column length 1.5 m, carrier gas N_2 regulated at 30 ml/min, flame ionisation detector) at 175°. The retention times are compared with those of 6-methyl octanoic acid and isooctanoic acid.

The usefulness of phosphoric acid treated polyester columns for the separation of free fatty acids has been proved by METCALFE[4].

Analysis of pharmaceutical preparations

We identified polymyxin B sulphate in three preparations: an ointment, containing 30 mg oxytetracycline and 1 mg polymyxin B sulphate in 1 g vaseline base; an ointment containing 5 mg dexamethasone, 20 mg polymyxin B sulphate, 15 mg tyrothrycin, 100 mg neomycin sulphate, 4 mg 5-chloro-*p*-(diethylaminoethoxyphenyl) benzthiazole in a base of glycerol triacetate, barium sulphate and silicone oil; and an aerosol preparation containing 625 mg neomycin sulphate, 625 mg zinc bacitracin and 20 mg polymyxin B sulphate in a chlorofluorohydrocarbon propellent.

Polymyxin B is extracted from the ointments by stirring 3 g ointment with 15 ml water in a beaker for 5 min on a hot plate. The cold solution is filtered and transferred to an Amberlite IR C50 column. The flow rate is regulated at 1 ml/min. The resin is washed with 100 ml water at a rate of 5 ml/min. The polymyxin B is eluted from the resin with 100 ml 10% solution of sodium chloride in methanol–water (50:50) at a rate of 1 ml/min. The methanol in the eluate is evaporated on a water bath and the remaining aqueous solution is extracted three times with 10 ml *n*-butanol. The butanol is washed with 10 ml water to remove the sodium chloride and evaporated in a hot air stream. The residue is dissolved in 0.5 ml 5.6 *N* hydrochloric acid and analysed as described.

The aerosol preparation is analysed in a similar way: after the propellents have been removed, polymyxin B sulphate is extracted from the mixture by stirring with 10 ml water. The mixture is filtered and the clear solution analysed on the Amberlite IR C50 column as described above.

Discussion

As polymyxin antibiotics essentially have the same antibacterial spectra and are active in similar concentrations[5], microbiological methods cannot be used for the specific identification of polymyxin B sulphate. Similarly direct colour reactions on peptides, as described by ROETS AND VANDERHAEGHE[6], cannot be used, all polymycins give the same colour reactions for the polymyxins. No convenient thin-layer chromatographic methods for the separation of the different polymyxins have been found in the literature. Only polymyxin B, D and M are separated by the method described by IGLOY AND MISZEI[7,8]. We also were unable to resolve the polymyxins B and E by thin-layer chromatography.

An amino acid analysis appears to be the only method of specifically identifying polymyxin B. Table I shows clearly how a qualitative amino acid analysis can distinguish polymyxin B from the other polymyxin antibiotics. This method is used by the British Pharmacopoeia[9] and by HOWLETT AND SELZER[10]. The former method consists in hydrolysis at 135° for 5 h and a paper chromatographic amino acid identification. The second method gave good results with a hydrolysis at 120° for 6 h and amino acid identification on silica gel. To avoid the appearance of ghost spots we prefer a hydrolysis at 110° for 22 h. The thin-layer chromatographic method on Cellulose G gives a very good separation of the four polymyxin B amino acids. The R_F values are: leucine 0.82, phenylalanine 0.68, threonine 0.36, α,γ-diaminobutyric acid 0.12.

A fatty acid analysis is necessary to prove the presence of the polymyxins B_1 and B_2. We found it impossible to separate the C_8–C_9 acids as ammonium salts by thin-layer chromatography and, as an identification as the dinitrophenylhydrazide[11] or hydroxamate[12] requires a lot of time, we prefer to use a gas chromatographic identification of the methyl esters. The retention time is 5 min for isooctanoic acid and 14 min for 6-methyloctanoic acid. We observed also the presence of a third acid, only present in low concentration (5% of the total acid), with the same retention time as *n*-octanoic acid (9 min).

In pharmaceutical preparations polymyxin B sulphate can be separated from neomycin sulphate and zinc bacitracin[13] or from tetracyclin[14] by thin-layer chromatography or electrophoresis. These methods are unable to distinguish polymyxin B from the other polymyxins. For a specific identification of polymyxin B sulphate an amino acid analysis is required. Therefore polymyxin B must be separated from the other ingredients. Elutions from thin-layer plates or electrophoresis strips gave unsatisfactory results. A good separation is obtained by using the weakly acid cation exchanger Amberlite IR C50 (sodium form). Polymyxin is isolated by eluting the resin with a 10% solution of sodium chloride in a methanol–water mixture[15]. After removal of the methanol, polymyxin B is further purified by extraction with butanol and analysed as described.

Some difficulties, however, can be encountered in analysing ointments containing polymyxin B sulphate and tyrothricin. Probably owing to the solubilising effect of glycerol triacetate tyrothricin is partly extracted from the ointment with water. Some faint ghost spots, belonging to hydrolysis products of tyrothricin, interfere with the polymyxin amino acid pattern.

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The separation of nucleic acids on basic polyamino acid–kieselguhr columns

A rapid, one-step procedure for the isolation of 5 S rRNA, in a highly purified form, from total nucleic acids using columns of basic polyamino acids adsorbed onto kieselguhr has been recently described[1]. These columns (BPAK) are easy to prepare and use, and are stable at room temperature. They can be rapidly regenerated after use and can be used repeatedly without loss of resolution or decrease in flow rate. A high flow rate (3–4 ml/min) can be used without loss of resolution, thus making the analytical procedure very rapid. The resolution obtained is easily reproducible and the columns are very stable. The columns are short, longer ones made of the same material having shown no advantages, and, in conjunction with a recording photometer, provide a fast and convenient analytical method. In view of these practical advantages, we wish to report the usefulness of these columns for the resolution of other species of nucleic acids.

Poly-L-arginine sulfate (mol. wt. 21,900), poly-L-lysine HBr (mol. wt. 20,000) and poly-L-ornithine HBr (mol. wt. 15,800) were purchased from Yeda-Miles Laboratories Inc., Rehovoth, Israel, and Hyflo Supercel (particle size 5–25 μ) from Serva, Heidelberg, G.F.R. Uniformly ^{14}C-labeled amino acids were products of the Department of Biology, C.E.A., Saclay. *Escherichia coli* K12 tRNA for the experiments with aminoacyl tRNA was obtained from Schwarz Bioresearch, Orangeburg N.Y., U.S.A., and from General Biochemicals, Chargrin Falls, Ohio, U.S.A.; amino acids from Calbiochem; membrane filters (MF 50) from Sartorius Membranfilter-Gesellschaft, Göttingen G.F.R.; and alumina (bacteriological grade) from Alcoa Company of America.

The *E. coli* K12 strain used in this work was grown under vigorous aeration in a New Brunswick Microferm fermentor in a medium which contained per l: 10 g Bacto Yeast, 10 g glucose, 17 g KH_2PO_4 and 21.8 g K_2HPO_4. The pH was adjusted to 7.4. The nucleic acids were extracted from exponentially growing cells by grinding the cells with alumina and subsequently treating them with phenol[2]. Suspensions of kieselguhr (8 g) in the starting buffer (60 ml) of the elution gradient, which were sufficient to give a column of 2 × 6.5 cm, were heated to boiling, with manual stirring, to remove air bubbles and then cooled in an ice–water mixture. Polyamino acids, at a concentration of 2 mg/ml, were dissolved in water, with the exception of polyarginine which was dissolved in 0.01 *M* HCl. Each column was made by adding the required volume of the polyamino acid solution to the cooled suspension of kieselguhr and the mixture was stirred well. The slurry was poured into a glass column (2 × 15 cm), fitted with a fritted disc which had been covered with a thin layer of Whatman Standard cellulose powder, and, for reasons of speed, packed under pressure. A layer consisting of kieselguhr (2 g) in buffer (10 ml) was added and a light plug of glass wool was placed on top of the column. The packed column was washed with starting buffer (150 ml) before the sample was applied. Chromatography was carried out using a linear elution gradient at room temperature, the elution buffers being supported on a platform 200 cm above the head of the column. The elution was monitored by an LKB Uvicord absorptiometer. Subsequently, the optical density of each of the fractions was measured at 260 nm in a Zeiss spectrophotometer PMQ II.

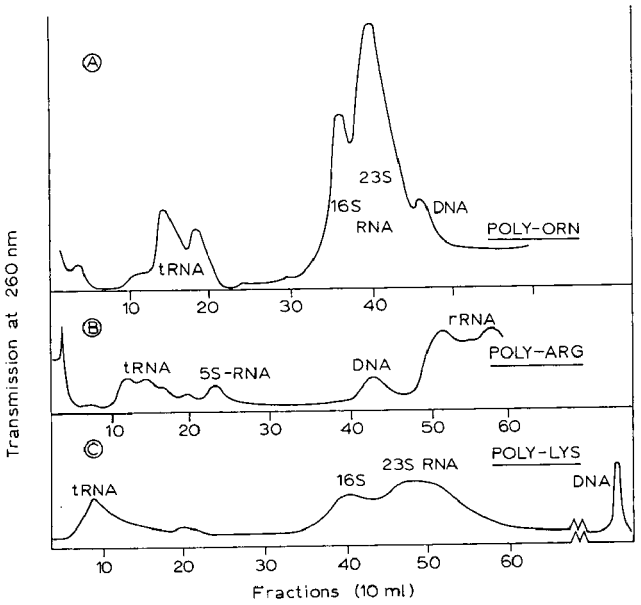

Fig. 1. Elution patterns of a nucleic acid mixture from basic polyamino acid–kieselguhr columns. The columns consisted of 6 mg polyamino acid and 8 g kieselguhr as described in the text and chromatography of the nucleic acid mixture (4–5 mg) was carried out at room temperature. The linear elution gradient, which was formed from 300 ml of starting buffer and a 300 ml reservoir of buffer and which was found to give optimal resolution of the nucleic acids, is shown for each column A = polyornithine, 0.5–3.0 *M* NaCl in 0.15 *M* phosphate (pH 6.7); B = polyarginine, 1.0 *M* NaCl in 0.15 *M* phosphate (pH 6.7)–5.0 *M* NaCl in 0.1 *M* Tris-HCl (pH 8.9); C = polylysine, 0.1 *M* NaCl in 0.15 *M* phosphate (pH 5.3)–1.6 *M* NaCl in 0.15 *M* phosphate (pH 6.7); DNA was then eluted with 3.0 *M* NaCl in 0.15 *M* phosphate (pH 6.7). The flow rate of the effluent through the cell of an LKB Uvicord was 3–4 ml/min and the transmission was plotted automatically every 20 sec. The peaks were identified as described in the text.

It was found that 6 mg of polyamino acid added to 8 g kieselguhr suffice for optimal fractionation of 5 mg nucleic acid. The elution gradients used for optimal resolution of the nucleic acid species on the three different columns are shown in Fig. 1. The identity of the peaks of the elution sequence was established by a comparative method. When applied to an MAK column, nucleic acids are resolved into a series of peaks whose nature and sequence is well known[3]. In our experiments, each of the known peaks eluted from an MAK column was chromatographed separately on each of the BPAK columns and the elution position of the various nucleic acids so found served to establish the elution sequence. It can be seen that the degree of resolution obtained with BPAK columns is different, in several respects, from that obtained with MAK columns, particularly in that 5S rRNA is well separated from 4S RNA. This latter separation was the subject of a recent communication[1]. The conditions of elution in all cases indicate that the nucleic acids are more tightly bound to BPAK columns than to MAK columns, presumably owing to the higher basicity of the former.

Under the conditions used, chromatography on polyarginine columns leads to considerable purification of DNA since rRNA is very tenaciously bound and can only

be partially eluted under conditions which do not lead to its degradation. On the other hand, the two rRNA species are eluted from columns of polylysine and polyornithine earlier than DNA: the reason for this reversal of elution sequence is not known, but a different mode of chromatography is indicated. In the case of polylysine columns DNA is very well separated from the rRNA and can only be displaced from the column by 3.0 *M* NaCl. However, in order to achieve this resolution a mildly acidic pH gradient was found to be essential; a salt gradient at a constant pH close to neutral did not lead to fractionation. Polylysine columns have already been used for the chromatography of DNA[4].

On polyarginine columns the tRNA peak is partially resolved. In order to investigate more closely the degree of resolution attained, ^{14}C-labeled aminoacyl tRNAs, the label being uniformly distributed in the amino acid moiety, were chro-

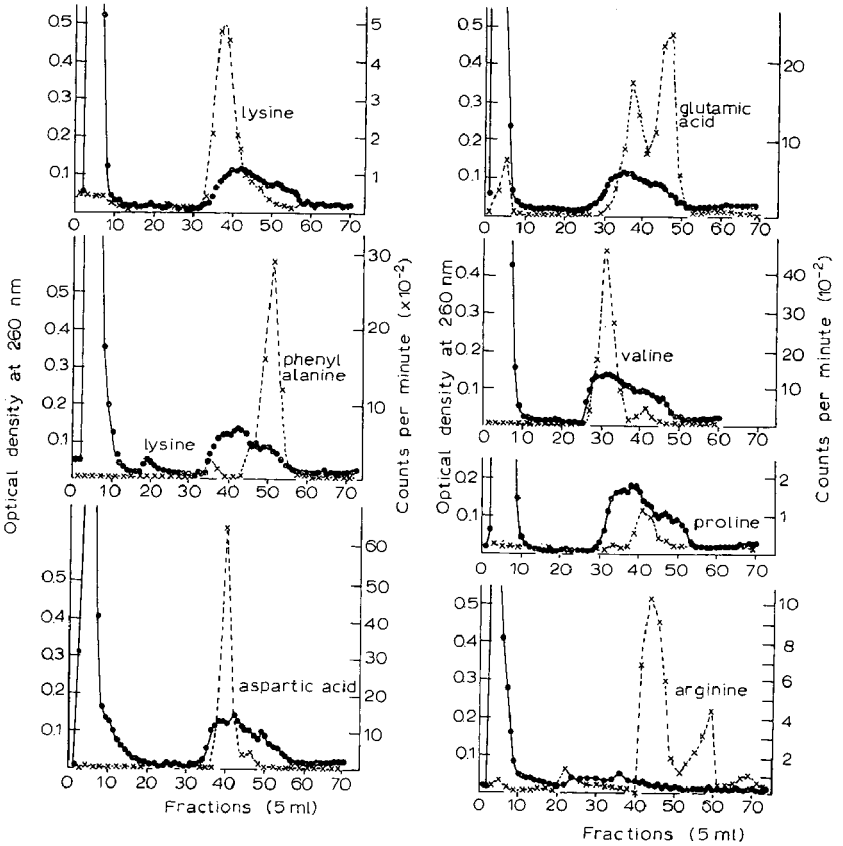

Fig. 2. Sequence of elution of ^{14}C-aminoacyl tRNA species from polyarginine columns. Stripped tRNA (5 mg) was aminoacylated as described[6], one of the twenty amino acids being uniformly labeled with ^{14}C. After phenol extraction and ethanol precipitation, the aminoacyl tRNAs were applied to a column, which was prepared from 6 mg polyarginine and 8 g kieselguhr, in 2 ml 0.4 *M* NaCl in 0.15 *M* phosphate, pH 6.0 (starting buffer). The column was eluted with a linear gradient formed from 250 ml starting buffer and 250 ml 2.5 *M* NaCl in 0.15 *M* phosphate, pH 6.0, the flow rate being 3–4 ml/min. The optical density (○—○) of each fraction was measured in a Zeiss spectrophotometer at 260 nm. Alternate fractions were adjusted to 5% with trichloroacetic acid, filtered and the filters were counted in a Packard scintillation counter (×– – – ×).

matographed. Elution patterns for seven amino acids are shown in Fig. 2. Six other amino acids whose elution profiles are not shown, were also found to be fractionated. A buffer at pH 6 was employed in order to minimize hydrolysis of the aminoacyl substituent. The resolution obtained was found to be at least as good as that found with MAK columns[5]. However, a surprising finding was that, in every case, 90% of the optical density was excluded whereas 100% of the counts/min were retained on the column, except in the case of glutamic acid. The potentially interesting implications of this finding are under further investigation.

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A simple technique for the estimation of mitochondrial glutamate–oxaloacetate transaminase in serum and tissues

It is thought that the determination of mitochondrial glutamate–oxaloacetate transaminase* (GOT II) activity in serum, during liver diseases, might give information concerning the degree of disturbed permeability of the mitochondria, and reveal the nature and severity of the underlying cellular damage[1–7].

Several methods[8–33] have been described for the separation of GOT into its mitochondrial and cytoplasmic isoenzymes. However, since the serum activity of GOT II is very low in most cases, and very labile[32,33], the characterisation of this isoenzyme is technically difficult, and therefore it is rarely used in clinical practice.

A simple and sensitive chromatographic method for the characterisation of GOT II in serum and tissue homogenates is described in this paper.

Methods

Columns (diameter 0.9 cm and length 15 cm) with a top feeding funnel, supplied by the Pharmacia Co. (Uppsala, Sweden), are used for chromatography.

Absorbent. DEAE Sephadex A 50 Medium. The ion exchanger (activated according to the directions of the Pharmacia Co.) is poured into the column, at room temperature, to give a final height of the settled suspension of 7–8 cm, and then repeatedly washed with 0.008 *M* Na phosphate buffer, pH 7.

The samples (sera or homogenates) are dialysed for 4 h in a continuous-flow apparatus, against 25 l of 0.008 *M* Na phosphate buffer, pH 7, at 4°. After dialysis, the total GOT activity is assayed. 1 ml of the dialysed sample is applied to the column at room temperature. After the sample has soaked into the column, elution is performed with 15 ml of 0.008 *M* Na phosphate buffer, pH 7. The entire chromatographic procedure is performed, at room temperature, within 15 to 30 min.

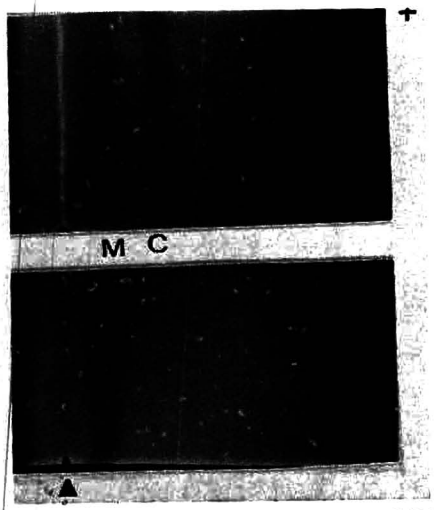
GOT activity is assayed in the eluate by the UV test (KARMEN's method modified by BERGMEYER[34]). If the enzyme activity in the sample is very low, it can be assayed by the MonoTest (Biochemica-Boehringer Co.), by dissolving the reagents in the eluate.

Results

The cytoplasmic isoenzyme of GOT (GOT I) is adsorbed by anion exchangers such as DEAE-Sephadex, while the mitochondrial isoenzyme (GOT II) remains unadsorbed. Therefore, only GOT II occurs in the eluate. GOT I activity can be calculated, if necessary, by subtraction of the mitochondrial activity from total GOT activity of the dialysed sample.

Sensitivity. Rat liver GOT II, obtained from washed mitochondria[36], and tested for purity by electrophoresis (Fig. 1), was diluted to give samples containing different amounts of GOT II activity. These samples were then chromatographed by the method described above. The results are reported in Table I. As little as 2.6 mU of GOT activity were completely recovered after chromatography.

* Glutamate–oxaloacetate transaminase (E.C.2.6.1.1.) = aspartate–aminotransferase = GOT. GOT I = cytoplasmic GOT; GOT II = mitochondrial GOT.



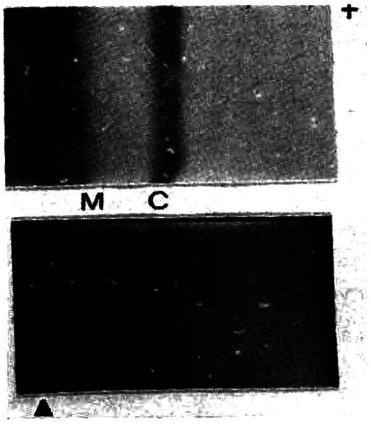


Fig. 1. The electrophoretic pattern of GOT from washed rat liver mitochondria shows no contamination by the cytoplasmic iso-enzyme (left, below). The electrophoretic technique used has been described by FIORELLI *et al.*[31]. The electrophoretic pattern of GOT in whole rat liver homogenate is shown for comparison (left, above).

Fig. 2. Electrophoretic pattern of GOT activity in rat liver cytoplasm, purified by cation-exchange column chromatography (right, below). Columns: diameter 0.9 cm, length 15 cm. Absorbent: CM cellulose C II (Whatman). Final height of the settled suspension: 8 cm. 1 ml of dialysed cytoplasm was applied to the column, and eluted with 15 ml of 0.008 *M* Na phosphate buffer, pH 7. The cytoplasmic isoenzyme is not adsorbed by the cation exchanger, and is therefore recovered in the eluate. The electrophoretic pattern of GOT in whole rat liver homogenate is shown for comparison.

Selectivity. Cytoplasmic GOT (which is usually contaminated by mitochondrial isoenzyme) was purified by chromatography on CM-cellulose. The degree of purification was tested by electrophoresis (Fig. 2). The eluate was then diluted to give samples containing different amounts of GOT I activity. These samples were then rechromatographed by the technique described above. The results are reported in Table II. No GOT activity was found in the eluates, even when as much as 450 mU of GOT were applied to the column.

Reproducibility. Five samples of the same serum* were chromatographed by

TABLE I

CHROMATOGRAMS OF DIFFERENT AMOUNTS OF GOT II

GOT activity applied to the column (mU)	*GOT activity recovered in the eluate (mU)*	*Recovery (%)*
95	93.6	98.5
47	46	97.8
27.6	26.13	94.6
4.2	4.28	101.9
2.6	2.68	103.4

* Serum from a patient with infectious hepatitis.

TABLE II

CHROMATOGRAMS OF DIFFERENT AMOUNTS OF GOT I

GOT activity applied to the column (mU)	*GOT activity recovered in the eluate (mU)*	*Recovery (%)*
18.6	0	0
51.75	0	0
103.5	0	0
225	0	0
450	0	0

TABLE III

VARIATIONS OF GOT ACTIVITY ON REPEATED CHROMATOGRAMS

Chromatogram	*GOT II activity in the eluate (mU)*
1	12.6
2	13.1
3	12.3
4	13.3
5	12.8

the technique described above. The reproducibility of GOT II activity in the samples proved to be satisfactory, as shown in Table III.

Discussion

The chromatographic technique described in this paper is sensitive enough to detect as little as 2.6 mU/ml of mitochondrial GOT activity. No contamination by cytoplasmic GOT has been found in the eluates by selectivity tests. The amount of mitochondrial GOT in samples is reproducible within narrow limits. This quantitative technique is more sensitive than the semiquantitative or qualitative methods reported by other authors. It is also much less time consuming than the method described by SCHMIDT *et al.*[30]. In fact, the continuous-flow apparatus permits good results with dialysis periods as short as 4 h. This is very important, since previous studies have shown that longer dialysis periods cause a striking loss of GOT II activity[36]. This might account for the data reported by SCHMIDT *et al.*[7], who were unable to detect GOT II activity in normal human serum, in contrast to BOYDE[28] and the data obtained in our laboratory by means of the technique described in this paper[35].

Moreover, the entire chromatographic procedure requires one elution only, and can be carried out, at room temperature, within 15–30 min. The simplicity of this method makes it suitable for clinical practice.

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Polyacrylamide gel electrophoresis and gel filtration of dyed polysaccharides

At present, polyacrylamide gel electrophoresis is a widely used method in the field of biopolymer chemistry. However, application of this useful and selective method for resolving mixtures of polysaccharides has hitherto been limited to glycoproteins[1] and mucopolysaccharides[2]. Recently, we have shown that the method can be applied to the analysis of neutral and uronic acid-containing polysaccharides[3]. The method possessed a satisfactory resolving power and reproducibility. Nevertheless, this technique had one substantial disadvantage; detection of polysaccharides

was rather difficult and insufficiently sensitive. The same disadvantage is inherent in the analytical gel filtration of polysaccharides on Biogels and Sephadex, because the most common phenol–sulphuric acid method of detection is time-consuming.

In recent years, information[4,5] concerning new types of reactive Procion dyes which form stable, covalent linkages with dyeing material has been reported. The use of these dyes in carbohydrate chemistry has also been described[6,7]. DUDMAN AND BISHOP[7], in particular, have conducted studies on the electrophoresis of dyed polysaccharides on cellulose acetate strips to eliminate the problem of detection. In the present work, the dyed polysaccharides were found to be satisfactorily resolved and characterised by the use of polyacrylamide gel electrophoresis and analytical gel filtration.

Experimental

Polysaccharides. The polysaccharides used in this study were samples which were available in our laboratory as follows: commercial samples of amylopectin, dextrans (mol. weight 15,000–20,000 and 60,000–90,000), and pectin; laminarin was isolated from *Laminaria japonica* as usual; sargassan[8], pelvetian[9], and zosterine[10] and its fragment (galacturonan) were also used.

Synthesis of the reactive dye. A solution of 3.6 g (0.02 moles) of cyanuric chloride (CC) in acetone (12 ml) was added over a period of 10 min at 0° to 6.2 g (0.01 moles) of Amido Black 10B (AB10B) dissolved in water (14 ml). The hydrochloric acid produced was neutralised with sodium carbonate. The mixture obtained was stirred for 2 h at room temperature, and the pH was then adjusted to 6.8 with phosphate buffer. The precipitate was filtered and dried *in vacuo* over P_2O_5 to furnish the dye CCAB10B; the yield was 5.6 g.

Dyeing procedure. The dyeing of the polysaccharide (50 mg) was achieved as described by DUDMAN AND BISHOP[7].

Electrophoresis. Polyacrylamide gels (4.6% acrylamide) were prepared according to the procedure of DAVIS[11]. The apparatus and technique for the electrophoresis of undyed polysaccharides has been described previously[3]. The following buffers were used: (1) boric acid (0.6 g) + EDTA sodium salt (1 g) + Tris (10 g) in 1 l of water (pH 9.2); (2) sodium tetraborate (2 g) + EDTA sodium salt (1 g) + Tris (10 g) in 1 l of water (pH 9.3).

Procedure. The solution (0.02–0.03 ml) of dyed polysaccharides (15–200 μg) in mixture of 15% Cyanogum 41 (0.7 ml) and buffer 1 or 2 (0.3 ml) was applied as layer on top of the gel, and voltage (200 V, 1 mA per tube) was immediately applied and the electropherogram run for 20–25 min. The voltage was then increased to 400 V, 7 mA per tube, and electrophoresis was continued under these conditions for 2.5–3 h; the migration of the bands was followed visibly. The coloured polysaccharide bands were sharp and easily visible on the electropherogram. The colour obtained was stable at least for 48 h.

Gel filtration. Biogel P ("Bio-Rad" Laboratories, Richmond, Calif., U.S.A.) were used for the gel filtration. The dyed polysaccharides (10–20 mg) or their mixtures dissolved in water (1–2 ml) were applied on the Biogel columns (2.0 × 25 cm) and eluted with water, and migration of the zones was followed visibly. Simultaneously, gel filtration of undyed polysaccharides was carried out under the same conditions. The fractions collected were tested using the phenol–sulphuric acid procedure[12].

Results and discussion

In the present study, the syntheses of dichlorotriazine dyes using cyanuric chloride and the chromophore groups: Amido Black 10B, Variaminic Blue and Congo Red, were carried out. The dyes CCAB10B, CCVB and CCCR, respectively, were obtained and used for dyeing polysaccharides according to the procedure of DUDMAN AND BISHOP[7]. It should be noted that the most satisfactory dye found was CCAB10B,

TABLE I

OPTICAL CHARACTERISTICS OF POLYSACCHARIDES DYED WITH CCAB10B

Compound	*Uronic acid content (%)*	λ_{max} *(mμ)*	$E_{1\%}^{1cm}$ *at* λ_{max}
CCAB10B dye	—	590	41.5
Laminarin	0	530	1.50
Amylopectin	0	530	2.15
Dextran ($15–20 \cdot 10^3$)	0	520	0.54
Dextran ($60–90 \cdot 10^3$)	0	525	0.55
Sargassan	20	520	0.74
Pelvetian	20	530	0.84
Zosterine[a]	60	600	0.23
Commercial pectin[a]	78–86	535	0.52
Galacturonan[a]	99.0	600	0.25

[a] Pretreated with CH_2N_2.

due to its good solubility in water and high extinction coefficient (Table I), while CCVB was poorly soluble in water and CCCR possessed a small extinction coefficient. The list of dyed polysaccharides and their optical characteristics are given in Table I. As can be seen from Table I, the incorporation of the dye into these polysaccharides was found to vary with the nature of polysaccharide. The dyed and undyed polysaccharides had a similar qualitative sugar composition, as was shown using acid hydrolysis and paper chromatographic examination. All of the dyed polysaccharides, which were sufficiently coloured, were subjected to electrophoretic examination. Because the samples were visible, it was a simple matter to experiment with variables to establish the most favourable conditions for electrophoresis. The most satisfactory results were obtained under the conditions described under *Experimental*. The sharpest bands of polysaccharides were observed in these circumstances. The results of the electrophoretic examination of the polysaccharide samples and some synthetic mixtures are shown in Figs. 1 and 2. Fig. 1 shows that commercial samples of amylopectin and dextrans afford several sharp and, broad bands to demonstrate hetrogeneity of these compounds on the contrary purified sargassan and pelcetian furnish sharp bands indicating a rather high homogereity of these polysaccharides. The separation of the synthetic mixtures was also excellent. Self-evidence, simplicity and reproducibility are characteristic proper- ties of the method. It is noteworthy that the dyed polysaccharides possessed higher electrophoretic mobility than the undyed ones. Nevertheless, the electrophoretic patterns were similar in both cases.

Gel filtration of dyed polysaccharides led to successful results. The separations of the mixture of dextrans and that of dextran (mol. weight 60,000–90,000) and pelvetian were run as models using Biogels P-60 and P-100 in the first and Biogel P-150 in the latter case. Satisfactory results were obtained in both cases. It is note-

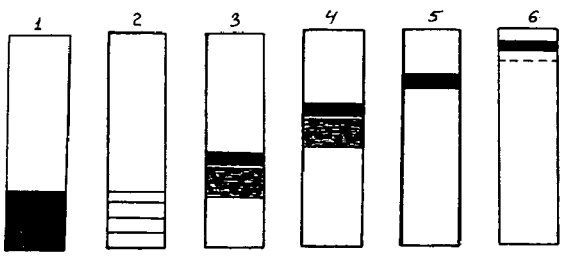

Fig. 1

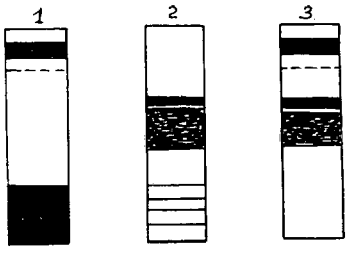

Fig. 2

Fig. 1. Polyacrylamide gel electrophoresis of dyed polysaccharides. 1 = laminarin; 2 = amylopectin; 3 = dextran (mol. weight 15,000–20,000); 4 = dextran (mol. weight 60,000–90,000); 5 = sargassan; 6 = pelvetian.

Fig. 2. Polyacrylamide gel electrophoresis of synthetic mixture of dyed polysaccharides. 1 = laminarin + pelvetian; 2 = amylopectin + dextran (60,000–90,000); 3 = dextran (60,000–90,000) + pelvetian.

worthy that the progress of gel filtration of dyed polysaccharides can be followed with a Uvicord and an automatic recorder to give the elution curve. Simultaneously, the elution curves of the gel filtration of parent polysaccharides were obtained and found to be similar.

The analytical value of gel filtration of the dyed polysaccharides is obvious. The samples were visible on the Biogel column immediately and, consequently, the process could be stopped at any time affording valid results rather than carrying out a long procedure using the phenol–sulphuric acid method of analysis of the separated fractions.

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An arrangement for simultaneous elution of one thin-layer chromatography plate with several solvents

In the "Vario-KS-Chamber"* described by GEISS AND SCHLITT[1] the chromatographic layer can be conditioned in a controlled manner, before and during development with certain chromatographically effective vapours. We describe here an accessory of this chamber, useful for the concurrent elution of a 20 × 20 cm TLC plate with up to 5 different single solvents.

The solvent tank of the Vario-KS-Chamber is exchanged for another which is divided into 5 smaller compartments. The solvent transport is obtained by 5 correspondingly smaller paper wicks for the solvent. Because of the different velocities of the different solvents through the layer, some adsorbent must be scraped from the plate, so that the layer, on the plate, appears as 5 rectangular bands 16.5 × 2.5 cm. This avoids the movement of faster solvents towards the bands of the slower solvents. Furthermore, in order to avoid mixing of vapour phases between neighbouring compartments, a sandwich slide, made of 6 polypropylene barriers of 1 mm thickness and glued together (see Fig. 1), is inserted between the bands before the elution.

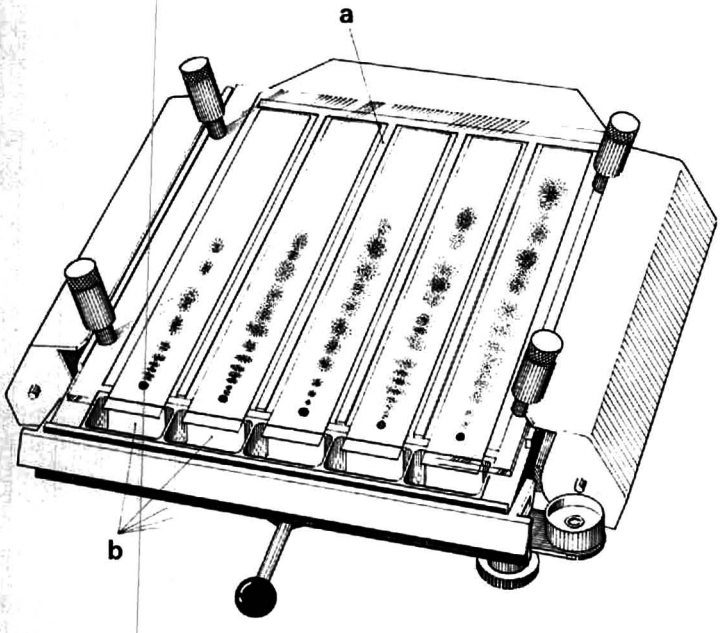

Fig. 1. Vario-KS-Chamber modified for elution with an orthogonal solvent gradient. (a) barriers; (b) solvent tanks.

Such an arrangement represents a form of gradient elution and is an "orthogonal" solvent gradient following the definition of NIEDERWIESER[2]; continuous gradient elution was first described by the same author[3].

* Manufacturer: Camag, Muttenz (Switzerland).

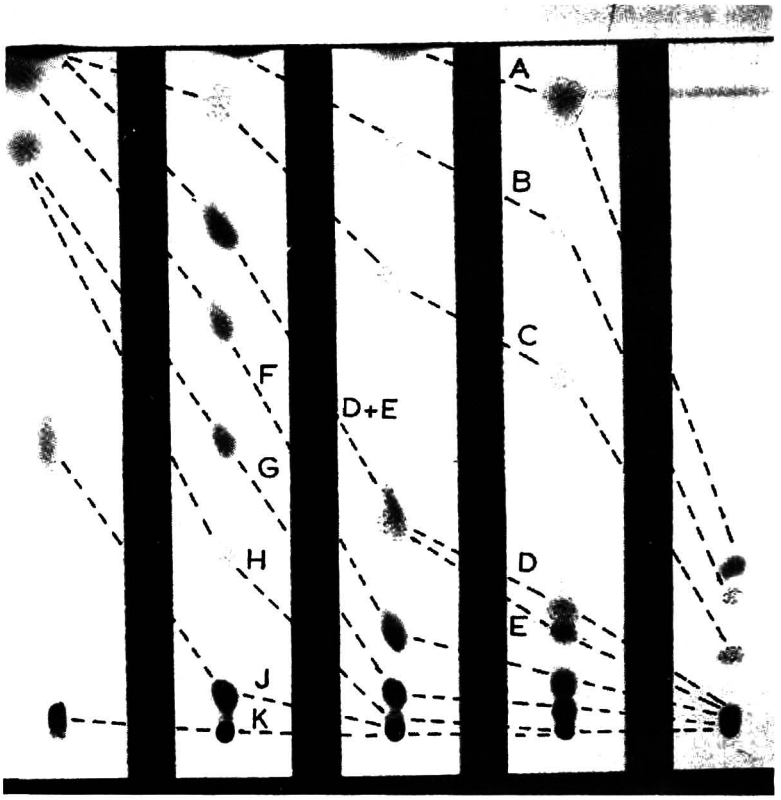

Fig. 2. Separation of a dye mixture on Silica Gel G, Merck. Solvents, from left to right: ethyl acetate; 1,2-dichloroethane; benzene; toluene and carbon tetrachloride; relative humidity 43%. Substances: Fat Red (A), *p*-methoxyazobenzene (B), Butter Yellow (C), Acetorange (D), Indophenol (E), F 11 Ciba (F), F 8 Ciba (G), F 22 Ciba (H), F 5 Ciba (J), F 34 Ciba (K).

This orthogonal solvent gradient is useful in new problems of separation in TLC when solvent systems have to be tested for their optimal effects. Five different solvents can be tested simultaneously on the same plate, one beside the other, under the same conditions. Naturally this gradient can be easily combined with all other gradients which can be used in the Vario-KS-Chamber.

In Fig. 2, a chromatogram of a complex dye mixture is shown; this could be obtained in 80 min. This is an example of the programmed resolution chromatography of a complex sample mixture whose components vary widely in polarity. It demonstrates, in addition, that for the separation of different groups of substances different solvents must be chosen. The pair Indophenol/Acetorange (D/E), for example, is only separated with toluene, under these conditions.

A further modification of the solvent tank makes it possible to work with a continuous solvent gradient. The barriers between the various liquid chambers are lowered in such a way that the various paper solvent wicks are able to exchange solvents between themselves in the gaseous phase before the beginning of the development. As very different mixtures are formed among the pure solvents, the R_F values are not changed in a stepwise but in a continuous way. NIEDERWIESER[3] obtained

such a gradient by combining the BN-Chamber with a complex arrangement of mixing reservoirs, which also allowed the control of the composition of the solvent along the gradient.

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Thin-layer chromatography of cyclic adenosine 3′, 5′-monophosphate on tetraborate-impregnated silica gel layers

A number of procedures for the separation of cyclic adenosine 3′,5′-monophosphate (c-AMP) from other adenine derivatives on thin-layer chromatograms have been published[1–3]. These procedures generally employ cellulose thin layers and a multi-component solvent system, *e.g.* *n*-butanol–acetone–acetic acid–ammonium hydroxide–water.

The tendency of borate ions to form complexes with the 2′,3′-*cis*-diol grouping on simple sugars[4] was utilised to develop a simple and efficient procedure for the separation of c-AMP from other adenine derivatives.

Methods

Plates were prepared using a slurry made up of Silica Gel GF_{254} (Fluka) (30 g) and 5% (w/v) aqueous sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$, 60 ml). Film thickness was 250 μ and the plates were heated at 110° for 30 min. Substrates (10 μl, 0.05% solution in 50% aqueous ethanol) were applied using a microsyringe. The developing solvent was 50% aqueous ethanol, and the development time was approximately 4 h for a 20 × 20 cm plate. After development the plates were dried at room temperature and viewed under UV light.

Results

The use of tetraborate-impregnated layers gave a very satisfactory separation of c-AMP from other adenine derivatives, as shown in Table I. c-AMP and theo-

TABLE I

TLC OF ADENINE DERIVATIVES ON TETRABORATE-IMPREGNATED LAYERS

Developing solvent: 50% aqueous ethanol.

Compound	R_F *value*
Adenosine	0.34
c-AMP	0.75
5′-AMP	0.06
3′-AMP	0.46
5′-ADP	<0.01
5′-ATP	<0.01
Theophylline	0.67

phylline can be further resolved by two-dimensional TLC, using water-saturated *n*-butanol as the developing solvent in the second direction. The R_F values of c-AMP and theophylline in this latter system are 0.05 and 0.44, respectively.

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Separation and detection of synthetic sweetners by thin-layer chromatography

A number of chromatographic procedures have been reported for the separation and identification of synthetic sweetners[1–11], but no report on the chromatography of the sweetners on Avicel thin layers* has been given so far as is known. Although both saccharin and dulcin are detectable by their UV absorption (or fluorescence) and other useful methods[1–3,6,8,10] with fairly high sensitivity, sactisfactory methods for the detection of cyclamate are rather poor.

The present paper describes the separation of the three sweetners on Avicel thin layers and their detection by a new sensitive method which consists of spraying with Pinacryptol Yellow reagent and UV irradiation.

Materials

Chromatographic materials. Avicel (or Avirin) is a microcrystalline cellulose manufactured by the American Viscose Division of FMC Co. (Marcus Hook, Pa., U.S.A.). Avicel SF, a finely powdered product of Avicel for use in TLC, was obtained from Funakoshi Pharm. Co. and Asahi Kasei Co. (Tokyo, Japan). Polyamide (Polyamide Woelm TLC), silica gel (Silica Gel G, Merck), alumina (Aluminum Oxide, Woelm neutral), and DEAE-cellulose (Serva DEAE-TLC) were also used for the TLC.

Reagents. The solvents used were purified by conventional methods to meet chromatographic standards. All other reagents were prepared from analytical reagent grade materials.

Preparation of the thin-layer plates

Twenty grams of Avicel SF were mixed in a glass homogenizer with 70 ml of water for *ca.* 60 sec to give a suspension. After deaeration with suction, the suspension was spread on 20 glass plates (10 × 10 cm) or 10 glass plates (10 × 20 cm) with a suitable applicator, pre-set to give 0.25-mm thick layers. The coated plates were kept horizontal and dried in air overnight, and stored in a desiccator containing silica gel.

The layers (0.25 mm thick) coated with the chromato-media other than Avicel SF were prepared by the conventional procedures and stored in the same way as above.

Application of samples and development of chromatoplates

The following sample solutions were used: a 0.06 *M* ethanolic solution of free cyclamate, a 0.02 *M* ethanolic solution of free saccharin, and a 0.02 *M* ethanolic solution of dulcin.

In order to determine the R_F values and examine the color tones, 0.06 μmole of cyclamate, and 0.02 μmole each of saccharin and dulcin were spotted on the starting line 1.5 cm from the edge of the plate (10 × 10 cm). The plate was developed ascendingly at 20–22° in a closed tank until the length of run was 7 cm. When the 20 × 10 cm plate was used, the samples were spotted on a starting line 2.5 cm from the edge of the plate and it was developed until the length of run was 12 cm.

* Avicel is a cellulose product for thin-layer plates and it is known to be superior in many respects to the cellulose products usually used for thin layers[12].

Detection of spots on the chromatoplates

The developed plates were examined by the following methods.

Pinacryptol Yellow–UV (Method I). The plate is sprayed with 0.1% (w/v) Pinacryptol Yellow solution in 95% ethanol and allowed to dry in the dark for 10 min, and then examined in transmitted UV light (3650 Å). Cyclamate appears as an orange fluorescent spot on a light greenish-blue background. Saccharin is a non-fluorescent orange spot and dulcin a dark violet spot.

Bromine–fluorescein–naphthylethylenediamine (Method II)[8]. After exposure to bromine vapor, the plate is sprayed with a 0.1% ethanolic solution of fluorescein, air-dried, and then sprayed with a 2% ethanolic solution of naphthylethylenediamine. Cyclamate appears as a yellow spot, saccharin is yellowish pink, and dulcin is yellowish orange on a dull orange background.

Methyl Red in phosphate buffer solution (Method III). The well dried plate is sprayed with a solution consisting of 1 part of 0.1% (w/v) ethanolic solution of Methyl Red and 2 parts of phosphate buffer solution (pH 7.0). Cyclamate and saccharin give reddish orange spots on a yellow background.

Silver nitrate–pyrogallol (Method IV)[1]. Silver nitrate (0.17 g) is dissolved in 1 ml of water and mixed with 5 ml of 10% NH_3; this solution is diluted with ethanol to 200 ml. The plate is sprayed with the solution prepared as above and then sprayed with a freshly prepared solution of 0.01% (w/v) ethanolic pyrogallol. Cyclamate appears as a transient white spot on a brown background.

UV absorption or fluorescence (Method V). The plate is examined in transmitted UV light (2537 Å), when saccharin appears as a blue fluorescent spot and dulcin as a dark spot.

Results and discussion

Separation and detection of sweetners on Avicel layers. The solvent systems suitable

TABLE I

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF SYNTHETIC SWEETNERS ON AVICEL LAYERS

Symbol	*Components*	*Ratio (v/v)*
A	AcOEt–conc. NH_3–acetone	1:1:8
B	DMF–EtOH–H_2O	5:4:1
C	Dioxane–pyridine–H_2O	7:2:1
D	Pyridine–EtOH–H_2O	6:3:1
E	Tetrahydrofuran–pyridine–H_2O	6:3:1

for the separation of cyclamate, saccharin and dulcin on the Avicel layers are summarized in Table I. On the small size (10 × 10 cm) plates, these solvents were fairly satisfactory in resolving the sweetners as small dense spots. The R_F values obtained are listed in Table II.

Five detection methods including the Pinacryptol Yellow reagent and other known procedures were examined and the detection limits of the sweetners by these methods were compared with each other. As can be seen in Table III, the sensitivity

TABLE II

R_F VALUES OF SYNTHETIC SWEETNERS ON AVICEL LAYERS

Sample	*Solvent system*				
	A	*B*	*C*	*D*	*E*
Cyclamate	0.15	0.74	0.29	0.47	0.31
Saccharin	0.31	0.83	0.39	0.64	0.41
Dulcin	0.82	0.91	0.80	0.79	0.89

TABLE III

DETECTION LIMITS (μg) OF SYNTHETIC SWEETNERS ON AVICEL LAYERS

Detection: I = Pinacryptol Yellow–UV; II = bromine–fluorescein–naphthylethylenediamine; III = Methyl Red in phosphate buffer; IV = silver nitrate–pyrogallol; V = UV absorption (or fluorescence).

Sample	*Method of detection*				
	I	*II*	*III*	*IV*	*V*
Cyclamate	1	2	4	8	—[a]
Saccharin	0.2	1	1	1	0.1
Dulcin	0.2	1	—	—	0.2

[a] Symbol — means that no spot was detected.

TABLE IV

R_F VALUES OF SYNTHETIC SWEETNERS ON LAYERS OF DIFFERENT CHROMATO-MEDIA

Chromato-media	*Solvent system*	*R_F value*		
		Cyclamate	*Saccharin*	*Dulcin*
Avicel SF	AcOEt–conc. NH_3–acetone (1:1:8)	0.04	0.23	0.88
Polyamide	Benzene–AcOEt–HCOOH[a] (5:10:2)	0.41	0.69	0.81
Silica gel	Acetone–10% NH_3 (9:1)	0.24	0.59	0.84
Alumina	*iso*-PrOH–conc. NH_3 (4:1)	0.14	0.30	0.70
DEAE-cellulose	0.5 *M* $HCOONH_4$ (pH 6.5)	0.74	0.36	0.53

[a] This solvent system was recommended by Dr. R. TAKESHITA.

TABLE V

DETECTION LIMITS (μg) OF SYNTHETIC SWEETNERS BY PINACRYPTOL YELLOW REAGENT ON LAYERS OF DIFFERENT CHROMATO-MEDIA

Sample	*Chromato-media*				
	Avicel SF	*Polyamide*	*Silica gel*	*Alumina*	*DEAE-cellulose*
Cyclamate	1	12	40	1	40
Saccharin	0.2	4	12	0.1	1
Dulcin	0.2	1	1	0.1	0.1

of the new method is comparable to that of the bromine–fluorescein–naphthylethylenediamine method which is the best one for detecting these three sweetners.

Colorations of the synthetic sweetners with Pinacryptol Yellow reagent. When the developed plate is allowed to stand in the dark for 10–20 min after being sprayed with the Pinacryptol Yellow reagent, cyclamate appears as an orange fluorescent spot on a greenish-blue background in long-wave (3650 Å) UV light. The color tone and intensity of the spot are unchanged for a long time, at least eight days, in the dark. The spots of saccharin with this reagent are similar to that of cyclamate in their color tones, but the lack of fluorescence of the saccharin spot distinguishes it from the cyclamate. Dulcin appears as a dark violet spot by this method. Its color tone and intensity are stable for a long time even in the light. The sensitivities of the reagent to both saccharin and dulcin are comparable to those of UV absorption or fluorescence as shown in Table III.

Detection of synthetic sweetners by Pinacryptol Yellow reagent on layers of different chromato-media. In order to examine the colorations given by the Pinacryptol Yellow reagent, cyclamate, saccharin and dulcin were chromatographed on the layers of different chromato-media. The solvent systems used to resolve the sweetners on the layers of each chromato-medium, together with the R_F values, are shown in Table IV. The experimental results shown in Table V indicate that these sweetners are the most sensitive to the reagent on Avicel and alumina layers, and the worst on silica gel layers.

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Chromatography of fat-soluble chloroplast pigments on preformed kieselguhr layers

In an earlier report[1], separations of chloroplast pigments from leaves and algae by chromatography on preformed flexible thin layers of cellulose, silica gel, alumina and polyamide were described. Precoated kieselguhr TLC sheets have very recently become available, and these are now evaluated for the separation of pigments extracted from leaves, algae and photosynthetic bacteria.

Experimental

Precoated Kieselguhr F_{254} TLC sheets with a 0.25-mm layer on aluminum backing are manufactured by E. Merck AG, Darmstadt, G.F.R. and distributed by Brinkmann Instruments, Inc., Westbury, N.Y., U.S.A. For adsorption chromatography, the sheets are dried for 15 min at 105° and cooled in air just before spotting. For reversed-phase partition chromatography, the sheets are soaked in a 7% (v/v) solution of Wesson Oil (a mixture of cottonseed plus soybean oils; Hunt-Wesson Foods, Inc., Fullerton, Calif., U.S.A.) in petroleum ether (60–110°), air dried a few minutes, and then oven dried at 75° for 30 min. After cooling, the sheet is spotted and developed with methanol–acetone–water (20:4:3) which has been equilibrated with the oil in a separatory funnel.

Ascending development for a distance of 15 cm was carried out by standing the sheets in the solvent held in the bottom of paper-lined, saturated, rectangular chambers covered with aluminum foil to retard the photo-decomposition of the pigments. Development times ranged from about 20 min for some solvents with unimpregnated sheets to about 50 min for the reversed-phase system.

Identification of the separated pigments was based on their colors, chromatographic sequence, absorption spectra in the visible region, and reaction to the vapors of concentrated HCl.

Pigments were extracted from spinach leaves by a method employing a blender as previously described[2]. The method used to extract pigments from *Chlorella pyrenoidosa* suspension has also been described[3]. For the extraction of the bacteria, this procedure was modified by using methanol–diethyl ether–petroleum ether (5:2:1) as the extraction solvent and dissolving the final residue in a small volume of diethyl ether–acetone (1:1) (for Chlorobium) and diethyl ether–petroleum ether (1:1) for Rhodospirillum. The treatment with boiling water was not required to increase the extractability of the pigments from Rhodospirillum.

Results and discussion

Separations by adsorption chromatography. Despite reports by other workers of successful separations of the pigments in extracts of leaves[4], algae[5] and bacteria[6], no solvent system was found in the present study which yielded a good separation of the chlorophylls and carotenoids from spinach or *Chlorella pyrenoidosa*. (These unsuccessful results were also obtained earlier[2,7,8] on home-made layers of Kieselguhr G.) All solvents employed by other workers were tested, plus many modifications which had proven sucessful with other types of preformed adsorbent layers[1]. In no case were

chlorophylls *a* and *b* cleanly separated, and streaked chlorophyll and carotenoid zones and extra green zones were usually obtained.

Many different solvents were tested for the separation of the four carotenoids in saponified[2] spinach and Chlorella extract, but only petroleum ether (20–40°) plus 10% acetone gave a complete separation, and then only at low loading (1–2 μl of saponified extract). The sequence of the separated zones was carotene (R_F 1.0), lutein (0.82), violaxanthin (0.68) and neoxanthin (0.39). The separated pigments were immediately cut from the sheet, eluted with ethanol, and their absorption spectra recorded in this solvent. The shapes and maxima of the spectra[2,8] indicated no rearrangement of the carotenoids. If the chromatogram is exposed to the vapors of HCl, neoxanthin and violaxanthin turn bluish green while carotene and lutein remain yellow.

With petroleum ether plus 1% *n*-propanol as the solvent, the major portions of the four carotenoids are well separated, but the lutein and violaxanthin zones have faint streaking tails.

Separations by partition chromatography. Development of spinach or Chlorella extract on Wesson Oil-impregnated kieselguhr sheets with methanol–acetone–water (20:4:3) resulted in the complete separation of the six major zones at loadings from 1–10 μl of extract: carotene (R_F 0.0), chlorophyll *a* (0.067), chlorophyll *b* (0.20), lutein (0.53), violaxanthin (0.80), neoxanthin (0.93). Visible absorption spectra of the eluted zones matched those reported for the pure pigments. Saponified extracts were also completely separated in this system.

If Wesson Oil is replaced with olive oil and oil-saturated methanol–acetone (20:1) is used as the developing solvent, R_F values are higher and the separation, although complete at the lowest loadings, is not so good.

The pigments of cultures of the green sulfur bacteria *Chlorobium thiosulfatophilum* and *Chlorobium limicola* yielded reversed-phase chromatograms with major spots at R_F 0.0 (orange) and R_F 0.35 (green). These spots were eluted with diethyl ether and their spectra identified the pigments as chlorobactene (491, 461, 435 nm)[9] and Chlorobium chlorophyll 660 (660, 431 nm)[10]. In addition to these, the following minor spots were noted: red-orange and green zones just above chlorobactene and a purple-grey zone at R_F 0.90 in the chromatograms of both organisms, and a blue zone, which was undoubtedly bacteriochlorophyll (see below), just below Chlorobium chlorophyll in *Chl. limicola* only.

The pigments of a culture of the non-sulfur purple bacterium *Rhodospirillum rubrum* separated on Wesson Oil-impregnated kieselguhr yielded a chromatogram with only two spots: spirilloxanthin (reddish pink, R_F 0.0; 528, 493, 463 nm[11], in ether) and bacteriochlorophyll (blue, R_F 0.30; 770, 576, 391, 358 nm[8], in ether). There was absolutely no indication of the presence of any other pigments as separated by STRAIN AND SVEC[12] from other cultures of *R. rubrum* in columns of powdered sugar. To check this, our extract was developed on a Baker preformed silica gel thin layer with petroleum ether plus 30% acetone and on unimpregnated kieselguhr with petroleum ether plus 10% acetone. In each case only two zones were resolved; spirilloxanthin streaked in both systems and bacteriochlorophyll formed a zone with double tails on the unimpregnated kieselguhr.

Attempts to reproduce the reversed-phase partition separations of EGGER[13] were unsuccessful, as they had been[7] on home-made layers of Kieselguhr G.

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Chromatographische Nachweisreaktionen von chinoiden Verbindungen

Als Chinone bezeichnet man Verbindungen, bei denen zwei, an einem aromatischen Kern stehende Wasserstoffatome durch zwei Sauerstoffatome ersetzt sind. Die meisten Chinone lassen sich leicht reduzieren und reoxidieren, was wir hier als Nachweisreaktion nach einer papier- oder dünnschichtchromatographischen Trennung benutzten.

Einige Farbreaktionen, die zur Charakterisierung chinoider Verbindungen im Schrifttum bereits beschrieben worden sind, eignen sich auch als Nachweis-Reagenzien für die Papier- und Dünnschicht-Chromatographie.

Beschreibung der Versuche

In den meisten Fällen zeichneten sich die chinoiden Verbindungen auf den entwickelten Chromatogrammen schon durch ihre Eigenfarbe aus. Eine Betrachtung im langwelligen UV-Licht ergab weitere Hinweise auf die mögliche Struktur des Chinons. So zeigten Benzochinon-Derivate keine, Naphthochinone dagegen eine stumpf-violette Fluoreszenz. Eine Ausnahme bildeten Furanonaphthochinone[10], die ähnlich wie die meisten Anthrachinon-Derivate eine leuchtende Fluoreszenzfarbe erkennen liessen.

Papierchromatographie. Bei Behandlung mit Ammoniakdämpfen zeigten Hydroxychinone auf dem Papier charakteristische Färbungen. Zum weiteren Nachweis wurden solche Flecke aus dem Papierchromatogramm ausgeschnitten, mit verdünnter KOH-Lösung eluiert und mit einer wässrigen Natriumdithionitlösung zum farblosen Hydrochinon reduziert.

Anschliessendes Schütteln der ungefärbten Lösung führte zur Reoxidation des Chinons, wodurch die chinoide Struktur der Verbindung bestätigt wurde. Trat mit Ammoniak keine typische Färbung auf, so wurden die farbigen Flecken mit Methanol eluiert und anschliessend mit Kalium- oder Natriumborhydrid (10%ige Lösung in Methanol) reduziert. Beim Schütteln der Lösung trat bei chinoiden Verbindungen eine Reoxidation ein (Tabelle I).

TABELLE I

REDUKTION EINIGER CHINONE MIT KBH_4

Verbindung	*Farbe*		
	In Methanol	*In KBH_4-Lösung*	*Geschüttelt*
p-Naphthochinon[a]	gelb	braunrot über farblos	braunrot
2-Hydroxynaphthochinon-(1,4)[a]	gelborange	farblos	gelbrot
Lapachol[a,b]	gelb	farblos	rötlich
Anthrachinon[a]	gelblich	farblos	gelbgrün
Tectochinon[a,b]	gelb	farblos	gelbgrün

[a] Fa. Carl Roth AG.
[b] Isoliert aus *Tectona grandis* (Teak).

TABELLE II

REAKTION EINIGER CHINONE MIT NH_3-DÄMPFEN AUF DÜNNSCHICHTCHROMATOGRAMMEN

Fertigplatte (Kieselgel F_{254} Merck AG). Laufmittel: Chloroform[7].

Verbindung	*Farbe*		
	Auf DC-Platten	*Mit NH_3*	*Nach Verdunsten von NH_3*
2-Hydroxynaphthochinon-(1,4)	orange	etwas intensiver	orange
Lapachol[6]	orange	kräftig rot	orange
S-4,4'-Dimethoxydalbergion[5,a]	gelb	grün	violet
Desoxylapachol[9,b]	gelb	violet	braun
1-Hydroxy-3-methylanthrachinon[c]	gelb	orange	gelb

[a] Isoliert aus *Dalbergia nigra* (Palisander).
[b] Isoliert aus *Tectona grandis* (Teak).
[c] Synthetisch hergestellt.

Dünnschichtchromatographie. Auch auf Dünnschichtplatten zeigten die getrennten Hydroxychinone nach der Behandlung mit Ammoniak typische Färbungen, die nach dem Verdunsten der Dämpfe wieder verschwanden.

Chinone, die auf Grund einer Allylgruppierung in der Seitenkette positiv auf den Dam–Karrer-Test[3] reagierten, gaben mit Ammoniak ebenfalls eine Farbvertiefung (Tabelle II).

Die Reduktion zum farblosen Hydrochinon liess sich durch Besprühen mit einer 10%igen Lösung (in Methanol) von Natriumdithionit bzw. Natriumborhydrid erreichen. Natriumborhydrid entfärbte dabei stärker als Natriumdithionit. Nach kurzem Trocknen an der Luft (Abzug) kehrte die ursprüngliche Farbe der mit Natriumdithionit besprühten Chinone wieder zurück, während die mit Natriumborhydrid behandelten nur sehr langsam reoxidiert wurden. Die Oxidation konnte durch Einbringen (1–2 min) der Platten in HCl-Dämpfe beschleunigt werden.

Die Reduzierung und anschliessende Reoxidation war weiterhin abhängig von der Zugehörigkeit zu den verschiedenen Gruppen. So liessen sich die Benzochinon-Derivate sehr schnell und leicht entfärben und ihre Farbe kehrte nach dem Trocknen auch als erste zurück. Die Anthrachinone konnten dagegen nicht vollständig zum farblosen Hydrochinon reduziert werden, vermutlich durch die Bildung farbiger Chinhydronverbindungen. Ihre Reoxidation dauerte dementsprechend länger. Die Naphthochinone nahmen dazwischen eine Mittelstellung ein.

Zum Vergleich wurde das gelbe Flavon Quercetin herangezogen. Bei diesem bewirkte Natriumborhydrid eine Farbvertiefung, die nicht rückgängig gemacht werden konnte (Tabelle III).

Zur weiteren Charakterisierung besprühten wir die entwickelten Chromatogramme mit einer 0.1%igen 2,4-Dinitrophenylhydrazinlösung (in 2 *N* äthanolischem HCl). Nach kurzem Trocknen wurden die Platten in NH_3-Gase gebracht[11]. Die untersuchten Benzochinon-Derivate bildeten mit 2,4-DNPH Kondensationsprodukte, die unter dem Einfluss von NH_3 violett oder grün wurden. Bei den Naphthochinon-Derivaten konnten diese Färbungen mit 2,4-DNPH nur bei Verbindungen mit min-

TABELLE III

REDUKTION EINIGER CHINONE AUF EINER DC-PLATTE

Fertigplatte (Kieselgel F_{254} Merck AG). Laufmittel: Chloroform.

	Derivat	*Farbe*				
		Auf dem Chromatogramm	*Nach dem Besprühen mit*		*Nach dem Trocknen bzw. nach HCl-Dampfbehandlung mit*	
			Na-dithionit	*Na-borhydrid*	*Na-dithionit*	*Na-borhydrid*
Benzochinon	S-4,4'-Dimethoxydalbergion[5]	gelb	fast farblos	farblos	gelb	gelb
Naphthochinon	Lapachol[6,a]	rot	fast farblos	farblos	rot	rosa
	Desoxylapachol[9]	gelb	fast farblos	farblos	gelb	rosa
	Plumbagin[4,b]	gelb	fast farblos	farblos	gelb	gelb
Anthrachinon	1-Hydroxy-3-methylanthrachinon[c]	gelb	blassgelb	fast farblos	gelb	gelb
	1,4-Dihydroxy-2-methylanthrachinon[c]	rot	blassrot	fast farblos	rot	rosa
Flavon	Quercetin[d]	gelb	gelb	gelb	gelb	stark gelb

[a] Isoliert aus *Tabebuia serratifolia* (Bethabara).
[b] Isoliert aus *Plumbago capensis* (Kap Bleiwurz, Wurzelrinde).
[c] Synthetisch hergestellt.
[d] Carl Roth AG.

TABELLE IV

REAKTION EINIGER CHINONE MIT 2,4-DINITROPHENYLHYDRAZIN AUF EINER DC-PLATTE

Fertigplatte (Kieselgel F_{254} Merck AG). Laufmittel: Chloroform.

Verbindung	*Farbe*		
	Auf dem Chromatogramm	*Mit 2,4-DNPH*	*Mit NH_3*
p-Benzochinon	gelbbraun	gelbbraun	violett
S-4,4'-Dimethoxydalbergion[5]	gelb	orange	grün
p-Naphthochinon	gelbbraun	bräunlich orange	grün
2-Hydroxynaphthochinon-(1,4)	orange	schwach rötlich	orangebraun
Lapachol[6]	rot	bräunlich	rot
Desoxylapachol[9]	gelb	rot	violett
α-Lapachon[6,a]	gelb	gelb	gelb
β-Lapachon[6,a]	orange	orange	bräunlich + etwas grünlich
β-Isopropenyl-dihydrofurano-naphthochinon[1,4,10,b]	gelb	gelb	bräunlich + grünlich
Makassar-chinon[1,c]	rot	rot	rot
Anthrachinon	—	gelblich	gelblich
1-Hydroxy-3-methylanthrachinon	gelb	gelb	rötlich

[a] Hergestellt aus Lapachol.
[b] Isoliert aus *Paratecoma peroba*.
[c] Isoliert aus *Diospyros celebica* (Makassar).

destens einem freien H-Atom in Nachbarschaft zu einer Keto-Gruppe deutlich beobachtet werden. Diese färbten sich under dem Einfluss von Ammoniakdämpfen ebenfalls grün oder violett. Alle untersuchten Anthrachinon-Derivate ergaben mit dem Reagenz keine Farbreaktionen. Lediglich Anthrachinon, das im Tageslicht kaum wahrzunehmen war, färbte sich durch 2,4-DNPH etwas gelb[7] (Tabelle IV).

Nähere Angaben über die Struktur des Chinons konnten auf den entwickelten Chromatogrammen auch durch den Craven-Test[2] und den Dam–Karrer-Test[3] gewonnen werden. Für den Craven-Test wurde die Platte mit einem Gemisch aus gleichen Teilen Äthanol (96%) und konzentriertem Ammoniak unter Zugabe von 0.5% Cyanessigsäureäthylester besprüht, begossen oder kurz in das Reagenz getaucht.

Eine grüne oder blaue Färbung deutet bei Benzo- und Naphthochinonen auf das Vorhandensein eines freien H-Atoms in Nachbarschaft zu einer Keto-Gruppe hin.

Beim Dam–Karrer-Test wurde das entwickelte Chromatogramm mit einer 10%igen äthanolischen KOH-Lösung besprüht, begossen oder in das Reagenz getaucht. Eine Farbvertiefung deutet auf eine Allylgruppierung in der Seitenkette des Chinons. Das Bedampfen mit NH_3 ergab den gleichen Effekt.

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Book Review

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Ancillary Techniques of Gas Chromatography, edited by L. S. ETTRE AND W. H. MCFADDEN, Wiley–Interscience, New York, 1970, 395 pp., price 165/- .

It is an interesting fact that gas chromatographers describe other techniques used in association with a gas chromatograph as "ancillary", yet as stated at the outset gas chromatography is "a separation technique" and it suffers many limitations as a method of identification. However the effectiveness of most techniques of identification depends upon seeing one compound at a time, as the book sets out to demonstrate, and the marriage with GC can produce a system of immense analytical potential, each partner contributing to the final result. Perhaps the word complementary would be more apt than ancillary and draw the attention of those analysts wedded to other physical techniques, because this should prove a useful volume to both gas chromatographers and mass spectroscopists etc.

The book is not confined to the problems of post-column interfacing with other instruments, but includes the various facets of pre-column reaction techniques such as pyrolysis, hydrogenation, etc., all neatly wrapped in separate chapters, the work of separate authors, each an authority in his sphere.

After a short introduction discussing general principles and flow patterns there follow three chapters devoted to pre-column methods. The first is a discussion on microreactors for investigation of chemical kinetics with particular reference to catalysed reactions. This is essentially a review, as indeed are the succeeding chapters, but in this case without giving any feeling that the authors own views are evident. The succeeding chapter on pyrolysis commendably covers a field about which a whole book can be written. The commonly used pyrolysis systems are described and compared, inherent problems are discussed, and reference is made to less common methods. Broad applications are illustrated by a well chosen selection of examples (a point which applies throughout the book) and demonstrates the value of the technique in dealing with non-volatile samples such as polymers. The third chapter in this group specifically devoted to pre-column reactions discusses their application to structural determination, particularly by variations on the theme of on-line hydrogenetics. Subtractive methods, ozonolysis and syringe techniques are also covered. This chapter emphasises the tremendous potential of gas chromatography when the user is prepared to forsake the "black-box" mentality and allies the method to some simple chemistry. Unlike the section on microreactors the author's own experience is markedly evident.

One of the milestones in the advance of organic analysis has been the coupling of mass spectrometry to gas chromatography but the problems of interfacing are not simple. These are treated in a lucid fashion and even the computer is not forgotten. The principles of mass spectrometry are discussed in sufficient depth for the gas chromatographer to communicate with the mass spectroscopist. In a similar fashion the problems of coupling IR and NMR are dealt with and here it is clear from each chapter that the interfacing problems are much greater, especially with NMR. As a

result, trapping techniques are more often employed. For this reason the reviewer feels that inclusion of a section devoted to preparative gas chromatography would have increased the value of an already useful book.

The last chapters on thin-layer chromatography and chemical identification again reflect the authority of the authors. Throughout, the book is well supplied with valuable literature references with one notable exception, when in mentioning the evils of dead space the author simply says: "There are some good suggestions in the literature"—he does not say where! However, this is a useful informative book which should stimulate the person who sees the gas chromatograph as a means to an end and not an end in itself.

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

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PRINCIPLES OF GEL CHROMATOGRAPHY

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SUMMARY

The principles of gel chromatography have been examined with regard to general chromatographic theory. It is shown that the elution volume for a solute is solely determined by the partition of a solute between the mobile and stationary phases and that various forms of diffusion only contribute to the width of the peak eluted. It is demonstrated that the treatment based on the diffusion model and the Giddings dynamic theory lead to practically identical results.

INTRODUCTION

Despite the considerable amount of work which has recently been carried out in the field of gel chromatography (other names: gel filtration, gel permeation chromatography and molecular sieve chromatography) some uncertainty concerning the interpretation of the fundamental processes involved still remains. Conflicting views prevail especially about the relative importance of diffusion and partition effects. It is therefore desirable to examine the principles of gel chromatography with regard to the laws of general chromatographic theory and, if possible, draw conclusions about the basic processes involved.

THEORY

Firstly the main results of the theory of linear partition chromatography are summarized. As shown in earlier work[1,2], the basic equations may be written as follows**:

$$v = \frac{\dot{\mu}}{v} = \frac{1}{1 + \frac{\gamma V_2}{V_1}} = w_1 \tag{1}$$

$$\mu_2(t) = \mu_2(0) + 2Dt \tag{2}$$

$$D = D' + D'' = D'_1 w_1 + D_2 w_2 + \frac{v^2 \gamma}{2 D_2 V_1 \left(\frac{\gamma}{V_1} + \frac{1}{V_2}\right)^3} \tag{3}$$

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** For symbols, see p. 211.

Eqn. 1 determines the peak mobility (relative peak velocity) in the column under steady state conditions and is valid for peaks of arbitrary form.

Eqn. 2 determines the broadening of the peak in the column under steady state conditions in terms of the variance. This formula is also valid for peaks of arbitrary form. The spreading is characterized by a spreading coefficient D, given by eqn. 3.

These equations are concerned with the situation in the column and are not directly applicable to normal experimental conditions in which the concentration distribution is determined as a function of the efflux volume. However, it may be shown[3] that the moments of the concentration distribution with respect to the efflux volume may be expressed in terms of the corresponding moments in the column. As a good approximation the following equations hold:

$$\mu_V = \theta \frac{L}{\omega} \tag{4}$$

$$\mu_{2V} = \left(\frac{\theta}{\omega}\right)^2 \mu_2 \left(\frac{L}{\omega}\right) \tag{5}$$

where, obviously, L/ω is the time it takes the peak to pass through the column.

Eqns. 1–3 are based on a fairly general model and take into account the various diffusion effects encountered in a chromatography column. This model also takes into account the solute distribution in the gel particles by using a stepwise approach in the treatment of the diffusion process in the particles. From this the continuous model is obtained by passing to the limit of infinitesimal steps. Eqns. 1–3 represent the results for a single step model, which, however, only differ insignificantly from the results obtained for the continuous model. A significant feature of the model is that the geometry of the column filling does not enter the treatment explicitly, but is taken into account by the volume to surface ratios V_1 and V_2. This is not surprising since the mass transfer between the mobile and stationary phases occurs at the surface separating the two phases.

We will next consider in more detail the implications of eqns. 1–5 on the mechanism of gel chromatography.

ELUTION VOLUME

In practical gel chromatography the position of a peak is determined by its elution volume, which is conventionally defined as the position of the maximum of the peak in the elution diagram. The elution volume is related to other column parameters by the formula[4]

$$V_e = V_0 + K_d V_i \tag{6}$$

Alternatively[5]

$$V_e = V_0 + K_{av}(V - V_0) \tag{7}$$

The last definition is more convenient since $V - V_0$, which is the total volume

of the stationary gel phase, is readily determined experimentally. Obviously, for a given solute K_d and K_{av} differ only by a constant factor.

Eqn. 7 provides an operational definition of the partition coefficient. It is therefore of great interest to compare eqn. 7 with eqn. 4, in which the equilibrium partition coefficient γ is used and which has been derived under non-static conditions. Rearranging eqn. 4 we get

$$\mu_V = \frac{L\theta}{\omega} = \frac{L\sigma V_1}{v} = L\sigma(V_1 + \gamma V_2) = V_0 + \gamma(V - V_0) \tag{8}$$

Thus, if μ_V is identified with the elution volume V_e (which is strictly valid only for symmetrical peaks) we find

$$\gamma = K_{av} \tag{9}$$

Eqns. 4 and 7 are based on a fundamental principle in chromatography, namely that only the solute present in the mobile phase contributes to the migration of the peak in the column. A mathematically rigorous form of this statement is given by eqn. 1. In the case of eqns. 4 and 7 some approximations are involved, which are necessary because of the distortion of the peak which occurs when it leaves the column, an effect which is not at present amenable to a rigorous mathematical treatment (*cf.* ref. 3).

Although there is substantial experimental evidence in support of eqn. 1 (the elution volume is in general independent of the elution rate) one may enquire under what conditions the solute distribution between the mobile and stationary phases depends on dynamic variables, such as the velocity v.

It should first be noted that the validity of eqn. 1 is a consequence of the linearity of the general diffusion equation

$$\frac{\partial c}{\partial t} = D_2 \frac{\partial^2 c}{\partial x^2} \tag{10}$$

which governs the diffusion in the stationary phase. If D_2 is concentration dependent the linearity of eqn. 10 is lost and eqn. 1 would no longer hold. This is of course also the case when γ depends on the concentration.

We can also ask whether γ is affected by the velocity gradient existing in the vicinity of the gel particles. Since it is normally assumed that the liquid in the immediate vicinity of the particle surface forms a stationary film, the problem reduces to the existence of an uneven partition of solute between the liquid which is flowing past and the film. This effect has been treated in detail in some recent articles[6,7]. However, in view of the low flow rates encountered in gel chromatography this effect appears to be negligible.

There is the further possibility that the partition volume V_2 depends on the velocity. This is the case if the thickness of the stationary liquid film depends on the velocity[8]. However, in general the film constitutes only a small fraction of the partition volume V_2 and therefore this effect is also expected to be rather small.

We may thus conclude that the elution volume of a peak is solely determined by solute partition between the mobile and stationary phases. This is normally inde-

pendent of the elution rate and is determined by the partition coefficient γ and the partition volume V_2 (V_1 can be expressed in terms of V_2). To treat the partition effect we need to dispose of only one of these parameters. However, as we shall see later, both parameters are needed when intraparticle diffusion is considered.

PEAK BROADENING

The broadening of a concentration peak in linear partition chromatography may be expressed in terms of the change of its variance with time and it then assumes the remarkably simple form of eqn. 2. This equation has the form of a diffusion equation and the generalized diffusion coefficient, or spreading coefficient, D, consists of two terms representing the longitudinal diffusion and chromatographic dispersion, respectively.

Longitudinal diffusion

The longitudinal diffusion in the mobile phase is due to the Brownian diffusion and diffusion arising from irregular flow (eddy diffusion). Considering these effects to be additive we may write

$$D'_1 = D_1 + kv \tag{11}$$

where the dependence of eddy diffusion on velocity has been taken into account explicitly. This dependence on velocity is obvious, since the eddy diffusion vanishes when v tends to zero. In the first order approximation the effect is proportional to velocity[9,10]. From eqn. 3 it follows that the contribution of longitudinal diffusion in the mobile phase is proportional to the mass fraction of solute in the mobile phase.

Longitudinal diffusion in the stationary phase is represented by the term $D_2 w_2$ in eqn. 3. This term is only significant if the column packing consists of particles having large dimensions in the axial direction of the column. If these dimensions are small the particles are surrounded by a solution of uniform concentration and this term should be omitted.

Chromatographic dispersion

The last term in eqn. 3, the chromatographic dispersion coefficient D'', represents the dispersion due to the finite rate of mass transfer between the mobile and stationary phases. Obviously this term vanishes when equilibration is instantaneous ($D_2 = \infty$). To bring out the physical significance of the various factors appearing in D'', we may rearrange the expression in eqn. 3 and get

$$D'' = \frac{v^2 \gamma V_2^2 w_1^2 (1 - w_1)}{2D_2} \tag{12}$$

We find that in this case geometrical factors are important, since D'' is directly dependent on the partition volume V_2, and thus on the dimensions of the particles used to fill the column. If the column packing consists of particles having a simple geometrical form, V_2 is directly computable. For instance, if the column packing consists of spherical particles with the radius r, we have

$$V_2 = \frac{\frac{4}{3}\pi r^3}{4\pi r^2} = \frac{1}{3} r \tag{13}$$

and eqn. 12 becomes

$$D'' = \frac{v^2 \gamma r^2 w_1^2 (1 - w_1)}{18 D_2} \tag{14}$$

In the treatment of partition effects in connection with the elution volume we found that it was sufficient to use only one of the parameters γ and V_2 to characterize the partition process. When diffusion effects are considered it is essential that the correct value of the partition volume V_2 is used. This also permits the consideration of those cases when the gel particles are non-uniform. For instance, when only the surface layer of a gel particle is accessible to the solution, the equilibration time is very much shorter than it would be for a uniform particle. This can be taken into account in eqn. 12 by assigning to V_2 a value comprising only the surface layer.

PEAK FORM AND END EFFECTS

The chromatographic theory used here is based on the moments of the concentration distribution in a peak and is not concerned with the form of these distributions. However, some information about the form of the concentration distribution may be obtained from the general form of eqn. 2. Since the spreading of a peak is governed by the law of diffusion, we can conclude that an initially sharp distribution (δ-function distribution) will give rise to a Gaussian peak. Similarly a peak which is initially Gaussian will remain so during the time it resides in the column.

In practice non-Gaussian peak forms are often encountered. This may be due to adsorption or other effects which make the process non-linear. However, even in the absence of non-linear effects non-Gaussian peaks may occur, and are then due to "end effects" occurring at the column ends. At the loading end the effect naturally depends on the loading conditions, and the magnitude of the effect can be reduced by using a proper loading procedure. Under normal operational conditions the most important factors which are responsible for non-Gaussian peaks are the finite width of the loading zone and the initial departure of the process from steady state conditions. The latter effect is probably also responsible for the initial skewness of the peaks.

At the effluent end, the peak is distorted when it leaves the column. A peak which is Gaussian within the column thus becomes non-Gaussian in the elution diagram. However, this effect is probably quite small, since the variance of the peak only undergoes a slight change when the peak leaves the column[3]. It is also possible that the "end effects" at the two ends of the column counteract each other, which results in nearly Gaussian curves in the elution diagram. The "end effects" are in general difficult to study analytically and it seems that numerical methods[11] are better suited for this purpose.

COLUMN EFFICIENCY

Perhaps the most direct way of indicating the efficiency of a chromatographic

column is to express the peak width relative to its position. In the elution diagram this would be given by the standard deviation of the peak divided by the elution volume. However, in order to avoid the root sign, we may use the square of this quantity instead, *i.e.*, the variance divided by the square of the elution volume. This is a dimensionless quantity and for an efficient column it should be as small as possible. Calling it the reduced dispersion and denoting it by S, we have

$$S = \frac{\mu_{2V}}{V_e^2} \tag{15}$$

Using eqns. 4 and 5 and rearranging with the help of eqns. 1–3 we get

$$S = \frac{2D_1}{Lv} + \frac{2D_2(1 - w_1)}{Lvw_1} + \frac{2k}{L} + \frac{vV_2^2 w_1(1 - w_1)}{LD_2} + \frac{\overset{0}{\mu}_{2V}}{V_e^2} \tag{16}$$

Eqn. 16 reflects the requirements for an efficient column operation immediately. Thus, an efficient column should be long and have a fine-grained filling (small V_2). The requirements for the elution rate are conflicting. For optimal conditions the sum of the terms representing longitudinal Brownian diffusion should be equal to the term representing the chromatographic dispersion. Omitting the longitudinal diffusion in the stationary phase we get for the optimal velocity

$$v = \frac{1}{V_2}\sqrt{\frac{2D_1D_2}{w_1(1 - w_1)}} \tag{17}$$

The efficiency also depends on the solute distribution between the mobile and stationary phases, the term representing the chromatographic dispersion having a flat maximum when the solute is equally distributed between the two phases. Finally, the efficiency depends on the initial peak width, represented by μ_{2V}^0. This term depends on the loading conditions and thus characterizes the efficiency of the overall chromatographic process, rather than the efficiency of the column.

The reduced dispersion defined by eqn. 15 also constitutes the basis of the theoretical plate concept, which is often used to express the efficiency of chromatographic columns. The plate height in a column, H, is given by

$$H = L\left(\frac{w}{4V_e}\right)^2 \tag{18}$$

Since the width w of the peak is usually taken to be four times the standard deviation, we have

$$H = LS \tag{19}$$

In the present study we prefer not to use the theoretical plate concept, since its use in chromatography is somewhat misleading. Its physical significance does not extend beyond that of a relative peak width and it does not represent a genuine analogy to the similar concept in the theory of distillation.

Finally, we shall compare the present theory with the dynamic theory of GID-

DINGS AND MALLIK[12]. Translating their expression for the plate height into our notations we get

$$H = LS = \frac{2D_1}{v} + \frac{2D_2(1 - w_1)}{vw_1} + \frac{1.2vV_2^2w_1(1 - w_1)}{D_2} + \sum_i \frac{1}{1/A_i + 1/C_{mi}v} \qquad (20)$$

We have assumed that the gel particles here are spherical, hence $d_p = 6V_2$, where d_p is the particle diameter in ref. 12.

A comparison with eqn. 16 shows that, apart from the term for the initial peak width, which is absent in eqn. 20, the results are almost identical. In the case of the term representing the chromatographic dispersion, there is a small difference in the numerical constant, and in the term representing the eddy dispersion some additional terms are included in eqn. 20. This close agreement between the two theories, which are quite different in approach, is of course very satisfactory and gives us confidence in the results obtained.

SYMBOLS

v = translational velocity of mobile phase
γ = partition coefficient
t = time
w_1, w_2 = mass fractions of solute in the mobile and stationary phase, respectively
D = spreading coefficient
D' = longitudinal diffusion coefficient
D'' = chromatographic dispersion coefficient
D_1, D_2 = diffusion coefficients in mobile and gel phase, respectively
k = eddy diffusion coefficient
V_1, V_2 = volumes per unit of interphase area of mobile and stationary phase, respectively
σ = interphase area per unit length of the column
L = length of the column
$\theta = v\sigma V_1$ = rate of solvent flow through the column
μ, μ_2 = the mean and the variance of the concentration distribution in a peak in the column, respectively
$\dot{\mu}, \dot{\mu}_2$ = time derivatives of μ and μ_2
ν = peak mobility
$\omega = v\nu$ = velocity of the peak
μ_V, μ_{2V} = the mean and the variance with respect to the efflux volume
V_e = elution volume
V_0 = void volume in the column
V_i = volume of the internal solvent in the gel
V = total volume of the column
S = reduced dispersion
H = height of one theoretical plate.

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CHROM. 4913

ROLE OF THE MOBILE PHASE PERMITTIVITY IN THE USE OF THE CAPACITANCE DETECTORS IN LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of the mobile phase permittivity on the sensitivity and linearity of detection by the capacitance detectors has been investigated theoretically. Relations have been derived between the response and the concentration for the interference and bridge methods. A higher mobile phase permittivity in comparison to that of the substance detected results in greater linearity and a lower sensitivity.

INTRODUCTION

The progress of modern liquid chromatography has been limited, to a great extent, by the parameters of the existing detectors. The detectors employed up to the present time have been critically evaluated recently[1]. There is no doubt that some new principles of detection will be studied. A detection method whereby a change in the real part of the complex permittivity

$$\varepsilon^* = \varepsilon' - j\varepsilon'' \tag{1}$$

is measured seems to be promising.

The aim of the present paper is to derive the dependence of the linearity and sensitivity of detection on the permittivity of the mobile phase.

The mobile phase acts as the dielectric of the measuring condenser of the capacitance detector, and the measuring circuit is arranged in such a way that the electrical signal is backed off to zero when pure mobile phase with a permittivity ε'_0 flows through it. If a component of the mixture analysed, having a different real part ε'_x, occurs in the mobile phase, the reactance of the condenser, $X = 1/\omega C$, will be changed by ΔX which results in a signal at the detector output.

A linear additivity relation for calculating the resultant permittivity, ε'_r, holds approximately,

$$\varepsilon'_r = c_x\varepsilon'_x + c_0\varepsilon'_0 \tag{2}$$

where c_x and c_0 are the volume fractions of the component under detection, with a a permittivity ε'_x, and of the mobile phase of permittivity ε'_0, respectively.

THE MEASURING CONDENSER

The measuring condenser has an invariable capacity C_k, given by the solid dielectric which keeps the electrodes in their respective positions, as well as by the stray capacity, and a variable capacity that is dependent on the permittivity of the liquid dielectric (*cf.* Fig. 1). Hence, the total capacity of the measuring condenser may be characterised by the equation

$$C = C_{\varepsilon'} + C_k \tag{3}$$

It is obviously desirable that the capacity C_k be small in comparison to $C_{\varepsilon'}$. A high capacity C_k decreases the sensitivity of the detection, but improves, to some extent, the linearity.

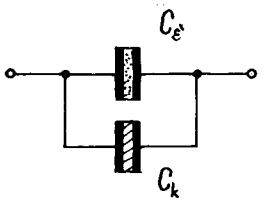

Fig. 1. An equivalent circuit for the measuring condenser of the capacitance detector.

THE RELATIONSHIP BETWEEN ε'_x AND ε'_0

If the component under detection is to bring about a change in the resultant permittivity, it is necessary that the permittivity of the former, ε'_x, be different from the carrier permittivity ε'_0; the greater the difference the larger the change at a given volume concentration. There are, in principle, two possible cases:

1. The mobile phase permittivity, ε'_0, is much higher than that of the component detected, ε'_x.

2. The permittivity of the component under detection is much higher as compared with that of the mobile phase.

Let us analyse the above cases with respect to the two groups of methods of measurement:

A. Interference methods;

B. Bridge methods.

Interference methods

The principle of the above methods is illustrated by the scheme shown in Fig. 2. A fixed frequency f_1 from an oscillator driven by a piezoelectric resonator is mixed in a mixer, M, with the frequency produced by the oscillator with the detecting condenser, whose capacity is given by eqn. 3. The high frequency components of the spectrum are rejected by a low-pass filter, F, so that the frequency component

$$|f_1 - f_2| = f_i \tag{4}$$

enters the frequency meter FM. Its output voltage is proportional to the frequency.

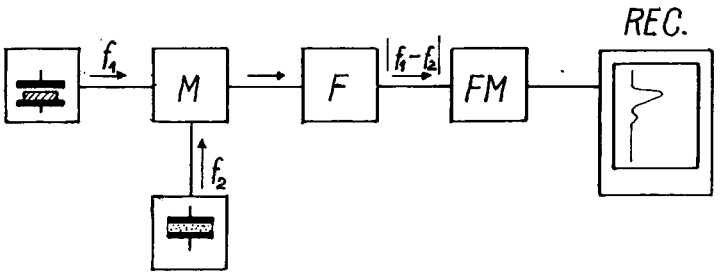

Fig. 2. Diagram of the measuring circuit used in the interference methods.

The relation between the frequencies f_1 and f_2 is chosen in such a way that the interference frequency equals zero when a pure mobile phase passes through the measuring condenser. Hence,

$$f_1 = f_{2(0)} = \frac{1}{2\pi\sqrt{L}\sqrt{C_{\varepsilon'_0} + C_k}} = k_1 \frac{1}{\sqrt{C_{\varepsilon'_0} + C_k}} \tag{5}$$

When the component detected occurs in the mobile phase, the permittivity of ε'_r will change, in compliance with eqn. 2, and the frequency f_2 will be:

$$f_{2(r)} = \frac{1}{2\pi\sqrt{L}\sqrt{C_{\varepsilon'_r} + C_k}} = k_1 \frac{1}{\sqrt{C_{\varepsilon'_r} + C_k}} \tag{6}$$

and the frequency meter FM will receive a frequency

$$f_i = |f_{2(0)} - f_{2(r)}| = k_1 \left| \left(\frac{1}{\sqrt{C_{\varepsilon'_0} + C_k}} - \frac{1}{\sqrt{C_{\varepsilon'_r} + C_k}} \right) \right| \tag{7}$$

In order to illustrate the role of the relation between ε'_0 and ε'_x, the data for plotting the graphs in Figs. 3 and 4 have been calculated from eqn. 7. If the measuring condenser has a capacity $C_{\varepsilon'} = 4$ pF with air and the invariable capacity is $C_k = 1$ pF, while the inductance L is chosen so that the frequency of the oscillator f_2 was 100 MHz in case of an empty condenser, it is possible to write:

$$f_2 = \frac{1}{2\pi\sqrt{L}\sqrt{(4+1)\cdot 10^{-12}}} = k_1 \cdot \frac{10^6}{\sqrt{5}} \tag{8}$$

i.e., $k_1 = 223.607$.

The plot in Fig. 3 illustrates the dependence of the interference frequency f_i on the volume fractions of the component within the range 0–0.10, for mobile phase and detected component permittivities of $\varepsilon'_0 = 10$ and $\varepsilon'_x = 2$, respectively.

The basic frequency with the mobile phase is:

$$f_1 = f_{2(0)} = k_1 \frac{1}{\sqrt{C_{\varepsilon'_0} + C_k}} = 34.921\ 509 \text{ MHz} \tag{9}$$

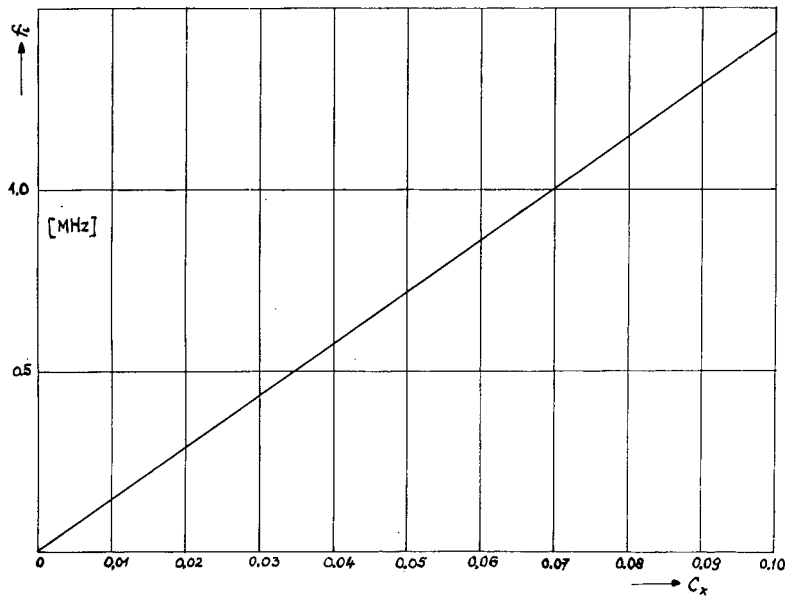

Fig. 3. Dependence of the interference frequency f_1 on the concentration with $\varepsilon'_0 = 10$ for the mobile phase and with $\varepsilon'_x = 2$ for the detected component.

The plot in Fig. 4 represents the dependence of the interference frequency f_i on the volume fraction within the range 0–0.10 for a mobile phase permittivity $\varepsilon'_0 = 2$ and the permittivity of the substance detected $\varepsilon'_x = 10$.

The basic frequency with pure mobile phase is:

$$f_1 = f_{2(0)} = k_1 \frac{1}{\sqrt{C_{\varepsilon'_0} + C_k}} = 74.535\ 597 \text{ MHz} \qquad (10)$$

It has been assumed in calculating the data for plotting the graphs that the same measuring condenser and inductivity L are employed, in both cases, while adjusting the fixed frequency f_i.

One can see on comparing the above diagrams that if the carrier permittivity ε'_0 is low in comparison to the permittivity of the component being detected, ε'_x, both the absolute and the relative sensitivity is higher than in the opposite case by about one order.

However, the situation is the reverse from the point of view of linearity. Higher values of the permittivity ε'_0 in comparison to ε'_x, results in a very good linearity being attained within a volume fraction range of 0–0.10. If a carrier liquid of low permittivity is used, the linearity may be regarded as satisfactory at very low concentrations. In the example given, *i.e.*, $\varepsilon'_0 = 2$ and $\varepsilon'_x = 10$, satisfactory linearity may be expected within volume fraction limits of 0–0.03; at a concentration of 0.10 a correction, by adding about 0.013, would be necessary.

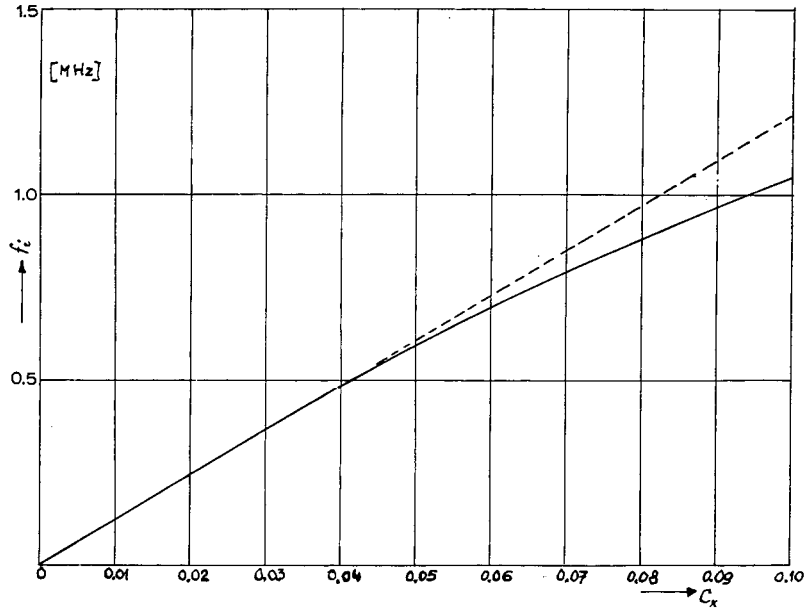

Fig. 4. Dependence of the interference frequency f_i on the concentration with $\varepsilon'_0 = 2$ for the mobile phase and with $\varepsilon'_x = 10$ for the detected component.

Bridge methods

If the impedance of the arm b–d in a balanced bridge as shown in Fig. 5 is changed by ΔZ_4, the voltage occurring across the diagonal a–b is given by:

$$\Delta\vec{U} = \vec{U}\cdot\left(\frac{\vec{Z}_4 + \Delta\vec{Z}_4}{\vec{Z}_3 + \Delta\vec{Z}_4 + \vec{Z}_4} - \frac{\vec{Z}_2}{\vec{Z}_1 + \vec{Z}_2}\right) \tag{11}$$

After a simple mathematical rearrangement we obtain:

$$\Delta\vec{U} = \vec{U}\cdot\frac{\Delta\vec{Z}_4}{\vec{Z}_4}\cdot\frac{\vec{Z}_1}{\vec{Z}_2}\cdot\frac{1}{\left(\frac{\vec{Z}_3}{\vec{Z}_4} + 1 + \frac{\Delta\vec{Z}_4}{\vec{Z}_4}\right)\cdot\left(\frac{\vec{Z}_1}{\vec{Z}_2} + 1\right)} \tag{12}$$

Let us introduce the presumption corresponding to maximum sensitivity of the bridge towards a change in the impedance of the arm Z_4:

$$\frac{\vec{Z}_1}{\vec{Z}_2} = \frac{\vec{Z}_3}{\vec{Z}_4} = 1 \tag{13}$$

Then, the voltage change ΔU across the bridge diagonal is given by:

$$\Delta\vec{U} = \vec{U}\cdot\frac{\Delta\vec{Z}_4}{\vec{Z}_4}\cdot\frac{1}{\left(4 + 2\cdot\frac{\Delta\vec{Z}_4}{\vec{Z}_4}\right)} \tag{14}$$

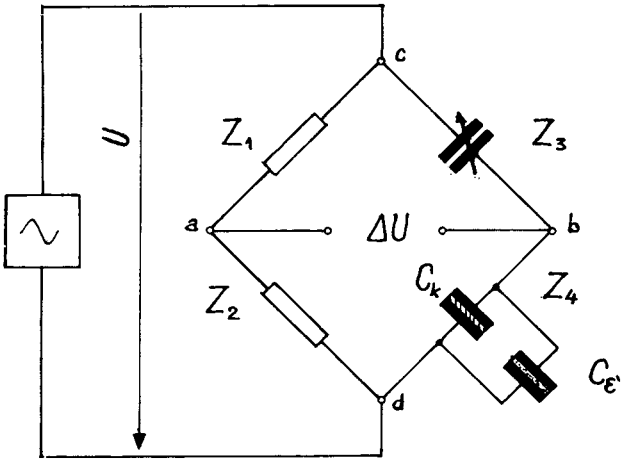

Fig. 5. Representative scheme of the measuring bridge.

If we assume that the impedance Z_4 is only represented by the reactance of the measuring condenser:

$$Z_4 = j\,X_4 = \frac{j}{\omega \cdot (C_{\varepsilon'0} + C_k)} \tag{15}$$

and

$$\Delta Z_4 = j\,\Delta X_4 = \frac{j}{\omega} \cdot \left(\frac{1}{C_{\varepsilon'0} + C_k} - \frac{1}{C_{\varepsilon'x} + C_k} \right) \tag{16}$$

Then

$$\frac{\Delta X_4}{X_4} = \frac{C_{\varepsilon'x} - C_{\varepsilon'0}}{C_{\varepsilon'x} + C_k} \tag{17}$$

On substituting the relation into eqn. 14, we obtain:

$$\Delta \vec{U} = \vec{U} \cdot \frac{(C_{\varepsilon'x} - C_{\varepsilon'0})}{(C_{\varepsilon'x} + C_k)} \cdot \frac{1}{\left[4 + \frac{2(C_{\varepsilon'x} - C_{\varepsilon'0})}{C_{\varepsilon'x} + C_k}\right]} \tag{18}$$

for the voltage change across the bridge diagonal.

This formula has been used to calculate the data for the plots shown in Figs. 6 and 7. The plot in Fig. 6 represents the dependence of the voltage ΔU for a bridge with an arm ratio $\vec{Z}_1/\vec{Z}_2 = \vec{Z}_3/\vec{Z}_4 = 1$, fed by a voltage $U = 1$ V, with the measuring condenser quoted in the above examples at $\varepsilon'_0 = 10$ and $\varepsilon'_x = 2$, on the concentration within volume fraction limits of 0–0.10.

Fig. 7 shows a plot for the same bridge, but for $\varepsilon'_0 = 2$ and $\varepsilon'_x = 10$.

In the second case, at $\varepsilon'_0 = 2$ and $\varepsilon'_x = 10$, the sensitivity is higher by a factor

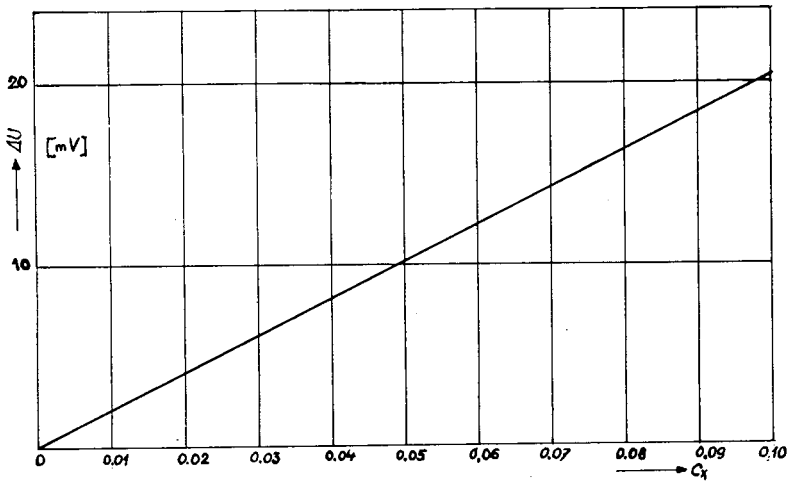

Fig. 6. Output voltage of the bridge with equal arms, fed by IV, showing the dependence on concentration ($\varepsilon'_0 = 10$, $\varepsilon'_x = 2$).

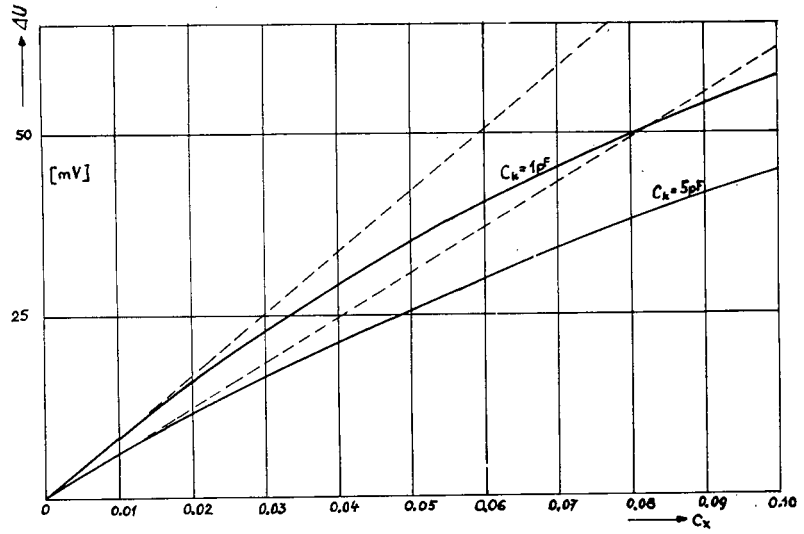

Fig. 7. Output voltage of the bridge with equal arms, fed by IV, showing the dependence on concentration ($\varepsilon'_0 = 2$, $\varepsilon'_x = 10$).

of 4 at concentrations up to 0.01. However, the linearity is substantially worse and may only be regarded as satisfactory up to a volume fraction of only 0.01.

When employing a mobile phase of high permittivity, ε'_0, in comparison to the permittivity of the component detected, ε'_x, excellent linearity may be attained, but at a somewhat lower sensitivity.

In Fig. 7 there is also a plot for the output voltage ΔU for the case where the invariable capacity $C_k = 5$ pF and the $C_{\varepsilon'}$ of the empty condenser is again 4 pF. It is apparent from the plot that although the sensitivity falls, the linearity increases.

CONCLUSION

In virtue of the present theoretical study it would seem that detection based on the principle of permittivity changes is promising for the construction of sensitive and linear detectors for modern liquid chromatography in the case where the mobile phase having a very different permittivity to that of the substance detected is used. Excellent linearity is attained when the permittivity of the mobile phase is substantially higher than that of the detected components, but the sensitivity is lower. Higher sensitivity is attained in the case when a mobile phase with a lower permittivity relative to the permittivities of components detected is used, however, the linearity is poorer and may only be regarded as satisfactory up to volume fraction concentrations of about 0.01.

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CHROM. 4920

PRESSURE INDUCED TURBULENCE IN A PACKED GAS CHROMATOGRAPHIC COLUMN

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SUMMARY

HETP *versus* carrier gas flow velocity curves have been measured on a low liquid load packed column operated under two different mean absolute pressures within wide flow velocity limits, using hydrogen as the carrier gas. In the case of the higher column pressure, a fall-off of the HETP occurred at a certain velocity, indicating the onset of turbulence in the flow of the carrier gas, whereas the corresponding curve obtained at the lower pressure displayed all the characteristics of laminar flow. The extent of turbulence was checked by measuring the characteristics of the dynamics of flow under the corresponding conditions.

INTRODUCTION

The idea that the laminar flow was the only practical kind of mobile phase motion in a packed or open gas chromatographic column was at one time widely accepted. However, it was seen in further investigations[2–4] that turbulent flow may also occur in the column, especially with packed columns, under conditions not very remote from the conventional ones.

A typical consequence of the onset of turbulence is a fall-off of the HETP at higher carrier gas velocities. However, this effect can also be interpreted in terms of GIDDINGS' coupling theory[5], and it is difficult to separate the coupling and the turbulence contribution from each other in terms of chromatographic measurements alone.

A great deal of valuable information on the role of turbulence in chromatography can be gained from papers published on the dynamics of flow of fluids[6–8]; the above papers clearly indicate that it is in a transitory streamlined-to-turbulent flow regime, rather than in a streamlined one, that many gas chromatography experiments are carried out with packed columns.

The present paper shows the role of the mean absolute column pressure as a turbulence inducing factor. Initially, the aim of the experiments presented here was to verify the concepts on the effect of the column length and the mean absolute column pressure on the course of the HETP *versus* flow velocity curve under streamlined flow conditions[9,10]. In measurements on a low liquid load packed column of a given length, operated at two different column outlet pressures, *i.e.*, at two different levels of mean

absolute column pressure, it turned out that while the results obtained at the lower absolute pressure were as expected for the whole range of flow velocities employed, there was a marked fall-off of the HETP at higher velocities in the measurement at the higher column pressure. Since it was suspected that this phenomenon stemmed from turbulence in the column, additional measurements were carried out on the dynamics of the carrier gas flow under the corresponding conditions.

EXPERIMENTAL

The measurement was carried out with a conventional column packing, 3 wt. % dinonyl phthalate on Chromosorb P 60–80 mesh, prepared by the slurry technique. The support (Carlo Erba, Italy) was screened till free from fines, dried at 200° for 2 h, and then coated with the stationary liquid (Griffin & George Ltd., Great Britain), using dichloromethane as the solvent. The column was a 0.75 m long stainless steel tube of 3 mm inner diameter, filled with 2.70 g of the above packing. Hydrogen was used as the carrier gas and a thermal conductivity detector was employed. Hexane was used as the solute, the samples being introduced in the form of about 40 μl charges of saturated hexane vapours in hydrogen with traces of air; a Zimmermann syringe (Zimmermann, D.D.R.) was employed. The injection port, column, and detector were kept at 40°.

The measurements proper were carried out on a Becker Multigraph 409 (Becker Delft, The Netherlands) adapted for work at high column inlet pressures and adjustable column outlet pressures; a more detailed description of the instrumentation used can be found in a previous paper[9]. The chromatograms were recorded by a Servogor RE 512 recorder (Goerz Electro G.m.b.H., Austria).

RESULTS AND DISCUSSION

The results of the measurements were processed in such a way as to produce the graphs in Fig. 1. Curves 1 and 2 were obtained at pressures of 1 and 3 atm, respectively, at the column outlet. Curves 1′ and 2′ represent the $j(P_i^2 - P_o^2)/2P_o$ *versus* $\bar{u}$ dependences corresponding to curves 1 and 2; P_i and P_o stand for the column inlet and column outlet pressures and j denotes the James-Martin compressibility factor.

The mean carrier gas velocity related to the HETP, $\bar{u}$, was determined from the ratio of the column length and the hold-up time of the air peak maximum. The apparent HETP, $\bar{H}$, was calculated by dividing the column length by the number of theoretical plates determined from the expression[11] $5.54(b/\Delta b_{1/2})^2$ where b and $\Delta b_{1/2}$ denote the distance of the solute peak maximum from the start line in the chromatogram and the peak width at the peak mid-height, respectively.

The data necessary for assessing the character of carrier gas flow were obtained by measuring the volume flow rate at the column outlet, using a soap-bubble flowmeter. The flow rate was expressed for the mean pressure and temperature in the column, employing the relation $\bar{v} = P'v'Tj/P_oT'$ where v' is the volume flow rate as measured at pressure P' and temperature T' and T is the column temperature. In order to make the data directly comparable with the results of chromatographic measurement, the column void cross-section, a, was calculated from several pairs of

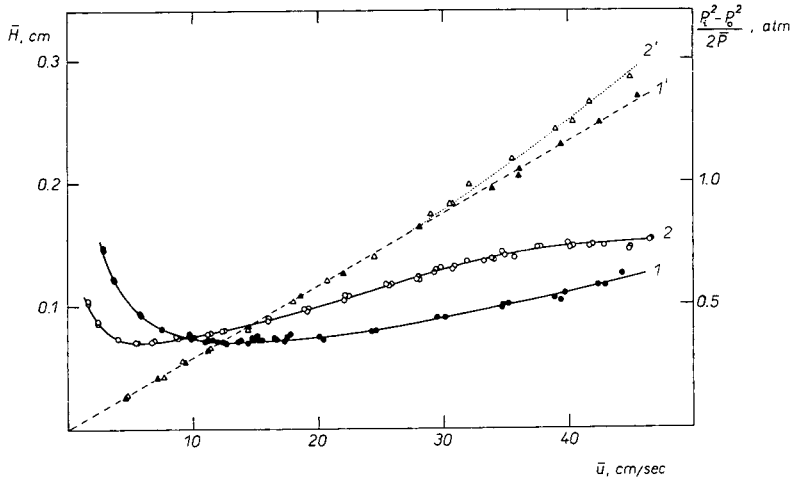

Fig. 1. $\bar{H}$–$\bar{u}$ curves and the corresponding $(P_i^2 - P_o^2)/2\bar{P}$ *versus* $\bar{u}$ plots obtained from the results measured for different levels of the mean absolute column pressure. Curves 1 and 2 correspond to P_o of 1 and 3 atm and P_i of within 1.08–2.36 and 3.02–4.45 atm, respectively, curves 1′ and 2′ have been constructed for the conditions corresponding to curves 1 and 2, respectively; P_i and P_o are the column inlet and the column outlet pressure and $\bar{P}$ denotes the mean column pressure.

results determined at a lower flow velocity by means of the air peak hold-up time and the respective volume flow rate measured directly. Thus, the former relation could be rewritten to read $\bar{u} = P'v'Tj/P_oT'a$. The $\bar{u}$ values so obtained were plotted against the corresponding values of the parameter $j(P_i^2 - P_o^2)/2P_o$. It turned out that the $\bar{u}$ values determined by the above procedure were practically identical to the corresponding $\bar{u}$ values obtained by dividing the column length by the gas hold-up time also in the region of higher velocities. According to Darcy's law, the above plot should be a straight line as long as the flow is laminar, while a deviation from linearity should indicate the onset of turbulence.

Curve 1 in Fig. 1 displays a typical course for a low liquid load packing column, operated under streamlined carrier gas flow conditions, the latter being evidenced by the course of line 1′. With carrier gas velocities up to 28 cm/sec, the change in the course of the $\bar{H}$ *versus* $\bar{u}$ curve upon the rise of the column outlet pressure from 1 to 3 atm (curve 2) is quite in accordance with expectation[9]; the concurrence of lines 1′ and 2′ indicates that the flow also remains laminar under the elevated mean column pressure within the above flow velocity region. However, further increase in the flow velocity results in a flattening of the respective part of curve 2. The point of the HETP fall-off on curve 2 clearly coincides with the incipience of the deviation from linearity of curve 2′. Hence, the above point on curve 2 indubitably corresponds to the onset of turbulence in the column.

The above situation can be accounted for in terms of the modified Reynolds number[6], defined by $Re = 2\bar{u}'r_p d_m/\eta_m$ where $\bar{u}'$ is the actual carrier gas velocity within the packing bed, r_p is the radius of the packing particles, and d_m and η_m are the density and the viscosity coefficient of the carrier gas, respectively. As the carrier gas density rises with increasing pressure while the viscosity remains essentially

unchanged, the modified Reynolds number increases proportionally to the pressure at a given flow velocity.

The mobile phase moves forward only in the interparticle space and is stagnant within the intraparticle voids. As the intraparticle void volume is accessible by diffusion, the velocity determined from the air peak hold-up time is lower than the actual flow velocity by the factor of the interparticle-to-total void volume ratio. Since the interparticle and intraparticle void volumes are approximately equal with low liquid load Chromosorb P[12], we shall assume that $\bar{u}' = 2\bar{u}$ in our case. The particle size of the 60–80 mesh support corresponds to an average particle radius of 0.0107 cm, and the viscosity coefficient of hydrogen amounts to about 0.92×10^{-4} Poise at 40°. The critical point on curve 2, corresponding to a $\bar{u}$ value of 28 cm/sec, corresponds to a mean absolute column pressure of 3.42 atm, *i.e.*, to a carrier gas density of 2.66×10^{-4} g/cm^3. For curve 1, the same velocity gives a pressure and density of 1.46 atm and 1.14 g/cm^3, respectively. Hence, the Reynolds numbers corresponding to the points given by $\bar{u} = 28$ cm/sec ($\bar{u}' = 56$ cm/sec) on curves 1 and 2 as well as 1′ and 2′ amount to 3.42 and 1.46, respectively. This is in good agreement with ERGUN's findings[6] indicating that turbulence begins at a Reynolds number of about 3 in packed columns.

The transitory laminar-to-turbulent flow region is very large, being completely turbulent[6] at a Reynolds number as large as about 3000, so that there are wide limits in which a fall of the steepness of the ascending branch of an $\bar{H}$–$\bar{u}$ curve may come about upon increasing the mean absolute column pressure. In this respect, the raising of the absolute column pressure under laminar and under turbulent flow conditions will result in opposite effects. This situation may be demonstrated by comparing the results obtained by measurements under streamlined flow conditions using H_2 carrier gas[9] with the results arrived at by HALÁSZ *et al.*[13] who worked with N_2 carrier gas. Owing to the density of nitrogen being more than one order higher than that of hydrogen and the viscosity only about twice that of hydrogen, the measurements by the latter authors were probably carried out within the above transitory region with respect to the higher flow velocities; the courses of their experimental points show a definite tendency towards domed curvature of the ascending branches of the $\bar{H}$–$\bar{u}$ curves measured at 4.0 and 6.0 atm outlet pressures, especially if less weight is given to the last three points of the 6.0 atm curve.

In open columns, turbulence begins at much higher Reynolds numbers. That is why the results of the measurements by HAZELDEAN AND SCOTT[14], also performed under elevated mean absolute column pressures, obey the concepts of the laminar carrier gas flow.

CONCLUSIONS

The raising of the mean absolute pressure in a gas chromatographic column by increasing the column outlet pressure leads to the onset of turbulence in the carrier gas flow. With a packed column, the turbulence becomes apparent at a modified Reynolds number value of about 3, which is in conformity with the conclusions reported by KNOX[3] for measurements in a different system.

The laminarity or the extent of turbulence of flow is indicated by the course of the $j(P_i^2 - P_o^2)/2P_o$ *versus* $\bar{u}$ plot; the incipience of the deviation from linearity

coincides with that of a fall-off of the HETP on the corresponding $\bar{H}$–$\bar{u}$ curve.

The conflicting findings with respect to the pressure dependence of the course of the ascending branch of the HETP *versus* flow velocity curve are probably due to the turbulence. The high propensity of the carrier gas flow towards pressure induced turbulence in packed columns may be a complicating factor in the determination of the gas phase and liquid phase mass transfer resistance coefficients by methods based on HETP measurements with gases of different density[15].

It also seems questionable as to what extent specific retention volumes remain independent of the carrier gas flow rate when measured under turbulent flow conditions and calculated using the James-Martin compressibility factor.

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CHROM. 4919

RADIOLYSIS PRODUCTS AS REFERENCE SUBSTANCES IN GAS CHROMATOGRAPHY

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SUMMARY

The possibility of using the many branched hydrocarbons, which are formed by radiolysis of an alkane, as reference substances in qualitative gas chromatographic analyses is suggested. The radiolysis spectra of *n*-alkanes are discussed in general with the aim of showing the rules for rapid and easy identification of the various products. The heavy parts of the radiolysis spectra of *n*-heptane and *n*-octane are described in detail as examples. Simple formulas for an easy and rapid calculation of the boiling point of any heavy radiolysis product are given in an Appendix.

INTRODUCTION

The wide development of gas chromatographic techniques at present allows the separation of very complex mixtures of compounds, even in the field of high-boiling point substances. These techniques find wide application in many fields and certainly have been used to a great extent in the study of the composition of crude oils. The difficulties met today in this kind of research often depend more on the lack of a sufficiently wide collection of reference substances than on the technical problems of separation. For example, in the case of the hydrocarbons, in which we are more directly interested, only a very small fraction of the many possible branched alkanes is available over C_7; the availability of alkenes is even scarcer.

We therefore propose the use of mixtures of hydrocarbons, which are formed by radiolysis of an alkane, as a source of reference substances and wish to demonstrate that this technique of standardisation may be applied very easily and offers wide possibilities.

Radiolysis of any alkane with n C atoms produces many saturated and unsaturated hydrocarbons with a number of C atoms v ranging from $v = 1$ to $v = 2n$. The formation of these compounds follows definite rules which we have deduced by means of much experimental work in the field of radiolysis[1] and which allows the prediction of the full spectrum of the radiolysis products of any alkane. In principle, therefore, an enormous number of heavy branched alkanes could be obtained, on demand, by radiolysis of a lighter hydrocarbon, properly chosen among the available products. However, the usefulness of a particular radiolysis product as a reference substance is conditioned by the ease with which the product can be immediately identified in

the radiolysis spectrum to which it belongs. This condition is fulfilled by practically all the radiolysis products of a normal alkane, but the radiolysis spectrum of a branched alkane is too complex to be of easy and immediate interpretation. For this reason we will restrict our description and discussion to the radiolysis spectra of *n*-alkanes in which linear and branched products are easily identifiable by their belonging to very characteristic groups and by their relative abundance inside the group.

The radiolysis spectra described here were obtained by irradiating the sample, contained in evacuated capillary ampoules, with ^{60}Co γ-rays at room temperature. It must be noted, however, that one may change some of the conditions, such as the shape of the ampoule, the nature of the ionising radiation (if α-rays or other very high "linear energy transfer" particles are ruled out) or the temperature, without substantially altering the shape of the spectra, at any rate, not to an extent which need be taken into account for the present purpose.

We believe that a sample of an *n*-alkane irradiated with a dose of 5–50 Mrad under conditions which may be varied considerably may be easily prepared today in any laboratory.

EXPERIMENTAL

The experimental conditions under which the chromatograms of Figs. 1 and 2 were obtained are given in Table I. It must be noted that these conditions were chosen so as to obtain sufficiently compact chromatograms which would give a comprehensive illustration of at least the heavy part of the radiolysis spectra. Obviously, much better resolution may be obtained under other conditions: especially for the products immediately following the parent alkane ("dehydrogenation products" and the first group of "synthesis products"; see further on), which are hidden under the tail of the giant parent-peak in the chromatograms of Figs. 1 and 2, but can easily be resolved in chromatograms obtained under different conditions.

Some further details about the preparation of the columns may be of interest. A soft glass tube (O.D. = 7.6 mm; I.D. = 3.5 mm) was drawn to the required length and thickness by means of an apparatus very similar to that described by DESTY *et al.*[2]. The capillary column was then: (a) filled with a 10% solution of NaOH; (b) kept at 110° for 60 min; (c) washed with hot, progressively less basic solutions down

TABLE I

EXPERIMENTAL CONDITIONS

Instrument	"Fractovap Model D" GLC unit (Carlo Erba)
Detector	Hydrogen flame ionisation
Vaporiser temperature	300°
Detector temperature	250°
Detector voltage	150 V
Sample size	~ 4 μl
Head split ratio	100/1
Column	100 m "attacked" glass capillary × 0.3 mm I.D.; 5% wt. squalane in *n*-hexane
Column flow	1.25 ml/min
Carrier gas	N_2
Temperature program	Isothermal at ~ 50° until after elution of *n*-hexane; then heated to 130° at 1°/min

to neutral reaction; (d) impregnated with a solution of suitable concentration of the chosen stationary phase. Resolving powers of up to 1000 theoretical plates/m were obtained on 100–150 m columns.

Similar results (or perhaps a little less satisfactory) were obtained by coating the internal surface of hard-glass capillary columns with carbon black before impregnation. The carbon deposition was obtained by thermal decomposition of CH_2Cl_2 vapours. The column was filled with a mixture of dry N_2–CH_2Cl_2 vapours, obtained by bubbling dry N_2 through liquid CH_2Cl_2 kept at 0°. The column was then sealed, heated to 450° and kept at this temperature for 1 h.

Very good results were also obtained with micro-packed columns prepared by drawing a glass tube to capillary dimensions (~ 0.50 mm diameter) previously filled up with Al_2O_3 powder (150–170 mesh, activated at 700° for 5 h). These columns, however, require rather high pressures of carrier gas and therefore were used by us for hydrocarbons with up to about six C atoms only. Many different stationary phases were tested and the best results were obtained with Ucon 50 LB 550 X (15 % solution in ether for impregnation). A column prepared with this stationary phase on attacked glass can resolve all the radiolysis products of *n*-decane, namely, hydrocarbons from methane up to a group of fifteen C_{20} isomers (except for the ethane–ethylene pair).

In general, all the columns described here show a behaviour — from the standpoint of retention times — which is intermediate between that of a packed gas–solid column and that of a normal capillary column for GLC. The behaviour of each column may be largely predetermined (on the basis of particular requirements) by means of a suitable choice of the degree of moisture of the solid support and of the quantity of liquid phase.

Our samples were prepared for irradiation by degassing them (by means of repeated freezing–evacuation–melting cycles) inside small glass capillaries (diameter = 1.5 mm; l = 45 mm), which were then sealed under vacuum and irradiated in a ^{60}Co γ-cell with a dose of up to 22 Mrad. The irradiated capillary was then introduced into a very small bulb-crusher which we had substituted for the customary inlet system of the gas chromatograph and broken in the stream of carrier gas*.

DISCUSSION

The radiolysis spectrum of an *n*-alkane with *n* C atoms may be subdivided into four parts: (1) the zone of "fragmentation products", which contains saturated and unsaturated hydrocarbons with the number of C atoms ranging from 1 to $n - 1$; (2) the zone of "dehydrogenation products" which contains unsaturated hydrocarbons with *n* C atoms; (3) the zone of "synthesis products", which contains saturated hydrocarbons with the number of C atoms ranging from $n + 1$ to $2n - 1$; (4) the zone of "dimerisation products" which contains saturated hydrocarbons with $2n$ C atoms. Each one of these parts is easily recognisable by the methods described below, each of them separately.

We will start our description with the group with the heaviest products because of its greater interest as a source of reference substances.

* This technique is very useful as it avoids the fractionation of the mixtures when precise quantitative analysis throughout all the spectrum is required. It is obviously unnecessary if only qualitative use of the radiolysis mixtures is made.

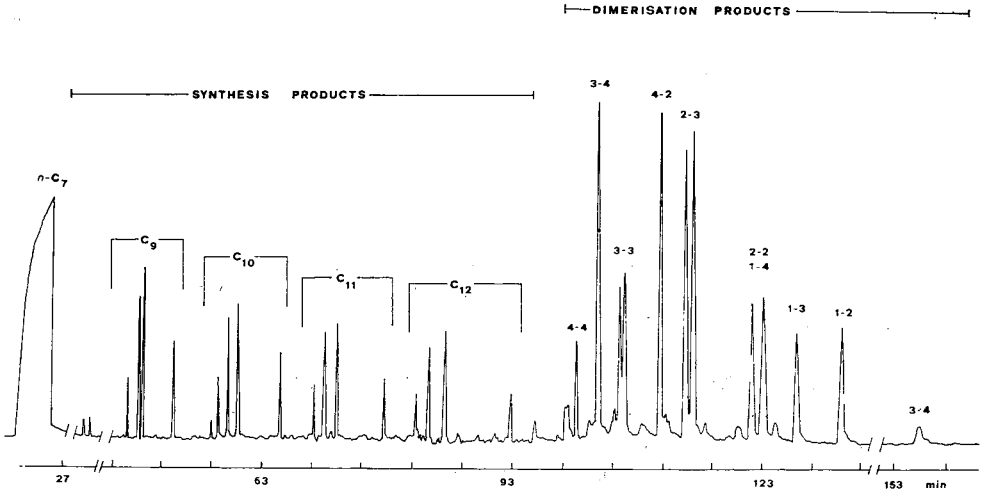

Fig. 1. γ-radiolysis spectrum of n-heptane. Zones of products heavier than the parent compound.

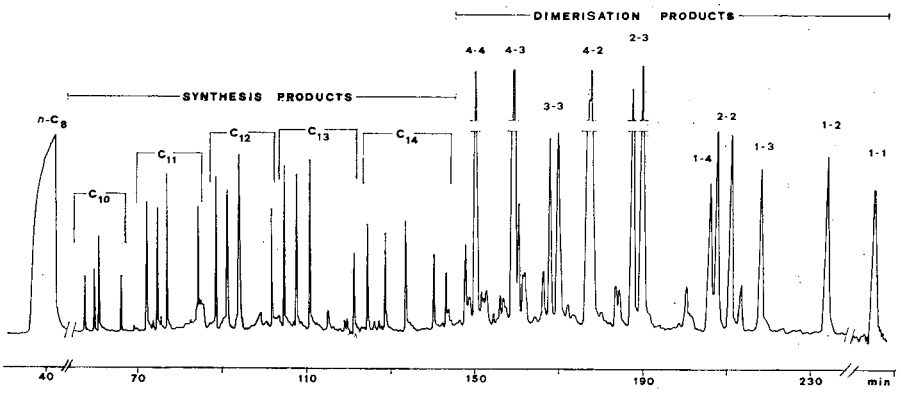

Fig. 2. γ-radiolysis spectrum of n-octane. Zones of products heavier than the parent compound.

Dimerisation products

As shown in Figs. 1 and 2, two distinctly separated zones appear in the chromatograms after the giant peak of the parent hydrocarbon; the first one is constituted by rather small peaks, given by "synthesis products", which are formed with low yields; the second one is constituted by much larger peaks (interrupted in Fig. 2), corresponding to the "dimerisation products". The separation of the two zones is clear cut.

The group of "dimerisation products" contains all the C_{2n} alkanes which may be formed by linkage of two individual parent molecules or — in other words — by addition of two parent radicals. In hexane, for instance, the following six C_{12} isomers are to be found: 4,5-diethyloctane, formed by linkage of two 3 positions (3–3); 3-ethyl,4-methylnonane, formed by linkage of the 2 and 3 positions (2–3); 4,5-dimethyldecane, formed by linkage of two 2 positions (2–2); 4-ethyldecane, formed by

linkage of the 1 and 3 positions (1–3); 5-methylundecane, formed by linkage of the 1 and 2 positions (1–2); and *n*-dodecane, formed by linkage of two 1 positions (1–1).

In general, in an *n*-alkane with *n* C atoms one finds $n(n+2)/8$ different dimers if *n* is even and $(n+1)(n+3)/8$ isomers for odd *n*. With nonpolar stationary phases (or of intermediate polarity), the dimers will be eluted according to the order of their boiling points, which may be easily calculated using the rules given in the Appendix. In general, the most branched isomer, which is formed by linkage of the two most central positions, will appear first and the linear one last. The peak of the linear dimer, which appears markedly later than the group of peaks of the branched isomers, is noticeably smaller, clearly marks the end of the chromatogram. It must be noted that a number of branched isomers contain two asymmetric C atoms and are made up of two equally abundant diastereoisomers. High resolution capillary columns, required in this kind of work, usually resolve the two diastereoisomers in a pair of nearby, almost equal (depending on the background) peaks. Three such pairs are shown in Fig. 2, corresponding to the 3–3, 3–2 and 2–2 dimers of *n*-octane. This fact, which causes an increase in the number of peaks, does not introduce any real complication in the identification of the products because of the ease with which a pair may be recognised as such. On the contrary, it sometimes represents a good check of the identification as, for instance, in the case of the dimers 1–4 and 2–2 of Fig. 2.

Another useful tool for checking the identification is the relative abundance of each isomer inside its group.

As a general rule, the dimers given by an *n*-alkane with an even number of C atoms may be subdivided into four groups of increasing abundance*

(1) the 1–1 dimer;
(2) the dimers formed by linkage of the 1 position to any other position;
(3) the dimers formed by linkage of two C atoms in the same position (other than the 1–1);
(4) the dimers formed by linkage of two different non-terminal carbons.

For an *n*-alkane with an odd number of C atoms the most central position is represented by one atom only instead of two. Consequently, the yields of the products formed by linkage of this position are lower and the corresponding chromatographic peaks markedly smaller (about half the size of those of the other isomers pertaining to the same group of abundance). The first peak, which is given by the isomer formed by linkage of two central carbons, is about four times smaller than the other ones of the same group (namely, group 3). A "corrected" internal distribution of the dimers given by an odd *n*-alkane may be obtained by multiplying by 4 the area of the first peak and by 2 those of the other peaks supposed to correspond to dimers given by the central carbon; the internal distribution obtained with these new data must coincide with that of the $n+1$ even *n*-alkane and therefore must follow the general rules just given above for even hydrocarbons.

*Synthesis** products*

Synthesis products are all the saturated hydrocarbons which may be formed by the substitution of a H atom of a parent molecule by an alkyl radical formed by

* Detailed quantitative information is given in ref. 1.
** Sometimes also called "intermediate" products.

fission of a C–C bond of the parent. In hexane, for instance, five groups of synthesis products are obtained, with 7 to 11 C atoms, and each group will be made up of 3 isomers, namely, the linear and two branched ones, corresponding to the substitution position of the hydrogen, 1, 2 or 3, respectively. In the group of products with 8 C atoms, for instance, we would find: 2-ethylhexane; 3-methylheptane; and *n*-octane. With nonpolar stationary phases (or of intermediate polarity), where the order of elution follows the order of boiling points of the products, the most branched isomer will appear first and the normal one last, somewhat apart from the others.

Only in the first group (the C_{n+1} products) is the order of elution somewhat different because the 2-derivative, which is characterised by the higher symmetry group $R–CH(CH_3)_2$, is the most volatile compound and is eluted first. The other ones are eluted successively following the order just mentioned for all the other groups.

Furthermore, the height ratios of the various peaks in each group are practically the same in all the groups except for the first (see ref. 1). As a results of this, the zone of synthesis products is consequently made up by the repetition of an *"a priori"* known number of very typically shaped groups of rather small peaks, which are positioned between the giant peak of the parent hydrocarbon and a group of much larger peaks, the dimers (see Figs. 1 and 2). Owing to these characteristics they cannot be confused by any means with other products and each one of the isomers is very easily identified in an unquestionable manner.

Unsaturated hydrocarbons are practically absent in this part of the spectrum except for the heaviest group of synthesis products (C_{2n-1} isomers) where they are present in amounts comparable to those of saturated isomers and may give rise to some uncertainty in the identification. The alkanes of this last group cannot, however, be used as reference substances in the spectra of hydrocarbons with $n > 6$ because of some overlapping of the most branched dimers (see Fig. 2).

Dehydrogeneration products

All the possible alkenes with one double bond and the same skeleton of C atoms as the parent alkane are contained in this group. The peaks corresponding to them do not appear in Figs. 1 and 2 but they are easily resolved with a liquid phase of intermediate polarity. In chromatograms obtained in this manner they are clearly distinguishable from the nearby synthesis products because of the very high yields with which they are formed. Nevertheless we think that these compounds cannot be of any use as reference substances because their identification is not as simple as it is for the dimerisation and synthesis products just discussed. Indeed, each alkene, except for the first, gives two chromatographic peaks, corresponding to the *cis* and *trans* forms, and the relative position of any peak is strongly determined by the operating conditions. Also, the relative abundances are not so markedly different as to provide a useful tool for identification.

Fragmentation products

All the *n*-alkanes with a number of C atoms smaller than that of the parent and the corresponding 1-alkenes are contained in this part of the spectrum. The alkanes are present at about the same molar concentration, except for methane and the C_{n-1} alkane, whose yields are markedly lower (3–4 times lower). The molar abundance of the 1-alkenes is about 30 % of that of the corresponding alkane. 2-Alkenes and some

acetylene are produced at such low yields that they do not interfere in the identification. Branched hydrocarbons are totally absent. It may be noted that all the "fragmentation products" are hydrocarbons easily available on the market; they may represent, however, a series of reference substances which one can easily and quickly prepare.

APPENDIX

The boiling temperature (b.p.) of any paraffin may be calculated starting from that of the normal isomer* by means of the empirical formula of GREENSHIELDS AND ROSSINI[3]:

$$\text{b.p. (isomer)} = \text{b.p. (normal)} - 4.50\ C_3/n^{1/2} - 5.72\ C_4/n^{1/2} + 15.87\ \Delta P_3/n^{1/2} + 72.93\ \Delta W/(n^2 - n) + 10.6\ P_4' \quad (1)$$

where n is the total number of C atoms; C_3 is the number of tertiary C atoms; C_4 is the number of quaternary C atoms; P_3 is the number of pairs of C atoms which lie three bonds apart from each other and $\Delta P_3 = P_3$ (isomer) $- P_3$ (normal); W (Wiener number) is the total number of bonds between all the possible pairs of carbons and ΔW is W (isomer) $- W$ (normal); P_4' is the number of quaternary carbon pairs separated by one carbon atom in the isomer.

It is well known that this empirical formula gives progressively less accurate approximations as the number of C atoms increases and sensible discrepancies between calculated and experimental boiling point may be already found for $n = 12$ when quaternary carbons are present. Radiolysis products of n-alkanes, however, never contain quaternary C atoms and their boiling point may be calculated with satisfactory approximation up to much larger molecular weights.

The computation of the parameters ΔP_3 and ΔW is a rather long and tedious exercise, especially when the boiling point of a number of compounds are to be calculated, as it is for a group of radiolysis products. Therefore we will give below some simple formulas whereby these parameters may be quickly calculated for the isomers we are interested in, namely, branched alkanes with only one or two tertiary carbons. These formulas have been obtained by expanding the double summations contained in ΔP_3 and ΔW and applying the expressions thus obtained in the cases that interest us. The results are collected in Table II.

Let us consider, first, a normal alkane with $n \geqslant 3$. We will have

$$P_3 = n - 3 \quad (2)$$

and

$$W = \sum_{2}^{n}{}_{h} \sum_{1}^{h}{}_{t} (h - t) = (n^3 - n)/6 \quad (3)$$

* The boiling points of normal alkanes are given in the Am. Petrol. Inst. Project 44 (College Station, Texas) up to C_{100}; the boiling point of branched alkanes are up to C_{12} only.

TABLE II

VALUES OF ΔP_3 AND ΔW FOR SYNTHESIS AND DIMERISATION PRODUCTS

Products with 1 tertiary carbon			*Products with 2 tertiary carbons*		
ΔP_3			ΔP_3		
	$k = \begin{cases} 2 \\ \\ n-l-1 \end{cases}$	$2 < k < (n-l-1)$		$l_1 = 1$	$l_1 > 1$
$l = 1$	0	1	$l_2 = 1$	3	4
$l > 1$	1	2	$l_2 > 1$	4	5
ΔW			ΔW		
	$-l(k-l)(N-k)$		$-N[(N+1)(n_1+n_2) - (n_1^2+n_2^2) - 2N]$		

Let us consider next:

(a) Branched alkanes with $n-l$ carbons in the main chain and one side chain of l carbons at the kth position. We will have

$$P_3 = n-1 \text{ if } 2 < k < n-l-1 \quad \text{and } l > 1 \tag{4}$$

$$P_3 = n-2 \text{ if } 2 < k < n-l-1 \quad \text{and } l = 1 \tag{5}$$

$$P_3 = n-2 \text{ if } k = 2 \text{ or } k = n-l-1 \text{ and } l > 1 \tag{6}$$

$$P_3 = n-3 \text{ if } k = 2 \text{ or } k = n-l-1 \text{ and } l = 1 \tag{7}$$

The Wiener number will be

$$W = \sum_{2}^{n-l} \sum_{1}^{h} (h-t) + \sum_{1}^{l} \sum_{0}^{h} (h-t) + \sum_{1}^{k-1} \sum_{1}^{l} (k-h+t) + \sum_{k+l}^{n-l} \sum_{1}^{l} (h-k+t) \tag{8}$$

where the first double summation refers to the main chain, the second one to the side chain and the third and fourth to the pairs, one atom of which is in the side chain and the second one on either one side or the other of the main chain. By calculating the summations and rearranging the results one obtains

$$W = (n^3-n)/6 - l(k-1)(n-k-l) \tag{9}$$

(b) Branched alkane with $n-l_1-l_2$ carbons in the main chain, l_1 carbons in a side chain at the k_1th position and l_2 carbons in another side chain at the k_2th position. In all the cases we are interested in, we also have $k_2 = k_1 + 1$; $3 \leqslant k_1 \leqslant (n-l_1-l_2-3)$. In these cases we will obtain

$$P_3 = n+2 \text{ if } l_1, l_2 > 1 \tag{10}$$

$$P_3 = n+1 \text{ if } l_1 = 1;\ l_2 > l_1 \tag{11}$$

$$P_3 = n \quad \text{if } l_1 = l_2 = 1 \tag{12}$$

and

$$W = W_1 + W_2 - \sum_{2}^{n-l_1-l_2} \sum_{1}^{h} (h-t) + \sum_{1}^{l_2} \sum_{1}^{l_1} (h+t+k_2-k_1) \tag{13}$$

where W_1 and W_2 are the Wiener numbers calculated by eqn. 9 for the two single branched alkanes with $n_1 = n - l_2$; $l = l_1$; $k = k_1$ and with $n_2 = n - l_1$; $l = l_2$; $k = k_2$ respectively; the third term subtracts the contribution of the pairs in the main chain, which has been considered twice in summing W_1 and W_2; the fourth term represents the contribution of the pairs with one atom in the first side chain and the second in the other one. By calculating the summations and rearranging the results, one gets

$$W = (n^3 - n)/6 - l_1(k_1 - 1)(n - l_1 - l_2 - k_1) - l_2k_1(n - l_1 - l_2 - k_1 - 1) - l_1l_2(n - l_1 - l_2 - 2) \quad (14)$$

Subtracting eqn. 3 from eqns. 9 and 14 we immediately obtain the required values of ΔW for cases (a) and (b) respectively.

The expressions of ΔW may be further simplified. Disregarding the official nomenclature, one may consider any product with only one tertiary carbon as the result of the addition of a C_l alkyl radical at the kth atom of the parent chain of N carbons. This gives $n - l = N$ and

$$\Delta W = -l(k - 1)(N - k) \quad (15)$$

Similarly, any product with two tertiary carbons may be considered as the result of the linkage of two C_N parent radicals by means of the n_1th carbon of the first and the n_2th atom of the second one (dimer n_1–n_2, using the nomenclature of the text and figures). This gives

$$n = 2N;\ k_1 = N - n_1 + 1;\ k_2 = N - n_1 + 2;\ l_1 = n_1 - 1;\ l_2 = n_2 - 1$$

and

$$\Delta W = -N[(N + 1)(n_1 + n_2) - (n_1^2 + n_2^2) - 2N] \quad (16)$$

As an example of the easy and immediate applicability of Table II let us consider the 3-4 dimer of n-decane: C_7H_{15}–$CH(C_2H_5)$–$CH(C_3H_7)$–C_6H_{13}. We have: $l_1 = 3$; $l_2 = 2$; $n_1 = 3$; $n_2 = 4$; $N = 10$ and therefore from the table $\Delta P_3 = 5$ and $\Delta W = -10[(11 \times 7) - (9 + 16) - 20] = -320$ which, inserted in eqn. 1, immediately gives the required boiling point.

ACKNOWLEDGEMENT

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CHROM. 4938

GASCHROMATOGRAPHISCHER NACHWEIS VON STICKSTOFFHALTIGEN PHARMAKA IN WÄSSRIGEN LÖSUNGEN MIT DEM STICKSTOFFDETEKTOR

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SUMMARY

Gas chromatographic detection of nitrogen-containing drugs in aqueous solutions by means of the nitrogen detector

The nitrogen detector (N-FID), a nitrogen-specific and nitrogen-selective version of the thermionic detector, offers many advantages in the qualitative and quantitative determination of nitrogen-containing drugs in aqueous solutions and especially in biological fluids. Without further preparation the excretion rate of known drugs can be followed by direct injection of the urine samples. Temperature-programmed screening analysis, necessary in the forensic field and in doping controls, is possible after a one-step extracting procedure. Optimal conditions for the use of the N-FID and columns adapted for the gas chromatography of aqueous solutions of drugs in the p.p.m. range are described.

EINLEITUNG

Die Gaschromatographie hat einen festen Platz beim Nachweis von basischen Pharmaka in Körperflüssigkeiten gefunden[1–4]. Wegen der geringen Konzentration der Wirkstoffe bzw. ihrer Metaboliten neben grossen Mengen an störenden Begleitstoffen ist jedoch eine umfangreiche und zeitraubende Probenvorbereitung nötig, die vor allem bei der Durchführung einer grossen Anzahl von Analysen stört. Die Verwendung von thermionischen Detektoren sollte diesen Aufwand wesentlich verringern. Es ist bekannt[5], dass bei der Auswahl von geeigneten Alkalisalzen, verglichen mit dem FID, ein empfindlicherer und zudem spezifischer Nachweis von Stickstoffverbindungen erreicht werden kann. Dass dieser Detektortyp bisher noch nicht auf dem Gebiet der Arzneimittelanalytik eingesetzt wurde, dürfte seinen Grund im Fehlen einer für Routineuntersuchungen geeigneten und kommerziell erhältlichen Konstruktion haben. Mit der neuerdings verfügbaren, als "Stickstoffdetektor" (N-FID) bezeichneten Version eines N-spezifischen thermionischen Detektors (Hewlett & Packard GmbH, Böblingen), prüften wir, ob die von uns angestrebte und für viele Fragestellungen wünschenswerte Reduzierung des Aufwands der Probenvorbereitung realisierbar ist.

EXPERIMENTELLES

Gaschromatographische Anordnung

Gerät. Für die gaschromatographischen Untersuchungen verwendeten wir das Modell 5755 B der Firma Hewlett & Packard GmbH, Böblingen, ausgerüstet mit einem Doppelkanalschreiber (Moseley H 10-7128 A) und einem elektronischen Integrator HP Modell 3370. Der Einbau des Stickstoffdetektors, Modell 15161 A erfolgte auf der A-Seite des FID. Anstelle des serienmässigen Detektordeckels verwendeten wir eine Eigenkonstruktion mit einer durch ein Nadelventil regelbaren Austrittsöffnung für die Brenngase. Ein in 50 Einheiten pro Umdrehung eingeteilter Antrieb (Duodial, Serie 2606) ersetzte den Regelknopf des Zehngangpotentiometers.

Säulen. Glassäulen; aus Alkaliboratglas; 2.5 mm i.D., 6.0 mm ä.D., vom Einspritzblock bis zum Detektoranschluss ohne Verbindungsstück durchgeführt; mit KOH gewaschener Glaswatte beidseitig auf *ca.* 1.5 cm Länge verschlossen, so dass die Injektion mit einer 10-μl Mikrospritze auf das Säulenmaterial erfolgt.

Säule 1: 1.06 m lang, 5 % Polyäthylenglykol 20 M (HP) auf Chromosorb W, AW-DMSCS, 80–100 mesh (HP). Der Träger wurde vor dem Imprägnieren mit flüssiger Phase mit 10 % methanolischer Kalilauge behandelt.

Säule 2: 1.50 m lang, 2 % Igepal CO-880 (HP) und 10 % Apiezon L (HP) auf Chromosorb P (Serva). Die Behandlung des Trägers entspricht Säule 1.

Betriebsbedingungen. Die Temperatur des Einspritzblocks bei Säule 1 war 190°, bei Säule 2 240°, die des N-FID 320°.

Für den Ofen ist die Temperatur wie bei den Abbildungen angegeben. Für das temperaturprogramm, siehe Tabelle I.

Gase: He, 55–60 ml/min; H_2, 25.5 $\pm$ 1.5 ml/min; und Luft, 180–200 ml/min.

Elektrometer- und Integratoreinstellung: Bereich, 10^2; Abschwächung, 1; Integratorausgang, 10 bzw. 20 mV; Neigungsdetektor, 1.0 mV im Anstieg, 0.3 mV im Abfall des Peaks; Geräuschdämpfung, 3.

TABELLE I

TEMPERATURPROGRAMM FÜR DEN OFEN

	Säule 1	*Säule 2*
Anfangstemperatur, °C	110	100
Isotherm nach der Injektion, min	1	1
Anstiegsrate, °C/min	8	8
Endtemperatur, °C	190	250
Isotherm bei der Endtemperatur, min	10	4

Reagenzien

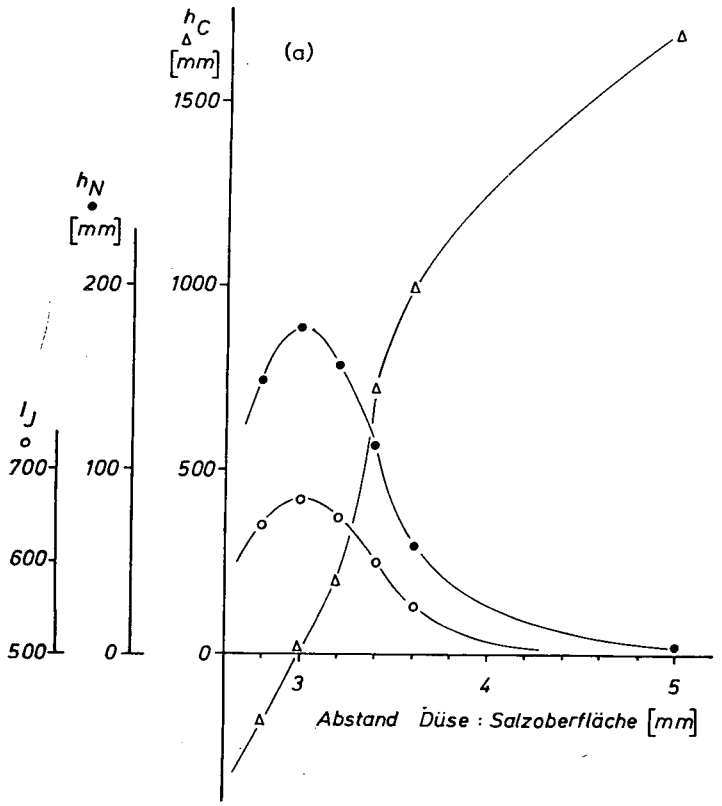
Die folgende Reagenzien wurden verwendet: Bidestilliertes Wasser; Diäthyläther, über Calciumhydrid aufbewahrt und vor Gebrauch frisch destilliert; Coffein, Ephedrin, *n*-Alkane (Merck); Amphetamin, Triglym (Triäthylenglykoldimethyläther) (EGA-Chemie); Methamphetamin, Dimethylamphetamin (Temmler), Niketham id, Pentamethylentetrazol und Heptaminol, isoliert aus Arzneimittelspezialitäten der

Firmen Ciba, Knoll und Delalaude; Cyclohexylacetamid, aus Cyclohexylamin und Essigsäureanhydrid dargestellt, mehrfach umkristallisiert.

Optimierung des Detektors

Nach dem Äquilibrieren der Gasströme und Temperaturen wird, vom unteren Totpunkt ausgehend, der Abstand der Elektrode zur Düse schrittweise vergrössert, anfangs in Intervallen von 0.2 mm, entsprechend 1/4 Umdrehung der Justierschraube, später in grösseren Abständen. Der Ionisationsstrom, gemessen in den Einheiten des Potentiometers, steigt mit grösser werdendem Abstand auf einen Maximalwert (I_{max}) an und fällt wieder ab. Er erreicht nach 2.5–3.5 Umdrehungen den Wert des unmodifizierten FID (I_0).

Nach jeder Veränderung des Abstandes werden, nachdem die Basislinie nicht mehr driftet, 2 μl der Eichlösung (10 p.p.m. Pervitin + 1 % Triglym in Wasser) eingespritzt. Sind die Signalhöhen der Kohlenstoff- und Stickstoffverbindungen bei drei aufeinanderfolgenden Injektionen gleich, so wird das Rauschen bei erhöhter Empfindlichkeit festgestellt. Dann kann die nächste Einstellung vorgenommen werden. Als Funktion des Abstandes werden der Ionisationsstrom (I_J), die Peakhöhen von Methamphetamin und Triglym (h_N, h_C), die Selektivität (S_N/C), sowie die untere Nachweisgrenze (UG) notiert und in einem Diagramm (s. Fig. 1) dargestellt. Der Stickstofferhöhungswert (ΔN) wird aus den N-Signalen bei I_0 und $I_{max.}$ berechnet



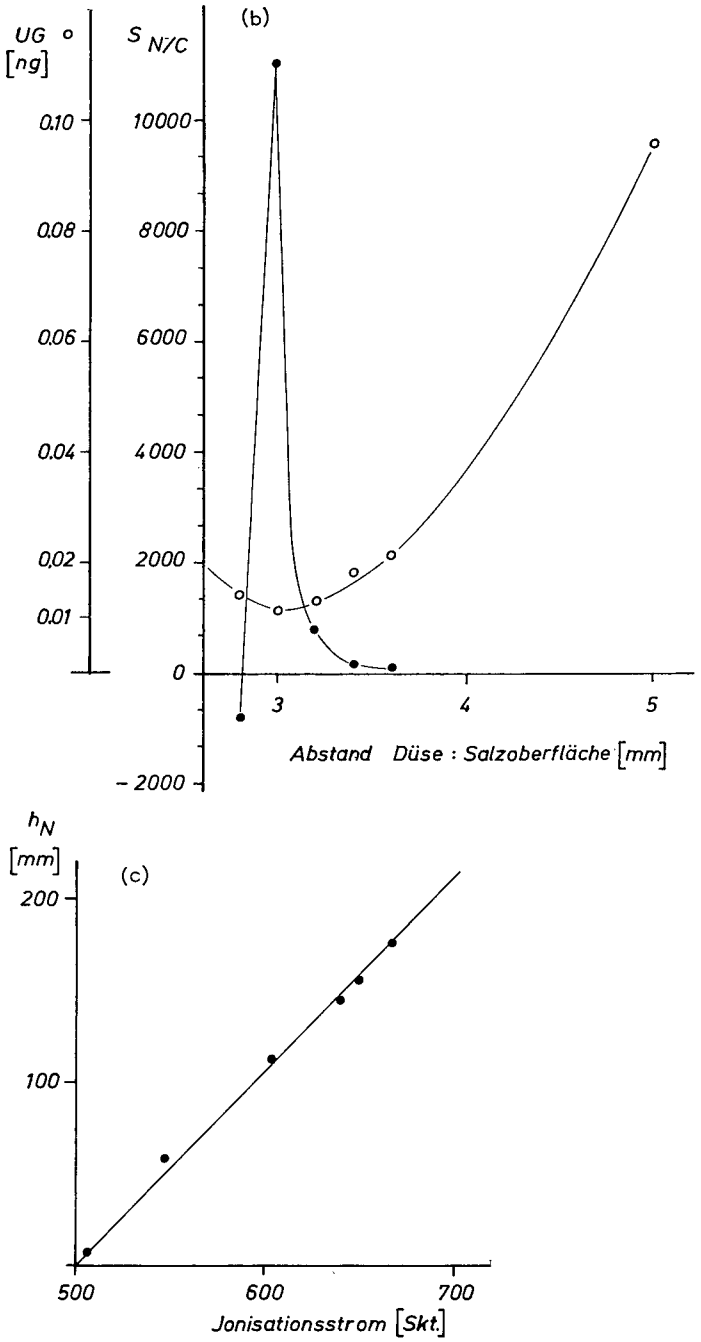


Fig. 1. (a) Ionisationsstrom, Peakhöhe des Methamphetamins und Peakhöhe des Triglyms als Funktion des Abstandes zwischen Düse und Salzoberfläche. (b) Selektivität und untere Nachweisgrenze als Funktion des Abstandes zwischen Düse und Salzoberfläche. (c) Peakhöhe des Metamphetamins als Funktion des Ionisationsstroms.

TABELLE II

WERTETABELLE ZU DEN FIG. 1a–c ABHÄNGIGKEIT DER PARAMETER DES N-FID VOM ABSTAND ELEKTRODE–DÜSE

Legende: u = Umdrehungen der Justierschraube, vom unteren Anschlag der Mechanik aus gezählt; d = Abstand zwischen Düse und Salzoberfläche in mm; I_J = Relativer Ionisationsstrom in Skalenteile; h_N = Peakhöhe des Methamphetamins in mm; h_C = Peakhöhe des Triglyms in mm; R_{min} = Breite des Rauschbandes in mm (h_N, h_C und R_{min} sind auf die Verstärkung $10^2 \times 1$ und 10 mV Integratorausgang berechnet); $S_N/_C = (h_N \cdot 1000)/h_C$; $UG = (2R_{min} \cdot G_N)/h_N$ [ng], wobei G_N = Menge an Methamphetamin in ng; $\Delta N = h_{N-FID}/h_{FID}$.

u	d	I_J	h_N	h_C	R_{min}	$S_N/_C$	UG	ΔN
0	2.8	640	144	−176	0.05	−820	0.014	36
0.25	3.0	668	176	16	0.05	11000	0.011	44
0.5	3.2	651	156	204	0.05	765	0.013	39
0.75	3.4	604	112	720	0.05	155	0.018	28
1.0	3.6	548	58	988	0.03	58	0.021	14.5
2.8	5.0	506	4	1680	0.01	2.5	0.096	—

(s. Tabelle II). Durch geringfügige Veränderungen der übrigen Parameter kann das Verhältnis der Signalhöhen zum Rauschen und die Selektivität weiter optimiert werden.

Probenvorbereitung und Injektionstechnik

In ein 15 ml fassendes Zentrifugenglas mit Schliff (NS 14.5) werden 3.2 g geglühtes Natriumsulfat eingefüllt. 5.0 ml Urin, 2.0 ml Äther und 0.5 ml 5 *N* Kalilauge werden nacheinander in dieser Reihenfolge zugegeben. Nach 10-minütigem mechanischen Schütteln wird 5 min bei 1000 r.p.m. zentrifugiert. Von der klaren ätherischen Phase werden 2 μl mit einer im Eisschrank vorgekühlten Spritze injiziert.

Lösungen der Wirkstoffe in Wasser werden direkt auf den Säulenanfang injiziert.

Bestimmung der Wiedergewinnungsraten

Eine wässrige Lösung mit einer Konzentration von 4 p.p.m. an Methamphetamin, Heptaminol, Ephedrin, Nikethamid und Pentamethylenetetrazol wird nach dem oben beschriebenen Verfahren extrahiert. Zum Vergleich werden weitere Proben mit festem Natriumkarbonat (250 mg/Probe) bzw. mit 0.5 ml konzentriertem Ammoniak alkalisiert.

Von der ätherischen Phase werden 2 μl auf die Säule 1 injiziert, von der wässrigen Phase verdünnt man 1 ml mit 2 ml Wasser und injiziert bei 5-fach erhöhter Empfindlichkeit 4 μl. (Eine Injektion der gesättigten Na_2SO_4-Lösung blockiert die Spritze!) Unter Berücksichtigung der Verdünnungsfaktoren erfolgt die Auswertung über den Integrator und nach der Höhenmethode. Hierbei wird die Summe der Wirkstoffe in der ätherischen und wässrigen Phase gleich 100 % gesetzt und der Anteil in den einzelnen Phasen berechnet.

Eichlösungen

Für Säule 1 war die Eichlösung 10 p.p.m. Methamphetamin und 1 % Triglym in bidestilliertem Wasser; für Säule 2 war sie 10 p.p.m. Amphetamin und 1 % Triglym in bidestilliertem Wasser.

ERGEBNISSE UND DISKUSSION

N-Spezifität und N-Selektivität

Zwei Bedingungen sind nach den Ergebnissen von KARMEN[6] zur Erzielung einer guten N-Spezifität notwendig:

Eine Verbrennung nahe dem Alkalisalz und ein geringer Abstand von Auffangelektrode zur Düse. Die vorliegende Detektorkonstruktion erfüllt diese Bedingungen, denn der in der Zylinderelektrode fixierte Rubidiumbromidkristall muss bis auf wenige Millimeter an die Düse angenähert werden, damit die Flamme die Kristalloberfläche berührt. Der wenig überstehende Rand des Platinzylinders wirkt als nahe der Verbrennungszone gelegener Kollektorring.

Der Detektor arbeitet wie ein FID, wenn die Flamme die Kristalloberfläche wegen eines zu gross gewählten Abstandes nicht erreicht. Das Verhältnis der Signale von Methamphetamin zu Triglym entspricht, unter Berücksichtigung der stoffspezifischen Korrekturfaktoren, den Gewichtsverhältnissen. Beim Absenken der Elektrode in die Flammenzone wird das N-Signal vergrössert. Die beginnende Behinderung der optimalen Verbrennung vermindert das C-Signal, das bei weiterer Verringerung des Abstandes sogar negativ wird (vergl. Fig. 2a–2c).

Parallel zu der Grösse des N-Signals verläuft der Grundionisationsstrom

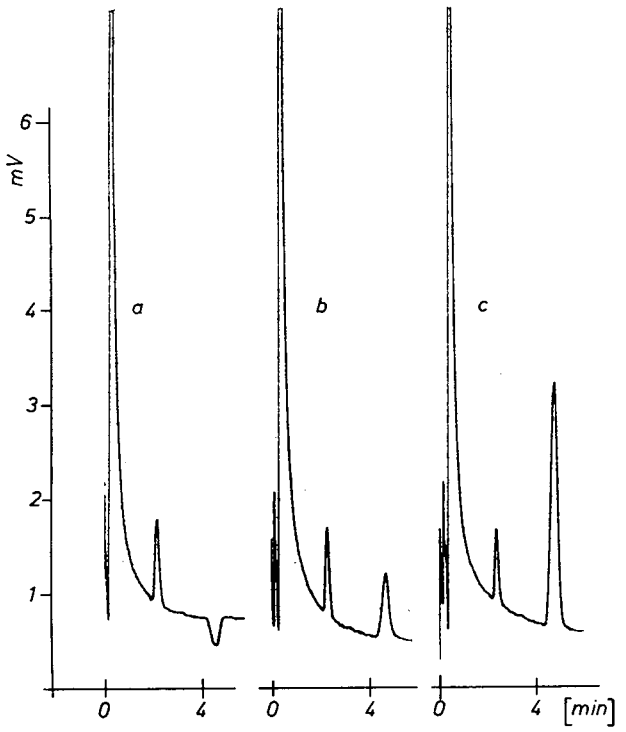


Fig. 2. Einfluss des Abstandes zwischen Düse und Salzoberfläche auf das Chromatogramm: (a) 2.8 mm, (b) 3.0 mm und (c) 3.4 mm Abstand. Retentionszeit: Methamphetamin, 2.0 min; Triglym, 4.6 min.

(Fig. 1c), der in relativen, willkürlich eingeteilten Einheiten des Zehngangpotentiometers abgelesen wird. Im Gegensatz zu den mit Salz-Sinterspitzen modifizierten FID ist diese Anordnung streng selektiv, denn sie unterdrückt die Kohlenstoffanzeige bis hin zu negativen Werten.

So erlaubt bereits die Variation des Abstandes die geeignete und zur Lösung eines Problems günstigste Selektivität einzustellen. Sie kann, angefangen von FID-Bedingungen bis zu einem Maximum, das bei einigem Optimierungsaufwand bei 1:3000 liegt, gewählt werden. Negative Werte für die Selektivität, die laut Definition aus negativen Kohlenstoff-Peaks resultieren, schieden für unsere Problemstellung aus, können aber unter Umständen für die Lösung anderer Probleme interessant sein.

Die untere Nachweisgrenze des N-FID

Die untere Nachweisgrenze ist ein wesentliches Charakteristikum eines Analysenverfahrens. Wie aus der Formel (siehe Tabelle I) hervorgeht[7], bestimmen bei vorgegebener Konzentration der Substanz zwei Messgrössen ihren Wert: die Signalhöhe und das Rauschen. Beide werden durch die Detektorparameter und, wenn auch in

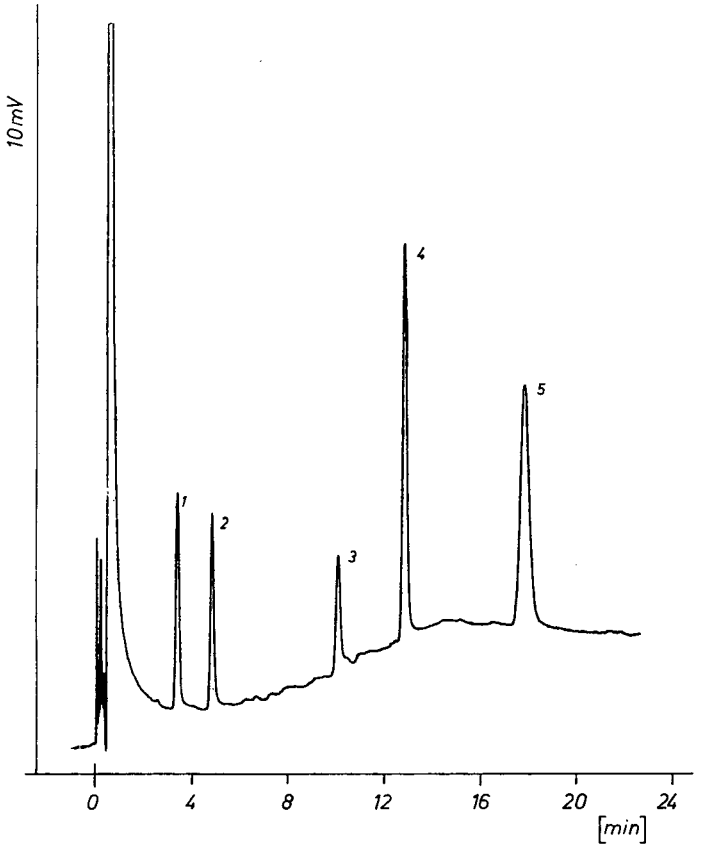


Fig. 3. Trennung einer wässerigen Lösung von Wirkstoffen auf Polyäthylenglykol 20 M (Säule 1, N = 840 für Methamphetamin). Konzentration: 5 p.p.m.; Einspritzmenge: 2 μl; 1 = Methamphetamin; 2 = Heptaminol; 3 = Ephedrin; 4 = Nikethamid; 5 = Pentamethylentetrazol.

unterschiedlichem Masse, durch die Qualität der Säule beeinflusst. Symmetrische Peaks und keine Verluste durch Adsorption sind Vorbedingungen für das Erreichen einer niedrigen Nachweisgrenze.

Auswahl des Säulenmaterials. Da unsere Untersuchungen auf die direkte Injektion von wässrigen Lösungen basischer Wirkstoffe, vor allem aus der Gruppe der Phenylalkylamine im 0.1–10.0 p.p.m.-Bereich hinzielten, überprüften wir die in der Literatur für die Chromatographie dieser Substanzen empfohlenen flüssigen Phasen und Säulenmaterialien. Hierbei ergaben zunächst nur die Polyäthylenglykoltypen mit wässrigen Testlösungen in diesem Konzentrationsbereich gute Ergebnisse (Fig. 3). Thermostabilere Phasen, wie Apiezon L und verschiedene Silikongummitypen, wiesen ein nicht mehr tolerierbares "tailing" auf. Eine Vorbehandlung des Trägermaterials durch Silylieren mit Dimethyldichlorsilan, durch Waschen mit alkoholischer Kalilauge oder Imprägnieren mit Kalilauge, konnte das "tailing" nicht genügend unterdrücken. Lediglich das Zumischen von Polyäthylenglykol 20 M oder Polyester verbesserte die Trenneigenschaften, ohne jedoch ganz die Trennleistungen der Polyäthylenglykol-

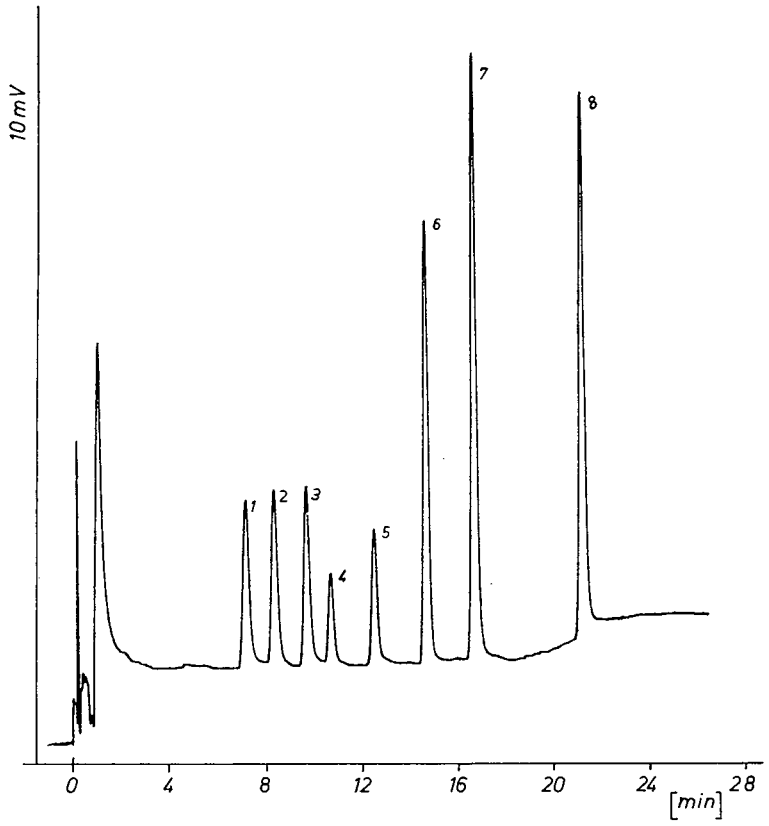


Fig. 4. Trennung einer wässerigen Lösung von Wirkstoffen auf Apiezon L–Igepal modifiziert (Säule 2). N = 680 für Methamphetamin. 1 = Amphetamin; 2 = Methamphetamin; 3 = Dimethylamphetamin; 4 = Cyclohexylacetamid (i.St.); 5 = Ephedrin; 6 = Niketham id; 7 = Pentamethylentetrazol; 8 = Coffein.

typen zu erreichen (Fig. 4). Die Eichkurven wässriger Lösungen von Methamphetamin, Nikethamid, Ephedrin, Heptaminol und Pentamethylentetrazol (Fig. 5) vrelaufen über den gesamten Konzentrationsbereich linear. Die Trennfähigkeit dieses Säulentyps wird durch wiederholte Injektionen wässriger Lösung nicht beeinflusst. Die theoretische Bodenzahl verändert sich über einen längeren Zeitraum nicht. Die gleiche Aussage gilt für die Injektion nicht getrockneter, mit Wasser gesättigter organischer Lösungsmittel, wie sie nach einem einmaligen Verteilungsschritt anfallen.

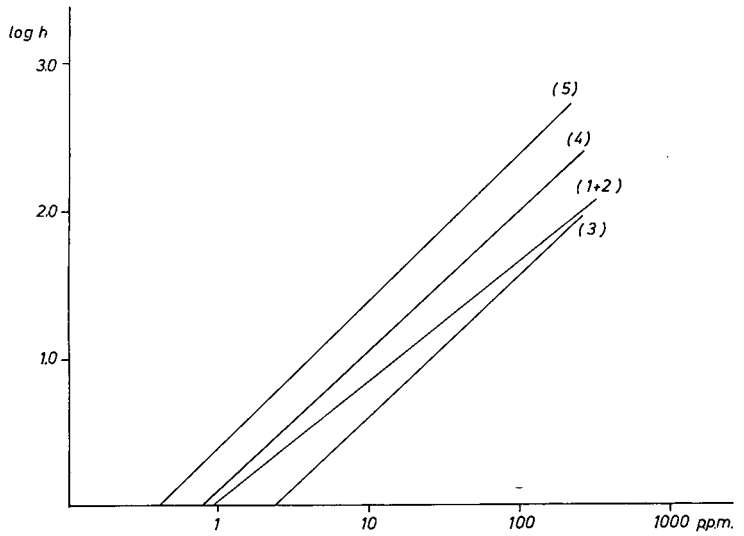


Fig. 5. Eichkurven von Methamphetamin (1), Heptaminol (2), Ephedrin (3), Nikethamid (4) und Pentamethylentetrazol (5).

Signal/Rauschverhältnis. Folgende Parameter beeinflussen das Signal/Rauschverhältnis: die Detektortemperatur, die Position des Salzes in der Kollektorelektrode, die Gasdurchflussmengen von Luft, Helium und Wasserstoff, sowie der Abstand der Elektrode zur Düse. Hierbei ist vorausgesetzt, dass die salzgefüllte Elektrode genau zentrisch über der Düsenbohrung montiert ist und somit auch bei einer Abstandsveränderung eine definierte Geometrie eingehalten wird.

Da mit Ausnahme des Elektrodenabstandes alle anderen aufgeführten Parameter nur in engen Grenzen verändert werden können, werden diese zweckmässigerweise fest eingestellt. So ist die Detektortemperatur durch die Beständigkeit der Silicongummiringe limitiert, die zum Abdichten der Glassäulen benutzt werden, obwohl eine Temperatursteigerung über die von uns gewählte obere Grenze von 330° hinaus noch eine Erhöhung des N-Signals ermöglichen würde. Das Rubidiumbromidsalz wird in dem Platinzylinder am besten so fixiert, dass dieser noch etwa 0.3 mm übersteht und somit als Kollektorring dient. Die Gasmengenströme sind so aufeinander abgestimmt, dass eine reduzierende und relativ kalte Flamme entsteht[8], Bedingungen, die ein hohes N-Signal unter weitgehender Unterdrückung des C-Signals begünstigen. In Tabelle II und in den Fig. 1a–c wurden bereits die Ergebnisse, die durch Veränderung des Elektrodenabstandes erhalten werden, zusammengestellt.

Es wird deutlich, dass die Höhe des N-Signals und die Breite des Rauschbandes von der Grösse des Grundionisationsstromes abhängen (s. Fig. 1c). Die Selektivität, die zu Beginn wegen des negativen Kohlenstoffpeaks ein negatives Vorzeichen besitzt, fällt vom Maximalwert mit steigendem Abstand rasch ab.

Die N-Erhöhungswerte, die von AUE *et al.*[5] und EBING[9] mit etwa 100 angegeben werden, führen nicht im gleichen Ausmass zu einer Verringerung der unteren Nachweisgrenze, da der alkalimodifizierte FID ein stärkeres Rauschen aufweist. Eine genaue Analyse des bei hoher Empfindlichkeit erhaltenen Rauschbandes (s. Fig. 6) zeigt, dass es aus drei Anteilen besteht: einem Rauschen mit einer Frequenz von 5–10 Hertz, einer plötzlichen Verschiebung der Basislinie um etwa 5–8 mm, einer Schwingung mit einer Frequenz von *ca.* 1/7 Hertz.

Fig. 6. Rauschen des N-FID: (a) ungefiltert, (b) mit Integrator und Geräuschdämpfung 3 und (c) mit geringem Überdruck im Verbrennungsraum, sonstige Parameter wie b. Bereich: 10 × 1; 0.5 mV Integratorausgang; Säule 1.

Das relativ schnelle Rauschen lässt sich mit der Inkonstanz der Flamme erklären. Elektronisch kann es mit Hilfe des Integrators unterdrückt werden (s. Fig. 6a und b). Das plötzliche Abweichen der Basislinie ist durch geringe Luftströmungen im Labor bedingt, wie sie z.B. schon durch langsames Vorbeigehen an dem Gerät in 1–2 m Abstand erzeugt werden. Das Öffnen der Labortür gibt zu extrem scharfen Peaks Anlass. Um von diesen geringen Luftdruckschwankungen unabhängig zu werden, ersetzten wir den üblichen Detektordeckel durch einen regelbaren Auslass. Wird das Abströmen der Brenngase behindert, so entsteht ein leichter Überdruck im Verbrennungsraum des N-FID. Hierdurch kann der Einfluss der äusseren Luftbewegung nahezu ausgeschlossen werden (s. Fig. 6c). Eine einfache, aber nicht ganz reproduzierbare Methode zur Reduzierung der Druckschwankungen ist das Verstopfen der Bohrungen im serienmässigen FID-Deckel mit Glaswolle.

Für die relativ langsamen und weniger intensiven Frequenzen von 1/7 Hertz fehlt uns zur Zeit eine Erklärung. Druckschwankungen der Brenngase können ausgeschlossen werden, da mehrfache Abwandlung der Gasversorgung keine Änderungen oder Verbesserungen ergaben.

Nach Optimierung aller Parameter und unter Ausschluss von Luftdruckschwankungen erzielten wir eine untere Nachweisgrenze von grössenordnungsmässig 10 pg pro Injektion. Dies stellt eine Verbesserung um den Faktor 10 dar, denn unter FID-Bedingungen erzielten wir auf dem gleichen Gerät eine Nachweisgrenze von 100 pg.

Der lineare Bereich

Die bei quantitativen Bestimmungen interessierenden Eichfunktionen für Methamphetamin, Heptaminol, Ephedrin, Nikethamid und Pentamethylentetrazol

sind in Fig. 5 wiedergegeben. Der lineare Bereich umfasst vier Zehnerpotenzen. Beginnend mit einer unteren Nachweisgrenze von 20 pg reicht er bis etwa 200 ng pro Injektion. Diese Werte hängen vom prozentualen Stickstoffgehalt und der Halbwertsbreite der Signale ab, die unter den angegebenen Analysenbedingungen etwa 10 sec beträgt. Liegen höhere Konzentrationen an stickstoffhaltigen Verbindungen vor, so kann durch Verdünnung der Proben oder durch Splitten des Trägergasstromes der lineare Bereich aufgesucht werden.

Analyse wässriger Lösungen

Wie aus den oben beschriebenen Ergebnissen hervorgeht, ist die wiederholte Injektion von wässrigen Lösungen der reinen Wirkstoffe ohne weiteres möglich. Die weit komplexere Zusammensetzung von Urin bewirkt Störungen, die jedoch bei isothermer Arbeitsweise und Optimierung aller Parameter des N-FID so weit umgangen werden können, dass der qualitative und quantitative Nachweis einzelner Wirkstoffe gelingt. So können u.a. oral aufgenommenes Amphetamin, Methamphetamin, Dimethylamphetamin, Nikethamid, Pentamethylentetrazol und Coffein einwandfrei im Urin nachgewiesen werden (vergl. Fig. 7).

Zur Verfolgung der Ausscheidungsgeschwindigkeit dieser stickstoffhaltigen Drogen erwies sich die hier beschriebene direkte Injektion von Urin als beste Lösung[10].

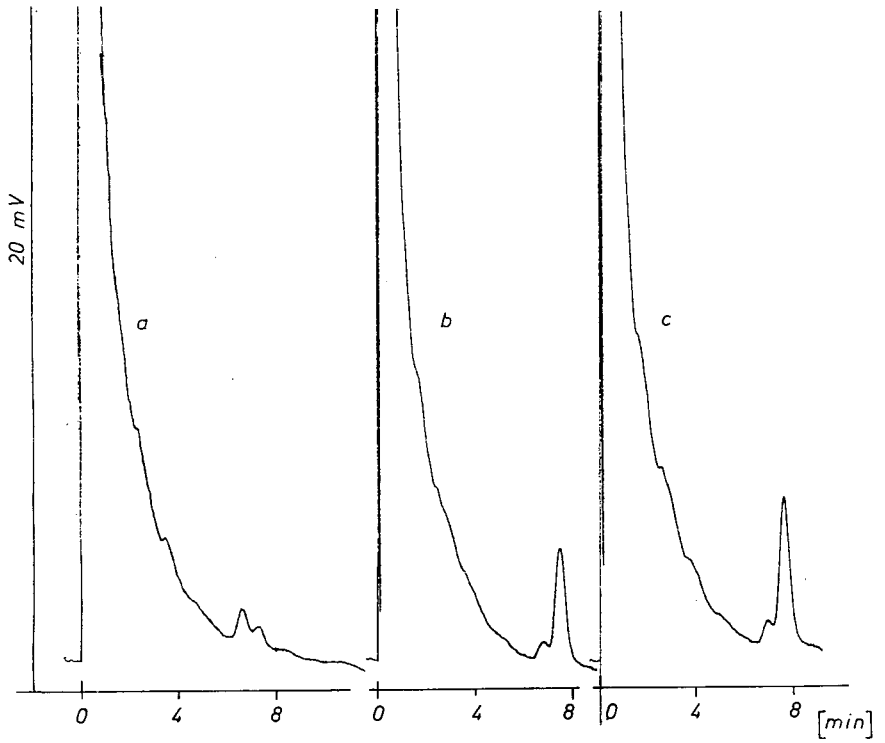


Fig. 7. Nachweis von Coffein nach oraler Applikation durch direkte Injektion von Urin auf die Säule. Säule 2, 220° isotherm, sonstige Parameter wie im experimentellen Teil angegeben. Coffeindosis: 300 mg. (a) Leerwert = 1.3 p.p.m.; (b) 90 min = 8.3 p.p.m. und (c) 150 min = 10.4 p.p.m.

Eine Übertragung auf andere Wirkstoffe ist möglich, da durch Wechsel der Polarität der flüssigen Phase oder durch höhere Trennleistung der benutzten Säulen störende Verbindungen abgetrennt werden können.

Diese Technik ist jedoch weniger geeignet bei temperaturprogrammierter Arbeitsweise, die bei Übersichtsanalysen wie Dopingkontrollen und forensischen Untersuchungen angezeigt ist. Bei der Vielzahl von Verbindungen, auf die geprüft werden soll, ist damit zu rechnen, dass Störsubstanzen und Wirkstoffe gleiche Retentionszeiten aufweisen. Ferner werden Zersetzungsprodukte von Rückständen aus dem Einspritzblock beim Abkühlen des Ofens im Säulenanfang festgehalten und bei nachfolgenden temperaturprogrammierten Analysen eluiert.

Diese Nachteile lassen sich jedoch vermeiden, wenn ein einfacher Verteilungsschritt durchgeführt wird. Durch Zusatz von wasserfreiem Natriumsulfat werden aus der alkalisierten wässrigen Phase die Wirkstoffe nahezu quantitativ in den Äther übergeführt (s. Tabelle III). Dabei werden aus dem Urin nur noch wenige, störende Verbindungen extrahiert, so dass vorhandene Drogen sicher nachweisbar sind. Die nach Applikation von pharmakologischen Normdosen zu erwartenden Konzentrationen von *ca.* 1–10 p.p.m. liegen deutlich über der Nachweisgrenze von etwa 0.05 p.p.m. (Fig. 8).

TABELLE III

WIEDERGEWINNUNGSRATEN VON METHAMPHETAMIN, HEPTAMINOL, EPHEDRIN, NIKETHAMID UND PENTAMETHYLENTETRAZOL

Auswertung: (a) Integrator, (b) Höhenmethode. GC-Bedingungen: Säule 1, sonstige Parameter siehe experimenteller Teil.

	Methamphetamin		*Heptaminol*		*Ephedrin*		*Niketamid*		*Pentamethylentetrazol*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
% Gehalt der ätherischen Phase über KOH-Lösung	100	100	99	98	98	96	100	99	98	96
% Gehalt der ätherischen Phase über Na_2CO_3-Lösung	98	97	89	91	100	90	99	100	99	95
% Gehalt der ätherischen Phase über NH_4OH-Lösung	100	99	100	98	100	100	99	99	94	94

Gegenüber den bisherigen Verfahren bedeutet die Reduzierung der Probenvorbereitung auf einen einfachen Verteilungsschritt ein wesentlicher Fortschritt, weil nicht nur der Arbeitsaufwand eingeschränkt, sondern auch Fehlermöglichkeiten vermieden werden, die mit einer in viele Einzelschritte unterteilten Analysenvorschrift verbunden sind.

DANK

Der Deutschen Forschungsgemeinschaft (M.D., L.J.) und dem Kuratorium für sportmedizinische Forschung (D.S., W.H.) schulden wir für die Unterstützung

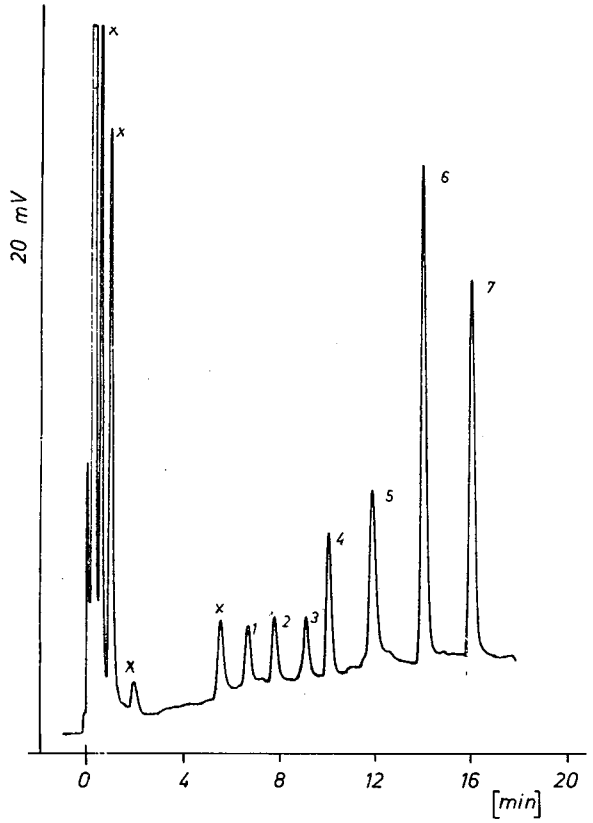


Fig. 8. Wiedergewinnung von Wirkstoffen aus Urin nach einem einfachen Verteilungsschritt. Zugemischt wurden: (1) Amphetamin, 1 p.p.m.; (2) Methamphetamin, 1 p.p.m.; (3) Dimethylamphetamin, 1 p.p.m.; (4) Cyclohexylacetamid (i.St.), 2 p.p.m.; (5) Ephedrin, 5 p.p.m.; (6) Niketamid, 3 p.p.m.; (7) Pentamethylentetrazol, 2 p.p.m.; (X) unbekannt.

der vorliegenden Arbeit Dank. Weiter danken wir den Temmler-Werken GmbH für die Überlassung von zahlreichen Vergleichssubstanzen.

ZUSAMMENFASSUNG

Der "Stickstoffdetektor" (N-FID), eine N-spezifische und N-selektive Version des thermionischen Detektors, bietet Vorteile für die qualitative und quantitative Analyse von stickstoffhaltigen Pharmaka in wässrigen, vor allem biologischen Flüssigkeiten. Unter Verzicht auf eine Probenvorbereitung kann bei isothermer Arbeitsweise die Ausscheidungsgeschwindigkeit von Wirkstoffen durch direkte Injektion der Urinproben bestimmt werden. Temperaturprogrammierte Übersichtsanalysen, wie sie bei forensischen Untersuchungen oder Dopingkontrollen anfallen, sind nach einem einfachen Verteilungsschritt möglich. Die Optimierung des N-FID sowie geeignete Säulen für die Gaschromatographie von wässrigen Lösungen mit Wirkstoffkonzentrationen im p.p.m.-Bereich werden beschrieben.

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CHROM. 4917

DETERMINATION OF N-METHYLAMINO ACIDS AND THEIR OPTICAL PURITY WITH AN AMINO ACID ANALYZER*

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SUMMARY

The ninhydrin color constants for N-methylamino acids obtained with a Model 120B Beckman amino acid analyzer were shown to increase 10–20 times to 18–93 % of those for the parent amino acid when the eluting buffer flow rate was decreased from 68 ml/h to 34 ml/h. The optical purity of N-methylamino acids can be established to within one part in 100 by determination with the analyzer of the diastereomeric dipeptides obtained by coupling L-alanine N-carboxyanhydride with the N-methylamino acid as devised for amino acids by MANNING AND MOORE.

INTRODUCTION

N-Methylamino acids react with ninhydrin[1–3], however, a higher temperature[2,4] and a longer reaction time[5,6] are required in order to obtain the maximum color development for these and the α-C-methylamino acids. EBATA *et al.*[5] investigated the determination of N-methylamino acids with a Hitachi KLA Type 2 amino acid analyzer and found that the color constants were 1.4–20 % of those for the parent amino acid, that many gave unsymmetrical peaks, and that the tedious addition of absorbances[7] method had to be used for the calculations. They therefore concluded that the amino acid analyzer was unsuitable for the determination of N-methylamino acids. Our need for a method of determining N-methylamino acids[8] has prompted an investigation of this problem. We have found that a standard Beckman Model 120B amino acid analyzer is a most satisfactory instrument for determining N-methylamino acids if it is operated with a half-normal buffer flow rate***. Under these conditions, the color constants are 18–93 % of those for the parent amino acid, and the usual $(H \times W)/C$ method of calculation[7] can be employed. The constants for two α-C-methylamino acids were also increased substantially.

A second obstacle encountered in developing our new method of synthesis of N-methylamino acids was establishing the optical purity of the products. The optical

* Supported by a grant from the Medical Research Council of Canada.
** Associate of the Medical Research Council of Canada.
*** We acknowledge the personal communication from Drs. C. H. HASSALL and J. S. DAVIES, University College, Swansea, that they had observed increased color yields for N-methylamino acids by decreasing buffer flow rates.

purity of amino acids has generally been determined using stereospecific enzymes such as the amino acid oxidases[9]. This method, unfortunately, is not applicable to N-methylamino acids because suitable enzymes are not available. A more recent approach to the determination of the isomers of amino acids involves the preparation of their diastereomeric dipeptides followed by separation and determination of these by some type of chromatography[10–14]. The method of MANNING AND MOORE[13] in which the dipeptides are prepared by reaction of the amino acid with an optically pure amino acid N-carboxyanhydride and subsequently determined with an amino acid analyzer seemed particularly attractive to us. This method was therefore investigated and it was found that it indeed could be used for determining the optical purity of N-methylamino acids.

MATERIALS AND METHODS

The amino acids were obtained from General Biochemicals Corp., Chagrin Falls, Ohio; sarcosine, N-methyl-DL-leucine, N-methyl-DL-alanine and α-C-methyl-DL-leucine from Cyclo Chemical Corp., Los Angeles, Calif.; α-aminoisobutyric acid from Sigma Chemical Co., St. Louis, Mo.; and L-alanine N-carboxyanhydride from Miles Laboratories Inc., Elkhart, Ind. N-Methyl-L-leucine, N-ethyl-L-leucine and N-methyl-L-phenylalanine were synthesized in our laboratory[8]. The amino acid analyzer was a standard Beckman Model 120B instrument equipped with a 0.9 × 50 cm column of AA-15 resin at 57°. Normal operation conditions refer to a 0.35 *N* sodium citrate buffer, pH 3.28, pumped at a flow rate of 68 ml/h with the ninhydrin solution being pumped at 34 ml/h. The absorption spectra of the products of the ninhydrin reaction with leucine, N-methylleucine and α-C-methylleucine were taken with a Unicam SP-800 recording spectrophotometer. The reaction was carried out in a boiling water bath for 30 min using the method of ROSEN[15].

The L-alanyl dipeptides for the determination of optical purity were prepared essentially as described by MANNING AND MOORE[13]. The sample (100 μmoles) was weighed into a 100 × 10 mm pyrex test tube and 1 ml of ice-cold 0.45 *M* borate buffer, pH 10.4 (prepared by adding 5 *N* KOH to 0.45 *M* boric acid at 0°) and one drop of octanoic acid were added. The tube was taken into a cold room (4°), L-alanine N-carboxyanhydride (12.7 mg; 100 μmoles) was quickly added, and the tube was shaken vigorously on a "Vortex Genie" stirrer for 2 min. The solution was brought to pH 2 with *N* HCl, diluted to 10 ml with water, filtered (Celite) and suitable aliquots were analyzed on the analyzer.

RESULTS AND DISCUSSION

Determination of N-methylamino acids

The results for the chromatography of a few representative N-methylamino acids on the long column of the amino acid analyzer operated under the normal conditions are illustrated in Fig. 1. It is seen that in agreement with a previous report[5], the peaks were all unsymmetrical, and moreover, that the color yields were very low since the sarcosine peak has a height about equal to that given by 0.05 μmole of glycine on our instrument. The variation of the constant $C = (H \times W)/c$ (H = net height of peak = absorbance; W = width of peak at half net height; c =

concentration) with concentration for one of these methylamino acids is shown in Fig. 2. It was obvious that the usual method of calculation using this constant could not be used. We had previously encountered a similar difficulty in the determination of ammonia by a modified procedure[16]. We had found, however, that this difficulty could be circumvented by using for the calculations a plot of $H \times W$ *versus* concentration, which had given a straight line. When this type of plot was made for the N-methylamino acids, as presented in Fig. 3, it was indeed found that straight lines resulted. Therefore, despite all the difficulties alluded to above, N-methylamino acids can be determined with the amino acid analyzer, albeit with only fair reproducibility, if a plot of $H \times W$ *versus* concentration is used for the calculations.

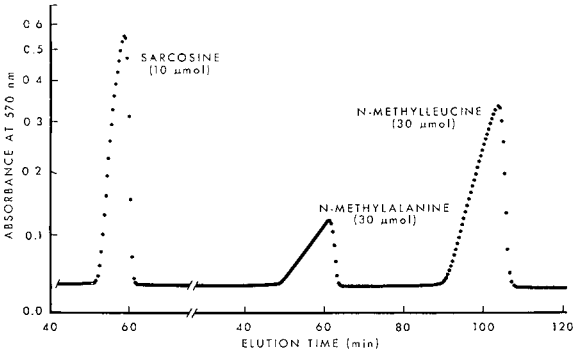

Fig. 1. Chromatography of N-methylamino acids under the normal operating conditions of the Beckman amino acid analyzer.

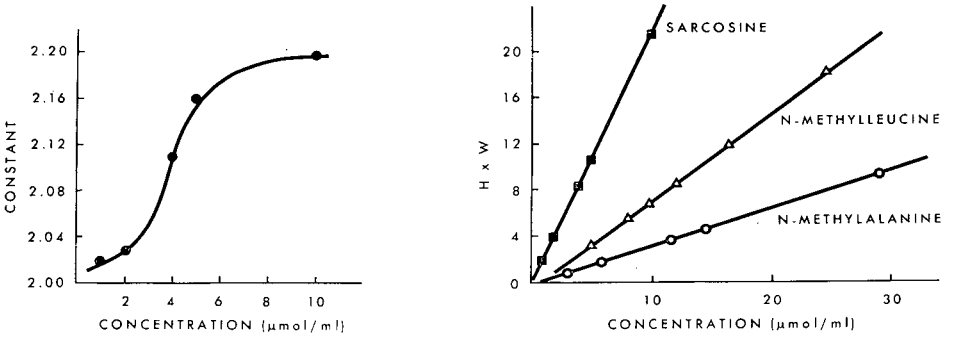

Fig. 2. Variation of constant ($H \times W/c$) with concentration for sarcosine under the normal operating conditions of the analyzer.

Fig. 3. Standard curves for the determination of N-methylamino acids under normal operating conditions of the analyzer.

Not being satisfied with the sensitivity of the method, we then verified that the reason for the low color yields, as suggested[5], was that the reaction of ninhydrin with N-methylamino acids was so much slower than with amino acids. It is seen in Fig. 4 that for sarcosine, the least hindered of all the N-methylamino acids, the reaction was not complete until after about 50 min. Under the same conditions, the reaction with

glycine was complete in 10–12 min. It has also been reported that the reaction of ninhydrin with an α-C-methylamino acid is unusually slow[4]. There was therefore no doubt in our minds that the low color yields obtained for these methylamino acids under the normal operating conditions of the analyzer were in large part due to the fact that the effluent stream remained in the reaction coil for too short a time. A second reason considered to account for the low color yields was the possibility that the colored products formed were not the same as those formed from amino acids and consequently that their absorbance maxima were not at the same wave length. This was therefore examined and it was found that over the range of 350–700 nm, the absorbance spectra of the solutions resulting from the reaction of ninhydrin with leucine, N-methylleucine and α-C-methylleucine were all identical, having maxima at 408 and 570 nm and minima at 458 nm.

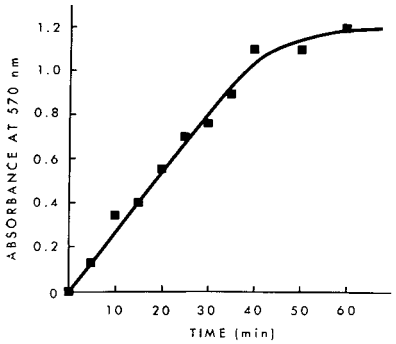

Fig. 4. Variation of the absorbance at 570 nm with time for the reaction of sarcosine with ninhydrin. 0.5-μmole samples of sarcosine were heated in a boiling water bath with cyanide–acetate buffer and ninhydrin reagent according to ROSEN[15]. At the designated times, the cooled mixtures were diluted with isopropanol–water (1:1) and the absorbance at 570 nm was read against a blank.

It was therefore decided to increase the reaction time in the analyzer by decreasing the buffer flow rate by one half, from 68 ml/h to 34 ml/h. This had the effect of increasing the reaction time by 50 % (the ninhydrin flow rate was not changed) and consequently doubling the constants for the amino acids. However, it also had the dramatic effect of increasing the constants for the N-methylamino acids by 10–20 times. The constants for α-C-methylalanine and α-C-methylleucine were also increased 5-fold and that for N-ethylleucine 30-fold. Furthermore, the constants were really constant, and the reproducibility was comparable with that for amino acids. The values of the constants for the N-methylamino acids now varied from 18–93 % of those for the parent amino acids. A comparison of the data for both flow rates appears in Table I.

There is a useful observation to be made from these results. The fact that α-C- and α-N-alkylamino acids react more slowly with ninhydrin can be made use of for identification purposes. A peak on the amino acid analyzer chart paper can be identified as being due to an alkylamino acid if, upon decreasing the buffer flow rate, the increase in the size ($H \times W$) of the peak is greater than the increase observed for an α-amino acid.

TABLE I

CHROMATOGRAPHIC DATA[a] FOR DIFFERENT ELUTING BUFFER FLOW RATES

A 0.9 × 50 cm column of AA-15 resin eluted with 0.35 *N* sodium citrate, pH 4.25, at 57°.

Compound	*Normal; 68 ml/h*		*Half-normal; 34 ml/h*	
	Constant	*Time (min)*	*Constant*	*Time (min)*
Sarcosine	3.0	30	36.3	59.5
Glycine	23.4	33	38.9	67
N-Methylalanine	0.36	28	7.5	54
α-C-Methylalanine[a]	1.8	33	10.2	62
Alanine	24.1	32.5	42.7	61
N-Methylleucine	0.74	34	15.7	63
N-Ethylleucine	0.03	32	1.0	60.5
α-C-Methylleucine	1.95	46.5	11.2	92.5
Leucine	23.9	51	40.5	102
N-Methylphenylalanine	0.66	61	23.5	106
Phenylalanine	22.7	79	40.6	157

[a] α-Aminoisobutyric acid.

Optical purity of N-methylamino acids

Since the details of the method of MANNING AND MOORE[13] were not available at the time, exploratory experiments were first carried out. The procedure using L-alanine N-carboxyanhydride described in MATERIALS AND METHODS is the one which was subsequently adopted in our laboratory for routinely determining the optical purity of amino acids. With amino acids, it gives results similar to those reported[13]*, that is, about a 90% yield in the coupling, small amounts of alanine and alanylalanine as by-products, and narrow separable peaks for the diastereomeric dipeptides. The reaction, however, does not proceed so smoothly with N-methylamino acids. It was found that much more alanine and alanylalanine resulted after the reaction of alanine N-carboxyanhydride with N-methylleucine than with leucine. This is due to the generally much lower reactivity of N-methylamino acids towards acylation reagents. This was demonstrated in this case by carrying out the coupling with a mixture containing seven parts N-methylleucine and one part leucine. The dipeptides were formed in a ratio of 1:2 (Ala·MeLeu:Ala·Leu).

A second signifiant difference which was observed when the method of MANNING AND MOORE was applied to N-methylleucine was that the peaks for the dipeptides of the latter were three times as wide (at the half-height) as those for the corresponding unmethylated peptides. This had the effect of decreasing the sensitivity of the method since the peaks of the two diastereomeric methylamino acid dipeptides were not so well separated. Instead of being able to detect one part of an isomer in 1000 parts of the other, as reported[13], we were only able to detect down to one part in 100. However, this was satisfactory for our purpose. The chromatographic data are given in Table II. Note that the N-methylamino acid isomers could actually be

* We are grateful to Dr. S. MOORE for sending us a copy of the manuscript before its publication.

TABLE II

CHROMATOGRAPHIC DATA FOR SOME L-ALANINEDIPEPTIDES

A 0.9 × 50 cm column of AA-15 resin eluted at a flow rate of 68 ml/h with 0.35 *N* sodium citrate, pH 3.28 for 85 min, followed by pH 4.25 buffer.

Compound	*Elution time (min)*	*Color yield ratio* L-D/L-L
L-Ala-L-Ala	144	
L-Ala-D-Leu	196	0.85
L-Ala-L-Leu	207	
L-Ala-D-MeLeu	180	1.06
L-Ala-L-MeLeu	170	
Ala	89	
Leu	141	
MeLeu	87	

determined in the presence of the parent amino acid since there would be no interference by any other peak.

It is worth noting a few of the very attractive features of this method of MANNING AND MOORE for determining the optical purity of amino acids. The amino acid in question need not be crystallized, nor purified, nor even isolated from a solution. The test can be run on a salt solution of the amino acid even in the presence of other amino acids if they or their coupling products do not overlap on the chromatogram with the dipeptide peaks. The information required is the relative color yields of the dipeptides which must be obtained from the racemate since they are different for each pair of diastereomers, and knowledge from some source of the order of elution of the diastereomers since this cannot be predicted.

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CHROM. 4914

THE RAPID SEPARATION OF NUCLEOTIDES IN CELL EXTRACTS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

Nucleotide profiles of cell extracts were determined using high pressure liquid chromatography. The nucleotide levels were quantitatively reproducible and the adenine levels as determined by the analyzer were in close agreement with those determined by an enzymatic cycling procedure. Optimal conditions were determined for the mono-, di- and triphosphates of the naturally occurring ribosides as well as for the sulfur analogs of some of these compounds. The peaks were identified by the use of internal standards, by comparison to chromatograms of standard solutions, by collection and identification of the fractions chemically and spectrophotometrically and by the use of an enzymic peak-shift technique. The latter method which utilizes the specificity of enzyme reactions with a nucleotide or class of nucleotides, can be used not only to verify peak identities but also to clarify or "unmask" a chromatogram. Chromatograms of cell extracts of red blood cells, homogenized schistosomes or murine leukemia or sarcoma cells were obtained in seventy minutes.

INTRODUCTION

In the study of the regulation of metabolic processes and how these processes are affected by drugs, it is important to be able to determine the concentrations of free nucleotides in cell extracts. Until recently, however, there has not been a highly sensitive rapid technique for determining the levels of these various nucleotides.

A liquid chromatographic system developed by Picker Nuclear which combines anion-exchange liquid chromatography under pressure with detection of the peaks by UV spectroscopy has enabled us to determine the nucleotide profile of cell extracts both qualitatively and quantitatively in a short time (70 min). The development of this instrument was made possible by the preliminary work of COHN[1] on ion-exchange chromatography, of ANDERSON[2], who devised a continuous column effluent monitoring system, of KIRKLAND *et al.*[3–5] and of HORVATH *et al.*[6–8], who investigated the operating parameters of high-pressure liquid chromatographic systems.

EXPERIMENTAL

Apparatus

A Picker (now manufactured by Varian) LCS-1000 nucleic acid analyzer was used. The double-beam UV detector operated at 254 mμ. The cylindrical flow cell was 1 mm in diameter and had a 10-mm pathlength. Either a 10-mV or a 1-mV Texas Instrument Servoriter recorder was used. The column, which was purchased from the Varian Corp., is 1.0 mm I.D., 3 m in length and is packed with a pellicular anion-exchange resin. A Gilson Model MF Mini-Escargot fractionator was placed in line to collect the fractions.

Mode of operation

The low-concentration eluent was 0.015 *M* KH_2PO_4 and the high-concentration eluent 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. A mixing chamber in the instrument was filled with low-concentration buffer to the 50-ml mark (the starting volume). The gradient flow rate in which the high concentration buffer was pumped into the mixing chamber was 6 ml/h. The column flow rate or flow of the gradient from the mixing chamber into the column was 12 ml/h. Full scale absorbancy ranges using a 1-mV recorder were from 0.002 to 0.064 absorbancy units and using a 10-mV recorder from 0.02 to 0.64 absorbancy units. For most samples, the UV output of 0.04 or 0.08 was used, although the output can be varied according to the sample size. All samples were introduced with a 10-μl Hamilton syringe.

Chemicals

Reagent grade KH_2PO_4 and KCl were purchased from Mallinkrodt. Standard solutions were prepared from commercially available nucleotides (purchased from P-L Biochemicals, Inc.) or from nucleotides prepared enzymatically in our laboratory. The hexokinase and pyruvate kinase were purchased from Sigma Chemical Co. and the phosphoenolpyruvate from Calbiochem Corporation.

Standard solutions

The standards used as references were the adenine and guanine nucleotides, one solution containing AMP*, ADP and ATP ($\sim$0.5 m*M* in each) and the other GMP, GDP and GTP (also $\sim$0.5 m*M* in each). These solutions were kept at $-5°$ and used qualitatively as references for retention times; however, for quantitation of peaks, freshly prepared solutions were used. Standard solutions of the pyrimidine nucleotides were prepared as well as cyclic 3′5′-AMP and cyclic 2′3′-GMP and 5′-monophosphate nucleotides of xanthine, hypoxanthine, 6-mercaptopurine, 6-methylmercaptopurine and 6-thioguanine.

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* The following abbreviations are used: AMP, ADP, ATP = adenosine 5′-mono-, 5′-di- and 5′-triphosphate; C-AMP = cyclic 3′,5′-adenosine monophosphate; GMP, GDP, GTP = 5′-phosphates of guanosine; C-GMP = cyclic 2′,3′-guanosine monophosphate; UMP, UDP, UTP = 5′-phosphates of uridine; CMP, CDP, CTP = 5′-phosphates of cytidine; TMP, TDP, TTP = 5′-phosphates of deoxythymidine; 6MMPRMP = 5′-monophosphate of 6-methyl mercaptopurineriboside; TGMP = 5′-monophosphate of 6-thioguanosine; XMP = 5′-monophosphate of xanthosine; IMP, IDP = 5′-phosphates of inosine; UDPG = uridine diphosphoglucose; UDPGA = uridine 5′-diphosphoglucuronic acid; NAD, NADH = the oxidized and reduced forms of nicotinamide-adenine dinucleotide.

Identification of peaks

Eluent peaks in cell extracts were identified in the following ways: (1) by collecting the peak and identifying the contents spectrophotometrically or chemically; (2) by injecting known standards along with the sample; (3) by comparing the extract chromatogram to that of known compounds; or (4) by known enzymatic reactions in which an enzyme added to the cell extract specifically converted one of the nucleotides present to another. The latter method showed the simultaneous disappearance of one substrate and the appearance of the corresponding product.

Quantitation of peaks

Since each nucleotide has its own molar extinction coefficient at 254 mμ, separate calibrations were required for quantitating each compound. Areas under the peak were determined by multiplying the height of the peak by the width at half height.

A control experiment was run to determine whether on the column there is any retention of compounds with subsequent bleeding out of these substances in later runs (ghosting). For this experiment a standard solution of the adenine nucleotides was run. The fractions and the effluent from the low concentrate wash were collected at $2\frac{1}{2}$-min intervals. A cell extract containing radioactive [^{14}C]adenine and [^{14}C]guanine nucleotides was then injected into the column and the effluent containing this sample was collected. The subsequent wash was collected separately. Another standard solution of adenine nucleotides was then run and this standard, and the wash after it, collected. All the collection tubes were washed with Bray's solution and the solution transferred to counting vials. The wash solutions and the standard solution run following the ^{14}C sample were found to have only background levels of radioactivity. A plot of the counts of the radioactive sample corresponded to the chromatogram from the analyzer (Figs. 1 and 2). Therefore, it was concluded that there is no observable retention of the nucleotides on the column and that distribution of the peaks determined by radioactivity corresponds well to the distribution of peaks as determined by UV absorption.

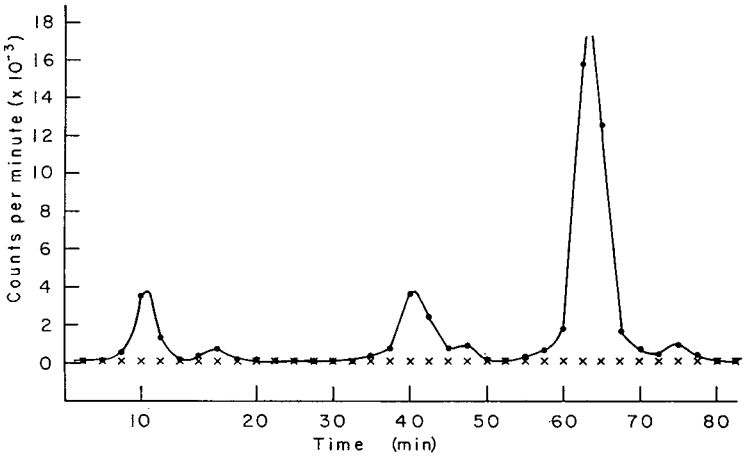

Fig. 1. Plot of counts (^{14}C) per minute *vs.* time of the schistosome sample. The sample is designated by dots and the control and washes by crosses.

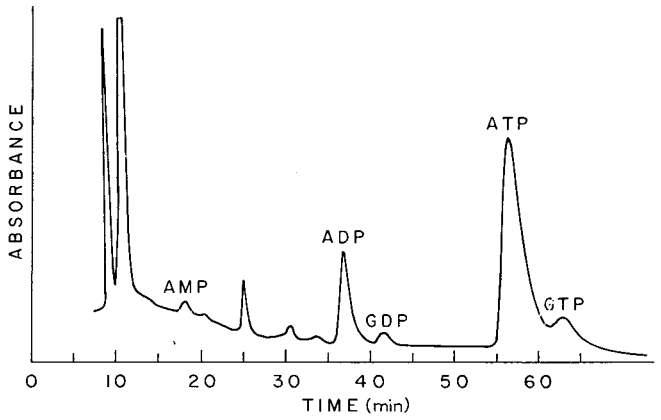

Fig. 2. Separation of nucleotides from homogenate of schistosomes. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 20 μl of extract of homogenate of schistosomes; UV output: 0.04.

RESULTS AND DISCUSSION

To obtain the optimum separation of nucleotides, a few operating parameters can be predicted, but most must be obtained by trial and error. The types of eluents used in conventional ion-exchange chromatography may also be used in a pellicular resin column under pressure, but with the latter methods the eluents are much less concentrated. The effect of temperature, flow rate, mode of elution, pH and salt concentration of the eluents has been discussed in detail for the separation of nucleosides and bases on a cation-exchange column by HORVATH AND LIPSKY[8], and for nucleotides on an anion-exchange column by HORVATH *et al.*[7]. In the present study, it was found that temperature and variations in pH (from 3.5–5.6) are less important than are the flow rates and salt concentration for optimizing the sharpness and separation of peaks.

Standards

Standard adenine and guanine nucleotides were analyzed routinely several times a week to check the reproducibility of the results and the stability of the instrument and column conditions. A representative chromatogram of these nucleotides is shown in Fig. 3. Fig. 4 shows a chromatogram of some of the important naturally occurring purine and pyrimidine nucleotides. The retention times of these standard solutions are given in Table I. It should be noted that retention times may vary from instrument to instrument and from column to column. Minor variations in retention times may also be caused by column conditions, fluctuations in the hydraulic system, and slight changes in purging procedure between runs. However, the relative order of peaks did not change and the small variations in retention times were correlated to the standard adenine and guanine nucleotide solutions which were run routinely. The order of the nucleotides is characteristically C, U, T, A and G for the mono- and diphosphates as well as the triphosphate nucleotides. Cyclic 3′,5′-AMP and cyclic 2′,3′-GMP had

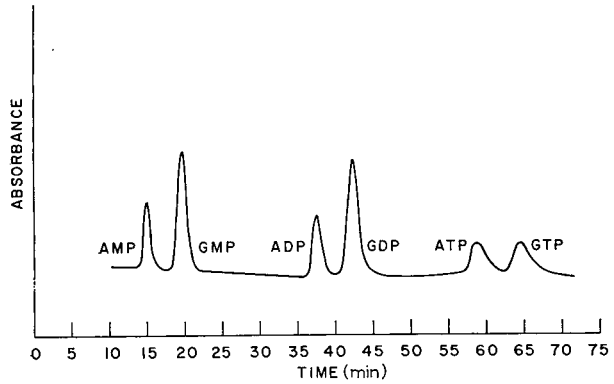

Fig. 3. Separation of mono-, di- and triphosphates of adenosine and guanosine. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: 3 μl of a mixture of ~ 0.05 m*M* AMP, ADP and ATP and 3 μl of a mixture of ~ 0.05 m*M* GMP, ADP and GTP; UV output: 0.08.

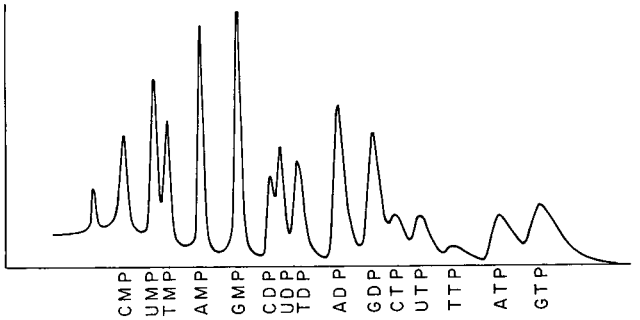

Fig. 4. Separation of mono-, di- and triphosphates of purine and pyridine ribosides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 2 μl of a solution of adenine nucleotides (~ 0.5 m*M* in each), 2 μl of a solution of guanine nucleotides (~ 0.5 m*M* in each), 2 μl of a solution of cytidine nucleotides (~ 0.2 m*M* in each), 2 μl of a solution of uridine nucleotides (~ 0.2 m*M* in each), and 2 μl of a solution of thymidine nucleotides (~ 0.2 m*M* in each); UV output: 0.08.

TABLE I

RETENTION TIMES (min) OF STANDARD NUCLEOTIDE SOLUTIONS

Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; UV output: 0.08.

Monophosphates		*Diphosphates*		*Triphosphates*	
CMP	10	NAD	8½	CTP	46
UMP	12	UDPG	24	UTP	49½
TMP	14	CDP	29	TTP	54
IMP	18	UDP	31	ATP	60
AMP	19	TDP	33	GTP	65
GMP	23	ADP	38		
C-AMP	24	NADH	39		
6MMPRMP	27	GDP	43		
TGMP	32	UDPGA	44		
XMP	34				

longer retention times than their linear analogs (Figs. 5a and b). This order of retention has also been found in conventional column chromatography using an anion-exchange column[9].

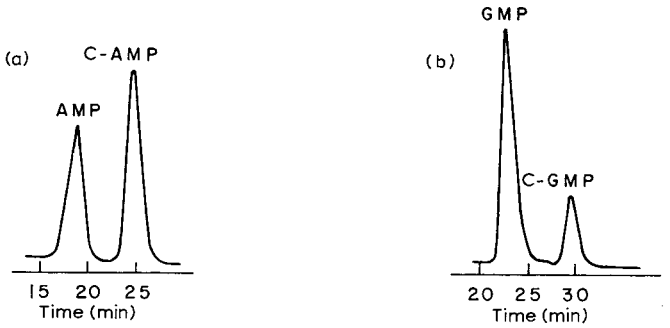

Fig. 5. Separation of cyclic AMP and GMP from their linear analogs. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: (a) 4 μl AMP and 1 μl C-AMP, (b) 4 μl GMP and 1 μl C-GMP; UV output: 0.16.

Optimal operating conditions

In order to find the optimal operating conditions for cell extracts, chromatograms of the adenine and guanine nucleotides were obtained in which the following conditions were varied one at a time: (1) temperature, (2) flow rates, (3) starting volume of low-concentrate eluent, (4) pH of eluent, (5) eluent composition, and (6) sample size (both volume and concentration).

Temperature, flow rates, starting volume, pH and salt concentration

It was found that 75° was the optimal temperature for peak sharpness, especially with the triphosphate nucleotides. Higher temperatures were not tried because of the well-known heat lability of di- and triphosphate nucleotides. The best resolution was possible using a column flow rate of 12 ml/h and a gradient flow rate of 6 ml/h. Increasing both the gradient and column flow rates increased peak sharpness and reduced retention time. These advantages, however, were offset by a disadvantage: these chromatograms were so crowded that there was not enough space in the cell extract chromatograms for good separation of all purine and pyrimidine nucleotide peaks (Fig. 6). The optimal starting volume of low-concentration buffer was 50 ml. Lowering the starting volume had the same advantages and disadvantages as increasing the flow rates. Changing the pH of the eluents affected the separation and peak sharpness, but to a much lesser degree. The optimal pH of both eluents for our work was 4.5. The salt concentration had a significant effect in both peak sharpness and resolution. It was found that the solutions suggested by SCHMUCKLER[10] of 0.015 *M* KH_2PO_4 in 2.2 *M* KCl were best for our purposes. Although formate solutions are used routinely in anion-exchange column chromatography, it has not been used in this instrument because of possible reaction of the formate with the stainless steel components[8].

Sample size

Sample volumes of 2 to 20 μl were injected into the column. Since we are limited to using a 10-μl syringe because of the instrument design, any volume larger than

10 μl had to be injected twice, thus doubling the possibility of sampling errors. Sample solution volumes in the range of 2 to 9 μl were the most satisfactory, giving the best quantitatively reproducible results. Solutions of nucleotides ranging from 0.01 mM to 1.0 mM were used with the natural nucleotides, and it was found that the concentrations were proportional to peak area. A plot of concentration *vs.* area is shown in Fig. 7.

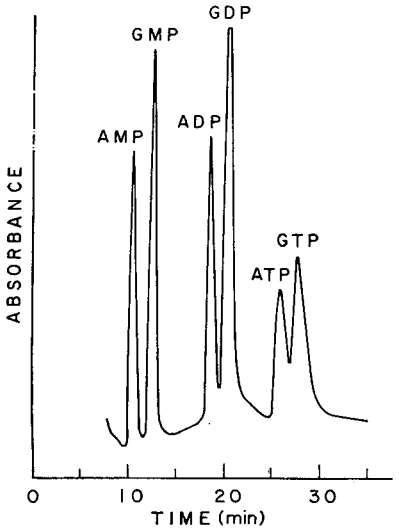

Fig. 6. Separation of adenine and guanine nucleotides with low starting volume of low-concentrate eluent and high flow rates. Starting volume: 35 ml; flow rates; 24 ml/h (gradient into column) and 12 ml/h (high-concentrate eluent into low); eluents: 0.015 M KH_2PO_4 and 0.25 M KH_2PO_4 in 2.2 M KCl; samples: 1 μl of a solution of adenine nucleotides and 1 μl of a solution of guanine nucleotides; UV output: 0.08.

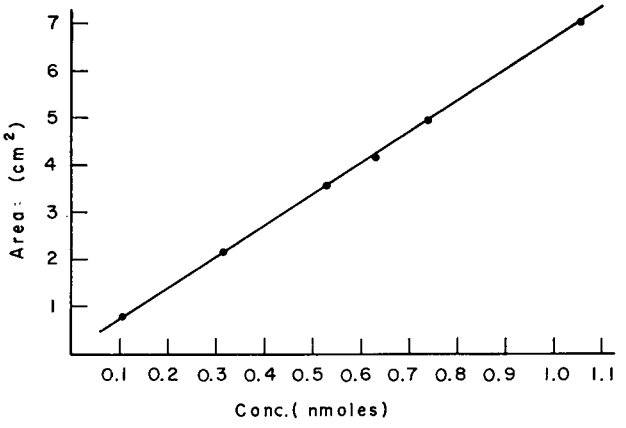

Fig. 7. Plot of concentration (in nmoles) of AMP *vs.* area (in cm^2) of AMP peak.

Eluents for constant eluent strength mode

For complete cell extracts, the gradient elution mode was used, but for the determination of either the mono-, the di- or the triphosphate nucleotides alone, the samples were run using a single concentration of eluent. For the separation of AMP and GMP, a solution of 0.10 *M* KCl gave good resolution; for ADP and GDP, 0.015 *M* KH_2PO_4 in 0.15 *M* KCl; and for ATP and GTP, 0.019 *M* KH_2PO_4 in 0.19 *M* KCl. The retention times were as follows: AMP, 11 min; GMP, 14½ min; ADP, 10 min; FDP, 12 min; ATP, 12 min; GTP, 16½ min.

Quantitation

In the quantitation of the adenine and guanine nucleotides, ten samples each of a standard solution of AMP and of GMP were run. The areas which were calculated by multiplying the height times the width at half height are tabulated in Table II.

TABLE II

TABULATION OF AREAS UNDER THE PEAKS OF STANDARD SOLUTIONS OF AMP AND GMP

The AMP solution was 6.08 × 10^{-5} *M* and the GMP solution was 5.85 × 10^{-5} *M*. The volume of sample solution injected ranged from 6–8 µl. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; UV output: 0.08.

AMP		*GMP*	
Spectrum No.	*Area/nmole*	*Spectrum No.*	*Area/nmole*
163	6.42	147	6.48
171	6.81	148	6.24
173	6.74	149	6.64
174	6.87	150	6.66
175	6.92	152	6.41
176	6.49	153	6.39
180	6.76	154	6.57
181	6.81	155	6.54
182	6.52	156	6.60
183	6.69	158	6.63
Mean	6.71	Mean	6.52
Standard deviation	0.18	Standard deviation	0.10
Coefficient of variation	2.8	Coefficient of variation	1.5

The standard deviation for AMP was 0.19 and for GMP was 0.10; the coefficient of variation for AMP was 2.6 % and for GMP, 1.5 %. The concentration of all adenine nucleotides in cell extracts was calculated by relating peak areas to that of the standard AMP and of all guanine nucleotides to that of GMP. A comparison of values of total adenine nucleotide content as determined by the analyzer and by an enzymatic method in four different blood samples showed close agreement and is shown in Table III. Samples were assayed by Dr. RALPH MIECH by an enzymatic cycling procedure specific for adenine nucleotides which employs ATP:GMP phosphotransferase, ATP:AMP phosphotransferase, pyruvate kinase and lactic acid dehydrogenase. Details will be published elsewhere.

TABLE III

TOTAL ADENINE NUCLEOTIDES (AMP + ADP + ATP) (nmoles/μl)

	Sample			
	H	*P*	*A*	*S*
Analyzer	0.387	0.344	0.354	0.229
Enzymatic analysis	0.393	0.332	0.348	0.230

Cell extracts

The free nucleotide content of cell extracts of murine Sarcoma 180 cells, murine leukemia cells (L5178Y), homogenized *Schistosoma mansoni* and human and rat red blood cells were determined. As in all nucleotide analyses, care in the preparation of the extract is of utmost importance in order to minimize the degradation of the nucleotides and to obtain valid results[11]. Samples of the chromatograms obtained in the study of the effect of purine antimetabolites on nucleotide levels of Sarcoma 180 cells are shown in Figs. 8 and 9. Fig. 2, as was noted previously in the control experiment for retention of nucleotides on the column, is typical of the chromatograms of extracts of homogenized schistosomes. Fig. 10 shows a chromatogram obtained in the study of the effect of antimetabolites on murine leukemia cells; Fig. 11 is representative of the identification of peaks in a chromatogram by the addition of known standard solutions. In this case, a solution of the uridine nucleotides was the standard used.

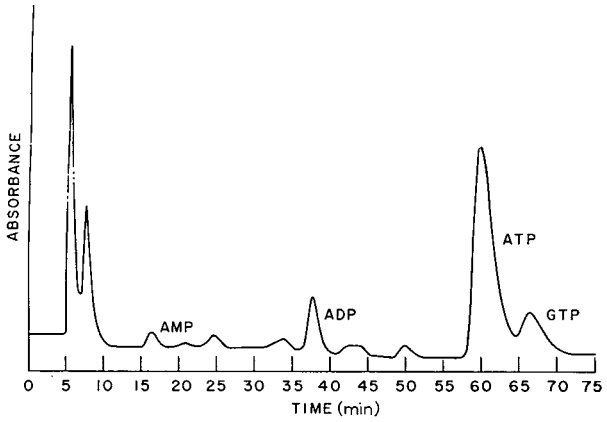

Fig. 8. Separation of nucleotides in cell extract of Sarcoma 180 cells. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 8 μl of a perchloric acid extract of Sarcoma 180 cells; UV output: 0.04.

"Enzymic peak-shifts"

"Enzymic peak-shifts" were used as a method of verifying peak identities. This technique utilizes the specificity of enzyme reactions with a nucleotide or class of nucleotides. An example of this procedure is the use of hexokinase (HK) and an excess of glucose to identify the ATP and ADP peaks[12].

$$\text{ATP} + \text{glucose} \xrightarrow[Mg^{2+}]{HK} \text{ADP} + \text{glucose-6-phosphate}$$

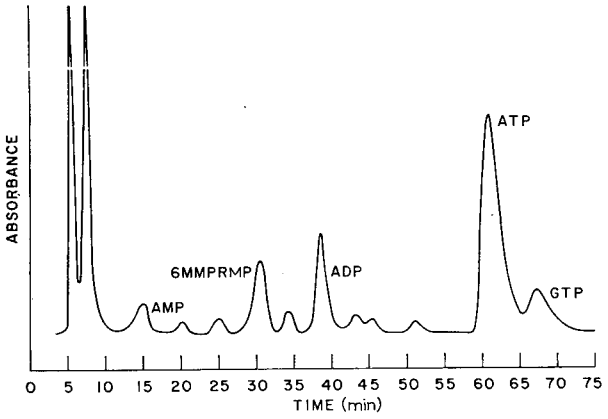

Fig. 9. Separation of nucleotides in extracts of Sarcoma 180 cells which had been treated for 1 h with an antimetabolite. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 8 μl of perchloric acid extract of drug-treated Sarcoma 180 cells; UV output: 0.04.

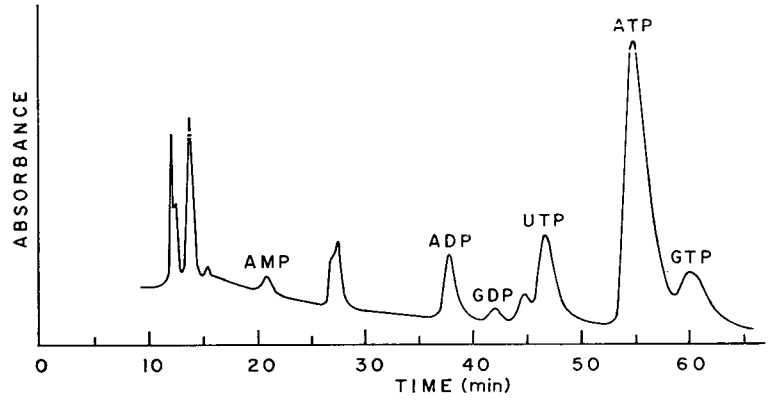

Fig. 10. Separation of nucleotides in a TCA extract of tissue culture of murine leukemia cells (L5178Y). Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 4 μl; UV output: 0.04.

In this reaction, the ATP peak disappeared and the ADP increased proportionally in size. The peaks for the guanine nucleotides and the AMP were not altered. Therefore, positive identification is established for the ATP and ADP peaks. In all the enzymic peak-shift reactions, the solutions were treated with trichloroacetic acid (TCA) to precipitate any protein or acid-insoluble material and to prevent accumulation of enzyme on the column. The nucleotides were in the TCA supernatant. The TCA was then removed by extraction with water-saturated diethyl ether. The chromatograms of standard solutions of extracted adenine and guanine nucleotides before and after the reaction with hexokinase are shown in Figs. 12 and 13. The same nucleotides were

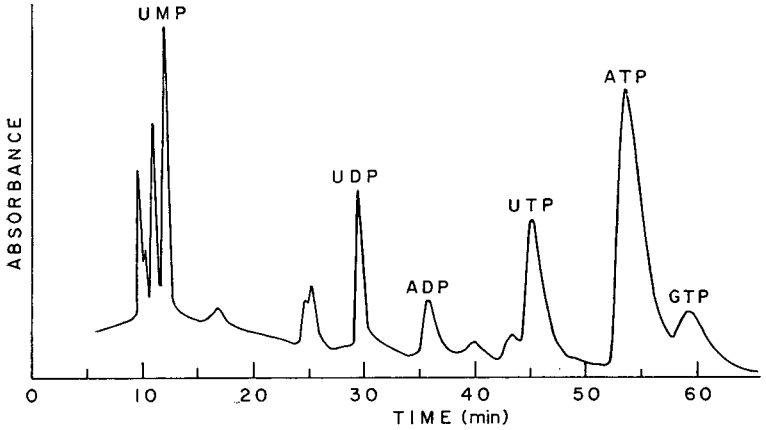

Fig. 11. Identification of uridine peaks by the addition of uridine nucleotides to a TCA extract of a tissue culture of murine leukemia cells (L5178Y). Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; samples: 4 μl cell extract and 1 μl solution of uridine nucleotides (mono-, di-, and triphosphate); UV output: 0.04.

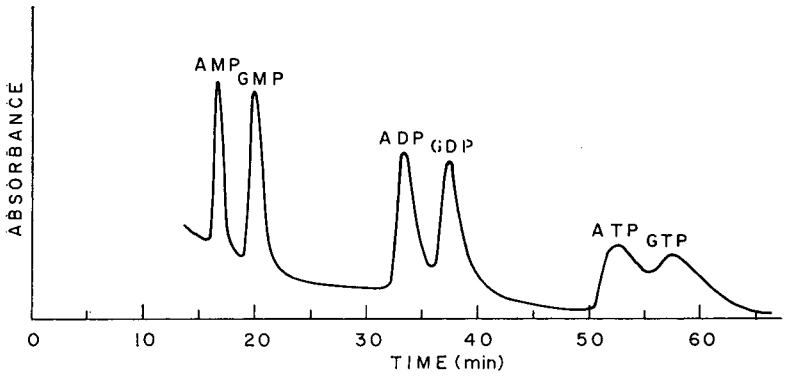

Fig. 12. Separation of a TCA extract of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents; 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 10 μl of a TCA extract of solutions of AMP, ADP and ATP and of GMP, GDP and GTP; UV output: 0.32 (1-mV recorder).

treated with pyruvate kinase (PK) and an excess of phosphoenolpyruvate (PEP) in the presence of magnesium chloride[13].

$$\text{ADP} + \text{PEP} \xrightarrow[Mg^{2+}]{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{GDP} + \text{PEP} \xrightarrow[Mg^{2+}]{\text{PK}} \text{GTP} + \text{pyruvate}$$

In this reaction, the diphosphates are phosphorylated to 5'-triphosphate nucleotides Fig. 14 is the chromatogram of an extract of a solution mixture after adenine and guanine nucleotides had been incubated with PK and PEP for 10 min. There are no

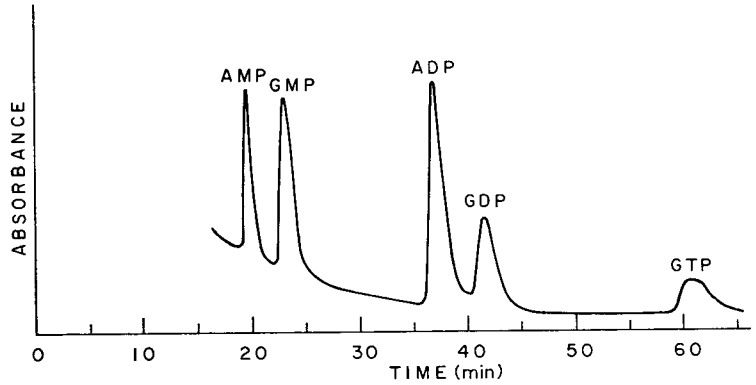

Fig. 13. Peak shifts caused by reaction of hexokinase and glucose on a solution of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; sample: 10μl of a TCA extract of reaction mixture; UV output: 0.32 (1-mV recorder).

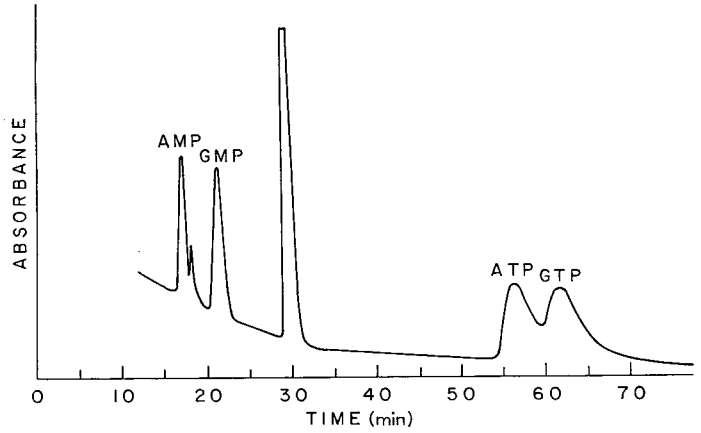

Fig. 14. Peak shifts caused by reaction of pyruvate kinase and phosphoenolpyruvate on a solution of adenine and guanine nucleotides. Starting volume: 50 ml; flow rates: 12 and 6 ml/h; eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl; Sample: 10 μl of a TCA extract of reaction mixture; UV output: 0.32 (1 mV).

diphosphate nucleotide peaks and the triphosphate nucleotide peaks have increased in size. The peak which has a retention time of 30 min may be PEP, but it has not been positively identified as yet.

"Enzymic peak-shift" reactions such as these are also useful in clarifying or "unmasking" a chromatogram. When one nucleotide is present in large quantity, it may hide the presence of small quantities of other nucleotides which have similar retention times. By completely removing the large peak, we are then able to show conclusively the nucleotides that are present. This technique is also helpful in the quantitation of a hidden peak or in determining the shape of a peak which otherwise can only be seen as a shoulder.

TABLE IV

ENZYMES THAT CAN BE USED IN "ENZYMIC PEAK-SHIFT" METHOD OF IDENTIFYING PEAKS IN CELL EXTRACT CHROMATOGRAMS

Trivial names	*EC No.*	*Systematic names*
Adenylate kinase	(2.7.4.3)	ATP:AMP phosphotransferase
ADP deaminase	(3.5.4.7)	ADP aminohydrolase
AMP deaminase	(3.5.4.6)	AMP aminohydrolase
Apyrase	(3.6.1.5)	ATP diphosphohydrolase
ATPase	(3.6.1.5)	ATP phosphohydrolase
Creatine kinase	(2.7.3.2)	ATP:creatine phosphotransferase
Deoxy-CMP deaminase	(3.5.4)	Deoxy-CMP aminohydrolase
Hexokinase	(2.7.1.1)	ATP:D-hexose 6-phosphotransferase
IMP cyclohydrolase	(3.5.4.10)	IMP 1,2-hydrolase
Pyruvate kinase	(2.7.1.40)	ATP:pyruvate phosphotransferase
UDPG dehydrogenase	(1.1.1.22)	UDPG glucose: NAD oxidoreductase

Other possible enzyme reactions

It is possible to use many other enzyme reactions in the "enzymic peak-shift" method of identifying nucleotide peaks. Almost any of the phosphotransferases (in the groups numbered 2.7.1 to 2.7.4 by the Enzyme Commission)* can be used with an excess of the appropriate substrate resulting in the disappearance of the ATP peak and the increase of the ADP peak[14]. The pyrophosphotransferases (2.7.6) can identify ATP and AMP and certain nucleotidylotransferases (2.7.7) react specifically with ATP, ADP, CTP, GDP, GTP, dTTP or the various sugar analogs of the uridine triphosphate nucleotides[14]. Some of the enzymes which act on acid anhydride bonds (3.6.1) such as ATPase and apyrase are also applicable for use in location on the chromatogram of ATP, ADP and AMP. The same is true for deoxy-CTPase for dCTP and dCMP[14].

AMP deaminase, ADP deaminase and deoxy-CMP deaminase (of the classification 3.5.4) can serve to characterize AMP and IMP, ADP and IDP, and dCMP and dUMP[14]. Uridine 5'-diphosphoglucose dehydrogenase with UDPG and NAD can be used to characterize not only the reactants, UDPG and NAD^+, but also the products, UDPGA and NADH[15].

In the case of a reversible reaction, this reaction can be coupled to a second enzymatic reaction to remove a product of the first reaction. If the second reaction is exothermic, the first reaction is driven to completion. If the second reaction is readily reversible, its equilibrium can be shifted to the right by adding an excess of substrate. A requirement in all such reactions is that neither the reactants nor the products absorb in the UV at 254 mμ.

One example of coupled enzymatic reactions is the reaction of ADP in the presence of adenylate kinase (AK) to form AMP and ATP[16], which can be driven to

* See Table IV for trivial and systematic names, and Enzyme Commission numbers of enzymes used in "enzymic peak-shifts".

completion in the presence of AMP deaminase. Since AMP deaminase converts AMP to IMP and NH_3[17], the products of the combined reactions are ATP, IMP, and NH_3.

$$2\ \text{ADP} \overset{\text{AK}}{\rightleftarrows} \text{AMP} + \text{ATP}$$

$$\text{AMP} \xrightarrow[\text{deaminase}]{\text{AMP}} \text{IMP} + \text{NH}_3$$

$$2\ \text{ADP} \xrightarrow[\text{AMP deaminase}]{\text{AK}} \text{ATP} + \text{IMP} + \text{NH}_3$$

Another procedure which involves the reaction of a product of the reversible reaction with another enzyme and excess substrate is the use of hexokinase (HK) and an excess of glucose in conjunction with the adenylate kinase reaction[12]. The ATP produced reacts with glucose until no ATP remains; thus, AMP and glucose-6-phosphate (G-6-P) accumulate.

$$2\ \text{ADP} \overset{\text{AK}}{\rightleftarrows} \text{ATP} + \text{AMP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G-6-P}$$

$$\text{ADP} + \text{glucose} \xrightarrow[\text{HK}]{\text{AK}} \text{AMP} + \text{G-6-P}$$

In both cases, these reactions are specific for the disappearance of the ADP. In the former reaction, two nucleotides accumulate, ATP and IMP, while in the latter only AMP is produced.

A third example of driving a reversible reaction in the desired direction by coupling it to another enzymatic reaction is the reverse of the previous reaction, the conversion of AMP and ATP in the presence of adenylate kinase to ADP. In the presence of creatine kinase (CK) and an excess of phosphocreatine[18], the reactions continue until there is no AMP or ADP in the reaction mixture; thus only ATP, phosphocreatine and creatine remain.

$$\text{AMP} + \text{ATP} \overset{\text{AK}}{\rightleftarrows} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{phosphocreatine} \overset{\text{CK}}{\rightleftarrows} 2\ \text{ATP} + 2\ \text{creatine}$$

$$\text{AMP} + 2\ \text{phosphocreatine} \xrightarrow[\text{CK}]{\text{AK}} \text{ATP} + 2\ \text{creatine}$$

Other coupled reactions for enzymic peak-shifts are possible, but the reactions outlined are especially applicable because of the high specificity of these enzymes for the adenine nucleotides. The reagents are readily available and inexpensive and none

of the added substrates or conversion products absorb in the UV at 254 mμ. In most cellular extracts, the concentrations of the adenine nucleotides are much greater than those of other nucleotides. Thus, "enzymic peak-shift" methods for the adenine nucleotides may be of especial value in "unmasking" peaks of the non-adenine compounds ordinarily hidden by the larger adenine nucleotide peaks. Work is proceeding to obtain the best possible conditions for carrying out these reactions and other conversions which can be used to characterize as many as possible of the nucleotide peaks.

Use of isotopes

The high-pressure liquid chromatographic system can be used in combination with isotopes for measuring radiospecific activities of nucleotides. This was demonstrated in the work with schistosomes in which the schistosomes were incubated with ^{14}C-labeled precursors to determine whether salvage pathways or *de novo* pathways were employed by these parasites in the metabolism of nucleotides[19]. In the experimental treatment of murine leukemia cells with antimetabolites, ^{35}S was incorporated into the drugs in order to determine both by the specific activity and the nucleotide profiles whether these drugs interfered with the metabolic processes at the nucleotide or the nucleic acid level. In the study of the effect of cancer chemotherapeutic agents on the Sarcoma 180 cells, time studies of the formation of the metabolites were carried out by means of paper chromatography and isotopes while the adenine and guanine nucleotide levels were determined quantitatively by the high-pressure liquid chromatographic system.

Limitations and possible improvements

A major limitation of this system is the UV detector which is only available at one set wavelength (254 mμ). In order to study analog nucleotides, especially 6-thio and 6-seleno purines (which absorb from 290–370 mμ), a second wavelength would be very useful. Other possible improvements include incorporation into the analyzer of a flow scintillation counter so that isotope studies could be carried out simultaneously with the separation. The development of procedures that would allow cell extracts to be run on a straight elution mode rather than a gradient would save the time now needed to condition the column between runs. It is not yet possible to distinguish between the deoxyribonucleotides and the ribonucleotides under the conditions we have used. A commonly employed device for solving this problem involves the use of borate ion in the elution system which would complex with the ribonucleotides and modify their migration[20,21]. Although we have not tested this method, it seems likely that separations can be effected using this technique.

In conclusion, high-pressure liquid chromatography can be an invaluable tool in the study of cell extracts. For non-volatile, polar compounds, it approaches the speed, efficiency and sensitivity that gas chromatography has for volatile, non-polar substances. This technique shortens the time and tedium previously involved in determining nucleotide levels. Therefore, it is possible to obtain quickly and easily nucleotide profiles which are qualitatively and quantitatively reproducible.

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CHROM. 4918

THE SEPARATION OF CELLODEXTRINS BY GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

The nature of the separation of the cellodextrin series, glucose–cellohexaose, on polyacrylamide and dextran gels has been investigated. It was found that the interaction between gel and solute increased with molecular weight, the effect being most pronounced with the dextran gel. One can anticipate that such adsorption effects will play an important role in all polar gel–solute systems when the solute has a conformation facilitating alignment with linear portions of the cross-linked network.

INTRODUCTION

The nature of the separation process for the cellodextrin series glucose–cellohexaose on polyacrylamide and dextrans gels has been studied as part of a general investigation of the structure and surface properties of water-swollen polymer networks. It was of interest to investigate the character of the interactive processes participating and the ability of such polar gels to separate rod-shaped molecules possessing little molecular flexibility[1].

Furthermore, the cellodextrins form an important homologous series useful in investigations concerned with the mechanism of carbohydrate depolymerization by enzymatic systems and by many chemical processes; they are also used extensively as model compounds in furthering our understanding of carbohydrate chemistry.

EXPERIMENTAL

Preparation of the cellodextrins

The cellodextrins were prepared by a modification of the method of MILLER *et al.*[2]. Whatman cellulose powder (CF-11), 90 g, was dissolved in 600 ml of fuming HCl at 0° and the solution allowed to warm to room temperature. It was then poured into 3600 ml of ice-cold distilled water and neutralized with 1260 g $NaHCO_3$. After standing for 24 h, the liquid was filtered to remove the fluffy, white precipitate

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and applied to a column of stearic acid-treated charcoal–Celite (dimensions 113 cm^2 × 800 cm). The stearic acid solution used was 2.5% in absolute ethanol. Gradient elution was performed with 20 l of water in one container connected through a siphon to 20 l of 50% absolute ethanol in a second container. Fractions were collected in an automatic collector (2100 × 18 ml). The carbohydrate contents of the fractions were measured using the orcinol reagent[3]. A typical fractionation is depicted in Fig. 1. The fractions were analyzed by thin-layer chromatography (TLC) using the system Kieselguhr-G buffered with 0.02 *M* sodium acetate and 65% isopropanol–ethyl acetate (1:1) as developer.

Fractions were combined according to the TLC results and the volume reduced in each case to about 200 ml by means of a rotary evaporator. The residues were

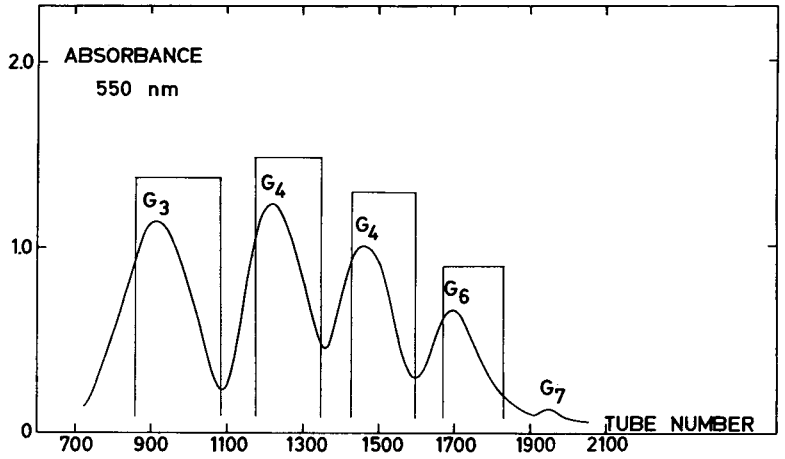

Fig. 1. Separation of cellodextrins, cellotriose–cellohexaose, on a carbon–Celite column obtained by ethanol–water gradient elution. The vertical axis represents the carbohydrate concentration determined by the orcinol method[3]. The rectangular zones represent the material isolated on the basis of the TLC analysis.

TABLE I

DIMENSIONS OF THE COLUMNS USED AT 25°

	Polyacrylamide P-2 (*ml*)	*Dextran G-15* (*ml*)
Settled bed volume	47.7	48.7
Void volume (Blue Dextran), V_0	17.1	19.25
Internal volume[a], V_I	28.7	17.0
Wt. of dry gel	15 g	~15 g
Column height	60 cm	
Cross-sectional area:	0.785 cm^2	
Flow-rate:	2 ml/h	
Sample volume:	0.1 ml	
Sample concentration:	1 mg/ml	

[a] Determined as the ordinate intercept of a plot of (V_e-V_0) *vs.* molecular weight for the cellodextrin series; this value agreed closely with that obtained from the elution of NaCl.

freeze-dried to yield approximately 4–5 g of each of the cellodextrins, cellotriose to cellohexaose.

Reagent-grade glucose and cellobiose were used to complete the series.

Column preparation

An accurately-weighed quantity of dried gel was allowed to swell in distilled water for 24 h. The slurry was de-gassed under high vacuum and the columns packed under gravity with continual addition of slurry to avoid layering defects in the columns. Subsequent to preparation each column was allowed to wash until a constant value of refractive index was obtained for the eluent. The columns were packed with polyacrylamide P-2, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif. U.S.A.) and Sephadex G-15, 40–120 μ (Pharmacia, Uppsala, Sweden). The final column dimensions are summarized in Table I. The pressure head (a Mariotte flask) was adjusted to provide a flow rate of about 2 ml/h at which it was judged, equilibrium conditions on the column would be approximated. All columns were thermostated to within $\pm$ 0.1° of the operating temperature required.

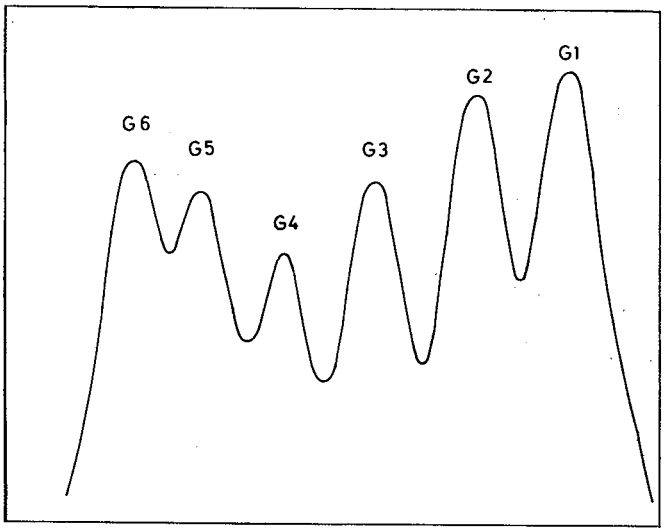

Fig. 2. Separation of cellodextrins, glucose–cellohexaose, on polyacrylamide P2 eluted with deionized water. The separation occurred within approximately one-third of the internal volume of the bed.

Sample application and detection

The sample solution (0.1 ml containing 0.1 mg solute) was applied to the gel surface with a micro pipette as a layer 1 mm deep. The solution was allowed to enter the bed, washed with successive portions of solvent and the column connected to the constant head device. The eluent was collected and the volume recorded to within $\pm$ 0.02 ml at regular intervals. A Waters Associates Model R4 differential, automatically-recording, refractometer was operated at $\times$ 8 attenuation. Under these conditions 90 % full-scale deflection was obtained at the sample maximum. The "dead"

volume between the end of the column and the refractive index cell was approximately 0.3 ml.

RESULTS AND DISCUSSION

Polyacrylamide gel

The separation of the cellodextrins on the P-2 column is illustrated in Fig. 2 for the mixture glucose–cellohexaose. By comparison with the total internal volume of the gel (V_I = 28.7 ml, 63% of settled bed volume) the separation of the six members of the series occurs over approximately 10 ml, *i.e.* about one-third of the internal volume.

The partition coefficient, K_D, is defined by:

$$K_D = \left(\frac{V_e - V_0}{V_t - V_0}\right)$$

where;

V_0 = void volume
V_t = total solvent volume in the column
V_e = elution volume for a given solute.

TABLE II

K_D AS A FUNCTION OF TEMPERATURE FOR THE CELLODEXTRINS ON POLYACRYLAMIDE P-2

Cellodextrin	*12° C*		*25° C*		*40° C*	
	K_D	$\Delta \ln K_D$	K_D	$\Delta \ln K_D$	K_D	$\Delta \ln K_D$
Glucose	0.92_6		0.92_1		0.91_5	
		0.080		0.084		0.088
Cellobiose	0.85_5		0.84_7		0.83_8	
		0.089		0.091		0.096
Cellotriose	0.78_2		0.77_3		0.76_1	
		0.097		0.099		0.105
Cellotetraose	0.71_0		0.70_0		0.68_5	
		0.090		0.093		0.098
Cellopentaose	0.64_9		0.63_8		0.62_1	
		0.079		0.078		0.082
Cellohexaose	0.60_0		0.59_0		0.57_2	

TABLE III

HYDRODYNAMIC PARAMETERS FOR CELLODEXTRINS IN AQUEOUS SOLUTION AT 25°

Cellodextrin	*Molecular weight (M)*	$[\eta]^a$ $(ml \cdot g^{-1})$	$D \cdot 10^{6a}$	V^a $(ml \cdot mole^{-1})$	L^a (Å)	r^b (Å)
Glucose	180	2.54	6.75	116	—	3.65
Cellobiose	342	2.74	5.16	222	14.6	4.85
Cellotriose	504	3.03	4.19	326	20.2	5.68
Cellotetraose	667	3.57	3.75	432	26.2	6.47
Cellopentaose	829	3.80	3.21	535	31.8	7.25
Cellohexaose	991	4.70	2.90	639	37.6	8.19

[a] Data of IHNAT AND GORING[1].
[b] Data of KURATH AND BUMP[10].

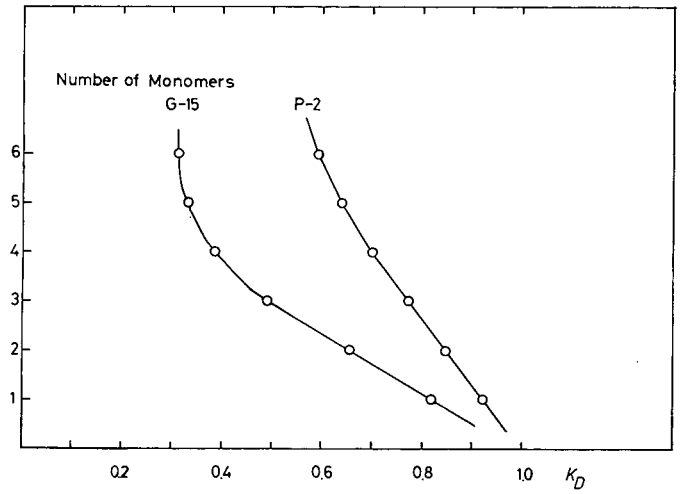

Fig. 3. Relationship between the number of chain units and K_D on polyacrylamide P2 and Sephadex G-15 with deionized water as eluant. The increasing curvature with increasing chain length indicates the influence of adsorption.

Values of K_D at various temperatures are summarized in Table II; Table III lists some pertinent structural and hydrodynamic parameters for the cellodextrins.

Fig. 3 shows the relationship between the number of chain units and the coefficient K_D. The relationship is linear for the compounds glucose–cellotetraose. Over the remaining part of the internal volume it appears that the separation may be only partly based on molecular size, there being a continuously varying relationship between chain length and K_D. It should be noted that plots of log (number of chain

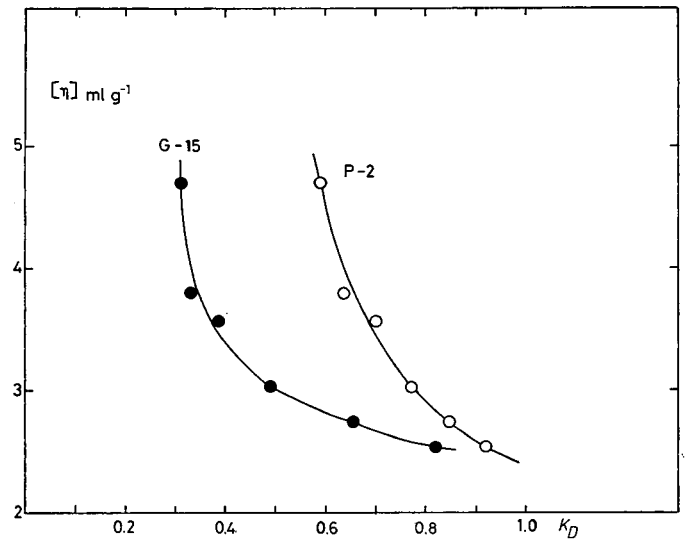

Fig. 4. Relationship between intrinsic viscosity and K_D for the cellodextrins in water.

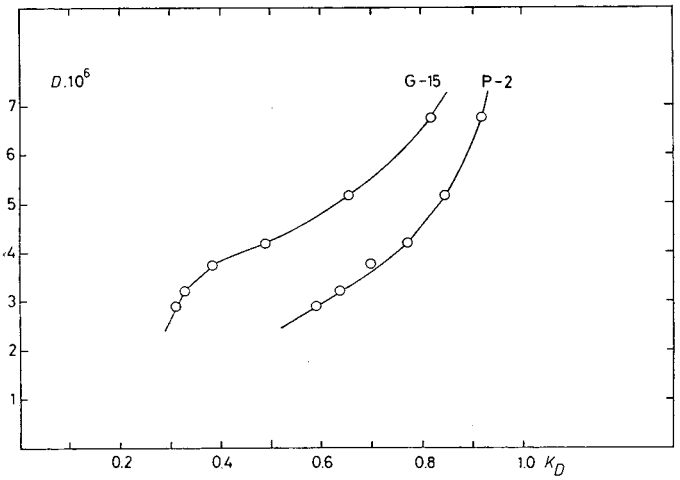

Fig. 5. Relationship between the free diffusion coefficient and K_D in water.

units) *versus* K_D have pronounced S-shaped forms. While the general shape of the curves in Fig. 3 is that expected from theory[6], the curvature is more pronounced and, as will be shown below, derives mainly from an increasing solute–gel interaction with increasing molecular weight; this phenomenon may be more important than is generally realised.

Plots of intrinsic viscosity and the free diffusion coefficient *versus* K_D are shown in Figs. 4 and 5, respectively. They illustrate that these hydrodynamic parameters are less suitable than the extended chain length for characterising the elution process. GIDDINGS *et al.*[6], using statistical mechanical methods with model systems, found that the mean external length of the molecule is more satisfactory than other molecular size paramenters for characterising partitioning in random, porous networks. However, the use of molecular weight or molar volume as a correlating parameter with K_D should be adequate for specifying partitioning within a fixed family of molecules.

Nature of the separation process

Much experimental evidence exists to show that sample migration in gel permeation is governed almost entirely by equilibrium considerations[4–6]. For example, as anticipated from theory[7], there is excellent agreement between the chromatographic partition coefficients and those derived from static equilibrium experiments; furthermore, the elution volumes normally show only a small dependence on flow rate for low molecular weight substances. Consequently one can assume that the elution volume of a given solute is a function of partition equilibrium and that diffusion is important only when considering the, usually small, peak dispersion (see below).

Assuming equilibrium, the free energy, ΔG°, required to transport a molecule from the solvent surrounding the gel particles to the solvent within the voids in the gel may be written:

$$\Delta G^\circ = -RT \ln \left(\frac{C_{\text{inside}}}{C_{\text{outside}}} \right) = -RT \ln K_D \qquad (1)$$

In the absence of enthalpy effects denoting interaction between gel and solute, we should expect:

$$\Delta G^\circ = -T\Delta S^\circ \qquad (2)$$

Here $\Delta G^\circ = R \ln \left(\frac{W_{\text{inside}}}{W_{\text{outside}}}\right)$,

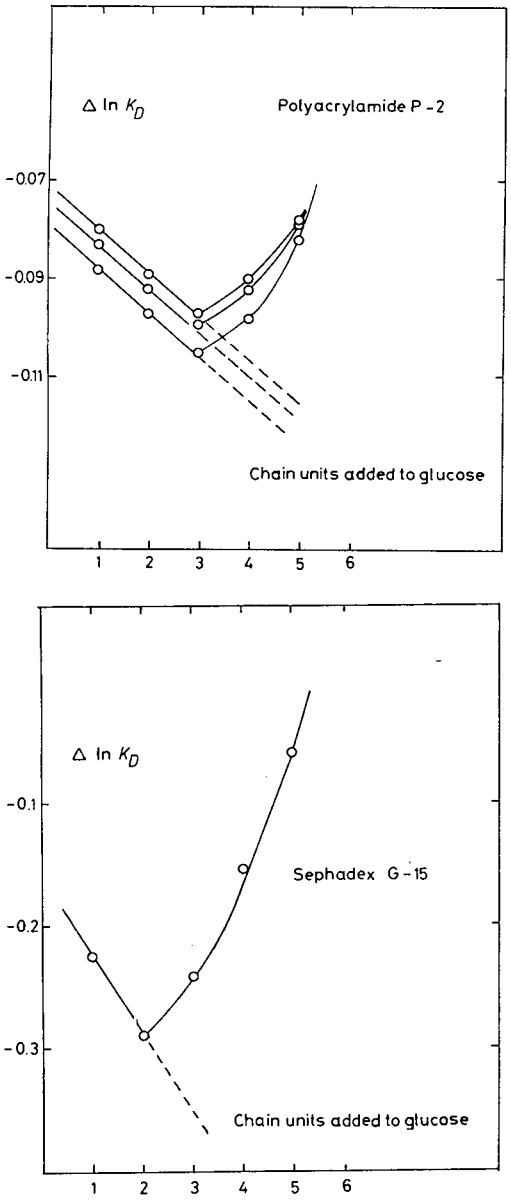

Fig. 6. $\Delta \ln K_D$ as a function of the number of chain units added to glucose in the cellodextrin series on: (a) polyacrylamide P2 at 40°, 25° and 12°; and (b) Sephadex G-15 at 25°.

where $(W_{inside}/W_{outside})$ is the ratio of possible configurations for the molecule within the pore compared with those in an equal volume of liquid bulk. This ratio is equal to K_D. A rod-shaped particle near a surface, for example, will be limited in rotational freedom and certain spatial configurations consequently forbidden; W_{inside} will thus be smaller than $W_{outside}$. Thus the partition function is proportional to the change in configurational entropy, as deduced by CASASSA[8] for randomly coiling chains.

As each anhydroglucose unit contributes a fixed amount to the molecular volume in an additive manner (see Table III), one would expect $\Delta \ln K_D$ to be constant for an homologous series such as the cellodextrins. Fig. 6 (a) shows that $\Delta \ln K_D$ is not constant, however, but at first decreases linearly with the addition of consecutive units and then increases rapidly; values of $\Delta \ln K_D$ are given in Table II. That $\Delta \ln K_D$ decreases rather than remaining constant is not entirely unexpected as the latter would only hold in the ideal case. The sharp upswing may be interpreted to mean that there are two opposing effects—on the one hand steric exclusion and on the other adsorption between gel and solute which occurs at higher molecular weight. The nature of the temperature dependence of K_D supports this view.

Temperature dependence of K_D

With interaction between gel and solute we have:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (3)$$

ΔH° may be estimated from the dependence of $\ln K_D$ on temperature (Fig. 7);

$$\frac{d \ln K_D}{dT} = \frac{\Delta H^\circ}{RT^2}$$

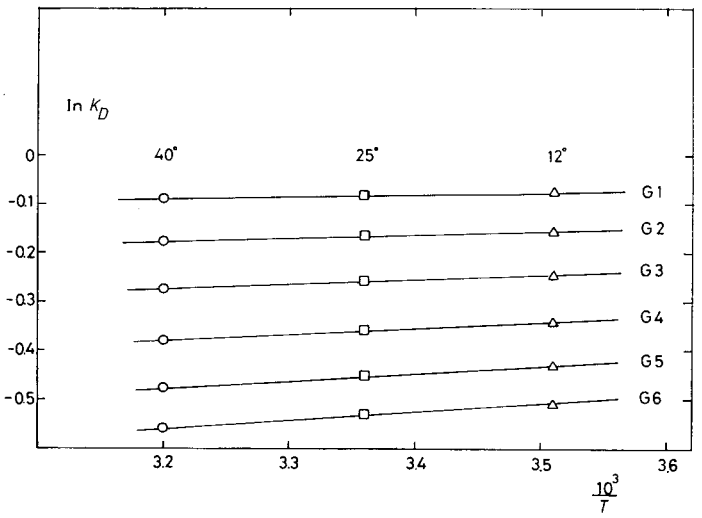

Fig. 7. The dependence of $\ln K_D$ on $1/T$ for the cellodextrin series on polyacrylamide P2 eluted with deionized water. The increasing slopes reflect negative enthalpies increasing as the series is ascended.

The entropy contribution follows from:

$$\Delta S^\circ = \left(\frac{\Delta H^\circ - \Delta G^\circ}{T}\right)$$

with ΔG° from Eqn. 1. Interpreting the changes in K_D with temperature in this way, the negative enthalpies (listed in Table IV) reflect an interaction with the gel which increases as the series is ascended. As the cellodextrins are rodlike, the frequency of interactive contacts will be proportional to the chain length; with increasing temperature the increased mobility results in elution at a smaller volume. One cannot exclude possible changes in the gel matrix with change in temperature. However, there was an insignificant change in V_I between 12° and 40°.

TABLE IV

ENTHALPY, FREE ENERGY AND ENTROPY PARAMETERS FOR CELLODEXTRINS ON POLYACRYLAMIDE P-2
ΔH° is calculated between 40° and 12°. The values of ΔG° and ΔS° are for 25°.

Cellodextrin	K_D (25°)	ΔH° ($cal \cdot mole^{-1}$)	ΔG° ($cal \cdot mole^{-1}$)	ΔS° ($cal \cdot mole^{-1}\ deg^{-1}$)
Glucose	0.92	−75	+50	−0.4
Cellobiose	0.85	−130	+100	−0.8
Cellotriose	0.77	−180	+155	−1.1
Cellotetraose	0.70	−230	+210	−1.5
Cellopentaose	0.64	−280	+265	−1.8
Cellohexaose	0.59	−305	+305	−2.0

The trend in K_D with increasing temperature is the opposite to that expected in comparison with the decreasing hydrodynamic volume as the temperature is increased. It has been established[9] that the cellodextrins behave hydrodynamically as rigid rods up to a temperature of at least 70°. There is, however, a progressive decrease in the degree of solvation which is manifested in the negative temperature coefficients of intrinsic viscosity. One would have anticipated an increase in K_D with increasing temperature and decreasing hydrodynamic volume if this change were responsible for the deviation from linearity in Fig. 3.

The hydrophilic character of polyacrylamide and cellodextrins leads one to expect such interactions with an increase in chain length. One can anticipate that in all polar gel–solute systems adsorption effects will play an important role when the solute has a conformation such that it can appropriately align with the linear portions of the cross linked-gel network.

The role of diffusion

The extent to which diffusion may influence the elution volume was examined. A known volume (50 ml) and concentration (0.3 $g \cdot dl^{-1}$) of a cellodextrin solution was added to a pre-swollen portion of gel (10 g in 50 ml water) and thoroughly mixed.

The decrease in cellodextrin concentration in the supernatant liquid was measured as a function of time employing the orcinol method[3] to measure the carbohydrate concentration. Equilibrium was rapid, final static concentrations being attained in less than 15 min for glucose, cellotetraose and cellohexaose. At the flow rate of the column (2 ml/h) this would correspond to steady state conditions

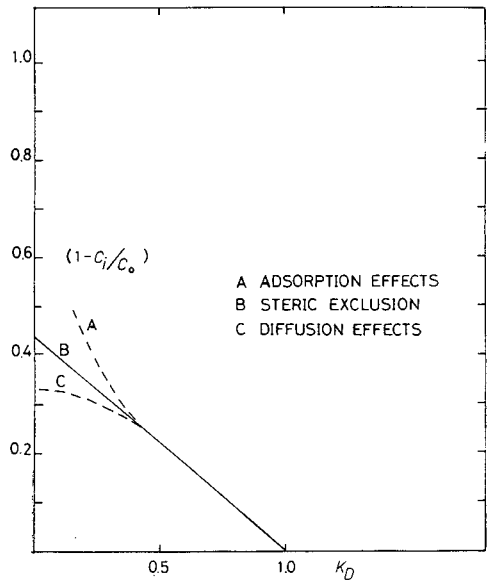

Fig. 8. Schematic diagram of the dependence of $(1-C_i/C_0)$ on K_D for static equilibrium experiments; C_i and C_0 are the initial and final concentrations of solution. Curve C indicates the behaviour when steady-state conditions are not attained when measuring K_D.

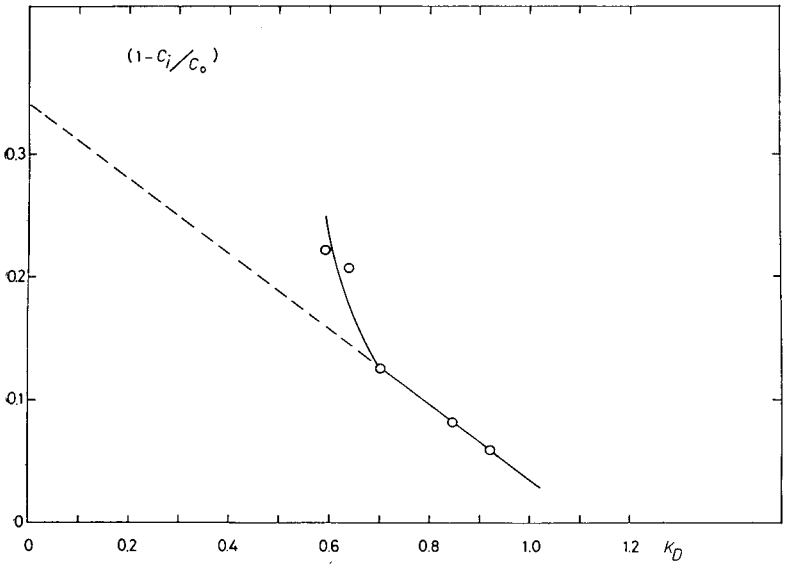

Fig. 9. Dependence of $(1-C_i/C_0)$ on K_D for the cellodextrin series in the system polyacrylamide P2/dionized water; *cf.* Fig. 8. The point for cellotriose is omitted.

subsequent to peak migration of less than 1.5% of the total bed volume. The coefficient K_D will thus approximate the equilibrium partition coefficient on elution.

Static equilibrium experiments

When a solution, volume V_i, concentration C_i, is mixed with a known weight of dry gel, there will be an increase in concentration of the supernatant solution at equilibrium which is proportional to the extent of steric exclusion of the solute from the swollen gel. Denoting the final concentration, C_0, we have[4]:

$$\left(1 - \frac{C_i}{C_0}\right) = \frac{V_g}{V_i}(1 - K_D)$$

where V_g is the internal volume of the gel; $K_D = C_g/C_0$ with C_g being the concentration inside the porous substrate. $(1-C_i/C_0)$ will be a linear function of K_D if diffusion effects are unimportant and the solute does not interact with the gel; these effects are depicted in Fig. 8.

TABLE V

K_D FOR CELLODEXTRINS–SEPHADEX G-15

Cellodextrin	K_D	$\Delta \ln K_D$
Glucose	0.82_0	
		0.225
Cellobiose	0.65_5	
		0.290
Cellotriose	0.49_0	
		0.241
Cellotetraose	0.38_5	
		0.154
Cellopentaose	0.33_0	
		0.059
Cellohexaose	0.31_1	

The initial and final concentrations were measured using a differential refractometer. 50 ml of a 0.3% solution of the cellodextrin were added to 10 g of the dry gel and allowed to equilibrate overnight. The results of these experiments are shown in Fig. 9 and again infer the influence of adsorption on K_D. The point for cellotriose is omitted as there was insufficient material for this measurement.

Dextran gel

The elution characteristics of the cellodextrins on Sephadex G-15 are similar to those for the polyacrylamide gel, except for the adsorption being much more pronounced. Although the bed volumes (Table I) of the gels were similar, the internal volume was substantially smaller (V_I = 17 ml; 37% of bed volume). The separation of the series, glucose–cellohexaose, occurred over approximately 9 ml, more than half of the internal volume, but separation as a linear function of chain length was restricted to the interval glucose–cellotriose (Fig. 3). Values of K_D are listed in Table V, and the behaviour of $\Delta \ln K_D$ as a function of increasing chain length is illustrated in Fig. 6(b).

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CHROM. 4924

LOWER LIMITS OF MOLECULAR WEIGHTS OF COMPOUNDS EXCLUDED FROM SEPHADEX G-25 ELUTED WITH AQUEOUS ACETONE MIXTURES

APPLICATION OF THE RESULTS TO THE SEPARATION OF THE COMPONENTS OF TANNIC ACID

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SUMMARY

Compounds of molecular weight greater than 900, 500, and 300 are excluded from Sephadex G-25 eluted with 40, 50 and 60% aqueous acetone, respectively. The lower exclusion limits were tested by showing that 40%, but not 50% or 60% aqueous acetone, separates the four phenolic components of tannic acid.

INTRODUCTION

Our early attempts to separate the polyphenolic constituents of aqueous extracts of tree leaves on the dextran gels Sephadex G-10 and G-25, using water as eluent, were unsuccessful, some of the polyphenols being strongly adsorbed on the gels. Promising results were obtained, however, when Sephadex G-25 was used in conjunction with aqueous ethanol or aqueous acetone, each of which is known to decrease adsorptive effects[1,2]. Because with 60% aqueous alcohol Sephadex G-25 swells about half as much as with water, SOMERS[1] considered that the molecular weight of a compound completely excluded from the gel would be about 2000, assuming that a molecular weight of about 5000 is excluded with water alone. This criterion was applied to the estimation of the molecular weights of wine tannins with 50% acetone[2] as eluent, and to the polyphenols of apple peel, separated with 60% ethanol[3].

We found that the colorimetric reagent Titan Yellow (mol. wt. 695) (refs. 4 and 5) was completely excluded from Sephadex G-25 in the presence of 50% acetone, so that there seems to be no simple relationship between the extent of swelling and the molecular weight excluded. We have therefore studied the behaviour of the Sephadex G-25–aqueous acetone system.

The volumes of 50% acetone necessary to elute from the column model compounds of different types were determined. The molecular weights of compounds completely excluded were found by plotting elution volume/column bed volume ($=V_e/V_b$) against log(molecular weight). The void volume (V_0) of the column was

determined with Blue Dextran (mol. wt. 2×10^6). The minimum molecular weight for a compound to be excluded from the column is given by the value of log(molecular weight) at the point of intersection of the lines V_e/V_b and V_0/V_b (Fig. 1).

The values for minimum molecular weights excluded were much less than expected, being approximately 900, 500 and 300 for the eluents 40, 50 and 60% acetone, respectively. Confirmation of the results was sought by testing tannic acid on the column with each eluent. Tannic acid is a mixture, consisting mainly of four phenolic compounds, the relative proportions of which may vary from sample to sample: gallotannin (mol. wt. approx. 1800), trigallic acid (mol. wt. 504), *m*-digallic acid (mol. wt. 342) and gallic acid (mol. wt. 180)[6–9] (Fig. 3). As expected, 40% acetone was the only eluent that separated the components. The order in which the components were eluted was confirmed in each experiment by two-way paper chromatography.

MATERIALS AND METHODS

Model compounds

For description see key to Fig. 1. From 1 to 5 mg of the purest reagents available were dissolved in 2.0 ml of the appropriate eluent.

Tannic acid

The tannic acid used was Hopkin & Williams "B.P., $C_{76}H_{52}O_{46} = 1701.2$". Because tannic acid is a mixture, neither a formula nor a molecular weight can properly be assigned to it. A large-scale separation of the components of the sample on Sephadex G-25 indicated that its composition was: gallotannin, 76.4%; trigallic acid and *m*-digallic acid, 13.8%; and gallic acid, 9.8%.

Acetone

Acetone was analytical reagent grade.

Determination of the minimum molecular weights excluded by Sephadex G-25

The column bed of Sephadex G-25 (Fine), volume approximately 200 ml, was made in a calibrated glass chromatographic column 35 cm × 3.25 cm I.D. The gel bed was washed to constant volume (V_b) with the chosen eluent and the void volume (V_0) determined with Blue Dextran. Effluents from the column were colllected in fractions of 2.0 ml and the elution volumes (V_e) at which the compounds first appeared, were measured.

The ratios V_0/V_b and V_e/V_b were plotted against log(molecular weight). Fig. 1 shows graphs for the three eluents.

Separation of the components of tannic acid

Tannic acid (10.0 mg in 2.0 ml of the appropriate eluent) was applied to the column and eluted with aqueous acetone. After diluting where necessary, each fraction of the eluent was treated with 1 ml Folin–Denis reagent and 2 ml saturated sodium carbonate solution, and after 45 min the optical densities of the solutions were measured in 1-cm cells at 725 nm. The optical density of each fraction, corrected for the initial dilution where necessary, was plotted against its elution volume (Fig. 2).

The order in which the components were eluted was confirmed by paper chromatography of the combined and concentrated fractions constituting a peak using Whatman No. 2 chromatography paper. The solvents applied were: first way, 6% acetic acid; second way, *sec.* butanol–acetic acid–water (14:1:5). The positions of the phenols were shown by dipping the chromatograms in a mixture of equal volumes of 0.3% ferric chloride solution and 0.3% potassium ferricyanide solution.

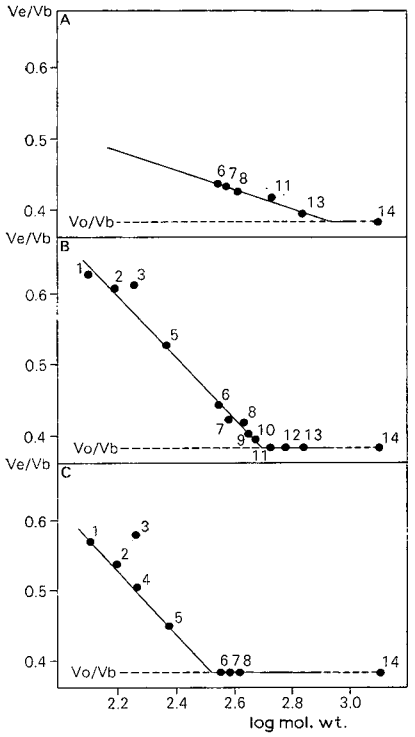

Fig. 1. Graph showing the lowest molecular weight for compounds to be excluded by Sephadex G-25 (Fine) with (A) 40%, (B) 50% and (C) 60% aqueous acetone as eluent. Model compounds: (1) pyrogallol (mol. wt. 120), (2) 2,2′-dipyridyl (mol. wt. 156), (3) gallic acid (mol. wt. 180), (4) glucose, (mol. wt. 180), (5) 4-(2-pyridylazo)resorcinol Na-salt (PAR, mol. wt. 237), (6) Phenol Red (mol. wt. 354), (7) *m*-Cresol Purple (mol. wt. 382), (8) Chlorophenol Red (mol. wt. 423), (9) Rhodamine 6G (mol. wt. 450.5), (10) Thymol Blue (mol. wt. 466), (11) Bromocresol Purple (mol. wt. 540), (12) Bromothymol Blue (mol. wt. 624), (13) Titan Yellow (mol. wt. 695), (14) Blue Dextran (mol. wt. 2×10^6). Indicators were detected by making the solutions alkaline; phenolic compounds by reacting with ferric chloride solution; 2,2′-dipyridyl was detected by adding a dilute solution of ferrous sulphate, followed by ammonia. The colorimetric reagents PAR and Titan Yellow were visible as such. Column bed volume (V_b), 187 ml; void volume (V_0), 72 ml; $V_0/V_b = 0.383$.

RESULTS AND DISCUSSION

The minimum molecular weights of the model compounds excluded by Sephadex G-25 when eluted with 40, 50 and 60% acetone are shown by the graphs of V_e/V_b

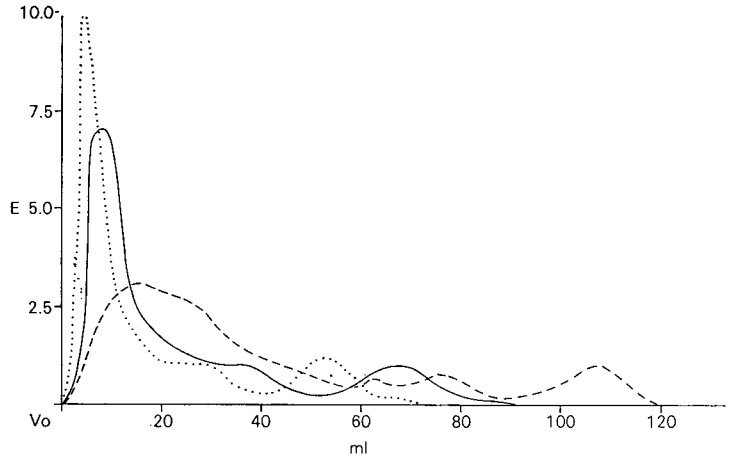

Fig. 2. Elution of the phenolic components of tannic acid with 40, 50 and 60% acetone. Optical densities shown are of the colours produced by the reaction of the Folin–Denis reagent with individual fractions. – – –, 40% acetone; —, 50% acetone; . . . . . , 60% acetone.

against log(molecular weight) to be approximately 900, 500 and 300, respectively. The values are probably within ± 50 of the true molecular weights.

As expected, an increase in the proportion of acetone in the eluent has a large effect in decreasing the limit of molecular weight for a compound to be excluded. When the eluent is 50% acetone the molecular weight limit is much less than the suggested value 2000 referred to earlier. It seems that compounds having a wide range of molecular weights greater than the limiting values might be separated on Sephadex G-25 by altering the relative proportions of acetone and water in the

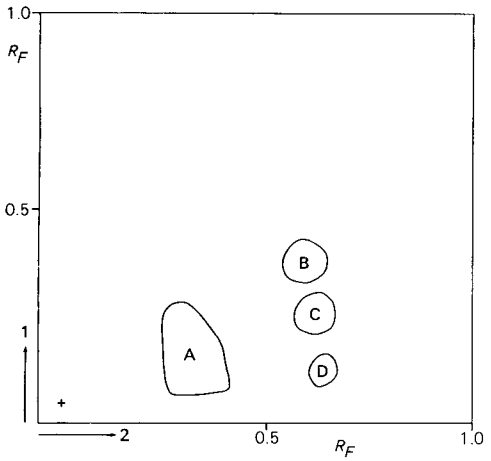

Fig. 3. Diagram of a typical two-dimensional chromatogram of tannic acid on Whatman No. 2 paper. Solvents: first way, 6% acetic acid; second way, *sec.*-butanol–acetic acid–water (14 : 1 : 5). Components were revealed by ferric chloride and potassium ferricyanide. A = gallotannin; B = gallic acid; C = *m*-digallic acid; D = trigallic acid.

eluent. Pharmacia Fine Chemicals (Uppsala, Sweden), manufacturers of Sephadex gels, give a fractionation range of 1000–5000 for peptides and globular proteins, and 100–5000 for dextrans separated on Sephadex G-25 (Fine) with water as eluent. Thus, by varying the proportions of water and acetone in the eluents, compounds of molecular weights between 900 and 5000 should be excluded. However, it is clear that the extent of adsorption increases as the proportion of acetone is decreased, particularly with polyphenols; the separation of the phenolic components of tannic acid with 40% acetone is favoured by adsorption. Paper chromatography of the separate peaks shows that gallotannin trails back slightly into trigallic acid, but a re-run of the almost pure trigallic acid fraction would separate the components completely. By choosing a suitably large column, and by a moderate increase of the quantity of tannic acid applied, the individual components can be isolated on a preparative scale.

The limits of molecular weights excluded with 50 and 60% acetone seem to be about 500 and 300, respectively, because the components of tannic acid are not separated by these eluents. Trigallic acid (mol. wt. 504) is masked by gallotannin in 50% acetone, and as shown by paper chromatography both trigallic acid and *m*-digallic acid (mol. wt. 342) are eluted with gallotannin by 60% acetone. Gallic acid was separated from the other components, with the eluents studied due not only to its small molecular weight, but chiefly to its adsorption by the gel.

Although these results indicate that Sephadex G-25 eluted with aqueous acetone behaves as a molecular sieve, the possibility was investigated that the compounds might also have been separated by partition chromatography, where the stationary phase could be considered as a water-rich phase, and the mobile eluent as an acetone-rich phase.

The model compounds, singly and in mixtures, were thus chromatographed on Whatman No. 1 paper, and on columns of cellulose, with 40, 50 and 60% acetone as eluents. The R_F values of all of the compounds on paper were between 0.9 and 1.0, and it was not possible to separate from one another the components of any mixture.

Chromatographing tannic acid with the above solvents again showed that no separation into its constituents had taken place. After column chromatography, all of the effluent fractions showed the presence of gallotannin, trigallic acid, *m*-digallic acid and gallic acid. In no case did the attempted separations take the same course as those of the experiments with Sephadex, and it would seem, therefore, that it is unnecessary to attempt to correct for a partition effect when assessing the results.

By using Sephadex G-25 with the eluents 40, 50 and 60% acetone, compounds can be separated into groups covering increasing molecular weight ranges, *i.e.* (a) up to 300, (b) from 300 to 500, (c) from 500 to 900, and (d) greater than 900. Having removed unwanted substances from one group, it is possible to separate the components of that group by re-running them on Sephadex G-25.

The method would seem to have useful possibilities in the separation of groups of components of plant extracts containing much polyphenolic material, *e.g.* vegetable tannins, in which many of the constituents, particularly those immobile in organic chromatographic solvents, and unresolved on paper chromatograms, are usually referred to as of high molecular weight. Our experience with Sephadex G-25 and

50% acetone indicates that a considerable amount of paper chromatographically unresolved polyphenolic material is not excluded from the gel, suggesting that the molecular weights of the components of such material may be less than 500.

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CHROM. 4910

A COMPARISON OF THIN-LAYER CHROMATOGRAPHIC ADSORBENTS, SUPPORTS AND DEVELOPING UNITS

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SUMMARY

Development of available silica gel adsorbents applied on glass plates, plastic films or glass fibers, yields reliable chromatographic data regardless of whether a glass tank, a sandwich unit, or a Gelman chamber is used. Depending on the type of sample and the user's need the presented data serve as a guide for the selection of a suitable system for a particular use.

INTRODUCTION

Those who use thin-layer chromatography are periodically importunated to discard the adsorbents they are currently using. They are urged to try a new, more versatile adsorbent, which, if used, allegedly will yield better chromatograms. In addition, they continually debate the choice of a developing unit and the optimal conditions for development of the chromatogram. Much of the data which follow were collected so that reasonable answers to these three questions could be obtained. Acid and basic drugs were chromatographed using many different adsorbents, developing units, and conditions for development. The data collected in these experiments were tabulated and are the basis for the subsequent discussion and conclusions.

EXPERIMENTAL

Equipment

Developing units. The following units were employed: (a) Developing tanks: 21 × 11 × 21 cm, glass, with ground glass lip and matching lid (hereafter referred to as *tanks*). (b) Chromatogram developing apparatus: sandwich unit introduced by the Eastman Kodak Co. (hereafter referred to as *trough*). (c) ITLC chromatography chamber: chromatographic chamber developed by the Gelman Instrument Company (hereafter referred to as *chamber*).

Plate-coating unit: A Desaga apparatus designed to coat five 20 × 20 cm plates was used.

Chemicals

All chemicals used for developing solutions and spray reagents were analytical grade. All drugs were obtained from cooperating drug manufacturers and were pharmaceutical grade chemicals.

Developing systems. The following systems were applied: (a) chloroform–acetone (9:1), (b) Davidow's, *i.e.* ethyl acetate–methanol–conc. ammonium hydroxide (85:10:5), (c) CBD, *i.e.* cyclohexane–benzene–diethylamine (75:15:10), and (d) 95 % ethanol.

Drug reference solutions. The acids were prepared as follows: 40 mg of the free acid were dissolved in 20 ml of absolute ethanol (sodium diphenylhydantoin being the only exception). For the preparation of the bases 40 mg of the salt were dissolved in 20 ml of absolute ethanol. If the salt was insoluble in ethanol, then the sample was dissolved in a minimal volume of 0.1 *N* sulfuric acid. This solution was then diluted to 20 ml with absolute ethanol.

Detection reagents. For the preparation of mercuric sulfate 5 g of mercuric oxide were suspended in 100 ml of distilled water. To this 20 ml of concentrated sulfuric acid were added with continuous mixing. The resulting mixture was cooled and diluted to 250 ml with distilled water.

The Dragendorff reagent consisted of 10 ml of a solution A, 10 ml of a solution B, 20 ml of glacial acetic acid and 100 ml of water. For the preparation of solution A 2.125 g of bismuth subnitrate were dissolved in 25 ml of glacial acetic acid and 100 ml of water; for solution B, 50 g of potassium iodide were dissolved in 125 ml of water.

The sodium nitrite reagent was prepared by dissolving 5 g in 100 ml of water.

Adsorbents. The adsorbents applied were: pre-coated glass plates, supplied by Brinkmann Instruments, Inc., Laboratory Products Department, Corning Glass Works, and Analtech, Inc.; pre-coated films, supplied by Brinkmann Instruments, Inc. and Eastman Kodak, Inc.; impregnated glass fiber, type SA, SAF of Gelman Instrument Co.; and glass-coated plates prepared in the laboratory from the following silica gel powders: Merck G, Corning C_3 (with binder), Corning C_5 (without binder), Merck GF, Merck HF, Silicar 7G, Silicar 7GF, Silicar 4GF, Camag GF, Whatman G41, Anasil B, Anasil S and Adsorbosil-2.

Procedure

Preparation of glass-coated plates

20 g of adsorbent were suspended in 50 ml of water and stirred until the gel appeared to be setting. Using a Desaga apparatus, this slurry was applied to five 20 × 20 cm glass plates. The plates were allowed to set at room temperature. After the coating had set, the plates were stored in a constant temperature (70° F) and constant humidity (40 %) room. Prior to sample application the plates were scribed (Chemical Rubber Company Scriber, Catalog No. 8705/975).

Spotting

All chromatograms were spotted with 3–4 μl of sample to give a 7.5-mm spot.

TABLE I

$R_F \times 100$ VALUES OF ACID AND NEUTRAL DRUGS — DEVELOPMENT IN SATURATED TANKS

	Amobarbital	*Phenobarbital*	*Pentobarbital*	*Secobarbital*	*Diphenyl-hydantoin*	*Glutethimide*	*Range*	*Median*
	Solvent: chloroform–acetone							
Pre-coated plates								
Brinkmann	18	14	21	24	12	41	29	20
Corning	20	13	20	25	16	42	29	20
Analtech	34	24	33	37	21	52	31	34
Self-coated plates								
Merck G	29	21	31	33	17	47	30	30
Corning C_3	20	12	19	25	15	44	32	20
Corning C_5	25	15	24	28	15	40	25	25
Films								
Kodak	33	24	36	36	20	49	29	35
Brinkmann	25	17	26	30	14	44	30	26
Sheet								
Gelman SA	65	53	63	69	46	78	32	64
	Solvent: Davidow's							
Pre-coated plates								
Brinkmann	43	20	46	47	45	76	52	46
Corning	56	37	62	69	69	89	52	66
Analtech	51	30	55	66	54	84	54	55
Self-coated plates								
Merck	55	32	58	59	57	84	52	58
Corning C_3	52	26	53	60	60	86	60	57
Corning C_5	61	33	65	66	66	92	59	66
Films								
Kodak	66	43	67	67	66	80	37	67
Brinkmann	50	20	58	67	63	94	74	63
Sheet								
Gelman SA	69	53	73	73	70	100	47	72

TABLE II

$R_F \times 100$ VALUES OF BASIC DRUGS — DEVELOPMENT IN SATURATED TANKS

	Chlorcyclizine	*Methaphenyline*	*Quinine*	*Propoxyphene*	*Carbinoxamine*	*Pheniramine*
	Solvent: Davidow's					
Pre-coated plates						
Brinkmann	64	57	67	71	55	50
Corning	84	71	84	93	68	59
Self-coated plates						
Merck G	78	81	76	84	60	61
Corning C_3	57	100	83	100	89	87
Films						
Kodak	66	66	56	69	64	62
Brinkmann	86	85	70	91	69	68
Sheet						
Gelman SA	88	90	71	92	81	79
	Solvent: CBD					
Pre-coated plates						
Brinkmann	41	47	03	32	30	26
Corning	40	46	02	57	20	24
Analtech	67	51	03	63	25	42
Self-coated plates						
Merck	59	63	03	70	40	50
Corning C_3	56	60	02	63	37	36
Corning C_5	60	63	02	82	38	52
Films						
Kodak	61	62	16	66	52	57
Brinkmann	57	59	02	73	37	46
Sheet						
Gelman SA	74	76	10	81	57	70

TABLE III

$R_F \times 100$ VALUES OF BASIC DRUGS ON VARIOUS ADSORBENTS — DEVELOPMENT IN SATURATED TANKS

Solvent: 95% ethanol.

	Antistine	*Cyclomethycaine*	*Clemizole*	*Covatin*	*Chlorcyclizine*	*Meclizine*	*Diatrine*	*Quinine*
Self-coated plates								
Merck G	14	19	61	35	31	72	10	22
Merck GF	11	14	58	25	21	65	15	20
Merck HF	13	15	57	27	22	68	15	26
Silicar 7G	32	25	58	46	32	74	40	26
Silicar 7GF	64	27	65	46	35	76	13	26
Silicar 4GF	63	34	64	64	61	78	58	18
Camag GF	11	10	46	26	17	58	18	33
Whatman G41	11	13	51	26	21	61	22	18
Anasil B	02	04	71	32	31	74	24	24
Anasil S	02	03	71	29	28	75	22	24
Adsorbosil-2	17	37	58	38	32	68	33	38
Sheet								
Gelman SA	13	18	75	43	43	80	15	31
Film								
Kodak	27	37	63	49	43	86	15	35

Clemizole	*Methamino-diazepoxide*	*Acetyl-phenazine*	*Carphenazine*	*Desmethyl-imipramine*	*Range*	*Median*
70	81	52	58	66	31	64
92	89	80	73	85	33	83
81	89	56	61	67	35	71
57	87	71	82	82	43	82
67	66	50	53	53	17	64
89	66	60	77	77	45	77
87	84	65	69	73	21	81
30	02	03	03	18	49	18
22	01	00	01	22	57	20
37	01	03	04	24	66	24
42	07	05	07	32	67	42
31	03	02	02	20	70	31
45	01	04	04	27	83	27
52	11	21	26	43	51	46
40	02	03	05	24	73	21
63	08	09	12	38	74	48

Pipradol	*Amol-anone*	*Azacy-clonone*	*Propoxy-phene*	*Levallor-phan*	*Benzocaine*	*Pyrathi-azine*	*Range*	*Median*
61	52	10	44	40	71	18	62	40
25	36	14	31	30	65	12	54	23
33	40	13	33	30	67	10	58	27
66	56	58	54	40	74	25	49	48
66	58	64	58	45	74	30	63	52
72	71	71	64	54	78	35	60	64
22	33	30	31	24	55	12	48	25
23	36	31	32	28	60	13	50	27
07	47	03	28	34	nd	05	72	26
nd	47	02	26	31	nd	08	73	25
60	48	55	48	53	66	29	51	48
79	57	08	49	44	77	22	72	44
49	57	23	54	44	52	27	71	43

Development

Tanks. "Saturated" means that two 4 × 20 cm blotter pads were placed at each end of the tank. Of the developing solution 100 ml were poured over the pads and into the bottom of the tank 1 h prior to developing the chromatogram. "Non-saturated" means that no blotter pads were present in the tank. The chromatogram was developed by placing it in the tank immediately after the solvent had been added.

Trough. The chromatogram was spotted and placed between the glass plates. The ground glass edges of the plates were coated with a thin film of petrolatum prior to use. The plates were then fastened together and the sandwich unit was inserted through the slit in the cover of the trough which had been filled with 100 ml of the developing solution.

Chamber. "Non-equilibrated" means that the chromatogram was developed immediately. With the chamber in an upright position, 25 ml of developing solvent were placed over the saturation pad in the center compartment of the chamber. Another 60 ml were placed in the bottom compartment. The spotted chromatogram was then placed in the chamber, the cover glass placed in position and the glass cover was sealed with Scotch tape. (Sheets and films were held in place by magnetic clips.) "Equilibrated" means that the chromatogram was placed in the chamber for 1 h prior to development of the chromatogram. During this period the chamber was horizontal, the developing solvent was in the bottom well and the pad was saturated with this solvent. The spotted chromatogram was placed in the chamber so that the solvent did not come into contact with the support. (Sheets and film were held in place by magnetic clips.) The chamber was closed with its glass cover and sealed with Scotch tape. After 1 h the chamber was placed in an upright position allowing the solvent to come into contact with the adsorbent. The chamber was secured in this upright position by the support rods of the unit.

RESULTS

The following tabulations summarize the results of many chromatographic determinations made to explore the effects of adsorbents, supports, developing chambers and operating conditions on the separation of acidic and basic drugs. Each datum represents the average of three replicate determinations. These were within ± 0.05 of the average. All R_F values whose difference was less than 0.05 were considered to be the same.

The "range" and "median" were determined for each chromatogram. "Range" refers to the difference in the R_F values between the lowest and highest spots. "Median" refers to the median R_F value. The "range" is used to assess the resolution of the system and the "median" the relative position of the band of substances on the chromatogram.

To evaluate the influence of adsorbents and supports on R_F values, the data presented in Tables I–III were accumulated.

Using different silica gel adsorbents, chromatographic data were obtained for non-basic drugs. These were developed in saturated tanks using chloroform–acetone or Davidow's solvent (Table I). In each of these developing solvents all the silica gel plates gave similar ranges and medians with the exception of the lower median obtained on one plate in Davidow's solvent. In chloroform–acetone, the ranges and

TABLE IV

$R_F \times 100$ VALUES OF ACID AND NEUTRAL DRUGS — DEVELOPING UNITS AND CONDITIONS OF DEVELOPMENT

	Amobarbital	*Phenobarbital*	*Pentobarbital*	*Secobarbital*	*Diphenyl-hydantoin*	*Glutethimide*	*Range*	*Median*
	Solvent: chloroform–acetone							
Saturated tanks								
Merck G							30	30
Gelman SA	See Table I						32	64
Kodak film							29	35
Unsaturated tanks								
Merck G	74	62	79	84	52	89	37	77
Gelman SA	79	61	81	86	49	92	41	83
Kodak film	76	51	75	78	62	83	32	76
Gelman chamber — non-equilibrated								
Merck G	54	36	53	62	29	73	44	54
Gelman SA	62	51	61	66	44	74	30	62
Kodak film	50	38	49	51	33	62	29	49
Gelman chamber — equilibrated								
Merck G	37	28	38	44	25	58	33	38
Gelman SA	62	52	61	66	47	75	28	62
Kodak film	37	26	38	42	26	50	24	38
Kodak trough								
Kodak film	55	41	53	56	33	77	44	54
	Solvent: Davidow's							
Saturated tanks[a]								
Merck G							52	58
Gelman SA							47	70
Kodak film							37	67

[a] Data for other than saturated tanks are not recorded because they gave R_F values which precluded determining valid range and median values.

medians on the two films were comparable to those on the plates. In Davidow's system both films gave medians comparable to those on the plates but the Brinkmann film gave the best resolution of any of the adsorbents studied. In both systems the ranges on the Gelman SA sheets were comparable to those on the silica gel plates, but the medians were higher.

Some basic compounds were developed in saturated tanks using Davidow's and

TABLE V

$R_F \times 100$ VALUES OF BASIC DRUGS — DEVELOPING UNITS AND CONDITIONS OF DEVELOPMENT

	Chlor-cyclizine	*Metha-phenylene*	*Quinine*	*Prop-oxyphene*	*Carbin-oxamine*	*Phenir-amine*
	Solvent: Davidow's					
Saturated tanks						
Merck G						
Gelman SA	See Table II					
Kodak film						
Unsaturated tanks						
Merck G	93	94	67	97	84	87
Gelman SA	93	87	49	92	71	73
Kodak film	90	91	86	92	91	90
Gelman chamber — non-equilibrated						
Merck G	85	82	56	94	74	66
Gelman SA	87	85	66	93	77	75
Kodak Film	73	74	60	74	72	71
Gelman chamber — equilibrated						
Merck G	85	88	59	91	71	67
Gelman SA	76	77	58	84	69	66
Kodak film	72	73	59	75	75	63
Kodak trough						
Kodak film	40	42	53	97	64	65
	Solvent: CBD					
Saturated tanks						
Merck G						
Gelman SA	See Table II					
Kodak film						
Unsaturated tanks						
Merck G	77	70	02	90	39	54
Gelman SA	81	74	06	85	53	65
Kodak film	80	84	04	91	56	70
Gelman chamber — non-equilibrated						
Merck G	54	56	06	80	38	49
Gelman SA	67	61	05	72	45	56
Kodak film	61	61	09	61	52	57
Gelman chamber — equilibrated						
Merck G	43	44	03	61	21	31
Gelman SA	58	54	04	63	41	49
Kodak film	54	55	08	51	40	46
Kodak trough						
Kodak film	87	88	13	88	61	67

CBD solvents (Table II). For plates developed in Davidow's solvent the best resolution was obtained on the Corning C_3 adsorbent. In the CBD solvent, the Corning C_5 was best. In both solvents the Brinkmann plate gave the lowest medians. The ranges and the medians on the Brinkmann film in both solvents were higher than those on the Brinkmann plate. In both solvents the Kodak film had the smallest range and in CBD it had the highest median. The range and median on the Gelman SA sheet in

Clemizole	*Methamino-diazepoxide*	*Acetyl-phenazine*	*Carphenazine*	*Desmethyl-imipramine*	*Range*	*Median*
					35	71
					21	81
					19	75
97	79	51	56	62	46	87
84	82	46	54	59	47	82
90	89	77	81	84	15	90
90	92	47	56	57	47	81
92	86	61	68	68	32	82
76	66	57	61	63	21	72
85	86	49	55	54	42	71
80	80	57	62	62	27	76
69	77	55	56	67	22	72
93	96	54	60	57	44	65
					67	42
					74	48
					51	46
52	62	05	04	28	88	52
65	64	08	11	44	75	65
59	78	04	11	37	86	62
45	—	07	08	29	74	42
51	60	07	09	32	67	56
48	59	16	18	34	52	52
40	35	03	05	15	58	26
47	—	08	09	34	54	47
40	43	10	13	26	47	45
60	72	18	22	46	75	61

Davidow's system were about the same as those of the Kodak film. In CBD the range on the sheet was similar to that on the plates but the median was higher than those on any plate or film.

Other basic drugs were developed in saturated tanks using 95 % ethanol (Table III). In this solvent the best resolution was obtained on the Anasil B and S plates and the Gelman SA sheet. The median was highest on Silicar 4GF but varied considerably on all the other adsorbents.

Using plates, films, and sheets, the influence of varying developing conditions on R_F values was investigated (Tables IV–VI). To facilitate the evaluation of these data they were collated and summarized in Table VII.

For each developing solvent, the ranges and medians on plates, sheets and films were usually higher in unsaturated tanks than in saturated tanks. In chambers after equilibration development of plates with the CBD, Davidow or chloroform–acetone system gave R_F values which were lower than those obtained following immediate development. In these three solvents, sheets and films gave the same R_F values when they were developed either immediately or after 1 h of equilibration. This same phenomenon pertained to all chromatograms developed in 95 % ethanol, whatever the supporting material was.

In tanks, saturated or unsaturated, the ranges on sheets and plates were generally comparable to and higher than those on the films. The highest median values were usually obtained on the sheets. In chambers, the ranges on sheets and films were less than those obtained on plates. Those on the film were generally slightly lower than

TABLE VI

$R_F \times 100$ VALUES OF OTHER BASIC DRUGS — DEVELOPING UNITS AND CONDITIONS OF DEVELOPMENT
Solvent: 95% ethanol.

	Antistine	*Cyclomethycaine*	*Clemizole*	*Covatin*	*Chlorcyclizine*	*Meclizine*	*Diatrine*	*Quinine*
Saturated tank								
Merck G								
Gelman SA	See Table I							
Kodak film								
Unsaturated tank								
Merck G	13	29	66	43	31	73	21	31
Gelman SA	13	17	74	42	42	81	13	28
Kodak film	32	37	71	54	41	83	15	28
Gelman chamber — non-equilibrated								
Merck G	12	22	65	33	29	75	24	25
Gelman SA	13	24	73	40	35	81	24	29
Kodak film	26	49	68	58	52	70	50	43
Gelman chamber — equilibrated								
Merck G	13	21	65	34	25	72	09	23
Gelman SA	08	22	74	43	48	80	08	30
Kodak film	30	44	62	52	46	65	45	39
Kodak trough								
Kodak film	39	64	97	79	73	95	71	62

those on the sheet. Median values were generally highest on the SA sheet. With the exception of Davidow's system, ranges on the film developed in the trough were usually comparable to those on plates and sheets developed in unsaturated tanks and, therefore, higher than those obtained in chambers, equilibrated or non-equilibrated. However, the medians on the films were higher. Following development of Kodak film in the trough, resolution was comparable to that obtained on plates or sheets in saturated tanks.

Whichever adsorbent and support was used, it took longer to develop the chromatogram in unsaturated tanks and in a sandwich cell than in the saturated tank or in a chamber[1]. The time required for a 10-cm development in each of the solvent systems used is given in Table VIII.

DISCUSSION OF RESULTS

Available silica gel adsorbents applied on glass plates, plastic films or glass fibers yield reliable chromatographic data regardless of the developing unit used. In a given laboratory with controlled humidity and temperature the R_F value can be reproduced[2] to within ± 0.05. No one adsorbent was optimal for all applications and no generalization as to selection for a particular application seems appropriate.

In saturated tanks, all the plates gave comparable ranges and medians. Supporting a given adsorbent on a film rather than on a plate resulted in comparable ranges but higher medians. The highest medians on all supports were those obtained

Pipradol	*Amol-anone*	*Azacy-clonone*	*Propoxy-phene*	*Levallor-phan*	*Benzocaine*	*Pyrathi-azine*	*Range*	*Median*
							62	40
							72	44
62	54	19	46	31	75	21	62	43
75	57	08	46	41	76	23	73	42
14	61	23	52	43	29	57	68	41
60	56	10	42	41	73	16	65	33
41	54	13	49	44	81	22	68	41
55	63	22	51	43	67	60	48	51
59	54	14	39	41	70	17	63	34
40	58	07	53	43	81	23	74	43
50	59	26	53	33	63	53	45	53
76	92	33	85	61	98	85	65	76

TABLE VII

DEVELOPING UNITS AND CONDITIONS OF DEVELOPMENT — SUMMARY OF DATA IN TABLES IV–VI

R = range; M = median.

	Tank				*Chamber*				*Trough*	
	Unsaturated		*Saturated*		*Non-equilibrated*		*Equilibrated*			
	R	*M*	*R*	*M*	*R*	*M*	*R*	*M*	*R*	*M*
Acids developed in chloroform–acetone										
Plate	37	77	30	30	44	54	33	38	—	—
Film	32	76	29	35	29	49	24	38	44	54
Sheet	41	83	32	64	30	62	28	62	—	—
Acids developed in Davidow's system										
Plate	—	—	52	58	—	—	—	—	—	—
Film	—	—	37	67	—	—	—	—	—	—
Sheet	—	—	47	72	—	—	—	—	—	—
Bases developed in Davidow's system										
Plate	46	87	35	71	47	81	42	71	—	—
Film	15	90	17	64	21	72	22	72	44	65
Sheet	47	82	21	81	32	82	27	76	—	—
Bases developed in CBD										
Plate	88	52	67	42	74	42	58	26	—	—
Film	86	62	51	46	52	52	47	45	75	61
Sheet	75	65	74	48	67	51	59	47	—	—
Bases developed in 95% ethanol										
Plate	62	43	62	40	65	33	63	34	—	—
Film	68	41	71	43	48	51	45	53	65	76
Sheet	73	42	72	44	68	41	74	43	—	—

TABLE VIII

TIME FOR 10-CM DEVELOPMENT

	Solvent system			
	Chloroform–acetone (9:1)	*CBD*	*Davidow's*	*95% ethanol*
Plate				
Unsaturated tank	33	43	35	63
Saturated tank	19	37	26	48
Sheet				
Unsaturated tank	46	55	43	106
Saturated tank	25	27	30	75
Film				
Unsaturated tank	50	55	65	108
Saturated tank	35	35	48	102
Plate				
Non-equilibrated chamber	34	28	35	60
Equilibrated chamber	25	28	23	42
Sheet				
Non-equilibrated chamber	33	32	30	80
Equilibrated chamber	30	30	26	70
Film				
Non-equilibrated chamber	48	48	47	110
Equilibrated chamber	41	45	40	104
Film				
Trough	55	50	50	103

on the sheets. The ranges on the sheets were equivalent to those on plates and films.

For a particular application, one adsorbent may be selected because its range or median is desirable. If extracts of normal biological samples are known to contain "artifacts" whose R_F values are very low and very high, a suitable system can be selected whose median is between these values. This selection would simplify the interpretation of the results because the spots due to the artifacts would not be in the same region as those due to any drugs.

The three developing units gave satisfactory data. Other reports also indicate that the development of drugs in unsaturated tanks gives a higher median and greater resolution than does the development in saturated tanks[3–5]. For plates, films, and sheets, development of drugs in a chamber generally results in lower medians and ranges than those obtained in unsaturated tanks. In the chambers, the lower ranges and medians obtained following immediate development indicate that the adsorbent is more saturated under these conditions than it is in an unsaturated tank, whereas after 1 h of equilibration in the chamber results are comparable to those obtained in a saturated tank.

Although originally designed for sheets, the sealed Gelman chamber was found to be satisfactory for developing plates and films. In this chamber the SA sheet and Kodak film are apparently rapidly saturated because equilibrating the units in the chamber for 1 h gave R_F data identical to those obtained when the chromatograms were developed immediately.

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QUANTITATIVE THIN-LAYER CHROMATOGRAPHIC ESTIMATION OF LABELED DOPAMINE AND NOREPINEPHRINE, THEIR PRECURSORS AND METABOLITES*

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SUMMARY

A simple quantitative separation method for combinations of sixteen ^{14}C- and ^{3}H-labeled catecholamines, their precursors and metabolites has been devised. The method uses precoated cellulose plates, diazotized *p*-nitroaniline as a spray detection agent and quantitates the labeled compounds by means of a liquid scintillation counter. The efficiency of the method was tested by examining differences of *in vitro* and *in vivo* metabolism of ^{14}C-labeled dopamine by control and enzyme-inhibited caudate nucleus.

INTRODUCTION

Thin-layer chromatographic (TLC) methods have been described for the separation and detection of catechol biogenic amines which permit rapid isolation and identification of small amounts of these compounds. However, the methods available are limited in the number of amines, their metabolites and precursors that can be examined and some are not suitable for use with radioisotopes[1–6].

This report, an extension of our previous publication[7], concerns a new two-dimensional TLC separation of a combination of the following compounds: DA, DHMA, DHPG, DOPA, DOPAC, EPI, HVA, MET, MHPG, 3MT, NE, NMN, OCT, TYM, TYR, and VMA**. The system uses precoated cellulose plates and allows a simultaneous, quantitative estimation of unconjugated ^{14}C- and ^{3}H-labeled compounds by means of a liquid scintillation counter. The method has been successfully applied to the analysis of DA metabolism in tissues such as: biopsies or tissue cultures of caudate nucleus and substantia nigra from rats, brain biopsies from patients

* A preliminary report was presented at Western Pharmacology Society Meeting, San Diego, Calif., U.S.A., January 1970.

** DA = dopamine, DHMA = 3,4-dihydroxymandelic acid; DHPG = 3,4-dihydroxy-phenylglycol; DOPA = 3,4-dihydroxyphenylalanine; DOPAC = dopacetic acid; EPI = L-epinephrine; HVA = homovanillic acid; MET = metanephrine; MHPG = 3-methoxy-4-hydroxyphenylglycol; 3MT = 3-methoxytyramine; NE = L-norepinephrine; NMN = normetanephrine; OCT = octopamine; TYM = tyramine; TYR = tyrosine; VMA = 3-methoxy-4-hydroxymandelic acid.

with Parkinson's disease and drug-induced dyskinesia models in rats and monkeys, all of which were incubated with labeled substrates. Thus it provides a simple radiometric assay for several enzymes involved in amine metabolism.

MATERIALS AND METHODS

Reference standards

The unlabeled reference standards were all from commercial sources and dissolved in 0.01 *N* HCL to make concentrations of 1 mg/ml. The μg amounts usually spotted on the chromatoplates were: DA (1), DHMA (1), DHPG (2.5), DOPA (0.5), DOPAC (1), EPI (1), HVA (1), MET (0.5), MHPG (1), 3MT (1), NE (1), NMN (0.5), OCT (0.5), TYM (1), TYR (1), and VMA (0.5). The ^{14}C-labeled DA (38 mCi/mmole) and ^{3}H-labeled DA (280 mCi/mmole) were purchased from Nuclear Chicago and dissolved in 0.01 *N* HCl.

Precoated plates

The 20 × 20 cm plates used were Avicel microcrystalline cellulose powder, glass backing, without fluorescent indicator (Merck, Darmstadt, distributed by Brinkmann Instrument Co., Westbury, N.Y., U.S.A.), 80 μ thick, or Cellulose Chromagram Sheet No. 6064, plastic backing, without fluorescent indicator (Eastman Co., Rochester, N.Y.), 160 μ thick. The Brinkmann plates were superior in quality and gave more uniform separation.

Solvents and solutions

All solvents and solutions used were analytical grade; 1-butanol, absolute methanol, and chloroform were obtained from Baker, ammonium hydroxide, formic acid, and glacial acetic acid from Mallinckrodt.

Detection reagent

DPNA* prepared from (a) *p*-nitroaniline (Eastman) 0.1 g dissolved in 2 ml concentrated HCl and diluted to 100 ml with water, (b) $NaNO_2$ (Mallinckrodt) 0.2 g dissolved in 100 ml water, and (c) K_2CO_3 (Mallinckrodt) 10 % solution, 20 g dissolved in 200 ml water. Equal volumes (usually 1 ml each) *p*-nitroaniline and $NaNO_2$ were placed in a beaker packed in ice. After 10 min 2 vol. K_2CO_3 solution were added to the beaker and mixed. The spray was applied to the plate using a small atomizer at a distance of 10 cm until the plates appeared translucent. Excess of reagent was avoided to preclude any diffusion of the spot and radioactivity.

Counting fluorophore

The scintillation counting fluorophore consisted of (a) naphthalene (100 g) scintillation grade, (b) PPO* (5 g), (c) POPOP* (0.2 mg) (all Packard Instrument Co.), and (d) *p*-dioxane (1 l) spectroquality (Mathison, Coleman and Bell). In a refrigerated counter the fluorophore had a tendency to freeze after several hours at the reduced temperature of the freezer.

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* DPNA = diazotized *p*-nitroaniline; PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-(5-phenyloxazolyl-2)-benzene.

Developing tanks

Glass, 10.75 × 9.25 × 2.75 in. inside dimensions, with lid (Kensco). Solutions of the sixteen reference standards were applied with a capillary to the cellulose chromatoplate at a point 1.5 cm from the base. The plates were developed in 2 dimensions with the solvent systems 1-butanol–methanol–1 *N* formic acid (60:20:20) in direction 1 and chloroform–methanol–1 *N* ammonium hydroxide (60:35:5) in direction 2. Between directions and after development, the plate was dried with a hair drier using warm air. The time for ascending development varied with the solvent system used, 3.5–4 h in direction 1 (plate layered direction) and 1.5–2 h in direction 2, for a height of 13 cm from the origin. The reference standards developed characteristic colors after exposure to DPNA spray. Lining the tank walls with filter paper moistened with solvent or saturating the chamber atmosphere did not enhance the separation.

When labeled compounds were used in this procedure the basic separation conditions were the same. However, radioactive standards or tissue extracts containing labeled material were applied to the origin along with the cold standards. After the separation and identification of spots with DPNA, the standard colored areas were scraped off and placed in individual counting vials containing 0.5 ml water. The scraped powder was gently swirled in the water and then 10 ml counting fluorophore was added and the samples were counted in a Packard liquid scintillation counter. The elution of the radioactivity from the cellulose into the fluorophore was 100 %.

RESULTS AND DISCUSSION

Separation and recovery

The R_F values of each compound and the spray-developed colors are presented in Table I. The values were obtained at 22° and represent the center of the spot. The separation of DA, its precursors and metabolites, is shown in Fig. 1. The spots remained small and round and there was no trailing of any of the standards. The amino acid DOPA has the lowest R_F value, the amines intermediate values and the acid metabolites the highest R_F values. A similar separation was observed for NE and its metabolites, with the acid and glycol metabolites having the highest R_F values (Fig. 2). Eleven compounds of interest in Parkinson's disease, drug-induced dyskinesias, and DOPA therapy can be separated well using this method (Fig. 3). However, it is difficult to separate DA from EPI and 3 MT from MET if these NE metabolites are present in a DA sample.

The method was examined for quenching effect and extraction recovery after separation. Because the labeled derivatives of many of the compounds in this study were not available, quenching was tested by adding 1000 c.p.m. each of ^{14}C-DA and ^{3}H-DA to the sixteen unlabeled reference standards that had been spotted on TLC in 32 separate 1 × 1 cm squares in amounts of 2–10 μg. After spraying and scraping, the individual samples were counted. The presence of the DPNA-sprayed standards did not appreciably affect the ^{14}C-DA since there was less than an average 5 % quenching of the sixteen compounds. However, the ^{3}H-DA was quenched an average of 63 %. Thu sthe present spraying and counting technique has limitations when using ^{3}H. Exposure of the developed plate to paraformaldehyde

TABLE I

R_F VALUES AND COLOR DEVELOPMENT OF REFERENCE STANDARDS

Solvents: direction 1, 1-butanol–methanol–1 *N* formic acid (60:20:20); direction 2, chloroform–methanol–1 *N* ammonium hydroxide (60:35:5).

Reference standard	*R_F value*		*Color after spraying with DPNA*
	Direction 1 (4 h)	*Direction 2 (2 h)*	
DA, its precursors and metabolites (see Fig. 1)			
DOPA	0.22	0.02	Magenta
TYR	0.38	0.08	Pink
DA	0.45	0.24	Magenta
OCT	0.50	0.31	Red
3MT	0.55	0.58	Blue
TYM	0.60	0.52	Pink
DOPAC	0.82	0.07	Magenta
HVA	0.90	0.34	Blue
NE, its precursors and metabolites (see Fig. 2)			
NE	0.35	0.12	Magenta
EPI	0.40	0.25	Magenta
NMN	0.45	0.41	Purple
MET	0.49	0.60	Purple
DHMA	0.60	0.05	Magenta
VMA	0.72	0.17	Purple
DHPG	0.68	0.56	Magenta
MHPG	0.68	0.71	Purple

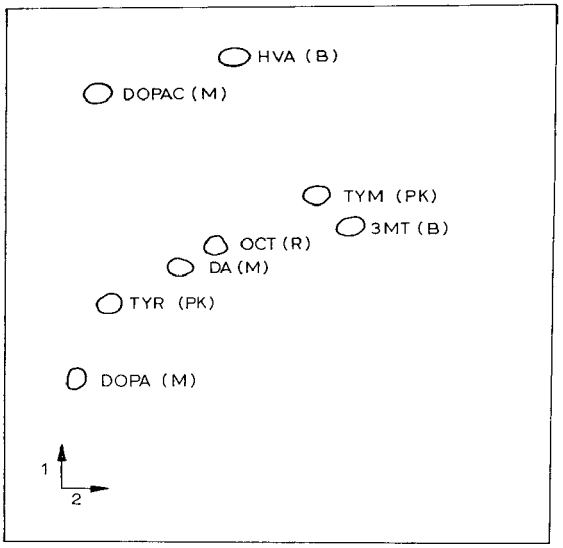

Fig. 1. Separation of DA, its precursors and metabolites. Plate: Eastman Cellulose No. 6064, without fluorescent indicator (160 μ), or Brinkmann microcrystalline cellulose (80 μ). Time: direction 1, 3.5–4 H; direction 2, 1.5–2 H. Solvents: direction 1, 1-butanol–methanol–1 *N* formic acid (60:20:20); direction 2, chloroform–methanol–1 N ammonium hydroxide (60:35:5). Spray: DPNA. Color development: M = Magenta, PK = pink, R = red, B = blue, and P = purple

gas and UV to visualize the standards by fluorescence[7] and/or autoradiography to locate the ^{3}H-labeled material can be used to eliminate the limitations caused by the quenching effect.

The elution recovery of pure ^{14}C-DA, ^{14}C-DOPA, and ^{14}C-NE standards separated and developed on TLC was uniformly 90 % or better. Autoradiography was used to check the efficacy of scraping only the spray-developed reference

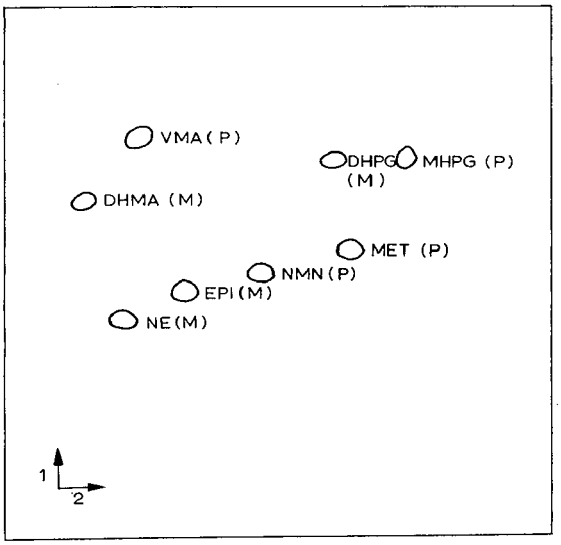

Fig. 2. Separation of NE and its metabolites. For conditions see legend to Fig. 1.

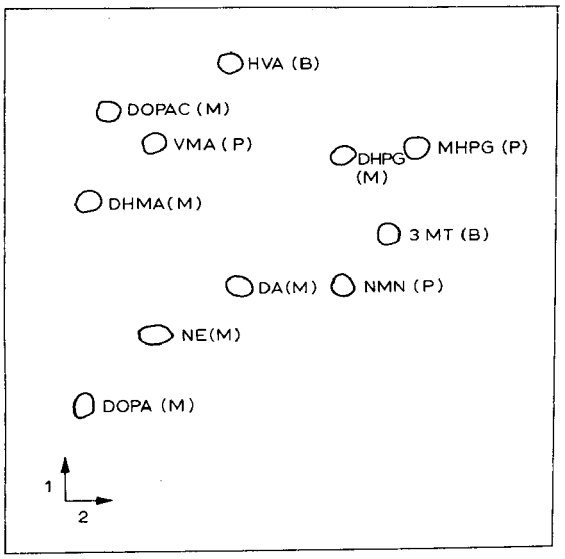

Fig. 3. Separation of compounds of interest in Parkinson's disease, drug-induced dyskinesias, DOPA therapy, etc. For conditions see legend to Fig. 1.

standard area. 91 % of ^{14}C counts applied from an *in vivo* mouse caudate nucleus experiment were recovered in the immediate area of the developed reference standards. The autoradiography showed some radioactivity on the periphery of the spots, so for complete recovery it was advisable in all experiments to scrape a short distance (1 mm) beyond the standard spot area.

Biological application

An application of the TLC separation to the metabolism of ^{14}C-DA by caudate nucleus showed metabolic differences in the rat *in vivo* and *in vitro*. Also, by inhibition of monoamine oxidase (MAO) and alteration of the normal caudate nucleus metabolism of DA *in vivo*, it was possible to show differences in metabolic products and give validity to the premise that the labeled substances formed were the metabolites mentioned.

For the *in vitro* studies, caudate nuclei were quickly dissected at 4° from brains of Sprague–Dawley rats sacrificed by decapitation. Slices not more than 0.5 mm thick were made from the caudates and an equal number of slices (approximately 50 mg) were placed in 5-ml vessels which contained 1 ml of Krebs phosphate medium[8], pH 7.4 gassed with 5 % CO_2–95 % O_2. After 15-min preincubation for temperature equilibration, the tissues were incubated for 20 min with 5×10^5 c.p.m. ^{14}C-DA. The tissues were removed from the vessels, rinsed in cold saline and homogenized with a Bronwill Biosonic Sonifier (Bronwill Scientific, Rochester, N.Y., U.S.A.) in 0.2 ml 0.2 *N* acetic acid which contained 1 μg of each reference standard of interest in the DA study. Aliquots were taken of the total homogenate and the centrifuged supernatant for counting, in order to measure ^{14}C-DA uptake and tissue extraction recovery. Aliquots of 10–25 λ of the tissue supernatant and Krebs medium, which had two drops of glacial acetic acid and 1 μg of each reference standard added, were spotted on the cellulose chromatoplate along with 1 μg DA and metabolite reference standards. The plates were developed and the radioactivity counted as described in MATERIALS AND METHODS. The lateral ventircle freehand injection technique of CLARK *et al.*[9], developed for mice, was adapted for use with rats under light ether anesthesia in the MAO inhibition study. In the examination of *in vivo* caudate nucleus metabolism of DA, 15 min after the injection of 2.5×10^5 c.p.m. ^{14}C-DA, in 5 λ pH 7.0 Krebs solution, into each ventricle, the rats were sacrificed by decapitation and the caudate nucleus rapidly removed at 4°. The tissue was rinsed in cold saline, weighed on a microbalance and frozen immediately over dry ice until analyzed, which was within 15 h of sacrificing.

The frozen tissue was broken up by the sonifier in 0.2 ml 0.2 *N* acetic acid after the addition of 1 μg DA and metabolite reference standard. Aliquots of this homogenate and the resulting supernatant, after centrifugation at $10{,}000 \times g$ and 0°, were taken for ^{14}C-DA uptake and tissue extraction data. Aliquots were spotted on the cellulose TLC as in the *in vitro* experiments mentioned above.

The results are shown in Table II. The relative amount of each compound is expressed as a percentage of the radioactivity of all the reference standard areas counted. Because of the small *N* value in this experiment it is not possible to include statistical differences between *in vivo* and *in vitro* metabolism of ^{14}C-DA by rat caudate nucleus but certain observations can be made.

The major metabolite found *in vitro* in both the tissue and in the Krebs medium

TABLE II

In vitro AND *in vivo* METABOLISM OF ^{14}C-DA BY RAT CAUDATE NUCLEUS

Reference standards	*Average % of recovered ^{14}C found in reference standards of interest, combined to total 100%*		
	In vitro[a]		*In vivo*[b] ($N = 2$)
	Tissue ($N = 3$)	*Medium* ($N = 3$)	
Origin	5.2 Range (1.7–9.3)	1.0 (0.9–1.2)	1.2 (0 2.5)
NE	0.4 (0–0.9)	0.9 (0.5–1.3)	1.9 (1.0–2.8)
DA	82.7 (74.5–93.2)	81.7 (74.5–89.1)	57.3 (52.1–63.6)
3 MT	0.2 (0–0.5)	0.2 (0–0.5)	5.1 (4.3–5.9)
DOPAC	11.6 (5.0–15.4)	14.8 (8.6–20.8)	13.4 (13.2–13.7)
HVA	0.1 (0–0.3)	1.4 (0.3–2.5)	22.5 (20.5–24.5)

[a] Incubated 20 min: 10–25 λ spotted on TLC plate.
[b] Sacrificed by decapitation 15 min after lateral ventricle injection.

was DOPAC. This has been reported by others in bovine[10] and rat brain homogenates[4,10] and rabbit caudate nucleus[11]. There was little HVA, 3 MT or NE formed. The apparent absence of labeled NE is interesting since UDENFRIEND AND CREVELING[12] found the caudate nucleus of two species (dog and bovine) to be almost as high in dopamine-β-oxidase activity as hypothalamus and comparable with the adrenal medulla. However, others have reported negligible conversion of labeled precursors to ^{14}C-NE by caudate nucleus of cat[13], rabbit[11] and rat brain homogenate[4].

The *in vivo* metabolic pattern is somewhat different. HVA was found to be the major catabolite, followed in decreasing order by DOPAC, 3 MT, and NE. In support of our findings, LAVERTY AND TAYLOR[14], using intraventricular administration of ^{3}H-DA, reported a metabolic pattern for the rat striatum, 1 h post injection which is similar to ours, with HVA the major catabolite. Also, in a review dealing with DA and brain function, HORNYKIEWICZ[15] stated that HVA is the main metabolic breakdown product of DA in brain.

To test the efficiency of this method further, we used a rapidly acting MAO inhibitor which has a strong effect on DA metabolism (Table III). After a 45-min pretreatment of mice with 100 mg/kg Pargyline no labeled HVA or DOPAC could be found in the caudate nucleus 15 min after the intraventricular injection of ^{14}C-DA. As expected, the relative amounts of 3MT greatly increased. Thus O-methylation was apparently the only major metabolic route available for the catabolism of the labeled DA.

The considerable advantage of this TLC procedure lies in the fact that with a rapid and uncomplicated sample preparation, combinations of sixteen different ^{14}C-or ^{3}H-labeled catecholamines, their precursors and metabolites can be estimated directly without preparing derivatives.

TABLE III

In vivo METABOLISM OF ^{14}C-DA BY CONTROL AND MAO-INHIBITED MOUSE CAUDATE NUCLEUS

Reference standards	*Average % of recovered ^{14}C found in reference standards of interest, combined to total 100%*		
	Control (N = 1)	*Pargyline 100 mg/kg Pre-Rx 45 min (N = 2)*	
Origin	0	0	0
NE	0	3.8	0
DA	55.7	68.5	75.2
3MT	6.8	27.7	24.8
DOPAC	19.1	0	0
HVA	18.8	0	0

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CHROM. 4926

ZUR IDENTIFIZIERUNG UND QUANTITATIVEN ANALYSE VON STROPHANTHUSGLYKOSIDEN

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(Eingegangen am 16. Juni 1970)

SUMMARY

A method for the identification and quantitative analysis of Strophanthus glycosides

A method for the separation and quantitative analysis of glycosides of Strophanthus seeds is described using MgO as adsorbent for thin-layer chromatography of alcoholic extracts of the seeds. In addition to the colorimetric evaluation of the Baljet reaction, a spectrophotometric measurement of the glycosides in concentrated sulphuric acid is suggested, which shows a higher sensitivity. The advantage of the latter method is that the adsorbent is dissolved directly in the measuring liquid, allowing quantitative measurement of the adsorbed glycosides without losses. Application of the method is demonstrated on the seeds of *Strophanthus kombé, gratus, hispidus* and *sarmentosus*. In addition, the distribution of glycosides in the seeds of *Strophanthus kombé* was investigated by means of this method.

EINLEITUNG

Über die Trennung von Strophanthusglykosiden mittels Dünnschicht-Chromatographie (DC) berichteten in jüngerer Zeit u.a. LUKAS[1], CORONA UND RAITERI[2], sowie HÖRHAMMER *et al.*[3]. Die beiden erstgenannten Autoren legen ihr Augenmerk vor allem auf den Nachweis des Hauptglykosides Strophanthosid und seiner Hydrolyseprodukte K-Strophanthin-β, Cymarin und Strophanthidin, deren Auftrennung aus K-Strophanthin-Handelspräparaten mit den angegebenen Methoden auch gelingt. HÖRHAMMER *et al.* berücksichtigen darüber hinaus auch Glykoside des Periplogenin- und Strophanthidol-Typ und berichten weiters über die Unterscheidung von alkoholischen Extrakten aus Samen verschiedener Strophantus-Arten.

Die quantitative Bestimmung der Hauptglykoside aus alkoholischen Extrakten von Strophanthussamen wird, insbesondere bei *Strophanthus kombé*, durch die Anwesenheit einer grossen Zahl glykosidischer Inhaltsstoffe erschwert. Bei unseren Untersuchungen über die Brauchbarkeit von MgO für die DC von Pflanzeninhaltsstoffen[4–6] schien auch die Auftrennung von Strophanthusglykosiden auf MgO-Schichten möglich. Über unsere diesbezüglichen Untersuchungen sowie über eine neue Methode zur quantitativen Bestimmung der Strophanthusglykoside in der Droge sei im folgenden berichtet.

EIGENE UNTERSUCHUNGEN

Extraktion der Glykoside

Üblicherweise werden die Strophanthussamen vor der Extraktion der Glykoside entfettet. Wie unsere Untersuchungen ergaben, werden durch die Behandlung mit Petroläther geringe Glykosidmengen, vor allem Cymarin, mit extrahiert. Da bei der Verwendung von MgO als Sorptionsmittel eine Vorentfettung nicht nötig ist, kann diese daher unterbleiben. Die Extraktion der feingemahlenen (Sieb V, ÖAB 9) und mit Seesand verriebenen Samen erfolgt mit 70 %igem Äthanol in einer kontinuierlich arbeitenden Apparatur durch 1 Std. Nach dieser Zeit sind keine messbaren Mengen von Glykosiden im Untersuchungsmaterial mehr nachweisbar.

Auftrennung des Glykosidgemisches mittels DC

Sorptionsmittel. Als Sorptionsmittel verwenden wir wegen des guten Trenneffektes und der Löslichkeit des Sorptionsmittels in Säure zwecks quantitativer Bestimmung Magnesiumoxid[4–6]. Die Beschichtung der Platten erfolgt wie üblich (Mengenangaben siehe EXPERIMENTELLER TEIL). Die Platten werden in 1.5-cm-breite Streifen eingeteilt, die Laufstrecke beträgt 15 oder 25 cm (Platten 20 × 30 cm), die

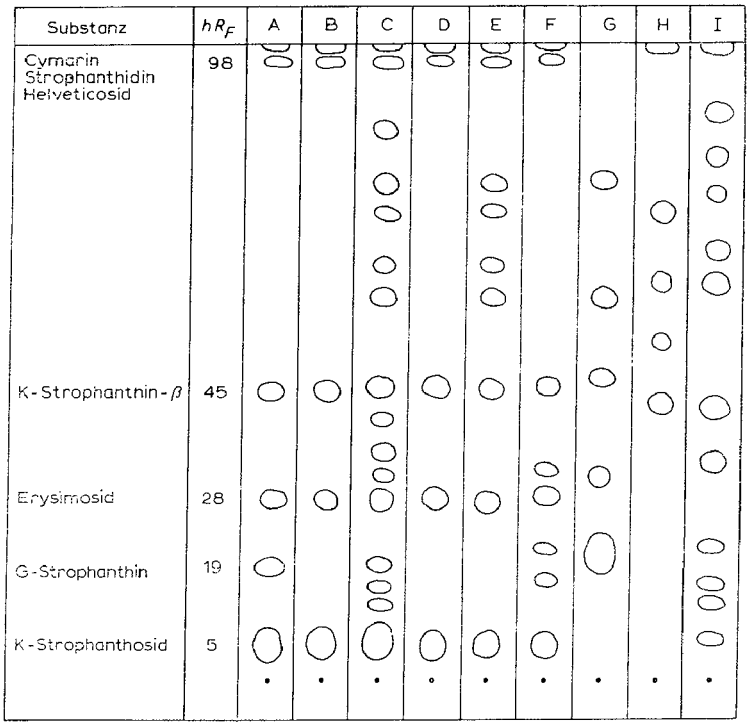

Fig. 1. Trennung von Strophanthusglykosiden mittels Dünnschicht-Chromatographie. Fliessmittelgemisch: Aceton–Wasser–Äthylacetat (4:0.6:5.4); Sorptionsmittel: MgO; Laufstrecke: 25 cm. A = Reinglykosidgemisch; B = K-Strophanthin (Handelspräparat); C = *Strophanthus kombé* I; D = *Strophanthus kombé* II; E = *Strophanthus kombé* III; F = *Strophanthus hispidus*; G = *Strophanthus gratus*; H = *Strophantus sarmentosus*, var. *major*; I = *Strophanthus sarmentosus*, var. *senegambiae*.

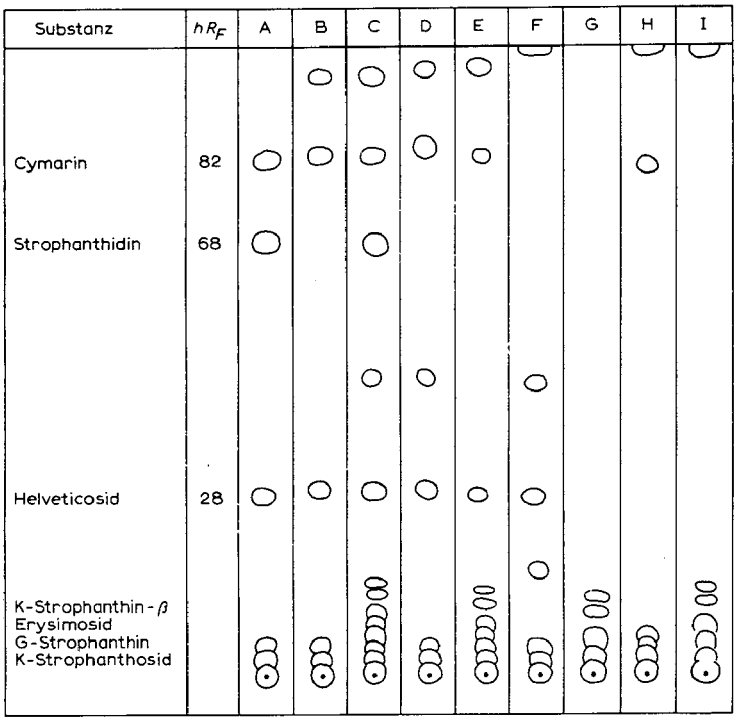

Fig. 2. Trennung von Strophanthusglykosiden mittels Dünnschicht-Chromatographie. Fliessmittelgemisch: Aceton–Wasser–Äthylacetat (4:0.2:5.8); Sorptionsmittel: MgO; Laufstrecke: 25 cm. A–I, s. Fig. 1.

Laufzeit 25 bzw. 90 min. Die Entwicklung der Chromatogramme erfolgt bei Zimmertemperatur und normaler Kammersättigung.

Fliessmittelgemische. Der beste Trenneffekt wurde mit dem Fliessmittelgemisch Aceton–Wasser–Äthylacetat erreicht. Der Anteil des Wassers ist dafür ausschlaggebend, ob die zuckerreicheren Komponenten oder die zuckerarmen bzw. zuckerfreien Komponenten besser aufgetrennt werden. Wie erwartet, lässt sich der alkoholische Gesamtextrakt aus Strophanthussamen auf einer Laufstrecke von 10 cm unter Verwendung nur eines Fliessmittelgemisches nicht befriedigend auftrennen. Wohl gelingt damit die Abtrennung der Hauptglykoside, doch zeigten weitere Untersuchungen, dass sich oftmals an einem Fleck (identischer R_F-Wert) bis zu drei Glykoside befinden. Wir verlängerten daher die Laufstrecke auf 15 bzw. 25 cm und benützten mehrere Fliessmittelgemische, die sich nur in der Menge des Wassers unterscheiden. Die optimale Auftrennung erfolgt bei einer Laufstretke von 25 cm. Bei der Verwendung des Fliessmittelgemisches "c" (s. Tabelle I) werden die zuckerreicheren Komponenten (unterer Teil des Chromatogrammes), bei Verwendung des Fliesmittelgemisches "d" die zuckerarmen bzw. zuckerfreien Komponenten (oberer Teil des Chromatogrammes) gut aufgetrennt (s. Fig. 1 und 2). Will man sowohl die zuckerreichen Glykoside wie auch die Aglykone gleichermassen befriedigend auftrennen, so entwickelt man eine Platte mit dem Fliessmittelgemisch "c" und eine andere mit dem Fliessmittelgemisch "d".

TABELLE I

ZUSAMMENSETZUNG DER FLIESSMITTELGEMISCHE ACETON–WASSER–ÄTHYLACETAT

Fliessmittel-gemisch	*Aceton–Wasser–Äthylacetat*	*Plattengrösse (cm × cm)*	*Laufstrecke (cm)*	*Trenneffekt*
a	4:0.7:5.3	20 × 20	15	Zuckerreiche Glykoside gut
b	4:0.4:5.6	20 × 20	15	Zuckerarme Glykoside gut
c	4:0.6:5.4	30 × 20	25	Zuckerreiche Glykoside gut
d	4:0.2:5.8	30 × 20	25	Zuckerarme Glykoside gut

Da je nach verwendeter Plattengrösse auch die Zusammensetzung des Fliessmittelgemisches geringfügig geändert werden muss (verschiedene Sättigung bei normaler und grösserer Küvette), zeigt Tabelle I die günstigsten Zusammenhänge zwischen Plattengrösse, Trenneffekt und Zusammensetzung des Fliessmittelgemisches.

Detektionsmittel. Die folgende Detektionsmittel wurden verwendet: (a) Kedde's-Reagens: Violette Flecke auf weissem Grund. Nachweisgrenze *ca.* 0.5 μg. (b) Jodlösung (1 %ig in Chloroform): Helle Flecken auf braunem Grund. Nachweisgrenze *ca.* 10 μg. Für die nachfolgende quantitative Bestimmung wird das Jod, das lediglich der Kennzeichnung dient, durch Erhitzen auf 120° über 10 min vertrieben. Eventuelle zurückbleibende Jodreste stören die spektroskopische Messung in konz. Schwefelsäure (s.u.) nicht.

Quantitative Analyse der Strophanthusglykoside

Die quantitative Analyse der Strophanthusglykoside erfolgt üblicherweise durch die colorimetrische Auswertung der Baljet-Reaktion. Durch eigene Untersuchungen (KARTNIG UND MIKULA[7]) konnte gezeigt werden, dass Glykoside des Sterol- und Triterpentypes in konzentrierter Schwefelsäure eine spezifische Absorptionsbande aufweisen, die zur quantitativen Bestimmung benützt werden kann. Selbst bei Glykosiden mit vier Zuckern tritt bei einer Menge bis zu 600 mg in 10 ml konz. Schwefelsäure keine Störung der Messung durch Verkohlungserscheinungen ein. Die Empfindlichkeit der spektralphotometrischen Messung in konz. Schwefelsäure ist grösser als die kolorimetrische Auswertung der Baljet-Reaktion. Voraussetzung ist verständlicherweise die Abwesenheit anderer organischer Verbindungen bei der Umsetzung mit Schwefelsäure, was jedoch durch die oben beschriebene Abtrennung mittels DC unschwer zu erreichen ist. Die quantitative Messung wird so durchgeführt, dass man den

TABELLE II

EXTINKTIONEN FÜR DIE EICHKURVEN DER WICHTIGSTEN STROPHANTHUSGLYKOSIDE

Substanz	*Baljet-Reaktion (495 nm)*			*Spektralphotometrische Messung in H_2SO_4*			
	25 μg	*50 μg*	*100 μg*	*nm*	*25 μg*	*50 μg*	*100 μg*
Strophanthosid	0.061	0.120	0.245	407	0.154	0.311	0.620
K-Strophanthin-β	0.085	0.174	0.352	407	0.255	0.508	1.010
Cymarin	0.120	0.245	0.490	415	0.265	0.540	1.080
Erysimosid	0.097	0.193	0.385	407	0.250	0.502	1.000
Strophanthidin	0.152	0.317	0.638	420	0.286	0.580	1.150

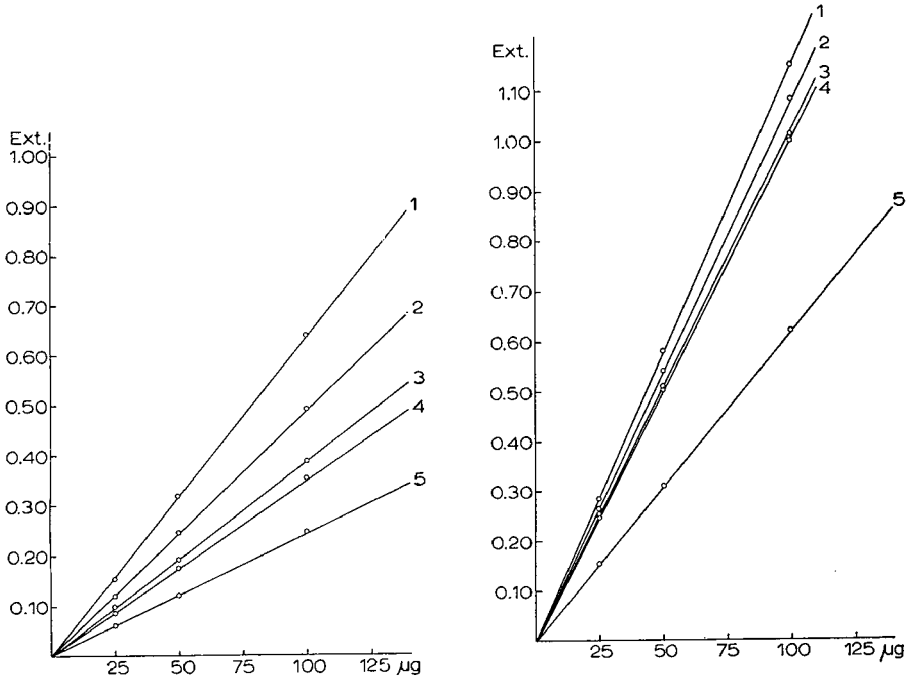

Fig. 3. Eichkurven von Strophanthusglykosiden erhalten durch Auswertung der Baljet-Reaktion. Messung bei 495 nm. 1 = Strophanthidin; 2 = Cymarin; 3 = Erysimosid; 4 = K-Strophanthin-β; 5 = K-Strophanthosid.

Fig. 4. Eichkurven von Strophanthusglykosiden. Spektroskopische Messung in konzentrierter Schwefelsäure. 1 = Strophanthidin (420 nm); 2 = Cymarin (415 nm); 3 = K-Strophanthin-β (407 nm); 4 = Erysimosid (407 nm); 5 = K-Strophanthosid (407 nm).

aus dem Chromatogramm abgeschabten Fleck in einem Reagensglas in 0.2 ml HCl konz. und 3 ml H_2SO_4 konz. löst und durch 15 min auf 60° erwärmt oder 2 h 30 min bei Zimmertemperatur stehen lässt. Sodann kühlt man ab und misst die klare Flüssigkeit bei der entsprechenden Wellenlänge gegen 0.2 ml konz. HCl + 3 ml konz. H_2SO_4, in der man eine der abgeschabten Zone entsprechende Menge MgO gelöst und ebenfalls 15 min auf 60° erwärmt hat.

Die Erfassungsgrenze der Messmethode liegt für Glykoside mit drei Zuckern bei 5 μg. Die Standardabweichung beträgt für Gesamtglykoside ± 1.4 %.

Der Vorteil dieses Messverfahrens liegt bei der Trennung auf MgO gegenüber der colorimetrischen Auswertung der Baljet-Reaktion darin, dass sich das MgO in 0.2 ml HCl + 3 ml H_2SO_4 völlig löst und somit das gesamte adsorbierte Glykosid zur Umsetzung und Messung gelangt. Die Eichkurven für freie und adsorbierte Glykoside decken sich. Sollte durch Auflösen einer zu grossen Menge MgO im Säuregemisch während der Umsetzungszeit $MgSO_4$ ausfallen, so kann dieses unschwer abzentrifugiert werden. Eine Beeinträchtigung der Messung tritt hierdurch nicht ein.

Untersuchung verschiedener Strophanthussamen sowie der Glykosidverteilung innerhalb des Samens

Die beschriebene chromatographische Auftrennung und quantitative Bestim-

mung der Strophanthusglykoside wurde an verschiedenen Mustern von *Strophanthus kombé* Samen sowie an Samen von *Strophanthus gratus, hispidus* und *sarmentosus* angewendet. Die Untersuchungen ergaben, dass *Strophanthus kombé* Samen verschiedener Herkunft Glykosidgemische unterschiedlicher Zusammensetzungen enthalten. So konnte z.B. in einer als "Mozambiquedroge" deklarierten Probe neunzehn glykosidische, Kedde-positive, Flecke nachgewiesen werden, während in einer als "Kilimandscharodroge" deklarierten Probe nur deren zwölf nachgewiesen werden konnten, wobei jedoch die Strophanthidinderivate in beiden Sorten vorhanden waren. Auch die Relationen der Glykosidmengen zueinander waren verschieden.

Die Auftrennung des alkoholischen Extraktes aus *Strophanthus gratus* Samen erbrachte das Vorliegen von vier weiteren, Kedde-positiven Flecken neben G-Strophanthin.

Aus *Strophanthus hispidus* konnten neun glykosidische Inhaltsstoffe nachgewiesen werden, wobei im Gegensatz zum Chromatogramm von *Strophanthus kombé* Cymarin und Strophanthidin nicht nachweisbar waren.

Von *Strophantus sarmentosus* standen die Samen zweier Varietäten (*major* und *senegambia*) zur Verfügung*. Die Extrakte beider Varietäten zeichneten sich durch das Fehlen von Erysimosid und K-Strophanthin-β aus, unterschieden sich jedoch auch von einander. Im Extrakt von Samen von *Strophanthus sarmentosus*, var. *major*, konnten nur sechs Kedde-positive Flecke gefunden werden, während aus der Varietät *senegambia* zwölf Flecke erhalten wurden.

An zwei Mustern von *Strophanthus kombé* Samen haben wir erstmalig die Verteilung der wichtigsten Glykoside innerhalb des Samens untersucht, wobei einerseits der Glykosidgehalt der Samenschale und andererseits der des Keimlings und Endosperms bestimmt wurde. Die Prozentzahlen in Tabelle III beziehen sich auf wasserfreie Probensubstanz.

TABELLE III

VERTEILUNG DER WICHTIGSTEN GLYKOSIDE IN *Strophantus kombé* SAMEN

Samen Strophanthus kombé[a]		*% Gesamt Glykoside*	*Strophanthosid*	*Erysimosid*	*K-Strophanthin-β*	*Cymarin*
Muster I	Samenschale	8.00	6.70	0.60	0.50	0.20
	Endosperm + Keimling	4.16	3.35	0.50	0.31	n.m.[b]
Muster II	Samenschale	7.25	5.65	0.80	0.60	0.20
	Endosperm + Keimling	3.85	3.05	0.47	0.33	n.m.[b]

[a] Der Fa. Dr. Siegfried, Zofingen, sei für die freundliche Überlassung von *Strophanthus kombé* Samen bestens gedankt.

[b] n.m. = nicht messbar; zu geringe Mengen.

Bei beiden untersuchten Mustern zeigte es sich, dass der %-Gehalt der Samenschale an Glykosiden annähernd doppelt so gross ist, wie der des Endosperm und Keimling. Die Verhältnisse der Glykosidmengen sind etwa gleich.

* Herrn Prof. Dr. T. REICHSTEIN sei für die grosszügige Überlassung der Samen von zwei *Strophanthus sarmentosus* Varietäten an dieser Stelle unser ergebener Dank ausgesprochen.

EXPERIMENTELLER TEIL

Extraktion

1 g Samen (pulv. Sieb V, ÖAB 9) wird mit 2 g Seesand verrieben und mit 50 ml Äthanol (70 %) in einer kontinuierlich arbeitenden Extraktionsapparatur durch 1 Std. extrahiert. Filtration durch ein kleines Faltenfilter in einem 50 ml Messkolben; Auffüllen mit Äthanol (70 %).

Dünnschichtchromatographie

Beschichten der Platten. 15 g MgO (Merck Nr. 5864) werden mit 65 ml Wasser angerührt und ausgestrichen. Schichtdicke, 0.25 mm. Trocknung bei 130° durch 30 min. Einteilung der Schicht in 1.5 cm-breite Bahnen. Laufstrecke, 15 oder 25 cm.

Auftragen des Extraktes. Vom 2 %igen, äthanolischen Extrakt werden zur quantitativen Bestimmung des Strophanthosides 50 μl, für die Bestimmung der übrigen Verbindungen 300 μl punktförmig aufgetragen.

Fliessmittelgemische. Für Fliessmittelgemische siehe Tabelle I.

Detektion. Die Detektion wurde durchgeführt mittels Kedde-Reagens (Stahl-Reagens Nr. 73)[8] oder Jod-Lösung (1 % in Chloroform).

Quantitative Auswertung

Colorimetrische Auswertung der Reaktion nach Baljet. Die abgeschabte Zone des Chromatogrammes in einem Zentrifugierglas mit 3 ml Methanol versetzen und 30 min stehen lassen; Zugabe von 3 ml Baljet-Reagens (5 ml 10 %ige wässrige NaOH-Lösung + 95 ml 1 %ige wässrige Pikrinsäurelösung); 30 min bei 20° stehen lassen, anschliessend zentrifugieren, dekantieren und bei 495 nm gegen Baljet-Reagens messen.

Spektroskopische Messung in konzentrierter Schwefelsäure. Die abgeschabte Zone des Chromatogrammes wird in einem Reagensglas mit 0.2 ml konz. HCl und darnach mit 3 ml konz. H_2SO_4 versetzt. Das Reaktionsgemisch wird gut durchgeschüttelt und 15 min bei 60° oder 2 Std. 30 min bei Zimmertemperatur stehen gelassen. Nach dem Abkühlen auf Zimmertemperatur misst man bei der dem Glykosid entsprechenden Wellenlänge (s. Tabelle II) gegen 0.2 ml HCl + 3 ml konz. H_2SO_4, denen man so viel MgO zusetzte, wie der abgeschabten Zone entsprach, und die man ebenfalls 15 min auf 60° erwärmt hatte.

ZUSAMMENFASSUNG

Es wird eine Methode zur qualitativen und quantitativen Analyse der Glykoside aus Strophanthussamen beschrieben, bei der der alkoholische Extrakt aus den Samen auf MgO-Schichten getrennt wird. Zur quantitativen Analyse wird neben der colorimetrischen Auswertung der Baljet-Reaktion die spektroskopische Messung der Glykoside in konz. Schwefelsäure vorgeschlagen, die sich durch grosse Empfindlichkeit auszeichnet. Der besondere Vorteil dieser Methode besteht darin, dass sich das MgO in der Messflüssigkeit löst und somit das gesamte adsorbierte Glykosid zur Messung gelangt. Als Anwendungsbeispiele wurden die Samen von *Strophanthus kombé, gratus, hispidus* und *sarmentosus* untersucht sowie die Verteilung der Glykoside in den Samen von *Strophantus kombé* bestimmt.

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CHROM. 4899

GEL CHROMATOGRAPHIC BEHAVIOR OF SOME METAL IONS

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SUMMARY

Some metal ions were chromatographed on Sephadex G-15 columns. Solutions of potassium chloride, sodium sulfate, sodium nitrate, and sodium perchlorate were used as eluting agents, *i.e.*, as background electrolytes. It was found that the elution volumes of the metal ions depend very much on the nature of the background electrolytes employed. These phenomena were explained in terms of the penetrating ability of the background electrolytes into the gel phase and direct and indirect interaction between the metal ions and the gel matrix.

INTRODUCTION

Gel chromatography has been developed as an extremely useful tool for the separation of macromolecules and the estimation of their molecular weights. Some recent investigations[1–13] have demonstrated that highly cross-linked gels are useful for the separation of small inorganic ions. Although the molecular-sieve effect due to the size difference of solutes is the main factor controlling the separation mechanism in gel chromatography, side effects such as adsorption[2,11], ion exclusion[5,6] and restricted diffusion[14] of solutes must be considered in some cases, especially for the separation of inorganic ions.

SAUNDERS AND PECSOK[1] have shown that the observed distribution coefficients of strong electrolytes in gel chromatography can be divided into additive cation and anion contributions. EGAN[2], and NEDDERMEYER AND ROGERS[3] have observed the effects of counter ions on the elution volumes of inorganic cations and anions.

The present investigation was undertaken to clarify the effect of the background electrolytes on the gel chromatographic behavior of various metal ions. Solutions of potassium chloride, sodium sulfate, sodium nitrate, and sodium perchlorate were used as eluting agents, *i.e.*, as background electrolytes.

EXPERIMENTAL

Sample solutions

Sample solutions of sodium, potassium, magnesium, calcium, strontium, barium

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manganese(II), cobalt(II), nickel(II), zinc, and cadmium ions were prepared by dissolving their chlorides in a solution of the same composition as that of the eluent used for the column operation. The sulfate of the copper(II) ion was used. The metal concentration of the sample solutions was 0.01 *M*.

0.1 *M* solutions of sodium nitrate and sodium perchlorate were prepared by a similar method. Solutions of Blue Dextran 2000 (Pharmacia Fine Chemicals, 0.1–0.2%) and tritiated water (Radiochemical Center) prepared by a similar method were used as standard materials of $K_d = 0$ and 1, respectively.

Eluents

Eluent I: 0.1 *M* potassium chloride–0.01 *M* hydrochloric acid solution. Eluent II: 0.1 *M* sodium sulfate–0.005 *M* sulfuric acid solution. Eluent III: 0.1 *M* sodium nitrate–0.01 *M* nitric acid solution. Eluent IV: 0.1 *M* sodium perchlorate–0.01 *M* perchloric acid solution.

Columns

Sephadex G-15 (Pharmacia Fine Chemicals, dry particle size 40–120 μ), which is a cross-linked dextran gel, was used as the bed material. The columns were 1.5 × 60 cm and 1.5 × 90 cm glass tubes (Shoei Glass).

The dry gel powder was suspended in an eluent and allowed to swell for two days. Undesirable fine particles were removed by decantation. The suspension of the swollen gel was deaerated under reduced pressure before use. The gel suspension prepared in this manner was poured into a vertical glass tube which had been partially filled with the eluent. After the gel bed had reached a height of about 5 cm, the outlet at the bottom was opened to allow the eluent to flow at a rate of approximately 30 ml/h. The addition of the gel suspension was continued until the gel bed reached the desired height. Bed volumes were adjusted to 100 or 150 ml. A disk of filter paper was placed on the top of the gel bed to protect the surface from disturbance. After packing, 500 ml of the eluent was passed through the column to settle the gel bed.

Procedure for elution

One milliliter of the sample solution was placed on the top of the bed just as the last few drops of the eluent soaked into the bed. As soon as the solution vanished into the bed, about 4 ml of the eluent were added and elution was started at a constant flow rate of 25–35 ml/h. The column temperature was kept at 20°. The effluent was collected in fractions of 1 ml with a Toyo Kagaku drop count fraction collector. Some fractions were chosen arbitrarily and their volumes were measured so that the fraction volume could be determined.

Concentrations of the samples or the standard substances in the effluents were determined as follows. Sodium ion: measurement of the radioactivity of the ^{22}Na used as a tracer. Potassium ion: flame photometric measurement. Other metal ions: chelatometric titration with EDTA. Chloride ion: argentometric titration. Blue Dextran 2000: measurement of absorbance at 630 mμ. Tritiated water: measurement of the radioactivity with a liquid scintillation counter.

Elution curves for nitrate, perchlorate and ammonium ion were recorded on a Japan Electron Optics liquid chromatograph Model JLC-2A equipped with detection columns packed with Sephadex G-15.

RESULTS AND DISCUSSION

In a previous paper[4] it was demonstrated that the degree of peak skewing of alkaline earth metal ions chromatographed on a Sephadex column decreases on using an acidic eluent containing both sodium chloride and hydrochloric acid. Therefore, acidic solutions of the background electrolytes were used as an eluent in this work. Distribution coefficients and elution orders of the metal ions are summarized in Fig. 1. Some of the elution curves of the metal ions obtained with eluents I, II, III, and IV are illustrated in Figs. 2, 3, 4 and 5, respectively. All of the columns used for the measurements of these elution curves had a bed volume of 100 ml. The distribution coefficient K_d is defined by eqn. 1,

$$V_e = V_0 + K_d \cdot V_i \tag{1}$$

where V_e is the elution volume of a given solute, V_0 the void volume outside the gel particles, and V_i the internal volume within the gel phase.

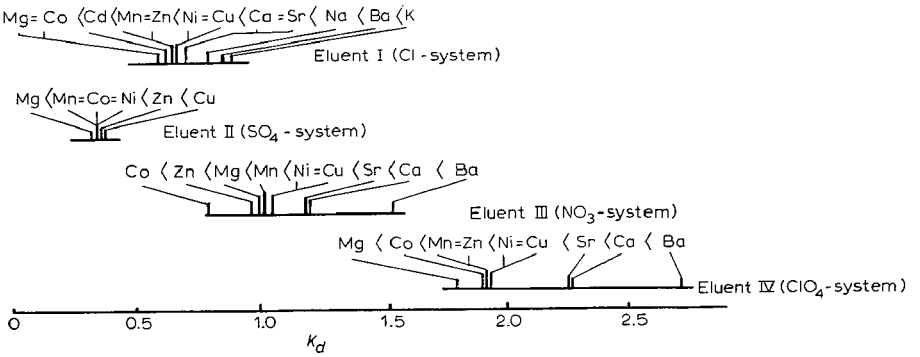

Fig. 1. K_d values of metal ions obtained by gel chromatography with eluents I, II, III and IV.

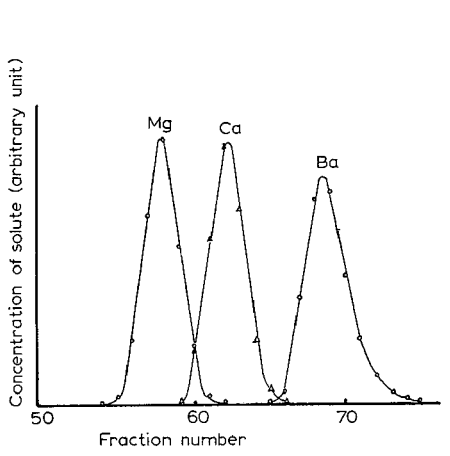

Fig. 2. Chromatograms obtained with eluent I. One fraction: 1.01 ml.

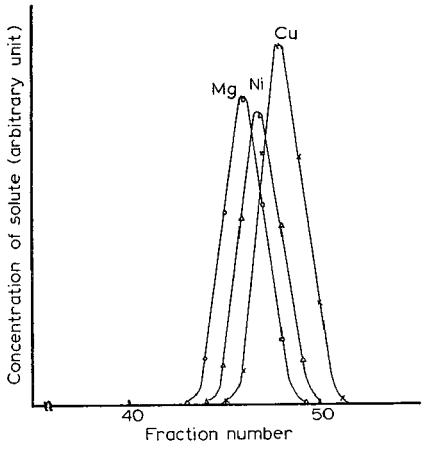

Fig. 3. Chromatograms obtained with eluent II. One fraction: 1.08 ml.

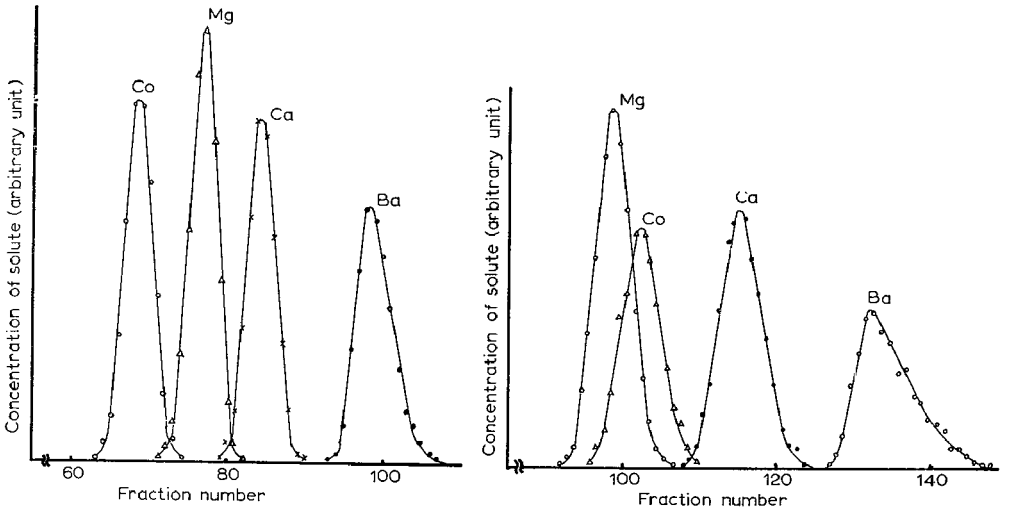

Fig. 4. Chromatograms obtained with eluent III. One fraction: 1.01 ml.

Fig. 5. Chromatograms obtained with eluent IV. One fraction: 1.11 ml.

When eluent I was used, all of the metal ions examined were eluted within the volume of $V_0 + V_i$, and their elution curves were symmetrical with the exception of the barium ion. As shown in Fig. 2, of the alkaline earth metal ions, magnesium, calcium and barium ion were separated from each other in this chloride system. However, the strontium ion was eluted at the same position as the calcium ion. When potassium chloride was used as a background electrolyte the elution peak positions of the potassium and chloride ion were determined by adding pure water as a sample and measuring the negative peaks of both the potassium and chloride ion.

When eluent II was used, magnesium, manganese (II), cobalt(II), nickel(II), zinc, and copper(II) ion were eluted very rapidly as compared with the results obtained with eluent I. All of their elution curves were symmetrical. However, as shown in Figs. 1 and 3, the separation of these metal ions was very poor in this sulfate system.

When eluent III was used, all of the metal ions examined were eluted slowly as compared with the results obtained with eluent I. Zinc, magnesium, manganese(II), nickel(II) and copper(II) ions were eluted nearly at the volume of $V_0 + V_i$, and the strontium, calcium, and barium ions were eluted more slowly. All of the elution curves of these metal ions except that of the barium ion were symmetrical. As shown in Fig. 4, the separation of the cobalt(II), magnesium, calcium and barium ions was almost complete in this nitrate system.

When eluent IV was used, all of the metal ions tested were eluted extremely slowly as compared with the results obtained with eluent I. All of the elution curves of the metal ions except that of the barium ion were also symmetrical. As shown in Fig. 5, magnesium, calcium and barium ions were completely separated in this perchlorate system.

In gel chromatography the molecular-sieve effect due to the size difference of solutes is the main factor controlling the separation mechanism. However, sometimes side effects such as adsorption will accompany the molecular-sieve effect. If there are no such side effects, the elution order of the metal ions should not be altered by variation of the composition of background electrolytes. The results obtained by the present work, as shown in Fig. 1, indicate the remarkable dependence of the elution order of the metal ions on the background electrolytes employed.

In the sulfate system the K_d values of the metal ions were very small. These phenomena may be closely connected with the gel chromatographic behavior of sulfate ion. It was found that sulfate ion is eluted faster than any of the metal ions employed in this work from a Sephadex G-15 column with eluent I. This result suggests that sulfate ion may be excluded from the gel to a considerably greater extent than that expected from the radius of its hydrated ion, 3.79 Å, reported by NIGHTINGALE[15]. Although the behavior of sulfate ion on a gel column cannot be explained quite satisfactorily at the present, the following discussion based on the idea of the polyfunctional character of the gel phase[4] will be possible. According to this idea the gel phase is divided into two regions, phase I and phase II. A background electrolyte can penetrate into phase II but not into phase I. When sodium sulfate is used as a background electrolyte, the volume of phase I is expected to become much larger than that in the chloride system. Since the metal ions accompanying the sulfate ion as a counter ion cannot penetrate into phase I, the K_d values of the metal ions must be considerably smaller than those obtained in the chloride system.

When eluents III and IV were used, the K_d values of most of the metal ions were larger than unity. These phenomena demonstrated that a side effect such as adsorption may play an important role in these systems. The adsorption effect may be explained in two ways. First, a given metal ion is directly adsorbed on the gel matrix. Second, the metal ion in question is adsorbed by counter ions which are preliminarily adsorbed on the gel matrix. If adsorption of the first type takes place, the similar results should also be observed when eluents I and II are used. The experimental results were contrary to the above assumption. The second type of adsorption seems to be similar to ion exchange. The gel chromatographic behavior of the metal ions in the nitrate and the perchlorate system can be explained by adsorption of this type. Sodium nitrate or perchlorate is adsorbed on the gel matrix and then a part of the sodium ions is exchanged for the metal ions in question.

Nitrate and perchlorate ion were eluted from a Sephadex G-15 column more slowly than the chloride ion when eluent I was employed. Since the radii of hydrated nitrate and perchlorate ions are larger than that of the hydrated chloride ion, the above fact suggests that nitrate and perchlorate ion are strongly adsorbed on the gel matrix. A similar adsorption effect can be seen in the behavior of the borate ion, which is strongly adsorbed on dextran gels[11].

The present authors have found that the K_d value of the magnesium ion obtained by its elution from a Sephadex G-25 column with eluent IV is nearly equal to that obtained on a Sephadex G-15 column. This can be explained by adsorption of the second type. The elution orders of the metal ions excepting the cobalt(II) and zinc ions in both the nitrate and the perchlorate system are very similar to each other. This fact also supports the above explanation.

The skewness of the elution curve of the barium ion can be interpreted in terms

of the interaction of the barium ion with the gel matrix as has been pointed out previously[4].

LAURENT AND KILLANDER[16] have used another distribution coefficient, K_{av}, defined as

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} = K_d \cdot \frac{V_i}{V_i + V_g} \tag{2}$$

where V_t is the total volume of the gel bed, and V_g the volume occupied by the gel matrix. Using an expression derived by OGSTON[17], they give

$$K_{av} = \exp\left[-\pi L(R_s + R_r)^2\right] \tag{3}$$

where L is the length of the gel skeleton per unit volume, and R_s and R_r denote the radii of a given solute and the gel skeleton, respectively. Eqn. 3 can be rewritten as

$$(-\log K_{av})^{1/2} = A \cdot R_s + B \tag{4}$$

where A and B are the constants for a given gel column.

The $(-\log K_{av})^{1/2}$ values of some metal ions obtained with eluent I are plotted against the R_s values of these ions reported by NIGHTINGALE[15] in Fig. 6, which shows a straight line without significant deviation for potassium, ammonium, sodium, nickel and magnesium ion. These metal ions do not form any chlorocomplexes. The K_{av} value for the chloride ion also falls on the same line. The plots for the barium, strontium, and calcium ions, however, do not fall on the straight line in spite of the lower stability constants of their chlorocomplexes. This may be ascribed to the adsorption of these ions on the gel matrix.

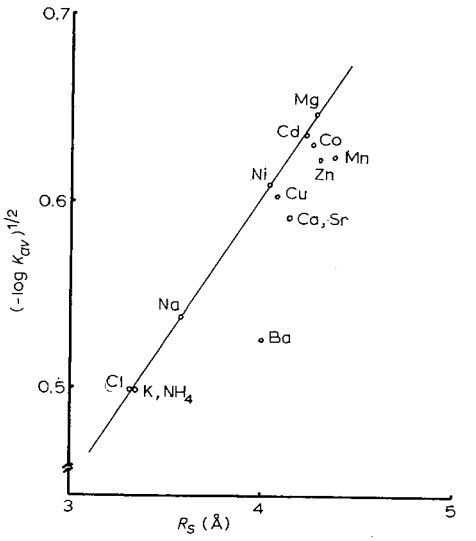

Fig. 6. Relationship between $(-\log K_{av})^{1/2}$ and R_s.

Since the properties of both the gel matrix and the solute ions are affected by the nature of the background electrolyte, it is necessary to satisfy the following requirements if one wants to correlate gel chromatographic data for sample ions with the sizes of their hydrated ions. (1) There are no side effects such as adsorption in the gel chromatographic process. (2) The background electrolyte can penetrate into the gel phase to a larger extent than the sample ions. (3) Both the cation and anion of the background electrolyte are not adsorbed on the gel matrix. (4) The sample ions do not form any complexes with counter ions of the background electrolyte.

Taking into consideration the above requirements it can be suggested that potassium chloride will be an excellent background electrolyte for gel chromatographic studies on various ions.

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THE EFFECT OF SAMPLE CONCENTRATION ON THE GEL CHROMATOGRAPHIC BEHAVIOR OF ALKALINE EARTH METAL IONS ON SEPHADEX COLUMNS

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SUMMARY

Magnesium, strontium and barium ions were chromatographed on a Sephadex G-15 column with a 0.1 *M* sodium chloride solution as an eluent. The shapes of elution curves and K_d values were found dependent on sample concentrations. This was explained in terms of the interaction of the solutes with the gel matrix and the variation in effective sizes of the solutes with the variation in ionic strength of the surrounding medium. The general features of the concentration dependence were discussed on the basis of two representative types of ion, the Mg-type and the Sr- or Ba-type. The former type is influenced by only a negligibly slight adsorption effect over a wide range of sample concentrations, while the latter type is markedly influenced by the adsorption effect, especially at lower sample concentrations.

INTRODUCTION

It has been demonstrated that gel chromatography on Sephadex columns is a useful technique for the investigation of inorganic compounds such as polyphosphates[1–6], polymeric ferric hydroxides[7,8], hydrated metal ions[2,6,9–14] and metal complexes[10,14]. The basic principle of this method has been explained in terms of steric exclusion or a sieving effect in the gel phase[15]. Larger molecules can penetrate into a smaller fraction of the internal volume of the gel phase and therefore are eluted earlier than smaller molecules.

However, the acceptance of exclusion principle alone as an explanation for the separation mechanism does not interpret all phenomena which are observed during elutions of inorganic compounds on columns packed with highly cross-linked Sephadex[2,9,11,12]. Other side effects resulting from the interaction of solutes with the gel matrix must be considered in some cases. The present work was undertaken to observe the sample-concentration dependence of elution curves of alkaline earth metal ions and followed by evaluation of the contribution of such side effects as adsorption to their chromatographic behavior. Magnesium, strontium and barium ions were chromatographed on a Sephadex G-15 column with a 0.1 *M* sodium chloride solution as eluent. The effect of sample concentrations on the chromato-

graphic behavior was explained in terms of the interaction of the metal ions with the gel matrix and the variation in the effective sizes of the solutes with the ionic strength of the media. The extent of the contribution of adsorption was found to be dependent on both sample concentration and the type of metal ion. Magnesium ions are influenced by only a negligibly slight adsorption effect over a wide range of sample concentrations, while strontium and barium ions are markedly influenced by the adsorption effect, especially at lower sample concentrations. The general aspects can be discussed on the basis of two representative types, the Mg-type and the Sr- or Ba-type, which are different in their affinity for the gel matrix.

EXPERIMENTAL

Sample solutions

All reagents used were of guaranteed grade from Wako Chemicals, unless otherwise stated. Sample solutions for magnesium, strontium, barium and EDTA ions were prepared by dissolving magnesium chloride, strontium chloride, barium chloride and disodium dihydrogen ethylenediaminetetraacetate in 0.1 *M* sodium chloride solution, respectively. Barium-133 available from the Radiochemical Center (Amersham, Great-Britain) was used as a tracer for barium ions.

Two standard solutions of Blue Dextran 2000 (Pharmacia Fine Chemicals 0.2%) and tritiated water (Radiochemical Center, used as a tracer) were also prepared by a similar method.

Eluent

The eluent used was 0.1 *M* sodium chloride solution.

Preparation of Sephadex columns

Sephadex G-15 (Pharmacia Fine Chemicals, particle size 40–120 μ) was suspended in the solution to be used as eluent and allowed to swell for 2 days. Undesirable fine particles were eliminated by repeated treatments *viz.* suspension, settling and decantation. A slurry of the prepared gel was poured into a column consisting of a glass tube, 1.5 × 60 cm, with a porous polystyrene disc at the bottom. A third of the column volume should be filled with the eluent before pouring the gel. After the gel had settled by allowing 500 ml of the eluent to flow through the column, excess gel above the desired height of the gel was removed, and a disc of filter paper (Tokyo Roshi 5 B) was placed on the top of the bed as a stabilizer. About 30 ml of a 0.01 *M* EDTA solution (disodium dihydrogen ethylenediaminetetraacetate in the eluent) was passed through the column to remove adsorbed impurities, and the bed was then washed again with 100 ml of the eluent before use. The total volume of the gel bed was adjusted to be 100 ml.

Procedure

One milliliter of the sample solution or standard solution was placed on the gel bed just as the last layer of the eluent soaked into the bed. Then the eluent was applied when the last portion of the sample solution vanished into the bed. The effluent was collected in fractions of 1.02 ml for magnesium, barium and EDTA ions and of 1.09 ml for strontium ions, at a flow rate of 20–30 ml/h maintained by hydrostatic pressure, with a Toyo Kagaku Drop Count fraction collector.

Unless otherwise stated all elutions were carried out at 20 ± 1°.

The amounts of solute in the fractions were determined as follows:

Blue Dextran. Measurement of absorbance at 620 mμ.

Alkaline earth metals. Complexometric titration with an EDTA solution, with the exception of the colorimetric determination[16] of magnesium at lower concentrations.

EDTA. Complexometric titration with a magnesium chloride solution.

Barium-133. Radioactivity measurement with a G. M. counter.

Tritium. Radioactivity measurement with a liquid scintillation counter.

Calculation of K_d values

The distribution coefficient, K_d, was calculated according to eqn. 1 (see also ref. 12),

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_e^{BD}}{V_e^{THO} - V_e^{BD}} \quad (1)$$

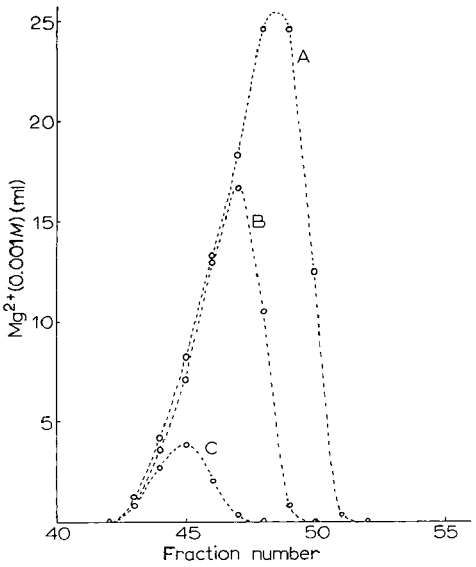
where V_0 is the void volume outside the gel particles, V_i the internal volume within the gel particles and V_e the elution volume of the sample corresponding to the maximum concentration of the elution peak. V_e^{BD} and V_e^{THO} represent the elution volumes of Blue Dextran (molecular weight = 2,000,000) and tritiated water, which were used as standard materials of $K_d = 0$ and 1, respectively.

RESULTS

In the course of a study[10] concerning the gel chromatographic behavior of magnesium and EDTA ions the present authors noticed that the elution patterns of magnesium and EDTA ions are dependent on the sample concentrations. Magnesium and EDTA ions gave symmetrical elution curves when 0.01 *M* sample solutions were eluted individually on a Sephadex G-15 column at room temperature (about 28°) with a 0.1 *M* sodium chloride solution. The K_d values were 0.62 and 0.22 for magnesium and EDTA ions, respectively. With increasing sample concentrations, however, the peak skewness for both ions became more marked and their elution volumes increased. An example is shown in Fig. 1 for EDTA ions.

It was also pointed out in a previous paper[12] that the chromatographic behavior of magnesium ions was different from that of calcium, strontium and barium ions. The appearance of the unsymmetrical elution curves for the latter three ions was explained in terms of the interaction of the solutes with the gel matrix. Therefore, magnesium, strontium and barium ions seem to be suitable samples for characterizing the interaction of the solutes with the gel matrix. Calcium is not included in this work, because it showed exceptionally marked peak skewing which could not be explained satisfactorily.

As shown in Fig. 2, the elution curve for magnesium ions at 0.01 *M* is symmetrical (S-shape). However, the K_d values increase with the increase of sample concentration. The appearance of unsymmetrical elution curves with sharp back edges (leading or L-shaped) is consistent with the preliminary observation for magnesium and EDTA ions described above. At extremely high sample concentrations shoulders



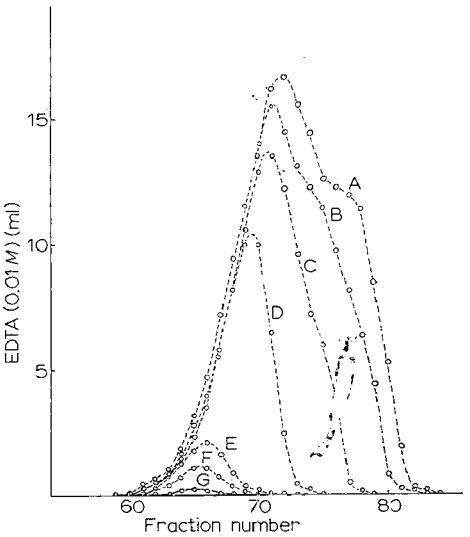


Fig. 1. Concentration dependence of elution curves of EDTA ions. Sample concentrations: A = 10^{-1} M; B = 5×10^{-2} M; C = 10^{-2} M.

Fig. 2. Concentration dependence of elution curves of magnesium ions. Sample concentrations: A = 2 M; B = 1.5 M; C = 1 M; D = 5×10^{-1} M; E = 10^{-1} M; F = 5×10^{-2} M; G = 10^{-2} M.

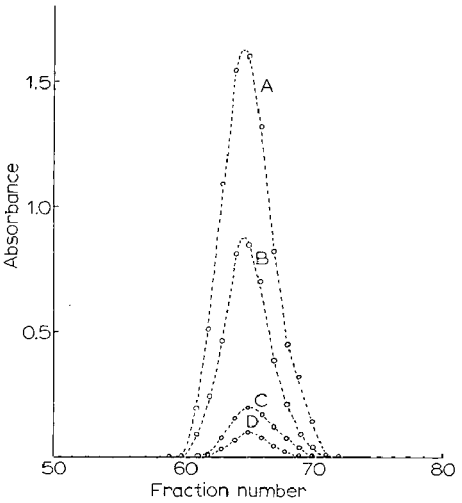


Fig. 3. Concentration dependence of elution curves of magnesium ions. Sample concentrations: A = 10^{-2} M; B = 5×10^{-3} M; C = 10^{-3} M; D = 5×10^{-4} M.

appear on the descending sides of the elution curves. On the other hand all elution curves at lower sample concentrations than 0.01 M are symmetrical and their elution positions remained unchanged in spite of the considerable variation in sample concentrations (Fig. 3).

The variation in the elution patterns for strontium and barium ions with sample concentrations is more complicated by the appearance of unsymmetrical elution curves with sharp front edges (tailing or T-shaped) at lower sample concentrations. As shown in Figs. 4 and 5 the elution curves for strontium ions belong to the T-shape in the concentration range of 10^{-1}–10^{-3} M and their K_d values increase with the decrease of sample concentrations. At higher sample concentrations than 0.1 M, however, the K_d values increase with the increase of sample concentrations, which is similar to the behavior of magnesium ions at sample concentrations higher than 0.01 M. Figs. 6 and 7 show the concentration dependence of barium ions. The experiments for barium ions in Fig. 7 were carried out using equal amounts of radioactive Ba-133 for each run. The general trend of variation in the elution patterns of barium ions is identical with that of strontium ions over a wide range of sample concentrations. However, the degree of variation in the K_d values of strontium ions at lower sample concentrations is somewhat less pronounced than that of barium ions. The elution curves for barium ions at higher sample concentrations appear as a whole to be composed of the T-shape. However, it becomes evident from quantitative analysis of the elution curves that such T-shape elution curves arise from the combined contributions of both T- and L-shapes. The front-to-back ratio[2], which is defined as the ratio of the front portion to the back portion of the peak width should be unity for a symmetrical elution peak, greater than unity for an L-shape elution peak and smaller than unity for a T-shaped elution peak. The front-to-back ratio of curve A in Fig. 6 is greater than unity for the top portion of the peak but, on the other hand is, smaller than unity for the lower portion.

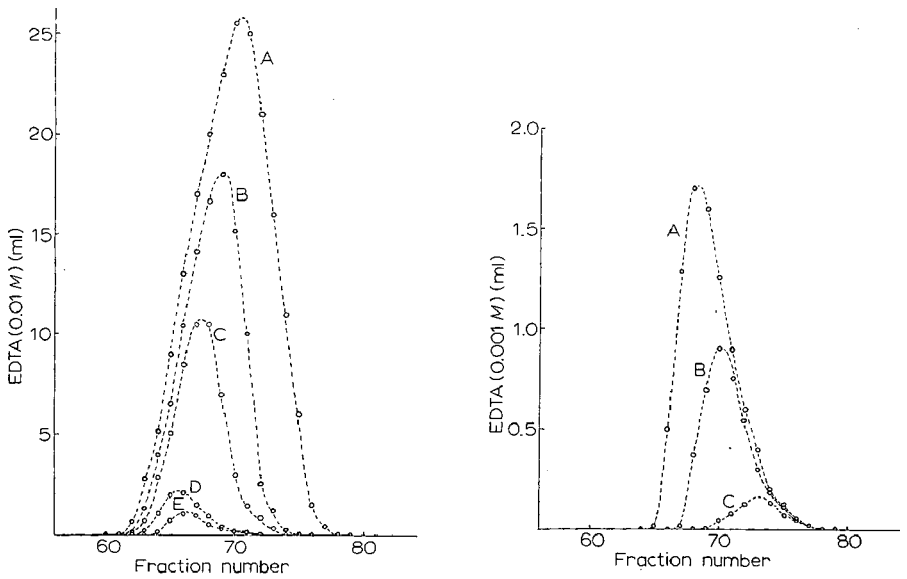


Fig. 4. Concentration dependence of elution curves of strontium ions. Sample concentrations: A = 2 M; B = 1 M; C = 5×10^{-1} M; D = 10^{-1} M; E = 5×10^{-2} M.

Fig. 5. Concentration dependence of elution curves of strontium ions. Sample concentrations: A = 10^{-2} M; B = 5×10^{-3} M; C = 10^{-3} M.

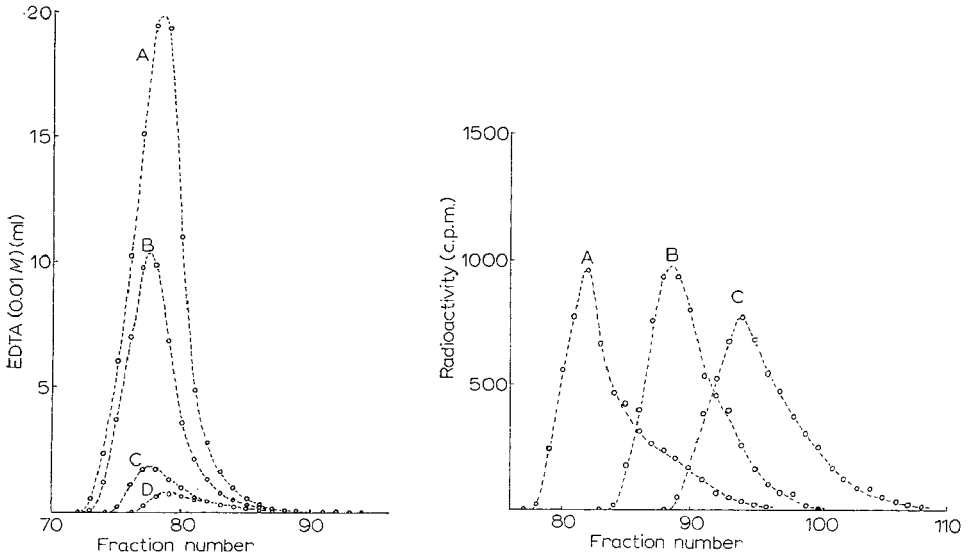


Fig. 6. Concentration dependence of elution curves of barium ions. Sample concentrations: A = 1 M; B = 5×10^{-1} M; C = 10^{-1} M; D = 5×10^{-2} M.

Fig. 7. Concentration dependence of elution curves of barium ions. Sample concentrations: A = 10^{-2} M; B = 10^{-3} M; C = 10^{-4} M.

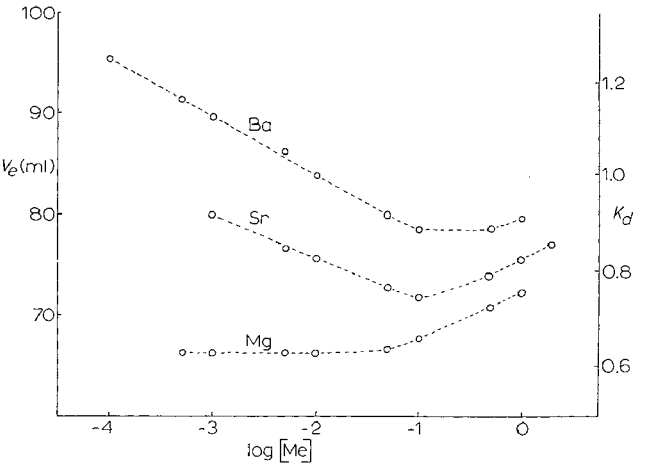


Fig. 8. Concentration dependence of the elution volumes or K_d values of magnesium, strontium and barium ions.

Therefore it is concluded that, although the peak skewing is still observed to a considerable extent, over the lower portion of the descending sides of the curves, even at relatively high sample concentrations, the top portion of the curve is primarily controlled by the effect which promotes the formation of the L-shape.

The overall picture for the three metals can be seen in Fig. 8, where the elution

volumes were plotted against the logarithms of the concentrations of the metal ion, [Me]. Barium ions at very low concentrations are eluted at the positions greater than the total liquid volume of the bed ($V_i + V_0 = 84$ ml). The slopes of V_e–log[Me] plots in Fig. 8 can be qualitatively correlated with the shapes of the corresponding elution curves in the following way:

$$\frac{dV_e}{d \log [Me]} = 0; \text{ S-shape}$$

$$\frac{dV_e}{d \log [Me]} < 0; \text{ T-shape}$$

$$\frac{dV_e}{d \log [Me]} > 0; \text{ L-shape}$$

Thus, the chromatographic behavior of magnesium ions can be described in terms of the combination of the S-shape and the L-shape, while strontium and barium ions are combinations of the T-shape and L-shape. It is a characteristic of strontium and barium ions that the minima appear in the intermediate region where two conflicting factors counteract to a comparable extent.

DISCUSSION

In most cases the gel chromatographic behavior of inorganic solutes can be explained in terms of the sieving effect based on their ionic sizes. A number of examples, however, are known which cannot be interpreted satisfactorily by the sieving effect alone. The possible side effects which may affect the chromatographic behavior of solute ions are classified into two categories. The first is ion exclusion which results in the faster elution of an ionic solute than that which could be expected from its ionic size. This has been successfully interpreted[2,11] in terms of the Donnan exclusion principle due to the presence of small amounts of negative charge (carboxylate ions) fixed on the Sephadex gel. Such phenomenon, however, can be eliminated by use of a suitable eluent containing a background electrolyte such as sodium chloride employed in this work. Secondly are the factors which cause the retardation of the solute on a column for a longer period than that which would be assumed from its ionic size. Such factors may be: (1) Coulombic interaction between the ionic solute and the fixed charge in the gel phase (ion exchange), and (2) chemical or physical adsorption of the solute on to the gel matrix (adsorption). Such interactions of the solute with the gel matrix may play an important role in determining the chromatographic behavior of the alkaline earth metal ions. Thus it is necessary to take into consideration the contribution of more than one factor in order to characterize the experimental results described above.

If only the sieving effect is operative and the effective size of the solute to be eluted is independent of the sample concentration one can expect the following experimental results: (a) The elution curve is symmetrical, corresponding to a linear isotherm. (b) The K_d value is independent of the sample concentration and always satisfies the relationship $0 \leqq K_d \leqq 1$. Most of the elution curves of the alkaline earth metal ions appear in the range of $0 \leqq K_d \leqq 1$ but it does not necessarily

mean the absence of the side effects. The contribution of the side effects can be estimated from the variation in both the K_d values and the shapes of elution curves. Since, as shown in Fig. 8, the general picture of the elution patterns varies greatly at concentrations near or around 0.1 M, the effect of sample concentrations will be discussed separately in the two sections concerning lower and higher sample concentrations below.

The effect at lower sample concentrations

Magnesium ions give the elution pattern expected from the contribution of the sieving effect alone. The effective size of the magnesium ions probably does not vary with sample concentration, because the total ionic strength of the medium is almost controlled by the contribution due to sodium chloride (0.1 M) in the eluent.

It is of interest that strontium and barium ions at lower sample concentrations give T-shaped elution curves and their elution volumes vary inversely with sample concentration. Such T-shape elution curves have more commonly been observed in adsorption chromatography[17] and accepted as evidence of a nonlinear isotherm of the convex type which can be related to the faster migration of the higher concentration portion of a solute band on the column. It therefore seems reasonable to discuss the gel chromatographic behavior of strontium and barium ions in terms of the interaction of the solutes with the gel matrix.

It has been reported[2,18] that, when pure water with no background electolyte was used as an eluent, the K_d values of inorganic electrolytes such as sodium chloride and calcium chloride were relatively small and increased with the increase of sample concentration, and moreover all the elution curves observed were unsymmetrical and L-shaped. From the additional fact that the elution patterns of uncharged substances such as glucose are not significantly dependent on sample concentrations, such a phenomenon was ascribed to the Donnan exclusion, resulting from the presence of negatively charged groups in the gel phase which exhibit a tendency to exclude anions from the gel phase, especially when small amounts of samples are applied to the gel bed. It is evident that the concentration dependence of strontium and barium ions in Figs. 5 and 7 is in marked contrast with the above observation. It can be ascribed to the difference in ionic strengths of the eluents used in both experiments. Since the eluent used in this work contains a sufficient amount of background electrolyte (0.1 M NaCl) to depress the action of the ion exchange sites, the variation in the K_d values of strontium and barium ions with sample concentrations cannot be ascribed to coulombic interaction between the solute ions and the gel matrix alone. Therefore, another mechanism of solute–gel interaction is required to explain the retardation of strontium and barium ions on the Sephadex column.

Some workers[18,19] have described a number of observations concerning the striking affinity of the dextran gel for aromatic compounds. Such abnormal adsorption of aromatic compounds has been explained in terms of the π-electron interaction between the solute molecules and the gel matrix. Of the inorganic oxo-acids hitherto investigated in our laboratory sodium salts of various oxo-acids of phosphorus[6] did not show any noticeable affinity for the gel matrix, showing a concentration dependence similar to that of magnesium ions. On the other hand molybdate and molybdophosphate[20] were markedly adsorbed on the Sephadex column. Thus, since

the retardation of the solutes on the Sephadex column takes place not only for cations but also for anions, the authors feel that such solute–gel interaction may not be based on the coulombic force in the main but on less electrostatic forces such as hydrogen bonding which is greatly dependent on the chemical structure of the solute. The hydroxy groups in the structures and the polarized water molecules in the hydration spheres of both solutes and Sephadex gel may play an important role in such an interaction. However, it is difficult at present to explain the difference between the adsorptive properties of such simple hydrated ions as alkaline earth metal ions. Therefore, it may be worthwhile to study the correlation between chemical structures and adsorptive properties of more complex inorganic compounds, such as has been discussed for aromatic compounds. The difference between elution behavior of phosphate and that of molybdate and molybdophosphate seems to be an interesting example to be characterized in the future.

The effect at higher sample concentrations

When sample concentrations became comparable with or larger than that of the eluent, the effect which promotes the formation of the L-shape (corresponding to a non-linear isotherm of the concave type) became predominant and the K_d values of the alkaline earth metal ions increased with sample concentration. Such a trend was also observed for anions such as EDTA, triphosphate and hypophosphate[6]. The general picture at higher concentrations is opposite to that at lower concentrations. Since the limiting K_d value or maximum K_d value at extremely high concentrations approaches but does not exceed unity, a primary contribution due to the sieving effect seems to be probable. It has been assumed[11,18] that there is a layer of immobile water molecules that firmly hydrate the gel matrix and are no longer able to act as a solvent available to ionic solutes. If the structure of the hydration layer breaks owing to the increase in ionic strength of the surrounding medium the effective internal volume available to the solutes becomes larger with sample concentration, which will consequently result in an increase in K_d values.

Another possibility is the variation in the effective sizes of the hydrated metal ions. It has also been assumed by EAKER AND PORATH[18] that the thickness of the hydration layer of an ionic solute or the effective size is dependent on the ionic strength of the medium. The fact that the extent of the variation in the K_d values of the alkaline earth metal ions decreases in parallel with the decreasing order of their sizes[21], Mg > Sr > Ba, can be explained on the basis of an additional assumption that the larger the thickness of the hydration layer of the metal ion, the greater the extent of the structure-breaking taking place.

It seems to be less probable that an alteration in the gel phase is a main factor. An increment, ΔV_i, in the internal volume may lead to an increment, $\Delta V_i \cdot K_d$, in the elution volume of a given metal ion. Therefore, the elution volume of a smaller metal ion might be expected to vary to a greater extent compared with that of a larger metal ion. However, no positive evidence for such an assumption can be seen in Fig. 8.

The appearance of the shoulders in Fig. 2 is probably attributable to the presence of multicomponents in solution. The formation of a magnesium chloro-complex such as $[MgCl_n]^{2-n}$ seems to be likely, though little information is available as to the chemical state of magnesium in such a concentrated solution. The effective

size of the magnesium chlorocomplex is assumed to be smaller than that of the corresponding hydrated ion, owing to the structure-breaking action of the ligands on the hydration layer.

It is concluded that sample concentration greatly affects the K_d values and the shape of the elution curves and that such concentration dependence can be classified into two representative types, the Mg-type and the Sr- or Ba-type, which differ in their affinity for the gel matrix .

Thus, if one tries to make a calibration curve between elution volumes and ionic sizes it is at first necessary to observe the sample-concentration dependence and to evaluate the contribution of side effects. The employment of an elution volume such as was obtained under the conditions corresponding to the region with negative slope in Fig. 8 should be avoided. A desirable criterion for such a purpose is that the elution curve is symmetrical and the slope in the V_e–log [Me] plot is zero.

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THIN-LAYER CHROMATOGRAPHIC BEHAVIOR OF A NUMBER OF METAL IONS ON DEAE-CELLULOSE IN THIOCYANIC ACID–ORGANIC SOLVENT MIXTURES

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SUMMARY

The thin-layer chromatographic behavior of 48 metals was investigated on a weakly basic anion exchanger, DEAE-cellulose, in binary solvent mixtures consisting of HSCN (0.1 to 2.1 *M*) and an organic solvent such as methanol, acetic acid, acetone, dioxane, etc. For the sake of comparison, the R_F values for the same metals on a microcrystalline cellulose, Avicel SF, in three solvent mixtures involving methanol, acetic acid and acetone are also presented. The addition of a protic solvent to the aqueous HSCN solution enhances the adsorption of many metals, in so far as the metals form stable thiocyanatocomplexes and their R_F values may be controlled predominantly by the ion-exchange mechanism. This is not the case for the nonprotic acetone–HSCN system, where mechanisms other than ion exchange may prevail to influence the R_F values of the metals. Useful multicomponent separations on DEAE-cellulose are presented to demonstrate the use of R_F measurements for predicting separations in these solvent systems.

INTRODUCTION

DEAE-cellulose along with other substituted celluloses has been extensively investigated in inorganic paper chromatography by LEDERER and his colleagues[1–8] and others[9–13]. Most of these works were carried out in aqueous acid or salt solutions, although LEDERER AND MOSCATELLI[2] investigated the influence of some organic solvents on the ion-exchange behavior of several metals on cellulose ion-exchange papers.

We have previously reported the thin-layer chromatographic (TLC) behavior of a number of metals on DEAE-cellulose in binary solvent systems consisting of HCl (or HNO_3) and an organic solvent[14,15], demonstrating the usefulness of DEAE-cellulose as an adsorbent in inorganic TLC. This work has been extended to obtain information about the chromatographic behavior of metals on DEAE-cellulose in binary solvent systems consisting of HSCN and an organic solvent. Generally, most metals exhibited a stronger adsorption on DEAE-cellulose in the mixed solvents than that in aqueous thiocyanate media[6,12], in the case where ion exchange is the mechanism involved in controlling the behavior of the metals. The solvent systems inves-

tigated allow a number of useful multicomponent separations to be conducted, particularly in methanolic HSCN media.

EXPERIMENTAL

Test solutions

For most metals 0.1 to 0.01 *M* stock solutions (0.1 *M* in HCl) were prepared from the chloride, oxide or metal. For Bi(III), Sb(III), Sn(IV), Zr and Hf, the acidity of the stock solutions was raised to 3 *M* HCl to prevent hydrolysis. 6 *M* HCl solutions were prepared for As(III) and Ti(IV) using the oxide and chloride, respectively. $AgNO_3$, $Pb(NO_3)_2$ and $TlNO_3$ were dissolved in 0.1 *M* HNO_3 to prepare 0.1 *M* stock solutions. Ammonium or sodium salts of Mo(VI), W(VI), Re(VII), Se(IV) and Te(IV) were each dissolved in deionized water to give 1–10 mg/ml. For niobium *ca.* 120 mg of Nb_2O_5 was fused with 3 g of $K_2S_2O_7$, and the resulting melt was taken up into 10 ml of 1 *M* tartaric acid.

Preparation of HSCN solution

A cation-exchange method[6] was used to prepare approximately 2.4 *M* HSCN solution, which was standardized both argentometrically and alkalimetrically and stored at 2° right up until use. This solution was used after an appropriate dilution with deionized water.

Preparation of thin-layer plates

A cellulose anion exchanger, MN-cellulose powder 300 DEAE (diethylaminoethylcellulose, Macherey, Nagel & Co.) was used as the adsorbent. Microcrystalline cellulose, Avicel SF(Avicel Sales Division, FMC Corp.) was also used. About 11 g of DEAE-cellulose were slurried with 100 ml of 1 *M* NH_4SCN solution, adjusted to pH 1.3 with dilute HSCN and stirred mechanically for 40 min. DEAE-cellulose was then washed with deionized water three times, each wash solution being removed by centrifugation. The DEAE-cellulose thus prepared was slurried with 73 ml of water and spread to a 250-μm thickness on five sheets of 200 × 200 mm glass plates. The plates were allowed to dry at 40° for 3 h after air-drying for 1 h and stored in a desiccator over a saturated KBr solution.

For the preparation of the microcrystalline cellulose plates, 20 g of Avicel SF was mixed with 75 ml of water and spread on 200 × 200 mm glass plates at a thickness of 250 μm. Avicel SF plates were dried and stored as above.

Application

A sample or stock solution, usually 0.5 μl, was applied to the plate 2.5 cm from one edge using a micropipette, and the plate was air-dried for 15 min before placing it in the chromatographic tank. The plate was then placed in the rectangular tank (21.5 × 21.5 × 10.5 cm) containing a solvent boat (20.5 × 2.2 × 1.8 cm) holding 40 ml of developing solvent and allowed to stand for 1 h to come to equilibration. Then the plate was immersed in the solvent and developed at room temperature until the solvent front had risen 15 cm from the start. All the solvent systems tested were binary, consisting of an organic solvent and HSCN of varying concentration. Among others, three organic solvents, *viz.*, methanol, acetic acid and acetone were tested in detail.

Detection

After development, the plate was dried under an IR lamp and metals were detected by spraying with the reagents below:

Hf, the rare earths, Sc, Th, U(VI), Y and Zr: 0.1 % aqueous arsenazo III solution.

Ag, Bi(III), Cd, Co(II), Cu(II), Fe(III), Hg(II), Ni, Pb(II) and Tl(I): dilute aqueous Na_2S solution.

Au, Ir(IV), Mo(VI), Pd(II), Pt(IV), Re(VII), Rh(III), Se(IV), Te(IV), and W(VI): 10 % $SnCl_2$ in 3 *M* HCl.

As(III), Mn(II) and W(VI): 10 % $AgNO_3$ in 10 % NH_4OH.

Al, Be, Ca, In and Mg: 0.1 % alizarin in 1 *M* NaOH.

Cd and Zn: 0.1 % PAN in ethanol followed by 1 *M* NaOH.

Ge, Sb(III) and Sn(IV): 0.05 % phenylfluorone in 95 % ethanol.

Ga: 0.5 % rhodamine B in 6 *M* HCl.

Sr and Ba: 0.1 % aqueous sodium rhodizonate followed by exposure to ammonia.

Nb: 0.1 % aqueous tannic acid.

Cr(III): 1 *M* NaOH followed by 10 % aqueous H_2O_2.

Ti(IV) and Ru(III): not used.

RESULTS AND DISCUSSION

R_F values for 48 metals chromatographed on DEAE-cellulose in methanol–HSCN media are illustrated in Fig. 1 as a function of HSCN concentration. The variation of the R_F values of the same metals with the volume concentration of methanol are shown in Fig. 2, where the HSCN concentration was kept constant at 1 *M*. The R_F values for the metals on DEAE-cellulose and on Avicel SF in methanol–, acetic acid–, and acetone–1 *M* HSCN media are given in Table I; the volume ratio of organic to aqueous was maintained constant at 3:1.

Adsorption behavior of metals

Among the metals tested, those which form stable thiocyanatocomplexes exhibit strong adsorption on the DEAE-cellulose over the HSCN concentration range tested (Fig. 1). Thus the R_F values for Zr, Hf, V(IV), Nb, Mo(VI), W(VI), Re(VII), U(VI), (Fe(III)), Co(II), the platinum group metals, Cu(II), Ag, Au, Zn, Cd, Hg(II), Ga, In, Sn(IV), Sb(III), and Bi(III) are generally low and mostly near or at start. Even metals which have comparatively high R_F values, *e.g.*, Re(VII), Fe(III), Co(II), Au, and Cd show increasing adsorption when the volume ratio of organic to aqueous is increased, their R_F values becoming almost zero in 95 % methanol media (Fig. 2). An exception is Ga, whose R_F values are less sensitive to the alcohol concentration. Obviously, the increating alcohol concentration reduces the concentration of the competitive thiocyanate ions and may facilitate the formation of thiocyanatocomplexes, thus allowing the thiocyanatocomplexes of the metals to be adsorbed easily on the ion-exchange sites of DEAE-cellulose[15].

The above mentioned metals have been shown to adsorb well on a strongly basic anion-exchange resin[16,17] and resin paper[6] from aqueous chloride–thiocyanate and pure HSCN solutions, respectively. In addition, the trends of the R_F values as

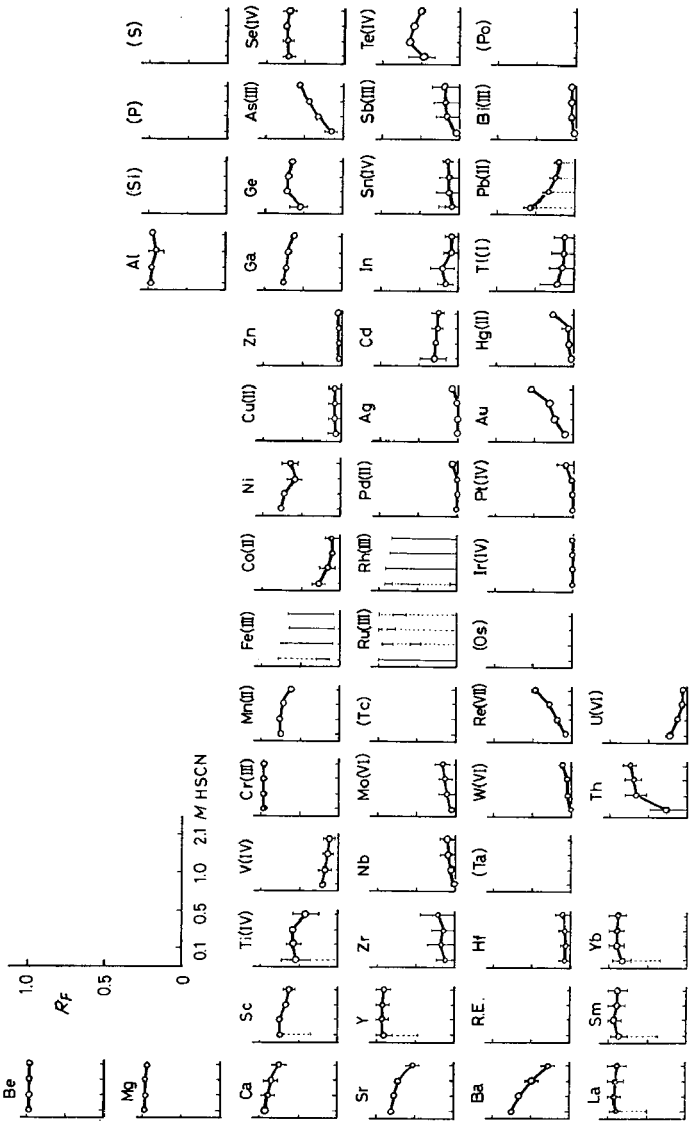

Fig. 1. R_F spectra of metals chromatographed on DEAE-cellulose in methanol–HSCN (3:1).

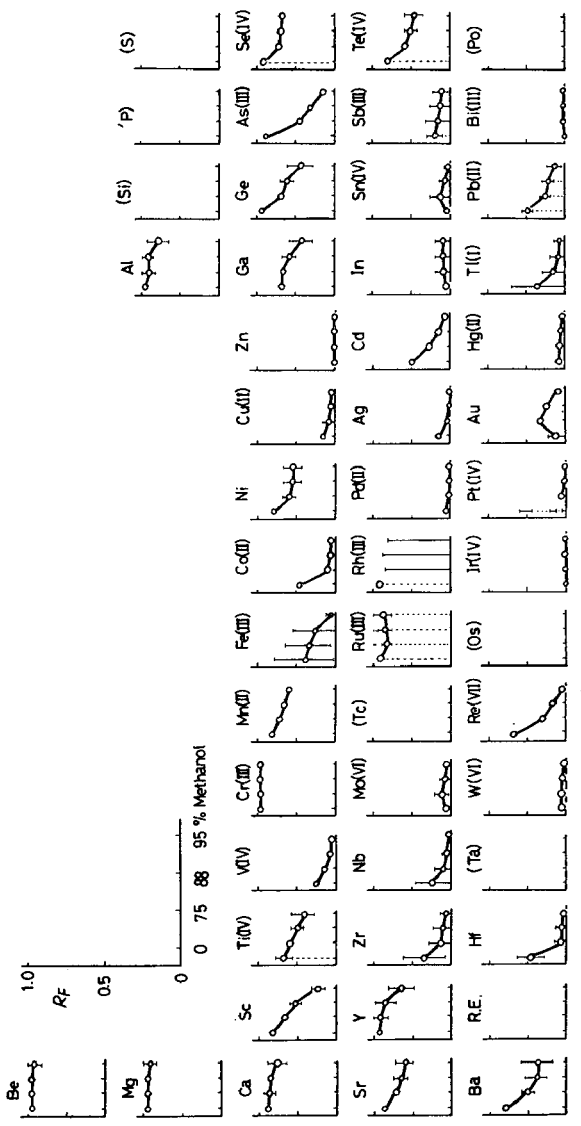

Fig. 2. R_F spectra of metals chromatographed on DEAE-cellulose in methanol–1 M HSCN.

TABLE I

R_F VALUES (× 100) ON DEAE-CELLULOSE AND AVICEL SF IN THREE DIFFERENT SOLVENT SYSTEMS

Metal	*Methanol–1 M HSCN (3:1)*		*Acetic acid–1 M HSCN (3:1)*		*Acetone–1 M HSCN (3:1)*	
	DEAE-cellulose	*Avicel SF*	*DEAE-cellulose*	*Avicel SF*	*DEAE-cellulose*	*Avicel SF*
Ag	0–5	61..93–96[a]	0–3	0–1..77	7–20	95–100
Al	82–100	89–97	39–53	49–59	89–100	58–91
As(III)	40–49		31–46..53	27–51	13–33, 41–51	43–51, 69–78
Au	27–36	92–97	7–42	81–90	89–97	95–100
Ba	41–57	45–61	47–60	58–66	23–63	36–54
Be	93–100	91–99	54–65	62–68	90–100	89–100
Bi(III)	0–5	77–87	0–3	0–3..15–29	7–16	45–63
Ca	77–93	75–83	49–61	54–61	89–100	45–69
Cd	19–33	81–89	0–5	43–53	59–91	95–100
Co(II)	3–15	87–97	0–3	49–57	87–96	97–100
Cr(III)	97–100	88–96	37–47	46–53	87–92	55–68..75–85
Cu(II)	0–15	87–96	0–32	0–4..64	30–41..91	95–100
Fe(III)	6–65	86–97	0–5	37–68	63–87	96–100
Ga	64–73	84–96	5–23	55–65	89–95	93–100
Ge	65–75	70–77	50–67	55–65	49–58	59–67
Hf	0–14	0–71	0–18	0–49	0–46, 89–94	0–74
Hg(II)	0–13	95–99	0–7	83–91	48–57..74–85	95–100
In	1–18	90–99	0–5	61–69	69–89	96–100
Ir(IV)	0–2		0–6		0–1	
La	78–100	77–87	35–51	35..41–54	62–91	46–78
Mg	95–100	81–91	55–67	52–60	86–91	45–73
Mn(II)	68–75	85–94	13–22	47–55	89–92	75–97
Mo(VI)	3–21	84–91	0–7	68–87	81–96	98–100
Nb	0–19	0–63	0–9	0–37	0–1..15	0–45
Ni	50–69	85–95	6–25	31–41	58–73	74–88
Pb(II)	0..18–32	39–53	0–10	0..49–56	0..43–56	94–100
Pd(II)	1–4	90–97	0–5	56..72–79	31–45	93–100
Pt(IV)	0–7	41–97	0–3	0–87	0–11..47	73–100
Re(VII)	24–35	77–88	19–27	65–73	55–72	91–98
Rh(III)	0–85	25–97	0–33	0–69	0–5..19–37..59–77	0–100
Ru(III)	0–2..77–89..95–100	61–97	0–35	0–75	0–2..69–81..88–92	20–100
Sb(III)	0–34	0–61	15–24	62–71	22–31..73–92	68–77, 91–100
Sc	61–71	86–93	5–15	58–65	87–92	97–100
Se(IV)	69–79	69–76	6–64	33–57	56–63	71–100
Sm	77–100	77–87	30–45	31..38–52	62–93	41–69
Sn(IV)	0–25	87–97	0–5	70–87	85–92	77–100
Sr	67–75	65–75	49–62	59–64	47–74	40–50
Te(IV)	53–65	55–64	0–23	2–37	32–55	40–47
Th	59–80	75–87	26–38	44–56	79–93	43–88
Ti(IV)	55–63	82–89	0–7..12	38–50	35..59–89	74–90
Tl(I)	0–29	0–27	0–33	0–3..49	0–37	0–32
U(VI)	3–10	91–97	0–2	51–58	44–55	97–100
V(IV)	7–20	88–97	0–5	51–60	57–72	96–100
W(VI)	7–11	81–87	0–1	67–72	0–1	99–100
Y	82–100	77–88	32–49	33..37–52	75–93	43–84
Yb	81–100	79–87	30–41	28..33–47	77–93	46–70
Zn	0–5	91–97	0–5	77–85	88–98	87–100
Zr	0–29	0–70	0–33	0–51	0–92	0–68

[a] ".." stands for weak streak.

a function of HSCN concentration in our methanol–HSCN system are very similar to those given by BAGLIANO *et al.*[6] for DEAE-paper in aqueous HSCN media. Taking into consideration the R_F values given by these authors for DEAE-paper and SB-2 resin paper the sequence of adsorption of thiocyanatocomplexes according to the systems involved is: anion exchange resin – aqueous HSCN > DEAE – methanolic HSCN > DEAE–aqueous HSCN.

The enhanced ion-exchange capability of DEAE-cellulose in mixed solvents over purely aqueous solvents has also been observed in metanol–HNO_3 (ref. 14) and methanol–HCl (ref. 15) systems.

Ion-exchange adsorption again seems to be the preponderant mechanism in determining the R_F values for metals forming thiocyanatocomplexes on DEAE-cellulose in acetic acid–HSCN media. As can be seen in Table I there are pronounced differences in the R_F values for metals forming thiocyanatocomplexes on DEAE-cellulose and on Avicel SF in acetic acid–HSCN medium, as is the case for the methanol–HSCN medium. R_F values on DEAE-cellulose are very low, near or equal to zero, while those on Avicel SF are fairly high. This is not the case for those metals, *e.g.*, the alkaline earths, the rare earths and Y, Cr(III), Ge, As(III), Se(IV) and Te(IV), which mostly do not form strong thiocyanatocomplexes. R_F values for the metals of this group do not differ appreciably from each other on DEAE-cellulose and Avicel SF.

In contrast to the protic solvent–HSCN systems, a great number of metals move upwards in acetone–HSCN media (Table I), irrespective of adsorbents, and there are actually no marked differences in R_F values on DEAE-cellulose and Avicel SF for most of the metals. A limited number of metals like W(VI), Ir(IV), Ag and Bi(III) remain at or near the start on DEAE-cellulose. Evidently, the diethylaminoethyl group does not function effectively in acetone–HSCN media, so that other mechanisms may prevail in determining the R_F values in this solvent system. The same observations are applicable in mixed solvent systems involving HCl and HNO_3.

Separation of metal ions

Knowledge of the R_F values for metals chromatographed on DEAE-cellulose in mixed solvent media will permit many separations of analytical interest to be conducted effectively. The methanol–HSCN system, particularly, may provide a number of useful separations, if we select a favorable concentration of HSCN and an appropriate volume ratio for a pair or group of metals. Results of multicomponent separations are illustrated in Fig. 3. The R_F value of a metal when chromatographed in a mixture does not differ from that obtained individually.

Even in solvent systems other than methanol– and acetone–HSCN interesting separations are feasible. For example in acetic acid–1 *M* HSCN (3:1):

W(VI) (0–3)–Re(VII) (22–30),
In(0–3)–Ga(6–17)–Al(42–54),
U(VI) (0–3)–Sc(7–13)–Th(31–41),

and in dioxane–1 *M* HSCN (3:1):

Ba(42–53)–Sr(67–76)–Ca(79–86)–Be(94–100),
Bi(III) (0–48)–Pb(II) (57–71),
Tl(I) (3–55)–Pb(II) (57–71),
Co(II) (10–28)–Cd(37–54)–Ni(81–89),
In(65–75)–Ga(90–100).

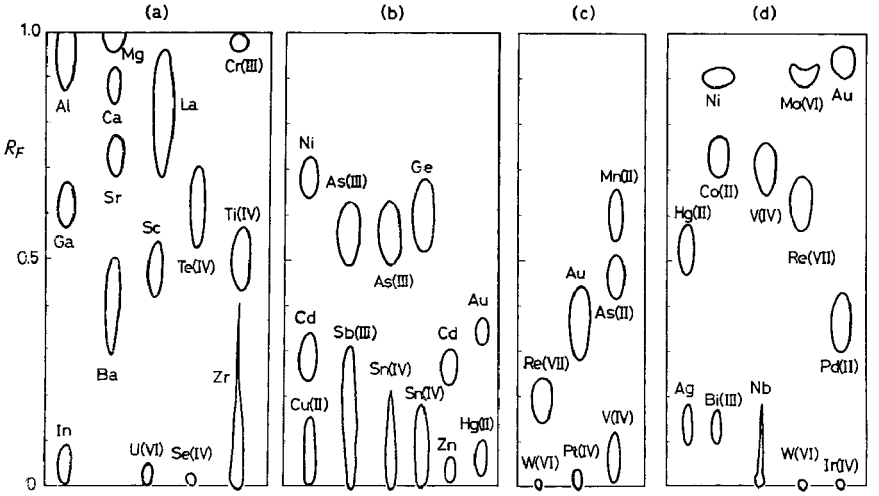

Fig. 3. Separations: (a) methanol–1 *M* HSCN (7:1), development time 30 min; (b) methanol–1 *M* HSCN (3:1), development time 55 min; (c) methanol–2.1 *M* HSCN (7:1), development time 30 min; (d) acetone–1 *M* HSCN (3:1), development time 40 min.

All of these separations were conducted on DEAE-cellulose. The values in parenthesis indicate the range of the R_F values ($\times 100$). When compared on the same volume ratio basis, the time necessary to achieve the separation in each solvent system is in the order: acetone (the most rapid) < dioxane < methanol < acetic acid.

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Notes

CHROM. 4900

The gas chromatographic behaviour of some isomeric derivatives of thiophene and furan

In this paper the gas chromatographic behaviour of the derivatives of thiophene and furan with different substituents on the 2 and 3 positions is examined on glass capillary columns with two different liquid phases. Previous work on these compounds is confined to the separation of the 2- and 3-isomers of acetylthiophene by PUCKNAT[1] at 125° with a 23 ft. × 1/4 in. column containing 20% Dow Corning F 1265 fluid on Diatoport, 60–80 mesh, and the separation of the 2- and 3-isomers of formylthiophene which were separated by OSTMAN[2] at 100° with an 10 ft. × 1/8 in. column containing 3% TCEPE on Aeropak, 80–100 mesh.

Experimental

Two glass capillary columns were used, containing, as liquid phases, trimer acid and Carbowax 20M. To obtain a uniform distribution of the liquid phase the two capillaries were precoated, the first one with a thin layer of carbon black[3] and the other with polytrifluorochloroethylene[4]. They were then coated, respectively, with 10 % trimer acid in methylene chloride and 15 % Carbowax 20M in methanol. Table I gives the column characteristics.

TABLE I

COLUMN CHARACTERISTICS

	Carbowax 20 M	*Trimer acid*
Length (m)	30	88
Internal diameter (mm)	0.32	0.29
Capacity ratio K'	3.8[a]	2.3[b]
Number of theoretical plates	50,000[a]	132,000[b]

[a] For *n*-tetradecane at 80°.
[b] For *n*-hexadecane at 130°.

All the measurements were carried out with a Carlo Erba gas chromatograph Model G.I. equipped with a flame detector and using N_2 as carrier gas.

The following pairs of isomers have been examined. Thiophene derivatives: 2-acetylthiophene[5], 3-acetylthiophene[6], 2-formylthiophene*, 3-formylthiophene[7], 2-trifluoracetylthiophene[8], 3-trifluoracetylthiophene[9], 2-benzoylthipohene[10], 3-benzoylthiophene[6], 2-acetylbenzothiophene[11], 3-acetylbenzothiophene[11]. Furane derivatives: 2-acetylfuran[5], 3-acetylfuran[12], 2-formylfuran*, 3-formylfuran[12], 2-benzoylfuran[5], 3-benzoylfuran[13]. The references give the methods used for their preparation. The experimental data measured on the two columns are reported in Table II. The influence of the liquid phase polarity (trimer acid, slightly polar, and Carbowax, more

* Technical product.

TABLE II

GAS CHROMATOGRAPHIC RESULTS FOR THE SEPARATION OF THE 2- AND 3-ISOMERS OF THIOPHENES AND FURAN DERIVATIVES

No.	*Compounds*	*Column temp. (°C)*		$\alpha = V_R'(2)/V_R'(3)$		K'		*Analysis time (min) (U opt.)*		$R^2/t \times 10^2$ (sec^{-1})	
		Carbowax 20M	*Trimer acid*	*Carbowax 20M*	*Trimer acid*	*Carbowax 20M*	*Trimer acid*	*Carbowax 20M*	*Trimer acid*	*Carbowax 20M*	*Trimer acid*
1	Acetylthiophenes	90	130	1.031	0.949	11.0	1.0	51	30	0.9	10.6
2	Formylthiophenes	80	80	1.138	0.987	8.8	2.4	41	73	8.6	0.1
3	Trifluoracetylthiophenes	80	80	1.393	1.181	4.5	1.1	21	33	8.4	13.7
4	Benzoylthiophenes	175	175	1.168	1.071	4.3	4.8	19	144	80.0	13.1
5	Acetylbenzothiophenes	170	170	1.082	1.040	4.4	5.2	20	156	30.0	2.2
6	Acetylfurans	65	65	0.942	1.033	10.8	2.0	50	60	2.8	0.3
7	Formylfurans	80	65	1.102	1.000	4.0	1.6	18	47	16.5	—
8	Benzoylfurans	170	170	1.462	1.210	4.5	1.6	21	48	416	8.3

polar) is shown by the values of the separation factor as given by the ratio of the corrected retention volumes of the isomers substituted on position 2 and position 3.

$$\alpha = V_R'(2) / V_R'(3)$$

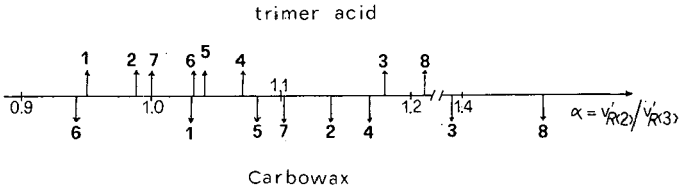

Fig. 1. Separation factors (α) on trimer acid and Carbowax columns for the compounds reported in Table II.

$\alpha < 1$ indicates a reversed elution order. By comparing the values for each couple of 2 and 3 isomers an inversion of the elution order ($\alpha < 1$) is observed for the acetyl and formyl thiophenes on trimer acid while with Carbowax the 2-isomer is eluted after the 3 isomer ($\alpha > 1$).

This behaviour can be explained by the fact that with these pairs of compounds the 2-isomer is more volatile and also more polar than the 3 isomer and consequently with the trimer acid column, which is less polar, the 2-isomer is eluted earlier, while with Carbowax, which is more polar, it is eluted after the 3 isomer.

The polarity and volatility act in opposite direction and on Carbowax the

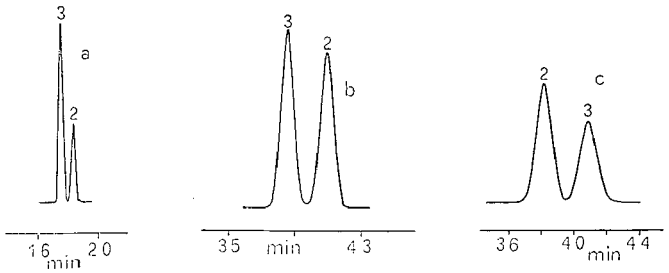

Fig. 2. Gas chromatographic separation on a Carbowax column of the 2- and 3-isomers of: (a) formylfuranes; (b) formylthiophens; (c) acetylfurans.

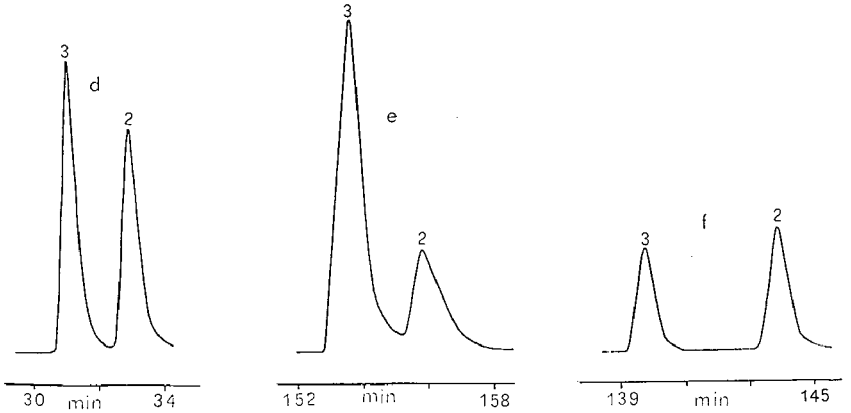

Fig. 3. Gas chromatographic separation on a trimer acid column of: (d) trifluoroacetylthiophenes; (e) acetylbenzothiophenes; (f) benzoylthiophenes.

first effect predominates and consequently the 2-isomer is retained more strongly than the 3-isomer.

For the other compounds, there is also an increase of α values on the Carbowax column with respect to the trimer acid, but since the 2-isomers are always eluted later from both the columns it is not possible to state if they are less volatile or more polar than the 3-isomer. With the formyl furans the values of α, very near to one, in the trimeric acid can be due to the fact that the 2-isomer is slightly more volatile than the 3-isomer but also more polar, as seen by their behaviour on the Carbowax column, and thus as the two effects almost compensate, the separation is very small. The stronger interaction observed from the gas chromatographic behaviour of thiophene and furan derivatives when a polar group is in position 2, as opposed to 3, can be explained from the stronger formation of hydrogen bonding with the free hydroxyl groups of the liquid Carbowax phase.

The α values on the liquid phases used for the compounds reported in Table II are plotted in Fig. 1. For all the compounds, except No. 6 (acetyl furan), separation factors on the trimer acid columns are less than those on Carbowax.

For the great majority of the compounds examined the gas chromatographic separation of the two isomers in 2 and 3 position is more easily achieved with a very polar liquid phase. With Carbowax the difference in polarity between the two molecules is enhanced. In a few cases, where the differences in vapour pressure are higher, as in the case of the acetylthiophenes, it is more convenient to use a less polar phase, such as the trimer acid; for the other pairs of compounds the vapour pressure differences are small and have an opposite effect to the polarity and consequently with Carbowax the α values differ more from unity. The Carbowax column in spite of a lower number of theoretical plates than the trimer acid column, generally gives a better separation with shorter retention times. This situation is shown from the last column of Table II where the ratio between the resolution and the analysis time measured under the best column conditions is reported. In Figs. 2 and 3 are shown some of the separations achieved.

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CHROM. 4908

Chromatographie an makroporösen Ionenaustauschern

Weit verbreitet ist die Benutzung von Ionenaustauschern mit Gelstruktur. Aus dem Bedürfnis, auch organische Substanzen wie Huminsäuren aus Wasser zu entfernen, sind makroporöse (makroretikulare) Ionenaustauscher entwickelt worden. Sie entstehen im allgemeinen unter Zusatz von Fällungsmitteln während der Polymerisation und bilden dadurch eine schwammartige starre Oberfläche aus.

Entscheidend für scharfe Auftrennungen ist eine möglichst schnelle Einstellung des Austauschgleichgewichts. Diesem Ziel kommt man bei Verwendung herkömmlicher Ionenaustauscher durch Verkleinerung der Korngrösse, Erhöhung der Temperatur und Verringerung des Vernetzungsgrades näher[1]. Da an der grossen und starren Oberfläche makroporöser Ionenaustauscher die für die Austauschgeschwindigkeit verantwortlichen Transportvorgänge schneller abzulaufen vermögen, war zu erwarten, dass an makroporösen Ionenaustauschern im Vergleich zu den gelartigen Ionenaustauschern bessere Trennungen auftreten. Mit speziellen Lewatit® Ionenaustauschern* haben wir entsprechende Untersuchungen vorgenommen.

Experimentelles

Die Auftrennungen des Substanzgemisches aus Uracil (I), Thymin (II) und Guanosin (III)[2] wurden an den stark sauren Ionenaustauschern Lewatit®-Merck S 1080 (70–150 mesh) und Lewatit® SP 1080 (70–150 mesh) durchgeführt, wobei der letztgenannte makroporös war. Die Trennungen fanden in temperierbaren Glassäulen von 1 m Länge und 1.5 cm Innendurchmesser statt. Das Füllen der Säulen geschah durch einfaches Sedimentieren bis zu einer Füllhöhe von 90 cm. Als Fliessmittel diente eine Pufferlösung aus 0.14 *M* Ameisensäure, die zugleich 0.35 *M* an Natriumchlorid war und mit konz. Ammoniak auf pH 3.7 eingestellt wurde. Für jede Trennung wurde ein Gemisch aus je 1 mg Uracil, Thymin und Guanosin gelöst in 1 ml Fliessmittel vorsichtig auf die Säule pipettiert. Die Durchlaufgeschwindigkeit blieb bei allen Untersuchungen konstant 1 ml/min. Die Detektion erfolgte mit einem Uvicord bei 254 nm. Die Thermostatisierung bei 10, 25, 38 und 50° war auf ± 1° genau.

TABELLE I

ZUSAMMENSTELLUNG DER VERSUCHSBEDINGUNGEN

	Lewatit®-Merck			
	S 1080 (gelartig)	*SP 1080 (makroporös)*	*M 1080 (gelartig)*	*MP 1080 (makroporös)*
Kapazität, mval/g	4.3	4.3	4.7	3.9
Korngrössenbereich des gequollenen Harzes, μ	120–360	160–310	190–320	165–310
Bettvolumen, cm^3	160	160	44	44
Durchflussgeschwindigkeit, ml/min	1	1	1	1
Substanzgemisch	Uracil (I) Thymin (II) Guanosin (III)		*p*-Aminobenzoesäure (IV) Salicylsäure (V) Gallussäure (VI)	
Fliessmittel	Ammoniumformiat pH 3.7		0.1 *M* Salzsäure in 50% Äthanol	

* Im Handel erhältlich bei E. Merck, Darmstadt.

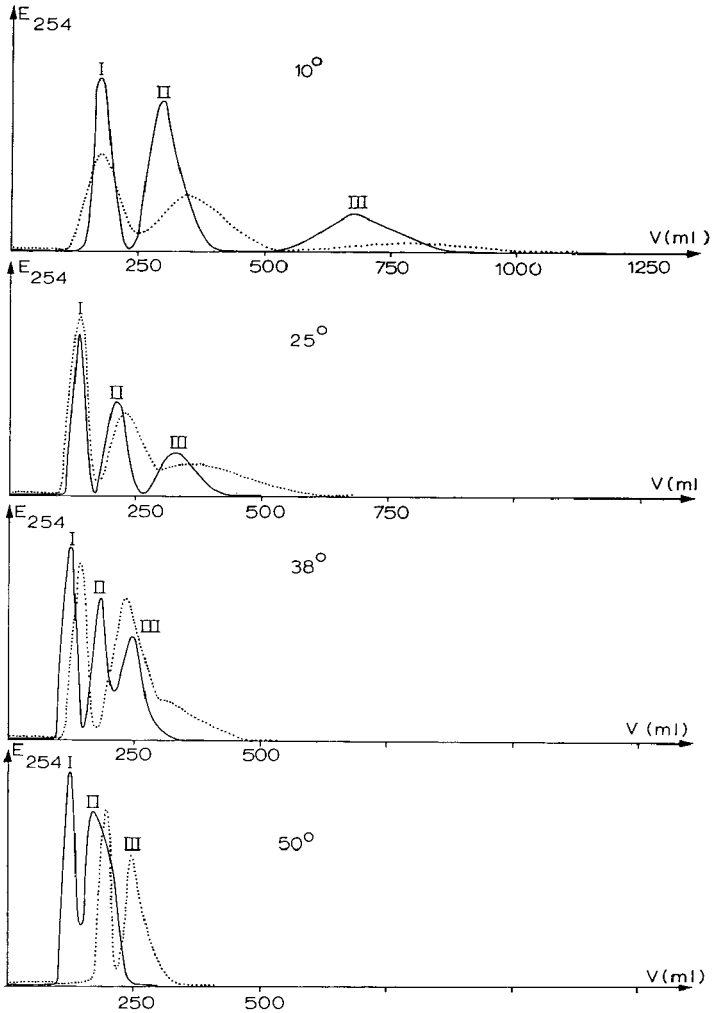

Fig. 1. Chromatographische Trennung von Uracil (I), Thymin (II) und Guanosin (III) an Lewatit® SP 1080 (———) bzw. Lewatit® S 1080 (. . . . .) bei verschiedenen Temperaturen.

Die Auftrennungen der aromatischen Säuren *p*-Aminobenzoesäure (IV), Salicylsäure (V) und Gallussäure (VI)[3] wurden an den stark basischen Ionenaustauschern Lewatit® M 5080 (70–150 mesh) und dem makroporösen Lewatit® MP 5080 (70–150 mesh) durchgeführt. Die Füllhöhe betrug hier 25 cm, als Fliessmittel diente 0.1 *M* Salzsäure in 50 % Aethanol. Für jede Trennung wurden je 10 mg der Einzelkomponenten gelöst in 1 ml Fliessmittel auf die Säule gegeben. Die übrigen Versuchsbedingungen stimmten mit den oben beschriebenen überein.

Ergebnisse und Diskussion

Ziel unserer Untersuchungen war es, die unterschiedlichen chromatographischen Eigenschaften von gelartigen und makroporösen Ionenaustauschern aufzuzeigen. Die vergleichenden Trennungen mussten daher unter denselben Versuchsbedingungen

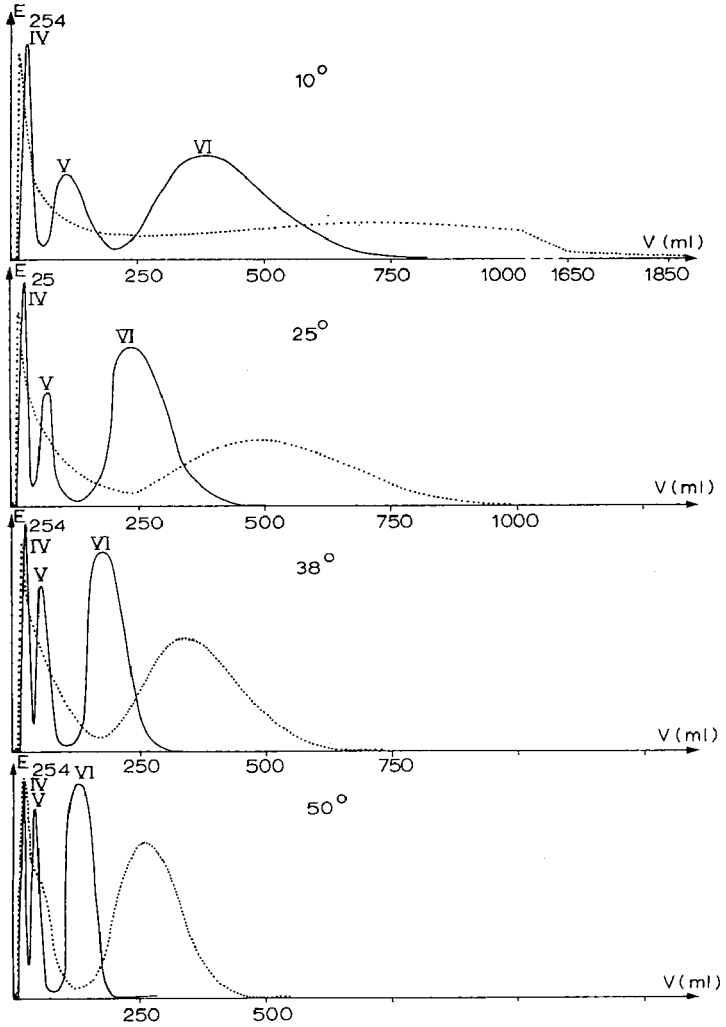

Fig. 2. Chromatographische Trennung von *p*-Aminobenzoesäure (IV), Salicylsäure (V) und Gallussäure (VI) an Lewatit® MP 5080 (———) bzw. Lewatit® M 5080 (. . . . .) bei verschiedenen Temperaturen.

ablaufen und ausserdem sollten die Kapazitäten und Korngrössenbereiche der gegenübergestellten Ionenaustauschertypen möglichst übereinstimmen. In Tabelle I sind die entsprechenden Daten für die verwendeten zwei Ionenaustauscherpaare zusammengestellt.

In der Fig. 1 sind die Trennungen von Uracil, Thymin und Guanosin an stark sauren Ionenaustauschern bei 10, 25, 38 und 50° wiedergegeben. Man erkennt, dass die Auftrennung des Substanzgemisches in jedem Falle an dem makroporösen Ionenaustauscher günstiger verläuft als an dem gelartigen. Weiterhin wird deutlich, dass durch Erhöhung der Temperatur und der damit einhergehenden Beschleunigung der Austauschkinetik die Elutionsvolumina abnehmen. Die maximale Trennschärfe tritt an dem makroporösen Lewatit® SP 1080 bei 25° auf, während bei 38 und 50°

die Peaks schon zu eng zusammenrücken. Die Trennungen an dem gelartigen Lewatit® S 1080 verlaufen zwar ähnlich, aber mit geringerer Auflösung und grösseren Elutionsvolumina.

Bei den basischen Ionenaustauschern machten sich in den chromatographischen Trenneigenschaften die Unterschiede zwischen makroporösen und gelartigen Ionenaustauschern noch deutlicher bemerkbar. Nach Fig. 2 werden *p*-Aminobenzoesäure, Salicylsäure und Gallussäure an dem makroporösen Lewatit® MP 5080 bei 38° am besten und bei den anderen Temperaturen hinreichend getrennt. An dem gelartigen Lewatit® M 5080 trennen sich *p*-Aminobenzoesäure und Salicylsäure nur bei 50° andeutungsweise auf. Gallussäure lässt sich bei allen Temperaturen abtrennen, wobei sich allerdings der Peak mit abnehmender Temperatur stark verbreitert. Allgemein verringern sich auch bei diesem Trennbeispiel die Elutionsvolumina mit steigender Temperatur.

Die Ursache für die besseren Trennungen an makroporösen Ionenaustauschern kann nur in der grösseren Porenweite und dem starreren Aufbau der Matrix liegen, wodurch bei den Austauschvorgängen die Gel-Kinetik zugunsten der Film-Kinetik zurückgedrängt wird. Weiterhin ist anzunehmen, dass bedingt durch die grosse Oberfläche an makroporösen Ionenaustauschern zusätzliche Verteilungsvorgänge eher auftreten als an gelartigen Ionenaustauschern.

Grössere Trennschärfe hat man bisher durch Verringerung der Korngrösse von gelartigen Ionenaustauschern bis herunter auf *ca.* 1 μ erreicht, wobei man einen grösseren Druckabfall in der Säule und damit Chromatographie unter Druckanwendung in Kauf nehmen musste. Makroporöse Ionenaustauscher eröffnen nach den hier vorliegenden Ergebnissen die Möglichkeit, dass gute Trennungen unter einfachen apparativen Bedingungen schon an Harzen mit Durchmessern von 100–300 μ zu erreichen sind.

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CHROM. 4911

Detection and estimation of cyclamate by thin-layer chromatography

With the rapid growth in the consumption of low-calorie and dietetic foods, a great deal of attention is being focussed on non-nutrition sweeteners. While a number of artificial sweeteners have been identified and developed, cyclamates and saccharin are the main sweeteners of commercial importance.

Chemically cyclamate is the sodium or potassium salt of cyclohexyl sulfamic acid which is susceptible to bromination due to its unsaturation[1-3]. KORBELAK[4] has separated and identified saccharin, cyclamates, dulcin and P-4000 on silica gel by thin-layer chromatography (TLC). KOJIMA[5] employed a $AgNO_3$–hydroquinone reagent for the identification of the salts of saccharin and cyclamate and used many solvent systems for their separation. SALO[6] used a mixture of acetylated cellulose and polyamide as the adsorbent for the separation of sweeteners and identified them under UV light. WOIDICH *et al.*[7] have separated saccharin and cyclamate on acetylated cellulose–polyamide adsorbent and employed dichlorofluorescein as the spray reagent before visualisation under UV light.

In our method, Silica Gel G is used as the adsorbent and fluorescein solution as the spray reagent after bromination. The thin-layer plates coated with Silica Gel G and containing cyclamate are subjected to bromine vapour when bromination takes place. The plate is then sprayed with 0.05% fluorescein solution when eosin is formed; unchanged fluorescein only remains on those parts of the layer where there are other compounds which can be brominated. In visible light, pink spots are seen on a yellow background. Detection by UV light is even more sensitive, the background fluorescence is quenched, and only the zones where there are cyclamates glow yellow green. Instead of the above procedure the cyclamate spots can also be developed on 0.04% sodium fluorescein impregnated Silica Gel G layers and then subsequently detected by bromination. The sensitivity is a little bit higher in this case.

The pink spots can be extracted with ethanol and the fluorescence measured quantitatively with a spectrophotometer or fluorimeter.

Experimental

Extraction of cyclamate from the sample. 50 ml of sample is acidified with 10 ml HCl and extracted with two 25 ml portions of ether. The combined ether extract is washed three times with 5 ml water. The ether solution is concentrated and made up to a volume of 10 ml.

If the sample is solid or semisolid 10 g of the sample is blended with 50 ml water, 10 ml HCl is added and the above procedure is then followed.

Preparation of the thin-layer plates. 10 × 20 cm glass plates are coated with a slurry of Silica Gel G in water (1:2) to a thickness of 250 nm and dried at room temperature for ½ h. The plates are activated at 110° for 1 h in an air oven and then in a desiccator.

Solvent system. Ethyl acetate–isopropyl alcohol–acetone–methanol–water (50:15:15:4:16).

Spray reagent. 0.05 g sodium fluorescein is dissolved in 10 ml absolute alcohol.

Preparation of standard cyclamate solution. 0.1 g of sodium cyclamate is dissolved in 100 ml of distilled water. 10 μl of this solution is equivalent to 10 μg of cyclamate.

Spotting and development. The Silica Gel G plates are spotted with 20 μl of the ethereal extract of the cyclamate along with standard cyclamate solutions containing 10, 20, 30, 40 and 50 μg of cyclamate leaving 1 cm from the base line. The plates are developed in the ascending manner with the mobile solvent mentioned above. When the solvent front reaches 10 cm from the starting point, the plate is removed from the solvent and dried in air for ½ h.

Detection of spots. Bromine vapour is applied to the plate by allowing bromine vapour from a litre flask containing one or two drops of bromine to pour over the surface; excess bromine should be avoided. The plate is kept at room temperature for 5 min to remove excess bromine and then sprayed with the 0.05% alcoholic fluorescein solution when pink spots on yellow background become visible ($R_F = 0.56$).

Quantitative analysis. The portions of the silica gel containing the pink spots are carefully scraped off with the sharp end of a knife and placed in test tubes. 10 ml of ethyl alcohol is added to each test tube and filtered. The filtrate is collected in 25 ml volumetric flasks. The residue is washed with ethanol and made up to volume with ethanol. The fluorescence is measured in a spectrophotometer (Zeiss type) at a wavelength of 540 mμ (Table I).

TABLE I

SPECTROPHOTOMETER READINGS AT 540 mμ

The unknown is calculated from the equation $y = -0.6141 + 1236.86x$.

Concentration of cyclamate (y) (μg)	*Optical density mean (x)*	*Standard deviation*	*C. V. (%)*
10	0.010	0.0014	14.0
20	0.016	0.0022	13.8
30	0.023	0.0038	16.5
40	0.034	0.0068	20.0
50	0.041	0.0094	22.0

The plot of concentration of cyclamate (in μg) is plotted against optical density and the unknown concentration of cyclamate is read by intrapolation from the standard line.

Conclusions

With the widespread use of cyclamate as an artificial sweetener in dietetic candies, jellies, canned fruits and particularly in carbonated beverages, a rapid and sensitive method is needed for its detection and quantitative estimation. The solvent system used here has the advantage that it separates cyclamate from other sweetening agents *viz.* sucrose ($R_F = 0.25$) and saccharin ($R_F = 0.19$).

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CHROM. 4927

Simple method of gradient elution in thin-layer chromatography of lipids

A number of techniques for gradient thin-layer chromatography (TLC) which are particularly useful for separating lipid mixtures having a wide range of polarity have been described[1,2]. This communication describes a simplified procedure for gradient elution which requires only the addition of a glass trough to the usual TLC equipment.

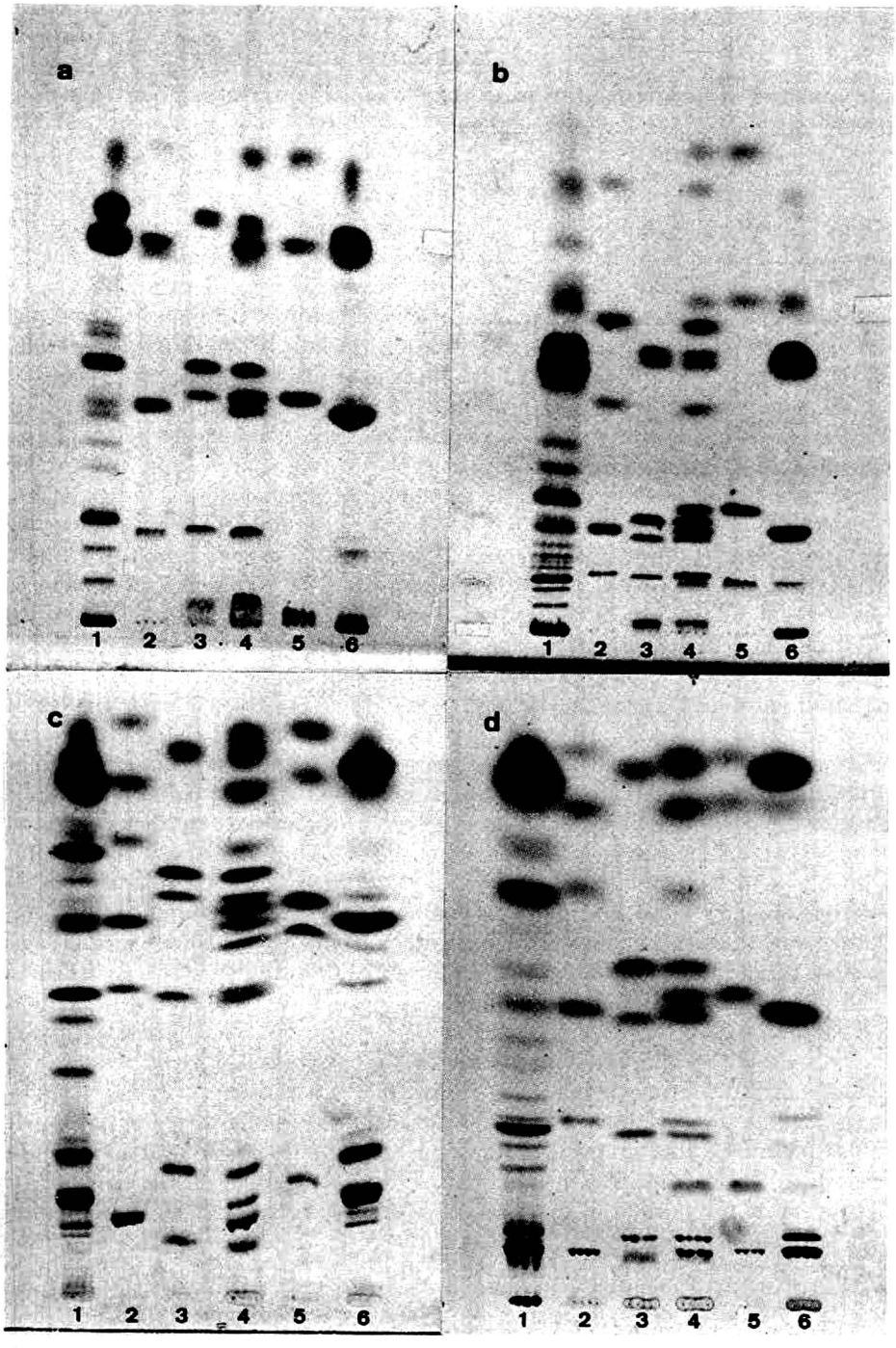
A tank and its filter paper liner are first saturated with a volatile non-polar solvent (the "atmospheric" solvent). The thin-layer plate is activated by heating, cooled to room temperature, and the samples applied. An excess of solvent is shaken onto the liner to help prevent desaturation while the tank is open. The trough is quickly placed on the bottom of the tank near the back. A relatively polar solvent ("trough" solvent) is added to the trough and the plate is placed in it. The trough solvent evaporates as it moves up the plate and is gradually replaced by atmospheric solvent. Both evaporation and influx are necessary to produce gradients sufficiently steep to be useful for most lipid analyses. The lipids to be separated are initially compressed into a thin band. As the polarity of the developing solvent mixture decreases, the more polar components lag behind.

Two examples each of neutral and total lipid separations are shown in Fig. 1 and a reasonably complete list of trough solvents potentially useful with this method is presented in Table I. Benzene, cyclohexane, and alkanes containing five to eight carbon atoms are good atmospheric solvents. Almost any combination of polar solvent in the trough and non-polar solvent in the atmosphere will produce a suitable gradient for neutral lipids. Also useful are trough solvents made by mixing two or more polar solvents, or a polar solvent and a non-polar solvent. Systems for the fractionation of total lipids are more difficult to devise (see note 4 below). One approach is the addition of about 0.5 volumes of methanol–formic acid–water (3:2:1) to one volume of moderately polar trough solvent, and about 0.3 volumes of methylene chloride, chloroform, or diethyl ether to one volume of atmospheric solvent. This gradient method has also been combined successfully with argentation TLC and reversed-phase TLC. In reversed-phase TLC the trough contains the less polar solvent. A system which has been used successfully is a stationary phase of 5% paraffin oil on Kieselguhr G, a methanol atmosphere, and butanone–acetonitrile (2:1) as the trough solvent.

The factors affecting (or effecting) separation interact in a complex fashion. Although the method is best described as gradient elution, in certain aspects it resembles polyzonal, flux gradient, and vapor-programmed TLC. Moreover, unlike other gradient elution methods, the polarity decreases with time and the composition of the solvent at the bottom of the plate is essentially constant. The method is not suitable for column chromatography, and the theoretical analyses of gradient elution by NIEDERWIESER AND HONEGGER[1] and SNYDER AND SAUNDERS[2] are not entirely applicable.

The following comments are intended as practical aids:

(1) The trough solvent must be volatile enough to evaporate from the plate, but not so volatile as to evaporate completely from the trough during chromatography.


a
1 2 3 4 5 6
b
1 2 3 4 5 6
c
1 2 3 4 5 6
d
1 2 3 4 5 6

In use, its composition may change slightly, but adjustments in trough volume or geometry are seldom necessary.

(2) Temperature is important because of its effect on volatility, and must be reasonably well controlled. Each trough solvent is best suited to a restricted temperature range, *e.g.*, diethyl ether to -5 to $+5°$.

(3) In a series of atmospheric solvents of similar polarity (*e.g.*, hydrocarbons), increased volatility flattens the gradient. Perhaps the major factor is decreased fractionation of the developing solvent by the adsorbant; with higher-boiling hydrocarbons (*e.g.*, heptane) a region of very steep gradient appears somewhere on the plate, resulting in a non-polar developing solvent above this region (see Fig. 2). This region is undoubtedly homologous to the secondary front(s) observed in polyzonal development, and the effect is important for many separations achieved with the present method. Defects in the plate and sometimes sample composition can affect solvent fractionation, leading to irregular lipid fractionation. For instance, in Fig. 1c the "hump" evident in column 5 appears to be due to the presence of stearic acid.

(4) The greater the difference in polarity between atmospheric and trough solvents, the greater the gradient. However, if gradients are desired that are steep enough to chromatograph both phospholipids and neutral lipids, the miscibility of the two solvent mixtures must be considered. The production of two phases at some time during the run is not always a problem. Indeed, a second phase does appear just above the origin with the system shown in Fig. 1c. The second phase can be eliminated by decreasing the proportions of the two most polar components of the trough solvent, but the region of very steep gradient in the middle of the plate also disappears. The separation of lipids of intermediate polarity is thus enhanced at the expense of both the neutral and polar lipid groups. The separation resembles that shown in Fig. 1d, which exhibits a single phase throughout. Again, decreased solvent fractionation is probably responsible.

(5) The mesh of the adsorbant affects the rate of development, and therefore the steepness of the gradient. Silica Gel G (Merck or MN) on 200-mm plates will produce approximately the same total gradient with a certain solvent system as does the finer mesh Adsorbosil 3 on 100-mm plates.

(6) Increasing the length of run by using a longer plate generally gives improved resolution only for lipids that chromatograph high on the shorter plates. However,

Fig. 1. Gradient elution chromatography of neutral lipids (a and b), and total lipids (c and d). Solvent systems are: (a) ethyl acetate in benzene; (b) diethyl ether in hexane–methylene chloride (10:1); (c) ethylene chloride–dimethoxyethane–methanol–formic acid–water (5:5:5:1:1) in heptane–methylene chloride (3:1); (d) hexane–2-propanol–water (5:7:1) in hexane. Plate b was chromatographed at 5°, the rest at room temperature (20–24°). Samples for a and b are: (1) total lipid extract of female *Ascaris lumbricoides*; the complexity of the neutral lipids of this nematode is due mostly to a large number of closely related glycoside esters; (2) in descending order, the compounds chromatographed are: cholesterol palmitate, cholesterol acetate, α-tocopherol, cholesterol, and 1-octadecylglyceryl ether; (3) triglyceride (double band of tristearin and tripalmitin, see 1b), 1,3-distearin, 1,2-distearin, 1-monostearin, and glycerol; (4) mixture of 2, 3, and 5; (5) squalene, methyl stearate, octadecanol, and stearic acid; (6) Total lipid extract of rat liver. Samples for c and d are identical except that 2 also contains sphingomyelin (lowest band), 3 contains phosphatidylethanolamine (second lowest band), and 5 contains phosphatidylcholine (lowest band). Plates 80 × 100 mm (projector slide cover glasses) coated with 0.2 mm layers of Adsorbosil 3 were developed to the top (about 20 min) with 2 ml trough solvent and 20 ml atmospheric solvent (tank volume 1 l). Plates were visualized by spraying with conc. sulfuric acid containing 3% conc. formalin, and charring on a 200° hot plate. Adsorbant and standards were purchased from Applied Science Laboratories, Inc., State College, Pa., U.S.A.

TABLE I

TROUGH SOLVENTS FOR USE FROM 0° TO 25°

The criteria for inclusion are volatility and low cost. Solvents are listed in order of their boiling points. Physical constants are those given in the *CRC Handbook of Chemistry and Physics.*

Solvent	*Mol. wt.*	*b.p.*[a]	ε[b]	*Solubility in water*[c] (*g*/100 *g*)	*Notes*[d]
Furan	68	32		"i"	ether
Methyl formate	60	34	8.5	30^{20}	ester
Diethyl ether	74	35	4.3	7.5^{20}	ether
Propene oxide	58	35		65^{30}	epoxide
Methylene chloride	85	40	9.1	2^{20}	alkyl chloride
Dimethoxymethane	76	44		d	acetal
Ethyl formate	74	54			ester
Diethylamine	73	56		∞	secondary amine
Acetone	58	57	21	∞	ketone
Methyl acetate	74	57	8.5	32^{20}	ester
Chloroform	119	61	4.8	1.0^{15}	alkyl chloride
Methanol	32	65	33	∞	alcohol, PLS
Tetrahydrofuran	72	66		∞	ether
Diisopropyl ether	102	68	3.9	$c.2^{20}$	ether, readily forms peroxides
Ethyl acetate	88	77	6.0	8.6^{20}	ester
1,3-Dioxolane	74	78		∞, d	acetal
Ethanol	46	78	26	∞	alcohol
n-Butylamine	73	78		∞	primary amine
2,2-Dimethoxypropane	104	80		d	ketal
Butanone	72	80	19	50^{20}	ketone
Methyl acrylate	86	80			ester, unsaturated
Acetonitrile	41	82	39	∞	nitrile, PLS
2-Propanol	60	82	18	∞	alcohol
tert.-Butanol	74	83		∞	alcohol
Diisopropylamine	101	84		8^{20}	secondary amine
1,2-Dimethoxyethane	90	85		∞	diether
Isopropyl acetate	102	89	5.6	3^{20}	ester
Triethylamine	101	90	3.1	1.5^{20}	tertiary amine

The following solvents may be used at room temperature when mixed with compounds listed above:

Water	18	100	80	∞	excellent solvent for non-lipids, PLS
Formic acid	46	101	48	∞	carboxylic acid, PLS
Nitromethane	61	101	39	10^{20}	lowest boiling nitroalkane, PLS
1,4-Dioxane	88	102	3.0	∞	diether
Pyridine	79	115		∞	tertiary amine
Acetic acid	60	118	6.2	∞	carboxylic acid

[a] Although vapor pressure is the important parameter, boiling point data are readily found for all these solvents. B.p. is misleading only for alcohols, whose volatility it underestimates.

[b] Dielectric constant is a convenient measure of "polarity" (elution power), but weighs hydrogen-bonding properties too lightly. Hence diethyl ether is more polar than methylene chloride. Solubility in water, and comparison of mol. wt. and b.p., afford other estimates of polarity.

[c] i = insoluble; d = decomposes.

[d] PLS = poor lipid solvent: does not by itself dissolve triglycerides sufficiently to prevent severe streaking during TLC.

the relative order of separation of different lipids (*e.g.*, diglycerides and sterols) is not necessarily constant during a run.

(7) Layer thickness exerts considerable effect because solvent efflux and influx vary with surface area, while developing solvent volume varies with layer volume. Thus, thicker layers give shallower gradients. Uniform layers are obviously important unless layer thickness is intentionally varied.

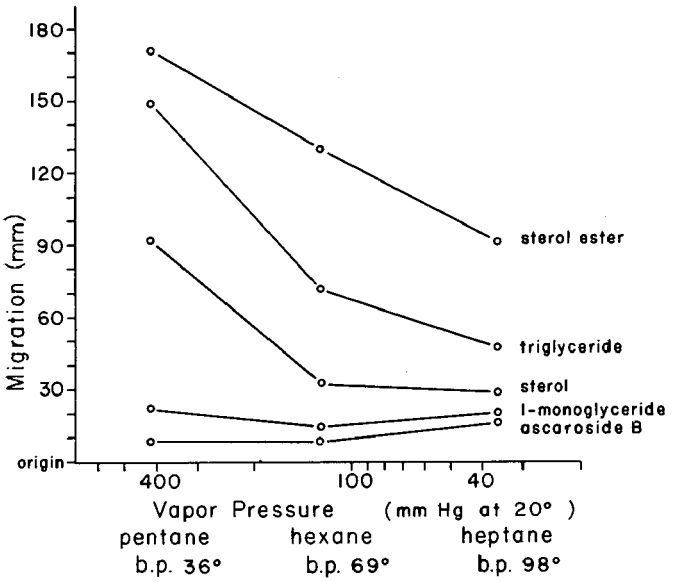


Fig. 2. The relation between the volatility of atmospheric solvent and the migration of lipids. Plates 200 mm long and coated with 0.25-mm layers of Silica Gel G (Merck) were developed to the top with ethyl acetate as trough solvent and the atmospheric solvent indicated.

Despite a large number of interacting variables, the separations are highly reproducible if the parameters are controlled. For some purposes the advantages of a continuous gradient, single development, and simple procedure may outweigh the advantages of direct control over solvent composition in multiple development systems or in continuous gradient systems requiring relatively complex apparatus.

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1 A. NIEDERWIESER AND C. C. HONEGGER, *Advan. Chromatog.*, 2 (1966) 123.
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Received June 23rd, 1970

News

Meeting

EASTERN ANALYTICAL SYMPOSIUM

The Eastern Analytical Symposium will be held at the Statler Hilton Hotel, New York City, N.Y., U.S.A. on November 18th, 19th and 20th, 1970. Sponsoring societies include the American Chemical Society, the Society for Applied Spectroscopy and the American Microchemical Society. For further details contact Melvin Goodman, Johnson and Johnson, 501, George Street, New Brunswick, N.J. 08903, U.S.A.

The portions of the programme of possible interest to "chromatographers" are given below:

Wednesday morning, November 18

APPLICATIONS OF ANALYTICAL TECHNIQUES IN WATER POLLUTION RESEARCH

L. L. CIACCIO, presiding

Sampling and analysis of Hudson River water, T. J. KNEIP, N.Y.U. Institute of Environmental Medicine, Sterling Forest, N.Y.

The monitoring of pesticides and lead in Lake Michigan, H. MANCY, University of Michigan, Ann Arbor, Mich.

Use of automated analytical techniques in the evaluation of a model stream, R. CARDENAS AND A. MOLOF, New York University, The Bronx, N.Y.

The analysis of secondary waste effluents, L. L. CIACCIO, General Telephone and Electronics Labs, Bayside, N.Y.

Wednesday afternoon, November 18

ADVANCES IN PERMEATION CHROMATOGRAPHY

J. N. LITTLE, presiding

The mechanism of the separation in permeation chromatography, E. F. CASASA, Mellon Institute, Pittsburgh, Pa.

Data handling in permeation chromatography, J. CAZES, Mobil Oil Research Center, Paulsboro, N.J.

The influence of permeation chromatography on polymer science, D. BLY, DuPont Co., Wilmington, Del.

Rigid glass substrates for permeation chromatography, W. HALLER, National Bureau of Standards, Washington, D.C.

COULOMETRY

A. J. BARD, presiding

Coulomb — the absolute chemical standard, G. MARINENKO, National Bureau of Standards, Washington, D.C.

Galvanic coulometry, P. A. HERSCH, Gould National Batteries, Minneapolis, Minn.

Coulometry in organometallic chemistry, M. D. MORRIS, University of Michigan, Ann Arbor, Mich.

Determination of total S, N and Cl and their specific detection in GLC effluents by microcoulometry, H. V. DRUSHEL, Esso Research Labs., Baton Rouge, La.

GENERAL PAPERS

D. L. NASH, presiding

Measurement of smoke density of TGA/photometric analysis, A. A. LOEHR AND P. F. LEVY, DuPont Co., Wilmington, Del.

High-resolution mass spectrometric investigation of treated solid waste and airborne particulates, J. L. SHULTZ, R. A. FRIEDEL AND A. G. SHARKEY, JR., Bureau of Mines, Pittsburgh, Pa.

Transmission and ATR infrared spectroscopy in the analysis of normal and diseased tissues, and certain biochemical compounds, F. S. PARKER, New York Medical College, New York.

Optical activity of Cu(II) complexes of the dipeptides with aliphatic side chains and a procedure for the analysis of mixtures of dipeptides differing in location of asymmetric center, B. VERMA AND Y. P. MYER, SUNY at Albany, N.Y.

Identification of compounds including polymers using molecular weight chromatography, D. G. PAUL AND C. E. BENNET, Chemalytics Corporation, Unionville, Pa.

Pulse polarographic determination of trace elements in alkali salt solutions, R. G. GREENE AND R. H. LANSING, Eastman Kodak Co., Rochester, N.Y.

Thursday, November 19

LIQUID–LIQUID CHROMATOGRAPHY WORKSHOP

P. W. ALMQUIST, presiding

Friday afternoon, November 20

ADVANCES IN AIR POLLUTANT ANALYSIS

R. K. STEVENS, presiding

Carbonate and noncarbonate carbon in atmospheric particles, P. K. MUELLER, R. W. MOSLEY AND L. B. PIERCE, Air Pollution Industrial Hygiene Laboratory, Department of Public Health, Berkeley, Calif.

Use of chemiluminescence for detection of ozone and excited oxygen, J. A. HODGESON AND K. J. KROST, National Air Pollution Control Administration, Raleigh, N.C.

Rotational microwave spectroscopy and air polution measurements, H. W. HARRINGTON, Hewlett-Packard, Palo Alto, Calif.

Measurement of hydrogen sulfide, sulfur dioxide and methyl mercaptan in ambient air by gas chromatography, A. E. O'KEEFE, R. K. STEVENS AND J. D. MULIK, National Air Pollution Control Administration, Cincinnati, Ohio.

GENERAL PAPERS

A. Z. CONNER, presiding

Solution thermodynamics of geometrically isomeric olefins by gas–liquid chromatography, R. L. STERN AND T. R. FAULKNER, Oakland University, Rochester, Mich.

The role of the solvent in ion-exchange, G. E. JANAUER, State University of New York at Binghamton, Binghamton, N.Y.

Quantitative aspects of spectrodensitometry of thin-layer chromatograms, J. C. TOUCHSTONE, S. S. LEVIN AND T. MURAWEC, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Pyrolytic analysis of functional group-containing organic compounds, S. F. SARNER AND E. J. LEVY, Chemical Data Systems, Oxford, Pa.

Analysis of α-hydroxy fatty acids and α-glyceryl ethers as their cyclic boronate derivatives, S. RAMACHANDRAN, R. F. KRUPPA AND R. S. HENLEY, Applied Science Laboratories, Inc., State College, Pa.

Vapor programming techniques for thin layer chromatography, H. S. HIRSCH, Brinkmann Instruments Inc., Westbury, N.Y.

Apparatus

A new, high performance recording spectrophotometer, the Cary 17, was introduced and demonstrated at the 21st Pittsburgh Conference in Cleveland, Ohio, March 1–6, 1970. The solid state instrument covers the wavelength range 186 nm to 2.65 μ. Also included are digital scanning, digitally coupled scan and chart drive.

For further information apply to the publisher under reference No. Chrom. N-282.

CHROM. 4979

A SOLID ELECTROLYTE DETECTOR FOR THE DETERMINATION OF TRACE AMOUNTS OF OXYGEN IN THE PRESENCE OF ARGON AND OTHER GASES

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Morganite Research and Development Ltd., Battersea, London S.W.11 (Great Britain)

(Received June 15th, 1970)

SUMMARY

A simple high-temperature electrochemical detector is described which is specific for oxygen gas. This feature enables the analysis of trace amounts of oxygen in argon to be carried out with the chromatographic column at normal temperatures. The response for oxygen is quantitative and exactly obeys the Nernst electrochemical equation. Other gases which react with oxygen at high temperatures, *e.g.* hydrogen and methane, give signals of opposite polarity to oxygen. The stability of the detector is dependent on the type of electrical connections to the sensing head. The use of a conductive reaction cement has been found to give connections which give a stable output with low electrical noise.

INTRODUCTION

The determination of trace amounts of oxygen, *ca.* 10^{-6} ml at s.t.p., in argon by gas–solid chromatography presents a number of difficulties. The two gases have almost identical retention times on columns of Linde 5A molecular sieve at room temperature[1] and on porous polymer packings, such as Porapak Q. Separation can easily be achieved when both gases are present in similar concentrations or as minor components, by cooling the column to $-72°$[2,3]. When oxygen is present as a trace component, the analysis is only possible when the column parameters are carefully optimised. However, chromatographic columns operating under these conditions give rise to characteristically long retention times and broad based peaks.

Indirect methods have been described in the literature in which hydrogen is used as carrier gas and oxygen in the sample is catalytically converted into water and separated by the usual chromatographic techniques[4–6]. Such methods, with thermal conductivity detectors, have sensitivities of the order of 10^{-4} ml oxygen.

Column materials which are specific for oxygen and give separation at room temperature, such as fire-brick impregnated with bull's blood, have been investigated[7]; but as materials containing haemoglobin are unstable, the column life is limited.

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Because of the lack of suitable column materials, detectors which are specific for oxygen have been reported. PHILLIPS *et al.* described a redesigned Hersch oxygen cell for the determination of oxygen in argon without complete separation and reported a limit of detection of 1×10^{-5} ml oxygen[8]. LITTLEWOOD *et al.* described an oxygen reaction coulometer incorporating a similar detection system for measuring changes in oxygen content caused by the introduction of combustible material[9].

High-temperature galvanic cells have been investigated for measuring the partial pressure of oxygen in hot gases[10,11]. These cells consist basically of a solid electrolyte containing oxygen vacancies, *e.g.* zirconia stabilized with calcium oxide, with electrodes in contact with opposite faces of the ceramic. The theoretical open circuit potential between the electrodes is related to the ratio of the oxygen partial pressures p_1/p_2 at the two electrodes by the Nernst equation:

$$E = \frac{RT}{nF} \ln \frac{p_1}{p_2} \qquad (1)$$

where

E = e.m.f. developed by the cell, V
T = temperature of the electrodes, °K
R = the universal gas constant
F = Faraday's constant
n = number of electrons transferred in the electrode process (4, for electrode reactions involving 1 gmole of oxygen).

The logarithmic dependence makes it possible to measure very small quantities of oxygen, and gases which react with oxygen at high temperatures.

This paper describes such a cell which has been developed in this laboratory for use as a specific detector for determining trace amounts of oxygen, hydrogen and methane in argon and other inert or non-reactive gases.

APPARATUS

The detector (Fig. 1) consists of a cylindrical tube, closed at one end, of impervious zirconium oxide stabilized with calcium oxide mounted into an aluminium block which carries inlet and outlet connections for the carrier gas. Two platinum electrodes are held in contact with the closed end by means of a ceramic reaction cement made from a mixture of orthophosphoric acid (0.4 ml, 25% v/v) and powdered stabilized zirconia (100 mesh, 2 g). (The cement is applied to the end of the tube with the electrodes in position and the arrangement slowly heated to 1100°C.) Purified helium carrier gas is fed into the zirconia tube through an alumina tube carrying the inner platinum electrode. The tube assembly is totally enclosed by a silica tube which carries the reference gas, either air or helium. The sensing head of the detector is heated by a wire-wound tube furnace at 1050°C. Electrical interference from the furnace is prevented by electrostatically screening the silica tube with platinum foil held at earth potential. Standing voltages developed between the electrodes are backed off with a simple potentiometer circuit and the out-of-balance signal is recorded with a potentiometric recorder (20 mV f.s.d.).

The detector is built into a glass and stainless steel chromatograph which incorporates a purification train, comprising molecular sieves, titanium sponge and

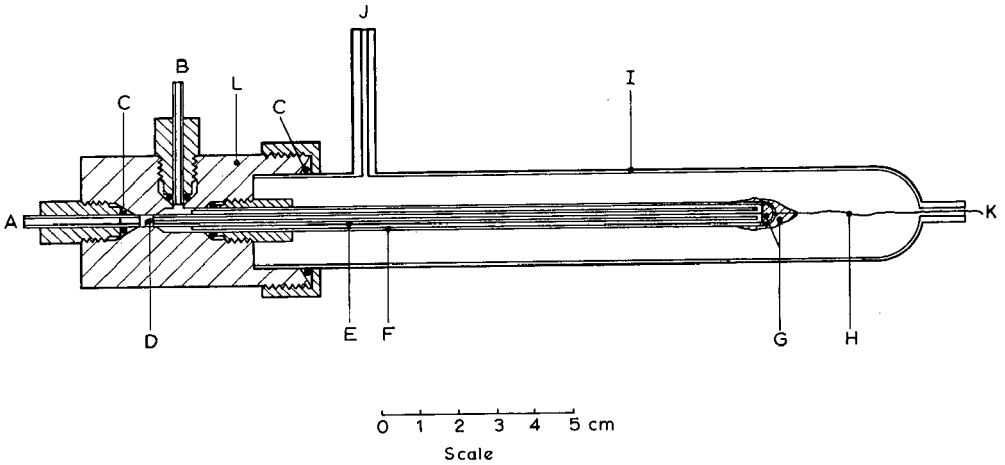

Fig. 1. Diagram of the oxygen detector. A = Carrier gas inlet; B = carrier gas outlet; C = silicone rubber O-rings; D = inner platinum electrode; E = alumina tube; F = zirconia-lime sheath; G = ceramic reaction cement; H = outer platinum electrode; I = silica tube and platinum foil; J = reference gas inlet, helium or air; K = reference gas outlet; L = aluminium block at earth potential.

"Hopcalite" columns with a sampling and low-pressure standardizing system similar to the one described by BERRY[12]. The chromatographic column consists of a coiled stainless steel tube (120 cm × 4 mm I.D.) containing 40–50 mesh molecular sieve 5A which has been activated at 350°C *in situ* for 24 h. The column is electrically heated and normally operates at 65°C.

RESULTS AND DISCUSSION

Dependence of standing voltage on flow of carrier gas, with air as reference gas

The results plotted in Fig. 2 show that the standing voltage tends to a maximum of 330 to 340 mV. The Nernst equation indicates that the voltage should be independent of flow rate provided that the partial pressure of oxygen in the carrier gas does not alter and the temperature of the electrodes remains constant. This discrepancy is due to a slight porosity of the electrolyte at high temperatures with the consequent

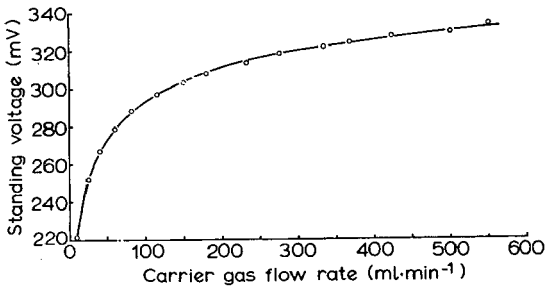

Fig. 2. Effect of carrier gas flow rate on the standing voltage with air as reference gas. Detector temperature, 1050°C.

diffusion of molecular oxygen from air into the carrier gas. The following treatment enables errors caused by the use of slightly pervious tubes to be calculated together with an accurate measurement of the partial pressure of oxygen in the ingoing carrier gas.

Diffusion of molecular oxygen through the electrolyte is primarily governed by the difference in partial pressures of oxygen inside and outside the tube. This is essentially constant provided that the partial pressure of oxygen in the carrier gas does not exceed 10^{-2} atm and thus the leak rate of molecular oxygen will be constant. If the carrier gas, at 1 atm pressure, flowing at V ml·min^{-1} contains v ml of oxygen per ml and if the leak rate of oxygen is u ml·min^{-1} then:

$$p_{O_2} = \frac{u}{V} + v \qquad (2)$$

where p_{O_2} is the partial pressure of oxygen in the carrier gas as measured by the detector.

The validity of this equation is shown by the straight-line graph when p_{O_2} is plotted against $1/V$ (Fig. 3). From the slope and intercept, the measured leak rate is 6.9×10^{-4} ml oxygen per min and the concentration of oxygen in the carrier gas with no leakage is 5×10^{-7} ml O_2 per ml of carrier gas. Hence by determining the partial pressures of oxygen in the carrier gas at a number of different flow rates the leak rate of oxygen through the electrolyte, or through any other part of the system, can be obtained together with an accurate value for the partial pressure of oxygen in the ingoing carrier gas.

As a result of this variation of voltage with flow, the chromatographic column must be sufficiently long to prevent any pressure pulses caused by the introduction of the sample from interfering with the response of the detector.

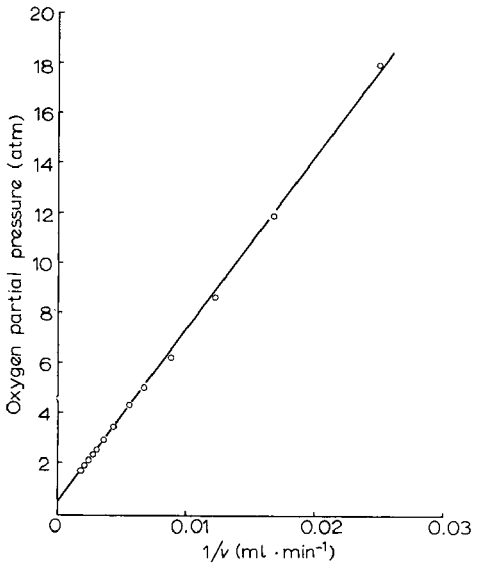

Fig. 3. Oxygen partial pressure of carrier gas as a function of flow rate.

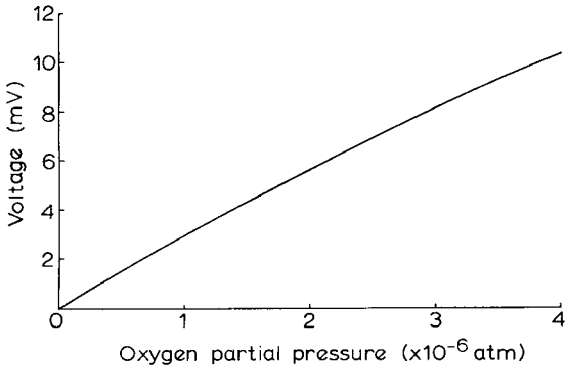

Fig. 4. Theoretical detector response for oxygen with rectangular peaks. Carrier gas p_{O_2}, 9.2 × 10^{-6} atm; detector temperature, 1050°C; reference gas, air.

Detector response

Due to the exact relationship between voltage and oxygen partial pressure (eqn. 1), it is possible to calculate the oxygen response of the detector. Consider a small quantity of oxygen, injected at reduced pressure, which remains as a discrete volume as it passes through the detector, then the expected peak height response, based on rectangular shaped peaks, would be as shown in Fig. 4, for the given conditions. The graph is not linear due to the logarithmic nature of the Nernst equation, but it does provide an acceptably linear part over the lower range, *i.e.* up to 3 × 10^{-6} atm. However, when a gaseous mixture containing oxygen is injected into a chromatographic system, dilution with the carrier gas occurs and therefore the detector response will be lower than that given by the theoretical curve. As the flow rate of the carrier gas and the oxygen peak elution time are known for this system (70 ml·min^{-1}, 19 sec), the reduced oxygen partial pressure of the injected gas can be calculated. Assuming that the detector signal is proportional to the oxygen partial pressure (true for pressures $\leqslant 3 \times 10^{-6}$ atm) and triangular peaks of constant width are produced, then the theoretical response can be recalculated. Fig. 5 shows that excellent agreement is

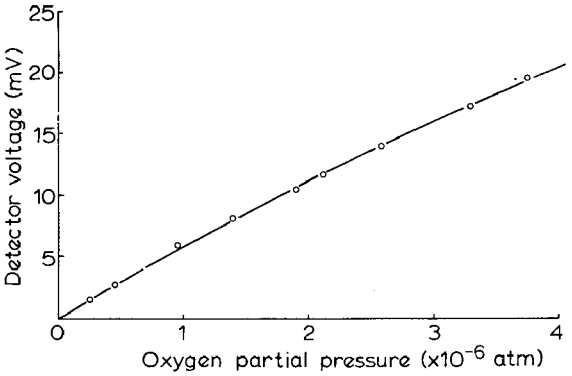

Fig. 5. Response curve for oxygen with triangular peaks. —, Theoretical curve; ○, experimental data.

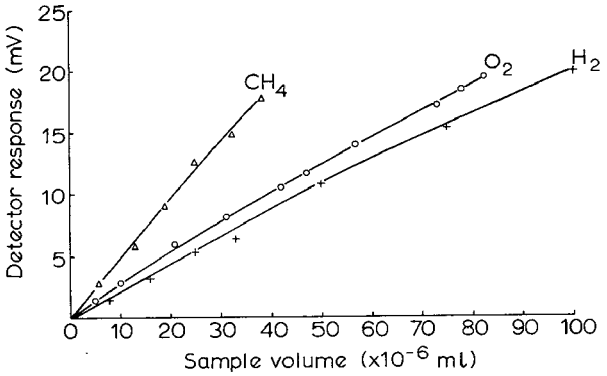

Fig. 6. Response for hydrogen, oxygen and methane with air as reference gas. The polarity of the oxygen signal has been reversed. Detector temperature, 1050°C; flow rate, 70 ml·min^{-1}.

obtained between the predicted response curve and the practical measurements (taken from oxygen data in Fig. 6).

Gas samples containing hydrogen and methane give signals of opposite polarity to oxygen, when they are eluted through the detector (Fig. 6). In this case these gases react with oxygen present in the carrier gas to produce a lowering of oxygen partial pressure. The sensitivity is greater for methane than hydrogen because both carbon and hydrogen atoms in methane are capable of reacting with oxygen.

Inert and unreactive gases such as argon and nitrogen do not produce signals and consequently the analysis of oxygen in argon can be carried out with the column operating at normal temperature and without separation. Calibration of the system when the components are not resolved is however rather more complicated. Argon samples which have a lower oxygen partial pressure than the carrier gas give signals of opposite polarity to that of the oxygen calibrating gas. For the case where the argon sample (1.1 ml) contains negligible oxygen, *i.e.* two orders of magnitude lower than the carrier gas, the maximum peak height signal would be expected to be $\approx$ 2.6 mV for the standard operating conditions. This corresponds to a negative error signal of

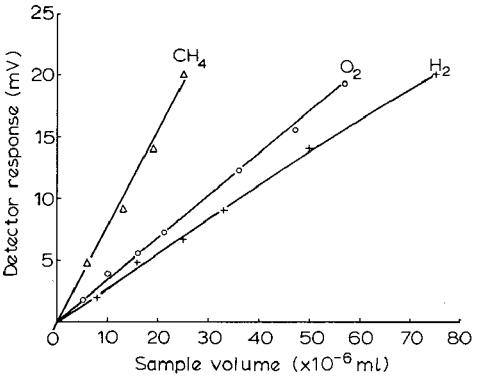

Fig. 7. Response for hydrogen, oxygen and methane with helium as reference gas. The polarity of the oxygen signal has been reversed. Detector temperature, 1050°C; flow rate, 70 ml·min^{-1}.

$\approx 1 \times 10^{-5}$ ml oxygen for argon samples having oxygen partial pressures equal to or greater than the carrier gas.

The sensitivity of the detector is governed by the oxygen partial pressure of the carrier gas and it has been shown that this is increased as the carrier gas flows through the detector due to diffusion of molecular oxygen. This can easily be reduced to negligible quantities by using pure helium carrier gas as reference. With this condition, the detector standing voltage only amounts to several mV and it is unaffected by temperature fluctuations. Assuming that the detector now operates with the carrier gas at its limiting purity, 5×10^{-7} atm oxygen, the oxygen sensitivity would be expected to be approximately ten times greater. Fig. 7 shows that the observed increase in sensitivity is a factor of two and suggests that leaks were occurring in the pipe-work prior to the detector. The required partial pressure of oxygen in the ingoing carrier gas to give this sensitivity is 4.5×10^{-6} atm.

The noise level of the detector operating in this mode is 0.2 mV, peak to peak, and the base-line drift is better than 0.4 mV·h^{-1}. These parameters are primarily governed by the purity of the carrier gas and the nature of the ohmic contacts at the sensing end of the solid electrolyte. The use of the ceramic reaction cement, which is conductive at high temperatures and forms a rigid contact between the electrodes and electrolyte, has been found to give good electrical stability. The cement is formulated to give sufficient through porosity to permit ready access of gas to the gas–electrolyte interface and is in addition capable of electrical conduction by transport of the oxide ion.

The minimum detectable volumes, corresponding to a signal/noise ratio of one, are: hydrogen 7×10^{-7} ml, oxygen 6×10^{-7} ml and methane 3×10^{-7} ml. The

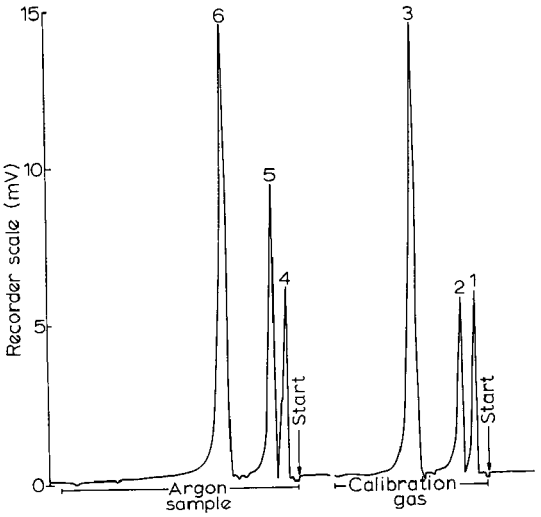

Fig. 8. Chromatogram of a 1.1-ml sample of argon and a calibration gas containing hydrogen, oxygen and methane. For the oxygen peaks the polarity of the signal has been reversed. Detector temperature, 1050°C; flow rate, 70 ml·min^{-1}; chart speed, 30 in.·h^{-1}; column, molecular sieve 5A at 65°C; reference gas helium. 1 = Hydrogen, 20×10^{-6} ml s.t.p.; 2 = oxygen, 16×10^{-6} ml s.t.p.; 3 = methane, 19×10^{-6} ml s.t.p.; 4 = hydrogen, 20×10^{-6} ml s.t.p.; 5 = oxygen, 35×10^{-6} ml s.t.p.; 6 = methane, 19×10^{-6} ml s.t.p.

chromatogram (Fig. 8) of a 1.1-ml sample of argon illustrates the sensitivity of the detector and the noise level at low concentrations.

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CHROM. 4936

A SIMPLE QUANTITATIVE METHOD FOR TRAPPING AND TRANSFER OF LOW CONCENTRATION GAS CHROMATOGRAPHIC FRACTIONS SUITABLE FOR USE WITH SMALL DIAMETER GLASS COLUMN SYSTEMS

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SUMMARY

A technique suitable for the quantitative trapping and transfer of fractions from glass porous layer open tubular columns is described. Fractions are collected in short U-traps of the cooled columns and may be rapidly and efficiently transferred to other glass columns for additional separation and identification by combined gas chromatography–mass spectrometry. High and low boiling compounds down to levels of 20 ng or lower can be handled with ease. A procedure for selectively reducing large interfering peaks is discussed. The method is especially useful for dealing with labile compounds, since there is minimal contact of the sample with hot metal surfaces at all stages.

INTRODUCTION

The analysis of complex mixtures by gas chromatography (GC) frequently necessitates the trapping out of fractions from a column either for subsequent analysis on a column of different polarity or for examination by some other analytical technique. Such complexity is encountered in the area of food flavour chemistry, where combined gas chromatography–mass spectrometry (MS) is, at the present time, the most widely used and versatile technique for studying the multi-component mixtures of volatile compounds isolated from various foodstuffs, usually in amounts at the p.p.m. levels. In most flavour essences only some of the regions of a complex chromatogram contain peaks (or groups of peaks) of olfactory significance and often these are present in relatively low concentrations.

One problem in analysing such essences is the following. Since high efficiency wall-coated or porous layer open tubular (PLOT) columns are normally used in the analysis of such mixtures, it is frequently not possible to inject enough sample onto the column to permit MS analysis of these low concentration components without impairing the separating power of the column by overloading.

Another problem which often arises in this type of analysis is caused by the presence of some components in such high relative concentrations that the mass

spectrum of a minor compound eluted on its tail is obscured. A high residual background from the large peak can make it very difficult to decipher a weak superimposed spectrum. This difficulty may sometimes, though not always, be overcome by analysing the mixture on a number of columns of different polarity.

One method of solving the first of the above problems is to inject a large sample onto a packed column and to trap out the desired region for rechromatography on a high efficiency column. If it is desired to trap out a very narrow region and minimise interference from large peaks surrounding the region of interest, repeated charges of sample may be applied to the capillary or PLOT column, the required fractions being concentrated in an appropriate trap and then rerun. Minor components of interest, which follow a large interfering peak, may be obtained relatively free of the latter by trapping out repeatedly fractions on the tail of the large component.

In recent years several authors[1–5] have described a variety of techniques for the collection and transfer of small amounts of samples eluted from packed and, to a somewhat lesser extent, from capillary columns[6]. When dealing with small diameter column systems there are two main requirements which a trapping and transfer technique should fulfil:

(a) Minimal loss of sample should take place during both the collection or concentration step and in the transfer stage.

(b) The trapped sample should be presented to the column in a sharp narrow band, so that the total available efficiency of the column is used.

This is particularly important where a minor component is trapped on the tail of a major peak.

In this paper, a technique is described for the trapping and transfer of fractions between glass PLOT columns which satisfies both of the above requirements. Recoveries are quantitative for compounds covering a wide boiling range at levels down to 20 ng or even lower. The methods permit the concentration of small peaks from a PLOT column, as well as the selective bypassing of large interfering peaks during the course of a run.

EXPERIMENTAL

Apparatus

In the work discussed here glass PLOT columns were prepared by a method recently described[7]. The fraction collecting traps were made from short straight lengths of the same PLOT tubing as follows. A piece of tubing, about 135 mm long, was heated in the middle over a micro bunsen flame and bent into the shape of a narrow U. Each open end was then turned down on the flame to give two short lengths (10 mm) at angles of 90° to the rest of the tube.

A Pye Model 104 gas chromatograph, equipped with a heated flame detector and a heated injection head, was used. A small volume injection system which fitted directly to the standard compression coupling of the Pye injection head, was made from a length of glass capillary tubing (6.25 mm O.D. × 1.05 mm I.D.) in the following manner. A length of capillary tube was carefully heated in a gas–oxygen flame and drawn out to give a narrow portion of approximately 1 mm diameter. The tube was then cut off to a length of 65 mm above the narrow portion so that when connected to

the injection port the narrow portion extended just below the bottom of the heated injection zone into the oven.

In order to make connections between the injection system and the column and between the column and a trap, short lengths (20 mm) of thin-wall PTFE tubing (bore 0.75 mm) were used in conjunction with 20-mm long hypodermic needles (21 gauge) from which the syringe adaptor fittings were cut off. The pieces of PTFE tubing were fitted carefully over the narrowed end of the injection system and over both ends of the column and any trap after softening the tubing over a very low bunsen flame. On cooling a tight leak-proof seal formed between the glass and PTFE tubing. A tiny spot of 'Araldite' resin was applied around each joint to reinforce it for operation at up to 220°. The cut-off ends of 21-gauge hypodermic needles were then inserted through approximately half the length of the connecting tube at one end of both the column and trap. A little 'Araldite' was placed arount the junction of the PTFE and the needle. Connection of the column to the injection system was then achieved by simply pushing the needle into the PTFE connection on the latter.

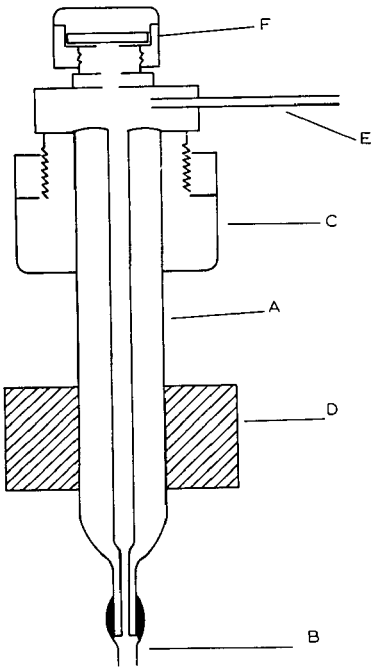


Fig. 1. Glass injection system. (A) Glass capillary tube (6.25 mm O.D. × 1.05 mm I.D.); (B) PTFE connection to column; (C) compression nut; (D) injection heater; (E) carrier gas inlet; (F) septum.

This method of connecting glass PLOT column systems possesses low dead volume and has provided a leak-proof injection system when tested with carrier gas pressures of 1.2 kg/cm^2 and column operating temperatures up to 220°. The injection system and column connection method are shown in Fig. 1.

The column, which was positioned in the oven by suspending it from a length of thin wire attached to a hook fixed to the roof of the oven, was connected to the FID and trap via a splitter system constructed from a three-way 1.55-mm O.D.

stainless steel coupling*. Two hypodermic needles, each 20 mm long, were silver soldered to two short lengths of stainless steel capillary tubing (10 mm × 1.55 mm O.D.) and were used to connect the PLOT column and trap to the coupling. The connection between the latter and the detector was made by silver soldering a 100-mm length of narrow stainless steel capillary tubing (0.5 mm O.D. × 0.3 mm I.D.) to a 10-mm length of 1.55-mm diameter tube. The narrow capillary was inserted through the line leading from the base of the detector into the oven, effectively reducing the dead volume of this portion. The split ratio between the FID and trap may be set to any required value by restricting the narrow capillary tube with suitable lengths of thin stainless steel wire. A split ratio of 9:1 in favour of the trap was used in the experiments described here.

A piece of thin-wall PTFE tubing, approximately 60 cm long, was connected to

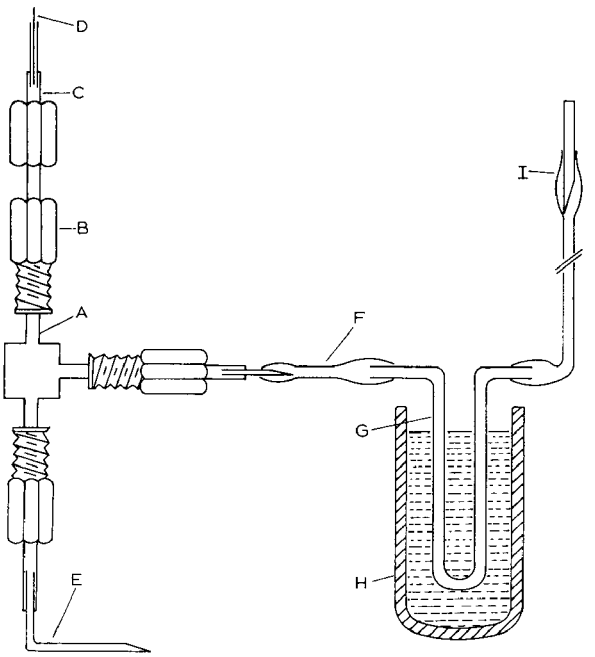


Fig. 2. Fraction collector and splitter arrangement. (A) 1.55 mm O.D. three-way connector; (B) connecting nut; (C) connection to FID; (D) restricting wire; (E) hypodermic connection to column; (F) PTFE connecting tube; (G) PLOT trap; (H) liquid nitrogen; (I) external blanking hypodermic connection.

the exit of the glass trap and led through a small hole bored through the roof of the chromatographic oven. This enabled the effluent from the column to be directed as desired during the course of a run to the FID by simply inserting a blanked-off hypodermic needle into the PTFE tubing outside the oven, thereby stopping the flow of carrier gas through the trap. The arrangement of the splitter and the trap is shown in Fig. 2.

* Simplifix Couplings Ltd., Maidenhead, Berks.

Trapping and transfer of fractions

Trapping of fractions is carried out by immersing the PLOT U-tube in a small Dewar flask of 30 ml capacity, which is filled with liquid nitrogen. If a number of peaks are to be collected at different times during a run with the splitter *in situ*, the U-tube is normally held in the coolant from the start of the run. When just one peak or group of peaks is to be trapped during a run, the splitter arrangement may be dispensed with and the trap connected directly between the column and the FID. In this case the trap is cooled just before the peak of interest emerges. To obviate the need to open the oven for insertion of the coolant under the trap, it is convenient to enclose the Dewar flask in a small wire cage, which can be raised or lowered within the oven by means of a length of thin copper wire protruding through a small hole in the roof of the oven. The wire is held in position by means of a small clamp fixed on the roof of the oven.

After collection of a fraction, the trap, still immersed in the liquid nitrogen, is removed from the oven and plugs of blanked off PTFE tubing are placed over each end. It may be stored indefinitely under these conditions without loss or contamination. For rechromatography of the trapped sample the column is disconnected from the injection block and the cooled trap inserted between the latter and the column. If the splitter is in position, the open end is blocked with a plug of blanked off PTFE tubing. The newly made connections are carefully checked for leaks. As soon as the oven attains the required starting temperature, the flask of coolant is removed by lowering from under the trap and the run allowed to proceed.

Contact of the sample with metal surfaces is kept to a minimum in the system described above, occurring only within the splitter and the hypodermic connectors, which possess a very low combined dead volume.

Trapping experiments were carried out with two different PLOT columns, a 12-m length coated with a 1% w/v solution of Carbowax 20M solution in methylene chloride and a 25-m length coated with a 0.2% w/v solution. The trap itself was also coated with a 0.2% solution of the same phase.

RESULTS AND DISCUSSION

The effectiveness of the trapping system was tested for a range of carrier gas flow rates from 2 to 20 ml/min using the 12-m column and diethyl ether as the test substance. Replicate charges (0.5 ml) of a vapour sample containing 0.2% v/v of diethyl ether in air were injected for each flow rate with the traps either cooled or uncooled. The chromatograph attenuation was increased by a factor of 100 when the trap was cooled. Comparison of peak heights showed the trapping system to be 100% efficient at 2–15 ml/min and 99.8% at 20 ml/min.

The quantitative aspect of the combined sample trapping and transferring process was investigated with a mixture of compounds having a range of polarities, consisting of a 0.5% v/v solution of undecane, *p*-xylene, *n*-pentanol and 2-octanone in diethyl ether. With the trap inserted directly between the 12-m column and the detector 0.2-μl charges of sample were injected onto the column at a temperature of 115° and a flow rate of 5 ml/min of helium. The ratio of each peak area relative to undecane was determined. The injection was repeated, but this time the trap was cooled immediately after elution of the undecane peak and the three components collected. The trapped fraction was then rechromatographed by the procedure de-

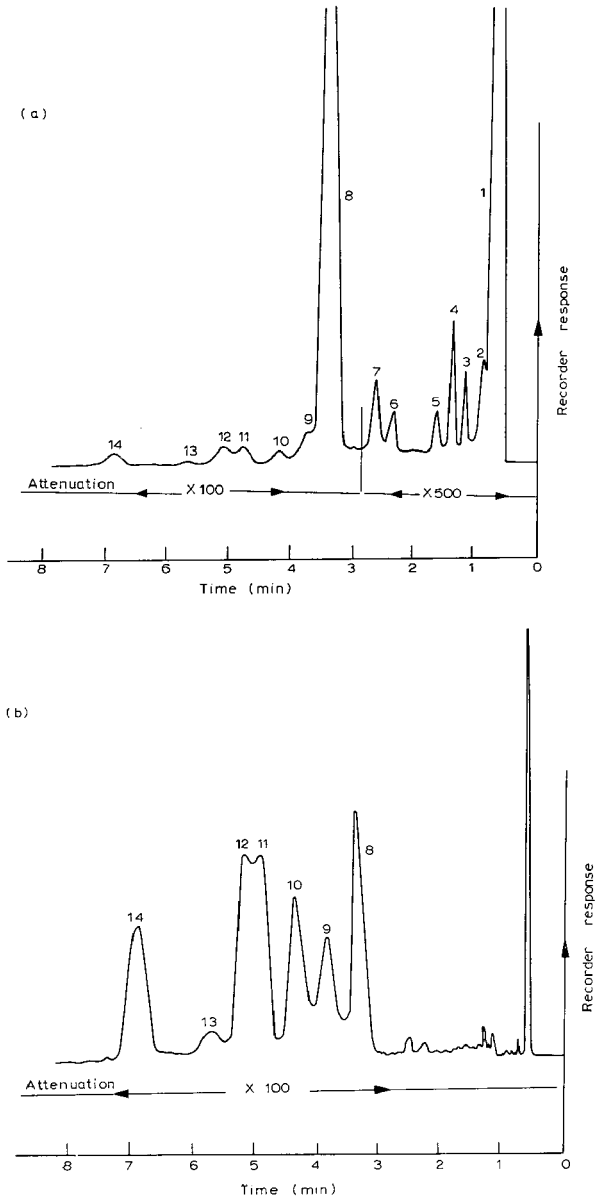


Fig. 3. Chromatograms of (a) a 0.5-μl injection of a synthetic mixture and (b) a rerun of a trapped fraction from a 5-μl injection showing a concentration of minor peaks. Column, 12 m × 0.5 mm PLOT, coated with 1% w/v Carbowax 20M; temperature 115°; flow rate, 6 ml/min helium.

scribed above and the peak area ratios relative to the undecane peak again determined. There was excellent agreement between the ratios obtained for both the trapped and untrapped runs in replicate experiments, giving overall trapping and transfer efficiencies in the range 97–100% for each of the three components. It has been possible

to obtain almost quantitative recoveries from the trapping and transfer of quantities as low as 20 ng of *n*-pentanol.

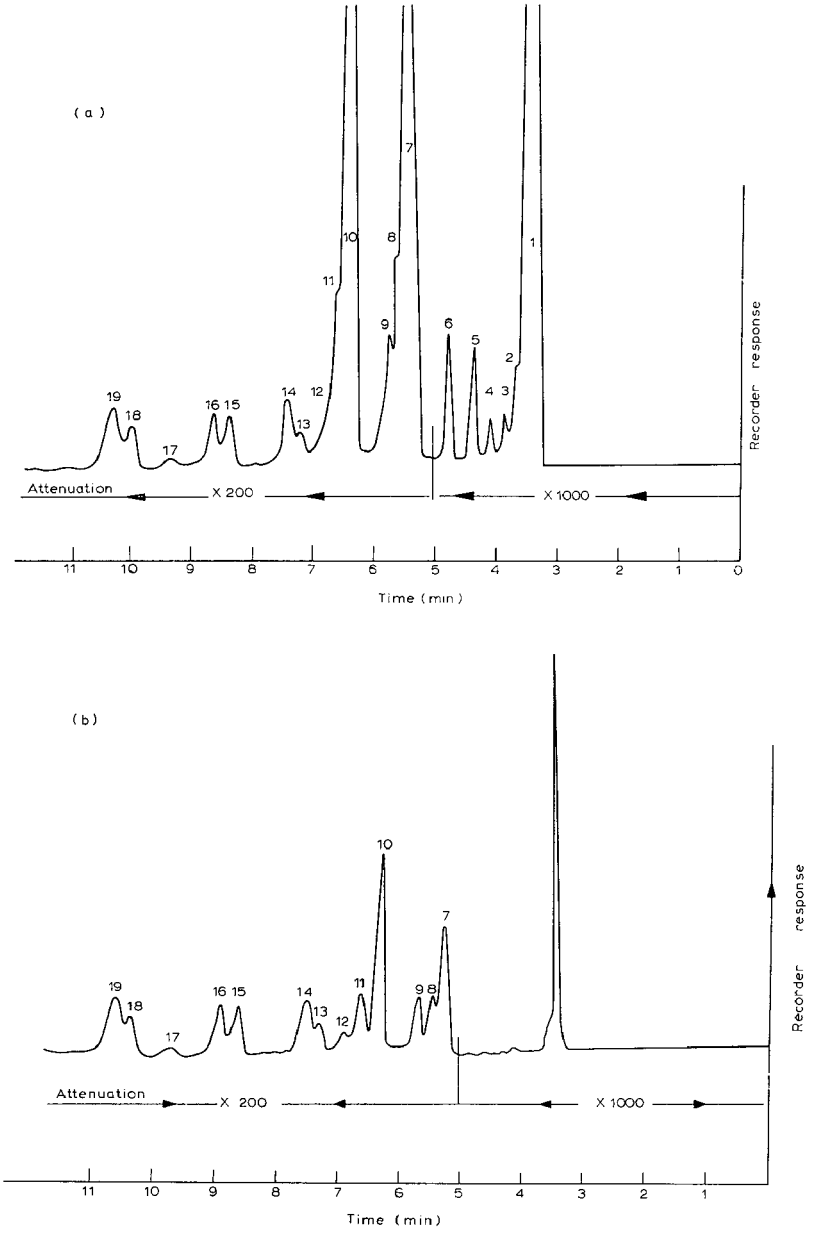
An example of concentrating an area of low concentration following a large peak is shown in chromatograms (a) and (b) in Fig. 3. Chromatogram (a) represents a 0.5-μl injection of a synthetic mixture of aliphatic alcohols, ketones and hydrocarbons in ether, corresponding to a weight of 50 μg for 2-octanone (peak 8) and between 0.2–1 μg for dodecane (9), *n*-hexanol (10), 4-octanol (11), 3-octanol (12) and *n*-heptanol (14). The column was then overloaded by injecting 5 μl of the same mixture. The coolant was applied to the trap just as the trailing edge of peak 8 came back on scale on the recorder chart, and the subsequent small peaks were collected. Chromatogram (b) shows the rerun of the trapped fraction under the same conditions. Peak 9 is seen to be much enhanced relative to peak 8 and the recoveries of the other minor components are clearly excellent, peak heights being enhanced by virtually the expected ratio of 10.

Fig. 4 represents a more complex situation, where two minor components, ethyl hexanoate (8) and *o*-ethylbenzene (9), eluted on the tail of a major component, *n*-pentanol (7), are followed by another major component, 2-octanone (10), on the tail of which occurs another minor component, dodecane (11). Fig. 4a shows the chromatography of a 0.2-μl sample injected with the splitter in position, but with the entire column effluent directed to the detector. With the trap cooled a second 0.2-μl sample was injected. The column effluent was initially directed to the detector by blocking off the flow through the splitter in the manner already described. As soon as the trailing edge of the major peak 7 came back on scale the effluent was directed through the trap to collect peaks 8 and 9. Just before the large peak 10 appeared, the trap was again blanked off until the latter appeared on scale and then the remaining peaks on the chromatogram were collected in the trap. These include *n*-hexanol (14), 4-octanol (15), 3-octanol (16), tridecane (18) and *n*-heptanol (19). Chromatogram 4b represents a rerun of the trapped components on the same column. The results of trapping and transferring can be seen to have caused no loss even in the resolution of incompletely separated peaks and the interference of the large peaks 7 and 10 with the minor components on their respective trailing edges has been markedly reduced. Fig. 4c shows the rerun of a fraction from a 1-μl sample of the same mixture trapped out as described above and indicates how small peaks can be concentrated from different regions of a chromatogram during the course of a run.

In the experiments described above vaporisation of the trapped fractions has been effectively achieved by simply allowing the trap to heat to oven temperature on removal of the coolant. Adequate vaporisation for compounds boiling up to around 300° is obtainable without difficulty. A sample containing 2 μg each of the *n*-paraffin series tridecane to heptadecane was injected on the 20-m column at 80° and programmed at 8°/min. From a second sample the five compounds were collected in the trap and then rechromatographed under the same conditions. The width at half height for each peak was identical for both the trapped and untrapped runs. If lower initial oven temperatures, *e.g.* 40–50°, are employed in the temperature-programmed analysis of wide boiling range mixtures it may be useful to assist vaporisation of the trapped sample by placing a beaker of hot water under the trap after removal of the coolant.

It may be necessary when dealing with mixtures of wide boiling range to trap out fractions at various times in the duration of a run. Consequently, the length of the cooled portion of the PLOT trap becomes increasingly less as the liquid nitrogen in

the Dewar evaporates and some loss of previously trapped lower-boiling compounds might be expected each time the carrier gas is bypassed through the trap to collect a fraction. In a relevant experiment 0.2 μl of diethyl ether were injected onto the 12-m column with the trap connected to the detector and immersed in a full flask of liquid nitrogen. The chromatograph was programmed from 70° at 3°/min with the attenuation set at $\times$ 50. No signal was registered on the recorder until the temperature reached



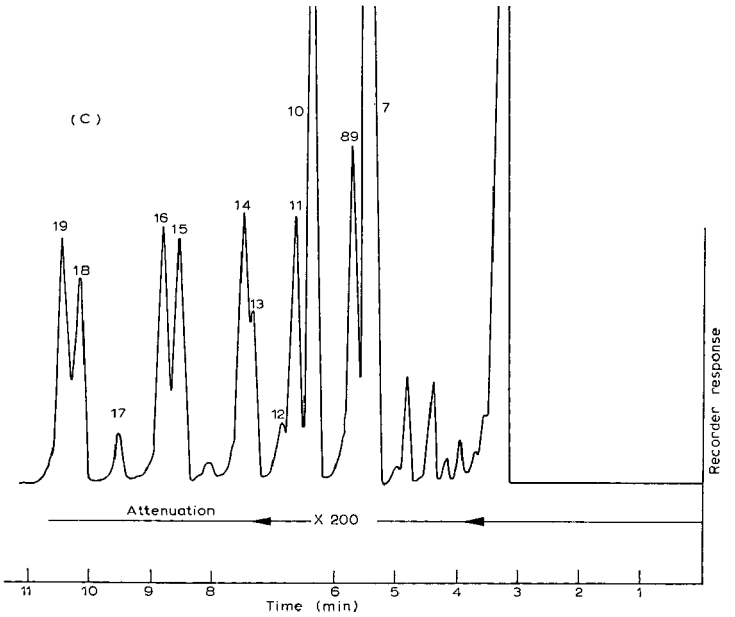


Fig. 4. Chromatograms of (a) a 0.2-μl synthetic mixture run without splitting; (b) a rerun of a trapped fraction from a 0.2-μl sample after selective reduction of peaks 7 and 10; (c) a rerun of a trapped fraction from a 1-μl sample showing a concentration of minor components. Column, 25 m × 0.5 mm PLOT, coated with 0.2% w/v Carbowax 20M; temperature, initially 80° (3 min), then programmed at 3°/min; flow rate, 2 ml/min helium.

195°, when the ether peak broke through in a sharp band, the trap having run completely dry.

The techniques presented in this paper have been developed particularly for use in the study of the complex mixtures represented by food volatiles which may contain labile compounds at the higher-boiling end. The minimal contact of the sample with large hot metal surfaces at all stages in collection and subsequent handling of trapped fractions is a particular advantage of the method and should make it applicable to a wide range of other problems involving labile compounds in trace amounts.

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CHROM. 4978

RAPID ELECTRON CAPTURE DETERMINATION OF CAFFEIC ACID AND QUERCETIN MOIETIES IN PLANTS*

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SUMMARY

A sensitive gas–liquid chromatographic method has been developed for the quantitative analysis of caffeic acid and quercetin moieties in plant leaf by means of electron capture detection. Caffeic acid, quercetin and their naturally occurring derivatives, chlorogenic acid and rutin, respectively, are directly extracted from plant materials with 1-propanol, and subjected to a transesterification–hydrolysis reaction to produce caffeic acid *n*-propyl ester and quercetin. After silylation of these compounds, the TMS derivatives are suitable for chromatography on 10% OV-101 in the case of caffeic acid and 1.5% OV-101 in the case of quercetin. The method requires about 5 h for their quantitative determination, and the approximate lower limits of detection in tobacco leaf samples are 20 ng caffeic acid and 300 ng quercetin.

INTRODUCTION

Caffeic acid and quercetin derivatives, for example chlorogenic acids and rutin, respectively, are among the most common soluble phenols in plant tissue[1]. Previous methods for analysis of these compounds were largely based upon absorption chromatography, paper chromatography, spectrophotometry and spectrophotofluorometry[2–4]. Gas chromatographic separation and detection of these and related phenolic moieties has recently been accomplished[5–9]. The use of volatile hydroxyl derivatives, especially trimethylsilyl (TMS) ethers, the development of stable silicone stationary column phases, and the sensitivity of flame ionization, argon ionization and thermal conductivity detectors facilitated this advance[5–9].

A quantitative gas chromatographic–flame detector method for caffeic acid moieties in tobacco was developed in our laboratories[10]. The major disadvantage of this method was the necessity for a preliminary separation of the plant phenol fraction. Compounds which have an affinity for electrons can often be detected at lower concentrations in an electron capture detector than in flame, argon ionization or thermal

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conductivity detectors[11–14]. In our present study the objectives were to: (1) extend the previous method to include an analysis for both caffeic acid and quercetin moieties in plant tissue, (2) increase the sensitivity of detection of these silylated phenolic derivatives by electron capture detection, and (3) shorten the analytical method by the elimination of a preliminary separation of the phenol fraction.

EXPERIMENTAL

Reagents

All chemicals were reagent grade unless otherwise specified. Acetonitrile and *n*-propyl alcohol were redistilled before use and the distillates were stored over anhydrous Na_2SO_4. Dry HCl gas was used. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Regis Chemical Co.*, Chicago, Ill. Chlorogenic acid hemihydrate, quercetin and rutin were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Kaempferol and myrcetin were obtained from K & K Laboratories, Inc., Plainview, N.J., and caffeic acid from Aldrich Chemical Co., Milwaukee, Wisc. Caffeic acid methyl ester and caffeic acid *n*-butyl ester were synthesized in our laboratories as previously described[10]. Caffeic acid *n*-propyl ester (new compound) was synthesized in the same manner as the methyl and *n*-butyl esters of caffeic acid[10]. The solid was recrystallized from methanol/water, m.p. 126° (uncorr.). C, H, found = 65.0, 6.3; C, H, calculated for $C_{12}H_{14}O_4$ = 64.9, 6.3.

Sample preparation

Some of the preliminary experimental work and most of the subsequent testing of the procedure for the preparation of samples for gas chromatography involved some or all of the steps given in the following analytical method for the determination of caffeic acid and quercetin moieties in plant tissues as esters, glycosides or in their free form.

Analytical method. Weigh 100 mg of freeze-dried tissue into an erlenmeyer flask and add 15 ml *n*-propanol. Insert a condenser and reflux 45 min. Filter the sample solution through Whatman No. 1 paper and collect the filtrate in a flask. Wash the residue several times with small portions of *n*-propanol and collect the washings in the same flask. Discard the insolubles. Pass dry HCl gas through the solution until it is saturated. Reflux this solution 1 h (cold-water condenser required), and then take to dryness on a rotary flash evaporator.

Add the following internal standards as weighed amounts sufficient to provide satisfactory peak heights during subsequent gas chromatography: Caffeic acid methyl ester, caffeic acid *n*-butyl ester, kaempferol and myrcetin. Add 1.00 ml acetonitrile and 1.00 ml BSTFA. Immediately cover the flask and swirl the mixture to effect complete solution. Transfer as much of the solution as possible (without washing) to a 5-ml sealable screw-type acylation tube, or a tube which can be sealed. Heat in an oil bath at 150° for 1 h. Cool to room temperature. Open the tube and inject 0.20–3.0 μl into the gas chromatograph.

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* Mention of proprietary materials in the text does not imply endorsement by the United States Department of Agriculture.

Gas chromatography

A Packard Model 7821 gas chromatograph was used in conjunction with a Packard Model 810 electron capture detector with tritium foil as the ionizing source.

In the analysis for caffeic acid moieties, the chromatograms were recorded on a Photovolt Microcord Model 44 recorder, using a chart speed of 6 in./h. A 6-ft. coiled glass column (4 mm I.D.) was used. The column was packed with 10% silicone stationary phase (OV-101) on 80–90 mesh Anakrom AS. The operating conditions were: inlet temperature, 220°; column temperature, 190°; detector temperature, 215°; and outlet temperature, 220°. Argon was used as a carrier gas at a flow rate of 100 cc/min. Detector voltage was 50 V.

In the analysis for quercetin moieties, the chromatograms were recorded on a Packard Model 561 recorder, using a chart speed of 10 in./h. The column size and packing were the same as that used for the caffeic acid analysis, except that 1.5% OV-101 was used. The operating conditions were: inlet temperature, 230°; column temperature, 220°; detector temperature, 220°; and outlet temperature, 230°. Carrier gas, flow rate and detector voltage were the same as those used for the caffeic acid analysis.

The peak areas were measured by planimetry at the retention times established with trimethylsilylated samples of caffeic acid *n*-propyl ester and quercetin. Retention times were calibrated by determining: (a) the relative peak positions of trimethylsilylated methyl, *n*-propyl and *n*-butyl esters of caffeic acid under the conditions specified for the 10% OV-101 column, and (b) the relative peak positions of trimethylsilylated kaempferol, quercetin and myrcetin under the conditions specified for the 1.5% OV-101 column. The peak areas of silylated caffeic acid *n*-propyl ester and silylated quercetin were compared with those from standard curves of peak areas from gas chromatographic analyses established with various amounts of chlorogenic acid hemihydrate and rutin added to a tobacco sample which had non-detectable amounts of caffeic acid and quercetin moieties. These samples were carried through the entire analytical method.

RESULTS AND DISCUSSION

Initial runs were made in which a high-phenol variety of tobacco leaf was extracted with either methanol, *n*-propanol or *n*-butanol, and, subsequently, prepared as described in the analytical method. GLC analyses of these samples were carried out by flame detection under the previously reported conditions[10]. Samples which were extracted and prepared with *n*-propanol and *n*-butanol yielded TMS-caffeic acid esters that were easily identifiable and there were few background peaks. However, methanol extractions and the preparation of TMS-caffeic acid methyl ester was not as suitable for quantitative GLC–flame detection analysis, because of greater background and overlapping peaks on chromatograms. *n*-Propanol was, therefore, selected for the extraction and preparation of samples for GLC–electron capture assay for caffeic acid and quercetin moieties in plant tissues. Tests showed that chlorogenic acid refluxed 1 h in *n*-propanol saturated with dry HCl was transesterified and yielded an equivalent amount of caffeic acid *n*-propyl ester; rutin treated in the same manner was hydrolyzed and yielded an equivalent amount of quercetin. Extraction of leaf samples with *n*-propanol and transesterification–hydrolysis with *n*-propanol saturated with dry HCl were, therefore, used in the analytical method.

TABLE I

RELATIVE PEAK POSITIONS[a] OF TMS DERIVATIVES OF CAFFEIC ACID ESTERS AND FLAVANOLS

GLC column conditions	*t_{R_1} component*	*t_{R_2} component*	*Relative peak position*
10% OV-101, 190°	Caffeic acid methyl ester	Caffeic acid *n*-propyl ester	0.91
	Caffeic acid *n*-butyl ester	Caffeic acid *n*-propyl ester	(−) 0.31
1.5% OV-101, 220°	Kaempferol	Quercetin	0.53
	Myrcetin	Quercetin	(−) 0.22

[a] Relative peak position = $(t_{R_2} - t_{R_1})/t_{R_1}$, where t_R = retention time of a component measured from the start.

The synthetically prepared methyl, *n*-propyl and *n*-butyl esters of caffeic acid were silylated and chromatographed according to the conditions given in EXPERIMENTAL. The relative peak positions of the TMS-methyl, *n*-propyl and *n*-butyl esters are given in Table I.

Because the anthracene and pyrene internal standards previously employed in flame detection of TMS-caffeic acid esters[10] were unresponsive in the electron capture detector, the methyl and *n*-butyl esters were used as internal standards for the assay of caffeic acid moieties as TMS-caffeic acid esters.

TMS-caffeic acid *n*-propyl ester was separated from peaks of other compounds in tobacco, tomato, peach and buckwheat leaf extracts. For example, caffeic acid moieties were added by spiking a tobacco sample low in caffeic acid moieties with

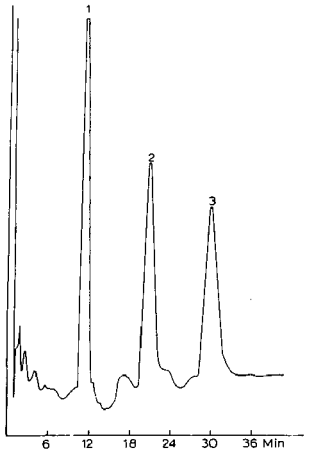


Fig. 1. Chromatogram of a 15-μg low-phenol tobacco sample spiked with 0.041 μg chlorogenic acid hemihydrate and subjected to the entire analytical method. Internal standards: 0.015 μg caffeic acid methyl ester (1) and 0.015 μg caffeic acid *n*-butyl ester (3). 2 = TMS-caffeic acid *n*-propyl ester assayed.

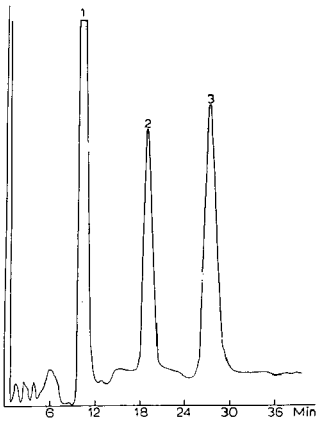
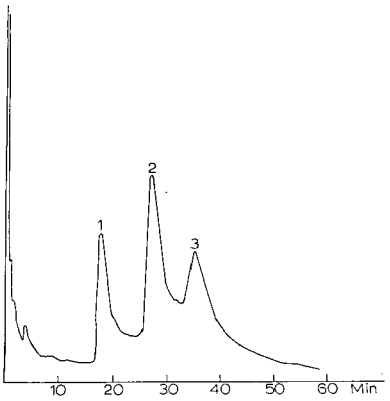


Fig. 2. Chromatogram of a 40-μg high-phenol tobacco subjected to the entire analytical method. Internal standards: 0.016 μg caffeic acid methyl ester (1) and 0.016 μg caffeic acid *n*-butyl ester (3). 2 = TMS-caffeic acid *n*-propyl ester assayed.



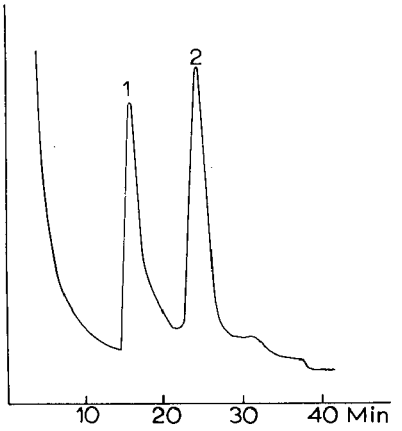


Fig. 3. Chromatogram of flavanols subjected to the silylation step of the analytical method. 1 = 0.45 μg kaempferol, 2 = 0.42 μg quercetin and 3 = 0.45 μg myrcetin.

Fig. 4. Chromatogram of a 75-μg low-phenol tobacco sample spiked with 1.50 μg rutin and carried through the analytical method. Internal standard: 0.75 μg kaempferol (1). 2 = TMS-quercetin assayed.

chlorogenic acid. The chromatogram obtained in Fig. 1 indicates the sensitivity of the analysis of caffeic acid moieties or derivatives, *e.g.* chlorogenic acid in tobacco leaf tissue. Similar results were obtained with other leaf samples. An advantage was the low level of background on chromatograms. Compared to the previous flame detector method for caffeic acid moieties in tobacco leaf[10], the electron capture method was more rapid, sensitive and had less background interference.

Leaves from tobacco (a high-phenol variety of *Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), peach (*Prunus persica*) and buckwheat (*Fagopyrum esculentum*) were analyzed for naturally occurring caffeic acid moieties. Chromatograms of 40 μg high-phenol tobacco (Fig. 2), 30 μg tomato and 30 μg peach all showed strong peaks corresponding to silylated caffeic acid *n*-propyl ester, but 50 μg buckwheat exhibited no detectable caffeic acid derivative.

Kaempferol, quercetin and myrcetin were silylated and chromatographed according to the conditions given in EXPERIMENTAL. The relative peak positions of the TMS-derivative of each of these compounds are given in Fig. 3 and Table I. The hydrocarbon 1,2:5,6-dibenz[*a*,*h*]anthracene was a suitable internal standard for the analysis of TMS-quercetin in preliminary runs in which a flame detector was used, but it was unresponsive to the electron capture detector. Kaempferol and myrcetin were, therefore, added prior to silylation to provide TMS-flavanol internal standards for the electron capture assay of quercetin moieties or derivatives (*e.g.* rutin) as TMS-quercetin.

A given quantity of silylated quercetin was two to three times more responsive with electron capture detection than it was with flame detection. There was also less background interference with electron capture than with flame detection when a high-phenol variety of tobacco leaf was prepared and assayed for quercetin. The increased sensitivity of TMS-quercetin detection with electron capture was substantial, but less than the parallel increased sensitivity noted with TMS-caffeic acid ester, *i.e.*,

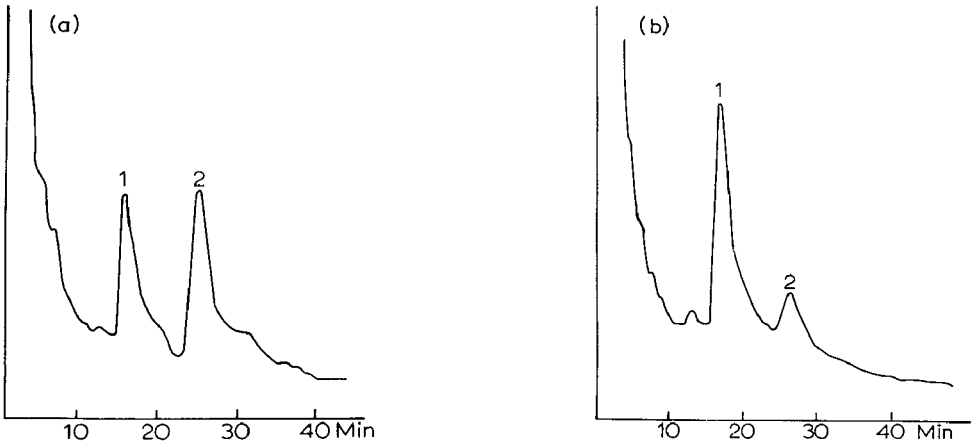


Fig. 5. (a) Chromatogram of 75 μg high-phenol tobacco carried through the entire analytical method. Internal standard: 0.45 μg kaempferol (1). 2 = TMS-quercetin assayed. (b) Chromatogram of 100 μg peach leaf carried through the entire analytical method. TMS derivatives assayed: kaempferol (1) and quercetin (2).

approximately twenty-fold. Apparently the TMS-caffeic acid ester contains a greater proportion of electrophilic sites than does TMS-quercetin.

TMS-quercetin was separated from peaks of other compounds in tobacco, tomato, peach and buckwheat leaf extracts. Quercetin moieties were added by spiking a tobacco leaf sample low in quercetin moieties with rutin. The chromatogram showed that the analytical method with electron capture provided a satisfactory method for analysis of quercetin moieties in tobacco leaf tissue with fairly low background on chromatograms (Fig. 4).

Leaves from a high-phenol variety of tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), peach (*Prunum persica*) and buckwheat (*Fagopyrum esculentum*) were analyzed for naturally occurring quercetin moieties. Chromatograms of 75 μg tobacco (Fig. 5a), 100 μg each of tomato, peach (Fig. 5b) and buckwheat all

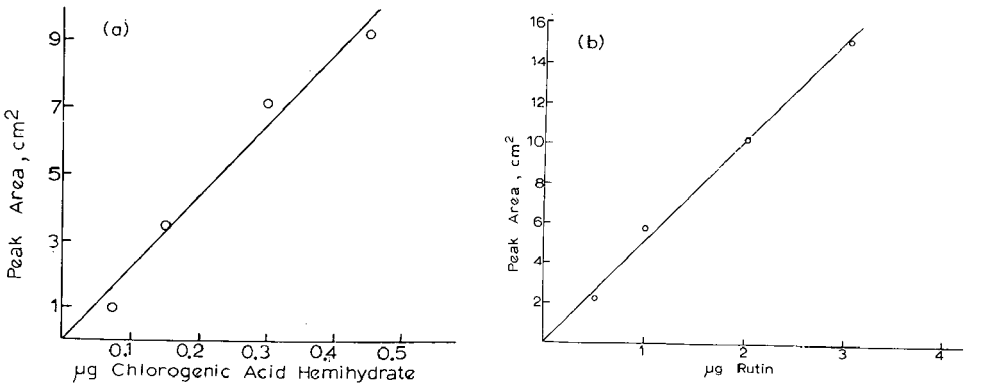


Fig. 6. (a) Recovery of chlorogenic acid hemihydrate (as TMS-caffeic acid *n*-propyl ester) spiked in 15 μg low-phenol tobacco. (b) Recovery of rutin (as TMS-quercetin) spiked in 100 μg low-phenol tobacco.

showed peaks corresponding to TMS-quercetin. In addition, the peach leaf (Fig. 5b) chromatogram showed the presence of naturally occurring kaempferol; this chromatogram was prepared without the use of a kaempferol internal standard.

Calibration curves for caffeic acid moieties (as chlorogenic acids) and quercetin moieties (as rutin) in leaf samples were plotted. In one case, for each analytical value 15 μg of a low-phenol tobacco variety sample was added to 0.10–0.50 μg standard chlorogenic acid hemihydrate (Fig. 6a). In the second calibration curve, each analytical value represented a 100-μg sample of the same tobacco variety which was spiked with 1.0–4.0 μg standard rutin (Fig. 6b). Similar calibrations were made with tomato, peach and buckwheat leaf samples.

A tobacco leaf sample high in plant phenols was analyzed five times for a determination of the precision of the analyses for caffeic acid and quercetin. The results and standard deviations obtained are as follows: caffeic acid = 1.32 $\pm$ 0.08; quercetin = 0.66 $\pm$ 0.08.

For comparison of GLC–electron capture analysis of caffeic acid and quercetin moieties in tobacco two other published methods were used for the analysis of tobacco leaf sample. In one case, a quantitative paper chromatographic–spectrophotometric analysis was performed for total chlorogenic acid isomers and rutin[15], and in the second case Arnow's nitrate–molybdate reagent[16], which yields a colored complex with *o*-dihydroxyphenols, was used. Assumptions[10] were made as follows: (1) caffeic acid and quercetin were not in the free state, and (2) chlorogenic acid isomers and rutin were the only caffeic acid and quercetin derivatives as well as the only *o*-dihydroxy plant phenols in the sample. Inspection of Table II shows the correlation

TABLE II

COMPARISON OF GLC ASSAY WITH OTHER METHODS FOR CHLOROGENIC ACID AND RUTIN IN A TOBACCO LEAF SAMPLE

Method	*Chlorogenic acid isomers (%)*	*Rutin (%)*	*Chlorogenic acid isomers plus rutin (%)*
GLC	0.30	0.23	0.53
Paper chromatography–spectrophotometry	0.38	0.10	0.48
Spectrophotometry (ARNOW's reagent)	0.58	0.16	0.74

among the results obtained by the three methods. In view of the dissimilarity of the analytical methodology and the necessary assumptions involved, the differences among the results do not seem great, and the use of the GLC–electron capture method for the estimation of the chlorogenic acid and rutin content of tobacco leaf seems valid.

The advantages of this method for the analysis of caffeic acid and quercetin moieties in plant leaf samples include: (1) a relatively short analytical period of approximately 5 h compared to a period about two times longer required for a quantitative conventional paper chromatographic analysis, (2) greater sensitivity than other GLC or paper chromatographic methods of specific plant phenol analysis[3,10] (*e.g.* approximately 20 ng caffeic acid and 300 ng quercetin are detectable), and (3) quantitation of results for these moieties in leaf samples.

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CHROM. 4893

THE HYDROLYSIS OF PROTEINS*

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SUMMARY

Experiments were made to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl with a view to developing a rapid hydrolysis procedure. The maximum yield for all of the protein amino acids was obtained at 145° ± 2° for the minimal time of 4 h. Essentially equivalent hydrolysis of ribonuclease was achieved at the two different hydrolysis conditions, *i.e.*, 110° ± 1° for 26 h, or 145° ± 2° for 4 h. The yields obtained were in good agreement.

INTRODUCTION

The particular method used for the hydrolysis of proteins prior to an amino acid analysis is of considerable importance since some amino acids are preferentially destroyed and the hydrolysis of others is incomplete. In view of the high precision attained in the gas–liquid chromatographic (GLC) analysis of amino acid mixtures, the nature of the hydrolytic conditions plays an increasingly important role and can be easily evaluated. The speed, precision, and accuracy of the GLC methods developed by GEHRKE *et al.*[1–7] make possible a thorough investigation of the various parameters involved in the quantitative hydrolysis of different proteins and their compositional characterization.

A hydrolysis reagent of broad specificity is required to break all of the possible peptide bonds which are found in natural products of varying complexity. The particular reagent used must be capable of cleaving all peptide bonds in a protein. Further, the peptide bonds must be accessible to the hydrolytic agent; however, two features of protein structure present difficulties in this respect. First, there is steric hindrance due to the bulky side chains of the aliphatic amino acids, and secondly, the macromolecular structure, *i.e.* that due to secondary and tertiary bonding of the protein,

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** Experimental data taken in part from doctoral research, University of Missouri, William Henry Hatch predoctoral research fellow. Now Associate Professor at Miami Dade Jr. College, Miami, Fla., U.S.A.

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prevents complete hydrolysis. The degree to which a protein molecule can unfold is limited by its secondary and tertiary structure; therefore, the hydrolysis reagent may react rapidly on one part of a protein molecule and slowly on another. This is evidenced by the number of different hydrolysis methods that are reported.

Differences in the stability of the various functional groups of amino acids necessitate a compromise among several experimental conditions in order to achieve the optimum hydrolysis of the protein. MOORE AND STEIN[8] reported that the best "all around" hydrolysis can be achieved by reaction for 24 h with 6 *N* HCl at 110° under conditions rigorously excluding oxygen, non-protein substances, and metals. Oxygen can be excluded by using a sealed tube hydrolysis technique. Generally, acid hydrolysis will yield over 95% recovery for aspartic and glutamic acids, proline, glycine, alanine, methionine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. However, tryptophan is completely destroyed, whereas 5–15% of threonine and serine are destroyed. Extrapolation to "zero-time" of hydrolysis can be done, but requires several different times of hydrolysis for each sample. GLC makes studies of "zero-time" hydrolysis practical. The peptide bonds of valine, isoleucine, and leucine are quite stable and thus a longer hydrolysis time is required to obtain maximum yield for these amino acids. WHITFIELD[9] has studied this problem and explained it in terms of steric factors. Extending the hydrolysis time to 70 h gives maximum yields[8] for these three amino acids. This, of course, results in lower yields for the other amino acids as compared to a 24 h hydrolysis time. As yet, no satisfactory method has been found for tryptophan, except alkaline, or enzymatic hydrolysis[8].

The purpose of this research was to study the rates and yields of protein hydrolysis and to determine the optimum reaction conditions which would give maximum yields of all twenty of the protein amino acids in the shortest possible time, using ribonuclease as a representative protein.

LITERATURE REVIEW

BRACONNOT[10], in 1820, first used sulfuric acid for the hydrolysis of a protein. The use of HCl as a hydrolytic agent was introduced by BOPP[11] in 1849. The hydrolytic agent commonly used today is HCl since the rate of peptide bond cleavage is increased in HCl over what it would be in sulfuric acid of equal concentration. An added advantage of HCl is that it can easily be removed from an amino acid mixture by evaporation. Protein samples are usually hydrolyzed with 2.5–5000 times their weight of 6 *N* HCl under reflux for 18–24 h.

The method of MACPHERSON[12] is generally recommended for large protein samples (*ca.* 0.2 g or larger). A protein sample which has been equilibrated under atmospheric conditions is weighed into a suitable round-bottomed flask which is fitted with a condenser. Concentrated A.R. HCl (36 w/w%) is added (*ca.* 20 ml/g protein), the protein is dissolved on a water bath at 35–40°, then sufficient hot doubly distilled water is added to bring the concentration of HCl to 20 w/w%. The solution is boiled gently under reflux for 24 h. The excess of HCl is removed under a partial vacuum and the sample is diluted to a suitable volume with 0.1 *N* HCl. An aliquot of this solution is then removed for classical amino acid analysis or GLC amino acid analysis.

The method of MOORE AND STEIN is in common use. A sample of air-dried or

lyophilized protein is placed in a 16 × 135 mm heavy-walled Pyrex tube (Corning No. 9860). The protein is suspended in 1 ml of 6 *N* HCl (a 1:1 dilution of concentrated reagent HCl with doubly distilled water). The sample is frozen by placing in a bath of ethanol and solid carbon dioxide. After freezing, the sample container is evacuated to below 50 μ, then sealed under vacuum. The hydrolysis is conducted at 110° ± 1° for 20 h or 70 h. Excess HCl is removed under vacuum at 40–45°, the sample is diluted to a known volume, and aliquots are removed for analysis. This technique or some modification of it is presently the preferred method for the hydrolysis of protein samples.

A serious problem associated with the acidic hydrolysis of proteins is the partial decomposition of some of the amino acids. The destruction of tryptophan is almost complete and a considerable loss of cysteine may occur. The breakdown of the other amino acids generally occurs to a lesser degree.

REES[13] reported in 1946 that hydrolysis with 6 *N* HCl for 24 h leads to a recovery of only 89.5% for serine and 94.7% for threonine. CORFIELD AND ROBSON[14] reported a 14% loss of serine in the hydrolysis of salmine. REES[13] and HIRS *et al.*[15] found the rate of decomposition of serine and threonine to vary with the purity of the HCl used in the acidic hydrolysis. However, an accurate determination of the threonine and serine content can be made by extrapolation to "zero-time" of hydrolysis if data are available for several different hydrolysis times. Examples of this technique were included in publications by HARFENIST[16] in 1953, by SMITH AND STOCKELL[17] in 1954, by HIRS *et al.*[15] in 1954, and by NOLTMANN *et al.*[18] in 1962.

There is a possibility that proline is degraded during acid hydrolysis. ELLIOT *et al.*[19] and ZUBER AND JAQUES[20] both suggested an empirical formula of $Arg_2Phe_2Pro_2Gly \cdot Ser$ for the peptide bradykinin from results based on amino acid analyses after acidic hydrolysis. BOISSONMAS *et al.*[21], however, synthesized bradykinin and found that the actual structure corresponded to the formula $Arg_2Phe_2Pro_3Gly \cdot Ser$. The variance between the formula determined from amino acid analysis and the actual formula may be due to the decomposition of proline during the acid hydrolysis prior to analysis.

LUGG[22] observed that pure tyrosine was not affected by heating it in acid at 100° for 20 to 30 h. LIGHT AND SMITH[23], however, reported that tyrosine was completely destroyed during the acid hydrolysis of the peptide Ala·Val·Gly·Tyr. SHEPHERD *et al.*[24] also obtained low recoveries of tyrosine from several peptides. This destruction was reduced but not eliminated when the samples were hydrolyzed under a nitrogen atmosphere. The decomposition of tyrosine may involve aspartic acid since tyrosine was quantitatively recovered from the peptide Val·Tyr·Pro but not from Val·Tyr·Pro·Asp. MUNIER[25] reported that tyrosine may be converted to 3-chlorotyrosine during hydrolysis by reacting with traces of chlorine in the HCl. This reaction could not, however, account entirely for the losses observed by HIRS *et al.*[15].

A large concentration of carbohydrates in the hydrolysis medium may seriously reduce certain amino acids. TRISTRAN[26] noted that arginine was extensively destroyed during acidic hydrolysis in the presence of carbohydrates with the amount of destruction being proportional to the concentration of carbohydrates, and BAILEY[27] reported losses of methionine as high as 20% in samples which were high in carbohydrate content. OSONO *et al.*[28] found that refluxing methionine with 10% HCl

resulted in the production of some homocystine, homocysteine, and glycine. LUGG[29] observed only a slight loss of cystine during acidic hydrolysis in the absence of carbohydrate; however, losses of 6 to 7% were noted in the presence of carbohydrates.

Lysine is considered to be the most stable of the diamino acids, but ISHII[30] reported a loss of 3% when lysine was heated at reflux with 20% HCl. The reported degradation products were aspartic acid, glycine, glutamic acid, and α-aminoadipic acid.

Steric hindrance by bulky side chain residues results in the slow release of some amino acids, particularly valine and isoleucine. Kinetic studies, by SYNGE[31] in 1945, and by HARRIS *et al.*[32] in 1956, clearly indicated hindrance by valine, leucine, alanine, and isoleucine and the yields for these amino acids which have been hydrolyzed for varying lengths of time were found to be a function of time. An accurate value for each of these amino acids can be determined by plotting yield as a function of hydrolysis time, and by drawing tangents to the maximums in the curves, then extrapolating to "zero-time".

The rates of decomposition of the amino acids during acidic hydrolysis are dependent on several factors including: the concentration of the hydrolyzing acid, the purity of the acid used, the time and temperature of hydrolysis, the presence of carbohydrates, aldehydes or metal impurities. Current methods represent a compromise among the several considerations mentioned above. The most common methods for the hydrolysis of proteins are outlined in two excellent review articles by LIGHT AND SMITH[33] and by MOORE AND STEIN[8].

Because of the considerable time required for the hydrolysis of proteins by the reported methods and the losses involved, it was considered highly important to investigate other procedures. From studies directed toward this goal, this research reports on a rapid hydrolysis method which can be completed in 4 h.

EXPERIMENTAL

Apparatus

A Varian Aerograph Model 2100 gas chromatograph with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and equipped with a Varian Model 20 recorder, and a Packard Instruments Co. Model 7300 dual column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder were used. Peak areas were determined with a digital readout integrator, Infotronics Model CRS 104.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump.

Pyrex 2.5 × 20 cm screw top culture tubes with teflon-lined caps (Corning No. 9826) were used as the reaction vessels for the hydrolysis.

Reagents

All reagents used were those specified by GEHRKE *et al.*[2].

The 6 *N* HCl solution in water was prepared by distilling a 6 *N* solution in an all-glass system and then adjusting the concentration of the constant boiling HCl to 6 *N* by the addition of doubly distilled water.

The 6 *N* HCl solution in *n*-butanol was prepared by bubbling anhydrous HCl into anhydrous *n*-butanol.

Ribonuclease A from bovine pancreas, crystallized five times, Type I-A, protease free, essentially salt free, activity of 70 Kunitz units per mg, was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Chromatography

The columns and chromatographic conditions were those as specified in ref. 2.

HYDROLYSIS METHODS

Hydrolysis of samples in 6 N HCl in water for GLC analysis

(1) Accurately weigh 10.0 mg of dry protein (ribonuclease) into the pyrex screw top with teflon-lined cap culture tube. (2) Flush tube with filtered N_2. (3) Add 10.0 ml of 6 *N* HCl in water to each tube. (4) Flush each tube again with N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (7) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in 1 ml of BuOH–HCl). (8) Add 1.5 ml of *n*-butanol 3 *N* in HCl per 1.0 mg of total amino acids, ultrasonic mix for 15 sec, esterify at 100° for 35 min, then dry at 60° under partial vacuum, and acylate as described in ref. 2.

Hydrolysis of samples in 6 N HCl in water for analysis by both GLC and classical ion exchange

(1) Accurately weigh 25.0 mg of dry protein (ribonuclease) into a large culture tube. (2) Flush tube with filtered N_2. (3) Add 25.0 ml of 6 *N* HCl in water to each tube. (4) Flush each tube again with N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (7) Accurately pipet 20.0 ml of 0.1 *N* HCl into each of the samples to dissolve the amino acid residue. Mix each sample thoroughly. (8) Draw a 5.0 ml aliquot of each sample and place in a $\bar{S}$ 125 ml flat-bottom boiling flask for GLC analysis, or analyze by classical ion exchange. (9) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (10) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in 1 ml of BuOH–HCl). (11) Add 1.5 ml *n*-butanol 3 *N* in HCl per 1.0 mg of total amino acids, ultrasonic mix for 15 sec, esterify at 100° for 35 min, then dry at 60° under a partial vacuum, and acylate as described in ref. 2.

Hydrolysis of samples by 6 N HCl in n-butanol

(1) Accurately weight 10.0 mg of dry protein (ribonuclease) into a large culture tube. (2) Flush tube with filtered nitrogen gas. (3) Add 15.0 ml of *n*-butanol 6 *N* in HCl (1.5 ml of BuOH–HCl per 1.0 mg of protein). (4) Flush reaction vessel again with filtered N_2. (5) Place teflon-lined cap on each tube and heat at 110° ± 1°, or 145° ± 2° in an oil bath for the specified time. (6) Add an appropriate quantity of I.S. (0.50 mg butyl stearate, dissolved in BuOH–HCl). (7) Dry the samples at 60° under a partial vacuum with a rotary evaporator. (8) Acylate as described by GEHRKE *et al.*[2].

RESULTS AND DISCUSSION

According to the direct esterification procedure reported by ROACH AND GEHRKE[6] the amino acids are esterified in *n*-butanolic HCl prior to their acylation with trifluoroacetic anhydride. The present procedure for the GLC analysis of a protein sample requires that the protein sample be hydrolyzed in 6 *N* HCl in water, dried, and then esterified with *n*-butanol 3 *N* in HCl. If complete hydrolysis of the protein were achieved in *n*-butanolic HCl, one of the steps in the GLC analysis of a protein hydrolysate could be eliminated since the *n*-butyl esters of the amino acids would be formed during the hydrolysis of the protein. Thus, studies were made to investigate the yields of hydrolysis of a model protein, ribonuclease, in 6 *N* HCl in *n*-butanol.

Samples of ribonuclease were hydrolyzed at 110° ± 1° in *n*-butanol 6 *N* in HCl for varying lengths of time. The experimental results from the GLC analysis of this hydrolysate are presented in Table I. A sample of ribonuclease was also hydrolyzed at 110° ± 1° in water 6 *N* in HCl for varying lengths of time. The GLC data are presented in Table II. Much higher yields were obtained for the samples hydrolyzed in aqueous 6 *N* HCl. Also, decomposition of all of the amino acids became a serious problem at hydrolysis times longer than 44 h with *n*-butanol 6 *N* in HCl, and very poor results were obtained on hydrolysis for 4 h to 10 h (Table I).

Since *n*-butanol 6 *N* in HCl was found to be unsuitable for the hydrolysis of proteins, it was decided to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl. Samples were hydrolyzed at 110° ± 1°, 145° ± 2°, and 175° ± 5° for 1 to 10 h. The optimum hydrolysis temperature in terms of rate of hydrolysis and a minimum of decomposition was found to be at 145° ± 2° for 4 h.

TABLE I

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with *n*-butanol 6 *N* in HCl for the specified time at 110° ± 1°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]				
	4 h	*10 h*	*24 h*	*44 h*	*70 h*
Alanine	2.26	3.61	6.54	9.14	3.31
Valine	0.81	1.72	3.69	5.27	3.48
Glycine	0.73	0.84	1.59	2.06	1.01
Isoleucine	0.27	0.47	0.73	1.23	1.50
Leucine	0.28	0.58	1.95	2.22	1.47
Proline	1.45	1.87	3.62	3.90	2.75
Threonine	1.34	1.59	2.59	2.20	1.24
Serine	3.53	3.48	3.98	3.29	1.81
Methionine[b]	—	—	—	—	—
Phenylalanine	0.61	0.94	2.52	2.90	2.36
Aspartic acid	2.93	4.33	8.52	7.00	3.80
Glutamic acid	3.06	4.31	8.15	7.10	4.56
Tyrosine[b]	—	—	—	—	—
Lysine	1.90	3.06	6.94	5.76	2.17

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml of *n*-butanol 6 *N* in HCl).

[b] The peaks obtained for methionine and tyrosine were too small to allow an accurate determination.

TABLE II

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 110° ± 1°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]					
	9½ h	*26 h*	*35 h*	*48 h*	*81 h*	*116 h*
Alanine	7.06	7.12	7.35	7.46	7.57	7.51
Valine	4.33	6.43	6.90	7.28	7.56	7.52
Glycine	1.60	1.65	1.66	1.66	1.69	1.68
Isoleucine	0.81	1.50	1.69	1.99	2.39	2.59
Leucine	1.83	1.91	1.96	1.99	2.02	2.01
Proline	2.78	3.09	3.12	3.14	3.19	3.17
Threonine	6.91	7.35	7.71	7.72	7.58	7.27
Serine	9.91	10.04	9.96	9.80	9.20	8.32
Methionine	3.47	3.39	3.36	3.44	3.17	2.93
Phenylalanine	2.59	3.05	3.17	3.22	3.25	3.29
Aspartic acid	13.23	13.39	13.74	13.97	13.96	13.99
Glutamic acid	11.13	12.08	12.43	12.04	12.49	12.33
Tyrosine	5.74	6.56	6.63	6.59	6.26	6.06
Lysine	8.88	10.15	10.44	10.59	10.61	10.75

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

TABLE III

AMINO ACID ANALYSIS OF RIBONUCLEASE AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed in a closed tube with 6 *N* HCl for the specified time at 145° ± 2°. EGA column only. *n*-Butyl stearate as internal standard. Ribonuclease, Type 1-A, Sigma Chemical Co. Analyses by GLC.

Amino acid	*Yield (w/w%)*[a]						
	2 h	*4 h*	*5 h*	*6 h*	*7 h*	*8½ h*	*9 h*
Alanine	7.30	7.28	7.24	7.37	7.40	7.54	7.45
Valine	6.25	7.15	7.16	7.31	7.41	7.54	7.55
Glycine	1.69	1.17	1.66	1.72	1.67	1.71	1.71
Isoleucine	1.40	1.94	2.07	2.23	2.26	2.53	2.55
Leucine	1.99	1.98	1.98	2.04	2.00	2.02	2.07
Proline	3.18	3.19	3.15	3.16	3.14	3.15	3.16
Threonine	7.73	7.62	7.52	7.36	7.11	7.18	7.17
Serine	10.25	9.66	9.40	8.82	8.52	8.15	8.01
Methionine	3.54	3.36	3.29	3.11	3.06	2.77	2.24
Phenylalanine	3.16	3.26	3.22	3.29	3.22	3.42	3.38
Aspartic acid	13.83	13.60	13.66	13.76	13.60	13.81	13.97
Glutamic acid	11.79	11.88	11.97	12.00	11.75	12.05	12.29
Tyrosine	6.57	6.80	7.00	7.26	7.29	6.17	6.47
Lysine	10.29	10.37	10.45	10.42	10.10	10.27	10.65

[a] Each value represents an average of two determinations at the macro level (10 mg total protein + 15 ml 6 *N* HCl).

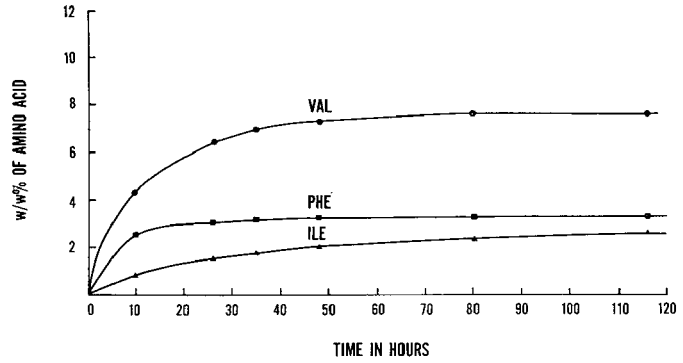

Fig. 1. Yields of the amino acids valine, isoleucine and phenylalanine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 110° ± 1°.

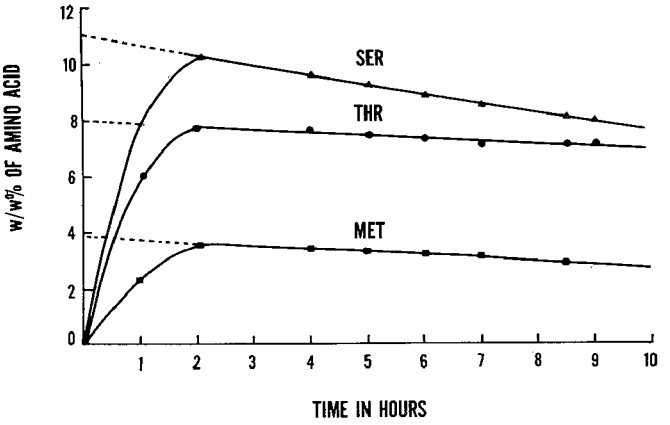

Fig. 2. Yields of the amino acids serine, threonine and methionine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 145° ± 2°.

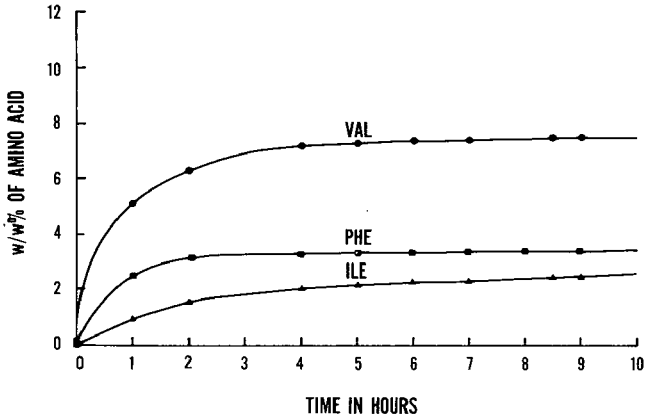

Fig. 3. Yields of the amino acids valine, isoleucine and phenylalanine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 145° ± 2°.

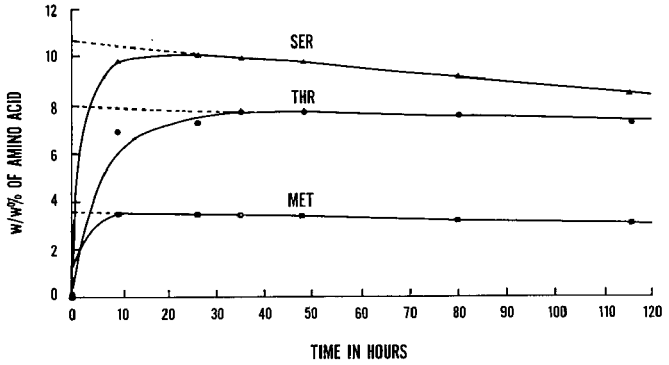

Fig. 4. Yields of the amino acids serine, threonine and methionine from ribonuclease as a function of hydrolysis time. Hydrolyzed in 6 *N* HCl at 110° ± 1°.

The GLC data in Table III were obtained from the analysis of samples of ribonuclease which had been hydrolyzed at 145° ± 2° for periods of time from 2 to 9 h. These data indicate that approximately equivalent hydrolyses of a protein can be achieved in 4 h at 145° ± 2° and in 24 h at 110°.

MOORE AND STEIN[8] reported that the best "all around" hydrolysis can be achieved with 6 *N* HCl for 24 h at 110° under conditions rigorously excluding oxygen, non-protein substances, and metals. These experimental conditions were selected to give maximum total recovery of the amino acids. Certain of the amino acids, however, undergo serious decomposition and the hydrolysis of others is incomplete. Maximum values for isoleucine, leucine, valine, and phenylalanine can be obtained from a plot of yield *versus* hydrolysis time. Also, an extrapolation to "zero-time" can be made to determine more accurate values for those amino acids which undergo serious decomposition, *i.e.* threonine, serine, and methionine.

Plots of yield *versus* hydrolysis time at 110° ± 1° for valine, isoleucine, phenylalanine, threonine, serine, and methionine from ribonuclease are shown in Figs. 1

TABLE IV

SELECTED AMINO ACIDS FROM THE AMINO ACID ANALYSIS OF RIBONUCLEASE

Amino acid	*Yield (w/w%)*		
	110° for 24 h	*145° for 4 h*	*Literature value*[15]
Valine	7.54[a]	7.55[a]	7.49
Isoleucine	2.54[a]	2.59[a]	2.67
Threonine	8.00[b]	8.00[b]	8.90
Serine	10.70[b]	11.05[b]	11.40
Methionine	3.54[b]	3.83[b]	4.00
Phenylalanine	3.30[a]	3.40[a]	3.51

[a] Values obtained by drawing a tangent to the maximum in a plot of yield of amino acid *versus* time of hydrolysis to obtain the maximum values.

[b] Values obtained by extrapolating to "zero-time" a plot of yield of amino acid *versus* time of hydrolysis.

TABLE V

AMINO ACID ANALYSIS OF BOVINE SERUM ALBUMIN AS A FUNCTION OF HYDROLYSIS TIME

Hydrolyzed with 6 *N* HCl for the specified time at 145° ± 2° in a closed tube, with norleucine as internal standard. Analyses by classical ion exchange.

Amino acid	*Yield (w/w%)*					
	2 h	*4 h*	*6 h*	*8 h*	*9 h*	*24 h*[a]
Alanine	4.95	5.00	5.14	4.83	4.91	4.76
Valine	4.15	4.46	5.10	4.79	4.89	4.56
Glycine	2.05	2.25	2.11	2.02	1.97	1.97
Isoleucine	1.18	2.12	2.27	2.16	2.22	2.04
Leucine	11.45	11.91	12.30	11.91	11.70	11.42
Proline	4.72	4.23	4.34	4.73	3.84	3.76
Threonine	4.97	5.07	5.05	4.91	4.93	4.97
Serine	3.64	3.73	3.47	3.08	3.40	3.55
Methionine	0.74	0.81	0.68	0.72	0.72	0.70
Phenylalanine	6.50	6.88	6.83	6.53	6.45	6.59
Aspartic acid	9.43	9.49	9.62	9.42	9.18	9.02
Glutamic acid	15.82	15.79	16.13	15.91	15.56	15.49
Tyrosine	5.57	5.92	5.34	5.38	5.15	5.53
Lysine	11.51	11.88	12.01	11.73	11.76	11.56
Histidine	4.00	4.43	4.28	4.16	4.06	4.09
Arginine	5.84	6.10	6.00	5.73	5.87	5.83
Tryptophan	—	—	—	—	—	—
Half-cystine	5.93	6.08	5.84	5.96	5.51	5.86

[a] Hydrolyzed with 6 *N* HCl for 24 h at 110° ± 1° in a closed tube, norleucine as internal standard. Analyses by classical ion exchange.

TABLE VI

RECOVERY OF AMINO ACIDS FROM A STANDARD MIXTURE

0.4 mg of *each* amino acid + 10 ml 6 *N* HCl heated for the specified time at 145° ± 2°. Each value represents an average of two independent analyses. Analyses by GLC.

Amino acid	*Recovery (%)*					
	2 h	*4 h*	*6 h*	*7 h*	*8 h*	*9 h*
Alanine	100.6	101.8	96.9	95.6	95.5	93.1
Valine	101.2	100.9	94.1	92.9	91.8	89.4
Glycine	100.6	99.7	95.5	92.9	90.3	87.7
Isoleucine	101.8	102.9	95.8	92.3	89.9	87.6
Leucine	101.2	98.8	94.9	92.8	91.5	89.2
Proline	99.4	94.6	89.9	88.7	89.9	88.9
Threonine	98.6	93.2	90.4	86.3	84.9	84.6
Serine	99.3	90.5	87.6	84.7	81.8	78.8
Methionine	98.1	90.6	79.2	73.6	71.7	75.5
Hydroxyproline	97.4	94.7	89.5	85.5	84.2	83.8
Phenylalanine	102.3	97.7	95.5	94.1	93.2	90.9
Aspartic acid	101.9	98.9	97.6	96.3	95.5	95.4
Glutamic acid	101.6	100.7	100.6	99.7	99.4	97.2
Tyrosine	102.6	100.0	98.7	94.2	93.5	90.9
Lysine	100.0	97.4	94.8	93.4	89.5	92.1
Arginine	95.2	88.1	85.7	76.2	77.1	65.2
Histidine	101.1	97.5	97.4	97.3	97.3	88.4
Cystine	102.8	97.1	100.0	85.7	77.1	74.3

and 4. Similar plots for hydrolysis of ribonuclease at 145° ± 2° are included in Figs. 2 and 3. Maximum values for threonine, serine, and methionine were obtained by extrapolating to "zero-time" as shown, whereas the maximum values for valine, isoleucine, and phenylalanine were obtained from the maximumin the curves. Maximum values for the protein amino acids from ribonuclease obtained in this way are included in Table IV. The agreement of the maximum values from the 110° curves with the corresponding values at 145° ± 2° indicates that hydrolysis under both sets of conditions gave essentially the same results. Also, both sets of data are in excellent agreement with the literature values.

After developing the set of "hydrolysis conditions" (145° ± 2° for 4 h) using ribonuclease as a model protein, several other proteins were then hydrolyzed under the same conditions and analyzed. The data for one of the proteins, bovine serum albumin, analyzed by classical ion exchange are given in Table V. The good agreement of the data for hydrolysis for 4 h at 145° ± 2° with 24 h at 110° shows that the hydrolysis of these proteins can be conducted equally well under these two sets of conditions.

Recovery data for GLC analysis of mixtures of the protein amino acids which had been heated for varying times at 145° ± 2° are included in Table VI. The recovery for all of the amino acids except arginine was excellent with a hydrolysis time of 2 h. On hydrolysis for 4 h, the losses in some cases were significant. Low recoveries were obtained for proline, threonine, serine, methionine, hydroxyproline, and arginine. Plots of yield *versus* hydrolysis time are required to obtain accurate results for these amino acids.

CONCLUSIONS

The use of *n*-butanol 6 *N* in HCl as a protein hydrolysis reagent would obviate one of the steps in the reported[2] GLC analysis of proteins since the *n*-butyl esters of the amino acids would be formed during the hydrolysis. Thus, these studies were initiated to investigate the yields on hydrolysis of a model protein, ribonuclease, in 6 *N* HCl in *n*-butanol. However, this reagent was found to be unsatisfactory since the rate of hydrolysis was much slower in this medium than it was in 6 *N* HCl in water, and the rates of decomposition of the amino acids were considerably faster.

Since *n*-butanol 6 *N* in HCl was found to be unsuitable for the hydrolysis of proteins, experiments were made to investigate the effect of temperature on the hydrolysis reaction in aqueous 6 *N* HCl with a view to developing a rapid hydrolysis procedure. The maximum yield for all of the protein amino acids was obtained at 145° ± 2° for the minimal time of 4 h. Essentially equivalent hydrolysis of ribonuclease was achieved at the two different hydrolysis conditions, *i.e.* 110° ± 1° for 26 h, or 145° ± 2° for 4 h. The yields obtained were in good agreement.

Plots of yield *versus* hydrolysis time *must* be made for *each* amino acid to obtain the best possible values for the amino acid composition of a protein. These plots are then extrapolated to "zero-time" for those amino acids which undergo decomposition; for those amino acids which are difficult to hydrolyze, the values are obtained from the maximum in the curves. Several protein samples must be hydrolyzed at different times to obtain all the data necessary to construct these plots. Therefore, a rapid hydrolysis method is needed which gives maximum values for the amino acids with a minimum of decomposition. Recovery studies as a function of hydrolysis time at

145° were made using standard amino acid mixtures; essentially complete recovery was obtained on hydrolyzing for 2 to 4 h. Pro, Thr, Ser, Met, OHPro, and Arg were the most sensitive to heat. This reported procedure allows one to rapidly hydrolyze several samples of a protein at 145° ± 2° and then to conveniently obtain the data by GLC for plotting these curves. The agreement among the data for the two different hydrolysis temperatures and times conclusively demonstrates that such plots can be made. Of special interest is a comparison of the data obtained for ribonuclease and bovine serum albumin for the 4 h hydrolysis at 145° ± 2° with the 24 h hydrolysis at 110° ± 1°. In almost every case a higher recovery was obtained for the 145° ± 2° hydrolysis.

With this method a protein can be essentially completely hydrolyzed in 4 h with a minimum of decomposition of the amino acids. Rapid hydrolysis of proteins coupled with quantitative GLC analysis of amino acids provides a powerful tool in protein research, biochemical, and nutritional investigations.

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AN EXPERIMENTAL STUDY OF AMINO ACID DEGRADATION UNDER OPEN FLASK HYDROLYTIC CONDITIONS

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SUMMARY

The authors suggest that the open flask system for acid hydrolysis of proteins could also be used to advantage when it is necessary to submit small quantities of pure and isolated proteins to acid hydrolysis. This method is simpler and offers a higher repeatibility, regarding the removal of dissolved oxygen, as against the sealed vial system. An experimental study of the behaviour of an artificial protein amino acid mixture, under standard open flask hydrolytic conditions, in constant boiling hydrochloric acid and in an oxygen-free atmosphere, is reported. The interaction of tryptophan and cystine under these conditions with formation of cysteine was investigated. It was found that this amino acid can severely interfere with the chromatographic separation of proline, when sodium buffers are employed in the elution of resin columns. The separation of these two amino acids has been achieved by employing a method which uses lithium buffers and which has been previously described by one of the authors for the separation of amino acids and related compounds in physiological fluids.

INTRODUCTION

Protein cleavage by acid hydrolysis in order to obtain amino acid solutions suitable for application to chromatographic analytical columns, is now usually accomplished, when isolated proteins are under investigation, according to the well known sealed vial system. On the other hand, when the amino acid analysis of the protein content of foods and feeds is required, owing to the necessity of working with larger samples, the hydrolysis of these proteinaceous materials is preferably carried out according to the open flask method, under a nitrogen atmosphere[1].

Since the latter method, from an operational point of view, is much easier and, as far as the dissolved oxygen removal is concerned, offers a better degree of repeatibility as against the sealed tube, it is suggested that, even for the hydrolysis of isolated proteins, whenever the sample quantity is not extremely low, the open flask method would be preferable.

Acid hydrolysis of proteins, even under controlled conditions, results in the loss, sometimes complete, of certain amino acids as HIRS *et al.*[2], TRISTRAM AND SMITH[3] and SMITH AND STOCKELL[4] have reported when employing the sealed tube method.

The experimental study reported here was therefore carried out in order to check the amino acid degradation as well as the reciprocal interference of some amino acids under open flask hydrolytic conditions. In particular, the behaviour of tryptophan and cystine by themselves as well as in the presence of each other was investigated.

The stability of tryptophan, under acidic conditions, when not in the presence of cystine and in the absence of oxygen, was confirmed[5–8].

The formation of cysteine as a product of the oxidation–reduction reaction between cystine and tryptophan was also confimed[6,9] and it was found that the formation of this compound from cystine can severely interfere with the determination of proline when using our method of protein amino acid analysis[10] employing Amberlite IR-120 crushed resin with the buffer elution system according to SPACKMAN *et al.*[11]. On the other hand, WAINER[12] has reported that, by employing the method of PIEZ AND MORRIS[13] for the protein amino acid analysis with Chromobead resin Type A, the cysteine peak merges with proline. Consequently, a study has been carried out in order to find a means of ridding the proline peak of cysteine. This was achieved by using the method for the determination of amino acids and related compounds in physiological fluids described in a previous paper by MONDINO[14], which employs an Amberlite IR 120 crushed resin column which is eluted with a lithium buffer system.

MATERIALS AND APPARATUS

Hydrochloric acid

36% analytical grade hydrochloric acid (Merck) diluted with water 1:1 (v/v) was twice distilled in a quartz Heraeus bidistiller; in this way constant boiling hydrochloric acid solution, about 6 *N*, was obtained.

Nitrogen

High purity grade nitrogen, containing less than 5 p.p.m. of oxygen was employed.

Pyrogallol solution

In order to obtain nitrogen absolutely free of oxygen, the high purity nitrogen was bubbled through a pyrogallol solution, which was obtained by dissolving 30 g of pyrogallol in 60 ml of warm water. To this solution, 160 ml of KOH in water (1:2, w/v) were added. 1 ml of the final solution is capable of absorbing between 10 to 12 ml of oxygen.

Amino acids

The following amino acids were employed: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, lysine, histidine and arginine; all were obtained from Fluka.

Amino acid solutions

The amino acids were dissolved in constant boiling hydrochloric acid at the amount of 750 nmoles of each amino acid per ml of solution. This concentration was chosen so that we obtained, in the case of the solution of the complete mixture,

the same order of magnitude of the proportion protein–acid as that usually employed in the sealed vial system (1/500).

Reaction flask

A 500 ml three neck reaction flask manufactured by Quickfit (catalog No. FR 500/4S/44A) was employed. The reflux condenser was inserted in the central neck and the nitrogen bubbler, catalog No. MF 5-ST 53/24, was introduced into one of the lateral necks. Through the other lateral neck, which was kept stoppered throughout the reaction time, the samples could be withdrawn. The flask was heated on an electric thermoregulated heating mantle, regulated in such a way that uniform and gentle boiling of the solution took place.

Rotating evaporator

A rotating film evaporator manufactured by Büchi, provided with a 50 ml evaporating flask, and heated by means of a water steam bath, was employed.

Amino acid analyser

An amino acid analyser "Aminolyzer", manufactured by the Optica Co. of Milan, was employed for the amino acid automatic analysis.

METHOD

The amino acid solutions, containing, as previously said, 750 nmoles of each amino acid per ml of solution, were prepared in volumes of 250 ml and then introduced into the hydrolysis flask. Before warming, the oxygen was removed from the solution and the apparatus, by bubbling the solution with the oxygen-free nitrogen at a flow rate of 2 ml per sec for 20 min. The heating was then started and the solution was brought to boiling point. Throughout the reaction time the nitrogen was kept bubbling at 1 ml per sec, the gas flow rate being measured by means of a rotameter.

Samples were withdrawn at zero time and at 24, 48 and 120 h by means of a 10 ml pipette, to which a "pro-pipette" was connected. Before the sample withdrawal, heating was discontinued in order to stop the boiling and to get a temperature drop in the solution down to 104–105°. The nitrogen bubbling during sampling was maintained and the top of the reflux condenser was stoppered in order to avoid a flow of air taking place through the open sampling neck of the reaction flask with a consequent contamination of the solution by oxygen.

The samples were kept in a freezer at −30°. Before processing for the amino acid analysis, they were allowed to stand some time at room temperature and then 1 ml, exactly measured, was introduced into the rotating film evaporator and evaporated to dryness. This operation was repeated 3 times, adding each time about 1 ml of distilled water. Finally, the residue was dissolved in the evaporator flask by addition of 1 ml, exactly measured, of 0.1 *N* HCl.

0.1 ml of this solution were then employed for the analysis of the amino acids on each column according to the method described by MONDINO[10] in a previous paper. In order to improve the precision and degree of accuracy in the evaluation of the proline peak, the sensitivity of the second colorimeter, reading at 440 nm, has been expanded to 0.5 O.D. units for a full scale deflection as against 2 O.D. sensitivity main-

tained on the first colorimeter, reading at a 570 nm wavelength. The cuvette light path was 20 mm long on both colorimeters.

In order to separate the peaks of proline and cysteine, which emerge together when sodium buffers are employed for the column elution, and which both have an absorbance maximum at 440 nm, the method for amino acid and related compound determination described by MONDINO[14] was employed. This method, applied to columns of Amberlite IR-120 crushed resin, employs lithium salt buffers as eluting agents. With this system the cysteine peak emerges well separated before the proline peak. The sensitivity on the colorimeter reading at 440 nm was set at 0.5 O.D. units for a full scale deflection and a cuvette having a 10 mm light path was employed.

Since in our analytical system, as previously reported[10,14,15], peak evaluation is possible by simply measuring the height of the peaks over the base line in mm, the peak heights of the chromatograms of the samples at 24, 38 and 120 h have been compared with the peak heights of the chromatograms run for the samples taken at zero time, which were considered equal to 100.

RESULTS AND DISCUSSION

In Table I the results of 5 experiments on the hydrolytic treatment of a mixture of the 18 protein amino acids are reported as single amino acid percentages of the initial quantity at 24, 48 and 120 h of treatment. Mean values, standard deviations and coefficients of variation are also reported.

Examining these results, it can be seen that aspartic acid, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine are stable under these hydrolytic conditions. Threonine and

TABLE I

RESULTS OF 5 EXPERIMENTS IN WHICH A MIXTURE OF THE 18 PROTEIN AMINO ACIDS HAS BEEN HYDROLYSED BY THE OPEN FLASK METHOD

M = mean; S.D. = standard deviation; c.v. = coefficient of variation.

	24h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	100.3	101.0	99.1	102.2	100.4	100.6	1.06	1.05
Threonine	98.1	98.8	96.8	97.6	97.8	97.8	0.73	0.74
Serine	94.3	95.0	94.0	95.3	95.8	94.8	0.73	0.77
Glutamic acid	99	99.4	97.7	100.6	98.5	99.0	1.08	1.09
Proline	116.8	120.8	113.4	115.6	114.0	116.1	2.94	2.53
Glycine	100.4	100.3	99	100.7	99.2	99.9	0.77	0.77
Alanine	101.0	100.4	99.2	100.6	99	100.0	0.89	0.89
Cystine	62.9	66.1	68.4	63.3	63.2	64.8	2.40	3.71
Valine	100.6	100.9	99.4	100.0	99.2	100.0	0.74	0.74
Methionine	101.5	99.9	101.4	98.6	99.3	100.1	1.28	1.28
Isoleucine	99.5	101.2	102.0	102.0	99.1	100.8	1.38	1.38
Leucine	99.8	100.2	101.8	100.9	98.8	100.3	1.13	1.13
Tyrosine	100.8	100.0	102.1	101.6	99.1	100.7	1.21	1.20
Phenylalanine	101.8	99.6	99.8	100.6	98.7	100.1	1.17	1.17
Tryptophan	55	55.7	54.8	54.8	55.9	55.2	0.52	0.94
Lysine	98.2	97.6	99.1	99.7	102.2	99.4	1.78	1.79
Histidine	99.1	99.6	99.5	99.5	102.1	100.0	1.21	1.21
Arginine	99.1	100.6	98.3	99.3	102	99.9	1.45	1.45

	48 h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	100.6	101.2	99.7	101	98.8	100.3	1	1
Threonine	95.3	93.7	95.4	96.6	96.8	95.6	1.24	1.30
Serine	87.5	88.4	89.1	90.2	90.6	89.2	1.27	1.43
Glutamic acid	99.5	98.8	96.9	99.6	101.4	99.2	1.62	1.63
Proline	128.7	128.8	131	134.3	127.7	130.1	2.64	2.03
Glycine	100.6	100.8	98.5	101.6	99.3	100.2	1.24	1.24
Alanine	100.6	100.2	98.7	101.7	99.4	100.1	1.15	1.15
Cystine	52.3	49.9	49	48.8	53.2	50.6	1.99	3.9
Valine	100.5	100.4	99.2	99.9	98.8	99.8	0.74	0.75
Methionine	98.6	101.6	99.1	101.3	100.2	100.2	1.32	1.31
Isoleucine	98.4	98.1	98	100	101.4	99.2	1.48	1.49
Leucine	99.2	100	99	100.5	101.5	100.1	1.02	1.02
Tyrosine	100.4	100.9	99.3	100.5	98.6	99.9	0.96	0.96
Phenylalanine	102	101.5	100	100.4	100.8	100.9	0.81	0.80
Tryptophan	38.8	41	39.9	40	40.1	40.0	0.78	2.00
Lysine	98.2	100	97.6	99.7	98.8	98.9	1	1.01
Histidine	98.4	101.8	99.1	101.1	98.6	99.8	1.54	1.55
Arginine	100.3	100	99.8	101.4	92.8	99.9	1.15	1.15
	120 h							
	1	*2*	*3*	*4*	*5*	*M*	*S.D.*	*c.v.*
Aspartic acid	101.1	101.2	102.2	100.1	98.3	100.6	1.48	1.47
Threonine	80.0	89.3	90.3	87.7	86.8	88.6	1.37	1.55
Serine	72.1	70.9	70	73.4	72.8	71.8	1.39	1.93
Glutamic acid	101.9	101	99.3	99.1	98.2	99.9	1.51	1.51
Proline	147.1	143.0	150.0	148.1	151.2	147.9	3.16	2.14
Glycine	101.8	101.3	101.6	101	98.1	100.8	1.52	1.51
Alanine	102.5	101	101.8	100.3	98.7	100.9	1.46	1.45
Cystine	38.9	36.9	37.2	36.5	36.6	37.2	0.98	2.63
Valine	101.2	100.8	100.9	100.1	98.7	100.3	1.00	1.00
Methionine	100.2	101.9	99.6	101	99.3	100.3	1.12	1.12
Isoleucine	99.9	101.4	100.3	98.7	98	99.7	1.34	1.34
Leucine	101.6	101.9	101.5	99.9	99.1	100.8	1.23	1.22
Tyrosine	102.1	102.7	100	99.5	99.2	100.7	1.59	1.58
Phenylalanine	102.3	101.1	100.9	100.5	98.5	100.7	1.38	1.37
Tryptophan	11.1	11.6	11.5	11.8	11.4	11.5	0.27	2.25
Lysine	100.5	101.5	101.8	99.5	99.4	100.5	1.11	1.10
Histidine	99.7	100.8	102.8	99.3	98.8	100.5	1.41	1.05
Arginine	98.6	98.9	99	101.2	99.1	99.4	1.04	1.05

serine are slightly degraded, while cystine and tryptophan are severely destroyed. The values of proline are shown to be increasing, due to an artifact which occurs when amino acid analysis is carried out under conditions which do not allow the separation of cysteine from this amino acid.

Considering the trend of the degradation curves of threonine and serine reported in Fig. 1, it can be assumed that the degradation of these two amino acids is free from any interference, at this concentration level, by other amino acids. This has been confirmed by treating threonine and serine alone under the same hydrolytic conditions and examining samples taken after 24, 48 and 120 h of boiling in an open flask. The results obtained in this way are in perfect agreement with those obtained when

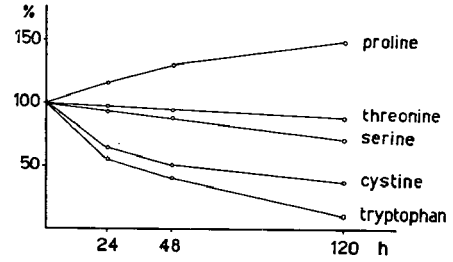

Fig. 1. Degradation curves (mean values of 5 results) of threonine, serine, tryptophan and cystine in the presence of each other and in the presence of all the other 18 protein amino acids. The artifacted curve of proline is also shown.

threonine and serine are treated in the presence of all the other protein amino acids.

The degradation curves of cystine and tryptophan, in the presence of all the other amino acids, are also illustrated in Fig. 1. The behaviour of these amino acids when treated separately, and alone, in the open flask under the conditions and at the concentration levels previously described, is completely different; both tryptophan and cystine are perfectly stable if boiled alone in hydrochloric acid in the absolute absence of oxygen. This result obtained in our open flask apparatus is in agreement with literature data[6] for the sealed tube system and can be taken as a test of the complete absence of oxidative conditions.

The behaviour of tryptophan and cystine in the presence of each other when

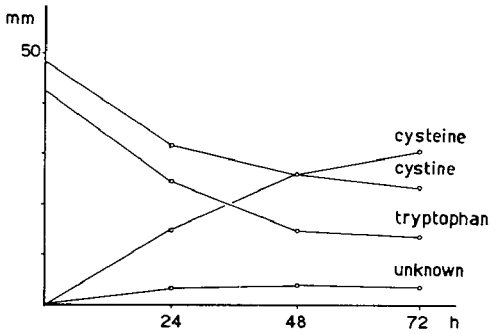

Fig. 2. Degradation curves of cystine and tryptophan treated in the presence of each other and in the absence of the other amino acids. The formation curves of cysteine and of another unknown reaction product are also illustrated.

treated for 72 h in the open flask, but in the absence of all the other amino acids has also been investigated. The results of this experiment are reported in Table II and in Fig. 2. The cysteine values were read on the second colorimeter at the 440 nm wavelength, as the absence of proline permitted the evaluation of cysteine.

Literature reports[6,9] state that, when tryptophan and cystine are both present in an acidic hydrolysis medium, in the absence of oxygen, sugars and metals, an oxidation–reduction equilibrium takes place, with the formation of 2 molecules of cysteine for every molecule of cystine and with consequent tryptophan oxidation. The oxidation–reduction reaction between tryptophan and cystine under the con-

TABLE II

RESULTS OBTAINED BY TREATING TRYPTOPHAN AND CYSTINE IN THE PRESENCE OF EACH OTHER AND IN THE ABSENCE OF THE OTHER AMINO ACIDS

The amino acid values are expressed as peak heights in mm over the base line of the chromatograms.

Time (h)	*Cystine*	*Tryptophan*	*Cysteine*	*Unknown*
0	48	42.5	0	0
24	32	24.5	15	3.5
48	26	15	26	4
72	23.5	13.5	30.5	3.5

ditions of our experiment gives rise, in addition to cysteine, to an unknown substance which, in the short column amino acid chromatogram eluted with sodium buffers, emerges just before tryptophan, as can be seen in Fig. 3.

With regard to cysteine, if the long column amino acid analysis is carried out by elution with sodium buffers, this amino acid cannot be separated, in that it has the same retention volume and the same absorbance maximum, when reacted with ninhydrin color reagent, as proline. The apparent increase in the proline values which can be seen in Table I and in Fig. 1, is satisfactorily explained in this way.

All our efforts, such as varying the column operational parameters, *e.g.* resin volume, temperature and pH, in our protein amino acid analysis method[10], in order to find suitable conditions for the separation of these two amino acids, were unsuccessful.

Since WAINER[12], who employed a different resin and the gradient buffer system of PIEZ AND MORRIS[13] for column elution, also did not succeed in obtaining this separation, it can be assumed that cysteine cannot be resolved from proline when sodium buffers are used as eluting media.

The resolution of this doublet became possible by employing the method for amino acids and related compounds in physiological fluids described by MONDINO[14] which uses lithium buffers for eluting Amberlite IR-120 crushed resin columns. Both

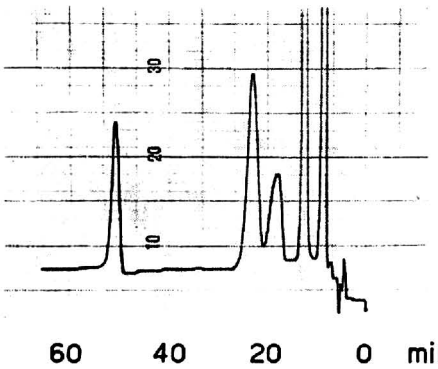

Fig. 3. Short column chromatogram of the reaction products which are formed when tryptophan and cystine alone are boiled for 48 h under hydrolytic conditions. The first two peaks from the left are ammonia and tryptophan. Just after tryptophan the peak of an unknown substance can be seen.

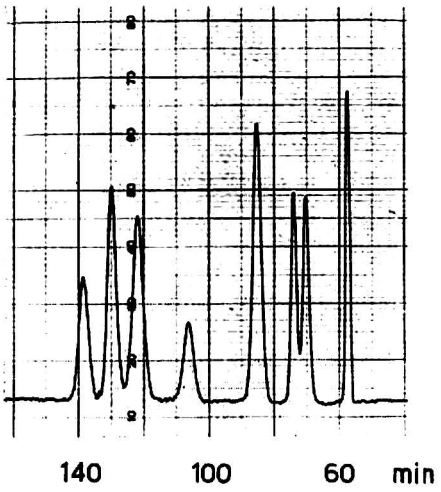

Fig. 4. Example of a separation with lithium buffers on Amberlite IR-120 crushed resin of proline from cysteine, originating from tryptophan. A sample of an 18 protein amino acid mixture after 48 h of hydrolytic treatment, has been chromatographed. The amino acids reported (200 nmoles load) are, from the left: alanine, glycine, proline, cysteine, glutamic acid, serine, threonine and aspartic acid, read on the second colorimeter at 440 nm, provided with a 10 mm light path cuvette and set at 0.5 O.D. for a full scale deflection.

peaks are read and recorded on the second channel when this is reading at 440 nm. In this system cysteine is eluted before proline as can be seen in Fig. 4, where an example of the separation of proline from cysteine with lithium buffers is shown.

It has to be stressed that, if a reducing sugar such as glucose, is added to the cystine–tryptophan mixture before acidic treatment, the formation of cysteine can be stopped.

Consequently, if a pure and isolated protein, containing in its molecule cystine and tryptophan residues, is submitted to acid hydrolysis by the open flask method in the total absence of sugars, metals and oxygen, the problem of the accurate determination of the proline residues, if present, can only be solved by employing lithium buffers to elute the resin columns of the automatic amino acid analyser.

Moreover, it has to mentioned that tryptophan, if present in a pure and isolated protein which does not contain in its chain any residue of cystine, can be directly determined by analysing the acid hydrolysate (provided that the previously described hydrolytic conditions are respected), without carrying out the hydrolysis of the protein in a basic medium.

Finally, some conclusions can be drawn with respect to the volumes of the solution submitted to hydrolysis and to the quantitity of protein which is available.

The volume of 250 ml which was employed in the above examples can be greatly reduced. Particularly, when sampling at intermediate hydrolysis times from a single flask is not required, the open flask method can be easily employed with volumes not exceeding 10 ml, provided that micro-chemistry glassware, easily available (Quickfit), is employed.

As far as the protein–acid ratio is concerned, it has to be stressed that any reduction of the concentration, which was chosen for the experiments reported here,

can be made, provided that the sensitivity limits of the analytical system available for the amino acid analysis are taken into consideration. As a matter of fact, a diminution of the protein–acid ratio will always improve the hydrolytic conditions, consequently minimising any interaction.

From this it can be deduced that, even in cases where only a few mg of protein are available for the investigation of the amino acid composition, the open flask method of acid hydrolysis can be adopted.

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CHROM. 4912

ISOTACHOPHORESIS

THE SEPARATION OF AMINO ACIDS

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SUMMARY

There are several instrumental methods of analysis available for amino acids. The most important ones are ion-exchange chromatography (STEIN AND MOORE), gas chromatography and paper chromatography. These techniques all have various disadvantages; the time for analysis may be long or the sample treatment complicated and non-reproducible or the detector may be poor.

Electrophoretic methods using gel or paper are also available for separating amino acids or their derivatives, but as in chromatography none of them is entirely satisfactory.

Hence separation of amino acids by isotachophoresis is being investigated and various approaches are described in this paper.

INTRODUCTION

The amphoteric character of amino acids is the main problem, but also an advantage, in all liquid separation systems, including isotachophoresis. In isotachophoresis the amino acids may be analysed either as anions or cations. Anion analysis has been chosen because there is a greater differentiation of the pK values of the carboxyl groups.

To ensure that the amino acids run as anions, high pH values are required in aqueous solutions, (*i.e.* at least pH 10), and even then the net charge of several of the amino acids is small. The higher the pH, the larger the proportion of the current carried by hydroxyl ions will be and the slower acids will be separated, unsatisfactorily, by zone electrophoresis. The pH in an aqueous solution is expressed by

$$K_a = \frac{[B]\,[H^+]}{[BH^+]}$$

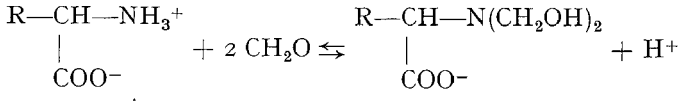
Here K_a is the acidic dissociation constant, and $[BH^+]$ and $[B]$ are the dissociated and undissociated forms of the buffer. Once the separation has reached a steady state

the pH is determined solely by the concentration in the leading solution of the ionised and unionised forms of the buffer, the mobilities of all the ions present in the leading solution and the mobilities and dissociation constants of the various zones.

EVERAERTS[1] has shown how the pH values may be calculated in detail. In general the concentration of each ion is lower than the preceeding one and the pH is higher. If the dissociation constants of succeeding zones are very different, however, there may be a rise rather than a fall of concentration and pH. The pH within the zones may differ by as much as 1 to 2 pH units from the pH of the leading electrolyte.

At the new pH (about 11), the hydroxyl ion concentration becomes 0.001 *M* which is actually comparable with that of the amino acid ions. The effective concentration of the hydroxyl ions is enlarged because their mobility is 4 to 5 times greater than that of an average amino acid ion. This thus causes an eluting effect. To avoid this problem the amino acids have to be transferred into compounds with a lower p*K* value, *i.e.* a less amphoteric character. Of the several methods of doing this, decarboxylation, esterification and deamination immediately spring to mind, replacing the amino group for a hydroxyl group is another possibility.

The method chosen is the equilibrium reaction with formaldehyde because of its non denaturating character, which can be of importance if preparative isotachophoresis is wanted; furthermore no sample treatment is necessary. The formaldehyde reacts with the amino group of the amino acids and decreases the p*K* value of this group. Thus a much lower pH is obtained with the same amount of ionised material. According to WHITE *et al.*[2] a possible explanation for the reaction is:



The shift in p*K* depends on the quantity of formaldehyde present.

The problem in using a formaldehyde solution is finding a buffer to work with at a pH of about 8. This buffer must have a great buffering capacity and not show any reaction with formaldehyde. Most buffers, suitable for this pH and buffering on cations, are based on an amine and therefore react with formaldehyde. So far good buffers have only been found among the tertiary amines.

A lower working pH has another advantage in that the eluting effects of the carbonate ion will disturb the analyses much less. The carbonate ion is present because carbon dioxide enters the system *via* the pressure valves for filling the capillary tube at high pH values or penetrates through the teflon capillary tube itself. Teflon is permeable to various gases, carbon dioxide being among them[3].

APPARATUS

The apparatus basically consists of a teflon capillary tube with a total length of 60 cm, an outside diameter of 0.75 mm and an inside diameter of 0.45 mm. This capillary tube is mounted between a cathode and an anode compartment, respectively. The effective length (*i.e.* from the injection point to the detector) is 50 cm.

A set of copper–constantan (0.001 in. diam.) thermocouples wound around the capillary tube and fixed with a suitable elastic glue are used as the detector. One thermocouple (giving the step-wise signal) is mounted with one junction on the cap-

illary tube. The temperature of the other junction is kept constant at 18°. The other thermocouple (giving the differential signal) is mounted with both junctions on the capillary tube[1].

The power supply consists of a 20 V d.c. power unit (Philips PE 1512), the voltage of which is lead to a 20 V d.c.–20 kV d.c. transformer (Spellman UHM 20 N 10 D). The output of the power unit as well as the input of the transformer are connected with a trigger unit because a current stabilised d.c. voltage is required.

The electrode compartments

The cathode consists of a platinum wire mounted in the compartment of the terminal electrolyte. This compartment is constructed[4] in such a way, that a sample can be injected into the apparatus with a syringe *via* a septum.

The anode compartment differs from the normal type used for isotachophoresis in that no membrane is present between the anode and the capillary tube. In this way pH effects, caused by the higher permeability of the membrane for protons, are avoided.

The electrode is mounted in a glass T piece. Another T piece is mounted in such a way that the rinsing water will not pass the electrode, so that none of the reaction products, formed at the anode, are able to enter into the capillary tube. These products could also enter into the capillary tube during the analysis, giving cause to disturbances.

Various reactions can possibly occur at the anode and are discussed below. Gas is not developed, at least not visibly.

Copper will go into solution at the anode as follows:

$$Cu - 3e \rightarrow Cu^{2+} + Cu^{+}$$

It can be seen that both ions are formed, because a red and a blue colour is formed near the electrode. Cu^{2+} can give $Cu(OH)_2$ which could be responsible for the dark slurry that is seen near the electrode.

The copper ions will undoubtedly form complexes with the buffer and the formaldehyde. For example, with pyridine both copper ions together can form some 9 positive complexes. Some of these will be stable enough to move in the opposite direction to that of the sample. It is evident that the same kind of complex formation can be expected with other buffers than pyridine.

The copper–buffer and copper–formaldehyde complexes, migrating to the cathode, could be changed into copper–amino acid complexes on meeting them in the capillary tube. Then again different charges would be possible.

Formaldehyde itself can easily be transformed into formic acid, in the presence of oxygen. This could also explain why no visible evolution of gas occurs. Formic acid can also be formed in the reaction of formaldehyde with the amino acids. This can be avoided by mixing a small amount of methanol in the solution.

By having a large volume between the exit of the capillary tube and the anode, the potential gradient over this compartment will be small. Thus the positive complexes mentioned above are not able to enter into the capillary tube easily.

Solutions

The capillary tube and the anode compartment are both filled with the same

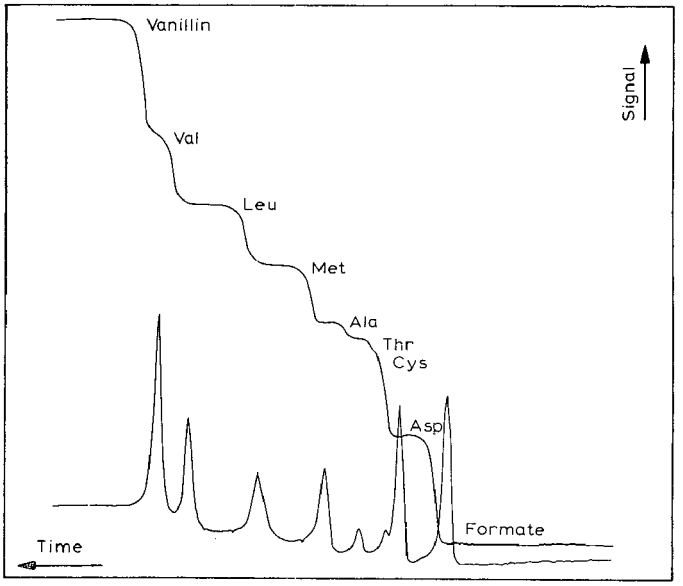


Fig. 1. Isotachophoretic analysis of some amino acids. For explanation see text.

solution, the leading electrolyte. This solution always contains 17.5% of formaldehyde. The buffer used is present in a concentration varying from 0.02 *M* to 0.06 *M*. The mixture of formaldehyde and buffer is first treated with a strongly basic ion exchanger, to remove foreign anions. Then the leading electrolyte and the anode solution are made by mixing the solution with an ion with a high net mobility like formate. The terminal electrolyte is made by mixing the buffer–formaldehyde solution with an ion with a low net mobility such as vanillin. Formate is used as leading ion because it can, as mentioned above, be formed during the analysis. Fig. 1 shows an example of an analysis of amino acids as it can be made at this stage.

It should be mentioned here that the electropherogram must be interpreted as follows. The stepwise curve (integral) gives the information about the zone temperature and this temperature is related to the ion species. The differential curve gives, from the distance between the peaks, the information about the concentration.

The conditions of the analysis were as follows: The leading electrolyte consisted of: *sym.* collidine 0.02 *M*; formic acid 0.01 *M*, and formaldehyde 17.5%; the pH of this solution is 7.1. The sample injected was 1 μl of a solution containing 0.4 mole of each of the following amino acids: aspartic acid, cysteine, threonine, alanine, methionine, leucine and valine dissolved in the mixture of collidine and formaldehyde.

CONCLUSIONS

Good buffers were found to be pyridine, 3-methylpyridine, *sym.* collidine and nicotine. The first two however have a p*K* value which is too low. Collidine is satisfactory however (about 7.2) and its reaction with formaldehyde is negligible. Nicotine has not been tested so far.

The solutions described above are satisfactory for qualitative analyses of amino acids. The method has yet to be developed for quantitative purposes.

ACKNOWLEDGEMENT

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CHROM. 4940

CHROMATOGRAPHIC AND ELECTROPHORETIC BEHAVIOR OF AMINOPHOSPHONIC ACIDS ON LAYERS OF CATION EXCHANGERS

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SUMMARY

The chromatographic and electrophoretic behavior of sixteen aminophosphonic acids was investigated on layers of weak and strong cation exchangers. The influence of the acidity of the eluent and of the form of the exchanger was also studied.

A comparison between the chromatographic characteristics of these aminophosphonic acids and the corresponding natural amino acids was made.

INTRODUCTION

Aminophosphonic acids have acquired importance in the past few years from a biological point of view[1,2]. They are not found in nature, with the exception of 1-aminoethylphosphonic acid[3]. In the pertinent literature we have not found any information about the chromatographic and electrophoretic behavior of the aminophosphonic acids. Furthermore, these compounds do not lend themselves to studies by adsorption or partition techniques due to their insolubility in the common organic solvents.

Ion-exchange chromatography seems particularly suitable for such compounds on account of their solubility in aqueous solvents and their acid–base characteristics. We have therefore deemed it useful, as a part of the research being carried on in our institute on the use of ion exchangers[4–7], to investigate the chromatographic and electrophoretic behavior of this class of compounds. The purposes of this study are: (a) to perfect methods for the separation of the aminophosphonic acids; and (b) to study their chemical properties by means of a comparison with the corresponding amino acids.

EXPERIMENTAL

Preparation of the layers

Layers having a thickness of 300 μ were used.

(a) Alginic acid. 6 g of the exchanger and 1.5 g of cellulose (No. 123 from Schleicher & Schüll) in 40 ml of water.

(b) Dowex 50 X4 (H^+ and Na^+ form). 4.5 g of 200–400 mesh resin and 4.5 g of cellulose in 40 ml of water.

(c) Dowex 50 X4 + CMCNa. 3 g of each exchanger in 40 ml of water.

(d) CMCNa (No. 132 from Schleicher & Schüll). 4.5 g in 50 ml of water.

Reagents and developers

Aqueous solutions (1%) of each aminophosphonic acid (Calbiochem) were prepared. These solutions were then diluted to obtain well-defined spots. The quantities of each compound (μg) are reported in Table I. A solution obtained by dissolving

TABLE I

R_F VALUES OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF (1) ALGINIC ACID, (2) DOWEX 50 X4 (H^+), (3) DOWEX 50 X4 (Na^+), (4) CMCMa AND (5) DOWEX 50 X4 (Na^+) + CMCNa, WITH WATER AS ELUENT

Aminophosphonic acid	*1*	*2*	*3*	*4*	*5*	*Amount (μg)*
1,4-Diaminobutylphosphonic	0.03	0.00	0.00	0.37	0.06	1.5
1,3-Diaminopropylphosphonic	0.03	0.00	0.00	0.53	0.12	1.5
1,2-Diaminoethylphosphonic	0.03	0.00	0.00	0.80	0.25	2.0
3-Aminopropylphosphonic	0.49	0.03	0.34	0.96	0.93	2.0
2-Aminoethylphosphonic	0.68	0.12	0.65	0.96	0.94	2.0
1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	0.74	0.19	0.40	0.96	0.94	1.5
1-Amino-2-phenylethylphosphonic	0.76	0.15	0.36	0.96	0.94	1.5
1-Aminomethylphosphonic	0.78	0.50	0.92	0.96	0.94	2.0
1-Aminoethylphosphonic	0.84	0.44	0.92	0.96	0.94	1.5
1-Aminopropylphosphonic	0.85	0.40	0.91	0.96	0.95	1.5
1-Aminobutylphosphonic	0.85	0.30	0.83	0.96	0.94	1.5
1-Aminopentylphosphonic	0.85	0.23	0.75	0.96	0.94	1.5
1-Amino-2-methylpropylphosphonic	0.85	0.32	0.92	0.96	0.94	1.5
1-Amino-1-methylethylphosphonic	0.85	0.32	0.91	0.96	0.94	1.5
2-Amino-4-phosphonbutyric	0.86	0.24	0.94	0.96	0.95	1.5
2-Amino-3-phosphonpropionic	0.95	0.62	0.96	0.96	0.96	1.5

0.1 g of ninhydrin in 50 ml of absolute alcohol, with addition of 10 ml of glacial acetic acid and 2 ml of collidine, was used as developer. After spraying, the plates were kept at 110° for 10 min. The only aminophosphonic acid to give a positive test with molybdate reagent was 2-aminoethylphosphonic acid.

Electrophoretic measurements

The electrophoretic measurements were made with a Camag apparatus for high potential electrophoresis, at a temperature of 18°. The electro-osmotic flow was measured with hydrogen peroxide.

RESULTS AND DISCUSSION

Effect of the exchanger

R_F values of sixteen aminophosphonic acids on layers of alginic acid, Dowex 50 X4 (H^+), Dowex 50 X4 (Na^+), CMCNa, and mixed layers of Dowex 50 X4 (Na^+) and CMCNa are reported in Table I.

On the alginic acid layers it can be observed that most aminophosphonic acids have an R_F value $\geqslant$ 0.68. Only diaminophosphonic acids and 3-aminopropylphosphonic acid, although this latter to a lesser degree, are adsorbed to any extent on alginic acid. Their separation from all the others is therefore possible.

On Dowex 50 X4 (H^+) there is much greater retention than on alginic acid. It is interesting to note the unusual behavior of 1-amino-2-phenylethylphosphonic acid and 1-amino-2(4-hydroxyphenyl)ethylphosphonic acid, which are retained more than would be expected on the basis of their acid–base characteristics.

From an analytical point of view the separation of 2-amino-4-phosphonbutyric acid (R_F = 0.24) from 2-amino-3-phosphonpropionic acid (R_F = 0.62) is interesting; these two acids correspond to glutamic and aspartic acids in which a carboxylic group has been replaced by a phosphonic one. The behavior of the diaminophosphonic acids is identical on both the H^+ and Na^+ forms of Dowex 50 X4. All other acids are held back to a lesser degree, even though the two acids containing phenyl groups are retained in an anomalous way on the Na^+ form.

On mixed layers of Dowex 50 X4 and CMCNa all aminophosphonic acids, with the exception of the three diaminophosphonic acids, move with the solvent front. It is interesting to note that the mixture of the two exchangers is influenced by the characteristics of the weak exchanger, as shown by the R_F values of aminophosphonic acids on layers of CMCNa alone.

On this latter layer, as the chromatogram of Fig. 1 shows, an excellent separation can be effected between the three diaminophosphonic acids and between these and all the other acids. The separation is also made easier by the well-defined spots we can obtain on this layer[8].

Effect of the eluent

The data of Table I show that separations of aminophosphonic acids, with the exception of the three diaminophosphonic acids, are possible only on layers of Dowex 50 X4 in the acid form. For this reason the data in Table II, obtained by using HCl of different concentrations as eluent, refer to this exchanger. With respect to the values

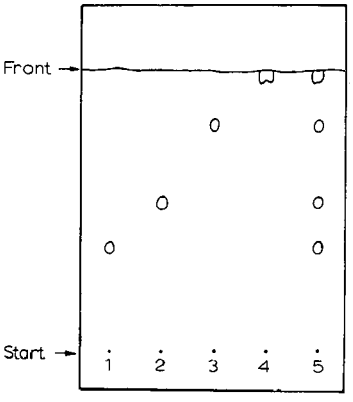


Fig. 1. Thin-layer chromatogram of aminophosphonic acids on CMCNa. 1 = 1,4-diaminobutylphosphonic; 2 = 1,3-diaminopropylphosphonic; 3 = 1,2-diaminoethylphosphonic; 4 = 3-aminopropylphosphonic; 5 = mixture.

TABLE II

R_F VALUES OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF DOWEX 50 X4 (H^+)

Development distance, 14.5 cm. Eluents: (a) HCl, 0.1 N; (b) HCl, 0.25 N; (c) HCl, 0.5 N; (d) HCl, 1 N.

No.	*Aminophosphonic acid*	*a*	*b*	*c*	*d*
1	1,4-Diaminobutylphosphonic	0.01	0.05	0.11	0.37
2	1,3-Diaminopropylphosphonic	0.02	0.06	0.15	0.41
3	1,2-Diaminoethylphosphonic	0.03	0.09	0.19	0.44
4	3-Aminopropylphosphonic	0.15	0.26	0.45	0.64
5	2-Aminoethylphosphonic	0.25	0.37	0.50	0.71
6	1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	0.21	0.22	0.26	0.38
7	1-Amino-2-phenylethylphosphonic	0.18	0.19	0.23	0.34
8	1-Aminomethylphosphonic	0.55	0.66	0.73	0.84
9	1-Aminoethylphosphonic	0.52	0.60	0.70	0.82
10	1-Aminopropylphosphonic	0.48	0.58	0.66	0.80
11	1-Aminobutylphosphonic	0.40	0.46	0.55	0.75
12	1-Aminopentylphosphonic	0.30	0.34	0.41	0.61
13	1-Amino-2-methylpropylphosphonic	0.44	0.50	0.55	0.76
14	1-Amino-1-methylethylphosphonic	0.44	0.50	0.55	0.76
15	2-Amino-4-phosphonbutyric	0.29	0.46	0.62	0.80
16	2-Amino-3-phosphonpropionic	0.48	0.59	0.69	0.86

obtained on eluting with water, we can observe a general increase in the R_F value, as was expected. Gradual resolution of the diaminophosphonic acids can be noted as the concentration of the acid in the eluent increases. It is interesting to draw attention to the behavior of the two aminophosphonic acids containing a phenyl group. These are not appreciably affected by a variation of the pH in the eluent, and this suggests that the mechanism determining their retention on the exchanger is not due solely to an ion-exchange process.

Correlation between R_F values and the characteristics of the aminophosphonic acids

The basicity of amino acids is intimately related to the position of the amino group in the molecule. It is therefore possible, without knowing the acid–base con-

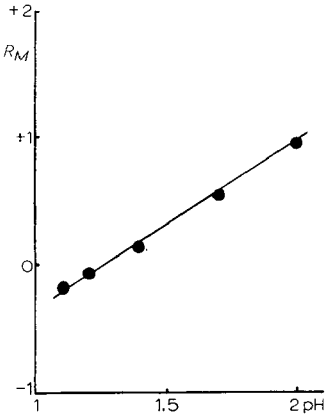


Fig. 2. R_M values *vs.* pH for diaminophosphonic acids on alginic acid thin layers. Eluents: HCl solutions.

stants, to forecast a basicity sequence for the various aminophosphonic acids on the basis of the position of the amino group with respect to the phosphonic group.

The sequence is: 1,4-diamino > 1,3-diamino > 1,2-diamino > 3-amino > 2-amino > 1-amino. R_F values on Dowex 50 X4 (H^+) are in agreement with this sequence, as the order of retention decreases starting with 1,4-diamino (see Table II), *i.e.* the R_F value increases with decreasing basicity. On the the other hand, on alginic acid the R_F values of the diaminophosphonic acids, though they are always lower than those of the monoamino acids, are equal to each other without depending on the acidity of the eluent (see Table I and Fig. 2).

On CMCNa, however, the differences in the acid–base characteristics of the diaminophosphonic acids are particularly evident, since they are distinctly separated on this layer (see Fig. 1).

With regard to the effect of the side chain, it is possible to observe with the increase in the number of carbon atoms a parallel increase in the retention capacity of the polystyrolic base exchangers.

On Dowex 50 X4 the R_F value sequence is: methyl > ethyl > propyl > butyl > pentyl.

Such a relationship is not respected with branched side chains, as the R_F values of 1-amino-2-methylpropylphosphonic acid and 1-amino-1-methylethylphosphonic acid show (see Table II). The sequence observed on Dowex is not respected on cellulose-type exchangers such as alginic acid.

Retention mechanism

Alginic acid. On this exchanger the relation[9]:

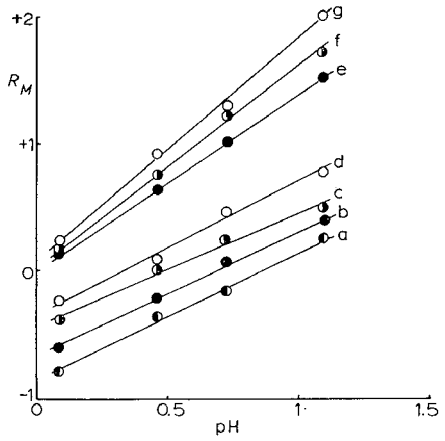
$$n\text{pH} = R_M + \text{constant} \qquad (1)$$

can be applied and the line in Fig. 2 is obtained, whose slope (1.32) indicates that these acids behave like divalent ions. The discrepancy between the theoretical and experimental values is probably due to the pH gradient along the layer[7]. We cannot affirm with certainty that the retention of the monoaminophosphonic acids is influenced only by the ion-exchange process, since eqn. 1 cannot be applied to them (due to their low retention with water), even if the agreement between the R_F values and the acid–base characteristics (3-amino > 2-amino > 1-amino) deems it probable.

Dowex 50 X4 (H^+). By applying eqn. 1 to chromatography on this exchanger it has been possible to show that the retention mechanism is not entirely an ion-exchange process. The progression of R_M/pH values for diaminophosphonic acids (curves e, f, g), 3-aminophosphonic acid (curve d), 2-amino-3-phosphonpropionic acid (curve a), and 2-amino-4-phosphonbutyric acid (curve b) are reported in Fig. 3. The slope for diaminophosphonic acids, falling between 1.4 and 1.7, indicates that on this exchanger too these amino acids behave like divalent ions. In the case of monoamino acids the slope, falling between 0.9 and 1.0, suggests that these compounds are present as monovalent ions.

As far as the other acids are concerned, it does not appear that the ion-exchange process is the predominant parameter in the retention mechanism, as shown by the curves reported in Fig. 4.

In particular, the greatest deviation from eqn. 1 is found for the two amino acids containing phenyl groups (curves f, g), and for acids having a linear side chain



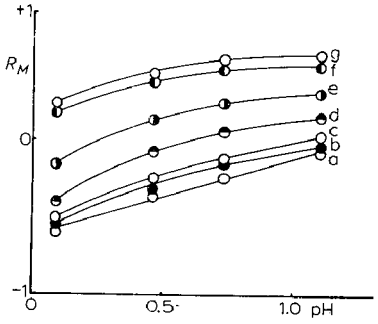


Fig. 3. R_M values *vs.* pH for some aminophosphonic acids on Dowex 50 X4 (H^+) thin layers. Eluents: HCl solutions. (a) 2-amino-3-phosphonpropionic; (b) 2-amino-4-phosphonbutyric; (c) 2-aminoethylphosphonic; (d) 3-aminopropylphosphonic; (e) 1,2-diaminoethylphosphonic; (f) 1,3-diaminopropylphosphonic; (g) 1,4-diaminobutylphosphonic.

Fig. 4. R_M values *vs.* pH for some aminophosphonic acids on Dowex 50 X4 (H^+) thin layers. Eluents: HCl solutions. (a) 1-aminomethylphosphonic; (b) 1-aminoethylphosphonic; (c) 1-aminopropylphosphonic; (d) 1-aminobutylphosphonic; (e) 1-aminopentylphosphonic; (f) 1-amino-2-(4-hydroxyphenyl)ethylphosphonic; (g) 1-amino-2-phenylethylphosphonic.

with five carbon atoms (curve d). As the length of the side chain decreases, the R_M/pH relation approaches its theoretical course. Nevertheless, the slope of the line (0.60) is distinctly less than its theoretical value of 1 (see curve a of Fig. 4) also in the case of 1-aminomethylphosphonic acid, which has only one carbon atom in the side chain. The other two acids having a branched side chain show the same curvilinear trend. Similar behavior was found by KNIGHT[10] in the separation of amino acids on ion-exchange papers consisting of polystyrolic matrices.

Comparison with amino acids

The use of aminophosphonic acids concerns, as far as can be discerned from the scarce data in the literature, their utilization in association with or as substitute for corresponding natural amino acids, and for this reason a comparison of the chromatographic behavior of these two classes of compounds was deemed useful.

The spots of natural amino acids (black) and the corresponding spots of aminophosphonic acids on layers of Dowex 50 X4 (H^+), eluted with 0.5 *M* HCl, are reported in Fig. 5. The distinct separation between the respective components of the two classes of substances appears evident from the chromatogram, as the aminophosphonic acids are retained less than the corresponding natural amino acids. This is in agreement with the more marked acid characteristics which the compound assumes when the carboxylic group is replaced by the stronger phosphonic group. Such a replacement does not however cause any variation in those chromatographic characteristics that depend upon the side chain of the molecule. In fact, in both classes of compounds, the sequence of R_F values for the five amino acids having a linear side chain containing from one to five carbon atoms follows an inverse order to the number of carbon atoms in the chain.

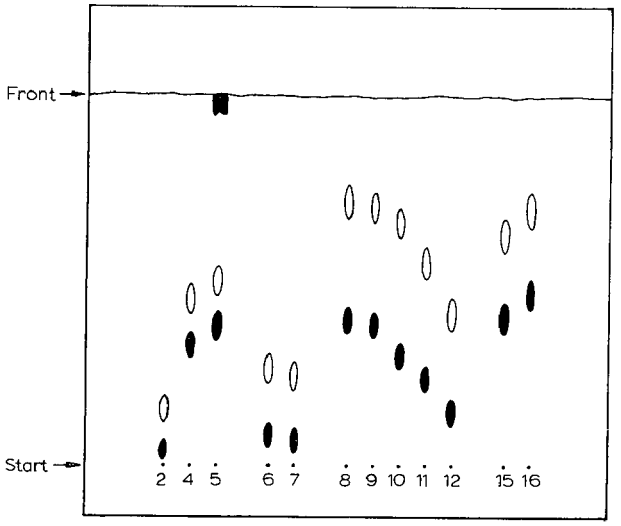


Fig. 5. Chromatographic behavior of aminophosphonic acids and the corresponding amino acids (black spots) on Dowex 50 X4 (H^+) thin layers. Eluent: 0.5 *N* HCl. The numbers refer to the aminophosphonic acids as reported in Table II. The spots relative to No. 5 refer, respectively, to β-alanine, 2-aminoethylphosphonic acid and taurine.

Moreover, considering the separation of β-alanine (R_F = 0.37) from 2-aminoethylphosphonic acid (R_F = 0.50) and from taurine (R_F = 0.96), we have a clear demonstration of the possibilities of ion-exchange chromatography where advantage is taken of the variations in the acid–base characteristics of the compounds due to the presence of a different acid group in the molecule.

TABLE III

MIGRATION DISTANCE (mm) OF AMINOPHOSPHONIC ACIDS ON THIN LAYERS OF ALGINIC ACID (AA) AND SODIUMCARBOXYMETHYLCELLULOSE (CMCNa)

Aminophosphonic acid	*AA*[a]	*CMCNa*[b]
1,4-Diaminobutylphosphonic	34	30
1,3-Diaminopropylphosphonic	34	25
1,2-Diaminoethylphosphonic	33	17
3-Aminopropylphosphonic	53	13
2-Aminoethylphosphonic	50	12
1-Amino-2-(4-hydroxyphenyl)ethylphosphonic	43	4
1-Amino-2-phenylethylphosphonic	43	2
1-Aminomethylphosphonic	43	−1
1-Aminoethylphosphonic	43	1
1-Aminopropylphosphonic	44	7
1-Aminobutylphosphonic	45	8
1-Aminopentylphosphonic	47	8
1-Amino-2-methylpropylphosphonic	46	9
1-Amino-1-methylethylphosphonic	46	8
2-Amino-4-phosphonbutyric	18	−16
2-Amino-3-phosphonpropionic	4	−35
H_2O_2	43	12

[a] Electric potential: 1100 V; time: 90 min; electrolyte: 1 *M* CH_3COOH.
[b] Electric potential: 600 V; time: 60 min; electrolyte: 0.1 *M* acetic buffer.

Electrophoresis

Migration distances of aminophosphonic acids on layers of alginic acid and CMCNa, using 1 *M* CH_3COOH and 0.1 *M* acetic buffer, respectively, as electrolytes, are reported in Table III. On both exchangers (and in particular on alginic acid) most aminophosphonic acids move only to a small extent in the applied electric field, taking into account the electro-osmotic flow (measured with H_2O_2). The behavior of 2-amino-4-phosphonbutyric acid and 2-amino-3-phosphonpropionic acid is an exception to this, as both show a distinctly anionic behavior and can be separated from all the others as well as from each other. The difference in the electrophoretic behavior of these two compounds increases as we use CMCNa instead of alginic acid as exchanger. On this latter exchanger most aminophosphonic acids show a predominately anionic character, with the exception of the most basic amino acids, such as the diamino acids, 3-aminopropylphosphonic acid, and 2-aminoethylphosphonic acid. This is also due to the higher pH of the electrolyte.

The fact that most aminophosphonic acids do not migrate, or at least migrate very little, under the influence of an electric field at pH = 4.85 indicates that, at such a pH, the amino acids, with the exception of the diamino acids, 2-amino-4-phosphonbutyric acid, and 2-amino-3-phosphonpropionic acid, are predominantly in the anionic form.

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CHROM. 4932

POLYVINYL ACETATE AS A STATIONARY PHASE IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The use of polyvinyl acetate as a stationary phase in thin-layer chromatography has been investigated using the derivatives of some organic compounds as the test substances. The phase gives very rigid layers and can be used for the separation of derivatives of homologous series as well as for the separation of derivatives of other types of organic compounds.

INTRODUCTION

In a study concerning the use of polyamide for thin-layer separations COPIUS PEEREBOOM used polyvinyl acetate as a binder[1]. During our investigations on the behaviour of 2,4-dinitrophenylhydrazones on thin layers of polyamide containing polyvinyl acetate, the R_F values of the 2,4-dinitrophenylhydrazones were found to depend to a large extent on the amount of polyvinylacetate used as binder for the polyamide layers. Presumably the polyvinyl acetate itself took part in the distribution of the compounds between the mobile and the immobile phases. It was then observed on substituting kieselguhr for the polyamide that the polyvinyl acetate itself acted as a convenient stationary phase for the separation of organic derivatives.

Some of the potentialities of this immobile phase for this purpose were then investigated.

EXPERIMENTAL

Preparation of plates

Measured amounts of polyvinyl acetate (Mowilith 50, Hoechst, G.F.R.) were dissolved in a mixture of 25 ml of methanol and 15 ml of ethyl acetate. The volume was adjusted to 60 ml with methanol and 25 g of Kieselguhr G (Merck) were suspended in the solution by shaking in an erlenmeyer flask. Chromatoplates were prepared using the standard Desaga equipment. The equipment should be cleaned immediately after use by rinsing with methanol until all polyvinyl acetate solution has been removed.

The plates were used after 3 h of drying at room temperature in the atmosphere of the laboratory.

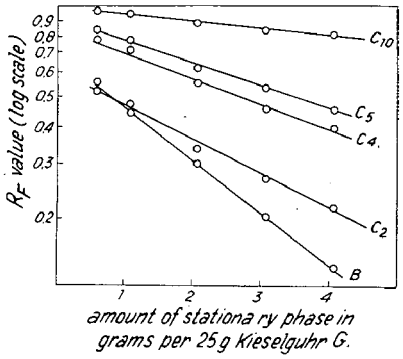


Fig. 1. Dependence of the R_F values of carbonyl 2,4-dinitrophenylhydrazones on the amount of immobile phase (polyvinyl acetate).

RESULTS AND DISCUSSION

Dependence of R_F and R_F^c values on the amount of polyvinyl acetate

A series of plates was prepared containing 0.5 to 4 g of polyvinyl acetate to 25 g of Kieselguhr G. A number of 2,4-dinitrophenylhydrazones were spotted at the bottom edge of the plates. The solvent consisting of a mixture of hexane–di-*n*-butyl ether–methanol–ethyl acetate (85:5:5:5) was allowed to rise to 11.5 cm above the point of application.

The results of these experiments are presented in Fig. 1 and it is seen that the log R_F values are linear with respect to the amount of stationary phase. It was shown earlier that perfect agreement between R_F values measured on different chromatograms can be obtained by using the standardization procedure[2–5] of GALANOS AND KAPOULAS. The relation found in Fig. 1 suggests that this correction procedure would apply to our data on different amounts of immobile phase by taking the log of the R_F values instead of the R_F values as such. The GALANOS AND KAPOULAS equation thus takes the form:

$$\log R_F^c = a \log R_F + b$$

The R_F values shown in Fig. 1 were then corrected using the R_F values found on 3 g polyvinyl acetate as the standard values. The results are shown in Table I.

TABLE I

R_F^c VALUES ON PLATES CONTAINING DIFFERENT AMOUNTS OF IMMOBILE PHASE, AFTER CORRECTION ACCORDING TO THE MODIFIED EQUATION OF GALANOS AND KAPOULAS

Solvent system B.

2,4-Dinitrophenyl-hydrazone of	*Amount of immobile phase per 25 g of Kieselguhr G (g)*				
	0.5	*1.0*	*2.0*	*3.0*	*4.0*
Butanal	0.56	0.50	0.49	0.46	0.46
Pentanal	0.62	0.56	0.56	0.54	0.52
Benzaldehyde	0.29	0.23	0.24	0.20	0.25

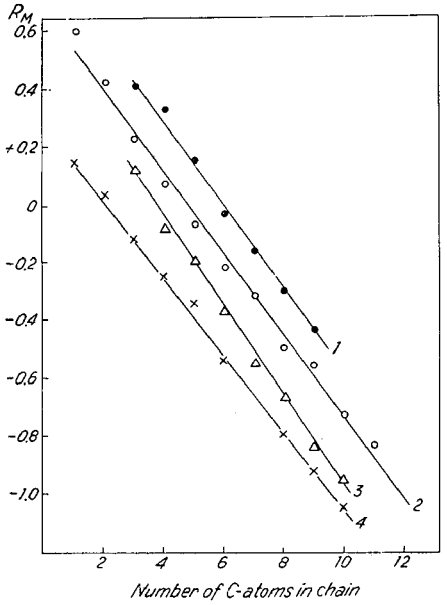
The R_F values of the 2,4-dinitrophenylhydrazones of ethanal and decanal were used as the reference compounds on all chromatograms, and it was from these values that those of butanal, pentanal and benzaldehyde were computed. As is shown in Table I, the values are nearly constant with the exception of those obtained on 0.5 g immobile phase which are significantly higher. It is clear that using the modified GALANOS AND KAPOULAS correction procedure, constant R_F values are obtainable even if there are slight variations in the amount of immobile phase present on the plates.

Behaviour of some organic derivatives on polyvinyl acetate

Two chromatographic systems were studied. One series of plates contained 2 g of polyvinyl acetate per 25 g of Kieselguhr G (M_2 plates), the other type contained 3 g of polyvinyl acetate for the same amount of support material (M_3 plates).

The solvent systems used were: System A/M_2 plates: hexane–di-*n*-butyl ether–methanol–ethyl acetate (65:10:10:15). System B/M_3 plates: hexane–di-*n*-butyl ether–methanol–ethyl acetate (85:5:5:5). Point of application: 1.5 cm from the bottom edge of the plate; front: 11.5 cm above the point of application.

2,4-Dinitrophenylhydrazones (2,4-DNPH's) and 2-diphenylacetyl-1,3-indanedione hydrazones (DAIH's). 2,4-DNPH's were prepared in the normal way. The DAIH's were prepared as follows: 3–5 mg of 2-diphenylacetyl-1,3-indanedione hydrazone was dissolved in 0.5 ml chloroform. To this solution about 3 mg of the carbonyl compound and a very small drop of concentrated hydrochloric acid were



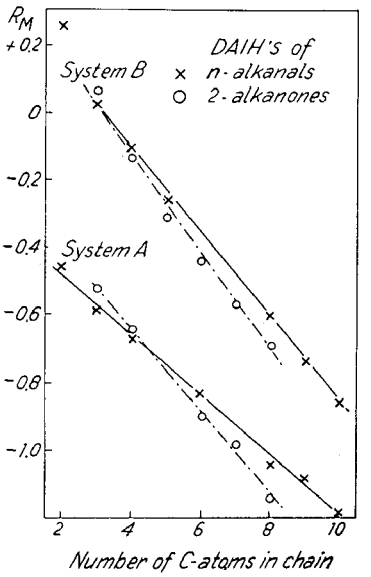


Fig. 2. Relation between R_M value and number of C atoms in chain for: (1) alk-2-enal 2,4-dinitrophenylhydrazones; (2) *n*-alkanals 2,4-dinitrophenylhydrazones; (3) 2-alkanones 2,4-dinitrophenylhydrazones; (4) *n*-alkanol 3,5-dinitrobenzoates in system B.

Fig. 3. Relation between the number of carbon atoms in the chain and the R_M value for aliphatic 2-diphenylacetyl indanedione hydrazones.

TABLE II

REGRESSION LINE EQUATION RELATING R_M VALUE AND CARBON NUMBER FOR SOME CARBONYL DERIVATIVES

	System	*Equation*
DAIH's of *n*-alkanals	A	$R_M = -0.0850\ C - 0.307$
	B	$R_M = -0.1250\ C + 0.392$
DAIH's of 2-alkanones	A	$R_M = -0.1239\ C - 0.148$
	B	$R_M = -0.1488\ C + 0.474$
2,4-DNPH's of *n*-alkanals	B	$R_M = -0.1394\ C + 0.668$
2,4-DNPH's of 2-alkanones	B	$R_M = -0.1631\ C + 0.681$
2,4-DNPH's of *n*-alk-2-enals	B	$R_M = -0.1454\ C + 0.870$

added. After standing overnight the solution was evaporated on a boiling water bath and the residue was taken up in 0.2–0.5 ml of chloroform. 1–2 μl of this solution was spotted on the plate. The derivatives have an intense yellow color and on the plates they exhibit a strong fluorescence in filtered UV light (363 nm)[6,7]. R_M values were calculated for each homologous series and the relation between R_M and the chain length for the particular series tested. The results are shown in Figs. 2 and 3. With the exception of some of the first members of the series a linear relation was found. The lines of best fit were calculated using least square methods and the results are shown in Table II.

System B was the most suitable for the separation of homologous series of aliphatic derivatives. The R_F values of the 2,4-DNPH's and the DAIH's of the same

TABLE III

R_F VALUES OF SOME 2,4-DNPH'S AND DAIH'S FROM AROMATIC CARBONYL COMPOUNDS

Compound	*2,4-DNPH's*		*DAIH's*	
	A	*B*	*A*	*B*
Furfural	0.52	0.13	—	—
	0.63	0.28		
5-Methylfurfural	0.59	0.18	—	—
	0.70	0.41		
5-Hydroxymethylfurfural	0.13	0.10	—	—
	0.34	—		
Vanillin	0.35	0.10	—	0.12
Isovanillin	0.43	0.11	0.39	—
Ethylvanillin	0.43	0.10	0.35	—
Benzaldehyde	0.65	0.20	—	0.26
p-Hydroxybenzaldehyde	0.37	0.10	—	0.03
o-Methoxybenzaldehyde	0.61	0.21	—	0.26
Phenylacetaldehyde	0.67	0.27	0.64	—
Phenylpropionaldehyde	0.67	0.29	—	0.34
Protocatechualdehyde	0.21	0.10	—	—
Gentisic aldehyde	0.23	—	—	—
Anisaldehyde	0.55	0.15	0.53	—
Cyclamenaldehyde	0.82	0.63	0.84	0.64
Cinnamaldehyde	0.62	0.20	0.61	—
p-Methoxyacetophenone	0.62	0.23	—	0.25
Benzophenone	—	0.49	0.70	—

carbonyl compound are not very different from each other. However the DAIH's have a much lower detection limit than the 2,4-DNPH's owing to their strong fluorescent colors which can be observed by inspection under filtered UV light. Table III contains the R_F values of a number of aromatic type derivatives and it is seen that system A in combination with system B separated a number of aromatic type derivatives.

3,5-Dinitrobenzoates. The 3,5-dinitrobenzoates of a number of aliphatic alcohols and phenols were prepared by reacting the individual compounds with 3,5-dinitrobenzoylchloride[8]. The regression line equation for the homologous series of aliphatic alcohols in relation to carbon number and R_M was found to be

$$R_M = -0.1312\ C + 0.279 \text{ for system B.}$$

The line of best fit together with the experimental points are shown in Fig. 2.

The regression line equation for system A was not calculated because of the very high R_F values found in this system.

The R_F values of some 3,5-dinitrobenzoates of phenols are shown in Table IV.

TABLE IV

R_F VALUES OF SOME 3,5-DINITROBENZOATES

3,5-Dinitrobenzoate of	R_F	
	System A	*System B*
2,5-Dimethylphenol	0.76	0.50
1-Naphthol	0.61	0.23
Thymol	0.82	0.39
m-Cresol	0.70	0.40
Phenol	0.65	0.29
Geraniol	0.88	0.85
Benzyl alcohol	0.67	0.35

Derivatives of amines. Amines were reacted with 2,4-dinitrochlorobenzene or 2,4-dinitrofluorobenzene to form the corresponding 2,4-dinitroanilides[8].

The regression lines relating carbon number and R_M value for the derivatives of homologous primary aliphatic amines were found to be:

$$\text{for system A: } R_M = -0.1195\ C - 0.049$$
$$\text{for system B: } R_M = -0.1456\ C + 0.800.$$

The first member of the series for system A, and the first two members for system B were excluded from the calculations. The resulting lines of best fit are shown in Fig. 4. A number of derivatives of other types of amines were separated in the two systems. The R_F values found are shown in Table V.

The homologues belonging to the series described in this paper are best separated from each other in system B. By lowering the amount of stationary phase and at the same time raising the polarity of the solvent a number of aromatic type derivatives can be separated, especially when present in a single mixture with aliphatics.

The running time of a chromatogram was 20–25 min which is usual in thin-layer chromatography. The reproducibility of R_F values was studied during the time the

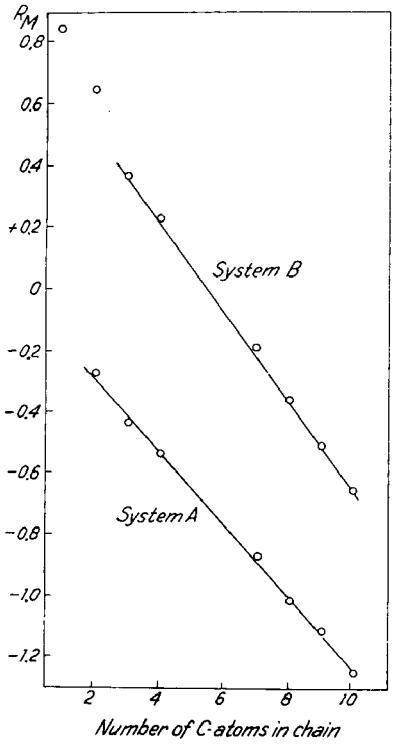


Fig. 4. Relation between carbon number and R_M value for 2,4-dinitroalkylanilides.

experiments were performed (about 18 months) using the 2,4-DNPH of pentanal in system B as a check and was found to be 0.122 R_F units.

However, using the correction procedure of GALANOS AND KAPOULAS the reproducibility was raised to 0.031 R_F units using the 2,4-DNPH's of ethanal and of decanal as reference compounds.

In conclusion, polyvinyl acetate seems to be a very useful addition to the range of thin-layer stationary phases. It gives very rigid layers which can be stored indefinitely. Separations can be obtained in a short time and R_F values are highly reproducible.

TABLE V

2,4-DINITROANILIDES OF SOME NON-ALIPHATIC TYPE AMINES

2,4-Dinitroanilide of	R_F	
	System A	*System B*
α-Naphthylamine	0.70	0.36
Benzidine	0.26	0.02
β-Phenethylamine	0.65	0.22
Cyclohexylamine	0.82	0.53
Aniline	0.66	0.28
p-Nitroaniline	0.50	0.06

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CHROM. 4958

DENSITOMETRY OF THIN-LAYER CHROMATOGRAMS USING POLAROID PROJECTION FILM

APPLICATION TO THE ANALYSIS OF MONOSACCHARIDES

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SUMMARY

A method for quantitative densitometry of thin-layer chromatograms using Polarcid Type 46-L projection film and a conventional thin-membrane densitometer is described. Standard curves prepared from fucose and glucose visualized with aniline–diphenylamine–phosphoric acid showed a linear relationship between the peak areas and the amount of sugar present on the chromatographic plate. The applicability of the method to biological materials is demonstrated by its successful use in determining plasma fucose concentrations. The method is applicable to the quantitative densitometry of chromatographic and electrophoretic systems where the properties of the substances to be analyzed and the physical characteristics of the separation system make direct densitometric analysis technically difficult.

INTRODUCTION

Materials separated on thin-layer chromatograms can be quantified either following elution from the chromatogram adsorbent or by an analytic method performed directly on the chromatographic plate, such as densitometry[1]. Densitometry has the advantage of simplicity but does suffer from limitations due to the physical characteristics of the chromatographic system. Densitometry of thin-layer chromatograms has been described for the analysis of amino acids[2], carbohydrates[3], lipids[4], steroids[5], alkaloids[6], drugs[7] and dyes[8], and the methods have been reviewed recently[9]. A number of instruments designed for thin-layer chromatogram densitometry are commercially available[10].

This communication describes a method of thin-layer chromatographic densitometry using photographs made on Polaroid Type 46-L Land projection film and a conventional thin-membrane strip densitometer. The method provides both a means of quantifying materials on thin-layer chromatograms and a permanent record of the appearance of the chromatogram. Photography on Polaroid projection film does not require a dark room, permits an immediate evaluation of the quality of the photograph

and provides a positive image of the chromatogram for scanning without the necessity of image reversal. The photograph can be taken immediately after color development and the densitometry performed when convenient since chromogen fading and background darkening of the original chromatogram with time are eliminated. The method requires a minimum amount of manipulation of the chromatogram, reducing the likelihood of disturbing the adsorbent surface. The photographs are resistant to mechanical damage and are stable for at least two years without special storage. The sensitivity of this method is as good as direct densitometry and the linearity of the response extends through a greater range of sample quantity than has been reported with direct densitometry of carbohydrates[3,11–13].

MATERIALS AND METHODS

L-Fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, sialic acid and aniline were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[$^{14}C_6$]Fucose was purchased from Calbiochem (Los Angeles, Calif.). Analytical grade methyl ethyl ketone, methanol, diphenylamine, 1-butanol, pyridine, hydrochloric acid, phosphoric acid and glacial acetic acid were purchased from Fisher Scientific Company (Boston, Mass.). Silica Gel G and Aluminum Oxide G were obtained from Brinkmann Instruments, Inc. (Westbury, N.Y.).

Two thin-layer chromatographic systems were used in this study[14]. Silica Gel G suspended in 0.1 *N* boric acid or Alusil (aluminum oxide–Silica Gel G, 1:1 w/w) suspended in distilled water was spread in 250-μ layers on 20 × 20 cm glass plates using an adjustable thin-layer chromatogram spreader. The plates were dried at room temperature and stored in a desiccator until used. Synthetic mixtures of sugars, reference standards of fucose and glucose, and neutralized acid hydrolysates of plasma proteins were applied along a line 2 cm from the lower edge of the plate in a volume of 1 μl using capillary microliter pipets. The plasma samples, the addition of L-[$^{14}C_6$]fucose to the plasmas for isotope dilution determination and the method for preparing the glycoprotein hydrolysates have been described[15]. In certain experiments, radioactively-labeled fucose was added to the fucose standards at a ratio of 100 d.p.m. of L-[$^{14}C_6$]fucose per microgram of fucose. The chromatographic plate was then placed in a developing tank containing a small amount of the solvent system, and allowed to equilibrate for 16 h. The Alusil plates were developed with 1-butanol–pyridine–0.1 *N* HCl (5:3:2) and the Silica Gel G plates were developed with methyl ethyl ketone–methanol–glacial acetic acid (6:2:2). Following equilibration, additional solvent was added until the fluid level rose sufficiently so that it was in contact with the adsorbent layer on the glass plate. The chromatogram was developed until the solvent had risen 15 cm from the lower edge of the plate. The chromatogram was removed from the tank and dried at room temperature. The chromatograms were sprayed evenly with the aniline–diphenylamine–phosphoric acid reagent[16] and heated in an oven at 105° for 15 min. This reagent gave good visualization with all the sugars used in this study; however, the sensitivity was somewhat greater for the hexoses than for fucose[12], while glucosamine was approximately 50% as reactive as the neutral sugars.

Photographs of the plates were made with a standard MP-3XL Industrial View Land Camera (Polaroid Corporation, Cambridge, Mass.) fitted with 150-W, 2800-K bulbs and the 127-mm lens. Photographs were made on Polaroid 4 × 5 Land Type 52

film (black and white prints) and on Polaroid Type 46-L Land projection film (black and white continuous contrast transparencies) for densitometry. The plates were placed on the surface of an unlighted conventional X-ray view box since this type of surface resulted in even base lines in the densitometric scans. A lens opening of F-22 to F-32, at $\frac{1}{2}$-sec exposure time, yielded optimal contrast and even base lines. The densitometer available for this study required that each plate be photographed in three sections in order to provide a large enough densitometric peak image for accurate measurement. Exposure of the chromatogram to the flood lights for 1 to 2 min did not change its appearance; however, after approximately 10 min exposure the chromatogram darkened. Identifying information was written on the Type 46-L Land projection film immediately after development, prior to drying of the emulsion. The Polaroid films were processed according to the manufacturer's instructions except that the Type 46-L Land projection film was fixed with Kodak fixer (Eastman Kodak Co., Rochester, N.Y.) for 30 sec because the Polaroid Dippit resulted in fine lines appearing on the film surface which adversely affected the quality of the densitometric scans. After rinsing for 1 h in running tap water, the projection film photographs were dried and cut into approximately 3-cm-wide strips and scanned in an Analytrol Recording Densitometer–Integrator (Beckman Instruments, Inc., Fullerton, Calif.) fitted with a 500-mμ peak wave length light filter, modified with a Scan-a-Tron attachment (Gelman Instrument Co., Ann Arbor, Mich.) and a B-2 logarithmic balancing cam. The slit size was 0.5 mm wide by 11 mm long; this length extended slightly beyond the visible boundaries of the chromogen images. All results included in this report are from scans that were carried out in the direction of development starting at the solvent front. Scanning could also be performed at right angles to the direction of development.

Curve areas were measured by planimetry. Following densitometry, those samples containing L-[$^{14}C_6$]fucose were scraped from the plate into counting vials, 0.5 ml of water was added and the radioactivity was measured with the liquid scintillation mixture of PROCKOP AND EBERT[17] and a Packard Tricarb liquid scintillation spectrometer. Control experiments indicated that the aniline–diphenylamine–phosphoric acid spray and the Silica Gel G particles did not interfere with the radioactivity determinations. The counting efficiency for chromatographed and unchromatographed L-[$^{14}C_6$]fucose was 50%. Recovery of L-[$^{14}C_6$]fucose from the chromatogram was 95% and the radioactivity was located entirely in the fucose spot.

Statistical analysis of differences among serum fucose levels was performed using standard methods with $P < 0.05$ as the level of significance. Linear regression analysis was employed to test for significance of the relationship between the area under the densitometry peaks and the amount of the substances on the chromatographic plate.

RESULTS

The Alusil plates yielded good results in resolving mixtures of the sugars present in plasma glycoproteins including fucose, mannose, galactose, glucosamine and sialic acid. Fig. 1 shows the separation of the sugars chromatographed in this system as recorded by scanning on Polaroid Type 46-L Land projection film. This sample contained a mixture of fucose, mannose, galactose, glucose, glucosamine and sialic

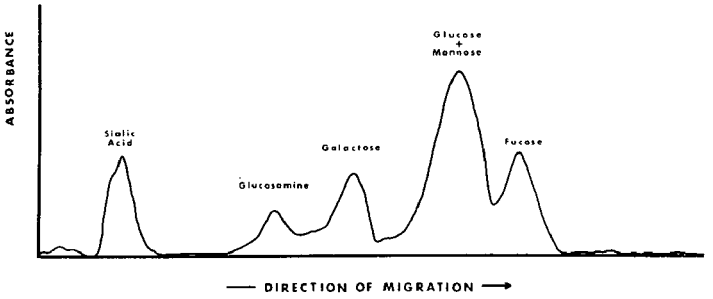

Fig. 1. Polaroid Type 46-L Land projection film densitometric analysis of a standard mixture of fucose, glucose, mannose, galactose, 5 $\mu g/\mu l$ each, and glucosamine and sialic acid, 10 $\mu g/\mu l$ each. Sample volume, 1.0 μl; adsorbent, Alusil; solvent, 1-butanol–pyridine–0.1 N HCl (5:3:2).

acid. The above sugars, except for glucose and mannose which had very similar R_F values, were well separated. A four-fold increase of the amount of the mixture placed on the chromatographic plate was accompanied by a similar increase in the areas of each densitometric peak, with the exception of sialic acid, which has an R_F of 0 (Fig. 2). Substances having low or high R_F values do not show a linear relationship between the amount on the chromatogram and the peak area when they are studied by direct densitometry[6,8].

Quantitative densitometric data were obtained for fucose and glucose chromatographed on Silica Gel G plates developed with methyl ethyl ketone–methanol–glacial acetic acid (6:2:2). Test solutions of each of the individual sugars were made at concentrations of 1–50 $\mu g/\mu l$. Known amounts of the sugars were placed on the

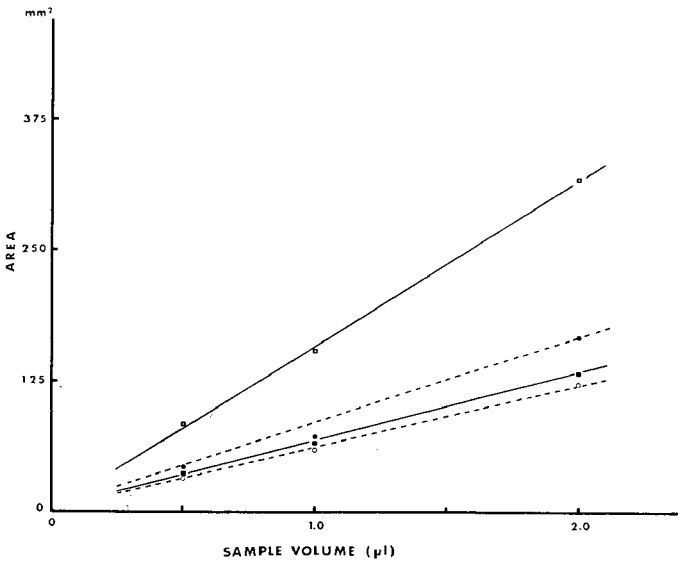

Fig. 2. Relationship of the Polaroid projection film densitometric peak areas to amount of sugar present on the chromatogram. The sugar mixture, adsorbent and solvent system were the same as in Fig. 1. Glucose–mannose peak (□—□), fucose peak (○— — —○), glucosamine peak (■—■), galactose peak (●— — —●).

chromatographic plates in a total volume of 1 μl, by using solutions of different sugar concentrations. Following chromatographic separation of the sugars, spraying with the aniline–diphenylamine–phosphoric acid reagent, Polaroid projection film photography and scanning, the areas under the curves were measured by planimetry, each value being the result of five planimeter readings, performed on duplicate scans. The data were analyzed to determine the relationship of the curve area to the amount of each sugar on the chromatographic plate. Fig. 3 shows the results of a study of

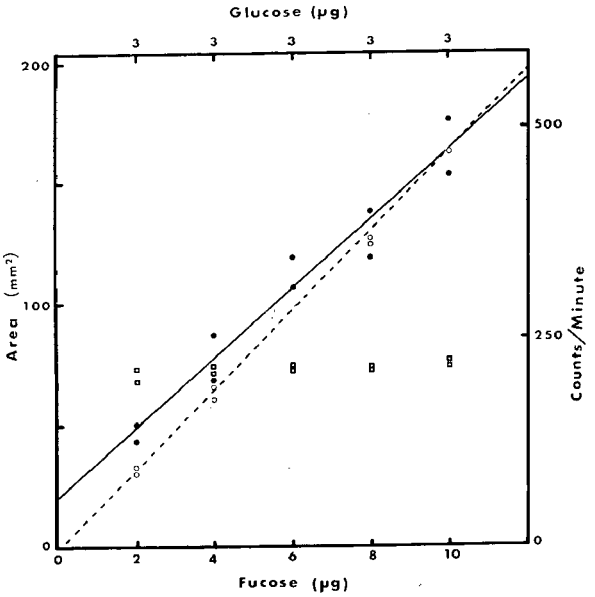

Fig. 3. Relationship of the Polaroid projection film densitometric peak area to the amount of fucose and glucose present on the chromatogram. Glucose (□), 3 μg and fucose (●), 2–10 μg per sample; L-[$^{14}C_6$]Fucose (○), 100 d.p.m. per μg fucose. Each point represents an individual determination. Densitometry and planimetry were performed in duplicate on each sample. The mean area of ten determinations of glucose was 74 mm^2 (S.D. = $\pm$2.0, S.E. = 0.8). The regression lines were fitted to the fucose peak area determinations (———) and L-[$^{14}C_6$]fucose radioactivity counts (— — —) by the method of least squares. The linear relationships were highly significant ($P < 0.001$).

standard fucose and glucose solutions with added L-[$^{14}C_6$]fucose. The amount of glucose placed on the chromatogram in each sample application was 3 μg and the amount of fucose on the chromatogram was varied as indicated. There is a linear relationship between the amount of fucose present and the areas under the densitometric peaks, and the regression line fitted to these points passes close to the origin although the origin did not fall within the 95% confidence limits of the slope. The reproducibility of the method on an individual chromatogram is demonstrated by the uniform peak areas obtained for the 3 μg of glucose present in each sample. As little as 1 μg of fucose or glucose could be measured by this method. When more than 25 to 30 μg of either sugar were placed on the chromatograms, the relationship between the amount of the sample and the area under the densitometric peak was no longer linear. In all our experiments with fucose and glucose standards performed as above,

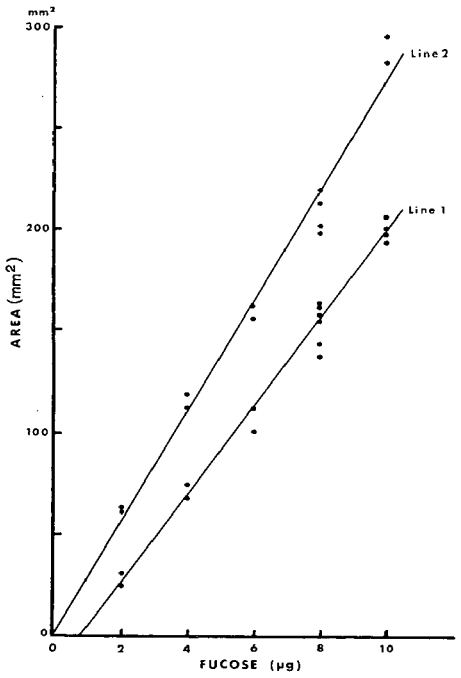

Fig. 4. Variation of the relationship between the Polaroid projection film densitometric peak areas to the amount of fucose present on the chromatogram following single and double spraying of the chromatogram with the aniline–diphenylamine–phosphoric acid spray reagent. Fucose (●), 2–10 μg per sample. Line 1, first spraying; line 2, second spraying. The regression lines were fitted to the experimental data by the method of least squares. The linear relationships were highly significant ($P < 0.001$).

a similar linear relationship was obtained and the regression lines passed close to the origin. The slope of the regression line was, however, variable.

Fig. 4 shows the results when a single chromatographic plate which contained standard amounts of fucose was sprayed, heated, photographed and scanned, then these steps repeated. In both instances, the relationship between the amount of fucose present and the area under the densitometric peaks was linear. The origin fell within the 95% confidence limits of the slope of line 2 but it was just outside of the 95% confidence limits of the slope of line 1. The slopes of the two regression lines were different, indicating that a primary source of variation between plates was in the staining reaction.

To test the usefulness of this method on biological material, human plasma protein-bound fucose levels were determined by combining radioisotope dilution and Polaroid 46-L projection film densitometry of plasma fucose isolated by thin-layer chromatography. In the experiments reported in this paper, the fucose-containing solutions were chromatographed on plates that also contained known amounts of fucose standards. The amount of fucose present on the chromatographic plate in the serum samples was determined by Polaroid 46-L Land projection film densitometry. The radioactivity present in the plasma fucose spots was counted and the fucose specific activity was calculated. As shown in Fig. 3, the ratio of radioactivity recovered

to the chromatographic peak area was constant when varying amounts of L-[$^{14}C_6$]-fucose and unlabeled fucose were chromatographed in a constant ratio. The plasma fucose concentration was determined by isotope dilution based on the amount of L-[$^{14}C_6$]fucose added to the plasma sample, the volume of plasma and the fucose specific activity. Table I shows that the levels of plasma fucose determined by the method of DISCHE AND SHETTLES[18] and by this method are not significantly different ($P > 0.20$).

TABLE I

PLASMA PROTEIN-BOUND FUCOSE CONCENTRATION MEASURED BY THE METHOD OF DISCHE AND SHETTLES AND BY THE POLAROID PROJECTION FILM DENSITOMETRY/ISOTOPE DILUTION METHOD

Significance of the difference in the fucose concentration determined by the two methods, $P > 0.20$.

Sample No.	*Fucose concentration (mg/100 ml)*	
	Dische and Shettles	*Densitometry/ isotope dilution*
1	5.8	8.4
2	5.8	7.2
3	8.0	7.1
4	5.6	7.7
5	9.2	8.0
6	10.5	9.7
7	6.2	5.7
8	9.8	7.0
9	13.8	9.9
10	18.0	8.2
11	9.0	11.7
12	8.6	11.9
13	9.6	7.9
14	8.6	8.2
Mean	9.2	8.5
S.D.	±3.4	±1.8
S.E.	±0.9	±0.5

DISCUSSION

Direct and conventional photographic[19] densitometric analysis of thin-layer chromatograms has been successful on a number of substances despite the problems inherent in using these methods with a non-transparent supporting material[20]. Direct transmittance and reflectance densitometry have both given satisfactory results in comparative studies[6,8]. The factors which affect the results of direct densitometry of thin-layer chromatograms include the following: the nature, thickness and moisture content of the absorbent layer, the solvent system, the rate of solvent flow, the R_F of the compounds, sample application size and shape, the direction of scanning, the measurement of the densitometric curve areas and the detection processes used for colorless compounds[5,8–10]. These factors were not systematically investigated, but the potential effects of these variables were taken into account in conducting the experi-

ments and no evidence was seen for these variables' causing unique problems in Polaroid projection film densitometry. This method gave satisfactory results in two thin-layer chromatographic systems with differing stationary and mobile phases and, while each chromatographic system would require a separate evaluation, it is probable that the method would be applicable to other systems.

Linear relationships have been found between the area of the densitometric peak and the amount of substance on the chromatographic plate in a number of systems[3,4,7,11–13,21]. Other investigators have obtained linearity by relating the square root of the area of the peak and the log of the amount of material on the chromatogram plate[5], the absorbance and the log of the amount of material on the chromatogram plate[22] or area of the peak and the square root of the amount of material on the chromatogram plate[6]. Our finding of a linear relationship between the peak areas and the amount of material on the plate, unless a certain maximum amount of substance is exceeded, is in agreement with direct densitometric studies of thin-layer chromatograms of carbohydrates[3,11–13,23]. We cannot predict the results of Polaroid projection film densitometry on chromatograms of materials where direct densitometry has not yielded linear relationships between peak area and amount of sample. It is of interest to note, however, that SHELLARD AND ALAM[6], working with a number of alkaloids, found that a linear relationship between peak area and amount of material on the plate was seen only when the chromatogram was rendered transparent and densitometry performed by transmittance. Photographing a chromatogram with Polaroid projection film may result in a similar transformation and therefore may yield linear relationships different from direct densitometry on the same chromatogram.

While the relationship between the amount of material present and the peak area was linear, there was a variation in the slopes of the regression lines fitted to the data from individual chromatograms. Differences in the degree of color development from plate to plate appeared to account for most of this variation; therefore each chromatogram must contain standards, in addition to the unknown samples. Investigators using direct densitometry have also reported that colorimetric reactions are the least reproducible step in the method[8,9]. In instances where the chromatographed compounds are colored, the results from replicate chromatograms would be more reproducible since variations due to photography and scanning are small. Differences in the exposure time, lens setting and illumination will affect the area of the curves obtained by densitometry from individual chromatograms, but if conditions are selected so that the photographs are representative of the actual appearance of the plate, and then the photographic conditions kept constant, duplicate photographs made with different film lots show good quantitative agreement. Differences in developing or fixing do not affect the results of this method. Scans performed on photographs before and after fixing gave identical results.

We did not do an extensive investigation of the effect of different colors on this method of densitometry, but the Polaroid projection film Type 46-L is panchromatic and would therefore be expected to record the visible spectrum satisfactorily. The colors of the glucose and fucose spots were blue when sprayed with the aniline–diphenylamine–phosphoric acid reagent. The color of glucosamine was yellow-brown and, while the spray reagent sensitivity was less than for neutral sugars, a linear relationship similar to that of neutral sugars was obtained with Polaroid projection film densitometry. Additional evidence that this method is applicable to materials

of other colors has been obtained by its use for quantitative lipoprotein electrophoresis stained red with Oil Red O[24].

The sensitivity of Polaroid projection film densitometry is similar to that of direct densitometric methods. Other workers have found the lower limits of sensitivity of the densitometry of carbohydrate thin-layer chromatography to be approximately the same as in our experiments[3,11,13] although direct densitometry of carbohydrates on thin-layer chromatograms with as little as 0.25 μg has been reported[12]. Spots barely visible on the chromatograms were easier to see in the photographs made with either Polaroid Land Type 52 black and white film or on the Polaroid Type 46-L Land projection film. The sensitivity of the method is most likely limited by the chromatographic system, the substance studied and the colorimetric reaction rather than by the photographic process.

The background against which the chromatogram is photographed is an important factor in the densitometric results. LUGG[20] presented the theoretical reason for the importance of background in the densitometry of non-transparent, non-homogeneous supporting media. He suggested that in reflectance densitometry, a material behind the object which reflected the portion of the incident light that was transmitted and scattered would improve contrast and the results of densitometry. Our findings and those reported by DALLAS[8] are consistent with this suggestion. Polaroid projection film densitometry was not tested with the use of transmitted light since uniform background lighting is required in order to avoid uneven backgrounds.

The use of a densitometer designed for analysis of small scale separations, such as the Microzone Densitometer (Beckman Instruments, Inc., Fullerton, Calif.), would save time and expense since an entire 20 × 20 cm chromatographic plate could be photographed on a single frame.

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CHROM. 4933

DÜNNSCHICHTCHROMATOGRAPHISCH-ENZYMATISCHER NACHWEIS UND ZUM WIRKUNGSMECHANISMUS VON CHLORKOHLENWASSERSTOFF-INSEKTIZIDEN

II. NACHWEIS DURCH HEMMUNG VON TRYPSIN

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SUMMARY

Thin-layer chromatographic–enzymatic identification and the mode of action of chlorinated hydrocarbon insecticides. II. Identification by inhibition of trypsin

It is shown that of the non-irradiated chlorinated hydrocarbons only dicofol and methoxychlor inhibit trypsin. After UV-irradiation on thin-layer plates all eighteen compounds studied show inhibiting activity against trypsin. Several unknown degradation products are formed by UV-irradiation. The amount of these substances may be variable, depending on environmental factors. It is unlikely that these new compounds are peroxides since reaction with KJ/starch and $Fe(SCN)_2$ gives negative results.

EINLEITUNG

Zur Vermeidung von Gefahren für Menschen, Tiere und Pflanzen bei Anwendung von Pflanzenschutzmitteln ist es nötig, das Schicksal der eingesetzten Wirkstoffe zu kennen und geeignete Nachweismethoden für sie und ihre Abbauprodukte zu finden. Ebenso wichtig ist es jedoch, die Wirkung dieser Verbindungen zu ermitteln, da nur aufgrund umfassender Kenntnis ihres Wirkungsmechanismus geeignete Schutzmassnahmen ergriffen werden können. Im Falle der schon lange als Insektizide bekannten Chlorkohlenwasserstoffe liegt bis heute zwar ein umfangreiches Material über Abbauwege und Metaboliten vor, doch kennt man noch immer nicht das stabilste Endprodukt der Abbaukette. Auch ist bisher noch immer relativ wenig über ihren Wirkungsmechanismus bekannt. Es ist daher nicht verwunderlich, dass man in neuerer Zeit zwar eine Abnahme der Schalendicke bei Vogeleiern durch DDT bzw. seine Abbauprodukte feststellte[1–6], der Grund dafür jedoch unbekannt blieb.

Es gilt heute allgemein als sicher, dass diese Substanzen primär auf das Nervensystem einwirken, wobei vor allem die sensorischen Nerven als Angriffspunkte angesehen werden. In welcher Weise die Chlorkohlenwasserstoff-Insektizide jedoch am

sensorischen Nerven eingreifen, ist bisher ebenfalls weitgehend ungeklärt. Zwar konnte gezeigt werden, dass DDT die K^+-Permeabilität des Nervengewebes erhöht[7] und mit Bestandteilen der Nervenmembranen Komplexe bildet[7,8], doch sagen diese Befunde noch nichts über den eigentlichen Wirkungsmechanismus aus.

In einer neueren Arbeit konnten MATSUMURA *et al.*[9] zeigen, dass DDT die ATPase der Nervenendigungen im Gehirn hemmt. Diese Befunde könnten einen Hinweis auf den Wirkungsmechanismus geben, da eine Hemmung der ATPase auch zu einer Hemmung des aktiven Ionentransports durch die Membranen führen dürfte.

Über die Wirkung der Chlorkohlenwasserstoffe auf Enzyme liegen bisher nur spärliche Untersuchungen vor. Lediglich das DDT ist in dieser Hinsicht etwas eingehender untersucht worden. In diesem Zusammenhang ist die Epoxidase-Induktion durch DDT[10,11] von besonderem Interesse. Leider sind auch hier die letztlichen Gründe für diese DDT-Wirkung unbekannt. Ebenso unbekannt ist die Ursache für die östrogene Aktivität von *o,p'*-DDT[12] und den durch DDT hervorgerufenen Hyperthyroidismus[13]. Auch der festgestellte Eingriff in den Aminosäurestoffwechsel und den Wasserhaushalt[14] lässt sich nicht eindeutig zuordnen. Die ebenfalls bekannte Blockierung von Atmungsfermenten und des Wasserstofftransfers wird *in vitro* durch eine Hemmung der Succinat-Dehydrogenase und Cytochromoxidase erklärt[14].

Weitgehendes Fehlen von enzymatischen Untersuchungen zur Wirkung der Chlorkohlenwasserstoff-Insektizide und ihrer Abbauprodukte lassen den Wirkungsmechanismus noch immer ungewiss erscheinen und mögliche Gefahren für die Umwelt unerkannt bleiben. Neuere Untersuchungen auf dünnschichtchromatographischer Basis konnten zeigen, dass die Chlorkohlenwasserstoffe auch die Rinderleber-Esterase beinflussen und nach UV-Bestrahlung zum Teil in sehr kräftige Esterase-Hemmer übergehen[15], obwohl alle bisher verfügbaren Daten gegen eine Hemmung der Acetylcholinesterase sprachen[14,16,17]. Wie im Biotest mit Drosophila-Imagines nach dem "dry-film"-Verfahren gezeigt werden konnte[18], verlieren die nach UV-Bestrahlung entstandenen Antiesterase-Substanzen die insektizide Wirkung der Originalwirkstoffe, bei Verfütterung an Drosophila-Larven nimmt diese hingegen zum Teil nur wenig ab[19], so dass Warnungen[18], sich auf den Biotest nach dem "dry-film"-Verfahren zu verlassen, mehr als berechtigt erschienen. Die künftige Rückstandsanalyse muss daher auch diese Abbauprodukte erfassen und wegen möglicher Gefahren berücksichtigen.

In einer Reihe von Untersuchungen soll die Wirkung von Chlorkohlenwasserstoff-Insektiziden und ihren UV-Abbauprodukten auf dünnschichtchromatographischer Basis auf einige Enzyme getestet werden, wobei zu prüfen ist, in welchem Umfange sich diese Verfahren zum Nachweis dieser Substanzklasse eignen. Gleichzeitig dienen diese Arbeiten der Aufdeckung möglicher Gefahrenquellen. Es soll hier über Untersuchungen zur Wirkung von Chlorkohlenwasserstoff-Insektiziden auf Trypsin berichtet werden.

MATERIAL UND METHODEN

Reagenzien

Die folgenden Reagenzien wurden verwendet: Kieselgel G nach Stahl mit *ca.* 13% $CaSO_4$, mittlere Korngrösse 10–40 μ; Aceton p.A.; Methylenchlorid p.A.; Cyclohexan p.A., nachgetrocknet mit Na_2SO_4; Methanol, getrocknet, p.A.; Chloroform

p.A.; Trypsin aus Rinderpankreas krist. lyophilisiert 2.0 U/mg (E.C. 3.4.4.4); N^{α}-Benzoyl-DL-arginin-4-nitroanilidhydrochlorid (Merck, Darmstadt, G.F.R.).

Enzym- und Substratlösung

Als Enzymquelle dient eine frisch bereitete Lösung von 250 mg Trypsin (E.C. 3.4.4.4) in 50 ml 0.03 *M* Phosphatpuffer pH 8.0, als Substrat eine ebenfalls frisch angesetzte Suspension von 400 mg N^{α}-Benzoyl-DL-arginin-4-nitroanilidhydrochlorid in 50 ml des gleichen Puffers. Die angesetzten Lösungen reichen für sechs Platten.

Insektizidlösungen und Dünnschichtchromatographie

Die untersuchten Wirkstoffe in analytischer Standardqualität werden mit Ausnahme von Hexachlorbenzol, das in Methylenchlorid gelöst wurde, als 1%ige Lösung in Aceton angesetzt, auf handgegossene Kieselgel G-Platten[15] aufgetragen und zur Bestimmung der Grenzkonzentration in Cyclohexan, zur Auftrennung der Bestrahlungsprodukte hingegen in Cyclohexan–Chloroform–Methanol (10:3:2) entwickelt.

Durchführung des enzymatischen Hemmtestes

Die Platten werden nach dem Entwickeln entweder sofort oder nach halbstündiger Bestrahlung mit ungefiltertem UV-Licht einer Hg-Analysen-Quarzlampe (Hanau) mit einem Abstand Strahler–Platte von 30 cm zunächst kurz mit Puffer und anschliessend mit der Trypsin-Lösung besprüht. Danach wird eine halbe Stunde bei 25° und etwa 90% Luftfeuchtigkeit inkubiert, anschliessend mit Substrat besprüht und etwa 1–2 Std. weiterinkubiert. Die endgültige Auswertung erfolgt, sobald die Platten unter Laborbedingungen nahezu trocken sind bei guter Beleuchtung.

ERGEBNISSE UND DISKUSSION

Untersuchungen zur Wirkung von Insektiziden auf Enzyme sind *in vitro* wegen der meist geringen Wasserlöslichkeit der Wirkstoffe stets etwas schwierig. Eine Applikation an lebende Tiere und anschliessende Bestimmung von Enzymaktivitäten schliesst den Nachteil der geringen Löslichkeit aus, birgt jedoch die Gefahr in sich, dass eine Verminderung der Aktivität verschiedene Ursachen haben kann und nicht unbedingt auf einer spezifischen Hemmung des untersuchten Enzyms beruhen muss. Dieser Einwand ist besonders dann berechtigt, wenn nicht gleichzeitig andere Parameter untersucht wurden. Bei Untersuchungen zur Wirkung von Pestiziden auf Enzyme auf dünnschichtchromatographischer Basis fällt das Problem der Löslichkeit nicht ins Gewicht, obwohl es zu einer direkten Wechselwirkung zwischen Enzym und Wirkstoff kommt. Nachteil dieses Verfahrens ist lediglich, dass man keine quantitativen Aussagen über Inhibitorkonstanten etc. machen kann. Leider ist diese Art der Untersuchung aus methodischen Gründen auf relativ wenig Enzyme beschränkt, da man für den Nachweis Reaktionsprodukte braucht, die entweder selbst gefärbt sind oder leicht in farbige Produkte überführt werden können. Man greift dabei in der Regel auf bekannte Testansätze zurück, die jedoch den Erfordernissen der Dünnschichtplatte entsprechend umgearbeitet werden müssen. Zum DC-Hemmtest mit Trypsin benötigt man eine relativ kräftige Substratsuspension, um später einen ausreichenden Kontrast zu erzielen. Aus dem gleichen Grund muss auch länger als normalerweise

TABELLE I

UNTERE NACHWEISGRENZEN DER CHLORKOHLENWASSERSTOFFE MIT UND OHNE UV-BESTRAHLUNG INFOLGE HEMMUNG VON TRYPSIN

Nachweisgrenze in μg; ( ) = nicht einwandfrei zu identifizieren.

Substanz	*Ohne UV-Bestrahlung*	*Mit UV-Bestrahlung*	*Substanz*	*Ohne UV-Bestrahlung*	*Mit UV-Bestrahlung*
DDT	(100)	6	Isodrin	(100)	6
DDD	(100)	6	Endrin	—	7
DDE	—	6	Aldrin	(100)	5
Dicofol	70	6	Dieldrin	—	7
Methoxychlor	70	6	Heptachlor	—	5
Perthan	—	6	Heptachlorepoxid	—	7
Hexachlorbenzol	—	5	Chlordan	—	9
Lindan	—	20	Isobenzan	—	6
Toxaphen	—	20	Endosulfan	—	7

bei Enzymreaktionen üblich bei hoher Luftfeuchtigkeit inkubiert werden, um möglichst viel Substrat zu spalten, das kontinuierlich in Lösung geht.

Unter den hier angewandten Bedingungen wird, wie aus Tabelle I hervorgeht, Trypsin nur von Dicofol und Methoxychlor gehemmt. Bei einer Auftragmenge von 70 μg kommt es an der Stelle, wo die Substanzen auf der Platte erscheinen, zu einer deutlichen Hemmung. DDT, DDE, Isodrin und Aldrin lassen bei einer Auftragsmenge von 100 μg zwar Flecken erkennen, doch sind diese nur schwach und nicht eindeutig als Hemmstellen zu identifizieren. Interessant ist jedoch, dass bei Isodrin am Start eine deutliche Trypsinhemmung auftritt, die jedoch auf Verunreinigungen des Wirkstoffes zurückzuführen sein dürfte.

Nach UV-Bestrahlung kommt es zu einer mehr oder weniger starken Trypsinhemmung durch alle untersuchten Wirkstoffe, wobei die nachzuweisenden Mengen zwischen 20 μg für Lindan und Toxaphen und 5 μg für Hexachlorbenzol, Aldrin und Heptachlor liegen (Tabelle I). Diese in der Regel um etwa eine Zehnerpotenz schlechtere Nachweisempfindlichkeit gegenüber dem Nachweis durch Esterasehemmung[15] verwundert jedoch nicht, da der Kontrast der weissen Hemmflecke auf hellgelbem Grund nur sehr mangelhaft ist. Damit eignet sich dieses Verfahren sicher nicht zum enzymatischen Routinenachweis für Chlorkohlenwasserstoff-Insektizide, obwohl eine Trypsinhemmung bei grösserem Kontrast sicher noch bei erheblich kleineren Auftragmengen festzustellen wäre. Die hier beschriebene Methode ist jedoch trotz des geringen Kontrastes sehr gut zum Screening auf Trypsininhibitoren anwendbar. Gleichzeitig zeigen die vorliegenden Ergebnisse eine neue, bisher unbekannte Wirkung von Abbauprodukten der Chlorkohlenwasserstoffe, deren Bedeutung für die Gesundheit an dieser Stelle jedoch nicht diskutiert werden soll. Ebenso erscheint es verfrüht, den Angriffspunkt dieser Substanzen am Enzym zu diskutieren, bevor nicht ein umfangreicheres Material über die Wirkung dieser Verbindungen auf Enzyme vorliegt. Weitere Arbeiten in dieser Richtung sollen folgen.

Zur Prüfung der Frage, ob durch die UV-Bestrahlung nur eines oder mehrere Abbauprodukte entstanden sind und ob es sich um Trypsininhibitoren handelt oder nicht, wurden 50 μg Substanz auf die Platten aufgetragen, mit UV bestrahlt und nach Chromatographie in verschiedenen Laufmitteln, von denen sich das hier aufgeführte

TABELLE II

hR_F-WERTE DER CHLORKOHLENWASSERSTOFF-INSEKTIZIDE NACH AKTIVIERUNG DURCH UV-BESTRAHLUNG

Substanz	*Aktivierung*			
	Vor Entwicklung	*Vor und nach Entwicklung*		*Nach Entwicklung*
DDT	0; 9; 13; 19	0; 2; 9; 13; 19;	94	94
DDD	0; 6; 22	0; 6; 9; 42;	91	91
DDE	0; 4; 9	0; 9; 41;	94	94
Dicofol	0; 6; 12; 18; 51	0; 6; 12; 38;	63	63
Methoxychlor	0; 5; 15	0; 5; 9; 34;	88	88
Perthan	0; 4; 18	0; 4; 18; 40;	93	93
Hexachlorbenzol	0; 5; 20	0;	96	96
Lindan	0	0;	88	88
Isodrin	0; 9; 15; 22; 31	0; 4; 9; 15; 31; 55;	95	95
Endrin	0; 10; 49	0; 5; 10; 41;	91	91
Aldrin	0; 6; 12; 18; 32; 45	0; 6; 12; 18; 32;	92	92
Dieldrin	0; 5; 11; 16	0; 5; 11;	89	89
Heptachlor	0; 12; 19; (38)	0; 12; (28);	94	94
Heptachlorepoxid	0; 8; 15; 22	0; 15; (64);	89	89
Chlordan	0; 10; 17; 22	0; 17; (29);	91	91
Isobenzan	0; 4; 9; 18	0; 18;	94	94
Endosulfan	0; 12	0; 25;	74; 92	74; 92
Toxaphen	0; 9; 16	0; 29;	93	93

als das geeignetste erwies, entweder sofort oder nach nochmaliger Aktivierung durch UV-Bestrahlung mit Enzym behandelt. Wie aus Tabelle II hervorgeht, entstehen eine Reihe von Abbauprodukten, deren Anteil sich allerdings von Versuch zu Versuch zum Teil stark ändern kann. Diese Änderungen dürften auf Umweltfaktoren wie Temperatur, Feuchte, Anzahl katalytisch aktiver Zentren auf dem Schichtmaterial etc. beruhen, die in diesem Zusammenhang jedoch nicht weiter untersucht wurden. Bei den in der Tabelle II nach der erneuten Bestrahlung auftretenden Hemmflecken mit den hohen hR_F-Werten handelt es sich um die nach der ersten UV-Behandlung unverändert gebliebenen Originalwirkstoffe, wie ein Vergleich zwischen Spalte 1, 2 und 3 der Tabelle II zeigt. Diese Ergebnisse, wonach die Flecke der unveränderten Chlorkohlenwasserstoff-Insektizide erst nach erneuter Aktivierung auftreten, stimmen mit denen aus Tabelle I sehr gut überein, wonach von den unbehandelten Wirkstoffen nur Dicofol und Methoxychlor bis zu einer Menge von 70 μg nachzuweisen sind. Da bei diesen Versuchen nur 50 μg aufgetragen wurden und über die Hälfte in Bestrahlungsprodukte übergegangen sein dürfte, wie aufgrund der Hemmintensität abgeschätzt wurde, ist es klar, dass ohne nochmalige Aktivierung keine Flecke in der Position der unveränderten Insektizide auftreten dürfen.

Allgemein zeigt sich, dass bei nochmaliger UV-Bestrahlung nach dem Entwickeln die Tendenz zur Hemmung verstärkt wird und neue Flecke auftreten, andere hingegen verschwinden. Ähnliche Erscheinungen konnten auch mit Rinderleber-Esterase festgestellt werden[20]. Das Sichtbarwerden von neuen Flecken nach nochmaliger Aktivierung dürfte darauf zurückzuführen sein, dass weniger wirksame Zwischenprodukte in das mögliche stark hemmende Endprodukt dieser Abbaureihe übergehen, das in diesem Laufmittel anscheinend am Start verbleibt. Ein befriedigendes Verfahren zur Auftrennung der Abbauprodukte konnte bisher noch nicht ge-

funden werden—eine Tatsache, an der auch deren Reinisolierung bisher scheiterte. Vor allem wirkt sich die starke Fahnenbildung sehr störend aus, da die Flecke vielfach in der Fahne untergehen. Auch eine Verminderung der Auftragmenge führte zu keiner verbesserten Auftrennung. Daher sind auch auf diesem Gebiet noch weitere Untersuchungen geplant.

Über die Struktur der UV-Bestrahlungsprodukte liegen bisher keine Anhaltspunkte vor, es dürfte sich jedoch sicher nicht um Peroxide handeln, die das Enzym oxydieren und auf diese Weise eine Hemmung vortäuschen. Von acht Wirkstoffen mit repräsentativen Vertretern aus jeder Gruppe der untersuchten Chlorkohlenwasserstoff-Insektizide wurden je 2 mg punktförmig auf eine Dünnschichtplatte aufgetragen und 1 Std. mit UV-Licht bestrahlt. Weder eine Behandlung mit Eisen-(II)-rhodanid noch mit KJ/Stärke-Lösung deutete auf entstandene oxydierende Bestrahlungsprodukte hin.

DANK

Mein besonderer Dank gilt Frau R. RAUBE für die sorgfältige Mitarbeit bei der Durchführung der Versuche.

ZUSAMMENFASSUNG

Es wird gezeigt, dass von den chlorierten Kohlenwasserstoffen nur Dicofol und Methoxychlor Trypsin hemmen. Nach UV-Bestrahlung der Dünnschichtplatten zeigen alle achtzehn untersuchten Wirkstoffe eine Hemmung gegen Trypsin. Durch die UV-Bestrahlung entstehen mehrere unbekannte Abbauprodukte. Der Anteil dieser Substanzen variiert mit den Umwelteinflüssen. Es dürfte sich bei diesen neuen Verbindungen nicht um Peroxide handeln, da eine Reaktion mit KJ/Stärke und Eisen-(II)-rhodanid negativ verläuft.

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CHROM. 4941

SEPARATION AND DETECTION OF CARBAMATES AND RELATED COMPOUNDS ON POLYAMIDE LAYERS

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SUMMARY

The separation and detection of 22 carbamates and related compounds including insecticidal or herbicidal materials, in current use, were investigated on polyamide layers. Suitable solvent systems and the R_F data obtained were reported.

A new spray reagent, Pinacryptol Yellow, was examined for the detection of these compounds and its usability was ascertained. A comparison was also made between the detection limits by UV absorption on polyamide and silica gel layers, and the superiority of the polyamide layers was confirmed.

INTRODUCTION

Polyamide has been used as a chromatographic substrate by a number of workers[1–6]. Recent developments include its use for the separation and identification of many kinds of substances containing hydroxyl groups such as phenolic compounds[2,3]. Substances containing of carbamate, urea, and anilide groups constitute a number of insecticidal or herbicidal materials and pharmaceuticals, and most of them contain a fundamental linkage $\begin{matrix} H & O \\ | & \| \\ -N- & C- \end{matrix}$ in the molecules. Each hydrogen and oxygen atom in this linkage could be concerned in hydrogen bonding with the $\begin{matrix} H & O \\ | & \| \\ -N- & C- \end{matrix}$ groups in the polyamide molecules.

This paper deals with the separation and detection of carbamates and related compounds including eighteen insecticidal or herbicidal materials in practical use and four synthesized materials for comparison, by thin-layer chromatography using polyamide.

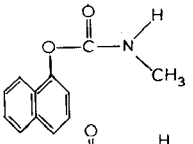
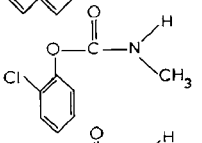
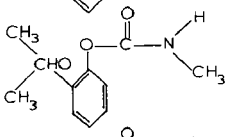
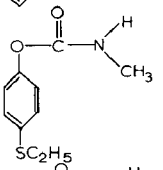
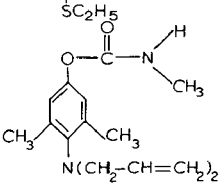
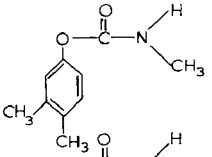
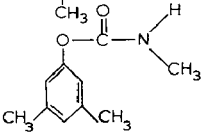
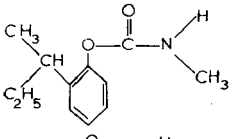
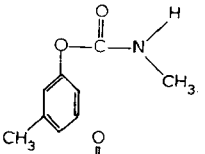
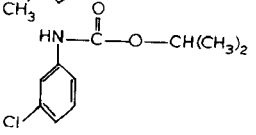
EXPERIMENTAL

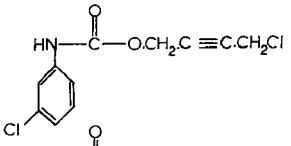
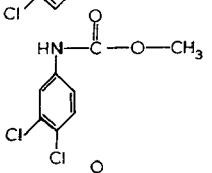
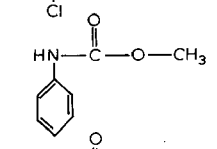
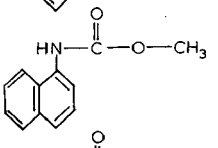
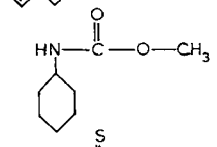
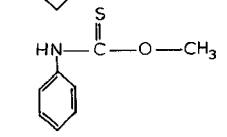
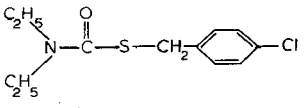
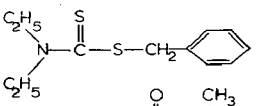
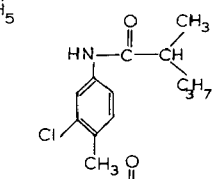
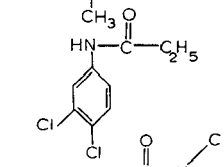
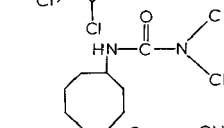
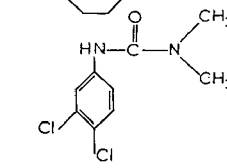
Materials

Wakō Polyamide B-10 which contains 10% anhydrous calcium sulfate (w/w)

TABLE I

CARBAMATES AND RELATED COMPOUNDS

Sample No.	*Chemical name*	*Chemical structure*	*Commercial name*
1	1-Naphthyl N-methylcarbamate		Denapon, Carbaryl Sevin, NAC
2	2-Chlorophenyl N-methylcarbamate		CPMC
3	2-Isopropoxyphenyl N-methylcarbamate		Bayer 39,007. Suncide, Baycon
4	4-Ethylmercaptophenyl N-methylcarbamate		EMPC, Tokisameito
5	4-Diallylamino-3,5-dimethylphenyl N-methylcarbamate		APC, Hydrole
6	3,4-Dimethylphenyl N-methylcarbamate		MPMC, Meobal
7	3,5-Dimethylphenyl N-methylcarbamate		Cosban, XMC
8	2-*sec.*-Butylphenyl N-methylcarbamate		Osbac, BPMC
9	3-Methylphenyl N-methylcarbamate		Tsumacide, MTMC
10	Isopropyl N-(3-chlorophenyl) carbamate		Chloro-IPC

11	4-Chloro-2-butynyl N-(3-chlorophenyl) carbamate		Carbyne, CBN
12	Methyl N-(3,4-dichlorophenyl) carbamate		Swep, MCC
13	Methyl N-phenylcarbamate		
14	Methyl N-α-naphthylcarbamate		
15	Methyl N-cyclohexylcarbamate		
16	Methyl N-phenylthiocarbamate		
17	S-p-Chlorobenzyl-N-diethyl thiolcarbamate		Saturn
18	S-Benzyl-N-diethyl dithiocarbamate		Cabac, TDW-39
19	N-(3′-Chloro-4′-methylphenyl)-2-methylpentaneamide		Solan, Dakuron
20	N-(3′,4′-Dichlorophenyl) propionamide		Stam, DCPA
21	3-Cyclooctyl-1,1-dimethylurea		Cycluron, Alipur-O
22	3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea		Karmex, Diuron

as a binder (Wakō Pure Chemical Ind. Ltd., Tokyo) was used for the thin-layer chromatography.

The reagents and solvents used in this investigation are of a specially refined grade.

The carbamates and related compounds used are as listed in Table I. Sample Nos. 1–12 and 17–22 were the commercial products of technical grade which were supplied with domestic manufacturers. If necessary, the samples were purified by conventional methods such as column chromatography and recrystallization.

Methyl N-phenylcarbamate (No. 13) and methyl N-α-naphthyl carbamate (No. 14) were synthesized from the corresponding isocyanate and methanol, respectively[7,8]. Methyl N-phenylthiocarbamate (No. 16) was synthesized from an equimolar amount of phenyl isothiocyanate and methanol by reacting them together at 120° for 5 h, and was purified by column chromatography and recrystallization to give needles, m.p. 88.0°. Methyl N-cyclohexylcarbamate (No. 15) was prepared by reacting an equimolar amount of cyclohexyl isocyanate and methanol at 50° for 2 h, and purified by recrystallization to give needles, m.p. 70.5°.

Preparation of the polyamide chromatoplates

Fourteen grams of Wakō Polyamide B-10 were mixed in a flask with 60 ml of distilled water and shaken for 30 sec to give a homogeneous suspension. This suspension was spread evenly on 5 glass plates (20 × 20 cm) with a suitable applicator, pre-set to give an 0.25-mm thick layer. The coated plates were kept horizontal and dried at 60–70° for 1 h and stored in a desiccator containing silica gel.

Application of the samples and development of chromatoplates

For the determination of R_F values, samples (1 μl of 1% solution in ethanol except for Nos. 3, 6, 7, 8, 9, 14, 16 and 22 which are each 2% solution) were spotted on the starting line 2.5 cm from the edge of the plate. The plate was developed in the ascending manner at 24–26° in a closed tank until the length of run was 12 cm.

Detection of spots on the chromatoplates

The following methods 1–6 were used according to the properties of the compounds.

(1) p-Nitrobenzenediazonium fluoroborate–KOH[9]. The plate is sprayed with 1 *N* KOH solution in ethanol, followed by spraying with a 10% ethanolic solution of ethylene glycol saturated with *p*-nitrobenzenediazonium fluoroborate. Only N-methylcarbamates with an O-aryl substitution give a blue-reddish violet spot against a whitish yellow background with this reagent.

(2) Bromine–fluorescein. After exposure to bromine vapor for about 15 sec, the plate is sprayed with an 0.2% (w/v) ethanolic solution of fluorescein. All of the compounds give a yellow spot against an orange-pink background in transmitted UV light.

(3) Rhodamine B–ultraviolet[10]. The plate is sprayed with Rhodamine B reagent which is prepared by dissolving Rhodamine B (0.2 g) in a mixture of 0.02 *N* $AgNO_3$ (50 ml) and 1 *N* HCl (50 ml) and filtering the resultant suspension. When the sprayed plate is irradiated with UV light for 30 min, most of the compounds give a violet spot against an orange background in transmitted UV light.

TABLE II

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF CARBAMATES AND RELATED COMPOUNDS ON POLYAMIDE LAYERS

Symbol	*Components*	*Ratio (v/v)*
A	H_2O–MeOH	5:5
B	H_2O–AcOH–MeOH	5:1:4
C	H_2O–acetone	6:4
D	H_2O–AcOH	6:4
E	H_2O–HCOOH–MeOH	4:1:5
F	H_2O–conc. NH_3–MeOH	3:1:6
G	H_2O–DMF	6:4
H	Petroleum ether–toluene–AcOH	7:2:1
I	Petroleum ether–xylene–AcOH	8:1:1
J	Cyclohexane–acetone	8:2

(4) UV absorption. The compounds having UV absorbing group(s) in their molecules are detectable as dark spots in transmitted UV light. For the sensitive detection of spots, a quick operation is necessary because most of the compounds are easily decomposed by UV irradiation.

(5) Silver nitrate–ultraviolet[11]. Silver nitrate (0.1 g) is dissolved in a mixture of 2-phenoxyethanol (10 ml) and water (1 ml), and the solution is diluted with acetone to 200 ml. The plate is sprayed with this reagent and irradiated with UV light for 15

TABLE III

R_F VALUES OF CARBAMATES AND RELATED COMPOUNDS ON POLYAMIDE LAYERS

Sample No.	*Solvent system*									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
1	0.42	0.52	0.57	0.46	0.63	0.61	0.62	0.44	0.34	0.51
2	0.62	0.68	0.67	0.61	0.75	0.82	0.72	0.49	0.40	0.54
3	0.73	0.78	0.74	0.70	0.81	0.82	0.80	0.55	0.48	0.60
4	0.52	0.61	0.59	0.54	0.70	0.72	0.61	0.54	0.46	0.60
5	0.32	0.71	0.40	0.89	0.90	0.62	0.35	0.75	0.70	0.78
6	0.60	0.70	0.65	0.63	0.74	0.74	0.71	0.58	0.49	0.61
7	0.58	0.69	0.65	0.63	0.74	0.73	0.70	0.60	0.52	0.67
8	0.58	0.67	0.59	0.58	0.73	0.73	0.60	0.66	0.59	0.74
9	0.67	0.76	0.70	0.68	0.78	0.76	0.74	0.56	0.47	0.63
10	0.28	0.39	0.40	0.33	0.50	0.54	0.39	0.70	0.65	0.79
11	0.22	0.33	0.35	0.30	0.47	0.50	0.36	0.54	0.47	0.67
12	0.20	0.29	0.34	0.29	0.41	0.44	0.30	0.52	0.44	0.60
13	0.56	0.66	0.64	0.60	0.71	0.70	0.69	0.57	0.48	0.61
14	0.39	0.51	0.53	0.44	0.58	0.59	0.59	0.60	0.52	0.58
15	0.81	0.88	0.70	0.68	0.73	0.87	0.74	0.98	0.89	0.91
16	0.35	0.41	0.41	0.34	0.48	0.58	0.48	0.65	0.57	0.65
17	0.34	0.40	0.36	0.32	0.53	0.62	0.39	0.96	0.86	0.95
18	0.19	0.24	0.27	0.18	0.38	0.49	0.28	0.96	0.84	0.93
19	0.21	0.33	0.32	0.28	0.48	0.50	0.30	0.52	0.44	0.72
20	0.23	0.33	0.36	0.33	0.47	0.47	0.33	0.28	0.21	0.50
21	0.71	0.77	0.72	0.69	0.80	0.81	0.71	0.57	0.48	0.61
22	0.33	0.45	—[a]	0.44	0.56	—	—	0.26	—	0.45

[a]Symbol — means that no experiment was performed.

min. The compounds having a phenyl group substituted with chlorine atom(s) are detectable by this method as a yellowish brown spot against a light brown background.

(6) Pinacryptol Yellow–ultraviolet[12]. The plate is sprayed with 0.1% (w/v) solution of Pinacryptol Yellow in 95% ethanol and air-dried in the dark. All of the compounds appear as dark greyish spots on a light blue background in transmitted UV light.

RESULTS AND DISCUSSION

The solvent systems suitable for the separation of the carbamates and related compounds on polyamide layers are summarized in Table II. They are roughly classified into two types, solvents A–G and H–J, according to the polarity of the constituent solvents. These solvents resolved all the compounds on the layers and the R_F values

TABLE IV

DETECTION LIMITS OF CARBAMATES AND RELATED COMPOUNDS ON POLYAMIDE LAYERS AND SILICA GEL LAYERS

Method 1 = *p*-Nitrobenzenediazonium fluoroborate–KOH;
Method 2 = Br_2–fluorescein;
Method 3 = Rhodamine B–UV;
Method 4 = UV absorption or fluorescence;
Method 5 = $AgNO_3$–UV;
Method 6 = Pinacryptol Yellow–UV.

Sample No.	*Method of detection*						
	1	*2*	*3*	*4*	*5*	*6*	*4*
	Polyamide						*Silica gel*[a]
				(in μg)			
1	0.02	0.3	3	0.1[c]	10	0.25	5
2	0.05	5	5	1	0.5	5	5
3	0.05	1	—	5	—	1	1
4	0.5	0.5	0.5	0.1	0.5	0.5	5
5	—[b]	0.5	—	0.5	5	0.5	1
6	0.5	0.5	30	5	—	1	5
7	0.05	0.5	30	10	—	5	10
8	0.05	5	30	1	—	5	5
9	0.05	0.5	50	5[c]	—	1	5
10	—	0.5	0.5	0.05	0.1	0.1	1
11	—	0.5	0.5	0.1	0.1	0.5	1
12	—	1	0.5	0.05	0.1	0.1	0.5
13	—	0.5	—	0.1	—	0.25	5
14	—	1	1	0.5[c]	1	0.5	5
15	—	0.5	80	—	—	20	100
16	—	0.5	1	0.05	1	0.05	0.1
17	—	0.5	1	0.5	50	5	20
18	—	0.5	0.1	0.05	1	0.5	0.5
19	—	0.5	0.5	0.01	0.1	0.01	10
20	—	5	0.5	0.05	0.1	0.05	20
21	—	0.5	50	—	—	5	20
22	—	3	0.5	0.01	0.05	0.05	5

[a] SilicAR TLC-7G (Mallinckrodt Chemical Works) was used.
[b] Symbol — means that no spot was observed even at 100 μg.
[c] These spots are fluorescent.

obtained are listed in Table III. Examination of the R_F values of the following pairs of samples, *viz.*, Nos. 1 and 14, Nos. 13 and 15, and Nos. 13 and 16 implies that the hydrogen bonding forces between both the $\begin{matrix} H & O \\ | & \| \\ -N- & -C- \end{matrix}$ groups in the carbamates and the polyamide molecules are feeble.

One of the characteristics of polyamide layers is to give a bright background in transmitted UV light, and this phenomenon provides a highly sensitive method of detecting UV absorbing substances on the chromatoplate[13].

A comparison is made between the detection limit of the spots by UV absorption (method 4) on polyamide layers and on silica gel layers. It can be seen from Table IV that the detection of the compounds containing UV absorbing groups in their molecules is more sensitive on polyamide layers than on silica gel layers.

With regard to other methods of detection, methods 1–3, and 5 can be used on the polyamide layers as well as on silica gel layers (see Table IV and EXPERIMENTAL).

The Pinacryptol Yellow reagent (method 6) which has been used by us for the detection of synthetic sweetners was here again applied to the detection of spots of carbamates and related compounds. By spraying with this reagent, all of the compounds gave a dark greyish spot against a light (fluorescent) blue background in transmitted UV light. The results shown in Table IV indicate that the applicability and sensitivity of the method are comparable to those of the bromine–fluorescein (method 2) or the UV absorption (method 4).

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CHROM. 4929

GAS CHROMATOGRAPHY OF INORGANIC VOLATILE CHLORIDES

A STUDY OF THEIR REACTIVITY TOWARDS CERTAIN COLUMN PACKING MATERIALS

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SUMMARY

The interaction of silicon tetrachloride, phosphorus oxychloride, phosphorus trichloride, tin tetrachloride, germanium tetrachloride, titanium tetrachloride, arsenic trichloride, carbon tetrachloride, antimony pentachloride and vanadium tetrachloride, upon the stationary phases: Kel-F oil 10, Apiezon L, Kel-F wax, Phasepak P, Silicone Rubber UC-W98, Silicone oil DC-550 and graphite was investigated.

With the exception of antimony pentachloride and vanadium tetrachloride, the chlorides tested showed no interaction with the above-mentioned phases.

INTRODUCTION

This investigation is a part of an extensive project, whose basic goal is to explore the application of GLC in the analysis of metals in alloys and oxides, based on the volatility of the corresponding metal chlorides.

The main problem in applying gas chromatography for the separation and eventually the quantitative determination of inorganic volatile chlorides is the choice of an appropriate liquid phase which does not react with these compounds. In the present work the overall stability of the following liquid phases: Kel-F oil 10, Kel-F wax, Apiezon L, Phasepak P, Silicone Rubber UC-W98, Silicone oil DC-550, and graphite against $SnCl_4$, $SiCl_4$, $GeCl_4$, VCl_4, $TiCl_4$, CCl_4, $POCl_3$, PCl_3, $AsCl_3$ and $SbCl_5$ is studied.

In general, reactivity may be a question of:

(a) interaction between solute and the liquid phase (sometimes due to the metallic tubing of the columns) resulting in the creation of interaction products;

(b) sorption of the solute on the solid support, or

(c) the way by which the solutes are dissolved in the liquid phase.

The term "reactivity" in this paper covers everything that causes the distortion or even disappearance of an expected peak.

The reactivity of inorganic volatile chlorides with respect to packing materials and, occasionally, the difficulty in determining the magnitude of this effect has often led to different opinions on the suitability of various substances that might be used as packing materials for the separation of inorganic chlorides.

For instance, FREISER[1] has reported the separation of $SnCl_4$ and $TiCl_4$ at 102° by using copper tubing packed with *n*-hexadecane coated Chromosorb. KELLER AND FREISER[2] and KELLER[3] have also studied the chromatographic behaviour of the above and of Nb(V) and Ta(V) chlorides in a column packed with squalane at a temperature of 200°. They also investigated the phases, *n*-octadecane, silicone oil, paraffin wax and Apiezon grease at temperatures between 100° and 200°, and they observed that both Apiezon grease and Silicone oil reacted with chlorides. HUTCHINSON[4], however, found that $AsCl_3$ could be separated from $SnCl_4$ using silicone oil on firebrick as the packing material with no apparent reaction.

WACHI[5] was unable to elute Sn(IV) and Ti(IV) chlorides at 125° and $FeCl_3$ at 325° using a stainless steel column packed with silicone grease or Apiezon M on ground C-22 Sil-O-Cel insulating brick, apparently owing to the reaction of the metal chlorides with the liquid phase and the walls of the column. TADMOR[6] also reported interaction between the chlorides and silicone wax, leading to several well-defined peaks.

Nevertheless, TADMOR[7] later proposed the separation of SCl_2, $PSCl_2$ and PCl_3 on Silicone oil DC-550 at 200° and ABE[8] described the qualitative separation of $SiHCl_3$, $SiCl_4$, BCl_3 and PCl_3 on various liquid phases, including silicone oil, on which he quantitatively separated $SiHCl_3$ from $SiCl_4$.

In another paper, TADMOR[9] studied the separation of $GeCl_4$, $SnCl_4$ and $AsCl_3$ and their interaction on a column packed with Sil-O-Cel C-22 insulating brick, either uncoated or coated with varying quantities of the liquid phase, by an isotopic exchange technique (hydrochloric acid labelled with ^{36}Cl). The chromatograms obtained with an uncoated support showed irregular peaks and imperfect separations. The incomplete recovery of the chlorides at the column's outlet was attributed to losses due to hydrolysis occurring within the column.

WILKE *et al.*[10] has reported on the determination of the purity of the tetrachlorides of silicon, tin and titanium by gas–liquid chromatography. They used nitrobenzene, silicone oil or Apiezon N on a diatomaceous support at 100°. They found that well resolved individual peaks for $SiCl_4$ and $TiCl_4$ could only be obtained by preinjecting a sufficient quantity of $TiCl_4$.

SIE *et al.*[11] in a study of the gas chromatographic separation of inorganic chlorides observed the appearance of a peak, attributed to hydrochloric acid, in a sample of gaseous chlorine after its contact with silicone grease.

In the present work the interaction between silicon tetrachloride, germanium tetrachloride, carbon tetrachloride, tin tetrachloride, titanium tetrachloride, arsenic trichloride, phosphorus trichloride, phosphorus oxychloride, vanadium tetrachloride and antimony pentachloride and the liquid phases Kel-F oil 10, Kel-F wax, Silicone oil DC-550, Silicone rubber UC-W98 and Apiezon L and Phasepak P is studied.

Study of the interaction between solutes and packing materials

Conclusions regarding the interaction between chlorides and liquid phases may be drawn from: (a) observation of the chromatograms obtained, (b) the changes, if any, occurring in the packing material after the experiments, and (c) the $\log V_g/\log p^0$ ratio.

The first thing to examine in studying the chromatograms is the stability of the base line and its eventual deviation from zero. The chromatograms can then be studied to ascertain the presence or absence of artifact peaks or the non appearance of expected peaks. One can then go on to observe the symmetry of the peaks, looking for tailing or skewing, so as to draw conclusions regarding the linearity of the partition isotherm.

As regards the appearance of the packing material at the end of a series of experiments, one should note any change that may have occurred in its colour or weight, or any other alteration. Possible retention of some of the solutes can be ascertained by checking whether there is any difference in the weight of the column before and after any long series of experiments. Finally, one can obtain indications as to whether or not the solutes are ideally dissolved in the liquid phase, without any change of their molecular structure, by examining the plot of log V_g *versus* log p^0, which should be a straight line with a slope equal to unity.

EXPERIMENTAL

Materials

Chlorides were obtained from commercial sources with the exception of germanium tetrachloride and vanadium tetrachloride which were prepared by chlorination of the corresponding metals. They were all purified by isothermal distillation and kept under absolutely anhydrous conditions.

Packing materials

(1) Kel-F oil 10, 10% w/w, on Celite 545
(2) Apiezon L, 10% w/w, on 45–60 mesh Chromosorb R
(3) Kel-F wax, 10% w/w, on Celite 545
(4) Phasepak P, 30–60 mesh
(5) Silicone rubber UC-W98, 10% w/w, on Diatoport S 80–100 mesh
(6) Silicone oil DC-550, 10% w/w on Celite 545
(7) Graphite purified Erg. B 6, 5% w/w, on glass beads 50–140 mesh

Operating conditions

Apparatus. A Hewlett-Packard chromatograph, Type 700, equipped with a Gow-Mac thermal conductivity detector with four W filaments was used. The instrument was modified in order to keep the oven temperature constant within $\pm$ 0.1°. From time to time the detector was washed with acetone in order to prevent alteration of response owing to deposits of hydrolysis or reaction products.

Columns. Glass coils of 183 cm length and an inside diameter equal to 4 mm were used. The columns were conditioned overnight at the maximum recommended temperature. The effluent end of the column was not connected to the detector during the conditioning period. All the columns were packed under anhydrous vacuum conditions by inserting a pyrex glass wool at one end of the column, applying vacuum to that end and adding the packing material at the other end of the column. Columns were vibrated and the vacuum continued until the packing material did not settle any further. Column oven temperatures used were at maximum *ca.* 30° below the maximum recommended temperature limit at isothermal conditions. The injection

port temperature was maintained at 50° above oven temperature, and that of detector was maintained equal to that of the oven.

Nitrogen, dried by passing through an activated molecular sieve and a phosphorus pentoxide trap, was used as the carrier gas at flow rates of between 10 and 100 ml/min so that the minimum height of a theoretical plate was obtained.

The column temperature was 60–190°. Bridge current T.C. detector was 150 mA.

The recorder used was a Kipp and Zonen, Type Micrograph BD2, 1 mV. The chart speed was 1 in. per min.

Injected volume of the sample was from 0.5 μl up to 10 μl injected with a Hamilton 701N syringe.

RESULTS AND DISCUSSION

Base line

In most of the chromatograms obtained the base line was stable and no deviation from zero was observed. However when $SbCl_5$ and VCl_4 were chromatographed a continuous displacement of the base line from its initial level appeared.

Artifact peaks

The $SiCl_4$, $AsCl_3$, $SnCl_4$, PCl_3, $POCl_3$ and CCl_4 behaved normally with all packing materials giving a single peak. In contrast to this, $SbCl_5$, VCl_4 and $TiCl_4$ showed abnormal behaviour. Thus, $SbCl_5$ when it was chromatographed with Apiezon L decomposed, yielding only one peak attributable to Cl_2. On silicone oil, a large number of well resolved unidentified peaks were obtained, while Phasepak P also gave a large number of peaks one of which was Cl_2. With graphite only one very distorted peak appeared. With Kel-F wax four peaks resulted, one of which was identified as Cl_2; while on Kel-F oil 10 no peak at all appeared, and the conclusion was drawn that the solute was completely retained by the liquid phase.

VCl_4, on silicone oil gave rise to two peaks. One of VCl_4, the second of $VOCl_3$. With Kel-F wax and Kel-F oil only a single but very distorted peak resulted.

$TiCl_4$ was completely retained by Apiezon L.

Due to their pronounced reactivity no further experiments were conducted with $SbCl_5$ and VCl_4. Graphite was used to overcome difficulties rising from the reactivity of these two compounds, upon the other liquid phases, but without complete success.

Symmetry of the peaks

Asymmetry of a peak may be due to one of two causes, according to the resulting shape. It may be the result of sorption on the solid support, which produces tailing, or may be attributed to a non-linear partition isotherm corresponding to a deviation from Raoult's law, *i.e.* variation of the partition coefficient with the concentration of the substance in the liquid phase. This results in skewing of the entire peak.

Tailing. Some small amount of tailing was observed in all experiments. This was clearly due to the fact that the solid supports being used were not silanised with the exception of Diatoport S. Of the packing materials studied, Kel-F wax on Celite gave the most symmetrical peaks with negligible tailing. There was a minimal amount of tailing on Phasepak P and on the liquid phase Silicone rubber on Diatoport S. The

two liquid phases Silicone oil and Kel-F oil on Celite only gave a very small amount of tailing though rather more was shown by Apiezon L, also on Celite.

Skewing. The peaks obtained using the stationary phase, Phasepak P and the liquid phases Apiezon L, Silicone rubber and Silicone oil on Celite showed no skewing except as noted.

With Kel-F oil 10 and Kel-F wax backward skewing of the peaks was observed in some cases, indicating a positive deviation from Raoult's law and a consequent increase of the partition coefficient in the gas and liquid phases due to an increase in the concentration of the chromatographed substance. More precisely Kel-F wax produced slight skewing of the $TiCl_4$ peak at a temperature of 100°.

Kel-F oil produced a pronounced skewing of the $TiCl_4$ peak and slight skewing of $SnCl_4$, $POCl_3$, $AsCl_3$ and CCl_4 peaks at 60°. Silicone oil resulted in a slight but quite noticeable skewing of the $POCl_3$, $AsCl_3$ and $TiCl_4$ peaks, only at 100°. This skewing appears when the sample size is relatively large, *e.g.* more than 2.5 μl of $POCl_3$ or $TiCl_4$. It was noticed that skewing is more pronounced when other chlorides have already been passed through the column. For instance, in the case of $AsCl_3$ with silicone oil at 150° it was observed that an injection of a 17 μl sample in a column that had not been used before for any other substances gave no skewing, whereas in a column through which $SnCl_4$, $SiCl_4$ and PCl_3 have been passed, the peaks started to skew as soon as the sample of $AsCl_3$ exceeded 2.5 μl.

Change of colour or weight of the packing material

No change in the colour of the listed packing materials was observed in all experiments, except in the case of titanium chloride, which caused darkening of the Apiezon L. Furthermore, after a great number of experiments no increase or decrease in the weight of the columns, could be observed. The check on the liquid phase was carried out by emptying the column, extracting the packing material with absolute ether, and weighing the residue. In a few cases there was a slight decrease in the weight when the column had been kept under working conditions for a long period at a relatively high temperature: this was clearly due to partial bleeding of the liquid phase.

Plot of log V_g versus log p^0

According to the relationship, $\ln V_g = -a \ln p^0 + C$, derived by HOARE AND PURNELL[12,13] the coefficient a is equal to the ratio of the heat of solution to the heat of vaporisation and is approximately unity in the case of liquid phases of similar polarity to the chromatographed substances. Consequently the plot of $\ln V_g$ *vs.* $\ln p^0$ is linear, with a slope of $a = 1$. Values of a greater or smaller than unity reflect deviations from ideal solution behaviour and are a measure of solute interaction with the liquid phase. Figs. 1–4 show the plot of log V_g *vs.* log p^0 for the systems studied.

On the liquid phase Kel-F oil 10 (Figs. 1 and 2) for the PCl_3 and $TiCl_4$ the plot of log V_g *versus* log p^0 is a straight line with a slope equal to unity or with a minimal deviation therefrom. Consequently ideal solution behaviour in this liquid phase can be deduced. In the case of $AsCl_3$, $SnCl_4$ and $GeCl_4$ the resulting plot is linear with a slope deviating 0–5° from unity, up to the value of log p^0 corresponding to a temperature of 70°. Beyond this the slope declines suddenly and this probably indicates that above this temperature these compounds cease to have ideal solution behaviour and interaction of the chlorides with the liquid phase takes place.

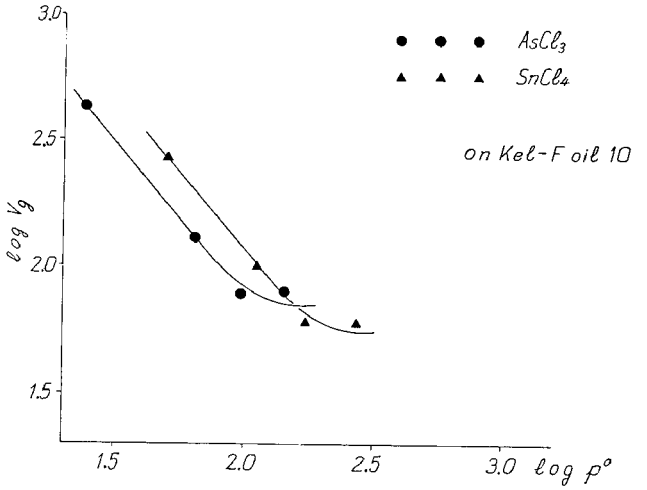

Fig. 1. Specific retention volume–vapour pressure plots for certain chlorides. Packing material: Kel-F oil 10, 10% w/w, on Celite 545.

Figs. 3 and 4 show the log V_g–log p^0 plots for the other phases. With only one exception these phases give plots that are linear, with a deviation from unity fluctuating between $+8°$ and $-7°$.

On the liquid phases silicone oil and Kel-F wax (Fig. 3) $AsCl_3$ shows considerable deviation, while $SnCl_4$ gives a linear plot that barely deviates from unity. $POCl_3$ and $TiCl_4$ on Kel-F wax show no deviation at all.

With the stationary phase Phasepak P (Fig. 3) deviations of $+4°$ and $-17°$ are given by $SnCl_4$ and $AsCl_3$, respectively.

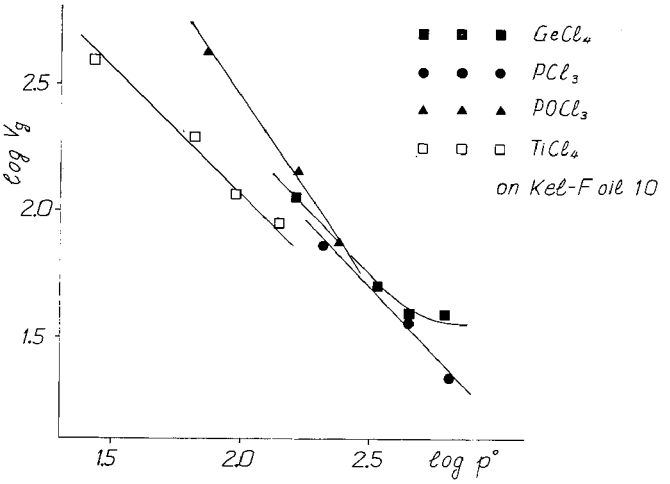

Fig. 2. Specific retention volume–vapour pressure plots for certain chlorides. Packing material: Kel-F oil 10, 10% w/w, on Celite 545.

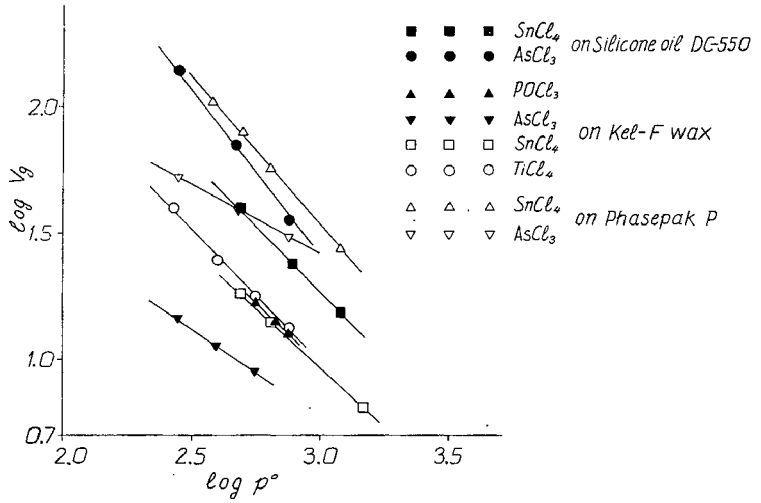

Fig. 3. Specific retention volume–vapour pressure plots for certain chlorides. Packing materials: (a) Silicone oil DC-550, 10% w/w, on Celite 545, (b) Kel-F wax, 10%, on Celite 545 and (c) Phasepak P 30–60 mesh.

With the liquid phases silicone rubber and Apiezon L (Fig. 4) the linear plots given by $AsCl_3$ and $SnCl_4$ show a slight deviation from unity, while for $POCl_3$ the slope of the plot $\log V_g$ *vs.* $\log p^0$ is equal to unity.

CONCLUSIONS

It has been shown in this paper that a number of organic partitioning phases, under particular operation conditions, do not interact with inorganic volatile chlorides

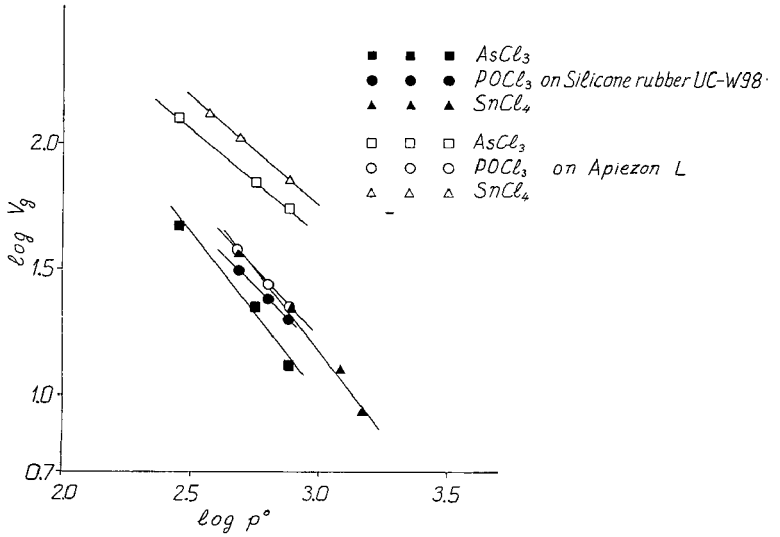

Fig. 4. Specific retention volume–vapour pressure plots for certain chlorides. Packing materials: (a) Silicone rubber UC-W98, 10% w/w, on Diatoport S and (b) Apiezon L, 10% w/w, on Celite 545.

and consequently they can be recommended for the quantitative separation and determination of these volatile compounds.

The absence of reactivity has been deduced from the fact that no artifact peaks appeared, the peaks were symmetrical with no skewing, so long as the columns were not overloaded, and had only slight tailing which was attributed to the non-silanisation of the solid support. In addition the plot of log V_g *vs.* log p^0 gave a straight line which is an indication of non reactivity between partitioning phase and solute.

Previously reported failures when using some of these phases for the same purpose can partially attributed to the fact that in the present work the experiments were done under anhydrous conditions and glass columns were used. CCl_4, $AsCl_3$ $SiCl_4$, $POCl_3$, PCl_3, $SnCl_4$, $GeCl_4$ and $TiCl_4$ behaved normally towards the partitioning phases tested. In contrast to these, $SbCl_5$ and VCl_4 reacted with almost all of them giving rise to a number of well resolved but unidentified peaks. The use of graphite as stationary phase was promising. Further experimental work is continuing on this system to see if better results may be achieved.

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CHROM. 4934

GEL CHROMATOGRAPHIC BEHAVIOR OF THE OXO ACIDS OF PHOSPHORUS

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SUMMARY

A number of oxo anions of phosphorus which contain one to four phosphorus atoms in the molecule were eluted with potassium chloride solutions on Sephadex columns. The K_d values of the oxo anions of phosphorus were measured as functions of the concentration and pH of the eluents, sample concentration and gel porosity. K_d values obtained by both the column and batch method were compared to each other.

The main conclusions are as follows. (1) When pure water was used as an eluent, extremely low K_d values were observed because of the electrostatic repulsion between the sample anions and the negatively charged gel matrix. (2) The K_d values increased with increasing concentration of potassium chloride. This may be due to the variation in the degree of hydration of sample anions. (3) At sample concentrations lower than 10^{-2} gram atom P per l the elution curves were symmetrical, while unsymmetrical elution curves were obtained at the higher sample concentrations. (4) The K_d values did not depend on the pH values of the eluent. (5) The most satisfactory separation of ortho-, di- and triphosphate anions was carried out on a Sephadex G-25 column. (6) The effective sizes of the oxo anions of phosphorus in aqueous solution were estimated from the gel chromatographic data.

INTRODUCTION

Some recent studies[1-8] have shown that gel chromatography is useful for the fractionation of macromolecules as well as for the separation of small inorganic ions. Although there are many arguments concerning the separation mechanism of inorganic species on a gel column, the molecular-sieve effect appears to be the major factor in many cases.

Since the family of oxo acids of phosphorus contains a number of compounds with different degrees of polymerization, investigation of their behavior in gel chromatography seems to be very appropriate for clarifying the separation mechanism of inorganic species on a gel column.

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In a previous paper[1] the present authors have demonstrated that oligophosphates can be eluted on a Sephadex G-25 column in order of decreasing molecular dimensions. ROGERS and his coworkers[2–4] have pointed out that both sample concentration and background electrolyte concentration play important roles in the gel chromatographic behavior of oligophosphates. FELTER *et al.*[5] and BOMBAUGH *et al.*[6] have fractionated some highly-polymerized linear phosphates using gel columns.

The purpose of the present investigation is to obtain fundamental information on the gel chromatographic behavior of a number of oxo acids of phosphorus which contain one to four phosphorus atoms in a molecule. The gel chromatographic investigation for a series of linear phosphates with degrees of polymerization higher than four will be reported in a subsequent paper[9]. In general the distribution coefficient K_d in gel chromatography is given by eqn. 1,

$$V_e = V_o + K_d \cdot V_i \qquad (1)$$

where V_e is the elution volume of a given solute, V_o the void volume outside the gel particles and V_i the internal volume within the gel phase. Although the molecular-sieve effect is a major factor for the separation mechanism, side effects such as ion exclusion[3,7], adsorption[8] and diffusion[10] must be considered in some cases. In order to estimate the contribution of these side effects the present authors measured K_d values for oxo anions of phosphorus as functions of concentration and pH of eluent, sample concentration and gel porosity, using Sephadex (a cross-linked dextran) as gel material and potassium chloride as the eluting agent, *i.e.*, background electrolyte. As has been pointed out in a preceding paper[11], potassium chloride is an appropriate background electrolyte for the gel chromatographic investigation of relatively large anions such as oxo anions of phosphorus. In the present study, the effective sizes of the oxo anions of phosphorus in aqueous solution were estimated from their gel chromatographic data as well.

Abbreviated notations proposed by BLASER AND WORMS[12] such as $\overset{1}{P}$ and $\overset{3}{P}$ are used in this paper. Ortho-, di-, tri-, tetra-, trimeta- and tetrametaphosphate are represented as P_1, P_2, P_3, P_4, P_{3m} and P_{4m}, respectively.

EXPERIMENTAL

Sample solutions

Sample solutions of seventeen oxo acids of phosphorus were prepared by dissolving the following salts in a solution of the same composition as that of the eluent used for the column operation:

$NaPH_2O_2 \cdot H_2O$ was used for $\overset{1}{P}$-acid; $Na_2PHO_3 \cdot 5H_2O$ for $\overset{3}{P}$-acid; $NaH_2PO_4 \cdot 2H_2O$ and KH_2PO_4 for P_1-acid; $Na_3P_2HO_5 \cdot 12H_2O$ for $\overset{2}{P}$–$\overset{4}{P}$-acid; $Na_2H_2P_2O_6 \cdot 6H_2O$ for $\overset{4}{P}$–$\overset{4}{P}$-acid; $Na_2P_2H_2O_5$ for $\overset{3}{P}$–O–$\overset{3}{P}$-acid; $Na_3P_2HO_6 \cdot 4H_2O$ for $\overset{3}{P}$–O–$\overset{5}{P}$-acid; $Na_4P_2O_7 \cdot 10H_2O$ for P_2-acid; $Na_5P_3O_8 \cdot 14H_2O$ for $\overset{4}{P}$–$\overset{3}{P}$–$\overset{4}{P}$-acid; $Na_4P_3HO_8 \cdot H_2O$ for $\overset{3}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$-acid; $(NH_4)_5P_3O_9 \cdot xH_2O$ for $\overset{5}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$-acid; $Na_5P_3O_{10} \cdot 6H_2O$ for P_3-acid; $Na_3P_3O_9 \cdot 6H_2O$ for P_{3m}-acid; $Na_6P_4O_{11} \cdot xH_2O$ for $\overset{4}{P}$–$\overset{4}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$-acid; $Na_4P_4O_{10} \cdot 4H_2O$

for $(-\overset{4}{P}-\overset{4}{P}-O-)_2$-acid; a sodium tetraphosphate solution was used for P_4-acid and $Na_4P_4O_{12}\cdot 4H_2O$ for P_{4m}-acid.

The salts of $\overset{1}{P}$-, $\overset{3}{P}$-, P_1-, P_2- and P_3-acids were commercial reagents, while the salts of $\overset{4}{P}-\overset{4}{P}$- (ref. 13), $\overset{3}{P}-O-\overset{3}{P}$- (ref. 14), $\overset{3}{P}-O-\overset{5}{P}$- (ref. 15), $\overset{3}{P}-O-\overset{4}{P}-\overset{4}{P}$- (ref. 16), P_{3m}- (ref. 17), P_4- (ref. 18) and P_{4m}-acid (ref. 19) and potassium Kurrol's salt (ref. 20) were prepared in the authors' laboratory. The salts of $\overset{2}{P}-\overset{4}{P}$-, $\overset{4}{P}-\overset{3}{P}-\overset{4}{P}$-, $\overset{5}{P}-O-\overset{4}{P}-\overset{4}{P}$-, $\overset{4}{P}-\overset{4}{P}-O-\overset{4}{P}-\overset{4}{P}$- and $(-\overset{4}{P}-\overset{4}{P}-O-)_2$-acid were supplied by BLASER AND WORMS.

Solutions of Blue Dextran 2000 (Pharmacia Fine Chemicals) and tritiated water (Radiochemical Center, Amersham, Great Britain), prepared by dissolving them in a solution of the same composition as that of the eluent were used as standard materials of $K_d = 0$ and 1, respectively. Potassium Kurrol's salt, $(KPO_3)_n$, which is a linear phosphate with an extremely high degree of polymerization (estimated to be about 10,000), was employed in place of Blue Dextran 2000 in some experiments. Potassium Kurrol's salt was dissolved in a dilute aqueous solution of lithium chloride and then added to the eluent employed, because it is difficult to dissolve it directly in an aqueous solution of potassium chloride[21].

Eluents

Pure water and 0.1, 0.5 and 1.0 *M* aqueous solutions of potassium chloride were used as eluents. Some of the potassium chloride eluent solutions were adjusted to definite pH values with suitable buffer solutions.

Columns

Several types of Sephadex, G-10 (particle size, 40–120 μ), G-15 (40–120 μ), G-25 (20–80 μ) and G-50 (20–80 μ), were used. Three glass tubes (Shoei Glass) of 1.5 × 60, 1.2 × 130 and 1.5 × 90 cm filled with 100, 120 and 150 ml of Sephadex gels, respectively, were used as columns. Gel beds were prepared by the method described in a preceding paper[11].

Procedure for elution

One milliliter of a sample solution was placed on top of the gel column and chromatographed by the procedure described in a preceding paper[11]. Sample concentrations were adjusted to 2×10^{-3}– 5×10^{-3} gram atom P per l, unless otherwise stated. The concentrations of the oxo acids of phosphorus in the effluents were determined colorimetrically by means of a molybdenum(V)–molybdenum(VI) reagent[22,23].

Determination of K_d by the batch method

In a 50 ml erlenmeyer flask with a glass stopper, 3.0 g of dry gel powder and 25.0 ml of a potassium chloride solution were mixed. The gel was allowed to swell for 24 h with occasional shaking. Then, 5.0 ml of a sample solution was added. The suspension was stirred for 1 h with a mechanical agitator. Preliminary experiments showed that no detectable concentration differences were found when the stirring was continued for 0.5, 1, 2, 3, 4 and 24 h. The gel was allowed to settle, and an aliquot of the solution was then withdrawn from the flask. Its phosphorus concentration was

determined colorimetrically. A similar method was employed to determine the void volume with Blue Dextran 2000 and potassium Kurrol's salt.

RESULTS AND DISCUSSION

Standardization of the columns

In gel chromatography the standardization of columns has a great advantage because results obtained with different columns can readily be compared with one another. Blue Dextran 2000 and tritiated water have been widely used to standardize gel chromatographic columns. Blue Dextran 2000 was weakly adsorbed on a highly cross-linked dextran gel to give an unsymmetrical elution curve. The maximum position of its elution peak at a concentration lower than 0.2% (w/w) is comparable with that of potassium Kurrol's salt, which is large enough to be excluded completely from the gel phase. In the present work Blue Dextran 2000 was usually employed because of the facility of its determination. When pure water was used as an eluent, however, the elution curve of Blue Dextran 2000 was so broad that its peak position could not be determined exactly. In such a case potassium Kurrol's salt was used as a standard material.

TABLE I

THE EFFECT OF ELUENT CONCENTRATION ON THE K_d VALUES

Gel, Sephadex G-25; bed volume, 150 ml.

Sample	K_d			
	Pure water	*KCl*		
		0.1 M	*0.5 M*	*1.0 M*
$\overset{1}{P}$		0.80	0.83	0.83
$\overset{3}{P}$		0.78	0.83	0.84
P_1	0.36	0.78	0.83	0.84
$\overset{2}{P}\text{–}\overset{4}{P}$		0.64	0.74	0.76
$\overset{4}{P}\text{–}\overset{4}{P}$		0.67	0.77	0.79
$\overset{3}{P}\text{–O–}\overset{3}{P}$		0.59	0.66	0.69
$\overset{3}{P}\text{–O–}\overset{5}{P}$		0.61	0.70	0.73
P_2	0.30	0.62	0.71	0.75
$\overset{4}{P}\text{–}\overset{3}{P}\text{–}\overset{4}{P}$		0.61	0.73	0.76
$\overset{3}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$		0.50	0.62	0.70
$\overset{5}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$		0.51	0.67	0.70
P_3	0.21	0.52	0.62	0.69
P_{3m}		0.52	0.67	0.70
$\overset{4}{P}\text{–}\overset{4}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$		0.46	0.62	0.64
$(\text{–}\overset{4}{P}\text{–}\overset{4}{P}\text{–O–})_2$		0.48	0.62	0.65
P_{4m}		0.46	0.60	0.65

Tritiated water was used to determine the total liquid volume in a column. However, some investigators[24,25] have demonstrated that its elution volume is slightly larger than the true total liquid volume, because isotope exchange of hydrogen atoms takes place very rapidly between water and dextran when one employs a highly cross-linked dextran gel. However, no special calibration due to this phenomenon was carried out in this work, because the correction term is negligibly small. Precision for the K_d values obtained in this work is within the range of $\pm$ 0.02.

Effect of eluent concentration

The K_d values of the oxo anions of phosphorus eluted with pure water and 0.1, 0.5 and 1.0 M solutions of potassium chloride from a Sephadex G-25 column are shown in Table I. The K_d values of the P_1-, P_2- and P_3-anions eluted with pure water are extremely low, as compared with those obtained by the elutions with the potassium chloride solutions. Since there are no appreciable differences between the V_o and V_i values obtained in the presence and the absence of potassium chloride as a background electrolyte, the low K_d values obtained in the absence of potassium chloride cannot be ascribed to the variation in the column parameters. The low K_d values may be explained, as has been pointed out by earlier workers[3,7], on the basis of the Donnan ion exclusion effect between the anionic solute species and a small number of fixed negative charges in the gel matrix which are believed to be carboxylate groups. In other words the penetration of the oxo anions of phosphorus into the gel phase is restricted by electrostatic repulsion between these oxo anions and the gel matrix.

Since the amount of fixed negative charges on the gel matrix is very small, the ion exclusion effect caused by the negative charges may be eliminated by the addition of potassium chloride to an eluent. NEDDERMEYER AND ROGERS[3], who observed the elution behavior of sodium chloride on a Sephadex column, have shown that a concentration of background electrolyte higher than 0.01 M is sufficient to eliminate the ion exclusion effect due to the gel matrix.

As shown in Table I, the K_d values of the oxo anions of phosphorus increase with increasing concentrations of potassium chloride. It is evident that the variations in the K_d values are not due to the column parameters, because both the V_o and V_i values did not depend upon potassium chloride concentrations, at least for the gel columns composed of Sephadex G-25. The fact that the extent of the variations in the K_d values for the monophosphorus species was small in comparison with those for the other species supports the above conclusion. If the variations in the K_d values are caused by the gel bed itself, for instance by the deformation of the gel particles, the same degree of variation will be observed. According to preliminary experiments in our laboratory the V_i values for the columns composed of Sephadex G-10 increased with increasing concentrations of potassium chloride in eluents. Therefore, one must take the dependence of the column parameters on the eluent concentration into consideration when a highly cross-linked gel is employed.

The K_d values of both the potassium and the chloride ion on a Sephadex G-15 column were higher than those of any of the oxo anions of phosphorus. Therefore, the oxo anions of phosphorus in the gel phase are always accompanied by the background electrolyte. In terms of phases I and II described in the preceding paper[11] the oxo anions of phosphorus can penetrate into phase II but not into phase I. This means that there may be no direct interaction between the oxo anions of phosphorus and the gel matrix.

TABLE II

EFFECT OF SAMPLE CONCENTRATION ON THE K_d VALUES

Gel, Sephadex G-25; bed volume, 100 ml for ^{3}P and P_3, 150 ml for the others; eluent, 0.1 *M* KCl.

Sample	K_d						
	Concn. of samples (gram atom P/l)						
	0.001	*0.005*	*0.01*	*0.05*	*0.1*	*0.5*	*1*
^{3}P	0.78	0.78	0.78		0.79		0.80
P_1	0.78	0.79	0.79		0.80		0.80
$^{4}P–^{4}P$	0.66	0.66	0.66	0.69	0.71		
P_2	0.62	0.63	0.63		0.69		
P_3	0.52	0.50	0.51	0.55	0.57	0.66	0.70
P_{3m}	0.52	0.52	0.52		0.58		0.69
$^{4}P–^{4}P–O–^{4}P–^{4}P$	0.46	0.46	0.46		0.50		0.66
$(–^{4}P–^{4}P–O–)_2$	0.48	0.48	0.47				
P_{4m}	0.46	0.46	0.45		0.50		0.63

Finally one must consider the deformation of the solute molecules. The degree of hydration in the oxo anions of phosphorus could decrease with increasing concentrations of potassium chloride in the eluents. This assumption leads to the conclusion that the K_d values of the oxo anions of phosphorus would become higher at the higher

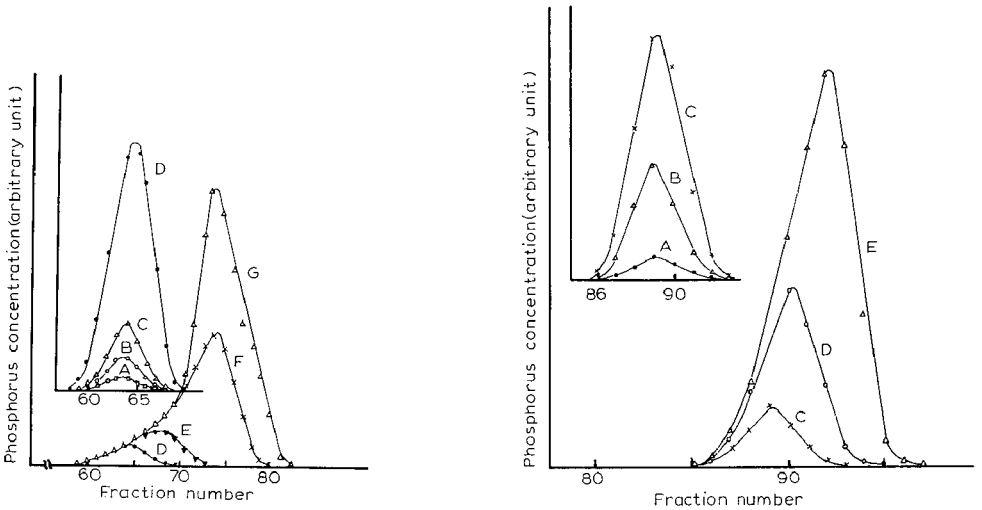

Fig. 1. Effect of sample concentration on the elution curves of the P_3-anion. Gel, Sephadex G-25; bed volume, 100 ml; eluent, 0.1 *M* KCl. Sample concentration (gram atom P per l): A = 0.001; B = 0.005; C = 0.01; D = 0.05; E = 0.1; F = 0.5; G = 1.0. One fraction = 1.03 ml.

Fig. 2. Effect of sample concentration on the elution curves of the $^{4}P–^{4}P$-anion. Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 *M* KCl. Sample concentration (gram atom P per l): A = 0.001; B = 0.005; C = 0.01; D = 0.05; E = 0.1. One fraction = 1.14 ml.

concentrations of potassium chloride. It has been pointed out[26] that the extent of the variations in the K_d values for non-electrolytes such as glucose, at different concentrations of a background electrolyte, are smaller than those for K_d values of ionic species. The K_d values of the oxo anions of phosphorus as well as alkaline earth metal ions[27] are dependent not only on the concentration of the eluents but also on the concentration of the sample solutions. These facts also support the above conclusion.

Effect of sample concentration

The effect of the sample concentration on the K_d values is given in Table II. This kind of effect is also of great importance in gel chromatography in evaluating the contribution of the molecular-sieve effect and other side effects, such as adsorption, to the separation process of solutes. The elution curves of the oxo anions of phosphorus tested all change in the same manner with the sample solution concentration. Two examples for the P_3- and $\overset{4}{P}\text{-}\overset{4}{P}$-anion are illustrated in Figs. 1 and 2. When the sample concentrations were lower than 0.01 gram atom P per l, the elution curves were symmetrical, and the elution peak positions did not change. However, when the concentrations were higher than 0.01 gram atom P per l, unsymmetrical elution curves with skewed leading edges were observed. The K_d values for these elution curves given in Table II were measured at the maximum concentrations. Elution patterns of this type are analogous to a concave adsorption isotherm in adsorption chromatography. However, the explanation based on adsorption seems to be unreasonable, because the K_d values of the oxo anions of phosphorus increase with increase of the sample concentrations, *i.e.*, the sample concentration dependence is contrary to that usually observed in adsorption chromatography. It seems to be more appropriate to explain the sample concentration dependence for the oxo anions of phosphorus in terms of the existence of oxo anions with different degrees of hydration.

Effect of pH of eluent solutions

The K_d values of the oxo anions of phosphorus eluted with 0.1 *M* potassium chloride solutions adjusted to pH 4.6, 7.0 and 9.2 from a Sephadex G-25 column are summarized in Table III. There is no significant pH dependence for the K_d values in columns a, b and c of Table III, but the K_d values obtained with the eluents buffered with borate are, in general, somewhat lower as shown in columns d and e. LINDQVIST[28] has demonstrated that the borate ion is strongly adsorbed on dextran gel. This phenomenon may be ascribed to the chemical reaction between the borate ion and dextran, because it is well known that the borate ion reacts with polyalcohols such as mannitol to form a strong acid. The electrostatic repulsion between the oxo anions of phosphorus and the borate anions adsorbed on the dextran gel leads to the decrease in the K_d values. This suggests that the buffering agent for the eluent should be selected carefully when one wants to separate solute molecules on the basis of the molecular-sieve effect.

The K_d values of ammonia and hydrochloric acid, when eluted with pure water through a Sephadex G-25 column, were reported to be 0.8 (ref. 29). This indicates that neither ammonium nor chloride ion is strongly adsorbed on the dextran gel. It has also been observed[29] that aromatic compounds are adsorbed to some extent on gel materials when eluted with pure water. However, it can be expected from earlier work[26] that the adsorption of aromatic compounds on dextran gel can be eliminated by the use of

TABLE III

THE EFFECT OF THE pH OF THE ELUENT ON THE K_d VALUES

Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 *M* KCl.

Sample	K_d				
	a *pH 4.6 phthalate–NaOH buffer*	*b* *pH 7.0 Tris buffer*	*c* *pH 9.2 NH_3–NH_4Cl buffer*	*d* *pH 7.0 borate buffer*	*e* *pH 9.2 borate buffer*
$\overset{1}{P}$	0.79	0.78	0.79	0.73	0.61
$\overset{3}{P}$	0.78	0.78	0.78	0.73	0.57
P_1	0.78	0.78	0.78	0.73	0.59
$\overset{2}{P}$–$\overset{4}{P}$	0.64	0.64	0.65	0.57	0.40
$\overset{4}{P}$–$\overset{4}{P}$	0.67	0.68	0.68	0.62	0.52
$\overset{3}{P}$–O–$\overset{3}{P}$	0.59	0.59	0.58	0.52	0.41
$\overset{3}{P}$–O–$\overset{5}{P}$	0.61	0.60	0.60	0.50	0.38
P_2	0.62	0.62	0.63	0.54	0.41
$\overset{4}{P}$–$\overset{3}{P}$–$\overset{4}{P}$	0.61	0.60	0.61	0.52	0.39
$\overset{3}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$	0.50	0.49	0.49	0.45	0.35
$\overset{5}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$	0.51	0.50	0.51	0.49	0.36
P_3	0.52	0.50	0.51	0.42	0.33
P_{3m}	0.52	0.52	0.52	0.44	0.33
$\overset{4}{P}$–$\overset{4}{P}$–O–$\overset{4}{P}$–$\overset{4}{P}$	0.46	0.46	0.47	0.42	0.32
$(-\overset{4}{P}$–$\overset{4}{P}$–O–$)_2$	0.48	0.48	0.48	0.42	0.30
P_{4m}	0.46	0.47	0.45	0.39	0.27

a background electrolyte. When P_1-, P_2- and P_3-anions were eluted with 0.1 *M* potassium chloride solutions containing 0.001, 0.005 and 0.01 *M* phthalate buffer from a Sephadex G-25 column, the K_d values of these anions did not change. This suggests that the phthalate anion is not adsorbed on the dextran gel. Since the K_d values listed in column b of Table III coincide well with those in columns a and c, it appears that the Tris buffer is also not adsorbed on the dextran gel.

The above discussion leads one to the conclusion that pH dependence of the K_d values of the oxo anions of phosphorus cannot be observed, when appropriate buffer agents are employed, in spite of the different dissociation states of the phosphorus oxo acids. The electrostatic effect appears to be a minor factor in the gel chromatographic behavior of the phosphorus oxo anions.

Effect of solute molecule diffusion

Experiments under static conditions were carried out to evaluate the contri-

TABLE IV

K_d VALUES OBTAINED BY THE BATCH AND COLUMN METHOD
Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 *M* KCl.

Sample	K_d	
	Batch	*Column*
P_1	0.78	0.78
P_2	0.61	0.62
P_3	0.51	0.52
P_{3m}	0.52	0.52
P_{3m}	0.46	0.46

bution of the diffusion of the solute molecules to the separation process on the column. If the separation of the solute molecules is only based on the molecular-sieve effect, the distribution coefficient K_d calculated from eqn. 2 should be equal to that obtained by the column method. Eqn. 2 can be derived on the assumption that the solute concentration within the gel phase is essentially the same as that in the external liquid phase. Then,

$$K_d = 1 - \frac{C_2 - C_1}{C_2} \cdot \frac{V_o' + V_i'}{V_i'} \qquad (2)$$

where C_2 is the concentration of the solute in equilibrium with a certain amount of Sephadex, C_1 is the concentration when no Sephadex is added, V_o' is the void volume of the solution and V_i' the internal volume. $V_o' + V_i'$ is considered to be equal to the total volume of the solution added. V_i' is calculated from the data for Blue Dextran 2000 or potassium Kurrol's salt as a standard material of $K_d = 0$.

The K_d values of some oxo anions of phosphorus obtained by the column and the batch method are shown in Table IV. There are no significant differences between the K_d values obtained by either method. This means that the effect of diffusion on K_d can be neglected in the gel chromatographic process under the conditions used in the present investigation.

TABLE V

K_d VALUES CLASSIFIED BY THE NUMBER OF PHOSPHORUS ATOMS IN A MOLECULE
Gel, Sephadex G-25; bed volume, 150 ml; eluent: 0.1 *M* KCl.

	Number of phosphorus atoms in a molecule			
	1	*2*	*3*	*4*
K_d	0.80–0.78	0.68–0.58	0.61–0.49	0.48–0.45

The elution order of the solute molecules and the effect of gel porosity

From all the results mentioned above it can be concluded that when the oxo anions of phosphorus are chromatographed on a Sephadex G-25 column with a 0.1 *M*

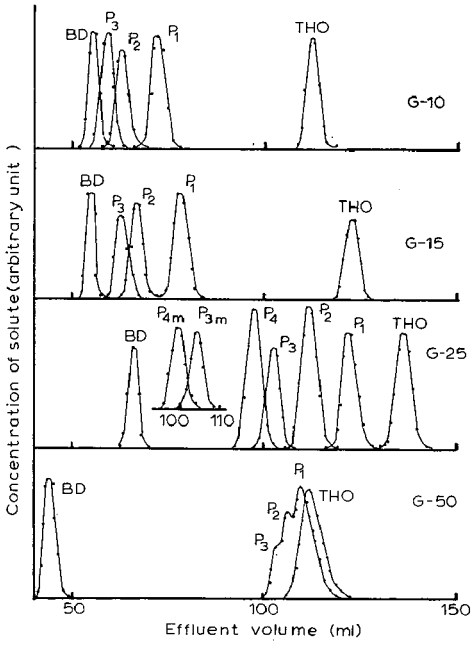

Fig. 3. Effect of gel porosity on the elution curves of P_1-, P_2-, P_3-, P_4-, P_{3m}- and P_{4m}-anions. Bed volume, 150 ml for G-10, G-15 and G-25 columns; 120 ml for the G-50 column. Eluent: 0.1 *M* KCl. BD = Blue Dextran 2000; THO = tritiated water.

potassium chloride solution, the separation mechanism is based only on the molecular-sieve effect. The order of the K_d values of the oxo anions of phosphorus obtained under the above conditions is roughly in agreement with that expected from their molecular dimensions as shown in Table V. This gel chromatographic method has been successfully applied to the group separation of ^{32}P-labeled oxo acids of phosphorus produced by neutron-irradiation of orthophosphates[30].

P_1-, P_2- and P_3-anion were eluted from Sephadex G-10, G-15, G-25 and G-50 columns with 0.1 *M* potassium chloride solutions. As shown in Fig. 3, the elution volume of each phosphate increases with increasing porosity of the gels. The Sephadex G-25 column gives the most satisfactory separation of P_1-, P_2- and P_3-anions.

Estimation of the size of the solute molecules

In recent years relationships between distribution coefficients and the molecular size of a solute have been studied by many investigators. As has been described in a preceding paper[11], LAURENT AND KILLANDER's model[31] can be expressed by eqn. 3.

$$(-\log K_{av})^{\frac{1}{2}} = A \cdot R_s + B \tag{3}$$

where A and B are constants for a given gel column and R_s is the radius of a solute molecule. K_{av} is defined as

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} = K_d \cdot \frac{V_i}{V_i + V_g} \tag{4}$$

where V_t is the total volume of the gel bed, and V_g the volume occupied by the gel matrix. For a Sephadex G-25 column $V_i/(V_i + V_g)$ was found to be equal to 0.862. If the elution process is controlled only by the molecular-sieve effect, one can calculate the R_s values of the solutes using eqn. 3.

As shown in Fig. 6 of the preceding paper[11], there is a linear relationship between the $(-\log K_{av})^{\frac{1}{2}}$ and the R_s values of potassium, chloride, sodium, nickel and magnesium ions. The K_{av} values of these ions were determined from an elution with a 0.1 M potassium chloride solution on a Sephadex G-15 column. The R_s values of these ions were taken from NIGHTINGALE's paper[32]. On the assumption that the linear relationship mentioned above can be used for the estimation of the molecular sizes of the oxo anions of phosphorus, the R_s values shown in Table VI were obtained from the K_{av} values of the oxo anions of phosphorus observed during an elution with a 0.1 M potassium chloride solution on a Sephadex G-15 column. Another set of the R_s values shown in Table VI was obtained by a similar method from the data for a Sephadex G-25 column. The results indicate that the oxo anions of phosphorus are considerably hydrated in aqueous solution.

TABLE VI

K_{av} VALUES AND MOLECULAR SIZE

Bed volume, 150 ml (* average K_{av} values obtained with both columns of bed volume 100 and 150 ml). Eluent, 0.1 M KCl.

Sample	*Sephadex G-15*		*Sephadex G-25*	
	K_{av}	R_s (Å)	K_{av}	R_s (Å)
$\overset{1}{P}$	0.39*	4.3	0.69	4.3
$\overset{3}{P}$	0.37	4.4	0.67	4.4
P_1	0.35*	4.5	0.67	4.4
$\overset{2}{P}\text{–}\overset{4}{P}$	0.22	5.6	0.55	5.5
$\overset{4}{P}\text{–}\overset{4}{P}$	0.24*	5.3	0.58	5.6
$\overset{3}{P}\text{–O–}\overset{3}{P}$	0.19*	5.7	0.51	5.8
$\overset{5}{P}\text{–O–}\overset{3}{P}$	0.20	5.7	0.52	5.7
P_2	0.20*	5.7	0.53	5.7
$\overset{4}{P}\text{–}\overset{3}{P}\text{–}\overset{4}{P}$			0.52	5.7
$\overset{3}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$	0.10	6.8	0.43	6.5
$\overset{5}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$	0.13*	6.4	0.44	6.4
P_3	0.13*	6.4	0.45	6.4
P_{3m}			0.45	6.4
$\overset{4}{P}\text{–}\overset{4}{P}\text{–O–}\overset{4}{P}\text{–}\overset{4}{P}$			0.41	6.8
$(\text{–}\overset{4}{P}\text{–}\overset{4}{P}\text{–O–})_2$			0.41	6.8
P_{4m}			0.40	6.9

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CHROM. 4935

GEL CHROMATOGRAPHIC BEHAVIOR OF LINEAR PHOSPHATES

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SUMMARY

Linear phosphates with degrees of polymerization of 1 to 13 were chromatographed on a Sephadex G-25 column with a 0.1 *M* potassium chloride solution and the following results were obtained. (1) The K_{av} values of the linear phosphates did not depend on pH of the eluents. (2) A linear relationship was observed between the K_{av} values and the logarithms of the degrees of polymerization. (3) Average degrees of polymerization of polyphosphate fractions determined by pH titration agreed approximately with those calculated from the gel chromatographic data.

INTRODUCTION

It is widely known that solute molecules are eluted from a gel chromatographic column in the decreasing order of their sizes when only the molecular-sieve effect is operative. This has stimulated many investigators[1-6] to find a relationship between the sizes and elution volumes of solute molecules. GRANATH AND KVIST[1] found that elution volumes of organic chain polymers were a linear function of the logarithms of their molecular weights and calculated average molecular weights of unknown samples using this relationship. This method has also been applied satisfactorily to the estimation of molecular weights of proteins or enzymes[2].

More recently, some investigators have attempted to treat the gel chromatographic behavior of solute molecules theoretically and derived many equations on the basis of the hypothetical structures for gel matrix, *viz.*, conical pores[7], rigid rods[8], cylindrical pores[9] and a mixture[10] of cones, cylinders and crevices. Distribution coefficients of some solute molecules were successfully correlated with their sizes when there were no side effects such as adsorption. It has been demonstrated in another paper[11] that the gel chromatographic behavior of a number of oxo anions of phosphorus, under the proper conditions, is based on the molecular-sieve effect, and the contribution of the side effects is almost negligible. Therefore, a theoretical study appears to be applicable to the gel chromatographic behavior of a series of linear phosphates (polyphosphates).

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This paper describes the gel chromatographic behavior of linear phosphates on cross-linked dextran gel columns. The relationship between their distribution coefficients and degrees of polymerization will be briefly discussed.

Abbreviated notations as P_1, P_2, P_3, etc. are used for ortho-, di-, triphosphate, etc. in this paper.

EXPERIMENTAL

A sodium polyphosphate glass with an average degree of polymerization $\bar{p} = 10$ was prepared[12]. Polyphosphate fractions with a $\bar{p}$ of 3–13 were prepared by fractional precipitation[13] from the sodium polyphosphate glass with a $\bar{p}$ of 10. The values of $\bar{p}$ of the polyphosphate fractions were determined by pH titration[14,15] with a Hirama Automatic Titrator. Individual linear phosphates with degrees of polymerization $p = 4$ to 13 were obtained as follows. The sodium polyphosphate glass with $\bar{p} = 10$ was chromatographed on an ion-exchange column in the manner described in a separate paper[16]. The linear phosphates with p up to 13 were clearly separated from one another. A fraction at the maximum concentration of each species was concentrated and desalted to the desired extent by adding dry Sephadex G-10 gel to it. To avoid hydrolysis of the linear phosphates a gel chromatographic run was started within 1 h after they were eluted from the ion-exchange column. The concentrations of sample solutions used for gel chromatography were 3×10^{-3} to 5×10^{-3} gram atom P per l for the individual phosphates and 0.01–0.02 gram atom P per l for the polyphosphate fractions.

The eluents used in this work were 0.1 *M* potassium chloride solutions containing the suitable buffer agents recommended in the preceding paper[11]. A 0.1 *M* potassium chloride–0.01 *M* hydrochloric acid solution (pH 2) was also employed as an eluent.

The Sephadex G-25 columns were identical with those used in the preceding paper[11].

RESULTS AND DISCUSSION

In the preceding paper[11] it was found that the elution curves for the oxo acids of phosphorus at sample concentrations lower than 0.01 g atom P per l are symmetrical and their elution volumes are within the total liquid volume of the column. As shown in Fig. 1, all of the elution curves for the linear phosphates with a p of 1–12 chromato-

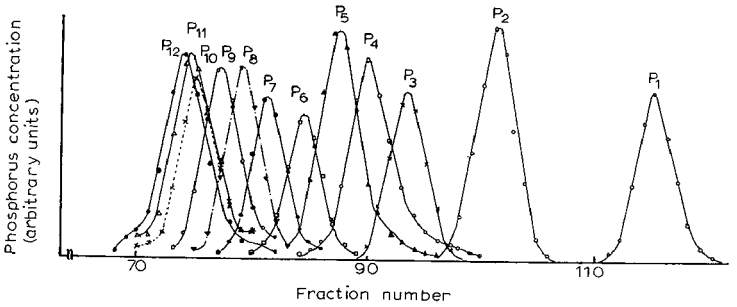

Fig. 1. Elution curves for linear phosphates. Gel, Sephadex G-25; bed volume, 150 ml; eluent 0.1 *M* KCl, pH 7.0. One fraction = 1.08 ml.

TABLE I

K_{av} AND R_s VALUES OF LINEAR PHOSPHATES AT VARIOUS ELUENT pH

Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 *M* KCl.

Sample	*Buffer and pH*							
	0.01 M HCl pH 2.0		*0.005 M phthalate pH 4.6*		*0.0025 M Tris pH 7.0*		*0.005 M ammonium pH 9.2*	
	K_{av}	R_s (Å)	K_{av}	R_s (Å)	K_{av}	R_s (Å)	K_{av}	R_s (Å)
P_1	0.67	4.4	0.67	4.4	0.70	4.2	0.69	4.3
P_2	0.54	5.6	0.54	5.6	0.53	5.7	0.54	5.6
P_3	0.44	6.4	0.44	6.4	0.45	6.4	0.44	6.4
P_4	0.39	6.9	0.40	6.9	0.40	6.9	0.40	6.9
P_5	0.36	7.2	0.35	7.3	0.36	7.2	0.38	7.0
P_6	0.32	7.6	0.33	7.6	0.33	7.6	0.34	7.5
P_7	0.28	8.0	0.29	8.0	0.29	8.0	0.29	8.0
P_8	0.26	8.3	0.26	8.3	0.26	8.3	0.27	8.2
P_9	0.24	8.6	0.24	8.6	0.24	8.6	0.23	8.7
P_{10}	0.21	8.9	0.21	9.0	0.21	9.0	0.21	8.9
P_{11}			0.20	9.2	0.20	9.2	0.19	9.2
P_{12}			0.18	9.4	0.20	9.2		
P_{13}			0.15	9.8				

graphed on a Sephadex G-25 column with an eluent of pH 7 are also symmetrical. The elution curves for some linear phosphates with higher p values have a relatively high background because of hydrolysis of the samples. The decomposition of the samples, however, is not so serious as to affect their elution positions because a greater part of each phosphate remained unchanged.

The K_{av} values of the linear phosphates at pH 2.0, 4.6, 7.0 and 9.2 are shown in Table I, together with their effective sizes R_s estimated according to the method described in the preceding paper[11]. The distribution coefficient K_{av} is defined by eqn. 1,

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \tag{1}$$

where V_e is the elution volume of the solute, V_t the total volume of the gel bed and V_o the void volume outside the gel particles. The R_s values of the linear phosphates increase, as expected, with increase of the degree of polymerization.

It is well known that linear phosphates with a p higher than 5 are hydrolyzed in aqueous solution to produce cyclic trimetaphosphate and smaller linear phosphates. The rate of formation of trimetaphosphate increases with increase of the acidity of solution. Therefore, it could be expected that the linear phosphates in acidic solution would have a configuration close to a six-membered cyclic structure. Deformation of this kind would be reflected in the variation in K_{av} values. As shown in Table I, however, there is no distinguishable variation in the K_{av} values of the linear phosphates in the wide pH range of the solutions.

Many investigators have found linear relationships between elution volumes of solute molecules and logarithms of their molecular weights or degrees of polymerization. As can be seen in Fig. 2, there is a similar relationship between the K_{av} values

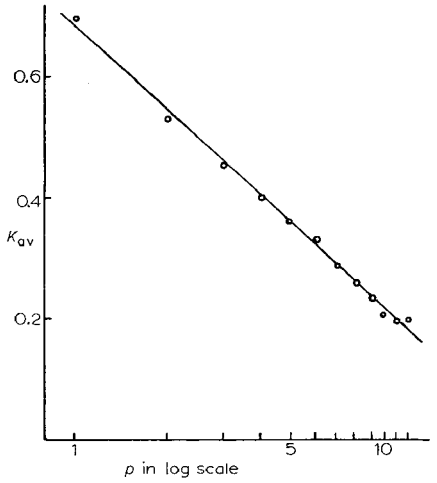

Fig. 2. Relationship between K_{av} and p. Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 M KCl, pH 7.0.

and the logarithms of the p values of the linear phosphates. Although this relationship is quite empirical, it is useful for estimating the molecular weights or distribution of the linear phosphates.

In order to test the applicability of the above relationship the following experiments were carried out. Several polyphosphate fractions, the $\bar{p}$ of which had preliminarily been determined by conventional pH titration, were chromatographed on a Sephadex G-25 column with a 0.1 M potassium chloride solution adjusted to pH 7.0. As an example, the elution curves for polyphosphates with $\bar{p}$ values of 3–13 are

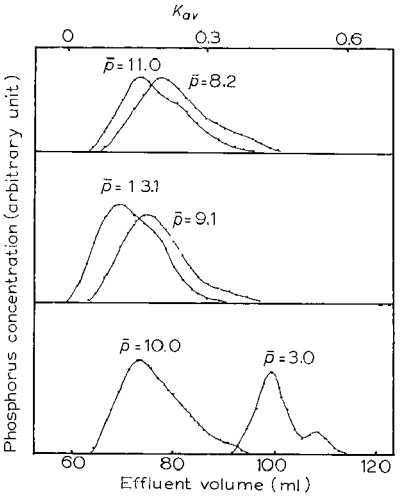

Fig. 3. Elution curves for some polyphosphate fractions. Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 M KCl, pH 7.0.

illustrated in Fig. 3. The $\bar{p}$ of a sample can be calculated from such an elution curve by eqn. 2,

$$\bar{p} = \frac{\Sigma x_i}{\Sigma(x_i/\bar{p}_i)} \tag{2}$$

where x_i denotes the content of phosphorus in the i-th fraction of the effluent and $\bar{p}_i$ is the average degree of polymerization of the linear phosphates in the i-th fraction, which can be determined from the linear relationship in Fig. 2. The $\bar{p}$ values of the

TABLE II

AVERAGE DEGREES OF POLYMERIZATION OF SODIUM POLYPHOSPHATE FRACTIONS DETERMINED BY GEL CHROMATOGRAPHY AND pH TITRATION

Gel, Sephadex G-25; bed volume, 150 ml; eluent, 0.1 *M* KCl (pH 7.0).

Gel chromatography on Sephadex G-25	*pH titration*
3.2	3.0
8.4	8.2
9.3	9.1
9.5	10.1
12.0	11.0
14.0	13.1

polyphosphate fractions calculated in this manner are listed in Table II. Good agreement is observed between the $\bar{p}$ values determined by both methods.

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Notes

CHROM. 4977

Oxygen–nitrogen separation on Cr_2O_3 gel

During studies on reversible adsorption of oxygen and nitrogen on various materials, we discovered the use of black Cr_2O_3 gel as solid adsorbent in the gas chromatographic separation of oxygen and nitrogen. This adsorbent has no advantages over the normally used molecular sieve, but it may be a less expensive alternative. The separation of a 0.1 ml air sample is shown in Table I.

TABLE I

SEPARATION OF AIR ON A Cr_2O_3 GEL COLUMN

Column dimensions: length, 1.50 m; I.D., 4 mm. Carrier gas: He, 30 ml/min. Detector: katharometer, 100°. Sample: 0.1 ml air.

Column temperature (°C)	*Retention times (min)*	
	O_2	N_2
25	1.03	1.96
50	0.91	1.44
75	0.82	1.15
100	0.74	0.95
125	0.68	0.81
150[a]		

[a] No complete separation; oxygen appears as a shoulder.

Experimental

Adsorbent preparation. The Cr_2O_3 gel was prepared by reduction of a solution of CrO_3 in water by ethanol, according to RUTHRUF *et al.*[1], and dried at 150° for 24 h. For column preparation we used the sieved fraction of 420–600 μm. The column was activated by heating at 350° for 4 h in a stream of He.

Instrumentation. A Becker Multigraph, type 409 gas chromatograph, was used with a four-filament katharometer as detector.

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CHROM. 4989

The gas–liquid chromatographic determination of 2-hydroxy-*s*-triazines*

In many biological systems, the 2-chloro-*s*-triazine herbicides give rise to the 2-hydroxy analogs as major metabolites. The latter compounds—which are also the products of photochemical decomposition[1]—are usually determined by TLC or spectrophotometry. Though gas–liquid chromatography has been employed successfully for the analysis of the parent triazines[2–7], their products of hydrolysis have not been amenable to this technique. The only report we found in the literature concerned the formation of TMS derivatives for identification purposes by MONTGOMERY *et al.*[8].

This study describes our search for a quantitative derivatization for several 2-hydroxy-*s*-triazines as well as the purification and analysis of soil and corn samples for Hydroxysimazine as a model compound

Derivatization

Several common silylation reagents were evaluated; of these, BSTFA [bis(trimethylsilyl)trifluoroacetamide)[9] gave the best results. When solvents such as acetonitrile or acetone were used, the reactions gave rise to three peaks. As may be expected, the fastest eluting peaks became more prominent with an increase in the temperature and/or an increase in the time of reaction. However, when BSTFA was used neat and at exactly controlled conditions, a single peak with very small amounts of side products was obtained for the 2-hydroxy analogs of Simazine, Atrazine, and Propazine. The trimethylsilylation was usually performed in a teflon-sealed, closed vial (Corning No. 2826) at 150° for 15 min, similar to a technique used by GEHRKE and co-workers[10]. Note: This reaction should always be carried out behind a safety shield with due caution.

Either these screw-cap vials or sealed capillaries can serve as a reaction vessel. As a sideline, the latter technique has been used to determine rather minute amounts of standards, as follows: A solution containing 2-hydroxy-*s*-triazine—and anthracene as an internal standard—was transferred into a pyrex capillary, the solvent evaporated, BSTFA added, the capillary sealed and kept at 150° for 15 min, then broken open and most of the reaction mixture injected into the gas chromatograph. One of the calibration curves obtained with the hydrogen flame detector is shown in Fig. 1; Hydroxyatrazine and Hydroxypropazine yield similar plots.

The trimethylsilylation of standards in screw-cap vials was conducted as described below in the analysis of a corn sample. Linear calibration curves were obtained again for all hydroxy-*s*-triazines mentioned.

Gas–liquid chromatography

Two columns have performed well with both the 2-chloro-*s*-triazine parent herbicides and the TMS derivatives of their 2-hydroxy analogs: 0.5% neopentylglycol

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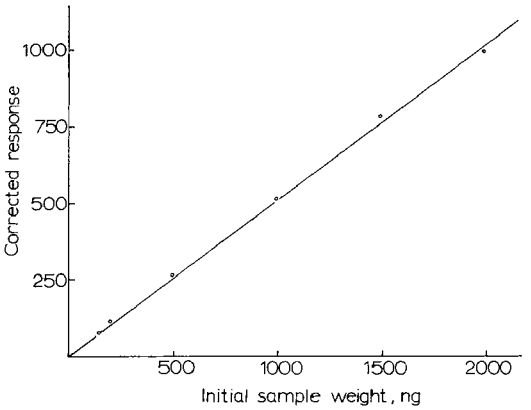

Fig. 1. Standard curve for Hydroxysimazine.

sebacate on 80/100 Chromosorb G-HP and 10% OV-17 on Chromosorb W-HP. Of these, the phenylmethylpolysiloxane OV-17 showed better stability and was used for all following experiments.

Analysis of corn samples

The following procedure uses modifications of the extraction method by PLAISTED AND THORNTON[11] and the purification steps reviewed by KNÜSLI[12]. Stem and leaves of a corn plant were frozen in liquid nitrogen and ground in a Waring blender. The material was spiked with various amounts of Hydroxysimazine, 1 g of each sample weighed into a flask, and 25 ml of methanol added. After ultrasonic stirring for 15 min, the liquid was drawn off with a pipet, the extraction repeated with fresh solvent, and the combined extracts taken to dryness on a rotary evaporator. The residue was redissolved in several (6–8 ml) portions of chloroform by ultrasonic stirring, and introduced into a dry, acidic alumina column (Brockman activity 1, Fisher Scientific Co.). Background material was eluted with more chloroform followed by 10 ml of 50% methanol in chloroform. Finally, the Hydroxysimazine was eluted with 25 ml of methanol and the collected fraction taken to dryness on the rotary evaporator. A column of the same size, 150 × 9 mm I.D., was packed with Silica Gel Grade 923, 100/200 mesh (Fisher Scientific Co.) in methanol, and the methanol eluted from the column with chloroform, thereby changing the appearance of the column from opaque white to colorless translucent. After the elution of the methanol and back-flushing with chloroform to remove residual air bubbles, the residue was introduced in the same manner as described for the alumina column. Some more interfering co-extractants were removed by chloroform and 5 ml of 5% methanol in chloroform; then the Hydroxysimazine was eluted with 10 ml of 25% methanol in chloroform and collected in a culture tube while the solvent was evaporated simultaneously by a stream of nitrogen. After evaporating to dryness, 0.1 ml of BSTFA was added, the tube sealed and placed in a 150° oil bath for 15 min, removed, and allowed to cool. The reaction products were chromatographed at 190° on a 6 ft. × 3 mm I.D. pyrex U-tube filled with 10% OV-17 on 80/100 mesh Chromosorb W-HP, and detected by flame ionization.

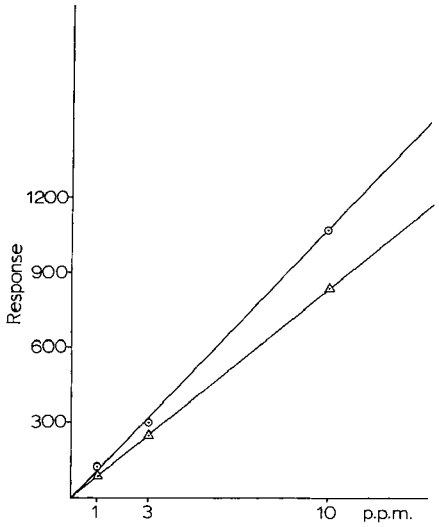

Fig. 2. Comparison of a standard curve of 2-Hydroxyatrazine to the recovery of the standard from corn. ⊙ = standard (single injection), △ = sample (average of three injections).

Analysis of soil samples

Menfro silt loam, a soil of medium organic content, was spiked with 1, 3, and 10 p.p.m. of Hydroxyatrazine. The soil samples were extracted with a mixture of conc. ammonium hydroxide–water–acetonitrile (4:10:86). The extract was taken to dryness on a rotary evaporator and processed as described above.

Results and discussion

The linear calibration curves which had been obtained for standards, promised a quantitative and sensitive method for the GLC determination of 2-hydroxy-*s*-

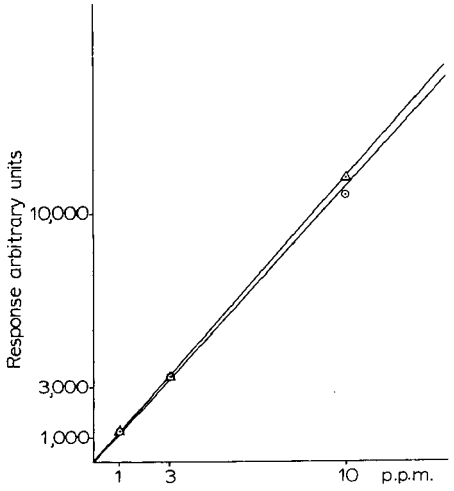

Fig. 3. Comparison of a standard of 2-Hydroxyatrazine to the recovery of the standard from Menfro silt loam. △ = standard (single injection), ⊙ = sample (average of three injections).

triazines as their trimethylsilyl derivatives. The determination of Hydroxyatrazine in spiked corn and soil samples, however, showed a minimum detectable level of not better than 1 p.p.m. due to the interference from co-extractants. Many components in the sample are apparently derivatized by BSTFA and the double column chromatographic cleanup does not remove them completely. Furthermore, the purification procedure is severely limited by the general low solubility of the hydroxytriazines. Fig. 2 shows a comparison of a standard curve to the recovery of the standard from corn and Fig. 3 shows the same for Menfro silt loam. In both figures, the sample data are averages of at least three chromatographic runs.

The 1 p.p.m. minimum detectable limit disqualifies the developed procedures for most residue analysis problems. They can be valuable, though, for certain metabolism studies, for confirmation of results obtained by other methods, and the like. If improved purification methods can be developed for biological extracts, the GLC determination of 2-hydroxy-s-triazines as trimethylsilyl derivatives should prove a significant improvement over existing methods.

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Analysis of indole acid derivatives by gas chromatography using liquid phase OV-101

It has been reported that gas–liquid chromatography (GLC) is the most versatile procedure for the analysis of acidic indoles; however, to be useful the free indole acids must be converted to more volatile derivatives[1–5], such as methyl esters[1–3,5,6], trimethylsilyl derivatives[7] or trifluoroacetyl derivatives[8]. The most commonly used stationary phases for the analysis of indole derivatives are: Versamid 900 (refs. 3, 5 and 7), an ethylenediamine linoleic acid polyamide resin; SE-30 (refs. 1 and 5–8), a dimethylsiloxane polymer; and HI-EFF 8BP (refs. 5 and 7), a polycyclohexanedimethanol succinate. In a comparative study, using the above stationary phases, it has been reported that none of the substrates had a resolution of 95% or higher for ten closely related indole methyl esters[5]. At the 95% level of resolution, Versamid 900 and HI-EFF 8BP resolved five and SE-30 four indole methyl ester pairs. Therefore, in many biological studies two stationary phases have to be used for the analysis of indole compounds. The objective of the present report is to demonstrate that one stationary phase (OV-101), and two derivatives (methyl esters and trimethylsilyl derivatives) will resolve nine of ten closely related acidic indoles.

The GLC work was carried out on a F & M Model 402 gas chromatograph equipped with a flame ionization detector. The column temperature was 200° and the flash heater and flame detector temperatures were kept at 250°. A 1.80-m U-shaped glass column with a 6-mm I.D. was used. The column was packed with Anakrom ABS 80/90 mesh (Analabs, Hamden, Conn., U.S.A.) coated with 5% OV-101, a liquid dimethylsiloxane polymer (Supleco, Bellefonte, Pa., U.S.A.). The carrier gas was helium at a flow rate of 75 ml/min.

The indole methyl esters were formed as described previously[5]. The trimethylsilylated (TMS) indole derivatives were formed via two procedures. About 5 mg of the desired indole acid were dissolved in 0.4 ml acetonitrile and 0.2 ml bis(trimethylsilyl)acetamide (BSA) were added under anhydrous conditions and kept at 70° as described by KLEBE *et al.*[9]. Samples were removed at various times and injected directly into the gas chromatograph. In the second method, the TMS derivatives were formed by adding 0.2 ml of bis(trimethylsilyl)trifluoroacetamide (BSTFA) instead of BSA. Although the action of BSTFA as a silylating agent is similar to BSA, BSTFA is more volatile than BSA and is usually eluted from the GC column near the solvent front. Both trimethylsilylating agents produce ethers from alcohols, esters from acids and displace nitrogen-bound protons[9].

Our first aim was to study the silyl donor strength of BSA and BSTFA. Fig. 1 is a representative example using indole-3-butyric acid, which is one of the more difficult indole acids to silylate completely. Silylation of the carboxyl group was accomplished in less than 1 min; however, silylation of the indole nitrogen atom was more difficult. Indole acids with shorter aliphatic carboxylic acid groups were not as difficult to silylate with BSA, except for indole-2-carboxylic acid, which was not completely silylated even after 120 min. However, BSTFA completely silylated both indole-3-butyric and indole-2-carboxylic acid within 10 min. All other tested indole acids were completely silylated in less time.

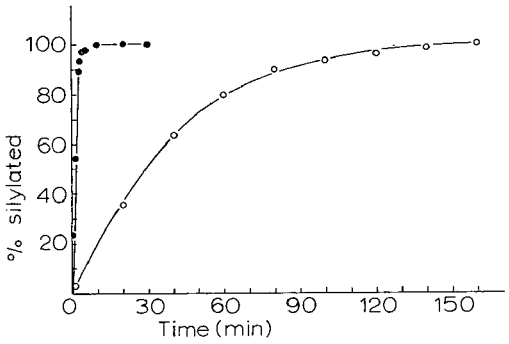

Fig. 1. Silyl donor strength of bis(trimethylsilyl)acetamide (BSA) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) using indole-3-butyric acid as substrate. Reaction mixture: 5 mg indole-3-butyric acid dissolved in 0.4 ml acetonitrile and either 0.2 ml BSA or 0.2 ml BSTFA at 70° under anhydrous conditions. Reaction with BSA (○—○) and BSTFA (●—●).

The GLC results for TMS and methyl ester derivatives of ten closely related indole acids, most likely found in biological material, are presented in Table I. All relative retention values (r) are given with respect to ethylindole-3-acetate, and their effective plate values (N) are calculated according to ETTRE[10]. The order of elution of the TMS and methyl ester derivatives was not the same. The total elution time of the TMS derivatives was about twice as long as that of the methyl esters, *viz.* 30 and 18 min, respectively. The effective plate values (N) on 5% OV-101 were about the

TABLE I

COMPARISON OF RELATIVE RETENTION (r), EFFECTIVE PLATE VALUE (N), AND RESOLUTION (R OF VARIOUS INDOLE TMS DERIVATIVES AND INDOLE METHYL ESTERS ON COLUMN SUBSTRATE OV-101

Column characteristics: column temperature, 200°; detector temperature, 250°; carrier gas, helium; column, 5% OV-101 on Anakrom ABS 80/90 mesh (for details see text); retention time ethylindole-3-acetate, 4.8 min.

Indole, TMS derivative	r	N	R	*Indole, methyl ester*	r	N	R
Indole-2-carboxylate	0.72	935		Indole-2-carboxylate	0.42	318	
			1.91				3.92
Indole-1-propionate	1.09	848		Indole-3-acetate	0.80	1063	
			2.30				0.18
Indole-3-acetate	1.49	905		Indole-5-carboxylate	0.82	653	
			1.69				0.52
Indole-3-carboxylate	1.85	1095		Indole-3-carboxylate	0.88	1290	
			1.98				0.30
Indole-3-propionate	2.32	1272		Indole-1-propionate	0.91	1373	
			0.20				1.87
Indole-5-carboxylate	2.37	1278		Indole-3-propionate	1.12	1110	
			3.92				3.28
Indole-3-butyrate	3.52	1765		Indole-3-butyrate	1.59	1745	
			0.61				0.38
Indole-3-lactate	3.73	1214		Indole-3-lactate	1.65	1628	
			2.19				4.73
Indole-3-glyoxylate	4.59	2490		Indole-3-glyoxylate	2.61	1834	
			3.79				1.42
Indole-3-acrylate	6.31	2190		Indole-3-acrylate	2.97	1695	

same for both derivatives; however, these N values were greater than those reported using Versamid 900 and HI-EFF 8BP as stationary phases[5].

For completeness of separation, or resolution (R), peak width at the base must be taken into account. The R values were calculated according to ETTRE[10]. Two adjacent peaks are 95% resolved when $R = 1$, and 99% resolved when $R = 1.5$. Only two pairs of the ten TMS derivatives were not resolved at the 99% level. Indole-3-propionate was not resolved from indole-5-carboxylate, and indole-3-butyrate was not resolved from indole-3-lactate. All other combinations could be resolved at the 99% level. Resolution of the methyl esters was not as good, only four adjacent indole pairs could be resolved at the 99% level; however, methylindole-3-propionate could be resolved from methylindole-5-carboxylate.

In summary, using TMS and methyl ester derivatives and the stationary phase OV-101, all indole acids could be resolved at the 99% level except indole-3-butyrate from indole-3-lactate. This makes stationary phase OV-101 superior to either Versamid 900, HI-EFF 8BP or SE-30 (ref. 5). It must be pointed out that the unresolved indole-3-butyrate indole-3-lactate pair can be resolved at the 99% level on either 3% HI-EFF 8PB or 3.5% Versamid 900 (ref. 5). It was found that BSTFA is more effective as a silyl donor than BSA and is, therefore, recommended for the formation of indole acid TMS derivatives.

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Increased resolution of polymers through longitudinal compression of agarose gel columns

EDWARDS AND HELFT[1] recently reported to have improved resolution through compressed beds of cross-linked dextran. Concurrently, we were measuring the effects of compression on a much more easily deformable gel, agarose, as a prelude to other investigations involving the molecular weight distributions of water soluble polymers. Since our results differed somewhat from those of EDWARDS AND HELFT, it was decided to make our findings the subject of this note; and, in addition, to introduce an alternate concept to resolution, namely, unit resolution.

Materials and methods

The components of the chromatographic system consisted of a 5-gallon polyethylene carboy (Nalge)*, a sample-injection valve (Chromatronix Model R60SV), a column fitted with one flow adapter (Sephadex type K25/45, I.D. 25 mm), a peristaltic pump (Buchler Instruments, type polystatic), and a differential refractive index monitor (Waters Model R-4 equipped with microcell). These components were connected in series with small bore (teflon or tygon tubing), approximately 0.031 in. I.D. between injection valve and detector. Chromatronix fittings joined separate pieces of tubing where necessary. The output of the refractive index monitor was fed into a potentiometric recorder (Leeds and Northrup Model W Speedomax).

Polyethylene glycol (PEG) 20,000 and 6,000 (Union Carbide) were mixed. The numbers following the name give the number average molecular weight specified by the manufacturer. A certain fraction of the PEG 20,000 (approximately 14% of the area under the refractive index against time curve) eluted at the void volume (V_0), while the remainder together with the PEG 6,000 polymer eluted at the total volume (V_T).

The column packing material was agarose gel (Sepharose 6B, lot No. 1929, purchased from Pharmacia Fine Chemicals, Inc.) which, according to specifications, has a molecular weight exclusion limit of 1×10^6 based on the elution of polysaccharides.

According to a procedure suggested by the manufacturer[2], the column was packed using a final head pressure of 30 cm of water. The column packing was permitted to stabilize by passing two column volumes of water through it and then lowering the flow adapter into place. The freely-settled height of the gel was 42.3 cm. The pump was set to deliver eluent in the range of 21.0–23.1 ml/h.

In each experiment, a 0.5 ml sample containing 2.5 mg of each polymer was dissolved in and eluted with singly-distilled water.

The gel remained stable for 166 h, during which time several samples were percolated through the gel. From this time until the end of the experiment, the gel appeared to compress in a stepwise manner after the sample had completely eluted through the column. Beyond 26% compression of the gel bed, the flow rate at the same pump setting began to decrease rapidly, so the experiments were discontinued.

* Mention of commercial items is for your convenience and does not imply endorsement by the U.S. Department of Agriculture over others of a similar nature.

Results

Chromatographers commonly use the parameters resolution (R_S) and height equivalent to a theoretical plate (H) to describe separating power and peak broadening in chromatographic columns.

Eqns. 1 and 2 define these parameters in terms of measurable quantities[3].

$$R_S = \Delta Z / 4\sigma_{AV} \quad (1)$$

$$H = \sigma^2 / L \quad (2)$$

where

σ is the quarter width of an elution peak
σ_{AV} is the arithmetic mean for σ's of two peaks
ΔZ is the distance between the means of two elution peaks
L is the column length.

The results of Table I indicate that a decrease in column length by compression is not equivalent to making a series of shorter columns by adding smaller amounts of the same gel under a constant packing procedure. These experiments altered the ratio of accessible internal volume to void volume for the gel. Hence, H is no longer a proportionality constant between σ^2 and L. Nevertheless, R_S as defined by eqn. 1 remains virtually constant, despite compression of the gel. Since a constant flow rate was maintained, even while compressing the gel, an appreciable saving of time was

TABLE I

PARAMETERS OF AN AGAROSE COLUMN AS A FUNCTION OF GEL COMPRESSION

L (cm)	V_0 (ml)	V_T (ml)	H_{AV} (mm)	$4\sigma_{AV}$ (ml)	t (h)[a]	ΔZ (ml)	R_S	L_R (cm)
38.8	51.3	149.6	0.718	17.7	7.82	98.3	5.55	6.99
38.8	51.3	152.0	0.870	20.0	7.95	100.7	5.04	7.70
37.0	42.9	139.2	0.803	17.5	7.55	96.3	5.50	6.78
34.9	34.1	128.4	0.949	16.5	6.88	94.3	5.72	6.10
31.2	23.3	112.2	1.37	16.6	6.71	88.9	5.36	5.82

[a] Measured from time of sample injection until trailing edge of last peak reaches baseline.

obtained as indicated in Table I. The values reported in the table are based on the commonly-accepted definition of R_S (ref. 4), whereas those in ref. 1 are based on the quarter width. In the course of these experiments, the column decreased in length from 38.8 to 31.2 cm. It appears we have effected this economy in time without causing significant diminution of the molecular weight range over which the gel separates. This is inferred from the relatively small change in σ_{AV} which we have obtained, but confirmation is needed by a separation of compounds within the complete operating range of the gel.

As an aid in comparing the gel compression experiments, the parameter unit resolution (L_R) may be used.

$$L_R = L / R_S \quad (3)$$

Table I shows that L_R decreases with gel compression, which indicates a more efficient separation. In addition, one can use L_R to calculate the minimum length of column required to separate two peaks, using data from an experiment in which incomplete or too great a separation occurs.

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CHROM. 5000

Thin-layer chromatography of adenine and its metabolites

When large quantities of adenine are administered to various mammalian species, a portion of it is oxidized by xanthine oxidase to 2,8-dihydroxyadenine (DHA) which is extensively deposited in the kidney[1,2]. 2-Hydroxyadenine (2HA) and 8-hydroxyadenine (8HA) have been shown to be the minor and major intermediate, respectively, in this metabolic pathway[3]. In this communication, a thin-layer chromatographic (TLC) procedure for their separation and identification is described.

Materials and methods

Adenine (Kay Fries) and DHA (Aldrich) were commercial products. Adenine was further purified by recrystallization from water. 2HA (isoguanine) was prepared from isoguanine sulfate (Sigma). 8HA was synthesized by the reaction of phosgene on 4,5,6-triamino pyrimidine[4]. Solutions of approximately 1 mg/ml were prepared. DHA and 2HA were dissolved in boiling mixture of morpholine–water (50:50) while adenine and 8HA were dissolved in dimethylsulfoxide. Solutions were prepared fresh prior to use. Samples were applied with 3 μl capillaries* (Microcaps, Drummond Scientific Co., Broomall, Pa.) and solvent evaporated by using a hot air gun.

TLC was carried out on precoated Avicel, microcrystalline Cellulose powder F glass plates with fluorescent indicator (Merck, Darmstadt, G.F.R.) using the following solvent systems: (1) *n*-butanol–methanol–water–conc. ammonia (60:20:20:1); (2) isopropanol–water–conc. ammonia (70:30:1); (3) *n*-butanol–morpholine–diethylene glycol (MCB)–water (45:15:10:30). All solvents used were reagent grade. Spots were located using a short wave UV lamp.

* Use of microsyringes with metallic parts should be avoided as additional spots are observed due to degradation.

TABLE I

R_F VALUES OF ADENINE AND ITS METABOLITES

Substance	*R_F values in solvent system*		
	S1	*S2*	*S3*
Adenine	0.39	0.53	0.54
DHA	0.06	0.18	0.24
2HA	0.13	0.27	0.36
8HA	0.29	0.44	0.47

Results

Adenine and its metabolites have been separated in all three solvent systems used. R_F values reported are average of triplicate determinations (Table I). Applications of this method in determining the stability of these compounds in alkaline medium and their identification and determination in biological materials is in progress.

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Recovery of lipids from thin-layer chromatography for radioassay

A popular method currently in use for recovery of lipids from thin-layer chromatography (TLC) plates involves the suspension of the silica gel scraped from the TLC plate in a scintillation solution containing a dioxane–water system[1]. However, the efficiency of this system for ^{14}C is only about 70–75% (refs. 1 and 2). The radioassay of lipids in a more conventional counting system containing 1 ml of added methanol is 96 ± 2% for neutral lipids and 78 ± 2% for phospholipids[3]. We have investigated this problem in the interest of finding a system which will give quantitative recovery of both neutral and phospholipids. Our observations with several different elution and counting systems are the basis of this report.

The radioactive compounds used, with one exception, were obtained from commercial sources (New England Nuclear Corp., Boston, Mass., U.S.A. and Applied Science Labs., Inc., State College, Pa., U.S.A.) and were chromatographically purified before use. [U-^{14}C]Sphingomyelin was prepared by the separation of phospholipids obtained from the liver and spleen of two rats injected with [3-^{14}C]serine and [1-^{14}C]-acetate. The sphingomyelin was separated by the TLC method of SKIPSKI *et al.*[4].

Liquid scintillation solvents which are capable of dissolving relatively large amounts of water have recently become available. We tested one of these (Aquasol, New England Nuclear Corp.) as our primary scintillator and solvent and compared the results with those obtained by elution of the lipids from silica gel and by suspension of the silica gel in the solvent mixture of SNYDER AND SMITH[2].

Neutral lipids are readily recoverable in several systems. Plates were developed in hexane–ether–acetic acid (90:10:1), and the unsaturated lipids visualized by exposure to iodine vapor[5]. In the case of saturated lipids two parallel spots were developed and one was identified by spraying with sulfuric acid. After sublimation of the iodine, the silica gel was either suspended directly in Aquasol or the solvent of SNYDER AND SMITH or the neutral lipid was eluted with three successive 5-ml portions of chloroform–methanol (3:2). The results are summarized in Table I. In all experiments the results were within 2–3% of the average value.

It was ascertained that up to 300 mg of silica gel could be added to Aquasol before the counting efficiency of a solution of [4-^{14}C]cholesterol was impaired.

TABLE I

RECOVERY OF NEUTRAL LIPIDS FROM TLC FOR RADIOASSAY

Eluent: chloroform–methanol (3:2) (3 × 5 ml).

Compound	*% Recovery*[a,b]		
	Aquasol	*Elution*	*Ref. 2*
[1-^{14}C]Cholesteryl stearate	98	97	90
[1-^{14}C]Tripalmitin	98	94	92
[16-^{14}C]Palmitic acid	95	90	84
[4-^{14}C]Cholesterol	100	100	93

[a] Average of three to five experiments.
[b] Similar results have been obtained with ^{3}H-labeled lipids.

The phospholipids presented the real problem since recovery of radioactivity by direct counting has been generally poor (70–80%). We found that phosphatidylcholine (PC) gave the poorest recovery in Aquasol and elution with the solvents used in developing the plates was little better. However, an elution system containing ammonia gave close to quantitative recovery of PC. Thus, we now have methods in our hands which will permit quantitative recovery of phospholipids for radioassay. The results with phospholipids are given in Table II. The plates were developed in

TABLE II

RECOVERY OF PHOSPHOLIPIDS FROM TLC FOR RADIOASSAY

Eluents: (A) chloroform–methanol–acetic acid–water (55:45:4:2); (B) chloroform–methanol–14 *M* ammonia (56:42:2).

Compound	*% Recovery*[a]			
	Aquasol	*Ref. 2*	*Elution A*	*Elution B*
[U-^{14}C]Phosphatidylglycerol	97	66	81	75
[U-^{14}C]Phosphatidylinositol	100	78	72	85
[U-^{14}C]Phosphatidylethanolamine	99	76	60	51
[U-^{14}C]Spingomyelin	98	—	90	85
[U-^{14}C]Phosphatidylcholine	70	83	74	96[b]

[a] Average of four to six experiments.
[b] Cumulative result of four elutions.

chloroform–methanol–7 *M* ammonia (230:90:15), and spots were visualized by exposure to iodine vapor or sulfuric acid spray. All results were within 3–5% of the average values.

The elution of PC from silica gel required at least three extractions with chloroform–methanol–14 *M* ammonia, (56:42:2). The percentages of counts eluted were 52, 38, and 6, respectively, in three successive extractions, so that even two elutions will give about 90% recovery of radioactivity.

Our results indicate that combination of a multipurpose scintillator, such as Aquasol, and elution can give complete radiorecovery of all classes of lipids from TLC plates.

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CHROM. 4931

Argentation thin-layer chromatography with silver oxide*

II. Amines, unsaturated and aromatic carboxylic acids

Recently a method of argentation thin-layer chromatography (TLC), with silver oxide on silica gel was shown to separate pyridine and some of its homologs[1]. For isomeric compounds, the order of the R_F values was shown to be the same as that of the pK_a values, the dipole moments, or the stability constants of known silver ion complexes of the corresponding pyridine homologs.

As argentation TLC using silver nitrate has been used for the separation of fatty acids, lipids, steroids, etc.[2], we tried to develop the method with Ag_2O for several other classes of compounds. As it has the advantage of allowing the use of a more polar solvent than chromatography with $AgNO_3$, it was thought that compounds such as amines and acids might be separated, and probably many others, as well as the compounds known to be separated in other forms of argentation chromatography.

Preparation of the plates

The Ag_2O plates were prepared as previously described[1]. Ag_2O/Silica Gel GF_{254} (Merck) plates were also prepared in the same way as some compounds can easily be identified by UV absorption on a fluorescent background, even with Ag_2O plates. In this work, only activated plates were used. The activation time was reduced to 45 min, giving better plates.

TABLE I

$R_F \times$ 100 VALUES OF SUBSTITUTED BENZOIC ACIDS, AND PHTHALIC, MALEIC AND FUMARIC ACIDS

Solvent I: ethanol–ethyl acetate–conc. NH_4OH (9:3:2); solvent II ethanol–water–conc. NH_4OH (10:1.2:1.6). The solvents were selected by preliminary scanning. The detection of the spots was by UV.

Substituent	*Solvent I*		*Solvent II*	
	Silica Gel $GF_{254} + Ag_2O$	*Silica Gel* GF_{254}	*Silica Gel* $GF_{254} + Ag_2O$	*Silica Gel* GF_{254}
2,6-di-OH	90	92	92	94
3,5-di-NO_2	88	91	91	92
2-OH	82	88	86	90
H	70	77	73	81
2,4-di-OH	65	83	69	85
3-OH	59	78	68	82
4-OH	50	80	64	84
Fumaric acid	26	41	38	61
Phthalic acid	20	30	29	46
Maleic acid	14	22	19	33

* This research was supported by a grant from the Fundação de Amparo à Pesquisa do Estado de São Paulo, and A. E. MAURO had a studentship from the same Foundation.

Carboxylic acids

We investigated some hydroxybenzoic acids, and phthalic, maleic and fumaric acids to test the method. The R_F values, solvents, and methods of detection are given in Table I.

The order of R_F values on the Ag_2O plates is 2,6-di-OH > 3,5-di-NO_2 > 2-OH > benzoic > 2,4-di-OH > 3-OH > 4-OH in both solvents (the numbers indicating substituents in the benzoic acid) and the separations are good. This is the same order as the acidity of the compounds and the inverse order of the pK_a values[5–7] and this order is maintained even if we include the dicarboxylic acids, if the pK_a value for the second carboxyl group[6] is used, giving: 4-OH > fumaric > phthalic > maleic, for the R_F values, and the inverse for the pK_a values.

It seems that the more dissociated the carboxylic acid is in our solvents (the carboxylate ion is stabilized by resonance or hydrogen bonding or both[3]) the further it is carried on the Ag_2O plate.

The order of dipole moments[4] (4-hydroxy > 2-hydroxy > 3-hydroxy; fumaric acid > 3-hydroxy > maleic acid > phthalic acid) is different from the order of the R_F values, but would explain the "anomalous" R_F of 2,4-dihydroxybenzoic acid. Considering the pK_a values of 2-hydroxy-, 4-hydroxybenzoic and benzoic acids, the difference in pK_a values, in pK_a units, is: 2-OH − H = −1.17 4-OH − H = +0.36.

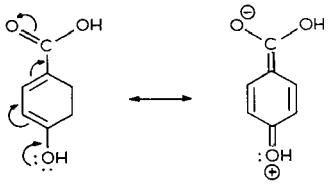
TABLE II

pK_a VALUES OF SUBSTITUTED BENZOIC ACIDS, AND PHTHALIC, MALEIC, AND FUMARIC ACIDS

Substituent	*pK_a*
2,6-di-OH	1.30[a]
3,5-di-NO_2	2.7–2.9[b]
2-OH	3.00[a]
H	4.17[a]
3-OH	4.08[c]
4-OH	4.53[c]
Fumaric acid (pK_2)	4.38[d]
Phthalic acid (pK_2)	5.408[d]
Maleic acid (pK_2)	6.23[d]

[a] Ref. 3. 2,4-Dihydroxybenzoic acid should have a pK_a value between 2-hydroxybenzoic and benzoic acids.
[b] Calculated from the known effect of NO_2 as substituent.
[c] Ref. 6.
[d] Ref. 7.

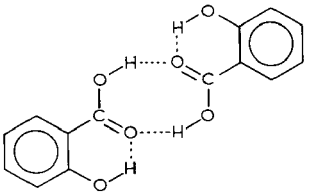
If there is additivity of the pK_a values, which is a reasonable hypothesis, the pK_a of 2,4-dihydroxybenzoic acid, having the contributions of the 2- and 4-hydroxy, should be approximately 4.17 − 1.17 + 0.36 = 3.36, *i.e.*, between 2-hydroxybenzoic acid and benzoic acid itself. That the R_F is between that of benzoic acid and 3-hydroxybenzoic acid can be understood if we consider the polarity of the compounds. The 4-hydroxy group, in the benzoic acid group, is an electron donor as the pK_a of 4-hydroxybenzoic acid clearly shows, and could be expected from resonance form (I) that opposes dissociation of the benzoic acid. The direction of electron movement



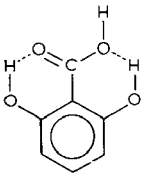
I

determines an increase in the dipole moment of the compound, and the increase in polarity has a lowering effect on the R_F value.

It should be noted that the dipole moments of the mono-hydroxybenzoic acids are in the order expected from the direction of the dipole moments (electronic effects) of the substituent groups. The order of the pK_a values is different because of hydrogen bonding, polar effects of substituents, and association[3] giving structures like II and III for 2-hydroxybenzoic acid and 2,6-dihydroxybenzoic acid and increasing the acidity of both to a value higher than the non-hydrogen-bonding forms would have.



II



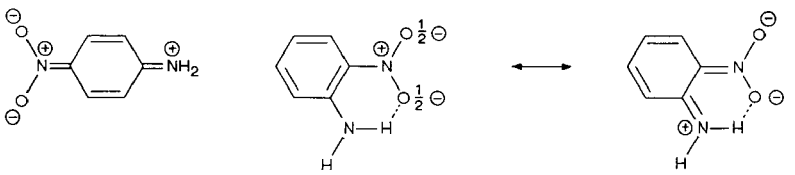
III

The inverse order of pK_a values shows that complexing has a major role in the mechanism of the chromatography of the investigated compounds.

Amines

We studied the chromatography of toluidines, chloroanilines and nitroanilines, comparing the *o*-, *m*-, and *p*-isomers in each case. This gives us a good understanding of substituent effect, as compared with aniline. In Table III the R_F values, solvents and method of identification are given.

The analysis of the R_F values on Ag_2O on Silica Gel GF_{254} plates shows clearly that, as in the case of the pyridines[1] and carboxylic acids, complexation (R_F values



IV V a V b

in the inverse order of pK_a values) is always present but, as the effect of substituents on polarity is quite different for Me (+I effect), Cl(−I effect) and NO_2 (−I, −M) and the steric effect of the substituents in the *o*-position is also important, there is always

TABLE III

$R_F \times 100$ OF SUBSTITUTED ANILINES

Solvent I: petroleum ether–ethyl acetate (1:1); solvent II: petroleum ether–ethyl acetate–benzene (6:3:1). Detection was done under a UV lamp or with ninhydrin reagent. If the chromatogram on Ag_2O was dried after development and left on the table, without further treatment, dark spots developed slowly for the toluidines and chloroanilines. The nitroanilines, if in a high concentration, could be seen as yellow spots.

Substituent	*Solvent I*		*Solvent II*	
	Silica Gel $GF_{254} + Ag_2O$	*Silica Gel* GF_{254}	*Silica Gel* $GF_{254} + Ag_2O$	*Silica Gel* GF_{254}
o-Cl	93	90	79	82
o-NO_2	85	81	68	65
o-CH_3	73	79	52	65
m-Cl	79	83	65	68
m-NO_2	69	65	50	48
m-CH_3	62	76	41	59
p-Cl	60	68	35	50
p-NO_2	56	59	32	37
p-CH_3	52	71	30	52
H	61	75	38	58

competition between the pK_a value and the dipole moment μ, the more polar compounds tending to have a lower R_F value.

Taking both these effects into account the order of the R_F values can easily be rationalized, and used as a measure of the extent of substituent effects.

The order of R_F values is: *o*-Cl > *o*-NO_2 > *m*-Cl > *o*-CH_3 > *m*-NO_2 > *m*-CH_3 > H > *p*-Cl > *p*-NO_2 > *p*-CH_3. It is interesting that for all 3 series, we had the R_F values in the order $o > m > p$. For the toluidines and chloroanilines, this is the inverse order of the pK_a values, but is not so for the nitroanilines, for which the pK_a values are in the order $m > p > o$[6,7], because of the resonance and steric effects in the p and o

TABLE IV

pK_a* VALUES AND DIPOLE MOMENTS (μ) OF SUBSTITUTED ANILINES

The pK_a values were taken from ref. 6, pp. 593–594 except for aniline, which was taken from ref. 7. The dipole moments are from ref. 6, p. 506; aniline and nitroanilines were taken from ref. 4; to have comparable results for each series, data was taken from the same author, wherever possible. References to the original articles are given in the above books.

Substituent	pK_a	μ
o-CH_3	4.42	1.58
m-CH_3	4.73	1.44
p-CH_3	5.08	1.31
o-Cl	2.62	1.77
m-Cl	3.32	2.66
p-Cl	3.81	2.97
o-NO_2	−0.28	4.45
m-NO_2	2.45	4.72
p-NO_2	0.98	7.10
H	4.58	1.52

positions, respectively, giving rise to the structures IV[8] and V. For the chloroanilines and nitroanilines, the R_F values are in the inverse order of the dipole moments[4] which gives a reasonable explanation, the more polar isomer having a lower R_F. Thus the order for the toluidines can be explained by the inverse pK_a order, *i.e.*, the isomer having more complexing power having a lower R_F.

The order of dipole moments for chloro- and nitroanilines is p-$NO_2 > m$-$NO_2 > o$-$NO_2 > p$-Cl $> m$-Cl $> o$-Cl; if we take the R_F values of each pair of anilines substituted by Cl and NO_2 in the same position, we have, as expected from the polarities: o-Cl $> o$-NO_2, m-Cl $> m$-NO_2, p-Cl $> p$-NO_2.

From the discussion above it is also easy to deduce that for each position, *o*, *m*, and *p*, we will always have $-Cl > -NO_2 > -CH_3$ for the R_F values. The fact that *m*-chloroaniline has a higher R_F than *o*-toluidine is only a consequence of the R_F values of the chloroanilines obeying the inverse order of polarities, while the R_F values of the toluidines obey the inverse order of the pK_a values. Aniline itself can be included in the toluidine series, with a pK_a higher than *o*-toluidine (steric effect) and lower than *m*- and *p*-toliudine (inductive effect).

What is more difficult to understand is that in the series of Cl and NO_2 substituted compounds, which seem to obey an inverse polarity law, when the substituent is at the same position, the R_F values of the *o*- and *m*-nitroanilines are higher than that of *m*- and *p*-chloroanilines, respectively. For *o*-nitroaniline it could be explained that the pK_a, which is much lower than all the others, is the reason for the compound being in the second place according to R_F value, because of its high polarity. For *m*-nitroaniline, where the resonance effect is supposed to affect the pK and polarity only to a small extent and the ratio of pK_a/μ is not so small, the explanation is not so good.

There are some small differences, especially in the position of the toluidines, on the pure silica gel plates, but in each series the relative positions are maintained, showing that, apart from the complexing, some of the mechanisms are the same in both cases.

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CHROM. 4930

Thin-layer chromatography of DANSYL-oestrogens

In a previous communication[1], we described the use of derivative formation with 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-chloride) as a means of determining oestriol in pregnancy urine. On viewing the fluorescent spots on a thin-layer plate, following chromatography of the DANSYL-oestrogens, one can observe any other oestrogen excreted in a significant amount, and we therefore considered it of value to record the behaviour of DANSYL-oestrogens on chromatography.

Table I shows the R_F values of 18 naturally-occurring oestrogens chromatographed both in the free state and as their DANSYL-derivatives.

TABLE I

R_F VALUES OF OESTROGENS CHROMATOGRAPHED ON KIESELGEL G PLATES, BOTH IN THE FREE STATE AND AS THEIR DANSYL-DERIVATIVES

The solvent was ethanol–chloroform (5:95).

Oestrogen	*R_F values*	
	Free compound	*DANSYL-derivative*
Oestrone	0.74	0.97
16-Keto-oestrone	0.59	0.12[a]
2-Methoxyoestradiol	0.53	0.79
16α-Hydroxyoestrone	0.46	0.83
16-Keto-oestradiol	0.46	0.83
2-Hydroxyoestrone	0.42	0.97
Oestradiol	0.38	0.83
11β-Hydroxyoestrone	0.29	0.80
2-Hydroxyoestradiol	0.25	0.83
17-Epioestriol	0.24	0.33
16-Epioestriol	0.24	0.32
2-Methoxyoestriol	0.15	0.25
6α-Hydroxyoestradiol	0.15	0.57
Oestriol	0.09	0.20
16,17-Epioestriol	0.09	0.21
6-Keto-oestriol	0.06	0.16
2-Hydroxyoestriol	0.045	0.25
6α-Hydroxyoestriol	0.03	0.14

[a] This is probably degraded. See comment in text.

In general, the only effect of the derivative formation is to raise the R_F value. An even higher elevation of R_F value is seen in the case of 2-hydroxy-oestrogens, which is presumably due to DANSYL-substitution on the phenolic 2-OH group as well as on the 3-OH. Thus, the retardation caused by the introduction of an extra hydroxyl group, seen among the free steroids, is not observed in the case of DANSYL-2-hydroxy-oestrogens.

Anomalous behaviour is also observed in the DANSYL-derivative of 16-keto-oestrone. This derivative would be expected to appear on the chromatogram with an R_F value slightly less than that of oestrone. Even if the derivative were to exist in the enolic form following the alkaline treatment, it should still have a moderately

high R_F value. Since, however, a spot is obtained with an R_F value of 0.12, it seems most probable that ring D undergoes fission under the conditions required for DANSYL-ation. It may, perhaps, be worth mentioning that 16-keto-oestrone, in common with 16-keto-oestradiol, may be chromatographed as DANSYL-16-epioestriol after reduction with borohydride.

In assessing the presence of unusual metabolites in pregnancy urine, it should be borne in mind that the DANSYL-ation reaction is not specific for oestrogens, but will occur with any phenolic OH group. This is not usually a serious drawback, and in more than 1000 oestriol determinations we have only twice been puzzled by the appearance of an unusual spot, with an R_F value similar to 16-epioestriol, in two patients receiving anticonvulsant drug therapy (diphenylhydantoin and luminal). That this spot was due to the drugs and not to the pregnancy was demonstrated when the same spot was obtained from a non-pregnant woman receiving similar treatment.

In pregnancy urine there is, however, a spot with an R_F value approximately half that of oestriol, that occurs to a minor extent in all pregnancies, but may reach high levels in some pathological conditions.

As we have mentioned previously[1], DANSYL-ation of oestrogens is applicable to pregnancy urines where oestrogen excretion is in excess of 1 mg per day. In non-pregnancy urines, the level of other phenolic material is comparable with, or in excess of, the amount of oestrogens, thus rendering differentiation difficult.

We wish to thank Dr. M. HARNIK, of Ikapharm, Ltd., for providing most of the oestrogens used in this study. We are grateful to Dr. J. FISHMAN, of the Institute for Steroid Research, Montefiore Hospital, New York, for giving us the 2-hydroxy oestrogens, and to Dr. H. L. DRYDEN, Jr., of G. D. Searle and Co., Chicago, for a generous gift of 11β-hydroxyoestrone.

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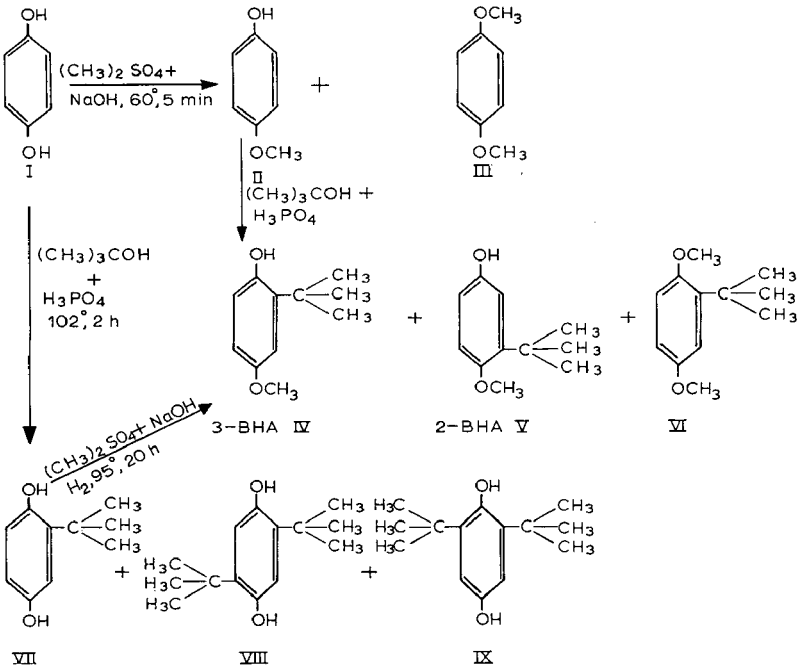
Studies on the synthesis of butylated hydroxyanisole

Butylated hydroxyanisole (BHA) is one of the best antioxidants employed for extending the shelf-life of fats and fatty foodstuffs. BHA[1] has better carry-through properties than other known preservatives like butylated hydroxy toluene and alkyl gallates, etc. BHA is either used alone or synergised with alkyl gallates or citric acid.

The two industrial processes employed for the preparation of BHA are: (a) methylation of hydroquinone (I) after the method of ROBINSON AND SMITH[2], when both *p*-hydroxyanisole (II) and 1,4-dimethoxybenzene (III) are formed. The reaction mass on treatment with *tert.*-butyl alcohol, using phosphoric acid as a condensing agent, gives a mixture of 3-*tert.*-butylhydroxyanisole (IV) and 2-*tert.*-butylhydroxyanisole (V) and 1,4-dimethoxy-*tert.*-butylbenzene (VI). (b) Hydroquinone (I) on butylation gives *tert.*-butylhydroquinone (VII) along with 2,5-di-*tert.*-butylhydroquinone (VIII) and 2,6-di-*tert.*-butylhydroquinone (IX). (VII) on methylation with dimethyl sulphate and aqueous sodium hydroxide in a hydrogen atmosphere (95°, 20 h) gave a mixture[3] of two BHA isomers (IV) and (V) along with (VI).



The yield of BHA is affected by various intermediates, the formation of which depends on different experimental factors like (1) molar concentration of reactants, (2) temperature of reaction, (3) time of reaction, etc. Even the ratio of 3- and 2-isomers of BHA varied between 80–90% and 2–20%, respectively, in the final product. In a continuous process the intermediate byproducts are not separated. In order to get the

required optimum yields of BHA, it becomes essential to identify rapidly and to estimate the various intermediate compounds.

3- and 2-BHA, mono- and di-*tert.*-butylhydroquinones have been investigated by gas chromatography (GC)[4–10], column chromatography (CC)[11,12], paper chromatography (PC)[13–21] and thin-layer chromatography (TLC)[22–38].

The present paper describes a rapid procedure for the separation and estimation of various compounds with only 2 μg formed in different synthetic routes of butylated hydroxyanisole.

Experimental

Materials and methods. The various compounds, *viz.*, *p*-hydroxyanisole (m.p. 53°), 1,4-dimethoxybenzene (m.p. 56°), *tert.*-butylhydroquinone (m.p. 129°)[39], 2,5-di-*tert.*-butylhydroquinone (m.p. 211–212°)[39,40], 2,6-di-*tert.*-butylhydroquinone (m.p. 102°)[39], 3-*tert.*-butylhydroxyanisole (m.p. 62–63°)[41], 2-*tert.*-butylhydroxyanisole (m.p. 65°)[42] and 1,4-dimethoxy-2-*tert.*-butylbenzene (b.p. at 12 mm 117–118°)[43] were synthesised after standard procedures and purified by subjecting them to vacuum distillation, column chromatography through alumina, and repeated crystallisations with petroleum ether (b.p. 40–60°). The compounds gave single spots on PC and TLC. All solvents were freshly dried and distilled. The temperature of irrigation was 22 $\pm$ 2°. Kodak photographic glass plates (35 $\times$ 22 cm) were used. The distance travelled by the solvent front in each case was 30 cm. The compounds were detected by a short-wave UV lamp "Chromatolite". A Hilger UV spectrophotometer was used for recording the percentage transmissions of 75% ethanolic solutions of individual compounds at suitable wavelengths.

Preparation of thin-layer plates, application of the compounds and irrigation of the plates. The slurry of the sorbent in chloroform–methanol was poured on the glass plates. The plates were dried at room temperature overnight and heated at 120 $\pm$ 2° in an oven for $\frac{1}{2}$ h before use. The plates were weighed before spotting, and average coating of the sorbent was recorded. The average coatings of sorbents (mg/cm^2) in the cases of Kieselgel G (Merck), neutral alumina with 20% $CaSO_4$ (Woelm, 200 mesh, B.S.S.), Kieselgel G + Kieselguhr G (75:25) were 7.2, 6.8 and 7.5, respectively.

An acetone solution of each standard sample (2 μl containing 2 μg) was spotted on the chromatoplates, 10 μl of the acetone solutions of the reaction products from processes (a) and (b) were also applied. The plates were irrigated with suitable solvents employing the ascending technique. The plates were viewed under UV light and migrations of compounds were noted. Fig. 1 is a typical chromatoplate showing the resolutions of various compounds formed during the preparation of BHA by synthetic routes (1) and (2). Table I gives the R_F values of various compounds.

Quantitative analysis. The individual compounds separated from different reaction products were scraped with a microspatula on a sintered funnel and extracted with 75% ethanol to obtain a known volume. The percentage transmissions were recorded against suitable wavelengths: hydroquinone, 299 mμ; *p*-hydroxyanisole, 243 mμ; 1,4-dimethoxybenzene, 290 mμ; *tert.*-butylhydroquinone, 245 mμ; 2,5-di-*tert.*-butylhydroquinone, 294 mμ; 2,6-di-*tert.*-butylhydroquinone, 288 mμ; 3-*tert.*-butylhydroxyanisole, 291 mμ; 2-*tert.*-butylhydroxyanisole 242 mμ; 2-*tert.*-butyl-1:4-dimethoxybenzene, 288 mμ. The amounts of each compound were calculated from the standard curves previously plotted for the individual compound.

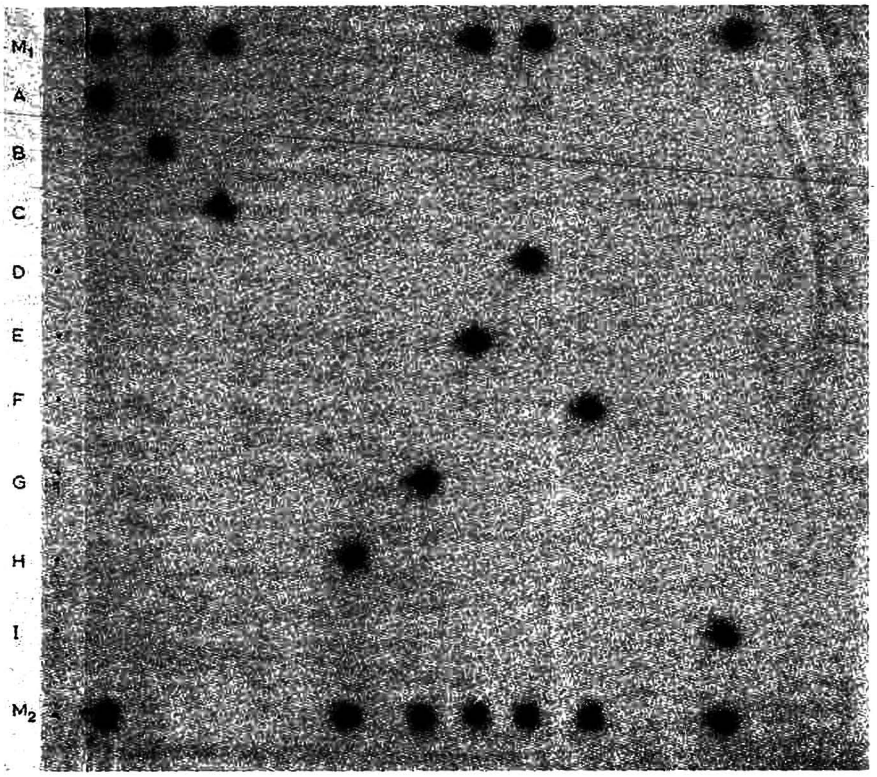


Fig. 1. Thin-layer chromatogram showing the resolutions of various compounds formed during the synthesis of butylated hydroxyanisole (BHA). M_1 = product from reaction (a); A = hydroquinone; B = *p*-hydroxyanisole; C = 1,4-dimethoxybenzene; D = 3-BHA; E = 2-BHA; F = *tert.*-butylhydroquinone; G = 2,6-di-*tert.*-butylhydroquinone; H = 2,5-di-*tert.*-butylhydroquinone; I = 1,4-dimethoxy-*tert.*-butylbenzene; M_2 = product from reaction (b). Sorbent: Kieselgel G. Solvent: chloroform–cyclohexane (1:1). Development: ascending.

Results and discussion

Hydroquinone was strongly adsorbed. Methylation of the hydroxyl group helps in the migration; thus *p*-hydroxyanisole was more mobile than hydroquinone and dimethoxybenzene had a higher R_F value than *p*-hydroxyanisole. Substitution of the *tert.*-butyl group in hydroquinone helps its mobility. 2,5-Di-*tert.*-butylhydroquinone had a lower R_F value than the 2,6-isomer. This was due to complete masking of one hydroxyl group by two *tert.*-butyl groups in the latter compound and thus helping in its mobility. 3-BHA migrated further than 2-BHA. It is observed that a compound with two electron-donating groups in vicinal positions in an aromatic ring will have a longer migration than the *meta*-substituted compound.

The compounds could be conveniently extracted with aqueous ethanol in the cold and the percentage transmissions at their respective maxima could be easily recorded, thus enabling quick estimations of all the compounds formed in the synthesis of butylated hydroxyanisole.

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TABLE I

R_F VALUES OF VARIOUS COMPOUNDS FORMED DURING THE SYNTHESIS OF BHA

Serial No.	*Compound*	*Sorbents/solvents*								
		Kieselgel G			*Neutral alumina–calcium sulphate (80 :20)*			*Kieselgel G–Kieselguhr G (3 :1)*		
		Cyclohexane–dioxane (9 :1)	*Benzene*	*Chloroform–cyclohexane (1 :1)*	*Petroleum ether–dioxane (9 :1)*	*Petroleum ether–cyclohexane (1 :1)*	*Chloroform–cyclohexane (1 :1)*	*Cyclohexane–dioxane (9 :1)*	*Chloroform–cyclohexane (2 :3)*	*Chloroform–petroleum ether (2 :3)*
1	Hydroquinone	0.11	0.03	0.05	0.09	0.00	0.02	0.06	0.04	0.03
2	*p*-Hydroxyanisole	0.19	0.13	0.12	0.28	0.20	0.10	0.21	0.21	0.14
3	1,4-Dimethoxybenzene	0.36	0.23	0.20	0.42	0.43	0.31	0.30	0.41	0.40
4	3-*tert.*-Butylhydroxyanisole	0.62	0.53	0.61	0.81	0.21	0.47	0.52	0.38	0.37
5	2-*tert.*-Butylhydroxyanisole	0.57	0.49	0.54	0.76	0.09	0.42	0.48	0.31	0.33
6	2-*tert.*-Butylhydroquinone	0.80	0.63	0.69	0.92	0.62	0.92	0.72	0.79	0.84
7	2,6-Di-*tert.*-butylhydroquinone	0.94	0.90	0.47	0.64	0.76	0.79	0.92	0.75	0.71
8	2,5-Di-*tert.*-butylhydroquinone	0.54	0.75	0.39	0.26	0.70	0.50	0.81	0.68	0.63
9	1,4-Dimethoxy-2-*tert.*-butylbenzene	0.90	0.69	0.86	0.90	0.83	0.96	0.76	0.80	0.82

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A method for the rapid chromatography of gibberellins

Thin-layer chromatography (TLC) has been used extensively since 1962 for the separation and purification of gibberellins[1–5]. Using the systems which have been described, separations are completed within 1½ to 2 h for a 15-cm solvent pathlength. This relatively rapid rate of separation results in a minimum of diffusion and excellent resolution. Gibberellins can be visualized on the chromatogram by spraying it with ethanolic sulfuric acid, heating for about 10 min and viewing the resulting fluorescent products under longwave UV light.

Although most gibberellins can be separated by adsorption chromatography, partition chromatography is necessary to separate mixtures of GA_1 and GA_3 and mixtures of GA_4 and GA_7. To accomplish these separations, chromatography plates are equilibrated overnight in the aqueous vapor of a two-phase solvent system and then developed in the organic phase.

The purpose of this report is to describe a system for the separation of gibberellins which offers the advantages of speed and convenience over conventional TLC. Excellent resolution and high sensitivity of detection characteristic of TLC are retained in this method. The chromatography medium used is a glass fiber paper impregnated with silica gel (ITLC Chromatography Medium, Gelman Instrument Co.).

The material can be cut and handled like paper. It is stable to the hot, acidic conditions required to induce fluorescence in gibberellins. Development is considerably faster than with conventional TLC, excellent resolution is obtained, and very small quantities of gibberellin can be detected. In addition, gibberellins can be recovered quantitatively for subsequent bioassay or chemical purposes.

Methods

ITLC chromatography medium is available in 20 × 20 cm sheets. These are maintained at 100° for at least 1 h to activate the silica gel. The sheets are cut to desired size, marked and spotted with sample in the usual manner employed for TLC, and developed by ascending adsorption or partition chromatography.

Adsorption chromatography is carried out in the usual manner. Sample is applied to the chromatogram and the sheet is placed in a chromatography tank containing a suitable volume of appropriate solvent.

Partition chromatography requires a 15-min equilibration in aqueous vapor before development in an organic solvent. The chromatography paper is sandwiched between two U-shaped polyethylene spacers about 2 mm in thickness (Fig. 1). These are in turn sandwiched between filter papers saturated with the aqueous phase of a two-phase solvent mixture. The chromatogram, spacers and filter papers are held between two glass plates and the complete assemblage is allowed to stand at least 15 min. The assemblage, still intact, is then placed into the developing solvent and the chromatogram is developed.

Visualization of gibberellins is effected generally in the same manner as with conventional TLC. The chromatogram is sprayed with ethanolic sulfuric acid (95:5) (ref. 4), heated at 100° for 3 min, and viewed under longwave UV light.

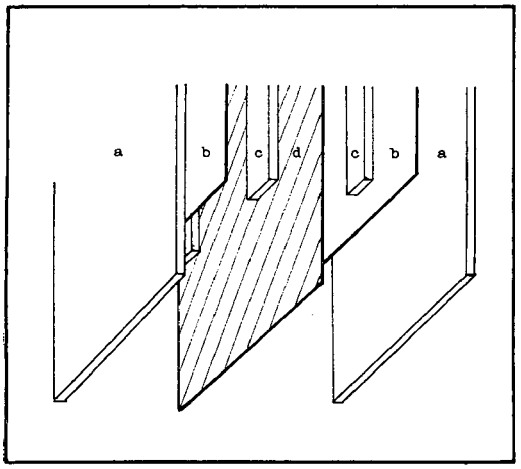

Fig. 1. Assemblage for equilibrating chromatograms prior to partition chromatography. (a) 5 × 20 cm glass plates; (b) 5 × 17 cm Whatman No. 1 filter paper, saturated with aqueous phase; (c) "U" shaped polyethylene spacer, *ca.* 2 mm thick, 5 × 17 cm outside dimensions; (d) glass fiber chromatography paper, 5 × 20 cm. Assemblage is held together with clips or rubber bands.

Results and discussion

The outstanding advantage of this system over TLC is in the speed of separation. Solvent pathlengths of 15 cm generally require 20 min or less for completion. The 15-min equilibration required for partition chromatography compares to overnight equilibration recommended for thin-layer systems[4].

Gibberellins have a greater mobility on this medium than they have on thin-layer chromatograms developed in the same solvent. Table I shows a comparison of R_F values for ten gibberellins developed on Silica Gel G plates in diisopropyl ether–acetic acid (95:5) with the R_F values obtained on silica gel-impregnated glass fiber paper developed in the same solvent mixture. Even the most polar gibberellin used in this study, GA_8, migrated approximately ten times as far on the glass fiber medium as on thin-layer plates and five of the ten gibberellins moved with the solvent front.

The R_F values of these gibberellins in additional solvent mixtures are shown in Table II. Solvents 1 and 2 are used for adsorption chromatography. Solvent 1 is a

TABLE I

COMPARISON OF GIBBERELLIN MOBILITY (R_F × 100) ON SILICA GEL-IMPREGNATED GLASS FIBER PAPER AND GLASS PLATE-SUPPORTED THIN LAYERS OF SILICA GEL G

Chromatograms were developed in diisopropyl ether–acetic acid (95:5).

	Gibberellin									
	A_1	A_2	A_3	A_4	A_5	A_7	A_8	A_9	A_{13}	A_{14}
Glass fiber	72	32	71	100	100	100	43	100	94	100
Glass plate	11[a]	4[a]	11[a]	37[a]	31[a]	37[a]	4[a]	75[a]	—	50

[a] From MACMILLAN AND SUTER[4].

TABLE II

MOBILITY ($R_F \times 100$) OF SEVERAL GIBBERELLINS ON SILICA GEL-IMPREGNATED GLASS FIBER PAPER DEVELOPED IN DIFFERENT SOLVENTS

Solvents: (1) benzene–acetic acid (95:5); (2) diisopropyl ether–acetic acid (95:5); (3) carbon tetrachloride–acetic acid–water (8:3:5); (4) chloroform–ethyl acetate–water–acetic acid (80:5:80:5); (5) chloroform–acetic acid–water (60:5:40). Solvents 3, 4 and 5 require 15-min equilibration in vapor of aqueous phase prior to development in organic phase.

Solvent	*Gibberellin*									
	A_1	A_2	A_3	A_4	A_5	A_7	A_8	A_9	A_{13}	A_{14}
1	19	12	20	70	61	72	5	97	50	78
2	72	32	71	100	100	100	43	100	94	100
3	0	0	0	70	47	60	0	100	24	82
4	39	0	30	85	100	67	9	100	20	70
5	63	—	77	—	—	—	43	100	—	98

good general purpose mixture for routine separations. Solvent 2 is useful for obtaining separation of relatively polar gibberellins.

Solvents 3, 4 and 5 are partition systems which require equilibration of the chromatogram before development. Solvent 3 is useful for the separation of GA_4 from GA_7; solvent 4 for the simultaneous separation of GA_4 from GA_7 and GA_1 from GA_3; and solvent 5 can be used for separating GA_1 from GA_3.

Figs. 2 and 3 illustrate examples of the practical uses to which this chromatography system can be put in the laboratory. Fig. 2 shows the results of a routine examination of the effluent from column chromatography purification of gibberellins.

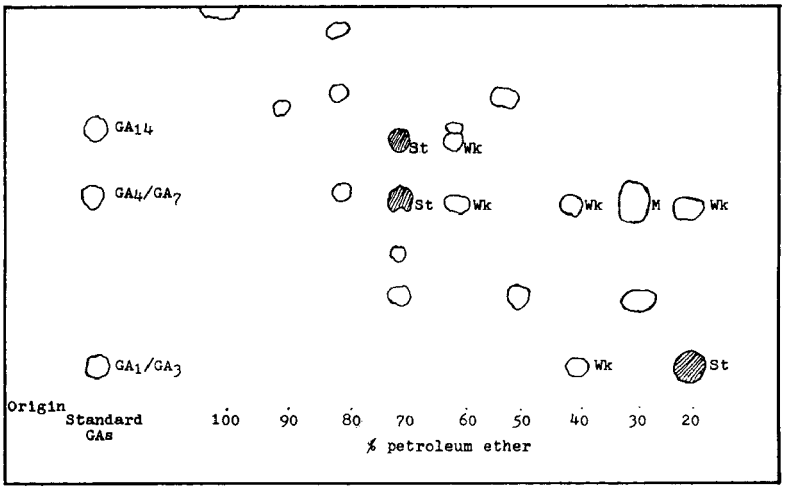

Fig. 2. Sample chromatogram showing fluorescence pattern obtained from effluent fractions of column chromatogram. Ethyl acetate-extractable organic acids from culture filtrates of *Gibberella fujikuroi* were chromatographed on a buffered Celite column[6] by stepwise elution with petroleum ether (60–110° boiling fraction) diluted with diethyl ether in 10% steps. The glass fiber paper chromatogram was developed in benzene–acetic acid (95:5). St = strong fluorescence; M = moderate fluorescence; Wk = weak fluorescence.

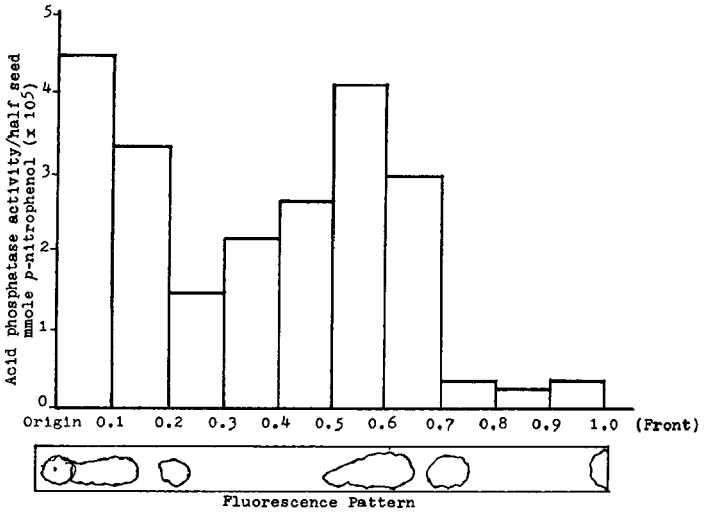

Fig. 3. Results of a bioassay for gibberellin-like substances eluted from a silica gel–glass fiber paper chromatogram. Ethyl acetate-extractable organic acids from culture filtrates of *Gibberella fujikuroi* were used as a source of gibberellin-like substances.

A buffered Celite column was loaded with organic acids produced by *Gibberella fujikuroi* and developed by stepwise elution with petroleum ether diluted with diethyl ether[6]. Portions of the effluent fractions were chromatographed on the glass fiber medium and the fluorescent pattern examined and recorded on this diagram.

Fig. 3 demonstrates how this material can be used for the purification of gibberellin-like materials in association with a bioassay. In this case, *Gibberella fujikuroi* was grown on a potato-dextrose broth medium and ethyl acetate-soluble organic acids were extracted from the culture filtrate[6]. A portion of the extract was chromatographed in solvent 1 and the chromatogram was cut into ten transverse strips, corresponding to ten R_F zones. A vertical strip was left on one side of the chromatogram and used for visualizing and locating gibberellin-like fluorescent materials. Each of the ten strips was washed with 10 ml acetone. The acetone was evaporated to dryness and a portion of the residue was tested in the barley endosperm acid phosphatase assay[7].

Two two major peaks of activity shown in Fig. 3 correspond in R_F values to GA_1/GA_3 and GA_4/GA_7. These are the most prevalent gibberellins in this extract when the fungus is grown under these conditions.

Subsequent examination of the strips of chromatographic material which had been washed with acetone revealed no fluorescent materials. Acetone appears to elute quantitatively gibberellin-like substances from this medium, without leaving an adsorbent residue in the eluate.

Conclusions

Silica gel-impregnated glass fiber paper is suitable for the purification of gibberellins and gibberellin-like materials. It can be used in connection with bioassays and for monitoring column effluents. It offers the advantages of speed and convenience over conventional TLC methods.

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