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AN EQUILIBRIUM THEORY FOR RARE-EARTH SEPARATION BY DISPLACEMENT DEVELOPMENT

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SUMMARY

A general equilibrium theory of chromatography is applied to the separation of rare earths by displacement development on a cation exchanger. The essential premises are local equilibrium, uniform sorbent properties, plug flow, absence of axial diffusion, stoichiometric exchange, and constant separation factors. Both development of a preadsorbed uniform band with a chelating agent and operation with chelation prior to loading are considered. The theory yields the distances and times required for resolution of any component from any other was well as the mobile-phase and stationary phase compositions at any point and time during development. Application of the theory to the separation of a fifteen-component rare-earth mineral, euxenite, illustrates the method.

INTRODUCTION

A highly successful process for preparative commercial separation of rare earths in ton quantities with high purity is displacement development with a chelating agent on a cation exchanger[1,2]. The characteristic and well-known features of this type of separation are that the mixture to be separated travels as a band of constant width, being displaced by a chelating development agent and itself displacing a retaining agent with which the column was presaturated; on its way through the column (or through a number of columns in series) the species within the band sort themselves out into individual zones of one species each, which all travel at the same rate and follow one another without interval. In practice, finite mass-transfer rates and unavoidable disturbances cause some overlap between the final zones, but under the simplifying premises usually employed the theory gives ideally sharp boundaries between the zones. A typical operation is with an ammonium-EDTA buffer as the development agent on a strong-acid cation exchanger with Cu^{2+} as the retaining ion,

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as developed by SPEDDING AND POWELL and their associates[1,3,4], to whose publications we refere for chemical mechanism, equipment, and practical details.

The development behavior is illustrated in Fig. 1 for the separation of a pre-adsorbed uniform band of a binary mixture. As development proceeds, the zone having the composition of the original mixture shrinks and eventually disappears, while zones of the two pure components grow on either side, all within the traveling band of constant length. The broken lines, superimposed on the column pictures, trace the traveling boundaries between the zones of different compositions.

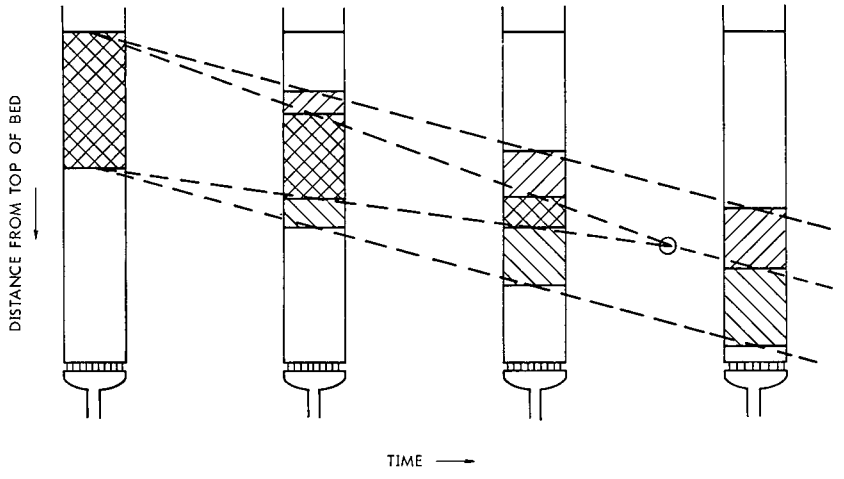

Fig. 1. Separation of binary mixture (schematic). (From D. B. JAMES, J. E. POWELL AND H. R BURKHOLDER[9].)

For optimum design, a knowledge of the distance and time a given mixture must travel to be resolved is obviously of key importance. Previous theoretical treatments, by SILLÉN[5] and SPEDDING, POWELL *et al.*[4,6–9], have given solutions for separations of binary and ternary mixtures but are not readily extended to more complex cases. A recent general theory of multicomponent chromatography under arbitrary initial and influent conditions[10], however, comprises displacement development as a special case and can be applied to provide the desired extension to any number of components, as will be shown here.

Computations with the formulas which follow are straightforward but become lengthy for systems with many components. For these a computer program that tabulates and plots the results has been made available[11]. The results calculated by this program for a fifteen-component separation will be shown here as an example.

Space limitations forbid us to give more than the mere outlines of derivations and proofs. For details reference to the original theory[10] must be made.

PREMISES

The usual assumptions of "equilibrium theories" of chromatography are made, namely, existence of local equilibrium at any point and time, uniform sorbent properties, plug flow, and absence of axial diffusion. We assume, moreover, that ion exchange is stoichiometric (*i.e.*, without change in total number of equivalents in either phase) and that the separation factors are constant. Volume changes of the ion exchanger are disregarded, and for convenience we only consider operations with a column of uniform cross section and with constant composition and flow rate of the development agent. These assumptions are essentially the same as in the earlier theoretical treatments, except that the premise of low solution concentration relative to the concentration in the ion exchanger, made by SPEDDING, POWELL *et al.*, is not needed.

Theories based on these premises are unrealistic in that they yield ideally sharp boundaries between the various zones of different compositions, boundaries that in actual operation are slightly diffuse. However, since practical rare-earth separations are conducted so that diffuse overlaps between zones are small compared to the band and zone widths, this idealization does not significantly impair the utility of the theory.

NOTATION AND DEFINITIONS

We shall consider the separation of an arbitrary n-component mixture. The species are numbered $1, 2, \ldots, n$ in the order of decreasing affinity for the ion exchanger in the presence of the chelating agent. Concentrations are given as equivalent ionic fractions x_i in the liquid and y_i in the ion exchanger ($i = 1, \ldots, n$). Thus, by definition

$$\sum_{i=1}^{n} x_i = 1 \quad \text{and} \quad \sum_{i=1}^{n} y_i = 1 \tag{1}$$

at any point in the band. Separation factors α_{1i} relative to the reference species 1 are defined as

$$\alpha_{1i} \equiv \frac{y_1 \, x_i}{x_1 \, y_i} \qquad (i = 1, \ldots, n) \tag{2}$$

(According to POWELL AND SPEDDING[4], these factors are virtually equal to the ratios of the stability constants of the respective chelates, chelation being the almost exclusive cause of selectivity.)

For mathematical convenience and greater generality, the derivations make use of a normalized and adjusted time variable, τ, defined as

$$\tau \equiv (tu_0 - z)C/\bar{C} \tag{3}$$

where t is the true time (say, in seconds), u_0 is the linear liquid flow rate (in cm/sec), z is the distance from the top of the bed (in cm), and C and $\bar{C}$ are the total concentrations (sums of concentrations of all components, in ionic equivalents per unit volume of column) in the liquid and ion exchanger, respectively. (The "adjustment" by $-z$ in eqn. (3) is the mathematically most convenient way of removing the premise $C \ll \bar{C}$ adopted in most earlier theories. Note that the adjusted time has the dimension of a length.)

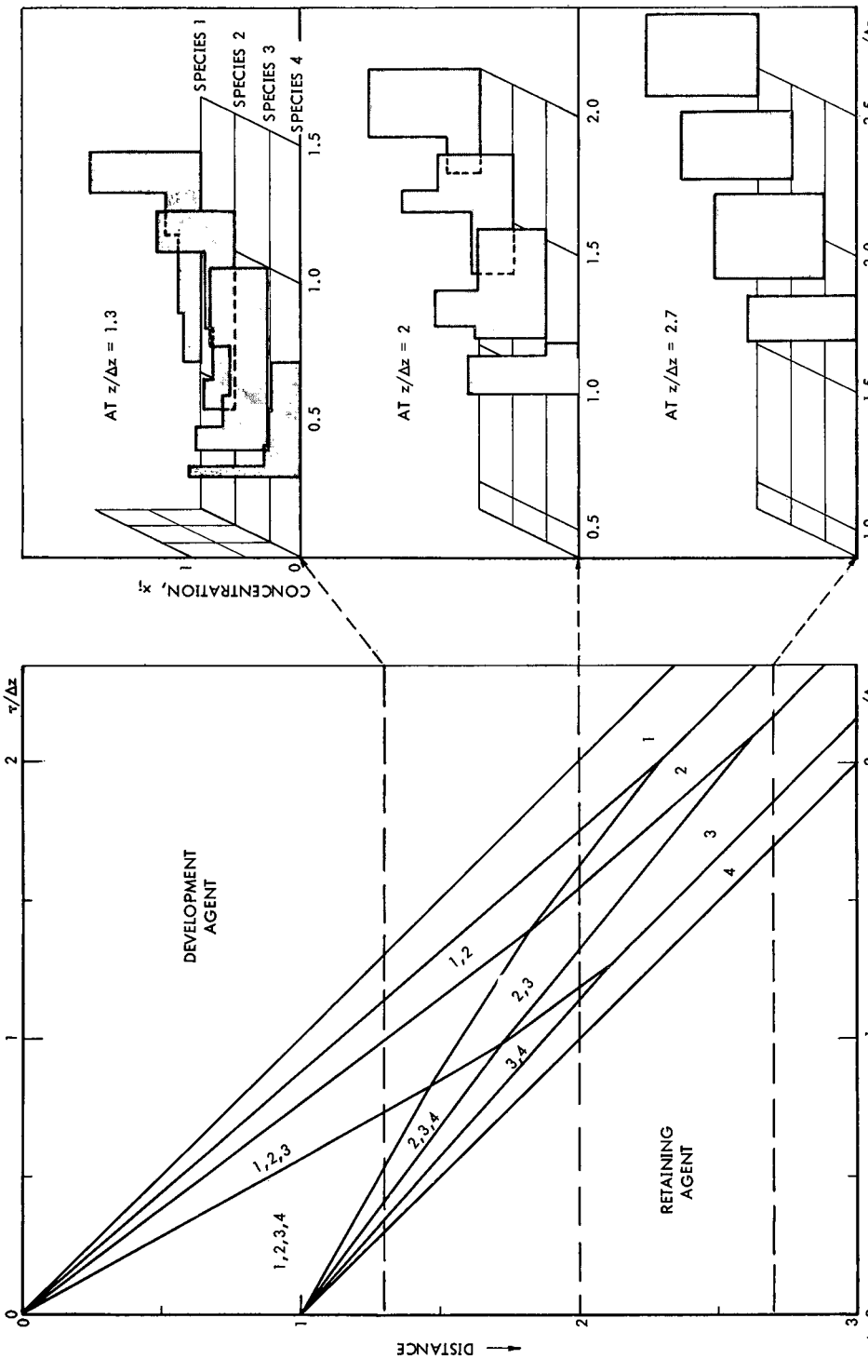

Fig. 2. Distance–time diagram and concentration histories at three different column levels for quaternary separation by development of uniform initial band. (Calculated for initial composition $y_1^\circ = 0.298$, $y_2^\circ = 0.239$, $y_3^\circ = 0.304$, $y_4^\circ = 0.159$ and separation factors $\alpha_{12} = 1.49$, $\alpha_{13} = 2.05$, $\alpha_{14} = 3.20$.)

The adjusted velocity, u_Δ, of a boundary between zones of different compositions is defined as

$$u_\Delta \equiv \partial z/\partial \tau|_\Delta \tag{4}$$

where Δ symbolizes the boundary. The definition of τ has been chosen so that the adjusted velocity of the rare-earth band (*i.e.*, of its front and rear boundaries) is unity.

OUTLINE OF THEORY

Distance–time diagrams

The derivations make use of geometrical constructions in "distance–time diagrams", which show the trajectories of boundaries between zones of different compositions. For easier mental translation into and from actual column operation, which is usually downflow, distance from the top of the bed is plotted downward; with the column pictures removed, Fig. 1 would thus immediately give a distance–time diagram. A diagram for a four-component case is shown in Fig. 2. The resolution distances and times, ultimately sought by the theory, are the coordinate values of the points at which trajectories intersect, and can thus be calculated once the velocities of the boundaries (trajectory slopes) are known.

Boundary velocities

A simple material-balance argument (amount entering minus amount leaving a column segment equals net change in content of segment), the starting point of theory in almost any text on chromatography, shows that a concentration step of an arbitrary species i advances at the true velocity $u_0 \Delta C_i/(\Delta C_i + \Delta \bar{C}_i)$, where ΔC_i and $\Delta \bar{C}_i$ are the concentration differences in the liquid and ion exchanger, respectively, across the step. (The usual equations differ in that they involve the fractional phase volumes; these cancel if, as is done here, the concentrations are expressed per unit volume of column rather than of the respective phase.) Conversion to adjusted velocity and equivalent fractions gives

$$u_\Delta = \Delta x_i/\Delta y_i \tag{5}$$

(where Δx_i and Δy_i are the differences across the step), as can be verified by eqns. (3) and (4). This relation holds for any species i.

Coherence

In multicomponent systems, a boundary between zones of different composition usually involves concentration variations of any number of species. If the boundary is to travel without splitting up into separate concentration steps, it is evident that eqn. (5) *must apply to all species simultaneously*, since the concentration steps of different species would otherwise travel at different velocities. Accordingly:

$$\Delta x_1/\Delta y_1 = \Delta x_2/\Delta y_2 = \ldots = \Delta x_n/\Delta y_n \tag{6}$$

for any given boundary. In the general theory[10], boundaries that meet this criterion are called *coherent*, and it is shown that and how coherent boundaries evolve from any arbitrary initial conditions. In the present case, the two initial composition

steps, at the front and rear of the uniform initial band, are noncoherent and break up into sets of coherent boundaries. Similarly, where two coherent boundaries cross, there is for an instant a noncoherent step, which immediately breaks up again into two coherent boundaries. All earlier theories have implied without proof that coherent boundaries are indeed formed.

h-Transformation and H-function roots

Except for the zone of the original mixture and those at the extreme rear and front of the band, where only species 1 and n, respectively, are present, the various transient compositions are not known beforehand. Eqn. (5) by itself is therefore not sufficient to calculate boundary velocities. This difficulty is best overcome with the so-called *h-transformation*, by which the set of composition variables $x_1,\ldots,x_n$ or $y_1,\ldots,y_n$ is replaced by a new set $h_1,\ldots,h_{n-1}$. [Only $n-1$ variables are needed since, in view of eqn. (1), only $n-1$ concentrations x_i or y_i can be varied independently.] The advantage of the new variables is that all boundary velocities and other quantities of interest can be calculated directly from the set of *h_i of the initial mixture alone*, without the need for computing intermediate compositions or properties.

The h_i are defined as the $n-1$ roots in h (or reciprocals of the roots in $1/h$) of the so-called "H-function":

$$\sum_{i=1}^{n} \left[\prod_{\substack{j=1 \\ j\neq i}}^{n} (h-\alpha_{1j})x_i \right] = 0 \tag{7}$$

or

$$\sum_{i=1}^{n} \left[\prod_{\substack{j=1 \\ j\neq i}}^{n} \left(\frac{1}{h} - \frac{1}{\alpha_{1j}}\right) y_i \right] = 0 \tag{8}$$

It can be readily verified that compositions $x_1,\ldots,x_n$ and $y_1,\ldots,y_n$ in equilibrium with one another give the same set of H-function roots. The roots are all real and fall into the intervals

$$\alpha_{1i} \leq h_i \leq \alpha_{1,i+1} \qquad (i = 1,\ldots,n-1) \tag{9}$$

For each species k absent from the respective composition, the polynomial (7) or (8) has a "trivial root" $h = \alpha_{1k}$. Thus

$$\begin{aligned} &h_1 = \alpha_{11} && \text{if } x_1 = 0, y_1 = 0 \\ &h_{i-1} = \alpha_{1i} \text{ or } h_i = \alpha_{1i} && \text{if } x_i = 0, y_i = 0 \qquad (1 < i < n) \\ &h_{n-1} = \alpha_{1n} && \text{if } x_n = 0, y_n = 0 \end{aligned} \tag{10}$$

The other, nontrivial roots are more conveniently calculated from the relations

$$\sum_{i=1}^{n} \frac{x_i}{h-\alpha_{1i}} = 0 \quad \text{or} \quad \sum_{i=1}^{n} \frac{y_i}{1/h-1/\alpha_{1i}} = 0 \tag{11}$$

obtained when eqn. (7) is divided by $\prod_{j=1}^{n} (h-\alpha_{1j})$, or eqn. (8) by $\prod_{j=1}^{n} (1/h-1/\alpha_{1j})$.

The roots are readily found by standard numerical procedures, since the functions in

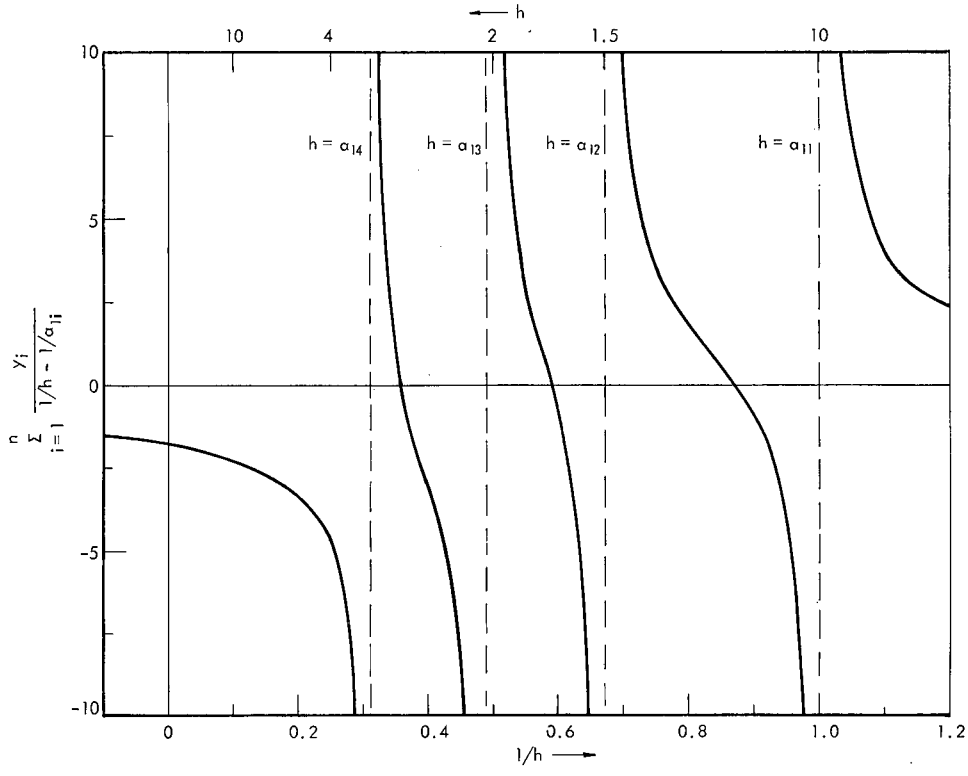

Fig. 3. Function $\sum_{i=1}^{n} y_i/(1/h - 1/\alpha_{1i})$ *versus* $1/h$. (Calculated for same composition and separation factors as in Fig. 2.)

h are single-valued and monotonic between their poles at the various $h = \alpha_{1i}$. An example of this behavior is shown in Fig. 3.

For two and three components, the roots can be given conveniently in an explicit form. Eqn. (11), solved for h, gives for two-component compositions

$$h = \alpha_{12}x_1 + x_2 \qquad \text{and} \qquad h = \frac{1}{y_1/\alpha_{12} + y_2} \tag{12}$$

and for three-component compositions

$$h_{1,2} = \tfrac{1}{2}\left[A \pm (A^2 - 4B)^{\frac{1}{2}}\right] \quad \text{and} \quad h_{1,2} = \frac{2}{\bar{A} \pm (\bar{A}^2 - 4\bar{B})^{\frac{1}{2}}} \tag{13}$$

where

$$A \equiv 1 - x_1 + \alpha_{12}(1 - x_2) + \alpha_{13}(1 - x_3)$$
$$B \equiv \alpha_{12}\alpha_{13}x_1 + \alpha_{13}x_2 + \alpha_{12}x_3$$
$$\bar{A} \equiv 1 - y_1 + (1 - y_2)/\alpha_{12} + (1 - y_3)/\alpha_{13}$$
$$\bar{B} \equiv y_1/\alpha_{12}\alpha_{13} + y_2/\alpha_{13} + y_3/\alpha_{12}$$

For the converse transformation, to obtain concentrations x_i or y_i from given sets of H-function roots, explicit equations can be written for any number of components:

$$x_j = \prod_{i=1}^{n-1}(h_i - \alpha_{1j}) \Bigg/ \prod_{\substack{i=1 \\ i\neq j}}^{n} (\alpha_{1i} - \alpha_{1j}) \qquad (14)$$

$$(j = 1, \ldots, n)$$

$$y_j = \prod_{i=1}^{n-1}\left(\frac{1}{h_i} - \frac{1}{\alpha_{1j}}\right) \Bigg/ \prod_{\substack{i=1 \\ i\neq j}}^{n}\left(\frac{1}{\alpha_{1i}} - \frac{1}{\alpha_{1j}}\right) \qquad (15)$$

These equations can be obtained as the solutions of the eqns. (11) with h_1, h_2, ..., h_{n-1} consecutively substituted for h.

Properties and behavior of H-function roots

Two of the properties of the H-function roots, examined in detail in the general theory, are of special importance in the present case. The first one is that *only one root varies across any coherent boundary*, and the second, that any boundary in the course of development is a root variation, propagated across the distance–time plane, that *existed initially or was introduced as an influent composition change.* A corollary of the second rule is that *no root values other than those of the initial and influent compositions will occur anywhere at any time.* An abbreviated proof of the first rule, applicable to the present case, is given in the Appendix, where it is also shown that the adjusted velocity of a coherent composition step with h_k as the varying root is given by

$$u_\Delta = h_k' \, h_k'' \prod_{\substack{i=1 \\ i\neq k}}^{n-1} h_i \Bigg/ \prod_{i=1}^{n} \alpha_{1i} \qquad (16)$$

where h_k' and h_k'' are the values of h_k on the two sides of the step. For the more complex proof of the conservation properties of the roots reflected in the second rule we must refer to the original theory[10].

The two initial composition steps, at the front and rear of the uniform initial band, involve variations of all H-function roots and thus are noncoherent. Upon development they break up into sets of coherent boundaries each involving variation of one root only. Within each set, the boundaries are in the sequence of increasing index numbers of their variable roots (seen in the direction of flow), since eqn. (16) with condition (9) gives the higher velocity for the boundary with variable root of higher index number. In the distance–time plane, each of the sets of boundaries appears as a bundle of trajectories having a common point of origin. The trajectories of the two bundles cross: each trajectory of a root variation Δh_k of the upstream bundle crosses all those of variations Δh_1, Δh_2, ..., Δh_{k-1} of the downstream bundle and eventually merges with that of Δh_k of the downstream bundle; the last is a merger rather than a cross-over because a single coherent boundary is formed from two coherent boundaries if these involve variations of the *same* root. The resulting overall trajectory pattern in the distance–time plane is shown schematically in Fig. 4.

The root values for the uniform initial zone can be calculated from its composition x_1°, ..., x_n° or y_1°, ..., y_n° from eqn. (11) and will be designated h_1°, ...,

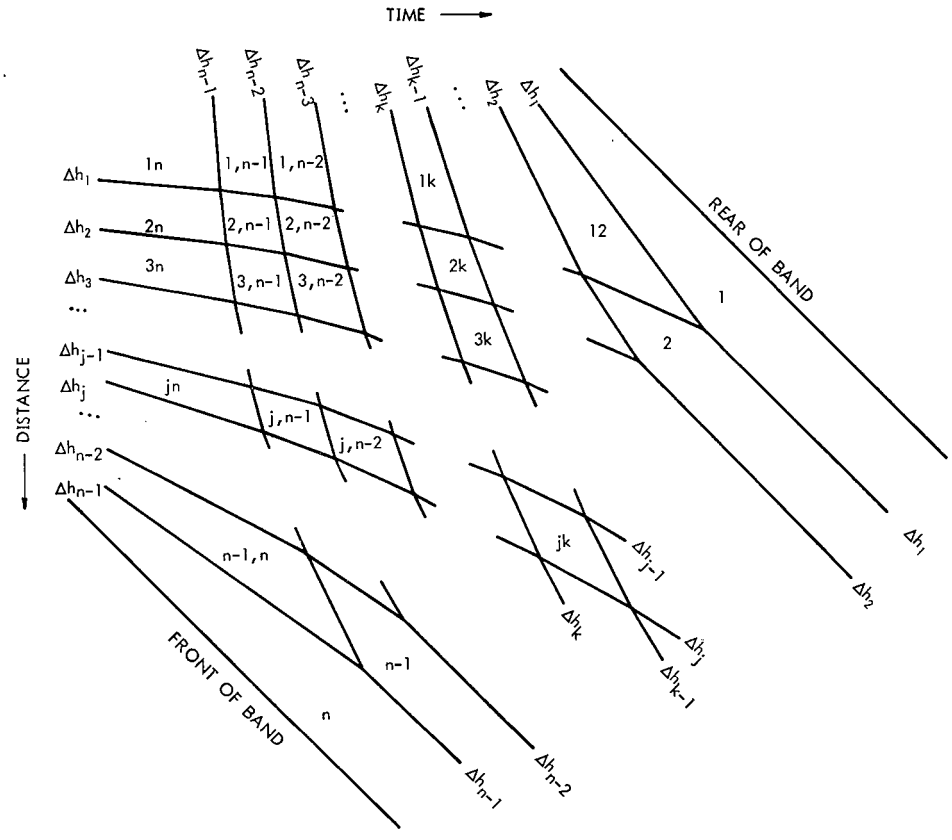

Fig. 4. Pattern of trajectories in distance–time plane (schematic). (Adapted from HELFFERICH AND KLEIN[10].)

$h_n^\circ{}_{-1}$. The root values at the extreme rear and front of the traveling band, where only species 1 and n, respectively, are present, are readily obtained from condition (10). At the rear, the absence of species 2, ..., n gives rise to the $n-1$ "trivial" roots

$$h_1 = \alpha_{1,i+1} \qquad (i = 1, \ldots, n-1) \tag{17}$$

the index numbers being dictated by condition (9). Similarly, at the front, the absence of species 1, ..., $n-1$ gives rise to the $n-1$ trivial roots

$$h_i = \alpha_{1i} \qquad (i = 1, \ldots, n-1) \tag{18}$$

Thus, any root h_k varies from $\alpha_{1,k+1}$ to h_k° across the Δh_k trajectory of the upstream bundle, and from h_k° to α_{1k} across the Δh_k trajectory of the downstream bundle; the region of h_k° narrows with progressing development and disappears when the two Δh_k trajectories merge.

Compositions of the zones

The composition of all the zones in the distance–time plane can now be calculated

from the roots h_i° of the initial mixture. We shall characterize the various zones by double numbers, jk referring to the zone between the trajectories of Δh_{j-1} and Δh_j of the downstream bundle and those of Δh_{k-1} and Δh_k of the upstream bundle ($j < k$). Using this convention, the jk zone is downstream of the trajectories of all Δh_i with $i < j$ of both bundles, upstream of the trajectories of all Δh_i with $i > k$ of both bundles, and between the upstream-bundle and downstream-bundle trajectories of all Δh_i with $j \leq i \leq k$. With the variations of the root values across the respective trajectories as noted earlier, the set of roots in the jk zone then is

$$\begin{aligned} h_i &= \alpha_{1i} && (i = 1, \ldots, j-1) \\ h_i &= h_i^\circ && (i = j, \ldots, k-1) \\ h_i &= \alpha_{1,i+1} && (i = k, \ldots, n-1) \end{aligned} \tag{19}$$

and, as conditions (10) show, only species $j, \ldots, k$ are present in the zone. The concentrations of these species can be calculated from eqns. (14) or (15), which give, with the appropriate substitutions,

$$x_l = \prod_{i=j}^{k-1}(h_i^\circ - \alpha_{1l}) \Bigg/ \prod_{\substack{i=j \\ i \neq l}}^{k} (\alpha_{1i} - \alpha_{1l}) \qquad (l = j, \ldots, k) \tag{20}$$

$$y_l = \prod_{i=j}^{k-1}\left(\frac{1}{h_i^\circ} - \frac{1}{\alpha_{1l}}\right) \Bigg/ \prod_{\substack{i=j \\ i \neq l}}^{k} \left(\frac{1}{\alpha_{1i}} - \frac{1}{\alpha_{1l}}\right)$$

Boundary velocities

The adjusted velocities of all boundaries can be calculated with equal ease from the H-function roots of the initial mixture. All that is needed is the substitution of the appropriate root values into the general equation (16). For the upstream trajectory bundle, generated by the composition step at the rear of the uniform initial band, one finds that the adjusted velocity of the variation Δh_k of the arbitary root h_k at the zone jk (*i.e.*, between the Δh_{j-1} and Δh_j trajectories of the downstream bundle) is

$$u_\Delta = \prod_{i=j}^{k} (h_i^\circ / \alpha_{1i}) \tag{21}$$

Similarly, one finds for the adjusted velocity of the variation Δh_j of the downstream bundle at the jk zone (*i.e.*, between the Δh_{k-1} and Δh_k trajectories of the upstream bundle):

$$u_\Delta = \prod_{i=j}^{k-1} (h_i^\circ / \alpha_{1,i+1}) \tag{22}$$

As Fig. 4 has shown, the Δh_k trajectory of the upper bundle crosses, in this sequence, those of $\Delta h_1, \Delta h_2, \ldots, \Delta h_{k-1}$ of the lower bundle, and the Δh_j trajectory of the lower bundle crosses, in this sequence, those of $\Delta h_{n-1}, \Delta h_{n-2}, \ldots, \Delta h_{j+1}$ of the upper bundle. Accordingly, the products in eqns. (21) and (22) lose one of their factors h_1°/α_{11}, h_2°/α_{12}, ... and $h_{n-1}^\circ/\alpha_{1n}$, $h_{n-2}^\circ/\alpha_{1,n-1}$, ... with each successive cross-over. Since condition (9) requires

$$h_i^\circ/\alpha_{1,i+1} < 1 < h_i^\circ/\alpha_{1i} \qquad (i = 1, \ldots, n-1) \tag{23}$$

the adjusted velocities of all boundaries of the upper bundle are larger than unity and decrease with each cross-over, whereas those of the lower bundle are all smaller than unity and increase with each cross-over. The adjusted velocity becomes unity when the two Δh_k trajectories (or Δh_j trajectories) of the two bundles finally merge.

Resolution distances and times

A glance at the distance–time diagram reveals that the zone jk is the last and farthest downstream to contain species j and k in the presence of one another. The development distance (*i.e.*, bed length) and adjusted time, z_{jk} and τ_{jk}, required for the resolution of these two species are thus given as the z and τ coordinate values of the point at which this zone disappears, that is, of the "southeast" corner of the zone in the distance–time diagram. Given the composition of the initial mixture and the width of its band, all resolution distances and times can thus be calculated in a straightforward manner by construction of the trajectory grid from the given points of origin of the bundles with the use of eqns. (21) and (22) for the trajectory slopes. The results of this calculation are listed in Table I. Derivations are provided in the Appendix.

Table I gives formulas for two types of operation, namely, development of a uniform initial band as in Figs. 1 and 2, and operation with chelation of the mixture prior to its introduction into a column initially loaded with only the retaining ion, as

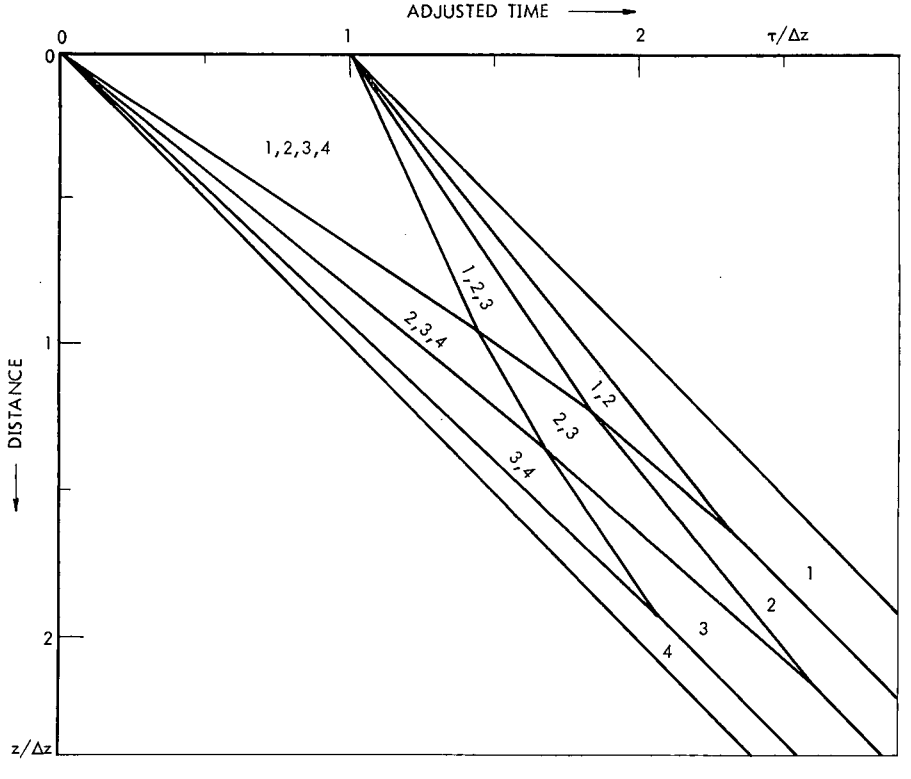

Fig. 5. Distance–time diagram for quaternary separation with chelation prior to loading. (Calculated for same original mixture and separation factors as in Fig. 2.)

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TABLE I

DISTANCES AND ADJUSTED TIMES FOR RESOLUTION

Development of uniform initial band	*Operation with chelation prior to loading*
Binary separations	
$z_{12} = \frac{\alpha_{12}}{\alpha_{12}-1}\Delta z$	$z_{12} = \left(\frac{1}{\alpha_{12}-1} + x_1^\circ\right)\Delta z$
$\tau_{12} = \left(\frac{1}{\alpha_{12}-1} + y_2^\circ\right)\Delta z$	$\tau_{12} = \frac{\alpha_{12}}{\alpha_{12}-1}\Delta z$
Ternary separations	
$z_{13} = \frac{\alpha_{13}}{\alpha_{13}-1}\Delta z$	$z_{13} = \frac{\alpha_{13}x_1^\circ + \alpha_{12}x_2^\circ + x_3^\circ}{\alpha_{13}-1}\Delta z$
$\tau_{13} = \frac{y_1^\circ + \alpha_{23}y_2^\circ + \alpha_{13}y_3^\circ}{\alpha_{13}-1}\Delta z$	$\tau_{13} = \frac{\alpha_{13}}{\alpha_{13}-1}\Delta z$
$z_{12} = \frac{\alpha_{12}\alpha_{13}(h_2^\circ-1)}{h_2^\circ(\alpha_{12}-1)(\alpha_{13}-1)}\Delta z$	$z_{12} = \frac{h_1^\circ(h_2^\circ-1)}{(\alpha_{12}-1)(\alpha_{13}-1)}\Delta z$
$\tau_{12} = \frac{\alpha_{12}\alpha_{13}(h_2^\circ-1)}{h_1^\circ h_2^\circ(\alpha_{12}-1)(\alpha_{13}-1)}\Delta z$	$\tau_{12} = \left(1 + \frac{h_2^\circ-1}{(\alpha_{12}-1)(\alpha_{13}-1)}\right)\Delta z$
$z_{23} = \left(1 + \frac{\alpha_{13}(\alpha_{13}-h_1^\circ)}{h_1^\circ(\alpha_{13}-1)(\alpha_{13}-\alpha_{12})}\right)\Delta z$	$z_{23} = \frac{h_2^\circ(\alpha_{13}-h_1^\circ)}{(\alpha_{13}-1)(\alpha_{13}-\alpha_{12})}\Delta z$
$\tau_{23} = \frac{\alpha_{12}\alpha_{13}(\alpha_{13}-h_1^\circ)}{h_1^\circ h_2^\circ(\alpha_{13}-1)(\alpha_{13}-\alpha_{12})}\Delta z$	$\tau_{23} = \frac{\alpha_{13}(\alpha_{13}-h_1^\circ)}{(\alpha_{13}-1)(\alpha_{13}-\alpha_{12})}\Delta z$
Multicomponent separations	
$k = 2,\ldots,n$: $z_{1k} = \frac{\alpha_{1n}}{\alpha_{1n}-1}\prod_{i=k}^{n-1}\left(\frac{\alpha_{1i}(h_i^\circ-1)}{h_1^\circ(\alpha_{1i}-1)}\right)\Delta z$	$z_{1k} = \frac{h_1^\circ}{\alpha_{1n}-1)}\prod_{i=2}^{k-1}\left(\frac{h_i^\circ}{\alpha_{1i}}\right)\prod_{i=k}^{n-1}\left(\frac{h_i^\circ-1}{\alpha_{1i}-1}\right)\Delta z$
$\tau_{1k} = \frac{1}{\alpha_{1n}-1}\prod_{i=2}^{n}\left(\frac{\alpha_{1i}}{h_{i-1}^\circ}\right)\prod_{i=k}^{n-1}\left(\frac{h_i^\circ-1}{\alpha_{1i}-1}\right)\Delta z$	$\tau_{1k} = \left[1 + \frac{1}{\alpha_{1n}-1}\prod_{i=k}^{n-1}\left(\frac{h_i^\circ-1}{\alpha_{1i}-1}\right)\right]\Delta z$
$j = 1,\ldots,n-1$: $z_{jn} = \left[1 + \frac{\alpha_{1j}}{\alpha_{1n}-\alpha_{1j}}\prod_{i=1}^{j-1}\left(\frac{\alpha_{1i}(\alpha_{1n}-h_i^\circ)}{h_i^\circ(\alpha_{1n}-\alpha_{1i})}\right)\right]\Delta z$	$z_{jn} = \frac{h_j^\circ}{\alpha_{1n}-\alpha_{1j}}\prod_{i=1}^{j-1}\left(\frac{\alpha_{1n}-h_i^\circ}{\alpha_{1n}-\alpha_{1i}}\right)\Delta z$
$\tau_{jn} = \frac{1}{\alpha_{1n}-\alpha_{1j}}\prod_{i=2}^{n}\left(\frac{\alpha_{1i}}{h_{i-1}^\circ}\right)\prod_{i=1}^{j-1}\left(\frac{\alpha_{1n}-h_i^\circ}{\alpha_{n1}-\alpha_{1i}}\right)\Delta z$	$\tau_{jn} = \frac{\alpha_{1n}}{\alpha_{1n}-\alpha_{1j}}\prod_{i=1}^{j-1}\left(\frac{\alpha_{1n}-h_i^\circ}{\alpha_{1n}-\alpha_{1i}}\right)\Delta z$
$j = 1,\ldots,n-1$; $k = 2,\ldots,n$; $j<k$: $z_{jk} = \frac{\alpha_{1j}\alpha_{1k}}{\alpha_{1k}-\alpha_{1j}}\left[\left(\frac{1}{\alpha_{1j}} - \frac{1}{h_k^\circ}\right)z_{j,k+1} + \left(\frac{1}{h_{j-1}^\circ} - \frac{1}{\alpha_{1k}}\right)z_{j-1,k} - \left(\frac{1}{h_{j-1}^\circ} - \frac{1}{h_k^\circ}\right)z_{j-1,k+1}\right]$	
$\tau_{jk} = \frac{1}{\alpha_{1k}-\alpha_{1j}}\left[(h_k^\circ-\alpha_{1j})\tau_{j,k+1} + (\alpha_{1k}-h_{j-1}^\circ)\tau_{j-1,\,k} - (h_k^\circ-h_{j-1}^\circ)\tau_{j-1,k+1}\right]$	

Δz = width of rare-earth band in (z,τ) diagram, measured long line of constant τ [for calculation, see eqn. (29)]; $\alpha_{23} \equiv y_2x_3/x_2y_3 = \alpha_{12}/\alpha_{23}$.

in Fig. 5. In general, the latter type of operation requires a smaller column length but a longer adjusted time for resolution of any given pair of species. Also, the "critical" pair, *i.e.*, that with the longest resolution distance or time, is not necessarily the same for the two types of operation.

For binary and ternary separations the formulas in Table I are explicit in terms of the initial composition of the mixture or its H-function roots, which are readily calculated from the composition by eqn. (13). For separations of higher order, explicit formulas can be given but are impractically lengthy; here Table I only lists those for resolution from species 1 and from species n and gives a recursion formula for calculating z_{jk} (or τ_{jk}) from previously calculated $z_{j-1,k+1}$, $z_{j-1,k}$, and $z_{j,k+1}$ (or corresponding τ values). The equations for the resolution distances in binary and ternary separations are identical with, or equivalent to, those given by SILLÉN[5] and SPEDDING, POWELL *et al.*[4,6-9], although the implicit ternary equations of the latter appear more complex since they lack the economy provided by the H-function roots. (All but the last of these publications, ref. 9, were confined to development of a uniform initial band.)

The initial condition in development of a uniform band calls for a comment. The formulas in Table I are calculated with the premise that no development takes place at any location z prior to that location's zero of adjusted time. That is, development at any z is presumed to start with a time lag z/u_0 relative to the top of the bed (see eqn. (3), solved for t at $\tau = 0$). This time lag is exactly the time needed for a nonsorbable agent to travel from the top to the location z. Accordingly, the premise is geared to operation with a column initially free of any development agent and with development by an agent essentially excluded by the ion exchanger. This corresponds closely to the conditions in practical separations.

Another point needing consideration is that the composition of the original rare-earth mixture provides the initial values $y_1^\circ, \ldots, y_n^\circ$ in development of a uniform presorbed band, but provides the influent values $x_1^\circ, \ldots, x_n^\circ$ in operation with chelation prior to loading. In the first case, the mixture is sorbed nonselectively in the absence of the chelating agent to give an ion-exchanger composition equal to that of the original mixture; as the front of the chelating anion penetrates the band at $\tau = 0$, slight and selective desorption occurs, to give a liquid-phase composition in equilibrium with, and differing from, the essentially unchanged ion-exchanger composition. In contrast, in the second case, the mixture enters the column at its composition $x_1^\circ, \ldots, x_n^\circ$ with the chelating agent providing selective sorption, so that an ion-exchanger composition in equilibrium with, and differing from, this liquid-phase composition is established at the top of the bed. Accordingly, when calculating the set of h_i° from the known composition of the original mixture, this composition should be taken as $y_1^\circ, \ldots, y_n^\circ$ in development of a uniform initial band, and as $x_1^\circ, \ldots, x_n^\circ$ in operation with chelation prior to loading.

The iterative calculation of the resolution distances and times for mixtures with many components, although straightforward, is lengthy if a computer is not available. However, if the establishment of safe design limits is sufficient, the following inequalities can be used. For development of a uniform initial band:

$$z_{jk} < \frac{1/\alpha_{1j}}{1/\alpha_{1j} - 1/\alpha_{1k}} \Delta z, \qquad \tau_{jk} < \frac{1/h_j^\circ}{1/\alpha_{1j} - 1/\alpha_{1k}} \prod_{i=j+1}^{k-1} \left(\frac{\alpha_{1i}}{h_i^\circ}\right) \Delta z \tag{24}$$

and for operation with chelation prior to loading:

$$z_{jk} < \frac{h_j^\circ}{\alpha_{1k}-\alpha_{1j}} \prod_{i=j+1}^{k-1} \left(\frac{h_i^\circ}{\alpha_{1i}}\right) \Delta z, \qquad \tau_{jk} < \frac{\alpha_{1k}}{\alpha_{1k}-\alpha_{1j}} \Delta z \tag{25}$$

[The right-hand sides of these inequalities are calculated with constant values of the velocities of Δh_j and Δh_k equalling the actual values at the zone jk, and therefore constitute upper limits because the actual velocity difference is larger initially and decreases with every crossing of other trajectories (see Fig. 6).]

The equations for the two types of operation reflect certain symmetry properties, more fully discussed in the original theory[10]: the equations for one type can be obtained from those of the other by interchange of x and y and of z and τ, replacement of u, h, and α by their reciprocals, and change of the indices i for the species to $n+1-i$ and for roots to $n-i$.

Notation in the original theory

A slightly different notation has been used here than in the original theory[10], where, to be consistent with the treatment of other kinds of operation, the development ion is species 1, the species of the mixture are 2, ..., $n-1$, and the retaining ion is n. An m-component separation then appears as an $(m+2)$-component problem and,

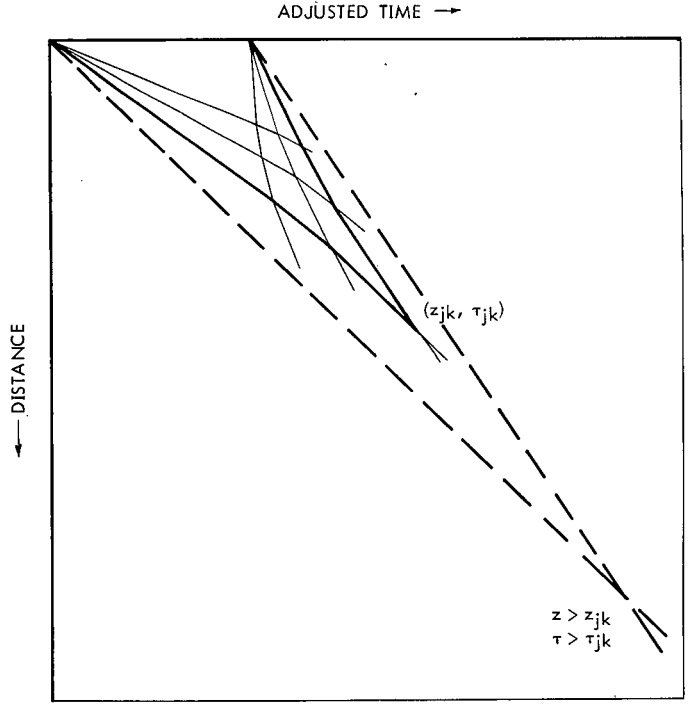

Fig. 6. Calculation of upper limits of resolution distances and times. Solid lines are actual trajectories; broken lines are fictitious linear trajectories used to calculate inequalities (see eqn. 25).

compared with the present treatment, all roots and separation factors (now relative to the development ion) are larger by a factor equalling the separation factor of the development ion and the first ion of the mixture. The invariance to the properties of the development and retaining ions and the attendant possibility of simplifying the notation as done here are discussed in the original.

COMPUTATION FROM OPERATING VARIABLES

The theory outlined above and the computer program RAREARTH[11] are in terms of normalized and adjusted variables. Practical application will require conversion from and to actual physical variables such as volumetric flow rate, column properties, ion-exchange capacity, true distances and times for resolution, etc.

In order to calculate normalized resolution distances and times (Z and T, in units of band width Δz) as well as adjusted boundary velocities u_Δ and compositions $x_1, \ldots x_n$ or $y_1, \ldots, y_n$ in the transient pattern, either by hand or with the program RAREARTH, only the separation factors α_{1i} and the fractional rare-earth concentrations in the original mixture are needed. As has been discussed by POWELL AND SPEDDING[4], the separation factors in rare-earth separations by ion exchange are essentially given by the ratios of the stability constants of the complexes formed with the development agent, stronger complexing resulting in lesser affinity for the resin. At least for standard development agents such as ethylenediaminetetraacetic acid (EDTA), the separation factors can thus be calculated from tabulated stability constants*. The fractional rare-earth concentrations in the original mixture are taken as $y_1^\circ, \ldots, y_n^\circ$ in development of a uniformly loaded initial band, and as $x_1^\circ, \ldots, x_n^\circ$ in operation with chelation prior to loading.

Because ion exchange with dilute solutions is essentially stoichiometric and because the exchange at the front and rear of the rare-earth band is complete, *i.e.*, involves complete conversion of the resin to and from the rare-earth form, the total concentration (in mequiv./cm^3 liquid phase) is the same in the influent (development agent), within the band, and in the effluent. The conversion of fractional liquid-phase concentrations x_i in the rare-earth band or effluent into actual concentrations c_i (in mequiv./cm^3 liquid phase) thus is

$$c_i = c\, x_i \qquad \text{(mequiv./cm}^3\text{ liquid phase)} \tag{26}$$

where c is the concentration of the development agent (in these units). The conversion to mequiv./cm^3 of column requires multiplication with the fractional intraparticle void volume, ε:

$$C = \varepsilon c \qquad \text{(mequiv./cm}^3\text{ column)} \tag{27}$$

The ion-exchange capacity (in mequiv./cm^3 of column) can be used directly as the total concentration, $\bar{C}$, of rare earths in the ion exchanger. The conversion of the fractional concentrations y_i to any desired units then is obvious.

The conversion of the respective normalized variables to true resolution distances and times and true boundary velocities requires, in addition to C and $\bar{C}$, a knowledge of the linear flow rate, u_0, and the (adjusted) band width Δz. The linear flow rate is the

* With the possible exception of yttrium, for which the separation factor may have to be determined experimentally.

volumetric flow rate $\dot{V}$ (in cm³/sec) divided by the cross-sectional area not occupied by the resin:

$$u_0 = \dot{V}/\varepsilon S \qquad \text{(cm/sec)} \tag{28}$$

where S is the cross-sectional area of the column (in cm²). The quantity Δz is the width of the rare-earth band measured along any line of constant τ in the distance-time diagram. The true width (*i.e.*, measured along a line of constant true time, t) of the band equals the total rare-earth charge, Q (in mequiv.), divided by the overall concentration $\bar{C} + C$ (total number of mequiv. per cm³ column) and the cross-sectional area, S. Conversion with eqn. (3) shows that the width along a line of constant τ then is

$$\Delta z = Q/\bar{C}S \qquad \text{(cm)} \tag{29}$$

The normalized resolution distances $Z \equiv z/\Delta z$ and $T \equiv \tau/\Delta z$, calculated by hand or obtained as output of the program RAREARTH, can now be converted into true distances and times. For distances, from eqn. (29),

$$z = \Delta z\, Z = QZ/\bar{C}S \qquad \text{(cm)} \tag{30}$$

and for times, with eqns. (3) and (27) to (30)

$$t = \frac{1}{u_0}\left(\frac{\bar{C}}{C}\tau + z\right) = \frac{\varepsilon S\,\Delta z}{\dot{V}}\left(\frac{\bar{C}}{\varepsilon c}T + Z\right) = \frac{\varepsilon Q}{\dot{V}}\left(\frac{T}{\varepsilon c} + \frac{Z}{\bar{C}}\right) \text{ (sec)} \tag{31}$$

Moreover, as can be shown from eqn. (3), the conversion of the adjusted boundary velocities, u_Δ, into true velocities u_Δ is

$$u_\Delta = \frac{u_0}{1 + \bar{C}/Cu_\Delta} = \frac{\dot{V}/\varepsilon S}{1 + \bar{C}/\varepsilon c u_\Delta} \quad \text{(cm/sec)} \tag{32}$$

A slight complication arises in operation with chelation prior to loading if the flow rate and concentration have different values, u_0' and C' (corresponding to $\dot{V}'$ and c'), during loading than during development. The calculation of the normalized variables (x_i, y_i, u_Δ, Z, T) is not affected, but some of the conversions to true physical variables are. The true time for loading will be

$$\Delta t' = Q/c'\dot{V}' \qquad \text{(sec)} \tag{33}$$

The conversion from adjusted or normalized time to true time (counted from the start of loading) then is

for $t \leq \Delta t'$ (corresponding to $T \leqslant 1 - \varepsilon c'Z/\bar{C}$)

$$t = \frac{(\bar{C}/C')\tau + z}{u_0'} = \frac{\varepsilon Q}{\dot{V}'}\left(\frac{T}{\varepsilon c'} + \frac{Z}{\bar{C}}\right)$$

for $\Delta t' \leq t \leq \Delta t' + \dfrac{z}{u_0}$ (corresponding to $1 - \varepsilon c'Z/\bar{C} \leq T \leq 1$)

$$t = \Delta t' + \frac{z - (\bar{C}/C')\,(\Delta z - \tau)}{u_0} = Q\left[\frac{1}{c'\dot{V}'} + \frac{\varepsilon}{\dot{V}}\left(\frac{Z}{\bar{C}} - \frac{1-T}{\varepsilon c'}\right)\right] \tag{34}$$

for $t \geq \Delta t' + \frac{z}{u_0}$ (corresponding to $T \geq 1$)

$$t = \Delta t' + \frac{z + (\bar{C}/C)(\tau - \Delta z)}{u_0} = Q\left[\frac{1}{c'\dot{V}'} + \frac{\varepsilon}{\dot{V}}\left(\frac{Z}{\bar{C}} + \frac{T-1}{\varepsilon c}\right)\right]$$

In addition, the conversions to true velocities and concentrations are affected as follows: for $t < \Delta t'$ (corresponding to $T < 1 - \varepsilon c' Z/\bar{C}$), u_0' (or $\dot{V}'$) is to be substituted for u_0 (or $\dot{V}$) in eqn. (32), and for $t < \Delta t' + z/u_0$ (corresponding to $T < 1$), c' and C' are to be substituted for c and C, respectively, in eqns. (26) and (32).

The relatively complex conversion to true time by eqns. (34) is a consequence of the fact that a flow-rate variation is instantaneously propagated through the column, the liquid being virtually incompressible, whereas a concentration variation in the liquid is propagated only at the rate of liquid-phase flow (assuming that the concentration $\bar{C}$ in the ion exchanger is not appreciably changed). As is illustrated in Fig. 7, the flow rate is u_0' to the left of the line $t = \Delta t'$, and is u_0 to the right of this line, while the concentration is C' to the left of the line $t = \Delta t' + z/u_0$ (which is also the line $T = 1$ and $\tau = \Delta z$), and is C to the right of this line. This leads to the three regions, separated by the two lines, and each covered by one of the three eqns. (34). (For

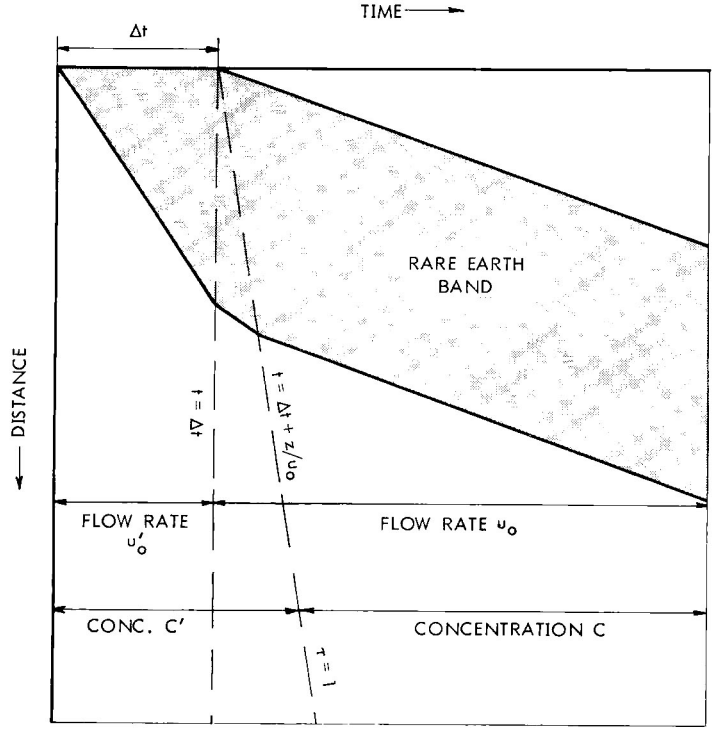

Fig. 7. Chelate loading with different flow rate and concentration (schematic). Changes in slope of band front correspond to loading with flow rate and concentration both twice as high as for development.

clarity, Fig. 7 greatly exaggerates the angle between the lines $t = \Delta t'$ and $t = \Delta t' + z/u_0$.)

An unexpected result is that the true distances (*e.g.*, of resolution) are invariant to changes of flow rate and concentration, since the conversion factor Δz, according to eqn. (29), is independent of these variables. This holds for development of a uniform initial band as well*.

It has been tacitly implied above that the separation factors are not altered by the concentration variation. If they are, an exact calculation, while entirely feasible, becomes quite complex. The computation must then be carried to $\tau = \Delta z$ (corresponding to $T = 1$) with the separation factors pertaining to c', and the composition profile along $\tau = \Delta z$ must then be used as the initial condition for a new calculation with the factors pertaining to c. In general, all boundaries existing at $\tau = \Delta z$, being coherent under the old set of separation factors, will be noncoherent under the new set and will therefore each give rise to a new set of coherent boundaries. This complication exceeds the faculties of the RAREARTH program. Fortunately, the actual concentration dependence of the separation factors of the rare earths is usually not larger than the uncertainty of their measurement, except possibly for lanthanide–yttrium systems. Also, the design is usually dictated by one of the longer resolution distances and times, and these are not much affected by somewhat different separation factors during loading: for species hard to separate, the loading time is but a small fraction of the resolution time and gives very little segregation. For most practical purposes, different separation factors during loading can therefore by ignored.

Another complication not covered here arises if one or several front or rear portions of the incompletely developed band are cut off at some intermediate stage or stages. Boundary trajectories that lead into the discarded portion will then be shifted, at the cut-off distance, to the cut-off point without change in slope, and none of the transient compositions will be altered. The detailed theory for this type of operation, which is of some practical importance, will be presented in a separate publication.

A PRACTICAL EXAMPLE

The practical application of the theory will be illustrated by the calculations for an ion-exchange separation of the rare earths of euxenite by displacement development with ethylenediaminetetraacetic acid (EDTA). A typical fractional composition of the fifteen rare earths of euxenite, a mineral rich in the yttrium earths, is shown in Table II. Table II also lists the separation factors relative to lanthanum (species 1) in the presence of EDTA as calculated from the EDTA stability constants given by WHEELWRIGHT *et al.*[13].

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* The independence in the range $C \ll \bar{C}$ is, of course, well known from simpler theories. What is shown here is that it extends beyond this range. A compensation of effects is involved. As is known from general chromatographic theory, the separation efficiency lessens with increasing mobile-phase concentration because the relative migration rates of the molecules of the species become more similar (*e.g.*, see ref. 12). In the present case, the numerical values of the resolution distances expressed in units of band length (at constant true time, as used by POWELL *et al.*) indeed increase, but this turns out to be compensated by the shorter length of the band, requiring lesser distances of relocation within the band upon separation, so that the true distances in cm are invariant.

TABLE II

TYPICAL RARE-EARTH COMPOSITION OF EUXENITE AND SEPARATION FACTORS OF EDTA COMPLEXES

Index number	*Element*	*Mole fraction*	*Separation factor* α_{1i}
1	Lanthanum	0.006	(1.000)
2	Cerium	0.015	4.68
3	Praseodymium	0.002	10.72
4	Neodymium	0.008	21.88
5	Samarium	0.010	67.61
6	Europium	0.001	93.33
7	Gadolinium	0.035	95.50
8	Terbium	0.016	457.1
9	Yttrium	0.622	691.8
10	Dysprosium	0.095	1,072
11	Holmium	0.035	3,890
12	Erbium	0.090	6,761
13	Thulium	0.015	22,380
14	Ytterbium	0.042	46,770
15	Lutetium	0.008	85,110

For a preparative separation on pilot-plant scale with a general-purpose strong-acid cation-exchange resin, realistic operating variables would be about as follows:

Amount of rare earths	$Q = 20{,}000$ mequiv.
Cross-sectional area of column	$S = 100$ cm^2
Fractional intraparticle void volume	$\varepsilon = 0.4$
Ion-exchange capacity	$\bar{C} = 2.0$ mequiv./cm^3 column
Concentration of development agent	$c = 0.10$ mequiv./cm^3 solution
Volumetric flow rate	$\dot{V} = 2.0$ cm^3/sec

Under these conditions, the band width is 100 cm. (Because $c \ll \bar{C}$, there is virtually no difference between the widths measured along lines of constant t and of constant τ, the "adjustment" of τ by $-z$ in eqn. (3) being negligible.) The time required to displace the band by a distance equal to its own length is $\Delta t = Q/c\dot{V} = 100{,}000$ sec $= 27.8$ h.

Distance–time diagrams for development of a uniformly loaded initial band and for operation with chelation prior to loading, calculated with the program RAREARTH from the data in Table II, are shown in Figs. 8 and 9. Scales of true distance and time have been added in accordance with the operating conditions listed above. For operation with prior chelation, the true-time scale presumes that the concentration and flow rate are the same for loading as for development. For reasons of scale, the diagrams do not extend to the point of resolution of gadolinium from europium, at $Z = 2.644$, $T = 2.601$ for development of a uniform initial band, and at $Z = 1.661$, $T = 2.619$ for operation with prior chelation.

A comparison of the distance–time diagrams for the two types of operation is instructive. In general, development of a uniform initial band requires longer distances but shorter times for resolution. (It should be noted, however, that the time required for loading the initial band is not included!) Development of a uniform band tends to give long resolution distances particularly for the species of low affinity for the resin (*i.e.*, species with a high index number), and operation with prior chelation tends to

give long resolution times particularly for species with high affinity (low index number). Thus, there is incentive to switch from the conventional development of a uniform band to chelated loading if excessive column length is required to resolve a critical pair of low-affinity species.

Another feature that comes out clearly in the example in Figs. 8 and 9 is that resolution of a major component from its neighbors tends to require long distances and times even if the respective separation factors are favorable. Thus resolution of the main component, yttrium (mole fraction 0.622), from terbium in development of a uniform band, and from dysprosium in operation with prior chelation, requires almost as long a distance and time as that of the pair europium–gadolinium, although the separation factors $\alpha_{Tb,Y} = 1.51$ and $\alpha_{Y,Dy} = 1.55$ are significantly larger than $\alpha_{Eu,Gd} = 1.023$.

APPENDIX

Root variations across coherent boundaries

The rule that only one H-function root varies across any coherent boundary can be derived, for displacement development, as follows. According to eqn. (5), the adjusted velocity of a concentration step of an arbitrary species j, regardless of the behavior of other species, is

$$u_A = \frac{x_j' - x_j''}{y_j' - y_j''} \tag{A.1}$$

where primes and double primes refer to the two sides of the step. Expressing the x_j and y_j in terms of h_i by means of eqns. (14) and (15) one finds

$$u_A = \frac{\prod_{i=1}^{n-1}(h_i' - \alpha_{1j}) - \prod_{i=1}^{n-1}(h_i'' - \alpha_{1j})}{\prod_{\substack{i=1 \\ i\neq j}}^{n} \alpha_{1i} \left\{ \prod_{i=1}^{n-1}[(h_i' - \alpha_{1j})/h_i'] - \prod_{i=1}^{n-1}[(h_i'' - \alpha_{1j})/h_i''] \right\}} \tag{A.2}$$

The condition of coherence is that u_A, and therefore the right-hand side of eqn. (A.2), will have the same value for all species $j = 1, \ldots, n$. The right-hand side of this equation will meet this condition if, and only if, all features distinguishing j from other species disappear. For this to be the case, all products in the numerator as well as in the denominator must have all factors but one in common. In view of the limits imposed on the root values by condition (9), this requires

$$h_i' = h_i'' \qquad \text{for all } i \neq k \tag{A.3}$$

where k may be any number $1, \ldots, n-1$. That is, all roots but one, h_k, must have the same value on both sides of the step. (At least one root must vary across the step, which otherwise would be nonexistent.) With this restriction, eqn. (A.2) is readily shown to reduce to eqn. (16).

This abbreviated proof presupposes that: (1) the step remains sharp, and (2) equalities $h_i'_{-1} = h_i''$ or $h_i' = h_i''_{-1}$, admitted by condition (9), do not occur.

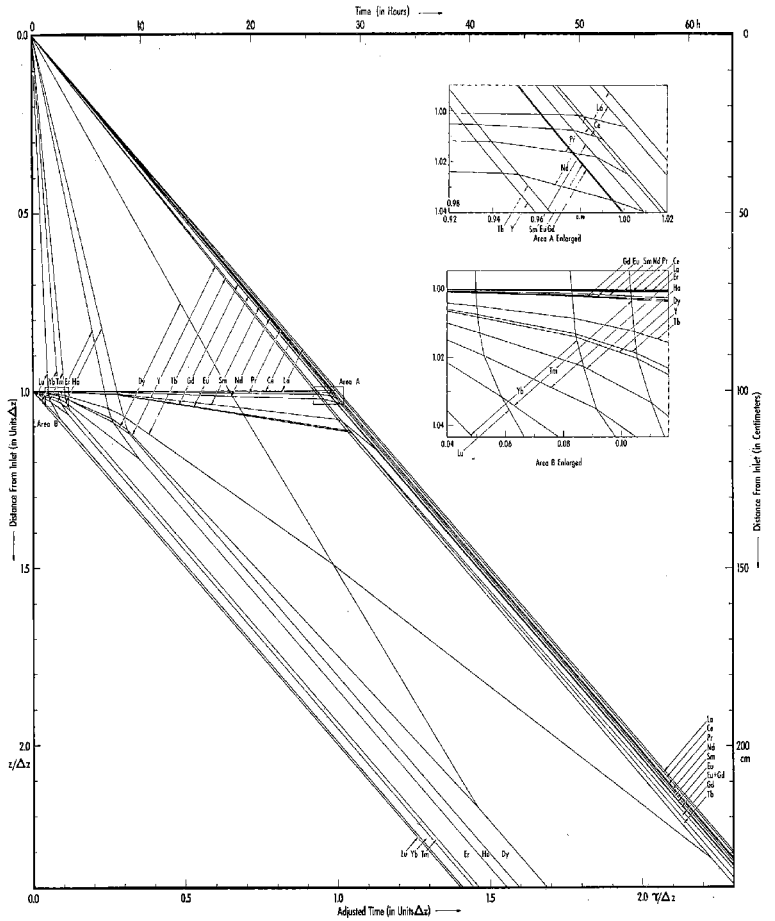

Fig. 8. Distance–time diagram of separation of rare earths of euxenite by development of uniform initial band.

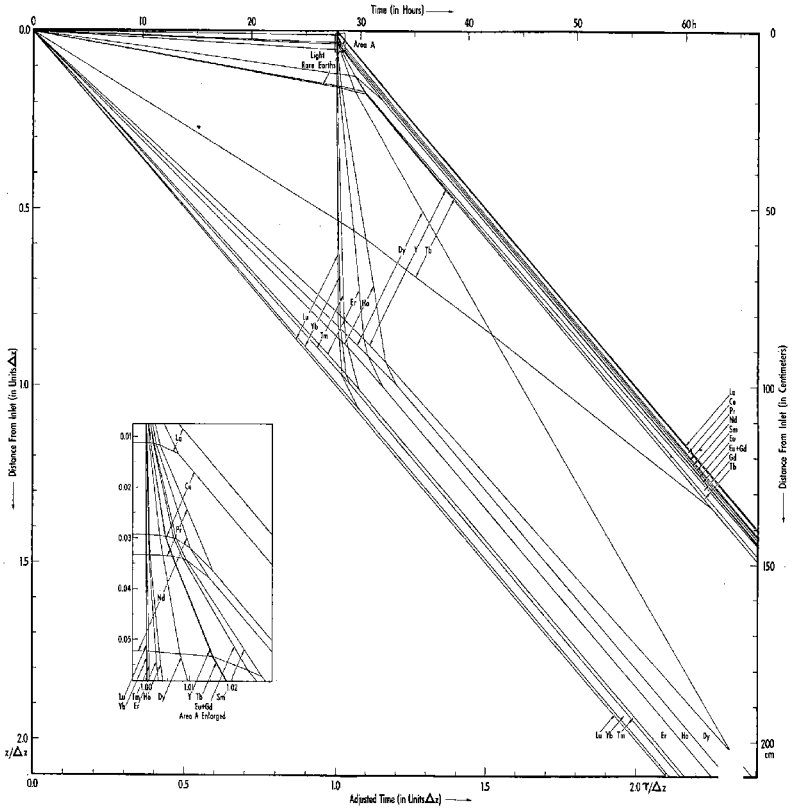

Fig. 9. Distance–time diagram of separation of rare earths of euxenite with chelation prior to loading.

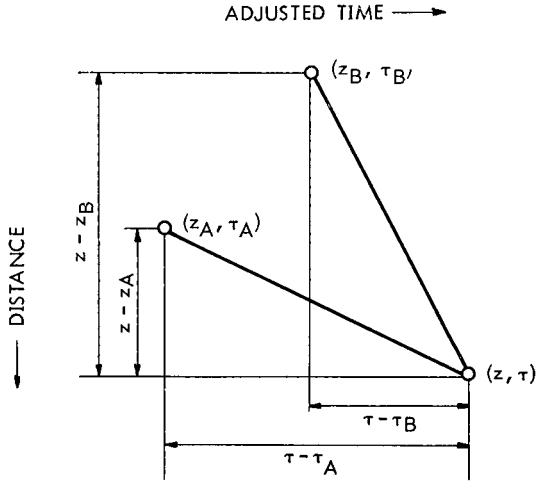

Fig. 10. Trajectories for triangle formula. (From HELFFERICH AND KLEIN[10].)

Calculation of resolution distances and times

The coordinates (z, τ) of intersection of two linear trajectories A and B, with points of origin (z_A, τ_A) and (z_B, τ_B) and slopes u_A and u_B, are calculated as follows. As Fig. 10 shows:

$$u_A = \frac{z - z_A}{\tau - \tau_A} \quad \text{and} \quad u_B = \frac{z - z_B}{\tau - \tau_B} \tag{A.4}$$

Solving for τ and z one finds

$$\tau = \frac{u_B\tau_B - u_A\tau_A - z_B + z_A}{u_B - u_A}$$

$$z = z_A + (\tau - \tau_A)u_A \quad \text{or} \quad z = z_B + (\tau - \tau_B)u_B \tag{A.5}$$

Appropriate values of u_A and u_B can be substituted by means of eqns. (21) and (22). The "triangle formula" (A.5) can then be used to calculate (z_{1n}, τ_{1n}), for which the substitutions are

$$z_A = \Delta z, \quad \tau_A = 0, \quad z_B = 0, \quad \tau_B = 0 \tag{A.6}$$

for development of a uniform initial band, and

$$z_A = 0, \quad \tau_A = 0, \quad z_B = 0, \quad \tau_B = \Delta z \tag{A.7}$$

for operation with chelation before loading. (Note that the band width in the τ direction is also Δz, since the slope of the trajectories of the front and rear boundaries of the band is unity.) Further values (z_{1k}, τ_{1k}) $(k = n-1, \ldots, 2)$ and (z_{jn}, τ_{jn}) $(j = 2, \ldots, n-1)$ are then calculated with the triangle formula as recursion formula, with substitution of the previously calculated $(z_{1,k+1}, \tau_{1,k+1})$ for (z_A, τ_A), or $(z_{j-1,n}, \tau_{j-1,n})$ for (z_B, τ_B). For the simpler expressions in binary and ternary separations, the root or roots can be expressed in terms of x_i or y_i by means of eqns. (12) or (13); this has been done in Table I.

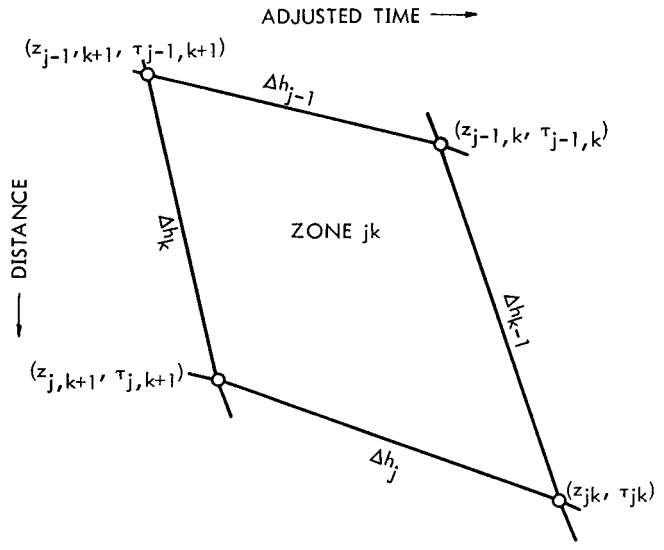

Fig. 11. Trajectories for four-point formula. (From HELFFERICH AND KLEIN[10].)

In principle, the various z_{jk} and τ_{jk} $(2 \leq j < k \leq n-1)$ in separations of four or more species can also be calculated with eqn. (A.5) as recursion formula. However, the "four-point" formulas in Table I, which allow sets of z_{jk} and of τ_{jk} to be generated independently of one another are more convenient. The formula for calculating z_{jk} from $z_{j-1,k}$, $z_{j,k+1}$, is obtained if the equations for the slopes of the four trajectories bounding the zone jk

$$\begin{aligned}
\frac{z_{jk} - z_{j,k+1}}{\tau_{jk} - \tau_{j,k+1}} &= \prod_{i=j}^{k-1} \frac{h_i^\circ}{a_{1,i+1}} \\
\frac{z_{jk} - z_{j-1,k}}{\tau_{jk} - \tau_{j-1,k}} &= \prod_{i=j}^{k-1} \frac{h_i^\circ}{a_{1i}} \\
\frac{z_{j,k+1} - z_{j-1,k+1}}{\tau_{j,k+1} - \tau_{j-1,k+1}} &= \prod_{i=j}^{k} \frac{h_i^\circ}{a_{1i}} \\
\frac{z_{j-1,k} - z_{j-1,k+1}}{\tau_{j-1,k} - \tau_{j-1,k+1}} &= \prod_{i=j-1}^{k-1} \frac{h_i^\circ}{a_{1,i+1}}
\end{aligned} \tag{A.8}$$

(see Fig. 11) and the continuity condition

$$\begin{aligned}
&(\tau_{jk} - \tau_{j,k+1}) - (\tau_{jk} - \tau_{j-1,k}) + (\tau_{j,k+1} - \tau_{j-1,k+1}) \\
&\quad - (\tau_{j-1,k} - \tau_{j-1,k+1}) = 0
\end{aligned} \tag{A.9}$$

are solved for the five unknowns, that is, for z_{jk} and the four time differences appearing in eqns. (A.8) and (A.9). The derivation of the formula for τ_{jk} is analogous.

SYMBOLS

$A, \bar{A}, B, \bar{B}$	parameters in eqn. (13)
c	concentration of development agent (mequiv./cm³ solution)
c'	total rare-earth concentration during loading of chelated mixture (mequiv./cm³ solution)
c_i	concentration of rare earth i in liquid phase in rare-earth band (mequiv./cm³ liquid phase)
C	total rare-earth concentration in liquid phase in rare-earth band (mequiv./cm³ column)
C'	$= \varepsilon c'$ (mequiv./cm³ column)
$\bar{C}$	ion-exchange capacity (mequiv./cm³ column)
h	argument of the H-function, eqns. (7) and (8)
h_i	i'th root of H-function
n	number of species in original mixture
Q	total amount of rare earths (mequiv.)
S	cross-sectional area of column (cm²)
t	time (sec)
T	$= \tau/\Delta z$
u_0	linear flow rate (cm/sec)
u_Δ	true velocity of boundary (cm/sec)
u_Δ	adjusted velocity of boundary
$\dot{V}$	volumetric flow rate (cm³/sec)
x_i	fractional liquid-phase concentration of rare earth i
y_i	fractional resin-phase concentration of rare earth i
z	distance from top of bed (cm)
z_{ij}	resolution distance of rare earths i and j cm
Z	$= z/\Delta z$
α_{ij}	separation factor of rare earths i and j cm
τ	adjusted time
τ_{ij}	adjusted resolution time of rare earths i and j cm
$h_i^\circ, x_i^\circ, y_i^\circ$	values of h_i, x_i, y_i in uniform initial band or in chelated mixture being loaded
$u_0', \dot{V}'$	values of u_0 and $\dot{V}$ during loading of chelated mixture
h_i', x_i', y_i'	values of h_i, x_i, y_i on upstream side of boundary
h_i'', x_i'', y_i''	values of h_i, x_i, y_i on downstream side of boundary
$\Delta C_i, \Delta \bar{C}_i, \Delta h_i, \Delta x_i, \Delta y_i$	variation of $C_i, \bar{C}_i, h_i, x_i, y_i$ across boundary
$\Delta t'$	time required for loading of chelated mixture (sec)
Δz	adjusted band width (cm)

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CHROM. 4465

CONTRIBUTION TO THE THEORY OF THE RETENTION INDEX SYSTEM

I. RETENTION INDICES USING PROGRAMMED-TEMPERATURE GAS CHROMATOGRAPHY

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SUMMARY

After examining the relationship between retention index and column temperature under isothermal conditions, we have shown in a recent publication that the temperature-dependence of the retention index may generally be determined by an Antoine-type equation:

$$I^{\text{stationary phase}}_{\text{substance}}(T) = A + \frac{B}{T + C}$$

After further investigation, we have established that the retention indices can be exactly calculated from programmed-temperature gas chromatography data with the following equation:

$$\frac{\int_{T_0}^{T_r} \left(A + \frac{B}{T + C}\right) dT}{T_r - T_0} = A + \left[\frac{2.3 \cdot B \cdot \log \frac{(T_r + C}{(T_0 + C)}}{T_r - T_0}\right]$$

A great advantage of this equation is that the data from normal hydrocarbons are not included in it.

INTRODUCTION

It is generally known that working without pure standard substances, the retention index system worked out by KOVÁTS[1] is the most important one among the qualitative evaluation methods of gas chromatography. The retention indices give a suitable foundation—beyond the application mentioned—for other investigations[2,3], and as a result of this recognition increasing interest has been manifested in recent years in some problems of the retention index system. Among these questions, the application of the retention index system is most prominent in programmed-temperature gas chromatography (PTGC). Although in this field the appropriate equations of VAN DEN DOOL AND KRATZ[4], GUIOCHON[5], HABGOOD AND HARRIS[6] and GIDDINGS[7],

among others, are available the differences in index experienced in some cases have stimulated the researchers working in this field to undertake further investigations. Also GOLOVNYA AND URALETZ[8], in addition to suggesting a new equation for using isothermal retention indices under PTGC conditions, are dealing with index-influencing factors and also are comparing different methods of index conversion.

In examining the relationship between retention index and column temperature under isothermal conditions, we recently demonstrated[9] that the temperature dependence of the retention index may generally be determined by an Antoine-type equation:

$$I^{\text{st. ph.}}_{\text{substance}}(T) = A + \frac{B}{T + C} \tag{1}$$

where

I = the symbol for the retention index

T = column temperature in °K

A, B and C = constants of the Antoine-type eqn. (1).

The three constants of the equation depend only on the quality of the substance and of the stationary phase and can easily be determined from experimental results[9]. Considering this information, we supposed that relying on eqn. (1), the index values in PTGC may be exactly described. In this paper we report on the results reached so far, based on data in the literature[4–8] and on our measurements.

THEORY

In our investigations we have been concerned only with cases in which the continuous temperature programming started simultaneously with the introduction of the sample and terminated only after the elution of the component examined; it may be described by a constant heating rate, β (°C/min). In the case examined, the following relationship is applicable, using the average value of integration:

$$I^{\text{st. ph.}}_{\text{substance}}(\text{PTGC}) = \frac{\int_{T_0}^{Tr} \left(A + \frac{B}{T + C}\right) \mathrm{d}T}{T_r - T_0} \tag{2}$$

where

T_r = retention temperature in °K

T_0 = initial temperature in °K.

Solution of the integral calculus in the numerator on the right-hand side of eqn. (2) yields:

$$\int_{T_0}^{Tr} \left(A + \frac{B}{T + C}\right) \mathrm{d}T = [AT + B \cdot \ln\ (T + C)]_{T_0}^{Tr} =$$

$$AT_r + B \cdot \ln (T_r + C) - AT_0 - B \cdot \ln (T_0 + C) = A\,(T_r - T_0) +$$

$$[\ln (T_r + C) - \ln (T_0 + C)] \cdot B = A\,(T_r - T_0) + B \cdot \ln \left(\frac{T_r + C}{T_0 + C}\right) =$$

$$A\,(T_r - T_0) + 2.3 \cdot B \cdot \log \left(\frac{T_r + C}{T_0 + C}\right) \tag{3}$$

Thus for eqn. (2) we may write:

$$I^{\text{st. ph.}}_{\text{substance}}(\text{PTGC}) = \frac{A\,(T_r - T_0) + 2.3 \cdot B \cdot \log\left(\frac{T_r + C}{T_0 + C}\right)}{T_r - T_0} =$$

$$A + \frac{2.3 \cdot B \cdot \log\left(\frac{T_r + C}{T_0 + C}\right)}{T_r - T_0} \qquad (4)$$

Eqn. (4) is suitable for the exact calculation of retention indices in PTGC.

EXPERIMENTAL

Our measurements were made using gas chromatographs (Carlo Erba Model C, D, GV and GI-452) with flame ionisation detectors in all cases. Carrier gas was N_2, while auxiliary gases were H_2 and O_2. Stationary phases were squalane; the silicone oils, SE-30, DC-200, DC-550, DC-702; Apiezon L; di-ethyleneglycol adipate; di-ethyleneglycol succinate; polyethylene glycol 1500 (PEG-1500); and polyethylene glycol 20 M.

As an example we present the calculation of benzene using PEG-1500 as stationary phase. The retention index of benzene on PEG-1500 under isothermal conditions is dependent on column temperature according to the following equation:

$$I^{\text{PEG-1500}}_{\text{Benzene}}(T) = 1\,718 - \frac{1\,032\,240}{T + 967} \qquad (5)$$

When the initial temperature, T_0 was 84°C, the retention temperature, T_r was 99°C. [$\beta = 4.5$°C/min]. The corresponding data are substituted into eqn. (4) and the following calculations are made.

$$I^{\text{PEG-1500}}_{\text{Benzene}}(\text{PTGC}) = 1\,718 + \frac{2.3 \cdot (-1\,032\,240) \cdot \log\left(\frac{372 + 967}{357 + 967}\right)}{372 - 357} =$$

$$1\,718 + \frac{2.3 \cdot (-1\,032\,240) \cdot \log\left(\frac{1\,339}{1\,324}\right)}{15} =$$

$$1\,718 + \frac{2.3 \cdot (-1\,032\,240) \cdot \log 1.0113}{15} =$$

$$1\,718 - \frac{2.3 \cdot 1\,032\,240 \cdot 0.0049}{15} =$$

$$1\,718 - 776 = 942 \qquad (6)$$

We compared data in the literature with that calculated by eqn. (6). The results are shown in Table I.

TABLE I

COMPARISON OF RETENTION INDEX DATA

Index units		
Literature data	*Calculated by eqn. (6)*	*Divergence from calculated value*
944[a]	942	+2
943[b]	942	+1
943[c]	942	+1

[a] VAN DEN DOOL AND KRATZ (ref. 4).
[b] GIDDINGS (ref. 7).
[c] GOLOVNYA AND URALETZ (ref. 8).

SYMBOLS

I = retention index
T = column temperature
T_0 = initial temperature
T_r = retention temperature
A, B and C = constants of Antoine-type equation
β = heating rate
st. ph. = stationary phase

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CHROM. 4435

BAND SPREADING IN MOLECULAR-SIEVE CHROMATOGRAPHY

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SUMMARY

Band spreading was studied in aqueous molecular-sieve chromatography systems using carbohydrate solutes. Bio-Gel P-2, Sephadex G-10, and Sephadex G-15 were used. P-2 gel was prepared in three size fractions by wet screening and elutriation. All gel preparations were characterized by extensive size measurements with a digital coding microcomparator. The band spreading behavior of solutes followed a theory based on the additivity of the mechanisms of axial dispersion and slow mass transfer. The molecular diffusion of solute in the gel phase was found to be the controlling mechanism of mass transfer (with substances of low molecular weight). A diameter equivalent to the most probable spherical volume was used in describing slow gel phase diffusion. Using the random walk theory of eddy diffusion, it was found that a diameter equivalent to the most probable specific area of the particles was best to describe axial dispersion.

INTRODUCTION

In all types of chromatography, band spreading is present and detracts from the efficiency of the separation process. Polymer applications of molecular-sieve chromatography (MSC) endeavor to order a disperse molecular weight preparation. The resulting chromatograms show the distribution of molecular weights as modified by band spreading. Other applications of MSC include the separation of solutes into semi-pure fractions. In these operations, band spreading leads to overlapping or cross-contamination of solutes. In both types of MSC operation, the mechanisms leading to band spreading are analogous if not identical.

The first theories of chromatography were descriptive, based on distillation plate models[1,2]. Later, by considering a continuously flowing system, it was possible to incorporate operating parameters such as particle diameter and eluent flow rate[3,4].

* A portion of a thesis submitted by NEALE POVEY in partial fulfillment of the requirements of The Institute of Paper Chemistry for the degree of Doctor of Philosophy from Lawrence University, Appleton, Wisc.; January, 1969.

The most recent theoretical approaches to the mechanisms of band spreading in MSC include coupling[5], flow profile[6], and diffusion-controlled partitioning[7].

The theory used with this work is based on previous developments of HAMILTON *et al.* concerning the behavior of ion-exchange systems[8]. Assuming that axial dispersion and mass-transfer effects can be combined linearly, eqn. (1) was derived[9]

$$H\alpha = \frac{2\,D_{ax}}{U} + \frac{2\,K_1^2\,\varepsilon U}{(K_1+\varepsilon)^2\,K_L} \tag{1}$$

Assuming that slow gel-phase diffusion-controlled mass-transfer resistance, eqn. (2) was derived[9].

$$H\alpha = 2\,\lambda d_p + \frac{K_1\,\varepsilon}{(K_1+\varepsilon)^2}\,\frac{d_p^2}{30\,D_g}\,U \tag{2}$$

where H = height of an equivalent theoretical plate, U = interstitial fluid velocity, D_g = gel-phase diffusion coefficient, D_{ax} = effective axial diffusivity, K_1 = distribution coefficient, d_p = diameter of a monodisperse gel fraction, ε = packing porosity, λ = 'eddy diffusion' coefficient, K_L = overall mass-transfer coefficient. The distribution coefficient is defined as

$$K_1 = \frac{V_e - V_0}{V_t}$$

where V_e = elution volume, V_0 = void volume, and V_t = total volume. The variable α is defined as

$$\alpha = \sqrt{1 - \frac{2}{N}} \tag{3}$$

where N = number of theoretical plates. This term was included because the extended plate theory of GLUECKAUF[3] was used in the derivations instead of the simpler theory of MARTIN AND SYNGE[1]. The factor α allows correct calculations to be made in relatively inefficient MSC systems, *ca.* less than 200 theoretical plates.

EXPERIMENTAL

One kilogram of 50–100 mesh Bio-Gel P-2 was fractionated in a Bauer-McNett fiber classifier. Three of the standard screen fractions were further fractionated in a 6×40 cm elutriation column. Ungraded Sephadex G-10 and G-15 gels were fractionated by repeated sedimentation and siphoning.

Gel hydration parameters

The water regain of the gels was measured by a method which combines filtration and vacuum distillation. The P-2 gel was also characterized by a method which measures the increased concentration of an excluded solute when the dry gel is swollen. The gel was swollen in an aqueous solution of a high polymer.

Dental dam method. Common filtration and centrifugation methods of measuring water regain do not satisfactorily account for pore water. This has been discussed by PEPPER *et al.*[10]. To circumvent this, all of the pore water was removed by vapor transport.

A quantity of dry gel was weighed and swollen to equilibrium in water. The

hydrated gel was then washed into a coarse, sintered-glass filter crucible. The bulk of the pore water was removed with suction; then a piece of dental dam was fastened over the crucible and the filtration continued for 20 min. The filter and contents were weighed, and the water regain was calculated using the equation

$$w_r = \frac{\text{wet gel (g)} - \text{dry gel (g)}}{\text{dry gel (g)}} = \frac{\text{hydration water (g)}}{\text{dry gel (g)}} \tag{4}$$

The dental dam served as a flexible barrier, making the entire gel sample subject to vacuum. As shown in Fig. 1, a definite change in the rate of water removal occurred after 20 min. It was inferred from this behavior that pore water was first removed, followed by hydration water. In applying this method to other materials, the weight loss *versus* time behavior must first be studied to ascertain the proper length of filtration.

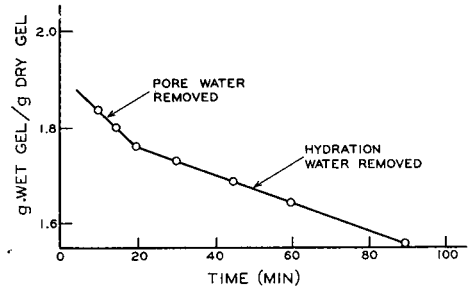

Fig. 1. Water loss during dental dam filtration of hydrated P-2 gel.

Data shown in Table I are an average of five determinations.

TABLE I

WATER REGAIN DATA

	w_r (g/g)	*Average deviation* (g/g)
Bio-Gel P-2 (excluded solute)	1.40	0.04
Bio-Gel P-2 (dental dam)	1.38	0.04
Sephadex G-15 (dental dam)	1.21	0.03
Sephadex G-10 (dental dam)	0.79	0.02

Excluded solute method. Through a simple mass balance, the following equation relating water regain to measurable parameters can be derived:

$$w_r = \frac{V_s \varrho \, (1 - c_0/c)}{M_{\text{gel}}} \tag{5}$$

where w_r = water regain, V_s = volume of solution initially added to dry gel, ϱ = density of solution, M_{gel} = mass of dry gel, c_0 = initial concentration of excluded solute, c = final concentration in interstitial fluid.

The results shown in Table I were obtained from five samples of ungraded Bio-Gel P-2. The solution was a preparation of Sephadex brand Blue Dextran 2000 at an initial concentration of about 375 μg/ml. The gel was removed from the interstitial fluid with a syringe filter, adding the solution directly to the spectrophotometer cells. The initial and final concentrations were determined from an absorbance calibration plot at 600 nm.

Specific volumes

The wet and dry specific volumes of the gel particles are defined as

$$\bar{V}_w = \frac{\text{volume of hydrated gel (ml)}}{\text{dry gel (g)}} \tag{6}$$

and

$$\bar{V}_d = \frac{\text{volume of dry gel (ml)}}{\text{dry gel (g)}} \tag{7}$$

These specific volumes can be used to calculate a useful parameter, the gel porosity, defined as

$$\varepsilon_g = \frac{\text{hydrated volume of gel}}{\text{total volume of gel}} \tag{8}$$

Using the relationship

$$\varepsilon_g = 1 - \frac{\bar{V}_d}{\bar{V}_w} \tag{9}$$

the specific volumes were calculated from measurements of sedimented volumes in graduated cylinders. The wet volume was measured in water, and the dry volume was measured in absolute ethanol, a nonswelling solvent for the gels. The porosity of 0.47 for random loose arrangements of spheres[11] was used to calculate actual gel volumes from the observed sedimented volumes. Data shown in Table II are an average of seven determinations.

TABLE II

WET AND DRY SPECIFIC VOLUME

Gel	$\bar{V}_w$ *(cm³g⁻¹)*	*Average deviation*	$\bar{V}_d$ *(cm³g⁻¹)*	*Average deviation*	ε_g
P-2	2.01	0.023	0.994	0.008	0.506
G-10	1.21	0.006	0.952	0.023	0.215
G-15	1.63	0.011	0.869	0.011	0.466
G-25	2.47	0.020	0.663	0.005	0.731

Gel diameter measurements

About 1500 diameters were measured from each of three P-2 gel preparations and about 1000 from the G-10 and G-15 preparations. Measurements were made with a digital coding microcomparator coupled with a data card punch. The data were compiled through the computer facilities at The Institute of Paper Chemistry. The

gel preparations were characterized by the various averages shown in Table V and additional distribution parameters which have been discussed elsewhere[9].

Solutes

The solutes were commercial preparations, purified by filtering the solutions with Darco G-60 activated carbon. Information pertinent to the solutes is given in Table III.

TABLE III

SOLUTES

Solute	*Abbreviation*	*Molecular weight*	*Diffusion coefficient, D_m ($25°$, H_2O, $cm^2sec^{-1} \times 10^6$)*
Glycine	GY	75	10.64
Glycerin	GC	95	9.4
Glucose	GLC	180	6.73
Sucrose	SUC	342	5.21
Raffinose	RAF	504	4.34
Schardinger α-dextrin	SAD	972	3.44
Schardinger β-dextrin	SBD	1,134	3.22
Sephadex brand Dextran 10	DEX	12,000	1.095

Chromatographic system

The equipment used in the experimental measurement of band spreading is shown in Fig. 2. The deaerator consisted of a heater and a bubble collector constructed

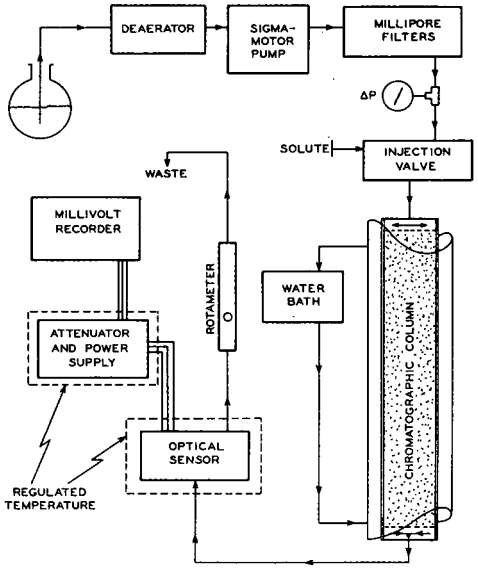

Fig. 2. Experimental chromatographic system.

from glass. A Sigmamotor Series T-8 pump was used with a duplex manifold of 3/16-in. O.D. Tygon tubing, formulation S 50 HL. Two Millipore filters were used, 8 μm and 3 μm. The injector valve was Chromatronix Model SV-8031 and the column was Chromatronix Model LC-1, a 1 × 20 in. column equipped with a water jacket. The detector was the Nestor/Faust Model 404 R.I. monitor. The optical head was packed with 200-μm glass beads to improve the dynamic response. Both the optical head and the power supply were temperature controlled to assure a steady base line.

The column was repacked between most of the temperature changes. This was done by filling the column with water and adding the gel as a slurry to a large funnel attached to the top of the column. In all of the major data runs, samples of 0.5 ml solution were used. Most solutes were injected at 5–7% concentrations (w/v). Dextran was used at 1–2%. The concentration of Schardinger β-dextrin was limited by its solubility, *ca.* 3%.

DATA REDUCTION

The height equivalent of a theoretical plate (HETP) of each curve was calculated from GLUECKAUF's equations[12].

$$N = \frac{8\,V_e\,(V_e - W_1)}{(W_1 + W_2)^2} \tag{10}$$

$$L' = L - \frac{V_f}{2A\varepsilon} \tag{11}$$

$$H = N/L' \tag{12}$$

where N = number of theoretical plates, V_e = corrected elution volume, L = measured length of packing, L' = corrected length, V_f = sample volume, ε = porosity, A = cross-sectional column area, and W_1 and W_2 are the leading and trailing widths of the elution curve measured at h/e.

Four to ten flow rates were used in the data runs, ranging from 0.15 to 5.0 ml/min. The data pertinent to each elution curve were recorded on punched cards and the analysis was made through computer programs. All the runs gave linear H or $H\alpha$ *versus* U plots as shown for the representative data in Fig. 3. The average correlation coefficient of all runs was 0.996. The slope and intercept were calculated from a least-squares analysis and these were used to calculate the following quantities:

$$\text{intercept} = H_0 \tag{13}$$

$$\frac{1}{K_L} = \frac{m\,V_0}{2\,V_t}\left(\frac{V_e - V_0}{V_e}\right)^2 \tag{14}$$

$$D_g = \frac{d_p^2\,V_0\,(V_e - V_0)}{30\,m\,V_e^2} \tag{15}$$

where d_p = particle diameter, V_e = peak elution volume, V_0 = void volume, V_t = total column volume, and m = the slope of the experimental $H\alpha$ *versus* U plot.

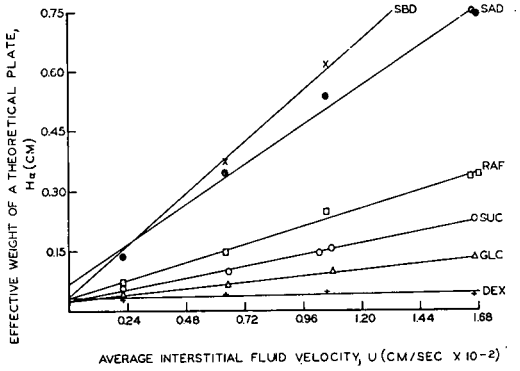

Fig. 3. Representative *Ha versus U* plot. (See Table III for abbreviations.).

RESULTS

Reproducibility

The column was repacked and the conditions were repeated four times. As shown in Table IV, there was good agreement between m, K_1, and ε. The large amount of variation of the parameter H_0 reflects the sensitivity of axial dispersion to changes in packing structure. The reproducibility data indicate that mass transfer parameters can be compared between packings but that only the gross axial dispersion behavior can be evaluated.

TABLE IV

REPRODUCIBILITY OF COLUMN PARAMETERS

70–80 mesh Bio-Gel P-2, 45°. Average correlation coefficient = 0.993. m = slope of *Ha versus U* (sec^{-1}); H_0 = intercept of *Ha versus U* (cm).

Run	*Raffinose*			*Glucose*			
	m	H_0	K_1	m	H_0	K_1	ε
13	11.50	0.079	0.291	4.34	0.068	0.412	0.354
46	11.71	0.040	0.296	5.03	0.029	0.414	0.365
47	11.76	0.052	0.288	4.89	0.044	0.414	0.381
48	11.33	0.041	0.293	4.93	0.034	0.412	0.368
Mean	11.58	0.053	0.292	4.80	0.044	0.413	0.367
Av. dev.	0.16	0.013	0.005	0.23	0.012	0.001	0.007
% dev.	1.4	25	1.7	4.8	27	0.24	1.9

Diameter averages

The following equations define the averages which were calculated for the gel preparations:

$$d_{sps} = \left(N \sum^{N} \left(1/d_n\right)\right)^{-1} \tag{16}$$

$$d_n = \sum^{N} d_n/N \tag{17}$$

$$d_{sv} = \left(\sum^{N} d_n^3\right)^{\frac{1}{3}} \Big/ N$$

$$d_a = \sum^{N} d_n^3 \Big/ \sum^{N} d_n^2$$

$$d_{wv} = \sum^{N} d_n^4 \Big/ \sum^{N} d_n^3$$

The relationship between the averages is shown schematically in Fig. 4 for the fractionated P-2 gel preparations. Table V is a more detailed presentation of the diameter data. The relative uniformity of diameter within the fractionated samples can be inferred from the ratios of volumetric to linear average.

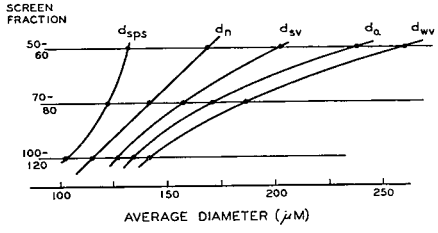

Fig. 4. Average diameters for fractionated gel.

TABLE V

DIAMETER AVERAGES

Preparation	d_{sps} *(μm)*	d_n *(μm)*	d_{sv} *(μm)*	d_a *(μm)*	d_{wv} *(μm)*
100–120, P-2	104.47	116.68	125.93	134.97	141.55
70–80, P-2	123.40	143.60	158.57	173.16	182.96
50–60, P-2	133.20	170.12	204.49	240.78	263.91
Sephadex G-10	72.48	76.06	79.77	83.61	87.38
Sephadex G-15	80.97	83.74	86.79	89.97	93.36

In order to ascertain the proper average for mass transfer, eqns. (1) and (2) were assumed to be exact. Values of K_L were calculated, and D_g was calculated using each of the averages. The experimental data were derived from band spreading measurements using three preparations of Bio-Gel P-2. Two solutes were used, glucose and raffinose; and runs were made at three temperatures, 15, 30, and 45°.

The deviations of D_g from group averages were used as one measure of the consistency of a diameter average. The deviations were normalized and combined until one value was obtained which was representative of the error associated with a given average. The results of this analysis are shown schematically in Fig. 5, abbreviated ΔD_g.

The overall mass transfer coefficient can be written

$$\log \frac{1}{K_L} = 2 \log d_p - \text{constant} \qquad (21)$$

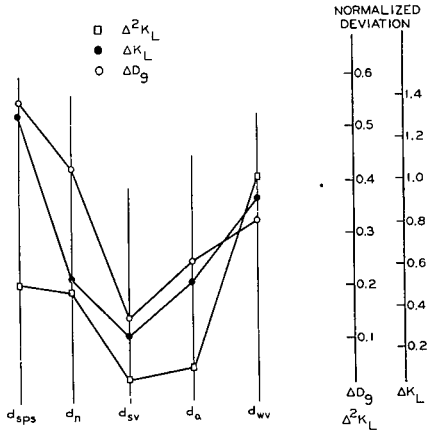

Fig. 5. Error analysis of diameter averages.

From this it can be seen that a log–log plot of $1/K_L$ *versus* d_p should have a slope of two. The slopes were calculated by least-squares analysis, and the results are shown in Fig. 5 as ΔK_L. Regardless of the slope, it should be the same for both solutes, *i.e.*, there should be no change in transfer mechanism. This analysis of error is shown in Fig. 5, abbreviated Δ^2K_L.

There was a minimum in the three measurements of consistency for the d_{sv} basis. This is not an unreasonable result since slow gel-phase diffusion should be best described by a volume averaged diameter.

Either d_{sps} or d_n is intuitively suggested by the random walk theories of eddy diffusion[13,14]. As shown in Fig. 6, there was a good linear correlation for both of these averages, except for the 70–80 mesh data. Different techniques were used for column packing in these runs, and it is reasonable that there is no agreement for these data.

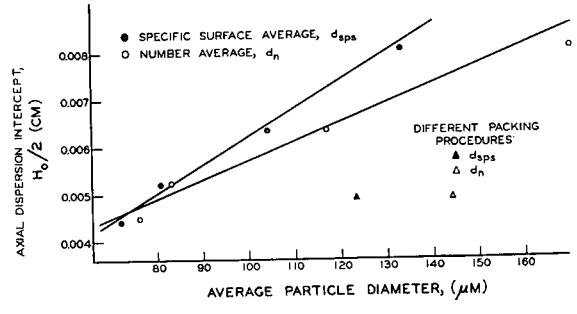

Fig. 6. Axial dispersion dependence on average particle diameter.

The regression parameters for the data shown in Fig. 6 are given in Table VI (excluding the 70–80 mesh data). The comparison is shown for the overall average eddy diffusion coefficient $<\lambda>$. The correlation coefficient is larger and the intercept is smaller for the d_{sps} line. There is better agreement between slope and $<\lambda>$ for d_{sps} than for d_n. From these comparisons, the conclusion is that the d_{sps} average gives a more consistent prediction of axial dispersion.

TABLE VI

DEPENDENCE OF AXIAL DISPERSION ON PARTICLE DIAMETER

	d_{sps} basis	d_n basis
Correlation coefficient[a]	0.998	0.991
Intercept[a]	3.5×10^{-4}	1.9×10^{-3}
Slope[a]	0.583	0.368
$<\lambda>$	0.620	0.557
$<\lambda>$ − slope	0.037	0.189

[a] Refers to data shown in Fig. 6.

Mass transfer

The dependence of mass transfer on particle diameter was given in eqn. (21). Theories dealing with slow film diffusion predict an exponent of 1 for d_p (ref. 8). Therefore, the slope of the log–log plot of $1/K_L$ *versus* d_p is a sensitive test for determining the controlling mechanism of mass transfer. The regression data in Table VII show that the slope is close to two in all cases, indicating that slow gel-phase diffusion was the controlling mechanism. The linearity of the relationships is an indication that there was not a change in the mechanism with variations in diameter.

TABLE VII

RELATION OF MASS TRANSFER COEFFICIENT TO PARTICLE DIAMETER

Condition	*Slope*[a]	*Intercept*	*Correlation coefficient*
15°, glucose	2.18	12.6	0.999
30°, glucose	1.92	11.0	0.984
45°, glucose	1.86	10.4	0.984
15°, raffinose	1.77	12.3	0.989
30°, raffinose	1.95	12.5	0.992
45°, raffinose	1.85	11.6	0.999

[a] Log $(1/K_L)$ *versus* log d_p.

The consistency of D_g can be seen from the data in Table VIII. There is good agreement except for some of the 100–120 mesh data at the higher temperatures. The slopes of the $H\alpha$ *versus* U plots were numerically small in these runs, making the calculations of D_g and K_L more susceptible to experimental error.

TABLE VIII

GEL-PHASE DIFFUSION COEFFICIENTS

Temperature (°C)	*Glucose ($D_g \times 10^7$)*			*Raffinose ($D_g \times 10^7$)*		
	100–120 mesh gel	*70–80 mesh gel*	*50–60 mesh gel*	*100–120 mesh gel*	*70–80 mesh gel*	*50–60 mesh gel*
15	3.13	3.02	2.85	1.00	1.04	1.01
30	4.82	5.25	3.73	1.72	1.79	1.14
45	6.40	7.84	7.03	3.02	2.85	2.97

Using the absolute rate theory of EYRING *et al.*[15], the activation energy of gel-phase solute diffusion can be calculated from the equation

$$\ln D_g = -E_a\left(\frac{1}{RT}\right) + \text{constant} \tag{22}$$

where D_g = calculated gel-phase diffusion coefficient, E_a = activation energy, R = gas constant, and T = absolute temperature.

The data shown in Table IX refer to solute behavior in the Bio-Gel P-2 systems. The activation energies increase in the expected way—with increasing molecular weight. All of the semilog plots of D_g *versus* $1/T$ were linear, confirming the conception of solute partitioning by localized restricted diffusion[16]. The activation energies are close to those reported for analogous gel systems, *cf.* 4.5–5.9 kcal/mole in ref. 17 and about 6.0 kcal/mole in ref. 18.

TABLE IX

VARIATION OF ACTIVATION ENERGY FOR DIFFUSION WITH SOLUTE

Solute	E_a *(kcal/mole)*
Glucose	5.34
Sucrose	5.90
Raffinose	6.50
Schardinger α-dextrin	6.53
Schardinger β-dextrin	6.73

Diffusivity retardation

The retardation ratio

$$\gamma = \frac{D_g}{D_m} \tag{23}$$

is useful in discussing the transport of solutes in gel systems. As shown in Table X, retardation is not sensitive to temperature. However, Tables X and XI show a high dependence of retardation on both solute and gel. There was a semilog correlation between the retardation ratio and solute molecular weight as shown in Fig. 7. It is of incidental interest that the intercepts of the lines in Fig. 7 agree only approximately with the equation of MACKEY *et al.*[19].

TABLE X

RETARDATION RATIOS, BIO-GEL P-2

	15°	*30°*	*45°*
Glucose	0.0587	0.0602	0.0671
Sucrose	0.0413	0.0492	0.0517
Raffinose	0.0307	0.0314	0.0429
Schardinger α-dextrin	0.0178	0.0263	0.0246
Schardinger β-dextrin	0.0145	0.0169	0.0210

TABLE XI

RETARDATION RATIOS, SEPHADEX GELS, 20°

	G-15	G-10
Glycine	—	0.0228
Glycerol	—	0.0266
Glucose	0.0717	0.0160
Sucrose	0.0376	0.0090
Raffinose	0.0297	0.0065
Schardinger α-dextrin	0.0122	0.0024

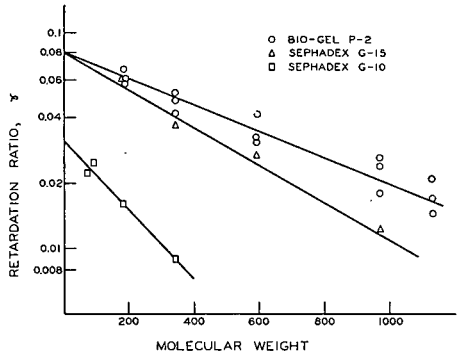

Fig. 7. Retardation ratio–molecular weight correlation.

$$\gamma = \frac{\varepsilon_g^2}{2 - \varepsilon_g} \tag{24}$$

based on a tortuosity model. The comparison is shown in Table XII.

TABLE XII

RETARDATION RATIOS PREDICTED BY THE RELATION OF MACKIE

	Predicted by eqn. (24)	Measured intercept
P-2	0.171	0.081
G-15	0.141	0.081
G-10	0.026	0.032

Solute partitioning was not found to be sensitive to flow rate or to any of the other experimental variables. The Sephadex gels were studied only at 20° so this conclusion cannot be completely generalized. All common colored materials (pH indicators, inks, Sephadex Blue Dextran 2000) were found to be chemically adsorbed on the Sephadex gels. Most of these materials were adsorbed on Bio-Gel P-2 with the exception of Na-Bromthymol Blue (Sargent) and Murexide (Polysciences). There was found to be an interaction between Schardinger α-dextrin and all of the Sephadex gels as shown in Table XIII by the abnormally large distribution coefficient for this solute.

TABLE XIII

EXPERIMENTAL DISTRIBUTION COEFFICIENTS

Solutes	*Gels*			
	P-2	*G-10*	*G-15*	*G-25*
Glycine	—	0.178	0.283	—
Glycerol	—	0.230	0.321	—
Glucose	0.410	0.186	0.287	0.361
Sucrose	0.361	0.136	0.241	0.328
Raffinose	0.291	0.082	0.179	0.287
Schardinger α-dextrin	0.271	0.119	0.289	0.371
Schardinger β-dextrin	0.243	—	—	—

There was found to be a good correlation between K_1 and molecular weight as shown in Fig. 8.

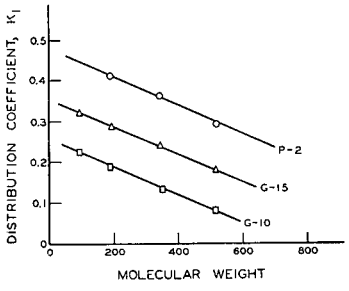

Fig. 8. Peak separation index correlation.

DISCUSSION AND CONCLUSIONS

Retardation of solute diffusivity in the gel matrix is the most important mechanism which causes band spreading in MSC systems. This is inherent in MSC since the same mechanisms are responsible for partitioning solutes according to molecular size. An interesting result of this relationship is that the bands which are eluted first from the column are spread less than subsequent bands. Since an excluded solute band does not involve gel phase transport, these bands are narrowest. This behavior was consistently observed and is shown in Table XIV for a representative series of curves.

TABLE XIV

REPRESENTATIVE SPREADING DATA

Solute	*Molecular weight*	*Distribution coefficient, K_1*	*Band spreading σ_v, (ml)*
Glucose	180	0.407	8.84
Sucrose	342	0.359	10.5
Raffinose	504	0.295	12.1
Schardinger α-dextrin	972	0.281	17.7
Schardinger β-dextrin	1,134	0.263	18.5
Dextran	12,000	0.0	2.94

This behavior agrees with observations in a polymer-related MSC system[20], but is contrary to the prediction of GIDDINGS[21].

Peak separation and band spreading are two effects which must be considered when designing a MSC separation. Two indices may prove useful in this respect,

$$I_{ps} = \frac{\Delta K_1}{\Delta \text{ molecular weight}} \tag{25}$$

$$I_{bs} = \frac{\Delta \log \gamma}{\Delta \text{ molecular weight}} \tag{26}$$

which are given in Table XV as calculated from data shown in Figs. 7 and 8. These indices are normalized measures of a gel's ability to separate molecules (I_{ps}) and of the increase in band spreading with increasing molecular weight (I_{bs}). As shown in Table XV, there is little difference between the abilities of the gels to separate molecules. However, the Sephadex gels caused more band spreading than did the Bio-Gel material. The reason for this is probably because the dextran matrix allowed more solute–gel interaction than did the acrylamide matrix. Thus, it would seem that the more inert a matrix is toward solutes, the better it will be in MSC applications.

TABLE XV

GEL EFFICIENCY PARAMETERS

I_{ps} = peak separation index; I_{bs} = band spreading index.

Gel type	$I_{ps} \times 10^4$	$I_{bs} \times 10^4$
P-2	3.65	6.07
G-15	3.54	8.67
G-10	3.50	15.9

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CHROM. 4473

LES APPLICATIONS DE LA THÉORIE DE LA CHROMATOGRAPHIE D'ADSORPTION LINÉAIRE DE SNYDER EN SÉRIE HÉTÉROCYCLIQUE

I. INFLUENCE DES EFFETS POLAIRES ET STÉRIQUES DE SUBSTITUANTS DIVERS SUR LES ÉNERGIES D'ADSORPTION D'HÉTÉROCYCLES THIAZOLIQUES SUR ALUMINE

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(Reçu le 6 mai 1969; manuscrit modifié reçu le 8 octobre 1969)

SUMMARY

Applications of SNYDER's *theory on linear adsorption chromatography to heterocyclic compounds. I. The influence of the polar and steric effects of various substituents on the adsorption energy of thiazoles on alumina*

SNYDER's theory of linear adsorption chromatography, that was applied to one hundred thiazole derivatives, made it possible to determine experimentally the adsorption energies of the compounds and to compare these with the adsorption energies calculated by means of fixed tables.

In a study on thiazoles containing one or two alkyl groups, this comparison enabled us to determine the variations in adsorption energy of the nitrogen atom of the ring due to the polarization effects and to the steric effects induced by the alkyl groups and to relate these effects to the constant relationships which exist between the polarization and steric effects of the substituents.

A similar investigation was made on 4-aryl thiazoles with various substituents in the 2-position. In this case variations in the adsorption energy of the molecules due to the polarization effects of the groups substituted *para* to the phenyl group with respect to the substituents in the 2-position were studied, and the mutual electronic interactions between the various groups were determined.

I. INTRODUCTION

Dans des mémoires précédents[1–3] nous avions pu établir des relations qualitatives entre le comportement chromatographique en couche mince et la structure de divers hétérocycles azotés appartenant à la série du thiazole, du benzothiazole, de la pyridine et de la pipéridine.

Nous avions notamment établi:

(a) une relation linéaire entre la mobilité sur Alumine GF_{254} et l'augmentation de la longeur de la chaine sur la position adjacente à l'atome d'azote (alcoyl-2 thiazoles, dialcoyl-2,4 thiazoles et alcoyl-2 benzothiazoles)[2];

(b) une relation linéaire entre la mobilité, dans un système chromatographique donné, et la polarité de ces bases hétérocycliques: $R_F = f(pK_a)$ en l'absence d'encombrement stérique important[3];

(c) une certaine relation entre la mobilité et l'effet inductif des substituants sur le cycle[1];

(d) enfin, l'influence prépondérante de l'encombrement stérique des substituants alcoyle autour de l'atome d'azote, responsable de l'adsorption de la molécule.

S'il ne nous a pas été possible de relier linéairement cet effet d'encombrement stérique aux constantes d'encombrement stérique E_s définies par TAFT[25] en cinétique chimique (par suite des différences essentielles entre les deux processus) il n'en n'a pas moins été permis de montrer l'identité du processus d'adsorption pour toutes les séries d'hétérocycles alcoylés en position-2 étudiées: pyridines, benzothiazoles, thiazoles, pipéridines, *tert.*-butyl-4 thiazoles, méthyl-4 thiazoles et nitro-4 thiazoles.

Désirant étudier quantitativement l'ensemble de ces phénomènes, nous avons recherché une méthode expérimentale susceptible de permettre une meilleure interprétation de ces relations: la théorie de la chromatographie d'adsorption linéaire de SNYDER[4–7] nous a semblé la plus appropriée pour réaliser cet objectif.

II. THÉORIE—RAPPELS

SNYDER[7] a établi une relation générale, pour la chromatographie en couche mince, entre l'affinité d'adsorption ou la distance de rétention linéaire du soluté, sa structure moléculaire et les différents paramètres chromatographiques tels que l'activité de l'adsorbant et le type d'éluant:

$$R_M' = \log \frac{V_a W_a}{V_S} + \alpha (S^0 - \varepsilon^0 A_S) + \Delta eas \tag{1}$$

Le point de départ de cette relation est l'expression du coefficient de distribution à l'équilibre K (cm^3/g) du processus chromatographique, en fonction des paramètres caractérisant l'adsorbant et le soluté:

$$\log K = \log V_a + \alpha(S^0 - \varepsilon^0 A_S) + \Delta eas \tag{2}$$

or, le coefficient K est relié au R_F par la relation:

$$R_F = \frac{1}{1 + \frac{W_a}{V_S} K} \tag{3}$$

bien connue en chromatographie.

La valeur R_F est à son tour relié au R_M' par l'équation de MARTIN:

$$R_M' = \log \left(\frac{1}{\xi R_F} - 1 \right) \tag{4}$$

dans laquelle ξ est un facteur de correction qui tient compte de l'existence d'un gradient

de concentration du solvant le long de la plaque. Sa valeur peut varier de 1.1 à 1.5 (bibl. 7), suivant la plus ou moins grande saturation de la cuve. Au cours de cette étude, nous avons pris $\xi = 1.1$.

La signification de chacun des termes est la suivante:

V_a est le volume correspondant à la surface active de l'adsorbant (cm^3/g).

W_a est le poids d'adsorbant sur la plaque (g).

V_S est le volume de solvant dans la cuve (cm^3).

α est le coefficient d'activité de l'adsorbant en prenant, pour référence, l'adsorbant calciné pour lequel $\alpha = 1$.

$S°$ est l'énergie d'adsorption du soluté (sans unité). Il ne dépend pour un adsorbant particulier que de la structure moléculaire du soluté.

$\varepsilon°$ est le pouvoir éluant du solvant ou du mélange utilisé pour le développement. Le pentane qui est pris comme solvant de référence a, par définition, un pouvoir éluant nul. Il représente l'énergie d'adsorption du solvant par unité de surface.

Dans le cas de systèmes binaires, des formules ont été établies par SNYDER[8], permettant de calculer le pouvoir éluant de ces mélanges en fonction de l'activité de la plaque, de la fraction molaire de la composante la plus polaire et de sa surface moléculaire.

A_S est un paramètre qui dépend de la grosseur de la molécule adsorbée. Il est proportionnel à la surface occupée par la molécule à la surface de l'adsorbant. C'est la surface moléculaire effective du soluté. Une unité A_S est égale à 8.5 $Å^2$ correspondant à la surface moléculaire calculée à partir des rayons de Van der Waal. Le benzène (dont la surface moléculaire est de 51 $Å^2$) est pris comme soluté de référence ($A_S = 6$). On peut considérer le A_S comme une propriété additive des différents groupements ou atomes présents dans une molécule et poser:

$$A_S = \sum_i a_i \tag{5}$$

Sur alumine les valeurs de A_S calculées par la relation précédente sont sensiblement égales aux valeurs expérimentales. Il n'en est pas de même sur silice, pour les groupements fortement adsorbés[7].

Des tables de valeurs de $\varepsilon°$, $S°$ et A_S ont été établies par SNYDER, pour l'alumine[9–11], pour un grand nombre de solvants et pour des groupements divers fixés à une chaine aliphatique ou à un cycle aromatique.

Quant au dernier terme de la relation (1), Δeas, c'est une fonction complèxe qui dépend de la structure du soluté, de la nature de l'éluant, de l'activité de l'adsorbant et des différentes interactions possibles entre soluté–solvant et soluté–adsorbant. Dans la majorité des cas où la structure des solutés est simple et où les éluants utilisés sont apolaires, ce terme est nul.

Quant à l'énergie d'adsorption du soluté $S°$, elle peut être déterminée soit expérimentalement, à l'aide de la relation (1), soit par le calcul, à l'aide de la relation (6)[4–7,12].

$$S° = \sum_i Q°_i - f(Q°_k) \sum^{i \neq k} Q°_i + \sum_i \sum_j q°_{ij} \tag{6}$$

$Q°_i$ est l'énergie d'adsorption caractéristique d'un des groupements i constituant le soluté; il est donné par les tables[7].

$\sum_{i} Q^\circ_i$ est la somme des énergies d'adsorption caractéristiques de tous les groupements constituant le soluté.

$f(Q^\circ_k) \sum_{i \neq k} Q^\circ_i$ représente la perte d'énergie d'adsorption du soluté par suite de la délocalisation des groupements i autres que k provoquée par la localisation d'un groupement k plus fortement adsorbé que les autres à la surface de l'adsorbant. En principe, $f(Q^\circ_k)$ est indépendant de la position des substituants par rapport à l'atome du cycle, mais plus les distances seront grandes, plus faible sera la valeur de $f(Q^\circ_k)$. Des tables de valeurs de cette fonction ont été également établies en série aromatique et en série aliphatique par SNYDER[7], en fonction de l'énergie d'adsorption des groupements et de leur position dans une chaine aliphatique. $\sum_{i} \sum_{j} q^\circ_{ij}$

Quant au dernier terme de la relation (6), il représente la variation d'énergie d'adsorption de la molécule due aux interactions entre les groupements i et j. Dans ce terme sont généralement compris les effets électroniques et stériques, les interactions chimiques entre deux groupements adjacents, les effets de non planéité des molécules etc. Le calcul de ce terme est complexe et n'est accessible que dans des cas simples où l'énergie d'adsorption d'un des deux groupements est faible (alcoyle ou halogéno).

Dans le cas de produits appartenant à la même famille, ce qui est notre cas, la relation (6) peut encore s'écrire:

$$S^\circ_{Th-i} = S^\circ_{Th} + [I - f(Q^\circ_k)] \cdot Q^\circ_i + \sum_{i} \sum_{j} q^\circ_{ij} \tag{6bis}$$

ou encore:

$$\sum_{i} \sum_{j} q^\circ_{ij} = \Delta S^\circ - 0.55\, Q^\circ_i \tag{6ter}$$

puisque

$$f(Q^\circ_k) = 0.45 \text{ pour } Q^\circ_k = 4.5.$$

ΔS° représente la variation d'énergie d'adsorption entre le thiazole substitué par un groupement i et le thiazole non substitué.

Q°_i est l'énergie d'adsorption du substituant sur le cycle.

Les relations précédentes, dont le bien fondé a déjà été vérifié pour plusieurs centaines de composés, seront successivement utilisées au cours de cette étude, ainsi que les deux relations suivantes:

$$R_M' = p_1 + \frac{\alpha_1}{\alpha_2} p_2 + \frac{\alpha_1}{\alpha_2} (R_M')_2 \tag{7}$$

en prenant:

$$p = \log \frac{V_a W_a}{V_S}$$

et

$$R_M' = \alpha \cdot A_S (\varepsilon_1 - \varepsilon_2) \tag{8}$$

La relation (7) relie linéairemant les valeurs R_M' d'un même soluté élué avec le même éluant sur deux adsorbants de même nature (silice ou alumine) mais d'activité

différente, tandis que la relation (8) exprime la variation du R_M' d'un même soluté élué sur le même adsorbant, mais avec deux solvants de pouvoir éluant différent.

Remarques

Pour que la relation (1) soit valable, un certain nombre de conditions doivent être remplies.

(a) Les valeurs R_F doivent toujours être pris entre 0.1 et 0.8 (bibl. 6).

(b) Pour pouvoir construire la droite des moindres carrés:

$$R_M' = p + \alpha \cdot f(S, E)$$

afin de déterminer les paramètres chromatographiques α et p, il faut au moins une dizaine de produits dont les énergies d'adsorption soient connues ou facilement calculables.

(c) On doit éviter, autant que possible, l'emploi de systèmes éluants binaires renfermant un faible taux d'un éluant dont le pouvoir est très élevé (éthers ou alcools), car dans ce cas, Δeas n'est pas nul et l'adsorption de certains solutés peut être modifiée (adsorption verticale et non plane des phénols)[7].

Avec ces restrictions la relation (1) permet en remplaçant S^0 par sa valeur tirée de (6), de calculer la valeur R_F de n'importe quel composé dans un système chromatographique donné.

III. PARTIE EXPÉRIMENTALE

Le mode opératoire ainsi que les R_F des thiazoles étudiés ont été mentionnés dans les mémoires précédents[1-3] à l'exception des alcoyl-5 et dialcoyl-2,5 thiazoles.

TABLEAU I

PRINCIPAUX SYSTÈMES CHROMATOGRAPHIQUES AVEC LEURS CARACTÉRISTIQUES SUR ALUMINE

Composés étudiés	*Bibl.*	*Adsorbants*	*Eluants et ε^0*	*R_F du mélange test Desaga*	*No. du système chromatographique*
Alcoyl-2 thiazoles	2	Alumine DF-5	Hexane–CH_2Cl_2 (6:1); $\varepsilon^0 = 0.16$	0.07, 0.12, 0.34	1
	Cette étude	Alumine GF_{254}	CCl_4; $\varepsilon^0 = 0.18$	0.1, 0.20, 0.49	2
Dialcoyl-2,4 thiazoles	2	Alumine DF-5	Hexane–CH_2Cl_2 (5:2); $\varepsilon^0 = 0.18$	0.1, 0.19, 0.53	3
	Cette étude	Alumine GF_{254}	CCl_4	0.1, 0.18, 0.45	4
Alcoyl-5 et dialcoyl-2,5 thiazoles	Cette étude	Alumine GF_{254}	CCl_4	0.16, 0.23, 0.46	5
Thiométhyl-2 thiazoles	Cette étude	Alumine GF_{254}	Hexane	0.02, 0.04, 0.06	6
	Cette étude	Alumine GF_{254}	Hexane–CH_2Cl_2 (9:1)	0.03, 0.07, 0.15	7
Aryl et diaryl thiazoles substitués ou non en -2	1	Alumine GF_{254}	Hexane–CH_2Cl_2 (8:2)	0.15, 0.45	8
	Cette étude	Alumine GF_{254}	Hexane–CH_2Cl_2 (9:1)	0.05, 0.1, 0.25	9
Hydroxy-2, amino-2 et mercapto-2 thiazoles	Cette étude	Alumine GF_{254}	Benzène; $\varepsilon^0 = 0.32$	0.88, 0.94	10a
Amino-2 aryl-4 thiazoles	1	Alumine G	Benzène; $\varepsilon^0 = 0.32$	0.57, 0.69, 0.86	10b
Hydroxy-2 et mercapto-2 aryl-4 thiazoles	Cette étude	Alumine GF_{254}	CH_2Cl_2; $\varepsilon^0 = 0.42$		11

Cependant afin d'avoir des résultats comparables et pour faciliter la reproduction de ces résultats, nous avons à nouveau chromatographié ces produits dans les systèmes indiqués dans le Tableau I.

Nous avons suivi la méthode décrite par STAHL et utilisé l'appareillage analytique Desaga No. 600[13] pour les plaques de 20 × 20 cm, en utilisant la technique verticale ascendante en atmosphère saturée. Le volume de solvant dans la cuve V_S est de 70 cm³. Les plaques d'alumine Merck GF_{254} sont préparées à partir de 30 g d'adsorbant dans 50 cm³ d'eau distillée, et activées 1 h à 130°. Le poids d'adsorbant par plaque W_a = 4.35 g et les dépôts sont de 2 μl pour des solutions à 5%. Les plaques ont été utilisées dans les 24 h après leur préparation afin de ne pas perdre leur activité. Celle-ci est beaucoup plus difficile à reproduire que dans le cas de la silice. Les écarts moyens entre les R_F sont de l'ordre de ± 0.02. Aussi, l'utilisation de plaques prêtes à l'emploi doit-elle présenter un avantage certain dans ce genre d'étude.

Détermination des paramètres chromatographiques

La première partie de notre étude a consisté à déterminer expérimentalement les paramètres chromatographiques α et $p = \log (V_a W_a / V_s)$.

Pour ce calcul, on a utilisé la méthode suivante: la relation (1) est appliquée à des composés témoins dont les énérgies d'adsorption et les A_S sur alumine et par suite les fonctions $f(S, E)$ sont connues ou facilement calculables, dans les systèmes chromatographiques choisis (tétrachlorure de carbone et pentane ou hexane additionné de chlorure de méthylène).

Ces composés sont les suivants: pyridine $S^0 = 6.4$, méthyl-2 pyridine $S^0 = 5.7$, diméthyl-2,6 pyridine $S^0 = 5.2$, quinoléine $S^0 = 6.8$, chloro-2 pyridine $S^0 = 5.3$, acétophénone $S^0 = 6.0$ benzophénone $S^0 = 6.2$, et aniline $S^0 = 6.3$.

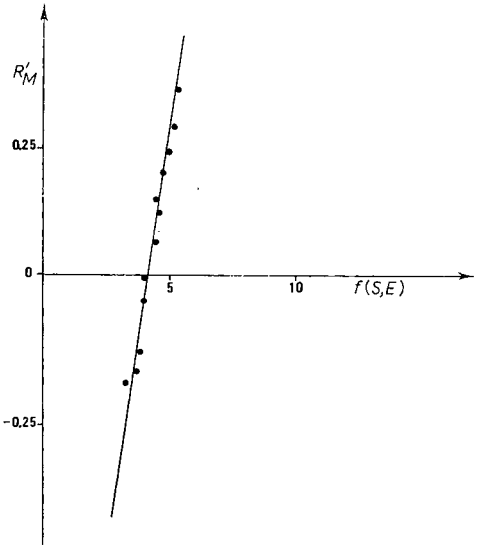


Fig. 1. Paramètres chromatographiques évalués à partir des mobilités des alcoyl-5 et dialcoyl-2,5 thiazoles, élués sur Alumine GF_{254} avec le tétrachlorure de carbone (α = 0.38, p = −1.45).

On a également pris comme témoin, le mélange test Desaga, à trois colorants dont les fonctions $f(S, E)$ sur alumine éluée avec le tétrachlorure de carbone, sont connues[7].

	$f(S, E)$
p-Aminoazobenzène ou bleu d'indophénol	6.2
Rouge soudan	5.07
Jaune soudan	4.24

Cependant, sur alumine GF_{254}, nous avons préféré prendre les valeurs suivantes: 6.1, 5.35 et 4 qui vérifient le mieux la relation (1).

Cette méthode nous a donné, pour l'ensemble des courbes utilisées, les résultats suivants:

$$\text{I} \quad \alpha_1 = 0.38 \pm 0.02 \text{ et } p_1 = -1.45 \pm 0.05$$

$$\text{II} \quad \alpha_2 = 0.45 \pm 0.02 \text{ et } p_2 = -1.85 \pm 0.05$$

Nos couches d'alumine présentaient donc, en moyenne, deux degrés d'activité, d'ailleurs reliés par la relation[7].

L'utilisation de ces paramètres dans la relation (1), nous a permis de calculer les énergies d'adsorption des différents thiazoles étudiés et, en premier lieu, celle du thiazole, trouvée égale à 5.8 alors que la valeur calculée à l'aide de la relation (6) est de 6.2. Cette variation de près de 7% représente la diminution d'énergie d'adsorption de la molécule, par suite des interactions électroniques mutuelles du soufre et de l'azote dans cette molécule.

Les énergies d'adsorption calculées d'après la relation (1) ne sont pas tout à fait constantes d'un système chromatographique à un autre et les variations sont d'autant plus grandes que le pouvoir éluant du solvant est fort (acétone, chlorure de méthylène, acétate d'éthyle, éthers, alcools), ceci est conforme aux observations de SNYDER[6] qui indique des variations de $\Delta S^\circ = \pm 0.4$ unité. Nous avons observé que ces variations étaient assez faibles ($\Delta S^\circ = \pm 0.2$) pour les systèmes chromatographiques dont le pouvoir éluant est inférieur ou égal à celui du tétrachlorure de carbone. Malheureusement, il n'est pas possible de chromatographier toute la série de composés dans ces systèmes. Toutefois, pour les composés dont les énergies d'adsorption sont élevées tels que les amino-2 thiazoles et à fortiori pour les hydroxy-2 et les mercapto-2 thiazoles, il est nécessaire d'utiliser des éluants plus forts, tels que le benzène ou le benzène additionné de 2 à 5% de méthanol ou le chlorure de méthylène. Mais, dans ce cas, les énergies d'adsorption ne correspondent plus aux valeurs qu'elles auraient dans des systèmes moins éluants. Ainsi, le S° de l'amino-2 phényl-4 thiazole est de 8.45 dans le benzène ($\varepsilon^\circ = 0.32$) et de 9 dans le tétrachlorure de carbone ($\varepsilon^\circ = 0.18$). Les énergies d'adsorption des amino-2 aryl-4 thiazoles calculées dans le système 10b devront être, de ce fait, majorées de 0.5 unité pour les ramener au système 7. De même, les énergies d'adsorption des hydroxy-2 et mercapto-2 aryl-4 thiazoles calculées dans le système 9 devront être majorées de 2 unités pour les ramener au système 7 pris comme système de référence, le benzène servant de solvant intermédiaire.

Une explication possible de ce phénomène est la tendance que manifestent les composés hydroxylés[9] ou les thiols à s'adsorber verticalement lorsque l'on augmente le pouvoir éluant du solvant. Cet effet serait moindre pour les composés aminés.

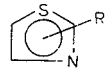
IV. DISCUSSION

Dans les thiazoles monosubstitués, la variation d'énergie d'adsorption de l'atome d'azote sous l'effet d'un substituant est représenté par $\sum_i \sum_j q^\circ_{ij}$ qui est donné par la relation (6 ter).

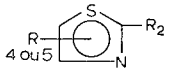
Dans le cas des alcoyl et des halogéno thiazoles, on peut considérer comme négligeable la variation d'énergie d'adsorption des groupements faiblement adsorbés et ne considérer que $\sum_j q^\circ_{ij}$.

Si l'on désigne par Δ, les différences entre les valeurs expérimentales des $\sum_j q^\circ_{ij}$ déterminées par la relation (6 ter) et les valeurs des q°_{ij} calculées par additivité des effets globaux dans les molécules monosubstituées correspondantes, ces différences représentent l'écart à l'additivité ou les variations d'énergie d'adsorption de l'atome d'azote, principal centre d'adsorption de la molécule, sous les interactions électroniques et stériques mutuelles des substituants sur le cycle.

Nous avons donc étudié successivement les variations d'énergie d'adsorption de l'atome d'azote dans les alcoyl (I) et dialcoyl thiazoles (II), dans les thiazoles diversement substitués en -2 et en -5 (III) et enfin dans les aryl-4 thiazoles diversement substitués en position-2 (IV).

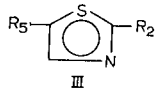


I



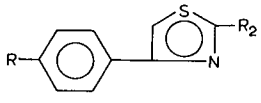
II

où $R_{2,4 \text{ ou } 5}$ = Me, Et, iPr et tBu.



III

avec R_2 et R_5 = Me, Ph, Br, Cl, SCH_3, NH_2 etc.



IV

avec R_2 = H, Cl, SCH_3, NH_2, OH et SH
et R = H, Cl, Br, CH_3, CH_3O et NO_2.

Effets électroniques et stériques des substituants alcoyle aux differentes positions du cycle thiazolique

Les variations d'énergie d'adsorption des composés du Tableau II sont liées à deux phénomènes principaux: d'une part aux effets stériques des substituants alcoyle en position -2 et -4 adjacentes à l'atome d'azote nucléaire, centre d'adsorption et d'autre part, aux interactions électroniques entre les groupements alcoyle en -2, 4 et -5 et ce centre.

Effets polaires des substituants alcoyle en -5. Les effets polaires des substituants alcoyle ne peuvent être évalués qu'à la position -5 caractérisée par l'absence d'encombrement stérique au niveau de l'azote.

TABLEAU II

ÉNERGIES D'ADSORPTION S^0 ET EFFETS POLAIRES ET STÉRIQUES ($\overset{j}{\Sigma} q^0_{ij}$) DES ALCOYL ET DIALCOYL THIAZOLES ISOMÈRES SUR ALUMINE GF_{254}

Solutés	*Systèmes et* R_M'[a]	S^0_{Th-i}[b]	ΔS^0[c]	$\overset{j}{\Sigma} q^0_{ij}$		Δ[f]	
				Exp.[d]	*Calc.*[e]		
Alcoyl-2 thiazoles	*2*						
Me	0.3	5.9	+0.1	+0.04			
Et	0.2	5.8	0	−0.04			
nPr	−0.03	5.35	−0.45	−0.45			
iPr	+0.01	5.4	−0.4	−0.4			
iBu	−0.07	5.3	−0.5	−0.5			
tBu	−0.2	5.1	−0.8	−0.8			
Néop	−0.1	5.3	−0.5	−0.5			
Alcoyl-5 thiazoles	*5*						
Me	0.42	6.5	0.7	0.65			
Et	0.35	6.4	0.6	0.55			
iPr	0.26	6.2	0.4	0.4			
tBu	0.14	5.9	0.1	0.1			
Dialcoyl-2,5 thiazoles	*5*						
Di Me-2,5	0.35	6.55	+0.75	0.65	0.89	−0.04	
Di Et-2,5	0.29	6.5	0.7	0.6	0.51	+0.09	
iPr-5 Et-2	0.20	6.3	0.5	0.45	0.96	+0.09	
Di iPr-2,5	0.05	5.95	−0.15	0.15	0	+0.15	
Me-5 tBu-2	0.01	5.8	0	−0.05	−0.15	+0.1	
Et-5 tBu-2	−0.1	5.6	−0.2	−0.25	−0.25	0	
iPr-5 tBu-2	−0.13	5.5	−0.3	−0.3	−0.4	+0.1	
Di tBu-2,5	−0.16	5.4	−0.4	−0.4	−0.7	+0.3	
Dialcoyl-2,4 thiazoles	*4*						
Di Me-2,4	0.2	6	+0.2	+0.1	0.08[g]	+0.02	
Me-4 Et-2	0.06	5.8	0	−0.1	0[g]	−0.1	
Me-4 iPr-2	−0.15	5.5	−0.3	−0.38	−0.36[g]	−0.02	
Me-4 tBu-2	−0.4	5.05	−0.75	−0.8	−0.76[g]	−0.04	
Me-2 tBu-4	−0.45	5	−0.8	−0.85	−0.76[g]	−0.09	
Et-2 tBu-4	−0.53	4.8	−1	−1.05	−0.84[g]	−0.21	
iPr-2 tBu-4	−0.87	4.1	−1.7	−1.7	−1.2[g]	−1.5	
Di tBu-2,4	−2	1.5	−4.3	−4.3	−1.6[g]	−2.7	

[a] $R_{M'} = p + \alpha (S^\circ - \varepsilon^\circ A_S)$ dans laquelle ε° est connu (*cf.* Tableau I), les A_S sont calculés à l'aide des tableaux de SNYDER[5-7] et α et p ont les valeurs déterminées précédemment.

[b] S° est calculé à l'aide de la retation (1).

[c] ΔS° est la différence entre l'énergie d'adsorption du thiazole substitué par un ou plusieurs groupements alcoyle et l'énergie d'adsorption du thiazole non substitué $S^\circ = 5.8$.

[d] $(\overset{j}{\Sigma} q^\circ_{ij})$ exp $= \Delta S^\circ - \varrho^\circ_i$ $(1 - f(\varrho^\circ_k) \# \Delta S^\circ - \varrho^\circ_i$ car ϱ°_i est faible. Pour les ϱ°_i et les A_S des groupements alcoyles, nous avons pris les valeurs suivantes:

Groupements alcoyles	*Alumine*	
	A_S	ϱ^0_i
Méthyle	1	0.06
Éthyle	1.4	0.04
Isopropyle	1.7	0
tert.-Butyle	2	0

[e] Dans le cas de dérivés polysubstitués $(\overset{j}{\Sigma} q^0_{ij})_{calc}$ est égal à la somme des $(\overset{j}{\Sigma} q^0_{ij})_{exp}$ des dérivés monosubstitués.

[f] Δ est égal à la différence entre les valeurs expérimentales et calculées des $(\overset{j}{\Sigma} q^0_{ij})$.

[g] Pour ce calcul, nous avons supposé que les $\overset{j}{\Sigma} q^0_{ij}$ des alcoyl-4 thiazoles étaient sensiblement les mêmes que les $\overset{j}{\Sigma} q^0_{ij}$ des alcoyl-2 thiazoles correspondants.

L'effet donneur des groupements alcoyle augmentant la densité électronique sur l'atome d'azote, on devrait observer une augmentation de l'énergie d'adsorption des dérivés alcoylés par rapport à la molécule non substituée, ce qui expérimentalement est bien constaté.

Mais si les variations d'énergie d'adsorption dans les alcoyl-5 thiazoles n'étaient dues qu'aux seuls effets polaires des substituants alcoyle, celles-ci pourraient, d'après SNYDER[4–14] s'exprimer quantitativement par la relation d'HAMMETT:

$$(q^{\circ}_{ij})_{-5} = \varrho_i \sigma_j \qquad (9)$$

dans laquelle ϱ_i mesure la sensibilité de l'énergie d'adsorption de l'azote nucléaire (i) aux interactions électroniques (j). Il est généralement négatif, ce qui implique une augmentation de l'énergie d'adsorption de i avec les substituants j dont les σ_j sont négatifs et qui augmentent, par conséquent, la densité électronique de i. Il est d'autre part proportionnel à l'énergie d'adsorption de i et sa valeur est plus importante sur alumine que sur silice.

La relation (9) devrait être constate pour toute la série étudiée puisque les σ_j sont sensiblement les mêmes pour tous les groupements alcoyle ($\sigma_j = -0.17$), mais pour les termes supérieurs, on constate que les énergies d'adsorption diminuent par rapport au méthyle par suite de l'encombrement de ces groupements modifiant l'adsorption de la molécule.

Il faut donc remplacer la relation (9) par la relation (9bis) qui tient compte de cet effet:

$$(q^{\circ}_{ij})_{-5} = \varrho_i \cdot \sigma_j + f(p) \qquad (9 \text{ bis})$$

Par comparaison avec la série pyridinique dans laquelle $\varrho_i = -2.7$ (bibl. 6), on doit avoir, en série thiazolique, un $\varrho_i \# -2.3$ et par suite $\varrho_i \cdot \sigma_j = 0.4$. Cette valeur est un peu inférieure à la valeur expérimentale (0.65) par suite de l'imprécision des mesures (S° à $\pm$ 0.2).

Si l'on admet que $f(p)$ est nul pour le groupement méthyle, on en déduit sa valeur pour les termes supérieurs, par simple différence à l'aide des valeurs des $(\sum^{j} q^{\circ}_{ij})_{-5}$ du Tableau II (*cf.* Tableau III).

Effets stériques. L'influence qualitative des effets stériques dans l'adsorption sur alumine des groupements alcoyle au voisinage d'un centre d'adsorption a été mise en évidence par de nombreux auteurs[15-17], notamment en série hétérocyclique: pyridine (bibl. 13, 14, 18–20 et cette étude), thiazole, benzothiazole, pipéridine[3], imidazole[21], dérivés du thiophène[14,18] et du furanne[14], cycloamines alcoylées en position -2[22], et en série aromatique: fluorénones et benzoquinones[14], *o*-alcoyl-phénols[19] et drogues basiques[23] etc.

Nous avons donc envisagé l'influence quantitative des effets stériques des groupements alcoyle dans les différentes séries d'alcoyl thiazoles étudiées.

La sensibilité de l'atome d'azote vis-à-vis des effets stériques de ces groupements, est, à cet égard, très significative. Les énergies d'adsorption des dérivés alcoylés en -2 ou en -2,4 sont, en effet, très inférieures à celles de leurs isomères en -5 ou en -2,5 correspondants.

Nous pouvons évaluer l'influence des effets stériques des groupements alcoyle -2 ou -4, en faisant l'hypothèse admise en série pyridinique par SNYDER[6] que les effets polaires des substituants sont sensiblement les mêmes en -2, en -4 ou en -5.

$$(\overset{j}{\Sigma}\, q^{\circ}_{ij})_{-2} \# (\overset{j}{\Sigma}\, q^{\circ}_{ij})_{-4} = (\overset{j}{\Sigma}\, q^{\circ}_{ij})_{-5} + f(E_{S'}) \qquad (10)$$

d'où l'on déduit $f(E_{S'})$ (*cf.* Tableau III).

$f(E_{S'})$ est de la forme $\beta_A E_{S'}$ où β_A est un paramètre qui caractérise, dans l'adsorption sur alumine, la sensibilité du couple "système chromatographique–soluté" aux effets stériques $E_{S'}$ des groupements alcoyle.

TABLEAU III

EFFETS STÉRIQUES DES GROUPEMENTS ALCOYLE SUR ALUMINE

Substituant alcoyle	*f(p)*	*f(Es')*
Méthyle	0	−0.6
Éthyle	−0.1	−0.6
Isopropyle	−0.25	−0.8
tert.-Butyle	−0.55	−0.9

Additivité des effets globaux des groupements alcoyle dans les molécules disubstituées. Ayant déterminé les principales interactions des substituants aux différentes positions du cycle avec le centre d'adsorption, il semblait intéressant de vérifier si ces interactions étaient additives ou non, dans le cas des dérivés disubstitués et cela, en les comparant aux valeurs expérimentales.

Les résultats de la dernière colonne du Tableau II nous montrent qu'il en est bien ainsi pour les dialcoyl-2,5 thiazoles d'une part, et les dialcoyl-2,4 thiazoles d'autre part, en l'absence d'encombrement stérique important, aux erreurs d'expérience près (Δ à $\pm$ 0.1).

Pour les groupements très encombrants (isopropyle et *tert.*-butyle) de part et d'autre du centre d'adsorption, l'énergie d'adsorption est beaucoup plus faible que ne le laisserait supposer l'additivité des effets de ces groupements pris séparément. Ainsi, avec le di-*tert.*-butyl-2,4 thiazole, l'encombrement stérique autour de l'atome d'azote est tel que celui-ci n'est pratiquement plus adsorbé.

Influence de substituants divers en position -2

Comme dans le cas des alcoyl-2 thiazoles, les variations d'énergie d'adsorption des thiazoles diversement substitués en -2 par des groupements amino, hydroxy, thiol, thiométhyle, halogéno, sont soumises aux effets polaires et stériques de ces groupements, mais également aux autres interactions possibles entre ces groupements et l'atome d'azote.

Ainsi, le méthyle et le chlore qui ont sensiblement la même taille, ont cependant des effets différents, la valeur plus négative du chlore par rapport au méthyle (−0.5) serait dûe d'après SNYDER[13], par comparaison avec la chloro-2 pyridine et la chloro-2 quinoléine, à des interactions électrostatiques entre les atomes de chlore et d'azote en position adjacente, beaucoup plus qu'à l'effet inductif de cet atome.

L'importance des effets stériques en -2 apparait également de façon significative dans le comportement des phényl-thiazoles, ainsi la différence entre le q^{0}_{ij} du phényl-5 thiazole et celui du phényl-2 thiazole est de +1, alors que l'effet inductif de ce groupement est faible, il en est de même pour le groupement méthyl-thio.

TABLEAU IV

EFFETS POLAIRES ET STÉRIQUES DES SUBSTITUANTS EN -2 DANS LE THIAZOLE, LE PHENYL-4 THIAZOLE ET LE DIPHENYL-4,5 THIAZOLE (SUR ALUMINE)

Dans ce tableau, comme dans les tableaux suivants, l'énergie d'adsorption, $S°$, du thiazole qui sert de référence, a été prise égale à 6.2, ce qui n'altère pas les valeurs de Δ de la dernière colonne du Tableau V, mais seulement les q^0_{ij} de +0.4.

Groupements en -2	$\sum_j q°_{ij}$			
	$Q°_i$[7]	*Thiazole*	*Phényl-4 thiazole*	*Diphényl-4,5 thiazole*
CH_3	0.06	−0.38	−0.28	−0.33
SCH_3	1.3	−1.01	−1.7	−1.7
Cl	0.2	−0.9	−1.7	−2.1
Br	0.33	−1.08	−1.9	−2.3
C_6H_5	1.85	−1.07	−1.6	−1.9
NH_2	4.4	+0.38	−0.1	+0.3
OH	7.4	+0.83	+1.7	—
SH	8.7	+0.5	+1.9	—

Le groupement cyclohexyle qui ne figure pas dans les tableaux, diminue également l'énergie d'adsorption de la molécule, malgré son caractère nucléophile.

Lorsque l'on substitue la molécule par un groupement phényle en -4, puis par un groupement phényle en -5, on peut également évaluer—toujours d'après la relation (6 ter)—les effets globaux (polaires et stériques) des substituants en -2 (*cf.* Tableau IV). Dans ce cas, $\Delta S°$ représente la différence entre l'énergie d'adsorption du phényl-4 thiazole ou diphényl-4,5 thiazole diversement substitué en -2 et le phényl-4 thiazole ou le diphényl-4,5 thiazole dans le deuxième cas.

Ces effets peuvent aussi être évalués à partir des effets globaux des substituants X en -2 dans le thiazole ($q°_{\mathrm{Th}}{}^{\mathrm{X}}{}_{\text{-2}}$ est donné par la première colonne du Tableau IV) et des interactions électroniques et stériques mutuelles Δ (tirées du Tableau V) entre les substituants en -2 d'une part, et le groupement phényle en -4 ou diphényl-4,5 thiazole d'autre part:

$$q°_{\varphi\text{-4 Th}}^{\text{X-2}} = q°_{\text{Th}}^{\text{X-2}} + \Delta_{\varphi\text{-4 Th}}^{\text{X-2}} \qquad (11)$$

Les effets globaux des groupements méthyl-thio, halogéno et phényle sont sensiblement les mêmes dans chacune des trois séries envisagées.

Variations de l'énergie d'adsorption de l'atome d'azote en fonction des substituants aux positions -2 et -4 du cycle thiazolique

Les valeurs expérimentales des énergies d'adsorption des aryl-4 thiazoles, substitués ou non en -2 du Tableau V, permettent de calculer d'après la relation (6 ter), les variations de l'énergie d'adsorption de l'atome d'azote du thiazole, sous les effets des substituants en -2 et des groupements aryl en -4.

Ces valeurs peuvent alors être comparées, dans la colonne suivante, aux valeurs calculées par additivité des variations d'énergie d'adsorption, sous les effets globaux des substituants en -2 d'une part, et des groupements aryl en -4 d'autre part:

$$(\sum_j q°_{ij})^{\text{-2,4}}_{\text{exp}} = (\sum_j q°_{ij})^{\text{-2}}_{\text{exp}} + (\sum_j q°_{ij})^{\text{-4}}_{\text{exp}} + (\Delta q°_{ij})^{\text{-2,4}} \qquad (12)$$

TABLEAU V

ENERGIES D'ADSORPTION ET EFFETS POLAIRES ET STÉRIQUES DES ARYL THIAZOLES DIVERSEMENT SUBSTITUÉES SUR ALUMINE

Solutés	*Systèmes et* R_M'	$S°_{Th\text{-}i}$	$\Delta S°$	$\sum_j q°_{ij}$		Δ
				Exp.	*Calc.*	
Aryl-2 thiazoles	*9*					
Phényl-2	0.52	6.15	−0.05	−1.07		
p-Chlorophényl-2	0.44	6.35	+0.15	−0.98		
p-Bromophényl-2	0.46	6.3	+0.1	−1.1		
p-Tolyl-2		6.25	+0.05	−1.02		
p-Méthoxyphényl-2	0.82	7.2	+1	−1.02		
p-Nitrophényl-2	1.15	8.05	+1.85	−0.57		
Aryl-5 thiazoles	*8*					
Phényl-5	0.31	7.3	1.1	+0.08		
p-Nitrophényl-5	0.9	8.4	2.2	−0.22		
Aryl-4 thiazoles	*8*					
Phényl-4	0.01	6.7	0.5	−0.52		
p-Tolyl-4	−0.3	6.5	0.3	−0.75		
p-Chlorophényl-4	−0.02	7.1	0.9	−0.23		
p-Bromophényl-4	−0.05	6.9	0.7	−0.5		
p-Méthoxyphényl-4	0.3	7.5	1.3	−0.7		
p-Nitrophényl-4	0.74	8.3	2.1	−0.32		
Methyl-2 phényl-4	−0.06	6.45	0.25	−0.8	−0.9	0.1
Polyaryl thiazoles	*8*					
Diphényl-2,4	−0.61	6.1	−0.1	−2.1	−1.6	−0.54
Diphényl-2,5	−0.09	7.7	1.5	−0.54	−1	0.46
Diphényl-4,5	0.20	8.3	2.1	0.06	−0.44	0.5
Triphényl-2,4,5	−0.07	7.4	1.2	−1.85	−1	−0.85
Méthyl-2 diphényl-4,5	0.01	8	1.8	−0.27	−0.32	0.05
Halogéno-2 thiazoles	*8*					
Chloro-2	−0.29[4]	5.4	−0.8	−0.91	−0.91	0
Bromo-2	−0.38[4]	5.3	−0.9	−1.08	−1.08	0
Chloro-2 phényl-4	−0.5	5.1	−1.1	−2.23	−1.43	−0.8
Chloro-2 *p*-tolyl-4	−0.5	5.3	−0.9	−2.06	−1.65	−0.4
Chloro-2 *p*-chlorophényl-4	−0.45	5.5	−0.7	−1.94	−1.14	−0.8
Chloro-2 *p*-bromophényl-4	−0.4	5.55	−0.65	−1.95	−1.4	−0.55
Chloro-2 *p*-méthoxyphényl-4	−0.3	5.95	−0.25	−2.4	−1.63	−0.75
Chloro-2 *p*-nitrophényl-4	0.55	8.2	+2	−0.53	−1.23	+0.7
Chloro-2 diphényl-4,5	−2	6.3	+0.1	−2.05	−0.84	−1.2
Thiométhyl-2 thiazoles	*5*					
Thiométhyl-2	−0.01	5.9	−0.3	−1.015	−1.015	0
Thiométhyl-2 phényl-4	−0.33	5.7	−0.5	−2.23	−1.53	−0.7
Thiométhyl-2 *p*-tolyl-4	−0.35	6.05	−0.1	−1.86	−1.76	−0.1
Thiométhyl-2 *p*-chlorophényl-4	−0.50	5.7	−0.5	−2.34	−1.24	−1.1
Thiométhyl-2 *p*-bromophényl-4	−0.50	5.7	−0.5	−2.41	−1.51	−0.9
Thiométhyl-2 *p*-methoxyphényl-4	+0.12	7.55	1.4	−1.23	−1.73	+0.4
Thiométhyl-2 *p*-nitrophényl-4	0.55	8.6	2.4	−0.73	−1.31	+0.58
Thiométhyl-2 diphényl-4,5	−0.25	7.3	1.1	−1.65	−0.955	−0.7

TABLEAU V *(suite)*

Solutés	*Systèmes et* R_M'	$S°_{Th\text{-}i}$	$\Delta S°$	$\sum_j q°_{ij}$ *Exp.*	*Calc.*	Δ
Amino-2 thiazoles	*10b*					
Amino-2 thiazole	0.95	9	2.8	0.38	0.38	0
Amino-2 phényl-4	0.24	9	2.8	−0.64	−0.14	−0.5
Amino-2 *p*-tolyl-4	0.05	8.7	2.5	−0.97	−0.37	−0.6
Amino-2 *p*-chlorophényl-2	0.14	8.9	2.7	−0.92	+0.15	−1.07
Amino-2 *p*-bromophényl-4	0.12	9.2	3	−0.55	+0.12	−0.43
Amino-2 *p*-méthoxyphényl-4	0.67	10.7	4.5	+0.06	−0.34	+0.4
Amino-2 *p*-nitrophényl-4	1	11.7	5.5	+0.65	+0.06	+0.6
Amino-2 diphényl-4,5	0.45	11	4.8	+1	1.1	−0.1
Hydroxy-2 thiazoles	*11*					
Hydroxy-2 thiazole	−0.10	11.1	4.9	0.83	0.83	0
Hydroxy-2 phényl-4	−0.25	12.5	6.3	1.2	0.3	0.9
Hydroxy-2 *p*-tolyl-4	−0.4	12.2	6	0.87	0.08	0.8
Hydroxy-2 *p*-chlorophényl-4	−0.2	12.6	6.4	1.2	0.6	0.6
Hydroxy-2 *p*-bromophényl-4	−0.19	12.6	6.4	1.1	0.33	0.8
Hydroxy-2 *p*-méthoxyphényl-4	−0.05	13.9	7.7	1.6	0.11	1.5
Hydroxy-2 *p*-nitrophényl-4	0.42	15.5	9.3	2.8	0.5	2.3
Mercapto-2 thiazoles	*11*					
Mercapto-2 thiazole	0.31	11.5	5.3	0.5	0.5	0
Mercapto-2 phényl-4	0.10	13.4	7.2	1.38	−0.02	1.4
Mercapto-2 *p*-tolyl-4	−0.19	13	6.8	0.95	−0.25	1.2
Mercapto-2 *p*-chlorophényl-4	0.64	15.1	8.9	2.9	0	2.9
Mercapto-2 *p*-bromophényl-4	0.55	15.2	9	3.07	0.27	2.8
Mercapto-2 *p*-méthoxyphényl-4	0.91	16.4	10.2	3.38	−0.22	3.6
Mercapto-2 *p*-nitrophényl-4	1.5	18	11.8	4.58	0.18	4.6

La différence entre ces valeurs ou écart à l'additivité peut être considérée comme une fonction représentant les variations de l'énergie d'adsorption de la molécule disubstituée en -2,4, sous les effets polaires, stériques et autres, des substituants en -2 et en -4.

$$(\Delta q°_{ij})^{-2,4} = (\sum_j q°_{ij})^{-2,4}_{\text{exp}} - (\sum_j q°_{ij})^{-2,4}_{\text{calc}} = f(\sigma, E_S' x) \quad \text{(12bis)}$$

Cette variation affecte non seulement l'énergie d'adsorption de l'atome d'azote, mais également celle des autres groupements présents dans la molécule.

Si $\Delta < 0$, cette diminution d'énergie d'adsorption est due principalement à un effet d'encombrement stérique pour les groupements autres que les groupements halogéno, cette influence est d'autant plus importante que les substituants en -2 ont un $\sigma_p < 0$.

Si $\Delta > 0$, l'acroissement d'énergie d'adsorption de la molécule peut être attribué à l'importance des interactions électroniques mutuelles entre les substituants en -2 qui ont un $\sigma_p < 0$ et l'atome d'azote.

C'est effectivement ce que l'on constate avec les hydroxy-2 et les mercapto-2 aryl-4 thiazoles, mais, il est vrai que dans ce cas, le groupement phényle en -4 favorise

les formes ol et thiol, plus fortement adsorbées sur alumine, alors que l'on observe le contraire avec les amino-2 aryl-4 thiazoles.

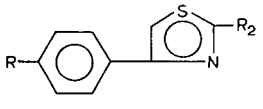
D'une façon générale, pour l'ensemble des composés étudiés, les Δ augmentent avec l'énergie d'adsorption du groupement en *para* et des groupements en -2. Ces variations semblent donc liées à plusieurs facteurs: à l'existence de formes tautomères, à une plus grande localisation des substituants à forte énergie d'adsorption, mais aussi à un mode d'adsorption différent dans le cas des hydroxy-2 et mercapto-2 thiazoles (adsorption verticale et non plane), ce qui rend hasardeux tout essai d'interprétation.

On peut néanmoins faire l'hypothèse que les variations d'énergie d'adsorption dues aux interactions électroniques entre les substituants en -2 et en -4 de l'atome d'azote sont sensiblement les mêmes que les molécules disubstituées en -2,5.

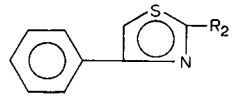
$(\sum^{j} q°_{ij})^{-2,4} \# (\sum^{j} q°_{ij})^{-2,5}$ pour les effets polaires.

Malheureusement, les données que nous possédons sur les aryl-5 thiazoles diversement substitués en -2 ne sont encore que fragmentaires, ce qui ne nous permet pas d'évaluer ce terme.

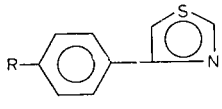
Variations de l'énergie d'adsorption de l'atome d'azote sous les effets polaires des substituants en para du groupement phényle en -4 en fonction des substituants en -2



IV



V



VI

Ces variations, rassemblées dans le Tableau V, sont calculées toujours d'après la relation (6 ter):

$$q°_{ij} = \Delta S° - 0.55\, Q°_i$$

dans laquelle $\Delta S°$ est cette fois la différence entre l'énergie d'adsorption du phényl-4 thiazole *para* substitué (IV) et du phényl-4 thiazole correspondant (V) et $Q°_i$ est l'énergie d'adsorption du groupement situé en *para* (R).

Dans le cas des aryl-4 thiazoles substitués en -2 cette relation peut également se mettre sous la forme:

$$q°_{ij} = q'°_{ij} + \Delta\, q°_{ij}$$

où $q'°_{ij}$ est la variation d'énergie d'adsorption sous l'influence des effets polaires des substituants (R) en *para* du phényl-4 thiazole non substitué en -2 (VI) (donné dans la troisième colonne du Tableau VI) et $\Delta q°_{ij}$ représente la variation d'énergie d'adsorption de la molécule, par suite des perturbations électroniques introduites par un substituant en *para* dans les aryl-4 thiazoles diversement substitués en -2 (IV).

TABLEAU VI

EFFETS POLAIRES DES SUBSTITUANTS EN *para* DES ARYL-4 THIAZOLES DIVERSEMENT SUBSTITUÉS À LA POSITION -2 ET LE RESTE DE LA MOLÉCULE

Groupements en para	$0.55\,Q°_i$	$\sum_j q°_{ij}$					
		H	*Cl*	SCH_3	NH_2	*OH*	*SH*
CH_3	0.03	−0.23	+0.17	+0.35	−0.33	−0.33	−0.43
Cl	0.1	+0.32	+0.3	−0.1	−0.2	0	1.6
Br	0.18	+0.02	+0.27	−0.18	+0.02	−0.28	1.6
CH_3O	1	−0.2	−0.15	+0.9	+0.7	+0.4	+2
NO_2	1.4	+0.2	+1.7	+1.5	+1.3	+1.6	+3.2

Ces $\Delta q°_{ij}$ peuvent être évalués pour chacune des séries d'aryl-4 thiazoles étudiés à partir des données du Tableau V en faisant la différence, à l'intérieur d'une même série, entre le Δ de la molécule substituée en *para* (IV) et le Δ de la même molécule non substituée en *para* (V).

Ces variations sont dans l'ensemble assez faibles, dans la série des aryl-4 thiazoles non substitués en -2 (VI).

L'introduction d'un méthyle en *para* provoque, malgré son effet donneur, une diminution de l'énergie d'adsorption de l'atome d'azote, excepté pour les molécules portant en -2 un groupement X ayant un effet inductif important (Cl, SCH_3).

Cette diminution d'énergie d'adsorption est plus importante pour les substituants amino, hydroxy et mercapto en -2, pour lesquels on peut supposer que les formes tautomères moins adsorbées sont favorisées. Les groupements halogéno en *para* n'ont pas beaucoup d'influence sur l'énergie d'adsorption de l'atome d'azote dans les aryl-4 thiazoles et les halogéno-2 aryl-4 thiazoles. L'effet inductif de ces substituants se manifeste néanmoins dans les thiométhylamino-2 et hydroxy-2 aryl-4 thiazoles avec lesquels on observe une diminution sensible de l'énergie d'adsorption. Dans les mercapto-2 aryl-4 thiazoles l'augmentation importante de l'énergie d'adsorption de la molécule est liée à la plus grande adsorption de la forme thiol de la molécule, favorisée par la présence de ces groupements (la variation d'énergie d'adsorption entre les deux formes étant approximativement de 1.5 unité).

Les variations $\sum_j q°_{ij}$ sont positives avec les groupements méthoxy et nitro et augmentent avec leur énergie d'adsorption. Ces variations importantes peuvent être dues à une augmentation de l'énergie d'adsorption de ces groupements soit par suite de leurs interactions électroniques avec le reste de la molécule, soit par suite d'une valeur plus importante de la fonction de localisation que celle que nous avons prise; $f(Q°_k)$ pour ces groupements pourrait donc être nul par suite de la localisation complète de ces groupements à la surface de l'alumine.

Ceci nous conduit à un résultat intéressant à savoir que lorsque le centre d'adsorption de la molécule se déplace de l'atome d'azote du cycle vers des substituants à la position -2, dont le $Q°_i \geqslant 1.3$, la localisation des substituants en *para* (dont le $Q°_i \geqslant 1.3$), donc suffisamment éloignés de ces centres, est complète.

V. CONCLUSION

La séparation, sur alumine faiblement activée d'une centaine de thiazoles diversement substitués aux positions -2, -4 et -5 du cycle, nous a permis de mettre en évidence les points suivants:

(1) Les effets stériques des substituants alcoyles en position *ortho* du centre d'adsorption sont prédominants. Une évaluation approximative de ces effets a pu être faite.

Ainsi, pour un groupement méthyle, la diminution d'énergie d'adsorption est de -0.5 ± 0.1, elle est le double pour un groupement *tert.*-butyle. A cette diminution d'énergie s'ajoute également une perte d'énergie due à une modification de l'adsorption de la molécule, par suite des effets stériques de ces groupements: pratiquement nulle pour le méthyle et l'éthyle, elle est de -0.55 pour le *tert.*-butyle.

(2) L'additivité des groupements alcoyle en -2 et -5 a été vérifiée, ainsi que dans la série des dialcoyl-2,4 thiazoles, pour les groupements peu encombrants.

Lorsque des groupements encombrants (isopropyle et *tert.*-butyle) sont de part et d'autre du centre d'adsorption, l'atome d'azote est de plus en plus masqué par ces groupements et la variation d'énergie d'adsorption de la molécule est plus élevée que celle attendue par additivité.

Ainsi, dans le di-*tert.*-butyl-2,4 thiazole, l'énergie d'adsorption de l'atome d'azote est pratiquement nulle.

(3) Les résultats encore fragmentaires obtenus avec les thiazoles diversement substitués en -5, ont néanmoins permis de relier les effets polaires de ces substituants aux σ_p de HAMMETT[24]. Cependant, la sensibilité de l'atome d'azote du thiazole, dans cette réaction, est moins sensible que dans le cas de la pyridine, par suite de sa plus faible énergie d'adsorption.

(4) L'étude des variations de l'énergie d'adsorption de l'azote sous l'effet combiné des substituants aryle en -4 et des substituants halogéno, méthyl-thio, hydroxy, amino et thiol en -2 a montré des différences assez sensibles entre ces variations et celles déterminées par le calcul, par additivité des variations produites par ces groupements pris séparément. La complexité de ces molécules ne permet pas de faire la part des différentes perturbations (électroniques, stériques, chimiques, etc.) introduites par ces groupements.

Quant à l'influence des substituants en *para* du phényl-4 dans les thiazoles diversement substitués en -2, il semble difficile de la relier aux effets polaires de ces groupements. En effet, par suite de leur éloignement vis-à-vis de l'atome d'azote, les effets dûs à la délocalisation de ces groupements sont plus faibles. Il en résulte, pour les groupements plus fortement adsorbés (CH_3O et NO_2) une augmentation de l'énergie d'adsorption de la molécule, indépendamment de leur caractère électron-attracteur ou électron-donneur.

Il ne faut également pas perdre de vue que l'impossibilité d'utiliser un système chromatographique unique, pour l'ensemble des composés étudiés (couches de même activité, éluées avec le même éluant) peut être la cause d'erreurs systématiques.

Néanmoins, cette étude, malgré ses limites, illustre les possibilités d'application de la théorie de SNYDER, à des molécules complexes et montre l'influence de quelques facteurs responsables de leur adsorption.

RÉSUMÉ

La théorie de la chromatographie d'adsorption linéaire de SNYDER, appliquée à une centaine d'hétérocycles thiazoliques, nous a tout d'abord permis de déterminer expérimentalement les énergies d'adsorption de ces composés puis de les comparer aux énergies d'adsorption estimées par le calcul à l'aide des tables établies.

Dans la série des alcoyl et des dialcoyl thiazoles, cette comparaison nous a permis de déterminer les variations d'énergie d'adsorption de l'atome d'azote du cycle sous les effets polaires et stériques de ces groupements et de relier ces effets aux constantes d'effets polaires et stériques des substituants.

Dans la série des aryl-4 thiazoles diversement substitués en position -2, nous avons également étudié les variations d'énergie d'adsorption de ces molécules sous les effets polaires des substituants en *para* du phényle en fonction des substituants en -2 et déterminé les intéractions électroniques mutuelles entre les divers groupements.

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CHROM. 4474

LES APPLICATIONS DE LA THÉORIE DE LA CHROMATOGRAPHIE D'ADSORPTION LINEAIRE DE SNYDER EN SÉRIE HÉTÉROCYCLIQUE

II. CAS DE LA SILICE; COMPARAISON AVEC L'ALUMINE

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SUMMARY

Applications of SNYDER's *theory on linear adsorption chromatography to heterocyclic compounds. II. Silica gel in comparison with aluminium oxide*

SNYDER's theory of linear adsorption chromatography has been applied to one hundred thiazoles, and we have experimentally determined the adsorption energy on silica gel and aluminium oxide (Merck).

By comparing these results with the adsorption energy estimated by calculations using data in fixed tables, we could determine the total interactions of different groups inside these molecules.

The interactions are additive, in the case of the groups that are weakly adsorbed (alkyl and halogen) and in the absence of steric hindrance on every side of the adsorption center.

The sensitivity of these molecules to the steric effects of the alkyl groups is more important on aluminium oxide than on silica gel.

In the series of the 4-aryl thiazoles with different groups in the 2-position we have also studied the variation of adsorption energy, under the effects, of the substituents in a position *para* to the phenyl group in the 4-position and of the substituents in 2-position.

This study allows us to relate these variations to the polar and steric effects of these substituents, but, the molecule being complex, other phenomena may also occur.

INTRODUCTION

L'étude précédente nous a permis de préciser les facteurs qui déterminent la séparation, sur alumine, des thiazoles diversement substitués aux positions -2, -4 et -5 du cycle et de déterminer les effets dûs aux interactions électroniques et stériques entre ces substituants et l'atome d'azote cyclique. Etant donnée la très grande similitude chimique et physique entre l'alumine et la silice et leur utilisation très générale

en chromatographie d'adsorption, il nous a semblé naturel de poursuivre avec la silice notre étude initiale sur l'alumine.

L'objet de ce mémoire est de montrer à l'aide des composés étudiés précédemment par application de la théorie de SNYDER, les similitudes et les différences éventuelles de leurs affinités d'adsorption pour ces deux adsorbants. Les relations, valables pour l'alumine, doivent être légèrement modifiées lors de l'emploi de la silice, par suite des différences fondamentales qui caractérisent le mode d'adsorption de ces deux supports. Ainsi, la relation générale qui permet le calcul du R_M' dans un système chromatographique donné devient[1]:

$$R_M' = \log \frac{V_a W_a}{V_s} + \alpha \left[\sum^{i} Q^\circ_i - f'(Q^\circ_k) \sum^{i \neq k} Q^\circ_i + \sum^{i} \sum^{j} q^\circ_{ij} - \varepsilon^\circ \sum^{i} a'_i \right] \qquad (1)$$

où S° a été remplacé par sa valeur calculée[1].

Tous les termes de cette relation ont la même signification que dans le cas de l'alumine mais $f'(Q^\circ_k)_{\text{silice}} = 0.4 f(Q^\circ_k)_{\text{alumine}}$[1]. Les variations d'énergie d'adsorption dues à la localisation d'un groupement dans une molécule sont en effet plus faibles sur silice que sur alumine. Cette fonction $f'(Q^\circ_k)$ (qui est égale à 0.18 pour un composé aromatique) est supposée indépendante de la position relative des groupements i par rapport à k. Cependant lorsque la distance entre les groupements délocalisés i et le groupement localisé k est supérieure ou égale à 6.2 Å, cette fonction décroit, car les variations de l'énergie d'adsorption de la molécule dues aux effets de localisation sont plus faibles[1].

De même, on a la relation[1]:*

$$(a'_i)_{\text{silice}} = (a_i)_{\text{calculé}} + 16 f(Q^\circ_k) \qquad (2)$$

(sur une couche de silice d'activité voisine de 0.6). Cette augmentation de la surface occupée par la molécule, sur la silice désactivée, est due à un effet de localisation du solvant ou à des différences d'énergie d'adsorption du solvant en différents points de la surface de l'adsorbant (bibl. I).

Ces différences entre la silice et l'alumine résident dans le fait que les groupements hydroxyle à la surface de la silice sont plus aptes à réagir avec les molécules adsorbées, pour donner lieu à une adsorption localisée, que les sites d'adsorption à la surface de l'alumine. SNYDER a montré que ces valeurs de a'_i décroissent lorsque l'activité de la couche augmente et que pour une couche d'activité standard ($\alpha = 1$): $a'_i = a_i$. La courbe de variation $\Delta a_i = f(Q^\circ_k)$ établie par SNYDER[1] montre que pour $Q^\circ_k \geqslant 4$, ce qui est le cas en série thiazolique, Δa_i est constant et égal à 7 (bibl. 1).

Des tables de valeurs de ε° (bibl. 2–5) de A_S (bibl. 3, 4), de S° (bibl. 3) et de Q°_i (bibl. 6) sur la silice permettent de calculer le R_M', de n'importe quel composé.

PARTIE EXPERIMENTALE

Le mode opératoire ainsi que les valeurs R_F des thiazoles étudiés ont été reportés dans des mémoires précédents. Mais comme pour l'alumine, ceux-ci ont à nouveau

* La valeur précédemment donnée par SNYDER[12] était de 14.5.

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TABLEAU I

PRINCIPAUX SYSTÈMES CHROMATOGRAPHIQUES AVEC LEURS CARACTÉRISTIQUES

Eluants	ε°	*Adsorbants*	R_F *du mélange test Desaga*	*Composés étudiés et références bibliographiques*	*No. du système et paramètres chromatographiques*
Hexane–CH_2Cl_2 (50:20)	0.2	SiO_2 GF_{254}	0.41; 0.45; 0.70	Dialcoyl-2,4 thiazoles[9] (R_F du thiazole 0.03)	1 ($a = 0.6$; $p = -1.05$)
Hexane–CH_2Cl_2 (50:20)	0.2	Film 301 R	0.07; 0.13; 0.39	Aryl-4 et halogéno-2 aryl-4 thiazoles (R_F du thiazole 0.26)	2 ($a = 0.6$; $p = -2.2$)
Benzène	0.25	SiO_2 GF_{254}	0.52; 0.58; 0.70	Composés témoins (R_F du thiazole 0.12)	3a ($a = 0.35$; $p = -0.85$) 3b ($a = 0.56$; $p = -1.2$)
$CHCl_3$	0.26	SiO_2 GF_{254}		Composés témoins (R_F du thiazole 0.32)	4 ($a = 0.66$; $p = -2$)
Hexane–acétone (55:15)	0.30	SiO_2 GF_{254}	0.10; 0.48; 0.55 0.62	Diaryl-4,5 thiazoles diversement substitués en -2	5 ($a = 0.43$; $p = -1.2$)
CH_2Cl_2	0.32	SiO_2 GF_{254}	0.35; 0.70; 0.92	Alcoyl-2 thiazoles diversement substitués en -2 (R_F du thiazole 0.3)	6a ($a = 0.55$; $p = -1.1$)
CH_2Cl_2	—	—		Alcoyl et dialcoyl thiazoles (R_F du thiazole 0.2)	6b ($a = 0.6$; $p = -1$)
CH_2Cl_2 + AcOEt	0.34	SiO_2 GF_{254}	0.10; 0.90; 0.95	Amino-2 thiazole et hydroxy-2 aryl-4 thiazoles (R_F du thiazole 0.62)	7 ($a = 0.52$; $p = -1.4$)
AcOEt	0.38	SiO_2 GF_{254}	—	Amino-2, hydroxy-2 thiazoles et composés témoins (R_F du thiazole 0.66)	8a ($a = 0.6$; $p = -1.2$) 8b ($a = 0.55$; $p = -1.35$)

été relevés afin de nous replacer dans des conditions identiques et faciliter la reproduction des résultats.

Les plaques de silice GF_{254} ont été préparées à partir de 30 g d'adsorbant dans 70 cm^3 d'eau distillée et activées 30 min à 100–110°. L'activité de la couche ne varie pratiquement plus, après ce laps de temps. Les plaques sont utilisées dans les 24 h après leur préparation. Le mélange test Desaga à trois colorants et le thiazole sont pris dans chaque cas comme solutés de référence.

Détermination des paramètres chromatographiques

Les solutés témoins choisis pour déterminer les paramètres chromatographiques ont été l'acétophénone ($S° = 6.2$), l'aniline ($S° = 6.6$), le *p*-anisidine ($S° = 8.6$) le

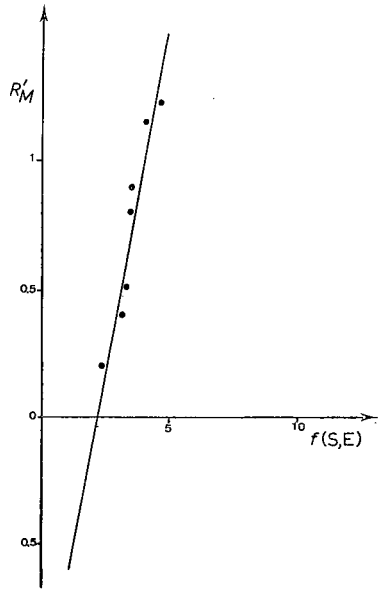

Fig. 1. Paramètres chromatographiques de la silice déterminés à partir des composés témoins. ($\alpha = 0.57$, $p = -1.2$). Éluant, le benzène.

p-nitrophénol ($S° = 8.5$) la pyridine ($S° = 7.7$) la chloro-2 pyridine ($S° = 6.5$) et le thiazole ($S° = 6.8$) dont les énergies d'adsorption et les A_S sur silice sont connus ou facilement calculables (éqn. 1) (*cf.* Fig. 1).

Les différents systèmes chromatographiques utilisés au cours de cette étude ainsi que les paramètres chromatographiques déterminés pour chaque série de composés, par la méthode de SNYDER précédemment décrite pour l'alumine, sont rassemblés dans le Tableau I. Les résultats obtenus montrent que sur onze systèmes cités, la moyenne des valeurs des paramètres chromatographiques obtenus sur sept d'entre eux (1, 3b, 6a, 7, 8a et 8b) est la suivante: $\alpha = 0.57$ et $p = -1.2$, valeurs assez voisines de celles trouvées par SNYDER[7] à partir des résultats de JANÁK[8]: $\alpha = 0.56$ et $p = -1.29$, sur silice avec l'hexane normal.

TABLEAU II

ENERGIE D'ADSORPTION ($S°$) ET EFFETS POLAIRES ET STÉRIQUES ($\sum_j q°_{ij}$) DES ALCOYL ET DIALCOYL THIAZOLES ISOMÈRES SUR SILICE GF 254

$S°$ du thiazole = 6.8 ± 0.2.

Solutés	*Systèmes et* R_M'		$S°_{th-i}$	$\Delta S°$	$\sum_j q°_{ij}$		Δ
					Exp.	*Calc.*	
Alcoyl-2 thiazoles	*6b*	*6a*					
Méthyl	0.47	0.37	7.2	0.4	0.3		
Éthyl	0.35	0.29	7.1	0.3	0.235		
n-Propyl	0.18	0.16	6.9	0.1	0.075		
Isopropyl	0.18	0.06	6.75	−0.05	−0.13		
tert.-Butyl	0.05	−0.1	6.4	−0.4	−0.53		
Alcoyl-4 thiazoles	*6b*						
Méthyl	0.40		7.1	0.3	0.2		
Éthyl	0.29		7	0.2	0.135		
Isopropyl	0.20		6.9	0.1	0.02		
tert.-Butyl	0		6.5	−0.3	−0.43		
Alcoyl-5 thiazoles	*6b*						
Méthyl	0.42		7.2	0.4	0.3		
Éthyl	0.44		7.3	0.5	0.435		
Isopropyl	0.40		7.35	0.55	0.47		
tert.-Butyl	0.35		7.4	0.6	0.47		
Dialcoyl-2,4 thiazoles	*1*						
Diméthyl-2,4	1.65		7.5	0.7	0.5	0.5	0
Méthyl-4 éthyl-2	1.4		7.3	0.5	0.235	0.435	−0.1
Méthyl-4 isopropyl-2	1.23		7.1	0.3	0.12	0.07	0.05
Méthyl-4 *tert.*-butyl-2	0.91		6.7	−0.1	−0.35	−0.35	0
Méthyl-2 *tert.*-butyl-4	0.96		6.7	−0.1	−0.33	−0.13	−0.2
Éthyl-2 *tert.*-butyl-4	0.70		6.4	−0.4	−0.6	−0.2	−0.3
Isopropyl-2 *tert.*-butyl-4	0.42		6.15	−0.65	−0.87	−0.56	−0.4
Di-*tert.*-butyl-2,4	−0.6		4.8	−2	−2.26	−0.96	−1.3
Dialcoyl-2,5 thiazoles	*6b*						
Diméthyl-2,5	0.67		7.6	0.8	0.6	0.6	0
Diméthyl-4,5	0.67		7.6	0.8	0.6	0.5	0.1
Méthyl-5 éthyl-2	0.44		7.4	0.6	0.435	0.535	−0.1
Méthyl-5 isopropyl-2	0.15		7.1	0.3	0.12	0.17	−0.05
Méthyl-5 *tert.*-butyl-2	−0.16		6.85	0.05	−0.18	−0.23	0.05

DISCUSSION

Influence des groupements alcoyle

L'examen des résultats du Tableau II nous montre que sur silice, l'effet d'un méthyle est sensiblement le même quelque soit sa position sur le cycle. Lorsque l'on passe aux termes supérieurs, l'énergie d'adsorption croit dans la série des alcoyl-5 thiazoles et décroit dans la série des alcoyl-2 et -4 thiazoles, par suite des effets stériques de ces groupements.

En reprenant les hypothèses précédemment émises pour l'alumine, on peut évaluer les effets stériques de ces groupements alcoyl-2 et -4. Ils doivent être sensiblement les mêmes pour les deux séries (*cf.* Tableau III). Si nous comparons les valeurs

TABLEAU III

EFFETS POLAIRES ET STERIQUES DES GROUPEMENTS ALCOYLE SUR SILICE

Groupements	*Effets polaires des substituants en -2,-4 ou -5*	*Effets stériques*	
		$\beta_S E'_S$	
	q°_{ij} *(exp.)*[a]	*−2*	*−4*
Méthyle	0.3	0	−0.1
Éthyle	0.43	−0.2	−0.3
Isopropyle	0.47	−0.6	−0.5
tert.-Butyle	0.47	−1	−0.9

[a] Calculé à l'aide de la relation $q^\circ_{ij} = \varrho_i \cdot \sigma_j = 0.35 \pm 0.05$ avec $\varrho_i = -2$.

ainsi évaluées, à celles obtenues pour l'alumine, nous obtenons la relation linéaire suivante:

$$(\beta_S E'_S)_{\text{silice}} = 2.6\ (\beta_A E'_S)_{\text{alumine}} + 1.45 \qquad (3)$$

E'_S étant la constante d'encombrement stérique des groupements alcoyle. β_S et β_A sont des paramètres qui dépendent des conditions expérimentales et de la sensibilité de l'atome d'azote du cycle à l'environnement stérique. Cette relation montre que la silice est moins sensible aux effets stériques des substituants alcoyle que l'alumine, ce que nous avions déjà montré par ailleurs[9,10]. Les différences entre la silice et l'alumine se font surtout sentir au niveau des substituants isopropyl et tertiobutyl en -4.

Dans la série des dialcoyl-2,4 thiazoles, les variations d'énergie d'adsorption de l'atome d'azote du cycle sous les effets polaires et stériques conjugués des substituants alcoyle en -2 et en -4 sont additives, excepté si l'encombrement stérique autour de l'azote croit successivement en -2 et en -4, c'est à dire, si les deux groupements alcoyle en -2 et en -4 sont deux isopropyle ou deux tertiobutyle ou l'un et l'autre.

Il faut, dans ce cas, faire intervenir un facteur de correction Δ tenant compte de la diminution d'énergie d'adsorption de l'atome d'azote due à la présence de ces deux groupements. Ce facteur est sensiblement le même pour deux isopropyle ou un isopropyle et un tertiobutyle ($\Delta = -0.3$); il est nettement plus élevé lorsque deux groupements tertiobutyle occupent les positions adjacentes à l'azote ($\Delta = -1.3$). (*cf.* Tableau IV). Ces variations sont également additives dans la série des dialcoyl-2,5 thiazoles.

Ce phénomène peut aussi être généralisé à d'autres hétérocycles azotés dialcoylés, dans des conditions expérimentales identiques. Cette loi d'additivité des effets

TABLEAU IV

ÉCARTS À L'ADDITIVITÉ DANS LA SÉRIE DES ALCOYL-2 *tert.*-BUTYL-4 THIAZOLES

Dialcoyl-2,4 thiazoles	*Δ(Dialcoyl-2,4 thiazoles)*	
	Alumine	*Silice*
Méthyl-2 *tert.*-butyl-4	−0.09	−0.2
Éthyl-2 *tert.*-butyl-4	−0.5	−0.3
Isopropyl-2 *tert.*-butyl-4	−1.5	−0.4
tert.-Butyl-2 *tert.*-butyl-4	−2.7	−1.3

TABLEAU V

EFFETS POLAIRES ET STÉRIQUES SUR SILICE DES SUBSTITUANTS EN -2 DANS LE THIAZOLE, LE PHÉNYL-4 THIAZOLE, ET LE DIPHÉNYL-4,5 THIAZOLE

Substituants en -2	$Q°_i$ *(système 1)*	*Thiazole*			*Phényl-4 thiazole*			*Diphényl-4,5 thiazole*[d]		
		$S°$[a]	$\Delta S°$[b]	$\sum_j q°_{ij}$[c]	$S°$	$\Delta S°$	$\sum_j q°_{ij}$	$S°$	$\Delta S°$	$\sum_j q°_{ij}$
H	0	6.8	0	0	7.8	0	0	9.8	0	0
CH_3	+0.11	7.2	0.4	0.31	8.1	+0.3	+0.2	9.8	0	−0.1
C_6H_5	+1.5	7.6	0.8	−0.43	8	+0.2	−1	10.2	0.4	−0.8
$-SCH_3$	+1.3	6.7	−0.1	−1.16	7.7	−0.1	−1.2	9.75	−0.05	−1.1
$-OCH_3$	+1.8	7	+0.2	−1.3		—			—	
Cl	−0.2	5.6	−1.2	−1.035	6.8	−1	−0.85	8.5	−1.3	−1.15
Br	−0.17	5.7	−1.1	−0.96		—			—	
SH	+0.67	7.7	+0.9	+0.35	9.1	+1.3	+0.75	11.1	+1.3	0.75
OH	4.2	10.2	3.4	−0.05	13.3	+3.5	+0.05	13.3	+3.5	0.05
NH_2	5.1	10.9	4.1	−0.1	12.1	+4.3	+0.1	14.5	+4.7	+0.5

[a] Calculée d'après la relation générale (éqn. 1) en prenant la moyenne des valeurs obtenues dans les systèmes 6a et 6b.

[b] $\Delta S°$ est la différence entre la molécule substituée en -2 et la molécule non substituée en -2.

[c] $\sum_j q°_{ij}$ est calculée à partir de la relation $\sum_j q°_{ij} = \Delta S° - 0.82\ Q°_i$.

[d] Les valeurs R_F des diaryl-4,5 thiazoles ont été déterminés dans le système 5, ceux des thiazoles diversement substitués en -2 dans les systèmes 6a et 6b et ceux des phényl-4 thiazoles diversement substitués en -2 dans les systèmes 2 et 7.

des groupements alcoyle recoupe des résultats théoriques, obtenus par la méthode HMO, des charges électroniques du thiazole substitué par des groupements méthyle[11].

Ces calculs ont, en effet, montré que la disponibilité du doublet de l'azote peut être obtenue par additivité d'incréments caractéristiques des substituants alcoyles portés par le cycle.

Influence des substituants en -2 en série thiazolique (thiazole, phényl -4 thiazole, diphényl-4,5 thiazole)

Les variations d'énergie d'adsorption de l'atome d'azote dans le thiazole, le phényl-4 thiazole et le diphényl-4,5 thiazole, dues à la présence de groupements divers à la position -2 manifestent une très grande sensibilité de l'atome d'azote du cycle sous les effets inductifs de ces substituants (*cf.* Tableau V). Ces variations sont sensiblement les mêmes pour les trois séries envisagées, excepté pour le groupement méthyle dont l'effet donneur se fait d'autant moins sentir que la molécule est moins basique.

Il en est de même pour le groupement phényle en -2 dont l'effet de conjugaison plus important dans les diphényl ou triphényl thiazoles, ajouté à un effet stérique supplémentaire, diminue de façon sensible l'énergie d'adsorption de la molécule.

Contrairement à l'alumine, la Silice G ou GF_{254}, légèrement acide est donc beaucoup plus sensible aux effets inductifs des substituants en -2 qu'à leurs effets stériques, par suite de la plus grande interaction acide–base entre le soluté et le support.

Influence des substituants sur le cycle thiazolique

Nous avons rassemblé dans le Tableau VI les énergies d'adsorption des aryl thiazoles ainsi que les variations d'énergie d'adsorption de ces molécules, par rapport au thiazole non substitué, sous l'influence des effets polaires (et stériques) des différents substituants. Ces variations sont comparées aux valeurs calculées par additivité des effets dus aux différents groupements pris séparemment.

Exception faite pour les dérivés nitrés en *para* des aryl-4 thiazoles diversement substitués en -2 dont les énergies d'adsorption semblent avoir été surestimées, et pour les composés en -2, 4 dont l'encombrement stérique est important, l'accord est bon entre les valeurs expérimentales et les valeurs calculées par additivité. Ce résultat est intéressant, car il rend plus facile l'appréciation du R_F d'une molécule polysubstituée. Il serait ainsi facile de calculer les R_F des aryl-5 thiazoles diversement substitués en -2 de la même façon que dans le cas des aryl-4 thiazoles.

Cette méthode a été appliquée aux thiométhyl-2 aryl-4 thiazoles dont nous avons calculé les R_F dans le système *2*.

Influence des substituants en para dans les aryl-4 thiazoles substitués ou non en -2

Les effets polaires des substituants en *para* ont été évalués de la même manière que dans le cas de l'alumine. Les résultats, rassemblés dans le Tableau VII montrent que ces effets sont sensiblement les mêmes pour un même groupement en *para* quel que soit le groupement à la position -2. D'autre part les variations observées ne sont pas du même ordre que les variations auxquelles on aurait pu s'attendre en se basant sur les σ_p de ces substituants. On en déduit que cette augmentation d'énergie d'adsorption est liée à l'énergie d'adsorption de ces groupements et non aux effets polaires qu'ils peuvent excercer sur l'atome d'azote du cycle.

TABLEAU VI

ENERGIES D'ADSORPTION ET EFFETS POLAIRES ET STÉRIQUES PAR RAPPORT AU THIAZOLE ET SUR SILICE DES ARYL-THIAZOLES DIVERSEMENT SUBSTITUÉS EN -2 OU EN *para* DU PHÉNYL-4

S° thiazole = 6.8.

Solutés	*Systèmes et* R_M'	S°_{th-i}	ΔS°	$\sum_j q^\circ_{ij}$		Δ
				Exp.	*Calc.*	
Aryl-4 thiazoles	2					
Phényl-4	0.20	7.8	1	−0.23		
p-Tolyl-4	0.37	8.3	1.5	+0.18		
p-Chlorophényl-4	0.29	8.15	1.35	+0.285		
p-Bromophényl-4	0.26	8.1	1.3	+0.21		
p-Méthoxyphényl-4	0.78	9.8	3.0	+0.27		
p-Nitrophényl-4	1.15	10.9	4.1	+0.57		
Chloro-2 aryl-4 thiazoles	2					
Phényl-4	−0.53	6.8	0	−1.065	−1.265	+0.2
p-Tolyl-4	−0.42	7	+0.2	−0.955	−0.855	−0.1
p-Chlorophényl-4	−0.58	6.8	0	−0.9	−0.75	−0.15
p-Bromophényl-4	−0.60	6.8	0	−0.95	−0.825	+0.125
p-Nitrophényl-4	0.05	8.7	1.9	−0.665	−0.765	+0.1
p-Méthoxyphényl-4	0.50	10	3.2	−0.265	−0.565	+0.3
Thiométhyl-2 phényl-4 thiazoles	2					
Phényl-4	−0.25	7.7	0.9	−1.39	−1.39	0
p-Tolyl-4	−0.05	8.15	1.35	−1.03	−0.98	−0.15
p-Chlorophényl-4	−0.1	7.95	1.15	−0.975	−0.875	−0.1
p-Bromophényl-4	−0.1	8	1.2	−0.95	−0.95	0
p-Méthoxyphényl-4	+0.5	9.75	2.95	−0.84	−0.89	0.05
p-Nitrophényl-4	+1	11.2	4.4	−0.29	−0.69	0.4
Divers	6					
Phényl-5	+1.2	8.3	+1.5	+0.3	0.3	0
p-Nitrophényl-5	+1.45	11.1	4.3	+0.73	0.73	0
Diphényl-4,5	−0.2	9.8	+3	0.54	+0.07	0.47
Diphényl-2,4	−0.9	8	1.2	−1.26	−0.66	−0.6
Diphényl-2,5	−0.32	9.4	2.6	+0.15	−0.13	+0.28
Triphényl-2,4,5	−0.7	10.2	3.4	−0.3	−0.36	+0.06
Mercapto-2 aryl-4 thiazoles	3a					
Phényl-4	−0.11	9.1	2.3	0.52	0.12	0.4
p-Tolyl-4	−0.23	9.05	2.25	0.38	0.53	−0.15
p-Chlorophényl-4	−0.21	9	2.2	0.585	0.635	−0.05
p-Bromophényl-4	−0.25	8.95	2.15	0.51	0.56	−0.05
p-Méthoxyphényl-4	0.06	10.8	4	0.72	0.62	+0.1
p-Nitrophényl-4		12.3	5.5	1.32	0.82	+0.5
Hydroxy-2 aryl-4 thiazoles	7					
Phényl-4	−0.25	11.3	4.5	−0.18	−0.28	+0.2
p-Tolyl-4	−0.15	11.5	4.7	−0.07	+0.13	−0.2
p-Chlorophényl-4	−0.33	11.5	4.7	+0.185	+0.235	−0.05
p-Bromophényl-4	−0.35	11.5	4.7	+0.16	0.16	0
p-Méthoxyphényl-4	0	13.4	6.6	+0.42	0.22	+0.2
p-Nitrophényl-4	+0.3	14.8	8	+0.93	0.42	+0.5
Amino-2 aryl-4 thiazoles	7					
Phényl-4	−0.1	12.1	5.3	−0.13	−0.33	0.2
p-Tolyl-4	−0.17	12.2	5.4	−0.12	0.08	−0.2
p-Chlorophényl-4	−0.2	12.1	5.3	0.035	0.185	−0.15
p-Bromophényl-4	−0.15	12.3	5.5	0.21	0.11	0.1
p-Méthoxyphényl-4	0.14	14	7.2	0.27	0.17	0.1
p-Nitrophényl-4	0.7	16.2	9.4	1.57	0.47	1.1

TABLEAU VII

EFFETS POLAIRES, DANS L'ADSORPTION SUR SILICE, DES SUBSTITUANTS EN *para* DES ARYL-4 THIAZOLES DIVERSEMENT SUBSTITUÉS EN -2

Groupements en para	$\sum_j q^\circ_{ij}$						
	$(I-0.4)\,f(Q^\circ_i)\cdot Q^\circ_i = 0.82\cdot Q^\circ_i$	*H*	*Cl*	SCH_3	*SH*	*OH*	NH_2
CH_3	−0.09	0.4	−0.1	+0.25	−0.15	0	0
Cl	−0.16	0.514	+0.16	+0.41	+0.06	0.26	+0.16
Br	−0.14	0.44	+0.11	+0.44	0	0.22	+0.3
CH_3O	1.5	0.5	+0.4	+0.55	+0.2	0.5	+0.4
NO_2	2.3	0.8	+0.9	1.2	+0.9	1.2	+1.8

Pour les groupements en *para* on peut considérer qu'étant donné leur éloignement du centre d'adsorption leur délocalisation est faible ainsi que leurs effets polaires.

Par suite, la variation d'énergie d'adsorption ΔS°, lorsque l'on passe de la molécule non substituée en *para* à la molécule substituée, est égale à l'énergie d'adsorption du groupement considéré $\Delta S^\circ = Q^\circ_i$, d'où $R_M' = \alpha(Q^\circ_i - \varepsilon^\circ \cdot a_i)$; avec le pentane et sur un adsorbant d'activité 1 on aurait: $R_M' = \alpha \cdot \Delta S^\circ = \alpha\, Q^\circ_i$. Ceci nous amène à attribuer une énergie d'adsorption positive de + 0.25 aux groupements halogéno. Nous trouvons une valeur de 0.2 pour l'énergie d'adsorption du groupe ment méthyle (la valeur donnée par SNYDER est de 0.11). Quant aux groupements plus fortement adsorbés comme le méthoxy et le nitro, les variations d'énergie d'adsorption de la molécule sont en moyenne de +1.9 pour le méthoxy et de +3 pour le nitro, ce

TABLEAU VIII

ENERGIE D'ADSORPTION DE L'ATOME D'AZOTE EN FONCTION DE L'ENCOMBREMENT STERIQUE

Solutés	$Q^\circ_{(-N=)}$ (± 0.2)	
	$Al_2O_{3exp.}$[a]	$SiO_{2exp.}$
Thiazole[a]	4.1	5.3
Benzothiazole	3.7	4.9
Méthyl-2 thiazole	3.6	5.3
Éthyl-2 thiazole	3.5	5.2
Isopropyl-2 thiazole	3.3	4.9
tert.-Butyl-2 thiazole	3	4.5
Diméthyl-2,4 thiazole	3.6	5.3
Di-*tert.*-butyl-2,4 thiazole	0	2.5
Chloro-2 thiazole	3.7	4.6
Bromo-2 thiazole	3.6	4.5
Thiométhyl-2 thiazole	3.2	4.4
Di-*tert.*-butyl-2,5 thiazole	2.9	4.4
Phényl-2 thiazole	3.1	4.9
Phényl-4 thiazole	3.6	5.1
Diphényl-2,4 thiazole	2.3	4.1
Amino-2 thiazole	3.8	4.5

[a] $S^0_{exp.}$ thiazole = 5.8.

qui correspond sensiblement aux énergies d'adsorption de ces groupements (1.83 et 2.8).

Ainsi, pour l'ensemble des dérivés substitués en *para* d'un phényl en position -4 et *à fortiori*, pour un substituant phényl en position -5 du thiazole, on peut considérer que, sur la silice, $f'(Q^\circ_k) = 0$.

Comparaison de l'influence des effets steriques des substituants en -2 et en -4 sur l'énergie d'adsorption de l'azote sur alumine et sur silice

La relation (4 bis) avec l'inclusion des effets polaires des substituants va nous permettre de calculer l'énergie d'adsorption de l'azote pour l'ensemble des composés étudiés. En effet, la variation d'énergie de l'atome d'azote $\Delta Q^\circ_{(-N=)}$ lorsque l'on passe de la molécule non substituée à la molécule substituée est reliée aux effets polaires et stériques des substituants par la relation:

$$\Delta Q^\circ_{(-N=)} = \sum^{j} q^\circ_{ij} = \varrho_i \cdot \sigma_j + \beta E_S \qquad (4)$$

d'où

$$\Delta Q^\circ_{(-N=)} - \varrho_i \cdot \sigma_j = f(E'_S) \qquad (4 \text{ bis})$$

Nous avons donc rassemblé dans le Tableau VIII, les valeurs pour la silice et pour l'alumine de l'énergie d'adsorption de l'atome d'azote dans différentes configurations, en faisant l'hypothèse, admise en série pyridinique par SNYDER, que les effets polaires des substituants sont sensiblement les mêmes aux différentes positions du cycle, ce qui permet de déterminer expérimentalement $\varrho_i \cdot \sigma_j$ par simple différence entre les q°_{ij} des substituants en -2 et en -5.

Les résultats du Tableau VIII font apparaître une très grande sensibilité de l'atome d'azote vis-à-vis de l'encombrement stérique des groupements environnants.

L'alumine est beaucoup plus sensible que la silice à ces effets, comme le montrent les résultats obtenus avec le méthyl-2 thiazole et le ditertiobutyl-2,4 thiazole. Il semblerait donc que le site d'adsorption auquel serait attaché l'atome d'azote adsorbé serait plus grand sur l'alumine que sur la silice, cela est au moins conforme à ce que l'on pense des différences entre les sites d'adsorption de ces deux adsorbants[5].

Les résultats obtenus en série pyridinique par SNYDER[6] ou avec d'autres hétérocycles azotés (benzothiazoles, pipéridines etc...) d'autre part, sont tout à fait semblables.

CONCLUSION

Au cours de cette étude, les points suivants ont pu être établis:

(1) Les variations d'énergie d'adsorption, sur silice et sur alumine, de l'atome d'azote cyclique, sous l'influence des effets polaires des substituants aux différentes positions du cycle, sont additives en l'absence d'effets stériques importants. Des facteurs de correction tenant compte de ces effets ont été proposés.

(2) On note la très grande sensibilité de l'atome d'azote du cycle vis-à-vis de l'encombrement stérique des groupements alcoyle, en position adjacente.

(3) Une sensibilité importante de la silice vis-à-vis des effets inductifs des substituants en -2, par suite des interactions acido-basiques entre le soluté et l'adsorbant.

(4) Sur silice, les variations d'énergie d'adsorption dûes aux effets polaires des substituants en *para* d'un phényle, sont pratiquement indépendantes des effets polaires que peuvent exercer ces substituants sur le reste de la molécule et de leur délocalisation par rapport à l'atome d'azote.

(5) L'alumine est, en général, plus sensible aux effets polaires et stériques des substituants sur le cycle, que la silice. Ce résultat est identique à celui observé par SNYDER[7] avec les dérivés de la pyridine.

(6) Enfin, des relations empiriques simples permettent de relier les variations d'énergie d'adsorption observées sur ces deux adsorbants.

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RÉSUMÉ

La théorie de la chromatographie d'adsorption linéaire de SNYDER a été appliquée à une centaine d'hétérocycles thiazoliques dont nous avons déterminé expérimentalement les énergies d'adsorption sur silice et alumine Merck.

La comparaison de ces résultats avec les énergies d'adsorption estimées par le calcul à l'aide des tables établies, nous a permis de déterminer les interactions globales des divers groupements à l'intérieur de ces molécules.

Ces interactions sont additives, dans le cas de groupements faiblement adsorbés (alcoyle et halogéno) et en l'absence d'encombrement stérique de part et d'autre du centre d'adsorption.

La sensibilité de ces molécules vis-à-vis des effets stériques des groupements alcoyle est plus importante sur alumine que sur silice.

Dans la série des aryl-4 thiazoles diversement substitués en position -2, nous avons également étudié les variations d'énergie d'adsorption, sous ces effets, des substituants en *para* du phényl en -4 et des substituants en -2.

Cette étude ne nous a pas permis de relier ces variations aux effets polaires et stériques des substituants car, étant donné la complexité de ces molécules, d'autres phénomènes peuvent également se produire.

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CHROM. 4467

GAS CHROMATOGRAPHIC BEHAVIOR AND CHEMICAL STRUCTURE OF METHYL PARATHION AND METHYL PARAOXON IN RELATION TO THEIR HOMOLOGS

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SUMMARY

Gas chromatography of methyl parathion and methyl paraoxon homologs on nonpolar phases shows that oxons elute before thions while the trend is reversed on polar phases. The pattern of elution of homologs shows linear relationship to chemical and thermodynamic properties as obtained from plots of retention value *versus* number of carbon atoms or number of methyl groups, and a plot of reciprocal of temperature *versus* log of retention time on polar and nonpolar phases shows straight relationships.

INTRODUCTION

Due to increased use of organophosphorus pesticides to replace the persistent organochlorine compounds, methods for their reliable determination are needed. The parent compounds are usually thionates which are essentially noninhibitory to cholinesterase but which are metabolized to toxic "oxons" which are invariably potent cholinesterase inhibitors; methods for their determination are therefore also of particular interest. Several reports of their separation by GLC are available. The general consensus is that "oxons" elute before "thions" on nonpolar liquid stationary phases, while the elution pattern is reversed on polar phases[1]; the opposite behavior has been also reported, however[2].

In order to clarify these conflicting data as well as to develop a method for the simultaneous separation of both types of compounds, a detailed study of the behavior of methyl parathion and methyl paraoxon homologs was undertaken; this study also permitted us to relate their chemical structures to their retention behavior.

EXPERIMENTAL

Methyl parathion (O,O'-dimethyl *p*-nitrophenyl phosphorothioate) and methyl paraoxon (dimethyl *p*-nitrophenyl phosphate) were prepared in the usual manner[3]. Parathion (O,O'-diethyl *p*-nitrophenyl phosphorothioate), *n*-propyl parathion (O,O'-dipropyl *p*-nitrophenyl phosphorothioate), and paraoxon (diethyl *p*-nitro-

phenyl phosphate) were received as analytical samples from the American Cyanamid Co., while *n*-butyl parathion (O,O'-dibutyl *p*-nitrophenyl phosphorothioate) and *n*-butyl paraoxon (*n*-dibutyl-*p*-nitrophenylphosphate) were obtained from Dr. T. R. FUKUTO of this department.

Gas chromatographic analyses were done as previously described[3] using Apiezon L and DEGS as liquid phases with a Hewlett-Packard Model 402 high-efficiency gas chromatograph equipped with hydrogen flame detector. The detector was modified for the thermionic detection of phosphorus by mounting a KCl pellet on the burner jet.

RESULTS AND DISCUSSION

Results of gas chromatography of a limited number of homologs of methyl parathion and methyl paraoxon on Apiezon L and DEGS columns are given in Table I.

TABLE I

RELATIVE AND OBSERVED RETENTION TIMES OF INDIVIDUAL INJECTIONS OF METHYL PARATHION, METHYL PARAOXON AND THEIR HOMOLOGS

Temperatures of flash heater and detector: 210°; flow rates of nitrogen, hydrogen, and air: 40, 21, and 300 ml/min, respectively.

Column temp. (°C)	*Relative and observed[a] retention times*						
	Methyl paraoxon	*Methyl parathion*	*Paraoxon*	*Parathion*	*n-Propyl parathion*	*n-Butyl paraoxon*	*n-Butyl parathion*
Column: 5% Apiezon L on Gas-Chrom Q 80/100, 2 ft. × 4 mm I.D.							
170	0.77 (1.85)	1.0 (2.40)	1.0 (2.45)	1.62 (3.90)	3.41 (8.2)	5.4 (13.0)	8.0 (19.3)
190	0.63 (0.85)	1.0 (1.35)	1.0 (1.35)	1.47 (2.00)	2.81 (3.80)	4.08 (5.50)	5.85 (7.9)
210	0.60 (0.40)	1.0 (0.65)	1.0 (0.65)	1.39 (0.90)	2.70 (1.75)	3.79 (2.45)	5.70 (3.70)
Column: 1.4% DEGS on Gas-Chrom Q 80/100, 2 ft. × 4 mm I.D.							
170	1.25 (2.65)	1.0 (2.10)	1.15 (2.40)	0.95 (1.95)	1.21 (2.55)	2.43 (5.1)	1.81 (3.80)
190	1.25 (1.25)	1.0 (1.00)	1.15 (1.15)	0.95 (0.95)	1.10 (1.10)	2.30 (2.30)	1.70 (1.70)
210	1.20 (0.56)	1.0 (0.47)	1.12 (0.53)	0.95 (0.44)	1.10 (0.52)	2.02 (0.95)	1.59 (0.75)

[a] Figures in parentheses denote the observed retention time in minutes.

Isothermal separation of these compounds on the Apiezon L column is shown in Fig. 1. On the Apiezon L column "oxons" elute before "thions" in all instances, while on the DEGS column this trend is reversed. Although *n*-propyl paraoxon was not available, from the behavior of the other compounds it would be expected to give a separate peak. These data, therefore, lend support to the observed gas chromatographic behavior of methyl paraoxon reported earlier[3].

However, under the conditions employed, methyl parathion and paraoxon could not be resolved when injected simultaneously on the Apiezon L column, and most of the compounds could not be resolved when injected simultaneously on the DEGS column. Although methyl parathion and paraoxon may be separable on other columns or by low-temperature GLC, their separation was not investigated further because they can be distinguished relatively easily by using a flame photometric detector in both phosphorus and sulfur modes.

Although the *n*-propyl and *n*-butyl homologs are not commercial insecticides,

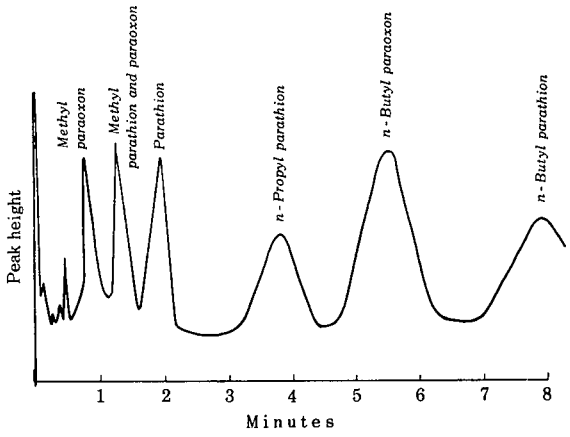

Fig. 1. Isothermal separation of methyl parathion and methyl paraoxon homologs. Column: 5% Apiezon L on Gas-Chrom Q 80/100, 2 ft. × 4 mm I.D.; temperatures of column, flash heater, and detector: 190, 210, and 200°, respectively; flow rates of nitrogen, hydrogen, and air: 40, 21, and 300 ml/min, respectively.

this system of separation could be extremely useful for studies in which the relationship of molecular dimensions, chemical structure, and biological activity are to be compared.

Although the resolution of this series of compounds was inferior on the DEGS column, the pattern of "oxons" eluting later than "thions" was as expected. However, the data present a very puzzling situation since parathion elutes before methyl parathion (also previously observed by BOWMAN AND BEROZA[4]) and paraoxon elutes before methyl paraoxon. HRIVNAK AND PASTOREK[5] also observed a shift of compounds containing a methyl group toward higher retention values on liquid phases of increasing polarity. Although no explanation can be advanced for this anomalous behavior on the basis of our present knowledge of the GLC behavior of organophosphorus compounds, the observation is significant since it points out that an attempt to correlate the behavior of organophosphorus compounds directly with that for saturated hydrocarbons in the literature could be very deceptive.

IDENTIFICATION AND BEHAVIOR OF COMPOUNDS

If the GLC column used for separation is sufficiently specific and has enough theoretical plates, each sample component can be theoretically separated from the others. If these compounds are not the same, there will usually be at least slight differences in retention volumes, indicated by the occurrence of a shoulder on the augmented peak or by rounding of the peak top accompanied by an increase in peak width.

Peak identifications from comparisons of retention values on a single column are, however, limited because the results are only characteristic but not specific. Specificity can be increased and further confirmation can be obtained by determining relative retentions on two columns having widely different polarities, or it can be buttressed by the use of selective detectors which are responsive only to certain atomic groupings[6]. Ancillary systems including chemical tests, TLC, cholinesterase inhibition,

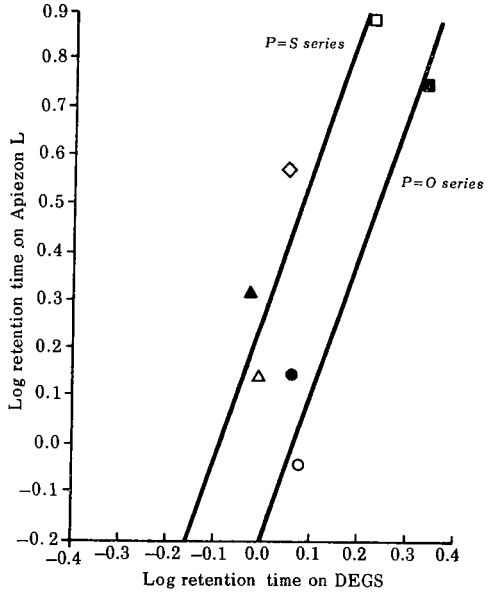

Fig. 2. Relationship between log retention time on DEGS and Apiezon L columns (data from Table I at 190°, legends as in Fig. 3).

mass spectrometry, and IR spectrometry can also be used for confirmation of identifications.

The uncertainties and ambiguities which arise in the use of coincidence methods by the use of a single column are perhaps greatest when working with compounds which have not been rigorously studied previously by GLC. The confusion can be further enhanced if some unexpected or unconsidered reaction or if some adsorption or chemical change occurs in the column.

The determination of retention data on columns of different polarities is very valuable for qualitative identification. This was first described by JAMES[7], who observed that when the retention volumes of aliphatic amines were plotted on two different columns, the respective points of each homologous series (primary, secondary, and tertiary) always gave a straight line. However, it was later demonstrated that it is more useful to plot the logarithm of the retention values than the actual values because in the latter case the graph becomes crowded in the region close to the origin[8–10]. Slopes of such graphs are characteristic of the chemical structure of the individual homologous series.

Using a log/log scale, the resulting plots are almost parallel to each other and now the intercepts are characteristic of the individual homologous series. Such a relationship for log retention time on Apiezon L *versus* DEGS, for example, is depicted in Fig. 2 for the dialkyl-substituted *p*-nitrophenyl phosphates and phosphorothioates.

The retention values of a homologous series can usually be related to various physical properties such as boiling points or to structural properties such as number of carbon atoms. The logarithms of retention times, relative retentions, or retention volumes *versus* such characteristics show a linear relationship. A plot of retention time

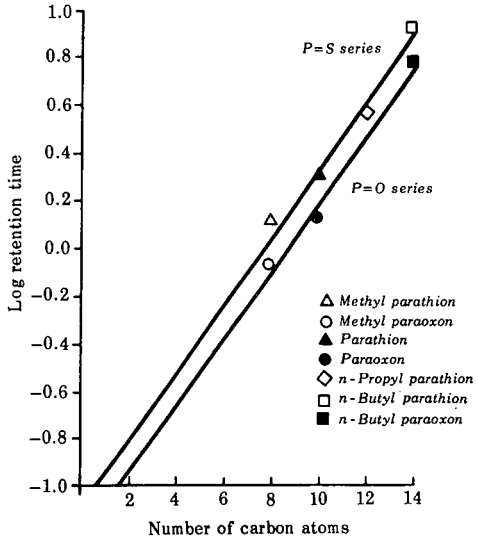

Fig. 3. Relationship between log retention time *versus* number of carbon atoms in the compound (data from Table I at 190° on Apiezon L).

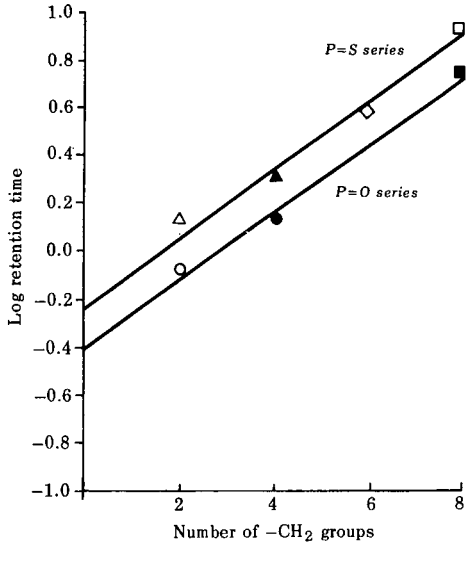

Fig. 4. Relationship between log retention time and number of substituted methylene groups in *p*-nitrophenylphosphorus esters (data and legend as in Fig. 3).

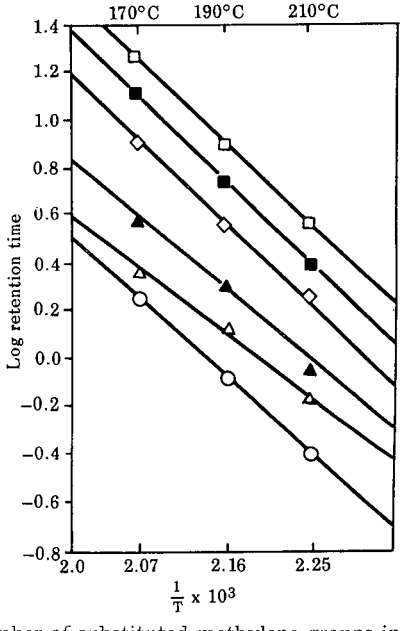

Fig. 5. Relationship between log retention time *versus* reciprocal of absolute temperature (data from Table I on Apiezon L, legends as in Fig. 3).

versus total number of carbon atoms is shown in Fig. 3. A linear relationship for both the P=S and P=O series is observed. Not only are the plots linear but also the slopes are parallel. The fact that the curves for the P=O and P=S series cross the abscissa at about two carbon atoms indicates that the retention of compounds with two carbon atoms will be zero, that is, the compounds will elute with the solvent. Because the solvent peak also has a measurable width, dimethyl or even trimethyl phosphate and phosphorothioates will not be resolved under these conditions, as is experimentally observed. Fig. 4 shows a plot of the number of methyl groups in *p*-nitrophenylphosphorus esters *versus* the logarithm of the retention time. The intersection of the plot with the *y*-axis indicates that all such compounds should show retention even when the number of methyl groups is zero.

Because of the validity of the Arrhenius equation, according to which the equilibrium constants of compounds in a homologous series should be linearly related to the reciprocal of the absolute temperature, it was informative to plot the logarithms of the retention times *versus* reciprocal absolute temperature. The ratio of the retentions of two solutes has been shown to be directly related to the ratio of their equilibrium constants. A linear relationship is observed for all the compounds (Fig. 5). These demonstrated relationships give considerable credence to the validity of the observed elution of methyl paraoxon before methyl parathion on the Apiezon L column and question the results of NAKATSUGAWA *et al.*[2], who reported the opposite behavior.

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A STANDARDIZED METHOD FOR THE IDENTIFICATION OF LICHEN PRODUCTS

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SUMMARY

A procedure for the routine identification of the products of lichen-forming fungi by thin-layer chromatography is described. Microextracts of plant fragments are chromatographed in three solvent systems. The spots of unknowns are assigned to R_F classes defined by the R_F values of marker controls of two lichen substances (atranorin and norstictic acid) chromatographed on every plate. The unknowns are tentatively identified by sorting (by R_F classes) punched cards summarizing microchemical data for all compounds previously studied. The preliminary identification is then confirmed by additional microchemical tests. The open-ended system can incorporate new and unknown compounds as well as information from other chromatographic systems. Data obtained by the standardized procedure are given for 104 products.

INTRODUCTION

The natural products of the lichen-forming fungi have become intimately involved in systematics and classification[1]. Some 300 specific substances have been reported from about 2,000 of the 18,000 described species of lichens[2]. Approximately 100 of these compounds are aromatic products that have been frequently mentioned in the taxonomic literature.

Today the lichen systematist faces the problem of learning to identify these natural products without a set procedure to guide him. An important paper by SANTESSON[3] records R_F values for spots of some 80 compounds on thin-layer chromatograms run in a variety of solvent systems. The compounds are grouped by chemical type and by certain color reactions and, R_F data are presented for chromatograms run in solvent systems best suited to each group. Several other papers devoted to the thin-layer chromatography (TLC) of lichen substances also treat specific groups of compounds such as the aliphatic acids[4] and aromatic aldehydes[5]. Several chromatographic solvents have been suggested in chemotaxonomic studies where a particular system was found to be convenient for distinguishing a limited number of specific compounds. While all of this information is extremely valuable for confirmatory identifications,

the worker surveying previously unstudied lichens requires R_F data for all lichen compounds in the same solvent systems. For example, if the lichen thallus produces coloration with *p*-phenylenediamine (PD), one may choose a solvent system recommended for PD-positive substances only to find that there are also PD-negative constituents present in the same plant and for which there are no R_F data for that solvent. It is then necessary to select other solvent systems, hoping by trial and error to find a suitable one. In spite of the intensive interest in lichen chemistry, no standardized system has been presented. The method described in this report attempts to overcome (1) the hit-and-miss aspects of microchemical identifications, (2) the difficulties due to varying R_F values, and (3) the problem of incorporating and retrieving microchemical information. The system described was developed for use by the lichenologists at Duke University. While the specific solvent systems and TLC plates used have been most successful in our laboratory, the general plan of the method can be modified and applied to any solvents used with any media.

In essence the system involves the following. Chromatographic data from three standard solvent systems for all compounds previously studied are stored on punched cards. The cards are keyed not to specific R_F values in the three solutions but rather to R_F classes the limits of which are determined by the R_F values of a control mixture consistently used as a marker on every chromatogram. Unknowns are identified by determining their R_F classes in the three solvent systems and then by sorting the punched cards for the known compounds belonging to all the same R_F classes.

These possibilities are then narrowed down by careful comparison of (a) actual R_F values with respect to the R_F values of the control marker spots, (b) color reactions, (c) appearance of the spot in short- and longwave UV light, (d) solubility, and any other pertinent data available. The final identification of the compound is achieved by appropriate confirmatory tests. Punched cards made for compounds that cannot be identified are added to the deck to permit the recognition of the same substances in species studied in the future.

MATERIALS AND METHODS

Sources of lichen products

Standards consisted of pure substances when these were available and of microextracts of herbarium specimens elsewhere.

Chromatographic materials and solvent systems

Chromatograms were developed in Brinkman tanks to a height of 10 cm on Merck Silica Gel F_{254} thin-layer plates that had been stored in a desiccator over $CaCl_2$–NaOH but not activated. The solvent systems are: solvent A: benzene–dioxane–acetic acid (90:25:4, 238 ml); solvent B: hexane–ethyl ether–formic acid (5:4:1, 200 ml); solvent C: toluene–acetic acid (85:15, 240 ml).

The benzene, hexane, and toluene were dried ($CaCl_2$), redistilled, and stored over sodium. The dioxane and anhydrous ethyl ether were passed through columns of alumina to remove peroxides. The ether was stored over sodium. The same solvent systems prepared without these purifications gave results sufficiently similar to those reported here to indicate that these precautions are not required for routine analyses.

The level of the solutions in the tanks was maintained constant, and the solutions

were replaced when the R_F values of the controls deviated significantly from their usual values. Atranorin and norstictic acid were selected for marker controls because of their ready availability and because of their behavior in the particular solvent systems used. A mixture of these two compounds is available in a single extract of many common species, for example *Parmelia perforata* (Jacq.) Ach. in North America and *P. acetabulum* (Neck.) Duby in Europe.

Eighteen spots were applied to the plates 2 cm from the bottom and 9 mm apart. On every plate the first, ninth, and eighteenth positions were used for controls. The controls on a plate were spotted from the same solution to eliminate R_F variation due to concentration.

Determination of R_F classes

The developed plates were air-dried, examined under UV light (254 nm and 366 nm), sprayed with 10% H_2SO_4, and heated at about 110° until colors developed. The colors of the spots were recorded as soon as the plates were taken from the oven.

In order to identify R_F classes, first the centers of the atranorin spots at positions 1 and 9 and at positions 9 and 18 are connected by a ruled line in pencil. The same is done for the norstictic acid spots. (Usually spots are slightly lower towards the center of the plate, the effect being more pronounced at lower R_F values, but sometimes the direction of variation is reversed.)

The distance between the origin and the solvent front was divided arbitrarily into eight regions measured with respect to the R_F values of the controls. The limits of the eight R_F classes are shown in Fig. 1. All R_F values for spots were recorded and

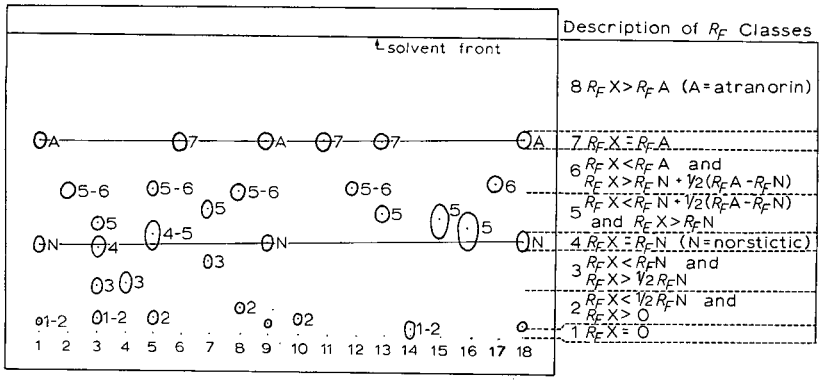

Fig. 1. A copy of a plate run in solvent C showing the controls of atranorin and norstictic acid at positions 1, 9, and 18, the horizontal penciled lines connecting the control spots, and the R_F classes of the spots of the principal compounds chromatographed. The number (or numbers) associated with each spot indicate its R_F class and the diagram to the right of the plate summarizes the R_F classes. The identities of the principal spots at each position on the plate are (1) atranorin (A), norstictic acid (N), and an unknown substance, an acetone extract of *Parmelia perforata*; (2) acetylportentol; (3) alectorialic acid (class 4) and barbatolic acid (class 3); (4) alectoronic acid; (5) anziaic acid (classes 4–5); (6) atranorin; (7) baeomycesic acid (class 5) and squamatic acid (class 3); (8) barbatic acid (classes 5–6); (9) atranorin (A) and norstictic acid (N), the control; (10) fumarprotocetraric acid and protocetraric acid (running together as a single spot); (11) chloroatranorin; (12) confluentic acid; (13) atranorin (class 7) and α-collatolic acid (class 5); (14) constictic acid; (15) cryptochlorophaeic acid; (16) 4-O-demethylbarbatic acid; (17) didymic acid; and (18) atranorin (A) and norstictic acid (N), the control.

TABLE I

TLC DATA FOR 104 LICHEN PRODUCTS CHROMATOGRAPHED IN THREE SOLVENT SYSTEMS

R_F classes			*Compounds*	*Example $R_F \times 100$ values (R_F of X/R_F of N, R_F of A[a])*			*Spot coloration with H_2SO_4 and heat, (*), pigmentation, color reactions, etc.*[b]
A	*B*	*C*		*A*	*B*	*C*	
1	1	1	Rhodocladonic	0	0	0	Red pigment; K+
1	2–3	2	Protocetraric	3/40, 68[a]	17/27, 54	5/31. 66	Dark gray*; PD+
1	3	2	Fumarprotocetraric	1–2/40, 68	24/28, 54	5/32, 67	Dark gray*; PD+
1	3	2	Thamnolic	2/42, 70	19/26, 53	12/32, 69	Brownish*; PD+
2	1	1–2	Constictic	8/42, 71	2/27, 58	3/31, 65	Orange-brown*; PD+
2	1–2	1	Erythrin	6–7/41, 71	5/27, 58	2/31, 67	Yellow*; C+
2	2	2	Salazinic	10/41, 71	6/27, 58	6/32, 66	Yellow*; K+, PD+
2	3	2	Polyporic	0–22/40, 68	0–14/23, 55	0–6/31, 67	Brownish pigment; pale*; K+
2	3	2	Unknown with stictic[c]	17/42, 67	15/27, 55	9/30, 67	Reddish gray*
2	3	3	Hypothamnolic	5/40, 68	21–22/26, 53	21–22/33, 68	Greenish*; K+, C+
2	3	3	Squamatic	9–10/40, 67	24/27, 59	25–26/31, 67–68	Yellow*; UV+++
2	4	2	Endocrocin	10/42–43, 70	26/25, 55	7/32, 67–68	Orange pigment; K+
2	4	2	Diploschistesic	17/43, 69	27/26, 53	11/31, 67	C+ blue
2	4	2	Siphulin	17/40, 67	25/24, 57	5–6/34, 70	C+ red
2	4–5	3	Physodalic	11/41, 71	30/26, 58	20/31, 66	Dark gray*; PD+
2	4–5	3	Haemathamnolic	16/41, 71	31/27, 58	29/33, 68	Yellow*; PD+
2	5	2	Pulvinic	13/42, 69	33/25, 56	7/32, 67	Yellow pigment; UV+ orange
2	5	2	Unknown with psoromic[d]	20/44, 70	32/26, 57	11/30, 67	Greenish*
2	5	2–3	Barbatolic	7/40, 69	35/25, 57	17/30, 65	Yellow*; PD+, K+
2–3	4–5	3	Umbilicaric	23/43, 68	27–28/25, 56	21/29, 65	Yellow*
3	2	2	Unknown with stictic[c]	23/42, 67	9/27, 55	11/29, 66	Yellow*; PD+
3	2	2	Variolaric	23/43, 68	8/25, 56	11/34, 69	Yellow or gray*
3	2	3	Stictic	31/37, 69	8/25, 54	22/31, 68	Orange-yellow*; PD+
3	2–3	3	Echinocarpic	26/40, 68	12/26, 53	18/31, 67	Reddish gray*; PD+
3	3	3	Galbinic	28/38, 65	16/27, 58	21/32, 67	Deep yellow*; PD+
3	3	5–6	Unknown triterpene(?)[c]	33/42, 62	26/29, 60	37/31, 67	Brownish*; UV−
3	4–5	3	Alectoronic	32/43, 71	30/27, 54	16/29, 64	Pale*; KC+, UV+++
3	5	3	Hypoprotocetraric	26/42, 70	37/27, 53	18/30, 69	Greenish brown or gray*
3	5	3	Physodic	28/43, 71	34/27, 57	21/31, 66	Greenish*; KC+
3	5	3	Gyrophoric	28/43, 71	35/27, 57	21/29, 66	Yellow or gray*; C+
3	5	3	Lecanoric	29/42, 71	37/27, 57	21/30, 64	Yellow*; C+
3	5	4–5	Lividic	33/42, 71	30/26, 57	32/30, 64	Greenish or green-brown*
3	5	5	Lobaric	27/41, 71	37/26, 57	35/30, 64	Pale green*; KC+
3	5	5	Virensic	24/37, 65	42/24, 57	35/29, 61	Gray black*; PD+, K+
3	5	5	Baeomycesic	36/40, 66	33/27, 54	44/31, 67	Yellow*; PD+
3	5	5	Psoromic	35/43, 69	37/26, 56	42/31, 66	Dull yellow or brown*; PD+

3	5	5	Picrolichenic	34/42, 71	35–36/26, 57	36/31, 66	Dull yellow or green*; KC+
3	5	5	4-O-Methylphysodic	37/42, 71	37/26, 57	42/31, 64	Gray green*; KC+
3	5–6	5	4-O-Methylgyrophoric	34/43, 69	41/26, 57	42/30, 65	Yellow*
3	6	5	Evernic	33/42, 71	45–46/27, 57	45/32, 67	Yellow*
3	6	5	Grayanic	35/43, 71	46–47/27, 57	44/33, 67	Brownish*
3–4	5	3	Olivetoric	36–37/43, 71	36/28, 61	23–24/31, 64	Yellow*; C+
3–4	5–6	4	Alectorialic	38–39/42, 71	42/27, 58	28/29, 64	Brown*; C+, KC+, PD+
3–6	1–3	1–4	Entothein	30–44/38, 66	0–15/23, 54	0–35/30, 66	Pigment; pale streak*
4	3	3	Strepsilin	40/49, 61	19/25, 57	24/30, 66	Pale*; C+ green
4	4	4	Norstictic				Yellow*; PD+, K+
4	5	3	Orsellinic[f]	39/40, 67	39–40/26, 57	25/31, 64	Yellow*; C+
4	5	4–5	Paludosic	38–39/40, 64–65	38/26, 57	30/28, 64	Brownish*; K+, KC+, (C+)
4	5	5	α-Collatolic	39/40, 65	33/26, 54	40/31, 65	Pale*; KC+
4	5	5	Glomelliferic	39/40, 66	38/25, 53	43/29, 65	Yellow*; KC+
4	5	5–6	Boninic	43/42, 69	38/27, 57	45–46/27, 61	Brownish purple*
4	6	4–5	Anziaic	38–39/40, 67	48/26, 56	33/30, 64	Yellow*; C+
4	6	5	4-O-Demethylbarbatic	39/41, 70	45/27, 55	36/32, 66	Yellow*; C+
4	6	5–6	Divaricatic	41/43, 70	49/27, 54	48/31, 67	Yellow*
4	6	5–6	Diffractaic	42–43/42, 70	48/27, 53	47–48/31, 66	Yellow*
4	6	5–6	Barbatic	42/43, 67	55/26, 59	47/30, 65	Yellow*
4	6	6	Sekikaic	41–42/40, 67	47/26, 57	50/31–32, 66	Brown*
4	6	6	Homosekikaic	42–43/40, 67	50/26, 57	50/28, 66	Yellow-brown*
4	6–7	6	Didymic	44/43, 67	55/27, 55	51/31, 67	Pinkish gray*; C+ blue-green
4	7	6	Imbricaric	40/39, 67	55/25, 57–58	52/33, 68	Yellow*
4	7	6	Stenosporic	41/39, 67	56/25, 57–58	51/32, 67	Yellow*
4	7	6	Perlatolic	41/39, 67	56/25, 57–58	51/31, 66	Yellow*
4	7	6	Sphaerophorin	41/38–39, 67	56/25, 57–58	51–52/32, 67	Yellow*
4–5	5	5	Microphyllinic	40/38, 68	32/25, 57	44–45/31, 64	Pale*; KC+
4–5	5	6	Unknown triterpene(?)[e]	47/43, 70	37/29, 60	53/31, 68	Brownish*; UV–
5	5	2–3	Orcinol	43/38, 68	33/25, 57	16–17/31, 64	Yellow*; C+
5	5	3	Rhodophyscin	44/40, 66	30–31/24, 56	18–19/27, 63	Pigment; greenish*; K+
5	5	3	Norlobaridone	47/39, 67	33–34/26, 57	23/31, 64	Greenish*; KC+
5	5	5	Cryptochlorophaeic	43/40, 67	38/26, 57	40/31, 66	Brown*; (K+), (C+), KC+
5	5	5–6	Confluentic	46–47/40, 67	34/28, 60	49/31, 65	Yellow*
5	5	6	Planaic	45/40, 64	35/25, 57	56/31, 66	Yellow*
5	5	6	Unknown with usnic[g]	52/40, 67	37/25, 57	51–52/29, 64	Greenish*
5	5–6	6	Merochlorophaeic	47/40, 67	43–44/26, 56	53/31, 64	Purple*; K+, KC+, (C+)
5	6–7	5	Thiophanic	49/40–41, 67	49/26, 56	48/33, 69	Yellow pigment
5–6	5–6	6	Zeorin	52/40, 67	37/25, 57	53/34, 70	Pink or pale brown*; UV–
5–6	6	6	Ergosterol	52/39, 65	47–48/25, 56–57	56–57/34, 70	Brown*
5–6	6	6	Ursolic	53/38, 67	43/25, 57	55–56/33, 69	Bright lavender*; UV–
5–6	6	6	Emodin	53/39, 65	43/24, 57	40/30, 66	Yellow pigment; K+; UV+++

(continued on p. 90)

TABLE I *(continued)*

R_F classes			Compounds	Example $R_F \times 100$ values (R_F of X/R_F of N, R_F of A[a])			Spot coloration with H_2SO_4 and heat, (*), pigmentation, color reactions, etc.[b]
A	*B*	*C*		*A*	*B*	*C*	
6	3–4	5–6	Acetylportentol	61–62/41, 67	27–28/33, 63	47/29, 64	Brown*; UV−
6	4	6	Unknown red pigment[h]	65/40, 70	31/31, 62	52–53/29, 66	UV+ orange
6	5	5	Methyl gyrophorate	58/43, 70	40/30, 57	42/31, 64	Yellow*; C+
6	5	6	Gangaleoidin	61/41, 69	34/25, 56	57/34, 70	Deep yellow*
6	5–6	6	Unknown triterpene(?)[i]	63/42, 67	47/29, 61	54/31, 67	Pink*; UV−
6	6	5	Ethyl orsellinate	59/41, 69	42.5/24.5, 56	43/31, 67	Yellow*; C+
6	6	6	Unknown triterpene(?)[j]	61/41, 66	31/25, 57	52/30, 67	Bright lavender*; UV−
6	6	6	Diploicin	62/41, 69	45/27, 54	55/31, 67	Very pale or colorless*
6	6	6	Thiophaninic	62/41, 69	49/24, 56	57/30, 67	Pigment; pale or colorless*
6	6	6	Usnic	62/40, 66	48/26, 54	57/29, 64	Greenish*; UV quench
6	6–7	7	Pannarin	62/41, 67	49–51/24, 53	65/31, 65	Dark gray*; PD+
7	5	6	Rhizocarpic	66–67/41, 67	34/24, 57	60/30, 68	Yellow pigment; UV+
7	5	6	Unknown pigment	67/41, 67	36/24, 57	59/30, 67	Yellow pigment
7	5–6	6	Epanorin	69/40, 68	38/23, 56	59/31, 66	Yellow pigment; UV+
7	6	6	Scrobiculin	61–62/39, 65	45–46/24, 56	51–52/32, 67	Brown*; (C+), KC+
7	6	6–7	Tenuiorin	66/39, 65	45/24, 56	66–67/32, 68	Yellow*
7	6	7	Lichexanthone	66/39, 65	45/24, 56	64/30, 64	Pale yellow*; UV+
7	6	7	Vulpinic	65/39, 65	50/24–25, 56	68/32, 68	Yellow pigment
7	6	7	Leprapinic	69/40, 68	45/25, 56	66/31, 66	Yellow pigment
7	6	7	Pinastric	69–70/41, 69	44/24, 55	65/32, 67	Yellow pigment
7	7	6	Vicanicin	64/39, 64	60/27, 60	64/35, 70	Pale*
7	7	7	Parietin	68/39, 66	57/27, 58–59	67/31, 66	Yellow pigment; K+; UV+++
7	7	7	Atranorin				Yellow*; PD+
7	7	7	Chloroatranorin	62/36, 62	53/25, 53	65/31, 65	Yellow*; PD+
7–8	8	8	Calycin	72/40, 69	52/23, 55	74–75/30, 66	Yellow pigment
8	8	8	Pulvinic dilactone	73/40, 69	62/24, 56	79/30, 69	Yellow pigment; UV+++

[a] The two numbers following the virgule (/) are measurements in millimeters to the norstictic acid line (R_F of N) and the atranorin line (R_F of A). (See Fig. 1.)

[b] Abbreviations for color reagents are: K = 10% aqueous KOH; PD = concentrated alcoholic *p*-phenylenediamine; C = saturated aqueous $Ca(OCl)_2$; KC = K followed by C.

[c] With two other unknowns and stictic acid in a number of species including *Usnea rubicunda* Stirt., for example.

[d] With atranorin and psoromic acid in *Argopsis* sp.

[e] This unidentified substance, which may be a triterpene, occurs in *Parmelia aurulenta* Tuck.

[f] This compound has been reported in lichens but not recently confirmed.

[g] In *Lecanora rubina* (Vill.) Ach.

[h] In *Arthonia rubrocinctum* Merr.

[i] This unknown substance, which may be a triterpene, occurs in *Pyxine caesiopruinosa* (Nyl.) Imsh.

[j] This unknown substance, which may be a triterpene, occurs in *Physcia* sp.

followed by the values measured to the norstictic acid and atranorin lines at the same horizontal position (see Table I).

Recording information on punched cards

A punched card, prepared for every compound, is keyed for the R_F classes in the three solvent systems and for an alphabetic sequence to the common chemical names. Additional data and specific R_F values, always expressed with the corresponding values of the norstictic acid and atranorin controls, are recorded directly on the cards. As an example, Fig. 2 shows the punched card for lobaric acid.

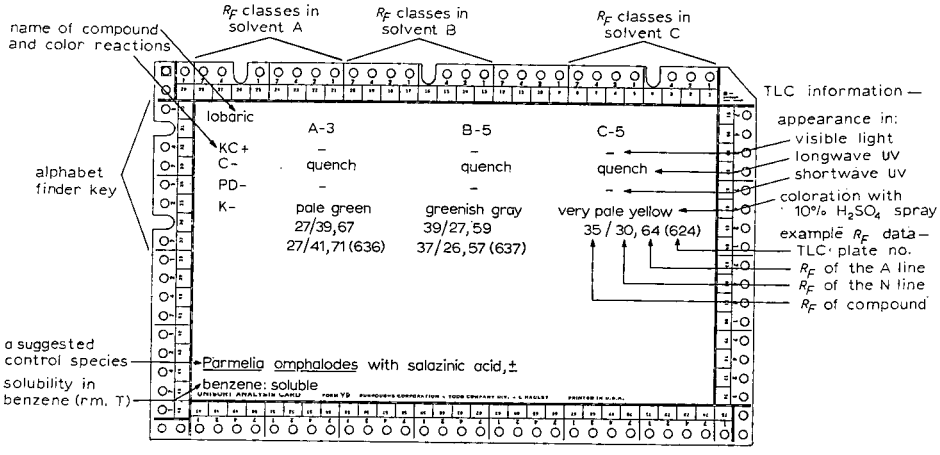

Fig. 2. An example of a punched card for a known compound showing the information stored.

Identification of compounds in crude extracts of lichens for chemotaxonomic surveys

Chemotaxonomic studies on lichens are usually based upon chemical analysis of fragments of herbarium specimens. In our laboratory the following procedure is used: cleaned plant material (1 cm^2 or less) is placed in a shell vial (Mercer Glass Works, Inc., N.Y.; shell vials, short style, 1/2 dr, 35×12 mm) numbered in India ink. The fragment is extracted three times by 5–10-min soakings in benzene at room temperature. The extracts are evaporated at about 50° on a microscope slide. The processes are repeated using acetone to extract the sample at elevated temperature (50°), and the acetone extracts are evaporated on a second slide. Usually many specimens are analyzed simultaneously, and the benzene and acetone extracts of each sample are chromatographed side-by-side on the thin-layer plate. As usual, at positions 1, 9, and 18, the controls of atranorin and norstictic acid are included.

To spot the chromatograms, a drop or two of acetone is flowed over the residue on the slide and collected in a micropipette. Three plates may be spotted simultaneously. The R_F values of the spots are recorded and the R_F classes determined. The punched cards make possible the identification of all previously studied compounds with the same R_F classes. Of the 104 compounds listed in Table I the maximum number of compounds with the same R_F classes is six. The additional information on the cards (Fig. 2) reduces the number of possibilities still further and suggests the best solution or solutions in which to rechromatograph the unknown with appropriate

standards. If the compound is not identifiable, a punched card for it is prepared and added to the deck.

RESULTS AND DISCUSSION

Table I summarizes the data obtained for the most common aromatic and triterpenoid products of lichens. The compounds are arranged according to increasing R_F classes so that those with the most similar R_F values in the three solvent systems are grouped together. It is unlikely that these R_F values could be reproduced exactly in another laboratory, since over a period of some months the R_F values of the controls of atranorin and norstictic acid change slightly. But it is expected that the R_F classes which depend only on the relative order of spots will be much more constant.

In the identification of unknowns from crude extracts of lichens containing several compounds it may be difficult to distinguish which spots on the chromatograms run in different solutions are due to the same compounds. Sometimes, one or more spots will be concealed by other spots in some solvents. Colorations with different spray reagents can sometimes help. Elution of a band run in one solvent and rechromatography of the eluted material in the three standard solvents usually solve the most difficult problems. And finally, the double extraction of the sample, first with benzene and then with acetone, often allows the identification of compounds that would be obscured in a simple acetone extract.

The system of punched cards described in this report can be varied in many ways and it can be expanded by adding new solvent systems and by the continual addition of cards for new compounds and unknowns encountered in surveys. Using this method, new students of lichenology in our laboratory have been able to identify many of the common aromatic lichen substances from their first chromatographic plates. The method gives meaning to the designation "unidentified substance" and assures that any particular unidentified substance will be recognized again if it is encountered in other species, allowing all such records to be cleared up when the substance is finally characterized.

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ANALYTICAL SEPARATION OF HORDEIN AND ITS FRACTIONS BY DISC ELECTROPHORESIS ON ACRYLAMIDE GEL

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SUMMARY

An analytical method requiring 200 to 500 μg of protein sample for the investigation of hordein and its fractions is described, in which disc electrophoresis on 7.5 to 20% acrylamide gels, in the presence of 6 and 2 *M* urea, is used. Among the currently used methods the proposed procedure is characterized by its very high resolution. Hordein is fractionated, at pH 4.6, into 6 clear and well-resolved fractions moving towards the cathode. In addition 3 to 4 other fractions, in very small quantities, can also be observed. The influence of different factors is studied with the view of obtaining optimum resolution. The protein discs were stained with Coomassie Brillant Blue R 250. The method was used in the investigation of the total hordein from "Winter barley 1337", crops 1963, and its fractions isolated by gel chromatography.

INTRODUCTION

The known analytical methods used for the characterization of hordein (prolamine of barley) require either a large amount of material, much time or special apparatus; furthermore, the resolving power of these methods is not satisfactory. They have been used for lack of better ones. The ultracentrifuge method, used by QVENSEL AND SVEDBERG[1], showed hordein preparations to be homogeneous. Tiselius electrophoresis applied to total hordein by BISERTE AND SCRIBAN[2] showed the presence of five fractions, two of which run together and are defined with difficulty as individual fractions. The quantity necessary for a separate determination in a modern apparatus for Tiselius-type electrophoresis is about 9–12 mg of hordein. The resolving power of paper electrophoresis was found to be much poorer when applied to hordein by KLAUSHOFER *et al.*[3]. The quantity of the sample necessary for analysis in this case was 1 mg, only three diffuse hordein fractions were found and these were hardly distinguished. The continuous carrier-free electrophoresis by HANNIG used as an analytical method by IVANOV *et al.*[4] had the same resolution and required the same quantity of the sample as the Tiselius electrophoresis. However, it showed better reproducibility. Fractionation of hordein in the presence of all the other barley proteins was attempted by electrophoresis on starch gel[5] and exhibited a very bad resolution. The method of separation of hordein fractions according to their different

molecular volumes, introduced by MESROB *et al.*[6] for preparative work, may also be used as an analytical method; however, the method was relatively slow and required more than 10 mg of protein sample.

The method of disc electrophoresis on acrylamide gel, suggested by ORNSTEIN AND DAVIS[7], was mainly developed for water-soluble proteins. An attempt has been made to use this method for the separation of barley proteins[8,9]. The results obtained so far were completely satisfactory, but they mainly cover the water-soluble basic proteins of barley[8] and the mixtures extracted by pyrophosphate buffer and 0.05 *M* acetic acid[9]. However, both solutions extract many other water-soluble proteins of barley and only some of the lowest molecular hordein components.

The aim of the present work was to develop a rapid analytical method with a high resolving power which can characterize the alcohol-soluble barley protein, defined by OSBORNE[10] as hordein. The disc electrophoresis method of ORNSTEIN AND DAVIS[7], with some modifications owing to the properties of hordein, was chosen. Hordein is only soluble in the presence of urea and in acetic acid at concentrations greater than 0.5 *M*. Hordein is not readily soluble in acetic acid at lower concentrations; in such cases turbid suspensions are obtained, from which a part of the protein precipitates very quickly. According to our preliminary investigations the solubility of hordein in the more dilute acetic acid solutions is selective, some lower molecular weight fractions being more soluble than the rest of the protein. Disc electrophoresis permits a separation of the hordein fractions, based on the electric charges and molecular volumes (and respectively on molecular weight).

MATERIALS AND METHODS

Acrylamide—pure, (Koch-Light, London).

Bisacrylamide (Fluka, Switzerland).

Tetramethylenediamine (TEMED), synthesized in our laboratory.

Dimethylamino-propionitril (DMAPN) (American Cyanamid Co., U.S.A.).

Urea—reagent grade, crystallized from methanol–water.

Total hordein and its fractions, separated according to molecular weight differences, were obtained by gel chromatography according to MESROB *et al.*[6].

Coomassie Brillant Blue R 250 was kindly provided by ICI, London.

The other reagents used were of "reagent grade" quality.

The disc electrophoresis procedure, described by ORNSTEIN AND DAVIS[7], was modified as follows.

The apparatus was prepared for use according to the detailed description given by DAVIS[11]. It consists of 6 tubes of 5 mm I.D. and 65 mm in length arranged in a circle around the cylindrical graphite electrodes. Both electrode vessels were placed in such a manner, that the anode vessel was above that of the cathode. It was supplied with direct current by a stabilized electronic amplifier. In accordance with ORNSTEIN'S theoretical basis of disc electrophoresis[12], K^+ was chosen as a fast cation, while the glycine and the acetate anion were used as the slow cation and counterion respectively. With this ion system and the hordein cation a narrow triple boundary can be achieved.

Urea was incorporated in all the gels; the urea concentration for 10% acrylamide gel was 6 *M* and for an acrylamide concentration of 20%—2 *M*. The pH values of the solutions was determined directly with a pH-meter, the computation of these values

for solutions containing so much urea being very difficult. The actual pH value for the large-pore gel was 6.3 and for the small-pore gel 4.6. Because of the considerable amount of urea in the gel solutions, DMAPN was added also to the large-pore upper gel, which led to complete polymerization of that gel even in the presence of the protein solution.

The following stock solutions were necessary for the preparation of the different gels (for 7.5% acrylamide in the small-pore gel).

(A)	1 *N* Potassium hydroxide	48.0 ml
	Glacial acetic acid	17.2 ml
	TEMED	4.0 ml
	Water added to make	100.0 ml
(B)	1 *N* potassium hydroxide	48.0 ml
	Glacial acetic acid	2.87 ml
	TEMED	0.46 ml
	Water added to make	100.0 ml
(B1)	(2 times higher concentration than B)	
	2 *N* potassium hydroxide	24.0 ml
	Glacial acetic acid	2.87 ml
	TEMED	0.46 ml
	Water added to make	50.0 ml
(C)	Acrylamide	30.0 g
	Bis-acrylamide	0.8 g
	Water added to make	100.0 ml
(D)	Acrylamide	10.0 g
	Bis-acrylamide	2.5 g
	Water added to make	100.0 ml
(D1)	(2 times higher concentration than D)	
	Acrylamide	10.0 g
	Bis-acrylamide	2.5 g
	Water added to make	50.0 ml

(E) Riboflavin, 4 mg in 100 ml of water. To each 5 ml of this solution 100 μl of DMAPN are added.

(E1) (2 times higher concentration than E)
4 mg of riboflavin in 50.0 ml of water. To each 2.5 ml of this solution 100 μl DMAPN are added.

Note: The solutions containing DMAPN only have a lifetime of 2–3 days.

(AP) 28 mg of ammonium persulfate was dissolved in 5 ml of water (this was mixed fresh daily).

The following solution (C1) was also used for the preparation of 20% acrylamide in the small-pore gel:

(C1)	Acrylamide	3.4 g
	Bis-acrylamide	7.8 mg
	Water added to make	10.0 ml

Those solutions not already mentioned regarding shelf-life can be stored in brown glass bottles in a refrigerator for more than a month. On account of its low stability it is recommended that the addition of urea to the solutions is done daily.

Preparation of the small-pore gel (7.5%)

2.5 ml of solution A, 5.0 ml of solution C, 7.2 g of urea and 5.0 ml of solution AP were taken and water was added to make 20.0 ml.

0.8 ml of the above mixture was poured into each tube, and a layer of *ca.* 2 mm water was formed over the gel and the solution then polymerized in UV light.

Preparation of the large-pore middle gel (3.5%)

0.50 ml of solution B, 1.0 ml of solution D, 0.5 ml of solution E and 1.44 g urea were mixed with water to make 4.0 ml. 0.2 ml of this mixture was poured into each tube which was then layered with *ca.* 2 mm of water and polymerized in UV light.

Preparation of the large-pore upper gel (3.5%)

0.5 ml of solution B1, 1.0 ml of solution D1, 0.5 ml of solution E1 and 1.44 g of urea mixed with water to make 4.0 ml. 0.1 ml of this solution was poured into each tube and 0.1 ml of 6 *M* aqueous urea solution containing 0.5 mg of hordein preparation was added; the mixture was then polymerized in UV light.

Preparation of the small-pore gel (20%)

3.5 ml of solution C1, 1.0 ml of solution A and 0.72 g of urea were made up to 6 ml with AP solution. The polymerization was carried out as before and the large-pore middle and upper gels were prepared according to the above description.

Buffer for the electrode vessels consisted of 26.3 g glycine, 8.0 ml glacial acetic acid and water was added to 1000 ml.

The introduction of gel solutions into the apparatus and the polymerization was carried out according to DAVIS[11]. Electrophoresis was carried out at 140 V. At the start the potential was increased till the current density reached 5 mA per tube, but after 10 min a current density of 3 mA per tube was used. The apparatus was kept at room temperature. The considerable thermostability of hordein made additional cooling of the apparatus unnecessary.

The current was passed for 100 min. The gels were removed from the tubes and colored according to CHRAMBACH *et al.*[13]. The gels were placed in test tubes with about 10 ml 12.5% solution of trichloroacetic acid as fixing agent for the protein. After 30 min of fixing, the gels were transferred to a 0.05% solution of Coomassie Brillant Blue R 250 (a 1% aqueous solution of the dye diluted 20 times with a 10% trichloroacetic acid solution) where the gel remains for 60 min. Washing the colored gels with a 10% trichloroacetic acid solution led to well-outlined discs on a clean background. The intensity of the coloring increased in the following 2 days.

The photometric measurements were performed on a Jouan Model E.S.A. C.-5-57 photometer for paper electropherograms, adapted by making an aperture on the carrier table and reducing the slit to 0.4 mm.

RESULTS AND DISCUSSIONS

The possibility of carrying out the disc electrophoresis in 0.5 *M* and 1 *M* acetic acid media as in the case of known electrophoretic methods for hordein separation was first examined. This resulted in only two diffuse discs being obtained, a large amount of protein remaining in the upper gel as stationary material. In order to

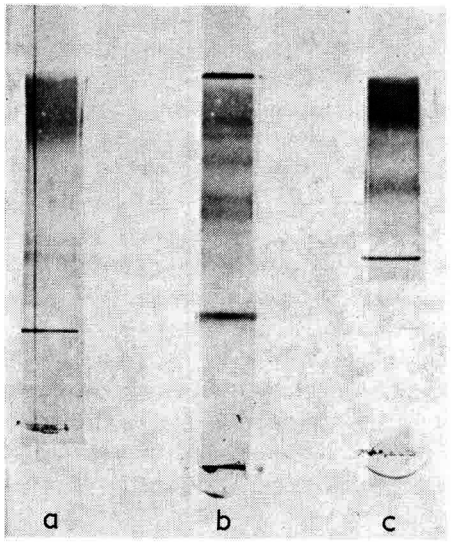

Fig. 1. Total hordein resolved in a 7.5% acrylamide gel in the presence of urea. All the other data are as given for the standard method of electrophoresis. The samples were separated at the following concentrations of urea: a = 8 *M*; b = 6 *M*; c = 4 *M*.

achieve a good separation of hordein into its fractions, electrophoresis in the presence of urea was attempted. The effect of different concentrations of urea in the gel was studied; 6 and 8 *M* concentrations of urea (Fig. 1) were found to be most suitable.

Since the density of the gel influenced the fractionation of the protein into

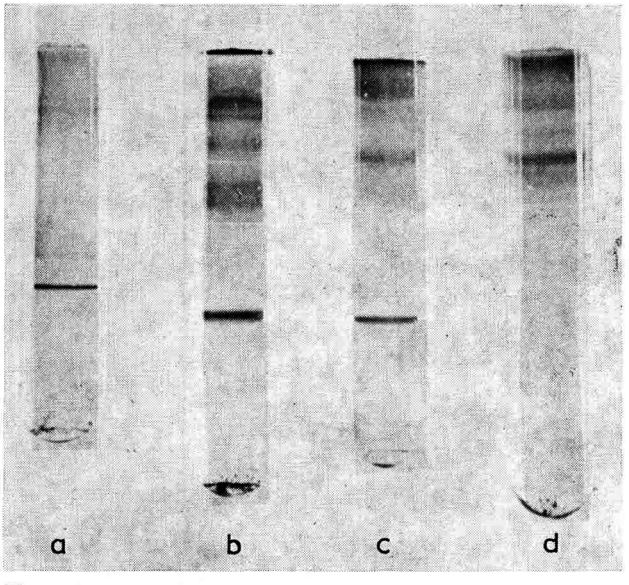

Fig. 2. Total hordein resolved by the described standard disc electrophoresis with 6 *M* urea, the concentration of the acrylamide in the small-pore gel being: a = 5%; b = 7.5%; c = 10%; d = 20% acrylamide with 2 *M* of urea.

components differing in molecular volume, the disc electrophoresis was investigated at different concentrations of acrylamide in the lower gel. The choice of different concentrations was limited, because the polymerization of acrylamide became worse in the presence of 6 *M* urea in the gel solution. With a concentration of more than 10% acrylamide, the gels were soft and unstable. 20% acrylamide in the lower gel was achieved by use of 2 *M* of urea. Disc electropherograms of hordein at different percentages of acrylamide in the small-pore gel are shown in Fig. 2.

The effect of current density and duration was studied maintaining the other conditions standard.

It was shown, that the optimal time for electrophoresis was 100 min. During this time the fastest fractions moved up to 35–40 mm. In the first 20 min the protein hardly reached the boundary of the lower gel.

At a higher current density (5 mA per tube) distorted discs were obtained. At 3 mA per tube the electrophoresis proceeds without increasing the temperature and yields good discs. The influence of the pH values of the different gels on the running and the separation of the hordein fractions was also investigated. At pH values of 8.4 for the lower gel and 10.3 for the upper gel, the mobility of the hordein fractions was negligible. Only two strong diffuse discs moved 5 mm towards the anode in 200 min. At a pH of 6.6 in the lower gel (8.3 in the upper gel) the movement was only 7–8 mm towards the cathode in 100 min. In this case the discs were also very diffuse.

The internal resistance of the small-pore gel exerts a sharp separation influence on the components of the hordein mixture, because of the large intervals of their molecular weights (and respectively their molecular volumes) from 15,200 to 350,000[14]. As can be seen from Fig. 2 the slow fractions were separated better in the gel with 5% acrylamide than in the gel with 10%. On the other hand, the fastest fractions were separated by a higher density in the small pore gel. Thus a gel with 7.5% acrylamide was chosen as the standard resolution method for total hordein. Study, however, of the purity of the different hordein fractions gives us grounds for recommending the use of 5% and 7.5% gels for the slow components of the hordein and 10% and 20% for the fast ones; especially in the case of fraction 4, where the most suitable gel was 20% acrylamide, but the buffer system should contain only 2 moles of urea (see Fig. 3). This is possible, because of the comparatively high solubility of this fraction in a 2 *M* urea solution.

It can be seen that under these experimental conditions the hordein fractions were, in general, separated more slowly. An attempt was made to accelerate the electrophoresis by increasing the current density. This, however, led to distortion of the discs as a result of increased temperature. An optimum value for the current density was established at 3 mA per tube for the buffer system employed and the standard conditions of electrophoresis.

In order to obtain good separation, the duration of electrophoresis should be 100 min. With some hordein preparations, especially when taken in larger quantities, complications were noted in the normal polymerization of the upper large-pore gel. In order to avoid this difficulty the use of Sephadex G-25 instead of acrylamide gel as an anticonvectional medium in the upper space of the tube was investigated. This was accomplished by introducing dry Sephadex G-25 into the protein solution being studied. The dextran swelled instantly and filled the liquid volume with a gel of sufficient stability. The results from this method of introducing the protein sample

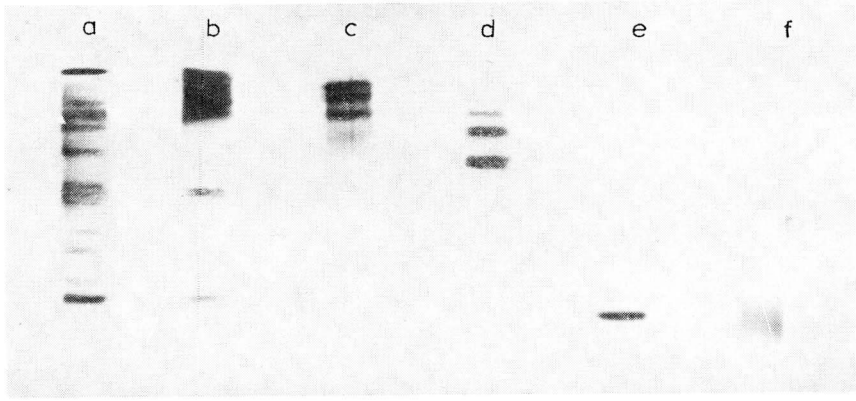

Fig. 3. Disc electrophoretic pattern according to the standard conditions: a = total hordein from "Winter barley 1337" crops 1963. Patterns due to fractions of the same hordein obtained by gel chromatography: b = fraction 1; c = fraction 2; d = fraction 3; e = fraction 4; f = fraction 4, resolved on 20% gel with 2 *M* of urea, for 220 min electrophoresis.

were similar to those obtained by the standard procedure. This method, however, allowed larger volumes of protein solutions to be employed.

The optimum quantity of protein for resolution per tube was found to be 500 μg

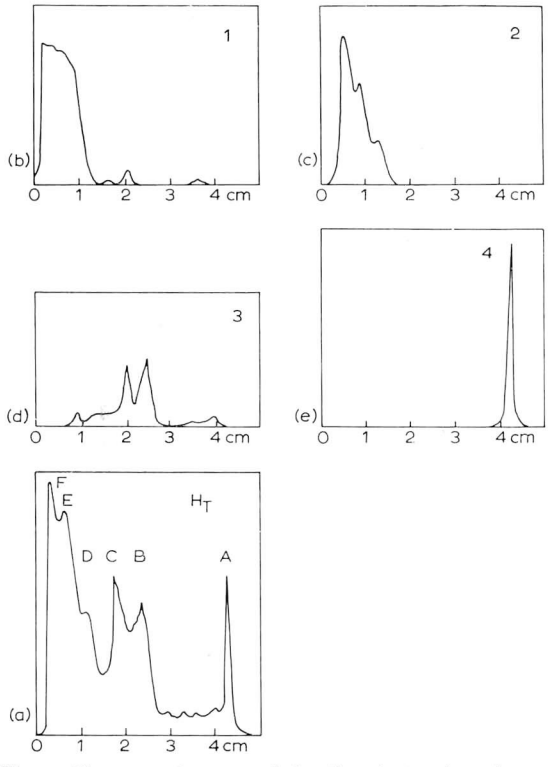

Fig. 4. Photometric scans of the disc electrophoretic separations given in Fig. 3. a–e as in Fig. 3; A–F *cf.* Table I.

of the total hordein; for the different hordein fractions an amount of 200–250 μg was quite sufficient. Satisfactory fractionation of the total hordein was established with even 300 μg to 1 mg.

The composition of total hordein isolated from "Winter barley 1337", crops 1963, by IVANOV *et al.*[4] was subjected to the described electrophoretic method of characterization. It was found to consist of 10 electrophoretically different fractions (Fig. 3).

It can be seen that hordein was resolved in 6 main fractions (A to F) and 4 secondary ones. Fraction C differed from the others in its strong red-violet color. Fractions E and F were grey-blue, and all others were greenish-blue. Fractions (1–4) also belonging to the same hordein, obtained by gel chromatography on Sephadex G-200[6], were separated by the same method. It can be seen from Fig. 3, that the fractions 1, 2 and 3 were not homogeneous but mixtures of the hordein fractions A–F in different ratios. These fractions were separated only to a certain degree. Thus, fraction 1 represented a protein zone with very little electrophoretic mobility and contained traces of A and B. Fraction 2 represented a mixture of D, E and F electrophoretic fractions. The mobility of these three fractions was very similar. Fraction 3 contained mainly B and C, and fraction 4 contained only the fraction A. The presence of the lower molecular weight fractions in the higher molecular weight ones is quite probably due to contamination in the gel chromatographic separation.

With a view to determining the amounts of individual fractions present in the total hordein, the discs obtained were photoscanned (Fig. 4). The disadvantage of this method of determination is the uncertainity of whether the different fractions were equally colored. The amounts of the different hordein fractions based on photometric determination are given in Table I.

TABLE I

PERCENTAGE OF THE DIFFERENT DISC ELECTROPHORETIC FRACTIONS IN TOTAL HORDEIN AND IN INDIVIDUAL HORDEIN FRACTIONS

Isolated on Sephadex G-200; amounts based on planimetry of curves given in Fig. 4.

Hordein preparation	*Electrophoretic fraction*					
	A	*B*	*C*	*D*	*E*	*F*
Total hordein	9.6	22.4	18.3	16.0	14.2	19.4
Fraction 1	1.2	1.8	slow moving material 97.0			
Fraction 2			12	13	66	9
Fraction 3	2	49	38	11		
Fraction 4	100					

The results obtained indicate that this method permits an electrophoretic analysis of hordein which gives better resolution than other known analytical methods. This procedure gives well-differentiated fractions combined with good reproducibility. The least amount of material necessary for the electrophoretic examination is 500 μg, the individual fractions requiring even less. The method does not require an expensive apparatus. The use of the Coomassie Blue dyeing procedure decreased the duration of the determination and increased the sensitivity of the method.

The Amidoschwarz 10B coloring technique leads to the dissolution of a considerable part of the protein discs because of the good solubility of hordein in acetic acid solutions. In trichloroacetic acid, however, hordein precipitates and the discs are clearly outlined.

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Notes

CHROM. 4477

Liquid–solid chromatography with open glass capillary columns Separation of 1-dimethylaminonaphthalene-5-sulphonyl amino acids

Open capillary columns are very commonly used for gas–liquid chromatographic separations of complex mixtures[1]. Alkali-treated glass capillary columns have proved particularly useful in fractionation of gaseous isotopic mixtures[2–4]; their extremely high resolving ability is a consequence of a very effective partition between the gas phase and solid microlayer of silica gel deposited on the inner glass surface[2].

The success of these columns in gas–solid chromatography and the wide range of applications of silica gel in liquid–solid chromatography prompted us to test alkali-treated glass capillary columns for submicroscale separations not feasible by gas chromatographic methods.

The present paper represents the first contribution to this problem, namely the fractionation of DNS*-amino acids. These compounds have been chosen because of the strong yellow fluorescence, which easily allows one to detect very minute amounts throughout the operation of the column and, after elution, on thin-layer chromatograms. Furthermore a series of solvent systems suitable for their fractionation on silica gel plates has been described[5,6].

Experimental

Preparation of capillary columns. Capillary columns of various lengths and diameters were prepared from Murano soft-glass tubes (2.0 mm I.D. and 6.0 mm O.D.) using the glass-tube drawing apparatus of DESTY *et al.*[7]. The columns filled with 2.5 *N* NaOH were kept at 100° for different periods of time (from 2 to 8 h), then washed with water until neutral, rinsed with acetone and dried in a stream of nitrogen.

Injector. With a few modifications to be discussed later, the injection apparatus (Fig. 1) is essentially the same as used in gas chromatography.

Separation of DNS-amino acids. The columns were equilibrated with benzene–pyridine–acetic acid (80:20:2), one of the solvent systems suggested by MORSE AND HORECKER[5] for thin-layer chromatography of DNS-amino acids. After equilibration the needle valve was set to give a 1:30 ratio of flow rate in the column to flow rate in the valve. The flow rate of the column was kept constant at 0.5 ml/h by applying a pressure from 20 to 100 Torr.

By means of an Hamilton microsyringe, 4 μl of a DNS-amino acid mixture containing 6 μmoles/ml of each derivative, dissolved in benzene–pyridine–acetic acid (80:20:2), were then injected into the column. Owing to the low ratio of flow rate in the column to flow rate in the needle valve, the actual amount of each compound entering the column was less than 1 nmole. Each falling drop emerging from the column was examined by thin-layer chromatography on pre-coated silica gel plates (Merck) developed with the above-mentioned benzene–pyridine–acetic acid solvent system.

* Abbreviation: DNS = 1-dimethylaminonaphthalene-5-sulphonyl.

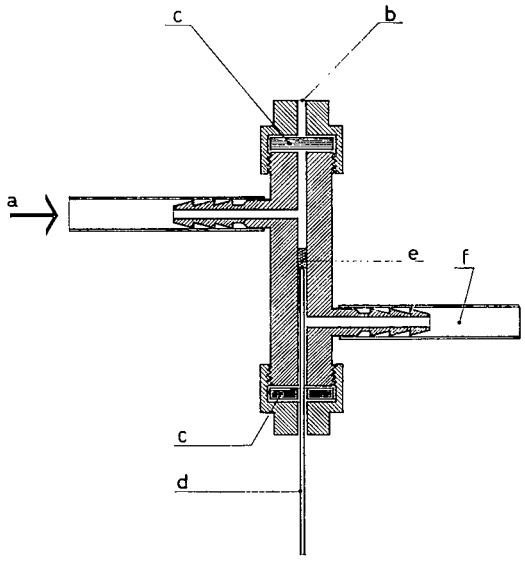

Fig. 1. Injector: a = solvent inlet, b = sample inlet, c = silicone rubber diaphragm, d = capillary column, e = platinum sponge, f = connection to needle valve.

R_F values were then plotted *vs.* time of appearance in the column eluate; in this way a qualitative elution pattern was obtained where each segment corresponds to the time interval during which one of the components emerges from the column. Such a "primitive" method of detection was quite satisfactory for this preliminary investigation, but the authors are aware that further developments will require the use of a highly sensitive detector capable of yielding a continuous recording of the solute concentration in the effluent liquid.

Results

Preliminary experiments showed that the method of introduction of the sample, as in gas–liquid chromatography, had a critical influence on the efficiency of the chromatographic procedure described in this paper. In fact, the use of an injection system without a needle valve, similar to that described by NYSTROM[8], gave poor results. By following the fluorescence of the samples traveling through the column, a remarkable broadening of the zones was apparent, even when the solutes were contained in a very small volume (0.2 μl).

With the use of a needle valve, which increases the flow rate between the injection site and the top of the column, sharp zones were obtained. Experiments run with different ratios of flow rate in the column to flow rate in the valve showed that a value of 1:30 was the most appropriate when the rate of flow of the column was 0.5 ml/h. The reproducibility of the chromatographic separations was improved when a small platinum sponge was inserted on top of the column (Fig. 1); this helped in getting a rapid mixing between sample and elution system.

The influence on the column efficiency of the size of the internal diameter was investigated by running a mixture of DNS-phenylalanine and DNS-glycine through columns treated 2 h with alkali and having identical lengths (10 m) but variable internal

diameters (0.23, 0.26 and 0.30 mm). In each case the flow rate was kept constant at 0.5 ml/h. The best results were obtained when the I.D. was 0.26 mm; in fact the time interval between the disappearance from the eluate of the fluorescence due to DNS-phenylalanine and the appearance of that due to DNS-glycine was 9, 14 and 4 min for columns with an I.D. of 0.23, 0.26 and 0.30 mm, respectively. In each case the zone width was unchanged.

Similarly, the effect of time of heating with alkali on the column efficiency was investigated. On 2-m-long columns, the two above-mentioned DNS-amino acids were not resolved when the attack with alkali had lasted only 2 h; with a 5-h treatment their separation corresponded to 5 min, a value which remained unchanged when the period of heating with NaOH was further extended.

Fig. 2 depicts the elution pattern of nine DNS-amino acids under the best

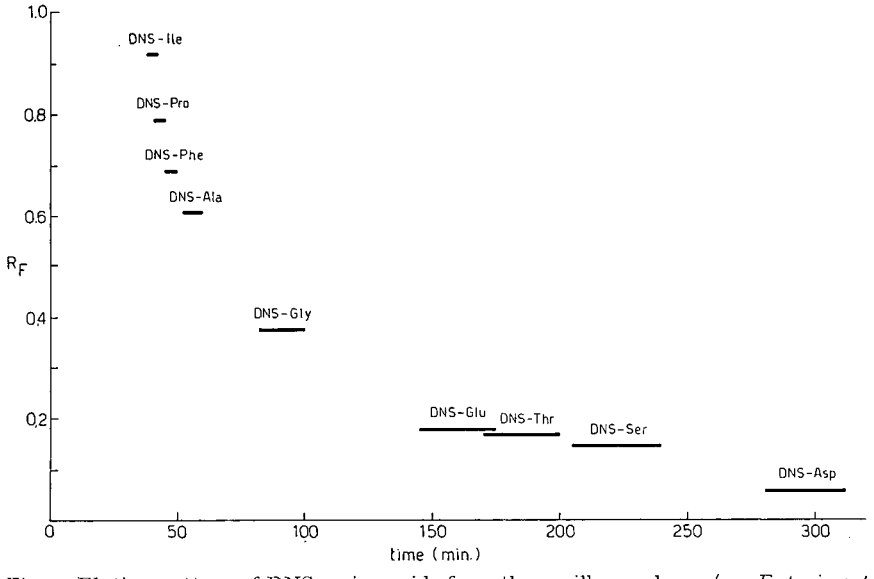

Fig. 2. Elution pattern of DNS-amino acids from the capillary column (see *Experimental*).

conditions worked out through the experiments reported above, namely: flow rate, 0.5 ml/h; ratio of flow rate in the column to flow rate in the needle valve, 1:30; I.D., 0.26 mm; length, 5 m; time of heating with alkali, 5 h. The separation of the nine derivatives is nearly complete, and, as compared to thin-layer chromatography in the same solvent system, is particularly good for those having low R_F values.

Discussion

The experiments reported in the present paper show that alkali-treated glass capillary columns can be used successfully for liquid–solid chromatographic separations. As compared to packed columns, these open columns work at exceedingly low pressure; therefore their length can be enormously increased, with the consequence that the number of theoretical plates, *i.e.* the resolution, attains very high values. This peculiar property is common to the capillary columns so widely used in gas–chromatographic analyses.

DNS-amino acids have proved particularly appropriate for this preliminary investigation, aimed to ascertain the potentiality of capillary columns in liquid–solid chromatographic separations. In fact the strong fluorescence of these compounds permits one to follow the formation of the zones and their behaviour throughout the whole operation and to detect in the eluate, without resorting to special equipment, the minute amounts of solute compatible with the capacity of capillary columns. Hence, a simple injection system, capable of yielding very sharp zones, has been developed and the main parameters (length and internal diameter of the column, extent of treatment with alkali) influencing the efficiency of the column have been fixed.

The reproducibility of the results has been excellent; in particular, with the solvent system used in the present investigation (benzene–pyridine–acetic acid), the same column can be used for subsequent runs of DNS-amino acid mixtures without impairing its efficiency. The positive results obtained in this first approach to the utilisation of capillary columns for liquid–solid separations are very encouraging. In fact, the high resolution, the speed of the operation and the requirement of minute amounts of sample make this technique very attractive. Furthermore, the availability of capillary columns coated with different types of adsorbent[9–12], as well as the obvious possibility of continuously recording the effluent using a sensitive detector, suggests potential applications for a very large number of analytical problems.

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A simplified buffer system for automated column chromatography of amino acids including methionine sulphone

A simplified buffer system for gradient elution in amino acid analysis was proposed recently by ELLIS AND PRESCOTT[1]. This system, for use on a 130-cm column of Technicon "Chromobeads, Type B", eliminated one of the original buffers, reduced the number of volumetric additions to the Autograd from 14 to 2, and involved the manipulation of only two Autograd valves. The gradient has been tested in these laboratories and the simplicity combined with resolution of the amino acids confirmed. However, in the amino acid analysis of proteins, cystine and methionine are usually determined as cysteic acid and methionine sulphone after acid hydrolysis of performic acid-oxidised protein. On adding these two amino acids to the standard mixture, and using the simplified buffer system, cysteic acid was frontally eluted as expected but methionine sulphone was not adequately resolved from aspartic acid. The effect of lowering the pH value of the initial eluting buffer has been studied.

Experimental

A Technicon amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey) was used. The 140-cm column was packed to a height of 126 cm with Technicon "Chromobeads, Type A" and was maintained at 60°. Buffers of pH 2.50 and 5.00 were prepared as directed by Technicon Instruments Co. Ltd. With only the first and sixth valves of the nine-chambered Autograd closed, a mixture of 280 ml of pH 2.50 buffer and 20 ml of methanol were placed in the section comprising chambers 1–5, and 240 ml of pH 5.00 buffer in section 6–9 such that each chamber contained 60 ml solution after reaching hydrostatic equilibrium. After applying the sample, dissolved in 0.1 *N* hydrochloric acid, to the column which was equilibrated with the pH 2.50 buffer without methanol, valves 1 and 6 were opened and pumping was commenced at a flow rate of 0.50 ml/min. After the emergence of histidine the eluting buffer was changed from the Autograd to pH 5.00 in order to elute arginine.

Results

Altering the pH of buffer No. 1 of the simplified system from pH 2.88 to 2.50, together with the inclusion of 6.67% by volume of methanol instead of 10% by volume of ethylene glycol, resulted in the separation of methionine sulphone from aspartic acid. The other common amino acids including the standard norleucine were still resolved, the only major differences being that cystine was brought closer to methionine and ammonia emerged sooner after phenylalanine. This system may therefore be used as a general one for hydrolysates of proteins with or without prior performic acid oxidation and retains the simplicity of the buffer system of ELLIS AND PRESCOTT[1].

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Teflon as a superior support for gas chromatography of organophosphorus pesticides and their metabolites

Although the elution pattern of a series of compounds is primarily dependent upon proper selection of liquid phase, in the GLC of polar compounds the proper selection of solid support is of major concern, especially when low-loaded columns are used. The surfaces of most supports are not completely inert and can interact with solutes and therefore can cause tailing, shifting retention, and occasionally even catalytic effects[1]. Undesirable adsorption characteristics of the support may be partially avoided by using higher loading if conditions permit, by using polar phases, and by conditioning with on-column treatment with hexamethyldisilazane. Effective suppression of support activity has also been achieved by coating the support with silver[2] or teflon emulsion[3].

These undesirable interactions are particularly noticeable when 1–2% of the liquid phase is used to coat the support, but it is fallacious to assume that they are absent on high-loaded columns, for even glass surfaces are not completely inert[4]. These effects are more noticeable when the liquid phase is nonpolar or less polar than the solute, which is then capable of reacting with the support through hydrogen bonding. Chemical reactions and isomerizations can also occur on the surface of support, much as they would with catalysts[5].

In this communication is described the behavior of some organophosphorus pesticides on various supports and a technique of using a teflon support to avoid these difficulties. This appears to be the first study of the use of a teflon support for organophosphorus pesticide analysis by GLC.

Materials and methods

Various solid supports including Chromosorb W 80/100 mesh, Chromosorb W HMDS-treated, Gas-Chrom Q, Polypak-1 80/100 mesh, "Haloport" teflon 30/60 mesh (Hewlett-Packard, F & M Scientific Div.), glass microbeads 80/120 mesh (Applied Science Laboratories, Inc.), Porapak Q 80/100 mesh (Water Associates, Inc.), and Supelcoport 80/100 mesh (Supelco, Inc.) were used. These supports were usually coated with liquid phase by batch coating but sometimes by the infiltration technique[6]. Teflon was coated by the in-place coating method.

The GLC analyses were reproducibly performed as previously described[7], using a Hewlett-Packard Model 402 high-efficiency gas chromatograph equipped with a hydrogen flame detector. The detector was modified for the thermionic determination of phosphorus by mounting a KCl pellet (Hewlett-Packard) on the burner jet.

Results and discussion

Methyl paraoxon could not be gas chromatographed on Chromosorb W with a coating of less than 5% SE-30. However, when Chromosorb W was coated by the infiltration technique[6] with 20% SE-30, adsorption effects were not observed. On the other hand, Porapak Q and Polypak-1 were unsatisfactory for use with organophosphorus compounds. The glass microbeads, with liquid phase loadings of 0.1–3% by both batch-coating and infiltration techniques, also were unsatisfactory as a support;

even methyl parathion, which elutes on most columns with minimum conditioning, was not gas chromatographed on these columns. It is difficult to explain this anomalous behavior, though the use of glass beads is more an art than a science.

The use of teflon as a column support resulted in symmetrical sharp peaks even at low liquid-phase loadings and column temperatures. A conditioning period of as little as 2 h was sufficient as well. Though the actual percentage of the liquid phase coated could not be determined with any precision, this offers no real difficulty for practical purposes. The handling of teflon is difficult due to its physical properties and its use in organophosphorus pesticide analysis by GLC has not been previously reported. KIRKLAND[8] studied its properties and devised a special way of coating, but it is cumbersome. The technique used here was as follows:

The glass column and the teflon support are cooled to 0°. The teflon is poured slowly in at one end of the column while a slight vacuum is applied to the other end, and the tube is gently vibrated with a pencil tip. Vigorous vibrations clog the column and produce dead spaces due to adhesion; once this occurs, it cannot be rectified by any amount of further vibration. The entire length of the column is first packed with teflon and then the desired amount of liquid phase in a volatile solvent is percolated through slowly under slight vacuum. The vacuum is continued for 2 h to remove the volatile solvent; otherwise, dead spaces will occur during conditioning. Thin plugs of silanized glass wool are lightly inserted in both ends of the column and, after conditioning for 2 h, the column is ready for use. Temperatures above 250° result in column deformation and noxious fumes are released; hence, use of the column is restricted to lower temperatures.

Gas-Chrom Q was found to be as effective as teflon at low liquid phase loadings (2–5% batch method); the glass columns were filled with gentle vibration sufficient to pack the column without discontinuities or shattering the coating on the particles. Supelcoport is comparable to Gas-Chrom Q support, but these supports differ from teflon in that longer conditioning periods are required to get constant response. It has also been reported in the gas chromatography of steroids that active sites in nonpolar columns may be reduced if a small amount of polar phase is introduced into the nonpolar phase[4]. This amount is generally not sufficient to alter the properties of the major phase. However, for organophosphorus compounds even the addition of as little as 0.2% polar phase adversely affected retentions. Another method of coating involved coating the support with 0.2% Epon resin followed by coating with the desired nonpolar phase; no significant advantage was found with this method, however. Thus it can be concluded that for the GLC of organophosphorus compounds, teflon is superior as the solid support.

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CHROM. 4470

Determination of bromacil by gas chromatography*

Available gas chromatographic procedures for the analysis of residues of the herbicide bromacil (5-bromo-3-*sec.*-butyl-6-methyluracil) imply the need for a microcoulometric titrating system coupled with a temperature-programming module[1,2] and/or short (13–24 in. long) gas chromatography columns[1,3] with the optional use of an electron-capture detector. Without doubt, some laboratories are not equipped with the microcoulometric system, and under ordinary usage conditions the average length of a gas chromatography column varies from 4 ft. to 6 ft. with a column diameter in the range of $\frac{1}{8}$ to $\frac{1}{4}$ in.

Bromacil residues in plant material, soil, and water can be determined with a conventional gas chromatography column and an ordinary type of gas chromatograph equipped with an electron-capture detector as discussed below.

Materials and methods

Gas chromatograph. Aerograph, Model 204-B, tritium foil electron-capture detector (250 mC); column temperature 200°, injection port temperature 200°, detector temperature 200°; range 10, attenuation 2; nitrogen carrier gas flow rate 30 ml/min.

Gas chromatography column. Gas-Chrom Q, 80–100 mesh, coated with a mixture of 3% QF-1 fluorosilicone (FS 1265), 10,000 cS, and 2% DC-200 silicone, 12,500 cS, packed in a $\frac{1}{8}$ in. O.D. × 5 ft. spiral borosilicate glass column; the column should be conditioned at 225° for at least a 24-hour period before it is used.

Bromacil, recrystallized, was furnished through the courtesy of E. I. du Pont de Nemours and Co., Inc., Wilmington, Dela. A solution of bromacil in ethyl acetate (Mallinckrodt, Nanograde), 0.2 ng/μl, gave a linear detector response within the examined range of 0.2–1.2 ng (see Fig. 1).

Water samples containing suspended soil particles, soil, and rice plant seedlings were used in this study. The preparation of the samples included the extraction and

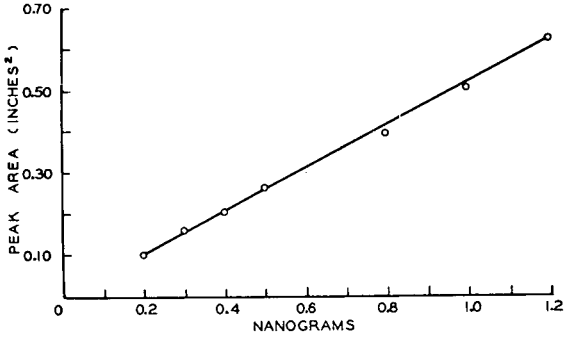

Fig. 1. Linearity curve for bromacil.

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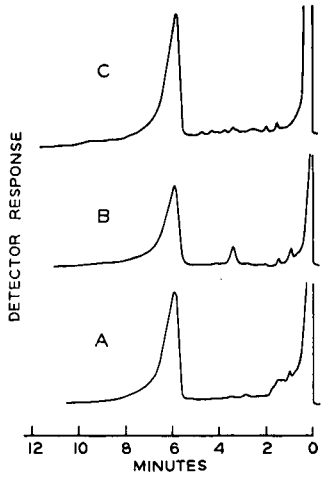

Fig. 2. Analysis of bromacil residues from 100-g samples of soil and 125-ml samples of water. Curve A: bromacil standard, 1 ng. Curve B: water sample, 1 μl of 10 ml ethyl acetate solution (10 ml ethyl acetate solution equivalent to 125 ml original water sample); water contained 0.06 p.p.m. bromacil. Curve C: soil sample, 3 μl of 10 ml ethyl acetate solution (10 ml ethyl acetate solution equivalent to 100 g soil sample); soil contained 0.04 p.p.m. bromacil.

cleanup procedures of PEASE[1,2] and JOLLIFFE *et al.*[3]; the final cleanup step for all samples was accomplished on a Florisil column instead of by the charcoal cleanup procedure suggested by JOLLIFFE *et al.*[3]. Because of the presence of soil particles in the water samples, sufficient sodium hydroxide (previously dissolved in a minimum amount of distilled water) was added to each water sample to make a 1% sodium hydroxide concentration, to insure efficient extraction of the bromacil residue from the samples[3]. The final extracts were made to suitable volumes with ethyl acetate (Mallinckrodt, Nanograde) for analysis by gas chromatography.

Representative gas chromatography curves in Fig. 2 illustrate the results of the analysis of bromacil residues from 100-g samples of soil and 125-ml samples of water. Samples fortified with bromacil gave recovery values of 85–89%. The limit of detectability, under the conditions used, was 0.005 p.p.m.

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CHROM. 4440

The separation of alkanes on Sephadex LH-20 and its application to a rock extract

The separation of alkanes by preferential sorption into inorganic molecular sieves (Union Carbide 5A and 7A) has been shown to be an important technique in the analysis of alkanes of geological interest[1,2]. Sephadex LH-20 (Pharmacia), a methylated dextran gel, which behaves as a molecular sieve, has also been used to separate alkane mixtures when used in column chromatography[3,4]. This paper describes more fully the potential of LH-20 in separating cyclic alkanes from acyclic alkanes of similar molecular weight.

Method and results

The starting materials were a mixture (A) prepared from alkanes of various structures (Table I) and a naturally occurring mixture (B) of branched and cyclic alkanes isolated by usual methods[5] from an organic extract of Green River Shale.

TABLE I

COMPOSITION OF MIXTURE A

The alkanes were present in approximately equal amounts.

Group	*Carbon numbers and names*
n-Alkanes	12, 14, 16, 18, 22, 24, 26, 28
Isoalkanes and anti-isoalkanes	13, 15, 16, 18, 24
Acyclic isoprenoid alkanes	15-farnesane, 19-pristane, 30-squalane
Cyclohexyl alkanes	16, 18, 19, 21
Tetracyclic alkanes	19-androstane, 27-cholestane

A slurry of LH-20 (approx. 90 g) was made with an acetone–chloroform mixture (1:1) and sedimented into a silicone-treated glass chromatography column (200 cm × 1.5 cm). The column was equilibrated by elution with solvent mixture (200 ml) and allowed to stand for 24 h. The gel then occupied 150 cm (270 ml) of the column.

Mixture A (9.7 mg in 0.5 ml petroleum ether b.p. 40–60°) was pipetted onto the top of the column of gel and allowed to be absorbed. Solvent-washed cotton wool was inserted onto the gel surface and the solvent mixture added. Eluant (100 ml) was run off from the bottom of the column and then fractions of 1.1 ml were collected (flow rate 40 ml/h). The alkane mixture occurred in fractions 14 to 63. Each of these fractions was evaporated at 40° under a nitrogen stream and redissolved in benzene (20 μl) containing internal standards of cyclohexylnonane (1 μl/ml) and dotriacontane (2 mg/ml). Analyses were carried out by GLC on a 10 ft. × $\frac{1}{8}$ in. O.D. column of 3% OV-1 on 100–120 mesh Gas-Chrom Q (Applied Science), programmed at 10°/min from 70° to 290°.

The amounts of each component in each fraction were calculated as percentages of amounts in the original mixture (*e.g.* Fig. 1) and the number of the fraction containing the maximum concentration of an alkane was plotted against the carbon number of that alkane (Fig. 2), the fraction numbers being on a logarithmic scale. There was no

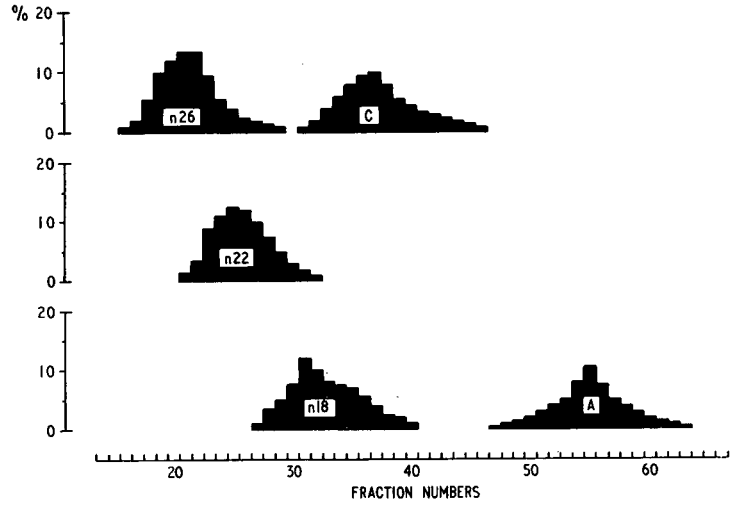

Fig. 1. Percentage of original sample present in fractions. n-26 = n-hexacosane; n-22 = n-docosane; n-18 = n-octadecane; C = cholestane; and A = androstane.

separation of normal from monomethyl alkanes and these alkanes (in Fig. 2) lie on a straight line (n). The cyclohexyl alkanes also produced a straight-line plot (c) displaced to higher fraction numbers. Between these two lines are the acyclic isoprenoid alkanes: squalane, pristane and farnesane. The tetracyclic alkanes cholestane and androstane were strongly retarded in elution from the column. It is seen, from the data presented, that the molecular sieving effect which separated n-hexacosane from n-octadecane is subordinate to another factor, possibly a solvent–solute interaction, which allows separation of n-octadecane from androstane.

The sample of Green River Shale branched and cyclic alkanes (28 mg in 0.25 ml

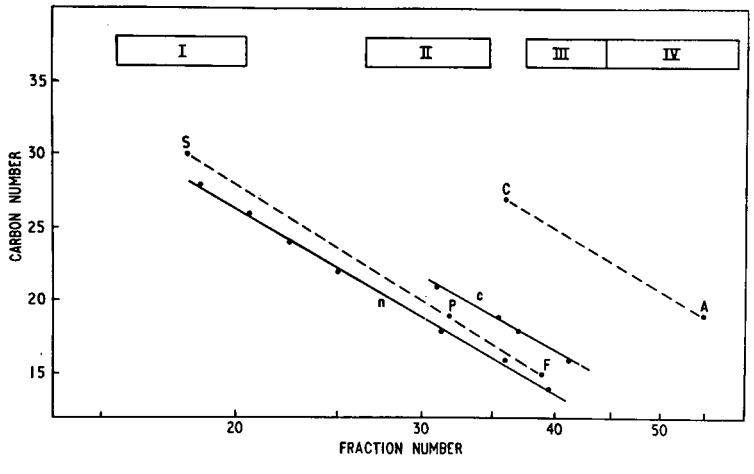

Fig. 2. Position of elution of the n-alkane series (n), the cyclohexyl alkane series (c), squalane (S), pristane (P), farnesane (F), cholestane (C) and androstane (A) and the fractions I–IV plotted against carbon number.

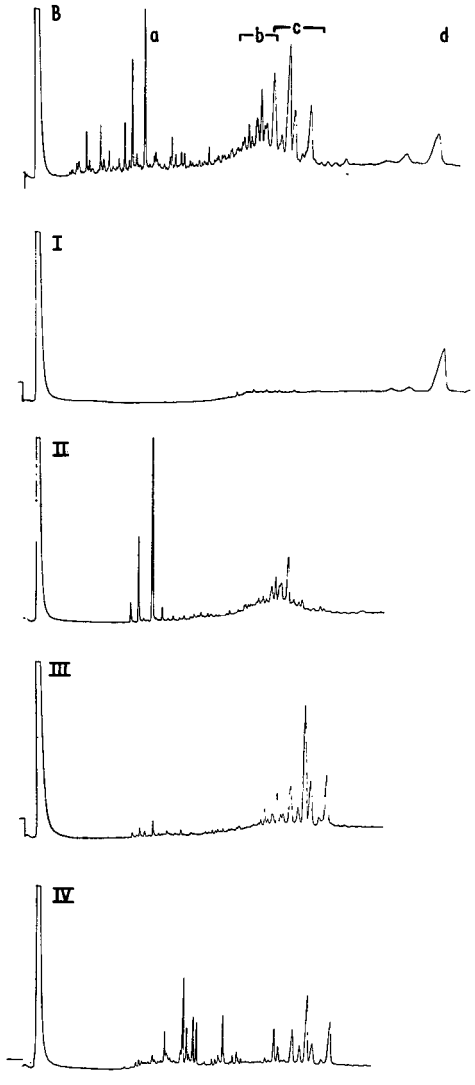

Fig. 3. GLC traces of the Green River Shale branched and cyclic alkanes fraction (mixture B) containing phytane (a), steranes (b), triterpanes (c) and perhydro-β-carotane (d) and the subsequent fractions I–IV.

petroleum ether) was passed through the column and appeared in fractions 16 to 60. After concentration and re-solution in benzene (without internal standards) analysis by GLC was carried out as before. Inspection of the gas chromatographs of each fraction allowed collection into larger fractions as follows:

I (16–20), containing only perhydro-β-carotane (d),
II (27–34), containing phytane (a), pristane, and steranes (b),
III (38–44), containing triterpanes (c),
IV (45–59)

Information on the identity of the alkanes was derived from the publications of previous workers[5–7]. The gas chromatograms of the original alkane mixture, and of the subsequent fractions I–IV, are shown in Fig. 3. Again it was seen that polycyclics were retarded relative to linear alkanes of the same carbon number, so that triterpanes were eluted after phytane. The separation also allowed the concentration of a group of alkanes in fraction IV with carbon numbers between 18 and 24 which had been minor constituents of the original mixture. From their behaviour on the column, it is believed that they have tri- and tetracyclic structures.

This method can be used advantageously for preliminary separation and concentration of alkanes prior to preparative GLC and GLC–mass spectrometry, or for the separation of components which have similar retention times on GLC. There appears to be no chemical breakdown as happens with methods involving refluxing but the large amounts of solvents evaporated in the processing may cause loss of volatile alkanes of low molecular weight.

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CHROM. 4434

The demonstration of tryptophan, tyrosine and carbohydrate-containing proteins in disc electrophoresis gels

The application of histochemical techniques for the localization of proteins in disc electrophoresis gels has been primarily limited to nonspecific protein-staining dyes, for example Amido Black, and to methods for the demonstration of enzymatic activity. Specific histochemical methods are available for the localization of amino acid components of proteins[1,2]. Certain proteins are known for their relative abundance of specific individual amino acids. The present study was initiated in order to localize in disc electrophoresis gels the components of amyloid, a pathologic protein which is unique among fibrous proteins in containing trytophan as a prominent amino acid constituent. Several other proteins are known to be either devoid of tryptophan, *e.g.* collagen, or to have a high tryptophan or tyrosine content, *e.g.* lysozyme. The purpose of the present paper is to describe the adaptation of two histochemical techniques, *i.e.* methods for detecting tryptophan and tyrosine on disc electrophoresis gels. In addition, proteins containing carbohydrates are also demonstrable by histochemical methods, for example, by the periodic acid Schiff (paS) reaction. The methods for this reaction that have been previously described, however, have not been found to be consistently reproducible.

Materials and methods

Human serum was used in all experimental techniques, and the sample was dissolved in 10% glucose in saline immediately before application to the gel. The electrophoretic macrotechnique was that described by DAVIS[3] and ORNSTEIN[4] and the microtechnique was that described previously (ref. 5)*. To improve diffusion conditions, the gels were placed on a shaking machine during each reaction step. The gels are made up in the following manner: concentration gel (3.5 cm long) contains 3.5% acrylamide, 0.875% N,N'-methylenebisacrylamide (Bis), 0.5 mg% riboflavin, and 59 m*M* Tris–phosphate buffer (pH 6.9); separation gel (7.0 cm long) contains 6.5% acrylamide, 0.175% Bis, 0.0575 vol. % TEMED, 3.75 mg% $K_3Fe(CN)_6$, 70 mg% ammonium persulfate and 0.375 *M* Tris–HCl buffer (pH 8.8); and electrode buffer contains 49.5 m*M* Tris–glycine (pH 8.4). Densitometric recording of stained gels is as described[6].

Staining procedures

Tryptophan. 100 μl of human serum are applied to standard gels 5 mm in diameter. After electrophoresis the gels are stained by treatment for 1 h at 20° using 1 g *p*-dimethylaminobenzaldehyde, 30 ml of glacial acetic acid, 10 ml of conc. HCl; then for 15 min at 20° using 40 ml of glacial acetic acid, 0.3 ml of 1 *N* HCl, 0.7 ml of freshly prepared 1% sodium nitrite; and finally for 30 min at 20° using 1.2 g picric acid, 10 ml of glacial acetic acid and water was added up to 100 ml. If crystallization occurs on the surface, gels are placed in glacial acetic acid until the crystals are dissolved.

* Combined macro–micro disc electrophoresis equipment: Boskamp, 5304 Hersel/Bonn, G.F.R.

Thereafter the gels are rinsed in running water for 5 h. Densitometric recording was made at 626 mμ.

Tyrosine. 1.5 μl of human serum are applied to micro columns 1 mm in diameter. After electrophoretic separation, the gel threads are treated for 16 h at 4° in a mixture of 12.5% acetic acid and 13.8% sodium nitrite (1:1); for 3 × 30 min in degassed distilled water at 20°; for 30 min at 20° using a freshly prepared solution containing 1 g 1-amino-8-naphthol-4-sulfonic acid (S-acid, K and K Laboratories, Jamaica, N.Y.), 1 g KOH, 1 g ammonium sulfamate, 70% ethanol added up to 100 ml and then for 3 × 30 min at 20° in 0.1 *N* HCl. All steps were carried out in light-excluding containers.

Carbohydrates. To give well-stained bands after electrophoresis on 5-mm gels, a staining procedure using the paS reaction was applied. The gels were treated for 16 h at 4° using 2.5 g sodium-*m*-periodate, 86 ml of H_2O, 10 ml of glacial acetic acid, 2.5 ml of conc. HCl and 1 g trichloroacetic acid; for 8 h at 4° with several changes of a solution of 90 ml of water, 10 ml of glacial acetic acid and 1 g trichloroacetic acid; for 16 h at 4° with Schiff's reagent (Merck); and for 2 × 2 h at 4° with 1 g potassium disulfite

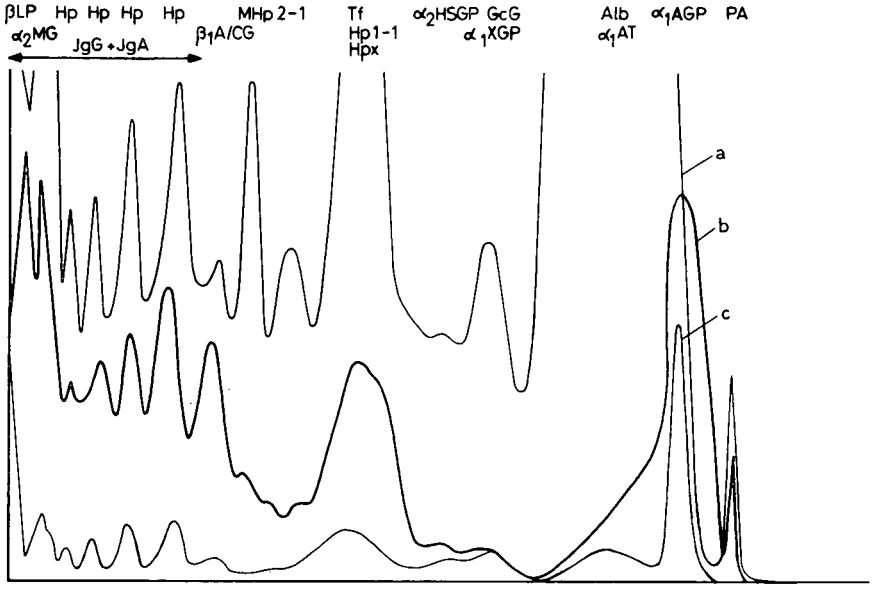

Fig. 1. Protein characterization after polyacrylamide gel electrophoresis. Technical details are described in the text; tryptophan and carbohydrate contents are according to SCHULTZE AND HEREMANS[7]. Abbreviations see p. 119. (a) Standard Amido Black stain; (b) Tryptophan detection (Try = tryptophan content); (c) Detection of glycoproteins (CH = carbohydrate content). Acid α_1 glycoprotein (CH, 41.4%; Try, 1.4%) is uncovered from the dominating albumin, which has a very low carbohydrate and tryptophan content (CH, 0.08%; Try, 0.13%). α_1 Antitrypsin (CH, 12.4%; Try, 0.55%) can be specifically demonstrated by the paS method. Note the considerable differences in the curve profiles in the post-albumin and post-transferrin region (Gc globulin CH, 4.2%; Try, 0.8%; α_2HS glycoprotein CH, 13.4%; Try, 0.82%). Increasing base line of tryptophan tracing in the last third of the electropherogram is caused by immunoglobulin G, which has a high tryptophan but low carbohydrate content (CH, 2.9%; Try, 3.83%). The position of several proteins, localized mainly by immunological methods[8], is indicated in the graph.

crystals, 20 ml of conc. HCl and 980 ml of water. Densitometric recording was made at 543 mμ. After completing the paS reaction the gels can be stained in 0.5% Amido Black–5% acetic acid for 2 h at 20°. After rinsing in running water for several minutes, they are destained in 2% acetic acid for about 16 h.

Discussion

The usefulness of specific techniques for amino acid constituents of proteins could be demonstrated in the previous study by the fact that certain constituents of serum, *e.g.* immunoglobulin G, are known to have a high tryptophan but a low carbohydrate content (Fig. 1); this is also true of the protein, amyloid, which initiated the present study. The ability to localize these proteins and to distinguish them from other proteins not having similar characteristics is extended by the present techniques. The method described for tryptophan has an absolute specificity only for indole-containing proteins[1]. In the case of the tyrosine reaction, only phenol-containing proteins will react[2]. The specificity of the paS reaction is not as great as those for the previously described methods for amino acids. The paS reaction will demonstrate not only carbohydrate constituents containing vicinal hydroxyl components but also any protein having an N-terminal serine or an α-hydroxy carboxylic acid. Despite the relative unselectivity of this method, however, the most significant reaction with proteins obtained will be those containing carbohydrates. The above methods have a much greater selectivity than methods using Sudan Black for phospholipids or lipoproteins which are capable of staining many nonlipid substances.

The use of the micro gel technique[5] in the demonstration of tyrosine (Fig. 2) was necessitated by the fact that during the chemical reaction nitrous oxide is released and the gas thus formed produced bubbles in routine gels. This was not the case when the micro method was used, since the small diameter of the gel permitted rapid diffusion of gas and eliminated, to a large extent, entrapment of gas bubbles within the gel.

Although, on a theoretical basis, methods for sulfydryl and disulfide com-

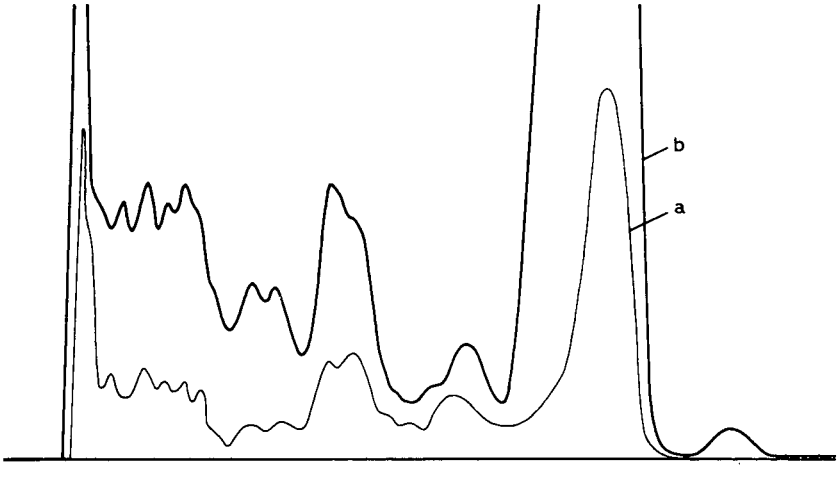

Fig. 2. Tyrosine reaction after microelectrophoresis of human serum. Technical details are as described in the text. (a) Tyrosine reaction; (b) Standard Amido Black stain.

ponents of proteins (cysteine and cystine) should be applicable to proteins demonstrated in the gel following electrophoresis, production of disulfide interchange during electrophoresis is known to occur. Usually this is prevented during electrophoresis by the incorporation in the sample of 0.1% thioglycollic acid. The incorporation of this reagent, however, would also interfere with the histochemical demonstration, since the histochemical reagent would react with unreacted thioglycollic acid still present in the gel.

Abbreviations

Alb = Albumin
α_1AT = α_1 Antitrypsin
α_1AGP = α_1 Acid glycoprotein
α_1XGP = α_1 X glycoprotein
α_2HSGP = α_2 HS glycoprotein
α_2MG = α_2 Macroglobulin
βLP = β Lipoprotein
β1A/C G = β1A/C globulin
Gc G = Gc globulin
Hpx = Haemopexin
Hp = Haptoglobin
IgA = Immunoglobulin A
IgG = Immunoglobulin G
MHp 2-1 = Monomeric haptoglobin 2-1
Pa = Praealbumin
Tf = Transferrin

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Separation of fatty acids, phospholipids and chloroplast pigments on Sephadex LH-20

The development of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) has further extended the application of gel filtration to the separation of lipid-soluble substances[1-8].

In this investigation, the potential of Sephadex LH-20 for the separation of both artificial and naturally occurring mixtures of fatty acids, phospholipids and chloroplast pigments was further explored. Some of the results have been briefly reported[9].

Materials and methods

Solvents. The solvents used were A.R. grade (British Drug Houses Ltd., Great Brittain). The chloroform as supplied contained 2% by vol. of ethanol and 0.05% by vol. of water.

Materials chromatographed. Suitable quantities of the various substances listed in Table I were dissolved in 2 ml of appropriate solvent prior to fractionation.

Column preparation. Sephadex LH-20 was suspended overnight in the eluent of choice and transferred to a separating funnel into which a glass stirrer was inserted. The gel was allowed to percolate rapidly, with constant stirring, into a vertical glass chromatography column which had been filled with solvent and across the end of which was fused a sintered glass disk (porosity 1). When the level of gel in the glass column had reached the required height, sand (1 cm approx.) was placed on top to prevent the gel floating in eluent of specific gravity greater than that of the Sephadex LH-20. Samples were applied by layering under the solvent on top of the column. The sand facilitated application of the samples without disturbing the gel. Column dimensions are given in Figs. 1 and 2, and the quantities of the material applied, in Table I. The chromatographic behavior of the polar lipids was compared with that

TABLE I

MATERIALS FRACTIONATED ON SEPHADEX LH-20

Materials	*Quantities applied to columns (mg)*	*Suppliers and sources*
Acetic acid	30	British Drug Houses Ltd., England
n-Butyric acid	40	British Drug Houses Ltd., England
Caproic acid (pure)	50	Koch-Light Labs. Ltd., England
Stearic acid (99% +)	80	Nutritional Biochemical Corp., U.S.A.
Linolenic acid	80	Nutritional Biochemical Corp., U.S.A.
Phospholipids (from bovine milk)	80	Extracted as described by DODGE AND PHILIPS[10]
Tributyrin	40	British Drug Houses Ltd., England
Tristearin	60	British Drug Houses Ltd., England
Chloroplast pigments *(Poa trivialis)*	—	Extracted as described by STRAIN *et al.*[11] from 1 g of plant tissue

of triglycerides which are apparently separated on Sephadex LH-20 by molecular sieving[5]. Fractionations were carried out at room temperature (approx. 18°) at a flow rate of 1 ml/min and the effluent was collected in 5-ml fractions.

Examination of column effluent. Fatty acids were estimated under CO_2-free N_2 by electrometric titration, to pH 10 with 0.01 *M* NaOH, of suitable quantities (containing 1–5 μequiv. of acid) of each effluent fraction, in the presence of 3 ml of propan-2-ol[12], using a Radiometer titrator, type TTT1*c*, coupled to a Radiometer titrigraph, type SBR2*c* (Radiometer, Copenhagen, Denmark). Triglycerides were estimated as described by MOORE[13]. The phospholipid content of the effluent was monitored by estimation of the phosphorus[14] in suitable portions (containing 0.2–2 mg of phospholipid) of each effluent fraction, following evaporation of the solvent and incineration on a microdigestion stand (Gallenkamp & Co. Ltd., London) for 1 h in 1 ml 60% (w/v) perchloric acid. The elution patterns of the chloroplast pigments (Fig. 2) were monitored spectrophotometrically.

Consecutive fractions corresponding to selected regions of the elution diagrams were evaporated to dryness under nitrogen and dissolved in 0.1 to 0.5 ml of solvent. The individual phospholipids[15–19] and chloroplast pigments[20] of these pooled fractions were identified by TLC on Silica Gel G (Merck).

Results

When chloroform only was used as column eluent, tristearin, tributyrin, stearic, capric, butyric and acetic acid were separated (Fig. 1) into well-defined peaks (elution volumes, V_e: 65, 85, 225, 320, 450 and 575 ml, respectively). When linolenic acid was included in the above mixture it was not separated from stearic acid. The capric acid

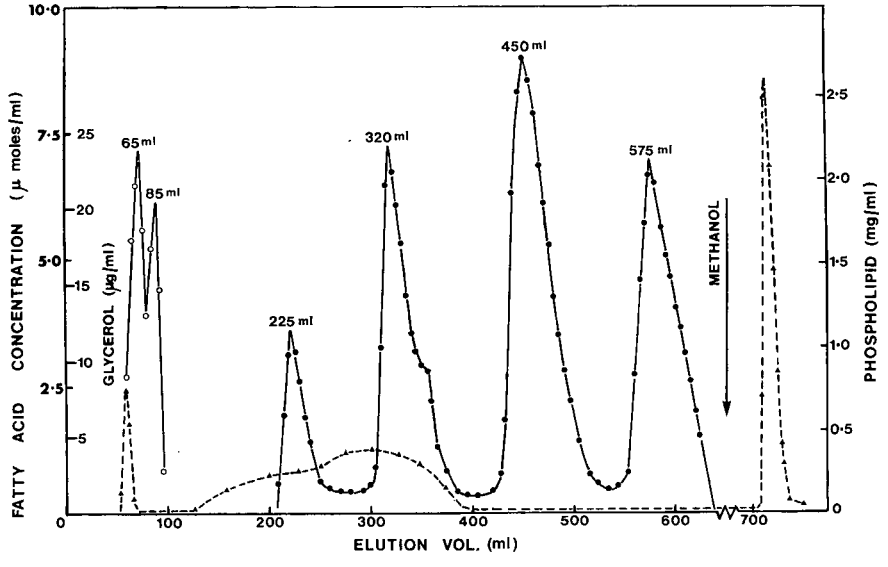

Fig. 1. Fractionation on Sephadex LH-20 columns (54 cm × 2.4 cm I.D.) equilibrated with chloroform, of an artificial mixture of bovine milk phospholipids (▲- - - -▲), triglycerides (○—○) and fatty acids (●—●). Following removal of the fatty acids the column was eluted with methanol. Elution volumes: tristearin, 65 ml; tributyrin, 85 ml; stearic acid, 225 ml; capric acid, 320 ml; butyric acid, 450 ml; acetic acid, 575 ml. Further details are given in the text.

appears to have contained a fatty acid contaminant as indicated by the inflection in its elution curve (Fig. 1), which was also observed on chromatography of capric acid on its own. The recoveries of the individual fatty acids and triglycerides were approximately 85%.

On fractionation of a preparation[10] of milk phospholipids (Table I) on its own, or in the presence of fatty acids (Fig. 1), the phosphatidylinositol was eluted as a sharp peak (<10% of phospholipid applied) near the void volume (V_e, 60 ml) followed by a wide phospholipid band (V_e, 140 to 395 ml; 50% of phospholipid applied) containing the sphingomyelin and most of the lecithin applied. On subsequent elution of the column with methanol (Fig. 1) or 20% by vol. of methanol in chloroform, a further 40% of the phospholipids applied were eluted as a large peak which contained the phosphatidylserine, phosphatidylethanolamine and some lecithin.

On columns equilibrated with a solvent of higher polarity, *viz.* chloroform containing 20% by vol. of methanol and 1.25% by vol. of water[21], very poor resolution of the polar lipids was obtained. Over 90% of the phospholipids of milk were eluted as a single peak (V_e, 55 ml) near the void volume, closely followed by the fatty acids. Stearic, capric and butyric acid were eluted as three ill-defined peaks (V_e, 70, 85 and 96 ml, respectively). On the other hand the separation of the triglycerides was largely unaffected by the polarity of the eluent.

On fractionation (Fig. 2) of an extract[11] containing chloroplast pigments (Table I) on columns equilibrated with petroleum ether (b.p. 40–60°), β-carotene and the chlorophyll artifact phaeophytin *a* emerged near the void volume (V_e, 24 ml), followed by a wide carotenoid band (V_e, 30 to 60 ml) containing lutein. On subsequent elution of the column with petroleum ether–diethyl ether (80:20) a large peak (V_e, 82 ml), containing chlorophylls *a* and *b* as well as violaxanthin and neoxanthin, was obtained. When the chloroplast pigments were applied to columns equilibrated with 2% by vol. of diethyl ether in petroleum ether (b.p. 40–60°), β-carotene, the chlorophylls and lutein were eluted as a large peak (V_e, 24 ml) near the void volume. On continued washing with this solvent, violaxanthin gradually leached off the column (V_e, 70 to 96 ml) followed by neoxanthin (V_e, 104 ml).

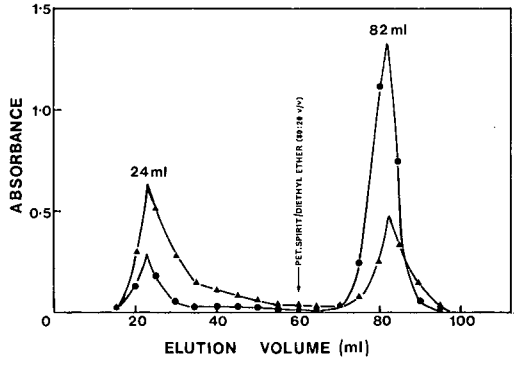

Fig. 2. Fractionation of chloroplast pigments from *Poa trivialis* (1 g) on Sephadex LH-20 columns (25 cm × 2.4 cm I.D.) equilibrated with petroleum ether (b.p. 40–60°) and eluted with petroleum ether–diethyl ether (80:20). ●—●, absorbance due to chlorophylls at $\lambda_{max.}$ = 640–660 mμ. ▲—▲, absorbance due to carotenoids read at 470 mμ to avoid contribution from absorbance due to chlorophylls. Further details are given in the text.

Discussion

The separation of lipid-soluble substances on Sephadex LH-20 is influenced by several chromatographic mechanisms[2,3,5,6,8,22,23] including molecular sieving, interaction with the gel matrix and liquid–liquid partition chromatography. The pronounced changes in the elution volumes of the fatty acids, phospholipids and chloroplast pigments as a result of increasing the polarity of the column eluents indicate that the adsorptive characteristics of the gel[3,5,6,8] played a major role in the resolution of the polar lipids. However, the elution of the fatty acids in order of decreasing molecular weight, from Sephadex LH-20 columns equilibrated with solvent of high polarity, suggests that molecular sieving also contributed to these fractionations.

The separation (Fig. 1) of the individual fatty acids (C_2, C_4, C_{10} and and C_{18}) is considerably better than that obtained in concurrent studies by ADDISON AND ACKMAN[6]. These investigators report that lauric (C_{12}) and behenic (C_{22}) acids were eluted, from Sephadex LH-20 columns equilibrated with chloroform, as a single band; behenic acid was located at the beginning of the band and most of the lauric acid at the end. While the improved separation now described may be partly attributed to the use of larger columns, the two reports are anomalous. The impaired resolution of the fatty acids, as a result of increasing the polarity of the column eluent, indicates that the chloroform used by ADDISON AND ACKMAN[6], which was relatively free of polar constituents (0.0027% by vol. of water), should have yielded better separation of individual fatty acids than that obtained with the chloroform (2% by vol. of ethanol and 0.05% by vol. of water) employed in the present study.

The behavior both of phospholipids and fatty acids on Sephadex LH-20 appears to be similar to that reported by NYSTRÖM AND SJÖVALL[24] for methylated Sephadex. However, better separation of the polar lipids appears to have been obtained in the present study.

In agreement with the results of MAXWELL AND WILLIAMS[7] little or no fractionation of chloroplast pigments or phospholipids was obtained on columns equilibrated with chloroform containing over 20% by vol. of methanol. However, milk phospholipids were separated into three fractions on columns equilibrated with chloroform. Further reduction in the polarity of solvent resulted in fractionation of chloroplast pigments.

The separations described suggest that chromatography on Sephadex LH-20 may be used as a preliminary step in the purification of individual phospholipids and chloroplast pigments. Furthermore, Sephadex LH-20 may prove useful for structural studies of lipids by providing a convenient approach to the separation of residual triglycerides, diglycerides[5], monoglycerides and fatty acids produced during controlled enzymic hydrolysis of lipids (*cf.* GARTON[25]).

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Porous glass as an adsorbent in thin-layer chromatography

A limited study of porous glass (Corning, Code 7935) was undertaken to determine whether initial results warranted further investigation of its potential use as another suitable TLC adsorbent. The intent of this preliminary study of porous glass adsorbent was to ascertain some of its properties, characteristics, and behavior in chromatographing selected pharmaceuticals by the "open column" method.

The literature shows a moderate number of published methods utilizing porous glass for chromatography, mostly gas–liquid and gas–solid procedures. Methods dealing with its use as a TLC adsorbent are very few. Porous glass, fused in the form of plates (Corning, Code 7930), has been used to characterize water-soluble inks[1]. The authors showed that treating the porous glass plates with acidic fluoride solutions or with boiling methanol produced a medium which gave chromatographic separations different from those with untreated porous glass. Powdered glass was used as a TLC adsorbent to separate three dyes. Comparison of results with those obtained by using silica gel and aluminum oxide showed differences in R_F values[2]. A publication in 1964 described the chromatography of three waxes, using porous glass as a TLC adsorbent[3]. The adsorbent was made from porous glass plates (Corning, Code 7930) ground to 200–250 mesh, mixed with plaster of Paris, and applied to plates in the manner described by STAHL[4]. Beeswax, bayberry wax, and spermaceti were compared for TLC development on the ground porous glass adsorbent, Silica Gel G, and aluminum oxide. Results indicated that porous glass produced more spots which were equal to or more distinct than those with Silica Gel G or aluminum oxide. ROUSER *et al.* separated beef brain lipids by two-dimensional TLC with porous glass adsorbent[5]. Three additional methods used porous glass to separate lipids, sugars, and phenols*.

Porous Glass Adsorbent (Corning) is the product of an intermediate phase in the manufacturing process for Vycor® glass. The borosilicate is treated with acid to leach out most of the boron. The process creates a porosity with diameter size of 30–40 Å and a surface area of 200–350 m^2/g. This results in an opalescent product of about 96% silica which is particle sized to about 300 mesh, and which has 24% by volume of void space and a pH of about 4.7 as a 10% aqueous slurry (10% aqueous slurry of Silica Gel G has a pH of about 5.8). Acidic silanol groups produce the surface phenomena; these form hydrogen bonds with electron-donating groups, making it possible to separate acidic, unsaturated, and neutral compounds[6].

Development time with organic solvents in many cases is several times faster than with other conventional adsorbents. Development time for porous glass with water as solvent is about equal to that for Silica Gel G. A 1. in × 3 in. microplate takes about 4 min to develop, using chloroform or ether. The coating can be heated to 450° without change of structure or properties. The product is said to adsorb ambient gases, vapors, and smoke, which may be removed with a 10% spray of hydrogen peroxide followed by reactivation[6]. Binders recommended for plate application are finely divided silica particles (Cabo-sil®, Cabot Corporation, Boston, Mass.), colloidal silica (Ludox SM®, E. I. duPont de Nemours and Company, Inc.), or Boehmite

* Received from Research Department, Corning Glass Works, Corning, N.Y. 14830, U.S.A.

alumina. Calcium sulfate is also used when more water is desired in the adsorbent[6]; however, it is thought to decrease surface area by plugging up some of the pores, resulting in a decrease of load capacity[7].

This study did not include quantitative work, but it has been reported that desired compounds can be determined in the presence of the adsorbent, after zones of interest are scraped off, because settling occurs readily, leaving no suspension in solvent[6]. However, one communication indicated that in eluting compounds from adsorbent, yellow pigments were obtained and caused interference in subsequent measurements such as IR. The analyst believed that the porous glass was catalyzing the polymerization of solvents to produce colored species and that the chromatography by porous glass was not essentially different from that by silica gel[8]. According to another view, the yellow substances were a result of adsorption of organic contaminants from the atmosphere due to the higher adsorptive activity of porous glass adsorbent[7].

Abrasion resistance is claimed to be higher than that of conventional adsorbents[6]. Experience in this laboratory indicated the opposite to be true, although reasonable care will prevent "dust off".

Rapid settling and low load capacities are reported to be disadvantages of porous glass compared to silica gel or aluminum oxide, but purity, uniformity of pore structure, absence of suspension in eluting solvents, its rigid structure, and its low visible background are advantages for TLC work[7].

Experimental

Initial work tentatively supported the previous report that porous glass TLC was not essentially different from that with Silica Gel G in chromatographing pharmaceuticals. Therefore, the experimental design was restricted to comparison with Silica Gel G TLC. Only plates of 1 in. $\times$ 3 in. (microslides) were used since they could be made in large numbers and because it was assumed that TLC phenomena would be essentially identical to those obtained with 8 in. $\times$ 8 in. plates, except on a smaller scale.

In preparing plates, 1 part of the absorbent was generally mixed with about 1.1 parts of water when using porous glass containing 12% calcium sulfate binder, or with about 1.3 parts of water when using porous glass containing 3% Boehmite alumina fiber. (In the latter case, the aqueous phase should also contain 8.3 parts of 5% ammonium hydroxide as a deflocculent.) Speed is essential after the slurry has been made to avoid settling problems.

Plates were coated with the Desaga–Brinkmann apparatus to a thickness of about 0.20 mm. (Higher load capacities are obtained with thicker coatings.) The plates were heated at 200° for 30 min to activate the adsorbent and remove all ammonia, then cooled, and stored in a desiccator. Spotting was done with a 10-μl syringe.

Reported solvent systems should be tried first, but it may be necessary to devise one. Suggested load capacities are from 0.1 to 5 μg.

Examples for four pharmaceutical groups follow.

Steroids

0.1 μg each of estrone, estradiol, equilin, testosterone, and progesterone were applied. The solvent system used was benzene–ethyl acetate–water (6 ml : 4 ml : 2 drops).

The spots were visualized by 10% sulfuric acid spray followed by heating at about 300° (hot plate). Spots also show intense fluorescence under UV light. See Fig. 1.

In this example Silica Gel G gave more compact spots, and porous glass with calcium sulfate was second best. Some streaking occurred with both porous glass runs (stretched spots). More polar solvents were tried; they gave tighter spots but with less resolution. Differences of resolution might be due to the degree of adsorbent activation. Both porous glass types showed spots that fluoresced more intensely than on the Silica Gel G plate. A porous glass plate (calcium sulfate) spotted with 10 ng of each of the steroids gave color spots that could still be easily detected.

Barbiturates

1 μg each of amobarbital, mephobarbital, and phenobarbital was applied. The solvent system used was benzene–acetone–methanol–water (8.5 ml:0.75 ml:0.75 ml:1

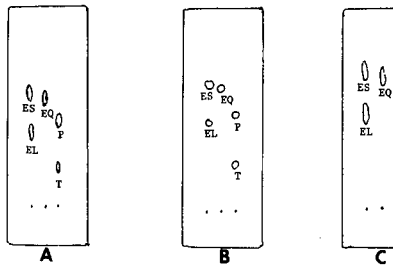

Fig. 1

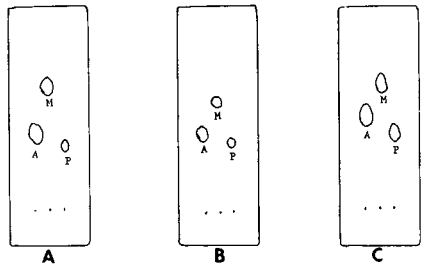

Fig. 2

Fig. 1. Comparison of TLC of steroids on (A) porous glass with $CaSO_4$, (B) Silica Gel G, and (C) porous glass with Boehmite alumina. ES = Estrone; EL = estradiol; EQ = equilin; T = testosterone; P = progesterone.

Fig. 2. Comparison of TLC of barbiturates on (A) porous glass with $CaSO_4$, (B) Silica Gel G, and (C) porous glass with Boehmite alumina. A = Amobarbital; M = mephobarbital; P = phenobarbital.

drop). The spots were visualized by UV light extinction after a 1 *N* sodium hydroxide spray. See Fig. 2.

Again, the Silica Gel G plate gave tighter spots, but the UV extinction contrast was better with the porous glass plates. Generally there was not much difference between the two adsorbents.

Aspirin, phenacetin, and caffeine

1 μg of each was applied. The solvent system used was water-washed ether–water-washed chloroform–methanol (8 ml:2 ml:2 drops). The spots were visualized by UV extinction for phenacetin and caffeine followed by heating on hot plate (about 300°), after which aspirin fluoresces strongly under UV. See Fig. 3.

It was uncertain whether the better resolution for aspirin using porous glass with $CaSO_4$ was due to differences in activation, binders, or adsorbent characteristics. The test was tried again on all three types of adsorbent microslides after they had been sprayed with a 10% ammonium hydroxide solution and activated at 100° and at 200°, respectively, for 30 min each. The results showed no essential difference from the illustrated chromatograms. Untreated Silica Gel G (no ammonia or heat activation)

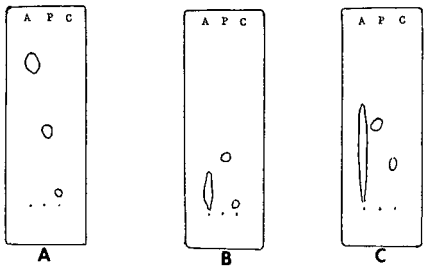

Fig. 3

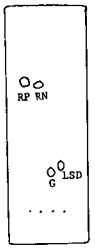

Fig. 4

Fig. 3. Comparison of TLC of aspirin (A), phenacetin (P), and caffeine (C) on (A) porous glass with $CaSO_4$, (B) Silica Gel G, and (C) porous glass with Boehmite alumina.

Fig. 4. Comparison of TLC of the alkaloids reserpine (RP), rescinnamine (RN), ergotamine (G), and LSD on Silica Gel G and on two types of porous glass. Spots did not move with either type of porous glass adsorbent. Only Silica Gel G microplate is illustrated.

again did not resolve aspirin. Contrast in detection by UV extinction was better with both types of porous glass plates than with Silica Gel G. As a note of interest, in one chromatogram where the spotting sequence was A to P to C, aspirin was detected in trace amounts in the phenacetin and caffeine columns even though the spotting syringe was rinsed twice before each compound was spotted. It is estimated that aspirin was detected at a contamination level of less than 0.01 μg.

Alkaloids

0.1 μg each of reserpine, rescinnamine, ergotamine, and LSD was applied. The solvent system used was water-washed chloroform–acetone (2:8). The spots were visualized by UV fluorescence. See Fig. 4.

No movement occurred with either type of porous glass, even when pure acetone was used, reflecting the higher acidity of the porous glass compared to Silica Gel G. Differences in resolution between porous glass and Silica Gel G are indicated for weakly alkaline substances.

Discussion

Porous glass adsorbent showed a TLC behavior essentially similar to that of Silica Gel G for five steroids, three barbiturates, phenacetin, and caffeine. Steroid spots were somewhat elongated when porous glass was used. Differences noted with aspirin appeared to be due to a combination of binder and adsorbent characteristics. Differences for the four alkaloids tested were due to the more acidic nature of porous glass. In all cases, speed of development was two or three times faster with porous glass when organic solvents were used.

Unfavorable aspects of porous glass are its rapid settling, low load capacity, and the reported formation of yellow substances which interfere when the adsorbent is eluted for subsequent quantitative determination (IR). Low load capacity may be due to the relatively small pore size (30–40 Å diameter) which does not contribute much usable surface area to the adsorbent for those organic molecules which are about 10 Å or more in diameter. This large molecular size and surface tension leave little room for those molecules to move in and out of the pore openings. Silica Gel G

is superior to porous glass in chromatographing spots compactly. The two adsorbents are not equivalent.

Porous glass adsorbent may be useful for TLC when a more acidic adsorbent is preferable, when better detection contrast is required, when speed of development is a consideration, or, in certain instances, when resolution with Silica Gel G is unsatisfactory. Further investigation is warranted.

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CHROM. 4421

Enhanced photoemulsion sensitivity at low temperatures used in radiochromatography

Recently a method of detecting tritium and radiocarbon in thin-layer radiochromatography has been developed. By adding scintillators to the thin-layer media, the small β particle energies are converted into light (β-radioluminescence)[1,2]. The detection sensitivity is greatly increased by lowering the temperature when detecting the light by photographic methods[3,4] but not by photomultiplier detection[2,5]. Upon lowering the temperature from 20° to −78° in the applied scintillators, anthracene and 2,5-diphenyloxazole, an increase in detection sensitivity less than 5% is found by photomultiplier detection, while for photographic detection a factor of *ca.* 25 is quoted for the sensitivity increase.

Thus we conclude that the film material, which in fact has been cooled down together with the radiochromatograms, is responsible for the main temperature variation in the overall detection sensitivity. In the film emulsions, back reactions might be prominent, either reducing the extent of latent image formation or producing

a fading of the latent images. The last process could be shown to be negligible[6]. A temperature-sensitive induction of the latent image was then hypothesized.

The mechanisms involved in latent image formation have been thoroughly investigated[7,8], and the low-temperature sensitivity enhancement can be explained according to a suggested model. Electrons released in the silver halide microcrystals by the impinging photons may fall into very shallow traps. New traps are formed at the same sites by successive neutralization of the electrons by Ag^+ from the lattice. These formed silver traps are unstable, and stabilization is reached only if this cycle is rerun, thus giving a site with two silver atoms in its close vicinity. This trap now is considered to be stable and acts as a nucleation center for silver atoms. The lifetime of the first unstable silver trap is temperature dependent, which at low photon intensities determines the rate of formation of stable nucleation centers.

In the following we shall very briefly report on some experiments and results so far as they are relevant to radiochromatography. A broader presentation is given elsewhere[6]. In order to make sure that the greatly increased detection sensitivity by photographic detection cannot be ascribed to temperature variations in scintillator efficiency, pure light exposures were done. Two temperatures, 20° and −78°, were chosen for convenience, and an electroluminescent light source (peak intensity at 470 nm) was used. To obtain the same optical density (O.D. = 0.5) in the film emulsion after a 5-h exposure at 20°, the light intensity had to be about eighty times larger than at −78° (PR X-Omat Estar Medical X-ray film). When the intensity of the electroluminescent light was increased by a factor 1000, no differences in the optical densities could be observed for exposures (5 sec) at the two different temperatures.

Lastly, a radionuclide scintillator system was used as a light source. [^{3}H] or [^{14}C]glucose solutions were pipetted onto Eastman K301R2 layers and dried. Anthracene was added by spraying a saturated benzene solution onto the layers until no further increase in β-radioluminescence output (peak intensities at 450 and 470 nm) could be measured using a photomultiplier detector.

For our [^{3}H]anthracene system, the largest part of the exposure results from

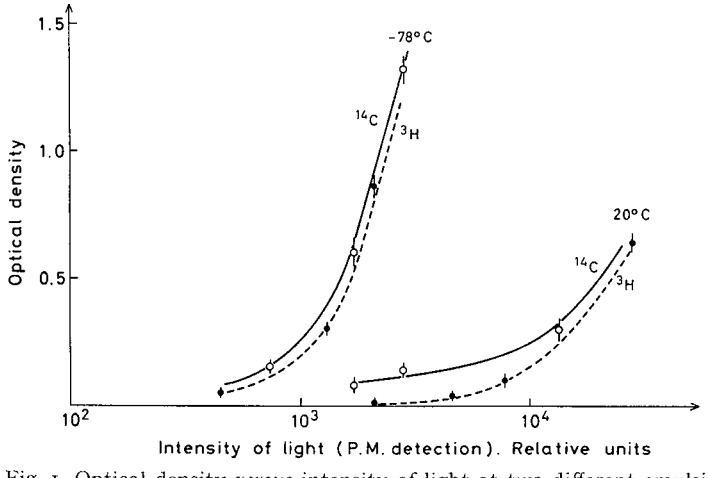

Fig. 1. Optical density *versus* intensity of light at two different emulsion temperatures. Further details are given in the text.

scintillations and only very little from direct electron exposure of the film. Yet the induction of latent images seems to be less temperature dependent than for electroluminescent exposure (twelve times increase as compared to eighty times, same exposure time). According to the model, however, a pulsed light source (lifetime of excited states in anthracene $\simeq 2 \cdot 10^{-8}$ sec) would give a less temperature-sensitive induction of latent images.

For the [^{14}C]anthracene exposures, β-particles as well as photons reach the emulsion. In Fig. 1 the [^{3}H]- and [^{14}C]anthracene systems are compared for the two temperatures, 20° and −78°. The abscissa indicates the proper photon intensity (I) measured with a photomultiplier. We notice that [^{14}C]- and [^{3}H]anthracene systems give slightly different O.D.–log I curves. On the one hand we have an additional direct β-electron exposure from the [^{14}C]anthracene system while, on the other hand, we have fewer but more intense scintillations. Both factors would give a less temperature-sensitive induction of the latent images, which is also seen in the figure, at least for low intensities.

To sum up, two main findings seem important to radiochromatography. Firstly by exposure at −78° rather than at 20°, the emulsion sensitivity is increased eighty times if the photons are randomly distributed in space and time (PR X-Omat Estar Medical X-ray film and Kodirex X-ray film at 470 nm and very low intensities). Secondly, the more uneven the distribution of the photons in space and time (depending on the speed of the scintillator) the less temperature-dependent the emulsion sensitivity will be.

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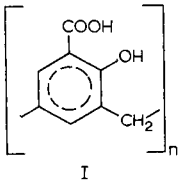
CHROM. 4475

Fractionation of poly(methylene-2-hydroxybenzoic acid) by preparative layer chromatography

In our work on biologically active synthetic polymers, poly(methylene-2-hydroxybenzoic acid) [I] which showed a reasonable anti-inflammatory activity on localized edema caused by bradykinin[1] has been examined.



I

To investigate whether such a property should be attributed to the polymer as a whole or to some fraction of it, we developed a fractionation procedure using preparative layer chromatography. Satisfactory results were obtained using Silica Gel H as sorbent and a mixture of benzene–acetic acid–water (2:2:1) as eluent. Each fraction was removed from the chromatoplate together with the sorbent and eluted using a column. The molecular and the equivalent weights were determined for each fraction.

Experimental

Materials and methods. A Shandon equipment for preparative layer chromatography was used. Chromatoplates (100 × 20 cm) were prepared by the method described by STAHL[2], with Silica Gel H (Merck) as sorbent. A 750-μ layer was used on all plates which were then placed for 3 h in the pre-drying rack, heated at 110° for 1 h and stored in a desiccator. The chromatographic tank was lined with filter paper and equilibrated for 4–5 h before use.

Benzene–acetic acid–water (2:2:1) was employed as the solvent system. The solvents were mixed in a separatory funnel and allowed to equilibrate for 30–40 min. The benzene phase was placed at the bottom of the chromatographic tank, and the aqueous phase was placed in a beaker inside the tank.

TABLE I

CHARACTERIZATION OF POLY(METHYLENE-2-HYDROXYBENZOIC ACID) (PMSA) FRACTIONS

Fraction	R_F *value* (× 100)	*Equiv. wt.*	*Mol. wt.*
1	0– 4	158.8	1100
2	5–18	160.9	800
3	19–27	165.3	550
4	28–38	173.8	450
5	39–46	143.8	288[a]
PMSA	—	163.9	650
Salicylic acid	78	138.2	138

[a] This is the molecular weight of 3,3'-dicarboxy-4,4'-dioxydiphenylmethane.

About 0.3 g of polymer previously solubilized in 2 ml of an acetone–water (10:1) mixture was deposited along the length of each plate. The chromatoplates were developed in the stainless-steel preparative chromatotank which held one chromatoplate rack with five plates. In this way it was possible to fractionate 1.5 g of polymer at the same time. The plates were developed for 14–16 cm (*ca.* 45 min) by the ascending method.

After complete drying, five fractions, with R_F values as reported in Table I, were observed as bands on the plate by means of UV light at 366 mμ (Engelhard Hanovia Model 16).

Extraction and characterization of fractions. The fractions were removed from the plates and transferred into glass tubes (2 × 30 cm) drawn at one end. The fractions were dissolved in ethanol; by adding dilute HCl a precipitate was obtained. After centrifugation and repeated washing with water, the fractions were dried under vacuum at 40°; each fraction was collected as a white powder.

The equivalent weight was determined by a potentiometric titration[3] of the carboxylic group of each monomeric unit with potassium methylate as titrant and pyridine–benzene–methanol (2:2:1) as solvent*.

Table I shows the equivalent weights of the fractions and related molecular weights determined in methanol by an isopiestic method (Hitachi Perkin-Elmer, Model 115).

Discussion

The results summarized in Table I show that the polymer fractionation is a function of the molecular weight of each fraction. The procedure developed allows one to obtain reasonable quantities of each fraction in a relatively short time. By using a set of five plates, it is possible to fractionate 1.5 g of polymer in about 2 h. This simple technique can also be satisfactorily used for the molecular weight fractionation of other similar polymers.

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* The higher values of the equivalent weight as compared with the equivalent weights calculated from formula I can be explained by the presence of hydroxymethylenic groups, as confirmed by NMR[4].

CHROM. 4476

Chromatography and detection of some β-D-glucosides

The successful detection of phenolic glucosides on paper chromatograms has depended on the presence of an aglucone which either shows chemical reactivity, especially towards coupling reagents, or interacts with UV light[1–9]. The use of ^{14}C-labelled glucose or aglucone, supplemented by techniques to confirm the presence of both moieties, has also assisted in the detection of glucosides[10–15]. On thin-layer chromatoplates *p*-coumaryl alcohol 4-O-glucoside, syringin and coniferin have been located using antimony pentachloride in carbon tetrachloride[16]. Whilst investigating the metabolism of simple phenols and their β-D-glucosides in plants we experienced some difficulty in finding a sensitive reagent for locating glucosides on thin-layer plates. Whilst arbutin and the β-D-glucosides of catechol and resorcinol were readily detected with 0.25% ferric chloride/potassium ferricyanide in 50% aqueous alcohol and other reagents, an alternative reagent had to be sought for the remaining β-D-glucosides used in the study. By increasing the proportion of phosphoric acid to aniline in a reagent used to detect mono- and disaccharides on paper chromatograms[17] a sensitive locating reagent for β-D-glucosides on thin-layer plates of silica gel was obtained.

Materials and methods

Thin-layer chromatography. TLC plates, 20 cm × 20 cm of Silica Gel G (Merck), 0.25 mm thick, were prepared using a BTL applicator. The plates were air-dried for 4 h, stored for 12 h and then activated at 120° for 1 h. The plates were allowed to cool over granular silica gel before use. Solutions of the glucosides in rectified spirit (100 or 1000 μg/ml) were applied to the plates and the plates developed using one of the five solvent systems described below.

The plates were dried at 120° for 10 min and then sprayed with the detecting reagent. After 45 min at 125–130°, the plates were viewed with filtered UV light (Hanovia Fluorescent Lamp, Model 11). The glucosides were visible as light fluorescent spots against a violet background.

Reagents. The aryl β-D-glucosides were available from another study and will be reported on elsewhere. Solvents and other chemicals used in the chromatography and detection of the β-D-glucosides were of normal reagent grade and used without further purification. Diethyl ether was saturated with water before use.

Developing solvents. The following solvent systems were used: (I) dimethylformamide–chloroform (2:4.5); (II) *n*-butanol–methanol–chloroform (3:1:6); (III) *n*-butanol–ether (1:4.5); (IV) *n*-butanol–dichloromethane (1:2); (V) *n*-butanol–chloroform (1:1.5).

Detecting reagent. A solution of 15% (v/v) aniline in *n*-butanol (20 ml) was added to a solution (50 ml) of 30% (v/v) of concentrated phosphoric acid (88–90%, s.g. 1.75) in *n*-butanol. The mixture was shaken well in order to disperse the precipitate formed initially and the clear solution was used as the detecting reagent. If difficulty was experienced with obtaining a clear solution the reagent was filtered before use.

TABLE I

R_F VALUES OF ARYL β-D-GLUCOSIDES

Glucoside	*Solvent system*				
	I	*II*	*III*	*IV*	*V*
Phenyl	0.41	0.36	0.30	0.28	0.24
2-Chlorophenyl	0.46	0.42	0.37	0.35	0.29
3-Chlorophenyl	0.46	0.40	0.36	0.29	0.26
4-Chlorophenyl	0.43	0.38	0.31	0.28	0.24
2,4-Dichlorophenyl	0.47	0.44	0.38	0.34	0.30
2,6-Dichlorophenyl	0.57	0.52	0.47	0.40	0.39
2,4,5-Trichlorophenyl	0.52	0.49	0.51	0.39	0.35
Pentachlorophenyl	0.62	0.57	0.55	0.46	0.44
4-Tolyl	0.45	0.39	0.29	0.30	0.27
4-Methoxyphenyl	0.40	0.36	0.21	0.27	0.23
4-Cyanophenyl	0.39	0.34	0.21	0.24	0.21
4-Nitrophenyl	0.39	0.35	0.26	0.27	0.23
2-Hydroxyphenyl	0.32	0.33	0.29	0.25	0.20
3-Hydroxyphenyl	0.26	0.25	0.28	0.23	0.17
4-Hydroxyphenyl	0.26	0.23	0.23	0.19	0.15
Tetrachloro-4-hydroxyphenyl	0.12	0.29	0.38	0.25	0.21

Discussion

The main part of our study was concerned with the formation of glucosides in plants treated with synthetic phenols. It was only necessary, therefore, to concern ourselves with chromatographic separation of the parent phenol from its glucoside and other metabolites and sugars which might occur in the treated plant. For this purpose developing solvents such as isopropanol–0.88 ammonia (9:1) and *n*-butanol–acetic acid–water (12:3:5) were adequate, however, the chromatographic separation of individual glucosides from each other with these systems was poor. This is not surprising in view of the dominant hydrophilic character of the glucose moiety. Some results of an investigation of some other solvent systems for improved separation are presented in Tables I and II.

Mixtures of simple alcohols or dimethylformamide with chloroform, dichloro-

TABLE II

R_F VALUES OF β-D-THIOGLUCOSIDES

Thioglucoside	*Solvent system*				
	I	*II*	*III*	*IV*	*V*
Phenyl	0.51	0.43	0.32	0.34	0.33
4-Tolyl	0.54	0.47	0.36	0.37	0.35
4-Chlorophenyl	0.52	0.44	0.38	0.36	0.33
4-Nitrophenyl	0.48	0.41	0.33	0.36	0.30
Ethoxythiocarbonyl	0.53	0.46	0.41	0.39	0.34
N-Methylthiocarbamoyl	0.31	0.26	0.19	0.16	0.13
N,N-Dimethylthiocarbamoyl	0.42	0.35	0.10	0.23	0.19

methane or diethyl ether proved to be the most effective for partial resolution of the glucosides investigated. The resolving power of a solvent mixture improved with the use of alcohols of increased chain length; at the same time the mobility of the glucosides was depressed. The mobility of the glucosides was greatest in solvent systems based on diethyl ether as a component and least with chloroform. The R_F values of the glucosides were depressed with increasing proportion of diethyl ether, dichloromethane or chloroform in the mixture with a slight improvement in resolution. On the other hand the addition of acetic acid or methanol enhanced the R_F values of the glucosides but caused some deterioration in resolving power.

The reagent used for the detection of the glucosides differs from that of HIMES *et al.*[17] in so far that it contains a considerable excess of phosphoric acid. Reducing the proportion of the solution of concentrated phosphoric acid in *n*-butanol to 40 ml leads to the formation of a bulky precipitate; the precipitate can be removed by filtration and the filtrate used without loss of sensitivity. Larger reductions in the quantity of phosphoric acid in the spray not only lead to precipitate formation but to loss of sensitivity of the reagent towards glucosides, but not towards glucose. All glucosides were visible at the 0.1–0.2 μg level as fluorescent spots under filtered UV light whilst larger amounts (1.0–2.0 μg) of glucoside were visible as grey-brown spots on a white background under ordinary daylight conditions.

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CHROM. 4483

GAS CHROMATOGRAPHIC SEPARATION OF DIBASIC ALKANOIC ACID DIMETHYL ESTERS AND THE THREE ISOMERIC PHTHALIC ACID DIMETHYL ESTERS WITH A POLYAMIDE LIQUID PHASE

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SUMMARY

The complete gas chromatographic separation of the dimethyl esters of dibasic alkanoic acids (C_3–C_{12}) and the three isomeric phthalic acids was carried out using a fluoren-9-one and N-66/6 2-stage column. The greater degree of polarity of the stationary phases (N-66/6 > N-6I > N-12 and NGS > PEGS > PEG-6000) that was expected was in accordance with the shorter retention times of dimethyl azelate on these stationary phases. The differences in the elution order of the three isomeric phthalic acids on each stationary phase were interpreted from electronic theory and hydrogen bonding. Plotting the logarithm of the retention times of the diesters relative to adipic acid diester *vs.* carbon numbers on all stationary phases investigated gave the same straight lines, with negligible deviations.

INTRODUCTION

A technique recently described for the analysis of copolyamides is the gas chromatographic resolution of the polymer hydrolysate[1]. The liberated diacids in the hydrolysate were esterified with BF_3–methanol, and the diesters were gas chromatographed following the extraction. Using this technique, the esters of sebacic and terephthalic acids were not separated. The gas chromatographic separation of the three isomeric dimethyl phthalates and the dimethyl sebacate were carried out using a Benton-34–Carbowax column[2]. However, the dimethyl esters of dodecadionic acid which was frequently encountered in polyamides has not been examined.

KOMERS AND BAZANT[3,4] used Provina packing coated with 10.8% erythritol for separation of isomeric dimethyl phthalates (*o*-, iso- and terephthalates). The following comparative retention volumes were determined: *o*-, 1.42; iso-, 1.00; and tere-, 0.88. The dimethyl esters of dibasic alkanoic acids in the range C_4 to C_{13} were separated with 5% PEG-6000 on firebrick C-22 (ref. 5), and the dimethyl esters of

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adipic, azelaic, sebacic and *o*-phthalic acids were resolved with 15% Ultramoll III or Resoflex[6]. The dibasic acid *n*-propyl esters in the range C_3 to C_{12} were also investigated[7]; however, they did not allow the separation of the dimethyl esters of dibasic alkanoic acids and the three isomeric phthalic acids on the same chromatogram.

BROOKS *et al.*[8] used a polyamide resin, Versamide 900, as stationary liquid phase for the separation of resin acid methyl esters (L-pimaric, abietic, pimaric and dehydroabietic acids). Versamide-900 was also used for the separation of dinitrobenzene isomers[9].

The primary objective of this research was the complete gas chromatographic separation of the dimethyl esters of dibasic alkanoic acids (malonic to dodecadionic acid) and the three isomeric phthalic acids using a single sample on the same chromatogram. The secondary objective was to ascertain whether any polyamides have the properties desirable for stationary liquid phases in gas chromatography.

MATERIALS AND METHODS

Stationary phases

Polyhexamethylene isophthalamide (N-6I, m.p. 175°) and polyhexamethylene adipamide–capramide copolymer (N-66/6, 30/70 w/w, m.p. 186°) were prepared from the corresponding nylon salts and caprolactam. Polylauramide (N-12, m.p. 182°) was kindly supplied by the Toyo Rayon Co. Ltd., and polyethyleneglycol 6000(PEG-6000, m.p. 63°), neopentylglycol succinate polyester (NGS, m.p. 63.5°), polyethyleneglycol succinate polyester (PEGS, m.p. 73°) and fluoren-9-one (m.p. 84°) were obtained from Nishio Kogyo Co. Ltd. The melting points of these polymers were measured with a Yanagimoto micromelting-point apparatus, model MP-S2.

Columns

For preparation of column packings, Dia-solid M (60–80 mesh) was used. The amount of liquid phases was 5% by weight in all cases. A stainless-steel tube, 2 m long and 3 mm I.D., was used for each column. In preparing the packings, polyester, polyether and fluoren-9-one phases were applied in an acetone solution and polyamide phases in a benzyl alcohol solution. All columns were conditioned at 240° for 24 h except for the fluoren-9-one column which was conditioned at 170°.

Gas chromatography

A Shimadzu gas chromatograph, model GC-4APTF, with dual flame ionization detectors and a linear temperature programmer was used. The injection port and the detector oven were maintained at 240°; the flow rate of the carrier gas was 60 ml of N_2 per min. The column temperature was maintained isothermally at 120, 140, 160, 180 and 200°, or programmed from 80 or 100° at a rate of 2°/min.

Dibasic alkanoic acids (malonic acid (C_3) to dodecadionic acid (C_{12})) and the three isomeric phthalic acids were commercial products of reagent grade and esterified with a dry HCl-methanol mixture. For the determination of retention times, all these esters were analyzed either separately or in various mixtures on prepared packings.

RESULTS AND DISCUSSION

The main results of the measurements and calculations are recorded in Figs. 1 and 2 and Tables I–III. The corrected retention times of dimethyl azelate on the stationary phases investigated at different temperatures are listed in Table I. Considering the data in Table I, it could be stated that the retention times on N-66/6 and NGS were shorter than on N-12 and PEG-6000. This was in accordance with the expected greater degree of polarity of N-66/6 and NGS, which could be explained by differences in the structures of these stationary phases[10].

The relative retention times of the dimethyl esters of the three isomeric phthalic acids and sebacic acid *vs.* azelaic acid are given in Table II. The gas chromatograms of

TABLE I

RETENTION TIMES OF DIMETHYL AZELATE

Stationary phase	*Retention time (min)*	
	140°	*160°*
PEG-6000	38.3	14.5
PEGS	32.0	13.1
NGS	10.9	4.5
N-12	21.0	11.9
N-6I	12.7	5.6
N-66/6	9.7	4.1
Fluoren-9-one	6.0	—

TABLE II

RELATIVE RETENTION TIMES OF THE THREE ISOMERIC PHTHALIC ACIDS AND SEBACIC ACID DIMETHYL ESTERS

Stationary phase	*Programmed temperature (2°/min)*	*Relative retention time*				
		Az[a]	*p*-[b]	*o*-[c]	*m*-[d]	*Se*[e]
PEG-6000	100–200	1.00	1.09	*m*- + *o*- 1.15		1.13
NGS	80–180	1.00	1.03	*m*- + *o*- 1.06		1.14
PEGS	100–200	1.00	*o*- + *p*- 1.07		1.12[a]	1.15
N-66/6	80–160	1.00	1.06	1.16	1.11	1.17
N-12	80–180	1.00	*p*- + *o*- 1.09		*Se* + *m*- 1.15	
N-6I	100–180	1.00	1.11	*m*- + *o*- 1.17		1.21
Fluoren-9-one	80–150	*Az* + *p*- 1.00		*m*- + *o*- 1.06		1.18

[a] Azelaic acid dimethyl ester.
[b] Terephthalic acid dimethyl ester.
[c] Phthalic acid dimethyl ester.
[d] Isophthalic acid dimethyl ester.
[e] Sebacic acid dimethyl ester.

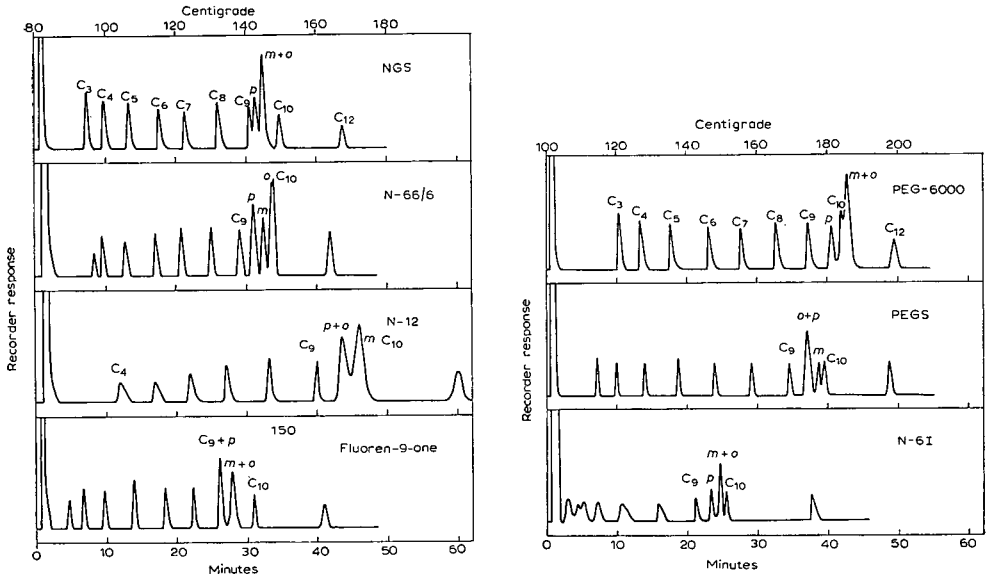

Fig. 1. Gas chromatograms of diesters on single columns. Programmed at 2°/min; flow rate of carrier gas 60 ml N_2/min. C_3 = malonic acid dimethyl ester; C_4 = succinic acid dimethyl ester; C_5 = glutaric acid dimethyl ester; C_6 = adipic acid dimethyl ester; C_7 = pimelic acid dimethyl ester; C_8 = suberic acid dimethyl ester; C_9 = azelaic acid dimethyl ester; C_{10} = sebacic acid dimethyl ester; C_{12} = dodecadionic acid dimethyl ester; *o* = phthalic acid dimethyl ester; *m* = isophthalic acid dimethyl ester; *p* = terephthalic acid dimethyl ester.

all dibasic acid dimethyl esters on the stationary phases studied are shown in Fig. 1.

On PEG-6000, NGS and PEGS, the retention times of sebacic acid dimethyl ester relative to those of azelaic acid dimethyl ester were almost identical using these three stationary phases; however, those of each of the three isomeric phthalic acid (*o*-, iso- and tere-) dimethyl esters were different on each phase. Isophthalic and *o*-phthalic acids or terephthalic and *o*-phthalic acids were not separated using programmed gas chromatography, although they were incompletely separated by isothermal gas chromatography. The elution order is as follows: tere-, iso- and *o*- on PEG-6000; tere-, *o*- and iso- on NGS; and *o*-, tere- and iso- on PEGS. This phenomenon could be interpreted by electronic theory, and the differences among the coherences of the stationary phases with *o*-phthalic acid dimethyl ester might be greater than with iso- or terephthalic acids.

On N-66/6, N-6I and N-12, the elution order using isothermal gas chromatography is as follows: tere-, iso- and *o*- on N-66/6 and N-6I; and tere-, *o*- and iso- on N-12. As the melting points of the polyamide stationary phases were close to each other, this elution difference was perhaps related to the stereo structure of the phthalic isomers and to the distance between adjoining amide linkages of the polyamide stationary phases. The hydrogen bonding of *o*-phthalic acid dimethyl ester with the N-12 liquid phase might be so weak that the retention time for *o*-phthalic acid dimethyl ester was shorter than that for isophthalic acid dimethyl ester.

These results of testing the stationary phases enabled us to conclude that the newly introduced stationary phase N-66/6 was efficient enough to separate the three

TABLE III

RELATIVE RETENTION TIMES ON A 2-STAGE COLUMN

Stationary phase		*Programmed temperature*	*Relative retention time*						
First	*Second*	(2°/min)	*Az*[a]	*p*-[b]	*m*-[c]	*o*-[d]	*o*- + *m*-	*Se*[e]	*o*- + *Se*
NGS	N-66/6	100–200	1.00	1.04			1.10	1.16	
N-66/6	NGS		1.00	1.06	1.10	1.15		1.16	
Fluoren-9-one	N-66/6	100–160	1.00	1.03	1.08	1.12		1.17	
N-66/6	Fluoren-9-one		1.00	1.06	1.11				1.16

[a] Azelaic acid dimethyl ester.
[b] Terephthalic acid dimethyl ester.
[c] Isophthalic acid dimethyl ester.
[d] Phthalic acid dimethyl ester.
[e] Sebacic acid dimethyl ester.

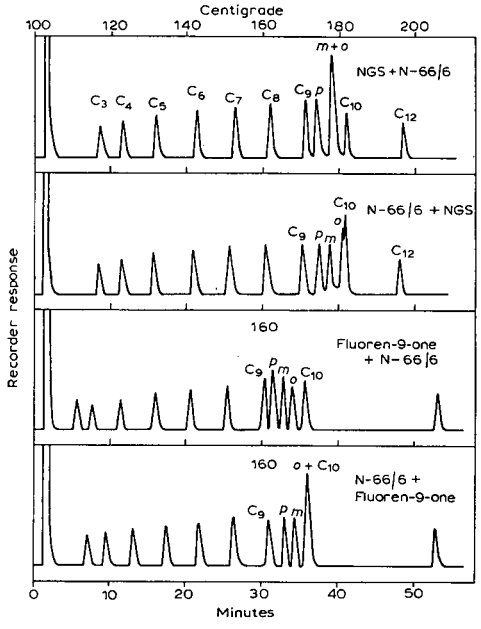

Fig. 2. Gas chromatograms of diesters on 2-stage columns. Conditions and abbreviations are identical to those in Fig. 1.

isomeric phthalic acid dimethyl esters. For separation of dibasic alkanoic acids, a 2-stage column was needed. The results are shown in Table III and Fig. 2. Complete separation of dibasic acid dimethyl esters was attained with the 2-stage column of fluoren-9-one (first stage) and N-66/6 (second stage). The column length of each stage was 2 m. Reverse combination of these stages resulted in different separations. This phenomenon could be attributed to the difference of the carrier gas flow distribution inside the column caused by the gas compressibility. As the average flow rate in the first stage was lower and the retention time was longer than in the second stage, it was assumed that the retention time on the 2-stage column was strongly affected

by the first-stage column. This supposition was proved from the consideration of the relative retention times in Tables II and III. The additivity of the relative retention times between the 2-stage column and corresponding individual columns was observed on the fluoren-9-one and N-66/6 2-stage column.

The peak shapes and the separation factors of diesters on polyamide stationary phases below their melting point were nearly identical to those over the melting points; the shapes on N-12 and N-6I were rather broad and those on N-66/6 sharp. From these results we assumed the following. First, the adsorption mechanism of diesters to the stationary liquid phases rather than dissolution of diesters in the stationary phases was responsible for the separation. The dissolution mechanism in the stationary phase was also considered over the melting point. Second, below their melting point crystallinity of the polyamides might affect the peak shape of diesters. The effect of aging upon retention times on polyamide phases during the serial analyses was within the limits of reproducibility of individual determinations; for example, the N-6I phase had a good thermal stability even after 100 h at 200°.

Plotting the logarithm of the retention times of dibasic alkanoic acid diesters relative to adipic acid diester *vs.* carbon numbers on all phases investigated gave straight lines. The equation obtained from these lines was:

$$\log(\text{relative retention time}) = \frac{\log 5.2}{4} \times (\text{carbon number}) + \log 0.083$$

This equation was identical, with negligible deviations, for all lines on the stationary phases. This phenomenon implied that the values of the relative retention times of the homologous series (the standard was also selected among them) were not affected by any stationary phases which were efficient enough for separation of diesters. Plotting the relative retention times against the number of carbon atoms using programmed gas chromatography (Fig. 1) also produced nearly straight lines for every stationary phase used.

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CHROM. 4380

ISOLATION OF AGAROSE AND GRANULATION OF AGAR AND AGAROSE GEL

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SUMMARY

A method has been developed for the isolation of agarose from "Difco" bactagar by means of ammonium sulphate and acetone. A modification of PHILIPSON's method was used for granulation of agar and agarose. A sprayer with removable discs allows one to prepare bead-shaped granules of any size required at gel concentrations of 2–7 %. The method may be a highly effective laboratory technique.

INTRODUCTION

Physicochemical properties and concentration of gel, on the one hand, and the size and shape of the granules, on the other hand, determine the effectivity and the conditions for application of the gel filtration method. Sephadex and Biogels are known not to fractionate macromolecules of molecular weight exceeding $4 \cdot 10^5$ (*e.g.*, macroglobulin, viruses, nucleic acids). POLSON[1] in 1961 suggested that granulated agar gel with large particles should be used for fractionating proteins of high molecular weight, but the carboxyl and sulphate groups of agaropectin present in the agar are responsible for extensive sorption and, consequently, separation on the agar column depends upon the ionic strength of the buffer[2]. HJERTÉN[3,4] succeeded in applying agarose gel, which is the natural component of agar, for separation of viruses and nucleic acids.

At present granulated agarose is commercially manufactored in Sweden ("Sepharose") and in the U.S.A. ("Sagarose")[5]. However, in practice, laboratory-made granulated agarose gel is often required. All the methods of isolation and granulation of agarose suggested so far have a common drawback: isolation of pure agarose requires costly reagents (*e.g.*, cetylpyridinium chloride) and granulation is time-consuming[1,3,6–8].

The present paper describes (1) a modification of the method of AJITSKY AND KOBOZEV[9] used for preparation of agarose from bactagar "Difco" and (2) a modification of the method of PHILIPSON AND BENGTSSON[6], which is highly effective for granulating agar and agarose gel.

ISOLATION OF AGAROSE

Agarose was isolated from "Difco" bactagar by fractionation with ammonium sulphate (AS) (reagent-grade). Preliminary experiments revealed that complete precipitation of agar occurs between 0.18 and 0.47 saturation with AS. To fractionate the agar, 1 l of hot saturated AS solution was added to 2 l of hot 2 % agar with stirring (*i.e.* to 0.33 saturation) and left for 30 min in a hot water bath. Then the mixture was poured into preheated centrifuge tubes and centrifuged at 2500 r.p.m. for 15 min without cooling. After centrifugation the supernatant was separated; the dense sediment (Fraction I) was washed with cold water to remove residual gel and heated in 1 l of water till completely dissolved. Two volumes of acetone were then added to the resulting solution, and it was left standing overnight at 4°. The white friable flakes of the sediment were repeatedly washed with distilled water to remove AS completely. The disappearence of AS was estimated by quantitative reaction of the supernatant with $BaCl_2$. Fraction I, devoid of AS, was dehydrated with acetone and dried at room temperature. The supernatant remaining after Fraction I had been sedimented in the centrifuge was left standing overnight at 4° with three volumes of acetone. The sediment which remained on removal of the acetone was repeatedly washed with distilled water to remove AS (Fraction II). Washed Fraction II was also dehydrated with acetone and air-dried. Unlike Fraction I, Fraction II sediments less readily in cold distilled water; therefore, it was sedimented in the centrifuge at 2500 r.p.m. for 30 min. Both fractions isolated from agar after dehydration look like white powder and readily dissolve in water; however, a 1 % solution of Fraction II forms a weaker gel than Fraction I and agar.

To identify Fractions I and II, the degree of electroendosmosis in electrophoresis of rabbit serum in gels of French agarose (L'Industrie Biologique Française) was compared to that in "Difco" agar and our Fractions I and II. All these gels were tested simultaneously in the same apparatus in veronal buffer (pH 8.6 $\pm$ 0.05) at E = 8 V/cm for 1 h. After electrophoresis the gel was dried, fixed and stained with

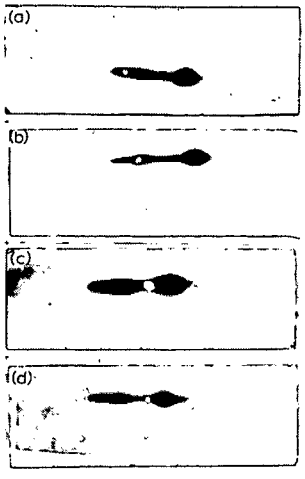

Fig. 1. Electrophoretograms of rabbit serum. (a) French agarose. (b) Agarose (Fraction I). (c) Agaropectin (Fraction II). (d) Difco agar.

Amino Black 10B. The electrophoregrams (Fig. 1) clearly show that in Fraction I the cathode displacement of protein is only slightly greater than in agarose whereas in the agar and, particularly, in Fraction II electroendosmosis is much more pronounced. Besides, Fraction II always shows worse protein separation as compared to agar and diffusion of proteins in the opposite direction of electrophoresis, which indicates strong sorption of proteins in Fraction II. The results of the above test indicate that probably Fraction I is agarose and Fraction II is ionisable agaropectin.

GRANULATION

A modification of the method of PHILIPSON AND BENGTSSON[6] was used for granulation of agar and agarose. A hermetically sealed 1-l metal tank (Fig. 2a) is attached to a holder fixed to a heavy slab*. The lower outlet of the tank is connected through a double-stroke valve to a sprayer** with removable brass discs which have a conical orifice. Compressed nitrogen, kept between 2–4 atm, is fed into the tank filled with melted agar or agarose which is then squeezed through the sprayer. The granulated gel enters a 20-l cylinder containing 15 l of a water–ether mixture (1:1) which has been precooled to 2°. The mixture is continuously blended by a stirring rod which is connected by a flexible shaft to a motor outside the hood***. After the granules have sedimented, the ether is removed and the granules are washed repeatedly to remove residual ether. They are then sieved through standard sieves under a strong stream of water.

This granulating device can be used to obtain granules from agar and agarose solutions at concentrations from 2 to 7 %. The granules sieved through 80–200 mesh appear bead-shaped in the phase-contrast microscope (Fig. 3). Depending on the size of granules required and on gel concentration, the diameters of the orifice of the brass discs fixed in the sprayer may be from 1 to 3.5 mm. The optimal diameter is decided

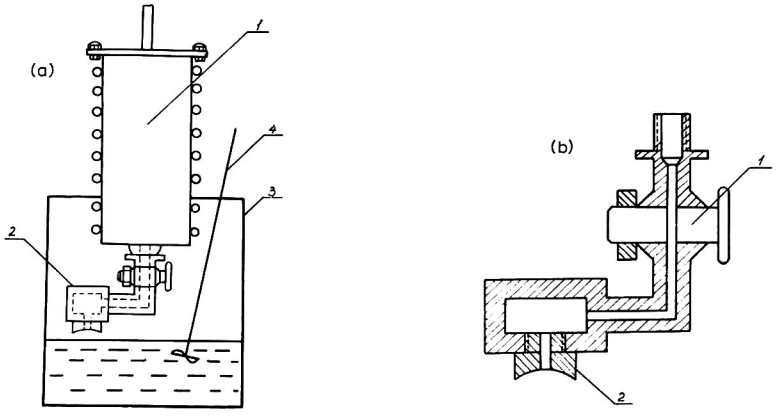

Fig. 2. (a) Device for making spherical agarose granules. 1 = hermetically sealed tank encircled with heating tube; 2 = sprayer; 3 = cylinder containing water–ether mixture; 4 = stirring rod. (b) Agarose sprayer. 1 = valve; 2 = removable brass discs with a conical orifice.

* The outer surface of the tank is encircled with a helical heating tube containing hot water.
** The design of the sprayer was suggested by S. B. KOOST.
*** Constant stirring is necessary to destroy the interfase to permit the granules to pass freely between the ether and water.

empirically as the size of granules at a given pressure and concentration decreases with the increase in the diameter of the orifice in the disc, except at some point at which the process of granulation ceases to give way to the liquid discharge of the gel. For more concentrated solutions, a bigger orifice is required.

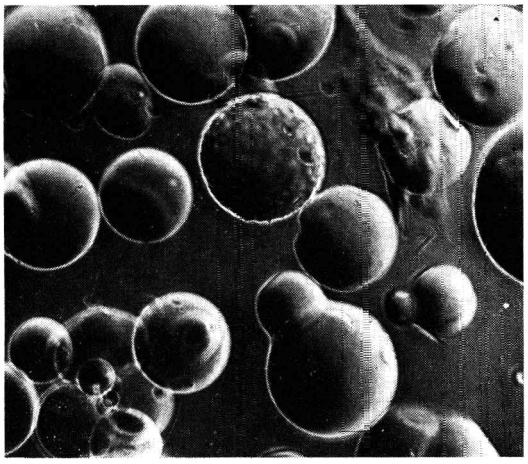

Fig. 3. Granules of 5% agarose, 80–200 mesh.

DISCUSSION

Agar is a mixture of two polysaccharides: agarose with a mol. wt. of 120000 and devoid of ionisable groups, and agaropectin with a mol. wt. of 12000 and containing acidic carboxyl and sulphate groups[10]. It is natural that gels of agarose and agaropectin display different behaviours in the column for gel filtration; the neutral agarose may serve as a "molecular sieve" whereas agaropectin is bound to have some undesirable properties and also accounts for considerable electroendosmotic flow in the direction of the cathode which is always present in electrophoresis in the agar gel. In the agarose gel no electroendosmosis is observed, which makes electrophoresis a convenient method of testing the purity of agarose.

Many methods of isolating agarose have been described. The procedure suggested by ARAKI[7] and modified by HJERTÉN[3] is based on agar acetylation by acetic anhydride in pyridine. Agarose prepared by this procedure has an admixture of agaropectin and has weaker gel-forming properties than agar because of partial hydrolysis of polysaccharide. Later HJERTÉN suggested the use of quarternary ammonium salts, particularly cetylpyridinium chloride, which sediments agaropectin by reacting with the acidic groups of the latter[4]. POLSON *et al.*[8] used polyethyleneglycol with a mol. wt. of 6000 to fractionate agar. Agarose obtained by the two methods mentioned above contains minimum quantities of sulphur and has the same gel-forming properties as agar; but unlike the latter, agarose gel shows practically no electroendosmosis. Both these methods require expensive reagents and are time-consuming. Another mode of separating polysaccharides is based on sedimenting agar by concentrated salt solutions and organic solvents[11]. AJITSKY AND KOBOZEV[9] fractionated commercial agar with ammonium sulphate. At 0.16 saturation, a dark-brown sediment, which

these authors believed to be agaropectin, precipitated from agar. By increasing the concentration of AS to 0.26, these authors succeeded in isolating the second fraction, agarose, from agar.

According to our data, sedimentation of polysaccharides from "Difco" bactagar starts only at 0.18 AS saturation. Agaropectin, as may be deduced from electroendosmosis (Fig. 1), sediments after agarose. This was to be expected because, on salting out polysaccharides with a component of higher molecular weight, agarose was first to precipitate. "Difco" bactagar differs from commercial agar in that the agarose isolated from it forms no large flakes at 0.33 AS saturation and that centrifugation is required to sediment it. It is important that the molten solutions should be treated with acetone to extract the yellow pigment.

As was mentioned above, in agarose obtained at 0.33 AS saturation, electroendosmosis is somewhat higher than in the French agarose preparation. However, no appreciable improvement in the electrophoretic properties of agarose was achieved when the precipitation range of Fraction I was limited to 0.24 AS saturation. On the other hand this procedure led to a twofold decrease in agarose yield. It should be noted that the concentration of AS required for agarose precipitation is determined experimentally for various kinds of agar[8] because of extensive differences in the content of agaropectin, which is the result of different methods of purification and various natural sources of agar.

The effectivity of separation of macromolecules in molecular sieves largely depends on the shape and size of granules. If the granules are spherical, the columns are packed more homogeneously, the buffer passes through at a greater rate and the substance in the columns becomes denser more slowly, which makes the columns fit for repeated use. On the other hand, the smaller the size of granules the less the void volume and the greater the total working surface of the granules are. Hence, when the separation of fractions of close molecular weight is required the smallest granules should be used[12].

The primary procedures of agar granulation described were based on mechanical grinding of gel in cold. But due to irregular shape of the particles, the column becomes dense very quickly and the flow rate of the buffer becomes very low. In 1964 HJERTÉN[4] and PHILIPSON AND BENGTSSON[6] independently suggested the method of emulgation for obtaining spherical agar granules. Under favourable conditions agar gel forms an emulsion of the water/oil type in non-polar organic solvents. According to HJERTÉN, a mixture of benzene, toluene and some polysaccharide emulsion stabilizer is added to a hot agarose solution; the solution is then stirred and gradually cooled in a high-speed mixer and spherical gel granules are formed. PHILIPSON AND BENGTSSON suggested that to obtain spherical granules the hot agar solution should be squeezed under pressure in the Zeitz filter through a needle into a mixture of water and ether. The latter procedure is more efficient as neither an expensive emulgator nor a complex mixer is required; the disadvantage of the method is that it is impossible to prepare small granules and to granulate agar at a concentration exceeding 5 % as at higher concentrations the agar congeals in the needles. In addition, any drops of cold ether on the point of the needle also cause agar to congeal in the needle. The second drawback of the method of PHILIPSON AND BENGTSSON is the condition that agar should be squeezed in small portions as the granules agglutinate the water–ether interface.

The system which has been suggested in this paper does not have the above

drawbacks. The sprayer with removable discs allows the preparation of granules of any required size at gel concentrations from 2 to 7 %. As the sprayer device is sufficiently large and the orifice is rather big, the spraying of 1 l of agar requires no more than 2 min, hence no cooling of the water–ether mixture is necessary and the gel does not congeal in the sprayer. Also in our device the pressure of nitrogen has less effect on the size of granules than does squeezing the solution through a needle. The large cylinder and constant stirring prevent the granules from agglutinating on the water–ether interface, thereby allowing large volumes of gel to be discharged into the mixture.

ACKNOWLEDGEMENTS

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CHROM. 4478

SEMI-AUTOMATIC CHROMATOGRAPHIC DETERMINATION OF NEUTRAL LIPIDS

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SUMMARY

A new type of detector for liquid chromatographic columns is described based on the continuous evaporation of the eluate and redissolution of residues in a fixed solvent that is fed to a recording diffractometer. Some of the limitations of the technique and its application to the quantitative determination of neutral lipids are described.

INTRODUCTION

Although liquid chromatographic techniques are essential in much biochemical research, particularly in the lipid field, there is still a dearth of automatic recording detectors suitable for such separations. The recording diffractometers so useful for separations using a single solvent are virtually useless when applied to systems involving a solvent change or gradient elution. We have therefore combined the principles of solvent evaporation described earlier as a basis for solute detectors[1] and that of continuously dissolving the residue in a single solvent that is passed to a recording diffractometer.

In this paper we describe an apparatus developed along these lines and its application to a particular lipid separation required in metabolic studies[2]. The instrument has the following advantages:

(1) It can detect and quantitatively determine as low as 10 to 20 μg of various lipids at one-half its full sensitivity under routine working conditions (see Table I).

(2) So long as silica gel chromatography and evaporation of solvent under an inert gas (N_2 or CO_2) do not alter the lipids under study, then the present form of the apparatus is suitable.

(3) After measurement of the concentration of the lipid, the total quantity is available for further study if this is desirable, *e.g.* for determination of carbon-14 content of a fraction.

GENERAL CONSIDERATIONS

In the system described here, the total eluate from a column is evaporated on a moving inert plastic tape (Mylar or Teflon tape, 1.375 in. wide). The residue is then dissolved in a suitable solvent which passes continuously through a recording refractometer (Type R4, manufactured by Waters Associates). The change in refractive index of the solvent is shown on a potentiometric recorder, the magnitude of change being a function of the concentration of the lipid.

Successful operation of the device is dependent upon: (1) making certain that the tape is clean, (2) having rapid and complete evaporation of eluates from the column, (3) preventing continuous redissolution of eluted lipid by succesive volumes of eluate, (4) ensuring that the solvent which redissolves the lipid is maintained at a constant level of purity (which specifically includes constant concentration of dissolved gas), (5) having a constant rate of flow of solvent on the tape followed by its complete removal, (6) having complete flow control of column eluate, and (7) having controlled tape speed.

OPERATION OF THE APPARATUS

The plastic tape moves through a continuously changing solvent bath in chamber A (Fig. 1) to be washed and then it proceeds over rollers through the gas lock (1) to chamber B. In this chamber, the tape is guided below the horizontal through a drying oven (7). The tape, now carrying eluate residues, emerges from the oven and is returned to the horizontal plane by a roller before leaving chamber B.

Immediately after entry into chamber B, the tape receives the eluate from the column (2). By crimping the tape into a trough by guides on the outside edges of the drying oven, the eluate is contained in the center of the tape. Nitrogen or other inert gas is forced (via 3) over a 50-W heater in the oven and through fine holes lining the sides of the base of the oven, so that the heated gas strikes the tape and is deflected towards the central pool of eluate. Two heating elements and gas streams at different temperatures are necessary for complete control of the solvent evaporation.

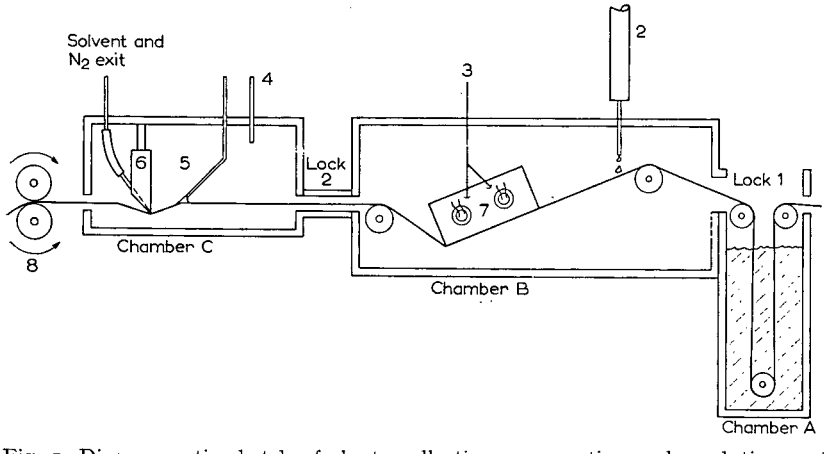

Fig. 1. Diagrammatic sketch of eluate collection, evaporation and resolution system. For key, see text.

Column eluate flows on to the tape in pulses. The pulsation coupled with the slope at which the tape is held prevents the following solvent from redissolving the residue from earlier portions of eluate. The solvent pool "buds off" under these conditions, producing discrete separate large drops which remain separate as they evaporate. Eluates which form crystalline residues show the residue as small circles after evaporation is complete.

An exhaust vacuum applied to the drying chamber removes the evaporated solvents.

For both collection of the material and observation of changes in refractive index, the tape moves into the redissolving chamber C through gas lock 2. Nitrogen saturated with the solvent used to redissolve the residue is continuously passed through C (via 4) to completely exclude air and to prevent loss of solvent in the redissolving phase and removal from the chamber.

The redissolving solvent flows to the tape via a Teflon applicator (5), 1 in. wide. This is at such a height above the tape that at the flow rate employed, the solvent forms a bridge between the applicator and the tape.

After passing under the applicator, the tape is inclined downwards to the solvent pick-up unit (6). This inclination is produced by having the pick-up unit press the tape down into a gap between two guide plates whose upper surfaces match the convex base of the pick-up unit. The pick-up unit pressing on the tape is a heavy metal section whose convex base is also cut and polished to a fine edge. At the lowest point of the base is an orifice (0.040 in.) that connects to the negative pressure produced by the pseudo-aspirator at the base of the condenser described later.

The negative pressure sucks the solvent, plus nitrogen, from the chamber up to a liquid–gas separator mounted above the refractometer. The separator unit is a glass Y-shaped tube which allows the solvent to flow from the bottom to the refractometer while the nitrogen passes to the aspirator. If the refractometer is not used, an appropriate vertical length of tubing is needed to permit the pick-up and Y tube combination to function and deliver samples to collection vials. Unless some negative liquid pressure is available at the base of such a liquid–gas separation unit, it cannot function. Flow rate of liquid from the Y tube is easily adjusted by maintaining the exit port at an appropriate level below the separator.

When the refractometer is employed, the ambient temperature in the Y tube must be greater than the internal temperature of the refractometer. Careful adjustment of the negative pressure determined by the level of the exit port is also necessary. Because the solvent contains nitrogen these adjustments must be properly made or small gas bubbles will form in the refractometer and render the instrument useless. Fortunately, gas bubbles are recognizable by pulses in the recording and so are easily differentiated.

TAPE DRIVE

The tape is drawn through the apparatus by a capstan (8, Fig. 1) having one metal and one rubber roller, driven by a synchronous motor via gearing to provide a range of speeds (minimum 1 in./min). The pressure exerted by the rollers of the capstan is sufficient to ensure that changes in tape resistance to movement will not cause changes in tape speed.

Cog tape drives, normally used for ensuring constant tape speed, were avoided because of the higher tape cost and increased difficulty in tape manipulation within the instrument. By using the cheaper unperforated tape, it is practical to use the tape only once.

In addition, using a tape only once is necessary when isotopic labelling is used, since failure to completely remove material containing label from the tape does not then cause any error in subsequent usage.

SOLVENT FLOW SYSTEM

The system consists of a still located at floor level. Vapor from the still rises through a partially insulated column to ceiling height and enters a condenser. The condensed liquid flows into a reservoir with two exits (1, Fig. 2)

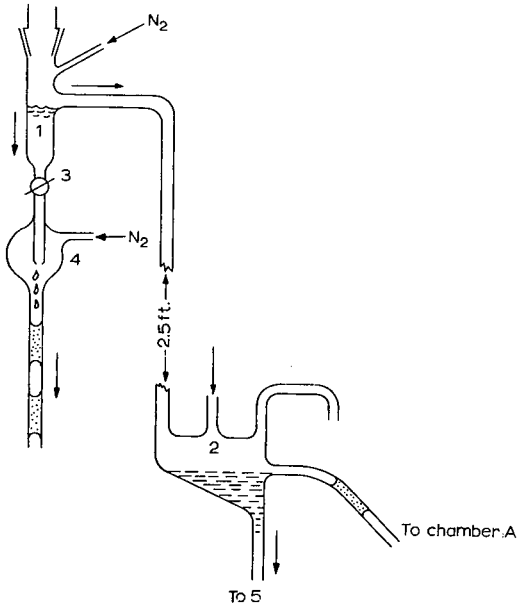

Fig. 2. Diagram of solvent system. For key, see text.

From the upper exit (an overflow), the solvent flows to a constant level reservoir (2) which feeds the applicator (5, Fig. 1). Adjustment of the height of this reservoir provides the desired flow rate to the tape. Overflow from reservoir 2 runs into the tape washing unit (Chamber A, Fig. 1). Overflow from here is returned to the still.

The second exit from reservoir 1 is fitted with a fine teflon stopcock to control flow (3, Fig. 2), the condensate then passes into the aspirator (4). The flow from the stopcock is set at a level such that discrete slugs of liquid form in the exit tube and draw nitrogen in through the side tube. The negative pressure so obtained is used to "lift" the redissolving solvent from the tape.

This simple system for producing constant flow and constant negative pressure was found satisfactory for providing the absolute constancy of flow needed. It has

additional advantages which are due to the use of the still as the source of driving power. First, constant distillation of the redissolving solvent through a fractionation column assures a minimum of change in the redissolving solvent. Secondly, the adjustments that control flow rates are working against gravity. Therefore, once set and locked they can be expected to remain constant; only changes in flow resistance can then alter flow rates. By adjusting the distillation rate to exceed flow through the aspirator and the redissolving unit, *only* the amount of fluid entering the washing chamber is subject to change as a function of room temperature and/or main voltage changes which may affect distillation rates.

It should be noted, however, that temperature of the redissolving solvent affects its viscosity. Thus its flow rate through a fixed resistance at a constant pressure head will change. Ambient temperature changes around these lines must therefore either be controlled or flow rate measured as part of the calibration procedure.

The flow rate of the redissolving solvent is in the range 0.6 to 0.8 ml/min. Since the refractometer is measuring concentration, fluctuations in rate of more than 1 % or 0.0006 to 0.0008 ml are undesirable during the analysis. A change in flow rate could be reflected as a significant change in calibration value. Equally important—changing flow rates cause much "noise" and poor baselines in the recordings. No inexpensive mechanical pumps have been found capable of this degree of constancy at such low flow rates.

The importance of flow rate constancy becomes apparent when the manner of operation of the refractometer is considered. The instrument is best described as a differential refractometer in the sense that it reads the difference in refractive index (at a sensitivity of 10^{-6}) between the refractive index of fluid in the reference cell and the sample cell. If the solvents in the two chambers are maintained constant, a zero reading (arbitrarily set on the recorder scale) results.

The refractive index of a liquid is altered, at *constant* temperature, by any impurity, *e.g.* dissolved gases, liquids, or solids. The change of refractive index is a function of (a) the concentration of impurity, and (b) the refractive index of the dissolved impurity.

Thus a change in flow rate of the redissolving solvent during the period in which lipid is being redissolved will change the calibration.

OPERATION OF THE COLUMN

The column used consists of a glass tube 46 cm long, 6 mm I.D., containing 4 g of silica gel. The base of the column is fitted with an ultrafine Teflon stopcock, the volume between the sintered glass filter at the column base and the stopcock being 0.02 ml. The column was kept at constant temperature by passing water at 19° through a concentric jacket. The top of the column terminated in a standard taper (10/30) male joint, below which was fitted a Teflon collar. The solvent line was connected via a Teflon cylinder into whose base was cut a female standard taper. This cylinder was secured to the column top by adjustable connection to the teflon collar. The cylinder was bored out to a diameter of 0.040 in. which connected with two ports. The stainless-steel solvent line from the column pump was fitted to one of these ports and a small needle valve to the other. In this way, any gas at the top of the column could be extruded through the valve by the solvent.

ห้องสมุด กรมวิทยาศาสตร์

Into the solvent line either a manifold fed by several pumps, or a mixing chamber, were connected so that both gradient elution or discrete changes of solvent could be used.

The glass tubing leading from the base of the Teflon stop-cock at the bottom of the column was drawn to a fine point. Eluate dropped into a narrow short funnel (9 mm I.D. at top, capillary at base). The funnel fitted into a 2 cm length of teflon tubing which in turn was connected to the stainless-steel (I.D. 0.040 in., O.D. 0.0655 in.) tubing which directed the eluate to the tape. The base of the tubing was held about 5 mm from the tape.

Such a multi-diameter line, in which a portion is capillary tubing, will retain fluid flowing through it until the fluid seeping into the larger diameter tubing below the capillary exerts sufficient negative pressure to pull air through the capillary base of the funnel. This is true provided, of course, that the flow rate into the funnel is not too great to prevent a break. By this simple means, a pulsating flow rate can be developed from the constant flow rate of eluate issuing from the base of the column.

Solvent pumps of various types have been employed. To date the most satisfactory in our hands have been the constant infusion pumps manufactured by the Harvard Apparatus Company. These are no more than glass syringes driven by constant-speed drives and serve well for solvents other than ethyl ether when high-resistance columns are used; for low-resistance columns they are satisfactory even with ether. When the higher pressures necessary for high-resistance columns are used, then syringes with Teflon plungers must be utilised, otherwise the low viscosity of lipid solvents will permit considerable leakage between the piston and cylinder walls.

Only Teflon, glass and stainless steel, can be used in these pumping systems. Plastics other than Teflon will continuously leach plasticizers into the solvents even after months of use.

The standard squeeze bottle, unless made of Teflon, cannot be used to store solvents employed in making lipid extracts to be analysed with this system. Metals other than stainless steel appear to release oil into the solvents; the oil apparently penetrates the metal during the machining operations and leaches out slowly.

PROBLEMS POSED BY ELUTION SOLVENTS

Because the detection system employed is sensitive to any compound soluble in the redissolving solvent, the minimum of non-volatile residue in the eluting solvents is critical; even hexane requires passage through large silicic acid columns followed by distillation.

As a purity test, 10 ml of hexane were evaporated on to a small area of the tape; when no signal was obtained, the hexane was considered sufficiently pure. Ether and methanol of analytical reagent grade usually passed this test at the first stage of the development of the system without further purification. Later a more dependable methanol ("Nanograde" provided by Mallinckrodt) has proven satisfactory with limitations (see below).

There are several escapes from the dilemma of working in a range where the solvents employed can, by the limits of purity they possess, interfere with the observations the analyst is attempting. These are: (1) blank runs, (2) solvent purification and (3) working in a restricted range.

The blank run approach is relatively simple but requires precision of operation and careful preplanning so as to minimise non-productive runs. In Fig. 4 is presented a recording from a blank run. It can be seen that there are no significant peaks recorded until methanol was used in the eluant sequence.

In this run, had the tape been stopped as each eluant came from the column, a small peak would have been seen. But since this peak, with these particular solvents, would not have exceeded 0.02 sq. in., it became invisible within the baseline variations when stretched out over the 15 min of elution and evaporation. The methanol in the eluate, however, appears to extract from the column that portion of impurity in the prior eluates which is absorbed by the column.

Successful application of the blank run principle requires, therefore, (a) precision operation (b) operation of a suitable column in a fashion which (1) minimises the solvent needed, and (2) moves the desired fraction off the column in as sharply defined fashion as possible.

It is not usually necessary to use a column capable of separating the components of 5 mg of assorted lipids when observations at the 10 to 50 μg level are to be made. This was the case in the work on which this description is based. A reduced column size reduces the solvent volume proportionately. At appropriate levels of choice of column and solvent volume, the level of impurity in the solvents employed in our studies would not interfere in the accuracy of determining 10 to 50 μg of lipid.

COLUMN COMPOSITION AND USE

With some grades of silica gel, regeneration of the column is possible, a suitable grade is "ultra pure" silica gel (0.05–0.2 mm) (Brinkman Instrument Company). Regeneration is performed by passing three column volumes of methanol, followed by a similar volume of ethyl ether through the column. Refilling the column with hexane or 5 % ethyl ether in hexane completes the procedure.

With continued use of the overall system, it has been found necessary to develop criteria for (a) properly constructed columns and (b) the continued ability of a column to function. It is not yet certain that the following criteria will prove permanently satisfactory. As of this date, however, they have resolved several situations and seemingly prevented potential difficulty. They are:

(1) When a column is constructed, several blank runs are made. Unless the column conforms (see Fig. 4) to expected blank run standards, it is discarded. If it conforms, frequent later runs are made to assure that the blank run values are maintained.

(2) An artificial mixture of known compounds (in our case sterol ester and free sterol) is placed on the column. If the results of this test conform to the expected values, the column is ready for use on unknown mixtures.

(3) A column use history is maintained. Failure to conform to expectations on test runs terminates use of the column. Several columns have functioned well through 50 determinations.

CHOICE OF "REDISSOLVING SOLVENT"

The sensitivity of the refractometer assay system is at a maximum when the solvent used has a refractive index far removed from that of the solutes. The most

suitable such solvent from the refractive index aspect for most blood and tissue lipids is methanol. Unfortunately, however, triglycerides are poorly soluble in methanol. The best compromise of both refractive index, solubility etc. is ethanol. A composite solvent such as chloroform–methanol cannot be employed if a still is used for solvent distribution and re-use. "Constant boiling" mixtures prove difficult because changes in barometric pressure alter the output of the still.

Test system

Because of our biological interests, development of the overall system was limited to accurate separation and measurement of sterol esters, triglycerides, free sterols and free fatty acids as discrete *classes*.

The elution sequence employed in the first evaluation of the analytical system was as follows:

(1) 5 % ethyl ether in hexane was used to fill the column after regeneration and to dissolve the lipid samples applied to the column.

(2) After application of the lipids to the column, 5 % ether in hexane was passed through for 10 min at the rate of 1 ml/min.

(3) Continuous gradient elution was then begun by adding 100 % ether to the 90-ml volume of 5 % ether in hexane in the mixing chamber at a constant rate.

(4) When the peak corresponding to the free sterols was seen, gradient elution was stopped and 100 % methanol was put through the column at a lower rate. The resulting peak would be expected to contain free fatty acids, residual phospholipids and possibly other very polar lipids. This fraction in the first stage of development was used only to prove recovery of lipids by tracer analysis.

The material present in each peak was determined by adding known quantities of specific substances to the standard extract described below. Defined quantities of each material had previously been deposited directly on the tape so that detector sensitivity could be predicted.

By finding a specific peak increased by the expected area, it was demonstrated (a) that the peak contained the substance and (b) that the added material was totally eluted from the column. Then by maintaining constant all volumes of solvent and the rates of change of solvent in the gradient elution, it became possible to predict within 2 to 3 min when a peak could be expected to appear. Sterol peaks were also identified by the Liebermann–Burchard reaction.

As a reference standard of biological mixture of lipids instead of a synthetic mixture, 50 ml of human plasma which had been deep frozen were extracted with chloroform–methanol (2:1). The extract was evaporated to an oil which was then re-extracted with chloroform. The chloroform was reduced in volume and dry acetone added. The mixture was evaporated to small volume and acetone again added. This procedure was repeated three times. The final volume reduction gave an oil.

The oil was then extracted with dry acetone. To the acetone extract, $MgCl_2$ was added to precipitate phospholipids. The concentration employed was 0.5 ml of a 95 % ethanol solution (50 g $MgCl_2$ per 100 ml) per 10 ml of acetone. The dissolved phospholipids were permitted to precipitate overnight at 4°. Removal of the phospholipids was completed by filtration through a sintered glass filter covered by a 2-cm-thick layer of ground filter paper. The final product was dissolved in 5 % ether in hexane. Although this procedure can be quantitative, no effort was made in

this preparation to recover all of the desired lipids available in the sample. The prime concern was exclusion of the phospholipids because of their interference with neutral lipid separation.

Aliquots of the extract ranging from an equivalent of 0.015 ml of plasma to 0.125 were assayed with the elution sequence described above. The calibration curves resulting for the sterol esters, triglycerides and free sterol fractions are presented in Fig. 3. The free fatty acid levels were not observed because (a) they were low and (b) at this stage of development of the procedure the blank methanol fraction showed erratic peaks due to solvent contaminants.

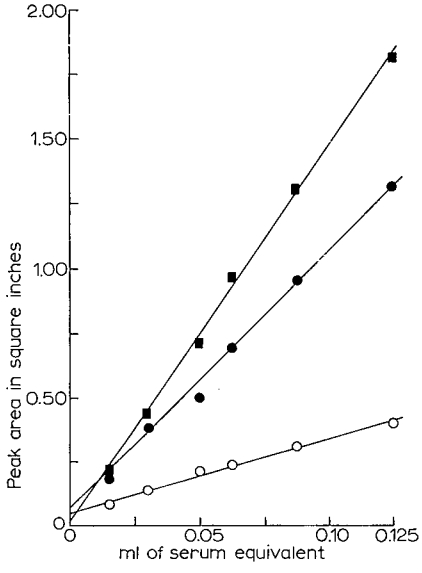

Fig. 3. The relationship between the area of the peak in square inches and the quantity of serum equivalent of the extract described in the text. Solid squares represent triglycerides; solid circles represent sterol esters; open circles represent free sterol.

As Fig. 3 shows, the device showed a linear response for each of the three lipid classes. However, only the triglyceride line passed through the origin. Computation of linearity by the method of least squares gave intercepts of 0.038, 0.050, and 0.010 sq. in. for the sterol ester, free sterol and triglyceride lines, respectively.

Theoretically, each of these lines should pass through O. A blank run through a column to which no extract has been added showed no peaks at the expected times on the recording.

Explanations of the observed discrepancy *at the time* were (a) that the solvents were *not* completely free of residue and (b) that when a lipid class moved down the column it carried with it an accumulation of residue that was not moved in similar fashion by the solvents alone.

Another possibility could have been the incomplete removal of solvent from the lipids. Retention of solvent, if it is constant and directly related to the amount of lipid deposited on the tape, would fit the observations. It was, however, difficult to accept this possibility. Neither rate of evaporation of samples of lipids nor the nature of solvent employed to deposit a particular sample on the tape was found to influence

the resulting signal. Had retained solvent contributed to the size of the signal, then deposition of a sterol ester sample on the tape dissolved in methanol should have produced a markedly different peak from the same amount of ester when it was deposited in a chloroform solution. Checks of this sort were applied early in development of the system and found to give the desired result: to wit, constant area per unit of lipid irrespective of solvent employed.

It was not until the refractometer was improved and a different system of elution was employed that it became clear that solvent impurity was the primary source of the difficulty. This is discussed below.

As one part of the original calibration of the system, sterol esters, triglycerides (as olive oil) and cholesterol were added to serum aliquots. The area obtained for each peak was corrected by subtraction of the area expected for the serum aliquot alone.

It was found in the initial study that with an ethanol flow of 0.78 ml/min, with the original refractometer at one-half its full sensitivity, that 0.02 sq. in. peaks were easily seen. In Table I are presented the final calibration values for that stage of development.

TABLE I

MICROGRAMS PER SQUARE INCH OF PEAK OF THREE LIPID CLASSES

Sterol ester	268 μg
Triglyceride	390 μg
Free cholesterol	206 μg

TABLE II

AMOUNT OF LIPID IN THE SERUM EXTRACT

	According to Dr. Kritchevsky (*mg/100 ml*)	*New system* (*mg/100 ml*)
Sterol esters	156	161
Triglycerides	595	579
Free cholesterol	51	58

Through the kindness of Dr. DAVID KRITCHEVSKY, an independent analysis of the three major classes of lipids in the serum extract was obtained. The sterol ester and free sterol areas obtained by use of the new system were corrected, for this comparison, by the value of the area at the intercept; the triglyceride value was not corrected. Comparative results are shown in Table II.

The values for the sterol esters and triglycerides lie within the probable error of both techniques. The free cholesterol values are significantly different and illustrate the inherent problem of any assay system of the type being described. The higher free cholesterol value obtained by using the refractive index method is undoubtedly due to the well known fact that other substances besides cholesterol are eluted by the solvent mixture which elutes cholesterol from a column of this type.

The completion of this series of studies satisfied us as to the usefulness of the

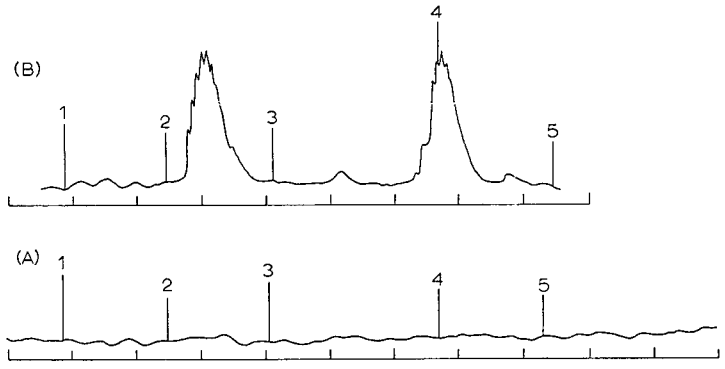

Fig. 4. Presented in this figure are tracing of column calibrating experiments done at maximal sensitivity. (A) represents a blank run and illustrates the peak seen when methanol strips the column of residue deposited by the earlier eluants. The vertical lines at the base of each tracing represent 10-minute intervals. The vertical, numbered lines represent the onset of pumping the following eluants through the column: (1) 12.0 ml of 5% ethyl ether in hexane; (2) 12.0 ml of 20% ethyl ether in hexane; (3) 20.0 ml of 50% ethyl ether in hexane; (4) 12.0 ml of 50% ethyl ether in methanol; (5) 12.0 ml of methanol. All eluants, except methanol, were pumped at 0.84 ml/min. Methanol was pumped at 0.4 ml/min. (B) 97.2 μg of cholesterol and 114 μg of cholesterol linoleate were placed on the column. The expected peaks were 1.13 and 0.98 sq. in., respectively. The areas found were 1.02 and 0.104. The column was regenerated by passing through 30 ml of methanol, 30 ml of ethyl ether and 15 ml of 5% ether in hexane. The column volume was 9 ml.

TABLE III

REPRESENTATIVE CALIBRATION VALUES DETERMINED ON NEW INSTRUMENT EMPLOYING PLANIMETER ACCURATE TO 0.01 sq. in.

The varying concentrations of purified cholesterol (via dibromination) and cholesterol linoleate (synthetic) were placed on the tape through the same funnel system used for column eluates. A wash of 0.3 ml of 5% ether in hexane was used to ensure complete deposit of standard on the tape. Redissolving solvent flow rate was 0.714 ml/min. Instrument sensitivity was maximal.

Micrograms	*Square inches*	*Micrograms per square inch*
Cholesterol determinations		
97.2	1.163	83.6
121.5	1.463	83.0
145.8	1.673	87.1
48.6	0.550	88.4
24.3	0.293	82.9
97.2	1.093	88.9
Calibration value = 85 ± 2.8 μg/sq. in.		
Cholesterol linoleate determination		
28.5	0.250	114.0
57.0	0.500	114.0
85.5	0.763	112.1
114.0	0.890	128.1
142.5	1.190	119.7
171.0	1.520	112.5
Calibration value = 116.7 ± 6.2 μg/sq. in.		

overall system and now a more sensitive refractometer has been obtained which provides a more stable baseline. A typical run is shown in Fig. 4. Calibration values are listed in Table III.

ACKNOWLEDGEMENT

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CHROM. 4469

THE LIQUID SPECTRORADIOCHROMATOGRAPH—AN AUTOMATED METHOD FOR THE SIMULTANEOUS SEPARATION, IDENTIFICATION, PURIFICATION, ESTIMATION AND RADIOASSAY OF THE COMPONENTS OF A MIXTURE OF UNSAPONIFIABLE LIPIDS

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SUMMARY

An automated system which we have termed the Liquid Spectroradiochromatograph, capable of monitoring the effluent from a liquid chromatography column for solute concentration, radioactivity and ultraviolet and visible light absorption, is described. Its usefulness in the analysis of mixtures of unsaponifiable lipids is discussed.

INTRODUCTION

Many studies on the biosynthesis of unsaponifiable lipids in plants, animals or bacteria involve feeding a radioactively labelled precursor (*e.g.*, [^{14}C]acetate or [^{14}C]-mevalonate) to the organism or enzyme system, and the subsequent extraction of the lipids and isolation of the unsaponifiable material. Separation of the unsaponifiable lipids is usually accomplished by column chromatography using stepwise elution and the arbitrary fractions, each of which may contain several components, are further resolved and analysed qualitatively to determine the nature of the components and quantitatively to determine the mass and radioactivity of these components[1,2]. As the chromatographic separations and subsequent analyses are time-consuming and often tedious, the construction of automated equipment capable of carrying out these procedures rapidly and reliably would be of considerable advantage to workers in this field.

This paper describes such a method; after the components of a lipid mixture have been separated by automated gradient elution chromatography, they are automatically assayed for UV or visible light absorption, radioactivity and mass before they are collected separately in an automatic fraction collector. The entire equipment is shown in Fig. 1.

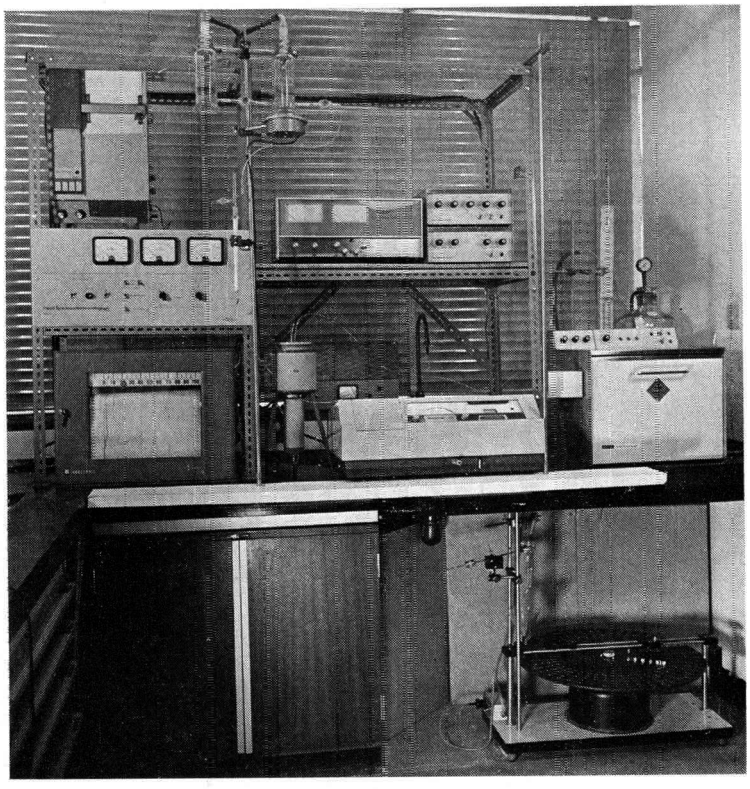

Fig. 1. Overall view of the instruments.

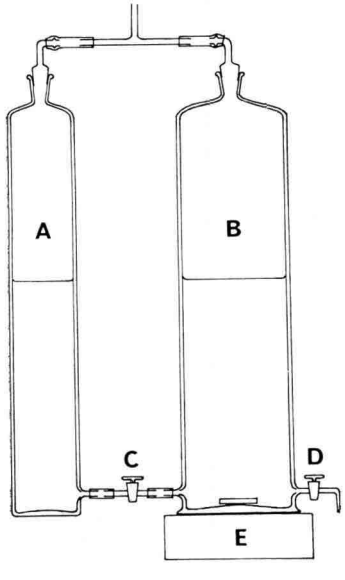

Fig. 2. Construction of the gradient elution apparatus. For key, see text.

MATERIALS AND METHODS

Gradient elution apparatus

The apparatus (Fig. 2) is based on those of PARR[3] and BOCK AND LING[4]. It consists of a reservoir (A) attached to a mixing chamber (B). The mixing chamber (B) consists of a cylindrical glass vessel (5 cm I.D.; 500 ml volume) fitted with a B24 Quickfit ground-glass socket and two side arms sited diametrically opposite to each other 1 cm from its base. Each side arm is fitted with a ground-glass tap, one of which (C) is connected by means of a short piece of nylon tubing [3.5 mm bore, Baird & Tatlock (London) Ltd.] to the side arm of the reservoir (A), the other (D) being connected through a length of nylon tubing (1.9 mm bore) to the sample injection device. The reservoir (A) is a cylindrical glass vessel fitted with a B24 Quickfit ground-glass socket and with a short glass side arm positioned 1 cm from its base. Four such vessels with I.D. of 5, 4, 3 and 2.5 cm, respectively, are available for use as the reservoir, enabling four different gradients to be obtained (see below).

During use, the reservoir (A) contains the polar solvent (*e.g.*, diethyl ether) and the mixing chamber (B) the non-polar solvent (*e.g.*, light petroleum, b.p. 40–60°) used in the filling of the chromatography column. At the beginning of the chromatographic analysis, the levels of the solvents in the two chambers are normally equal and the taps (C and D) are opened simultaneously. Should the particular separation require it, however, the level of the solvent in the mixing chamber could be higher than that in the reservoir. In this case, the chromatogram is started by opening the tap (D) and allowing the non-polar solvent to pass through the column until the level in the mixing chamber falls to that in the reservoir when the other tap (C) is opened and the gradient commenced.

Mixing of the two solvents is accomplished by a magnetic stirrer (E) positioned underneath the mixing vessel. Care is taken not to stir too vigorously so as to avoid vortex formation; this would raise the effective level of solvent in the mixing chamber and upset the shape of the gradient. The ground-glass sockets of both vessels are fitted with B24 adaptors connected by rubber tubing to a glass T-piece which is in turn similarly connected to a 20-l aspirator bottle. This bottle is fitted with a mercury manometer and the air pressure inside can be raised by means of a bicycle pump. This simple device enables a constant head of pressure to be maintained throughout the chromatography on the solvent passing through the column; the flow rate is therefore constant.

When the 5 cm I.D. reservoir (A) is used in conjunction with the mixing chamber (B), a linear gradient is obtained. The concentration (C_v) of the polar solvent in the solvent mixture leaving the mixing chamber after a volume (V) has flowed through the apparatus is given by the equation:

$$C_v = C_2 + (C_1 - C_2)\frac{V}{V_{\text{total}}}$$

where C_1 is the concentration of the polar solvent in the reservoir (A), C_2 is the concentration of the polar solvent initially present in the mixing chamber and V_{total} is the total volume of liquid in both vessels[5].

When the reservoir vessel (A) has an I.D. of 2.5, 3 or 4 cm, a concave expo-

nential gradient is obtained, the shape of which can be calculated by the equation:

$$C_v = C_1 - (C_1 - C_2)\left(1 - \frac{V}{V_{\text{total}}}\right)^P$$

where V, V_{total}, C_v, C_1 and C_2 have the above meanings and P is the ratio of the cross-sectional area of the reservoir (A) to that of the mixing chamber (B).

Sample injection device

The sample injection device (Fig. 3) consists of a piece of 0.2-in. bore glass tubing ending in a Bio Quickfit cone; the overall length is 3.5 in. Two side arms of the same gauge tubing are attached, diametrically opposite to one another, half an inch above the cone. One is fitted with a ground-glass tap (F) and is bent downwards at a right angle. The other side arm has an upward right-angle bend and is tapered to fit tightly into the 1.9-mm bore nylon tubing bringing solvent from the gradient elution apparatus. A rubber injection septum (G), such as is used in glass gas–liquid chromatography columns, is firmly plugged into the end of the tubing which is opposite to the cone. The ground-glass cone fits into the Bio Quickfit socket of the chromatography column. The injection device is held securely to the column by passing elastic bands over its side arms and attaching them to the hooks (H) on the side of the column.

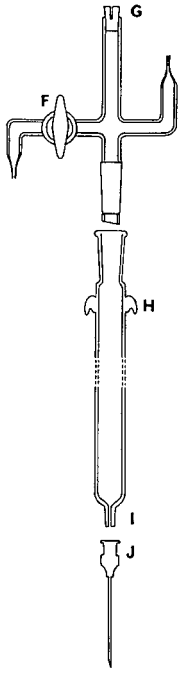

Fig. 3. Sample injection device and column assembly. For key, see text.

Chromatography column

Several glass chromatography columns have been made for use with this apparatus. Each has a Bio Quickfit ground-glass socket at one end to receive the sample

injection device and a cone (I) specially ground to connect with a Luer fitting hypodermic needle (J) at the other. In some cases, this ground cone is replaced by a 1-cm length of 3-mm bore nylon tubing fitted to the end of the column, which has been narrowed by pulling out in a flame; this size of nylon tubing fits perfectly into the Luer socket of the hypodermic needle. The columns differ from one another in respect of their I.D. (0.5 cm to 1.0 cm) and lengths (10 cm to 30 cm). The 1.5-in. hypodermic needles are of stainless steel (G19, Philip Harris and Co., Birmingham, England). They fit tightly into the 1-mm bore flexible nylon tubing [Baird & Tatlock (London) Ltd.] used to interconnect the column effluent monitoring devices. This tubing is sufficiently narrow to minimise the delay time between a solute arriving at the different monitoring stages (volume of tube = 0.785 ml/m).

Column filling and sample injection

The chromatography column is filled with adsorbent, irrespective of the column length, to within 2 cm of the bottom edge of the ground-glass socket. The remaining 2 cm of column space are filled with anhydrous sodium sulphate. Thus when the sample injection device is fitted, the only dead space filled with solvent is the volume of the Quickfit joint plus that of the 0.5 in. of tube up to the side arms of the injection device, a total volume of 2 ml. Developing solvent passes from the gradient elution apparatus through the 1.9-mm bore nylon tubing to the tapless side arm of the injection device. Should the level of solvent rise too high in the main tube of the injection device, the excess can be run off through the other side arm by opening the tap (F); this is necessary only during the initial setting-up of the apparatus.

The mixture to be chromatographed is injected on to the column once the flow rate of solvent from the gradient elution apparatus through the column is constant and the effluent monitoring devices are stabilised and set to register zero response. The sample is dissolved in the minimal volume of the non-polar solvent, present initially in the mixing chamber of the gradient elution apparatus. This solution is taken into a 2-ml graduated glass syringe fitted with a 6-in. stainless-steel hypodermic needle. The needle is pushed through the rubber septum (G) and into the layer of anhydrous sodium sulphate which diffuses the sample over the whole cross-sectional area of the column. The plunger of the syringe is slowly depressed to deliver a known aliquot of the sample (usually 1 ml) and the needle is then withdrawn.

Detection of light-absorbing solutes

On emerging from the column, the eluate passes in turn via 1-mm bore nylon tubing to a number of monitoring devices (Fig. 4). The first of these is a Beckman DB recording spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife, Scotland). This can be set at any wavelength within the range 205–770 nm and will continuously monitor the transmittance of the eluate at this wavelength throughout a chromatographic separation, recording the trace on the Honeywell multi-channel recorder. Alternatively, the spectrophotometer can be used to monitor the absorption spectra of eluted solutes, its output being fed, in this instance, to the Beckman potentiometric strip-chart recorder which is fitted with a Beckman scale expansion accessory, enabling the instrument to be used for solutions of low concentration.

The eluate from the chromatography column is fed by 1-mm bore nylon tubing into a flow cell mounted in the sample compartment of the spectrophotometer. The

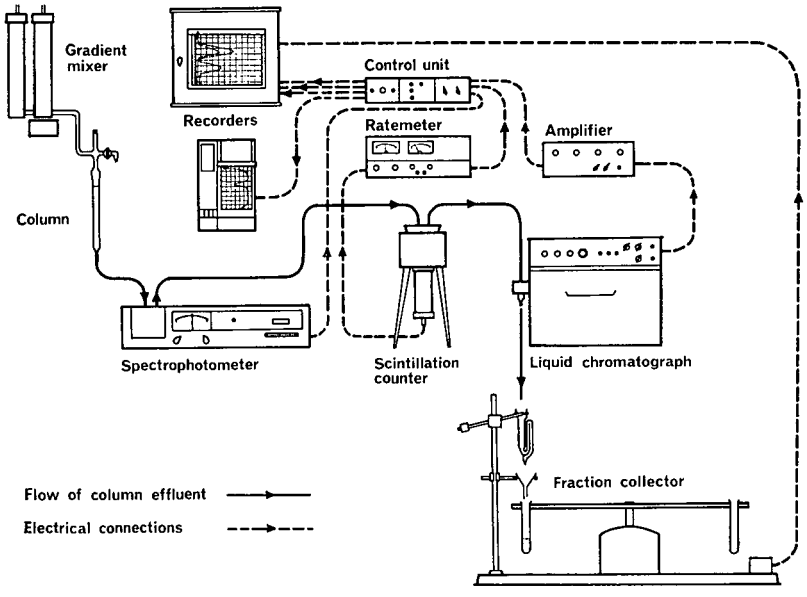

Fig. 4. Arrangement of the instrumentation, showing the flow of solvent and the electrical connections.

reference compartment contains a 0.5-cm blank cell containing the appropriate solvent (*e.g.*, light petroleum). The sample flow cell is constructed from two plates of spectroscopic grade silica held apart by a metal spacer machined to give a 0.5-cm light path through the sample. The total volume of the cell, defined by the size of the cavity through the spacer, is 0.25 ml. The inlet and outlet ports, at the bottom and top of the cell respectively, consist of lengths of stainless-steel tubing cut from G19 1.5 in. hypodermic needles brazed into drilled holes in the metal spacer. These connect with the 1-mm bore nylon tubing through which the eluate enters and leaves the spectrophotometer, passing through light-tight holes drilled in the hinged lid of the sample compartment. The silica–metal joints of the flow cell are solvent tight under the small positive pressures used, and are maintained with the epoxy resin 'Araldite' [CIBA (A.R.L.) Ltd., Duxford, Cambridge, England]. This flow cell has recently been replaced by a 0.5-cm path length Chandos Type 42 silica flow cell (Chandos Intercontinental Ltd., Stockport, Cheshire, England).

Radioassay of components in eluate

After leaving the spectrophotometer, the eluate from the column is monitored for radioactivity by passing it through an NE 808 flow cell (Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland) positioned against the face of an I.D.L. scintillation counter [type 663, Isotope Developments Ltd., Beenham, Reading, Berks., England (now Nuclear Enterprises Ltd.)] by means of a special mounting assembly supplied by Nuclear Enterprises Ltd. Good contact between the face of the flow cell and that of the photomultiplier of the scintillation counter is maintained by a thin film of Nujol. The whole assembly is mounted in a lead shielding unit (type 722, Isotope Developments Ltd.). The output from the scintillation counter is fed into

a transistorised ratemeter and high-voltage unit (type 7070, Isotope Developments Ltd.); this unit also supplies the high voltage (1400 V) to the scintillation counter. The output (linear or logarithmic) from the ratemeter is fed into one of the channels of the Honeywell recorder.

The flow cell consists of a flat spiral glass tube containing europium-activated calcium fluoride crystals. It has a capacity of approximately 0.5 ml and can be used for the continuous monitoring of β-emitters either in organic liquids or vapours at temperatures of up to 150° if required. The makers quote figures of 1.55 % counting efficiency for ^{3}H with a background of 3.0 c.p.s. and 16.6 % efficiency for ^{14}C with a background of 1.34 c.p.s.; our results bear out these figures. The inlet and outlet ports are slightly modified in order that they can be connected to the standard 1-mm bore nylon tubing used to carry the column eluate. The pieces of glass tubing constituting these ports are reduced in length to approximately 1 cm and the Luer fitting of a G19 1.5 in. stainless-steel hypodermic needle is fitted over each and bonded with Araldite. The length of each needle is reduced to 1 cm prior to fitting so that the modified flow cell fits into the mounting assembly. Suitable lengths of 1-mm bore nylon tubing are firmly pushed over each stainless-steel tube and led out of the mounting assembly and surrounding lead shield through light-tight exit ports in order to carry the column eluate flow to and from the scintillation counter.

Detection of organic compounds in eluate

Organic compounds in the column eluate are detected by means of a Pye liquid chromatograph (Series 1, W.G. Pye and Co. Ltd., Cambridge, England), which also gives an idea of their relative quantities.

In this instrument, the column eluate is led into a very narrow stainless-steel tube whose end is bevelled. A high-precision stainless-steel wire moves at constant speed past the end of the tube, within the bevel, and is continuously coated with a very thin film (approximately 10 μl over 4.5 cm of wire) of the column eluate. The vast majority of the eluate passes on and is collected by the fraction collector. After being coated, the wire passes through an evaporator oven which is held at a temperature slightly higher than that of the boiling point of the chromatography solvent and which is swept by argon gas; the solvent is removed, leaving any solute adhering to the wire. The wire travels into a pyrolyser oven set, usually, at 700°. Here the organic solute on the wire is pyrolysed and the volatile pyrolysis products are swept by a stream of argon (30 ml/min) into an argon ionisation detector. The resulting signal is amplified and the output from the detector amplifier is fed to the multi-channel Honeywell recorder.

The performance of the system depends to a considerable extent upon the cleanliness of the moving wire. Cleaning the wire is effected by passing the wire through a cleaner oven also set at 700° and swept by a stream of argon (40 ml/min) before it is coated with chromatographic eluate.

Collection of fractions

After the eluate has passed through the liquid chromatograph, and a small sample has been removed by the moving wire, the remainder passes downwards through a metal funnel fitted with 1.9-mm bore flexible nylon tubing. An increase

in the tube diameter is made at this point because the pressure head established at the top of the chromatography column is lost at the wire-coating stage and bubbles of air tend to be introduced. The flow of the eluate is much improved by using the wider bore tube.

The fraction collector (Beaumaris Instrument Co., Beaumaris, North Wales) holds 150 15-ml tubes in three concentric rows, and the sample tube changer is controlled by the syphon balance arm method, the syphon having a capacity, and therefore the fraction a volume, of 3 ml. This method of control is preferred to that of drop-counting because the fraction volumes are constant irrespective of the nature of the eluting solvent. Every time the syphon empties and the fraction is changed, a microswitch is closed to complete the circuit of the event marker pen in the Honeywell recorder, thus giving a permanent record of the fraction changes.

Recorders and control assembly

Two recorders are used as part of the instrumentation, a Honeywell ElectroniK 15 multi-point strip chart recorder (4-channel; Honeywell Controls Ltd., Newhouse, Lanarkshire, Scotland) and a Beckman 100504 U linear potentiometric strip chart recorder fitted with a Beckman S151060 scale expansion accessory (both from Beckman Instruments Ltd., Glenrothes, Fife, Scotland).

The Honeywell recorder is a continuous balance potentiometer which measures and records the magnitude of a number of process variables. In this case, three process variables are fed into the four channels of the recorder, the spectrophotometer output into channel 4, the scintillation counter ratemeter output into channel 2 and the liquid chromatograph argon ionisation amplifier output into both channels 1 and 3. Each separate channel has a print-out cycle of 10 sec and each record is printed on the chart paper as a series of dots, a different colour being used for each channel. The chart speed used is 12 in./h. The operation pen (event marker) of the recorder is connected to the event marking output of the fraction collector and thus records the fraction changes.

The Beckman recorder, a continuous pen recording instrument, is included for use in following parameter changes which may be too fast for the multi-point recorder. It can, of course, be used for only one channel at a time, but the control unit has switches to connect it to any of the three instrument outputs. Its most useful function is for the rapid monitoring of the absorption spectrum of an eluted fraction. When the whole of the instrumentation is not in use, this recorder can still be used with the spectrophotometer for normal laboratory purposes. Indeed, any of the individual instruments may be used independently of the others as the liquid flow system is so simple and versatile.

In addition to switching the various instrument outputs to the appropriate recorder, the control unit also has an important function as a voltage attenuator. This facility is necessary because while the 0–10 mV output from the liquid chromatograph argon ionisation amplifier is matched to the -1 to $+10$ mV Honeywell recorder input, the outputs from the scintillation counter ratemeter and the spectrophotometer are both of the order of 0–100 mV. The attenuators used for these two channels, each consisting of a simple resistor network, nominally reduce each of the voltages to one tenth of their value, but are variable by turret switches and further resistors to give 2, 5 and 10 times effective "amplification" of the reduced signals.

This attenuation is not necessary for the Beckman recorder as it accepts an input voltage of 0–100 mV. It is necessary, however, to use the 10 × sensitivity setting on the scale expansion accessory when monitoring the output from the liquid chromatograph argon ionisation amplifier with this recorder. The control unit also has three meters (Fig. 1) which continuously display the responses of the spectrophotometer, scintillation counter and liquid chromatograph, respectively.

An additional function of the control unit is to reverse the polarity of the input from the spectrophotometer to the Honeywell recorder as the former measures transmittance rather than absorbance. This signal, now having a negative value, is brought back within the range of the recorder by a backing voltage supplied by a mercury battery fitted with a continuously variable attenuator. This variable resistor is used as a "set zero" control for the spectrophotometer record. The linear recorder now records the percentage of light absorbed by the eluate in the spectrophotometer flow cell, and its extinction and hence the concentration of a known solute may be calculated from this.

RESULTS AND DISCUSSION

While the equipment has been used with considerable success in studies on unsaponifiable extracts from several natural sources, its performance can best be assessed when using a standard mixture of defined components. Thus the apparatus has been used to separate and assay the isoprenoid compounds in a mixture containing per millilitre of light petroleum solution: [^{14}C]squalene (specific activity 1×10^4 d.p.m./mg), 1.0 mg; phytoene, 25 μg; β-carotene, 40 μg; lanosterol, 2.5 mg; [^{14}C]-cholesterol (specific activity 1×10^4 d.p.m./mg), 2.5 mg; and [^{14}C]ergosterol (specific activity 1×10^4 d.p.m./mg), 2.5 mg.

The column used for the separation had an I.D. of 1 cm and was packed to a depth of 20 cm with aluminium oxide (Woelm acid-washed, deactivated with water to Brockmann activity grade III) by allowing the adsorbent to settle out from a slurry in light petroleum (b.p. 40–60°). The sample (1 ml) was added to the column which was developed first with 30 ml of light petroleum, run in from the mixing chamber (B). This vessel had an I.D. of 5 cm and contained, initially, 250 ml of light petroleum. After 30 ml of light petroleum had passed down the column, gradient elution was commenced by opening the tap (C) and allowing the more polar solvent from the reservoir (A) to run into the mixing chamber. This reservoir had an I.D. of 3 cm and contained an initial charge of 80 ml of 40 % diethyl ether in light petroleum (v/v).

As there was only one component in the standard mixture that absorbs visible light (β-carotene, λ_{max} in light petroleum at 448 nm), the spectrophotometer was set throughout at a wavelength of 280 nm to monitor phytoene (λ_{max} at 285 nm) and ergosterol (λ_{max} at 282 nm, both in light petroleum) and was used at 450 nm only to follow the elution of the yellow β-carotene band. The passage of ^{14}C-labelled solutes through the scintillation counter was monitored with the photomultiplier operating at 1400 V. The count-rate range used was 0–400 c.p.s. and the time constant on the ratemeter was 2.5 sec. As solutes of known radioactivity were being eluted from the column, the linear output mode of the ratemeter was used; the logarithmic output is used when the range of radioactivity to be recorded is not known. The portion of the eluate stream that entered the liquid chromatograph was carried by the wire,

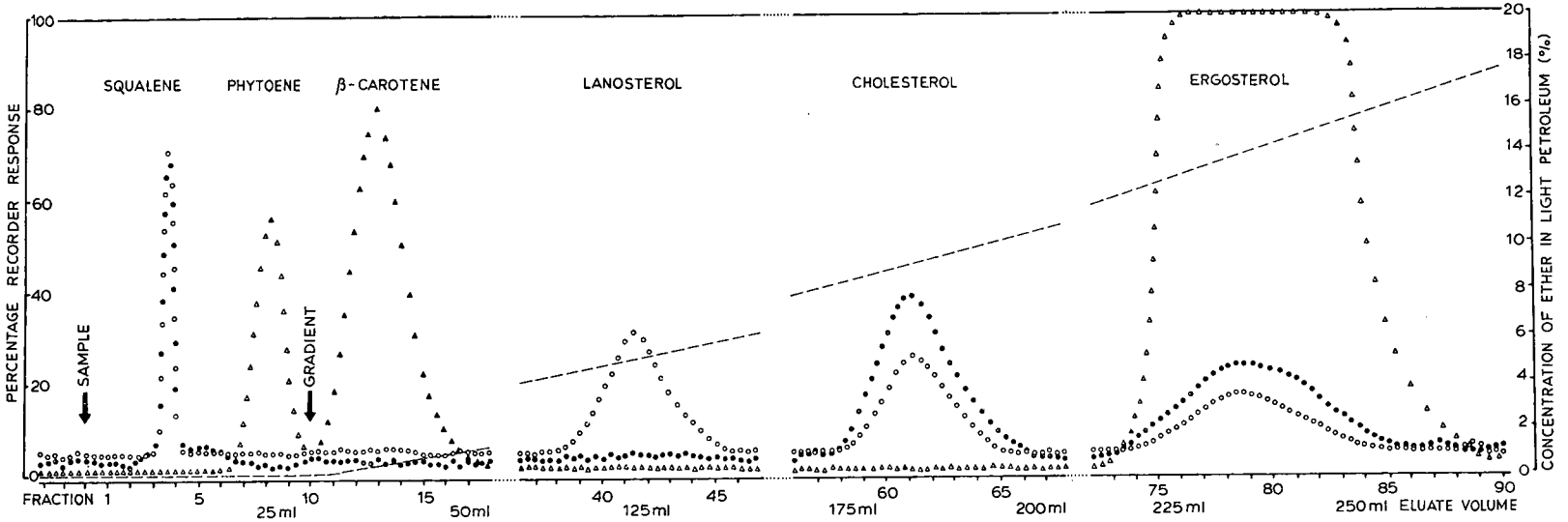

Fig. 5. Tracing of multi-point recorder chart after the analysis of the standard test mixture (see text for details). Key: △△△, absorption at 285 nm and ▲▲▲, absorption at 450 nm (from spectrophotometer); ●●●, radioactivity (from scintillation counter); ○○○, concentration of solute (from liquid chromatograph); – – –, composition of solvent (percentage of ether in light petroleum).

moving at a speed of 8 in./sec, first into the evaporator oven, which was held at a temperature of 80° in order to remove the solvent, and then into the pyrolyser oven set at 700°. A detector voltage of 1250 V gave the best response and an amplifier attenuator setting of $\times 3$ ensured that the scale of the response was acceptable to the recorder.

The results of the analysis are shown in Fig. 5. Squalene is the first compound to leave the column, being eluted as a sharp band with light petroleum and appearing in fraction 4. The recorder peaks from the scintillation counter and the liquid chromatograph are coincident, indicating that the squalene is radioactive. The combination of these two monitoring devices thus results in a reliable method of detecting squalene and assessing its radioactivity and is more rapid than either detecting squalene on thin-layer chromatograms of random column fractions and scanning the thin layer for radioactivity[1,2] or reducing to the corresponding saturated hydrocarbon and identifying this compound and measuring its radioactivity on a gas radiochromatograph[6].

Phytoene leaves the column after 25 ml of light petroleum have passed through and appears in fractions 8 and 9. While an insufficient amount is present to be detected by the liquid chromatograph at the setting used, its high molar extinction coefficient ($\varepsilon = 50 \times 10^3$ at 285 nm) leads to a strong response (55 % full scale) by the spectrophotometer. Similarly, the strong absorption of visible light by β-carotene ($\varepsilon = 134 \times 10^3$ at 448 nm) leads to a large (80 %) response by the spectrophotometer although the liquid chromatograph does not record the presence of this solute at the sensitivity employed. The β-carotene is eluted with 0–2 % diethyl ether in light petroleum (v/v) and appears in fractions 12 to 15.

Lanosterol, on the other hand, has no conjugated double bonds in its molecule and cannot be detected by light absorption but only by the liquid chromatograph. It is eluted from the column with 4–6 % ether in light petroleum in fractions 40 to 45. Previous methods of lanosterol detection have depended on evaporating the solvent from the column fractions and carrying out a Liebermann–Burchard test on the residue; lanosterol yields a yellow colour (maximal absorbance at 458 nm), the intensity of which is at its maximum after 40 min (at 25°)[7]. The Liebermann–Burchard test has also been the basis of previous methods for the detection of cholesterol, which yields a blue-green colour in the test. The liquid chromatograph, however, is able to respond to cholesterol and the coincidence of this response and that from the scintillation counter shows that the cholesterol is radioactive. It is eluted from the alumina column with 8–10 % ether in light petroleum and appears in fractions 59 to 66. The ergosterol, which is eluted from the column with 12–16 % ether in light petroleum as fractions 75 to 88, is also radioactive. This sterol, however, absorbs UV light ($\varepsilon = 12.3 \times 10^3$ at 282 nm); the spectrophotometer response is very high compared with those for phytoene and β-carotene since a relatively high concentration of radioactive ergosterol is included in the standard sample mixture.

Throughout the analysis, the spectrophotometer gives an extremely stable response. In contrast to this, the outputs from the liquid chromatograph and the scintillation counter show random variations, but only to the extent of ± 1.5 % and ± 2.5 % of the full-scale deflections respectively at the sensitivities used. The sensitivity of these instruments can be increased to respond to smaller quantities of solute and to lower ^{14}C activities. This results, however, in a loss of signal-to-noise

ratio so that the interpretation of small peaks in the record becomes rather difficult. The nature of the chromatographic separation is such that the bands of the more strongly adsorbed solutes are broader than those of components eluted earlier from the column. There is not, however, any significant difference in the width of the peaks representing the responses of different instruments to a given solute, indicating that the volume and shape of the flow cells are satisfactory in that they do not allow diffusion of the solutes in the solvent stream. The coincidence of the responses from different instruments to the same component shows that the bore of the tubing used to connect the flow cells and the volumes of the flow cells themselves are small enough to eliminate any significant time-lag. The retention volumes of given components eluted from standard columns are sufficiently reproducible to be used for the identification of solutes by their adsorption characteristics[8].

The prime function of the instrument, as it is used in the authors' laboratory, is in the routine qualitative analysis of lipids, especially unsaponifiable lipids such as the terpenoid hydrocarbons, carotenoids and sterols, extracted from biological systems which have been incubated with radioactive substrates. The recorded responses from the spectrophotometer and the scintillation counter, while quantitative, are only sometimes given absolute quantitative significance as the light-absorbing and radioactively labelled components are often subjected to further purification prior to assay by standard methods. The liquid chromatograph record is only semiquantitative because of the variable nature of the pyrolysis products of different solutes; standardisation of the liquid chromatograph for individual components is required for its results to be absolutely quantitative.

The instrumentation described in this paper is capable of carrying out not only the separation and identification of the components of a lipid mixture but also the estimation of the quantity and radioactivity of these components. The analysis is complete in about 2.5 h and is thus much more rapid than was possible by previous methods in which the fractions were first collected and then analysed individually. While the equipment is completely automatic and will operate unattended, the analysis of a mixture of completely unknown quantitative proportions requires some attention on the part of the operator in order that the sensitivities of the various monitoring devices can be adjusted to give responses of acceptable magnitude.

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CHROM. 4481

APPLICATION OF THIN-LAYER CHROMATOGRAPHY FOR IMPROVING THE CONTINUOUS TNT PROCESS

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SUMMARY

Thin-layer chromatographic separations were performed on TNT samples removed throughout the process in order to study and improve the continuous TNT process. Nitrator samples were chromatographed to examine the effects of changes in nitrating conditions on reaction products. Separation of the product TNT showed that two impurities were formed during the purification, and TLC was used to relate the amounts of these impurities produced under different operating conditions and thus to establish which conditions were required to eliminate their formation.

INTRODUCTION

The use of thin-layer chromatography (TLC) for the analysis of TNT has been reported by YASUDA[1]. In his paper, YASUDA describes the separation techniques and results of the analysis of purified TNT obtained using the three-stage nitration (batch) process. Recently, a continuous-process TNT plant was constructed at the Radford Army Ammunition Plant (RAAP). In this continuous process[2], toluene is fed to the first nitrator and concentrated nitrating acids to the higher nitrators. The organic phase proceeds through the process in which the toluene is successively nitrated to mono-, di-, and trinitrotoluene. The acid flows countercurrent to the nitrotoluenes and becomes weaker as a result of nitration. Thus, the rate of nitration in the lower nitrators is controlled by using progressively weaker acids.

The impure TNT from the last nitrator is washed with water to remove entrained acids and then is treated with sodium sulfite (Sellite), a process called Selliting, in two successive washers to remove the asymmetrical β- and γ-isomers. The process was originally designed to carry out Selliting at a pH of about 8.5, using soda ash for pH adjustment and control. After the Sellite treatment, the purified TNT is again washed with water and then dried and flaked.

When the continuous plant at RAAP was begun, TLC techniques were investigated to aid studies on process and product control and to increase understanding of the reactions occurring at each step in the process. TLC analyses were performed on samples taken at various stages of nitration and purification. The separation tech-

niques used were considerably different from those previously reported. The support used in the present study was silica gel with a starch binder; it was easily prepared and had an excellent adhesion to glass plates. The spray reagent found most suitable for visualizing the TLC fractions was ethylenediamine. This reagent has also been used for the spectrophotometric determination of polynitroaromatic compounds[3].

MATERIALS AND METHODS

Apparatus and reagents

Silica gel with a starch binder and a fluorescent indicator was purchased from Brinkmann Instruments, Inc.; applicator and glass plates (200 × 200 mm) were purchased from Kensington Scientific Co. All solvents were reagent grade, and no further purification was necessary; ethylenediamine was reagent grade. A 25-μl syringe was used to deliver aliquots of the sample to the support.

Experimental

Preparation of thin-layer plates. Thirty grams of support were added to 75 ml of warm water which was constantly agitated. The water temperature was then raised to near boiling to assure that the starch binder was well mixed. A 275-μ gate was used to control plate thickness, and the plates were oven dried at 110° for 2 h before use.

Separation procedure. Best separation results on finished TNT and nitrator samples were obtained with a benzene–cyclohexane–ethyl acetate mixture (50:45:5). A more polar solvent system of benzene–ether–ethanol (50:30:20) was used to separate oxidation products from the nitrator vessels. A few drops of ammonium hydroxide were added to the latter solvent system to prevent tailing.

No single solvent system was found which could separate all nitration products in one separation. However, a two-dimensional separation using benzene–cyclohexane (75:25) as a second solvent at 90° after an initial development with benzene–cyclohexane–ethyl acetate (50:45:5) gave satisfactory results. A two-dimensional separation of an extremely impure TNT sample is shown in Fig. 1.

Nitration studies. Sampling and sample preparation. Approximately 1 ml of the organic phase was pipetted from the nitration separator vessels into a preweighed 25-ml volumetric flask containing 3–5 ml of water. The flask was reweighed, and the sample was diluted to volume with acetone. A 15-μl aliquot of the sample was placed about 1.5 in. from the bottom of the plate. If a two-dimensional separation was to be made, the aliquot was placed in the lower left-hand corner. Care was taken to prevent broadening of the aliquot spot. Development was made in a saturated chromatographic tank containing 100 ml of the solvent system. Approximately 1.5 h were required for the front to travel 15 cm.

The developed chromatogram was viewed first using short-wavelength UV light to determine if the separation was adequate. The plate was next sprayed with ethylenediamine; and red, brown, yellow and blue spots developed showing the locations of the various fractions.

Purification. Sampling and sample preparation. A clean metal rod was used to remove TNT at various purification stages. The metal rod was dipped into the decanter vessels and, upon withdrawal, the TNT solidified and was removed from the rod. One

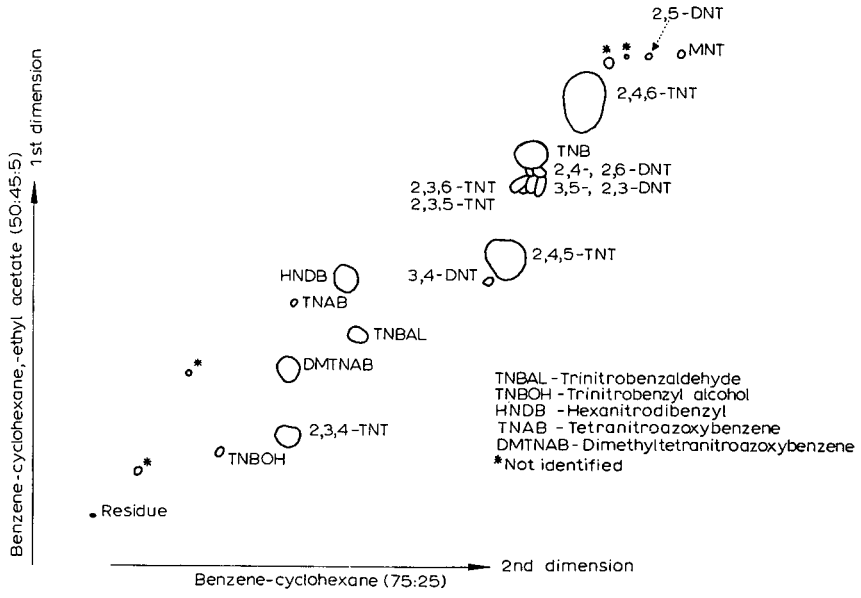

Fig. 1. A two-dimensional separation of an impure TNT sample.

gram of the solidified TNT was placed in a 25-ml volumetric flask, dissolved in acetone and diluted to volume.

Investigation of Sellite variables. The process variables, pH and solution density were studied in a complete 2^n factorial experiment to relate these variables to complete isomer removal and by-product formation. The effects of the different pH and density levels were determined by TLC analyses of TNT samples taken during the investigation.

RESULTS AND DISCUSSION

Nitrator studies

Samples from all nitrators were analyzed by TLC to identify the various nitration and oxidation products formed and to determine the nature of the reactions occurring at each step of the process.

Nitration products. The progression of nitration through mono-, di-, and tri-stages can be seen in Fig. 2. The mononitrotoluenes from separators 1 and 2 were not separated into *ortho-*, *meta-*, and *para*-isomers under the conditions used. All the other major nitration products were identified, as well as some of the minor fractions. The small amounts of TNT in nitrators 1 and 2 result from the addition of the acidic wash water from the purification to nitrator 2. This is done both to recover the TNT in the wash water and to dilute further the nitrating acids in these nitrators to control the rate of nitration.

The predominant fractions in nitrator 1 are the mononitrotoluenes, while nitrators 2 and 3 contain large quantities of 2,6- and 2,4-DNT. The DNT decreases as nitration continues through nitrators 4 and 5 and usually cannot be seen by TLC at nitrator 6. Corresponding to the decrease in DNT, the 2,4,6-TNT increases as do the 2,3,4 (β-) and 2,4,5 (γ-) isomers of TNT. In nitrator 6, the major fraction is

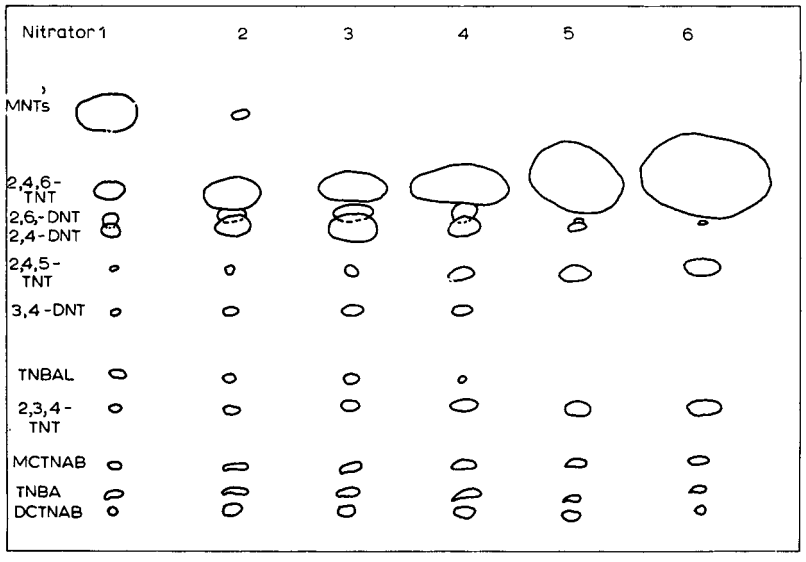

Fig. 2. A separation of the products in the organic phase of nitration.

2,4,6-TNT and about 3.5 % of the β- and γ- isomers. The DNT content is generally less than 0.2 %.

Oxidation products. The second part of the nitration study was designed to identify the type and quantity of oxidation products produced in nitration. Fig. 2 also shows a separation of the oxidation products present in the organic phase.The major oxidation products were found to be 2,2'-dicarboxy-3,3',5,5'-tetranitroazoxy benzene (DCTNAB) known as "white compound", a monocarboxy tetranitroazoxy benzene (MCTNAB), and trinitrobenzoic acid (TNBA). These fractions were identified from their IR spectra and R_F values on thin-layer plates. Quantitative methods have been developed for these fractions and will be described in a later paper. Several minor oxidation products were also separated and are believed to be nitrocresols[4–7].

Purification studies

As indicated above, the crude TNT entering the purification process is primarily 2,4,6-TNT with small amounts of the β- and γ-TNT isomers and lesser amounts of DNT. Some TNBA and DCTNAB are also present in the crude TNT. The amount of these oxidation products varies with nitrating conditions. In the first step of purification (water wash), TNBA readily decarboxylates to trinitrobenzene (TNB) which remains with the TNT throughout purification.

Removal of the β- and γ-isomers of TNT is accomplished by treating the water-washed TNT with an aqueous solution of sodium sulfite. Separation of samples following Selliting showed that two products were formed as the β- and γ-isomers were removed. These products are not normally found in TNT manufactured by the batch process. The presence of these new products was undesirable, since they lowered the freezing point of the product TNT, and occasionally they were produced in sufficient amounts to cause the TNT to be rejected. Therefore, it was necessary to identify these products in an effort to reduce or eliminate their formation. Using IR and

NMR analysis of these products from TLC separations, one was identified as hexanitrodibenzyl (HNDB). The second has not yet been positively identified but is believed to be 2,2′-dimethyl-3,3′,5,5′-tetranitroazoxy benzene (DMTNAB). The formation of at least one of these compounds, HNDB, would be expected to be pH dependent; therefore, the pH was considered to be an important variable in the Selliting process.

Although a quantitative method was neither developed for HNDB nor for DMTNAB, TLC was an excellent and rapid semi-quantitative analytical method. Best estimates from standards on thin-layer plates showed that HNDB was present at 0.5 % or higher in TNT initially produced at RAAP. No measurements were made for DMTNAB.

The experiment designed was carried out on one of the operating TNT lines on four successive days with two levels of operational conditions being run each day (one in the morning and one in the afternoon). Fig. 3 shows a separation of samples of equal weight obtained under the two extreme experimental conditions in the study. It can be readily seen that at low levels, little HNDB and DMTNAB were produced. There was also a corresponding increase in the freezing point of the product TNT made

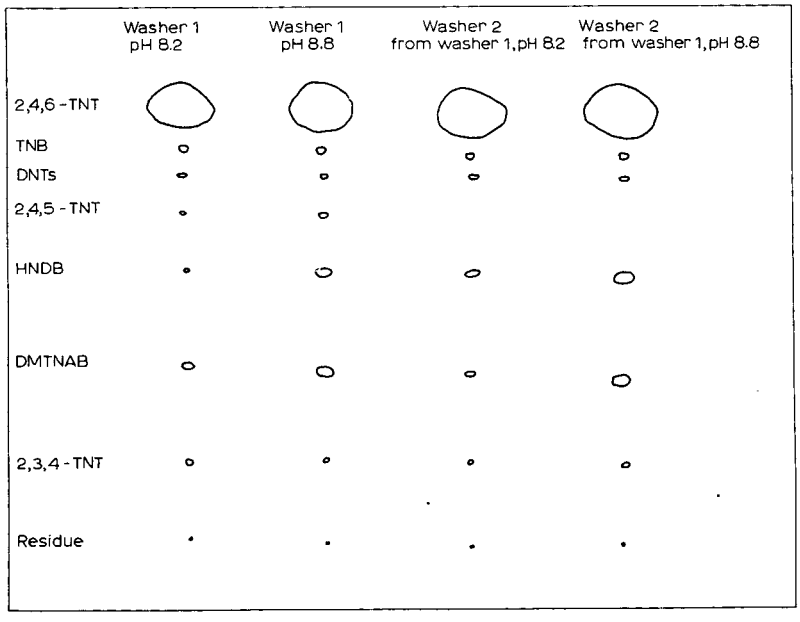

Fig. 3. A separation of samples under extreme conditions in the Selliting study.

under these conditions. The effect of the density of Sellite (concentration) over the range investigated was small. Therefore, pH was the primary parameter of the process affecting formation of HNDB and DMTNAB.

Since lowering the pH from 8.8 to 8.2 considerably reduced the amount of HNDB and DMTNAB, lowering the pH even further should eliminate their formation. However, attempts to do this on an operating line led to incomplete separation of the TNT and the aqueous phases which resulted in improper operation of Sellite

washers and separators. The cause of this poor separation was attributed to the release of CO_2 from the soda ash used for pH control as the pH in washer 1 was allowed to go below about 8.0.

Sellite studies in the laboratory

In an effort to overcome some of the problems encountered with the soda ash–Sellite system, a study of Selliting was begun in the laboratory. A sodium *meta*-bisulfite–Selite mixture was used to determine the effects of Selliting at pH 5. Although no HNDB or DMTNAB were produced under these conditions, removal of the isomers was not adequate for production of high quality TNT. Therefore, Selliting at higher pH values was tried.

The use of soda ash with *meta*-bisulfite to maintain a pH of 7 was attempted. Under these conditions, some HNDB and DMTNAB were formed and gassing occurred. Thus, this system could not be used. The purification system which held the most promise of success was a Sellite-*meta*-bisulfite mixture at near neutral pH. In laboratory studies, this system worked extremely well, with good isomer removal and no HNDB or DMTNAB formation. Separations for the conditions described above are shown in Fig. 4.

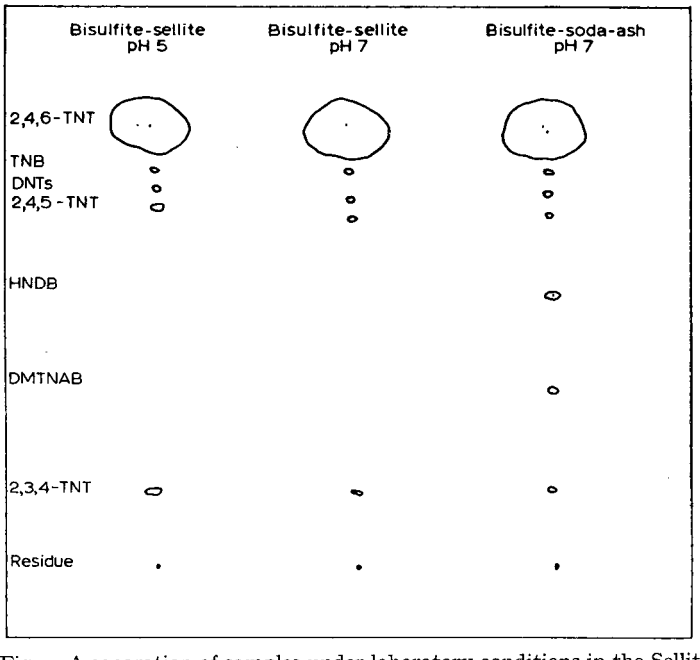

Fig. 4. A separation of samples under laboratory conditions in the Selliting study.

Sellite–meta-bisulfite studies

Following the successful demonstration of the Sellite–*meta*-bisulfite system in the laboratory, an evaluation of the system was made on one of the operating lines. A solution of Sellite, rather than soda ash, was fed to the first Sellite washer to control the pH. By using Sellite, the pH of the washer could be controlled over the range of 6.5 to 9.0 with proper physical operation of the washer and separator. A solution of

Sellite only, or a mixture of Sellite and *meta*-bisulfite, was fed to Sellite washer 2 and, in turn, to washer 1. In this manner, the pH of washer 2 could be controlled at any level between 7 and 9, the range to be investigated. It was found that controlling the pH of washer 1 at about 7.5 and washer 2 at about 8.0 was optimum in terms of completeness of removal of β- and γ-isomers and minimization of the formation of HNDB and DMTNAB.

By using only a solution of Sellite in washer 2, the pH of this washer was consistently maintained at 9.0. Under these conditions and controlling washer 1 at pH 7.5 with Sellite, isomer removal was essentially complete and only a small amount of HNDB ($< 0.20\,\%$) and DMTNAB were produced. Thus, with the aid of TLC techniques, a marked improvement was made in both purification operations and quality of product TNT as a result of eliminating the use of soda ash and lowering the pH at which Selliting occurred.

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CHROM. 4489

ZUR BEEINFLUSSUNG DER DÜNNSCHICHTCHROMATOGRAPHIE INSEKTIZIDER PHOSPHORSÄUREESTER UND DEREN NACHWEIS MIT DER BENZHYDROXAMSÄURE-REAKTION DURCH ULTRAVIOLET-STRAHLUNG

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SUMMARY

Influence of ultraviolet irradiation on the thin-layer chromatography of insecticidal phosphoric acid esters and on their detection by the benzohydroxamic acid reaction

While chromatographing insecticidal phosphates on thin layers and after ultraviolet irradiation, secondary components are produced. The type and amount of these substances may be variable depending on environmental factors. Nitro aromatic substituted phosphates hydrolyse to some extend to their nitro phenols. These should be responsible for the enhancement of their detection by benzohydroxamic acid reagent after ultraviolet irradiation. In the case of parathion, no paraoxon is developed after ultraviolet irradiation.

EINLEITUNG

In einer kürzlich erschienenen Arbeit[1] untersuchten wir die Eignung einer von EPSTEIN UND DEMEK[2] beschriebenen Reaktion zwischen Hydroxamsäuren und Cholinesterasehemmern. Im Fall der von uns[1] benutzten Benzhydroxamsäure interpretierten wir den Reaktionsablauf in Analogie zu dem Vorschlag von EPSTEIN UND DEMEK[2]. Demzufolge würde Benzhydroxamsäure bevorzugt Phosphorsäureester spalten, wobei sie selbst in Phenylisocyanat umgewandelt wird, das seinerseits weitere Benzhydroxamsäure abfängt. Somit steht an jenen Orten nicht mehr ausreichend Benzhydroxamsäure zur Verfügung, um chromogenes Substrat (2-Azobenzolnaphthyl-(1)-acetat) zum Farbstoff zu spalten. Diese Flecke bleiben also weiss.

Durch 15 Min. währende UV-Bestrahlung der Monothio- und Dithiophosphorsäureester vor der Reaktion konnte der Nachweis in vielen Fällen empfindlicher oder überhaupt erst möglich gemacht werden. Andere Autoren[3–6] berichten Analoges bei der Untersuchung der Hemmaktivität insektizider Phosphorsäureester gegenüber Cholinesterase. Sie deuten diese Erscheinung so, dass nur von der durch UV-Bestrahlung entstehenden O-analogen Verbindung, dem "Oxon", die eigentliche Hemmwirkung ausgeht. Einige wenige bisher veröffentlichte Experimentalarbeiten scheinen dieser Interpretation Recht zu geben (z.B. Lit. 7 und die Sekundärliteratur in Lit. 4).

Bei unseren Resultaten[1] fällt auf, dass die schlechten Nachweisempfindlichkeiten der Monothiophosphorsäure-nitrophenylester Parathion, Parathion-methyl, Fenitrothion und Chlorthion durch 15 Min. UV-Bestrahlung nicht verbessert werden können. Erst nach einstündiger Bestrahlung sind gerade noch 5 μg dieser Wirkstoffe erkennbar.

ERGEBNISSE

Wir untersuchten die Verhältnisse zunächst beim Parathion eingehender. Nach ein- oder zweistündiger UV-Bestrahlung von 5–50 μg des Parathions am Start der Dünnschichtplatte sind nach dem Chromatographieren je nach Probenmenge und Umweltfaktoren maximal drei Flecke mit Rhodamin B sichtbar, von denen derjenige mit dem grössten R_F-Wert auf Grund des Vergleiches mit drei verschiedenen Fliessmitteln (vgl. Tabelle I) als Parathion identifiziert wurde. Der R_F-Wert des letzten Fleckes ist in System I und III kleiner, in System II grösser als derjenige des Paraoxons.

TABELLE I

MITTLERE hR_F-WERTE VON 30 μg 1–2 Std. UV-BESTRAHLTEM PARATHION UND VERGLEICHSSUBSTANZEN

	System I	*System II*	*System III*
Parathion bestrahlt	64, 49, 24	67, —[a], 25	45, 33, 20
Parathion	64	67	45
Paraoxon	36	13	25
p-Nitrophenol	24	25	20

[a] Zweiter Fleck nicht sichtbar.

Er stimmt überein mit dem R_F-Wert des *p*-Nitrophenols. Beide Flecke werden mit dem Nitrogruppen-Sprühreagens[8] charakteristisch sichtbar, und zwar der erste blaugrün, der andere gelb. Mit dem Thiono-Reagens[9] und dem Schwefel-Reagens wird der erste Fleck angefärbt. Bei grosser Aufgabemenge ist zuweilen der zweite Fleck mit dem Thiono-Reagens zu erkennen. Mit Benzhydroxamsäure-Reagens erscheinen die Originalsubstanz als weisser sowie *p*-Nitrophenol als gelblicher (wegen des alkalischen pH im Reagens) Fleck. Bei sehr grossen Mengen kann auch die mittlere Komponente sichtbar werden. Wesentlich geringere Mengen *p*-Nitrophenol als Parathion hemmen noch die 2-Azobenzolnaphthyl-(1)-acetat-Spaltungsreaktion des Nachweises mit Benzhydroxamsäure-Reagens (vgl. Tabelle II).

TABELLE II

UNTERE NACHWEISGRENZEN DER NITROARYL-PHOSPHORSÄUREESTER UND IHRER HYDROLYTISCHEN SPALTPRODUKTE MIT DEM BENZHYDROXAMSÄURE-REAGENS

Substanz	μg
Parathion	25
Parathion-methyl	25
p-Nitrophenol	1
Fenitrothion	50
3-Methyl-4-nitrophenol	3
Chlorthion	50
Chlorthionfolgeprod. v. $hR_F' = 22$	*ca.* 12

TABELLE III

RELATIVE hR_F'-WERTE DER KOMPONENTEN, DIE NACH EINSTÜNDIGER UV-BESTRAHLUNG VON JE 30 μg NITROARYL-PHOSPHORSÄUREESTER MIT VERSCHIEDENEN DETEKTIONSMITTELN ERKANNT WURDEN

Chromatographie-System IV.

Wirkstoff	*Rhodamin-B-Reagens*	*Benzhydroxamsäure-Reagens*	*Nitrogruppen-Reagens*		*Phosphor-Reagens*	*Thiono-Reagens*	*Schwefel-Reagens*
			Blau	*Gelb*			
Parathion[c]	120, 68, 30[a]	118, 32[a]	116	31[a]	114, 66, 0	118, 67	118
Parathion-methyl	87, 57, 31[a]	88, 30[a]	86	31[a]	85, 55, 40, 8	88	87
Fenitrothion	90, 64, 32[b]	94, 30[b]	91	33[b]	90, 64, 38, 20, 0	91	91
Chlorthion	86, 22	89, 25	88	25	87, 33, 0	80	87

[a] *p*-Nitrophenol ergab im Mittel einen Wert von 30.
[b] 3-Methyl-4-nitrophenol ergab im Mittel einen Wert von 34.
[c] Paraoxon erscheint hier mit dem Mittelwert 36.

Praktisch analog verhalten sich die anderen nitrogruppenhaltigen Phosphorsäureester Parathion-methyl, Fenitrothion und Chlorthion (vgl. Tabelle III). Für Fenitrothion war die Vergleichssubstanz 3-Methyl-4-nitrophenol zur Hand.

Schliesslich wurden diejenigen Mono- und Dithiophosphorsäureester, deren Nachweis mittels Benzhydroxamsäure-Reagens sich laut unserer früheren Arbeit[1] durch 15 Min. UV-Bestrahlung wesentlich verbessern liess, mit Rhodamin B untersucht. Nur die Wirkstoffe, deren Chromatogramme nach einstündiger UV-Bestrahlung des Startfleckes mehr Flecke als nur die Originalkomponenten aufwiesen, wurden anschliessend dem Test mit den übrigen spezifischen Sprühreagentien unterworfen. Die Ergebnisse sind in Tabelle IV zusammengestellt. Es wurde in System IV chromatographiert. Zum besseren Vergleich wurde stets der Farbstoff Sudan III (Schering) als innerer Standard zugegeben. Die R_F-Werte der Komponenten wurden durch den zugehörigen Wert des Standards dividiert und mit dem Faktor 100 multipliziert.

Bei dieser Gruppe fällt auf, dass bei der Detektion durch Benzhydroxamsäure-Reagens nur jeweils eine Komponente – zweifellos die Originalwirkstoffe – sichtbar wurden. Dagegen wurden (bei 30 μg Aufgabemenge) mit den anderen Sprühreagentien im bestrahlten sowie unbestrahlten Falle daneben oft noch weitere Komponenten nachgewiesen.

Bei allen in dieser Arbeit beschriebenen Versuchen waren die Ergebnisse nicht immer exakt reproduzierbar. In den Tabellen sind die maximal aufgefundenen Komponenten eingetragen.

DISKUSSION DER ERGEBNISSE

Aus den Versuchsergebnissen leiten wir ab, dass sich in vielen Fällen nicht erst nach experimenteller UV-Behandlung teilweise Umwandlungen der Phosphorsäureester abspielen, sondern schon bei der gewöhnlichen Dünnschichtchromatographie. Diese Schlussfolgerung wird auch durch die Feststellung gestützt, dass die Grössenordnungen der dünnschichtchromatographisch entstandenen Nebenkomponenten wesentlich über denen der gaschromatographisch festgestellten Spurenverunreinigungen liegen.

Bei der Dünnschichtchromatographie der nitrogruppenhaltigen aromatisch substituierten insektiziden Phosphorsäureester Parathion, Parathion-methyl, Fenitrothion und Chlorthion entstehen – besonders nach UV-Einwirkung – hauptsächlich die entsprechenden Nitrophenole als hydrolytische Spaltprodukte sowie je ein weiteres Produkt, das wir auf Grund des Verhaltens gegenüber den Nachweisreagentien für einen isomerisierten Ester halten müssen[10]. Im Falle des Parathions entstand niemals das O-Analoge, also Paraoxon, in nachweisbarer Mengen, d.h. entsprechend den Detektionsgrenzen nicht über 1.5 % (bezogen auf die Gesamtprobenmenge).

Durch die Entstehung der Nitrophenole kann der Nachweis mit Benzhydroxamsäure-Reagens empfindlicher werden, da noch wesentlich geringere Nitrophenolmengen das zur Hydrolyse des 2-Azobenzolnaphthyl-(1)-acetats nötige Alkali binden. Bei den anderen Phosphorsäureestern liessen sich keine Folgeprodukte finden, die beim Nachweis mittels Benzhydroxamsäure-Reagens interferieren. Es entstehen aber auch hier Folgeprodukte, die besonders mit dem sehr empfindlichen Phosphor-Reagens[11] entdeckt werden. Speziell bei den mit diesem Sprühmittel nachgewiesenen Komponenten mit geringem hR_F'-Wert dürfte es sich um ein- oder zweibasische Phosphorsäure-

TABELLE IV

RELATIVE hR_F'-WERTE DER KOMPONENTEN, DIE OHNE ODER MIT EINSTÜNDIGER BESTRAHLUNG VON 30 μg PHOSPHORSÄUREESTER MIT VERSCHIEDENEN DETEKTIONSMITTELN ERKANNT WURDEN

Chromatographier-system IV. b = bestrahlt; u = unbestrahlt.

Wirkstoff	*Bestrahlung*	*Rhodamin B-Reagens*	*Benzhydroxamsäure-Reagens*	*Phosphor-Reagens*	*Thiono-Reagens*	*Schwefel-Reagens*
Fenchlorphos[a]	u	134, 106	140	138	136, 103	141, 110
	b	138, 107	140	140, 122–93, 68, 0	138, 105	140, 108
Fenthion	u	117	120	118, 50, 35, 15, 0	121	120
	b	120, 49, 34, 15	123	117, 48	121, 13	121, 83, 52, 36, 16
E 838	u	63, 15	63	61, 28, 18	60	59, 17–0
	b	62, 15	65	59, 38, 26, 17	60, 37, 14	60, 48, 27, 15
Bromophos	u	140	137	138, 0	142	144
	b	139, 80	139	143, 0	143	145, 82, 60
Bromophos-äthyl	u	161	164	164	162	163
	b	159, 132, 74	159	166, 0	164	165, 58
Carbophenothion[b]	u	144	142	141	145, 118	143
	b	143, 139–57	146	141	144, 132–31	146
Carbophenothion-methyl[b]	u	129	133	131	129	127
	b	127, 103–49	128	134, 0	129, 98–40	134
Phenkapton	u	157	152	158	157	155
	b	155, 131, 118–68	153	158	150, 120–75	151
Thiometon	u	127	128	127, 58	130, 66	132, 64
	b	127, 57	128	129, 58, 8	128, 67, 8	130, 68
Phorat	u	148	148	147, 32, 0	152, 77	149
	b	148, 76, 23	150	145, 30, 0	151, 76, 26	152, 78, 30

[a] 2,4,5-Trichlorphenol ergibt den mittleren Wert 90.

[b] *p*-Chlorthiophenol, Schuchardt, "*ca.* 99%, F. 51–52°" erscheint in zwei Flecken mit den Werten 198 und 175, von denen der zweite vermutlich die Hauptkomponente darstellt.

alkylester handeln. Der mengenmässige Anteil der Folgeprodukte variiert (von nicht bis deutlich nachweisbar) je nach den Umweltbedingungen (Temperatur, Feuchte, Anzahl katalytisch aktiver Zentren auf dem Schichtmaterial der betreffenden Charge, etc.) bei der Chromatographie und bei der Vorbehandlung. Die oft recht unterschiedlichen Absolutwerte des inneren Standards sind dafür deutlicher Hinweis. Ferner sind Bestrahlungsintensität und -dauer für den jeder Komponente eigenen Verlauf der Konzentration–Zeit-Kurve für die Folgeprodukte von Bedeutung[7]. Mangels eines anderen Hinweises müssen wir vorläufig annehmen, dass durch die vorstehend beschriebenen Faktoren in unterschiedlichem Masse Abbaureaktionen gefördert oder auch gebremst werden. Es ist denkbar, dass die Konzentrationen der die 2-Azobenzolnaphthyl-(1)-acetat-Spaltung fördernden Komponenten durch UV-Behandlung besonders schnell verringert werden.

EXPERIMENTELLES

Die Platten wurden wie in Lit. 1 beschrieben hergestellt. Trennstrecken 10 cm. Chromatographie: System I, Cyclohexan–Dioxan (7:3), Sandwich-Verfahren; System II, Cyclohexan–Essigester (3:1), Sandwich-Verfahren; System III, Heptan–Aceton (8:2), Sandwich-Verfahren; System IV, Cyclohexan–Dioxan (8:2), Normalverfahren in nicht ausgekleidetem Trog.

Die UV-Bestrahlung der aufgegebenen Proben erfolgte am Start mit der Desaga-UV-Lampe (UVIS Best.-Nr. 131 100) mit Licht von 254 und 366 nm in 9.5 cm Abstand.

Reagentien

Rhodamin-B-Reagens: 0.01 %ige wässrige Lösung von Rhodamin B; Betrachtung bei 254 nm.

Benzhydroxamsäure-Reagens: beschrieben in Lit. 1.

Nitrogruppen-Reagens: beschrieben in Lit. 8.

Phosphor-Reagens: beschrieben in Lit. 11.

Thiono-Reagens: 0.5 %ige wässrige Lösung von $PdCl_2$. Auf 25 ml dieser Lösung wurden 15 Tropfen 25 %ige HCl gegeben.

Schwefel-Reagens: Sprühlösung 1, 1 %ige wässrige Lösung von Tetrabromphenolphthaleinsäureäthylester-Kaliumsalz, vor dem Gebrauch 1:5 mit Aceton verdünnt; Sprühlösung 2, 2 %ige wässrige Lösung von $AgNO_3$, vor dem Gebrauch 1:3 mit Aceton verdünnt; Sprühlösung 3, 10 %ige wässrige Lösung von Zitronensäure, vor dem Gebrauch 1:2 mit Aceton verdünnt.

DANK

Für experimentelle Mitarbeit bin ich Frau A.-M. TURGAY zu besonderem Dank verpflichtet.

Ferner danke ich den Firmen American Cyanamid, Bayer, Cela, Dow Chemicals, Geigy, Sandoz und Stauffer für die freundlicherweise überlassenen Wirkstoffe.

ZUSAMMENFASSUNG

Während der Dünnschichtchromatographie und nach UV-Bestrahlung insek-

tizider Phosphorsäureester entstehen Sekundärstoffe. Art und Menge dieser Substanzen variieren mit den Umgebungseinflüssen. Bei nitroaromatisch substituierten Phosphorsäureestern erfolgt in gewissem Grade Hydrolyse zu den Nitrophenolen. Auf diese Stoffe wird die Erhöhung der Nachweisempfindlichkeit für nitroaromatische Phosphorsäureester durch länger währende UV-Bestrahlung vor der Benzhydroxamsäure-Reaktion zurückgeführt. Aus Parathion entwickelte sich dabei kein Paraoxon in nachweisbaren Mengen.

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STUDIES ON THE RELATIONSHIP BETWEEN MOLECULAR STRUCTURE AND CHROMATOGRAPHIC BEHAVIOUR

XVI. THE BEHAVIOUR OF SOME SUBSTITUTED INDANOLS ON LAYERS OF CELLULOSE IMPREGNATED WITH SIMPLE AMIDES

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SUMMARY

Indanols have been chromatographed on thin layers of cellulose impregnated with simple amides as stationary phases and hexane as the mobile phase. The R_F values have been shown to vary with the impregnation coefficient of the mobile phase. Differences in the behaviour of the amides have been correlated with differences in their molecular dimensions. The effects of molecular structure on the chromatographic behaviour are reflected in the successful separation of 4-indanol and its homologues from the 5-indanol series.

INTRODUCTION

A recent investigation[1] has shown that the highest degree of reproducibility of R_F values in reversed-phase thin-layer chromatography is obtained when the impregnant is dissolved in the solvent used to slurry the impregnant support and the resultant sample-loaded chromatograms are eluted in a double saturation chamber (*i.e.* a glass sandwich made of the chromatoplate, a glass former and a cover plate enclosed in a disposable polythene bag which served as the chromatographic chamber[2]). In this investigation indanols were used as model compounds, the mobile phase being aqueous ethanol (37.5 % v/v) with ethyl oleate supported on cellulose as the stationary phase.

The R_F values for the compounds were shown to be less reproducible if the stationary phase was chromatographed into the support medium and/or when the chromatograms were eluted in conventional tanks.

Where a high degree of reproducibility of R_F values was obtained it was attributed to constancy of the A_M/A_S ratio in the equation[3]

$$\alpha = \frac{A_M}{A_S}\left(\frac{1}{R_F} - 1\right) \qquad (1)$$

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In cases where the A_M/A_S term is constant an alternative chromatographic parameter, the R_M value, has been derived[4], *viz.*:

$$R_M = \log\left(\frac{1}{R_F} - 1\right) \quad (2)$$

This term, which is more directly related to the partition coefficient, α, than is the simple R_F term, has been used to substantiate the validity of the MARTIN additivity principle[5]. The R_M theory, as applied to paper chromatography has been exhaustively reviewed[6]. It has also been used successfully in relating the molecular structure of phenols to their thin-layer chromatographic behaviour[7–13].

Eqns. (1) and (2) may be combined, *viz.*:

$$R_M = \log \alpha - \log A_M + \log A_S \quad (3)$$

Provided that A_M remains constant, α being constant by definition, then linear plots of R_M *vs.* $\log A_S$ (or more specifically the log of the concentration of the stationary phase in the solvent used for slurrying the support mechanism) should indicate the constancy of the A_S term. For methylated phenols chromatographed on layers of cellulose impregnated with formamide[14,15] or with N-methylated formamides[15], using hexane as the mobile phase, this has been shown to be approximately so.

In the present investigation, the chromatographic system of cellulose impregnated with amides as the stationary phase and hexane as the mobile phase has been re-investigated using a different type of solute in an attempt to confirm our previous findings[15]. Indanols, phenols in which one side of the molecule bears a five-membered ring, were chosen for the investigation because we wished to observes if any stereo-specific interaction in the alignment of the phenolic molecule with the amide surface resulted from the presence of the fused ring.

EXPERIMENTAL

Chromatography

Full details of the experimental procedure are given in previous papers[14,15]. Cellulose (15 g MN 300 HR) was slurried with solutions (65 cm^3) of amides (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 *M*) in acetone. The mixture was used to coat glass plates (5 × 20 cm × 20 cm) at an applied layer thickness of 0.3 mm.

The chromatoplates, spotted with indanols (1 μl of 0.25 % solutions in cyclohexane), were eluted with hexane (40 ml) in our double saturation chamber[2].

The visualisation of the indanols with alkaline potassium permanganate was carried out as previously reported[1].

2,6-Dimethylphenol was used as an internal standard on all plates from which R_F values of indanols were obtained. The high degree of reproducibility ($\pm$0.01 R_F unit) for this internal standard was as reported in earlier papers[14,15]. The R_F values of the indanols were also reproducible to $\pm$0.01 R_F units. These R_F values (the mean of at least 5 determinations) and their R_M values are quoted in Tables I–III.

The determination of the amounts of amide above and below the solvent front on eluted chromatograms

In order to determine the onset of double fronting[14,15] the amounts of amide above and below the solvent front were determined.

TABLE I

R_F AND R_M VALUES OF SUBSTITUTED INDANOLS ON THIN LAYERS OF CELLULOSE IMPREGNATED WITH FORMAMIDE

Concentration of formamide in slurrying solvent (moles litre^{-1}).

Key	*Indanol*	*0.5*		*1.0*		*2.0*		*3.0*		*4.0*		*5.0*		*6.0*	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
1	4-Indanol	0.64	−0.250	0.60	−0.176	0.39	+0.195	0.23	+0.525	0.17	+0.689	0.13	+0.826	0.11	+0.908
2	1-Methyl-	0.79	−0.545	0.74	−0.455	0.55	+0.087	0.40	+0.176	0.28	+0.410	0.21	+0.586	0.16	+0.716
3	2-Methyl-	0.79	−0.545	0.74	−0.455	0.55	−0.087	0.40	+0.176	0.28	+0.410	0.21	+0.580	0.16	+0.716
4	5-Methyl-	0.90	−0.955	0.86	−0.788	0.73	−0.432	0.63	−0.231	0.54	−0.070	0.44	+0.105	0.35	+0.269
5	6-Methyl-	0.78	−0.550	0.75	−0.478	0.57	−0.123	0.43	+0.140	0.32	+0.327	0.25	+0.535	0.19	+0.630
6	7-Methyl-	0.72	−0.410	0.68	−0.327	0.50	0.000	0.35	+0.269	0.24	+0.501	0.18	+0.659	0.14	+0.789
7	7-*tert.*-Butyl-	0.94	−1.194	0.91	−1.004	0.83	−0.699	0.76	−0.500	0.68	−0.327	0.60	−0.176	0.55	−0.087
8	5,7-Di-*tert.*-butyl-	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—
9	5,7-Di-*tert.*-butyl-3-methyl-	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—
10	5-Indanol	0.48	+0.025	0.47	+0.051	0.28	+0.410	0.17	+0.689	0.12	+0.865	0.09	+1.005	0.07	+1.124
11	1-Methyl-	0.68	−0.327	0.64	−0.250	0.47	+0.051	0.33	+0.301	0.23	+0.525	0.17	+0.689	0.14	+0.789
12	3-Methyl-	0.68	−0.327	0.64	−0.250	0.47	+0.051	0.33	+0.301	0.23	+0.525	0.17	+0.689	0.14	+0.789
13	4-Methyl-	0.75	−0.478	0.73	−0.432	0.56	−0.105	0.42	+0.140	0.32	+0.327	0.25	+0.479	0.19	+0.630
14	6-Methyl-	0.79	−0.575	0.78	−0.550	0.60	−0.176	0.46	+0.070	0.36	+0.258	0.28	+0.410	0.21	+0.580
15	7-Methyl-	0.65	−0.269	0.60	−0.176	0.43	+0.123	0.29	+0.389	0.20	+0.602	0.14	+0.789	0.12	+0.865
16	6-*tert.*-Butyl-	1.00	—	1.00	—	1.00	—	1.00	—	0.97	−1.509	0.94	−1.194	0.90	−0.955

TABLE II

R_F AND R_M VALUES OF SUBSTITUTED INDANOLS ON THIN LAYERS OF CELLULOSE IMPREGNATED WITH N-METHYL FORMAMIDE

Concentration of N-methyl formamide in slurrying solvent (moles litre^{-1}).

Key	*Indanol*	*0.5*		*1.0*		*2.0*		*3.0*		*4.0*		*5.0*	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
1	4-Indanol	0.30	+0.368	0.16	+0.716	0.10	+0.954	0.08	+1.040	0.07	+1.024	0.00	—
2	1-Methyl-	0.43	+0.123	0.23	+0.525	0.14	+0.789	0.11	+0.908	0.08	+1.040	0.06	+1.195
3	2-Methyl-	0.43	+0.123	0.23	+0.525	0.14	+0.789	0.11	+0.908	0.08	+1.040	0.06	+1.195
4	5-Methyl-	0.65	−0.269	0.39	+0.195	0.21	+0.580	0.14	+0.789	0.12	+0.865	0.10	+0.954
5	6-Methyl-	0.45	+0.087	0.23	+0.525	0.13	+0.826	0.10	+0.954	0.11	+0.820	0.10	+0.954
6	7-Methyl-	0.42	+0.140	0.22	+0.550	0.10	+0.954	0.07	+1.124	0.06	+1.195	0.00	—
7	7-*tert.*-Butyl-	0.75	−0.478	0.50	0.000	0.30	+0.368	0.21	+0.580	0.17	+0.689	0.14	+0.789
8	5,7-Di-*tert.*-butyl-	1.00	—	1.00	—	0.95	−1.276	0.92	−1.061	0.87	−0.827	0.82	−0.659
9	5,7-Di-*tert.*-butyl-3-methyl	1.00	—	1.00	—	1.00	—	0.95	−1.276	0.92	−1.061	0.89	−0.907
10	5-Indanol	0.25	+0.478	0.14	+0.789	0.08	+1.061	0.06	+1.195	0.04	+1.380	0.00	—
11	1-Methyl-	0.40	+0.176	0.20	+0.602	0.12	+0.865	0.08	+1.091	0.06	+1.195	0.00	—
12	3-Methyl-	0.40	+0.176	0.20	+0.602	0.12	+0.865	0.08	+1.091	0.06	+1.195	0.00	—
13	4-Methyl-	0.49	+0.017	0.28	+0.410	0.15	+0.750	0.12	+0.865	0.09	+1.005	0.06	+1.195
14	6-Methyl-	0.52	−0.035	0.31	+0.348	0.17	+0.689	0.13	+0.826	0.10	+0.959	0.08	+1.040
15	7-Methyl-	0.38	+0.213	0.18	+0.659	0.10	+0.954	0.07	+1.124	0.06	+1.195	0.00	—
16	6-*tert.*-Butyl-	0.75	−0.478	0.73	−0.432	0.54	−0.070	0.38	+0.213	0.30	+0.368	0.24	+0.500

TABLE III

R_F AND R_M VALUES OF SUBSTITUTED INDANOLS ON THIN LAYERS OF CELLULOSE IMPREGNATED WITH N,N'-DIMETHYL FORMAMIDE

Concentration of N,N'-dimethyl formamide in slurrying solvent (moles-litre^{-1}).

Key	*Indanol*	*0.5*		*1.0*		*2.0*		*3.0*		*4.0*	
		R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
1	4-Indanol	0.28	+0.410	0.13	+0.826	0.09	+1.005	0.06	+1.195	0.00	—
2	1-Methyl-	0.43	+0.123	0.21	+0.580	0.13	+0.826	0.08	+1.061	0.05	+1.279
3	2Methyl-	0.43	+0.123	0.21	+0.580	0.13	+0.826	0.08	+1.061	0.05	+1.279
4	5-Methyl-	0.50	0.000	0.28	+0.410	0.18	+0.659	0.12	+0.865	0.08	+1.061
5	6-Methyl-	0.42	+0.140	0.20	+0.602	0.13	+0.826	0.07	+1.124	0.06	+1.195
6	7-Methyl-	0.40	+0.176	0.19	+0.630	0.09	+1.005	0.06	+1.195	0.00	—
7	7-*tert.*-Butyl-	0.65	−0.269	0.44	+0.105	0.28	+0.410	0.19	+0.630	0.13	+0.826
8	5-7-Di-*tert.*-butyl-	1.00	—	1.00	—	0.82	−0.658	0.74	−0.455	0.64	−0.250
9	5,7-Di-*tert.*-butyl-3-methyl	1.00	—	1.00	—	0.90	−0.955	0.84	−0.721	0.76	−0.500
10	5-Indanol	0.18	+0.630	0.11	+0.908	0.07	+1.124	0.04	+1.380	0.00	—
11	1-Methyl-	0.32	+0.327	0.19	+0.630	0.11	+0.908	0.07	+1.124	0.04	+1.380
12	3-Methyl-	0.32	+0.327	0.19	+0.630	0.11	+0.908	0.07	+1.124	0.04	+1.380
13	4-Methyl-	0.41	+0.149	0.23	+0.518	0.14	+0.789	0.10	+0.954	0.06	+1.195
14	6-Methyl-	0.52	−0.035	0.28	+0.410	0.17	+0.689	0.11	+0.908	0.07	+1.124
15	7-Methyl-	0.30	+0.368	0.17	+0.689	0.10	+0.954	0.06	+1.195	0.00	—
16	6-*tert.*-Butyl-	0.81	−0.609	0.64	−0.250	0.40	+0.070	0.34	+0.288	0.28	+0.410

The chromatoplates, lacking the solute spots, were prepared and eluted as described above. The mobile phase was allowed to rise 12.5 cm (±0.5 cm) above the normal point of application of the solutes. They were then removed from the sandwich chamber and the mobile phase was allowed to evaporate. After this, bands (2 cm in width) were removed from the layers and transferred into flasks containing sodium hydroxide (25 cm^3 of 1.0 N). Ethylene glycol was added and the flask and its contents were heated under conditions of total reflux for 2 h. This resulted in the hydrolysis of the amide. After this time the reaction products together with flask and condenser washings were transferred to erlenmeyer flasks and the excess sodium hydroxide was determined with standard hydrochloric acid. From the results obtained, the amount of sodium hydroxide used in the reaction and hence the amount of amide present in each band was determined.

DISCUSSION

The effect of the amide loading

The results quoted in Tables I–III show that the R_F values of the indanols

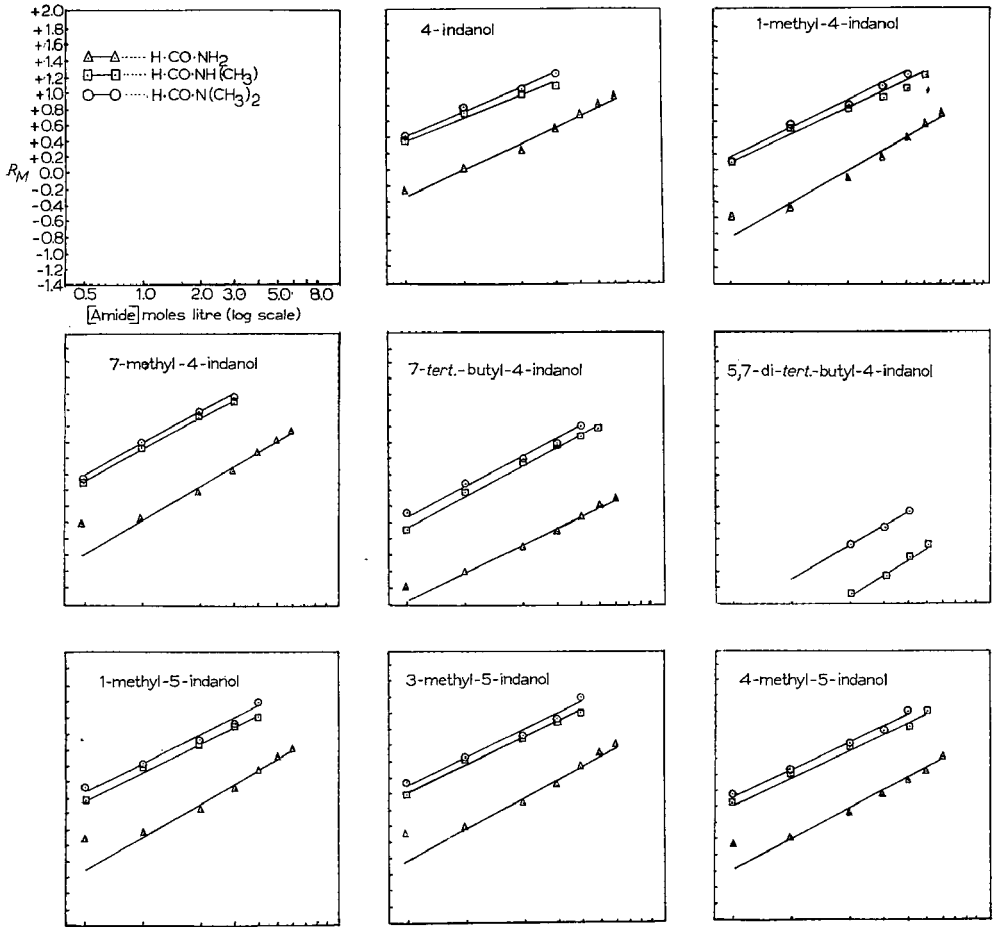
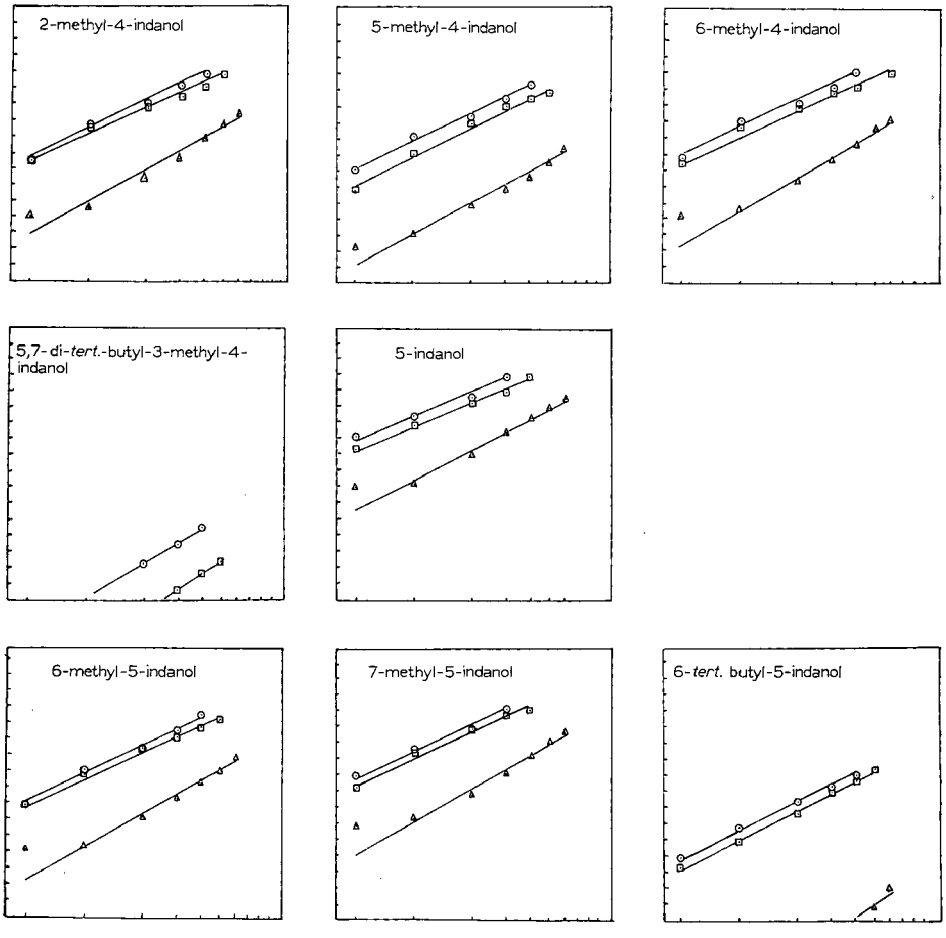


Fig. 1. R_M values (subst. indanols) *vs.* concentration of amide in the slurrying solvent (log scale).

decrease with an increase in the concentration of the amide in the solvent used to slurry the support medium, *i.e.* with an increase in the impregnation coefficient of the stationary phase. This is to be expected because an increase in the impregnation coefficient is reflected in an increase in the A_S term of eqn. (1). This behaviour parallels the behaviour of the methylated phenols in the same systems[15].

Plots of R_M values of the indanols *vs.* concentration of the amide (log scale) in the slurrying solvent are shown in Fig. 1 from which it can be seen that they are linear over the bulk of the range of concentrations studied. This can be taken as proof of the validity of eqn. (3) and evidence for the constancy of the A_S term (or at least the A_M/A_S ratio) in the system studied.

Deviations from linearity, however, occur at very low or very high amide loadings, the points of deviation for each amide studied being identical with those obtained when the methylated phenols were investigated in the same systems[15], *i.e.* they are independent of the nature of the solutes investigated. This confirms that these deviations are a consequence of the chromatographic phases or more specifically the stationary phase, *i.e.* the deviations at low concentration being attributed to incomplete coverage of the cellulose by the stationary phase while those at high con-



centrations were considered to be a consequence of excess stationary phase being sloughed off the cellulose and pushed ahead of the mobile phase so resulting in the phenomenon of double fronting[15].

The former of these views is difficult to prove but evidence for the latter was forthcoming from two sources,

(a) the R_F values of the solutes (indanols and methylated phenols) tending towards a minimum,

(b) visual inspection of the plates after evaporation of the mobile phase showed that the region beyond the solvent front remained damp at high impregnation coefficients, the extent of the damp zone increasing with an increase in the impregnation coefficient.

From the results in Tables I–III and from Fig. 1, it is apparent that, for the three amides studied, the points at which the linear part of each curve begins and ends are not the same but that they occur at progressively higher impregnation coefficients *viz.* N,N'-dimethylformamide < N-methylformamide < formamide. This confirms our findings for the same systems when methylated phenols were used as the solutes[15].

Because fixed volumes of molar concentrations of the amides in acetone were used to slurry fixed amounts of cellulose, it is to be expected that, for a given concentration of amide in the slurrying solvent, the same number of molecules of each amide would be present on the cellulose and hence that the points of deviation from linearity should coincide for each amide. That this is not so suggests that either our visual appraisal of the onset of double fronting is at fault, or that, while the number of molecules of the stationary phase will have some importance in determining the R_F values, some other effect is superimposed on this primary factor. Here, we suggest that this addition effect will be related to the molecular dimensions of the molecules of the stationary phases.

In order to assess the creditability of these suppositions it was necessary, in the first instance, to determine chemically the amount of amide present above and below the stationary phase. This was done by the hydrolytic method described above.

Layers bearing a high concentration of amide were prepared. For each amide and for each impregnation coefficient investigated (0.5 *M* increments) they were divided into two groups. The first of these were not eluted but the layers were divided into bands, each band was removed from the plate and the total amount of amide in each band was determined. A uniform distribution of the amide over the layer was observed for each concentration.

The second group of plates was eluted in the usual way, the mobile phase was allowed to evaporate and the layers were again divided into bands and the amide content of each band was determined hydrolytically. The results here fell into two groups.

(a) Where the impregnation coefficient was lower than the values shown in Table IV there was little or no difference between the amide concentration in the bands below or above the solvent front, *i.e.* the distribution of the amide over the layer is constant.

(b) Where the impregnation coefficient for the amide was greater than the values shown in Table IV then the amide concentration below the solvent front was fairly uniform whereas the amide concentration in the bands above the solvent front were

variable. In the band immediately above the solvent front this concentration was at its highest for the layer, decreasing with an increase in the distance of the band above the solvent front. Furthermore, the higher the initial impregnation coefficient of the layers of this category the greater the amount of amide found in the zones beyond the solvent front. These results clearly confirm the existence of the phenomena of double fronting.

The results quoted in Table IV, however, show that in the case of N-methylformamide and N,N'-dimethylformamide the onset of double fronting, as determined by chemical means occurred at a slightly lower impregnation coefficient than that expected from the R_F values though there is generally good agreement between the two sets of values.

It is now necessary to correlate these impregnation coefficients with the molecular dimensions of the amides. Two possible dimensions could be used: (a) the molar volume of the amide; (b) the parachor of the amide.

The former, because it is normally determined at the boiling point of the liquid to be investigated, was considered to be inappropriate for consideration in a chromatographic system in which the chromatograms were run at temperatures well below the boiling points of the liquid stationary phases used.

The parachor was therefore chosen as being the more appropriate molecular dimension. Its use for chromatographic systems consisting of liquid stationary phases which are members of the same homologous series is particularly significant because it represents the relative molar volume for each stationary phase when it is measured under conditions of unit surface tension, *i.e.* the molecular interactions of the stationary phases will be approximately equal and the spreadability of the phases over the support will be the same.

From values quoted in a standard text[16] we have computed the parachors of the three amides. From the ratios of these we have calculated, relative to formamide, the impregnation coefficients (*i.e.* their molar concentrations in the slurrying solvent) at which double fronting should begin (Table IV). These values show excellent agreement with (a) those obtained by the constancy of R_F values and (b) those obtained chemically for the onset of this phenomenon.

From these results we can rationalise the observed differences in the points of deviation from linearity for the three amides. Thus, for the same impregnation coefficient, formamide because it has the smallest molecular dimensions will cover a smaller

TABLE IV

MOLARITY OF AMIDE IN THE SLURRYING SOLVENT AT WHICH THE PHENOMENON OF DOUBLE FRONTING OCCURS

(a) = based on point at which R_F values become constant; (b) = determined by the hydrolytic methods; (c) = determined from ratios of parachor values for the amides.

Amide	*Molarity*		
	(a)	*(b)*	*(c)*
Formamide	6.0	6.0	6.0
N-Methylformamide	5.0	4.5	4.5
N,N'-Dimethylformamide	4.0	3.5	3.5

area of the cellulose than will either of the other two amides. Therefore it will require a higher impregnation coefficient of this substrate than either of the other two before either the cellulose can be regarded as being fully covered, (*i.e.* before the lower break point of the linear part of the curve is reached), or before double fronting becomes a problem (*i.e.* the upper break point of the linear part of the curve is reached). Similarly a higher impregnation coefficient of N-methylformamide than of N,N'-dimethylformamide is needed to reach the same points. These views therefore confirm our thesis that the molecular dimensions of a stationary phase are of considerable importance in determining the appropriate amount of stationary phase to be used in order to devise a chromatographic system in which the mechanism can be considered to be a simple partition system beween two liquid phases. The significance of these observations cannot be stressed too highly, particularly if the results of the chromatographic investigations are to be used either for the correlation of chromatographic behaviour of solutes with their molecular structures, or if they are to be used to interpret the various interactions which are likely to occur between the three components of the chromatographic system, *viz.* solute, mobile phase and stationary phase.

The chromatographic behaviour of the indanols

In the first instance the indanols investigated are best considered as being a part of a homologous series.

An important observation is that the R_F values of 4-indanol in all systems are higher than the comparable values for the isomeric 5-indanol. The ΔR_M values for these two (within the limits of experimental error and within the limitations of the R_M theory[6]) are comparable with the ΔR_M values for the pair 2-methylphenol and 4-methylphenol for the same system[15]. Because we have already shown that the ΔR_M values for the latter pair are a result of steric hindrance of the hydrogen bonding between the phenolic proton and the carbonyl oxygen of the amide substrate, it is reasonable to suppose that such a steric effect exists between the *peri*-CH_2 group of the fused ring and the hydroxyl group. Evidence for such an interaction in naphthols, tetralols and anthrols has been given by MARCINKIEWICZ *et al.*[17]. Using the system ethyl oleate–aqueous ethanol, GRAHAM[1] observed slight separation of these two isomeric indanols but the ΔR_M values for the two were much smaller than those reported here. Undoubtedly the reason for the difference in the results obtained in the two investigations is the easier solvation of the phenolic group by the free molecules of the mobile phase in the earlier system[1] compared with the more difficult solvation of the same group by the support stabilised molecules of the stationary phases (*i.e.* the amides) used in the present investigation. In the 5-indanol, of course, the fused ring is remote from the phenolic group and hence does not interfere with the latter's hydrogen bonding with the stationary phase.

The importance of the presence of absence of this effect is seen when we consider the methyl substituted compound of each parent indanol. The general effect of methylation of the parent compound is to increase the R_F value of the substituted compound relative to the parent. This effect is superimposed on the other constitutive effects already existing in the molecule. Thus the methylated 4-indanols all have higher R_F values than the corresponding isomeric methylated 5-isomers and the ΔR_M value approximate to the ΔR_M values attributed to the *ortho* effect of the *peri*-CH_2 group.

This is of significance because in the only previous attempted separation of these compounds by thin-layer chromatography it was observed that the small *peri ortho* effect observed for the parent compound lost its significance in the methylated compounds[1].

Over and above this, the increase in the R_F values of the methylated compounds is dependent on the position of the methyl group relative to the hydroxyl group. Thus the 5-methyl-4-indanol has higher R_F values than its isomers. Similarly the R_F values of the 4-methyl-5-indanol and the 6-methyl-5-indanols are higher than their isomers. These higher values can undoubtedly be attributed to the fact that the methyl group is *ortho* to they hydroxyl group in these compounds and hence they exert a steric effect on the approach of the phenolic group to the amide substrate thereby reducing the hydrogen bond interaction between the two.

The ΔR_M values for the *ortho*-methyl group in the *o*-methyl-5-indanols compared with the non *ortho* isomers are of the order expected for such a group from our previously observed results for simple phenols. In the case of the *o*-methyl-4-indanol, however, the ΔR_M values for the *ortho*-methyl group is much higher than expected for a single *ortho*-methyl group, but it must be remembered that in this compound the phenolic group is sterically hindered on both sides, on the one side by the *peri* CH_2 group and on the other by the methyl group. The greater than expected result is in accord with our previous finding that the steric effect of the second methyl group in 2,6-dimethylphenol (compared with 2-methylphenol) is much greater than that of the first (*i.e.* 2-methylphenol compared with 4-methylphenol)[15].

Ortho effects aside, some other effects are worthy of consideration. Firstly, the chromatographic behaviour of the methyl group is independent of the nature of the ring (*i.e.* aromatic or alicyclic) into which it is substituted. This is an important observation because the major difference between the methyl group in the aromatic ring and the same group in the alicyclic ring lies in differences in their electronic effects consequent upon the group being part of a conjugated system in the former and not in the latter. This suggests that hyperconjugation as a contributory constitutive effect plays little part in the chromatography of these compounds, thus confirming the similar observation for these compounds in a different chromatographic system by GRAHAM[1]. Other workers[17,18] have attempted to use the concept of hyperconjugation as a constitutive effect in explaining observed chromatographic behaviour of phenolics but alternative explanations of their results have been expressed[19]. Overall, therefore, and supported by a rational review of the phenomenon of hyperconjugation[20], the existence of this effect as a constitutive chromatographic parameter must be treated with some caution.

Finally, in connection with the methylated indanols, we observe that the 7-isomer has the lowest R_F values and hence is just separable from the other non *ortho* methylated isomers.

Changing the nature of the substitutent from a methyl group to a *tert.*-butyl group has the result of increasing the size of the non-polar part of the molecule and hence its solvation by the non-polar mobile phase. This causes an increase in R_F values. This is to be expected from our previous studies in the methylated phenols[15] and from the application of the MARTIN additivity principle[5]. The relationship between the size of a substituent and its steric effect is clearly shown in the results for 7-*tert.*-butyl-4-indanol and 6-*tert.*-butyl-5-indanol. In the former the steric effect results

from the smaller *peri*-CH_2 group whilst in the latter it stems from the *tert.*-butyl group and hence this latter compound has the higher R_F value of the two.

Consideration of Tables I–III and of Fig. 1 show that the R_F values of all the compounds studied depend upon the nature of the amide investigated; the values obtained on formamide being far higher than the values for the other two amides. The differences in values for the indanols on N-methylformamide and N,N'-dimethylformamide, however, are much smaller. This behaviour again parallels the behaviour of the methylated phenols[15] in the same systems. Such behaviour is turned to advantage in the case of the two compounds, 5,7-di-*tert.*-butyl-4-indanol and 5,7-di-*tert.*-butyl-3-methyl-4-indanol (compounds 8 and 9 in Tables I–III). Although listed as two compounds, the original sample was supplied as a single compound (No. 8). When this was chromatographed on N-methylformamide and N,N'-dimethylformamide at high impregnation coefficients the sample was resolved into two distinct spots of equal size and the problem was to identify them. The ΔR_M values in the two systems were too small to suggest a tri-*tert.*-butyl compound but suggested the possible presence of a methyl group in the compound with the higher R_F values. The original compound 8 was synthesised by butylation of 4-indanol obtained from coal tar fractions and it is known that the 4-indanol is often contaminated with 3-methyl-4-indanol[21]. Thus butylation of impure 4-indanol would yield a mixture of compounds 8 and 9. For this reason we have tentatively identified compound No. 9 as the 5,7-di-*tert.*-butyl-3-methyl-4-indanol. However, it must be stated that the presence of the methyl group in the 3 position will result in a steric effect on the OH group–substrate hydrogen bond. This was taken into consideration in attempting to identify compound No. 9. The ΔR_M values of compounds 8 and 9 are of the order expected for an *ortho*-methyl group. Hence, though positive identification of the structure of compound 9 on the basis of its R_F values above is not possible, it seems probable that the compound has been correctly identified.

CONCLUSIONS

The results of this investigation show that the chromatographic behaviour of indanols on amides is dependent upon: (a) the impregnation coefficient of the amide used, (b) the molecular dimensions of the amides, (c) hydrogen bonding between the proton of the phenolic group and the carbonyl oxygen atom of the substrate, (d) steric effects inherent in the molecules to be separated.

The above factors are all combined to given successful resolution of the 4-indanol series from the corresponding 5-indanols.

ACKNOWLEDGEMENT

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Notes

CHROM. 4496

Gas chromatographic separation of cresol, xylenol and chloroaniline isomers

In a previous communication[1], work was reported on the gas chromatographic separation of pyridine homologues, chloroanilines and toluidines. This was employed as an analytical technique during studies on separation of the isomers of these compounds by solvent extraction. Since then, work on the use of solvent extraction for such separations has been extended to the cresol and xylenol isomers. This necessitated a method of quantitative analysis for the isomers present in the solvent phases. Several previously described liquid phases were studied for the gas chromatographic separation of the isomers but none was found to be satisfactory.

TABLE I

RETENTION TIMES AND CONDITIONS

Condition		*Compound*	*Retention time (min)*
Stationary phase and column length	*Temperature (°C)*		
30% Dispersol CWL; 5 ft.	180	*o*-cresol	9.0
		p-cresol	12.0
		m-cresol	15.0
		2,6-xylenol	5.5
		2,4-xylenol	10.0
		2,5-xylenol	10.0
		2,3-xylenol	12.0
		3,4-xylenol	16.0
		3,5-xylenol	16.0
20% Carbowax 1500; 5 ft.	180	*o*-chloroaniline	4.0
		p-chloroaniline	7.0

The possible use of Dispersol CWL (a product of ICI Ltd.) as a liquid phase for GLC was studied and it was found to give good resolution of the cresol and xylenol isomers. The use of this material as a liquid phase for GLC has not previously been reported.

In our previous communication[1], separation of the chloroaniline isomers was described using polyphenyl ether, diglycerol and glycerol as liquid phases. During subsequent work, it has been found that Carbowax 1500 is superior to any of these phases for this separation.

Experimental

Instrumentation. A Pye Series 104 dual flame ionisation, temperature-programmed chromatograph Model 24 was used.

Column preparation. Two parts by weight of Dispersol CWL and one part by weight of solid potassium hydroxide were dissolved in aqueous methanol and applied to Chromosorb W (80–100 mesh) as solid support. Celite was employed as solid support for the Carbowax 1500.

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Gas chromatography of sulfur mustard and its analogs

Vesicants such as bis(2-chloroethyl) sulfide (sulfur mustard) and 2-hydroxyethyl-2'-chloroethyl sulfide (sulfur half-mustard) have been studied extensively as clinically useful anti-tumor agents[1–3] and as alkylating agents in studies of the mechanism of cytotoxicity with DNA *in vitro* and *in vivo*[4–7]. A recent review on the chromatography of alkylating agents[8] discussed the paper and thin-layer chromatography of sulfur mustard and its analogs, but there appears to be no established procedure for gas chromatographic determination of these compounds. The present report concerns the development of a gas chromatographic method for the analysis of sulfur mustard, half-mustard, and a major hydrolysis product of both, bis(2-hydroxyethyl) sulfide (thiodiglycol).

Bis (2-chloroethyl) sulfide and thiodiglycol were obtained from K & K Laboratories, Inc., Plainview, N.Y. 2-Hydroxyethyl-2-chloroethyl sulfide was synthesized by the reaction of ethylene dichloride with sodium mercaptoethanol as described by SELIGMAN *et al.*[3]. The final product was purified by distillation at 48°, 0.08 mm; its naphthyl urethan derivative melted at 96.5–97.5° (ref. 9).

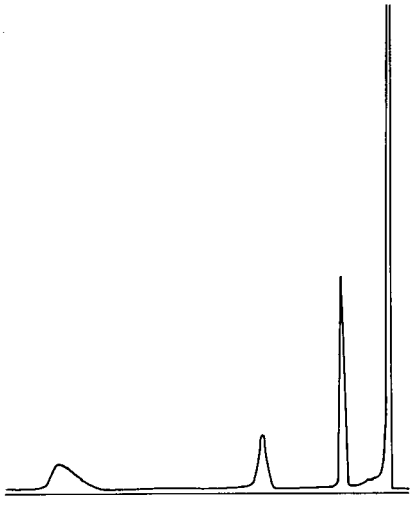

Fig. 1. Isothermal separation of sulfides. Elution order: (first) sulfur mustard, half-mustard, thiodiglycol. The initial off-scale peak is methylene chloride.

Glass columns, 1.5 meter by 0.2 cm (I.D.), were packed with 100–120 mesh Gas-Chrom Q coated with 3% cyclohexanedimethanol succinate (Hi-Eff 8bp, Applied Sciences) and cured overnight at 240° with a low helium flow. Isothermal analyses were made using an Aerograph 600-B gas chromatograph with a hydrogen flame ionization detector and a glass inlet port liner. The column and inlet temperatures were 120° and 170°, respectively, with a helium flow rate of 35 ml/min. Samples were injected as dilute solutions in methylene chloride; the separation obtained is shown in Fig. 1. A linear relationship between peak area and sample size was obtained, as illustrated for sulfur mustard in Fig. 2.

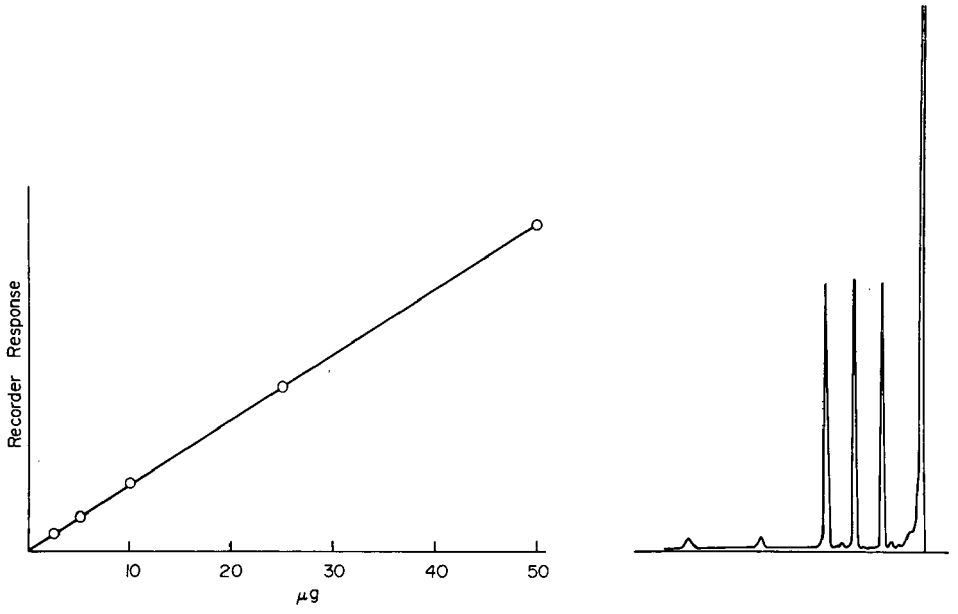

Fig. 2. Standard curve for sulfur mustard. Peak area response is plotted against amount injected.

Fig. 3. Programmed-temperature separation of sulfides. Elution order the same as in Fig. 1 (main peaks). Late-eluting components are impurities.

Temperature programming both shortened the analysis time and eliminated the peak distortion observed with thiodiglycol in Fig. 1. Fig. 3 shows the results obtained using a Perkin-Elmer Model 900 gas chromatograph, injection port and detector temperatures of 240° and 260°, respectively, and a column temperature linearly programmed from 110° to 230° at 8°/min. The helium flow rate was 40 ml/min.

There was no indication of a need to "saturate" these columns with the sample materials. The detector responses per microgram of sulfide injected was the same for the first run in the morning as for the tenth subsequent run.

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CHROM. 4480

The separation of sodium salts of sulphocarbazole acids by thin-layer chromatography

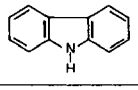
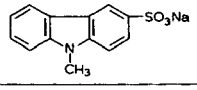
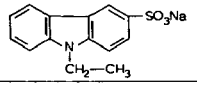
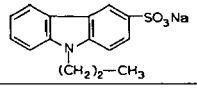
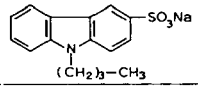
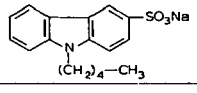
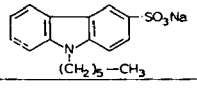
Carbazole is a basic substance for organic derivatives which are important in the chemical industry. Large quantities of carbazole occur in and are obtained from coal tar. Sodium derivatives of sulphocarbazole are widely used in industry, and a rapid and simple method of identifying them during processing is desirable. Apart from that, the relationships between R_F and the molecular structure of the compounds examined is very interesting. The present paper deals with the conditions for the separation of these substances by adsorption thin-layer chromatography.

Experimental

The separation of the above substances was examined on a series of adsorbents using different solvent systems in order to establish an optimal chromatographic system. The most suitable adsorbent for the separation of these compounds was Silica Gel GF 254 (Merck) coated on glass plates. The adsorbent layers were prepared as suggested by the manufacturers[1] and the plates were activated by heating at 135° for 2 h.

TABLE I

CHROMATOGRAPHED SUBSTANCES

No.	*Structural formula*	*Chemical name*
1		Carbazole
2		Sodium salt of *n*-methyl sulphocarbazole
3		Sodium salt of *n*-ethyl sulphocarbazole
4		Sodium salt of *n*-propyl sulphocarbazole
5		Sodium salt of *n*-butyl sulphocarbazole
6		Sodium salt of *n*-pentyl sulphocarbazole
7		Sodium salt of *n*-hexyl sulphocarbazole

The solutes (concentration, 0.04 mole/l) were spotted with calibrated 4-μl pipettes. Table I presents a list of the compounds examined and their structure formulae.

The chromatograms were developed using 1- and multi-component solvent systems by the ascending technique. The following 1-component solvents were used as the mobile phase: (1) water, class AB* according to the classification proposed by PIMENTAL AND MCCLELLAN[2,3]; (2) methanol, class AB; (3) propanol, class AB; (4) acetone, class B; (5) chloroform, class A; (6) trichloroethylene, class A; (7) benzene, class N; (8) toluene, class N; (9) carbon tetrachloride, class N.

Ethyl acetate–methanol–formic acid–pyridine (80:10:10:10) and (75:7.5:7.5:10), ethyl acetate–methanol–formic acid–piperidine (80:10:10:10) and ethyl acetate–methanol–formic acid–morpholine (80:10:10:10) were used in multi-component solvent systems. All the solvents were dried with silica gel[4], heated at 300°. The results are presented as chromatographic spectra for 1- and multi-component solvent systems.

Results

Separation by adsorption TLC depends on the type of substance used, on the solvent or solvent system, and especially on the adsorbent because of the importance of the differentiation of strengths involved in the chromatographic system consisting of adsorbent–separated substance–solvent.

Fig. 1 presents the results of the separation of substances using a 1-component mobile phase.

As shown no separation of the chromatographed substances takes place. The highest R_F values are obtained for the solvents of class AB according to EWELL *et al.*[2] and PIMENTAL AND MCCLELLAN[2,3]. The lowest R_F values are obtained using the mobile phase of classes A, B and N. The course of the carbazole spectrum deviates from that of its derivative, which can probably be explained by the lack of a steric effect in this substance.

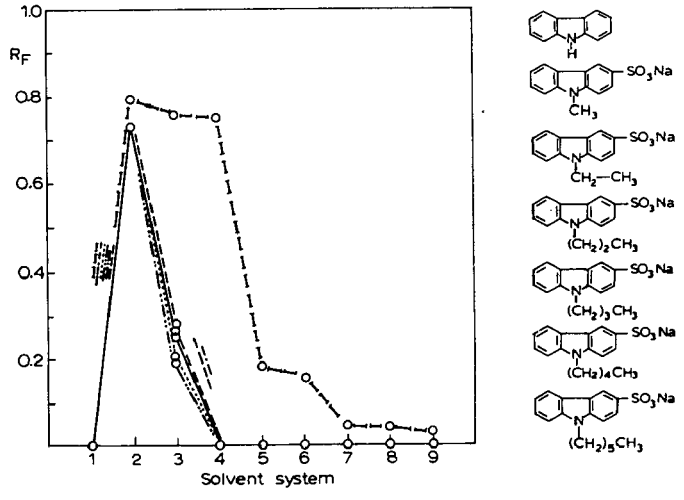


Fig. 1. Chromatographic spectra of carbazole and derivatives of sulphocarbazole obtained using a 1-component mobile phase: 1 = water; 2 = methanol; 3 = propanol; 4 = acetone; 5 = chloroform; 6 = trichloroethylene; 7 = benzene; 8 = toluene; 9 = carbon tetrachloride.

Sodium derivatives of sulphocarbazole differ only by the quantity of CH_2-groups; all the substances have active SO_3Na-groups and a nitrogen atom and can react with OH-groups on the silica gel surface by forming a hydrogen bond of a different energy. Apart from these reactions, others can occur which are typical of this kind of adsorption (specific or nonspecific interaction[5–8]).

No strong interactions from the 1-component mobile phases used are observed due to the strong adsorption of the substances on the silica gel surface and, consequently, to the lack of more differentiated adsorption capacities, probably affected by the formation of hydrogen bonds between a nitrogen atom and OH-groups on the silica gel surface. Thus, the compounds examined show similar R_F values (see Fig. 1).

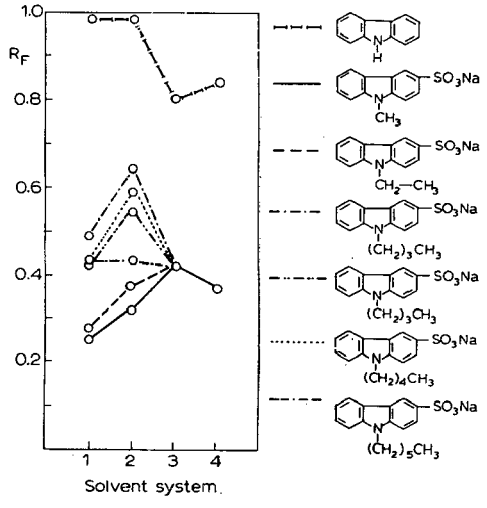


Fig. 2. R_F values of carbazole and of derivatives of sulphocarbazole obtained using a multi-component mobile phase: 1 = ethyl acetate–methanol–formic acid–pyridine, (80:10:10:10); 2 = ethyl acetate–methanol–formic acid–pyridine, (75:7.5:7.5:10); 3 = ethyl acetate–methanol–formic acid–piperidine, (80:10:10:10); 4 = ethyl acetate–methanol–formic acid–morpholine, (80:10:10:10).

The results obtained using a multi-component solvent system as the mobile phase are presented in Fig. 2. Using a multi-component mobile phase, differentiations of adsorption capacities of separate components of this mixture are observed. Consequently, different actions occur between the solvent molecules and the substances separated, on the one hand, and the adsorbent and the substances, on the other.

As the properties of the components of the mobile phase differ, a competitive displacement of the adsorbed molecules from the adsorbent surface will take place, which results in the separation of the chromatographed compounds. As shown in Fig. 2, a satisfactory separation of the compounds takes place only in one case using ethyl acetate–methanol–formic acid–pyridine (75:7.5:7.5:10) as mobile phase. This phenomenon is associated with the adsorption of a substance from multi-component solvent systems which is still not well understood. The above results show that a

satisfactory separation of the derivatives of sulphocarbazole acid is available using a mixed solvent of definite structure, as a mobile phase.

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CHROM. 4482

A quantitative analysis of sorbose by thin-layer chromatography in the presence of some frequently occurring monosaccharides

Thin-layer chromatography is utilized extensively for the analysis of substances including sugars and sugar mixtures. Several authors[1–10] have described solvent systems which separate the various components of monosaccharide mixtures containing, among others, sorbose, by using variously prepared layers. The R_F values given by the authors show that the determination of sorbose is most frequently disturbed by fructose and mannose and more rarely by glycose and xylose. The best separation of sorbose from the other monosaccharides by unidirectional development has been published by WALDI[8] and by LATO and co-workers[9,10]. The two-dimensional chromatograms published by FIGGE[3] also show a good separation; however, it is well known that for quantitative determinations the unidimensional technique is more preferable.

Our present paper describes a method which allows the separation of sorbose from some frequently occurring monosaccharides by unidirectional development and its quantitative analysis.

Separation

30 g of Kieselgel G are mixed with 70 ml of 1/15 M phosphate buffer (pH 7), and a layer 0.25 mm thick is prepared from this mixture. The plates are then dried for 1 h at 100° after spreading. On the plates divided into strips are applied 25–50 μg of the aqueous solution of the following monosaccharides in 10 μl: sorbose, glucose,

galactose, mannose, fructose and xylose. For development the solvent acetone–ethyl acetate–acetic acid–water (50:50:25:5) is used. The solvent front is permitted to reach a height of about 15 cm from the starting point, and then the plate is removed, dried in air, and the development is repeated in the same solvent till the solvent front reaches a height of 15 cm. After drying at 100°, the plate is sprayed with the reagent α-naphthol.

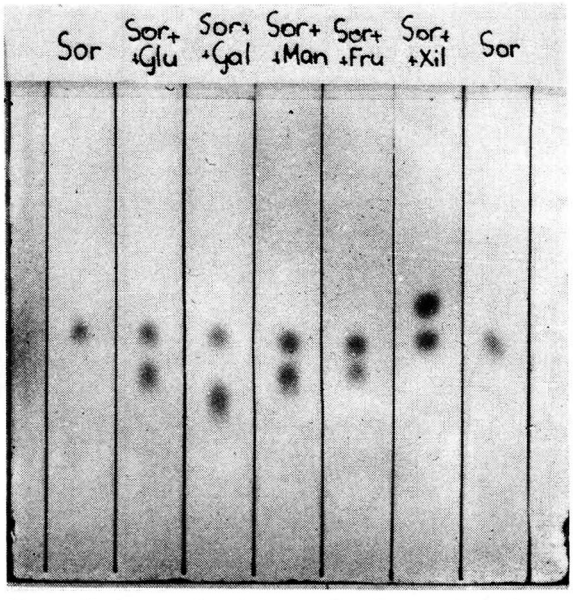

Fig. 1. Sorbose and mixtures of sorbose and various monosaccharides on a Kieselgel G sheet prepared with a phosphate buffer (pH 7) after a single development in the solvent acetone–ethyl acetate–acetic acid–water (50:50:25:5). Spray reagent: α-naphthol.

Fig. 1 shows the chromatogram of pure sorbose and that of a mixture of sorbose and the various monosaccharides after the first development. The spots are already separated after the first development, and, after the second development, shown in Fig. 2, the separation is complete.

Quantitative determination

Quantitative determination is carried out after the above-mentioned second development. The calibrating curve is prepared as follows: 100, 75, 50 and 25 μg of sorbose are applied to the parallel strips of the plate and developed as described above. The two strips at the edges of the sheet are sprayed with the reagent α-naphthol with the rest of the plate covered by a glass plate. The spot with the R_F value corresponding to sorbose is scratched off or sucked off from the undeveloped strips of the plate with a suitable device and is transferred to a small G4 glass filter. The glass filter is fixed in a 2-ml test tube with a glass stopper, and the spot is eluted in several portions with a total of 2 ml of distilled water, the liquid being sucked off after the addition of each portion. Elution is carried out at room temperature with several minutes between additions of the eluting portions. Using the 2-ml eluate, an anthrone color reaction is carried out using 0.2 % anthrone reagent with sulfuric acid (95 wt. %) as described

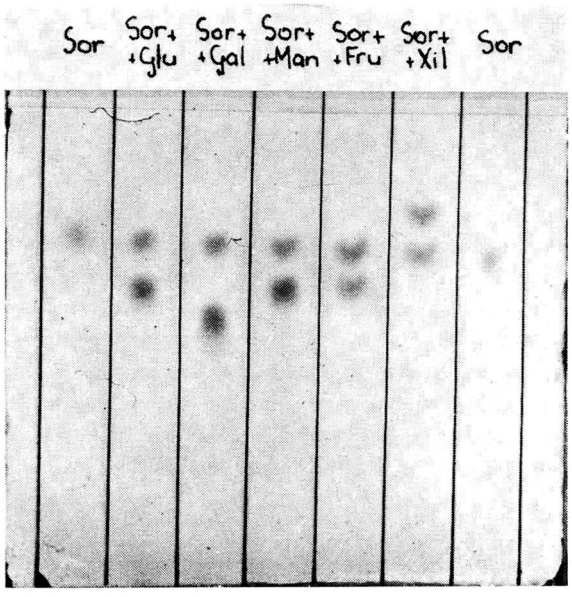

Fig. 2. Sorbose and mixtures of sorbose and various monosaccharides on a Kieselgel G sheet prepared with a phosphate buffer (pH 7) after a double development with the solvent acetone–ethyl acetate–acetic acid–water (50:50:25:5). Spray reagent: α-naphthol.

in the literature[11]. To the 2-ml eluate cooled in ice 4 ml of the anthrone reagent are added in small portions and then heated for 10 min on a boiling water bath. After cooling on an iced water bath, the extinction is measured photometrically at 620 nm. The blank test is prepared by dropping a solution containing no sorbose on one of the strips, by eluting the spot having the R_F value equal to that of sorbose and by further treatment used for the spots containing sorbose. By this method we obtained the following extinction values corresponding to the different quantities of sorbose (Table I).

The averages of these extinction values were presented graphically as a function of

TABLE I

A COMPARISON OF EXTINCTION VALUES OBTAINED APPLYING VARIOUS QUANTITIES OF SORBOSE

Quantity of sorbose applied to plate (μg)	*Extinction values*	*Average*
100	0.330, 0.335, 0.302, 0.310, 0.334	0.322
75	0.254, 0.246, 0.220, 0.218, 0.262	0.240
50	0.124, 0.148, 0.174, 0.170, —	0.154
25	0.095, 0.092, 0.080, 0.073, 0.085	0.085

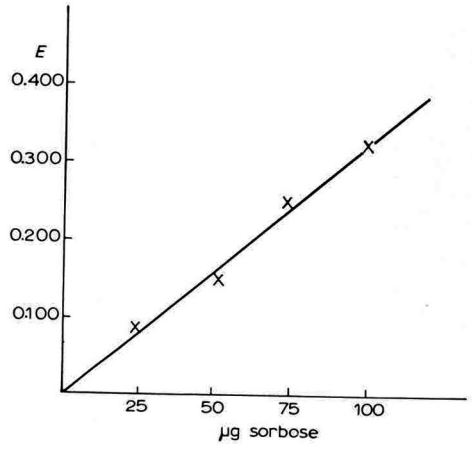

Fig. 3. Calibration curve obtained by plotting the average of the extinction values *versus* quantity of sorbose applied to plate.

concentration. The calibration curve obtained in this way is shown in Fig. 3. The unknown sorbose concentration is found with the aid of this calibration curve.

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CHROM. 4351

Determination of degradation kinetics of chlorzoxazone by thin-layer chromatography

In 1955 GARRETT AND CARPER[1] demonstrated the use of chemical kinetics to predict the stability of pharmaceuticals. However, due to the complex nature of most pharmaceuticals and to the complexity of degradation products, in many cases classical analytical methods proved to be too inaccurate and imprecise for such studies. Studies on degradation kinetics of newly developed chlorzoxazone-N-methyl-*d*-glucamine (chlorzoxazone-NMG) in nonaqueous solvents have been problematic. Spectrophotometric analysis was impossible due to the overlap of the absorbance spectra of the degradation products with that of chlorzoxazone (Fig. 1). Due to its low solubility in water-immiscible solvents and to the additional interference of breakdown products, chlorzoxazone could not be separated from its decomposition products by solvent extraction. Although a gas chromatographic method has been reported for the separation and identification of chlorzoxazone[2], it would be unsuitable for this formulation which is heat labile. Thus, TLC, which was initially used pharmaceutically[3] and whose use in this field has been reviewed by COMER AND COMER[4], offers a solution to such problems.

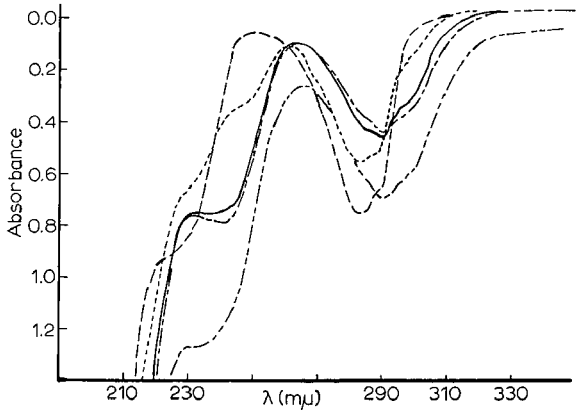


Fig. 1. Spectra of chlorzoxazone and its degradation products. — —, chlorzoxazone and also chlorzoxazone solution in PEG 200 containing sorbitol solution and glycerine (curves superimposed); - - - -, chlorzoxazone-NMG formulation immediately after preparation; ———, chlorzoxazone-NMG formulation after 4 h at 100°; — — — —, chlorzoxazone-NMG formulation after 6 at 100°; — — — — —, chlorzoxazone-NMG formulation after 24 h at 100°.

The purpose of this investigation was to develop a simplified and accurate TLC method for the quantitative determination of our heat-labile chlorzoxazone formulations in the presence of decomposition products and to demonstrate the applicability of the method for conducting chemical kinetic studies on the degradation of complex pharmaceutical formulations.

Materials and methods

Chemicals and stability formulations. Chlorzoxazone (trademark Paraflex) was

generously supplied by McNeil Laboratories, Inc. (Fort Washington, Pa.), and N-methyl-*d*-glucamine (NMG) was obtained from K and K Laboratories, Inc. Methanol was spectro grade, while other chemicals and solvents were reagent or U.S.P. grade. Stability data are based on the formulation in Table I; other formulations studied differed in concentrations of NMG, sorbitol solution, glycerine and in the conditions under which they were prepared.

TABLE I

CHLORZOXAZONE-NMG FORMULATION FOR STABILITY STUDIES

Chemical	*Amount*
Chlorzoxazone	5.0 g
N-Methyl-*d*-glucamine	6.0 g
Sorbitol solution (U.S.P.)	5.0 ml
Sodium metabisulphite	0.1 g
Polyethylene glycol 200 q.s.	100.0 ml

Spectra of successively degraded formulations. One-ounce flint glass bottles, each containing about 25 ml of the formulation shown in Table I, were tightly sealed with aluminum foil and placed in an 100° oven. Samples were withdrawn at different time intervals and, after dilution with methanol, were scanned directly using a Perkin-Elmer Model 202 spectrophotometer. The spectra are shown in Fig. 1.

Adsorbents and solvents. Several adsorbents (Table II) were examined for selection by a standard procedure. As the stationary phase to be used in further studies, Silica Gel HF_{254} (Brinkmann Instrument, Inc.) was chosen on the basis of easy visualization of chlorzoxazone spots under UV light, stronger binding of the gel to the glass plates and no interference in the analytical procedure.

TABLE II

EVALUATION OF ADSORBENTS FOR THIN-LAYER CHROMATOGRAPHY

Adsorbent	*Binding of adsorbent*	*Detectability of chlorzoxazone*			*Contaminating ions*[a]
		Visible light	*Short wave UV light*	*Long wave UV light*	
Aluminium Oxide G	Poor	No	No	No	Ca^{2+}
Silica Gel G	Poor	No	No	No	Ca^{2+}
Silica Gel H	Good	No	No	No	None
Silica Gel GF_{254}	Good	No	Yes	No	Ca^{2+}
Silica Gel HF_{254}[b]	Good	No	Yes	No	None

[a] Taken from the labels of the containers.
[b] Adsorbent selected for use in TLC.

Because chlorzoxazone is soluble in methanol but is sparingly soluble in chloroform, several mixtures of these solvents (Table III) were examined. The solvent mixture, a 9:1 ratio of chloroform–methanol, which gave an R_F value of 0.58 (Table III) was selected as the mobile phase for further studies. Using a standard TLC method

TABLE III

R_F VALUES OF CHLORZOXAZONE FOR VARIOUS SOLVENT MIXTURES

Ratio chloroform–methanol	*Approximate R_F values*[a]
19:1	0.32
9:1[b]	0.58
4:1	0.87
7:3	1.00
3:2	1.00

[a] Each value is an average of two experiments.
[b] Solvent mixture selected for use in TLC.

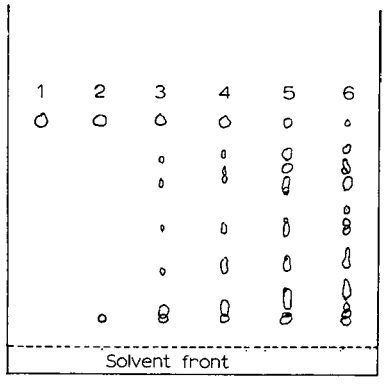
and Silica Gel HF_{254} as the stationary phase, chloroform–methanol (9:1) afforded greater separation of chlorzoxazone from degradation products as well as from other ingredients in the formulation.

Preparation of thin-layer plates. Standard 20 × 20 cm chromatographic plates were coated with a 0.25-mm layer of the adsorbent using a Desaga Model SII applicator (Brinkmann Instrument, Inc.). The plates were prepared and activated according to instructions from Brinkmann Instrument, Inc. The activated plates were prewashed with the solvent system, dried and stored in a dessicator.

Development of chromatograms. The samples to be assayed were diluted fivefold with methanol. For each formulation, 20 μl of the diluted solutions were applied as four 5-μl spots to the previously prepared chromatograms, and as many as 16 spots (4 formulations) could be run on each chromatogram. These chromatograms were developed in Desaga rectangular trough chambers lined with Whatman No. 3MM filter papers. The chambers were saturated with the solvent vapor by being allowed to stand for about 20 min before use. The solvent front was permitted to rise approx. 14 cm from the bottom edge of the plates. These plates were air dried, and the chlorzoxazone spot was marked under shortwave UV light. Numerous breakdown products were visualized by placing the resolved chromatograms in a saturated iodine chamber for about 15 min (Fig. 2). The size of the areas marked was kept constant for each spot in order to maintain a constant adsorbent blank.

Removal and elution of spots. A simple scraping and eluting unit was obtained by modifying an assembly described by MOTTIER AND POTTERAT[5]. The glass portion of a medicinal dropper (10 cm long) was used for this purpose. The collars were filed off, and one side of this open end was flattened in a flame to give a smooth scraping surface. The narrow end of the dropper, firmly packed with glass wool, was attached to a vacuum line. Each area was scraped off the plate and completely drawn into this assembly by the vacuum. The assembly was supported above a volumetric flask. The chlorzoxazone was eluted with 8–9 ml of methanol without any transfer of the adsorbent, and the volume made up to 10 ml with methanol. These solutions were filtered through teflon filter discs having a 5-μ pore diameter (Millipore LSWP01300). The absorbances of these solutions were recorded at 238 mμ using a Beckman-DU spectrophotometer. The readings were corrected with the adsorbent blank (methanol)[6], and the concentrations of the drugs were determined from the calibration curve.

Preparation of the calibration curve. Solutions of chlorzoxazone were prepared



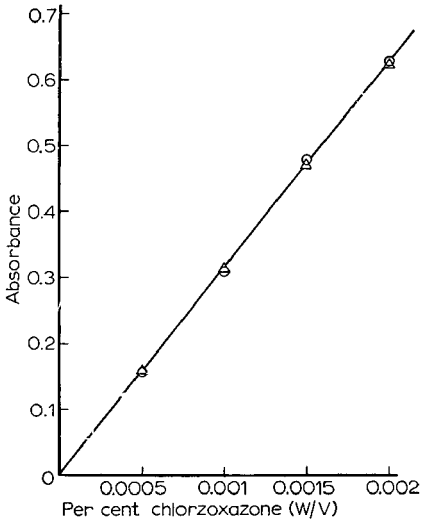


Fig. 2. Thin-layer chromatogram showing separation of chlorzoxazone from its solutions and from its degradation products (each position represents 5 μl of 1.0% chlorzoxazone after dilution with methanol). 1 = chlorzoxazone; 2 = chlorzoxazone solution in PEG 200 containing sorbitol solution and glycerine; 3 = chlorzoxazone-NMG formulation immediately after preparation; 4 = chlorzoxazone-NMG formulation after 4 h at 100°; 5 = chlorzoxazone-NMG formulation after 6 h at 100°; 6 = chlorzoxazone-NMG formulation after 24 h at 100°.

Fig. 3. Calibration curves for chlorzoxazone. ○—○, after TLC-elution; △—△, after direct dilution.

in polyethylene glycol 200 (PEG 200) containing 2.5% each of sorbitol solution, propylene glycol and glycerine. These solutions were diluted with methanol to give different concentrations of chlorzoxazone. Each solution was resolved using the TLC method previously described, and after filtration the absorbance was measured. The readings were used to prepare a calibration curve.

A similar calibration curve was prepared by direct dilution of chlorzoxazone with methanol using absorbance readings at the same wavelength. Both these curves are shown in Fig. 3.

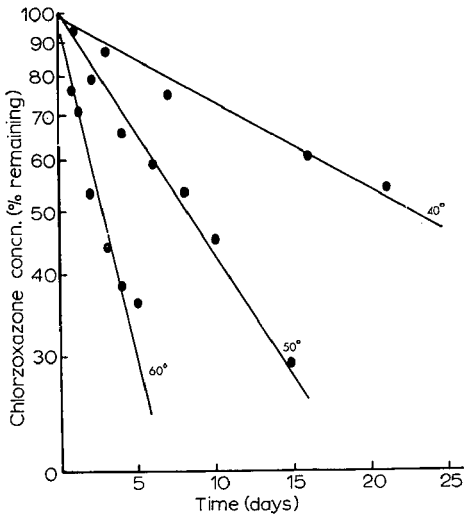
Reproducibility and recovery by TLC. Chlorzoxazone solution (5 w/v %) was prepared in PEG 200 containing a 5 % (v/v) sorbitol solution. The solution was assayed by the TLC-elution method and by a direct method in which the test solution was diluted with an appropriate amount of methanol and absorbances were read at 283 mμ. The results are shown in Table IV.

TABLE IV

COMPARISON OF THE TLC METHOD WITH THE DIRECT DILUTION METHOD

Method	*Replications (No.)*	*Actual value (mg/ml)*	*Mean assay value (mg/ml)*	*Standard deviation (mg/ml)*
Direct dilution	12	50.0	50.00	±0.15
TLC	12	50.0	49.77	±0.33

Accelerated stability studies. Chlorzoxazone-NMG formulations were placed in 40, 50 and 60° ovens. The samples were withdrawn at predetermined time intervals and assayed by the TLC–elution method. Data are shown graphically in Fig. 4. An Arrhenius plot, constructed for the rate constants calculated from Fig. 4, is shown in Fig. 5.



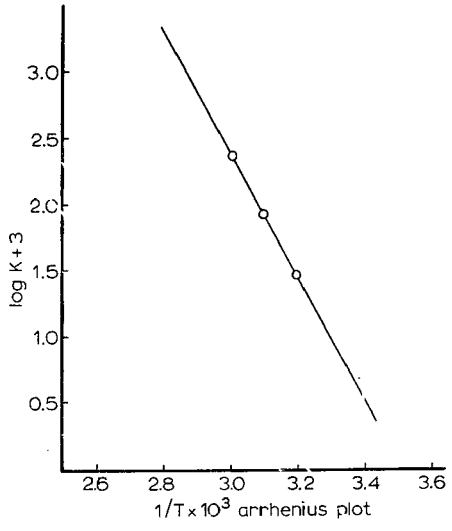


Fig. 4. Apparent first-order plots for degradation of chlorzoxazone-NMG formulation.

Fig. 5. Arrhenius plot for chlorzoxazone-NMG formulation.

Results and discussion

Two factors, *i.e.* the resolution of the active ingredient in the formulation and a satisfactory procedure for the quantitative analysis of this ingredient, were essential for the successful utilization of TLC in the study on degradation kinetics of complex pharmaceuticals.

Fig. 2 shows the resolution of chlorzoxazone and of its formulations at different levels of degradation. It is apparent from this figure that the spots of other products in the formulation neither overlapped nor interfered with the chlorzoxazone spots.

Excellent recovery was made possible by the simple collecting unit which could scrape, remove and elute the chlorzoxazone spots in the same unit without transfer. Almost complete recovery (99.5 %) of chlorzoxazone showed the accuracy of this TLC–elution method (Table IV). The small loss (0.5 %) was well within the limits of experimental error. The accuracy of the method was also apparent from the superimposed calibration curves (Fig. 3) based on direct dilution of chlorzoxazone and its recovery after being chromatographed. The low value of the standard deviation (99.5 $\pm$ 0.66, see Table IV) also indicated the high precision of this method.

The TLC–elution method was used successfully for studying the degradation kinetics of chlorzoxazone-NMG formulations. Fig. 4 shows good first-order degradation plots of the formulation under study. Rate constants of degradation calculated from Fig. 4 gave a good Arrhenius plot (Fig. 5). Using this plot it was possible to

predict the stability of the formulation at any desired temperature. The predicted values were well within the limits of experimental error. Using this TLC–elution method, the stability of other chlorzoxazone-NMG formulations could also be predicted with similar accuracy and precision.

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Book Review

CHROM. 4479

Techniques of Thin-Layer Chromatography in Amino Acid and Peptide Chemistry, 2nd English ed. (second revision), by G. PATAKI, Ann Arbor–Humphrey Science Publishers, Ann Arbor and London, 1969, 252 pp., price $ 18.75.

This book was first published in German in about 1965 and all references within the body of the book are prior to this date. The English translation appeared in 1967 and the so-called English edition (revised) is a reprint with the addition of an updated bibliography. The author is at pains to make this clear whereas the publisher appears to be equally determined to hide the fact.

The author's work is universally known and respected and there is little doubt that it was remarkably up-to-date when originally published. However, its omissions are now glaringly evident with but two pages devoted to dansyl derivatives, nothing on dansyl-Edman subtractive methods or on what are now the standard chromatographic methods for these compounds. Likewise the comments on the TLC of blood amino acids are now entirely incorrect.

In spite of the long delay in the appearance of this translation, it is full of irritating errors such as "protein is fused into a quartz tube with acid" instead of "hydrolysed in a sealed tube under pressure", etc. In my view the publishers have done the author a great disservice in bringing out a poor translation of a five-year-old work under the guise of a second revised 1969 edition.

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IVOR SMITH

News

Apparatus

New from Phoenix Precision Instrument Co. (Philadelphia, Pa., U.S.A.) is the *anaerobic cell* assembly, a new accessory for its dual-wavelength scanning spectrophotometer. The anaerobic cell accessory was developed for spectrophotometric measurements in an oxygen-free environment. Outstanding features include a unique stop-cock, cell-plunger assembly that provides for purging of the cell and the subsequent addition of a reagent under inert environmental conditions. A special housing cover that excludes outside light, so that measurement can be made under room light conditions, is also a feature of the anaerobic cell accessory.

For further information apply to the publisher under reference No. Chrom. N-256.

Manufacturers' Literature

New literature on the Series 2000 and 3000 full size circular scale and the 8000 full size strip chart *recorder/controllers* is available from Barber-Colman (Rockford, Ill., U.S.A.). All of these instruments are null balance and incorporate a Zener solid state reference source. A wide variety of control forms are available, including proportional or proportional plus rate plus reset. The bold face indicating scale of the 2000 and 3000 series can be read at a distance of more than 50 ft. The strip chart 8000 series incorporates a full 12 in. indicating scale with a 11 in. calibrated width. Also included in the 8000 series is a quick change multipoint instrument which gives the user a record of up to 24 points and the advantage of six additional switches for signal indication.

For further information apply to the publisher under reference No. Chrom. N-253.

We gather from Newsheet No. 1 July 1969 from Fluorochem Ltd. (Glossop, Derbyshire) that a variety of *thermal stable polyesters* are available and that they solicit inquiries for other stationary phases.

For further information apply to the publisher under reference No. Chrom. N-258.

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CHROM. 4497

GAS CHROMATOGRAPHY USING THE VAPOUR OF ORGANIC SOLVENTS AS CARRIER GASES

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SUMMARY

The vapour of organic solvents, such as ethanol, benzene and carbon tetrachloride was used as a carrier gas in gas chromatography. The effect of the vapour on the retention volume was examined at the liquid phase of polyethylene glycol, dioctyl phthalate and silicone oil. The experimental value of (gaseous diffusion coefficient for N_2 and octane)/(that for carbon tetrachloride and octane) was 2.3, which showed good coincidence with the calculated values.

INTRODUCTION

The carrier gases in gas chromatography (GC) usually used are permanent gases, while in some cases the vapour of formic acid[1] or water[2–5] is added to the permanent gas to reduce tailing in order to obtain reproducible and quantitative results. The use of water[6,7] or carbon dioxide[8] as a carrier gas has been recommended in the analysis of polar compounds, and the values of the partition coefficients of the samples obtained using the latter carrier gases vary in comparison with those obtained using permanent carrier gases.

This study attempts to use the vapour of organic solvents as carrier gas in GC, such as in other chromatographic methods that use many development solvents, and examines the effect of organic vapours on partition and diffusion coefficients in comparision with nitrogen as carrier gas.

EXPERIMENTAL

Apparatus

Fig. 1 shows the schematic flow system of a gas chromatograph consisting of three parts: (1) gas generator part, (2) separation column and (3) condensing part of organic carrier vapour. The three parts were maintained at constant temperature, and the connecting gas lines were heated with tape heaters. The 400-ml cupper tanks (A and B in Fig. 1), resistant column (D) (I.D. 4.5 mm) and a thermal conductivity

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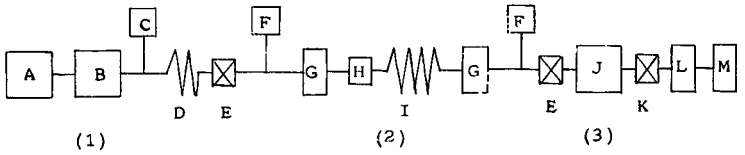


Fig. 1. Flow system consisting of (1) gas generator part, (2) separation column and (3) condensing part of organic carrier vapour. A = generator tank, B = buffer tank, C = pressure gauge, D = resistant column, E = needle valve, F = compound pressure gauge, G = detector, H = injector, I = column, J = trap for condensing of organic vapour, cooled with dry ice–methanol or water, K = stopcock, L = $CaCl_2$ trap, M = vacuum pump.

detector having tungsten filaments of 22 Ω were connected in order. Columns were of cupper tube, I.D. 4.5 mm, and supports were 40–60 mesh of firebrick C-22. Temperature of the column was maintained at 125°. Silicone oil (DC-550), polyethylene glycol, dioctyl phthalate and other reagents were extra pure grade.

Procedure

The generator tank (A) contained approximately 300 ml of organic solvent. The system was evacuated by vacuum pump at room temperature and after evacuation the stopcock (K) was closed. Then the generator part (1) was heated to an adequate temperature (80–110°) at which the vapour of the solvent reached a pressure of 1–2 kg/cm^2. The flow rate of the solvent vapour was adjusted by controlling the inlet and the outlet pressures of the column, using needle valves (E). The column pressure and the temperature of the generator (1) were adjusted for a minimum noise level at the baseline. In some experiments the condensing part (3) was immersed in a water bath which was regulated at constant temperature, and the flow rate of organic carrier vapour was controlled by the temperature difference between generator part and condensing part without using needle valves. Before operating the gas chromatograph, the system was allowed to condition for several hours to reach an equilibrium of the organic vapour with the coating liquid. The flow rate was calculated by dividing the dead volume of the separation column by the retention time of hydrogen at the organic carrier vapour.

The vapour of carbon tetrachloride, benzene or ethanol was employed as a carrier gas. In most cases, 0.1–5 μl of a sample solution were injected. The gas chromatograph was usually operated at the reduced pressure.

Ratio of retention volume (RRv)

The ratio of the retention volume is given by dividing the retention volume (*Rv*) of a sample with an organic carrier vapour by a standard retention volume of the same sample with nitrogen carrier gas. Since a retention volume of octane vapour with nitrogen carrier gas was independent of the mean absolute pressure, from 150 to 800 mm Hg, the retention volume of each sample was taken as the standard retention volume when the outlet pressure of a column was 1 atm.

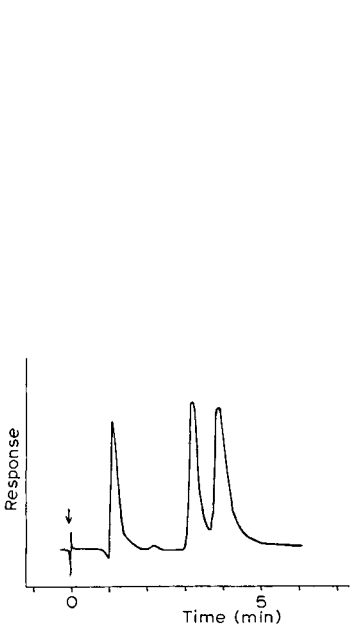
RESULTS AND DISCUSSION

The gas chromatograph using organic vapour as a carrier gas gave an excellent baseline and sensitivities which were nearly equal to the ordinary gas chromatograph

with nitrogen carrier gas. An example of the chromatogram with organic carrier vapour is shown in Fig. 2.

Relation between Rv and mean absolute column pressure

It is shown in Fig. 3 that the retention volumes of *n*-octane and toluene depended on the absolute column pressure, while the retention volume of *n*-propanol did not. The higher the mean absolute column pressure, the more soluble was the vapour of organic solvent into a liquid phase according to Henry's law. Then it is suggested that the nature of the silicone oil was somewhat altered due to the dissolution of carbon tetrachloride vapour in it. The tendency of the retention volumes shown in Fig. 3 might be explained by the above reason.



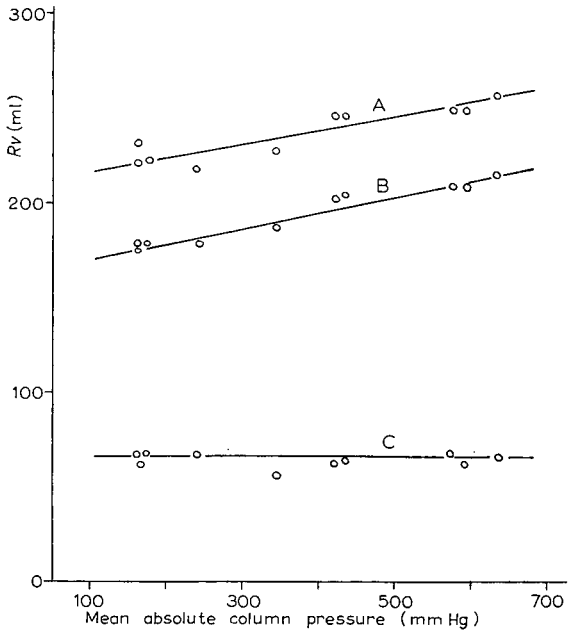


Fig. 2. Chromatogram using CCl_4 vapour as a carrier gas. Column, silicone oil 25% on firebrick C-22, temperature, 125°; column pressure, P_i, 515 mm Hg; P_o, 145 mm Hg; flow rate, 60 ml/min; sample, 0.5 μl of a mixture of toluene, *n*-octane and *n*-propanol.

Fig. 3. The relation between the mean absolute column pressure and retention volume with CCl_4 carrier vapour. *A* = *n*-octane, B = toluene, C = *n*-propanol. Column, silicone oil 25% on firebrick C-22; temperature, 125°; flow rate, 32–20 ml/min.

Second virial coefficients for carrier vapours and solute vapours

The second virial coefficient (B_{12}) characterizes the interaction between unlike molecules. The calculated values of B_{12} for CCl_4–*n*-octane, N_2–*n*-octane, CCl_4–toluene and N_2–toluene are −1020, −220, −1010 and −241 $cm^3 \cdot mole^{-1}$, respectively. They were calculated using the empirical formulas proposed by GUGGENHEIM AND MCGLASHAN[9]. Since the values of the second virial coefficients for carbon tetrachloride vapour and solute vapours are more negative than coefficients for nitrogen and solute vapours, the state of the former gas deflects more than the latter from that of an ideal gas.

TABLE I

RATIO OF RETENTION VOLUMES WITH TWO DIFFERENT ORGANIC CARRIER VAPOURS AND THREE DIFFERENT LIQUID PHASES

Sample	*Liquid phase*	*Polyethylene glycol*		*Dioctyl phthalate*		*Silicone oil*	
	Organic carrier vapour	C_2H_5OH	C_6H_6	C_2H_5OH	C_6H_6	C_2H_5OH	C_6H_6
Ethanol					1.7		1.9
n-Propanol		0.9	1.0	0.8	1.2	0.7	1.4
n-Octane		0.7	0.8	0.8	1.0	0.5	1.3
Toluene		0.7	0.9	0.7	1.0	0.6	1.1

It is reasonable to assume that the gas state of other organic carrier and solute vapours, same as carbon tetrachloride and solute vapours, deflects from an ideal gas state in comparison with nitrogen and solute vapours.

Ratio of retention volume with two different organic carrier vapours and three different liquid phases

It was suggested that the two factors, which were mentioned above, had an important effect upon GC with the organic carrier vapour. The experimental values shown in Table I were given mostly under the following experimental conditions: column 170 cm × 4.5 mm I.D.; liquid phase 25w/w % on firebrick C-22; temperature 125°; column pressure, P_i, 280–150 mm Hg; P_o, 120–60 mm Hg; flow rate, 20–40 ml/min.

The *RRv* with the ethanol carrier vapour on silicone oil is remarkably smaller than one, and the *RRv* with benzene carrier vapour on polyethylene glycol was comparatively smaller than the *RRv* with benzene carrier vapour on silicone oil. The result is that the *RRv* is relatively small when the polarity of the organic carrier vapour has the reverse polarity of the liquid phase.

The sample of alcohols generally showed a large *RRv* with benzene carrier vapour in comparison with other sample. It is suggested that a thin adsorption layer of benzene existed on the surface of liquid phase, and this delayed transfer of the sample into the gas phase. The phenomenon of the surface adsorption of organic vapour, solute, on the surface of liquid phase in gas chromatography with helium carrier gas was also reported by MARTIN[10], MARTIRE[11] and PECSOK *et al.*[12]. The existance of the surface layer was suggested in GC using a gas mixture of water vapour and helium as a carrier gas[4].

It is also suggested that the small *RRv* of *n*-propanol with ethanol carrier vapour is due to the retention of the sample in the surface adsorption layer and/or to the interaction between *n*-propanol and ethanol in the gas phase. The small values of octane and toluene with ethanol carrier vapour are explained by the altered nature of the liquid phase resulting in the dissolution of ethanol into it.

Gas diffusion coefficient (D_{gas})

An equation relating the height equivalent to a theoretical plate (H) with the linear gas velocity (u) is[13]

$$H = 2\lambda d_p + \frac{2\gamma D_{\text{gas}}}{u} + \frac{8}{\pi^2} \cdot \frac{k'}{(1 + k')^2} \cdot \frac{d_f^2}{D_{\text{liq}}} \cdot u$$

The symbols are defined as in the report of BOHEMEN AND PURNELL[14]. For convenience in discussion, the equation is written more simply

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

The linear gas velocity at the minimum H was about 5.2 cm/sec with carbon tetrachloride carrier vapour but 7.4 cm/sec with nitrogen carrier gas under the following experimental conditions: column (170 cm × 4.5 mm I.D.); silicone oil, 25w/w% on firebrick C-22 (40–60 mesh); temperature, 125°; column pressure, P_o, 80 mm Hg; sample, *n*-octane.

The values of A evaluated by the usual graphical method for the data obtained in the above experimental condition were negative. Since negative values of A are unacceptable to the theory, the eddy diffusion term, A, might be flow dependent, and the van Deemter equation should be written

$$H = (A + B)/u + Cu \qquad (1)$$

Eqn. 1 is proposed by BOHEMEN AND PURNELL[14]. According to eqn. 1 the plot of Hu *vs.* u^2 should be linear, of slope C and intercept $A + B$. Fig. 4 shows such a graph for the data obtained under the above experimental condition.

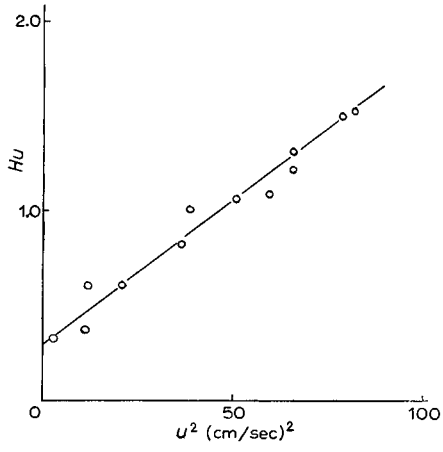


Fig. 4. Plot of Hu *vs.* u^2. Column, silicone oil 25% on firebrick C-22 (40–60 mesh); temperature, 125°; organic carrier vapour, CCl_4; sample, *n*-octane (1 μl).

Assuming $\lambda = 2$, the value of A was calculated, then the value of B was evaluated. The value of B_1/B_2 is equal to $D_{gas,1}/D_{gas,2}$, which is defined as the relative diffusion coefficient. The experimental value obtained by dividing the D_{gas} for nitrogen and *n*-octane by the D_{gas} for carbon tetrachloride and *n*-octane was 2.3. This experimental value of relative diffusion coefficient for *n*-octane–carrier vapour pairs shows good coincidence with the calculated value using the empirical formula of GILLILAND[15], 2.5, and using the formula derived by HIRSCHFELDER *et al.*[16], 2.0. We believe that GC using the vapours of organic solvents as a carrier gas will prove to be a new tool for the determination of gaseous diffusion coefficients.

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CHROM. 4490

SEMICONDUCTOR PHOTODETECTORS AND ELECTRICAL NOISE IN OPTICAL PHOTODENSITOMETRIC EQUIPMENT FOR QUANTITATIVE ASSESSMENT OF THIN MEDIA CHROMATOGRAMS

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SUMMARY

This paper considers the electrical noise originating in photodetector devices and associated amplifier equipment of photodensitometric instruments intended for quantitative assessment of thin media chromatograms. From a comparison of optical and electrical noise values the minimum intensity of illuminating light is calculated, which is necessary to meet a prescribed performance standard. Photomultiplier tubes and solid-state PIN-diodes are compared and it is shown that it is feasible to use these devices for high grade photodensitometric work. Advantages accrue both in the electrical and mechanical design of the instrument, and there is a resultant decrease in the expected costs plus an easier mode of operation.

INTRODUCTION

In two recent papers[1,2] the authors discussed optical background noise and its implications for the ultimate sensitivity and accuracy of photodensitometric methods for the quantitative assessment of thin media chromatograms. Measures designed to decrease the amount of optical noise present in various densitometric arrangements were described and analysed. If, however, the optical noise is reduced to very low levels, another source of noise becomes important, and this may then limit the performance of the system. This other noise source is generated in the photoelectrical conversion unit and the subsequent electronic circuitry. It is an analysis of this aspect of noise to which this paper is devoted.

THE SOURCES OF ELECTRICAL NOISE

The most important source of electrical noise is actually the photodetector itself. Physically this noise is caused by random fluctuations in the number and energy of

the charge carriers liberated by the incident light energy. The detailed mode of generation of the noise signal, however, varies considerably from one type of photodetector to another. It is not the purpose of this paper to consider these questions in detail; it will be left to the interested reader to look for specific information in the specialized literature.

The amplitude of the noise signal generated by a photodetector device is one of its most important characteristic parameters. Together with the radiant conversion sensitivity it determines the detection threshold obtainable, provided that—and this is usually the case—the noise of the subsequent amplifiers can be made equal to or smaller than the noise originating in the photodetector itself. From elementary considerations it can be shown that only the amplifier stages immediately following the detector need to be considered; the remainder of the circuit can usually be neglected in this context.

From an electrical point of view, the photodetector can be represented by an ideal signal generator i_s, a noise generator i_ν, an internal impedance Z_i, and a load impedance Z_L (Fig. 1). The photodetector devices most suited for the present application can electrically be considered as current generators. It is, therefore, convenient to draw the equivalent diagram in Fig. 1 on this basis. The current generators shown are assumed to have infinite impedance. The internal dynamic impedance of the detector is represented by Z_i, connected in shunt to the terminals of the equivalent generators.

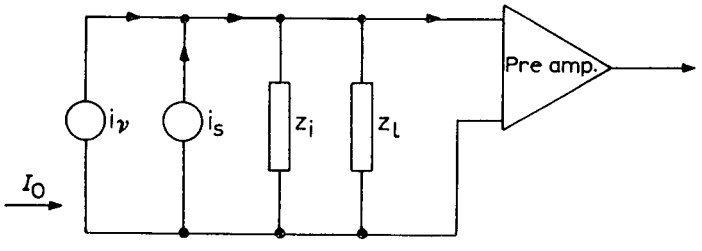

Fig. 1. Equivalent circuit of photoelectric convertor. i_ν = noise current generator; i_S = signal current generator (both ideal with infinite impedance); I_0 = incident light intensity; Z_i = internal impedance; Z_L = load impedance.

Since we are concerned here with slowly varying signals containing only very low frequency components, Z_i can be considered as a pure resistance R_i and the same also holds usually for the load impedance, Z_L, which includes, of course, the amplifier input impedance. For the best energy transfer from the photodetector to the amplifier chain, R_i should be equal to R_L. For current generating devices with high values of R_i, this rule may prove impractical. In these cases, R_L should be as large as is consistent with noise and supply voltage requirements.

There is a noise voltage across the terminals of any resistor, the r.m.s. value of which is determined by the relation:

$$e_{\nu_R} = 0.126 \sqrt{R \cdot \Delta F} \cdot \mu V \tag{1}$$

or in terms of noise current

$$i_{\nu_R} = 0.126 \sqrt{\frac{\Delta F}{R}} \cdot 10^{-3} \mu A \tag{1a}$$

In this equation R is the resistance value in kΩ, and ΔF the bandwidth of the system in kHz; the resistor is supposed to be at room temperature. The value of e_{vR} is the minimum value, compatible with a given resistance at room temperature. If this resistance is connected across the input terminal of an amplifying element, the noise measured at the output is always larger than the value which can be calculated from eqn. 1. The reason is that any amplifying element generates some noise of its own. This increase in noise as compared with that due to the input resistance is expressed by the noise figure of the amplifier, *e.g.*, a noise figure of 3 dB means that the total noise power at the output could be thought of as being produced by an ideal noise free amplifier with two times the noise power of the input resistor, that is $\sqrt{2}$ times e_{vR}, at the input. The noise figure of a given amplifier is a function of the input resistance R_1; for a certain value of R_1 it shows a usually rather flat minimum, to both sides of which it increases. Typical values for a well-designed amplifier at moderate values of R_1 up to a few MΩ are 3 dB, though rather lower values may be achieved. At very high values of R_1 (order of tens of MΩ are required for some types of photodetectors) usually higher noise figures have to be considered.

From the electrical point of view it is convenient to express the noise produced by the photodetector itself in terms of a noise figure, related to the circuit resistance (R_i in parallel with R_L), chosen for optimum conditions.

This figure states how many times the noise current (or voltage) generated by the photodetector exceeds the thermal noise of the total circuit resistance.

If this noise figure is larger than the noise figure of the associated amplifier, only the noise contribution from the photodetector has to be considered. This is the case with photomultiplier tubes with their high built-in current amplification; with solid-state photodevices an individual examination is necessary.

THE OPTICAL NOISE EQUIVALENT POWER

The intrinsic noise of the photodetector itself is the limiting threshold for the lowest light intensity which may still be detected with some degree of reliability. For these and related considerations on the optical side of the device, it is, however, preferable to express the noise output in optical units. To this purpose, the optical noise equivalent power (NEP) is introduced; it defines an optical input intensity (in W or lumens) that produces an output signal of the detector which is equal to the r.m.s. value of the detector noise signal at a bandwidth of 1 Hz.

The noise equivalent power of different types of photodetectors may differ by several orders of magnitude. With high grade photomultiplier tubes values down to about 10^{-16} W may be obtained. (Much lower values are available in special purpose tubes; for general application, however, the cost of these devices is almost prohibitive.) With solid-state devices the lower limit lies at present at NEP values of the order of 10^{-11} to 10^{-14} W. The minimum detectable light intensity of phoultomtiplier tubes is, therefore, several orders of magnitude lower than that of solid state devices.

PHOTOMULTIPLIERS *versus* SOLID-STATE DEVICES

Up until now photodensitometric devices have always employed photomultiplier tubes. Despite their high sensitivity and excellent noise performance, photomultiplier

tubes have several drawbacks when compared with solid-state devices. These disadvantages make the use of the latter attractive.

High sensitivity photomultiplier tubes are rather expensive. In addition, they need sophisticated and, therefore, expensive accessories. Their high-voltage supply has to be precisely regulated and divided among the dynodes. The tube has to be protected against optical overloading, which may lead to irreversible changes in the characteristics and even to self destruction.

Among the different types of solid-state photodetectors it appears that the PIN-diode (p-intrinsic-n) is best suited to the requirements of the present problem[3]. At present there are under development solid-state photodevices with built-in (avalanche) current multiplication. These devices will, when (commercially) available, reduce the advantages of the conventional photomultiplier still further. As compared with photomultiplier tubes we find on the credit side that these diodes are much smaller and more rugged; mounting problems are, therefore, considerably eased. The voltage requirements are modest, being of the order of 20 V, and adequate stabilization does not present a problem. The spectral sensitivity characteristic is much flatter than that of most photomultipliers and their long term stability is much improved. Magnetic interference is of no concern and optical overloading is easily tolerated. On the debit side their slower response is of little consequence for the purposes envisaged in the present requirements. Their low output signal at high impedance levels requires, however, careful design of the associated amplifier equipment in order to keep the noise figure low. High input impedance–low noise junction field effect transistors appear to be the best solution for this purpose.

Both photomultiplier tubes and PIN-diodes are essentially current-devices with very high internal impedance. Their noise contribution at a given bandwidth ΔF is, therefore, basically determined by the dark current i_D according to the relation:

$$i_r^2 \simeq 1.6\, i_D \cdot \Delta F \cdot 10^{-10}\ \mu\text{A} \qquad (\Delta F[\text{kHz}],\ i[\mu\text{A}]) \tag{2}$$

The luminous sensitivity β of a photomultiplier tube or any other high impedance photoelectric device is defined as the change in output current per lumen (or W) of incident radiant flux. Using this conversion factor we obtain the noise equivalent light power N_0:

$$N_0 \simeq \frac{i_r^2}{\beta \Delta F} = \frac{1.6\, i_D}{\beta} \cdot 10^{-13}\ \text{W} \qquad (\beta[\mu\text{A/W}]) \tag{3}$$

Eqn. 2 holds under the assumption that the noise originating in the photodevice is essentially white. White noise is caused by completely random fluctuations—in this case the instantaneous number of charge carriers (electrons, holes)—around an average value. Characteristic for white noise is a spectral power density, which is constant throughout the relevant range of frequencies as is the case with white light. In the "white" region the total noise power is therefore proportional to the bandwidth of the signals.

In most electronic elements, however, the power density of the noise increases sharply at very low frequencies. Below a certain crossover frequency the spectral power density instead of being constant rises with $1/f$ (f = frequency). This results in a considerable increase in noise in the very low frequency region (above the value calculated from eqn. 2).

THE OVERALL NOISE EQUIVALENT LIGHT INTENSITY

The above-mentioned dependency of the noise at very low frequencies (called the "flicker noise region") leads to a considerable increase in noise for signals with very low frequencies. Typically the crossover frequency between the $1/f$ portion and the "white" part of the spectrum is of the order of a few hundred Hz. For this reason, it is advisable to transpose very low frequency signals to a higher position along the frequency axis. In optical information retrieval systems this is most easily accomplished by chopping the light beam at a rate which is well above the crossover frequency. The chopping frequency acts as a carrier, modulated by the original signal. In this way, the original signal is shifted to a higher frequency region of the spectrum, where the noise of the photodetector and associated amplifier equipment is lower. Chopping the light beam, therefore, not only bypasses the problems associated with d.c. amplification, but also improves the signal-to-noise ratio, provided the chopping frequency is sufficiently high. A drawback of chopping the input light beam is that it actually reduces the incident light signal and, therefore, the output signal level of the photodetector by 1/2. It can be shown that by using special (so called "synchronous") detection techniques following the pre-amplifier stage, half of the noise energy can be suppressed. The result is equivalent to a $\sqrt{1/2}$ decrease of the light loss caused by chopping. Alternating the light beam between two detectors working into a common differential input amplifier instead of straight interrupting of the beam offers a similar improvement. In general, however, the gain of these more sophisticated procedures does not warrant the expense; (see also ref. 2).

The chopping frequency has to be removed at a point of suitably high signal level by demodulation and smoothing the signal, so that a replica of the original light signal is restored. The required bandwidth of the chopped signal for a fixed slit scanning device is

$$\Delta F > \frac{V}{W} \tag{4}$$

Here V is the transport velocity of the paper perpendicular to the slit and W the width of the slit. For a flying spot-scanning device this becomes (for a square-shaped or circular spot with no overlap)

$$\Delta F > \frac{V}{W} \cdot \frac{B}{W} = \frac{VB}{W^2} \tag{5}$$

where B is the width of the scan. The term V/W represents the number of scanning lines per second and B/W the number of independent points per line.

After amplification the chopped signal has to be demodulated (rectified). To remove the chopping frequency, the demodulated signal is filtered and smoothed. The bandwidth of the signal after this operation is approximately $\Delta F/2$. Frequently, however, further filtering and integrating is advisable, resulting in an effective output bandwidth $\Delta F_0 \ll \Delta F/2$. The result is a decrease in noise by a factor $\sqrt{2\Delta F_0/\Delta F}$. The reason for this is that it is the final bandwidth which counts and it is of no importance when the bandwidth limitation (filtering) is performed.

With N_0 being the noise equivalent power of the photodetector, we obtain an equivalent input light intensity

$$I_{v_E} = N_0 \sqrt{\Delta F} \tag{6}$$

If two independent photodetectors are used as for example in the double beam device now being designed in our laboratories, the electrical noise power is increased by a factor of 2 and the noise amplitude by $\sqrt{2}$. This is because the noise contributions of both photodetectors are independent and non-correlated. This is true regardless of whether difference or ratio forming of the two signals is employed. In eqn. 6, therefore, a factor of 2 has to be added under the square root sign.

If the preamplifier noise contribution cannot be neglected, it has to be included in expression (6). Let us assume that the preamplifier increases the total noise power by a factor of K as compared to the photodetector alone. Then the factor K also needs to be inserted under the square root sign in eqn. 6.

$$I_{v_E} = N_0 \sqrt{2\Delta FK} \tag{7}$$

OPTICAL AND ELECTRICAL NOISE

In two recent papers[1,2] we have discussed steps which help to reduce the optical noise of chromatogram scanning devices. In order, however, to make full use of the low optical noise values now obtainable, the total noise equivalent light intensity (for the electrical noise) has to be equal to or smaller than the optical noise amplitude.

$$I_{v_E} \leqslant I_{v_{\text{opt}}} \tag{8}$$

The optical noise differs from the electrical noise in that it is essentially multiplicative; it may, therefore, be expressed as a constant fraction of the light intensity I_0 at the photodetector input.

$$I_{v_{\text{opt}}} = \bar{\bar{v}}_{\text{opt}} \cdot I_0 \tag{9}$$

Similarly, the useful signal S_u is determined by the product of I_0 and the absorbance α_c of the investigated zone.

$$S_u = c \cdot \alpha_c \cdot I_0 \tag{10}$$

The lowest value of α_c, which may be determined with a given accuracy and reliability, is given by the ratio σ of useful signal-to-noise. Considering for the moment only the optical noise, we have

$$\alpha_c \geqslant \frac{\sigma \cdot \bar{\bar{v}}_{\text{opt}}}{c} \tag{11}$$

This relation is valid if condition (8) is achieved. Assuming now, as a limiting case, that the equality sign in eqn. 8 is valid, we can write for the overall noise signal:

$$I_{v_{\text{tot}}} = \sqrt{2} \cdot I_{v_E} \tag{12}$$

Using eqns. 7 and 11, I_0 can now be expressed as a function of the minimum value of α_c to be detected. As before, we have to postulate a minimum signal-to-noise ratio σ. Here we have, however, to take into account the fact that the electrical output signal is reduced due to chopping. Assuming simple chopping and subsequent rectifi-

cation, the reduction factor is as previously described 1/2. Taking account of these considerations, we find for an arrangement using two separate photodetectors:

$$a_c \geqslant \frac{\sigma \cdot 4N_0 \sqrt{\Delta F \cdot K}}{I_0 \cdot c} \approx \frac{4\sigma N_0 \sqrt{\Delta F \cdot K}}{I_0} \qquad (c \approx 1) \tag{13}$$

Relation (13) permits the determination of the light intensity I_0 at the photodetector input, which has to be maintained for a given sensitivity and accuracy. The latter is mainly determined by the value of σ. In crude approximation, the accuracy in % is equal to $100/\sigma$. Since according to eqn. 4 ΔF is proportional to the scanning speed, one possibility of improving the resolving power of the instrument is to reduce the speed across the scanning beam.

A NUMERICAL EXAMPLE

The best way to illustrate the results obtained so far is probably to calculate a numerical example. Let us consider transmission measurements on Whatman No. 3 paper, with optical density 3.4. The corresponding transmittance is $10^{-3.4} \simeq 3 \cdot 10^{-4}$. A high grade PIN-diode is supposed to be used as photodetector. Its NEP value N_0 is 10^{-13} W; the noise figure K of the amplifier is assumed to be 2 dB $\simeq$ 1.6, and the bandwidth of the chopped signal $\Delta F \simeq 160$ Hz. This latter value is based upon 4 lines per sec scanning speed and 40 points per line. Narrow-band filtering and integration of the demodulated signal is in this example disregarded. If this can also be applied it results in further improvements in the signal-to-noise ratio and the obtainable resolving power. The load resistor of the photodiode has to be reasonably large (of the order of 10^7 Ω), if the value of K mentioned is to be obtained.

A high performance double beam scanning device is assumed with an optical noise value $\bar{\nu}$ below 10^{-3}; the required accuracy for the weakest signal is to be of the order of 10%, corresponding to a signal-to-noise ratio $\sigma \geqslant 10$. The weakest change in absorbance which may be detected with this accuracy is, therefore, about $a_{c\,\min} \approx 10^{-2}$ natural units or about $4 \cdot 10^{-3}$ in optical density (decimal) units.

Introducing these parameters into eqn. 13, we obtain for the light intensity required at the photodetector input:

$$I_0 \geqslant \frac{4 \cdot 10 \cdot 10^{-13} \cdot 16}{10^{-2}} = 6.4 \cdot 10^{-9} \tag{14}$$

The principle sources of attenuation of the light beam are the optical density of the paper and the spectral band pass filter required to limit the energy of the beam to the absorption band of the substance investigated. Let us assume that the scanning beam is permitted to cover a spectral band about 3 nm wide[2]. The fraction of the total visible light energy radiated by the source, which falls into this band, depends upon the type of source used and upon the spectral position (colour) of the absorption band. Together with the losses in the filtering device used (interference filter, wide band monochromator), about 0.1% may be considered as a typical value. These two factors together result, therefore, in an attenuation of approximately $3 \cdot 10^{-4} \times 1 \cdot 10^{-3} = 3 \cdot 10^{-7}$.

Further, we have to consider the losses in the remainder of the optical system and the fact that only a small spatial part of the total light flux of the lamp can be

really utilized. Altogether, the useful radiant flux may be estimated to about 5% of the total light output.

With these estimates, we obtain a required optical output of the lamp in the visible region of the spectrum equal to:

$$I_{tot} \approx 6.4 \cdot 10^{-9} \cdot \frac{1}{3 \cdot 10^{-7} \cdot 5 \cdot 10^{-2}} \approx 0.43 \text{ W} \quad (15)$$

This value may, of course, easily be obtained even with a certain power reserve, using a light source in the range of 100 to 300 W input power. It, therefore, appears that the use of semiconductor photodetectors in this field, which up to now has been an exclusive domain of photomultiplier tubes, is feasible and promising even for photometric equipment with very high performance standards. Careful design of the optical system with a view to efficient utilisation of the light flux of the lamp is, of course, a prerequisite. Integration over the whole zone area and smoothing, as considered in refs. 1 and 2, reduce the effective bandwidth ΔF of the system and should, therefore, bring about a further improvement of the obtainable sensitivity by a factor of 5 or more.

ACKNOWLEDGEMENTS

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CHROM. 4468

RAPID DETERMINATION OF THE MOLECULAR-WEIGHT DISTRIBUTION OF ETHYLENE GLYCOL OLIGOMERS BY THIN-LAYER CHROMATOGRAPHY AND THEIR PHOTOMETRIC EVALUATION*

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SUMMARY

Thin-layer chromatography, coupled with the direct photometric measurement of the spots visualized with iodine vapour, offers a suitable means of estimating the molecular-weight distribution of oligomeric polyethylene glycol derivatives. The present work is restricted to the lower oligomers in order to permit a comparison with gas chromatographic data, but more interest is attached to the resolution of higher oligomers, for which gas–liquid chromatography is unsuitable.

INTRODUCTION

The use of programmed-temperature gas chromatography is restricted to volatile derivatives in the determination of the molecular-weight distribution (MWD) of polyethylene glycol compounds having the general formula $RO(CH_2CH_2O)_nR'$, where R and R′ are *e.g.* hydrogen atoms, alkyl groups, or alkylaryl groups and n is the degree of polymerization. Admittedly, the substitution of trimethylsilyl groups for the OH groups raises the volatility of some derivatives and permits the resolution of oligomers with molecular weights of nearly 1000 under suitable conditions[1]. However, with less volatile derivatives, such as those in which R and R′represent *p*-(1,1,3,3-tetramethylbutyl)phenyl groups, progressively poorer results are obtained as the molecular weight exceeds 700–800. This emerges from a comparison with the corresponding molecular-weight distribution obtained by column chromatography[2].

For the determination of the MWD of less volatile higher members, one must resort to liquid–solid chromatography, and very good results have in fact been obtained by thin-layer chromatography in the fractionation of derivatives with R = *p*-*n*-nonylphenyl and R′ = H, with n values of up to 16 (see ref. 3), and in the determination of the MWD of compounds with n values of up to 14 by the circular technique and the densitometric evaluation of a photograph of a suitably visualized chromatogram[4]. Furthermore, compounds with n values of up to 27 have been separated in the case of the series $RS(CH_2CH_2O)_nCH_2CH_2SR$ with R = R′ = *tert*-hexyl[5].

The aim of the present study was to examine the accuracy of TLC, coupled with direct photometric evaluation in the determination of the MWD of ethylene glycol oligomers. The visualizing agent was iodine vapour which has been used recent-

* Translated by Express Translation Service.

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ly for the paper chromatographic detection of these compounds[6]. The advantages of iodine vapour over the usual sprays are as follows:

(a) no damage to the plate and no spot enlargement;

(b) inextensive and transient adsorption on the silica, giving a uniform surface, with little difference from the initial one;

(c) the generally pale brown spots on a white background are easy to measure photometrically;

(d) iodine vapour is often non-destructive, because it can be removed under reduced pressure.

The main disadvantage of iodine vapour is that the fading time of the spots varies with the nature of the substance. However, the problems of accuracy introduced by this fact into the photometric evaluation can be minimized by optimizing the geometry and the saturation of the tank, the time of exposure, and the "fixation" of the visualized plate.

The work was done on two types of compounds having the formula $RO(CH_2CH_2O)_nR'$, one type with R = R' = phenyl, and the other type with R = *p-tert.*-nonylphenyl and R' = H, the value of n ranging up to 17. The results were checked against gas chromatographic data.

EXPERIMENTAL

The diphenyl derivatives, whose mean degree of polymerization $\bar{n}$ was about 7.3 and 10.3, were prepared as described before[1]. Single oligomers with n = 6, 8, and 9 with purities of 99.5, 98.8, and 98.0%, respectively, determined by gas chromatography, were synthesized in the laboratory, while the derivatives with R = *p-tert.*-nonylphenyl and R' = H, with $\bar{n}$ = 5.4 and 6.4 were commercial products (ex Chemische Werke Hüls). The compound with n = 3 (93.0%) was obtained by fractionation of a mixture with $\bar{n}$ = 3.3 (see ref. 7) by column chromatography.

The silica adsorbent (Merck, type G) was applied in 40–20-μ thick layers to glass plates measuring 15 × 5 cm and 15 × 2.5 cm. This was done by means of an applicator with a fixed slit of 200 μ. A photometric check on the resulting plates was found to be essential and the more irregular ones were rejected. The plates were dried by raising the temperature to 180° in 1 h and by maintaining this temperature for ½ h. This was done to avoid blister formation in the silica layer.

40 μg samples were applied to the plates as a 1% solution in acetone with a 10-μl Hamilton microsyringe, so as to form a spot with a diameter not exceeding 3 mm. The spots were eluted to a distance of 12.5 cm by the ascending technique with various solvents (chromatographic grade, C. Erba, Milan) and their mixtures, the plates having been placed in cylindrical Desaga tanks (20 × 5 cm) equilibrated with solvent vapour.

The spots were then visualized in similar tanks in a horizontal position; the atmosphere in these was saturated with iodine vapour by placing a few iodine crystals at the bottom. The plates were kept in the tanks for at least 15 min. Each plate was then immediately covered with a glass plate of the same size, and an adhesive tape was stuck around the edge to minimize evaporation of the iodine in order to preserve the colour of the spots.

The plates were then evaluated photometrically with a Joyce Chromoscan, the

TABLE I

DATA OBTAINED FROM THE THIN-LAYER CHROMATOGRAPHIC AND THE GAS CHROMATOGRAPHIC ANALYSIS OF AN ARTIFICIAL BINARY AND A TERNARY MIXTURE OF OLIGOMERS OF THE TYPE $RO(CH_2CH_2O)_nR'$, R = R′ = PHENYL

$\bar{x}_n$ is the mean molar fraction in %, and s_n is the corresponding mean square deviation, based on three determinations.

n	*Composition of the mixture by*		
	Gravimetry x_n	*GLC* x_n	*TLC* $\bar{x}_n \pm s_n$
8	52	54.0	50.5 ± 2.1
9	48	46.0	49.5 ± 2.1
6	33	37.3	25.3 ± 1.5
8	36	34.0	37.2 ± 1.8
9	31	28.7	37.5 ± 2.0

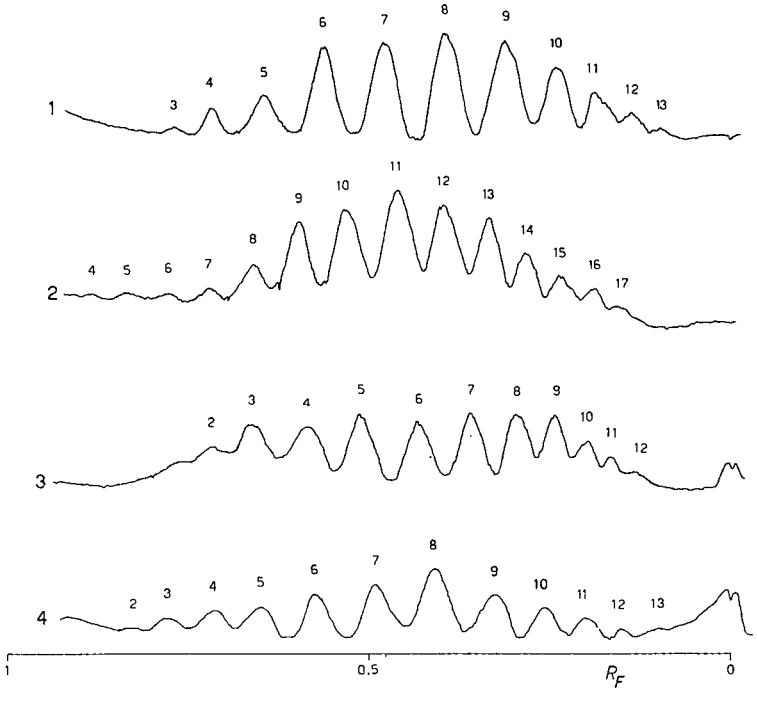

Fig. 1. Tracings obtained for the thin-layer chromatograms of polyethylene glycol derivatives of the type $RO(CH_2CH_2O)_nR'$. Temperature 25°. The numbers over the peaks give the n values. Curve 1: R = R′ = phenyl; $\bar{n}$ = 7.3; solvent–butanone. Curve 2: R = R′ = phenyl; $\bar{n}$ = 10.5; solvent:butanone–water (90:10). Curve 3: R = *p-tert.*-nonylphenyl, R′ = H; $\bar{n}$ = 5.4; solvent: butanone–water (95:5). Curve 4: R = *p-tert.*-nonylphenyl, R′ = H; $\bar{n}$ = 6.5; solvent:butanone saturated with water.

TABLE II

COMPARISON OF DATA OBTAINED BY THE THIN-LAYER CHROMATOGRAPHIC AND BY THE GAS CHROMATOGRAPHIC ANALYSIS OF POLYETHYLENE GLYCOL DERIVATIVES

n is the degree of polymerization; $\bar{n}$(calc.) is the number average degree of polymerization found from the molecular-weight distribution; $\bar{x}_n$ is the mean molar fraction in %; and s_n is the corresponding mean square deviation, based on four determinations.

n	$RO(CH_2CH_2O)_nR'$, $R = R' =$ *phenyl*				$RO(CH_2CH_2O)_nR'$, $R =$ *p-tert.-nonylphenyl*			
	$\bar{n} = 7.3$		$\bar{n} = 10.5$		$\bar{n} = 5.4$		$\bar{n} = 6.5$	
	GLC x_n	*TLC* $\bar{x}_n \pm s_n$	*GLC* x_n	*TLC* $\bar{x}_n \pm s_n$	*GLC* (*R'* = *TMS*) x_n	*TLC* (*R'* = *H*) $\bar{x}_n \pm s_n$	*GLC* (*R'* = *TMS*) x_n	*TLC* (*R'* = *H*) $\bar{x}_n \pm s_n$
1	—	—	—	—	traces	—	—	—
2	1.30	—	0.93	—	0.99	2.41 ± 0.90	0.99	traces
3	2.00	1.02 ± 0.21	1.05	—	14.02	9.69 ± 0.28	4.11	3.12 ± 0.82
4	4.44	3.85 ± 0.26	1.18	1.07 ± 0.48	25.60	17.04 ± 0.70	9.46	8.37 ± 0.12
5	8.56	7.78 ± 0.49	1.30	1.62 ± 0.21	23.76	21.21 ± 0.64	17.54	13.99 ± 1.23
6	18.46	18.09 ± 0.92	2.33	2.32 ± 0.31	15.07	17.75 ± 1.00	18.78	17.72 ± 0.39
7	21.93	21.84 ± 0.62	5.59	3.39 ± 1.27	9.59	13.41 ± 0.11	16.39	17.83 ± 1.18
8	20.08	19.85 ± 0.43	9.30	9.81 ± 0.61	5.77	10.45 ± 0.59	13.98	16.71 ± 0.54
9	13.04	15.48 ± 0.33	14.26	14.45 ± 1.23	3.17	4.79 ± 0.68	9.21	11.36 ± 0.53
10	6.20	7.73 ± 0.45	17.16	18.07 ± 1.79	1.68	2.17 ± 0.10	6.39	6.32 ± 0.74
11	2.38	3.11 ± 0.34	15.68	16.40 ± 1.42	0.35	1.08 ± 0.26	3.15	3.70 ± 0.25
12	0.99	1.03 ± 0.27	13.49	13.68 ± 1.29	—	traces	—	0.88 ± 0.17
13	0.62	0.22 ± 0.05	9.08	8.92 ± 0.86	—	—	—	traces
14	—	—	5.63	4.92 ± 0.74	—	—	—	—
15	—	—	1.98	3.33 ± 0.15	—	—	—	—
16	—	—	0.86	1.84 ± 0.20	—	—	—	—
17	—	—	0.18	0.18 ± 0.06	—	—	—	—
$\bar{n}$(calc.)	7.20	7.44	10.21	10.51	5.18	5.69	6.61	6.75

light absorption of the plates being recorded by an apparatus capable of measuring the transmission. The maximum sensitivity was found at 430 nm (obtained with a glass filter) and a slit of 5 × 0.5 mm. The linear response of the recorder in the absorption was in the range 0–1.5 and the symmetry of the triangular peaks permitted the amount of each component of the mixture to be estimated by measuring the areas.

The samples were also subjected to programmed-temperature gas chromatographic analysis under the same conditions as used before[1]. The compounds with R = *p-tert.*-nonylphenyl and R' = H were first converted into their trimethylsilyl (TMS) derivatives.

RESULTS AND DISCUSSION

The relative error in the MWD was determined by analyzing known mixtures of diphenyl derivatives with $n = 6$, 8, and 9, after finding a linear relationship between the amount adsorbed on the plate and the light absorption of the resulting spot, rendered visible with iodine vapour. The linearity of this relationship extended up to 40-μg samples, with a 5% accuracy (relative mean square deviation of the slope).

Table I shows the results obtained by TLC and GLC for a binary and a ternary

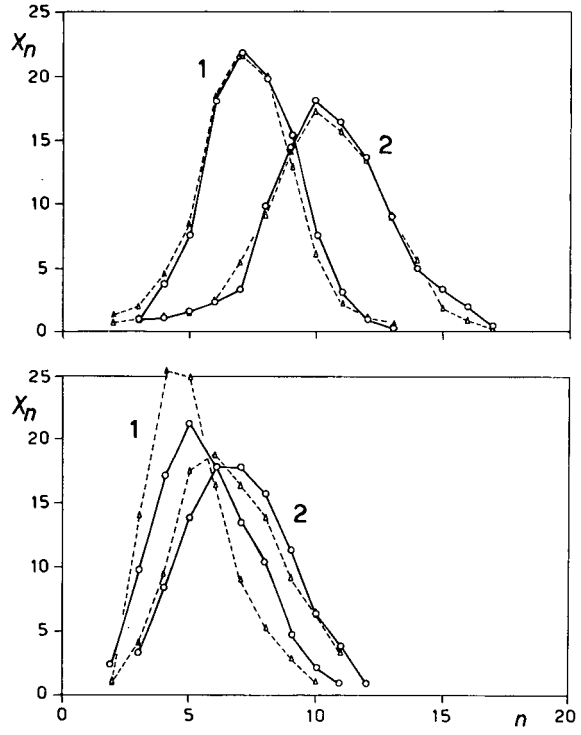

Fig. 2. Molecular-weight distributions as determined by TLC (circles and full line) and by GLC (triangles and broken line) for polyethylene glycol derivatives of the type $RO(CH_2CH_2O)_nR'$ with R = R′ = phenyl (upper figure) and $\bar{n}$ = 7.3 (1), $\bar{n}$ = 10.5 (2); and with R = *p-tert.*-nonylphenyl, R′ = H (lower figure) and $\bar{n}$ = 5.4 (1) and $\bar{n}$ = 6.5 (2). n is the degree of polymerization, and x_n is the molar fraction in % (*cf.* Table II).

mixture; the molar fractions are expressed in %. For the binary mixture, the values are comparable with the actual composition, but indicate a distortion in favour of the higher oligomer (the reactivity of iodine seems to be proportional to n). For the ternary mixture, on the other hand, systematic errors, particularly for $n = 6$, were caused by the fact that light absorption of the spot decreased with time (15–20% in the first 10 min, as compared to a 1–2% decrease for the $n = 9$ compound in the same period). This peculiarity leads to low values for those members of the mixture with a low or a medium molecular weight, but this can be minimized by doing the photometric scanning within 2–3 min after visualization.

Fig. 1 shows some thin-layer chromatograms obtained for derivatives of both types. The spot at the start line in the case of the monononylphenyl derivatives is due to intermediates and by-products of the industrial synthesis.

The data listed in Table II indicate good agreement between TLC and GLC as regards the molecular-weight distribution of the two series of compounds. This applies particularly to the distribution at higher degrees of polymerization, where the distortion at lower n values is less apparent (*cf.* Fig. 2). Furthermore, the precision is almost always lower (in %) for compounds at the beginning of the distribution curve than for those at its end.

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CHROM. 4503

SEPARATION OF ALKYL GALLATES AS COMPLEXES WITH CINCHONINE AND STRYCHNINE

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SUMMARY

The molecular complexes of various alkyl gallates with cinchonine and strychnine are reported. It was possible to separate and characterize a gallate up to 2 μg as its complex from a mixture.

INTRODUCTION

Alkyl gallates are widely employed for extending the shelf life of various fatty food stuffs and in combination with butylated hydroxy anisole work as excellent synergists. Against certain bacteria *viz. Salmonera narasino*, *Saccharomyces cerevisiae* etc. they show microbicide action which increases with the increasing chain length of the alkyl radical[1]. They are used as polymer stabilizers[2] and possess prophylactic and therapeutic antiradiation properties[3]. The latter effect is due to the binding of free radicals in the cells and inhibition of the activity of oxidizing enzymes[4].

The molecular complexes of gallic acid with caffeine, theophylline and theobromine have been reported by thermal analysis[5]. Nicotine gallate is known to be insecticidal in nature[6].

The separation and identification of alkyl gallates have been reported by employing different chromatographic techniques. Gas chromatography was carried out by using the columns packed with 1.5% SE-30, 0.7% QF-1 and 1.0% neopentyl glycol succinate polyester[7]. Reversed-phase partition chromatography[8], paper chromatography[9–18], centrifugal chromatography[19] on Whatman No. 3 paper, and thin-layer chromatography using 10% acetylated cellulose[20], alumina[21], silica gel[22–35] and polyamide powder[36–38] as adsorbents have been employed both for their qualitative and quantitative studies.

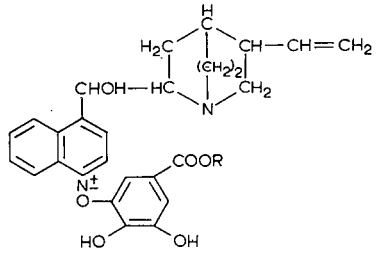
Great difficulty was faced by the present authors in the distinct resolution and identification of *sec.*- and *tert.*-alkyl gallates from their mixtures by employing thin-layer chromatography; even two-dimensional thin-layer chromatography was not helpful. This was due to the very close proximity of R_F values of branched-chain alkyl gallates, superimposing each other.

TABLE I

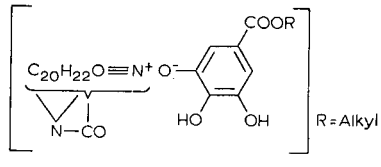
THE R_F VALUES OF VARIOUS GALLATE–ALKALOID MOLECULAR COMPLEXES

Sample No.	*Complexes with cinchonine*							
	Complexes of cinchonine–strychnine with the following gallates	*Adsorbents*	*Kieselgel G impregnated with 2% cinchonine*			*Magnesium silicate impregnated with 2% cinchonine*		
		Solvents	*Chloroform–acetic acid (85:15)*	*Carbon-tetrachloride–methyl alcohol (4:1)*	*Benzene–acetic acid (4:1)*	*Ethylene-dichloride–methyl alcohol (9:1)*	*Ethylene-dichloride–acetic acid (9:1)*	*Chloroform–acetic acid (9:1)*
1	Methyl gallate		0.26	0.23	0.15	0.04	0.22	0.51
2	Ethyl gallate		0.31	0.25	0.21	0.07	0.41	0.83
3	*n*-Propyl gallate		0.38	0.27	0.24	0.09	0.56	0.87
4	Isopropyl gallate		0.25	0.06	0.08	0.00	0.00	0.00
5	*n*-Butyl gallate		0.47	0.30	0.26	0.12	0.61	0.89
6	*sec.*-Butyl gallate		0.19	0.12	0.07	0.00	0.00	0.00
7	*tert.*-Butyl gallate		0.14	0.16	0.06	0.00	0.00	0.00
8	Isoamyl gallate		0.42	0.22	0.28	0.16	0.65	0.93
9	*n*-Octyl gallate		0.55	0.34	0.35	0.23	0.93	0.94
10	*sec.*-Octyl gallate		0.09	0.12	0.06	0.00	0.00	0.04
11	*n*-Dodecyl gallate		0.68	0.36	0.39	0.29	1.00	0.97

It was observed by the present authors that both cinchonine and strychnine invariably formed 1:1 molecular complexes with alkyl gallates *viz.* methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec.*-butyl, *tert.*-butyl, isoamyl, *n*-octyl, *sec.*-octyl and *n*-dodecyl gallate. This was confirmed by varying the gallate–alkaloid ratios and by recording their UV absorption spectra in ethanol.



Cinchonine–alkyl gallate complex.



Strychnine–alkyl gallate complex.

In literature no work on the molecular complexes of alkyl gallates with alkaloids using any of the chromatographic techniques is reported. The present paper describes the resolution and characterization of alkyl gallates up to 2 μg as their molecular complexes with cinchonine and strychnine employing a thin-layer chromatographic technique. The gallates could be conveniently estimated by measuring the percentage transmission of the eluted spots at 272 mμ.

Complexes with strychnine											
Kieselgel G–Kieselguhr (75:25) impregnated with 2% cinchonine			*Kieselgel G impregnated with 2% strychnine*			*Magnesium silicate impregnated with 2% strychnine*			*Kieselgel G–Kieselguhr G (75:25) impregnated with 2% strychnine*		
Ethylenedichloride–methyl alcohol (4:1)	*Ethylenedichloride–acetic acid (4:1)*	*Chloroform–acetic acid (9:1)*	*Chloroform–acetic acid (4:1)*	*Chloroform–methyl alcohol (9:1)*	*Benzene–methyl alcohol–acetic acid (45:8:4)*	*Chloroform*	*Chloroform–acetic acid (4:1)*	*Benzene–acetic acid (9:1)*	*Chloroform–methyl alcohol (9:1)*	*Chloroform–acetic acid (9:1)*	*Carbontetrachloride–methyl alcohol (7:3)*
0.32	0.49	0.15	0.24	0.31	0.18	0.19	0.55	0.09	0.48	0.07	0.45
0.38	0.64	0.24	0.32	0.34	0.21	0.24	0.85	0.22	0.52	0.12	0.50
0.40	0.80	0.31	0.41	0.39	0.25	0.27	0.90	0.32	0.55	0.15	0.52
0.17	0.19	0.03	0.04	0.09	0.15	0.00	0.06	0.00	0.16	0.03	0.40
0.44	0.86	0.35	0.49	0.40	0.35	0.29	0.90	0.40	0.56	0.18	0.52
0.15	0.21	0.03	0.03	0.11	0.17	0.00	0.05	0.00	0.17	0.04	0.38
0.18	0.22	0.02	0.06	0.10	0.18	0.00	0.05	0.00	0.18	0.02	0.39
0.34	0.91	0.38	0.55	0.40	0.40	0.33	0.90	0.42	0.57	0.23	0.47
0.47	0.91	0.48	0.66	0.46	0.51	0.41	0.92	0.94	0.65	0.25	0.54
0.18	0.20	0.03	0.05	0.08	0.14	0.00	0.07	0.00	0.16	0.03	0.35
0.49	0.94	0.53	1.00	0.56	0.60	0.50	0.94	1.00	0.76	0.29	0.57

EXPERIMENTAL

Preparation of alkyl gallates

Gallates of methyl, ethyl, propyl, isopropyl, *n*-butyl, *sec.*-butyl, *tert.*-butyl and isoamyl alcohols were prepared by refluxing a mixture of dry gallic acid (1 mole), alcohol (5 moles), toluene (10 moles), *p*-toluene sulfonic acid (0.05 moles) and sulfuric acid (0.5 mole), respectively, for 5 h in a heating mantle. After distillation of the solvent and excess alcohol under vacuum, the mass was extracted twice with ether and washed with cold water. The solution was dried over anhydrous magnesium sulfate, the solvent distilled off and the ester crystallized from ethanol (yields 68–72%). The gallates of *n*-octyl, *sec.*-octyl and dodecyl alcohols were prepared by converting gallic acid into galloyl chloride by treatment with thionyl chloride. Alcohol (1 mole), galloyl chloride (1.1 moles) and pyridine (5 moles) were kept on the water bath for 2 h with frequent stirring and were poured over crushed ice. The crystalline mass was filtered and washed with a minimum amount of cold water. The gallates were crystallized as needles from benzene with yields between 62–65%. Their melting points were as follows: methyl gallate, 200°; ethyl gallate, 160°; *n*-propyl gallate, 148°; isopropyl gallate, 123–124°; *n*-butyl gallate, 135°; *sec.*-butyl gallate, 259°; *tert.*-butyl gallate, 248°; isoamyl gallate, 143°; *n*-octyl gallate, 95–96°; *sec.*-octyl gallate, 170°; dodecyl gallate, 97°. The purity of the compounds was established by twodimensional reversed-phase paper chromatography in which single spots were obtained.

Preparation, spotting and irrigation of plates

Kodak photographic glass plates, 22 × 22 cm, were used. Thin films on the plates were prepared as follows. A fine slurry of the adsorbent (30 g) in ethanol–water (1:1, 60 ml) was prepared; it was poured on the glass plates which were tilted from

side to side. The plates were dried overnight at room temperature and activated at 120° for half an hour. The plates were weighed, and the average coating of the adsorbents was recorded. The thin-layer coatings in the cases of Kieselgel G, magnesium silicate and Kieselgel G–Kieselguhr G (3:1) were 4.32, 7.11 and 3.82 mg/cm², respectively.

The plates were impregnated with cinchonine and strychnine by irrigating them with their respective 2% chloroform solutions. The alkaloid-coated plates, after evaporation of the solvent, were immediately spotted with 2 μg of each ester with a standard microcapillary. The plates were irrigated at 24 $\pm$ 1° with appropriate solvents, using an ascending system. The gallate–alkaloid complexes were light gray in color and could be easily located. Fig. 1 is a typical chromatoplate showing the resolutions of complexes of gallates with cinchonine. The R_F values are given in Table I.

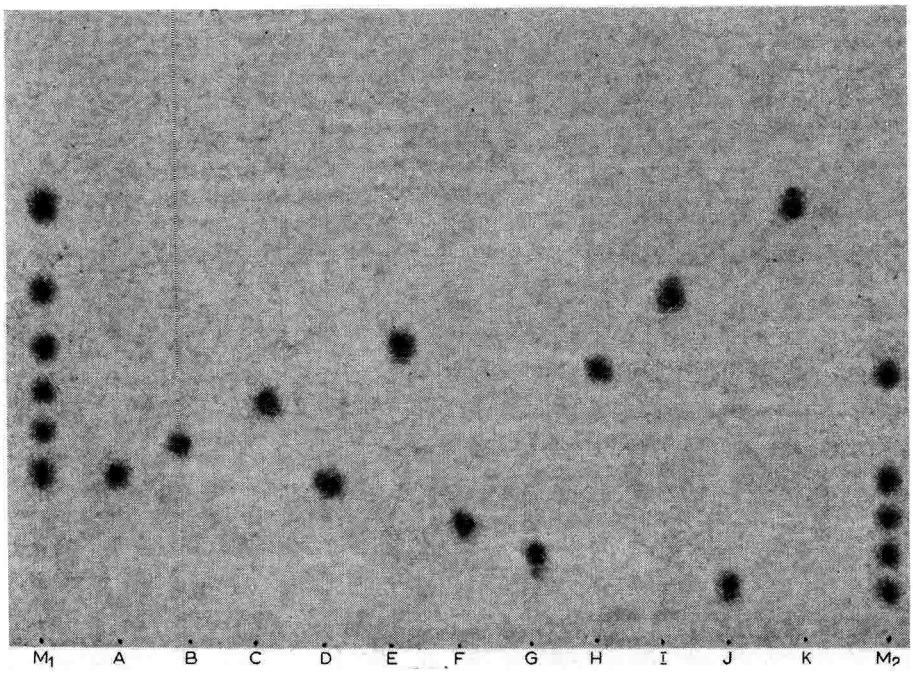


Fig. 1. Thin-layer chromatogram showing the resolutions of molecular complexes of alkyl gallates with cinchonine (Ci). A = methyl gallate–Ci; B = ethyl gallate–Ci; C = propyl gallate–Ci; D = isopropyl gallate–Ci; E = *n*-butyl gallate–Ci; F = *sec.*-butyl gallate–Ci; G = *tert.*-butyl gallate–Ci; H = isoamyl gallate–Ci; I = *n*-octyl gallate–Ci; J = *sec.*-octyl gallate–Ci; K = *n*-dodecyl gallate–Ci; M_1 = mixture of A, B, C, E, I and K; M_2 = mixture of J, G, F, D and H. Adsorbent: Kieselgel G containing 2% cinchonine. Solvent: chloroform–acetic acid (85:15) with 2% cinchonine; system: ascending.

Quantitative estimation of alkyl gallates

The spots of known and unknown quantities of gallates were separately scrapped with a microspatula and extracted with a known volume of 90% ethanol. The percentage transmission of each solution was recorded at 272 mμ, and the quantity of the gallates was calculated.

Discussion and results

When the alkyl gallates with their mixture were run on untreated Kieselgel G and magnesium silicate plates employing different solvent systems, very close R_F values, overlapping each other particularly in the cases of *sec.*- and *tert.*-alkyl gallates, resulted. It was not possible to separate these branched-chain alkyl gallates even by two-dimensional thin-layer chromatography. The typical R_F values of various gallates on untreated plates were as follows:

Adsorbent Kieselgel G; solvent system: petroleum ether 60–80°–acetic acid (5:1); A, 0.16; B, 0.18; C, 0.21; D, 0.14; E, 0.26; F, 0.13; G, 0.12; H, 0.25; I, 0.30; J, 0.13; K, 0.39.

Adsorbent magnesium silicate; solvent system: carbon tetrachloride–acetic acid (10:1); A, 0.08; B, 0.10; C, 0.11; D, 0.06; E, 0.13; F, 0.06; G, 0.05; H, 0.13; I, 0.21; J, 0.06; K, 0.26.

Kieselgel G and Kieselgel G–Kieselguhr G (3:1) proved to be excellent adsorbents for the complexes studied. Incorporation of Kieselguhr G greatly helped in the migration of complexes. The spots were quite distinct in both cases, and there was no tailing. Magnesium silicate required more time for irrigation and was not helpful in good resolutions of isopropyl gallate, *sec.*-butyl gallate, *tert.*-butyl gallate and *sec.*-octyl gallate complexes.

Polarity of solvents played an important role. Higher polarity favored increase in the movement of complexes and hence better resolutions of mixtures.

In general the R_F values of the complexes of alkyl gallates containing normal alkyl groups increased with the rise in their hydrocarbon radicals. Molecular complexes of esters having branched-chain alkyl groups with both cinchonine and strychnine had lower mobilities compared to their straight-chain analogues. This apparently showed a steric influence of the branched-chain molecular complexes on their migrations. The alkyl gallates could be conveniently characterized and estimated up to 2 μg.

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CHROM. 4417

THIN-LAYER CHROMATOGRAPHIC SEPARATION ON LAYERS HEAVILY LOADED WITH POWDERED SCINTILLATORS FOR LUMINESCENCE DETECTION OF RADIONUCLIDES

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SUMMARY

Experiments are reported demonstrating that interference is not necessarily caused in a chromatographic separation on standard thin layers by the admixture of powdered scintillators. The study is of importance in connection with a sensitive method of radionuclide detection by β-radioluminescence (either scintillation fluorography on photographic film material or by direct photoelectric detection). Kieselguhr-, silica gel-, and cellulose thin layers were prepared containing various proportions of either an inorganic or organic scintillator (zinc silicate or anthracene, respectively). Mixtures of carbohydrates, phospholipids, and amino acids were tested as representative systems with suitable layers and developing solvents.

INTRODUCTION

A successful chromatographic fractionation on thin layers of adsorbents containing powdered scintillators would be advantageous for the detection of radionuclides by means of β-radioluminescence (scintillation fluorography, or -autoradiography).

This method[1-7] is based on the conversion of the β-particle energy into light upon absorption in a suitable scintillator. The high reflection coefficients of Al_2O_3- and SiO_2-powders (standard thin-layer media) means that a large proportion of the emitted photons manage to escape from the chromatogram and can be used for localization of the radio-labelled spots and measurement of their activities.

The method is particularly useful for low-energy radionuclides such as tritium (^3H) and radiocarbon (^{14}C) (refs. 1, 4–7), the two most important radio labels. In the case of these nuclides only a small proportion of the electrons are capable of leaving the chromatogram and therefore direct electron detection of the spots is normally much slower than with the β-radioluminescence method.

There are several types of β-radioluminescence detection. They may be grouped into liquid scintillation, gel scintillation, and solid scintillation methods. In solid scintillation detection two different techniques may be employed for preparing the layer:

(a) The scintillator is added to the thin-layer plates (in the form of a solution or suspension) by a spraying technique following the completion of the separation procedure. After drying, either film- or photomultiplier detection is applied.

(b) The scintillator is added to the adsorbent before the preparation of the thin-layer plates. With this method much larger amounts of scintillators may be added[4–7]. Detection is as above.

The alternative (b) is normally preferred, provided the scintillator is chemically inert and the fractionation is obtained without difficulty. The advantages are less work in preparing the plates and a much better reproducibility in the nuclide quantization.

The purpose of these experiments has been to investigate whether the addition of scintillators (anthracene or zinc silicate) to some thin-layer media would hinder a successful separation. The separation properties of a thin-layer medium depend upon several parameters, such as grain size, pore diameter and volume, density, and surface area[8]. In addition, the chromatographic fractionation may be modified, for example, by mixing adsorbents, by using buffer solutions instead of water for dispersion, or, finally, by impregnation or heat activation[9]. Small variations in other parameters, such as those of the experiment itself, might give some degree of fluctuation in the results obtained[10,11].

It is now reported how some selected systems retained or modified their separation performances upon admixture with a scintillator.

EXPERIMENTAL

Kieselgel 7431 G Merck, Kieselguhr 8129 G Merck, and cellulose powder MN 300* were used as adsorbents. Zinc silicate (B.D.H.) or anthracene (purity 99%)**, were used as scintillator additives. Layer thickness was kept close to 0.25 mm, the plate dimension was 7.5 × 15 cm. The plates were coated with the layer by means of a Quickfit demonstration kit. Solute samples were applied in volumes from 1 to 10 μl. Ascending development for a distance of 10–12 cm was used. A reference plate was always developed together with the scintillator-loaded plate to avoid, as far as possible, any difficulty in establishing the reproducibility of thin-layer chromatography[8–11]. The selected chromatographic systems and visualization methods are presented in Table I.

Anthracene layers

The anthracene available was in large crystals which required grinding before preparing the layers. The adsorbent and the anthracene were thoroughly mixed before and after addition of the suspension liquid. 50% amounts of anthracene only were used. Table II shows the proportions used for mixing the layer suspension (sufficient for approximately 2 plates 7.5 × 15 cm).

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** Schuchardt, Munich, G.F.R.

TABLE I

SELECTED CHROMATOGRAPHIC TEST SYSTEMS AND VISUALIZATION

	Carbohydrates	*Phospholipids*	*Amino acids*
Test systems			
Compound	Rhamnose, xylose, arabinose, mannose, glucose, and galactose (Eastman Organic Chemicals)	Lecithin, unpurified (A/S Norsk Soyamelfabrik)	Cystine, glycine, leucine, lysine (Shandon standard solutions)
Adsorbent	Kieselguhr G[a]	Silica Gel G	Cellulose and Silica Gel G
Developing solvent	Butanol–acetone–water (40:50:10)	Chloroform–methanol–water (65:25:4)	Chloroform–methanol–17% ammonia (40:40:20)
Color reagent and reaction			
Reagent	Phthalic acid–aniline in acetone	Molybdenum reagent	Ninhydrin reagent
Adsorbent only	Brownish	Bluish	Violet
Adsorbent + anthracene	Brownish	Bluish with blue-grey background	Red-grey
Adsorbent + zinc silicate	Pale brownish	Bluish	Orange

[a] Pre-treated with 0.2 *M* NaH_2PO_4.

TABLE II

ANTHRACENE LAYER COMPONENTS

Anthracene (%)		*Anthracene* (g)	*Adsorbent*	*(g)*	*Suspension liquid*	*(ml)*
0	Reference	0	Kieselguhr G	4	0.2 *M* NaH_2PO_4	7
50		1.5	Kieselguhr G	1.5	100% ethanol	8[b]
0	Reference	0	Silica gel G	3	50% ethanol	7
50		1	Silica gel G	1	96% ethanol	6
0	Reference	0	Cellulose	1.5	50% ethanol	8
50		1	Cellulose	1	96% ethanol	7

[b] 0.2 *M* NaH_2PO_4 was applied to the layer by spraying *ca.* 3 ml 0.2 *M* NaH_2PO_4 per plate.

Zinc silicate layers

The zinc silicate available seemed to be of variable grain size. We tried to get it more homogeneous by grinding. The zinc silicate was used in amounts of 25, 50, 75 and 100%. Table III shows what proportions were used when mixing the layer suspensions (for approximately 2 plates 7.5 × 15 cm).

RESULTS AND DISCUSSION

The effect of the addition of anthracene and zinc silicate to the selected systems was examined; the experimental results are presented in Tables IV and V, where the

TABLE III

ZINC SILICATE LAYER COMPONENTS

Zinc silicate			*Adsorbent*	*(g)*	*Suspension liquid (ml)*	
(%)		*(g)*				
0	Reference	0	Kieselguhr G	4	0.2 *M* NaH_2PO_4	7
50		2	Kieselguhr G	2	0.2 *M* NaH_2PO_4	10
25		1	Kieselguhr G	3	0.2 *M* NaH_2PO_4	9.5
0	Reference	0	Silica Gel G	3	50% ethanol	7
50		1	Silica Gel G	1	50% ethanol	6
75		2	Silica Gel G	0.7	50% ethanol	8.7
100		3	Silica Gel G	0	50% ethanol	10
0	Reference	0	Cellulose	1.5	50% ethanol	8
50		0.8	Cellulose	0.8	50% ethanol	8

TABLE IV

R_F VALUES WITH AND WITHOUT ANTHRACENE IN THE LAYER

Brace shows unseparated compounds.

Compounds in mixture applied		*Amount (μg)*	*R_F values on adsorbent only (reference)*	*R_F values with 50% anthracene in the layer*
Carbohydrates			*Kieselguhr G*[a]	
Rhamnose		2	0.93	0.92
Xylose		2	0.70	0.55
Arabinose		2	{ 0.50	{ 0.35
Mannose		2		
Glucose		2	0.36	0.27
Galactose		2	0.24	0.18
Phospholipids			*Silica Gel G*	
Impure lecithin		150		
Unknown component	I		0.98	0.87
Unknown component	II		0.84	0.78
Unknown component	III		0.66	0.67
Unknown component	IV		0.51	0.47
Unknown component	V		0.41	—
Unknown component	VI		{ 0.30	{ 0.27
Unknown component	VII		{ 0.25	{ 0.23
Unknown component	VIII		0.13	0.12
Amino acids			*Cellulose*	
Leucine		1.3	0.85	0.91
Glycine		0.8	0.50	0.54
Cystine		2.4	0.32	0.35

[a] Pre-treated with NaH_2PO_4.

R_F values of the same components separated on layers with and without scintillator are shown. Figs. 1 and 2 illustrate the separation.

Anthracene

The separation properties (and the developing rate) were not influenced by the addition of anthracene to the layer. The R_F values however, were displaced a little

TABLE V

R_F VALUES WITH AND WITHOUT ZINC SILICATE IN THE LAYER

Brace shows unseparated components.

Compounds in mixture (applied)	*Amount (μg)*	*R_F values on adsorbent only (references)*	*R_F values on layers with 50% zinc silicate*	*R_F values on layers with 25-, 75- and 100% zinc silicate*	
Carbohydrates		*Kieselguhr G*[a]		*25%*	
Rhamnose	4	0.90	0.68	0.82	
Xylose	4	0.61	0.58	0.72	
Arabinose	4	0.42	0.45	0.62	
Mannose	4				
Glucose	4	0.31	0.30	0.56	
Galactose	4	0.22		0.47	
Phospholipids		*Silica Gel G*			
Impure lecithin	100				
Unknown component I		0.98	0.82		
Unknown component II		0.85	—		
Unknown component III		0.67	0.65		
Unknown component IV		0.36	0.45		
Unknown component V		0.17	(0.27–0.18)		
Unknown component VI		0.06	0.03		
Amino acids				*75%*	*100%*
Leucine	2.6	0.50	0.68	0.63	0.78
Glycine	2.1	0.26	0.36	0.44	0.59
Cystine	4.8		—	0.38	
Lysine	3.6	0.10	0.25	—	
Amino acids		*Cellulose*			
Leucine	2.6	0.79	0.69		
Glycine	2.1	0.37	0.33		
Cystine	0.21	0.21	0.09		

[a] Pre-treated with 0.2 M NaH_2PO_4.

as Table IV and Fig. 1 show. This generally minor influence of anthracene on the separation properties of silica gel has been reported earlier by LÜTHI AND WASER[4].

Zinc silicate

Admixtures of zinc silicate caused some changes in the separation properties as Table V and Fig. 2 show. Developing time increased considerably (*e.g.* 2 h per 10 cm for phospholipids on the silica gel layer with 50% zinc silicate).

Zinc silicate in Kieselguhr G layers resulted in a smaller spread in the R_F values of the carbohydrates, as shown in Fig. 2, and a fainter coloring of the spots when sprayed with the color reagent.

Zinc silicate had no effect on the separation of the impure lecithin on the Silica Gel G layers, but the R_F values were displaced. Of the amino acids tried on this layer, cystine and glycine did not separate though they had different R_F values. Glycine formed a two-edged tail.

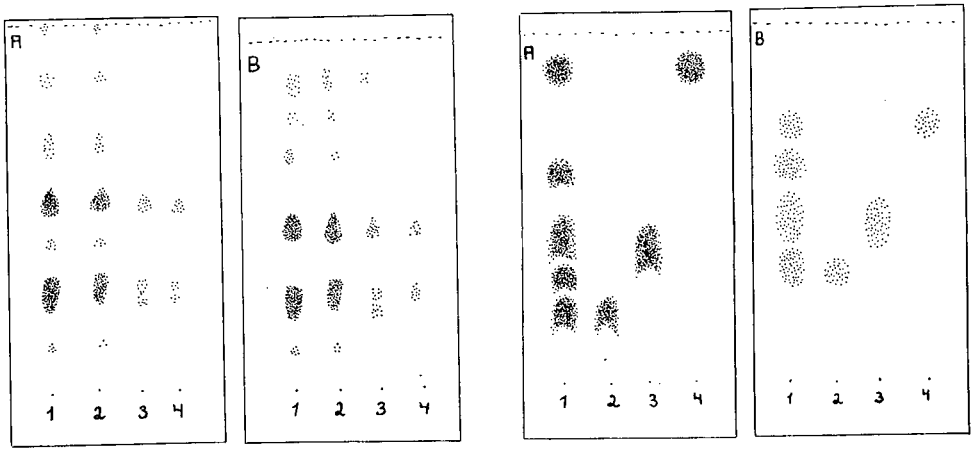

Fig. 1. Comparison between separation of phospholipids (impure lecithin) on Silica Gel G layers with and without anthracene addition. A = 100% Silica Gel G (reference); B = 50% Silica Gel G, 50% anthracene. Developing solvent: chloroform–methanol–water (65:25:4). Amounts of lecithin applied: (1) 150 μg, (2) 100 μg, (3) 10 μg, (4) 5 μg.

Fig. 2. Comparison between separation of carbohydrates on Kieselguhr G layers with and without zinc silicate addition. A = 100% Kieselguhr G pre-treated with 0.2 *M* NaH_2PO_4 (reference); B = 50% Kieselguhr G, 50% zinc silicate pre-treated with 0.2 *M* NaH_2PO_4. Developing solvent: *n*-butanol–acetone–water (40:50:10). (1) A mixture (from top to bottom) of: rhamnose 4 μg, xylose 4 μg, arabinose 4 μg, mannose 4 μg, glucose 4 μg and galactose 4 μg; (2) galactose 4 μg; (3) mannose 4 μg, arabinose 4 μg; (4) rhamnose 4 μg.

The effect of adding zinc silicate to the cellulose layers was studied using the same amino acids and developing solvent as was used in the Silica Gel G layers. Cystine and glycine did not separate in this case either, and glycine formed the same sort of tail as on silica gel plus zinc silicate layers.

CONCLUSION

Addition of anthracene to the layers leads to only small displacements of the R_F values of the compounds separated. Zinc silicate addition, however, resulted in changed separation properties for some of the selected systems.

The systems studied were chosen on the basis of common chromatographic practice and no special adjustments were tried. The experiments are somewhat preliminary. One may suppose that more accurate adjustments in selected systems will result in improved performances as compared to those demonstrated so far.

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EVALUATION OF SOLVENTS USED IN SUMMARIZED CHROMATOGRAMS

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SUMMARY

Based on some concepts from the Information Theory, the solvents used for antibiotic substances by ISHIDA *et al.* and BETINA were evaluated relative to each other by means of summarized chromatography.

The information that a summarized chromatogram system supplies should depend mainly on three factors: (1) distribution of the R_F of the reference substances in each solvent; (2) interrelationship between the R_F values that a given substance shows in the different solvents; and (3) experimental error.

Thus, admitting a constant error, it was found that among the 11 solvents tested, those that gave the most information were water; butanol; 3% aq. ammonium chloride; benzene–methanol (4:1) and butanol–methanol–water (4:1:2) with and without helianthin. The phenolic solvents were less informative; ethyl acetate is mediocre in all cases.

Although the information is very high in some of the 11 solvents tested (up to 85% of H_{max}), only water supplies information independent of the others, which are redundant among each other.

INTRODUCTION

The papergram or summarized chromatogram procedure is usually used in our laboratories for the rapid identification of antibiotic substances. The solvents used include those suggested by ISHIDA *et al.*[1], and, more recently, those suggested by BETINA[2] have also been employed. We have been interested in evaluating these solvents in order to determine which supplies more and which supplies less information.

According to the Information Theory, all R_F values in a solvent must be equally probable for this solvent to provide the maximum information ($H_{s(max)}$). Furthermore, to obtain the maximum information from a summarized chromatogram

TABLE I

EXPERIMENTAL R_F VALUES × 100

Solvent systems used: 1 = butanol satd. with water; 2 = 3% ammonium chloride; 3 = 80% phenol; 4 = 80% phenol in NH_3 atm.; 5 = acetone–water (1:1); 6 = butanol–methanol–water (4:1:2) + 0.15 g helianthin; 7 = butanol–methanol–water; 8 = benzene–methanol (4:1); 9 = water; 10 = ethyl acetate satd. with water; 11 = benzene satd. with water. Solvents 1 to 9 correspond to the system of ISHIDA *et al.*; solvents 9,1,10 and 11 correspond to BETINA's system.

Antibiotics	*Solvent system*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Actinomycin	92	25	100	84	43	90	89	88	20	92	15
Aminocidin sulphate	0	87	4	77	6	23	3	0	27	0	0
Chloramphenicol	86	85	91	90	93	88	89	45	86	85	9
Chlortetracycline hydrochloride	28	51	81	82	31	64	56	0	43	0	0
Colistin sulphate	0	94	65	96	49	54	24	14	14	0	0
Cycloheximide	72	87	92	91	91	80	80	59	93	70	13
Demethyltetracycline	25	31	60	73	30	45	43	0	28	27	0
Dihydrostreptomycin sulphate	0	95	8	95	0	57	0	0	0	0	0
Etamycin	93	33	90	94	92	95	94	85	39	93	15
Filipin	95	0	97	87	94	93	92	0	0	75	0
Griseofulvin	90	0	96	91	80	90–45	92	94	0	95	89
Leukomycin tartrate	94	82–0	96	95	94	93	94	93	28	95–0	15
Lincomycin hydrochloride	38	100	90	95	89	71	66	65	100	0	0
Meticilin	34	97	95		97	49	43		95	0	0
Mikamycin A	97	0	95		92	98	97	23	3	98	15
Mikamycin B	91	13	95	81	94	96	95	55	25	97	27
Misionin	72	0	93	86	67	79	72	50	0	0	0
Neomycin	0	95	0	77	0	45	0	0	0	0	0
Nystatin	6	0	84	68	0	55	53	0	0	0	0
Oxacillin	46	88	93	96	95	66	68	0	94	58–0	0
Paromomycin	0	92	0	81	0	22	0	0	0	0	0
Penicillin G-Na	40	94	88	94	91	62	64	0	100	0	0
Penicillin V-K	37	88	88	94	94	57	62	0	100	0	0
Polymyxin B sulphate	0	97	67	96	0	67	52	0	0	0	0
Puromycin	49	65	92	95	86	73	67	43	18	0	0
Pyrrolidinemethyltetracycline	7	65	83	82	43	55	36	25	37	0	0
Rifamycin O	96	0	94	100	93	100	100	100	65–0	100–0	54–0
Rifamycin S	95	28	93	94	92	93	94	83–0	77	92	69
Rifamycin SV-Na	91	43	94	93	81	92	91	91–0	79	100	50
Spiramycin base	86	94	100	100	93	94	88	0	7	99–0	0
Staphylomycin	93	45	100	0	93	94	92	18	34	100–0	0
Streptomycin sulphate	0	100	0	85	0	36	0	0	4	0	0
Terramycin base	25	74	92	100	54	67	55	10	52	0	0
Tylosin base	92	92–0	92	100	92	87	85	35	43	100–0	0
Viomycin sulphate	0	100	0	100	0	49	0	0	0	0	0

($H_{p(\max)}$), the R_F obtained with a solvent must be independent of the R_F in another solvent.

Observation of the R_F values obtained with a group of substances and several solvents, such as the data published by BETINA[2], or those shown in Table I, suggests that the distribution of the R_F values is not equiprobable because extreme R_F values occur more frequently than median ones. In this case the graphical representation of the frequency as a function of R_F would give a U-shaped curve.

With regard to the independence of the R_F value of a solvent as regards another solvent, if the functional chromatographic mechanism is the same, we should expect

some relation between them, although it may be complicated or unknown. An example of this is the F factor found by CONNORS[3] which relates the R_F values of uracil derivatives, barbituric acids, inorganic phosphates, etc. in different solvents when the functioning mechanism is partition. Later, SOCZEWINSKI[4] used R_M values to find the same factor.

A third variable that affects the information supplied by a papergram is the experimental error, which, according to the Information Theory, establishes the number of signals, n, to be considered, that is the number of discernible R_F values.

The present report deals essentially with the study of the R_F distribution for which the experimental error can be ignored; however, it is convenient to mention it here.

In the methods described here, the error is undoubtedly greater than in conventional chromatography because data obtained with substances that are minor components in broths of variable composition are compared with those obtained from pure reference substances. In the symposium organized by the Chromatography Group of the Czechoslovak Chemical Society at Liblice, in June 1961, an error of 10% was admitted for conventional chromatograms. We have found variations of up to 0.2 R_F; in addition it was found that the error depends on the solvent. Water showed the least error (variations of 0.1 R_F) while acetone–water (1:1) showed the greatest error, which we consider a question of speed and equilibrium of the chromatographic process. We believe that expression of the R_F values for these methods would be more logical with only one decimal place ($n = 10$) rather than two decimal places ($n = 100$) which is more commonly used.

Assuming the same experimental error for all solvents, the maximum information given by each would be:

$$H_{s(\max)}\, n = \log_2 n \text{ (bits)}$$

If a chromatographic system consists of m solvents and the condition of independence is fulfilled, the maximum theoretical information that it can supply would be:

$$H_{p(\max)} = m \log_2 n$$

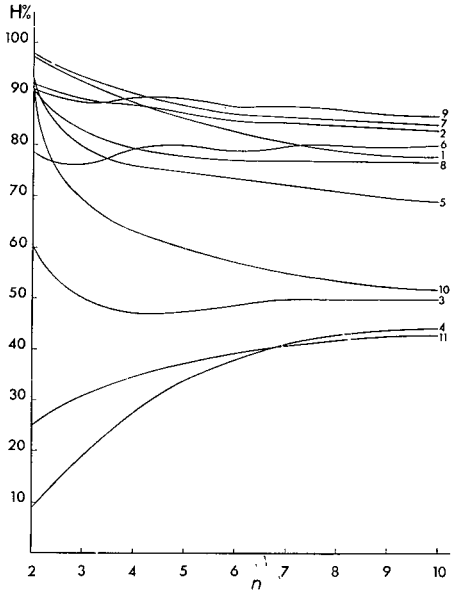
Supposing we had 2,000 reference antibiotics to identify one unknown, we would need $\log_2 2{,}000 = 10.96 \cong 11$ bits of information.

If $n = 100$, $H_{s(\max)} = \log_2 100 = 6.66$ bits so that we would only need two solvents.

If $n = 10$, $H_{s(\max)} = \log_2 10 = 3.32$ bits, and we would need four solvents to solve the problem. Experience has shown that a problem like this is unlikely to be solved with just a few solvents so that the real information obtained (H_p) would be less than the $H_{p(\max)}$ that we have just calculated.

The procedure chosen to study the distribution of the experimental data in each solvent consisted of dividing the R_F scale into a number of equal sectors and considering the R_F data within each sector as equal signals. This allowed us to calculate the information of the solvent (H_s) for n signals and to relate it to $H_{s(\max)}$ for the same n.

$$H\% = \frac{H_s}{H_{s(\max)}} \cdot 100$$



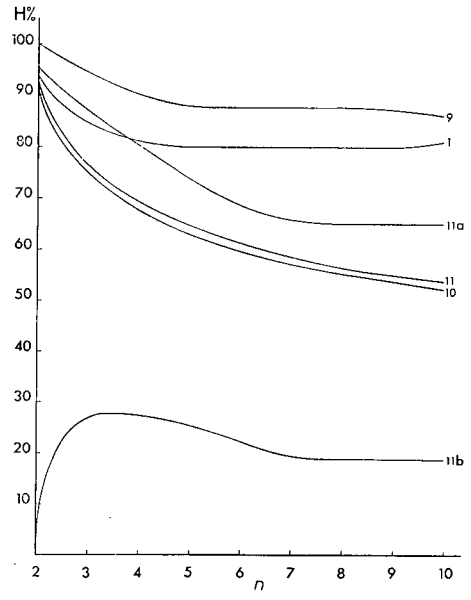


Fig. 1. $H\% = f(n)$ with our data. Numbers correspond to the solvents listed in Table I.

Fig. 2. $H\% = f(n)$ with data published by BETINA. Numbers correspond to the solvents in Table I, (11a) benzene considering only fungal antibiotics, (11b) *idem* with no fungal antibiotics.

The H_s value was calculated according to the formula of SHANNON AND WEAVER[5]:

$$H_s = \Sigma p_i \log_2 p_i$$

p_i being the probability of the appearance of each signal.

We calculated $H\%$ for each solvent and for a series of n values and plotted $H\% = f(n)$, thus obtaining curves that enabled us to evaluate the solvents relative to each other (see Figs. 1 and 2). When making the comparisons, we chose the interval of n values from 6 to 10 in order to obtain a more accurate evaluation, bearing in mind the number of experimental data and former considerations about the experimental error.

The experimental values used were partly taken from those published by BETINA (only those for the four basic solvents, 62 data each), and partly those obtained by us using the same solvents and the solvents proposed by ISHIDA *et al.* (in all 11 solvents, 35 data each), (Figs. 1 and 2).

Our data were obtained by a technique described elsewhere[6], for which the containers and quantities of solvent are smaller than the ones used by BETINA. These details are not considered important, since the problem has been studied from a comparative point of view.

The information obtained with each of the 11 solvents studied is shown in Fig. 1. It is possible to see a group of solvents that give quite a high $H\%$ (between 77 and 87%); these include water, *n*-butanol–methanol–water (4:1:2), 3% aq. ammonium chloride, *n*-butanol saturated with water and benzene–methanol (4:1). The ace-

TABLE II

χ^2 VALUES

Solvent systems compared	χ^2		
	Our data	BETINA's *data*	
Satd. butanol *vs.* water	0.00	0.53	
3% Aq. ammonium chloride *vs.* water	1.44		
Benzene–methanol (4:1) *vs.* water	1.83		
Butanol–methanol–water (4:1:2) + 0.15 g helianthin *vs. idem* without helianthin	18.48		
Butanol–methanol–water (4:1:2) *vs.* satd. butanol	10.87		
Butanol–methanol–water (4:1:2) *vs.* water	2.61		
Satd. butanol *vs.* satd. ethyl acetate	10.29	31.30	
Satd. ethyl acetate *vs.* water	0.29	0.70	
Satd. benzene *vs.* water	2.45	0.34	1.82[a]
Satd. benzene *vs.* satd. butanol	5.48	16.60	8.49[a]
3% Aq. ammonium chloride *vs.* benzene–methanol (4:1)	7.34		
3% Aq. ammonium chloride *vs.* butanol–methanol water (4:1:2)	4.81		
3% Aq. ammonium chloride *vs.* satd. butanol	12.93		
Satd. butanol *vs.* benzene–methanol (4:1)	8.68		
3% Aq. ammonium chloride *vs.* satd. ethyl acetate	7.93		
Satd. ethyl acetate *vs.* satd. benzene		18.10	

[a] Values obtained considering only fungal antibiotics.

tone–water (1:1) solvent supplies slightly less information and finally four solvents (ethyl acetate saturated with water, 80% aq. phenol, 80% aq. phenol in an ammonia atmosphere and benzene saturated with water) supply little information (between 43 and 52%).

We obtained Fig. 2 from the data published by BETINA for his four basic solvents. This is similar to Fig. 1 in the sense that water and butanol are in the group of solvents supplying more information, while ethyl acetate and benzene are in the group supplying minor information.

Almost half of the antibiotics used by BETINA are produced by fungi. Compared with this, we only used a few antibiotics of that origin. To take this difference into account we studied the data obtained by BETINA for antibiotics produced by fungi

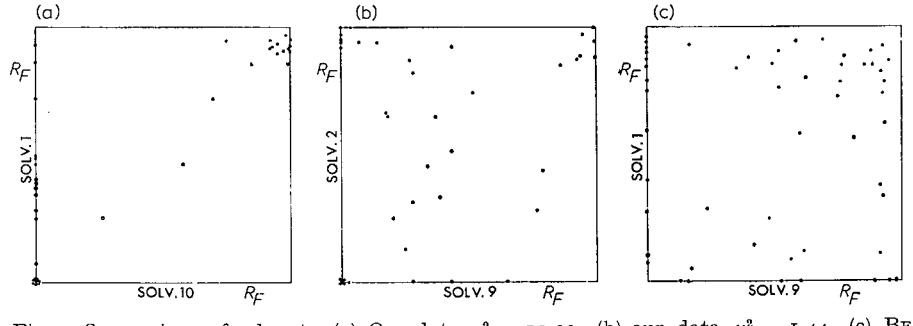


Fig. 3. Comparison of solvents. (a) Our data $\chi^2 = 10.29$, (b) our data $\chi^2 = 1.44$, (c) BETINA's data $\chi^2 = 0.53$.

and those from other sources separately. With respect to water, butanol and ethyl acetate, similar results were obtained for both groups of antibiotics, but benzene gives more information in the group of antibiotics from fungi (65%) than in the other group (20%). The explanation for this is that fungi produce simpler substances than actinomycetes and bacteria and they are often aromatic.

By applying the chains of MARKOFF[7], we can find how much the solvent information decreases due to redundance, but we do not consider that we have sufficient experimental data. However we can tell whether there is independence between the information given by two solvents if we present the R_F data of each of the substances graphically and afterwards test the independence hypothesis by the χ-square method. For this we divide the diagram into four equal sectors of 0.5 R_F per side. Fig. 3 shows the R_F distribution obtained with some pairs of solvents and Table II summarizes the χ^2 data obtained with the solvents that supplied a great deal of information.

Establishing the 0.05 significance level ($\chi^2 = 3.84$), we can only accept the independence hypothesis for the pairs when one of the solvents is water.

CONCLUSIONS

Among the solvents studied by BETINA, water and butanol supply more information. Benzene is only useful in the case of antibiotics produced by fungi; ethyl acetate is mediocre in all cases.

Some of the solvents proposed by ISHIDA *et al.* in addition to butanol and water are also very informative. These are 3% aq. ammonium chloride, benzene–methanol (4:1), butanol–methanol–water (4:1:2) with and without helianthin. The phenolic solvents were less informative.

Although much information is obtained in some of the eleven solvents tested (up to 87% of H_{max}), only water supplies information independent of the others, which are redundant among each other.

ACKNOWLEDGEMENT

We wish to express our thanks to Dr. FERMIN J. ALFONSO for his kind advice and discussion of the present paper.

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CHROM. 4488

CHEMICAL AND CHROMATOGRAPHIC ISOLATION OF *K*-CASEIN*,**

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SUMMARY

Improved chemical and chromatographic methods for the isolation of electrophoretically pure *K*-casein have been developed. The chemical method is simple, mild, reproducible, and can be completed in about one day. Contact of the protein with reactive chemicals is minimized. The use of 6.6 *M* urea in the first step apparently does not adversely affect the *K*-casein. The remaining treatments, addition of Ca^{2+}, pH adjustment, dialysis, and centrifugation are mild. The method gives a good yield of *K*-casein (1.5 g/15 g of pI casein). A very pure *K*-casein was obtained by DEAE-cellulose chromatography of the chemically isolated protein. The polyacrylamide and starch gel urea electrophoretic gel patterns of chemically prepared *K*-casein show that the protein is electrophoretically pure and the gel zone patterns of *K*-casein reduced with 2-mercaptoethanol show the characteristic bands as reported by others. The amino acid composition, and content of sialic acid and phosphorus are comparable with the reported values. A single symmetrical sedimenting peak of 13.5 S was obtained on ultracentrifugation in 0.076 *M* Tris–0.005 *M* citrate buffer, pH 8.6.

INTRODUCTION

In protein research, the important objective is to obtain a preparation in pure form. The isolation procedure should be simple, involve mild treatment of the protein, and give a good yield of the final preparation. DEAE-cellulose column chromatography and Sephadex gel filtration have been used as the major means of purifying the caseins[1–3]. One of the most common chemical fractionation procedures in use for isolation of the *K*-casein is the urea–sulfuric acid method[4], which involves the use of 7 *N* H_2SO_4. The *K*-casein obtained by this method contains major impurities of

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** Experimental data taken in part from doctoral thesis of K. K. TRIPATHI, University of Missouri, 1968.

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α_{s1}- and β-caseins. A further purification is usually made by ethanol precipitation[5].

This research reports a simple, reproducible, two-step chemical procedure for isolation of K-casein, chromatography on DEAE-cellulose and comparisons with K-caseins isolated by other chemical and chromatographic methods. Characterization was by polyacrylamide (PGUE), and starch gel urea electrophoresis (SGUE), chemical and amino acid analyses, sedimentation velocity, and gel-zone electrophoresis after reduction with 2-mercaptoethanol (2ME).

EXPERIMENTAL

The procedures for the preparation of the K-casein are outlined as follows and in the accompanying flow diagram. The essentials of the isolation of K-casein include the urea fractionation of isoelectric casein into the α_{s1}-K-casein complex with β- and γ-casein remaining in the supernatant. The α_{s1}-K-casein complex is then fractionated with 0.25 M $CaCl_2$ at pH 7.0, followed by DEAE-cellulose column chromatography for further purification.

FLOW DIAGRAM

CHEMICAL FRACTIONATION OF K-CASEIN

Isoelectric casein (15 g) from pooled milk, twice precipitated at pH 4.6 and washed.

1 — Disperse in 400 ml H_2O. Dissolve in 6.6 M urea, dilute to 4.6 M urea. Allow to stand 8 h at 2°, pH 4.6 Centrifuge at 3000 r.p.m. for 30 min.

- Supernatant (β- and γ-casein). Use for preparation of β- and γ-casein.
- Precipitate (α_{s1}-K-casein gel).

2 — Repeat step 1.

- Supernatant. Discard.
- Precipitate (α_{s1}-K-casein gel).

3 — Disperse in 250 ml H_2O. Add 0.1 N NaOH dropwise with stirring to pH 8.5. Adjust to pH 7.0 with 0.1 N HCl. Make 0.25 M in $CaCl_2$ with 4 M $CaCl_2$. Maintain at pH 7.0 with 0.1 N NaOH. Centrifuge at 3000 r.p.m. for 30 min.

- Precipitate Ca-α_{s1}-, and trace of β-casein. Use for α_{s1}-casein preparation.
- Supernatant (enriched-K-casein).

Dialyze, lyophilize and repeat step 3.

4

- Precipitate. Discard.
- Supernatant (enriched K-casein).

5 — Centrifuge at 30,000 r.p.m. for 30 min.

- Precipitate (Ca-α_{s1}-casein). Discard.
- Supernatant (K-casein).

6 — Dialyze *vs.* distilled H_2O at 2° using 4 changes at 4 to 8 h each.

Chemically prepared K-casein.
Further purified by DEAE-cellulose chromatography.

Chemical method

Fifteen grams of isoelectric casein were suspended in 400 ml of distilled water and dissolved in urea solution (6.6 M). The solution was diluted to 4.6 M in urea and

held until the precipitate settled (approximately 8 h). The supernatant, containing the β- and γ-caseins, was decanted and discarded. The precipitate, containing the α_{s1}-*K*-casein complex, was washed with distilled water and again dissolved in urea solution (6.6 *M*). The solution was diluted to 4.6 *M* in urea. The supernatant was decanted and discarded. The gelatinous α_{s1}-*K*-casein complex was dispersed in 250 ml of distilled water and was dissolved by the dropwise addition of 0.1 *N* NaOH with constant stirring to pH 8.5, which was not exceeded. The solution was readjusted to pH 7.0 with 0.1 *N* HCl, and sufficient 4 *M* $CaCl_2$ was slowly added to make the resulting solution 0.25 *M* in $CaCl_2$. The pH was maintained at 7.0 by the addition of 0.1 *N* NaOH. The suspension was centrifuged at 3000 r.p.m. for 30 min. The supernatant (*K*-casein) was saved and the precipitate, Ca-α_{s1}- and β-caseinates, was discarded. The supernatant was thoroughly dialyzed until free of Ca^{2+}, lyophilized, and then the $CaCl_2$ treatment was repeated as above. The suspension was centrifuged at 30,000 r.p.m. for 30 min. The supernatant, containing the *K*-casein, was dialyzed against distilled water until free of Ca^{2+}, then lyophilized. All of the operations were carried out at 2–4°. The yield was at least 1.5 g of electrophoretically pure *K*-casein.

The chemically prepared *K*-casein was purified by both DEAE-cellulose column chromatography and 75% ethanol 1 *M* in NH_4Ac methods to determine if an improvement in the purity of the isolated *K*-casein could be obtained.

Chromatography on DEAE-cellulose

DEAE-cellulose with 0.70 mequiv./g exchange capacity, obtained from Bio-Rad Laboratories, was used. The anion exchanger was regenerated and thoroughly washed. A 25 × 4.5 cm column was used, and equilibrated with 2 l of 0.03 *M* glycine–NaOH buffer (pH 8.0) containing 2 *M* urea, at a flow rate of 250 ml/h. A soft gradient stepwise elution was made with successively higher concentrations of NaCl (0.05–0.25 *M*) in the glycine–NaOH buffer. The effluent was continuously monitored and recorded by a Vanguard automatic UV analyzer, Model 1056. The effluent fractions were dialyzed against distilled water for 24 h with changes each 4 h, then lyophilized. In addition, the methods of ZITTLE AND CUSTER[4] and MCKENZIE AND WAKE[5] were used for the isolation of *K*-casein preparations which were then compared with the chemically and chromatographically prepared *K*-casein described in this paper. Also, the ethanol–NH_4Ac method of MCKENZIE AND WAKE[5] was used for further purification of the chemically prepared *K*-casein.

PGUE and SGUE characterization

The prepared *K*-caseins were characterized by PGUE and SGUE. The details of the electrophoresis, gel concentration, sample loading, and buffer conditions, etc., have been described by GEHRKE *et al.*[6]. The chemically and chromatographically prepared *K*-caseins were also reduced with 2ME (0.2 v/v%) in Tris (hydroxymethyl)-aminomethane (THAM) Na_2EDTA–boric acid buffer pH 9.2 for PGUE. The same concentration of 2ME in Tris–citrate buffer pH 8.6 was used for SGUE. A comparative study of the *K*-caseins prepared by the different methods was made by PGUE.

Amino acid analysis

The amino acid composition of the *K*-casein preparation was determined with a Technicon automatic amino acid analyzer using the procedure described by PIEZ

AND MORRIS[7]. Thirty milligrams of duplicate samples, dried over P_2O_5 for several hours, were hydrolyzed for 24 h with 6 *N* HCl at 110° in a sealed tube under nitrogen. The HCl was removed with rotary evaporation at room temperature or by lyophilization. The separations were made on a 0.6 × 133 cm column of 22 micron spherical polynuclear sulfonic acid resin beads (Chromobeads A) at 60° using citrate buffers of pH 2.875, 3.80 and 5.00 for the gradient elution. The data are given in Table I.

TABLE I

WEIGHT PER CENT OF AMINO ACIDS IN *K*-CASEIN

Each value is an average of 2 independent determinations. Chemical preparations.

Amino acid	*Per cent*	*Amino acid*	*Per cent*
Aspartic acid	7.94	Methionine	1.92
Threonine	6.64	Isoleucine	5.98
Serine	5.63	Leucine	6.04
Glutamic acid	19.03	Tyrosine	7.13
Proline	7.88	Phenylalanine	3.52
Glycine	1.17	Lysine	7.18
Alanine	4.86	Histidine	2.18
Valine	5.36	Arginine	3.65
Half-cystine	0.75		
		Total	96.86

Chemical analysis

The sialic acid content of the *K*-casein was determined by WARREN's thiobarbituric acid method[8]. Thirty milligrams were hydrolyzed at 80° for 1 h in 10 ml 0.1 *N* H_2SO_4 to release the sialic acid. Sialic acid (concentrated assay, 16.5% sialic acid) was used as the standard. The standards and unknown were carried through the same procedure.

The phosphorus content was determined by the phosphomolybdovanadate method. The standards were prepared to contain 0.5, 1.0, 1.5, and 2.0 mg of P_2O_5/100 ml from KH_2PO_4. The standards and samples were read on a Beckman Model DU spectrophotometer at 400 nm against a distilled water blank.

Sedimentation analysis

The chemically prepared *K*-casein was studied by means of ultracentrifugation. The sedimentation velocity experiments were made at 20° with a rotor speed of 59,780 r.p.m. A valve type 4°/12 mm synthetic boundary cell was used.

RESULTS AND DISCUSSION

The PGUE patterns of the chemically prepared *K*-casein and *K*-caseins obtained by four other different methods are presented in Fig. 1. The sample loading for the chemically prepared *K*-casein was 0.7 mg and for the other preparations 0.25 mg each. It is noteworthy that the gel zone patterns for the chemically prepared *K*-casein at about 3 times the loading showed absence of contamination, as was observed for the chromatographically[9] or ethanol–NH_4Ac purified samples (Fig. 1). Further, the chemically prepared *K*-casein gave nearly the same gel zone pattern as for the chro-

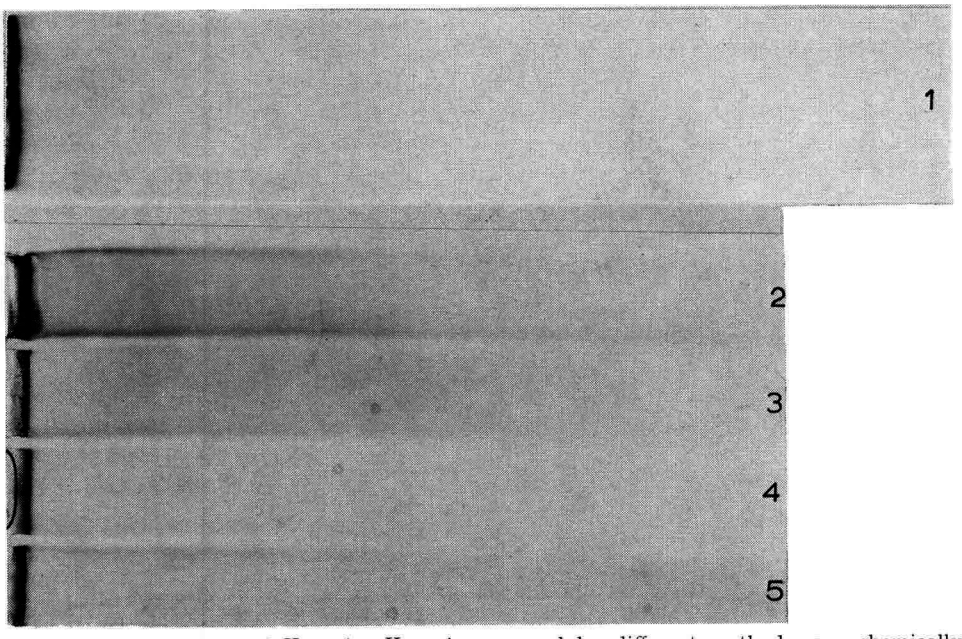

Fig. 1. PGUE patterns of *K*-casein. *K*-casein prepared by different methods. 1 = chemically prepared; 2 = chemically prepared, purified by ethanol–ammonium acetate precipitation; 3 = purified by DEAE-cellulose column chromatography; 4 = McKenzie and Wake[5]; 5 = Zittle and Custer method, with ethanol–NH_4Ac purification[4]. PGUE conditions: 7% cyanogum, 4.5 *M* urea, pH 9.2. Slot 1: 0.7 mg sample. Nos. 2–5, 0.25 mg. Run vertically for 5 h, 200 V, at 18°. Buffer was 0.083 *M* in THAM, 0.003 *M* in Na_2EDTA, and 0.012 *M* in H_3BO_3.

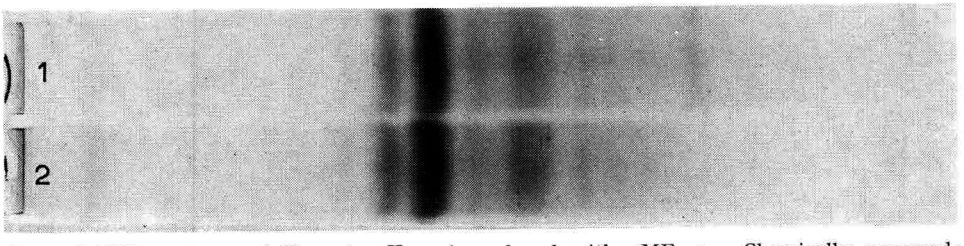

Fig. 2. PGUE patterns of *K*-casein. *K*-casein reduced with 2ME. 1 = Chemically prepared; 2 = purified by DEAE-cellulose column chromatography. PGUE conditions: 7% cyanogum, 4.5 *M* urea, pH 9.2, 0.2% 2ME, 0.7 mg sample. Run vertically for 5 h, 200 V, at 18°. Buffer was 0.083 *M* in THAM, 0.003 *M* in Na_2EDTA, and 0.012 *M* in H_3BO_3.

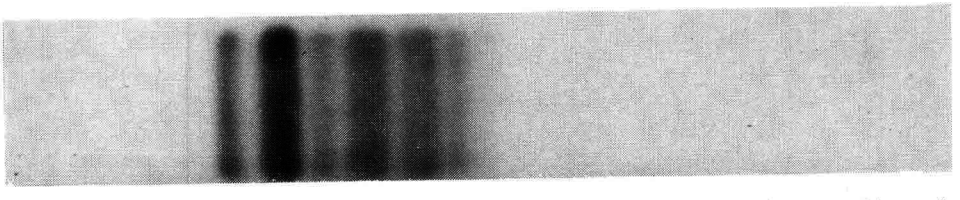

Fig. 3. SGUE pattern of *K*-casein. *K*-casein reduced with 2ME. SGUE conditions: 16% starch, 6 *M* urea, pH 8.6, 0.2% 2ME, and 0.7 mg sample. Gel buffer was 0.076 *M* Tris–0.005 *M* citrate and the bridge buffer was 0.3 *M* Na-borate. Run vertically for 18 h, 200 V, at 5°.

TABLE II

CHEMICAL ANALYSES OF K-CASEIN

Each value is an average of 4 independent analyses. Chemical preparations.

	NANA	*Phosphorus*
Per Cent[a]	1.89 ± 0.02	0.32 ± 0.01

[a] Based on sample vacuum dried over P_2O_5.

matographically purified K-casein when reduced with 2ME (Fig. 2). The SGUE pattern of the chemically prepared K-casein reduced with 2ME (Fig. 3) shows the characteristic bands for K-casein as reported in the literature. All of the preparations were considered electrophoretically pure.

The amino acid composition (Table I), and the sialic acid and phosphorus content (Table II) of the chemically prepared K-casein are in agreement with the reported values for K-casein.

The analytical ultracentrifuge pattern of chemically prepared K-casein is shown in Fig. 4. A single peak was observed. The sedimentation coefficient in 0.076 M Tris–0.005 M citrate buffer at pH 8.6 was found to be 13.5 S.

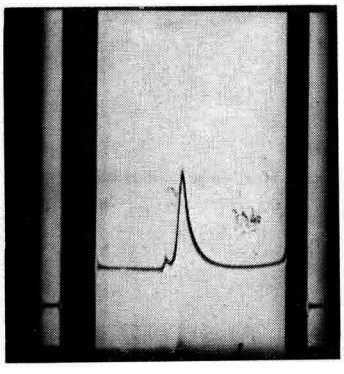

Fig. 4. Ultracentrifuge pattern of K-casein. Sedimentation of K-casein at 59,780 r.p.m., using a Valve Type synthetic boundary cell with 4°/12 mm sector, temperature 20°. 0.076 M Tris–0.005 M citrate buffer, pH 8.6, 0.7% chemically prepared K-casein, bar angle 60°, 1500 sec.

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CHROM. 4499

MOLECULAR WEIGHT DETERMINATIONS AND THE INFLUENCE OF GEL DENSITY, PROTEIN CHARGES AND PROTEIN SHAPE IN POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY

The use of polyacrylamide gels as molecular sieves and electrophoresis as a transport mechanism provides a reliable procedure for estimating the molecular weight of proteins. A series of multimers derived from urease and from bovine serum albumin have been employed as standards. The method described may be used for proteins with a wide range of molecular weights.

INTRODUCTION

It has been pointed out by SMITHIES[1], RAYMOND AND NAKAMICHI[2], TOMBS[3], SHAPIRO *et al.*[4], ZWAAN[5], and BLATTLER[6] that information about the size of protein molecules can be obtained from electrophoresis in polyacrylamide gels. The rationale resembles that for molecular weight determinations by Sephadex chromatography. A complication is the variable charge density characteristic of proteins. ZWAAN[5] and SHAPIRO *et al.*[4] have provided two different approaches to reduce or eliminate the varying influence of charge. The work presented here is an investigation of other factors important to quantitative electrophoresis.

To avoid varying charge effects, a polymerized sample of bovine serum albumin (BSA), or polymerized urease, was used as a standard.

EXPERIMENTAL

Materials and methods

The urease was prepared essentially by the method of SUMNER[7] and DOUNCE[8] as modified by GORIN *et al.*[9]. The extracting solution contained 160 ml acetone, 0.01 *M* β-mercaptoethanol, 0.001 *M* Na_2EDTA and deionized water in a volume of 500 ml. The urease was stored as crystals in the acetone–citrate solution. Under these con-

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ditions urease slowly polymerizes. A significant factor in the success of this work is the utilization of appropriate protein standards and in order to employ proteins of different size but as similar as possible, polymerized proteins were utilized. It is realized that in the proteins chosen, shapes and charges do vary somewhat. Urease and BSA were utilized since these proteins polymerize slowly with age.

CREETH AND NICHOL[10] first studied the associated forms of urease and found evidence for the formation of dimers and trimers of urease by ultracentrifuge studies. We have repeated and confirmed their work and related the ultracentrifuge patterns to the patterns obtained by electrophoresis studies. The greater sensitivity of electrophoresis yielded additional components which were characterized by electrophoresis and found to be the tetramer and pentamer of urease. Higher multimers of urease are evidently insoluble since solutions of polymerized urease often contain precipitates. It is well known that preparations of BSA often contain dimers, trimers and higher multimers of BSA.

We used a commercial sample of BSA which was aggregated to an unusual extent since at least six components were present. This degree of polymerization was probably due, in part, to the age of the sample since it was several years old. A study of the components revealed that they formed an arithmetic polymerization sequence (see Fig. 3 and ref. 11). All components could be reduced to a single band identical with BSA monomer.

The electrophoresis experiments were carried out in a vertical gel apparatus* permitting all the samples to be run in the same gel under identical conditions. The experiments were conducted in a buffer containing Tris, 6 g/l and Na_2EDTA, 0.6 g/l. The gels were formed from cyanogum** and polymerized with the conventional catalyst system of TMED (tetramethylethylenediamine) and ammonium persulfate. To polymerize 150 ml of solution, 0.10 g of $(NH_4)_2S_2O_8$ was used. Sufficient TMED was used to complete polymerization in 15–20 min. This usually required 15–20 μl of TMED solution. The polymerization was carried out in nitrogen. The gels were prepared by adding cyanogum to a fixed volume of water so that, for example, a 5% gel contained 5 g of cyanogum and 100 ml of water. The addition of 1 g of acrylamide to 100 ml of water increased the volume to approximately 101 ml and this relationship was found to be approximately linear up to 15 g of acrylamide per 100 ml. Our experience has shown that cyanogum has too much bisacrylamide for use in making gels denser than 10%. Formulations containing less of the cross linking reagent yield gels that are more supple and less fragile.

The succinylated BSA was prepared as described by CHERRY[12] and HABEEB *et al.*[13]. Succinic anhydride was used at 0° and the pH was maintained at 7.5–8.0 with a pH stat. The reduced succinylated BSA was first succinylated and then reduced in 0.5 *M* β-mercaptoethanol and 6 *M* guanidine hydrochloride. α-Urease (240,000) was prepared as described by BLATTLER *et al.*[14].

RESULTS

Fig. 1 is a plot of the maximum gel "pore" size of polyacrylamide gel *vs.* gel

* This instrument is commercially available from the Biochemical Instrument Co., 881 Oakway Rd., Eugene, Ore. 97401, U.S.A.

** E-C Apparatus Corporation, 222 South 40th Street, University City, Philadelphia, Pa. 19104, U.S.A.

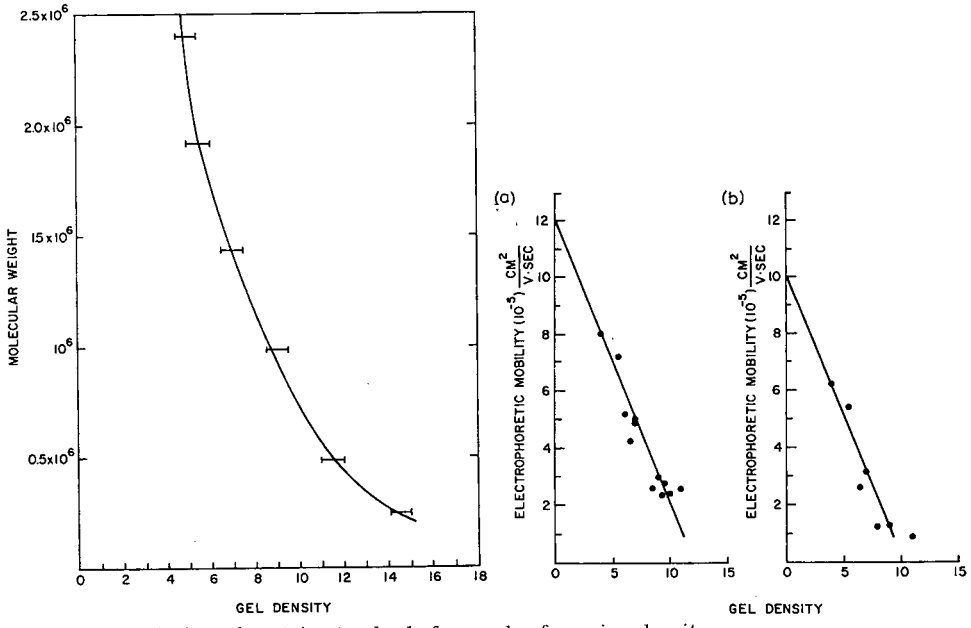

Fig. 1. The exclusion of protein standards from gels of varying density.

Fig. 2. The electrophoretic mobility of BSA at varying gel densities. (a), BSA monomer, zero intercept: $12.0 \cdot 10^{-5}$ cm^2/V·sec; (b), BSA dimer, zero intercept: $10.1 \cdot 10^{-5}$ cm^2/V·sec.

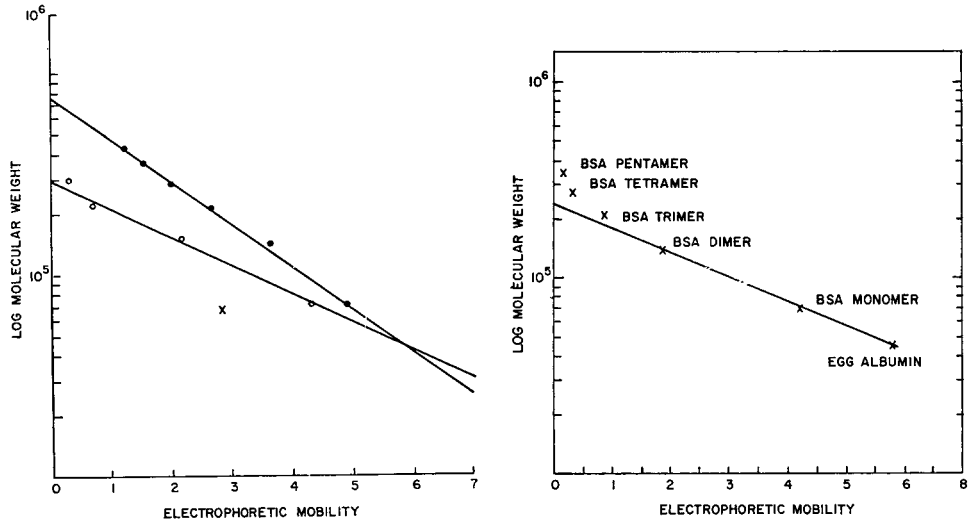

Fig. 3. Relation of molecular weight of BSA and derivatives to electrophoretic mobility in 5% gel. (●—●), native BSA; (○—○), succinylated BSA; × = succinylated reduced BSA.

Fig. 4. Relation of molecular weight to electrophoretic mobility in 9% gel.

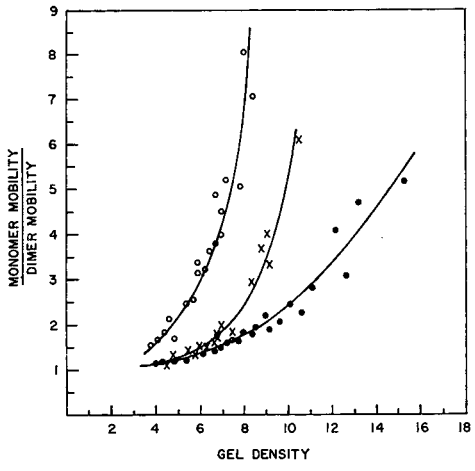

Fig. 5. Ratios of mobilities for monomer–dimer pairs at various gel densities. (○—○), urease monomer/urease dimer; (●—●), BSA monomer/BSA dimer; (×—×), α-urease/native urease.

density. The "pore" size is represented by protein molecular weight. The protein standards used were catalase, urease and the higher multimers of urease. They were subjected to electrophoresis through polyacrylamide gels ranging in density from 4–15% in increments of 0.125%. It was found that urease with a molecular weight of 480,000 was excluded by gels of 11–12%. In 11% gels the urease will enter the gel but the electrophoretic mobility is exceedingly slow. In 12% gels the urease remains at the origin.

In Fig. 2 are graphs of the electrophoretic mobility of BSA monomer and dimer as a function of gel concentration. The curves are approximately linear with indistinguishable slopes but different intercepts.

In Fig. 3 is a graph of the logarithm of the molecular weight of BSA multimers plotted against the electrophoretic mobility in a 5% gel. Mobility is expressed in arbitrary units. The sample of BSA utilized contained multimers up to hexamers.

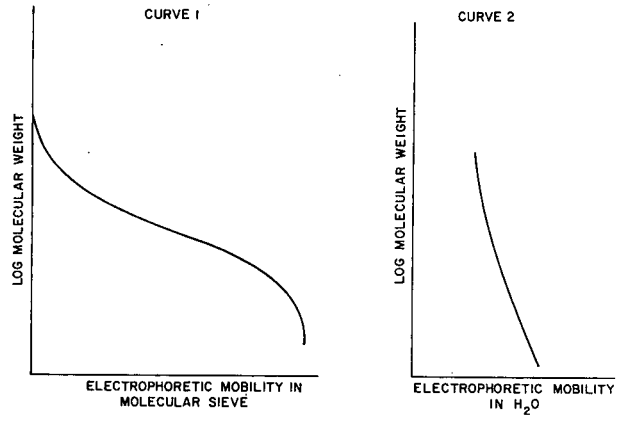

Fig. 6. A comparison of electrophoretic mobilities in gels and in water.

Included in Fig. 3 is a curve for succinylated BSA and BSA multimers in 5% gel. For both sets a linear relationship was obtained. If the same BSA sample was succinylated, and then reduced, only a single component was obtained. Clearly, succinylated BSA has a lower mobility than the native protein and the succinylated, reduced derivative moves even more slowly.

Fig. 4 is a similar plot but represents the course of electrophoresis in a 9% gel. In this denser gel there is a notable deviation from linearity for the higher multimers. In 7% gels there is a slight deviation from linearity for BSA pentamer and higher multimers. There was some indication that small proteins in dilute gels deviate from linearity in the opposite direction.

TABLE I

MIGRATION RATIOS FOR β-LACTOGLOBULIN A AND B

Gel density (%)	*Electrophoretic mobility*		*B/A*
	A	*B*	
6	8.55	7.93	0.929
9	8.25	7.55	0.916
11	8.30	7.73	0.931
12	6.30	5.75	0.922

When the ratios of the mobilities of a protein monomer–dimer pair were measured at various gel concentrations it was noted that characteristic curves were obtained as illustrated in Fig. 5.

In Table I similar data for β-lactoglobulin A and B are presented. The relative mobilities of these two forms are independent of gel densities. This indicates a difference in charge but similarity in size and shape.

DISCUSSION

The exclusion limit for polyacrylamide gels shown in Fig. 1 is useful for experimental design and is analogous to the void volume in gel chromatography. The limits indicated are approximate since the shape of the protein may affect the results. For example, the pentamer of BSA (350,000) cannot enter a 9% gel but urease (480,000) can penetrate the gel although its mobility is low. Presumably the pentamer is less compact than urease. However, the exclusion limit does yield a semi-quantitative estimate of molecular weight.

The electrophoretic mobilities of BSA monomer and dimer are a linear function of gel density. The curves deviate slightly from linearity with dense gels very near the exclusion limit. The intercept is governed by the size and shape of the protein and the gel density in which it has zero mobility. The slope of the line is determined largely by the charge on the protein.

The mobility of the dimer is approximately 80% that of the monomer in aqueous solution. By increasing the gel density from zero to 5% the mobility of the monomer becomes 70% that of the dimer and in a 7.5% gel the dimer mobility is 50% that of the monomer. Such considerations indicate that effective use of poly-

acrylamide gels as molecular sieves requires denser gels than normally used. In order to accentuate the difference in mobility between two similar proteins with a small difference in size it is necessary to use gels approaching the exclusion limit of the larger protein. The mobility of BSA dimer in a 10% gel is approximately 10% that in buffered water. This difference in mobility results in sharp bands and thus dense gels improve band sharpness as well as resolution.

By using a multimeric series of one protein the quantitative relationship between molecular weight and mobility can be explored directly without compensating for charge difference in standards. Succinylation of such a protein series produces a set of proteins with higher electrostatic charge and larger size.

When the same experiment is carried out in a denser gel the relationship may be no longer linear. The larger proteins migrate unexpectedly faster. This effect is even more noticeable for proteins whose size is close to the exclusion limit of the gel. In general there was little deviation from linearity if the protein had a molecular weight smaller than half the exclusion limit.

The general character of electrophoretic mobility is illustrated by curve 1 of Fig. 6. The curve is sigmoid but has a linear region in the middle. Since the frictional coefficient is proportional to $M^{-\frac{1}{3}}$ in aqueous solution the mobility of a monomer should be $2^{\frac{1}{3}}$ or 1.21 times that of the dimer. In Fig. 5 the monomer–dimer ratio for BSA approaches this value of 1.21 in 5% gels. This indicates once again the relative inefficiency of dilute gels as molecular sieves. In aqueous solution the electrophoretic mobility E is proportional to the charge C and frictional coefficient F, so that $E \sim CF$. However, in diffusion $F \sim 1/(M)^{\frac{1}{3}}$ and thus, $E \sim C/M^{\frac{1}{3}}$. Thus an 8-fold difference in mass produces only a 2-fold change in mobility. Curve 2 of Fig. 6 represents this situation. The curve rises steeply as does the right-hand portion of curve 1 because the mobility of small proteins in a dilute gel is little affected by the gel. Larger proteins are strongly influenced by the sieving action of the gel and their mobility is much less than that in aqueous solution. In electrophoresis experiments a 10% difference in charge produces a 10% difference in mobility. Due to the relation $1/M^{\frac{1}{3}}$ a 10% difference in mass produces a negligible effect on mobility. The molecular sieving effect of polyacrylamide gels makes the difference in mass as measurable as a difference in charge.

In this work it was found that, in a 5% gel, proteins with molecular weights between 50,000 and 500,000 tend to fall in the linear region of curve 1. In a 10% gel the upper limit for linearity is approximately 150,000 and may extend as low as 15,000 for the lower limit. In the ZWAAN technique one measures the mobility of a protein in a dense gel and divides by the mobility in a dilute gel. This is equivalent to dividing curve 1 by curve 2 in Fig. 6. Since curve 2 is approximately linear, as is the center portion of curve 1, the result is an approximately linear curve.

It has long been known that proteins could be graded in size by comparing relative mobilities in molecular sieve gels of increasing selectivity. SMITHIES[1] employed starch gels and later RAYMOND[2] used polyacrylamide gels. Nevertheless, this technique has not been widely used in spite of the fact that it is very simple and no compensation need be made for charge difference between proteins. Fig. 5 shows relative mobility ratios for three monomer–dimer protein sets. If only one experiment had been run the low mobility of the dimer might have been attributed to lack of charge instead of larger size. The same approach may be used to show that suspected genetic

variants have the same molecular weight. This is illustrated by the data in Table I for β-lactoglobulin A and B. The relative mobilities of these two components do not change with gel density and hence indicate similar size, but dissimilar charge. The data indicate that the B form has 92% as much charge as the A form. If the difference is due to a single ionizable group this indicates a charge of 13 for the A form and 12 for the B form.

CONCLUSION

Molecular weight determination by molecular sieve electrophoresis is a reliable procedure for the preliminary characterization of minor components.

Variation in charge density can be more reliably compensated for than can variation in shape but the results indicate that shape differences must be rather extreme to affect the results. The technique described is particularly useful for the study of genetic variants or homologous sets of proteins.

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THIN-LAYER CHROMATOGRAPHY OF METAL IONS ON CELLULOSE IMPREGNATED WITH THIOCYANATE SALTS OF LIQUID ANION EXCHANGERS

I. EXPERIMENTAL PARAMETERS INVOLVED IN THE SYSTEM AMBERLITE LA-2-THIOCYANATE/AQUEOUS AMMONIUM THIOCYANATE

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SUMMARY

A selection of metal ions (Ag(I), Cu(II), Cd(II), Bi(III) and Mn(II)) have been chromatographed on supports impregnated with the liquid anion exchanger, Amberlite LA-2-thiocyanate, formed from the free amine by an indirect method via the chloride form or by a direct method, *i.e.* with thiocyanic acid, using aqueous ammonium thiocyanate as the mobile phase. Cellulose is preferred to silica gel as the support. The R_F values are independent of the method used to convert the free amine to the salt form. The R_F values are shown to vary with the impregnation coefficient of the stationary phase and with the composition of the mobile phase. A high degree of reproducibility of R_F values is obtainable in the system studied.

INTRODUCTION

Using thiocyanate media as mobile phases, BRINKMAN *et al.*[1] have chromatographed six metal ions on substrates (silica gel thin layers and paper) impregnated with the thiocyanate salts of long-chain (secondary, tertiary and quaternary) amines. From their preliminary results, these authors concluded that further work on these systems was justified. In contrast, others[2,3] have examined a wider range of metal ions on paper impregnated with tertiary amines: tri-*n*-butylamine, tri-*n*-octylamine and tri-isooctylamine, only. For a given mobile phase, little difference in behaviour of the metal ions was observed using these three tertiary amines. This illustrates, as observed by BRINKMAN *et al.*[1], that the character of the amine group rather than the nature of the substituent hydrocarbon chain is the primary factor governing the chromatographic behaviour of the metal ions in the systems investigated[1–3]. None of these works, however, included a systematic investigation of the experimental

conditions necessary to produce a chromatographic system from which the highly reproducible R_F values necessary for the unambiguous characterisation of the metal ions separated could be obtained.

For these reasons, before making a systematic investigation into liquid ion-exchange chromatography of metal ions using aqueous thiocyanate media, we investigated a number of chromatographic parameters likely to affect the behaviour of the metal ions in the systems studied. Our object in this work was to establish conditions which give a high degree of reproducibility of R_F values that are essential in order to gain insight into the mechanisms of the chromatographic system, characterise metal ions according to their R_F value, and develop systems which can be used for the quantitative determination of the ions separated[4,5].

Before beginning our experimental work, it was pertinent to consider which type of long-chain amine would be most suitable for an investigation into the parameters which govern the system. Primary and quaternary amines which show the extremes of ion exchange character, as exemplified by BRINKMAN *et al.*[1], were considered to be unsuitable. We therefore selected a secondary amine–Amberlite LA-2, converted to the thiocyanate form, as the impregnant because we think that results obtained using this anion exchanger of intermediate strength would show a close affinity with the results expected from the use of both primary amines and quaternary compounds.

EXPERIMENTAL

The exact molecular weight of the amine–Amberlite LA-2 (370) was determined by a method described earlier[6]. This enabled us accurately to prepare solutions of the amine in chloroform (0.1–0.5 *M*). From these, solutions of the thiocyanate salts were prepared by one of the two following methods: (a) By an indirect method in which the amine hydrochloride was first formed by the method described by BRINKMAN AND DE VRIES[7]. Conversion to the amine hydrochloride was shown to be 100%. The amine hydrochloride was treated with an equal volume of ammonium thiocyanate solution (3 *M*) and the mixture was allowed to stand overnight, after which the aqueous phase was separated from the organic phase. This procedure was repeated. The amine thiocyanate was then washed with three equal volumes of distilled water and filtered through a pad of anhydrous magnesium sulphate to form a clear solution. (b) By a direct method in which thiocyanic acid (1.5 *M*) was prepared by passing potassium thiocyanate (2 *M*) through the cation resin 'Zeo-carb 226' in the hydrogen form[8]. The acid solution obtained was shaken with a solution of Amberlite LA-2 in chloroform. The phases were separated and the procedure repeated, so forming the anion exchanger according to eqn. 1.

$$R_1R_2NH + HSCN = (R_1R_2NH_2)^+SCN^- \qquad (1)$$

The anion exchanger was washed three times with deionised water and filtered through a pad of anhydrous magnesium sulphate. This conversion was found to be 98%. A concentrated solution of ammonium thiocyanate (9 *M*) in deionised water was prepared. The exact molarity of this solution was found by titrating a dilute solution (5 ml made up to 250 ml with deionised water) against silver nitrate (0.1 *M*) using

ferric alum indicator. Calculated volumes of the standardised solution were used to prepare the stock solutions of ammonium thiocyanate (0.1–7.0 *M*), used as the mobile phases.

Cellulose (15 g of MN-300-HR) or silica gel (30 g of Merck G) were slurried with a solution (70 ml), of the appropriate molarity, of the impregnant in chloroform, and the resulting suspension was used to prepare the chromatolayers (5 × 20 × 20 cm) at an applied thickness of 0.3 mm using a Shandon apparatus. They were dried in air for 20 min to allow the chloroform to evaporate.

Solutions of 1 μl containing 1 μg of five metal ions (Ag(I), Cu(II), Cd(II), Bi(III) and Mn(II)) were applied to the layers by the technique previously described[9]. The chromatograms were eluted with solutions of ammonium thiocyanate of the appropriate molarity in our double saturation chamber (*i.e.* our polythene bag technique[9,10]) at a constant temperature of 25 ± 0.1°, until the solvent front had travelled a fixed distance (12.5 ± 0.5 cm) from the point of application of the metal ions. The eluted layers were then dried in an air oven at 120° for 20 min and sprayed either with PAN (1-(2-pyridylazo-)2-naphthol) solution (0.1% in ethanol). Then they were exposed to ammonia vapour to visualise Mn(II) or with Dithizone (0.1% in chloroform) to visualise the remaining ions. R_F values were computed in the usual way.

RESULTS AND DISCUSSION

Choice of support

Silica gel and cellulose were investigated as possible supports for the impregnant

TABLE 1

A COMPARISON OF THE R_F VALUES OBTAINED USING SILICA GEL OR CELLULOSE AS SUPPORTS FOR THE IMPREGNANT AMBERLITE LA-2-THIOCYANATE OR USING AMBERLITE LA-2 THIOCYANATE PREPARED EITHER BY THE INDIRECT METHOD OR BY THE DIRECT METHOD

a = results obtained from silica gel impregnated with Amberlite LA-2-thiocyanate (0.1 *M*) prepared by the indirect method; b = results obtained from cellulose impregnated with Amberlite LA-2-thiocyanate (0.1 *M*) prepared by the indirect method; c = results obtained from cellulose impregnated with Amberlite LA-2-thiocyanate (0.1 *M*) prepared by the direct method.

Metal ion	*Method*	*Molarity of ammonium thiocynate*								
		0.1	*0.5*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Ag(I)	a	0.00	0.03	0.08	0.32	0.62	0.76	0.83	0.89	0.91
	b	0.00	0.02	0.08	0.30	0.58	0.74	0.85	1.00	1.00
	c	0.00	0.02	0.09	0.32	0.57	0.78	0.86	1.00	1.00
Cu(II)	a	0.00	0.02	0.07	0.17	0.34	0.54	0.74	0.82	0.88
	b	0.00	0.02	0.04	0.13	0.24	0.42	0.64	0.72	0.81
	c	0.00	0.02	0.05	0.12	0.20	0.42	0.60	0.75	0.83
Cd(II)	a	0.00	0.04	0.04	0.14	0.25	0.39	0.58	0.70	0.81
	b	0.00	0.00	0.00	0.04	0.08	0.18	0.30	0.44	0.58
	c	0.00	0.00	0.00	0.06	0.11	0.21	0.34	0.43	0.60
Bi(III)	a	0.00	0.00	0.00	0.02	0.06	0.16	0.29	0.47	0.60
	b	0.00	0.00	0.00	0.02	0.04	0.06	0.18	0.32	0.49
	c	0.00	0.00	0.00	0.02	0.05	0.08	0.18	0.28	0.48
Mn(II)	a	0.78	0.97	0.95	0.92	0.91	0.91	0.93	0.95	0.97
	b	0.81	0.70	0.58	0.48	0.46	0.41	0.42	0.44	0.46
	c	0.80	0.68	0.56	0.48	0.44	0.42	0.42	0.46	0.49

(prepared by the indirect method). The R_F values (the mean of at least 6 determinations) obtained are shown in Table Ia and b. Each R_F value was reproducible to $\pm$ 0.04 R_F units for the silica gel system and $\pm$ 0.02 for the cellulose system.

It can be seen that, whilst the R_F values are higher on the silica gel than on the cellulose support, the R_F value trends on the two supports are similar, suggesting that each substrate is completely covered with the impregnant so that we can regard the system as one in which the distribution of the solutes is between the thiocyanate mobile phase and the liquid anion exchanger impregnant. In the event of incomplete coverage of the layers with the impregnant, we would not expect the observed comparability of the results because of a competitive interaction between the solutes and the supposedly inert support[11–13].

Similar observations concerning the higher R_F values for impregnated silica gel layers compared with impregnated cellulose layers when each bore the same impregnation coefficient of the same impregnant (Primene JM-T-hydrochloride or tri-*n*-butylphosphate) have been reported[6,10]. Two possible reasons exist for this behaviour. Firstly a higher weight:volume ratio of substrate to impregnant exists in the case of silica gel compared with cellulose; this effectively reduces the impregnation coefficient of the former[10]. Secondly, it has been shown that the impregnant is more firmly held on the former[10]. This results in a less effective uptake of the impregnant from the slurrying solvent by the silica gel during the preparation of the plates. These two effects are superimposable.

We confirmed the observation of BRINKMAN *et al.*[1] that the solvent front on the silica gel layers was fairly irregular. On cellulose layers, however, we found that the solvent front was uniform except at very high molarities of the mobile phase. Two other disadvantages were noticed when silica gel was used as a substrate which was not apparent when cellulose was used. Firstly, the presence of iron in the silica gel resulted in an interaction between the iron and the mobile phase, causing the iron to be precipitated on the layers as a red thiocyanate complex. This colour could possibly interfere with the identification of many metal ions which form red complexes with PAN or other chromogenic reagents. Secondly, the mechanical stability of the layers formed from silica gel as a support for the stationary phase was observed to be very inferior to that of cellulose layers. When silica gel layers were placed in the eluent, the silica gel at the bottom of the plate often flaked off, increasing the elution time considerably. This also leads to irregular solvent fronts. Often normal movement of the eluent caused the silica gel to flake, ruining the plate. This is in accordance with the findings of other workers[6,10].

For these reasons, preference was given to the choice of cellulose as a support for our subsequent investigations.

Mode of formation of the thiocyanate form of the liquid anion exchanger

Table Ib and c shows the results obtained when the amine is converted to the liquid anion exchanger, *i.e.* the salt form, by the indirect and direct methods. The former method suffers from the disadvantage that in its final form the exchanger may not be present exclusively as the thiocyanate salt but as a mixture of the thiocyanate and the chloride forms[14]. The disadvantage inherent in the latter process is the instability of free thiocyanic acid which necessitated its preparation afresh on each occasion it is used.

From Table Ib and c it appears that the R_F values, within the limits of experimental error, are largely independent of the method used for the preparation of the ion exchanger. In subsequent work, we therefore decided to adopt the simpler, more convenient method of formation of the liquid anion exchanger from the free amine, namely the indirect method.

In conjunction with this work it is significant to note that neither WAKSMUNDSKI AND PRZESZLAKOWSKI[2] nor PRZESZLAKOWSKI[3] appear to have preformed the anion exchanger from the free amine prior to the impregnation of the paper. In spite of this, they discuss their results in terms of an anion exchange mechanism between anionic thiocyanate complexes of the metal ions and the thiocyanate salt form of the amine[3]. This discrepancy between their chromatographic practice and the theoretical discussion of their results can be reconciled only if one assumes the *in situ* formation of the amine salt during the development of their chromatograms. For this to happen, however, the presence of protons in the system are essential. Since ammonium thiocyanate is the salt of a strong acid and a weak base, it is reasonable to suppose that some degree of hydrolysis of the salt will occur in aqueous solution with the formation of thiocyanic acid, thus enabling *in situ* formation of the anion exchanger to occur.

We consider that this is likely to be so, for the pH range of the aqueous ammonium thiocyanate varies from pH 5.8 (0.1 *M*) solution to pH 5.2 (7.0 *M*) solution, *i.e.* all the solutions used by us and by the above workers have an acid pH.

From the above it is clear that *in situ* formation of the amine salt probably does occur; but when it does, it must result in the formation of a concentration gradient of the mobile phase over the stationary phase. Such behaviour is not consistent with a high degree of reproducibility of R_F values, and this must be regarded as a real weakness in the interpretation of the data obtained by WAKSMUNDSKI AND PRZESZLAKOWSKI[2] and PRZESZLAKOWSKI[3]. Such a weakness is obviated in our work by our making the liquid anion exchanger before the preparation of the chromatolayers.

The variation of the R_F values with variations in the composition of the mobile phase

In general, we see that an increase in the R_F values (Table Ia, b and c) of most of the metal ions occurs with an increase in the molarity of the ammonium thiocyanate in the mobile phase. This pattern, however, is not observed in the case of Mn(II) which shows an initial decrease in values with an increase in the molarity of the mobile phase with a slight increase in the values at high molarities of the mobile phase. The overall patterns of the R_F values obtained by us agree with those obtained by other workers[1-3].

The effect of the variation of the amine–salt concentration on the thin layers

It has been shown that the R_M values obtained from reversed-phase thin-layer chromatograms are linearly related to the cross sectional area (A_s) of the stationary phase according to eqn. 2 (refs. 6, 11, 12).

$$R_M = \log_{10} A_s + \text{constant} \qquad (2)$$

If the $\log_{10} A_s$ term is directly related to the log of the concentration of the

TABLE II

R_F AND R_M VALUES OF SOME METAL IONS ON CELLULOSE LAYERS IMPREGNATED WITH VARYING CONCENTRATIONS OF AMBERLITE LA-2-THIOCYANATE WITH AMMONIUM THIOCYANATE (4.0 M) AS THE ELUENT

		Loading of Amberlite LA-2-thiocyanate (molarity with respect to –NH)							
		0.0	*0.1*	*0.15*	*0.2*	*0.25*	*0.3*	*0.4*	*0.5*
Cd(II)	R_F	1.00	0.19	0.15	0.13	0.11	0.10	0.06	—
	R_M	—	+0.630	+0.728	+0.826	+0.908	+0.954	−1.279	—
Cu(II)	R_F	1.00	0.43	0.34	0.28	0.24	0.19	0.10	—
	R_M	—	+0.122	+0.288	+0.410	+0.501	+0.630	+0.954	—
Ag(I)	R_F	1.00	0.74	0.69	0.62	0.58	0.53	0.45	0.37
	R_M	—	−0.454	−0.341	−0.213	−0.140	−0.052	+0.087	+0.231
Time of run (min)		45	70	85	100	120	150	170	200

stationary phase in the solvent used to slurry the cellulose then the latter should also be linearly related to the R_M values[15].

The results in Table II show that an increase in the impregnation coefficient of the stationary phase results in a decrease in the R_F values. For the three metals studied, the plots of R_M *vs.* the log of the impregnation coefficient are linear, giving a series of parallel lines. This suggests that the variation in R_M values with the variation in the impregnation coefficient is a property of the impregnant rather than one of a particular ion. After a given plate loading (0.25 M) there is a break in the parallel lines and the R_M value plots show an upward trend. At loadings above this point, the metal ion spots become more diffuse, the time taken for the solvent front to travel a fixed distance increases and the solvent front becomes very ragged. All these features have been previously reported for reversed-phase thin-layer chromatography of metal ions chromatographed on thin layers of cellulose impregnated with Primene JM-T-hydrochloride using hydrochloric acid as the mobile phase[6]. It is of interest to note that similar alterations in the shapes of the R_M *vs.* impregnation coefficient curves have also been observed in reversed-phase paper chromatography when tri-*n*-butylamine was the impregnant[2,3]. In these investigations, however, the authors studied very high impregnation coefficients and found a downward R_M trend at *ca.* 25% impregnation coefficient. This corresponds approximately to an impregnation coefficient of 1.5 M.

In an earlier paper we have shown that the void spaces in filter paper result in an approx. 5-fold higher degree of impregnation compared with thin-layer systems[16]. We have also shown that the molar volume of the impregnant, as well as its molar concentration, is of importance in governing the points at which deviation from linearity occurs in chromatographic systems[15]. Considering both these facts we find that the point of deviation in our system and that observed by WAKSMUNDSKI AND PRZESZLAKOWSKI[2] and PRZESZLAKOWSKI[3] become reconciled.

The comparability of the present results with those obtained for the Primene JM-T-hydrochloride–hydrochloric acid system[6] confirms that the point of deviation from linearity occurs as a result of polymerisation of the stationary phase[4].

From Fig. 1, it appears that there is no particular advantage to be gained

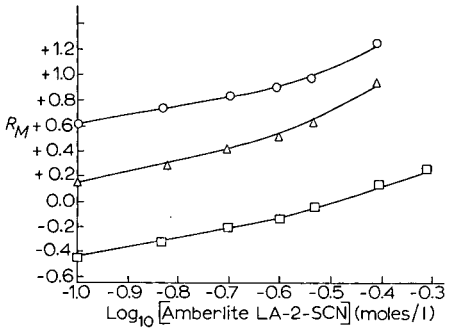

Fig. 1. R_M values of metal ions *vs.* $\log_{10}$ concentration of Amberlite LA-2-thiocyanate in the slurrying solvent. ○—○ = Cd(II); △—△ = Cu(II); ⊡—⊡ = Ag(I).

from using impregnation coefficients greater than 0.1 *M* with respect to the amine functional group, even though higher impregnation coefficients lower the R_F values. Therefore for further work on the system Amberlite LA-2-thiocyanate–aqueous ammonium thiocyanate, we used an impregnation coefficient of 0.1 *M*.

Reproducibility of the R_F values

The results quoted in the foregoing sections were the mean of at least 6 determinations suggesting that some degree of reproducibility of R_F values is obtainable in the system Amberlite-LA-2-thiocyanate–aqueous ammonium thiocyanate. However, it was considered that before we could use the R_F values obtained in the system either for the characterisation of metal ions or for a discussion of the mechanisms involved in the separation of the metal ions in the chromatographic system investigated, it was necessary to re-assess the degree of reproducibility of the R_F values of the metal ions once the experimental conditions discussed above had been standardised. Table III shows the degree of reproducibility of the R_F values obtained for Mn(II). This ion was chosen because the preliminary experiments had shown that the R_F values obtained for it lay in the region of meaningful R_F values, *i.e.* 0.1–0.9 (ref. 16).

TABLE III

THE CHROMATOGRAPHIC BEHAVIOUR OF MANGANESE(II) IONS IN CELLULOSE LAYERS IMPREGNATED WITH AMBERLITE LA-2-THIOCYANATE (0.1 *M*) PREPARED BY THE INDIRECT METHOD WITH VARYING CONCENTRATIONS OF AQUEOUS AMMONIUM THIOCYANATE AS THE MOBILE PHASE

	Concentration of NH_4SCN (molarity)						
	1	*2*	*3*	*4*	*5*	*6*	*7*
R_F (Mn(II))	0.58	0.47	0.44	0.41	0.42	0.44	0.46
S.D. ($\times 10^{-2}$)	1.1	0.96	0.89	0.67	0.73	0.81	0.95
Average length of spot (cm)	2.1	1.6	1.4	1.5	1.5	1.7	1.9
Time of run (min) ($\pm$ 5 min)	50	65	75	85	100	115	135
No. of R_F values determined	120	120	120	120	120	120	120

Assuming a Gaussian distribution of R_F values about the mean, then 68% of the R_F values should lie within the standard deviation and 95% of the R_F values should lie within twice the standard deviation. From these results it may be concluded that the reproducibility of the system is of the order of $\pm$ 0.02 R_F units because the standard deviation is of the order 0.007–0.01. This was confirmed by the fact that on all subsequent plates the standard manganese spot was never more than 0.02 R_F units above or below the value shown in Table III.

CONCLUSIONS

In the reversed-phase thin-layer chromatography of metal ions on supports impregnated with the liquid anion exchanger Amberlite LA-2-thiocyanate using aqueous ammonium thiocyanate as the mobile phase we find that: (a) cellulose is to be preferred to silica gel as a support for the impregnant, (b) the R_F values are independent of the method used to convert the free amine to the appropriate salt form, and (c) the R_F/R_M values vary with the degree of impregnation of the support with the stationary phase and with the composition of the mobile phase. It is possible to obtain a high degree of reproducibility of R_F values in the system.

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THIN-LAYER CHROMATOGRAPHY OF METAL IONS ON CELLULOSE IMPREGNATED WITH THIOCYANATE SALTS OF LIQUID ANION EXCHANGERS

II. THE SYSTEMATIC INVESTIGATION OF METAL IONS IN THE SYSTEM AMBERLITE LA-2-THIOCYANATE/AQUEOUS AMMONIUM THIOCYANATE

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SUMMARY

Sixty-eight metal ions have been chromatographed on thin layers of cellulose impregnated with Amberlite LA-2-thiocyanate using aqueous ammonium thiocyanate (0.1–7.0 *M*) as mobile phases. Most of the ions chromatographed exhibited the extremes of chromatographic behaviour in that they either remained at the point of application or they migrated with the solvent front. Chromatographic distribution was observed for VO^{2+}, Mn(II), Ni(II), Cu(II), Ag(I), Cd(II), Tl(I), Pb(II) and Bi(III). Where distribution of the metal ions occurred the results are explained in terms of an anion-exchange mechanism.

INTRODUCTION

Using thiocyanate media as mobile phases, metal ions have been chromatographed on substrates (paper or thin layer) impregnated with organophosporous compounds, both neutral (*i.e.* tri-*n*-butyl phosphate[1,2] and tri-isooctyl phosphate[3]) and cationic (*i.e.* bis(diethylhexyl)orthophosphoric acid[3]), long chain tertiary amines[4,5] and the thiocyanate salts of long chain amines[6,7].

In a previous paper[7] we considered the effects of a number of experimental parameters which were likely to affect the behaviour of metal ions when these are chromatographed on the thiocyanate form of the secondary amine—Amberlite LA-2 using ammonium thiocyanate solutions (0.1–7.0 *M*) as eluents. From these studies it was concluded that:

(a) Cellulose is to be preferred to silica gel as the support medium for the liquid ion exchanger.

(b) The R_F values obtained are independent of whether the amine is converted

to the salt form directly or indirectly, *i.e.* by forming the amine hydrochloride initially and using this to form the thiocyanate salt.

(c) The R_F values depend upon the degree of impregnation of the support with the amine salt.

By controlling the experimental conditions it was shown that a high degree of reproducibility of R_F values of the metal ions was obtained. Using the strictly standardised conditions discussed in the previous paper, we have extended our investigation to a systematic study of the behaviour of 68 metal ions on thin layers of cellulose impregnated with the thiocyanate form of the secondary amine—Amberlite LA-2 with aqueous ammonium thiocyanate of appropriate molarities as eluent system. The results of these studies are reported in this paper.

EXPERIMENTAL

Solutions of the metal ions (1 mg/ml) were prepared as previously described[8]. The standardisation of the amine, the preparation of the amine solution (0.1 *M* in chloroform), the preparation of the salt form of the amine by the indirect method and the preparation of the aqueous ammonium thiocyanate solutions, (0.1–7.0 *M*) have also been described[7,9].

We used the method discussed in an earlier paper[7] for the preparation of the impregnated layers (Cellulose MN 300 HR + Amberlite LA-2-thiocyanate (0.1 *M*), the application of metal ions to the layer and the subsequent elution of the chromatolayers in our double saturation chamber—the polythene bag technique[10,11].

The eluted plates were either viewed under ultraviolet light whilst still wet, inspected under visible light or sprayed with one of the following chromogenic reagents in order to identify the position of the metal ions on the chromatolayers:

(1) 1-(2-pyridyl-azo)-2-naphthol(PAN) (0.1% w/v in ethyl alcohol);

(2) 8-hydroxyquinoline (0.1% w/v in ethyl alcohol);

(3) diphenyldithiocarbazone (dithizone) (0.1% w/v in chloroform);

(4) (2,7-bis-(O-arsono-phenyl-azo)-;1,8-dihydroxy-naphthalene-;3,6-disulphonic acid (sodium salt) (Arsenazo III) (0.1% in water);

(5) Potassium iodide solution (0.5 *M*);

(6) Acidic stannous chloride solution.

The appearance of the metal ions on the layers after the appropriate treatment are listed in Table I.

RESULTS AND DISCUSSION

The R_F values, being the mean values of at least 6 determinations are given in Table II. A spot of Mn(II) was applied to each plate as an internal standard. The R_F value of this standard has been shown to be highly reproducible[7] (*i.e.* to $\pm$ 0.02 R_F units). The data given in Table II were obtained from plates on which the R_F values of the standard were reproducible to within its predetermined mean value.

Using these highly reproducible R_F values we have investigated the system in light of the possible mechanisms *viz.*:

$$M^{n+} + m(SCN)^- \leftrightharpoons M(SCN)^{x-} \quad \text{where } \chi^- = (m-n), \tag{1}$$

$$M(SCN)^{x-} + x[R_2NH_2]^+(SCN)^- \leftrightharpoons [R_2NH_2]_x{}^+M(SCN)^{x-} + x(SCN)^- \tag{2}$$

TABLE I

METHODS USED FOR THE IDENTIFICATION OF METAL IONS ON CHROMATOLAYERS

Treatment of plates	*Metals*	*Colour*
Coloured complexes without any treatment	TiO_2	pale yellow
	Fe(III)	blood red
	Co(II)	turquoise
	Ru(III)	black
	Pd(II)	yellow
	UO_2^{2+}	pale yellow
	Bi(III)	pale yellow
UV wet plate	VO^{2+}	dark spot
	ZrO^{2+}	dark spot
	Nb(V)	dark spot
	Hg(II)	dark spot
	Ta(V)	dark spot
	Pt(II)	dark spot
	Pt(IV)	dark spot
	Rh(III)	dark spot
Spray plates with PAN and expose to ammonia vapour	Mn(II)	red spot
	Ni(II)	red spot
	Zn(II)	red spot
	Ga(III)	red spot
	Mo(VI)	purple spot
	In(III)	red spot
Spray with Oxine and expose to UV	Alkaline earths	fluorescent spot under UV
	Al(III)	
	Cr(III)	
Dithizone	Cu(II)	pink-red spot
	As(III)	orange spot
	As(V)	orange-yellow spot
	Ag(I)	pink spot
	Cd(II)	pink spot
	Sn(II)	orange spot
	Sb(III)	orange spot
	Bi(III)	orange spot
	Au(III)	orange spot
	Hg(II)	orange spot
	Se(IV)	orange spot
	Te(IV)	orange spot
Arsenazo(III)	Sc(III), Y(III)	green spot on blue background
	Lanthanides, UO_2^{2+}, Th(IV)	green spot on blue background
Potassium iodide	Tl(I)	faint yellow spot in visible light, dark spot under UV
Stannous chloride	W(VI)	dark spot under UV, pale yellow spot in visible light

TABLE II

R_F VALUES OF METAL IONS CHROMATOGRAPHED ON CELLULOSE IMPREGNATED WITH AMBERLITE LA-2-THIOCYANATE/AQUEOUS AMMONIUM THIOCYANATE

Ion	*Molarity of ammonium thiocyanate solution*								
	0.1	*0.5*	*1.0*	*2.0*	*3.0*	*4.0*	*5.0*	*6.0*	*7.0*
Block elements									
Alkali metals									
Li	[a]								
Na	[a]								
K	[a]								
Rb	[a]								
Cs	[a]								
Alkaline earths									
Be	1.00	1.00	1.00	1.00	1.00	1.00	streaked		0.00
Mg	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ca	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sr	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ba	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
First row transition elements									
Sc(III)	1.00	1.00	1.00	streaked		0.00	0.00	0.00	0.00
TiO^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
VO^{2+}	0.00	0.02	0.05	0.06	0.07	0.10	0.12	0.15	0.17
Cr(III)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mn(II)	0.81	0.70	0.58	0.48	0.44	0.41	0.42	0.44	0.46
Fe(III)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Co(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ni(II)	0.69	0.76	0.85	1.00	1.00	1.00	1.00	1.00	1.00
Cu(II)	0.00	0.03	0.07	0.17	0.34	0.52	0.74	0.79	0.81
Zn(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Second row transition elements									
Y(III)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ZrO^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nb(V)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mo(VI)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ru(IV)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rh(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pd(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ag(I)	0.00	0.02	0.08	0.31	0.58	0.74	0.85	1.00	1.00
Cd(II)	0.00	0.00	0.00	0.04	0.08	0.18	0.30	0.44	0.58
Third row transition elements									
Hf(IV)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ta(V)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
W(VI)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Re(VI)	[a]								
Os(VII)	[a]								
Ir(III)	[a]								
Pt(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Au(III)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hg(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

TABLE II *(continued)*

Ion	*Molarity of ammonium thiocyanate solution*								
	0.1	*0.5*	*1.0*	*2.0*	*3.0*	*4.0*	*5.0*	*6.0*	*7.0*
Some p-block elements									
Al(III)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ga(III)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
In(III)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tl(I)	0.74	0.71	0.69	0.76	0.87	1.00	1.00	1.00	1.00
Sn(II)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sn(IV)	[a]								
Pb(II)	0.32	0.30	0.32	0.52	0.69	0.83	0.95	1.00	1.00
As(III)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
As(V)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Sb(III)	[a]								
Bi(III)	0.00	0.00	0.00	0.03	0.04	0.05	0.18	0.32	0.58
Se(IV)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Te(IV)	Streaked at all concentrations								
The lanthanides	Excluding Pm(III) which was not chromatographed, all had R_F values of 1.00.								
Some actinides									
Th(IV)	Streaked at all concentrations								
U(VI)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] These elements were chromatographed but could not be detected on the eluted chromatogram by the spray reagents normally used for their detection[8,10].

i.e. one involving the anionic thiocyanate complexes of the metals, and

$$M^{n+} + n(SCN)^- \leftrightharpoons M(SCN)_n \quad (3)$$

$$n[R_2NH_2]^+(SCN)^- + M(SCN)_n \leftrightharpoons [R_2NH_2]_m{}^+M(SCN)_{m+n}{}^- \quad (4)$$

i.e. one involving the uptake of a neutral thiocyanate complex of the metal ion[5].

From Table II, it can be seen that the ions can be classified into three groups according to their chromatographic behaviour, *viz.*, (a) ions which travel with the solvent front; (b) ions which remain at the point of application at all concentrations of ammonium thiocyanate investigated; (c) ions which lie between the extremes of (a) and (b).

(a) Ions which travel with the solvent front

Ions which fall into this category include the alkaline earths, some p-block elements and the lanthanides. Apart from the first member of the second row transition metals (*i.e.* Y (III)), the only transition element which falls into this group is Cr(III). The behaviour of this last ion is of interest in the interpretation of the possible mechanisms involved in the system. Thus these elements either (a) form complexes (neutral or anionic) which are not retarded by the anion exchanger of intermediate strength used in this investigations or (b) do not form such complexes under the conditions of the chromatographic experiments conducted by us.

Cr(III) is known to form a series of well-defined thiocyanato complexes of general formula

$$[Cr(III)(H_2O)^{6-n}(SCN)^n]^{3-n}$$

where n = 1–6 (refs. 12 and 13).

The anionic complexes of Cr(III) (*i.e.* these compounds in which n = 4–6) have been successfully chromatographed on anion exchangers[14,15]. The neutral complex (*i.e.* n = 3), however, was hardly retarded at all. This overall behaviour is in contrast with our failure to retard the Cr(III) ion at any ammonium thiocyanate molarities. A similar failure to retard this ion on the stronger tertiary amine exchangers has been reported[5]. BAGLIANO *et al.*[16] using either thiocyanic acid or mixtures of thiocyanic acid and aqueous potassium thiocyanate as eluents have chromatographed Cr(III) on filter paper, anionic cellulose exchange paper and anionic resin impregnated paper. For the most part they found little differences in R_F values, which with one exception were greater than 0.8 R_F units.

These results strongly suggest that the $[Cr(H_2O)_6]^{3+}$ is, under the conditions of the systems studied, kinetically inert so that replacement of the water ligand with the thiocyanate ligand does not take place and consequently the ion travels close behind the solvent front.

BAGLIANO *et al.* did not comment on the comparability of the behaviour of the $[Cr(H_2O)_2]^{3+}$ on the normal cellulose and on the modified (*i.e.* chemically or impregnated) celluloses. We suggest that some form of interaction with the common factor, *i.e.* the cellulose matrix, is responsible for the behaviour observed by them—possibly with residual carboxylic acid groups in the cellulose functioning as weak cation exchangers for the cation. That such a behaviour is not observed either by us or by others using reversed-phase systems is taken as evidence for the complete coverage of the cellulose by the liquid anion exchanger[7,9].

Overall, we here present evidence for the failure of the Cr(III) ion to form anionic thiocyanate complexes in the chromatographic systems studied. Whilst, by implication, it is possible that a similar behaviour is responsible for the failure of the remaining ions in this group to be retarded by the ion-exchange mechanism, we have no evidence here to discount the second possibility outlined above, namely the part played by the strength of liquid anion exchanger in governing retardation of the ions. (This effect will be considered in a subsequent paper.)

(b) Ions which remain at the point of application

The metals which remained at the point of application in all ammonium thiocyanate solvent systems are for the most part transition elements. It is particularly noticeable that the whole of the second row transition elements, with the exception of Ag(I and Cd(II) are completely retarded, as are all those elements of the third transition series which could be positively identified on the chromatogram.

The strong absorption of these ions could be due either to the formation of strongly absorbed complexes or the formation of insoluble thiocyanates. The former appears to be the more likely explanation because a number of crystalline salts of the second and third transition series have been prepared containing the metal and the thiocyanate ion as the complex anions[17,18].

The low instability constants of $[Au(SCN)_4]^-$, $[Hg(SCN)_4]^{2-}$, $[Co(SCN)_4]^{2-}$ and

$[Zn(SCN)_4]^{2-}$ (ref. 19), also suggest that these metals remain at the point of application because of the formation of strongly absorbed anionic complexes.

(c) Ions which show chromatographic distribution

Eqns. 2 and 4 suggest that the interaction between the metal ions and the stationary phase involves either ion exchange (eqn. 2) or an association which results in the neutral complex being converted to an anionic form on the exchanger (eqn. 4). Thus the latter mechanism appears to differ from the former in degree only, *i.e.* in the manner of uptake for, after the initial uptake, the metal species is held in its anionic form. Consequently its subsequent displacement from the layers is likely to be in the same form. This is a reasonable supposition for if the anionic form is kinetically stable on the liquid ion exchanger in the presence of a relatively small number of $(SCN)^-$, it will also be stable in a solution rich in such ions, *i.e.* we are of the opinion that it is not possible to distinguish kinetically between the labile $(SCN)^-$ on the liquid ion exchanger and those present in the mobile phase. Therefore the system can be treated simply as one in which, whilst accepting the possibility of the original uptake of the species as a neutral complex, its subsequent removal will be one of ion exchange and as such will therefore depend to a large extent on the concentration of the displacing anion, the $[SCN]^-$ in the mobile phase.

Fig. 1 shows the R_F spectra of those metal ions, all of which are known to form

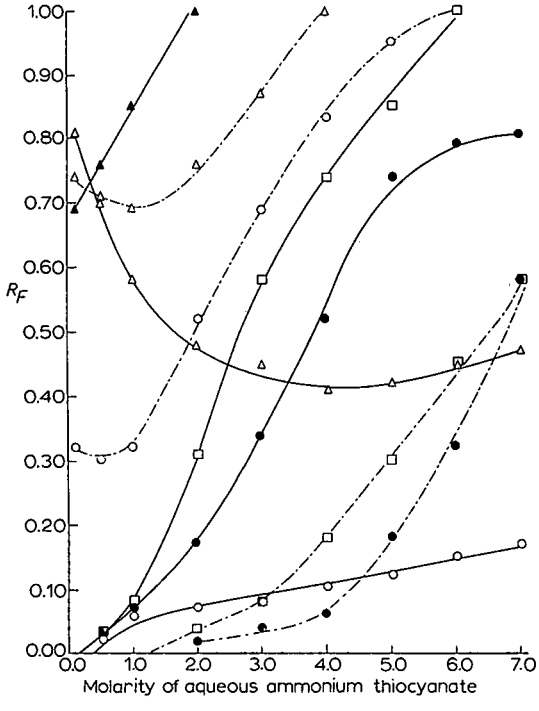

Fig. 1. R_F values of metal ions *vs.* concentration of aqueous ammonium thiocyanate mobile phase (moles (▲—▲), Ni(II); (△—△), Mn(II); (△—·—△), Tl(I); (⊙—⊙), VO^{2+}; (⊙—·—⊙), Pb(II); (⊡—⊡), Ag(I); (⊡—·—⊡), Cd(II); (●—●), Cu(II); (●—·—●, Bi(III).

anionic thiocyanate complexes[20], which migrate in the systems studied. For the greatest number of ions, it is seen that the R_F values increase with an increase in the $(SCN)^-$ concentration of the mobile phase thus giving credence to the above supposition. In the case of Pb(II), Tl(I) and Mn(II), however, the R_F values initially fall with increasing $(SCN)^-$ concentration, then they go through a minimum before showing a rise at the highest $(SCN)^-$ concentration studied. This behaviour for Mn(II) is interpreted as being indicative of the change in the nature of the Mn(II) ion in the system from the cationic Mn^{2+} through the neutral species to the anionic thiocyanate form. Evidence for the probable presence of mixed species is based on the nature of the spot which was somewhat diffuse at low $(SCN)^-$ concentrations becoming more compact at higher concentrations[7] suggesting the presence of more than one species in the system and that during the chromatographic process a slow equilibrium occurred between the species.

The behaviour of Be(II) and Sc(III) is of interest in the above context. These ions, at low $(SCN)^-$ concentration move with the solvent front but at certain concentrations (5.0–6.0 *M* for Be(II) and 2.0–3.0 *M* for Sc(III) they formed streaks from the point of application in the direction of the travel of the mobile phase. Above these concentrations they remained at the point of application. Both these ions are known to form neutral complexes[20] so that it may be that at the intermediate concentration an equilibrium exists between the simple Be(II) and Sc(III) ions and the neutral complexes but that at high concentrations they exist entirely as the neutral form which, having associated with the liquid anion exchanger, becomes retarded. A similar equilibrium between species probably accounts for the streaking of Te(IV) and Th(VI).

In the foregoing discussion, it is presumed that the displacement of the anionic metal complex from the ion exchanger is a result of a mass action effect in which the equilibrium illustrated by eqn. 2 is driven to the left by an excess of the simple $(SCN)^-$. However, alternative mechanisms can be postulated in which the displacer is a combination of the simple $(SCN)^-$ with the undissociated ammonium thiocyanate, *viz.*:

$$[R_2NH_2]_x{}^{+}M(SCN)^{x-} + x(SCN)^- + xNH_4SCN \rightleftharpoons x[R_2NH_2]^+SCN^- \cdot NH_4SCN + M(SCN)^- \quad (5)$$

or

$$[R_2NH_2]_x{}^{+}M(SCN)^{x-} + x(SCN)^- + xNH_4SCN \rightleftharpoons [R_2NH_2NH_4]_x{}^{2+}(SCN)_2{}^- + M(SCN)^- \quad (6)$$

Evidence for the probable existance of these mechanisms (eqns. 5 and 6) has been put forward by GOTO[21], who has made the following observations concerning the extraction of ammonium thiocyanate from water into a solution of a quaternary ammonium thiocyanate salt, Quatamin T-08 in toluene:

(a) ammonium thiocyanate in excess of the stoichiometric amount is absorbed from the aqueous phase by the organic phase and the amount so absorbed increases with the increase in the concentration of the thiocyanate in the aqueous phase.

(b) log–log plots of the distribution coefficients of the ammonium thiocyanate *vs.* the concentration of the extractant in the organic phase indicated the formation of a 1:1 complex between the extractant and the ammonium thiocyanate.

(c) The infrared spectrum of the organic solution of the extractant, after its equilibration with the aqueous phase containing varying concentrations of the ammonium thiocyanate, showed absorption bands which clearly indicated the presence of the ammonium radical in the organic phase. The intensities of these bands increased with an increase in the concentration of the ammonium thiocyanate in the aqueous phase.

It is difficult to decide from chromatographic evidence alone if the simple $(SCN)^-$ is responsible for the displacement of the anionic metal complexes from the liquid anion exchanger or if it is displaced by a combination of the simple $(SCN)^-$ and the undissociated ammonium thiocyanate. However, it is felt by the present authors that the work of GOTO[21], coupled with the proposal of the formation of a similar complex during the extraction of $Co(SCN)^{2-}$ into tri-*n*-octylamine from acidic thiocyanate media[22] presents strong evidence for the probability of the mechanisms exemplified by eqns. 5 and 6 occurring in our chromatographic system.

(d) A comparison with other ion-exchange systems

In general, the behaviour observed by us for those metals which showed chromatographic distribution in our system is comparable with that observed by other workers[4–6].

An advantage of our system over that proposed by WAKSMUNDSKI AND PRZESZLAKOWSKI[4] and PRZESZLAKOWSKI[5] lies in our use of much smaller impregnation coefficients to achieve comparable results; thus it appears that the R_F spectra shown by these workers were obtained on layers impregnated with 100% of the impregnant, tri-*n*-butylamine, compared with our use of a low impregnation coefficient (0.1 *M*) of our secondary amine.

The R_F values obtained by BRINKMAN *et al.*[6] are higher than those obtained by us for the same impregnation coefficient. This, as already discussed by us[4], is almost certainly because of the use of silica gel as the impregnant support by these workers, though it is accepted that some differences between our respective systems may be a direct result of their use of acidified thiocyanate media.

It is difficult to make a direct comparison of the results obtained by us with results obtained for the elution of metal ions from anion-exchange resins by other workers for frequently these workers have eluted their columns with mixed eluent systems[23–25]. However, TURNER *et al.*[26] and MAJUMDRA AND MITRA[27] both used simple thiocyanate media. Significantly both sets of workers report the precipitation of Cu(II) as a black precipitate on the resin. A similar behaviour was reported for Hg(II) (ref. 26) and for VO^{2+} (refs. 26 and 27) though TURNER *et al.*[26] reported that the latter could be eluted with thiocyanate (8.0 *M*). This behaviour is in marked contrast with our observations on the chromatographic distribution of Cu(II) and VO^{2+} in our systems. Thus the use of liquid ion exchangers would appear to have certain advantages over the use of resins for these two elements. Surprisingly both groups of workers[26,27] claim that Ni(II) was not absorbed in their system whilst MAJUMDRA AND MITRA[27] stated that Tl(I) and Th(IV) were not absorbed. This again contrasts with our observed retention of Ni(II) and Tl(I) and streaking of Th(VI).

It is not possible at this juncture to compare the results obtained by us on thin layers of liquid anion exchangers with the results obtained by other workers using, in the main, modified cellulose anion exchangers[16,28,29] because they used

acidified thiocyanate media. Such a consideration will therefore be deferred until a subsequent paper. However, we do observe that for many of the metal ions which we report as having migrated with the solvent front, BAGLIANO *et al.*[16] report some retardation. The reason for this is probably, as given for Cr(III) above, the presence of residual carboxylic acid groups on the cellulose matrix retarding the ions by a cation exchange mechanism. This is another reason for favouring the use of liquid anion exchangers as impregnants rather than the use of modified celluloses.

(e) The separation of metal ions

From the results obtained, it is obvious that the separation of metal ions in the system Amberlite LA-2 thiocyanate/aqueous ammonium thiocyanate will have little application in the characterisation of metal ions on the basis of their R_F values. Notwithstanding this, however, a practical potential exists for the technique in the purification of any of the ions which show chromatographic distribution from those which illustrate the extremes of chromatographic behaviour, *i.e.* those which have R_F values of either 1.00 or 0.00. Such a technique has been used by HAMAGUCHI *et al.*[23–25].

CONCLUSIONS

The chromatographic behaviour of metal ions on thin layers of cellulose impregnated with the liquid anion exchanger Amberlite LA-2-thiocyanate using aqueous ammonium thiocyanate (0.1–7.0 *M*) as stationary phases can be explained in terms of an anion exchange mechanism. The system, however, appears to have little value as a means of characterising the metal ions on the basis of their R_F values.

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Notes

CHROM. 4487

Analysis of the products of hydroammonolysis of acetone

The hydroammonolysis of acetone is the basis of the industrial production of 2-propyl amine which is an important intermediate in the pesticide industry[1]. In the raw product obtained under the conditions of industrial production, several compounds are present besides the expected main components. When the reaction mixture is separated into two fractions, the first one will contain the low-boiling main constituents while the second one contains mainly the high-boiling by-products. The amount of the latter is 5–8 wt% for the given procedure. It was important for the purpose of controlling the process to determine the amount of the main product and the nature of the by-products. Gas chromatography seemed to be most suited to perform this task. Owing to the remarkable differences in the physical and chemical properties of the compounds to be separated, a combined detector and programmed-temperature technique was necessary to achieve satisfactory separation.

Experimental

Gas chromatographic measurements were carried out on a Carlo Erba Model C.ATC/t–type gas chromatograph. In the case of detection with a thermistor, the following experimental conditions were used: column, spiral of copper, length 1966 mm, I.D. 6 mm; packing, 20 wt% Carbowax 1550 on Celite 545, average particle diameter 0.315–0.400 mm; length of packing, 1952 mm (plug of nickel gauze of 7–7 mm length); carrier gas, hydrogen, inlet pressure 1.18–1.15 atm, outlet pressure 1.00 atm, flow rate at the outlet 80–100 ml/sec. Temperature of the column was 56–62°, and temperature of the evaporator was 60–90°. Measuring limit of the chart recorder was 2.5 mV; heating current of the detector, 18–20 mA; paper speed, 1.25 cm/sec; and sensitivity of the detector, 1/128–1/4.

In the case of flame ionisation detection, a Carlo Erba Model D-type gas chromatograph was used. The following experimental conditions were found to be best: column, U-shaped stainless-steel tube, length 800 mm, I.D. 6 mm; packing, 32 wt% Carbowax 1550 on Celite 545 with average particle diameter of 0.315–0.400 mm; carrier gas, nitrogen; inlet pressure, 1.15 atm; auxiliary gases, hydrogen with an inlet pressure of 1.35 atm and oxygen with an inlet pressure of 2.2 atm. Temperature of the column was 60–150°, and temperature of the evaporator, 80–170°. Measuring limit of the chart recorder was 2.5 mV; paper speed, 1.25 cm/sec; sensitivity of the detector, 1/12800–1/400. In most cases the sensitivity was adjusted to 1/6400. Samples were introduced by a 10-μl Hamilton syringe with 0.1-μl graduation.

The conditions of programmed-temperature gas chromatographic measurements differed from the above by the following factors: temperature of the evaporator, 190°; temperature of the column for 8 min after sample introduction, 84°, before being raised at a rate of 16°/min for 5 min. After that followed an isothermal portion at 164° until completion of the separation.

Part of the compounds were available in the pure form for the purpose of

TABLE I

RETENTION DATA OF COMPOUNDS FOUND IN TWO FRACTIONS

A = raw product of the chromatographic separation at 56°, measurement by thermistor; B = raw product of the chromatographic separation at 141°, measurement by flame ionisation detector; C = residue of the chromatographic separation at 56°, measurement by thermistor; D = residue at 131°, measurement by flame ionisation detector; E = residue at programmed temperature, measurement by flame ionisation detector.

No.	*Compound*[a]	*A (56°)*	*B (141°)*	*C (56°)*	*D (131°)*	*E*
1	*n*-Hexane	0.12	0.10	—	—	—
2	Cyclohexane	0.12	0.10	—	—	—
3	Ammonia	0.12	—	—	—	—
4	Isopropylamine	0.15	0.37	—	—	0.36
5	Di-isopropylamine	0.23	0.39	0.23	0.38	0.38
6	Acetone	0.34	0.54	—	0.54	0.54
7	Isopropanol	1.00	1.00	1.00	1.00	1.00
8	Water	3.01	—	3.01	—	—
9	Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one)	1.65	1.19	1.67	1.20	1.21
10		—	1.37	—	1.35	1.38
11	Methyl isobutyl ketone	—	1.50	—	1.55	1.60
12	*n*-Propanol	—	1.50	—	1.55	1.97
13	Triacetonine	—	0.78	—	0.79	0.80
14	2-Methyl-4-pentanol	—	0.84	—	0.85	0.85
15	Mesityl oxide	—	0.84	—	0.85	0.87
16		—	0.89	—	0.89	0.89
17	Di-isobutyl ketone	—	0.89	—	0.89	0.92
18	2,6-Dimethyl-4-heptanol	—	0.89	—	0.89	0.93
19		—	0.96	—	0.96	0.97
20	Triacetone diamine	—	1.00	—	1.00	1.00
21	Diacetone amine	—	1.03	—	1.04	1.04
22	Acetonine isomers	—	1.06	—	1.06	1.07
23		—	—	—	1.09	1.09
24		—	—	—	1.09	1.12
25	2,4-Diamino-2-methyl pentane	—	—	—	1.18	1.19
26	Diacetone alcohol	—	—	—	—	1.31
27	Diacetone-alkamine	—	—	—	—	1.31
28		—	—	—	—	1.48
29	Triacetone amine	—	—	—	—	1.83

[a] Compounds 10, 16, 19, 23, 24 and 28 have not been identified.

identifying the separated compounds. A number of compounds were prepared in the course of the present work in the pure form according to methods taken from the literature[2–4]. Very often the mixtures obtained in the reactions were used.

Results and discussion

The results of the evaluation of the chromatograms obtained at various temperatures and on programmed-temperature column by a standard addition method are summarized in Table I.

In Table I are given the compounds found in the two fractions by means of different detectors. Retention data are related to substance 7 for substances 1–12 and to substance 20 for compounds 13–29. In Table II the amounts of the main constituents of the sample are given in %.

TABLE II

MAIN CONSTITUTENTS OF THE SAMPLE

Constitutent	*Amount (in %)*
Ammonia	5.3
2-Propylamine	44.3
Di-isopropylamine	11.2
Acetone	1.8
2-Propanol	4.1
Water	28.8
By-products	4.5

The formation of the great number of compounds is due to simultaneous and consecutive reactions. Compounds 3–8 are starting materials or main products. Tri-isopropylamine was also expected as a product[5], but it could not be identified in the mixture. This is in accordance with the experience of KUFFNER *et al.*[6–9] who have proved that this compound can be produced under special conditions only and is not formed during hydroammonolysis.

Most of the by-products are formed during the reaction of acetone with ammonia, condensation of acetone, and reduction reactions brought about by hydrogen. Previous work indicates the formation of normal and cyclohexane[10]. According to the same source also the formation of *n*-hexylamine can be expected, although this could not be found in the mixture by a standard addition method. Propyl alcohol may be produced from isopropyl alcohol by isomerisation.

The method described seemed to be best for the gas chromatographic analysis of the given complicated system.

On the basis of the method elaborated for the quantitative analysis of the product, the optimum conditions of the hydroammonolysis reaction of acetone could be determined.

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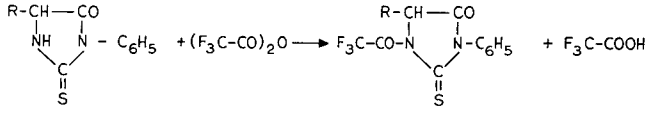
CHROM. 4500

Gas chromatographic analysis of amino acids as trifluoroacetylated phenylthiohydantoins*

The analysis of the 3-phenyl-2-thiohydantoin (PTH) derivatives of amino acids, used for the first time by EDMAN in the study of proteins[1,2], is commonly carried out by paper and thin-layer chromatography[3,4]. Only few significant papers on the gas chromatographic determination of the PTH derivatives of amino acids can be found in scientific literature[4]. It is as yet impossible to carry out a complete gas chromatographic examination of these compounds that would be very useful in the sequence analysis of peptides. The greatest difficulties arise from their insufficient volatility that makes it necessary to use high temperatures, glass columns, very small amounts of stationary phase, very long analysis times, etc.

We thought it possible to overcome in part these drawbacks by decreasing the polarity of the PTH's through their N-trifluoroacetylation:



Preparatoin of the N-trifluoroacetylated PTH's of amino acids

The PTH's of glycine, α-alanine, valine, leucine, isoleucine and proline were prepared by the EDMAN method[1]. The purity of the products obtained was checked spectrophotometrically according to the procedure described by SJÖQUIST[6]. To about

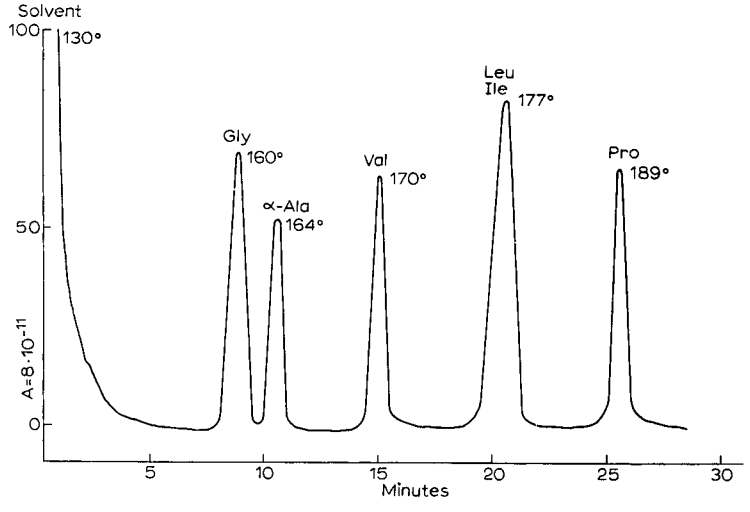


Fig. 1. Chromatogram obtained with the mixture of the six amino acids analysed.

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3 mg of each PTH 2 ml of methylene chloride and 0.3 ml of TFAA were added. The solution was kept at room temperature for 30 min and then analysed by gas chromatography.

Gas chromatographic procedure

A Varian-Aerograph 1520 B gas chromatograph with a flame ionisation detector was used. The analytical conditions were as follows: stainless-steel columns, 1.5 m by 3 mm I.D.; carrier gas, nitrogen, 20 ml/min; support, acid-washed, 68–80 mesh Chromosorb W; stationary phase, 5% S.E. 30; injector temperature, 220°; detector temperature, 250°; programmed oven temperature, initial value at 130°, from the initial value to 150° at 4°/min, from 150° to the end at 2°/min; amount of sample injected, 1 μl. An example of chromatogram is shown in Fig. 1.

The results of this preliminary research, carried out on the simplest amino acids, show that trifluoroacetylation allows the gas chromatographic analysis of their PTH derivatives (even with stainless-steel columns) at relatively low temperatures, even if a 5% concentration of the stationary phase is used. All the amino acids are completely separated except leucine and isoleucine which give one peak.

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CHROM. 4501

A rapid chromatographic method for the estimation of lysine

Lysine is frequently the most limiting essential amino acid in human and non-ruminant diets based on plant foods, though these foods are usually adequate in content of the remaining basic amino acids[1]. For this reason, plant breeders and animal nutritionists have shown considerable interest in this amino acid. Many of these scientists work in small laboratories without access to the more sophisticated equipment necessary for automated amino acid analyses, and in these situations there is a requirement for rapid methods employing simple apparatus.

Several methods for estimating lysine are presently in use. These are based on ion-exchange chromatography[2–9], gas–liquid chromatography[10], microbiological assay[11], enzymatic decarboxylation[12,13], paper chromatography[14], thin-layer chromatography[15], reaction with fluorodinitrobenzene[16,17] and reaction with picric acid[18]. Undoubtedly, the ion-exchange techniques, properly applied, are the most dependable and subject to least error, but with these methods the large number of ninhydrin-positive compounds normally present in hydrolysates of feeds or physiological solutions sets a lower limit to the time necessary for a clean separation of lysine from the remaining compounds on the column, particularly ornithine. Only when weak cation exchange resins have been employed has this latter separation been satisfactory in runs of less than 1 h duration[6], but these resins are not as dependable as strong cation-exchange resins for routine analysis[19].

This paper describes a simple manual chromatographic method which permits at least one lysine estimation per hour. By completely or partly automating the procedure this performance is improved. The method has been employed to estimate lysine in rumen liquor, silage liquor, deproteinized plasma, and hydrolysates of feed, faeces and bacteria.

Materials and methods

Column conditions. Resin type, Amberlite IR-120; resin particle diameter, $40 \pm 7\ \mu$; column resin height, 15 cm; column resin diameter, 0.6 cm; column temperature, 35°; rate of elution, up to 60 ml/h.

Buffer solutions. (i) pH 2.0 buffer (1.0 *N* sodium concn., 0.1 *M* citrate concn.). Per litre: sodium hydroxide (97%), 41 g; concentrated hydrochloric acid, 84 ml; citric acid, 21 g; phenol, 1 g. (ii) pH 3.1 buffer (1.0 *N* sodium concn., 0.1 *M* citrate concn.). Per litre: sodium hydroxide (97%), 41 g; concentrated hydrochloric acid, 53 ml; citric acid, 105 g; phenol, 1 g; BRIJ 35, 5 g. Adjust with conc. HCl as necessary.

Acid ninhydrin reagent. Dissolve 2.5 g ninhydrin (indantrione hydrate) in 160 ml of glacial acetic acid. Add 40 ml of 6 *M* phosphoric acid[20–23].

Procedure. Before use, the column is washed with 1 *N* NaOH and then equilibrated with pH 2.0 buffer. Of the sample 0.5–10.0 ml in pH 2.0 buffer are applied to the column, and 1-ml portions of pH 2.0 buffer are used to wash the walls of the column before pH 3.1 buffer is introduced and the elution commenced. The elution buffer is forced through the column at a rate of 40–60 ml/h, using nitrogen pressure or a simple pump. The eluate is collected in 2.4-ml fractions. To each fraction is added 4 ml of the acid ninhydrin reagent, and the mixture is heated in boiling water for

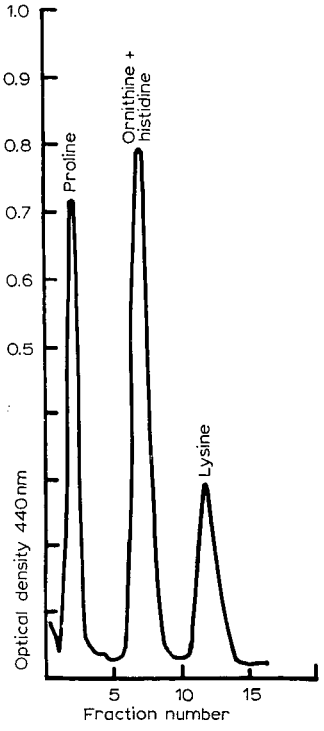

Fig. 1. Chromatogram of deproteinized calf plasma.

exactly 15 min. Each sample is then rapidly cooled to room temperature, and the optical density (O.D.) of the yellow lysine–ninhydrin complex is measured at 440 nm in 1-cm cuvettes on a Unicam SP 600 spectrophotometer.

Results and discussion

Fig. 1 shows a chromatogram of deproteinized calf plasma. Of the more commonly occurring acid ninhydrin positive amino acids, most difficulty was experienced in separating lysine from both ornithine and histidine. At citrate concentrations less than 0.5 *M*, lowering the pH improved the ornithine–lysine separation, but a less satisfactory separation of lysine and histidine was achieved. Using the procedure described above, proline, diaminopimelic acid and hydroxylysine were eluted before histidine and ornithine which eluted together, cleanly separated from lysine. Under these conditions, the "pH 5.5 ninhydrin" reagent of MOORE AND STEIN[24] was found to be unsatisfactory, probably due to the strong buffering capacity of the elution buffer.

As shown in Fig. 2, several amino acids formed a coloured complex when reacted with acid ninhydrin under the conditions described earlier. The E^{M}_{440} for arginine was found to be about 1.2 moles^{-1} cm^{-1}, but as suggested by CHINARD[20], this colour was probably due to contaminants. During the first 15 min of heating, the yield of the lysine–ninhydrin complex increased rapidly, but only a slow increase in colour yield was obtained thereafter. Accordingly, a 15-min heating period was adopted as standard procedure. The coloured complex thus formed was stable at room temper-

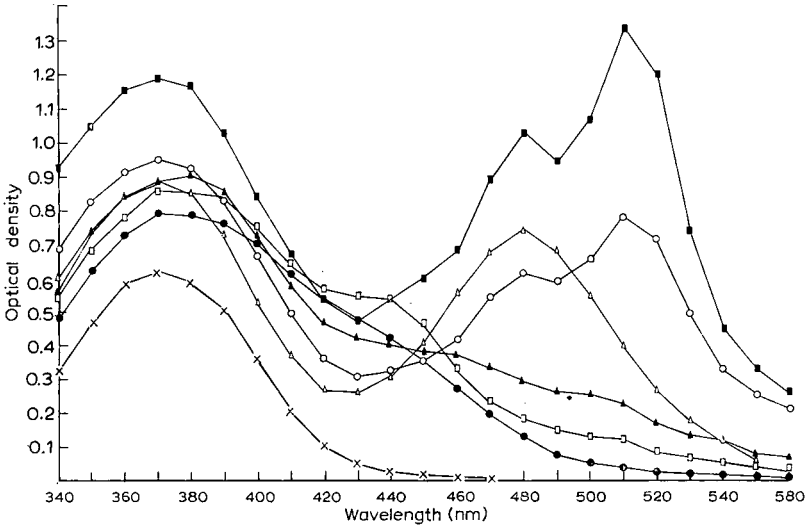

Fig. 2. Absorption spectra of products obtained by heating several amino acids with acid-ninhydrin reagent for 15 min at 100°. Symbols (in brackets μmoles/6.4 ml): ○—○, proline (0.5); ●—●, diaminopimelic acid (DAPA) (1.0); □—□, hydroxylysine (1.0); ■—■, ornithine (0.5); △—△, histidine (20.0); ▲—▲, lysine (1.0); ×—×, pH 3.10 buffer (blank) (—).

ature for at least 60 min. Under these conditions a straight-line relationship was established between O.D. and lysine concentration, in the range 0–3.5 μmoles lysine/6.4 ml, the molar extinction coefficient, E^{M}_{440}, for the lysine–ninhydrin complex being 2.64×10^3 moles^{-1} cm^{-1}. The recovery of added standard lysine from the column was 100%.

When the lysine contents of various acid hydrolysates and deproteinized physiological solutions were estimated by both the present method and the standard automated Technicon amino acid analyser, no significant difference was found between the values obtained.

One of the main advantages of this method is that although it was developed as a manual technique employing very simple and general laboratory equipment, it is faster than most of the more sophisticated automated procedures and no less accurate. Indeed, by increasing the number of columns to three and using a staggered loading procedure it is possible to perform one analysis every 30 min. Furthermore, the fact that ammonia and many other "pH 5.5 ninhydrin" positive compounds give no coloured complex under these low pH conditions means that the method is less prone to interference from unidentified compounds in complex media.

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CHROM. 4506

Behaviour of histones in exclusion chromatography and gel electrophoresis in relation to their molecular weights

The system of gel exclusion chromatography introduced by PORATH AND FLODIN[1] with dextran gels and later extended to polyacrylamide gels has been used with success in separating fractions of the histones[2–8]. As far as the authors are aware, however, none of these investigations was carried out to establish the molecular weights in the manner described by WHITAKER[9] and ANDREWS[10] for many well-characterised proteins. This study was therefore undertaken because knowledge of this parameter is essential information for understanding the structure of deoxyribonucleohistone.

Experimental

As standards, the following proteins were used (with their molecular weights given in parentheses): bovine serum albumin (67 000 and containing a little dimer); hen ovalbumin (45 000); bovine chymotrypsinogen (25 670) and trypsin (24 300); horse heart myoglobin (17 800); egg lysozyme (14 400); bovine pancreatic ribonuclease (13 700); horse heart cytochrome *c* (12 450); bovine insulin (5900) and salmine (about 4000). The following histone fractions were prepared from calf thymus (for nomenclatures see ref. 11): F1, (I); F2B, (IIb2); F2A2, (IIb1); F3, (III); and

F2A1, (IV), and the extra histone F5, (F2C) from chicken erythrocyte nuclei[12,13] kindly given by Dr. E. W. JOHNS.

The chromatography was carried out on columns (3 × 60 cm) of G-75 and G-100 dextran (Sephadex) and P-60 and P-100 polyacrylamide (Biogel) at room temperature and a flow rate of about 24 ml/h maintained by a peristaltic pump. Fractions of 3–4 ml were collected in weighed tubes and the fraction size checked by weighing every 5th or 10th tube. In any given run the weights agreed to within 0.3%. The sample, generally 1–3 mg, was dissolved in 2 or 3 ml of the solvent for application. The columns were run with 0.01 or 0.02 *N* HCl, and in some instances with 0.02 *N* HCl–0.1 *M* KCl. Acrylamide gel electrophoresis was carried out by the modification described for histones by JOHNS[14].

Results

The results of chromatography on Biogel P-100 are shown in Fig. 1 in the usual form of a semi-log plot. In 0.02 *N* HCl the results were very similar and those on Sephadex and on P-60 Biogel were also in general similar but showed poorer resolution

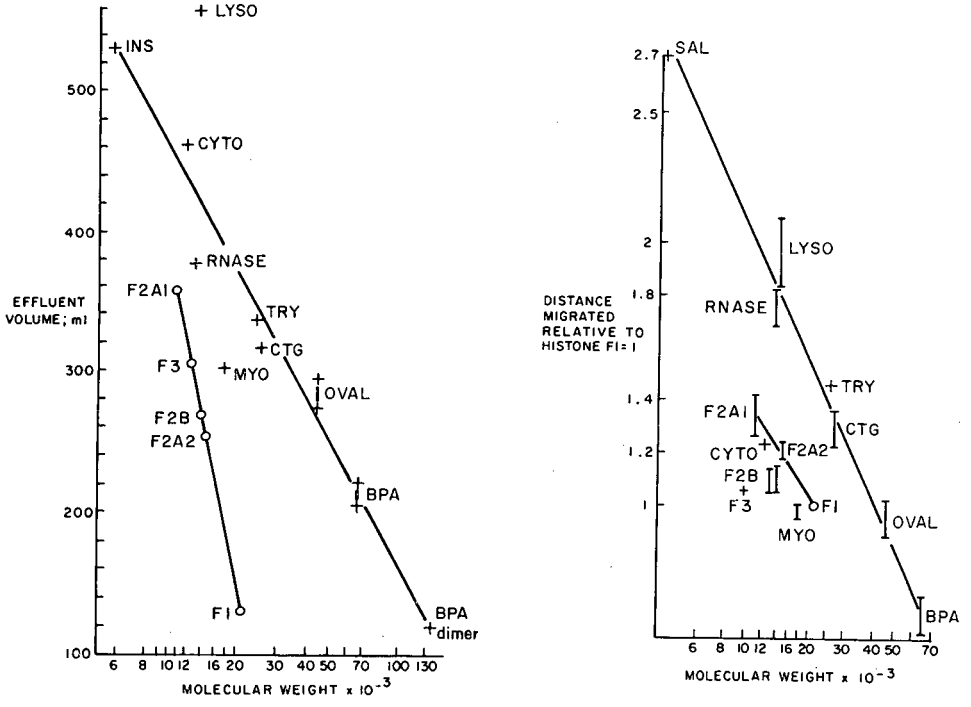

Fig. 1. Exclusion chromatography of standard proteins and histones on Biogel P-100 in 0.01 *N* HCl. Abbreviations: INS, insulin; LYSO, lysozyme; CYTO, cytochrome *c*; RNASE, ribonuclease; TRY, trypsin; CTG, chymotrypsinogen; OVAL, ovalbumin; BPA, bovine plasma albumin; MYO, myoglobin; F2A1, F3, F2B, F2A2 and F1, histone fractions. For the histone molecular weights (11 000–21 000) adopted here, see the text.

Fig. 2. Behaviour of histones and other proteins in acrylamide gel electrophoresis at pH 2.4. Abbreviations as in Fig. 1, and in addition: SAL, salmine. For the histone molecular weights (11 000–21 000) adopted here, see the text.

or a greater deviation of the standard proteins from the straight line plot. The effluent volumes of the proteins were all higher when run in 0.02 *N* HCl–0.1 *M* KCl solvent on Sephadex G-100.

A useful standard curve on P-100 was given by the following proteins: serum albumin dimer (a higher proportion of this peak could be produced by adding 10^{-3} *M* mercuric chloride to the albumin) and monomer, ovalbumin, chymotrypsinogen, trypsin, cytochrome *c* and insulin. On Sephadex G-100, serum albumin dimer was excluded, whereas cytochrome *c* and insulin were well off the line. On both columns ribonuclease and myoglobin were also well off the line under these acid conditions.

When related to these curves, the molecular weights of the histone fractions lay in the range 67 000–36 000 on Sephadex G-100 and from 125 000–22 000 on Biogel P-100, and the order of decreasing apparent weight was: F1, F5, F2A2, F2B, F3 and F2A1, though F2A2, F2B and F3 are not definitely distinguishable.

In acrylamide gel electrophoresis, the histones also migrate in a similar order, with F1 the slowest and F2A1 the fastest. They all migrate slowly, however, in comparison with several of the larger standard proteins such as ovalbumin, chymotrypsinogen or trypsin. Fig. 2 shows a semi-log plot of the molecular weight and the electrophoretic migrations, collected from many separate runs, in which all the distances are referred to histone F1 as standard.

Discussion

The histones behave anomalously in gel exclusion chromatography. None of the apparent molecular weights, ranging from 22 000 to 125 000, agree with the values from N- and C-terminal amino acid measurements[11,15], approximate though these are, or with the values from ultracentrifugal studies[16–18] or with those for fractions F2B and F2A1 now established by the amino acid sequences[19,20]. These last three methods of measurement put the whole group of histones within the range of 10 000 to 25 000 molecular weight, so that one limited result of the exclusion chromatographic studies is an indication of the relative sizes of the different fractions.

Taking the molecular weight of 21 000 as correct for histone F1 (I)[16], and 11 000 for F2A1 (IV)[19], the results here give values for F2A2, F2B and F3 in the range 15 000 to 13 000, and for fraction F5 (F2C), which was determined only on a G-100 column, a value of 18 000.

The interpretation of the observed behaviour of the histones on these columns can be considered under four headings.

Aggregation. Low ionic strength, acid conditions were chosen here to avoid the aggregation which is favoured by high salt and neutral or alkaline pH. A partial aggregation would give rise to chromatographic peaks of the monomeric forms eluting later from the columns, but this did not occur. Moreover, aggregate bands were not seen in acrylamide gel electrophoretic patterns of these histone fractions, although slow-running bands can be readily produced by prior treatment under known aggregating conditions.

Ionic interaction with the column material. Acidic group ionisation in Sephadex and also in Biogel owing to slight hydrolysis of amide groups would be strongly depressed by operation of the columns at pH near 2, as was done here. Moreover, the presence of acidic groups would lead to retardation of the basic histones, which is the opposite of the observed effect.

Hydrophobic interactions. Interactions of this sort may depend very much on amino acid sequence and conformation. However, the histones do not have a much lower content of hydrophobic amino acids, as defined by HATCH[21], than that of the standard proteins used.

Conformation. Owing to their high net positive charges and the rupture of the α-helical sections, the histones are largely unfolded under the acid conditions used. Prolyl residues produce permanent bends in polypeptide chains which would be important determinants of their behaviour in gel pores. It is noteworthy that the higher the proline content of the histone fraction, the more readily it is excluded from the gel. Thus the order of elution and the proline content (as moles %) are: F1 (9.2); F5 (6.7); F2A2–F2B–F3 (4.5–4.8–5.0) and F2A1 (1.0). No other amino acid (except to a less marked extent, alanine) shows any direct correlation with elution behaviour. Interference with the ability to penetrate the gel is also suggested in their electrophoretic mobilities. In spite of high net positive charges per unit weight, especially in fraction F1, they are retarded when compared with some larger proteins of lower net charge (Fig. 2).

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CHROM. 4502

Microdetection of kusum oil

Recent interest in kusum oil, a fat available from the seeds of the species *Schleichera trijuga*, that is a member of the family *Sapindaceae*, centres upon some prominent features of the oil. The fat is an oleic-rich oil distinguished by the presence of a wide range of fatty acids from acetic to behenic[1–4] and by a remarkably low content of linoleic acid[1]. The oil is further characterised by a high Reichert–Meissl value (R.M.V.) and a high Kirschner value (K.V.)[5]. These figures taken in conjunction with the low Polenske value (P.V.) and the buttery consistency of the oil might render the oil difficult to detect if used as a butter adulterant. Thus, an occasion may arise when the oil in possible admixture with such fats or, maybe, for some other compelling reasons, needs to be identified. Detection or determination of the arachidic acid which is present in a fairly high proportion[1,2] in the oil may, perhaps, indicate the presence of kusum oil, but the mere presence of arachidic acid, in such cases, cannot be considered conclusive for the presence of kusum oil. Proper solution of the problem requires a method that is conclusive and, at the same time, sensitive enough to detect microquantities of the oil with minimum interference in a reasonable time, and it is expected that the method be preferably simple. Now, a notable feature of the oil is the characteristic presence of a cyanogenic compound in a concentration reported to be between 0.03% and 0.5% expressed as HCN[1,6]. The cyano compound has recently been studied by KUNDU *et al.*[3,4] and is shown to be cyanoglyceride (the first and so far the only lipid known to have been characterised in a naturally occurring oil). This communication utilises this unique feature of the oil and devises a procedure that involves the use of chromatography and copper acetate–benzidine acetate as a reagent for the detection of cyanide ion in the vapour phase and that is applicable for detecting microquantities of the oil.

Experimental

Apparatus and reagents

The apparatus used were 10-ml test tubes, 100-ml erlenmeyer flasks, micropipettes, ordinary pipettes, glass plates (20 × 20 cm, 10 × 20 cm), chromatographic chambers, etc. The glassware was thoroughly cleansed and dried prior to use.

The reagents used were 1 *N* aqueous sodium hydroxide, 9 *N* sulphuric acid, chloroform (Merck), Silica Gel G (Merck), petroleum ether (60–66°, C.P.), diethyl ether (C.P., dry and distilled, peroxide-free), acetic acid (A.R.), iodine crystals (Merck). The sample was *ca.* 5% solution in chloroform, and reference substances were *ca.* 1% solution in chloroform. Copper acetate–benzidine acetate reagent was prepared[7] by mixing equal volumes of a 3% solution of copper acetate in water and a 1% solution of benzidine in *ca.* 10% acetic acid solution, prior to use.

Preliminary detection

About 0.1 g of the sample (although lower amounts may be allowed) is taken in a clean 10-ml test tube. The contents are warmed with the sodium hydroxide solution, acidified with sulphuric acid in the cold, and the vapour evolved is allowed to come in contact with a piece of filter paper soaked with the copper acetate–benzidine acetate reagent. The presence of the cyano compound (and hence, generally, of kusum oil) is indicated by the exposed zone of the filter paper being coloured blue.

Confirmation of the presence of kusum oil

A 0.2-ml solution of the crude oil in chloroform (approx. 10 mg) is applied in the form of a band on a silica gel layer (0.8 mm thick) spread uniformly over a glass plate and fractionated into classes, using the solvent system, *n*-hexane–diethyl ether–acetic acid (75:25:1) in a perfectly flat-bottom chromatographic chamber closed at the top with a lid and equilibrated inside with the solvent vapour. Suitable reference substances consisting of a triglyceride, a diglyceride, a fatty acid and a sterol are chromatographed side by side under identical conditions for comparison. The solvent front is allowed to ascend to about 15 cm. The plate is then taken out of the chamber, the solvent removed by evaporation and the bands are located by exposure to iodine vapour. The triglyceride band is marked and the adsorbed iodine removed from the plate by warming. The marked band is then scraped off the plate into a 100-ml erlenmeyer flask, extracted with warm chloroform and filtered into another 100-ml erlenmeyer flask. The solvent is removed and the procedure described under preliminary detection is repeated with the residual mass. The presence of kusum oil is confirmed by the blueing of the exposed zone of the filter paper.

Discussion

The procedure described above is capable of detecting micro quantities of the oil. The sensitivity of the method comes out to about 25 μg with respect to the cyano compound expressed as HCN; the value has been calculated on the basis that 10 mg of the oil have been used and the concentration of the cyano compound in the oil is about 0.25% (mean of the reported values) expressed as HCN. Possible effects of the presence of several selected oils were noted, using model mixtures. Thus, butter fat (fat with comparable R.M.V., P.V. and K.V.), coconut oil (*Cocos nucifera*, oil with high P.V. but low R.M.V., K.V. and I.V.), peanut oil (*Arachis hypogaea*, oil with low R.M.V., P.V. and K.V. but medium I.V.) and safflower oil (*Carthamus tinctorius*, oil with low R.M.V., P.V. and K.V. but high I.V.) were respectively fortified with graded amounts (10, 5, 2 and 1%, by weight) of the kusum oil in chloroform solution, and the procedure given above was repeated in each case. Appropriate control experiments were performed side by side for comparison. Only the oils with added kusum oil gave satisfactory positive response to the test within the limit recorded. The procedure described is applicable in general to any cyano compound capable of liberating HCN under the conditions of the experiment and, in the case of seed oils, is generally indicative of the presence of kusum oil but, on chromatographic resolution as above, becomes specific for kusum oil as so far no other oil is known to contain cyanoglycerides.

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CHROM. 4504

Separation of neptunium, plutonium, americium and thorium by anion exchange

The separation of Np(IV) and Pu(III) by anion exchange from nitric acid[1–3] as well as Pu(IV), U(VI) and Am(III)[4] has been reported. Because Np(VI), like U(VI), does not form strong anionic complexes, the separation of Np(VI) and Pu(IV) can be carried out by using the agents which hold the neptunium in hexavalent and plutonium in tetravalent state. The difference in the distribution coefficients for Pu(IV) and Th(IV) in nitric acid solutions[5–7] gives the possibility for their separation, too.

This paper describes investigations carried out on the separation of Np(VI) and Th(IV) from Pu(IV) by anion exchange from nitric acid solution. Americium has also been included in the separation scheme.

TABLE I

SEPARATION OF Am(III), Np(VI), Th(IV) AND Pu(IV)

Column: 15 cm × 0.11 cm². Exchanger: Dowex 1 X4, 100–200 mesh. Temperature: 50°. Flow rate: 1 ml/min/cm².

Element	*No. of samples*	*Percent of elements eluted in fractions*			
		7.2 M HNO_3 (5 ml)	*7.2 M HNO_3 (10 ml)*	*4.0 M HNO_3 (25 ml)*	*0.35 M HNO_3 (10 ml)*
^{241}Am	9	100.1 ± 1.2	⩽0.01	[a]	[a]
^{237}Np	9	[a]	99.3 ± 1.4	⩽0.05	⩽2
^{234}Th	9	~0.02	~0.04	100.3 ± 1.3	[a]
^{239}Pu	9	[a]	⩽0.06	⩽0.09	100.0 ± 0.7

[a] The activity of these radionuclides could not be detected. About 30% of ^{233}Pa elutes in the neptunium fraction and 70% in thorium fraction.

Experimental

Reagents and radionuclides. The exchanger Dowex 1 X4 (100–200 mesh) in nitrate form was used.

The purification and the preparation of the Am and Pu solutions were carried out as described previously[4]. The isolation of ^{234}Th from $UO_2(NO_3)_2 \cdot 6H_2O$ was done as described earlier[8]. ^{237}Np was in the form of nitrate in HNO_3. The amount of ^{241}Am and ^{234}Th used were at the tracer levels. From $4.2 \cdot 10^{-3}$ to 0.45 mg of ^{237}Np and $1.6 \cdot 10^{-3}$ to 1.7 mg of ^{239}Pu were used. All chemicals were of p.a. purity.

Determination of radionuclides. ^{239}Pu, ^{237}Np and ^{241}Am were measured with a Tracerlab Model P-12 alpha scintillation detector. The ^{234}Th was measured with a Nuclear Chicago GM counter. In the presence of the alpha-emitters mentioned, it is possible to measure ^{234}Th over an absorber of 8 mg/cm² aluminium. However, because of the presence of beta-emitters of ^{233}Pa (which is produced by alpha decay of ^{237}Np), ^{234}Th–^{234}Pa were measured over an absorber of 99.6 mg/cm² of aluminium.

The purity of ^{239}Pu, ^{237}Np and ^{241}Am was checked, *i.e.* the content of each one in the other two was determined by alpha spectrometric analysis using a Tracerlab,

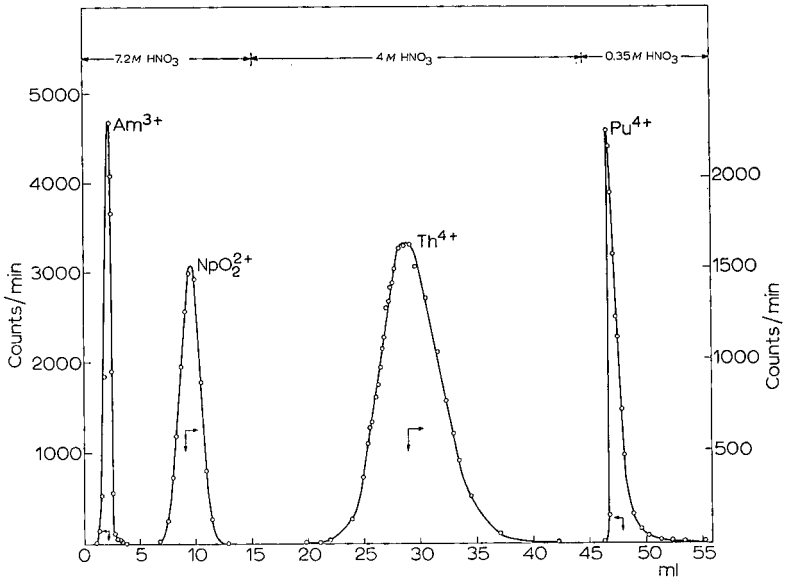

Fig. 1. Separation of Am(III), Np(VI), Th(IV) and Pu(IV) (tracer amounts of Am and Th, 40 μg of ^{237}Np and 1.5 μg of ^{239}Pu) on Dowex 1 $X_4(NO_3^-)$, 100–200 mesh, at 50°. Column: 15 cm × 0.11 cm^2. Flow rate: 1 ml/min/cm^2.

Model RLD-1, Frisch Grid chamber with a Model 20623 RCL 256 channel pulse height analyser.

Check of the valency state of Np in nitric acid solutions was made spectrophotometrically[9,10] with a Unicam SP-500 spectrophotometer.

Sample preparation. The solution of the radionuclides in 1–2 *M* HNO_3 were treated with $NH_2OH \cdot HCl$ (0.025–0.05 *M*) for 15–30 min and then $NaNO_2$ (0.05–0.10 *M*) was added. After 30 min the solution is adjusted to 7.2 *M* HNO_3, thermostated for 30 min at 50° and transferred to the column, pretreated with a 7.2 *M* HNO_3 and thermostated at 50°. The amount of sample transferred to the column (15 cm × 0.11 cm^2) should not exceed 1.6 ml.

Results and discussion

It was reported that Np(V) in nitric acid higher than 3 *M* in the presence of NO_2^- oxidises to Np(VI)[11]. Under the same conditions Pu exists as Pu(IV). The anion-exchange behaviour of neptunium in strong nitric acid containing $NaNO_2$ was identical to the behaviour of Np(VI) which was prepared by oxidising Np with Ag^{2+} or Ce^{4+}. Spectrophotometric analysis of a neptunium solution in 4–7 *M* HNO_3 containing 0.1 *M* $NaNO_2$ have shown that Np(V), at 50°, was oxidised to Np(VI).

Np(VI) elutes from the anion-exchange column approximately like U(VI). The distribution coefficients determined by the column method[12] for 7.2 *M* HNO_3 at 50° for Np(VI) and U(VI) are 10.4 and 7.0, respectively.

On the basis of these data as well as on the basis of the data reported[4], the separation of Am(III), Np(VI) and Pu(IV) was carried out (Fig. 1). The elution of

Th(IV) from the column has been achieved with 4 M HNO_3. Results are shown in Table I.

As it is seen from Table I satisfactory separation of given radionuclides is obtained. However, when a sample contained about 0.2 M H_2SO_4, only 90% of ^{237}Np was recovered.

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Letter to the Editor

CHROM. 4505

Dear Sir,

the paper by DIMOV AND SHOPOV (*J. Chromatog.*, 44 (1969) 170–172) mentions the method for predicting I^S values by means of structural increments described by myself in several papers[1–7].

The authors compare the differences between calculated and experimental values given in my paper found in *J. Chromatog.*, 23 (1966) 1–17, page 4, Table II with differences determined by their own method of index calculation. The authors overlooked that this table was only given to point out the existence of increments of the second order for the calculation of I^S values of double- and multiple-branched hydrocarbons where the branches are close together within the molecule. This is to be seen from the sentence: "Durch zusätzliche Inkremente für die 2,3- und 2,2-Dimethylalkane werden auch die Indexwerte der Dimethylalkane vorausberechenbar". In other papers published during the years 1964, 1966, 1967 and 1968, I defined these increments of second order which are to compensate for interactions between functional groups, in more detail in refs. 5 and 6. Using these additional increments for the calculation of the $I_{\text{theor.}} - I_{\text{exp.}}$ in the Table of DIMOV AND SHOPOV, the differences become small or vanish completely.

The greatest accuracy of prediction of I^S values is of course not obtained with smaller molecules, for example below C_8. Because the I^S values of hydrocarbons with carbon numbers up to C_{10} have been measured by several authors (MATUKUMA[8], HIVELY AND HINTON[9] and LOEWENGUTH AND TOURRES[10]) with high accuracy, the calculation of I^S values for practical applications is especially useful for greater molecules, although the rules of calculation are valid approximately even for smaller molecules.

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Book Reviews

CHROM. 4495

Ion Exchange and Solvent Extraction of Metal Complexes, edited by Y. MARCUS AND A. S. KERTES, John Wiley & Sons Ltd., London, New York, Sydney, Toronto, 1969, 1037 pp., price 315/—.

This book provides a comprehensive account of both the principles of ion exchange and solvent extraction, the interaction and formation of metal complexes in aqueous and non aqueous conditions and their study by ion-exchange and solvent extraction techniques.

The first three chapters set the scene for the book discussing electrolyte solution in aqueous and non aqueous media and the formation of complexes in solution. Following a general account (Chapter 4) of ion exchangers, the cation and anion exchange of metal complexes is dealt with in Chapters 5 and 6. The remainder of the book is concerned with solvent extraction, the quantitative physical chemistry in Chapter 7 and extraction processes, *i.e.* compound formation, solvation and ion pair formation, in Chapters 8–10. A discussion of synergistic extraction follows and the concluding chapter considers several examples of the application of distribution methods in both ion exchange and solvent extraction for study of metal complexes in solution. Extensive useful appendices of solvent properties, commercial ion-exchange data and distribution charts of extraction of elements are included.

The book can only be described as the definitive work for this branch of chemistry and the several thousand references up to 1967 contained therein show its comprehensive nature. The book will undoubtedly appeal to not only graduates and research workers studying metal complex formation using ion-exchange or solvent extraction techniques but also will find a place on the book shelves of those who use both techniques to effect extraction and separation on a more practical level. A wealth of data describing ion-exchange and solvent extraction systems permits rationalisation of extraction and separation problems in the light of recent knowledge. Particularly the compilation in the appendix of the distribution data for metal ions between aqueous solution and both resinous exchangers and solvents provides an easy guide to what separations are feasible and under what conditions. The relevant chapters in the book provide a basic understanding of the chemistry of the particular system. Of especial interest is the informative chapter (Chapter 11) on synergism in solvent extraction systems which gives a useful classification of such systems and follows logically from the preceding chapters discussing the various types of solvent extraction of single extractant or solvating agent systems.

Particular likes of the reviewer were the emphasis in Chapter 7 on the usefulness of physical measurements to assist characterisation of new or little known extractants and in Chapter 8 the comment that the physical properties of the extractants may vary from the anhydrous state in the diluent to the water saturated equilibrated state encountered in distribution studies. It is important to realise this as correct interpretation of the distribution data can hinge on the physical state of the extractant in the organic phase.

Unfortunately there are an irritating number of misspellings in the book and although the reviewer did not find any, it tends to suggest that there may be similar errors in the formulae and equations.

Priced at 315/— this book is unlikely to be purchased by individual research workers but libraries of laboratories engaged in studies of metal complexes in solution would do well to consider purchasing more than one copy as the demand for the book will surely be heavy.

Warren Spring Laboratory, Stevenage (Great Britain) D. S. FLETT

CHROM. 4494

A Programmed Introduction to Gas–Liquid Chromatography, by J. B. PATTISON, Heyden & Son., London, and Sadtler Research Labs., Inc., Philadelphia, 1969, 303 pp., price 45/—, $ 4.95.

This book constitutes a unique approach to the teaching of the basic principles of gas chromatography to persons with no prior knowledge of the technique. The approach is to give a short background to a particular aspect of GC, for example, the stationary phase and then to proceed through a series of questions and answers. If the student selects a wrong answer he is referred to a page on which his error is explained; he continues until he reaches the correct answer and then moves onto the next question. In this way the student is led through the subject in a series of simple stages.

It is claimed to provide a "simple, cheap answer to the training problem of the busy laboratory", teaching "your staff how to set up, efficiently operate and interpret the results from a GLC instrument". How well does it achieve these claimed objectives and precisely who will benefit from its use? To try and answer these questions the book has been used to introduce newcomers, at various levels of academic ability and analytical background, to GLC in the reviewer's laboratory. The book seems to justify its claims well for the initial training of routine operators who are simultaneously gaining practical experience at the bench. It has been found to be simple, clear and direct in its teaching of the basic principles. Providing one does not expect more than it sets out to give its use can be strongly recommended. One should not expect it to be a substitute for more formal text-books, rather it is an introduction to them. The reviewer believes this book will be very welcome to routine GC laboratory supervisors with a recurrent operator training problem; it is, for them and their trainees, an effective, painless introduction to GLC.

Esso Research Centre, Abingdon, Berks. (Great Britain) S. G. PERRY

News

CORRECTION

We are happy to report that when some time ago we stated that all further issues of the Camag Bibliography Service on Thin-Layer Chromatography would be charged for, we were in error (*cf.* Vol. 42, No. 4, p. 568). We gather that there is no intention to charge for future issues distributed by Camag, Muttenz, Switzerland. The charge to which we referred concerns only the United Kingdom and does in no way prejudice how Camag or its other distributors handle this matter.

Apparatus

Phoenix Precision Instrument Co., Philadelphia, Penn. introduces a *Vibrating Platinum Electrode Accessory* for the measurement of oxygen concentrations in solutions and designed especially for use with the Phoenix Dual-Wavelength Scanning Spectrophotometer.

It can easily be adapted to the cell housing of the Spectrophotometer and it can be used with single or double cell holders in the dual-wavelength or split beam modes. The cover plate of the Vibrating Platinum Electrode Accessory has provisions for direct connection of a syringe or needle injection through a septum for light-tight operation.

For further information apply to the publisher under reference No. Chrom. N-236.

Manufacturers' Literature

Over the last three years the Universal Fisher Engineering Company have been developing continuous chromatography as a process technique. It is now practicable to continuously refine a raw product stream in production scale quantities, using chromatographic methods.

Gas chromatography refining

The use of chromatography adds several important new dimensions to the processing of volatile chemicals. (1) The selection of column solution properties, *e.g.* polarity, allow separations not possible by distillation. (2) The low concentrations of sample in the vapour phase at temperatures greatly below the sample boiling point. This allows the processing of thermally labile products with minimum decomposition. (3) Plant operating conditions for a continuous separation may be calculated from data quickly obtained with a pilot analytical column. This minimizes plant start-up time when either the feedstock or the product purity requirements are changed.

Liquid chromatographic refining

Continuous liquid–liquid and liquid–solid chromatography may be similarly applied to plant scale separations. Two specific advantages of liquid chromatography are: (1) Thermally labile compounds may be processed. (2) The control of solution properties of the mobile phase also, gives enhanced specificity to the separating power of the system.

Applications

The fields in which continuous chromatography is presently being applied cover the following:

(1) *Gas chromatography*. Essential oils—pinenes, rosewood oil, terpenes, orange oils. Hydrocarbon isomers—heptenes, xylenes, cyclopentane. Fatty acids—esters. Higher alcohols—glycols. Pharmaceutical purifications.

(2) *Liquid chromatography*. Molecular weight distribution in sugars. Separation of carbohydrates using deionised water and ion-exchange resins.

Equipment available

Universal Fisher Engineering can presently offer a range of three equipments. The feed through-put rates that may be obtained, range from $\frac{1}{2}$ lb./day to 20 lbs./day.

For further information apply to the publisher under reference No. Chrom. N-259.

The latest issue of Perkin-Elmer Analytical News is devoted entirely to gas chromatographic applications. There are two papers both concerned with the analysis of barbiturates in biological material. The reports suggest that Apiezon L packing is more satisfactory for high concentrations whilst neopentyl glycol adipate is more suitable for trace quantities.

For further information apply to the publisher under reference No. Chrom. N-240.

The JEOL News 1969 Vol. 7C No. 2 contains descriptions of (i) mass analysis of organic compounds with photographic plate detection, (ii) measurements with a gas chromatographic–mass spectrographic combination method employing the JMS-OLS mass spectrometer, and (iii) automatic analysis of mass spectral data with the JMA-1C-O.

For further information apply to the publisher under reference No. Chrom. N-239.

The Spring issue of Analytical Advances, the house journal of Hewlett-Packard Company, describes in some detail the recently announced 7600A automated gas chromatography system and the Series 7620A gas chromatographs.

For further information apply to the publisher under reference No. Chrom. N-238.

The availability of a new report, entitled *The Influence of Gel Permeation Chromatography on Polymer Science and Technology* by Dr. RAYMOND F. BOYER of the Dow Chemical Company has just been announced. The article discusses the unique features of the method, the need for molecular weight distribution measurement in science and industry, and the relative advantages and disadvantages of several current methods for molecular weight distribution of polymers.

For further information apply to the publisher under reference No. Chrom. N-237.

Erratum

Characterization of fiber parameters in agarose gels by light scattering. B. ÖBRINK, *J. Chromatog.*, 37 (1968) 329.

(1) An error has been made in the calculation from the light scattering measurements of the M/L values of the agarose fibers. The correct M/L range should be 3.94×10^{-13}–5.38×10^{-13} g/cm, which is about three times larger than the corresponding one obtained by gel chromatography. However, this discrepancy is not necessarily incompatible with the conclusion that the light scattering measurements are in agreement with the results of gel chromatography. The steric exclusion, operating in gel chromatography, depends largely on the fraction of the fibers with low M/L values. Light scattering measurements, on the other hand, yield weight-average M/L values, *i.e.* depend more on the fibers with high M/L ratios. A heterogeneity with respect to fiber diameter may explain the discrepancy observed.

(2) Centrifugation of agarose solutions before light scattering measurement was carried out for 60 min, not 20 min.

Department of Medical Chemistry,
University of Uppsala, Sweden

BJÖRN ÖBRINK

J. Chromatog., 45 (1969) 147–149.

Page 149, in the address of GERHARD BUNDSCHUH, G.F.R. should read G.D.R.

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