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RESOLUTION IN THIN-LAYER CHROMATOGRAPHY WITH SOLVENT OR ADSORBENT PROGRAMMING

COMPARISONS WITH COLUMN CHROMATOGRAPHY AND NORMAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A general theory of sample resolution has been developed for various TLC techniques in which the composition of the developing solvent or adsorbent varies throughout separation. For the separation of complex samples containing many components of widely differing adsorptivities, it is predicted that gradient-layer thin-layer chromatography (TLC) should provide generally better separation than gradient elution TLC, and the latter technique should in turn be superior to polyzonal TLC. However, these generalizations must be qualified by certain practical considerations. The advantages of vapor-programmed TLC as recently described appear to be open to question. At the present time none of these TLC techniques can compete with gradient elution from columns as regards separation speed and resolution in a single separation.

INTRODUCTION

In the separation of a sample by liquid–solid chromatography (LSC) one can choose from among a variety of different techniques: normal column chromatography or thin-layer chromatography (TLC), stepwise or gradient elution from columns or on plates (TLC), continuous or multiple development TLC, adsorbent gradients (TLC), and so forth. Apart from experimental convenience and the equipment available for separation, the main factors in the selection of a given procedure are separation speed and sample resolution. Until recently, however, comparisons of different LSC techniques with respect to relative speed and resolution have been difficult to make. Prior to 1967 little existed in the way of adequate theory or relevant experimental data. Recent experimental and theoretical studies[1–4] have clarified this situation with respect to normal column and TLC techniques, including continuous and multiple development TLC. It now appears that column separations in these cases have a significant advantage over TLC with respect to both separation speed and resolution, when each procedure has been experimentally optimized.

The difficulty in adequately separating many multicomponent samples by normal LSC (*i.e.* where the same solvent and adsorbent are used throughout separation) has given rise to a number of special techniques: stepwise or gradient elution from

columns, analogous solvent-programming techniques in TLC, gradient-layer TLC, polyzonal TLC, etc.*. Solvent programming in columns (gradient or stepwise elution) has been examined recently[6] with respect to separation speed and resolution, but comparable studies of related TLC procedures (see reviews of refs. 5 and 7) are so far lacking. In this paper we will develop a simple, idealized theory of resolution for these latter TLC procedures. Since separation time in TLC is normally fixed within narrow limits, we can ignore the possibility of simultaneously varying separation speed. In this attempt we recognize that the complexity of practical TLC systems (the latter techniques in particular)—and the resulting approximations which are required in any practical theory—will limit the validity of our final conclusions. On the other hand, this same experimental complexity simultaneously precludes the easy generalization of experimental TLC data and the direct comparison of different techniques. In the final analysis simple theory is necessary as an initial guide in attempting to understand these various chromatographic systems—particularly with regard to comparisons between different TLC and column procedures.

GENERAL THEORY

Resolution in single-solvent separations with a fixed stationary phase (*i.e.* normal column or TLC procedures) is best defined by the relationship

$$R_s = \Delta d/2(\sigma_A+\sigma_B) \tag{1}$$

At the end of separation, Δd is the spacing between the centers of two adjacent, adsorbed bands (A and B), and σ_A and σ_B are their widths (standard deviations of the Gaussian curves). Eqn. (1) is directly applicable in TLC separations. For elution from a column, the quantities Δd, σ_A and σ_B are measured immediately before elution of the two bands from the column. For two narrow, closely adjacent bands—the case of greatest interest—it can be assumed that $\sigma_A = \sigma_B$, and $k_A \approx k_B$ (k_A and k_B are the partition ratios for bands A and B; *i.e.* the ratio of total A or B in the stationary phase to total A or B in the mobile phase during separation). With these approximations it can be shown readily (*e.g.* ref. 2) that

$$R_s = (1/4)\ [(k_A/k_B)-1]\ \sqrt{N'}\ [k_B/(1+k_B)] \tag{1a}$$
$$\qquad\quad (a) \qquad\quad (b) \qquad\quad (c)$$

Here N' is the number of theoretical plates in the bed length that have been traversed by A or B at the end of separation. For elution from a column, N' is equal to the total number of plates (N) in the column. For separations by TLC, N' is equal to $R_F \cdot N$, where R_F refers to the average distance migrated by A and B relative to the solvent front, and N is the number of plates in the adsorbent bed behind the solvent front. Resolution is seen in eqn. (1a) to be a product of three essentially independent factors: (a) a separation selectivity factor, (b) a bed efficiency factor, and (c) a function of the partition ratio of B (or A, since $k_B \approx k_A$). The optimization of separation selectivity and efficiency in LSC has been discussed in detail[4,8]. For a given set of experimental conditions (*i.e.* single solvent and adsorbent), the optimum value of k_B is 2 so that $R_F = 1/3$ and factor (c) is equal to $2/3$ (but see discussion of ref. 8).

When a given sample contains many components of widely differing migration

* For a description of these special TLC techniques see ref. 5 and following sections of this paper.

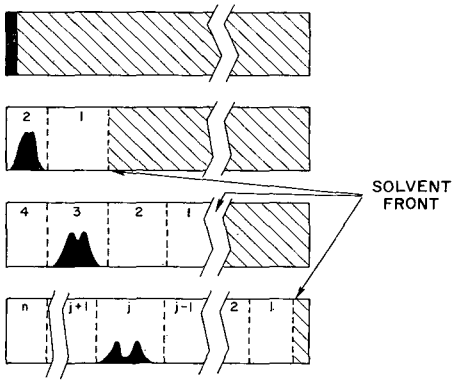
rates (k values), eqn. (1a) predicts that weakly adsorbed components will be poorly resolved; *i.e.* factor (c) is small. Similarly, eqn. (1a) predicts that in TLC strongly adsorbed components will also be poorly resolved; *i.e.* R_F and N' are small. In elution from columns, strongly adsorbed bands are well resolved but require excessive separation times (this is equivalent to a loss in resolution per unit time). This general problem can be overcome by a systematic change in sample component k values during separation, such that each component is separated under optimum conditions (*i.e.* $k \approx 2$). Sample k values can be changed by variations in temperature (temperature programming), solvent (stepwise or gradient elution, polyzonal TLC, etc.) or adsorbent (gradient layer TLC). In column chromatography solvent programming is preferable to other techniques as a means of varying k during separation[6]. For similar reasons it can be argued that temperature programming in TLC is not as effective as solvent programming*. The present theoretical treatment will therefore ignore the possibility of TLC temperature programming.

Experimental TLC separations are subject to a number of complications which would be quite difficult to include in a theoretical treatment of the present kind. These complications include the transfer of solvent between plate and vapor phase during separation, the development of solvent concentration gradients (*i.e.* varying ratios of solvent to adsorbent) across the plate in the direction of solvent flow, solvent demixing during separation, temperature effects (heat of wetting), and changes in adsorbent activity during separation (see ref. 4, sect. 13.2E). In the present examination, unless otherwise noted, each of these effects is ignored. This means that we assume all solvent transfer to the plate occurs by capillary flow up the plate, and the ratio of solvent to adsorbent at any point behind the solvent front is constant. These approximations have a significant effect on the validity of final quantitative data furnished by our theory, but any qualitative conclusions do not appear to be seriously compromised.

Gradient elution thin-layer chromatography

By gradient elution TLC we mean a separation in which the composition of solvent entering the bottom of the plate changes with time (as in gradient elution from columns). We can approximate any such continuous solvent program by a series of individual solvents 1, 2...i...n of volumes V_1, V_2...V_i...V_n and average partition ratios (for a given pair of bands A and B) k_1, k_2...k_i...k_n. A given pair of sample bands A and B will be carried a certain distance along the adsorbent bed as a result of the passage of the first solvent volume (V_1) through the two band centers (see Fig. 1). Similarly the two bands will be carried still further along the adsorbent bed by passage of solvent 2 through the band centers. Finally, this process will end when the front of solvent 1 reaches the end of the adsorbent bed (or some arbitrary point short of the bed end). At this time the two bands will be surrounded by some intermediate solvent j. Assuming that k_n is reasonably small, the two bands will have migrated a significant distance along the bed; *i.e.* neither R_F nor N' will be zero. If k_1 is reasonably large, and if k decreases by regular steps in going from solvent 1 to n, migration of two bands will begin when $(k/1+k)$ decreases significantly below one and will accelerate as k approaches zero (thus keeping the two bands well ahead of the very strong solvents in the latter part of the solvent program (for which k equals zero). Consequently throughout separation the factor (c) of eqn. (1a) will be significantly

* This assumes no vaporization of mobile phase as temperature is increased. Flux gradient TLC represents an exception.



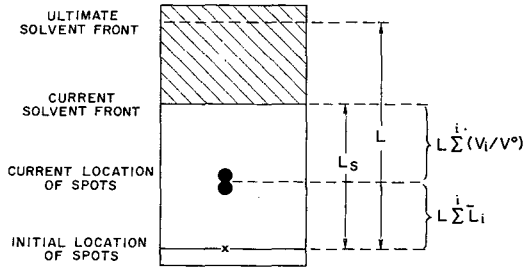


Fig. 1. Separation of a pair of sample components in gradient elution TLC.

Fig. 2. Gradient elution TLC after passage of the i-th solvent through the two bands of interest.

greater than zero. In this way significant sample resolution is maintained for both weakly and strongly adsorbing sample components, as long as factor (a) is not close to zero. It is only required that $k_1 > 0$ and $k_n/(1+k_n) < 1$.

The calculation of resolution in gradient elution TLC proceeds in essentially the same way as for solvent programming in column elution (see discussion of ref. 6). We begin by allowing the initial solvent volume V_1 to pass completely through the band centers of A and B. As a result the two bands migrate some distance L_1 along the bed, given by

$$L_1 = L\,(V_1/V^0)/k_1 \tag{2}$$

L is the total length of the bed (more accurately, the total length of the bed which lies between the initial point of sample application and the final solvent front), and V^0 is the volume of total solvent required to wet the bed length L. We next allow the second solvent volume V_2 to pass completely through the band centers of A and B. As a result the two bands migrate an additional distance L_2 through the bed. This process is continued until the front of solvent 1 reaches the end of the bed or some predetermined point which marks the end of separation. For any solvent i, the fractional distance $L_i/L = \bar{L}_i$ migrated by A and B as a result of the passage of solvent i through the two band centers is given as

$$\bar{L}_i = \frac{V_i/V^0}{k_i} \tag{2a}$$

The total distance migrated by the solvent front L_s, after passage of solvent i through the two band centers, is given by eqn. (2b) (see Fig. 2).

$$L_s/L = \overset{i}{\Sigma}\,(V_i/V^0) + \overset{i}{\Sigma}\,\bar{L}_i \tag{2b}$$

At the completion of separation, L_s/L equals one, so the condition that the two bands will be surrounded by solvent j at the conclusion of separation (see Fig. 1) is

$$\overset{j-1}{\Sigma}\,[(V_i/V^0) + \bar{L}_i] < 1 < \overset{j}{\Sigma}\,[(V_i/V^0) + \bar{L}_i] \tag{2c}$$

The distance migrated by the two bands as a result of the passage of some fraction of

the j-th solvent through the band centers ($L_{j'}$) is calculated as follows. After the passage of solvents 1 through $(j-1)$ across the band centers, the distance lying between the front of solvent 1 and the end of the bed (or final solvent front) ΔL is given by

$$\Delta L/L = 1 - \sum^{j-1} [(V_i/V^0) + \bar{L}_i] \tag{3}$$

The average R_F value of the two bands in solvent j, $(R_F)_j$, is seen to be given by

$$\begin{aligned}(R_F)_j &= L_{j'}/\Delta L \\ &= 1/(1 + k_j)\end{aligned} \tag{3a}$$

The quantity $\bar{L}_{j'} = L_{j'}/L$ can be defined, and from the above relationship

$$\bar{L}_{j'} = (\Delta L/L)/(1 + k_j) \tag{4}$$

with $(\Delta L/L)$ calculable from eqn. (3). The average R_F value of the two bands is then given as $(\sum^{j-1} \bar{L}_i) + \bar{L}_{j'}$. The resolution R_s developed as a result of the migration of the two bands the total distance $\bar{L}_1 + \bar{L}_2 + \ldots \bar{L}_{j-1} + \bar{L}_{j'}$ can now be calculated in the same way that R_s has previously been calculated for solvent programming in elution from columns (see ref. 6):

$$(R_s)^2 = \frac{N\,[(k_A/k_B) - 1]^2 \cdot Q^2}{16} \tag{5}$$

N is the total number of theoretical plates in the bed length L, and the quantity Q^2 is given as

$$Q^2 = \frac{\left[\sum^{j} \left(\underset{1}{\overset{i \leqslant m \leqslant j}{}}\right) G_m \bar{L}_i k_i/(1 + k_i)\right]^2}{\sum^{j} \left(\underset{1}{\overset{i \leqslant m \leqslant j}{}}\right) G_m^2\, \bar{L}_i} \tag{5a}$$

The summation over $\bar{L}_i$ ends with the term $\bar{L}_{j'}$ (note the similarity of eqn. (5a) above and eqn. (6c) from ref. 6). The band compression factor G_m is given as

$$G_m = k_{m+1}\,(1 + k_m)/k_m\,(1 + k_{m+1}) \tag{5b}$$

Eqn. (5a) permits us to calculate the number of effective theoretical plates NQ^2 in a separation by gradient elution TLC as a function of a particular solvent program (series of values of V_i and k_i for different sample bands) and value of N.

One of the more important characteristics of a solvent program in gradient elution TLC is its ability to provide comparable sample resolution (*i.e.* comparable values of NQ^2) for components of differing relative adsorption (differing values of k_1). In the case of gradient elution from columns, it has been shown[6] that so-called logarithmic solvent programs (eqn. 5c) give equal resolution for all sample components except those that are very weakly adsorbed (*i.e.* k_1 small):

$$\log k = \log k_1 - b(V/V^0) \tag{5c}$$

Here V is the total volume of solvent which precedes solvent of partition ratio k, k_1 refers to the k value for the first solvent in the program, and b is seen to be a measure of gradient steepness; *i.e.* how fast k changes with solvent volume V. We have calculated Q^2 of eqn. (5a) (by computer) as a function of b for various logarithmic solvent programs in gradient elution TLC (V_i small). The results of this calculation are shown in Fig. 3a as Q^2 *vs.* R_F and in Fig. 4a as Q^2 *vs.* $\log k_1$. Assuming a typical

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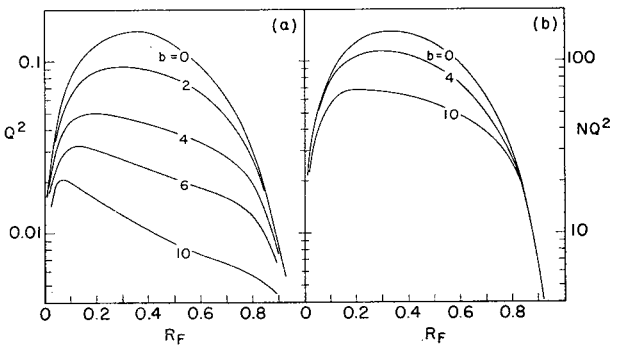


Fig. 3. Resolution in gradient elution TLC (a) and gradient-layer TLC (b) as a function of gradient steepness b (logarithmic programs with V_i small).

value of N in TLC equal to 1000 (ref. 2) we have also indicated values of $\tilde{N}Q^2$ (so-called "effective theoretical plates") in Figs. 3 and 4.

What is the significance of these calculated values of Q^2 and NQ^2? First, the data of Figs. 3a and 4a show how the number of effective theoretical plates NQ^2 (and sample resolution) varies with the steepness of the solvent gradient b, differences in compound adsorptivity k_1 and the distance migrated along the plate (R_F). We see that average resolution along the plate (*i.e.* NQ^2) decreases with increasing steepness of the solvent gradient, just as in gradient elution from columns[6]. At the same time, however, a greater range of sample components (a greater range in k_1 values) can be separated with comparable resolution (Fig. 4a). Second, we see that a logarithmic solvent program does not provide equal values of NQ^2 for all sample components in TLC, in contrast with the case of gradient elution from columns[6]. To a certain extent this reflects "end effects" which are beyond our control in actual practice (*i.e.* the inevitable approach of R_s to zero for R_F values close to zero or one). A similar phenomenon was encountered in gradient elution from columns[6] for the case of weakly adsorbed sample components. For steep solvent gradients ($b > 4$) there is a systematic decrease in NQ^2 with increasing R_F over most of the plate (Fig. 3a). This could be corrected (*i.e.* NQ^2 made more nearly constant) by changing the form of the solvent

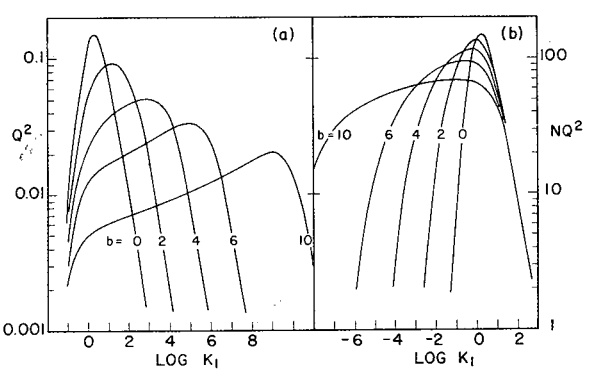


Fig. 4. Resolution in gradient elution TLC (a) and gradient-layer TLC (b) as a function of gradient steepness b (logarithmic programs with V_i small).

program so that log k changes more rapidly with V in the latter part of the program (relative to a logarithmic solvent program). Finally, the values of NQ^2 which are provided by gradient elution TLC with steep solvent gradients ($b > 4$) are rather small (generally less than 30 effective plates). By contrast, gradient elution from columns easily provides in excess of 400 effective plates for all sample components, with comparable separation times[6]. Thus gradient elution TLC is very much less efficient than gradient elution from columns, when the latter is properly optimized, just as normal column elution is more efficient than normal TLC (*i.e.* fixed solvent throughout separation). Alternatively, comparable separations by gradient elution from columns can be obtained in much less time than by gradient elution TLC.

Adsorbent gradients in thin-layer chromatography

By an adsorbent gradient in TLC we mean a systematic change in the composition of the adsorbent (or adsorbent activity) along the plate. Adsorbent gradients can exist either parallel to the direction of solvent flow, or at right angles to solvent flow (see ref. 5). Both techniques have been referred to as "gradient-layer TLC". The use of adsorbent gradients at right angles to solvent flow is equivalent to using several different plates of varying adsorbent composition for an initial survey of optimum TLC separation conditions. This technique is outside the scope of the present treatment. The use of an adsorbent gradient parallel to solvent flow provides still another means of dealing with the problem of multicomponent sample separation in TLC. Thus if the initial part of the plate consists of very weak adsorbent, strongly adsorbing sample components will be separated on this section of the plate, and less strongly adsorbing components will pass through without separation. If the remainder of the plate is composed of adsorbent of continuously increasing activity (stronger retention of all sample components), the remaining sample components will be retained in various parts of the bed and separated there. In this way both strongly and weakly adsorbing sample components can be resolved in a single separation.

The calculation of resolution in gradient-layer TLC proceeds in much the same way as for gradient elution TLC. We begin with a bed divided into segments 1, $2 \ldots i \ldots n$ of relative lengths ($L_i = L_i/L$) $\bar{L}_1, \bar{L}_2 \ldots \bar{L}_i \ldots \bar{L}_n$, and corresponding k values (for a particular pair of adjacent bands A and B) $k_1, k_2 \ldots k_i \ldots k_n$. Passage of some volume V_1 of solvent (same solvent throughout) will suffice to carry the two bands to the end of bed segment 1, with

$$V_1/V^0 = \bar{L}_1 k_1 \tag{6}$$

Similarly, passage of some volume V_2 of solvent suffices to carry the two bands to the end of bed segment 2. The total distance migrated by the solvent front L_s, after passage of the two bands through the first i bed segments is given by eqn. (2b). The condition that the two bands will lie in bed segment j at the end of separation is given by eqn. (2c). The distance migrated by the two bands in the j-th bed segment is given by eqn. (4), and the average R_F value at the end of separation is equal to $({}^{j}\bar{\Sigma}^{1}\, L_i) + \bar{L}_{j'} = \bar{L}$ (as for gradient elution TLC). The resolution R_s developed as a result of the migration of the two bands the total distance $({}^{j}\bar{\Sigma}^{1}\, L_i) + \bar{L}_{j'}$ can now be calculated in essentially the same way that R_s was calculated in gradient elution from columns or in gradient elution TLC. The resulting expression is the same as that given in eqns. (5) and (5a), except that the band compression factor G_m is given by

$$G_m = (1 + k_m)/(1 + k_{m+1}) \tag{7}$$

The latter expression can be derived in the same way as eqn. (5b) was derived originally in ref. 6. These relationships allow us to calculate the effective theoretical plates NQ^2 in gradient-layer TLC as a function of sample adsorptivity k_1 and the adsorbent activity program (k as a function of position on the plate). In Figs. 3b and 4b we have plotted the results of such a calculation for a logarithmic adsorbent activity program (*i.e.* $\log k = \log k_1 + b[x/L]$, where x is the distance along the plate from the point of sample application). The data of Figs. 3b and 4b show a general resemblance to the calculations for gradient elution TLC: steeper adsorbent activity gradients (larger values of b) provide less resolution of adjacent bands, but a wider range of sample components can be separated; the logarithmic adsorbent activity program does not provide exactly equal resolution at all positions on the plate; sample resolution is significantly poorer than comparable separations by gradient elution from columns. The major difference between gradient elution and gradient-layer TLC is generally better resolution by the latter technique. For large values of b and conditions that provide significant resolution for a comparable range in k_1 values, gradient-layer TLC can offer from two to three times more effective plates than in the case of gradient elution. Thus our simple theory predicts that gradient-layer TLC is superior to gradient elution TLC.

Although our simple theory indicates a clear-cut superiority of gradient layer TLC over gradient elution TLC, in actual practice this will not always be the case. The problem is an experimental one, namely the great difficulty in preparing adsorbent gradients with $b > 4$ (see below). When $b < 4$, the relative advantage of gradient layer TLC with respect to resolution is considerably reduced (*i.e.* NQ^2 for gradient layer TLC approaches that for gradient elution TLC at similar values of b). Furthermore, the limitation $b < 4$ reduces the range in sample components (*i.e.* k_1 values) which can be separated on a single plate, which is a further limitation on gradient layer TLC.

We should note in passing that adsorbent gradients can be achieved in several different ways. An active adsorbent (*e.g.* silica gel) can be mixed with varying proportions of an inactive solid (*e.g.* Kieselguhr). This is a simple procedure, but the maximum range in k values (proportional to concentration of strong adsorbent) is limited to about 10^2. Greater dilution of the strong adsorbent would result in too low a capacity and overloading of the initial part of the plate. The adsorbent bed can also be exposed to solvent vapors in special devices[9,10] which permit different vapors to contact different parts of the plate, creating an adsorbent activity gradient across the plate. Presumably a similar device, loaded with adsorbent of varying water content, would also permit varying deactivation of the plate with water vapor (*e.g.* ref. 11).

Polyzonal thin-layer chromatography

In this technique the advantages of gradient elution TLC can be obtained by using a multicomponent solvent mixture of a type which will undergo solvent demixing during separation (see discussion of ref. 5). In the simplest case, that of a two component solvent system $\underline{a}$–$\underline{b}$ ($\underline{b}$ the stronger adsorbing solvent), selective adsorption of $\underline{b}$ occurs during the advance of solvent $\underline{a}$–$\underline{b}$ up the plate. When the difference in solvent strengths of $\underline{a}$ and $\underline{b}$ (*i.e.* their relative adsorptivities or ε^0 values; see ref. 4) is sufficiently great, a secondary solvent front will be observable between the main

solvent front and the point of sample application. These two solvent fronts are termed the α front (primary front) and β front (secondary front), respectively. The composition of solvent lying between the α and β fronts (α zone) will be pure $\underline{a}$, while the composition of solvent between the β front and the point of sample application (β zone) will be the original solvent $\underline{a}$–$\underline{b}$. The k value of a given compound will be greater in the α zone than in the β zone. As a result, strongly adsorbing sample components will tend to migrate in the β zone, with significant resolution, while weakly adsorbing components will migrate in the α zone (again with significant resolution). Thus polyzonal TLC appears to offer the same advantages as gradient elution TLC. As many as three separate solvent fronts (α, β, δ) have been achieved in polyzonal TLC[5].

To appreciate the differences between polyzonal TLC and gradient elution TLC as regards sample resolution, we will compare resolution in each case for the simplest possible analogous systems: a two component solvent mixture $\underline{a}$–$\underline{b}$ in polyzonal TLC *vs.* gradient elution first with $\underline{a}$ then with $\underline{c}$ ($\underline{c}$ chromatographically equivalent to $\underline{a}$–$\underline{b}$, except no solvent demixing). We will make the following assumptions:

(1) equal lengths for the final α and β zones in the polyzonal TLC case;

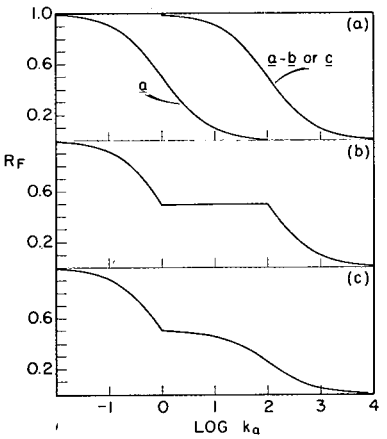
(2) equal volumes of $\underline{a}$ and $\underline{c}$ pass through the point of sample application in the gradient elution case;

(3) the solvent level in contact with the bottom of the plate coincides with the point of sample application (but note the additional possibilities discussed in ref. 5);

(4) solvent demixing is quite pronounced, so that the β front sharply divides solvent of original composition $\underline{a}$–$\underline{b}$ from pure $\underline{a}$ (the α zone); sample k values will therefore change abruptly across the β front by some large factor.

In our model calculation we will further assume an arbitrary (large) ratio of k values in the two zones (α and β): $k_\alpha/k_\beta = 100$. Similarly we will assume for solvents $\underline{a}$ and $\underline{c}$ that $k_{\underline{a}}/k_{\underline{c}} = 100$. First, consider sample R_F values in pure $\underline{a}$ or pure $\underline{c}$ ($\equiv \underline{a}$–$\underline{b}$). These are plotted in Fig. 5a *vs.* values of $k_{\underline{a}} = k_a$. For the same compound (*i.e.* a given value of $k_{\underline{a}}$), R_F values are of course smaller in solvent $\underline{a}$ than in solvents $\underline{c}$ or $\underline{a}$–$\underline{b}$. Next consider R_F values as a function of $k_{\underline{a}}$ in our two-solvent polyzonal TLC system. For compounds with $k_{\underline{a}}$ values less than one, the R_F value of the compound in pure $\underline{a}$ is greater than 0.5, and the compound will always migrate in the α zone. Consequently for $k_{\underline{a}} \leqslant 1$, R_F values in our model polyzonal TLC system will be equal to $1/(1+k_{\underline{a}})$; *i.e.* identical to R_F values which would result if pure $\underline{a}$ was the developing solvent throughout separation. Similarly compounds with $k_{\underline{a}}$ values greater than 100 (R_F in solvent $\underline{a}$–$\underline{b} \leqslant 0.5$) will always migrate in the β zone, and their R_F values will be equal to $1/(1 + 0.01\ k_{\underline{a}})$; *i.e.* the same as would result for solvent $\underline{a}$–$\underline{b}$ (or $\underline{c}$) in the absence of solvent demixing. Sample components with $k_{\underline{a}}$ values between 1 and 100 will migrate unresolved at the β front. R_F *vs.* $k_{\underline{a}}$ is plotted in Fig. 5b for our model polyzonal TLC system.

In the case of gradient elution TLC with solvent $\underline{a}$ followed by solvent $\underline{c}$, compounds with $k_{\underline{a}}$ values less than one always migrate in the $\underline{a}$ solvent zone, and their R_F values are the same as in elution with pure $\underline{a}$ throughout separation. Compounds with $k_{\underline{a}} > 1$ migrate a certain distance across the plate as a result of the passage of solvent $\underline{a}$ across the band center (Eqn. 2). Then these bands are overtaken by solvent $\underline{c}$, compressed by the factor G_m (Eqn. 5b), and migrate a certain distance as a result of passage of $\underline{c}$ through the band center. The total distance migrated by a band with $k > 1$ can be calculated as in the preceding section on gradient elution TLC



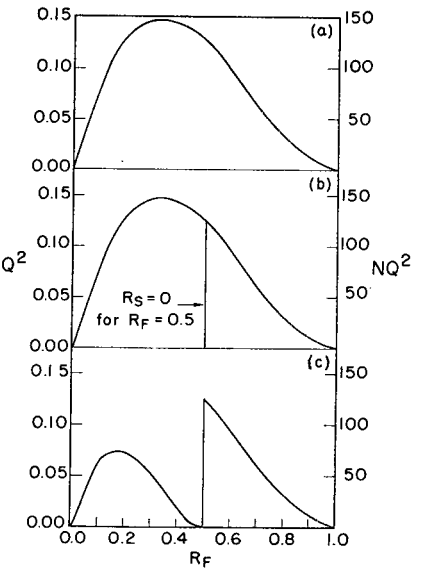


Fig. 5. R_F values *versus* k_a in normal TLC (a), polyzonal TLC (b) and gradient elution TLC (c); model system with two solvents ($k_a/k_b = 100$).

Fig. 6. Resolution in normal TLC (a), polyzonal TLC (b) and gradient elution TLC (c); same systems as in Fig. 5.

(Eqns. 2a–4). The resulting R_F *vs.* k_a plot is shown in Fig. 5c.

Now consider the evaluation of resolution in these two cases; *i.e.* NQ^2 *vs.* k_a in polyzonal and gradient elution TLC. First, we must calculate NQ^2 in single-solvent TLC as a function of k: $NQ^2 = NR_F\, k/(1 + k)^2$ (Eqn. 1a). This function is plotted *vs.* R_F in Fig. 6a and *vs.* k_a in Fig. 7a. Now in polyzonal TLC, the resolution of compounds with k_a less than one is the same as for single-solvent elution with *a* throughout. Similarly, the resolution of compounds with k_a greater than 100 is the same in polyzonal TLC as for single-solvent elution with *a*–*b* throughout, assuming no solvent demixing (see above discussion of R_F values in polyzonal TLC). For compounds with $1 \leqslant k_a \leqslant 100$, NQ^2 is equal to zero since these compounds are bunched together with R_F equal 0.5. A plot of NQ^2 *vs.* R_F is thus essentially the same for our model polyzonal TLC case (Fig. 6b) as for normal TLC elution (Fig. 6a). The corresponding plot of NQ^2 *vs.* k_a (polyzonal TLC) is shown in Fig. 7b.

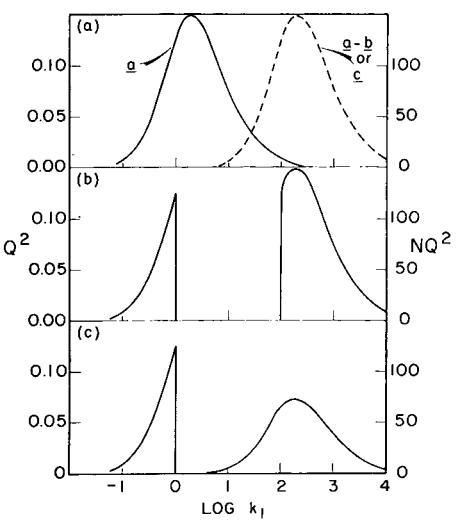
In the case of gradient elution with *a* followed by *c*, compounds with $k_a \leqslant 1$ again have the same resolution as for development with solvent *a* throughout. For compounds with $k_a > 1$, resolution can be calculated as previously described (Eqn. 5). Plots of NQ^2 *vs.* R_F and NQ^2 *vs.* k_a are shown in Figs. 6c and 7c, respectively.

A simple comparison of NQ^2 *vs.* R_F in Fig. 6 suggests that equivalent resolution is provided by normal (single-solvent) and polyzonal TLC, with somewhat inferior resolution for gradient elution TLC. This is an oversimplication, however, as shown in Fig. 7. Here we see that gradient elution TLC provides adequate resolution for the same range of k_a values (*i.e.* sample components) as provided by polyzonal TLC, plus significant resolution for components with $10 \leqslant k_a \leqslant 100$, where polyzonal TLC provides no resolution whatsoever. Thus in comparing the two techniques, we see

that gradient elution TLC provides significant resolution for a greater range of sample components, at the price of somewhat lower resolution than polyzonal TLC for some of these components. Judged from the standpoint of comparable resolution for all sample components (which is one of the main objectives of special techniques for multicomponent samples), gradient elution TLC is superior to polyzonal TLC*. We also see in Fig. 7a that two normal TLC separations (with solvents *a* and *c*) provides better overall sample resolution than either of the two other techniques.

Vapor-programmed thin-layer chromatography

This is a recently introduced technique[9,10] for carrying out TLC separations on a plate which has been initially exposed to the vapors of a series of different solvents. Because of varying deactivation by adsorbed solvent, the activity of the plate varies from one end to the other. Exposure of a plate section to a strong solvent such as methanol leads to highly deactivated adsorbent and small k values, and vice versa for weak solvents such as the hydrocarbons. In principle vapor-programmed TLC could be used in the same way as gradient-layer TLC, and the resulting theory of resolution would be the same for these two techniques (Figs. 3b and 4b would describe vapor-programmed TLC). Actually vapor-programmed TLC has been suggested[9] for the separation of difficultly separable mixtures (similar k values), rather than multicomponent mixtures of widely different k values. In pursuit of this objective, it has been suggested that the adsorbent activity should decrease in the direction of solvent flow (negative value of b), rather than increase as in normal gradient-layer TLC.



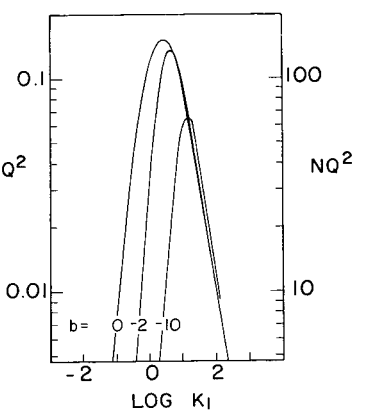


Fig. 7. Resolution in normal TLC (a), polyzonal TLC (b) and gradient elution TLC (c); same systems as in Fig. 5.

Fig. 8. Resolution in gradient-layer TLC with negative adsorbent gradients.

* However for multicomponent solvent systems where several fronts are formed and the individual fronts are not sharply defined (*i.e.* there is a continuous transition from one zone to the next over a finite part of the TLC plate), it can be shown that polyzonal TLC gradually merges into gradient elution TLC so far as relative resolution is concerned. That is, in this case gradient elution and polyzonal TLC provide comparable resolution for similar values of b.

Fig. 8 shows the calculated resolution for such negative activity gradients. We see that average resolution (NQ^2) decreases for increasingly negative b values, and the range of sample components (*i.e.* range of k values) that can be efficiently separated (large NQ^2) also decreases as b becomes more negative. Thus a negative activity gradient of this type actually works in the opposite direction to that desired. A negative activity gradient does provide greater displacement of peak centers for bands near the center of the plate, because bands tend to migrate more rapidly (relative to the solvent front), the further they progress along the plate. This gives the appearance of improved sample resolution in some cases, particularly when the bands are well resolved in the absence of an adsorbent activity gradient. As shown in Fig. 8, however, real resolution (as measured by the ability to separate closely adjacent bands in high purity) becomes poorer for negative activity gradients.

In addition to a negative adsorbent activity gradient for vapor-programmed TLC, an alternation of active and inactive adsorbent sections along the plate has also been suggested[9]. This is quite similar to the negative solvent gradient or "polarity reversal" that has been used (*e.g.* ref. 12) to provide greater displacement of closely adjacent elution peaks. The principle of the latter technique is as follows. As soon as the first of two bands leaves the column, a much weaker solvent overtakes the second band, freezing it at the end of the column. Eventually a stronger solvent is used to elute the second band, and the two bands then appear as widely separated peaks in the elution chromatogram. It must be emphasized strongly that the latter technique—and the alternation of adsorbent activity in vapor-programmed TLC—does not provide any real gain in resolution. The relative contamination of each band by the other is the same despite their differing positions in the chromatogram. The only possible advantage of this artificially enhanced peak separation is that the two bands may be more easily recovered at the end of separation, with less chance of further intermixing as a result of manipulation during recovery.

In summary, vapor-programmed TLC could be a useful alternative to gradient-layer TLC, if the solvent vapors provide decreased adsorbent activity in the direction of development. The use of negative adsorbent activity gradients and the alternation of adsorbent activity appears to work at cross purposes to improved sample resolution in the general case.

DISCUSSION

The present theoretical treatment suggests that the four TLC techniques we have examined can be arranged in an order of decreasing general performance: gradient-layer TLC (best), gradient elution TLC, polyzonal TLC and vapor-programmed TLC (worst). With suitable modification, however, vapor-programmed TLC should provide separations comparable to those by normal gradient-layer TLC. On the other hand, practical considerations make it difficult to achieve the full potential of gradient layer TLC, so that in many cases gradient elution TLC will be the preferred technique. None of these TLC techniques can compete with stepwise or gradient elution from columns in terms of speed or sample resolution, when the column procedures have been fully optimized[6,8]. Likewise none of these TLC procedures ever exceeds normal (single-solvent) TLC with respect to maximum resolution: *i.e.* $Q^2 = 0.15$ for k equal 2. As the solvent or adsorbent gradients become less steep ($b \rightarrow 0$),

the resolution of these various procedures approaches that of normal TLC. As a result the separation of multicomponent samples (large range in k values) with maximum resolution (large NQ^2) is best carried out by compositing the results of several different normal TLC separations (*e.g.* Fig. 7a—with solvents *a* and *c*—*vs.* Fig. 7b or 7c). Each of these individual TLC separations can be varied to provide optimum values of k (equal 2) for the different pairs of closely adjacent bands in the sample, so that NQ^2 for the overall separation is equal to about 150 effective plates. Sample resolution could be further improved by as much as a factor of 6 (to about 1000 effective plates NQ^2) by carrying out the individual separations with continuous development[4], but this would involve a prohibitive amount of work for a given sample when several such separations are required.

It should be emphasized that we have focused attention on effective plates (NQ^2) and ignored separation selectivity $[(k_A/k_B) - 1]$; see eqn. (1a). While there is no reason to expect that separation selectivity will be consistently better in any one of these special TLC systems (*i.e.* gradient-layer *vs.* gradient development, etc.), separation selectivity will in general not be the same. Thus it is quite possible to observe better sample resolution with a technique that provides a smaller value of NQ^2, as a result of larger differences in $[(k_A/k_B) - 1]$. In general, however, we should expect better sample resolution in those techniques where NQ^2 is predicted to be larger. The choice of gradient-layer over gradient development TLC for a given separation is therefore likely to be correct, but will not always be so.

Another point which should be stressed is that many complex samples do not require a high separation efficiency (NQ^2 value) for their satisfactory separation, because k_A/k_B is relatively large for all pairs of adjacent bands. Even relatively inefficient procedures (*e.g.* polyzonal TLC) can provide adequate separations in such cases. Therefore the ability of a given technique to provide reasonable separation of a particular multicomponent mixture is not an accurate criterion of the overall utility of that procedure for more difficult separations.

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A COMPARISON OF THE MASS DETECTOR AND GAS DENSITY DETECTOR FOR QUANTITATIVE ANALYSIS

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SUMMARY

A gas chromatograph was equipped with a gas density detector and mass detector in series. A comparison was made between the repeatability of response of the two detectors over a wide gas flow rate range, and at a fixed flow rate. The linear dynamic range, limit of detection and response time of the gas density detector were measured. The value of the gas density detector for relative composition analyses, assuming a response based on molecular weight, was assessed and compared with results obtained using the mass detector.

INTRODUCTION

The use of the mass detector for quantitative analysis has recently been demonstrated[1]. It has been shown that quantitative results can be obtained with a precision of 1%.

A gas density balance designed for use as a chromatographic detector was introduced by MARTIN AND JAMES in 1956[2]. Since only a few papers have subsequently been published on this type of detector, it is pertinent to discuss the device in detail. The gas density balance is a non-destructive detector and by passing column effluent first into this detector and then into a mass detector, a direct comparison of the performance of the two detectors is possible. The results of this work are described below.

DISCUSSION

The original Martin gas density balance was constructed from a solid copper block. Other workers[3] have constructed skeletal types of balance from copper tubing, and have compared some of the characteristics of the two models. The overall conclusions were that the original block detector gives less noise, but has a smaller

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linear dynamic range than the skeletal model. However, both detectors have a linear dynamic range greatly in excess of that required for gas chromatography.

The skeletal gas density balance is far easier to construct. Other minor modifications to the original MARTIN AND JAMES design have been proposed by several workers[3–7], but these concern mainly the construction of the detector and do not substantially alter the performance characteristics. A gas density balance for use at high temperatures has also been described[8].

The sensitivity of the detector will depend on the volume of the main conduits, the power dissipated by the heater, and the nature of the carrier gas. A gas of low thermal conductivity will increase the temperature gradient along the wire, and for this reason nitrogen is generally used as carrier gas. However, for the analysis of materials of molecular weight similar to that of nitrogen, a different gas should be used, in order to maintain reasonable sensitivity. The detector is temperature sensitive and excellent thermostatting is required. For a correctly balanced detector, the response is completely flow insensitive. The constituents of mixtures under analysis do not come into contact with the heated filament of the detector. The response of the detector is predictable from a knowledge of molecular weights. By correcting the peak areas corresponding to each of the constituents of a mixture, using the expression:

$$f = \frac{M_X}{M_X - M_C} \tag{1}$$

where M_X and M_C are the molecular weights of the constituent X, and of the carrier gas, respectively, the percentage composition of the mixture by weight, is obtained directly:

$$\%\ \text{w. of component X} = \frac{A_X f_X}{\Sigma_1^j Af} \cdot 100 \tag{2}$$

where A = peak area.

Thus no experimental calibration of the detector is required, and the response should be linear for all materials[9,10]. Clearly the gas density balance has many of the properties of an ideal detector for quantitative analysis.

Although the Martin gas density balance is not commercially available, a simplified version based on a design by NERHEIM[11] is manufactured by the Gow-Mac Instrument Company*. GUILLEMIN AND AURICOURT have published several papers[12–15] on the performance of the Gow-Mac gas density detector.

These authors set out to define the optimum operating conditions for quantitative analysis. The effect of the ratio of the reference and analytical gas flow rates on sensitivity was studied. The performance of the detector was examined using several different carrier gases, both permanent gases, and those of high molecular weight such as the halogenated alkanes. The effect of temperature on detector sensitivity was studied. The linearity of the detector was briefly examined, but no definitive study was undertaken. Results of the quantitative analyses of a number of mixtures of low boiling halogenated hydrocarbons, calculated using eqn. 2, were in good agreement with the true mixture compositions. Each mixture was analysed

* Gow-Mac Instrument Company, New Jersey.

three times, at one sample size only. Useful data may be found in a paper by WALSH AND ROSIE[16]. The effect of changing various parameters on the detector output was studied. Several two-component mixtures, and one five-component mixture were analysed, and the bias of the results varied between 1.1 and 2.2%. No information is given on the precision of the determinations. In the present work the effect of gas flow rate on detector response, the repeatability of response, and the reliability of the detector as a quantitative device is assessed.

EXPERIMENTAL

A Shandon KG 2 chromatograph was fitted with a Gow-Mac gas density detector Model 091. The gas outlet of the gas density detector was connected with a short length of $\frac{1}{8}$ in. O.D. stainless steel tubing to a mass detector, situated outside the chromatograph oven. The tube was heated resistively. A detailed description of the mass detector has previously been published[17]. The responses of the detectors were monitored with a Honeywell dual pen potentiometric recorder. The mass detector output was fed to the 10 mV channel, and the gas density detector to the 1 mV channel. Two chromatograms were thus obtained for each analysis. The chromatograms were not completely superimposed due to the finite time taken for a component to traverse the distance between the two detectors. This is illustrated in Fig. 1.

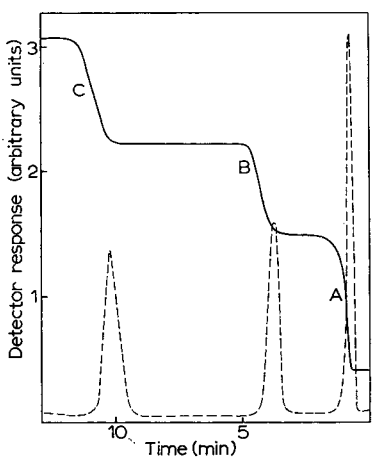


Fig. 1. Chromatogram from mass detector–gas density detector system. A = ethyl acetate; B = *n*-propyl acetate; C = *n*-butyl acetate; ———, mass detector response; - - - - -, gas density detector response.

Preparation of samples

Prior to use, all compounds were checked for impurities by conventional gas chromatographic techniques. The majority of aliphatic hydrocarbons were found to be of high purity, and minor amounts of branched alkanes in normal alkanes were removed by treatment with molecular sieve. The acetates, ketones, and aromatics, which were in general better than 98% pure, were distilled before use. In addition,

TABLE I

OPERATING CONDITIONS

Column	Ref. E
Carrier gas	Nitrogen
Injection temperature	154°
Column temperature	104° ± 0.1°
Delivery tube temperature	34° ± 1°
Nominal sample size	1.2 μl
Gas density detector	
Bridge current	150 mA
Sensitivity	× 200
Mass detector range	1 mg f.s.d.

impurities in several of these compounds were removed by preparative scale chromatography, using a Wilkens Autoprep chromatograph. The lower alcohols and ketones were dried by standing over molecular sieve, and the water content checked using a Martin gas density balance. Mixtures were prepared by weighing directly into sample bottles, which were completely filled, and stored in a refrigerator when not in use. Samples for analysis were withdrawn with a syringe through a septum fitted to the bottles.

The effect of flow rate on the relative response of the gas density detector was measured by varying the total flow rate through the detector (*i.e.* the flow rate to the mass detector) over the range 15–250 ml min^{-1}. The requirement that there must be a substantially greater flow rate through the reference arm of the detector than through the analytical arm was met over the whole flow rate range. The same three-component acetate mixture was analysed three times at each of 24 different flow rates. The conditions of analysis are given in Table I.

Column Ref. E is 4 m × 4 mm I.D. stainless steel containing 20% PEGA on 72–85 mesh Chromosorb G.

The percentage composition of the mixture was calculated at each flow rate from the chromatograms. The results from the mass detector were calculated directly from step height measurements. The gas density detector results were calculated from peak area measurements, corrected using eqn. 2. Mean percentage compositions embracing the whole flow rate range are quoted in Table II.

Comparison of the results obtained using the two detectors reveals a similar trend in bias, although the values are significantly greater on the gas density detector.

TABLE II

QUANTITATIVE RESULTS (WIDE FLOW RATE RANGE)

x_0 = true % composition; $\bar{x}$ = mean % weight of n determinations; σ = standard deviation; V = coefficient of variation (%).

Component	*Mass detector*					*Gas density detector*			
	x_0	$\bar{x}$	σ	V	*% bias*	$\bar{x}$	σ	V	*% bias*
Ethyl acetate	33.49	33.04	0.63	1.93	−1.37	32.68	2.4	7.34	−2.42
n-Propyl acetate	31.64	31.38	0.28	0.90	−0.85	31.05	1.8	5.80	−1.86
n-Butyl acetate	34.87	35.57	0.67	1.89	+1.99	36.26	2.0	5.52	+3.84
				1.57	|1.40|			6.62	|2.34|

TABLE III

QUANTITATIVE RESULTS (FIXED GAS FLOW RATE)

Component	*Mass detector*					*Gas density detector*			
	x_0	$\bar{x}$	σ	V	*% Bias*	$\bar{x}$	σ	V	*% Bias*
Ethyl acetate	33.29	33.07	0.414	1.25	−0.66	34.04	1.42	4.43	+2.25
n-Propyl acetate	30.92	30.66	0.158	0.52	−0.87	30.21	0.76	2.52	−2.33
n-Butyl acetate	35.78	36.26	0.404	1.11	+1.34	35.75	1.44	3.85	−0.08
				0.96	|0.96|			3.60	|1.55|

The standard deviations of the gas density detector results are all of the same order and are much greater than those obtained using the mass detector. Repeatability can be defined numerically in terms of the coefficient of variation. If the coefficient of variation is $n\%$ then the repeatability of 19 out of 20 results is $\pm n\%$. The repeatability of the mass detector response is $\pm$ 1.6% and the Gow-Mac gas density detector $\pm$ 6.6%. The very much higher value obtained with the latter detector may result from the difficulty of precisely assessing peak areas by peak height and width measurements. The precision of area measurements by this method was measured in a different series of experiments. The coefficient of variation was 2.9% for 31 analyses. For the mass detector the change in bias with flow rate was $5 \times 10^{-3}\%$ per ml min^{-1}. For the gas density detector the value was $1 \times 10^{-2}\%$ per ml min^{-1}, and although this value is greater than that for the mass detector, for practical purposes the relative responses of both detectors are flow independent.

A similar acetate mixture was analysed 15 times under the conditions given in Table I, at a single gas flow rate (analytical gas flow 49 ml min^{-1} and reference gas flow 77 ml min^{-1}). The results are summarised in Table III.

Comparison with Table II shows that the performance of both detectors is much improved. Coefficients of variation have been halved and the bias of the results considerably decreased. Repeatability of response of the gas density detector is $\pm$ 3.6%, whereas it is $\pm$ 1% for the mass detector.

TABLE IV

OPERATING CONDITIONS

Column	Ref. E
Carrier gas	Nitrogen
Injection temperature	154°
Column temperature	101°
Delivery tube temperature	34°
Analytical gas flow rate	45 ml min^{-1}
Reference gas flow rate	60 ml min^{-1}
Sample sizes	0.1–5 μl
Gas density detector	
Filament current	125 mA
Sensitivity	× 500 to × 50
Temperature	101°
Mass detector	
Ranges	100 μg–5 mg
Temperature	24°

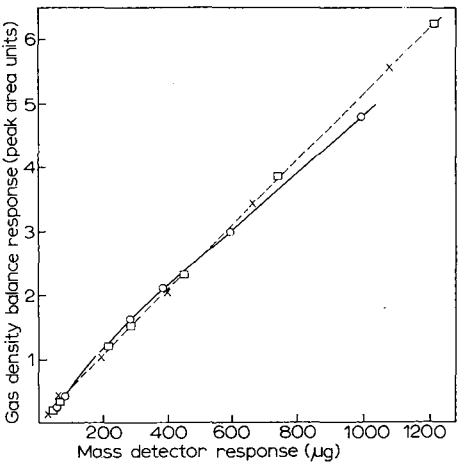
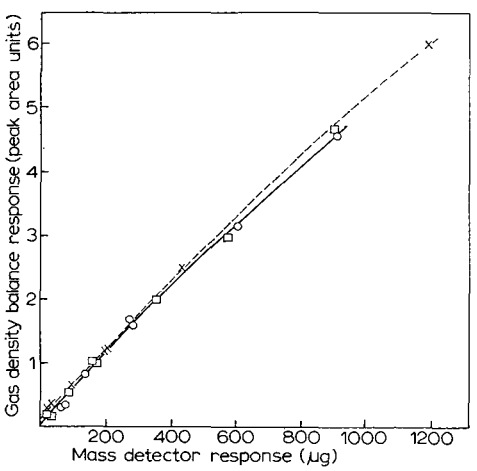


Fig. 2. Gas density detector calibration curve. ○, benzene; □, toluene; ×, ethylbenzene.

The response of the gas density detector to a variety of organic compounds, over a range of sample sizes, was measured under the conditions given in Table IV. Conditions were chosen such that the mass detector was operated within the range known to give a linear response, and the gas density detector to give optimum behaviour.

The results are expressed graphically, by plotting the weight of each compound detected by the mass detector against the (corrected) peak area obtained from the gas density detector. Peak areas were in general measured with a digital integrator, but for comparison some areas were in addition calculated from peak height and



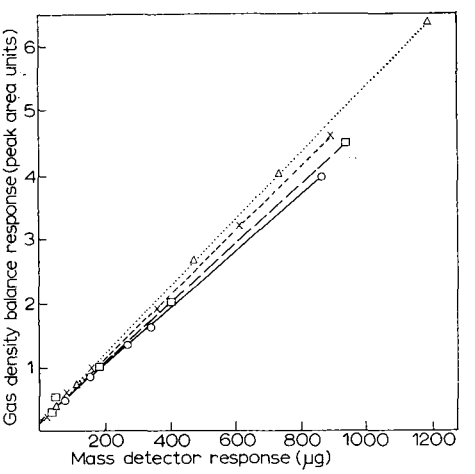
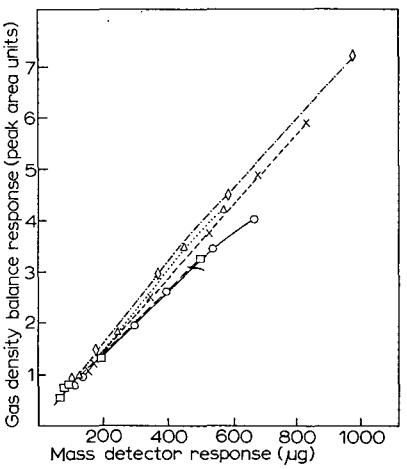


Fig. 3. Gas density detector calibration curve. ○, methyl ethyl ketone; □, methyl *n*-propyl ketone; ×, methyl *n*-butyl ketone.

Fig. 4. Gas density detector calibration curve. ○, methyl acetate; □, ethyl acetate; ×, *n*-propyl acetate; △, *n*-butyl acetate.



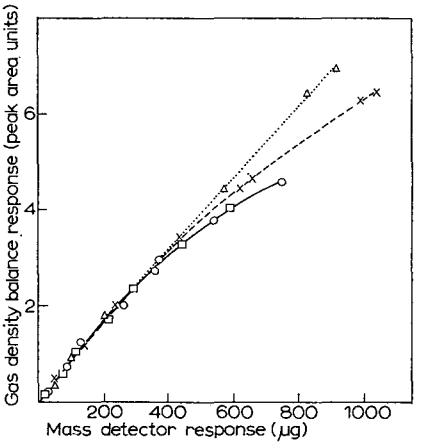
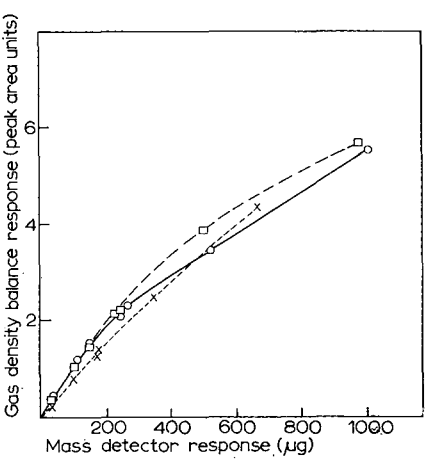


Fig. 5. Gas density detector calibration curve. ○, *n*-heptane; □, *n*-octane; ×, ethyl acetate; △, methyl ethyl ketone; ◇, benzene.

Fig. 6. Gas density detector calibration curve. ○, cyclohexane; □, *n*-octane; ×, carbon tetrachloride; △, dichloroethylene.

width measurements. These results are discussed below. Although each compound formed part of a mixture, the results are absolute in the sense that response is expressed in terms of detected weight and not percentage composition. The composition of the mixtures in no way affects the results, and all components were well resolved. Each Fig. (2–8) shows the response of the detector to each of the components of a mixture. Since corrected peak areas were used, all curves on all figures should be coincident and linear, assuming an ideal detector response. In practice it is difficult



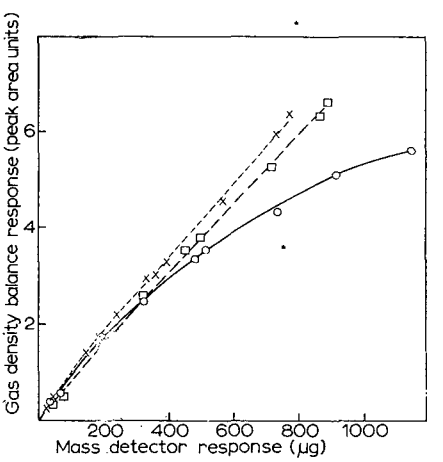


Fig. 7. Gas density detector calibration curve. ○, 2,2,4-trimethylpentane; □, *n*-octane; ×, 1-octene.

Fig. 8. Gas density detector calibration curve. ○, *n*-octane; □, butylene oxide; ×, dioxan.

to ensure complete reproducibility of operating conditions from day to day, so that it is only reasonable to expect coincidence of the curves obtained from a single mixture, *i.e.*, the curves on any one figure should be coincident, but not necessarily have the same slope as the remaining figures. The only compounds to give a linear response over the whole range investigated were toluene, ethylbenzene, and the acetates. In general all compounds gave a linear response over a fairly limited range (about 10^1). All response curves were virtually coincident at low sample sizes, but became progressively divergent as the sample size increased. 1-Octene gave a significantly lower response, attributed at least in part to the presence of partially resolved impurities. All peaks in all determinations were reasonably symmetrical, so that deviations from linearity as a result of inaccurate area measurement are unlikely. Most figures were constructed from digital integrator results, which were compared with manual peak area measurements: identical response patterns emerged. It is

TABLE V

PERCENTAGE COMPOSITION ANALYSES

Compound	*Composition*			*Response*		*Fig.*
	x_0	$\bar{x}_M$	$\bar{x}_D$	R_{DO}	R_{DM}	
n-Pentane[a]	22.50	22.30	21.62	0.96	0.97	—
n-Hexane	17.57	18.40	17.55	1.00	0.96	
n-Heptane	14.67	15.16	15.69	1.07	1.03	
n-Octane	16.45	16.46	16.81	1.02	1.02	
n-Nonane	28.82	27.69	28.32	0.99	1.02	
Benzene	41.33	41.35	40.56	0.98	0.98	2
Toluene	31.00	30.94	31.50	1.02	1.02	
Ethylbenzene	27.67	27.72	27.94	1.01	1.01	
Methyl ethyl ketone	42.20	43.05	42.63	1.01	0.99	3
Methyl *n*-propyl ketone	25.69	25.75	25.67	1.00	1.00	
Methyl *n*-butyl ketone	32.10	31.20	31.70	0.99	1.00	
Methyl acetate	23.72	21.46	20.62	0.87	0.96	4
Ethyl acetate	23.47	25.50	24.16	1.03	0.95	
n-Propyl acetate	22.82	23.03	23.58	1.03	1.02	
n-Butyl acetate	29.99	30.02	31.64	1.06	1.05	
n-Heptane	18.44	18.61	18.25	1.00	0.98	5
n-Octane	13.77	13.82	13.45	0.99	0.99	
Ethyl acetate	23.68	23.92	24.43	1.03	1.03	
Methyl ethyl ketone	16.49	16.28	16.29	0.99	1.00	
Benzene	27.63	27.37	27.59	1.00	1.00	
Cyclohexane	19.30	19.39	19.02	0.99	0.99	6
n-Octane	15.87	16.14	16.54	1.04	1.02	
Carbon tetrachloride	34.92	34.79	33.80	0.97	0.97	
Dichloroethylene	29.92	29.68	30.63	1.02	1.03	
2,2,4-Trimethylpentane	38.03	38.25	40.56	1.07	1.06	7
n-Octane	35.59	35.53	37.09	1.04	1.04	
1-Octene	26.38	26.23	22.35	0.85	0.85	
n-Octane	35.64	36.26	37.17	1.04	1.02	8
Butylene oxide	35.71	36.20	33.81	0.95	0.94	
Dioxan	28.65	27.54	29.02	1.01	1.05	

[a] Column and gas density detector at 66°.

reasonable to conclude therefore that deviations from linearity are a real effect. A sample chromatogram, which shows the analysis of a 1 μl sample of a ketone mixture, is shown in Fig. 1.

A linear gas density detector response is not a sufficient criterion for satisfactory quantitative performance. On Fig. 5 for example, all components of the mixture give a linear response to about 500 μg, but only the *n*-heptane and *n*-octane curves coincided, *i.e.* only these two materials give identical absolute response. This is not the case for the remaining constituents. To obtain satisfactory quantitative results the detector must give a response linear with concentration and equal for all materials, at all sample sizes: even with heptane the absolute response decreases with sample size (see Table VI).

For each mixture, using the linear and coincident portion of the response curves, and for a homologous series of alkanes the mean values of the percentage weights detected by the gas density detector were calculated from the corrected peak areas ($\bar{x}_D$ values). These results are given in Table V. The mean percentage weights of the components in each mixture were also calculated from the mass detector results ($\bar{x}_M$ values) and using these as a standard, the response of the gas density detector with respect to the mass detector was found. The response factor was defined as:

$$R_{DM} = \frac{\bar{x}_D}{\bar{x}_M} \tag{3}$$

All mixtures were of known composition (x_0 values), so that in this particular case, a check could be made on the response factors obtained using the mass detector as standard. The response factor was defined as:

$$R_{DO} = \frac{\bar{x}_D}{x_0} \tag{4}$$

The factors R_{DM} should be the more reliable, since losses due to evaporation of sample before injection, and column adsorption effects, are eliminated.

The relative composition analyses given by the gas density detector operated within the linear and coincident portion of the response curve, were satisfactory. The standard deviations of the response factors were 4.8×10^{-2} for R_{DO} and 4.2×10^{-2} for R_{DM}.

The effect of calculating the relative amounts of the various components of a

TABLE VI

EFFECT OF SAMPLE SIZE ON DETECTOR RESPONSE

Weight of material detected (μg)	*GDD response ($cm^2\mu g^{-1}$)*	*% heptane detected*	
		MD	*GDD*
129	0.779	18.71	18.54
143	0.768	18.93	18.35
259	0.695	18.29	17.98
400	0.657	18.65	17.20
523	0.651	18.70	16.69
670	0.597	18.77	16.31

mixture when response curves are not coincident is shown in the following example (Table VI). The absolute response of the gas density detector is the ratio of the gas density detector (GDD) and mass detector (MD) responses, *i.e.*, is area per unit weight. The table gives the response for maximum sensitivity. The true percentage weight of the component (*n*-heptane) was 18.44%.

The absolute response of the gas density detector to *n*-heptane decreased as sample size was increased. Similar effects occurred for the remaining constituents of the mixture, but to different extents. As a result the proportion of *n*-heptane detected by the gas density detector changed with sample size.

The lower limit of detection, Q_0, of the gas density detector was calculated using the YOUNG equation[18].

$$Q_0 = \frac{2R_nM}{PF} \tag{5}$$

where

R_n = noise level (mV)
M = amount of component (mmole)
P = peak area (mV min)
F = flow rate (ml min^{-1})

The noise level (R_n) of the detector was measured on the maximum sensitivity. The response to very small amounts of *n*-octane (in terms of peak area, P) was measured, and the absolute masses of the samples (in mmoles) were obtained from the mass detector. It is reasonable to assume that the response of the gas density detector is linear and predictable over a small range in the region of the limit of detection, and hence by using eqn. 5 a value for the lower limit of detection was

TABLE VII

SOME GAS DENSITY DETECTOR CHARACTERISTICS

Type of detector	*Response time (sec)*	*Limit of detection (mmole ml^{-1})*	*Compound*	*Reference*
MARTIN	3	4×10^{-6}	Pentanol	19
NERHEIM filament	8	1×10^{-6}	Butane	11
NERHEIM thermistor	8	2×10^{-7}	Butane	11
Gow-Mac filament	—	3×10^{-8}	Carbon tetrachloride	12
Pneumatic bridge		5×10^{-6}	—	20

calculated. The lower limit of detection = 6.4×10^{-7} mmole ml^{-1}. Under the conditions of the experiment this represents a mass limit of detection of 0.6 μg. The upper limit of detection exceeds that normally required for gas chromatography, and certainly exceeds the capacity of column Ref. E.

The response time of the gas density detector was measured by the SCHMAUCH procedure[19]. The value was determined at room temperature for benzene and ether, with an analytical gas flow rate of 51 ml min^{-1} and reference flow of 75 ml min^{-1}.

The response time was 11 sec, a value which can be obtained from the following equation:

$$\tau = \frac{V_D}{F} \tag{6}$$

where V_D is the detector dead volume and F the carrier gas flow rate. For the gas density detector $V_D = 8$ ml and hence at 51 ml min^{-1}, the response time is 9.5 sec.

Literature values for limits of detection and response time for some gas density detectors are given in Table VII and agree well with those quoted above.

CONCLUSIONS

For all materials examined the gas density detector gave a response close to the calculated value, over a small concentration range. Provided that the detector is used within this range excellent quantitative results can be obtained. It is however not obvious when this limit is exceeded. The linear dynamic range of the detector does not approach the dynamic range, and is species dependent. The detector is very stable and has a reasonable lower limit of detection. Response time is rather long, although satisfactory for most packed column analyses. Notwithstanding these limitations the detector is one of the most suitable commercially available devices for quantitative work. The precision and accuracy of the results obtained from the mass detector under the same conditions were significantly better.

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CHROM. 4232

CALIBRATION OF GAS CHROMATOGRAPHIC DETECTORS USING THE MASS DETECTOR

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SUMMARY

The mass detector was used to confirm the predictability of response of the Martin gas density balance, to calibrate a katharometer, and to measure limits of detection. The mass detector was operated in parallel with two destructive detectors, the flame thermocouple and flame ionisation detectors, and response curves were constructed for several different compounds.

INTRODUCTION

It has been established that the response of the mass detector is species independent, and that its response is linear over a wide operating range[1]. The detector thus offers an excellent, rapid and reliable means of calibrating other detectors[2]. It is not necessary to prepare carefully weighed-out mixtures, and the amount of material injected into the chromatograph need not be accurately known. Calibration errors arising from effects such as irreversible adsorption on the column are eliminated, and the only precaution necessary is to ensure that there is no leakage or condensation of material between the detector undergoing calibration, and the mass detector. A detector may be calibrated using either a single substance, or a number of components simultaneously. A detector may be calibrated absolutely, since the absolute adsorption efficiency of the mass detector at any flow rate is readily determined, or it may be calibrated relative to a pure standard material. The response of the Gow-Mac gas density detector toward a number of materials has been studied by this technique[3,4]. Several other detectors have been calibrated with the aid of the mass detector, and the results are presented herein. The Martin gas density balance was calibrated to confirm that response is a function of molecular weight. A katharometer was calibrated to demonstrate the value of the technique for use with a detector of completely unpredictable response. Destructive detectors must be operated in parallel with the mass detector. The flame thermocouple detector was calibrated by this means. Detectors such as the flame ionisation detector, whose sensitivities differ significantly from that of the mass detector, must also be calibrated in parallel, the major portion of the split effluent stream being fed to the less sensitive detector.

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The mass detector is of value in the determination of limits of detection, since the amount of material present in the region of the detection limits is readily obtained from the mass detector response.

EXPERIMENTAL

The Martin gas density balance

The absolute and relative responses, and linear dynamic range of the Martin gas density balance towards a number of compounds were determined in a manner analogous to that previously described for the Gow-Mac gas density detector[3]. Operating conditions are given in Table I.

TABLE I

OPERATING CONDITIONS FOR CALIBRATION OF THE MARTIN GAS DENSITY BALANCE

Apparatus	Shandon KG 2
Column	Ref. E[a]
Column temperature	101°
Carrier gas	Nitrogen
Analytical gas flow rate	50 ml min^{-1}
Reference gas flow rate	50 ml min^{-1}
Sample sizes	0.2–5 μl
Gas density balance	
filament current	1.9 A
sensitivity	$\times 10^3$, $\times 500$
Mass detector	
ranges	1–5 mg
temperature	24°

[a] Column details are given in Table XIII.

For each component of a mixture, a graph was plotted of response of the gas density balance (corrected peak area) against the mass detector response (weight adsorbed). In all cases a straight-line relationship was found, *i.e.* the Martin gas density balance gave a linear response at least over the range investigated (about 10^2). In addition the slopes of the lines were identical for all components of a mixture, they passed through the origin and the response per unit weight (the sensitivity) was identical at all sample sizes (see *e.g.*, Table III). There were, however, small variations in response per unit weight from one mixture to another, but these can be attributed to the day-to-day fluctuation of conditions (temperature, flow, rate, etc.). This is borne out by the observation that a given compound, analysed at different times, gave a slightly different response per unit weight. The results are shown in Figs. 1–6 for all the mixtures containing more than two components. The coefficient of variation of the absolute response factors ($cm^2\ \mu g^{-1}$) for 137 determinations was 5.1%.

The mean percentage weight of each component in the mixture $\bar{x}_D$ over a wide mass range was found and compared with that obtained from the mass detector ($\bar{x}_M$). These values, together with the true percentage weight (at injection), x_0, are given in Table II.

Excellent quantitative results were obtained for all samples. The standard deviations of the relative response factors were 2.2×10^{-2} for R_{DO} and 1.8×10^{-2} for

TABLE II

QUANTITATIVE ANALYSIS USING THE MARTIN GAS DENSITY BALANCE

$R_{DM} = \bar{x}_D/\bar{x}_M$; $R_{DO} = \bar{x}_D/x_o$.

Compound	*Composition*			*Response*		
	x_o	$\bar{x}_M$	$\bar{x}_D$	R_{DO}	R_{DM}	*Fig.*
Benzene	38.45	38.90	39.05	1.02	1.00	1
Toluene	33.22	33.14	33.11	1.00	1.00	
Ethylbenzene	28.33	27.96	27.84	0.98	1.00	
Methyl ethyl ketone	35.88	36.72	37.16	1.04	1.01	2
Methyl *n*-propyl ketone	38.21	38.93	38.30	1.00	0.98	
Methyl *n*-butyl ketone	25.92	24.34	24.54	0.95	1.01	
Ethyl acetate	39.33	39.71	40.34	1.03	1.02	3
n-Propyl acetate	31.32	31.18	30.54	0.98	0.98	
n-Butyl acetate	29.36	29.11	29.12	0.99	1.00	
n-Heptane[a]	22.79	23.33	23.30	1.02	1.00	4
n-Octane	15.38	15.38	15.40	1.00	1.00	
Ethyl acetate	21.54	21.00	21.76	1.01	1.04	
Methyl ethyl ketone	16.10	15.74	15.47	0.96	0.98	
Benzene	24.19	24.55	24.07	1.00	0.98	
Cyclohexane	21.17	20.57	21.07	1.00	1.02	5
n-Octane	16.65	16.17	16.71	1.00	1.03	
Carbon tetrachloride	34.54	35.34	34.94	1.01	0.99	
Dichloroethylene	27.64	27.92	27.28	0.99	0.98	
n-Octane	26.74	27.63	27.44	1.02	1.01	6
Butylene oxide	28.80	28.56	27.01	0.94	0.95	
Dioxan	44.45	43.81	45.55	1.03	1.04	
Benzene	52.51	52.31	52.02	0.99	0.99	
Toluene	47.49	47.69	47.98	1.01	1.01	
n-Butyraldehyde[b]	39.80	39.24	38.96	0.98	0.99	
Methyl ethyl ketone	60.20	60.76	61.05	1.02	1.01	
Isopropyl alcohol	40.12	42.40	42.18	1.06	1.00	
Nitromethane	59.88	57.60	57.82	0.94	1.00	
Water[c]	54.02	47.30	47.29	0.88	1.00	
Ethyl alcohol	45.98	52.70	52.71	1.12	1.00	
Ethyl alcohol[d]	53.63	54.25	53.68	1.00	0.99	
n-Propyl alcohol	46.37	47.75	46.32	1.00	1.01	
n-Propyl alcohol[d]	52.83	54.91	54.59	1.04	1.00	
n-Butyl alcohol	47.17	45.09	45.41	0.94	1.00	
n-Butyl alcohol[d]	52.95	53.42	51.74	0.98	0.97	
n-Amyl alcohol	47.05	46.58	48.26	1.02	1.03	
n-Propyl alcohol[d]	44.94	45.36	45.57	1.02	1.00	
Methyl *n*-propyl ketone	55.06	54.64	54.43	0.98	1.00	

[a] See also Table III.
[b] Column temperature, 66°.
[c] Column Ref. D at 70°.
[d] Column Ref. D at 140°.

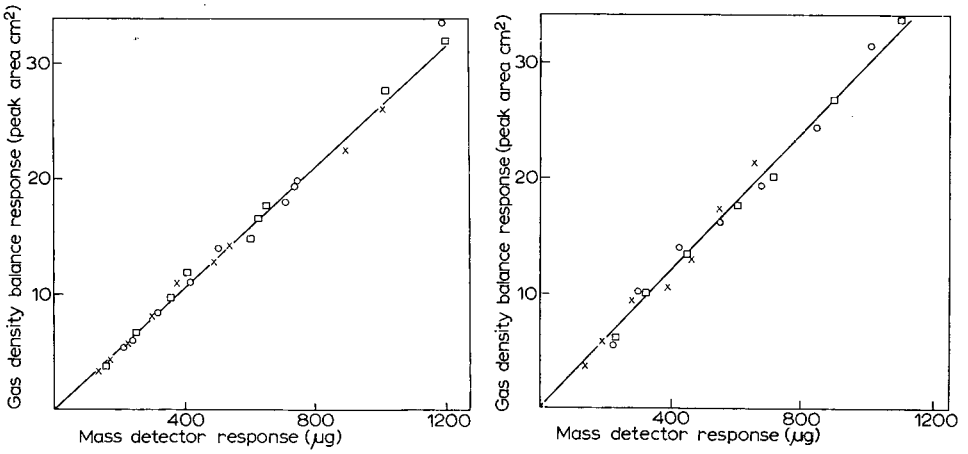


Fig. 1. Response curve for the gas density balance. ○, Benzene; □, toluene; ×, ethylbenzene.

Fig. 2. Response curve for the gas density balance. ○, Methyl ethyl ketone; □, methyl *n*-propyl ketone; ×, methyl *n*-butyl ketone.

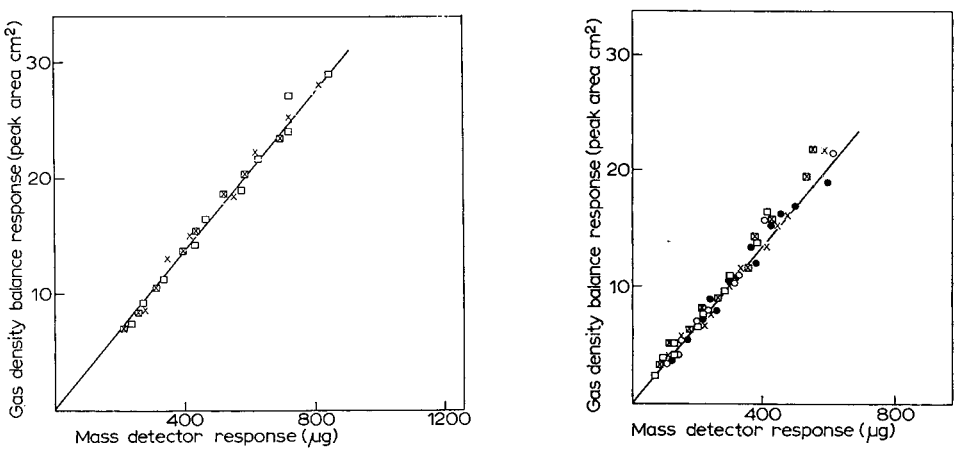
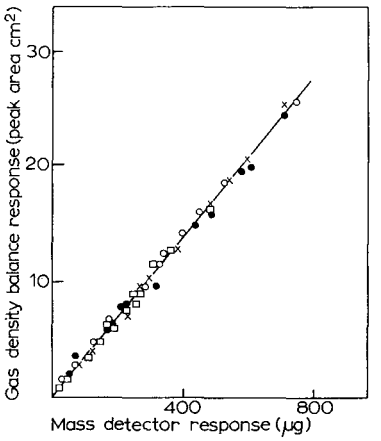


Fig. 3. Response curve for the gas density balance. ×, Ethyl acetate; □, *n*-propyl acetate; ⊠, *n*-butyl acetate.

Fig. 4. Response curve for the gas density balance. ×, *n*-Heptane; □, *n*-octane; ○, methyl ethyl ketone; ⊠, ethyl acetate; ●, benzene.

R_{DM}. Absolute response values for *n*-heptane, for a variety of sample sizes, are given in Table III.

Comparison with the Gow-Mac gas density detector results[3], obtained under similar conditions, reveals that the Martin gas density balance used is the less sensitive, by a factor of about 30. However, the absolute response of the Martin gas density balance is constant whereas the Gow-Mac detector response depends on sample size. The Martin detector will therefore give reliable relative composition data over a wide



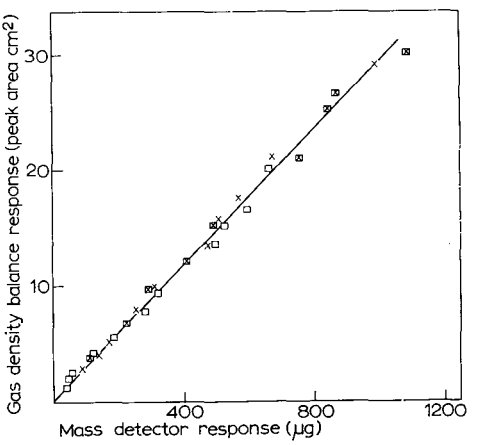


Fig. 5. Response curve for the gas density balance. ○, Cyclohexane; □, *n*-octane; ×, carbon tetrachloride; ●, dichloroethylene.

Fig. 6. Response curve for the gas density balance. ×, *n*-Octane; □, butylene oxide; ⊠, dioxan.

sample size range, but the Gow-Mac detector will only give accurate results within a limited range.

The repeatability of the relative composition results was determined over the whole sample size range used: the coefficient of variation of 165 determinations was 2.1%. The same value was obtained for the mass detector repeatability. A similar calculation for the Gow-Mac detector is meaningless, since response is concentration dependent; even the result for single sample size was significantly greater, at 3.6%. The overall bias of the Martin gas density balance results was 0.5%, *i.e.* 1.5% absolute bias. Very similar values were obtained for the mass detector bias. Bias values for the Gow-Mac detector increased as sample size increased. Bias values for a single sample size are similar to the Martin and mass detector values quoted above.

TABLE III

ABSOLUTE RESPONSE VALUES FOR *n*-HEPTANE

GDB = gas density balance; MD = mass detector. $x_0 = 22.79$.

Weight of material detected (μg)	*GDB response ($cm^2\ \mu g^{-1}$)*	*% heptane detected*	
		MD	*GDB*
124	0.0241	22.95	23.78
175	0.0251	23.20	23.88
213	0.0239	23.38	23.59
254	0.0251	23.51	21.43
300	0.0246	23.50	23.74
338	0.0249	22.69	23.26
411	0.0235	23.57	24.29
436	0.0246	23.02	22.72
479	0.0240	23.47	22.95

TABLE IV

OPERATING CONDITIONS FOR CALIBRATION OF A GOW-MAC KATHAROMETER

Apparatus	Shandon KG2
Column	Ref. E
Column temperature	101°
Carrier gas	Nitrogen
Analytical gas flow rate	51 ml min^{-1}
Reference gas flow rate	51 ml min^{-1}
Sample sizes	0.1–1 μl
Katharometer	
filament current	150 mA
sensitivity	× 500 to × 50
temperature	101°
Mass detector	
ranges	100 μg–1 mg
temperature	24°

The lower limit of detection was determined by the procedure previously described[3]. The lower limit of detection was 6.3×10^{-6} mmole ml^{-1}, representing a mass limit of detection of 8 μg. The Gow-Mac detector exceeds this value by a factor of 10, although it is more sensitive by a factor of 30. The discrepancy is a result of the lower noise level on the Martin detector. The upper limit of detection exceeds that normally required for gas chromatography.

The response time of the Martin detector, at a flow rate of 50 ml min^{-1} was 3.5 sec. The value quoted by SCHMAUCH[5] is 3 sec.

Calibration of a katharometer

A Gow-Mac katharometer type 9285D fitted with tungsten–rhenium filaments was placed in series with the mass detector. The operating conditions are given in Table IV.

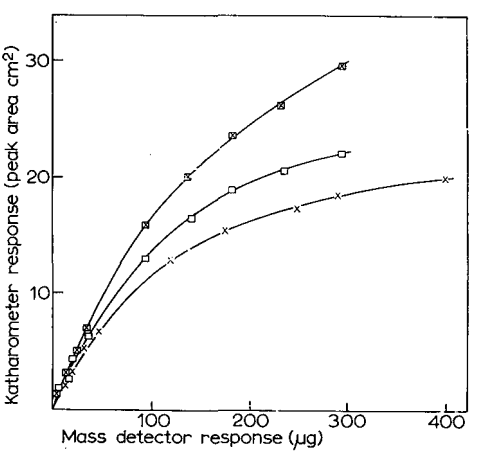


Fig. 7. Response curve for a katharometer. ×, Benzene; □, toluene; ⊠, ethylbenzene.

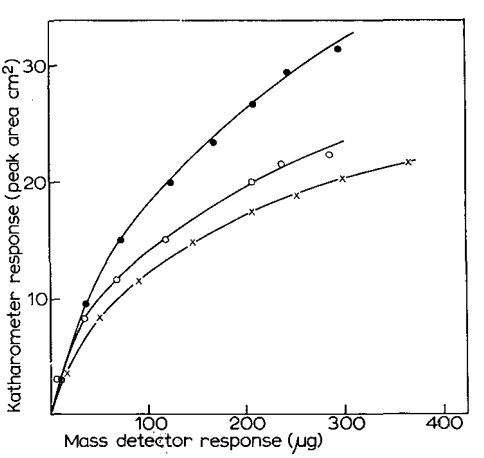


Fig. 8. Response curve for a katharometer. × Methyl ethyl ketone; ○, methyl *n*-propyl ketone; ●, methyl *n*-butyl ketone.

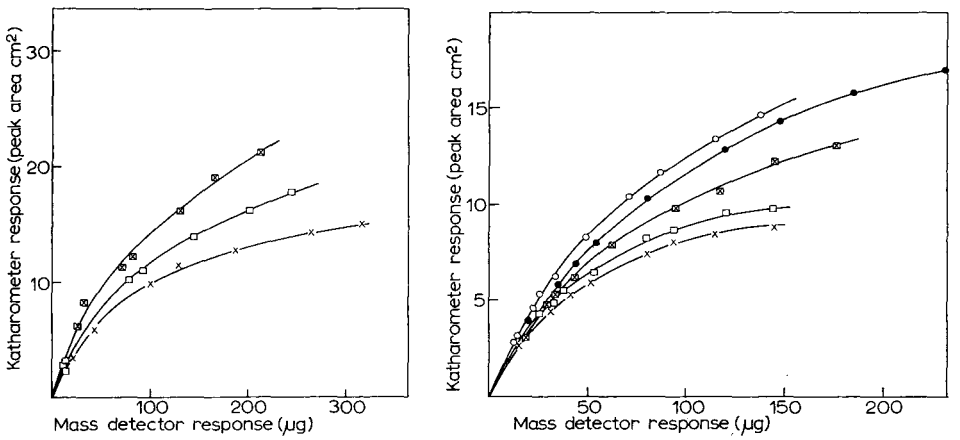


Fig. 9. Response curve for a katharometer. ×, Ethyl acetate; □, *n*-propyl acetate; ⊠, *n*-buty acetate.
Fig. 10. Response curve for a katharometer. ×, *n*-Heptane; □, *n*-octane; ⊠, ethyl acetate; ○, methyl ethyl ketone; ●, benzene.

A series of mixtures, including the same compounds listed in Table II, was analysed covering the mass range of 10–300 μg per component. Since the response of a katharometer is not predictable when nitrogen is used as carrier gas, the results are most satisfactorily expressed graphically. Response curves are shown as plots of peak area, obtained from the katharometer, against the weight of component, determined by the mass detector. Each figure shows the response of the detector to the constituents of each mixture (Figs. 7–13). All compounds, except carbon tetrachloride, gave a response of similar pattern, namely a gradual fall in sensitivity as sample size was

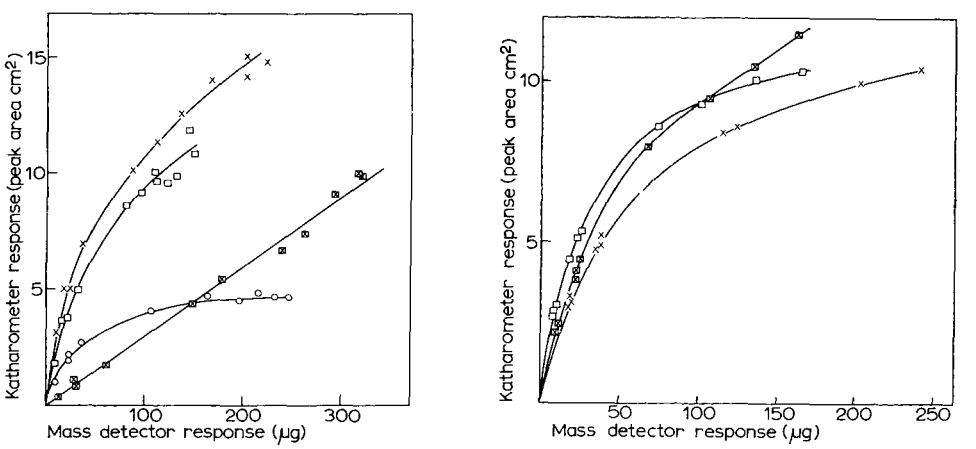


Fig. 11. Response curve for a katharometer. ×, Cyclohexane; □, *n*-octane; ⊠, carbon tetrachloride; ○, dichloroethylene.
Fig. 12. Response curve for a katharometer. ×, 2,2,4-Trimethylpentane; □, *n*-octane; ⊠, 1-octene.

TABLE V

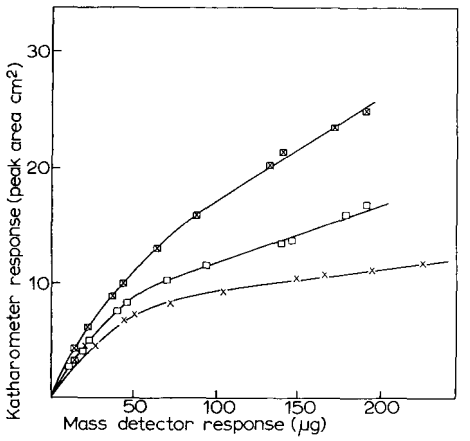
RESPONSE FACTORS OF A SERIES OF COMPOUNDS WITH RESPECT TO *n*-HEPTANE

Compound	*Area/unit weight* ($cm^2\ \mu g^{-1}$)	*Weight response w.r.t. heptane* (R_W)[a]	*Molar response w.r.t. heptane*		*Fig.*
			R	R_M	
n-Heptane	0.79	1.00	1.00	1.00	15
Benzene	1.11	1.41	1.81	1.24	7
Toluene	1.33	1.69	1.84	1.62	
Ethylbenzene	1.64	2.09	1.97	2.17	
Methyl ethyl ketone	1.28	1.63	2.27	1.38	8
Methyl *n*-propyl ketone	1.42	1.81	2.11	1.68	
Methyl *n*-butyl ketone	1.80	2.29	2.30	2.29	
Ethyl acetate	0.98	1.25	1.42	1.17	9
n-Propyl acetate	1.21	1.54	1.51	1.55	
n-Butyl acetate	1.43	1.82	1.57	1.99	
n-Heptane	0.81	1.03	1.03	1.03	10
n-Octane	0.90	1.15	1.01	1.21	
Ethyl acetate	1.01	1.29	1.46	1.21	
Methyl ethyl ketone	1.27	1.62	2.25	1.34	
Benzene	1.18	1.50	1.93	1.39	
Cyclohexane	1.07	1.36	1.62	1.26	11
n-Octane	0.96	1.22	1.07	1.30	
Carbon tetrachloride	−0.30	−0.38	−0.25	−0.62	
Dichloroethylene	0.38	0.48	0.50	0.47	
2,2,4-Trimethylpentane	0.77	0.98	0.86	1.04	12
n-Octane	0.93	1.18	1.04	1.24	
1-Octene	0.93	1.18	1.06	1.24	
n-Octane	0.91	1.16	1.02	1.20	13
Butylene oxide	1.13	1.44	2.00	1.31	
Dioxan	1.72	2.19	2.49	2.04	
Benzene	0.57	1.78	—	1.39	—
p-Cymene	1.53	3.60	—	2.04	

[a] w.r.t. = with respect to.

increased. Carbon tetrachloride was the only material to give a response linear with concentration, but for all sample sizes the response was negative. A chromatogram of the mixture containing carbon tetrachloride is shown in Fig. 14.

n-Heptane was used as a reference standard, and the response of pure *n*-heptane (99.99%) was measured over the mass range of 50 to 170 μg: the response curve is shown in Fig. 15. The response of any other compound with respect to *n*-heptane can be calculated using the appropriate calibration curve. The following response factors have been calculated and are listed in Table V: area response per unit weight of material ($cm^2\ \mu g^{-1}$), and response with respect to *n*-heptane by weight (R_W) and in molar proportions (R) for 100 μg of material. The alternative way of expressing molar



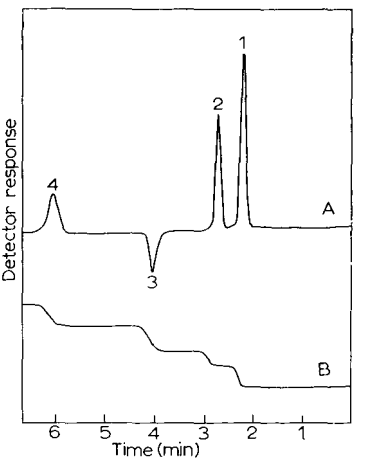


Fig. 13. Response curve for a katharometer. ×, *n*-Octane; □, butylene oxide; ⊠, dioxan.

Fig. 14. Chromatogram of a four-component mixture. A = response of katharometer; B = response of mass detector. 1 = cyclohexane; 2 = *n*-octane; 3 = carbon tetrachloride; 4 = dichloroethylene.

response is to read directly, from the response curve, the response per mole and express this value relative to one mole of the standard material (R_M values).

The weight response factors do not follow any trends. Molar response factors are about unity for simple paraffins; simple aromatics approach two, and halogenated compounds give very low values. The difference between benzene and *p*-cymene is striking.

A response curve in the region of the lower limit of detection was constructed. The limit of detection in terms of peak area was estimated from the point at which the extrapolated response curve cut the noise level of the detector: the weight, and hence the concentration of material represented by this peak area was estimated from the mass detector response. The lower limit of detection for *n*-heptane was 8×10^{-8}

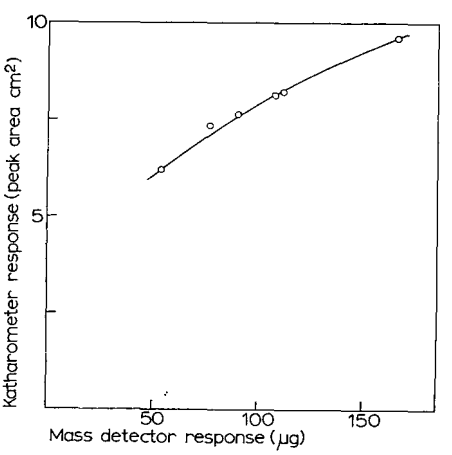


Fig. 15. Response curve for a katharometer. *n*-Heptane.

mmole ml^{-1} (0.5 μg). The upper limit of detection was estimated from Fig. 15 and was 1×10^{-4} mmole ml^{-1} (150 μg).

Although the sensitivity of the detector ($cm^2\ \mu g^{-1}$) is species and concentration dependent, it is similar to, and a little greater than that of the Gow-Mac gas density detector for many materials.

Calibration of a flame thermocouple detector

The calibration of a destructive detector can be carried out by placing the detector in parallel with the mass detector. The flame thermocouple detector has a sensitivity the same order as the mass detector, so that by splitting the column effluent in approximately equal proportions, a reasonable response will be obtained from each detector. Ideally the ratio of the amounts of material reaching the two detectors will

TABLE VI

OPERATING CONDITIONS FOR CALIBRATION OF THE FLAME THERMOCOUPLE DETECTOR

Apparatus	Pye Panchromatograph
Column	Ref. A
Column temperature	50°
Carrier gas	Nitrogen
Flow rate	
major stream	60 ml min^{-1}
minor stream	33 ml min^{-1}
Sample sizes	0.3–3.5 μl
Flame thermocouple	
thermocouple	Pt–Pt/Rh
cold junction	23°
Hydrogen flow rate	50 ml min^{-1}
Air flow rate	250 ml min^{-1}
Mass detector	
ranges	1–5 mg
temperature	23°

be in the ratio of the flow rates at the detectors. However, it may arise that the split ratio is dependent on gas viscosity and hence will be different for different materials; it may also depend on the concentration of material. Such variations in split ratio will interfere with the calibration of the detector if it is to be used subsequently in the absence of a stream splitter. Using approximately equally split streams, and small concentrations of material in the carrier gas, and for materials of a similar nature, variations in split ratio should be negligible compared with the errors resulting from peak area measurements. The linearity of a flame thermocouple detector was determined using a 2:1 splitter, and the quantitative analysis of a two-component mixture was carried out.

A Pye Panchromatograph flame ionisation detector chamber was modified to take a flame thermocouple detector. The cold junction of the detector was maintained at room temperature, and placed in a large block of expanded polystyrene to minimise random temperature fluctuations. The output of the detector was fed directly to a 10 mV potentiometric recorder, without amplification. The standing thermocouple, emf, was backed off with a simple potential divider driven by a 1.5 V battery. Operating conditions are given in Table VI.

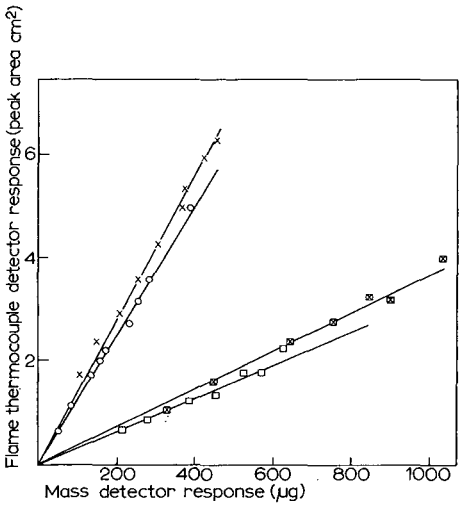


Fig. 16. Response curve for a flame thermocouple detector. Major stream to flame thermocouple detector; ○, benzene; ×, toluene. Minor stream to flame thermocouple detector; □, benzene; ⊠, toluene.

A two-component mixture was analysed several times covering the mass range of 200 μg–2 mg per component, firstly with the major stream, and then with the minor stream to the flame thermocouple detector. For each set of runs a response curve of peak area against weight detected by the mass detector was plotted (Fig. 16). In all cases the response of the flame thermocouple detector varied linearly with sample size. For the materials analysed the heats of combustion were, for practical purposes, identical. The response curves for the two materials at each split ratio should therefore coincide if response is based solely on heats of combustion. The heats of combustion, and the slopes of the response curves, obtained from Fig. 16, are given in Table VII.

Since the streams were split, the slopes of the response curves do not represent absolute sensitivities. An estimate of the split ratio is given by the ratio of the flow rates at the two detectors and is 60/33, *i.e.* 1.82:1. The split ratio can be calculated from the ratio of the weight of injected material and the weight of material detected by the mass detector. A 1 μl sample of the mixture will contain 0.30 mg of benzene. With the major stream to the mass detector 0.20 g of benzene was detected, *i.e.* the split ratio was 2:1. The same value was obtained for toluene. This method relies on injection of a known amount of sample and no loss of material within the column.

TABLE VII

FLAME THERMOCOUPLE DETECTOR RESPONSE

Compound	*Heat of combustion (kcal g^{-1})*	*Response (cm^2 μg^{-1})*	
		Major stream	*Minor stream*
Benzene	10.02	0.013	0.032
Toluene	10.15	0.014	0.036

Since the detector gives a linear response with respect to concentration, an estimate of the split ratio for each material can be obtained from the response curves. Using Fig. 16, the response of the flame thermocouple detector for each material at the 500 μg level was found, and the split ratio calculated from the differences in response when the major and minor streams were interchanged: *e.g.* for 500 μg of benzene detected by the mass detector, with the major stream to the mass detector, and with a split ratio of n:1,

$$\frac{500}{n} = 1.6\,k \tag{1}$$

where k is a proportionality constant.

For the minor stream to the mass detector:

$$500 = 6.25\,\frac{k}{n} \tag{2}$$

from which $n = 1.98$, *i.e.* the split ratio is 1.98:1. For toluene $n = 1.97$, *i.e.* the split ratio is 1.97:1. Thus the absolute sensitivity of the detector was 0.0065 $cm^2\,\mu g^{-1}$ for benzene and 0.0070 $cm^2\,\mu g^{-1}$ for toluene. The limit of detection (without amplification of the thermocouple output) was 5.9×10^{-5} mmole ml^{-1} for benzene.

The percentage composition of the mixture of benzene and toluene was calculated directly from the ratios of the peak areas, corrected for heats of combustion ($\bar{x}$ values). The composition of the mixture was also estimated using the experimentally determined response factors ($\bar{x}_E$ values). The results are given in Table VIII.

TABLE VIII

QUANTITATIVE ANALYSIS USING THE FLAME THERMOCOUPLE DETECTOR

Compound	*Mass detector*			*Flame thermocouple detector*		
	x_0	$\bar{x}$	*V (%)*	$\bar{x}$	$\bar{x}_E$	*V (%)*
Benzene	37.48	37.86	0.8	35.38	36.76	2.9
Toluene	62.52	62.14	—	64.62	63.24	—

More accurate results were obtained using the experimentally determined response factors rather than those based on heats of combustion. The coefficient of variation of the results was significantly greater than the mass detector results.

Calibration of a flame ionisation detector

The use of the mass detector for calibration purposes is not restricted to detectors of comparable sensitivity. It is possible, using a stream splitting device, to calibrate detectors of much greater sensitivity. To demonstrate this, a flame ionisation detector was calibrated. The conditions of operation are given in Table IX.

The response of the detector toward methyl propionate, toluene, and chlorobenzene was determined: the results are illustrated in Fig. 17 as plots of peak area against weight detected by the mass detector. From the slopes of the response curves, the response per unit weight for each compound was found, and hence the response

TABLE IX

OPERATING CONDITIONS FOR CALIBRATION OF A FLAME IONISATION DETECTOR

Apparatus	Pye Panchromatograph
Column	Ref. B
Column temperature	100°
Carrier gas	Nitrogen
Column flow rate	48 ml min^{-1}
Flame ionisation detector	
voltage	50 V
hydrogen flow rate	50 ml min^{-1}
air flow rate	250 ml min^{-1}
sensitivity	10^{-9}, 10^{-8}
Mass detector	
temperature	23°

relative to one component as standard calculated. These values, together with the coefficients of variation (V) of the response factors, are given in Table XI.

The response factors must be corrected for the contribution of the stream splittings if the detector is to be used in the absence of the splitter. Since the response for each material was linear, it follows that the splitting ratio remained constant over the concentration range covered, but was not necessarily the same for all the components in the mixture. The splitting ratio was determined for each compound individually, under conditions as near as possible to those used in the linearity experiment. The mass detector was connected firstly to the minor stream, and a number of injections of identical size made. The detector was then attached to the major stream and the experiment repeated. The splitting ratio was calculated from the mean value of the step heights in each experiment. The results are given in Table X.

The responses per unit weight obtained from the calibration curves (Fig. 17) were corrected using the values given in Table X, and compared with published response data[6] (Table XI).

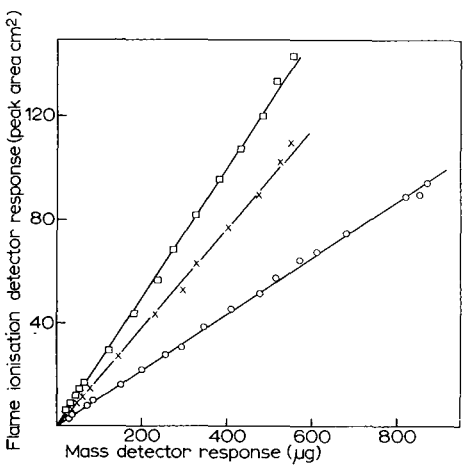


Fig. 17. Response curve for a flame ionisation detector. ○, methyl propionate; □, toluene; ×, chlorobenzene.

TABLE X

SPLITTING RATIOS IN FLAME IONISATION–MASS DETECTOR SYSTEM

Compound	*Mean detected weight (mg)*		*Ratio*
	Major stream	*Minor stream*	
Methyl propionate	6.928	0.1045	66.3:1
Toluene	6.249	0.0946	66.1:1
Chlorobenzene	8.428	0.1178	71.5:1

TABLE XI

FLAME IONISATION DETECTOR RESPONSE

Compound	*Response per unit weight*		*Relative response*	*Corrected response*	*Published response*
	($cm^2\ \mu g^{-1}$)	*V (%)*			
Methyl propionate	0.108	4.2	0.44	0.44	0.40
Toluene	0.247	3.8	1.00	1.00	1.00
Chlorobenzene	0.192	5.1	0.78	0.72	0.69

TABLE XII

QUANTITATIVE ANALYSIS USING THE FLAME IONISATION DETECTOR

Compound	x_0	$\bar{x}_M$	$\bar{x}_E$	σ	*V (%)*	*Bias*
Methyl propionate	33.51	33.05	33.09	0.81	2.45	−0.42
Toluene	30.41	30.35	30.35	0.44	1.45	−0.06
Chlorobenzene	36.08	36.60	36.56	0.66	1.81	+0.48

TABLE XIII

COLUMN DETAILS

Reference	*Stationary phase*		*Inert support*		*Length and I.D.*	*Material*
	Type	*%*				
A	Apiezon L	7.5	Chromosorb G	80–100	1.1 m × 3 mm.	Stainless steel
B	PEG 20M	15	Chromosorb G	60– 80	1.1 m × 3 mm	Stainless steel
D	PORAPAK Q	—	—	100–120	0.56 m × 3 mm	Stainless steel
E	PEGA	20	Chromosorb G	72– 85	4.0 m × 4 mm	Stainless steel

Using the response factors obtained from the calibration curves, the mean percentage composition of the mixture was calculated ($\bar{x}_E$ values) and compared with the results obtained from the mass detector ($\bar{x}_M$ values).

The coefficient of variation of the absolute response (area per unit weight) for 45 determinations was 4.4%, and the coefficient of variation of the percentage composition was 1.9%. Very similar values were obtained with the Martin gas density balance.

The specifications of the various columns used in this work are given in Table XIII.

CONCLUSIONS

The Martin gas density balance gives excellent quantitative results over a wide range of sample sizes. No deviations from linearity were observed, and all responses were predictable on a molecular weight basis. Its performance is entirely satisfactory and it may be used with confidence.

When nitrogen is used as carrier gas, a katharometer should be calibrated for all materials at all concentrations. This may be conveniently carried out using the mass detector.

A flame thermocouple detector was calibrated using the mass detector and response was shown to be linear over the concentration range investigated. The linearity of response of a flame ionisation detector toward several compounds was confirmed. Comparison of the response factors with literature values showed good agreement, even though the operating conditions were not identical.

The mass detector is a useful device for rapidly and reliably calibrating both non-destructive and destructive detectors.

ACKNOWLEDGEMENT

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RESPONSE OF THE ALKALI FLAME IONIZATION DETECTOR TO HALOGEN COMPOUNDS

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SUMMARY

The response of the alkali flame ionization detector to halogen compounds, as well as the effect of the halogen compound structure on the molar response, have been determined using various alkali metals. An increase in response, as compared with the flame ionization detector, occurs with sodium with chloro and bromo compounds and with sodium and potassium in the case of iodo compounds. The molar responses of various monobromo compounds are approximately equal. The response to the chlorobenzenes increases proportionately to the number of chlorine atoms in the molecule.

INTRODUCTION

The alkali flame ionization detector (AFID) is a detector which is selective for compounds containing phosphorus[1,2], halogens[1,2], nitrogen[3,4], arsenic[4,5], and sulphur[6]. The sensitivity of detection is dependent on a number of working parameters, *e.g.* the flow rate of the gases[1], the species of alkali cation[7,8], and the detector geometry[3]. With respect to the time stability of the detector response, the most important parameter is the way the alkali metal salt is placed in the flame; the most convenient method seems to be the use of a jet tip made of compressed salt[9]. The performance of the above type of AFID has already been described for compounds containing phosphorus[9–12], nitrogen[11–13], and sulphur[6].

The selectivity of the AFID to halogen compounds was described as early as in the first papers on this detector[1,2]. However, it would seem from the literature that the AFID response to this type of compound depends essentially on the detector design. For instance, it has been found by JANÁK AND SVOJANOVSKÝ[14] that the detector is sensitive, selectively, to halogen containing compounds when using all alkali metals; similarly, KARMEN[15] has found that the relative sensitivity towards compounds with phosphorus and chlorine varies only a very little with the use of various alkali metals. On the other hand, GIUFFRIDA *et al.*[7] found that the response

to halogen compounds may be suppressed by the use of KCl or KBr; HARTMANN[9] has even found that the response to chloro compounds may be, under certain conditions, negative. The aim of the present work was to follow in greater detail the response of an AFID, with a compacted alkali metal salt jet tip, to halogen compounds.

EXPERIMENTAL

The AFID used had a jet tip of a compressed alkali metal salt and its design has been described by us earlier[6]. This detector was built-in into a CHROM 3 Gas Chromatograph (Laboratory Equipment, N.E., Prague). A 68 cm long stainless steel column of 0.6 cm open diameter, packed with 5 wt % of polyethylene glycol-on-Sterchamol (80–100 mesh) was used for the chromatography of the model halogen compounds. The column temperature was 90°. The compounds chromatographed were dissolved in hexane and injected with a Hamilton microsyringe.

The flow rates of the carrier gas and air were 60 and 660 ml/min, respectively, the hydrogen flow rate was varied so as to obtain the required background current value.

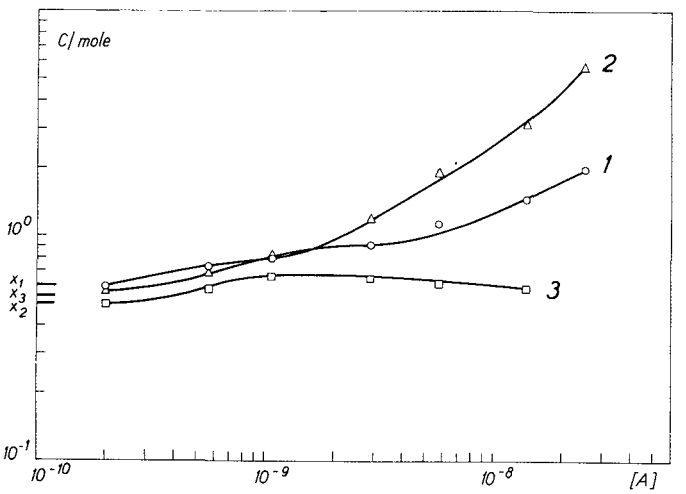

Fig. 1. Dependence of the response on the background current using a sodium salt. 1 = Chlorobenzene; 2 = bromobenzene; 3 = iodobenzene. x_1, x_2, and x_3 = FID ionization efficiencies for chlorobenzene, bromobenzene, and iodobenzene, respectively, at optimum H_2 and N_2 flow rates.

RESULTS

Figs. 1–3 show the dependence of the molar responses of chloro-, bromo-, and iodobenzene on the background current when using Na, K, and Cs salts. In case of the sodium salt (Fig. 1) an enhancement in response, as compared to the FID, comes about with all three compounds, and the response to chloro- and bromobenzene rises in dependence on the background current, while the response to iodobenzene displays a maximum in the region 1–2 $\times$ 10^{-9} A. The response increases in the sequence: I $<$ Cl $<$ Br compound.

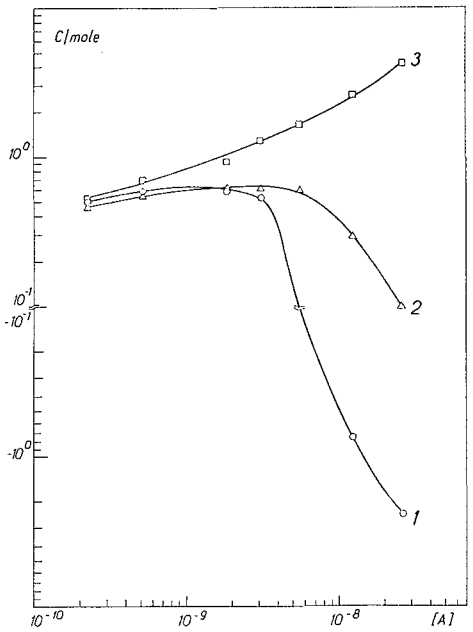

Fig. 2. Dependence of the response on the background current using a potassium salt. 1 = Chlorobenzene; 2 = bromobenzene; 3 = iodobenzene.

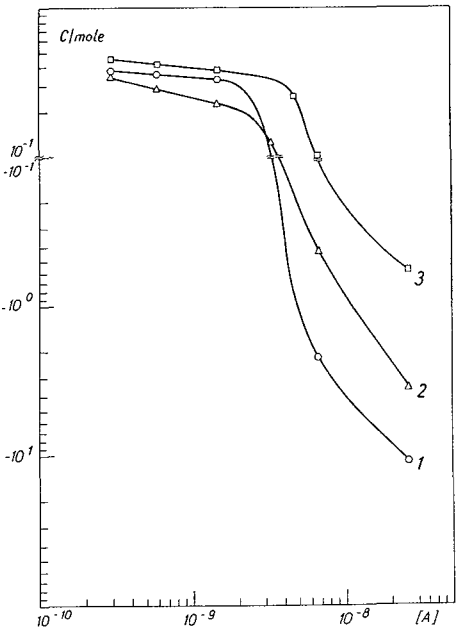

Fig. 3. Dependence of the response on the background current using a caesium salt. 1 = Chlorobenzene; 2 = bromobenzene; 3 = iodobenzene.

Unlike the sodium salt, where an increase in the ionization current occurs with all the halogen benzenes on passing them through the detector, the use of the potassium salt (Fig. 2) leads to an increase in the ionization current over the whole region of background currents investigated only with iodobenzene. With chloro- and bromobenzene a decrease in the ionization current occurs at a certain background current value, the latter being lower for chlorobenzene than for bromobenzene; the response is negative under these conditions.

With caesium salt a decrease in the ionization current occurs with all three halogen benzenes, and the background current incidental to a negative response increases in the order: Cl < Br < I.

It is apparent from Figs. 1–3 that the courses of the background current dependence of the response to the individual halogen benzenes are considerably different. In order to prove whether this course is invariable for a given halogen, the above relationships were followed for various bromo compounds (bromocyclohexane, bromotoluene, and bromocymene) employing sodium and potassium salts. These relationships, relative to that of bromocyclohexane which was used as a reference compound, are illustrated in Figs. 4 and 5. In both cases, *i.e.* both with Na and K the response to the individual bromo compounds is different within the region of lower background currents. When employing potassium salts the courses are different up to approx. 1.5×10^{-8} A, when the response to all the three bromo compounds

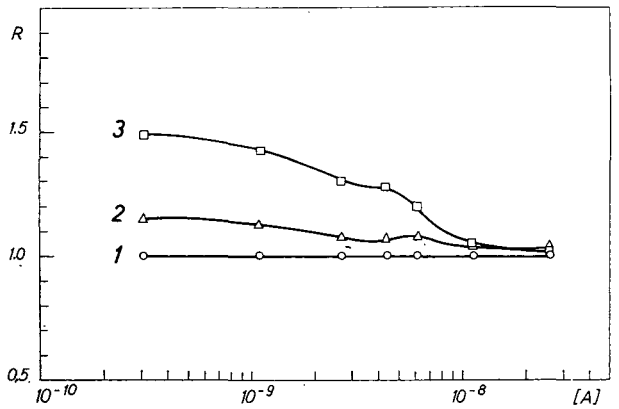

Fig. 4. Dependence of the response on the background current using a sodium salt. 1 = Bromocyclohexane; 2 = bromotoluene; 3 = bromocymene; R = relative response.

becomes negative. At higher background currents, when a decrease in the ionization current occurs, the responses are approximately the same. When a sodium salt is used the courses of the response to the above compounds are different up to about 1×10^{-8} A.

The effect of structure on the response was investigated on a number of monobromo derivatives of benzene, toluene, cyclohexane, cymene, and styrene, at a background current of 1×10^{-8} A (Table I), using a sodium salt. The ionization efficiencies of the above bromo compounds, expressed in Coulombs/mole compound, are, under the given conditions, approximately equal.

Table I also summarizes the ionization efficiencies of mono-, di-, tri-, and tetrachlorobenzene. As a single burner AFID is also sensitive to carbon compounds the response to chlorobenzene is not solely due to the presence of the chlorine atom.

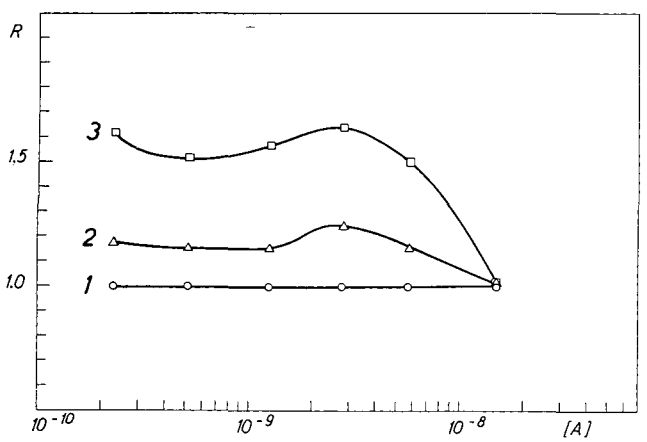

Fig. 5. Dependence of the response on the background current using a potassium salt. 1 = Bromocyclohexane; 2 = bromotoluene; 3 = bromocymene; R = relative response.

TABLE I

MOLAR RESPONSE OF SOME HALOGEN COMPOUNDS

Compound	*Ionization efficiency*	
	in Coulombs/ mole	*in Coulombs/ gram atom Cl*
Chlorobenzene	1.38	1.38
1,4-Dichlorobenzene	2.79	1.40
1,3,5-Trichlorobenzene	4.10	1.37
1,2,4,5-Tetrachlorobenzene	5.53	1.38
Bromocyclohexane	2.4	
4-Bromotoluene	2.5	
β-Bromostyrene	2.2	
Bromobenzene	2.4	
2-Bromocymene	2.5	

The contribution of the carbon skeleton (benzene) was therefore subtracted from the ionization efficiency found for the molecule of the benzene chloro derivatives. It can be seen from Table I that the ionization efficiency so obtained increases in relation to the number of chlorine atoms in the molecule of the substance under investigation, the contribution of the individual chlorine atoms being equal.

DISCUSSION

It seems from the dependence of molar response of bromo compounds on the background current that the differences in the detector responses occur within a region where the ionization efficiency of the AFID is not very different from that of the FID for the given flow rates of the gases. In the background current region, where the responses to compounds, with a given heteroatom, are considerably different (either with respect to the magnitude or direction) the molar responses are approximately equal. In the case of the bromo compounds and sodium salt the limiting current is about 1×10^{-8} A above this value the response begins to increase sharply. With potassium salts, it is about 1.5×10^{-8} A when the response becomes negative. A similar course is shown by sulphur compounds, where the differences in response are exhibited up to values of 4×10^{-10} A, when the response becomes, in the case of potassium salts, negative.

It can be inferred from the data obtained that, with this type of detector, the mechanism of response appurtenant to the AFID is overlapped by the mechanism characteristic of the FID. This effect is especially remarkable at lower background currents (lower alkali metal concentration in the flame and lower ionization of the metal) when the molar response increases with the number of carbon atoms in the molecule. At higher background currents (higher alkali metal concentration and higher degree of ionization) there is an apparent preponderance of the AFID mechanism, and, therefore, the molar responses for a given element are approximately equal.

The response of an AFID of the above type to chloro and bromo compounds can be regarded as selective in view of the enhanced response, as compared to the FID, only in the case of sodium salts, while in the case of iodo compounds it is so merely for potassium salt. In other cases the response is dependent on the cation in the sense that the background current at which a negative response occurs varies according to the atomic number of the cation used. In the case of Rb salt the response curve is similar to that with potassium.

Recently, LAKOTA AND AUE[16] reported results for halogen compounds with this type of AFID which were, in certain respects, similar to the phenomena quoted in the present paper. Using rubidium salts, they always obtained a negative response for chloro compounds, while the response to compounds containing Br, I, N, and P was positive.

It is very interesting to compare our results with the findings of HAYHURST AND SUGDEN[17] who followed the changes in the concentration of electrons in the flame in the presence of alkali metals and halogens. They found that in the case of sodium there is an increase in the concentration of electrons in the flame with all halogens (except fluorine), according to the order: $I < Cl < Br$; in the case of potassium this is so only with iodine, and in the case of caesium there is no increase in the concentration of electrons with any halogen. These findings bear a striking qualitative resemblance to the results quoted in the present work.

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CHROM. 4229

A NEW UNIVERSAL RADIOIONIZING DETECTOR FOR GAS CHROMATOGRAPHY: MODEL DNW

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SUMMARY

The basic parameters characterizing the new construction of a detector—model DNW—are presented in this paper. This detector can be used as a cross-section, argon or electron-capture type detector. Nickel-63 was used as the source of ionizing radiation in the detector. The detector can work at temperatures up to 400°.

INTRODUCTION

In addition to such classical detectors for gas chromatography as the flame-ionizing detector and the catharometer, three types of radioionizing detector are currently being used. They are designated according to the type of physical phenomenon which plays the most important role in the detectors and are: the cross-section detector, the argon detector and the electron-capture detector.

In recent years—as a result of the investigations of several research laboratories dealing with the construction of such devices—a characteristic form for the active space has been worked out for all of these detectors.

The cross-section type detector is generally characterized by the cylindrical configuration of the electrodes. The mutual distance between them is chosen in such a way that homogeneity of the electrical field inside the active space of the detector is assured[1,2].

The argon detector can be constructed in a similar manner to the cross-section type which was developed by BOTHE[3], or it can have the electrode configuration which was proposed by LOVELOCK[4]. In the latter type of the geometrical configuration, the electrical field extending between the detector electrodes is not homogeneous and causes the increased production of metastable atoms around the electrodes. In consequence, the sensitivity and dynamic range of the detector signal increase.

The electron-capture type detector, especially if it operates under d.c. supply

conditions, should be such that the configuration and form of the electrodes and active space ensure the non-homogeneity of the electrical field which is necessary for ionic recombination.

All the above-mentioned configurations of the active space can be obtained easily in the detector described in this paper. The required nature of the detector response is achieved by changing the end of the appropriate electrode, and the use of a suitable carrier gas and voltage supply.

CONSTRUCTION OF THE DETECTOR

The detector is shown in Fig. 1. It consists of a head (Fig. 1a) in which there are electrodes, 2 and 3, and insulators, 5, 6 and 7. The casing, 8, is screwed into the head. The casing is fitted with a supporting collar, 9.

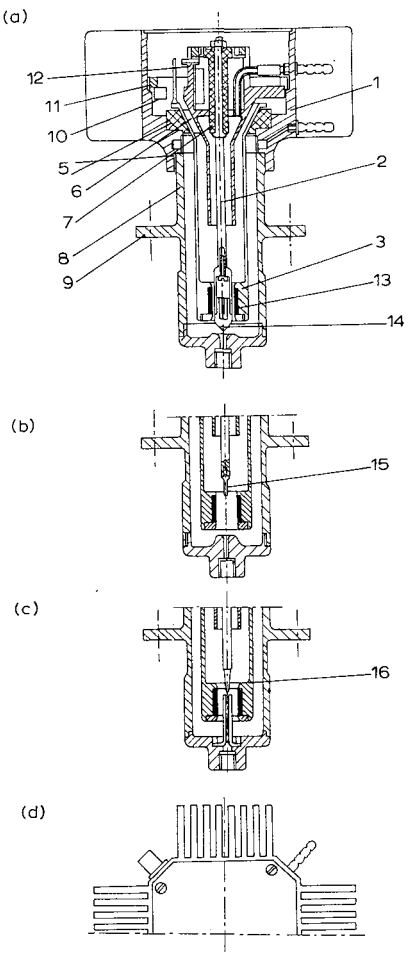

Fig. 1. Cross-section of the detector–Model DNW.

The detector is fastened on the thermostat in such a way that its active space is within the thermostat while the detector head is outside. The electrode insulators which are in the detector head are made of teflon. The detector head has cooling fins (Fig. 1d) which ensure a practically constant temperature for the insulators 5 and 6. Insulator 7 is inside the electrostatic screen, 10, which is fixed in the detector head by means of a lock-nut, 11. Insulator 7 is cooled by water which flows through a brass cylinder, 12, placed in the screen, 10. Such a cooling system for the insulators makes it possible to keep their temperature constant to that of the thermostat, inside which the active space of the detector is situated.

The geometrical configuration of the active space corresponding to a particular detector type is attained by changing the end of electrode 2. The active space of the detector is inside the radiation source, 13, which is placed on the end of electrode 3. ^{63}Ni is used as the source of beta particles in the detector model DNW.

Fig. 1a shows the geometrical configuration of the active space corresponding to a cross section-type detector. The end of electrode 2 is a cylinder, 14. The distance between its surface and the inner surface of radiation source is 1 mm.

Fig. 1b shows the geometrical configuration of the active space corresponding to an electron-capture type detector. The electrode 2 ends with the terminal 15, the size of which ensures such an non-homogeneity of the electrical field inside the detector that its sensitivity for electronegative components reaches its optimum.

The geometrical configuration of the active space of the argon detector is shown in the Fig. 1c. In this case the electrode 2 is terminated by the terminal 16 the shape of which permits the ionization of gas to commence inside the detector at a fairly low voltage supply.

A view of the detector head is shown in Fig. 1d. On the side of the detector head are the leads for the voltage supply and the electrometer, as well as the inlet and outlet for the cooling water and the outlet for the carrier gas.

THE WORKING CONDITIONS FOR THE DETECTOR

The use of an appropriate carrier gas and an adequate voltage supply are necessary factors for the instigation of any definite physical phenomena in the active space of the detector.

The cross-section type detector shown in Fig. 1a can work with hydrogen or helium as the carrier gas with a voltage supply of 100 V.

The electron-capture type detector (Fig. 1b) has been examined with nitrogen as carrier gas and with a d.c. voltage supply. With the sizes of terminal 15 (Fig. 1b) of electrode 2 used, the optimum voltage supply was 9 V.

The argon-type detector (Fig. 1c) works at voltage of 1000 to 1500 V with argon as the carrier gas.

THE DEPENDENCE OF THE IONIZATION CURRENT OF THE DETECTOR ON THE TEMPERATURE

The idea behind the construction of the detector in question was, apart from its universality, to increase detector stability at variable temperatures. This property of the detector has been achieved by placing the insulators of the electrodes 2 and 3

outside the active space of the detector, which is situated within the variable temperature zone of the thermostat. In the various designs of radioionizing detectors met so far, the insulators of the electrodes are situated near the active space of the detector, so they are subject to variable temperatures. A change of temperature of the insulators causes them to have different electrical resistances which in turn means a change in leakage currents that influence the "drift" of the zero line. Hence it would appear that by assuring a steady temperature for the insulators, the stability of the detector increases. The "drift" of the zero line then only results from changes in the physical property of the gases with respect to their different temperatures.

Figs. 2, 3 and 4 show the percentage changes of the ionization current of the detector when working as a cross-section, electron-capture and argon-type detector with varying temperatures. When water flows through the cooling cylinder 12 (Fig. 1a), the response of the detector is designated "with cooling". When the detector works without water flowing through the cylinder and is cooled only by natural air circulation we designated it "without cooling".

Fig. 2 shows the change of ionization current of the detector with configuration of the electrodes according to Fig. 1a, with helium as carrier gas. The voltage supply is 100 V. It is sufficient to secure the response of the detector within the saturation current.

In Fig. 2, curve 1 shows the response of the detector with cooling, and curve 2—without cooling. In this case, the limited efficiency of the cooling system on insulator 7 is surprising; the stability of the zero line of the detector with cooling is only about 20% better than without cooling.

In Fig. 3 the results of analogous investigations of the electron-capture type detector with the geometrical configuration of the electrode as shown in Fig. 1b are presented. The detector was examined with nitrogen as carrier gas with a d.c. voltage supply. In this case, the increase of the temperature of the active space of the detector gives a rise in the ionization current. The above dependence does not agree with the

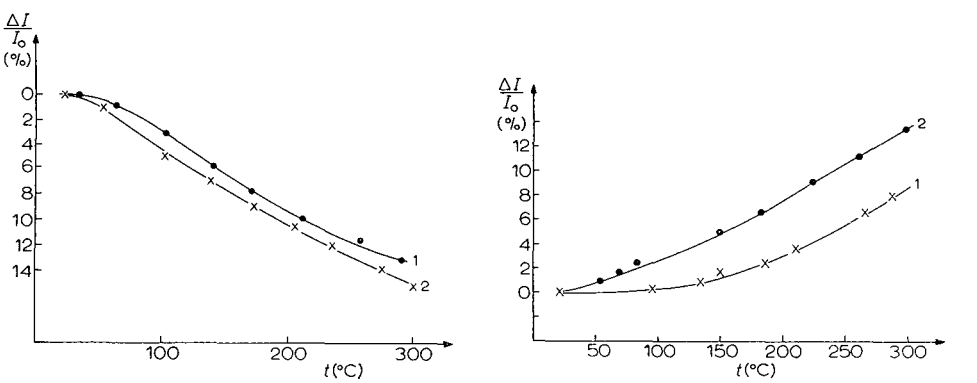

Fig. 2. Dependence of the cross-section type detector current on its temperature, carrier gas–He: V_g = 26 ml/min; voltage supply = 100 V; I = $1.6 \cdot 10^{-10}$ A. Curve 1 = with cooling; curve 2 = without cooling.

Fig. 3. Dependence of the electron-capture type detector current on its temperature; carrier gas–N_2: V_g = 50 ml/min; voltage supply = 9 V; I = $2 \cdot 10^{-9}$ A. Curve 1 = with cooling; curve 2 = without cooling.

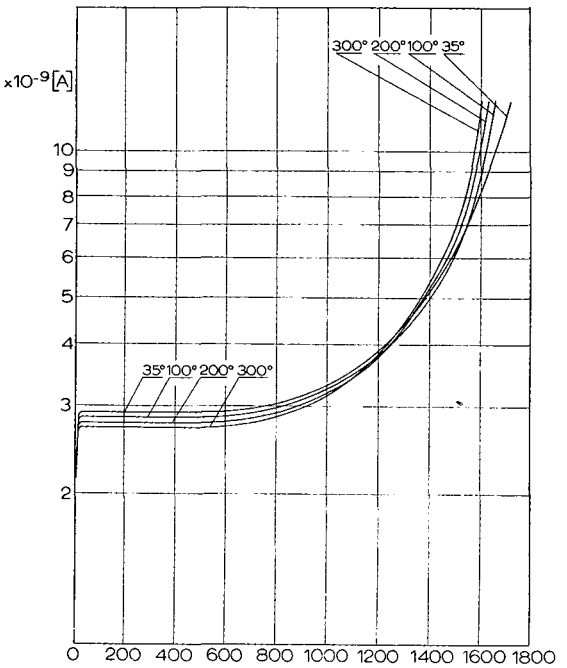

Fig. 4. Current–voltage characteristics of the argon-type detector at different voltages supplies; carrier gas = argon; V_g = 50 ml/min.

expected one and is rather difficult to explain. In Fig. 3, curve 1 shows the response of the detector with cooling, and curve 2—without cooling. As is seen the effectiveness of the cooling system is better than with the cross-section type detector. When the detector is operated with cooling, its drift zero line is smaller by about 50% than without cooling.

Different results have been obtained for the argon-type detector (as in Fig. 1c). Fig. 4 shows the current–voltage characteristics of this detector which works with argon as carrier gas, different temperatures of the active space being used.

For a voltage supply up to 600 V, an increase of the temperature in the detector results in the expected decrease of the saturation of the ionization current.

With higher voltage supply, gas amplification occurs in the detector. The value of the gas amplification coefficient depends exponentially upon the temperature. Thus, enhancement of the detector temperature causes an increase in its ionization current. It can be seen in Fig. 4 that these characteristics overlap one another in the voltage supply range of 1000 to 1400 V.

The comparison of the relative changes in the ionizing current for the detector with and without cooling is shown in Fig. 5. When the detector is supplied with 100 V and argon is used as carrier gas, the effectiveness of the additional cooling is better than when the detector works under the same conditions with helium as carrier gas. In this case, the stability of zero line is better by about 55% when the detector is cooled (curve 2, Fig. 5).

For a voltage supply of 1100 V the drift of the zero line of the detector with

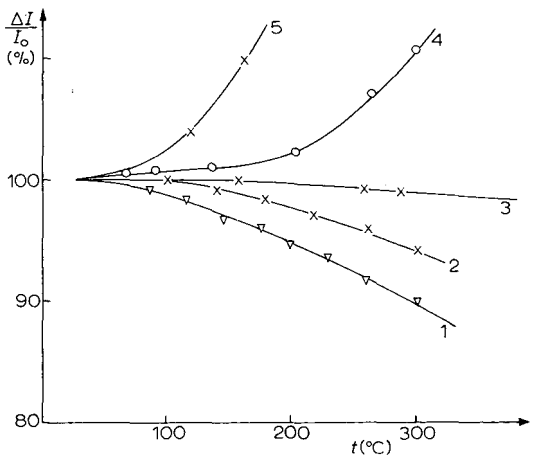

Fig. 5. Dependence of the argon-type detector current on its temperature; carrier gas–argon; V_g = 50 ml/min. Curve 1 = voltage supply 100 V with cooling; curve 2 = 100 V without cooling; curve 3 = 1100 V with cooling; curve 4 = 1500 V with cooling; curve 5 = 1500 V without cooling.

cooling—according to Fig. 4—is smaller; and at 300° amounts to 2% (curve 3, Fig. 5).

When the argon detector operates at a higher voltage, the effectiveness of the cooling system is the highest. Curve 4 (Fig. 5) is with respect to the detector with a voltage up to 1500 V with cooling, and curve 5 is without cooling.

CONCLUSIONS

After analysing the results presented in Figs. 2–5 with regard to the operation of the DNW Model detector, one can say that the drift of the zero line of the detector is connected with the flow of current through the insulators and depends upon the different properties of the gases at variable temperatures. The value of the leakage current depends upon the voltage at which the detector works and on the temperature of its insulators. It has been observed that the value of this current also depends upon the kind of gas flowing through the detector.

On the basis of the results presented it can be stated that the detector—Model DNW has very good thermal stability, particularly when it is operated with additional cooling.

Another practical advantage of the present detector is the possibility of working at up to 400° and of using three types of detectors in one *viz.* the cross-section type, electron-capture type and argon-type detector.

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CHROM. 4225

THE IDENTIFICATION OF SESQUITERPENE HYDROCARBONS FROM GAS–LIQUID CHROMATOGRAPHY RETENTION DATA

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SUMMARY

The identification of sesquiterpenes by gas–liquid chromatography is evaluated. Retention indices (obtained using sesquiterpene standards rather than *n*-alkanes) were found to be the most reproducible form of GLC data. Retention data for fifty-five sesquiterpenes and a number of saturated hydrocarbons obtained by hydrogenation of sesquiterpenes are reported.

INTRODUCTION

Until recently the study of the sesquiterpenes from natural sources has been plagued by separation problems since most plants produce complicated mixtures of very similar compounds. With the increased use of GLC[1–3] and adsorption chromatography on selective phases (such as $AgNO_3$–silica)[3–6] many sesquiterpenes of unquestioned purity have been isolated. However, these unsaturated compounds generally are quite unstable and thus it is virtually impossible to keep large numbers of authentic samples for GLC comparison.

Among the goals of our research are biogenetic studies using radiolabeled sesquiterpene precursors in whole plants and plant extracts. In connection with these studies we required analytical techniques which would allow us to identify the components of gross mixtures available in only small amounts (10 μg–10 mg). We felt that GLC on a number of different columns (with appropriate standardization procedures) should serve to identify the major sesquiterpene hydrocarbons in such mixtures.

At present, the less numerous, and more thoroughly studied, monoterpene hydrocarbons can be identified unambiguously by GLC on one or two columns (*e.g.* ref. 7). The retention data from such studies have generally been given in KOVATS' indices or as retention times relative to a standard terpene. We felt that the more numerous sesquiterpenes** could be identified in a similar manner if retention data were obtained on a sufficient number of distinct selective phases.

* Undergraduate research participant, University of Washington, 1968–1969.

** At present at least a hundred distinct sesquiterpene hydrocarbons have been characterized. However, this probably accounts for only one third of the possible structures. Thus the retention data for a component of a mixture must also be precise (and dependable) enough to indicate that a new substance has been isolated.

METHODS AND MATERIALS

Materials

The sesquiterpenes were either obtained through the generosity of other workers in the field or by isolation from the appropriate essential oils. The identity and purity of these samples were verified through IR and NMR spectroscopy.

H. U. DAENIKER (Givaudan Corp.) supplied α-cedrene, β-cedrene, valencene, and thujopsene. G. OURISSON (Institut de Chimie, Strasbourg, France) supplied longifolene, cyperene, caryophyllene, α-gurjunene, and calarene. V. HEROUT (Institute of Organic Chemistry and Biochemistry, Czech. Acad. of Science) supplied $\beta(\gamma)$-bisabolene, cyperene, γ-muurolene, ε-muurolene, γ-cadinene, β-bourbonene, humulene, the santalenes, and γ-amorphene. S. DEV (National Chemical Laboratory, Poona-8, India) supplied α-himachalene, β-himachalene, α-copaene, and β-selinene. L. WESTFELT (Swedish Forest Products Research Laboratory, 114 86 Stockholm, Sweden) supplied longicyclene, α-muurolene, and γ-muurolene. J. E. MCMURRY (Univ. of California, Santa Cruz) supplied cyclosativene, sativene, and isosativene. Y. HIROSE AND Y. OHTA (The Institute of Food Chemistry, Osaka, Japan) supplied α-ylangene, δ-cadinene, α-amorphene, and α-muurolene.

δ-Selinene was obtained from β-selinene by treatment with formic acid. α-Curcumene was isolated from the oil of *Curcuma aromatica*. α-Copaene, α-curcumene, longifolene, β-farnesene, β-curcumene, γ-curcumene, γ-cadinene, and δ-cadinene were isolated from the needle oil of *Chamaecyparis nootkatensis*[8]. β-Selinene and α-selinene were isolated from oil of celery. α-Zingaberene was isolated from oil of ginger. α-Muurolene and δ-cadinene were isolated from rectified cade oil. Humulene and caryophyllene were isolated from wild ginger oil. Zizaene, cyclocopacamphene, β-vetivenene, 4βH-nootkata-1(10),7(11),8-triene, and the antipodes of 5αH-selina-4(14),7-diene, and 5αH-selina-4(14),7(11)-diene were isolated from oil of vetiver and this work will be the subject of another communication.

Methods

The analyses were performed on a F & M Scientific Series 700 laboratory chromatograph (Hewlett-Packard) equipped with WX filaments. The columns used were 16–50 ft. × 0.125 in. The stationary phase loading was 0.5–3% on silanized Chromosorb G. The columns had between 300–800 theoretical plates per foot under normal operating conditions (80–200°, 15–40 cc/min flow of helium).

The sesquiterpenes and *n*-alkane standards were introduced as 2–10% solutions in *n*-decane. The injection volumes were 0.1–0.6 μl, delivered with a 1 μl Hamilton syringe. KOVATS' indices were calculated using *n* (even) *n*-alkanes or by the method given below. The odd *n*-alkanes generally lie on a different line than the even ones on a plot of *n vs.* log t_R'. KOVATS' indices[9] were calculated only when the peaks for the unknown and co-injected standards corresponded to those obtained from 0.02–0.2 μl of a 5% solution of the pure substances. Careful studies indicated that overloading effects (skewing to longer retention time, peak broadening, etc.) could not be detected until the injection volume was 0.9 μl of a 5% solution. Thus all measurements were obtained from traces on which all of the important peaks were Gaussian and far below maximum loading.

The method of calculating KOVATS' indices (using two sesquiterpenes as

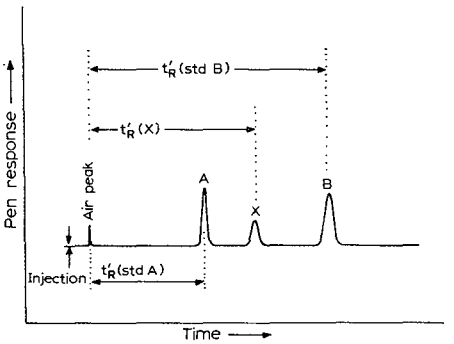


Fig. 1. Schematic GLC trace.

standards, A and B) and relative retentions is shown below, together with an illustrative trace (Fig. 1):

$$RR_X \text{ (rel. to A)} = \frac{t'_R \text{ (X)}}{t'_R \text{ (std A)}}$$

$$I_X = I_{\text{std A}} + \left(\frac{\log t'_R \text{ (X)} - \log t'_R \text{ (std A)}}{\log t'_R \text{ (std B)} - \log t'_R \text{ (std A)}}\right)(I_{\text{std B}} - I_{\text{std A}})$$

RESULTS AND DISCUSSION

The first problem was to determine the most reproducible form of retention data. Preliminary studies indicated that relative retention times (from $RR = 0.4$–2.0) could be reproduced ($\pm$ 0.8%) on a single column as long as the temperature was kept with a 5° range and the flow rates were controlled to $\pm$ 10%. However, relative retentions could not be reproduced as well on other columns containing the same stationary phase. KOVATS' indices were more reproducible from column to column but varied significantly with temperature (see Table I). We found that $\Delta I/\Delta T \sim +1.1$ for sesquiterpenes on Carbowax, and that $\Delta I/\Delta T$ increased on polar columns and was somewhat less on less selective non-polar phases. In all cases, the KOVATS'

TABLE I

KOVATS' INDICES OF SELECTED SESQUITERPENES ON CARBOWAX 20 M

Compound	*I*		*ΔI*
	132°	*205°*	
Longicyclene	1518.8	1598.5	80
α-Copaene	1521.5	1593	71.5
α-Cedrene	1597.5	1689	91.5
Longifolene	1600.0	1697	97
Caryophyllene	1618.5	1695.5	77
Humulene	1681.5	1765	83.5
Valencene	1725.5	1801	75.5
α-Muurolene	1726	1792	66
γ-Cadinene	1762	1835.5	73.5

indices increased with increasing temperature. Under normal laboratory conditions the oven temperature can be reproduced to $\pm 3°$ and thus KOVATS' indices could be reproduced to only $\pm$ 3.5 units (out of 1400–1900 units) with all other sources of error discounted.

The temperature dependence variation in KOVATS' indices[1] within the sesquiterpenes is much less ($\Delta I/\Delta T = +$ 1.1 $\pm$ 0.25 for the entire group) and thus KOVATS' indices can be reproduced to $\pm$ 1 unit by using sesquiterpenes as standards rather than using even *n*-alkanes as is usually done. Our usual procedure consisted of determining (in the usual manner[9]) the KOVATS' indices of a number of sesquiterpenes on a single phase on one day so as to minimize temperature variation. From then on, the indices of additional sesquiterpenes were determined using sesquiterpenes as standards (see *Methods*). We generally used α-copaene–α-cedrene–humulene–γ-cadinene mixtures for standardization. The indices for sesquiterpenes on seven different stationary phases are collected in Table II. In addition, approximate indices can be calculated for other sesquiterpenes from GLC traces in the literature if the traces also show: (1) the air peak and peaks due to two sesquiterpenes for which standardized indices are available; or (2) peaks due to three sesquiterpenes for which standardized indices are known. Approximate KOVATS' indices obtained in this way are included in Table II, and are indicated by an asterisk. Chromatographic data from refs. 1–3 were used for these calculations. A trace of the hydrocarbon portion of the oil of *Mentha piperita* on Apiezon L[3] was particularly informative. The trace included peaks for ylangene, humulene, and γ-cadinene which were used for standardization. The indices calculated for β-bourbonene, caryophyllene, γ-muurolene, α-muurolene, and δ-cadinene (also appearing in this oil) were within $\pm$ 3 units (average) of the values obtained by us.

Examination of the data presented in Table II quickly shows that all of these sesquiterpenes can be distinguished by the use of only two to three different phases. Thus other workers should be able to confirm the identity of these substances by co-injection with two identified sesquiterpenes appearing in Table II. The types of selectivity displayed by various stationary phases will be discussed in detail in a later communication. However, some correlations deserve comment at this point. In every case except the himachalenes the *exo*-methylene isomers are eluted after the corresponding trisubstituted olefins. Comparison of the indices for α-curcumene and β-curcumene (or δ-cadinene) reveals an increasing affinity (and therefore retention) for aromatics in going from Apiezon L to Carbowax and particularly DEGS. There are many other cases of position reversals not only on changing stationary phase but even with temperature changes on a single phase (see longicyclene–α-copaene, longifolene–caryophyllene–calarene, thujopsene–santalene, valencene–β-bisabolene–α-muurolene all on Carbowax 20M).

In the structure elucidation of sesquiterpenes it is a common practice to identify the carbon skeleton of a new sesquiterpene by hydrogenation to the fully saturated ring system. The saturated hydrocarbons are usually identified from their physical properties and by comparison of thick-film IR spectra with the published standards[11]. However, this method is not foolproof. The IR spectra of saturated hydrocarbons are particularly devoid of useful detail for such comparisons and these substances have very low rotatory powers. In addition, the hydrogenations involved usually introduce new asymmetric centers and the resulting products are mixtures of dia-

TABLE II

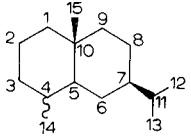
MODIFIED KOVATS' INDICES OF SESQUITERPENES

The values for the sesquiterpenes indicated by an asterisk are approximate only, since they are obtained from traces in the literature[1–3]. Values in parentheses have been obtained from a single trace only.

		Apiezon L 155°	*SF-96* 170°	*SE-30 (UC-W98)* 130°	*DC-710*[d] 165°	*QF-1* 132°	*Carbowax 20 M* 132°	*Carbowax 20 M* 165°	*Carbowax 20 M* 205°	*DEGS* 160°
1	Cubebene*	1368								
2	α-Longipinene*			1359.5						
3	α-Ylangene	1401.5	1396.1		1454.5			1538.5		1653
4	β-Elemene*	1410								
5	α-Bourbonene*	1410								
6	α-Copaene	1410.2	1400.5	1378.5	1459	1447	1521.5	1551.3	1593	1665
7	Cyclosativene	1411.9	1399.7					1549		(1684)[c]
8	Longicyclene	1417.1	1409.1	1371	1454	1465	1518.8	1554	1598.5	1684
9	Cyclocopacamphene	1417.8				1467.2		1555.4		1685.6
10	β-Bourbonene	1418.3	1411.7	1386	1477.3	1477.5	1547	1586.5	1618	1714
11	β-Farnesene	1429.2				1509		1668		1818.5
12	Sativene	1434.7	1420.7					1594.5		(1738)[c]
13	Cyperene	1446.6	1432.5	1398	1501	1493	1562	1606	1650	1736.5
14	α-Gurjunene		1435.2	1413	1500.5	1471	1558	1591	1633	1712.5
15	Caryophyllene	1451.7	1445.3	1417.5	1523	(1587)	1618.5	1655.5	1695.5	1835.5
16	Longifolene	1464.0	1440.2	1404	1517.5	1520	1600	1643	1697	1802.5
17	Isosativene	1464.4	1440.9					1639		(1797)[c]
18	Calarene	1466.0	1459.7	1435	1535.5	1513	1618	1655.5	1700	1806
19	β-Ylangene*			~1417.5						
20	β-Copaene*			~1422.5						
21	α-Cedrene	1473.4	1445.0	~1414	1516	1518	1597.5	1640	1689	1788.5
22	Thujopsene	1476.1	1458.3	1430.5	1542.3	1540	1643	1684.2	1732	1858.5
23	Aromadendrene*	~1477								
24	α-Maaliene*	~1477								
25	γ-Curcumene	1481.9				1532.5				
26	β-Cedrene	1482.4	1454.8	1421	1533.5	1539.5	1624.5	1670	1714	1834.5
27	α-Curcumene	1483	1480.4	(1475)	1589	1557.5		1787.5	(1814)	1992.5
28	ε-Muurolene	1484.8	(1474.0)	(1445)	(1561.5)	1552.5	(1675.5)	1713.8	(1759.5)	1893.5
29	Humulene	1487.2	1476.6	1446.8	1561.5	1583.5	1681	1719	1765	1929.5
30	Santalene(minor)		1459.5	1441	1535	1522	1644.5	1671	1702	1830
31	Santalene (major)		1470.5	1454	1548	1533.5	1658	1683	1714.5	1843.5
32	Selina-4(14), 7-diene[a]	1491.9	1475.7			1542		1694		1852.5
33	δ-Selinene[a]	1504.5						1728.5		
34	γ-Muurolene	1505.7				1545		1725		1889
35	γ-Amorphene	1506.4				1544.5		1724		1896.5

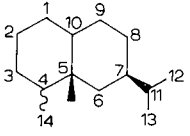
36	α-Himachalene	1508.0		1444	1561.5	1533.5	1662.5	1704.5	1755	1870
37	α-Amorphene	1509.5	1491.7		1582.5	1535		1724.5		1897
38	Zizaene	1511.6	1481.8			1562		1706.3		1879.5
39	β-Bisabolene	1512.9	1510.3	1496.5	1592.5	1548	1726	1745.5	1772	1909.5
40	β-Curcumene	1513.6	1510.4			1547.5		1756		1922.5
41	α-Zingaberene		(1479.6)		1583.5			1738	(1762)	
42	Valencene[b]	1525.6	1508.8	1457	~1600	1581	1725.5	1760	1801	1948
43	β-Himachalene	1529.7		1491	1607.5	1578	1717	1752.5	1799	
44	β-Selinene[a]	1530.2	1506.3		1595	1597.5		1766.5	1815.5	1958
45	γ-Bisabolene	1531.3	(1505)		1601			1765.5	1815	
46	α-Muurolene	1531.3	1507.7	1495	~1600	1558.5	1726	1752.5	1792	1927.5
47	α-Pyrovetivene[b]	1533.9	1521.5					1817		2026.0
48	α-Selinene[a]*	1534.5								
49	ε-Bulgarene*	1538								
50	δ-Cadinene	1546.4	(1526.4)	~1504[d]*	1628.5			1784	1818	1959
51	Calamenene*	~1550								
52	γ-Cadinene	1554.9	1523.5	1506.5	1623.5	1587	1762	1792.3	1835.5	1978.5
53	Selina-4(14), 7(11)-diene[a]	1572.0				1611.5		1816.3		(2018)[c]
54	Selina-3,7(11)-diene[a]*	1580								
55	β-Vetivenene[b]	1583.0	1563.3					1885		2111

[a] Selinane (eudesmane) hydrocarbons are designated using THEOBALD's system of nomenclature based on the numbering shown[10]:

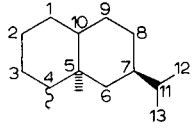


α-selinene [= 3,11-diene], β-selinene [= 4(14), 11-diene] and δ-selinene [= 4,6-diene].

[b] Eremophilane and nootkatane sesquiterpenes are named based on the following structure projections and numbering systems.



Eremophilane



Nootkatane

Valencene = 4β*H*-nootkata-1(10),11-diene; β-vetivenene = 4β*H*-nootkata-1,7(11),9-triene; α-pyrovetivene = 4β*H*-nootkata-1(10),7(11),8-triene.

[c] Extrapolated from measurement at 175° on the DEGS column: α-copaene (1692.1), cyclosativene (1711.8), sativene (1766.7), isosativene (1827.9), selina-4(14),7-diene (1888.3), zizaene (1923.4), β-bisabolene (1935.0), δ-cadinene (1990.4), γ-bisabolene (1997.6), γ-cadinene (2015.0), α-curcumene (2018.6), selina-4(14),7(11)-diene (2049), and α-pyrovetivene (2074.0).

[d] A 15° increase leads to KOVATS' indices from 7.5–15 units higher than those listed. $(\Delta I/\Delta T)_{ave} = 0.75$.

TABLE III

KOVATS' INDICES OF HYDROGENATION PRODUCTS OF SESQUITERPENES

Hydrogenations were performed using Adam's catalyst in all cases. The solvent was acetic acid unless otherwise specified.

Compound[a]	*Hydrogenation product of*	*Retention index*			
		Apiezon L 155°	*SF-96 170°*	*Carbowax 20 M 165°*	*DEGS 175°*
Farnesane	β-farnesene	1367.6		1354	1350
Bisabolane I	β-bisabolene	1458.4		1501	1557
Bisabolane II[b]	β-curcumene[b]	1463.6		1520.7	1586.5
Copaane I	α-copaene	1477.2	1455.4	1598.8	1740
Copaane II[c]		1437.3	1420.6	1550	1676
Muurolane I	α-muurolene	1479.2	1454.7	1582	1713
Muurolane II		1535.6	1505.6	1659	1808.5
Cadinane I	γ-cadinene[d]	1503.2	1483.4	1618	1760
Cadinane II		1493.5	1478.4	1597	1728
Amorphane I	α-amorphene	1489.8	1463.4	1593	1718.2
Amorphane II		1524.4	1498.4	1648.5	1794.2
Calarane	calarene	1508.1	1489.3	1670.5	1845.5
4,5αH-Eudesmane = selinane	β-selinene	1539.6	1509.5	1664.5	1819.4
Nootkatane	valencene	1530.6	1500.9	1648	1796.2
Vetivane I = nootkatane	[e]	1529.8	1500.4	1649	1797
Vetivane II = 7-epinootkatane		1553	1518.7	1683	1843.5
Cedrane I	β-cedrene	1521.3	1479.4	1662	1841.5
Cedrane II		1509.1	1470.3	~1651	~1826
Zizaane	zizaene	1513.1	1479.4	1660	1842.5

[a] When several diastereomeric products were obtained they are listed in order of decreasing proportion in the mixture obtained.

[b] The two bisabolanes are obtained in a 1:1 ratio from β-bisabolene. Hydrogenation of β-curcumene gives a 1:1.7 mixture of bisabolanes I and II respectively.

[c] Produced in higher yield when hydrogenation is performed with benzene as the solvent.

[d] δ-Cadinene yields cadinane II, cadinane I, and muurolane II as the major hydrogenation products.

[e] From either β-vetivenene or α-pyrovetivene.

stereomers—the exact composition varying with the placement of the double bonds in the original sesquiterpene. Here again, precise and reproducible GLC retention data could offer a more dependable basis for identification. To this end, we have hydrogenated some common sesquiterpenes and determined the KOVATS' indices of the resulting hydrocarbons. The products obtained from the hydrogenation reactions were GLC resolvable mixtures of diastereomers in all cases for which inspection of Dreiding models suggested nonstereospecific hydrogenation. The compositions of these mixtures and the structure assignments for the components will be the subject of another communication. Table III gives the indices for the major saturated products obtained. KOVATS' indices for the hydrogenation products showed temperature

dependence similar to that of the sesquiterpenes themselves; for this reason the indices were calculated using sesquiterpene standards.

Preliminary examinations of monoterpenes and oxygenated sesquiterpenes indicate that highly reproducible retention indices can be obtained by the internal standardization method used above for the sesquiterpene hydrocarbons. Extensions of Tables II and III and tables of retention indices for other natural products will be published periodically in the Chromatographic Data section of this journal.

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CHROM. 4210

THE DETERMINATION OF SOME SUBSTITUTED UREA HERBICIDE RESIDUES IN SOIL BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A method is described for the direct determination of eight substituted urea herbicides at the 0.1 to 1.0 p.p.m. level in soil using electron-capture gas chromatography. Residues of linuron were determined at 0.1, 0.5 and 1.0 p.p.m. in three soils of different organic matter content. Benzomarc, chlorbromuron, diuron, fluometuron, metobromuron, metoxymarc and neburon were determined at 0.1 and 1.0 p.p.m. in a single soil. The mean recovery for linuron was 88%. The recoveries obtained for the other seven herbicides ranged from 73 to 104%.

INTRODUCTION

Methods for the analysis of substituted urea herbicide residues in soil have been reported using a variety of techniques. Some methods involve hydrolysis of the herbicide to produce an aniline derivative which is determined colorimetrically after diazotisation and coupling to produce an azo dye[1-3]. Thin-layer chromatography has been used to measure residues of several urea herbicides in soil[4]. Other workers have used gas chromatography to measure aniline derivatives after hydrolysis of the herbicide[5,6]. Halogenated aniline derivatives have been used to improve the sensitivity and specificity of residue determinations using electron-capture gas chromatography[7,8]. More recently the gas chromatography of twelve unchanged substituted urea herbicides has been described[9]. Eight of these compounds containing either three fluorine, two chlorine or one bromine atom were considered suitable candidates for residue methods based on their measurement by direct electron-capture gas chromatography. This paper describes a method for the determination of linuron residues in soil that is also suitable for benzomarc, chlorbromuron, diuron, fluometuron, metobromuron, metoxymarc and neburon in soils.

EXPERIMENTAL

Materials

The following herbicides were evaluated:

Benzomarc	N-benzoyl-N-(3,4-dichlorophenyl)-N',N'-dimethylurea
Chlorbromuron	N-(4-bromo-3-chlorophenyl)-N'-methoxy-N'-methylurea
Diuron	N'-(3,4-dichlorophenyl)-N,N-dimethylurea
Fluometuron	N'-(3-trifluoromethylphenyl)-N,N-dimethylurea
Linuron	N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea
Metobromuron	N'-(4-bromophenyl)-N-methoxy-N-methylurea
Metoxymarc	N'-(3,4-dichlorophenyl)-N-(4-methoxybenzoyl)-N',N'-dimethylurea
Neburon	N-butyl-N'-(3,4-dichlorophenyl)-N-methylurea

Some properties of the soils are listed in Table I.

TABLE I

CHARACTERISTICS OF SOILS

Source	*Parent material*	*Texture*	*% organic carbon*	*% clay (<0.002 mm)*	*pH in water (1:2.5)*	*Cation exchange capacity (mequiv./100 g)*
Hollow (Weed Res. Org.)	Calcareous gravel	Sandy loam	1.9	15.6	7.1	11
Trawscoed	Alluvium from greywacké	Silty clay loam	3.7	32.6	6.2	12
Helmshore	Boulder clay	Clay loam	12	6.6	6.3	18

Fortification of the soil

Aqueous solutions were prepared containing 2.5, 12.5 and 25 μg herbicide in 2.5 ml. Solutions of neburon were prepared in 10% methanol to overcome its low water solubility. Portions of 25 g of air dry soil were weighed into shallow 7-cm aluminium dishes and 2.5 ml of aqueous herbicide solution was added with a pipette uniformly over the surface of the soil. The fortified soil now containing approximately 9% moisture was allowed to air dry naturally and was extracted after an interval of one

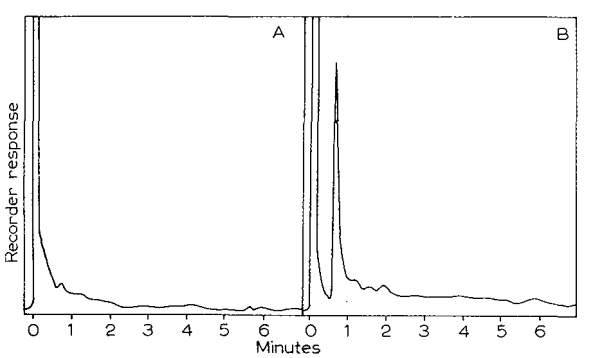

Fig. 1. Chromatogram of (A) control Hollow soil and (B) control Hollow soil fortified with 1 p.p.m. linuron.

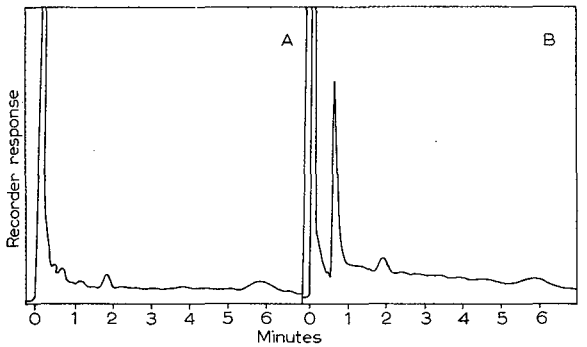

Fig. 2. Chromatogram of (A) control Trawscoed soil and (B) control Trawscoed soil fortified with 1 p.p.m. linuron.

week. The soil was mixed with a spatula once during this time to facilitate drying. The average moisture content of the 'air dry' soil at the time of extraction was 1.2, 2.2 and 3.3% for Hollow, Trawscoed and Helmshore respectively.

Extraction procedure

Air dried soil (25 g) was placed in a stoppered 250-ml conical flask with 50 ml of re-distilled methanol and shaken on a wrist-action shaker for 1 h. After shaking, the soil slurry was allowed to settle and the supernatant liquid was filtered through a fluted Whatman No. 1 filter paper into a stoppered tube. As soon as 10 to 15 ml of filtrate had been collected, the filter funnel was removed and the tube was stoppered to prevent evaporation. A 5-ml aliquot was transferred with a pipette to a 100-ml stoppered conical flask, a clean glass bead was added and the solution was concentrated to about 0.5 ml under reduced pressure in a water bath at 50°. The flask was then taken from the water bath and the remaining methanol removed with a gentle stream of dry air. The residue was dissolved in 5 ml of redistilled 2,2,4-trimethylpentane; 0.5 g of anhydrous sodium sulphate was added and the flask was stoppered and shaken vigorously for 1 min. Aliquots of this solution were taken for gas chromatography.

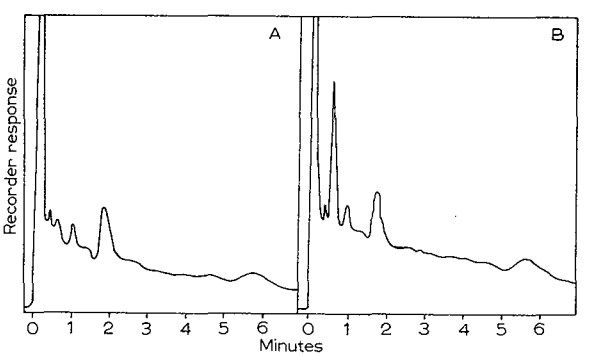

Fig. 3. Chromatogram of (A) control Helmshore soil and (B) control Helmshore soil fortified with 1 p.p.m. linuron.

Gas chromatography

A Varian Aerograph 1520 gas chromatograph was used fitted with the electron-capture detector previously described in detail[9]. The Aerograph electron-capture detector was not found to be suitable. The performance of other electron-capture detectors has not been evaluated but it seems probable that any design that includes glass in its specification will not be suitable.

The operating conditions were as follows: Column, 1.5 m × 3.5 mm O.D. stainless steel packed with 5% E301 (methyl silicone) on 60–80 mesh Gas-Chrom Q; flow rate, 50 ml/min oxygen-free nitrogen; injector temperature, 265°; column temperature, 150°; (140° used for fluometuron), detector temperature, 200°; detector voltage, 90 V d.c.; sensitivity, × 1; attenuation, 4 and 8; recorder, Leeds and Northrup Speedomax W; chart speed, 30 in./h.

The ends of the column were packed with steel wool that had been washed with hexane. A stainless steel injector insert was fitted and injections were made with the tip of the needle just entering the column. It is essential to exclude glass from the system and to adhere strictly to the conditions described[9].

Calibration standards

Solutions of herbicides containing 1 mg/ml were prepared in re-distilled methanol. Using a Hamilton syringe, 50 μl of the solution was transferred to a 100-ml volumetric flask and diluted to volume with re-distilled 2,2,4-trimethylpentane. This solution, containing 2.5 ng herbicide in 5 μl was diluted with 2,2,4-trimethylpentane to give a range of standards containing 0.1–1.0 ng herbicide in 5 μl. The graph of log peak height *vs.* log nanograms herbicide was essentially linear for each compound over the range 0.1 ng to 1.0 ng. The 2,2,4-trimethylpentane solutions obtained from extracts of the soil were diluted where necessary so that the standard injection volume of 5 μl contained a herbicide concentration within the calibration range.

TABLE II

THE RECOVERY OF LINURON FROM THREE SOILS FORTIFIED WITH THREE HERBICIDE CONCENTRATIONS

Soil	*Amount added (p.p.m.)*	*Amount found (p.p.m.)*	*% recovery*	
			Mean	*S.D.*
Hollow	0	0.05	—	—
	0.1	0.08[a]	80	3.1
	0.5	0.43	86	0.0
	1.0	0.79	79	1.7
Trawscoed	0	0.08	—	—
	0.1	0.10	100	11.7
	0.5	0.52	104	4.0
	1.0	0.91	91	1.7
Helmshore	0	0.30	—	—
	0.1	0.08	80	16.2
	0.5	0.38	76	5.3
	1.0	0.94	94	6.9

[a] Corrected for blank values.

RESULTS

In Tables II and III the recoveries given are based on three replicates at each level.

TABLE III

THE RECOVERY OF SEVEN SUBSTITUTED UREA HERBICIDES FROM TRAWSCOED SOIL FORTIFIED WITH TWO HERBICIDE CONCENTRATIONS

Herbicide	*Amount added (p.p.m.)*	*Amount found (p.p.m.)*	*% recovery*	
			Mean	*S.D.*
Benzomarc	0	0.06	—	—
	0.1	0.07[a]	70	4.6
	1.0	0.77	77	4.6
Chlorbromuron	0	0.03	—	—
	0.1	0.08	80	29.5
	1.0	0.84	84	12.3
Diuron	0	0.03	—	—
	0.1	0.08	80	9.7
	1.0	0.86	86	6.7
Fluometuron	0	0.02	—	—
	0.1	0.10	100	10.4
	1.0	0.90	90	3.5
Metobromuron	0	0.06	—	—
	0.1	0.08	80	3.5
	1.0	0.73	73	8.0
Metoxymarc	0	0.06	—	—
	0.1	0.09	90	15.0
	1.0	0.99	99	16.0
Neburon	0	0.04	—	—
	0.1	0.09	90	9.1
	1.0	1.04	104	7.6

[a] Corrected for blank values.

The method developed for linuron was applied to seven other substituted urea herbicides and determinations were made on Trawscoed soil fortified with 0.1 and 1.0 p.p.m. herbicide.

Samples of Hollow soil fortified with approximately 1 p.p.m. linuron were stored for two years and analysed during storage. The results are shown in Table IV.

DISCUSSION

Three soils of widely different organic matter content were chosen in order to test the method with soils likely to produce different background responses. The method of extraction was primarily developed for linuron and extraction experiments were first carried out on soil fortified with this herbicide. Dichloromethane, methanol,

TABLE IV

THE ANALYSIS OF HOLLOW SOIL FORTIFIED WITH LINURON AND STORED FOR TWO YEARS
(A) Stored air dry; (B) stored deep frozen (−10°) (14% moisture).

Date	*Amount linuron found (p.p.m.)*	
	A	*B*
23. 1.67	1.12	1.12
2.10.67	1.11	1.13
21. 2.69	0.97	1.03

acetone and dichloromethane containing 10% acetone or 10% methanol were evaluated as extraction solvents by shaking 25 g of field treated soil with 50 ml of solvent for 1 h and overnight for 16 h. Of these solvents, methanol gave the highest recovery of linuron with the lowest background from coextracted material. Extraction with methanol for 1, 2 and 3-h periods showed that increased recoveries were not obtained by shaking for more than 1 h. Extraction of linuron from moist soil (14 to 16% moisture) with methanol gave low recoveries but on mixing the soil with its own weight of anhydrous sodium sulphate before extraction, recoveries were similar to those obtained with dry soil.

Fortification of the soil with herbicides in volatile solvents immediately prior to extraction gave consistent recoveries of around 100% but using the fortification method adopted for this work, lower recoveries were generally obtained. It is therefore considered important to leave an interval of at least one week or longer after fortifying a soil before attempts are made to measure extraction efficiency. The results in Table IV illustrate the storage stability of soil samples containing linuron. In Table II the mean recoveries differed somewhat between levels and between soils. The standard deviation of the linuron recoveries tended to rise with increasing soil organic matter. Some of the variations obtained in the recovery experiments may be due in part to the difficulty of selecting a method for reproducibly fortifying soils with herbicides that bears a reasonable resemblance to practical field application procedures. Since replicate injections of linuron standards gave a coefficient of variation of 5.1%, some of the variations in the recoveries may be assigned to the final measurement and probably reflect the chromatographic instability of this compound.

In Table III the standard deviations have been calculated at both levels for each of the seven herbicides. Some of these figures, *e.g.* for chlorbromuron and metoxymarc, are high in comparison with those for linuron, but the method may not be ideal for all of the herbicides tested as it was basically developed for linuron. However, it is likely that only minor modifications would be necessary to establish satisfactory procedures for these herbicides. It cannot be judged whether the standard deviations obtained in this work are typical of those obtained by other workers for herbicide residue methods, since standard deviations for recoveries at individual levels are rarely if ever quoted in the literature.

Linuron, diuron, neburon and metoxymarc were not resolved from each other on the 5% E301 column and it would be necessary to confirm the identity of residues of unknown origin by an alternative technique. Thin-layer chromatography meth-

ods[4,10,11] would probably be suitable. In order to improve the sensitivity of the method a clean-up procedure would be required particularly for very high organic matter soils. The analyses reported in this work were obtained without clean-up. The minimum detectable linuron concentration in all three soils was considered to be 0.05 p.p.m. The minimum detectable levels of diuron, fluometuron, neburon, metoxymarc and benzomarc were slightly lower than 0.05 p.p.m. because of the greater sensitivity of the electron-capture detector to these compounds[9].

The method has been in routine use for over two years in this laboratory, analysing soil samples for linuron residues. Soil samples from the field are sieved, subsampled and allowed to air dry naturally. They are stored at room temperature while awaiting analysis.

This method has considerable advantages over existing methods for estimating some substituted urea herbicides in soil. The extraction from the soil is simple and rapid and final measurement is made of the original herbicide. It avoids the preliminary separations often required to distinguish the original molecule from aniline derivatives that may also be present in the soil.

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GAS–LIQUID AND THIN-LAYER CHROMATOGRAPHY OF PHORATE, DISULFOTON AND FIVE OF THEIR OXIDATION PRODUCTS

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SUMMARY

Phorate, phorate sulfoxide, phorate sulfone, phoratoxon, phoratoxon sulfoxide, phoratoxon sulfone, disulfoton, disulfoton sulfoxide, disulfoton sulfone, disulfoton oxygen analog, disulfoton oxygen analog sulfoxide and disulfoton oxygen analog sulfone were resolved by gas–liquid chromatography with temperature programming on a column containing Chromosorb W AW-DMCS coated with 5% stabilized DEGS. The limit of detection of the organophosphorus compounds after thin-layer chromatography was determined for eleven detection methods. An enzyme inhibition method with 5-bromoindoxyl acetate as substrate was found to be the most sensitive method. Bromocresol Green–$AgNO_3$ reagent detected all the compounds at the sub-microgram level. Sixteen mobile phases and six sorbents were compared for the TLC resolution of the organophosphorus compounds. The mobile phase methanol–benzene (10:90) with the sorbent MN-Kieselgel G-HR gave the best resolution of phorate, disulfoton and their oxidation products.

INTRODUCTION

A large number of column supports and stationary phases have been used for the gas–liquid chromatographic (GLC) analyses of organophosphorus pesticides and their metabolites[1–11]. McLEOD *et al.*[11] separated phorate and five of its metabolites with a column of 5% DEGS on 80–100 mesh HMDS-treated Chromosorb W. An oven temperature of 150° was used for the phorate and phoratoxon separation and 195° for the phorate sulfoxide, phorate sulfone, phoratoxon sulfoxide and phoratoxon sulfone separation.

Many mobile phases and sorbents have been evaluated for the thin-layer chromatographic (TLC) resolution of organophosphorus pesticides[12–20]. BLINN[17] resolved phorate and five of its oxidation products on thin-layer plates with a 1.75% methanol in chloroform mobile phase. MENZER AND DITMAN[19] employed a 2.5% methanol in chloroform mobile phase with Silica Gel G for the resolution of disulfoton, phorate and five of their oxidation products.

Many reagents have been used for the detection of organophosphorus compounds on paper and thin-layer chromatograms. WATTS[21] recently published an extensive review on the chromogenic spray reagents for organophosphorus pesticides.

A comprehensive study on the GLC and TLC of phorate, phoratoxon, phorate sulfoxide, phorate sulfone, phoratoxon sulfoxide, phoratoxon sulfone, disulfoton, disulfoton oxygen analog, disulfoton sulfoxide, disulfoton sulfone, disulfoton oxygen analog sulfoxide and disulfoton oxygen analog sulfone is reported here.

EXPERIMENTAL

Organophosphorus compounds

Phorate, phoratoxon, phorate sulfoxide, phorate sulfone, phoratoxon sulfoxide and phoratoxon sulfone were obtained from American Cyanamid Company. Disulfoton, disulfoton oxygen analog, disulfoton sulfoxide, disulfoton sulfone, disulfoton oxygen analog sulfoxide and disulfoton oxygen analog sulfone were obtained from Chemagro Corporation. The phorate and disulfoton were analytical grade, 97.8 and 96.8%, respectively, while their oxidation products were technical grade. Phorate and disulfoton were dissolved in hexane while the oxidation products were dissolved in acetone. All standards were diluted with hexane to give appropriate concentrations for GLC and TLC analyses.

Gas–liquid chromatography

A Varian Aerograph 2100 gas chromatograph fitted with a phosphorus detector was operated as follows: (1) 55 cm × 2 mm bore capillary U-shaped glass column containing Chromosorb W (80–100 mesh) coated with DC-200 and QF-1 (0.4 g of DC-200 and 0.6 g of QF-1 per 10 g of Chromosorb W); the nitrogen, hydrogen and compressed air flow rates were 20, 14 and 170 ml per min, respectively; the injector, column and detector temperatures were 200, 190 and 210°, respectively. (2) 110 cm × 1 mm bore capillary U-shaped glass column containing Chromosorb W AW-DMCS (high performance, 80–100 mesh) coated with 5% (w/w) stabilized DEGS; the nitrogen, hydrogen and compressed air flow rates were 20, 14 and 170 ml per min, respectively; for phorate and its oxidation products, the injector and detector temperatures were 190° and the column temperature was programmed at 2° per min from a starting temperature of 162° to 182° and then isothermal at 182°, for disulfoton and its metabolites the injector and detector temperatures were 195° and the column temperature was programmed at 1° per min from a starting temperature of 173° to 193° and then isothermal at 193°. The column supports were coated according to the method of MENDOZA *et al.*[22].

Thin-layer chromatography

The following sorbents and water were used to prepare the thin-layer plates: MN-Kieselgel G-HR (1:2, w/v), Silica Gel G (1:2, w/v), SilicAR TLC-7 (1:2, w/v), Aluminum Oxide G (1:2, w/v), Adsorbosil-M-2 (1:2.25, w/v), and Silica Gel H (1:2.42, w/v). The sorbents were shaken with the required amount of water and spread, 400 μ or 250 μ thick, with a Desaga applicator on acetone-rinsed glass plates (20.5 cm × 20.5 cm). Silica Gel G thin-layer plates were also prepared with pH 6 buffer[17]. The freshly coated plates were allowed to stand at room temperature for the following

TABLE I

TLC MOBILE PHASES

No.	*Composition*	*No.*	*Composition*
1	Chloroform	9	Methanol–benzene (5.0:95.0)
2	Methanol–chloroform (1.0:99.0)	10	Methanol–benzene (7.5:92.5)
3	Methanol–chloroform (1.5:98.5)	11	Methanol–benzene (10.0:90.0)
4	Methanol–chloroform (2.0:98.0)	12	Methanol–benzene (15.0:85.0)
5	Methanol–chloroform (2.5:97.5)	13	Acetone–hexane (20.0:80.0)
6	Methanol–chloroform (3.0:97.0)	14	Acetone–hexane (25.0:75.0)
7	Methanol–chloroform (4.0:96.0)	15	Cyclohexane–acetone–chloroform (70.0:25.0:5.0)
8	Methanol–chloroform (5.0:95.0)	16	Acetone–benzene (15.0:85.0)

periods: MN-Kieselgel G-HR, 10 min; Silica Gel G, 45 min; SilicAR TLC-7, 10 min; Aluminum Oxide G, 10 min; Adsorbosil-M-2, 30 min, and Silica Gel H, 45 min. The plates were then placed in a vertical position in an oven at 110° for 1 h. The mobile phases (Table I), prepared from glass distilled solvents, were placed in the glass chambers (Arthur H. Thomas Co.) 10 min or 1 h, in the case of mobile phases containing methanol, before developing the plates. Filter paper liners were placed in the chambers, when methanol was a constituent of the mobile phase. The plates were developed at room temperature. The organophosphorus compounds were applied 1.5 cm from the bottom of the thin-layer plate and developed until the mobile phase had reached a line drawn at a predetermined distance, usually 15 cm, from the starting point. The organophosphorus compounds were detected on the thin-layer plates by one of the methods shown in Table II. The reproducibility of the hR_F values[23], effect of thickness of sorbent and length of solvent travel on resolution of the organophosphorus compounds were studied.

RESULTS AND DISCUSSION

GLC tracings obtained with the organophosphorus compounds are shown in Figs. 1 and 2. Phorate, disulfoton and their five oxidation products were resolved

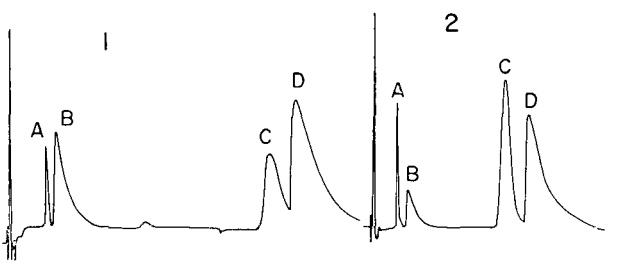

Fig. 1. GLC tracings obtained from the chromatography of disulfoton, phorate and some of their oxidation products on Chromosorb W coated with DC-200 and QF-1. Tracing 1: (A) 2×10^{-3} μg disulfoton, (B) 4×10^{-3} μg disulfoton oxygen analog, (C) 1.8×10^{-2} μg disulfoton sulfone, (D) 1×10^{-1} μg disulfoton oxygen analog sulfone. Tracing 2: (A) 2×10^{-3} μg phorate, (B) 1×10^{-2} μg phoratoxon, (C) 1×10^{-2} μg phorate sulfone, (D) 1×10^{-1} μg phoratoxon sulfone. Detection with Varian phosphorus detector.

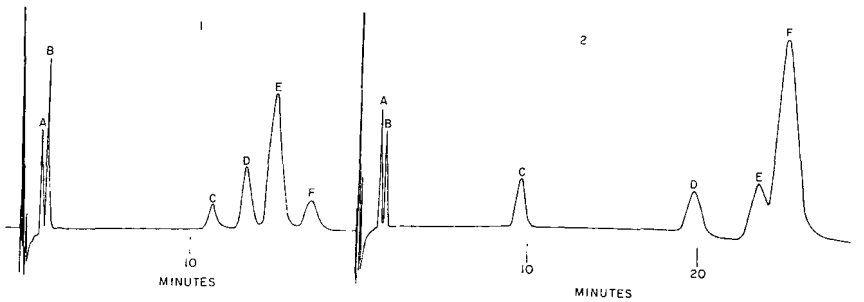

Fig. 2. GLC tracings obtained from the chromatography of disulfoton, phorate and some of their oxidation products on Chromosorb W AW-DMCS high performance coated with 5% DEGS. Tracing 1: (A) 1×10^{-3} μg phorate, (B) 2.5×10^{-3} μg phoratoxon, (C) 5×10^{-4} μg phorate sulfoxide, (D) 1×10^{-2} μg phoratoxon sulfoxide, (E) 5×10^{-3} μg phorate sulfone, (F) 2×10^{-3} μg phoratoxon sulfone. Tracing 2: (A) 1.5×10^{-3} μg disulfoton, (B) 1.2×10^{-3} μg disulfoton oxygen analog, (C) 3×10^{-3} μg disulfoton sulfoxide, (D) 6×10^{-2} μg disulfoton oxygen analog sulfoxide, (E) 1×10^{-2} μg disulfoton sulfone, (F) 4×10^{-2} μg disulfoton oxygen analog sulfone. Detection with Varian phosphorus detector.

on the 5% DEGS column. Phorate sulfoxide, phoratoxon sulfoxide, disulfoton sulfoxide and disulfoton oxygen analog sulfoxide were not detected when chromatographed on the DC-200 and QF-1 column. The oxygen analogs did not tail on the DEGS column. Temperature programming of the column had the advantage that the parent pesticide and its five oxidation products were resolved in a single run whereas McLEOD *et al.*[11] had to use two runs to resolve the six compounds. The 5%

TABLE II

LIMIT OF DETECTION (μg) OF THE ORGANOPHOSPHORUS COMPOUNDS ON MN-KIESELGEL G-HR THIN-LAYER PLATES, 450 μ THICK

Key: (A) Enzyme inhibition[24]; (B) palladium chloride[17]; (C) Bromocresol Green–$AgNO_3$[25]; (D) iodoplatinate[26]; (E) Brilliant Green–bromine[14]; (F) bromine–ferric chloride–2-(*o*-hydroxyphenyl)benzoxazole-UV[27]; (G) bromine–ferric chloride–2-(*o*-hydroxyphenyl)benzoxazole–Congo Red[27]; (H) 2,6-dibromobenzoquinone-4-chloroimide[29]; (J) iodine[30]; (K) 4-(*p*-nitrobenzyl)pyridine[31].

Compound	*Detection method*										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
Phorate	0.075	0.5	0.2	0.1	0.5	1	1	1	0.5	2	>20[b]
Phorate sulfoxide	0.075	0.5	0.2	2	0.5	1	1	1	0.5	>20	>20
Phorate sulfone	0.005	0.5	0.5	5	0.5	1	1	1	0.5	>20	>20
Phoratoxon	0.025	2	0.2	0.5	5	5	5	5	10	2[a]	>20
Phoratoxon sulfoxide	0.010	5	0.5	5	>20	>20	>20	>20	>20	>20	>20
Phoratoxon sulfone	0.005	5	0.5	5	>20	>20	>20	>20	>20	>20	>20
Disulfoton	0.075	2	0.5	0.2	0.5	1	1	1	0.5	2	>20
Disulfoton sulfoxide	0.050	0.5	0.5	1	0.5	1	1	1	0.5	>20	>20
Disulfoton sulfone	0.400	2	0.5	2	1	1	1	1	0.5	>20	>20
Disulfoton oxygen analog	0.050	2	0.5	0.5	2	5	5	5	20	2[a]	>20
Disulfoton oxygen analog sulfoxide	0.025	5	0.5	5	>20	>20	>20	>20	>20	>20	>20
Disulfoton oxygen analog sulfone	0.400	5	0.5	>20	>20	>20	>20	>20	>20	>20	>20

[a] Spot only visible for about 10 min.
[b] Did not test quantities above 20 μg.

TABLE III

hR_F[a] VALUES OBTAINED WHEN PHORATE AND ITS OXIDATION PRODUCTS WERE CHROMATOGRAPHED ON MN-KIESELGEL G-HR, 400 μ THICK

Mobile phase: No. 11 (*cf.* Table I).

Compound	*Plate No. 1*		*Plate No. 2*		*Plate No. 3*		*Plate No. 4*		*Plate No. 5*		*Av.*
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	
Phorate	82	83	85	85	84	84	79	79	83	82	82.7 ± 2.0
Phorate sulfone	71	71	73	73	71	71	69	69	71	71	71.0 ± 1.7
Phoratoxon	62	62	66	65	60	61	61	61	61	62	62.1 ± 1.9
Phorate sulfoxide	46	46	51	51	42	43	49	49	45	45	46.7 ± 2.9
Phoratoxon sulfone	39	39	44	43	35	35	43	43	37	38	39.6 ± 3.4
Phoratoxon sulfoxide	22	21	26	26	20	20	23	23	21	21	22.3 ± 2.2

[a] Ratio of distance travelled by the substance and the mobile phase front × 100.

DEGS column has been found to be satisfactory for a number of other oxygen analogs of organophosphorus pesticides (unpublished results).

Results of TLC detection methods are shown in Table II. The enzyme inhibition method of MENDOZA *et al.*[24] (A) was the most sensitive. The palladium chloride method[17] (B) detected all the organophosphorus compounds tested. The compounds were detected immediately and the sensitivity was increased if the plates were subsequently sprayed with 5 *N* NaOH. The Bromocresol Green–$AgNO_3$ spray reagent[25] (C) detected small quantities but at times it was difficult to clear the background with the acetate buffer spray. To decrease the background, one-half the recommended amount of Bromocresol Green was used in the spray. The iodoplatinate reagent[26] (D) detected all the compounds tested except disulfoton oxygen analog sulfone and is particularly useful because the reagent is stable indefinitely. Methods (E)[14], (F)[27], (G)[27], (H)[28] and (I)[29] are useful for the detection of phorate, disulfoton and their sulfoxide and sulfone but did not detect the oxygen analog sulfoxide and the oxygen analog sulfone at the 20 μg level. The iodine spray[30] (J) detected only phorate, phoratoxon, disulfoton and disulfoton oxygen analog and the oxygen analogs

TABLE IV

EFFECT OF MOBILE PHASE, THICKNESS OF SORBENT (μ) AND LENGTH OF RUN (cm) ON THE hR_F VALUES OF PHORATE AND ITS OXIDATION PRODUCTS

Compound	*12*[a]				*11*			
	400 μ		*250 μ*		*400 μ*		*250 μ*	
	15 cm	*10 cm*	*15 cm*	*10 cm*	*15 cm*	*10 cm*	*15 cm*	*10 cm*
Phorate	89	90	85	86	85	90	88	89
Phorate sulfone	76	79	78	77	75	80	78	79
Phoratoxon	65	70	75	71	67	72	71	71
Phorate sulfoxide	45	52	70	60	53	56	61	58
Phoratoxon sulfone	41	45	63	51	44	45	52	48
Phoratoxon sulfoxide	35	35	47	37	27	26	31	23

[a] For mobile phases, see Table I.

TABLE V

hR_F VALUES OBTAINED WHEN PHORATE AND ITS OXIDATION PRODUCTS WERE CHROMATOGRAPHED ON 400 μ

Compound	*Silica Gel G (buffer)*[a]							*Silica Gel G (water)*[a]					*Silica Gel H*[a]							
	1[c]	*2*	*3*	*4*	*5*	*13*	*15*	*4*	*9*	*11*	*12*	*13*	*3*	*4*	*5*	*6*	*9*	*10*	*11*	*14*
Phorate	67	72	73	73	71	19	53	81	73	77	80	83	72	75	80	77	70	74	74	74
Phorate sulfone	31	53	61	63	65	15	33	63	55	66	69	40	47	58	72	66	55	60	60	32
Phoratoxon	15	37	51	53	57	18	35	43	37	55	60	47	23	37	63	57	41	50	50	33
Phorate sulfoxide	11	21	35	37	47	6	16	23	19	37	47	30	7	17	45	35	23	35	37	14
Phoratoxon sulfone	4	13	27	29	42	5	13	16	15	31	41	19	3	11	39	29	19	30	33	14
Phoratoxon sulfoxide	0	5	10	11	21	1	4	5	3	17	31	3	1	3	16	10	5	11	18	3

[a] Length of mobile phase travel was 15 cm.
[b] Length of mobile phase travel was 12 cm.
[c] For mobile phases, see Table I.

were only visible for about 10 min. At the 20 μg level, 4-(*p*-nitrobenzyl)pyridine[31] (K) did not detect any of the organophosphorus compounds tested on the MN-Kieselgel G-HR or Silica Gel G (water) plates. GETZ AND WHEELER[20] noted that the sensitivity may be reduced by a factor of 5 if the 4-(*p*-nitrobenzyl)pyridine or tetraethylenepentamine are off color. This may account for the failure to detect any of the organophosphorus compounds tested, although the tetraethylenepentamine was filtered through charcoal as recommended[20]. GUTH[16] did not detect 10 μg of phorate but did detect 5 μg of disulfoton on Kieselgel GF_{254} with the 4-(*p*-nitrobenzyl)-pyridine reagent. The TCNE reagent (2% tetracyanoethylene in benzene), DDG reagent (2% 2,3 dichloro-5,6-dicyano-1,4-benzoquinone in benzene) and chloranil (1% tetrachloro-*p*-benzoquinone in benzene) have been used to detect sulfoxides, sulfones and sulfides[32] but failed to detect, at the 20 μg level, any of the organophosphorus compounds used in this study.

The main difficulty in the resolution by TLC of phorate, disulfoton and five of their oxidation products is in separating the sulfone from the oxygen analog and the sulfoxide from the oxygen analog sulfone. Detection method B was used for all

TABLE VI

hR_F VALUES OBTAINED WHEN DISULFOTON AND ITS OXIDATION PRODUCTS WERE CHROMATOGRAPHED ON 400 μ THICK PLATES

Compound	*Silica Gel G*[a]		*Silica Gel H*[a]		*MN-Kieselgel G-HR*[a]			*SilicAR TLC-7*[a]	
	4[b]	*12*	*9*	*10*	*9*	*11*	*13*	*10*	*11*
Disulfoton	78	79	76	77	83	85	69	82	83
Disulfoton sulfone	60	65	55	59	55	71	29	63	70
Disulfoton oxygen analog	45	61	48	49	41	62	35	55	64
Disulfoton sulfoxide	21	44	23	25	15	35	15	28	41
Disulfoton oxygen analog sulfone	17	41	21	21	12	30	11	26	35
Disulfoton oxygen analog sulfoxide	5	31	4	8	3	19	2	13	24

[a] Length of mobile phase travel was 15 cm.
[b] For mobile phases, see Table I.

THICK PLATES

MN-Kieselgel G-HR[a]								*SilicAR TLC-7*[a]								*Aluminum Oxide G*[a]								*Adsorbosil-M-2*[b]			
4	*6*	*7*	*8*	*9*	*11*	*12*	*13*	*3*	*4*	*9*	*10*	*11*	*12*	*13*	*15*	*1*	*4*	*11*	*12*	*13*	*14*	*16*		*1*	*3*	*13*	*15*
89	91	91	91	83	84	89	73	79	75	77	76	79	73	58	65	78	84	82	84	89	68	80		85	85	88	73
72	83	84	87	56	73	76	30	67	65	63	62	68	69	34	34	71	84	82	84	37	40	69		55	73	55	65
57	74	79	83	20	63	65	35	51	55	45	50	59	65	37	37	70	84	82	84	49	46	63		43	76	64	66
35	56	66	75	8	46	45	21	32	37	31	35	43	53	17	17	56	84	82	84	24	21	39		23	57	38	50
27	48	59	70	6	39	41	12	27	31	24	27	37	47	14	14	—	84	82	84	2	20	36		8	48	21	38
9	20	32	45	2	23	35	4	7	10	7	12	23	35	0	3	—	81	75	84	1	1	0		3	17	6	12

studies on hR_F values of the organophosphorus compounds. Table III shows the reproducibility of hR_F values for phorate and the five oxidation products when chromatographed in duplicate, on five MN-Kieselgel G-HR plates. Although there was some variation in the hR_F values from plate to plate, all plates gave good resolution of the compounds.

The effects of sorbent thickness and length of run on the resolution of phorate and its oxidation products are shown in Table IV. Mobile phase No. 11 gave superior results to that obtained with No. 12. Phorate and its oxidation products were adequately resolved on both the 250 and 400 μ thick layers when chromatographed with mobile phase No. 11.

The hR_F values reported in Tables V and VI were obtained from single chromatographic separations and are a guide in the selection of conditions for the TLC of phorate, disulfoton, and their oxidation products. The hR_F values obtained for phoratoxon and disulfoton oxygen analog were greater than the hR_F values obtained for phorate sulfone and disulfoton sulfone with mobile phase No. 13. The hR_F values which were obtained for phorate and five of its oxidation products are shown in Table V. The following combinations of sorbent and mobile phase gave the best resolution of phorate, phorate sulfoxide, phorate sulfone, phoratoxon, phoratoxon sulfoxide and phoratoxon sulfone: Silica Gel G (buffer) with mobile phases No. 3 or No. 4; MN-Kieselgel G-HR with mobile phase No. 4, No. 6 or No. 11 and Silica Gel H with mobile phase No. 10. On SilicAR TLC-7 with mobile phases No. 9 or No. 10 phorate and its oxidation products were resolved but the spots tended to streak. Aluminum Oxide G did not give satisfactory resolution with any of the mobile phases. The mobile phases moved much slower in the sorbent Adsorbosil-M-2 than in the other sorbents and so the length of the run was terminated after 12 cm. Of the mobile phases tested No. 13 provided the best resolution with Adsorbosil-M-2. The hR_F values which were obtained with disulfoton and its oxidation products are shown in Table VI. The following combinations of sorbent and mobile phase gave the best resolution of disulfoton, disulfoton sulfone, disulfoton oxygen analog, disulfoton sulfoxide, disulfoton oxygen analog sulfone and disulfoton oxygen analog sulfoxide: Silica Gel H with mobile phase No. 10 and MN-Kieselgel G-HR with mobile phase No. 11.

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CHROM. 4231

CHROMATOGRAPHIE SUR COUCHES MINCES DES COMPOSÉS ORGANIQUES DU PHOSPHORE

II. CHROMATOGRAPHIE DES COMPOSÉS NEUTRES

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SUMMARY

Thin-layer chromatography of organophosphorous compounds. II. Chromatography of neutral compounds

Further studies on organophosphorous compounds have been carried out. A new adsorbent–solvent system is described which permits a satisfactory separation of several neutral organophosphorous compounds (phosphine, phosphite, phosphate, phosphonate, phosphinate and phosphine oxide). The visualization reagent, which was not suitable for the detection of these neutral compounds, has also been improved.

INTRODUCTION

Dans une récente publication[1] nous avons donné différents couples adsorbants–solvants adaptés à la séparation d'un certain nombre de composés organiques du phosphore. Cependant, les solvants cités ne permettent pas l'analyse de tous les organophosphorés. Afin d'élargir le domaine d'application de la méthode, nous avons classé ces composés en trois catégories que nous avons étudiées séparément.

D'abord les composés avec le même groupement phosphoré et des radicaux identiques mais dont le degré d'estérification ou d'alkylation est différent, comme par exemple les phosphates neutres $(RO)_3PO$ et acides $(RO)_2P(O)(OH)$ et $(RO)P(O)(OH)_2$. La chromatographie de ces composés a fait l'objet de la précédente communication[1].

Puis les composés avec radicaux identiques et même degré d'alkylation ou d'estérification mais des groupements phosphorés différents. Ce sont les organophosphorés neutres R_3P, R_3PO, $(RO)_3P$, $(RO)_3PO$ etc. L'analyse de ces composés fait l'objet de la présente étude.

Enfin, les composés avec même groupement phosphoré et même degré d'alkylation ou d'estérification mais différents radicaux. Ce peut être la série des phosphates neutres ou celle des phosphonates ou celle des phosphinates.

Dans la catégorie des organophosphorés neutres, nous avons étudié la séquence suivante: R_3P (phosphine), $(RO)_3P$ (phosphite), R_3PS (sulfure de phosphine), $(RO)_3PO$ (phosphate), $(RO)_2P(O)R$ (phosphonate), $R_2P(O)(OR)$ (phosphinate) et R_3PO (oxyde

de phosphine) avec R = C_6H_5, C_4H_9, et C_8H_{17}. Nous avons ainsi pu comparer le comportement de la série aliphatique avec celui de la série aromatique.

PARTIE EXPÉRIMENTALE

La connaissance des propriétés chimiques[2], physiques et spectroscopiques[3,4] des composés organophosphorés neutres nous a permis de progresser rapidement dans la recherche du solvant et de l'adsorbant. Ces molécules organophosphorées possèdent toutes un certain pouvoir de donneurs d'électrons lié à la présence des groupements phosphoryle P=O ou thiophosphoryle P=S. En outre, les différences de polarité des liaisons P–O–C et P–C et les propriétés intramoléculaires et intermoléculaires de ces composés entraînent une variation de la solubilité dans les solvants polaires, de la viscosité et des autres propriétés[2] dans l'ordre suivant:

$$R_3P > (RO)_3PO > (RO)_2RPO > (RO)P(O)R_2 > R_3PO$$

Ces considérations nous ont conduits à guider notre choix vers un adsorbant acide et un solvant neutre moyennement polaire.

Choix de l'adsorbant

Nous avons abouti rapidement dans ce choix en utilisant l'ensemble Desaga qui permet d'obtenir des couches avec gradient d'adsorbant, d'activité ou de pH. Nous avons pu vérifier qu'un adsorbant acide (silice ou oxyde d'aluminium acide Merck) convient mieux qu'un adsorbant alcalin (alumine basique), alors qu'un adsorbant neutre (Kieselguhr ou oxyde d'aluminium neutre) ne permet pas de séparer ces composés avec les solvant utilisés. Notre choix s'est porté sur la silice qui permet une meilleure révélation avec le réactif utilisé à cet effet.

TABLEAU I

INFLUENCE DE LA NATURE DE LA SILICE ET DES CONDITIONS OPÉRATOIRES SUR LE TEMPS DE DÉVELOPPEMENT

Solvant: hexane–acétone (150:50). Développement de 10 cm à 25°. Nous utilisons 200 ml de solvant afin d'avoir encore une certaine quantité de solvent dans la cuve lorsque le papier filtre est imbibé.

Conditions de développement	*Temps de développement (min)*			
	Silice HR	*Silice H*	*Silice G*	*Plaque "DC Merck"*
(1) 200 ml de solvant, 1 h 30 min de saturation	13	19	20	30
(2) 200 ml de solvant, 1 h 30 min de saturation (cuve tapissée de papier filtre)	8	12	13	18
Même solvant après 4 h 30 min de saturation	9	14	16	24

Nous avons déjà signalé l'influence du type de silice utilisée lors de la chromatographie des phosphines[5], mais elle est ici beaucoup plus importante car certaines des substances étudiées ont des R_F très voisins. Nous avons étudié l'influence de la silice sur la chromatographie en préparant des couches d'adsorbant de 0.25 mm d'épaisseur avec les silices HR, H, G et PF_{254} Merck et en utilisant des plaques "DC Merck" vendues dans le commerce sur support de verre et sur support d'aluminium. Les couches que nous utilisons le plus fréquemment sont préparées à partir de 15 g de silice

TABLEAU II

INFLUENCE DES CONDITIONS DE DÉVELOPPEMENT SUR LES R_F D'ORGANOPHOSPHORÉS NEUTRES

La chromatographie est réalisée sur couche de silice HR (0.25 mm) à 25° avec le solvant hexane–acétone (150:50).

Substances ($R = C_6H_5$)	*Solvant dans les conditions 1*[a]		*Solvant dans les conditions 2*[a]	
	Plaques obtenues à partir de 15 g de silice et 50 ml H_2O	*Plaques obtenues à partir de 20 g de silice et 60 ml H_2O*	*Plaques obtenues à partir de 15 g de silice et 50 ml H_2O*	
			1 h 30 min de saturation	*4 h 30 min de saturation*
R_3PO	0.16	0.15	0.10	0.09
$R_2P(O)(OR)$	0.32	0.30	0.21	0.19
$RP(O)(OR)_2$	0.45	0.45	0.37	0.32
$P(O)(OR)_3$	0.53	0.52	0.45	0.40
R_3PS	0.59	0.59	0.51	0.47
$(RO)_3P$	0.87	0.73	0.61	0.57
R_3P	0.93	0.84	0.68	0.63

[a] Voir le Tableau I.

et 50 ml d'eau distillée pour cinq plaques de 20 × 20 cm. Nous les avons comparées à des couches obtenues dans les mêmes conditions avec 20 g de silice et 60 ml d'eau. Les résultats concernant cette influence de l'adsorbant sont donnés dans les Tableaux I, II et III.

Choix du solvant

Les solvants qui se sont révélés les plus favorables sont composés d'un solvant non polaire (hexane, cyclohexane) additionné à un solvant plus polaire (acétone,

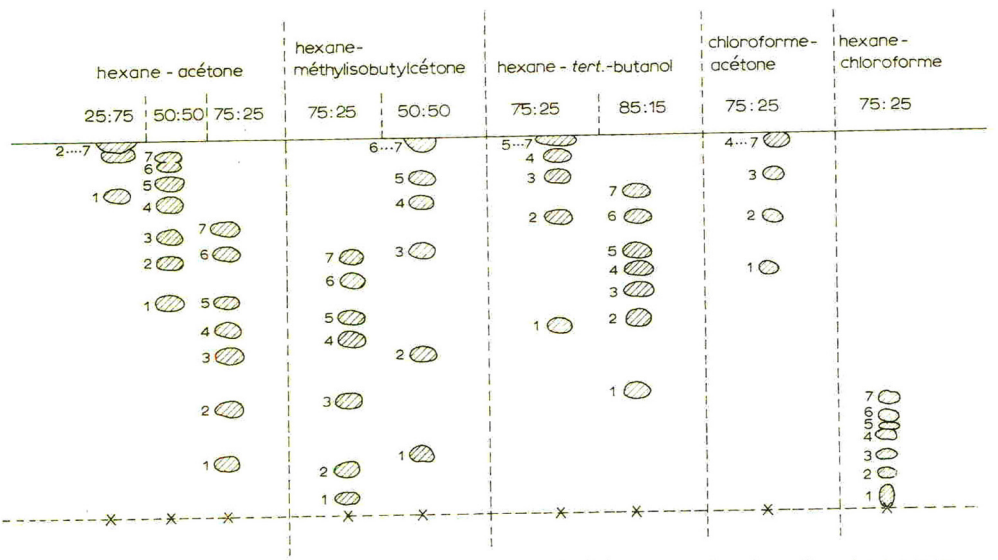

Fig. 1. Influence de la composition du solvant (nature, polarité, etc.) sur la séparation des organophosphorés neutres par chromatographie sur couches de silice H de 0.25 mm d'épaisseur. 1 = R_3PO; 2 = $R_2P(O)(OR)$; 3 = $(RO)_2P(O)R$; 4 = $(RO)_3PO$; 5 = R_3PS; 6 = $(RO)_3P$; 7 = R_3P ($R = C_6H_5$).

TABLEAU III

INFLUENCE DE LA NATURE DE LA SILICE SUR LES R_F D'ORGANOPHOSPHORÉS NEUTRES

La chromatographie est réalisée à 25° sur des couches de silice de 0.25 mm d'épaisseur préparées à partir de 15 g de silice et 50 ml d'eau. Le solvant est le mélange hexane–acétone (150:50) dans une cuve tapissée de papier filtre et utilisé après 1 h 30 min de saturation.

Substances ($R = C_6H_5$)	*Silice HR*	*Silice H*	*Silice G*	*Plaque "DC Merck"*
R_3PO	0.10	0.07	0.08	0.08
$R_2P(O)(OR)$	0.21	0.16	0.18	0.11
$RP(O)(OR)_2$	0.37	0.25	0.28	0.23
$(RO)_3PO$	0.45	0.33	0.35	0.29
R_3PS	0.51	0.38	0.41	0.35
$(RO)_3P$	0.61	0.52	0.56	0.49
R_3P	0.68	0.58	0.64	0.54

méthylisobutylcétone ou alcool butylique tertiaire) dans un rapport variant de 50:50 à 80:20 suivant les solvants et les séparations désirées. En effet, en variant la nature et les proportions relatives de ces deux types de solvants, il est possible d'améliorer la séparation de certains composés par rapport à d'autres (Fig. 1). Cependant, le solvant qui convient le mieux à la séparation de tous les organophosphorés neutres avec même radical, R, est le mélange hexane–acétone (75:25).

Ces solvants présentent un avantage appréciable pour les analyses: c'est la rapidité du développement (Tableau I) et la netteté des séparations obtenues (Fig. 2), mais ils présentent l'inconvénient de dissoudre les graisses qui assurent sur le couvercle rodé l'étanchéité de la cuve.

Choix du révélateur

Nous avons légèrement modifié la composition du réactif molybdique–perchlorique donnée dans la précédente communication[1]. Nous avons porté de 50 ml à

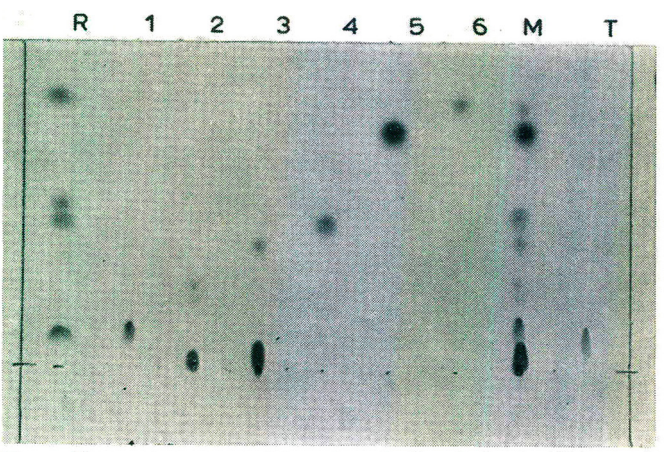

Fig. 2. Chromatogramme obtenu lors de l'analyse d'un mélange de composés organophosphorés neutres sur une plaque de silice "DC Merck" du commerce. Solvant: hexane–acétone (65:35). 1 = R_3PO; 2 = $R_2P(O)(OR)$ (avant purification) contenant $R_2P(O)(OH)$ (tache au départ); 3 = $RP(O)(OR)_2$ impur contenant $RP(O)(OH)_2$ (tache au départ); 4 = $(RO)_3PO$; 5 = $(RO)_3P$; 6 = R_3P ($R = C_6H_5$). R = référence; M = mélange; T = témoin.

TABLEAU IV

R_F COMPARÉS DE COMPOSÉS ORGANOPHOSPHORÉS NEUTRES ALIPHATIQUES ET AROMATIQUES (25°)

Substances	*Hexane–acétone (150:50)*		*Hexane–acétone (150:50) (papier filtre)*		*Cyclohexane–acétone (150:50)*	
	HR	*DC*	*HR*	*DC*	*HR*	*DC*
Oxydes de phosphines						
$R = C_6H_5$	0.13	0.13	0.07	0.06	0.11	0.16
$R = C_4H_9$	0.09	0.09	0.05	0.05	0.07	0.10
$R = C_8H_{17}$	0.21	0.24	0.13	0.15	0.17	0.26
Phosphinates						
$R = C_6H_5$	0.29	0.28	0.18	0.16	0.30	0.32
$R = C_4H_9$		0.27		0.14		0.31
$R = C_8H_{17}$		0.30		0.22		0.37
Phosphonates						
$R = C_6H_5$	0.59	0.38	0.30	0.23	0.40	0.46
$R = C_4H_9$	0.57	0.37	0.30	0.22	0.37	0.39
$R = C_8H_{17}$		0.37		0.28		0.46
Phosphates						
$R = C_6H_5$	0.66	0.46	0.38	0.28	0.65	0.56
$R = C_4H_9$	0.60	0.45	0.37	0.28	0.48	0.48
$R = C_8H_{17}$		0.48		0.32		0.53
Sulfure de phosphine						
$R = C_6H_5$	0.73	0.50	0.41	0.29	0.75	0.65
Phosphites						
$R = C_6H_5$	0.81	0.76	0.54	0.46	0.88	0.87
$R = C_4H_9$	1.00	0.95	0.69	0.60	0.98	0.98
Phosphines						
$R = C_6H_5$	0.91	0.85	0.59	0.52	0.93	0.93
$R = C_4H_9$	1.00	1.00	0.72	0.66	1.00	1.00
$R = C_8H_{17}$		1.00	0.77	0.70	1.00	1.00

80 ml la quantité d'acide perchlorique dans un litre de révélateur et nous y avons ajouté 25 ml d'acide sulfurique concentré. En effet, certaines de ces substances, notamment les phosphonates et phosphates, sont difficiles à hydrolyser pour être transformées en phosphomolybdate par le réactif molybdique puis en oxyde de molybdène bleu par action d'un réducteur comme l'hydrogène sulfuré; et c'est pourquoi nous avons augmenté la concentration en acide perchlorique du révélateur. Quant à l'addition de l'acide sulfurique elle permet d'obtenir un fond jaune qui contraste beaucoup plus avec le bleu des spots que l'ancien fond marron.

DISCUSSION ET EXPLOITATION DES RÉSULTATS

Il ressort de cette étude que l'on ne peut pas qualifier de valeur absolue le R_F attribué à une substance sans spécifier toutes les conditions opératoires. Ce problème est ici d'autant plus important que nous utilisons des solvants très volatils et qui dissolvent facilement les graisses. Cet inconvénient de la mauvaise reproductibilité des R_F dans le temps, avec le matériel habituel, peut être limité en utilisant des substances

témoins ou même être évité en utilisant une cuve spéciale dont l'étanchéité est assurée par une gorge contenant du mercure.

Cette reproductibilité assurée ou approchée n'empêche pas l'obtention de R_F différents, pour une même substance, avec les différentes silices utilisées. La cause en est d'abord, certainement, la nature et la pureté de la silice et ensuite la différence de granulation entre les couches. Les couches de silice vendues sur plaque d'aluminium ou de verre ont une granulation plus fine et plus resserrée qui entraîne un temps de développement plus long mais ont l'avantage de donner des taches plus fines. Les supports d'aluminium se sont révélés très intéressants car ils peuvent être découpés facilement pour servir en radiochromatographie ou être utilisés du point de vue quantitatif comme les bandes de papier.

Du point de vue pratique cette méthode permet la séparation et l'analyse, rapides et efficaces, de composés très souvent utilisés en raison de leurs propriétés extractives[2]. Il est également possible de séparer avec ces adsorbants et solvants des isomères du genre phosphonate $(RO)_2RP(O)$ et phosphite $(RO)_3P$ (Tableau IV).

Du point de vue théorique nos résultats confirment les propriétés de ces composés car nous retrouvons, pour les R_F, sur le chromatogramme la même séquence:

$R_3P > (RO)_3P > R_3PS > (RO)_3PO > (RO)_2RPO > (RO)R_2PO > R_3PO$

que celle caractérisant les autres propriétés de ces composés, que ce soit dans la série aliphatique ou dans la série aromatique. Ce résultat est en rapport avec la "basicité" et la solubilité dans les solvants de ces composés. En effet, ces organophosphorés entrent en compétition avec l'acétone (solvant à caractère "basique") pour former des liaisons hydrogène avec les sites–OH de la silice. Et les constantes d'équilibre des complexes ainsi formés gouvernent en grande partie la migration des substances sur le chromatogramme. Le rôle de l'hexane est de diminuer le pouvoir éluant de l'acétone.

REMERCIEMENTS

Nous tenons à remercier Monsieur P. CHABRIER, Directeur de Recherches au C.N.R.S. qui, en nous procurant une partie de ces composés, nous a permis de limiter nos synthèses à un nombre restreint de composés et nous a permis ainsi d'accélérer cette étude.

RÉSUMÉ

Nous avons poursuivi les recherches entreprises dans le domaine des organophosphorés. Nous avons mis au point un couple adsorbant–solvant qui permet une séparation très convenable des différents organophosphorés neutres (phosphine, phosphite, phosphate, phophonate, phosphinate et oxyde de phosphine). Nous avons également amélioré le réactif de révélation qui n'était pas assez puissant pour la détection de ces composés neutres.

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CHROM. 4248

VERFAHREN ZUR ERZIELUNG GUT REPRODUZIERBARER R_F-WERTE BEI SERIENMÄSSIG DURCHGEFÜHRTER DÜNNSCHICHT-CHROMATOGRAPHIE

ROUTINEMETHODE ZUR IDENTIFIZIERUNG INSEKTIZIDER CHLORKOHLENWASSERSTOFFE

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(Eingegangen am 16. Juni 1969)

SUMMARY

A method yielding high reproducible R_F values suitable for thin-layer chromatography in series. Routine method for identification of chlorinated hydrocarbon pesticides

A general method is described. By using preferably monocomponent solvents R_F values of high reproducibility are obtainable. Maximum deviations from the mean value are not more than 3 hR_F, mean deviations from the mean values are equal to 0.8 hR_F, standard deviations are of $\pm$ 1.2. The technique needs cheap and simple laboratory equipment only, and a large number of plates may be chromatographed daily in routine analysis. This method is applied to the identification of at least sixteen chlorinated hydrocarbon pesticides by their R_F values.

EINLEITUNG

Auf dem 3. Internationalen Symposium über die "Reproduzierbarkeit in der Papier- und Dünnschichtchromatographie", 2.–5. Oktober 1967 in Liblice, wurde heftig über die Frage diskutiert, inwieweit die—nur durch besondere Massnahmen erreichbare—gute Reproduzierbarkeit der R_F-Werte angestrebt werden sollte. Zur dünnschichtchromatographischen Screening-Analyse von völlig unbekannten Proben, in denen Komponenten aus einem Spektrum von viel mehr als zehn Stoffen erwartet werden, müssen die R_F-Werte zur Identifizierung herangezogen werden, da bereits die Unterbringung aller Vergleichsstoffe den Platz für die Proben auf einer Dünnschichtplatte stark einengt. Oft differieren die R_F-Werte etlicher der erwarteten Komponenten bei solchen Screening Tests nur wenig. Besonders aus diesem Grunde ist eine gleichbleibende Trennleistung des dünnschichtchromatographischen Systems und eine geringe Streuung der R_F-Werte unabhängig von tages- oder jahreszeitlich bedingten Klimaschwankungen und vom Experimentator notwendig. Mehrere Au-

toren[1-19,27] berichteten über folgende, auf die Reproduzierbarkeit Einfluss nehmenden Faktoren: Qualität (Porosität, Chargenabhängigkeit) des Adsorbens, Schichtdicke, Reinheitsgrad der Fliessmittel, Kammersättigungsgrad, Chromatographiertechnik, Kammervolumen, Abstand des Startes vom Eintauchspiegel, Temperatur, Vorbedampfung durch Wasser (Feuchte) und Fliessmittelkomponenten. Besonders die Arbeiten von GEISS *et al.*[4,5,9-11] befassten sich grundlegend mit den wichtigsten Faktoren der Vorbedampfung. In der von ihnen entworfenen KS-Kammer*[5,12] können unterschiedliche Vorbedampfungsverhältnisse nebeneinander auf der gleichen Dünnschichtplatte geschaffen werden. In der von diesen Autoren gleichfalls beschriebenen GS-Klimakammer**[5] kann auf einer Platte einheitlich unter exakt kontrollierbaren Bedingungen chromatographiert werden. Beide Kammern sind vergleichsweise teuer und werden für das klimakontrollierte Chromatographieren durch jeweils eine einzige Platte u.U. über mehrere Stunden gegenüber der zeitlich nachfolgenden Dünnschichtplatte blockiert.

Für die serienmässige dünnschichtchromatographische Identifizierung vieler Chlorkohlenwasserstoffinsektizidwirkstoffe in unbekannten Proben mit hohem Plattendurchsatz pro Tag entwickelten wir daher ein Verfahren, das den Gebrauch der genannten kostenaufwendigen Kammern vermeidet. Auch bei unserem Verfahren werden alle für die Reproduzierbarkeit wesentlichen Faktoren exakt kontrolliert. Man kann sie variabel nach Wunsch einstellen und damit das Trennergebnis in der beabsichtigten Weise optimieren.

In dieser Veröffentlichung sind unsere zwei Jahre währenden Erfahrungen mit diesem Verfahren berücksichtigt. POGACAR *et al.*[20] bedienten sich der Tube-Dünnschichtchromatographie für reproduzierbares Arbeiten. Wir beschäftigten uns ebenfalls mit dieser Methode. Für das Variieren der Schichten in Röhren schien uns jedoch die Ausarbeitung einer gut kontrollierbaren Schichtherstellungsmethode zu aufwendig. Ferner lässt sich bei den Tubes das Klima während der Probenaufgabe nicht kontrollieren. Aus diesen Gründen wendeten wir uns wieder planen Schichten zu.

Zur Beurteilung der Leistungsfähigkeit eines dünnschichtchromatographischen Analysenverfahrens sind Chlorkohlenwasserstoffinsektizide ein besonders geeigneter Prototyp, da die Trennung dieser Verbindungen hauptsächlich auf ihrer Adsorptions–Desorptionswechselwirkung mit dem Adsorbens selbst, weniger auf verteilungschromatographischen Prozessen beruht. Die erstgenannte Wechselwirkung wird begreiflicherweise durch adsorbierte Feuchtigkeit (oder andere Vorbedampfung) besonders empfindlich beeinträchtigt, wie bereits von REICHEL[7] gezeigt wurde. Bei einigen von REICHEL nicht untersuchten Chlorkohlenwasserstoffinsektiziden ist die R_F-Wert-Abhängigkeit auch bei geringen Änderungen im Feuchtegehalt so gross, dass wir uns nicht entschliessen konnten, ein feuchteklimatisiertes Labor als Klimatisierungskammer für die Schichten zu benutzen. Erfordert eine Trennung besonders hohe Feuchten, dann wäre die Methode von REICHEL dem Laboratoriumspersonal kaum zuzumuten, und schliesslich müssten in die meisten europäischen Laboratorien mit unverhältnismässig hohem Kostenaufwand hochwertige Klimaanlagen erst nachträglich installiert werden. Darüberhinaus ist die Gleichmässigkeit von Temperatur und Feuchte in derart grossen Räumen technisch noch immer problematisch.

Nachstehend wird zunächst das dünnschichtchromatographische Rahmen-

* Beziehbar von der Firma Camag, Muttenz, Schweiz.
** Beziehbar von der Firma Desaga, Heidelberg.

verfahren, mit dem sich hohe Reproduzierbarkeit im Serienbetrieb erzielen lässt, beschrieben und begründet. Im Anschluss werden die Arbeitsvorschrift zur Identifizierung der Chlorkohlenwasserstoffinsektizide und die damit erreichbaren Ergebnisse mitgeteilt.

RAHMENVERFAHREN

(1) Das Sorptionsmaterial wird stets von der gleichen Quelle unter gleicher Signatur bezogen. Das Schüttgewicht der gewünschten Siebfraktion (s. Abschn. 2) wird festgestellt. Aus den Dimensionen der Glassplatte und der gewünschten ("Pseudo"-) Schichtdicke (nicht exakt zutreffende Annahme: die Packungsdichten bei der Ermittlung des Schüttgewichtes und auf der fertigen Platte seien gleich) ergibt sich die Gewichtsmenge des pro Platte anzuwendenden Sorptionsmaterials. An sehr feuchten Tagen sollte das Gut unmittelbar vor der Ermittlung des Schüttgewichts 1 h bei 80° am Rotationsverdampfer evakuiert werden. Im Falle einer im Schüttgewicht von der Bezugscharge abweichenden Folgecharge muss eine korrigierte Menge Sorptionsmittel pro Plattenfläche angewendet werden. Nach einiger Übung (vgl. Abschn. 3 und 4) erhält man innerhalb einer Platte eine gleichmässigere Schichtverteilung, als wir sie je mit dem Streichverfahren herstellen konnten. Darüberhinaus ist eine bessere Kontrolle über die Identität der Schichtdicken der Platten untereinander gegeben. Über die Gleichmässigkeit solcher Platten gibt Tabelle I Auskunft. Auf jenen

TABELLE I

PRÜFUNG DER GLEICHMÄSSIGKEIT DER SCHICHTEN VON GEGOSSENEN DÜNNSCHICHTPLATTEN

Vergleich der hR_F-Werte von Isobenzan auf 11 Bahnen verteilt über die gesamte Breite von 20 × 20 cm-Platten, durchgeführt an drei verschiedenen Tagen.

Platte	*Bahn-Nr.*											*Mittel-wert*	*Mittl. Abweichung vom Mittelwert*	*Grösste Abweichung*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*			
1 (23. Febr.)	28	28	28	29	29	29	29	29	30	30	30	29	0.5	1
2 (27. Febr.)	29	29	29	29	30	30	30	30	30	30	33	30	0.6	3
3 (28. Febr.)	30	31	31	30	30	30	29	29	29	31	31	30	0.6	1

Platten war die Korngrössenverteilung noch weniger eng fraktioniert, als wir das heute tun. Die Homogenität unserer jetzt hergestellten Platten ist nur wenig schlechter als die von kommerziellen Fertigplatten.

(2) Man verwende Siebfraktionen mit Korngrössenverteilung nur innerhalb eines Intervalls von 10 μm. Geeignet sind die Fraktionen 50–60 μm, 40–50 μm, 30–40 μm, 20–30 μm. Bei Anwendung engfraktioniertem Materials wird gute Haltbarkeit der Schicht sowie Konstanz der Wanderungsgeschwindigkeit der Fliessmittelfront (Abweichung der Geschwindigkeiten von Platte zu Platte $< 1\%$!) erreicht und die Zahl der Platten, die bei der Trennung nicht die Normen (Auswahlbedingungen s. Abschn. 10!) erfüllen, wird verringert. Mit der Wahl der Fraktion werden die chromatographische Entwicklungszeit (kleine Korngrösse → kleine Fliessmittelgeschwindigkeit) und die Trennung kritischer Paare beeinflusst. Dieser Effekt ist bisher wenig

beachtet worden. Er übertrifft den Einfluss des angewendeten Suspensionsmittels (z.B. Chloroform statt Wasser) auf die Homogenität der Schicht und damit auf die Güte der Trennung, wie er von VERSINO *et al.*[21] berichtet wird. Mit bestimmten Suspensionsmitteln wird dort nur eine möglicherweise für die Haftung—und damit die homogene Kapillaraktivität—günstigere Korngrössenverteilung entlang der Dickenkoordinate der Schicht erreicht.

(3) In einem 75 ml-Erlenmeyerkolben wird die für eine Platte abgewogene Menge Sorptionsmaterial mit dem optimal ermittelten Volumen Suspensionsmittel (Wasser, organisches Lösungsmittel, Gemisch Wasser–organisches Lösungsmittel je nach Art des Schichtmaterials) versetzt. Das Suspensionsmittelvolumen ist optimal, wenn die Suspension einerseits nicht von der Platte fliesst und sich andererseits leicht visuell homogen über die gesamte Fläche verteilen lässt.

(4) Die Suspension wird 1 min geschüttelt und dann mit einem Schwung entlang einer 20 cm-Kante auf die sorgfältig gesäuberte (s. Abschn. 11!) Glasplatte gegossen. Durch mehrfaches Hin- und Herkippen der Glasplatte über einen dunklen Untergrund wird das Sorptionsmaterial visuell erkennbar gleichmässig verteilt. Sofort anschliessend wird die Platte auf einer austariert waagerechten Ebene zugfrei zum Lufttrocknen abgelegt. Trockenzeit: im allgemeinen (bei gewöhnlichen Suspensionsmitteln): über Nacht. Die waagerechte Unterlage kann z.B. mit Hilfe einer grossen dicken Glasplatte auf einem Nivellierdreieck in einem zugfrei abgedichteten Laborabzug erstellt werden.

(5) Alle lufttrockenen Platten werden nach Abkratzen der erforderlichen Ränder und Markierung der Ziellinie zunächst 16 h (über Nacht) über H_2SO_4 $\varrho = 1.84$ vorgetrocknet und damit in den gleichen Ausgangszustand—unabhängig vom Raumklima—gebracht. Wir tun dies in Packungen von 10 Stück in dem Desaga-Trockengestell, welches in einen luftdicht verschliessbaren (Glas-) Kasten der Abmessungen $25 \times 25 \times 25$ cm so eingestellt wird, dass die Platten sich senkrecht über der Oberfläche der Säure befinden. Alle 1–2 Tage muss das spezifische Gewicht der Säure geprüft und ggf. neu eingestellt werden. Eine Hitzetrocknung fördert die Tendenz zum Abblättern der Schichten; sie ist ferner weniger gleichförmig, wenn nicht ein Ventilatorumluftrockenschrank verwendet wird. Aber auch dort ist der Wasserentzug an den Platten weniger gut kontrollierbar. Ausserdem wird beim Überführen der Platten in das nächste Vorbehandlungssystem das dort herrschende Gleichgewicht durch heisse Platten stärker gestört. Starke Änderung der Vortrockungszeit kann eine Verschlechterung der Schichthaftung sowie einen hemmenden Einfluss auf die Wanderungsgeschwindigkeit des Fliessmittels ausüben.

(6) Anschliessend erfolgt eine stets gleichbleibende Zeit lang die Äquilibrierung der Platten mit einem bestimmten Luftfeuchtegehalt, der sich als vorteilhaft für die beabsichtigten Trennungen erweist. Dies geschieht in einem abgedichteten, in jedem Labor leicht selbst herzustellenden Kasten der ungefähren Grösse $1 \times 0.5 \times 0.5$ m. Wir benutzen den in Fig. 1 abgebildeten Glaskasten. Für das Photographieren wurde die Vordertür abgenommen. In der rechten hinteren Ecke der Kammer befindet sich auf einem Sockel ein offener PVC-Bottich der Grundfläche mindestens 25×25 cm, einer Höhe von mindestens 20 cm und mit einem durch die rechte Aussenwand der Kammer geführten (und abgedichteten) Ablasshahn. Der Bottich ist zu Dreivierteln mit der Klimaschwefelsäure gefüllt. Das Flügelrad an der rechten Seitenwand wird mit geringer, stets gleichbleibender Umdrehungszahl von einem rechts ausserhalb angebrachten Rührwerksmotor angetrieben. Eine genaue Feuchtigkeitskontrolle mit dem

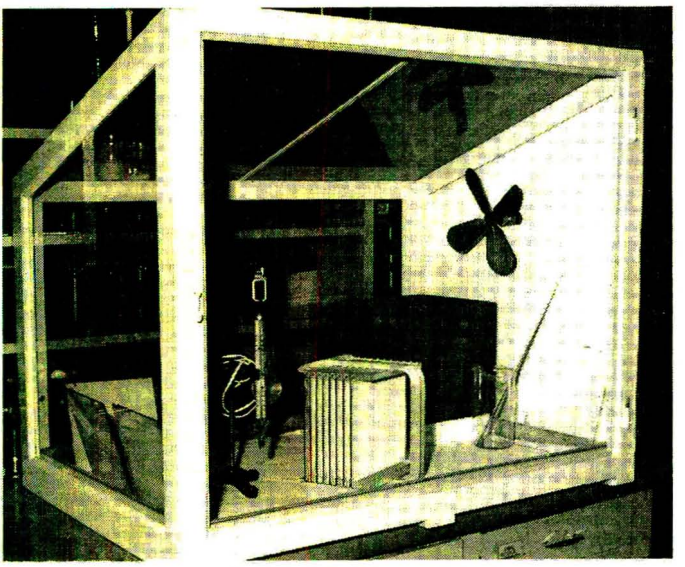


Fig. 1. Klimatisierungskammer für Dünnschichtplatten.

im Hintergrund sichtbaren Hygrometer ist nicht möglich. Wir ermitteln mit ihm lediglich die zur Äquilibrierung nötige Zeit nach dem Einstellen der Platten (z.B. im Vordergrund). Die genaue Feuchtekontrolle geschieht durch Spindeln (im allgemeinen einmal täglich: abends) der Dichte der Klimaschwefelsäure, die durch Zugiessen von wenigen Millilitern Wasser bzw. H_2SO_4 unter Rühren (vgl. Glasstab vor dem Bottich!) stets genau eingestellt wird. Oft bewährt sich eine H_2SO_4 von $\varrho = 1.35$. Sie ruft eine rel. Feuchte von ca. 47% hervor. Das Einstellen von Feuchtigkeitswerten $< 15\%$ relativ verbietet sich, da dann Einflüsse der Raumluft innerhalb der wenigen Sekunden der Überführung der Platten zur nächsten Behandlungsstufe (s. Abschn. 7!) nicht mehr vermieden werden können und somit die Reproduzierbarkeit schlechter wird. Wir äquilibrieren meist $1\frac{1}{2}$ h vor der Probenaufgabe. $\frac{1}{2}$ h vorher braucht die Kammer, um bei laufendem Ventilator ein Feuchtegleichgewicht herzustellen.

(7) Zur Probenaufgabe wird die Analysenplatte schnell in die sog. "Präparierbox" (Desaga, Best.-Nr. 1240 80) überführt. Diese Box wird schon $\frac{1}{4}$ h vorher und während des Auftragens mit einem Strom von 400 ml N_2/min gespült. Der Stickstoff passiert unmittelbar davor zwei Waschflaschen mit H_2SO_4 derselben Dichte wie diejenige in der Klimakammer. Das Aufbringen der Proben 3.0 cm vom unteren Rand Rand entfernt erfolgt mittels Kapillarpipetten von konstantem Volumen (1 oder 3 μl) durch ein Loch der Auftrageschiene dieser Box. In Hinsicht auf die leichte Zersetzlichkeit mancher Pflanzenschutzmittelwirkstoffe und zur Erzielung möglichst niedriger Nachweisgrenzen ist eine geringe Verweilzeit der Proben am Start wünschenswert. Dies ist bei den aufwendigen Kammern von GEISS nicht realisierbar. Unser Verfahren erfüllt diesen Wunsch. Raumlufteinflüsse während der Probenauftragezeit, die die Vorklimatisierung der Platten merkbar beeinträchtigen, werden auf die beschriebene Weise weitgehend vermieden. Selbstverständlich ist auch die Dichte der Klimaschwefelsäure in den Waschflaschen alle 1–2 Tage zu überprüfen.

(8) Chromatographiert wird im Sandwich-Verfahren, System Camag. Als Konterplatten werden entweder unbehandelte oder gleicherweise beschichtete Glasplatten verwendet, welch letztere der gleichen Vorbehandlung unterworfen waren wie die Analysenplatten. Die genau senkrechte Aufstellung erfahren die 2 mm-Sandwiches auf genau waagerecht austarierter Unterlage. Stets frisches Fliessmittel wird vor dem Einstellen der Sandwiches in die Tröge immer genau 1 cm hoch eingefüllt. Im Sandwich-Verfahren ist die grösste Gewähr für die Konstanz der Verhältnisse während der Chromatographierzeit gegeben. Besonders Temperatureinflüsse wirken sich hier nur wenig aus. Das System ist unumschränkt anwendbar beim Arbeiten mit Einkomponentenfliessmitteln. Viele Mehrkomponenten-Systeme zeigen oft unerwünschte Entmischungserscheinungen, die überdies die Reproduzierbarkeit beeinträchtigen können. An einer Möglichkeit zur reproduzierbaren Chromatographie mit allen Mehrkomponenten-Fliessmittelsystemen wird z.Zt. gearbeitet.

(9) Die Raumtemperatur ist bei allen Schritten des Verfahrens innerhalb eines Intervalls von 5° zu halten.

(10) Für die Identifizierung einer bestimmten Stoffgruppe wird ein Vertreter dieser Klasse von mittlerem R_F-Verhalten und chemisch-physikalischer Beständigkeit ausgewählt. Diese Standardsubstanz wird auf jede Analysenplatte einmal für eine mittlere Bahn aufgetragen. Aus einer grossen Zahl von Versuchen (8–10) wird dessen Soll-R_F-Wert gemittelt. Dabei wird auch die Sollzeit ermittelt, die die Fliessmittelfront zwischen Probenaufgabeort und Ziellinie benötigt. Nur solche Platten gelangen zur Auswertung, deren Standard nicht mehr als $\pm$ 1 vom hR_F-Sollwert und deren Laufzeit nicht mehr als $\pm$ 2% von der Sollzeit abweichen. Eine Relativierung der R_F-Werte ist im allgemeinen für die Reproduzierbarkeit unergiebig, da viele Substanzen auf Störeinflüsse bei der Dünnschichtchromatographie in sehr unterschiedlichem Ausmass reagieren (also $\Delta R_{F(st)} \neq \Delta R_{F(a,b\ldots)}$). Auch die von BRENNER *et al.*[22] vorgeschlagene Standardisierung über die R_M-Werte: $R_M = \log (1/R_F - 1)$; $(R_M)_i - (R_M)_{st} = (R_k)_{i\ st}$ verbessert die Ergebnisse gegenüber den ursprünglichen R_F-Werten nicht, vermutlich weil die Trennung der Chlorkohlenwasserstoffinsektizide nur in geringem Masse auf flüssig–flüssig-Verteilungsprozessen beruht. Die Ausschussquote der nicht auswertbaren Platten liegt nicht höher als 30%, neuerdings—seitdem wir durchweg engfraktioniertes Sorptionsmaterial verwenden—sogar erheblich niedriger. Es folgen Durchschnittsangaben betreffend die Reproduzierbarkeit über Monate hinweg:

Selbstgegossene Platten

Grösste Abweichung vom Mittelwert	*ca.* 3 hR_F
Durchschnitt der mittleren Abweichungen vom Mittelwert ($\overline{M.A.}$)	*ca.* 0.8 hR_F
Mittlere Standardabweichung ($\bar{s}$)	*ca.* 1.2

Fertigplatten

Grösste Abweichung vom Mittelwert	*ca.* 2 hR_F
Durchschnitt der mittleren Abweichungen vom Mittelwert ($\overline{M.A.}$)	*ca.* 0.4 hR_F
Mittlere Standardabweichung ($\bar{s}$)	*ca.* 0.9

(11) Zur Reinigung der Glasplatten wird zunächst die Schicht mit einem Holzspatel abgestreift und unter fliessendem, ca. 40° warmem Wasser gespült. Mit einem

Reinigungsmittel wie "Vim" (ohne Sand) und einer weichen Bürste wird bis zur befriedigend ausfallenden "Wasser-Ablauf-Probe" (ohne Schlieren!) gesäubert, mit destillirtem Wasser nachgespült und in das Desaga-Abtropf-Gestell aus Metall gebracht. Dort werden die Platten noch beidseitig mit Methanol abgespült, ehe sie trocknen. Zweckmässigerweise trägt man bei der Reinigung Gummihandschuhe.

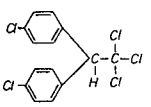
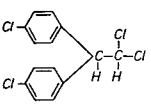
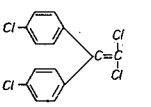
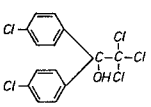
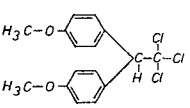
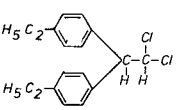
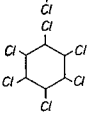
(12) Nach dem beschriebenen Chromatographier-Verfahren wurden auch Fertigplatten der Firma Merck: DC-Alu-Folien erfolgreich eingesetzt.

VERFAHREN ZUR IDENTIFIZIERUNG DER CHLORKOHLENWASSERSTOFFINSEKTIZIDE

In Tabelle II sind die verwendeten Wirkstoffe beschrieben.

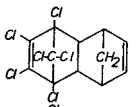
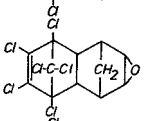
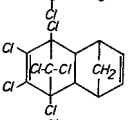
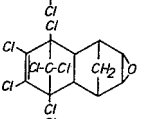
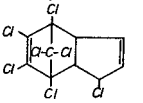
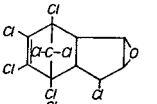
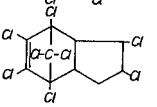
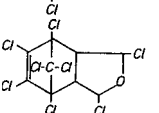
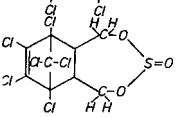
TABELLE II

VERWENDETE WIRKSTOFFE

Wirkstoffe	*Formel*	*Herkunft*	*Verwendete Qualität*
DDT		Schering "techn."	Umkristallisiert aus Methanol und Tetrachlorkohlenstoff Schmp. 105–107°
DDD		Geigy, Basel "99.8%"	Original Schmp. 109–110°
DDE		Geigy, Basel "rein, 99.3%"	Original Schmp. 89–90°
Dicofol		BBA, Braunschweig "Kelthane, rein"	Original Schmp. 76–77°
Methoxychlor		Geigy, Basel "rein, 99.8%"	Original Schmp. 89°
Perthan		Rohm & Haas "techn."	Auf Tonplatten gereinigt Schmp. 55–58°
Hexachlorbenzol		BBA, Braunschweig	Original Schmp. 226–227°
Lindan		Marktredwitz "*ca.* 99%"	Original Schmp. 113–114°

(Vortsetzung auf S. 88)

TABELLE II *(Vortsetzung)*

Isodrin		BBA, Braunschweig "rein"	Original Schmp. > 200°
Endrin		Schering "techn. 99%"	Umkristallisiert aus Methanol Zers. 200°
Aldrin		Shell "techn. 94%"	Umkristallisiert aus Aceton und Heptan–Methanol Schmp. 103–104°
Dieldrin		Marktredwitz *"ca.* 96%"	Umkristallisiert aus Methanol und Heptan–Methanol Schmp. 173–174°
Heptachlor		Cela "72%"	Umkristallisiert aus Butanol und 50%igem Alkohol Schmp. 93–94°
Heptachlorepoxid		Velsicol "96.2%"	Original Schmp. 161–162°
Chlordan		Velsicol "reference grade"	Original
Isobenzan		Shell "Telodrin techn."	Umkristallisiert aus Benzol Schmp. 122–124°
Endosulfan		Shell "rein, Isomere I + II im Verh. 2:1"	Original Schmp. 86–96°
Toxaphen	chloriertes Camphen	Merck "techn. ca. 95%"	Original

System I

4.0 g Kieselgel G suspendiert in 15 ml Essigester für eine 20 × 20 cm-Platte; Vorklima: H_2SO_4 $\varrho = 1.35$; Konterplatte analog; Fliessmittel: Cyclohexan; Strecke: 10 cm sollen in 42 min durchflossen sein; Standard: Isobenzan, Sollwert $hR_F = 30$; hR_F-Wert-Schema: Tabelle III.

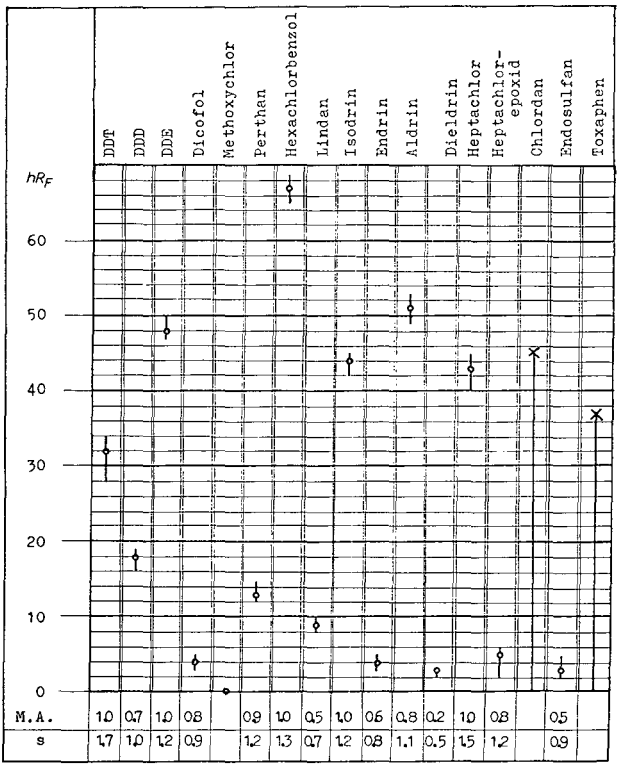
System II

3.26 g Magnesiumsilikat DC Woelm 30–40 μm, suspendiert in 15 ml 90%igem Äthanol für eine 20 × 20 cm-Platte; Vorklima: H_2SO_4 $\varrho = 1.35$; Konterplatte analog*; Fliessmittel: Cyclohexan; Strecke: 10 cm sollen in $2\frac{1}{2}$ h durchflossen sein; Standard: DDD, Sollwert $hR_F = 25$; hR_F-Wert-Schema: Tabelle IV.

* Bei diesem System wurde erstmals eine zweite Analysenplatte als Konterplatte mit der ersten zu einem Sandwich vereinigt. Beide Platten hatten stets ideale Sollwerte und gute R_F-Werte. Nahezu 50% Zeit- und Materialeinsparung!

TABELLE III

hR_F-WERTE DER CHLORKOHLENWASSERSTOFF-INSEKTIZIDE IM SYSTEM I



System III

Polyamid-DC-Alufolie F 254, Merck, Schichtdicke 0.15 mm, 20 × 20 cm; Vorklima: H_2SO_4 $\varrho = 1.35$; Konterplatte: 7.5 g Polyamid DC Woelm, suspendiert in 45 ml Chloroform–Methanol (2:3) wurden auf 5 Platten gestrichen. Vorklima: analog Fertigplatte; Fliessmittel: 80%iges Äthanol; Strecke: 10 cm sollen in 3 h durchflossen sein; Standard: Lindan, Sollwert $hR_F = 35$; hR_F-Wert-Schema: Tabelle V.

System IV

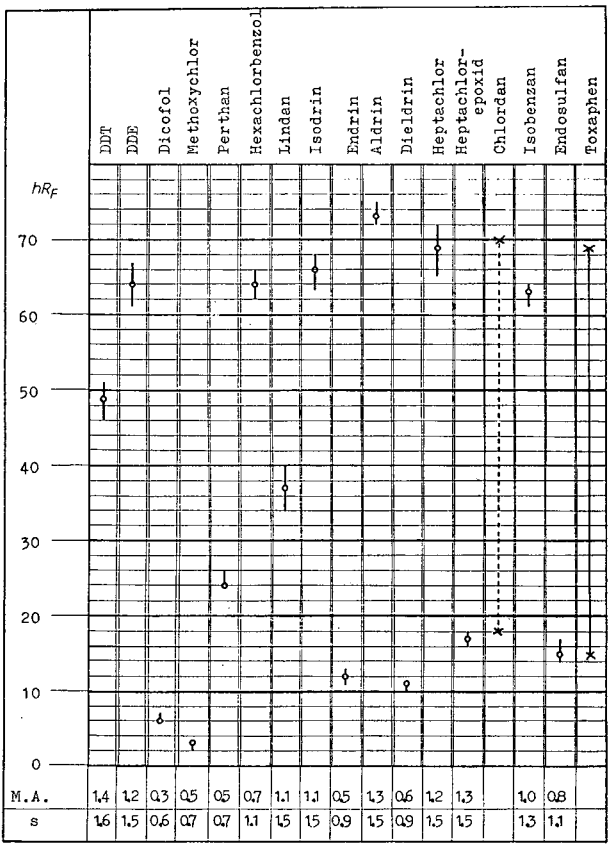
Kieselgel-DC-Alufolie, Merck, Schichtdicke 0.25 mm, 20 × 20 cm; Vorklima: H_2SO_4 $\varrho = 1.35$; Konterplatte: unbeschichtete Glasplatte; Fliessmittel: Cyclohexan–Aceton (9:1); Strecke: 10 cm sollen in 50 min durchflossen sein; Standard: Isobenzan, Sollwert $hR_F = 67$; hR_F-Wert-Schema: Tabelle VI.

Die Flecke wurden sichtbar gemacht mit nachstehenden Sprühmitteln nebeneinander oder alternativ:

(a) Mit 0.25 mg Rhodamin B pro 20 × 20 cm imprägnierte Platten wurden leicht mit *ca.* 20 ml Wasser aus *ca.* 40–50 cm Entfernung besprüht. Betrachtet wird unter einer UV-Lampe, deren Strahlenquelle von $\lambda = 254$ nm 15 cm entfernt ist. Untere Nachweisgrenze bei 0.1 μg.

TABELLE IV

hR_F-WERTE DER CHLORKOHLENWASSERSTOFF-INSEKTIZIDE IM SYSTEM II



(b) *Ca.* 15 ml 0.005% wässrige Rhodamin B-Lösung wurde aus *ca.* 35 cm Entfernung gesprüht. Anschliessend erfolgte Nachsprühen mit *ca.* 1 ml 10%iger Sodalösung. Erkennung wie unter (a). Untere Nachweisgrenze 0.3–0.1 μg.

(c) In eine Lösung von 0.2 g Na in absolutem Äthanol wird kurz vor dem Sprühen 0.1 g N,N-Dimethyl-*p*-phenylendiaminhydrochlorid zugefügt. *Ca.* 5 ml dieser Lösung werden im Abstand von *ca.* 35 cm auf die Platte gesprüht, danach noch reichlich Wasser. Nach 1–20 min Bestrahlung mit ungefiltertem UV-Licht (evtl. ist nachzufeuchten) sind bei Tageslicht braune Flecke auf sandfarbenem Untergrund sichtbar. Untere Nachweisgrenze 0.1–0.6 μg.

RESULTATE UND DISKUSSION

In den Tabellen III–VI sind für die Chromatographiersysteme I–IV die hR_F-Mittelwerte der Substanzen, gekennzeichnet durch ein ○, eingetragen. Die daran anschliessenden senkrechten Striche geben die jeweiligen festgestellten Streuamplituden (also grösste Abweichung vom Mittelwert nach beiden Seiten) wieder. In der vorletzten

TABELLE V

hR_F-WERTE DER CHLORKOHLENWASSERSTOFF-INSEKTIZIDE IM SYSTEM III

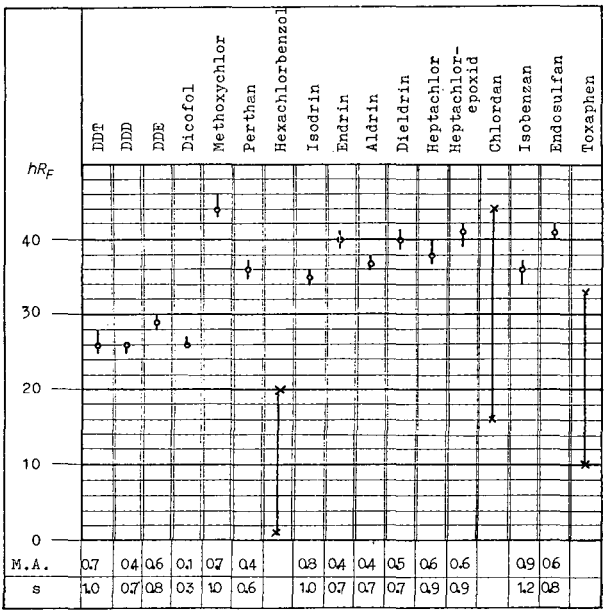
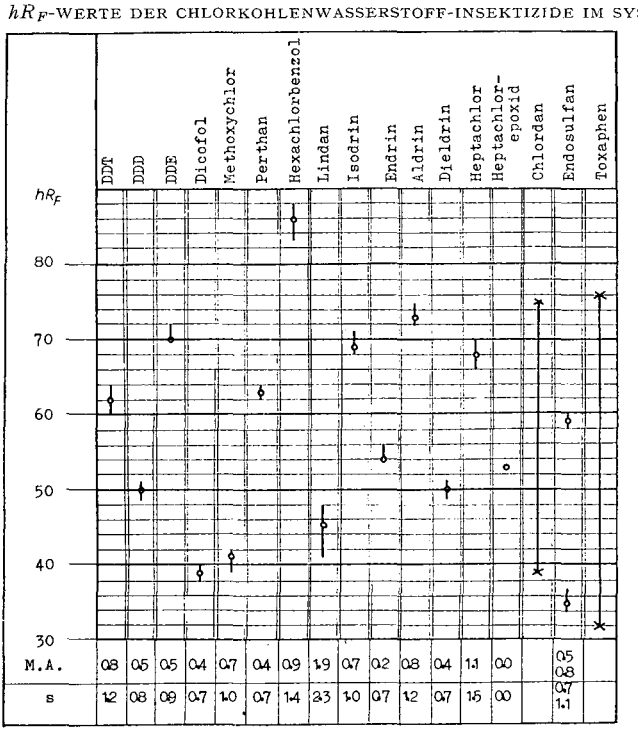


TABELLE VI

hR_F-WERTE DER CHLORKOHLENWASSERSTOFF-INSEKTIZIDE IM SYSTEM IV



Spalte charakterisiert die mittlere Abweichung vom Mittelwert ($\overline{M.A.}$; in hR_F-Einheiten) die Häufigkeit und das Ausmass der Streuungen. In der untersten Spalte finden sich die den einzelnen Wirkstoffen zugehörigen Standardabweichungen.

$$s = \sqrt{\frac{\Sigma\,(hR_F - \overline{hR_F})^2}{n-1}}$$

Allen Angaben liegen die Werte zugrunde, die an mindestens 10 verschiedenen Tagen gewonnen wurden.

Mit diesen Systemen können alle der in jeweils einem der vier Systeme interferierenden Komponenten letztendlich voneinander unterschieden werden. So werden die in System I interferierenden Isodrin–Heptachlor bei den Systemen II und III schon besser differenziert. Endrin, Endosulfan, Dieldrin, Dicofol und Heptachlorepoxid—in System I schwer unterscheidbar—sind in II oder III gut bis befriedigend, in IV z.T.

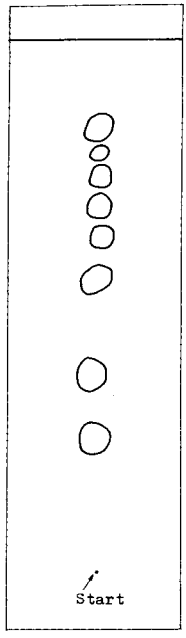


Fig. 2. Dünnschichtchromatogramm von Chlordan an Magnesiumsilikat. Fliessmittel: Cyclohexan.

sehr gut voneinander getrennt. DDT–Isobenzan trennen sich in II und III gut; für Dieldrin–Endrin ist System IV nötig. Bei Gegenwart von Chlordan und Toxaphen sind die übrigen Wirkstoffe nicht erkennbar. Chlordan zeichnet sich vor Toxaphen dadurch aus, dass es sich im System II in 8 Komponenten zerlegen lässt (vgl. Fig. 2), während Toxaphen in allen Systemen ein Band darstellt. Die Bereiche, in denen solche "Bänder" zu erwarten sind, werden in den Tabellen III bis VI mit $\times$—$\times$ gekennzeichnet.

Selbstgegossene Polyamidplatten gelangen uns bisher ohne Binder nur mit zu geringer Haftfestigkeit, so dass gut reproduzierbare Werte nur mit Fertigplatten er-

halten wurden. Anwendung einer beschichteten Konterplatte ist hier vorteilhaft, um die Chromatographierzeit erträglich zu halten.

Zur guten Fixierung der Alu-Folien hat sich ein kleiner Trick bewährt: Ein Tropfen Fliessmittel zwischen Glasplatte und glatter Folienseite hält die Folie schon bei der Probenaufgabe in Position.

Die beschriebene Kombination der 4 Systeme soll als ein Muster gelten, wie man mindestens 16 Vertreter einer Wirkstoffklasse dünnschichtchromatographisch mit grosser Sicherheit bereits auf Grund ihrer R_F-Werte identifizieren kann. Durch Vortrennen schon auf Grund markanter Löslichkeitsunterschiede etwa nach der Art, wie bei THIER UND BERGNER[23] angegeben, kann man einige Pflanzenschutzmittelwirkstoffklassen voneinander trennen, z.B. die Chlorkohlenwasserstoffinsektizide von den Phosphorsäureesterinsektiziden. Für die letzteren hat übrigens GUTH[24] einen Trennungsgang nach Art der klassischen Analyse auf dünnschichtchromatographischem Wege ausgearbeitet. Wir halten unser R_F-Wert-System für vielseitiger und sicherer, da es auch nach Hinzukommen neuer Vertreter dieser Wirkstoffklasse oder bei Einbeziehen weiterer Metaboliten unbeeinträchtigt funktioniert und zudem den Vorzug der übersichtlichen Einfachheit besitzt.

Wir glauben, dass auch in Gegenwart von nennenswerten Mengen an Pflanzeninhaltsstoffen in den Proben die R_F-Werte wenig beeinflusst werden. Zumindest wurde dies von MENDOZA *et al.*[25] für die Phosphorsäureesterwirkstoffe nachgewiesen.

Kürzlich[26] wurden Pflanzenschutzmittel aller Wirkstoffklassen an Florisil, einem Magnesiumsilikat, chromatographiert. Die bekannte Chargenunterschiedlichkeit des Florisils zwang erwartungsgemäss diese Autoren, ein Farbstoff-Testgemisch mit zu chromatographieren. Nicht zuletzt wegen seines basischen Charakters halten wir Florisil—ebenso wie die meisten Al_2O_3-Sorten—für das Chromatographieren mancher leicht zersetzlicher Wirkstoffe nicht für geeignet.

DANK

Besonderer Dank gebührt Frl. R.-M. ARLT und Frau A.-M. TURGAY für äusserst geschickte und sorgfältige Mitarbeit. Ferner danke ich den in Tabelle II genannten Firmen für die freundliche Überlassung der Wirkstoffe.

ZUSAMMENFASSUNG

Es wurde ein Rahmenverfahren zur Erzielung sicher reproduzierbarer R_F-Werte mit vorzugsweise einkomponentigen Fliessmitteln beschrieben. Die grössten Abweichungen vom Mittelwert betragen nicht mehr als 3 hR_F, die mittleren Abweichungen vom Mittelwert 0.8 hR_F, die mittleren Standardabweichungen $\pm$ 1.2. Das Verfahren benötigt nur einfache und billige Laborhilfsmittel und erlaubt einen hohen Plattendurchsatz pro Tag für Routineuntersuchungen. Mit hilfe dieses Rahmenverfahrens wurde ein kombiniertes System zur Identifizierung von mindestens 16 Chlorkohlenwasserstoffinsektiziden auf Grund ihrer gesicherten R_F-Werte entwickelt.

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CHROM. 4255

DÜNNSCHICHTCHROMATOGRAPHISCH-ENZYMATISCHER NACHWEIS UND ZUM WIRKUNGSMECHANISMUS VON CHLORKOHLENWASSERSTOFF-INSEKTIZIDEN

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SUMMARY

Thin-layer chromatographic–enzymatic identification and the mode of action of chlorinated hydrocarbon insecticides

1. It is shown that chlorinated hydrocarbons influence the activity of bovine liver esterase.

2. Most of the compounds inhibit the enzyme, but DDT, DDD, DDE, Perthane, and methoxychlor activate it.

3. After UV-irradiation on thin-layer plates all compounds show strong inhibition activity against bovine liver esterase.

4. Almost all of the chlorinated hydrocarbons studied are decomposed by UV-irradiation as was shown by their R_F-values obtained from thin-layer chromatography on Silica Gel G. Although DDT inhibits bovine liver esterase after UV-irradiation its insecticidal activity is lost, and it is supposed that this effect is due to inability of the new compound to penetrate the insect.

EINLEITUNG

Chlorierte Kohlenwasserstoffe sind schon relativ lange als Insektizide bekannt. Wenn sie auch heute immer mehr von den weniger persistenten Organophosphaten und Carbamaten verdrängt werden, haben sie ihre Stellung dennoch auf einer Reihe von Anwendungsgebieten behaupten können.

Über ihren Wirkungsmechanismus liegen trotz jahrzehntelanger Forschung nur recht spärliche Informationen vor, während es, hervorgerufen durch das grosse Interesse der Öffentlichkeit an der Detoxifizierung dieser äusserst persistenten Insektizide, über den Abbau im allgemeinen und den Stoffwechsel im besonderen zahlreiche Publikationen gibt.

Man weiss mit ziemlicher Sicherheit, dass diese Substanzen primär auf das Nervensystem einwirken, wobei das DDT in dieser Beziehung am besten untersucht ist. Ältere Arbeiten[1,2] nahmen an, dass eher die motorischen als die sensorischen Nerven die Angriffspunkte darstellen, doch konnte inzwischen das Gegenteil bewiesen werden.

Hohe DDT-Konzentrationen von 1000 p.p.m. zeigten zwar bei amerikanischen Schaben eine Wirkung auf motorische Nerven und Muskelfasern, niedrige von 0.01 p.p.m. hingegen wirkten weder auf die genannten Gewebe noch auf das Zentralnervensystem, hatten andererseits aber einen starken Effekt auf die sensorischen Nerven[3,4]. In welcher Weise DDT jedoch am sensorischen Nerven eingreift, ist noch völlig ungeklärt. Zwar konnte gezeigt werden, dass es die K^+-Permeabilität des Nervengewebes erhöht[5] und mit Bestandteilen der Nervenmembranen Komplexe bildet[5,6], doch wird damit noch nichts über den eigentlichen Wirkungsmechanismus ausgesagt.

Noch weniger ist über den Wirkungsmechanismus der anderen Chlorkohlenwasserstoffe bekannt. Lediglich für Lindan wurden Veränderungen der Zellstruktur im Blut, im Fettkörpergewebe, in der Nervensubstanz und den Zellen fast aller Organe nachgewiesen[7,8].

TABELLE I

NAME UND STRUKTUR DER UNTERSUCHTEN CHLORKOHLENWASSERSTOFFE

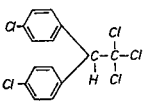
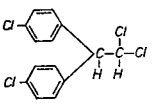
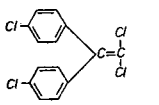
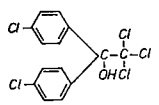
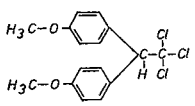
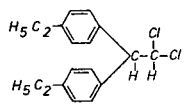
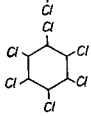
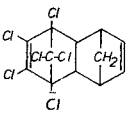
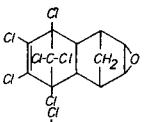
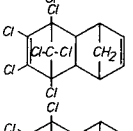
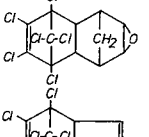
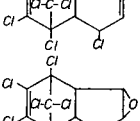
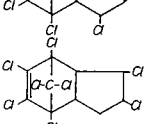
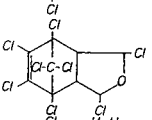
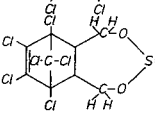
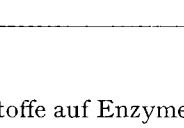
Trivial-Name	*Chemische Bezeichnung*	*Strukturformel*
DDT	2,2-Bis-(*p*-chlorphenyl)-1,1,1-trichloräthan	
DDD (= TDE)	2,2-Bis-(*p*-chlorphenyl)-1,1-dichloräthan	
DDE (DDT-Olefin)	2,2-Bis-(*p*-chlorphenyl)-1,1-dichloräthylen	
Dicofol (Kelthan)	1,1-Bis-(*p*-chlorphenyl)-2,2,2-trichloräthanol	
Methoxychlor	2,2-Bis-(*p*-methoxyphenyl)-1,1,1-trichloräthan	
Perthan	2,2-Bis-(*p*-äthylphenyl)-1,1-dichloräthan	
Hexachlorbenzol	1,2,3,4,5,6-Hexachlorbenzol	
Lindan	γ-1,2,3,4,5,6-Hexachlorcyclohexan	

TABELLE I *(Vortsetzung)*

Isodrin	1,2,3,4,10,10-Hexachlor-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-endo-dimethannaphthalin	
Endrin	1,2,3,4,10,10-Hexachlor-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-5,8-endo-dimethannaphthalin	
Aldrin	1,2,3,4,10,10-Hexachlor-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-exo-dimethannaphthalin	
Dieldrin	1,2,3,4,10,10-Hexachlor-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-5,8-exo-dimethannaphthalin	
Heptachlor	1,4,5,6,7,8,8-Heptachlor-3a,4,7,7a-tetrahydro-4,7-endomethylen-inden	
Heptachlor-Epoxid	1,4,5,6,7,8,8-Heptachlor-2,3-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-endomethylen-inden	
Chlordan	1,2,4,5,6,7,8,8-Octachlor-2,3,3a,4,7,7a-hexahydro-4,7-endomethylen-inden	
Isobenzan (Telodrin)	1,3,4,5,6,7,8,8-Octachlor-1,3,3a,4,7,7a-hexahydro-4,7-methanisobenzofuran	
Endosulfan (Thiodan)	6,7,8,9,10,10-Hexachlor-1,5,5a,6,9,9a-hexahydro-6,9-methan-2,4,3-benzoedioxathiepin-3-oxid	
Toxaphen	chloriertes Camphen	

Über die Wirkung der chlorierten Kohlenwasserstoffe auf Enzyme liegen bisher nur spärliche Untersuchungen vor. DDT soll in den Aminosäurestoffwechsel eingreifen, den Wasserhaushalt stören sowie Atmungsfermente und den Wasserstofftransfer blockieren[9]. Unter anderem kommt es *in vitro* zu einer Hemmung der Succinat-Dehydrogenase und Cytochromoxidase[9].

Während die insektiziden Organophosphate und Carbamate durch ihre Hemmung der Cholinesterase einen Hinweis auf ihren Wirkungsmechanismus geben, der mit den beobachteten Vergiftungssymptomen übereinstimmt und diese Hemmeigenschaft in der Analytik dieser Verbindungen eine grosse Bedeutung gewinnt, sprechen alle bisher verfügbaren Daten gegen eine Hemmung der Cholinesterase durch chlorierte Kohlenwasserstoffe[9,10]. Bei Modellversuchen zum Nachweis esterasehemmender

Insektizide mit Benzhydroxamsäure konnte EBING[11] jedoch zeigen, dass auch eine Reihe von Chlorkohlenwasserstoffen mit dieser Modellsubstanz wie Esterasehemmer reagieren und auf diese Weise nachzuweisen sind. Es soll hier über erste Untersuchungen zur Wirkung von Chlorkohlenwasserstoff-Insektiziden auf Rinderleber-Esterase auf dünnschichtchromatographischer Basis berichtet werden.

MATERIAL UND METHODEN

Reagenzien

Kieselgel G nach Stahl mit *ca.* 13% $CaSO_4$—mittlere Korngrösse 10–40 μ (Merck, Darmstadt); Methylenchlorid p.A.; Cyclohexan p.A.

Enzym- und Substratlösung

Die Enzympräparation erfolgte in Anlehnung an ACKERMANN[12], doch wurde in einem Bühler-Homogenisator mit 0.02 *M* Phosphatpuffer pH 7.0 statt des Wassers 2 min bei Höchstgeschwindigkeit homogenisiert und 10 min bei 10 000 × *g* zentrifugiert. Zur Verdünnung des Rohextraktes wurde der gleiche Puffer genommen. Als Substrat diente eine kurz vor dem Besprühen angesetzte Mischung aus einer äthanolischen β-Naphthylacetat-Lösung und einer wässrigen Echtblausalz B-Lösung in den von ACKERMANN[12] angegebenen Konzentrationen.

Dünnschichtchromatographie und Insektizidlösungen

Die untersuchten Wirkstoffe in analytischer Standardqualität (Tabelle I) wurden als 0.1%ige Lösung in Methylenchlorid angesetzt und auf handgegossene Kieselgel G-Platten (5.7 g Kieselgel G werden in 15 ml Wasser suspendiert, auf eine gut gereinigte Glasplatte im Format 20 × 20 cm aufgebracht und über Nacht bei Zimmertemperatur getrocknet) aufgetragen und in Cyclohexan entwickelt.

Durchführung des enzymatischen Hemmtestes

Die Platten werden nach dem Entwickeln entweder sofort oder nach halbstündiger Bestrahlung mit ungefiltertem UV-Licht einer Hg-Analysen-Quarzlampe (Hanau) mit einem Abstand Strahler-Platte von 30 cm mit Enzymlösung besprüht und in der von ACKERMANN[12] beschriebenen Weise weiterbehandelt. Die Beurteilung erfolgt im Falle der Aktivierung kurz nach dem Besprühen mit Substrat, im Falle der Hemmung zu einem späteren Zeitpunkt. Die Platten sind anfangs vor übermässiger direkter Lichteinstrahlung zu schützen.

Biotest zur Wirkung von unbehandeltem und UV-bestrahltem DDT

Die Versuche wurden in Glaszylindern (7.5 × 11.5 cm) durchgeführt, auf deren Innenoberfläche der Wirkstoff aus einer Acetonlösung gleichmässig verteilt wurde. Die eine Hälfte der Ansätze blieb unbehandelt, die andere Hälfte wurde 30 min mit ungefiltertem UV-Licht bestrahlt, wobei das UV-Licht von oben in die Zylinder eingestrahlt wurde. Pro Testansatz wurden 50 Drosophila genommen.

ERGEBNISSE UND DISKUSSION

Unter den hier angewandten Bedingungen reagieren, wie aus Tabelle II her-

TABELLE II

UNTERE NACHWEISGRENZEN DER CHLORKOHLENWASSERSTOFFE MIT UND OHNE UV-BESTRAHLUNG INFOLGE HEMMUNG BZW. AKTIVIERUNG VON RINDERLEBER-ESTERASE

Nachweisgrenze in μg; a = Aktivierung; s = Fahnenbildung.

Substanz	*Ohne UV-Bestrahlung*	*Mit UV-Bestrahlung*	*Substanz*	*Ohne UV-Bestrahlung*	*Mit UV-Bestrahlung*
DDT	5 a	1	Isodrin	50	0.25
DDD	5 a	1	Endrin	1	0.3
DDE	6 a	1	Aldrin	10	0.25
Dicofol	1	1	Dieldrin	1	1
Methoxychlor	5 a	1	Heptachlor	1	0.25
Perthan	2 a	1	Heptachlorepoxid	1 s	0.3 s
Hexachlorbenzol	—	1	Chlordan	5 s	1 s
Lindan	1	0.25	Isobenzan	5	0.25
Toxaphen	1 s	1 s	Endosulfan	2	0.3

vorgeht, grundsätzlich alle untersuchten Chlorkohlenwasserstoffe ausser Hexachlorbenzol mit Rinderleberesterase. Diese Ergebnisse stehen im Gegensatz zu der bisher vertretenen Ansicht (vergl. Lit. 9, 10 und 13), dass chlorierte Kohlenwasserstoffe keinen Einfluss auf die Cholinesterase haben. Die Chlorkohlenwasserstoffe DDT, DDD, DDE, Perthan und Methoxychlor aus der DDT-Gruppe zeigen im Gegensatz zu den anderen chlorierten Kohlenwasserstoff-Insektiziden die Eigentümlichkeit, die Leberesterase zu aktivieren. Diese Aktivierung äussert sich auf der Dünnschichtplatte in der Weise, dass an den Stellen, wo sich die genannten Substanzen befinden, sehr schnell rötliche Flecken auf sich langsam färbendem hellen Untergrund auftreten. Sie verschwinden jedoch nach einiger Zeit, wenn die Substratspaltung auf der Platte weiter fortgeschritten ist. Aus diesem Grunde entgingen sie anfänglich dem Nachweis, da die Dünnschichtplatten erst nach abgeschlossener Färbung ausgewertet wurden, während später eine kontinuierliche Auswertung vorgenommen wurde. Diese Beobachtungen konnten inzwischen durch eingehendere Untersuchungen mit Humanserum-Esterase bestätigt werden[14], und auch BOGUSZ[15] konnte an Arbeitern, die mit Chlorkohlenwasserstoffen in Berührung gekommen waren, ähnliche Tendenzen nachweisen, wenn uns die Daten auch nicht signifikant zu sein scheinen.

Durch Aktivierung mit UV-Bestrahlung kann der Nachweis der Chlorkohlenwasserstoff-Insektizide zum Teil erheblich gesteigert werden (Tabelle II), wobei alle untersuchten Wirkstoffe—auch die oben genannten aus der DDT-Gruppe—ausnahmslos eine Hemmung der Leberesterase bewirken. Die Nachweisgrenzen liegen dabei mit und ohne Aktivierung durch UV-Bestrahlung erheblich unter den beim chemischen Nachweis mit Benzhydroxamsäure erzielten Werten[11]. Aus dem Verhalten der Insektizide beim Nachweis mit Esterase und den teilweise extremen Unterschieden in der Nachweisempfindlichkeit ist anzunehmen, dass die Reaktionen mit Benzhydroxamsäure und Esterase nach völlig verschiedenen Mechanismen ablaufen. Ob es sich im Falle der Esterasebeeinflussung durch Chlorkohlenwasserstoffe um eine Reaktion der Substanzen mit dem aktiven Zentrum oder um eine Bindung an allosterische Orte handelt, ist zur Zeit nicht zu entscheiden.

Während bei Organophosphat-Insektiziden eine Aktivierung durch UV-Bestrahlung und gesättigte wässrige Bromlösung gleichermassen gut möglich war[12], führt eine Behandlung der Platten mit gesättigter wässriger Bromlösung im Falle der

chlorierten Kohlenwasserstoffe zum völligen Misserfolg, da dann nur noch wenige Verbindungen (DDT, DDE und Dicofol) nachzuweisen sind. Auch eine Verminderung der Schichtdicke führte zu einem wesentlich schlechteren Nachweis. Der Einfluss grösserer Schichtdicken wurde hingegen nicht untersucht. Eine ähnliche Abhängigkeit der Nachweisempfindlichkeit von der Schichtdicke der Platten fanden auch MENDOZA *et al.*[16] beim Nachweis von Organophosphaten mit Esterase.

Die zum Teil erhebliche Steigerung der Nachweisempfindlichkeit nach UV-Bestrahlung lässt auf eine Veränderung des Moleküls schliessen, die sich in einer Änderung des R_F-Wertes bemerkbar machen müsste. In Tabelle III sind die hR_F-Werte der Chlorkohlenwasserstoff-Insektizide bei UV-Bestrahlung vor und nach dem Entwickeln in Cyclohexan gegenübergestellt. Es zeigt sich, dass alle Wirkstoffe mehr oder weniger stark verändert wurden; denn alle bleiben bei vorheriger Behandlung am Start zurück, während sie bei nachfolgender Behandlung die üblichen R_F-Werte zeigen. Lediglich bei Lindan und Isobenzan findet man neben dem Abbauprodukt grosse Mengen der Ausgangssubstanz. Ob Methoxychlor, Endosulfan und Dieldrin sowie Chlordan und Heptachlorepoxid Veränderungen erfahren haben oder nicht, ist aufgrund des R_F-Wertes in nur einem verwandten Laufmittel nicht zu entscheiden, was

TABELLE III

hR_F-WERTE DER CHLORIERTEN KOHLENWASSERSTOFF-INSEKTIZIDE BEI AKTIVIERUNG DURCH UV-BESTRAHLUNG VOR UND NACH DEM ENTWICKELN IN CYCLOHEXAN

Substanz	*Aktivierung*		*Substanz*	*Aktivierung*	
	vor Entwicklung	*nach Entwicklung*		*vor Entwicklung*	*nach Entwicklung*
DDT	0	43	Isodrin	0	63
DDD	0	25	Endrin	0, 2	3.5
DDE	0	61	Aldrin	0, 2	64
Dicofol	0	3.4	Dieldrin	0	4
Methoxychlor	0	1	Heptachlor	0	55
Perthan	0	18	Heptachlorepoxid	0	0, 6, 13, 28
Hexachlorbenzol	0	85			
Lindan	0, 14	14	Chlordan	0	0, 23, 34, 48, 62
Toxaphen	0, 20	0, 12, 20, 28, 45	Isobenzan	0, 4, 44	44
			Endosulfan	0, 4	0, 6

im Rahmen dieser Arbeit allerdings auch von untergeordneter Bedeutung ist. Für eine Reihe von Chlorkohlenwasserstoffen liegen Untersuchungen über die Wirkung von UV-Bestrahlung vor. DDT zersetzt sich beispielsweise unter HCl-Abspaltung[17,18], wobei auch oxydative Vorgänge ablaufen—z.B. Bildung von 4,4'-Dichlorbenzophenon[18]—während Lindan beständig sein soll[10], was mit den vorliegenden Ergebnissen weitgehend übereinstimmt. Aldrin wird durch UV-Bestrahlung in Dieldrin überführt, das seinerseits bei feiner Verteilung in eine unbekannte Substanz übergehen soll[19]. Eine andere Arbeit (Lit. 20) berichtet, dass UV-Licht unterhalb 270 nm aus Dieldrin ein an der Doppelbindung stehendes Chloratom abspaltet und die gebildetete Pentachlorverbindung für Mäuse vierfach toxischer, für Fliegen hingegen halb so toxisch ist. Ähnliches gilt auch für Aldrin. Diese Ergebnisse korrelieren mit den hier berichteten sehr gut; denn die durch UV-Bestrahlung entstandenen Verbindungen sind stärkere

TABELLE IV

BEEINFLUSSUNG DER INSEKTIZIDEN WIRKUNG VON DDT DURCH UV-BESTRAHLUNG

Auswertung nach 24 h.

Aufwandmenge	*O-Kontrolle*	*Normal*		*UV*	
		10 µg	*20 µg*	*10 µg*	*20 µg*
Zahl der toten Tiere	5	35	47	7	9
Zahl der geschäd. Tiere	—	15	3	—	—

Esterasehemmer, was sich bei Mäusen, denen die Substanz peroral appliziert wird, in einer grösseren Toxizität der Bestrahlungsprodukte bemerkbar macht, während sie bei Fliegen schlechter einzudringen scheint. Über die Wirksamkeit dieser durch UV-Bestrahlung aus den Chlorkohlenwasserstoffen entstandenen Substanzen als Esterasehemmer liegen bisher noch keine Informationen vor.

Da UV-Bestrahlung bei den meisten chlorierten Kohlenwasserstoffen zu einer signifikanten Erhöhung der Anticholinesterase-Aktivität führte (Tabelle II), lag die Vermutung nahe, dass die eigentliche Wirkung dieser Insektizide nach Ausbringung teilweise auf einer solchen Aktivierung durch UV-Bestrahlung beruht. Es wurde deshalb für DDT untersucht, welchen Einfluss eine UV-Bestrahlung auf die insektizide Wirkung hat. Die Ergebnisse sind in Tabelle IV zusammengefasst. Es zeigt sich, dass die insektizide Wirkung nach UV-Bestrahlung trotz Bildung von Verbindungen, die die Leber-Esterase *in vitro* stärker hemmen, drastisch vermindert ist. HARRISON *et al.*[21] finden dagegen bei der Untersuchung handelsüblicher Formulierungen einer Reihe von Chlorkohlenwasserstoffen—darunter auch DDT—für DDT und einige andere Substanzen dieser Gruppe unter Einfluss von UV-Licht (künstliche Bestrahlung und Sonnenlicht) eine sehr grosse Persistenz.

Ähnliche, wenn auch nicht so ausgeprägte Tendenzen wie beim DDT, lassen sich auch bei den anderen Chlorkohlenwasserstoffen beobachten. Orientierende Vorversuche haben ergeben, dass lediglich bei Dicofol, Dieldrin und Heptachlorepoxid kein Unterschied zwischen behandelten und unbehandelten Ansätzen zu existieren scheint, während bei allen übrigen Chlorkohlenwasserstoff-Insektiziden durch UV-Behandlung eine Verminderung der insektiziden Aktivität einzutreten scheint. Ob die Ursache für die verminderte Aktivität der meisten Verbindungen in einer fehlenden Aufnahme dieser entstandenen Antiesterase-Substanzen in den Körper zu suchen ist, oder wir uns über den Wirkungsmechanismus völlig falsche Vorstellungen machen, muss späteren Untersuchungen mit markierten Wirkstoffen vorbehalten bleiben. Da durch die Bestrahlung aber wahrscheinlich polare Substanzen entstanden sind, dürfte wohl eher eine verminderte Aufnahme als Ursache für die verminderte insektizide Aktivität in Frage kommen.

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Mein besonderer Dank gilt Frau R. RAUBE für die sorgfältige Mitarbeit bei der Durchführung der Versuche sowie den Firmen Cela, Geigy, Marktredwitz, Merck, Rohm & Haas, Schering, Shell und Velsicol für die Überlassung der Wirkstoffe.

ZUSAMMENFASSUNG

1. Es wird gezeigt, dass chlorierte Kohlenwasserstoffe die Aktivität der Rinderleber-Esterase beeinflussen.

2. Die meisten Verbindungen hemmen das Enzym, während DDT, DDD, DDE, Perthan und Methoxychlor es aktivieren.

3. Nach UV-Bestrahlung der Dünnschichtplatten zeigen alle Verbindungen eine starke Hemmung der Rinderleber-Esterase.

4. Fast alle untersuchten chlorierten Kohlenwasserstoffe werden, wie an Hand der R_F-Werte nach Dünnschichtchromatographie an Kieselgel G gezeigt wird, durch UV-Bestrahlung zersetzt. Obwohl DDT die Rinderleber-Esterase nach UV-Bestrahlung hemmt, verliert es seine insektizide Wirkung, und es wird angenommen, dass dieser Effekt auf der Unfähigkeit der neuen Verbindung beruht, in das Insekt einzudringen.

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CHROM. 4251

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF THE β-FLUOROETHYL ESTER OF XENYL ACETIC ACID (M 2060) IN THE TECHNICAL PRODUCT AND IN 2% LIQUID FORMULATIONS*

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SUMMARY

Two methods, based on preliminary thin-layer chromatography, have been developed for determining the β-fluoroethyl ester of xenyl acetic acid in the technical product and the 2% liquid formulation.

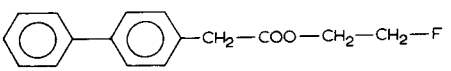
The acaricide, or a convenient amount of the formulation, is dissolved in acetone and the solution is spotted on a thin-layer chromatographic plate.

After development and identification by means of a UV lamp, the M 2060 area is isolated, detached and eluted. In the case of the determination of the active ingredient in the technical product, hydrolysis is conducted on the eluate residue, followed by titration of the resulting xenyl acetic acid.

In the liquid formulation, dehalogenation of the eluate residue is carried out with sodium biphenyl and fluorine is determined colorimetrically, by means of cerium nitrate and alizarin complexone, under special conditions.

INTRODUCTION

The β-fluoroethyl ester of xenyl acetic acid (M 2060)** is a compound that was discovered by the Agricultural Research Institute of the Montecatini Edison Compa-



ny, and is characterized by an extremely high level of activity against the eggs of mites even when used in mid-winter treatments[1,2].

This is the outstanding property which distinguishes M 2060 from any other acaricide on the market. In view of its effectiveness against pests and its high toxicity for warm-blooded animals, this product was formulated for winter use only, and for

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** Italian patent No. 710,046, granted June 27, 1966. Proposed common name (ISO) for the active ingredient: fluenethyl.

simultaneous control of the hibernating stages of aphids, scales, Psyllae and mites, and consists of 2% active ingredient in mineral oil.

The method, developed for the determination of the active ingredient in the technical product and the formulation, is based on a combination of thin-layer chromatography with a final determination procedure which is both specific and accurate. This combination has already been successfully adopted for the analysis of the most varied types of product[3–6] and has often permitted analytical difficulties, arising from the presence of products with very similar physico-chemical properties, to be overcome.

In the technical product, M 2060 is separated by thin-layer chromatography, eluted and hydrolyzed. The xenyl acetic acid formed is titrated with alkali, according to a method already used for organic acid esters[7–9].

In the case of the 2% liquid formulation, the evaluation procedure of the active ingredient had to be modified, due to the fact that a thorough separation of the acaricide from formulants is not practicable by means of thin-layer chromatography and this causes interference in the final volumetric titration. To obviate this, the determination of M 2060 *via* its fluorine is carried out after chromatography.

The compound is dehalogenated with sodium biphenyl[10] and the resulting fluoride is treated with cerium nitrate and alizarin complexone, in order to obtain a coloured compound which can be subjected to photometric determination[11,12].

It was thought less advisable to use the colorimetric method for the determination of the active ingredient in the technical product because this procedure provides less accurate and precise results than those obtained by volumetric titration, and this may have some bearing on the analysis of highly purified products.

EXPERIMENTAL

Determination of M 2060 in the technical product

Of thoroughly homogenized sample 2.5 g is weighed accurately into a 50 ml volumetric flask, dissolved in acetone and diluted to volume. With a controlled pipette, 1 ml of acetone solution is applied as a uniform thin streak (14 cm long and 3 cm from bottom edge) to a glass plate (20 × 20 cm) covered with a 1 mm layer of Silica Gel $HF_{254+366}$. The outside of the tip of the pipette is rinsed with a few drops of acetone by means of a glass capillary.

After the solvent has completely evaporated, the chromatogram is developed with *n*-hexane–ethyl acetate (9:1), in a saturated chamber, until the solvent front reaches 3 cm from the upper scored line. This operation is repeated four times. When the plate is completely dry, it is exposed to UV light in order to visualize the area containing the M 2060 ($R_F = 0.45$). This area is marked, allowing a safety margin depending on the presence of other compounds and the silica gel from around the zone considered is completely removed with a microscope slide. The M 2060 area is then scraped off and transferred quantitatively onto a 10 G 4 Jena glass crucible mounted on a vacuum assembly, and eluted with 30–40 ml acetone.

The solvent is allowed to remain in contact with the adsorbent for a few minutes before filtering. It is filtered, under vacuum, directly into a 100 ml Kjeldahl flask. After each wash with acetone, the vacuum is released and the silica gel in the crucible is carefully stirred with a glass rod. Using a rotating evaporator, the filtrate

is cautiously evaporated at 35° (maximum) nearly to dryness; 20 ml methanol is added followed by 10 ml methanolic 2 *N* KOH. A few glass beads are introduced and the flask is placed under a condenser and gently refluxed for 1 h. The solution is transferred to a 400 ml beaker, washed with up to 150 ml water and concentrated to about 30 ml; a further 100 ml water is added and the solution is boiled again to 50 ml in order to eliminate all the methanol. It is then transferred to a 250 ml separatory funnel, washed with 50 ml water, neutralized to phenolphthalein with HCl and 5 ml excess is added.

The hydrolysate is extracted with three 50 ml portions of ethyl ether, after stirring each time for 1 min, and allowing the phases to separate thoroughly. The ether extracts are combined and washed with three 10 ml portions of a saturated solution of NaCl, and filtered through a small cotton plug into a 300 ml erlenmeyer flask. The separatory funnel and the cotton are washed with a further 50 ml ether. The solvent is evaporated to dryness by means of the rotating evaporator and the last traces of HCl are removed by a current of air.

Acetone (14 ml) and CO_2-free water (6 ml) are added to dissolve the residue, 2 further drops of phenolphthalein are added and the solution is finally titrated with 0.01 *N* NaOH solution.

Determination of M 2060 in the liquid formulation

Of the homogenized formulation 1.25 g is weighed into an acid weighing bottle, transferred to a 25 ml volumetric flask, dissolved in acetone and diluted to volume. 1 ml of the acetone solution is applied to the thin-layer plate, by means of a controlled pipette, in a uniform thin streak 11 cm long, 3 cm from the right side of the plate and 3 cm from its bottom edge. A glass capillary is used to rinse the outside of the tip of the pipette with a few drops of acetone. On the left of the streak, 2 cm from the edge of the plate, a small amount (0.15 ml) of sample solution with the addition of 1 mg M 2060 is spotted; the purpose of this is to permit better detection of the M 2060 area.

Chromatographic development, as described for the determination of the active ingredient in the technical product, is then carried out. The area corresponding to the M 2060 is marked and eluted with acetone. Using the rotating evaporator, the filtrate is carefully evaporated at 35° (maximum) to dryness. 10 ml toluene and the contents of a sodium biphenyl bottle (South-Western Analytical Chemicals) are added to the residue with stirring. After 2 min the excess reagent is destroyed with 2 ml water. The mixture is cooled to room temperature, and the contents of the Kjeldahl flask are transferred to a separatory funnel and extracted with four 20 ml portions of water. The water extracts are collected in a 100 ml volumetric flask, neutralized first with conc. HCl, then with dilute acid, using 2 drops of phenolphthalein as indicator. The solution is diluted to volume, stirred and filtered through a Whatman filter paper No. 42 into a dry erlenmeyer flask. 25 ml of filtrate is transferred to a 50 ml volumetric flask. 0.1 *N* NaOH is first added and then 0.1 *N* HCl dropwise, for decoloration of the indicator. 2 ml pH 4 buffer solution is added; (60 g sodium acetate trihydrate in 500 ml water; 115 ml glacial acetic acid; diluted to 1 l), then 5 ml 0.001 *M* cerium nitrate solution (0.4342 g cerous nitrate hexahydrate in water, diluted to 1 l), 10 ml acetonitrile and 5 ml 0.001 *M* alizarin complexone solution (77 mg of 1,2-di-

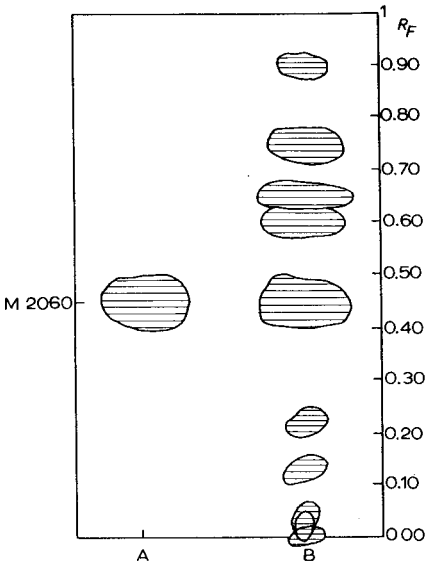

Fig. 1. Thin-layer chromatography of M 2060. Silica Gel $HF_{254+366}$, 1 mm thick. Solvent system: *n*-hexane–ethyl acetate (90:10); No. 5 migrations. Identification by UV (= 366 nm). A = pure M 2060; B = pure M 2060 plus the impurities contained in the technical product.

TABLE I

RESULTS OF M 2060 DETERMINATIONS ON SAMPLES OBTAINED BY ADDING VARYING AMOUNTS OF DIFFERENT IMPURITIES IN THE TECHNICAL PRODUCT TO KNOWN AMOUNTS OF PURE PRODUCT

M 2060 expected (mg)	*% calc.*	*M 2060 found (mg)*	*% found*
28.0	55.5	27.2	54.0
28.0	55.5	27.3	54.1
28.9	54.5	29.4	55.5
28.9	54.5	29.3	55.3

TABLE II

RESULTS OF M 2060 DETERMINATIONS ON ARTIFICIALLY-DECOMPOSED TECHNICAL PRODUCT

Method 1: thin-layer chromatography, hydrolysis and volumetric titration (%)	*Method 2: thin-layer chromatography, dehalogenation and fluorine determination (%)*
52.9	53.0
52.8	52.6
53.1	52.6
52.6	51.9
	51.7
	52.2

TABLE III

RESULTS OF M 2060 DETERMINATIONS ON SAMPLES OF PURE AND TECHNICAL PRODUCTS, ACCORDING TO THE CHROMATOGRAPHIC–VOLUMETRIC METHOD

	Pure M 2060 (%)	*Technical M 2060 (%)*
	100.7	95.6
	100.1	95.4
	100.5	94.7
	100.8	94.7
	100.1	95.7
	100.7	95.5
	100.2	94.2
		94.5
		94.8
		94.9
		95.0
		95.5
		95.5
		94.9
Mean	100.4%	95.1%
Standard deviation	0.29	0.47
Mean deviation	0.26	0.41

hydroxyanthraquinone-3-methylamine N,N-diacetic acid dissolved in 0.04 ml ammonium hydroxide 22° Be and 6 ml water; 0.03 ml glacial acetic acid; diluted to 200 ml. Store in dark). The reactants are mixed by swirling and diluted to 50 ml with water. After 60 min, the absorbance at 617 nm is measured against a reagent blank, in 1 cm cuvettes, and the amount of fluorine is determined from a calibration curve obtained by adding the reagents to known amounts of NaF solutions. Beer's law is obeyed up to a concentration of 25 μg fluorine in 50 ml.

TABLE IV

RESULTS OF M 2060 DETERMINATIONS ON A SAMPLE OF A 2% LIQUID FORMULATION, ACCORDING TO THE CHROMATOGRAPHIC–COLORIMETRIC METHOD

Analysis	*% M 2060*
1	1.78
2	1.75
3	1.78
4	1.68
5	1.76
6	1.73
7	1.74
8	1.73
9	1.73
10	1.69
11	1.71
Mean	1.73
Standard deviation	0.033
Mean deviation	0.024

RESULTS AND DISCUSSION

Of the large number of solvent mixtures tested, *n*-hexane–ethyl acetate gave the best separation of M 2060. The impurities normally present in the technical product do not interfere, as they are easily separated by thin-layer chromatography (Fig. 1).

Table I shows the results obtained by volumetric determinations on M 2060 samples to which the impurities present in the technical product had been expressly added.

Both methods, volumetric and colorimetric, can be used for the determination of the active ingredient in the technical product. However, since the latter shows a slightly lower degree of reproducibility, preference should be given to the former.

Table II gives the results obtained by analysing, according to the methods proposed, a sample of thermally-decomposed technical material.

Tables III and IV give the results of M 2060 determinations on samples of pure and technical products and a 2% liquid formulation.

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CHROM. 4241

USE OF THE ACID DYE TECHNIQUE FOR QUANTITATION OF PHARMACEUTICAL AMINES ELUTED FROM THIN-LAYER CHROMATOGRAMS

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SUMMARY

A quantitative thin-layer chromatographic procedure coupled with the acid dye technique for the estimation of the amount of eluted sample is described for the analysis of mixtures of pharmaceutical amines. With the five amines studied, the over-all recovery after application, development, adsorbent removal, elution and color development was 97.1% with an average coefficient of variation of 0.7% (based on ten chromatoplates having six replicates on each). Analysis of three binary mixtures of amines by two operators showed no appreciable difference between the two, with average assay values for each of 100.6% and 100.1%.

INTRODUCTION

The acid dye technique is a general procedure for the quantitative analysis of organic amines, with many modifications having been described[1]. The most common involves extraction of the ion-pair formed between the amine and an indicator dye from an aqueous buffered system into an organic phase and spectrophotometric measurement of the organic layer. The method has been shown to be rapid, accurate and precise, and to afford a relatively high and similar sensitivity for many amines regardless of other functional or chromophoric groups present[2,3]. Neutral, acidic or weakly basic materials as well as most excipients do not interfere.

Many pharmaceutical preparations contain mixtures of amines which often require separation prior to determination of the individual components. Thin-layer chromatography offers a rapid means for the separation, hence a combination of this procedure with the acid dye technique was investigated as a general method for the analysis of mixtures of amines. In addition, the results of the study serve to demonstrate the precision and accuracy that may be obtained with quantitative thin-layer chromatography.

EXPERIMENTAL

Reagents and materials

Thin-layer chromatography. Chromatoplates, 20 × 20 cm were coated to a thickness of 250 μ using a slurry of 35 g of Silica Gel DSF-5 (Camag) with 67 ml of water for five plates. The developing solvent used was chloroform–methanol–conc. ammonia (100:8:1). In this solvent system, the approximate R_F values for ephedrine, cyclizine, propoxyphene, chlorpheniramine and diphenhydramine were 0.16, 0.62, 0.66, 0.67 and 0.82, respectively. Detection was by short-wave UV light.

Acid dye technique. A buffer solution of pH 8 (McIlvaine) was prepared by mixing 97.3 ml 0.2 *M* Na_2HPO_4 with 2.7 ml 0.1 *M* citric acid. For the Bromthymol Blue (BTB) buffer solution sufficient Bromthymol Blue was dissolved in buffer of pH 8 to give a molar ratio of dye to drug of 10–13:1 when using 5 ml of BTB buffer solution and 5 ml of sample solution. The BTB buffer solution (250 ml) was shaken with 50 ml of photometric solvent before use. The photometric solvent consisted of benzene (AR) or benzene (AR) containing 1% by volume of isoamyl alcohol (AR).

Reference solutions. Methanolic solutions of chlorpheniramine maleate, cyclizine monohydrochloride, diphenhydramine hydrochloride, ephedrine sulfate and propoxyphene hydrochloride having concentrations in the order of 6–8, 9, 8, 10 and 12.5 μg/μl, respectively.

Sample solutions of mixtures. Methanolic solutions of each two-component mixture were prepared in the concentrations stated above for the reference solutions.

Procedure

Spotting the sample. An Agla micrometer syringe was filled and clamped securely in a vertical position. The chromatoplate was raised mechanically so that the needle tip pierced the silica gel layer and touched the backing glass plate. The syringe needle had been blunted by careful filing so as to allow the liquid to flow smoothly from the syringe onto the silica gel layer. Ten microliters of sample solution were applied in one operation by smoothly turning the micrometer head.

Chromatography and recovery of sample. Chromatography was carried out in filter paper lined jars and the solvent was allowed to travel 15 cm (about 45 min). The plates were air dried and sample spots located under short-wave UV light. A sharp stylus was used to outline the sample spot about 2 mm from the outer edge. Each sample spot was removed from the plate using a modified medium porosity sintered glass funnel of 10 mm diameter. The outlet end was drawn out to provide a tip about 3.5 cm in length and 1 mm I.D. The upper end was joined about 1.5 cm above the sintered glass disc to a 7 cm length of 4 mm O.D. glass tubing bent at 45° at the midway point. Suction was applied to the outlet end, and the inlet end (having a short length of tygon tubing projecting 0.5 cm beyond the end) applied to the plate for removal of the silica gel layer. For the blank determination, an area similar to the sample was removed.

Elution of the sample and quantitative determination. Following removal of the sample spot, a solvent reservoir (glass tubing 8 mm I.D. × 12 cm with an outlet of 4 mm O.D. tubing) was connected through the tygon tubing to the upper end of the sintered glass funnel. The outlet end of the funnel was then inserted through a rubber

stopper into a centrifuge tube*. Gentle suction was provided by inserting a syringe needle (connected to vacuum) through the rubber stopper into the centrifuge tube. For a number of simultaneous determinations, several of these assemblies were connected to a common vacuum manifold system using syringe needles and tygon tubing for connecting linkages.

The sample was eluted with 5 ml of 1% (v/v) HCl flowing from the reservoir through the silica gel into the centrifuge tube. The amount of vacuum was adjusted to give an elution time of about 10 min. To the eluate were added 5 ml of buffer solution, 5 ml of BTB buffer solution and 10 ml of photometric solvent. The tube was shaken vigorously for 1 min (alternatively, a mechanical device which tumbled 15 tubes end-over-end at 100 r.p.m. for 5 min was found convenient for processing a number of tubes simultaneously). The tubes were centrifuged for 2 min and the supernatant decanted carefully into a clean dry cuvette for measurement of absorbance at 410 mμ against a blank prepared similarly from silica gel removed from the plate.

RESULTS AND DISCUSSION

Experimental conditions for the acid dye technique were such that approximately 75–100 μg of drug in the 5 ml of eluate gave a satisfactory absorbance at 410 mμ when partitioned with BTB from the buffered aqueous phase into 10 ml of photometric solvent. Each drug examined followed Beer's Law over the concentration range of interest, as shown in Table I. Benzene containing 1% isoamyl alcohol by volume was used as extracting solvent for ephedrine since the sensitivity is enhanced about 50% by the use of this solvent compared to benzene alone. Benzene alone was satisfactory for the other drugs as there was no appreciable difference in sensitivity or precision of assay between the two solvents.

For each drug, the dye concentration was chosen so that at the optimum concentration of drug the molar ratio of dye to drug was about 10 to 1. For most compounds at a fixed concentration, the observed absorbance is independent of the concentration of dye providing the molar ratio of dye to drug is greater than about 2 to 1. This is true for all the compounds examined in this study except ephedrine. With ephedrine,

TABLE I

ABSORBANCE OF STANDARD SOLUTIONS CONTAINING VARYING CONCENTRATIONS OF DRUG

Propoxyphene hydrochloride		*Cyclizine monohydrochloride*		*Chlorpheniramine hydrochloride*		*Diphenhydramine hydrochloride*		*Ephedrine sulfate*	
Concn. (μg/5 ml)	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*	*Concn. (μg/5 ml)*	*Absorbance per 100 μg*
61.1	0.355	34.7	0.565	31.6	0.465	24.3	0.576	51.5	0.500
81.4	0.409	52.0	0.567	47.9	0.481	48.6	0.595	72.0	0.539
101.8	0.411	69.4	0.578	63.8	0.483	72.9	0.642	82.3	0.522
122.1	0.409	86.7	0.596	79.8	0.493	97.2	0.638	102.9	0.539
142.5	0.401	104.1	0.595	95.7	0.491	121.4	0.618	113.2	0.537
				111.7	0.490			133.8	0.520

* Cat. No. 15846, Wilkens-Anderson Co., Chicago.

the observed absorbance increases with increase in dye concentration. However, Beer's law is obeyed at any fixed concentration of dye under the experimental conditions described. Therefore it is essential to use the same solution of dye for each sequence of analyses in order to achieve accurate and precise results for ephedrine.

The major requirement of quantitative thin-layer chromatography, whether involving measurement of sample amount by spot area, by densitometry or by elution, is the application of a known amount of sample in a reproducible manner. FAIRBAIRN AND RELPH[4] recently have reported on the errors obtained in the production of the initial spot. In a review of the literature, they have pointed out typical examples where coefficients of variation are at least 5% or more, and coefficients of variation of 5–7% were obtained by those authors using the same technique on model systems. The error was shown to arise mainly from creep-back on the syringe needle during application of the solution to the chromatoplate. This difficulty could be largely overcome by the technique of forcible ejection of liquid from the syringe onto the plate, giving coefficients of variation of about 2%.

In the present study, creep-back on the syringe needle also was noted, especially with methanolic solutions. When using the hanging drop technique (*i.e.*, a small drop of liquid is produced on the tip of the needle and the chromatoplate touched to the drop and this sequence repeated until the desired volume is applied), only about 80% of the material reached the plate in some instances. The remainder stayed on the outside of the syringe needle. Consequently the technique was used of securely clamping the micrometer syringe in a vertical position, then mechanically raising the chromatoplate so that the needle point pierced the silica gel and rested against the glass plate. In this manner, solution from the syringe would flow directly on to the adsorbent layer. With most syringe needles, the length of the tip keeps the aperture above the chromatographic layer. At certain times, a droplet of liquid would form in the opening and reach a considerable size before touching the layer and being adsorbed. In such cases, creeping of the solvent up the outside of the needle would be significant (but occurring at irregular times). By reducing the length of the needle point by filing (and maintaining the bevel angle unchanged), the aperture of the syringe needle opened directly into the adsorbent layer. In this way, the solution would flow directly into the adsorbent without creep-back.

Following development of the chromatogram, spots were located under short-wave UV light, then the adsorbent containing each sample was sucked off the plate into the modified sintered glass funnel. The tip of tygon tubing served adequately to remove all the adsorbent of interest from the plate. Five milliliters of 1% (v/v) acid was found to be more than adequate for elution of each amine from the adsorbent. Additional solvent passed through the system gave a blank reading with the eluate. The volume of 5 ml of eluting solvent was convenient for use with the experimental conditions for the acid dye technique, *i.e.*, sample in 5 ml aqueous solution, 10 ml total of aqueous buffer and indicator dye solution, and extraction of the ion-pair into 10 ml of photometric solvent. The total volume of aqueous and organic solvent (25 ml) then allowed convenient mixing or extraction in a 42 ml centrifuge tube which could be used with a bench top centrifuge. It was noted that a higher sensitivity was achieved using 1% (v/v) HCl as eluting solvent compared with 0.1 *N* H_2SO_4. With propoxyphene, for example, the sensitivity was about 15% greater using 1% (v/v) HCl whereas with ephedrine the sensitivity was about 5% greater. Both eluting solvents resulted

in the same over-all recovery from the chromatogram. Therefore 1% (v/v) HCl was adopted as solvent of choice for elution.

Comparison of the absorbance obtained using 5 ml of eluate from a blank chromatogram against the absorbance from 5 ml of 1% (v/v) HCl showed no elution of material from the adsorbent layer which would interfere in the quantitative measurement. Nevertheless, a blank from the chromatogram was always used when analyzing samples recovered from the same plate. Thus the acid dye technique overcomes the problem of interference encountered when quantitation is by direct UV absorbance of the eluate. SPENCER AND BEGGS[5] in a detailed study of errors occurring in analysis by UV measurement found that fines eluted from silica gel gave a significant contribution to the over-all absorbance. This interference could be eliminated only by filtration through a 0.45 μ synthetic membrane filter. Likewise, MORRISON AND CHATTEN[6] observed that the amount of barbiturate eluted from a chromatoplate was grossly overestimated when measured by direct UV absorbance. The problem was overcome by aqueous elution followed by extraction of the mercury salt into chloroform and determination of the mercury by a dithizone procedure.

TABLE II

RECOVERY OF SAMPLE FROM CHROMATOPLATE

Compound spotted	*Operator*	*Micrograms spotted*	*% recovered*		*No. of Determinations*
			Av.	*S.D.*	
Chlorpheniramine maleate[a]	A	79.76	98.9	0.8	6
	A	64.60	97.1	0.8	6
Chlorpheniramine maleate[b]	A	79.76	98.0	0.4	6
	A	64.60	97.8	0.8	6
Cyclizine monohydrochloride[b]	A	85.90	96.7	0.8	5
	B	85.90	96.8	0.6	5
Diphenhydramine hydrochloride[b]	A	90.63	97.7	0.5	6
Ephedrine sulfate[b]	A	82.75	95.0	0.7	6
Propoxyphene hydrochloride[b]	A	128.14	96.1	1.0	5
	B	128.14	96.5	0.8	5

[a] Sample chromatographed just off origin with neutral developing solvent. Layer removed from both application and sample zone.

[b] Sample chromatographed in the ammoniacal solvent system. Layer removed from sample zone only.

Table II shows typical recoveries and reproducibility with the five amine drugs used in the study. The weight of drug applied was the amount theoretically delivered by the Agla syringe for each setting of 10 μl on the micrometer. No attempt was made to calibrate the syringe for absolute volume delivered, although the amount was within 3% of that found by dilution of the sample solution with a macropipet and measurement of the concentration. The recovery of sample from the chromatoplate was determined by comparison with the amount delivered directly from the micrometer

syringe into a centrifuge tube. For delivery, the aperture of the syringe needle was touched to the inside wall of the tube and 10 μl expelled in one continuous motion. In each analysis, the average absorbance from four such tubes was used for determination of recovery from the chromatoplate. The top entry in Table II shows a duplicate set of six recoveries obtained by chromatographing the sample just off the origin (using a neutral developing solvent) and removing the layer containing the sample as well as the application zone. The second entry for chlorpheniramine in Table II shows the recovery and reproducibility are essentially the same for samples before and after chromatography. Results for the other compounds in Table II show a similar degree of recovery and reproducibility. No appreciable difference occurred between operators using the same experimental technique. For all determinations, the average recovery amounted to 97.1%, with a coefficient of variation of 0.7%.

TABLE III

ANALYSIS OF TWO-COMPONENT MIXTURES OF AMINES[a]

Mixture No.	*Operator*	*Components*	*Micrograms spotted*[c]	*% recovered*			
				Spot 1	*Spot 2*	*Spot 3*	*Av.*
1	A	Diphenhydramine hydrochloride	78.86	102.0	103.4	101.4	102.3
		Ephedrine sulfate	109.91	98.6	97.7	99.5	98.6
1	B	Diphenhydramine hydrochloride	79.45	102.2	102.0	101.6	101.9
		Ephedrine sulfate	100.75	100.0	98.7	99.1	99.3
2	A	Diphenhydramine hydrochloride	76.39	102.3	100.4	—	101.4
		Chlorpheniramine maleate	100.91	98.5	100.4	98.8	99.2
2	B	Diphenhydramine hydrochloride	75.95	100.2	99.4	99.8	99.8
		Chlorpheniramine maleate	101.03	98.6	99.5	100.0	99.4
2[b]	—	Diphenhydramine hydrochloride	75.95	100.0	99.8	100.0	99.9
		Chlorpheniramine maleate	101.03	96.1	96.6	97.8	96.8
3	A	Chlorpheniramine maleate	101.84	100.8	102.0	101.4	101.4
		Ephedrine sulfate	100.32	100.6	101.3	101.1	101.0
3	B	Chlorpheniramine maleate	104.32	98.9	101.1	99.5	99.8
		Ephedrine sulfate	102.82	100.0	100.4	100.0	100.1

[a] Three volumes of sample solution spotted at separate locations and the recovery of each compared to the average of three reference standards on the same chromatoplate.
[b] Chromatoplate spotted and chromatographed, then left overnight before elution.
[c] Theoretical amount contained in each 10 μl of sample solution.

Table III, showing the results of analyses of several two-component mixtures of amines, demonstrates the accuracy and precision that may be achieved by the described experimental procedure. Results between operators were in good agreement, with the largest difference amounting to 1.6%. The average recovery for all spots (excluding 2[b]) was 100.3% with a coefficient of variation (CV) of 1.2. The average recovery for all spots by operator A was 100.6% (CV = 1.5) while that for operator B was 100.1% (CV = 1.1). Since the above data were calculated from the results of three separate sample spots compared to the average of three standards on the same chromatoplate, the true variability under conditions of assay will be greater. Nevertheless,

the combination of the acid dye technique with quantitative thin-layer chromatography offers a relatively rapid and precise means for the analysis of mixtures of amines.

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CHROM. 4252

DETECTION OF ADULTERATION OF BUTTER FAT (GHEE) BY THE RANDOM REARRANGEMENT REACTION AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A new method, involving the use of the effect of the random rearrangement reaction in fats has been developed which detects 5–10% (w/w) of adulterants such as hydrogenated groundnut, tallow and mohua (Mowrah) fats in butter fat (ghee). The method consists of the isolation of the trisaturated glycerides (GS_3) of rearranged pure and adulterated ghee by silver nitrate–silica gel thin-layer chromatography, and separation of the isolated GS_3 into individual glyceride components by reversed phase chromatography on liquid paraffin coated thin layers of Kieselguhr G using acetone–methanol–acetic acid (60:40:0.5) as developing solvent. Some GS_3 components of ghee increase after rearrangement and the presence of the above adulterants further increases their concentration. Tallow and mohua (Mowrah) fats increase the concentration of the GS_3 components of butter fat more than hydrogenated groundnut fat after randomisation. A prominent difference in the occurrence of the fatty acids, principally C_{12} to C_{16}, also exists between some GS_3 components of rearranged pure butter fat (ghee) and rearranged adulterated butter fats.

When both hydrogenated groundnut and mohua (Mowrah) fats are adulterants, the C_{12} to C_{16} acids of some GS_3 components of pure butter fat become more concentrated after the random rearrangement. With tallow as adulterant, however, the concentration of the C_{12} to C_{16} acids in some GS_3 components having similar mobility compared to pure butter fat significantly decreases after random rearrangement.

Variations in the concentrations of the trisaturated glyceride components, including constituent fatty acids, between pure butter fat and adulterated butter fats are better visualised when the fats are randomly rearranged than without rearrangement.

INTRODUCTION

One type of rearrangement reaction in glycerides involves the inter and intra molecular exchange of acyl radicals of the glycerides, with or without a catalyst at suitable temperatures. When a triglyceride mixture (natural or synthetic) is subjected

to rearrangement, a mixture of glycerides is formed in which the distribution of the acyl groups is statistical or random and the overall glyceride composition of the rearranged products differs from the original combination. The alteration in glyceride composition of some natural oils after random rearrangement was readily detected, with the help of TLC, by CHAKRABARTY *et al.*[1,2] and by PRIVETT *et al.*[3]. CHAKRABARTY *et al.* have also suggested that the rearrangement reaction involving the randomisation principle may be utilised for detecting adulteration of an oil (fat with other oils) or fat by considering the changes in pattern that are likely to occur with respect to the difference in number of component glycerides and their concentration in the pure and adulterated glyceride oils before and after random rearrangement. The use of the rearrangement reaction for detecting groundnut oil in mustard oil, in conjunction with TLC, has been reported by CHAKRABARTY *et al.*[4].

The present paper describes the detection of adulterants such as hydrogenated groundnut, tallow and mohua (Mowrah) fats in butter fats (ghee) at the 5–10% level by first conducting the random rearrangement reaction and then adopting the TLC technique. It should be stated that these adulterants have been chosen for a comparison of the efficacy of the present method with an earlier report[5] by some of us and for the extension of our research on the development of methods for detection of adulteration in oils and fats some of which have been reported[5,6].

EXPERIMENTAL

Random rearrangement reaction and isolation of the rearranged products

The method adopted was essentially that of CHAKRABARTY *et al.*[7]. Pure butter fat (ghee) and butter fat (ghee) containing 5–10% (w/w) of fats like hydrogenated groundnut, mohua (Mowrah) and tallow were dissolved separately in *n*-hexane so as to form a 60% solution (w/w) and agitated by a magnetic stirrer with 0.4% sodium methoxide (based on the weight of the fat solution) for 30 min in a small conical flask. The catalyst was destroyed by 1:3 HCl and the products were taken up in ether and the ether layer was washed free of HCl by distilled water. The ether solution, after drying over anhydrous sodium sulphate, was filtered and the ether removed in nitrogen atmosphere. The fats were purified from methyl esters of mono- and diglycerides that might be present in the rearranged fats by preparative adsorption silica gel TLC.

Isolation of the trisaturated glycerides (GS_3) from pure and randomly rearranged fats

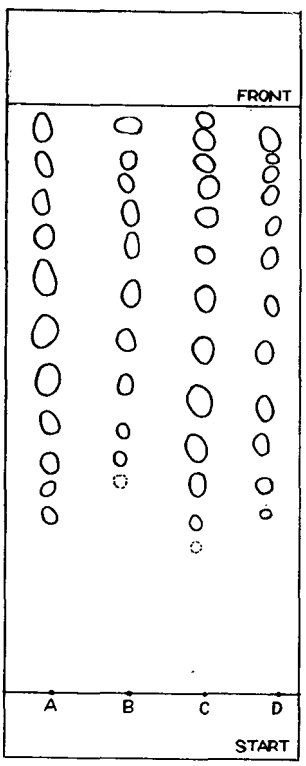
GS_3 was isolated from identical quantities of pure and adulterated randomised products, according to the method of CHAKRABARTY *et al.*[5], by elution with $CHCl_3$ containing 0.5% acetic acid from $AgNO_3$–Silica Gel G TLC plates.

Separation of total glycerides and trisaturated glyceride (GS_3) components of pure and adulterated randomised and unrandomised butter fat (ghee) samples

Separation was achieved by eluting twice with a solvent system consisting of acetone–methanol–acetic acid (60:40:0.5) on liquid paraffin impregnated Kieselguhr G thin-layer plates, and the glyceride components were detected as blue violet spots by iodine vapour followed by a spray of a 2% solution of starch in 20% ethanol[5].

Identification of the component fatty acids in some individual trisaturated glycerides (GS_3) of fats (ghee) both before and after rearrangement

Some GS_3 components having identical positions on the chromatograms but differing in concentration were scraped from plates and saponified with 2 *N* methanolic KOH and extracted with petroleum ether (40–60°) to remove paraffin. They were then acidified with 1:3 HCl and extracted again with diethyl ether. After washing 2 to 3 times with a few millilitres of water the ether was removed in nitrogen atmosphere and the fatty acids left were weighed and dissolved in benzene to give 1% solutions. The benzene solutions were then applied, in the form of spots, to a paraffin impregnated Kieselguhr G layer and eluted with acetic acid (90%) saturated with liquid paraffin. The spots were detected as before by iodine vapour and starch solution.



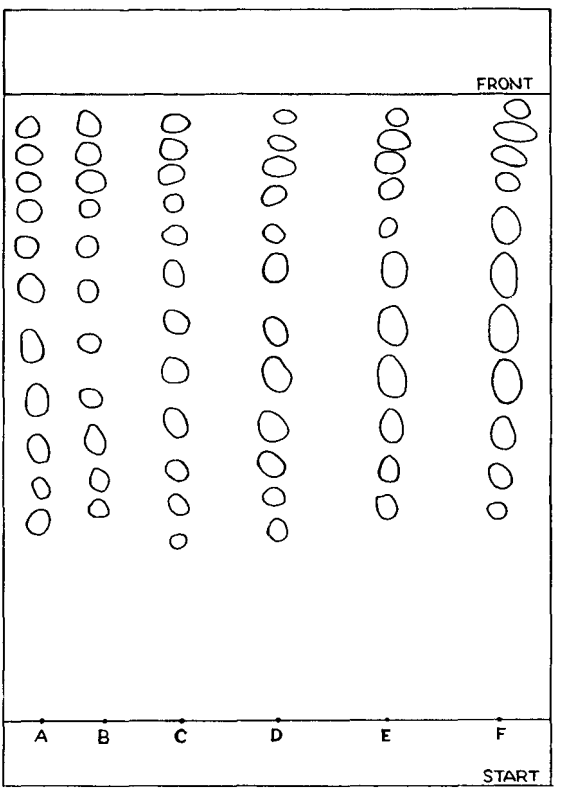


Fig. 1. Separation of total glycerides. (Total amount spotted, 60 μg). A = butter fat (ghee); B = randomised butter fat (ghee); C = butter fat adulterated with 5% hydrogenated groundnut fat before randomisation; D = butter fat adulterated with 5% hydrogenated groundnut fat after randomisation.

Fig. 2. Separation of total glycerides. (Total amount spotted, 60 μg.) A = butter fat (ghee); B = randomised butter fat (ghee); C = butter fat adulterated with 5% mohua (Mowrah) fat before randomisation; D = butter fat adulterated with 5% mohua (Mowrah) fat after randomisation; E = butter fat adulterated with 5% tallow before randomisation; F = butter fat adulterated with 5% tallow after randomisation.

TABLE I

CHARACTERISTICS OF THE SAMPLES INVESTIGATED

Sap. value = saponification value, R.I. = refractive index, R.M. = Reichert–Meissl value, R.P. = Reichert–Polenske value.

Sample	*Iodine value*	*Sap. value*	*R.I. at 40°*	*R.M.*	*R.P.*	*Slip point (°C)*
1 Butter fat (Ghee)	31.0	222.0	1.4533	30.2	1.6	28.5
2 Randomised butter fat (ghee)[a]	—	—	—	—	—	31.5
3 Hydrogenated groundnut fat	56.1	185.1	1.4594	—	—	41.0
4 Randomised hydrogenated groundnut fat[a]	—	—	—	—	—	36.0
5 Mohua (Mowrah) fat	60.5	190.0	1.4600	—	—	22.0
6 Randomised mohua (Mowrah) fat[a]	—	—	—	—	—	31.0
7 Tallow	44.3	196.5	1.4583	—	—	49.0
8 Randomised tallow[a]	—	—	—	—	—	46.5
9 Butter fat adulterated with 5% hydrogenated groundnut fat before randomisation	32.4	220.8	1.4554	28.2	1.5	30.0
10 Butter fat adulterated with 5% hydrogenated groundnut fat after randomisation[a]	—	—	—	—	—	32.0
11 Butter fat adulterated with 10% hydrogenated groundnut fat before randomisation	33.6	218.5	1.4559	26.3	1.4	30.5
12 Butter fat adulterated with 10% hydrogenated groundnut fat after randomisation[a]	—	—	—	—	—	32.0
13 Butter fat adulterated with 5% mohua (Mowrah) fat before randomisation	32.2	220.4	1.4605	28.7	1.4	29.0
14 Butter fat adulterated with 5% mohua (Mowrah) fat after randomisation[a]	—	—	—	—	—	32.0
15 Butter fat adulterated with 10% mohua (Mowrah) fat before randomisation	33.7	218.8	1.4608	27.6	1.4	29.5
16 Butter fat adulterated with 10% mohua(Mowrah) fat after randomisation[a]	—	—	—	—	—	30.5
17 Butter fat adulterated with 5% tallow before randomisation	31.8	221.0	1.4558	28.4	1.4	29.0
18 Butter fat adulterated with 5% tallow after randomisation[a]	—	—	—	—	—	35.5
19 Butter fat adulterated with 10% tallow before randomisation	32.6	219.7	1.4561	26.5	1.4	29.5
20 Butter fat adulterated with 10% tallow after randomisation[a]	—	—	—	—	—	35.5

[a] The analytical characteristics, excepting slip point ,were not determined because such characteristics generally remained unaltered after the rearrangement reaction.

RESULTS

The slip points of the fats before and after randomisation by the A.O.C.S.[8] method are indicated in Table I.

The chromatographic separations of the total glycerides of pure and adulterated

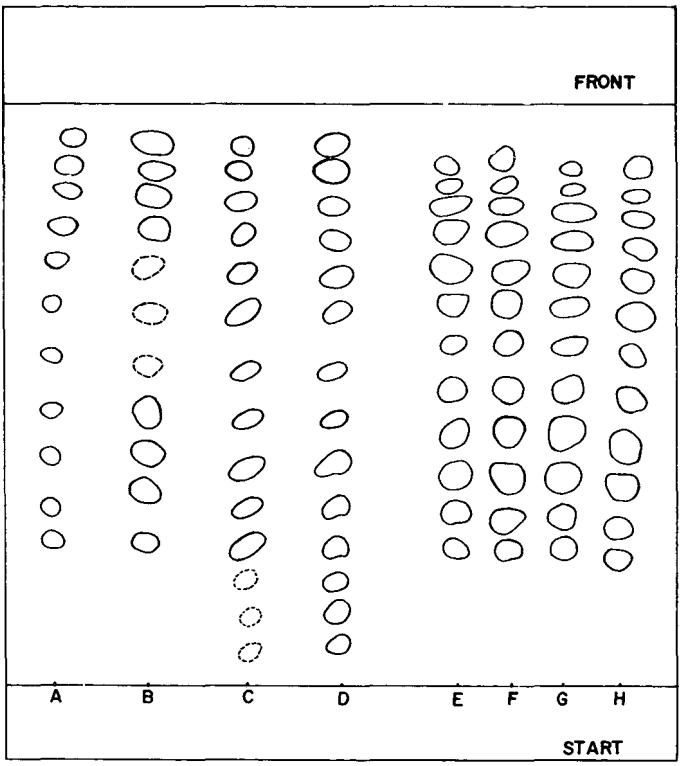


Fig. 3. Separation of trisaturated glycerides. (Total amount spotted, 80 μg.) A = butter fat (ghee); B = randomised butter fat (ghee); C = butter fat adulterated with 5% hydrogenated groundnut fat before randomisation; D = butter fat adulterated with 5% hydrogenated groundnut fat after randomisation; E = butter fat adulterated with 5% mohua (Mowrah) fat before randomisation; F = butter fat adulterated with 5% mohua (Mowrah) fat after randomisation; G = butter fat adulterated with 5% tallow before randomisation; H = butter fat adulterated with 5% tallow after randomisation.

butter fat (ghee) samples before and after the randomisation reaction are shown in Figs. 1 and 2.

The chromatogram of the trisaturated glycerides of pure and adulterated butter fats before and after random rearrangement is shown in Fig. 3.

The relative concentrations of the fatty acids present in the selected trisaturated glyceride components of pure butter fat and butter fat adulterated with the hydrogenated groundnut, tallow and mohua (Mowrah) fats separated by reverse phase TLC are shown in Table II (A–D). The + sign in these tables indicates the approximate concentration. An increased number of + signs denotes increased concentration. Separations of fatty acids by TLC are shown in Figs. 4–7.

DISCUSSION

The chromatographic separations of total glycerides of butter fat (ghee) and adulterated butter fats (*vide* Figs. 1 and 2) indicate the influence of the random

TABLE II

QUALITATIVE DETECTION OF COMPONENT FATTY ACIDS

Samples of trisaturated glycerides	*Fatty acids*								
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
(A) Fraction 11 numbered from the top										
Butter fat (ghee)				+++	+++	+++	+++	+++		
Randomised butter fat (ghee)				++	++	++	++	+		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat before randomisation				+++	+++	+++	+++	++		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat after randomisation				++++	++++	++++	++++	+		
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat before randomisation				+	+	++	++			
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat after randomisation				+	+	+	+			
Butter fat (ghee) adulterated with 5% tallow, before randomisation				+	+	+	+	+		
Butter fat (ghee) adulterated with 5% tallow, after randomisation				++++	++++	++++	++++			
(B) Fraction 10 numbered from the top										
Butter fat (ghee)				++	++	++	++	+		
Randomised butter fat (ghee)				++	++	+++	++	+		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat before randomisation				+	+	++	++	+		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat after randomisation				+++	+++	++++	++++	++	+	
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat before randomisation				+	+	++	+	+		
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat after randomisation				+	+	++++	+	+		
Butter fat (ghee) adulterated with 5% tallow before randomisation				++	+++	++	++			
Butter fat (ghee) adulterated with 5% tallow after randomisation				+	+	+	+			
(C) Fraction 4 numbered from the top										
Butter fat (ghee)				+++	+++	+++	+++			
Randomised butter fat (ghee)				++++	++++	++++	++++	++		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat before randomisation				+	+	+	+			

(continued on p. 122)

TABLE II *(continued)*

Samples of trisaturated glycerides	*Fatty acids*									
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{2}
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat after randomisation					++++	++	+++++	++		
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat before randomisation					+	+	+			
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat after randomisation				+	++	++	++			
Butter fat (ghee) adulterated with 5% tallow before randomisation				+	+	++++	++++			
Butter fat (ghee) adulterated with 5% tallow after randomisation						+	+			
(D) Fraction 3 numbered from the top										
Butter fat (ghee)				++	+++	+++	+++	+++		
Randomised butter fat (ghee)				+	++	++	++	++		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat before randomisation				+	++	++	++	++		
Butter fat (ghee) adulterated with 5% hydrogenated groundnut fat after randomisation					+	+	+	+		
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat before randomisation				+	+	+	+	+		
Butter fat (ghee) adulterated with 5% mohua (Mowrah) fat after randomisation					++++	++++	++++	++++		
Butter fat (ghee) adulterated with 5% tallow before randomisation				++	+++	+++	+++	+++		
Butter fat (ghee) adulterated with 5% tallow after randomisation				+	+	+	+	+		

rearrangement reaction on the alteration in the composition of glycerides. The concentrations of some of the constituent glycerides of pure butter fat increase when adulterated with hydrogenated groundnut, mohua (Mowrah) and tallow when compared with pure butter fat after randomisation. This observation suggests the presence of adulterants in butter fat.

However, commensurate with our previous observations[5] the detection of adulteration becomes easier and more conclusive when the trisaturated glycerides are first isolated from the pure and adulterated butter fats before and after randomisation and then resolved into their components by reversed phase TLC.

The concentration of some of the GS_3 components of pure butter fat and adulterated butter fats increases after randomisation. It is also interesting to note

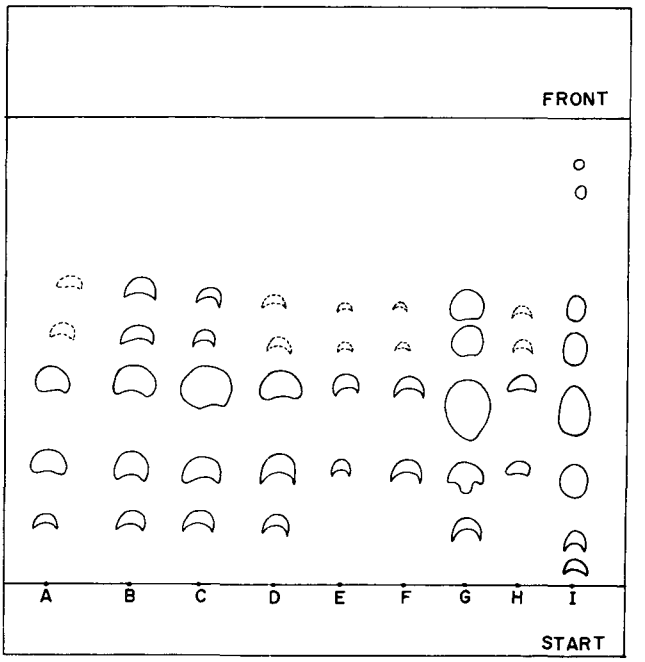


Fig. 4. Separation of fatty acids of trisaturated glycerides (fraction 11 from the top). (Total amount spotted, 30 μg.) A = pure butter fat (ghee); B = randomised butter fat (ghee); C = ghee adulterated with 5% hydrogenated groundnut fat before randomisation; D = ghee adulterated with 5% hydrogenated groundnut fat after randomisation; E = ghee adulterated with 5% mohua oil before randomisation; F = ghee adulterated with 5% mohua oil after randomisation; G = ghee adulterated with 5% tallow before randomisation; H = ghee adulterated with 5% tallow after randomisation; I = standard fatty acid mixture (C_6–C_{22}).

that some GS_3 components of the adulterated rearranged fats are more concentrated than the corresponding GS_3 components of randomised pure butter fat and unrandomised adulterated butter fats. The increase in concentration, however, depends on the type of adulterant. Thus tallow, on account of its inherently typical glyceride composition, and mohua (Mowrah) fat, because of the higher quantity of saturated fatty acids (C_{16} to C_{18}) compared with hydrogenated groundnut fat, markedly increase the concentration of trisaturated glyceride components of randomised butter fat having identical mobilities. The increase in concentration contributed by the hydrogenated groundnut fat is less, presumably owing to the lower content of total saturated fatty acids, notably C_{16}, compared with tallow and mohua and also due to the presence of *trans*-oleic acids which may behave differently from the other two fats during randomisation. The pronounced difference between the pure randomised butter fat and the adulterated randomised butter fats, with respect to the concentration of their trisaturated glyceride spots, allows the rapid detection of adulterants in butter fat.

The identification of fatty acids of some selected GS_3 components of pure butter fat and adulterated fats, both before and after randomisation, reveals some interesting features (*vide* Table II).

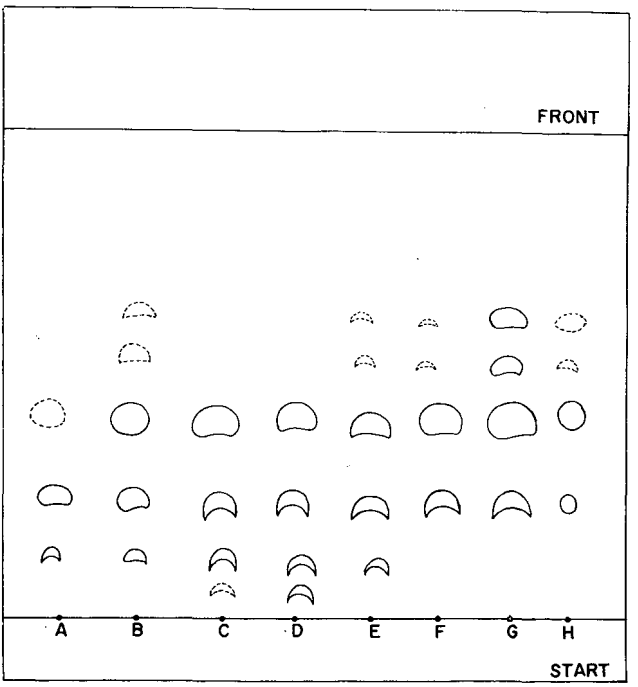


Fig. 5. Separation of fatty acids of trisaturated glycerides (fraction 10 from the top). (Total amount spotted, 30 μg.) A–H are as in Fig. 4.

The presence of C_8 to C_{20} fatty acids and their relative concentrations again depends on the nature of the fats. Thus in the trisaturated glycerides (GS_3) (fraction 11 numbered from the top) of the samples it appears that C_{10} to C_{16} acids of randomised ghee are less concentrated than pure ghee. But when ghee contains hydrogenated groundnut fat and tallow as adulterants, the C_{10} to C_{16} fatty acids become more concentrated than in ghee after randomisation. Before randomisation the fatty acid content of ghee containing hydrogenated groundnut fat is similar to that of unrandomised ghee and the C_{10} to C_{16} fatty acids in the case of ghee containing tallow are less concentrated than in both ghee and randomised ghee. On the other hand, the concentration of C_{10} to C_{16} fatty acids in ghee containing mohua (Mowrah) fat before and after randomisation is much less than in ghee and randomised ghee.

Similarly in the trisaturated glyceride fractions (numbered 10 from the top) some difference in the concentrations of the fatty acids is noted. Thus C_{10} to C_{18} fatty acids of ghee containing hydrogenated groundnut fat after randomisation are observed to be more concentrated than in ghee, randomised ghee and an unrandomised mixture of ghee and hydrogenated groundnut fat. Ghee containing tallow after randomisation shows lower amounts of C_{10} to C_{16} fatty acids than ghee, randomised ghee and an unrandomised blend of ghee and tallow. Furthermore, C_{20} fatty acid has been detected in randomised ghee containing hydrogenated groundnut fat. In the case of ghee adulterated with mohua, it should be noted that after randomisation

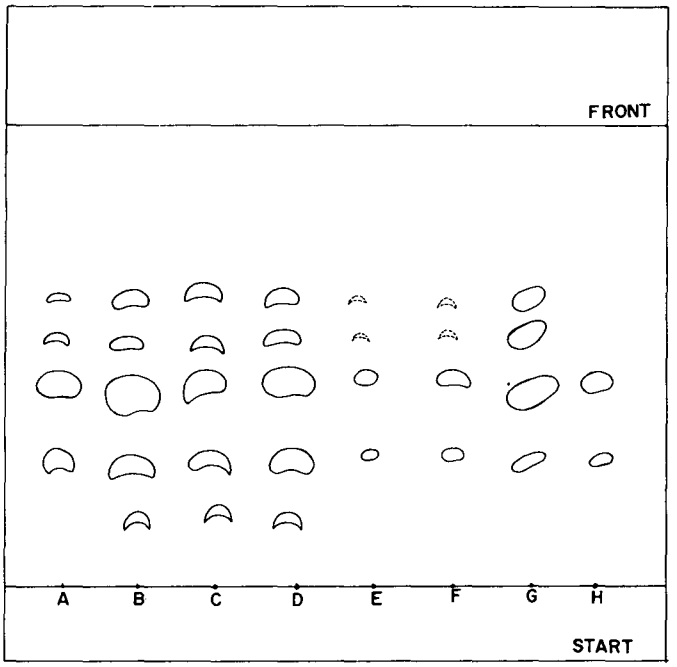


Fig. 6. Separation of fatty acids of trisaturated glycerides (fraction 4 from the top). (Total amount spotted, 30 μg.) A–H are as in Fig. 4.

only the C_{14} fatty acid becomes more concentrated than in the unrandomised mixture, ghee and randomised ghee.

The trisaturated glyceride fraction (numbered 4 from the top) of ghee shows that the concentration of C_{10} to C_{16} fatty acids is much less than the corresponding fraction of randomised ghee which, in addition to the above acids, contains C_{18} fatty acid. In the trisaturated glyceride (fraction 4 from the top) of ghee containing hydrogenated groundnut fat the C_{10} fatty acid is not detected after randomisation and there is less C_{14} fatty acid than in ghee but more C_{16} fatty acid than in randomised ghee and ghee. Before randomisation the presence of tallow in ghee is found to increase the amounts of C_{14} and C_{16} fatty acids of ghee. After randomisation the amounts of the said acids are remarkably less compared with those of randomised ghee and ghee; the C_{10} and C_{12} fatty acids were also not detectable. Ghee containing mohua shows C_{10} to C_{16} fatty acids in greater amounts after randomisation than in the unrandomised mixture but less than in ghee and randomised ghee.

C_{10} to C_{18} fatty acids of the trisaturated glyceride component of ghee (numbered 3 from the top) are more concentrated than the corresponding fatty acids of the trisaturated glycerides of randomised ghee. Ghee adulterated with hydrogenated groundnut fat indicates that there are less C_{10} to C_{18} acids than those in ghee but they are almost similar in concentration to those of randomised ghee. After randomisation of ghee containing hydrogenated groundnut fat C_{12} to C_{18} fatty acids become much less concentrated than even in randomised ghee. Ghee containing mohua after

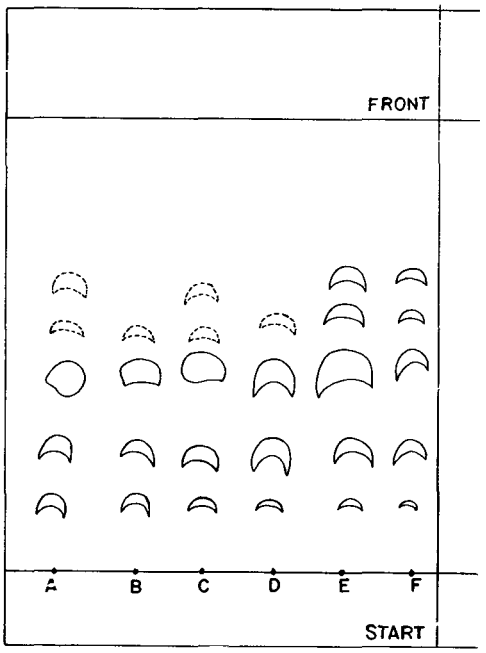


Fig. 7. Separation of fatty acids of trisaturated glycerides (fraction 3 from the top). (Total amount spotted, 30 μg.) A = ghee adulterated with 5% hydrogenated groundnut fat before randomisation; B = ghee adulterated with 5% hydrogenated groundnut fat after randomisation; C = ghee adulterated with 5% mohua before randomisation; D = ghee adulterated with 5% mohua after randomisation; E = ghee adulterated with 5% tallow before randomisation; F = ghee adulterated with 5% tallow after randomisation.

randomisation contains principally C_{12} to C_{18} fatty acids in greater amounts than in randomised ghee. But after randomisation the same acids become less concentrated than in ghee, randomised ghee and ghee containing mohua. The C_{10} to C_{18} fatty acids of ghee containing tallow appear to be more concentrated before randomisation than those of randomised ghee. Thus the variation in concentration of some component fatty acids of the trisaturated glyceride spots of ghee and adulterated ghee before and after randomisation corroborate further the detection of adulterants like hydrogenated groundnut fat, mohua (Mowrah) and tallow in ghee.

In this connection it is also important to note that while slip points (*vide* Table I) fail to distinguish pure butter fat from butter fats containing 5 to 10% hydrogenated groundnut fat and mohua after randomisation, the TLC separation of glycerides and the fatty acids thereof, is capable of detecting the influence of the rearrangement reaction on alterations in the glyceride pattern of the fats and consequently facilitates the identification of adulterants in butter fats (ghee).

The combination of the rearrangement reaction and TLC appears, therefore, to be a convenient method for the detection of adulterants in butter fat (ghee) and it can be inferred that the method is likely to be useful in the detection of adulteration in other fats. It is also possible to visualise the possibility of quantitative evaluation of the various glycerides separated, or of fatty acids thereof, by combining selective

enzymatic hydrolysis, gas–liquid chromatography, spectroscopy or other methods with TLC. Such attempts are under way by the present authors.

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CHROM. 4233

NEW SOLVENT SYSTEMS FOR THIN-LAYER CHROMATOGRAPHY OF AFLATOXINS

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SUMMARY

Three new solvent systems have been developed for the thin-layer chromatography (TLC) of aflatoxins B_1, B_2, G_1, and G_2. Solvent system "A" is an analytical adsorption chromatography system which changes the usual resolved sequence of aflatoxins to B_1, G_1, B_2, and G_2. Solvent "B" is a liquid partition chromatography system which is very good analytically and has the resolution needed to make its use for preparative TLC practical. The third TLC solvent system, "C", is an adsorption chromatography system which is excellent both analytically and preparatively. Solvent system "C" requires two developments or three hours of continuous development to give the maximum degree of resolution.

INTRODUCTION

Aflatoxins are toxic metabolites produced by some strains of the fungus *Aspergillus flavus*[1,2], which under the proper conditions of temperature and humidity will grow quite well on grains, peanuts, and other organic material. These metabolites have been implicated in intoxications of domestic animals which have been fed moldy feed[3,4]. Several papers have been published concerning aflatoxins which report techniques for the isolation and purification of sufficient quantities of the aflatoxins to meet other research requirements. The technique of thin-layer chromatography (TLC) has found extensive use in these research projects. The most important advantages of TLC are its resolving power, sensitivity, and speed. It is sensitive enough to detect nanogram quantities and has sufficient resolving power to separate milligram quantities of closely related aflatoxins into the following sequence: aflatoxin B_1, B_2, G_1 and G_2. Most of the solvent systems which have been reported in the literature relative to aflatoxin fractionation by TLC resolve these components into this same sequence. Chloroform–methanol systems with variations from 2 to 7% methanol have been reported by several laboratories[5–8]. Other solvent systems which provide an analytically effective separation include chloroform–acetone (90:10 or 85:15)[9], diethyl ether–

methanol–water (96:3:1)[10], the upper phase of benzene–ethanol–water (46:35:19)[11] and a partition system using benzene–formamide–water[12]. A special system for the purification of aflatoxin G_1 has been used to demonstrate that pure aflatoxin G_1 has a blue, rather than a green, fluorescence. This system of LIJINSKY AND BUTLER[13] is chloroform–diethyl ether–acetic acid (2:2:1). Recently, a two-dimensional system for TLC of aflatoxins has been reported by PETERSON AND CIEGLER[14] in which the plate is developed first in chloroform–acetone (90:10) and then in ethyl acetate–isopropanol–water (10:2:1). This system separates aflatoxin B_2 and G_1 better than the others and provides a way to purify aflatoxin G_2, which has been most difficult. All of these have been tried in this laboratory following the conditions described in the respective papers. The results of these comparison experiments have served to encourage publication of the solvent systems developed in this laboratory. The purpose of this paper is to report the effectiveness of three new solvent systems for TLC of aflatoxins.

METHODS

A thin layer of Silica Gel H* or HR* plus G-HR* (1:1, w/w) ($^1/_4$, $^1/_2$ or 1 mm) was applied to clean glass plates with an adjustable Desaga applicator*. The plates were air dried overnight and activated by heating to 120° for 1 h before being used or stored in a large desiccator. All the solvents were analytical reagent or reagent grade and the chloroform was redistilled before use. Most chromatograms were developed by the ascending method in the dark[15] at room temperature (25°) in unlined, unequilibrated glass tanks[16] containing freshly mixed solvent. Two variations of the continuous development technique were used with solvent system "C". The first procedure was with the B-N Chamber*,[17], in the horizontal position. Good results could also be obtained by placing the top of the regular development tank ajar after the solvent front has ascended about three-fourths of the way up the plate to allow for solvent evaporation from the top of the plate and continuous development. This was done in a hood. Fluorescence of the aflatoxins was visualized with the UV lamp** having a primary wave length of 365 mμ which corresponds with the excitation wave length for all four major aflatoxins[18]. UV absorption spectra of ethanol solutions of aflatoxins B_2 and G_1 were obtained by using a Beckman DB-G spectrophotometer equipped with a recorder. The crude aflatoxin mixture, obtained by Dr. A. C. PIER of this laboratory from Mr. PETER ROGOVIN, Northern Utilization Research and Development Division, Peoria, Ill., was part of the same batch used for studies of acute intoxication in swine[19].

RESULTS AND DISCUSSION

In the course of our mycotoxin research[19], three new solvent systems were developed which added to the value of TLC as a procedure for isolation and purification of aflatoxins.

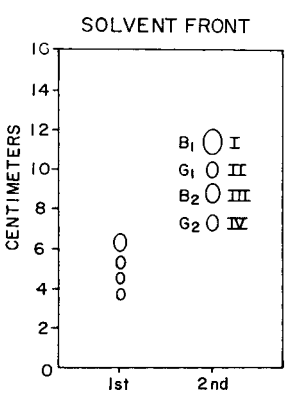
* Distributed by Brinkmann Instruments, Westbury, N.Y.
** Burton Ultraviolet Lamp, Model 19-0, Santa Monica, Calif.

Solvent system "A"

The high resolving power of TLC has proven very useful in characterization problems involving closely related compounds such as the aflatoxins. This was demonstrated again when it was found that the order or sequence of the aflatoxin series was different in this system from that in the others. Solvent system "A" consisting of methylene chloride–trichloroethylene–*n*-amyl alcohol–formic acid (80:15:4:1) changed the resolved order of the aflatoxins from B_1, B_2, G_1, G_2 to B_1, G_1, B_2, G_2. It required about 50 min for the solvent front to ascend a distance of 16 cm from the origin in an unlined, unequilibrated tank at room temperature (25°). The best chromatograms were obtained using $^1/_4$ mm layers of a mixture of Silica Gel HR and G-HR (1:1, w/w) as the adsorbent. The TLC plates were activated at 120° for 1 h and cooled before the sample was applied. Resolution of the four major aflatoxins was accomplished by one development, but was improved by a second development (Fig. 1). Reversal of aflatoxins B_2 and G_1 from their usual order was demonstrated by scraping off the four major fluorescent bands into separate tubes, eluting and rechromatographing with another TLC solvent system such as solvent system "C" or chloroform–acetone (90:10). Results of a typical experiment are illustrated in Fig. 2. In addition, the UV absorption spectra of fluorescent spot No. II from solvent system "A" had absorption maxima (in ethanol) at 243, 257, 264 and 362 mμ, which corresponded to those for aflatoxin G_1 standard[20]. Fluorescent spot No. III had absorption maxima (in ethanol) at 223, 265 and 362 mμ, which corresponded to those for aflatoxin B_2 standard[6].

Solvent system "B"

This is a liquid partition system which works well analytically. The TLC plates were sprayed with 12 ml of a solution of *tert.*-butyl alcohol–formic acid–water (10:1:25) per 8 × 8 in. plate and air dried again for 30 min. The plates were then ready for sample application and development in the ascending direction in an unlined, unequilibrated tank at room temperature (25°). One development required about 35 min and accomplished excellent resolution of the fluorescent toxins. The time of development varies



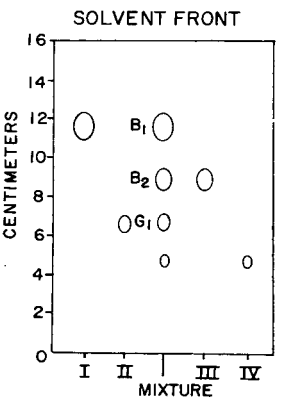
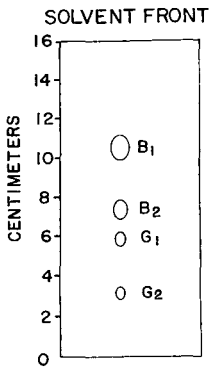


Fig. 1. Separation of aflatoxins on Silica Gel HR plus G-HR (1:1, w/w) after one and two developments with solvent "A".

Fig. 2. Separation of aflatoxins on Silica Gel H after two developments with solvent "C". Purified fractions are from TLC system "A", as in Fig. 1. I = aflatoxin B_1; II = G_1, mixture of four aflatoxins; III = B_2; and IV = G_2.



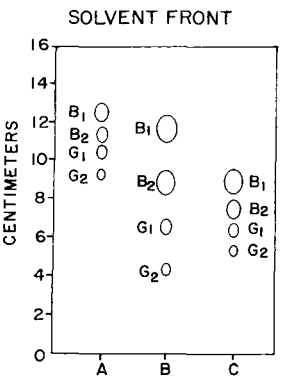


Fig. 3. Separation of aflatoxins on Silica Gel H after one development with solvent "B".

Fig. 4. Comparison of resolution of aflatoxins after two developments in the respective solvent systems. A = Chloroform–methanol (97:3, v/v) on Silica Gel G-HR; B = solvent "C" on Silica Gel H; C = chloroform–acetone (90:10, v/v) on Silica Gel G-HR.

with the thickness of the layer, the amount of spray used, the drying conditions, and the time between spraying and spotting sample. Best results were obtained with thin layers of Silica Gel H at $^1/_4$ mm thickness (Fig. 3). Solvent system "B" consisted of xylene–*tert.*-butyl alcohol–formic acid (94:5:1). The boiling points of *tert.*-butyl alcohol and xylene are low enough so that they can be removed from the aflatoxin by evaporation under vacuum with a Roto-Vap* at a temperature of 45°.

Solvent system "C"

Solvent system "C" provides a high level of resolution between all four major aflatoxins which makes preparative TLC much more practical and effective. In most of the TLC systems published to date for the separation of aflatoxins, the toxic fluorescent compounds are so close together (Figs. 4A and C) that it makes the use of these solvent systems for preparative TLC of aflatoxins rather tedious if not impractical. The highest quality chromatograms using solvent system ""C" were obtained with $^1/_4$ mm thin layers of Silica Gel H processed by continuous development in the B-N chamber for 3 to 4 h or by using the usual ascending technique and multiple development. Maximum resolution required two developments with each one taking about 75 min (Fig. 4B). Solvent system "C" consists of chloroform–trichloroethylene–*n*-amyl alcohol–formic acid (80:15:4:1). In 1962, VAN DER ZIJDEN *et al.*[15] reported using 72 large TLC plates to get 40 mg of aflatoxin B_1. Recently, HANNA AND CAMPBELL[21] reported a preparative procedure using 2 mm thick layers of Silica Gel PF_{254} with $CaSO_4$ as the adsorbent layer and developed with 15% acetone in chloroform. They applied 270 mg of solids per plate which contained 20 to 40 mg of aflatoxin B_1 and recovered an aflatoxin preparation of 95% purity. We were able to put 150 mg of crude solids on each large TLC plate of 1 mm thickness and after subsequent development in solvent "C" and elution obtained 60 to 70 mg of purified aflatoxin B_1. System "C" is very good, both analytically and preparatively, for the isolation and purification of aflatoxins B_1, B_2, G_1 and G_2.

* Buchler Instruments, Fort Lee, N.J.

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CHROM. 4215

CHROMATOGRAPHY OF LYSOSOMAL ENZYMES ON HYDROXYLAPATITE

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SUMMARY

Soluble proteins were extracted from Triton WR 1339-filled rat liver lysosomes and were chromatographed on hydroxylapatite by stepwise elution with increasing concentrations of phosphate buffer (pH 6.8). The distribution of the activities of the cathepsins A, B, C, and D; β-galactosidase; α-mannosidase; β-glucuronidase; β-N-acetylglucosaminidase; acid phosphatase and arylsulfatase in the resulting fractions was determined. Ammonium sulfate fractionation of lysosomal proteins was combined with separation on hydroxylapatite in an attempt to improve the resolution of the cathepsins. Lowering the pH of the phosphate buffers to 5.8 greatly increased the affinity of lysosomal proteins for hydroxylapatite. By a combination of these manipulations fractions were obtained which contained cathepsin D without contamination by other cathepsins.

INTRODUCTION

The lysosome is now known to contain about 40 enzymatic species with the capacity to degrade all the major classes of biological macromolecules. These enzymes act in combinations determined by their specificity to digest individual complex molecules within the lysosome. Precise definition of these digestive pathways in terms of their individual enzymes depends, at least in part, on separation of the enzyme components of the lysosome. This is particularly true of the pathway of protein catabolism for which only limited knowledge exists concerning the contribution of each of the cathepsins to proteolysis. Availability of purified lysosome fractions allows application of conventional chromatographic methods to the separation of the enzymes in the lysosome. The properties of lysosomal enzymes in several chromatographic systems (DEAE-cellulose, Sephadex G-200, CM-cellulose) have been described elsewhere[1]. The usefulness of hydroxylapatite for fractionation of lysosomal enzymes was suggested by the work of HAYASE[2], who partially purified lysosomal lipase with hydroxylapatite and demonstrated its capacity to resolve lysosomal protein into a number of distinct zones. Descriptions of the properties of hydroxylapatite[3,4] also suggest its high resolving power and its adaptability to either analytical or preparative operations. In view of the complexity of the mixture of proteins extractable from

lysosomes, hydroxylapatite seems particularly well suited to be a general method for separating lysosomal enzymes. This paper describes the fractionation of soluble lysosomal enzymes on hydroxylapatite columns with special emphasis on the behavior of the cathepsins. Factors that limit the capacity of hydroxylapatite to resolve complex mixtures of proteins are discussed.

EXPERIMENTAL

Materials

Hydroxylapatite (Bio-gel HT) in 1 m*M* sodium phosphate buffer, pH 6.8, was purchased from Bio-Rad Laboratories. Cytochrome *c*, type III, and hemoglobin, type I, were purchased from Sigma Chemical Co., N-CBZ-L-glutamyl-L-phenylalanine, benzoyl-L-arginine amide hydrochloride, and glycyl-L-tyrosinimide acetate were purchased from Mann Research. Materials used for the automated analyses are given by BECK AND TAPPEL[5].

Fractionation and concentration of lysosomal protein

Triton WR 1339-filled lysosomes were prepared from rat liver by the method of MAHADEVAN AND TAPPEL[6]. Soluble protein was extracted from these lysosome preparations by two methods. In the first method, protein was precipitated by bringing the lysosome fraction to 100% saturation with solid ammonium sulfate. Protein was collected by centrifugation at 13 000 × *g* for 20 min. Because of the high density of sucrose in these lysosome preparations, the precipitated protein floated to the surface during centrifugation. The subnatant liquid was removed by suction and the protein was resuspended in 1 m*M* sodium phosphate buffer, pH 6.8. Membranes were removed from the protein fraction by centrifugation at 95 000 × *g* for 45 min. The clear supernatant fraction was dialyzed overnight against 1 m*M* sodium phosphate buffer, pH 6.8. The dialyzed solution was concentrated 25-fold by vacuum filtration through dialysis tubing. In the second method, part of a batch of Triton-filled lysosomes was brought to 100% saturation with ammonium sulfate. From the remainder of the batch, three fractions of lysosomal protein were obtained by fractional precipitation between 0–30, 30–60, and 60–90% saturation of ammonium sulfate. Precipitated protein was collected by centrifugation and separated into membrane and soluble portions as described above. All fractions were dialyzed overnight against 1 m*M* sodium phosphate buffer, pH 6.8.

Column operation

Hydroxylapatite beds were poured either in hand-blown glass columns (0.9 × 15 cm) with glass wool bed supports or in a Pharmacia K 15/30 column. Protein was eluted from the columns by stepwise application of increasing concentrations of sodium and potassium phosphate buffers. Chromatographic separation of lysosomal proteins was carried out at 4°.

Enzyme and protein determinations

Protein concentration was determined by measuring the absorbance at 280 mμ and by the method of MILLER[7]. Both methods were standardized with bovine serum albumin. Acid phosphatase, β-N-acetylglucosaminidase, arylsulfatase, β-glucuronidase

and β-galactosidase activities were determined by the automated methods of BECK AND TAPPEL[5]. Measurements of α-mannosidase activity were made by automated analysis in the same manner as the above enzymes using a method adapted from CONCHIE AND FINDLAY[8].

Cathepsin A activity was measured by the method of IODICE *et al.*[9]. Cathepsin B was measured by the method of GREENBAUM AND FRUTON[10]; liberated ammonia was collected by the microdiffusion method of SELIGSON AND SELIGSON[11]. The procedure of METRIONE *et al.*[12] was used to measure cathepsin C activity. Cathepsin D determinations were performed according to GIANETTO AND DE DUVE[13].

RESULTS

The extraction of soluble protein from Triton-filled lysosomes was monitored by measurement of protein content at each step. Since the separation of the cathepsins was of especial interest, the activities of these enzymes were followed throughout this procedure. These measurements (Table I) indicated that the recovered soluble lysosomal proteins were enriched in cathepsins B, C, and D, while constituting only about 20% of the original protein. There was considerable loss of total cathepsin A activity in this procedure and, thereby, a decline in the specific activity of this enzyme. The process by which the proteins were concentrated resulted in some further loss of protein and a decline in the total and specific activities of the cathepsins. Only traces of catheptic activity were associated with the membrane fraction.

The pattern produced by the stepwise elution of lysosomal protein from hydroxylapatite consisted of twelve distinct zones (Fig. 1). In a trial operation of the column, Triton WR 1339 showed no tendency to bind to hydroxylapatite even at low phosphate concentrations and appeared in the void volume. The large initial peak obtained by the elution of soluble lysosomal proteins with 1 m*M* sodium phosphate buffer was due to the presence of Triton. The completeness of the recovery of protein from the column was difficult to determine due to the interference of Triton with the protein determinations. However, HAYASE[2] has shown that lysosomal protein was eluted by buffers up to 0.6 *M*, but not by buffers of higher concentration even with extended washing. Thus, it is likely that the lysosomal protein was completely eluted from this column.

Representative fractions from each of the protein peaks were assayed for the activities of lysosomal enzymes and the resulting values are presented as percent of

TABLE I

EXTRACTION OF SOLUBLE PROTEIN FROM TRITON-FILLED LYSOSOMES

	Volume (ml)	*Protein*		*Cathepsin A*			*Cathepsin B*			*Cathepsin C*			*Cathepsin D*		
		mg	%	*S.A.*[a]	*T.A.*[b]	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%
Original	340	221	100	168	1115	100	0.9	5.8	100	136	451	100	5	34	100
Dialyzed soluble	125	59	27	160	281	25	2.5	4.4	76	231	204	45	37	66	197
Concentrated	3.9	46	21	64	88	8	2	2.7	46	163	112	25	21	29	9

[a] Specific activity expressed as mμmoles of product/mg protein/min.
[b] Total activity expressed as μmoles of product/min/ml × ml.

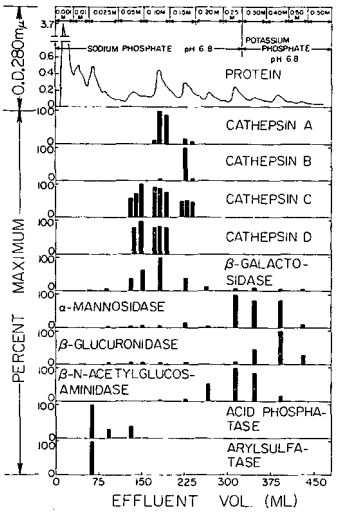

Fig. 1. Chromatographic separation of lysosomal enzymes. Concentrated lysosomal protein (35 mg) was applied to a 1.5 × 16 cm hydroxylapatite column. The flow rate was 35 ml/h and elution was by stepwise increases in phosphate buffer concentration. The fraction volume was 3 ml and protein was monitored by measuring absorbance at 280 mμ. Activities are presented as percent of maximum specific activity for each enzyme.

maximum specific activities (Fig. 1). The distribution of enzyme activities in these fractions reveals the capacity of hydroxylapatite to separate the components of complex protein mixtures. Acid phosphatase and arylsulfatase, for example, are maximally active in fractions which are virtually free of the other enzymes measured. Even in regions of the protein pattern that contain a number of enzymes, useful partial separations (*e.g.* of cathepsins A and B, of β-glucuronidase and β-N-acetylglucosaminidase) were achieved. Cathepsins B and D were completely separated. Several of the enzymes were partly concentrated in one or a few peaks and were partly separated from the other enzymes, although none of the enzymes was completely separated from all of the others.

The catheptic enzymes were all eluted within a narrow range of phosphate concentration and their distributions, especially those of cathepsins B, C, and D, overlap considerably. A series of experiments was undertaken to determine if the elution of the cathepsins from hydroxylapatite could be modified to improve the separation of these enzymes. Another portion of soluble lysosomal proteins, including those prepared by fractional precipitation with ammonium sulfate, were used for these experiments. The further concentration of protein by vacuum filtration after ammonium sulfate precipitation proved to be not only disadvantageous but unnecessary. The great affinity of hydroxylapatite for proteins in the presence of low phosphate concentrations makes it possible to apply proteins to these columns in very dilute solutions; protein is effectively concentrated during application because of its adsorption in a narrow zone of the column bed. The concentration step was, therefore, abandoned in the second method of preparation of lysosomal protein. The distribution of catheptic activities in these preparations indicated that a partial separation of the cathepsins had been achieved by ammonium sulfate treatment (Table II).

TABLE II

AMMONIUM SULFATE FRACTIONATION OF TRITON-FILLED LYSOSOMES

	Volume (ml)	*Protein (mg)*	*Cathepsin A*			*Cathepsin B*			*Cathepsin C*			*Cathepsin D*		
			S.A.[a]	*T.A.*[b]	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%
Original	270	135	312	129		26	11		1380	285		295	122	
$^2/_3$ Original	176	90		86	100		7	100		190	100		81	100
100% sat.	42	29	175	15	18	59	5	72	1223	54	28	565	49	61
$^1/_3$ Original	88	45		43	100		4	100		95	100		41	100
60–90% sat.	33	3.3	860	9	20	52	0.5	14	3420	17	18	414	4	10
30–60% sat.	11	4.5	13	0.2	0.4	0	0	0	653	4	5	531	7	18
0–30% sat.	11	2.8	19	0.2	0.4	15	0.1	4	308	1	1	181	1	4

[a] Specific activity expressed as mμmoles of product/mg protein/min.
[b] Total activity expressed as μmoles of product/min/ml × ml.

The protein solutions were equilibrated with the appropriate 1 m*M* phosphate buffer and applied to small (0.9 × 2.5 cm) columns. Elution was carried out by stepwise application of 3 or 5 ml of each of the buffers in a series. The eluent in each step was collected as a single fraction and analyzed for protein (280 mμ absorbance) and for the activity of the cathepsins.

Fig. 2 (A, B) indicates the effect of the pH of sodium phosphate buffer on the elution of lysosomal protein. At pH 6.8 the pattern of cathepsin elution is similar to that presented in Fig. 1. However, at pH 5.8 the cathepsins are more strongly adsorbed and are eluted only with the application of higher phosphate concentration. In similar

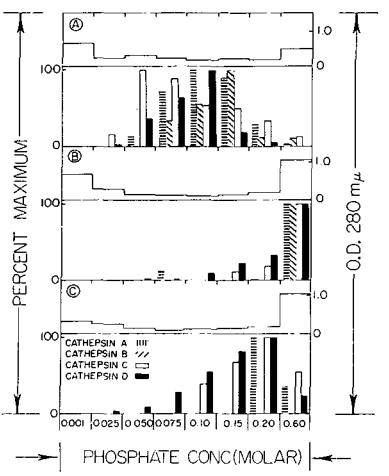

Fig. 2. Effect of pH on elution of cathepsins from hydroxylapatite. Protein was obtained by ammonium sulfate fractionation of Triton-filled lysosomes (see Table II). Samples containing 5 mg of lysosomal protein were applied to 0.9 × 2.5 cm hydroxylapatite columns and eluted by stepwise application of increasing concentration of phosphate buffer. Sodium phosphate was used at each step except the last, which was 0.6 *M* potassium phosphate. Activities are presented as percent of maximum specific activity for each enzyme. (A) Protein from 100% ammonium sulfate fraction eluted with pH 6.8 phosphate buffer. (B) Protein from 100% ammonium sulfate fraction eluted with pH 5.8 phosphate buffer. (C) Protein from 30–60% ammonium sulfate fraction eluted with pH 5.8 phosphate buffer.

experiments, sodium phosphate at pH 7.8 and potassium phosphate buffer at pH 6.8 produced elution patterns like that produced by sodium phosphate at pH 6.8.

Attempts were made to utilize this pH effect to enhance the separation of the cathepsins on hydroxylapatite. The protein precipitated between 30 and 60% saturation of ammonium sulfate was relatively richer in cathepsin D than were the other preparations and was free of cathepsin B. Elution of this protein from hydroxylapatite with sodium phosphate buffer at pH 5.8 yielded some fractions which contained only cathepsin D and which were free of other cathepsins (Fig. 2C).

DISCUSSION

The results reported here illustrate both the advantages and limitations inherent in the use of hydroxylapatite for the separation of complex protein mixtures. The separation of as many as ten enzymes into four distinct groups in a single step is clearly advantageous. Further, hydroxylapatite chromatography, which is not based on the ionic properties of the solutes, emerges as a useful supplement to ion-exchange methods. BECK *et al.*[1] have recently described the separation of rat liver lysosomal enzymes on DEAE-cellulose, Sephadex G-200, and CM-cellulose and the resolving power of hydroxylapatite compares quite favorably with that achieved by their methods. Several nonproteolytic lysosomal enzymes were partly or completely separated from other enzymes on hydroxylapatite. For example, acid phosphatase and arylsulfatase can be clearly separated from a number of other enzymes. BECK *et al.*[1] showed that arylsulfatase and acid phosphatase can be resolved on DEAE-cellulose. Thus, the combination of these chromatographic methods could purify and separate the two enzymes. Similarly, β-N-acetylglucosaminidase, β-glucuronidase, and arylsulfatase appear in the same region of the eluent from a DEAE-cellulose column. Yet these three enzymes are well separated from each other by either CM-cellulose or hydroxylapatite chromatography. Likewise, CM-cellulose will separate β-glucosidase from β-N-acetylglucosaminidase on one hand and from a combination of β-galactosidase and β-glucuronidase on the other. Separation of β-galactosidase and β-glucuronidase is readily achieved on hydroxylapatite. These examples suggest the usefulness of hydroxylapatite for the separation of the soluble lysosomal enzymes when combined with ion-exchange methods.

None of the above chromatographic systems, either separately or in combination, yielded good separations of the cathepsins. The combination of ammonium sulfate fractionation and "batch" type elution at pH 5.8 and pH 6.8 from small hydroxylapatite columns resulted in partial separations of the cathepsins (Fig. 2). Recent experiments in this laboratory (LIAO, unpubl. results) have indicated that it may be possible to combine DEAE-cellulose and Sephadex G-100 to separate partly the four cathepsins.

The exact mechanism by which proteins are adsorbed to hydroxylapatite and displaced from it by phosphate ions has not been defined. From studies of the effects of various anions and cations on the elution of proteins from hydroxylapatite[3,4,14], it appears that protein molecules, as well as phosphate ions, are bound to the calcium sites of the hydroxylapatite crystal. BERNARDI AND KAWASAKI[15] have recently proposed that the adsorption of proteins to hydroxylapatite is due to the interaction of their carboxyl groups with calcium sites on the hydroxylapatite crystal. Their

conclusion is based on the observation that only polypeptides containing free carboxyl groups are strongly adsorbed to hydroxylapatite. While their data establish the interaction of carboxyl groups with hydroxylapatite they do not exclude interactions by other groups. In fact, phosphoproteins have an unusually high affinity for hydroxylapatite[16]. Their hypothesis is also not necessarily consistent with their own observation that denatured proteins are poorly adsorbed to hydroxylapatite or with the results reported here on the increased affinity of proteins for hydroxylapatite at lower pH values.

Despite these uncertainties about the mechanism of hydroxylapatite chromatography, it is clear that the protein–hydroxylapatite interaction is decreased by increased phosphate ion concentration. At low phosphate concentrations, proteins are strongly adsorbed to hydroxylapatite. As phosphate concentration increases, the interaction between protein and hydroxylapatite is reduced and the rate of protein migration begins to approach that of the solvent phase. If the shift from strong to weak interaction between a particular protein and hydroxylapatite is produced by a small increase in phosphate concentration, the protein will be eluted within a narrow solvent zone. In practice, however, the behavior of proteins on hydroxylapatite is more complex and the poor separation of the cathepsins described above exemplifies the principal limitation of this method. The cathepsins were eluted from the column within a narrow range of phosphate concentration and were difficult to resolve on hydroxylapatite by small increases in phosphate concentration. This is due in part to the formation of multiple "false peaks" by the same protein. This phenomenon is evident in the pattern shown in Fig. 1.

The formation of false peaks is probably due to the tendency of proteins to be retarded or readsorbed by hydroxylapatite after their initial elution. Because of this a given protein will not be completely eluted by a phosphate ion concentration high enough to cause its desorption. With further increase in phosphate concentration, more of the retained protein is eluted in a second solvent zone to produce a false secondary peak of the same solute in the effluent. This results in mixing of proteins that would otherwise be separable if critical elution concentration were the only operational factor.

It is difficult to explain this retention of a protein by lower regions of a column after the protein has been initially eluted. HJERTEN[4] observed that a portion of the migrating zone of the colored protein ceruloplasmin was readsorbed to the hydroxylapatite column and could not be eluted by extended washing with the same buffer. We have observed the same phenomenon with cytochrome *c*. The bulk of adsorbed cytochrome *c* is eluted from hydroxylapatite by 0.15 *M* sodium phosphate buffer (pH 6.8). However, some protein becomes distributed along the length of the column and is resistant to extended elution with 0.15 *M* buffer. Most, but not all of the retained cytochrome *c* can be eluted by 0.2 *M* buffer. This suggests that the retained protein may be even more tightly bound than it was prior to its initial elution.

The readsorption of protein could be explained if phosphate concentration in the lower portion of the column were to fall below the critical elution concentration. Since hydroxylapatite has an affinity for phosphate ions as well as for protein, it is possible that phosphate ions are removed from solution during the passage of buffer through the column. The effect of this would be a deterioration of the advancing front of increased phosphate concentration. The phosphate ion concentration would then

fall below the level at which the protein had been desorbed. At this point the protein would be readsorbed. TISELIUS *et al.*[3] reported that phosphate ions are retarded by hydroxylapatite and we have also found this to be the case; effluent phosphate buffers approach only about 80% of the phosphate concentrations of applied buffers. This suggests that the ionic environment within the hydroxylapatite column is complex and that the migration of a protein cannot be expected to follow the simple pattern that would be obtained if sharp interfaces between the steps of phosphate concentration could be maintained.

Even with the uncertainties and limitations of hydroxylapatite chromatography, its large capacity to resolve complex protein mixtures makes it an effective method for the analysis of the enzymatic complement of lysosomes. The combination of hydroxylapatite with ion-exchange methods offers many possibilities for the separation and purification of lysosomal enzymes.

ACKNOWLEDGEMENTS

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CHROM. 4247

CHROMATOGRAPHY AND ZONE ELECTROPHORESIS OF INORGANIC IONS DISSOLVED IN FUSED SALTS

V. ELECTROPHORETIC BEHAVIOUR OF SOME METAL IONS DISSOLVED IN MOLTEN PERCHLORATES*

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SUMMARY

The mobilities of alkali metal ions in a $LiClO_4$–$KClO_4$ eutectic at 300° increase as their crystalline radius decreases while the opposite occurs for alkaline earth metal ions dissolved in the same solvent.

The slope of the mobility curve *vs.* ionic radius of the alkaline earth metal ions decreases in a $LiClO_4$–$Mg(ClO_4)_2$ melt at 300° and is completely reversed in pure $Mg(ClO_4)_2$ at 270°.

From the data obtained in molten perchlorates, as well as from results reported by other authors in molten nitrates, some general considerations and predictions on the mobility of a given cation dissolved in various fused salts having the same anion but different cations or *vice versa*, are reported and discussed.

INTRODUCTION

As shown by electrophoretic experiments in molten alkali nitrates[1–3] interactions between dissolved metal ions and the anions of the solvent depend very much upon the nature of the alkali metal ion of the solvent itself. These interactions decrease as the crystalline radius of the alkali metal ions of the solvent decreases. However, strong association reactions occur even in fused lithium salts as indicated by the anionic behaviour of some metal ions dissolved in these solvents[1].

To obtain a better insight on anion–cation interactions it seemed interesting to compare the electrophoretic behaviour of metal ions dissolved in fused salts having the same anion but cations of different electrical charge. The use of fused solvents other than the uni–univalent salts is restricted because many salts have melting points which are too high for electrophoretic experiments on glass fiber paper while others decompose or are very difficult to dehydrate. However, magnesium perchlorate could be used as it yields an anhydrous melt at 270°.

In this paper the electrophoretic behaviour of alkali and alkaline earth metal

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ions dissolved in a molten $LiClO_4$–$KClO_4$ eutectic and $Mg(ClO_4)_2$ are reported and discussed.

The use of perchlorates as fused solvents has till now received very little attention; only a few papers have been published in this field[4–7]. Thus electrophoretic experiments in fused perchlorates also give more information on these solvents.

EXPERIMENTAL

$KClO_4$ and $Mg(ClO_4)_2$ were Merck reagents grade, while anhydrous lithium perchlorate was obtained according to a procedure described elsewhere[4]. The $LiClO_4$–$KClO_4$ eutectic (76% moles of $LiClO_4$; m.p. 207°) and the $LiClO_4$–$Mg(ClO_4)_2$ mixture (32% moles of $Mg(ClO_4)_2$; m.p. $\sim$ 235°) were obtained by mixing the salts in suitable quantities and drying them again under vacuum at 110°. Preliminary chromatographic controls were performed to ascertain whether precipitation of the oxides or interaction with the support occurs.

The electrophoretic experiments were performed in an apparatus similar to that described in a previous paper[8], the experimental conditions being similar to that reported in ref. 1. The strip of glass-fiber paper was chromatographically impregnated and left for conditioning overnight. When $Mg(ClO_4)_2$ was used as electrolyte, the strip was directly impregnated by dipping it in molten $Mg(ClO_4)_2$ contained in a dry-box filled with anhydrous nitrogen.

A suitable mixture of alkali nitrates was used as catholite (alkali perchlorates cannot be employed since the alkali metals discharged at the cathode react with the solvent explosively). This mixture was controlled experimentally so that this substitution did not affect the composition of the solvent, since no trace of nitrate ions ($<$ 0.01 mg) was found in the neighbouring vessel, into which the strip dipped.

The alkali and alkaline earth metal ions were detected radiometrically by using ^{22}Na, ^{42}K, ^{86}Rb, ^{134}Cs, ^{45}Ca, ^{89}Sr and ^{131}Ba as tracers. A Gaussian distribution of the activity was obtained by scanning the strip every 0.5 cm for alkali metal ions and every 0.2 cm for alkaline earth metal ions, owing to their lower mobility. Tl(I) ion was detected as sulphide.

RESULTS AND DISCUSSION

Table I shows the electrophoretic mobilities of the alkali and alkaline earth metal ions, and thallium(I) dissolved in fused $LiClO_4$–$KClO_4$ at 300°. Measurements of mobility of other inorganic ions such as Pb(II), Cd(II), Co(II), Ni(II) and Cu(II) were not carried out because chromatographic experiments showed that these ions did not travel with the front of the solvent, thus suggesting the low solubility of their oxides*.

Owing to the lack of knowledge of electroosmotic flow in molten media, mobility data could represent the apparent, rather than the true mobility of the species under examination.

As shown in Fig. 1 the mobilities of alkali metal ions decrease as their crystalline

* The attempt to hinder the formation of metal oxides by adding ammonium perchlorate as acid to the molten solvent (according to the report in our previous paper[1]) was unsuccessful, since ammonium perchlorate decomposes very quickly at this temperature.

TABLE I

ELECTROPHORETIC MOBILITIES (cm^2/V sec) OF ALKALI, ALKALINE EARTH METAL IONS AND THALLIUM IN MOLTEN $LiClO_4$–$KClO_4$ AT 300°

Ion	$u \times 10^4$	*Crystalline ionic radius (Å)*
Na(I)	3.6 ± 0.1	0.97
K (I)	3.0 ± 0.1	1.33
Rb (I)	2.6 ± 0.1	1.47
Cs (I)	2.4 ± 0.1	1.67
Tl (I)	2.7 ± 0.1	1.15
Ca (II)	0.45 ± 0.01	0.99
Sr (II)	0.80 ± 0.01	1.12
Ba (II)	1.22 ± 0.01	1.34

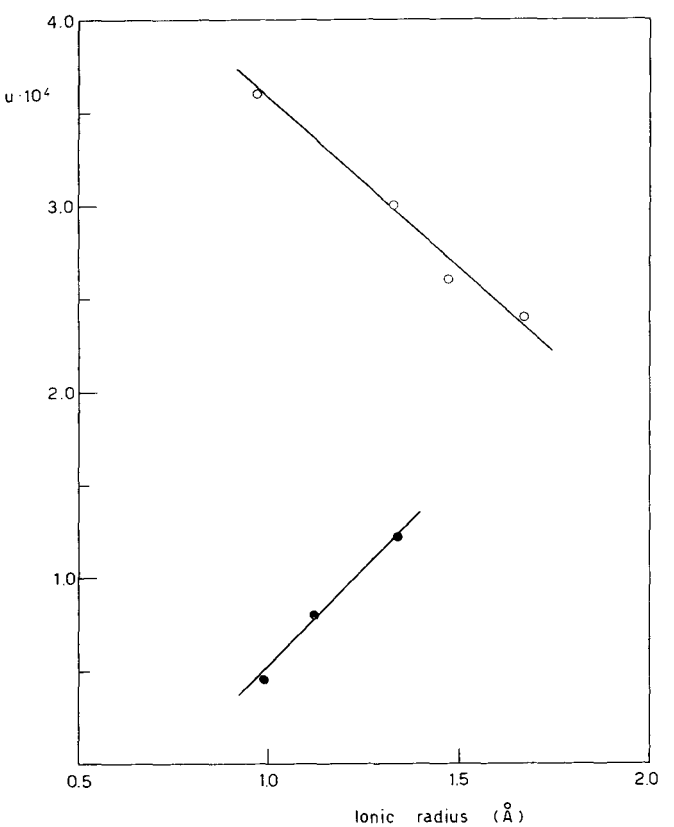

Fig. 1. Electrophoretic mobilities (cm^2/V sec) of Na^+, K^+, Rb^+ and Cs^+ (circles) and Ca^{2+}, Sr^{2+} and Ba^{2+} (black dots) in a molten $LiClO_4$–$KClO_4$ eutectic at 300° as a function of crystalline ionic radius.

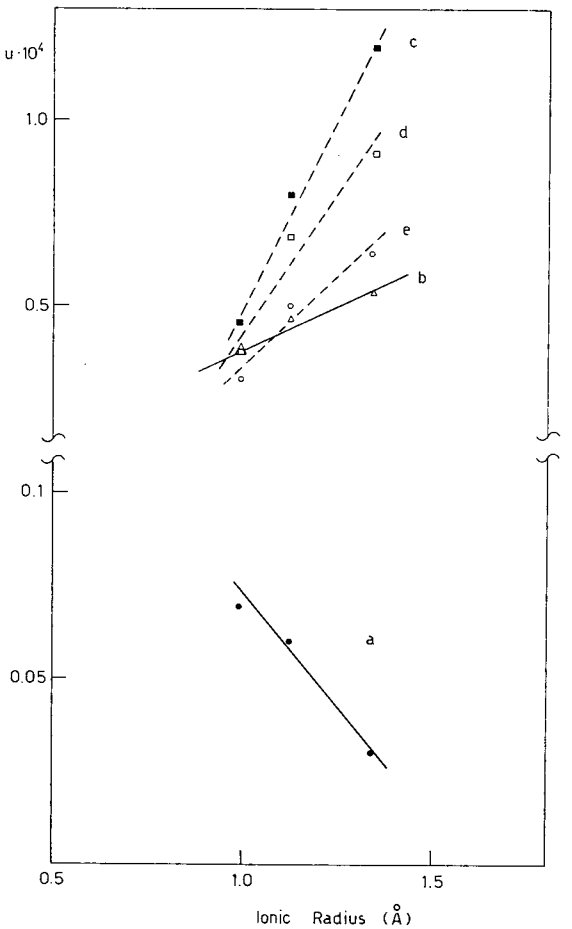

Fig. 2. Electrophoretic mobilities (cm^2/V sec) of alkaline earth metal ions, as a function of crystalline ionic radius, in various molten media. (a) Pure $Mg(ClO_4)_2$ at 270°; (b) $LiClO_4$–$Mg(ClO_4)_2$ mixture at 300°; (c, d, e) $LiClO_4$–$KClO_4$ eutectic at 300°, 270° and 240° respectively.

radius increases while the opposite occurs for the alkaline earth metal ions (an analogous trend was also observed by FORCHERI[9] in molten nitrates).

The opposite behaviour of the alkali and alkaline earth metal ions can be related to the presence of the Li ion in the solvent. This ion, owing to its small crystalline radius, interacts more strongly than the other alkali metal ions with the anion of the solvent. Thus, in molten $LiClO_4$ or in fused systems with high concentrations of Li ions, the interactions of Na^+, K^+, Rb^+ and Cs^+ with the anion of the solvent are weak and their mobility decreases with their increasing crystalline radius. On the other hand, the higher electrical charge of the alkaline earth metal ions makes them more reactive than the Li ion. Since interaction is stronger the smaller the alkaline earth metal ion, the mobility decreases as their crystalline radius decreases.

Taking into account the behaviour of the alkali metal ions in alkali nitrates[2], a decreasing mobility with increasing crystalline radius can also be expected for

TABLE II

ELECTROPHORETIC MOBILITIES ($cm^2/V\ sec \cdot 10^{-4}$) OF ALKALINE EARTH METAL IONS DISSOLVED IN $LiClO_4$–$Mg(ClO_4)_2$ MIXTURE AT 300° AND PURE $Mg(ClO_4)_2$ AT 270°

Ion	*$LiClO_4$–$Mg(ClO_4)_2$ (300°)*	*$Mg(ClO_4)_2$ (270°)*
Ca (II)	0.37 ± 0.01	0.07 ± 0.01
Sr (II)	0.47 ± 0.01	0.06 ± 0.01
Ba (II)	0.53 ± 0.01	0.03 ± 0.01

alkaline earth metal ions in a solvent whose cation interacts with its own anion more strongly than with the tracer alkaline earth metal ions. To test this hypothesis, one of the smaller, *i.e.* the more interacting alkaline earth metal ions, was selected as cation of the solvent, and electrophoretic experiments were performed either in pure $Mg(ClO_4)_2$ at 270° or in $LiClO_4$–$Mg(ClO_4)_2$ mixture at 300°.

The values obtained are reported in Table II. In Fig. 2 these data are plotted *vs.* ionic radii of the ions. Data obtained in $LiClO_4$–$KClO_4$ at 240°, 270° (Table III) and 300° are also reported for comparison (dashed curves). These temperatures were selected since the comparisons among different fused systems (in our case $LiClO_4$–$KClO_4$ eutectic and pure $Mg(ClO_4)_2$) are usually made at the same fixed number of degrees above their melting points or at the same ratio between working temperature and melting point.

It can be seen from Fig. 2:

(a) That the mobilities of alkaline earth metal ions in the $LiClO_4$–$KClO_4$ eutectic increase with temperature; however, the slope of the mobility curves of these ions is not greatly affected by temperature changes;

(b) When pure $Mg(ClO_4)_2$ is employed a reversal of the slope is obtained, in agreement with our previous expectations;

(c) When $LiClO_4$–$Mg(ClO_4)_2$ is employed the slope of the mobility curves is lowered with respect to that obtained in $LiClO_4$–$KClO_4$, but not reversed, owing to the low concentration of Mg^{2+}.

It must be pointed out that the different behaviour of the alkaline earth metal ions in $LiClO_4$–$KClO_4$ and in $Mg(ClO_4)_2$ cannot be ascribed to viscosity differences between these solvents, since variations in viscosity can increase or decrease, but not reverse the slope of the mobility curves. Thus the inversion of the slope of the mo-

TABLE III

ELECTROPHORETIC MOBILITIES ($cm^2/V\ sec$) OF ALKALINE EARTH METAL IONS IN MOLTEN $LiClO_4$–$KClO_4$ AT 240° AND 270°

Ion	*$u \times 10^4$ (240°)*	*$u \times 10^4$ (270°)*
Ca (II)	0.30 ± 0.01	0.37 ± 0.01
Sr (II)	0.50 ± 0.01	0.68 ± 0.01
Ba (II)	0.64 ± 0.01	0.91 ± 0.01

bility curves must be ascribed to the different interacting forces of the cations of the solvents.

The electrophoretic values obtained in molten perchlorates for alkali and alkaline earth metal ions as well as what was previously reported in the literature for alkali metal ions in molten nitrates[1-3] allow us to make some general predictions about the influence of the cation and the anion of the solvent on the mobility of the dissolved metal ions.

Let us consider the following two cases:

(1) Two or more tracer metal ions are dissolved in a given molten salt.

(2) A given metal ion is dissolved in two or more molten salts having the same anion but different cations or *vice versa*.

In the first case the possibilities are the following:

(a) The cation of the solvent interacts with its own anion more strongly than the dissolved metal ions (*e.g.* alkali metal ions in a molten lithium salt or alkali and alkaline earth metal ions in a molten magnesium salt). In this case the interactions of the dissolved metal ions with the anion of the solvent are weak; their mobilities are thus expected to decrease with increasing crystalline radius (in agreement with Stokes' law).

(b) The cation of the solvent interacts with its own anion less than the dissolved metal ions (*e.g.* alkali and alkaline earth metal ions in a molten caesium salt). In this case, owing to the strong interaction with the anions of the solvent, the mobilities of the dissolved metal ions are expected to decrease as their crystalline radius decreases.

(c) The associating power of the cation of the solvent is intermediate to that of the dissolved metal ions (*e.g.* Li^+, Na^+, K^+, Rb^+ and Cs^+ in a molten potassium salt). In this case the mobilities are expected to increase from Li^+ to K^+ and decrease from K^+ to Cs^+; thus the curve of mobility *vs.* crystalline ionic radius exhibits a maximum for the cation of the solvent (see ref. 2).

Predictions on the variation of the mobility of a given metal ion dissolved in different molten solvents (case 2) are more complicated since differences in the viscosity of the solvents must be taken into account. However, if the viscosity values are known, some predictions can also be made, taking into account the deviations from the product $u \times \eta = K$, obtained from Stokes' law for ideal systems. For example if we consider a given metal ion (*e.g.* K^+) dissolved in different fused salts with the same anion (*e.g.* $LiNO_3$, KNO_3 and $CsNO_3$), the interaction of this ion with the nitrate anion will increase from KNO_3 to $CsNO_3$ and decrease from KNO_3 to $LiNO_3$. Thus the product $u \times \eta$ in the various solvents is not constant and is expected to increase as the interacting power of the cation of the solvent increases:

$$u^{K+}_{CsNO_3} \times \eta_{CsNO_3} < u^{K+}_{KNO_3} \times \eta_{KNO_3} < u^{K+}_{LiNO_3} \times \eta_{LiNO_3}$$

where u^{K+} is the mobility of the potassium ion in the system under consideration which has a viscosity value η.

As regards the mobility of a given cation in various fused solvents having the same cation but different anions the product $u \times \eta$ is expected to decrease as the interaction of the tracer cation with the anion of the solvent increases.

The mobilities of transition metal ions will probably show an even more complicated dependence on the solvent employed, owing to the possibility of them forming "true" complex ions.

We think that predictions on the mobility of a given anion in various molten salts having the same cation but different anion or *vice versa* could also be made by considering similar interactions to those previously reported for the mobility of the cations. Some preliminary experiments on the electrophoretic mobilities of various inorganic anions dissolved in a molten $LiClO_4$–$KClO_4$ eutectic at 300° (ref. 10), showed that all these species move towards the cathode. In some cases (*e.g.* the nitrate anion) this "cationic" mobility is very high ($3.3 \cdot 10^{-4}$ cm^2/V sec) thus justifying the use of nitrate mixtures in the cathode vessel in our electrophoretic experiments with fused perchlorates. "Cationic" mobility of the dissolved anions can be related to their stronger interaction than perchlorate anion with Li ions of the solvent. This is also in agreement with the well-known weak associating power of the perchlorate anion in aqueous solution.

In conclusion, from the knowledge on the ionic interactions, some predictions on the electrophoretic behaviour of the ionic species in molten salts can be made; on the other hand, useful information on the association reactions occurring in molten salts can be obtained from the electrophoretic mobilities of inorganic ions.

However, more exhaustive data are necessary for a more quantitative knowledge of these associations, which are of fundamental importance for the study of the properties of ionic melts.

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CHROM. 4223

ANALYSIS OF THYROID PROTEINS BY MEANS OF MICRO DISC ELECTROPHORESIS

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SUMMARY

A micro disc electrophoresis technique was used to separate the soluble thyroid proteins at a neutral pH. Samples of 27 S, 19 S and 12 S proteins were prepared by centrifugation in sucrose density gradients and the electrophoretic separation patterns of these proteins were recorded by direct densitometry of the gels after staining with Amido Black.

The relations between the amount of 27 S, 19 S and 12 S and the optical densities of the stained protein discs were analysed.

The contribution of serum proteins to the 3–8 S peak in the density gradient centrifugation pattern was studied by electrophoresis of the thyroid proteins from perfused and non-perfused glands. It was found that the "heavy" part of the 3–8 S peak from non-perfused glands mainly represents serum proteins. However, after perfusion the 3–8 S region still contains four electrophoretically distinct bands in addition to the 19 S and 12 S bands.

The colloid extracted by micropuncture from one rat thyroid follicle was analysed by the method described. The migration rate of the single recorded protein was very similar to that of 19 S thyroglobulin.

INTRODUCTION

It is a well established fact that the major protein component of the thyroid gland is 19 S thyroglobulin. It is also generally accepted that the thyroglobulin is stored in the follicular lumen and a large number of observations indicate that thyroglobulin is the predominant constituent of the colloid. For example, the spectral absorbance properties of the colloid have been found to be the same as those of thyroglobulin solution[1–3] and fluorescein-coupled thyroglobulin antiserum has been observed to stain the colloid[4,5]. Furthermore, light and electron microscopic autoradiographic observations strongly indicate that the iodoproteins synthesized in the follicular cells are stored in the follicular lumen[6–8].

However, none of the methods used is specific for 19 S thyroglobulin. For example, both larger and smaller soluble thyroid iodo-proteins exert thyroglobulin antigenecity[9]. Furthermore, autoradiographic studies with labeled iodine have low

specificity since not only thyroglobulin but also its 12 S precursor protein can be iodinated[10–12].

Proteins other than iodoproteins are also probably present in the follicular lumen. Proteolytic activity has been found in extracts obtained by micro-puncture of single follicles of rat thyroids[13]. According to BALFOUR *et al.*[14], sera from patients with Hashimoto's and other thyroid diseases contain antibodies against an antigen of the colloid distinct from thyroglobulin.

Thus, the colloid evidently contains thyroglobulin-like proteins and probably other substances as well but detailed information of the composition of the colloid is lacking. This must be considered as a notable deficiency in our knowledge of thyroid physiology. For example, studies on thyroglobulin synthesis in this[15,16], and other laboratories[17,18], strongly indicate that the 19 S thyroglobulin formation is completed intracellularly, but without data on the composition of the colloid it is not possible to determine if precursor proteins are also secreted into the follicular lumen. Also having a bearing on the colloid composition is the question whether any hydrolysis of thyroglobulin occurs in the follicular lumen or whether the hormone release is the result of exclusive intracellular thyroglobulin degradation by lysosomal enzymes[19–23].

Against this background, it was deemed important to obtain information on the composition of the colloid in the follicular lumen. The only way to isolate the colloid, without substantial contamination by extrafollicular or intracellular components, seemed to be by micropuncture of the thyroid follicles. This, in turn, made it necessary to adapt a micromethod capable of separating the proteins extracted from a single or a few follicular lumina.

This paper presents the results, obtained by a micro disc electrophoresis technique at a neutral pH, of a separation and identification of the soluble thyroid proteins. The reliability of a quantitative determination of the protein by direct densitometry of the gels is analysed. The usefulness of the technique for the analysis of colloid from a single follicle is also illustrated. A more comprehensive account of the protein composition of the follicular colloid will be presented in another paper.

MATERIAL AND METHODS

Micro disc electrophoresis

The electrophoresis technique used is a microscale modification[24] of the original disc electrophoretic method of ORNSTEIN[25] and DAVIS[26].

The inner walls of 5 μl glass capillaries (Microcaps, Drummond Sci. Co., Broomall, Pa., U.S.A.) were coated with 0.07% methyl cellulose (Methocel, Dow Chem. Co., Midland, Mich., U.S.A.) in 30% formic acid–formaldehyde solution (1:5)[27].

Stock solutions. Stock A: pH 6.8, 8.69 g Tris + 0.63 ml N,N,N′,N′-tetramethylethylenediamine + 3.6 *N* H_2SO_4 + H_2O to 100 ml and a pH of 6.8.

Stock B: 10 g acrylamide (Eastman Organic Chemicals) + 200 mg N,N′-methylenebisacrylamide + H_2O to 37.5 ml.

Stock C: 1 mg riboflavin + 50 ml H_2O.

Stock D: pH 6.8, 5.98 g Tris + 0.46 ml N,N,N′,N′-tetramethylethylenedi-

amine + 1.0 *N* HCl + H_2O to 100 ml and a pH of 6.8.

Stock E: 3 mg riboflavin + 50 ml H_2O.

Preparation of the lower gel. One ml of (0.5 ml Stock A + 1.5 ml Stock B) + 1.0 ml Stock C gives a 10% solution.

Preparation of the upper gel. One ml of (0.5 ml Stock D + 1.5 ml Stock B) + 1 ml H_2O. One ml of this solution is added to 1 ml Stock D. One ml of this solution is added to 0.8 ml H_2O + 0.2 ml Stock E. The final concentration of the upper gel will be 2.5%.

Electrode buffer, pH 8.3. 3.0 g Tris + 14.4 g glycine + H_2O to 500 ml. A drop of Bromphenol Blue is added.

The electrophoresis was carried out at +4° and the voltage gradient over the gel was kept at 20 V and 40 V during the stacking and separation phases, respectively. The run was terminated when the Bromphenol Blue band had migrated 7 mm into the separation gel.

After the run, the gels were immediately pushed out of the capillaries into 80% ethanol. The proteins were stained for 5 min in 0.5% Amido Black solution in 7.5% acetic acid. After staining, the gels were destained in 7.5% acetic acid and stored in 7.5% acetic acid containing a small amount of Amido Black. The extinction of the storage solution was about 0.15 at 620 mμ.

Densitometry

The optical density of the stained protein discs in the gel was recorded after destaining in the storage solution for 1 hour and 45 minutes. A Schnell Photometer II (Zeiss, Jena, East Germany) equipped with a modified UR 400 Linear Logarithmic Integrator Recorder (Vitatron N.V., Dieren, Holland) was used for the recording[33]. For densitometric measurements the gel was mounted in a slit (20 × 0.5 mm) in a brass plate which was placed between two glass plates. By means of a motor, the gel was scanned at a speed of 2.17 mm/min, and the recording ratio between the paper and gel was 92. The gel was magnified 32 times on the photometer screen. An adjustable slit permitted the recording of the entire width of the gel image or a fraction of it. A red filter, Wratten No. 25, was inserted in the light path.

Photographs of the gels were taken using a Liesegang enlarger with parallel light and Ilford N-50 half tone backed plates.

Preparation of the thyroid proteins

The thyroid protein samples used as standards were prepared as shown in Fig. 1. Thyroid glands were excised from guinea pigs and rats after thorough perfusion of the anaesthetized animals with large volumes of phosphate buffered saline (PBS). The glands were pooled in cold PBS, weighed and minced. Homogenisation was performed in a glass homogenizer equipped with a tightly fitting Teflon pestle. The homogenate was centrifuged at 700 × *g* for 10 min and the resulting supernatant was centrifuged at 105 000 × *g* for 90 min.

An aliquot of the supernatant from the second centrifugation (S II) was stored at −20° until used for electrophoresis and protein determination. The proteins in the remaining S II supernatant were separated by centrifugation on either a 4.5 ml or a 14 ml continuous sucrose density gradient, 10–25%, prepared with PBS in 5 ml or 15 ml Spinco tubes respectively. After centrifugation at 75 000 × *g* for 16 h (5 ml

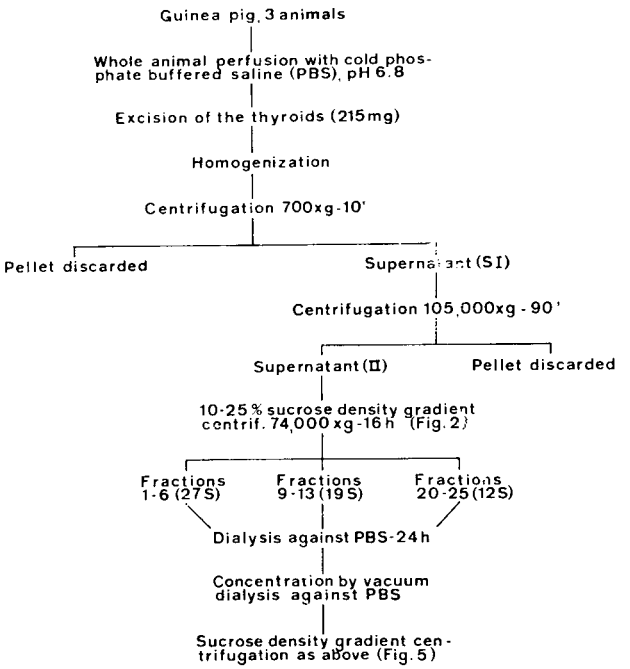

Fig. 1. Scheme for the preparation of the soluble proteins from guinea pig and rat thyroids.

tube) or at 150 000 × *g* for 14 h (15 ml tube) the tube was punctured and about 40 fractions were collected. Every second fraction was diluted with 0.8 ml PBS and the optical density at 280 mμ was measured. Every second, non-diluted fraction was analysed by disc electrophoresis. The samples used for electrophoresis were diluted in such a way that they all contained approximately the same amount of protein as judged from the absorption figures at 280 mμ.

In the guinea pig series, fractions were pooled from three regions of the density gradient. These fractions are referred to as 27 S, 19 S and 12 S (see Fig. 2). The pooled fractions were dialysed against PBS for 24 h and concentrated by vacuum dialysis at +4°. In the rat series, fractions from the 19 S region of the density gradient were pooled and handled in parallel with the guinea pig series. The pooled fractions from both series were further purified by another sucrose density gradient centrifugation as described above. The density gradient patterns were recorded at 280 mμ and the top fractions from each peak were analysed by electrophoresis.

Extraction of colloid

The extraction of colloid was performed by a micropuncture technique similar to that described by DE ROBERTIS[13] and SELJELID[31].

In rats, the isthmus of the thyroid was used for the puncture, in guinea pigs the right lobe was the most suitable part of the gland. Under light sodium pentobarbital anaesthesia the gland was surgically exposed under a dissecting microscope and gently freed from surrounding connective tissue. The extraction of colloid was performed through a glass cannula with a 10–15 μ wide, tapered tip and adapted to

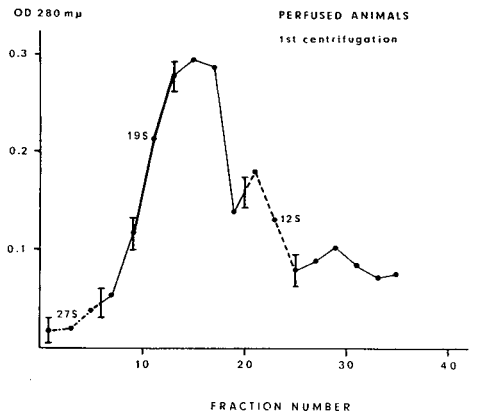

Fig. 2. Density gradient centrifugation pattern of the soluble proteins from guinea pig thyroids. The fractions were pooled and recentrifuged from the three regions (27 S, 19 S and 12 S) of the gradient indicated.

a Fonebrune micromanipulator. The colloid was extracted by negative pressure over the tip of the cannula produced by a glass syringe connected to the cannula *via* a low-elastic tube-system. The extracted colloid samples were immediately poured out into 5 or 10 μl of cold PBS.

RESULTS AND DISCUSSION

The main component of a sample of soluble thyroid proteins is the 19 S thyroglobulin molecule, which has a molecular weight of near 2/3 million. In addition to this molecule the heavier 27 S molecule and the lighter 12 S, 7 S and 4 S components are found in different amounts[9]. The wide range of molecular weights makes it difficult to design a separation system which gives optimal separation of all the proteins which could be expected in the colloid.

Under the separation conditions found to be most satisfactory (as regards voltage, separation time etc.) separation gels of 5.5 to 7.5% acrylamide were suitable for the separation of the larger molecules, 27 S and 19 S, while the smaller units, 12 S to 4 S, were preferably separated on 15 to 25% gels. As the amount of protein extracted from a single follicle is too small to be divided into several portions for analysis in different systems a 10% gel was chosen as a compromise for the survey analysis of the colloid proteins.

Since the resistance, and consequently the heat production, increases rapidly during the stacking of the proteins in the spacer gel[25], the voltage gradient was kept low during this phase of the electrophoresis (20 V). When the reference substance (Bromphenol Blue) was visible in the separation gel as a distinct band, the voltage gradient was increased to 40 V. This augments the speed of the separation and reduces the effect on the separation pattern of the time-dependent free diffusion.

In an alkaline environment, thyroglobulin is easily degraded into 12 S subunits[28]. To eliminate the effect of the basic pH's originally described for the method (pH 9.8, ref. 26, and 8.8, ref. 24) the pH of the separation gel was adjusted to 6.8.

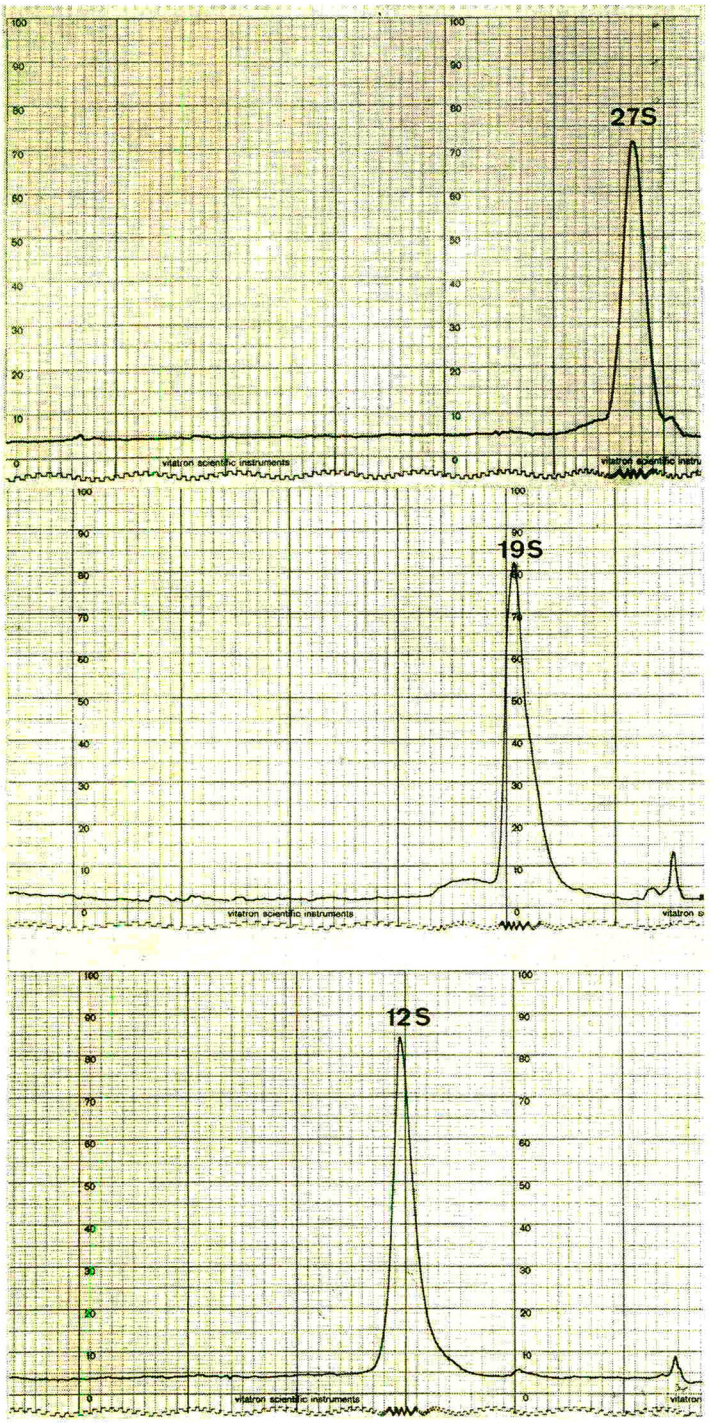

Fig. 3. Densitometric recordings of the electrophoretic separation patterns for 27 S, 19 S and 12 S proteins.

In this system no degradation of 27 S, 19 S or 12 S components was observed during the electrophoresis (Fig. 3).

Qualitative analysis of the electrophoretic separation patterns

The soluble thyroid proteins from guinea pigs and rats were used. Guinea pigs were chosen because their thyroids contain considerable amounts of stable 12 S protein[29]. In order to eliminate the disturbing influence of serum proteins, the animals were perfused with cold PBS prior to the excision of the glands. This was considered important owing to the fact that one of the 4 S components extracted from the thyroid gland has very similar electrophoretic properties to those of serum albumin[9].

The density gradient centrifugation pattern of the soluble guinea pig thyroid proteins is shown in Fig. 2. After electrophoretic analysis it was obvious that the peaks of the density gradient pattern did not represent single protein components (Fig. 4). Therefore the proteins of each one of the 27 S, 19 S and 12 S regions of the gradient were further purified by a second density gradient centrifugation.

Recentrifugation of the fractions in the 27 S region gave no distinct peak in the optical density pattern of the gradient (Fig. 5). However, on disc electrophoresis

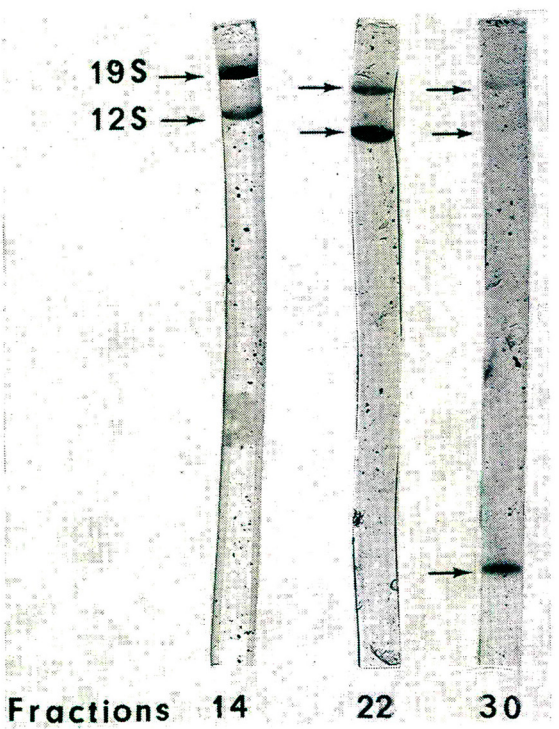

Fig. 4. Photographs of the gel separation patterns resulting from electrophoresis of fractions No. 14, No. 22 and No. 30 in Fig. 2. The part migrating most slowly is the major component in fraction No. 14, from the 19 S peak, and the second, faster migrating band predominates in fraction No. 22, from the 12 S peak. In fraction No. 30 a very fast migrating band predominates, but very faint bands corresponding to 19 S and 12 S are also visible.

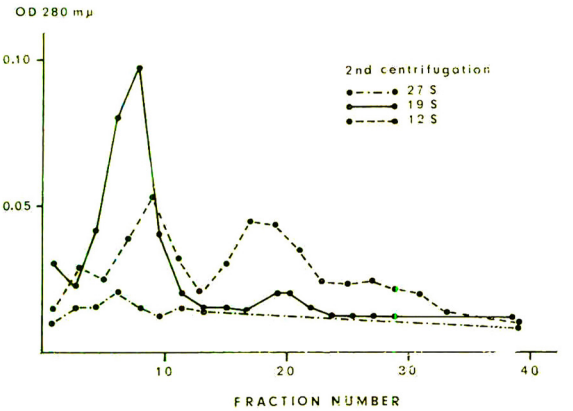

Fig. 5. Density gradient patterns of the recentrifuged 27 S, 19 S and 12 S samples indicated in Fig. 2. Fraction No. 4 from the 27 S gradient, fraction No. 9 from the 19 S gradient and fraction No. 18 from the 12 S gradient were subjected to electrophoretic analyses.

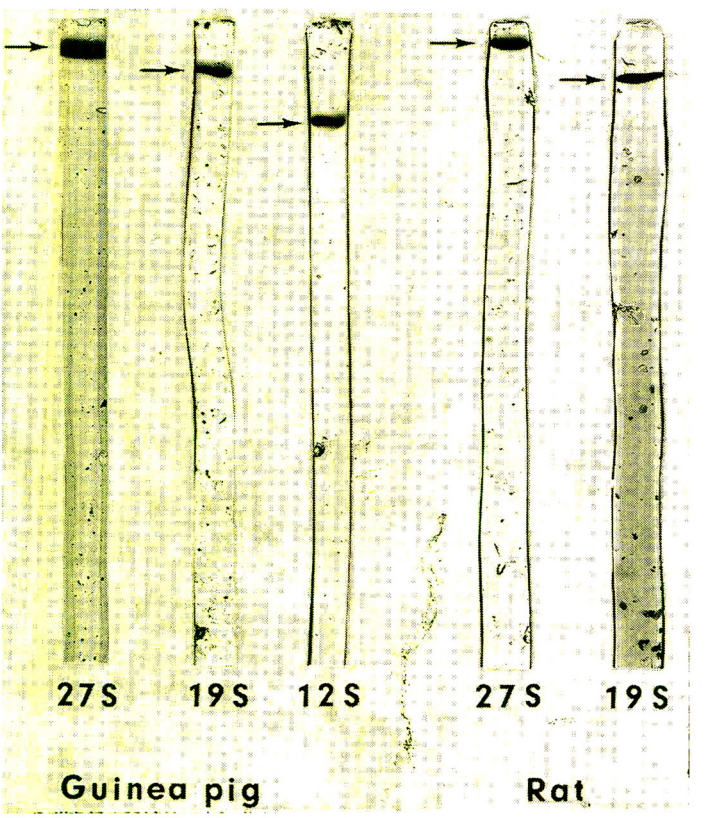

Fig. 6. Left: Three photographs of gel patterns obtained by electrophoresis of purified guinea pig thyroid proteins: fraction No. 4, No. 9 and No. 18 in Fig. 5. Right: The electrophoretic separation patterns of similarly purified rat thyroid proteins from the 27 S and 19 S density gradient regions.

the protein of the first four fractions of the 27 S gradient migrates as one band, distinct from the 19 S particles (Fig. 6).

The optical density pattern of the second 19 S gradient shows two peaks (Fig. 5). Samples from the faster sedimenting larger peak display only one electrophoretic band, most probably 19 S thyroglobulin (Fig. 6).

The extinction pattern obtained from the recentrifugation of the 12 S proteins shows two peaks (Fig. 5). The components of the larger, slowly sedimenting peak move in a single band on electrophoresis and very probably constitute 12 S protein (Fig. 6).

Density gradient centrifugation of the soluble rat thyroid proteins, which was run simultaneously with that of the guinea pig proteins, resulted in a small extinction peak in the 27 S region and a large peak in the 19 S region but no visible 12 S peak. Electrophoretic separation of the proteins in these peaks resulted in a single band from each peak, moving at the same rate as the 27 S and 19 S components of the guinea pig proteins (Fig. 6).

Electrophoretic analyses of the guinea pig thyroid proteins in the fractions from the 3–8 S region of the gradient display a marked heterogeneity. The protein

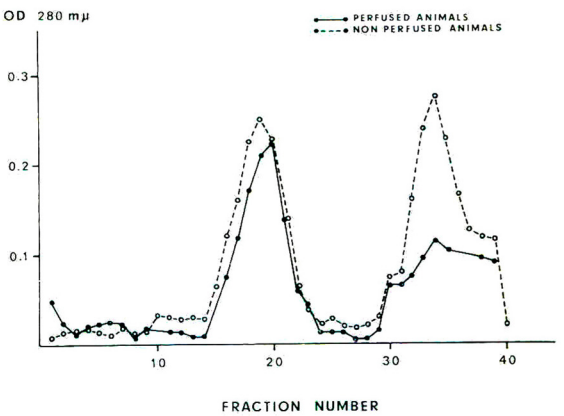

Fig. 7. Density gradient separation patterns representing the distribution of the soluble proteins in thyroid homogenates from perfused (●—●) and non-perfused (○— —○) guinea pigs.

components of the fastest fractions of the 3–8 S region (Fig. 7, fractions 30–34), split up into a group of four bands which do not separate completely on 15% gel (Fig. 8a). In addition to this group of proteins the electrophoretic pattern also shows a smaller amount of faster migrating proteins which form a broader zone without discrete bands. The latter group of proteins are, however, the major protein components in the 3–8 S region of the density gradient pattern produced by proteins extracted from non-perfused thyroids (Fig. 8b). The later fractions from the 3–8 S peak (Fig. 7, fractions 36–40) display one major component which migrates just behind the reference substance in the 10% separation gel (Fig. 9).

Small amounts of 12 S and 19 S proteins were found in all the fractions from the 3–8 S peak.

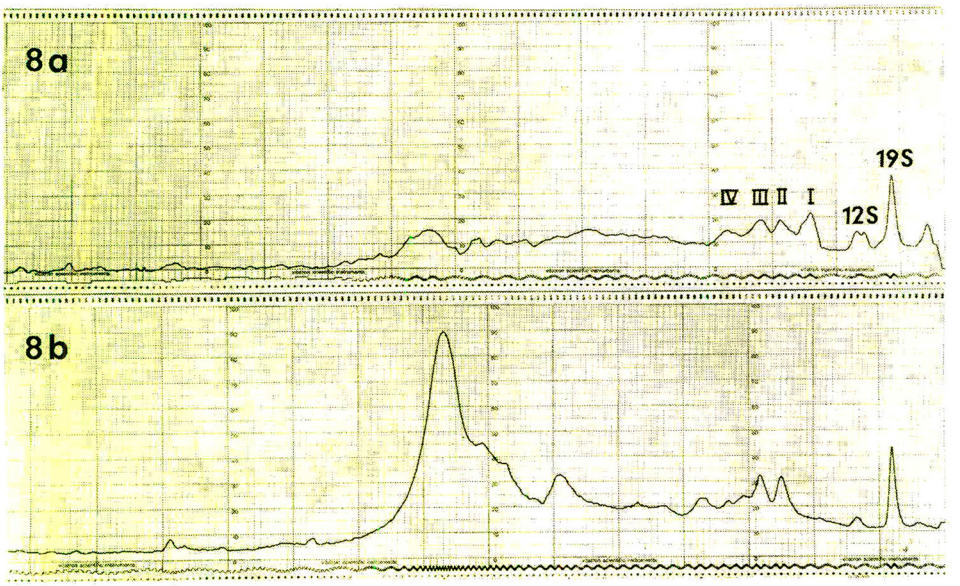

Fig. 8. Densitometric recordings of the gel separation pattern of fractions in the 3–8 S region of the gradients in Fig. 7. (8a) Perfused animals. Fraction No. 32, analysed on 15% separation gel. 19 S and 12 S particles form the two slowest migrating bands. The 12 S band is preceded by four bands (I–IV) of unknown nature. Faster migrating proteins are spread over a zone without discrete bands. (8b) Non-perfused animals. Fraction No. 32, analysed on 15% separation gel. The fastest migrating bands are the predominant constituents of this fraction. These proteins probably represent serum proteins.

Quantitative determination of the thyroid proteins

The Zeiss photometer employed in this study has previously been used for quantitative estimation of electrophoresis separation patterns[32]. The Vitatron integrator unit was modified in such a way that the inverse extinction, caused by the stained band against the light background could be recorded[33].

The contrast between the stained band and the gel was increased about 2-fold by the red filter. In addition, the contrast could be increased by about 10% by narrowing the adjustable slit in the image plane of the photometer since the central

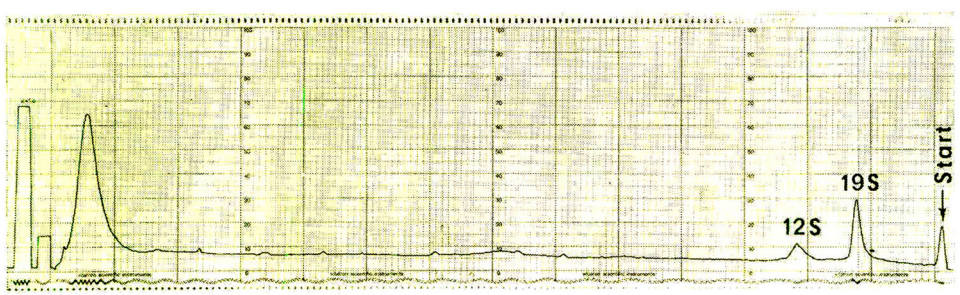

Fig. 9. Densitometric recording of the gel separation pattern of proteins in fraction No. 36 in Fig. 7. The proteins were analysed on 10% separation gel.

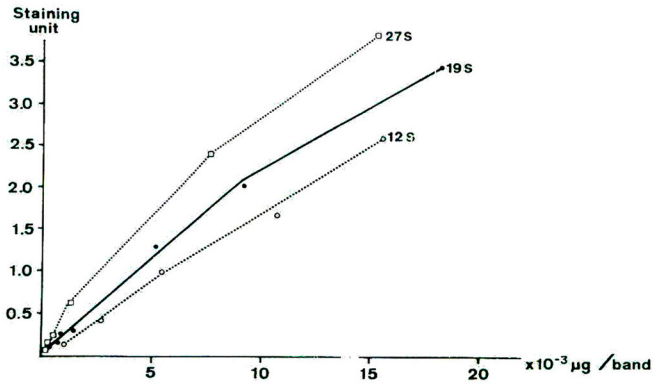

Fig. 10. Diagram showing the relation between the amount of 27 S, 19 S and 12 S particles (μg) and the optical densities of the resulting protein discs after electrophoresis in a 10% separation gel. The staining unit was calculated from S.U. $= a \cdot 1/b \cdot E$ where a is the number of integration impulses; b, the height of the external standard which varies with the sensitivity adjustment; E the fixed values of the external standard (1.0 and 0.2).

part of the stained band has a higher optical density than the lateral parts of the band, provided that the stain is evenly distributed in the protein disc.

In order to calibrate the staining against the amount of protein in a band, electrophoretically homogeneous samples of the 27 S, 19 S and 12 S proteins were prepared. The protein concentration of the samples was determined from their absorption at 280 mμ and at a neutral pH. The extinction coefficient employed was

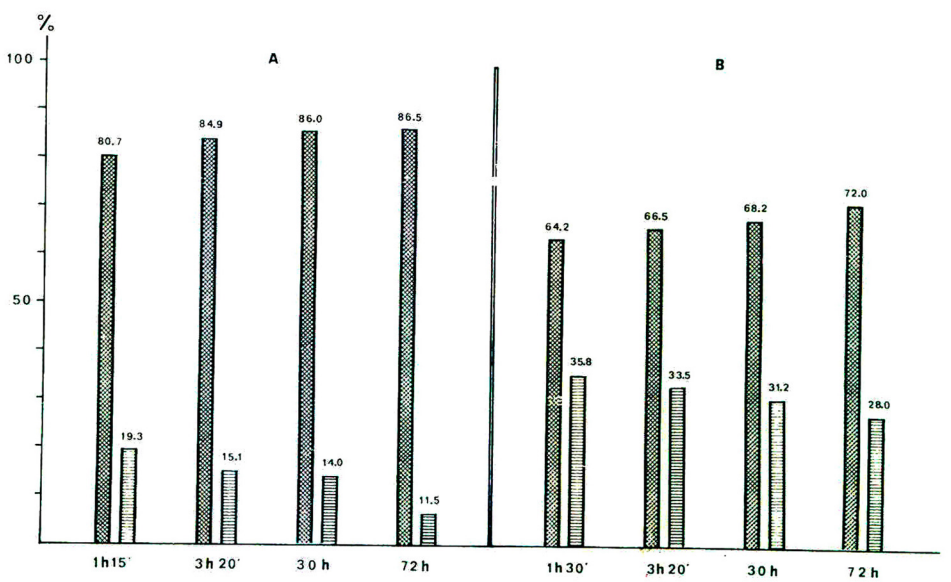

Fig. 11. The relative distribution of stain between a 19 S and a 12 S protein disc in the same gel at increasing destaining periods. In gel A and gel B the same trend of the stain distribution is visible.

$E^{1\%}_{1cm}$ = 10.4 (ref. 30). (This value of the extinction coefficient is an approximation as it has been determined on a mixture of proteins extracted from hog thyroids.) After electrophoresis and staining the optical density was recorded on the densitometer and a staining unit was calculated on the basis of the number of integration impulses/band and the sensitivity adjustment of the recorder (Fig. 10).

The interrelation between the amount of protein and the optical density of the stained protein in a band is linear for all three species at low concentrations of protein. At higher concentrations of protein/band the quotient between the optical density and the protein concentration decreases.

In order to assess to what degree the densitometric recordings are affected by the diffusion rate of the staining molecules from the protein particles, a series of experiments was performed in which the percentage distribution of the stain between

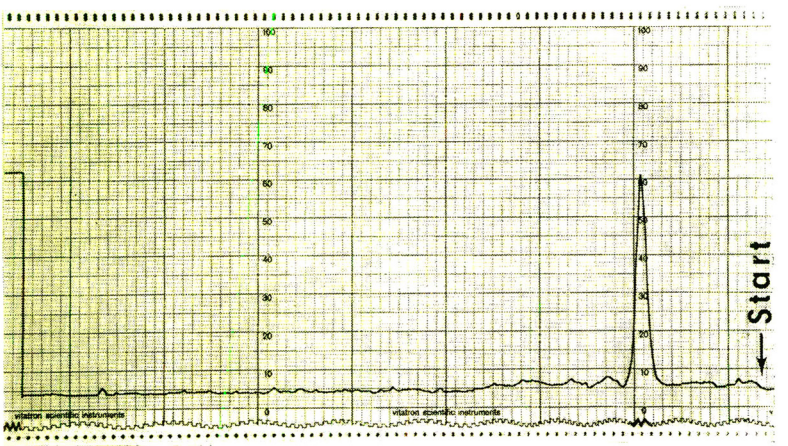

Fig. 12. Densitometric recording of the separation pattern of the colloid extracted from one large follicle of the rat thyroid. This colloid was analysed in a 10% separation gel. The migration rate of the band is very similar to that of 19 S particles of the reference samples. No other components could be recorded in this gel.

a 19 S and a 12 S band in the same gel was calculated after different destaining intervals. It was found that the relative amount of stain in the 19 S band increases during the destaining. This is the case both when the initial concentration of 19 S is high relative to 12 S (Fig. 11, A) and when there is a more even distribution of the two molecules in the sample (Fig. 11, B). After destaining for 1 h and 45 min, however, there is practically no shift in the per cent distribution of stain between the 19 S and 12 S bands. Thus, this destaining artefact does not reduce the validity of the densitometric recordings.

Disc electrophoresis of the colloid

Fig. 12 shows an example of an electrophoretic separation pattern of the colloid extracted from one follicle in the isthmus of a rat thyroid. The migration rate of the single visible band corresponds to that of 19 S thyroglobulin in the reference samples.

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ION-EXCHANGE PAPER CHROMATOGRAPHY OF INORGANIC IONS

XXV. SOME CONSIDERATIONS ON THE DETERMINATION OF THE "CHARGE" OF A METAL ION BY ION-EXCHANGE EQUILIBRIA

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SUMMARY

Ion exchangers with few reactive groups such as cellulose sulphonic acid paper do not distinguish between ions of different charges in the law of mass action equation. It is suggested that this is due to the large distance between the exchange groups. Similar results are obtained with papers impregnated with very dilute solutions of liquid ion exchangers.

INTRODUCTION

BOYD *et al.*[1] reviewed the question of ion-exchange equilibria in 1947 and pointed out that the equilibria may be considered as a type of law of mass action equation although other approaches are also possible (*e.g.* the Freundlich isotherm). From this paper it was however clear that the charge of the adsorbed cation can influence adsorption as in a law of mass action equation. TOMPKINS AND MAYER[2] have shown that law of mass action equations may also be used for complexing equilibria on ion exchangers, *e.g.*, for the elution of rare earth ions with citrate solution.

Since then confirmation of this principle in ion exchange has been obtained several times, not only with the usual ion-exchange resins but also with inorganic ion exchangers[3]; liquid ion exchangers also behave in the same way. A simple equation relating the R_F value, the "charge" of the ion and the concentration of the eluent electrolyte was proposed for ion-exchange resin papers[4].

$$x\ \mathrm{pH} = R_M + \text{constant}$$

Usually the concentrations were not "corrected" by multiplication with the activity coefficient as this was deemed presumptive in a system where there are certain to be concentration gradients and mass gradients along the paper strip. In spite of the rather empirical approach straight line relationships between $-\log [H^+]$ and R_M were obtained with ion-exchange resin papers[4], zirconium phosphate papers[5], ammonium tungstophosphate paper[6], papers impregnated with liquid ion exchangers[7], and alginic acid thin layers[8], to mention a few.

In all these cases the tangent which should indicate the "charge" of the cation was usually around 1 for monovalent cations, 1.4 to 2 for divalent cations, 2 to 2.7 for trivalent cations and 3 to 3.7 for tetravalent ones.

To summarise: all the evidence seems to indicate that the cations combine with the cation exchangers by a mass action type of equation where the charge of the cation can be considered identical with the charge exhibited in simple solid salts.

We have recently found one exception to this rule. $Co(NH_3)_6^{3+}$, on sulphonic resin papers, behaves like a pentavalent cation[9]. This could be explained by assuming outer-sphere complex formation between the sulphonic groups of the resin and the

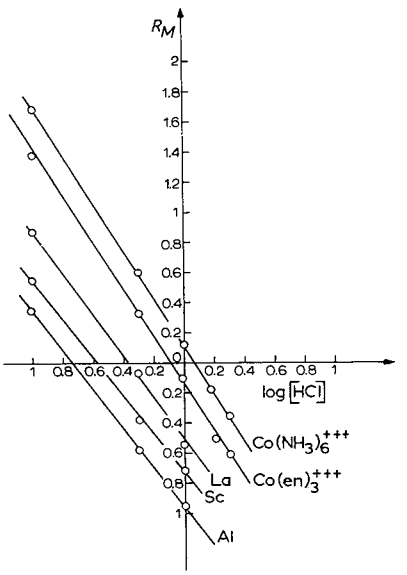

Fig. 1. R_M *versus* log [HCl] plots for Macherey-Nagel sulphonic acid cellulose papers. Slopes for $Co(NH_3)_6^{3+}$ = 1.59; $Co(en)_3^{3+}$ = 1.57; La = 1.2; Sc = 1.27; and Al = 1.31.

$Co(NH_3)_6^{3+}$. It should be mentioned here that anomalous results with certain ions have been observed previously, *e.g.*, the strong adsorption of $Rh(H_2O)_6^{3+}$ on sulphonic resin papers and the seemingly irreversible adsorption of Fe(III) on cellulose phosphate papers. However in both cases complex formation between the resin and the metal ions seemed evident.

While studying the remarkable behaviour of $Co(NH_3)_6^{3+}$ we discovered another exception to the usual adsorption of cations on ion exchangers. This behaviour is exhibited by ion exchangers with only a few ion-exchange groups on a non-exchanging surface and is the topic of the present paper.

RESULTS

While working with $Co(NH_3)_6^{3+}$ we thought that it would be interesting to examine its behaviour on cellulose sulphonic acid paper (Macherey-Nagel): the results

TABLE I

THE SLOPES OF THE R_M *versus* LOG [$HClO_4$] PLOTS FOR THE MACHEREY-NAGEL CELLULOSE SULPHONIC ACID PAPER

Data from F. SARACINO[11].

Metal ion	*Slope*
Al(III)	1.65
Sc(III)	1.22
Y(III)	1.7
La(III)	1.3
Co(II)	1.1
Ni(II)	1.07
Cu(II)	1.36
Zn(II)	1.0
Mn(II)	1.04
Cd(II)	1.23
UO_2^{2+}	1.24

were very different from those on sulphonic resin papers, namely the "charge" obtained was around 1.6 instead of 4.75. Unexpectedly low results were also obtained for the slopes of the R_M *versus* log [$HClO_4$] plots, derived from the R_F data, for several multivalent cations eluted on cellulose sulphonic acid papers with perchloric acid (results already published by SARACINO[11]). An example of such plots is shown in Fig. 1, while Table I collects all the results for di- and trivalent ions. It can be seen that the slopes obtained are much lower (in absolute values) than expected. These substituted cellulose papers have one obvious difference in their properties from the resins, namely, the few sulphonic groups are spaced rather far apart (one on each sugar moiety). We thought therefore that the abnormal results obtained could be attributed to this property.

In order to confirm this assumption, two series of experiments were carried out. Rare earths (known to be "uncomplexed" by chloride ions) were eluted with hydrochloric acid on Macherey-Nagel (MN) cellulose sulphonic acid paper and on Whatman

TABLE II

SLOPES OF THE R_M *versus* LOG [HCl] PLOTS ON MACHEREY-NAGEL CELLULOSE SULPHONIC ACID PAPER

Metal ion	*Slope*
La(III)	1.4, 1.2
Ce(III)	1.3
Pr(III)	1.45
Nd(III)	1.8
Sm(III)	1.4
Eu(III)	1.4
Gd(III)	1.7
Tb(III)	1.8
Dy(III)	1.3
Ho(III)	1.8
Er(III)	1.9
Tm(III)	1.3
Yb(III)	1.85
Lu(III)	1.6

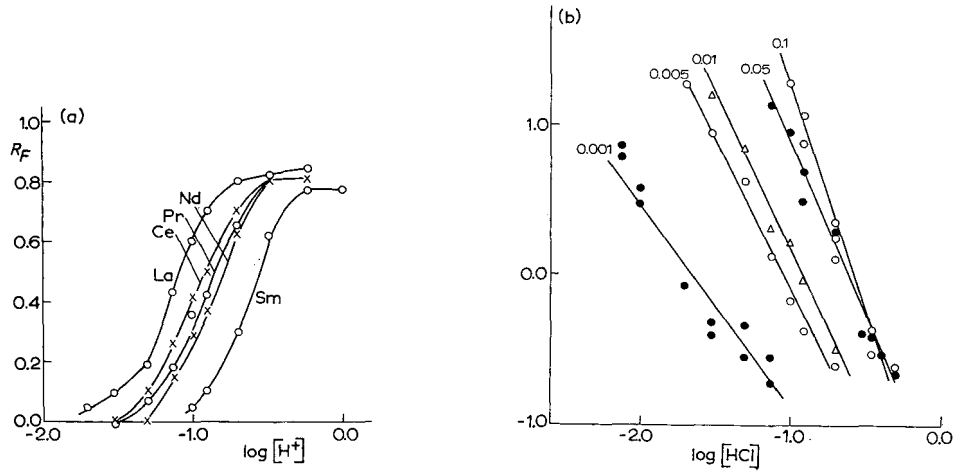

Fig. 2. (a) R_F *versus* log [H+] plots for papers impregnated with 0.005 *M* HDEHP. (b) R_M *versus* log [H+] plots for papers impregnated with various concentrations of HDEHP. Data for La(III) as given in Table III.

No. 1 paper loaded with very low amounts of di-(2-ethylhexyl)orthophosphoric acid (HDEHP), a well-known liquid cation exchanger extensively used in reversed-phase chromatography[7].

The reversed-phase experiments were carried out on paper treated with HDEHP solutions down to 0.001 *M* in cyclohexane. Although the actual amount of extractant loaded on the paper was unknown, it was reasonably assumed to be roughly proportional to the concentration used, so that the surface density of active exchange groups on the paper increases as the concentration of HDEHP decreases in the treating solution.

The slopes of the straight lines which best fitted the R_M *versus* log [HCl] plots for the MN paper and HDEHP-treated paper are reported in Tables II and III, respectively, for the latter together with their confidence limits, calculated from a statistical approach at the 95% probability level. A representative plot for HDEHP-treated paper is shown in Fig. 2. While the MN paper results confirm that the "charges" for the rare earths are far below 3, the HDEHP-treated paper results show

TABLE III

SLOPES OF R_M *versus* LOG [HCl] PLOTS FOR PAPER IMPREGNATED WITH VARIOUS AMOUNTS OF HDEHP

Metal ion	*HDEHP concentration in the impregnating solution*				
	0.1 M	*0.05 M*	*0.01 M*	*0.005 M*	*0.001 M*
La(III)	−3.02 ± 0.47	−2.29 ± 0.23	−2.12 ± 0.46	−1.99 ± 0.26	−1.34 ± 0.18
Ce(III)	−2.82 ± 0.58	−2.68 ± 0.21	−2.40 ± 0.35	−2.17 ± 0.51	−1.40 ± 0.18
Pr(III)	−2.98 ± 0.37	−2.47 ± 0.36	−2.20 ± 0.56	−2.10 ± 0.43	−2.06 ± 0.30
Nd(III)	−3.08 ± 0.61	−2.40 ± 0.41	−2.28 ± 0.73	−2.00 ± 0.41	−2.27 ± 0.52
Sm(III)	−3.11 ± 0.69	−2.69 ± 0.35	−2.66 ± 0.53	−2.12 ± 0.62	−1.71 ± 0.48

TABLE IV

DISTANCES MOVED BY METAL IONS IN $HClO_4$ BY HIGH VOLTAGE ELECTROPHORESIS

Paper: Whatman No. 1. Experiments conducted at 3–4° for 2 h at 300 V. Camag apparatus.

Metal ion	*Distance in mm corrected for electro-osmotic flow (with H_2O_2)*		
	0.1 N $HClO_4$	*0.5 N $HClO_4$*	*1.0 N $HClO_4$*
Tl(I)	93	76	61
UO_2^{2+}	45	35	35
Bi(III)	46	49	50
Cd(II)	60	56	52
Pb(II)	74	57	57
Cu(II)	61	47	50
Fe(III)	52	45	47
Co(II)	61	56	51
Ni(II)	62	56	51
Mn(II)	70	57	50
Zn(II)	62	57	51
Al(III)	69	56	49
Y(III)	67	59	52
La(III)	75	65	58
Zr(IV)	28 comet	37 comet	38 comet
Th(IV)	49	55	55

that such "charges" actually decrease as the HDEHP loading is lowered, ranging from about 3 for the greatest HDEHP amounts to below 2 for paper treated with 0.001 *M* HDEHP solutions.

Two hypotheses could explain such a decrease in the observed "charges" with decreasing surface density of the active exchange groups. On the one hand, the distance among the groups may be large enough to prevent a cation from interacting with the number of active groups as required by its actual ionic charge.

On the other hand, the relatively few groups may be insufficient to destroy the outer-sphere complexes built around the multivalent cations in the original solution. Paper electrophoretic experiments have repeatedly shown that in hydrochloric acid solutions[10] there is little difference in speed between trivalent metal ions such as Al(III), and divalents such as Cu(II), Ni(II) or Co(II). This cannot be entirely explained by chloride complexes such as $AlCl^{2+}$ since the same behaviour was observed in other, certainly non-complexing, media; results obtained with perchloric acid are reported in Table IV.

Either being the reason, the data reported above show that a slope very different from that expected can be obtained with ion exchange materials, under suitable circumstances. We should not be surprised if an analogous situation is soon found with the very similar extraction equilibria.

In planning the above experiments, we also hoped to demonstrate an effect on the slopes due to the increasing size of the hydrated radius of the different rare earths. Unfortunately, the results obtained suffer from too large an uncertainty for this purpose, as, probably due to the non-uniformity of both the stationary phases and to their low capacity, either the detection or shape determination of the eluted spots were often impaired.

Larger differences among hydrated ion sizes will probably make ions behave

in rather different ways and this could improve separation factors. That is why we believe that systems such as those considered in this work are rather promising, in spite of the low ion-exchange capacities.

Finally, we would like to propose a new nomenclature for ion-exchange systems. Exchangers having active sites far enough apart as to show such effects as the ones dealt with in this work should be called "dilute" exchangers, while those which actually differentiate between different valencies in the law of mass action should be called "concentrated" ones. This still leaves the terms strong and weak, for denoting the strength of the reactive group as is the usual practice.

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Notes

CHROM. 4258

Der Einfluss der Molekülform auf die Temperaturabhängigkeit der Retentionsindizes isomerer Alkane

Die Retentionsindizes isomerer Alkane hängen, wenn auch nur in geringem Masse, von der Temperatur ab[1–4]. Nachdem gezeigt werden konnte[5], dass der Reten-

TABELLE I

AUF DIE LÄNGE EINER C–C-EINFACHBINDUNG a_1 BEZOGENE QUADRATISCHE MITTELWERTE DER RADIEN K_R^2, EXPERIMENTELL BESTIMMTE $(\Delta I/10°)_{ex}$[4] UND BERECHNETE $(\Delta I/10°)_{th}$ TEMPERATURKOEFFIZIENTEN DER RETENTIONSINDIZES DER ISOMEREN ALKANE C_6 BIS C_9

Substanz	K_R^2	$(\Delta I/10°)_{ex}$	$(\Delta I/10°)_{th}$	*Substanz*	K_R^2	$(\Delta I/10°)_{ex}$	$(\Delta I/10°)_{th}$
Hexane				Nonane			
n-6	1.4132	0.00	0.00	n-9	2.3657	0.00	0.00
2-M-5	1.2510	0.12	0.18	2-M-8	2.2196	0.30	0.16
3-M-5	1.1965	0.50	0.24	3-M-8	2.1216	0.35	0.27
2,2-DM-4	1.0370	0.80	0.85	4-M-8	2.0724	0.30	0.32
2,3-DM-4	1.0895	1.00	0.58	3-Ä-7	1.9743	0.45	0.43
Heptane				4-Ä-7	1.9251	0.45	0.49
n-7	1.7271	0.00	0.00	2,2-DM-7	1.9768	0.45	0.43
2-M-6	1.5672	0.14	0.18	2,3-DM-7	1.9267	0.65	0.48
3-M-6	1.4865	0.35	0.27	2,4-DM-7	1.9264	0.20	0.48
3-Ä-5	1.4059	0.62	0.35	2,5-DM-7	1.9755	0.30	0.43
2,2-DM-5	1.3288	0.67	0.72	2,6-DM-7	2.0736	0.25	0.32
2,3-DM-5	1.3273	1.01	0.73	3,3-DM-7	1.8300	1.15	0.94
2,4-DM-5	1.4074	0.32	0.35	3,4-DM-7	1.8288	0.80	0.95
3,3-DM-5	1.2487	1.45	1.14	3,5-DM-7	1.8776	0.55	0.69
2,2,3-TM-4	1.1701	1.55	1.55	4,4-DM-7	1.7809	1.15	1.20
Octane				2-M-3-Ä-6	1.7796	1.05	1.20
n-8	2.0449	0.00	0.00	2-M-4-Ä-6	1.8284	0.55	0.95
2-M-7	1.8913	0.27	0.17	3-M-3-Ä-6	1.6833	1.75	1.70
3-M-7	1.7984	0.37	0.27	3-M-4-Ä-6	1.7308	1.55	1.46
4-M-7	1.7673	0.27	0.31	2,2,3-TM-6	1.6845	1.60	1.70
3-Ä-6	1.6744	0.37	0.41	2,2,4-TM-6	1.7330	1.35	1.45
2,2-DM-6	1.6464	0.59	0.48	2,2,5-TM-6	1.8308	0.65	0.94
2,3-DM-6	1.6142	0.72	0.65	2,3,3-TM-6	1.6357	1.85	1.95
2,4-DM-6	1.6449	0.53	0.49	2,3,4-TM-6	1.6833		1.70
2,5-DM-6	1.7377	0.30	0.34	2,3,5-TM-6	1.7808	0.85	1.20
3,3-DM-6	1.5230	1.30	1.12	2,4,4-TM-6	1.6842	1.60	1.70
3,4-DM-6	1.5525	1.12	0.97	3,3,4-TM-6	1.5870	2.20	2.20
2-M-3-Ä-5	1.5214	1.60	1.13	3,3-DÄ-5	1.5857	2.90	2.21
3-M-3-Ä-5	1.4306	1.99	1.60	2,2-DM-3-Ä-5	1.5866	2.10	2.21
2 2,3-TM-5	1.4010	1.66	1.75	2,3-DM-3-Ä-5	1.5382	2.70	2.46
2,2,4-TM-5	1.4931	1.18	1.27	2,4-DM-3-Ä-5	1.6342	1.80	1.96
2,3,3-TM-5	1.3698	2.14	1.92	2,2,3,3-TeM-5	1.4431	2.70	2.95
2,3,4-TM-5	1.4612	1.50	1.44	2,2,3,4-TeM-5	1.5391	2.25	2.45
2,2,3,3-TeM-4	1.2500	2.62	2.54	2,2,4,4-TeM-5	1.5885	1.90	2.20
				2,3,3,4-TeM-5	1.4906	2.65	2.71

tionsindex (unpolarer) isomerer Verbindungen eine Funktion des quadratischen Mittelwertes des Radius und der Dichte bzw. der Zahl der durch drei C–C-Bindungen getrennten Kohlenstoffatome ist, liegt es nahe zu untersuchen, ob auch der Temperaturkoeffizient der Retentionsindizes eine Funktion dieser Grössen ist.

Der quadratische Mittelwert des Radius R eines Moleküls ist durch folgende Beziehung definiert:

$$\bar{R}^2 = \frac{1}{N} \sum_{i=1}^{N} \bar{r}_i^2 \qquad (1)$$

Hierbei sind N die Zahl der (gleichen) Atome bzw. Atomgruppen und r_i der Abstand des i-ten Atoms bzw. der i-ten Atomgruppe von Schwerpunkt des Moleküls. Der mit 2/3 M (M $\triangleq$ Molekulargewicht) multiplizierte Wert von $\bar{R}^2$ entspricht dem mittleren Trägheitsmoment eines Moleküls. Den grössten Radius haben jeweils die n-Alkane; der Radius wird umso kleiner, je kompakter ein Molekül gebaut ist. Unter Benutzung des Modelles der "frei drehbaren Valenzwinkelkette" kann man die quadratischen

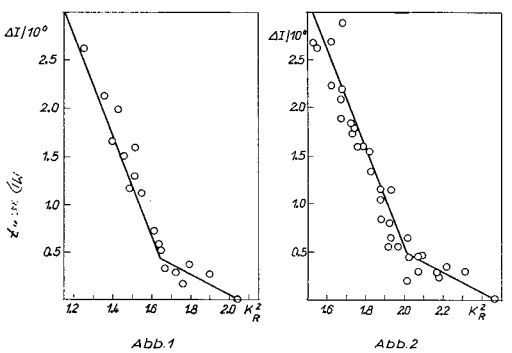

Abb. 1 Abb. 2

Fig. 1. Die Abhängigkeit der Temperaturkoeffizienten der Retentionsindizes der isomeren Octane vom quadratischen Mittelwert des Radius.

Fig. 2. Die Abhängigkeit der Temperaturkoeffizienten der Retentionsindizes der isomeren Nonane vom quadratischen Mittelwert des Radius.

Mittelwerte der Radien einfach berechnen[6]. Die auf die Länge einer C–C-Einfachbindung a_1 bezogenen quadratischen Mittelwerte der Radien $\bar{R}^2/a_1^2 = K_R^2$ der isomeren Alkane C_6 bis C_9 sind in Tabelle I zusammengestellt.

Trägt man die von TOURRES[4] an Squalan bestimmten Temperaturkoeffizienten der Retentionsindizes als Funktion des quadratischen Mittelwertes des Radius bzw. von K_R^2 auf, so erhält man den in Fig. 1 für die Octane und in Fig. 2 für die Nonane dargestellten Zusammenhang*.

Diese Abhängigkeit lässt sich für die isomeren Alkane C_6 bis C_9 durch folgende Beziehung näherungsweise darstellen:

$$\Delta I/10^0 = 5.2(0.85\, K_{R_0}^2 - K_R^2) \quad \text{für } K_R^2 < 0.81\, K_{R_0}^2 \qquad (2)$$

* Es sei erwähnt, dass POLLMANN[7] für die Druckabhängigkeit der Viskosität isomerer Hexane und Heptane vom quadratischen Mittelwert des Radius[6] einen ganz ähnlichen Kurvenverlauf erhält. Siehe auch Lit. 8.

und

$$\Delta I/10^0 = 1.1(K_{R_0}{}^2 - K_R{}^2) \qquad \text{für } K_R{}^2 > 0.81\, K_{R_0}{}^2 \tag{3}$$

$K_{R_0}{}^2$ ist jeweils der Wert des isomeren *n*-Alkans.

Die mit Gleichung (2) und (3) berechneten Temperaturkoeffizienten sind in Tabelle I den experimentellen Werten gegenübergestellt.

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CHROM. 4272

A method for the prediction of retention indices of hydrocarbons on squalan

At present, the retention indices (I) are the means most widely used for identification of chromatographic peaks. These indices, like the boiling temperature, density and other physicochemical characteristics of substances, are experimentally determined. Even for one and the same substance, these values depend on the conditions of the analysis. Due to the great number of variables in GC, a long time is required for the experimental determination of I.

In this paper we develop a new method for the calculation of I in which no experimental chromatographic data are necessary. This allows a very precise prediction of the retention of the isoparaffins and naphthenes on squalan.

In the analysis of natural products and especially of oil cuts, which contain components similar in their properties, there are two problems to be solved. Firstly, the most complete resolution and exact identification is required in order to make the second step—quantitative analysis. There are different techniques for the determination of the degree of resolution, which are not the subject of the present paper. KOVATS' indices[1-3] are the most popular and successful means for the identification of the peaks in a chromatogram. They are calculated from the experimentally-determined V_N of the components of the mixture and the V_N of the reference n-paraffins added.

There are several methods[1,4,5] for the determination of I, based on the indices obtained for similar substances on the same stationary phase. In one of these methods[1], the difference between the indices of two substances is determined by the empirical relationship:

$$\Delta I \simeq 5\Delta t_b^\circ \qquad (1)$$

According to this relationship, the indices do not depend on the temperature of the analysis, which is not true. Apart from this, it is only valid for paraffin isomers.

Another method for the determination of I has been developed by SCHOMBURG[4,5]. He uses the experimentally-determined indices of different isomeric hydrocarbons in order to obtain what he calls the "homomorphic" factor. By means of this factor, he calculates ΔI between an n-paraffin and the corresponding isoparaffin using the formula:

$$I_i^A = I_z^A + \Sigma H_i^A \qquad (2)$$

where I_i^A is the retention index of an isomer on a nonpolar stationary phase; I_z^A the index of the corresponding n-paraffin, and H_i^A is the "homomorphic" factor. This method also does not allow another way of determining the influence of the temperature of the analysis on the resolution except an experimental one.

The other methods for calculating ΔI concern the differences in I with the change in the stationary phases.

The new method for calculating the retention indices of hydrocarbons on squalan requires knowledge of the vapour pressures of the substances analysed at the temperature of the analysis, their molecular weights and densities. The method is based on the fact that squalan is a non-specific medium with respect to the solution of paraffins and naphthenes[6]. This means that the non-ideality of the solution is

compensated in sign and magnitude by the ratios of the molecular volumes of the solutes mentioned. According to an earlier paper[6] on a phase for which

$$\log \gamma' \leqslant 0.0 \pm 0.03 \text{ units} \tag{3}$$

where γ' is the deviation from ideality of the solution after correction with $V_{\text{mol}_{i,s}}$, the relative retention of two solutes, $r_{i,s}$, can be expressed by the relation:

$$r_{i,s} = \frac{p_s^\circ \cdot V_{\text{mol}}}{p_i^\circ \cdot V_{\text{mol}_i}} \tag{4}$$

In this equation, p° is the vapour pressure for the substances i and s at the temperature of the analysis, and V_{mol} are their molecular volumes. In analogy with the KOVATS' formula, we can write the following equation for $I_{\text{theor.}}$:

TABLE I

RETENTION INDICES OF ISOPARAFFINS AND NAPHTHENES

No.	*Hydrocarbon*	$I_{theor.} - I_{exp.}$	$I_{p^\circ} - I_{exp.}$	$I_{(8)} - I_{(9)}$	$I_{(4)} - I_{exp.}$	$I_{(1)} - I_{exp.}$
1	2,2-Dimethylbutane	−6	—	4.5	—	30
2	Cyclopentane	0.5	—	3.5	—	—
3	2,3-Dimethylbutane	−7	—	2	−15	20
4	2-Methylpentane	−1	—	1	0	—
5	3-Methylpentane	−4	—	1	0	11
6	2,2-Dimethylpentane	−4.5	7	2	11.5	20
7	Methylcyclopentane	−3	−16.5	2.5	—	—
8	2,4-Dimethylpentane	−3	7	0	—	—
9	2,2,3-Trimethylbutane	−12	−4	1.5	−8	25
10	3,3-Dimethylpentane	−10.5	−5	0	−4	18
11	Cyclohexane	−1	−21	2.5	—	—
12	2-Methylhexane	−1	3	0	−4	18
13	1,1-Dimethylcyclopentane	−7	−10.5	—	—	—
14	2,3-Dimethylpentane	−7	−4	2	−20	13
15	3-Methylhexane	−3.5	−1	0	7	9
16	1-*cis*-3-Dimethylcyclopentane	−2.5	−7.5	—	—	—
17	1-*trans*-3-Dimethylcyclopentane	−10	−15	—	—	—
18	3-Ethylpentane	−5.5	−5	0.5	—	10
19	1-*trans*-2-Dimethylcyclopentane	−7	−14	—	—	—
20	1-*cis*-2-Dimethylcyclopentane	−7	−19	—	—	—
21	2,2-Dimethylhexane	−3.5	7	—	8	12
22	1,1,3-Trimethylcyclopentane	−10.5	−8	—	—	—
23	Methylcyclohexane	−9	−21	2.5	—	—
24	2,5-Dimethylhexane	−2.5	6.5	1.5	−1	—
25	Ethylcyclopentane	−4.5	−17.5	—	—	—
26	2,4-Dimethylhexan	−4	4	2	3	—
27	2,2,3-Trimethylpentane	−9	−2.5	1.5	—	20
28	3,3-Dimethylhexane	−7	0	0	0	9
29	2,3,4-Trimethylpentane	−8.5	−4	—	—	—
30	2,3,3-Trimethylpentane	−13	−10	—	−37	—
31	2,3-Dimethylhexane	−5.5	−2	—	−25	10
32	2-Methyl-3-ethylpentane	−7	−3.5	—	—	—
33	2-Methylheptane	−1	4	—	1	3
34	4-Methylheptane	−2	1.5	—	—	—
35	3,4-Dimethylhexane	−7	−4.5	—	−28	13
36	1-*cis*-2-*trans*-4-Trimethylcyclopentane	−11	−14.5	—	—	—
37	3-Methyl-3-ethylpentane	−12.5	−10	—	—	—
38	3-Ethylhexane	−4	0	—	—	—
39	3-Methylheptane	−2.5	0	—	0	5

$$I_{\text{theor.}} = 100 \cdot z + 100 \frac{\log (p_{z^\circ-1} \cdot V_{\text{mol}_{z-1}} / p_{i^\circ} \cdot V_{\text{mol}_i})}{(p_{z^\circ-1} \cdot V_{\text{mol}_{z-1}} / p_{z^\circ} \cdot V_{\text{mol}_z})} \quad (5)$$

where z and $z - 1$ are the carbon numbers of the standard n-paraffins and i, the corresponding hydrocarbons.

Using this formula, the retention indices of many isoparaffins and naphthenes were calculated using tabulated data for p° and d^4 taken from ref. 7. The calculated values of $I_{\text{theor.}}$ were compared with those obtained experimentally or listed in the literature[8]. In order to assess the accuracy of the new method, we give, instead of the indices themselves, several differences (δ) in their values. The first column of Table I gives the difference between $I_{\text{theor.}}$ calculated by eqn. (5) and I obtained experimentally, with an accuracy of 0.5 index units. In the second column is given the difference between I calculated only from the values of p° and $I_{\text{exp.}}$. The third column shows the differences between $I_{\text{exp.}}$ obtained by two different authors[8,9]. The fourth column gives the differences between the results of SCHOMBURG's method and $I_{\text{exp.}}$; the last column shows the differences between the indices from eqn. (1) and $I_{\text{exp.}}$.

The results in Table I show an extremely good agreement between the theoretically and experimentally obtained values for the retention indices. Compared to the methods described in refs. 1, 4 and 5, where experimental data are used, the new method is more accurate. The calculation of I based only on the values of p°, assuming that $\gamma_{i,s}^\circ = 1$, is not precise; the values obtained show deviations in different directions. Another advantage of the new method is that it allows the calculation of the indices of the naphthenes.

The newly-developed method has the advantage that, for the calculation of I, physicochemical characteristics of the analysed hydrocarbons which are precisely determined, are used. Besides this, $I_{\text{theor.}}$ accurately shows the effect of the temperature of the analysis on the relative resolution. The deviations, when present, are always negative. This means the absence of random error.

The advantages of the new method would allow the planning of the conditions of an analysis and the prediction of the resolution at different temperatures. All these factors will considerably shorten the time for the preliminary analysis and the time for the identification of the peaks of hydrocarbons separated on squalan.

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Analysis of free phytosterols by gas chromatography using liquid phase OV-101

The most often used stationary phase for the analysis of phytosterols by gas–liquid chromatography (GLC) is the dimethyl silicone polymer SE-30[1–6] but this stationary phase gives an excessive amount of tailing with free sterols[3] and poor component resolution even when an acid-washed, silanized support in a glass column is used[6]. Therefore, the GLC determination of free phytosterols on SE-30 is not considered suitable and derivatives such as esters[3,5,7] and ethers[8] including trimethylsilyl ethers[5–7] are generally used. A number of new silicone stationary phase for GLC have become available and of special interest for the analysis of free phytosterols is OV-101, a liquid dimethylsiloxane polymer with 0% phenyl subsitution. Used in this investigation were the biologically important phytosterols: cholesterol (cholest-5-en-3 β-ol), campesterol (24 α-methylcholest-5-en-3 β-ol), β-sitosterol (24 α-ethylcholest-5-en-3 β-ol) and stigmasterol (24 β-ethylcholest-5, 22-dien-3 β-ol) (Applied Science Laboratory, State College, Pa., U.S.A.).

All GLC work was carried out on a F & M Model 402 equipped with a flame ionization detector. The columns were 1.80 m U-shaped glass with a 6 mm I.D. and packed with Anakrom ABS 80/90 mesh (Analabs, Hamden, Conn., U.S.A.) coated with 3% OV-17, OV-22, and OV-101 (Supelco, Bellefonte, Pa., U.S.A.) and Anakrom ABS 60/80 mesh coated with 5% SE-30. The column temperature was 250° and the flash heater temperature was kept at least 25° above that of the column. The flame detector temperature was 300°. Helium was the carrier gas at a flow rate of 100 ml/min. Ethyl acetate was chosen as the solvent because it showed minimum tailing. Samples of 2–10 μg of sterol either alone or in a mixture dissolved in ethyl acetate were injected with a 10 μl Hamilton syringe. Cholestane was used as the internal standard. The trimethylsilyl (TMS) ether derivatives were formed as described by KLEBE *et al.*[9].

The GLC results for the four phytosterols and their TMS ethers on three liquid phases are presented in Table I. Stationary phase OV-17, phenylmethyl silicone, which has a 50% phenyl substitution was also tested but the results were very similar to those obtained with OV-22 and, therefore, are not shown in Table I. All relative retention values (r) are given with respect to cholestane, and their effective plate values (N) are calculated according to ETTRE[10]. The order of sterol elution was the same for all liquid phases: cholesterol, campesterol, stigmasterol and β-sitosterol. Liquid phase OV-101 at the 5% level gave the best over-all results and higher or lower column loadings did not improve peak separation.

For the four phytosterols SE-30 had N-values of 796–1490, OV-22 had N-values of 641–780, and OV-101 had N-values of 1810–2200. The TMS ether derivatives of the four sterols gave higher N-values on SE-30, somewhat lower N-values on OV-22, and about the same N-values on OV-101. Liquid phase OV-101 gave superior N-values in all cases even if the TMS ether derivatives were compared.

For completeness of separation, or resolution (R), peak width at the base must be taken into account. Resolution was calculated according to ETTRE[10]. If $R = 1$, the two peaks are 95% resolved and if $R = 1.5$, the two peaks are better than 99% resolved from each other. The three liquid phases can be compared directly if it is assumed that $R = 1$ is an acceptable resolution. OV-22 is undesirable for the analysis

TABLE I

COMPARISON OF RELATIVE RETENTION (r), EFFECTIVE PLATE VALUE (N), AND RESOLUTION (R) OF VARIOUS PHYTOSTEROLS AND THEIR TMS ETHER DERIVATIVES ON THREE GAS CHROMATOGRAPHIC COLUMN SUBSTRATES

Column characteristics: column temperature 250°, detector temperature 300°, carrier gas, helium at a flow rate of 100 ml/min, column support Anakrom ABS (for details see text).

Compound	*Substrates*								
	5% SE-30[a]			*5% OV-22*[b]			*5% OV-101*[c]		
	r	N	R	r	N	R	r	N	R
Free sterol									
Cholestane	1.00	—		1.00	—		1.00	—	
			5.58			6.32			6.62
Cholesterol	1.93	1490		2.69	752		1.90	1853	
			2.47			1.96			2.68
Campesterol	2.56	1201		3.64	641		2.46	1810	
			0.71			0.87			1.31
Stigmasterol	2.80	796		4.15	752		2.77	2130	
			1.16			0.95			1.87
β-sitosterol	3.24	1241		4.56	780		3.10	2200	
Sterol, TMS ether									
Cholestane	1.00	—		1.00	—		1.00	—	
			7.80			4.42			5.73
Cholesterol	2.35	1599		2.08	577		2.33	1934	
			2.74			1.70			2.27
Campesterol	3.09	1644		2.75	570		3.11	1947	
			0.85			0.59			1.07
Stigmasterol	3.37	1547		3.03	638		3.42	2113	
			1.56			0.91			1.02
β-sitosterol	3.94	1648		3.49	653		3.95	2140	

[a] Retention time cholestane 11.6 min.
[b] Retention time cholestane 6.8 min.
[c] Retention time cholestane 12.7 min.

of free phytosterols or their TMS ethers. SE-30 gave a resolution of 95% for three of the four free sterols and a 99% resolution for two of the four components. OV-101 had the best resolution—at least 95% for all components and a 99% resolution for three of the four free sterols. The TMS ether derivatives gave somewhat poorer resolution on OV-101 than did the free sterols, but all components were resolved at the 95% level. SE-30 resolved three of the four TMS ethers at the 99% level, however, campesterol and stigmasterol could not be resolved at the 95% level.

Highly symmetrical peaks were obtained with OV-101 and it was found that this liquid phase was superior to SE-30 in every respect for the analysis of free phytosterols and their TMS ethers. But no claim can be made that complete optimum conditions were obtained for each liquid phase but the results presented in this note are representative of our experience with these liquid phases which were tested under comparable conditions and which permitted evaluation of relative merits. This GLC procedure has been employed in the analysis of biological samples[11].

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Silylation and gas–liquid chromatographic analysis of an aqueous polyhydric alcohol mixture

As surveyed elsewhere[1,2] catalytic high pressure hydrogenolysis of sucrose has been studied in several countries. The process, in general, is carried out in water or a water–alcohol solution. The reaction product is an aqueous polyhydric alcohol mixture and it is impossible to evaporate the water completely. This fact explains why the well-known silylation method of SWEELEY *et al.*[3], so often applied for the analysis of polyhydric alcohols[4], has not been used, as far as we know, for the quantitative analysis of the mentioned hydrogenolysis products.

Recently Pierce Chem. Co. announced Tri-sil 'Z' (a mixture of trimethylsilylimidazoles in dry pyridine) as a silylation reagent for hydroxy and polyhydric compounds in either dry or aqueous solution.

This note reports on the use of Tri-sil 'Z' for the quantitative GLC analysis of the reaction product formed during catalytic high pressure hydrogenolysis of sucrose.

It is emphasized that the purpose of this study was not to develop optimum GLC conditions, but to investigate the applicability of the silylation method to our problem.

Materials and methods

Catalytic hydrogenolysis of sucrose[2]. A 250 ml autoclave, equipped with a

vertically pulsating, electromagnetic stirrer, was charged with 50 g sucrose; 5 g catalyst $CuO–CeO_2–SiO_2$; 0.5 g $Ca(OH)_2$ and 75 ml $CH_3OH–H_2O$ (75:25 w/w). Reaction conditions were: 235°; 200 atm hydrogen pressure; 30 min reaction time. The cooled hydrogenolysis product was filtered off and part of it was distilled to evaporate CH_3OH and H_2O. 10–20 wt. % of water remains in the distilled hydrogenolysis product.

Preparation of the trimethylsilyl derivatives. 1 ml Tri-sil 'Z' was added according to the directions mentioned on the vial to 10–15 mg of the distilled hydrogenolysis product. 25 μl was injected into the gas chromatograph.

Gas chromatography. Apparatus: F & M 810 with a thermal-conductivity cell. Column: 1.5-m stainless steel tube (4 mm I.D.) packed with 20 wt. % Silicone Rubber U.C.W. 98 on 60–80 mesh Diatoport S. Column conditioned with Silyl 8 (Pierce Chem. Co.).

Results and discussion

The following GLC conditions were found to be suitable: carrier gas, hydrogen 60 ml/min; column temperature, 125°–275° with programming at 8°/min; injection port, 280°; detector temperature, 300°. Fig. 1 shows a typical chromatogram.

A number of synthetic polyhydric alcohol mixtures were prepared and dissolved in $CH_3OH–H_2O$. These mixtures were then distilled, silylated and chromatographed in the same way as the hydrogenolysis product. Peak areas were measured with a planimeter and the relative calibration factors calculated.

It was found that: The silylation seems to be complete; the reproducibility of the method is very good; the silylated samples remain unchanged for at least 24 h; the relative errors are $\leqslant 5\%$. A detailed analysis of the hydrogenolysis product is reported in Table I.

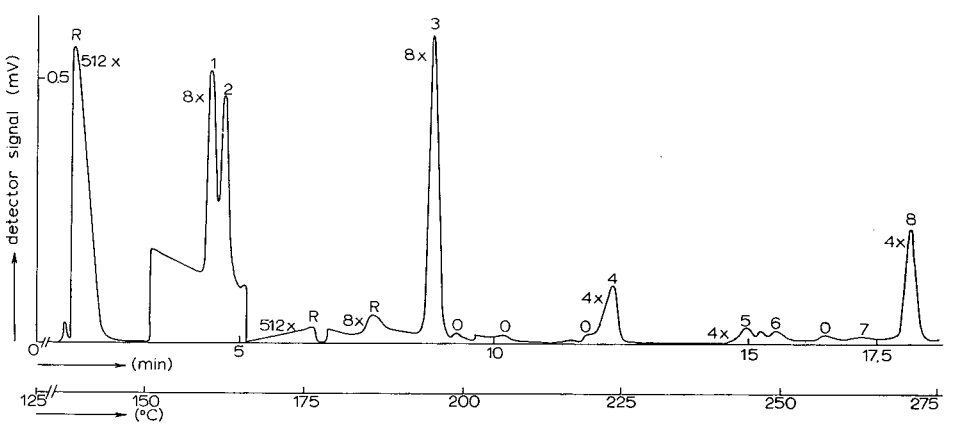

Fig. 1. Gas chromatogram of the silylated reaction product formed during catalytic high pressure hydrogenolysis of sucrose. Column: 1.5-m tube packed with 20 wt. % Silicone Rubber U.C.W. 98 on 60–80 mesh Diatoport S. Carrier gas: hydrogen 60 ml/min. Components: 1 = ethylene glycol; 2 = propane-1,2-diol; 3 = glycerol; 4 = tetritols; 5 = pentitols; 6 = methylfructofuranosides; 7 = dehydrated hexitols; 8 = hexitols; O = unknown; R = compounds of Tri-sil 'Z'.

TABLE I

DETAILED ANALYSIS OF THE REACTION PRODUCT FORMED DURING CATALYTIC HIGH PRESSURE HYDROGENOLYSIS OF SUCROSE

Components	*Weight (%)*
Ethylene glycol	15.9
Propane-1,2-diol	21.1
Glycerol	43.0
Tetritols	5.4
Pentitols	1.3
Methyl-D-fructofuranosides	1.3
Dehydrated hexitols	0.2
Hexitols	10.1
Unknown	1.7
Total	100.0

We concluded that: the silylation method can be applied to the GLC analysis of the hydrogenolysis products described; the accuracy is comparable with other GLC methods[1]; one of the advantages of the handsome silylation method compared with acetylation is the saving of time both in sample derivatization and reduction of retention times. With the silylation method it is possible to analyse unreacted saccharides like sucrose, fructose and glucose. This is quite impossible with acetylation.

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Automated molecular-sieve chromatography of polysaccharides

Molecular-sieve chromatography (MSC) is a useful technique for investigating the polymeric properties of acidic polysaccharides[1,2]. In earlier studies[3,4], elution patterns were obtained by collecting many small fractions which were then screened tediously by a colorimetric method[5].

A considerable range of agarose gels and porous glass or silica beads is now available for studies of high molecular weight polymers. This range will probably increase in the future. Manufacturer's data for such products are usually obtained from tests with characterised proteins, dextrans, or polystyrene fractions; their performance with other types of polymer (*e.g.* acidic polysaccharides) frequently differs[6] and must therefore be evaluated.

The different basic types of agarose preparations (gelled beads, and granular, physically disintegrated gels) vary fundamentally in chromatographic characteristics. Similar considerations apply to porous glass beads, which appear to be offered as two distinct types—"Bio-Glas" (Bio-Rad Laboratories, California) and "Haller Glass" (Corning CPG glass). The choice of a molecular-sieve with a suitable pore-size distribution has a significant influence on the degree of separation attainable[7]. Although HEITZ AND KERN predicted[8] that separation efficiency will eventually be determined by molecular coil dimensions rather than by experimental refinement, it seems reasonable to suppose that, for complex branched molecules with associated steric and charge effects, the most suitable molecular-sieve and the optimum chromatographic parameters for a particular analysis can best be found by experiment. In addition to variables such as the eluant composition and flow-rate, particle size of the molecular-sieve etc., the column shape[9] can be important—in addition to the column dimensions—if optimum results are to be obtained for polymer systems that have not previously been characterised rigorously. There are no standard methods for the preparation, purification, and analysis of polysaccharides. Successful procedures are frequently established only by series of successive refinements, and conditions devised for one polysaccharide system should not be taken automatically as optimum for others, even if they are similar in type or origin.

In all chromatographic techniques there have always been two time-consuming tasks: evaluating new or modified materials[10], and establishing optimum conditions for their use with different classes of compounds. At the present time, the extent of these committments in MSC clearly calls for an automated method of monitoring the effluent from the columns continuously, *e.g.* by differential refractometry[11]; spectroscopy[12]; radioactive labelling[13]; flame-ionisation, conductivity[14,15], and differential vapour-pressure[16] detectors; polarography[17]; and, most interesting of all, a return to true chromatography[18].

Selective dyes such as Toluidine Blue[19,20], Alcian Blue[21], and Mucicarmine[22] have long been used in differential staining reactions for electrophoresis and chromatography, and the recent resurgence of attention to mucopolysaccharides (glycosaminoglycans) has led[23] to renewed interest in dyed complexes.

DUDMAN AND BISHOP[18] observed that the reactivity of "Procion" dyes with polysaccharides was proportional to the number of primary hydroxyl groups present;

a pure polygalacturonan was completely resistant to the dyes tested, and a polysaccharide containing *ca.* 30% uronic acid was dyed only to a slight extent. Tests on the behaviour of acidic gum polysaccharides were not reported. The uronic acid content of such materials frequently falls within the 5–25% range, but some botanical genera *(Khaya, Sterculia)* contain up to *ca.* 50%.

It was therefore of interest to evaluate the broad range of application of this colorimetric technique by studying the extent of the reactions of the dyes Procion Blue M3G and Procion Brilliant Red M2B (kindly provided by I.C.I. Ltd., Dyestuffs Division, Manchester) with a wide range of acidic gum polysaccharides from the *Acacia, Albizia, Araucaria, Azadirachta, Combretum, Khaya, Lannea* and *Sterculia* genera. Although the extent of reaction, as indicated by DUDMAN AND BISHOP[18], appears to be inversely proportional to the uronic acid content, those gums with the highest uronic contents available to us (*Brachychiton diversifolium*[24,25], 51%; *Khaya senegalensis*[26], 43%) nevertheless were dyed to an extent adequate for their colorimetric detection in the eluate from MSC columns containing the agarose gels "Bio-Gel A5" and "Sepharose 4B". For polysaccharides with low uronic acid content the amount of dyestuff used can be controlled, so that the molecular weight of the natural product is not significantly increased. Dextrans are also readily dyed; the characterised fractions available commercially can continue to be used as calibration standards for relative measurements[6] if more valid standards of closely similar chemical structure, characterised by fundamental methods, are not available.

Unfortunately, the use of Procion dyes is not of general applicability: a carrageenan did not react in the dyeing process.

The use of dyed polysaccharides (we prefer the use of Procion Brilliant Red M2B) leads to a simple method of monitoring the behaviour of the native polysaccharides during molecular-sieve chromatography, or for evaluating—with a selected, characterised polysaccharide—the performance of a molecular-sieve column. The dyed sample (1–3 mg) in 2 *M* sodium chloride (0.5 ml) is applied with care to the top of a silanised glass column (35 × 1.5 cm I.D.) containing an appropriate molecular sieve (porous glass or silica; agarose, dextran, or polyacrylamide gels). A suitable eluant is 1 *M* sodium chloride, at 0.5–1 ml/min; automated analyses then take about 2 h. The column effluent is passed directly via capillary tubing into the cell (modified to permit continuous flow) of a colorimeter (Unicam SP 1300). The photo-cell signal is fed via an impedance-matching device (Vibron 33B electrometer) into a recorder (Kent, 1 mV, chart speed 2 in./h). By increasing the recorder input resistor from 1 to 2.5 Ω the maximum signal from the photo-cell gives a recorder deflection of 10 in. This particular experimental combination of instruments is, of course, not unique. Micro-flow colorimeters (Phoenix MFC 800) give an optical path-length of 20 mm for a cell hold-up volume of only 0.035 mm, if increased sensitivity is essential.

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Säulen- und papierchromatographischer Nachweis einer Säure- und Phosphatase-stabilen Phosphorverbindung in Pflanzen

Phosphonoverbindungen wurden erstmalig 1959 in biologischem Material entdeckt[1]. Die danach beschriebenen Vorkommen beschränkten sich ebenfalls auf das Tierreich, wo sie inzwischen bei Vertretern von vier verschiedenen Tierstämmen nachgewiesen werden konnten. Die weitaus meisten Arbeiten wurden jedoch an *Tetrahymena pyriformis*, einem holotrichen Ciliaten, durchgeführt[2-4], der bis zu 13% des aufgenommenen Phosphats in Phosphonosäuren einbauen kann[2].

Bisher sind von dieser völlig neuen Substanzklasse Aminoäthylphosphonsäure (AEP)[1-4] und 2-Amino-3-Phosphonopropionsäure (APP)[4] sowie die N-Methylderivate von AEP[5] aus biologischem Material bekannt geworden. Charakteristikum der Phosphonosäuren ist ihre stabile kovalente P–C-Bindung, die sich—im Gegensatz zu allen anderen im Organismus vorkommenden organischen Phosphorverbindungen wie Phosphatestern, Säureanhydriden etc.—weder durch herkömmliche Phosphatasen noch durch mehrtägiges Kochen in 6 *N* HCl spalten lässt.

Die bisherigen Informationen liessen vermuten, dass Phosphonoverbindungen nur bei Tieren vorkommen. Vorliegende Arbeit weist jedoch das Vorkommen einer Ninhydrin-positiven, Säure- und Phosphatase-stabilen Phosphorverbindung säulen- und papierchromatographisch in zwei einzelligen Algen nach, so dass das Ausmass der Verbreitung dieser Substanzklasse damit noch nicht zu übersehen ist.

Die Grünalge *Pseudochlorella aquatica*—aus der Algensammlung des Pflanzenphysiologischen Instituts der Universität Göttingen—wurde mit ^{32}Phosphat in 300 ml-Kulturröhren bei 16–18° und Dauerschwachlicht im Chlorella-Nährmedium[6] inkubiert. Nach drei Tagen wurden die Zellen 15 min bei 4000 × *g* zentrifugiert, in etwa 250 ml 6 *N* HCl suspendiert und 48 h bei 110° am Rückfluss gekocht. Zur Entfernung der Salzsäure wurde das Rohhydrolysat mehrfach am Rotationsverdampfer im Wasserstrahlpumpenvakuum bei etwa 40° bis nahe zur Trockne eingedampft und jeweils mit Wasser aufgenommen. Vor dem letzten Eindampfen wurde das Hydrolysat zur Entfernung verkohlter Reste filtriert und der Rückstand gut mit Wasser gewaschen. Das Filtrat wurde schliesslich zur Trockne eingedampft und in 10 ml 1 *N* Ameisensäure aufgenommen und nach Auszählen eines Aliquots am Endfensterzählrohr auf eine neutralgewaschene Dowex 50-H-Säule (1 × 25 cm) gebracht und mit zweimal 5 ml 1 *N* Ameisensäure eingewaschen. Dann wird mit Wasser weitereluiert und Fraktionen von 5 ml gesammelt. Ab Fraktion 21 wird mit 0.6 *N*, ab Fraktion 51 mit 2 *N* HCl weitereluiert.

In dem Bereich, in dem bei Tetrahymena 2-Amino-3-Phosphonopropionsäure und 2-Aminoäthylphosphonsäure[7] sowie bisher nichtidentifizierte andere Phosphonosäuren[8] von der Säule eluiert werden, erscheint die neue Verbindung nicht. Erst bei Elution mit 2 *N* HCl kommt sie mit der Front zusammen mit Cystein–Cystin und einigen anderen Aminosäuren von der Dowex 50-H-Säule. Die unbekannte Substanz ist, wie Papierchromatogramme in fünf Laufmitteln zeigten, in ihrer Radioaktivität homogen. Sie bleibt in basischen Laufmitteln am Start zurück, läuft dagegen, wie aus Tabelle I hervorgeht, in sauren Laufmitteln gut ab. Dieses Verhalten wurde auch

TABELLE I

hR_F-WERTE DER ISOLIERTEN PHOSPHORVERBINDUNG SOWIE AEP, N-TRIMETHYL-AEP (nach Lit. 5) UND Pi ALS VERGLEICH IN VERSCHIEDENEN LAUFMITTELN BEI ABSTEIGENDER CHROMATOGRAPHIE AUF S & S 2040 B

1 = *n*-Butanol–Eisessig–Wasser (52:14:35); 2 = Methanol–Ammoniak–Wasser (7:1:2); 3 = Äthanol–Ammonium-Acetat pH 3.8 (5:2); 4 = *tert.*-Butanol–Methyläthylketon–Diäthylamin–Wasser (10:5:1:10); 5 = *tert.*-Butanol–Methyläthylketon–Ameisensäure (90.8%)–Wasser (10:5:1:10).

Substanz	*Laufmittel*				
	1	*2*	*3*	*4*	*5*
X	24	0	83	0	83s
AEP	31	64	78	69	75
TM-AEP	—	—	—	32	—
Pi	59	—	—	—	—

zur Abtrennung der nach der Säulenchromatographie in der Fraktion noch enthaltenen Aminosäuren ausgenutzt. Schon nach Chromatographie in den beiden ersten Systemen aus Tabelle I sind keine Verunreinigungen mehr nachzuweisen. Radioaktivität und Ninhydrinanfärbung stimmen völlig überein.

Nach Passieren aller fünf Systeme wurde nochmals im System I chromatographiert, mit 0.01 *N* HCl eluiert und nach mehrfachem Eindampfen am Rotationsverdampfer in 0.2 *M* Na-Acetat–0.1 *M* $MgCl_2$ pH 4.8 aufgenommen, die Probe geteilt und ein Teil mit saurer Phosphatase (Boehringer), der andere ohne Phosphatase als Kontrolle 2 h bei 37° inkubiert und dann beide Proben im System I erneut chromatographiert. Kontrolle und Phosphatase-Ansatz stimmten in Radioaktivität und Ninhydrinanfärbung völlig überein, ein Freisetzen von Phosphat war nicht festzustellen, so dass die ^{32}P-markierte Substanz nicht nur gegen Säure sondern auch gegen Phosphatase beständig ist und damit ein weiteres Kriterium der Phosphonosäuren erfüllt.

Die Identität dieser neuen Verbindung ist noch ungeklärt. Dem Verhalten an der Dowex 50-Säule nach könnte es sich um N-Trimethyl-AEP (TM-AEP)[5] handeln, das ebenfalls sehr spät von der Säule kommt, doch sind damit weder die Anfärbbarkeit mit Ninhydrin noch der R_F-Wert (Tabelle I) vereinbar.

TABELLE II

AUFNAHME VON ^{32}PHOSPHAT UND EINBAU IN SÄURE- UND PHOSPHATASE-STABILE BINDUNG DURCH PSEUDOCHLORELLA

	Radioaktivität (in i.p.m.)	*nM*
Angebotene Menge ^{32}Phosphat	$2.2 \cdot 10^9$	$1.5 \cdot 10^6$
Aufgenommene Menge ^{32}Phosphat (= Säurehydrolysat)	$5.4 \cdot 10^6$	$3.7 \cdot 10^3$
Einbau in Säure-stabile Bindung (Dowex 50-Fraktionen)	$1.03 \cdot 10^5$	70.0

Die Papierchromatographie hatte gezeigt, dass die in Frage kommenden Fraktionen in ihrer Radioaktivität homogen sind, so dass die Radioaktivität nach der säulenchromatographischen Reinigung der Einbaurate an Phosphat in die Säurestabile Phosphorverbindung gleichgesetzt werden darf. Wie aus Tabelle II hervorgeht, werden 2% des aufgenommenen Phosphats in die neue Substanz eingebaut, wobei zu berücksichtigen ist, dass das Chlorella-Nährmedium für Pseudochlorella nicht optimal ist und daher nur ein mittelmässiges Wachstum der Kultur erfolgte. Wesentlich geringere Einbauraten werden jedoch für die seltenen Phosphonosäuren von Tetrahymena[8] beobachtet, und auch AEP wird unter weniger günstigen Wachstumsbedingungen von Tetrahymena in nicht viel grösserem Umfange synthetisiert[7]. Für die zweite untersuchte Alge, *Astasia longa* (1204-17a), liegen die Einbauraten in der gleichen Grössenordnung wie bei Pseudochlorella. Die hier gefundene Verbindung stimmt mit der aus Pseudochlorella säulen- und papierchromatographisch völlig überein. Ob diese neue Verbindung mit jener nichtidentifizierten Säure-stabilen, von HORIGUCHI in einem zusammenfassenden Referat[9] erwähnten Phosphorverbindung aus Euglena identisch ist, bleibt abzuwarten.

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CHROM. 4265

The separation of phenolic glucosides by gel filtration

The naturally-occurring phenolic glucosides are heterogenous according to their structural components and to their molecular size. In different natural sources the main phenolic glucoside is always accompanied by a few other phenolic glucosides and carbohydrates. The methods of separation and purification of phenolic glucosides therefore attract great interest.

In order to separate the phenolic glucosides and carbohydrates which occur in the bark and leaves of *Populus tremula*, we applied the method of gel filtration on Sephadex G-25 with water as eluent. In our previous paper[1], we established that it is possible to separate the glucosides salicin and tremuloidin from carbohydrates. Salicin and tremuloidin adsorb in a manner which does not conform with the theory of gel filtration. The phenolic glucoside salicin (mol. wt. 286) has an adsorbing aromatic ring in its molecule and the distribution coefficient is higher than expected. Tremuloidin (2-benzoylsalicin, mol. wt. 390) is adsorbed more strongly than salicin because of two aromatic rings in the molecule.

In continuing our studies on the separation of phenolic glucosides, we applied the method of gel filtration to more complex mixtures of the naturally-occurring phenolic glucosides.

The phenolic glucosides tested and their structures, are listed in Table I.

TABLE I

PHENOLIC GLUCOSIDES AND THEIR STRUCTURES

Glucoside	*Structure*	*Mol. wt.*
Salicin	2-oxy-benzylalcohol-β-D-glucopyranoside	286
Populin	6-benzoylsalicin	390
Tremuloidin	2-benzoylsalicin	390
Fragilin	6-acetylsalicin	328
Salireposide	2,5-dioxy-benzylalcohol-β-D-(6-O-benzoyl)-glucopyranoside	406
Grandidentatin	*cis*-cyclohexandiol(1,2)-1-β-D-(2-*p*-cumaroyl)-glucopyranoside	424
Triandrin	3-(4-hydroxyphenyl)-2-propen-1-ol-1-β-D-glucopyranoside	312

We isolated from different natural sources three additional phenolic glucosides besides salicin: tremuloidin, populin and salireposide.

The freshly-obtained bark of various Salicaceae species *(Populus tremula, Salix alba, Salix repens)* was extracted with 96% ethanol and the extract treated with an excess of basic lead acetate. The mixture was filtered and the lead removed from the clear filtrate by treatment with hydrogen sulphide. The resulting clear solution was concentrated under reduced pressure. After evaporation, crystals were collected and examined by means of paper or thin-layer chromatography.

In our experiments we also tested samples of the phenolic glucosides, fragilin, triandrin and grandidentatin.

Filtration experiments were carried out using Sephadex G-25 and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Most of the experiments were performed on Sephadex columns with a dimension of 1.104 cm. The dry Sephadex was allowed to swell in water or in a mixture of water and organic solvents used as an eluent. The substances to be tested were dissolved in an appropriate eluent and put on the column in a volume of 2–3 ml. The concentration of the tested substances varied between 10 and 15 mg. All experiments were carried out in 12–18 h at room temperature. The flow rate was approximately 2 ml/10 min.

The fractions were analysed using Millon's reagent (H. THIEME[2]; B. DOBROWOLSKA AND K. TWARDOWSKA[3]). Our investigation showed that, instead of Millon's reagent, concentrated sulphuric acid can be used as a colorimetric reagent for the determination of phenolic glucosides which give a red-coloured complex with sulphuric acid (only salireposide gives a yellow complex[4]). The absorption maximum of the coloured complex salicin–sulphuric acid is at 510 mμ. Concentrated sulphuric acid (3 ml) was added to the fraction to be tested (1 ml). After 20 min at room temperature the optical density was measured at 510 mμ.

Fractionated glucosides were identified by means of thin-layer chromatography on silica gel in a system of chloroform–methanol (4:1)[5].

Besides the distilled water for gel filtration, mixtures of water–ethanol (9:1), water–methanol (9:1) and 96% ethanol were used as eluents. Consequently, our experiments are divided into three groups.

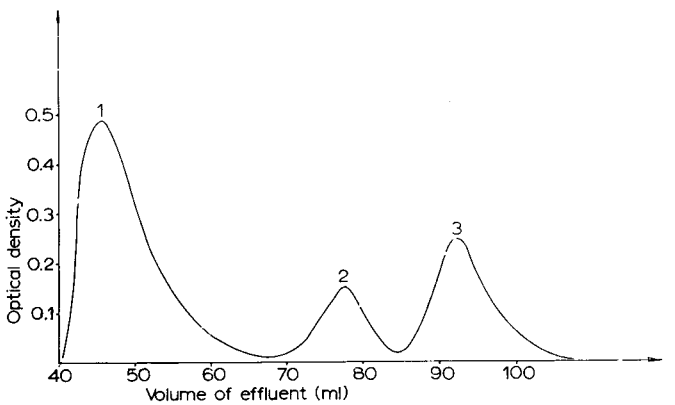

Fig. 1. Separation of phenolic glucosides on Sephadex G-25 in distilled water. Bed dimensions: 1.104 cm. Sample: 2 ml containing 10 mg of glucosides. Flow rate 2 ml/10 min. 1 = salicin; 2 = populin; 3 = salireposide.

Separation of phenolic glucosides on Sephadex G-25 with distilled water as an eluent

With distilled water as an eluent we separated salicin, populin (or tremuloidin) and salireposide (Fig. 1). Fragilin (mol. wt. 328) is collected in the same fractions as salicin (mol. wt. 286), which indicates the possibility of separating fragilin from populin (or tremuloidin) and salireposide, but not from salicin. With water as an eluent it is impossible to separate populin (6-benzoylsalicin, mol. wt. 390) from tremuloidin (2-benzoylsalicin, mol. wt. 390).

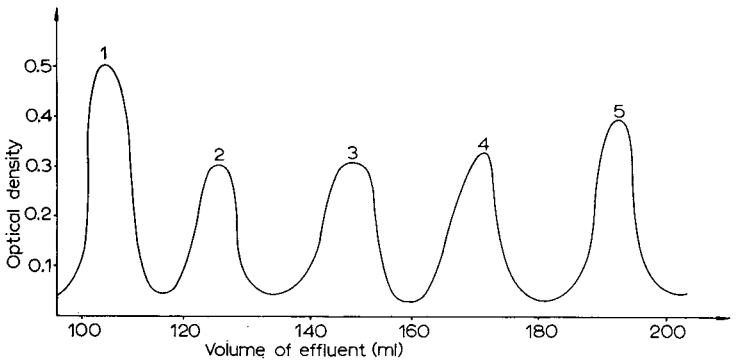

Fig. 2. Separation of phenolic glucosides on Sephadex G-25 in water–methanol (9:1). Bed dimensions: 1.104 cm. Sample: 3 ml containing 3 mg of each glucoside. Flow rate 2 ml/10 min. 1 = salicin; 2 = populin; 3 = tremuloidin; 4 = grandidentatin; 5 = salireposide.

Separation of phenolic glucosides on Sephadex G-25 with water–methanol (9:1) as an eluent

In order to increase the low solubility of phenolic glucosides in water, we performed some experiments with a mixture of water and methanol (Fig. 2). The results obtained show the possibility of separating populin (6-benzoylsalicin, mol. wt. 390) from tremuloidin (2-benzoylsalicin, mol. wt. 390); tremuloidin is adsorbed more strongly than populin. With a mixture of water and methanol, we succeeded in separating salicin, populin, tremuloidin, salireposide and grandidentatin. Similar results were obtained when elution was carried out with water–ethanol (9:1).

Separation of phenolic glucosides on Sephadex LH-20 with 90% ethanol as an eluent

With ethanol as an eluent on Sephadex LH-20, we separated salicin, populin, tremuloidin and salireposide. The results obtained are basically similar to the results obtained on Sephadex G-25, but the separation volumes are smaller (Fig. 3).

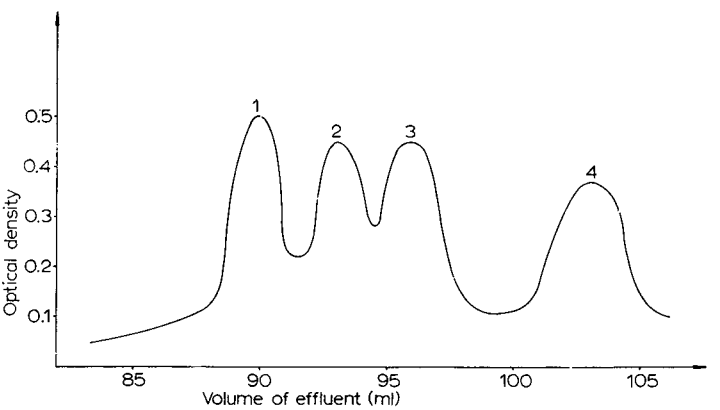

Fig. 3. Separation of phenolic glucosides on Sephadex LH-20 in ethanol. Bed dimensions: 1.106 cm. Sample: 3 ml containing 4 mg of each glucoside. Flow rate 2 ml/15 min. 1 = salicin; 2 = populin; 3 = tremuloidin; 4 = salireposide.

Gel filtration on Sephadex G-25 and Sephadex LH-20 may give good separation of phenolic glucosides. The phenolic glucosides adsorb in a manner which does not conform with the theory of gel filtration and leads to higher elution volumes than can be expected from the molecular size. The adsorption effect is related to the structure of the phenolic glucosides and increases with the number of aromatic rings included in the structure of glucosides. Therefore, populin or tremuloidin (mol. wt. 390) which have two adsorbing aromatic rings in their molecule, are adsorbed more strongly than salicin (mol. wt. 286). The adsorption effect also depends on hydroxyl groups included in the agluconic part of the glucosides: hydroxyl groups in the molecule seem to increase adsorption. None of the glucosides tested are irreversibly adsorbed onto the column.

When elution was carried out with distilled water, we separated salicin, populin (or tremuloidin) and salireposide. Better results in separation were obtained on Sephadex G-25 with water–ethanol (9:1) or water–methanol (9:1). With these eluents, we separated salicin, populin, tremuloidin, salireposide and grandidentatin. The separation of populin from tremuloidin is completely eliminated when elution is carried out with distilled water. In all experiments fragilin and triandrin were eluted simultaneously with salicin.

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CHROM. 4271

Separation of acrylate, methacrylate, and tiglate ions by paper chromatography

A solution of phenol and water is an extremely useful solvent for both paper and thin-layer chromatography and has been used as such for years. The effectiveness of this solvent can be enhanced, in certain instances, by the addition of a third component.

Although phenol and water will not begin to separate sodium acrylate from sodium methacrylate, the addition of methyl acetate to the system changes the results significantly. This modified solvent will also effect the separation of sodium methacrylate from sodium tiglate, the free acids of which are often found together in the defensive scent fluid of certain ground beetles[1].

A convenient preparation for this solvent is to mix 30 ml of liquefied phenol (88%), 10 ml of methyl acetate free of any acid, and 3 ml of distilled water. At ordinary room temperatures only one layer should be obtained. A chamber equilibration time of 1 h is adequate but a minimum solvent flow on the paper of 15 cm, requiring about 3 h, is needed to achieve a clean separation.

After the run the paper should be dried for 1 h in a stream of warm air before being dipped in 0.015% Methyl Red in 2-propanol or 95% ethanol. Within 1 min, yellow spots will appear on the pink background and should be marked immediately as they tend to fade in time. Alternatively a dip of 0.03% dichlorofluorescein in 95% ethanol followed by viewing under UV light can be used for detection. This second method is more sensitive, being able to detect spots applied from 0.03 M solutions of any of the three salts. However, this sensitivity also reveals streaking below each spot and in addition causes the spot to appear somewhat larger than those disclosed by Methyl Red.

Even though less sensitive, Methyl Red picks out just the core of each spot and none of the streaking, thus improving the apparent separation of the individual ions. With the phenol–methyl acetate–water solvent already described, R_F values of 0.22 for sodium acrylate, 0.32 for sodium methacrylate, and 0.41 for sodium tiglate are commonly observed. Both detecting agents give about the same values while potassium salts will yield slightly higher R_F values. Whatman No. 1 paper and ascending flow were used throughout this investigation.

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Thin-layer chromatography of C_{19}-steroid 2,4-dinitrophenylhydrazones on polyamide

Since the reaction of 2,4-dinitrophenylhydrazine with ketosteroids[1,2] has been shown to be quite useful for the qualitative and quantitative analyses of ketonic C_{19}-steroids[2–4], the isolation of corresponding derivatives by thin-layer chromatography (TLC) gained considerable interest. So far, however, the separation of epimeric C_{19}-steroid 2,4-dinitrophenylhydrazones by TLC on silica gel or alumina presented some difficulties, thus calling for the elaboration of new chromatographic systems.

By using reversed-phase TLC on polyamide and suitable solvent systems, adequate resolution of closely related C_{19}-steroid derivatives may be obtained. For such purposes, 5–25 μg of 2,4-dinitrophenylhydrazones are dissolved in chloroform and applied to the polyamide layer (0.1-mm thickness, Selecta-Fertigplatten Nr. 1600, Schleicher & Schüll, 3354 Dassel, G.F.R.) by means of a capillary syringe. Diffuse spots should be concentrated to a narrow band on the starting line by ascending chromatography in methylene chloride. Chromatograms are developed by the ascending technique in a paper-lined chamber filled with the mobile phase.

Table I contains the R_F values of numerous C_{19}-steroid 2,4-dinitrophenylhydrazones in different solvent systems. All R_F values represent the mean of at least three determinations. As can be seen, the derivatives of epimeric C_{19}-steroids such as 17β- or 17α-hydroxy-4-androsten-3-one (testosterone and epitestosterone), 3α- or 3β-hydroxy-5α-androstan-17-one (androsterone and epiandrosterone), 3α- or 3β-hydroxy-5β-androstan-17-one (etiocholanolone and epietiocholanolone) and 3α- or 3β-hydroxy-4-androsten-17-one (3α- and 3β-hydroxy-4-androstenone) are readily separated in every solvent system. By combination of reversed-phase TLC on poly-

TABLE I

THIN-LAYER CHROMATOGRAPHY OF C_{19}-STEROID 2,4-DINITROPHENYLHYDRAZONES ON POLYAMIDE

Solvent systems: 1 = methylene chloride–methanol (2:8); 2 = 1,2-dichloroethane–methanol (2:8); 3 = trichloroethylene–methanol (2:8); 4 = methylene chloride–methanol–water (5:7:3); 5 = trichloroethylene–methanol–water (20:64:16); 6 = acetone–methanol–acetic acid (20:80:0.5); 7 = carbon tetrachloride–acetone–methanol–water (5:10:40:10); 8 = acetone–ethanol–water (5:16:4).

Derivative of	R_F *value in solvent system*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Testosterone	0.22	0.30	0.34	0.16	0.25	0.17	0.09	0.11
Epitestosterone	0.47	0.53	0.53	0.33	0.39	0.39	0.23	0.28
Androsterone	0.65	0.69	0.66	0.50	0.52	0.55	0.40	0.53
Epiandrosterone	0.40	0.43	0.44	0.26	0.39	0.26	0.14	0.20
Etiocholanolone	0.71	0.77	0.72	0.62	0.61	0.68	0.54	0.72
Epietiocholanolone	0.66	0.71	0.67	0.54	0.56	0.60	0.43	0.56
3α-OH-4-Androstenone	0.53	0.67	0.64	0.49	0.57	0.67	0.39	0.57
3β-OH-4-Androstenone	0.67	0.56	0.55	0.37	0.54	0.51	0.23	0.38
11β-OH-Androsterone	0.64	0.66	0.65	0.53	0.60	0.67	0.44	0.59
11β-OH-Etiocholanolone	0.71	0.72	0.68	0.60	0.65	0.71	0.54	0.73
Dehydroepiandrosterone	0.38	0.41	0.42	0.24	0.36	0.26	0.14	0.19
3β-Chloro-dehydroepiandrosterone	0.66	0.68	0.65	0.48	0.57	0.66	0.38	0.58

amide with TLC on Silica Gel G in chloroform–dioxan (94:6)[5], the isolation of most C_{19}-steroid 2,4-dinitrophenylhydrazones becomes feasible, facilitating the estimation of these compounds in biological extracts[5,6]. At the same time, individual derivatives in discrete spots may be quantitated after TLC on polyamide by means of direct densitometry with a spectrodensitometer (Model SD 3000, Schoeffel Instr. Corp., Westwood, N.J., U.S.A.). The estimation of C_{19}-steroid 2,4-dinitrophenylhydrazones by this procedure, the sensitivity of which approximates 10 ng, as well as its application to the analysis of C_{19}-steroids in biological material, will be presented in a forthcoming communication.

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CHROM. 4245

Rapid quantitation of Δ^4-3-ketosteroids by thin-layer densitometry

Thin-layer densitometry of steroids has recently received increased attention. Such methods are based either on staining of free substances after chromatography[1] or the formation of coloured derivatives[2–4]. The present paper describes the quantitation of Δ^4-3-ketosteroids by use of their quench effect upon the fluorescence at 254 mμ provided by a suitable dye in the adsorbent.

Methods

For chromatography Analtech (Wilmington, Del., U.S.A.) plates with a 250 μ thick layer were used. The coating material was Silica Gel GF_{254} with fluorescence at 254 mμ. In a special scoring device (Schoeffel Instr. Corp., Westwood, N.J., U.S.A.) the thin layer was divided into lanes of 1 cm width. Because of the double beam operating system of the densitometer only alternate lanes were loaded with a mixture of Δ^4-3-ketosteroids. The blank lanes served as reference for the instrument. The plates were then developed in a suitable solvent system such as chloroform–dioxane (94:6) yielding adequate separation of various steroids.

Direct quantitation was performed by means of a Schoeffel spectrodensitometer, Model SD 3000 (Schoeffel Instr. Corp., Westwood, N.J., U.S.A.). A quartz

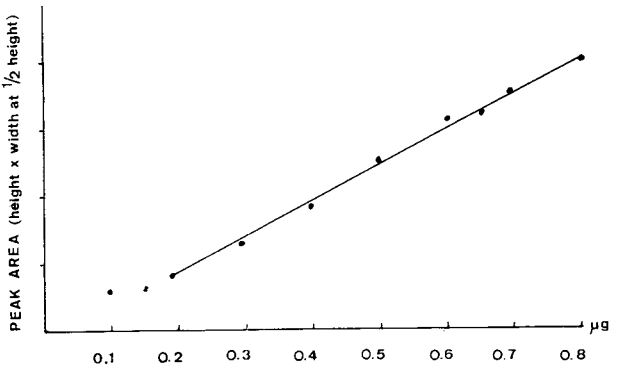

Fig. 1. Calibration curve of the densitometer (wavelength 254 mμ) with recorder full scale at optical density 0.5.

monochromator provided UV light of 254 mμ wavelength that passed through two slits. The reference and the sample lane of the plate moved through the UV light beams and the emitted light was registered in an optical density computer, Model SDC 300 (Schoeffel Instr. Corp.) connected with an integrating 10 in. strip recorder, Model SDR 303 (Schoeffel Instr. Corp.). Whereas free areas of the sample lane appeared as a high positive base line the steroid spots became visible as negative peaks (Fig. 3). Peak areas were calculated by triangulation (height × width at 1/2 height) and evaluated for standard curves.

Results

Amounts of 0.1 μg Δ^4-3-ketosteroid could still be clearly detected. At a full scale deflection of the recorder at different optical density values a satisfactory linearity of standard curves was achieved for concentrations between 0.2 and 8.0 μg

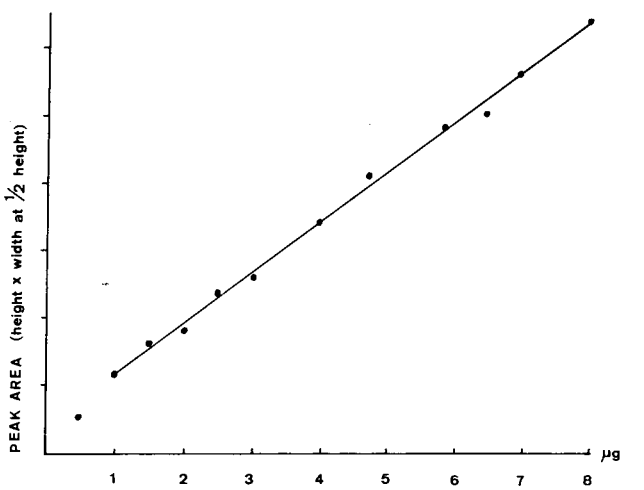

Fig. 2. Calibration curve of the densitometer with recorder full scale at optical density 1.0.

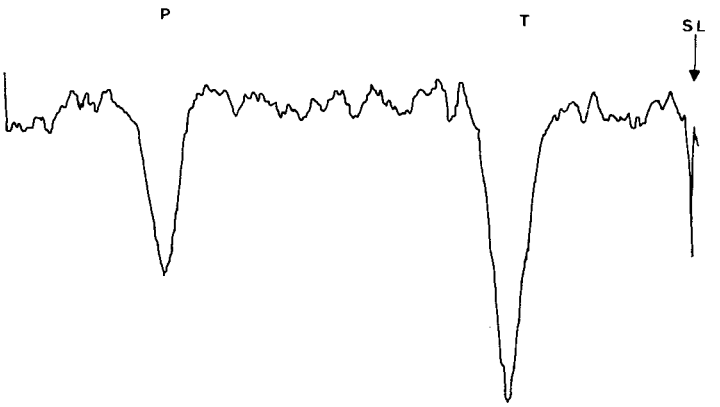

Fig. 3. Recording of 2 μg testosterone (T) and 1 μg progesterone (P) after chromatography in chloroform–dioxane (94:6), SL = starting line.

(Figs. 1 and 2). Fig. 3 demonstrates the recording of a chromatogram with 1 μg progesterone and 2 μg testosterone after development in chloroform–dioxane (94:6).

The present method was adopted for rapid estimation of free progesterone in peripheral plasma of pregnant women. Results of this investigation will be published in a forthcoming paper.

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CHROM. 4250

New thin-layer chromatographic solvent systems for separating steroid hormones

Three years ago we began research to determine if steroid hormones were normally present in cow's milk. It led to the development of two one-dimensional TLC systems for rapidly separating common reference sex and adrenal hormones. During this period several different TLC solvent systems were reported for separating steroid hormones[1–5]. But since our solvent systems are different, they may be helpful to other investigators.

Methods

All solvents were ACS grade and used as obtained from the manufacturer. They were: anhydrous diethyl ether, petroleum ether, 1-butanol, and formic acid. Silica Gel G (according to Stahl) was acquired from Brinkmann Instruments, Inc., N.Y. The steroid hormones (Figs. 1 and 2) used in this investigation were purchased from Mann Research Laboratories, Inc., except 4-androsten-17α-ol-3-one, which was acquired from Steraloids, Inc. Plates 0.25 mm thick were prepared using a suspension

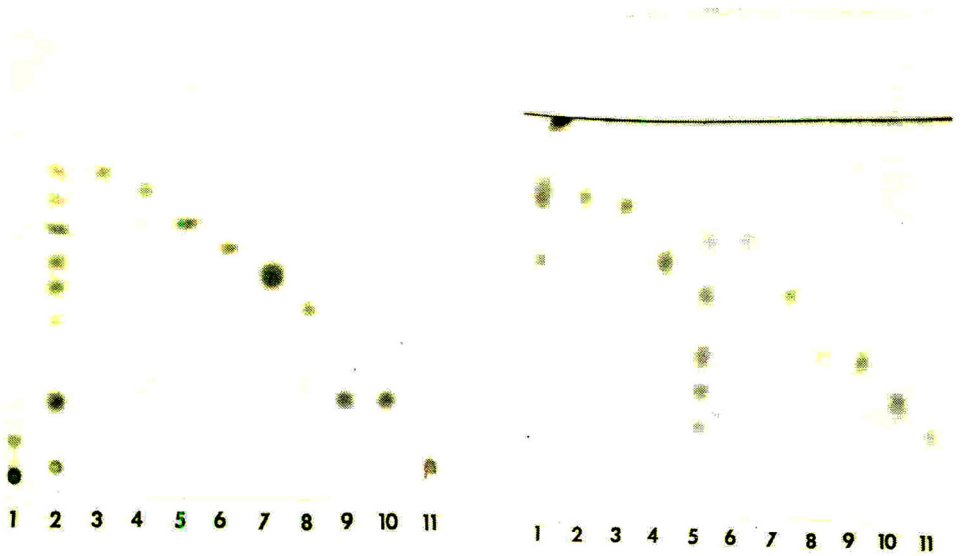

Fig. 1. Separation of sex hormones in system 1. Reference samples: (1) mixture of corticosteroids, (2) mixture of sex hormones, No. 3–11, (3) 1,3,5(10)-estratriene-3,17β-diol 17-acetate, (4) 1,3,5(10)-estratrien-3-ol-17-one 3-acetate, (5) 1,3,5(10)-estratrien-3-ol-17-one, (6) 1,3,5(10)-estratriene-3,17α-diol, (7) 1,3,5(10)-estratriene-3,17β-diol, (8) 4-pregnene-3,20-dione, (9) 4-androsten-17α-ol-3-one, (10) 4-androsten-17β-ol-3-one, and (11) 1,3,5(10)-estratriene-3,16α,17β-triol.

Fig. 2. Separation of corticosteroids in system 2. Reference samples: (1) mixture of sex hormones, (2) 4-androsten-17α-ol-3-one, (3) 4-androsten-17β-ol-3-one, (4) 1,3,5(10)-estratriene-3,16α,17β-triol, (5) mixture of corticosteroids, No. 6–11, (6) 4-pregnene-17α,21-diol-3,11,20-trione 21-acetate, (7) 4-pregnen-21-ol-3,20-dione, (8) 4-pregnene-17α,21-diol-3,11,20-trione, (9) 4-pregnene-11β,17α,21-triol-3,20-dione, (10) 4-pregnene-11β,21-diol-3,20-dione, and (11) 4-pregnen-21-ol-3,11,20-trione.

of 30 g Silica Gel G in 60 ml of 0.01 *M* sodium carbonate[6]. Approximately 1 h before use, a plate was activated at 120° and a developing chamber was saturated with vapors of its solvent.

The reference hormones were dissolved in chloroform–methanol (2:1) and applied to the plates with disposable micropets. All compounds were spotted at a concentration of *ca.* 5 μg, except 4-pregnene-3,20-dione which was about 10 μg.

The two developing solvent systems were: (1) petroleum ether–diethyl ether–formic acid (100:100:2), and (2) petroleum ether–diethyl ether–1-butanol–formic acid (100:50:30:2). Both systems moved 15 cm from the point of spotting. The spots were observed after spraying the plate with H_2SO_4–H_2O (1:1) and heating in an oven at 120° until the organic material colored and charred.

Results and discussion

The hormones were initially grouped as sex and adrenal. System 1 (Fig. 1) was developed to separate sex hormones: system 2 (Fig. 2), adrenal hormones. Excellent separation of the common sex hormones except the α- and β-isomers of 4-androsten-17-ol-3-one (No. 9 and 10) and 1,3,5(10)-estratriene-3,16α,17β-triol (No. 11) is seen in Fig. 1. But in Fig. 2, 1,3,5(10)-estratriene-3,16α,17β-triol (No. 4) separates cleanly with five of the six corticosteroids. Identical R_F values are observed for 4-pregnene-17α,21-diol-3,11,20-trione (No. 8) and 4-pregnene-11β,17α,21-triol-3,20-dione (No. 9). Also, the isomers of 4-androsten-17-ol-3-one (No. 2 and 3) do not separate with system 2.

An interesting observation was recorded in our laboratory. When TLC plates containing reference steroid hormones were sprayed with a H_2SO_4 solution and heated in an oven, colors developed which could be associated with certain hormones. But when extracts from bovine adrenal glands and corpora lutea were chromatographed in these solvents, compounds with identical R_F values to the reference hormones were seen; however, they did not color. This observation cannot be satisfactorily explained.

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CHROM. 4234

Partition data of cephalosporins determined by means of reversed-phase thin-layer chromatography

In a previous paper a reversed-phase thin-layer chromatography method was shown to be a suitable technique for the determination of partition data of penicillins[1].

The reversed-phase TLC method previously described and discussed[1] is used in the present work for the determination of partition data of cephalosporins. Silicone oil was used as the stationary phase. An aqueous mobile phase was used (sodium acetate veronal buffer at pH 7.4) alone or in various proportions with acetone. The structures of the tested cephalosporins are indicated in Table I. The spotting and detection of the compounds were performed as previously described[1].

Results and discussion

The cephalosporins migrated practically without tailing as round spots as can be seen in Fig. 1. It is interesting to note that the iodine azide solution, suitable for penicillins, was ineffective in detecting the cephalosporins. The transformation of R_F values into R_M values permitted one to obtain a series of data where positive and negative R_M values derive from R_F values respectively smaller and greater than 0.5. Therefore higher and/or positive R_M values indicate compounds more lipophilic than those represented by a lower and/or negative R_M value. The plots of the R_M values versus the composition of the mobile phase are represented in Fig. 2. For each compound there was a range of linear relationship between R_M values and composition of the mobile phase. The straight lines of Fig. 2 were calculated by means of the least-square method from the R_M values in the range of linearity. Therefore only the R_M values corresponding to acetone concentrations between 0 and 10–12–16% were used. At higher concentrations the R_M values started to show the tendency of the compounds to migrate with the solvent front. For the lowest (most hydrophilic) compound

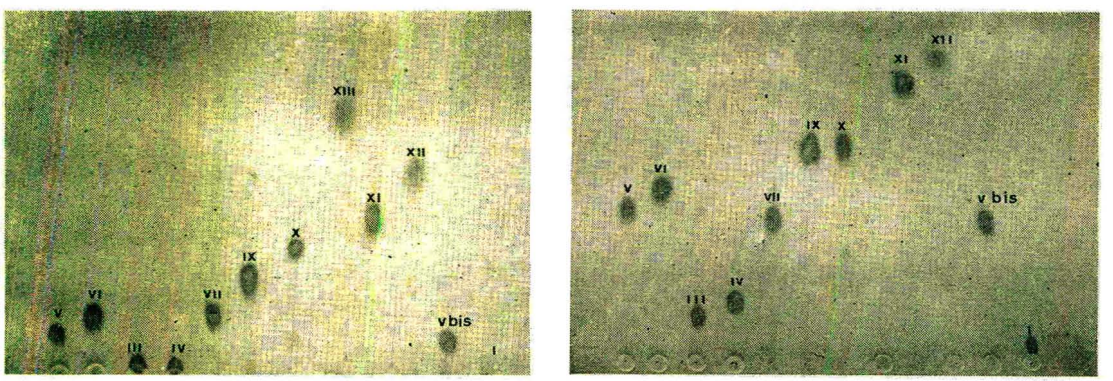


Fig. 1. Reversed-phase TLC of some cephalosporins. Stationary phase: silicone oil on Silica Gel G layer. Mobile phase: buffer in (a) and acetone-buffer 10% in (b). Detection: potassium permanganate alkaline solution. Amounts: 1 μg of each compound. The compounds are indicated as in Table I. Compound V bis corresponds to acid Cephaloram. In (b) compound XIII is no longer detectable as it migrated with the solvent front.

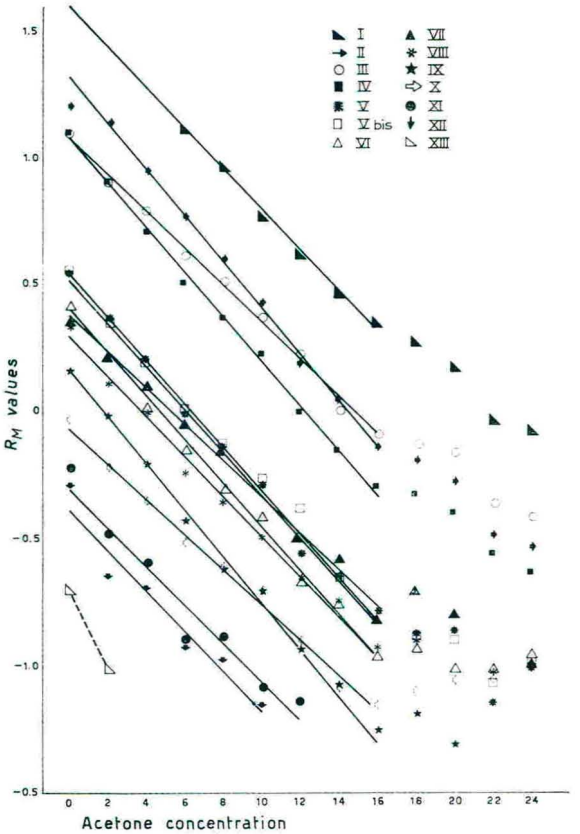


Fig. 2. The R_M values are plotted against the composition of the mobile phase. The straight lines were calculated by means of the least-squares method, except in the case of the lowest compound. Here the points were connected by a dotted line. Each point represents the mean of eight determinations. The R_M values corresponding to acetone concentrations higher than 24% are not reported. The cephalosporins are indicated as in Table I. Compound V bis corresponds to acid Cephaloram.

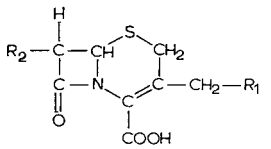
there were only two available points because with higher acetone concentrations in the mobile phase it migrated with the solvent front. In Fig. 2 these two points were simply connected by a dotted line. The R_M value indicated in Table I is therefore the experimental R_M value with buffer as the mobile phase. The highest (most lipophilic) compound I did not move until a certain acetone concentration was reached. By means of the equations of the straight lines of Fig. 2 the interpolated or extrapolated R_M values at 0% were calculated. In this way it was possible to obtain, for each compound, a R_M value in a standard system of water–silicone oil.

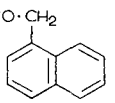
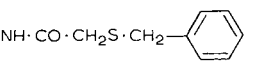
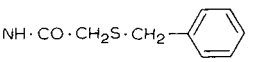
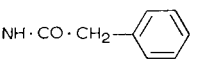
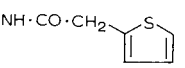
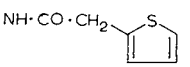
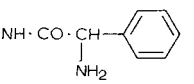
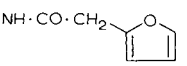
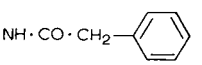
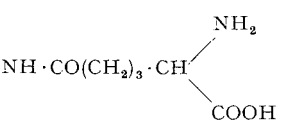
The calculated R_M values are reported in Table I. They permit the study of the influence of substituent groups on the partitioning of cephalosporins. The lipophilic character of compound II is more and more decreased by the substitution of the naphthyl moiety in the side chain with a benzene, a thiophene or a furan ring, as in compounds V, VI and IX respectively. The hydrophilic character of compound V is

TABLE I

LIST OF THE CEPHALOSPORINS ACCORDING TO THE DECREASING LIPOPHILIC CHARACTER OF THEIR MOLECULES, AS EXPRESSED BY THEIR CALCULATED R_M VALUES

The experiments were carried out with the sodium and potassium salts or with the free acids. Cephaloram was used both as sodium salt and free acid.



Compounds	R_1	R_2	R_M *value*
I (acid)	O·CO·CH_3	NH·CO·$(CH_2)_6$·CH_3	1.60
II (acid)	O·CO·CH_3		1.32
III (sodium salt)	N_3		1.08
IV (sodium salt)	O·CO·CH_3		1.08
V (sodium Cephaloram)	O·CO·CH_3		0.54
VI (sodium Cephalotin)	O·CO·CH_3		0.40
VII (acid)	N_3		0.37
VIII (acid Cephaloglycin)	O·CO·CH_3		0.29
IX (acid)	O·CO·CH_3		0.16
X (acid)	OH		−0.07
XI (acid)	O·CO·CH_3	NH·CO·CH_2Cl	−0.31
XII (7-amino-cephalosporanic acid)	O·CO·CH_3	NH_3^+	−0.39
XIII (potassium Cephalosporin C)	O·CO·CH_3		−0.71

increased by the introduction in the side chain, as in compound VIII, of a NH_2 group or by the substitution of the $OCOCH_3$ group with an OH one, as in compound X, or of the benzene ring with a Cl atom, as in compound XI. The substitution of a $OCOCH_3$ group with a N_3 does not seem to influence the R_M value in a significant way. This was noted for compounds IV and III and for VI and VII, respectively. However at higher acetone concentrations the compounds with the N_3 group seem to be less hydrophilic than those with the $OCOCH_3$ group.

The data of the present work confirm the existence of a linear relationship between R_M values and composition of the mobile phase. This relationship, first noted by SOCZEWINSKI AND WACHTMEISTER[2], is useful because it permits the calculation, from the range of maximum accuracy, of the R_M values for all the compounds in a standard system. It can be noted that the acidic form and the sodium salt of Cephaloram practically give the same results. This was also observed in the case of penicillins[1,3]. The considerations about the influence of the substituents on the partitioning of cephalosporins, as indicated by their R_M values, permit conclusions to be drawn which are in agreement with those of other investigators. The lipophilic character of the naphthyl substitution in comparison with a benzene and a thiophene ring was shown by HANSCH *et al.*[4] and IWASA *et al.*[5]. The hydrophilic character of the substitution of a H atom, an $OCOCH_3$ group and a benzene ring respectively with an NH_2, an OH and a Cl atom was also shown by IWASA *et al.*[5].

It can be noted that the iodine–azide solution, which is effective in detecting phenylthiohydantoins[6], thiophosphoric esters[7] and penicillins[3], did not succeed in detecting the spots of cephalosporins.

The advantages of the present method and its suitability for studying the relationship between partition data and biological activity of drugs have already been pointed out[1].

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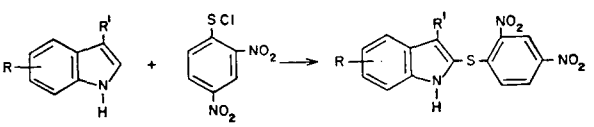
CHROM. 4280

Indoles and auxins

V. Separation of 2,4-dinitrophenylthio-derivatives of naturally occurring indoles by thin-layer chromatography*

Numerous solvent systems are available for the chromatographic separation of naturally occurring indoles and their R_F values are often used to indicate the identity of these compounds.

In our attempts to find derivatives of indoles that can be used as additional chromatographic evidence for their characterization, we prepared a number of 2,4-dinitrophenylthio-(DNPS**) derivatives of indoles occurring naturally[1]. Most 3-substituted indoles, like tryptophan and some related compounds (*cf.* ref. 2, 3), react with 2,4-dinitrophenylsulfenyl chloride (DNPSCl) under acidic conditions, to give 2-(2',4'-dinitrophenylthio)-3-indolyl derivatives as the single reaction product. It is conceivable, however, that indoles suitably substituted for electrophilic attack in the benzene moiety (hydroxyl group) may form different derivatives. This is currently under investigation.



In this note, we describe the thin-layer chromatography of these DNPS-derivatives and show that indoles can be separated chromatographically after their DNPS-derivatives are prepared from mixtures.

Compounds

DNPSCl was purchased from Fluka A.G. and ethyl 2,4-dinitrophenylsulfenate was prepared by allowing DNPSCl to dissolve in ethanol (*cf.* methanolysis of *o*-nitrophenylsulfenyl chloride[4]). Analysis, m.p. (124°; ref. 5) and the NMR spectrum[6] agree with the structure and the values reported. DNPS-indole derivatives were used (a) as crystalline materials[1] or (b) from a freshly prepared crude reaction mixture: The indole derivative (~ 10 mg) in dichloromethane (2 ml) and 99% formic acid (1 ml) was added to an equimolar amount of DNPSCl in dichloromethane (2 ml) and this mixture was kept for 1–2 h at 25°. Samples were applied directly to the thin-layer sheets, carefully dried to remove formic acid and the chromatograms developed. Reaction mixtures containing tryptamine derivatives were neutralized (sodium bicarbonate) before spotting. Treated in this fashion, all indoles mentioned in Table I give one major spot on the chromatograms with usually small amounts of unreacted DNPSCl present. The following compounds were not included in Table I because either more than one compound was formed (gramine, 3-indoleacrylic acid, 3-indolepyruvic acid, 3-indoleacetaldehyde) or because reaction with DNPSCl was too slow to be of practical use under these conditions (3-indolecarboxaldehyde, 3-indolecarboxylic acid).

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* NRCC No. 10851. For part IV see *Phytochemistry*, in press.

** Abbreviation used: DNPS = 2,4-dinitrophenylsulfenyl or 2,4-dinitrophenylthio.

TABLE I

R_F VALUES (× 100) FOR 2,4-DINITROPHENYLTHIO-DERIVATIVES OF NATURAL INDOLES

Reaction product of DNPCl with	*Solvent system*[a,b]										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*	*L*
3-Indoleacetamide	56	22	49	27	86	77	45	9	—	—	—
Ethyl 3-indoleacetate	89	75	85	73	87	86	90	73	—	—	—
3-Indoleacetonitrile	78	55	80	63	86	87	80	57	—	—	—
Tryptophol	75	32	85	73	85	84	76	74	—	—	—
3-Indoleacetone	90	62	83	60	90	85	85	64	—	—	—
Melatonin	70	28	50	25	90	78	53	7	—	—	—
3-Indoleacetic acid	20	7	13	8	76	66	9	~o	—	—	—
3-Indole-γ-butyric acid	44	16	36	~o	78	73	33	8	—	—	—
3-Indole-β-propionic acid	40T	15	~o	~o	81	72	29T	7	—	—	—
3-Indoleacetylaspartic acid	5	~o	~o	~o	31T	14T	~o	~o	86	61T	60T
3-Indole-L-lactic acid	7	~o	~o	~o	81	34	~o	~o	93	70	77
5-Hydroxy-3-indoleacetic acid	8	~o	7	~o	66	26	~o	~o	94	74	80
Tryptamine	9	7	37T	30T	68	42	10T	~o	65	57	63
Serotonin	8	~o	7	~o	63	24	~o	~o	57	26	46
N-Methyltryptamine	8	~o	~o	~o	55	44	4	~o	59	62	65
Bufotenine	45	18	6	~o	58	47	16	~o	43T	53T	61T
3-Indoleacetyl-ε-L-lysine	~o	~o	~o	~o	45	10	~o	~o	24	11	15
2,4-Dinitrophenylsulfenyl chloride[c]	95	90	88	81	84	80	89	86	95	95	90
Ethyl 2,4-dinitrophenylsulfenate	97	92	88	81	84	81	91	86	94	94	91

[a] A = diisopropyl ether–dimethylformamide (80:20); B = diisopropyl ether–dimethylformamide (90:10); C = hexane–acetone (50:50); D = hexane–acetone (60:40); E = chloroform–methanol (50:50); F = benzene–methanol (75:25); G = chloroform–methanol–carbon tetrachloride (10:1:9); H = chloroform; I = ethyl acetate–isopropanol–water–formic acid (65:25:5:2); K = chloroform–methanol–acetic acid (80:15:2); L = chloroform–methanol–acetic acid (70:30:0.5).

[b] ~o = spot remains on starting line or R_F value <3 usually with tailing from origin. T = Tailing.

[c] Streaking or multiple spot formation is observed in most solvents. The R_F value for the most intensely colored spot is reported in the Table.

Chromatography

The solvent systems used and the R_F values for the DNPS-indole derivatives on Eastman Chromatogram silica thin layer sheets (6061) are given in Table I.

All DNPS-derivatives are self indicating on the chromatograms because of their yellow colors. The limit of detection is ~2 μg. No significant change of the yellow color was observed, when the DNPS-derivatives, on developed chromatograms were sprayed with Ehrlich's or Salkowski's reagent or with a 2,4,7-trinitro-9-fluorenone or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone solution.

Application for indole mixtures

The DNPS-derivatives were prepared from a mixture of 3-indoleacetonitrile, 3-indoleacetamide, 3-indoleacetic acid and ethyl 3-indoleacetate (2 mg each) as described above except that an excess of DNPSCl (2 equiv.) was used, a condition that would be encountered with a natural sample. The large excess of unreacted DNPSCl makes this mixture unsuitable for direct chromatographic comparison with authentic materials. Ethanol was therefore added to convert DNPSCl into the corresponding sulfenate ester, a compound that gives a single spot at R_F values higher than the

DNPS-indole derivatives in most solvents. After chromatography of this treated mixture in solvent D the R_F values for the four DNPS-derivatives were found identical to standard compounds either alone or in mixture.

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Polyamide–silica gel thin-layer chromatography of food preservatives

The chromatography of food preservatives has been studied by numerous investigators. The separation of these preservatives on thin layers of cellulose acetate-polyamide[1], cellulose[2] and silica gel[3] has been reported, but there is no report on separation by polyamide–silica-gel layers. In a previous report[4], better separation of red food dyes was obtained with polyamide–silica gel layers; therefore, this method was further applied to separate ten preservatives. For comparison, the thin-layer chromatography of only polyamide and of only silica gel is also described.

Experimental

Preparation of polyamide–silica gel mixed layer. Ten grams of polyamide (ε-polycaprolactam CM 1007S of Toyo Rayon Co., Tokyo, Japan) were dissolved in 80 ml of 90% formic acid; then 20 ml of distilled water were added. After gentle warming (below 40°) and stirring, a homogeneous solution was obtained. It was then cooled to room temperature, and 52 g of Silica Gel G (E. Merck) were added. Of the previous solution 200 ml were poured into a dish (14.5 × 19.5 × 2.5 cm) into which a glass plate (12 × 14 × 0.1 cm) was dipped. Both sides of the glass were covered homogeneously.

The glass was hung for 2 min over the dish to let the excess solution drain off. It was then air dried for 3 h and heated at 100° for 30 min. These layers can be stored for a long period.

Preparation of polyamide layer. Instead of 10, 20 g of polyamide were dissolved before proceeding as described in the previous method, but without adding Silica Gel G.

Preparation of silica-gel layer. Dilute slurries of Silica Gel G (45 g to 100 ml of water) were sprayed at 2 kg/cm^3 pressure from a distance of 20 cm onto 8 horizontal glass plates (12 × 14 cm) which were then dried at 100° for 30 min. The thickness of the layers was about 250 μ.

Chromatographic procedure. A 0.2% alcoholic solution of samples was applied to the starting line 1.5 cm from the bottom of the layer, and the plate was developed by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min before use.

Visualization. The layers were sprayed with a 0.07% alcoholic solution of Rhodamine B, and deep-violet spots could be observed under UV light at 366 mμ.

Results and discussion

R_F values obtained with two solvent systems are given in Table I. It is interesting to note that the R_F values of the *p*-hydroxybenzoic acid ester in the two solvent systems are reversed. In the nonaqueous system (solvent I), the R_F values increase with an increase in the molecular weight of the esters, *i.e.* the order of R_F values is the same as that obtained by previous workers[1–3]. However, in the aqueous system (solvent II), the R_F values decrease with an increase in the molecular weight.

In both solvent systems, when using polyamide–silica gel mixed layers the R_F values are lower and separation is better than when polyamide and silica gel layers are employed. A 10-cm ascent from the origin is more rapid using the mixed layers

TABLE I

CHROMATOGRAPHIC DATA

Solvent I: *n*-hexane–benzene–glacial acetic acid (1:1:1); solvent II: water–28% ammonia solution (20:5). a, R_F value obtained on polyamide-silica gel layer; b, silica gel layer; c, polyamide layer.

No.	Substance	Solvent I			II		
		a	*b*	*c*	*a*	*b*	*c*
1	Methyl *p*-hydroxybenzoate	0.30	0.73	0.61	0.55	0.91	0.78
2	Ethyl *p*-hydroxybenzoate	0.35	0.77	0.69	0.46	0.88	0.70
3	Propyl *p*-hydroxybenzoate	0.41	0.78	0.74	0.34	0.79	0.59
4	Isopropyl *p*-hydroxybenzoate	0.44	0.78	0.74	0.32	0.77	0.60
5	Butyl *p*-hydroxybenzoate	0.49	0.79	0.78	0.24	0.69	0.49
6	Isobutyl *p*-hydroxylbenzoate	0.53	0.80	0.81	0.21	0.69	0.50
7	Sorbic acid	0.88	0.87	0.98	0.73	0.95	0.94
8	Benzoic acid	0.84	0.89	0.98	0.64	0.96	0.88
9	Salicylic acid	0.66	0.84	0.62	0.49	0.96	0.74
10	Dehydroacetic acid	0.74	0.96	0.80	0.62	0.95	0.85
Time required (min)[a]		90	25	240	130	15	180

[a] Time required to ascend 10 cm from origin.

than using polyamide layers. The content (16%) of polyamide in this polyamide–silica gel mixed layer is higher than that in the previous report (12%)[4] for getting a more stable layer.

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CHROM. 4284

Polyamide–kieselguhr thin-layer chromatography of yellow food dyes

The thin-layer chromatography of food dyes has been studied by numerous investigators. The separation of synthetic food dyes by thin layers of cellulose[1], starch[2], silica gel[3], aluminum oxide[4] and polyamide[5] has been reported. Recently, a better separation of red food dyes was obtained by CHIANG[6] with a mixed polyamide–silica gel thin layer. Therefore, further modification of this method was tried. In this note, the separation of five yellow food dyes and three harmful yellow dyes (auramine, metanil yellow and picric acid) by mixed polyamide–kieselguhr thin-layer chromatography is described. For comparison, the thin-layer chromatography on only polyamide and on only kieselguhr is also described.

Experimental

Preparation of polyamide–kieselguhr mixed layer. Ten grams of polyamide chip (Nylon 6, type 1022B of UBE Industrial Ltd., Osaka, Japan) were dissolved in 80 ml of 90% formic acid; then 20 ml of distilled water were added. After warming (below 40°) and stirring, a homogeneous solution was obtained. It was then cooled to room temperature, and 40 grams of Kieselguhr G (E. Merck) were added. Of the previous solution 200 ml were poured into a dish (14.5 × 19.5 × 2.5 cm) into which a glass plate (12 × 14 × 0.1 cm) was dipped. Both sides of the glass were covered homogeneously. The glass was hung for 2 min over the dish to let the excess solution drain off. It was then air dried for 3 h and heated at 100° for 30 min.

Preparation of polyamide layer. Instead of 10 g, 20 g of polyamide were dissolved before proceeding as described in the previous method, but without adding Kieselguhr G.

Preparation of kieselguhr layer. Dilute slurries of Kieselguhr G (45 g in 100 ml of water) were sprayed at 2 kg/cm^3 pressure from a distance of 20 cm onto 8 horizontal glass plates (12 × 14 cm) which were then dried at 100° for 30 min. The thickness of the layers was about 250 μ.

TABLE I

CHROMATOGRAPHIC DATA

Solvent I: methanol–acetone–water–30% sodium acetate solution–ethylenediamine (10:10:20:5:2); solvent II: ethanol–water–ether–5% NH_4Cl solution–ethylenediamine (15:15:10:5:2). a, R_F value obtained on polyamide–kieselguhr layer; b, kieselguhr layer; c, polyamide layer.

No.	Dyes	Solvent I			Solvent II		
		a	b	c	a	b	c
1	Naphthol yellow S	0.66	0.97	0.45	0.69	0.98	0.54
2	Yellow AB	0.11	0.82	0.02	0.35	0.88	0.10
3	Yellow OB	0.05	0.79[a]	0.01	0.23	0.84	0.10
4	Tartrazine	0.91	0.97	0.84	0.88	0.85	0.80
5	Sunset yellow FCF	0.38	0.98	0.59	0.81	0.94	0.78
6	Metanil yellow	0.31	0.88	0.13	0.53	0.92	0.43
7	Auramine	0.73	0.96	0.35[a]	0.76	0.96	0.49
8	Picric acid	0.53	0.98	0.77[a]	0.60	0.95	0.52
Time required (min)[b]		75	30	300	150	90	600

[a] Tailing.
[b] Time required to ascend 10 cm from origin.

Chromatographic procedure. A 0.5% alcoholic solution of Yellow AB, Yellow OB and auramine and a 0.5% water solution of other dyes were applied to the starting line 1.5 cm from the bottom of the layer, and the plate was developed by ascending techniques. The chamber had been equilibrated with the respective solvent for 30 min before use.

Results and discussion

The R_F values obtained with two solvent systems are given in Table I. It has been found that the results show better separation and sharp spots with polyamide–kieselguhr mixed layers than with polyamide and kieselguhr layers. Also a 10-cm ascent from the origin is more rapid using the mixed layers than when polyamide layers are employed. In the mixed layer, polyamide serves as a strong binder and makes the layers very durable and easy to handle. Both sides of the glass are independent of each other, and chromatography can be performed simultaneously on both sides. The addition of a small amount of salt and ethylenediamine in the solvent mixture is essential to break hydrogen bonding between polyamide and dyes. Oil-soluble dyes of Yellow AB and Yellow OB are rather difficult to separate because of the close similarity of their structures.

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Valylprolylarginine: a ninhydrin negative tripeptide

Ninhydrin is the most popular location reagent for amino acids and peptides, since its first application to paper chromatography[1]. For peptides, however, the sensitivity of the reagent is known to decrease as the molecular weight of the peptide increases. A further disadvantage is that ninhydrin produces no colour with cyclic peptides or the peptides whose terminal α-amino groups are acetylated or similarly substituted. In this communication, we wish to present a new example showing an additional limitation of the use of ninhydrin for the location of peptides on filter paper.

In the course of our investigations[2,3] on the primary structure of stem bromelain (EC 3.4.4.24), we isolated a glycoundecapeptide from the peptic digest of the parent glycoprotein. The amino acid sequence now determined is: Ala–Arg–Val–Pro–Arg–Asn–Asn(sugar)–Glu–Ser–Ser–Met, where sugar stands for the carbohydrate moiety present. When the tryptic digest of the glycopeptide was subjected to paper chromatography (pyridine–acetic acid–*n*-butanol–water, 10:3:15:12, by volume) or paper electrophoresis with pyridine acetate buffers at pH 3.5 and 6.5 (ref. 4), the occurrence of a peptide fragment that did not produce a ninhydrin colour at all was demonstrated; on the other hand, it gave positive stainings with both Sakaguchi reagent and the peptide reagent, *tert.*-butyl hypochlorite–*o*-tolidine–potassium iodide[5,6]. The fragment gave an amino acid composition of valine, proline and arginine in 1:1:1 ratio. The subtractive method for Edman degradation[7] indicated valine to be the amino group terminus, while carboxypeptidase B-DFP (EC 3.4.2.2, Worthington Biochemical Corp., Freehold, N.J., U.S.A.) revealed only arginine: thus the fragment must have been a tripeptide, Val–Pro–Arg. Chemical synthesis of L-Val–L-Pro–L-Arg was then kindly performed for us by Dr. S. SAKAKIBARA of the Institute for Protein Research, Osaka University, Osaka, Japan. The product, upon chromatography and electrophoresis on paper, was found to behave in exactly the same way as that shown by the isolated tripeptide, giving practically no colour development on spraying with an 0.2% ninhydrin solution in water-saturated *n*-butanol, by dipping in 0.2% ninhydrin solution in acetone, or by using ninhydrin–cadmium reagent[8]. Faintly positive colour development was noted only when a spot test was made directly on the filter paper with as much sample as 0.1 μmole per spot.

To eliminate the possibility of an interaction between the tripeptide and the filter paper in the ninhydrin reaction, the colour development was carried out in solution, instead of on paper. The synthesized tripeptide was chromatographed on Hitachi spheric resin No. 2610 (0.9 × 50 cm) using a Hitachi Model KLA-3B amino acid analyzer with the solvent system for acidic and neutral amino acids. The ninhydrin colour produced for the tripeptide, as recorded at 570 mμ, was compared with that for norleucine which was used as an internal standard. Several other di- and tripeptides were also analyzed in the same way. As shown in Fig. 1, Val–Pro–Arg gives an extraordinarily low value, indicating that poor ninhydrin colour production is an intrinsic property of this tripeptide. The poor colour production was not due to the amino terminal valyl residue, because longer and shorter valyl peptides were found to give reasonably high colour values (Fig. 1).

Further experiments were carried out to see whether the negative result of

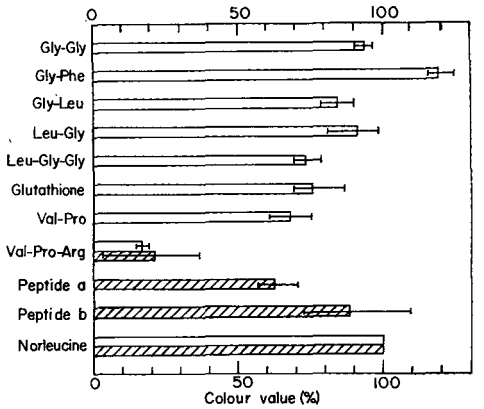

Fig. 1. The ninhydrin colour values of the peptides in solution. Clear bars are the values obtained by the amino acid analyzer (see text); hatched bars are those by the method of YEMM AND COCKING[9]. The value for norleucine was taken as 100%. Peptide a = Val–Pro–Arg–Asn–Asn-(sugar)–Glu–Ser–Ser–Met; peptide b = Ala–Arg–Val–Pro–Arg–Asn–Asn(sugar)–Glu–Ser–Ser–Met. Both peptides were isolated from the proteolytic digest of stem bromelain.

ninhydrin test on filter paper was due to poor reactivity of the tripeptide, or due to the fact that the product was colourless, even though it was formed. Val–Pro–Arg and Leu–Gly–Gly were spotted on filter paper and sprayed with ninhydrin reagent. After heating the paper, only the leucyl peptide stained. Both materials were then eluted from the paper with water and the eluates were hydrolysed in 6 *N* HCl at 105° for 20 h. The amino acid analysis of the hydrolysates showed that leucine was almost completely lost from the leucyl peptide, but there was a good recovery of glycine; while for the valyl peptide, valine as well as proline and arginine remained almost intact. The results indicate that ninhydrin hardly reacts with the α-amino group of Val–Pro–Arg under the conditions employed.

Very poor colour production by ninhydrin has also been observed with the tripeptide, Ile–Pro–Pro, which was obtained by tryptic hydrolysis of a decapeptide isolated from the venom of *Agkistrodon halys blomhoffii* (private communication from Dr. H. KATO, Institute for Protein Research, Osaka University). By analogy, it seems that its being a tripeptide with a bulky aliphatic side chain group on the amino group terminus, followed by a prolyl residue, is significant in reducing the reactivity toward the ninhydrin reagent on filter paper.

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Détermination de l'acide aminé N-terminal d'une protéine par application de la méthode d'Edman au matériel fixé sur gel de polyacrylamide

Au cours de notre travail portant sur la séparation des deux isoenzymes du trypsinogène de mouton[1], nous avons été amené à mettre au point une technique de détermination du résidu N-terminal des protéines fixées sur gel de polyacrylamide en utilisant non pas la réaction avec le fluorodinitrobenzène (FDNB) comme l'a décrite CATSIMPOOLAS[2] mais bien celle au phénylisothiocyanate (PITC) selon la méthode bien connue d'EDMAN. C'est cette technique que nous allons décrire en détail.

Sur des gels à 15% d'acrylamide à pH 2.5 préparés selon CHOULES ET ZIMM[3] sont placés 200 à 300 μg de protéine afin de charger ceux-ci au maximum mais sans avoir toutefois de recouvrement des bandes. L'électrophorèse s'effectue pendant 4 h sous une différence de potentiel de 80 V. Comme la réaction avec le PITC se réalise en milieu organique basique le pH des gels a été modifié progressivement par agitation pendant 15 min dans un tampon phosphate 0.5 *M* pH 8.7, puis 30 min dans le même tampon auquel on ajoute progressivement un volume égal de pyridine. Ce processus a pour effet d'éviter autant que possible un éclatement des gels lors de la réaction avec le phénylisothiocyanate en milieu organique. A ce moment les gels sont à nouveau agités 1 h en présence d'un mélange composé d'un volume de tampon phosphate et d'un volume de solution de PITC à 5% dans de la pyridine. Ils sont alors placés dans une solution à 2.5% de PITC dans de la pyridine pendant une nuit. Leur aspect et leur conformation changent, ils durcissent et perdent leur transparence.

Le lendemain les gels sont lavés au benzène, ensuite recouverts de 100 ml de benzène et de 10 ml d'eau; le mélange est agité et remplacé jusqu'à ce que le support reprenne sa forme initiale et soit complètement décoloré. Les tranches de gels correspondant aux dérivés phénylthiocarbamylés des protéines sont alors découpées par comparaison avec des gels colorés au FDNB ou au bleu de Coomassie[4] et dont l'électrophorèse a été réalisée en parallèle. Une vingtaine de tranches sont réunies, séchées sous vide et broyées afin d'être réduites en poudre; ce matériel est extrait 5 fois au benzène puis séché afin d'éliminer les traces résiduelles de solvant organique. L'hydrolyse ménagée libérant le PTH-acide aminé correspondant à l'extrémité N-terminale de la protéine est effectuée sur cette poudre à l'aide d'acide chlorhydrique 1 *N* à 100° pendant 90 min dans un tube à bouchon rodé. Après refroidissement on extrait 5 fois la solution acide par de l'acétate d'éthyle. Les phases organiques réunies contenant les PTH-acides aminés, sauf ceux de l'arginine et de l'histidine, sont alors lavées 3 fois par agitation en présence d'une quantité minimum d'eau. L'identification des PTH-acides aminés se réalise par chromatographie sur papier selon la méthode de SJOQUIST[5]. Elle

peut s'effectuer également très facilement par hydrolyse acide des PTH-acides aminés[6]. La solution dans l'acétate d'éthyle est évaporée à sec dans un tube à hydrolyse; après addition d'un ml de HCl 6 *N*, le tube est scellé sous vide et l'hydrolyse effectuée à 150° pendant 24 h. L'acide aminé N-terminal ainsi régénéré est déterminé à l'aide de l'analyseur automatique d'acides aminés.

Cette technique appliquée aux deux bandes obtenues lors de l'électrophorèse sur gel d'acrylamide du trypsinogène de mouton met en évidence, à côté de glycine, artefact inhérent à l'hydrolyse acide des PTH-acides aminés, de la valine et de la phénylalanine et cela pour chaque bande.

Ce résultat a été confirmé par réaction avec le fluorodinitrobenzène selon la technique de CATSIMPOOLAS[2].

Chacune des deux bandes séparées par électrophorèse est donc constituée par du trypsinogène. Des mesures d'activité trypsique potentielle l'ont confirmé. L'activité plus élevée du matériel élué de la bande rapide ainsi que des essais d'électrophorèse réalisés avec et sans persulfate, catalyseur de polymérisation nous ont permis de conclure à l'action oxydante de celui-ci[7] dans les conditions opératoires utilisées ici.

L'intérêt de cette méthode est de permettre de caractériser, à partir de quantités minimes, les divers constituants séparés par électrophorèse sur gel d'acrylamide ainsi que de recouper et de confirmer les résultats obtenus par la méthode de SANGER appliquée au même matériel.

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Fractionation of cobra venom by electrofocusing

Electrofocusing has been well known for several years[1–3]. The development of this technique has recently been made possible by the availability of ampholine. In recent months, several substances, which were previously difficult to obtain by classical means of fractionation, have been purified by this new technique. A general review and several very interesting applications of this technique were recently described at the *XVIIth Annual Colloquium on the Protides of the Biological fluids (Bruges 1969)* which was partially devoted to this problem[4].

As part of our investigations on Cobra venom components[5,6], it seemed that it would be of interest to study the application of this method to our material. Because of its high resolving power, electrofocusing is usually applied as one of the last steps in a fractionation process. However, its direct application to a mixture may provide interesting indications regarding the isoelectric points of the constituents and sometimes lead to the isolation of one of these in an almost pure state. This new fractionation method was therefore applied to the venom of a Formosan cobra *(Naja naja atra)*. The experiments were carried out in an LKB analytical column.

A density gradient was formed with sucrose solutions. The ampholine carriers consisted of a mixture of low molecular weight aliphatic polyamino- polycarboxylic acids (available from LKB Produktor, Stockholm). A 1% concentration in a total volume of 110 ml was used.

Focusing was carried out for 72 h at a temperature of 4°, the voltage being initially adjusted to maintain a maximum input power of 2 W. After 24 h running, the voltage was fixed at 750 V for the next 48 h; the current finally dropping to less than

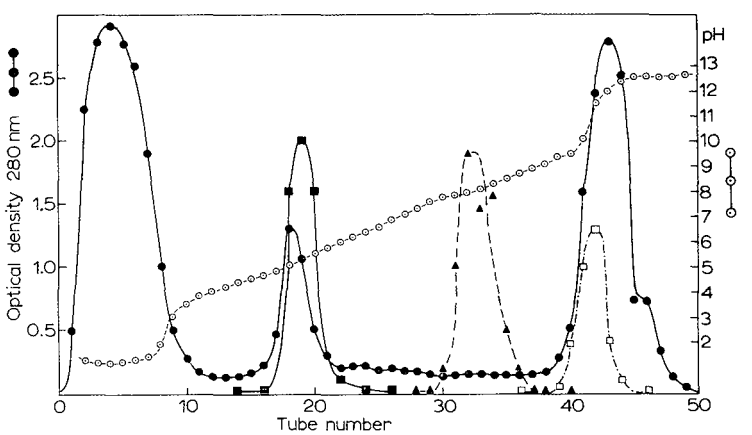

Fig. 1. Electrofocusing of *Naja naja atra* venom; pH range 3–10. ●—●, absorbance at 280 nm; ⊙—⊙, pH; □—□, toxicity[7]. Arbitrary enzymatic activities are plotted as follows: ■—■, phospholipase A (E.C. 3.1.1.4.) detected by estimation of the lecithin degradation products by thin-layer chromatography; ▲—▲, L-amino acid oxidase (E.C. 1.4.3.2.) estimated by the ZELLER[8] manometric method.

1 mA. At the end of the run, the column was emptied and 2 ml fractions were automatically collected. Absorbancies at 280 nm, pH and enzymatic activities were determined on each tube without removing the ampholines and sucrose.

As shown in Figs. 1 and 2, electrofocusing is a very useful technique for the separation of cobra venom components. All the enzymatic activity seemed well preserved after fractionation except that of the hyaluronidase and phosphodiesterase. Nearly all the enzymes were focused at sufficiently different pH's, which permitted their separation by a second run in a narrower pH range.

Electrofocusing, in association with another fractionation procedure, could lead

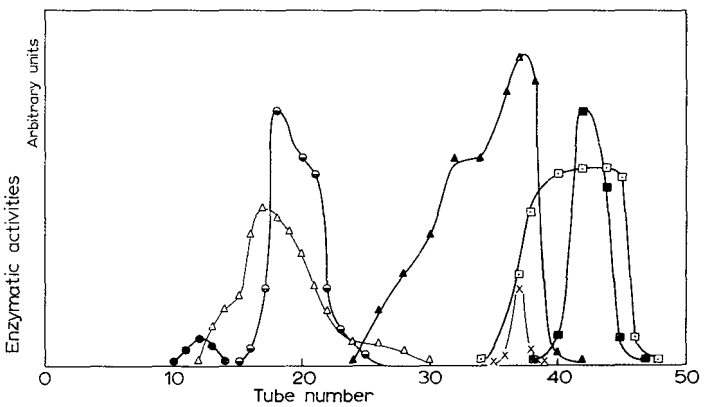

Fig. 2. Electrofocusing of *Naja naja atra* venom. Activities are represented as follows: ●—●, hyaluronidase (E.C. 4.2.99.1.) estimated by the TOLKSDORF turbidimetric method[9]; △—△, phosphomonoesterase, estimated by the LEVINTHAL spectrophotometric method[10]; ◒—◒, cholinesterase (E.C. 3.1.1.8.) determined by the KRAMER colorimetric method[11]; ▲—▲, 5′-nucleotidase (E.C. 3.1.3.5.) tested by the BABKINA method[12]; ×—×, phosphodiesterase (E.C. 3.1.4.1.) determined by the BOMAN spectrophotometric method[13]; ■—■, endonuclease estimated by the SHAPIRA method[14]; ⊡—⊡, inhibitor of anaerobic glycolysis tested manometrically on Ehrlich ascites cells[15].

to an easy means of purification of any one of these enzymes. This new technique is certainly an invaluable analytical method for p*I* determination. With regard to its application to preparative work, two points remain to be elucidated. Firstly, whether the enzymatic and physiological activities are wholly conserved, and secondly, the problem of the removal of sucrose and "ampholine" from small proteins and large peptides. Further investigations are being conducted in this field.

We are most indebted to Mrs. KEPPENS and to Mr. COOMANS for their technical assistance. These experiments were carried out in the Biochemistry Laboratory of the I.I.F.-I.M.C., C.E.R.I.A., Brussels. We thank the Province de Brabant and the F.W. Breth Foundation, New York, for the financial and moral support given to this work.

Note added in proof

Since this manuscript was completed a paper has been published[16] dealing with the fractionation by isoelectric focusing of a crotalidae snake venom *(Agkistrodon rhodostoma)*.

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CHROM. 4267

Stability trends of some 1:1 and 2:1 malonato and 1,1-cyclobutanedicarboxylato cobalt, nickel, copper and zinc chelates*

In the course of investigation of the stability of metal chelate species formed with the anion of 1,1-cyclobutanedicarboxylic acid (a ligand analogous to the malonate anion), determination of the stoichiometric formation constants of 1:1 and 1:2 metal chelate species of the 3*d* transition metals (cobalt, nickel, copper and zinc) involving both CBDA and malonate anions was undertaken at 25° and $I = 0.1$ (KNO_3). With malonate K_1 values, the results were in fair agreement with results of others[1,2]; and with CBDA anion, behavior was sufficiently different from that of malonate to warrant comment.

The method used in this work has been described previously[3]; thus, it need not be redescribed. The results are compared (Table I) with results of YASADA *et al.*[2] (in parenthesis).

TABLE I

STEP FORMATION CONSTANTS OF 1:1 AND 1:2 CHELATE SPECIES (AT 25°; $I = 0.1$)

Metal (II) ion	*Malonato species*		*CBDA species*	
	K_1	K_2	K_1	K_2
Co	$9.0 \pm 0.1 \times 10^2$	30 ± 3	$1.6 \pm 0.2 \times 10^2$	10 ± 5
Ni	$1.88 \pm 0.02 \times 10^3$ (1.6×10^3)	47 ± 3	$2.2 \pm 0.2 \times 10^2$	10 ± 5
Cu	$1.04 \pm 0.01 \times 10^5$ (1×10^5)	840 ± 10	$1.03 \pm 0.01 \times 10^5$	1300 ± 100
Zn	$9.3 \pm 0.1 \times 10^2$ (5×10^2)	30 ± 5	$3.4 \pm 0.1 \times 10^2$	30 ± 10

Considering the relatively high ionic strength (0.1) in our experiments, it would be invalid to compute and report thermodynamic stability constants for comparison with other data at $I \rightarrow 0$. A glance at the variety of miscellaneous malonate chelate stability data compiled by SILLÉN AND MARTELL[4] reveals recorded values of $\log^T K_1$(Cu) ranging from 5.60 to 5.80, as well as other uncertainties, and the fact that K_2 values in metal malonate systems generally have not been reported. However, in the case of copper malonate at 20°, $I = 0.1$ (KNO_3), GELLES AND NANCOLLAS[1] reported $K_1 = 0.65 \times 10^5$ and $\beta_2 = 5.4 \times 10^7$, yielding $K_2 = 8.3 \times 10^2$ in excellent agreement with our 8.4×10^2. Their value for K_1, however, is substantially lower than ours (1.04×10^5), as well as that of YASADA *et al.*[2].

It is noteworthy that, although 1:1 malonate and CBDA chelates of copper (II) are nearly identical in stability, both the 1:1 and 1:2 CBDA chelates formed in the cases of cobalt, nickel and zinc are much less stable than corresponding malonate species. In changing the ligand from malonate to the analogous, but more constrained, CBDA entity, a reversal in stability of 1:1 nickel and zinc chelate species occurs.

* Work was performed in the Ames Laboratory of the U.S. Atomic Energy Commission. Contribution No. 2570.

With malonate the order of affinity of cation for ligand is Cu $\gg$ Ni $>$ Zn $\cong$ Co; with CBDA the observed order is Cu $\gg$ Zn $>$ Ni $>$ Co.

From the relative magnitudes of β_2 values it is apparent that CBDA buffer solutions would be highly effective eluants in cation-exchange elution separations of copper and nickel. In view of the nearly identical affinities of Cu(II) and Ni(II) for Dowex 50-X8 cation-exchange resin, for example, elution of copper and nickel with ammonium CBDA should afford a separation factor as large as 6×10^4. Indeed, it has been possible in this laboratory to achieve quantitative recovery of copper from nickel or zinc by selective elution of from 0.1 to 2.0 mmoles of Cu(II) from 2 mmoles of binary mixture on 40–50 mesh Dowex 50-X8 resin beds, 2 mm in diameter and 60 mm long, using 60–100 ml of 0.1 *M* ($(NH_4)_{1.5}H_{0.5}$) CBDA (followed by 300 ml of distilled water) at 5 ml/min.

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Book Review

CHROM. 4230

An Introduction to Chromatography on Impregnated Glass Fibre, by FREDERICK C. HAER, Ann Arbor Science Publishers, Ann Arbor, 1969, 173 pp., price $ 15.00.

This plastic, spirally-bound book is ostensibly designed to promote the use of commercially produced sheets of silica gel impregnated glass fibre and its associated equipment in so-called "Instant Thin Layer Chromatography". Faster analysis times and greater sensitivity compared with conventional thin layer chromatography are two of the claims made for this material although no substantiating evidence is offered.

The lay-out of the book follows the stereotyped pattern of most recent monographs on thin layer chromatography. The first half gives an elementary account of basic theory and technique, though the style in which it is written would make it difficult for the beginner to digest at first reading. Statements to the effect that chloroform is a less polar solvent than benzene (p. 29) is contrary to TRAPPE's well-known eluotropic series (p. 17) and deserves a few words of explanation. The remainder of the book is devoted to applications, written mainly in the form of recipes, in which coated glass fibre sheets have been used. Electrophoretic procedures are also included.

In general, the standard of the photographs is poor and much more care could have been taken over the presentation of the chromatograms. For a book of this quality, the price is unreasonable and therefore this publication cannot be recommended.

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News

Meetings

SEMINAR ON LIQUID AND GEL PERMEATION CHROMATOGRAPHY

A regional seminar on Liquid and Gel Permeation Chromatography, presented by Becker Delft N.V. and Waters Associates, will be held on October 27th, 1969, in the Rotterdam room of the Rotterdam Hilton Hotel.

Contact: Becker Delft N.V., Instrumenten- en Apparatenfabriek, 259 Vulcanusweg, Phone 01730 - 25903, P.O. Box 219, Delft, The Netherlands.

EASTERN ANALYTICAL SYMPOSIUM

The *1969 Eastern Analytical Symposium* will be held from November 19th to 21st, 1969, at the Statler Hilton Hotel in New York City.

The technical sessions will cover topics in the field of spectroscopy, chromatography, biomedical analyses and miscellaneous subjects, such as analysis for chromosome imbalance, solvation energies on pH scales in non-aqueous solvents, quantitative NMR, computer controlled experimentation, automatic clinical analyzers, and new sampling techniques for atomic absorption and emission.

There will also be a workshop devoted to electro-analytical methods and computer-calculators followed by demonstration experiments conducted by exhibitors at the symposium. As in previous years, the symposium will include technical film sessions, an employment bureau, and a large exhibit of instrumentation and equipment related to spectroscopy and analytical chemistry.

This annual meeting is sponsored by the analytical groups of the New York and New Jersey Sections of the American Chemical Society; Baltimore-Washington, Delaware Valley, New York and New England Sections for applied spectroscopy; and the American Microchemical Society.

Further information is available from RICHARD J. KNAUER, Armco Steel Corp., Advanced Materials Division, P.O. Box 1697, Baltimore, Md. 21203 (U.S.A.).

SECOND INTERNATIONAL AIR POLLUTION CONFERENCE

INTERNATIONAL UNION OF AIR POLLUTION PREVENTION ASSOCIATIONS

The Second International Air Pollution Conference will be held December 6th–11th, 1970 at the Sheraton Park Hotel, Washington, D.C., U.S.A. The Air Pollution Control Association (U.S.A. and Canada) is the host association for the 1970 conference. The Second International Air Pollution Conference will include the conference, an exposition and a number of social events. The General Conference Chairman is Mr. JOSEPH W. MULLAN. The co-chairmen of the Program Comittee are Mr. JOHN S. LAGARIAS and Prof. ARTHUR C. STERN.

The Program Committee invites submission of proposals to present papers at the Conference. The schedule for submission and review of proposals and papers is as follows: July 1969–January 1970—submission of proposals to present papers; deadline, January 31st, 1970. February 1970–April 1970—review of proposals by Program Committee for acceptance; deadline, April 30th, 1970. May 1970–July 1970—submission of papers; deadline, July 31st, 1970. August 1970–November 1970—translation, preprint preparation and reproduction; preparation of final program. November 1970–December 1970—preprint distribution and printing of final program.

The Conference will consist of plenary sessions at which only specifically solicited papers, reports and speeches will be presented in either English, French, German, Japanese or Spanish, and at which there will be simultaneous translation into these five languages; and concurrent sessions for papers chosen from among those submitted in response to this invitation. The concurrent sessions will be limited to presentations and simultaneous translation in English and French, the two official languages of the International Union. Preprints will be in both languages.

It is presently planned to have concurrent sessions in the following six subject areas: (1) Air pollution chemistry and physics—papers on sampling, analysis, instrumentation, aerosols, and on effects on non-biological systems. (2) Air pollution meteorology—papers on transport, diffusion, modeling, forecasting, and stacks. (3) Air pollution medicine and biology—papers on effects on people, animals, and vegetation; and on air quality criteria. (4) Air pollution engineering—papers on sources, their engineering control and control equipment. (5) Air pollution control administration—papers on legislation, regulations, inspection, and control program operation; and on air pollution standards. (6) Air pollution surveys—papers reporting community and area studies.

Proposals to present papers should include a provisional title for the paper, an outline limited to 200 words and the names, titles, affiliations of the proposed authors and address of the principal author. Proposals may be submitted in the language of the author. Proposals to present papers should be sent to: Professor ARTHUR C. STERN, Program Co-Chairman, Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina, P.O. Box 630, Chapel Hill, N.C. 27514, U.S.A.

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Paper Chromatography

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CHROM. 4270

THE USE OF GAS CHROMATOGRAPHIC DETECTORS FOR MOLECULAR WEIGHT DETERMINATIONS

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SUMMARY

The molecular weights of the constituents of mixtures may be determined, after chromatographic separation, using a detector whose response is a function of molecular weight. Various methods are discussed and results presented for a mass detector/gas density balance system, by which molecular weights may be determined from a single analysis.

INTRODUCTION

An ideal procedure for the determination of the molecular weights of the constituents of a mixture would comprise the separation of the constituents by gas chromatography, and at the same time, the use of the detector response to determine the molecular weight of each component as it emerged. Present methods require pure and isolated materials: ebullioscopic and cryoscopic methods are straightforward, but give only moderate accuracy. Mass spectrometry gives very accurate results but demands expensive equipment. Using a gas chromatographic detector whose response depends solely on molecular weight, the accuracy of the method will depend on the accuracy to which peak areas can be measured. The response of the gas density balance[1], the jet stream detector[2], and the ultrasonic detector[3] is a function of molecular weight. The gas density balance has been used by several workers for molecular weight determinations[4–9], but no data have been published using the remaining detectors.

DISCUSSION

The response of the gas density balance is given by the equation:

$$A = kq \frac{M_x - M_c}{M_x} \quad (1)$$

where q = amount of component x, M_x = molecular weight of x, A = peak area, and M_c = molecular weight of carrier gas. The proportionality constant k, can be found by measuring the response to a known amount of a pure material of known

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molecular weight. By injecting under identical conditions a known amount of an unknown material, its molecular weight can be calculated, using eqn. 1. In practice this method is open to a number of serious objections. It is not possible merely to separate the constituents of a mixture in the gas chromatographic column, and to determine the molecular weight of each component as it emerges, since values for q for each component are not known. Both the standard material and the unknowns are required in the pure isolated state. In addition it is particularly difficult to inject a known amount of material into the apparatus, and to ensure that no fraction is lost before reaching the detector. It is difficult to maintain precisely the same experimental conditions over the period of time required for calibration and subsequent analysis of unknowns. A satisfactory practical procedure for the determination of molecular weights using a gas density balance was first carried out by LIBERTI *et al.*[5]. To an unknown mixture is added a compound of known molecular weight, M_s, and the mixture is analysed in the conventional manner. For a two-component mixture, two peaks of areas A_x and A_s for the unknown and standard, respectively, are obtained:

$$A_{x1} = kq_{x1}\frac{M_x - M_1}{M_x} \tag{2}$$

and

$$A_{s1} = kq_{s1}\frac{M_x - M_1}{M_s} \tag{3}$$

where M_1 = molecular weight of carrier gas 1.

The experiment is repeated using a carrier gas of different molecular weight, M_2, to give:

$$A_{x2} = kq_{x2}\frac{M_x - M_2}{M_x} \tag{4}$$

and

$$A_{s2} = kq_{s2}\frac{M_s - M_2}{M_s} \tag{5}$$

It is not essential to inject precisely the same quantity of the mixture in each series of runs since the ratio:

$$\frac{q_{x1}}{q_{s1}} = \frac{q_{x2}}{q_{s2}} \tag{6}$$

and by combining eqns. 2 to 5

$$\frac{A_{x1}(M_s - M_1)}{A_{s1}(M_x - M_1)} = \frac{A_{x2}(M_s - M_2)}{A_{s2}(M_x - M_2)} \tag{7}$$

The A values are obtained directly from the peak areas of the chromatograms, and all molecular weights are known except M_x, which can be calculated. Using nitrogen and hydrogen as the two carrier gases, molecular weights to about 4% of the true values were obtained for materials of molecular weight about 150 (ref. 5). Similar results were obtained by REVEL'SKII *et al.*[6] using nitrogen and argon. In an attempt to improve upon the accuracy of the results, PARSONS[7] used one carrier gas of molecular

weight lower than the unknown, and the other carrier gas of molecular weight higher than the unknown (*e.g.* nitrogen and dichlorodifluoromethane). Errors of the order of 1–2 % are quoted in the published data[7]. Molecular weight determinations based on the LIBERTI scheme, although giving acceptable results suffer from the disadvantage that column conditions must remain constant for the duration of the two sets of runs, although it is no longer necessary to know the amount of sample injected, or to work with pure isolated materials. The need to change the carrier gas is tiresome, but is not regarded as a very serious disadvantage.

An alternative method for determining molecular weights using the gas density balance was devised by PHILLIPS AND TIMMS[8]. Eqn. 1 is rearranged and rewritten:

$$PV = \frac{KA}{M_x - M_c} \tag{8}$$

where P and V are the pressure and volume of a vapour x, and K a constant. Pressure–volume (P–V) measurements are made on the vapour, which is then passed into a gas density balance. K is found using a material of known molecular weight. The method gives molecular weights, in general to within 1 % of the true values, for materials of boiling point up to about 200°. The P–V equipment requires considerable skill to operate and the determination of a single molecular weight is fairly time consuming. Pure isolated materials are required. Preparative chromatography or other methods of purification must therefore be employed before molecular weight determinations can be carried out.

A chromatographic method for the determination of molecular weights based on the measurement of the increase in flow rate which occurs as a component emerges from a column was proposed by SCOTT[10]. The gas volume, ΔV, occupied by m grams of solute vapour is given by the equation:

$$\Delta V = m \frac{K}{K+1} \times \frac{22.4 \times 10^3}{M} \times \frac{T}{273} \tag{9}$$

where K = partition coefficient and M = molecular weight of solute.

For a two-component mixture, containing one material of known molecular weight, M_s:

$$\frac{\Delta V_s}{\Delta V_x} = \frac{M_x m_s}{M_s m_x} \tag{10}$$

provided that $K \approx K + 1$.

If the detector responds solely to flow rate changes:

$$\frac{\Delta V_s}{\Delta V_x} = \frac{A_s}{A_x} \tag{11}$$

where A_s and A_x are peak areas representing the standard and the unknown, respectively. The molecular weight of the unknown is given by:

$$M_x = \frac{A_s m_x M_s}{A_x m_s} \tag{12}$$

It is essential to know the weights of the injected materials, which when using syringe injection implies that the densities of the standard and the unknown

must be known. The assumption that $K \gg 1$ will give rise to negligible errors provided that retention times are long and are similar for the standard and the unknown.

The flame thermocouple detector[11] is sensitive to both flow rate changes and changes in temperature caused by the presence of an eluted material. These two effects can be isolated by preventing the material from reaching the detector. The detector will then respond only to flow rate changes. SCOTT used the following system to accomplish this effect. The exit of a normal partition column was attached to a length of empty tubing which itself was attached to a column containing activated charcoal. A substance, on emerging from the partition column, produced a flow rate change which was detected as a positive peak by the flame thermocouple detector. On entering the adsorption column the material was totally adsorbed, resulting in a flow rate decrease, which was detected as a negative peak. By using the adsorption peak area rather than the partition peak area, the assumption that $K \gg 1$ is removed. Using the results quoted by SCOTT[10], the molecular weights of a number of materials determined by this method have been calculated, and are quoted in Table I.

TABLE I

MOLECULAR WEIGHT DETERMINATIONS (FLAME THERMOCOUPLE DETECTOR)

Compound	*Detector response (area/unit weight)*	*Molecular weight*		*Bias*
		Calculated	*True*	
n-Hexane (standard)	5.8	86.2	86.2	Standard
Carbon tetrachloride	3.2	156.2	153.8	+ 2.4
Chloroform	4.1	122.0	119.5	+ 2.5
Dichloroethylene	5.3	94.4	97.0	− 2.6
n-Butyl chloride	5.3	94.4	92.7	+ 1.7
Ethyl acetate	5.6	89.3	88.1	+ 1.2
Ether	6.8	73.5	74.2	− 0.7
Acetone	8.6	58.2	58.1	− 0.1

Errors of the order of 2 % are encountered. On the assumption that the detector is responding only to flow rate changes, the major errors arise from the difficulty of injecting known weights of each material, and of measuring the resulting peak areas.

The requirement that the amount of injected material must be known (and hence densities known) is common to all of the detectors which can be used for molecular weight determinations, and constitutes the major limitation and error source in the determination of molecular weights by gas chromatography.

It has been established that the mass detector will give reliable quantitative analyses over a wide range of operating conditions, and that response is proportional to mass. If the mass detector is operated in conjunction with a detector responding to molecular weight changes, then the amount of material present is obtained directly from the mass detector response. A knowledge of the amount of material injected, its density, and the percentage composition of the mixture is not required, and losses of material within the column do not affect the results. It was demonstrated by BEVAN AND THORBURN[4] that by using a gas density balance and the mass detector in series, the molecular weights of the constituents of an unknown mixture could be found in a single run. Two chromatograms are obtained: the mass detector will give values of

q (eqn.1) for each material, and the gas density balance the corresponding values of A. The value of k is found by adding to the mixture a compound of known molecular weight. It is not necessary to add a precisely measured amount of standard. The only requirement is the same for any conventional quantitative analysis, namely that resolution of the components should be complete. It would appear that the use of the mass detector in conjunction with the gas density balance offers an ideal method for the determination of molecular weights. There are, however, two factors which limit the method:

(i) the calculation of a molecular weight depends on the accuracy with which a peak area and a step height can be measured, as with any other method involving gas chromatographic detectors.

(ii) the change in response of the gas density balance for species of different molecular weight is a maximum when values of M_c and M_x (eqn. 1) are of the same order: but the absolute response of the detector is a minimum when M_c and M_x are similar, and zero when they are equal. As the values of M_c and M_x diverge it becomes more and more difficult to distinguish between the responses of compounds of similar molecular weight; in the limiting case $(M_x—M_c)/M_x = 1$, and the molecular weight term disappears. The effect is shown graphically in Fig. 1 for a number of carrier gases covering the molecular weight range 4 to 121. It will not be possible to determine the molecular weight of a material with certainty if its molecular weight is at a point on or approaching the plateau of the curve. Consider the curve for nitrogen. It should be possible to determine the molecular weight of any material up to about 120, including values below that of nitrogen, but with decreasing certainty as the molecular weight increases. Over about 120, even a small discrepancy in the measurement of A will result in an error in the value of $(M_x—M_c)/M_x$, and the error in M_x itself will be grossly magnified.

For a detector to be of value for molecular weight determinations it is essential that the response depends only on molecular weight changes. It has previously been shown that the response of the Gow-Mac gas density detector is a function of molecular weight within certain limits[12]. It is generally accepted and has recently been demon-

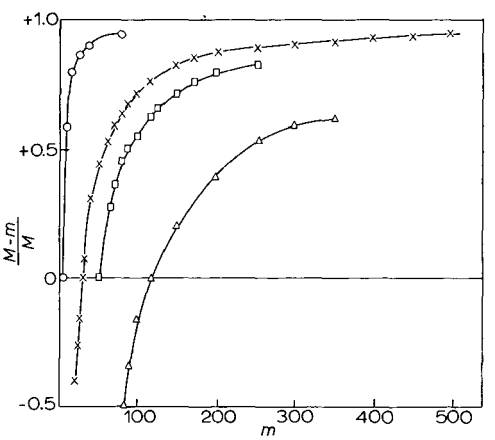

Fig. 1. The effect of carrier gas on molecular weight determinations. m = molecular weight of component; M = molecular weight of carrier. ○, helium; ×, nitrogen; □, carbon dioxide; △, dichlorodifluoromethane.

strated[13] that the Martin gas density balance gives a response predictable on a molecular weight basis, over a wide concentration range for all materials, and this detector was therefore used in the present work.

The fall in precision with increasing molecular weight limits the value of the gas density balance–mass detector combination. Combination of the mass detector with the flame thermocouple detector operated as an anemometer overcomes this limitation, since although the response of the flame thermocouple decreases as molecular weight increases, it does so linearly and hence a marked fall in precision does not occur. Using a combination of partition and adsorption to create flow rate changes, it would appear to be necessary to operate the two detectors in parallel. However, the incorporation of a stream splitting device may interfere with the flow rate pattern as a material is eluted. Preferably, the two detectors should be operated without the need for a stream splitter. This may be accomplished by replacing the adsorption column with a second short partition column. Elution from the main partition column will give a positive detector response; the material is then partitioned on the second column, after which the gas stream is deflected to the mass detector, by means of a two-way tap. Such a procedure could only deal with widely separated components, and the condition that $K \gg 1$ must be satisfied. An alternative method which would be satisfactory for multi-component mixtures is to trap all components on the adsorption column, and after completion of the run, place this column in front of the partition column, in a chamber sufficiently hot to quantitatively desorb all material; the run is repeated using the mass detector in place of the flame thermocouple detector.

EXPERIMENTAL

The molecular weights of a number of materials have been determined using the Martin gas density balance in series with the mass detector. Operating conditions are given in Table II.

The linearity of response of the gas density balance toward each sample was checked by covering a reasonable concentration range and plotting graphs of detector response (peak area) against the mass detector response (weight adsorbed). The molecular weight can be calculated from the slopes of the curves, since the slope gives A/q (eqn. 1) directly. However, more precise values can be obtained by calculating the mean value of A/q.

Several two-component mixtures were analysed, and the molecular weight

TABLE II

OPERATING CONDITIONS

Apparatus	Shandon KG2
Carrier gas	Nitrogen
Analytical gas flow rate	51 ml min^{-1}
Reference gas flow rate	51 ml min^{-1}
Gas density balance filament current	1.9 A
Sensitivity	$\times 10^3$
Mass detector ranges	1–5 mg

of each component calculated assuming that the remaining component was the standard. The molecular weight values given in Table III are the mean of about ten determinations. Bias values are given in terms of molecular weight, and not as percentage error.

TABLE III

MOLECULAR WEIGHT DETERMINATIONS (GAS DENSITY DETECTOR)

Compound	*Mean molecular weight*	*Standard deviation*	*True molecular weight*	*Bias*
Water[a]	17.5	—	18.0	— 0.5
Ethyl alcohol	43.8	—	46.1	— 2.3
Water[a]	18.0	0.13	18.0	zero
Ethyl alcohol	46.1	0.63	46.1	zero
Methyl alcohol[b]	> 28	—	32.0	*ca.* — 2
Ethyl alcohol	52.9	—	46.1	+ 5.8
Ethyl alcohol[c]	45.2	0.61	46.1	— 0.9
n-Propyl alcohol	62.3	1.70	60.1	+ 2.2
n-Propyl alcohol[c]	59.1	3.27	60.1	— 1.0
n-Butyl alcohol	75.9	6.06	74.1	+ 1.8
Isopropyl alcohol[d]	59.4	4.67	60.1	— 0.7
Nitromethane	61.8	5.22	61.0	+ 0.8
n-Propyl alcohol[c]	59.7	3.03	60.1	— 0.4
Methyl *n*-propyl ketone	87.1	6.35	86.1	+ 1.0
n-Butyraldehyde[e]	70.8	7.29	72.1	— 1.3
Methyl ethyl ketone	73.4	4.65	72.1	+ 1.3
Isopropyl alcohol[d]	60.4	—	60.1	+ 0.3
n-Propyl alcohol	59.8	—	60.1	— 0.3
Benzene[d]	76.5	2.41	78.1	— 1.6
Toluene	94.7	4.57	92.1	+ 2.6
n-Heptane[f]	90.9	15.23	100.2	— 9.3
n-Octane	128.4	24.25	114.2	+ 14.2
n-Heptane[d]	107.6	—	100.2	+ 7.4
n-Nonane	144.2	—	128.5	+ 15.7
n-Octane[f]	98.6	23.24	114.2	— 15.6
n-Nonane	159.8	29.75	128.5	+ 31.3

[a] Column D at 70°. (For column details, see Table VI.)
[b] Column E at 70°.
[c] Column D at 140°.
[d] Column E at 101°
[e] Column E at 68°.
[f] Column H at 106°.

The variations of bias and standard deviation with molecular weight are shown in Figs. 2 and 3 respectively. Clearly, accuracy and precision are inadequate over a molecular weight of about 100. In the region of 100, values are as good as those obtained by LIBERTI *et al.*[5], and become progressively better as molecular weight decreases.

For a relative composition analysis using the gas density balance, the molecular

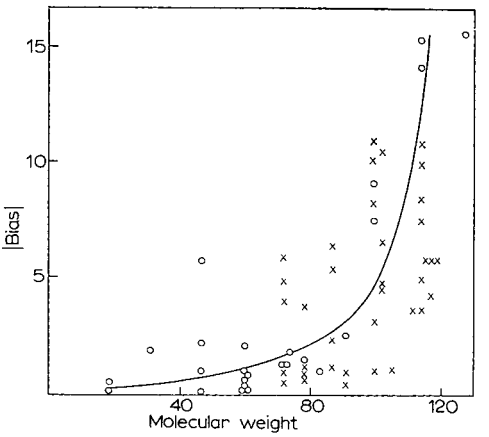

Fig. 2. Variation of bias with molecular weight. O, two-component mixtures; ×, multicomponent mixtures. For column details, see Table VI.

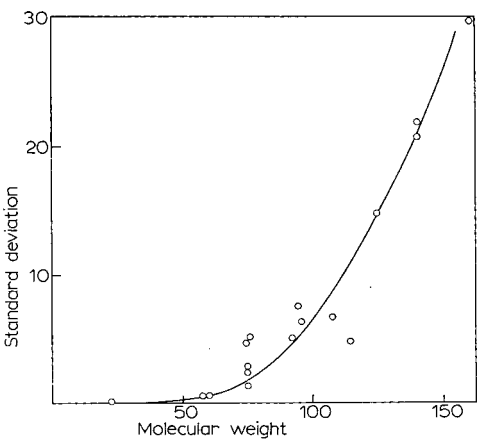

Fig. 3. Variation of standard deviation with molecular weight.

TABLE IV

ANALYSIS OF FATTY ACID MIXTURE

x_0 = true % composition; $\bar{x}_M$ = observed % composition using mass detector; $\bar{x}_D$ = observed % composition using gas density balance.

Compound	*Observed molecular weight*	*Monomer mol. wt.*	x_0	$\bar{x}_M$	$\bar{x}_D$
Water (standard)	18.0	18.0	—	—	—
Formic acid	54.8	46.0	—	—	—
Formic acid	54.8	46.0	62.04	60.79	56.74
Acetic acid	82.2	60.0	37.96	39.21	43.26

weight of the components of the mixture must be known. The analysis of mixtures containing free fatty acids cannot be carried out using correction factors based on simple molecular weights, since the lower members of the series dimerise. The degree of dimerisation is dependent on temperature and pressure, so that the correction factors will depend on the conditions under which the analysis is carried out. The molecular weight of formic acid was estimated using water as a standard, and under the same conditions the percentage composition of a formic acid–acetic acid mixture was calculated. The results are given in Table IV.

TABLE V

MOLECULAR WEIGHT DETERMINATIONS (GAS DENSITY DETECTOR)

Compound		*True molecular weight*	*Mean detected molecular weight*				
			1	*2*	*3*	*4*	*5*
Cyclohexane	1	84.2	—	82.9	86.7	105.6	
Dichloroethylene	2	97.0	98.9	—	102.7	132.9	
n-Octane	3	114.2	109.3	106.9	—	154.8	
Carbon tetrachloride	4	153.8	108.9	106.5	113.6	—	
Benzene		78.1	—	78.9	79.2		
Toluene		92.1	91.0	—	92.5		
Ethyl benzene		106.2	103.7	105.1	—		
Methyl ethyl ketone		72.1	—	77.0	72.6		
Methyl *n*-propyl ketone		86.1	79.8	—	80.5		
Methyl *n*-butyl ketone		100.2	99.1	110.4	—		
Methyl ethyl ketone		72.1	—	71.2	65.7	68.1	69.1
Benzene		78.1	79.3	—	71.2	74.3	75.6
Ethyl acetate		88.1	102.7	100.5	—	93.5	75.8
n-Heptane		100.2	111.3	108.7	94.0	—	103.0
n-Octane		114.2	125.0	121.6	102.9	110.8	—
Ethyl acetate		88.1	—	93.8	90.5		
n-Propyl acetate		102.1	95.3	—	98.2		
n-Butyl acetate		116.2	111.9	122.0	—		
Ethyl acetate		88.1	—	82.1	79.8		
n-Propyl acetate		102.1	112.8	—	98.3		
n-Butyl acetate		116.2	138.6	122.0	—		
Butylene oxide		72.1	—	63.4	66.1		
Dioxan		88.1	110.8	—	94.8		
n-Octane		114.2	140.4	104.1	—		

TABLE VI

COLUMN DETAILS

Reference	*Stationary phase*		*Inert support*		*Length (m)*	*I.D. (mm)*
	Type	%	*Type*	*B.S. Mesh*		
D	Porapak Q	—	—	100–120	0.56	3
E	PEGA	20	Chromosorb G	72–85	4	4
H	ApL	20	Chromosorb G	72–85	2	4

The mass detector gives excellent quantitative results, but the gas density balance results are only fair.

An advantage of the determination of molecular weights by gas chromatography is that pure isolated materials are not required. The analysis of multi-component mixtures represents a more realistic situation than the analysis of a two component mixture in which one material is the added standard. For the multi-component mixtures listed in Table V each component in turn was taken as the standard, and the mean molecular weight of all the remaining constituents calculated. Thus for an n-component mixture, there will be n standards and $(n-1)$ values for the mean molecular weight of each component. It is not valid to calculate the mean of the $(n-1)$ molecular weights, to give a single value, since the different standards used to calculate the values all have different molecular weights themselves, and hence fall on different parts of the curve shown in Fig. 1.

The variation of bias with molecular weight is shown in Fig. 2. Details of the columns used in this work are given in Table VI.

CONCLUSIONS

By operating the gas density balance and mass detector in series, satisfactory molecular weight values may be obtained for materials within a given molecular weight range. The range is determined by the molecular weight of the carrier gas. Using nitrogen as carrier, satisfactory molecular weights were obtained over the range 18 to 100, for both two-component mixtures and multi-component mixtures.

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CHROM. 4278

TRACE DETERMINATION OF PHENOLS BY GAS CHROMATOGRAPHY AS THEIR 2,4-DINITROPHENYL ETHERS*

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SUMMARY

Dinitrophenylation of phenols by reaction with 1-fluoro-2,4-dinitrobenzene is shown to yield derivatives amenable to gas chromatography and displaying strongly electron-capturing properties. Various methods of preparation are compared.

INTRODUCTION

Water palatability studies and pesticide residue analysis are two areas in which the gas chromatographic determination of trace quantities of phenols is currently attracting much interest. Free phenols can be determined directly by gas chromatography[1,2], but in general their properties preclude detection at the nanogram level. The usual approach to the development of sensitive phenol detection methods, which we have also followed, has been to prepare derivatives that are amenable to gas chromatography and detectable at the nanogram level with either electron-capture, flame-thermionic or flame-photometric detectors.

Phosphorylation of phenols by reaction with dimethyl phosphorochloridothionate has been used to prepare derivatives amenable to detection with the flame-thermionic or flame-photometric detectors[3]. In our experience, however, removal of excess reagent is difficult and the yield of the phosphorylated product is low when microgram quantities of phenols are reacted in solution, particularly in the case of the less acidic phenols.

In preparing electron-capturing derivatives of phenols, esters such as trifluoroacetates[4] and chloroacetates[5], and ethers formed by reaction with the reagent α-bromo-2,3,4,5,6-pentafluorotoluene[6] have proved useful. In a recent paper[7] the use of 1-fluoro-2,4-dinitrobenzene for the preparation of strongly electron-capturing derivatives of aromatic amines was described. In this present study, the same reagent was used to prepare the dinitrophenyl ether derivatives of the phenols. These derivatives display useful gas chromatographic and electron-capturing properties. A comparative study of techniques for preparing these phenol derivatives is also described.

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EXPERIMENTAL

Preparation and gas chromatography of 2,4-dinitrophenyl ethers

2,4-Dinitrophenyl ethers of some 40 phenols were prepared in milligram amounts using the procedure of REINHEIMER *et al.*[8]. 5 μl quantities of solutions containing about 5 ng of derivative in acetone were injected on to a GE XE-60 chromatographic column described previously[7], with electron-capture detection. Sub-dilutions were also injected to determine the detection limits for the various compounds.

Derivative formation with microgram quantities of phenols

The following three procedures were used to prepare derivatives from 10 μg quantities of phenols.

(i) 4 ml of acetone containing the phenol(s), 0.1 ml of a saturated sodium methoxide solution in methanol and 1 ml of 1-fluoro-2,4-dinitrobenzene solution (1 % w/v in acetone) were pipetted into a 10 ml pear-shaped flask and refluxed for 30 min. The solution was added to 25 ml of an aqueous sodium hydroxide solution (2.5 % w/v), washed in with a little water, and the mixture extracted with 25 ml of chloroform. The chloroform extract was dried with anhydrous sodium sulphate and evaporated to low volume[8]. The residue was then dissolved in acetone and a 5 μl aliquot injected on to the gas chromatographic column for comparison with a series of standards.

(ii) 25 ml of water containing the phenol(s), 0.1 ml of a saturated sodium methoxide solution in methanol and 1 ml of a 1-fluoro-2,4-dinitrobenzene solution (1 % w/v in acetone) were shaken together in a separator for 2 min. 25 ml of sodium hydroxide solution (2.5 % w/v) and 10 ml of *n*-hexane were added and the solution was shaken for 1 min. The *n*-hexane layer was separated and dried by passage through a short column of anhydrous granular sodium sulphate and a 5 μl aliquot examined by gas chromatography.

(iii) An acetone solution containing the phenol(s) was spotted on to a thin-layer chromatoplate coated with a 250 μm thick layer of one of the following adsorbents (Merck): Kieselguhr G, Silica Gel G or Aluminium Oxide G, or alternatively on one of the following papers (Whatman): filter paper No. 541, silica gel loaded paper S.G. 81, aluminium hydroxide loaded paper A.H. 81 or glass fibre paper G.F. 81. The layer or paper was then sprayed with a saturated solution of sodium methoxide in methanol (7 ml was required to spray 400 sq. cm to the required level) followed by a similar quantity of a solution of 1-fluoro-2,4-dinitrobenzene (4 % w/v in acetone). The layer or paper was sandwiched between two glass plates clipped tightly together and was heated in an oven at 190° for 40 min. When cool the layer in the area of interest was removed, either by scraping the layer from the plate in the case of the thin-layer adsorbents or by cutting out the spot area where a paper was used. The layer adsorbents were transferred to short columns plugged with cotton wool and the derivatives eluted with two 5 ml portions of acetone. The papers were directly added to tubes containing 10 ml of acetone together with two drops of water with resultant extraction of the derivatives. The yield of derivative from each sample was determined by injecting aliquots of the final solutions on to the gas chromatographic column for comparison with standards.

RESULTS

Table I shows the retention time data and sensitivity to electron-capture detection of the 2,4-dinitrophenyl ethers on a Silicone GE XE-60 column. Linear calibration curves were obtained over the range of 1–10 ng. Table II shows the yields of derivatives obtained by the various preparative procedures; these yields are expressed as percentages of the theoretical yield assuming 10 μg of phenol to react completely to form the corresponding ether. pK_a values (in aqueous solution) for the various phenols examined, were obtained from literature sources and are included in Table II.

DISCUSSION

The elution sequence of 2,4-dinitrophenyl ethers (see Table I) is influenced by the nature of the phenolic portion of the molecule and, under the conditions specified, spans a sufficiently wide time range to be of value in phenol characterisation. The *ortho, meta, para* sequence of elution—the "*ortho* effect"—normally associated with the gas chromatography of free isomeric phenols is also displayed by their 2,4-dinitrophenyl ethers although the separation of the isomeric ethers is poorer than that displayed by the corresponding phenols. The electron-capturing properties of the derivatives do not appear to arise from the phenolic portion of these molecules but are associated with the presence of the aromatic nitro groups. The apparent differences in sensitivity (see Table I) arise as a result of peak broadening associated with increasing retention time.

The electron-capturing properties of 2,4-dinitrophenyl ethers compare favourably with phenol derivatives prepared by reaction with chloroacetic anhydride[5] or α-bromo-2,3,4,5,6-pentafluorotoluene[6]. The elution sequence, in terms of the parent phenols, is different with each of the three derivatives and the 2,4-dinitrophenyl ethers apparently give better separations than the other two groups of compounds. Because of the relatively low volatility of the 2,4-dinitrophenyl ethers, it was necessary to operate the columns at temperatures close to the maximum permissible with electron-capture detectors using tritium as the radioactive source. The absence of any signs of decomposition and the known stability of ethers would indicate that separations at higher temperatures would be possible provided a suitable electron-capture detector were used (*e.g.* one based on ^{63}Ni). This probably opens up the prospect of gas chromatography of the dinitrophenyl derivatives of polyhydric phenols which are known to be formed readily[8], but were not examined in this work as it was assumed that their retention times would be excessive at 215°.

The results in Table II indicated that the nature of the phenol, together with the procedure used for derivative formation greatly influenced the yield of 2,4-dinitrophenyl ether. Under reflux conditions, with acetone as the solvent, increasing yield was obtained as the acid strength of the phenol increased, whereas a reaction in cold aqueous conditions resulted in a reversal of this behaviour. Under the forcing conditions encountered with the "sandwiched layer" technique, higher yields were generally obtained, with the exception of reactions carried out on an alumina thin layer. The more acidic phenols in general gave the highest yields of derivative but the support exerted a marked influence on the reaction. The most useful support material for

TABLE I

RETENTION TIME DATA AND ELECTRON-CAPTURE SENSITIVITY OF 2,4-DINITROPHENYL DERIVATIVES OF PHENOLS

Conditions: 1% GE XE-60 and 0.1% Epikote 1001 on Chromosorb G, acid-washed, dimethylchlorosilane coated, 60–80 mesh. Temperature: 215°. Column material: glass 140 cm in length, 1.5 mm I.D.

Parent phenol	*Retention time relative to 1-naphthol derivative*	*Sensitivity*[a] *to electron-capture detection* ($g \times 10^{-9}$)	*Parent phenol*	*Retention time relative to 1-naphthol derivative*	*Sensitivity* ($g \times 10^{-9}$)
Phenol	16	0.10	4-*tert.*-Butylphenol	38	0.10
o-Cresol	17	0.05	4-Allylphenol	38	0.1
4-Fluorophenol	19	0.10	4-*sec.*-Butylphenol	40	0.10
m-Cresol	20	0.05	4-*tert.*-Pentylphenol	50	0.10
2,5-Xylenol	20	0.05	4-Bromophenol	56	0.10
p-Cresol	21	0.05	2,4-Dichlorophenol	58	0.10
Thymol	23	0.05	Eugenol	62	0.10
2-Isopropoxyphenol	23	0.05	4-Iodophenol	92	0.10
3,5-Xylenol	23	0.05	3,5-Dimethyl-4-methylthiophenol	94	0.10
4-Ethylphenol	28	0.05	4-*tert.*-Octylphenol	95	0.20
2-Methoxyphenol	29	0.05	1-Naphthol	100 (8.2 min)	0.20
3-Ethyl-5-methylphenol	29	0.05	2,4,5-Trichlorophenol	102	0.20
2-Chlorophenol	29	0.05	2-Naphthol	141	0.20
4-Isopropylphenol	31	0.10	4-Cyclohexylphenol	153	0.30
3,4-Xylenol	31	0.10	2-Nitrophenol	168	0.50
3-Chlorophenol	33	0.10	3-Nitrophenol	178	0.50
3,5-Di-*tert.*-butylphenol	36	0.10	4-Nitrophenol	238	0.50
4-Chlorophenol	38	0.10	4-Benzylphenol	285	0.40

[a] Sensitivity expressed as the weight of derivative producing a peak with height equivalent to 10% full-scale deflection at an amplification producing a noise level of 5% f.s.d.

TABLE II

COMPARATIVE STUDY OF PROCEDURES FOR 2,4-DINITROPHENYL ETHER FORMATION

Procedures: (1) Refluxing in acetone solvent; (2) cold aqueous reaction; (3) sandwiched layer reaction, chromatoplate coated with Kieselguhr G; (4) sandwiched layer reaction, chromatoplate coated with Silica Gel G; (5) sandwiched layer reaction, chromatoplate coated with Aluminium Oxide G; (6) sandwiched layer reaction, filter paper No. 541; (7) sandwiched layer reaction, silica gel loaded paper SG 81; (8) sandwiched layer reaction, aluminium hydroxide loaded paper AH 81; (9) sandwiched layer reaction, glass fibre paper GF 81.

Compound	pK_a	% *Yield (average of six determinations)*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Phenol	10.0	6	15	4	27	0	40	51	35	1
4-Fluorophenol	9.88	5	25	10	34	0	43	46	41	6
4-Chlorophenol	9.42	23	20	41	57	0	65	69	64	7
4-Bromophenol	9.34	38	19	62	52	0	64	63	59	19
4-Iodophenol	9.10	61	20	91	62	0	72	76	76	44
2,4-Dichlorophenol	7.82	34	0	32	46	0	44	57	54	0
4-Nitrophenol	7.20	54	0	100	51	0	27	100	66	80

general screening was the paper loaded with silica gel which gave high yields with the minimum dependence on the acidity of the phenol.

The "sandwiched layer" reaction can be used to prepare many derivatives simultaneously – a 20 × 20 cm plate can be spotted with fifty or more samples and after reaction each derivative may be eluted separately. Another advantage of this type of reaction is that only a relatively small amount of the reagent is present on a single spot and the problem of removal of excess reagent encountered frequently with other types of preparation does not usually arise.

The methods described are being applied in this laboratory to the detection and determination of residues of certain pesticides, such as carbamates, which yield phenols on hydrolysis.

ACKNOWLEDGEMENTS

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CHROM. 4257

PHYTANIC ACID L-MENTHYL ESTERS

GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF DIASTEREOISOMERS

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SUMMARY

L-Menthyl esters of a naturally occurring (marine) phytanic acid, of an all-synthetic phytanic acid, and of a phytanic acid known to be of 3D, 7D, 11D configuration were compared by GLC. As in the pristanates, the LDD and DDD diastereoisomers were the farthest apart, but in the marine phytanate only two diastereoisomers, presumably LDD and DDD, could be detected. A general guideline is proposed that in the GLC of L-menthyl esters of isoprenoid fatty acids with 2-methyl and 3-methyl substituents the LDD and DDD diastereoisomers will be, respectively, the first and last to elute.

INTRODUCTION

The initial demonstration of the separation of diastereoisomers of phytol-derived pristanic (2,6,10,14-tetramethylpentadecanoic) and phytanic (3,7,11,15-tetramethylhexadecanoic) acids by gas–liquid chromatography (GLC) of the methyl esters gave only two GLC peaks in each case[1]. It was inferred that if one peak in each case coincided with the corresponding ester of the authentic "all-D" isomer prepared from a bacterial (*Halobacterium cutirubrum*) lipid[2], then the other peak should be the other diastereoisomer (respectively, 2L,6D,10D-pristanate and 3L,7D,11D-phytanate) formed through the reduction of the double bond in phytol (3,7D,11D,15-tetramethyl-2-hexadecen-1-ol). Subsequently, a discrepancy was discovered between optical rotation and GLC data for a pristanic acid of marine origin. This led to an examination by GLC of the diastereoisomers of various samples of pristanic acids as their L-menthyl

esters, and the demonstration of the occurrence in the marine pristanic acid of lesser amounts of two new diastereoisomers in addition to those of the LDD and DDD configuration[3]. It was also shown that these particular diastereoisomers were essentially absent from two samples of pristanic acids isolated from ruminant fats; the latter were therefore considered to be closely linked to a direct origin from phytol.

The new diastereoisomers were believed to be the DLL and LLL diastereoisomers formed by oxidation of pristane (2,6,10,14-tetramethylpentadecane) in the marine lipid food web. This process could not occur with the phytol-derived hydrocarbon phytane (3,7,11,15-tetramethylhexadecane) since terminal oxidation either regenerates the common diastereoisomers of phytanic acid, or fatty acids of completely new and different structures. Only minor amounts of phytane occur, relative to pristane, in some marine organisms[4,5], but higher proportions are reported in others[6]. Phytane is well-known as a component of petroleums[7–9] but not necessarily of petroleum precursor systems[10]. Small amounts are also found in terrestrial animal lipids[6,11]. However, phytenic acids with double bonds in other than the 2-position are known to occur in biological systems[12], and various C_{20} phytol-derived hydrocarbons with one or more double bonds in various positions have been reported as components of marine lipids[13]. Instead of terminal oxidation, the migration of a double bond along the chain might cause racemization of the asymmetric centers originally present in phytol (7D and 11D)[14] and thus produce other diastereoisomers in addition to the expected LLD and DDD forms. It was therefore considered worthwhile examining the L-menthyl esters of the phytanic acid described above[15] for the presence of these other diastereoisomers.

EXPERIMENTAL

The isolation of the marine phytanic acid has been described in detail[15], as has the total synthesis of ethyl phytenate[16] from which all-synthetic phytanic acid was prepared by hydrogenation and saponification. Farnesanoic (3,7,11-trimethyldodecanoic) acid containing four diastereoisomers was prepared from farnesol by procedures similar to those described elsewhere[15]. L-Menthyl esters were prepared by reacting the acid chlorides of the acids (generally prepared by refluxing with freshly distilled $SOCl_2$) with L-menthol in the presence of pyridine according to recognized procedures, strict attention being paid to anhydrous conditions. Gas–liquid chromatography was carried out as described elsewhere[17] with high-efficiency open-tubular columns coated with butanediol-succinate polyester.

RESULTS AND DISCUSSIONS

The methyl esters of the marine pristanic and phytanic acids, analysed on a system of two columnscoupled in series and capable of giving 80,000 theoretical plates, are partially resolved into two components as shown in Figs. 1 and 2. Analysis of these marine samples on a single column of half this efficiency has been illustrated elsewhere[18]. Analyses of the corresponding L-menthyl esters (single columns) are also shown in Figs. 1 and 2, including the results of coinjection of reference authentic all-DDD L-menthyl phytanate with the marine L-menthyl phytanate esters.

These results suggest that in the marine phytanic acid there are effectively only

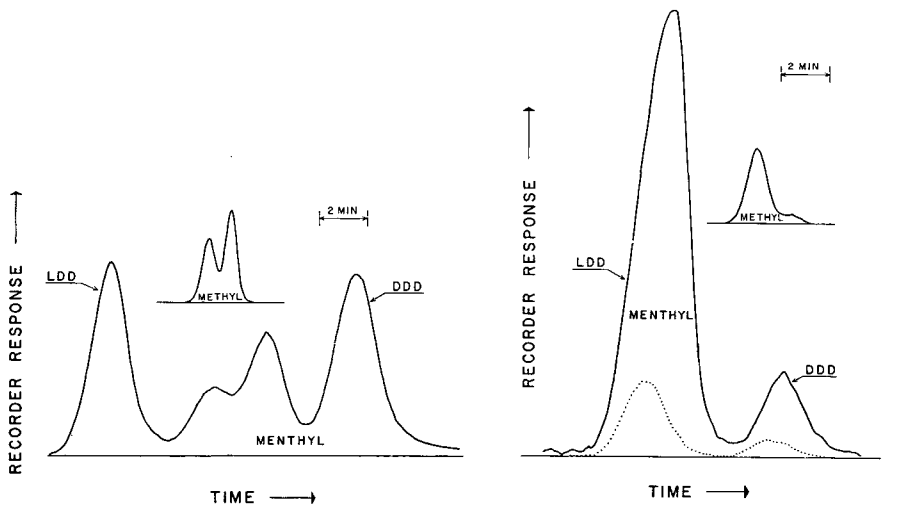

Fig. 1. Comparison of GLC analyses of methyl esters of marine pristanic acid (insert; analysis on coupled columns of approximately 90,000 theoretical plates) with menthyl esters (bottom, analysed on a single column of approximately 40,000 theoretical plates).

Fig. 2. Comparison of methyl (insert) and menthyl (solid line) esters of marine phytanic acid. GLC details as in Fig. 1. Dotted line shows peak area ratio when a small amount of authentic menthyl DDD phytanate was added to sample of marine origin.

two diastereoisomers present, the LDD predominating. The area percentages (Disc Instruments integrator) from the GLC analyses are:

	Methyl	Menthyl
LDD	89	89.2
DDD	11	10.8

The optical rotation measured for this sample (as the methyl ester) was $[\alpha]_D^{25} = -3.58°$. Using the observed $[\alpha]_D^{25}$ values of $+3.78°$ for the DDD diastereoisomer and $-0.60°$ for phytanate derived from phytol (an equal mixture of LDD and DDD respectively[2, 19 20], a value of $-4.98°$ may be calculated for the LDD diastereoisomer. From these values of $[\alpha]_D^{25}$ for the LDD and DDD diastereoisomers it may then be calculated that the observed rotation of $-3.58°$ would result from a mixture of 84 % LDD and 16 % DDD diastereoisomers; these calculated proportions may thus be taken as a reasonable confirmation of the GLC area results. The resolution of the LDD and DDD menthyl phytanates is generally inferior to that of the corresponding pristanates or to that which might be obtained on coupled columns, and this analysis might not disclose other phytanate diastereoisomers at a level of 2–3 % or less even should these occur between the LDD and DDD peaks. Previous results for diastereoisomer ratios in marine and terrestrial phytanates, and terrestrial pristanates, based on methyl esters[1, 17, 18, 21] are in all probability correct, but published pristanate ratios for samples of marine origin[1, 18] may be suspect.

The purely synthetic phytanic acid, previously examined by GLC as methyl

and ethyl esters[20], was also converted to L-menthyl esters. As shown in Fig. 3 this material gives a triplet peak system in ratios of 1 : 2 : 1, with the LDD and DDD diastereoisomers of marine origin coinciding with the first and last peaks. The shape of this triplet peak is similar in all of the known methyl, ethyl, and L-menthyl ester forms for purely synthetic acids, and very similar in aggregate to the composite quartet of peaks observed for synthetic farnesanoic (3,7,11-trimethyldodecanoic) acid in the form of the L-menthyl ester[17].

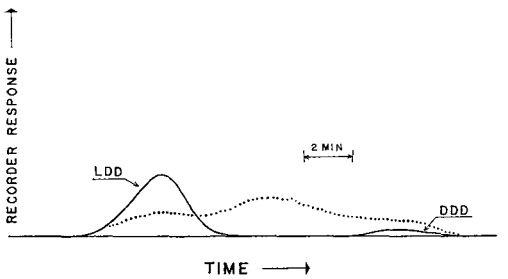

Fig. 3. Comparison of GLC analyses of menthyl esters of all-synthetic phytanic acid (dotted line) and the phytanic acid of marine origin. Single GLC column.

In Table I are listed three synthetic isoprenoid fatty acids (which are racemic at each appropriate methyl branch), the types of esters which have been examined by GLC, and the elution pattern observed on GLC. In the methyl esters enantiomeric pairs are present, but these become diastereoisomers when converted to L-menthyl esters. Pristanic acid of marine origin is included since four components of the eight are known. It is apparent that there is a possible extension to the L-menthyl esters of the guideline that optical isomers of isoprenoid fatty acids having the same configuration at all methyl-branched carbons will elute last when run as methyl esters by GLC[20]. In the L-menthyl esters it is possible that the "all-D" configuration will definitely elute last, whereas in the methyl esters this guideline always included the enantiomeric "all-L" configuration. Not unexpectedly, in the four L-menthyl esters of pristanic acid (incomplete), and eight of phytanic acid (complete), the LDD diastereoisomer is known to elute first, since it is likely that the proximity of the first methyl branch removed from the carboxyl group gives it maximal interaction with the L-menthyl moiety. This also could be a general guideline. Such relationships have been studied for other ester systems[22,23].

This view can be extended to argue that the modification of the volatility or polarity by the L-menthyl alcohol which confers diastereoisomeric properties to the enantiomeric isoprenoid acids will not be exact, but that subtle interactions will alter the nominally additive properties as tabulated for multiple-branched acids of another series[20]. The resolution of the L-menthyl ester of synthetic 3,7,11-trimethyldodecanoate into four components by GLC indicates that a complete investigation of such effects through synthesis of the simpler related materials with only one asymmetric center in the fatty acid, and of the specific diastereoisomers with two asymmetric centers in the fatty acids, would be technically feasible, although a challenging problem. Extension of this to the materials with three asymmetric centers in the fatty

TABLE I

SUMMARY OF COMPARATIVE SEPARATIONS OF DIASTEREOISOMERS OF ISOPRENOID FATTY ACIDS, AS METHYL AND L-MENTHYL ESTERS, WITH PARTICULAR REFERENCE TO THE ORDER OF ELUTION OF CERTAIN DIASTEREOISOMERS WITH OPPOSITE CONFIGURATIONS AT THE FIRST METHYL SUBSTITUENT

Fatty acid and sources[a]	*Methyl ester diastereoisomers*				*L-Menthyl ester diastereoisomers*			
	Number expected	*Observed format*	*Postion of*		*Number expected*	*Observed format*	*Postion of*	
			LD(D)	*DD(D)*			*LD(D)*	*DD(D)*
2,6,10-Trimethylundecanoic, (probable), synthetic and natural	2	—	—	—	4	1:2:1 triplet	?	last
3,7,11-Trimethyldodecanoic, synthetic and natural	2	1:1 doublet	first	last	4	1:2:1 triplet[b]	?	last
4,8,12-Trimethyltridecanoic, synthetic and natural	2	1:1 doublet	first	last	4	—	—	—
2,6,10,14-Tetramethylpentadecanoic, natural	4	1:1 doublet	in first peak	in last peak	8	four peaks	first	last
3,7,11,15-Tetramethylhexadecanoic, synthetic and natural	4	1:2:1 triplet	second	last	8	1:2:1 triplet	in first peak	in last peak

[a] Natural includes chemical preparations from phytol and *H. cutirubrum*.
[b] Center peak partially resolved[17].

acid chain would be correspondingly more difficult, but the separations achieved for the natural marine L-menthyl pristanates suggest that the pristanates would be more promising than the phytanates as materials for study.

Exact GLC retention data is not relevant since there were minor variations in operating conditions (except in the instances of GLC runs shown in figures as superimposed); ECL values for L-menthyl esters (relative to methyl esters of normal aliphatic acids) also varied for a given column with age. Typical data have been listed elsewhere[17] for most esters, but comparative ECL values of the four L-menthyl pristanates were 22.35, 22.43, 22.44 and 22.51 on a 150 ft. (50 m) BDS column operated at 150° and 40 p.s.i.g. helium, while the LDD and DDD L-menthyl phytanates had ECL values of 23.50 and 23.65 under different conditions of 170° and 50 p.s.i.g. on another BDS column. The prolonged retention times for L-menthyl esters preclude practical analysis on two high-efficiency columns coupled in series.

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GAS CHROMATOGRAPHIC ANALYSIS OF AMINES IN VOLATILE SUBSTANCES OF *STREPTOCOCCUS LACTIS**

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SUMMARY

The qualitative composition and quantitative relationships of amines in the volatile components of 6-, 24-, and 30-h cultures of a strain of *Streptococcus lactis*, in the logarithmic growth phase, was studied chromatographically using retention indices.

The 21 amines found were identified as follows: primary amines—methylamine, ethylamine, isopropylamine, butylamine, isobutylamine, *tert.*-butylamine, amylamine, isoamylamine; secondary amines—dimethylamine, diethylamine, dipropylamine, diisopropylamine, diisobutylamine, *n*-butylisopropylamine, pyrrolidine, piperidine; tertiary amines—trimethylamine, triethylamine, tripropylamine. The qualitative composition of amines remained constant as the culture grew, but a sharp change in the quantities of the individual amines in the 6-, 24-, and 30-h cultures was observed. This pointed to the formation of amines during the metabolism of the bacterial cells and suggested the existence of special fermentative systems involved in the synthetic production of amines. Thus a fundamentally new phenomenon that of synthesis of secondary and tertiary amines by microorganisms has been discovered.

INTRODUCTION

It is generally agreed that aliphatic amines are formed on decarboxylation of amino acids[1–3]. This is true, however, only for primary amines, whereas secondary and tertiary amines can be formed only through alkylation processes that are as yet unknown with the exception of methylation. We have previously shown[4–8] that the volatile components of food products contain a great number of organic bases many of which were rather unexpectedly found to be secondary and tertiary aliphatic amines.

It was thus considered of interest to determine whether the formation of these amines was due to vital functions or was the result of disintegration of decayed organisms. A study of the composition and kinetics of amine accumulation in the volatile components of quickly growing organisms was therefore undertaken; the

* Translated by A. L. PUMPIANSKY, Moscow.

strain of *Str. lactis* being chosen for the investigation. The organic bases were identified by gas chromatography by a procedure previously described[9,10].

MATERIAL AND METHODS

The strain of *Str. lactis* was maintained on wort agar slants at 37°. Cultivation took place in twenty-four 2 l conical flasks each containing 400 ml of sterilised skim milk, used as nutrient, inoculated with two agar slants of microorganisms.

At intervals of 6-, 24-, and 30- h (logarithmic phase of growth), fermentation was stopped by means of a 5 % solution of mercuric chloride (5 ml per 400 ml of culture medium). The 24- and 30-h portions were centrifuged and precipitated to yield 535 and 545 g, respectively. The 6-h portion looked like milk and 500 ml of it was investigated without centrifugation. The volatile compounds were isolated *in vacuo* as previously described at 30–33° under 3–4 mm pressure[4]. With the 6-h culture, the volatile compounds were trapped under 15 mm pressure. The compounds removed, together with water vapour, were condensed in three traps containing a 10 % solution of trichloroacetic acid in absolute ethanol. The traps were cooled with dry-ice and acetone. The volatile components of the 24- and 30-h cultures were allowed to distil over for 24 h, those of the 6-h specimen for 8 h. Special investigation showed it not expedient to prolong the time of isolation. After distillation under vacuum the amount remaining was 250, 152, and 159 g for the 6-, 24-, and 30-h cultures, respectively.

After each run the contents of the traps were combined and vapour-distilled under nitrogen to remove alcohol, neutral and acidic compounds. The distillate (about 1 l) was discarded, the contents of the traps treated with KOH solution up to pH 10, the organic bases were distilled under nitrogen and were absorbed by 0.2 *N* HCl. The hydrochloric solution (*ca.* 1 l) was evaporated to dryness at 30–35° in a rotary evaporator and the remaining hydrochloric salts were dried under vacuum to yield 6.7, 10.0 and 10.6 mg of 6-, 24-, and 30-h specimens, respectively. The free organic bases were analysed by gas chromatography, each sample consisting of 4.5 mg of hydrochloric amine salts, 6 μl of distilled water, 50 μl of chromatographically pure dodecane and a small piece of solid KOH[11].

Analyses of the amine solutions in dodecane were made at 100° with a gas chromatograph equipped with a flame ionisation detector and nitrogen flow rate of 20–60 ml/min using four 1.5 m × 4 mm glass columns packed with the detergent 'Novator'[9] coated with liquid phases of various polarity: 10 % of tristearin; 5 % of liquid paraffin with 2 % of KOH, 10 % of Tween-80 and 10 % of polyethylene glycol 1000.

The sample (5 μl) was injected into the column with a Hamilton syringe (10 μl). Gas chromatographic parameters were computed in terms of retention indices[12].

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained on a column with liquid paraffin for amine mixtures isolated from the volatile components formed during the logarithmic phase of growth of *Str. lactis* for 6-, 24-, and 30-h. Comparative data demonstrate that the volatile components of these bacteria contain a large amount of organic bases. It is also quite evident that their proportions change with time (compare peaks 4, 12, and 17).

The amine content was estimated in terms of the peak area calculated according to the formula $S = ht$, where h is the height of the peak and t the retention time.

In the absence of absolute calibration this method fails to determine the true content of every amine in the mixture, but it can be used to follow changes in concentration of a particular amine in the sample under examination and, in our case, to obtain kinetic evidence concerning the character of the change in the composition of the amines as the *Str. lactis* grows.

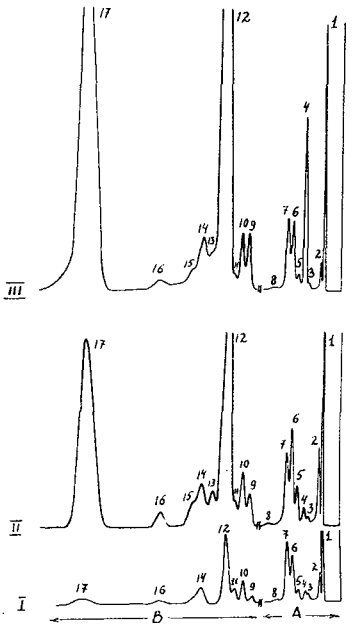

Fig. 1. Chromatogram of amines found in the volatile products resulting from the metabolism of *Str. lactis* on the 150 × 0.4 cm column packed with detergent 'Novator'; liquid phase: 5% liquid paraffin with 2% KOH. Temperature: 100°; nitrogen flow rate: 26 ml/min (section A) and 55 ml/min (section B). I = the 6-h culture; II = the 24-h culture; III = the 30-h culture. 1 = trimethylamine; 2 = dimethylamine + methylamine; 3 = ethylamine; 4 = diethylamine + isopropylamine; 5 = not identified; 6 = diisopropylamine; 7 = triethylamine, 8 = not identified; 9 = isobutylamine; 10 = dipropylamine; 11 = butylamine; 12 = not identified + pyrrolidine, 13 = isoamylamine; 14 = diisobutylamine + piperidine; 15 = amylamine; 16 = tripropylamine; 17 = not identified.

Table I lists the amines identified in the volatile compounds of these bacteria after 6-, 24-, and 30-h (logarithmic growth phase) as well as the change in their composition in the mixture during the above periods of time.

It will be seen from Table I that the mixture contains 21 amines. However, their actual number might prove to be much higher because the analytical conditions used permitted the chromatograms to register only those amines whose retention index did not exceed 1200 units. This is the index of dodecane that was used as a solvent for the introduction of the amines into the chromatograph.

Chromatograms were analysed by the method already described involving the equations derived for correlating the retention index with the number of carbon

TABLE I

CONCENTRATION CHANGES OF THE AMINES IN THE VOLATILE COMPOUNDS OF *Str. lactis*
Amine concentration is given as relative percentage.

No.	*Amines*	*Culture growth period (hours)*		
		6	*24*	*30*
Primary				
1	Methylamine	5.2	0.7	0.1
2	Ethylamine	0.3	0.1	0.1
3	Isopropylamine	traces	0.2	0.2
4	Butylamine	1.5	0.2	0.1
5	Isobutylamine	0.8	0.7	0.7
6	Amylamine	—	0.4	0.4
7	Isoamylamine	0.7	0.7	0.1
8	Aliphatic amine	1.9	1.3	0.2
Secondary				
9	Dimethylamine	0.1	1.2	0.1
10	Diethylamine	2.5	0.5	3.6
11	Dipropylamine	4.0	1.2	0.7
12	Diisopropylamine	9.4	2.8	1.3
13	Diisobutylamine	—	1.2	0.6
14	Aliphatic amine	15.9	50.6	56.1
15	Pyrrolidine	2.9	0.4	0.1
16	Piperidine	5.6	traces	0.1
17	Not identified	0.2	0.5	traces
Tertiary				
18	Trimethylamine	25.7	7.2	2.8
19	Triethylamine	18.3	2.0	1.7
20	Tripropylamine	0.7	1.3	0.3
21	Aliphatic amine	4.1	27.0	30.9

atoms and the boiling point of the substance[9,10]. This method led to the identification of seventeen of the organic bases of the 21 amines listed in Table I. Regularities, previously discovered, made it possible to determine the character of the functional group and to estimate the boiling point of three of the unidentified amines (Table I, Nos. 8, 14, 21; Fig. 1, peaks 5, 12, 17, respectively).

This was done by determining the value of ΔI that is specific and rather constant for primary, secondary, and tertiary amino groups. The data used for the identification of these three amines are given in Table II. The values of ΔI presented in Table II characterise No. 8 as a primary, No. 14 as a secondary, and No. 21 as a tertiary aliphatic amine. Knowing the character of amine it is possible to estimate its boiling point from corresponding equations for primary, secondary, and tertiary amines. These equations hold for aliphatic amines with a normal or isocarbon chain.

Table II (column 8) shows the calculated boiling points.

The data on the character of the carbon chain of the amines under investigation were obtained by equations correlating the index value with the number of carbon atoms in the molecule derived by us for *n*-aliphatic primary, symmetrical secondary and tertiary amines. The experimental values for the retention indices of the amines being investigated were substituted into these equations. The deficit in the number of carbon atoms points to branched carbon radicals.

TABLE II

DATA USED TO DECIPHER UNIDENTIFIED AMINES

I^{TS}_{100}, $I^{L.P.}_{100}$, I^{TW}_{100}, I^{PEG}_{100} = The indices of the amines at the temperature of 100° on columns with tristearin,

No. of amine in Table I and standards	I^{TS}_{100}	$I^{L.P.}_{100}$	I^{TW}_{100}	I^{PEG}_{100}	$\Delta I^{L.P.-TS}$	ΔI^{PEG-TW}
8-Unidentified	541	645	611	784	104	173
tert.-Butyl amine	550	647	598	780	97	182
14-Unidentified	822	822	909	966	0	57
Isopropyl-*n*-butylamine	821	820	904	963	−1	59
n-Propyl-isobutylamine	823	819	897	952	−4	55
21-Unidentified	984	981	1007	1025	−3	17

It can be deduced from Table II that the three amines all contain isocarbon radicals. It was also suggested that amine No. 8 could be *tert.*-butylamine, as the retention index values for isopropyl- and isobutylamines are different[10]. To prove this suggestion a study was undertaken of the behaviour of a standard of *tert.*-butylamine in all the liquid phases; the values of the retention indices are given in Table II. The agreement between the values of indices and boiling points permitted amine No. 8 to be identified as *tert.*-butylamine.

Amine No. 14 was identified structurally by referring to the calculated retention indices of some isomers with seven atoms. It was found in this way that the closest values to that of amine No. 14 were those of *n*-butylisopropylamine and *n*-propyl-isobutylamine. The two amines were synthesised and their indices were measured on the four columns. The retention values and boiling points showed amine No. 14 to be *n*-butylisopropylamine. The tertiary amine No. 21, boiling at 166° and having ten carbon atoms might have one or several isocarbon chains. It was not possible to identify this amine because it has many isomers and, according to literature, at least four of them boil at about 166° (ref. 13).

It is evident from Table I and chromatograms on Fig. 1 that the qualitative composition of organic bases isolated from the volatile products of *Str. lactis* metabolism was constant during the whole period of growth investigated. On the other hand, a sharp change in the quantitative relationships was observed over the same period. It was of interest to follow these changes during the growth of *Str. lactis*. In the 6-h culture, taken as a reference point, about one-half of the total amines was represented by trimethylamine and triethylamine; in the 30-h culture their percentage was drastically lowered (almost by ten times) with a corresponding increase of *n*-butylisopropylamine and a tertiary amine with 10 carbon atoms whose total percentage rose up to 87 (Table I).

It is of interest compare our results with the data reported by WEURMAN[14] on the composition of volatile amines in milk. Using paper chromatography he found

liquid paraffin, Tween-80 and polyethylene glycol 1000, respectively.

B.p. of amine calc. according to formula:	*Lit b.p.*	*The number of carbon atoms (n) according to formula:*	*Identity*
$\text{b.p.} = \frac{I^{L.P.}_{100}}{4.0} - 116 = 45$	45	$n = \frac{I^{L.P.}_{100} - 386}{100} = 2.6$	*tert.*-Butylamine
$\text{b.p.} = \frac{I^{L.P.}_{100}}{4.0} - 85 = 121$	124 126	$n = \frac{I^{L.P.}_{100} - 176}{100} = 6.5$	Isopropyl *n*-butylamine
$\text{b.p.} = \frac{I^{L.P.}_{100}}{4.0} - 79 = 166$		$n = \frac{I^{L.P.}_{100} - 146}{86} = 9.7$	Tertiary amine with 10 carbon atoms

methylamine, ethylamine, butylamine, and dimethylamine and suggested the presence of pyrrolidine. Our experiments showed a rapid decrease in the amount of pyrrolidine during the growth of the bacteria. It is therefore thought that the presence of this amine was due to its availability in the milk initially. The data presented in Table I and Fig. 1 cannot be used to draw any conclusions regarding the mechanism of formation of secondary and tertiary amines. It can only be said that the monoalkylamines do not undergo a gradual (stepwise) alkylation to tertiary amines as shown by the differences in, say, the concentrations of diethylamine and triethylamine. The same is observed to be true for isobutylamines and diisobutylamines.

The qualitative composition of the amines isolated from the volatile products of *Str. lactis* metabolism is quite varied involving primary, secondary, and tertiary amines with a normal or isocarbon chain, pyrrolidine and piperidine.

An interesting peculiarity of the amine composition is its relatively high content of various secondary and tertiary amines such as diisopropylamine, diethylamine, diisobutylamine, *n*-butylisopropylamine, and a tertiary amine with ten carbon atoms.

Decarboxylation of free amino acids can account for such primary aliphatic amines as methylamine, ethylamine, isobutylamine, and isoamylamine whose precursors may be, correspondingly, glycine, alanine, valine and leucine. Pyrrolidine may result, similarly, from proline. However, the mechanism of the formation of most of the amines is not clear. The presence of such structurally simple primary amines as *n*-butylamine, *n*-amylamine, isopropylamine and, even more so, of secondary and tertiary amines cannot be explained solely by decarboxylation of amino acids.

The presence of secondary and tertiary amines of varied structure and their increasing percentage during the growth of the lactobacilli proves that these amines result from the metabolism of these bacteria. One might, therefore, suggest the existence of special enzymes involved in the synthesis of these amines.

It is to be noted that the qualitative composition of the amines in volatile

products of *Str. lactis* metabolism differs from that found by us in other food products[4–8].

Both the origin of the amines and the role they play in the vital activity of the organisms are not clear. It could be suggested that the amines provide a peculiar defence for the microorganisms; in which case the qualitative and quantitative composition of the amines in the volatile components of various microorganisms could be specific for every particular species.

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DIRECT ESTERIFICATION OF THE PROTEIN AMINO ACIDS

GAS–LIQUID CHROMATOGRAPHY OF N-TFA *n*-BUTYL ESTERS*

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SUMMARY

The reaction conditions necessary for the "direct esterification" of the protein amino acids to their *n*-butyl esters are described. All of the amino acids were quantitatively esterified in *n*-butanol 3 *N* in hydrochloric acid at 100° with the exception of isoleucine. This "direct esterification" method with *n*-butanol permits a rapid derivatization and analysis by gas–liquid chromatography of the protein amino acids, thus, one of the major disadvantages of the earlier reported method has been removed.

The amino acids were observed to dissolve very slowly in *n*-butanol 6 *N* in hydrochloric acid even when the samples were subjected to ultrasonic mixing. Fairly rapid dissolution occurred in 1.5 *N* hydrochloric acid but a longer esterification time was noted. The optimum concentration of hydrochloric acid was found to be 3 *N* because the amino acids dissolved quickly in this solution with ultrasonic mixing and short esterification times were obtained. The more insoluble amino acids were broken up by ultrasonic mixing, thus increased rates of solution and esterification to the *n*-butyl esters were achieved. The effect of temperature over the range of 90 to 120°, on the rate of esterification was investigated but little effect on the relative molar response values was observed. However, the time of esterification was quite significant for two amino acids, tryptophan and isoleucine. Nineteen of the amino acids were quantitatively esterified with *n*-butanol 3 *N* in hydrochloric acid in 15 min at 100°, but 35 min were required for the esterification of isoleucine. However, with the longer esterification time, tryptophan underwent some decomposition (*ca.* 15%).

An esterification time of 35 min is recommended for samples which are to be analyzed for all of the amino acids including isoleucine and tryptophan; however, only 15 min are required if a slight error in the absolute value for isoleucine is permissible.

In quantitative work, a reference calibration mixture is analyzed under exactly the same experimental conditions as are the samples, thus correction is made for any

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breakdown of tryptophan and the response values of all of the other amino acids are placed on the same basis.

Using the "direct esterification" method, recoveries of amino acids from a mixture averaged 99.7% with an average relative standard deviation of 0.92%. This "direct esterification" method allows a rapid, precise, and accurate derivatization of the protein amino acids to their N-TFA *n*-butyl esters with a minimum of transfers and sample handling. A complete gas–liquid chromatographic analysis of the protein amino acids, including derivatization and chromatography, can be completed in less than 1 h.

INTRODUCTION

Gas–liquid chromatographic (GLC) methods have proved to be most useful for the analysis of amino acids in biological substances because of their speed and sensitivity. Since the low volatilities of the amino acids has prevented their direct analysis by GLC, suitable volatile derivatives of the amino acids must be prepared. In 1962, ZOMZELY *et al.*[1] investigated the N-trifluoroacetyl (N-TFA) *n*-butyl esters as possible derivatives. LAMKIN AND GEHRKE[2] reported in 1965 that the most suitable derivative with respect to volatility and chromatography for the GLC analysis of the natural protein amino acids is the N-TFA *n*-butyl ester. The experimental conditions for quantitative derivatization and chromatographic requirements for separation were detailed by GEHRKE AND STALLING[3] in 1967. This was followed by a recent monograph by GEHRKE *et al.*[4] which presents macro, semimicro, and micro methods, reagents, sample preparation, instrumental and chromatographic requirements, and sample ion-exchange cleanup for the quantitative GLC analysis of the protein amino acids as their N-TFA *n*-butyl esters.

The chromatographic properties of several derivatives of the protein amino acids, in addition to the N-TFA *n*-butyl esters, have also been evaluated. Among the derivatives are the trimethylsilyl derivative introduced by RÜHLMANN AND GIESECKE[5] in 1961 and studied more recently by GEHRKE *et al.*[6,7]; the N-acetyl *n*-amyl esters by JOHNSON *et al.*[8]; and the N-trifluoroacetyl methyl esters by SAROFF AND KARMEN[9], by HAGEN AND BLACK[10], by CRUICKSHANK AND SHEEHAN[11], and by DARBRE AND ISLAM[12]. Each of these derivatives has proved to be useful for GLC separations, but none has received the intensive study regarding quantitative analysis as has the N-TFA *n*-butyl ester. An excellent review was written by BLAU[13] in 1968 on the analysis of amino acids by GLC covering the areas of derivatives, methods, and applications.

The N-TFA *n*-butyl esters of the amino acids are less volatile than the N-TFA methyl esters. The N-TFA methyl esters are so volatile that losses can easily occur during the derivatization procedure especially if the samples are concentrated after derivatization. DARBRE AND ISLAM[12] report that there is danger of loss of the more volatile N-TFA methyl esters when the samples are dried for longer than 4 min under vacuum at the temperature of an ice bath. Recent investigations[14] resulted in losses of *ca.* 10% for the more volatile N-TFA methyl esters when the samples were dried under a stream of nitrogen at room temperature; whereas insignificant losses were obtained for the N-TFA *n*-butyl esters dried under the same conditions. Only

ca. a 10% loss occurred for the more volatile N-TFA *n*-butyl esters when evaporated at 100°.

During the past eight years, GEHRKE and co-workers[2–4,15] have developed a quantitative single general method for the determination of the twenty protein amino acids and at least forty other non-protein amino acids as their N-TFA *n*-butyl esters.

MUSSINI AND MARCUCCI[16] reported the esterification of the amino acids using diazobutane, but the most common method for preparing esters of the amino acids with the higher alcohols is the reaction of the amino acids with the alcohol in the presence of anhydrous hydrogen chloride. Direct formation of the higher alkyl esters of the amino acids has been a problem because of the lack of solubility of cystine and some of the other amino acids in the higher alcohols. BLAU AND DARBRE[17] suggested dissolving the amino acids in a small amount of anhydrous trifluoroacetic acid before adding the *n*-BuOH·HCl to obviate the solubility problems associated with cystine and some of the others. STALLING *et al.*[18] reduced the solubility problems by first making the methyl esters by direct esterification with methanolic HCl, then interesterification with *n*-butanolic HCl. These workers found that all of the amino acid methyl ester hydrochlorides were soluble in this reagent.

The quantitative GLC methods developed by GEHRKE and co-workers at the University of Missouri have proved to be most satisfactory except that the total derivatization time (methyl ester formation and interesterification) requires about 3.5 h for completion. Thus, in view of the number of laboratories which use this chromatographic method, it was highly desirable to have a faster procedure. From studies directed toward this goal, this research reports a simple, rapid, direct esterification method for preparing the N-TFA *n*-butyl esters of the protein amino acids with *n*-butanol 3 *N* in HCl which can be completed in 15 min. Also, the solubility problems associated with cystine and some of the other amino acids were removed.

EXPERIMENTAL

Apparatus

A Varian Aerograph Model 2100 gas chromatograph with a four-column oven bath, four flame ionization detectors, two dual differential electrometers, and equipped with a Varian Model 20 recorder, and a Packard Instruments Co. Model 7300 dual column gas chromatograph with hydrogen flame detectors and equipped with a Honeywell Electronik 16 strip chart recorder were used. A digital readout integrator (Infotronics, Model CRS 104) was used for determining peak areas.

Solvents were removed from the samples with a CaLab rotary evaporator, "cold finger" condenser, and a Welch Duo-Seal vacuum pump, or the samples were taken just to dryness with a stream of dry filtered nitrogen gas. The filters for purification of the N_2 gas contained activated charcoal and $CaSO_4$.

Pyrex 16 × 75 mm glass screw top culture tubes with teflon lined caps (Corning No. 9826) were used as the reaction vessel for the acylation reactions.

Reagents

All of the amino acids used in this study were obtained from Mann Research Laboratories, Inc., New York, N.Y. 10006, or Nutritional Biochemicals Corp., Cleveland, Ohio, and were chromatographically pure.

Methanol and butanol were "Baker Analyzed" reagents. The trifluoroacetic anhydride was obtained from Distillation Products Industries, Rochester, N.Y. 14603, and was an "Eastman Grade" chemical. Acetonitrile, a "Baker Analyzed" reagent of "Nanograde" purity, was stored over drierite in a bottle with a ground glass stopper. Anhydrous HCl, 99.0% minimum purity, was obtained from the Matheson Company, Joliet, Ill., 60434.

The methanol, *n*-butanol, and methylene chloride were redistilled from an all-glass system and stored in all-glass inverted top bottles to protect from atmospheric moisture. The methanol was first refluxed over magnesium turnings, and the methylene chloride and *n*-butanol over calcium chloride before distillation. The anhydrous HCl gas was passed through a H_2SO_4 drying tower before bubbling into the butanol or methanol.

Columns

Stabilized grade ethylene glycol adipate was obtained from Analabs, Inc., Hamden, Conn. 06518 and coated on 80–100 mesh acid-washed (A.W.) Chromosorb W which had been dried at 140° for 12 h as described by ROACH AND GEHRKE[19]. The EGA column material was packed in 1.5 m × 4 mm I.D. glass columns.

The OV-17 and OV-22 siloxane substrates were purchased from Supelco, Inc., Bellefonte, Pa. 16823. The support material for these columns was 80–100 mesh high-performance (HP) Chromosorb G. The OV-22 glass columns were 1.0 m × 4 mm I.D. The OV-22 columns gave essentially the same results as the OV-17 columns reported earlier. Thus, OV-17 and OV-22 columns can be used interchangeably.

The column packing was prepared by first adding a known amount of support material to a 500-ml ribbed round-bottom flask; then adding the solvent used to dissolve the stationary phase until the liquid level was about 1/4 in. above the support material. The solvent used to dissolve EGA was dry acetonitrile, and the OV-17 or OV-22 substrates were dissolved in dry methylene chloride. The stationary phase was weighed into a small erlenmeyer flask, dissolved in the appropriate solvent, and then transferred to the flask containing the support. The flask containing the support and stationary phase was placed in a 60° water bath, and the solvent was *slowly* removed with a rotary evaporator over a time period of 45 min under partial vacuum. This ensured a uniform coating of the stationary phase on the support material.

ANALYTICAL DERIVATIZATION METHOD

Macro method (1–20 mg total)

Pipet an appropriate aliquot of a protein hydrolysate, amino acid mixture, or ion-exchange cleaned physiological fluid[4] into a 125 ml flat-bottom flask. Add an *exact* amount (0.2–4 mg) of *n*-butyl stearate, ornithine or other internal standard (I.S.) solution to each flask[4].

Dry under a partial vacuum on an all-teflon rotary evaporator placed in a 60° water bath.

Add 1.5 ml of *n*-BuOH 3 *N* in HCl per 1.0 mg of total amino acids, mix on an ultrasonic mixer for at least 15 sec at room temperature, esterify at 100° for 15 min in an oil or sand bath.

Evaporate to dryness under a partial vacuum at 60° on a rotary evaporator.

Add 3 ml of anhydrous methylene chloride and 1 ml of trifluoroacetic anhydride (TFAA) for each 10 mg of amino acids and thoroughly mix.

Transfer an appropriate aliquot to a Corning No. 9826 culture tube with teflon-lined screw cap. The amount need not be exact since an I.S. is included.

Acylate at 150° for 5 min in the closed tube placed behind a safety shield.

Semimicro (~ 2 mg total) and micro method (1–200 μg total)

Pipet an appropriate aliquot of sample into a Corning No. 9826 culture tube or micro reaction tube. Add an appropriate *exact* amount of ornithine or other internal standard[4].

Dry by passing a stream of filtered dry N_2 gas over the sample at 100°.

Add 150 μl of *n*-BuOH 3 *N* in HCl per 100 μg of total amino acids. Mix on an ultrasonic mixer for at least 15 sec at room temperature. Esterify at 100° for 15 min.

Evaporate to dryness at 100° using a stream of dry, filtered N_2 gas or by simply placing on a sand bath and allow to evaporate.

Azeotrope water with 150 μl of CH_2Cl_2.

Add 60 μl of CH_2Cl_2 and 20 μl of TFAA for each 100 μg of amino acids (minimum final volume of 80 μl).

Acylate at 150° for 5 min in a closed tube with teflon-lined screw cap placed behind a safety shield. The sample is ready for chromatography.

If the amount of starting sample is only 25–250 ng of each amino acid, concentrate the acylated sample to 50 μl by opening the vial and allow to evaporate by gentle warming. In this case use an acylation solution of 0.2 μl TFAA/40 μl CH_2Cl_2. Inject 4–8 μl of the concentrated sample.

RESULTS AND DISCUSSION

In 1968, GEHRKE *et al.*[15] presented an EGA and OV-17 dual column system for the separation of the N-TFA *n*-butyl esters of all twenty protein amino acids. Recently, ROACH AND GEHRKE[19] reported the use of stabilized EGA coated on dried (140° for 12 h) 80–100 mesh A.W. Chromosorb W for the rapid quantitative analysis of all the protein amino acids except arginine, histidine, and cystine. Also, ROACH *et al.*[20] reported the use of either OV-22 or OV-17 coated on 80–100 mesh, HP Chromosorb G for the rapid quantitative determination of histidine, arginine, tryptophan, and cystine. The gas–liquid chromatographic separations are now so rapid that only 15 to 25 min are required for a complete analysis of the twenty protein amino acids.

The major drawback to the method which was reported earlier for the GLC analysis was the considerable time required for interesterification. Formation of the methyl esters of the amino acids required 30 min and the interesterification required 2.5 h. Because of the considerable time involved in methyl ester formation, then interesterification to the *n*-butyl esters, a concerted effort was made to develop a "direct esterification" procedure.

STALLING *et al.*[18] reported that cystine and some of the other amino acids were difficult to dissolve in *n*-butanol which was 1.25 *N* in HCl. These authors noted that a fine white precipitate formed on the bottom of the container when *n*-BuOH·HCl was added to a dried sample of the amino acids. The present authors also noted this precipitate and considered it important to investigate the effect of HCl concentration

and ultrasonic mixing on the solubility of the amino acids. Ultrasonic mixing at room temperature for about 15 sec resulted in complete dissolution of the white precipitate. The ultrasonic mixing was of primary importance to the esterification reaction since the amino acid precipitates were readily broken up, thus the rates of solution and reaction were significantly enhanced in *n*-butanol 3 *N* in HCl. A study was then made to determine the effects of other reaction conditions: the HCl concentration, the reaction time, and the temperature.

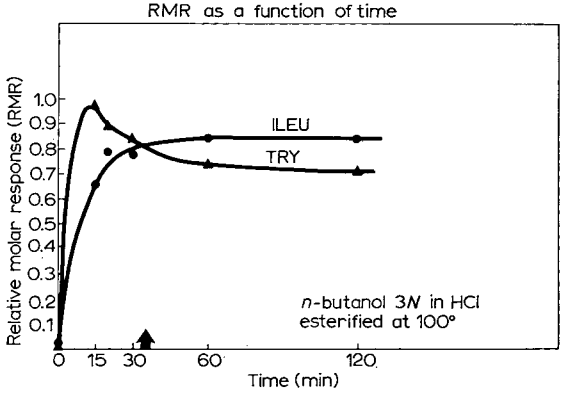

Fig. 1. *RMR* values for isoleucine and tryptophan as a function of esterification time.

TABLE I

THE RELATIVE MOLAR RESPONSE OF AMINO ACIDS AS A FUNCTION OF ESTERIFICATION TIME[a]

Amino acid	*Relative molar response*[b] *(a.a./glu.)*				
	15 min	*20 min*	*30 min*	*60 min*	*150 min*
Alanine	0.54	0.51	0.51	0.53	0.54
Valine	0.71	0.70	0.72	0.71	0.72
Glycine	0.43	0.42	0.42	0.42	0.43
Isoleucine	0.70	0.78	0.80	0.84	0.84
Leucine	0.83	0.81	0.81	0.82	0.82
Proline	0.73	0.71	0.71	0.70	0.71
Threonine	0.64	0.61	0.63	0.63	0.63
Serine	0.55	0.53	0.52	0.53	0.52
Methionine	0.54	0.50	0.52	0.51	0.52
Hydroxyproline	0.76	0.74	0.74	0.74	0.75
Phenylalanine	1.15	1.13	1.13	1.11	1.12
Aspartic acid	0.92	0.91	0.91	0.90	0.91
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Tyrosine	0.95	0.96	0.97	0.95	0.87
Lysine	0.86	0.84	0.85	0.84	0.84
Histidine	0.63	0.63	0.64	0.63	0.61
Arginine	0.62	0.64	0.63	0.62	0.59
Tryptophan	0.97	0.88	0.77	0.74	0.63
Cystine	0.88	0.92	0.92	0.91	0.90

[a] Direct esterification in *n*-butanol 3 *N* in HCl at 109°. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents an average of two determinations.

Isoleucine was found to be the most difficult amino acid to esterify and tryptophan was partially decomposed in *n*-BuOH·HCl, but tryptophan undergoes decomposition in any acidic media. Since isoleucine was the most difficult amino acid to esterify and some losses were observed for tryptophan, these two amino acids were selected as models for further experiments. The samples were derivatized as described in the Section ANALYTICAL DERIVATIZATION METHOD.

The effect of the concentration of HCl on the esterification reaction was investigated first. Solutions of 1.5, 3.0, and 6.0 *N* HCl in *n*-butanol were studied. In all experiments, 1.5 ml of *n*-BuOH·HCl was added for each 1.0 mg of total amino acids. The amino acids dissolved very slowly in 6 *N* HCl, whereas 1.5 *N* HCl dissolved the samples, but longer times were required.

TABLE II

THE RELATIVE MOLAR RESPONSE OF AMINO ACIDS AS A FUNCTION OF ESTERIFICATION TEMPERATURE[a]

Amino acid	*Relative molar response*[b] *(a.a./glu.)*			
	90°	*100°*	*110°*	*120°*
Alanine	0.53	0.53	0.51	0.52
Valine	0.72	0.72	0.70	0.73
Glycine	0.44	0.44	0.42	0.43
Isoleucine	0.80	0.82	0.82	0.83
Leucine	0.80	0.82	0.81	0.84
Proline	0.71	0.72	0.71	0.74
Threonine	0.63	0.64	0.64	0.65
Serine	0.54	0.55	0.55	0.56
Methionine	0.56	0.55	0.54	0.55
Hydroxyproline	0.76	0.75	0.74	0.76
Phenylalanine	1.13	1.14	1.14	1.16
Aspartic acid	0.89	0.90	0.89	0.91
Glutamic acid	1.00	1.00	1.00	1.00
Tyrosine	0.95	0.96	0.96	0.97
Lysine	0.86	0.85	0.84	0.88
Histidine	0.61	0.62	0.64	0.67
Arginine	0.62	0.61	0.62	0.64
Tryptophan	0.90	0.88	0.81	0.77
Cystine	0.85	0.89	0.90	0.92

[a] Direct esterification in *n*-butanol 3 *N* in HCl for 35 min. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents a single determination.

A plot of the relative molar response (*RMR*) values for isoleucine and tryptophan as a function of time of esterification is given in Fig. 1. The *RMR* for glutamic acid was arbitrarily assigned a value of unity, and the response values of each amino acid relative to glutamic acid, $RMR_{\text{a.a./glu.}}$, were calculated as follows:

$$RMR_{\text{a.a./glu.}} = \frac{A_{\text{a.a.}}/g_{\text{a.a.}}/MW_{\text{a.a.}}}{A_{\text{glu.}}/g_{\text{glu.}}/MW_{\text{glu.}}}$$

where $A_{\text{a.a.}}$ = area in counts of amino acid peak, $g_{\text{a.a.}}$ = grams of amino acid in sample, and $MW_{\text{a.a.}}$ = molecular weight of amino acid.

A direct esterification time of 35 min was selected to give maximum *RMR* values for isoleucine and all of the other amino acids with the exception of tryptophan.

The *RMR* values for the amino acids given in Table I were determined at five different esterification times (15 to 150 min). These data show that excellent results can be achieved with an esterification time of 15 min for all of the amino acids except isoleucine. Note that the maximum *RMR* value for tryptophan was obtained for a 15-minute "direct esterification". The maximum *RMR* for isoleucine was obtained after esterification for *ca.* 1 h but little change in *RMR* values resulted after 45 min. Direct esterification of isoleucine at 100° for 15 min yielded an average *RMR* value of about 85% of the maximum value. Since the w/w% recovery depends not only upon the *RMR* of the experimental sample, but also upon the *RMR* for the calibration mixture taken through the complete method, a recovery of 100% can be achieved when both the sample and the calibration mixture are esterified for 15 min under *exactly* the same reaction conditions.

TABLE III

RECOVERY OF AMINO ACIDS IN A MIXTURE BY DIRECT ESTERIFICATION

Amino acid	*Milligrams*[a]		*Recovery* (%)[b]
	Added	*Found*	
Alanine	0.4456	0.435	97.6
Valine	0.5856	0.566	96.7
Glycine	0.3756	0.367	97.7
Isoleucine	0.6560	0.644	98.2
Leucine	0.6560	0.650	99.1
Proline	0.5756	0.564	97.9
Threonine	0.5956	0.591	99.2
Serine	0.5356	0.525	98.0
Cysteine	0.6060	0.591	97.5
Methionine	0.7460	0.746	100.0
Hydroxyproline	0.6556	0.652	99.4
Phenylalanine	0.8260	0.819	99.2
Aspartic acid	0.6656	0.687	103.2
Glutamic acid	0.7356	0.747	101.5
Tyrosine	0.9060	0.912	100.7
Lysine	0.7310	0.748	102.3
Histidine	0.7760	0.771	99.4
Arginine	0.8710	0.908	104.2
Tryptophan	1.0210	1.158	113.4[c]
Cystine	1.2016	1.233	102.6

[a] Each value represents an average of three independent determinations. *n*-Butyl stearate as internal standard. Esterified for 35 min at 100° in *n*-butanol 3 *N* in HCl.

[b] Recovery based on amino acid calibration mixture taken through complete method with interesterification.

[c] High value due to loss of tryptophan in calibration mixture during interesterification for 2.5 h at 100°.

The maximum *RMR* value for tryptophan was obtained on direct esterification for 15 min at 100°. After reaching a maximum, the *RMR* value decreased with time due to the decomposition of tryptophan in the acidic *n*-butanol solution. An average of about 85% of the maximum *RMR* for tryptophan was obtained with a 35-minute esterification. An average recovery of more than 95% was obtained, however, when the sample and calibration mixture were reacted under the same experimental con-

ditions (100° for 35 min). However, samples should be esterified directly at 100° for 15 min to obtain the most accurate tryptophan values. It is well known that tryptophan undergoes decomposition during acidic hydrolysis, thus analysis for tryptophan in acidic hydrolysates is useless. For samples which contain no tryptophan, direct esterification for 35 min at 100° is recommended to assure quantitative esterification of isoleucine.

The effect of esterification temperature on the *RMR* values was investigated in the range of 90 to 120°. The data in Table II demonstrate that the temperature at which the esterification was conducted influenced the *RMR* values only slightly. A temperature of 100° ±5° was selected as the esterification temperature. Using isoleucine and tryptophan as model compounds, the conditions for direct esterification were determined. The next step in the study was to apply these reaction conditions to a mixture containing the twenty protein amino acids. Aliquots containing known

TABLE IV

RELATIVE MOLAR RESPONSE OF N-TFA *n*-BUTYL ESTERS OF AMINO ACIDS[a]

Amino acid	*Relative molar response*[b]				
	1	*2*	*3*	*Av.*	*RSD* (%)
Alanine	0.523	0.518	0.513	0.518	0.97
Valine	0.725	0.731	0.735	0.730	0.69
Glycine	0.427	0.438	0.434	0.433	1.29
Isoleucine	0.829	0.823	0.835	0.829	0.72
Leucine	0.838	0.831	0.827	0.832	0.67
Proline	0.732	0.717	0.724	0.724	1.04
Threonine	0.641	0.636	0.629	0.635	0.95
Serine	0.532	0.540	0.538	0.537	0.78
Cysteine	0.458	0.454	0.446	0.453	1.35
Methionine	0.562	0.551	0.557	0.557	0.99
Hydroxyproline	0.763	0.757	0.745	0.755	1.21
Phenylalanine	1.139	1.140	1.142	1.140	0.14
Aspartic acid	0.917	0.909	0.914	0.913	0.44
Glutamic acid	1.000	1.000	1.000	1.000	—
Tyrosine	0.957	0.946	0.952	0.952	0.58
Lysine	0.863	0.859	0.846	0.856	1.04
Histidine	0.623	0.619	0.630	0.624	0.89
Arginine	0.643	0.637	0.653	0.644	1.26
Tryptophan	0.851	0.840	0.832	0.841	1.13
Cystine	0.932	0.958	0.941	0.944	1.40

[a] Direct esterification in *n*-butanol 3 *N* in HCl at 100°. *RMR* of glutamic acid assigned a value of 1.

[b] Each value represents a single determination.

amounts of the amino acids (~ 5 mg) were dried, an excess of *n*-butanol 3 *N* in HCl (1.5 ml per 1.0 mg total amino acids) was added, the samples were esterified for 35 min, and then acylated with TFAA in a closed tube at 150° for 5 min as described by GEHRKE *et al.*[4]. The calibration mixtures were prepared by first forming the methyl esters of the amino acid and then interesterifying and acylating as described by GEHRKE *et al.*[4]. The recovery data obtained from this experiment are given in Table III. Excellent recovery was obtained for all of the amino acids indicating that the direct esterification of the amino acids was essentially complete. The high value for

tryptophan in the sample resulted from the low *RMR* for tryptophan in the calibration mixture. This resulted from heating tryptophan in an acidic *n*-butanolic solution for a total of 3 h with the corresponding losses.

The *RMR* values for the amino acid derivatives which were obtained using the direct esterification procedure are presented in Table IV. The precision of the data is excellent as evidenced by the relative standard deviations, which in most cases are *ca.* 1% or less.

CONCLUSIONS

The reaction conditions for the "direct quantitative esterification" of the protein amino acids have been established. These experiments conclusively demonstrate that GLC analyses of the amino acids can be made precisely, accurately, and rapidly when the N-TFA *n*-butyl esters of the amino acids are formed directly in *n*-butanol 3 *N* in HCl. A significant advantage of the N-TFA *n*-butyl ester derivative over the N-TFA methyl ester derivative is that no losses occur on concentration at room temperature, whereas serious losses of the methyl esters would occur. Further, the losses of the butyl ester derivative would be minimal if any leaks occurred during derivatization. A complete GLC analysis of the protein amino acids, including derivatization and chromatography, can be completed in less than 1 h. This GLC method should fill an important need of scientists who are faced with the problem of amino acid analyses of biologically important substances.

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CHROM. 4287

THE ASSAY OF GLUTETHIMIDE IN PLASMA

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SUMMARY

A gas–liquid chromatographic method for the estimation of glutethimide in plasma is described. The method allows the estimation of concentrations of the drug as low as 0.3 μg/ml. Results are presented showing plasma levels of the drug up to twenty-four hours after a therapeutic dose.

INTRODUCTION

Glutethimide (α-ethyl-α-phenyl glutarimide)* is a widely used non-barbiturate hypnotic. A sensitive method for the assay of this drug in plasma was required in order to compare plasma levels in normal subjects with those achieved in subjects in various clinical states after a therapeutic dose of the drug, and to assess the efficacy of different methods of treating glutethimide poisoning.

Various methods have been published for the determination of glutethimide in plasma. Some depend on the determination of the drug by UV spectrophotometry[1–3], others on the use of GLC[4–6]. None of these methods was found to be adequate for the assay of glutethimide in plasma from subjects who had received a normal dose of glutethimide (250–500 mg). It has been shown with radioactive glutethimide that the concentration of the drug in plasma may be less than 1 μg/ml 5 h after a dose of 250 mg[7]. Of the available methods those in which determination is by UV spectrophotometry suffer from the disadvantage that the non-specific absorbance due to normal plasma constituents may be high in comparison with the absorbance due to the drug. This problem has not been overcome by any of the different extraction procedures used. Of the GLC methods available that of WINSTEN AND BRODY[6] was found capable of determining glutethimide in plasma only at concentrations higher than 10 μg/ml. The method of FINKLE[4] was capable of determining accurately levels of more than 3 μg/ml. The method of KORZUN *et al.*[5] was found to be unsuitable owing to the use of an excessively high column temperature which caused bleeding of the liquid phase from the column with consequent reduction of the sensitivity. In addition, the use of dimethylformamide as a solvent for the glutethimide caused progressive deterioration of the column owing to reaction between the solvent and the liquid phase, a polyethylene glycol, Carbowax 20 M.

* Doriden®.

The method described in this article is suitable for the assay of glutethimide in plasma down to levels of 0.3 μg/ml and has been used to assay blood samples taken from volunteers up to 24 h after oral administration of 500 mg of glutethimide.

MATERIALS AND METHODS

Chromatography

A Pye Series 104 gas chromatograph fitted with a dual flame ionisation detector head was used in this work, so that it could be used as a dual column–dual detector instrument. Thus two separate determinations could be carried out simultaneously.

Glass columns (9 ft. × 1/4 in. O.D.) were used. They were inactivated before use by rinsing with dichlorodimethylsilane, followed by methanol. They were air dried.

Chromosorb G* (100–120 mesh) was used as the support. It was prepared by washing for one hour with several volumes of concentrated HCl. The acid was removed by repeated washing with distilled water. The fine particles were removed by decanting after the last wash. The material was dried at 110°. It was then inactivated by refluxing for 12 h in twice its volume of a mixture of 10% trimethylsilane and 10% diethylamine in hexane. The material was dried by suction, washed with *n*-propanol, followed by petroleum ether (60–80°) and then dried at 110°.

The liquid phase used was Carbowax 20M*. The required volume of a 0.5% solution of Carbowax 20M in chloroform (1 ml/g Chromosorb G) was added to the support material and the chloroform was removed under vacuum at 70° using a rotary evaporator. The coated support, when completely dry, was packed into the columns under nitrogen at a pressure of 14 p.s.i., with vibration. Columns were preconditioned by heating at 210° for 48 h with a slow flow of nitrogen through the column. Operating conditions were as follows: column oven 200°; detector oven 260°; carrier gas nitrogen, flow rate 100 ml/min; hydrogen flow rate 80 ml/min; air flow rate 800 ml/min. All samples were dissolved in tetrahydrofuran. 1 μl samples were injected into the column. Glutethimide had a retention time of 12 min under these conditions.

Extraction procedure

Duplicate 1 or 2 ml plasma samples were extracted by shaking for 10 min with 10 ml redistilled dichloromethane. After centrifugation at 3000 r.p.m. for 5 min the aqueous phase was removed and the organic phase containing the drug washed with 4 ml 1 *N* NaOH, followed by 4 ml 1 *N* HCl and then 4 ml distilled water. After each wash tubes were centrifuged in order to ensure adequate separation of the phases, before the aqueous phase was removed. A measured aliquot of the organic phase (usually between 5 and 7 ml) was then transferred to a 10 ml conical Quickfit testtube and the dichloromethane removed on a rotary evaporator, at 30°. The tubes were then sealed with a rubber disc and aluminium cap of the type used in sealing vials. The residue was then redissolved in 100 μl of tetrahydrofuran which was introduced into the tube through the seal with a (50 μl) Hamilton syringe. When the solvent had been added tubes were stored at 0°. These precautions were necessary in order to prevent concentration of the sample through the evaporation of the solvent.

* Applied Science Laboratories, Inc.

Standards, for the calibration of the instrument, were prepared by drying a suitable volume of a solution of glutethimide in ethanol on to a conical testtube and were then treated in the same way as the samples. The detector response was calibrated each day.

It was found that the concentration of glutethimide in each sample could be accurately estimated by the measurement of the height of the peak above the extrapolated baseline, provided that not more than 1 μl samples were injected. With constant injection volume peak width is constant and triangulation of the peak is therefore unnecessary.

In order to test the recovery of glutethimide from plasma known volumes of the standard solution of glutethimide in ethanol were dried on to testtubes. The residue was then dissolved in 1 or 2 ml of plasma from normal volunteers who had not taken glutethimide for at least two weeks.

In order to assess the usefulness of the method in assaying plasma levels of glutethimide after a therapeutic dose of the drug, six normal volunteers swallowed two tablets (500 mg) approximately 1 h after a light breakfast. Blood samples were taken by venepuncture at 1/2, 1, 2, 4, 6, 8, 12 and 24 h after the dose had been taken. The heparinised blood was centrifuged within 15 min and the plasma was frozen and stored at $-20°$ until the assay was carried out.

RESULTS AND DISCUSSION

The reproducibility of the response of the detector to injections of 1 μl samples of glutethimide at two different concentrations (10 and 100 ng/μl) was determined. The standard deviation at both concentrations was found to be 3.3%. The response of the detector to glutethimide was shown to be linear over the range 2.5 ng–1 μg. The smallest quantity that can be accurately measured is 2.5 ng.

The recovery of glutethimide from plasma is between 90 and 100%. Details are shown in Table I. Lower concentrations than 0.3 μg/ml glutethimide in plasma cannot be estimated because of interference by normal plasma constituents.

Using this method it was found possible to assay glutethimide in blood samples

TABLE I

RECOVERY OF GLUTETHIMIDE FROM PLASMA

Concentration of glutethimide in plasma (μg/ml)	*Plasma volume extracted (ml)*	*Apparent concentration (μg/ml)*	*% recovery*
0.5	1	0.45	90
0.5	1	0.5	100
1	1	0.95	95
1	1	0.90	90
2.5	1	2.5	100
2.5	1	2.3	92
5	1	4.5	90
5	1	4.7	94
10	1	9.5	95
10	1	9.7	97

taken from subjects who had taken the drug between 30 min and 24 h beforehand. Results are shown in Table II. Fig. 1 shows a typical tracing from chromatography of (a) a normal, drug-free plasma extract and (b) an extract of plasma containing glutethimide.

Analysis of the results of duplicate assays of plasma samples indicate that the overall error in the method is 8%.

TABLE II

PLASMA GLUTETHIMIDE LEVELS (μg/ml) AFTER INGESTION OF 500 mg OF GLUTETHIMIDE IN TABLET FORM BY SIX SUBJECTS

The results of duplicate assays on each sample are shown.

Subject	*Time (h)*							
	1/2	*1*	*2*	*4*	*6*	*8*	*12*	*24*
SWMH	1.4	3.2	3.6	2.6	1.6	1.5	1.1	0.5
	1.4	3.7	3.6	2.5	1.8	—	1.0	0.5
CMcM	1.2	4.4	7.9	4.4	3.8	3.1	2.0	0.4
	1.4	4.9	6.2	4.1	3.8	3.3	2.0	0.4
AMW	0.9	3.5	2.6	2.2	1.7	1.4	0.9	0.7
	1.2	4.3	3.6	2.2	—	1.4	1.4	0.9
PMG	4.0	6.5	4.8	2.4	1.7	1.5	1.1	0.4
	3.7	4.5	5.2	2.6	1.6	1.4	1.0	0.4
RKR	0.8	3.2	2.9	2.1	1.5	1.3	1.1	0.6
	0.9	3.5	3.1	—	—	1.4	1.2	0.7
GK	<0.3	0.7	1.3	1.6	2.8	2.4	2.1	0.8
	<0.3	0.8	1.4	1.6	2.9	2.4	2.1	0.8

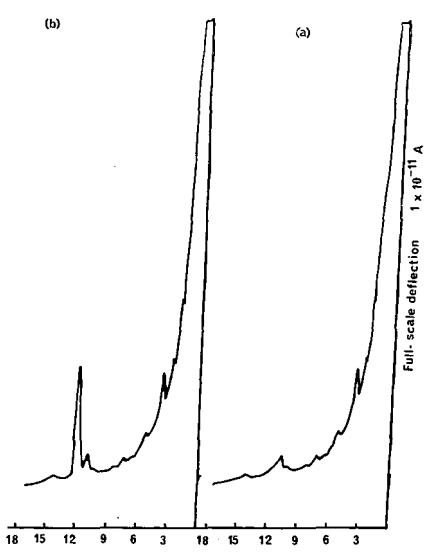

Fig. 1. Tracing obtained by chromatography of (a) extract from normal plasma and (b) extract from plasma containing glutethimide (1 μg glutethimide per ml plasma).

It can be seen from Table II that plasma levels vary considerably between subjects. Peak plasma levels were attained more rapidly in some subjects than in others, the time of the maximum concentration ranging from 1 to 6 h. The peak levels varied from 2.9 to 7.1 μg/ml. This variation could not be accounted for by differences in body weight of the subjects. At 24 h after the dose plasma levels ranged from 0.4 to 0.8 μg/ml. The sensitivity of the method was adequate for the assay of all samples except one. In the subject from whom this sample was taken the drug appeared to be absorbed very slowly and in the 30 min sample less than 0.3 μg/ml of glutethimide could be detected.

The results suggest that the rate and extent of absorption of glutethimide from tablets is variable. Irregularities in the plasma decay curves indicate that absorption continues throughout the first twelve hours and that the concentration of the drug in the plasma during this period is dependent both on the rate of absorption from the gut and on the rate of its elimination from the plasma by metabolism and excretion.

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CHROM. 4269

IDENTIFICATION AND DETERMINATION OF ORGANOMERCURIAL FUNGICIDE RESIDUES BY THIN-LAYER AND GAS CHROMATOGRAPHY

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SUMMARY

A study has been made of the thin-layer and gas–liquid chromatographic characteristics of the dithizonates of a number of organomercurial fungicides in common use. A method is given for the extraction of these fungicides from apples, potatoes and tomatoes and their identification and determination, as their dithizonates, by thin-layer and gas chromatography.

INTRODUCTION

Organomercury compounds have been in use as fungicides in agriculture and horticulture on a fairly large scale for a number of years. In certain countries they are also used by the wood pulp industry to prevent slime formation. The main agricultural use in the U.K. is as a dressing for seeds of cereal crops but seeds of beet and mangolds and seed potatoes are often similarly protected by the compounds. Organomercurial sprays are applied to apple and pear trees to prevent scab and similar sprays are used extensively on tomatoes, especially glass-house crops. Currently there is some interest in the mercury residue problem but concern has also been expressed from time to time about the contamination of our general environment[1–3].

Studies on the translocation of these compounds and the residues occurring in foodstuffs have been recently reviewed[1]. Most of the mercury residue work carried out so far has consisted of the determination of mercury as Hg by the traditional methods such as acid digestion of the sample followed by estimation of the resultant inorganic mercury as the dithizonate[4]. These methods, however, give no indication of the chemical nature of the mercury compound present in the sample or even whether it is in an organic or inorganic form. The nature of the mercury compound present is actually of some importance as the toxicities of individual mercury compounds differ considerably. Methylmercury compounds, for instance, are far more toxic than their phenylmercury analogues. However, very little work has been done on identification and determination of individual organomercurials. Some has been carried out in Sweden, a country which has experienced serious mercury pollution problems associated with the use of the compounds in agriculture and its large wood pulp

industries. WESTÖÖ[5,6] has investigated thin-layer and gas–liquid chromatographic methods for estimating methylmercury compounds in fish, meat, liver and eggs and showed incidentally that gas chromatographic separation of some organomercurials, as their dithizonates, was possible. This present study examines the TLC and GLC systems required to separate and identify most of the organomercury compounds in common use in agriculture and horticulture, including the alkyl-, alkoxyalkyl- and arylmercury compounds. The application of these techniques to the identification and determination of these compounds in potatoes, apples and tomatoes is also described.

THIN-LAYER CHROMATOGRAPHY

The following types of organomercury compounds were examined:

Me–Hg–X	MeO–Et–Hg–X	Phenyl–Hg–X
Et–Hg–X	EtO–Et–Hg–X	Tolyl–Hg–X

The nature of X affects the properties of the compound. In general, when X is an anion such as sulphate, nitrate or acetate, the compound tends to be ionic and water soluble. When X is a halogen or dicyandiamide the compound tends to be non-polar and soluble in organic solvents. As all these variations of X are likely to be encountered in practice, it is difficult to devise a single TLC system suitable for separating all these compounds. If, for example, silica gel plates are used with an organic solvent as the mobile phase, then the chlorides and diphenylmercury move up the plate and the more salt-like compounds remain at the origin line. Visualisation can be achieved satisfactorily with a spray of 0.05% dithizone in chloroform, but efficient TLC separation of the intact organomercurials was judged unlikely to be successful for all the compounds likely to be encountered.

As the actual identity of the X moiety is not so important from a residue point of view, and as its identity can usually be established by simple chemical tests, the chromatography of the dithizonates was investigated. All the mercury compounds examined readily yielded characteristic stable intensely yellow to red complexes with dithizone on simply shaking the organomercurial, in solid form or in solution, with a chloroform solution of dithizone until a slightly green colour indicated an excess of reagent. Inorganic mercury compounds which may be present give the usual mercury di(dithizonate): diphenylmercury is at least partially converted to phenylmercury dithizonate. A number of solvent systems were tried with silica gel and alumina absorbents and the R_F values for the most satisfactory combinations are given in Table I. This shows that by appropriate selection, all the dithizonates can be clearly separated and identified. The dithizonates of methyl- and ethylmercury compounds are, perhaps, the least well separated of the spots but any doubt as to the identity of these two compounds can be easily resolved by the gas chromatographic procedures detailed below. Visualisation is not a problem; as little as 2 μg of these compounds are self-indicating as yellow or red spots.

Nearly all the commercially available samples of organomercury compounds used in this study were found to contain varying quantities of inorganic mercury and other organomercury compounds including diphenylmercury. TLC was found very useful for isolating pure specimens of the organomercury compounds as standards.

TABLE I

R_F VALUES × 100 OBTAINED BY TLC OF DITHIZONATES OF ORGANOMERCURY COMPOUNDS

Systems: (1) silica gel, hexane–acetone (9:1);
(2) silica gel, hexane–acetone (19:1)
(3) silica gel, hexane–acetone (93:7);
(4) silica gel, light petroleum–acetone (9:1);
(5) alumina, hexane–acetone (19:1);
(6) alumina, light petroleum–acetone (19:1);

Layer thickness: 250 μ.

Dithizonate	*System*					
	1	*2*	*3*	*4*	*5*	*6*
Methylmercury	64	48	57	77	89	86
Ethylmercury	64	51	62	78	91	87
Methoxyethylmercury	32	16	25	44	58	49
Ethoxyethylmercury	44	23	34	55	71	67
Phenylmercury	48	34	46	62	72	69
Tolylmercury	52	40	53	69	79	76
Mercury di-dithizonate	19	9	17	28	19	15

The same solvent systems were employed but chromatoplates 500 μm thick were used so that much larger amounts of the dithizonates could be applied. Appropriate areas of absorbent from the developed plate were then scraped off and the pure organomercury dithizonate eluted with diethyl ether.

GAS–LIQUID CHROMATOGRAPHY

WESTÖÖ[5] in his work on methylmercury compounds in fish used 10% Carbowax columns with electron-capture detection to show that various alkylmercury compounds, including their dithizonates, could be separated by GLC. TERAMOTO *et al.*[7], also working with methylmercury compounds, used a 25% diethylene glycol succinate column. These and a number of other stationary phases on various supports, with electron-capture detection, have been investigated. Again it was found much more convenient to use the dithizonates of the compounds under study. In general, the more polar phases such as Carbowax 20M and ethylene glycol adipate, on Chromosorb G, were found to give good separations but had a distinct tendency to produce tailing peaks on the chromatograms. By far the most satisfactory column consisted of 2% of polyethylene glycol succinate on Chromosorb G. Typical retention times for this column are given in Table II. The dithizonates of the various alkyl- and alkoxyalkylmercury compounds have fairly short retention times but are clearly separated from one another. Sensitivity is good and the system can easily detect 0.05 ng of these compounds. By contrast the arylmercury dithizonates had relatively long retention times with peaks that were correspondingly broader at the base. The peaks corresponding to phenylmercury dithizonate and tolylmercury dithizonate were also slightly asymmetrical; this type of peak appears to be an inherent characteristic of the arylmercury dithizonates, for which no obvious reason could be found. It is very marked on some types of column. Stationary phases such as Apiezon L, Silicone GE SE-52, Cyanosilicone GE XE-60, Carbowax 1500M and ethylene and diethylene

TABLE II

TYPICAL GLC RETENTION TIMES FOR ORGANOMERCURY DITHIZONATES

(I) 2 % polyethyleneglycol succinate on Chromosorb G (acid-washed, DMCS-treated, 60–80 mesh) in glass columns 1.5 m long, 3 mm I.D.; carrier gas, nitrogen.

Dithizonate	*Column temperature* (°C)				
	140	*150*	*160*	*170*	*180*
Methylmercury	3.8	2.8	2.2	1.6	1.2
Ethylmercury	6.6	4.6	3.6	2.7	2.0
Ethoxyethylmercury	17.0	11.6	8.7	6.2	4.9
Methoxyethylmercury	17.4	12.0	8.7	6.2	4.9
Tolylmercury	—	—	—	29.0	19.5
Phenylmercury	—	—	—	42.0	27.0

(II) 1 % polyethyleneglycol succinate on Chromosorb G (acid-washed, DMCS-treated, 60–80 mesh) in glass columns, 1.2 m long, 3 mm I.D.; carrier gas, nitrogen.

Dithizonate	*Column temperature* (°C)	
	170	*180*
Tolylmercury	6.4	3.2
Phenylmercury	10.0	5.0

glycol succinates, on Chromosorb W, G or Q as support, all showed this feature to some extent. Teflon, 40–60 mesh, was probably the best support but has certain intrinsic disadvantages. Direct "on column" injection tended to minimise this effect and was used throughout. Nevertheless, excellent reproducibility of these peaks for the arylmercury dithizonates was obtained on the polyethylene glycol succinate column referred to above and 1 ng of these compounds could be readily detected. A shorter column, containing only 1 % of polyethylene glycol succinate, specifically for the arylmercury dithizonates, was also useful in that shorter retention times were obtained together with narrower peaks on the chromatogram. Typical retention times obtained by use of this column are also given in Table II. This system would readily detect 0.5 ng of these arylmercury compounds.

Mercury compounds are known to "poison" tritiated foil detectors. The tritium source is a very weak β-emitter and almost any coating deposited on the foil will reduce emission. This effect is even more marked when the coating has a high electron capturing potential, as is the case with mercury, and can result in emission falling to zero. In preliminary work, it was found that injections of large amounts of these mercury compounds at oven temperatures of 190° or higher led to rapid deterioration of detector response because of this effect. Sensitivity could be fairly easily restored by cleaning the foil gently with a mild abrasive polish[8] but this was clearly to be avoided if possible. Operation at temperatures below 150° reduced this effect to negligible proportions but it was far more satisfactory, in the interests of obtaining reasonable retention times, to maintain an oven temperature of 180° and restrict the mercury content of injections to 100 ng or less.

APPLICATION OF METHODS TO SAMPLES

The methods described by WESTÖÖ[5,6] were designed mainly for detecting residues of methylmercury compounds in fish. The organomercurial is converted to the chloride or bromide by treatment with hydrochloric or hydrobromic acids and then extracted with toluene. Clean-up of the extract is effected by conversion of the mercurial into a water-soluble form such as the hydroxide, sulphate or cysteine derivative followed by acidification and back extraction into benzene. Good recoveries were claimed for methylmercury compounds but these methods are not suitable for the detection of alkoxyalkyl compounds which are usually very unstable in even dilute acids. Further, the use of an aqueous system as a means of extraction appeared likely to give inadequate penetration into vegetable and fruit material and it is on these crops that organomercurial fungicides may be used in the U.K. and in countries from which we import these foodstuffs.

A method was sought, principally for potatoes, tomatoes and apples, by which all the organomercurials, including the alkoxyalkyl, could be extracted unchanged with good solvent penetration of the sample. The possibility of using an acetone solution of dithizone was examined and showed promise but clean-up of the initial extract proved difficult. This was overcome by conversion of the organomercury dithizonate to the water-soluble nitrate by extraction of the compound into 1% aqueous silver nitrate. The aqueous solution was then treated with potassium thiocyanate, filtered and the organomercury thiocyanate extracted with toluene. This procedure gave 60 to 70% recoveries for the alkyl and alkoxyalkyl compounds but poor recoveries for the arylmercurials.

An efficient method for the extraction and clean-up of all the organomercurials was finally developed using a slightly alkaline solution of cysteine hydrochloride in propan-2-ol. (The use of a slightly alkaline solution is essential if the alkoxyalkyl compounds are to be recovered unchanged.) The extract was then washed with diethyl ether or toluene and the organomercurials extracted with a diethyl ether solution of dithizone.

This method was applied to potatoes, tomatoes and apples and gave recoveries of 85 to 95% for samples spiked with 1.0, 0.1 and 0.01 p.p.m. of methyl-, ethyl- and ethoxyethylmercury as their chlorides, and 5 and 0.5 p.p.m. of phenyl- and tolylmercury acetates.

Method

The method described here was found suitable for potatoes, tomatoes and apples but could obviously be applied to other foodstuffs. In the case of apples and potatoes, the residues to be determined will in most cases be concentrated in the skin or outer layers. Samples of apples and potatoes are therefore coarsely peeled and the thick peel chopped to provide material for analysis. Mercury residues in tomatoes tend to be distributed more evenly in the fruit. Five grams of chopped peel of apples or potatoes, or 5 g of the macerated fruit in the case of tomatoes, are macerated with a mixture of 10 ml of propan-2-ol and 5 ml of alkaline cysteine hydrochloride solution (1% aqueous solution adjusted to pH 8.0 by the addition of 5 *N* ammonia solution). After allowing the liquor to settle, the clear layer is decanted and the extraction repeated twice more with further portions of extractant solutions. The combined

extracts are then centrifuged at 2500 r.p.m. for 5 min. The clear liquor is separated, diluted with 700 ml of 4% sodium sulphate solution and the solution washed with three 50-ml portions of diethyl ether. It was found that, at this stage, potatoes gave a gelatinous precipitate but this remained in the ether layer and could be discarded without apparently affecting appreciably the recovery of mercury compounds. The organomercurials are then extracted from the aqueous solution using three 25-ml portions of a 0.005 % solution of dithizone in diethyl ether. The combined extracts are then dried by passage through a short column of granular anhydrous sodium sulphate and concentrated to a suitable volume, usually 5 ml, in a Kuderna-Danish evaporator. The final solution can then be examined by TLC using silica gel as absorbent and a mixture of hexane and acetone, 93:7, as developing solvent (system 3 in Table I). If the results indicate that it is appropriate, then one of the other systems in Table I can also be tried. The final solution is also injected on to the first of the gas chromatographic columns described in Table II. The shorter column described in Table II should also be used if arylmercury compounds are present.

ACKNOWLEDGEMENT

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CHROM. 4290

ERFAHRUNGEN MIT AKTIVITÄTSGRADIENTEN IN DER DÜNNSCHICHTCHROMATOGRAPHIE

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(Eingegangen am 16. Juli 1969)

SUMMARY

Experiences with activity gradients in thin-layer chromatography

It is shown that some particular separations (hypnotics, dyes) described in the literature were likely to be accomplished not by "parallel" pre-loading gradients of the layer but by other effects, *e.g.* favourable activity, increased solvent transport. In all cases examined, equivalent results could be obtained without any gradient. When chromatograms are developed in unsaturated N-chambers with binary solvent mixtures whose components are very different in polarity, contrary to prior assumptions no parallel gradients but slightly antiparallel ones are formed (*e.g.* with benzene–ethanol, 97:3). Several examples show that it is not at all the same whether a chromatogram is developed by pure or by ethanol-stabilized chloroform.

EINLEITUNG

NIEDERWIESER[1] hat kürzlich ein rationelles System für die Bezeichnung von Gradienten bei chromatographischen Verfahren vorgeschlagen (Fig. 1). Die Richtung des Gradienten wird dabei auf die Fliessrichtung der mobilen Phase bezogen; die Pfeile zeigen in Richtung zunehmender Mobilität der zu chromatographierenden

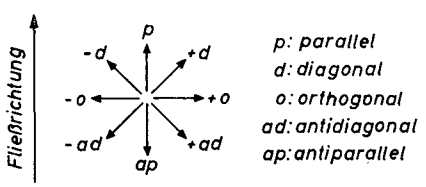

Fig. 1. Rationelle Bezeichnung der Richtung von Gradienten. Bezug: Fliessrichtung.

Substanzen. Ein paralleler Gradient liegt z.B. vor, wenn die "Aktivität" einer DC-Platte in Laufrichtung abnimmt. NIEDERWIESER[1,2] zeigte ebenfalls, dass die parallele Anordnung prinzipiell unvorteilhaft ist, weil die Flecken in Laufrichtung auseinandergezogen werden (siehe dazu auch Lit. 3). DE ZEEUW[4–10] hingegen hat über eine Reihe von speziellen Trennungen berichtet, deren Gelingen er ausdrücklich dem

jeweiligen Vorliegen eines parallelen Aktivitätsgradienten zuschreibt, der nahe beieinanderliegende Substanzen unter Trennung auseinanderziehe. Solche parallelen Gradienten sollen sich z.B. immer bilden, wenn in ungesättigten N-Kammern mit Fliessmittelgemischen aus Komponenten unterschiedlicher Polarität entwickelt wird; deshalb seien in solchen Kammern "theoretisch verbesserte Trennungen zu erwarten"[6]. Wir haben eine Reihe der Versuche nachgearbeitet, konnten aber in keinem Fall die speziellen Trenneffekte auf das Vorliegen von p-Gradienten zurückführen.

GRADIENTEN UND UNGESÄTTIGTE N-KAMMERN

DE ZEEUW hat in einer ungesättigten N-Kammer zehn Barbitursäuren mit stabilisiertem Chloroform–Aceton (90:10) getrennt[7]. Wir erhielten unter diesen Bedingungen annähernd die gleiche Trennung (Fig. 2a; vergl. mit Fig. 3 von Lit. 7). Dass diese Auftrennung nicht an die ungesättigte N-Kammer und p-Gradienten gebunden ist, zeigt Fig. 2b: Diese gegenüber Teilbild (a) eher noch verbesserte Auftrennung wurde auf einer Platte erzielt, die in einer Vario-KS-Kammer mit stabilisiertem Chloroform–Aceton (90:10) 15 Min. homogen vorbedampft und dann mit dem gleichen Gemisch 80 Min. durchlaufend chromatographiert wurde. Die Trennung beruht also in beiden Fällen – ausgehend von einer "Grundtrennung" – auf dem erhöhten Fliessmitteldurchsatz, der in unserem Falle durch die Durchlauftechnik und im Falle der ungesättigten N-Kammer[7] durch die Abdampfung des Fliessmittels von der Schicht in die Kammer erfolgte (s. dazu Fig. 4a). Wenn man die Trennbilder in der gesättigten und der ungesättigten N-Kammer (z.B. Fig. 3 links und rechts in Lit. 7) vergleicht, stellt man fest, dass alle R_F-Werte in der ungesättigten Kammer um den Faktor 1.8 grösser sind, die Trennung ist also lediglich durch den um den Faktor 1.8 grösseren Fliessmitteldurchsatz "herausentwickelt" worden*. Die vorerwähnte Grundtrennung beruht auf der zufällig günstigen Kombination Chloroform–Äthanol–Aceton im richtigen Mischungsverhältnis. Dies zeigte sich, nachdem wir in der Vario-KS-Kammer erfolglos eine Reihe anderer Vorbedampfungs- und Fliessmittelkombinationen probiert hatten. Die Trennung wird schon schlechter, wenn man statt des stabilisierten Chloroforms reines benutzt.

Um die Verteilung der polaren und unpolaren Komponenten eines binären Fliessmittelgemisches auf der Schicht vor und nach der Entwicklung zu bestimmen, haben wir durch eine geeignete Vorrichtung (s. EXPERIMENTELLER TEIL) in unterschiedlichen Höhen "trockene" und fliessmittelfeuchte Schichtproben entnommen und gaschromatographisch analysiert. Wir zogen das Gemisch Benzol–Äthanol dem von Chloroform–Aceton vor, um vor Zersetzungserscheinungen sicher zu sein. Die wichtigsten Ergebnisse sind in den Fig. 3a und b zusammengefasst.

Es zeigte sich, dass sich in der ungesättigten N-Kammer bei der Entwicklung mit einem Fliessmittelgemisch wie Benzol–Äthanol (97:3) nicht etwa – wie in der

* Kürzlich wurde die Trennung einiger Hypnotika auf Celluloseschichten beschrieben[11], die, wenn man nur die angegebenen R_F-Werte in Betracht zieht, etwa ebenso gut wäre wie die Trennung durch DE ZEEUW[7], in natura aber wegen prohibitiv ungünstiger Fleckform deutlich unterlegen ist. Die dort[11] beklagte Nichtreproduzierbarkeit kann sehr einfach durch Feuchtekontrolle behoben werden. Zudem wird bei hoher Feuchte (~ 80% r.F.) die Fleckform wesentlich besser. Nach unseren Ergebnissen erzielt man auf diesen Schichten die besseren Resultate bei 80% r.F. und mit Benzol als Fliessmittel.

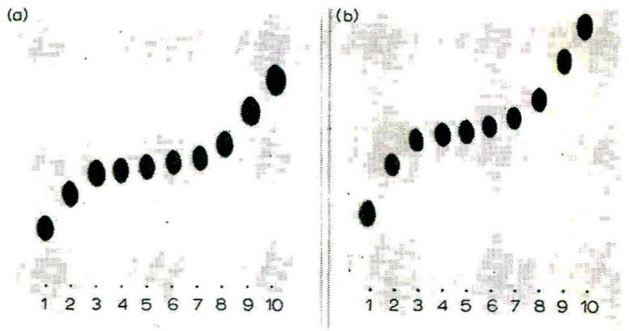

Fig. 2. Trennung von Hypnotika auf Kieselgel HF (Merck). (a) ungesättigte N-Kammer; Fliessmittel, stab. Chloroform–Aceton (9:1); 40% r.F. (b) Vario-KS-Kammer; Fliessmittel, stab. Chloroform–Aceton (9:1); 15 Min. homogene Vorbedampfung; Durchlaufchromatographie, Σ 100 Min.; offener Schieber; 35% r.F. 1 = Heptobarbital, 2 = Phenobarbital, 3 = Cyclobarbital, 4 = Allobarbital, 5 = Butobarbital, 6 = Pentobarbital, 7 = Itobarbital, 8 = Secobarbital, 9 = Hexobarbital, 10 = Methylphenobarbital.

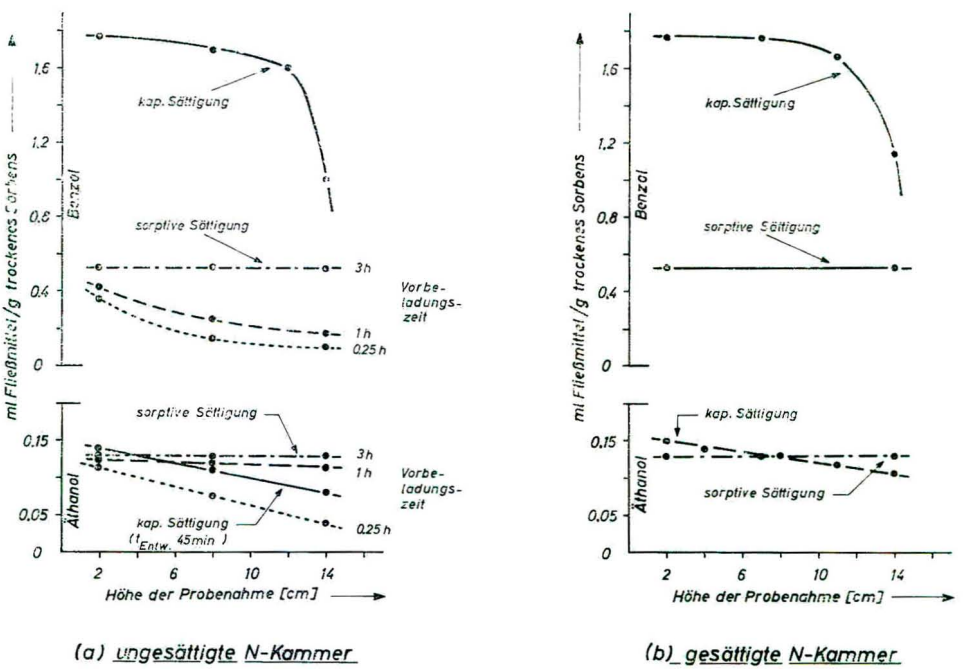

Fig. 3. Fliessmittelkonzentrationen vorbedampfter und entwickelter Kieselgelschichten. Kieselgel G (Merck); Fliessmittel, Benzol–Äthanol (97:3). Sorptive Sättigung: Aus dem gesättigten Gasraum maximal adsorbierbare Fliessmittelmenge. Kapillare Sättigung: Fliessmittelbeladung der Schicht nach der Entwickling. (a) Ungesättigte N-Kammer (Volumen 3 l) ohne Filterpapierauskleidung. Entwicklung darin sofort nach Einfüllen des Fliessmittels begonnen. (b) Gesättigte N-Kammer mit Papierauskleidung.

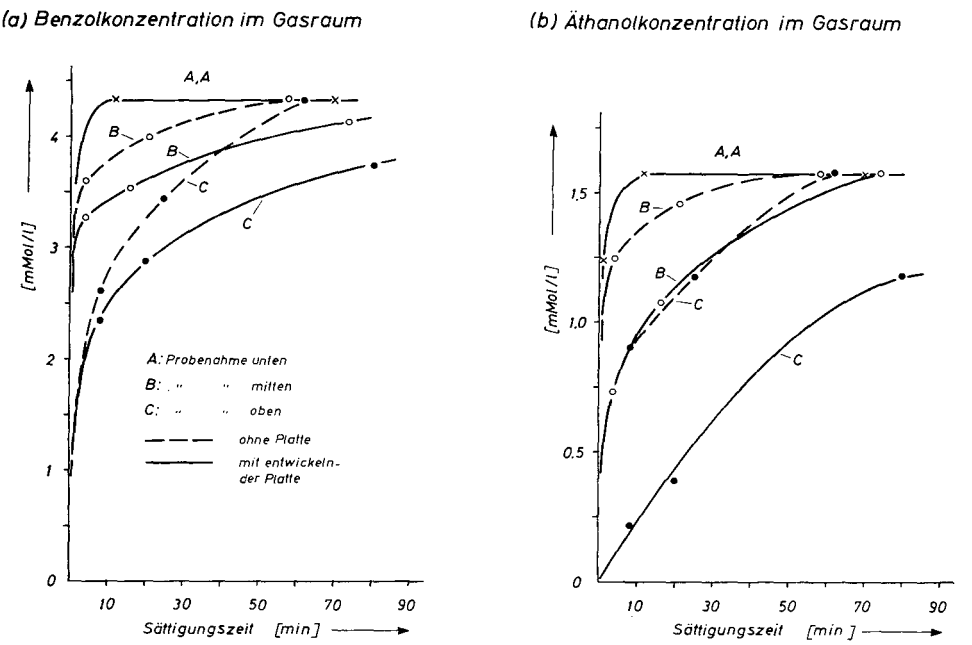

Fig. 4. Kammersättigungen. Probenahmestellen auf Höhe A = 2.5 cm, B = 9 cm und C = 16 cm. Kieselgel G (Merck); ungesättigte N-Kammer; Fliessmittel, Benzol–Äthanol (90:10). (a) Einstellung der Kammersättigung bei Benzol. (b) Einstellung der Kammersättigung bei Äthanol. In der gesättigten Kammer ist die Kammersättigung für beide Komponenten $\leqq$ 5 Min. nach Einfüllen des Fliessmittels und Schütteln der Kammer erreicht.

Literatur vermutet[7] – ein paralleler, sondern ein schwach antiparalleler Gradient ausbildet (Fig. 3, "kapillare Sättigung"). Nach der Entwicklung befindet sich also "oben" auf der Platte weniger Äthanol pro Gramm trockene Schicht als unten. Das bei Ende der Entwicklung gemessene Konzentrationsprofil verlief auch während der Entwicklung analog. Für die Ausbildung des antiparallelen Äthanolgradienten geben wir folgende Erklärung: Für das obere Kammerdrittel verläuft die Sättigungskurve* des Äthanols wesentlich flacher als die des Benzols (Kurve C, durchgezogene Linie in Fig. 4a und b). Dies ist darauf zurückzuführen, dass die unteren und mittleren Teile der Schicht aus dem von der Flüssigkeitsoberfläche aufsteigenden Gasgemisch schon bevorzugt die polarere Komponente herausadsorbieren, und infolgedessen oben ein an Äthanol relativ verarmtes Gemisch ankommt (vgl. Fig. 4a und b). Erst mit dem Erreichen der Kammersättigung – in allen Zonen der Kammer und für alle Komponenten – wird der Gradient ausgeebnet (sorptive Sättigung). Die dazu nötige Zeit (=2 Std.) ist beträchtlich länger als die Entwicklungszeit in der ungesättigten Kammer (45 Min.).

Die oberen Schichtpartien haben zwar – wie richtig festgestellt wurde[7] – eine längere Zeit als die unteren zur Verfügung, während der sie vor dem Eintreffen der

* Der Kammersättigung.

Front mit der Gasphase in Kontakt sind; es steht ihnen aber auch im entsprechenden Gasraumabschnitt weniger Äthanol zur Verfügung als den unteren. Schliesslich darf nicht übersehen werden, dass auch die kapillar aufsteigende Flüssigkeitssäule mit dem davor befindlichen Gasraum in Wechselwirkung tritt, ein Effekt, den wir nicht weiter messend verfolgten.

Aus den Fig. 3a und b lassen sich noch weitere Erkenntnisse über die Verteilung der Fliessmittelkomponenten auf der Schicht in verschiedenen Phasen des chromatographischen Prozesses gewinnen. Es fällt zunächst auf, dass auch in der gesättigten Kammer nach der kapillaren Sättigung noch ein flacher ap-Gradient für Äthanol vorliegt (Fig. 3b).

In der ungesättigten N-Kammer* wird der Gradient der Äthanolvorbeladung mit zunehmender Vorbedampfungszeit flacher. Er ist nach 3 Std. nivelliert. Zu diesem Zeitpunkt ist auch die sorptive Sättigung erreicht (Fig. 3a). Die Werte für die sorptive Sättigung in der gesättigten Kammer (wo sie sich natürlich schneller einstellt) und der ungesättigten Kammer sind naturgemäss gleich, nämlich *ca.* 0.13 ml Äthanol und 0.53 ml Benzol pro Gramm Sorbens. Durch die Entwicklung steigt dann die Benzolkonzentration um den Faktor 3 auf *ca.* 1.8 ml/g an (kapillare Sättigung); interessanterweise bleibt aber die Äthanolkonzentration bei sorptiver und kapillarer Sättigung** etwa gleich. Daraus ist zu schliessen, dass für dieses Fliessmittelgemisch die Gleichgewichtskonzentration des Äthanols in der stationären Phase gegenüber dem flüssigen Fliessmittel einerseits und gegenüber seiner Gasphase andererseits annähernd die gleiche ist.

Sehen wir uns noch einige Zahlenwerte an. Die Äthanolkonzentration, bezogen auf Benzol, beträgt im flüssigen Fliessmittel 3 Vol. %, bei sorptiver Sättigung steigt sie (in der Schicht) auf *ca.* 20% und fällt bei der Entwicklung (kapillare Sättigung) durch den Verdünnungseffekt des aufsteigenden Fliessmittels wieder auf 8%. Infolgedessen sind $8 - 3 = 5\%$ Teil der stationären Phase. Die vorgenannten 0.13 ml Äthanol/g Sorbens im Volumenelement der benetzten Schicht verteilen sich also zu *ca.* 0.09 ml/g auf die stationäre und zu *ca.* 0.04 ml/g auf die mobile Phase. Diese Zahlen gelten wieder nur für den unteren Schichtbereich, wo die mobile Phase sicher die Zusammensetzung des Fliessmittels hat, weil eventuelle "Fronten" der Fliessmittelzusammensetzung schon vorbeigewandert sind.

BRINGEN PARALLELE GRADIENTEN VORTEILE?

Mit der Vario-KS-Kammer lassen sich der Schicht über Vorbedampfung Aktivitätsgradienten verschiedenster Anordnung aufprägen[3,12]. DE ZEEUW hat mit einer Variante unseres KS-Kammertyps ("VP-chamber") die Trennung des Gemisches Indophenol (1), Nitroanilin (2), Sudanrot (3), Buttergelb (4) studiert[10], wobei er durch abwechselnde Vorbedampfung mit Benzol und Chloroform über Brems- und Beschleunigungszonen einen (über alles) parallelen Gradienten einstellte und durchlaufend entwickelte. Der damit erhaltenen guten Trennung (Fig. 6b) stellte er schlechtere Chromatogramme, erhalten in gesättigten N-Kammern mit reinem Benzol bzw. Benzol–Chloroform, gegenüber und zog daraus den Schluss, dass die Trennverbesserung

* Die Platte wurde sofort nach Einfüllen des Fliessmittels eingestellt und ohne Eintauchen der Schicht, d.h. ohne Entwicklung, vorbedampft.

** Gesättigte Kammer, sofortiger Entwicklungsbeginn, unterer Schichtbereich.

auf den (über-alles-)p-Gradienten und die spezielle Kombination von Beschleunigungs- und Bremströgen zurückzuführen sei.

Dieser Vergleich bringt hinsichtlich der beobachteten Trennleistung insofern kein objektives Bild, als die ausgenützten Trennstrecken bei den beiden Arbeitstechniken sehr verschieden sind; d.h. die Durchlaufchromatographie muss – bei kleinen R_F (< 0.4) und sonst gleichen Bedingungen – immer die bessere Trennung bringen.

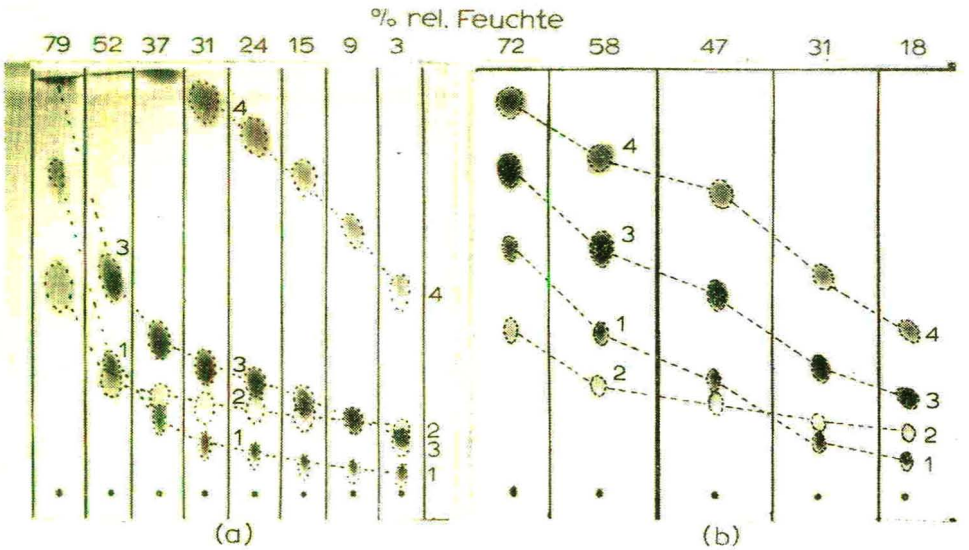

Fig. 5. Trennung von Indophenol (1), Sudanrot (2), *p*-Nitroanilin (3) und Buttergelb (4) auf Kieselgel G (Merck). Vario-KS-Kammer; Feuchte-o-Gradient; Fliessmittel: (a) Benzol; Durchlaufchromatographie, (b) reines Chloroform.

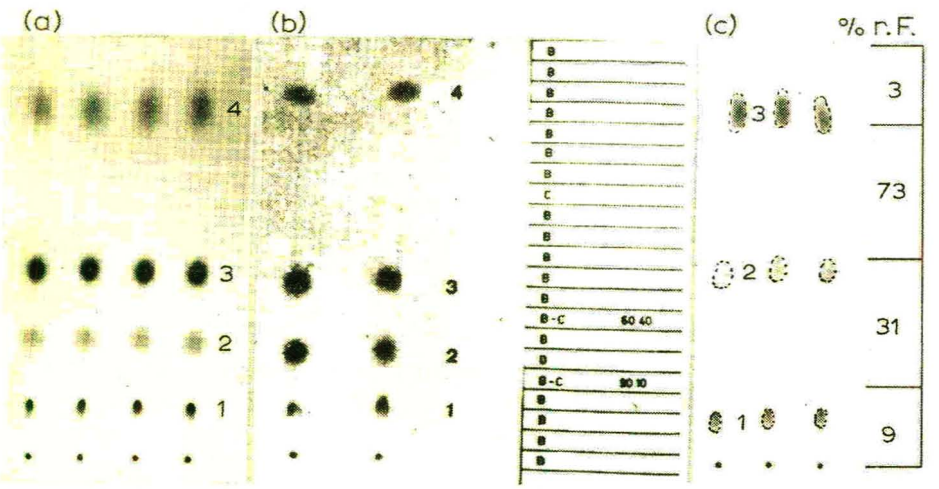

Fig. 6. Variierte Trennung des Farbstoffgemisches von Fig. 5 auf Kieselgel G (Merck). (a) KS-Variokammer; Fliessmittel, reines Chloroform; 24% r.F.; Durchlaufchromatographie, 160 Min. (b) Reproduktion Chromatogramm VP-Chamber von DE ZEEUW; Fliessmittel, Benzol; 29% r.F.; Vorbedampfung wie angezeichnet. (c) Vario-KS-Kammer; Fliessmittel, Benzol; Aktivitätsverteilung wie angezeichnet. Manipulation der Trennung (a) in Fig. 5. Die Trennung von Fig. 6a wurde im Gegensatz zu (b) ohne jeden Gradienten erzielt.

Beim Nacharbeiten dieser Trennbeispiele zeigte sich, dass sowohl mit reinem Chloroform als auch mit reinem Benzol als Fliessmittel ohne jeglichen Aktivitätsgradienten in Fliessrichtung, d.h. ohne jede Vorbedampfung mit Benzol oder Chloroform, ausgezeichnete Trennungen möglich sind, wenn man nur die richtige Arbeitsfeuchte, d.h. die richtige Aktivität, wählt. Dies illustrieren die Fig. 5a und b.

Mit *Benzol* als Fliessmittel (Fig. 5a) ist die Trennung in der Reihenfolge (v. oben nach unten) 4–3–2–1 nur zwischen 24–37 % r.F. möglich (DE ZEEUW: 29 % r.F.). Bei *ca.* 45 % r.F. können 1 und 2, bei 9 % r.F. können 2 und 3 nicht voneinander getrennt werden. Oberhalb 45 % r.F. ist die Reihenfolge 4–3–1–2, unterhalb 9 % r.F. 4–2–3–1.

Mit *reinem Chloroform* als Fliessmittel ist eine perfekte Trennung auch ohne Durchlaufchromatographie möglich und zwar, wie wieder der orthogonale Aktivitätsgradient der Fig. 5b zeigt, oberhalb 47 % r.F. in der Reihenfolge 4–3–1–2 und unterhalb 30 % in der Reihenfolge 4–3–2–1. Bei etwa 40 % r.F. ist keine Trennung möglich. Die Vario-KS-Kammer gestattet, den gesamten Aktivitätsbereich auf der gleichen Platte mittels eines Quergradienten ("o-Gradient" in der Terminologie von NIEDERWIESER) durchzutesten. Im vorliegenden Fall kann man sich zudem aussuchen, in welcher Reihenfolge man die Substanzen getrennt haben will. Die Trennung bei 24 % ist – aus optischen Gründen – mit der Durchlaufchromatographie weiter herausentwickelt worden (Fig. 6a) und unterscheidet sich kaum von dem Bild von DE ZEEUW (Fig. 6b). Alle nebeneinanderliegenden Teilbilder der Fig. 5b kann man auch einzeln (und mit *ca.* 40 % geringeren R_F-Werten) in der gesättigten N-Kammer erhalten, wenn nur die jeweils erforderliche Luftfeuchtigkeit herrscht.

Gradienten in Fliessrichtung können in der Praxis durchaus von Nutzen sein, z.B. wenn man die (aufgrund anderer Effekte getrennten) Substanzen aus irgendeinem Grund gleichmässiger auf die Trennstrecke verteilen will. Einige Beispiele dazu findet man bei Lit. 3. Einen Fall von extremer Manipulation der Flecklage zeigt Fig. 6c. Die drei Flecken (3, 2 und 1) sind hier optimal über die Trennstrecke verteilt;

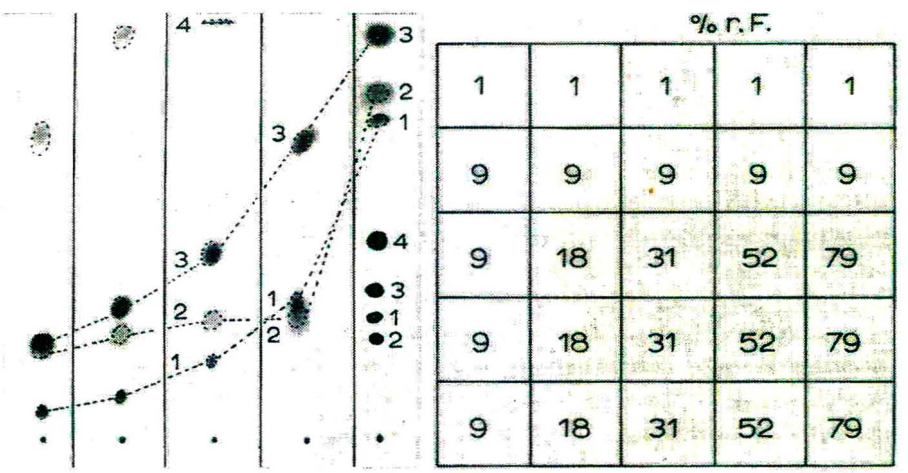

Fig. 7. Trennung der Farbstoffe von Fig. 5 auf Kieselgel G (Merck). Vario-KS-Kammer, Mosaikeinsatz; Fliessmittel, Benzol; Durchlaufchromatographie, 3 Std. Bei der Trennung ganz rechts kam es während der Entwicklung zu einer Inversion. In der Zone niedriger Aktivität (79% r.F.) war die Reihenfolge 2–1–3–4, in der oberen Zone (9; 1% r.F.) wurde sie dann zu 1–2–3–(4) umgekehrt.

jedoch handelt es sich nur um eine Scheinverbesserung der Trennung von Fig. 6a und zu einem bestimmten Grade auch der von Fig. 5b, ganz rechts: Eine schon bestehende Grundtrennung wurde nur (mehr oder weniger proportional) gedehnt. Das bedeutet nicht, dass in den vergrösserten Zwischenräumen auch mehr zu trennende Verbindungen aufgenommen werden können.

In Fig. 7 sind die chromatographischen Bedingungen der Fig. 5a etwas abgewandelt worden: Um zu vermeiden, dass die obersten Flecken bei der Durchlaufchromatographie an den Rand hinauswandern (was für Buttergelb (4) dann doch nicht ganz verhindert werden konnte), wurde mit den beiden oberen Trogreihen durch sehr niedrige Feuchten gebremst (Überlagerung eines orthogonalen durch einen antiparallelen Gradienten). Die interessanteste Beobachtung bei diesem Versuch war, dass sich die Fleckreihenfolge innerhalb der gleichen Reihe (ganz rechts) während der Entwicklung umkehrte: Über den drei untersten Trögen (79–79–79 % r.F.) wurde aufgetrennt in der Reihenfolge 4–3–1–2. Beim Überstreichen des ap-Gradienten 79–9 % stellten sich wieder die Aktivitätsbedingungen ein, die zur Reihenfolge (4)–3–2–1 führten.

Die beobachteten Inversionen sind auf die relative Unempfindlichkeit der R_F-Werte des *p*-Nitroanilins (2) gegen Aktivitätsänderungen zurückzuführen. Am Rande sei vermerkt, dass diese Inversionen nur bei gipshaltigen Kieselgelen auftreten. Auf gipsfreien Kieselgel-Schichten erhält man bei allen Feuchten die Reihenfolge 4–3–2–1.

IST CHLOROFORM GLEICH CHLOROFORM?

Handelsförmigem Chloroform sind zur Stabilisierung einige Prozente Äthanol beigemischt. Der wesentlich elutionskräftigere Alkohol verändert dabei radikal die chromatographischen Eigenschaften, die reinem Chloroform eigen sind, eine Tatsache, die in der Praxis häufig übersehen oder missachtet wird*. Wir haben früher[12] schon einmal darauf beruhende Scheinanomalien des Chloroforms aufgeklärt. Zur Illustration dieses Effektes seien hier noch einige Beispiele angeführt:

(1) Wäre die Platte, die zum Chromatogramm der Fig. 5b führte, nicht nur mit reinem Chloroform entwickelt sondern auch vorbedampft worden, hätte man bei unveränderter Trennung lediglich um 30–40 % niedrigere R_F-Werte erhalten. Wird indessen mit stabilisiertem Chloroform vorbeladen (und entwickelt), so wird der unterlegte orthogonale Aktivitätsgradient völlig eingeebnet; ausserdem bricht die Trennung von 4–3–1 zusammen (Fig. 8).

(2) In der Literatur ist die Feststellung zu finden[13], es spiele bei der Trennung der vorerwähnten Hypnotika keine Rolle, ob reines oder stabilisiertes Chloroform als Fliessmittel verwendet werde. Die Fig. 9a und b zeigen das Gegenteil. In der gesättigten N-Kammer wird die Platte wieder durch den Alkoholanteil des Chloroforms bei der Vorbeladung desaktiviert, weshalb die Substanzen wesentlich höher laufen als mit reinem Chloroform. Ein Vergleich der Fig. 9a und b mit denen der Literatur macht deutlich, dass dort mit stabilisiertem Chloroform entwickelt wurde. Die Bilder 9a und b zeigen schliesslich, dass die R_F-Werte der Hypnotika mit zunehmender

* *Editor's note*: Already in the first separations of acetylamino acids on silica gel columns by MARTIN *et al.* the impurities in the chloroform used played an essential role.

Feuchte, d.h. fallender Aktivität, wie zu erwarten steigen, was ebenfalls im Widerspruch zu Angaben der Literatur[13] steht, nach denen die R_F-Werte durch ein Maximum gehen sollen.

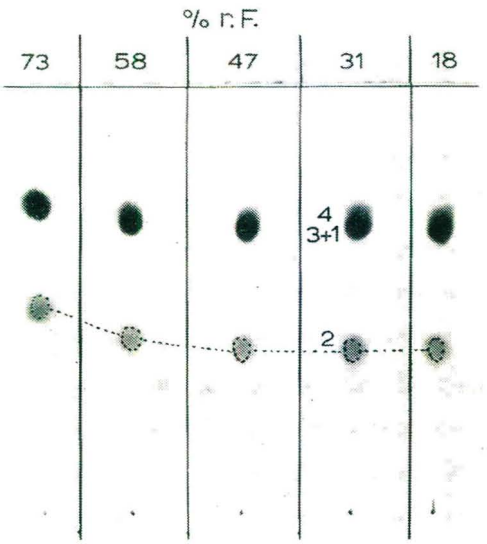

Fig. 8. Trennung der Farbstoffe von Fig. 5 auf Kieselgel G (Merck). Vario-KS-Kammer, orthogonaler Aktivitätsgradient; Vorbedampfung mit stabilisiertem Chloroform (30 Min.); Fliessmittel, reines Chloroform. Das Chromatogramm ist zu vergleichen mit Fig. 5b. Die in Chloroform enthaltenen 1–2% Äthanol haben die Aktivitätsstufen eingeebnet und ausserdem die Trennung verdorben.

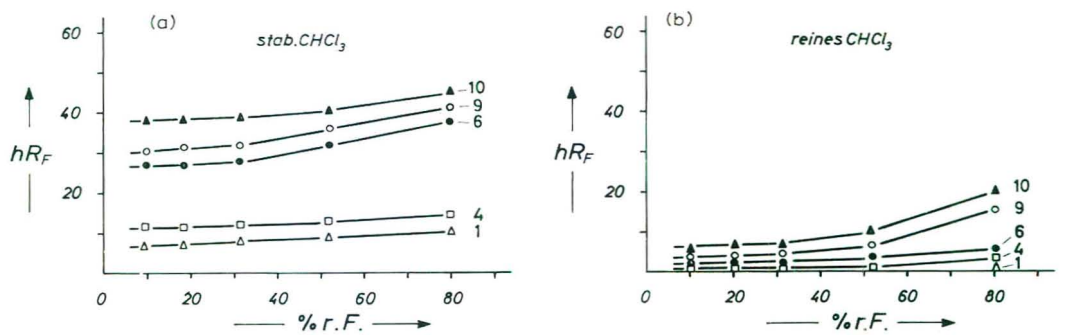

Fig. 9. Einfluss der Feuchte (Aktivität) und der Reinheit des Chloroforms auf die Chromatographie von Hypnotika. Kieselgel HF (Merck); Vario-KS-Kammer; orthogonaler Aktivitätsgradient; 10 Min. Vorbedampfung mit dem Fliessmittel. Bezeichnung der Substanzen wie in Fig. 2. Fliessmittel: (a) stabilisiertes Chloroform, (b) reines Chloroform.

EXPERIMENTELLER TEIL

Die Platten wurden in üblicher Weise gestrichen und getrocknet. Die meisten Chromatogramme wurden mit der Vario-KS-Kammer (Hersteller Fa. Camag, Muttenz, Schweiz) entwickelt. Das Handelschloroform wurde durch Filtration über eine Aluminiumoxidsäule (Aktivität I Brockmann) äthanolfrei (< 0.05 %) gemacht (GC-

Kontrolle auf 1 m-Polypak-1-Säule, 170°). Die Bestimmung der Zusammensetzung der mobilen Phase geschah auf folgende Weise: Mit einem verschliessbaren Schabelöffel wurden von in N-Kammern befindlichen Platten von der trockenen oder feuchten Schicht durch Öffnungen im Deckel je etwa 30 mg-Proben entnommen und in 3 ml N,N-Dimethylformamid eingebracht. Die Lösungen wurden gaschromatographisch auf einer 1 m-Polypak-Säule bei 170° analysiert. Die entnommene Menge Sorbens wurde nach Abdampfen des Lösungsmittels (bei 120°) und Klimatisierung um 50 % r.F. ausgewogen. Genauigkeit des Gesamtverfahrens ± 5–10 % rel.

DANK

Wir danken Frau E. MARAFANTE für die sorgfältige Ausführung der Versuche.

ZUSAMMENFASSUNG

Es wird gezeigt, dass einige in der Literatur beschriebene spezielle Trennungen (Hypnotika, Farbstoffe) wahrscheinlich nicht durch parallele Vorbedampfungsgradienten sondern durch andere Effekte, wie günstigen Wassergehalt der Schicht, erhöhten Fliessmitteldurchsatz zustande gekommen sind und dass man in allen geprüften Fällen ohne Gradienten gleich gute Resultate erhalten kann. Bei der Entwicklung von Chromatogrammen mit Fliessmittelgemischen aus zwei Komponenten unterschiedlicher Polarität bildet sich in ungesättigten N-Kammern entgegen früheren Annahmen kein paralleler sondern ein schwach antiparalleler Gradient aus (Beispiel: Benzol–Äthanol, 97:3). Am Beispiel der Hypnotika-Trennung wird erneut dargelegt, dass es keineswegs gleichgültig ist, ob man mit reinem oder mit äthanolstabilisiertem Chloroform chromatographiert.

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CHROMATOGRAPHIC PROPERTIES OF SOME CYCLIC α-IMINO ACIDS HOMOLOGOUS TO PROLINE, AND THEIR DNP-, DNS- AND PTH-DERIVATIVES

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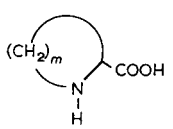
SUMMARY

The chromatographic behavior of the homologous cyclic α-imino acids of ring sizes ranging from four- to eleven-members (including proline) on ion-exchange and thin-layer systems is described. The validity of the Martin relation in the homologous series was verified. This was further cogently demonstrated by the "non-fit" of the isomeric but non-homologous α-imino acid 4,4 dimethylproline. The 2,4-dinitrophenyl- (DNP-), 5-dimethylaminonaphthalenesulfonyl- (DNS-), and phenylthiohydantoin- (PTH-) derivatives of these imino acids were also separated by TLC. Within certain limitations, the Martin derivation also appeared to be linearly related to the number of methylene groups in some of these derivatives.

INTRODUCTION

We have recently described[1] the synthesis of a homologous series of α-imino acids (*1d–1i*) which represent the higher membered ring homologs of L-proline (*1c*), the naturally occurring α-imino acid of protein origin. Whereas the occurrence in the plant kingdom of the six-membered pipecolic acid (*1d*) and its analogs have been adequately documented[2–4], the possible presence in nature of the homologous medium ring compounds, namely hexahydroazepine-2- carboxylic acid (*1e*), octahydroazocine-



1a $m = 1$ *1f*, $m = 6$
b, $m = 2$ *g*, $m = 7$
c, $m = 3$ *h*, $m = 8$
d, $m = 4$ *i*, $m = 9$
e, $m = 5$

2-carboxylic acid (*1f*), octahydroazonine-2-carboxylic acid (*1g*), decahydroazecine-2-carboxylic acid (*1h*), and azacycloundecane-2-carboxylic acid (*1i*), respectively the

seven-through-eleven-membered cyclic α-imino acids, has not been investigated, although a rational biochemical basis for their occurrence cannot presently be advanced.

The very first member* of this cyclic α-imino acid series, aziridine-2-carboxylic acid (*1a*), is strictly a synthetic chemical entity known to be unstable and therefore not isolable except as the ester or as salts[5,6]. The next higher homolog, L-azetidine-2-carboxylic acid (*1b*), is relatively stable, has been isolated from, and constitutes the major soluble nitrogenous nonprotein component of the Liliacae[7–9]. *1b*, a proline antagonist which inhibits protein synthesis, substitutes for L-proline in the protein of *Escherichia coli* and of the seedlings of the mung bean, *Phaseolus aureus*[10]. *1b* is also incorporated into the actinomycin molecule when added to the culture medium of *Streptomyces antibioticus* or of *S. chrysomallus* to give new biosynthetic actinomycins[11].

The possibility that α-imino acids of medium ring size might occur as natural constituents or metabolites of plants or of other organisms does not appear so tenuous if one considers the wide variety of structurally unique amino acids of non-protein origin that have been discovered in recent years[2–4]. For example, the imino acid of unknown structure recently isolated by BLUNDEN AND CHALLEN[12] from *Salix fragilis* leaf galls might possibly correspond to one of these in our series, although additional evidence is obviously required. In order to facilitate their detection and identification, if and when their presence is suspected, we have prepared a number of derivatives of these homologous α-imino acids, namely, the DNP-, DNS- and PTH-derivatives, and herewith record their chromatographic properties in TLC systems. The chromatographic properties of the parent α-imino acids are also described.

EXPERIMENTAL

The DNP- and DNS-derivatives of the imino acids were prepared by slight modifications of procedures described in standard reference works[13,14]; however, the preparative procedure for the imino acid phenylthiohydantoins differed from the usual methods. 4,4-Dimethyl-DL-proline, a proline analog where the 4-position (the position where proline, as peptidyl proline, is usually hydroxylated) is blocked by methyl groups, was also synthesized in the course of this work**. L-Azetidine-2-carboxylic acid, L-proline, the latter's DNP- and DNS-derivatives, and L- and DL-pipecolic acids were purchased from Calbiochem (Los Angeles, Calif.), Nutritional Biochemicals Corp. (Cleveland, Ohio), or Aldrich Chemical Co. (Milwaukee, Wisc.).

Paper chromatography was accomplished on Whatman No. 1 paper using the descending technique. The availability of commercial pre-coated TLC plates dictated their selection for thin-layer chromatography in order that others wishing to duplicate our results would not be faced with quality differences of homemade plates as they may exist in different laboratories. However, laboratory-coated TLC plates were also used. Selection of solvent systems and adsorbants were based on (a) their ability to separate each member of the homologous series, and (b) where possible, previous history of their use for separation and identification of amino acids in protein hydroly-

* Glyoxalimine (*1*, $m = 0$), theoretically the lowest member of this series, is not a ring system in the classical sense and will not be considered.

** The description of these procedures as well as certain other analytical data will be presented elsewhere.

sates[14,15], in consideration of possible future application for contemplated biochemical studies. For ion-exchange chromatography of the free α-imino acids a Beckman-Spinco Model 120 Amino Acid Analyzer, column size 0.9 × 63 cm with AA-15 resin, flow rate 18.6 ml/h was used. The sodium citrate buffers were of pH 3.28 (± 0.01) and 4.25 (± 0.01), and the buffer change was programmed to take place at 90 min. The mixture applied contained 1 μmole of each imino acid and 0.1 μmole each of the internal markers, aspartic acid, glycine and tyrosine, so that a direct visual comparison of the relative extinctions of the ninhydrin generated chromophores at 440 and 570 mμ could be made by inspection. For visualization of the free α-imino acids, the paper or thin-layer chromatograms were sprayed in the usual manner with 0.3 % ethanolic ninhydrin reagent which is available commercially in aerosol dispensers. Proline and dimethylproline gave yellow colors, and pipecolic acid the usual violet color. All the others in the homologous series gave mauve colors, although the seven membered *1e* had a somewhat yellowish tinge. The DNP-derivatives were all visible as yellow spots (they were also fluorescencequenching when the TLC adsorbant also contained a UV fluorescing background), while the DNS-derivatives all fluoresced bright yellow. The PTH-derivatives were visualized under a UV light source by quenching of the fluorescent background. The limits of detectability were of the same order of magnitude reported for the DNP-, DNS and PTH-derivatives of other amino acids[14].

RESULTS AND DISCUSSION

Chromatographic separation of the free α-imino acids

Fig. 1 shows the elution profile of the homologous series of cyclic α-imino acids from the 4-membered L-azetidine-2-carboxylic acid (*1a*) to the 11-membered azacycloundecane-2-carboxylic acid (*1i*) from an ion-exchange column. Not surprisingly, the imino acids were eluted in the order of increasing molecular weights. The positions of the individual imino acids were checked by subjecting each one, singly, to ion-

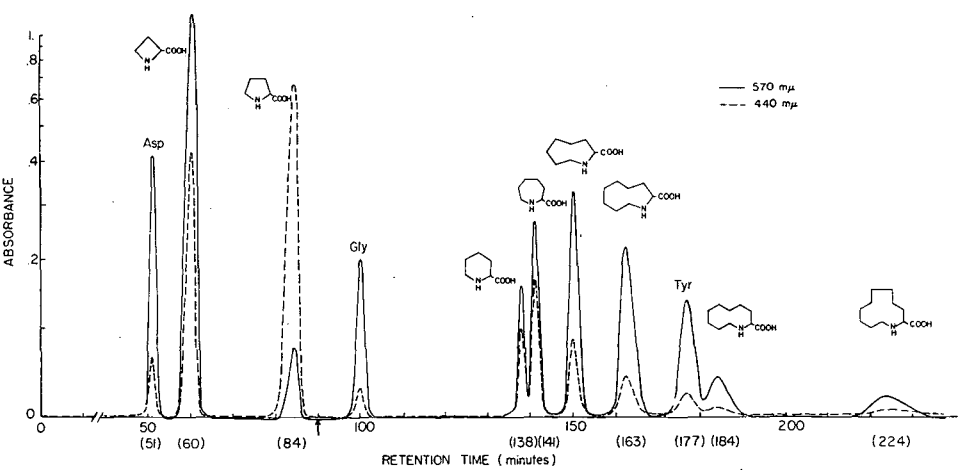


Fig. 1. Chromatographic behavior of the homologous series of cyclic α-imino acids containing four to eleven members in the ring on an ion-exchange column. Their retention times can be compared to that of the marker amino acids, aspartic acid, glycine and tyrosine. Buffer change at the arrow.

exchange chromatography. No overlaps or crossovers were noted. The considerable lag in the elution time with concomitant broadening of the peak of the highest molecular weight member of this series, *viz.*, azacycloundecane-2-carboxylic acid (*1i*), is due largely to its molecular size and lipophilicity since its p*K*a (10.1) is not much different from that of the 9-membered octahydroazonine-2-carboxylic acid (*1g*, p*K*a 10.5). The marker amino acids, aspartic acid, glycine and tyrosine served to orient the positions of some of the naturally occurring amino acids to the elution profile of the cyclic imino acids.

A number of paper and TLC systems were used to separate this homologous series of α-imino acids, and these are recorded in Table I. The relatively long periods required for a run (up to 18 h) and the observed broadening of the applied spots make paper chromatographic separations less appealing than the TLC method.

TABLE I

SEPARATION OF HOMOLOGOUS CYCLIC α-IMINO ACIDS BY PAPER AND THIN-LAYER CHROMATOGRAPHY

Paper chromatography on Whatman No. 1 paper; TLC on Silica Gel HF_{254}, laboratory-coated (solvent F), or precoated Silica Gel F_{254} plates (E. Merck, A.G., distributed by Brinkmann Instruments Inc., Westbury, N.Y.) (solvent G). Solvents: A = acetic acid–*tert.*-amyl alcohol–H_2O (1:10:2); B = *tert.*-amyl alcohol sat'd with H_2O; C = butyl acetate–ethanol–triethylamine–H_2O (5:5:2:3); D = 2-butanone–*n*-butyl alcohol–triethylamine–H_2O (10:10:2:5); E = 2,6-lutidine–*tert.*-amyl alcohol sat'd with H_2O (1:1) (ref. 16); F = *n*-butyl alcohol–acetic acid–H_2O (4:1:1); G = *n*-propyl alcohol–H_2O (7:3). Development time: 18 h for solvents A, B and E; 7 h for solvent C; 6 h for solvent D; ~3 h for solvents F and G.

Number of methylene groups (m)	*α-Imino acid*	*R_F values in solvents*						
		Paper chromatography					*TLC*	
		A	*B*	*C*	*D*	*E*	*F*[a]	*G*[b]
2	L-Azetidine-2-carboxylic acid	0.05	0.04	0.13	0.05	0.16	0.15	0.30
3	L-Proline	0.09	0.07	0.19	0.06	0.23	0.17	0.36
4	DL-Pipecolic acid	0.15	0.13	0.28	0.10	0.29	0.22	0.43
5	Hexahydroazepine-2-carboxylic acid	0.23	0.20	0.34	0.17	0.39	0.28	0.51
6	Octahydroazocine-2-carboxylic acid	0.32	0.28	0.42	0.27	0.46	0.33	0.56
7	Octahydroazonine-2-carboxylic acid	0.40	0.39	0.52	0.42	0.55	0.39	0.62
8	Decahydroazecine-2-carboxylic acid	0.46	0.49	0.69	0.64	0.63	0.45	0.67
9	Azacycloundecane-2-carboxylic acid	0.56	0.57	0.75	0.73	0.70	0.52	0.72
4	4,4-Dimethyl-DL-proline	—	—	—	—	—	—	0.55[a]

[a] Average of 6 determinations S.D. ≤ 0.02

[b] Average of 12 determinations. S.D. ≤ 0.02.

The presence of a unique ring homology here prompted us to evaluate the validity of the Martin relation[14] in this series of α-imino acids. As can be seen from Fig. 2, the Martin derivation, R_M*, was indeed a linear function of the number of methylene groups, *m*, in the imino acid molecule. Surprisingly, this relationship held true in a ternary solvent system F, as well as in the binary system G. This is indicative of minimal solvent demixing in these TLC systems. Extension of the straight lines in both directions allows the prediction of the R_F values of the lower and higher membered homologs of this series. Thus, the lowest cyclic homolog of proline, aziridine-2-carboxylic acid (*1a*), may be expected to have R_F values of 0.11 of 0.26 in solvent systems

* Related as the function of R_F as follows: $R_M = \log (1/R_F - 1)$.

F and G, respectively. The instability of this imino acid in the free form renders this prediction purely academic; nevertheless, the possibility remains that certain of its more stable derivatives might well relate similarly when compared to the corresponding derivatives of the higher members. The larger ring homologs, namely, those α-imino acids containing ten, eleven or twelve methylene groups are expected to be chemically more stable, and similar projections might give, for example, R_F values of 0.78 and 0.59 in solvents F and G for the as yet unreported azacyclododecane-2-carboxylic acid (I, $m = 10$).

It is noteworthy that 4,4-dimethylproline, a dialkylated proline which is isomeric with the 7-membered hexahydroazepine-2-carboxylic acid (*Ie*) and which may be considered to contain four methylene groups in the molecule, did not fall on the straight line defined by the Martin relation. In fact, it behaved in solvent G as though it contained *six* methylene groups (however, *vide infra*).

TLC of the homologous DNP-, DNS- and PTH-α-imino acids

Table II lists the R_F values of the homologous series of DNP-, DNS- and PTH-α-imino acids in a number of TLC systems. DNP-DL-Pipecolic acid behaved quite anomalously in the two systems described, its R_F values deviating considerably from that expected for its position in the homologous series. DNP-L-Pipecolic acid behaved similarly. Sampling errors were eliminated as possible causes. This deviation

TABLE II

SEPARATION OF DNP-, DNS- AND PTH-DERIVATIVES OF HOMOLOGOUS CYCLIC α-IMINO ACIDS BY TLC

Silica Gel F_{254}, pre-coated plates for the DNP-, pre-coated silica gel without fluorescent indicator (E. Merck, A.G.) for the DNS-imino acids, and MN-Polyamid-DC 11 UV_{254} (Macherey, Nagel and Co., distributed by Brinkmann Instruments, Inc.), laboratory-coated plates, for the PTH-derivatives. The DNS-imino acids were applied as piperidinium salts, except for DNS-dimethylproline which was applied as the cyclohexylamine salt. Solvents: H = benzene–pyridine–acetic acid (40:10:1); I = 95% ethanol–benzene–H_2O (95:125:10); J = abs. ethanol–benzene–H_2O (95:125:10); K = heptane–*tert.*-butyl alcohol–acetic acid (20:5:8); L = carbon tetrachloride–benzyl alcohol–acetic acid (7:2:1); M = *n*-propyl alcohol–H_2O (1:2); N = abs. ethanol–H_2O (1:1); O = methanol–H_2O (2:1).

Number of methylene groups (m)	*Derivative of*	*R_F values in solvents*								
		DNP			*DNS*		*PTH*			
		H[a]	*I*[b]	*J*[c]	*K*[a]	*L*[a]	*M*[d]	*N*[d]	*O*[d]	
2	L-Azetidine-2-carboxylic acid	0.11	0.36	0.34	0.17	0.37	0.40	0.48	0.55	
3	L-Proline	0.21	0.41	0.41	0.24	0.48	0.39	0.46	0.54	
3	DL-Proline	0.21	0.42	0.41	0.24	0.48	—	—	—	
4	DL-Pipecolic acid	0.56	0.59	0.63	0.39	0.66	0.36	0.43	0.51	
5	Hexahydroazepine-2-carboxylic acid	0.49	0.55	0.58	0.42	0.65	0.27	0.32	0.41	
6	Octahydroazocine-2-carboxylic acid	0.59	0.63	0.66	0.48	0.71	0.23	0.26	0.34	
7	Octahydroazonine-2-carboxylic acid	0.68	0.68	0.72	0.53	0.76	0.19	0.21	0.28	
8	Decahydroazecine-2-carboxylic acid	0.74	0.73	0.78	0.56	0.78	0.15	0.15	0.21	
9	Azacycloundecane-2-carboxylic acid	0.75	0.73	0.80	0.58	0.81	0.12	0.11	0.15	
4	4,4-Dimethyl-DL-proline	0.36	0.49	0.52	0.37	0.59	0.25	0.30	0.41	

[a] Average of 8 determinations. S.D. ≤ 0.02.
[b] Average of 2 determinations. S.D. ≤ 0.02.
[c] Average of 3 determinations. S.D. ≤ 0.02.
[d] Average of 5 determinations. S.D. ≤ 0.02.

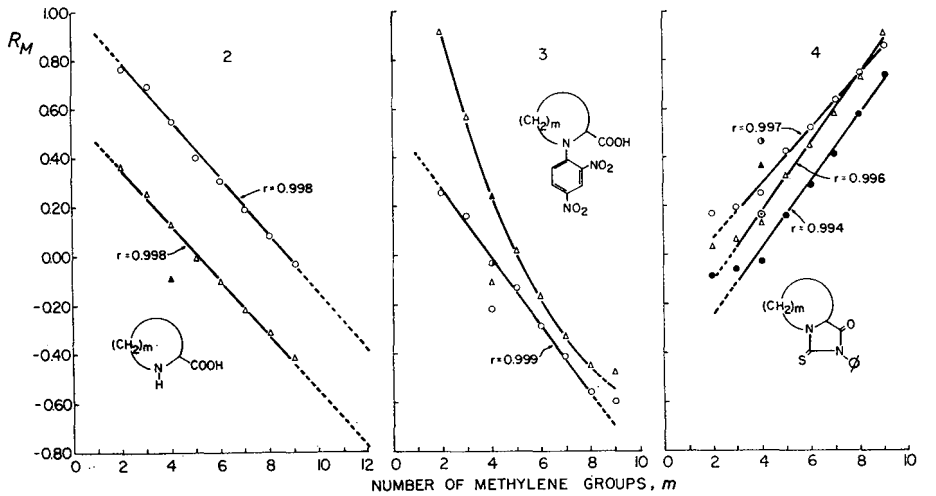


Fig. 2. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the α-imino acid molecule represented by the inset structure. ○—○ = Chromatographed in solvent F; △—△ = solvent G. ▲ = 4,4-Dimethyl-DL-proline in the latter system. The lines were drawn using the method of least squares; the correlation coefficients (r) are indicated. From the dashed extensions the theoretical R_M values for the yet unreported series of α-imino acids with 2, 10, 11, or 12 methylene groups can be found.

Fig. 3. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the homologous DNP-α-imino acids. △—△ = Solvent H; ○—○ = solvent J. ▲ and ◑ = DNP-derivative of 4,4-dimethyl-DL-proline in solvents H and J, respectively. Note that it is not possible to extend the line beyond $m = 9$ since linearity is not preserved here. The straight line was drawn using the method of least squares except that points at $m = 4$ and $m = 9$ (for DNP-pipecolic acid and DNP-azacycloundecane-2-carboxylic acid, respectively) were not included; the curved line was drawn through the points △ (except at $m = 4$, and $m = 9$).

Fig. 4. The Martin derivation (R_M) plotted as a function of the number of methylene groups (m) in the α-imino acid PTH-derivatives. ○—○ = solvent M; △—△ = solvent N; ●—● = solvent O. ◑, ▲ and ⊙ = PTH-derivative of 4,4-dimethyl-DL-proline in solvents M, N and O, respectively. The experimental points at $m = 2$ were not included in drawing the straight lines.

is reflected in the plot of R_M *vs.* number of methylene groups in the molecule (Fig. 3). Although a non-linear relation is observed for the homologous series in solvent H, a fairly linear relation obtains in solvent J. Both DNP-pipecolic acid ($m = 4$) and DNP-azacycloundecane-2-carboxylic acid ($m = 9$) fell outside their expected positions on the straight line. On the other hand, the points for DNP-dimethylproline coincided on the line (assuming four methylene groups) *in the expected positions for DNP-pipecolic acid* and might easily be mistaken for the latter. This appears to be purely fortuitous, as, it is recalled, dimethylproline itself as the free imino acid did not behave in this manner (see Fig. 2 above).

The PTH-derivatives of the homologous α-imino acids were not easily separable on TLC plates of silica gel, alumina or cellulose, and homologs differing by one methylene group overlapped each other in the variety of solvent systems tried. Efficient separations were achieved, however, on a polyamide matrix (Table II) (*cf.* WANG *et al.*[17]). The behavior of the PTH-derivatives on polyamide plates was reminiscent of reversed-

phase paper chromatography[18] in that the smaller molecular weight, less lipophylic members of the homologous series moved faster than the larger molecular weight highly lipophylic members. The Martin relation was again found to be valid (Fig. 4), and the correlation was quite satisfactory if the phenylthiohydantoin of azetidine-2-carboxylic acid ($m = 2$) was excluded.

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THIN-LAYER CHROMATOGRAPHY OF MALTO-OLIGOSACCHARIDES*

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SUMMARY

Procedures for the separation, location and elution of malto-oligosaccharides are described. Best separations were obtained on glass plates (20 × 46 × 0.5 cm) coated with Kieselguhr G, serially developed in solvents of varying proportions of 1-butanol, pyridine and water. Major zones were temporarily visualized by exposing the plates to iodine vapors. The located zones were eluted from the thin-layer plates into fiberglass filter pieces with water. Radioactivity of the zone can be determined directly by placing the filter in a toluene-based scintillation fluid and counting in a scintillation counter. After radioactivity determination, the carbohydrates can be eluted from the filter with water and quantitatively measured.

INTRODUCTION

A procedure for the separation, recovery and determination of both carbohydrate content and radioactivity of malto-oligosaccharides was required as part of a study of *in vivo* starch synthesis[1]. Paper chromatographic separations require several days, and generally are only capable of separating malto-oligosaccharides of degree of polymerization (DP) below 10 (*cf.* refs. 2 and 3). Several thin-layer chromatographic procedures have been reported[4–6]; but these procedures have been designed primarily for the separation and qualitative or quantitative measurement of the fractions directly on the thin-layer plate[5]. This communication describes a thin-layer chromatographic system suitable for the separation of malto-oligosaccharides with DP of up to 20–25. A convenient procedure for the location and elution of zones will be described.

* Purdue Agricultural Experiment Station Journal Paper No. 3711.
** This study was begun when one of us (J.C.S.) was a visiting professor of Horticulture at The Pennsylvania State University, University Park, Pa.

MATERIALS AND METHODS

Equipment

The Shandon 500 Chromatank* chambers were obtained from Colab Laboratories, Inc., Chicago Heights, Ill. Carbohydrates were streaked on the thin-layer plates with a Micro Spray Pistol obtained from Brinkmann Instruments, Inc., Westbury, N.Y. A fixed thickness applicator was also obtained from Brinkmann Instruments, Inc.

Materials

Kieselguhr G was a product of E. Merck, Darmstadt, G.F.R. Solvents were of reagent grade and used as received. The malto-oligosaccharide mixture, prepared by partial acid hydrolysis of amylose, was a gift from Dr. H. F. ZOBEL.

Procedures

A Kieselguhr G layer 250 μ thick was spread on glass plates 20 $\times$ 46 $\times$ 0.5 cm. The plates were allowed to dry at room temperature overnight prior to use. The malto-oligosaccharide mixture was solubilized in 90% dimethylsulfoxide (DMSO) and aliquots (0.5–20 mg) were applied as narrow streaks 2 cm above the lower edge

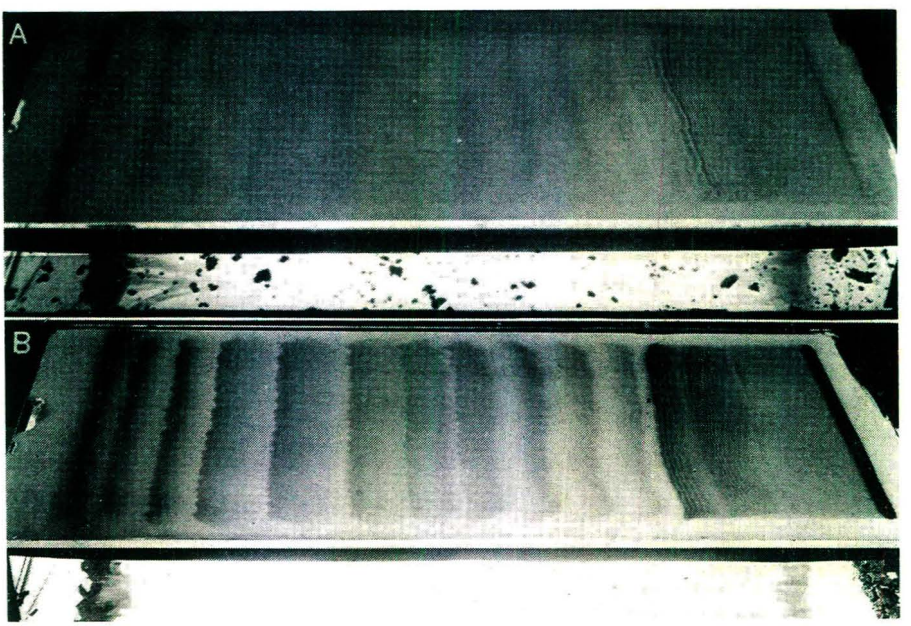

Fig. 1. (A). Developed thin-layer chromatogram exposed to iodine vapors. A 6 mg sample of malto-oligosaccharides was separated as described for the large plates in Table II. (B). The plate pictured in (A) after spraying with 20 ml of detection reagent (18 ml of 95% ethanol, 1.0 ml concentrated H_2SO_4 and 1.0 ml of anisaldehyde) and heating at 100° for 25 min[4].

* Mention of a trademark name, proprietory product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

of the plate. The plates were developed in solvents containing various proportions of 1-butanol, pyridine and water as will be noted in the results.

The malto-oligosaccharide zones were visualized by spraying with 20 ml of anisaldehyde–H_2SO_4–ethanol (1:1:18) spray reagent and heating at 100° for 15 to 30 min[4]. The malto-oligosaccharides so detected could not be eluted for the determination of radioactivity and carbohydrate contents. In order to elute the carbohydrate zones from the plates the zones were located by placing the plate, adsorbent side down, over a glass tray containing iodine crystals (Fig. 1A). The malto-oligosaccharides complex with the iodine vapors more rapidly than the Kieselguhr, and thus show up as light yellow bands across the plate which can be marked on the back of the glass. The zone should contain at least 100 μg of malto-oligosaccharides in order to show up well. When the plate is removed from the presence of the iodine vapors, the color disappears rapidly and the malto-oligosaccharides are left unchanged.

The malto oligosaccharides can be eluted from the plate by first removing a streak of Kieselguhr 0.5–1.0 mm wide from between each zone. Place the plate in a humid chamber. The malto-oligosaccharides can be chromatographed off the plate with water and into Whatman GF-82 fiberglass paper pieces (1.5 mm × 4 cm) (Fig. 2).

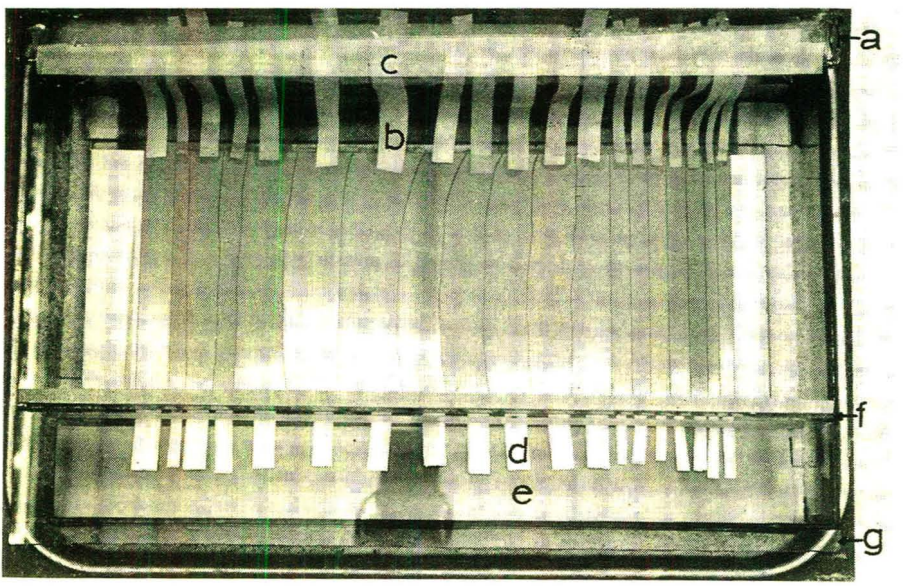

Fig. 2. Elution apparatus (51 cm long × 37 cm wide × 10 cm high) was constructed from 3 mm thick window glass held together with polyethylene tape. Water is carried by capillarity from a chromatograph trough (a) to the zones by wicks (b) cut from Whatman No. 54 chromatograph paper. Strips of teflon are taped to the cover glass (c) and top of the wall (not shown) where they contact the wicks. Fiberglass filter pieces (d) are placed on a glass plate covered with a sheet of teflon (e). The teflon is used to inhibit any transfer of compound from the paper. A movable glass piece (f) with a strip of teflon on the lower edge holds the filters firmly in contact with the chromatograph zones and also makes it possible to maintain high humidity around the chromatogram even if the cover glass (g) above the fiberglass filters is removed to allow drying. To aid in maintaining high humidity a shallow pan of water (not shown) was placed directly below the thin-layer chromatogram. The entire apparatus is kept in a metal tray to facilitate handling.

TABLE I

R_F VALUES OF MALTO-OLIGOSACCHARIDES SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Glass plates 5 × 20 cm and 20 × 45 cm were coated with a 250 μ thick layer of Kieselguhr G. The maltonatively narrow streaks 16 cm long were applied to the large plates. All solvent compositions are given

Solvents	*Degree of polymerization*									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Small plates										
70:20:10	0.86	0.56	0.25	0.08	0.03	0.01				
60:30:10	0.90	0.76	0.57	0.37	0.18	0.10	0.06	0.04	0.03	
50:40:10	0.92	0.83	0.77	0.53	0.31	0.19	0.12	0.08	0.05	
40:50:10	0.94	0.90	0.81	0.70	0.52	0.36	0.25	0.18	0.12	0.10
65:20:15[d]	0.89	0.78	0.60	0.39	0.20					
50:40:20[d]	0.93	0.89	0.84	0.78	0.70	0.63	0.55	0.44	0.36	0.30
60:20:20[c]	0.90	0.82	0.70	0.54	0.37	0.23	0.16	0.10	0.07[b]	0.03
50:30:20	0.93	0.90	0.86	0.79	0.70	0.61	0.51	0.39	0.29	0.20
45:35:20	0.95	0.91	0.87	0.81	0.74	0.67	0.61	0.53	0.44	0.35
40:40:20	0.96[a]	0.93[a]	0.90[a]	0.85	0.80	0.73	0.65	0.54	0.44	0.34
35:45:20	0.96[a]	0.94[a]	0.89[a]	0.85	0.79	0.73	0.68	0.59	0.50	0.41
30:50:20	0.96[a]	0.92[a]	0.90[a]	0.87	0.83	0.78	0.71	0.63	0.52	0.42
50:28:22	0.93	0.88	0.82	0.75	0.67	0.58	0.50	0.39	0.31	0.23
45:33:22	0.97	0.92	0.86	0.80	0.74	0.69	0.64	0.58	0.50	0.44
40:38:22	0.93[a]	0.89[a]	0.84	0.81	0.77	0.73	0.68	0.62	0.55	0.49
39:39:22	0.95[a]	0.90[a]	0.85	0.82	0.78	0.73	0.68	0.62	0.57	0.50
30:48:22	0.93[a]	0.90[a]	0.85[a]	0.81	0.78	0.73	0.66	0.61	0.53	0.45
42:34:24	0.96[a]	0.91[a]	0.87	0.83	0.78	0.75	0.70	0.65	0.59	0.52
40:36:24	0.92	0.88	0.83	0.80	0.76	0.73	0.68	0.64	0.60	0.55
38:38:24	0.92	0.87	0.82	0.77	0.75	0.67	0.60	0.54	0.49	0.44
50:20:30[c]	0.88	0.81	0.67	0.49	0.31	0.25	0.18	0.14	0.11	0.08
40:30:30	0.94	0.90	0.86	0.82	0.79	0.74	0.68	0.62	0.55	0.49[b]
60:40:30[d]	0.92	0.90	0.84	0.77	0.71	0.64	0.57	0.49	0.39	0.31
Large plates										
45:35:20	0.94	0.90	0.86	0.81	0.77	0.69	0.61	0.51	0.42	0.34
40:38:22	0.93	0.90	0.86	0.81	0.75	0.70	0.62	0.53	0.45	0.37
65:20:15[d]	0.87	0.77	0.61	0.42	0.24	0.14	0.09	0.05	0.03	0.02
60:40:30[d]	0.91	0.87	0.83	0.77	0.69	0.61	0.53	0.42	0.33	0.25
50:40:20[d]	0.94	0.91	0.87	0.81	0.74	0.65	0.55	0.44	0.33	0.25

[a] Denotes detectable zones which were very close together.
[b] Very dark narrow zones composed of several different size malto-oligosaccharides. Thus all zones
[c] These solvents separated into two phases during chromatography.
[d] These solvents were used in the earlier study and are included for comparison.

RESULTS AND DISCUSSION

WEILL AND HANKE[4] reported the separation of malto-oligosaccharides up to DP 9 on thin-layer plates coated with Kielselguhr G. One of their better solvents was composed of 1-butanol–pyridine–water (13:4:3). In our laboratory this solvent separated malto-oligosaccharides of DP 5 or smaller but the larger components remained near the origin. These same workers also reported the separation of malto-oligosaccharides to DP 10 with a 1-butanol–ethanol–water (5:3:2) solvent. We found that this system resolved components to DP 7; but the larger malto-oligosaccharides appeared as a smear up the lower half of the plate. HUBER *et al.*[6] reported the separation of megalosaccharides up to about DP 35 using continuous TLC. In this separation components smaller than DP 19 were chromatographed off the plate. Their optimum

oligosaccharides, dissolved in 90% dimethylsulfoxide, were applied as a narrow band 1 cm long. Alter-
in the order 1-butanol–pyridine–water.

11	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
0.07	0.06	0.04	0.03									
0.22	0.17	0.14	0.09	0.06[a]	0.05[a]	0.03[a]	0.02[a]					
0.02												
0.15	0.10[a]	0.07[a]	0.05[a]	0.04[a]	0.03[a]							
0.28	0.22	0.16	0.13	0.10	0.08	0.07						
0.27	0.19	0.15	0.11[a]	0.08[a]	0.06[a]	0.06[a]						
0.34	0.26	0.20	0.16	0.12	0.09	0.08						
0.34	0.26	0.20	0.15[a]	0.12[a]	0.09[a]							
0.19[b]	0.16	0.14	0.10	0.08	0.05							
0.35	0.29	0.22	0.18	0.15	0.12	0.10[a]	0.09[a]					
0.42	0.36	0.29	0.24	0.20	0.16							
0.41	0.35	0.28	0.22	0.17	0.12	0.10[a]	0.08[a]					
0.37	0.31	0.25	0.20	0.15								
0.44	0.35	0.28	0.22	0.16	0.13[a]	0.11[a]	0.09[a]					
0.48	0.41	0.34	0.27	0.22	0.18	0.15[a]	0.14[a]	0.13[a]				
0.38	0.34	0.29	0.25	0.21								
0.47[a]	0.38[a]	0.33[a]	0.28[a]	0.23[a]	0.19[a]	0.16[a]						
0.23	0.21	0.19	0.15	0.12	0.09	0.07						
0.28	0.23	0.18	0.15	0.12	0.10	0.08	0.04					
0.31	0.26	0.22	0.17	0.12	0.10	0.08	0.06	0.05	0.04	0.04	0.03	0.03
0.01												
0.18	0.17[b]	0.13	0.10	0.07	0.06[a]	0.05[a]	0.04[a]	0.02[a]				
0.19	0.14	0.10	0.08	0.04								

below this are of unknown degrees of polymerization.

separations were obtained on thin-layer plates coated with a mixture of three parts Silica Gel G and one part Kieselguhr G. A solvent system of 1-propanol–nitromethane–water (5:2:3) was considered to be the best. When this system was tested in our laboratory there was incomplete separation and considerable smearing between zones.

Preliminary studies indicated that malto-oligosaccharides larger than DP 12 could be separated with 1-butanol–pyridine–water solvents in ratios of (6:4:3) and (5:4:2). We have reported partial separations of malto-oligosaccharides derived from pullulanase digestion of β-amylase limit dextrins by using long plates (45 × 20 cm) and multiple developments[1]. The plates were first developed 23 cm in 1-butanol–pyridine–water (5:4:2), then to 32–34 cm in (6:4:3) and finally to 43–44 cm in (13:4:3). The plates were completely dried between each development. During the above study[1] we noticed that quite often the components DP 13 to DP 17 were

TABLE II

R_F VALUES OF MALTO-OLIGOSACCHARIDES SEPARATED BY UNIDIMENSIONAL MULTIPLE THIN-LAYER CHROMATOGRAPHY

Small plates prepared as described in Table I were developed their entire length 1, 2 or 4 times in 1-butanol once 25 cm in (39:39:22); once 30 cm in (50:28:22) and finally 40 cm in (70:20:10). All plates were dried

Solvent	*Times developed*	*Degree of polymerization*											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*[c]	*8*	*9*	*10*	*11*
Small plates													
40:40:20	1	0.92[a]	0.89[a]	0.84	0.80	0.75	0.69	0.61	—	0.53	0.44	0.34	0.27
40:40:20	2	—[b]	—[b]	0.99[a]	0.98[a]	0.96[a]	0.94[a]	0.91	0.86	0.85	0.76	0.65	0.56
40:40:20	4	—[b]	—[b]	—[b]	0.96[a]	0.95[a]	0.94[a]	0.93[a]	0.92	0.90	0.85	0.79	0.71
Large plates													
Plate 1		0.95	0.89	0.83	0.75	0.65	0.58	0.49	—	0.42	0.35	0.29	0.25
Plate 2		0.87	0.84	0.74	0.67	0.59	0.53	0.46	—	0.41	0.35	0.30	0.26

[a] Same as in Table I.
[b] Zones crowded together near the front.
[c] Relative R_F value of a DP 8 standard chromatogramed on the same plate.

concentrated into a very narrow region. In an effort to find a solvent composition which can be used for optimum separation we have tested a number of systems composed of varying proportions of 1-butanol, pyridine and water chosen from points on a tri-linear graph. These solvents were first tested on 5 × 20 cm plates coated with Kieselguhr G. Several mixtures resulted in good separations of malto-oligosaccharides (Table I). Multiple development of the small plates could be used to resolve the larger components (Table II). In an effort to obtain maximum separation on a single chromatogram, large plates were serially developed in several solvent combinations. One of the better separations utilized two developments (18 and 25 cm) with a solvent shown to move the larger malto-oligosaccharides followed by development 2/3 of the distance with a solvent capable of separating the intermediate size components. The final full plate development separated the smallest compounds (Table II and Fig. 1).

If the separated malto-oligosaccharides are to be removed from the Kieselguhr G, a non-destructive method for the location of the zones is required. Iodine vapors

TABLE III

RECOVERY OF MALTOSE FROM THIN-LAYER CHROMATOGRAM PLATES

Maltose (0.5–4.0 mg) was streaked on a 20 × 20 cm thin-layer plate. After drying 2 × 20 cm zones containing the maltose were eluted with water into fiberglass filters. The maltose was then eluted from the filters into 10 ml scored tubes and the quantity recovered measured by the phenol–H_2SO_4 procedure[9].

Maltose added (mg)	*Maltose found (mg)*	*Recovered (%)*
0.0	0.04[a]	
0.5	0.45	90.0
1.0	0.87	87.0
2.0	1.83	91.5
4.0	3.99	99.7

[a] This amount in the blank was substracted from each standard sample.

nol–pyridine–water solvents. The large plates were serially developed once 18 cm in (39:39:22); between developments

12	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*
0.20	0.15	0.12	0.09	0.08											
0.44	0.35	0.27	0.22	0.16	0.13	0.10[a]	0.09[a]	0.07[a]							
0.62	0.50	0.40	0.28	0.22	0.15	0.12[a]	0.10[a]	0.08[a]	0.07[a]	0.06[a]					
0.25	0.24	0.24	0.23	0.22	0.21	0.20	0.19	0.19	0.18	0.18	0.17	0.16	0.16	0.15	0.12
0.25	0.24	0.23	0.23	0.21	0.19	0.17	0.16	0.14	0.13	0.11	0.09	0.07	0.06	0.04	0.02

have been used to visualize temporarily amino acids on paper chromatograms[7] and steroids on thin-layer chromatograms[8]. The malto-oligosaccharide zones complexed with the iodine vapors when sufficient carbohydrate was present (4–20 mg on a 45 × 20 cm plate). The zones appear as faint yellow bands and are best seen when viewed with light from below (Fig. 1A).

Once the zones are located they can be conveniently eluted from the plate into pieces of chromatograph paper or fiberglass filters (Fig. 2). Maltose and malto-oligosaccharides essentially move with the water front and are completely transferred to the filter in one development (Tables III and IV). Thin-layer chromatograms con-

TABLE IV

RECOVERY OF MALTO-OLIGOSACCHARIDES FOLLOWING CHROMATOGRAPHY

Samples of a mixture of malto-oligosaccharides (4–20 mg) were streaked on 20 × 45 cm thin layer plates. The chromatograms were serially developed once to 23 cm in the 1-butanol–pyridine–water (50:40:20) solvent, once to 33 cm in the (60:40:30) solvent and once to 41 cm in the (65:20:15) solvent. The zones were detected with iodine vapor. The Kieselguhr was scored between each zone and the malto-oligosaccharides eluted into fiberglass filters with water until saturated and then removed. New filters were added for the second elution. For the third elution the zones were scraped off the plate and put in 10 ml scored tubes. Water was added and heaten 20 min in a boiling water bath. After centrifugation an aliquot was taken for carbohydrate determination. The total carbohydrate content was measured as described in Table III.

Malto-oligosaccharide added (mg)	*Malto-oligosaccharide recovered in all zones*							
	1st elution		*2nd elution*		*3rd elution*		*Sum*	
	(mg)[a]	%	*(mg)*[a]	%	*(mg)*[a]	%	*(mg)*[a]	%
4.0	3.21	80.3	0.02		0	0	3.23	80.7
6.0	5.24	87.3	0.40		0	0	5.64	94.0
10.0	8.34	83.4	1.80	18.0	0	0	10.14	101.0
20.0	16.49	82.4	1.66	8.3	0.27	1.3	18.42	92.1

[a] All samples were corrected for the slight contamination in the Kieselguhr G. A zone above the front was eluted and used as a blank.

taining between 4 and 20 mg of malto-oligosaccharide mixture were developed by a three-development system. The zones were visualized with iodine and eluted with water. Over 80 % of the carbohydrate was recovered in the first elution (Table IV). The small amount of carbohydrate not eluted the first time was confined to the origin. With such a system one can measure the radioactivity of a zone by placing the dried filter, plus carbohydrate, in a toluene based scintillation fluid and counting as previously described[1]. After counting, the filter can be removed, and dipped twice in toluene to remove the scintillators. The carbohydrates can be eluted from the filter with water and quantitatively measured[1].

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THE IDENTIFICATION OF RUBBER COMPOUNDING INGREDIENTS USING THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

During 1964–1965, we examined and reported the utility of thin-layer chromatography for the identification of certain rubber compounding ingredients. These ingredients included amine antioxidants and antiozonants, phenolic antioxidants, guanidines, accelerators, and the amines obtained from accelerators. Each of these groups was examined in an effort to determine the solvent and indicator systems most suitable for the separation and, hence, identification of the largest possible number of compounds in each of the above categories. As a result, the systems we have chosen and described herein should make it possible to identify most of the above named types of compounding materials manufactured in the United States.

INTRODUCTION

The five general methods of analysis that have been employed for the identification of rubber compounding materials are spot tests, liquid column chromatography, paper chromatography, and UV and IR spectroscopy. One of the earlier and most comprehensive procedures available was published by ZIJP[18] in 1956. ZIJP subjected extracts of the vulcanizates to various preliminary treatments after which the compounding ingredients or their residues were separated and identified using paper chromatography. Somewhat later, in 1959, GACZYNSKI AND STEPIEN[7] published a paper chromatographic method for the identification of six most commonly used accelerators (Poland) and N-phenyl-2-naphthylamine.

The analysis of vulcanizates was reviewed in 1959 by BURGER[3] and more recently in 1961 by AULER[2]. AULER examined the various methods that had been used for the determination of antioxidants, antiozonants and accelerators with special emphasis on paper chromatography. He evaluated the methods of MIKSCH AND PRÖLSS[11] and ZIJP, refined portions of their methods, and included some of his own work.

* A description of this work has been presented at a meeting of the Division of Rubber Chemistry, American Chemical Society, Miami Beach, Fla., May 4th–7th, 1965, and at a technical symposium of the Akron Rubber Group, Inc., October 28th, 1966.

Since the paper chromatographic work of MIKSCH AND PRÖLSS, ZIJP and AULER, the introduction of commercially available apparatus and standard absorbent materials for TLC have provided a new, rapid, and more widely applicable technique for chromatographic separations and identifications. Surprisingly only a few reports[9,13–15,17] concerning the application of TLC to the identification of compounding ingredients had appeared prior to 1965 and these dealt with a limited number of materials. It was our purpose, therefore, to examine the general usefulness of TLC for the identification of a wide range of compounding ingredients and to develop systems suitable for their identification.

Many schemes, including the excellent procedure by ZIJP, have been presented for the isolation of compounding ingredients from a vulcanizate and the subsequent treatments necessary prior to chromatography or other forms of analysis. Many of these procedures are adequate for a given problem. It is, however, impossible to anticipate all situations, and some of the more challenging rubber compounds might well require development of new procedures. The scope of the work we are reporting does not include additional schemes for the isolation of compounding ingredients from vulcanizates.

Since our original presentation, several other workers have published work dealing with the TLC analysis of compounding ingredients. We have included references to recent work of which we have become aware.[1,4–6,8,10,12]

EXPERIMENTAL

TLC equipment commercially available from Research Specialties Company and Brinkmann Instruments, Inc., was used for these studies. Silica Gel G from E. Merck AG., Darmstadt, was used for layers. A 2:1 weight ratio of water and Silica Gel G was used to make the slurries for the layer preparation. The layers were dried for 1 h at 110° in a forced air oven and cooled in a desiccator where stored until used.

Reasonably constant R_F values are obtained if the layer thickness is kept between 200 to 300 μ, the sample does not exceed the layer capacity, and chamber saturation is maintained. Our layer thickness was kept between 250 to 300 μ and the sample sizes in most instances were kept between 35 to 45 μg. In general, samples of 5 to 15 μg are used for the above layer thickness. However, the variation in R_F values for our sample size range was found to be small and caused no problem in identification work. A larger sample size was, in fact, found to be more advantageous

TABLE I

MANUFACTURERS OF RUBBER CHEMICALS

Code	*Manufacturer*	*Code*	*Manufacturer*
a	DuPont	i	C. P. Hall
b	UniRoyal	j	Ethyl Corporation
c	Vanderbilt	k	Shell
d	Harwick	m	American Cyanamid
e	Eastman	n	Catalin
f	Universal Oil Products	o	Neville
g	Goodyear	p	Dow
h	Monsanto	q	Pennsalt

TABLE II

APPROXIMATE R_F × 100 VALUES AND COLORS FOR AMINE ANTIOXIDANTS AND ANTIOZONANTS

Development on Silica Gel G with benzene–acetone–concentrated ammonium hydroxide (100:5:0.1) as solvent; indicator: benzoyl peroxide. For code to manufacturers, see Table I.

No.	*Amine*	*Trade name*	R_F × 100	*Color*
1	N-Phenyl-1-naphthylamine	Neozone A (a)	72	Blue-green
2	N-Phenyl-2-naphthylamine	Neozone D (a)	67	Purple
3	*p*-(*p*-Tolyl-sulfonylamido)-diphenylamine	Aranox (b)	18	Orange
4	Nonylated diphenylamines	Polylite (b)	79, 74	Blue and gray
5	Octylated diphenylamines	Age Rite Stalite (c)	78, 74, 71	Blue and gray
6	Octylated diphenylamines	Age Rite Stalite S (c)	77	Blue-gray and brown
7	Octylated diphenylamines	Octamine (b)	79, 76	Blue-gray and brown
8	4,4′-Dimethoxy-diphenylamine	25% of Thermoflex A (a)	51	Red
9	4-Isopropoxy-diphenylamine	Age Rite Iso (c)	64	Green-brown
10	4-Isopropylamino-diphenylamine	Nonox ZA (d)	31	Brown
11	N,N′-Di-isopropyl-*p*-phenylenediamine	Tenamene 5 (e)	7	Violet
12	N,N′-Di-*sec.*-butyl-*p*-phenylenediamine	Tenamene 2 (e)	15	Violet
13	N,N′-Bis(1,4-dimethylpentyl)-*p*-phenylenediamine	Eastozone 33 (e)	21	Violet
14	N,N′-Bis(1-ethyl-3-methylpentyl)-*p*-phenylenediamine	UOP 88 (f)	47	Violet
15	N,N′-Bis(1-methylheptyl)-*p*-phenylenediamine	UOP 288 (f)	21	Violet
16	N-Isopropyl-N′-phenyl-*p*-phenylenediamine	Flexzone 3C (b)	31	Brown
17	N-Cyclohexyl-N′-phenyl-*p*-phenylenediamine	Flexzone 6H (b)	37	Brown
18	N-(1-Methylheptyl)-N′-phenyl-*p*-phenylenediamine	UOP 688 (f)	47	Brown
19	N,N′-Diphenyl-*p*-phenylenediamine	Age Rite DPPD (c)	42	Yellow
20	N,N′-Di-2-naphthyl-*p*-phenylenediamine	Age Rite White (c)	46	Pink
21	Mixture of diaryl-*p*-phenylenediamines	Wingstay 100 (g)	59, 55, 51	Yellow
22	Polymerized 1,2-dihydro-2,2,4-trimethyl-quinoline	Age Rite Resin D (c)	54, 45, 34, 26, 23, 12, 8, 5, 3	Gray, blue and brown
23	6-Dodecyl-1,2-dihydro-2,2,4-trimethyl-quinoline	Santoflex DD (h)	67, 40	Tan
24	6-Ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline	Santoflex AW (h)	44	Brown-green
25	High-temperature diphenylamine–acetone reaction product	BLE (b)	65	Blue and violet
26	High-temperature diphenylamine–acetone reaction product	Age Rite Superflex (c)	65	Blue and violet
27	Low-temperature diphenylamine–acetone reaction product	Aminox (b)	61	Purple
28	Low-temperature N-phenyl-2-naphthylamine–acetone reaction product	Betanox Special (b)	62, 58	Green-brown
29	Aldol-1-naphthylamine	Age Rite Resin (c)	60, 44, 33, 21, 10	Blue, brown and yellow
30	Butyraldehyde–aniline condensation product	Antox (a)	68, 42, 28	Brown and red-brown
31	N,N′-Diphenylethylenediamine	Stabilite (i)	39	Brown
32	N,N′-Diphenylpropylenediamine	Stabilite L (i)	45	Green-brown
33	N,N′-Di-*o*-tolylethylenediamine	Stabilite Alba (i)	47	Red
34	2,4-Toluenediamine	7.5% of Neozone C (a)	2	Brown
35	4,4′-Diaminodiphenylmethane	Tonox (b)	4	Brown
36	Phenothiazine		51	Green

when working with unknown extracts. In the case of multicomponent materials, the sample size was increased to yield reasonable quantities of the components. Saturation of the developing tank atmosphere was ensured by placing filter paper wet with solvent on the tank walls. The development distance was 15 cm in all cases except for the guanidines, which were developed through a distance of 10 cm. The sample, as a 1 % solution, was placed on the layer by means of a platinum wire loop[16] bent at a right angle to the stem of the wire which had been fused into a glass tubing handle. The volume of solution picked up by a loop about 1 mm in diameter was 3.5 to 4.5 μl. The wire loop method of sample application was preferred since the wire was easily cleaned in a burner flame and gave the required reproducibility for qualitative analysis. All samples were used as obtained from the suppliers. A list of the suppliers is given in Table I.

Amine antioxidants

A solvent of benzene–acetone–conc. ammonium hydroxide (100:5:0.1) was used for the one-dimensional development of the materials listed in Table II and shown in Figs. 1, 2, and 3. After evaporation of the developing solvent, the samples were indicated by spraying with a 4 % solution of benzoyl peroxide in benzene. Two-dimensional developments were obtained by developing in the first direction with the benzene–acetone–ammonium hydroxide solvent, drying the plate in a 50° vacuum

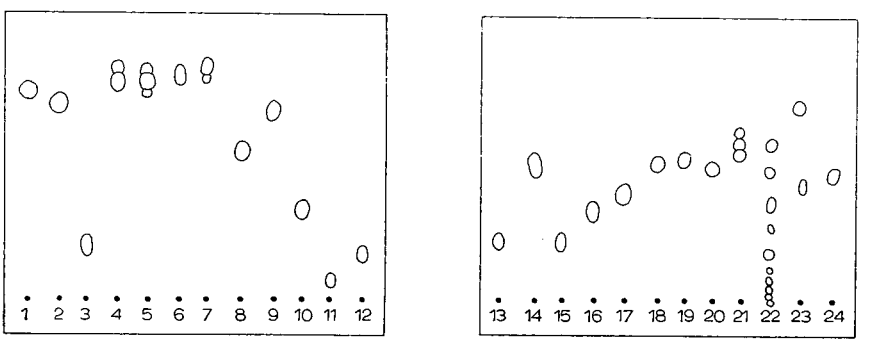


Fig. 1. Separation of amine antioxidants and antiozonants by one-dimensional thin-layer chromatography. Solvent: benzene–acetone–concentrated ammonium hydroxide (100:5:0.1); development distance: 15 cm; indicator: 4% benzoyl peroxide in benzene. 1 = N-Phenyl-1-naphthylamine; 2 = N-phenyl-2-naphthylamine; 3 = *p*-(*p*-tolylsulfonylamido)-diphenylamine; 4 = nonylated diphenylamines; 5 = octylated diphenylamines; 6 = octylated diphenylamines; 7 = octylated diphenylamines; 8 = 4,4′-dimethoxydiphenylamine; 9 = 4-isopropoxydiphenylamine; 10 = 4-isopropylaminodiphenylamine; 11 = N,N′-diisopropyl-*p*-phenylenediamine; 12 = N,N′-di-*sec.*-butyl-*p*-phenylenediamine.

Fig. 2. Separation of amine antioxidants and antiozonants by one-dimensional thin-layer chromatography. Solvent: benzene–acetone–conc. ammonium hydroxide (100:5:0.1); development distance: 15 cm; indicator: 4% benzoyl peroxide in benzene. 13 = N,N′-bis(1,4-dimethylpentyl)-*p*-phenylenediamine; 14 = N,N′-bis(1-ethyl-3-methylpentyl)-*p*-phenylenediamine; 15 = N,N′-bis(1-methylheptyl)-*p*-phenylenediamine; 16 = N-isopropyl-N′-phenyl-*p*-phenylenediamine; 17 = N-cyclohexyl-N′-phenyl-*p*-phenylenediamine; 18 = N-(1-methylheptyl)-N′-phenyl-*p*-phenylenediamine; 19 = N,N′-diphenyl-*p*-phenylenediamine; 20 = N,N′-di-2-naphthyl-*p*-phenylenediamine; 21 = mixture of diaryl-*p*-phenylenediamines; 22 = polymerized 1,2-dihydro-2,2,4-trimethylquinoline; 23 = 6-dodecyl-1,2-dihydro-2,2,4-trimethylquinoline; 24 = 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline.

oven at 150 to 250 mm mercury for a few minutes, cooling the plate to room temperature, and then developing in the second direction with cyclohexane–acetone–conc. ammonium hydroxide (100:5:0.1) (Fig. 4).

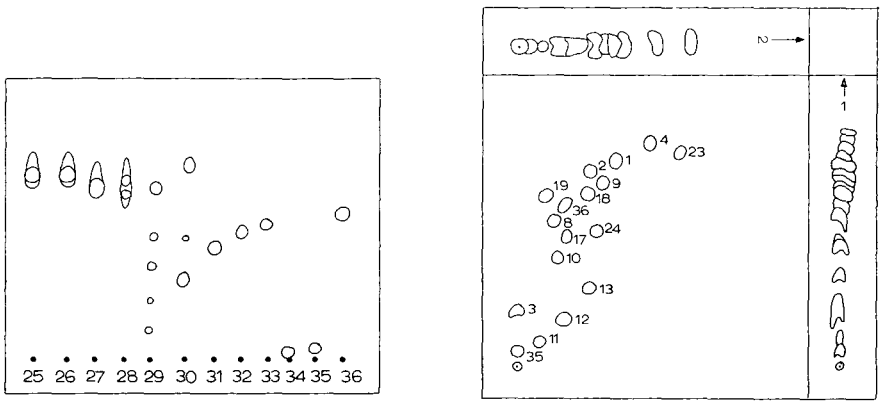


Fig. 3. Separation of amine antioxidants and antiozonants by one-dimensional thin-layer chromatography. Solvent: benzene–acetone conc. ammonium hydroxide (100:5:0.1); development distance: 15 cm; indicator: 4% benzoyl peroxide in benzene. 25 = high-temperature diphenylamine–acetone reaction product; 26 = high-temperature diphenylamine–acetone reaction product; 27 = low-temperature diphenylamine–acetone reaction product; 28 = low-temperature N-phenyl-2-naphthylamine–acetone reaction product; 29 = aldol-1-naphthylamine; 30 = butyraldehyde–aniline condensation product; 31 = N,N′-diphenylethylenediamine; 32 = N,N′-diphenylpropylenediamine; 33 = N,N′-di-*o*-tolylethylenediamine; 34 = 2,4-toluenediamine; 35 = 4,4′-diaminodiphenylmethane; 36 = phenothiazine.

Fig. 4. Separation of a mixture of seventeen amines by two-dimensional development. Solvents: (1) benzene–acetone–conc. ammonium hydroxide (100:5:0.1); (2) cyclohexane–acetone–conc. ammonium hydroxide (100:5:0.1). Indicator: 4% benzoyl peroxide in benzene.

The developing solutions used here, while good for purposes of identification, do not resolve the many fractions found in several of the multi-component reaction mixtures. For example, the many components in the octylated and nonylated diphenylamines and the amine–acetone reaction products (Samples 4, 5, 7, 25, 26, and 27)

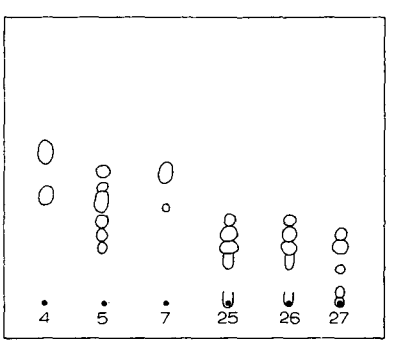


Fig. 5. Separation of amine antioxidants and antiozonants by thin-layer chromatography. Solvent: cyclohexane–benzene–acetone (100:10:1); development distance: 15 cm; indicator: 4% benzoyl peroxide in benzene. 4 = nonylated diphenylamines; 5 = octylated diphenylamines; 7 = octylated diphenylamines; 25,26 = high-temperature diphenylamine–acetone reaction products; 27 = low-temperature diphenylamine–acetone reaction product.

TABLE III

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR AMINE ANTIOXIDANTS AND ANTIOZONANTS

Development on Silica Gel G with cyclohexane–benzene–acetone (100:10:1) as solvent; indicator: benzoyl peroxide. For code to manufaturers, see Table I.

No.	*Amine*	*Trade name*	$R_F \times 100$.	*Color*
4	Nonylated diphenylamines	Polylite (b)	53, 39	Blue and brown
5	Octylated diphenylamines	Age Rite Stalite (c)	48, 42, 37, 30, 25, 21	Blue, gray, brown and yellow
7	Octylated diphenylamines	Octamine (b)	46, 34	Blue-gray and brown
25	High-temperature diphenylamine–acetone reaction product	BLE (b)	29, 25, 19, 13, 0	Blue and violet
26	High-temperature diphenylamine–acetone reaction product	Age Rite Superflex (c)	29, 25, 19, 14, 0	Blue and violet
27	Low-temperature diphenylamine–acetone reaction product	Aminox (b)	25, 20, 12, 4, 0	Blue and violet

TABLE IV

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR PHENOLIC ANTIOXIDANTS

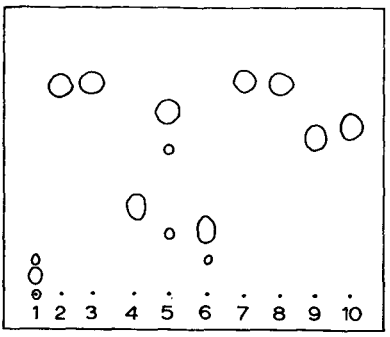
Development on Silica Gel G with benzene as solvent; indicator: 2,6-dichloroquinonechlorimide. For code to manufacturers, see Table I.

No.	*Phenol*	*Trade name*	$R_F \times 100$	*Color*
1	Hydroquinone monobenzyl ether	Age Rite Alba (c)	7	Blue
2	2,6-Di-*tert.*-butyl-phenol	Ethyl 701 (j)	72	Violet
3	2,6-Di-*tert.*-butyl-4-methyl-phenol	Ionol (k)	74	Creamy yellow
4	2,6-Di-*tert.*-butyl-α-methoxy-4-methyl-phenol	Ethyl 762 (j)	30	Violet-yellow
5	2-α-Methylcyclohexyl-4,6-dimethyl-phenol	Nonox WSL (d)	64	Creamy yellow
6	Butylated hydroxyanisole	Tenox BHA (e)	22	Blue
7	Butylated hydroxytoluene	Tenox BHT (e)	74	Creamy yellow
8	4,4′-Bis(2,6-di-*tert.*-butyl-phenol)	Ethyl 712 (j)	74	Yellow
9	2,2′-Methylene-bis(4-methyl-6-*tert.*-butyl-phenol)	Antioxidant 2246 (m)	62	Yellow
10	2,2′-Methylene-bis(4-ethyl-6-*tert.*-butyl-phenol)	Antioxidant 425 (m)	59	Gray-blue
11	2,2′-Methylene-bis(6-α-methyl-cyclohexyl-4-methyl-phenol)	Nonox WSP (d)	62	Yellow
12	4,4′-Methylene-bis(6-*tert.*-butyl-2-methyl-phenol)	Ethyl 720 (j)	41	Red-violet
13	4,4′-Methylene-bis(2,6-di-*tert.*-butyl-phenol)	Ethyl 702 (j)	76	Red-brown
14	4,4′-Butylidene-bis(6-*tert.*-butyl-3-methyl-phenol)	Santowhite Powder (h)	19	Blue
15	2,2′-Thio-bis(4-methyl-6-*tert.*-butyl-phenol)	CAO-6 (n)	72	Yellow-brown
16	4,4′-Thio-bis(6-*tert.*-butyl-2-methyl-phenol)	Ethyl 736 (j)	45	Red-brown
17	4,4′-Thio-bis(6-*tert.*-butyl-3-methyl-phenol)	Santowhite Crystals (h)	28	Blue-violet
18	4,4′-Thio-bis(3,6-di-*sec.*-amyl-phenol)	Santowhite L (h)	64	Blue and gray
19	N-Butyl-*p*-amino-phenol	Tenamene 1 (e)	3	Blue
20	Mixture of *tert.*-octyl and *tert.*-butyl cresols	Wingstay T (g)	73	Violet
21	Fortified phenol	Zalba Special (a)	69	Red-brown
22	Alkylated phenol	Naugawhite (b)	69,60	Yellow
23	Alkylated phenol	Nevastain B (o)	64, 29	Blue
24	Alkylated phenol	Stabilite White (i)	76, 59, 50	Blue-gray and brown
25	Alkylated phenol	Age Rite Superlite (c)	69, 54, 47, 43, 39, 30, 26, 18, 9	Violet, blue and gray
26	Substituted styrenated phenol	Phenox (p)	58, 26, 10	Blue
27	Substituted styrenated phenol	Wingstay S (g)	58, 26, 10	Blue
28	Substituted styrenated phenol	Age Rite Spar (c)	58, 26, 10	Blue
29	Substituted styrenated phenol	Wingstay V (g)	67, 61, 56 50, 44, 28, 17, 9	Blue and cream
30	Tri(nonylated phenyl) phosphite	Polygard (b)	63, 52, 48, 39, 22, 12	Blue, gray, salmon, white
31	Phosphited polyalkylpolyphenol	Age Rite Geltrol (c)	76, 69, 63, 48, 41, 19, 12, 3	Blue, gray, salmon, violet

can be better resolved using a solvent such as cyclohexane–benzene–acetone (100 : 10 : 1((Fig. 5 and Table III).

Phenolic antioxidants

The phenolics (Table IV) were developed with benzene (Figs. 6, 7, and 8). The samples were visualized by spraying with a buffer solution containing 23.4 g sodium tetraborate and 3.3 g sodium hydroxide in 1 l of water followed by a spray of freshly prepared 0.1 % 2,6-dichloroquinonechlorimide in methanol.



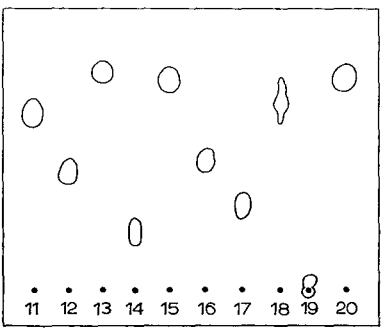


Fig. 6. Separation of phenolic antioxidants by thin-layer chromatography. Solvent: benzene; development distance: 15 cm; indicators: (1) borate buffer; (2) 0.1 % 2,6-dichloroquinonechlorimide in methanol. 1 = hydroquinone monobenzyl ether; 2 = 2,6-di-*tert.*-butylphenol; 3 = 2,6-di-*tert.*-butyl-4-methyl-phenol; 4 = 2,6-di-*tert.*-butyl-α-methoxy-4-methyl-phenol; 5 = 2-α-methylcyclohexyl-4,6-dimethyl-phenol; 6 = butylated hydroxyanisole; 7 = Butylated hydroxytoluene; 8 = 4,4′-bis(2,6-di-*tert.*-butyl-phenol); 9 = 2,2′-methylene-bis(4-methyl-6-*tert.*-butyl-phenol); 10 = 2,2′-methylene-bis(4-ethyl-6-*tert.*-butyl-phenol).

Fig. 7. Separation of phenolic antioxidants by thin-layer chromatography. Solvent: benzene; development distance: 15 cm; indicators: (1). borate buffer; (2) 0.1 % 2,6-dichloroquinonechlorimide in methanol. 11 = 2,2′-methylene-bis(6-α-methylcyclohexyl-4-methyl-phenol); 12 = 4,4′-methylene-bis(6-*tert.*-butyl-2-methyl-phenol); 13 = 4,4′-methylene-bis(2,6-di-*tert.*-butyl-phenol); 14 = 4,4′-butylidene-bis(6-*tert.*-butyl-3-methyl-phenol); 15 = 2,2′-thio-bis(4-methyl-6-*tert.*-butyl-phenol); 16 = 4,4′-thio-bis(6-*tert.*-butyl-2-methyl-phenol); 17 = 4,4′-thio-bis(6-*tert.*-butyl-3-methyl-phenol); 18 = 4,4′-thio-bis(3,6-di-*sec.*-amyl-phenol); 19 = N-butyl-*p*-amino-phenol; 20 = mixture of *tert.*-octyl and *tert.*-butyl cresols.

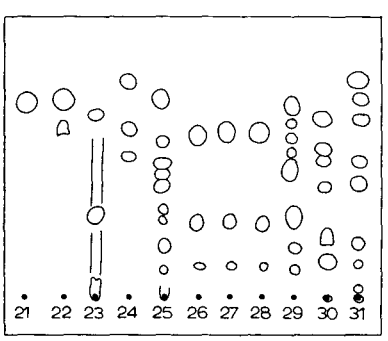


Fig. 8. Separation of phenolic antioxidants by thin-layer chromatography. Solvent: benzene; development distance: 15 cm; indicators: (1) borate buffer; (2) 0.1 % 2,6-dichloroquinonechlorimide in methanol. 21 = fortified phenol; 22, 23, 24, 25 = alkylated phenols; 26, 27, 28, 29 = substituted styrenated phenols; 30 = tri(nonylated phenyl) phosphite; 31 = phosphited polyalkylpolyphenol.

TABLE V

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR PHENOLIC ANTIOXIDANTS

Double development on Silica Gel G with *n*-hexane–benzene (8:1) as solvent; indicator: 2,6 dichloroquinonechlorimide. For code to manufacturers, see Table I.

No.	*Phenol*	*Trade name*	$R_F \times 100$	*Color*
2	2,6-Di-*tert.*-butyl-phenol	Ethyl 701 (j)	58	Violet
3	2,6-Di-*tert.*-butyl-4-methyl-phenol	Ionol (k)	57	Creamy yellow
8	4,4'-Bis(2,6-di-*tert.*-butyl-phenol)	Ethyl 712 (j)	27	Yellow
13	4,4'-Methylene-bis(2,6-di-*tert.*-butyl-phenol)	Ethyl 702 (j)	25	Red-brown
15	2,2'-Thio-bis(4-methyl-6-*tert.*-butyl-phenol)	CAO-6 (n)	30	Yellow-brown
20	Mixture of *tert.*-octyl and *tert.*-butyl cresols	Wingstay T (g)	11, 16, 56, 65, 69, 74, 83	Violet, blue and gray
21	Fortified phenol	Zalba Special (a)	5, 47	Red-brown

Since several samples were found to travel similar distances when eluted with benzene a second procedure was devised. The plate was developed with *n*-hexane–benzene (8:1) for a distance of 15 cm, dried in the vacuum oven, and then again developed 15 cm in the same direction with the same solvent system (Fig. 9 and Table V).

Guanidines

Acetone containing 1 % concentrated ammonium hydroxide was employed to resolve three guanidines (Table VI). The samples were detected by spraying with 4 % sodium hypochlorite (Fig. 10).

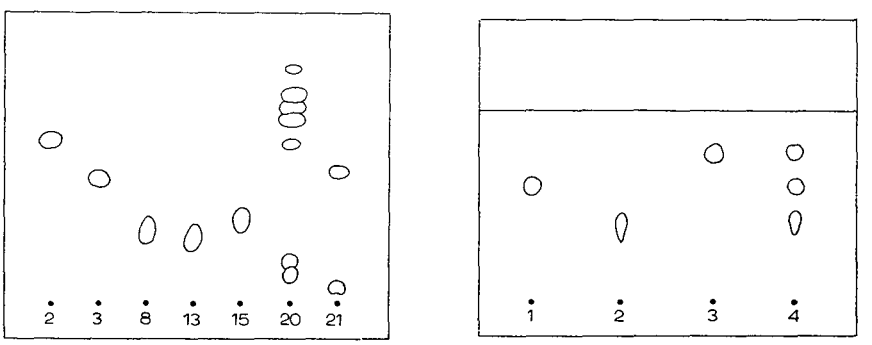


Fig. 9. Separation of phenolic antioxidants by thin-layer chromatography. Solvent: *n*-hexane–benzene (8:1); development distance: 15 cm (twice); indicators: (1) borate buffer; (2) 0.1 % 2,6-dichloroquinonechlorimide in methanol. 2 = 2,6-di-*tert.*-butyl-phenol; 3 = 2,6-di-*tert.*-butyl-4-methyl-phenol; 8 = 4,4′-bis(2,6-di-*tert.*-butyl-phenol); 13 = 4,4′-methylene-bis(2,6-di-*tert.*-butyl-phenol); 15 = 2,2′-thio-bis(4-methyl-6-*tert.*-butyl-phenol); 20 = mixture of *tert.*-octyl and *tert.*-butyl cresols; 21 = fortified phenol.

Fig. 10. Separation of guanidines by thin-layer chromatography. Solvent: 1 % conc. ammonium hydroxide in acetone; development distance: 10 cm; indicator: 4 % aqueous sodium hypochlorite. 1 = diphenylguanidine; 2 = di-*o*-tolylguanidine; 3 = triphenylguanidine; 4 = mixture.

TABLE VI

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR GUANIDINES

Development on Silica Gel G with 1 % concentrated ammonium hydroxide in acetone as solvent; indicator: sodium hypochlorite.

No.	*Guanidine*	$R_F \times 100$	*Color*
1	Diphenylguanidine	63	Red-brown
2	Di-*o*-tolylguanidine	43	Red-brown
3	Triphenylguanidine	80	Yellow-brown

Accelerators

A mixture of benzene–ethyl acetate–acetone (100:5:1) was used to chromatograph the accelerators listed in Table VII and shown in Figs. 11, 12, and 13. Those compounds containing a readily liberated amine were sprayed with 4 *N* hydrochloric acid and placed in a 130° oven for approximately 15 min. The samples were then sprayed with 0.5 % solution of ninhydrin in ethanol containing 10 % acetic acid and 0.5 %

TABLE VII

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR ACCELERATORS

Development on Silica Gel G with benzene–ethyl acetate–acetone (100:5:1) as solvent; indicators: ninhydrin (1–17 and 22–28) and bismuth nitrate (18–21). For code to manufacturers, see Table I.

No.	*Accelerator*	*Trade name*	$R_F \times 100$	*Color*
1	Tetramethylthiuram monosulfide	Unads (c)	27	Red
2	Tetrabutylthiuram monosulfide	Pentex (b)	53	Red-brown
3	Tetramethylthiuram disulfide	Methyl Tuads (c)	41	Red
4	Tetraethylthiuram disulfide	Ethyl Tuads (c)	57	Red
5	Dipentamethylenethiuram tetrasulfide	Tetrone A (a)	61, 56	Purple
6	Cyclic thiuram	Conac T (a)	17, 12, 8	Yellow-brown
7	Piperidinium pentamethylenedithiocarbamate	Accelerator 552 (a)	54	Purple
8	Zinc dimethyldithiocarbamate	Methyl Zimate (c)	49	Red
9	Zinc diethyldithiocarbamate	Ethyl Zimate (c)	64	Red
10	Zinc dibutyldithiocarbamate	Butyl Zimate (c)	77	Red-brown
11	Copper dimethyldithiocarbamate	Cumate (c)	63	Yellow-green
12	Bismuth dimethyldithiocarbamate	Bismate (c)	32	Red
13	Selenium dimethyldithiocarbamate	Methyl Selenac (c)	56, 51	Red
14	Tellurium diethyldithiocarbamate	Tellurac (c)	60, 48	Red
15	Cadmium diethyldithiocarbamate	Cadmate (c)	47	Red
16	Lead diethyldithiocarbamate	Ethyl Ledate (c)	54	Red
17	2-Benzothiazyl-N,N-diethylthiocarbamyl sulfide	Ethylac (q)	29	Red
18	2-Mercaptobenzothiazole	MBT (a)	25	Yellow-brown
19	Benzothiazyl disulfide	MBTS (a)	44	Yellow-brown
20	Zinc benzothiazyl sulfide	Zetax (c)	24,0	Yellow-brown
21	2-Mercaptothiazoline	2-MT (m)	11	Yellow
22	N-*tert.*-Butyl-2-benzothiazole sulfenamide	Santocure NS (h)	45	Creamy yellow
23	N,N-Diispropyl-2-benzothiazole sulfenamide	DIBS (m)	56	Yellow-brown
24	N-Cyclohexyl-2-benzothiazole sulfenamide	Santocure (h)	48	Orange-brown
25	N-Oxydiethylene-2-benzothiazole sulfenamide	Amax (c)	24	Red-violet
26	2-(2,6-Dimethyl-4-morpholinothio) benzothiazole	Santocure 26 (h)	31, 23	Tan
27	N,N-Dimethylcyclohexylamine salt of dibutyldithiocarbamic acid	RZ-50A (h)	72	Red-brown
28	1,1-Methylene-dipiperidine–carbon disulfide reaction product	R-2 Crystals (h)	streaks	Purple

cadmium acetate and returned to the oven for color development. The thiazoles were indicated with a 5 % solution of bismuth nitrate in 1 *N* nitric acid. Since benzothiazole disulfide does not react with the bismuth nitrate until reduced to 2-mercaptobenzothiazole, it was sprayed first with 0.5 % ammonium sulfide. The excess ammonium sulfide was removed by placing the plate in a 50° vacuum oven at 150 to 250 mm mercury for a few minutes. The thiazoles are satisfactorily indicated after the ninhy-

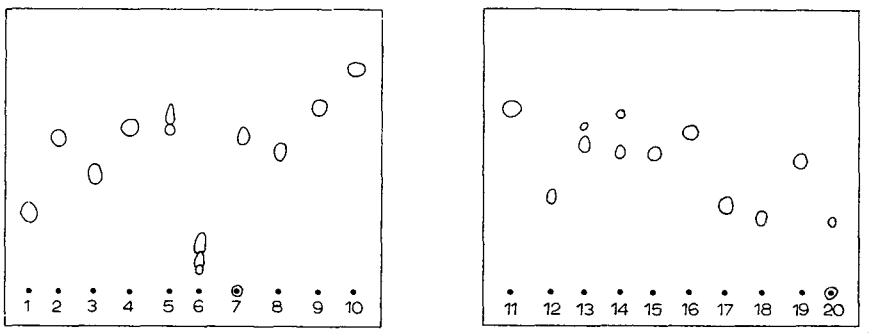


Fig. 11. Separation of accelerators by thin-layer chromatography. Solvent: benzene–ethyl acetate–acetone (100:5:1); development distance: 15 cm; indicators: (1) 4 *N* hydrochloric acid; (2) 0.5 % ninhydrin in ethanol containing 10 % acetic acid and 0.5 % cadmium acetate. 1 = tetramethylthiuram monosulfide; 2 = tetrabutylthiuram monosulfide; 3 = tetramethylthiuram disulfide; 4 = tetraethylthiuram disulfide; 5 = dipentamethylenethiuram tetrasulfide; 6 = cyclic thiuram; 7 = piperidinium pentamethylenedithiocarbamate; 8 = zinc dimethyldithiocarbamate; 9 = zinc diethyldithiocarbamate; 10 = zinc dibutyldithiocarbamate.

Fig. 12. Separation of accelerators by thin-layer chromatography. Solvent: benzene–ethyl acetate–acetone (100:5:1); development distance: 15 cm; indicators: for 11–17: (1) 4 *N* hydrochloric acid; (2) 0.5 % ninhydrin in ethanol containing 10 % acetic acid and 0.5 % cadmium acetate; for 18–20: 5 % bismuth nitrate in 1 *N* nitric acid. 11 = copper dimethyldithiocarbamate; 12 = bismuth dimethyldithiocarbamate; 13 = selenium dimethyldithiocarbamate; 14 = tellurium diethyldithiocarbamate; 15 = cadmium diethyldithiocarbamate; 16 = lead diethyldithiocarbamate; 17 = 2-benzothiazyl-N,N-diethylthiocarbamyl sulfide; 18 = 2-mercaptobenzothiazole; 19 = benzothiazyl disulfide; 20 = zinc benzothiazyl sulfide.

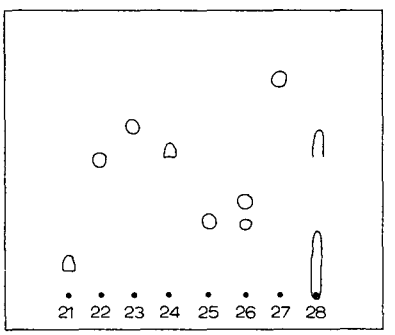


Fig. 13. Separation of accelerators by thin-layer chromatography. Solvent: benzene–ethyl acetate–acetone (100:5:1); development distance: 15 cm; indicators for 21: 5 % bismuth nitrate in 1 *N* nitric acid; for 22–28: (1) 4 *N* hydrochloric acid; (2) 0.5 % ninhydrin in ethanol containing 10 % acetic acid and 0.5 % cadmium acetate. 21 = 2-mercaptothiazoline; 22 = N-*tert*-butyl-2-benzothiazole sulfenamide; 23 = N,N-di-isopropyl-2-benzothiazole sulfenamide; 24 = N-cyclohexyl-2-benzothiazole sulfenamide; 25 = N-oxydiethylene-2-benzothiazole sulfenamide; 26 = 2-(2,6-dimethyl-4-morpholinothio)benzothiazole; 27 = N,N-dimethylcyclohexylamine salt of dibutyldithiocarbamic acid; 28 = 1,1′-methylenedipiperidine–carbon disulfide reaction product.

drin treatment of other compounds on the same plate if they have been masked from the ninhydrin spray by covering with a glass plate. Since one of the sulfenamide amines *tert.*-butylamine, does not yield an especially intense color, the benzothiazole portion can be indicated, after the 4 *N* hydrochloric acid treatment, by further treatment with ammonium sulfide and bismuth nitrate. A second solvent system that has been used to some advantage for accelerators is benzene–ethyl acetate–*n*-butanol (50:1:1).

Amine hydrochlorides

A mixture of *n*-butanol–water–formic acid (5:1:1) was used to separate the amines (Table VIII). After development, the solvent was removed by heating the plate in a 130° forced air oven. When dry, the plate was sprayed with the ninhydrin solution used for the accelerators and returned to the oven to bring out the colors (Fig. 14).

TABLE VIII

APPROXIMATE $R_F \times 100$ VALUES AND COLORS FOR AMINE HYDROCHLORIDES

Development on Silica Gel G with *n*-butanol–water–formic acid (5:1:1) as solvent; indicator: ninhydrin.

No.	*Amine*	$R_F \times 100$	*Color*
1	Dimethylamine	16	Red
2	Diethylamine	25	Red
3	Diisopropylamine	35	Yellow-brown
4	Di-*n*-butylamine	52	Red-brown
5	*tert.*-Butylamine	38	Creamy-yellow
6	Cyclohexylamine	42	Orange-brown
7	Aniline	48	Pink
8	Piperidine	23	Purple
9	Morpholine	18	Red-violet
10	2,6-Dimethylmorpholine	29	Tan

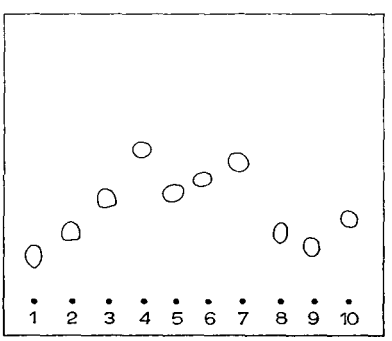


Fig. 14. Separation of amine hydrochlorides by thin-layer chromatography. Solvent: *n*-butanol–formic acid–water (5:1:1); development distance: 15 cm; indicator: 0.5% ninhydrin in ethanol containing 10% acetic acid and 0.5% cadmium acetate. 1 = dimethylamine; 2 = diethylamine; 3 = diisopropylamine; 4 = di-*n*-butylamine 5 = *tert.*-butylamine; 6 = cyclohexylamine; 7 = aniline; 8 = piperidine; 9 = morpholine; 10 = 2,6-dimethylmorpholine.

RESULTS AND DISCUSSION

In all cases, solvent systems giving the greatest range of R_F values have been chosen. The developing distance in all but one case was 15 cm to give additional space as well as separation since rather large numbers of samples were considered in certain groups. Indicating reagents giving a wide range of colors have also been chosen to permit the identification of the compound in many instances by both color and travel distance. Approximate descriptions of the colors obtained for the compounds we examined have been presented in the accompanying tables. Most of the colors will vary somewhat depending upon the conditions of and time after indication. Many satisfactory indicating reagents other than those chosen by us are available and most can be found in the references we have cited. Approximate R_F values have been tabulated; however, an identification is best made by comparison to known compounds. An unknown compound is identified when, after adjusting the sample spot size to match that of the known, it travels the same distance and gives the same color as the known. If even higher probability is desired, the above procedure may be repeated with a different suitable developing solvent.

Amine antioxidants and antiozonants

The amines are generally quite well spaced with a one-dimensional development (Figs. 1, 2, and 3). One-dimensional development has also been found sufficient to resolve the common commercial mixtures. However, several antioxidants and antiozonants are often mixed for increased effectiveness and some situations may require the use of a two-dimensional development. In Fig. 4, the separation of a mixture of seventeen antioxidants and antiozonants by two-dimensional development is shown. It should be possible to separate and identify almost any combination of antioxidants and antiozonants using the above two solvent mixtures or modifications thereof. The small amount of ammonium hydroxide was added to the developing solutions to minimize the tendency of certain compounds to tail.

Phenolic antioxidants

It has been noted that several of the samples (2, 3, 7, 8, 13, 15, 20, and 21) travel nearly identical distances when eluted with benzene. Samples 3 and 7 not only travel identically, but color the same. Thus BHT (butylated hydroxy toluene) is apparently 2,6-di-*tert.*-butyl-*p*-cresol (IR spectra confirm this conclusion). The 8:1 *n*-hexane–benzene double development separates all but samples 2 and 3 and also shows the several components present in sample 20. The only difference between samples 2 and 3 is a methyl group in the *para* position and a separation of the two would be expected to be somewhat difficult. In this situation, the use of an indicating reagent giving a wide range of colors becomes useful. Since it is not especially common to use more than one phenolic antioxidant in a compound, the above or any other pairing should not be a particularly great problem.

One note of caution: certain components of the complex mixtures, including the phosphites (samples 21 to 31) lag in color development when buffered 2,6-dichloroquinonechlorimide is used as indicator. This can be overcome by warming the plate after application of both spray reagents.

The phosphites were included with the phenolics since they are also non-staining.

Guanidines

Only three compounds are involved and no problems are encountered in the separation.

Accelerators

Since only one or two accelerators are generally used in a rubber compound, a TLC identification should not be too difficult. However, more complex curing systems containing three or four accelerators have been used for certain special applications and the identifications, as expected, become more difficult with increasing sample complexity.

Often it is very difficult and frequently impossible to isolate identifiable amounts of undecomposed or unreacted accelerators from a vulcanizate. In such instances, one can only identify the remaining fragments and reaction products and attempt to reconstruct the original accelerator system based on a knowledge of compounding and the use for which the vulcanizate was intended.

Amine hydrochlorides

A 5:1:1 mixture of *n*-butanol–water–formic acid gave the best separation of the ten amine hydrochlorides. Variation of the ratio of *n*-butanol to water and formic acid (*e.g.*, 3:1:1 to 6:1:1) varies the relative positions of the compounds and may be used to advantage if an overlapping pair is observed. One difficulty experienced with ninhydrin was the light color, a creamy yellow, obtained with *tert.*-butylamine as mentioned above for N-*tert.*-butyl-2-benzothiazole sulfenamide.

CONCLUSIONS

TLC has been shown to be a rapid, reliable, relatively simple, and inexpensive analytical technique of great utility for the identification of rubber compounding ingredients as illustrated above. Although an exhaustive accumulation of all antioxidants, antiozonants and accelerators available in the United States was not made, a major portion of the various types of compounds available were examined to show the general utility of TLC for separation and identification.

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CHROM. 4273

ION-EXCHANGE CHROMATOGRAPHY OF TISSUE NUCLEOTIDES

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SUMMARY

A procedure has been devised for the isolation of soluble tissue nucleotides by ion-exchange chromatography on 1.0 × 90 cm columns of DEAE-Sephadex A-25, acetate form, using consecutive concave concentration gradients of the volatile salt triethylammonium acetate at pH 4.7. The procedure gives a high degree of resolution of nucleotides in tissue extracts, and eluate samples can be freed of salt and water with a minimum of manipulation, by storage at reduced pressure and room temperature over sodium hydroxide and phosphorus pentoxide. The method is applicable to the analysis of extracts from a variety of tissues.

INTRODUCTION

Many procedures have been described for the separation of tissue nucleotides by ion-exchange chromatography (see the reviews by SAUKKONEN[1], GRAV[2] AND MANDEL[3]). The materials which have been employed for such separations include the polystyrene-based ion-exchangers (see, for example, the procedures described by HURLBERT *et al.*[4], ANDERSON *et al.*[5] and INGLE[6]), DEAE-cellulose*,[7–9], TEAE-cellulose[9], ECTEOLA-cellulose[10,11], DEAE-Sephadex[12] and PEI-cellulose[13,14].

Most of the published procedures, unfortunately, possess certain inherent disadvantages. For example, most of the procedures which utilize the polystyrene ion-exchangers involve the use of acidic eluants, and some acid-labile materials could be

* The following abbreviations will be employed: A-X = an uncharacterized metabolite of adenosine; C-X_1, C-X_2 and C-X_3 = uncharacterized metabolites of cytidine; DEAE-cellulose and DEAE-Sephadex = diethylaminoethyl derivatives of cellulose and Sephadex, respectively; ECTOELA-cellulose = ion-exchanger prepared by the reaction of cellulose with epichlorhydrin and triethanolamine; FGAR = formylglycineamide ribonucleotide; FMP, FDP and FTP = the 5′-mono-, -di- and -triphosphates, respectively, of formycin; Me6MPR-P = 6-(methylmercapto)-purine ribonucleoside 5′-phosphate; 6MPR-P = 6-mercaptopurine ribonucleoside 5′-phosphate; PEI-cellulose = polyethyleneimine-cellulose; TEAE-cellulose = triethylaminoethyl-cellulose; U-X_1, U-X_2 and U-X_3 = uncharacterized metabolites of uridine. Other abbreviations used are those permitted by *J. Biol. Chem.*, 244 (1969) 2.

destroyed during chromatography. In addition, 6-thiopurine derivatives in particular are very strongly bound to polystyrene-based exchangers by non-ionic forces, resulting in unpredictable elution behavior of these substances and necessitating the use of relatively drastic elution conditions (see ref. 15); since much of the work of this laboratory involves carcinostatic thiopurine compounds, these ion-exchangers would not be suitable for our purposes.

Non-ionic interactions between ion-exchangers and the materials being separated can be minimized by the use of exchangers with a cellulose matrix, and a number of procedures using such materials have been described (see, for example, refs. 8–14). These materials, however, also suffer from a number of drawbacks; their ion-exchange capacities are limited (see PETERSON AND SOBER[16]), requiring the use of relatively large columns, and, until recently, they have been available only in a fibrous form, leading to difficulty in the preparation of uniformly-packed columns, a difficulty which is further compounded by the large column beds required.

A further disadvantage of many procedures is that relatively high concentrations of salt and/or acid are required for the complete elution of the nucleotides; the removal of salts from column eluates in order to facilitate characterization and detailed analysis of the eluted compounds involves the time-consuming manipulation of large numbers of samples. This difficulty has been minimized in some procedures by the use of volatile eluants such as ammonium or triethylammonium salts (see, for example, refs. 8, 12, 17 and 18).

Another problem which frequently arises is that while some procedures can achieve excellent resolution of mixtures of authentic nucleotides, they give much less satisfactory separations of nucleotides in tissue extracts, presumably as a result of the presence in these extracts of salts, sugar phosphates, lipids, etc.

In view of the problems inherent in the use of the best of the available procedures, a study was undertaken to devise a method which would achieve the resolution of most if not all of the soluble tissue nucleotides in a single chromatographic run; would employ an ion-exchange material which did not exhibit the disadvantages of the polystyrene and cellulose materials as noted above; and would employ an eluant salt which could be removed from column effluents with a minimum of manipulation.

DEAE-Sephadex A-25 was chosen as the ion-exchanger, since it possesses a high exchange capacity, exhibits minimal non-ionic interaction with both naturally-occurring and synthetic purine and pyrimidine derivatives, and is available in the form of uniform beads, thus facilitating the preparation of uniformly packed columns. For reasons that have been discussed elsewhere[6,7,19,20], concave concentration gradients at constant pH were employed; initial studies showed that maximal resolution of tissue nucleotides could be achieved at pH 4.70. The volatile salt triethylammonium acetate was selected as the eluant, since it is easily prepared as needed from triethylamine and acetic acid, both of which are readily available in high purity; it buffers well at the pH employed; it exhibits minimal absorption of UV light; and it is readily removed from eluant samples at room temperature and reduced pressure[12,17].

Results which have been obtained with this procedure have been included in a number of earlier reports[21–25], and a brief description of the method has been published[26]. This report presents the details of the procedure.

EXPERIMENTAL

Materials

DEAE-Sephadex A-25 was purchased from Pharmacia (Canada) Ltd. Triethylamine (reagent grade) was purified within four weeks of use by refluxing for 3 h with 2,4-diaminophenol dihydrochloride ("Amidol") (3 g/l), followed by distillation at atmospheric pressure (see PARISH[27]). [8-^{14}C]-6-Mercaptopurine was purchased from New England Nuclear Corp., and was stored frozen in saline saturated with hydrogen sulfide at pH 8; before use, the suspensions were acidified to pH 2, hydrogen sulfide was removed with a stream of nitrogen, and the pH was then adjusted to 7.5–8.0 with sodium hydroxide. (Under these conditions, no decomposition of the drug could be detected after two years, *cf.* ref. 28.) Other radioactive materials were obtained from Schwarz BioResearch Co. Other chemicals were obtained from commercial suppliers, and were of the highest available purity.

Procedures for the maintenance and propagation of the Ehrlich ascites carcinoma have been described elsewhere[15].

Preparation of the ion-exchanger

DEAE-Sephadex A-25 (purchased in the chloride form) was allowed to hydrate overnight in distilled water, and was then washed by decantation ten times with 0.5 *N* sodium hydroxide and three times with water. The ion-exchanger was then suspended in 2 *M* sodium acetate pH 4.5–5.5, packed into a column and washed with the same buffer until the effluent was free of chloride ion. The material was then transferred to a large beaker and suspended in 0.04 *M* triethylammonium acetate, pH 4.70 ± 0.05 ("starting buffer"); the suspension was allowed to settle for 15 min, and the milky supernatant fluid was siphoned off. The procedure was repeated until all of the "fines" had been removed. The ion-exchanger was prepared shortly before use; it can be regenerated and re-used, but has generally been discarded after one use.

Preparation of the columns

Columns were made from 1 × 120 cm Pyrex tubing, and were "siliconized" by treatment with a solution of dimethyldichlorosilane in benzene* according to the supplier's instructions. (The use of coated columns is not required for optimal resolution of tissue nucleotides, but greatly facilitates the cleaning of the columns.) A short section of gum rubber tubing was attached to the bottom of the column, and closed off with a clamp. The column was then filled with starting buffer, and a column support was prepared by inserting a 2 cm square of surgical gauze into the column with a long glass tube, to form a "pocket" at the bottom of the column, and dropping eight to ten glass beads (diameter, 5 mm) into the "pocket" through the tube. A slurry of prepared ion-exchanger was added to a reservoir fitted to the top of the column, and the outlet was then opened. The exchanger was allowed to settle to a height of 90 to 93 cm, while the flow rate through the column was maintained at 50 ml/h by means of a metering pump**. Excess ion-exchanger was then removed, and starting buffer (at least 300 ml) was pumped through the column at the same flow rate to ensure complete equilibration. Immediately before application of the sample, the top

* "Column coating", Cat. No. 81900, BioRad Laboratories, Richmond, Calif., U.S.A.
** Instrument MiniPump, Milton Roy Instrument Ltd., Philadelphia, Pa., U.S.A.

5–6 cm of the column bed were stirred up with a glass rod, and allowed to settle by gravity; this ensured a uniform bed surface. A disc of filter paper was placed on top of the column bed to minimize disturbance of the surface during sample application.

Tissue extracts

Liver. Mice were killed, and readily-accessible portions of the liver were rapidly excised and frozen by compression between blocks of dry ice. The frozen livers were extracted with 0.4 *M* perchloric acid (1.5 ml/g of tissue), using a glass homogenizer fitted with a Teflon pestle; the homogenate was centrifuged, and the residue was re-extracted with perchloric acid (1.0 ml/g of tissue). The two extracts were combined and neutralized with potassium hydroxide; the neutralized extract was stored in ice for 60 min and centrifuged to remove potassium perchlorate. The final extract was used immediately, or stored at $-20°$ for up to four days.

Tumor cells. For studies of the metabolism of 6-mercaptopurine, the drug (6 μmoles per mouse, 1.1 μC per μmole) was administered to mice, seven days after implantation of the Ehrlich ascites carcinoma, by intraperitoneal injection. Two hours after administration, the animals were killed, the tumors were rapidly drained through an abdominal incision into chilled saline and the cells were collected immediately by centrifugation at 2°. The cells were extracted with perchloric acid by the general procedure described for liver.

In other experiments with tumor cells, the cells were collected, washed and incubated with the appropriate precursors in FISCHER's medium[29] as described elsewhere[30]. At the completion of the incubation period, the cells were collected by centrifugation, and extracts were prepared as described above.

Chromatography

Samples were applied to the column by means of a syringe and a length of polyethylene tubing. Material adhering to the sides of the column was washed into the column bed with several small portions of starting buffer, and elution was then begun.

The elution scheme employed is presented in Table I. Gradient 1, which elutes nucleotides through GMP, and 2, which continues elution through ADP, were run to completion. Gradient 3 was run until the elution of ATP was complete, and was then discontinued; "gradient" 4 was then pumped through the column to elute GTP as a sharp band. For the preparation of a stock 2 *M* triethylammonium acetate solution, glacial acetic acid (230 ml) and distilled water (1200 ml) were stirred at 2°, and cold purified triethylamine (210 ml) was added in portions; the mixture was stirred until homogeneous, and diluted to 2 l. Working solutions were prepared by dilution of the stock solution to 90 % of the desired volume, dropwise addition of triethylamine to pH 4.70 ± 0.05 and dilution to volume. Fractions of approximately 5 ml were collected at an initial flow rate of 45 to 50 ml/h, maintained with the pump; the flow rate was decreased at intervals to compensate for the increased resistance to flow which developed during chromatography, in order to prevent undue compaction of the column bed.*

* The resistance to flow and the flow rate fluctuate considerably during the first 3–4 h of operation of the column, and the column must be watched carefully during this period. During the balance of the chromatography, however, changes in flow rate are slight and gradual.

TABLE I

ELUTION SCHEME FOR CHROMATOGRAPHY OF NUCLEOTIDES

Gradient No.	*Mixing chamber*	*Reservoir*
1	600 ml 0.04 *M* buffer[a] (1000 ml bottle[b])	450 ml 0.35 *M* buffer (500 ml conical flask[d])
2	440 ml 0.35 *M* buffer (500 ml bottle[c])	260 ml 1.00 *M* buffer (250 ml conical flask[d])
3	700 ml 1.00 *M* buffer (1000 ml bottle[b])	260 ml 1.40 *M* buffer (250 ml conical flask[d])
4	200 ml 1.40 *M* buffer containing 1.00 *M* triethylammonium formate	

[a] "Buffer" refers to triethylammonium acetate, pH 4.70 ± 0.05.
[b] Cat. No. 1240, Corning Glass Works, Corning, N.Y.; I.D., 9.5 cm.
[c] Cat. No. 1550, Corning Glass Works Corning, N.Y.; I.D., 7.5 cm.
[d] Cat. No. 4980, Corning Glass Works, Corning, N.Y.

Recovery of eluted materials

The appropriate eluate fractions were pooled, transferred to a petri dish, and frozen to a slush at −20°. Water and salt were removed from the frozen samples by storage in vacuum desiccators over solid sodium hydroxide and phosphorus pentoxide at reduced pressure (20–50 μ) at room temperature for 16–24 h. Residual salt was removed by dissolving the residue in water and repeating this procedure.

Identification of eluted materials

Nucleotide materials in column eluates were identified on the basis of a minimum of three of the following criteria: (1) UV absorption spectra, (2) coincident elution from the column with authentic samples added to the tissue extracts, (3) co-chromatography with authentic compounds by paper chromatography in at least two solvent systems, (4) incorporation of radioactivity into the eluted materials following exposure of the tissue to appropriate radioactive precursors, (5) identification of the purine or pyrimidine moiety after treatment of the eluted material with *Crotalus terrificus* venom, and (6) specific enzymic assays, where applicable.

RESULTS

The chromatographic profiles presented in the accompanying figures are typical of the results obtained in more than 150 chromatographic experiments.

Fig. 1 depicts the chromatographic analysis of an extract of tumor cells which had been exposed to [^{14}C]cytidine, C-X_1, C-X_2 and C-X_3 are all cytidine-containing compounds, but have not been characterized as yet. Similarly, U-X_1, U-X_2 and U-X_3 are uridine-containing compounds; although they have not been characterized in detail, the presence of uridine and the apparent net charge of these metabolites suggest that the first two may be UDP-sugar and/or UDP-aminosugar derivatives, while U-X_3 may be UDP-glucuronate.

Figs. 2 and 3 represent the analysis of extracts of tumor cells which had been incubated with [^{14}C]adenine and [^{14}C]guanine, respectively. Peak A-X is an un-

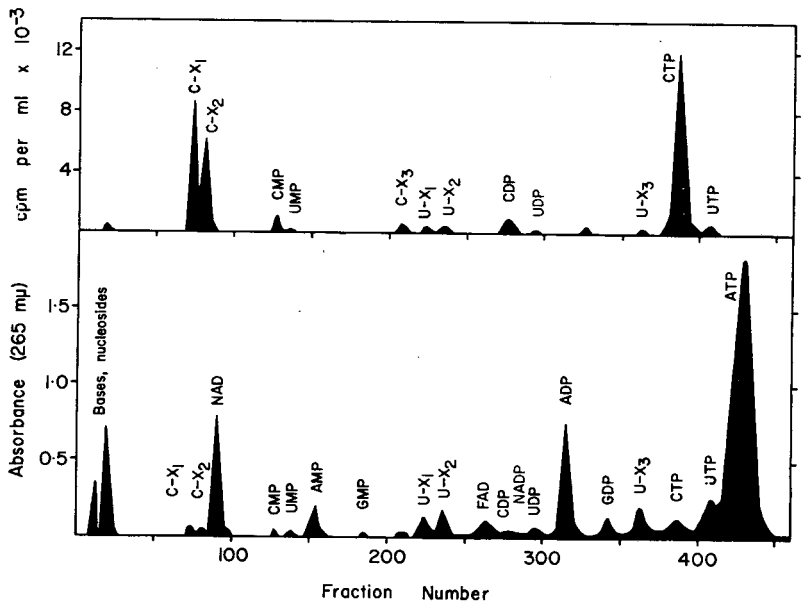

Fig. 1. Chromatography of an extract of tumor cells previously exposed to [^{14}C]cytidine. Tumor cells (5 g, wet weight) were incubated with [2-^{14}C]cytidine (23 μC per μmole, 0.04 μC per ml) for 30 min. The cells were extracted and the extracts were analyzed as described in the section EXPERIMENTAL. (GTP was not eluted in this experiment.)

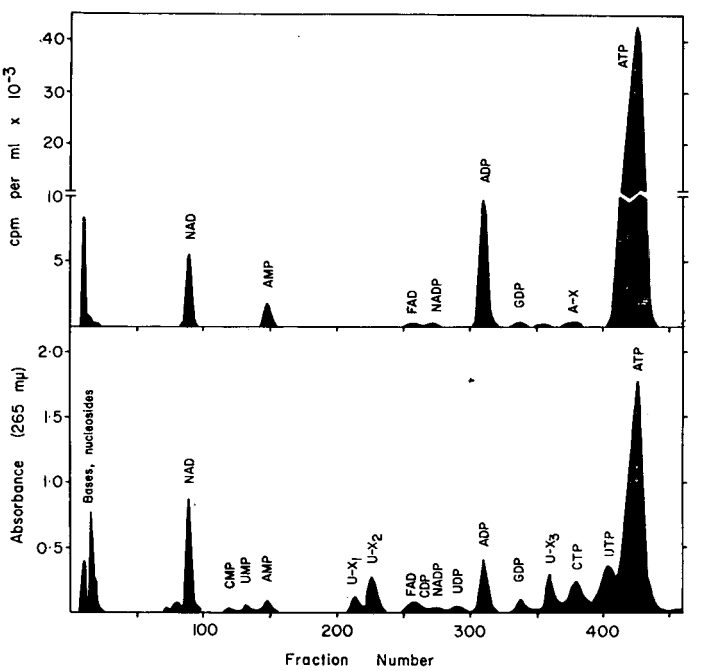

Fig. 2. Chromatography of an extract of tumor cells incubated with [^{14}C]adenine. The conditions were the same as for Fig. 1, except that the cells were incubated with [8-^{14}C]adenine (53 μC per μmole, 0.1 μC per ml) for 60 min. (GTP was not eluted in this experiment.)

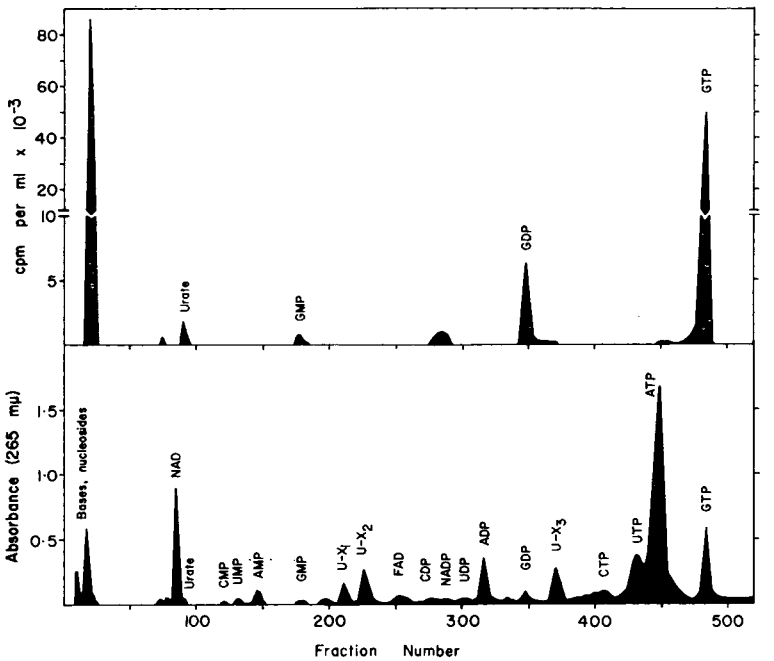

Fig. 3. Chromatography of an extract of [^{14}C]guanine-labeled cells. The conditions were the same as for Fig. 1, except that the cells were incubated with [8-^{14}C]guanine (29 μC per μmole, 0.1 μC per ml) for 2 h.

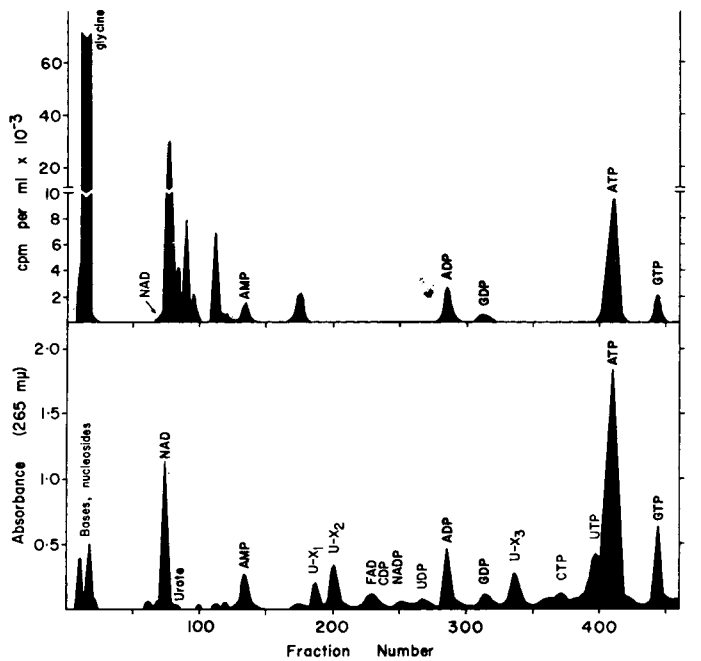

Fig. 4. Chromatography of an extract of tumor cells previously exposed to [^{14}C]glycine. The conditions were the same as for Fig. 1 except that the cells were incubated with [1-^{14}C]glycine (33 μC per μmole, 0.2 μC per ml) for 90 min.

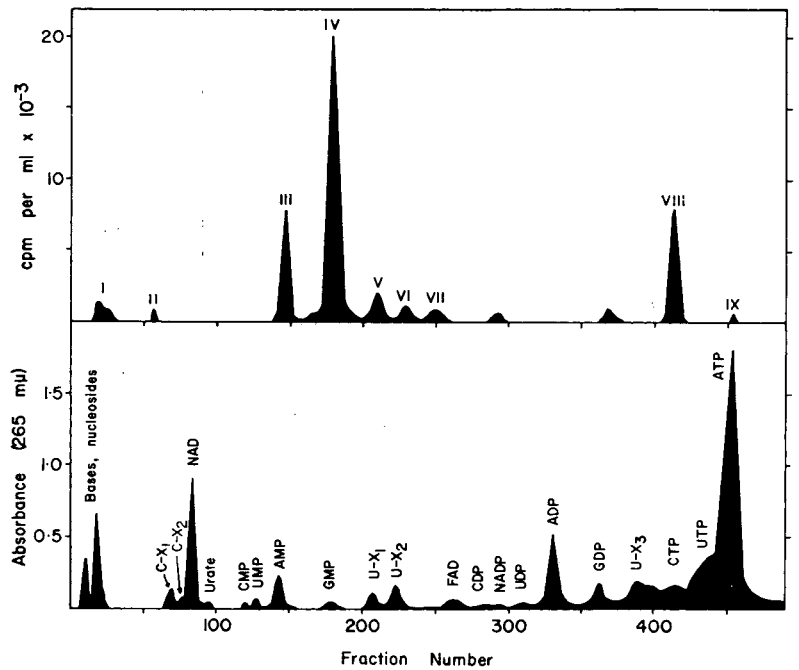

Fig. 5. Chromatography of an extract of tumor cells after treatment with [^{14}C]6-mercaptopurine. The details of this experiment are presented in the section EXPERIMENTAL. The standard chromatographic procedure was employed, except that the column was washed with 100 ml of 0.35 *M* buffer between gradients 1 and 2. (GTP was not eluted in this experiment.)

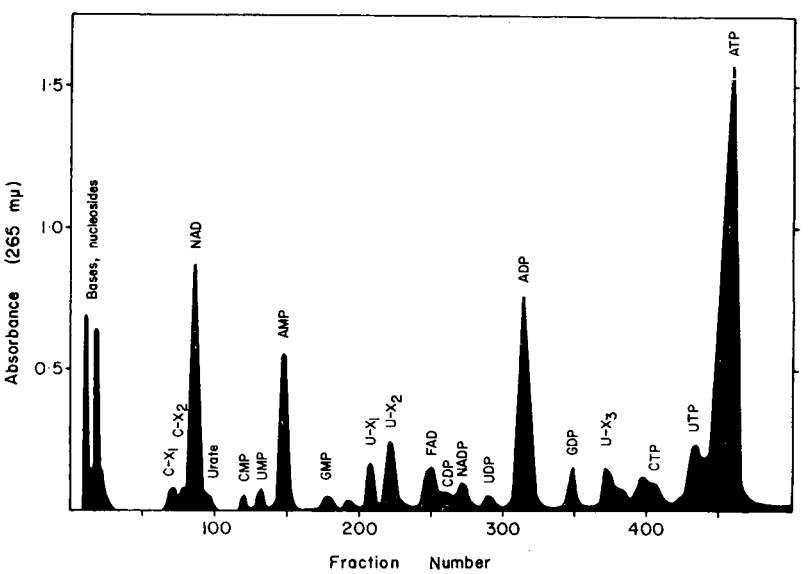

Fig. 6. Chromatography of an extract of mouse liver. For details, see the section EXPERIMENTAL

characterized adenosine derivative; the peaks which are not identified represent metabolites which have not yet been examined in detail.

Fig. 4 shows the results of the chromatography of an extract of tumor cells which had been incubated with [^{14}C]glycine, and indicates the degree of resolution which can be achieved with this procedure. Most of the peaks which are eluted between fractions 75 and 130 have not been characterized; some may be intermediates in purine synthesis *de novo*, since many of these peaks are diminished or absent when cells which have been treated with 6-(methylmercapto)purine ribonucleoside, a known inhibitor of this pathway[31], are treated in the same manner[32].

Fig. 5 presents the results of an experiment in which [^{14}C]mercaptopurine was administered to tumor-bearing mice 2 h before the collection and extraction of the cells. Peak I consist of at least three compounds, including unchanged 6-mercaptopurine; peaks III and IV have been identified by procedures which have been described previously[15,33], as 6-(methylmercapto)purine ribonucleoside 5'-phosphate and 6-mercaptopurine ribonucleoside 5'-phosphate, respectively; and peak VIII has been identified as 2-hydroxy-6-mercaptopurine ribonucleotide[34] ("thioXMP"). The other peaks have not been characterized at the present time*. (One or more of these other metabolites may be derivatives of 2-amino-6-mercaptopurine ("thioguanine")—see SCANNELL AND HITCHINGS[35].) The "shoulder" on the leading edge of peak IV and the two peaks between VII and VIII have been observed in some, but not all, experiments, and their status as metabolites of 6-mercaptopurine is uncertain.

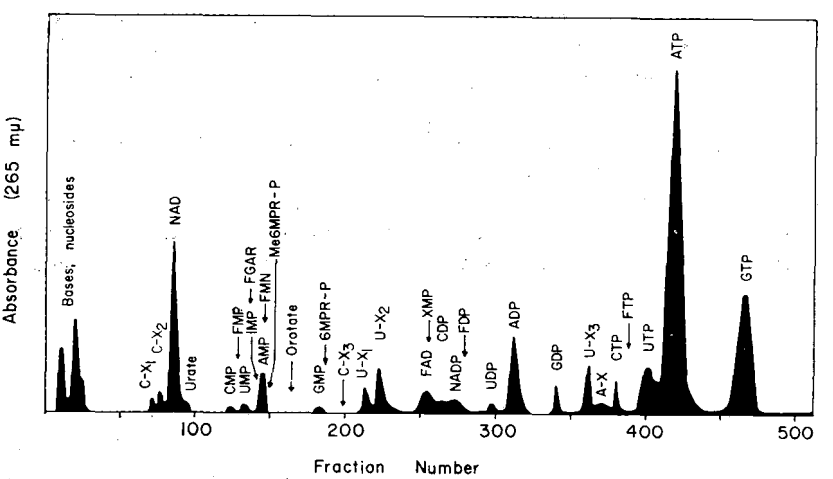

Fig. 7. Composite chromatographic profile. For explanation, see text.

Fig. 6 presents a chromatographic profile obtained with an extract of mouse liver. The procedure has also been applied successfully to the analysis of extracts of blood cells[36] and skin fibroblasts[24]; extracts of other tissues have not been examined in this laboratory, but studies in other laboratories have shown that the method can be employed successfully in the analysis of extracts of rat uteri[37] and bacterial cells[38].

* Peak IX coincides precisely with the ATP peak and may well be the result of a trace contamination of the 6-mercaptopurine preparation with [^{14}C]hypoxanthine.

Fig. 7 presents a composite chromatographic profile, summarizing the results of studies performed in this laboratory over the past four years, and showing the elution positions of a number of purine and pyrimidine derivatives.

DISCUSSION

The chromatographic procedure described in this report achieves a high degree of resolution of soluble nucleotides in extracts of a variety of tissues, although the original objective, that of achieving complete resolution of these nucleotides in a single chromatographic run, has not been realized. For most purposes, the degree of resolution achieved with this method will be adequate; in some instances, however, rechromatography of selected portions of the column eluates may be required.

The procedure as described here has been successfully employed in the analysis of up to 30 ml of extract, derived from up to 10 g of tumor cells or 5 g of liver, without significant impairment of resolution. The chromatography of larger volumes of tissue extracts, or of extracts containing very high levels of salt, leads to some loss of resolution (see ref. 24). The analysis of extracts derived from larger amounts of tissue can also lead to diminished resolution; this is observed primarily in the nucleoside triphosphate region, and is mainly a consequence of broadening of the ATP peak.

Most nucleotides are eluted in volumes of 50–75 ml, and column eluates can be freed of salt and water with a minimum of manipulation. The procedure gives highly reproducible results; the elution position of a given nucleotide rarely varies by more than five fractions, and in most instances chromatographic profiles obtained in experiments carried out under comparable conditions are virtually superimposable. Tissue nucleotides appear to be very stable during the chromatographic procedure; the absence of an increase in background radioactivity in the later stages of the experiments depicted in Figs. 1–5 strongly suggests that little or no breakdown of nucleotides occurs under the conditions employed.

This procedure, which was originally devised for studies of the metabolism of carcinostatic purine analogues by tumor cells, offers a number of advantages over the existing procedures, and should be of considerable value in studies of many aspects of nucleotide metabolism.

ACKNOWLEDGEMENTS

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CHROM. 4276

RAPID SEPARATION OF NUCLEOSIDES AND NUCLEOTIDES BY CATION-EXCHANGE COLUMN CHROMATOGRAPHY

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SUMMARY

Three methods for separation of nucleosides and nucleotides on the cation exchanger AG 50W–X4, at alkaline pH, are described. Two of the methods, one for the deoxy-series and one for the ribo-series, separate the nucleotides together and the nucleosides into the individual components. Regeneration of the columns is not required for re-use. The third method separates completely all nucleotides and nucleosides. All separations are complete in one and a half to two hours.

INTRODUCTION

Methods used for the determination of the sequence of oligonucleotides include digestion by various enzymes and separation, identification and quantitation of the products. Following removal of terminal phosphate groups the enzyme used is usually an exonuclease such as snake venom phosphodiesterase (EC 3.1.4.1.) which releases the terminal bases as nucleosides and the internal bases as nucleotides[1–3]. The large number of oligonucleotides released by partial degradation of even the smallest nucleic acids[4] necessitates that the methods of analysis for sequence be both rapid and quantitative. Recently a number of methods for separation of nucleosides or nucleotides have been reported[5–9]. Two of these methods[5,8] are rapid, that is, can be accomplished in 1 to 1.5 h. The method described by BLATTNER AND ERICKSON[5] is applicable only to nucleotides and that of UZIEL *et al.*[8], for nucleosides, has been developed for use with a highly automated chromatographic system and will not separate nucleotides from nucleosides under the conditions described. We have designed procedures for both the ribo- and deoxyribo-series in which a mixture of nucleotides and nucleosides can be separated by column chromatography within 1.5 to 2 h. The procedures have the additional advantages that the columns do not need regenerating but can be used many times, the sample material can contain enzymes and buffer salts and the eluting solvents are volatile.

EXPERIMENTAL

The cation exchanger used is AG 50W-X4, minus 400 mesh (Calbiochem, Los Angeles, Calif., U.S.A.). The resin was washed twice with 10 vol. of 1 *N* HCl,

three times with 10 vol. of concentrated NH_4OH followed by three washes with 10 vol. of the elution buffer to be used[5]. Fines were removed by decantation.

Nucleosides and nucleotides were purchased from Calbiochem and enzymes from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. *E. coli* alkaline phosphatase (EC 3.1.3.1.) was assayed with sodium *p*-nitrophenylphosphate (British Drug Houses Ltd.) and snake venom phosphodiesterase (EC 3.1.4.1.) with calcium bis-*p*-nitrophenylphosphate (Sigma Chemical Co.) Bovine spleen phosphodiesterase (EC 3.1.4.1.) was not assayed prior to use since it was used in excess.

Column preparation and elution

Chromatography columns were glass, 11 mm I.D. with internally threaded ends (Ace Glass Incorporated, Vineland, N.J., U.S.A.). They were fitted with a polyethylene filter disc, pore size 100 μ, and a nylon bottom drip adapter with a Luer fitting which connected to Chromatronix (York Instrument Company, New York, N.Y., U.S.A.) teflon tubing adaptors. These column end fittings eliminated dead volume at the end of the column so that mixing of the eluted material was minimized. Columns were packed with a 50 % slurry of the resin and washed with the corresponding elution buffer for 1 h prior to use. Columns stored for even a few hours were routinely washed 1 h before use. Flow rate was maintained with the use of a Buchler polystaltic pump at 70–100 ml/h. The internal threading of the glass columns was required to withstand the back pressure generated by the pump. All columns were operated at room temperature. Sample volumes ranged from 0.02 to 2.2 ml. Enzyme digestion mixtures were adjusted to the pH of the elution buffer and loaded directly on the column. The sample was allowed to soak completely into the resin bed then the column eluted immediately. The UV absorption of the column effluent was determined with a Gilford 2000 spectrophotometer at 260 mμ, or an Isco model UA-2 UV monitoring system at 254 mμ. Two milliliter fractions were collected.

Sample preparation

Commercial nucleosides and nucleotides of the ribo- and deoxyribo-series were dissolved in the appropriate chromatography buffer to give a final solution of 0.5–0.8 A_{260} units or 5–17 A_{260} units per nucleotide or nucleoside.

Deoxyoligonucleotides obtained from DNase 1 digests were fractionated according to chain length[10] and the fractions digested with 0.5 units of *E. coli* alkaline phosphatase per 30–100 A_{260} units dissolved in 0.05 *M* Tris–HCl buffer pH 8.0, for 6 h at 37°, total volume 2.0 ml. Phosphatase was removed by four phenol extractions followed by four ethyl ether extractions to remove the excess phenol. The ether was removed by evaporation in a water bath at 50° for 15 min. 1.5 ml of the phosphatase treated material was adjusted to pH 8.8 with 0.1 *M* Trizma base (Sigma Chemical Company, St. Louis, Mo., U.S.A.), pH unadjusted, containing 0.03 *M* $MgCl_2$, and 0.2 units of snake venom phosphodiesterase were added and the mixture incubated for 12 h at 37°. A second 1.5 ml sample of the phosphatase treated material was mixed with 0.4 ml of 0.5 *M* sodium acetate buffer pH 6.5 and to this 1 unit of spleen phosphodiesterase was added and the mixture incubated for 24 h at 37°. Following adjustment of the pH for chromatography, as described above, these samples were applied directly to the chromatographic columns.

Chromatographic systems

Three different chromatographic elution conditions have been developed.

Separation of 3'- or 5'-deoxynucleotides and deoxynucleosides. Column, 42 cm by 11 mm; elution buffer, ammonium formate prepared by adjustment of 0.3 *M* NH_4OH to pH 9.2 with concentrated formic acid.

Separation of 3'- or 5'-ribonucleotides and ribonucleosides. Column, 60 cm by 11 mm, buffer, ammonium formate prepared by adjustment of 0.3 *M* ammonium hydroxide to pH 8.9 with concentrated formic acid.

Fractionation of all four deoxynucleosides and 5' deoxynucleotides. Column, 42 cm by 11 mm; buffer, ammonium formate prepared by adjustment of 0.015 *M* ammonium hydroxide to pH 3.2 with formic acid. Following elution of the first five components with this buffer a second ammonium formate buffer, prepared by adjustment of 0.3 *M* ammonium hydroxide to pH 9.2 with formic acid, is applied.

A Radiometer, Model TTT 1c pH meter, calibrated at 20° and standardized at pH 4, 7 or 10 daily, was used for all pH measurements.

All buffers must be freshly prepared since being volatile they are susceptible to pH change upon standing. The elution buffers are the same as those used for loading the columns and the first two columns automatically regenerate themselves with elution and no further regeneration is required. Nucleotides and nucleosides were distinguished by phosphate analysis and the separated components identified by spectral analysis.

RESULTS

Separation of 3'- or 5'-deoxynucleotides and deoxynucleosides and 3'- or 5'-ribonucleotides and ribonucleosides

The chromatographic separation of two mixtures of 5'-deoxynucleotides and deoxynucleosides is shown in Fig. 1a and two mixtures of 5'-ribonucleotides and ribonucleosides in Fig. 1b. Both sets of mixtures contained identical components in equal proportions but differed in concentration by a factor of 20 for the deoxy compounds, and 12 for the ribo compounds. All four nucleotides were eluted together in these systems and the four nucleosides then separated into the individual components in the elution order thymidine or uridine, cytidine, guanosine and adenosine. The separated components were analyzed quantitatively by pooling the peaks, adjustment of the pH and spectral analysis. The results presented in Table I list both recoveries and the volume of the fractions eluted from the column for the largest sample. The results shown in Fig. 1a and b and Table I are those obtained by the optimal conditions listed under EXPERIMENTAL. Variations in column length, column diameter, flow rate, molarity and pH of the eluting buffer were all investigated. The separation is extremely pH dependent. Variations of as little as 0.1 pH units will affect the elution position of deoxyguanosine so that it will interfere with either the deoxycytosine or deoxyadenosine separations; similarly for guanosine in the ribo-series. Longer columns improve the distance of separation between individual components but also increase the time required for the separation. Oligonucleotides and inorganic phosphate co-chromatograph with the mononucleotide fractions.

Fig. 1c shows an identical chromatogram to that in Fig. 1b except for the addition of deoxythymidine to the mixture of ribomononucleotides and mononucleosides

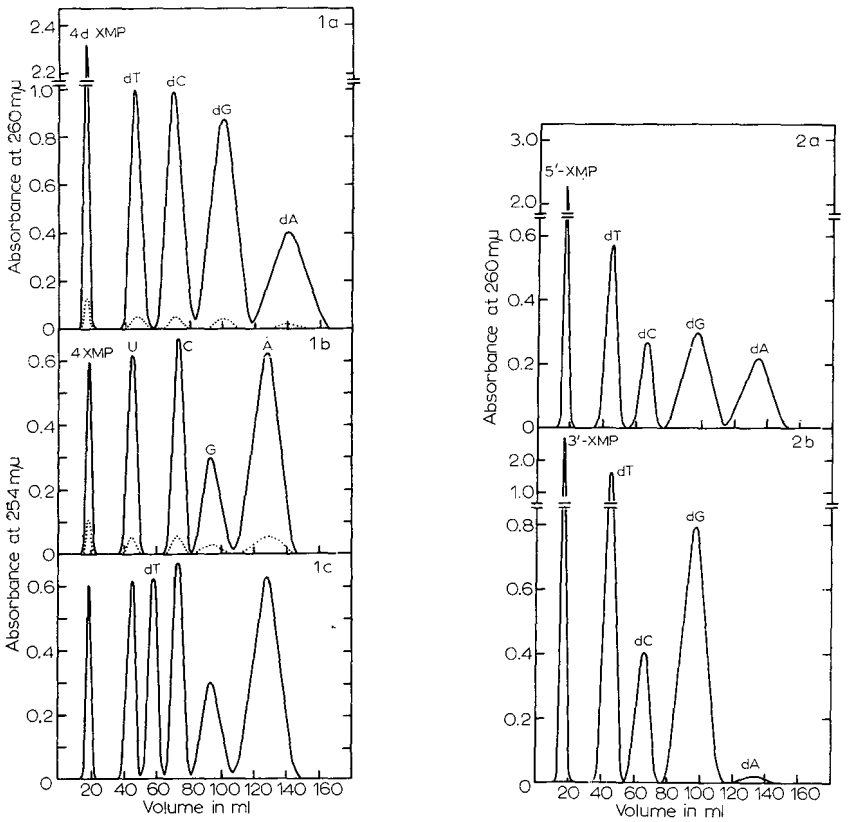

Fig. 1. (a) Chromatography of 5′-deoxynucleotides and deoxynucleosides. A mixture containing the four 5′-deoxynucleotides and four deoxynucleosides was loaded on a 42 × 1.1 cm column and eluted with a 0.3 *M* ammonium formate buffer, pH 9.2.———, 46.80 absorbance units (260 mμ) of mixture in 1.0 ml sample volume. ·····, 2.34 absorbance units (260 mμ) of mixture in 0.05 ml sample volume. (b) Chromatography of 5′-ribonucleotides and ribonucleosides. A mixture containing the four 5′-ribonucleotides and four ribonucleosides was loaded on a 60 × 1.1 cm column and eluted with 0.3 *M* ammonium formate buffer, pH 8.9.———, 36.0 absorbance units (260 mμ) of mixture in 0.6 ml sample volume.·····, 3.0 absorbance units (260 mμ) of mixture in 0.05 ml sample volume. (c) Fractionation of deoxythymidine in the ribonucleoside chromatographic system. To a mixture of 36.0 absorbance units (260 mμ) of 5′-ribonucleotides and ribonucleosides 7.0 A_{260} units of deoxythymidine were added. The total mixture was chromatographed as described in (b). Sample volume: 0.8 ml.

Fig. 2. (a) Chromatography of a snake venom phosphodiesterase digest of the trinucleotides from a DNase I digest of *E. coli* K 12 DNA. The fractionation was performed as described in Fig. 1a. Sample volume: 1.0 ml. (b) Chromatography of a spleen phosphodiesterase digest of the trinucleotides from a DNase I digest of *E. coli* K 12 DNA. The fractionation was performed as described in Fig. 1a. Sample volume: 2.2 ml.

applied to the column. The deoxythymidine was eluted in a position intermediate between uridine and cytidine and this has an advantage in the sequential analysis of ribo-oligonucleotides which may have small DNA oligonucleotide contaminations in that the deoxythymidine will show up as an individual peak.

In studies of the composition of oligodeoxynucleotides or oligoribonucleotides

TABLE I

QUANTITATIVE ANALYSIS OF THE 5′-NUCLEOTIDES AND NUCLEOSIDES SEPARATED BY CHROMATOGRAPHY ON DOWEX 50

The analyses correspond to the chromatographic separations of the 46.8 A_{260} unit sample shown in Fig. 1a and the 36.0 A_{260} unit sample shown in Fig. 1b. The fractions containing each component were pooled and the total absorbance measured at the pH of the buffer. The figures in parentheses indicate the number of determinations.

Components	*% recovery ± S.E.M.*	*Volume of fraction*
5′-Deoxynucleotides	99.4 ± 0.06 (4)	8.8
dT	99.0 ± 0.03 (4)	15.8
dC	100.0 ± 0.02 (4)	21.5
dG	101.6 ± 0.03 (4)	37.0
dA	98.4 ± 0.07 (4)	43.8
5′-Ribonucleotides	99.5 ± 0.06 (3)	7.4
U	101.0 ± 0.09 (4)	12.4
C	100.8 ± 0.06 (4)	20.0
G	100.0 ± 0.03 (4)	27.8
A	98.9 ± 0.11 (4)	37.0

the first deoxynucleotide or ribonucleotide peaks eluted can be conveniently fractionated into their four components by the procedure of BLATTNER AND ERICKSON[5]. This allows a complete nucleotide/nucleoside analysis within a period of 2 to 2.5 h. The chromatogram shown in Fig. 2a shows results of a phosphomonoesterase and snake venom phosphodiesterase digest of the trinucleotides isolated from a DNase 1 digest. Fig. 2b shows a similar separation of a phosphomonoesterase and spleen phosphodiesterase digest of the same material. Comparing the separations with those obtained in Fig. 1a they are seen to be identical and the salts and enzymes present in the enzyme incubation medium do not interfere with the chromatographic elution. In these experiments the mononucleotides were desalted by sublimation *in vacuo* and subsequently fractionated according to BLATTNER AND ERICKSON[5]. The results are presented in Table II.

Fractionation of all four deoxynucleosides and 5′-deoxynucleotides

A second method of approach for a complete nucleotide/nucleoside separation is shown in Fig. 3. The first elution buffer elutes the four deoxynucleotides and deoxythymidine. Following the appearance of adenylic acid the buffer is changed and a peak of absorbance appears immediately following the application of the second buffer due to the change in salt concentration. A similar effect has been noted previously by KHYM AND UZIEL[11]. The diesterase enzymes do not interfere with the separation. Recoveries from this column are better than 98%.

DISCUSSION

The separation of nucleosides at high pH values on a cation exchanger where the total charge of the nucleosides is nil (for A and C) or partially negative (for G, T and U) is due to preferential non-ionic interactions with the resin[12]. The partial negative charge of the hydroxyl groups of guanosine, deoxyguanosine, uridine and thymidine

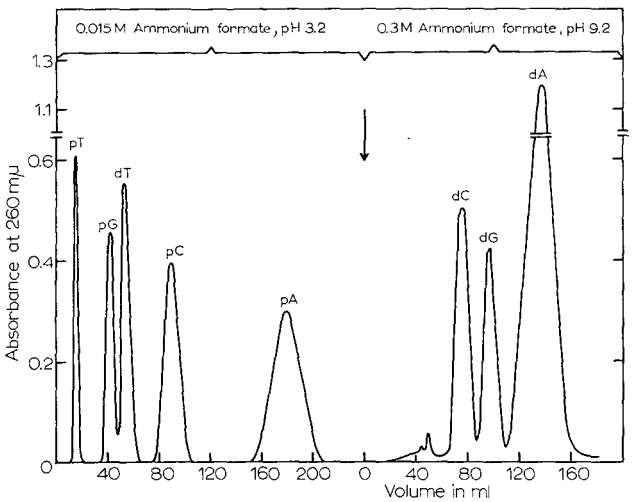

Fig. 3. Separation of all four deoxynucleosides and 5′-deoxynucleotides. A mixture of the eight components totalling 80 absorbance units (260 mμ) was loaded on a 42 × 1.1 cm column in 0.015 *M* ammonium formate buffer, pH 3.2. Sample volume: 1.0 ml. This buffer was then used for elution of the first five components and the second elution buffer, 0.3 *M* ammonium formate, pH 9.2, applied.

TABLE II

ANALYSIS OF THE 5′ AND 3′ ENDS OF THE TRINUCLEOTIDES ISOLATED FROM A DNase I DIGEST OF *E. coli* K 12 DNA

The analysis corresponds to the chromatographic separations shown in Figs. 2a and b. The separated components were pooled and the total absorbance measured at pH 2 (pH 1 for guanine) and the concentrations calculated from the extinction coefficients of the compounds. The 5′-deoxynucleotides from the snake venom phosphodiesterase digest (Fig. 2a) were desalted by evaporation and sublimation *in vacuo* and analyzed according to BLATTNER AND ERICKSON[5].

Components	*5′ end (Fig. 2a)*		*3′ end (Fig. 2b)*	
	μmoles	%	*μmoles*	%
dT	0.275	25.1	0.920	38.0
dC	0.087	7.9	0.410	16.9
dG	0.426	38.9	1.050	43.4
dA	0.307	28.0	0.040	1.7
Total	1.095	99.9	2.240	100.0
pT	0.627	28.3		
pC	0.416	18.8		
pG	0.797	35.9		
pA	0.378	17.0		
Total	2.218	100.0		

at the pH used results in the differential elution of all the components of the mixture. Together with the differences in pK values the additional methyl group of deoxythymidine may explain the increased retention of dT at pH 8.9, a factor used for the separation of this nucleoside from uridine.

The first two systems described, the separation of 3′- or 5′-deoxynucleotides and deoxynucleosides and the separation of 3′- or 5′-ribonucleotides and ribonucleosides, are on column systems which do not require any regeneration. This allows a very fast turn-over of experiments. A complete separation of all four deoxynucleosides and deoxynucleotides on the single column by two buffers at two different pH values requires regeneration of the column and equilibration with the first buffer before re-use. An advantage with all methods is that the enzymes and ions in the digestion mixtures do not interfere with the separations and quantitative analyses. Attempts to determine the actual elution position of the enzyme by using ten-fold excess of enzyme and measuring enzyme activity and protein were unsuccessful. However, the columns have been re-used up to twelve times without repacking. The separation of deoxythymidine from the ribonucleosides provides an easy method for identification of DNA contamination in RNA preparations. The pH at which the elutions occur is in the most stable range for the purine glycosidic links. This is compared to the disadvantage of elution of purine nucleosides and nucleotides with acidic solutions[8]. However, the main advantages of the procedures described above are the rapid separations achieved in 1.5 to 2 h, the quantitative recoveries of the material and the accuracy of the method which allows very small quantities of material to be quantitatively measured (see Fig. 1). The method is currently being applied to the analysis of deoxyribonucleotides from DNase 1 digests (Table II).

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CHEMICAL FORMS OF IODINE IN CARRIER FREE PREPARATIONS OF $Na^{131}I$

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SUMMARY

Various forms of iodine have been separated chromatographically and their R_F values determined. The formation of unidentified components in the systems: $^{131}I^-/I_2$, $^{131}I^-/IO_3^-$, $^{131}I^-/IO_4^-$, has been investigated as a function of the concentration of the reagent, the pH of the medium, the aging time of the solution and the temperature, by employing a paper chromatographic method.

INTRODUCTION

Radioactive iodine-131 is obtained by irradiating tellurium: $^{130}Te(n,\gamma)^{131}Te \xrightarrow{\beta} {}^{131}I$. The final form of the preparation is as $Na^{131}I$ in a reducing solution: $Na_2S_2O_3$, Na_2CO_3, $NaHCO_3$ and $Na^{131}I$ in NaOH (refs. 1 and 2).

In addition to the known stable forms: IO_3^-, IO_4^- and I_3^-, commercial preparations of iodide can also contain a certain quantity of unidentified noniodide components[3].

On investigating the valency state of the iodine, obtained by neutron irradiation of tellurium, the percentage of oxidised and reduced forms of iodine was found to vary considerably depending on the chemical form of the target and the pH value of the medium in which the irradiated target is dissolved[4]. It was found that part of the radioiodine can be in the form of unstable IO_2^- ions. This form is fixed by the addition of inactive IO_3^-, IO_4^- and I_2, as carriers, to a neutral solution of the irradiated target, in which it remains stable for a long time.

The chemical behaviour of iodine in very small concentrations differs considerably from its known behaviour in macro quantities. Unstable oxidation products such as HIO and HIO_2 are much more stable in carrier-free solutions[5].

In preparations of high specific activity, the free hydrogen and OH-radicals built up by water radiolysis change the chemical form of the radioisotopes[6]. We have also observed this phenomenon on investigating radioiodide preparations produced at the Boris Kidrič Institute by one-dimensional ascending chromatography. Investigation on preparations from Oak Ridge have shown that after aging for 4 weeks the percentage of unidentified components considerably increases[3]. Various workers have

given these components different names: "Extraneous band", "S-material", and "Unknown"[7-9].

In medicine, iodine is used for examining the function of the thyroid gland and for treating some forms of hyperthyreosis. In biology, it is used for the radiobiological investigations of enzymes. As the thyroid only fixes iodine in the form of iodide, the use of a radioiodide preparation in medicine for diagnostic and therapeutic purposes can, in cases where other forms of iodine are present, lead to wrong conclusions.

The purpose of this work is to investigate the conditions which bring about the formation of unidentified components in radioiodide preparations and to attempt to identify them.

The reactions: I^-/IO_3^-, I^-/IO_4^- and I^-/H_2O_2 have been investigated as a function of the concentration of the reacting agent, the pH of the medium, aging and temperature.

EXPERIMENTAL

An ascending paper chromatographic method was used, which according to the literature data, is suitable for investigating slow exchange reactions (I^-/IO_3^-)[10,11] and instantaneous exchanges (I_2/I^-)[12], in which the exchange is completed prior to chromatographic separation.

This method allows for quantitative separation of the substances present in very low concentrations. Carrier-free amounts of the radioisotope behave as macro quantities, while the R_F values remain constant up to concentrations of 10^{-12} moles/l.

Mixtures of various concentrations of the components tested, I^-/IO_3^- and I^-/IO_4^-, were made. The pH values were adjusted by means of suitable buffer solutions. The samples investigated at room temperature as a function of time and heated at high temperatures were sealed in quartz ampoules.

In order to investigate the fast exchange reactions in the system I_2/I^- and observe the appearance of higher polyiodides (I_5^-, I_7^-, I_9^-)[13], equal volumes of I_2 in nitrobenzene or benzene solutions of fixed concentrations were equilibrated with aqueous solutions of NaI. The phases were separated by centrifugation.

In all the experiments the NaI solutions were labelled with iodine-131 produced in the Boris Kidrič Institute. The specific activity of the reaction mixture amounted from several tens to several hundreds of mCi/ml.

Aliquots of 1–5 μl of each sample were taken, depending on the specific activity of the sample, and spotted on a 30 × 2.5 cm chromatographic strip at a distance of 2.5 cm from the end of the strip.

Whatman papers No. 1, No. 3 MM, No. 31 and washed Whatman No. 1 paper were examined (the paper was washed with 2 *N* HCl by ascending chromatography and then with distilled water until the reaction to Cl ion disappeared). As the type of paper did not have any effect on the separation of the forms of iodine investigated, except in the case when washed Whatman No. 1 paper was used and where the R_F values were slightly higher, we used Whatman No. 1 chromatographic paper for our investigations.

Several different solvent mixtures for the separation procedures were investigated, for example: *n*-butanol–acetic acid–water (78:5:17); isoamyl alcohol saturated with 2 *N* NH_4OH; *n*-butanol–dioxan–2 *N* NH_4OH (50:12.5:37.5), methanol–water (70:30).

The best separation of the unidentified forms of iodine was obtained with the following solvent mixtures: butanol saturated with 3 *N* NH_4OH and butanol–ethanol–2 *N* NH_4OH (5:1:2).

The chromatograms were developed in glass cylinders at room temperature for about 17 h. After the chromatograms had been developed and dried, the distribution of the radioactivity along the chromatogram was determined by scanning the strip under the window of a GM counter, with constant 2-pi geometry, at 1/2 cm intervals. The percentage of each form of iodine was determined by measuring the radioactivity on the chromatography strips relative to the total activity of the band or by determining the areas covered by the curves of the separated components obtained with an instrument for automatic activity registration.

SEPARATION OF THE COMPONENTS AND DETERMINATION OF THEIR R_F VALUES

The R_F value of each form of iodine, which can occur in radioiodide preparations, was determined. $Na^{131}IO_3$ was obtained by oxidising the radioiodide solution with hypochlorite in a slightly acid medium and by addition of a small quantity of inactive iodide (as carrier). Oxidation to $Na^{131}IO_3$ is fast and quantitative. The $Na^{131}IO_3$ obtained in this way is further oxidised to $Na^{131}IO_4$ by introducing chlorine in strong alkaline solutions[14]. $Na^{131}I_3$ is obtained by dissolving I_2 in a NaI solution labelled with $Na^{131}I$.

The R_F values of each particular form (I^-, I_3^-, IO_3^-, IO_4^-) were identical with those obtained with the mixture of all forms on chromatographic separation.

The formation of unidentified components in the solution was observed in several solutions of radioiodide produced by the Boris Kidrič Institute after various

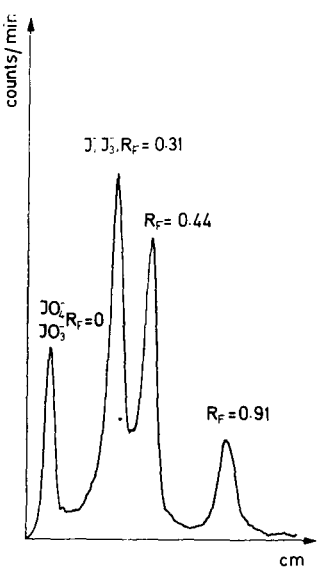


Fig. 1. Chromatographic separation of stable forms of iodine IO_3^-, I^-, I_3^- from unidentified species. Solvent: butanol saturated with 3 *N* NH_4OH, for 17 h on Whatman No. 1 chromatographic paper.

TABLE I

EFFECT OF AGING THE $Na^{131}I$ PREPARATION ON THE FORMATION OF STABLE OXIDATION FORMS OF IODINE AND FORMS WITH AN R_F VALUE OF 0.44

Reducing solution	*pH of the solution*	*Activity mC/ml*	*On production day*		*30 days later*	
			Unidentified $R_F = 0.44$ (%)	IO_3^- (%)	*Unidentified* $R_F = 0.44$ (%)	IO_3^- (%)
NaOH	10.7	7	0	2	3	2
NaOH	10.7	14	1	0	2	1
NaOH	8.7	8	2	2	2	2
NaOH	8.0	3	0	1	5	4
$Na_2S_2O_3$	9.2	9	2	1	6	3
$Na_2S_2O_3$	8.0	15	2	0	24	13
$Na_2S_2O_3$	8.0	4	1	0	8	5
$Na_2S_2O_3$	8.0	4	4	0	10	6
$Na_2S_2O_3$	7.8	15	2	0	24	13
$Na_2S_2O_3$	7.6	14	3	0	10	13
$Na_2S_2O_3$	7.4	9	3	1	4	7
$Na_2S_2O_3$	7.2	15	2	3	25	10

periods of time. Apart from the above mentioned stable forms, there are two unidentified forms of iodine whose R_F values are 0.44 and 0.91.

Using the solvent mixture butanol saturated with 3 N NH_4OH, we separated four components with the following R_F values: IO_3^- and IO_4^-, $R_F = 0$; I^- and I_3^-, $R_F = 0.31$; and two unidentified spots, $R_F = 0.44$ and 0.91. The butanol–ammonia solvent system mentioned above was used in all subsequent investigations.

Fig. 1 shows a chromatogram for the separation of the stable forms of iodine from the unidentified forms.

In order to discover the reason for the variation in the percentages of the oxidised and unidentified forms of iodine, we analyzed the radioiodide preparations in a reducing medium of $Na_2S_2O_3$ and in NaOH solutions to determine the dependence on the pH of the medium, the specific activity, and the aging time of the preparation. Results of these investigations are shown in Table I.

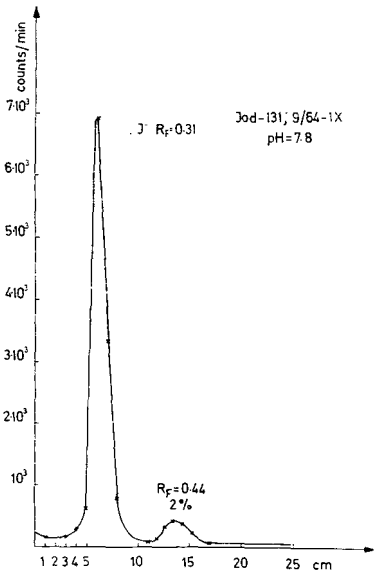
Table I shows that the unidentified component with $R_F = 0.44$ and the stable oxidised form IO_3^- are mainly formed in the reducing solution $Na_2S_2O_3$ (on standing, the dilute solution decomposes), at lower pH values and in preparations of higher specific activity.

The unidentified form with $R_F = 0.91$ appears in negligible amounts in solutions which have aged only a short time.

Most of the radioiodide preparations investigated, with pH above 10, were stable even after long storage and they only contained iodide.

Figs. 2 and 3 show the distribution of radioactivity over a chromatogram obtained by analysing the $Na^{131}I$ solution in $Na_2S_2O_3$, pH = 7.8, and with an activity of 15 mCi/ml. The percentage of the unidentified form whose R_F value is 0.44 increases to 24 % after aging for 30 days, while the percentage of the oxidised form increases to 13 %.

The spectrometric analysis of the chromatogram strips, by means of a 256-channel analyzer, confirmed that the activity of the unidentified forms belongs to iodine-131.



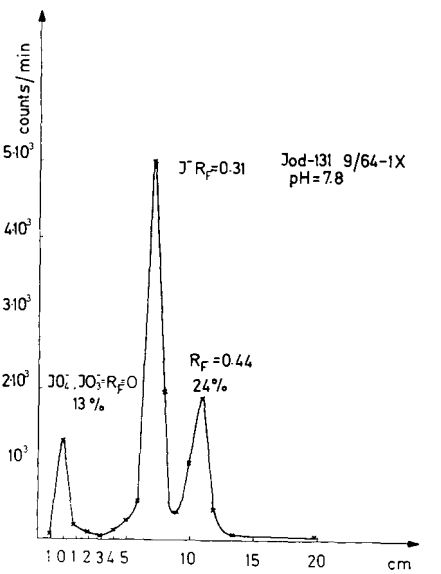


Fig. 2 Chromatogram showing the distribution of activity for each form of iodine in the radioiodide preparation YVI-131/Ix produced at the Boris Kidrič Institute, Vinča. Chromatogram of preparation on the production day.

Fig. 3. Chromatogram showing the distribution of activity of each form of iodine in a radioiodide preparation of YVI-131/Ix. Preparation aged for 30 days.

INVESTIGATION OF THE CONDITIONS UNDER WHICH THE UNIDENTIFIED FORMS WITH R_F VALUES 0.91 AND 0.44 ARE FORMED

Oxidation of iodide to I_2 with iodate

In acid medium this reaction proceeds according to the following equation:

$$5I^- + IO_3^- + 6H^+ \rightarrow 3I_2 + 3H_2O \qquad (1)$$

Mixtures of the reacting components of the following concentration ratios of IO_3^- to I^- were made: $5 \cdot 10^{-2}$ $M/1 \cdot 10^{-2}$ M; $5 \cdot 10^{-2}$ $M/1 \cdot 10^{-5}$ M; $5 \cdot 10^{-2}$ $M/1 \cdot 10^{-8}$ M; $5 \cdot 10^{-2}$ M/carrier free; $5 \cdot 10^{-5}$ $M/1 \cdot 10^{-5}$ M. Solutions with pH values of 1.2, 3.4 and 7 were investigated.

Isotopic exchange between iodides and iodates is a comparatively slow reaction and depends on the pH of the solution. In neutral medium no exchange takes place at room temperature. Exchange occurs only in acid medium and the exchange rate decreases sharply with decreasing acidity of the solution.

Aliquots from the solutions with a pH of 1.2 were taken, at room temperature, at intervals from 0.5 to 72 h, and from the solutions with a pH of 3.4 they were taken at intervals from 0.5 to 96 h. Neutral solutions of the reacting components were heated from 8 to 60 h at 228°; aliquots for analysis being taken after the solution had cooled to room temperature.

Investigation results of the iodide-iodate exchange and the formation of unidentified forms under the above conditions are given in Table II.

TABLE II

ACTIVITY DISTRIBUTION BETWEEN THE IODATE AND UNIDENTIFIED IODINE FORMS IN THE $^{131}I^-/IO_3^-$ SYSTEM AS A FUNCTION OF THE pH OF THE MEDIUM, CONCENTRATION AND TIME

pH of the solution	% IO_3^-							% $R_F = 0.44$							% $R_F = 0.91$							*Concentration ratio of* IO^{3-}/I^-
	0.5 h	*3 h*	*6 h*	*24 h*	*48 h*	*72 h*	*96 h*	*0.5 h*	*3 h*	*6 h*	*24 h*	*48 h*	*72 h*	*96 h*	*0.5 h*	*3 h*	*6 h*	*24 h*	*48 h*	*72 h*	*96 h*	
1.2	7	9	13	—	—	65	—	0	0	0	—	—	0	—	10	20	23	—	—	8	—	$5\cdot10^{-2}/1\cdot10^{-2}$
	7	7	10	—	—	38	—	0	0	0	—	—	0	—	9	9	9	—	—	4	—	$5\cdot10^{-2}/1\cdot10^{-5}$
	6	6	6	—	—	38	—	0	0	0	—	—	0	—	6	6	8	—	—	4	—	$5\cdot10^{-2}/1\cdot10^{-8}$
	0	0	0	—	—	20	—	0	0	0	—	—	0	—	0	0	0	—	—	12	—	$5\cdot10^{-2}/^{131}I^-$
	0	0	0	—	—	10	—	0	0	0	—	—	0	—	0	0	0	—	—	10	—	$5\cdot10^{-5}/1\cdot10^{-5}$
3.4	5	—	5	11	11	—	11	0	—	0	0	0	—	0	8	—	8	8	8	—	8	$5\cdot10^{-2}/1\cdot10^{-2}$
	7	—	7	11	11	—	11	5	—	5	16	16	—	21	1	—	1	8	12	—	20	$5\cdot10^{-2}/1\cdot10^{-5}$
	6	—	6	11	16	—	16	0	—	0	7	14	—	19	1	—	1	8	15	—	18	$5\cdot10^{-2}/1\cdot10^{-8}$
	0	—	7	11	—	—	—	0	—	0	2	—	—	—	0	—	0	2	—	—	—	$5\cdot10^{-5}/^{131}I^-$
	0	—	0	3	—	—	—	0	—	0	0	—	—	- -	0	—	0	0	—	—	—	$5\cdot10^{-5}/1\cdot10^{-5}$

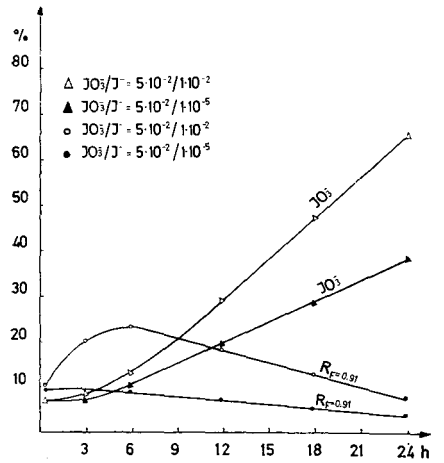


Fig. 4. Percentage content of $^{131}IO_3^-$ and the component with $R_F = 0.91$ in an $IO_3^-/^{131}I^-$ system as a function of the $^{131}I^-$ concentration and aging time of the solution at pH = 1.2.

It can be seen that the unidentified forms of iodine with R_F values 0.91 and 0.44, appearing in carrier-free radioiodide solutions, form on isotopic exchange between iodide and iodates.

The component with an R_F value of 0.44 is formed only in solutions at pH 3.4 for iodide concentrations less than $1\cdot10^{-5}$ M. Under these conditions exchange proceeds to 15% after 96 h. If the iodide concentration exceeded $1\cdot10^{-5}$ M this form did not appear; however, in acid medium at pH 1.2, although the exchange reaches the highest percentage, up to 65% after 72 h, this form was not present.

The component with an R_F value of 0.91 (it moves with the solvent front) is formed in solutions at pH = 1.2 and 3.4 for all iodide concentrations cited. The percentage of this component depends on the aging time and iodide concentration, as shown in Fig. 4.

As Fig. 4 shows the radioactivity of this component increases in the beginning but after several hours of aging it decreases, while the iodate content permanently increases, being faster with larger iodide concentrations.

In the above ratios of the reacting agents, the iodate ion was always in excess.

TABLE III

EXCHANGE RATE OF $^{131}I^-$ AND IO_3^- IN NEUTRAL MEDIUM AT 228°

Heating time (h)	*Concentration (moles/l)*		*Exchange degree* x/c	*Exchanged part* $x/c(1+b/a)$	*Nonexchanged part* $1-x/c(1+b/a)$
	$NaIO_3$	$Na^{131}I$			
8	$5\cdot10^{-2}$	$1\cdot10^{-2}$	0.07	0.091	0.91
20	$5\cdot10^{-2}$	$1\cdot10^{-2}$	0.19	0.228	0.77
41	$5\cdot10^{-2}$	$1\cdot10^{-2}$	0.35	0.420	0.58
60	$5\cdot10^{-2}$	$1\cdot10^{-2}$	0.47	0.560	0.44
41	$5\cdot10^{-2}$	$1\cdot10^{-5}$	0.00	—	—
41	$5\cdot10^{-2}$	$1\cdot10^{-8}$	0.00	—	—

If the iodide ion was in excess in the reaction mixtures, the presence of the components with R_F values 0.91 and 0.44 was not detected by chromatographic analysis. Analysis of these solutions furnish data only for the presence of triiodide ion (R_F = 0.31).

In neutral medium, isotopic exchange is, even at a temperature of 228°, a slow reaction, and it is only obtained for the concentrations of the reacting components $1 \cdot 10^{-2}$ *M* $Na^{131}I/5 \cdot 10^{-2}$ *M* $NaIO_3$. The results are shown in Table III.

On the basis of the exchange rate equation[10],

$$R = -\frac{2.3}{t(a+b)} \log\left[1 - \frac{x}{c}\left(1 + \frac{b}{a}\right)\right] \text{ l·mol}^{-1}\text{h}^{-1} \qquad (2)$$

where: t = time; x/c = exchange degree; $x/c(1 + b/a)$ = exchanged part, $1 - x/c(1 + b/a)$ = unexchanged part, and from the experimental data in Table III, we calculated the rate constant for the reverse bimolecular isotopic exchange reaction: $Na^{131}I + NaIO_3 \rightarrow NaI + Na^{131}IO_3$. R = 0.216 l·mol^{-1}h^{-1}.

Oxidation of iodide with periodate

In solutions with a pH > 3, the reaction between iodides and periodates proceeds according to the equation:

$$IO_4^- + 2I^- + H_2O \rightarrow IO_3^- + I_2 + 2OH^- \qquad (3)$$

As seen in equation (3), the components appearing in the solution are: IO_4^-, IO_3^-, I^- and I_2. By chromatographic separation with the system of solvents described

TABLE IV

ACTIVITY DISTRIBUTION BETWEEN THE IODATE AND THE UNIDENTIFIED IODINE FORMS IN THE $^{131}I/IO_4^-$ SYSTEM AS A FUNCTION OF THE pH OF THE MEDIUM, THE CONCENTRATION AND TIME

pH of the solution	*Time*	*Content of* IO_3^- (%)	*Content of* $R_F = 0.91$ (%)	*Content of* $R_F = 0.44$ (%)	*Ratio of* IO_4^-/I^-
3.4	2 min; 1 h; 24 h	8; 36; 95	20; 0; 0	0; 0; 0	$5 \cdot 10^{-2}/1 \cdot 10^{-2}$
		5; 9; 10	0; 2; 2	0; 8; 10	$5 \cdot 10^{-2}/1 \cdot 10^{-5}$
		4; 4; 7	2; 2; 2	7; 7; 13	$5 \cdot 10^{-2}/1 \cdot 10^{-8}$
5.5–7.5	2 min; 1 h; 24 h	80; 100; —	0; 0; —	0; 0; —	$5 \cdot 10^{-2}/1 \cdot 10^{-2}$
		38; 56; 100	2; 2; 0	12; 6; 0	$5 \cdot 10^{-2}/1 \cdot 10^{-5}$
		— 7; 61	— 2; 6	— 10; 3	$5 \cdot 10^{-3}/1 \cdot 10^{-5}$
		7; 11; 11	0; 2; 2	7; 60; 60	$5 \cdot 10^{-3}/1 \cdot 10^{-8}$
10	2 min; 1 h; 24 h	52; 100; —	11; 0; —	0; 0; —	$5 \cdot 10^{-2}/1 \cdot 10^{-2}$
	2 min; 1 h; 24 h	0; 0; 5	0; 0; 0	0; 0; 0	$5 \cdot 10^{-5}/1 \cdot 10^{-5}$
	2h at 95°	93; — —	0;	0;	$5 \cdot 10^{-5}/1 \cdot 10^{-5}$
	2 min; 1 h; 24 h	17; 71; 100	5; 2; 0	8; 3; 0	$5 \cdot 10^{-2}/1 \cdot 10^{-8}$
	— — 24 h	—; —; 0	—; —; 0	—; —; 0	$5 \cdot 10^{-5}/1 \cdot 10^{-8}$
	2 h at 95°	59	0	0	$5 \cdot 10^{-5}/1 \cdot 10^{-8}$
	2 min; 1 h; 24 h	13; 21; 75	11; 15; 3	15; 19; 6	$5 \cdot 10^{-2}/^{131}I^-$

earlier, the activity for IO_3^- and IO_4^- is determined as the sum at the starting point of the chromatogram; the iodate does not separate from the periodate. In these investigations we have also used a solvent which separates IO_3^- from IO_4^- (ref. 14); the chromatogram was developed in separate experiments using both solvents. In this way we could obtain results for all the components present.

Table IV shows the results of these investigations, under various conditions of pH, concentration of reacting agent, and aging time. The iodide and periodate concentrations varied. The iodide concentration gradually decreased to carrier free radioiodide concentration, while periodate occurred in excess.

Results showed that isotopic exchange between iodides and periodates does not take place in solutions with pH = 3.4; 5.5–7.5 and 10. The unidentified form whose R_F value is 0.44 appears in solutions with the above pH values if the iodide concentration is below $1 \cdot 10^{-5}$. In neutral solutions for the concentration ratio $1 \cdot 10^{-8}$ *M* I$^-$/$5 \cdot 10^{-3}$ *M* IO_4^- the percentage of the unidentified form, with $R_F = 0.44$, increases even as far as 60 % of the total radioactivity. The form with $R_F = 0.91$ was present under all the above conditions, irrespective of the iodide concentration.

TABLE V

RATE OF IODATE FORMATION ACCORDING TO REACTION (3)
0.06 *M* $NaIO_4$, 0.12 *M* NaI labelled with $^{131}I^-$. Precipitation with $Ba(NO_3)_2$ at 60°.

	$NaIO_4$ added to $Na^{131}I$ solution in presence of $Ba(NO_3)_2$	*Radioactivity of precipitate as a function of time of addition of $Ba(NO_3)_2$ to reaction mixture after*					*Precipitation in presence of NH_4NO_3 ($pH = 7$) after 600 sec*
		4 sec	*10 sec*	*30 sec*	*90 sec*	*160 sec*	
imp./min	1.185	6.153	11.788	16.203	16.500	16.580	2.612

In order to obtain more data for the conditions in which the unidentified form of iodine appears, we investigated the rate of iodate formation, according to equation (3), by separating the iodate produced from the reaction mixture, using $Ba(NO_3)_2$. The radioactivity of the iodate precipitate was determined as a function of the time of the addition of barium salt as precipitating agent. By treating the mixture of radioiodide and barium nitrate with inactive periodate solution, a slightly radioactive precipitate is obtained (periodate reacts with iodide before barium precipitates periodate from the reaction mixture).

If barium nitrate is added after the solution of radioiodide and inactive periodate is left to react for some time, one obtains an iodate precipitate whose activity depends on the time when the $Ba(NO_3)_2$ was added to the reaction mixture. Results are shown in Table V.

As can be seen from the results, the activity of the iodate increases and after some time it remains constant. On addition of barium nitrate the primary reaction (3) in which iodate is formed stops, because periodate precipitates as well as iodate. Further formation of iodate proceeds according to the secondary reactions:

$$OH^- + I_2 \rightarrow I^- + IOH \quad (4)$$

$$3IOH \rightarrow 2HI + HIO_3 \quad (5)$$

The rate of the secondary reaction decreases with decreasing pH of the solution. Under our experimental conditions the addition of barium salts decreases the pH of the solution from 10 to 6.

According to the results obtained by MAGNIER *et al.*[15], who investigated the mechanism of reaction (3), only the elementary iodine formed in this reaction is radioactive and is produced from the I ion. Iodine atoms in an iodate molecule are exclusively formed from periodates and they are inactive. Radioactive iodate is then formed from radioactive I_2 by the secondary reactions (4) and (5).

If precipitation with barium nitrate in the presence of ammonium nitrate (pH = 7) proceeds even after 10 min, the radioactivity of the precipitate obtained is weak and the solution above the precipitate is radioactive, its colour is brown and is characteristic of the presence of an I^- and I_2 mixture.

Oxidation of iodide with H_2O_2

In addition to iodate and periodate as radiochemical impurities in radioiodide preparations, hydrogen peroxide also occurs as the product of water radiolysis, especially in preparations of high specific activity. On oxidising carrier-free radioiodide preparations with H_2O_2, we also found, by chromatographic separation, in addition to iodates some unidentified components with $R_F = 0.44$ and 0.91. As in cases of iodide oxidation with iodate and periodate, the form with $R_F = 0.44$ is present only in solutions in which the iodide concentration was below $1 \cdot 10^{-5}$, while the component with $R_F = 0.91$ appears in solutions for all the iodide concentrations investigated. On oxidising a radiochemically pure preparation with a 3 % solution of H_2O_2 in acid medium (2 N H_2SO_4), a component containing 13 % of the activity and with $R_F = 0.44$ appears within 10 min of aging. After 40 min the activity of this form increases to 24 % and does not change even after aging for 24 h. The activity of iodate is constant and amounts to about 7 %.

The effect of the concentration ratio $I_2/^{131}I^-$ on the appearance of the unidentified component with $R_F = 0.91$.

Further investigations of the properties of the unidentified components have shown that, on extraction with organic solvents, the component with $R_F = 0.44$ remains in the aqueous phase.

The unidentified form which on chromatography has an R_F value of 0.91 goes quantitatively to the organic phase together with the triiodide ion ($R_F = 0.31$).

It is known that the solvents in which iodine solubility is greatest, favour the formation of higher polyiodides. In our investigations nitrobenzene was chosen as such a solvent. The aqueous phase, NaI, labelled with $^{131}I^-$ was equilibrated with the organic phase, I_2 in nitrobenzene. Two series of experiments were made. In the first series the concentrations of $[I^-] > [I_2]$ and in the second series $[I^-] < [I_2]$, the concentration of NaI was $1 \cdot 10^{-1}$ and $1 \cdot 10^{-5}$ M respectively. The I_2 concentration varied from $1 \cdot 10^{-1}$ to $1 \cdot 10^{-5}$ M. In this case we assumed that the exchange between the iodide and iodine atom, which are components of the polyiodide, is instantaneous[12]. After separating the phases, the organic phase of the solutions was investigated chromatographically. In the case where the ratio $[I] > [I_2]$, it was found that only the triiodide ion with $R_F = 0.31$ was present. Under these conditions, the unidentified form with $R_F = 0.91$ does not appear.

In the solutions where the concentration ratio was $[I_2] > [I^-]$, an unidentified form with an R_F value of 0.91 was found in addition to the triiodide ion in the organic phase. The percentage of this form decreases with increasing I_2 concentration. Results of these investigations are shown in Fig. 5. As shown in the diagram, the amount of the component with $R_F = 0.91$ increases with decreasing I_2 concentration, so that for $1 \cdot 10^{-4}$ M I_2 it is 73 % of the total radioactivity, while the amount of the triiodide ion is proportionally smaller. The aqueous phase contained only iodide.

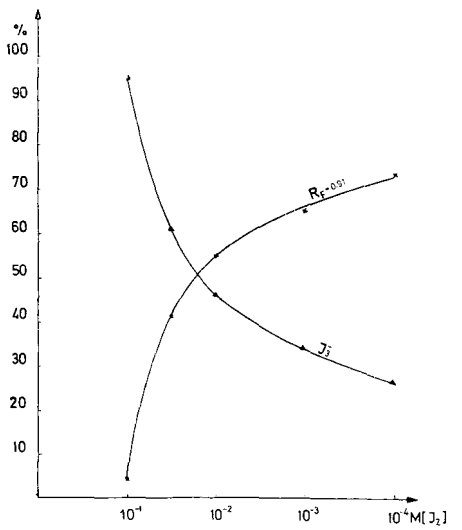


Fig. 5. Percentage content of $^{131}I_3^-$ and component with $R_F = 0.91$ in the organic phase of the system I_2 in nitrobenzene, aqueous solution of $Na^{131}I$, as a function of I_2 concentration. $[I_2] > [I^-] = 1 \cdot 10^{-5}$ M.

The conditions for the formation of this form were also investigated by equilibrating the aqueous phase $Na^{131}I$ with an organic phase of I_2 in benzene. The ratio of the concentrations of I^- in aqueous phase to I_2 in benzene was the same as that in the preceding experiments with nitrobenzene.

Both phases were investigated chromatographically after they had been separated. The unidentified form with $R_F = 0.91$ only accounts for ~ 5 % of the total activity for the largest concentration of I_2 ($1 \cdot 10^{-1}M$ $I_2/1 \cdot 10^{-5}M$ I^-), while 95 % was found in the form of triiodide ion with $R_F = 0.31$. The aqueous phase contained only iodide.

Results have shown that solutions of benzene whose dielectric constant is much smaller (2.3) compared with that of nitrobenzene (36.1) are not suitable for the formation of a component with an R_F value of 0.91.

CONCLUSIONS

Chromatographic investigations have shown that, besides the stable forms of iodine, two unidentified components occur in radioiodide preparations, depending on the aging period. With the solvent mixture butanol saturated with 3 N NH_4OH,

stable forms of the two unidentified components could be separated chromatographically. The R_F values of the various forms were: IO_3^-, IO_4^- = 0.0; I^-, I_3^- = 0.31; unidentified forms = 0.44 and 0.91.

In order to investigate the conditions under which the unidentified components are formed, radioiodide solutions were oxidised with iodate, periodate and hydrogen peroxide. The concentrations of the reacting components were for iodide: $1 \cdot 10^{-2}$ *M* to carrier free, while the amounts of IO_3^- and IO_4^- were higher than the same for respective I^- concentrations *viz*: $5 \cdot 10^{-2}$, $5 \cdot 10^{-3}$ and $5 \cdot 10^{-5}$ *M*.

The unidentified component with R_F = 0.44 is formed in the solutions when the iodide concentration is below $1 \cdot 10^{-5}$ *M*; in the system $^{131}I^-/IO_3^-$ at pH = 3.4, and in the system $^{131}I^-/IO_4^-$ in weakly acid, neutral and alkaline media. If the iodide concentration is above $1 \cdot 10^{-5}$ *M* this form is not present.

The component with R_F = 0.91 is formed on oxidation with iodate in acid solution and with periodate in acid, neutral and alkaline media, in presence of excess IO_3^- and IO_4^-, for all iodide concentrations investigated.

The rate constant *R* for the isotopic exchange $^{131}I^-/IO_3^-$ in neutral medium at 228° was determined for the concentrations $1 \cdot 10^{-2}$ *M* $^{131}I^-/5 \cdot 10^{-2}$ *M* IO_3^- and its value is 0.216 $l \cdot mol^{-1}h^{-1}$.

Under our experimental conditions no exchange occurs between $^{131}I^-/IO_4^-$. As the formation of unidentified components is also followed by the presence of iodate, the rate of iodate formation in the system $^{131}I^-/IO_4^-$ has been investigated. Iodine atoms in the iodate molecule formed from the periodate are inactive. Active iodate is formed from $^{131}I_2$ by secondary reactions.

On extraction of reaction mixtures with nitrobenzene, the unidentified form with R_F = 0.91 goes quantitatively to the organic phase together with the I_3 ion, while the unidentified component with R_F = 0.44 remains in aqueous phase together with the I^- and IO_3^- ions.

On equilibrating the aqueous phase $Na^{131}I$ with the organic phase, I_2 in nitrobenzene: when $[I_2] > [I^-]$ in addition to I_3^- in the organic phase an unidentified form with R_F = 0.91, also appears as a result of instantaneous isotopic exchange, while the aqueous phase contains only iodide.

According to the results obtained, it can be assumed that in nitrobenzene solutions a mixture of polyiodides is formed: I_3^- (R_F = 0.31) and an unidentified form with R_F = 0.91 which is probably a mixture of higher polyiodides (I_5^-, I_7^- and I_9^-).

The unidentified form which remains in the aqueous phase, after extraction with organic solvents, and has an R_F value of 0.44, is stable in neutral solutions even after a long time. On addition of acid it disappears, while the amount of IO_3^- and the form with R_F = 0.91 increase to the same amount. It does not reduce with $Na_2S_2O_3$, and the effect of sulphites and thiouracyl is negligible. Electrophoretic behaviour of this component shows that it is an anion whose migration differs from the known forms IO_3^-, IO^- and polyiodide. This form appears as an intermediate product on oxidation of iodide or I_2 to iodate, in solutions of low concentration where the reaction rate is reduced.

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CHROM. 4260

ZUR PAPIERCHROMATOGRAPHISCHEN TRENNUNG DER PLATINMETALLE UNTER BESONDERER BERÜCKSICHTIGUNG DES OSMIUMS

1. TEIL. ALKOHOLE, KETONE UND ÄTHER ALS FLIESSMITTEL

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SUMMARY

Paper chromatographic separation of platinum metals with special reference to osmium Part I. Alcohols, ketones and ethers as solvents

The paper chromatographic behaviour of osmium in known compounds has been examined using alcohols, ethers and ketones as solvents. The R_F values obtained when using different solvent mixtures have been compared with those obtained for other platinum metals. A relationship was observed between the R_F values and the distribution coefficients α of the chloro- and bromo-osmium complexes.

EINLEITUNG

Über die papierchromatographische Trennung der Platinelemente liegen bereits zahlreiche Veröffentlichungen vor, so dass durch Anwendung der in den verschiedenen Arbeiten geprüften Laufmittelzusammensetzungen praktisch jede gewünschte Trennung innerhalb der Platinmetallgruppe erreicht werden kann. Man vergleiche hierzu die Arbeiten von LEDERER, BLASIUS, PFEIL u.a.[1–14]. Osmium und Ruthenium werden bei diesen Trennungen im allgemeinen durch Destillation entfernt während Rh, Pd, Ir und Pt in den Rückstandslösungen zum Nachweis kommen[15]. Eine Reihe von Arbeiten befassen sich aber auch mit der gemeinsamen Trennung der Platinmetalle ohne diese vorausgehende Ru- und Os-Destillation[4,5,8,10,11].

Bei kritischer Durchsicht der Literatur fällt nun auf, dass die Angaben über die papierchromatographische Abtrennung des Osmiums von den Platinmetallen wenig genau sind. Während in manchen Arbeiten keinerlei Hinweise über die für die Trennung benutzte Osmiumverbindung zu finden sind, lässt die beispielsweise sehr allgemein gehaltene Angabe über eine Verwendung von Osmiumchlorid als Auftragslösung manche Fragen offen. Es ist aus den Angaben nicht ersichtlich, ob der wasserlösliche Komplex $Na_2[OsO_2Cl_4]$ oder die durch Eindampfen im HCl-Strom aus konz. HCl gebildete Verbindung $Os(OH)Cl_3$ vorliegen. Da das ebenfalls zitierte $OsCl_4$ in vielen Laufmitteln schwer löslich ist, dürfte es bei den Versuchen sehr wahrscheinlich nicht verwendet worden sein.

Bei Trennungen, wie sie in der Geochemie und Radiochemie anfallen, kommt naturgemäss gerade der Auftragslösung und deren Herstellung entscheidende Bedeutung zu. Es wurde deshalb das Laufverhalten von genau definierten und reproduzierbar herstellbaren Osmiumverbindungen mit einer Reihe von Fliessmitteln systematisch untersucht und mit dem Verhalten der bei entsprechenden Bedingungen in einem Mehrstoffgemisch vorliegenden Platinmetallverbindungen verglichen. Als Laufmittel wurden einmal die in der Literatur für die Trennung der Platinmetalle als geeignet eingestuften Stoffe geprüft. Zum anderen wurden auch Lösungsmittel in die Untersuchungen einbezogen, die sich bei Versuchen über die Flüssig–Flüssig-Extraktion des Osmiums[16] durch hohe Verteilungskoeffizienten α auszeichneten. Da die Papierchromatographie im Prinzip als Verteilungschromatographie aufgefasst werden kann, sollten nämlich gerade Lösungsmittel mit grossen α-Werten in Übereinstimmung zu MARTIN UND SYNGE[17–19] für die Trennung geeignete grosse R_F-Werte aufweisen.

VERSUCHSDURCHFÜHRUNG

Für die Trennung verwendete Osmiumverbindungen

Osmium wurde bei den verschiedenen Versuchen als $H_2[OsCl_6]$, $H_2[OsBr_6]$ und als eine beim Ansäuern des in NaOH übergegangenen Osmiumtetroxids entstandene Verbindung chromatographiert. Folgendes ist zu diesen Verbindungen anzumerken.

$H_2[OsCl_6]$

Der Chlorokomplex des Osmiums ist nach zwei Methoden darstellbar: Einmal nach REMY[20] durch Reduktion von Osmiumtetroxid mit konzentrierter HCl (spez. Gew. >1.16):

$$OsO_4 + 10\ HCl \longrightarrow H_2[OsCl_6] + 2\ Cl_2 + 4\ H_2O$$

Zum anderen nach GILCHRIST[21] in mässiger Wärme durch Reduktion von Osmiumtetroxid mit 20%iger Salzsäure unter Zusatz einiger Milliliter Äthanol.

Der zweite Weg ist zur Herstellung der für papierchromatographische Untersuchungen vorgesehenen Auftragslösungen vorzuziehen. Die Reduktion lässt sich nämlich schneller und auch mit weniger Salzsäure durchführen, so dass das Einengen eines grossen HCl-Überschusses entfällt. Wichtig ist vor allem, dass die salzsaure Lösung bei der Reduktion mindestens 3 Std. leicht über dem Kochpunkt gehalten wird. Bei zu niedriger Temperatur wird nämlich Osmiumtetroxid nur zum Teil in den stabilen gelben Hexachlorokomplex umgewandelt, was bei der weiteren Verarbeitung zu Osmiumverlusten führen kann.

$H_2[OsBr_6]$

Der Hexabromokomplex des Osmiums bildet sich leicht und schnell beim Erhitzen von Osmiumtetroxid in 20%iger Bromwasserstoffsäure unter Zusatz von Äthanol. Die Lösung des Komplexes lässt sich analog zum Chlorokomplex ohne merkliche Osmiumverluste einengen; zur Vermeidung von Verlusten, die bei einer evtl. unvollständigen Umwandlung eintreten können, empfiehlt sich jedoch auch hier das Anschalten einer Vorlage.

Angemerkt sei, dass die Hexachloroverbindung des Osmiums stabiler als der entsprechende Bromokomplex ist. Bei den papierchromatographischen Versuchen kann deshalb $H_2[OsBr_6]$ im Gegensatz zu $H_2[OsCl_6]$ im Fall oxidierender Einflüsse des Laufmittels nicht angewandt werden.

HCl angesäuerte Lösung von OsO_4/NaOH

Das papierchromatographische Verhalten der OsO_4/NaOH-Anlagerungsverbindung unterscheidet sich charakteristisch von dem der Halogenidkomplexe des vierwertigen Osmiums. Dies dürfte darauf zurückzuführen sein, dass die nach Gl. (2) entstandene (orange bzw. braunrot gefärbte) Anlagerungsverbindung

$$OsO_4 + 2\ NaOH \longrightarrow 2\ NaOH \cdot OsO_4 \text{ bzw. } Na_2[OsO_4(OH)_2] \qquad (2)$$

mit HCl nach Gl. (3) reagiert und hierbei einen Komplex mit sechswertigem Osmium bildet

$$2\ NaOH \cdot OsO_4 + 6\ HCl \longrightarrow Na_2[OsO_2Cl_4] + Cl_2 + 4\ H_2O \qquad (3)$$

Möglicherweise führt auch das aus NaOH und HCl entstandene NaCl mit OsO_4 und HCl unter Chlorentwicklung zu $Na_2[OsO_2Cl_4]$, wie es analog von der Reaktion des KCl mit OsO_4 und HCl her bekannt ist.

Der $Na_2[OsO_2Cl_4]$-Komplex zeigt nicht nur ein von den Chloro- und Bromokomplexen des Osmiums abweichendes charakteristisches Laufverhalten, sondern auch aufgrund der VI. Wertigkeit des Osmiums eine merkliche Instabilität gegenüber Wertigkeitsänderungen. Dies wirkt sich beispielsweise dadurch aus, dass das als $Na_2[OsO_2Cl_4]$ gelaufene Osmium mit Thioharnstofflösung leicht in den purpurroten $[Os(NH_2CSNH_2)_6]^{3+}$-Komplex übergeht, während beim Chloro- und Bromokomplex erwärmt und mit $SnCl_2$ reduziert werden muss. Mit stark salzsauren Laufmitteln ist ausserdem mit der Möglichkeit einer Reduktion auf dem Chromatogramm zu rechnen, bei der nach Gl. (4) der Osmium-IV-Chlorokomplex entsteht, der ein von der Auftragslösung abweichendes Laufverhalten hat und mit der salzsauren Thioharnstofflösung nur schwer angefärbt werden kann.

$$[OsO_2Cl_4]^{2-} + 4\ H^+ + 4\ Cl^- = [OsCl_6]^{2-} + Cl_2 + 2\ H_2O \qquad (4)$$

Platinmetallverbindungen

Die Platinmetalle wurden in Form folgender Verbindungen zusammen mit den Osmiumkomplexen aufgetragen: $RuCl_3 \cdot (1\text{–}3\ H_2O)$; $Na_2[PdCl_4]$; $Na_2[PtCl_6] \cdot 6H_2O$; $Na_3[RhCl_6] \cdot 12H_2O$ und $H_2[IrCl_6]$ bzw. das nach Reduktion mit Hydrazindichlorid erhaltene $H_3[IrCl_6]$. Die aufgetragene Lösungsmenge enthielt jeweils etwa 4 μg.

Nachweis der Platinmetalle

Während im Fall des Osmiums beim Wandern der Chloro- und Bromokomplexe bereits deren Eigenfarbe für den Nachweis herangezogen werden konnte, musste der Sauerstoffkomplex des Osmiums mit Thioharnstoff entwickelt werden. Ruthen liess sich durch die Blaufärbung des Thioharnstoffkomplexes, der sich beim Erhitzen des Papiers rasch bildet, nachweisen.

Zur Entwicklung von Palladium und Platin fanden die Farbreaktionen mit Rubeanwasserstoff Anwendung. Während bei Palladium sofort eine Gelb- bis Orangefärbung entsteht, muss bei Platin erwärmt (purpurrote bis rosa Farbe) bzw. mit $SnCl_2$ (Gelbfärbung) oder $SnCl_2$+KJ (rötliche Farbe) behandelt werden. Rhodium konnte durch Besprühen mit einer KJ-haltigen salzsauren $SnCl_2$-Lösung durch eine rötliche Farbreaktion festgestellt werden.

Der Iridiumnachweis erfolgte mittels der intensiven braunen Farbe des Ir(IV), das sich bei oxidierend wirkenden Laufmitteln aus Ir(III) von selbst bzw. durch Besprühen des Papiers mit einer H_2O_2-haltigen salzsauren Lösung bildet.

Chromatographische Methode

Das Laufverhalten der Platinmetalle wurde in der Hauptsache mit der Rundfiltermethode, die erstmals MAJUMDAR UND CHAKRABARTTY[8] zur Trennung der Platinmetalle einsetzten, bestimmt. Einmal ist die Messgenauigkeit dieses Verfahrens aufgrund der relativ schmalen Zonen grösser als bei der absteigenden oder aufsteigenden Chromatographie. Zum anderen können die nach der Rundfiltermethode erhaltenen R_F-Werte einem Verfahren zugrundegelegt werden, das papierchromatographische Trennungen im Makromassstab erlaubt[22].

Bei Diskussion von R_F-Werten darf nicht übersehen werden, dass sich die nach verschiedenen Methoden beobachteten Werte merklich voneinander unterscheiden können. Die mit bestimmten Fliessmitteln erreichbaren Unterschiede in den R_F-Werten verschiedener Elemente bleiben jedoch bei Änderung der Laufverfahren annähernd erhalten, so dass eine Übertragung auf Trennprobleme oft qualitativ möglich ist.

VERSUCHSERGEBNISSE

Alkohole

Die mit verschiedenen Alkoholen nach der Rundfiltermethode erhaltenen R_F-Werte sind in den Tabellen I–III zusammengestellt. Die Bedingungen wurden bei den Einzelversuchen gleich gehalten: Papier 2043 b Mgl nach Potterat (Schleicher & Schüll), Durchschnitt 30 cm; Laufmittel 15 ml Alkohol+3 ml 8 N HCl. Aus den Tabellen I–III ist ersichtlich, dass die verschiedenen Osmiumverbindungen mit den meisten Alkohol–Salzsäuregemischen ein gutes Laufverhalten zeigen. Die R_F-Werte liegen bis auf wenige Ausnahmen in der Grössenordnung von 0.7–1.0. Von den Osmiumverbindungen wandert der Chlorokomplex am schärfsten. Die R_F-Werte sind in der Reihe abgestuft: $H_2[OsBr_6] > H_2[OsCl_6] > 2NaOH \cdot OsO_4$ (salzsauer).

Innerhalb der homologen Reihe der primären Alkohole ist eine geringe Tendenz zur Abnahme der R_F-Werte mit zunehmender C-Zahl zu erkennen. Diesen Effekt zeigen sehr ausgeprägt $RuCl_3$, $H_2[IrCl_6]$ bzw. $H_3[IrCl_6]$, $Na_3[RhCl_6]$, $Na_2[PdCl_6]$ und in geringem Masse auch $H_2[PtCl_6]$. Das schlechte Verhalten des Octanols geht auf die schlechte Mischbarkeit mit 8 *N* HCl zurück, die zur Bildung zweier Phasen Anlass gibt.

Die bei Zugabe von Bromwasserstoffsäure mit den Alkoholen erreichbaren R_F-Werte der Osmiumverbindungen liegen etwas höher als bei Zugabe von HCl, wie Tabelle IV veranschaulicht.

TABELLE I

R_F-WERTE PRIMÄRER HOMOLOGER ALKOHOLE

Bedingungen s. Text.

Alkohol + 8 N HCl	*Verbindung*								
	$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4 + HCl$	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
Methanol	0.92–1.0	0.85–1.0	0.82–0.93	0.74 –0.83	0.74 –0.84	0.83 –1.0	0.83–1.0	0.87 –0.96	0.76 –0.92
Äthanol	0.94–1.0	1.0[b]	0.85–0.94	0.54 –0.70	0.46 –0.57	0.87 –0.93	0.97–1.0	0.54– 0.72	0.465–0.645
Äthanol[a]	0.79–1.0	0.77–1.0	0.83–1.0	0.0 –0.56	0.099–0.5	0.71 –0.79	0.79–0.92	0.73 –0.94	0.42 –0.89
Propanol	0.95–1.0	0.0 und 1.0	0.8 –0.9	0.21 –0.67	0.26 0.41	0.78 –0.9	0.95–1.0	0.255–0.73	0.256–0.46
Butanol	0.83–0.97	0.0 und 1.0[c]	0.79–0.89	0.0 –0.48	0.114–0.34	0.68 –0.765	0.76–0.91	0.66–0.58	0.128–0.41
Amylalkohol	0.82–0.95	0.97–1.0	0.62–0.87[d]	0.051–0.38	0.097–0.24	0.5 –0.62	0.71–0.89	[e]	0.046–0.218
Octanol	[f]	[f]	0.27–0.68[g]	0.0 –0.243	0.108–0.212	0.235–0.35		0.0 –0.415	0.0 –0.286

[a] 12.6 ml Äthanol + 1.4 ml 10 *N* HNO_3.
[b] Mit Nachschleier bis 0.87.
[c] Hauptmenge bleibt am Startpunkt und nur eine geringe Menge läuft mit Laufmittelfront.
[d] Geringe Mengen bleiben am Startpunkt.
[e] Schmiert sehr stark.
[f] Nicht brauchbar.
[g] Schmiert von 0.68–1.0.

TABELLE II

R_F-WERTE SEKUNDÄRER, TERTIÄRER, UNGESÄTTIGTER UND MEHRWERTIGER ALKOHOLE

Bedingungen s. Text.

Alkohol + 8 N HCl	*Verbindung*								
	$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4$ + HCl	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
Isopropylalkohol	0.88 –0.98	0.91–1.0	0.76–0.87	0.156–0.35	0.18 –0.284	0.78–0.87	0.86–0.95	0.25 –0.395	0.22 –0.35
Isobutylalkohol	0.835–0.93	0.97–1.0	0.73–0.81	0.127–0.215	0.168–0.23	0.67–0.74	0.80–0.87	0.178–0.44	0.17 –0.25
Isopentylalkohol	0.83 –0.89	0.86–1.0	0.68–0.80[d]	0.132–0.27	0.106–0.112	0.54 –0.65	0.75–0.88	0.146–0.19[f]	0.11 –0.24
Trimethylcarbinol[a]	0.75 –0.87	0.77–0.9	0.68–0.81	0.046–0.127	0.108–0.184	0.64–0.72	0.55–0.63	0.73 –0.83[g]	0.054–0.196
Butanol-(2)	0.865–0.965	[c]	0.72–0.84	0.0 –0.215	0.066–0.152	0.64–0.745	0.64–0.745	0.078–0.235	0.0 –0.184
Allylalköhol	0.83 –0.92	0.90–0.97	0.75–0.89[e]	0.26 –0.65	0.24 –0.75	0.95–0.98	0.85–0.95	0.53 –0.91[h]	0.44 –0.81
Äthylenglykol[b]	0.99 –1.0	0.99–1.0	0.99–1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] Trimethylcarbinol zeigt für die verwendeten Platinmetallverbindungen ein ausgezeichnetes Laufverhalten.
[b] Alle aufgetragenen Substanzen laufen mit der Laufmittelfront.
[c] Startpunkt und R_F-Wert 1.0; zwei deutlich sichtbare Zonen.
[d] Und 0.96–1.0; zwischen den Zonen etwas verschmiert.
[e] Und 0.98–1.0; zwischen den Zonen etwas verschmiert.
[f] Schmiert bis 0.91.
[g] Und 0.104–0.23; zwischen den Zonen verschmiert.
[h] Schwerpunkt bei 0.87 mit Nachschleier.

TABELLE III

R_F-WERTE CYCLISCHER ALKOHOLE

Bedingungen s. Text.

Alkohol + 8 N HCl	*Verbindung*								
	$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4$ + HCl	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
Cyclohexanol	0.89 –1.0	0.95–1.0[a]	[b]	0.034–0.26	0.066–0.165	0.585–0.705	0.705–0.95	[c]	0.031–0.196
Benzylalkohol	0.125–0.192 0.59 –0.89	0.65–0.94	0.54–0.69 0.98–1.0	0.144–0.34	0.133–0.224	0.41 –0.49	0.52 –0.72	0.104–0.83	0.104–0.22
Tetrahydrofurfuryl-alkohol	1.0	1.0	1.0	0.92 –1.0	1.0	0.97 –1.0	1.0	0.85 –1.0	0.85 –0.94[d]

[a] Geringe Mengen auch am Startfleck.
[b] Schmiert über das ganze Chromatogramm.
[c] Schmiert bis 0.8; Hauptmenge bei 0.052–0.21.
[d] Mit Vorschleier von 0.94–0.97.

TABELLE IV

R_F-WERTE DER PLATINVERBINDUNGEN BEI VERWENDUNG VON ALKOHOL–HBr (MIND. 48 %, $d = 1.50$)-GEMISCHEN ALS FLIESSMITTEL

Verbindung	*Propanol*		*n-Butanol*	
	8 N HCl	*HBr*	*8 N HCl*	*HBr*
$H_2[OsCl_6]$	0.95–1.0	0.93 –1.0	0.83 –0.97	0.98–1.0
$H_2[OsBr_6]$	0.0 und 1.0	1.0	0.0 und 1.0[a]	0.99–1.0
$2NaOH/OsO_4$ + HCl	0.8 –0.9	1.0	0.79 –0.89	0.99–1.0
$RuCl_3$	0.21–0.67	0.224–0.56	0.0 –0.48	0.0 –0.54
$Na_3[RhCl_6]$	0.26–0.41	0.072–0.186	0.114–0.34	0.62–0.188
		und 0.37 –0.49		und 0.25–0.40
$Na_2[PdCl_4]$	0.78–0.9	0.85 –0.98	0.68 –0.765	0.81–1.0
$Na_2[PtCl_6]$	0.95–1.0	0.99 –1.0[b]	0.76 –0.91	0.99–1.0[c]
$H_2[IrCl_6]$	0.255–0.73	0.25 –0.437[d]	0.166–0.58	0.0 –0.35[e]
$H_3[IrCl_6]$	0.256–0.46	0.207–0.38	0.128–0.41	0.0 –0.3

[a] Hauptmenge bleibt am Startpunkt und nur eine geringe Menge läuft mit Laufmittelfront.
[b] Nachschleier von 0.84–0.99.
[c] Mit Nachschleier bis 0.66.
[d] Mit Vorschleier von 0.437–0.714.
[e] Mit Vorschleier bis 0.69.

Ketone

Auch bei den Ketonen sind die R_F-Werte der niederen Glieder am höchsten, wie aus Tabelle V hervorgeht. Bemerkenswert sind die Unterschiede zwischen den nach der absteigenden Methode und dem Rundfilterverfahren erhaltenen R_F-Werten.

Äther

Die mit Dioxan und Tetrahydrofuran als Laufmittel gemessenen R_F-Werte nehmen mit der HCl-Konzentration zu (s. Tabelle VII). Für eine Trennung der Platinmetalle sind die geprüften Äther jedoch nicht geeignet, da die R_F-Werte der Platinmetallverbindungen sehr ähnlich liegen. Allein Rhodium zeigt R_F-Abweichungen, die bei Anwendung einer Tetrahydrofuran–HCl-Mischung (15 ml Äther + 3 ml 8 *N* HCl) zur Abtrennung dieses Metalls von den übrigen Platinmetallen benutzt werden kann.

DISKUSSION

Praktische Bedeutung der Versuche

Durch die Untersuchung wird bewiesen, dass der Chloro- und Bromokomplex des Osmiums und die wahrscheinlich als $Na_2[OsO_2Cl_4]$ vorliegende Osmiumverbindung, die beim Ansäuern des $2NaOH/OsO_4$-Addukts mit Salzsäure entsteht, mit vielen Fliessmitteln ein definiertes Laufverhalten zeigen. Die R_F-Werte der Osmiumkomplexe unterscheiden sich bei den einzelnen Laufmitteln oft merklich voneinander, so dass eine genaue Angabe der den R_F-Werten zugrundeliegenden Osmiumverbindungen unbedingt erforderlich ist.

Bei Auswahl der für eine Trennung vorgesehenen Verbindung des Osmiums muss

TABELLE V

R_F-WERTE EINIGER EINFACHER KETONE

A = Absteigende Methode; R = Rundfiltermethode.

Laufmittel	*Verfahren*	*Verbindung*							
		$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4 + HCl$	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
15 ml Aceton + 3 ml 8 *N* HCl	R	0.97 –1.0	0.0 0.98 –1.0	0.0 0.97 –1.0	0.0 0.32 –0.75	0.267–0.465	0.97 –1.0[e]	0.89 –1.0		0.325–0.48
14 ml Aceton + 4 ml H_2O + 2 ml konz. HCl[a] + 0.4 g Thioharnstoff	R	1.0	1.0	0.0 0.39 –0.71	0.33 –0.42	0.57 –0.74	0.69 –0.75	0.66 –0.76 und 1.0	1.0	0.71 –0.81
19 ml Aceton + 1 ml HCl (d = 1.18)	A	0.64 –0.90	0.90 –1.0			0.067–0.178	0.69 –0.83	0.75 –1.0	0.72 –0.90	0.027–0.178
14 ml Aceton + 7 ml 10 *N* HNO_3	R	0.60 –0.69	0.70 –0.785	0.61 –0.68	0.091–0.164	0.08 –0.25	0.64 –0.70	0.54 –0.60	0.55 –0.64[f]	0.56 –0.62 0.65 –0.71
15 ml Methyläthylketon + 3 ml 8 *N* HCl	R	0.76 –0.88	0.93 –1.0	0.80 –0.97	0.28 –0.48	0.165–0.25	0.76 –0.77	0.75 –0.79	0.18 –0.24 0.75 –0.80	0.167–0.255 0.74 –0.80
14 ml Methyläthylketon + 6 ml HCl (d = 1.18)	A	0.84 –0.92	0.92 –0.81					0.76 –0.85	0.73 –0.83	0.21 –0.31
15 ml Methyl-*n*-propylketon + 3 ml 8 *N* HCl	R	0.49 –0.63	0.53 –0.59 0.66 –0.74 0.79 –0.89	0.52 –0.90	0.11 –0.185	0.092–0.15	0.39 –0.41	0.41 –0.435		
14 ml Methyl-*n*-propylketon + 6 ml HCl (d = 1.18)	A	0.86 –0.97			0.09 –0.12	0.093–0.15	0.54 –0.66	0.82 –0.93 0.34 –0.36	0.10 –0.73	0.11 –0.174
15 ml Methyl-*n*-butylketon + 3 ml 8 *N* HCl[b]	R	0.355–0.885	0.495–0.57 0.69 –0.82	0.59 –1.0	0.069–0.148	0.066–0.132	0.31 –0.35	0.51 –0.6 0.78 –0.87	0.068–0.59	0.104–0.136 0.32 –0.35
14 ml Methyl-*n*-amylketon + 6 ml konz. HCl (d = 1.19)[c]	R	0.218–0.45	0.418–0.67	0.159–1.0	0.168–0.195	0.158–0.17	0.15 –0.168	0.192–0.20	0.212–0.24 0.164–0.202	0.17 –0.208
14 ml Methyl-*n*-amylketon + 6 ml HCl (d = 1.19)[d]	A	0.114–0.27	0.29 –0.74	0.16 –0.40	0.021–0.049 0.064–0.077	0.021–0.037	0.053–0.071	0.095–0.114	0.095–0.138	0.029–0.061

[a] Laufmittel nach Lit. 8.
[b] Laufmittel entmischt sich beim Laufen.
[c] Bis auf Osmium zeigen alle Platinmetalle einen sehr schmalen R_F-Bereich. Nach Lit. 9 ist dieses Laufmittel gut zur Trennung der Chloride der Platinmetalle, einschliesslich Gold und Silber, geeignet.
[d] Laufmittelfront 37.8 cm vom Startpunkt entfernt.
[e] Nachschleier bis 0.92.
[f] Nachschleier bis 0.51.

TABELLE VI

R_F-WERTE EINIGER ALIPHATISCHER UND CYCLISCHER KETONE

A = Absteigende Methode; R = Rundfiltermethode.

Laufmittel	*Verfahren*	*Verbindung*								
		$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4 + HCl$	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
Methyl-isobutylketon (10 ml + 10 ml 10 *N* HCl)	A	0.67 –0.80	0.80 –0.91	0.72–0.85	0.53 –0.66	0.60 –0.72	0.0 0.54 –0.65	0.65 –0.78	0.58 –0.76	0.58 –0.76
Methyl-isopentylketon (15 ml + 3 ml 8 *N* HCl)	R	0.73 –0.84	0.51 –0.65	0.68–0.83	0.91 –1.0	0.96 –1.0	0.76 –0.83	0.78 –0.875	0.78 –1.0	0.95 –1.0
Cyclopentanon (15 ml + 3 ml 8 *N* HCl)	R	0.60 –0.79	0.75 –0.85 0.9 –1.0	0.59–1.0	0.57 –0.61	0.188–0.29	0.56 –0.57	0.58 –0.59	0.158–0.51	0.16 –0.27
Cyclohexanon (15 ml + 3 ml 8 *N* HCl)	R	0.69 –0.89 0.93 –0.97	0.435–0.50 0.88 –0.99	0.89–0.98	0.163–0.249[b]	0.108–0.186	0.44 –0.46	0.65 –0.73 0.84 –0.95	0.108–0.88	0.10 –0.178
Aceton (20 ml) + Acetylaceton (2 ml) + 2 *N* HCl (0.6 ml)	A	0.34 –0.66	0.395–0.81		0.0 –0.46[c]	0.0 –0.064	0.83 –1.0	0.315–0.36[d]	0.3 –0.66[e]	0.0
Aceton (4 ml) + Acetophenon (10 ml) + HCl (6 ml, $d = 1.19$)[a]	R	0.204–0.358	0.50 –0.91		0.065–0.074	0.556–0.81	0.101–0.194	0.164–0.286	0.105–0.146[f]	0.073–0.11

[a] Laufmittel nach Lit. 9.
[b] Vorschleier bis 0.6.
[c] Schmiert.
[d] Laufverhalten sehr gut.
[e] Mit Nachschleier.
[f] Nachschleier von 0.047–0.105.

TABELLE VII

R_F-WERTE MIT ÄTHER–HCl-MISCHUNGEN

Aufgetragene Substanz	*Fliessmittel*					
	Tetrahydrofuran–HCl (15:3)			*Dioxan–HCl (15:3)*		
	0.1 N	*2 N*	*8 N*	*0.1 N*	*2 N*	*8 N*
$H_2[OsCl_6]$	0.66–0.74	0.89–0.92	0.94–0.97	0.68–0.80	0.73 –0.75	0.96–1.0
$H_2[OsBr_6]$			0.95–1.0	0.76–0.77	0.735–0.76	1.0
$2NaOH/OsO_4$ + HCl		0.98–1.0[a]	0.94–0.97	0.65–0.68	0.74 –0.76	0.99–1.0
$RuCl_3$	0.0	0.86–0.88	0.94–0.97	0.64–0.67	0.7 –0.81	0.89–1.0
	0.63–0.66					
	0.52–0.58					
$Na_3[RhCl_6]$	0.65–0.68	0.69–0.78	0.55–0.65	0.65–0.67	0.69 –0.71	0.81–0.91
	0.73–0.78					
$Na_2[PdCl_4]$	0.69–0.71	0.88–0.89	0.92–0.95	0.66–0.68	0.70 –0.72	0.93–1.0
$Na_2[PtCl_6]$	0.69–0.71	0.86–0.88	0.94–0.95	0.63–0.67	0.7 –0.73	0.93–1.0
$H_2[IrCl_6]$	0.65–0.68	0.87–0.88[b]	0.95–0.96	0.65–0.71	0.68 –0.71	0.98–1.0
$H_3[IrCl_6]$	0.63–0.66	0.87–0.875[c]	0.96	0.65–0.68	0.70 –0.72	0.92–1.0

[a] Sehr schwach nachweisbar.
[b] Mit Nachschleier von 0.75–0.87.
[c] Mit Nachschleier von 0.67–0.87.

berücksichtigt werden, dass das Laufverhalten des beim Ansäuern mit Salzsäure aus den $2NaOH/OsO_4$-Addukt gebildeten Komplexes im Vergleich zum Chloro- oder Bromokomplex bei manchen Fliessmitteln weniger gut ist. Es liegt eine Neigung zur Bildung von Doppelzonen bzw. Doppelflecken und zum Verschmieren vor, die wahrscheinlich auf die Instabilität der VI. Wertigkeitsstufe des Osmiums im $Na_2[OsO_2Cl_4]$-Komplex zurückgeht.

Der Vergleich des Osmium-Laufverhaltens mit dem entsprechenden Verhalten der übrigen Platinmetalle zeigt, dass mit den meisten Alkohol–HCl- und Keton–HCl-Fliessmitteln Osmium in Form der Halogenokomplexe besser wandert als die entsprechenden Komplexe der übrigen Platinmetalle.

Zusammenhang zwischen R_F-Werten und Verteilungskoeffizienten der Flüssig–Flüssig-Extraktion

Die mit zunehmender Zahl der Kohlenstoffatome bei Alkoholen und Ketonen gemessene Abnahme der R_F-Werte der Chloro- und Bromokomplexe des Osmiums steht in gewisser Analogie zu Ergebnissen der Flüssig–Flüssig-Extraktion dieser Komplexe. Die Untersuchungen stellen somit einen Beitrag zur Frage des zwischen Verteilungskoeffizienten α ($=C_o/C_w$) der Flüssig–Flüssig-Extraktion und R_F-Werten der Papierchromatographie bestehenden Zusammenhangs dar, auf den MARTIN UND SYNGE[17] erstmals hinwiesen. Bei Rückführung der Papierchromatographie auf einen kontinuierlichen fraktionierten Verteilungsprozess ist nämlich anzunehmen, dass bei einer Verteilung von Molekeln zwischen stationärer und beweglicher Phase von der in der beweglichen Phase löslicheren Molekelart eine grössere Zahl in die Fliessmittelphase pro Zeiteinheit übergeht und so schneller wandert. Zwischen R_F und α kann somit ein Zusammenhang erwartet werden, demzufolge hohe Verteilungskoeffizienten mit relativ grossen R_F-Werten verbunden sein sollten; s. hierzu auch CERRAI[23] und OHASHI u.a.[24].

Der Vergleich der hier erhaltenen R_F-Werte mit den Verteilungskoeffizienten α der Flüssig–Flüssig-Extraktion des Osmiums[16] zeigt nun, dass dieser Zusammenhang für die Extraktion und für das papierchromatographische Verhalten der Osmium–Chloro- und Bromokomplexe gegeben ist:

Einmal sind sowohl die Verteilungskoeffizienten α als auch die R_F-Werte bei Anwendung von Alkoholen und Ketonen als Extraktions- bzw. Fliessmittel bei den Bromokomplexen des Osmiums grösser als bei den Chlorokomplexen. Zum anderen nehmen die Verteilungskoeffizienten und die R_F-Werte bei Alkoholen geringfügig mit der Zahl der Kohlenstoffatome des Extraktions- bzw. Fliessmittels ab. Der im Gegensatz zu der relativ starken R_F-Abnahme der anderen Platinmetalle—siehe hierzu auch Lit. 14—stehende leichte R_F-Rückgang des Osmiums bei Erhöhung des Alkohol-Molekulargewichts wird somit auch bei der Extraktion beobachtet. In Übereinstimmung zum R_F/α-Zusammenhang ist bei Ketonen ein erheblicher Rückgang der R_F-Werte und der Verteilungskoeffizienten mit Zunahme der Kohlenstoffanzahl feststellbar, wie die Gegenüberstellung der Messwerte in Fig. 1. veranschaulicht.

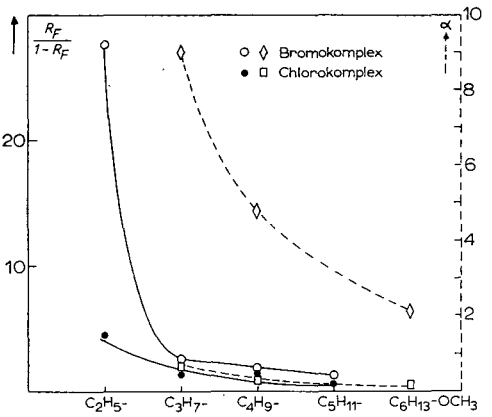

Fig. 1. Vergleich der R_F-Werte (eigentlich $R_F/(1-R_F)$-Werte) und Verteilungskoeffizienten[16] des Osmiumchloro- und -bromokomplexes bei Anwendung von Ketonen als Fliess- bzw. Extraktionsmittel.

Zusammenfassend ist festzustellen, dass sich Osmium in Form der Chloro- und Bromokomplexe zu papierchromatographischen Trennungen heranziehen lässt. Durch Vergleich mit Ergebnissen von Extraktionsversuchen konnte ausserdem die Gültigkeit des von MARTIN UND SYNGE postulierten Zusammenhangs zwischen R_F-Werten und Verteilungskoeffizienten für den Chloro- und Bromokomplex des Osmiums erkannt werden.

ZUSAMMENFASSUNG

Das papierchromatographische Verhalten des in definierten Verbindungen vorliegenden Osmiums wurde bei Anwendung von Alkoholen, Äthern und Ketonen untersucht und die mit verschiedenen Fliessmitteln erhaltenen R_F-Werte mit den R_F-Werten der übrigen Platinmetalle verglichen. Zwischen R_F-Werten und Verteilungs-

koeffizienten α der Flüssig–Flüssig-Extraktion konnte für die Chloro- und Bromokomplexe des Osmiums, vor allem bei Ketonen, ein Zusammenhang festgestellt werden.

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ZUR PAPIERCHROMATOGRAPHISCHEN TRENNUNG DER PLATINMETALLE UNTER BESONDERER BERÜCKSICHTIGUNG DES OSMIUMS

2. TEIL. MISCHSYSTEME UND SALZE ALS FLIESSMITTEL

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SUMMARY

The paper chromatographic separation of platinum metals with special reference to osmium. Part II. Mixed systems and salts as solvents

The paper chromatographic behaviour of the platinum metals has been examined by using various mixed systems, hydrochloric acid and solutions of inorganic salts as solvents. The gap in the paper chromatographic data concerning osmium was closed. A saturated sodium chloride solution proved to be specially suitable for the separation of platinum metals. The sequence of separation is: $Ir^{3+} = Rh > Ru > Pd > Pt > Os$.

EINLEITUNG

Im 1. Teil der über die papierchromatographische Trennung der Platinmetalle durchgeführten Arbeit wurde gezeigt, dass die definiert hergestellten Halogenokomplexe des Osmiums und unter besonderen Bedingungen auch die OsO_4/NaOH-Anlagerungsverbindung papierchromatographisch bearbeitet werden können[1].

Im folgenden werden nun die Ergebnisse weiterer Untersuchungen angeführt, deren Ziel eine weitgehende papierchromatographische Auftrennung aller sechs Platinmetalle ist. Diese Untersuchungen wurden erforderlich, da über die papierchromatographische Abtrennung des Osmiums in der Literatur keine genauen Angaben vorliegen (s. Literatur im Teil 1).

VERSUCHSDURCHFÜHRUNG

Die Durchführung der Versuche erfolgte analog zur vorangehenden Arbeit. Als Osmiumverbindungen wurden somit die nach den angegebenen Vorschriften darstellbaren Komplexe $H_2[OsCl_6]$, $H_2[OsBr_6]$ sowie der beim HCl-Ansäuern aus der OsO_4/NaOH-Anlagerungsverbindung (wahrscheinlich) gebildete Komplex $Na_2[OsO_2Cl_4]$

TABELLE I

R_F-WERTE VERSCHIEDENER LAUFMITTELMISCHUNGEN

Laufmittel	*Verfahren*	*Verbindung*								
		$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4 + HCl$	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
Methanol (7 ml) + Ameisensäure (7 ml)	A	0.59 –0.80	0.53 –0.68	0.0 –0.62[e]	0.35 –0.51			0.58–0.78	0.33 –0.58	
Methanol (14 ml) + Pyridin (4 ml) + Toluol (2 ml)	A	0.0 –0.48	0.41 –0.58		0.25 –0.86	0.18 –0.69[d]	0.55–0.7	0.38–0.45	0.35 –0.48[e]	0.0 –0.134
Isobutylalkohol (5 ml) + Methanol (5 ml) + konz. HCl (5 ml)	R	0.79 –0.89	0.79 –0.87	0.71–0.79	0.50 –0.61	0.54 –0.62	0.72–0.78	0.69–0.77	0.75 –0.85[f] 0.53 –0.66	0.50 –0.63
Methanol (5 ml) + *tert.*-Butylalkohol (5 ml) + konz. HCl (5 ml)	R	0.74 –0.82	0.78 –0.79	0.65–0.74	0.51 –0.62	0.60 –0.67	0.70–0.74	0.70–0.80	0.71 –0.77[g]	0.60 –0.68
n-Butanol (10 ml) + Essigsäure (2 ml) + Acetessigester (1 ml) + Aqua dest. (2 ml)	R	0.0 0.126–0.37	0.136–0.24 0.6 –0.65	0.0 –0.42[h]	0.206–0.26	0.115–0.172 0.172–0.264	0.23–0.28	0.10–0.23	0.051–0.235	0.032–0.172
Isopentylalkohol (5 ml) + Methanol (5 ml) + konz. HCl (5 ml)	R	0.80 –0.88	0.87 –0.90	0.73–0.83	0.54 –0.65	0.59 –0.65	0.72–0.786	0.80–0.86	0.62 –0.72[i]	0.59 –0.685
Isopentylalkohol (16 ml) + Tetrahydrofurfuryl-alkohol (4 ml) + konz. HCl (10 ml) (d = 1.19)	A	0.71 –0.84	0.79 –0.95	0.75–0.84	0.26 –0.48	0.25 –0.33	0.58–0.65	0.71–0.78	0.26 –0.355	0.26
Isopentylalkohol[a] (5 ml) + Methanol (5 ml) + Pyridin reinst (5 ml) + konz. HCl (5 ml)	R	0.0 –0.48	0.34 –0.53	0.0 –0.6[j]		0.48 –0.56	0.59–0.62	0.58–0.61	0.41 –0.52	0.40 –0.47
Äthylacetat (12 ml)[b] + konz. HCl (6 ml) + Essigsäure (2 ml)	A	0.54 –0.69	0.59 –0.74	0.50–0.66	0.2 –0.30	0.26 –0.35	0.4 –0.52	0.56–0.62	0.64 –0.71[k]	0.27 –0.37

[a] Das Laufmittel entmischt sich während des Laufens; es sind deutlich zwei Laufmittelfronten zu erkennen.
[b] Laufmittel nach Lit. 4.
[c] Schwerpunkt bei 0.46.
[d] Schmiert.
[e] Mit Nachschleier.
[f] Nachschleier bis 0.53.
[g] Nachschleier von 0.61–0.71.
[h] Schmiert.
[i] Vorschleier von 0.72–0.85.
[j] Hauptmenge bei 0.47–0.53 und 0.0.
[k] Mit Nachschleier, der bis zum dreiwertigen Ir reicht.

TABELLE II

R_F-WERTE BEI ANWENDUNG VON HCl ALS FLIESMITTEL

Konzentrationsbereich 0.01–10 *N* HCl. Rundfiltermethode.

Aufgetragene Verbindung	*HCl-Konzentration*								
	0.01 N	*0.05 N*	*0.1 N*	*0.5 N*	*1 N*	*2 N*	*5 N*	*8 N*	*10 N*
$H_2[OsCl_6]$	0.93–0.985	0.94 –0.985	0.94 –1.0	0.89–0.98	0.885–0.95	0.83 –0.90	0.785–0.88	0.72 –0.786	0.637–0.76
$H_2[OsBr_6]$	0.88–0.96	0.854–0.92	0.846–0.935	0.79–0.88	0.78 –0.86	0.71 –0.79	0.61 –0.705	0.50 –0.587	0.40 –0.506
$2NaOH/OsO_4$ + HCl	0.0 –0.125[a]	0.168–0.286	0.347–0.52	0.77–0.89	0.825–0.90	0.815–0.905	0.82 –0.89	0.69 –0.80	0.60 –0.74
$RuCl_3$	0.90–0.96	0.89 –0.945	0.905–0.96	0.88–0.96	0.89 –0.96	0.85 –0.92	0.95 –1.0	0.91 –0.965	0.85 –0.95
$Na_3[RhCl_6]$	0.95–0.99	0.93 –1.0	0.906–1.0	0.93–1.0	0.895–1.0	0.88 –1.0	0.97 –1.0	0.944–1.0	0.953–1.0
$Na_2[PdCl_4]$	0.88–0.95	0.92 –1.0	0.93 –0.985	0.92–0.99	0.93 –0.98	0.865–0.935	0.83 –0.9	0.724–0.79	0.66 –0.743
$Na_2[PtCl_6]$	0.93–1.0	0.96 –1.0	0.93 –1.0	0.92–1.0	0.89 –1.0	0.855–0.92	0.826–0.902	0.767–0.835	0.713–0.80
$H_2[IrCl_6]$	0.92–0.97[b]	0.92 –0.95[c]	0.91 –0.97	0.90–0.97	0.88 –0.1	0.84 –0.895[d]	0.815–0.88[e]	0.71 –0.79[f]	0.69 –1.0
$H_3[IrCl_6]$	1.0	1.0	1.0	1.0	1.0	1.0	0.99 –1.0	1.0	0.92 –1.0

[a] Vorschleier bis 0.81.
[b] Vorschleier 0.97–1.0.
[c] Schmiert bis 1.0.
[d] Vorschleier bis 1.0.
[e] Mit Vorschleier von 0.88–1.0.
[f] Vorschleier 0.79–1.0.

aufgetragen. Die Platinmetalle wurden als $RuCl_3 \cdot 1–3\ H_2O$, $Na_2[PdCl_4]$, $Na_2[PtCl_6] \cdot 6\ H_2O$, $Na_3[RhCl_6] \cdot 12\ H_2O$, $H_2[IrCl_6]$ bzw. $H_3[IrCl_6]$ eingesetzt und über Farbreaktionen nachgewiesen. Da in einigen Fällen die nach verschiedenen Verfahren gemessenen R_F-Werte voneinander abwichen, wurde neben der vor allem benutzten Rundfiltermethode[2,3] bei einigen Fliessmitteln auch die absteigende Methode zur R_F-Bestimmung herangezogen.

VERSUCHSERGEBNISSE

Mischsysteme

Die R_F-Werte verschiedener Lösungsmittelmischungen sind in Tabelle I zusammengestellt. Man erkennt, dass z.B. beim teilweisen Ersatz eines alkoholischen Fliessmittels durch organische Verbindungen keine merklichen Änderungen der mit den reinen Alkohol–HCl-Mischungen erreichbaren R_F-Unterschiede eintreten. In der Tabelle sind auch die mit dem von NODDACK UND SANIR[4,5] angegebenen Laufmittelgemisch erhaltenen R_F-Werte eines Platinmetallgemisches einschliesslich der den verschiedenen Osmiumverbindungen zugehörigen neuen Werte zusammengestellt. Die R_F-Werte zeigen folgende Abstufung: Ir(IV) $\approx$ Os $\approx$ Pt $>$ Pd $>$ Rh $\approx$ Ir(III) $\geqslant$ Ru.

Salzsäure

Tabelle II gibt eine Übersicht über die mit verschieden konzentrierter HCl als Fliessmittel erhaltenen R_F-Werte. Bei den Chloro- und Bromokomplexen des Osmiums nehmen die R_F-Werte mit der HCl-Konzentration ab, während die beim Ansäuern des $2NaOH \cdot OsO_4$-Addukts erhaltene Verbindung ein R_F-Maximum bei 1–5 *N* HCl aufweist.

Salze als Fliessmittel bzw. Laufmittelzusätze

Da sich nach NODDACK UND SANIR[4,5] eine gesättigte NH_4Cl-Lösung als Laufmittel für die Abtrennung des Platins einsetzen lässt, wurde das Laufverhalten anderer Salze in die Untersuchungen einbezogen.

NH_4Cl

Die mit der Rundfiltermethode bei Anwendung einer gesättigten Lösung von Ammonchlorid erhaltenen R_F-Werte sind in Tabelle III angegeben. Im Gegensatz zu den oben zitierten Angaben[4,5] konnte Platin nicht von den übrigen Elementen der Platingruppe abgetrennt werden. Die R_F-Werte deuten jedoch eine Trennmöglichkeit des Osmiums an.

Natriumchlorid

Reine NaCl-Lösungen ohne Zusätze. In Tabelle IV sind die mit verschieden konzentrierten NaCl-Lösungen erhaltenen R_F-Werte zusammengestellt. Man erkennt, dass gesättigte NaCl-Lösung ein geeignetes Laufmittel für die Trennung der aufgetragenen Platinmetallverbindungen darstellt. Die R_F-Werte sind in der Reihe abgestuft: Rh $>$ Ru $>$ Pd $>$ Pt $>$ Ir(IV) $=$ Os. Mit 1 *M* NaCl-Lösung lassen sich die verschiedenen Osmiumverbindungen $H_2[OsCl_6]$, $H_2[OsBr_6]$ und $2NaOH \cdot OsO_4(HCl)$ gut trennen.

TABELLE III

R_F-WERTE MIT GESÄTTIGTER NH_4Cl-LÖSUNG ALS LAUFMITTEL

Auftragslösung	*R_F-Wert*
$H_2[OsCl_6]$	0.0 –0.085 (überwiegender Teil des Os)
	0.82–0.87
$H_2[OsBr_6]$	0.67–0.73
$2NaOH/OsO_4$ + HCl	0.83–0.90
$RuCl_3$	0.92–0.99
$Na_3[RhCl_6]$	0.95–1.0
$Na_2[PdCl_4]$	0.84–0.91
$Na_2[PtCl_6]$	0.85–0.93
$H_2[IrCl_6]$	0.97–1.0
$H_3[IrCl_6]$	0.97–1.0

TABELLE IV

R_F-WERTE IN ABHÄNGIGKEIT VON DER KONZENTRATION DES FLIESSMITTELS NaCl
Rundfiltermethode.

Auftragslösung	*NaCl-Konzentration*			
	0.1 M	*1 M*	*5 M*	*Gesättigt*
$H_2[OsCl_6]$	0.91–0.97	0.84–0.91	0.65 –0.83	0.63 –0.74
$H_2[OsBr_6]$	0.82–0.90	0.71–0.79	0.365–0.47	0.34 –0.44
$2NaOH/Os_4$ + HCl	0.0 –0.188	0.0 –0.37	0.70 –0.83[a]	0.66 –0.82
$RuCl_3$	0.89–0.99	0.84–0.91	0.73 –0.81	0.9 –0.95
$Na_3[RhCl_6]$	0.92–0.98	0.89–0.97	0.9 –1.0	1.0
$Na_2[PdCl_4]$	0.85–0.95	0.87–0.93	0.8 –0.87	0.8 –0.88
$Na_2[PtCl_6]$	0.89–0.99	0.84–0.91	0.73 –0.81	0.725–0.786
$H_2[IrCl_6]$	0.91–0.95	0.85–0.90	0.7 –0.75	0.64 –0.73
	und 0.95–1.0	und 1.0	und 1.0	
$H_3[IrCl_6]$	1.0	1.0	1.0	1.0

[a] Nachschleier 0.38–0.7.

Laufmittel mit Zusatz von gesättigter NaCl-Lösung. Über die Eigenschaften von mit gesättigten NaCl-Lösungen versehenen Laufmitteln orientiert Tabelle V. Durch den NaCl-Zusatz werden (beispielsweise im System Isobutylalkohol–Methanol–HCl) nur geringfügige Änderungen der R_F-Werte hervorgerufen.

In Tabelle VI sind noch einige R_F-Werte zusammengestellt, bei denen das Laufmittel Methanol–HCl auf einem mit 1 *M* NaCl-Lösung getränkten Papier nach Eintrocknen der NaCl-Lösung verwendet wurde. Die R_F-Werte sind im Vergleich zum nicht mit NaCl vorbehandeltem Papier um 0.1–0.2 Einheiten kleiner. Bei Vergrösserung der HCl-Konzentration nehmen besonders die R_F-Werte der Osmiumverbindungen zu.

Lithiumchlorid

Aus den R_F-Werten der Tabelle VII ist abzulesen, dass mit gesättigten LiCl-Lösungen ein schlechteres Laufverhalten der Osmiumkomplexe als mit gesättigten

TABELLE V

R_F-WERTE VON MIT GESÄTTIGTER NaCl-LÖSUNG VERSEHENEN FLIESSMITTELN

Rundfiltermethode.

Fliessmittel	*Auftragslösung*								
	$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4$ + HCl	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$N_2[IrCl_6]$	$H_3[IrCl_6]$
Ges. NaCl (10 ml) + 25% $NaNO_2$ (5 ml)	0.63–0.75	0.38–0.495	0.029–0.19			0.76–0.88		1.0	1.0
Ges. NaCl (10 ml) + Ameisensäure (5 ml)	0.71–0.81	0.55–0.59	0.0 0.75 –0.83[a]	0.9 –0.97	0.95–1.0	0.78–0.86	0.74–0.82	0.74–0.78[b] 0.97–1.0	0.97–1.0[c]
Ges. NaCl (10 ml) + Formaldehyd (5 ml)	0.67–0.77	0.46–0.55	0.72 –0.81[d]	0.82–1.0	0.95–0.98[e]	0.78–0.86	0.75–0.82	0.7 –0.77	0.98–1.00
Ges. NaCl (5 ml) + Isobutylalkohol (5 ml) + Methanol (5 ml) + konz. HCl (5 ml)	0.85–0.94	0.87–0.96	0.82–0.90	0.67–0.76	0.79–0.86 0.38–0.65	0.82–0.90	0.87–0.94	0.84–0.95	0.71–0.86
Isobutylalkohol (5 ml) + Methanol (5 ml) + konz. HCl gesättigt mit NaCl (5 ml)	0.73–0.81	0.74–0.89	0.69 –0.77	0.47–0.56	0.54–0.62 0.29–0.37	0.69–0.75	0.73–0.84	0.56–0.67[f] 0.73–0.80	0.54–0.70

[a] Am Startfleck geringe Mengen.
[b] Zwischen den Zonen verschmiert.
[c] Nachschleier bis 0.92.
[d] Nachschleier bis 0.0.
[e] Nachschleier bis 0.85.
[f] Zwischen den Zonen diffus verteilt.

TABELLE VI

ABHÄNGIGKEIT DER R_F-WERTE VON DER HCl-KONZENTRATION AUF MIT 1 *M* NaCl VORBEHANDELTEM PAPIER

Rundfiltermethode. Laufmittel: 7 ml Methanol + 3 ml HCl.

Auftragslösung	*HCl-Konzentration*		
	0.1 N	*2 N*	*8 N*
$H_2[OsCl_6]$	0.56–0.72	0.65–0.75	0.75 –0.88
$H_2[OsBr_6]$	0.52–0.64	0.62–0.71	0.77 –0.87
$2NaOH/OsO_4$ + HCl	0.0 0.58–0.73	0.0 0.43–0.46 0.67–0.76	0.0 0.55 –0.63 0.72 –0.86
$RuCl_3$	0.0 0.7 –0.785	0.0 0.67–0.82	0.0 0.66 –0.77
$Na_3[RhCl_6]$	0.60–0.69	0.59–0.71	0.62 –0.705
$Na_2[PdCl_4]$	0.59–0.68	0.60–0.69	0.425–0.505 0.75 –0.83
$Na_2[PtCl_6]$	0.69–0.78	0.71–0.76	0.81 –0.88
$H_2[IrCl_6]$	0.59–0.69 0.69–0.77	0.69–0.74 0.49–0.59	0.62 –0.77 0.78 –0.85
$H_3[IrCl_6]$	0.67–0.75 0.58–0.67	0.49–0.56 0.59–0.69	0.63 –0.7

NaCl-Lösungen erhalten wird. Mit einer Mischung aus Ameisensäure, Dioxan und ges. LiCl-Lösung kann Rhodium und Palladium von den übrigen Platinmetallen abgetrennt werden.

DISKUSSION

Auch diese Versuche bestätigen das definierte Laufverhalten der nach den angegebenen Vorschriften hergestellten Chloro- und Bromokomplexe des Osmiums. Sie zeigen aber auch die Bedeutung der Angabe der bei papierchromatographischen Versuchen aufgetragenen Osmiumverbindungen. Besonders deutlich sind die R_F-Unterschiede der verschiedenen Osmiumkomplexe beispielsweise bei Anwendung einer NaCl–$NaNO_2$-Lösung als Laufmittel. Es gilt in diesem Fall: $H_2[OsCl_6]$, R_F = 0.63–0.75; $H_2[OsBr_6]$, R_F = 0.38–0.495; $Na_2[OsO_2Cl_4]$, R_F = 0.029–0.19.

Für die Abhängigkeit des R_F-Wertes der aufgetragenen Anlagerungsverbindung $2NaOH \cdot OsO_4$ von der HCl-Konzentration sind sehr wahrscheinlich die Umsetzungen

$$Na_2[OsO_4(OH)_2] + 8HCl \rightleftharpoons H_2[OsO_2Cl_4] + Cl_2 + 2NaCl + 4H_2O \quad (1)$$

und

$$H_2[OsO_2Cl_4] + HCl \rightleftharpoons OsCl_3(OH) + Cl_2 + H_2O \quad (2)$$

verantwortlich. Unter der Annahme, dass sich in der stehenden Phase das Osmium als $H_2[OsO_2Cl_4]$ und in der beweglichen Phase als $OsCl_3(OH)$ befindet, lässt sich für den mit dem R_F-Wert zusammenhängenden Verteilungskoeffizienten

$$\alpha = \frac{OsCl_3(OH)}{H_2[OsO_2Cl_4]} \quad (3)$$

TABELLE VII

R_F-WERTE BEI ANWENDUNG VON LiCl-HALTIGEN GEMISCHTEN FLIESSMITTELN

Rundfiltermethode

Fliessmittel	*Auftragslösung*								
	$H_2[OsCl_6]$	$H_2[OsBr_6]$	$2NaOH/OsO_4 + HCl$	$RuCl_3$	$Na_3[RhCl_6]$	$Na_2[PdCl_4]$	$Na_2[PtCl_6]$	$H_2[IrCl_6]$	$H_3[IrCl_6]$
LiCl (ges.)	0.21–0.345	0.047–0.134	0.29–0.41	0.97 –0.1	0.98–1.0	0.39–0.476	0.356–0.49	0.30–0.39 1.0	1.0
5 ml LiCl (ges.) + 5 ml Ameisensäure + 5 ml H_2O	0.70–0.81	0.47 –0.56	0.0 0.73–0.82	0.94 –1.0	0.96–1.0	0.75–0.83	0.76 –0.85	0.72 –0.78 1.0	1.0
3 ml LiCl (ges.) + 10 ml Tetrahydrofuran + 5 ml Ameisensäure	0.89–0.95	0.93 –1.0	0.95–0.91 0.92–0.97	0.265–0.60	0.37–0.46	0.71–0.77	0.83 –0.90	0.86–0.95	0.85–0.94
3 ml LiCl (ges.) + 10 ml Dioxan + 5 ml Ameisensäure	0.90–0.94	0.90–0.93	0.90–0.94	0.92 –0.95	0.61–0.68	0.78–0.84	0.92	0.92–0.94	0.92–0.94

über die Gleichgewichtskonstante der Reaktion (2) eine Zunahme mit der [HCl]-Konzentration entsprechend Gl. (4) ableiten:

$$\log \alpha = \log K + \log [HCl] + \text{const.} \quad (4)$$

Da der bewegliche Osmiumkomplex $OsCl_3(OH)$ mit zunehmender HCl-Konzentration nach Gl. (5) in den Chlorokomplex des Osmiums überführt wird

$$OsCl_3(OH) + 3HCl = H_2[OsCl_6] + H_2O \quad (5)$$

nähert sich das Laufverhalten der als $2NaOH \cdot OsO_4$ aufgetragenen Verbindung bei hohen HCl-Konzentrationen dem Verhalten des Osmium-Chlorokomplexes. Das mit HCl als Fliessmittel im Bereich von 1–5 *N* HCl im Fall des $2NaOH \cdot OsO_4$-Addukts beobachtete R_F-Maximum (s. Tabelle II) findet somit eine Erklärung.

ZUSAMMENFASSUNG

Das papierchromatographische Verhalten der Platinmetalle wurde bei Anwendung von verschiedenen Mischsystemen, Salzsäure und anorganischen Salzlösungen als Laufmittel untersucht und die bisher bestehende papierchromatographische "Osmium-Lücke" geschlossen. Für die Trennung der Platinmetalle erwies sich eine gesättigte Natriumchloridlösung als besonders geeignet, wobei als Reihenfolge der Trennung gilt: $Ir^{3+} = Rh > Ru > Pd > Pt > Os$.

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Notes

CHROM. 4294

Une méthode ultrasensible de mesure de l'oestriol par chromatographie en phase gazeuse avec capture d'électrons

Formation de 16,17-diheptafluorobutyrate de 3-méthyl-oestriol

La faible quantité d'oestrogènes contenue dans le plasma en dehors de la grossesse humaine rend leur dosage très délicat. Depuis quelques années cependant des méthodes spécifiques et très sensibles ont été publiées pour l'oestrone et l'oestradiol libres plasmatiques, ayant recours, soit à la double dilution isotopique[1,2], soit à la chromatographie en phase gazeuze avec capture d'électrons[3–5], soit à la fixation protéique compétitive[6–8]. Cependant à part quelques résultats publiés par WOTIZ *et al.*[5] aucun auteur n'a à notre connaissance mis au point de technique de sensibilité équivalente pour l'oestriol plasmatique.

Préparation et identification du dérivé

Dans un premier temps, nous avons tenté d'obtenir et d'identifier des cristaux d'un dérivé fluoré du méthyl-oestriol, le 16,17-diheptafluorobutyrate de 3-méthyl-oestriol (oestratriène-3β,16α,17β-triol) ($DHFBMe_3$). Ce dérivé a été préparé selon la méthode de VAN DER MOLEN *et al.*[9]: 100 mg de 3-méthyl-oestriol sont dissous dans 2 ml de tétrahydrofurane auquel on ajoute 0.5 ml d'anhydride heptafluorobutyrique (Fluka) et quelques gouttes de pyridine. Après agitation le tube bouché est gardé 2 h à l'obscurité puis évaporé sous azote à 45°. Le résidu sec après évaporation est dissous dans 3 ml de méthanol aqueux à 70 % et extrait 5 fois par 2 ml d'hexane. Les fractions hexane mélangées sont recristallisées 3 fois dans benzène/hexane. On obtient ainsi des cristaux blancs allongés se groupant en buisson et dont le point de fusion est de 92°.

L'heptafluorobutyrate de testosterone (HFBT) utilisé comme standard interne est préparé de la même manière.

La nature exacte du composé formé a pu être précisée. Les chromatographies sur couche mince de gel de silice dans divers systèmes n'ont montré qu'une seule tache absorbant en UV.

La chromatographie en phase gazeuse avec capture d'électrons donne un seul pic avec les colonnes 1 % XE-60 ou 1 % QF 1 sur Gas Chrom Q. De même un seul pic a été observé en ionisation de flamme après chromatographie sur une colonne de 2 % XE-60 sur chromosorb WAWO-MC.

La spectrographie de masse réalisée à Utrecht grâce au Dr. VAN DER MOLEN a montré que le composé d'un poids moléculaire de 694 possède 2 résidus heptafluorobutyriques. Il correspond donc à la formule $C_{27}H_{24}O_5F_{14}$.

La microanalyse a indiqué une teneur en gramme pour 100 g de produit brut de 46.61 pour C (calculée 46.65), 3.62 pour H (calculée 3.46), 37.37 pour F (calculée 38.30).

Il s'agit donc incontestablement de 16,17-diheptafluorobutyrate de 3-méthyl-oestriol (Fig. I).

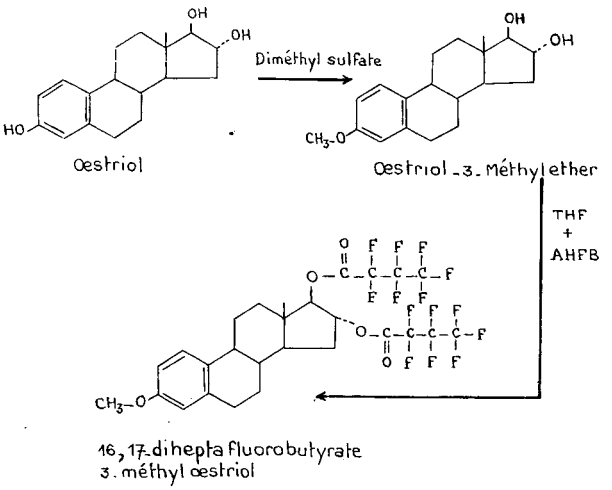

Fig. 1. Formation du 16,17-diheptafluorobutyrate de 3-méthyl-oestriol (oestratriène-3β,16α,17β-triol).

Pour la préparation de microquantités (de l'ordre du ng) de dérivé, la technique est modifiée de la manière suivante: l'extrait sec est repris dans 0.1 ml de tetrahydrofurane + 10 μl d'anhydride heptafluorobutyrique et laissé la nuit à l'obscurité.

On obtient ainsi un dérivé qu'il est possible de mesurer par chromatographie en phase gazeuse avec capture d'électrons de façon très sensible et précise.

Mesure par chromatographie en phase gazeuse

La mesure est effectuée dans un appareil F et M Biomédical 402 équipé d'un

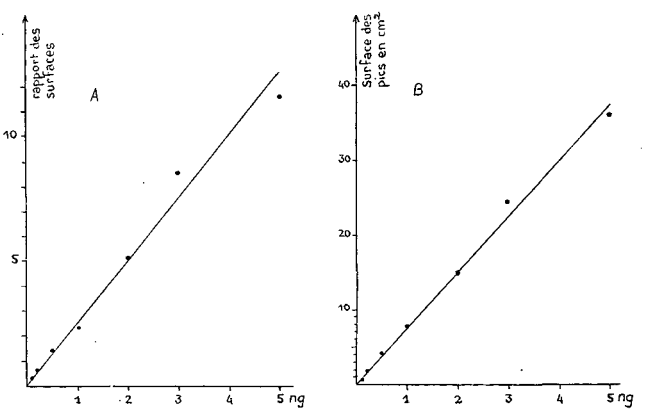

Fig. 2. A, Courbe de calibration du 16,17-diheptafluorobutyrate de 3-méthyl-oestriol indiquant le rapport des surfaces de diverses quantités de ce dérivé (0.1 à 5 ng) et d'une quantité fixe (1 ng) d'heptafluorobutyrate de testosterone. B, Surfaces exprimées en cm^2 des pics obtenus en réponse à l'injection de quantités croissantes de 16,17-diheptafluorobutyrate de 3-méthyl-oestriol. La sensibilité de l'appareil étant de 1/64 correspondant à un courant de $2.56 \cdot 10^{-10}$ V pour une déflection complète de la pointe de l'enregistreur.

détecteur avec source de tritium et contenant une colonne de verre siliconé en U de 60 cm × 4 mm remplie de Gas Chrom Q imprégné de QF 1 à 1 %. La température du four est à 170°, le flash-heater à 210°, le détecteur à 210°. L'intervalle de pulsation est de 150 μsec. Un mélange argon–méthane (95:5) est utilisé comme gaz vecteur avec un débit de 75 ml/min et comme gaz de purge avec un débit de 225 ml/min.

On utilise comme standard interne l'heptafluorobutyrate de testosterone. Le diheptafluorobutyrate de méthyl-oestriol ($DHFBMe_3$) a par rapport à celui-ci un temps de rétention de 0.45 soit 8 et 17 min environ. La courbe de calibration (Fig. 2A) montre que le rapport de surface des pics de $DHFBMe_3$ et de HFBT est égal à 1 quand la quantité de ce dernier dérivé est 3 fois supérieure à celle du précédent. Pour des quantités de 0.1 à 5 ng de $DHFBMe_3$ la réponse du détecteur est linéaire (Fig. 2B). Une quantité aussi faible que 0.1 ng de $DHFBMe_3$ correspondant à $1.8 \cdot 10^{-7}$ mmole d'oestriol libre donne une réponse nettement supérieure aux variations de la ligne de base et très aisément mesurable.

Conclusion

Nous disposons ainsi d'une micro-méthode ultra-sensible et spécifique de mesure de l'oestriol dans les milieux biologiques et plus particulièrement le plasma. Des résultats préliminaires[10] sur l'application de cette méthode aux extraits plasmatiques ont déjà permis de constater que cette technique donnait des résultats positifs et reproductibles au cours de la phase lutéale du cycle menstruel aussi bien que dans le plasma des femmes enceintes.

La spectrographie de masse a été réalisée à Utrecht (Pays Bas) par le Docteur VAN DER MOLEN, la micro-analyse a été effectuée par le service central de micro-analyse du C.N.R.S.

Ce travail a été réalisé avec l'aide du C.N.R.S. (R.C.P. No. 150).

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* Service du Prof. J. VARANGOT.

CHROM. 4309

Gas–liquid chromatographic determination of benzene metabolites

The metabolism of benzene has been extensively studied by various investigators, including WILLIAMS[1] and NAKAJIMA AND TOMIDA[2]. However, determining benzene metabolites spectrophotometrically and radiochemically was so difficult that, after PARKE AND WILLIAMS[3], no one repeated the comprehensive experiment on benzene metabolism. Now, a gas–liquid chromatographic technique is available for determination of these metabolites.

Materials and methods

A male Japanese white rabbit, weighing 3.5 kg, was injected subcutaneously with benzene, 1.0 ml/kg, mixed with an equal volume of sesame oil. Urine was collected every 12 h for 3 days after the benzene injection. Urinary phenols were extracted into diethyl ether and subjected to GLC; if these solutions were dilute it was necessary first to concentrate them by freeze-drying. Conjugated phenols were extracted after hydrolysis in 6 *N* sulfuric acid at 100° for 1 h[4].

In agreement with PORTEUS AND WILLIAMS[5,6], phenol and catechol were found to be excreted early; hydroquinone was excreted later. A description of the other metabolites will be given in another paper.

Benzene and phenols (phenol, catechol, resorcinol, hydroquinone, pyrogallol, hydroxyhydroquinone, phloroglucine) were dissolved in diethyl ether in a concentration of 10 mg/ml; 1 μl of this solution was employed for the determination, using an Hitachi gas–liquid chromatograph, Model K 53. DL-Phenylmercapturic acid and *trans, trans*-muconic acid were also dissolved in hot ethyl alcohol and determined by GLC as well.

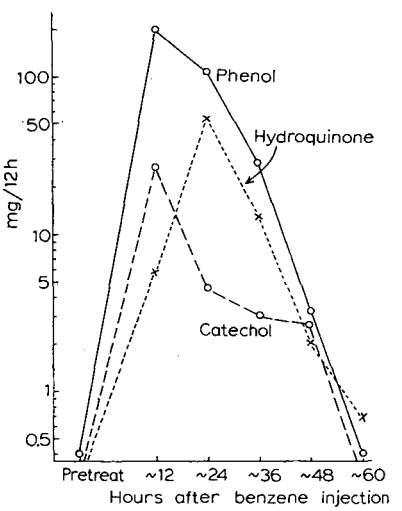

Fig. 1. Urinary excretion of main benzene metabolites in a rabbit after a subcutaneous 1.0 ml/kg benzene administration.

The apparatus consisted of a glass column (3 mm I.D., 2 m long) filled with Chromosorb G AW 80/100 mesh, coated with 3 % Silicone OV-17 and pretreated with dimethyldichlorosilane as support, had a hydrogen-flame ionization detector and used nitrogen, 60 ml/min, as carrier gas. The sample was run on the column treated with hexamethyldisilazane.

TABLE I

Compounds	*Retention time (min)*
Benzene	0.4
Phenol	0.7
Catechol	1.3
Resorcinol	1.9
Hydroquinone	1.9
Pyrogallol	3.0
Hydroxyhydroquinone	4.5
Phloroglucine	7.9
DL-Phenylmercapturic acid	0.7
trans,trans-Muconic acid	1.9

Benzene and phenols, but not resorcinol and hydroquinone, were clearly determined. Because resorcinol and hydroquinone have been difficult to fractionate by GLC[7,8] (see Table I), they should be separated by a chemical procedure[3]. After the initial determination of benzene and phenols in the sample by GLC, saturated lead acetate was added to the solution and the pH was adjusted to 9 with 2 *N* ammonia solution. Hydroquinone, just remaining in solution at pH 9, was determined when the supernatant was first centrifuged and then subjected to GLC. Resorcinol was calculated from the difference between the peaks of total resorcinol–hydroquinone and hydroquinone. Retention times were the same for *trans,trans*-muconic acid and hydroquinone and for DL-phenylmercapturic acid and phenol. Because *trans,trans*-muconic acid and DL-phenylmercapturic acid were barely soluble in diethyl ether, the resorcinol–hydroquinone and phenol peaks were practically uncontaminated. After crystallization, DL-phenylmercapturic acid was determined by GLC and *trans,trans*-muconic acid by spectrophotometry[5].

Discussion

Benzene metabolites have been determined by GLC, but this technique has only been applied to the identification of monophenol in an experiment *in vitro*[9]. Also the determination of phenols, with the exception of triphenol, has been attempted by this technique[7,10,11]. However, all the benzene metabolites are difficult to determine.

Our newly developed technique has enabled us to determine mono-, di- and triphenols with one column without destruction of phenols and to perform the experimental work on benzene metabolites with ease.

We thank Professor S. IMAMURA for financial support, the Central Laboratory of the Chugai Pharmaceutical Co. for generously supplying us with glucuronides

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CHROM. 4300

A protection device for temporary power failure to automatic fraction collectors

One problem encountered in operating a conventional fraction collecting device is caused by a temporary power failure while the instrument is unattended. If a photoelectric or thermistor tripping mechanism is used in the volumetric device of the instrument and if the level of liquid passes this detection device during the power failure the fraction collector will never empty without manual resetting even if the power is restored. Such short term power failures that prevent the volumetric device from operating can be just as disastrous as long term power failure. However, it is possible to provide a safety mechanism for a fraction collector to overcome this problem of short term power interruption by modifying the instrument circuit so that the volumetric unit will automatically empty whenever the power is turned on.

The operation of the volumetric unit of many fraction collectors is such that a photocell or thermistor activates a small relay which in turn activates a mechanical emptying mechanism. If a time-delay relay which is opened by the same power source as the instrument is connected in parallel with this relay, it will trigger the emptying mechanism each time that power is turned on. After the time-delay period for the relay has passed, it will open and the fraction collector will function in the normal manner until the power is again interrupted.

This modification has been successfully employed with the Gilson Medical Electronics Model VL Linear Fraction Collector. The time-delay relay used was a Bliss-Eagle CG10AG DPDT* unit with a 0.06–10 sec time delay. The installation is shown in the simplified schematic (Fig. 1). With the circuit illustrated, the emptying

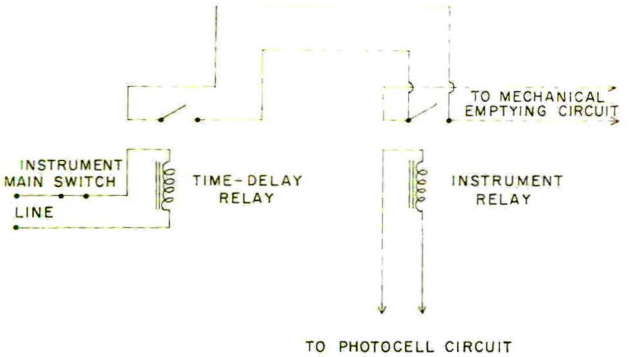

Fig. 1. Schematic diagram showing position of time-delay relay in fraction collector circuit. Shown in power-on position. Time-delay setting of relay 1.0 sec.

mechanism is activated: (i) when the photocell circuit is activated at the moment the correct volume has been collected, or (ii) whenever the power is returned to the main circuit. The operation of the instrument with this adaptation insures that collection after the power returns is started with an empty volumetric device. If power interruptions are extensive then many fractions will be lost. However, this modification will guard against the loss of any more fractions after the power returns.

A similar modification should be possible on most fraction collectors which do not have a similar device already built-in.

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* Available from Electrosonic Corporation, Toronto, Ont., Canada.

CHROM. 4299

Modification of the controls of the Beckman amino acid analyzer*

Two simple modifications to the Beckman 120C amino acid analyzer were of considerable value in this laboratory. The first of these allowed the analyzer to make both buffer changes automatically during analyses of physiological fluids. The second

* From Bureau of Medicine and Surgery, Navy Department, Research Tasks MR005.09.0026 and MR005.20-0288B. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

modification allowed any or all of the buffer pumps to be run independently of the automatic controls of the analyzer.

Analysis of basic amino acids by the MOORE AND STEIN procedure requires a buffer change in order to elute arginine[1]. Although the Beckman Model 120C amino acid analyzer* used in this laboratory is capable of making a buffer change during either the acidic or the basic analysis, it cannot make both buffer changes automatically. Since a large number of physiological samples are assayed in the laboratory, a means was devised which allowed the changes to be made automatically. The cam and pivot arm were removed from the "Motovalve" (Fig. 1). A new pivot arm,

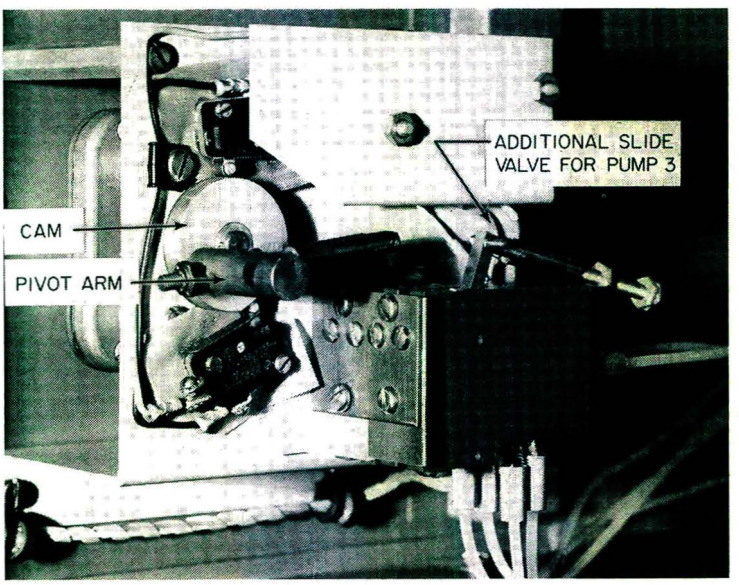

Fig. 1. Photograph of modified "Motovalve" assembly.

machined to the dimension given in Fig. 2, was connected to the cam and the cam and pivot arm assembly were reinstalled. The support bracket was extended with a brass plate to hold a second slide valve for pump 3. Being secured in the slotted position on the pivot arm allows the valve actuator to be disconnected during column 3 analyses, which would otherwise interfere with the regeneration of column 4 (for neutral and acidic amino acids).

The addition of an interval time for the control of pumps 2, 3, and 4 and switches to allow selection of any one or all pumps permits their operation independent of the analyzer controls. This modification is useful for regeneration of column(s) during an analysis with automatic shutdown of the pump; it also allows the pumps to be used for other purposes. The standard controls of the Beckman 120C amino acid analyzer do not permit the automatic control of pumps not used during the analysis.

* Mention of commercial products herein does not constitute endorsement by the Department of the Navy.

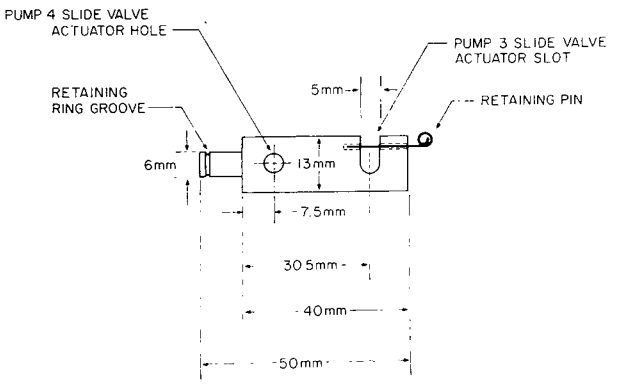

Fig. 2. Pivot arm assembly. The dimensions given are approximate and should be determined for each instrument.

The electrical schematic for the control device is shown in Fig. 3. It is connected to the analyzer circuitry at the points indicated with reference to the manufacturer's instruction manual. Switches SW-2, 3, and 4 select the corresponding pumps whose operation is to be timed by the interval selected on the timer T, with a range up to 3 h.

The modifications, described above, allow complete automatic analyses of both basic and acidic ninhydrin positive compounds and the operation of pumps 2, 3, and 4 independently of the analyzer controls. Both changes should be generally applicable for use with similar instruments.

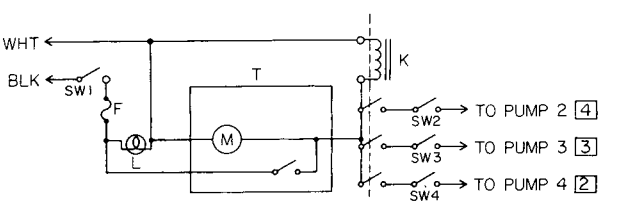

Fig. 3. Electrical scheme for control device. Arrows refer to points where connections are made into the Beckman circuit. Manual A-TB-029, June, 1966. L = indicator light, T = interval timer, 0–3 h (industrial timer Mod. PBM-3H), K = relay, 3 pst, 115 V a.c., F = fuse, 5 A, SW1, SW2, SW3, SW4 = switch SPST toggle.

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CHROM. 4206

Révélation colorée des spots de phénylthiohydantoïne d'acides aminés*

L'analyse de la séquence des acides aminés des chaînes peptidiques selon la méthode d'Edman implique l'identification des dérivés phénylhydantoïne (PTH) acides aminés. Nous proposons, ci-après, une technique nouvelle d'identification de ces dérivés par révélation à la ninhydrine des spots de PTH d'acides aminés obtenus après chromatographie sur couche mince. Cette méthode est applicable sans perturbation du protocole classique d'Edman et peut permettre de réduire à un le nombre de chromatographies nécessaires à chaque stade de la dégradation.

Partie experimentale

Matériel

Composition du réactif: 100 mg de ninhydrine (Merck ou B.D.H.), 5 ml de collidine (Merck), q.s.p. 100 ml éthanol absolu. Couche mince de gel de silice: Eastman type K 301 R. Solutions de phénylthiohydantoïne (PTH) (Mann Research) à 0.005 μmole/μl dans l'acétate d'éthyle pur.

Technique de coloration

On dépose sur la couche mince, activée à 110° pendant 15 min 1 μl de chaque PTH, soit 0.005 μmole. Après séchage à 110° pendant 5 min, on pulvérise la solution de réactif ninhydrine-collidine. Cette pulvérisation doit être très fine mais assez abondante pour imprégner complètement la plaque**. La couche mince est ensuite portée à l'étuve à 110° pendant 15 min. On note alors des colorations qui apparaissent dans un ordre caractéristique (Tableau I).

TABLEAU I

COLORATIONS SPÉCIFIQUES DES PTH-ACIDES AMINÉS

PTH-acides aminés	*Temps d'apparition (min)*	*Colorations*
Sérine	1	Rouge violacé
Glycine	1.5	Orange intense
Alanine	1.5	Rouge violacé
Méthionine sulfone	2	Brun
Cystine	3	Rose intense
Acide cystéique	3	Rose pâle
Asparagine	4	Jaune clair
Glutamine	4	Brun vert
Méthionine	4.5	Brun
Acide glutamique	4.5	Brun foncé (halo bleu)
Histidine	4.5	Jaune faible
Acide aspartique	4.5	Rose
Arginine	4.5	Jaune très faible
Tryptophane	5	Jaune intense
Tyrosine	5	Jaune paille
Thréonine	5	Brun clair
Lysine (ε-)	7	Rose très faible
Proline	8	Brun très faible
Phénylalanine	9	Jaune très faible
Leucine et isoleucine	15	Gris très faible
Valine		Incolore

* Contribution No. 26 du Département et du Groupe de Recherches.

** Des essais de trempage (dipping) ont été réalisés sur bandelettes Eastman. Ils ont conduit au même résultat.

Les spots atteignent leur maximum de coloration au bout de 15 min et la conservent pendant 30 min. Par la suite, les teintes pâlissent surtout à la lumière, et disparaissent au bout de quelques jours. La PTH-valine ne se colore absolument pas; les PTH de la phénylalanine (jaune), de la lysine (rose), de la leucine et de l'isoleucine (gris) sont presque imperceptibles à ce niveau de concentration. La coloration se développe également par application d'un spot de PTH acide aminé sur une plaque déjà traitée au réactif et séchée: la coloration apparaît après un nouveau passage à l'étuve.

Expériences complémentaires

Essais sur la composition du réactif. Ces essais ont été réalisés sur couche mince Eastman de la même manière que précédemment.

Ninhydrine à 0.4% dans l'acétone: les couleurs sont beaucoup plus pâles et se révèlent plus lentement.

Ninhydrine à 0.4% dans l'éthanol 95%: les couleurs sont toujours plus pâles mais leur durée d'apparition est plus réduite.

Ninhydrine à 0.4% dans l'éthanol 95% tamponnée à pH 5.5 par un tampon acétate 1 *M*: la variation de pH ne semble pas influencer le temps d'apparition ni les couleurs des PTH. Les résultats sont assez semblables à ceux obtenus avec une solution de ninhydrine non tamponnée.

La collidine seule ni le chauffage seul ne permettent aucune révélation.

Essais sur la composition du support. Gel de silice: Le gel est appliqué sur des plaques de verre à l'aide de l'étaleur "Camag". L'épaisseur de la couche est de 250 μ.

Gel de silice avec indicateur fluorescent et avec plâtre (Camag).

Gel de silice avec indicateur fluorescent et sans plâtre (Prolabo).

Gel de silice sans indicateur fluorescent, avec plâtre (Merck). Les colorations dans ces trois cas ne se développent que très lentement (1 h à 110° minimum), elles sont beaucoup moins intenses et plus fugaces, et évoluent rapidement, en une journée, vers une teinte uniforme jaune. Pourtant la nature et la composition du support ne semblent pas modifier l'apparition de coloration.

Papier Whatman 3 MM. Ce papier (lavé éventuellement à l'acide acétique) et séché à l'étuve pendant une heure, donne des colorations en 15 min. Les colorations sont intenses mais certaines PTH ne se différencient que très mal. Par exemple Asp, Glu, Asn, Gln, Met, Leu, Ile, Phe, Lys, sont beaucoup plus ternes que sur couches minces.

Sur ces deux types de support les résultats sont donc moins bons qu'en utilisant les couches minces Eastman.

Essais sur d'autres substances que les PTH. Les concentrations et le mode opératoire pour ces substances ont été les mêmes que ceux appliqués aux PTH-acides aminés.

Thiourée: pas de coloration.

Phénylthiourée et diphénylthiourée: pas de coloration.

Thiocyanate de sodium: pas de coloration.

Phénylisothiocyanate: légère coloration rose.

Les expériences suivantes montrent que (i) la coloration n'est pas due à l'acide aminé mais à la PTH (ii) elle se produit avec les PTH d'acides aminés issues de la dégradation aussi bien qu'avec les PTH standard.

Coloration après chromatographie de l'acide aminé libre et de la PTH qui en dérive (Essais avec Ala, Glu, Tyr, Lys)

Sur la même ligne de départ nous déposons 1 μl de PTH à 20 μmoles/ml et 1 μl de l'acide aminé correspondant à une concentration de 20 μmoles/ml. La couche mince est développée ensuite dans le système V de JEPPSSON ET SJÖQUIST[1]. Après révélation à la ninhydrine–collidine nous pouvons constater que l'acide aminé libre n'a pas migré et qu'il s'est coloré en violet alors que la PTH correspondante migre et a pris sa couleur spécifique.

Dégradation d'Edman sur deux peptides

La méthode d'EDMAN *et al.*[2] est appliquée à deux dipeptides de synthèse, glycylproline et glycylalanine (1 mg). Après coupure à l'acide trifluoracétique et évaporation du réactif, on n'extrait pas la thiazolinone mais on provoque sa cyclisation par HCl en présence du résidu restant. L'acide chlorhydrique est évaporé sous azote et le résidu est repris par 20 μl d'acétate d'éthyle. 1 μl est prélevé pour la chromatographie sur plaque Eastman dans le solvant V de JEPPSSON ET SJÖQUIST[1].

Après révélation à la ninhydrine–collidine nous constatons pour le dipeptide glycylalanine: la PTH glycine a migré et s'est colorée en orange alors que l'acide aminé alanine n'a pas migré et s'est coloré en violet; pour le dipeptide glycylproline: la PTH glycine a migré et elle est orange, alors que la proline sur la ligne de départ est colorée en jaune (coloration spécifique de l'acide aminé proline par la ninhydrine).

Application à l'identification des PTH-acides amines

L'utilisation de cette technique, conjointement à la chromatographie sur couche mince, permet une identification des PTH-acides aminés plus rapide et plus aisée dans une majorité des cas. En effet, (a) les acides aminés qui donnent lieu aux teintes les plus intenses et les plus contrastées sont parmi ceux qui se trouvent le plus abondamment dans les protéines; (b) le temps d'apparition d'une couleur contribue à l'identification du spot; (c) lors de la chromatographie des PTH-acides aminés dans les solvants IV et V (Lit. 1) on constate que l'ensemble se répartit en plusieurs groupes de R_F semblable; à l'intérieur de chaque groupe les PTH acides aminés de R_F voisins présentent des teintes différentes. Par exemple dans le solvant V on note à partir du point d'application la répartition suivante:

Groupe I ($R_F \simeq 0.05$) PTH Asn, jaune clair; PTH Gln, brun vert; PTH Ser, rouge violacé; et PTH Met O_2, brun.

Groupe II ($R_F \simeq 0.20$) PTH Glu, brun foncé; PTH Asp, rose; PTH Lys, rose très faible; PTH Tyr, jaune; et PTH Thr, brun.

Groupe III ($R_F \simeq 0.25$) PTH Try, jaune; et PTH Gly, orange intense.

Groupe IV ($R_F \simeq 0.42$) PTh Ala, rouge violacé; PTH Met, brun; et PTH Phe, jaune très faible.

Groupe V ($R_F \simeq 0.58$) PTH Leu, gris très faible; et PTH Ile, gris très faible. La proline et la valine pouvant être identifiées par leur R_F caractéristique.

On voit que dans chaque groupe une teinte identifie une PTH (d'autant mieux qu'un mélange standard est utilisé parallèlement).

La différenciation entre leucine et isoleucine reste cependant impossible par cette méthode. On peut donc adopter le protocole suivant: Après extraction de la PTH dans l'acétate d'éthyle[2], on dépose sur une bandelette Eastman K 301 R 1 μl ou plus de cet extrait (correspondant à 5 nM) et on colore immédiatement. Si aucune

coloration n'apparaît, on continue l'identification de la manière classique (trois chromatographies dans trois mélanges différents). Si une coloration se développe elle permettra de sélectionner un solvant de partage. Après chromatographie en présence d'un mélange standard de PTH, la plaque est séchée puis lue sous UV courts, photographiée, enfin révélée à la ninhydrine. Cette dernière opération, si elle fournit une coloration moins bonne que la première, permet cependant de confirmer l'exactitude du résultat.

L'utilité de ce protocole est confirmée par l'exemple suivant: lors du 3ème cycle de la dégradation de la chaîne α de l'hémoglobine humaine, on a obtenu avant chromatographie un spot rouge violacé très caractéristique de la sérine ou de l'alanine. La chromatographie, en accord avec l'expérience qui montre que la réaction d'EDMAN a un très faible rendement pour la sérine, révèle à l'UV une tache extrêmement faible et qui n'aurait pas permis seule l'identification du PTH-acide aminé. L'ensemble des deux résultats permet un choix en faveur de la PTH-sérine.

Il semble donc que cette méthode de coloration, plus sensible et de mise en oeuvre plus aisée que celle au réactif de Grote[3] puisse fournir une aide précieuse lors de l'étude de la structure primaire d'une protéine, tant par l'apport d'un test complémentaire que par la diminution du nombre des chromatographies nécessaires à l'identification du PTH-acide aminé.

Des tentatives d'extension de la méthode et d'interprétation du phénomène sont en cours.

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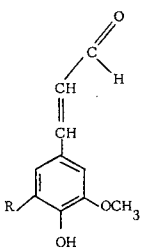
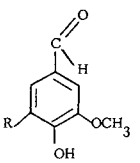
CHROM. 4296

Simple semi-quantitative estimation of sinapyl and certain related aldehydes in wood and in other materials

In the course of an inquiry into the possible aetiological factors of nasopharyngeal tumours among the Chinese and the Kenyans[1], the smoke of Chinese incense and of anti-mosquito coils was tested for known carcinogenic constituents. On thin-layer chromatography a spot was detected which gave a red colour with sulphanilic acid[2]. The compound giving this reaction was identified as sinapyl aldehyde (I)[3]. This is a known constituent of lignins in angiospermous woods[4]. Sinapyl aldehyde was detected in condensates of smoke and in extracts of powdered sandal wood (*Santalum album*) the main constituent of incense, in coconut shell powder (*Cocos nucifera*, the major constituent of anti-mosquito coils) and in a series of hard woods, both in extracts with methanol at room temperature and in condensates obtained by dry distillation[3]. Such preparations contained also coniferyl (II) and syringic (III) aldehydes and vanillin (IV). Furfuraldehyde was obtained on dry distillation, while 3,5-dimethoxy-1,4-benzoquinone was detected in beech extracts.





I R = OCH_3, sinapyl aldehyde III R = OCH_3, syringic aldehyde

II R = H, coniferyl aldehyde IV R = H, vanillin

Distillates from gymnospermous woods contained coniferyl aldehyde, vanillin and furfural, but not I or III.

Hard-wood dust came under suspicion also in connection with tumours of the nasal cavities among furniture workers[5]. Sinapyl aldehyde (or its epoxide) may be of interest in this connection. Glycidaldehyde, the epoxide of acrolein, the simplest α,β-unsaturated aldehyde, has been reported to be carcinogenic for mice and for rats[6,7].

In view of the desirability of screening various materials for the content of I and of the other compounds, the micro-distillation method of STAHL[8] was adapted and proved convenient.

About 10–20 mg of the finely powdered material is placed into the wider part of a Pasteur pipette having in the capillary part a constriction and a small bulb. The tube is sealed at the wide end and heated in a horizontal position in a metal block essentially as described by STAHL. The aldehydes sublime into the capillary part of the tube, protruding from the block; the more volatile constituents being absorbed by the water that distills first and acts as a condensing compartment. The block used

TABLE I

R_F VALUES AND COLOUR REACTIONS OF WOOD ALDEHYDES

TLC on Silica Gel G. Solvent system: *n*-hexane–diethyl ether–dichloromethane–glacial acetic acid (4:3:2:1). Spray reagents: A = sulphanilic acid (1 % in 30 % acetic acid); B = 2,4-dinitrophenylhydrazine (0.5 % in 2 *N* HCl); C = phloroglucinol (2.5 % in 3 *N* HCl).

Compound	R_F	*Reagents*		
		A	*B*	*C*
Sinapyl aldehyde	0.35	Red	Orange	Purple
Syringic aldehyde	0.48	Yellow	Orange	Peach
Coniferyl aldehyde	0.56	Orange	Orange	Purple
Vanillin	0.70	Yellow	Orange	Peach
Furfural	0.80	Red	Orange	Grey

had seven cylindrical depressions and allowed six specimens to be heated at the same time and the temperature read on a thermometer. When wood dust is tested, this becomes somewhat charred; the temperature at which the aldehydes come over is about 300° which is maintained for 1.5 h. The capillaries are then eluted with methanol, and appropriate aliquots applied to thin-layer plates (250 μ thick) prepared from Kieselgel G (Merck & Co.), activated at 105° for 90 min. The plates were run in an acidic solvent and the compounds detected as shown in Table I. By serial dilutions

TABLE II

YIELDS OF ALDEHYDES (μg/g) OBTAINED BY THE METHOD DESCRIBED

	Sinapyl	*Syringic*	*Coniferyl*	*Vanillin*
Eucalyptus sp.	3000	3000	150	100
Fagus Sylvatica L. (Beech)	800	800	250	250
Tectona Grandis (Teak)	700	500	600	450
Santalum Album L.	600	500	300	200
Quercus Robur L. (Oak)	500	600	250	200
Chinese Incense	500	600	250	250
Indian Incense	100	200	100	50
Cocos Nucifera L.	300	500	300	300
Juniperus Procera Hochst	0	0	450	600
Larix Decidua (Larch)	0	0	600	600

and comparison with standard solution the content of the respective aldehydes in various woods and other materials was semi-quantitatively estimated (Table II). The values were substantially higher than in methanolic extracts of these materials.

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Synergistic complexation effects in the thin-layer chromatography of certain triglycerides on silica impregnated with silver nitrate

The co-ordination of olefins with certain metal ions and with silver ions in particular has long been recognised and studied. Using a partition method WINSTEIN AND LUCAS[1] showed that the complexation of silver with olefins and acetylenes was both reversible and rapidly attained. The nature and physical chemistry, including the correlation with bond order, steric hindrance and electron releasing effects have been studied by FUENO *et al*[2], TRAYNHAM AND SCHNERT[3] and by GARDNER *et al.*[4]. NICHOLS[5] first proposed that the distribution between a hydrocarbon solvent and a methanolic silver nitrate solution might be used to separate *cis–trans* isomers. This was later confirmed experimentally for fatty acid esters by SCHOFIELD *et al.*[6]. The order of stability of silver complexes according to KIRCHER[7] is cyclopropenes > *cis*-olefins > alkynes > *trans*-olefins > allenes. This variation in stability constants forms the basis of chromatographic separation of unsaturated lipid materials. The chromatographic separation of lipids using argentation has been reviewed in detail by MORRIS[8] and only the main points will be mentioned herein.

MORRIS[9] and DE VRIES[10] reported simultaneously that adsorbents impregnated with silver nitrate separate lipids according to the degree and geometry of their unsaturation, using thin-layer chromatography and column chromatography. At the same time BARRETT *et al.*[11] reported the separations of glycerides on thin layers impregnated with silver nitrate. Further work has been described by DE VRIES AND JURRIENS[12], GUNSTONE AND PADLEY[13] and by DEN BOER[14]. The order of separation of triglycerides in benzene–ether mixtures was reported by GUNSTONE AND PADLEY[13] as 000, 001, 011 and 002, 111, 012, 112, 022, 003, 122, 013, 222, 113, 023, 123, 223, 033, 133, 233 and 333 where the numbers refer to the numbers of *cis* double bonds in the fatty acid chain. The separations are complicated where there are isomers present, R_F values being a function of the number of double bonds as well as the stereochemistry. DE VRIES[15] has shown that *trans* double bonds give rise to significantly higher R_F values than *cis* in triglycerides, MORRIS[8] has reported on the effect of the position of the double bond in the fatty acid chain and RENKONEN AND RIKKINEN[16] on the effects of the position of the unsaturated fatty acid in the triglyceride. In addition to the molecular solute variables indicated above there are variables due to the chromatographic conditions. DALLAS[17] has discussed the general factors which affect R_F values in adsorption TLC and STEIN AND SLAWSON[18] silver nitrate impregnated chromatography in particular.

JONES[19] has described how certain olefin hydrocarbon such as cyclooctene complex very strongly with silver ions. The present communication considers the synergistic complexation of silver with benzene and cyclooctene and cyclohexene and subsequent reduction of retention of certain triglycerides on silver nitrate impregnated silica thin-layer plates using benzene–cyclic olefin eluants. The effects are related to those described by FEATHERSTONE AND SORRIE[20] on the solubility of straight chain olefins–silver fluoroborate solution in benzene. The effects of increase in chain length in the linear and cyclic olefins are similar.

Experimental

Triglycerides: tristearin, trielaidin, triolein, trilinolein and trilinolenin all being of 95–100% purity. These were made up as 0.5% w/v solutions in chloroform with 0.005% BHT as antioxidant and stored at 4°.

Solvents: cyclooctene (*cis*[21]), cyclohexene and benzene, all of Analytical Reagent grade.

TLC materials: Silica Gel G (Merck); silver nitrate A. R.

Spray reagent: dichlorofluorescein, 0.01% in methanol.

Plates: 10 × 2.5 cm.

Procedure

The thin layers of impregnated Silica Gel G were prepared following the technique of BARRETT *et al.*[11] slurrying the silica gel (30 g) with 60 ml of a 12.5% w/v solution of silver nitrate, giving a layer 300 μ thick with 20% w/w $AgNO_3$. The resulting plates were activated by heating at 110° for 45 min and then stored over silica gel, in the dark, until required. The triglyceride solutions were applied in 1 μl aliquots, which was found to be the optimum loading and the chromatogram developed at 20° in the appropriate solvent mixture. The walls of the tank were lined with filter paper to provide an atmosphere saturated with solvent, to improve reproducibility. The chromatograms were dried at 60°, in a current of air, taking care to remove all the solvent; cyclooctene being particularly difficult to dry, taking about 10 min for complete removal of the solvent. The chromatograms were visualised by spraying with dichlorofluorescein reagent and examination under 365 nm UV light.

The results obtained are reproducible if determinations are carried out using the same batch of plates, there are some batch variations which give rise to differences in R_F values.

Results

All the results given in the tables, if expressed graphically, produce smooth curves.

Discussion

The results reported in Tables I and II show that little or no movement of certain triglycerides takes place in the pure cyclic olefins or in pure benzene, whilst higher R_F values are obtained in mixtures of the two. This indicates that synergistic

TABLE I

R_F VALUES USING THE CYCLOOCTENE–BENZENE SOLVENT SYSTEM AT 20°

Values are the mean of 3 determinations.

Compound	*% benzene*												
	0	*2*	*5*	*10*	*15*	*40*	*50*	*60*	*70*	*80*	*90*	*95*	*100*
Tristearin	0	0.80	0.82	0.87	0.87	0.86	0.90	0.88	0.90	0.79	0.75	0.71	0.64
Trielaidin	0	0.78	0.84	0.94	0.93	0.92	0.87	0.91	0.92	0.75	0.64	0.59	0.31
Triolein	0	0.82	0.85	0.93	0.92	0.90	0.92	0.90	0.91	0.74	0.64	0.57	0.11
Trilinolein	0	0.85	0.81	0.80	0.29	0.04	0.04	0.05	0.05	0.04	0.05	0.07	0
Trilinolenin	0	0.86	0.84	0.81	0.64	0	0	0	0	0	0	0	0

TABLE II

R_F VALUES USING THE CYCLOHEXENE–BENZENE SOLVENT SYSTEM AT 20°

Values are the mean of 3 determinations.

Compound	% *benzene*												
	0	*2*	*5*	*10*	*15*	*40*	*50*	*60*	*70*	*80*	*90*	*95*	*100*
Tristearin	0.12	0.16	0.40	0.59	0.71	0.98	0.98	0.84	0.82	0.79	0.71	0.70	0.64
Trielaidin	0.18	0.29	0.20	0.28	0.44	0.76	0.74	0.64	0.60	0.54	0.50	0.40	0.31
Triolein	0.06	0.23	0.20	0.24	0.32	0.69	0.69	0.64	0.56	0.50	0.36	0.30	0.11
Trilinolein	0.05	0.15	0.21	0.31	0.30	0.33	0.35	0.29	0.09	0.04	0.06	0.06	0
Trilinolenin	0.06	0.12	0.16	0.16	0.18	0.10	0.09	0.07	0.07	0.04	0.05	0.05	0

TABLE III

EFFECT OF THE NUMBER OF DOUBLE BONDS UPON THE R_F VALUES

5 % benzene in cyclohexene, 20°; results obtained on a single plate.

Compound	*Number of double bonds*	R_F *value*
Tristearin	0	0.350
Trielaidin	3 (*trans*)	0.215
Triolein	3 (*cis*)	0.200
Trilinolein	6	0.185
Trilinolenin	9	0.140

TABLE IV

TIME (min) FOR THE SOLVENT MIXTURE TO RUN 8 cm

Mixture	% *benzene*											
	0	*2*	*5*	*10*	*15*	*40*	*50*	*60*	*70*	*80*	*90*	*100*
Cyclooctene	35	34.5	35	34.5	31	21.5	20	16.5	16.0	15	12	10.5
Cyclohexene	19	15	10.5	11	10.5	10.5	10.5	10.5	10.5	11.0	10.0	10.5

complexation of the silver in the thin-layer plates with benzene and cyclic olefin is taking place, thus releasing the triglycerides into the mobile phase. Similar synergistic effects have been reported earlier in a study of the solubility of straight chain olefins in aqueous silver fluoroborate in the presence of aromatic hydrocarbons by FEATHERSTONE AND SORRIE[20]. The synergistic effect was found to be larger the longer the chain length of the olefin. In the present work a similar effect is found with cyclic olefins as R_F values are considerably higher in cyclooctene–benzene mixtures than in cyclohexene–benzene mixtures. A linear relationship was observed between the number of double bonds present in the triglycerides studied and R_F values as illustrated by the results recorded in Table III.

At the loading used very little tailing was observed and satisfactory separations of the triglycerides were obtained using the appropriate mixture of solvents, from the

tables, for the compounds under examination. Whilst the systems employed do not give a separation of the triglycerides as great as that obtained by BARRETT *et al.*[11] using chloroform (99.5) and acetic acid (0.5) it does provide some information on the nature and number of double bonds present in triglycerides.

The effect of temperature between 4 and 20° upon the R_F values was examined and found to be slight, the major effect being an increase of running from 20 to 45 min at the lower temperature. The running time was also dependent on the solvent composition (Table IV); this effect is under further investigation.

The control of R_F values using the synergistic complexation effect would appear to allow the development of novel separations of lipids by argentation chromatography.

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Interference of ethoxyquin in the thin-layer chromatographic estimation of aflatoxin

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) can be used as a chemical preservative (antioxidant) for the purpose of retarding oxidative destruction of carotenes, xanthophylls, and vitamins A and E in certain foods, forage crops, and animal feeds. We have lately been informed that it might give fluorescence on thin-layer chromatograms resembling that of aflatoxin[1]. Since we have been involved in aflatoxin methodology for almost three years this question seemed to be worthy of investigation. In order to find out to what extent the presence of ethoxyquin can confuse thin-layer chromatographic identification of aflatoxin B_1, a number of chromatographic systems developed for aflatoxin determinations were applied.

Experimental

Samples. Four samples of commercial-grade ethoxyquin, received from different sources were examined: EQ I = ethoxyquin, unidentified source; EQ II = ethoxyquin, Koch-Light Laboratories Ltd., Great Britain; EQ III = santoquin, Monsanto Company, U.S.A.; and EQ IV = kurasan, Východočeské chemické závody Synthesia, Czechoslovakia.

A standard of aflatoxin B_1 (3 μg/ml in chloroform) was used for comparison of the R_F values and the intensity of fluorescence of the ethoxyquin spots. The ethoxyquin samples were dissolved in chloroform in concentrations giving an intensity of fluorescence of the same order as the aflatoxin standard.

Chromatography. To compare the R_F values of aflatoxin B_1 and ethoxyquin three kinds of thin-layer chromatoplates were used: 0.4 mm and 0.25 mm thick layers of Silica Gel G (Merck), activated 2 h at 100°, and commercial Silufol 254 UV plates, Kavalier, Czechoslovakia. Aflatoxin B_1 (0.03 μg) and ethoxyquin solutions (10 μl) were spotted separately in alternate order and together in order to observe their resolution. Ten different solvent systems were employed:

S1: 3 % methanol in chloroform[2,10];
S1a: ethyl ether following the S1 system[3];
S2: 5 % methanol in chloroform[4];
S3: 7 % methanol in chloroform[5];
S4: benzene–95 % ethanol–water (46:35:19) two phase[5];
S5: benzene–95 % ethanol–water (46:35:19) upper phase;
S6: 10 % acetone in chloroform, equilibrated[6];
S7: 10 % acetone in chloroform, unequilibrated[7];
S8: 15 % acetone in chloroform[8];
S9: chloroform–acetone–*n*-hexane (85:15:20)[9].

Developed chromatoplates were examined under an analytical UV lamp (366 mμ).

In order to determine whether fluorescence of ethoxyquin origin will also occur when its solution is run through a column such as is used in certain methods for a clean up procedure for aflatoxin extracts[10], columns consisting of silica gel 0.05–0.2 mm (Merck) and anhydrous sodium sulphate were prepared.

The ethoxyquin samples, alone or together with aflatoxin, were put on the columns, and subsequently eluted with 150 ml volumes of petroleum ether, ethyl ether, and 3 % methanol in chloroform. The eluates were evaporated to dryness and dissolved in a small volume of chloroform and spotted on the TLC plates, and examined in UV light.

The following spray reagents were used: 5 % nitric acid in water, and 1 % 2,6-dibromoquinone-4-chloroimide in ethanol.

Spectrophotometry. The Unicam SP 800A Spectrophotometer, with 10-mm quartz cells was used. The UV absorption spectrum for four samples of ethoxyquin (concentration 0.15 mg/ml in 0.5 *N* HCl containing 1 % of ethanol) was drawn up according to CHOY *et al.*[11]. The spectra of the fluorescent fractions recovered from the thin-layer plates, in concentrations corresponding to 0.15 mg/ml of ethoxyquin were also determined. Thin-layer plates were coated with Silica Gel H (Merck), 0.8 mm thick, and dried for 2 h at 100°. Ethoxyquin samples were dissolved in chloroform to a final concentration of 3 mg/ml, and 0.5 ml portions of these solutions were applied in a line across the chromatoplate; the plates were then developed twice, up to a distance of 15 cm from the base line, in 5 % methanol in benzene. The blue fluorescent bands of ethoxyquin impurities were marked under UV light, scraped from the plates, and extracted five times with 1 % ethanol in 0.5 *N* HCl up to 10 ml volume.

Results and discussion

The fluorescence of ethoxyquin solutions is known, and quantitative methods for it based on this property have been published[12,13]. In the literature available, however, no publication was found to describe its fluorescence on thin-layer plates.

Initially it was noted that the chloroform solution of EQ I, in a concentration of 0.2 mg/ml, gave fluorescent spots of the same colour and intensity as 3 μg/ml of aflatoxin B_1. At the start only the 0.4 mm Silica Gel G plates and the S1 system were involved. It soon turned out that in order to obtain similar spots from the remaining ethoxyquin samples, much higher concentrations were required: about 6 mg/ml for EQ II; 3 mg/ml for EQ III, and 1 mg/ml for EQ IV. This observation suggested it was not ethoxyquin itself which was responsible for this fluorescence. In fact, when the plates were sprayed with the 2,6-dibromoquinone-4-chloroimide reagent[14], the spots of ethoxyquin appeared high above the fluorescent spots, having R_F values in different solvent systems varying from 0.75 to 0.95. Yet it was also possible to see the blue fluorescence emitted by true ethoxyquin, but only when the wet plates were inserted under the UV lamp. This was obtained when the plates were developed in the S4 and S5 solvent systems. The fluorescence disappeared swiftly as the plates dried off.

When the chloroform solutions of the four ethoxyquin samples were applied in the above concentrations on plates coated with an 0.25 mm layer of Silica Gel G or on Silufol sheets, the fluorescent spots corresponding to EQ II, EQ III, and EQ IV were not compact, and tailing occurred. The concentrations of ethoxyquin samples finally applied on 0.25 mm thin-layer plates and Silufol sheets were as follows: EQ I, 0.1 mg/ml; EQ II, 0.3 mg/ml; EQ III, 0.3 mg/ml and EQ IV, 0.2 mg/ml. The R_F values in different solvent systems, for aflatoxin B_1 and fluorescing spots derived from ethoxyquin impurities are presented in Table I. In some solvent systems, particularly S8 and S9, the fluorescent spots of ethoxyquin origin tended to divide

into two separate spots, suggesting that it was not just one chemical compound responsible for the fluorescence.

As is shown in Table I chromatograms developed in certain solvent systems exhibit similar R_F values for the blue fluorescent spots of aflatoxin B_1 and impurities of ethoxyquin origin. As a result of this fact there is the possibility of mistake when the sample analysed for aflatoxin contains an additive of commercial ethoxyquin. In order to differentiate the aflatoxin fluorescence, and that from impurities accompanying ethoxyquin, a second development of the thin-layer plates in ethyl ether is to

TABLE I

COMPARISON OF THE R_F VALUES FOR AFLATOXIN B_1 (Afl), AND THE FLUORESCENT SPOTS OF ETHOXYQUIN ORIGIN (EQ), SPOTTED ON DIFFERENT THIN-LAYER PLATES AND DEVELOPED IN A SERIES OF SOLVENT SYSTEMS

Solvent system	*Silica Gel G 0.4 mm*		*Silica Gel G 0.25 mm*		*Silufol sheets*	
	Afl	*EQ*	*Afl*	*EQ*	*Afl*	*EQ*
S1	0.48	0.59	0.52	0.59	0.34	0.22
S1a	0.49	0.77	0.54	0.80	0.36	0.30
S2	0.63	0.73	0.63	0.69	0.53	0.38
S3	0.70	0.77	0.70	0.76	0.58	0.49
S4	0.46	0.67	0.42	0.68	0.30	0.35
S5	0.33	0.57	0.30	0.49	0.29	0.30
S6	0.37	0.46	0.64	0.69	0.29	0.12
S7	0.66	0.74	0.70	0.80	0.45	0.17
S8	0.48	0.52	0.46	0.53	0.32	0.12
S9	0.33	0.48	0.35	0.48	0.20	0.10

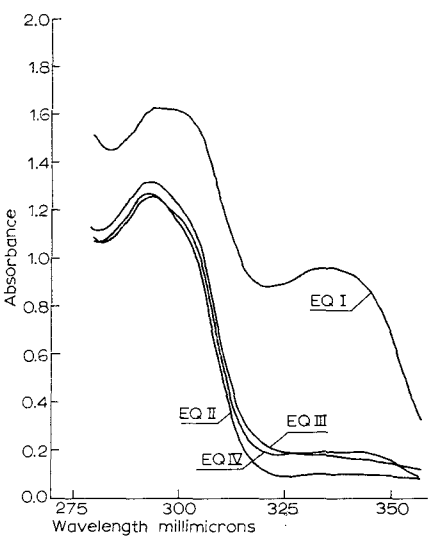

Fig. 1. The UV absorption spectra of the four ethoxyquin samples, concentration 0.15 mg/ml in 0.5 *N* HCl with 1 % ethanol.

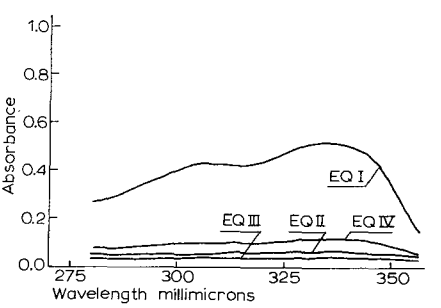

Fig. 2. The UV absorption spectra of the fluorescent fractions recovered from the thin-layer plates, derived from the four ethoxyquin samples at the concentrations equal to 0.15 mg/ml, extracted with 1 % ethanol in 0.5 *N* HCl.

be recommended, as well as spraying the plates with 5 % nitric acid. After the application of this spray reagent, the fluorescence of aflatoxin B_1 changes to yellow, while the blue fluorescence of ethoxyquin impurities remains unchanged.

The behaviour of the blue fluorescence derived from ethoxyquin samples was also examined in the column chromatography process applied for aflatoxin purification. It was found that the fluorescent compounds present in ethoxyquin samples, were not eluted from the silica gel column with petroleum ether, but were quantitatively recovered by means of ethyl ether, and the third eluate methanol–chloroform was entirely free of them. On the other hand, aflatoxin B_1 remained on the column until the 3 % methanol in chloroform was applied, which eluted all the aflatoxin introduced to the column. This means that in case of the presence of ethoxyquin in a sample analysed for aflatoxin contamination, the run through the column for the clean up procedure will exclude the fluorescent compounds accompanying ethoxyquin from the final solution applied to the thin-layer plates.

Spectrophotometric estimations were also performed in order to furnish additional proof of the statement, concluded from the chromatographic examination, that it was not ethoxyquin which gave the permanent, blue fluorescence on the thin-layer plates. The UV absorption spectra for the four ethoxyquin samples, in dilute HCl solutions, show absorption in the range from 280 to 305 mμ as indicated in Fig. 1. The spectra of fluorescent fractions were definitely different in this range as is shown in Fig. 2. The concentration of the fluorescent compounds recovered from the thin-layer plates corresponded to the concentration of ethoxyquin samples introduced in Fig. 1. The blue fluorescent bands moved 5.5 to 7 cm from the base line, the width of them being different for the various samples: 1.5 cm for EQ I; 0.5 cm for EQ II; 0.8 cm for EQ III; and 1 cm for EQ IV.

The spectrophotometric evaluation of both ethoxyquin samples and the fluorescent fractions derived from these samples confirmed the results of the chromatographic findings that it was not ethoxyquin itself which exhibits aflatoxin-like blue fluorescence, but unidentified chemical impurities accompanying this compound.

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A sensitive method of documentation of porphyrin and hemin thin-layer chromatograms

In thin-layer chromatography of porphyrin esters[1-5] there is a real need for a method which permits, by a special but simple technique, the preparation of permanent records of the porphyrins on the chromatogram, which are excited to red fluorescence by long-wave UV light. The documentation should also be of high sensitivity and permit the simultaneous recording of the porphyrins and hemin as a complementary procedure to the simultaneous separation of these cyclic tetrapyrroles as methyl esters on silica gel thin-layer plates[3,4]. By using a camera with a normal lens plus specific filters and powerful UV lamps arranged in a special apparatus we obtained good reproducible photographs in black and white as well as slides in color. Light values for different films are presented. The method can be conveniently used to estimate the composition of a porphyrin mixture separated on a thin-layer plate or card, independent of viewing it directly under UV radiation.

Experimental

Thin-layer chromatography of the porphyrins, as their methyl esters, was carried out on silica gel plates (as described for Silica Gel H[1]) and several other precoated plates or cards[2], *e.g.* Silica Gel F_{254} plates (Merck, Darmstadt, Germany), Silica Gel plates DSF-A (Camag, Muttenz, Switzerland), and silica gel aluminum cards (Riedel-de Häen, Seelze-Hannover, Germany). The standard solvent system in analytical work for the separation of porphyrin esters according to their number of carboxylic acid methyl ester groups consists of benzene–ethyl acetate–methanol (85:13.5:1.5)[3]. Before separating the porphyrin esters in this solvent system a short run once or twice in a chloroform–methanol (130:20) solvent mixture is recommended for the formation of a narrow starting line[4]. The details of the chromatographic methods have been described earlier[1-4] and are presented fully elsewhere[5].

The porphyrins used were isolated from cell suspensions of *Achromobacter metalcaligenes*[6] on Silica Gel F_{254} plates and identified by their absorption spectra in chloroform[2,5]. Protohemin, obtained from Fluka (Buchs, Switzerland), was esterified, then purified by TLC[1] and analyzed by spectrophotometry[3].

The setup for photographing the porphyrins on silica gel thin-layer chromatograms is sketched in Fig. 1. By means of the data given in the legend to Fig. 1 the setup is easy to construct. Primarily it consists of two Desaga UV lamps 13 1000 (366 nm) (Desaga, Heidelberg, Germany), a camera and lens plus specific filters, a camera bracket and a base plate. The cameras and objectives used in this study are: an Exacta Varex IIa® (Ihagee Kamerawerk, Dresden, Germany) with a Jena T lens (1:2.8; f = 50 mm); and a Zeiss Ikon Contaflex (Zeiss Ikon, Stuttgart, Germany) with a Zeiss Tessar lens (1:2.8, f = 50 mm) and an additional A28 lens (f = 1 m). The room must be completely darkened for the photographic recording.

The filters found empirically suitable both for black and white and color films were studied for their efficiency for the photographic documentation of porphyrins by spectrophotometry as shown in Fig. 2. The following B + W filters (Johannes Weber KG, Wiesbaden, Germany) are employed for the photographic recording of the

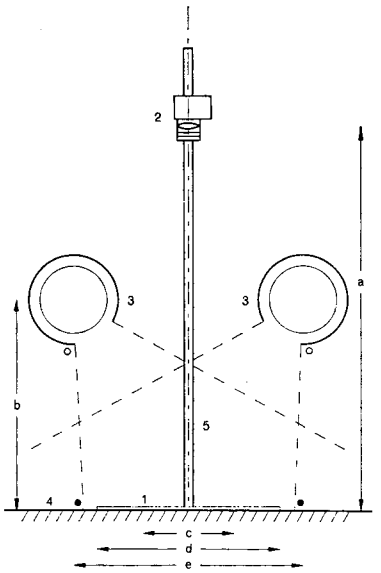

Fig. 1. Front view of the setup for fluorescence photography of porphyrins on silica gel thin-layer chromatograms (1). The other parts are: A single lens mirror reflex camera with a normal photographic objective (2), the filters are given in Table I; two Desaga UV lamps 131000 (366 nm) (3); a mat-black base plate (4); and a stand for vertical adjustment of the camera holder. The distance from the lens to the chromatogram is 55 cm (a) for plates 20·20 cm (d), and 40 cm for plates (cards) 10·20 cm (c). This variation in the distance has no influence on the light values given in Table I. The distance of the UV lamps (3) from the base plate is 23 cm (b), and from the optical axis 12.5 cm (e/2). The UV lamps are placed in such a way that the lower edge of each lamp (○) is projected on the black base plate 1 cm beyond the edge of a silica gel plate 20·20 cm (●). For photographic data (filter pack, light values and exposure times) see Table I.

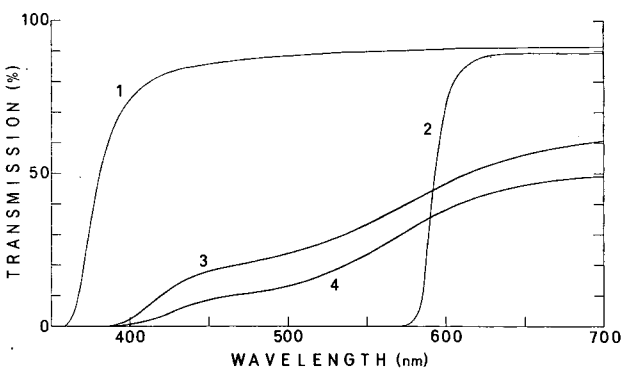

Fig. 2. Optical density in the visible range of the filters used for porphyrin photography in silica gel thin-layers. The filters were measured in a double beam Hitachi–Perkin Elmer EPS-3T spectrophotometer. 1 = 010; 2 = 090; 3 = 010 + KR 12; 4 = 010 + KR 12 + KR 6. For the origin and description of the filters see text.

fluorescence of porphyrins on thin-layer chromatograms: 010 = UV-Haze; 090 = light red; KR 12 and KR 6 for color correction against bluish tinge.

The photographic materials used in this laboratory are as follows:

Black and white negative: Ilford FP4 (ASA 125, DIN 22), developed in Ciba-Ilford Microphen; Agfa Isopan ISS21 (ASA 100).

Black and white reversal: Agfa Dia-Direct (ASA 32, DIN 16).

Color negative: Agfa CN S (ASA 80, DIN 20).

Color reversal: Agfacolor CT 18 (ASA 50), Kodachrome II (ASA 25, DIN 15), Kodachrome X (ASA 64, DIN 19).

The photographic detection of the red-fluorescent porphyrins on thin-layer chromatograms is achieved by using the arrangement shown in Fig. 1 and the conditions described in its legend in conjunction with the filters and light values listed in Table I.

TABLE I

LIGHT VALUES AND FILTERS FOR PHOTOGRAPHY OF PORPHYRIN–SILICA GEL THIN-LAYER CHROMATOGRAMS IN COMBINATION WITH THE SETUP SHOWN IN FIG. 1

Film	*Light value at lens aperture f/5.6 Exposure index 100 ASA (21 DIN)*	*Exposure time (min)*	*Filters*
Black and white negative	− 1	1	
	− 5[a]	16	090
Black and white reversal	− 3.5	6	090
Color negative	− 2	2	010 + KR 12 + KR 6
Color reversal	− 2	2	010 + KR 12
	− 2.5	3	010 + KR 12 + KR 6

[a] This value has to be used for the simultaneous recording of hemin.

Results and discussion

A method is described for the photographic recording of porphyrins on chromatograms under long-wave UV light. The data given in Table I were obtained using the following silica gel preparations: Silica Gel H, pre-coated F_{254}, and DSF-A plates and DC aluminum cards SI (see *Experimental*). The light values in Table I, ascertained with new UV lamps, have a range of ± 0.5 and will be reduced by using older radiation sources. The data depend on the properties of the UV lamps and are only valid for the radiation of the Desaga 13 1000 lamp.

For black and white photographs, the light red filter (090) is the most favorable, as it has a transmission of 89% in the region of the emission maxima[7] of the porphyrin methyl esters between 625 and 635 nm (Fig. 2). Since no transmission is observed with the 090 filter for light of wavelengths below 600 nm, as shown by spectrophotometry (Fig. 2), the additional use of the UV filter is not necessary. Other filters which are useful for recording the fluorescence of porphyrin thin-layer chromatograms are the dark yellow, yellow-orange, orange-red, and dark red B + W filters. However, the dark red filter 091[8] does not show an equal sensitivity for the fluor-

escent light emitted by the different porphyrin esters; the transmission at 620 nm is 11%, and 55% at 635 nm.

For color films, the filter packs given in Table I are especially suitable for the original true recording of fluorescent porphyrins. With the filter combination, 010 + KR 12 + KR 6 (resp. 010 + KR 12), light of wavelengths below 600 nm was absorbed preponderately allowing a high sensitivity in the detection of the porphyrins, and resulting in dark red spots with a blue background on the slides in spite of long exposure times (Table I). The color reproduction of the Kodachrome II slides is more natural if correction filters KR 12 + KR 6 are used. However, for other color reversal films, such as Agfacolor CT 18, the KR 12 filter will do.

Use of the red 090 filter for black and white exposures results in the sensitivity of the recording exceeding that when the chromatogram is viewed by the human eye in the detection of very low concentrations of porphyrin methyl esters on silica gel layers under the powerful UV lamps arranged as in Fig. 1. The minimum amount of porphyrin ester recorded by photography is 0.001 μg. Fig. 3, which shows a chromatogram with increasing amounts of coproporphyrin, indicates that the intensity of fluorescence is dependent on the topical distribution of the substance in the thin-layer. The photogram represents a summation reproduced from three films at different chromatographic times: (a) after application; (b) after formation of a narrow starting line; and (c) after running. The spots exhibit the greatest intensity of fluorescence in position b.

Photography of porphyrin thin-layer chromatogram without filters[1] required a

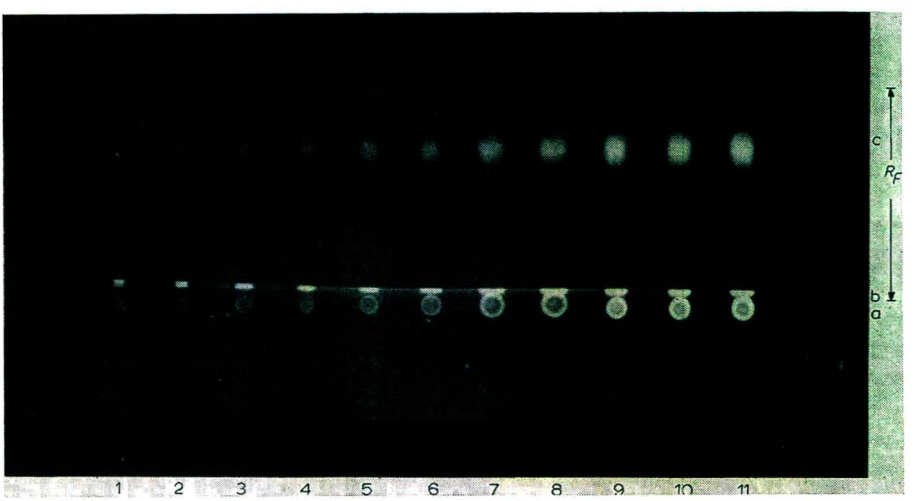

Fig. 3. Summation photogram of increasing amounts of coproporphyrin III tetramethyl ester on a Silica Gel F_{254} plate, which was photographed (film Ilford FP4, aperture $f/5.6$, shutter speed 40 sec) in three chromatographic situations: (a) after application of the coproporphyrin ester in chloroform solution by self-filling micro-pipettes; (b) after a two-fold short development of the plate in chloroform–methanol (130:20) for the formation of a narrow starting line; (c) after running in a fresh solvent mixture of benzene–ethyl acetate–methanol (85:13.5:1.5). For the production of this photograph, the films from situations a, b and c were pasted upon one another. The amounts in μg of coproporphyrin applied are: 1 = 0.003; 2 = 0.007; 3 = 0.014; 4 = 0.022; 5 = 0.029; 6 = 0.043; 7 = 0.072; 8 = 0.100; 9 = 0.207; 10 = 0.311; 11 = 0.414.

relatively high concentration of about 0.2 $\mu g/cm^2$, and resulted in a record in which the fluorescent spots show black against the white background of the plate[1,9], analogous to the record in Fig. 4c.

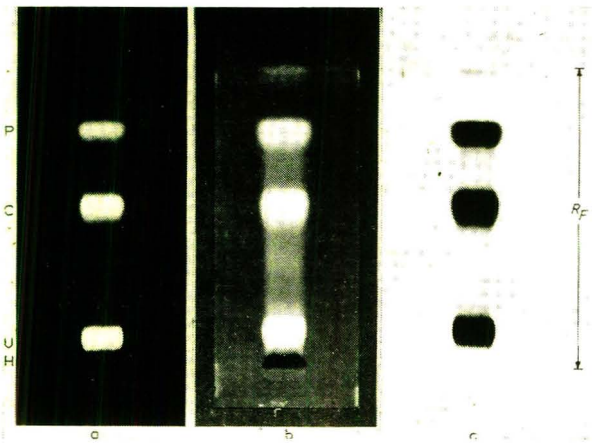

Fig. 4. Silica Gel F_{254} thin-layer chromatogram with proto-, copro-, uro-porphyrin and hemin methyl ester (P, C, U and H), developed in the solvent systems described in the legend of Fig. 3, but in a saturated atmosphere. The chromatogram was photographed with a black and white negative film for the documentation of porphyrins only (a) and for the additional detection of hemin (b). Recording of porphyrins as dark spots on white background was achieved with a black and white reversal film. Using filter 090, the film, lens aperture and exposure time are: a (Ilford FP_4, $f/5.6$, 30 sec), b (Ilford FP_4, $f/4$, 3 min), c (Agfa Dia-Direct, $f/4$, 9 min). Color photography of the same chromatogram with Agfacolor CT 18 was carried out under the following conditions: filter pack 010 + KR 12, aperture $f/5.6$, and exposure time 2 min.

Fig. 4 shows three different photographs of the same porphyrin and hemin chromatogram. The actual photographic conditions are given in the legend. The reproduction under the conditions of Fig. 4b permits the simultaneous record of hemin, which can be seen well in daylight as a brownish-black spot on the plate. Fig. 5 is the reproduction of a chromatogram of porphyrins from a bacterial culture (*Achromobacter metalcaligenes*), recorded using both a black and white negative (A) and a reversal film (B). Differences in the concentrations of the porphyrins separated on the chromatogram of about 1:400 (Fig. 5) could be detected by taking photographs under UV radiation with the technique reported here. The growth and incubation conditions of the bacterial system and the preparation of porphyrin methyl esters from it have been described in previous paper[6].

When photographing a series of porphyrin chromatograms we prefer records according to the Figs. 3, 4a and 5A, which give a natural impression of the fluorescence as well as possible in black and white. As shown in Figs. 3 and 5 the photographs allow an approximate estimation of the relative concentrations present in the porphyrin zones on the chromatograms.

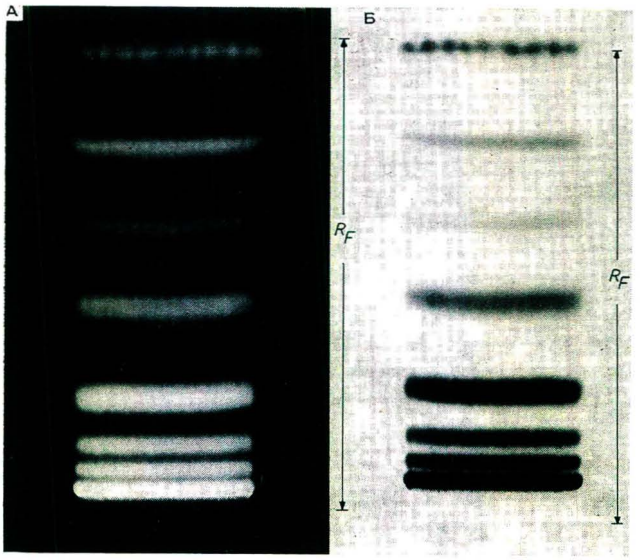

Fig. 5. Thin-layer chromatogram of porphyrin methyl esters (P-ME) prepared from cultures of *Achromobacter metalcaligenes* grown in a special medium with added δ-aminolevulinic acid[6]. According to Figs. 4a and c the photographic data were as follows: A, Ilford FP_4, *f*/5.6, 30 sec; B, Agfa Dia-Direct, *f*/4, 6 min. The porphyrin stripes from the start up to the front are: octa-, hepta-, hexa-, penta-, tetra-, tri- and dicarboxylic(proto) P-ME. The spots at the solvent front are lipids[2,4]. Results from a spectrophotometric analysis showed the difference in the concentration between tricarboxylic and uro P-ME to be about 1:400.

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Separation of fructosans by thin-layer chromatography

During a study of the metabolism of fructosans in explants of Jerusalem artichoke tubers[1] a rapid chromatographic system for the separation of the lower members of the fructosan series was needed. Although systems have been worked out for the separation of fructosans either by paper chromatography[1,2] or by gel-filtration[3], these methods are too time-consuming for the simultaneous analysis of numerous samples. A thin-layer chromatographic one-dimensional separation technique on cellulose plates was therefore developed, which allows the separation within 8 h of fructose, sucrose and the first seven homologues of the fructosan series.

Thin-layer plates were prepared as follows: 22 g of cellulose (MN 300, Macherey, Nagel & Co., G.F.R.) were mixed in a Waring-blendor with 145 ml of 33 m*M* K_2HPO_4 solution, and the suspension spread on glass plates (5 × 20 cm) using a Desaga spreader (Desaga, Heidelberg) set at a thickness of 0.4 mm. After drying for 24 h at room temperature, 15 μl of a fructosan mixture (100 mg/ml), obtained from Dahlia tubers by water extraction, were applied to the start line by means of a motor-driven Hamilton syringe. After drying with warm air, the plates were chromatographed in one dimension using either of the following solvents:

Solvent A: *n*-propanol–ethyl acetate–water (75:10:15);

Solvent B: *n*-propanol–ethyl acetate–water (60:10:30).

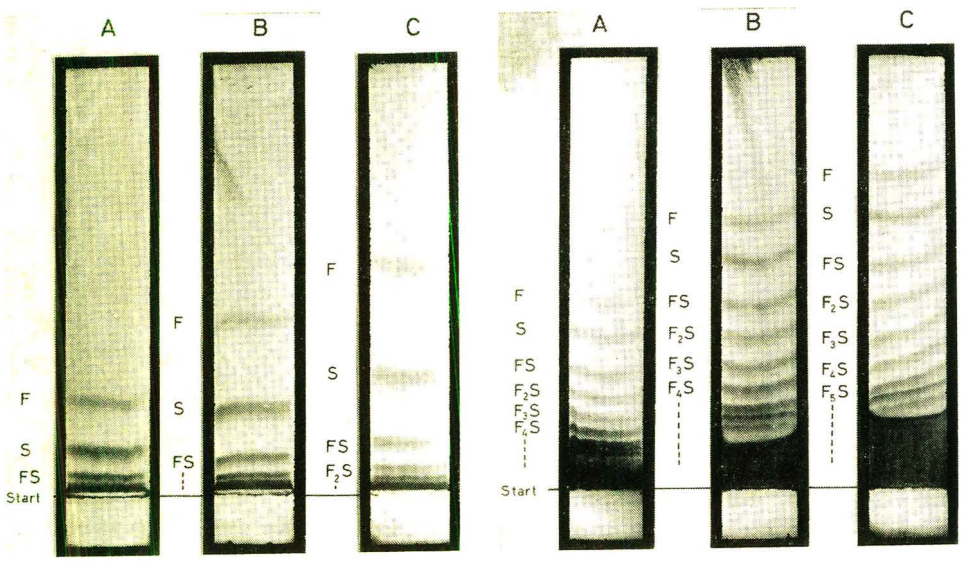

Fig. 1. Separations of fructosan mixtures by thin-layer chromatography in solvent A. The plates were stained with naphthoresorcinol[4]. (A) after one development; (B) after two developments; (C) after three developments.

Fig. 2. Separations of fructosan mixtures by thin-layer chromatography in solvent B. The plates were stained with naphthoresorcinol[4]. (A) after one development; (B) after two developments; (C) after three developments.

The chromatograms were routinely developed twice in the same solvent up to 16 cm above the start line. The plates were dried with warm air before the second development. Each run lasted about 3½ h. For documentation the plates were stained with naphthoresorcinol[4] and photographed on orthochromatic films.

Figs. 1 and 2 show the separations obtained using solvents A and B, respectively; in addition they illustrate the effect of multiple developments in one dimension using a single solvent. As can be seen solvent A is the most efficient for the separation of fructose, glucose, sucrose and FS*, while solvent B is more convenient for separation of the S, FS, F_2S . . . F_7S series.

As already mentioned the plates were coated with suspensions of cellulose in 33 mM K_2HPO_4. Cellulose plates prepared without phosphate gave a similar separation pattern, but severe tailing was usually observed. An evaluation of the effect of varying the phosphate concentration revealed that minimal tailing was obtained when the phosphate concentration was kept between 25 and 60 mM. Higher phosphate concentrations resulted in decreased R_F values.

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* Abbreviations used: F = fructose; S = sucrose; FS = 1-fructosylsucrose; F_2S = (1-fructosyl)$_2$sucrose; F_nS = (1-fructosyl)$_n$sucrose.

CHROM. 4292

Dünnschichtchromatographisch-enzymatischer Nachweis phosphororganischer Insektizide

Zum dünnschichtchromatographischen Verhalten einiger weiterer Insektizide

In einer früheren Arbeit[1] ist über eine empfindliche dünnschichtchromatographisch-enzymatische Methode zum Nachweis insektizider Organophosphate sowie über die Möglichkeiten zur Steigerung der Nachweisempfindlichkeit schwacher bzw. indirekt hemmender Cholinesteraseinhibitoren durch "Aktivierung" berichtet worden. Das insbesondere für Rückstandsuntersuchungen gut geeignete Verfahren kann unter

geringfügiger Variation der Arbeitsbedingungen prinzipiell auf alle cholinesterase-hemmenden Verbindungen angewendet werden. Über das Verhalten einer Reihe phosphororganischer Insektizide gegenüber verschiedenen Laufmitteln und verschiedenen Aktivierungsverfahren wird in der vorliegenden Arbeit berichtet.

Material und Methodik

Pestizide. An Pestiziden standen die in Tabelle I aufgeführten Thiono- bzw. Dithiophosphorsäureester, an direkt hemmenden Phosphorsäureestern "Phosdrin" und "Metasystox R" in unterschiedlichen Reinheitsgraden (22.2–99 %) als analytische Standards der Firma PolyScience Corporation, Evanston, Ill., sowie der Phosphonsäureester "Butonate" zur Verfügung. Von den Präparaten wurden Stammlösungen mit 1 mg Pestizid/ml Chloroform hergestellt. Die Einstellungen der Endkonzentrationen erfolgten durch entsprechendes Verdünnen mit Chloroform.

TABELLE I

TRIVIALNAME UND CHEMISCHE BEZEICHNUNG DER UNTERSUCHTEN ESTERASEINHIBITOREN

Trivialname	*Chemische Bezeichnung*
Butonate	O,O-Dimethyl-(1-*n*-butyryloxy-2,2,2-trichloräthyl)-phosphonat
Co-Ral	O,O-Diäthyl-O-(3-chlor-4-methyl-cumarin-7-yl)-thionophosphat
Coroxon	O,O-Diäthyl-O-(3-chlor-4-methyl-cumarin-7-yl)-phosphat
Disyston	O,O-Diäthyl-S-2-(äthylmercapto)-äthyldithiophosphat
Ethion	O,O,O′,O′-Tetraäthyl-S,S′-methylen-bis-dithiophosphat
PO-Ethion	O,O,O′,O′-Tetraäthyl-S,S′-methylen-bis-thiophosphat
Guthion	O,O-Dimethyl-S-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl-methyl)-dithiolphosphat
Gutoxon	O,O-Dimethyl-S-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl-methyl)-thiolphosphat
Malathion	O,O-Dimethyl-S-(1,2-dicarboxyäthyl)-dithiophosphat
Malaoxon	O,O-Dimethyl-S-(1,2-dicarboxyäthyl)-thiolphosphat
Metasystox R	O,O-Dimethyl-S-2-(äthylsulfinyl)-äthylthiolphosphat
Methyltrithion	O,O-Dimethyl-S-(*p*-chlorphenyl-thiomethyl)-dithiophosphat
PO-Methyltrithion	O,O-Dimethyl-S-(*p*-chlorphenyl-thiomethyl)-thiolphosphat
Phorate	O,O-Diäthyl-S-(äthylmercaptomethyl)-dithiophosphat
PO-Phoratesulfon	O,O-Diäthyl-S-(äthylsulfonylmethyl)-thiolphosphat
Phosdrin	O,O-Dimethyl-O-(1-carbomethoxy-1-propen-2-yl)-phosphat
Ronnel	O,O-Dimethyl-O-(2,4,5-trichlorphenyl)-thionophosphat
Ronnoxon	O,O-Dimethyl-O-(2,4,5-trichlorphenyl)-phosphat

Von den Thiono- und Dithiophosphorsäureestern wurden die PO-Verbindungen durch Oxydation mit Brom nach folgender Vorschrift dargestellt: 1 mmol der Thionoverbindung wird in 5 ml Äthanol gelöst. Unter Schütteln gibt man tropfenweise gesättigte wässrige Bromlösung bis zum Auftreten einer bleibenden schwachen Gelbfärbung zu. Man lässt die Lösung noch *ca.* 30 min bei Zimmertemperatur stehen und beseitigt danach den Bromüberschuss durch tropfenweise Zugabe von 0.1 *M* Natriumthiosulfatlösung bis zur Entfärbung. Die PO-Verbindungen werden durch drei- bis fünfmaliges Ausschütteln mit je 50 ml Chloroform extrahiert. Der Chloroformextrakt wird mit entwässertem Natriumsulfat getrocknet und das Lösungsmittel im Vakuum abdestilliert. Stammlösungen (1 mg Pestizid/ml) und Endkonzentrationen werden durch Verdünnen mit Chloroform hergestellt.

Die Durchführung der chromatographischen Trennung, der Aktivierung sowie des enzymatischen Hemmtestes erfolgte in der früher beschriebenen Weise[1].

Ergebnisse und Diskussion

Chromatographie. Die geringe Polarität einiger Thiono- und Dithiophosphorsäureester macht es erforderlich, zu den bewährten Fliessmittelkombinationen aus Benzol und Aceton noch *n*-Hexan als unpolare Komponente hinzuzuziehen. Die ermittelten R_F-Richtwerte sind in Tabelle II zusammengefasst.

TABELLE II

R_F-WERTE VON ACHTZEHN PHOSPHORORGANISCHEN WIRKSTOFFEN AUF KIESELGEL G IN VERSCHIEDENEN LAUFMITTELSYSTEMEN

Wirkstoff	*Laufmittel*						
	Benzol	*n-Hexan–Benzol (2:1)*	*n-Hexan–Benzol–Aceton*		*Benzol–Aceton*		
			15:10:1	*10:10:1*	*19:1*	*9:1*	*4:1*
Butonate	0.08	0.00	—	—	0.29[a]	0.40	0.61
Co-Ral	0.15	0.00	0.33[a]	0.40[a]	0.70	0.81	0.95
Coroxon	0.00	0.00	0.00	0.00	0.05	0.13	0.38[a]
Disyston	0.64[a]	0.00	0.72	0.77	0.95	1.00	1.00
Ethion	0.69[a]	0.06	0.88	0.91	0.97	1.00	1.00
PO-Ethion	0.00	0.00	0.00	0.00	0.20	0.27[a]	0.52[a]
Guthion	0.09	0.00	0.07	0.25[a]	0.40[a]	0.66	0.88
Gutoxon	0.00	0.00	0.00	0.00	0.14	0.20[a]	0.41[a]
Malathion	0.00	0.00	0.12	0.36[a]	0.68[a]	0.82	0.95
Malaoxon	0.00	0.00	0.00	0.00	0.23[a]	0.30[a]	0.53
Metasystox R	0.00	0.00	0.00	0.05	0.20	0.33[a]	0.60
Methyltrithion	0.80	0.31[a]	0.74	0.80	0.91	1.00	1.00
PO-Methyltrithion	0.00	0.00	0.00	0.00	0.05	0.11	0.32[a]
Phorate	0.71	0.00	0.61[a]	0.70	0.95	1.00	1.00
PO-Phoratesulfon	0.00	0.00	0.00	0.00	—	—	—
Phosdrin	0.00	0.00	0.00	0.00	0.05	0.34[a]	0.53[a]
Ronnel	0.95	0.43[a]	0.84	0.90	1.00	1.00	1.00
Ronnoxon	0.00	0.18[a]	0.07	0.18	0.43[a]	0.52[a]	0.74

[a] Zum Nachweis besonders geeignete Laufmittelsysteme.

Durch die Kombination *n*-Hexan–Benzol (2:1) ist es u.a. auch möglich, die stark unpolaren Verbindungen wie "Ronnel" und "Methyltrithion" in R_F-Bereiche zu bringen (0.43 bzw. 0.31), die für die semiquantitative Bestimmung günstig sind.

Aktivierung von Thionophosphorsäureestern mit Bromdampf. Wie bereits in den früheren Untersuchungen[1] festgestellt wurde, erweist sich die Oxydation der Thionophosphorsäureester mit Bromdampf auch in dieser Untersuchungsreihe in den meisten Fällen als nicht optimal (Tabelle III). Auch ein Teil der direkthemmenden PO-Verbindungen wird durch die Behandlung mit Bromdampf in seiner Nachweisempfindlichkeit mehr oder weniger beeinträchtigt. Phosdrin lässt sich nach Brombehandlung wie das DDVP und das Dibrom auch in Mengen von > 1 μg nicht mehr nachweisen. Von diesen beiden Esteraseinhibitoren unterscheidet sich das Phosdrin dahingehend, dass es auch nach Aktivierung mit wässriger Bromlösung nicht mehr

TABELLE III

NACHWEISGRENZEN FÜR PHOSPHORORGANISCHE WIRKSTOFFE OHNE UND MIT AKTIVIERUNG (ng)

Wirkstoff	*Ohne Aktivierung*	*Aktivierung mit*			
		Bromdampf 30 sec	*Bromwasser 15 min*	*UV-Bestrahlung 20 min*	*Ammoniaklösung 15 min*
Butonate	k.N.[a]	k.N.	k.N.	k.N.	5
Co-Ral	k.N.	5	1	1	k.N.
Coroxon	0.05	0.5	0.05	0.05	0.1
Disyston	k.N.	7	5	50	k.N.
Ethion	k.N.	0.5	0.5	0.1	k.N.
PO-Ethion	0.1	0.2	0.2	0.1	0.1
Guthion	k.N.	0.2	0.05	1.0	k.N.
Gutoxon	0.02	0.2	0.02	0.02	0.02
Malathion	k.N.	0.5	0.1	10	k.N.
Malaoxon	0.05	0.5	0.05	0.05	0.05
Metasystox R	10	10	10	10	10
Methyltrithion	k.N.	0.5	0.05	1	k.N.
PO-Methyltrithion	0.5	2	1	0.5	1
Phorate	k.N.	5	5	50	k.N.
PO-Phoratesulfon	1	5	2	1	2
Phosdrin	0.1	k.N.	k.N.	1	1
Ronnel	k.N.	1	0.05	5	k.N.
Ronnoxon	0.05	0.5	0.05	0.05	0.1

[a] k.N. = kein Nachweis

erfasst werden kann. Dieses Verhalten kann neben den R_F-Werten als typisches Merkmal zur Identifizierung des Phosdrins herangezogen werden.

Aktivierung von Thionophosphorsäureestern mit wässriger Bromlösung. Die Aktivierung der Thionophosphorsäureester mit wässriger Bromlösung erweist sich auch in dieser Untersuchungsreihe als das günstigere Verfahren. Alle untersuchten Verbindungen lassen sich nach Aktivierung mit Bromwasser im Pikogramm- bzw. im unteren Nanogrammbereich nachweisen. Direkthemmende Verbindungen werden, bis auf Phosdrin, in ihrer Nachweisempfindlichkeit nicht beeinflusst.

Aktivierung von Thionophosphorsäureestern durch UV-Bestrahlung. Durch UV-Bestrahlung wird bei dem Thionophosphorsäureester "Co-Ral" und dem Dithiophosphat "Ethion" eine gute Aktivierung erzielt. Als weniger geeignet erweist sich die Aktivierung durch UV-Bestrahlung bei Bromophos, Disyston, Guthion, Malathion, Methyltrithion, Phorate und Ronnel (Tabelle III). Aus vergleichenden Untersuchungen, bei denen die entwickelten Chromatogramme vor Durchführung des enzymatischen Testes einmal mit Bromwasser allein und einmal mit UV-Licht bestrahlt und anschliessend mit Bromwasser behandelt worden sind, geht hervor, dass die Dithiophosphorsäureester Guthion, Malathion und Methyltrition offenbar durch UV-Licht in grösserem Umfange zerstört werden. Nach UV-Bestrahlung werden bei gleichen Konzentrationen und gleichen äusseren Bedingungen kleinere und schwächer ausgebildete Hemmflecken erhalten (Tabelle IV).

Aktivierung von Butonate mit Ammoniak. "Butonate", der Buttersäureester des Phosphonats 'Trichlorphon", kann durch Behandlung mit verdünnter Ammoniaklösung über das Trichlorphon in den starken Esteraseinhibitor DDVP umge-

TABELLE IV

NACHWEISEMPFINDLICHKEIT VON THIOPHOSPHORSÄUREESTERN NACH AKTIVIERUNG MIT BROMWASSER BZW. UV-BESTRAHLUNG UND ANSCHLIESSENDER BROMWASSERBEHANDLUNG

—, Nachweis negativ; +, Nachweis schwach positiv; ++, Nachweis positiv; +++, Nachweis stark positiv.

Wirkstoff	*ng/Fleck*	*H_2O/Br_2*	*UV-Bestrahlung $+H_2O/Br_2$*	*ng/Fleck*	*H_2O/Br_2*	*UV-Bestrahlung $+H_2O/Br_2$*
Dimethoat	10	—	+	100	+	++
Ethion	0.1	—	+	0.5	+	+++
Disyston	5	+	+	50	++	++
Methylparathion	0.05	++	++	2	+++	+++
Parathion	0.01	++	++	0.5	+++	+++
Phorate	5	—	+	50	++	++
Ronnel	0.05	++	++	2	+++	+++
Bromophos	0.05	++	—	2	+++	++
Guthion	0.05	++	—	2	+++	+
Malathion	0.5	++	—	20	+++	+
Methyltrithion	5	++	—	50	+++	+

wandelt werden, wobei sich eine Einwirkungsdauer von 10 bis 15 min in Bezug auf die Nachweisempfindlichkeit des Butonats als optimal erweist.

Die vorliegenden Untersuchungen bestätigen, dass insektizide Organophosphate sowie die Thionogruppierung enthaltende Phosphorsäureester und der Phosphonsäureester "Butonate" nach Anwendung optimaler Aktivierungsverfahren mit hoher Empfindlichkeit durch die Kombination der Dünnschicht-Chromatographie mit dem enzymatischen Hemmtest nachgewiesen werden können. Diese Nachweisverfahren ermöglicht unter Einbeziehung des R_F-Wertes, des Verhaltens einer Verbindung gegenüber verschiedenen Aktivierungsverfahren sowie des Auftretens bestimmter esterasehemmender Hauptmetabolite eine Identifizierung unbekannter Esteraseinhibitoren. Nach Erkennung des Inhibitors ist weiterhin in einem vom jeweiligen Inhibitor abhängigen Konzentrationsbereich—allgemein handelt es sich dabei um den unteren Nachweisbereich—durch visuellen Fleckenvergleich eine semiquantitative Bestimmung der Inhibitormenge möglich.

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The chromatographic identification of substituted urea herbicides

Although many procedures have been described for the determination of residues of substituted urea herbicides, adequate methods for the identification of residues of these compounds have not been reported. Analytical procedures based on hydrolysis to the aniline derivative such as those of DALTON AND PEASE[1], KIRKLAND[2], GUTENMAN AND LISK[3], WEBLEY AND MCKONE[4] and HENKEL[5] can at best only distinguish between compounds in which the substituents in the aniline ring vary. Thin-layer and paper chromatographic procedures have been proposed by MAJOR[6], ABBOTT *et al.*[7], GUTH *et al.*[8], KATZ AND FASSBENDER[9], FINOCCHIARO AND BENSON[10] and HENKEL[11] but in each case only a few of the commercially available ureas were considered. The gas chromatographic behaviour of twelve ureas is reported by MCKONE AND HANCE[12] but the resolution under the conditions described was not adequate for identification.

The object of the work described here was to evaluate the TLC behaviour of eleven urea herbicides in a range of solvent systems and to select suitable systems which when used in conjunction with gas chromatography[1-,13] would provide a means for the identification and determination of residues of these compounds.

Materials and methods

Thin-layer plates (20 × 20 cm) were coated with a 0.3 mm layer of silica gel (Merck Kieselgel PF 254–366) and activated at 110° for 1 h. One reverse phase system was used in which the activated plates were impregnated by allowing a 5 % v/v solution of liquid paraffin BP in hexane to run to the top of the plate. The solvent was evaporated at 40°.

The developing solvents evaluated were:

(1) Chloroform
(2) Dichloromethane
(3) Diethyl ether
(4) Dichloromethane–diisopropyl ether (4:1)
(5) Chloroform–ethanol (19:1)[8]
(6) Dichloromethane–ethanol (19:1)[8]
(7) Chloroform–acetone (9:1)[7]
(8) Chloroform–acetone (7:3)[7]
(9) Hexane–acetone (9:1)[7]
(10) Hexane–acetone (7:3)[7]
(11) Acetone–*n*-hexane–benzene (1:2:5)[10]
(12) Chloroform–nitromethane (1:1)[11]
(13) Chloroform–glacial acetic acid (60:1)[9]
(14) Ethanol–water (4:6) with paraffin impregnated plates[14]

Spots of solution (5 μl) containing 5 μg of each herbicide were applied 2 cm from the edge of the plates. The solvents were allowed to run for 15 cm after which the plates were dried and viewed under UV light from a Hanovia "Chromatolite".

TABLE I

TLC R_F VALUES AND GAS CHROMATOGRAPHIC RETENTION TIMES FOR ELEVEN UREA HERBICIDES

System	*Fluometuron*	*Monolinuron*	*Buturon*	*Monuron*	*Metobromuron*	*Diuron*	*Linuron*	*Metoxymarc*	*Neburon*	*Benzomarc*	*Chlorbromuron*
1	0.14	0.48	0.15	0.12	0.51	0.14	0.58	0.13	0.38	0.14	0.56
2	0.07	0.24	0.19	0.06	0.25	0.07	0.32	0.07	0.17	0.07	0.33
3	0.22	0.48	0.53	0.15	0.51	0.16	0.51	0.16	0.50	0.15	0.52
4	0.21	0.58	0.57	0.18	0.59	0.20	0.62	0.21	0.54	0.19	0.63
5	0.43	0.71	0.66	0.42	0.72	0.42	0.72	0.42	0.68	0.70	0.72
6	0.50	0.63	0.57	0.49	0.61	0.51	0.68	0.44	0.61	0.48	0.73
7	0.39	0.62	0.60	0.33	0.64	0.39	0.68	0.38	0.59	0.34	0.68
8	0.58	0.70	0.75	0.51	0.72	0.58	0.74	0.57	0.75	0.58	0.74
9	0.04	0.11	0.07	0.03	0.11	0.04	0.11	0.04	0.10	0.05	0.10
10	0.34	0.44	0.40	0.27	0.43	0.34	0.48	0.33	0.49	0.35	0.48
11	0.15	0.41	0.44	0.11	0.43	0.17	0.44	0.13	0.38	0.14	0.44
12	0.63	0.81	0.84	0.54	0.82	0.64	0.86	0.64	0.85	0.78	0.85
13	0.22	0.38	0.31	0.22	0.38	0.22	0.43	0.23	0.32	0.30	0.46
14	0.53	0.58	0.54	0.60	0.53	0.46	0.41	0.44	0.18	0.42	0.38
GC retention times (min)	0.24	0.49	0.50	0.51	0.68	0.95	0.95	0.95	0.95	0.97	1.39

Results and discussion

The R_F values obtained are given in Table I, together with the retention times for the gas chromatographic system reported by McKONE AND HANCE[12]. The best resolutions obtained by the thin-layer systems were given by solvents 1, 2, 10, 13, 14 each of which could resolve a mixture of all the ureas into four groups. Of these systems 13 and 14 are probably best. The four groups produced by system 13 contain (a) fluometuron, monuron, diuron, Metoxymarc; (b) buturon, neburon, Benzomarc; (c) monolinuron, metobromuron; (d) linuron, chlorbromuron, while the groups produced by system 14 contain (a) neburon; (b) diuron, linuron, Metoxymarc, Benzomarc, chlorbromuron; (c) fluometuron, buturon, metobromuron; (d) monolinuron, monuron.

Thus the use of these two chromatographic systems would allow the identification of any of the ureas except that diuron cannot be resolved from Metoxymarc and linuron cannot be resolved from chlorbromuron. None of the thin-layer systems studied will resolve these two pairs. However, if the gas chromatographic system is also used then linuron and chlorbromuron can be separated although diuron and Metoxymarc would still not be resolved.

Using the extraction procedure of McKONE[13] levels of 0.5 p.p.m. may be easily visualised on thin-layer plates and for soils low in organic matter the limit of detection is 0.1 p.p.m. or less.

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The detection of sulphonamido groups

Many sulphonamides of pharmacological interest also contain an aromatic amino group, which can be readily detected by diazotization and coupling, or by the formation of a Schiff base with an aldehydic reagent (such as *p*-dimethylaminobenzaldehyde in acid solution)[1,2]. Such reactions are used widely to estimate antibacterial sulphonamide drugs. The sulphonamide group itself is little used in analytical procedures, although a method involving chlorination and subsequent reaction with iodide has been described[3].

4-Ethylsulphonylnaphthalene-1-sulphonamide (ENS), which induces an acute proliferative response in the mouse bladder[4], lacks an aromatic amino group. For studies of its metabolism[5], a colour reaction based on the sulphonamido group itself had to be devised. It was observed that chromatograms of sulphonamides such as ENS showed yellow spots when they were sprayed with ethanolic, non-acidified *p*-dimethylaminobenzaldehyde (DAB) and then left in ammonia vapour. This paper describes the development of this finding into a satisfactory reagent for the detection of ENS and other compounds with unsubstituted sulphonamido groups on chromatograms.

Experimental

Reagent. 1.5 g *p*-dimethylaminobenzaldehyde or *p*-dimethylaminocinnamaldehyde (British Drug Houses Laboratory Reagent Grade) was dissolved in 75 ml ethanol and 25 ml ammonia, S.G. 0.88, was added. Ethanol and ammonia were British Drug Houses Analar.

Chromatography. Paper chromatograms were run on Whatman No. 1. Thin-layer chromatograms were 0.25 mm of Silica Gel N or Alumina G (Macherey, Nagel) on glass plates.

Results

Reagent. Spots of ENS sprayed with DAB developed a yellow colour with ammonia vapour very slowly. It was found that ammonia solution incorporated in the ethanolic spray coloured the spots yellow immediately. The concentrations of the reagents were not critical. A mixture of one part ammonia (S.G. 0.88) with three parts 2% DAB in ethanol was suitable; with more ethanol in the spray the spots faded more rapidly; with more aqueous component the colour did not appear so quickly. The reagent was considerably more sensitive if allowed to age for about 1 h before use; it was stable for at least a month. The spots began to fade slowly after about 5 min, particularly if spraying had been light. They could be regenerated by further spraying with the reagent.

If the same molar concentration of methylamine, diethylamine or triethylamine was used in place of ammonia, the reagent retained the ability to detect sulphonamido groups, though it was not as satisfactory. Replacement of ammonia by sodium hydroxide did not give an effective reagent, though addition of sodium hydroxide to the ammoniacal reagent did not prevent the development of a colour with sulphonamides.

p-Dimethylaminocinnamaldehyde (DAC) can replace DAB in the reagent. DAC gives red spots on an orange background. The yellow on white chromatograms with DAB reagent are less affected by uneven spraying, and detection of faint spots can be made with more confidence. Aldehydes, such as resorcylaldehyde, which are activated by hydroxy instead of dimethylamino groups do not appear to be suitable for detection of the sulphonamido group in the manner described, although in acid conditions they react well with the aromatic amino group[6].

Compounds detected. All compounds with unsubstituted sulphonamido groups tested gave a yellow colour when sprayed with the standard reagent on silica gel, alumina or paper.

TABLE I

DETECTION LIMITS OF SULPHONAMIDES CHROMATOGRAPHED ON SILICA GEL THIN LAYERS IN TOLUENE–ETHYL ACETATE (1:1)

Compound	*Detection limit* (μg)	R_F *value*
4-Ethylsulphonylbenzene-1-sulphonamide	0.5	0.25
4-Ethylsulphonylnaphthalene-1-sulphonamide	0.2	0.44
4-Methylsulphonylnaphthalene-1-sulphonamide	0.2	0.40
4-Ethylthionaphthalene-1-sulphonamide	0.2	0.59
Naphthalene-1-sulphonamide	0.4	0.57
p-Toluene-sulphonamide	1.0	0.55
N^4-Acetylsulphanilamide	1.0	0.06
4-Piperonyl-2,3,5,6-tetrafluorobenzene sulphonamide	0.3	0.77
4-(Cyclohexylamino)-2,3,5,6-tetrafluorobenzene sulphonamide	0.3	0.75
1-Oxo-3-(3′-sulphamyl-4′-chlorophenyl)-3-hydroxyisoindoline; chlorthalidone ("Hygroton", Geigy)	0.5	0.15
p-(Tetrahydro-2H-1,2-thiazin-2-yl)benzene sulphonamide dioxide; sulthiame	0.4	0.23

Table I shows the limits of detection for a number of chromatographed sulphonamides, with R_F values. The concentrations detected were less than 5 mM in each case. The compounds were applied in 1 μl spots to a 0.25-mm thick silica gel chromatogram run in toluene–ethyl acetate (1:1).

On alumina chromatograms, the reagent was slightly more sensitive. Less than 5 mM solutions could also be detected on paper chromatograms, run in butanol–1 N ammonia (1:1) for example, when the sulphonamides were applied in 5 μl spots. The spots were more stable on paper than on thin layers.

From Table I it can be seen that sulphonamido groups have been detected when: (a) attached to benzene or naphthalene rings, (b) the aromatic ring hydrogens are completely substituted, (c) the *para* position is substituted with either electron-attracting or electron-donating groups.

1-Ethylsulphonylnaphthalene and 1,4-diethylsulphonylnaphthalene contain the sulphonyl but not the sulphonamido group. They were not detected by the reagent.

Yellow colours were given by sulphathiazole and sulphapyridine, in which the sulphonamido group is substituted with a heterocyclic ring. The colours developed slowly as the chromatograms dried in the air and became neutral. These sulpha-drugs

also contain aromatic amino groups which can react with DAB in neutral or acid conditions. N-Acetyl- and N-methyl-ENS, and N-(2′-pyridyl)ethylthionaphthalene-1-sulphonamide were not detected by the reagent; each contains a substituted sulphonamido group, but lacks the aromatic amino group.

Discussion

CROWELL AND MCLEOD[7] have shown that the imine Me_2N–Ph–CH=NH is the dominant species in an ammoniacal methanolic solution of DAB, and that the hydrobenzamide can be formed from imine molecules with the elimination of ammonia. Reaction products such as these may be involved in the detection of sulphonamido groups by *p*-dimethylaminobenzaldehyde under the conditions used; it was found that the reagent was considerably more effective when allowed to stand before use. The finding that ammonia can be replaced by aliphatic amines without abolishing the activity of the reagent suggests that in some cases imine formation may not be involved. Diethylamine, for example, does not appear to react with DAB; dilution with *n*-hexane precipitates unchanged DAB from its solution in diethylamine.

The reaction appears sensitive enough for it to be useful in chromatographic studies of the metabolism of biologically active compounds with unsubstituted sulphonamido groups. A wide range of such substances seem likely to react in view of the successful detection of a variety of types of sulphonamide, which has been demonstrated.

Thanks are due to Dr. S. G. DE BAKER for a gift of 4-piperonyl- and 4-cyclohexylamino-2,3,5,6-tetrafluorobenzene sulphonamides, to Mr. TURNER for many of the other sulphonamides, and to Prof. D. B. CLAYSON for his interest.

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CHROM. 4304

The separation of isoasparagine from asparagine

The best available synthesis of L-isoasparagine involves the treatment of carbobenzoxy-L-asparagine with acetic anhydride followed by ammonia, and removal of the carbobenzoxy group[1]. Several recrystallizations of carbobenzoxy-L-isoasparagine are recommended[1] to remove carbobenzoxy-L-asparagine. Since such treatment cannot remove all traces of the unwanted material, we have devised an electrophoretic procedure (analytical scale) and a chromatographic procedure (analytical and preparative) to permit the preparation of L-isoasparagine free from L-asparagine.

Paper electrophoresis at pH 3.0 (0.1 *M* acetic acid plus formic acid to attain the proper pH) resulted in good separation of the isomers of asparagine (obtained by treatment of the carbobenzoxy-L-asparagines with 30 % HBr in acetic acid for 1 h), which, as indicated in DU VIGNEAUD's procedure[1] can be distinguished by the color of their reaction products with ninhydrin. In this system, asparagine moved as fast to the cathode as picric acid (used to follow the rate of migration) moved to the anode. Isoasparagine moved about twice as fast as asparagine. This is in accordance with the relative acidity of the two carboxyl groups[2] of aspartic acid ($pK = 1.995$ and 3.910).

Ion-exchange chromatography on an automatic amino acid analyzer by the accelerated system of SPACKMAN[3] (buffer flow rate of 50 ml/h) provided excellent separation of isoasparagine from asparagine: Asparagine eluted after 107 min with pH 3.25 citrate buffer while isoasparagine eluted in the position of isoleucine when the pH 4.25 buffer was used for elution. The color yield with ninhydrin of isoasparagine was 57 % of that of norleucine.

These procedures indicated that the first crop of crystals from the DU VIGNEAUD synthesis[1] contained 8 % asparagine. Analysis of a second crop from the mother liquor showed that it contained only 9 % isoasparagine. With the ion-exchange system described it is possible to prepare large amounts of isoasparagine free from asparagine by removing the latter with pH 3.25 buffer and recovering the isoasparagine from the pH 4.25 eluate. Removal of the citrate buffer salts by standard methods yielded pure isoasparagine.

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Book Reviews

CHROM. 4211

Ion-Exchange, edited by JACOB A. MARINSKY, Marcel Dekker, New York, 1969, 250 pp., price $ 12.75, £ 6.2.0.

This is the second volume of a series with the full title *Ion Exchange: A Series of Advances*. In judging it, one is naturally tempted to compare it with Volume I, which appeared in 1966. Unfortunately, this comparison is a disappointing one in several ways. First, the present volume is somewhat pedestrian, and lacks the brilliant exposition so evident in Volume I. Secondly, whereas the contents of Volume I made a closely knit whole, the topics dealt with in the six chapters of Volume II form a curious mixture bearing little relationship one with another. One has the feeling that the series is not being planned as a whole but rather that ideas for the subject matter of chapters are being evolved as the series itself evolves. Thus we find chapters dealing with ion-exchange in glasses, in molten systems, and in zeolites, as well as the synthesis of ion-exchange resins, the interaction between organic ions and ion-exchange resins, and the chromatography of sugars and their derivatives.

Of these topics, only the last three are likely to be of any interest at all to those whose primary interest is chromatography, and only the last one is likely to be of direct interest. Unfortunately, it is also the shortest, comprising a mere 22 pages. Curiously, despite the generic title of the series, the methods described in this chapter are not based on ion exchange but on partition chromatography, though ion-exchange resins are used as the substrate.

Nevertheless the chapter, which is largely a summary of recent work by SAMUELSON and his school in a field he has made characteristicly his own, has great merit. The impact on the reader of the chromatograms illustrated is similar to that made many years ago by the now classical chromatograms obtained by MOORE AND STEIN for the separation of amino acids by ion-exchange chromatography. Clearly, SAMUELSON has developed this technique to a point where it has become a valuable and powerful method for the separation of sugars on an analytical scale.

The book is well presented and misprints are so few as to be nearly (but not quite) non-existent. The price is high, as with all books published in the U.S.A., and in view of its limited interest to readers of the Journal of Chromatography, it must be their personal decision whether they consider this interest is sufficient to tempt them to spend £ 6.2.0 on it.

The Permutit Co. Ltd.,
London (Great Britain)

T. R. E. KRESSMAN

CHROM. 4262

Modern Methods for the Separation of Rarer Metal Ions, by JOHANN KORKISCH, Pergamon Press, Oxford, 1969, 620 pp., price £ 7.10.0.

The first 27 pages describe the separation techniques, *viz.* ion-exchange chromatography (including electrophoresis), liquid–liquid extraction, distillation and coprecipitation. Evidently not much can be said in such a short space but the chapter seems adequate as an introduction. The rest of the book is divided into chapters according to groups of rarer metals: actinides, rare earths, lithium, rubidium, caesium and francium, beryllium, radium, gallium, indium and thallium, etc., and in each chapter the separations that can be obtained with the above techniques are discussed. On the whole an admirably useful reference book for analytical- and radiochemists.

We have only two strong objections to the way the book is written: (1) It is not sufficiently critical and somewhat out of date. This is more serious in one chapter than in another. Some sections only contain the literature up till 1965 with few later additions and some list obsolete preliminary papers along with completely worked out analytical methods without explanatory comments. (2) In some chapters, *e.g.* the platinum metals, a short summary of the solution chemistry of the metal ions should have been included and the separations discussed from this point of view.

In spite of these shortcomings the book fills a gap in the literature as there is no survey of this kind more recent than 1958.

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CHROM. 4167

Gas Chromatography, by O. E. SCHUPP III, Vol. 13 of *Technique of Organic Chemistry*; edited by E. S. PERRY AND A. WEISSBERGER, Interscience, New York, 1968, 437 pp., price £ 7.15.0.

The contents of this volume are rather unbalanced; some parts are comprehensive, whilst other topics have been sadly neglected. Could this be a case of editing to fit the requirements of the series? Incidentally, throughout the book, no reference is made to the other volumes in the series, despite their obvious relevance to GC (for instance, Vol. 10, *Fundamentals of Chromatography*).

The omission of a section on the engineering requirements of chromatograph construction is justifiable, since most workers now use commercial equipment. However, the author has almost ignored the existence of capillary columns and has not dealt adequately with the simpler theory of chromatographic separation. Instead, he has dealt in depth with packed column design—his section on solid supports is partic-

ularly informative—and the chapter on theory is devoted almost exclusively to the effects of all conceivable variables on the efficiency of packed columns. Evolving from this chapter, a theme is developed through the rest of the book: that one should always aim to work at maximum efficiency. Another subject which is dismissed in a couple of pages is the use of spectroscopic techniques to supplement GC in qualitative analysis; mass and infra-red spectrometers, directly linked to gas chromatographs, are now so popular that more detail is warranted.

It is difficult to recommend this as a first book for beginners in the field of gas chromatography. It would appear that the introductory chapter was written as a necessary chore; the language is often confusing (even to a practising chromatographer), and specific terms such as "back-flushing", "overloading" and "non-selective" are used with no regard to the reader's probable ignorance of them.

Most illustrations have been reprinted from the literature. Although generally of a good standard, some have been taken out of context and contain details not explained by the text. The concluding chapter, headed *Qualitative Analysis* is another example of poor editing; it includes pages of information concerning solute resolution which would have fitted better into an earlier chapter. The index is just adequate.

Published in 1968, this book takes into account developments up to early 1967; some of the few references to the 1967 literature have been added as stop-press items. This is noticeable in the section on ancillary techniques, where important modern equipment such as the Curie-point pyrolyser is not mentioned, and in the chapter on detectors where all data on electron capture detectors refer to tritium sources, although nickel-63 is briefly referred to.

In conclusion, this volume, though certainly not outstanding, is probably no worse than several others written on the subject, and could be quite useful as a basic text, especially to those who are efficiency-conscious.

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CHROM. 4301

SECOND CROSS VIRIAL COEFFICIENTS OF BENZENE–GAS MIXTURES FROM HIGH PRESSURE SOLUBILITY MEASUREMENTS

A COMPARISON WITH GAS CHROMATOGRAPHIC VALUES*

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SUMMARY

Values of second cross virial coefficients have been determined from solubility measurements at high pressures for binary mixtures of benzene with He, H_2, N_2, Ar, CH_4, and C_2H_4. The results of these measurements are compared with values obtained using newly developed high pressure gas–liquid chromatographic methods. The agreement between second cross virial coefficients obtained by these two different methods is found to be good.

INTRODUCTION

In recent years there has been considerable interest in the use of gas–liquid chromatography (GLC) as a means of determining thermodynamic properties of two component systems[1–10]. In these studies, the variation of net retention volume of a pure sample with carrier gas pressure is used to determine both the activity coefficient at infinite dilution of the sample component in the stationary liquid phase and the second cross virial coefficient representing deviations from ideality in the gas phase caused by intermolecular pair interactions between molecules of the sample component and those of the carrier gas. Comparisons of activity coefficients obtained using GLC methods and those obtained from static vapor pressure measurements show good agreement[3,5]. Second cross virial coefficient data, determined using other methods, also show good agreement in the few cases where data are available for comparison[8,9,11]. In general, however, there is a paucity of second cross virial coefficient data available for comparison to GLC values, particularly for systems involving gases of moderate complexity such as methane and ethylene whose solubility in the stationary liquid phase cannot be neglected, thus necessitating the introduction of approximate correction factors in GLC second cross virial coefficient determinations[9,10].

Values of second cross virial coefficients involving one solute, benzene, in a series of gases ranging in complexity from helium to ethylene, have been determined

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from solubility measurements at high pressures and are reported here. Temperatures of these measurements (32°, 35°, 50°) have been chosen to maximize comparison with existing GLC values.

EXPERIMENTAL

The experimental technique used here for determining second cross virial coefficients is a modification of a method used by PRAUSNITZ AND BENSON[12]. The method entails measuring the concentration of benzene vapor in equilibrium with its liquid phase in the presence of a compressed inert gas. The flow system used in these experiments is shown schematically in Fig. 1.

The gas to be studied is allowed to flow under high pressure from its storage cylinder (A) through a Matheson Model 3 pressure regulator (B) into a cylindrical steel equilibrium cell (C) containing liquid benzene. A detailed drawing of the equilibrium cell is shown in Fig. 2. In use, the cell is filled with benzene to a level approximately 2 in. from the top. The high pressure gas entering the bottom of this cell is dispersed into small bubbles by a fritted glass sparger (pore size 14 μ) to insure rapid equilibration between the gas and benzene as the bubbles pass upwards through the liquid phase. There were no problems with benzene entrainment or splashing at the surface provided adequate dead space was provided over the liquid surface. The temperature of this cell was controlled to within $\pm 0.05°$ by a constant temperature oil bath (D).

The vapor–gas mixture leaving the cell passes through a heated section of tubing (E) and is expanded through a heated Matheson stainless steel needle valve (F) into a series of three cold traps (G) which are suspended in a dry ice–acetone bath while

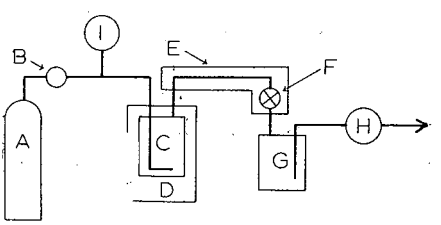

Fig. 1. Schematic diagram of equipment.

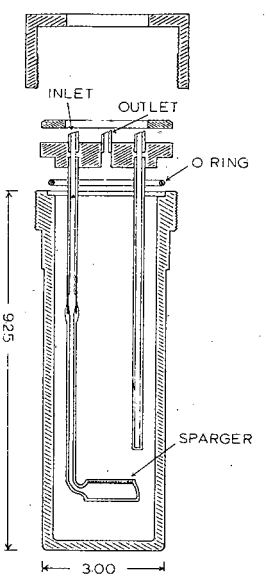

Fig. 2. Equilibrium cell. (Dimensions in inches.)

the run is in progress. The temperature of the tubing (E) and valve (F) is maintained at a temperature exceeding that of the equilibrium cell to prevent condensation from occurring until the gas–vapor mixture is expanded into the cold traps. After passing through the traps, the gas stream passes through an American Meter Co. Model AL 17-1 wet-test meter (H) which measures the volume of essentially pure diluent gas at ambient temperature and pressure which has flowed through the system during a given experimental determination. The pressure of the gas–vapor system in the high pressure section between the regulator (B) and the expansion valve (F) is measured with a 1000 lb./sq. in. range bourdon gauge (I) which was calibrated against an Aminco Model 47-2221 dead-weight tester.

The experimental procedure followed in using this apparatus is very simple. The system containing benzene is loaded with the gas to be studied at the pressure of interest and is allowed to come to equilibrium over a period of at least 12 h at the desired temperature. The valve (F) is then adjusted to give the desired flow rate (0.5 l/min–1.0 l/min) with the system being allowed to vent directly to the atmosphere for a period of approximately 30 min to insure attainment of steady state conditions. The traps are then lowered into the cold bath and the flow of the gas–vapor mixture is immediately diverted to the trapping system and wet-test meter. After a period of flow, the flow is again vented to the atmosphere, the traps are sealed, and removed from the cold bath. The quantity of benzene in the cold traps is then determined gravimetrically while the volume of gas is measured at ambient pressure and temperature. Flow rates and durations of flow were adjusted for each run so that generous and approximately constant amounts of benzene (1–3 g) were trapped during each run in order to minimize weighing errors. In all cases, nearly all the benzene collected was found in the first trap, with the second trap never containing more than 1 % of the amount of benzene found in the first trap. No measurable amounts of benzene were ever detected in the third trap. Since the vapor pressure of benzene at dry ice–acetone temperature is insignificant ($\sim 10^{-2}$ mm Hg), it can be confidently assumed that the traps were essentially 100 % efficient in removing benzene vapor from the gas stream. Thus, the mole fraction of benzene in the gas phase in the equilibrium cell at the pressure of the run is readily determined from the measured mass of benzene collected and the volume of accompanying gas.

RESULTS AND DISCUSSION

A single determination of the mole fraction of benzene in a given diluent gas at a known pressure is sufficient information to allow the second cross virial coefficient for benzene and that gas to be determined provided that: (a) the solubility of that gas in benzene is known at that pressure; (b) the second virial coefficients of pure benzene vapor and pure gas are known at the temperature of the experiment; and (c) the pressure is not so great as to require the introduction of third or higher virial coefficients for the description of the gas phase properties of the system in the equilibrium cell.

The fugacity of benzene (component 2) in the liquid phase, $\bar{f}_2^L$, is related to the mole fraction of dissolved gas (component 1), X_1, and the total hydrostatic pressure of the system, P, by the relation:

$$\bar{f}_2^{L} = \gamma_2^{L}(1 - x_1)P_2^{\circ}\Phi_2^{\circ} \exp\left[\frac{V_2^{\circ(L)}(P - P_2^{\circ})}{RT}\right] \quad (1)$$

Here P_2° and Φ_2° are the vapor pressure and fugacity coefficient of pure benzene at temperature T, while γ_2^{L} and $V_2^{\circ(L)}$ are the activity coefficient and molar volume of benzene in the liquid phase. For small mole fractions of dissolved gas such as encountered in these experiments, γ_2^{L} can be taken to be unity without introducing appreciable error. The fugacity of benzene in the gas phase, $\bar{f}_2^{G}$, can be expressed in terms of the mole fraction of benzene in the gas phase, y_2, its fugacity coefficient, Φ_2 and pressure P, as:

$$\bar{f}_2^{G} = \Phi_2 y_2 P \quad (2)$$

The condition for equilibrium is that $\bar{f}_2^{G} = \bar{f}_2^{L}$, so that the vapor phase solubility of benzene in a compressed gas is related to the hydrostatic pressure of the system by:

$$y_2 = \frac{(1 - x_1)P_2^{\circ}}{P}\left(\frac{\Phi_2^{\circ}}{\Phi_2}\right) \exp\left[\frac{V_2^{\circ(L)}(P - P_2^{\circ})}{RT}\right] \quad (3)$$

If contributions arising from third and higher virial coefficients are neglected the fugacity coefficient of benzene vapor in the gas mixture can be expressed as:

$$\ln \Phi_2 = \frac{2}{V}[y_2 B_{22}(T) + y_1 B_{12}(T)] - \ln Z \quad (4)$$

where y_1 represents the mole fraction of diluent gas in the gas phase, V is the molar volume of the gas phase mixture, and the symbols $B(T)$ denote second virial coefficients representing deviations from ideality caused by pair interactions between molecules of the species designated by subscripts. Z represents the compressibility factor of the gaseous mixture.

Thus, a given experimental measurement of the mole fraction of benzene in the vapor phase with a particular diluent gas at a known temperature and pressure can be used to evaluate the ratio of fugacity coefficients, Φ_2°/Φ_2 in eqn. (3). The second cross virial coefficient $B_{12}(T)$ for that particular benzene–gas mixture can then be evaluated from this ratio of fugacity coefficients in an iterative fashion using eqn. (4) with a trial value for $B_{12}(T)$ to make initial estimates of the molar volue, V, and compressibility factor, Z. In the computations that follow, fugacities have been used with Henry's law constants obtained from refs. 13–20 to calculate x_1 of eqn. (3) as a function of pressure for the various benzene–gas systems, Values of pure component second virial coefficients used in these calculations are found in refs. 21–34. P° was obtained at the desired temperatures from data found in ref. 35 while values of the molar volume of liquid benzene at these temperatures were taken from ref. 36.

Table I lists experimentally determined mole fractions of benzene vapor in the various gas mixtures. Fig. 3 is a graphical representation of the data in Table I. In Fig. 3 it is seen that not only does the mole fraction of benzene increase at a given pressure going from the relatively nonpolarizable gas He to ethylene, whose molecules are very polarizable, but further, that in the case of ethylene, the mole fraction passes

TABLE I

VAPOR COMPOSITION IN BINARY SYSTEMS CONTAINING BENZENE

Gas	*Temperature* (°C)	*Total pressure* (*atm*)	*Mole fraction of benzene* (y_2)
He	50	15.2	0.0225
		36.3	0.00930
		36.3	0.00934
		49.5	0.00687
		50.2	0.00680
		64.3	0.00534
		64.3	0.00519
		64.3	0.00526
H_2	50	22.4	0.0169
		39.0	0.0105
		39.0	0.0104
		39.0	0.0105
		39.4	0.0102
		50.7	0.00856
		50.7	0.00862
		50.7	0.00854
		56.6	0.00770
		57.2	0.00770
		63.8	0.00714
Ar	32	23.7	0.00950
		23.7	0.00968
		36.9	0.00696
		36.9	0.00723
		51.3	0.00608
		51.3	0.00606
		64.0	0.00569
		64.0	0.00577
	50	24.9	0.0173
		36.5	0.0139
		36.5	0.0142
		50.3	0.0118
		50.3	0.0116
		64.6	0.0100
		64.6	0.0105
N_2	35	22.9	0.0106
		22.9	0.0108
		22.9	0.0107
		37.0	0.00773
		37.0	0.00779
		37.0	0.00772
		37.0	0.00777
		37.0	0.00773
		37.0	0.00753
		50.3	0.00680
		50.3	0.00673
		50.3	0.00676
		64.6	0.00601
		64.6	0.00597
		64.6	0.00606
	50	23.4	0.0173
		36.4	0.0142
		36.4	0.0141

(*continued on next page*)

TABLE I (*continued*)

Gas	*Temperature* (°C)	*Total pressure* (*atm*)	*Mole fraction of benzene* (y_2)
		49.9	0.0118
		49.9	0.0115
		62.8	0.0102
		62.8	0.0103
		63.4	0.0101
CH_4	50	21.5	0.0224
		36.7	0.0164
		36.7	0.0167
		52.1	0.0147
		52.1	0.0146
		54.5	0.0143
		64.5	0.0146
		64.5	0.0144
C_2H_4	50	23.4	0.0209
		23.8	0.0205
		30.2	0.0180
		30.2	0.0186
		37.0	0.0177
		37.0	0.0174
		46.2	0.0177
		46.2	0.0174

through a minimum at approximately 40 atm and increases with total pressure beyond this point.

Fugacity coefficients for benzene in the various gases have been calculated from the data of Table I using eqn. 3. Fig. 4 shows the values obtained at 50° plotted as a function of pressure.

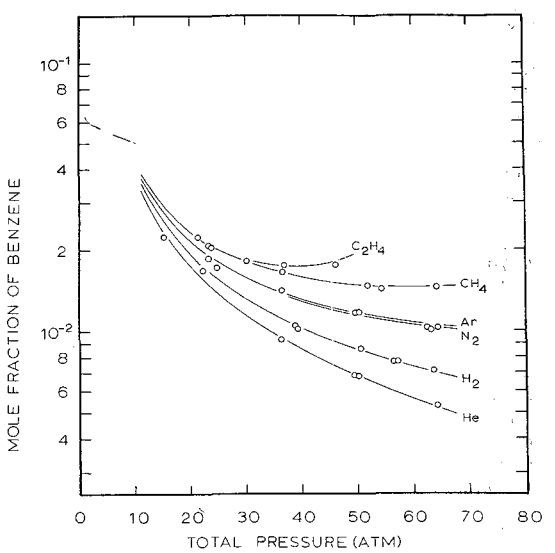

Fig. 3. Vapor phase solubility of benzene in the gases He, H_2, N_2, Ar, CH_4, and C_2H_4 at 50°. (The solid line represents computed values of mole fraction based on experimentally determined second cross virial coefficients listed in Table II.)

Second cross virial coefficients for the various benzene-gas mixtures corresponding to these fugacity coefficients have been calculated using eqn. 4. For each system, excellent agreement was found to exist among values of $B_{12}(T)$ obtained at different pressures. Averages of the values obtained for each benzene–gas system are listed in Table II. Second virial coefficients obtained from GLC methods are also listed in this table for comparison. It is seen that with one exception, a value measured for helium, the second cross virial coefficients obtained here agree within experimental error with values derived from GLC work.

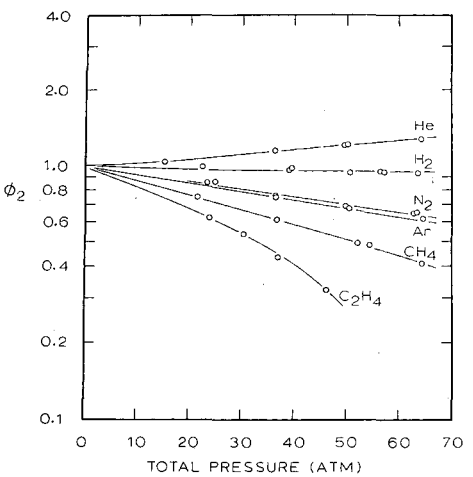

Fig. 4. Fugacity coefficients of benzene in He, H_2, N_2, Ar, CH_4, and C_2H_4 at 50°. (The solid line represents computed values of fugacity coefficients based on the experimentally determined second cross virial coefficients listed in Table II.)

Since the experimental methods used here are completely different from those used in the GLC method, the values for second cross virial coefficients listed here represent an independent check of the results obtained from GLC measurements. The

TABLE II

SECOND CROSS VIRIAL COEFFICIENTS FOR BENZENE WITH VARIOUS GASES

Gas	*Temperature* (°C)	$B_{12}(T)$ (*cm³/mole*)	$B_{12}(T)$ *GLC* (*cm³/mole*)
He	50	+ 67 ± 4[a]	+ 57 ± 8[b], +49 ± 8[b]
H_2	50	+ 4 ± 3	− 5 ± 8[b]
N_2	35	− 97 ± 3	−104 ± 10[c]
N_2	50	− 85 ± 3	− 87 ± 8[b], −94 ± 10[c]
Ar	32	−122 ± 3	−135 ± 10[c]
Ar	50	− 95 ± 3	− 85 ± 8[b], −90 ± 10[c], −88[d]
CH_4	50	−171 ± 3	−155 ± 15[b]
C_2H_4	50	−282 ± 5	—

[a] Experimental error is expressed as average deviation.
[b] Ref. 8.
[c] Ref. 7.
[d] Ref. 5.

good agreement between values obtained by these two methods strongly attest the fact that gas chromatographic methods represent a powerful tool for studying gas phase interactions between dissimilar molecules.

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CHROM. 4302

UNTERSUCHUNGEN ÜBER DIE TRENNUNG VON NITROPHENOLEN UND NITROANISOLEN DURCH GASCHROMATOGRAPHIE

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SUMMARY

Separation of nitrophenols and nitrophenyl methyl ethers by gas chromatography

The gas chromatographic behaviour of nitrophenols and nitrophenyl methyl ethers was studied on polyester (PEGA, BDS, PEGS, NPGS) and silicone (XE-60, QF-1, SE-30) columns. The elutions on polyester type columns were affected by hydrogen bonding between studied compounds and liquid phases. On these columns nitrophenols with intramolecular hydrogen bonds had shorter retention times than related nitrophenyl methyl ethers.

EINLEITUNG

Phenole stellen, insbesondere wenn sie durch Nitrogruppen substituiert sind, beträchtlich polare Verbindungen dar, die bei der Trennung durch Gaschromatographie eine Reihe von Schwierigkeiten bereiten können.

Zwecks Erniedrigung der Adsorption dieser Verbindungen an dem Trägermaterial der Trennsäule besteht die Möglichkeit, die Phenole vor dem Chromatographieren in weniger polare Verbindungen um zu setzen, wie z.B. in Trimethylsilyläther[1,2], Methyläther[3–6], Acetate[7,8] und Trifluoracetate[9].

Unter den freien Phenolen wurden Alkylphenole[10–17], sowie Chlorphenole[18,19] und Nitrophenole[20] durch Gaschromatographie abgetrennt.

Gegenstand dieser Arbeit ist das Studium der gaschromatographischen Trennung von Isomeren der Mononitro- und Dinitrophenole und der von ihnen abgeleiteten Methyläther auf verschiedenen stationären Phasen.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

EXPERIMENTELLER TEIL

Gaschromatograph

Die chromatographischen Arbeiten wurden auf dem Gerät Fractovap Modell GI (Carlo Erba, Milan) durchgeführt, das mit einem Flammenionisationsdetektor ausgerüstet war. Als Trägergas gelangte Stickstoff zur Anwendung.

Chromatographische Trennsäulen

Für die Bestimmung der relativen Retentionszeiten wurden gläserne Trennsäulen in einer Länge von 1 m und mit einem Durchmesser von 2.2 mm benutzt. Bei Verwendung polarer Polyester-Trennflüssigkeiten als stationäre Phase haben wir als Träger Chromosorb W, und bei Verwendung stationärer Silikon-Phasen silanisiertes Chromosorb W verwendet; die Korngrösse betrug in beiden Fällen 60–80 mesh. Der Träger wurde mittels der stationären Phase in einer Menge von 5 Gew.-% imprägniert, wobei wir bei den polaren Polyester-Trennflüssigkeiten noch 1 Gew.-% Orthophosphorsäure hinzugefügt haben. Die Säulen wurden vor der Benutzung unter mässigem Durchfluss des Trägergases bei 200° während 8 Std. konditioniert.

Säulenfüllung

Chromosorb W, Polyäthylenglykoladipat (PEGA), Butandiolsuccinat (BDS), Polyäthylenglykolsuccinat (PEGS), Neopentylglykolsuccinat (NPGS), Cyanoäthylmethylsilikonfett XE-60 (XE-60), Fluorosilikonöl QF-1 (QF-1) und Methylsilikonfett SE-30 (SE-30) waren Handelspräparate der Fa. Carlo Erba, Milan.

Nitrophenole

Durch Nitrierung des 3-Nitrophenols gemäss BANTLIN[21] wurde ein Gemisch des 2,3-, 3,4- und 2,5-Dinitrophenols erhalten, das durch die Bariumsalze getrennt wurde. Durch Nitrierung des 2-Nitrophenols gemäss HOLLEMAN[22] wurde ein Gemisch des 2,4- und 2,6-Dinitrophenols erhalten, das durch fraktionierte Präzipitation der Natriumsalze mittels Bariumchlorid getrennt wurde. Durch Hydrolyse des 3,5-Dinitroanisols gemäss HANTZSCH[23] wurde das 3,5-Dinitrophenol erhalten.

Nitroanisole

Durch Einwirkung von Natriumalkoholat auf 1,3,5-Trinitrobenzol gemäss REVERDIN[24] wurde 3,5-Dinitroanisol hergestellt. Die übrigen Anisole wurden durch Einwirkung von Diazomethan auf die entsprechenden Mono- und Dinitrophenole in der Weise hergestellt, dass man eine 5%ige Lösung von Diazomethan in Äther, zubereitet gemäss ARNDT[25], in einem 10%igen Überschuss nach und nach in kleineren Anteilen und unter Rühren zu einer 10%igen Lösung des entsprechenden Phenols in Äther zugegeben hat. Das Reaktionsgemisch wurde 2 Std. bei Zimmertemperatur stehengelassen, dann auf das halbe Volumen eingeengt und mit einer 2%igen Natriumcarbonatlösung durchgeschüttelt. Die Ätherschicht wurde abgetrennt, mit wasserfreiem Natriumsulfat getrocknet und durch Destillieren eingeengt. Der Destillationsrückstand wurde via ein geeignetes Lösungsmittel auskristallisiert.

ERGEBNISSE UND DISKUSSION

Die relativen Retentionsvolumen der Nitrophenole und der abgeleiteten Nitroanisole auf stationären Polyester-Phasen werden in Tabelle I angeführt. Bei der gas-

TABELLE I

RELATIVE RETENTIONSVOLUMEN DER NITROPHENOLE UND NITROANISOLE AUF STATIONÄREN POLYESTER-PHASEN BEI 200°

Verbindung	5% *PEGA* 1% H_3PO_4	5% *BDS* 1% H_3PO_4	5% *PEGS* 1% H_3PO_4
2-Nitrophenol	0.09	0.10	0.09
2-Nitroanisol	0.27	0.28	0.27
3-Nitrophenol	2.91	2.56	3.04
3-Nitroanisol	0.18	0.21	0.17
4-Nitrophenol	4.82	4.02	5.00
4-Nitroanisol	0.32	0.34	0.31
2,3-Dinitrophenol	2.53	2.38	2.90
2,3-Dinitroanisol	2.94	2.86	3.00
2,4-Dinitrophenol	1.00	1.00	1.00
2,4-Dinitroanisol	3.40	3.28	3.36
2,5-Dinitrophenol	0.80	0.79	0.78
2,5-Dinitroanisol	1.69	1.70	1.59
2,6-Dinitrophenol	1.53	1.53	1.56
2,6-Dinitroanisol	0.83	0.86	0.80
3,4-Dinitrophenol	>20[a]	>20[a]	>20[a]
3,4-Dinitroanisol	2.91	2.84	2.82
3,5-Dinitrophenol	>20[a]	>20[a]	>20[a]
3,5-Dinitroanisol	1.27	1.30	1.15

[a] Nicht eluiert nach 45 Min.

chromatographischen Trennung der Nitrophenole auf stationären Polyglykol-Phasen haben wir festgestellt, dass die Wasserstoffbindungen zwischen der stationären Phase und der phenolischen Gruppe[20] einen grossen Einfluss auf die Retentionsreihenfolge ausüben. Die Stärke der Wasserstoffbindung zwischen der phenolischen Gruppe und den Hydroxylgruppen der stationären Phase wird durch die Stellung bzw. durch die Stellungen der Nitrogruppe beeinflusst, die bei der Beziehung zwischen der Struktur und den chromatographischen Eigenschaften der dem Studium unterworfenen Verbindungen eine sehr wichtige Rolle spielen. Darüber hinaus entstehen Wasserstoffbindungen, wenn auch relativ schwächere, auch zwischen der Nitrogruppe und den Hydroxylgruppen der stationären Phase. Der Einfluss dieser Bindungen kann ganz besonders in jenen Fällen zum Ausdruck gelangen, wenn der Wasserstoff der phenolischen Gruppe durch eine Methylgruppe ersetzt wird, oder wenn die phenolische Gruppe eine intramolekulare Wasserstoffbindung bildet, bzw. eine sterische Hinderung aufweist.

Vom Gesichtspunkt der Möglichkeit einer direkten gaschromatographischen Trennung der Dinitrophenole (ohne eine Überführung in flüchtigere Derivate) ist die Nitrogruppe in Stellung 2 oder 6, oder auch in Stellung 2,6 sehr bedeutsam. Diese Dinitroderivate verhalten sich infolge der intramolekularen Wasserstoffbindung (2,3-, 2,4- und 2,5-Dinitrophenol) auf Polyester-Säulen wie Mononitrophenole, sie werden sogar vor dem 3- und 4-Nitrophenol eluiert. Die Erhöhung der relativen Flüchtigkeit, wenn sich die Nitrogruppe in *ortho*-Stellung befindet, gelangt am meisten in der Reihe der Mononitrophenole zum Ausdruck; 2-Nitrophenol wird auf Polyglykol-Säulen sogar vor dem Phenol[20] eluiert. Die Möglichkeit eines direkten Chromatographierens von Dinitrophenolen, bei denen sich wenigstens eine Nitrogruppe in

ortho-Stellung befindet, würde bei der Analyse einiger als Pesticide verwendeten Dinitrophenole[26–29] ausgenützt.

Dinitrophenole, bei denen kein "*ortho*-Effekt" oder keine sterische Hinderung der phenolischen Gruppe zu verzeichnen ist (3,4- und 3,5-Dinitrophenol), treten durch eine hohe Verzögerung oder Adsorption auf der gaschromatographischen Säule hervor.

Die Abhängigkeit zwischen der Retentionsreihenfolge des Nitrophenols und des von diesem abgeleiteten Nitroanisols auf Polyester-Säulen ist sehr eindeutig: die von Nitrophenolen abgeleiteten Methyläther, die sich durch den "*ortho*-Effekt" auszeichnen, weisen längere Retentionszeiten auf als die ursprünglichen Nitrophenole. Diese Tatsache wird bereits durch die obenangeführten Schlussfolgerungen über die Bedeutung der Nitrogruppe in *ortho*-Stellung für die Gaschromatographie der Nitrophenole bestätigt. Auf dem Chromatogramm in Fig. 1 ist zu ersehen, wie 2-Nitroanisol hinter dem 2-Nitrophenol eluiert. Interessant ist auch die Elution des 2,6-Dinitroanisols, die mit der Tatsache zusammenhängt, dass es sich beim 2,6-Dinitrophenol um eine sterische Hinderung und nicht um einen "*ortho*-Effekt" handelt.

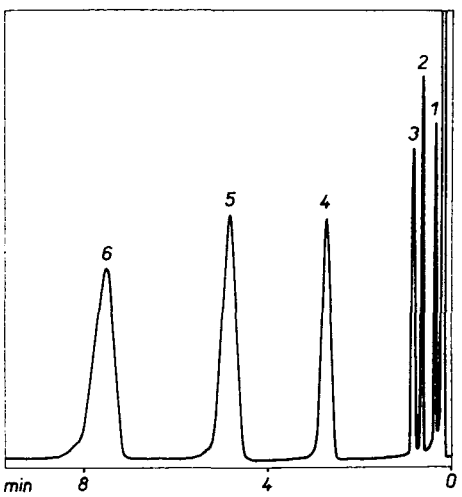

Fig. 1. Chromatogram einiger Nitrophenole und Nitroanisole. 1 = 2-Nitrophenol; 2 = 3-Nitroanisol; 3 = 2-Nitroanisol; 4 = 2,6-Dinitroanisol; 5 = 2,6-Dinitrophenol; 6 = 3-Nitrophenol Trennsäule: 5 % NPGS und 1 % H_3PO_4 bei 185°.

Auf Polyglykol-Säulen ist es daher nicht notwendig, zwecks Erhöhung der relativen Flüchtigkeit jene Dinitrophenole in Methyläther zu überführen, bei denen ein "*ortho*-Effekt" vorhanden ist, weil die intramolekulare Wasserstoffbindung an sich dem Molekül des Nitrophenols eine grössere relative Flüchtigkeit verleiht als es das Ersetzen des phenolischen Wasserstoffs durch die Methylgruppe imstande ist. Bei den übrigen Nitrophenolen hat allerdings die Methylierung der phenolischen Gruppe Bedeutung. Es betrifft diesfalls vorzugsweise die 3,4- und 3,5-Dinitrophenole. Die relative Flüchtigkeit der von ihnen abgeleiteten Methyläther liegt nämlich auf dem Niveau der übrigen Dinitroanisole, auch wenn sich diese Dinitrophenole durch eine grosse Verzögerung auszeichnen.

Auf Grund der Retentionsreihenfolge der Nitroanisole besteht die Möglichkeit, eine bestimmte, wenn auch (im Vergleich mit den freien Phenolen) weitaus schwächere Wasserstoffbindung zwischen dem ätherischen Sauerstoff und den Hydroxylgruppen der stationären Phase anzunehmen. Bei den Mononitroderivaten wächst die Interaktion in Richtung der 3-, 2- und 4-Isomere, was sich mit dem Elektroneneffekt der Nitrogruppe in den angeführten Stellungen in Übereinstimmung befindet (beim 2-Isomer liegt eine teilweise sterische Hinderung des ätherischen Sauerstoffs vor). Unter den Dinitroanisolen weist das 2,6-Isomer die schwächste Interaktion mit der stationären Phase auf (sterische Hinderung), die stärkste Interaktion hingegen das 2,4-Isomer (Konjugation der beiden Nitrogruppen).

Die relativen Retentionsvolumen der Nitroanisole auf Silikon-Säulen sind in Tabelle II enthalten. Der Unterschied zwischen den Retentionsangaben auf diesen Säulen sinkt mit dem Gehalt an polaren Gruppen in der stationären Phase. Auf Silikon-Säulen haben freie Nitrophenole in Übereinstimmung mit Lit. 30 asymmetrische Retentionswellen dargeboten.

TABELLE II

RELATIVE RETENTIONSVOLUMEN DER NITROANISOLE AUF STATIONÄREN SILIKON-PHASEN

Verbindung	*5% XE-60* 200°	*5% QF-1* 200°	*5% SE-30* 180°
2-Nitroanisol	0.25	0.26	0.36
3-Nitroanisol	0.18	0.20	0.33
4-Nitroanisol	0.28	0.31	0.43
2,3-Dinitroanisol	2.59	2.07	1.80
2,4-Dinitroanisol	2.48	2.03	1.76
2,5-Dinitroanisol	1.21	1.04	1.05
2,6-Dinitroanisol	0.66	0.61	0.62
3,4-Dinitroanisol	2.34	1.89	1.67
3,5-Dinitroanisol	1.00	1.00	1.00

ZUSAMMENFASSUNG

Es wurden die gaschromatographischen Eigenschaften von Nitrophenolen und Nitroanisolen auf Polyester- (PEGA, BDS, PEGS, NPGS) und Silikon- (XE-60, QF-1, SE-30) Trennsäulen einer Untersuchung unterzogen. Auf Polyester-Säulen wird die Trennung durch Wasserstoffbindungen zwischen der stationären Phase und den zu chromatographierenden Verbindungen beeinflusst. Auf solchen Säulen weisen Nitrophenole mit intramolekularen Wasserstoffbindungen eine höhere relative Flüchtigkeit auf als die von ihnen abgeleiteten Nitroanisole.

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CHROM. 4320

DETERMINATION OF PROSTAGLANDINS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Two procedures are described for the determination of prostaglandins B, E and F by gas–liquid chromatography. In the first, keto prostaglandins are quantitatively dehydrated, esterified, and acetylated to give products stable to gas chromatography, while F prostaglandins are rapidly analyzed as trimethylsilyl ether-trimethylsilyl esters. In the second procedure, a mixture of prostaglandins is separated into B, E and F class fractions by column chromatography. The B class of prostaglandins, both that present originally and that formed from E prostaglandins by base treatment, is resolved according to degrees of unsaturation by gas chromatography of their acetylated methyl esters. The F prostaglandins are similarly resolved by gas chromatography of their trimethylsilyl ether-methyl esters.

INTRODUCTION

Prostaglandins, pharmacologically active derivatives of prostanoic acid ($C_{20}H_{38}O_2$) have been determined by thin-layer chromatography (*e.g.* refs. 1–3), paper chromatography[4], enzymatic assay[5], fluorescence[6], optical rotatory dispersion and absorption spectroscopy[7], as well as by their effects on smooth muscle preparations *in vitro* and/or blood pressure *in vivo* (ref. 8, review). The trihydroxy (F) prostaglandins have been analyzed by gas chromatography of their trimethylsilyl ether-methyl esters[9], but keto prostaglandins of the E type have not been successfully gas chromatographed without decomposition[2].

Those methods above which are most sensitive, *e.g.*, fluorescence and enzymatic assay, are also least selective in that they do not distinguish between prostaglandins of differing degrees of unsaturation. The highly selective techniques of reversed-phase and thin-layer chromatography lack the desired sensitivity, while the spectroscopic methods suffer interference from many biological materials. The bioassay methods have extreme sensitivity, but complete selectivity requires highly elaborate procedures. The present report is concerned with attempts to develop a sensitive and selective gas chromatographic method for the determination of a variety of classes of prostaglandins.

METHODS

Materials

Prostaglandins E_1, E_2 and $F_{1\alpha}$ were provided through the courtesy of Dr. JOHN PIKE, the Upjohn Company, Kalamazoo, Mich. Prostaglandins B_1, B_2, $F_{2\alpha}$ and $F_{2\beta}$ were synthesized from the appropriate E prostaglandins by base treatment[7] or sodium borohydride reduction[10], respectively.

Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide, distilled into ether and used immediately under nitrogen. Pyridine was distilled from barium oxide and stored over NaOH pellets. Acetic anhydride and dioxane were redistilled shortly before use.

Gas chromatography liquid phases and solid supports were obtained from Applied Sciences Laboratories, State College, Pa., as were 14% BF_3 in methanol, *n*-tetracosane and methoxyamine hydrochloride. Anhydrous diethyl ether was Mallinckrodt AR grade. Silylating reagents were from Pierce Chemical Company, Rockford, Ill. Isopropenyl acetate was obtained from Aldrich Chemical Company, Cedar Knolls, N.J. All other chemicals were the best grades available from Fisher Scientific Company, Raleigh, N. C.

A Perkin-Elmer Model 900 instrument equipped with hydrogen flame ionization detectors, a 1 mV recorder and adapted for direct on-column injection into glass columns was used for the gas chromatography. Mallinckrodt SilicAR CC-4 silicic acid (100–200 mesh) was washed with chloroform, activated at 105° for 2 h, and stored in glass bottles in a desiccator over Drierite until used for column chromatography. Silica Gel GF plates (Analtech), 250 μ thickness, were activated at 105° for 1 h shortly before use, and were developed in lined tanks.

Prostaglandin concentrate

The starting material for the fractionations to follow was an ethyl acetate solution of reference prostaglandins intended to simulate fraction "h" of ÄNGGÅRD[11]. The mixture was loaded onto a column of silicic acid[1] and washed with 20 ml of *n*-hexane–ethyl acetate (7:1) per gram of adsorbent. The prostaglandin "concentrate" was then eluted *in toto* with 40 ml of ethyl acetate–acetone (95:5) per gram of silicic acid. The two fractions were examined by thin-layer chromatography in system D-IV[1].

Preliminary screening of concentrate by gas chromatography

A rapid, qualitative or semi-quantitative procedure was needed for the detection of minute amounts of E and/or F prostaglandins in a variety of samples. Although this could be accomplished by a combination of thin-layer chromatography and bioassay[3], the following approach was developed because of lack of facilities for bioassay.

An aliquot of concentrate from the above procedure was dried with a nitrogen stream, dissolved in 0.5 ml of anhydrous methanol, and treated with 0.5 ml of 14% BF_3 in methanol at 60° in a closed tube for 10 min. The mixture was cooled, diluted with 5 ml of water, and extracted twice with ethyl acetate. The ethyl acetate solution was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated to dryness with a stream of nitrogen.

The residue was treated for 10 min at room temperature with 0.1 ml of a freshly prepared solution of 0.1% 2,4-dinitrobenzenesulfonic acid in isopropenyl acetate. A few grains of anhydrous potassium carbonate were added, and after 15 min aliquots of the solution were examined by gas chromatography as described below.

A second aliquot of the original concentrate was taken to dryness under nitrogen and treated with 0.1 ml of trimethylsilylimidazole–bis(trimethylsilyl)trifluoroacetamide (2:1) for 20 min at room temperature. Aliquots of this reaction mixture were also examined by gas chromatography.

Gas–liquid chromatography of both of the above reaction mixtures was accomplished on a 2 m × 0.5 cm column packed with 3% OV-1 silicone on 100–120 mesh Gas-Chrom Q. The injection port, column, and detector manifold temperatures were 240°, 230° and 280°, respectively. The helium flow rate was 60 ml/min.

Column fractionation of prostaglandins into classes

If the above screening procedures indicated the probable presence of prostaglandins, the remaining concentrate was separated into three fractions. Up to 200 μg of total prostaglandins were loaded onto a column of 1 g of SilicAR CC-4 slurried in *n*-hexane–ethyl acetate (2:1).

A and B prostaglandins (if present) were eluted with 25 ml of *n*-hexane–ethyl acetate (2:1), giving Fraction I. Type E prostaglandins were eluted with 30 ml of *n*-hexane–ethyl acetate (2:3) (Fraction II), and F prostaglandins were eluted with 30 ml of ethyl acetate–acetone (95:5) (Fraction III). This procedure was evaluated with 50 μg each of PGB_1, PGE_1 and $PGF_{1\alpha}$; each fraction was collected in 5 ml portions and examined by thin-layer chromatography in solvent A-I[2].

Derivatization of Fraction I

The dry residue from Fraction I was esterified with diazomethane and the reagent was removed with a stream of nitrogen. The methylated derivative was treated overnight at room temperature with 100 μl of acetic anhydride–pyridine (3:5). One milliliter of water was added, and the mixture was extracted three times with diethyl ether (total 1.5 ml); the ether extract was washed with water, dried over anhydrous sodium sulfate, and filtered. The ether was removed with a nitrogen stream and the residue was dissolved in a few microliters of methylene chloride for gas chromatographic examination.

Derivatization of Fraction II

The dry residue was dissolved in 0.5 ml of ethanol and treated with 0.5 ml of 1 *N* aqueous NaOH at 40° for 30 min. The solution was cooled to room temperature, diluted with 2.5 ml of water, and acidified with HCl. The reaction products were extracted into ethyl ether, washed once with water and taken to dryness under nitrogen, then esterified and acetylated as described for Fraction I.

Derivatization of Fraction III

The dry residue was esterified with diazomethane, the reagent was blown off, and 10 μl of methylene chloride containing 0.30 μg of *n*-tetracosane were added as an internal reference. Forty microliters of trimethylsilylimidazole–bis(trimethylsilyl)-

trifluoroacetamide (2:1) were added and the mixture was let stand for 20 min at room temperature.

If it was desired to remove the unreacted reagent, 1 ml of methylene chloride was added. The solution was washed first with 2 ml of 0.1 *M* potassium bicarbonate buffer pH 7.5, then with 2 ml of water. The methylene chloride phase was dried over anhydrous sodium sulfate, filtered, and concentrated under nitrogen.

Gas–liquid chromatography of methyl esters

The derivatives from Fractions I, II and III were chromatographed on a 2 m by 0.2 cm glass column packed with 3% cyclohexanedimethanol succinate (CHDMS) on 100–120 mesh Gas-Chrom Q. The helium flow rate was 45 ml/min and the detector manifold was at 280°. The column and injection port temperatures were 230° and 240°, respectively, for the acetylated fractions and 185° and 195°, respectively, for the trimethylsilyl ethers. Peak areas were measured as the product of peak height times the width at half the height.

RESULTS

The screening procedure

Thin-layer chromatography in systems A-I and M-I[2] of the products from BF_3–CH_3OH treatment of 100 μg samples of PGE_1 and PGE_2 revealed no detectable starting material remaining. Using the methyl esters of PGE_1, PGE_2 and PGB_1 produced with diazomethane[2] as reference compounds, it was found that the only detectable products from BF_3–CH_3OH treatment co-chromatographed with methyl-PGB_1 and were separated from authentic methyl-PGE_1 and methyl-PGE_2. The methanolysis products gave a positive reaction with the hydroxylamine–ferric chloride spray[12] indicative of esters. The BF_3–CH_3OH treatment thus apparently dehydrated the PGE as well as esterifying it. The dehydration product expected from

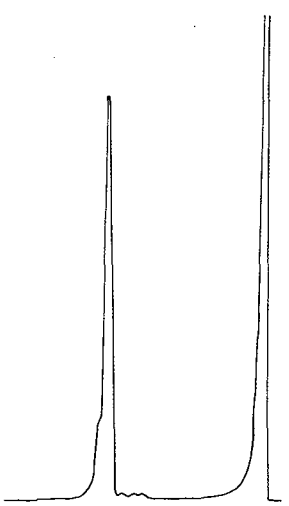

Fig. 1. Gas chromatography of PGE_1 and PGE_2 derivatives on OV-1 at 230°.

acid conditions is PGA[7], but PGA and PGB are not resolved in these solvent systems[1] and the product was not unequivocally identified.

Gas chromatography of the acetylated products on OV-1 gave a single major peak. However, the derivatives of PGE_1 and PGE_2 were not resolved on this column (Fig. 1). $PGF_{1\alpha}$ gave two peaks eluting well after the PGE derivatives under these conditions; accordingly, the F prostaglandins were determined as silylated derivatives.

Prostaglandins $F_{1\alpha}$, $F_{2\alpha}$ and $F_{2\beta}$ each gave a single peak on GLC of their

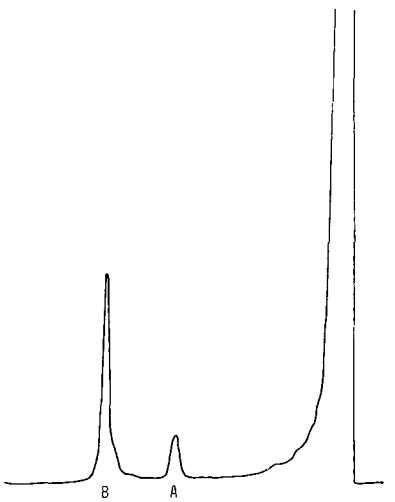

Fig. 2. Gas chromatography of silyl ether-silyl esters on OV-1 at 230°. Peak A = $PGF_{2\beta}$, B = $PGF_{1\alpha}$ and $PGF_{2\alpha}$.

silylation products. While the silyl ether-silyl ester of $PGF_{2\alpha}$ was separated from that of $PGF_{2\beta}$, no separation of silylated $PGF_{1\alpha}$ and $PGF_{2\alpha}$ was achieved (Fig. 2). Prostaglandins E_1 and E_2 did not give stable derivatives with the silylation reagent, and hence did not interfere with detection of F prostaglandins. It is not known whether the E prostaglandins failed to react under these conditions or the derivatives did not elute from the column.

Column fractionation

Complete separation of the three prostaglandin classes was routinely achieved through use of the volumes of eluting solvents indicated earlier. However, recovery of the E class in Fraction II free of contamination by dehydration products required that the ethyl acetate eluant be initially free of acetic acid. This was easily insured by passing it through a small column of F-20 Alumina (Applied Sciences) shortly before use. If this was done, the recoveries of PGE reported for SilicAR CC-4 by ANDERSEN[1] were readily achieved.

The recovery of $PGF_{1\alpha}$ was estimated by gas chromatography, after methylation and silylation of identical aliquots of a $PGF_{1\alpha}$ solution. Half of the aliquots were first chromatographed on SilicAR CC-4 as above, and half were not. On this basis the recovery of $PGF_{1\alpha}$ in Fraction III varied from 92–97%.

Prostaglandins of the B type

The acetylated methyl esters of PGB_1 and PGB_2 were apparently stable under the conditions used and gave resolvable, single peaks on CHDMS (Fig. 3). Acetylated methyl-PGB_1 was stable in dry methylene chloride for several weeks at 0°, and was used as a comparison standard for quantitation of the PGB obtained from Fractions I and II. No significant difference between the detector response to acetylated methyl esters of PGB_1 and PGB_2 was observed, and the limit of detectability for either was approximately 10 ng (for 1 sq. cm peak area).

Prostaglandins of the F type

Tetracosane was included as an internal standard as it was found difficult to store the trimethylsilyl ethers of PGF for long periods without hydrolysis. If this

Fig. 3. Gas chromatography of acetylated methyl-PGB on CHDMS at 230°. Peak A = PGB_2; B = PGB_1.

were not the case, the silyl ether of methyl-$PGF_{1\beta}$ or of methyl-$PGF_{2\beta}$ might be used advantageously as internal standards, since they have not been found occurring naturally[8]. Under the conditions used in this study, the detector response per microgram of $PGF_{1\alpha}$ derivatized was found to be 0.75 ± 0.03 (S.D., three comparisons) times the response per microgram of *n*-tetracosane. The separation achieved is illustrated in Fig. 4.

DISCUSSION

A wide variety of procedures was tested in attempts to form a silylated derivative of PGE suitable for gas chromatography. Hexamethyldisilazane and trimethylchlorosilane in pyridine, bis(trimethylsilyl)acetamide with and without trimethylchlorosilane, trimethylsilylimidazole, bis(trimethylsilyl)trifluoroacetamide and Tri-Sil Z, alone and in various combinations, were applied to PGE_1 or methyl-PGE_1 but in every case the products either did not elute from OV-1 and CHDMS or gave multiple peaks. Attempts were also made to protect the keto group of methyl-PGE_1

by reacting it with methoxyamine in pyridine[13], but the methyl oxime decomposed on gas chromatography. The decomposition of the acetylated methyl ester of PGE_1 reported previously[2] was also observed in the present study; an example of this decomposition is shown in Fig. 5.

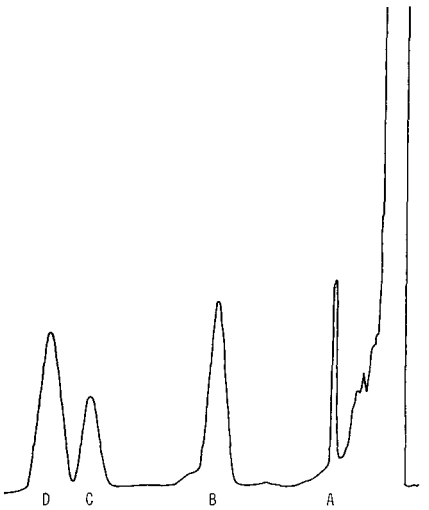

Fig. 4. Gas chromatography of silyl ether-methyl esters on CHDMS at 185°. Peak A = *n*-tetracosane internal standard; B = $PGF_{2\beta}$; C = $PGF_{2\alpha}$; D = $PGF_{1\alpha}$.

The conditions used for the screening procedure permit the detection of approximately 5–10 ng of either E or F prostaglandins. The preliminary steps leading to preparation of a prostaglandin concentrate require varying amounts of time depending on the nature of the starting material; thus, a tissue extract (*e.g.*, ref. 11) requires a more elaborate work-up than a sample of semen (*e.g.*, ref. 9). Starting from the concentrate, the complete derivatization and gas chromatography procedure for the keto prostaglandins requires about 90 min, while that for F prostaglandins in-

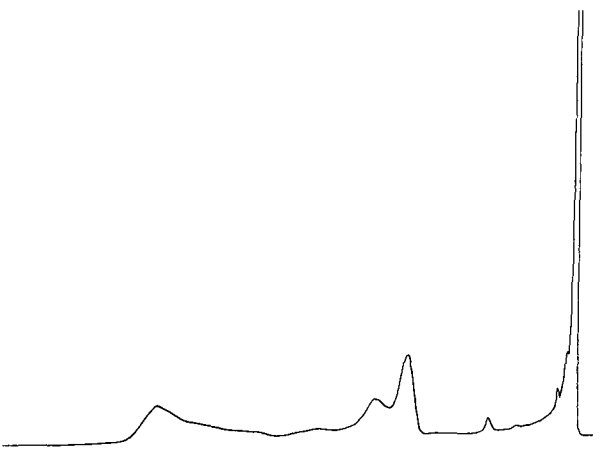

Fig. 5. Gas chromatography of methyl-PGE_1 acetate on CHDMS at 230°.

volves less than 1 h. The lack of selectivity toward unsaturation shown by OV-1 under these conditions is not a disadvantage for screening purposes, as it results in an increased sensitivity to a mixture of prostaglandins of the same class.

Fraction I from the silicic acid column may contain both A and B mono-keto mono-hydroxy prostaglandins[1]. Authentic samples of PGA were not available during the present study, so no data on their gas chromatographic properties are given. There is a tendency for PGE to dehydrate to PGA on handling, so some PGA is to be expected in Fraction I. This could be determined by dividing Fraction I into two portions, gas chromatographing the acetylated methyl esters made before and after treatment with NaOH. The amount of PGB present after NaOH treatment minus the amount present originally would thus be a measure of PGA.

Acetylation of methyl-PGB was accomplished under mild conditions with acetic anhydride–pyridine rather than with isopropenyl acetate and acid as in the screening procedure. Under certain circumstances the latter reagent will react with ketones[14] to give an enol-acetate, and it was considered advisable to avoid this potentiality in the quantitative studies.

The procedure used for analysis of F prostaglandins is a modification of that described by BYGDEMAN AND SAMUELSSON[9]. They used hexamethyldisilazane and trimethylchlorosilane in pyridine to form the trimethylsilyl ethers, evaporating the excess solvent and reagents prior to gas chromatography. This results in a precipitate of ammonium chloride, which occasionally may adsorb small amounts of the derivative. The silylating reagent used in the present work was chosen because it does not "tail" badly on gas chromatography and hence need not be removed from the reaction products; in addition, there is no adsorptive precipitate formed, and the same reagent can be used to form the silyl ether-silyl ester derivatives in the screening procedure. In regard to the latter, it was found that this silylating reagent was less sensitive to traces of moisture than were the others tested.

The trimethylsilyl ether-methyl esters of PGF were chromatographed on CHDMS at a liquid phase loading of 0.5% by the previous workers[9]. The loading was increased to 3% in the present work in order to increase column life. However, the use of a 0.2 cm diameter column here in place of the 0.5 cm used previously[9] gave approximately the same total amount of liquid phase in a 2 m length, permitting a column temperature of 185° in both studies.

The much greater retardation of the acetylated PGB than of trimethylsilylated PGF by CHDMS was not unexpected. This stationary phase has a very high affinity for keto groups (methyl heptadecyl ketone elutes after methyl stearate); moreover, trimethylsilyl groups tend to interfere with the interaction between the ester groups in the stationary phase and the oxygenated functions in the sample[15].

It was somewhat surprising that the more unsaturated prostaglandin F derivatives eluted from a polyester liquid phase ahead of their less unsaturated analogues. Under the conditions used, London dispersion interactions appear to outweigh dipole-induced dipole interactions. This phenomenon may be related to the temperature used, since resolution of PGB_1 from PGB_2 and of $PGF_{1\alpha}$ from $PGF_{2\alpha}$ was found to be diminished by lowering the temperature. A possibly related effect is seen with the methyl esters of ω-3 eicosatrienoic and ω-6 eicosatetraenoic acids on diethylene glycol succinate. At 220° the tetraene elutes after the triene, but at 170° the order is reversed[16].

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CHROM. 4319

BILE ACIDS

XXVIII. GAS CHROMATOGRAPHY OF NEW BILE ACIDS AND THEIR DERIVATIVES

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SUMMARY

The new phases OV-1 and OV-17 are compared with QF-1 in the gas chromatographic analysis of 80 methyl 5β- and 5α-cholanoates and their complete trimethylsilyl (TMSi) ethers. The 5α-cholanoates were slower than their 5β-isomers in elution from the columns by factors of 1.22, 1.11, and 1.20 for QF-1, OV-1, and OV-17, respectively. Methyl α-muricholate can be effectively separated on OV-17 from methyl cholate; the complete TMSi ethers of deoxycholate, cholate, α-, β-, and ω-muricholates can be separated on OV-17 from the TMSi ether of hyocholate. OV-17 resembles PhSi-35 in its polarity and selectivity.

INTRODUCTION

The coupling of gas chromatography with mass spectrometry[1] has provided a new tool for the determination of structure of the components from the effluent of the gas chromatograph. With this new tool has arisen a need for stationary phases with exceptional thermal stability and low bleed rate. A new series of silicones containing 0–65 % phenyl groups in place of methyl groups in dimethylpolysiloxane polymers has provided phases of particular interest to the mass spectrometrist. HORNING *et al.*[2,3] have reported on the use of OV-1 and OV-17 for the separation of urinary acids as the methyl ester trimethylsilyl (TMSi) derivatives and the TMSi derivatives of a number of steroids; SUPINA *et al.*[4] have reported on the evaluation of six of these silicones for the separation of lipids. KUKSIS[5] has studied the chromatography of seven bile acids as their methyl esters on 1 % OV-17, and concluded that this phase is similar in retention characteristics to PhSi-35. The studies reported here present the results of gas chromatographic analysis of a series of 80 bile acids as their methyl esters and their TMSi derivatives on the silicone phases OV-1 and OV-17 in comparison with the fluorosilicone QF-1. Mass spectra were determined to ascertain the structures of the materials studied.

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MATERIALS AND METHODS

Gas chromatography

An F and M Model 402 gas chromatograph fitted with a hydrogen flame detector was used isothermally in these studies. The columns were silanized glass U-shaped tubes (6 ft. × 4 mm, I.D.) packed individually with 3 % OV-1, OV-17, or QF-1 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Since the silicones* OV-1 and OV-17 are reported to be stable up to 350° and 375°, respectively, the following conditions were used: column, 260°; flash heater, 275°; detector, 275°; helium, 40 p.s.i. at a flow rate of 40 cc/min. With the fluorosilicone QF-1 the conditions were: column, 230°; flash heater, 245°; detector, 245°; helium, 40 p.s.i. at a flow rate of 40 cc/min. Methyl deoxycholate was used as internal standard. Relative retention time, R_t, relates to methyl deoxycholate (1.00) or its TMSi derivative (1.00). Absolute retention times of methyl deoxycholate were: on QF-1, 29.0 min; on OV-1, 38.4 min; on OV-17, 44.0 min. For the TMSi derivative of methyl deoxycholate the retentions were: QF-1, 10.0 min; OV-1, 27.3 min; OV-17, 14.2 min.

Mass spectrometry

An LKB Model 9000 Gas Chromatograph Mass Spectrometer fitted with molecule separators of the Becker Ryhage type was used as reported previously[6]. A silanized coiled glass column (6 ft. or 8 ft. × 0.25 in., O.D.) packed with 3 % OV-1, OV-17, or QF-1 on Gas-Chrom Q was used for gas chromatography under the following conditions: ion source, 310°; ionizing energy, 70 eV; ionizing current, 60 μA; for QF-1: flash heater, 240°; column, 215°; molecule separator, 255°; for OV-1 or OV-17: flash heater, 285°; column, 260°; molecule separator, 290°.

Bile acids and derivatives. Cholic, deoxycholic and hyodeoxycholic acids were gifts of the Wilson Laboratories, Chicago, Ill. 3β-Hydroxy-Δ^5-cholenoic acid was obtained from Mann Research Laboratories. All other 5β-acids reported in Table I were available in this laboratory from current or previous studies. Methyl 3β,6β-dihydroxy-5α-cholanoate was a generous gift of Dr. PETER ZIEGLER, Canadian Packers Ltd., Toronto, Canada. All other 5α-acids and their methyl esters were prepared in these laboratories[6–9]. In general, methyl esters of bile acids were obtained from the free acid by treatment with diazomethane. Samples of the methyl esters were prepared for gas chromatography in acetonitrile. TMSi ethers were prepared from the methyl esters with a mixture of trimethylsilyl chloride, hexamethyldisilazane, and dry pyridine as reported by MAKITA AND WELLS[10] and discussed by WELLS *et al.*[11]. The solutions were made up to a concentration of 1–2 μg of methyl ester per μl. After the mixture had stood for a minimum of 10 min on the warm top of the oven, a sample of 1–2 μl of solution was injected into the gas chromatograph. Samples were discarded after 24 h, although decreased amounts of TMSi ethers could be detected as long as four days after preparation.

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* The stationary phases OV-1 and OV-17 are commercially available polymers of dimethylpolysiloxane and phenylmethylpolysiloxane (50 % phenyl), respectively. QF-1 is a commercially available fluorosilicone. Thus, OV-1 is a non-polar phase comparable to SE-30, and OV-17 has polarity intermediate between OV-1 and QF-1.

RESULTS

Table I contains the relative retention times of the 5β- and 5α-cholanoates. SJÖVALL[12], MAKITA AND WELLS[10], KUKSIS[13], and OKISHIO AND NAIR[14] have previously presented data for esters of some bile acids on QF-1; the data of Table I present for the first time a rather complete comparison of retention times for the 5β-derivatives and their new 5α-analogs. Where comparisons can be made, agreement is good between the values of Table I and the published values for the fluorosilicone QF-1, although the data were obtained from columns at different temperatures and different concentrations of the phase on the support. The data in Table I show smaller relative retention times for the non-polar OV-1 than for the other phases. The poorer selectivity of OV-1 for ketones is especially reflected in the grouping of the monoketo monohydroxy derivatives with the diols and the monoketo-diols or diketo monohydroxy derivatives with the triols.

From an analysis of 45 methyl 5β-cholanoates most of the generalizations discussed by KUKSIS[5] are confirmed. Thus, on the more selective fluorosilicone, QF-1, the order of elution of monohydroxy derivatives (in increasing R_t) is C_{12}, C_7, C_6 and C_3 with elution of the axial derivative prior to the equatorial alcohol. With the less selective silicone OV-1 all derivatives are grouped rather closely with little distinction between the axial and equatorial hydroxyl groups at C_{12}, C_7, C_6 or C_3. Results from the column of OV-17 tend to approach that of QF-1, but also show less ability to differentiate between the conformational or configurational isomers.

Among the 5β-dihydroxy derivatives eluted from QF-1, four diaxial diols (7α,12α; 6β,12α; 3β,12α; 3β,7α) are eluted before the equatorial-axial 3α,12α-diol, methyl deoxycholate. The axial 12α- and 7α-hydroxyl groups are shielded by the side chain and the C_{18} angular methyl groups, and the A/B *cis* configuration of the ring structure, respectively, and hence are unlikely to interact appreciably with the liquid phase. The 6β-hydroxyl group encounters 1,3-diaxial interference especially with the C_{19} angular methyl group. This difference is not found in the 5α-series and will be discussed below.

Two pairs each of equatorial/axial:diaxial (3α,12α/3β,12α; 3α,7α/3β,7α) and of diequatorial:equatorial/axial (3α,7β/3α,7α; 3α,6α/3α,6β) diols are eluted from QF-1 as predicted, *i.e.*, the equatorial/axial diol follows the diaxial diol, and is followed by the diequatorial diol. Although, the phases OV-1 and OV-17 are unable to distinguish well between the axial and equatorial 3-hydroxyl groups in the 3,12- and 3,7-diols, the 3α,6-diols are distinguished on QF-1, OV-1 or OV-17.

The four monoketo esters are eluted from QF-1, OV-1 and from OV-17 as predicted in the order of increasing R_t: C_{12}, C_7, C_6, and C_3. On OV-17 the 3,7-dione is eluted prior to the 3,12-dione, whereas the reverse is the case on QF-1. The monohydroxy monoketo derivatives are eluted within a narrower range on OV-17 than on QF-1, and are eluted with or slightly later than the diketones from each phase. The Rohrschneider constants (Table II) for the three phases[4] suggest that a better separation of ketones from alcohols may not be expected for OV-17 over QF-1. Figs. 1 and 2 show the separation of methyl lithocholate, 3-dehydrolithocholate, deoxycholate, ursodeoxycholate, chenodeoxycholate, and hyodeoxycholate on OV-1 and OV-17, respectively. The peaks are well defined and separated on OV-1; the 3α,7-diols are not separated on OV-17. KUKSIS[5] was unable to separate urso- and hyodeoxycholic acids

TABLE I

RELATIVE RETENTION TIMES OF METHYL CHOLANOATES

All relative retention times are referred to methyl deoxycholate = 1.00. Absolute times of elution of methyl deoxycholate are: QF-1, 29.0 min; OV-1, 38.4 min; OV-17, 44.0 min. Methyl deoxycholate was injected simultaneously with each of the esters.

Substituent	*5β*			*5α*			*Ratio 5α/5β*		
	QF-1	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*
None	0.15	0.35	0.20	0.18	0.40	0.24	1.20	1.14	1.20
12α	0.27	0.55	0.39	0.37	0.60	0.49	1.37	1.09	1.21
12β	0.31	0.56	0.41	—	—	—	—	—	—
7α	0.34	0.60	0.44	0.40	0.64	0.51	1.18	1.07	1.16
7β	0.36	0.59	0.46	0.43	0.67	0.52	1.19	1.14	1.13
6β	0.38	0.62	0.47	—	—	—	—	—	—
6α	0.45	0.65	0.52	—	—	—	—	—	—
3β	0.44	0.64	0.51	0.55	0.72	0.60	1.25	1.13	1.18
3α	0.49	0.62	0.52	0.50	0.72	0.58	1.02	1.12	1.12
12-Keto	0.49	0.57	0.43	0.60	0.63	0.51	1.23	1.11	1.19
7-Keto	0.57	0.58	0.46	0.72	0.66	0.57	1.26	1.14	1.24
6-Keto	0.78	0.64	0.58	—	—	—	—	—	—
3-Keto	1.00	0.71	0.61	1.06	0.76	0.71	1.06	1.07	1.16
7α,12α	0.70	0.91	0.84	0.90	0.99	0.99	1.29	1.09	1.18
6β,12α	0.77	0.94	0.91	—	—	—	—	—	—
3β,12α	0.86	1.00	1.00	1.16	1.16	1.20	1.35	1.16	1.20
3β,7α	0.94	1.06	1.10	1.29	1.18	1.34	1.37	1.11	1.22
3α,12α	1.00	1.00	1.00	1.07	1.12	1.19	1.07	1.12	1.19
3α,7α	1.18	1.08	1.14	1.22	1.18	1.27	1.04	1.09	1.11
3α,6β	1.20	1.11	1.21	—	—	—	—	—	—
3α,7β	1.27	1.06	1.13	—	—	—	—	—	—
3α,6α	1.50	1.20	1.32	—	—	—	—	—	—
3β,6β	—	—	—	1.27	1.20	1.29	—	—	—
7,12-Diketo	1.45	0.91	0.85	1.97	0.96	1.06	1.36	1.06	1.25
3,12-Diketo	2.86	1.05	1.29	3.52	1.15	1.51	1.23	1.10	1.17
3,7-Diketo	3.05	1.04	1.21	3.96	1.17	1.52	1.30	1.17	1.26
12-Keto-7α	—	—	—	1.40	1.03	1.12	—	—	—
7-Keto-12α	1.10	1.13	0.88	1.56	1.09	1.13	1.42	0.96	1.28
12-Keto-6β	1.19	0.99	1.11	—	—	—	—	—	—
12-Keto-3α	1.61	1.00	1.12	—	—	—	—	—	—
7-Keto-3α	1.80	1.03	1.20	1.97	1.23	1.42	1.09	1.19	1.18
7-Keto-3β	—	—	—	2.12	1.26	1.41	—	—	—
3-Keto-12α	1.82	1.07	1.17	2.23	1.19	1.41	1.23	1.11	1.21
3-Keto-7α	2.40	1.23	1.43	2.32	1.27	1.60	0.97	1.03	1.12
3-Keto-6α	2.93	1.32	1.59	—	—	—	—	—	—
3β,7α,12α	1.90	1.63	2.08	2.78	1.88	2.71	1.46	1.15	1.30
3α,7β,12α	2.20	1.52	2.07	—	—	—	—	—	—
3α,7α,12α	2.33	1.66	2.26	2.68	1.77	2.73	1.15	1.07	1.21
3α,6β,7β	2.33	1.68	2.30	2.10	1.78	2.34	0.90	1.06	1.02
3α,6β,7α	2.42	1.84	2.65	—	—	—	—	—	—
3α,6α,7β	2.67	1.69	2.25	—	—	—	—	—	—
3α,6α,7α	3.14	1.85	2.56	—	—	—	—	—	—
7-Keto-3α,6α	1.78	1.48	1.59	—	—	—	—	—	—
7-Keto-3α,12α	3.51	1.59	2.27	4.62	1.95	2.89	1.32	1.23	1.27
12-Keto-3α,7α	3.85	1.73	2.57	—	—	—	—	—	—
7,12-Diketo-3α	4.55	1.50	2.18	—	—	—	—	—	—
3-Keto-7α,12α	4.79	1.88	2.79	6.10	1.96	3.12	1.27	1.04	1.12
6-Keto-3α,7β	—	—	—	2.06	1.52	1.88	—	—	—
7-Keto-3β,12α	—	—	—	4.50	1.85	2.87	—	—	—
3,7-Diketo-12α	5.38[a]	1.54	2.31	7.14[b]	1.84	3.04	1.32	1.20	1.32
3,12-Diketo-7α	—[b]	1.91	3.30	—[b]	—[b]	—[b]	—	—	—
3,7,12-Triketo	6.40	1.39	2.25	9.24	1.64	2.98	1.44	1.18	1.32

[a] The peak was not well formed, so this value is tentative.

[b] This material is retained too long on the column to provide meaningful data.

TABLE II

ROHRSCHNEIDER CONSTANTS OF THREE SILICONE PHASES

For a more detailed discussion on these constants and the method of acquisition, see ref. 15.

Phase	*X*	*Y*	*Z*	*U*	*S*
OV-1	0.16	0.20	0.50	0.55	0.48
OV-17	1.30	1.66	1.79	2.53	2.47
QF-1	1.09	1.86	3.00	3.94	2.41

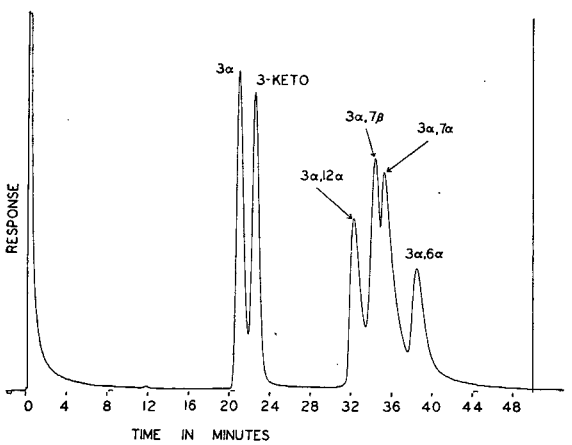

Fig. 1. Chromatographic separation of methyl 5β-cholanoates on 3% OV-1. A mixture of methyl lithocholate (3α), 3-keto-5β-cholanoate (3-keto), deoxycholate (3α,12α), ursodeoxycholate (3α, 7β), chenodeoxycholate (3α,7α) and hyodeoxycholate (3α,6α) was injected into a column of 3% OV-1 on Gas-Chrom Q at 260°. The time of elution of methyl deoxycholate differs from that stated in Table I since a different column was used in this separation.

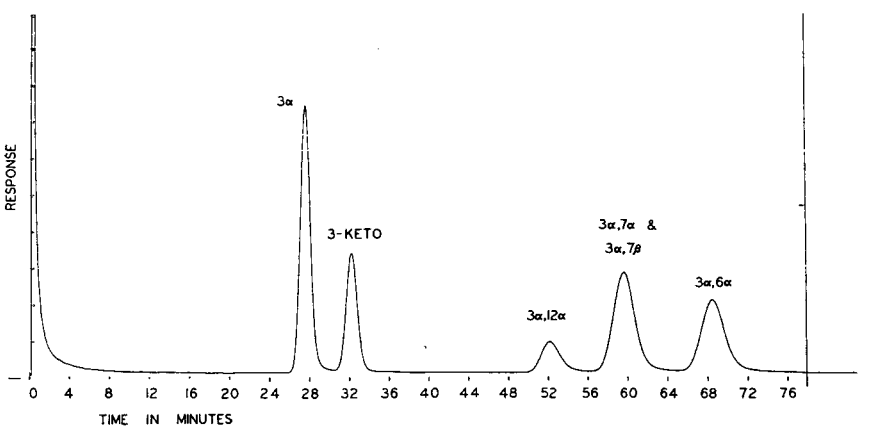

Fig. 2. Separation of methyl 5β-cholanoates on 3% OV-17. The same mixture of esters used for Fig. 1 was injected into a column of 3% OV-17 on Gas-Chrom Q at 260°. Chenodeoxycholate (3α,7α) and ursodeoxycholate (3α,7β) are not separated on this phase.

as their methyl esters on a 4-ft. column of 1 % OV-17 at 235°, or by programmed elution from 230–280°.

Among the trihydroxy derivatives the triaxial triol (3β,7α,12α) is eluted before the equatorial-diaxial triol (3α,7α,12α) from each of the phases, but the non-specific OV-1 shows little distinction between them; QF-1 offers the best separation. However, the diequatorial axial triol (3α,7β,12α) is eluted before cholate on each phase. The order of elution of the muricholates (3α,6,7-triols) varies. From QF-1 the order (6β,7β; 6β,7α; 6α,7β; 6α,7α) differs from that on OV-1 (6β,7β; 6α,7β; 6β,7α; 6α,7α) or that on OV-17 (6α,7β; 6β,7β; 6α,7α; 6β,7α). Thus, of the trihydroxy acids present in rat bile OV-17 offers a separation of α-muricholate (3α,6β,7α-triol, R_t 2.65) from cholate (R_t 2.26), a separation not attained on QF-1 because of the relatively large amount of cholate.

In the 5α-series Rings A and B are *trans* and the 3α- and 3β- substituents are axial and equatorial, respectively, the reverse of the 5β-series. This difference is immediately evident in their order of elution from QF-1 and OV-17, but not from the non-selective OV-1. Three examples appear as contradictions to the generalization that selective phases retain the 5α-cholanoates longer than the corresponding 5β-cholanoates: the ester of the 7-keto-12α-ol on OV-1, and the esters of the 3-keto-7α-ol and the 3α,6β,7β-triol on QF-1. From the ratios of the relative retention times (5α/5β) the longer average retention times of the 5α-derivatives on each of the three phases has been calculated to be: for QF-1, 1.22 (range 0.90–1.49); for OV-1, 1.11 (range 0.96–1.23); and for OV-17, 1.20 (range 1.02–1.32); *i.e.* on the average a 5α-cholanoate would be expected to have a retention time 1.22 times that of the 5β-derivative on QF-1, 1.11 on OV-1 and 1.20 on OV-17*.

Table III contains the relative retention times of the TMSi ethers of the methyl 5β- and 5α-cholanoates. Formation of the TMSi derivatives of the hydroxy esters results in greater volatility as the etherified nucleus tends to approach the hydrocarbon nature of methyl cholanoate; *i.e.* on QF-1 the slowest TMSi derivative of the triols (3α,6α,7β, R_t 1.88) was eluted as the tris-TMSi derivative in 18.8 min. Despite this shorter elution time the retention times of the TMSi ethers respond dramatically to the conformation of the substituents. In general those TMSi derivatives of the 5α-series are retained longer than the corresponding TMSi derivative of the 5β-series in which an axial substituent of the 5β-series appears as an equatorial substituent in the 5α-series. This is particularly true of the 3β(e),5α-derivatives; *i.e.* 3β; 3β,7α; 3β,12α; and 3β,7α,12α-hydroxy-5α-cholanoates are each retained longer than the corresponding derivatives of the 3β(a)-5β-cholanoates. On the other hand the TMSi derivatives of the 3α-ol, 3α,7α-diol, 3α,7α,12α- and 3α,6β,7β-triols of the 5α-cholanoates are eluted from each of the phases before their respective 5β-cholanoates. HOSHITA *et al.*[16] have reported that the TMSi derivatives of methyl *allo*-cholate, *allo*-chenodeoxycholate, and *allo*-deoxycholate each exhibit retention times on 1 % SE-30 of 0.96 relative to that of the TMSi ethers of the corresponding 5β-cholanoates. HOFMANN AND MOSBACH[17] have reported retention times of 0.74 on 0.5 % Hi-Eff 8B and 0.91 on 1 % QF-1 for the TMSi derivative of methyl *allo*-deoxycholate relative to the TMSi ether of methyl deoxycholate. The present studies show that the TMSi ether of methyl

* These ratios also indicate a lower precision in measurement of R_t for those substances eluted substantially later than the standard; *i.e.*, the ratio of R_t for 5α/5β derivatives is generally larger than the average value for substituted ketones.

TABLE III

RELATIVE RETENTION TIMES OF TRIMETHYLSILYL ETHERS OF METHYL CHOLANOATES

All relative retention times are referred to the bis(trimethylsilyl) ether of methyl deoxycholate = 1.00. Absolute times of elution of this derivative are: QF-1, 10.0 min; OV-1, 27.3 min; OV-17, 14.2 min. This derivative was injected simultaneously with each of the TMSi derivatives listed.

Substituent	*5β*			*5α*			*Ratio 5α/5β*		
	QF-1	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*	*QF-1*	*OV-1*	*OV-17*
12α	0.59	0.58	0.62	0.66	0.60	0.64	1.12	1.03	1.03
12β	0.55	0.54	0.56	—	—	—	—	—	—
7α	0.65	0.61	0.63	0.62	0.57	0.57	0.97	0.94	0.91
7β	0.71	0.71	0.75	0.81	0.78	0.83	1.14	1.10	1.11
6β	0.63	0.62	0.63	—	—	—	—	—	—
6α	0.67	0.65	0.72	—	—	—	—	—	—
3β	0.83	0.81	0.86	1.20	0.82	1.20	1.45	1.01	1.40
3α	0.91	0.85	0.93	0.89	0.72	0.92	0.98	0.85	0.95
7α,12α	0.72	0.69	0.60	0.63	0.63	0.53	0.88	0.91	0.88
6β,12α	0.79	0.80	0.69	—	—	—	—	—	—
3β,12α	1.02	1.00	0.90	1.35	1.23	1.19	1.32	1.23	1.32
3β,7α	0.96	0.96	0.87	1.14	1.08	1.00	1.19	1.13	1.15
3α,12α	1.00	1.00	1.00	0.93	1.18	1.22	0.93	1.18	1.22
3α,7α	1.09	1.04	1.00	1.00	0.84	0.92	0.92	0.81	0.92
3α,6β	1.08	1.10	0.98	—	—	—	—	—	—
3α,7β	1.24	1.21	1.14	—	—	—	—	—	—
3α,6α	1.20	1.12	1.08	—	—	—	—	—	—
3β,6β	—	—	—	1.40	1.33	1.31	—	—	—
3β,7α,12α	0.96	0.96	0.79	1.18	1.01	0.79	1.23	1.05	1.00
3α,7α,12α	1.05	1.09	0.90	1.00	1.05	0.87	0.95	0.96	0.97
3α,6β,7α	1.05	1.12	0.84	—	—	—	—	—	—
3α,6α,7α	1.38	1.15	1.11	—	—	—	—	—	—
3α,6β,7β	1.44	1.45	1.18	1.41	1.44	1.17	0.98	0.99	0.99
3α,6α,7β	1.88	1.92	1.71	—	—	—	—	—	—
12-Keto-7α	—	—	—	2.43	0.90	1.18	—	—	—
7-Keto-12α	2.74	0.93	1.35	3.42	0.97	1.54	1.20	1.04	1.14
12-Keto-6β	2.13	0.95	1.27	—	—	—	—	—	—
7-Keto-3α	3.65	1.34	1.98	4.16	1.41	2.30	1.14	1.05	1.16
3-Keto-12α	4.10	1.09	1.67	3.04	1.17	1.85	0.74	0.93	1.11
3-Keto-7α	3.81	1.17	1.84	4.12	1.08	1.60	1.08	0.92	0.87
12-Keto-3α	3.19	1.33	1.91	—	—	—	—	—	—
3-Keto-6α	5.04	1.32	1.99	—	—	—	—	—	—
12-Keto-3α,7α	4.37	1.72	1.97	—	—	—	—	—	—
7-Keto-3α,12α	4.95	1.59	2.03	5.45	1.62	1.95	1.10	1.02	0.96
3-Keto-7α,12α	5.84	1.35	1.68	5.07	1.09	1.45	0.87	0.81	0.92
3,7-Diketo-12α	12.5	1.56	3.10	12.7	1.78	3.76	1.02	1.14	1.21
7,12-Diketo-3α	4.55	1.88	3.38	—	—	—	—	—	—
7-Keto-3β,12α	—	—	—	7.00	1.96	2.46	—	—	—

allo-deoxycholate is eluted before the comparable derivative of methyl deoxycholate on 3% QF-1, but not on OV-1 or OV-17. The data in Table III also show shorter relative retention times of the TMSi ethers of the 5α-cholanoates of the 7α-ol, 7α,12α-diol, and 3-keto-7α,12α-diol on each of the three phases, the 3-keto-12α-ol on QF-1 and OV-1, the 3-keto-7α-ol on OV-1, and the 7-keto-3α,12α-diol on OV-17. The mass spectra of the TMSi ethers of methyl 3-keto-7α,12α-dihydroxy-5α- and 5β-cholanoates

have been analyzed in support of the structure of the former derivative and are clearly bis TMSi ethers[6]. In contrast the TMSi ether of the 3α-hydroxy-7-keto-5α-cholanoate is not eluted more rapidly than its 5β-analog on either of the three phases. The TMSi derivative of the 3α,7β-diol is retained a little longer than that of the 3α,6α-diol on each of the three phases, whereas the 3α,6α-diol is retained longer than the 3α,7β-diol on each phase. The derivatives with the longest relative retention times are those of the keto compounds; by mass spectrometry[6] no evidence was found for the formation of enolic TMSi ethers.

The relative retention times of the TMSi ethers of the trihydroxy 5β-cholanoates indicate that α- and β-muricholates (3α,6β,7α- and 3α,6β,7β-triols) are separable on QF-1, OV-1, or OV-17. Although the trimethylsilyl ethers (TMSi) of these triols are not separable from cholate on QF-1 or OV-1, they are separated on OV-17 (Fig. 3). Fig. 3 also shows the separation of the TMSi ethers of β-muricholate from cholate and α-muricholate in a mixture of these materials in the concentration reported[18,19] in rat bile (0.76:10:0.99). The area under the peaks of elution was measured with an Infotronics Model CRS-108 integrator and the results supported the ratio of injected material.

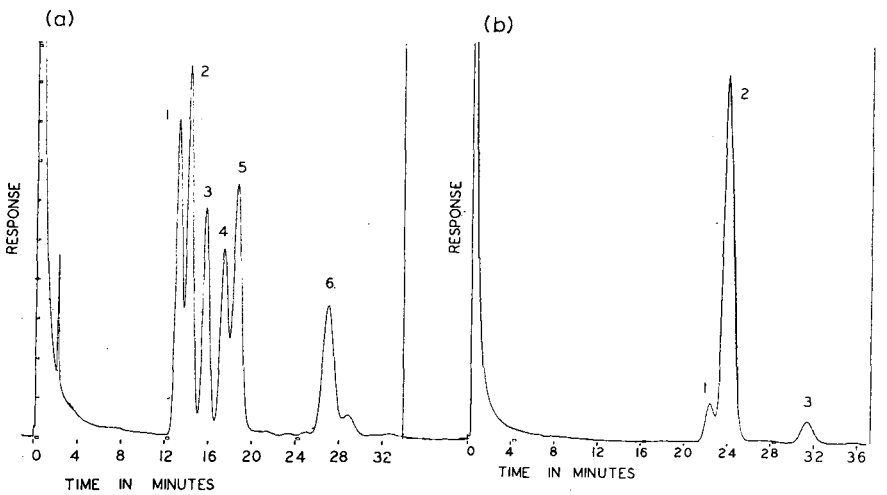

Fig. 3. Separation of TMSi ethers of methyl trihydroxy 5β-cholanoates on OV-17. (a) A mixture of methyl α-muricholate (1), cholate (2), deoxycholate (3), hyocholate (4), β-muricholate (5), and ω-muricholate (6) was converted to the completely silylated ethers and separated on 3% OV-17 on Gas-Chrom Q at 260°. Methyl deoxycholate was used as an internal standard. (b) A mixture of methyl α-muricholate (1) (3α,6β,7α), cholate (2) (3α,7α,12α), and β-muricholate (3) (3α,6β,7β) prepared in a ratio of 0.99:10.0:0.76 as reported for rat bile was completely silylated and separated on 3% OV-17 on Gas-Chrom Q at 260°. A different column of OV-17 was used in this separation.

In 1962 CLAYTON[20–23] proposed that the retention times of a polysubstituted steroid with noninteracting substituent groups was a product of the retention time of the unsubstituted nucleus and a series of constant factors characteristic of each substituent and its position in the molecule. SJÖVALL[12] has shown the concept can be extended to the bile acid series. Data in Table IV extend SJÖVALL's observations on

TABLE IV

RELATIONSHIPS BETWEEN NATURE OF SUBSTITUENTS AND RELATIVE RETENTION TIMES OF SUBSTITUTED METHYL CHOLANOATES

Substituent	*QF-1*				*OV-1*				*OV-17*			
	5α		5β		5α		5β		5α		5β	
	Found	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*	*Found*	*Calcd.*
None	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00	—	1.00
12α	2.06	—	2.07	—	1.50	—	1.57	—	2.04	—	1.95	—
12β	—	—	2.07	—	—	—	1.60	—	—	—	2.05	—
7α	2.22	—	2.27	—	1.60	—	1.71	—	2.13	—	2.20	—
7β	2.39	—	2.40	—	1.68	—	1.69	—	2.17	—	2.30	—
6β	—	—	2.53	—	—	—	1.77	—	—	—	2.35	—
6α	—	—	3.00	—	—	—	1.86	—	—	—	2.60	
3β	3.05	—	2.93	—	1.80	—	1.83	—	2.50	—	2.55	—
3α	2.78	—	3.27	—	1.80	—	1.77	—	2.42	—	2.60	
12-Keto	3.33	—	3.27	—	1.58	—	1.63	—	2.13	—	2.15	—
7-Keto	4.00	—	3.80	—	1.65	—	1.66	—	2.38	—	2.30	—
6-Keto	—	—	5.20	—	—	—	1.83	—	—	—	2.90	—
3-Keto	6.11	—	6.67	—	1.90	—	2.03	—	2.96	—	3.05	—
7α,12α	5.00	4.57	4.67	4.70	2.48	2.40	2.60	2.68	4.13	4.34	4.20	4.29
6β,12α	—	—	5.13	5.24	—	—	2.69	2.78	—	—	4.55	4.58
3β,12α	6.44	6.28	5.73	6.06	2.90	2.70	2.86	2.87	5.00	5.10	5.00	4.97
3β,7α	7.17	6.77	6.27	6.65	2.95	2.88	3.03	3.13	5.58	5.32	5.50	5.61
3α,12α	5.94	5.73	6.67	6.77	2.80	2.70	2.86	2.78	4.96	4.93	5.00	5.07
3α,7α	6.78	6.17	7.87	7.42	2.95	2.88	3.08	3.03	5.29	5.15	5.70	5.72
3α,6β	—	—	8.00	8.27	—	—	3.17	3.13	—	—	6.05	6.11
3α,7β	—	—	8.47	7.85	—	—	3.03	2.99	—	—	5.65	5.98
3α,6α	—	—	10.00	9.81	—	—	3.43	3.29	—	—	6.60	6.76
7,12-Diketo	10.9	13.3	9.7	12.4	2.40	2.61	2.60	2.71	4.42	5.07	4.25	4.95
3,12-Diketo	19.6	19.6	19.1	21.8	2.88	3.00	3.00	3.31	6.29	6.30	6.45	6.56
3,7-Diketo	22.0	25.6	20.3	25.3	2.93	3.31	2.97	3.37	6.33	7.04	6.05	7.02

12-Keto-7α	7.78	7.39	—	—	2.58	2.53	—	—	4.67	4.54	—	—
7-Keto-12α	8.78	8.24	7.34	7.87	2.73	2.48	3.23	2.61	4.71	4.86	4.40	4.49
12-Keto-6β	—	—	7.94	7.85	—	—	2.83	2.89	—	—	5.55	5.05
12-Keto-3α	—	—	10.7	10.7	—	—	2.86	2.88	—	—	5.60	5.59
7-Keto-3α	10.9	11.1	12.0	12.4	3.08	2.97	2.94	2.94	5.92	5.76	6.00	5.98
7-Keto-3β	11.8	12.2	—	—	3.15	2.97	—	—	5.88	5.95	—	—
3-Keto-12α	12.4	12.1	12.1	13.8	2.98	2.85	3.06	3.19	5.88	6.04	5.85	5.95
3-Keto-7α	12.9	13.6	16.0	15.1	3.18	3.04	3.51	3.47	6.67	6.30	7.15	6.71
3-Keto-6α	—	—	19.5	20.0	—	—	3.77	3.78	—	—	7.95	7.43
3β,7α,12α	15.4	13.9	12.7	13.8	4.70	4.32	4.66	4.91	11.3	10.9	10.4	10.9
3α,7β,12α	—	—	14.7	16.2	—	—	4.34	4.69	—	—	10.4	11.7
3α,7α,12α	14.9	12.7	15.5	15.4	4.43	4.32	4.74	4.76	11.4	10.5	11.3	11.2
3α,6β,7β	11.7	—	15.5	19.8	4.45	—	4.80	5.29	10.2	—	11.5	14.1
3β,6β,7α	—	—	16.1	18.8	—	—	5.26	5.35	—	—	13.3	13.4
3α,6α,7β	—	—	17.8	23.5	—	—	4.83	5.56	—	—	11.3	15.5
3α,6α,7α	—	—	20.9	22.3	—	—	5.29	5.63	—	—	12.8	14.9
7-Keto-3α,12α	25.7	22.9	23.4	25.7	4.88	4.46	4.54	4.61	12.0	11.7	11.4	11.7
12-Keto-3α,7α	—	—	25.7	24.3	—	—	4.94	4.94	—	—	12.9	12.3
7,12-Diketo-3α	—	—	30.3	40.7	—	—	4.29	4.80	—	—	10.9	12.9
3-Keto-7α,12α	33.9	27.0	31.9	31.3	4.90	4.56	5.37	5.44	13.0	12.9	14.0	13.1
7-Keto-3β,12α	25.0	25.1	—	—	4.63	4.46	—	—	12.0	12.1	—	—
3,7-Diketo-12α	39.7	45.3	—	—	—	—	4.40	5.29	—	—	11.6	13.7
3,12-Diketo-7α	—	—	—	—	—	—	5.46	5.66	—	—	16.5	14.4
3,7,12-Triketo	51.3	81.2	42.7	82.9	4.10	4.94	3.97	5.49	12.4	13.5	11.3	15.1

QF-1 and include results on the newer phases, OV-1 and OV-17. With the exception of polyfunctional derivatives with long retention times (*e.g.* di- and triketonic esters) agreement between calculated and found values is generally better for the 5β- than the 5α-derivatives. Small errors in calculation in reference to methyl cholanoates are multiplied several times to magnify apparent differences between the calculated and found values. SJÖVALL[12] has commented on the potential interactions between the glycolic hydroxyl groups of the muricholic acids (3α,6,7-triols), and has suggested that such interactions may account for the discrepancies observed with these materials. Similar calculations have been made for the TMSi derivatives in Table III; in general, agreement between calculated and found values is reasonable for the phases OV-1 and OV-17. The data calculated from QF-1 indicate the trend, but the variance between calculated and found values probably reflects a combination of multiple calculations and variation in oven temperature from day to day.

From data at hand it is possible now to demonstrate the effects of change in a given phase by increasing the content of the "polar" group (phenyl, in this case). Thus, data in Table V compare relative retention times from OV-1 and OV-17 with those of SE-30 from VANDENHEUVEL AND BRALY[25], and phases of polysiloxanes prepared for and studied by SJÖVALL, *et al.*[24] with 20 and 35 mole percent of phenyl in place of methyl groups. The relative retention times from OV-17 and PhSi-35 are remarkably similar for all derivatives except the monoketodihydroxy- and diketomonohydroxy-cholanoates. The superior selectivity of ketones by QF-1 is evident

TABLE V

COMPARISON OF RELATIVE RETENTION TIMES OF METHYL 5β-CHOLANOATES ON FIVE DIFFERENT PHASES

Substituent	*OV-1, 3 %* *260°*[a]	*PhSi-20* *215°*[b]	*PhSi-35* *215°*[b]	*OV-17, 3 %* *260°*[a]	*QF-1, 3 %* *230°*[a]	*SE-30, 0.5%* *208°*[c]
None	0.35	0.23	0.20	0.20	0.15	—
12α-	0.55	0.42	0.38	0.39	0.31	0.52
12-Keto	0.57	0.45	0.42	0.43	0.49	—
3α-	0.62	0.54	0.49	0.52	0.49	0.63
3-Keto	0.71	0.62	0.58	0.61	1.00	—
3α,12α-	1.00	1.00	1.00	1.00	1.00	1.00
3,12-Diketo	1.05	1.27	1.25	1.29	2.86	—
3α,7α-	1.08	1.16	1.18	1.14	1.18	1.10
3α,7β-	1.06	1.12	1.16	1.13	1.27	1.09
3α,7-Keto	1.03	1.14	1.21	1.20	1.80	1.04
3,7-Diketo	1.04	1.16	1.21	1.29	3.05	—
3α,6α-	1.20	1.34	1.30	1.32	1.50	1.22
3α,7α,12α-	1.66	2.20	2.32	2.26	2.33	1.72
3α,12α-7-Keto	1.59	2.20	2.36	2.27	3.51	—
3α,7α-12-Keto	1.73	2.62	2.73	2.57	3.85	—
3α-7,12-Diketo	1.50	2.18	2.34	2.18	4.55	—
3,7,12-Triketo	1.39	2.03	2.30	2.25	6.40	—
3α,6α,7α-	1.85	2.41	2.62	2.56	3.14	—
3α,6β,7β-	1.68	2.20	2.24	2.30	2.33	—

[a] This paper.
[b] Ref. 24.
[c] Calculated from data from. ref. 25.

from the data of Table V; however, the failure to separate the 3-keto-cholanoate from deoxycholate on QF-1 can be resolved with OV-1, OV-17, PhSi-20 or PhSi-35. As the phenyl content of these methylpolysiloxanes is increased, the separation of isomeric alcohols is improved[5] and tends to approach the fluorosilicone QF-1.

Relative retention times of eleven TMSi derivatives on five different phases are compared in Table VI. The TMSi ether of cholate is eluted earlier from OV-17 than from any of the other phases. The TMSi ethers of chenodeoxycholate and deoxycholate are not separated on OV-17 or the triphasic column of OKISHIO *et al.*[14,26]. The triequatorial ω-muricholate (3α,6α,7β-triol) is retained the longest on each column. Clearly, the choice of phases must be based upon the desired objectives. These data confirm the desirability of a preliminary separation of bile acids according to the number of substituent groups before chromatography as their TMSi ethers[5].

TABLE VI

COMPARISON OF RELATIVE RETENTION TIMES OF TRIMETHYLSILYL ETHERS OF METHYL 5β-CHOLANOATES ON VARIOUS PHASES

Substituent	*OV-1* 260°[a]	*OV-17* 260°[a]	*QF-1* 230°[a]	*QF-1* 210°[b]	*QF-1* *SE-30*; *NGS*[b]	*SE-30* 238°[c]
12α-	0.58	0.62	0.59	—	—	0.63
3α-	0.85	0.93	0.91	0.94	0.91	0.95
3α,12α-	1.00	1.00	1.00	1.00	1.00	1.00
3α,7α-	1.04	1.00	1.09	1.09	1.00	1.17
3α,7β-	1.21	1.14	1.24	1.06	1.27	1.35
3α,6α-	1.12	1.08	1.20	1.13	1.18	1.25
3α,7α,12α-	1.09	0.90	1.05	1.12	1.04	1.20
3α,6α,7α	1.15	1.11	1.38	1.41	1.41	—
3α,6α,7β-	1.92	1.71	1.88	2.09	2.32	—
3α,6β,7α-	1.12	0.84	1.05	—	—	—
3α,6β,7β-	1.45	1.18	1.44	—	—	—

[a] This paper.
[b] Calculated from data from ref. 26.
[c] Calculated from data from ref. 25.

In these studies* the conditions of MAKITA AND WELLS[10] have been used to form the completely silylated ethers of these esters, as opposed to the procedures of SJÖVALL[12], ENEROTH *et al.*[27,28] and BRIGGS AND LIPSKY[29] for the formation of partial derivatives. GRUNDY *et al.*[30] have identified fecal bile acids as complete silyl ethers on SE-30 or DC-560, but have not published retention times of these derivatives. VANDENHEUVEL AND BRALY[25] have reported on the gas chromatography of methane sulfonates and mixed silyl ethers of bile acids. OKISHIO *et al.*[14,26] have reported favorable results from a column of mixed phases (QF-1, SE-30 and NGS). EVRARD AND JANSSEN[31] have utilized a column of 1 % JXR to study fecal bile acids after oxidation of their methyl esters to the respective keto derivatives.

* Mass spectrometry has been carried out on most of the derivatives in Tables I and III; a manuscript on this subject is in preparation.

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SEPARATION OF PERMITTED AND NON-PERMITTED SOLVENTS FOR USE IN FOODSTUFFS BY GAS CHROMATOGRAPHY AND THE USE OF A SOLID SAMPLER FOR THE ESTIMATION OF RESIDUAL SOLVENTS IN OILS AND OLEORESINS

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SUMMARY

A simple method for the estimation of residual solvents in oils and oleoresins has been developed. This method incorporates the use of a solid sampler which enables only the volatile solvent to be added to the top of the gas chromatographic column, the residual oil being retained in the sample holder, thus preventing contamination of the stationary phase.

INTRODUCTION

Solvents are used in the food industry in two main ways: firstly for the extraction of oils and oleoresins, fixed or otherwise and in the decaffeination of coffee, where the solvent is subsequently removed; and secondly as carriers for food flavourings, vitamins, colorants, etc., where the solvent is essential to ensure adequate mixing of such substances into the foodstuff. The solvents concerned become ingredients of the foodstuff and are consumed; water and ethanol are commonly used. As from November 1969 the Solvents in Food Regulations 1967 (Statutory Instrument 1967, No. 1582) come into force. These regulations permit only ethanol, diethyl ether, ethyl acetate, glycerol and its mono-, di- and tri-acetates, propan-2-ol and propane-1,2-diol to be used as solvents in foodstuffs, where the solvent will form an ingredient of the foodstuff and be consumed as such.

Most of the work published on the detection of solvent residues in foodstuffs has been done on the estimation of traces remaining in solvent-extracted meals, oils and oleoresins. Direct extraction with a suitable solvent and gas chromatographic examination of the extract have been used[1,2]. Gas chromatographic examination of the headspace of oleoresins under suitable conditions has also been employed[3]. The most widely used method where applicable is steam distillation and extraction of the distillate with a suitable extractant and examination of this by gas chromatography[4,5]. In the case of residual solvent in oils direct injection of the oils into the gas chromato-

graph has been used[6] but the problem of column contamination greatly restricts this method. Steam distillation and extraction are lengthy techniques with risk of losses and a quicker method has been sought for the detection of low boiling solvents in oils, oleoresins and other foodstuffs.

Recently the use of solid samplers in gas chromatography has found wide applications in many fields. The main advantage is that there is no masking by a large diluent peak of trace components in a sample. The injection of sintered glass pellets on which the sample has been evaporated has been used for injecting volatile fatty acids[7]. Automatic continuous systems of solid sample injections have been used for the introduction of steroids in small ferrous metal capsules which are subsequently removed from the top of the columns by a solenoid-activated electromagnetic field[8]. Similar systems have been employed using stainless steel gauzes onto which samples have been evaporated and which are dropped from a rotating wheel at appropriate intervals onto the top of a heated column[9]. Encapsulation of samples in polythene or indium capsules which are subsequently melted with the release of volatiles from the sample have been used successfully[10,11]. The use of modified syringes where the sample is held in a needle for a short time and withdrawn through a valve if necessary has been described[12–14]. Sealing of a sample in a glass capillary which is subsequently crushed on top of the gas chromatographic column has also been reported to give reproducible results[15,16]. A solid sampler of this latter type was thought to be most suitable for use in the food field. However, the removal of the crushed capillary and contents following release of the volatiles without interruption of the gas flow

TABLE I

POSSIBLE SOLVENT RESIDUES IN FOOD PRODUCE

	Boiling point
Permitted solvents	
Ethyl acetate	77.2
Ethyl alcohol	78.5
Glycerol	290.0
Glycerol monoacetate	158.0
Glycerol diacetate	280.0
Glycerol triacetate	259.0
Monopropylene glycol	189.0
Propan-2-ol	82.3
Other solvents likely to be used	
Amyl acetate	142.0
Benzyl alcohol	205.0
Butane-1,3-diol	204.0
Butyl acetate	124.0
Diethyl tartrate	280.0
Ethyldigol	202.0
Hexane	68.7
Isopropyl myristate	192.6/20 mm Hg
Methanol	64.7
2-Methoxyethanol	124.0
2-Methylpentane-2,4-diol	206.5
Propan-1-ol	97.2
Triethyl citrate	294.0

TABLE II

RETENTION TIMES OF A VARIETY OF SOLVENTS, INCLUDING THOSE PERMITTED IN FOOD, ON AN ANTAROX CHROMATOGRAPHIC COLUMN

Injection volume: 1 μl of solution in methanol.

Temperature (°C)	*Solvent*	*Retention time* (*min*)
150	Ethyldigol	1.7
	Propane-1,2-diol	1.5
	Butane-1,3-diol	2.7
	2-Methylpentane-2,4-diol	1.7
	Benzyl alcohol	4.7
	Isopropyl myristate	10.6
168	2 Methylpentane-2,4-diol	0.9
	Butane-1,3-diol	1.5
	Benzyl alcohol	2.5
	Isopropyl myristate	5.2
	Glycerol triacetate	5.2
190	Butane-1,3-diol	0.7
	Isopropyl myristate	2.3
	Benzyl alcohol	1.2
	Glycerol triacetate	2.3
	Glycerol diacetate	2.4
	Glycerol monoacetate	4.0
	Diethyl tartrate	7.2
	Triethyl citrate	8.8

would be necessary to prevent contamination of the column by higher boiling components. Such an application has now been developed in this laboratory.

The work discussed in this paper describes the gas chromatographic separation of a wide range of solvents of a type used in food manufacture and the development of a solid sampler in which the sample is sealed in a glass capillary. This sampler has been used to estimate the more volatile solvents; hexane and trichloroethylene in fixed oils and oleoresins have been quantitatively determined.

EXPERIMENTAL

Chromatographic separation of solvent residues

Several stationary phases have been reported in the literature for use in the separation, identification and estimation by gas–liquid chromatography of solvent mixtures. RAWLINSON AND DEELEY[17] used a stationary phase consisting of 8% butane-1,4-diol succinate on hexamethyldisilazane-treated Chromosorb W (80–100 mesh) for the separation of high-boiling esters. WATTS AND HOLSWADE[6] used 10% didodecyl phthalate on Chromosorb P (60–80 mesh) for the separation of hydrocarbon solvents and there are reports on the use of 25% Apiezon L on Celite (60–80 mesh) for the separation of a variety of residual solvents. The solvents used at present and those likely to be used in the future are listed in Table I. This shows that the range of boiling points of these solvents may vary from 50° to 320°. It is therefore likely that more than one type of column will be required in the separation of the variety of possible solvent residues. Many stationary phases were investigated and Tables II

TABLE III

RETENTION TIMES OF A VARIETY OF SOLVENTS, INCLUDING THOSE PERMITTED IN FOOD, ON A PORAPAK S CHROMATOGRAPHIC COLUMN

Injection volume: 1 μl of solution in methanol.

Temperature (°C)	*Solvent*	*Retention time* (*min*)
150	Methanol	1.7
	Ethanol	2.7
	Ethyl acetate	9.0
	Propan-2-ol	4.7
	Propan-1-ol	6.7
	Hexane	10.6
200	Methanol	1.3
	Propan-2-ol	1.7
	Amyl acetate	13.3
	Propan-1,2-diol	5.6
	Butane-1,3-diol	18.6
	2-Methylpentane-2,4-diol	28.6
	Ethyldigol	37.0
	2-Methoxyethanol	3.7
	Benzyl alcohol	38.5
	Glycerol	40.0
220	Glycerol monoacetate	39.0
	2-Methylpentane-2,4-diol	12.4
	Benzyl alcohol	16.0
	Ethyldigol	14.6
	Butane-1,3-diol	8.3
	Amyl acetate	8.9
	Butyl acetate	5.7

and III give the retention times of a number of solvents on the two most successful columns. Table II shows the retention time for a mixture of solvents, using an Antarox column consisting of 8 % Antarox CO-990 absorbed on AW-DMCS Chromosorb W (80–100 mesh) and Table III gives retention times using a Porapak S column (100–120 mesh). Two other stationary phases were also used with some success. A column of 5 % FFAP (free fatty acid phase) on AW-DMCS Chromosorb G (80–100 mesh) acted very similarly to the Antarox column but the use of 25% Apiezon L on Celite (60–80 mesh) gave too long retention times and accurate estimation of trace quantities was not possible. All the columns used were 185 cm long and had an external diameter of 3 mm. Standard solutions of all the solvents were prepared at the 10 p.p.m. level in methanol and 1 μl samples were injected onto the top of the column. A Philips PV 4000 chromatograph was used for this work. The Antarox column was very successful in the separation and determination of most of the higher boiling solvents but in the case of solvents with boiling points of $< 100°$ the eluting peaks became involved with the tail from the methanol. However, the Porapak S column gave excellent separation of the low boiling esters, hydrocarbons and alcohols.

Solvent residues in edible oils

The problem of solvent residues in edible oils exists for all solvent-extracted edible oils which are then refined for human consumption. In the case of olive oil or

castor oil three solvents have been generally used in the past for extraction. These are hexane, trichloroethylene and carbon disulphide. Of these, the latter is now no longer used and the most popular extraction solvent is hexane, trichloroethylene being used to a very limited extent. It is probable that in "residue olive oil", *i.e.* oil extracted from the pressed pulp of the olive, and "which has undergone a complete refining process including deodorization ("stripping") at a temperature which is more than twice as high as the solvent's distillation temperature, no trace of solvent should be found". However, the present method of analysis, that of direct injection onto the top of the column, is not at all satisfactory as it rapidly changes the character of the stationary phase and greatly affects the sensitivity of the detection system. The use of a solid sampler (Fig. 1), however, gets over the problem as only the volatiles are injected onto the top of the column and continuous injection of samples for several weeks has little or no effect on the stationary phase.

Method

The solid sampler (Fig. 1) is connected to a gas chromatographic system con-

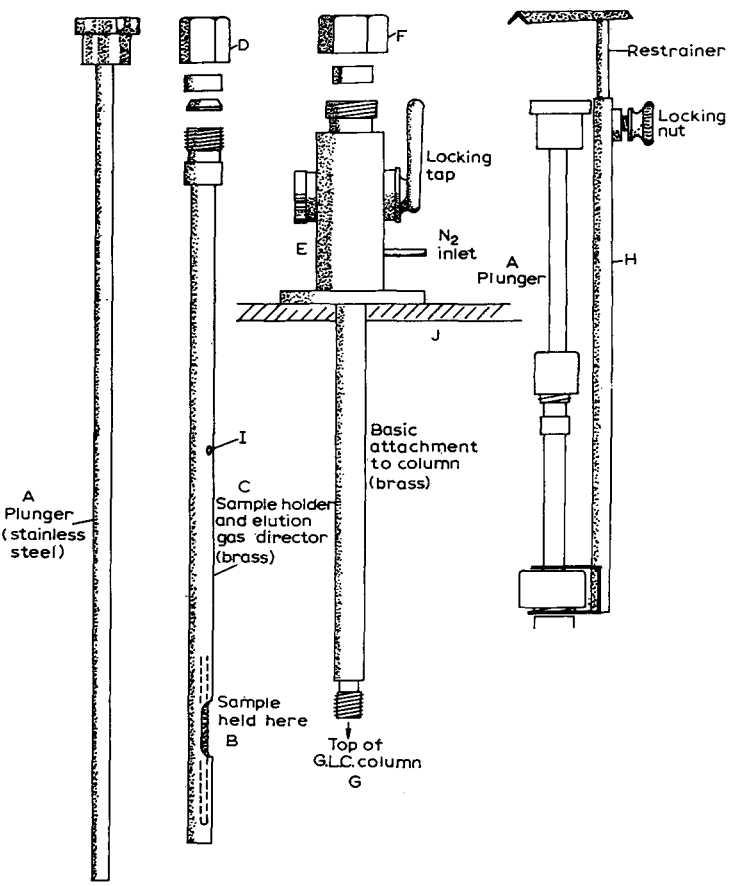

Fig. 1. Gas–liquid chromatography solid sampler.

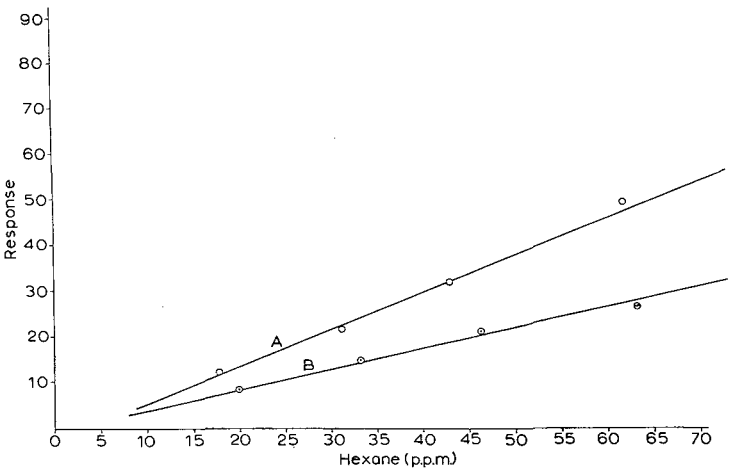

Fig. 2. Response of the flame-ionisation detector to hexane and trichloroethylene in olive oil. A = hexane in olive oil; B = trichloroethylene in olive oil.

sisting of a 185-cm Porapak S (100–120 mesh) column of 3 mm O.D. operated at 200°. Hexane and trichloroethylene residues are estimated in olive oil and castor oil.

The method of handling the sample is determined by its viscosity. Olive oil is injected into a capillary tube 12.5 mm long and sealed at one end, using a 10 μl microsyringe. Castor oil is transferred to the capillary using an Agla syringe. The tube is sealed at the other end and placed in compartment B of the solid sampler (Fig. 1). The plunger A is inserted into the top of the cylinder C and an airtight seal is made by fastening nut D. The assembled cylinder and plunger are then inserted in the outer cylinder E, and an airtight seal is made by fastening nut F. The outer cylinder E passes through the top of the oven J of the chromatograph and is connected to the chromatographic column G. With the carrier gas supply switched on and restrainer H in position to prevent ejection of the plunger, the sample is left in compartment B for 3 min to reach equilibrium. The plunger is then depressed until the glass capillary

TABLE IV

RECOVERY OF *n*-HEXANE AND TRICHLOROETHYLENE FROM OLIVE OIL

Solvent	*Added (p.p.m.)*	*Recovered (p.p.m.)*	*Retention time (min)*	*Recovery (%)*
Hexane	5	4.5	2.6	90
	10	9.1	2.6	91
	20	18.2	2.6	91
	50	49.1	2.6	98
	100	99	2.6	99
Trichloroethylene	5	4.7	4.0	95
	10	9.8	4.0	98
	50	50	4.0	100
	100	100	4.0	100

is broken and then raised again until the bottom of the plunger is just above the level of the vapour outlets I. This allows the volatile vapours from the sample to pass through I down onto the column. The plunger is kept in position by adjustment of restrainer H. The components in the sample are detected using a flame-ionisation detector. When the analysis is complete, F is loosened and C and A are removed together and the remaining glass and oil held in B are removed and the next sample is placed in position.

Fig. 2 shows the response of the flame-ionisation detector to olive oil samples containing standard hexane and trichloroethylene residues and Table IV shows the recoveries obtained for hexane and trichloroethylene for various concentrations of these solvents in olive oil. Traces down to 5 p.p.m. (of these solvents in oils) can be estimated and continuous injection over a number of weeks does not affect the column or the retention time of the solvents.

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CHROM. 4293

THE DETERMINATION OF CHLORINATED HYDROCARBONS USING THE NEGATIVE ALKALI FLAME DETECTOR RESPONSE*

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SUMMARY

Under specific instrumental conditions, the alkali flame detector will give negative peaks for chlorine-containing compounds, and positive peaks for Br-, I-, N-, or P-containing compounds. In such a mode, the detector can be used for qualitative and quantitative determination of chlorides. It discriminates against carbon compounds by approximately three orders of magnitude, detecting a minimum amount of 1 ng of chlorobenzene. The negative response can be used to detect chlorinated hydrocarbon pesticide residues in soil at levels between 0.01 and 10 p.p.m. without purification of an exhaustive hexane extract.

INTRODUCTION

Gas–liquid chromatography combined with electron capture detection is by far the most successful method in the determination of chlorinated hydrocarbon residues. The method possesses superior sensitivity, and, if proper purification procedures are followed, yields quantitative results. In the determination of very low amounts of residues, however, special problems arise. When extraneous peaks originating from co-extractants start to appear in greater number in the chromatogram, there is usually some doubt shed on the identity of the peaks supposedly representing chlorinated hydrocarbons. Although the use of a different type of GLC column will generally dispell some of the doubt, the accuracy of the analysis is by no means assured. To complicate matters further, none of the commonly employed means of establishing identity (IR, MS, etc.) are easy to use with very low pesticide levels.

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To a much lesser degree than the electron capture detector (ECD), the alkali flame detector (AFD, 'thermionic detector', 'Karmen–Giuffrida detector', 'phosphorus detector', refs. 1 and 2) has been used for the detection of volatile halides (refs. 3–9 and others). Most authors agree that its response to halogen is inferior to phosphorus; and that the ECD is by far preferable to the AFD for the analysis of chlorinated hydrocarbons (*e.g.* ref. 17).

It is a well-known fact to most analysts working with the AFD that it may produce negative peaks at certain flow conditions. The reason for this behavior is unknown.

This study was designed primarily to investigate this effect and to find instrumental conditions, at which a satisfactory negative response for chlorides in general and chlorinated hydrocarbon insecticides in particular, could be obtained; maintaining at the same time the positive response of the detector toward other heteroatoms (Br, I, N, P) and carbon compounds in general. Such detector characteristics would make it feasible to distinguish the volatile chlorides from any other compounds appearing on the chromatogram. As a secondary objective, the application to crude (non-purified) soil extracts and a subsequent attempt at quantitation were considered important.

Preliminary experiments

Former studies of the AFD[9] had shown that different elements exhibit response maxima in different areas of the flame. Small changes in the instrumental parameters (*i.e.* the H_2, N_2, and air flow, the shape and size of the electrode, and the shape of the alkali source) can have a marked influence on the response of various species. In order to arrive at the best conditions for an optimal (negative) response/noise ratio, the electrode and alkali source dimensions were evaluated in preliminary experiments. Single loop platinum electrodes of inner diameters 3, 5 and 7 mm were used, matched with alkali sources similar to one described earlier[10]. Ceramic beads coated with rubidium sulfate, pressed and drilled rubidium and potassium chloride pellets (ref. 11, compare with ref. 12), and pressed and drilled rubidium sulfate pellets were tested. The latter, although the most difficult to prepare, gave the best results and were used exclusively for later studies. High-speed drilling was used to obtain bores between 1 and 4 mm in 1 mm steps. This produces a variety of flame shapes from a tall and narrow flame to a short and broad one. Twelve combinations of electrode and alkali source were evaluated with chlorobenzene, bromobenzene, iodobenzene, benzylamine, and tri-*n*-butylphosphate, and the direction and maximum of response measured over electrode distances ranging from 1 to 10 mm above the alkali source at a variety of flow conditions.

Negative response could be obtained for each of the active elements at particular conditions. The exact location and the maximum of the negative range, however, depended to a great deal on the particular alkali source (bead or pellet), and were not *exactly* reproducible with another source of approximately the same dimensions. This was obviously caused by the fact that, in our experiments, the surface structure and dimensions of a pellet were not completely reproducible, and the shape and the alkali content of the flame varied accordingly.

We decided from these preliminary studies to use a 1 mm bore pellet and a 7 mm I.D. electrode for the detection of chlorides.

EXPERIMENTAL

A Barber-Colman 5320 gas chromatograph was modified as follows: The aquarium pump air supply was replaced with a high pressure air cylinder with regulator and needle valve; the hydrogen supply was taken from a tank through a differential flow controller and a flow equalizer-activated carbon filter arrangement. The Variac-controlled heating system of the column bath was replaced by a lab-made thermostat, and the injection port modified by drilling through the injection block and extending the glass column up to the septum, to allow direct on-column injections into a 'swept-septum' configuration.

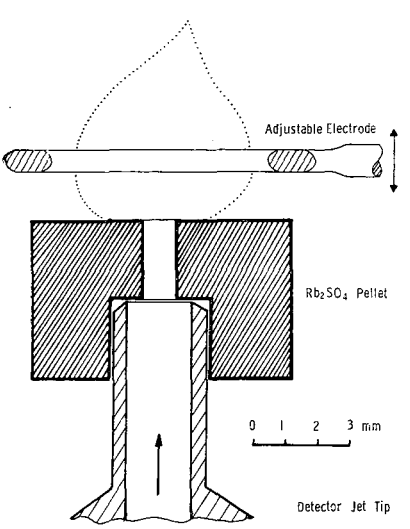

Fig. 1. Alkali-flame detector modification.

Fig. 2. Detector selectivity. Electrode: 7 mm I.D., set 4 mm above the pellet, + 240 V. Pellet: pressed Rb_2SO_4, 1 mm bore. Flow rate: H_2 33, N_2 50, air 215 ml/min.

A coiled pyrex column of 1.7 m length and 3.5 mm I.D. was filled with a mixed stationary phase suitable for pesticide analysis, 9.8% DC-200 + 15.8% QF-1 (50/50 w/w, see ref. 13) on Anakrom ABS, 90/100 mesh, prepared in fluidized bed. The basic design of the AFD with a vertically adjustable electrode as described earlier[14], was used with the following modifications: A steel cylinder surrounded the flame area; it was cut to allow free movement of the electrode. This electrode was a slightly flattened single loop of 7 mm I.D., made from platinum wire 1 mm thick. The alkali source was a pressed, high-purity rubidium sulfate pellet (K & K, 99.9% Rb_2SO_4, fractionally recrystallized from double distilled water). With a screw-type press similar to models used in IR work, pellets were produced from finely ground, slightly moist rubidium sulfate. The pressing and high-speed drilling of the pellets takes some practice before a satisfactory product can be obtained. A good pellet, however, will perform for several months without apparent deterioration. A scheme of the detector is shown in Fig. 1.

Optimization of detector response

The height of the electrode and the flow rates (especially H_2) were changed in small increments while repeatedly injecting a mixture of chlorobenzene, bromobenzene, and iodobenzene in hexane, until an optimal response/noise ratio for negative chlorine response had been obtained (*e.g.* Fig. 2). In a typical case, the pellet bore was 1.0 mm and the electrode approx. 1.5 mm (1.4–1.6 mm) above the Rb_2SO_4 surface. The hydrogen flow was 33, nitrogen 50, and air 215 ml/min. These conditions were then used to determine response factors for all other tested elements (Fig. 3).

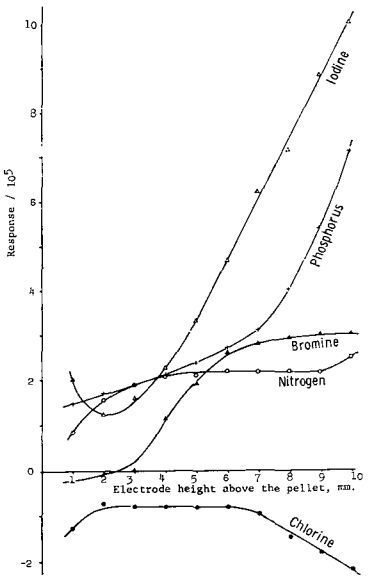

Fig. 3. Effect of electrode height. Electrode: 7 mm I.D. Pellet: pressed Rb_2SO_4, 1 mm bore. Potential: + 240 V. Flow rate: H_2 33, N_2 50, air 215 ml/min. Injections: 1 μl of hexane solutions containing 1 μl/ml each of chlorobenzene, bromobenzene, iodobenzene and benzylamine and 10 μg/ml of tri-*n*-butylphosphate.

The response was calculated as

$$R = \text{Peak area } (\text{cm}^2) \cdot \frac{\text{attenuation}}{\text{sensitivity}} \cdot \frac{1}{\text{weight injected (g)}}$$

in cm^2/g for all elements except phosphorus. To keep phosphorus within the limits of Fig. 3, its response was calculated in cm/0.1 g. For purposes of comparison, 1 cm^2 is roughly equivalent to 7×10^{-6} Coulombs.

Determination of selectivity ratio

Essentially the same approach as reported earlier[14] was used to illustrate the practical performance which can be expected from such a system. At conditions optimized for negative chlorine response, Lindane was compared to *n*-octadecane on a

weight basis. Taking the FID response as a standard, the selectivity ratio (SR) was calculated as in the following example:

$$SR\left(\frac{\text{Lindane}}{\text{octadecane}}\right) = \left(\frac{A_{Cl}}{A_C}\right)_{\text{alkali flame}} \times \left(\frac{A_C}{A_{Cl}}\right)_{FID}$$

wherein A_{Cl} and A_C represent the peak areas obtained from the chlorocompound and the hydrocarbon, respectively.

Soil spiking and analysis

Menfro silt loam with no history of pesticide treatment (courtesy of Dr. BILLY TWEEDY, Department of Plant Pathology, University of Missouri, Columbia) was spiked with 10, 1, 0.1 and 0.01 p.p.m. of Lindane, Aldrin and heptachlorepoxide, as well as Dieldrin at double and *p,p'*-DDT at five times this concentration (heptachlor was inadvertently omitted). Samples of the spiked soil (approx. 25 g) were placed into thimbles and extracted for 16 h in a Soxhlet or 8 h in a Goldfisch apparatus (Fisher Scientific Company, St. Louis, Mo.) with hexane. The resulting extracts were taken to a volume of 1 ml in a Kuderna-Danish evaporator, and 1 μl injected. In case of the 0.01 p.p.m. spiked soil, the extract was concentrated to 0.1 ml. Solutions of pesticide standards (Fig. 4, Parathion was added to show the positive response for phosphorus) were used to determine linearity of the detector (Fig. 5) and the recovery in the soil extracts. After chromatographic separation at 190° (Fig. 6), the area of the peaks was determined for use in a calibration curve (Fig. 7).

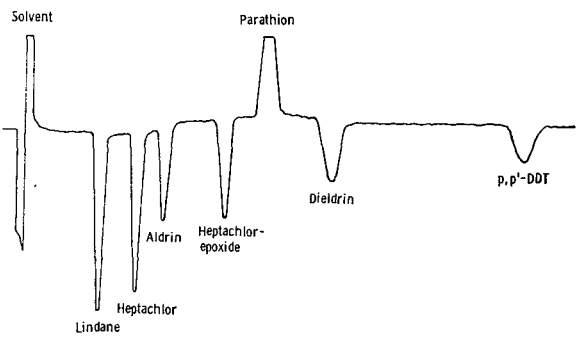

Fig. 4. Standard mixture of pesticides. Electrode: 7 mm I.D., set 1.5 mm above the pellet. Pellet: pressed Rb_2SO_4, 1 mm bore. Potential: + 240 V. Flow rate: H_2 33, N_2 50, air 215 ml/min. Column: 9.8% DC-200 + 15.8% QF-1 (50/50 w/w) on Anakrom ABS, 90/100 mesh, prepared in fluidized bed. Column bath 190°, injection port 220°, detector 210°. Injection: 1 μl of a hexane solution containing 10 p.p.m. of each of the chlorinated hydrocarbons and 1 p.p.m. of Parathion.

RESULTS AND DISCUSSION

Considering the simplicity of the alkali flame detector design, it is amazing how many different modes of performance it is capable of assuming. In this study, some basic parameters were altered in preliminary experiments in order to find a system, which would produce negative response to chlorine containing compounds with a response/noise ratio acceptable for pesticide residue analysis. Several configurations showed promise; Fig. 1 shows the principal configuration of the detector version finally chosen for detailed evaluation.

After this system had been optimized for chlorine response, the minimum detectable amount of chlorobenzene was approx. 1 ng. The minimum detectable amounts of several chlorinated hydrocarbons were somewhat lower, but still approximately three orders of magnitude higher than those obtainable with an ECD. The response of the ECD, however, depends to a much higher degree on the amount of halogen substitution than the AFD, whose response is proportional to the amount of halogen introduced.

While the AFD is clearly inferior to the ECD in terms of sensitivity, it can provide a qualitative test for the presence of chlorine in a compound available only in trace quantities. Obviously, this test will be erroneous if the compound contains phosphorus in addition. The results obtainable from compounds which contain other

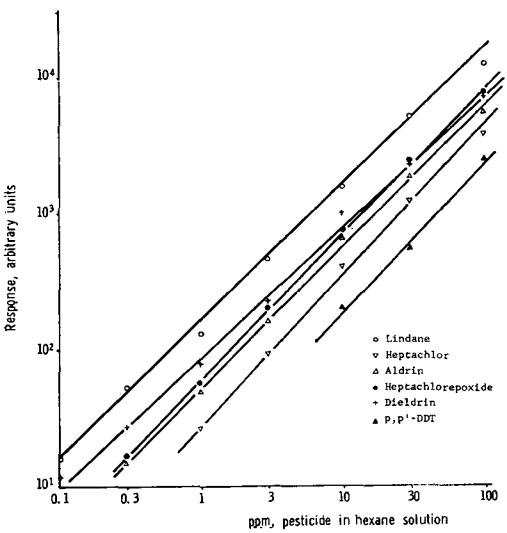

Fig. 5. Linear range of detector response. Electrode: 7 mm I.D., set 1.5 mm above pellet. Potential: + 240 V. Pellet: pressed Rb_2SO_4, 1 mm bore. Flow rate: H_2 33, N_2 50, air 215 ml/min. 1 μl injections.

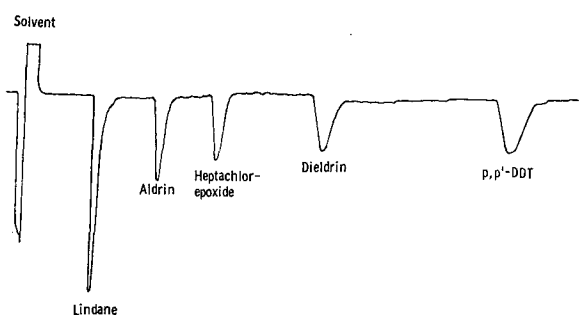

Fig. 6. Crude soil extract. Menfro Silt Loam spiked with 1 p.p.m. each of Lindane, Aldrin, heptachlorepoxide, 2 p.p.m. of Dieldrin and 5 p.p.m. of *p,p'*-DDT. Soxhlet extraction with hexane. Electrode: 7 mm I.D., set 1.5 mm above the pellet. Pellet: pressed Rb_2SO_4, 1 mm bore. Potential: + 240 V. Flow rate: H_2 33, N_2 50, air 215 ml/min. Column bath 185°, injection block 220°, detector 200°.

halogens or nitrogen, besides chlorine, would suffer from some degree of ambiguity, depending on the particular conditions involved. In actual residue analysis, however, these cases are relatively rare and the analyst is more often faced with the question, whether a particular peak in the chromatogram represents a chlorinated hydrocarbon residue or an unknown (and unwanted) co-extractant. For these cases, the AFD, functioning in a negative mode for chlorine, should allow an easy decision. A similar answer can be obtained from the microcoulometric detector, which does not, however, distinguish between the halogens.

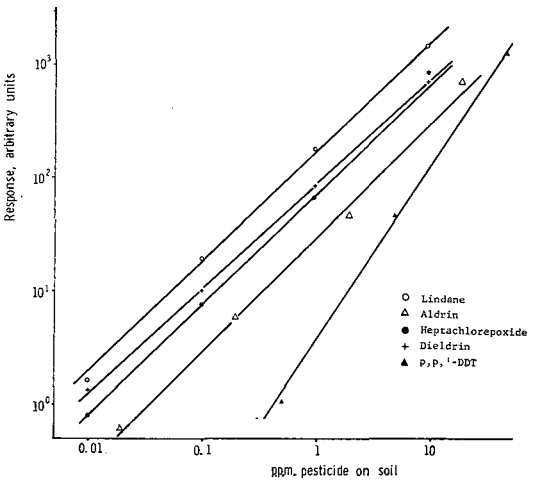

Fig. 7. Chlorinated hydrocarbons from soil. Menfro Silt Loam spiked with a mixture of Lindane, Aldrin, heptachlorepoxide, Dieldrin and *p,p'*-DDT (weight ratio 1:1:1:2:5); Soxhlet extracted with hexane. Electrode: 7 mm I.D., set 1.5 mm above the pellet. Pellet: pressed Rb_2SO_4, 1 mm bore. Potential: + 240 V. Flow rate: H_2 33, N_2 50, air 215 ml/min. Column: 9.8% DC-200 + 15.8% QF-1 (50/50 w/w) on Anakrom ABS, 90/100 mesh, prepared in fluidized bed. Column bath 185°, injection port 220°, detector 200°.

Fig. 2 shows an example of the detector set to distinguish clearly between chlorine and the other halogens. (Fluorine is not comparably enhanced in the AFD.) Once the detector dimensions and flow conditions are given, the distance of the electrode from the Rb_2SO_4 surface, 'electrode height', is the determining parameter for size and sign of the response and the consequent discrimination against other elements (Fig. 3). It should be emphasized that such response profiles can vary a great deal with small changes in detector dimensions and flow conditions. For the chosen set of conditions, however, chlorine response was always negative, bromine either negative or positive and the other elements were positive throughout the range of electrode heights. The question what setting to use in a particular analysis depends on a number of factors, such as the response/noise ratio and the selectivity ratio towards unwanted co-extractants. For example, the analysis for hydrocarbons in soil (Figs. 6 and 7) called for a high selectivity ratio towards carbonaceous material and low-noise characteristics, which were best achieved with an electrode height around 1.5 mm.

The detector linearity was tested with a variety of chlorinated hydrocarbon standards (Fig. 4; Parathion is included to show the positive response for phosphorus).

The results of this study are satisfactory (Fig. 5), with aberrations apparently due to changes in detector sensitivity during the course of the experiment. For accurate measurements, an internal standard may have proved advantageous.

The 'solvent peak' on the chromatograms (Figs. 2, 4, and 6) shows a negative and a positive part. This is due to a disturbance of the detector, since smaller amounts of carbon compounds give symmetric, positive peaks. If the solvent peak is kept on scale, its area is almost entirely positive.

The discrimination of the AFD against carbon compound background lies in a range, which could permit gas chromatographic analysis of unpurified extracts from various types of samples, provided their residue content were not extremely low. As a measure of this discrimination, the selectivity ratio (SR) based on a comparison with the response characteristics of the ubiquitous hydrogen flame detector, was measured with Lindane and octadecane as model substances for a chlorine and a carbon compound.

The value obtained was SR (Lindane/octadecane) = 3100, which stems from the ratio of responses from the AFD obtained for equal weight amounts (1040) and the same ratio from a regular FID (0.33). This particular approach to characterize selectivity was chosen from an entirely practical viewpoint. The SR values depend, of course, on the response of the standard in the FID and will, therefore, vary with the choice of the standard. They provide, however, a good practical indication of the amount of interference which can be expected to arise from volatile co-extractants; or the selectivity to be gained for chlorocompounds *vs.* hydrocarbons when the analysis is switched from a FID to an AFD.

Although phosphorus response is still greater than chlorine response – as seen from SR (Lindane/tri-*n*-butylphosphate) = 0.105 at conditions optimized for chlorine – the figure is much better than values found in the literature, which generally range two or three orders of magnitude lower. Using different pellet and electrode dimensions, this value could quite conceivably be shifted further in favor of chlorine.

To test the detector with an actual analysis, the common case of a soil analysis for chlorinated hydrocarbons was chosen. No purification of the extract was attempted; however, the initial extraction was done in a Soxhlet or Goldfisch apparatus with hexane for an extended period of time; a procedure designed to minimize the amount of co-extractants[15,16]. Since continuous extractions run practically unattended, the amount of time spent for sample preparation was minimal.

As can be seen from Figs. 6 and 7, the results obtained would satisfy the requirements for a screening analysis. The recoveries for all pesticides except *p,p'*-DDT are acceptable at the 10–0.1 p.p.m. levels (80–100%). The scope of this work did not include the analysis of vegetables, fruits, tissues, or biological fluids, and none of these types of analyses were attempted. Judged from the results of a study involving nitrogen containing herbicides with similar selectivity ratios[14], such an investigation would be definitely worthwhile.

It should be noted in the context of routine analysis, that we found the AFD quite temperamental in the described mode. It is easily disturbed, prone to sudden baseline shifts, and constant attention of the operator is required in the high-sensitivity ranges. Although the device is simple (and inexpensive), it takes time to adjust the conditions for optimal performance. Thus, it will definitely not replace the EC detector for quantitative analyses.

In summary, the AFD in its described version, may have applications for the qualitative and semi-quantitative analysis of volatile halides in screening and identity tests.

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CHROM. 4288

SINGLE-STAGE CLEAN-UP OF ANIMAL TISSUE EXTRACTS FOR ORGANOCHLORINE RESIDUE ANALYSIS

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SUMMARY

The use of column chromatography in the clean-up of hexane extracts of animal tissues for pesticide analysis is described, using dry, partially deactivated alumina and silica columns to remove fat and other unwanted materials. By limiting the fat loading of the columns, complete elution of organochlorine residues, free of substances interfering in gas chromatographic analysis, is achieved.

Two types of alumina, of different activities, and one type of silica have been tested and the order of elution of fourteen organochlorine residues determined. Alumina, activated at 800° and partially deactivated with 5% by weight of water, was found to be the most efficient. Silica columns were more effective in the separation of pesticides by differential elution.

INTRODUCTION

The methods most commonly used in pesticide analysis for the removal of fats and other interfering substances from solvent extracts of dairy products and animal tissues involve a liquid–liquid partition stage followed by a column chromatography stage. The first typically employs hexane and acetonitrile[1,2], or hexane and dimethylformamide (DMF)[3], and several successive extractions are made into the more polar solvent (acetonitrile or DMF). This solvent is then diluted with a large volume of water and the pesticides are transferred back to hexane, which is finally passed through an adsorbent column in the second stage to remove the final traces of fats and pigments. For the second stage, Florisil[2] or partially deactivated alumina[3] is used, and with Florisil it is possible to obtain a useful separation[4] of organochlorine residues into two groups by eluting with solvents of different polarity. Unfortunately the variability among batches of Florisil necessitates the testing of individual columns to determine the exact volumes of the two solvents required to produce the desired separation.

Single-stage clean-up of plant extracts can usually be achieved by column chromatography alone, but many animal tissue extracts contain too much lipid material for such clean-up to be sufficient for analysis by gas chromatography. The columns are normally prepared by slurrying Florisil or alumina into chromatographic tubes

with solvent, and the samples for clean-up are added to the solvent-wet columns. The efficiency of recovery of added residues from these columns is usually high.

In two-stage clean-up processes, a significant fraction of the total pesticide content of an extract is lost at the liquid–liquid partition stage, the percentage loss varying with the nature of the pesticide. Recovery from the column chromatography stage is usually complete, however, and the use of column chromatography alone would therefore offer an advantage in quantitative analysis. Furthermore, it is usual to obtain a relatively large final volume of extract of which only a few microlitres are taken for GLC analysis, the remainder being discarded if not required for other methods used in confirmation of identity. By concentrating the original extract to a suitable degree before clean-up, and obtaining a small volume of final solution, an appreciable saving of both adsorbents and solvents can be achieved. For this reason small columns have been developed, using fresh adsorbent for each sample. This also avoids the possibility of contamination between samples, which may occur if large columns are used repeatedly. The first part of this paper describes the use of dry alumina powder, in columns of small dimensions, which has been found to give results superior, both qualitatively and quantitatively, to the two-stage technique previously employed. In the second part, the alternative use of silica gel is discussed, this adsorbent being more useful than alumina in separating pesticides by differential elution.

EXPERIMENTAL

The following material was used: Glass columns, 45 cm long × 0.6 cm bore, drawn to a tip at the lower end, and plugged at the tip with glass wool, or solvent-washed cotton wool. Pipettes, graduated for content, 1 ml. Tubes, test or tapered centrifuge, graduated 10 × 0.1 ml, with glass stoppers. Flasks, graduated 1 ml, 10 ml and 20 ml, with glass stoppers. *n*-Hexane, 67–70°, redistilled and the 68–69° fraction collected. Tested to be free of residues detectable by electron capture GLC after evaporating 100 ml to 1 ml in a clean air-stream. Alumina powder, analytical grade aluminium hydroxide activated at 800° for 4 h, cooled in a desiccator, partially deactivated by shaking with 5% by weight of distilled water for 30 min and stored in a closed vessel. Silica gel, for chromatography, Merck No. 7754, 70–325 mesh (ASTM), dried at 120° for 2 h, cooled, and deactivated as for alumina.

The tissue extracts used in the investigations were obtained by Soxhlet extraction, for 30 min, of 5 g aliquots of animal tissue (previously ground to a dry powder with anhydrous AR grade crystalline sodium sulphate) using approximately 100 ml of hexane. The extracts were subsequently made up to 100 ml, and 50 ml evaporated to dryness in a tared dish to determine the extractable fat. The remaining 50 ml were evaporated and made up to smaller volumes, from 1 to 25 ml depending on the fat content, before clean-up.

ALUMINA COLUMNS

Alumina was prepared by activating aluminium hydroxide at 800° for 4 h and partially de-activating with 5% by weight of distilled water. Initially 2 g of the de-activated alumina was slurried into the columns with hexane, and the solvent drained

to the surface of the alumina, but these "wet" columns were found to be ineffective in retaining a high proportion of the lipid in fatty extracts. It is believed that the initial diffusion of the sample (1 ml applied to 2 g of alumina) into the interstitial hexane reduces the ability of the alumina to adsorb the lipid material. The use of columns prepared without slurrying ("dry" columns) enables the sample to be applied directly to the alumina surface and adsorbed into the upper 2–3 mm. These columns were found to be much more effective in retaining fat and pigments. The "channelling" effect, which may occur when dry columns of large dimensions are used, in which the solvent passes through the bed in an uneven manner (usually on one side only), does not occur with the narrow-bore tubing used for the method now described.

For extracts of fairly low fat content a single alumina column is often sufficient for satisfactory clean-up but for extracts of high fat content two identical columns are used serially. The first removes the greater proportion of the unwanted fat, and the eluate from this column is concentrated and transferred quantitatively to the second column. Each column is prepared by placing 2.0 $\pm$ 0.1 g of dry alumina powder (activated at 800°, partially deactivated with 5% by weight of water) in a 45 cm long $\times$ 0.6 cm bore chromatographic tube having a glass wool plug at its lower end. 1.0 ml of a hexane extract of animal tissue, containing up to 100 mg of extracted fat per millilitre, is placed on the alumina surface by means of a 1-ml pipette calibrated for content. (Pipettes calibrated for delivery of 1 ml of water are liable to give a drainage error when used for hexane solutions.) The extract is drained down one side of the glass column, and washed into the alumina by refilling the pipette with 1 ml of hexane and draining down the same side of the column. (A slight incline of the column from the vertical position is advantageous.) With a suitable receiver below the column a further 10 ml of hexane is added above the alumina, and additional volumes added as required for elution. The eluate is evaporated to 1 ml or slightly less in a stream of cold dry, filtered air and transferred by means of the 1-ml pipette to the surface of the second alumina column. One millilitre of hexane is added to the first receiver, removed by the same 1-ml pipette, and used to rinse the first millilitre into the second column. The elution from the second column is then made with ten or more millilitres of hexane and the eluate adjusted to a suitable volume by evaporation. Glass-stoppered test-tubes calibrated to 10 ml have been found very suitable, the final volume after evaporating being adjusted to 5 ml or 10 ml as required. When necessary, a reduction of the volume to 1 ml can be made in tapered graduated centrifuge tubes, but these have been found to be insufficiently accurate for the most precise work.

Results

Table I shows the typical order in which a number of organochlorine pesticides and metabolites were eluted from a single alumina column of the type described above. The eluates were collected in 1-ml fractions. With such a small total volume it is not convenient to separate the pesticides into two or more fractions, although subdivisions at 5 ml might be of value in confirming the identity of certain pesticides such as dieldrin and β-BHC, which would be recovered in the second fraction. (Silica columns, described in the second part of this paper, are more effective for this purpose.) It was found that an eluate of about 15 ml was necessary in order to elute β-BHC and DCBP (*p,p'*-dichlorobenzophenone) from the column.

TABLE I

PERCENTAGE RESIDUES IN 1-ml ELUATE FRACTIONS FROM A 2-g COLUMN OF ALUMINA (800° ACTIVATION, 5 % H_2O)

Abbreviations: *p,p'*-DCBP = *p,p'*-dichlorobenzophenone (= DBP); *p,p'*-MDE = 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (= DDMU); *p,p'*-DDE = 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; *p,p'*-TDE = 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (= DDD); *p,p'*-DDT = 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; *o,p'*-DDT = 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p'-DDT*
1				47		69				48	52		24	39
2	18			44		25				49	40	11	71	53
3	69		4	5		4				2	5	70	5	5
4	7		43	3	17	2		3		1	2	16		2
5	3		41	1	58			36			1	2		1
6	2		11		15		41	46				1		
7	1		1		6		48	15						
8					3		9							
9					1		2		4					
10		7							21					
11		25							36					
12		31							25					
13		26							11					
14		9							3					
15		2												
16														
17														
18														

If β-BHC and p,p'-DCBP are of no interest, or known not to be present, a total eluate of 11–12 ml is sufficient to ensure the elution of the other twelve residues. The percentages of the various residues eluted in successive 1-ml fractions will be rather variable, but several tests on the procedure using the same combination of organochlorines have shown that the order of elution is as shown in Table I, within ± 1 ml. Slight variations between individual columns may be expected, due to differences in internal diameter, and in the weight of alumina used.

The use of alumina activated at 500° and partially de-activated with 6% distilled water was considered as a possible alternative where separation of the residues into two groups is desirable. This adsorbent required 15 ml of hexane for the elution of approximately 95% of most residues (Table II) but endrin and heptachlor epoxide required a further 15 ml. Dieldrin, β-BHC, and DCBP were not eluted with hexane, or with 5% diethyl ether in hexane. The use of a hexane–diethyl ether solvent mixture with a higher proportion of ether is liable to elute a significant proportion of fat from tissue samples. In view of the incomplete recovery of some residues from this column, its use was not further investigated.

A series of six replicate pairs of alumina columns was tested by eluting 1 ml aliquots of a mixture of standard pesticides in 25 mg corn oil dissolved in hexane through each pair, according to the method described (using 800° alumina). The final extracts, adjusted to 10 ml, were analysed on two GLC columns and peak heights compared with those from a 1-ml aliquot of the standard mixture diluted to 10 ml. The GLC columns were 5 ft. long and 1/8 in. O.D., containing (a) a 10% coating of DC-200 silicone oil on 80–100 mesh Chromosorb W (AW/DMCS) and (b) 5% DC-200 plus 7.5% QF-1 fluorosilicone on the same support. The chromatograph used was an Aerograph Model 205-2B, fitted with electron capture detectors. The mean and standard deviations of the pesticide concentrations, expressed as a percentage of the values for the unprocessed standard solution, are given in Table III. The standard deviations of replicate injections of the standard solution were in the range of ± 1.2 to ± 1.6%, and the recoveries of the pesticides from the columns were found to be complete.

Using the dry alumina columns, extracts of seal blubber, which are difficult to clean up even with the normal two-stage procedure (involving also an appreciable loss of pesticide), gave excellent GLC chromatograms in which the return to base line following the solvent peak was rapid. Recoveries of a number of organochlorine pesticides added to 250 g of penguin fat (selected for its comparative freedom from pesticides) were quantitative, and similar to those obtained with corn oil. Comparison of GLC chromatograms obtained by the single-stage and two-stage techniques (the latter involving hexane–DMF partition[3], followed by a slurried alumina column) has confirmed the improved clean-up of the single-stage method.

For samples which contain only a small proportion of fat, resulting in the transfer of not more than about 10 mg of fat to the first column, it is usually sufficient to use this column alone for clean-up. The second column is necessary only for higher fat loadings.

Table IV shows the percentage recoveries of eight organochlorine pesticides added to two animal tissue extracts, of pike liver (*Esox lucius*) and penguin fat (*Aptenodytes forsteri*). Recoveries, within the limits of error of the determinations, were 100%, after allowing for the residues originally present. By comparison, re-

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TABLE II

PERCENTAGE RESIDUES IN 1 ml ELUATE FRACTIONS FROM A 2-g COLUMN OF ALUMINA (500° ACTIVATION, 6% H_2O)

For chemical identities of residues, see Table I.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p'-DDT*
1						4								
2				12		59				6	18			3
3				59		27				47	57		3	48
4				20		6				31	17		32	37
5				6		3				10	5		43	9
6				2		1				3	2		17	2
7	4			1						2	1		4	1
8	20									1			1	
9	35	Not eluted					Not eluted		Not eluted			5		
10	25		4									17		
11	12		11									28		
12	4		24									24		
13			26									14		
14			18									7		
15			10									4		
16			5					1				1		
17			2		2			3						
18					3			5						
19					3			6						
20					4			8						
21–30					88			77						

TABLE III

PERCENTAGE RECOVERY OF ADDED PESTICIDES FROM CORN OIL

Corn oil spiked with eight organochlorine pesticides, and 25 mg cleaned up on six pairs of 2-g alumina columns, 800°/5% H_2O type.

Pesticide	*Mean % recovery from corn oil*	*S.D.*
α-BHC	101.5	±1.3
γ-BHC	99.4	±1.7
Heptachlor	100.5	±2.2
Heptachlor epoxide	101.2	±2.7
Aldrin	100.8	±1.9
Dieldrin	101.0	±1.9
p,p'-DDE	101.0	±3.1
o,p'-DDT	101.9	±2.5

TABLE IV

PERCENTAGE RECOVERY OF ADDED PESTICIDES FROM ANIMAL TISSUE

S.D. = ±5%.

Pesticide	*% recovery from pike liver*	*% recovery from penguin fat*
α-BHC	97	100
γ-BHC	103	103
Heptachlor	97	106
Heptachlor epoxide	102	108
Aldrin	97	104
Dieldrin	105	108
p,p'-DDE	108	105
o,p'-DDT	104	109

coveries from spiked extracts of the same tissues cleaned up by the hexane–DMF process were low. The chromatograms for the penguin fat were unusable, the partition stage in the clean-up being difficult to achieve owing to emulsification. In the case of pike liver spiked with dieldrin, recoveries were 90% by the DMF process and 105% by the alumina column. Cod liver (*Gadus morrhua*) extracts similarly spiked gave 31% recovery by the DMF process and 103% by the alumina column. With some samples additional peaks have been detected on the chromatograms. These are possibly due to substances which, in the liquid–liquid partition process, remain in the discarded hexane extract or are removed by the aqueous wash, but their identity has so far not been established. The polychlorinated biphenyls, which are often found in wildlife samples, are eluted from the columns in the first 2–3 ml of hexane.

The single-stage method has proved very successful with a variety of samples, including seal blubber, fish liver and muscle, shellfish tissues and vegetable oil. A few types of sample may prove to be more difficult to clean up, however, and limitation of the column loading to 100 mg of extractable lipids is advised. If polar solvents are used in the initial extraction of tissue samples, the organochlorine residues must be transferred to hexane solution before using the alumina columns described. The

columns have not been tested with samples containing organophosphorus or other pesticides, and the technique should be fully examined before use in the analysis of such compounds.

An improved version of the elution column has now been developed in place of that used in the investigation. This has a 15-cm section of 0.7 cm bore at the lower end, and is joined at the upper end to a section of 2.5 cm bore, 8 cm long. It is thus possible to apply the 1 ml aliquots of sample directly to the upper surface of the alumina, and to add 20 ml of hexane for elution if necessary to the upper reservoir. The elution time for these modified columns is longer, owing to the smaller head of pressure available.

The advantages of the twin alumina column technique over the liquid–liquid partition method are (a) small quantities of solvent and adsorbent reduce operative costs, (b) alumina as an adsorbent is less variable than Florisil, (c) recoveries of the fourteen organochlorine residues tested are complete, in contrast to the experience with solvent partition, (d) results are more reproducible, (e) clean-up of fats is more effective, (f) samples can be processed more rapidly and with less laboratory space, (g) some residues lost in solvent partition are recovered by the new technique, and (h) the emulsion formation often experienced with liquid partition of fats is avoided.

SILICA COLUMNS

Columns of powdered silica have been reported[5] as being relatively inefficient for clean-up purposes, although KADOUM[6–8] has described a technique using small columns, in the solvent-wet form, for clean-up and separation of a number of pesticide residues. He found it necessary to use *n*-hexane and benzene in different proportions to elute substances of differing polarities, but thereby achieved a useful confirmatory technique. For samples of unknown pesticide content, however, such as wildlife specimens, this procedure could result in an inconveniently large number of extracts per sample for analysis, and a compromise would normally be accepted, by which several residues would be eluted with the most polar solvent mixture.

In the present investigation the use of silica columns containing 2 g of 70–325 mesh (ASTM) silica pre-wetted with hexane was found to result in a significant elution of fatty materials when a more polar solvent was applied to elute those pesticides not extracted by hexane. Columns identical to those previously described for alumina, but using 2 g of prepared silica in the dry form, were therefore tested. Some unidentified substances were found to be eluted from silica activated at 120° and partially deactivated by shaking with 5% (by weight) of distilled water, two of these substances being similar in retention time to α-BHC and γ-BHC when analysed by GLC using a DC-200 silicone column. Unlike α- and γ-BHC, however, they were eluted by hexane, whereas the two BHC isomers required 10% diethyl ether in hexane for elution.

The contaminants were removed by pre-washing the silica, before activation, in a Buchner funnel with hot distilled water, followed by a wash with diethyl ether, drying off by drawing air through the silica, and activating in an oven at 120° for 2 h. Partial deactivation by shaking for 30 min with 5% (by weight) of distilled water then produced the desired activity. Drying the silica at 300° was found to result in

a lower final activity, which was less suitable for the separation of organochlorine residues to be described.

The grade of silica found most effective for the purpose was Silica Gel No. 7754 (Merck), which is stated to have a mesh range of 70–325 (ASTM). Silica of this type, from which material passing a 200-mesh sieve had been removed, and silica from another supplier (Hopkin and Williams) stated to have an approximate mesh range of 100–200, were also found suitable for producing the separation between solvents described below, but the more rapid rate of elution of *p,p'*-DDT with these two types precluded the possibility of separating *p,p'*-DDT from other pesticides. The unsieved Merck silica was therefore finally selected for routine use, although columns of this material take longer for elution than the alumina columns previously described. Shorter columns, using less than 2 g of silica, are faster but give a less effective separation.

Results

The typical order of elution of fourteen organochlorine pesticides and related substances is shown in Table V. Two solvents are necessary for the elution, the first being *n*-hexane (10 ml) and the second 10% diethyl ether in hexane (10 ml). If the first 10 ml of hexane is followed by a further 10 ml of the same solvent, none of the substances in the second group (extracted by 10% ether in hexane) are eluted. Thus an excess of hexane is not critical, and it may be found convenient to collect 12–13 ml of eluate and reduce the final volume to 10 ml or 5 ml by evaporation. The polychlorinated biphenyls are eluted in the first 4 ml of hexane, and hexachlorobenzene, which is sometimes difficult to distinguish from α-BHC in GLC analysis, is eluted in the first 3 ml of hexane.

The fourteen substances listed in Table V are approximately equally divided between the two solvent fractions, but for wildlife samples in which the DDT group of substances and dieldrin, together with the PCBs, are often the only contaminants, the separation enables the PCBs, *p,p'*-DDE, *p,p'*-DDT and *o,p'*-DDT to be removed in the hexane, and *p,p'*-TDE and dieldrin in the 10% ether–hexane. Furthermore, it is possible (using the Merck grade of silica) to elute with 5 ml of hexane, thus removing the PCBs and *p,p'*-DDE, and following this with 10 ml of 10% ether–hexane, which elutes the *p,p'*-DDT with the *p,p'*-TDE and dieldrin. Any *o,p'*-DDT present will be split between the two fractions, but this is often an insignificant contaminant. Analysis by a single GLC column of the DC-200 or SE-30 silicone type is then sufficient for a complete analysis of both eluates, as the interference of PCBs with *p,p'*-TDE and *p,p'*-DDT is avoided. The interference of PCBs with *p,p'*-DDE is usually small.

The use of two silica columns in succession (as previously described for alumina) for cleaning up 1 ml aliquots of fat extracts, has been found fairly effective provided the fat content is not excessive, but the preferred technique is to use one alumina column (eluted with 20 ml of hexane) followed by a silica column using the two-solvent method to separate the pesticides into groups as described above. At least 100 mg of fat can be removed by this combination.

The efficiency of elution of pesticides from a silica column was examined by applying known weights of pesticide in 1 ml of hexane to the column, and eluting with 10 ml of hexane, followed by 10 ml of 10% ether in hexane, the results being compared on GLC analysis with those obtained from the original solution. Within the

TABLE V

PERCENTAGE RESIDUES IN 1-ml ELUATE FRACTIONS FROM A 2-g COLUMN OF SILICA GEL (120° ACTIVATION, 5% H_2O)

For chemical identities of residues, see Table I. Solvents: Fractions 1–10, hexane. Fractions 11–20, 10% ether in hexane.

Fraction No.	*α-BHC*	*β-BHC*	*γ-BHC*	*Hepta-chlor*	*Hepta-chlor epoxide*	*Aldrin*	*Diel-drin*	*Endrin*	*p,p'-DCBP*	*p,p'-MDE*	*p,p'-DDE*	*p,p'-TDE*	*p,p'-DDT*	*o,p' DDT*
1														
2						6								
3				17		69					22			
4				48		25				66	64			4
5				31						31	14			41
6				4						3			10	43
7													43	8
8													37	4
9													10	
10														
11														
12														
13														
14														
15								96				2		
16								4	64			6		
17	100	51	10		97		8		36			90		
18		41	62		3		63					2		
19		6	28				29							
20		2												

limits of the analytical errors, recoveries of all pesticides examined were found to be complete.

The efficiency of recovery from fat extracts, spiked with a number of pesticides and cleaned up on an alumina column followed by a silica column, has also been examined. In this instance the first 5-ml eluate of hexane was followed by a 10-ml elution with 10% ether in hexane, the eluates being analysed separately, and the concentrations of residues found being corrected for the presence of any similar residues in the original sample. As with the earlier tests of alumina columns, recovery of the added pesticides was complete.

The two-column alumina/silica gel method has been found very effective in cleaning-up hexane extracts of samples of seal blubber containing a number of PCB residues as well as *p,p'*-DDE, *p,p'*-TDE, *p,p'*-DDT and dieldrin. One-millilitre aliquots of the extracts, containing approximately 100 mg of extractable fat, are eluted on an alumina column with 20 ml of hexane, and the eluate is evaporated to 1 ml with a stream of air. This volume is then transferred quantitatively to the silica column as previously described. Hexane is used to elute the PCBs and *p,p'*-DDE, the first 5 ml being collected, and the remaining residues are eluted with 10 ml of 10% ether in hexane, which is subsequently reduced to 5 ml by evaporation.

The technique described has so far been employed only for organochlorine residues, and should be tested with standard solutions before the clean-up of extracts containing organophosphorus or other residues is attempted. It is also probable that the efficiency of clean-up will vary with the type of lipid material being removed. While it has been found possible to remove more than 200 mg of some types of extractable lipid, certain fish oils tend to elute more easily with hexane, and the column loading may have to be reduced below 100 mg. Alternatively an additional alumina column may be used. As the efficiency of recovery of the organochlorine residues is approximately 100%, the passage of the sample through a succession of columns does not result in any loss of these residues. The separation afforded by the silica columns has revealed the presence of residues of unknown origin in wildlife samples, these being in the 10% ether–hexane eluate, and thus distinguishable from PCBs. They have not so far been identified as any of the commonly used pesticides.

CONCLUSIONS

The twin-column chromatographic clean-up method described has several advantages over the more commonly employed liquid–liquid partition method. In particular, it gives 100% recovery of organochlorine compounds with a minimum use of solvents, and is more rapid and reproducible. Where pre-GLC sub-division of residues is not required, the twin alumina column version is preferred, but by replacing the second alumina column with a silica column a useful separation of PCB compounds from many organochlorine pesticides is obtained.

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CHROM. 4259

ANALYTICAL AND PREPARATIVE COLUMN CHROMATOGRAPHY OF NEUTRAL LIPIDS WITH CONTINUOUS MONITORING OF THE ELUATE BY A FLAME IONIZATION DETECTOR

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SUMMARY

A method is described for the fractionation of neutral lipids into classes by means of silicic acid column chromatography. Elution is performed with a concave gradient of ethyl ether in petroleum ether; the gradient is achieved by means of two metering pumps. This gradient is simple to obtain and can be easily modified for different requirements following our scheme of calculation. The effluent is monitored continuously by means of a hydrogen flame ionisation detector, a part of the effluent being continuously drawn off. Our method can be used for analytical purposes as well as for isolation of pure lipidic fractions.

The quantitative analysis of the components of a lipid mixture is performed following a weighing microtechnique, using a Cahn electrobalance.

Examples are reported of the separation of reference lipid mixtures of oils and tissue lipids and of oil solutions of steroids for pharmaceutical use.

Our method is also useful for detecting lipidic contaminants in solvents and materials for chromatography. The necessity of very carefully controlled solvents and materials to avoid contamination is underlined.

INTRODUCTION

Methods for the separation of neutral lipids into classes by column chromatography have been reported by many authors, where many different elution systems, stepwise[1–21] or with a continuous gradient[5,21–25], have been used together with different techniques for the analysis of the eluates, based in most cases on colorimetric measurements, weighing[5,8,9] or radioactivity measurements[15]. In a great majority of cases the use of colorimetric techniques specific for the analysis of various classes, while adequate for analytical purposes, does not, however, always decisively show whether the procedure is suitable for the isolation of a fraction of good purity.

In our research concerning the lipid composition of organs and tissues of animals under different experimental treatments[26], we developed a method for the fractiona-

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tion of neutral lipids into classes, attempting to satisfy preparative and analytical exigencies as well as those inherent with the application of the method, which we tried to automate as much as possible.

In order to achieve this aim, we studied a procedure which had to fulfil the following conditions:

(a) That it had to have a minimum of contamination of the eluate by the elution system and the apparatus;

(b) That it permitted the separation of a lipid mixture during one working day, which limits the quantity analyzed to suit the other parameters on which the analytical procedure depends. On the other hand the quantity of mixture analysed must be such as to permit a weighing technique for the determination of the main fractions;

(c) That it guaranteed good resolution during the separation, compatible with the composition of the mixtures studied;

(d) That the development of the column was achieved in a highly efficient manner with respect to the separation effects according to LAKSHAMANAN AND LIEBERMAN[27] and to practical technical arrangements, that is by a solvent mixture of continuous varying composition following a concave concentration gradient, as studied by us;

(e) That the composition of the eluates was controlled in an as efficient and practical way as possible, that is by a continuously automated system. Owing to the nature of the components under study, this was done by using an apparatus based on the continuous transport of an aliquot of the eluate through a hydrogen flame ionization detector, with registration of the responses as described in refs. 28–31.

Such a method for the fractionation of neutral lipids from organs and for the isolation of pure lipid fractions has been developed. Its whole realization, particularly with respect to the flexibility with which gradients are obtained and to the use of an automated detector for the eluted fractions, prompted us to describe this method, which might also be of interest for the solution of analogous problems.

EXPERIMENTAL

Solvents and materials for column chromatography

Petroleum ether for chromatography, boiling range 65–75°, was twice distilled before use after filtration through a silicic acid column. This solvent is subsequently called petroleum ether (P.E.) in the text. Ethyl ether was freed of peroxides by passage over basic aluminium oxide and double distillation. Double distilled methanol was used. Silicic acid (Mallinckrodt, 100 mesh) was sieved through a 300 mesh sieve and solvent treated as described by HIRSCH AND AHRENS[5]. According to HIRSCH AND AHRENS, analogous results can be obtained using Biorad silicic acid for chromatography when prepared as described by these authors. Silicic acid selected in this way is heated to 125° for 8 h in an oven, cooled in a desiccator and 9.38 ml water are added to 100 g of the product. The water content is theoretically 8.58 % but in reality it is a little higher, because, as mentioned by BÖTTCHER *et al.*[32], silicic acid even when heated for more than 2 h at 120° retains a practically constant quantity of water, about 1.2 %, as demonstrated by titration with Fisher reagent.

Chromatographic columns

LKB glass columns, type 4200, of diameter 9 and 12 mm, and 30 cm in length

are used, and columns of 6 and 25 mm diameter of the same length are also used. Analogous results are obtained with glass columns constructed in the laboratory, 11 mm in diameter, 27 cm length, terminating in two cones (No. 12) into which two teflon conical plugs designed for minimal dead volume are introduced. The connections between the columns and other apparatus are made by teflon tubes, O.D. approx. 1/16 in. (1.58 mm), I.D. 1–1.2 mm. In order to introduce the adsorbent in the columns, the latter are half filled with solvent, and silicic acid suspended in petroleum ether is gradually poured in, allowing the solvent to slowly flow out of the column. 5 g silicic acid are normally used for the 9 and 12 mm diameter columns giving a height of adsorbent in the column of 155 and 95 mm (H/d 17.3; 7.9) respectively; for the 6 mm diameter columns, a maximum of 3.5 g of silicic acid is allowed (H 250 mm, ratio H/d 42.0); for 25 mm diameter columns, 23 g of silicic acid (H 90 mm, ratio H/d 3.6) are normally used.

Sample preparation

The following compounds are used as reference lipids: squalane; cholesterol stearate or palmitate; tripalmitin; palmitic acid; cholesterol from ASI or Calbiochem, purified by us by column chromatography as described in this paper until a single component is obtained. Glyceryl distearate (a mixture of 1,2- and 1,3-isomers) and glyceryl monostearate were prepared by us from the commercial product according to QUINLIN AND WEISER[33] and successively rechromatographed. Testosterone propionate was of analytical grade, controlled by UV spectrophotometry and thin-layer chromatography; the natural olive oil was an authentic sample from our Institute. Mixtures of the reference lipids were prepared by weighing on a Cahn electrobalance, model Gram. Total lipids of rat liver were obtained by extraction with chloroform–methanol (2:1) according to FOLCH *et al.*[34]. The total lipid content of an aliquot of the extract was determined by weighing to a constant weight in a vacuum desiccator.

Charging the column

Direct method. The sample, dissolved in as small a volume as possible (1–2 ml of petroleum ether), is pipetted on to the column, and washed with the same solvent (3 × 1 ml), allowing a slow efflux by gravity. The empty part of the column is refilled with the solvent and the upper part is connected to the feeding system.

System with an injector. A simple and efficient injector is obtained by introducing into the small feeding tube, immediately before it enters the column, a three way LKB joint, type 3065 A, modified by substituting the glass wedge and the tube lock in one arm with a teflon wedge, terminating with a flat face on the other end, on which a grey rubber septum (Perkin-Elmer for GLC) is fixed by the nut. For the injection, a Hamilton precision syringe with teflon plunger (gas tight type) for the assurance of perfect fit, is used. Normally approx. 20 mg of neutral lipids are chromatographed in columns containing 5 g silicic acid, that is 4 mg/g (1/250), rising to as much as 7.5 mg/g. In the preparative columns the sample is approx. 85–100 mg for 23 g silicic acid.

Feeding system for the column

The solvent is pumped into the column by a metering pump, type CH MM 1-B 24

Milton Roy, with an adjustable flow rate (R_2 ml/min). The gradient of ether in the petroleum ether is obtained by pumping, with another metering pump with a flow rate R_1, a volume V_R of ether from the reservoir into a mixing chamber (equipped with a magnetic stirrer) containing a volume V_M of petroleum ether. The expression which gives the shape of the gradient as a function of the flow rates R_1 and R_2 and the volumes V_R and V_M is derived from WREN's general equation[35] described for U-connected vessels, as results from the following development.

In the most complete form, WREN's equation is

$$C_v = C_R - (C_R - C_M) \cdot \left(1 - \frac{v}{V_R + V_M}\right)^P \tag{1}$$

where

$$P = \frac{d_R^2 \cdot \rho_M}{d_M^2 \cdot \rho_R} \tag{2}$$

were C_R and C_M are the initial concentrations of the solvent forming the gradient (in this case ether) in the reservoir and in the mixing chamber; C_v is the concentration of the liquid which is present in M at the moment when a volume v has run off to feed the column; A_R and A_M are the areas of the reservoir (R) and mixing (M) vessels; d_R and d_M are the respective diameters and ϱ_M and ϱ_R are the densities of the liquids contained in M and R. The simplified form where

$$P = V_R/V_M = A_R/A_M \tag{3}$$

is used instead of

$$\frac{d_R^2 \cdot \rho_M}{d_M^2 \cdot \rho_R}$$

when the liquids have the same density or, on the other hand, when the movement of both liquids can be made independent of their density. As mentioned above, we have achieved this by transferring the liquids by means of two accurately regulated metering pumps. The flow rates of the two pumps R_1 (transfer) and R_2 (feed to column) are related to the volumes to be transferred during the same time interval by the simple expression

$$P = \frac{V_R}{V_M} = \frac{R_1}{R_2 - R_1} \tag{4}$$

P is the power of eqn. (1) and the gradient is characterized by its value. From eqn. (4) and the total volume of the eluate

$$V_T = V_R + V_M \tag{5}$$

it can be derived that

$$V_M = \frac{V_T}{P + 1} \tag{6}$$

and

$$V_R = P \cdot \frac{V_T}{P + 1} \tag{7}$$

For a concave gradient[27,35,36] $P < 1$*.

Applying these simple equations (1, 4, 5, 6, 7) one is able to calculate the experimental data necessary to obtain the chosen gradient.

In our experiments $P = 1/4$ was chosen, and thus $V_R = 1/5\ V_T$ and

$$V_M = \frac{4}{5} V_T \text{ and } \frac{R_1}{R_2 - R_1} = \frac{1}{5 - 1} = \frac{1}{4}$$

and $R_2 = 5$ and $R_1 = 1$. Therefore if the flow rate R_2 is fixed as 1 ml/min, R_1 is $1/5\ R_2$, *i.e.* 0.2 ml/min. For $C_M = 0$ (no solvent producing the gradient in the mixing chamber at time 0), eqn. (1) is simplified to

$$C_v = C_R - C_R \left(1 - \frac{v}{V_T}\right)^P \tag{8}$$

When the concentrations and volumes are expressed in percent,

$$C_v\,\% = 100 - 100 \left(1 - \frac{v}{100}\right)^P \tag{9}$$

from which equation, for $P = 1/4$, it is easy to draw the theoretical graphical expression of the gradient (Fig. 1a) or a table. After the definition of the gradient shape by the parameter P (R_1 and R_2), it only remains to establish the total volume of the eluate V_T, which is dependent on the chromatographic problem, that is on the column volume; V_R and V_M can then be obtained from eqns. (6) and (7).

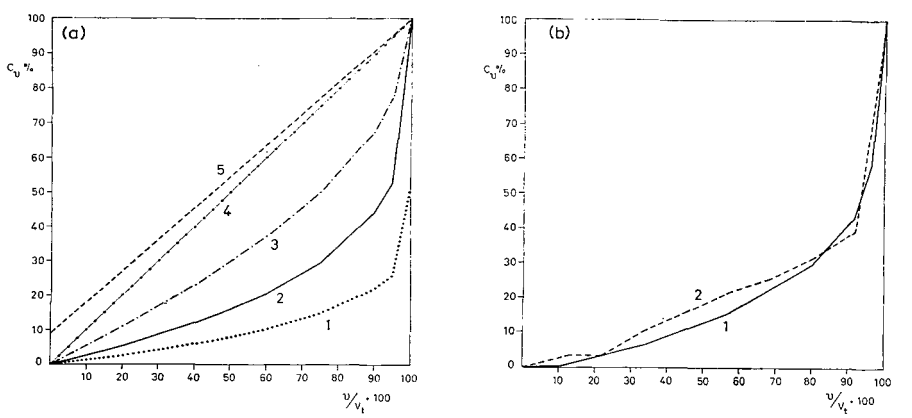

Fig. 1 (a) Theoretical gradient curves: (1) Concave $P = 1/4$, C_R 50 % ethyl ether (e.e.) in petroleum ether; (2) concave $P = 1/4$, C_R 100 % e.e.; (3) concave $P = 1/2$, C_R 100 % e.e.; (4) linear, C_R 100 % e.e.; (5) linear, $C_R = 100$ % e.e., from $C_M = 9.5$ % e.e. in petroleum ether. (b) Experimental gradient curves: (1) concave, $P = 1/4$, $C_R = 100$ % e.e. with pump, as in the text; (2) concave $P = 1/4$, $C_R = 100$ % e.e. with siphon communicating bottles.

* The concavity is further accentuated for $P \to 0$.

In most of our experiments, V_T was 430 ml, V_R 86 ml of ethyl ether, V_M 344 ml of petroleum ether, $R_2 = 1$ ml/min and $R_1 = 0.2$ ml/min. These volumes were chosen so as to obtain a gradient which permits a separation analogous to that obtained by stepwise feeding of the column, with mixtures of petroleum ether–ethyl ether (v/v) in the following proportions and amounts: 99:1, 80 ml; 96:4, 70 ml; 92:8, 150 ml; 75:25, 60 ml; ethyl ether 70 ml. To obtain a better separation of the hydrocarbon fraction, our gradient is preceded by a short elution with 30 ml of petroleum ether. Also a final elution with 60 ml of ethyl ether shortens the recovery time for the monoglycerides.

For columns of 25 mm diameter and approx. 25 g of silicic acid, the gradient is of the same type, with $V_T = 1865$ ml, $V_R = 373$ ml of ether and $V_M = 1492$ ml petroleum ether, preceded by an elution with 50 ml petroleum ether and followed by an elution with 260 ml ethyl ether (total development time 2175 min, *i.e.* 36 h and 15 min).

The experimental confirmation of the gradient curve is carried out by measuring the concentration of ethyl ether in petroleum ether at various points of the elution (at various values of $v/V_T \cdot 100$). In order to measure the concentration of ethyl ether it is coloured with Sudan III red and this permits an easy spectrophotometric determination of $C_v\%$ ($C_v\% = E/E_{tot} \cdot 100$)*

A gradient, obtainable with two vessels communicating at the bottom so that the respective areas were $A_R/A_M = 1/4$ ($d_R/d_M = 1/\sqrt{4}$) ($\varrho_R = \varrho_M$ approx.) was also studied, but we did not obtain regular or reproducible results (Fig. 1b).

Analysis of eluates

In order to monitor the various fractions eluted from the column as concentration peaks, a part of the eluate from the column is transferred onto a chain conveyor, which, after the solvents have been evaporated to dryness, introduces the solid residues into a hydrogen flame ionization detector. A Liquid Chromatography Detector (LCD) Mod. 5400 of Barber Colman, constructed according to principles described in refs. 28–30 is used. The operating conditions for the instrument are as follows: attenuation $\times$ 100 (input 3×10^{-10} A), with a 5 mV Leeds and Northrup Speedimax W recorder; nitrogen, hydrogen and air pressures 1.6, 2.2 and 3.8 kg/cm^2 respectively; chain speed regulator 9.7.

The splitting ratio was adjusted to 5–6%, and this was obtained by a glass overflow splitter of the type described by WOODS[37] but was of a smaller size and covered at the top by a teflon plug. Our design of the splitter assures much less contamination of the last fractions of the chromatogram, reducing evaporation and the deposition of a residue that remains as a ring in the central part of the splitter cup. The needle used was a Hamilton No. 726, with a Kel-F hub. Apart from changing the needle, the splitting rates can be varied by varying the height of the splitter in respect to the chain; this has also been reported by LUND AND KUNSMAN[38], who described a different type of overflow splitter

The main part of the eluate is directed to a time controlled fraction collector (Radirac LKB 3400) which is synchronised to start with the start of the chromatography; every fraction is normally regulated to 10 min; the flow R_2 being 1 ml/min,

* The values of $C_v\%$ are reported graphically on the ordinate, with respect to $v/V_T \cdot 100$ on the abscissa (Fig. 1b).

the corresponding whole fraction volume V_F is 10 ml; but since V_S is the volume removed by the splitter, $V_F' = V_F - V_S$ where V_F' = the actual fraction volume, collected in the tubes. V_F' and V_S are determined before the start of the experiment by a series of exact measurements, excluding losses by evaporation in the test tubes. In cases when a chromatogram lasts several hours and such losses cannot be avoided, the volume of the fraction is adjusted to the theoretical value V_F' with petroleum ether before quantitative and qualitative measurement are carried out on the fractions.

The qualitative control of the eluates is done by thin-layer chromatography with convenient aliquots of V_F', normally 1 ml per fraction or less for the main peak. This volume is evaporated to dryness in conical test tube and transferred, in a minimum volume of solvent, by a Hamilton microsyringe onto a plate, 20 × 20 cm, covered with Silica Gel G, thickness 0.5 mm.

The solvents used are (for those not previously specified, of analytical grade):

(a) Petroleum ether–ethyl ether–acetic acid (95:5:1) for less polar lipids (hydrocarbons and esters of sterols);

(b) Petroleum ether–ethyl ether–acetic acid (70:30:1) for neutral lipids;

(c) Chloroform–methanol–water (65:25:4) for more polar lipids (phospholipids).

Details of the technique are reported elsewhere[39].

The analysis of the eluates is performed by weighing. After confirmation of the identity of each fraction, 8 ml or less of each fraction volume V_F', corresponding to a single peak are pooled, evaporated to dryness under nitrogen in a water bath at 40° or, for bigger volumes, in a rotating evaporator under vacuum or in a nitrogen atmosphere. The residues are quantitatively transferred with 3–4 aliquots of 100 μl petroleum ether into tared teflon microcups of approx. 50 mg weight (teflon weighing cups No. 2034 of Cahn Instruments) and after evaporation of the solvent on a heated thermoregulated plate at 40°, they are stored for 24 h in a desiccator and then weighed on a Cahn electrobalance (Gram model). After keeping the samples for a further 24 h in the desiccator, the weight, which generally remains constant, is recorded.

The results are calculated for the total volume V_F of the fractions. The amount obtained for each single peak is referred to the total weight of the chromatographed substance, thus obtaining the percent composition of the mixture.

RESULTS AND DISCUSSION

The pattern of a chromatographic separation performed under the conditions described in the text is shown in Fig. 2. The graph is a reproduction of a record obtained by the liquid chromatography detector (LCD). The resolution is optimal for hydrocarbons, esters of sterols, and triglycerides. The fatty acid peak is also separated from triglycerides and cholesterol, but the resolution of the cholesterol and diglyceride peaks is incomplete. In spite of this, the separation obtained by us can be favourably compared with those of other authors[5,9,10,22].

The pattern of the separation is not essentially modified when a column of 12 mm is substituted by one of 9 or 6 mm diameter. With this last type of column the ratio H/d is about 42 instead of 7.9 for the 12 mm diameter column but there is no evidence of the advantages that are associated with the lengthening of the column[23,40]. Probably a higher H/d ratio or a higher flow ratio might be used; certainly the choice of the best conditions for a chromatographic separation is not a

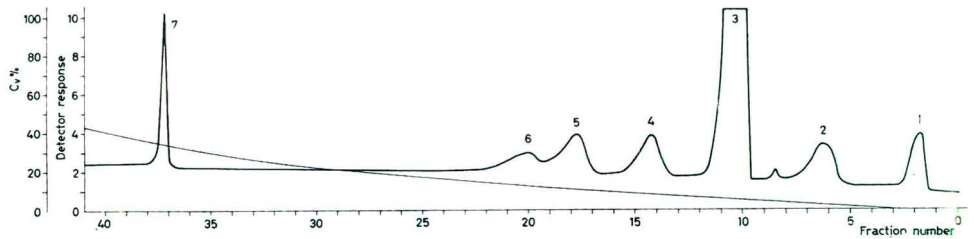

Fig. 2. Chromatogram of a reference lipid mixture. Column 12 mm diam. × 95 mm high, charged with 5 g silicic acid. Sample 20.23 mg. Detector sensitivity 3 × 10^{-10} A. Concave gradient (P = 1/4), C_R = 100 % ethyl ether in petroleum ether; column flow rate 1 ml/min. Splitting ratio: 6 % of the column flow by-passed to detector. Peaks 1 = squalane; 2 = cholesterol stearate; 3 = tripalmitin; 4 = palmitic acid; 5 = cholesterol; 6 = glycerol distearate; 7 = glycerol monostearate. The gradient curve is shown.

simple problem as discussed by HANAHAN *et al.*[41] in the case of the use of stepwise or continuous gradients in the column chromatography of phospholipids.

For example, a modification of the gradient, where it is subdivided into two parts, one concave of V_T = 215 ml utilized for 56 %, with C_R 50 % in ether and a successive linear gradient between 9 and 100 % of ether of V_T = 190 ml, utilized for 63 % to C_v = 66.5 %, does not improve the resolution of cholesterol and the diglycerides, but it does permit the elution of the monoglycerides in the gradient without the necessity of using pure ether at the end of the gradient (Fig. 3). It is

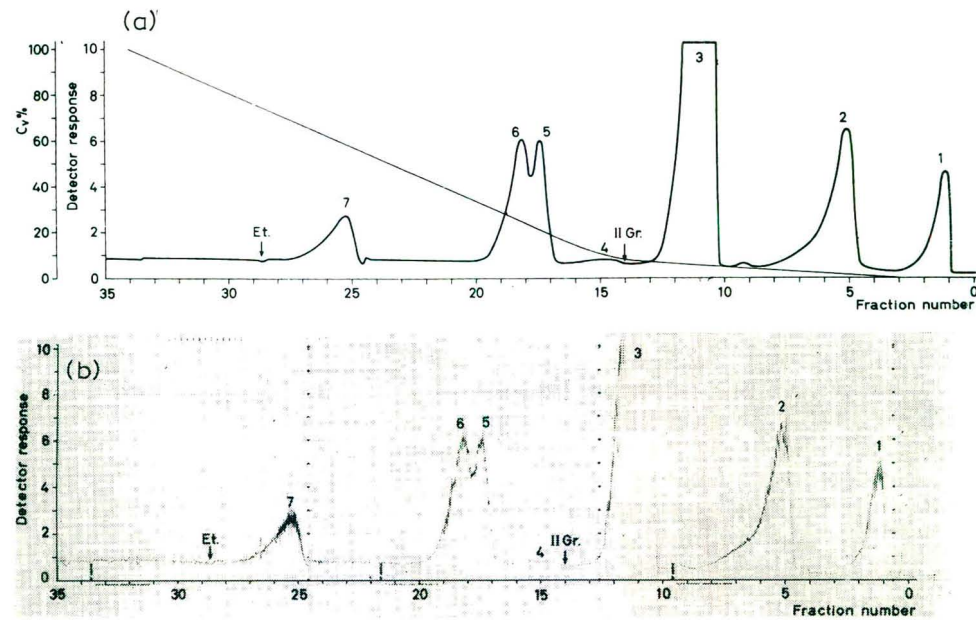

Fig. 3 (a) Chromatogram of a reference lipid mixture. Column 9 mm diam. × 160 mm high, charged with 5 g silicic acid. Sample 16.62 mg. Detector sensitivity 3 × 10^{-10} A. Concave gradient (P = 1/4) C_R = 50 %, ethyl ether in petroleum ether, 56 % used until C_v = 9.5 %, plus linear gradient C_R = 100 % ethyl ether in C_M = 9.5 % ethyl ether in petroleum ether. Peak order as in Fig. 2. The gradient curves are reported. (b) Direct reproduction of the recording obtained with the LCD for the chromatogram described in (a).

thus clear that for the isolation of certain fractions present in a certain quantity of eluate in more or less complex mixtures of lipids, more convenient gradients or combinations of gradients can be used for each separate case, thanks to the simplicity and flexibility of the system for the gradient preparation.

Our gradient permits, under conditions only requiring programming at the start, a better separation than was obtained in our preceding work[39] where a stepwise succession of solvents (ether–petroleum ether) analogous to that described by HIRSCH AND AHRENS[5] was used. The separation as far as the diglycerides are concerned is obtained with columns consisting of 5 g silicic acid, with 260–300 ml of the gradient, corresponding to 61–70% of its total volume: when substances of a polarity lying between that of di- and monoglycerides are not expected, the final elution with ether can be started after the diglycerides, stopping the gradient at whatever point desired.

The quantitative evaluation of this separation procedure was carried out by chromatographing pure lipids individually or in mixtures and determining them by weighing the corresponding fractions obtained from the chromatogram. The results are given in Table I, where the total and relative recoveries of the various components are reported with respect to the quantity of the mixtures of pure lipids and individual lipids chromatographed.

Commenting on these results it can be generally said that the total recovery is very good and higher than 93–95 %. This is also valid for the greater part of the individual components of the mixture. The use of a weighing procedure for the determination of the recovery and of the composition of the analysed mixture coupled with the control of purity of the fractions done by TLC demonstrates how, in a chromatographic procedure for the separation of lipids, it is extremely important to use highly purified solvents and accurately cleaned laboratory glassware. We found that solvents that were not purified (even chromatographic grade) contain lipid impurities (triglycerides, hydrocarbons, fatty acids and other products) in appreciably quantity. They are retained by the column when at low solvent polarity and are subsequently eluted by the gradient (Fig. 4). In many cases the contamination of the solvents, even of the purified ones, can be as much as 1–3 μg/ml (p.p.m.) and, when accurately evaluating the results, it is necessary to take into consideration the elution volume of a fraction and subtract from the total weight the amount calculated for the

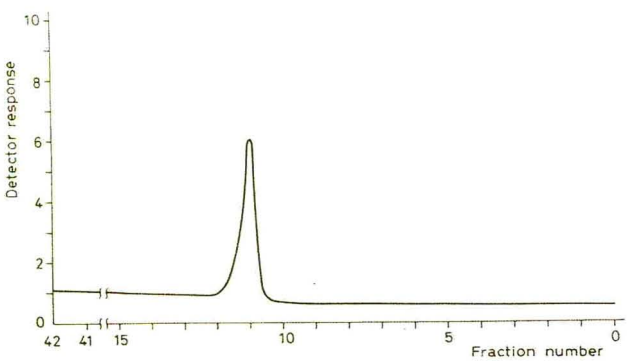

Fig. 4. Chromatogram of non-purified solvents: a peak of triglycerides is visible. Operating conditions as in Fig. 2.

TABLE I

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF REFERENCE AND IMPURE LIPIDS

Recovery is reported as per cent of the total chromatographed amount. In the second line theoretical in brackets.

Sample	*No.*	*Chromatographed amount mg*	*Hydrocarbons*	*Sterol esters*	*Triglycerides*	*Fatty acids*
Mixture of reference lipids	1	20.23	4.60	17.84	52.89	6.52
			4.84	19.92	57.96	6.65
			(95.04)	(89.56)	(91.25)	(98.04)
Mixture of reference lipids	2	18.17	4.57	4.62	61.47	6.55
			4.95	5.01	63.99	6.62
			(92.32)	(92.21)	(96.06)	(98.94)
Mixture of reference lipids	3	17.44	6.44	5.01	57.53	7.73
			5.90	5.22	64.16	7.22
			(109.15)	(95.98)	(89.67)	(107.06)
Mixture of reference lipids	4	20.26	4.83	7.10	62.20	4.87
			4.70	7.80	63.15	4.78
			(102.76)	(91.92)	(98.49)	(101.88)
Rat liver lipids	5	39.94	0.09	5.19	18.20	1.05
			—	—	—	—
Rat liver lipids	6	39.94	0.07	5.24	18.79	1.53
			—	—	—	—
Single reference and impure lipids						
Tripalmitin	7	12.02			95.42	2.49
					100.00	
					(95.42)	
Tripalmitin	8	12.21			91.98	2.84
					100.00	—
					(91.98)	
Tripalmitin	9	15.64			91.05	2.56
					100.00	—
					(91.05)	
Pure tripalmitin + palmitic acid	10	12.37			74.83	15.27
					82.69	17.31
					(90.49)	(88.21)
Pure tripalmitin	11	10.90			93.50	0.05
					100.00	—
					(93.50)	
Impure cholesterol stearate	12	50.00		94.98	0.26	1.02
				100.00	—	—
				(94.98)		
Impure mono- and di-glycerides	13	5.99			3.42	3.49
					—	—
Natural olive oil	14	19.94	0.92[a]	[a]	85.50	4.44
			—	—	—	—

[a] These two peaks were not completely resolved in this experiment: recovery is calculated together.

impurities in the solvent. The results reported in Table I have been calculated taking, when necessary, this observation into consideration.

The use of the chromatographic procedure for the analysis and purification of single lipids as described is shown in experiments carried out with tripalmitin defined as 99 % pure. As shown in Fig. 5, in fact a small but well separable peak of fatty acids is present. Thin-layer chromatography reveals these to be associated with another,

composition of mixtures is reported when it is known. The per cent recovery for each component is given

Sterols	*Diglycerides*	*Mono-glycerides*	*Phospho-lipids*	*Other fractions*			*Recovered %*	*Amount mg*
9.64 [a]	[a]			3.56	—	—	95.06	19.23
5.39	5.24							
(90.69)								
10.13 [a]	[a]	9.08		0.88	1.65	—	98.95	17.98
5.39	5.83	8.20						
(90.28)		(110.73)						
5.33	6.45	6.44					94.95	16.56
4.45	6.31	5.73						
(97.80)	(102.22)	(112.29)						
6.64	5.36	7.13		0.82	0.70		99.66	20.19
7.01	6.14	6.41						
(94.72)	(87.30)	(111.23)						
4.02	0.88	1.13	56.50				87.06	34.77
—	—	—	—					
3.81	0.75	0.95	56.08				87.22	34.83
—	—	—	—					
							97.92	11.77
							94.82	11.58
							93.61	14.64
							90.11	11.15
							93.56	10.19
				0.70			96.97	48.48
				—				
	41.18	47.69					95.79	5.73
	50.62	49.38						
	(81.35)	(96.58)						
4.22	3.01	1.43	—	—	—	—	99.52	19.85
—	—	—	—	—	—	—		

not identified, component of a polarity lying between that of triglycerides and fatty acids. These impurities are present to the extent of 2.5–2.8 % and they are no longer observed, either as a single component (Fig. 6) or in a mixture with fatty acids (experiments No. 10 and 11), in chromatograms of tripalmitin purified by us. Equally good results are obtained for the purification of mixtures of mono- and diglycerides (Fig. 7), where small quantities of triglycerides and fatty acids are revealed. The

purity of the fractions is in every case confirmed by thin-layer chromatography.

In certain cases it can be advantageous to use columns of larger dimensions for preparative purposes. The graph in Fig. 8 shows the separation of a complete mixture of neutral lipids on a column of 2.5 cm diameter. The gradient elution was carried out for approx. 24 h. The main fractions are well separated and the recovery is of the same order as that obtained on the 5 g column.

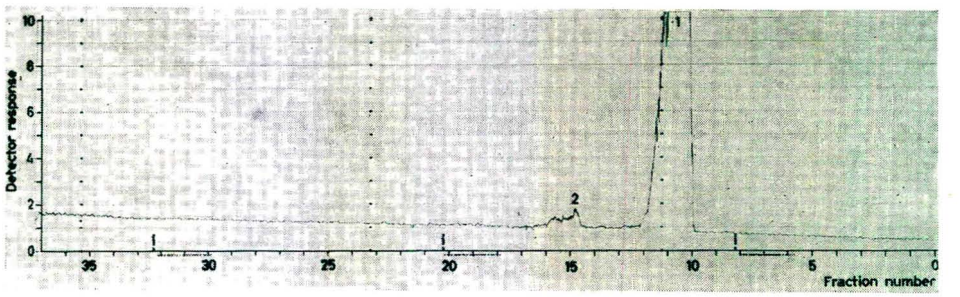

Fig. 5. Chromatogram of tripalmitin erroneously claimed as 99% pure. Direct reproduction of the recording obtained with the LCD. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 12.21 mg. Peaks 1 = pure tripalmitin; 2 = palmitic acid + unknown compound.

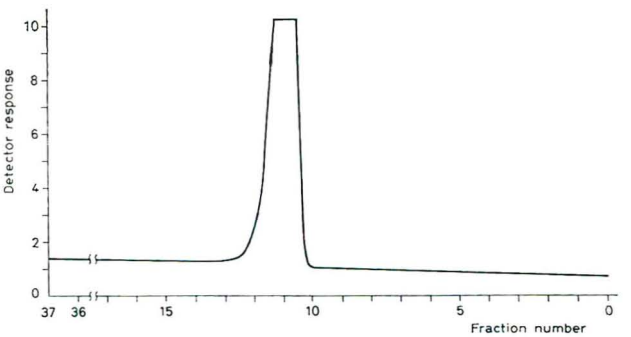

Fig. 6. Chromatogram of pure tripalmitin. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 10.89 mg. A single peak is present.

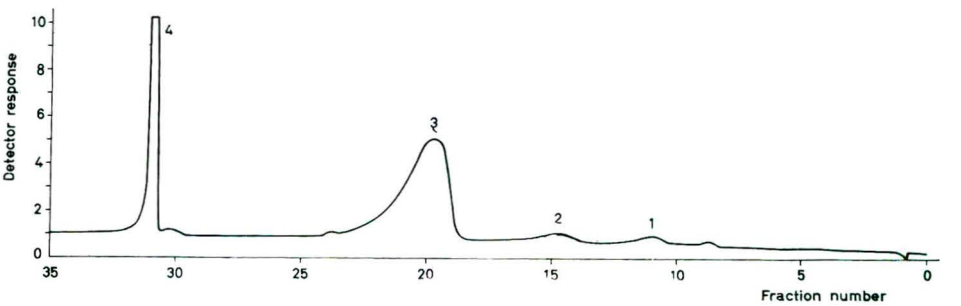

Fig. 7. Chromatogram of a mixture of glyceryl di- and monostearate. Operating conditions as in Fig. 2 except column 9 mm diam., 160 mm high; sample 5.98 mg. Peaks: 1 = tristearin; 2 = stearic acid; 3 = glyceryl distearate; 4 = glyceryl monostearate.

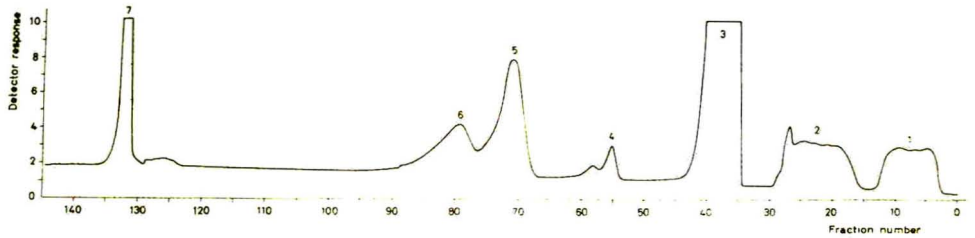

Fig. 8. Chromatogram of a reference lipid mixture. Column 25 mm diam. × 90 mm high, charged with 23 g silicic acid. Sample 85.64 mg. Detector sensitivity 3 × 10^{-10} A. Concave gradient ($P = 1/4$) C_R = 100% ether (373 ml) in petroleum ether (C_M = 1492 ml). Column flow rate 1 ml/min. Splitter ratio: 6% of the column flow to the detector. Peak order as in Fig. 2.

Application of this technique to the analysis of total lipids in rat liver, obtained by chloroform–methanol (2:1) extraction according to FOLCH *et al.*[34], is reported in Table I (experiments No. 5 and 6). The graph obtained for the separation is shown in Fig. 9. It is interesting to note the presence of 3 peaks containing esterified cholesterol in these natural lipids. We plan to study the components of these peaks and to try to establish a relation between the separation and the nature of the components. The load capacity is higher (about 40 mg of total lipids) as approximately half the weight is present as phospholipids, which are eluted at the end by methanol. The reproducibility of the data of the percent composition of the twice chromatographed extract is very good and the recovery can be considered as satisfactory, considering the fact that the extract investigated may contain some non-lipid components.

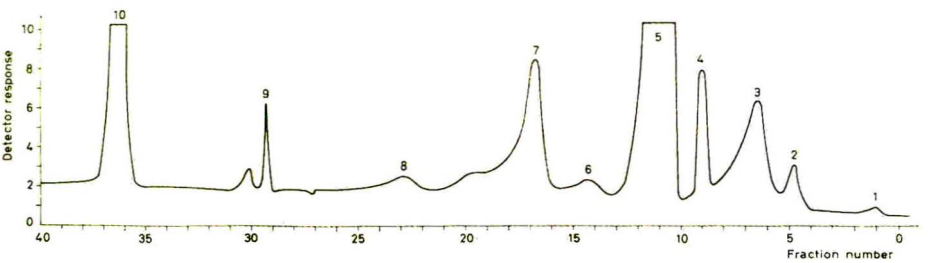

Fig. 9. Chromatogram of rat liver lipids. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 39.94 mg. Peaks: 1 = hydrocarbons; 2,3,4 = cholesterol esters; 5 = triglycerides; 6 = fatty acids; 7 = cholesterol; 8 = diglycerides; 9 = monoglycerides; 10 = phospholipids.

Examples of other important applications of this technique are given by experiment No. 14, concerning the separation of natural olive oil. The corresponding graph is reported in Fig. 10.

Fig. 11 shows the graph for a solution of testosterone propionate in neutral oil for pharmaceutical use (conc. 25 mg/ml). Clear resolution of the steroid, giving a well shaped peak lying between the di- and the monoglyceride peaks is shown. This permits a UV spectrophotometric or GLC determination. This application of our technique will be described in detail in another paper.

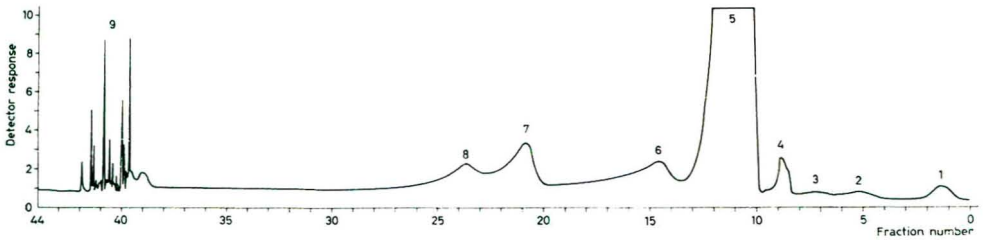

Fig. 10. Chromatogram of a natural olive oil. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 19.94 mg. Peaks: 1 = hydrocarbons; 2,3,4 = probably sterol esters; 5 = triglycerides; 6 = fatty acids; 7 = sterols; 8 = diglycerides; 9 = monoglycerides.

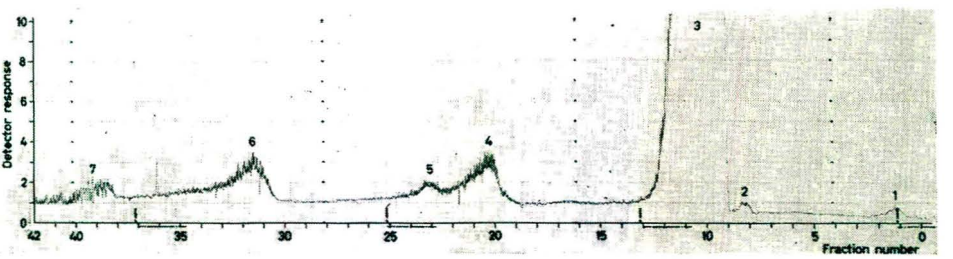

Fig. 11. Direct reproduction of the recording obtained with the LCD for chromatography of a solution of testosterone propionate in neutralised olive oil. Operating conditions as in Fig. 2, except column 9 mm diam., 160 mm high; sample 40 μl oil solution. Peaks: 1 = hydrocarbons; 2 = sterol esters; 3 = triglycerides; 4 = sterols; 5 = diglycerides; 6 = testosterone propionate; 7 = monoglycerides.

CONCLUSIONS

A method of this type is useful for the purification of single classes of neutral lipids in small quantities, for preparative purposes (for use as standards and for the preparation of derivatives for GLC etc.), and for the fractionation of lipid mixtures for analytical purposes, if one considers that colorimetric methods, as already reported in our preceding paper[39] may be employed in addition to the gravimetric method described. In any case contamination of the fractions with impurities from the solvent or the apparatus must be avoided. We think that we have demonstrated that by the use of an LCD and a weight microtechnique it is easily possible to detect impurities both qualitatively and quantitatively and abolish them if necessary. In our opinion this aspect of lipid chromatography has only been briefly considered by some authors, as for example by HORNING *et al.*[8] and has not been treated as extensively as it deserves.

Moreover a method of this type permits a chromatographic separation of lipids into classes under well standardized and reproducible conditions, with a minimum of assistance from the analyst, and a record is also obtained which permits easy appraisal of the quality of the separation. It is possible to observe the form of the peaks, the resolution, the column efficiency, in a manner similar to that done normally with

gas–liquid chromatography, applying the general considerations of liquid chromatography to lipid chromatography as described in refs. 40, 42–45.

Our results offer a useful contribution with respect to the results obtained by other authors[30,31] who have attempted to solve the difficult problem of using the LCD with microcolumns for quantitative analysis of lipids. It is one of the best real examples of the use of the LCD technique, for the analytical and preparative chromatography of lipids. The extensive use of one efficient splitter also permits one to envisage the possibility of further quantitative applications in the future.

ACKNOWLEDGEMENT

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GEL FILTRATION IN LIPOPHILIC SOLVENTS USING HYDROXYALKOXYPROPYL DERIVATIVES OF SEPHADEX

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SUMMARY

Liquid–gel chromatography involving a lipophilic derivative of Sephadex has been investigated in two solvent systems. The separations studied involve gel filtration mechanisms, and elution data are quoted for eighty compounds. Analysis of column performance characteristics has indicated high efficiency, good loading capacity and quantitative recovery of sample with little 'tailing'. The potential application of these techniques to purification and group separation has been evaluated in respect of both neutral and polar lipid fractions.

INTRODUCTION

The use of gel chromatography in aqueous media has been developed extensively for routine laboratory use. Its adaptation to less polar organic solvents is less advanced: few successful applications to lipid extracts have been reported. The properties of a number of gels suitable for lipophilic solvents (in particular the modified dextran, Sephadex LH-20) are reviewed by JOUSTRA *et al.*[1] and by NYSTRÖM[2].

Gel chromatography is a general term describing all processes involving exchange of solute between a liquid mobile phase and a stationary gel phase. The distribution of a compound between the mobile and gel phase is primarily governed by its partition coefficient. A secondary mechanism also influences elution: free diffusion of solute molecules is restricted to a fraction of the gel as a result of steric interaction with the gel matrix. The behaviour of such a gel–solvent system depends on the polarity of the matrix and the choice of eluting solvent. Three cases may be distinguished:

(i) Partition chromatography, where the gel is more polar than the solvent, and samples are eluted in order of their polarity.

(ii) Gel filtration, where the gel and solvent have the same polarity characteristics and elution is in order of decreasing molecular size of the solutes.

(iii) 'Reversed-phase' partition, where the gel is less polar than the solvent, and samples are eluted in inverse order of their polarity.

Normal Sephadex and aqueous solvents are closely matched in polarity, leading to separation by gel filtration. With lipophilic gels, polarity is variable in both

gel matrix and solvent: the resultant complications have been investigated by SJÖVALL *et al.*[3]. Polarity effects may include contributions due to hydrogen bonding, hydrophobic bonding, dipolar or ionic interactions, and dispersion forces. Consequently, it is extremely difficult to devise a system in which gel filtration is unaccompanied by other separation processes. For example, Sephadex G-25 in pure water, or LH-20 in ethanol, show a retention of aromatic and polyolefinic compounds, ascribed to dispersion forces[1,4]. Similarly, a lipophilic gel–benzene system, though showing good compatibility of polarity, may exhibit powerful hydrogen-bonding effects in addition to gel filtration.

In our investigations of terpenoids in green leaves of *Petasites hybridus*, the need arose for a method of isolating the sesquiterpenoids as a class. Gel filtration appeared to be a potentially effective technique, having regard to the fact that many accompanying lipids have considerably larger molecules[5]. Consequently, we undertook the present investigation of gel filtration systems involving the novel lipophilic derivative of LH-20 described by ELLINGBOE *et al.*[6,7].

EXPERIMENTAL

Preparation of gels

In preliminary work, Sephadex LH-20 was converted to the hydroxyalkoxypropyl-Sephadex of ELLINGBOE *et al.*[7,8]: 200 g of Sephadex LH-20 yielded 399 g of derivative. The olefin oxide used, Nedox 1114 (Ashland Oil and Refining Co., Minneapolis, Minn., U.S.A.), contains chains of 11–14 carbon atoms, and the gain in weight of the gel indicates 50 % content by weight of hydroxyalkyl groups. This derivative may be designated N1114-50 %-LH-20. (Other derivatives having different degrees of polarity can be made by using appropriate quantities of the olefin oxide[8]. Up to 60 % by weight of substituents may be incorporated by subjecting N1114-50 %-LH-20 to a second reaction step under the same conditions.) After solvent washing, the gels were dried *in vacuo* for 24 h. The dried product, unlike the starting material, was hydrophobic and waxy. The bead structure was intact*. Silanisation of glassware facilitated handling Sephadex gels of all types.

Material for high resolution columns was prepared using a fraction of Sephadex LH-20 (particles $< 53\ \mu$) that passed through a 300 B.S. mesh sieve. Sieving of N1114-50 %-LH-20 was not possible because of aggregation of beads. This fraction contains some very fine ($< 10\ \mu$) and colloidal materials which influence separation adversely. Derivatives with narrower particle size distributions have been prepared (*cf.* ref. 3) using the continuous flow differential sedimentation method of HAMILTON[9]. Starting material in this case was Superfine Sephadex G-25. This yielded four fractions, with bead sizes (in the dry state) of 10–16 μ, 17–23 μ, 24–30 μ, and 30–40 μ. These fractions were subsequently converted to LH-20-type derivatives using a method described by ELLINGBOE *et al.*[7].

Packing of columns

Gels were allowed to swell overnight in the appropriate solvent. Before packing, the gel slurry was freed from occluded air by immersion for 5–10 min in an ultrasonic

* The beads may be damaged in the reaction if a magnetic or mechanical stirrer makes contact with the walls of the reaction vessel[8].

bath. Glass columns were fitted with an extension, and part by filled with solvent. Pre-swollen coarse gel was added, to cover the lower end of the column to a depth of a few millimetres, and the gel slurry was added in one operation. A steady flow of solvent was started, and the gel allowed to settle under continuous vibration. Under these conditions, sedimentation of gel lasted 6 h and bed volume was stabilised after 24 h. No additional pressure was applied. It was essential for even packing that the column was vertical during settling of the gel.

After 24 h, the column had passed solvent to about three times the bed volume. A filter paper was placed on top of the gel. This protected the surface against mechanical disturbance, and appeared to improve the distribution of sample on the bed. Solvent washing was continued for a further 24 h before analytical work was commenced. Gel packing factors (volumes of column occupied by 1 g of swollen gel) are given for solvents suitable for gel filtration in Table I.

Columns for high-resolution chromatography were made up with a view to minimising the effect of dead spaces on peak broadening. Glass tubing 9 mm I.D. was fitted to a standard Teflon tubing connector (Glenco No. 3020 1/2 in. to B) via a collar that held fine Teflon fabric, which acted as a bed support, and showed less

TABLE I

VOLUME OF COLUMN PACKED BY 1 g OF N1114-50%-LH-20 AFTER SWELLING

Solvent systems suitable for gel filtration behaviour.

Solvent	*Gel packing factor (volume packed/g of dry gel)*
Benzene	3.75[a]
Tetrahydrofuran	3.71[a]
Chloroform	4.43
1,2-Dichloroethane	3.16
Benzene–isopropanol (75:25)	3.78[a]
Cyclohexane–isopropanol (75:25)	4.12

[a] Interchange of these solvents on-column is permissible.

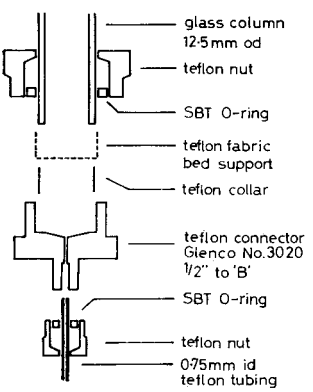

Fig. 1. Column end fitting. Teflon connector Glenco No. 3020 from Glass Engineering Co., Inc., Houston, Texas, U.S.A. Teflon fabric bed support 0659 SR25/45 from Pharmacia Fine Chemicals. O-rings cut from solvent-resistant rubber sheet type SBT, Esco (Rubber) Ltd., London.

tendency to clog than porous glass or Teflon discs. The connector was attached directly to the fraction collector by Teflon tubing (Fig. 1). As accurate determination of S.E.V. (see below) depended on accurate measurement of bed volume, such columns were calibrated volumetrically before packing. The bed volume could then be measured at any time to allow for variations arising from changes in temperature or flow rate.

Sample handling techniques

Samples of reference compounds were applied as test mixtures, of one to ten components, containing 0.05–10 mg (commonly 0.5 mg) of each component in a total of 0.1 ml of the eluting solvent: these quantities were well within the capacity of each column. Elution and collection of fractions was carried out on the BTL Chromafrac system using a peristaltic pump to deliver highly reproducible fraction volumes. Vinescol 23 fluorinated rubber tubing (Esco (Rubber) Ltd., London), used in the pump, was resistant to benzene and other solvents. All other fittings were glass or Teflon.

Samples were applied to the top of the column without mixing, by a careful procedure. Excess solvent was allowed to drain into the bed, and sample applied to the moist filter paper protecting the bed surface. The sample was washed below this surface by dropwise addition of 0.1 ml solvent before elution was started. This has been found to be the most reproducible method of sample application for analytical purposes.

The elution of samples was examined by collection of fractions (0.1–0.5 ml) and quantitative GLC, allowing separate determination of partly resolved components. Involatile compounds (plant pigments, glycerol esters and natural sterol esters) required quantitative TLC assay.

Retention data are recorded as the Standard Elution Volume, S.E.V.[5]. This is defined as the ratio between measured elution volume and total column volume, multiplied by 100. S.E.V. is a dimensionless quantity, numerically equal to 'percentage of total column volume'[3,7,8]. This serves to correlate results from different sources by reducing them to a standard form.

RESULTS AND DISCUSSION

The lipophilic dextran N1114-50 %-LH-20 has been investigated in two solvent systems, *viz.* benzene, and benzene–isopropanol (75:25). Elution data are recorded in Tables II–V, and certain regularities are illustrated in Fig. 2 (*cf.* also Fig. 5). Molecular shape has a very marked influence on the effective molecular size for gel filtration: linear compounds tend to be eluted earlier than branched or cyclic molecules. Members of a homologous series show a progression in S.E.V. values that is related to the logarithm of the molecular weight: this is more clearly exposed in Fig. 3 for the *n*-alkane series.

A detailed examination of the benzene–gel system (Tables II and III and Fig. 2) shows that compounds having a free hydroxyl group are retarded on the gel. Elution of such compounds occurs later than would be allowed by the purely steric mechanism of gel filtration, for which the limiting value is about 80 S.E.V. Other polar groupings, such as the carbonyl groups in progesterone, do not affect elution in the same way.

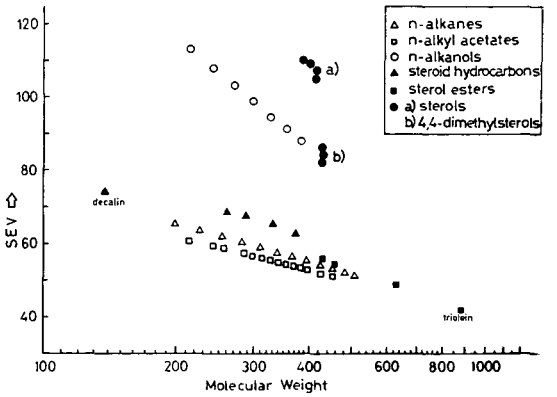

Fig. 2. Relationship between Standard Elution Volume (S.E.V.) and molecular weight for the N1114-50 %-LH-20/benzene system. Cyclised molecules (solid markings) tend to have higher S.E.V. values for a given molecular weight than straight-chain types (open symbols) except where steric hindrance of hydroxyl groups influences retention, *e.g.* the 4,4-dimethylsterols.

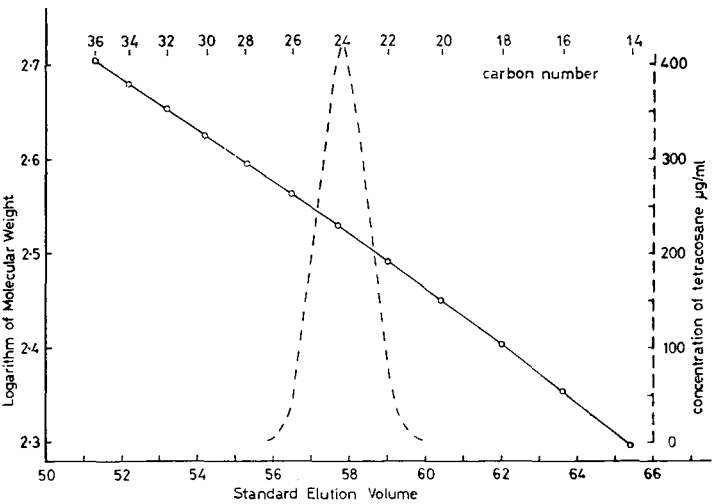

Fig. 3. Elutions of homologous series from N1114-50 %-LH-20/benzene columns. *n*-Alkanes display an almost linear relationship between S.E.V. and the logarithm of the molecular weight. The slope in the region from C_{24}–C_{36} is such that 10 % change in molecular weight (Δ log mol. wt. = 0.041) corresponds to a difference of 1.5 units in S.E.V. The broken line indicates the elution profile of tetracosane from the 100 cm column, showing the degree of overlap with neighbouring alkanes.

It appears that a hydrogen bonding effect is operating, with the gel acting exclusively as the basic component. In accord with this, retention of hydroxylic compounds is related to the environment of the hydroxyl groups (Table III). The *n*-alkanol series shows progressive gel filtration behaviour superimposed on the retention due to the hydroxyl group. Phenols are more strongly retained than aliphatic alcohols, and carboxylic acids are eluted too slowly for practical use under these conditions. There is no retention effect associated with aromaticity. Esters and ketones are eluted

TABLE II

S.E.V. VALUES OF NON-HYDROXYLIC COMPOUNDS ON THE N1114-50%-LH-20/BENZENE SYSTEM
Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.14 mm. Operating temperature: 18°.

Compound	*S.E.V.*	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
n-Hexatriacontane	51.3	506	α-Amyrin acetate	57.1	468
n-Tetradecane	65.4	198	Stigmasteryl acetate	55.2	454
Methyl lignocerate	53.4	382	Cholesteryl TMSi	55.9	458
Methyl behenate	54.5	354	Cholestan-3-one	60.0	386
Methyl arachidate	55.6	326	Cholest-4-ene-3,6-dione	58.6	398
Methyl stearate	56.8	298	Cholesteryl chloride	59.7	404.5
Methyl myristate	59.5	242	Progesterone	61.3	314
Methyl laurate	60.8	214			
			Isopetasin	56.9	316
Cholesteryl palmitate	49.0	624	Isopetasyl acetate	59.1	276
Cholesteryl butyrate	54.5	456	Isopetasone	61.3	232
Cholesteryl acetate	56.3	428	S-Petasin	54.2	334
Cholesteryl benzoate	54.0	490			
Cholestane	62.7	372	Tetralin	71.0	132
Coprostane	62.6	372	Decalin	73.8	138
5α-Cholane	65.3	330	Naphthalene	73.2	128
5α-Pregnane	67.7	288			
5α-Androstane	68.5	260			

TABLE III

S.E.V. VALUES OF HYDROXYLIC COMPOUNDS ON THE N1114-50%-LH-20/BENZENE SYSTEM
Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.14 mm. Operating temperature: 18°.

Compound	*S.E.V.*	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
Ceryl alcohol	88.1	382	Phytol	93.5	296
Lignoceryl alcohol	91.3	354	*trans,trans*-Farnesol	97.7	222
Behenyl alcohol	94.6	326	*cis,trans*-Farnesol	93.5	222
Arachidyl alcohol	99.0	298	Nerolidol	77.7	222
Stearyl alcohol	103.3	270	Isopetasol	119.6	234
Cetyl alcohol	108.2	242			
Myristyl alcohol	113.2	214	Methyl deoxycholate	156	406
			Methyl hyodeoxycholate	236	406
Cholesterol	110.2	386	Methyl ursodeoxycholate	134.5	406
Lanosterol	82.3	426	Methyl chenodeoxycholate	156	406
Dihydrolanosterol	84.5	428			
Cycloartenol	86.5	426	Estrone	360	270
Stigmasterol	105.1	412			
β-Sitosterol	107.4	414			
Campesterol	109.2	400			

earlier than might be expected on the basis of molecular weight. This effect was first observed by ENEROTH AND NYSTRÖM[10] for ketonic steroids on other lipophilic gels, in chloroform or methylene chloride, and has been illustrated further by SJÖVALL *et al.*[3].

On addition of an alcohol to the eluting solvent, hydroxylic compounds are selectively displaced from the gel (presumably through competitive hydrogen bonding): other compounds are not affected to any great extent (Fig. 4). At a level of 25% isopropanol in benzene, there is no observable polarity effect for cholesterol, nor for any

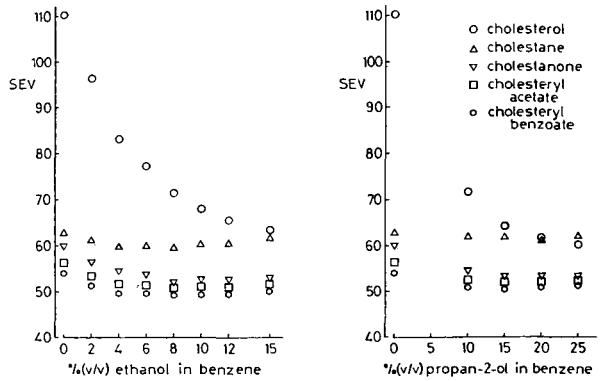

Fig. 4. Influence of alcohols in eluting solvents on the elution of cholestane derivatives.

TABLE IV

S.E.V. DATA FOR THE NI114-50%-LH-20/BENZENE–ISOPROPANOL (75:25) SYSTEM

Δ (S.E.V.) refers to [S.E.V. for benzene (Tables II and III) – S.E.V. for benzene–isopropanol]. Column: 100 × 0.9 cm I.D. Flow: 6 ml/h. HETP: 0.17 mm. Operating temperature: 18°.

Compound	*S.E.V.*	Δ (*S.E.V.*)	*Mol. wt.*	*Compound*	*S.E.V.*	*Mol. wt.*
n-Hexatriacontane	50.3	1.0	506	5α-Cholan-24-ol	63.2	346
n-Tetradecane	64.0	1.4	198	5α-Cholane-3α,24-diol	59.3	362
Octacosyl acetate	48.1	3.1	452	5α-Cholane-3α,12α,24-triol	59.9	378
Myristyl acetate	55.2	3.7	256	5α-Cholane-3α,7α,12α,24-tetrol	59.2	394
Ceryl alcohol	53.9	24.2	382			
Myristyl alcohol	62.2	51.0	214	Lithocholic acid (3α-OH)	58.6	376
Lignoceric acid	54.2	—	368			
Lauric acid	63.0	—	200	Deoxycholic acid (3α,12α-OH)	59.9	392
				Hyodeoxycholic acid (3α,6α-OH)	56.0	392
				Ursodeoxycholic acid (3α,7β-OH)	56.0	392
Cholesteryl acetate	53.3	3.0	428	Chenodeoxycholic acid (3α,7α-OH)	59.6	392
Cholesteryl benzoate	52.2	1.8	490			
Cholestan-3-one	54.5	5.5	386	Cholic acid (3α,7α,12α-OH)	59.4	408
Cholesterol	60.8	49.4	386			
Cholestane	62.8	−0.1	372	Gibberellin A_1	64.5	348
Coprostane	62.6	0	372	Gibberellin A_3	64.5	346
5α-Cholane	65.9	−0.4	330	Gibberellin A_4	61.6	332
5α-Pregnane	68.2	−0.5	288	Gibberellin A_5	59.1	330
5α-Androstane	69.3	−0.8	260	Gibberellin A_7	61.6	330
				Gibberellin A_9	56.8	316
Stigmasteryl acetate	52.1	3.1	454			
Stigmasterol	59.2	45.9	412			
Lanosterol	58.5	23.8	426			
Isopetasin	50.3	6.6	316			
Isopetasyl acetate	51.1	8.0	276			
Isopetasone	53.1	8.2	232			
Isopetasol	58.2	61.4	234			

of the more polar compounds studied. Free acids are not separated from their methyl esters. Higher levels of alcohol give rise to reversed-phase partition[7]. Data for the system benzene–isopropanol (75:25) are cited in Table IV. Non-hydroxylic compounds behave substantially as in the pure benzene system, but polar compounds no longer show distinctive behaviour (Fig. 5). No compound has been found to be retained longer than 80 S.E.V., and this is the nearest to ideal gel filtration encountered in the present study. The most apparent abnormality is the early elution of carbonyl compounds. A carbonyl group is equivalent to about eight methylene units in this system.

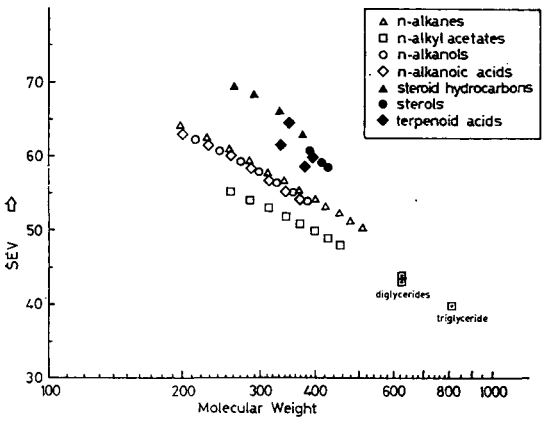

Fig. 5. Relationship between S.E.V. and molecular weight for the system N1114-50 %-LH-20/benzene–isopropanol (75:25).

Application of gel chromatography to biological separations

While deviations from ideality are substantial in the systems described, molecular size is clearly a major factor determining elution. Group separations, *e.g.* of lipids from lipoproteins, or glycerol esters from simple terpenoids, are thus potentially feasible. Gibberellins, for example, have closely related structures, and in gel filtration systems this results in similar S.E.V. values. The system benzene–isopropanol (75:25) elutes these compounds as a composite peak (Fig. 6). A correlation of elution data for

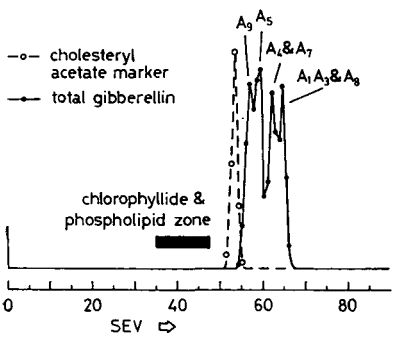

Fig. 6. Elution pattern of gibberellin mixtures in N1114-50 %-LH-20/benzene–isopropanol (75:25).

TABLE V

CORRELATION OF S.E.V. DATA FOR COMMON LIPIDS AND NATURAL PRODUCTS

Systems: A = N1114-50 %-LH-20/benzene. B = N1114-50 %-LH-20/benzene–isopropanol. (75:25) C = LH-20/benzene–ethanol (1:9).

Compound	*System*		
	A	*B*	*C*
Triolein	42.2	—	52.0
Tripalmitin	43.2	39.8	—
Lecithin[a]	—	36.8	—
Phosphatidic acid[a]	—	43.8	—
Distearin (2-OH)	—	43.2	—
Distearin (3-OH)	—	44.0	—
Monostearin (1,2-diOH)	280	55.4	—
Chlorophyll *a*	45.0	—	73.5
Chlorophyll *b*	44.8	—	70.0
β-Carotene	47.4	—	70.0
Squalene	52.0	51.0	58.5
β-Sitosterol	107.4	59.4	69.0
Cholesterol	110.2	60.8	70.0
Lanosterol	82.3	58.5	68.5
Petasin	56.9	50.3	76.5
S-Petasin	54.2	—	80.0

[a] These samples were not homogeneous.

compounds of biological origin is given in Table V. On unmodified Sephadex LH-20 (ref. 4), retention of aromatic compounds does not allow the separation of involatile compounds from those suitable for gas chromatography. This is best achieved by the N1114-50 %-LH-20/benzene system, which eliminates compounds of high molecular weight by elution in early fractions, and retains compounds of excessive polarity. A polar fraction so retained may be eluted subsequently by washing through with solvent containing alcohol. This effectively purges the column, which may then be re-used after equilibration with benzene.

Column performance

Columns were prepared with particular attention to high efficiency. The sieving of Sephadex LH-20 was most important in this respect, together with the design of the actual chromatographic system. Theoretical plate heights have been calculated from the elution of cholesteryl acetate[5] (Fig. 7). For standard columns (100 × 0.9 cm I.D.) 7000 plates have been obtained, corresponding to a HETP of 0.14 mm. Commercial Sephadex LH-20, unsieved, gives a HETP of 0.5 mm, and material fractionated by sedimentation has been reported[3] to give a HETP in the region of 0.1 mm. The elution of cholesteryl acetate was monitored by the collection of fractions (0.1 ml) and quantitative GLC: 25 determinations were required to construct each peak in Fig. 7.

Peaks eluted from dextran gels in general appear to be Gaussian in form, with little tailing. Tailing has been investigated by application of a high loading (Fig. 7). GLC analysis of the effluent was able to detect 10 p.p.m. of the applied sample:

30 p.p.m. was found in a fraction 3 S.E.V. units from the peak centre, and none was detectable at 3.2 S.E.V. units. These results have been substantiated using 30 μCi [24-^{14}C]cholic acid, which demonstrated little long-term retention of radioactivity, and minimal radioactive bleed after the sample had passed (Fig. 8). This capability is essential for efficient fractionation of radioactive extracts obtained in lipid metabolic studies.

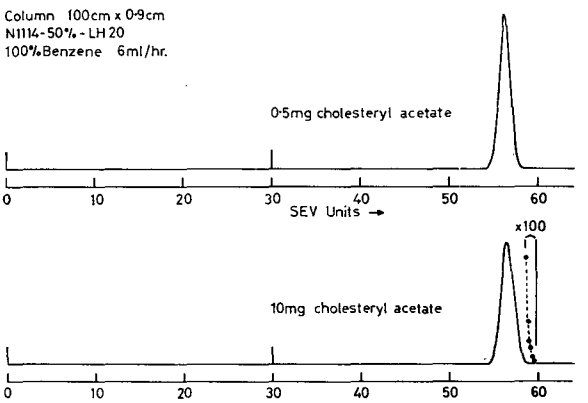

Fig. 7. Elution profile of cholesteryl acetate from N1114-50%-LH-20/benzene. The upper profile has a peak width at baseline of 2.7 S.E.V. units from which the HETP of 0.14 mm may be derived[11]. The lower profile shows the effect of a higher loading and a 100-fold scale expansion (broken line) illustrates the absence of serious 'tailing'.

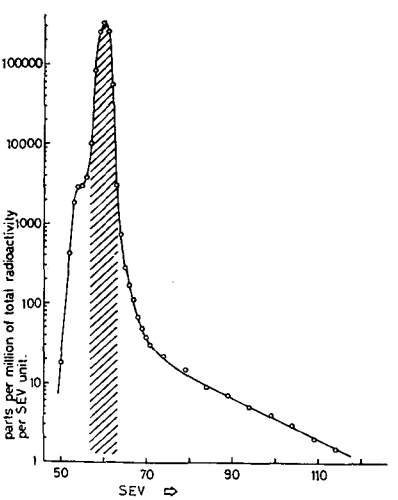

Fig. 8. Elution of 30 μCi [24-^{14}C]cholic acid (200 μg sample) from N1114-50%-LH-20/benzene–isopropanol (75:25). No inactive carrier was added to the sample. Recovery was measured as 102% of total activity administered, 62.28 × 10^6 d.p.m., 101% falling in the shaded region. The logarithmic scale permits an unbroken curve to be plotted to 1 p.p.m. of total activity. The hump at S.E.V. 54 represents an impurity not separable by TLC (0.7% of total activity).

Theoretical aspects

The theoretical aspects of gel chromatography have been discussed by GIDDINGS AND MALLIK[11], and zone dispersion in particular by BILLMEYER *et al.*[12]. HEITZ AND ČOUPEK have investigated experimental factors describing zone dispersion[13]. Factors determining separation have been considered by FLODIN[14] and SJÖVALL *et al.*[3]. It is observed that the HETP varies with the nature of the solute[12] as well as with the expected operating factors such as flow rate, particle diameter[3], and temperature[8]. In this investigation it has been found that for gel filtration processes, peak width is independent of elution volume (Table VI) and this result has also been reported by BOMBAUGH *et al.*[15] using polystyrene gels. Polarity partition processes follow the normal behaviour associated with chromatography.

TABLE VI

ZONE BROADENING EFFECTS IN GEL FILTRATION

Peak widths[10] (4σ) of *n*-alkanes on the N1114-50 %-LH-20/benzene system.

n-Alkane	*S.E.V.*	4σ	*n-Alkane*	*S.E.V.*	4σ
C_{34}	52.2	2.6	C_{22}	59.0	2.6
C_{32}	53.2	2.6	C_{20}	60.4	2.9
C_{28}	55.3	2.7	C_{18}	62.0	2.8
C_{26}	56.5	2.9	C_{16}	63.6	2.7
C_{24}	57.7	2.8	C_{14}	65.4	2.6

Compared with any process dependent on polarity, gel filtration is a weak method of separation. High column efficiencies are essential, and must be coupled with a collection or detection system appropriate to the small separation volumes frequently obtained. In some respects, dextran derivatives are considerably more effective than polystyrene media. They are also well suited to the technique of 'recycling chromatography'[16] as demonstrated by NYSTRÖM AND SJÖVALL[17], who obtained, in this way, efficiencies of over 50 000 theoretical plates using methylated Sephadex on 60 × 2.5 cm columns. N1114-50 %-LH-20 separates over a molecular weight range from 100 to 2000, and 30 000 plates would allow separation of compounds differing by 10 % in molecular weight. A similar separation on a polystyrene gel required 180 000 plates in a column of 160 ft.[15].

Practical aspects of gel chromatography

The 80 S.E.V. limit for gel filtration has many advantages in the application of gel chromatography to analytical liquid chromatography. Compounds are eluted more rapidly than in other techniques, for a given flow rate. Secondly, the column has no tendency for prolonged retention of samples, and may be used repeatedly. Re-use of a column is one of the major factors in the development of analytical chromatography. Reproducibility in analytical work is more attainable when there is no variation in column parameters from one trial to another. The results listed in Tables II–V were determined on a single column. S.E.V. values could be measured directly to a standard error of ± 2 %, and by correlation[5] with cholesteryl acetate (S.E.V. 56.3), to ± 0.5 %. As a corollary to reproducibility, predictability of results is also extremely good, and S.E.V. values may be taken from a qualitative analysis and used to estimate

actual elution volumes in a subsequent preparative separation. Gel chromatography is well adapted to preparative work, and separation is not affected by high sample loadings. Fig. 7 shows two peaks, at loadings of 0.5 mg and 10 mg respectively, without increase of peak width for the larger sample. The limit for a 9 mm column is about 30 mg, when peaks may be 10 % broader. Higher loadings may be tolerated if separation factors are sufficiently high. Fig. 9 compares loadings of 6 and 250 mg/cm^2; peak broadening is due mainly to the large sample volume required. The inert chemical nature of the modified dextran is another asset in preparative work. Recovery of compounds has been found quantitative: the most satisfactory evidence has been derived from studies of [^{14}C]cholic acid (Fig. 8), and of [^{14}C]cholesterol, which yielded similar results. It appears that gel chromatography may be generally preferable in the separation of labile substances, and biological extracts may be applied directly, without conversion to derivatives.

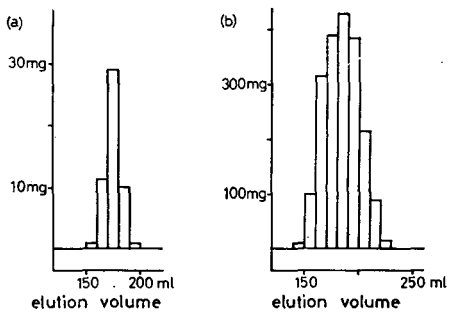

Fig. 9. Effect of high loading in preparative gel chromatography. Column 37 × 3.2 cm Sephadex LH-20 in chloroform; sample diphenyldiselenide (supplied by Dr. D. D. MacNicol). (a) 50 mg in 1 ml chloroform; (b) 2 g in 25 ml chloroform.

CONCLUSION

While it is apparent that the technique of gel filtration in lipophilic solvents does not give the same separating power as GLC, the method may be developed as a means of group separation for biological extracts. Gel chromatography in general has advantages of versatility, high efficiency and reproducibility. The inert chemical nature of the gel means that there is little or no irreversible adsorption of sample, allowing sensitive samples to be analysed, and permitting repeated use of columns. Conversion of polar samples to derivatives is not necessary, as solvent systems may be employed that are independent of sample polarity. Liquid chromatography is convenient for preparative work, and gel chromatography has in addition to the above advantages a high sample loading capacity.

ACKNOWLEDGEMENTS

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CHROM. 4317

POLYTETRAFLUOROETHYLENE LAYERS FOR THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

Polytetrafluoroethylene layers have been evolved whose properties are satisfactory for thin-layer chromatography (TLC). These properties include versatility of composition, rate of drying, uniformity, adherence to a support without binder, wettability, rate of development, and resolution of test-mixture components. The layers have been used to separate both inorganic and organic mixtures. Polytetrafluoroethylene is compatible in thin layers with numerous TLC adsorbents and thus can be a component of mixed-adsorbent or gradient-adsorbent layers or an inert diluent for other TLC adsorbents. The inertness of polytetrafluoroethylene layers is an advantage in their deposition from, development by, or visualization with highly corrosive liquids. Polytetrafluoroethylene layers are especially suitable for extraction chromatography; the extractant may be included in either the layer or the developer. A thin layer of polytetrafluoroethylene is a suitable solid support for liquid ion exchangers. Therefore, by TLC on polytetrafluoroethylene it should be possible to study the mechanisms of liquid ion exchange without the need to consider the reactivity of the liquid phases with the solid support.

INTRODUCTION

Polytetrafluoroethylene is often a solid support in gas chromatography[1] and less often in column chromatography of both inorganic[2] and organic compounds[3]. Many of the materials that are solid supports in gas and column chromatography are also used as solid phases for thin-layer chromatography (TLC), but polytetrafluoroethylene is not among them. Possibly, its lack of use in TLC is attributable to its nonwettability by some liquids.

The work described here demonstrates that polytetrafluoroethylene is a satisfactory and versatile solid phase for TLC. Three factors make its use in TLC possible: (1) it is lipophilic and thus is wettable by and swells in numerous organic liquids, (2) fluorochemical surfactants cause it to be wet by aqueous media, and (3) it is now

* Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

available in suitable particulate form. In this work, several potentially useful types of particulate polytetrafluoroethylenes and of surfactants were evaluated. Procedures were developed for preparing thin layers of polytetrafluoroethylene, and the layers were used for the TLC separation of inorganic and organic mixtures.

Compared with other TLC media, polytetrafluoroethylene layers have several advantages; the layers are: chemically inert; compatible with corrosive liquids that may be used as slurry media, developers, or reagents for visualization; and essentially noninterfering in the identification and quantitative measurement of resolved components.

PARTICULATE POLYTETRAFLUOROETHYLENE FOR USE IN TLC

Properties desired

To be a suitable solid phase for TLC, a material must have a particle size in the range about 1 to 25 μ[1]. The polytetrafluoroethylenes used for gas and column chromatography (*e.g.*, the Tee 6's) have particles that are too large (~ 105 to 120 μ). Those offered as lubricants meet the particle-size requirement and are the types that were evaluated.

Also, the material must be wettable by all the liquids used in the TLC system. The nonwettability of polytetrafluoroethylene by certain liquids is overcome by the use of fluorochemical surfactants.

Sources

Sources of particulate polytetrafluoroethylenes are limited. Table I lists sources of polytetrafluoroethylene and related types of lubricants potentially useful in TLC. Of these, the following were evaluated: TL-120 FEP, TL-126, Fluoroglide 200 lubricant (lots 1 and 2), and Fluoroglide 200 chromatography grade TWO218. The Fluoroglides were the most satisfactory; the supplier has indicated that the Fluoroglide 200

TABLE I

SOURCES OF POLYTETRAFLUOROETHYLENE AND RELATED TYPES OF PARTICULATE LUBRICANTS

Designation	*Approximate average particle size* (μ)	*Source*
Fluoroglide 200[a]	10	Chemplast, Inc., 150 Dey Rd., Wayne, N.J. 07470, U.S.A. (Distributor: Norrell, Inc., 721 Scott St., Memphis, Tenn., U.S.A.)
Fluoroglide 200, chromatography grade TWO218[b]	1	
TL-115	8 to 10	Liquid Nitrogen Processing Corp., 412 King St., Malvern, Pa., U.S.A.
TL-120 FEP[c]	0.7	
TL-126	8 to 10	
Whitcon 5, 6, 7, and 8	< 1	Whitford Chemical Corp., P.O. Box 515, Westchester, Pa. 19380, U.S.A.

[a] Two lots of this were obtained from Norrell, Inc.; their properties differed slightly.
[b] This material was obtained directly from Chemplast, Inc.
[c] Described by the vendor as 'fluorinated ethylene propylene', a material closely related to polytetrafluoroethylene.

chromatography grade TWO218 most likely is the form that will continue to be available[5].

Evaluation of properties

Particle size and shape of each of the materials evaluated were determined by electron microscopy. Figs. 1 and 3 show electron micrographs of the materials as received; the differences among them are readily evident. Figs. 2 and 3 give electron micrographs of the dry residues after the materials were layered from organic liquids.

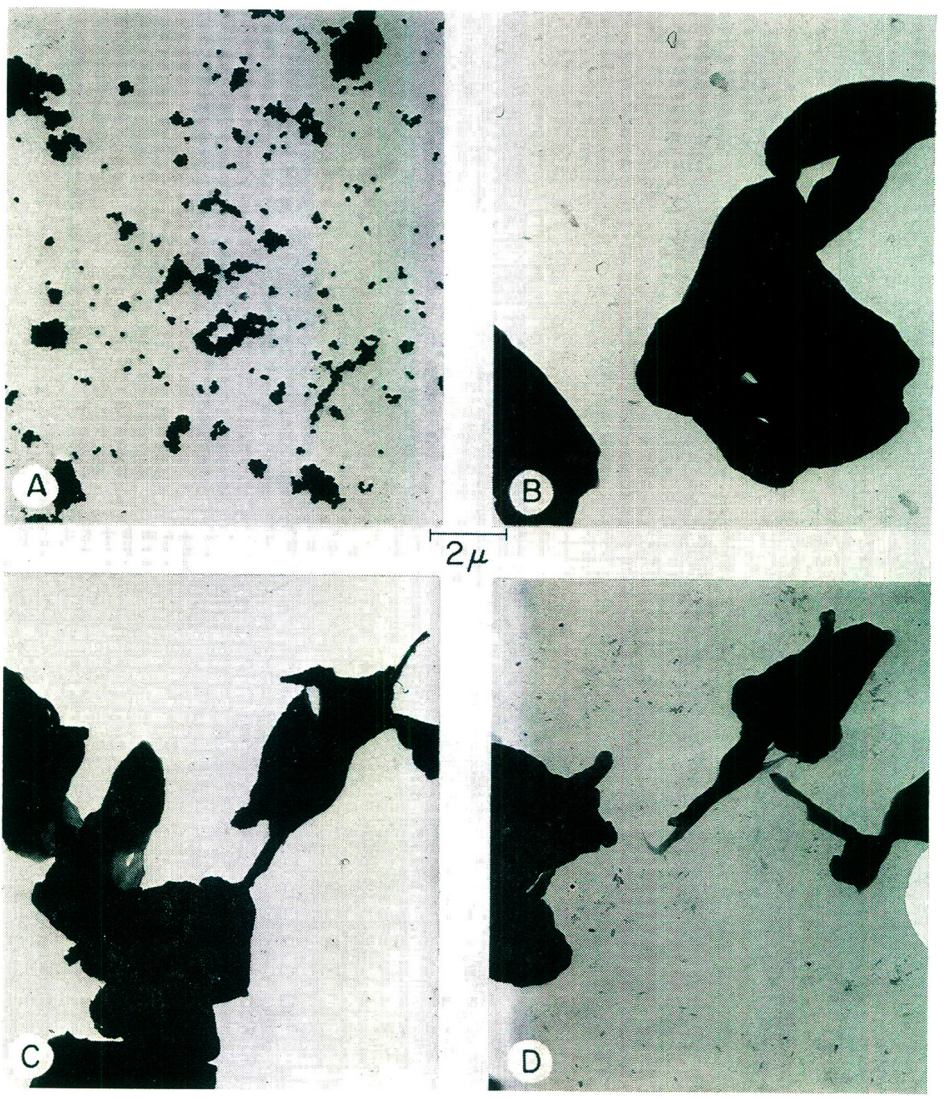

Fig. 1. Electron micrographs of particulate lubricants as received. A = 'fluorinated ethylene propylene' TL-120 FEP; B = polytetrafluoroethylene TL-126; C = polytetrafluoroethylene Fluoroglide 200, lot 1; D = polytetrafluoroethylene Fluoroglide 200, lot 2.

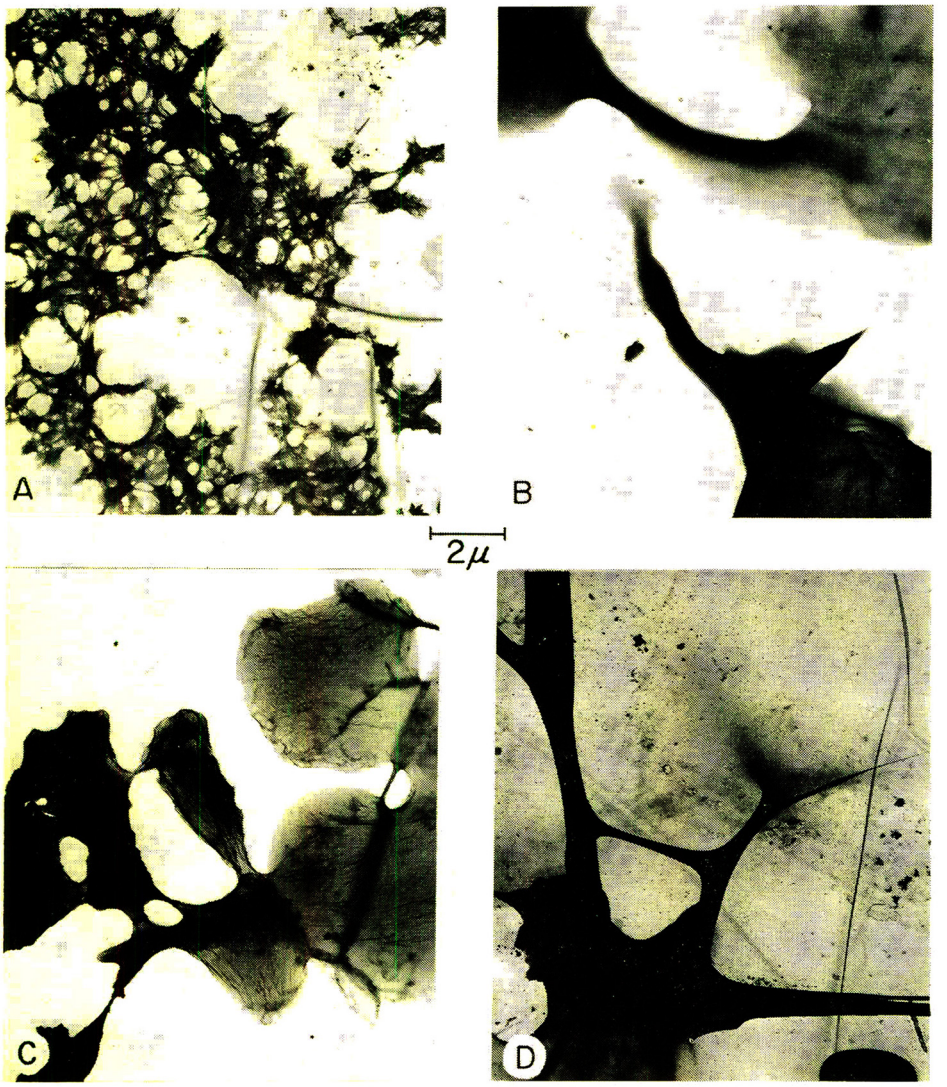

Fig. 2. Electron micrographs of dry residues from slurries of particulate lubricants in methyl isobutyl ketone. A = 'fluorinated ethylene propylene' TL-120 FEP; B = polytetrafluoroethylene TL-126; C = polytetrafluoroethylene Fluoroglide 200 lubricant, lot 1; D = polytetrafluoroethylene Fluoroglide 200 lubricant, lot 2.

These electron micrographs strikingly indicate that the forms of the particles are changed somewhat when the particles are deposited from a slurry in an organic liquid. The degree of change appears to be related to the degree of their swelling in the liquid and to the original size and shape of the particles.

The electron micrographs give clues as to the suitability of the materials for forming thin layers from slurries. The TL-120 FEP forms slurries of excellent quality, but the dry layers 'craze' excessively. Slurries of the TL-126 are difficult to pour or

to spread evenly because of lumping of the solid; the dried layers have a rough discontinuous surface. The inclusion of a fluorochemical surfactant with the TL-126 does not improve its performance. Each of the Fluoroglides gives both slurries and layers of good properties. The Fluoroglides differ among themselves in the amount of liquid required as a slurrying medium (TWO218 requires about one-third more than the others) and in the color of the layer formed (TWO218 gives the whitest).

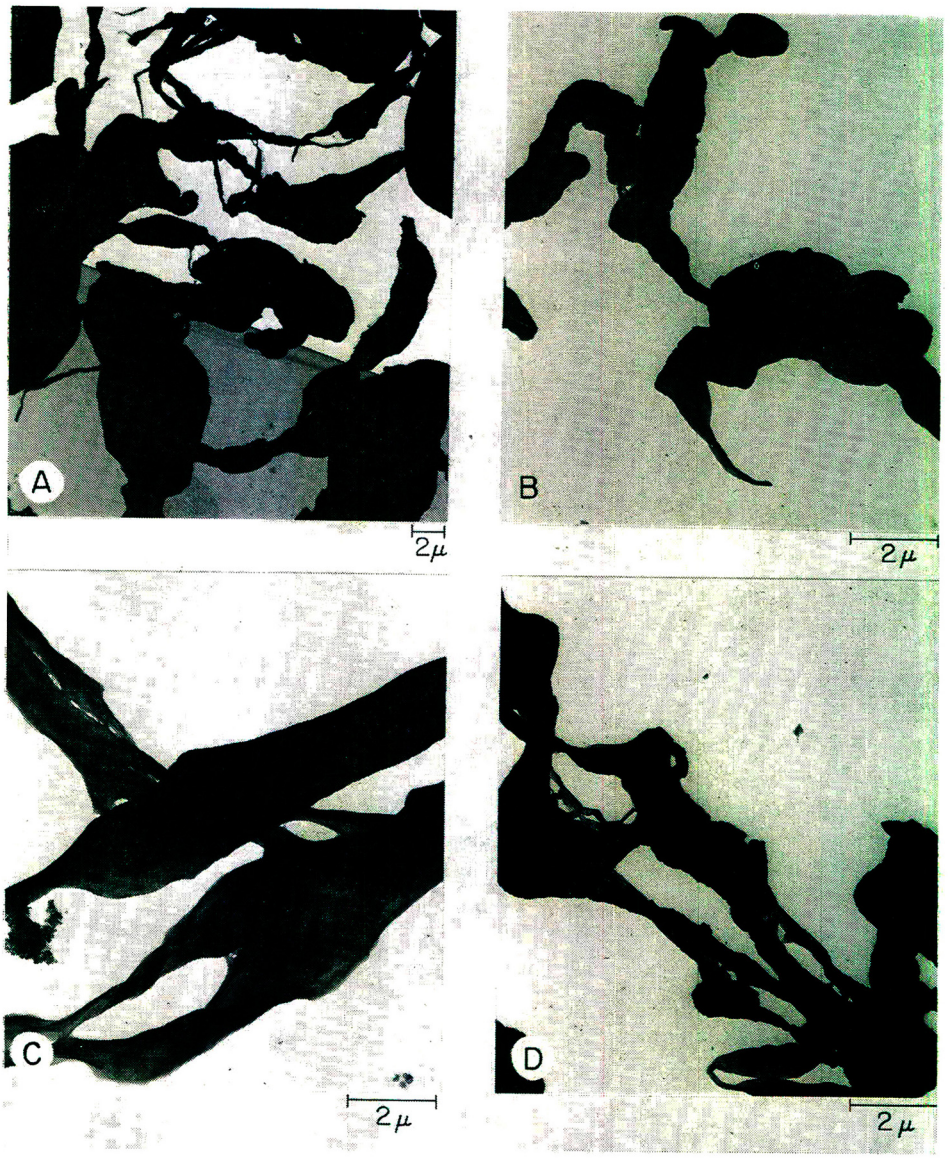

Fig. 3. Electron micrographs of Fluoroglide 200 chromatography grade TWO218. A and B = as received; C = dry residue from slurry in methyl isobutyl ketone; D = dry residue from slurry in perfluoro-2-butyltetrahydrofuran.

It appears that the great irregularity of shape and the strand-like tails of the particles of the Fluoroglides (especially TWO218) cause interlocking of the particles. Thus, the layers formed from them adhere to supports, do not craze, and provide interstices. Very likely, the interstices entrap some of the liquid phase of the slurry and also serve as capillary channels along which a developer can migrate.

The surface area of the 'fluorinated ethylene propylene' and of each of the polytetrafluoroethylenes was measured by nitrogen adsorption[6]. The results are:

Material	*Surface area, m^2/g*
TL-120 FEP	13.7
TL-126	1.0
Fluoroglide 200 (lot 1)	1.1
Fluoroglide 200 (lot 2)	0.9
Fluoroglide 200 (TWO218)	3.0

These values indicate the relative adsorptive capacities of the materials.

Swelling in organic liquids is a property of polytetrafluoroethylene that has received only limited attention[7,8]. This property is advantageous in the preparation of TLC layers, because it makes possible slurries that settle relatively slowly and therefore are easy to handle. Also, it facilitates the incorporation into the layers of certain added components such as liquid ion exchangers. The relative degree of swelling of TL-126 in 20 organic liquids and of Fluoroglide 200 (lot 2) in 42 organic liquids was measured. The liquids were representative of those that might be used as slurrying media or as developers. Among the liquids were alkanes, alkenes, arenes, alcohols, ketones, amines, fluorinated organic compounds, chlorinated hydrocarbons, dimethyl sulfoxide, and dimethylformamide. The amines, cyclic ketones, dimethyl sulfoxide, and dimethylformamide give curdy suspensions; secondary and tertiary alcohols and certain fluorinated organic compounds give chalky suspensions. The majority of the other liquids tested give gelatinous suspensions. The degree of swelling is least in the alkanes and greatest in the amines, dimethyl sulfoxide, and dimethylformamide. All the organic liquids tested readily wet polytetrafluoroethylene. The fluorinated organic compounds wet it very quickly and give slurries and layers of excellent qualities. Also, they perform well as developers and possibly should be studied further for this purpose. These results indicate that polytetrafluoroethylene can be layered from organic liquids and that the dry layers can be spotted, developed and visualized with organic liquids.

Solubility of polytetrafluoroethylene in organic liquids is of interest, because resolved components that are to be identified and measured quantitatively are often removed from a TLC layer by dissolution in an organic solvent. If the polytetrafluoroethylene also dissolves, it may interfere. The electron micrographs (Figs. 1–3) indicate that dry residues from slurries of the polytetrafluoroethylenes no longer consist of discrete particles but appear to be networks of interconnected amorphous structures with membranous 'feet' that seem to have been formed from a gel of the polytetrafluoroethylene. Therefore, the solubility of polytetrafluoroethylene in the four selected liquids *n*-nonane, benzene, methyl isobutyl ketone, and monofluorotrichloromethane (Genosolv A) was studied. Each liquid was brought into intimate contact with polytetrafluoroethylene long enough to cause dissolution if it occurred. Then the liquid phase was separated carefully from the polytetrafluoroethylene by multiple centrifugations, checked for the Tyndall effect, and analyzed by IR spectro-

metry. Only for the Genosolv A did the IR spectrum (Fig. 4) indicate solubility.

The IR and mass spectra of polytetrafluoroethylene are of particular interest in determining the extent of its interference in the quantitative measurement of resolved components directly on the polytetrafluoroethylene. The spectra are given in Figs. 4 and 5, respectively. Polytetrafluoroethylene has no IR absorption bands at wavenumbers greater than about 1500 cm^{-1}; therefore, that section of the spectrum is not shown in Fig. 4. The mass spectrum at the lower temperatures (150° and lower) is a low background; at the high temperatures (~ 310°) the bands are chiefly in the high-mass region. These IR and mass spectra indicate that it should be possible to obtain spectra of organic substances isolated on polytetrafluoroethylene layers, provided the ratio of the substance to the polytetrafluoroethylene is sufficiently high.

Its behavior in strong solutions of sodium hydroxide and of hydrofluoric acid was studied with Fluoroglide 200 (lot 2). When a fluorocarbon surfactant and a small amount of an organic solvent are incorporated in the aqueous slurrying medium,

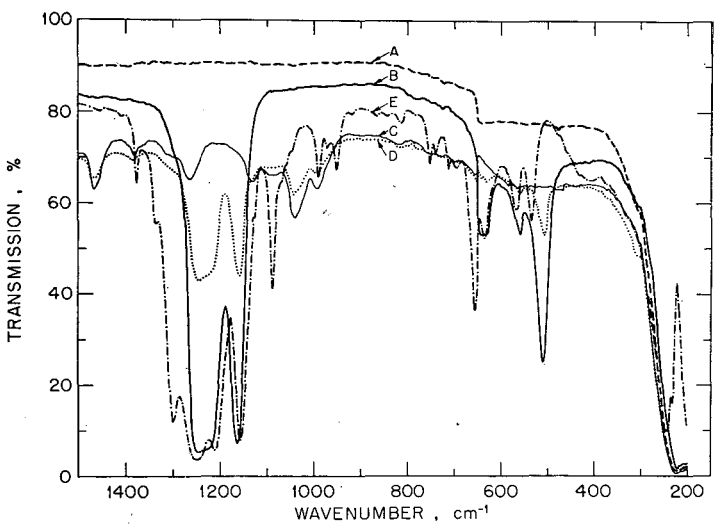

Fig. 4. Infrared spectra. A = potassium bromide blank; B = Fluoroglide 200 chromatography grade TWO218; C = monofluorotrichloromethane, Genosolv A; D = TWO218 in Genosolv A; E = fluorochemical surfactant FX-173.

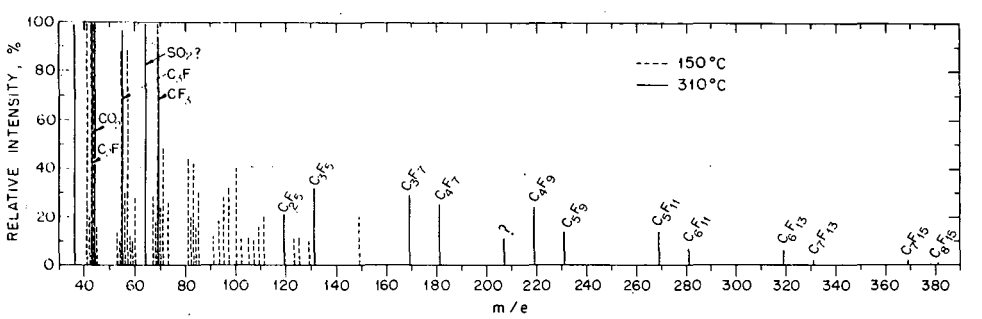

Fig. 5. Mass spectra of polytetrafluoroethylene Fluoroglide 200 chromatography grade TWO218 at 70 eV.

homogeneous suspensions of polytetrafluoroethylene are formed in strong solutions of sodium hydroxide (10 *M*) and of hydrofluoric acid (28 *M*). Layers can be made from the slurries. Because the layer from strong sodium hydroxide solution is hygroscopic, it has a gel-like appearance.

The purity of the three Fluoroglide 200 polytetrafluoroethylenes is indicated by IR and mass spectrometry to be high; the three samples, which were obtained over a period of two years, give essentially the same IR and mass spectra. Purification of the material is not required for its use as a TLC solid phase.

FLUOROCHEMICAL SURFACTANTS FOR USE WITH POLYTETRAFLUOROETHYLENE IN TLC

Properties desired

A surfactant suitable for use with polytetrafluoroethylene in TLC should be inert, nonvolatile, and soluble in the slurrying liquid, developer, and test solution. Compared with hydrocarbon type surfactants, fluorochemical surfactants are reported[9] to have the advantages of: chemical stability; effectiveness at smaller concentrations; greater effectiveness for aqueous systems; the same effectiveness in both organic and aqueous systems; stability to heat, acids, electrolysis, and strong oxidizers; depressing effect on the vapor pressure of solvents; and complexation in some cases.

A number of fluorochemical surfactants are available from the 3M Company[9]. These are stated to be anionic fluorochemicals that contain a completely fluorinated tail and a solubilizing group. Nonionic fluorochemical surfactants[10] may also be suitable; they were not studied.

Evaluation

Of the 3M Company's solid fluorochemical surfactants, the following were evaluated: FC-95, FC-98, FC-126, and FX-173. Of these, FX-173 was selected for use, because it is soluble in several organic solvents commonly used for slurrying TLC media and is slightly soluble in water. Also, on layers that contain FX-173 water migrates fastest and with a straight front; with the other three surfactants the front was not straight.

The IR spectrum of FX-173 is given in Fig. 4, where it can be compared with that of Fluoroglide 200 (TWO218). The FX-173 also has no bands at wavenumbers greater than 1500 cm^{-1}. Its spectrum is very similar to that of the Fluoroglides; its interference in the qualitative and quantitative measurement of resolved components should be minimal. The manufacturer has not revealed the chemical formula of FX-173. The IR spectrum indicates that it may be the sulfonate of a perfluorohydrocarbon, probably of the form $C_nF_{(2n+1)}SO_3^-X^+$, where X^+ is not H^+. Its IR spectrum is similar to, but not identical with, those of perfluoronated surface-active agents ('tensides') reported by HUMMEL[11].

The FX-173 can be used satisfactorily in any of a number of ways. It can be included in the slurry and thus become evenly distributed in the dry TLC layer. A solution of it in an organic liquid can be deposited on the layer only in the area to be spotted; in this way the amount and location in the layer are restricted to that just required to cause the layer to accept a test spot. It can be included in a test solution that otherwise might not wet the layer. Also, it can be included in the developer.

It was observed experimentally that FX-173 has some effectiveness as a binder. Polytetrafluoroethylene layers that contain it appear to adhere slightly better to Mylar film than do those without it.

PREPARATION AND PROPERTIES OF POLYTETRAFLUOROETHYLENE LAYERS

Slurries of polytetrafluoroethylene

Slurries of polytetrafluoroethylene are characterized by versatility. By the use of a surfactant, they may be prepared in aqueous as well as in organic vehicles. They may include any of a great variety of other TLC adsorbents. The miscibility of polytetrafluoroethylene in slurries with each of some 20 other adsorbents (both inorganic and organic) was demonstrated experimentally. The slurries may include other special solid or liquid agents such as binders, silver nitrate, fluorescing agents, acids, bases, liquid ion exchangers, and complexers.

The preparation of a slurry is simple. The polytetrafluoroethylene is added to a liquid phase that contains the other components of the slurry. An orbital sander is a useful tool for mixing a slurry in a volatile flammable liquid; a closed container that holds the slurry can be attached to the plate of the sander and, with the plate in a vertical position, agitated a few seconds to effect mixing.

The viscosity of polytetrafluoroethylene slurries can be regulated by varying the ratio of polytetrafluoroethylene to liquid. For Fluoroglide 200 (TWO218), a 1:3 w/v ratio of polytetrafluoroethylene to organic liquid usually gives a slurry of satisfactory consistency.

The rate of settling of polytetrafluoroethylene slurries is of interest relative to the possibility of segregation of components of the slurry after it is layered. Fig. 6

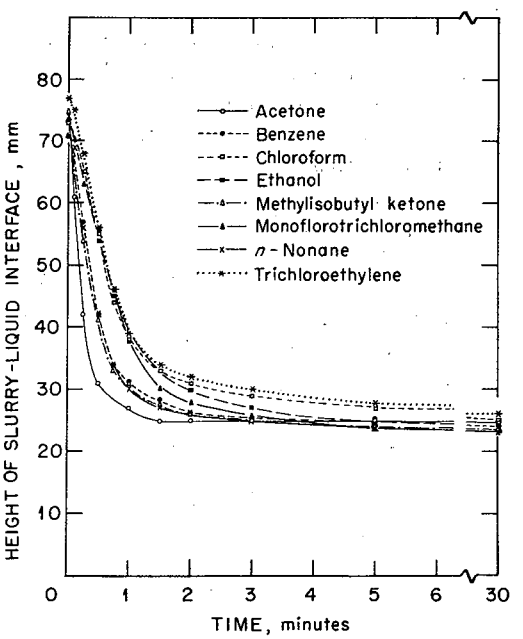

Fig. 6. Curves for the settling rate of Fluoroglide 200 lubricant (lot 2) from eight organic liquids.

gives rate-of-settling curves for Fluoroglide 200 (lot 2) in eight organic liquids. The TWO218 type settles less rapidly.

Application to the support is no problem with polytetrafluoroethylene slurries. By any of the usual means, they can be layered onto glass, vinyl and Mylar films, and metal supports and presumably also onto other types of TLC supports. No difficulty was encountered in preparing an 8 in. by 48 in. chromatofilm of polytetrafluoroethylene on Mylar film by means of a Desaga/Brinkmann applicator.

The rate of drying of the layer is a function of the volatility of the slurrying liquid and of the layer thickness. Apparently, some of the liquid phase of the slurry is permanently retained in the interstices of the polytetrafluoroethylene particles.

Marking of the layers to show the desired origin, development distance, and the like is done conveniently with a stylus or dissecting needle before the layers are thoroughly dry. Dots can be made that are readily evident when the layer is backlighted with a flashlight during the development.

Properties of layers

Adherence to the support is achieved with polytetrafluoroethylene layers without the use of binders. If care is used in wetting the layer with the liquid nitrogen, it is even possible to examine a binder-free polytetrafluoroethylene layer on a Mylar film support under liquid nitrogen without having the layer flake off the support.

The appearance of the layers under UV light depends partly on the other components present in the layer. It has been possible to easily observe resolved components that absorb, fluoresce, and phosphoresce after being excited with 254 or 366 nm UV light.

The rate of developer migration on polytetrafluoroethylene layers is a function of the usual variables such as layer thickness, type of developer, and nature of other components of the layer.

Solvent fronts move in a straight line both in the absence of wetting agent and when FX-173 is present.

Spotting of polytetrafluoroethylene layers is not a problem. The layers readily accept organic test solutions in the absence of a surfactant. With a surfactant present either in the layer or in the test solution, they accept aqueous test solutions.

The precision of R_F values of components isolated on polytetrafluoroethylene layers is comparable with that on other layers.

Mixed-adsorbent layers

The suitability of polytetrafluoroethylene as a component of mixed-adsorbent layers was mentioned above. It can be used not only as an active adsorbent, either alone or together with numerous other TLC adsorbents, but also as an inert diluent for other adsorbents or particulate solids that may be under study, for example, soils.

Gradient-adsorbent layers

Polytetrafluoroethylene was used satisfactorily to prepare gradient-adsorbent layers with polyamides. The layers were slurried from *n*-propanol and were used to separate aromatic and heterocyclic hydrocarbons by development with *n*-propanol. The layers were used with the gradient in each of the three possible directions. Be-

TABLE II

EXAMPLES OF MIGRATIONS AND SEPARATIONS ON THIN LAYERS THAT CONTAINED POLYTETRAFLUOROETHYLENE

Substance chromatographed	*Layer*			*Developer*	*Substances*	
	Solid phase (A)	*Slurry vehicle (B)*	*A : B (w/v)*		*Migrating*	*Resolved*
Inorganic substances						
Pr^{3+}, Nd^{3+}, Ho^{3+}	FG200(1)–FX-173 (30:1)	Acetone	1:2	0.2 to 1 *M* HF–0.5 *M* HDEHP[a] in acetone	All	Possibly
	FG200(1)–FX-173–HF (conc.) (30:1:0.25)	MIBK[b]	1:2	Acetone	All	None
Ni^{2+}, Fe^{3+}, Pr^{3+}, Nd^{3+}, Ho^{3+}	FG200(1)–FX-173–HF (conc.) (30:1:0.25)	MIBK	1:2	0.5 *M* HDEHP in MIBK	Fe^{3+}, Nd^{3+}, Ho^{3+}	Fe^{3+}, (Ni^{2+}, Pr^{3+}), Nd^{3+}, Ho^{3+}
	FG200(1)–FX-173 (30:1)	0.1 *M* HDEHP in cyclohexane	1:2	0.4 *M* HCl in H_2O	Ni^{2+}, Pr^{3+}, Nd^{3+}, Ho^{3+}	Fe^{3+}, Ni^{2+}, (Pr^{3+}, Nd^{3+}, Ho^{3+})
	FG200(1) or (2)	0.4 *M* HCl in cyclohexane	1:2	0.1 *M* HDEHP in cyclohexane	Fe^{3+}	Fe^{3+}, (Ni^{2+}, Pr^{3+}, Nd^{3+}, Ho^{3+})
Ni^{2+}, UO_2^{2+}, Fe^{3+}	TWO218	*n*-Butanol	1:3	*n*-Butanol–H_2SO_4 (conc.)–HF (conc.)–H_2O (100:9.5:5:85.5)	All	Ni^{2+}, UO_2^{2+}, Fe^{3+}
	TWO218	Acetone	1:3	Acetone–HCl (conc.)–HF (conc.)–H_2O (90:5:1:4)	UO_2^{2+}, Fe^{3+}	Ni^{2+}, UO_2^{2+}, Fe^{3+}
29 radioisotopes of metal ions	TWO218–FX-173 (3:0.025)	MIBK–HNO_3 (conc.) (9:0.1)	1:3	0.5 *M* HDEHP in MIBK	27	Many[c]
Organic substances						
Fluoranthene, benzo-[*g,h,i*] perylene	TWO218	FC-75[d]	1:2	FC-75	Fluoranthene	Fluoranthene, benzo[*g,h,i*]-perylene
	TWO218–Woelm polyamide (2:1, 1:1, and 1:2)	*n*-Propanol	1:2	*n*-Propanol	Fluoranthene	Fluoranthene, benzo[*g,h,i*]-perylene

Ten aromatic and heterocyclic hydrocarbons	TWO218–Aviamide-6 (1:4)	*n*-Propanol	1:3	*n*-Propanol	All	Eight (ten distinguishable by luminescence properties[c])
Old solution of corticosterone in methylene chloride	TWO218–Aviamide-6 (1:4)	*n*-Propanol	1:3	Methanol	Four	Five
6-Chloro-9-(3'-diethylamino-2'-hydroxypropylamino)-methoxyacridine dihydrochloride	TWO218	Carbon tetrachloride–ethanol (19.8:0.2)	1:3	Carbon tetrachloride–ethanol (19.8:0.2)	Two	Three
17β-Estradiol, estrone	TWO218	Chloroform–methanol (97:3)	1:3	Chloroform–methanol (97:3)	17β-Estradiol, estrone	Neither
Androsterone, 11-dehydrocorticosterone	TWO218	Methanol	1:3	Methanol	Androsterone, 11-dehydrocorticosterone	Neither (both moved with solvent front)
Sodium cacodylate	TWO218–FX-173 (30:1)	Acetone	1:3	Perfluorodiethylpropyl amine	A contaminant	Contaminant from sodium cacodylate
Cacodylic acid, sodium cacodylate	TWO218	*n*-Hexane–acetone (5:1)	1:3	*n*-Hexane–acetone (5:1)	Cacodylic acid, sodium cacodylate	Neither (both moved with solvent front)

[a] HDEHP = di-(2-ethylhexyl)orthophosphoric acid.
[b] MIBK = methyl isobutyl ketone.
[c] Data to be reported in detail in a separate article.
[d] FC-75 = perfluoro-2-butyltetrahydrofuran.

cause of the different rates of migration of *n*-propanol along layers of polytetrafluoroethylene and polyamide, the solvent front was curved rather than straight when the gradient was in the horizontal direction.

Potential of polytetrafluoroethylene layers for extraction TLC

Polytetrafluoroethylene layers have considerable potential for use in extraction TLC. Because polytetrafluoroethylene swells in and is wet by most organic liquids, a homogeneous immobile phase of an extractant can be obtained on a thin layer of polytetrafluoroethylene. Also, the extractant may be included in the developer rather than in the layer. By changing the location of the extractant, the order of migration of the resolved components can be reversed, and versatility can thus be introduced in the manner of use of the layer in extraction chromatography. This reversal was demonstrated experimentally by the resolution of metal ions with the liquid ion exchanger di-(2-ethylhexyl)orthophosphoric acid (HDEHP). By means of polytetrafluoroethylene layers, it should be possible to use TLC as a means to study liquid ion exchange without the need to consider the reactivity of the liquid phases with the solid support. No doubt many column chromatographic separations made on polytetrafluoroethylene supports[2] can be scaled down by means of thin layers of polytetrafluoroethylene.

Comparison with other chromatographic media

No detailed study has been made to compare polytetrafluoroethylene layers with other chromatographic media. However, in several separations polytetrafluoroethylene layers were observed to be superior. The discreteness of resolution of metal ions with a developer that contained HDEHP was very much greater with polytetrafluoroethylene layers than with polyethylene layers or with Gelman ITLC-SA medium when all the experimental conditions except the type of layer were identical. The resolution of metal ions with the developer *n*-butanol–H_2SO_4 (conc.)–HF (conc.)–H_2O (100 :9.5 : 5 : 85.5) was greater on a polytetrafluoroethylene layer than on a paper strip[12].

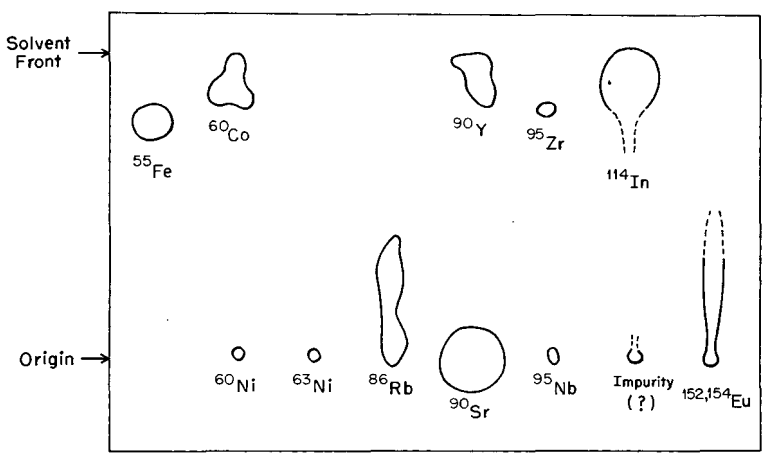

Fig. 7. Map of autoradiograms of thin-layer chromatograms of some inorganic ions on a polytetrafluoroethylene TWO218 layer developed with ~ 0.5 *M* HDEHP in methyl isobutyl ketone.

SEPARATIONS ON THIN LAYERS OF POLYTETRAFLUOROETHYLENE

Table II gives examples of numerous migrations and separations that have been achieved on thin layers that contained polytetrafluoroethylene. In a separate paper, the separations of the radioisotopes and of the hydrocarbons will be discussed; maps of chromatograms that illustrate these latter separations are given in Figs. 7 and 8, respectively. These examples indicate the versatility of polytetrafluoroethylene as a solid phase for TLC.

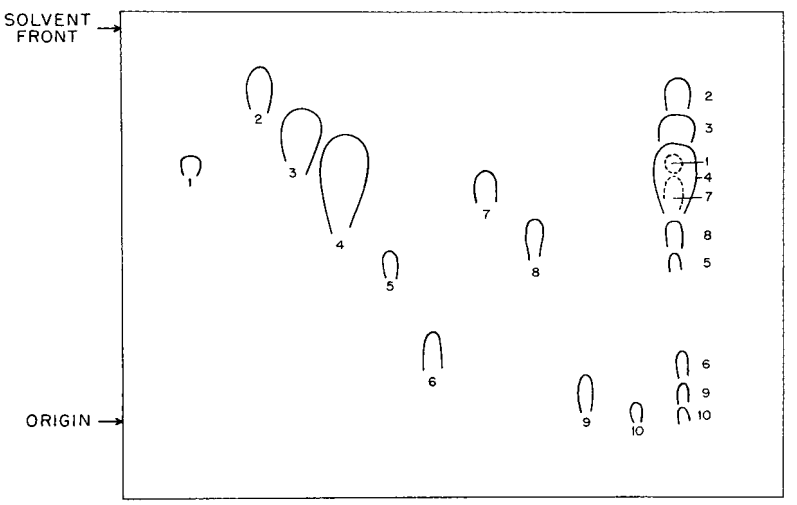

Fig. 8. Map of a thin-layer chromatogram of aromatic and heterocyclic hydrocarbons on TWO218–Aviamide-6 (1:4) developed with *n*-propanol. 1 = *o*-hydroxydiphenyl; 2 = naphthalene; 3 = fluorene; 4 = phenanthrene; 5 = pyrene; 6 = chrysene; 7 = anthracene; 8 = fluoranthene; 9 = benzo[*e*]pyrene; 10 = benzo[*g*,*h*,*i*]perylene.

The applicability of polytetrafluoroethylene layers in separation systems that contain hydrofluoric acid[13–14] is of special interest. Table II includes two examples of this application. Also, with aqueous hydrofluoric acid solutions (0.9 to 28 *M*) as developers, the resolution of amino acids on polytetrafluoroethylene layers was attempted. Some evidence existed for the migration of the amino acids, but their detection was difficult because of the hydrofluoric acid content of the layer. Further work is planned to investigate the TLC behavior of biochemicals in systems that contain hydrofluoric acid.

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CHROM. 4303

UNTERSUCHUNG DER AUS MUSKELGEWEBE UND FLEISCHERZEUGNISSEN EXTRAHIERBAREN NICHTFETT- UND FETTSÄUREN MITTELS DÜNNSCHICHT- UND GASCHROMATOGRAPHIE

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(Eingegangen am 30. Mai 1969; geänderte Fassung am 24. Juli 1969)

SUMMARY

Examination of non-fatty and fatty acids extractable from muscle tissue and meat products by thin-layer and gas chromatography

This paper presents a procedure for extraction and chromatography of the non-esterified acids occurring in meat and meat products. Thin-layer (TLC) and gas–liquid chromatography (GLC) are used for qualitative and quantitative evaluations of aliphatic and fatty acids. Only the aliphatic acids (*e.g.* lactic, succinic, pyruvic, fumaric etc.) could be separated and identified by thin-layer chromatography. Two solvents were used for the separations on cellulose layers and another for those on the Kieselguhr–silica gel layer. The acids were detected with benzidine $NaJO_4$. The stained spots gave a bluish-violet or yellow colour, depending on the nature of the acid. The gas chromatographic separations of methyl esters were performed on Reoplex 400 (10 %) and on Apiezon M (15 %). The separations on Reoplex were obtained by programming the temperature at 3°/min from 60° to 170°. Methyl esters of fatty acids could easily be assayed at an isothermal temperature (235°) on Apiezon M. Known and previously unknown aliphatic acids could be detected.

EINLEITUNG

Bisher durchgeführte Säureuntersuchungen an Fleisch und Fleischwaren konzentrierten sich vor allem auf die Bestimmung der Veränderungen im Milchsäuregehalt[1,2]. In einzelnen Muskelfleisch-extrakten konnte neben Milchsäure auch Glykol- und Bernsteinsäure aufgefunden werden[3,4]. In verschiedenen tierischen Geweben wurden weitere Säuren papierchromatographisch[5] nachgewiesen, jedoch nicht identifiziert.

In Rohwürsten konnte mittels chemischer Methoden neben Milchsäure auch Brenztraubensäure[6] bestimmt werden, ausserdem wurden gaschromatographisch[7] einige während der Reifung gebildeten freien Fettsäuren nachgewiesen. Die während der Lagerung von Fleisch[8,9], wie auch der Reifung von Wursterzeugnissen[10] entstan-

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denen freien Fettsäuren werden für die Beurteilung der Qualität derartiger Erzeugnisse in immer grösserem Umfange herangezogen.

Es wurde deshalb eine Methode erarbeitet, bei welcher die in Fleisch und Fleischerzeugnissen vorkommenden nicht veresterten Säuren mittels Dünnschicht- und Gaschromatographie nachgewiesen und bestimmt werden können.

METHODIK

Aufarbeitung der Extrakte

Die zur Extraktion von Säuren aus Muskelgewebe geeigneten Verfahren mit 96 %igem Äthanol, bzw. 0.6 *N* $HClO_4$, wurden zunächst miteinander verglichen und an die spezielle Fragestellung angepasst (Analysenschema s. Fig. 1). Bei der Extrak-

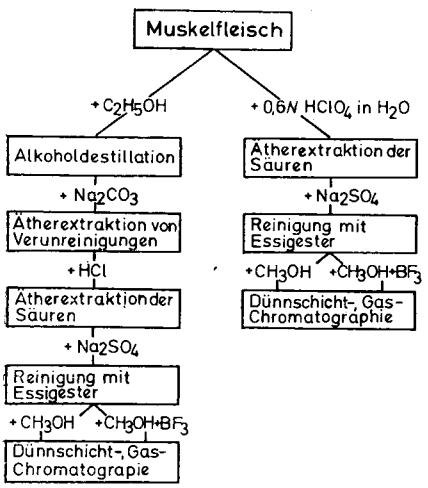

Fig. 1. Vergleich der Aufarbeitung von Säuren bei Extraktion aus Muskelgewebe mit 96 %igem Äthanol bzw. 0.6 *N* Perchlorsäure.

tion mit heissem Alkohol wurde die Menge so bemessen, dass das portionsweise zugegebene zerkleinerte Gewebe (100 g) damit vollständig bedeckt war. Nach dem Abkühlen verblieben die mit Alkohol versetzten Proben für eine Nacht im Kühlschrank. Die am Rotavapor eingeengten, und mit halbgesättigter Sodalösung alkalisch gemachten Extrakte wurden anschliessend zur Entfernung von Begleitstoffen mit Äther ausgeschüttelt. Nach dem Ansäuern mit HCl konnten die aus dem Gewebe stammenden Säuren in Äther überführt werden. Die mit Na_2SO_4 getrocknete ätherische Lösung wurde nun eingedampft und der in wenig Essigester aufgenommene Rückstand abfiltriert. Der erneut eingeengte Extrakt konnte zur Chromatographie in 1 ml Methanol gelöst werden.

Bei der Extraktion von Säuren mit 0.6 *N* $HClO_4$ wurde das Gewebe (100 g) zusammen mit dem Extraktionsmittel homogenisiert und die abfiltrierte säurehaltige Lösung mit Äther ausgeschüttelt. Bei der weiteren Aufarbeitung konnte entsprechend der oben beschriebenen Methode verfahren werden (vgl. Fig. 1).

Chromatographie der Säuren

Dünnschichtchromatographie. Die Dünnschichtchromatographie der Säuren erfolgte auf Trennschichten, bei welchen Kieselgur und Kieselgel im Verhältnis 1:1 gemischt wurde. Zur Trennung der aufgetragenen Säuren wurde das Laufmittel Benzol–Äthanol–Ammoniak (25 %) (10:20:5) eingesetzt[11]. Ausserdem wurden die extrahierten Säuren auf Celluloseschichten mit den hierfür zusammengestellten Laufmitteln: *n*-Butanol–Ameisensäure–Wasser (10:7:1) (obere Phase) und Äthanol–Ammoniak (25 %)–Wasser (35:7:2) chromatographiert.

Zum Nachweis der Säuren wurde bei silikathaltigem Beschichtungsmaterial mit Bromkresolgrün besprüht und zur speziellen Erkennung der Fettsäuren mit Jod bedampft. Auf Celluloseschichten konnten Säuren durch aufeinander folgendes Besprühen mit (a) 0.5 %iger äthanolischer Benzidinlösung und (b) 0.1 %iger wässriger $NaJO_4$-Lösung sichtbar gemacht werden. Wichtig ist, dass die angegebene Reihenfolge beim Aufsprühen eingehalten wird. Nach dem Besprühen von (a) lässt man kurze Zeit antrocknen. Ketosäuren konnten mit Dinitrophenylhydrazin (DNPH), gelöst in 3 N H_2SO_4, nachgewiesen werden. Für Celluloseschichten war der Nachweis bei Verwendung einer 0.05 %igen DNPH-Lösung und für silikathaltige Platten bei 0.3 %iger DNPH-Lösung am empfindlichsten.

Gaschromatographie. Die Veresterung der extrahierten Säuren erfolgte mit Methanol und Bortrifluorid als Katalysator. Bortrifluorid wurde in 14 %iger methanolischer Lösung von der Firma Serva (Heidelberg) bezogen. Die Methylierung der extrahierten Säuren erfolgte jeweils durch Vermischen von einem Teil Säureextrakt mit einem Teil des Methylierungsreagenzes. Die Proben blieben eine Nacht bei Zimmertemperatur verschlossen stehen und wurden dann nach MAZLIAK UND SALSAC[12] ohne weitere Aufarbeitung der Gaschromatographie unterworfen. Für Referenzuntersuchungen und zur Erstellung von Eichkurven mussten die in Methanol gelösten Säuren entsprechend verestert werden. An Stelle von Milchsäure wurde deren Calzium-Salz methyliert. Eine vorherige Entfernung des Kations durch Ionenaustauscher erwies sich als nicht erforderlich.

Die gaschromatographischen Trennungen wurden mit einem Fraktometer F 20 der Firma Perkin-Elmer durchgeführt. Die Methylester der aliphatischen Nichtfettsäuren, wie auch der Fettsäuren, liessen sich auf der polaren Säule Reoplex 400[13] (10 % auf Chromosorb W) trennen. Zur Identifizierung der extrahierten Fettsäuren wurden deren Methylester auch auf der apolaren Säule Apiezon M (15 % auf Kieselgur) chromatographiert. Die Trennungen auf Reoplex 400 erfolgten bei Temperaturprogrammierung zwischen 60 und 170°, bei einer Anstiegsrate von 3°/min. Die Methylester der Fettsäuren mit mehr als 16 C-Atomen erschienen im isothermen Nachlauf bei 170°. Auf Apiezon M wurde isotherm bei 235° gefahren. Die Identifizierung wurde neben dem Vergleich mit Reinsubstanzchromatogrammen durch Mitchromatographieren der vermuteten Methylester (Peakerhöhung) durchgeführt. Das Trägergas Stickstoff hatte eine Strömungsgeschwindigkeit von 35 ml/min. Die Strömungsgeschwindigkeit für Wasserstoff betrug 33 ml/min und für synthetische Luft 350 ml/min. Bei Apiezon M wurde mit einer Stickstoffströmung von 25 ml/min gearbeitet.

Der Nachweis der abgetrennten Substanzen erfolgte im FID-Detektor. Die Temperatur des Einspritzblocks betrug 250°. Injiziert wurden Flüssigkeitsmengen von 0.2–2.0 μl. Die Konzentration der verwendeten Referenzlösungen lag zwischen

0.15 und 2.0%, je nach Empfindlichkeit des Detektors für den injizierten Säuremethylester. Der Papiervorschub des Schreibers betrug 0.5 cm/min.

ERGEBNISSE UND DEREN INTERPRETATION

Vergleich der Extraktionsmethoden mit Äthanol bzw. 0.6 N $HClO_4$

Bei der äthanolischen Aufarbeitung der Säuren war der Anteil mitextrahierter Begleitstoffe, die auf Ninhydrin und diazotierte Sulfanilsäure positiv reagierten, grösser als im $HClO_4$-Extrakt. Perchlorsäureextrakte enthielten dagegen mehr durch Jodadsorption nachweisbare Verunreinigungen. Obwohl beide Verfahren zur Aufarbeitung von Säuren aus tierischem Gewebe eingesetzt werden können, war die Reproduzierbarkeit der Ergebnisse bei äthanolischer Extraktion besser und die Aufarbeitung schonender, so dass wir diesem Verfahren den Vorzug gaben.

Säurenachweis mit Benzidin–$NaJO_4$

Die beim Säurenachweis mit Benzidin und $NaJO_4$ erzielte Farbtönung erwies sich für einzelne Säuren bzw. Säuregruppen als spezifisch und variierte hinsichtlich des Farbtons zwischen violett, blau und gelb bis weiss. Eine violette bis blau-violette Farbe ergab sich bei der auf den Nachweis sehr empfindlich reagierenden Säuregruppe: Malein-, Bernstein-, Fumar-, Citronen-, Aconit-, Malon-, und Adipinsäure. Eine blaue bis blassblaue Farbe wurde bei: Glykol-, Milch-, β-Hydroxybutter- und 3-Hydroxybuttersäure erhalten. Zwischen den beiden genannten Gruppen lagen hinsichtlich ihres Farbtons Oxal- und Glutarsäure. Eine gelbliche Tönung konnte für die Ketosäuren: Oxalessig-, Brenztrauben-, Glyoxyl-, und Ketoglutarsäure erzielt werden. Ketoglutar- und Lävulinsäure erschienen manchmal zunächst auch mit blauer, später verblassender Farbe. Fettsäuren präsentierten sich als gelblich-weisse Flecken. Palmitolein- und Ölsäure erschienen ebenfalls manchmal zunächst mit hellblauem, später verblassendem Farbton.

Die erzielte Anfärbung dürfte darauf beruhen, dass das zunächst aufgesprühte Benzidin an säurefreien Stellen bis zu gelblich-bräunlichen Produkten oxidiert wird, an säurehaltigen Stellen jedoch unter Mitwirkung der Säuren gefärbte Oxidationsprodukte ergibt. $NaJO_4$ ist anderen Oxidationsmitteln vorzuziehen. Die Absorptionsmaxima der mit Säuren erzielten Farbkomponenten liegen zwischen 500 und 650 nm (s. Fig. 2). Einzelne Säuren lassen sich schon ab 10 γ Substanz auf der Platte damit nachweisen. Dieser Farbnachweis für Säuren eignet sich nicht für silikathaltige Trägerschichten, da hier offenbar die Kieselsäure die Oxidation des Benzidins stört. Da die Farbreaktion spezifisch ist, kann sie neben dem R_F-Wert einen wichtigen Hinweis bei der Identifizierung von Säuren darstellen. Das Reagenz wurde weder bei FERRAZ UND RELVAS[14] noch bei STAHL[15] zum Nachweis von Säuren benannt, so dass angenommen wird, dass es bisher hierfür nicht beschrieben wurde.

Dünschichtchromatographie der Säuren

Die verwendeten Laufmittel und Beschichtungsmaterialien ermöglichten eine dünnschichtchromatographische Trennung der nicht zur Fettsäurereihe gehörenden Säuren. Bei Chromatographie auf Celluloseschichten wurde das verwendete Laufmittel Äthanol–Ammoniak–Wasser (35:7:2) günstig durch das saure Laufmittel *n*-Butanol–Ameisensäure–Wasser (10:7:1) (obere Phase) ergänzt (vgl. Fig. 3 und 4).

Beide Laufmittel entsprechen in ihrer Zusammensetzung den bei der Dünnschichtchromatographie von Tricarbonsäurecyclussubstraten eingesetzten Laufmittel[16].

Die aus Fleisch und Fleischerzeugnissen extrahierbaren Säuren konnten im oben erwähnten alkalischen Laufmittel in vier Hauptfraktionen aufgetrennt werden, die als Fraktion A–D bezeichnet wurden (vgl. Fig. 3). Fraktion A enthält Spuren von

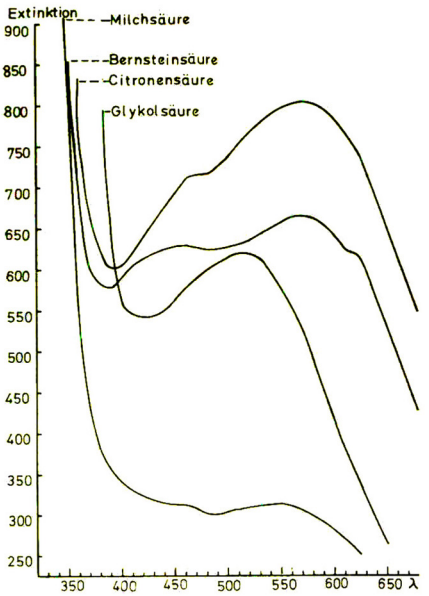

Fig. 2. Extinktionskurven der Reaktionsprodukte, die bei der Umsetzung von Carbonsäuren mit Benzidin und anschliessender Oxidation mit $NaJO_4$ entstehen.

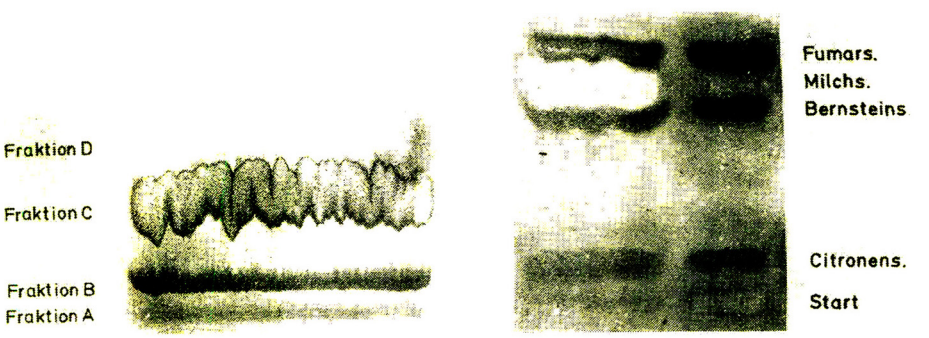

Fig. 3. Dünnschichtchromatographische Trennung von Carbonsäuren aus geräucherter Mettwurst (äthanolischer Extrakt). Beschichtungsmaterial: Cellulose. Laufmittel: Äthanol–Ammoniak–Wasser (35:7:2). Sprühmittel: Benzidin/$NaJO_4$. Fraktion A: Citronensäure sowie nicht identifizierte Verbindungen; Fraktion B: Bernsteinsäure + Fumarsäure; Fraktion C: Milchsäure + Glykolsäure (Spuren); Fraktion D: Fettsäuren + 3-Hydroxybuttersäure + Lactylmilchsäure.

Fig. 4. Dünnschichtchromatographische Trennung von Carbonsäuren aus Muskelgewebe. Links: äthanolischer Extrakt, rechts: Reinsubstanzen. Beschichtungsmaterial: Cellulose. Laufmittel: *n*-Butanol–Ameisensäure–Wasser (10:7:1) (obere Phase). Sprühmittel: Benzidin/$NaJO_4$.

Citronensäure und eventuell noch weitere Verbindungen. Fraktion B besteht aus Bernstein- und Fumarsäure. Fraktion C enthält als Hauptbestandteil Milchsäure. Spuren von Glykolsäure können hier ausserdem vorhanden sein. In Fraktion D können ausser den höheren Fettsäuren auch Lactylmilchsäure und 3-Hydroxybuttersäure anwesend sein.

Zur Trennung der in Muskelgewebe und Fleischerzeugnissen vorkommenden Hydroxysäuren: Milch-, Glykol- und 3-Hydroxybuttersäure eigneten sich besonders Mischschichten aus Kieselgur und Kieselgel im Verhältnis 1:1, bei Verwendung von Benzol–Äthanol–Ammoniak (10:20:5) als Fliessmittel. Hiermit konnten die höheren

TABELLE I

R_F-WERTE VON CARBONSÄUREN AUS MUSKELGEWEBE UND FLEISCHERZEUGNISSEN
Trägerschicht: Cellulose.

Identifizierte Säuren	*Laufmittel*				*Bemerkungen*
	n-C_4H_9OH–HCOOH–H_2O (10:7:1)		C_2H_5OH–NH_4OH (25%)–H_2O (35:7:2)		
	Gef. Säure	*Reinsubst.*	*Gef. Säure*	*Reinsubst.*	
—	0.07	—	—	—	Säurenachweis positiv
Citronen-	0.15	0.15	0.09	0.08	—
Glykol-	0.51	0.58	0.46	0.46	Häufig verdeckt durch Milchsäure
Bernstein-	0.58	0.66	0.27	0.31	—
Milch-	0.66	0.66	0.51	0.60[a]	—
	—	0.81	—	0.79[b]	—
Fumar-	0.72	0.77	0.33	0.41	—
Brenztrauben-	0.79	0.75	—	—	Leicht zersetzbar
Höhere Fett-	0.91	0.94	0.92	0.94	Nur als Gruppe nachweisbar

[a] Hauptfleck.
[b] 2. Milchsäurefleck (Lactylmilchsäure).

TABELLE II

R_F-WERTE VON CARBONSÄUREN AUS MUSKELGEWEBE UND FLEISCHERZEUGNISSEN
Trägerschicht: Kieselgur und Kieselgel im Verhältnis 1:1.

Identifizierte Säuren	C_6H_6–C_2H_5OH–NH_4OH (25%) (10:20:5)		*Bemerkungen*
	Gef. Säure	*Reinsubst.*	
Bernstein-	0.10	0.17	R_F-Wert mehrerer Säuren
Glykol-	0.31	0.29	Häufig verdeckt durch Milchsäure
Milch-	0.33	0.36[a]	—
	—	0.50[b]	—
	0.59	0.62[c]	—
3-Hydroxybutter-	0.49	0.53	—
Höhere Fett-	0.75	0.77	Nur als Gruppe nachweisbar
—	0.94	—	Positiv mit J_2 und DNPH

[a] Hauptfleck.
[b] Lactid (sehr schwach).
[c] Lactylmilchsäure (schwach).

Fettsäuren besser und schärfer als auf Celluloseschichten von den übrigen Säuren abgetrennt werden. Die dünnschichtchromatographisch identifizierten Säuren wurden in Tabelle I und II mit den entsprechenden Laufmitteln und Beschichtungsmaterialien zusammengestellt.

Gaschromatographie der Säuren

Methodische Ergebnisse. Bei den durchgeführten Untersuchungen wurde entgegen den Angaben von CANVIN[13] festgestellt, dass Fumarsäuredimethylester bei Chromatographie auf Reoplex (400) unmittelbar vor Bernsteinsäuredimethylester aus der Säule austritt. Bei zitiertem Autor erschien Fumarsäuredimethylester auf der gleichen Säule wesentlich später, und zwar erst nach dem Dimethylester des Isocitronensäurelactons. Der letztgenannte Dimethylester wurde seinerseits, bei den von uns durchgeführten Untersuchungen, erst nach Linolsäuremethylester (Peak Nr. 22 in Fig. 5–7) aus der Säule eluiert. Es muss angenommen werden, dass unter den von CANVIN[13] angegebenen Methylierungsbedingungen ein spät austretendes Pyrazolin aus Fumarsäuredimethylester entstanden ist, dessen mögliche Entstehung auch von MCKEOWN UND READ[17] diskutiert wurde.

Die von LANGNER[18] beobachtete Zersetzung von Milchsäure beim Methylieren mit Methanol und Bortrifluorid als Katalysator konnte unter den angegebenen Bedingungen nicht festgestellt werden. Die Zersetzung darf nicht auf Bortrifluorid als Katalysator, sondern muss auf die anschliessende Hitzebehandlung zurückgeführt werden.

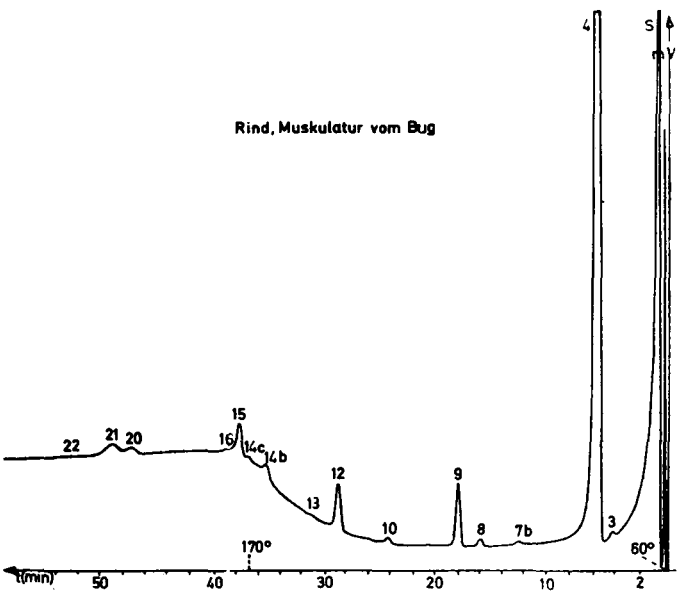

Fig. 5. Gaschromatographie der Methylester der aus Bugmuskulatur des Rindes extrahierten Säuren auf Reoplex 400. Technische Angaben s. Text. Erklärung der durchnummerierten Peaks: 3 = Brenztrauben-; 4 = Milch-; 7b = 3-Hydroxybutter-; 8 = Fumar-; 9 = Bernstein-; 10 = Laurin-; 12 = unbekannt; 13 = Myristin-; 14b und c unbekannt; 15 = Palmitin-; 16 = Palmitolein-; 20 = Stearin-; 21 = Öl-; 22 = Linolsäuremethylester.

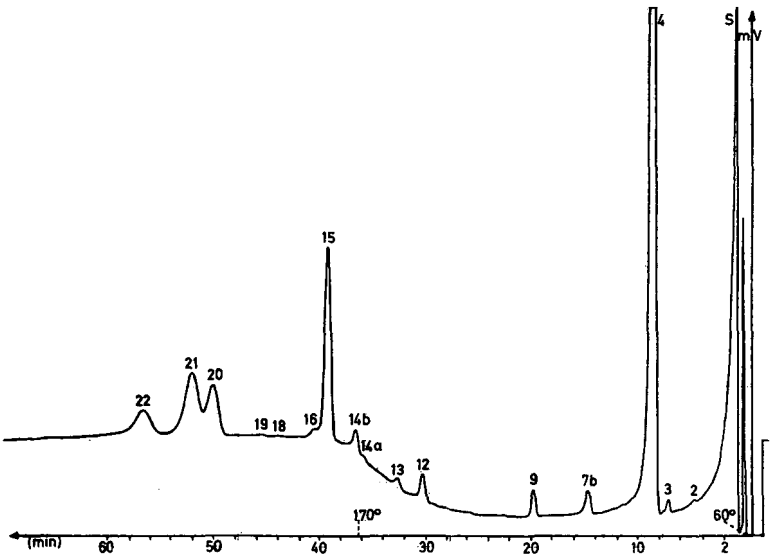

Fig. 6. Gaschromatographie der Methylester der aus Brustmuskulatur von Hähnchen extrahierten Säuren auf Reoplex 400. Technische Angaben s. Text. Erklärung der Peaks, s. Fig. 7.

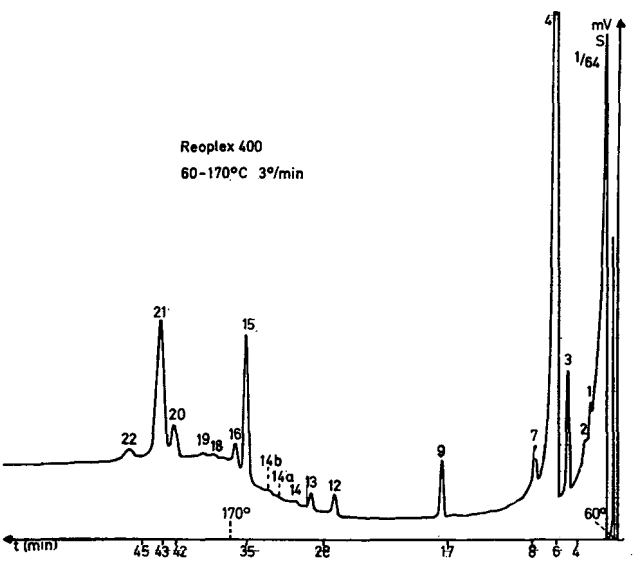

Fig. 7. Gaschromatographische Trennung der aus geräucherter Mettwurst extrahierten Säuren nach Überführung in Methylester auf Reoplex 400, mit Temperaturprogrammierung. Erklärung der durchnummerierten Peaks: 1 = Butter- (?); 2 = Capron-; 3 = Brenztrauben-; 4 = Milch-; 7 = Capryl-; 7b = 3-Hydroxybutter-; 9 = Bernstein-; 12 = unbekannt; 13 = Myristin-; 14 = Myristolein-; 14a, b und c unbekannt; 15 = Palmitin-; 16 = Palmitolein-; 18 = Heptadecan-; 19 = Heptadecen- (?); 20 = Stearin-; 21 = Öl-; 22 = Linolsäuremethylester.

Identifizierung von Säuren in Fleisch und Fleischerzeugnissen. Auch bei der gaschromatographischen Untersuchung der methylierten Säuren konnte Milchsäure (Peak Nr. 4) als mengenmässig wichtigste Säure bei Muskelgewebe und daraus hergestellten Erzeugnissen nachgewiesen werden (vgl. Fig. 5–7). Bei den Nichtfettsäuren soll auf das Vorkommen von Brenztraubensäure (Peak Nr. 3), 3-Hydroxybuttersäure (Peak Nr. 7b) und Bernsteinsäure (Peak Nr. 9) hingewiesen werden. In geringer Menge konnte auch Fumarsäure (Peak Nr. 8) in der Muskulatur frisch geschlachteter Tiere aufgefunden werden. Als die mengenmässig wichtigsten freien Fettsäuren in Fleisch und Fleischerzeugnissen konnten Palmitinsäure (Peak Nr. 15), Stearinsäure (Peak Nr. 20), Ölsäure (Peak Nr. 21) und Linolsäure (Peak Nr. 22) ermittelt werden. Diese wurden sowohl auf der polaren Säule Reoplex 400 (Fig. 5–7) wie auch auf der apolaren Säule Apiezon M (Fig. 8) identifiziert.

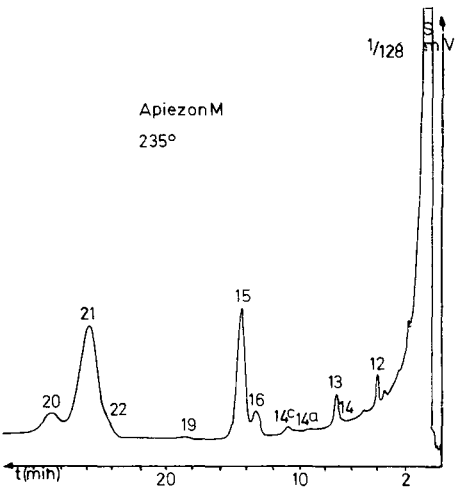

Fig. 8. Gaschromatographische Trennung der aus geräucherter Mettwurst extrahierten Säuren nach Überführung in Methylester auf Apiezon M, isotherm. Erklärung der Peaks, s. Fig. 7.

Die Menge einzelner nichtveresterter Säuren, die in der Muskulatur von verschiedenen Tierarten und in verschiedenen Geweben nachweisbar ist, dürfte grösseren Schwankungen unterworfen sein. So konnten bei vergleichbaren chromatographischen Bedingungen für die aus der Bugmuskulatur vom Rind (Fig. 5) extrahierten Fettsäuren wesentlich geringere Anteile als für die Gehalte an freien Fettsäuren, die aus der Brustmuskulatur von Hähnchen (Fig. 6) stammten, festgestellt werden.

Im weiteren sollen nun vor allem die quantitativen Veränderungen der aufgefundenen Säuren während der Bearbeitung und Lagerung von Muskelgewebe und Fleischerzeugnissen bestimmt werden.

ZUSAMMENFASSUNG

Die im Gewebe frisch geschlachteter Tiere, wie auch in Fleischerzeugnissen anwesenden kurzkettigen nicht veresterten, aliphatischen Nichtfett- und Fettsäuren

wurden mit Äthanol extrahiert und zur Chromatographie aufgearbeitet. Die Identifizierung der Säuren erfolgte mittels Dünnschicht- und Gaschromatographie. Für die Dünnschichtchromatographie von Säuren auf Celluloseschichten wurden zwei Laufmittel und ein spezifisches Sprühmittel beschrieben. Ein weiteres Laufmittel wurde direkt aus der Literatur übernommen. Zur Gaschromatographie von aliphatischen Nichtfett- und Fettsäuren eignete sich unter Anwendung der Temperaturprogrammierung Reoplex 400 als flüssige Phase. Die Fettsäuren wurden ausserdem isotherm auf Apiezon M chromatographiert. In Muskulatur konnte, neben zahlreichen bekannten Säuren, auch die bisher nicht erwähnte Fumarsäure nachgewiesen werden.

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CHROM. 4285

DETECTION OF CHLORPROMAZINE AND THIORIDAZINE METABOLITES IN HUMAN ERYTHROCYTES

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SUMMARY

Chlorpromazine and thioridazine metabolites were detected in bound and unbound forms in the red blood cells of patients receiving these drugs.

The metabolites were separated by thin-layer chromatography and identified, by comparison to reference compounds, as: unmetabolized chlorpromazine (CPZ), CPZ mono-demethyl, CPZ di-demethyl, CPZ sulfoxide, mono-demethyl CPZ sulfoxide, di-demethyl CPZ sulfoxide; unmetabolized thioridazine (TH), TH ring sulfoxide, TH side-chain sulfoxide, demethyl TH side-chain sulfoxide.

By visual comparison of the size and color intensity of the spots with those of standards at various concentrations, the content of free metabolites in 10 cc of packed red cells was, in most cases, equivalent to their concentration in 4 ml of plasma. In blood samples from patients on long-term medication, free chlorpromazine metabolites were present in the entire red cell fraction of the sample at a higher concentration than in the corresponding plasma fraction.

The chromatographic patterns formed by the plasma extracts showed large daily variations in the concentration of the free metabolites as well as in the ratio unbound/bound forms. More reproducible patterns were obtained from the extraction of the free metabolites present in red cells.

INTRODUCTION

Although the literature on psychoactive phenothiazine derivatives is particularly extensive, little is known relative to the metabolic picture and to the distribution of these drugs in blood.

Studies on the pharmacological properties of the phenothiazines and their metabolites in animals[1,2] and in humans[3–7] indicate that the therapeutic effects of these compounds are related to the metabolic pathways and to the rate of biotransformation which the drug undergoes. Inter-patient and intra-patient variations in response to phenothiazine therapy have been studied mainly in relation to excretion patterns and

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correlated to phenolic and conjugated metabolites[8]. Analyses of serum or plasma[9–16], or whole blood[17], have been limited to the determination of total drug metabolites detectable by the adopted analytical procedure, or to the separation and identification of some metabolites by thin-layer chromatography[18–20]. Technical difficulties in the detection of the submicrogram amounts of these compounds present in blood have been overcome by gas chromatography[21–23]. Recently CURRY AND MARSHALL[24] and CURRY[25] reported on the determination by gas chromatography of plasma levels of chlorpromazine, the sulfoxide and the mono- and di-demethyl derivatives in a group of patients receiving various dosages of the drug. The results showed a large variation in the plasma concentration of the drug in patients receiving similar dosages. Only the unbound forms of the four compounds were determined.

It has been observed that plasma levels of unbound metabolites, at a given time, do not necessarily reflect the concentration of the active compound(s) at the site of action and consequently may not be directly related to the therapeutical effect of the drug[26]. The concentration in plasma of the active unbounds forms of the drug, available for diffusion into the sites of action, is influenced by many factors: absorption of the compound, binding to plasma and tissue proteins and degree of reversibility of the complexes formed, transport across membranes, degree of interaction at level of the sites of action and biotransformation of the drug into metabolites with specific pharmacological activity[27].

Perhaps a better understanding of individual variations in response to phenothiazine therapy could be achieved by studying the complete picture of the distribution of the drug and its metabolites in bound and unbound forms, in both plasma and blood cells.

SALZMAN AND BRODIE[10] postulated that chlorpromazine is bound to serum proteins to a great extent. Differences in the degree of plasma protein binding of chlorpromazine and two metabolites have been observed *in vitro*: chlorpromazine and the mono-demethyl derivative showed a considerable protein binding with respect to the sulfoxide[28]. When chlorpromazine and chlorpromazine sulfoxide were incubated for 1 h with whole blood[29], different concentrations were found in the plasma, red cell and platelet fractions. These variations were related to the relative degree of protein plasma binding of the two compounds. Further experiments, *in vitro*, confirmed the uptake of some phenothiazines by red cells and platelets[30,31].

Organic bases of high lipid solubility are known to penetrate the human erythrocyte at rates depending on their lipid-to-water partition[32]. Studies on the distribution of drugs in blood have led to the observation that red cell concentration of sulfanilamide exceeds its plasma level[33]; acetazolamide accumulates and persists in the erythrocytes independently of its concentration in plasma[34]. No reference has been found in the literature concerning the presence of phenothiazine derivatives in human blood cells.

This study, prompted by previous observations[35], was designed as a complement to a project currently in progress in this laboratory on the distribution of psychoactive phenothiazine compounds in blood. It is limited to the detection of phenothiazine derivatives in the red cells of patients receiving various dosages of chlorpromazine or thioridazine and to the identification of some of their metabolites.

EXPERIMENTAL

Material

Blood samples were obtained from patients hospitalized in Cleveland State Hospital and receiving 800–1200 mg of chlorpromazine or 600–800 mg of thioridazine *pro die*.

The patients received the same medication for at least four weeks prior to sampling. No other drugs which could interfere with the analytical procedure adopted were administered. Blood specimens were collected in the morning, 90 min after ingestion of the drug. Patients from the same hospital receiving no phenothiazine derivatives served as control subjects.

Reagents and reference solutions

All reagents were of analytical grade purity. The diethyl ether contained 0.0001 % peroxides.

The Folin–Ciocalteu reagent (Fisher Scientific Co.) was diluted with an equal part of distilled water prior to use.

Reference solutions were prepared from the following chlorpromazine and thioridazine metabolites: chlorpromazine, CPZ mono-demethyl, CPZ di-demethyl, CPZ sulfoxide, mono-demethyl CPZ sulfoxide, di-demethyl CPZ sulfoxide, 7-hydroxy-chlorpromazine, 8-hydroxy-chlorpromazine; thioridazine, TH demethyl, TH ring sulfoxide, TH side-chain sulfoxide, TH disulfoxide, TH disulfone and demethyl TH side-chain sulfoxide.

Stock solutions were prepared by dissolving 10 mg of each substance in 10 ml of 95 % ethyl alcohol. The solutions were stable for ten weeks when stored in the dark at 4°. Aliquots of the stock solutions were used to prepare working standard solutions at various concentrations which ranged from 0.25 to 1 mg %. The alcoholic solvent was evaporated under nitrogen; the residue, dissolved in 0.001 *N* hydrochloric acid, was extracted according to the procedure adopted for the biological samples.

Extraction method

Free metabolites. Chlorpromazine, thioridazine, and their metabolites, were extracted from the biological samples into diethyl ether. The sample, made alkaline by addition of 1 *N* sodium hydroxide (pH 9–10), was shaken twice with 2 vol. of ether for 2 min, and twice with 5 vol. of ether for 10 min. Each ether phase, as soon as it was separated from the aqueous phase, was transferred to a flask containing 2–5 ml portions of 1 *N* hydrochloric acid and shaken for 10 min. The aqueous acidic phases were collected, made alkaline (pH 12) and re-extracted with two 25-ml portions of methylene chloride. The organic phases were combined and evaporated to dryness under a stream of nitrogen.

Bound metabolites. 5 *N* sodium hydroxide was added to the aqueous residue after extraction of the free metabolites. The final concentration of sodium hydroxide was 3 % (w/v). The flask containing the sample was placed for a few minutes in hot water and gently shaken in order to remove traces of ether remaining in the residue. It was then heated in a boiling water bath for 1 h. After cooling, the extraction was continued as outlined above for the free metabolites.

Recovery of chlorpromazine and thioridazine metabolites. In order to validate the

extraction method adopted, aliquots of the reference solutions corresponding to a content of 7 and 15 μg of substance were transferred to a test tube. The ethanol was evaporated under nitrogen. Three milliliters of 0.001 N hydrochloric acid were added to the test tube to dissolve the residue. The absorption spectrum of this solution was determined over the range of 200 to 400 mμ on a Beckman DB-G recording spectrophotometer. The solution was then neutralized and transferred to a flask containing 10 ml of phenothiazine free plasma or 20 ml of red cell hemolysate. The extraction was continued as for the biological samples. After evaporation of the methylene chloride, the residue was dissolved in 3 ml of 0.001 N hydrochloric acid. The absorbance of this solution was determined again, *versus* a blank sample prepared from the same plasma or hemolysate under identical experimental conditions. The recovery of the added substance was calculated from the difference between the initial and the final readings of the acidic solution at the wavelength of maximum absorption of the compound analyzed. Correction was made for the loss occurring during the various steps of the extraction procedure. Decomposition of the compounds was checked by thin-layer chromatography.

Chromatographic method

Merck Silica Gel G plates without fluorescence indicator, 20 × 20 cm, 0.25 mm, used during the experiments, were obtained from Brinkmann Instruments.

The plates were re-activated for 30 min at 100° and used the same day. Two parallel lines were marked 13 and 14 cm from the line of the application points which was 3 cm from the base of the plate. The silica gel between the two parallel lines was scraped off. A series of vertical parallel strips 2 cm wide was also scribed on each plate. The chromatographic tank was previously saturated for 1 h with the solvent. Two plates containing biological extracts and reference solutions were developed simultaneously in each tank. The chromatographic mixture was used for only one development. The chromatographic systems used were:

System I: acetone–12 N ammonium hydroxide (100:7);

System II: 95 % ethyl alcohol–ethyl acetate–glacial acetic acid–water (15:60:15:10);

System III: chloroform–absolute methanol–glacial acetic acid (90:10:10).

The development was allowed to proceed until the solvent front had reached 13 cm from the line of the application points. Then the plates were overrun for 10 min, removed from the tank, air-dried, sprayed lightly with the Folin–Ciocalteu reagent and observed for color development. A subsequent spray of 50 % sulfuric acid (v/v) was applied after 5 min and the color development observed again at room temperature as well as after heating the plate at 100° for a few minutes.

Procedure

Blood specimens collected in EDTA tubes were centrifuged for 10 min at 2500 r.p.m. The plasma and the buffy coat were removed and the red cells washed three times with 100 ml of 0.9 % sodium chloride solution each time. The third washing solution was subjected to extraction. The washed red cells were hemolyzed by adding an equal volume of distilled water and by shaking the test tube for a few minutes. Six extracts were prepared from each blood sample analyzed:

Extract A: hemolysate obtained from 10 cc of packed red cells;
Extract B: 10 ml of plasma;
Extract C: 4 ml of plasma;
Extract D: third washing solution (see above);
Extract E: hydrolysate from the residue after extraction A;
Extract F: hydrolysate from the residue after extraction B.

After evaporation of the methylene chloride, each residue was dissolved in acetone and quantitatively spotted on the chromatographic plate. Aliquots of the reference solutions mentioned above were extracted with the same procedure and chromatographed simultaneously with the biological extracts. Samples of phenothiazine-free plasma and hemolysate were also included in the analysis.

RESULTS AND DISCUSSION

Extraction procedure and recovery studies

Analytical methods previously used for the extraction of the phenothiazine derivatives from biological material involved the use of *n*-heptane[10,16], ethylene dichloride[36], carbon tetrachloride[37], chloroform[19,38] or methylene chloride[8,22]. When these solvents were used for recovery studies of the pure substances from aqueous alkaline solutions, the figures obtained showed considerable discrepancies with respect to the relative amounts of sulfides and sulfoxides recovered. Furthermore, they proved to be unsuitable for the extraction of the red blood cell hemolysates because of the tendency for gel formation which caused a considerable percentage of organic solvent to be lost during the extraction operations. The use of a single large volume of solvent to extract successively small portions of the sample resulted in an extract containing considerable amounts of normally occurring substances which interfered with the chromatography of the phenothiazine derivatives. Re-extraction of chlorpromazine and thioridazine from chloroform or methylene chloride in hydrochloric acid at various concentrations proved to be unsuitable, as the partition between the two phases is in favor of the organic solvent. Re-extraction in dilute sulfuric acid yielded poor recoveries of the sulfoxide forms. Unidentified green spots were observed in the chromatograms of thioridazine-containing samples when the concentration of the sulfuric acid was increased.

Extraction with diethyl ether[6,11] was preferred despite the content of peroxides present in the commercially available product. Attempts to remove the peroxides were only partially successful: the recoveries of the sulfide forms of the two drugs after a single 10 min-extraction were inconsistent and the oxidation to the corresponding sulfoxides was still considerable. By reducing the contact time between sample and ether to 2 min and by re-extracting each ether phase with the acid solution as soon as it was separated from the aqueous phase, no oxidation of the sulfide forms to the corresponding sulfoxides was noted.

Tables I and II show respectively the recoveries of 7 and 15 μg of chlorpromazine and thioridazine metabolites added to 10 ml of phenothiazine free plasma or to 20 ml of hemolysate, when extracted according to the procedure described.

Hydrolysis of the bound metabolites

During preliminary experiments, acid or alkaline hydrolysis was applied to por-

TABLE I

RECOVERY OF KNOWN AMOUNTS OF CHLORPROMAZINE METABOLITES FROM DRUG-FREE PLASMA AND HEMOLYSATE[a]

Compound	*7 μg added*		*15 μg added*	
	Plasma	*Hemolysate*	*Plasma*	*Hemolysate*
Chlorpromazine	68 ± 3.4	60 ± 2.2	79 ± 5.6	65 ± 4.2
CPZ mono-demethyl	76 ± 6.5	72 ± 5.6	86 ± 3.7	79 ± 5.1
CPZ di-demethyl	77 ± 3.1	69 ± 5.2	82 ± 6.1	76 ± 3.8
CPZ sulfoxide	97 ± 1.5	93 ± 4.4	98 ± 1.1	91 ± 1.9
Mono-demethyl CPZ sulfoxide	96 ± 2.3	91 ± 3.1	101 ± 2.1	96 ± 4.4
Di-demethyl CPZ sulfoxide	89 ± 4.8	88 ± 3.2	99 ± 1.3	94 ± 2.5
7-Hydroxy-chlorpromazine	62 ± 5.6	61 ± 4.6	74 ± 3.8	71 ± 3.6
8-Hydroxy-chlorpromazine	75 ± 3.6	77 ± 4.8	81 ± 1.4	79 ± 3.8

[a] Each figure is the average of four determinations.

TABLE II

RECOVERY OF KNOWN AMOUNTS OF THIORIDAZINE METABOLITES FROM DRUG-FREE PLASMA AND HEMOLYSATE[a]

Compound	*7 μg added*		*15 μg added*	
	Plasma	*Hemolysate*	*Plasma*	*Hemolysate*
Thioridazine	73 ± 2.1	69 ± 5.1	76 ± 4.3	78 ± 2.4
TH demethyl	85 ± 4.2	82 ± 3.3	83 ± 1.5	80 ± 3.7
TH ring sulfoxide	97 ± 3.3	98 ± 2.3	102 ± 2.4	99 ± 2.1
TH side-chain sulfoxide	95 ± 3.5	92 ± 2.1	99 ± 2.1	101 ± 2.7
TH disulfoxide	61 ± 3.8	59 ± 4.6	65 ± 4.5	67 ± 3.5
TH disulfone	101 ± 3.6	97 ± 2.2	96 ± 3.1	98 ± 3.7
Demethyl TH side chain sulfoxide	81 ± 2.3	79 ± 3.7	89 ± 4.7	86 ± 3.5

[a] Each figure is the average of four determinations.

tions of the aqueous residues after extraction of the free metabolites from the plasma samples. The use of hydrochloric acid or sulfuric acid at various concentrations led to higher quantities of unmetabolized chlorpromazine and thioridazine than the alkaline hydrolysis, and to lower quantities of the corresponding sulfoxides. Recovery studies on the available reference compounds added to phenothiazine-free plasma, showed that the sulfoxides are, by the action of the strong acid, partially converted to the corresponding sulfides, depending on the concentration of the acid and on the time of hydrolysis. Blank samples to which thioridazine metabolites were added and plasma samples from patients receiving thioridazine, when hydrolyzed with sulfuric acid or hydrochloric acid, yielded green unindentified derivatives[39].

Recoveries of the pure substances, added to phenothiazine-free samples hydrolyzed with 3 % sodium hydroxide, were similar to those shown in Tables I and II.

Chromatography of the extracts

Reference compounds. The R_F values of chlorpromazine and thioridazine metabolites in the three chromatographic systems used are tabulated respectively in

Tables III and IV together with the color reactions formed with the Folin–Ciocalteu reagent and the subsequent spray with the sulfuric acid solution, at room temperature and after heating the plate at 100°.

TABLE III

R_F VALUES (× 100) AND COLOR REACTIONS OF CHLORPROMAZINE METABOLITES

Compound	*System No.*			*Reagents*	
	I	*II*	*III*	*Folin*	H_2SO_4
CPZ di-demethyl	90	72	43	Fuchsia	
Chlorpromazine	85	50	40	Fuchsia	
Di-demethyl CPZ sulfoxide	79	42	11	—	Pink
7-Hydroxy-chlorpromazine	73	66	23	Violet	
CPZ mono-demethyl	60	61	52	Fuchsia	
CPZ sulfoxide	51	13	15	—	Pink
8-Hydroxy-chlorpromazine	41	66	25	Blue-violet	
Mono-demethyl CPZ sulfoxide	25	33	23	—	Pink

TABLE IV

R_F VALUES (× 100) AND COLOR REACTIONS OF THIORIDAZINE METABOLITES

Compound	*System No.*			*Reagents*		
	I	*II*	*III*	*Folin*	H_2SO_4	*100°*
Thioridazine	83	66	48	Blue-green		
TH demethyl	75	86	56	Blue-green		
TH disulfone[a]	70	40	27	—	—	—
TH ring sulfoxide	54	23	17	—	Blue violet	
TH side-chain sulfoxide	45	20	10	Pink		
Demethyl TH side-chain sulfoxide	33	45	21	Pink		
TH disulfoxide	29	6	4	—	—	Pink

[a] For the localization of the spot, see test.

The Folin reagent was useful for the identification of the sulfide forms of the two drugs. The subsequent spray with sulfuric acid resulted in color reactions with the sulfoxide forms and in an intensification of the colors produced by the Folin reagent. Under the chromatographic conditions described, the sulfoxide derivatives of the two drugs (0.5–1 μg) formed colored spots at room temperature, with the exception of thioridazine disulfoxide, which develops a pink color only after heating the plate. Thioridazine disulfone did not give any color formation and was localized as a blue fluorescent spot when the untreated plate was observed in UV light.

Blood extracts. The results obtained by the analyses of the bound and unbound metabolites in the red cell and plasma fractions from the blood samples of 50 patients in chlorpromazine and 24 patients in thioridazine therapy are tabulated respectively in Tables V and VI, and in Tables VII and VIII. The extraction of each sample was repeated twice at intervals of two days. The resultant extracts were chromatographed in two different systems. System I was used for the chromatography of the extracts from the first sample, independently of its content in chlorpromazine or thioridazine. The extracts from the second sample of blood were chromatographed in System II if

TABLE V

CHROMATOGRAPHIC PATTERNS OF UNBOUND AND BOUND CHLORPROMAZINE METABOLITES IN THE RED CELL AND PLASMA FRACTIONS OF FIFTY BLOOD SAMPLES (EXTRACTS A, B, E AND F)

Patient No.	*Unknown*[a]	*CPZ di-demethyl*	*CPZ mono-demethyl*	*Chlorpromazine*	*Di-demethyl CPZ sulfoxide*	*Mono-demethyl CPZ sulfoxide*	*Unknown*[a]	*CPZ sulfoxide*
1		A B F		A B F	B F			A B F
2		A B F		A B F				
3		B F			F			F
4		A B		A B F				A B F
5		A B		A B F				
6	B	A B E F	A B F	A B E F	A B F	B F		A B F
7		A B		A B F				
8		B						B
9		A B F		A B F				
10		B			F			F
11		A B		A B F				
12	E	A B F		A B F	B F			A B F
13		A B		A B F	B			A B F
14		A B F	F	A B F				
15		A B F		A B F				
16	E	A B F		A B F	B F			A B F
17		A B		A B F				
18	E	A B F		A B F	B F			A B F
19	E	A B F		A B F	B F			A B F
20		A B		A B F				
21	E	A B F		A B F	B F			A B F
22		A B F		A B F				
23		B			F			B F
24	B E	A B E F	B F	A B E F	A B F	B	B F	A B F
25		A B F		A B F				
26		B						B
27		A B F		A B F				
28		A B F		A B F				
29		A B		A B F				
30	E	A B F		A B F	B F			A B F
31		A B F		A B F				
32	E	A B F		A B F	B F			A B F
33		A B		A B F				
34		A B F		A B F				
35	E	A B		A B F	B F			A B F
36		B			F			F
37		A B		A B				
38		A B F		A B F	B F			A B F
39	E	A B F		A B F	B F			A B F
40		A B		A B				
41		A B		A B				
42		A B F		A B F				
43		A B F		A B F	B F			A B F
44		A B F		A B F	B F			A B F
45		A B F		A B F				
46		A B		A B				
47	E	A B E F	A B E F	A B E F		A F	B F	A B F
48		A B		A B F				
49	E	A B		A B F	B F			A B F
50		B						B

[a] See test.

TABLE VI

CHLORPROMAZINE METABOLITES DETECTED IN EXTRACTS A, B, E AND F AND OCCURRENCE OF THE SPOTS (R_F VALUES IN SYSTEM II)

Groups and % of cases	*R_F values (× 100) of the spots*	*Reference compounds*	*% appearance of the spots within each group*[a]			
			Unbound metabolites		*Bound metabolites*	
			Extract A	*Extract B*	*Extract E*	*Extract F*
Group A	72	CPZ di-demethyl	—	100	—	14
14 % (7 patients)	42	Di-demethyl CPZ sulfoxide	—	—	—	57
	13	CPZ sulfoxide	—	57	—	57
Group B	72	CPZ di-demethyl	100	100	—	50
48 % (24 patients)	61	CPZ mono-demethyl	—	—	—	4
	50	Chlorpromazine	100	100	—	83
Group C	81	Unknown[b]	—	—	62	—
32 % (16 patients)	72	CPZ di-demethyl	100	100	—	75
	50	Chlorpromazine	100	100	—	100
	42	Di-demethyl CPZ sulfoxide	—	94	—	87
	13	CPZ sulfoxide	100	100	—	100
Group D	81	Unknown[b]	—	67	67	—
6 % (3 patients)	72	CPZ di-demethyl	100	100	100	100
	61	CPZ mono-demethyl	67	100	33	100
	50	Chlorpromazine	100	100	100	100
	42	Di-demethyl CPZ sulfoxide	67	67	—	67
	33	Mono-demethyl CPZ sulfoxide	33	67	—	67
	22	Unknown[c]	—	67	—	67
	13	CPZ sulfoxide	100	100	—	100

[a] Figurs were rounded for simplicity.
[b] See test.
[c] See test.

TABLE VII

CHROMATOGRAPHIC PATTERNS OF UNBOUND AND BOUND THIORIDAZINE METABOLITES IN THE RED CELL AND PLASMA FRACTIONS OF TWENTY-FOUR BLOOD SAMPLES (EXTRACTS A, B, E AND F)

Patient No.	*Thioridazine*	*Unknown*[a]	*TH ring sulfoxide*	*TH side-chain sulfoxide*	*Demethyl TH side-chain sulfoxide*
1			A B F	A B F	
2	A B		A B E F	A B E F	
3	A B F	B F	A B E F	A B E F	A B F
4			A B F	A B	
5	A B		A B E F	A B F	
6			B	B	
7	A B		A B F	A B F	
8	A B E F	B F	A B E F	A B E F	A B F
9	A B		A B E F	A B E F	A B F
10	A B		A B F	A B	
11	A B		A B E F	A B E F	A B F
12			A B	A B	
13	A B		A B E F	A B	
14			B F	B F	
15	A B E F	B F	A B E F	A B E F	A B F
16			B		
17	A B		A B E F	A B E F	A B F
18	A B		A B F	A B	
19	A B		A B E F	A B E F	A B F
20			A B F	A B F	
21	A B		A B F	A B	
22	A B E F	B F	A B E F	A B E F	A B F
23			A B	A B F	
24	A B		A B E F	A B F	

[a] The spot is pink after Folin reagent. Its R_F value in System III was 39.

chlorpromazine had to be analyzed and in System III if the sample contained thioridazine. The use of different chromatographic systems for the second development was necessary because of the close R_F values of chlorpromazine and chlorpromazine di-demethyl in System III; System II did not give satisfactory separation between thioridazine ring sulfoxide and thioridazine side-chain sulfoxide.

Only metabolites detected in plates developed in two different systems were included in the results. Analyses of the third-washing solutions were always negative.

Chlorpromazine metabolites. The chromatographic patterns resulting from the analysis of blood samples from patients receiving chlorpromazine were grouped according to the absence of any free chlorpromazine metabolites in the red cell fraction (Group A), or to the presence, in this fraction, of unmetabolized chlorpromazine together with: chlorpromazine di-demethyl (Group B), or chlorpromazine di-demethyl and chlorpromazine sulfoxide (Group C), or chlorpromazine di-demethyl and chlorpromazine sulfoxide in addition to other metabolites (Group D). Table VI shows the percentage of appearance of the metabolites detected in Extracts A, B, E and F, within each of these groups.

Patients in Groups A, B, and C were receiving different dosages of chlorpromazine, varying between 800 and 1200 mg *pro die*. No considerable differences were noted in these patients with respect to the terms of medication. The three patients in Group D were in chlorpromazine therapy for longer periods of time. Two of them, at the time

TABLE VIII

THIORIDAZINE METABOLITES DETECTED IN EXTRACTS A, B, E AND F, AND OCCURRENCE OF THE SPOTS (R_F VALUES IN SYSTEM I)

Groups and % of cases	*R_F values (× 100) of the spots*	*Reference compounds*	*% appearance of the spots within each group*[a]			
			Unbound metabolites		*Bound metabolites*	
			Extract A	*Extract B*	*Extract E*	*Extract F*
Group A	54	TH ring sulfoxide	—	100	—	33
12% (3 patients)	45	TH side-chain sulfoxide	—	66	—	33
Group B	54	TH ring sulfoxide	100	100	—	60
21% (5 patients)	45	TH side-chain sulfoxide	100	100	—	60
Group C	83	Thioridazine	100	100	19	25
67% (16 patients)	60	Unknown[b]	—	25	—	25
	54	TH ring sulfoxide	100	100	75	100
	45	TH side-chain sulfoxide	100	100	56	75
	33	Demethyl TH side-chain sulfoxide	50	50	—	50

[a] Figures were rounded for simiplicity.

[b] The spot is pink after Folin reagent. Its R_F value in System III was 39.

of sampling, were receiving 800 mg, and the third 1000 mg of the drug *pro die*.

Unbound and bound chlorpromazine metabolites were absent in the red cell fractions from the seven patients in Group A. Analysis of 4 ml of plasma was negative. The concentration of free and bound forms in 10 ml of plasma was at the limits of detection. The sulfoxide form of chlorpromazine di-demethyl appeared in Extracts F in four samples, together with chlorpromazine sulfoxide. Unmetabolized chlorpromazine was absent in all the plasma extracts or its concentration was below the limit of detection. In these patients, chlorpromazine di-demethyl was constantly the major metabolite present in free form in plasma.

The chromatographic patterns obtained from the 24 patients in Group B showed, as a common characteristic, the presence in the red cell fraction of free unmetabolized chlorpromazine and the di-demethyl derivative. The two metabolites were also present in free form in the extracts from the corresponding plasma (Extracts B) in all the samples, and simultaneously in bound form in twelve samples of plasma. Extracts E were negative. No sulfoxides were detected in any of the extracts analyzed in this group. As estimated by visual comparison of the size and color intensity of the spots, unmetabolized chlorpromazine appeared to be the major metabolite present in free form in red cells as well as in plasma.

Free chlorpromazine sulfoxide was present together with unmetabolized chlorpromazine and the di-demethyl derivative in the red cell fraction of all the samples in Group C. Chlorpromazine and its sulfoxide were constantly detected in free and bound form in the plasma extracts. The total free chlorpromazine content in red cells appeared to be equivalent to 4 ml of plasma. The analysis of the bound forms in red cells was negative with respect to the metabolites identified in the other fractions; an unidentified derivative was detected in ten of the sixteen samples analyzed in this group. It formed a pink-violet spot when the sprayed plate was heated at 100°. Its R_F values were: 97 in System I, 81 in System II, and 63 in System III. The spot was absent in chromatograms of similar extracts from phenothiazine-free samples.

The same unknown metabolite was present in Extracts B and/or E from the patients in Group D. It was detected in free form in plasma and in bound form in red cells in the blood samples of these patients over a period of five months. Unmetabolized chlorpromazine and the di-demethyl derivative were present in free and bound form in the red cells and plasma of all the three patients in this group. Chlorpromazine mono-demethyl appeared in the two plasma fractions (Extracts B and F) in all samples, in free form in the red cells of two patients, and in bound form only in one of these samples. Chlorpromazine sulfoxide was also constantly detected in Extracts A, B and F. It represented the major metabolite within the sulfoxide derivatives detected. The sulfoxide forms of the two demethyl derivatives appeared randomly in Extracts A or B or F. Hydrolysis of the red cells yielded only sulfide forms. A metabolite, constantly detected in the two plasma extracts of two patients in this group, remained unidentified. It formed a violet spot at room temperature with the Folin reagent. Its R_F values were: 10 in System I, 22 in System II and 24 in System III.

In all the three patients the level of total free chlorpromazine material in 10 cc of red cells was equivalent or higher than in 10 ml of plasma. The bound forms in plasma appeared to be also at a higher concentration than the free forms.

As mentioned above, the daily dosages of these three patients were below the maximum reached in some of the cases studied: at the time of sampling, they had been

in chlorpromazine therapy at various dosages for longer periods of time ranging from six to eight months.

A characteristic pattern resulted from the analysis of the blood sample from one patient in this group. During the experiments it had been observed that each free metabolite detected in the red cell fraction was constantly present also in the extract from the corresponding 10 ml of plasma. In the case mentioned, mono-demethyl chlorpromazine sulfoxide was detected in free form in the red cells (Extract A) and in bound form in plasma (Extract F), but it was absent as a free metabolite in the plasma extract B (Fig. 1). Chlorpromazine mono-demethyl appeared in all the four fractions and distinctly at a higher concentration in free form in red cells. Unmetabolized chlorpromazine and the di-demethyl derivative were also present in Extracts A, B, E and F.

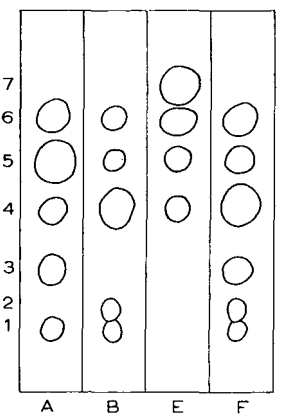

Fig. 1. Chromatographic patterns of Extracts A, B, E and F from a blood sample of a patient in chlorpromazine therapy (1000 mg/day).

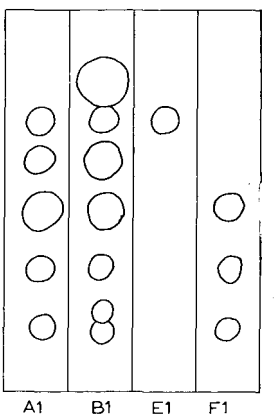

Fig. 2. Chromatographic patterns of Extracts A, B, E and F (reported in the test and in the figure as: A1, B1, E1 and F1) from a blood sample from the same patient, five days later.

The analyses of the two samples were performed on 10.6 cc of packed red cells and on 12.1 ml of plasma, representing the entire content of the samples. The plates were developed in System II. The spots, in order of increasing R_F values, correspond to: CPZ sulfoxide (1), unknown (2), mono-demethyl CPZ sulfoxide (3), chlorpromazine (4), CPZ mono-demethyl (5), CPZ di-demethyl (6), unknown (7).

The predominant spot in the two plasma fractions (B and F) corresponded to unmetabolized chlorpromazine; in the red cell fraction, the major free metabolite was the mono-demethyl derivative.

The analysis repeated on a second blood sample after two days, according to the procedure adopted, yielded similar patterns. The analysis was performed again five days later and then at intervals of weeks for five months.

In Fig. 2 are shown, for comparison purposes, the chromatographic patterns resulting from the extraction of the sample obtained from this patient five days after the analysis represented in Fig. 1.

Mono-demethyl chlorpromazine sulfoxide appeared as a free metabolite in plasma (Extract B1), and nearly at the same concentration as in red cells (Extract A1).

The level of chlorpromazine mono-demethyl in plasma (B1) increased with respect to Extract B, while its concentration in the red cell fraction (A1) appeared to be lower than in Extract A. Correspondingly, the bound form of this metabolite disappeared from plasma and from red cells. Unmetabolized chlorpromazine and the di-demethyl derivative were the only bound forms of sulfides present respectively in plasma and in red cells.

In this sample, free unmetabolized chlorpromazine was the major metabolite detected in the red cell fraction. The major spot in the plasma extract (B1) showed the same chromatographic properties as the unknown detected in Extract E (Fig. 1).

A comparsion of the chromatographic patterns of each fraction in Fig. 1 and in Fig. 2 showed also evident variations in the relative concentrations of total chlorpromazine derivatives, free and bound, in plasma as well as in red cells. These differences were more considerable with respect to the distribution and to the concentrations of the various metabolites between the four fractions, rather than with respect to the level of total chlorpromazine material present in each of the two different samples of blood. Extract B1 in Fig. 2 showed an evident increase in the plasma concentration of total free metabolites with respect to the analogous Extract B in Fig. 1; concomitantly the level of the bound forms in plasma (Extract F1) decreased with respect to Extract F in Fig. 1. Lower levels were also noted in the content of the bound forms in red cells. A more reproducible pattern appeared to be obtained from the extraction of the free metabolites present in red cells.

Similar variations were noted in many samples when the analyses were repeated at intervals of weeks under identical experimental conditions. The more apparent discrepancies observed were related to the concentration of the various metabolites free in plasma and to the ratio unbound/bound forms in this fraction.

Thioridazine metabolites. The results from the analysis of blood samples from patients in thioridazine therapy were grouped according to the absence of any free thioridazine metabolites in the red cell fraction (Group A), or to the presence, in this fraction, of thioridazine ring sulfoxide and thioridazine side-chain sulfoxide (Group B), or to the presence of the two sulfoxides together with unmetabolized thioridazine (Group C) (Table VIII).

The extracts from the patients in Group A formed poor patterns. Free thioridazine ring sulfoxide was present in the plasma extracts (10 ml) in all three cases. The side-chain sulfoxide was detected in this fraction in two samples. Extracts A, C and E were negative.

Contrary to the patterns formed by chlorpromazine, the two thioridazine sulfoxides appeared in free form in red cells before the unmetabolized drug and the demethyl derivative could be detected. Ring and side-chain sulfoxides were present simultaneously as free metabolites in the red cells and in the corresponding 10 ml of plasma, in the five patients in Group B. No bound metabolites were detected in Extracts E.

The chromatographic patterns of the red cell fractions from the sixteen patients in Group C showed the presence of unmetabolized thioridazine; in all the samples it was detected constantly together with the two sulfoxides. The corresponding plasma extracts showed the same free forms. In eight samples demethyl thioridazine side-chain sulfoxide was present, in free form, in red cells and in plasma, and simultaneously in bound form in the plasma fraction. Extracts F contained thioridazine ring sulfoxide in all the cases.

Contrary to the patterns formed by the bound chlorpromazine metabolites in red cells, the two sulfoxide derivatives of thioridazine were detected in the fractions E of some samples. In no case was the content of free metabolites in red cells higher than in 4 ml of plasma. The concentration of bound forms in plasma appeared to be lower than the level of the free metabolites.

In all the blood samples analyzed, thioridazine ring sulfoxide represented apparently the major metabolite; nevertheless, the higher sensitivity of this metabolite to the color developers used must be taken into consideration, when compared with the other thioridazine reference compounds studied.

CONCLUSIONS

From the results obtained during the experiments, it appeared that the concentration of unbound forms of chlorpromazine and thioridazine metabolites in red cells reflects the level of the total drug present in plasma.

Plasma levels appeared not to be a reflection of the administered dosage, but rather to be depending on the terms of medication. Plasma protein binding did not limit the diffusion of the drug derivatives into red cells, as shown by comparison of the chromatographic patterns obtained from these two fractions.

In patients receiving various dosages of the drug for periods of time ranging between four and nine weeks, the concentration of chlorpromazine and thioridazine metabolites in 10 cc of packed red cells was estimated, by visual comparison of the size and color intensity of the spots, to be equivalent to the drug content in 4 ml of plasma.

Samples from patients in longer-term medication (chlorpromazine) yielded more intense patterns, regardless of the administered dosage. In some of these samples, free forms of chlorpromazine were detected in the red cell fraction at a higher concentration than in 10 ml of plasma; correspondingly the ratio unbound/bound forms in plasma was exceptionally reversed, the bound metabolites appearing at a higher concentration than the free forms.

In the 96% of the total samples analyzed (74 patients) the distribution of chlorpromazine and thioridazine metabolites in the four fractions (unbound and bound forms in red cells, and unbound and bound forms in plasma) appeared to be as follows: plasma free forms > plasma bound forms > red cell free forms > red cell bound forms. In patients in long-term chlorpromazine therapy, and depending on the day of sampling, this relation was: plasma bound forms > red cell free forms > plasma free forms > red cell bound forms (Fig. 1), or it was: plasma free forms > red cell free forms > plasma bound forms > red cell bound forms (Fig. 2).

The variation in the relative concentration of the various metabolites in each of the four fractions did not appear to affect the total drug content in the whole sample.

The most apparent daily variations in the chromatographic patterns formed by the two drugs studied in each of the four fractions analyzed were related to the level of the free metabolites in plasma. As these variations appeared to be inconsistently depending on the day of sampling, any approach made in order to establish a correlation between plasma level of the drug and patient response to the phenothiazine therapy should be reviewed. Clearly, such a correlation cannot rely exclusively on the determination of the plasma level of unbound metabolites present in any given sample.

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CHROM. 4314

THIN-LAYER CHROMATOGRAPHIC SEPARATIONS OF CYCLOKETONE 2,4-DINITROPHENYLHYDRAZONES*

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SUMMARY

Ten cycloketone 2,4-dinitrophenylhydrazones have been chromatographed by thin-layer techniques using Eastman Chromagram sheets coated with silica gel and developed with benzene in Eastman sandwich apparatus. Good separations of all test compounds from binary mixtures with the standard cyclohexanone 2,4-dinitrophenylhydrazone are reported as well as good separations of more complex mixtures of the test compounds.

INTRODUCTION

Many investigators have applied a variety of thin-layer chromatography techniques to a large group of carbonyl 2,4-dinitrophenylhydrazones (2,4-DNPHs). These compounds are particularly well suited to such studies because of the ease of their preparation and their intense orange-yellow color which makes visualization procedures unnecessary.

In all these works, only scattered reference is made to the separation of cycloketone 2,4-DNPHs. A most comprehensive study of TLC separations of carbonyl 2,4-DNPHs was made by BRÜMMER AND MÜLLER-PENNING[1], in which they investigated nearly 250 compounds using a Kieselguhr G adsorbent with a developing solvent of petroleum ether–chloroform–ethyl acetate (30:3:1). Reported among the many R_B values (R_F relative to butter yellow) from this study were those of five cycloketone 2,4-DNPHs: cyclopentanone, cyclohexanone, cycloheptanone, cyclooctanone and cyclopentadecanone.

DENTI AND LUBOZ[2] have reported R_{for} values (R_F values relative to the R_F of formaldehyde) for the 2,4-DNPHs of cyclopentanone and cyclohexanone using ad-

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sorbent layers of Silica Gel G and Alumina G (both alone and impregnated with silver nitrate) with four different solvent mixtures. ZAMOJSKI AND ZAMOJSKA[3] also determined R_{for} values for these two compounds in a study which included a large number of aliphatic aldehydes and ketones using silica gel with a benzene developer. Both groups obtained good separations for these two compounds. JART[4] included R_F values for cyclopentanone and cyclohexanone 2,4-DNPHs along with those for 70 other carbonyl compounds*. SLOOT[5] used alumina impregnated with silver nitrate to separate several 2,4-DNPHs including those of cyclopentanone, cyclohexanone, 3-methylcyclopentanone and 2-methylcyclopentanone.

As far as can be determined, no other studies have included a homologous series of aliphatic cycloketone 2,4-DNPHs as does the study upon which this report is based**.

EXPERIMENTAL

Materials

All of the cycloketone 2,4-DNPHs used in this experiment were prepared by the method of SHRINER *et al.*[7] and were recrystallized from methanol until the melting points agreed with those found in the literature[7,8].

Solvents used for recrystallization, spotting, and developing were all "reagent grade".

Solutions of the test compounds were spotted on 20 × 20 cm Eastman Chromagram Sheets (Type 6061) with a stated silica gel coating thickness of 100 μ.

Benzene was used exclusively as the solvent for development.

Procedure

Considerable effort was expended to insure standardization of procedure. The following steps were followed carefully in the collection of all data presented.

(1) Solutions of the 2,4-DNPHs used for spotting were made by dissolving 100 mg of the test compound in 100 ml of a chloroform–methanol (1:1) mixture. Since all the spotting solutions contained the same solvent mixture, the variable effect of different spotting solvents on spot mobility noted by KIDDER AND DEWEY[9] was avoided.

(2) All Chromagram sheets were activated for 60 min in a drying oven at 100–110° before spotting. Activated sheets were stored in a desiccator until spotted.

(3) Spots were applied to the 20 × 20 cm activated Chromagram sheets with a micropipet. Each spot contained 1.5–2.0 μl of each compound in the spot. The spot diameter was kept below 3 mm.

(4) Spots were located 20 mm from the bottom edge of the sheet and 20 mm from each side. The distance between adjacent spots was 16 mm. Each sheet contained spots of nine binary mixtures (the 2,4-DNPHs of the standard cyclohexanone and another of the series), a spot of the standard alone, and a spot of one of two complex mixtures. The complex mixtures consisted of five compounds, the derivatives of all the odd-numbered cycloketones or the derivatives of all the even-numbered cycloketones. One such sheet is illustrated after development in Fig. 2.

* *Editor's note.* There are ten to twenty other papers separating those two compounds.
** *Editor's note.* For a similar separation by PC see ref. 6.

(5) Spotted sheets were heated for 5 min at 100–110° in a drying oven to insure complete evaporation of the spotting solvent. Spotted sheets not developed immediately were stored until used in a desiccator.

(6) The spotted sheets were developed in the Eastman Chromagram Developing Apparatus (6071). Since four such sandwich-type chambers were available, four sheets were developed simultaneously. All development was over a distance of 150 ± 4 mm using benzene as the developing solvent.

RESULTS AND DISCUSSION

Comparison of R_F values seems more meaningful when they are reported relative to an internal standard. Because cyclohexanone was available in largest amounts in our laboratory, its 2,4-DNPH was chosen as a standard. The R_F value of the standard (R_S) was then defined as:

$$R_S = \frac{\text{migration distance of cycloketone 2,4-DNPH}}{\text{migration distance of cyclohexanone 2,4-DNPH}}$$

The mean R_F values and the mean R_S values for each of the ten cycloketone derivatives included in this study are reported in Table I with their standard deviations. Each of the values in the table represents the mean for twenty separate runs. Only those sheets on which the R_F value of the standard was 0.22 or less were included in this summary.

Precision of results

Throughout the experimental procedure, Chromagram sheets were activated, spotted, and developed in groups of four. It was expected, therefore, that the precision within such a group should be very high. In fact, however, considerable deviations were found not only from group to group, but also from sheet to sheet within a group. On some sheets the adsorbent ability of the silica gel layer seemed high, and all the R_F values were low. On other sheets the adsorbent ability of the silica gel was apparently low and the R_F values of all compounds were relatively higher. The R_F value of the standard cyclohexanone 2,4-DNPH varied from 0.16 to 0.41 with 0.22 as the median value.

TABLE I

R_F AND R_S VALUES FOR CYCLOKETONE 2,4-DNPHS

2,4-DNPH of	$R_F \pm \sigma$	$R_S \pm \sigma$
Cyclobutanone	0.13 ± 0.02	0.61 ± 0.02
Cyclopentanone	0.15 ± 0.01	0.73 ± 0.02
Cyclohexanone	0.20 ± 0.02	1.00
Cycloheptanone	0.28 ± 0.02	1.41 ± 0.04
Cyclooctanone	0.28 ± 0.02	1.43 ± 0.04
Cyclononanone	0.33 ± 0.02	1.71 ± 0.05
Cyclodecanone	0.40 ± 0.04	2.06 ± 0.08
Cycloundecanone	0.44 ± 0.04	2.23 ± 0.11
Cyclododecanone	0.49 ± 0.04	2.53 ± 0.12
Cyclopentadecanone	0.53 ± 0.04	2.70 ± 0.12

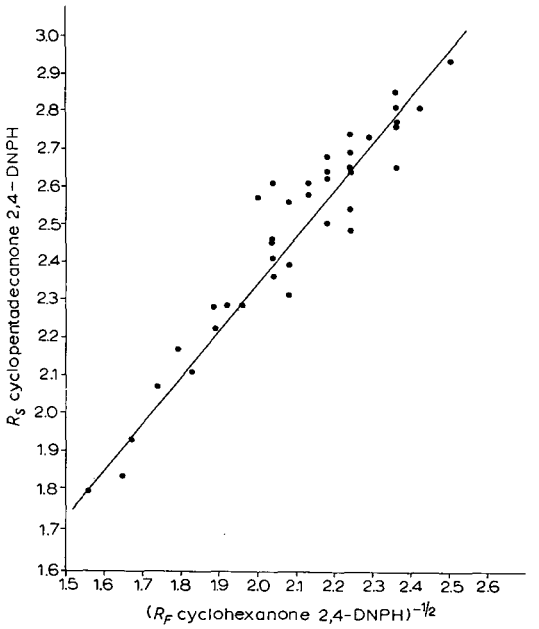

Fig. 1. Graph of the R_S value for cyclopentadecanone 2,4-DNPH plotted against the reciprocal of the square root of the R_F value of the standard.

On those sheets on which the R_F value of the standard was high, the R_S values of all test compounds were lowered with the exception of cyclobutanone and cyclopentanone 2,4-DNPHs, which increased slightly with increasing R_F value of the standard. Fig. 1 shows a graph of the R_S value for cyclopentadecanone 2,4-DNPH plotted against the reciprocal of the square root of the R_F value of the standard.

We conclude that despite the rigorous standardized procedure followed, differences in R_F and R_S values from sheet to sheet are caused by differences in the Chromagram sheets themselves. In a recent article, JOLLIFFE AND SHELLARD[10] also found a high variance ratio between the values taken from different Chromagram sheets from the same pack and suggested that the lack of reproducibility may be due to a lack of constancy in the pH of the silica gel layer which they report varies from 6.5 to 4.0.

Whatever the reason for variation in values, the R_F value of the standard seems to reflect rather accurately the adsorbent activity of the sheet. For these reasons, in compiling the data for Table I, only those sheets were considered on which the R_F value of the standard was at or below the arbitrary value of 0.22, which included the most active half of the forty sheets developed in this study.

Separations

Each member of the series of compounds tested separated clearly from the standard. In addition, all compounds whose structure varied by two or more carbon atoms were separable. This was illustrated experimentally by the good separations of the complex mixtures composed of all the odd-numbered or of all the even-

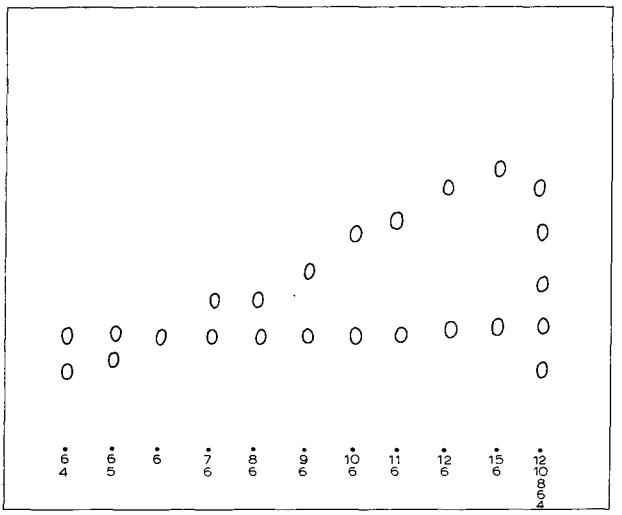

Fig. 2. Separation of mixtures of 2,4-DNPH derivatives of the following cycloketones: cyclobutanone (4); cyclopentanone (5); cyclohexanone (standard) (6); cycloheptanone (7); cyclooctanone (8); cyclononanone (9); cyclodecanone (10); cycloundecanone (11); cyclododecanone (12); cyclopentadecanone (15).

numbered cycloketone derivatives (see Fig. 2). Adjacent members of the series can be separated with the exception of the derivatives of cycloheptanone and cyclooctanone, cyclodecanone and cycloundecanone, and cycloundecanone and cyclododecanone.

Of special interest were the R_F and R_S values for the 2,4-DNPHs of cycloheptanone and cyclooctanone. Within experimental error these values were identical. It is noted that in their comprehensive study of TLC separations of carbonyl 2,4-DNPHs, BRÜMMER AND MÜLLER-PENNING[1] also obtained very similar R_B values for these two compounds (0.96 and 1.00). We are unable to suggest a reason for the apparent elevation of values for the cycloheptanone compound relative to the other members of the series.

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CHROM. 4315

THE THIN-LAYER CHROMATOGRAPHY OF ALKYL AND HALOGEN SUBSTITUTED BENZALDEHYDE 2,4-DINITROPHENYLHYDRAZONES

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SUMMARY

The thin-layer chromatographic separation of alkyl and halogen substituted benzaldehyde 2,4-dinitrophenylhydrazones has been studied using a zinc carbonate adsorbant on glass plates and a 90% carbon disulfide–10% chloroform solvent mixture as the eluant. The halogen substituted benzaldehyde 2,4-dinitrophenylhydrazones were separated from the standard compound, benzaldehyde 2,4-dinitrophenylhydrazone. Of the alkyl derivatives examined only 4-isopropylbenzaldehyde 2,4-dinitrophenylhydrazone separated.

The adsorptive properties of thin layers of zinc carbonate were investigated and an eluotropic series was established.

INTRODUCTION

In an earlier paper[1] we reported the results of our thin-layer chromatographic study of several monosubstituted benzaldehyde 2,4-dinitrophenylhydrazones (2,4-DNPH). Nine of the nineteen compounds reported in that study could not be separated from binary mixtures with benzaldehyde 2,4-DNPH. All of these inseparable compounds contained alkyl or halogen substituents. In recent related studies, JART AND BIGLER[2] reported that the 4-chloro-, 4-bromo-, and 4-methylbenzaldehyde 2,4-DNPHs were not separable from benzaldehyde 2,4-DNPH ($R_{\text{benzaldehyde}}$ values of 0.99, 0.99 and 1.03, respectively). FREYTAG AND NEY[3], however, reported the separation of 3-methyl- and 4-methylbenzaldehyde 2,4-DNPHs from benzaldehyde 2,4-DNPH on chromatoplates of Kieselgel G.

We now wish to report the separation of seven of the nine alkyl and halogen substituted compounds from binary mixtures with benzaldehyde 2,4-DNPH.

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EXPERIMENTAL

Materials

The 2,4-DNPH derivatives of the benzaldehydes reported in this study were all prepared by the procedure of SHRINER *et al.*[4] and were recrystallized from 95% ethanol until the melting points agreed with the literature values.

The developing solvents and the zinc carbonate (B4312) adsorbant were "Baker Analyzed" Reagent Grade.

Preparation of the chromatographic plates

A 250 μ layer of zinc carbonate adsorbant was spread on glass plates (200 × 200 mm) with a Desaga adjustable applicator (Model 3-11-S). The slurry consisted of 70 ml of distilled water and 25 g of zinc carbonate that contained 5% of a soluble starch binder. The plates were air dried for 24 h, activated in a drying oven at 110° for 1 h, and stored in a desiccating cabinet until used.

Development of the chromatographic plates

Standard Desaga developing tanks were used. Each was lined with Whatman No. 1 filter paper and presaturated with solvent for 1 h. All plates were developed over a distance of 150 mm.

RESULTS AND DISCUSSION

A report on the thin-layer chromatographic separation of mixtures of 2,4-DNPH derivatives of aliphatic aldehydes with zinc carbonate chromatoplates[5] prompted us to investigate this novel adsorbant with our alkyl and halogen substituted compounds.

Separation of binary mixtures

The thin-layer chromatographic separation of alkyl and halogen substituted benzaldehyde 2,4-DNPHs relative to the standard compound, benzaldehyde 2,4-DNPH, is reported in Table I. The values presented are defined relative to the standard compound in the following manner:

$$R_{\text{benzaldehyde}} = \frac{R_F \text{ substituted benzaldehyde 2,4-DNPH}}{R_F \text{ benzaldehyde 2,4-DNPH}}$$

Seven of the nine substituted benzaldehyde 2,4-DNPHs that were chromatographed as binary mixtures were separable from the standard compound, benzaldehyde 2,4-DNPH. This may be attributed to the polar nature of the substituent in the case of the halogen substituted compounds, all of which were found to migrate slower than the standard.

The methyl substituted derivatives gave R_F values close to that of benzaldehyde 2,4-DNPH and when chromatographed as mixtures were inseparable from the standard.

4-Isopropylbenzaldehyde 2,4-DNPH was found to be separable, migrating faster than benzaldehyde 2,4-DNPH. This can be attributed to the greater aliphatic

TABLE I

$R_{benzaldehyde}$ VALUES OF SUBSTITUTED BENZALDEHYDE 2,4-DNPHs

Apparatus and conditions: tank, 90 % carbon disulfide–10 % chloroform, glass plate, zinc carbonate.

Substituent	$R_{benzaldehyde}$
4-F	0.74
4-Br	0.78
4-Cl	0.79
3-Cl	0.84
3-Br	0.86
3-F	0.87
3-CH_3	1.00
4-CH_3	1.00
4-$CH(CH_3)_2$	1.10

TABLE II

PRECISION OF $R_{benzaldehyde}$ VALUES OF SUBSTITUTED BENZALDEHYDE 2,4-DNPHs

Substituent	*S.D.*
4-F	± 0.019
4-Br	± 0.020
4-Cl	± 0.020
3-Cl	± 0.012
3-Br	± 0.015
3-F	± 0.015
3-CH_3	± 0.009
4-CH_3	± 0.008
4-$CH(CH_3)_2$	± 0.022

TABLE III

ELUOTROPIC SERIES OF SOLVENTS FOR ZINC CARBONATE

Apparatus and conditions: tank, glass plate, zinc carbonate.

Eluant	R_F *benzaldehyde 2,4-DNPH*
Carbon disulfide–acetone (85:15)	0.92
Acetone–cyclohexane (50:50)	0.87
Tetrahydrofuran	0.87
Acetone	0.84
Dioxane	0.82
Benzene–ethyl acetate (80:20)	0.77
Benzene	0.76
Carbon disulfide– chloroform (90:10)	0.66
Toluene–cyclohexane (75:25)	0.56
Benzene–cyclohexane (60:40)	0.48
Carbon tetrachloride	0.24
Carbon disulfide–benzene (95:5)	0.15
Cyclohexane	0.05
Carbon disulfide	0.00

nature of the substituent which increases the solubility of the derivative in the eluant and decreases the adsorbant–derivative interaction.

Precision of experimental work

The precision of this experimental work is expressed in terms of the standard deviation of 9–12 $R_{\text{benzaldehyde}}$ values for each of the compounds studied. These are presented in Table II. These standard deviations lie within the limits of $\pm$ 0.05 which is generally considered acceptable. The precision of a larger number of determinations was obtained by taking the standard deviation of some 50 R_F values of benzaldehyde 2,4-DNPH. It was found to be $\pm$ 0.033, which although less precise, lies within the limits normally found satisfactory.

Eluotropic series of solvents (ref. 6)

An eluotropic series, a series of solvents in order of increasing eluting power, was established for the zinc carbonate adsorbant. This information was obtained from the data collected in the selection of the optimum solvent system and is shown in Table III. The eluting power of the solvent is reported in terms of the R_F value of the standard compound, benzaldehyde 2,4-DNPH—the larger the R_F value, the greater the eluting power of the solvent.

ACKNOWLEDGEMENT

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CHROM. 4295

THE CHROMATOGRAPHIC PURIFICATION OF HUMAN KAPPA-CASEIN*

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SUMMARY

The fractionation of human casein was shown to be more difficult than that of caseins from ruminants. As human casein is digested by rennin, although less satisfactorily than with other caseins already studied, the purification of a κ-like fraction was attempted.

Fractionations obtained by precipitation or chromatography gave rise to rennin-sensitive components. Two κ-like fractions could be characterized by chromatography on DEAE-cellulose in the presence of dissociating agents.

INTRODUCTION

Starch gel urea electrophoresis of human casein, in the presence of mercaptoethanol, showed it to have a very complicated composition[1]. Though isoelectric human casein was found to be heterogeneous some time ago[2], very few attempts at fractionation have been made and purified fractions corresponding to the α-, β- or κ-components of cow casein have not been obtained until now.

The digestion of human casein by rennin (EC 3.4.4.3), which has been investigated by ALAIS AND JOLLÈS[3], suggested the presence of a component similar to the κ-fraction of bovine casein which is the specific substrate of this enzyme. Preliminary fractionation experiments by chromatography on ion-exchange columns[4] allowed these authors to prepare a fraction rich in the κ-component from human casein. More recently, MALPRESS AND SEID-AKHAVAN[5] have also chromatographed human casein and found two main fractions: one of them was precipitated by calcium and the other was rennin-sensitive; they were similar to bovine α- and κ-caseins but their electrophoretic behaviour was different and indicated that they were always heterogeneous. More recently, NAGASAWA *et al.*[6] have published the results of an incomplete fractionation of human casein; their data were different from those of the

* 21st Communication on caseins; 20th communication, J. JOLLÈS, P. JOLLÈS AND C. ALAIS, *nature*, 222 (1969) 668.

above mentioned authors and did not mention the presence of a calcium-sensitive component.

This paper is concerned with the fractionation of human casein, the main aim being the isolation of a rennin-sensitive component. As the precipitation methods most commonly used in the study of bovine casein only gave poor results with human casein, chromatography on anion exchange columns in the presence of dissociating agents was tried.

EXPERIMENTAL

Human casein was prepared from fresh pooled milk. As previously reported[1,3], its preparation was more difficult than that of casein from ruminants; thus our previously described method was employed[1].

The following precipitation procedures used for the preparation of cow κ-casein were tried in the case of human casein: (1) precipitation by calcium chloride and ethanol, according to McKENZIE AND WAKE[7]; (2) precipitation by sulphuric acid in the presence of urea, according to ZITTLE AND CUSTER[8].

The chromatographic fractionations were done on DEAE-cellulose columns, at pH 7, either by a salt gradient or by stepwise elution. Because of the strong tendency of casein components to form complexes, a dissociating agent must be added in a fairly high concentration. We used an imidazole buffer containing 3.3 *M* urea[9] and a phosphate buffer containing 20% dimethylformamide[10].

Starch gel electrophoresis in a horizontal cell was performed according to WAKE AND BALDWIN[11] with a Tris–borate buffer solution, pH 9.3, containing 4.5 *M* urea and 0.03 *M* mercaptoethanol.

The rennin sensitivity was determined by the action of rennin (0.7 μg crystalline rennin/ml) on 0.5% casein fractions at pH 6.7 and 25°; the release of non-protein nitrogenous substances (NPN) was determined in the 12% trichloracetic acid (TCA) filtrate[12].

RESULTS

Rennin sensitivity of human casein

We prepared twelve lots of human casein from twelve different pooled milk samples (1–2 l) and found that the rennin sensitivity varied considerably from one batch to another. Table I indicates that three lots were not digested at all and three others only to a very slight extent (about 0.3% NPN). Six lots released NPN in quantities lower or about the same as with bovine casein. We also observed that two lots were highly soluble in 12% TCA.

In the following fractionation experiments, we only used batches Nos. 4 and 9; they had a rather low solubility in TCA and gave a good release of NPN which postulated the existence of a κ-like component. The electrophoretic patterns are presented in Fig. 1.

Fractionation of human casein by precipitation

Fractionation with calcium and ethanol was attempted with human casein in 4% solution. The first stages occurred as expected (precipitation by calcium chloride

TABLE I

SENSITIVITY OF HUMAN CASEIN TO RENNIN: LIBERATION OF NON-PROTEIN NITROGEN (NPN) CONTAINING SUBSTANCES

Casein		*NPN*[a]	
Lot No.	*Solution mg N/ml*	*Before rennin action*	*Increase after 60 min*
1	2.47	2.46	0.35
2	0.921	1.35	0
3	1.88	2.11	0.92
4	2.45	1.97	0.90
5	2.21	5.04	0
6	2.38	8.60	1.38
7	2.02	1.80	0.57
8	2.27	20.35	0.27
9	1.93	1.36	1.5
10	2.31	3.14	0.37
11	2.40	3.91	0
12	1.16	4.24	0.87

[a] Percentage of total nitrogen; rennin 0.7 μg/ml, pH 6.7.

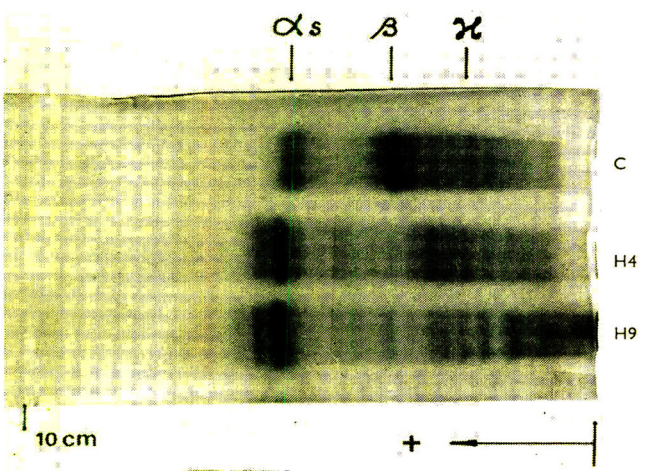

Fig. 1. Starch gel electrophoresis of human (H) and cow (C) caseins; (0.76 *M* Tris–citric acid buffer, pH 8.6, in the presence of 7 *M* urea; 15 h; 15 V/cm in the presence of mercaptoethanol).

and then sodium sulphate to give fractions a and b). However, no immediate precipitation was observed when ethanol was added in the presence of ammonium acetate; at this stage bovine casein gives the characteristic rubbery precipitate of the κ-fraction. The human casein solution floculated slowly at room temperature; the precipitate was separated by centrifugation, washed with 50 % ethanol and dried under vacuum (fraction d).

The four separated fractions representing 92 % of the treated human casein were found in the following proportions (calculated as % recovered substances):

Fraction (a): precipitated by calcium 49.7 %;
Fraction (b): soluble in sodium sulphate 4.4 %;
Fraction (c): soluble in ethanol 36.3 %;
Fraction (d): precipitated by ethanol 9.5 %.

Fractions (a) and (b) do not release any NPN when digested by rennin; fraction (c) releases a small quantity. However, fraction (d) is strongly attacked by rennin; the amount of NPN is 9.4 %, the solution becomes turbid but floculation does not occur. Electrophoresis revealed that all these fractions are heterogeneous and, in particular, the rennin-sensitive component (d) gives rise to several bands.

In the case of bovine casein, a simplified method with sulphuric acid and urea can be used to obtain the κ-rich fraction[8]. With human casein, the precipitations occurred in the ordinary way. We separated fraction (a) by precipitation with sulphuric acid at pH 1.4, from the soluble fraction (b). The former was not digested by rennin; the latter was strongly attacked with a release of 7.3 % of the total nitrogen as NPN, but the electrophoretic pattern remained heterogeneous. An attempt to purify (b) by ethanol treatment gave a solution which was insoluble in water at pH 6.7 and in the electrophoretic buffer solutions.

Fractionation of human casein by column chromatography on DEAE-cellulose in the presence of urea

As fractionation on DEAE-cellulose with an imidazole–HCl buffer, pH 7.0, containing 4.5 *M* urea and a NaCl gradient gave good results with bovine casein, this method was applied to human casein. Three attempts were made, each with some modification, in order to improve the separation. Fig. 2A and Table II summarize

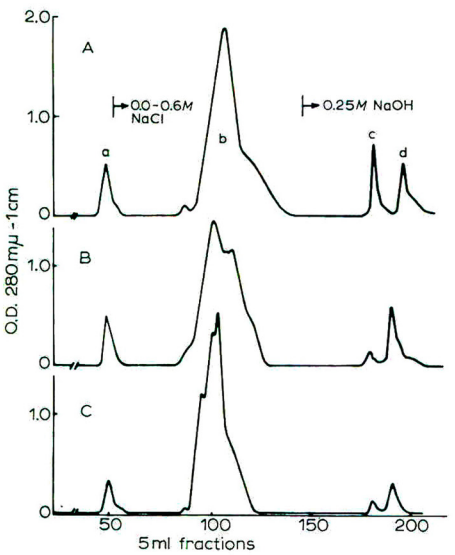

Fig. 2. Chromatograms obtained on DEAE-cellulose (50 × 3 cm column) of: (A) = 1 g human casein; (B, C) chromatograms of peak (b); 0.02 *M* imidazole–3.3 *M* urea buffer, pH 7.

TABLE II

FRACTIONATION OF HUMAN CASEIN ON DEAE-CELLULOSE COLUMNS IN THE PRESENCE OF UREA[a]

Fraction	*Chromatogram A (1 g human casein)*		*Chromatogram B (0.73 g fraction b of A)*	*Chromatogram C (0.60 g fraction b of B)*		*Chromatogram D (0.15 g fractions c and d of A + B + C)*	
	Yield	*NPN*	*Yield*	*Yield*	*NPN*	*Yield*	*NPN*
a	8		6	4		0	
b	81	(1) 0.5 (2) 1.5	87	92	(1) 1.5 (2) 6.0 (3) 10.2	85	1.5
c	6	7.3	1	0.7		9	15.6
d	5	0	6	3.3		6	0.4
Total yield	92		95	90		70	

[a] The yields of the fractions are expressed as % of total dry substances eluted from the column (after dialysis). NPN = Non-protein nitrogen liberated by rennin (0.7 μg/ml) at pH 6.7 expressed as % of total nitrogen. Fractions a are insoluble at pH 6.7; fractions b were subdivided into 2 or 3 parts according to their pattern (Fig. 2).

the results of the third experiment. A minor fraction (a) was eluted with the void volume; it contained a substance insoluble at pH 7.0 in the absence of urea. Over the course of the NaCl gradient, a very small peak appeared first (less than 1 % of the eluted substances) followed by a wide asymmetrical peak which could be divided into 2 parts; the first part which was the more abundant, was hardly digested by rennin, while from the second part more NPN could be obtained than from whole human casein. About one-tenth of the casein was not eluted by the NaCl gradient. NaOH permitted the recovery of two well-separated fractions; the first (c) was still neutral and strongly rennin-sensitive, the solution becoming opalescent; the second (d) was alkaline and not attacked by rennin at pH 6.7.

The substances corresponding to the large peak eluted in the gradient (b) were chromatographed twice more (see Figs. 2B and 2C and Table II). Any further satisfactory fractionation was unobtainable and we did not try higher urea concentrations in case they caused irreversible denaturation.

Fig. 3 shows the starch gel electrophoretic patterns of the substances eluted in

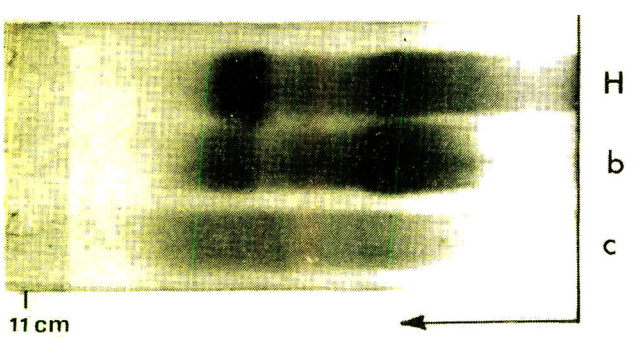

Fig. 3. Starch gel electrophoresis in the presence of mercaptoethanol, of human casein (H) and of the fractions obtained during chromatography on DEAE-cellulose reported in Fig. 2 (b and c); for conditions, see Fig. 1.

the chromatogram in Fig. 2B. The fraction eluted by NaOH, which is not completely soluble in water at pH 6.7, seems to have been partly denatured.

Table II shows that the three parts observed in the large peak of the chromatogram (C) are attacked by rennin in different ways. The third part (tubes No. 106–120) contains a protein fraction which is highly rennin-sensitive (10.2 % of NPN released) and which becomes milky; it represents about 6 % of the original human casein.

A fourth chromatogram (D) was performed with a mixture of fractions (c) and (d) eluted by NaOH. Surprisingly most of this substance was eluted by the NaCl gradient; it was not easily attacked by rennin. Only one-tenth of the substance was eluted by NaOH and was neutral; this fraction (D-c) was highly rennin-sensitive as shown in Table II.

In conclusion, we were able to separate two human κ-like fractions with different chromatographic behaviour.

Fractionation of human casein by column chromatography on DEAE-cellulose in the presence of dimethylformamide

Dimethylformamide has a dissociating effect on protein complexes; it has already been used in the fractionation of bovine casein with stepwise elution by NaCl[10]. The method was applied to human casein; Figs. 4 and 5 and Table III present the results of a chromatogram. In spite of a final elution by NaOH, only 77 % of the substances put on the column were recovered. Table III also shows that important differences in the absorbancy of each component at 280 mμ must exist.

Components with a low electrophoretic mobility were separated in fraction (a) which was not attacked by rennin, while fractions (c) and (d) eluted by 0.15 and 0.25 *M* NaCl were rennin-sensitive. It was shown that they mainly contained the most mobile components electrophoretically. However, it was the alkaline fraction (g) which gave the highest release of NPN with rennin; this human casein fraction did not appear to contain any fast moving components, but only slow moving components in a blurred zone. As in previous experiments, rennin-sensitive fractions were found in 2 different zones of the chromatogram; and furthermore, their composition as determined by electrophoresis was different.

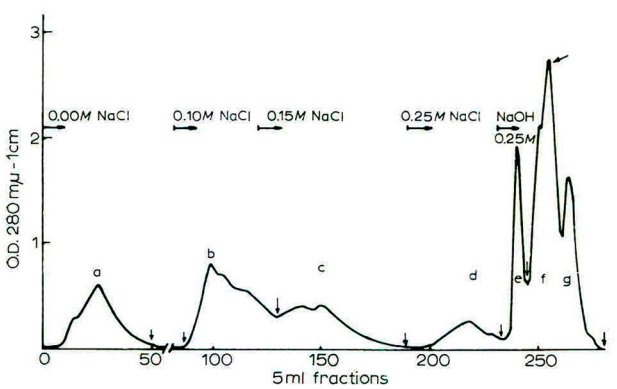

Fig. 4. Chromatography on DEAE-cellulose (36 × 2 cm column) of 1 g human casein; 0.02 *M* phosphate–0.2 *M* boric acid buffer, pH 7, with 20 % dimethylformamide.

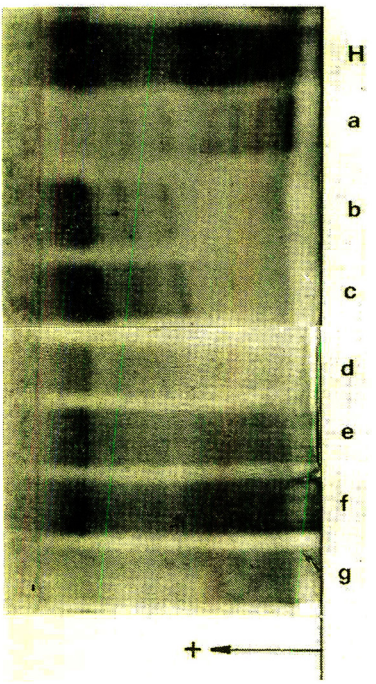

Fig. 5. Starch gel electrophoresis in the presence of mercaptoethanol, of human casein (H) and of fractions obtained during chromatography on DEAE-cellulose reported on in Fig. 4 (a–g); for conditions see Fig. 1.

TABLE III

FRACTIONATION OF HUMAN CASEIN ON DEAE-CELLULOSE COLUMNS IN THE PRESENCE OF DIMETHYLFORMAMIDE

Fractions	*Dry substance*[a] *(after dialysis)*	*% of eluted substances*	*% of total optical density*	*Action of rennin*[b]
a	165	21.5	12	0
b	167	21.7	19	0.9
c	124	16.0	13	4.1
d	73	9.5	6	4.7
e	49	6.4	8	0.3
f	121	15.7	26	1.3
g	71	9.2	16	7.2
Total	770 mg	100	100	

[a] From 1 g human casein.
[b] Non-protein nitrogen liberated by rennin at pH 6.7 expressed as % of total nitrogen.

DISCUSSION

The digestion by rennin and the alteration of the electrophoretic pattern in the presence of mercaptoethanol[1] strongly suggest the existence of a κ-like component

in human casein; its purification, however, seems to be difficult. We cannot at the present time give a precise definition of human κ-casein, but we were able to isolate some fractions which are certainly very rich in a κ-component as they gave rise, after rennin digestion, to an insoluble peptidic 'paracasein' moiety[13] and to a NPN release which is similar or higher than the NPN release from purified bovine and sheep κ-caseins[14] under the same conditions of digestion: human κ-fractions: 7.3 to 15.6 % of total N; bovine κ-casein: 8.5 % of total N; sheep κ-casein: 13.8 % of total N.

As we separated several highly rennin-sensitive human casein fractions, the question arises as to whether there really are different human κ-caseins or only genetic variants. During the fractionation of bovine casein on DEAE-cellulose, κ-casein was eluted by a neutral buffer and it was suggested that the rennin-sensitive alkaline fraction would be a denatured form of bovine κ-casein[9]. In the case of human casein, the situation is not identical; two peaks were eluted by NaOH but the first one was still neutral and it gave rise to a high NPN release.

The hypothesis of the existence of several human κ-caseins could explain the differences among the two κ-fractions previously described by MALPRESS AND SEID-AKHAVAN[5] and by NAGASAWA *et al.*[6]. Although no NPN data were indicated by these authors, it is possible to compare their fractions with those which we have separated by chromatography.

Other fractionation experiments concerning human κ-casein are in progress.

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CHROM. 4322

FRACTIONATION OF OLIGONUCLEOTIDE ISOPLITHS BY ELECTROPHORESIS ON POLYACRYLAMIDE GELS

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SUMMARY

A new procedure has been described for the analysis of an enzymatic digest of [^{32}P]RNA. It has been shown that oligonucleotides of equal chain length (isopliths) may be subfractionated into components of different composition by electrophoresis on polyacrylamide gels. Separation appears to be due primarily to differences in net charge. The distribution of components within the gel was determined by slicing the gel and counting ^{32}P by Cerenkov radiation in a scintillation counter. Since the method is simple and rapid it lends itself to the analysis of many samples and may be useful as a 'fingerprinting' procedure for RNA.

INTRODUCTION

We have recently described a method for comparing RNA molecules in which selected oligonucleotides derived from them were analyzed by column chromatography[1]. Although a limited number of compositional isomers* were separated by the technique employed at that time, the use of appropriate double isotope labels permitted the demonstration of significant differences between 18S and 28S ribosomal RNA molecules. This has established the usefulness of the experimental approach. We have now investigated a different method for separating compositional isomers which, because of its speed and increased resolution, may extend the number of RNA species which can be compared in this manner.

In the present study we have shown that oligonucleotides of equal size (isopliths) may be subfractionated into their compositional isomers by electrophoresis on polyacrylamide gels in acidic buffers. Other workers have shown that these gels are capable of separating high molecular weight RNAs[2–4], large RNA fragments[5] and oligonucleotides[6,7] on the basis of molecular size. Under the conditions which we have employed, the separation of compositional isomers appears to be due to differences in

* For purposes of this discussion, the term 'compositional isomer' is used to denote a class of oligonucleotides whose members have identical nucleotide compositions, but not necessarily identical sequences. No other generally accepted term is in use at the present time.

their net charge. Since the running time is short (2–3 h) and many samples can be analyzed concurrently, the procedure should be particularly useful for comparing RNA molecules by their oligonucleotide 'fingerprints'.

Techniques for separating oligonucleotides have been the subject of a recent review by RUSHIZKY AND SOBER[8].

MATERIALS AND METHODS

Preparation of ^{32}P-labeled ribosomal RNA

Ribosomal RNA was prepared from Ehrlich ascites tumor cells, grown in the peritoneal cavity of mice[9] for eleven days. Each of two animals was injected with 1.5 mCi of [^{32}P]H_3PO_4 in 0.5 ml of spinner culture medium (Grand Island Biological, neutralized with NaOH). After a period of 40 h the animals were sacrificed and the tumor cells collected. The cells were washed in cold saline and cytoplasmic extract was prepared by a method similar to that described for HeLa cells[10]. For 10 min prior to their disruption, the cells were allowed to swell in a hypotonic medium containing 0.01 *M* Tris-HCl, 0.01 *M* NaCl, 0.003 *M* $MgCl_2$, pH 7.4. The cells were disrupted with a stainless steel Dounce homogenizer, and nuclei removed by centrifugation. Ribosomal RNA was purified from the cytoplasmic extract by a hot phenol-detergent method[11]. Transfer RNA and any contaminating DNA was removed by precipitating the high molecular weight RNA with 2.5 *M* LiCl[12]. The precipitate was washed twice with 1 *M* LiCl. The initial specific activity of the purified RNA preparation was 6×10^6 d.p.m./mg.

Enzymatic hydrolysis of RNA

Seven milligrams of ^{32}P-labeled ribosomal RNA and 200 units of ribonuclease T1 (Sankyo, obtained from Calbiochem, Los Angeles) in 2 ml of 0.05 *M* Tris-HCl, pH 7.4, were incubated at 37° for 3 h. HCl was added (final concentration, 0.1 *N*) to hydrolyze cyclic phosphodiester bonds. After 30 min, the incubation mixture was neutralized with NaOH, and further maintained at 37° for 16 h. The digestion was terminated by the addition of three volumes of 10 *M* urea.

Chromatography of enzymatic digest of RNA at neutral pH

The products of digestion were separated into isopliths (fragments of equal chain length) by chromatography on DEAE-Sephadex at neutral pH. The conditions of chromatography and preparation of the ion exchanger have been described previously[1]; further details are given in the legend to Fig. 1.

Concentration and desalting of oligonucleotides

Appropriate fractions from the neutral pH chromatogram, corresponding to isopliths of the desired chain length, were pooled and the oligonucleotide material was concentrated as follows. Zinc chloride (1 ml of a 0.5 *M* solution) was added to 100 ml of pooled eluate. The pH of the solution was adjusted to 8–9 by the addition of NaOH. The solution was allowed to stand with occasional stirring for 20 min, during which time a zinc hydroxide precipitate formed. The suspension, combined with a water rinse of the container, was centrifuged at 1500 × *g* for 2 min. The pellet was suspended in 15 ml of water and centrifuged in a 15 ml graduated conical tube.

At this stage, the oligonucleotides were tightly bound to the zinc hydroxide gel. Nucleotides were released and simultaneously the zinc was removed in the following way. The gel was well dispersed in 2.5 ml water; 0.95 ml of oxalic acid (0.5 *M*) was quickly added, and the tube immediately mixed vigorously. The addition of the acid solubilized the gel, and after a few seconds delay, zinc oxalate precipitated. After 15 min, the zinc oxalate was removed by centrifugation (2000 × *g* for 5 min) leaving the oligonucleotides and approximately 10 m*M* oxalic acid (pH 2) in the supernatant fluid. Although satisfactory for many applications, the ionic strength was still too high for electrophoresis on polyacrylamide gels.

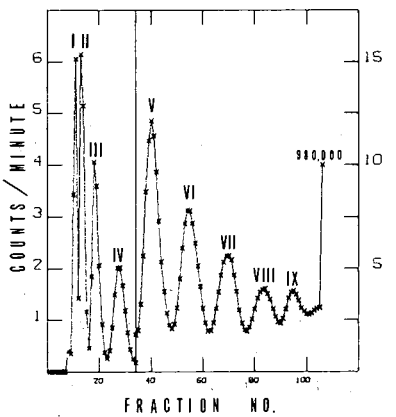

Fig. 1. Chromatography at neutral pH of a ribonuclease T_1 digest of ^{32}P-labeled ribosomal RNA. The digest was applied to a DEAE-Sephadex A-25 column (0.9 × 20 cm) and eluted with a linear gradient of NaCl (0.17–0.28 *M*) in 7 *M* urea–0.02 *M* Tris-HCl (pH 7.4) at 0.5 ml/min. Total volume of gradient was 1 l. Left ordinate: counts per min × 10^{-5}; right ordinate: (× 10^{-4}) refers to fractions to the right of the vertical line. The final fraction combines counts eluted from the column by 0.5 *M* NaCl.

The remaining oxalic acid was removed by dialysis. The solution was transferred to Visking dialysis tubing (8/32) and dialysed for a total of 3 h against three changes of water (2 l each). Dialysis tubing was prepared by soaking it in water for 5 min and rinsing it immediately before use. At the completion of dialysis, the pH of the sample was 4–5. The sample was dried in an Evapo-mix (Buchler Instruments, Fort Lee, N.J.) at a temperature below 40°. The nucleotides were dissolved in 50–100 μl of 5 *M* urea solution.

Electrophoresis of oligonucleotides on polyacrylamide gels

Polyacrylamide gels were prepared using the following reagents: acrylamide (recrystallized from chloroform as described by LOENING[2]); N,N'-methylenebisacrylamide; N,N,N',N'-tetramethylethylenediamine (TEMED); these were obtained from Eastman Organic Chemicals. Stock solutions contained: (a) acrylamide, 12.5%, bisacrylamide, 0.125% (both w/v); (b) TEMED-acetate, 10% (TEMED adjusted to pH 7 with acetic acid); (c) ammonium persulfate, 10% (w/v), freshly prepared. For 40 ml of 8% acrylamide gel mixture, 25.6 ml of (a), 0.2 ml of (b), and 14.0 ml of water were combined. The mixture was degassed for several seconds under vacuum,

then 0.2 ml of (c) was added and the preparation gently mixed. Acrylic plastic tubes (0.625 cm I.D. by 18 cm) were filled to a height of 15 cm. After 1 h, polymerization was complete and the gels were electrophoretically equilibrated with the required buffer (see RESULTS) for 16 h at 250 V.

For electrophoresis, 20–50 μl of oligonucleotide solution was layered above a vertical gel. Upper and lower reservoirs were filled with buffer and the whole apparatus was air-cooled with a 10 in. fan in a 4° room. At an applied voltage of 400 V, the current flow was 2–6 mA/gel, depending upon the particular buffer used. Other details are given in the legends to the figures.

Following electrophoresis, gels were fractionated into 1.4 mm thick disks using a semi-automatic slicer[13]. Removal of the gel from the plastic tube was facilitated by using a stream of water from a fine steel tube connected to a syringe. The gel was transferred to a trough filled with 50% glycerol for lubrication, then to the slicer. Disk-shaped slices were collected in plastic scintillation vials. The slices were immersed in 1 ml of 0.02 *M* NH_4OH, and the vials counted directly in a Beckman LS-250 scintillation counter. Efficiency of counting ^{32}P by Cerenkov radiation[14] was about 45% under these conditions.

Composition of oligonucleotides

The composition of oligonucleotides separated by gel electrophoresis was determined after eluting them from the gel slices. Eighty to 90% of the counts were recovered in the NH_4OH which had been added to the scintillation vials. Appropriate eluates were pooled and 0.1 ml of zinc chloride (0.5 *M*) was added per 10 ml. The zinc hydroxide precipitate which formed was allowed to stand for 20–30 min, then collected by centrifugation. The zinc was removed with oxalic acid as described above. The supernatant fluid was made 0.3 *N* with respect to KOH, and oligonucleotides were hydrolyzed for 18 h at 37°. The solution was diluted to 5 ml with water and acidified by the addition of 0.2 ml of 3 *M* formic acid. It was slowly filtered through a bed of activated charcoal (0.2 ml of 30%, supported on a 1 in. Whatman GF/A glass fiber filter). The charcoal was washed with water and the nucleotides eluted with 5 ml of ethanol–1 *M* NH_4OH (1:1). The ethanol–ammonia was removed under vacuum on a Buchler Evapo-mix, following which the mononucleotides were dissolved in 100 μl of ethanol–ammonia.

Mononucleotides were separated by high voltage paper electrophoresis on a flat-plate apparatus (Savant Instruments, Hicksville, N.Y.). The samples, to which were added 100 μg of each of the four ribonucleotides, were applied to Whatman No. 3 MM paper as 2 cm streaks. The buffer system was 0.1 *M* sodium citrate, pH 3.9 (measured at 23°). Voltage gradients of 40–50 V/cm were applied for 2–2.5 h. Under these conditions, the nucleotides were well separated. Using UV absorbance as a guide, the nucleotide spots were cut out. The paper was cut into 3 mm squares and counted in glass vials containing 15 ml of scintillator (Liquifluor, 160 ml to 3.8 l of toluene, Pilot Chemicals).

Other materials

Urea solutions used for column chromatography were purified to remove an insoluble residue which otherwise contaminated the desalted oligonucleotide solutions. To 1 l of a 10 *M* solution of urea (J. T. Baker Chemicals), 10 ml of 0.5 *M* zinc chloride,

2 ml of 5 *M* NaOH and 2 ml of Norit (30%) were added. Zinc hydroxide precipitated and the suspension was permitted to stand overnight. The urea solution was filtered through a Whatman GF/A glass fiber filter, then through a Millipore membrane (0.45 μ pore size). The final product had an absorbance of 0.01 units at 260 nm and 0.10 units at 230 nm; a 7 *M* solution did not interfere with the precipitation of oligonucleotides by zinc hydroxide (Table I), and little insoluble material accompanied the concentrated oligonucleotides.

Graphs were plotted by a Calcomp Incremental Plotter with a CDC G-20/3100 computer system.

RESULTS

Concentration and desalting of oligonucleotides

Oligonucleotides eluted from a DEAE-Sephadex column were concentrated and desalted as described in METHODS prior to analysis by gel electrophoresis. The recovery at each stage of the purification scheme is shown in Table I, expressed as percent recovered at each step. The overall recovery is the product of the individual recoveries. In two separate experiments the overall recovery ranged from 66–90%. A critical step in the procedure is the addition of oxalic acid. The zinc hydroxide gel must be well dispersed in water in order to obtain its complete dissolution before the zinc oxalate precipitates. Losses associated with dialysis seemed to be due predominately to handling and not through the membrane itself.

TABLE I

RECOVERY OF OLIGONUCLEOTIDES DURING DESALTING (%)

Procedure	*Oligonucleotide size*			
	Tetra	*Penta*	*Hexa*	*Hepta*
Pooled sample	100	100	100	100
Zinc hydroxide precipitation	99	99	98	99
Zinc oxalate precipitation	87	81	88	89
Dialysis	85	82	85	88
Overall recovery	73	66	73	78
Overall recovery (in a separate experiment)	90	80	87	

Stability of oligonucleotides

Two experiments were carried out to assess the stability of oligonucleotide isopliths during their purification and analysis. The results are shown in Fig. 2. In one experiment, tetranucleotides were concentrated as described above and, after dialysis, they were mixed with cold marker oligonucleotides and re-chromatographed on DEAE-Sephadex. The results shown in the lower panel of Fig. 2 indicate that almost all the material co-chromatographed with marker tetranucleotides. In another experiment, pentanucleotides were eluted from a polyacrylamide gel after electrophoresis under conditions similar to Fig. 4A, then re-chromatographed with marker oligonucleotides. As shown in Fig. 2 (upper panel), approximately 95% of the nucleotide material chromatographed with pentanucleotides.

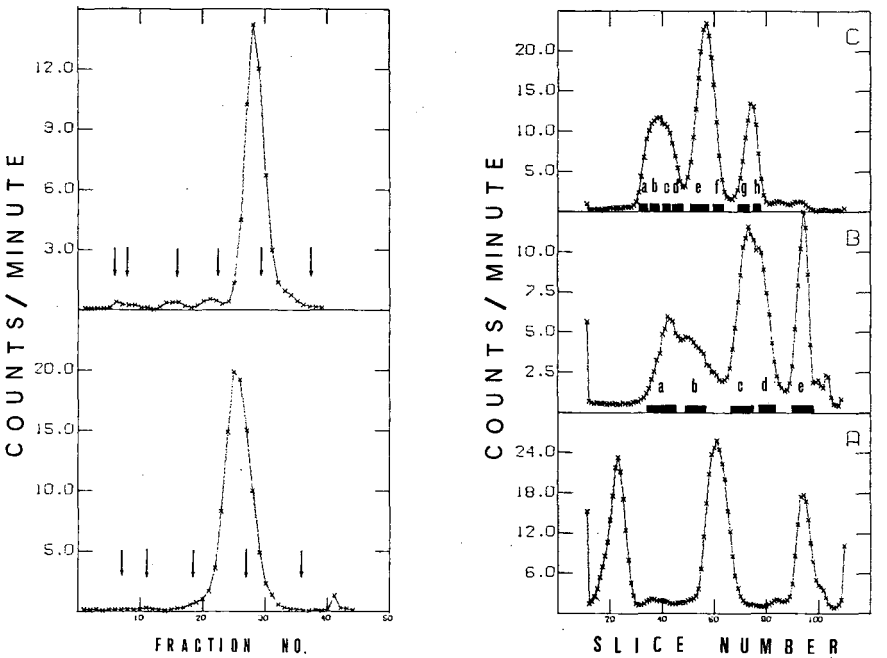

Fig. 2. Re-chromatography of recovered tetra- and pentanucleotides. Lower panel: tetranucleotides from a ribonuclease T_1 digest of ^{32}P-labeled rRNA were separated by chromatography as in Fig. 1, then concentrated and desalted as described in METHODS. Following the dialysis step, the nucleotides were combined with an unlabeled mixture of oligonucleotides (prepared by limited alkaline hydrolysis of rRNA) and re-chromatographed on DEAE-Sephadex. Upper panel: a similar experiment in which pentanucleotides were electrophoresed on polyacrylamide gels as described in METHODS, eluted from the sliced gel with 0.1 *M* formic acid, and chromatographed as above. In both figures, the arrows indicate the positions of the marker oligonucleotide peaks. Ordinate: counts per min $\times 10^{-2}$.

Fig. 3. Electrophoresis of tetranucleotides on polyacrylamide gels. Tetranucleotides (peak IV of Fig. 1) were concentrated and desalted as described in METHODS. One hundred micrograms of oligonucleotide, dissolved in 40–50 μl of 5 *M* urea, were layered over each of three polyacrylamide gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 120 min, 0.1 *M* formic acid; (B) 100 min, 0.05 *M* Na citrate, pH 3.2; (C) gel was modified to contain 4% acrylamide, 0.04% bisacrylamide, 6.7 *M* urea, and 0.15 *M* formic acid to give a final pH of 3.3; the lower buffer reservoir contained urea and formic acid, the upper only formic acid; running time was 110 min. In this and subsequent figures, the applied voltages were 400 V; current flows were 2–6 mA/gel; anode is on the right; following electrophoresis, the gel was sectioned into disk-shaped slices, starting at the top of the gel; where gels are plotted as starting at slice No. 11, this indicates that slices 1–11 have been counted together; similarly, any gel slices beyond No. 110 have been pooled; horizontal bars indicate fractions which have been pooled for nucleotide composition analysis (see Table II); all ordinates, counts per min $\times 10^{-2}$.

Electrophoresis of oligonucleotide isopliths

The results of a series of experiments designed to test the use of polyacrylamide gels for electrophoresis of oligonucleotides are shown in Figs. 3–8, and the composition of selected peaks is listed in Table II. Tetra- to nonanucleotide peaks from the chromatogram shown in Fig. 1 were selected for analysis on gels. Several acidic buffer systems were compared, ranging from 0.1 *M* formic acid (pH 2.3 at 23°) to Na citrate, pH 3.4. The effect of chain length on mobility may be estimated by comparing Figs.

3A, 4A, 6A, 7A, 8A and 8B. These are tetra- to nonanucleotides, respectively, which have been electrophoresed in 0.1 *M* formic acid, 8% acrylamide gels. Fairly small differences in overall mobilities were observed. For example, if we compare components with one uridylate residue, we see that these are recovered at approximately slice No. 60. The mobilities may therefore be estimated from their running times, which range from 120 min for tetranucleotides to 155 min for heptanucleotides. This suggests that chain length plays a relatively minor role in separations under these conditions.

The most striking feature of these electropherograms is the differences in mobilities within isopliths, in some cases greater than five-fold. These differences are most distinct in gels run in 0.1 *M* formic acid. From inspection of the nucleotide compositions (Table II), it is clear that the predominant factor governing mobility is the number of uridylate residues per chain. Adenylate and cytidylate residues contribute

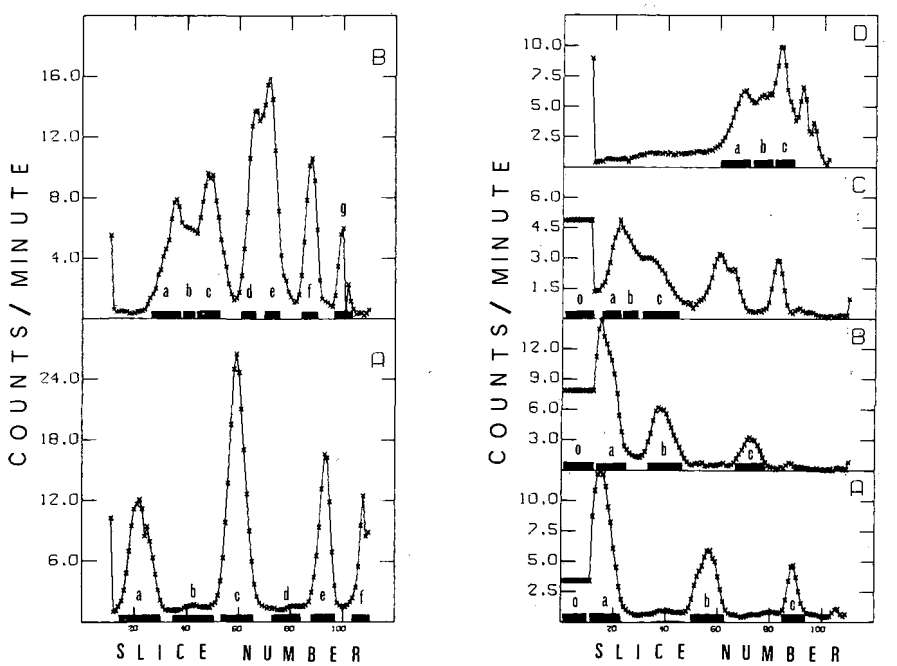

Fig. 4. Electrophoresis of pentanucleotides. Approximately 80 μg of desalted pentanucleotides (from peak V of Fig. 1) were layered over polyacrylamide gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 130 min, 0.1 *M* formic acid; (B) 105 min, 0.05 *M* Na citrate, pH 3.2. See also the legend to Fig. 3.

Fig. 5. Electrophoresis of dephosphorylated pentanucleotides. The 3′-terminal phosphates of desalted pentanucleotides (peak V of Fig. 1) were removed with alkaline phosphatase. Two hundred micrograms of pentanucleotides were incubated with 5 μg of Worthington phosphatase in 50 μl of 0.2 *M* Tris, pH 7.4. The sample was incubated at 37° for 3.5 h, following which it was dialysed against 2 l of water for 1 h. The nucleotide solution was concentrated under vacuum and dissolved in 100 μl of 5 *M* urea. Twenty-five micrograms of the dephosphorylated pentanucleotides were layered over each of four polyacrylamide gels and subjected to electrophoresis. Buffers and running times used were as follows: (A) 0.1 *M* formic acid, 130 min; (B) 0.05 *M* Na citrate, pH 2.9, 105 min; (C) 0.05 *M* Na citrate, pH 3.2, 105 min; (D) 0.05 *M* Na citrate, pH 3.4, 120 min. See also the legend to Fig. 3.

TABLE II

NUCLEOTIDE COMPOSITIONS OF OLIGONUCLEOTIDES

The two sets of numbers refer to duplicate nucleotide analyses performed on each oligonucleotide peak.

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(i) Tetranucleotides, pH 3.2, Fig. 3B					
(a)	0.32	0.48	0.15	0.05	GC_2A
	0.24	0.60	0.12	0.04	
(b)	0.27	0.32	0.34	0.06	GC_2A; GCA_2
	0.23	0.33	0.38	0.06	
(c)	0.26	0.38	0.12	0.24	GC_2U; GCAU
	0.26	0.39	0.10	0.26	
(d)	0.25	0.16	0.33	0.26	GCAU; GA_2U
	0.24	0.15	0.35	0.25	
(e)	0.25	0.16	0.10	0.48	GCU_2; GAU_2
	0.23	0.18	0.10	0.48	
(ii) Tetranucleotides, urea, Fig. 3C					
(a)	0.22	0.58	0.13	0.07	GC_3; GC_2A
	0.22	0.63	0.11	0.04	
(b)	0.22	0.50	0.22	0.06	GC_2A
	0.23	0.53	0.21	0.03	
(c)	0.23	0.30	0.41	0.05	GCA_2
	0.23	0.31	0.41	0.05	
(d)	0.23	0.20	0.48	0.09	GCA_2
	0.24	0.17	0.47	0.12	
(e)	0.23	0.39	0.11	0.27	GC_2U; GCAU
	0.24	0.36	0.16	0.24	
(f)	0.24	0.19	0.29	0.28	GCAU
	0.25	0.20	0.31	0.24	
(g)	0.24	0.17	0.10	0.49	GCAU; GCU_2 (?)
	0.26	0.21	0.17	0.36	
(h)	0.26	0.13	0.18	0.43	GCAU; GAU_2 (?)
	0.27	0.12	0.22	0.39	
(iii) Pentanucleotides, 0.1 M formic acid, Fig. 4A					
(a)	0.20	0.49	0.29	0.02	GC_3A; GC_2A_2
	0.23	0.47	0.28	0.03	
(b)	0.21	0.41	0.27	0.11	GC_2A_2; GC_2AU
(c)	0.19	0.39	0.22	0.21	GC_2AU
	0.19	0.39	0.21	0.21	
(d)	0.21	0.25	0.18	0.36	$GCAU_2$
(e)	0.19	0.24	0.15	0.42	$GCAU_2$
	0.19	0.25	0.15	0.40	
(f)	0.18	0.12	0.10	0.60	GCU_3; GAU_3
	0.21	0.13	0.11	0.55	
(iv) Pentanucleotides, pH 3.2, Fig. 4B					
(a)	0.20	0.65	0.12	0.04	GC_3A
	0.20	0.66	0.12	0.02	
(b)	0.20	0.41	0.36	0.03	GC_2A_2
	0.19	0.46	0.32	0.03	
(c)	0.22	0.26	0.48	0.05	GCA_3; GC_2A_2
	0.22	0.28	0.47	0.03	
(d)	0.21	0.46	0.11	0.22	GC_2AU; GC_3U
	0.21	0.46	0.11	0.22	
(e)	0.18	0.25	0.36	0.21	GCA_2U
	0.20	0.25	0.35	0.20	
(f)	0.19	0.24	0.16	0.40	$GCAU_2$
	0.19	0.25	0.19	0.37	
(g)	0.19	0.12	0.12	0.56	GCU_3; GAU_3
	0.19	0.13	0.14	0.55	

(*continued on next page*)

TABLE II (*continued*)

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(v) Dephosphorylated pentanucleotides, 0.1 M formic acid, Fig. 5A					
(o)	0.05	0.51	0.20	0.24	GC_2AU
	0.04	0.57	0.19	0.20	
(a)	0.04	0.43	0.31	0.22	GC_2AU
	0.02	0.43	0.31	0.24	
(b)	0.03	0.33	0.18	0.45	$GCAU_2$
	0.02	0.34	0.18	0.46	
(c)	0.02	0.16	0.14	0.68	GCU_3; GAU_3
	0.04	0.18	0.15	0.63	
(vi) Dephosphorylated pentanucleotides, pH 2.9, Fig. 5B					
(o)	0.08	0.43	0.21	0.28	GC_2AU
(a)	0.04	0.40	0.32	0.23	GC_2AU
	0.03	0.41	0.30	0.25	
(b)	0.06	0.27	0.25	0.42	$GCAU_2$
	0.00	0.34	0.18	0.47	
(c)	0.08	0.14	0.18	0.61	(?)
	0.04	0.14	0.16	0.67	
(vii) Dephosphorylated pentanucleotides, pH 3.2, Fig. 5C					
(o)	0.07	0.38	0.48	0.07	GC_2A_2
	0.08	0.46	0.37	0.09	
(a)	0.05	0.56	0.18	0.21	GC_2AU
	0.10	0.44	0.24	0.22	
(b)	0.04	0.49	0.24	0.24	GC_2AU
	0.06	0.47	0.23	0.25	
(c)	0.05	0.30	0.39	0.27	GCA_2U
	0.07	0.27	0.39	0.26	
(viii) Dephosphorylated pentanucleotides, pH 3.4, Fig. 5D					
(a)	0.01	0.62	0.16	0.21	GC_2AU
	0.01	0.48	0.24	0.27	
(b)	0.01	0.44	0.30	0.26	GC_2AU
	0.02	0.35	0.34	0.29	
(c)	0.04	0.31	0.28	0.37	$GCAU_2$
	0.03	0.26	0.31	0.40	
(ix) Hexanucleotides, 0.1 M formic acid, Fig. 6A					
(a)	0.15	0.68	0.14	0.04	GC_4A
	0.17	0.62	0.17	0.04	
(b)	0.19	0.40	0.38	0.03	GC_3A_2; GC_2A_3
	0.16	0.37	0.42	0.04	
(c)	0.15	0.57	0.11	0.18	GC_3AU
	0.19	0.53	0.12	0.17	
(d)	0.17	0.30	0.34	0.19	GC_2A_2U
	0.18	0.29	0.35	0.18	
(e)	0.20	0.30	0.20	0.31	GC_2AU_2
	0.20	0.27	0.20	0.33	
(f)	0.16	0.17	0.15	0.52	$GCAU_3$
	0.18	0.18	0.15	0.50	
(x) Hexanucleotides, 0.1 M acetic acid, Fig. 6B					
(a)	0.16	0.56	0.24	0.04	GC_3A_2; GC_4A
	0.16	0.56	0.24	0.04	
(b)	0.18	0.40	0.25	0.17	GC_2A_2U; GC_3AU
	0.16	0.36	0.33	0.15	
(c)	0.19	0.28	0.20	0.33	GC_2AU_2
	0.19	0.26	0.21	0.33	
(d)	0.20	0.18	0.26	0.37	GCA_2U_2
	0.20	0.17	0.26	0.37	
(e)	0.15	0.15	0.10	0.59	GCU_4; $GCAU_3$

(*continued on next page*)

TABLE II (*continued*)

Peak	*Distribution of* ^{32}P				*Composition*[a]
	G	*C*	*A*	*U*	
(xi) Hexanucleotides, 0.2 M acetic acid, Fig. 6C					
(a)	0.21	0.68	0.07	0.04	(?)
(b)	0.18	0.37	0.37	0.09	(?)
(c)	0.17	0.57	0.08	0.18	GC_3AU; GC_4U
	0.17	0.55	0.10	0.18	
(d)	0.18	0.27	0.37	0.18	GC_2A_2U
	0.18	0.27	0.35	0.20	
(f)	0.19	0.26	0.21	0.34	GC_2AU_2
	0.20	0.26	0.22	0.32	
(g)	0.19	0.17	0.17	0.48	$GCAU_3$
	0.19	0.18	0.13	0.50	
(xii) Hexanucleotides, pH 3.2, Fig. 6D					
(a)	0.14	0.65	0.16	0.05	GC_4A
(b)	0.14	0.46	0.33	0.07	GC_3A_2
(c)	0.15	0.45	0.21	0.18	GC_3AU
	0.17	0.47	0.19	0.17	
(d)	0.16	0.29	0.34	0.21	GC_2A_2U
	0.18	0.28	0.36	0.18	
(e)	0.16	0.18	0.17	0.48	$GCAU_3$
	0.17	0.18	0.17	0.48	
(xiii) Heptanucleotides, 0.1 M formic acid, Fig. 7A					
(a)	0.09	0.37	0.36	0.17	GC_2A_3U; GC_3A_2U
	0.15	0.37	0.33	0.15	
(b)	0.14	0.17	0.12	0.02	GC_5A
	0.12	0.72	0.12	0.04	
(c)	0.15	0.43	0.39	0.03	GC_3A_3
(d)	0.15	0.58	0.14	0.15	GC_4AU
	0.12	0.57	0.13	0.16	
(e)	0.16	0.27	0.28	0.29	$GC_2A_2U_2$
	0.13	0.30	0.28	0.29	
(f)	0.15	0.23	0.21	0.40	GC_2AU_3; GCA_2U_3
	0.11	0.27	0.21	0.41	
(xiv) Octanucleotides, 0.1 M formic acid, Fig. 8A					
(a)	0.12	0.62	0.22	0.04	GC_5A_2
	0.12	0.62	0.22	0.04	
(b)	0.12	0.46	0.27	0.14	GC_4A_2U
(c)	0.11	0.56	0.16	0.16	GC_5AU
(d)	0.12	0.46	0.27	0.15	GC_4A_2U
	0.13	0.47	0.25	0.15	
(e)	0.10	0.36	0.36	0.18	GC_3A_3U
(f)	0.12	0.35	0.28	0.25	$GC_3A_2U_2$
	0.13	0.35	0.27	0.25	
(g)	0.13	0.22	0.23	0.42	$GC_2A_2U_3$
	0.17	0.23	0.24	0.36	

[a] Composition of an oligonucleotide peak was deduced from the distribution of ^{32}P amongst the four nucleotides, taking chain length and a single guanylate residue per molecule (except the dephosphorylated pentanucleotides) into consideration. In those cases where peaks appear to be a mixture of compositional isomers the two major species are identified. (?) = a complex mixture whose composition cannot be deduced.

proportionately less to mobility, and hence are less well resolved. In order to increase the separation of cytidylate and adenylate-containing oligonucleotides, buffers of higher pH were utilized. This is shown in Fig. 3B for tetranucleotides, which appears to be an improvement over Fig. 3A. Definite increase in resolution of some penta-

nucleotide peaks was obtained in citrate buffer, pH 3.2 (Fig. 4B) as compared to 0.1 *M* formic acid (Fig. 4A). However, amongst hexanucleotides, buffers of higher pH caused crowding together of components (Fig. 6D *vs.* 6A). Unusually sharp peaks were observed with acetic acid buffers (Figs. 6B, 6C and 7B). The explanation for this is not known.

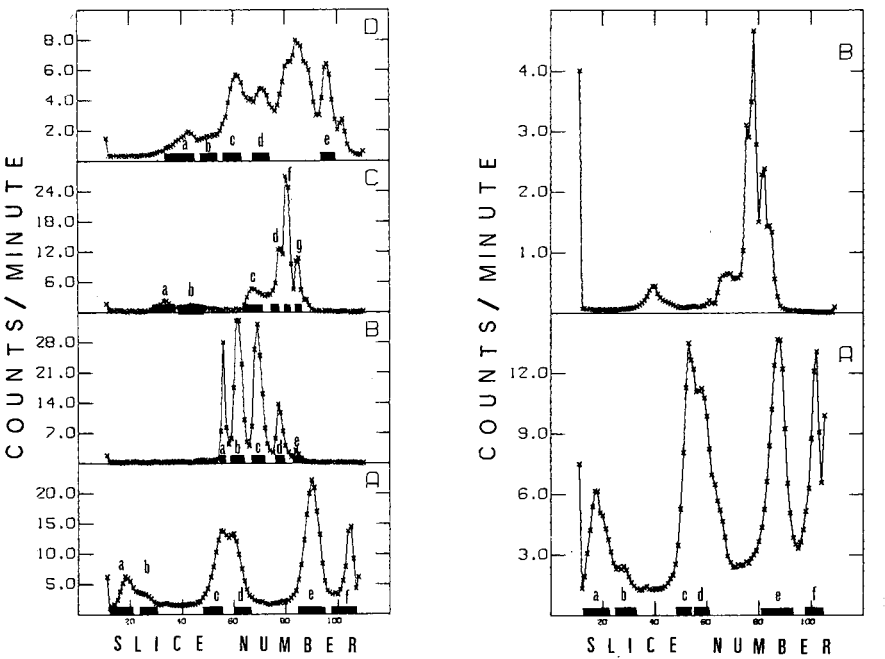

Fig. 6. Electrophoresis of hexanucleotides. Approximately 80 μg of desalted hexanucleotides (peak VI of Fig. 1) were layered over each of four gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 140 min, 0.1 *M* formic acid; (B) 125 min, 0.1 *M* acetic acid; (C) 130 min, 0.2 *M* acetic acid; (D) 110 min, 0.05 *M* Na citrate, pH 3.2. See also the legend to Fig. 3.

Fig. 7. Electrophoresis of heptanucleotides. Approximately 60 μg of desalted heptanucleotides (peak VII of Fig. 1) were layered over each of two gels and subjected to electrophoresis. Running times and buffers used were as follows: (A) 150 min, 0.1 *M* formic acid; (B) 140 min, 0.2 *M* acetic acid. See also the legend to Fig. 3.

The effect on mobility and resolution of removing the 3'-terminal phosphate from pentanucleotides was examined (Fig. 5). The pentanucleotide-tetraphosphates migrated more slowly than the corresponding pentanucleotides, but no marked increase in resolution was detected. Such a statement must be qualified, however, since the nucleotide compositions suggest that the dephosphorylation may not have gone to completion, and some components seem to have been lost at the origin of the gels. In another experiment, the effect of urea in the buffer system was tested. The result, shown in Fig. 3C, is little different than in the absence of urea (Fig. 3B).

DISCUSSION

We have explored the usefulness of polyacrylamide gels as a supporting medium for the electrophoretic separation of oligonucleotides. Although (deoxy)oligonucleotides have been successfully fractionated on such gels[6], the separations were based upon size and utilized the 'sieving' action of the gels. Our method was designed to subfractionate oligonucleotide isopliths into their compositional isomers on the basis of differences in net charge. Initially, a discontinuous buffer system was used to concentrate oligonucleotides for application to the gels, but this was unsatisfactory. This led to the development of the zinc hydroxide–oxalic acid dialysis procedure which has proven to be rapid and reliable.

Amongst the electrophoresis buffer systems tested, 0.1 *M* formic acid has been the simplest and most useful. Many of the compositional isomers were resolved in this system. We have no information about separation on the basis of sequence. The incomplete separation of isomers containing cytidylate from those containing adenylate residues led to an investigation of other buffer systems. Some increase in resolution with higher pH was obtained in the case of pentanucleotides (and probably tetranucleotides) but not with hexanucleotides. The inclusion of high concentrations of urea to suppress non-ionic binding of purine nucleotides to the polyacrylamide gave no detectable increase in resolution. We have adopted the formic acid system for most purposes.

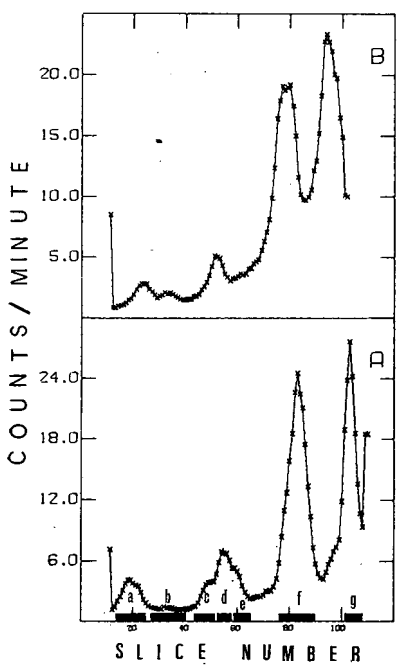

Fig. 8. Electrophoresis of octa- and nonanucleotides. Eighty-five micrograms of octanucleotides and 70 μg of nonanucleotides (peaks VIII and IX Fig. 1, respectively) were electrophoresed on polyacrylamide gels equilibrated with 0.1 *M* formic acid. (A) octanucleotides for 155 min; (B) nonanucleotides for 160 min. See also the legend to Fig. 3.

The present procedure was developed as part of a method for characterizing complex RNA molecules by their oligonucleotide 'fingerprints'[1,15]. Because of the short time required for electrophoresis and the simplicity of fractionating and counting gels, the method seems well suited to this purpose. Although ^{32}P has been used in this study, the ease of elution of labeled oligonucleotides from the gel should permit the use of ^{3}H and ^{14}C for double-label experiments where two RNA species are to be compared directly[1].

These data can be examined to see if they provide any evidence for heterogeneity of the ribosomal RNA molecules. For this analysis, the combined molecular size of 18S + 28S RNA is assumed to be equivalent to 7000 nucleotides. A specified heptanucleotide would therefore be 7/7000 = 1/1000 of the weight of the RNA if it occurred only once per 18S + 28S RNA molecule. If a heptanucleotide peak could be identified that was less than 1/1000 of the weight of the total, unfractionated RNA, this would mean that it occurred less than once per 18S + 28S RNA molecule and would imply heterogeneity in these molecules. When the appropriate corrections for recoveries and isotope decay are applied to peak b of Fig. 7A, the counts in this peak are close to 1/1000 of the total. Thus, this peak probably has a unique sequence, but does not provide evidence for heterogeneity amongst ribosomal RNA molecules. Similar calculations carried out on the minor octa- and nonanucleotide peaks (Figs. 8A and B) lead to the same conclusion. Such evidence, of course, does not rule out the possibility that some heterogeneity exists.

In conclusion, a method has been described for subfractionating oligonucleotides. It is rapid and lends itself to the analysis of several samples simultaneously. Ready recovery of the separated isomers from the gel should permit counting of double-labeled (^{3}H and ^{14}C) samples, and further analysis of the oligonucleotides where necessary.

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CHROM. 4305

IONENAUSTAUSCH BEI HOHEN KONZENTRATIONEN DER ÄUSSEREN LÖSUNG

I. MITT. BEITRÄGE ZUM MECHANISMUS DER SORPTION VON Tl(III) AN DOWEX 50 X8 AUS LÖSUNGEN HOHER KONZENTRATION AN Cl^--, Br^-- UND J^--IONEN

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SUMMARY

Ion exchange at high concentrations of the mobile phase. Part I. The influence of high concentrations of Cl^-, Br^- and I^- ions on the absorption mechanism of Tl(III) on Dowex 50 X8

The absorption of Tl(III) from highly concentrated solutions of various metal halides on Dowex 50 X8 has been examined. The partition coefficients were shown to increase at a concentration of > 2 moles/l MCl, > 0.5 moles/l MBr and > 0.5 moles/l MI. In the presence of 5 M $NaClO_4$, halogen concentrations of $\geqslant 0.1$ moles/l cause a large increase in absorption. These findings and considerations on the invasion of electrolytes show that the increase in K_d should be ascribed explicitly to the absorption of anionic complexes of the type TlX_4 by Dowex 50 X8. Conditions for the absorption are a sufficient concentration of ligands in the resin phase, as well as a high concentration of the mobile phase. Organic solvents cause desorption of the anionic complexes from Dowex 50 X8. The results obtained may indisputably be explained by the assumption that an exchange takes place between the little hydrated TlX_4^- ions and the organic matrix of the resin.

EINLEITUNG

Während die Anwendung von Anionenaustauschern auch bei hohen Konzentrationen der äusseren Lösung in der analytischen Praxis und zum Studium von Lösungsgleichgewichten allgemein verbreitet ist, fand die Untersuchung des Sorptionsverhaltens von Kationenaustauschern bei hohen Konzentrationen erst in den letzten Jahren zunehmendes Interesse. Es wurden bei diesen Untersuchungen einige interes-

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sante Effekte beobachtet, die den bisherigen Anschauungen über den Kationenaustausch widersprechen.

(1) Die Elutionsordnung in der Reihe der Alkali- und Erdalkaliionen kehrt sich bei Konzentrationen des Grundelektrolyten von > 7 Mol/l um[1–4].

(2) Die Verteilungskoeffizienten für viele, vor allem mehrfach geladene Kationen steigen bei Konzentrationen der äusseren Lösung von 3–5 Mol/l erneut an, wobei z.B. für Ionen wie Sc(III) oder Zr(IV) aus 8–9 N $HClO_4$ K_d-Werte von 10^4–10^5 (ml/g) erreicht werden[2,4–8].

(3) Eine Reihe von Elementen wie Au(III), Ga(III), Fe(III) und Tl(III) sowie Jod und Astatin werden aus HCl oder HBr, wo sie stabile Komplexe des Typs MX_4^- bilden, von Kationenaustauschern sorbiert. Ihre Sorption nimmt mit der Konzentration des Grundelektrolyten zu[2,5,9–17]. Unter analogen Bedingungen werden diese Elemente von Anionenaustauschern mit hohen Verteilungskoeffizienten sorbiert.

Eine Theorie, die eine umfassende Deutung dieser Anomalien des Sorptionsverhaltens von Kationenaustauschern im Gleichgewicht mit Lösungen hoher Elektrolytkonzentration erlaubt, konnte aus dem bisher vorliegenden experimentellen Material nicht abgeleitet werden. Besondere Schwierigkeiten bereitet dabei die Interpretation der Aufnahme anionischer Komplexe durch Kationenaustauscher. So steht z.B. der Ansicht, dass Ionen des Typs MX_4^- durch eine Extraktionswirkung der organischen Matrix des Harzes sorbiert werden[10], die Meinung gegenüber, dass die Bildung von Ionenassoziaten mit der funktionellen Gruppe des Harzes vom Typ $(RSO_3H_2^+)$ (MX_4^-) als Ursache der Sorption anzusehen ist[13,14,16,18].

Die vorliegende Arbeit ist deshalb dem Mechanismus der Sorption anionischer Komplexe an Kationenaustauschern gewidmet, da dieser Effekt auf Grund der hohen Selektivität der Sorption neben dem theoretischen auch praktisches Interesse besitzt. Am Beispiel der Sorption von Tl(III) am Harz Dowex 50 X8 untersuchten wir eine Reihe von Faktoren, die in der Literatur bisher weniger beachtet wurden, wie den Einfluss von Kation und Anion des Grundelektrolyten und von organischen Solventien, sowie die Sorption aus gemischten Elektrolytlösungen konstanter Ionenstärke.

EXPERIMENTELLER TEIL

Ionenaustauscher

Dowex 50 X8 (100–200 mesh) mit einer Kapazität von 5.02 mäquiv./g (H-Form) wurde in der üblichen Weise eingefahren, anschliessend mit HCl–Cl_2 behandelt, mit reichlich Wasser gewaschen und an der Luft getrocknet.

Lösungen

Zur Herstellung der Lösungen wurden p.a.-Chemikalien und bidestilliertes Wasser verwendet. Die Gehaltsbestimmung erfolgte argentometrisch nach Volhard.

^{204}Tl-Lösung. 5 mC $^{204}TlNO_3$ mit einem Trägergehalt von 2 mg Tl wurden in 5 ml verdünnter HCl in der Hitze gelöst und mit einem Cl_2-Überschuss oxidiert. Das dreiwertige Thallium wurde durch Zutropfen von Ammoniaklösung als $Tl(OH)_3$ gefällt, der Niederschlag abzentrifugiert und mit reichlich Wasser chloridfrei gewaschen. Man löste ein wenig 1 N H_2SO_4. Ein Aliquot dieser Stammlösung wurde mit Wasser verdünnt und als Indikator jeweils 1 Tropfen verwendet.

Arbeitsmethode

Die Verteilungskoeffizienten wurden im Batch-Verfahren bestimmt. Jeweils 50 oder 100 mg des lufttrockenen Harzes und 2–5 ml Lösung wurden mit einem Tropfen der $^{204}Tl(III)$-Lösung versetzt und zur Einstellung des Sorptionsgleichgewichtes drei Tage mit einer Schüttelmaschine gut durchmischt. Die Tl(III)-Konzentration betrug $\leqslant 3.2 \times 10^{-6}$ Mol/l. Anschliessend wurde vom Harz abgetrennt und die Aktivität in einem Aliquot der Lösung mit einem G–M-Zählrohr gegen einen Aktivitätsstandard gemessen. Die Selbstabsorption wurde durch Messung einer Reihe von Standards korrigiert. Der Verteilungskoeffizient ergab sich nach

$$K_d = \frac{\%\ \text{Sorption}}{\%\ \text{Lösung}} \times \frac{\text{Volumen der Lösung}}{\text{Harzmenge}} \left(\frac{\text{ml}}{\text{g}}\right)$$

ERGEBNISSE

System Tl(III) – MCl – H_2O – Dowex 50 X8

In Tabelle I sind die Ergebnisse der Sorption von Tl(III) aus Lösungen einiger Metallchloride zusammengefasst. Nach Durchlaufen eines Minimums bei einer Konzentration von 1–2 Mol/l beobachtet man für alle untersuchten Chloride mit Ausnahme von NH_4Cl ein Wiederansteigen der Verteilungskoeffizienten. Der steile Anstieg von K_d bei Konzentrationen von > 5 Mol/l LiCl steht offenbar im Zusammenhang mit der starken Zunahme des Aktivitätskoeffizienten von LiCl[19].

TABELLE I

SORPTION VON Tl(III) AN DOWEX 50 X8 AUS CHLORIDHALTIGEN LÖSUNGEN

Nr.	*Konz. MCl (Mol/l)*	*Verteilungskoeffizient (ml/g)*					
		HCl	*LiCl*	*NaCl*	*KCl*	*NH_4Cl*	*$CaCl_2$*
1	0.1	$1.18 \cdot 10^1$	$1.56 \cdot 10^2$	$8.15 \cdot 10^1$	$1.43 \cdot 10^2$	$5.61 \cdot 10^1$	$2.44 \cdot 10^1$
2	0.5	2.71	$1.33 \cdot 10^1$	$1.20 \cdot 10^1$	$1.84 \cdot 10^1$	$1.04 \cdot 10^1$	$1.05 \cdot 10^1$
3	1	<1	7.69	4.10	$1.50 \cdot 10^1$	5.82	4.10
4	2	—	3.88	9.34	$2.55 \cdot 10^1$	5.51	$1.42 \cdot 10^1$
5	3	3.76	7.94	$2.01 \cdot 10^1$	$2.81 \cdot 10^1$	5.27	$1.83 \cdot 10^1$
6	4	—	$2.36 \cdot 10^1$	$2.42 \cdot 10^1$	$1.48 \cdot 10^1$	3.66	$2.82 \cdot 10^1$
7	5	4.17	$2.93 \cdot 10^1$	$2.68 \cdot 10^1$	—	4.22	$3.78 \cdot 10^1$
8	6	—	$5.52 \cdot 10^1$	—	—	—	$2.70 \cdot 10^1$
9	7	5.26	$8.95 \cdot 10^1$	—	—	—	—
10	8	6.67	$1.5 \cdot 10^2$	—	—	—	—
11	10	6.81	—	—	—	—	—

System Tl(III) – MBr – H_2O – Dowex 50 X8

Die Ergebnisse zeigt Tabelle II.

Im Gegensatz zum Chloridsystem steigt K_d bereits bei einer Konzentration von 0.5 Mol/l MBr an.

System Tl(III) – MJ – H_2O – Dowex 50 X8

Die Sorption von Tl(III) an Dowex 50 X8 aus Lösungen einer Jodidkonzentra-

TABELLE II

SORPTION VON Tl(III) AN DOWEX 50 X8 AUS BROMIDHALTIGEN LÖSUNGEN

Nr.	*Konz. MBr (Mol/l)*	*Verteilungskoeffizient (ml/g)*					
		HBr	*LiBr*	*NaBr*	*KBr*	NH_4Br	$CaBr_2$
1	0.5	$1.10 \cdot 10^1$	$2.52 \cdot 10^1$	$4.07 \cdot 10^1$	$2.08 \cdot 10^2$	$3.87 \cdot 10^1$	$1.40 \cdot 10^2$
2	1	$2.46 \cdot 10^1$	$3.70 \cdot 10^1$	$1.01 \cdot 10^2$	$3.9 \cdot 10^2$	$1.12 \cdot 10^2$	$3.36 \cdot 10^2$
3	2	$5.01 \cdot 10^1$	$1.48 \cdot 10^2$	$2.04 \cdot 10^2$	$8.21 \cdot 10^2$	$2.30 \cdot 10^2$	$5.55 \cdot 10^2$
4	3	$9.85 \cdot 10^1$	$2.02 \cdot 10^2$	$3.63 \cdot 10^2$	$1.08 \cdot 10^3$	$4.15 \cdot 10^2$	$8.60 \cdot 10^2$
5	4	$1.32 \cdot 10^2$	—	$5.37 \cdot 10^2$	$8.78 \cdot 10^2$	$5.40 \cdot 10^2$	$1.16 \cdot 10^3$
6	5	$1.63 \cdot 10^2$	$7.08 \cdot 10^2$	$7.58 \cdot 10^2$	—	$7.94 \cdot 10^2$	$1.25 \cdot 10^3$
7	6	$1.66 \cdot 10^2$	$1.81 \cdot 10^3$	$1.03 \cdot 10^3$	—	—	$1.27 \cdot 10^3$
8	7	—	$9.95 \cdot 10^2$	—	—	—	$1.29 \cdot 10^3$

tion von 0.5–7 Mol/l ist in Fig. 1 dargestellt. Wie im Falle der bromidhaltigen Lösungen steigt K_d oberhalb einer Konzentration von 0.5 Mol/l MJ kontinuierlich an.

Systeme mit konstanter Ionenstärke

System Tl(III) — 8 Mol/l (H,Li) Cl — H_2O — Dowex 50 X8. Wie Fig. 2 zu entnehmen ist, nimmt die Sorption von Tl(III) bei konstanter Chloridionenkonzentration mit dem molaren Verhältnis $Li^+ : H^+$ zu. Das bestätigt die Ergebnisse von Tabelle I und beweist einen wirklichen Kationeneffekt.

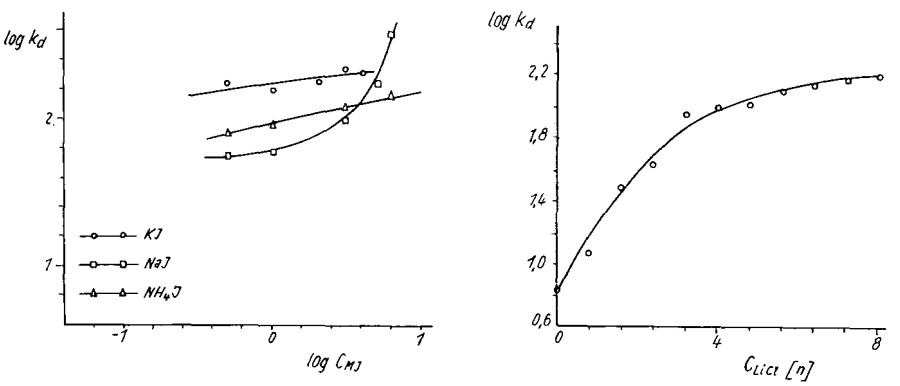

Fig. 1. Sorption von Tl(III) an Dowex 50 X8 aus Lösungen verschiedener Alkalijodide.

Fig. 2. Sorption von Tl(III) an Dowex 50 X8 aus 8 *M* (H, Li)Cl-Lösung.

System Tl(III) — 5 Mol/l Na(ClO_4,X) — H_2O — Dowex 50 X8 ($X^- = Cl^-, Br^-, J^-$). Die Ergebnisse über die Sorption von Tl(III) an Dowex 50 X8 aus 5 *M* $NaClO_4$-Lösung in Gegenwart von Mikromengen Halogenionen zeigt Fig. 3.

Man sieht, dass bereits ein Gehalt von 10^{-4} Mol/l J^-, sowie 10^{-2} Mol/l Br^- bzw. Cl^- zu einem Anstieg der Sorption führt. Der Anstieg erfolgt dabei in einem Kon-

zentrationsbereich, in dem auch in der wässrigen Phase Komplexe vom Typ TlX_4^- gebildet werden[20,21]. In Übereinstimmung mit der Reihe der Komplexstabilitäten

$$TlJ_4^- > TlBr_4^- > TlCl_4^-$$

beginnt die Sorption des Jodidkomplexes bei der geringeren Ligandenkonzentration.

Einfluss von organischen Solventien. Fig. 4 zeigt den Einfluss von Methanol, Äthanol, Aceton und Isopropanol auf die Sorption von Tl(III) an Dowex 50 X8 aus 2 *M* NaBr.

In allen Fällen beobachtet man nach einem schwachen Anstieg einen starken Abfall von K_d bei höheren Gehalten an organischen Lösungsmitteln. Die Desorptionswirkung nimmt mit der Kettenlänge des organischen Restes in der Reihe Methanol < Äthanol ≈ Aceton < Isopropanol zu.

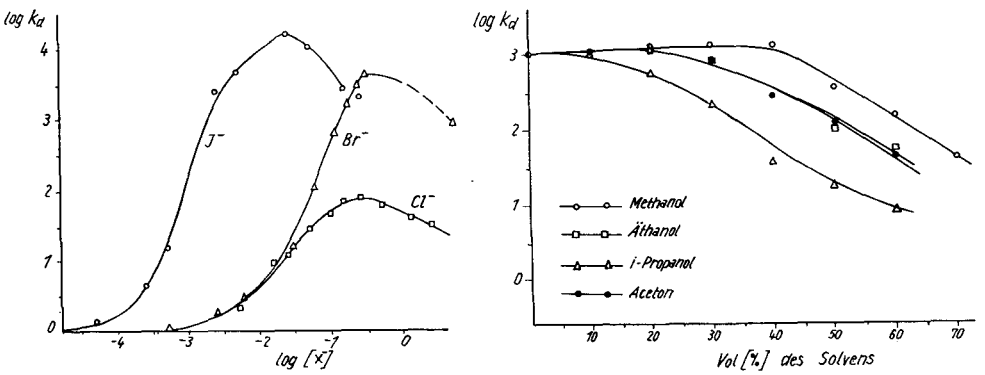

Fig. 3. Sorption von Tl(III) an Dowex 50 X8 aus 5 *M* $NaClO_4$ in Gegenwart von Mikromengen an Halogenionen.

Fig. 4. Abhängigkeit der Sorption von Tl(III) an Dowex 50 X8 aus 2 *M* NaBr vom Gehalt an organischen Solventien.

DISKUSSION

Aus den experimentellen Ergebnissen ergeben sich folgende für die Diskussion des Mechanismus der Sorption wesentliche Befunde:

(1) Die Sorption von Tl(III) an Dowex 50 X8 steigt bei Konzentrationen > 2 Mol/l MCl, > 0.5 Mol/l MBr und > 0.5 Mol/l MJ mit der Konzentration des Grundelektrolyten an.

(2) Die Verteilungskoeffizienten von Mikromengen Tl(III) an Dowex 50 X8 nehmen in der Reihe HX < LiX < NH_4X < NaX < KX zu ($X^- = Cl^-$, Br^-, J^-).

(3) Die Sorption von Tl(III) an Dowex 50 X8 aus Lösungen von Metallhalogeniden hoher Konzentration steigt in der Reihe MCl < MBr < MJ an ($M^+ = Na^+$, K^+, NH_4^+).

(4) In Systemen hoher Ionenstärke (5 *M* $NaClO_4$) führt bereits die Zugabe von Mikromengen an Halogenionen (> 0.1 Mol/l) zu einer Erhöhung der Verteilungs-

koeffizienten des Tl(III) an Dowex 50 X8. Die wirksamen Halogenidkonzentrationen sind von der gleichen Grössenordnung, wie sie zur Bildung von anionischen Komplexen vom Typ TlX_4^- in wässrigen Lösungen notwendig sind.

(5) Organische Solventien bewirken Desorption von Tl(III) aus Lösungen von NaBr an Dowex 50 X8, wobei die Desorptionswirkung in der Reihe Methanol < Äthanol ≈ Aceton < Isopropanol zunimmt.

Aus den Befunden (1) und (4) können wir schliessen, dass Tl(III) durch Kationenaustauscher bei hoher Ionenstärke der äusseren Lösung und in Gegenwart von Halogenionen einer Konzentration > 0.1 Mol/l in Form neutraler oder anionischer Komplexe, wahrscheinlich des Typs TlX_4^- ($X^- = Cl^-$, Br^-, J^-), sorbiert wird.

Die erste Voraussetzung für die Existenz anionischer Komplexe in der Harzphase ist aber eine zur Komplexbildung ausreichende Ligandenkonzentration. Auf Grund der beträchtlichen Stabilität der Tl(III)–Halogenid-Komplexe sind dazu Konzentrationen von > 0.1 Mol/l ausreichend.

Der Übergang des Grundelektrolyten in die Harzphase wird durch das Donnangesetz beschrieben. Danach gilt im Falle eines 1:1-Elektrolyten für den molalen Donnankoeffizienten

$$\lambda = \frac{m_{MX}}{\bar{m}_{RM} + \bar{m}_{MX}} \left(\frac{\gamma\pm}{\bar{\gamma}\pm}\right)^2 \qquad (1)$$

In Formel (1) bezeichnen

m_{MX} = molale Konzentration von MX,

$\bar{m}_{RM}$ = molale Konzentration der funtionellen Gruppe des Harzes,

$\lambda = \bar{m}_{MX}/m_{MX}$ – molaler Donnankoeffizient des Elektrolyten MX,

$\gamma\pm$ = mittlerer molaler Aktivitätskoeffizient von MX.

Die überstrichenen Symbole gelten für die Harzphase. Man sieht, dass λ mit der Konzentration des Grundelektrolyten zunimmt und ausserdem von der Kapazität und der Quellung des Harzes abhängig ist. Wie in Fig. 5 am Beispiel der Aufnahme von HBr durch Dowex 50 X8 gezeigt ist, erreicht λ bei einer Konzentration von 1–2 Mol/l für starke Elektrolyte MX und Harze vom Typ des Dowex 50 X8 Werte von ⩾ 0.1, entsprechend einer Konzentration des äusseren Elektrolyten in der Harzphase

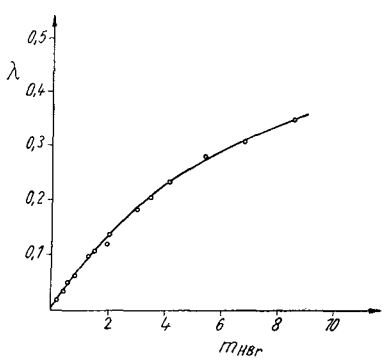

Fig. 5. Abhängigkeit des molalen Donnankoeffizienten von HBr an Dowex 50 X8 von der HBr-Molalität in der wässrigen Phase[29].

von $>$ 0.1 Mol/l. Damit sind die Bedingungen für die Existenz neutraler und anionischer Komplexe des Tl(III) in der Harzphase bereits bei einer Konzentration der äusseren Lösung von $\approx$ 1 Mol/l MX gegeben. Daraus ergibt sich zwangsläufig, dass kationische Formen von Tl(III) in der Harzphase unter diesen Bedingungen nicht mehr beständig sind. Der Wiederanstieg der Sorption bei Konzentrationen von 1–2 Mol/l ist damit der Aufnahme anionischer Komplexe zuzuordnen. Auf Grund der höheren Harzinvasion von Bromiden und Jodiden gegenüber Chloriden und der grösseren Stabilität ihrer Komplexe mit Tl(III) verschiebt sich das Minimum nach niederen Konzentrationen der äusseren Lösung (Befund 1) und tritt bei den Alkalijodiden praktisch nicht mehr auf.

Ein direkter Nachweis für die Aufnahme anionischer Komplexe durch Kationenaustauscher wurde am Beispiel des $FeBr_4^-$ erbracht[12].

Die Tatsache, dass K_d mit der Konzentration des Grundelektrolyten weiter ansteigt, weist aber daraufhin, dass eine weitere Voraussetzung für die Aufnahme anionischer Komplexe durch Kationenaustauscher eine hohe Konzentration der äusseren Lösung ist.

Der Zustand von Harzphase und Lösung bei Erhöhung der Konzentration des Grundelektrolyten ist gekennzeichnet durch:

Abnahme der Wasseraktivität in beiden Phasen;

Dehydratisierung der Ionen;

Abnahme der Quellung des Harzes;

Zunahme der Elektrolytinvasion und der Festionendichte im Harz.

Die Abnahme der Wasseraktivität und die Dehydratisierung der Ionen begünstigt die Bildung von Ionenassoziaten in beiden Phasen. Deshalb können wir annehmen, dass der Grundelektrolyt und die funktionelle Gruppe des Harzes bei Erhöhung der Konzentration in zunehmendem Masse in die undissoziierte Form übergehen. Nimmt man nun die Extraktion der Komplexe TlX_4^- durch die organische Matrix des Harzes an, so sollte die zunehmende Abschirmung der funktionellen Gruppe bei Ansteigen der Konzentration des äusseren Elektrolyten die Wechselwirkung der komplexen Anionen mit der Matrix begünstigen, da bei vollständiger Dissoziation der funktionellen Gruppe besonders in Harzen hoher Festionendichte diese Wechselwirkung durch die Abstossung

$$RSO_3^- \leftrightarrow TlX_4^-$$

erschwert ist.

Das Ergebnis von TITZE UND SAMUELSON, die eine höhere Sorptionsfähigkeit von Dowex 50 X8 für $FeCl_4^-$ bei einer 55%igen Desulfonierung des Harzes feststellten, wird bei dieser Betrachtungsweise verständlich[10]. Dagegen steht es im Widerspruch zu einer Assoziatbildung mit der funktionellen Gruppe.

Die innere Oberfläche eines Kationenaustauschers kann man in hydrophile und hydrophobe Bereiche aufteilen, die im gequollenen Harz statistisch verteilt sind Die nichtsulfonierten Mikrobereiche mit hydrophoben Eigenschaften, von denen offenbar der Matrixeffekt ausgeht, werden bevorzugt organische Moleküle oder schwach hydratisierte Ionen anlagern.

Aus der Literatur ist bekannt, dass TlX_4^--Ionen nicht hydratisiert sind[22]. Die Matrixwirkung sollte deshalb in der Reihe $TlCl_4^- < TlBr_4^- < TlJ_4^-$ zunehmen.

In der gleichen Reihenfolge beobachten wir eine Zunahme der Verteilungs-

koeffizienten (Befund 3). Da die abschirmende Wirkung der Gegenionen auf die funktionelle Gruppe in der Reihe $H < Li < Na < K$ zunimmt, sollte auch die Sorption bei gleicher Konzentration des Grundelektrolyten in der Reihe $HX < LiX < NaX < KX$ ansteigen. Das steht in Übereinstimmung mit Befund 2. Dass es sich hierbei um einen wirklichen Kationeneffekt handelt, der durch die unterschiedliche Abschirmung der funktionellen Gruppe bedingt ist, zeigt auch Fig. 2.

Diskutieren wir nun die erhaltenen Ergebnisse vom Standpunkt der Autoren, die die Bildung von Assoziaten $(RSO_3H_2^+)$ (TlX_4^-) annehmen. Die Sorption wird bei einem solchen Mechanismus abhängig sein von Radius und Ladung des zu sorbierenden Anions und von der Stärke der Wechselwirkung des Kations M^+ mit der funktionellen Gruppe des Harzes.

Man sollte deshalb erwarten, dass die Sorption der komplexen Anionen des Tl(III) in der Reihe $TlJ_4^- < TlBr_4^- < TlCl_4^-$, d.h. mit Abnahme des Anionenradius zunimmt. Dies steht im Widerspruch zu Befund 3. Ausserdem sollte man erwarten, dass mehrfach geladene Anionen wie ZnX_4^{2-} oder CdX_4^{2-}, die am Anionenaustauscher sorbiert werden, auch von Kationenaustauschern bei hohen Konzentrationen der äusseren Lösung aufgenommen werden. Die Selektivität des Kationenaustauschers beschränkt sich aber auf Anionen des Typs MX_4^-, was dazu deutlich im Widerspruch steht[9,11].

Da ZnX_4^{2-} und CdX_4^{2-} auf Grund ihrer zweifach negativen Ladung stark hydratisiert sind, wird ihre geringe Sorption am Kationenaustauscher verständlich, wenn man einen Matrixeffekt annimmt.

Die Bildung von Assoziaten der funktionellen Gruppe mit der Komplexsäure $HTlX_4$ bedeutet die Anlagerung eines Überschussprotons an die RSO_3H-Gruppe. Da man die Sorption von Tl(III) bereits aus 1 *M* HBr beobachtet, müsste man annehmen, dass unter diesen Bedingungen ein Teil der RSO_3H-Gruppen protonisiert sind. Nach Angaben der Literatur hat RSO_3H den Charakter einer starken Säure[23]. Ausserdem haben IR-spektroskopische und DK-Messungen an Ionenaustauschern vom Typ Dowex 50 gezeigt, dass bei einer Wasseraufnahme von mehr als 3 H_2O pro RSO_3H-Gruppe die Ablösung des Protons von der funktionellen Gruppe erfolgt[24–27]. Die Bindung des Protons bzw. anderer Metallkationen erfolgt über Wasserstoffbrücken von Wassermolekülen der primären Hydrathülle. Das bedeutet, dass unter Bedingungen, bei denen man Sorption anionischer Komplexe beobachtet, die funktionelle Gruppe des Harzes praktisch vollständig dissoziiert ist. Damit wird die Anlagerung eines Überschussprotons und die Ausbildung fester Assoziate von $HTlX_4$ mit der funktionellen Gruppe recht unwahrscheinlich.

Interessant ist in diesem Zusammenhang, dass Tl(III) aus HCl durch TBP und verschiedene Äther in Form der Säure $HTlCl_4$ extrahiert wird. Dabei ergab sich, dass $HTlCl_4$ in der organischen Phase praktisch vollständig dissoziiert vorliegt und lediglich eine Verknüpfung des Protons an die P=O- bzw. C=O-Gruppen des Extraktionsmittels je nach der Säurekonzentration über H_2O-Moleküle oder direkt erfolgt[21,28]. Berücksichtigt man, dass die makroskopischen DK-Werte organischer Extraktionsmittel geringer als die der Harzphase sind, so kann man die Dissoziation der überführten Komplexsäure $HTlCl_4$ auch im Harz annehmen.

Interessant ist auch der Einfluss von organischen Solventien auf die Sorption komplexer Anionen durch Kationenaustauscher. Die Verteilungskoffizienten steigen bei Zugabe von 10–20 % Alkohol oder Aceton schwach an und fallen bei höheren Gehalten um nahezu 2 Grössenordnungen ab (Fig. 4).

Mit Erhöhung des Anteils an Alkohol sinkt die DK der äusseren Lösung ab, dadurch nehmen die Dehydratisierung der Ionen und die Neigung zur Bildung von Ionenassoziaten zu. Das schwache Ansteigen von K_d hat seine Ursache in der zunehmenden Abschirmung der funktionellen Gruppe des Harzes. Bei hohen Gehalten an Alkohol nimmt der organische Charakter der wässrigen Phase weiter zu, was unserer Meinung nach infolge weitgehender Dehydratisierung der Ionen zu einer Verringerung der Selektivität der Sorption für wenig hydratisierte Ionen, wie z.B. TlX_4^-, führt. Dass es sich bei diesem Effekt um einen Abfall der Selektivität handelt, unterstreicht die schwache Zunahme der Harzinvasion des Grundelektrolyten mit der Alkoholkonzentration[29].

Einen ähnlichen Einfluss beobachtet man bei der Sorption von $FeCl_4^-$, $AuCl_4^-$ und $TlCl_4^-$ an Anionenaustauschern. KORKISCH und Mitarbeiter prägten dafür den Ausdruck CIESE-Effekt und nehmen an, dass sich zwischen der Komplexsäure HMX_4 und dem Alkohol oder Keton feste Assoziate bilden[30,31].

Gegen die Bildung solcher Assoziate spricht, dass $HTlX_4$ in Extrakten dissoziiert vorliegt, die Desorption bereits bei einem molaren Verhältnis von H_2O: Isopropanol von < 20 beginnt und im System Alkohol–HCl–H_2O selbst bei Konzentrationen von 80% Alkohol keine merkliche Ionenassoziation beobachtet wurde[32,33].

Zusammenfassend kann man sagen, dass die hier erhaltenen Ergebnisse ohne Widerspruch durch die Annahme einer Wechselwirkung der wenig hydratisierten komplexen Anionen TlX_4^- mit der organischen Matrix des Harzes erklärbar sind. Dagegen steht die Annahme einer Assoziation der komplexen Säure $HTlX_4$ mit der funktionellen Gruppe des Harzes im Widerspruch zu experimentellen Resultaten dieser Arbeit und Ergebnissen der Literatur. Weitere Untersuchungen über den Einfluss der Harzstruktur sind aber notwendig, um einen solchen Mechanismus eindeutig auszuschliessen.

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ZUSAMMENFASSUNG

Die Sorption von Tl(III) aus Lösungen verschiedener Metallhalogenide hoher Konzentration an Dowex 50 X8 wurde untersucht. Dabei ergab sich, dass die Verteilungskoeffizienten bei einer Konzentration von > 2 Mol/l MCl, > 0.5 Mol/l MBr und > 0.5 Mol/l MJ ansteigen. In Gegenwart von 5 M $NaClO_4$ bewirken Halogenkonzentrationen von $\geqslant 0.1$ Mol/l eine starke Zunahme der Sorption. Wie diese Ergebnisse und Betrachtungen über die Elektrolytinvasion zeigen, ist der Anstieg von K_d eindeutig der Aufnahme anionischer Komplexe vom Typ TlX_4^- durch Dowex 50 X8 zuzuordnen. Voraussetzungen für die Sorption sind eine ausreichende Ligandenkonzentration in der Harzphase, sowie eine hohe Konzentration der äusseren Lösung. Organische Solventien bewirken Desorption der anionischen Komplexe vom Dowex 50 X8. Die erhaltenen Ergebnisse lassen sich ohne Widerspruch bei Annahme einer Wechselwirkung der wenig hydratisierten TlX_4^--Ionen mit der organischen Matrix des Harzes deuten.

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CHROM 4307

SEPARATION OF RARE EARTH ELEMENTS BY GAS CHROMATOGRAPHY OF THEIR CHLORIDES

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SUMMARY

Gas–solid chromatography separations of lanthanide chlorides were carried out on the glass capillary columns at $\leqslant 250°$ using aluminium trichloride vapours (40–170 mm Hg) as a component of the carrier gas. $AlCl_3$ forms volatile complexes with rare earth chlorides, and at the same time, modifies the surface of the column.

INTRODUCTION

Up to the present time, gas chromatographic separations of rare earth elements have only been carried out by using volatile chelates–trisdiketonates (including fluoroderivatives) with branched carbon chains. The general use of the GLC techniques[1,2] reported might not be possible owing to the complicated synthesis of the compound involved. Inorganic rare earth substances, as such, have not been employed in gas chromatography because of their low volatility.

Recently GRUEN AND ØYE[3] obtained spectroscopic evidence that heating neodymium trichloride in aluminium trichloride vapours (temperature range 200–800°) results in a much higher concentration of neodymium in the gas volume than that corresponding to the vapour pressure of neodymium chloride itself. They suggested that this might be due to the formation of volatile complexes of the general formula $Nd(AlCl_4)_nCl_{3-n}$. In the present work an attempt was made to utilize such species for the separation of rare earths by gas–solid chromatography. Preliminary experiments showed the formation of volatile complexes with aluminium chloride to be a common property of the chlorides of lanthanide elements. To prevent the dissociation of the complexes a mixture of an inert gas with the Al_2Cl_6 vapours was used as carrier gas. The latter served also for dynamic modification of the surface of the glass capillary column.

EXPERIMENTAL

Apparatus

Gas chromatography was performed in the apparatus shown in Fig. 1. Tube furnaces (1) (2) (3) and (4) maintained the required stepped temperature distribution

along a glass tube (5). This consisted of several welded sections; the spiral one (2.5 m × 1 mm I.D.) placed in a thermostat (1) served as the chromatographic column. Nitrogen (flow rate 12 ml/min), after passing through a flow meter (8), passed through a molecular sieve desiccant (9) and was saturated with aluminium chloride vapour in a tube (6). This was completely filled with the solid chloride and situated in furnace (4) where the temperature was 20–30° higher than in the thermostat (3), whose temperature corresponded to the required vapour pressure of Al_2Cl_6. The supersaturation arising was quickly destroyed by partial condensation of the vapours and thus a steady concentration was maintained. We used an Al_2Cl_6 partial vapour pressure between 40 and 170 mm Hg, the temperature of the furnace (3) being 138–155°.

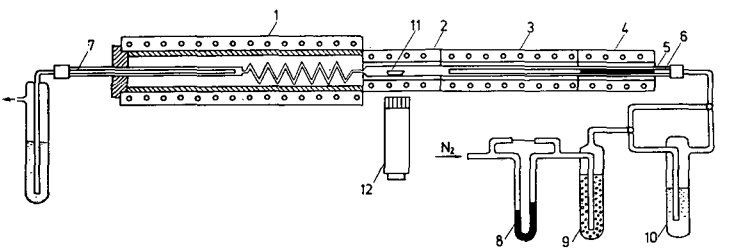

Fig. 1. The chromatographic apparatus (see text for description).

Sample introduction

The sample solution with radioactive tracers was evaporated in a glass boat and the residue was then evaporated to dryness first with concentrated nitric acid and then with concentrated hydrochloric acid. The boat was put into furnace (2) which is maintained at about 180°. Nitrogen, saturated with thionylchloride vapour in vessel (10) was passed over the sample for several minutes to dehydrate the rare earth chlorides. The tube (6) with solid aluminium chloride was then inserted and the carrier gas was passed through the system for 5 min. After stopping the flow the temperature of furnace (2) was raised rapidly to 500° after which the carrier gas was again supplied. The volatile compounds sublimed into the carrier gas, the rate of the process being followed by a chart recording the counting rate from a scintillation counter (12) with a Na(Tl)I crystal. The sample was introduced into the column in about 1 min.

The gaseous complexes of the rare earth elements condensed with aluminium chloride at the exit of the thermostat (1) in a tube (7) inserted into tube (5). One cm of tube (7) was extracted every 2 min from the thermostat and thus fractions of the condensate were collected. The chromatogram was observed by measuring the intensity of β and/or γ radiation along tube (7).

The isotopes ^{144}Ce, ^{143}Pr, ^{147}Pm, ^{155}Eu, $^{148,150}Gd$, ^{160}Tb, ^{159}Dy, $^{165,167}Tm$, ^{166}Yb and ^{170}Lu without or with several micrograms of a carrier were used.

Volatile complexes

Runs with separate elements showed that at an Al_2Cl_6 vapour pressure of $\approx$ 100 mm Hg the chlorides of Ce, Pr, Pm, Gd, Tb, Dy, Tm, Yb and Lu rapidly form volatile complexes, which can be transported by the carrier gas in the broad range of temperature (150–500°) investigated. The retention time was found to decrease

with increasing atomic number. Peculiar behaviour was observed in the case of europium which sublimed very slowly from the boat and was found to be distributed all along the gas conduit.

We suppose that the volatility of lanthanide chlorides in the presence of aluminium chloride can be accounted for by the formation of compounds of the general formula $LnAlCl_6$ (where Ln is the lanthanide element). This composition is suggested by analogy with the stoichiometry of vapour complexes such as $NaAlCl_4$[4], $BeAlCl_5$[5], $FeAlCl_6$[6] and $KLnCl_4$[7] reported earlier. These last compounds are rather strong bonded, their stability increasing from Lu to La. NOVIKOV AND GAVRYUCHENKOV[7] have pointed out that, as a general rule, complex compounds are formed in the vapour phase by those chlorides which themselves incline to dimerization, and that the double compounds are often more stable towards dissociation than any of the related dimeric species. Dimers of rare earth trichlorides were reported to be reasonably stable, the heat of dimerization being from 32 to 48 kcal/mole[7,8]. These data give additional support to the suggested existence of $LnAlCl_6$ complexes.

Thus the peculiar behaviour of europium, as compared with other lanthanides, can evidently be accounted for by the instability of europium trichloride. This decomposes upon heating to yield $EuCl_2$[9] which does not form dimeric molecules in vapour state[8]. Ytterbium is also capable of forming a dichloride, however, it showed 'normal' behaviour. This is explained by the big difference between the enthalpies of decomposition from the trichloride to dichloride: these are reported[10] as 12 kcal/mole and 24 kcal/mole for liquid $EuCl_3$ and $YbCl_3$, respectively. The value for $SmCl_3$ is 27 kcal/mole and so samarium would be expected to behave like other lanthanides.

Retention time vs. temperature and Al_2Cl_6 pressure

Generally speaking, one may expect the retention time of the complex to depend on the column temperature in a complicated way. With an increase of temperature two factors producing opposite effects are operative. Firstly, the desorption step is accelerated, making the retention time of the complex shorter. On the other hand and for the same reason, the modifying action of the Al_2Cl_6 vapour becomes weaker, which can result in an increase of the effective heat of adsorption of the complex.

The temperature dependence of the position and shape of the emerging peak is shown in Fig. 2 for ^{160}Tb with Al_2Cl_6 partial vapour pressure of 115 mm Hg. The

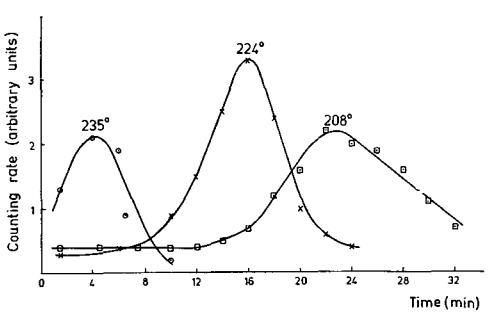

Fig. 2. Temperature dependence of terbium chromatogram (4 μg of carrier). Al_2Cl_6 partial vapour pressure 115 mm Hg, nitrogen flow rate 12 ml/min.

heat of adsoption for the terbium complex was calculated from the retention time data using a molecular kinetics equation[11]. The values obtained, *viz.* 30.4 kcal/mole at 208°, 31.0 kcal/mole at 224° and 30.3 kcal/mole at 235° are within the limits of experimental error and hence the peak shift is mainly due to the accelerated desorption of the complex molecules.

The retention time depends on the partial vapour pressure of the aluminium chloride in the carrier gas. At a fixed column temperature the elution can be made faster and the peak narrower by increasing Al_2Cl_6 vapour pressure. The data obtained for ^{160}Tb chloride at a column temperature of 220° are shown in Fig. 3. The Al_2Cl_6 vapour pressure equation used was that given in ref. 12. The strong dependence of the retention time on the quantitative composition of the carrier gas seems to support the suggestion about the dynamic modification of the glass surface by adsorption of aluminium chloride vapours[13,14].

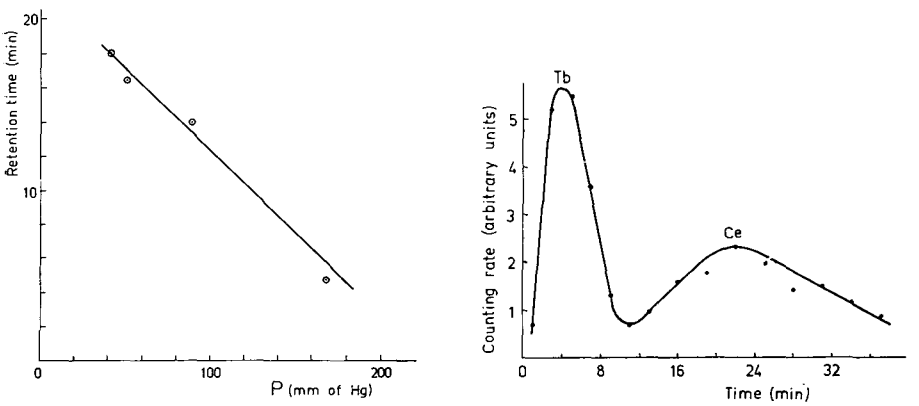

Fig. 3. Retention time of terbium at 220° as a function of Al_2Cl_6 partial vapour pressure. Nitrogen flow rate 12 ml/min.

Fig. 4. Separation of a terbium–cerium mixture. Column temperature 250°, Al_2Cl_6 partial vapour pressure 40 mm Hg, nitrogen flow rate 12 ml/min.

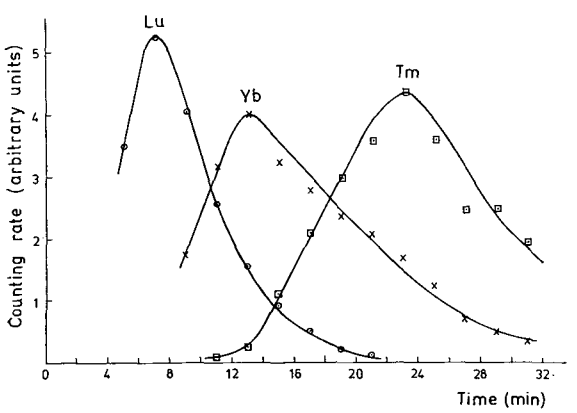

Fig. 5. Separation of a thulium–ytterbium–lutecium mixture. Column temperature 168°, Al_2Cl_6 partial vapour pressure 40 mm Hg, nitrogen flow rate 12 ml/min.

Separations

Satisfactory separations of some rare earth mixtures were achieved in spite of using a very short capillary column. The chromatogram of a ^{144}Ce–^{160}Tb mixture is given in Fig. 4. Fig. 5 shows the result of an experiment with the neighbouring heavy lanthanides—Tm, Yb and Lu. In this case the fractions of condensate were analysed for the isotope content by measuring the most characteristic and abundant γ-rays of the above elements in the < 0.5 MeV region with a Ge(Li) spectrometer. The separation factors obtained, $\alpha_{Lu/Yb} = 1.8$ and $\alpha_{Yb/Tm} = 1.7$, are considerably better than those for the same elements in ion-exchange chromatography[15,16] and in GLC of chelates[1,2,17]. Still higher separation factors were achieved with reversed phase partition chromatography[18] but with an inverted sequence of elution.

CONCLUSION

The evident advantages of the technique described are the very simple synthesis of the volatile compounds in the course of sample introduction and the possibility of working with both trace and macroquantities of elements.

High separation factors make the method promising and allow one to hope that good separations can be achieved. Work is in progress to determine the optimum conditions for separation of mixtures of the lanthanide and transuranium elements.

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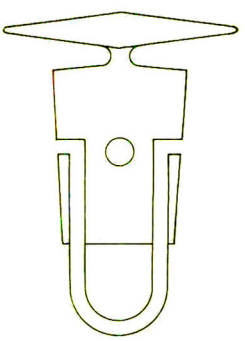
Notes

CHROM. 4343

A simple gas chromatographic injection system

In order to obtain optimum resolution in gas chromatographic analysis, it is necessary to inject the sample gas into a carrier gas flow in such a manner that the sample remains as nearly as possible as a disc or cylinder of gas sandwiched between the carrier. Conventional gas injection systems either use several stopcocks, which effectively divert the carrier through a sample holder tube, or are rather expensive. The cheaper systems suffer from the fact that several stopcocks must be operated, resulting in a certain degree of spreading out or diffusing of the sample disc. This necessitates the use of a longer column that would otherwise suffice.

We have for some time made use of a simple 'home-made' stopcock which we find minimizes the diffusion of the sample disc, and achieves good resolution with columns up to 20% shorter than have hitherto been necessary.



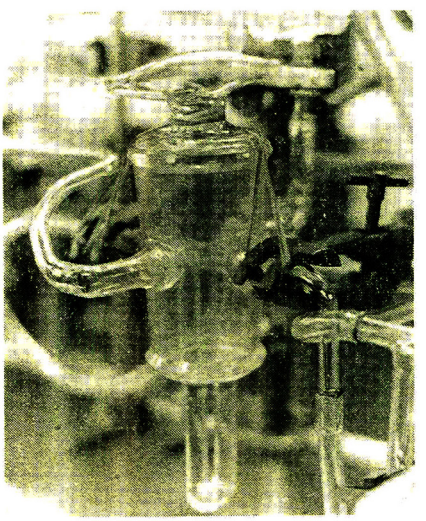


Fig. 1. Vertical cross-section through barrel of the injection stopcock.

Fig. 2. Injection stopcock.

The stopcock key is shown in Fig. 1. This key has two bores, one of which is a 'straight-through' bore which normally allows passage of the carrier gas from reservoir to chromatography column. The second bore—shaped like a letter U—provides the sample holder volume, which can be filled in the normal way. Our model had a sample volume of about 12 ml.

The stopcock in position in a vacuum line is shown in Fig. 2. Once filled the key can be quickly turned so that the sample is compressed to a disc and carried directly to the chromatographic column. The improvement in peak shape that results from using this technique is shown in Fig. 3.

The stopcock key was made by Mr. T. J. MAPLE (Chemistry Department, Queen Mary College) and the barrel was supplied and the grinding performed by 'Clemwoods', 20–24 Beaumont Road, Chiswick, W. 4.

One of us (D.J.M.L.) wishes to thank the University of London for a maintenance grant.

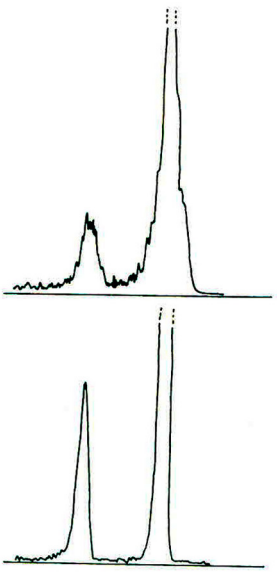


Fig. 3. Radio-chromatographic traces of the separation of HT (right-hand peak) and CH_3T (left-hand peak) on an activated silica gel column, under comparable conditions. Conventional injection procedures were used for the top trace. The bottom trace was obtained using the injection stopcock described in this note. The improvement in peak shape can be seen quite clearly.

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CHROM. 4306

Quantitative gas chromatographic determination of 5,5-dimethyl-2,4-oxazolidinedione

5,5-Dimethyl-2,4-oxazolidinedione (DMO) is a weak acid whose acid hydrogen linked with nitrogen can be titrated with soda in the presence of phenolphthalein as described by STOUGHTON[1]. This simple method for DMO evaluation has been adopted in control laboratories where relatively concentrated solutions (*e.g.*, 2.5 %) are analysed. Unfortunately, when the quantitative estimation is required in biological fluids such as urine, plasma, and tissue homogenates, this method presents insuperable difficulties. These are mainly due to the small amount of substance present and the great variety of other matter which interferes with the titration in such fluids.

BUTLER AND WADDEL have developed a selective extraction method for DMO from biological liquids using a borate buffer, and a quantitative determination of DMO based on characteristic UV absorption[2-4]. We have already proved that this method is valid[5-8].

Apart from BUTLER AND WADDEL'S method and the radioisotopic evaluation of [2-^{14}C]DMO[9,10], no other procedures for the determination of DMO in biological fluids have been described in the literature.

The purpose of this study was to investigate the gas-chromatographic behaviour of DMO and see whether it might be applied to the determination of this product. It is a well-known fact that the most reliable method at present available for calculating body and muscle intracellular pH is based on DMO distribution in the body, and the quantitative determination of this material is thus a prerequisite[2,6,10].

Equipment and working conditions

A Carlo Erba Fractovap GV gas chromatograph equipped with a flame ionization detector and stainless steel columns, 2 m × 2 mm I.D., was used. The columns were filled with 10 % butanediol succinate on Gas-Chrom Q (90–100 mesh), 10 % Carbowax 20 M on Chromosorb W (60–80 mesh), and 2 % SE-30 on Chromosorb W (60–80 mesh).

The working conditions for the 10 % butanediol succinate on Gas-Chrom Q column are as follows: column temperature 213°; detector temperature 130°; injector temperature 263°; nitrogen flow 20 ml/min.

Synthesis of 5,5-dimethyl-2,4-oxazolidinedione

The DMO was prepared according to STOUGHTON'S method[1] starting from the methyl ester of α-hydroxy-isobutyric acid and anhydrous urea in the presence of sodium and anhydrous ethyl alcohol.

The final product, when crystallized three times from benzene and dried in a vacuum at 40°, proved to be pure from a gas chromatographic standpoint. The corrected m.p. was 76–77°. Elementary analysis for nitrogen gave N_2 = 10.87 % (theoretical N_2 = 10.85 %).

Solutions in distilled water were injected into the gas chromatograph.

Synthesis of 5-methyl-2,4-oxazolidinedione (MO)

MO was synthesized by the same method as DMO starting from the methyl

ester of lactic acid. The final product when distilled in a vacuum at 142° at 1 mm Hg also proved to be pure from a gas chromatographic angle. The m.p. was 49°. Elementary analysis for nitrogen gave N_2 = 12.03 % (theoretical N_2 = 12.17 %).

Solutions in distilled water were injected into the gas chromatograph.

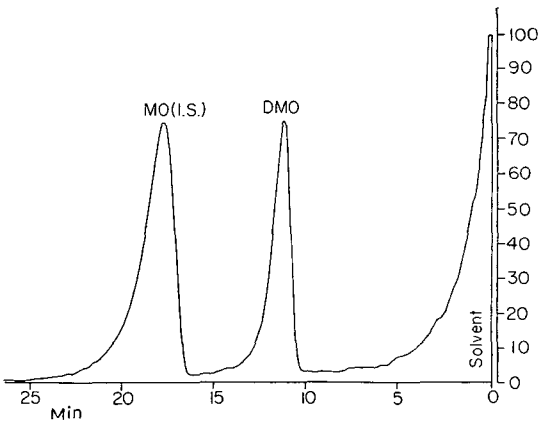

Fig. 1. Gas chromatogram showing a separation between DMO and MO (internal standard). Analytical conditions: column, 2 m × 2 mm I.D., filled with 10 % butanediol succinate on Gas-Chrom Q (90–100 mesh); column temperature = 213°; evaporator temperature = 263°; detector temperature = 130°; nitrogen flow = 20 ml/min; flame ionization detector.

Results and discussion

All three columns used gave satisfactory peaks both with DMO and MO. The best separation of both peaks was obtained with the column containing 10 % butanediol succinate on Gas-Chrom Q (90–100 mesh).

When this column was used under the conditions described above the DMO retention time, relative to that of MO, was 0.64 (Fig. 1). Although the molecular weight of MO is less than that of DMO, it is retained in the column longer probably because the MO molecule has a higher polarity than the DMO molecule.

Results obtained by analysing a number of solutions with various MO/DMO weight ratios are reported in Table I. Fig. 2 shows that there is a linear correlation between area ratios (A_R) and weight ratios (W_R). The best straight line was obtained by the least squares method using an Olivetti P 101 computer. The standard deviation of the mean weight factor of response ($\bar{f} = 2.59 \pm 0.088$) shows that the analytical method adopted is highly reproducible. These data indicate that MO may be used as an internal standard for the quantitative determination of DMO. The smallest amount of DMO which could be determined quantitatively was 1 μg.

In conclusion, the gas chromatographic evaluation of DMO using MO as an internal standard appears to be highly sensitive. In addition, it is superior to WADDEL AND BUTLER's UV-spectrophotometric method as regards selectivity.

In view of these properties we believe that the gas chromatographic method may be used instead of the costly radioisotopic method even when bioptical samples are available.

The method described here has already been applied[11] to the measurement of

TABLE I

AREA RATIOS (A_R) BETWEEN MO AND DMO AND RESPONSE WEIGHT FACTORS ($\bar{f}$) OBTAINED BY GAS CHROMATOGRAPHIC ANALYSIS OF 6 SOLUTIONS CONTAINING DIFFERENT MO/DMO WEIGHT RATIOS(W_R)

Analytical details are given in Fig. 1.

Solution	$W_R = MO/DMO$	$A_R = MO/DMO$	$\bar{f}$
1	7.16	2.79	2.68
2a	5.37	2.22	2.42
2b	5.37	2.01	2.67
3	3.58	1.40	2.56
4	2.39	0.89	2.68
5	1.79	0.70	2.54
6a	1.19	0.46	2.60
6b	1.19	0.46	2.60

$\bar{f} \pm$ d.v. $= 2.59 \pm 0.088$.

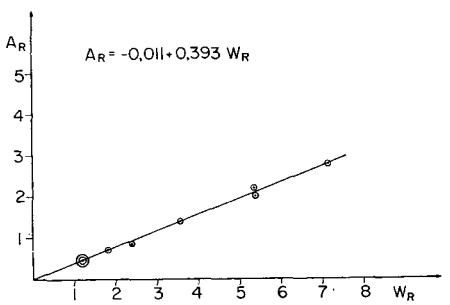

Fig. 2. Linear correlation between area ratios (A_R) and weight ratios (W_R) of the solutions reported in Table I. The equation of the best straight line was obtained by the least squares method.

the intracellular pH of human muscle in normal and pathological subjects; further experiments will be reported soon.

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CHROM. 4331

Gas–liquid chromatographic separation of heptono-1,4-lactones as trimethylsilyl derivatives*

The separation of glyconolactones is of interest in studies of the pulping and bleaching of wood products[1], in gas–liquid chromatographic procedures for the analysis of uronic acids[2] and neutral glycoses[3], and for the identification of aldonic acids in natural products[4]. The present investigation was initiated in order to devise a convenient method for the detection and tentative identification of aldoheptoses in bacterial cell walls[5]. The procedure involves the identification of the heptono-1,4-lactone produced by mild oxidation of the aldoheptose released on hydrolysis of the starting material.

Paper chromatographic[6] and ion-exchange chromatographic[7,8] methods for the separation of aldonic acids and their lactone derivatives have been reported, however, the separation of heptonolactones is probably best achieved by gas–liquid chromatography of their trimethylsilyl derivatives[2,3,9]. The characterization of trimethylsilylated glyconolactones, isolated by gas–liquid chromatography, has been made by their mass spectrographic analysis[10].

All sixteen possible heptono-1,4-lactones were prepared by applying the Kiliani–Fischer cyanohydrin synthesis (see the review by HUDSON[11]) to all eight of the possible D-aldohexoses. Each aldohexose gave rise to the expected two epimeric heptonic acids which, after lactonization[3] and trimethylsilylation, were separated by gas–liquid chromatography and the two components were each collected. The IR spectrum of every collected derivative showed an absorption band in the carbonyl region at 1784 cm^{-1}, characteristic of a saturated 1,4-lactone structure. The identity of each component was established by gas–liquid chromatography of its corresponding hepta-O-acetylheptitol derivative, prepared by acetylation of the borohydride reduction product of the detrimethylsilylated heptonolactone, using authentic hepta-O-acetylheptitols as reference standards[12].

Table I records the gas chromatographic retention times of the sixteen 2,3,5,6,7-penta-O-trimethylsilylheptono-1,4-lactone derivatives relative to 2,3,5-tri-O-trimethylsilyl-D-ribono-1,4-lactone. The separation of the derivatives on several types of column packing materials was investigated but none provided a complete separation of all sixteen heptonolactone derivatives and the neopentylglycol sebacate polyester liquid phase column appeared to give the most satisfactory separations. Although some overlap of the peaks due to the heptonolactone derivatives occurs, the epimeric derivatives arising from each parent aldohexose following the cyanohydrin reaction were in each case well separated from each other.

The tentative identification of aldoheptoses in the hydrolyzates of bacterial cell walls and other biological materials can be made using the gas chromatographic procedure described above to analyse the heptonolactone components produced by mild oxidation[3] of the aldoheptose components in the hydrolyzates. All the trimethylsilyl derivatives of the heptono-1,4-lactones have greater retention times than the

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TABLE I

GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF 2,3,5,6,7-PENTA-O-TRIMETHYLSILYLHEPTONO-1,4-LACTONES

Parent aldohexose	*2,3,5,6,7-Penta-O-trimethylsilylheptono-1,4-lactone derived by cyanohydrin synthesis on an aldohexose (TMS derivative)*	*Relative retention time*[a]
D-Allose	D-Glycero-D-*allo*-heptono-1,4-lactone	3.52
	D-Glycero-D-*altro*-heptono-1,4-lactone	2.98
D-Altrose	D-Glycero-D-*gluco*-heptono-1,4-lactone	2.00
	D-Glycero-D-*manno*-heptono-1,4-lactone	3.64
D-Glucose	D-Glycero-D-*gulo*-heptono-1,4-lactone	3.90
	D-Glycero-D-*ido*-heptono-1,4-lactone	3.29
D-Mannose	D-Glycero-D-*galacto*-heptono-1,4-lactone	2.30
	D-Glycero-D-*talo*-heptono-1,4-lactone	2.73
D-Gulose	D-Glycero-L-*galacto*-heptono-1,4-lactone	1.95
	D-Glycero-L-*talo*-heptono-1,4-lactone	2.14
D-Idose	D-Glycero-L-*ido*-heptono-1,4-lactone	3.63
	D-Glycero-L-*gulo*-heptono-1,4-lactone	2.68
D-Galactose	D-Glycero-L-*manno*-heptono-1,4-lactone	3.40
	D-Glycero-L-*gluco*-heptono-1,4-lactone	2.24
D-Talose	D-Glycero-L-*altro*-heptono-1,4-lactone	2.66
	D-Glycero-L-*allo*-heptono-1,4-lactone	2.24

[a] Relative retention time quoted with reference to 2,3,5-tri-O-trimethylsilyl-D-ribono-1,4-lactone (= 1.00). Retention time 3.5 min.

trimethylsilyl derivatives of hexonolactones and pentonolactones so that the aldoheptoses may be analyzed in the presence of aldohexoses and aldopentoses.

Experimental

Synthesis of heptono-1,4-lactones. To a solution of aldohexose (0.5 g) in water (4 ml) cooled to 0° was added a fresh solution of sodium cyanide (0.25 g) in water (3 ml) and the mixture was kept at 5° for 24 h. The reaction mixture was boiled gently for 5 h and water was added at intervals to maintain a total volume of about 10 ml. The cooled solution was passed down a column of Rexyn 101 (H^+) ion-exchange resin (25 ml) and the eluate and washing after concentration to near dryness were treated with a few drops of 2 *N* hydrochloric acid and were reconcentrated to dryness under reduced pressure, below 60°. The residue containing mixed heptono-1,4-lactones was used for gas chromatographic studies.

Gas–liquid chromatography. Separation of the 2,3,5,6,7-penta-O-trimethylsilylheptono-1,4-lactone derivatives was made with a Hewlett-Packard Model 402 gas chromatograph with a hydrogen flame detector, and fitted with glass U tubes (5 ft. × 6 mm × 3 mm I.D.) packed to each end with 10% (w/w) neopentylglycol sebacate polyester on 80–100 mesh acid-washed Chromosorb W. The column was maintained at 190° and development was made with helium at a flow rate of 90 ml/min. The flash heating system was not used and in preparative runs the derivatives were collected from the exit of the column in drawn-out glass tubes (10 cm × 3 mm I.D.) in which the compounds were readily condensed.

The heptono-1,4-lactones (*ca.* 10 mg) were trimethylsilylated in acetonitrile (0.2 ml) by treatment at 80° for 2 h with bis(trimethylsilyl)trifluoroacetamide con-

taining 1% trimethylchlorosilane (0.2 ml) (Regisil®, Regis Chemical Co., Chicago, Ill.) and the mixture was injected directly onto the top of the gas chromatographic column packing material.

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CHROM. 4339

A new mold inhibitor for amino acid analyzer buffers

To help safeguard against mold formation, it is customary to add inhibitors in the preparation of citrate buffer solutions for an automatic amino acid analyzer. The more commonly used inhibitors are pentachlorophenol and octanoic acid (*n*-caprylic acid).

In our laboratory we have observed that these are not very effective under our particular conditions.

In trying new inhibitors, we found that diethylcarbonate (ethylcarbonate) is more effective and convenient. Diethylcarbonate is used in Germany as preservative for beverages and liquid foods. It is insoluble in water and miscible with ethyl alcohol. When emulsified in water it will decompose slowly to CO_2 and ethyl alcohol.

Application

Dissolve 1 ml of ethyl carbonate in 10 ml of ethyl alcohol. Transfer 1 ml to 500 ml of distilled water and use this emulsion for the last rinse of the flask that will receive the prepared buffer, leaving it in contact for at least 5 min.

To the rinsed flask add 1 ml per l of buffer and immediately fill it with the prepared buffer. If stored in a tightly closed container, this solution can be kept indefinitely. During the normal operation time, or with intermittent work, the preservative acts fairly well.

The brand of diethyl carbonate we used was 'Bayacovin' (Bayer), specially developed for the 'cold sterilisation' of beverages.

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CHROM. 4321

A quick separation of the ε-N-methyl-lysines using the amino acid analyzer*

In view of the demonstration of the presence of ε-N-methyl-lysine in flagellar proteins[1], histones[2–6] and plasma[7], ε-N-dimethyl-lysine** in histones[3–5] and ε-N-trimethyl-lysine** in histones[4] and cytochrome *c*[8], it is desirable that a simple and quick method for the identification and estimation of these compounds be available. The standard amino acid analyzer systems do not separate the methyl-lysines, therefore various modifications of these have been used[2–8]. We have found that a short column of Aminex A-5 resin (Bio-Rad Laboratories, Richmond, Calif.) eluted with a pH 6.48 buffer at 25° is ideal for the separation of the methyl-lysines since it separates them from each other and from all of the protein amino acids.

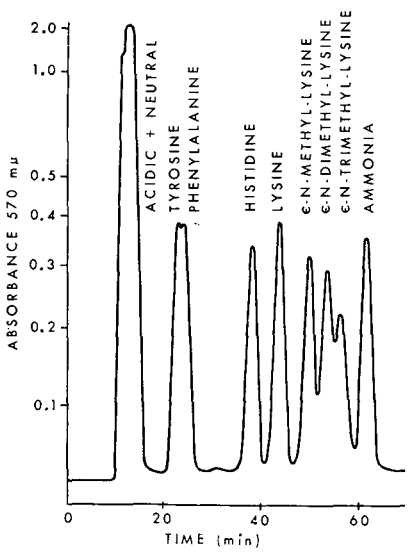

Fig. 1. Chromatography of the ε-N-methyl-lysines in the presence of a calibration mixture on a 15 × 0.9 cm column of Aminex A-5 resin eluted with 0.35 *N* sodium citrate, pH 6.48, at 25°. Buffer flow rate, 68 ml/h. The mixture contained, except for ammonia, 0.25 μmoles of each component.

The separations achieved with a Beckman model 120B amino acid analyzer are illustrated in Figs. 1 and 2. Fig. 1 is the curve obtained when the eluent was pumped through at the normal operation flow rate of 68 ml/h[9], while Fig. 2 is the curve obtained at a flow rate of 34 ml/h. It is seen that the slower system (120 min) gives a complete separation of all the basic components of the mixture, but that an adequate separation is also obtained with the faster (60 min) system. Both curves are readily reproducible since the positions of emergence of the peaks are not significantly altered

* Supported by a grant from the Medical Research Council of Canada.

** Though these terms are in common usage, the correct names are: ε-N,ε-N-dimethyl-lysine and ε-N,ε-N,ε-N-trimethyl-lysine.

by minor variations of the pH of the eluting buffer. If a pH 5.28 eluent is used, ε-N-dimethyl-lysine and ε-N-trimethyl-lysine emerge together, but all peaks are still completely separated from each other, with histidine appearing after ammonia. It is worth noting that the 440/570 mμ absorbance ratios of lysine and ε-N-methyl-lysine are similar, as are those of ε-N-dimethyl-lysine and ε-N-trimethyl-lysine, but that the ratios for the two pairs are very different.

A complete summary of the color constants and elution times obtained for the Aminex A-5 resin system as well as other systems appears in Table I. It should be

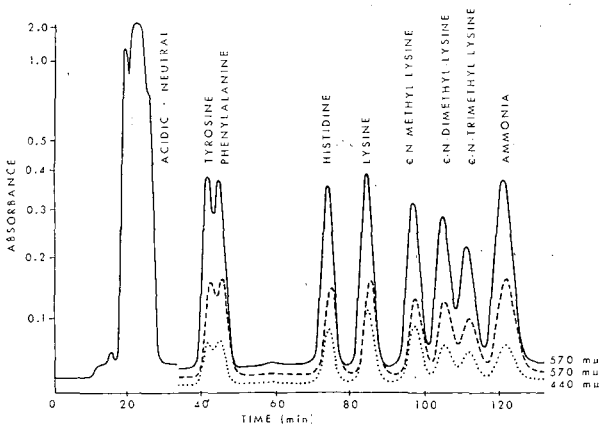

Fig. 2. Same as in Fig. 1, except a buffer flow rate of 34 ml/h.

TABLE I

DATA FROM THE BECKMAN AMINO ACID ANALYZER

Compound	*Eluting buffer, 0.35 N sodium citrate; Ninhydrin flow rate, 34 ml/h*					
	Eluent pH 5.28, 68 ml/h; 57°				*Eluent pH 6.48*[a], *34 ml/h; 25°*	
	0.9 × 50 cm, AA-15 resin		*0.9 × 7 cm, PA-35 resin*		*0.9 × 15 cm, Aminex A-5 resin*	
	Time (min)	*Constant*[b]	*Time (min)*	*Constant*[b]	*Time (min)*	*Constant*
L-Lysine	164	22.06	25	23.80	84	43.5
ε-N-Methyl-L-lysine[10]	178	20.15	25	21.02	97.5	39.7
ε-N-Dimethyl-L-lysine[c]	178	19.48			105	39.2
ε-N-Trimethyl-L-lysine[d]	159	18.48			111.5	35.5
DL-Homolysine[13]	228	22.77	33	24.77		

[a] Prepared by adding NaOH to pH 5.28 buffer.

[b] Mean of five determinations for different amounts. Reproducibility was within ± 3 %.

[c] Solution prepared by refluxing α-N-acetyl-ε-N,ε-N-dimethyl-L-lysine·$2H_2O$[11] in 2 *N* HCl for 1.5 h.

[d] Solution prepared by heating α-N-benzoyl-ε-N,ε-N,ε-N-trimethyl-L-lysine gold salt[12] in 6 *N* HCl in a sealed tube at 110° for 72 h.

noted that the variation of the constants obtained with different systems is significant enough not to be ignored in calculations.

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CHROM. 4324

A new accelerated fully automated system for amino acid analysis by ion-exchange chromatography

In a recent paper we reported on the development of a chromatographic system which separates the amino acids usually present in acidic protein hydrolysates within 63 min[1]. This was mainly achieved by improved column technologies and reduction of extra column contributions to band spreading in fittings and analytical system.

Since then, an improved resin has been developed for this system and precision data have been generated. Also, a final manifold for the peristaltic valve and pump has been assembled and evaluated.

Improved resin

The original resin used in the system tended to pack down during the first three runs and the top fittings of the columns had to be adjusted in order to avoid any dead space detrimental to resolution. The back pressure in the column for the separation of the acidic and neutral amino acids reached 600 p.s.i.

The new resin does not pack after loading and the back pressure in the columns has been reduced by about 50 % when resin beads of comparable size distribution are used. Its basic chemical composition remains unchanged, but the new resin has been subjected to a special hardening procedure. It shows excellent mechanical strength. The chromatographic performance of the improved resin is very similar to the original material (see Fig. 1). In order to improve the resolution of the basic amino acids, the resin bed in the short column was lengthened to 4.5 cm and the time for the whole chromatographic cycle extended to 65 min.

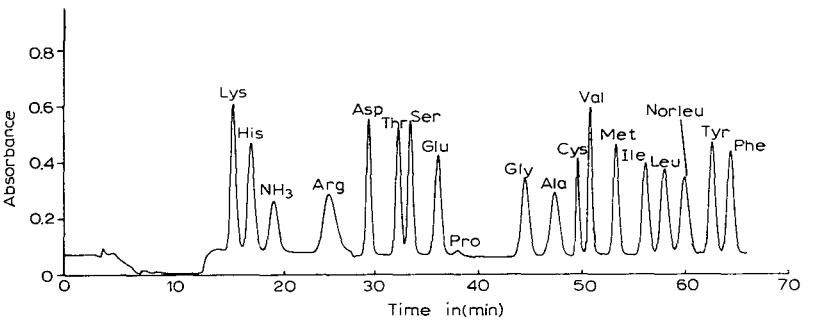

Fig. 1. Chromatogram of an amino acid standard representing one complete automatic cycle. The sample contained 0.03 μmoles of each amino acid. Absorbance read at 570 mμ.

The manifold

The complete flow diagram of the system manifold is represented in Fig. 2. The column effluents are first fed into a segmented stream of 0.2 *M* hydrazine sulfate solution. Regular segmentation with nitrogen can be provided by means of an 'air bar' or by a pressurized loop configuration in which nitrogen is pumped from a large pump tube into a small pump tube. Thus a gas bubble is released from the pressurized smaller tube whenever a roller bar of the peristaltic pump is lifted from the tubes. The peristaltic valve, the principle and function of which have been described previously[2], determines which of the two column effluents goes to the analytical system and which goes to waste. Valve positions 4 through 7 are assigned this control function. Only after passage through the valve is the column effluent combined with the ninhydrin reagent.

Buffer feed for the separation of the acidic and neutral amino acids to positive displacement pump 2 is controlled by valve positions 9, 10, 11, 13 and 14; in other words, in any valve position only one of these lines is opened. Positions 8 and 15 control the flow to the short column via positive displacement pump 1 (Fig. 2).

Another useful modification has been incorporated with the new procedure. The absorbance signal produced by the bulk of the acidic and neutral amino acids initially eluted from the short column was difficult to handle by an automatic integrator. Therefore, a segmented stream of water, instead of the column effluent, is now pumped into the analytical system until lysine, the first basic amino acid, is eluted from the column. Valve positions 16 and 17 were selected to perform this switch. Valve positions 18 to 20 control the flow of ninhydrin or wash solutions—sodium hydroxide and methylcellosolve—into the analytical system.

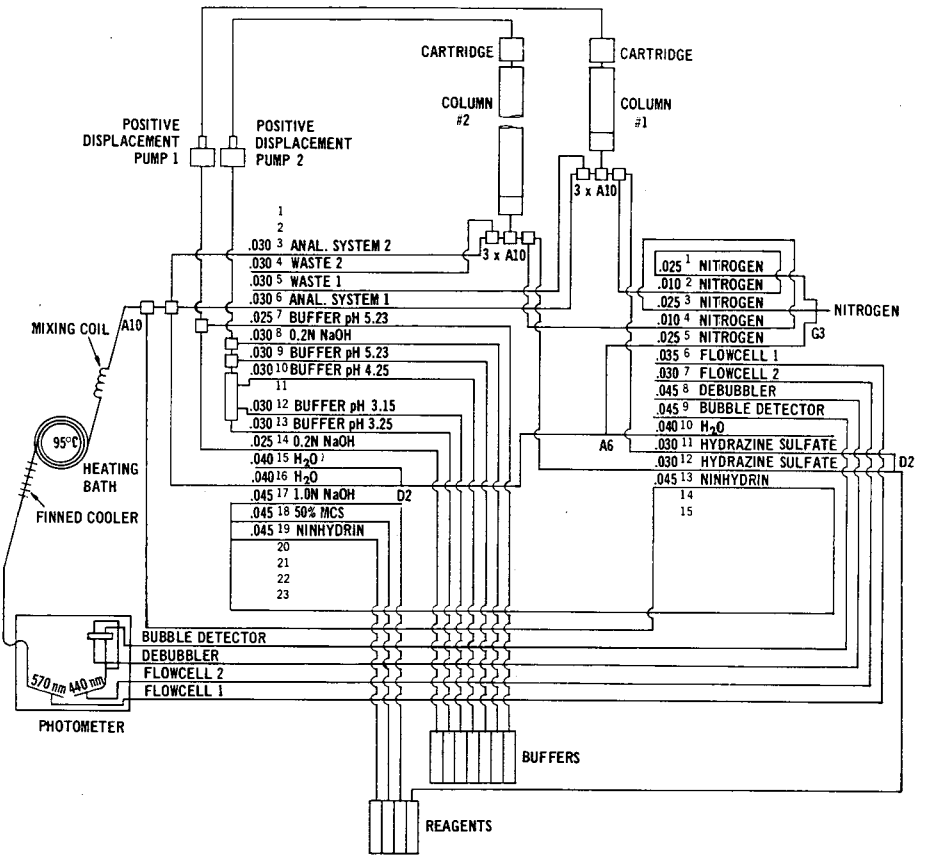

Fig. 2. Complete flow diagram of system. Manifold of peristaltic valve on the left, manifold of peristaltic pump on the right side. Internal diameter of pump tubes given in inches.

A standard heating bath coil of 12 m length and 1.6 mm I.D. is used for the development of the analytical reaction.

Precision data

The precision of the procedure was established with ten consecutive chromatograms. Samples were applied automatically and peak areas and retention times were determined with an electronic digital integrator (Table I).

For the identification of individual amino acids by means of retention time good precision of this parameter is essential. In order to establish confidence limits for a single measurement of retention time the standard deviation has to be multiplied with a factor τ which depends on the number of observations and the confidence limit desired. For ten observations and a confidence limit of 99 % (*e.g.* 99 % of all measured values will fall within this limit) the value for τ is 3.24. With the described system, when this test is applied to the most critical case, the identification of threonine and serine, the confidence limits do not overlap.

TABLE I

RT = retention time; σ_{RT} = standard deviation of retention time; C.V.(area) = coefficient of variation of peak areas; C.V.(peak height) = coefficient of variation of peak heights.

	RT (*sec*)	σ_{RT} (*sec*)	*C.V.* (*area*) (%)	*C.V.* (*peak height*) (%)
Lys	544	2.5	1.15	1.51
His	1048	3.6	0.63	1.13
Arg	1506	7.2	1.67	1.67
Asp	1763	6.2	2.51	2.07
Thr	1935	6.9	1.12	1.61
Ser	2006	7.5	1.40	1.86
Glu	2167	8.5	0.96	0.57
Gly	2680	12.2	0.60	1.98
Ala	2854	13.2	0.72	1.59
Cys	2973	7.3	1.74	4.14
Val	3047	8.6	1.93	2.00
Met	3200	9.5	1.42	3.08
Ile	3370	10.9	0.78	0.95
Leu	3481	11.8	0.59	0.64
Norleu	3601	13.5	1.46	1.98
Tyr	3757	10.7	1.44	1.71
Phe	3863	12.2	2.10	1.22

The data obtained for the retention times indicated that peak height precision should be good. The peak height precision was therefore calculated, and proved acceptable.

The system has been successfully operated for a period of several months and is now being field tested by a number of independent users.

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CHROM. 4328

Chromatography of fish pituitary extracts on Sephadex G-100

A significant advance in endocrinological research with chromatography has been made possible by the introduction of cross-linked dextran polymers known as Sephadex (Pharmacia, Uppsala, Sweden). The development of the technique of molecular sieving on Sephadex by PORATH AND FLODIN[1] and PORATH[2,3] offered a simple and rapid method for the fractionation of water-soluble substances. Since then Sephadex has been extensively used for the separation and purification of bovine growth hormone (DELLACHA AND SONENBERG[4]), human growth hormone (REISFELD *et al.*[5], ROOS *et al.*[6]) and porcine growth hormone (PAPKOFF *et al.*[7]). Purification of a gonadotrophic factor of the hypophysis of the carp (*Cyprinus carpio* L.) has been achieved by FONTAINE AND GERARD[8]. Recently in 1968 YAMAZAKI AND DONALDSON[9,10] purified salmon pituitary gonadotrophin by gel filtration on Sephadex G-100. In the present investigation, separation of the water-soluble protein of the pituitary extracts of some fish into different components has been attempted by gel filtration on Sephadex G-100. Fish at their different stages of gonadal maturation have been chosen for this. They also differed in their spawning habits, *i.e.*, puntius (*Puntius gonionotus* Bleeker) and tilapia (*Tilapia mossambica* Peters) spawn very easily in captivity whereas grass carp (*Ctenopharyngodon idellus* Cuv. and Val.), bighead carp (*Aristichthys nobilis* Richardson) and silver carp (*Hypophthalmichthys molitrix* Cuv. and Val.) do not spawn at all in captivity.

TABLE I

PROPERTIES OF THREE GRADES OF SEPHADEX

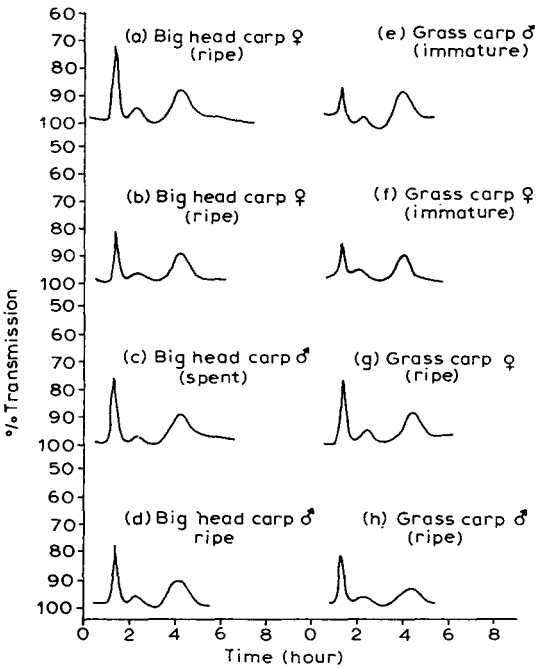
Type	*Water regain (g/g)*	*Size of molecule completely excluded (mol. wt.)*	*Suitable loading for fractionation (g/g dry gel)*	*Swelling factor (ml/g)*
G-50	5.0	8 000–10 000	0.18	10
G-100	10.0	100 000	—	20
G-200	20.2	200 000	0.18	40

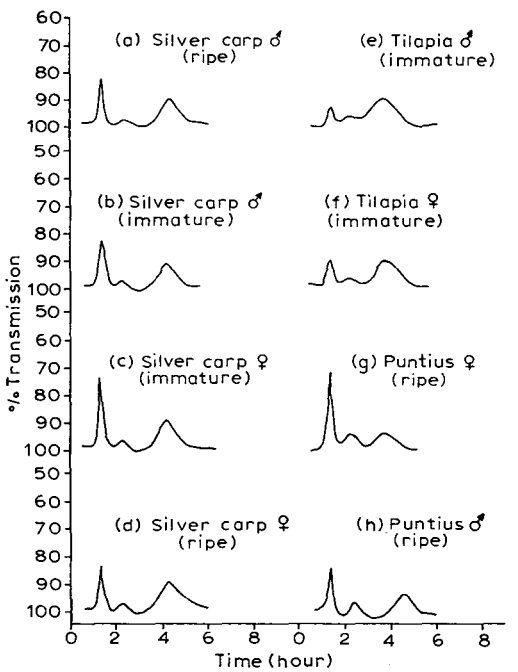
Pituitary glands of sexually immature and mature males and females of the above-mentioned fish and also of tilapia hybrid male (*Tilapia mossambica* Peters × *Tilapia hornorum* Trewavas) were homogenized with a Tri-R Tissue Homogenizer in the following eluants: 0.1 *M* sodium chloride; 0.02 *M* acetic acid (SANFELIPPO AND SURAK[11]); 0.2 *M* ammonium acetate (AURBACH AND POTT[12]); acetic acid–pyridine–water, 60:15:25 (PORATH AND LINDER[13]); phosphate–Tris–EDTA buffer (pH 4.0); lithium borate buffer, pH 8.6 (2.2 g lithium hydroxide and 23.75 g boric acid made 2 l with water). Then these were centrifuged in a Super Supragyro centrifuge at 4000 r.p.m. for about 15 min. The supernatant was directly applied to different Sephadex columns, 42 × 0.9 cm diameter, equilibrated with the particular eluants. Three different grades of Sephadex have been used, the properties of which are listed in Table I (after GORDON AND EASTOE[14]).

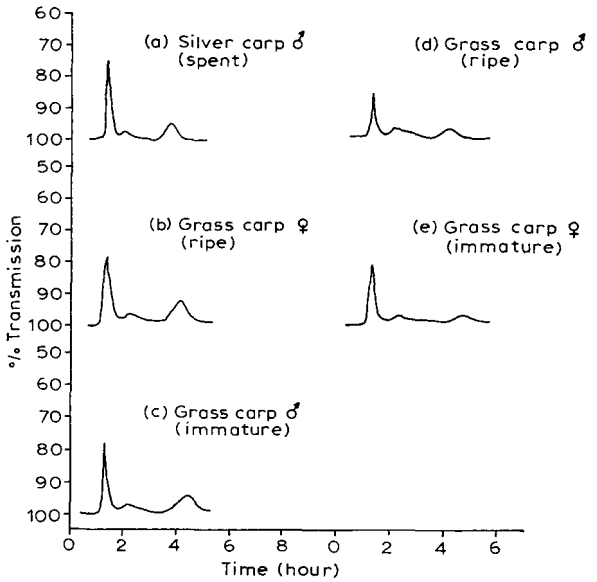
TABLE II

DETAILS OF THE FISH AND THEIR PITUITARY USED FOR CHROMATOGRAPHY

Fig. No.	*Eluent*	*Total dry weight of pituitaries (mg)*	*Fish*	*No. of fish*	*Range in length (cm)*			*Range in weight (g)*			*Sex*	*Condition of gonad*
					Min.	*Max.*	*Mean*	*Min.*	*Max.*	*Mean*		
1a	0.2 *M* ammonium acetate	6.7	Big head carp	1	—	—	75.5	—	—	4550	♀	Ripe
1b		4.8	Big head carp	1	—	—	64.5	—	—	4070	♀	Ripe
1c		5.0	Big head carp	1	—	—	64.0	—	—	3630	♂	Spent
1d		4.6	Big head carp	1	—	—	63.3	—	—	3290	♂	Ripe
1e		5.0	Grass carp	12	29.1	32.5	37.1	282	567	390.4	♂	Immature
1f		4.8	Grass carp	9				—	—		♀	Immature
1g		5.5	Grass carp	1	—	—	82.7	—	—	7390	♀	Ripe
1h		3.5	Grass carp	1	—	—	60.0	—	—	3010	♂	Ripe
2a	0.2 *M* ammonium acetate	4.5	Silver carp	1	—	—	60.0	—	—	2900	♂	Ripe
2b		3.5	Silver carp	1	—	—	53.0	—	—	2150	♂	Immature
2c		3.8	Silver carp	1	—	—	58.0	—	—	2600	♀	Immature
2d		4.4	Silver carp	1	—	—	58.3	—	—	2690	♀	Ripe
2e		8.2	Tilapia hybrid (*T. mossambica* × *T. hornorum*)	12	23.6	29.5	27.0	216	526	404.8	♂	Immature
2f		8.3	*Tilapia mossambica*	28	14.3	16.9	15.3	60	115	80	♀	Immature
2g		7.5	Puntius	6							♀	Ripe
2h		5.5	Puntius	6	24.1	30.5	27.2	183	409	281	♂	Ripe
3a	0.1 *M* sodium chloride	4.7	Silver carp	1	—	—	56.5	—	—	2540	♂	Spent
3b		5.5	Grass carp	1	—	—	77.0	—	—	5150	♀	Ripe
3c		4.0	Grass carp	1	—	—	68.0	—	—	3750	♂	Immature
3d		3.7	Grass carp	1	—	—	65.0	—	—	2930	♂	Ripe
3e		3.4	Grass carp	1	—	—	56.0	—	—	2970	♀	Immature


(a) Big head carp ♀ (ripe)
(e) Grass carp ♂ (immature)
(b) Big head carp ♀ (ripe)
(f) Grass carp ♀ (immature)
(c) Big head carp ♂ (spent)
(g) Grass carp ♀ (ripe)
(d) Big head carp ♂ ripe
(h) Grass carp ♂ (ripe)
% Transmission
Time (hour)


(a) Silver carp ♂ (ripe)
(e) Tilapia ♂ (immature)
(b) Silver carp ♂ (immature)
(f) Tilapia ♀ (immature)
(c) Silver carp ♀ (immature)
(g) Puntius ♀ (ripe)
(d) Silver carp ♀ (ripe)
(h) Puntius ♂ (ripe)
% Transmission
Time (hour)



Figs. 1–3. Sephadex G-100 chromatograms of fish pituitary extracts.

Mixtures of these in different proportions were also tried, with no success, to get better separation. The best separation was obtained with Sephadex G-100 alone.

Elution was carried out at room temperature at about 10 ml/h and was recorded with a LKB 4701 A Uvicord recorder at 253.7 nm at a recording speed of 10 mm/h. Of all the eluants tried, the best separations were obtained with 0.2 *M* ammonium acetate and 0.1 *M* sodium chloride. 0.02 *M* acetic acid did not give sharp peaks nor did the phosphate–Tris–EDTA buffer. With pyridine the recorder did not work. The fractions of the pituitary extracts eluted with lithium borate buffer were again examined by horizontal electrophoresis to see whether they further resolve into more components. But unfortunately no bands were obtained.

For most of the mature and big fish, an individual pituitary was usually sufficient for one run, but when it was not sufficient, pituitaries from a few fish of the same sexual developmental stage were mixed together for the run. Details on length, weight and gonadal condition are given in Table II.

Pituitary extracts showed positive ninhydrin reaction and so did the different eluted fractions. Figs. 1–3 show the chromatograms of the pituitary extracts. These clearly indicate that the water-soluble protein of the pituitary extracts has been fractionated into three distinct components. The biological activity of these fractions is being tested on fish and other animals. This study will be described in detail elsewhere (SINHA[15]).

Thus there are three eluted peaks all of which show very close resemblance in all the recordings, despite the fact that they are of different fish, of different spawning habits and of different sex, at their different stages of gonadal maturation. Because of the differences in the weight of the sample of the pituitary, no direct comparison can be made of the amount of the different eluted peak component at different stages. Yet it seems obvious that the difference may be purely quantitative, *i.e.*, different

amount of a single component, or partly qualitative, *i.e.*, difference in the relative amount of components. It is perhaps because of this similarity that very high percentages of positive results have been obtained when pituitary materials were injected in experimental fishes even though the donor and recipient were of different species (HASLER *et al.*[16]; CLEMENS AND SNEED[17]; YAMAZAKI AND DONALDSON[9,10]; YASHOUV *et al.*[18]).

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CHROM. 4326

Gel filtration chromatography for the quantitative purification and stabilization of cellulases of turbid solutions

The evaluation of cellulase activity of rumen liquor, silage extracts and some filtrates of fungal growth media (crude enzyme preparations) is complicated by the turbidity of the solutions. The turbidity interferes with established colorimetric enzyme assays. Purification of the assay medium subsequent to the incubation and prior to the colorimetric reducing sugar determination[1,2], although possible[3,4], was found to be too cumbersome for routine analysis. Instead we adapted existing qualitative gel filtration chromatographic techniques[5] to the quantitative purification of turbid crude enzyme preparations.

Experimental

Columns consisted of 4.5 × 15 cm precision bore glass tubing with sintered glass retaining plate. The tubing was collapsed below the sintered glass plate to give minimum hold-up of liquid. A 2 mm layer of silver sand was placed on the sinter and the columns were filled with suspensions of swollen Sephadex G-75 (bead form 65–100 mesh). The bed was allowed to settle at a buffer flow rate of 10 ml/h. The operating buffer flow rate was 20 ml/h and the columns were located in a room at 21° ± 1°. The load maximum was 3.5 mg of protein. A sodium phosphate buffer, pH 6.3, was used as eluting solvent (0.01 *M* in total phosphate, 0.1 *M* in NaCl and 0.005 *M* in NaN_3). The cellulase activity eluted in the 30–80 ml elution fraction. For routine use fractions were collected in graduate cylinders. At the completion of the cellulase elution, the columns were regenerated by increasing the flow rate to maximum possible. Regeneration was completed in 90–120 min.

Cellulase activity of the crude enzyme preparation was established by the method of FELSTENSTEIN[6] using ion-exchange columns[7] to purify the assay medium after incubation. The crude enzyme preparations were applied to the column. In some instances purified C_1 (activity toward insoluble cellulose) and C_x cellulase[8] (activity towards soluble cellulose) (*Trichoderma viride*) were added. The results are shown in Table I.

TABLE I

RECOVERY OF CELLULASE ACTIVITY FROM GEL FILTRATION COLUMNS

Source of crude enzyme preparation	*Amount of protein applied to column (mg)*	*Cellulase activity applied to column*		*Recovery of cellulase activity*	
		C_x units[b]	*C_1 units*[b]	*C_x*	*C_1*
Filtered rumen	3.1	42.3	5.0	97	98
liquor	1.1	17.6	1.3	96	93
Aqueous silage	3.4	21.1	16.2	103	102
extracts	2.0	13.0	11.8	104	100
Trichurus cylindricus	2.9	165.2	100.0	97	101
culture filtrate[a]	0.5	37.4	17.1	94	98

[a] A 6 day culture was used, wheat bran being the major growth substrate. The filtrate was dark brown and turbid.

[b] For definition of units, see ref. 8.

Results

The recovery of cellulase activity from the columns was complete. Added activity was also recovered quantitatively. The method is applicable to protein solutions having a high concentration of cellulase activity as well as to those having low cellulase activity. The phosphate ions did not interfere with the incubation nor with the color development. In general the level of activity in the eluted fraction was sufficiently high, eliminating the need for the concentration of the eluent. When concentration and removal of buffer salts was necessary, a Diaflo ultra filtration cell (Amicon Corporation) was used. The columns have been reused for a number of months and no degeneration has been observed.

The C_1 cellulase activity of filtered rumen liquor was reported to be low and variable[6]. In our preliminary studies[9] the instability of the C_1 enzymes was recognized. Purification by gel filtration stabilized the activity. Speed in filtration and application to the column was essential. Three day storage of purified rumen cellulase preparations was facilitated. The lyophilized enzyme preparation appears to be retaining full activity for indefinite periods of time.

The procedure appears lengthy when viewed in terms of time which elapses between application of the crude enzyme preparation and obtaining the purified enzyme solution, however, the actual working time in fact is small. The columns are of simple, inexpensive construction and a number of them can be set up requiring the part-time attention of a single operator only. Purification of the assay medium subsequent to incubation by ion-exchange chromatography or chemical manipulation of a much larger number of samples (*e.g.* assays are usually carried out in multiples) was found to be much more time consuming, and in the case of chemical manipulation, much less accurate. Besides, the advantage of stabilizing the cellulases from rumen fluid would not be realized.

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CHROM. 4329

A simple thin-layer chromatography method for separating the ergotoxine alkaloids

Paper chromatography with reversed phase technique has been successfully employed for the separation of the ergotoxine components[1-3]. SCHLIENTZ *et al.*[1] have also reported the separation of α-ergokryptine from β-ergokryptine by such a method. HOHMANN AND ROCHELMEYER[4] employed cellulose thin layers impregnated with formamide for separation of an ergotoxine mixture, while PROCHÁZKA *et al.*[5] could separate these ergopeptides by wide-porous silica gel thin layers that were treated with formamide and made alkaline with ammonia. MCLAUGHLIN *et al.*[6] reported that thin-layer plates with Aluminum Oxide G as the stationary phase separated ergocornine from ergokryptine.

Silica Gel G on thin-layer plates with fluorobenzene solutions as the mobile phase were tested for their ability to separate the ergotoxine components. The procedure and results are described.

Experimental

Hexafluorobenzene and pentafluorobenzene were obtained from Imperial Smelting Corp. (N.S.C.) Ltd., Bristol, England. Ergocornine, ergocristine, ergocristinine, α-ergokryptine and $\alpha(\beta)$-ergokryptine were a gift from A. Hofmann, Sandoz AG, Basel, Switzerland. An ergotoxine mixture was obtained locally. All other chemicals were reagent grade.

Glass sheets 20 × 20 cm or 20 × 40 cm were employed. Silica Gel G according to Stahl was applied at 0.25 mm thickness. Sample solution (prepared: 2 μg alkaloid/μl $CHCl_3$) was applied at the origin in quantities of 2 to 8 μg.

Glass chromatography chambers were lined with No. 3 Whatman chromatography paper that was thoroughly saturated with the solvent system. Ascending development was employed. Development time varied from 7 h for the 40 cm plates with the hexafluorobenzene mobile phase to 2.7 h for the 20 cm plates with the pentafluorobenzene mobile phase.

The composition of each mobile phase was hexafluorobenzene or pentafluorobenzene–dimethylformamide–absolute ethanol (13:1.9:0.1). Both solutions may be used many times over a period of weeks without loss of resolution.

The ergotoxine components on the developed plates were detected by their fluorescence in UV light and by their staining blue with Ehrlich's reagent (5% *p*-dimethylaminobenzaldehyde in concentrated HCl).

Results and discussion

The R_F values of the ergotoxine components are shown in Table I. The hexafluorobenzene mobile phase separated $\alpha(\beta)$-ergokryptine into two components. It also separated the ergotoxine mixture and the standard blend into three components with R_F values the same as pure ergocristine, ergocornine and ergokryptine. However, pentafluorobenzene mobile phase did not resolve ergocornine from ergokryptine. Both systems separated the pharmacologically active ergotoxines from the inactive, stereoisomeric ergotinines.

TABLE I

SILICA GEL G THIN-LAYER CHROMATOGRAPHIC SEPARATION OF ERGOTOXINE AND ERGOTININE COMPONENTS

Solvent systems: I = Hexafluorobenzene–dimethylformamide–ethanol (13:1.9:0.1). II = Pentafluorobenzene–dimethylformamide–ethanol (13:1.9:0.1).

Alkaloid	R_F *value*	
	I	*II*
Ergocristine	0.26	0.44
α-Ergokryptine	0.30	0.48
β-Ergokryptine	0.23	0.48
Ergocornine	0.33	0.48
Ergocristinine	0.35	0.71
Ergokryptinine	0.43	0.82
Ergocorninine	0.40	0.78

By employing easily prepared Silica Gel G thin layers on plates 40 cm in length with the hexafluorobenzene mobile phase very small quantities of ergotoxine may be resolved into its components. Developed plates dry well at room temperature without any fan. Spots on the chromatogram are almost as small as the original spot, and there are no diffuse borders or tailing.

While pentafluorobenzene mobile phase does not separate the ergotoxine components, it is useful in separating the ergotoxines from other alkaloids such as ergotamine (R_F 0.20) and ergosine (R_F 0.25) that are commonly found in the same culture or sclerotium.

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Editor's note: The literature cited is incomplete. Complete lists can be found in *Bibliography of Paper and Thin-layer Chromatography*, (special volume of the *Journal of Chromatography*).

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CHROM. 4333

The detection of steroids by silicotungstic acid on Gelman sheets

Silicotungstic acid was first reported as a spray reagent for steroids by KRITCHEVSKY AND KIRK[1]. Only infrequent use was made of the reagent, probably because of the degradation of paper accompanying heating of the sprayed chromatogram[2,3]. Thin-layer adsorbents are generally acid stable, however, so a reexamination of the utility of this reagent seems appropriate.

Methods and materials

The stationary phases for these studies included Gelman instant thin-layer chromatography (ITLC) type S.A., S.G., and A. Chromatograms were run in Gelman ITLC chambers. Development reagents used were 15 % w/v of silicotungstic acid (Bakers Analyzed Reagent) in water, and 15 % w/v of phosphotungstic acid (Mallinckrodt Analytical Reagent) in water. The ITLC sheets were developed by dipping the sheets in the reagent, then heating 15 min at 110° in the oven. The sheets were spotted with 2.0 μl pipets. The solvent system was chloroform–acetone (30:1) (both Baker Analyzed Reagent). Hydrocortisone, estradiol, progesterone, testosterone, estrone, cortisone, estriol, and pregnanediol, were donated by Parke-Davis Research Labs, Ann Arbor, Mich. These were dissolved in carefully redistilled dioxane.

Results

The nature of the adsorbent had little or no effect on the sensitivity of the reagent. A comparison of the sensitivity for various steroids on the various adsorbents of silicotungstic and phosphotungstic acids is shown in Table I. Sheets were heated to maximum color development (15 min, 150°) and the color faded on additional heating. Colors produced are shown in Table II. The reagents were prepared at concentrations of 0.1, 1, 5, 10, 15, and 25 % w/v and compared. A concentration of 15 % was sufficient for optimum development. R_F values in the chosen solvent system are indicated on Table III. Without activation, the R_F values on Type A were too high to be useful.

TABLE I

SENSITIVITY

Steroid	*Minimum amount detected (g)*	
	Silicotungstic acid	*Phosphotungstic acid*
Hydrocortisone	2.4×10^{-7}	2.4×10^{-7}
Estradiol	2.4×10^{-7}	2.4×10^{-7}
Progesterone	2.4×10^{-7}	2.4×10^{-7}
Testosterone	2.4×10^{-7}	2.4×10^{-7}
Estrone	6.0×10^{-8}	2.4×10^{-7}[a]
Cortisone	2.4×10^{-7}	5.0×10^{-7}
Estriol	2.0×10^{-7}[a]	5.0×10^{-7}
Pregnanediol	2.4×10^{-7}	2.4×10^{-7}

[a] 8.0×10^{-8} g were detected on the Type A sheets.

TABLE II

COLOR WITH SILICOTUNGSTIC ACID

Steroid	*Type SA*	*Type SG*	*Type A*
Hydrocortisone	chocolate brown	grey	chocolate brown
Estradiol	red-tan	red-brown	red-brown
Progesterone	yellow-brown	grey	grey
Testosterone	yellow-brown	grey	grey
Estrone	orange-brown	red	red
Cortisone	yellow-brown	grey	yellow-brown
Estriol	red-brown	red	red
Pregnanediol	yellow-brown	grey	grey

TABLE III

R_F VALUES[a]

Steroid	*Type SA inactivated*	*Type SA activated*[b]	*Type SG inactivated*	*Type SG activated*[b]	*Type A activated*[b]
Hydrocortisone	0.01	0.01	0.10	0.12	0.02
Estradiol	0.34	0.35	0.81	0.90	0.68
Progesterone	0.72	0.70	0.95	0.95	0.95
Testosterone	0.35	0.34	0.83	0.82	0.85
Estrone	0.64	0.60	0.95	0.93	0.82
Cortisone	0.05	0.04	0.28	0.26	0.00
Estriol	0.01	0.02	0.10	0.15	0.01
Pregnanediol	0.20	0.16	0.67	0.64	0.62

[a] Using chloroform–acetone (30:1).
[b] Activated by heating 45 min at 150°.

Discussion

It is apparent that silicotungstic acid, which has been criticized as low in sensitivity on cellulose paper[2], is equally as good as the commonly employed phosphotungstic acid on thin-layer plates. Characteristic colors produced by the various steroids should prove useful in establishing the identity of the compounds under study.

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CHROM. 4246

Polyamide layer chromatography

XXII. Separation of quinones

In 1969, ENDRES[1] showed that the quinoid compounds could not be separated by polyamide powder column chromatography because of their irreversible sorption to polyamide powder. Then the powder was acetylated and it was shown that this powder could be used to separate the quinones. Later, GRAU AND ENDRES[2] used acetylated polyamide powder to prepare thin-layer plates on which several quinones, including benzoquinone, naphthoquinone and anthraquinone, could be separated.

Contrary to ENDRES' work, EGGER AND KLEING[3] separated the lipoquinones without difficulty by free polyamide thin-layer chromatography. They showed the excellence of the method by separating ubiquinones differing in two isoprene units. CHAN AND CROW[4] also separated quinoid pigments from insects by free polyamide thin-layer chromatography and stated that this was an excellent medium for polyhydroxyanthraquinones.

We acetylated the polyamide layer prepared according to WANG AND WANG[5] and compared it with free polyamide layers using identical solvent systems.

Materials and methods

Polyamide layers were prepared according to WANG AND WANG[5]. All reagents and solvents were Wako fine chemicals (Osaka, Japan).

One milligram of each quinone was dissolved in 1 ml of methanol, and an aliquot of this sample solution was transferred to the polyamide layer.

Acetylation of polyamide layers (ref. 6). Twenty milliliters of acetic anhydride were mixed with 7 ml of anhydrous pyridine and this solution was poured into a porcelain dish (diameter 20 cm). A part of the polyamide layer (15 × 15 cm) was soaked in the solution for 5 min, hung at room temperature for one day and finally dried at 70° for 30 min. Then the layer could be used or stored. There were no apparent changes even after 3 months of storage.

The completeness of the acetylation was checked by spraying the layer with ninhydrin solution[7]. Before acetylation the layer gave a violet color when sprayed with ninhydrin solution, while a completely acetylated layer showed no color change. The terminal amino group of the ε-polycaprolactam chain is responsible for the ninhydrin color reaction.

Chromatography. The chromatographic techniques were the same as those used in the earlier work[5].

Detection. The detection was made by UV irradiation (254 mμ) to identify naphthoquinone and anthraquinone as quenched spots. Then the chromatogram was sprayed with concentrated ammonia and irradiated with UV light which showed the benzoquinone as a quenched spot.

Results and discussion

In Figs. 1 and 2 the photographs of chromatograms of three quinones on acetylated and non-acetylated polyamide layers are shown, while Table I shows the

TABLE I

R_F VALUES OF QUINONES

Solvent systems: 1, methanol–water (9:1); 2, acetone–water–acetic acid (20:2.5:0.5); 3, ethyl acetate–acetic acid–carbon tetrachloride (18:2:6); 4, water–acetic acid (9:1); 5, ethyl acetate–acetic acid (9:1); 6, *n*-hexane–chlorobenzene–acetic acid (4:1:0.5); 7, methanol; 8, methanol–water (1:1). A, free polyamide layer; B, acetylated polyamide layer. T = tailing.

	Solvent															
	1		*2*		*3*		*4*		*5*		*6*		*7*		*8*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Benzoquinone	0.82	0.85	0.85	0.85	0.88 T	0.87 T	0.79	0.81	0.95	0.96	0.41	0.36	0.67	0.67	0.38	0.43
Naphthoquinone	0.71	0.72	0.67	0.67	0.91	0.92	0.20 T	0.20 T	0.96	0.97	0.70	0.70	0.71	0.77	0.42	0.48
Anthraquinone	0.48	0.46	0.35	0.22	0.85	0.85	0.02	0.02	0.93	0.95	0.70	0.71	0.57 T	0.58 T	0.07 T	0.08 T

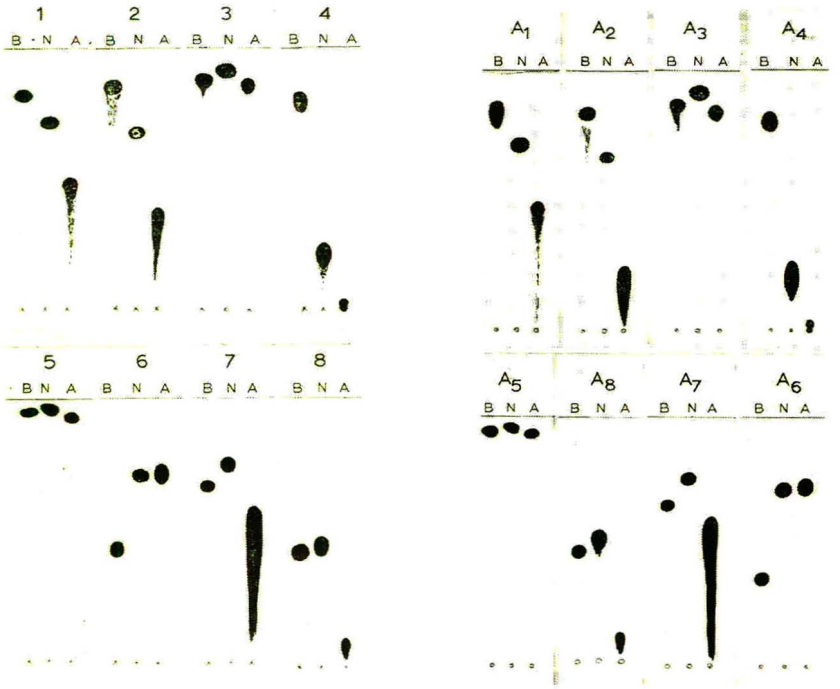

Fig. 1. Chromatograms of three quinones on acetylated polyamide layers. The numbers on the plates are the same as those in Table I. B = benzoquinone; N = naphthoquinone; A = anthraquinone.

Fig. 2. Chromatograms of three quinones on free polyamide layers. For explanation see Fig. 1.

R_F values of the quinones in eight solvents. The R_F values are almost identical on both types of layers. The tailing is not influenced by the acetylation. If the solvent system is suitable, the spots are circular on both types of layers. Our results are consistent with those of EGGER AND KLEING[3] and CHAN AND CROW[4] showing that free polyamide can be used for the separation of quinones.

Finally it should be pointed out that solvent 4 gives a good separation of the three quinones although naphthoquinones tail. Separation by two-dimensional development is also possible.

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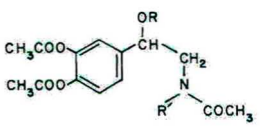
Thin-layer chromatography of some acetylated adrenaline derivatives*

In view of the importance of adrenaline and its derivatives, in biology and medicine, considerable interest has developed, during the past two decades, in chromatographic procedures for the separation and identification of these compounds. The problems encountered in the chromatography of these substances arise from many sources. In general, catecholamines such as adrenaline are insoluble in non-polar solvents, and their solutions in aqueous media tend to be unstable under both alkaline and acidic conditions. Several attempts have been made, in the past, to overcome these problems by the formation of suitable derivatives, in which the phenolic hydroxyl and the amine groups are protected.

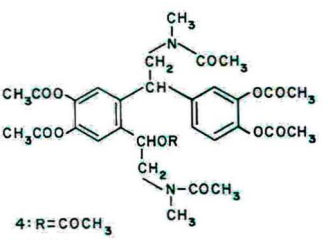
The trimethylsilyl derivatives, which have been extensively used in gas chromatographic studies with the catecholamines (*cf.* review by PIERCE[1]), and the trifluoroacetates[2] are sensitive to moisture and hydroxylic solvents and as such are unsuitable for paper or thin-layer chromatography. The dansyl derivatives of a number of phenylalkylamines have recently been prepared for both mass spectroscopy and TLC[3,4]. Whilst fairly extensive use has been made of the acetyl derivatives of a number of biogenic amines for paper and gas chromatographic studies[5–10], the TLC properties of these derivatives do not appear to have been studied extensively. WALDI has reported a procedure in which adrenaline, noradrenaline and other arylalkylamines can be chromatographed on silica gel layers after acetylation[11]; the method was adapted to the quantitative estimation of these compounds[11]. Procedures for the TLC of the acetyl derivatives of tyramine and dopamine have also been reported[12,13].

During the course of recent investigations[14,15] in these laboratories into the action of strong acids on catecholamines, such as adrenaline (*cf.* refs. 16 and 17),



1: R=H; R'=CH_3
2: R=$COCH_3$; R'=CH_3
6: R=R'=H

3



4: R=$COCH_3$
5: R=H

* Issued as NRCC No. 10974.

the acetyl derivatives of a number of phenylalkylamines including triacetyladrenaline (1), tetraacetyladrenaline (2), pentaacetyladnamine (3), heptaacetyladrepine (4), hexaacetyladrepine (5) and triacetylnoradrenaline (6) were synthesized. This communication reports the results of some investigations into the TLC properties of these compounds.

Experimental

Acetyl derivatives

The acetyl derivatives were prepared by the methods described in the literature: triacetyladrenaline (1)[18]; tetraacetyladrenaline (2)[19]; pentaacetyladnamine (3)[20]; heptaacetyladrepine (4)[14,15]; hexaacetyladrepine (5)[14,15] and triacetylnoradrenaline (6)[21].

Thin-layer chromatographic procedure

Plates. Commercially available Merck Silica Gel F-254 plates (20 × 20 cm) (thickness, 0.25 mm) were used.

Solvents. S_1: *n*-Butyl acetate–methanol (8:2);
S_2: Benzene–methanol (8:2);
S_3: *n*-Butyl acetate–methanol (9:1);
S_4: Chloroform–acetone (1:1);
S_5: Cyclohexane–acetone (3:7);
S_6: *n*-Butanol–acetic acid–water (4:1:1);
S_7: Chloroform–pyridine (9:1).

Detection. (a) The developed chromatoplates were exposed to ammonia fumes for 30–60 min. The acetyl derivatives 1, 2, 4, 5 and 6 were detected as dark brown spots on a colourless background. Pentaacetyladnamine (3) gave a reddish-brown spot.

(b) The developed chromatoplates were sprayed with 40 % (v/v) orthophosphoric acid. The plates were then heated at 110° for 7 min; cooled, sprayed with 5 % ethanolic phosphomolybdic acid and heated at 110° for 5 min. The acetyl derivatives were observed as greyish-brown spots on a pale blueish-green background (*cf.* WALDI[11]).

Results and discussion

The R_F values of the acetyl derivatives 1–6 in the seven different solvent systems

TABLE I

THIN-LAYER CHROMATOGRAPHY OF SOME ACETYLATED ADRENALINE DERIVATIVES (R_F VALUES)

Compound No.	*Average R_F value*[a] *in solvent system*						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
1	0.55	0.58	0.37	0.49	0.51	0.76	0.41
2	0.61	0.65	0.46	0.69	0.69	0.75	0.79
3	0.69	0.67	0.51	0.70	0.72	0.85	0.76
4	0.46	0.49	0.21	0.34	0.32	0.70	0.40
5	0.38	0.45	0.14	0.13	0.14	0.75	0.10
6	0.56	0.47	0.35	0.38	0.49	0.78	0.14

[a] R_F values obtained on silica gel plates (thickness = 0.25 mm).

S_1–S_7 are given in Table I. It can readily be seen from Table I that by selecting a suitable solvent system satisfactory separations of the compounds can be obtained. Acetylation of adrenaline often leads to mixtures of products including the tri- and tetraacetyl derivatives 1 and 2; the solvent systems S_4, S_5 and S_7 readily separate such mixtures. Similarly acetylation of adrepine often gives mixtures of the hepta- and hexaacetyl derivatives 4 and 5 which are well differentiated by systems S_4, S_5 and S_7.

Both methods described for locating the spots on the developed chromatoplates work satisfactorily for all the compounds investigated. The ammonia-fuming method, although slow, offers the advantage of simplicity. The acetyl groups are presumably removed by ammonolysis to give the free catechol derivatives which readily undergo oxidation to dark coloured products. Chromogenic reagents, such as alkaline potassium ferricyanide, which are commonly used for the detection of catecholamines can also be used for the detection of the acetyl derivatives, after preliminary fuming with ammonia.

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CHROM. 4325

Separation of the herbicide C-6989 and some related compounds by thin-layer chromatography

p-Nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether (C-6989) (Fig. 1) is a new pre-emergent herbicide which has shown promise in soybeans[1,2] and peanuts[3]. It is active against a wide variety of annual broadleaf and grass weeds.

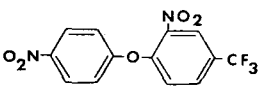

Fig. 1. Structure of C-6989.

Prior to conducting metabolic studies of C-6989 in plants, it was necessary to investigate the chromatographic behavior of this compound and its possible metabolites. Optimum separation of C-6989 and seven possible metabolites was obtained using two-dimensional thin-layer chromatography on Eastman Chromagram Sheet 6060, developing twice in benzene in one direction and twice in benzene–1,2,3-trichloropropane (3:2) in the perpendicular direction.

The compounds were detected either as colored spots or as blue absorbing spots when the sheet was exposed to short wavelength UV radiation (254 mμ).

Experimental

Thin-layer sheets precoated with 100 μ silica gel with fluorescent indicator (Eastman Chromagram Sheet 6060) were used throughout the study.

Ten micrograms of C-6989 and the seven possible metabolites were each spotted 2 cm from the bottom of the sheet and developed in one of the following solvent systems: (I) benzene, either once or twice; (II) benzene–1,2,3-trichloropropane (3:2), either once or twice; (III) chloroform; (IV) benzene–1,2-dichloroethane (1:1). The chromatograms were dried at room temperature in a forced air hood.

For two-dimensional chromatography, a mixture of 5 μg of each of the eight compounds was spotted 2 cm from the right edge and 2 cm from the bottom of the sheet and developed twice in solvent I, then turned 90° and developed twice in solvent II.

Results and discussion

Table I shows the colors of the eight compounds and their approximate R_F values in the various solvents used. The detection limit with the UV light was less than 1 μg.

Fig. 2 illustrates the results of the two-dimensional technique which is being used with metabolic studies of C-6989 in this laboratory.

When radioactive C-6989 is used in studies the separated compounds are eluted and the radioactivity quantified using liquid scintillation counting or some other method. In the case of concentrations below the visible detection limits, the radioactive spots are located by exposing the sheet to X-ray film for a specified time (two

TABLE I

COLOR AND R_F VALUES OF C-6989 AND SEVEN POSSIBLE METABOLITES IN FOUR SOLVENT SYSTEMS

Solvent systems: I = Benzene.
II = Benzene–1,2,3-trichloropropane (3:2).
III = Chloroform.
IV = Benzene–1,2-dichloroethane (1:1).

No.	*Compound*	*Color*	*Solvent system*					
			I (once)	*I (twice)*	*II (once)*	*II (twice)*	*III*	*IV*
1	*p*-Nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether (C-6989)	None	0.58	0.72	0.68	0.82	0.70	0.54
2	*p*-Nitrophenyl-α,α,α-trifluoro-2-amino-*p*-tolyl ether	None	0.36	0.57	0.59	0.76	0.68	0.45
3	2-Nitro-4-trifluoromethylphenol	Light yellow	0.33	0.56	0.51	0.71	0.52	0.42
4	*p*-Aminophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether	Light brown	0.16	0.36	0.40	0.59	0.51	0.30
5	*p*-Aminophenyl-α,α,α-trifluoro-2-amino-*p*-tolyl ether	Light brown	0.11	0.24	0.30	0.45	0.41	0.17
6	*p*-Nitrophenol	Yellow	0.05	0.12	0.17	0.24	0.15	0.08
7	2-Amino-4-trifluoromethylphenol	Light brown	0.04	0.08	0.05	0.09	0.11	0.08
8	*p*-Aminophenol	Brown	0.00	0.03	0.05	0.05	0.04	0.03

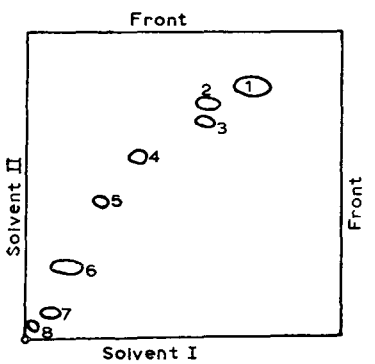

Fig. 2. Two-dimensional thin-layer chromatogram of C-6989 and seven possible metabolites. Solvent systems: (I) benzene (twice); (II) benzene–1,2,3-trichloropropane (3:2) (twice). Compounds: see Table I for identification.

weeks in this laboratory) and development of the X-ray. The spots are then located and eluted for quantification.

The compounds used in this study were supplied by CIBA Agrochemical Company, Division of CIBA Corporation, Vero Beach, Fla.

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Biochemistry of sphingolipids

XXII. Paper chromatographic separation of different types of cerebrosides and oligoglycosylceramides

In a previous paper[1] we described some new paper chromatographic systems for the characterization of glycosphingolipids. Some of these systems were applied to the identification of these substances in biological material[2–7].

As can be seen from the literature only a few reports on paper chromatography of sphingolipids have been published[8–15].

The main purpose of the present study is the characterization of different groups of glycosphingolipids by this technique.

Experimental

Isolation and column chromatography of sphingolipids. For the isolation of sphingolipids, various human and mammalian body tissues (*e.g.* brain, kidney, spleen, liver, aorta) and fluids (blood serum, erythrocytes, milk) were extracted by the procedure described by SUZUKI[16]. The lower phase of the extracts was hydrolyzed with 1 *N* methanolic potassium hydroxide for 48 h at 38°. The mixture of crude sphingolipids was isolated and fractionated on Florisil, DEAE-cellulose and silicic acid columns, and the fractions containing sphingolipids were further purified by column chromatography or preparative thin-layer chromatography.

Paper chromatography. Three types of impregnated paper were used:

(a) Commercial silica gel paper—Schleicher and Schüll No. 288.

(b) Whatman No. 3 paper impregnated with silica gel[17].

(c) Commercial silica gel paper—Schleicher and Schüll No. 288 impregnated with 0.05 *M* sodium tetraborate[18,19].

Solvent systems. These were as follows:

System 1: chloroform–methanol–96 % ethanol–water (120 : 16 : 4 : 2).
System 2: chloroform–methanol–water (120 : 16 : 1).
System 3: chloroform–methanol–water (120 : 18 : 1).
System 4: chloroform–methanol–water (120 : 20 : 1).
System 5: chloroform–methanol–water (120 : 25 : 2).
System 6: chloroform–methanol–12.5 % aqueous ammonia (100 : 30 : 4)
System 7: tetrahydrofuran–diisobutylketone–water (45 : 9 : 4).
System 8: chloroform–acetone–acetic acid (120 : 32 : 4).
System 9: chloroform–acetone-acetic acid (95 : 57 : 4).
System 10: chloroform–acetone–acetic acid (104 : 48 : 4).
System 11: chloroform–methanol–acetic acid (95 : 57 : 10).
System 12: chloroform–acetone–propionic acid (67 : 72 : 10).

Further experimental modifications of the above mentioned solvent systems are given in Table I.

The samples were spotted as a narrow line (1–3 cm) on chromatograms (18 × 18 cm) at a distance of 1–1.5 cm from the end of the paper and developed in an appropriate solvent system.

TABLE I

EXPERIMENTAL CONDITIONS FOR PAPER CHROMATOGRAPHY OF DIFFERENT TYPES OF CEREBROSIDES AND OLIGOGLYCOSYLCERAMIDES

Solvent system	*Designation in Figs. 1, 4 and 6*	*Technique*	*Type of paper*	*Solvent front (cm from the starting line)*	*Remarks*
1	I	ascending	b	15	unsaturated chamber
2	II	ascending	a	15	unsaturated chamber
3	III	ascending	a	15	unsaturated chamber
4	IV	ascending	a	15	unsaturated chamber
5	V	ascending	a	15	unsaturated chamber
6	VI	ascending	a	15	unsaturated chamber
7	VII	ascending	a	15	unsaturated chamber
3	VIII	horizontal	a	25	unsaturated chamber
8	IX	ascending	b	15	unsaturated chamber
9	X	ascending	b	15	unsaturated chamber
9	XI	ascending	a	15	unsaturated chamber
10	XII	ascending	b	15	unsaturated chamber
11	XIII	ascending	a	15	unsaturated chamber
12	XIV	ascending	a	15	chamber saturated overnight
11	XV	ascending	a	—	4 h development
3	XVI	ascending	c	15	unsaturated chamber
5	XVII	ascending	c	15	unsaturated chamber
2	XVIII	horizontal	c	25	unsaturated chamber

Detection. The following reagents were used:
(a) 0.001% Rhodamine B in 0.25% *M* K_2HPO_4[1].
(b) Chlorine–benzidine–potassium iodide reagent[1].
(c) 0.02% Cresyl violet in 1% acetic acid[1].
(d) 0.05% Pinacryptol yellow in water[1].
(e) Periodic acid–Schiff's reagent[19].

Results and discussion

A schematic representation of the chromatographic behaviour of the different types of glycosphingolipids in the solvent systems studied is given in Figs. 1, 4 and 6.

Systems I–VIII (Fig. 1) were generally used for the separation of the total spectrum of cerebrosides (monoglycosylceramides) and oligoglycosylceramides. In all of these systems the most pronounced effect on the migration of the individual fractions is produced by the differences in the number of hexose moieties in the molecule of the compounds. Cerebrosides have the highest mobility, and oligoglycosylceramides (tetraglycosylceramides–aminoglycosphingolipids) the slowest.

Monoglycosyl- and diglycosylceramides could be further separated into the fractions with unsubstituted and hydroxy fatty acids. The latter fraction has a slower mobility. The systems I, II, III and VIII, in particular, are very effective for this purpose (Fig. 1).

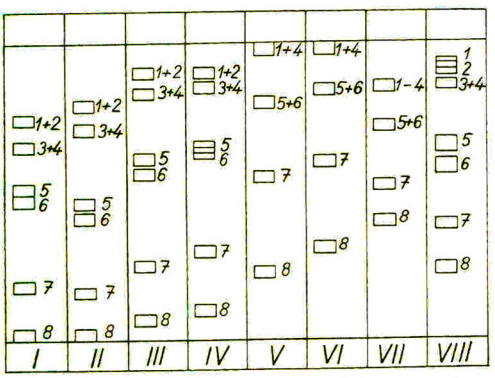
Increasing concentrations of methanol or water in the solvent mixtures resulted in increased migration of all fractions (*e.g.* systems IV and V).. In these systems monoglycosyl- and diglycosylceramides run close to the solvent front. On the other hand, triglycosylceramides and higher oligoglycosylceramides are well differentiated. De-

velopment by horizontal chromatography[19] (system VIII) permits a better separation of the monoglycosyl- and diglycosylceramides in contrast to the technique over short distances.

However, it should be emphasized that in this case a partial separation of the glucosylceramides and galactosylceramides could be obtained.

In system VII all monoglycosyl- and oligoglycosylceramide fractions are also very well separated, but without further subfractionation into unsubstituted and hydroxy fatty acid groups. If this system was combined with system III in a two-dimensional modification, an excellent separation was achieved (Fig. 3). System VI is practically equivalent to system V or VII.

Sulphatides and sphingomyelins migrated near to the position of the triglycosylceramides in systems I–VIII and gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1})* remained on the starting line. G_{M2} and G_{M3} run from the start in systems V–VIII and could be separated from each other. Mobility of both these fractions is lower than the migration rate of the tetraglycosylceramides. All these sphingoglycolipids could be



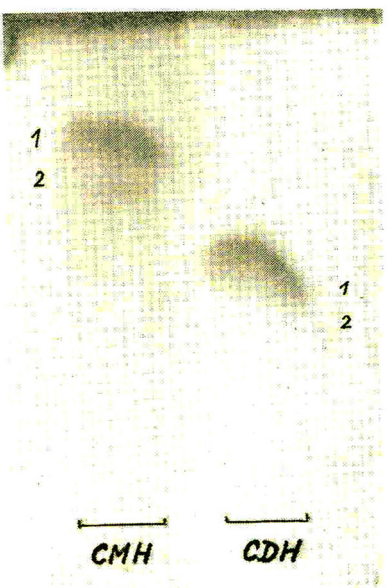


Fig. 1. Schematic representation of the migration of different fractions of cerebrosides and oligoglycosylceramides in solvent systems I–VII (see Table I). 1 = Monoglucosylceramides with unsubstituted fatty acids (from human spleen); 2 = monogalactosylceramides with unsubstituted fatty acids (from human adult brain); 3 = monoglucosylceramides with hydroxy fatty acids (from human kidney); 4 = monogalactosylceramides with hydroxy fatty acids (from human brain or kidney); 5 = diglycosylceramides with unsubstituted fatty acids (from human kidney); 6 = diglycosylceramides with hydroxy fatty acids (from human kidney); 7 = triglycosylceramides (from human kidney); 8 = tetraglycosylceramides (from human kidney).

Fig. 2. Separation of monoglycosylceramides (cerebrosides-CMH) and diglycosylceramides (CDH) isolated from the lipoproteins of fat globules of cow's milk. Solvent system: III. Detection: 1 = Monoglycosyl- or diglycosylceramides with unsubstituted fatty acids; 2 = monoglycosyl- or diglycosylceramides with hydroxy fatty acids.

* Nomenclature according to SVENNERHOLM[20].

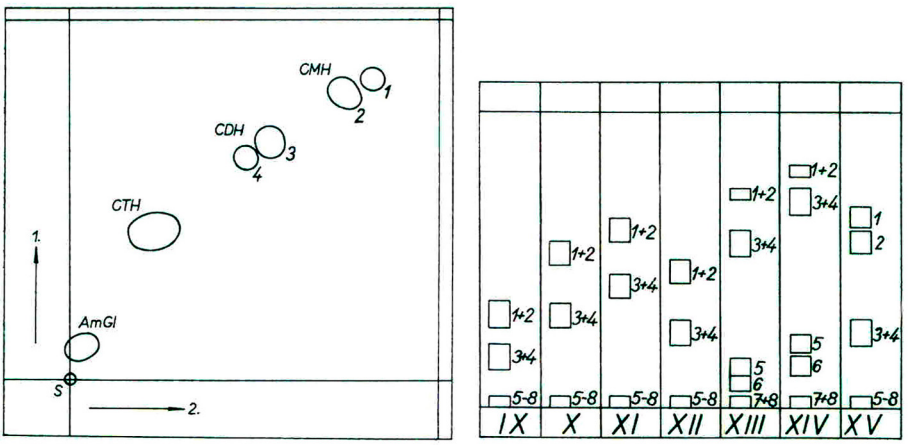


Fig. 3. Two-dimensional separation of monoglycosylceramides (cerebrosides) and oligoglycosylceramides. Paper: (a), 1st dimension: solvent system VII; 2nd dimension: solvent system III. Detection: (b), CMH = monoglycosylceramides (1 = Fraction with unsubstituted fatty acids, 2 = fraction with hydroxy fatty acids); CDH = diglycosylceramides (3 = fraction with unsubstituted fatty acids, 4 = fraction with hydroxy fatty acids); CTH = triglycosylceramides; AmGl = tetraglycosylceramides (aminoglycosphingolipids).

Fig. 4. Schematic representation of the migration of different fractions of cerebrosides and oligoglycosylceramides in solvent systems IX–XV. The nomenclature and origin of the fractions are the same as in Fig. 1.

Fig. 5. Separation of monoglycosylceramide (cerebroside) fraction isolated from adult human brain. Paper: (b), Solvent system IX. Detection: (a), 1 = Monoglycosylceramides with unsubstituted fatty acids; 2 = monoglycosylceramides with hydroxy fatty acids.

Fig. 6. Schematic representation of the migration of different fractions of cerebrosides and oligoglycosylceramides in solvent systems XVI–XVIII. The nomenclature and origin of the fractions are the same as in Fig. 1. ? = Monoglycosylceramides with unsubstituted fatty acids probably containing C_{16-20} fatty acids.

differentiated from monoglycosyl- and oligoglycosylceramides by the use of various detection reagents.

Systems IX–XV (Fig. 4) are especially advantageous for the differentiation of the unsubstituted and hydroxy fatty acid subfractions of monoglycosylceramides. System XIV could be also used for partial resolution of the diglycosylceramides.

The nature of both subfractions of the monoglycosyl- and diglycosylceramides is of a special interest. This type of sphingoglycolipid is only present in very small concentrations in normal adult human brain, but some other tissues and fluids, *e.g.* spleen, liver, kidney and blood serum contain relatively large amounts. SVENNERHOLM AND SVENNERHOLM[20] reported that monoglycosyl- and diglycosylceramides of human

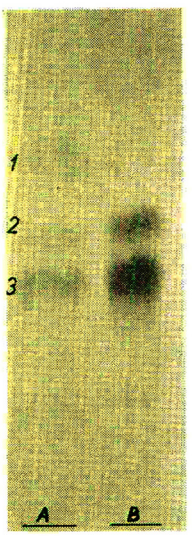


Fig. 7. Separation of monoglucosylceramides and monogalactosylceramides. Paper: (c), Solvent system XVIII. Detection: (e), A = Standards; B = monoglycosylceramide fraction isolated from human adult brain. 1 = Monoglucosylceramides with unsubstituted fatty acids; 2 = monogalactosylceramides with unsubstituted fatty acids; 3 = monogalactosylceramides with hydroxy fatty acids.

liver and spleen contain hydroxy fatty acids as shown on thin-layer chromatography. In contrast to these results SUOMI AND AGRANOFF[21] have found only traces of hydroxy fatty acids in the same materials. The authors suggested that the double spot is due to the differences in the chain lengths of the fatty acid residue. Similar observations were noticed by MIRAS *et al.*[22] in the diglycosylceramide fraction of human leucocytes.

On the other hand approximately equal proportions of monoglycosyl- and diglycosylceramides with unsubstituted fatty acids have been identified in human kidney[23].

Another criterion which could play an important role in the subfractionation of such substances is the nature of the hexose moiety. It is clearly evident that glucosylceramides have a somewhat higher mobility on thin-layer chromatograms than galactosylceramides. On paper chromatography the differences in migration are not so distinct, although in some solvent systems a partial separation was observed.

In our present study only monoglycosylceramides from adult human brain tissue and diglycosyl-, triglycosyl- and tetraglycosylceramides from human kidney were analysed. For this reason it must be pointed out that the presence of hydroxy fatty acids could probably explain the nature of the slower migrating spot.

Systems XVI–XVIII (Fig. 6) are very useful for the separation of glucosyl- and galactosylceramides.

As we have pointed out in a previous paper[19], it is possible that the fraction with the mobility between the spots of glycosyl- and galactosylceramides with unsubstituted acids probably corresponds to the glycosylceramides with unsubstituted fatty acids containing predominately C_{16-20} acids. Similar observations were reported by KEAN[24] after thin-layer chromatography on silica gel impregnated with sodium tetraborate. Monoglycosylceramides isolated from the spleen of a patient with Gaucher's disease formed a double spot, while glycosylceramides isolated from brain gangliosides gave only one compact spot with the same mobility as the lower spot of the spleen cerebrosides. It is very well known that the major fatty acid in brain gangliosides is stearic acid. On the other hand the upper spot of the glycosylceramides in spleen contains mainly fatty acids with 22–24 carbon atoms (78.5%)[21].

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Biochemistry of sphingolipids

XXVI. An attempt at the characterization of long-chain bases and their degradation products through their oxidative cleavage

The presence of dienic long-chain bases as native constituents for the spectrum of the sphingomyelin bases in biological material was firstly described by SWEELEY AND MOSCATELLI[1]. Analysis of the aldehydes obtained from plasma sphingomyelin bases by HIO_4 oxidation resulted in the authors finding a component that corresponded to a sphingosine chain of 18 carbon atoms, and was more unsaturated than 18:1. Recently KARLSSON[2-4] isolated a C_{18}-compound with an allylic group and double bonds in the 4 and 14 positions from the same material. Oxidative cleavage with lead tetraacetate and potassium permanganate and subsequent thin-layer and gas chromatography of the oxidation products (aldehydes and fatty acids) showed it to be identical with 1,3-dihydroxy-2-amino-erythro-*trans,cis*-octadecadiene. HIRVISALO[5] studied the type of double bonds in this derivative using partial reduction with hydrazine hydrate and thin-layer chromatography of the resultant compounds. Oxidative cleavage with periodate confirmed the position of the double bonds at C-4 and C-14. The same results were obtained later by POLITO *et al.*[6]. Argentation thin-layer chromatography of the DNP (dinitrophenyl) derivatives of the more unsaturated long-chain bases was described by MICHALEC[7]. Recently KARLSSON AND MÅRTENSSON[8] have also reported the presence of dienic bases in human kidney sphingoglycolipids.

This paper describes a relatively simple chromatographic method for the identification of these substances.

Experimental

Materials. DNP derivatives of the dienic long-chain base fraction were isolated from human blood serum sphingomyelins by preparative thin-layer chromatography

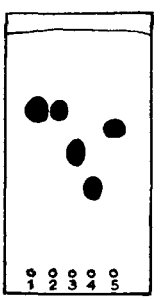

Fig. 1. Separation of the aldehydes obtained after the oxidative cleavage of DNP-derivatives of long-chain bases. Adsorbent: Silufol UV 254 impregnated with 5% $AgNO_3$. Solvent system: *n*-hexane–diethyl ether (85:15). Detection: Schiff's reagent. 1 = Saturated aldehydes (hexadecanal); 2 = saturated aldehydes from oxidized 4D-hydroxysphinganine (pentadecanal); 3 = dienic aldehydes from oxidized 3,5-sphingadiene (2,4-*trans*, *trans*-heptadecadienal); 4 = dienic aldehydes from oxidized 4,14-sphingadiene isolated from human blood serum sphingomyelins (2,12-*trans,cis*-hexadecadienal); 5 = monoenoic aldehydes from oxidized sphingenine (hexadecenal).

on Silufol UV 254 sheets impregnated with Ag^+ (ref. 7). Standard samples of some other substances (*e.g.* 3,5-sphingadiene etc.) were prepared in our laboratory or kindly donated by Dr. K. A. KARLSSON (University of Gothenburg, Sweden).

Oxidation of DNP derivatives of bases with lead tetraacetate. The DNP derivatives (dissolved in a small volume of benzene) were oxidized with lead tetraacetate reagent (500 mg of Pb_3O_4 dissolved in 5 ml of acetic acid at 80°) for 60 min at 60–70°. After cooling the reaction mixture was diluted with distilled water and the aldehydes produced were extracted with *n*-hexane. The hexane layer was washed twice with water and dried with Na_2SO_4. After evaporation of the solvent the residue was dissolved in a small volume of *n*-hexane.

Chromatography. An aliquot of this solution was spotted on Silufol UV 254 sheets (4 × 7.5 cm) impregnated with a 5 % solution of $AgNO_3$ and developed in *n*-hexane–diethyl ether (85:15). After drying the chromatogram the spots were located with Schiff's reagent.

Results and discussion

The separation scheme of the aldehydes is given in Fig. 1. As can be seen from this figure the highest mobility, in the solvent system used, was shown by the saturated aldehydes which are well separated from the monoenoic and dienic aldehydes.

The dienic aldehyde resulting from the oxidation of 3,5-sphingadiene (*trans,trans*-configuration) has a markedly higher mobility than the aldehyde originating from the dienic fraction of human blood serum sphingomyelins (4,14-octadecasphingadiene). This behaviour could be explained by the different position of the double bonds. The same effect was observed by LEES AND KORN[9] after the argentation thin-layer chromatography of positional isomers of long-chain unsaturated fatty acid methyl esters. According to these authors, methyl-9,12-octadecadienoate has a higher mobility than methyl-6,11-octadecadienoate. Another factor which could be responsible for this different mobility is the configuration of the double bonds. The *trans,trans*-isomers of methyl-9,12-octadecadienoate were observed recently by STROCCHI AND PIRETTI[10] to be more mobile than the *cis,trans*- or *trans,cis*-isomers.

Summing up, our results confirm the findings of other authors obtained by other analytical techniques. We conclude that the simple method of the oxidative cleavage of dienic DNP derivatives of long-chain bases with lead tetraacetate (or other oxidative reagents) with subsequent characterization of the resultant aldehydes on layers impregnated with Ag^+ can be used to explain, in many cases, the positional and geometrical configuration of the double bonds present in these substances.

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Eine kombinierte Nachweismethode mit Kaliumpermanganat und Ninhydrin in der Peptide mapping Technik

Bei der Chromatographie von Aminosäuregemischen oder Peptiden ist eine für einzelne Aminosäuren selektive Färbemethode von grossem Vorteil. Die Anforderungen, die an ein selektives Nachweisreagens gestellt werden, umfassen zusätzlich zu hoher Spezifität und grosser Empfindlichkeit einfache experimentelle Ausführung, lange Haltbarkeit der Reagenzien und vor allem eine Möglichkeit zur Kombination mit einer Übersichtsfärbung[1–4]. Ausgehend von einer Anfärbung der Serumproteine nach Papierelektrophorese mit Kaliumpermanganat in saurer Lösung[5] stellte sich uns die Frage, welche Proteinbausteine das Kaliumpermanganat reduzieren. Zahlreiche Untersuchungen an Aminosäuren und Oligopeptiden haben ergeben, dass nur Cystein, Cystin, Methionin, Tryptophan und Tyrosin im Protein für diese Reduktion verantwortlich sind. Dies veranlasste uns, Kaliumpermanganat auch als Sprühreagens zum Nachweis dieser 5 Aminosäuren auf Chromatogrammen zu verwenden.

Methodik

Kaliumpermanganat-Sprühreagens. Zu 100 ml dest. Wasser werden 5 ml einer wässerigen 2 %igen Kaliumpermanganatlösung und 20 ml einer 5 %igen wässerigen Schwefelsäure gegeben. In sauberen Glasgefässen ist dieses Reagens nahezu unbeschränkt haltbar.

Ninhydrin-Sprühreagens. 1 %ige Lösung von Ninhydrin in 96 %igem Äthylalkohol.

Anfärbung. Das trockene und gut gelüftete Chromatogramm wird mit dem Kaliumpermanganatreagens bis zur kräftigen Rosaviolettfärbung und bei Bedarf nach 45 Min mit Ninhydrin besprüht.

Ergebnisse

Nach dem Besprühen mit dem sauren Kaliumpermanganat erscheinen die Aminosäuren Cystein, Cystin, Methionin, Tryptophan und Tyrosin sofort als weisse Flecke auf dem von Kaliumpermanganat rosaviolett gefärbten Hintergrund (Fig. 1). Alle anderen Aminosäuren werden nicht sichtbar, da das Oxidationspotential des Mn(VII) bei dieser Acidität zur raschen Oxidation dieser Aminosäuren nicht ausreicht. Die hellen Flecke heben sich von dem zuerst rosavioletten, später ins Bräunliche wechselnden Hintergrund deutlich ab. Kurz nach dem Besprühen zeigen Papier- und

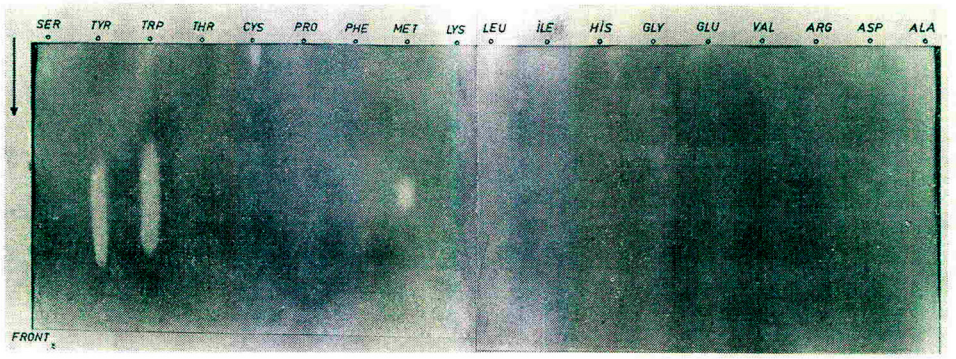

Fig. 1. Sichtbarmachung von Aminosäuren mit saurem Kaliumpermanganat auf einem Papierchromatogramm. Laufmittel: Äthanol–Wasser (77:23); Laufzeit: 3 Std.; Auftragsmenge: 10 μg. Tyr, Trp, Cys–SH, Met, treten sofort als helle Flecke auf rosaviolettem Hintergrund hervor.

Dünnschichtchromatogramme ein unterschiedliches Verhalten. Papierchromatogramme bleichen in etwa 45 Min. vollständig aus, jedoch ist wiederholtes Besprühen mit saurem Kaliumpermanganat ohne Verlust an Empfindlichkeit durchaus möglich. Auf Dünnschichtchromatogrammen nimmt der Hintergrund nach etwa 15 Min. eine beständige braune Farbe an.

Der Nachweis der Aminosäuren mit Ninhydrin auf Papier und dünnen Schichten wird durch vorhergehendes Besprühen mit dem sauren Kaliumpermanganatreagens nicht nachteilig beeinflusst (Fig. 2). Das Besprühen mit Ninhydrin erfolgt zweckmässig nach *ca.* 45 Min. Auf Papierchromatogrammen unterscheiden sich bei dieser Nachfärbung die Farbtöne einiger Aminosäuren kurz nach der Entwicklung bei 80° von denen der Erstanfärbung mit Ninhydrin. Auf organischen Dünnschichten erhalten die Positionen der Aminosäuren nach dem Besprühen mit Ninhydrin und Behandeln bei 80° eine karminrote Farbe, der braune Hintergrund bleicht erst beim Erwärmen aus. Die Nachweisgrenze des sauren Permanganatreagenzes liegt für die Aminosäuren bei 10^{-7} Mol/cm^2. Dies entspricht etwa der Empfindlichkeit des Ninhydrins[7].

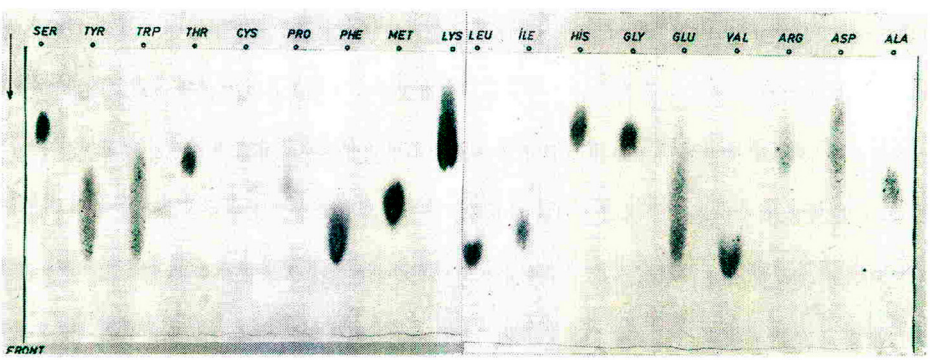

Fig. 2. Das in Fig. 1 gezeigte Chromatogramm, das zusätzlich nach etwa 45 Min. mit Ninhydrin überfärbt wurde. Sämtliche Aminosäuren werden mit Ninhydrin sichtbar.

Diskussion

Kaliumpermanganat in saurer Lösung wurde bereits zum unspezifischen Nachweis für organische Substanzen vorgeschlagen[6]. Bei Konzentrationen, wie sie normalerweise bei chromatographischen Untersuchungen biologischen Materials vorliegen, reagieren unter den angegebenen Bedingungen keineswegs alle organischen Substanzen. Im alkalischen Medium[4,8] zeigen Kaliumpermanganat-Sprühreagenzien zum selektiven Nachweis einzelner Aminosäuren gegenüber unserem Verfahren geringere Empfindlichkeit und sind zusätzlich bei weiteren Aminosäuren positiv. Überdies unterscheidet sich die Nachweisgrenze und die Oxidationsgeschwindigkeit für einzelne Aminosäuren im Alkalischen beträchtlich, wodurch der Aussagewert der Methode eingeschränkt wird.

Ein wesentlicher Vorzug des sauren Permanganates ergibt sich aus der Möglichkeit der Kombination mit der Ninhydrin-Besprühung als Übersichtsfärbung für Aminosäuren und Peptide. Vorteilhaft kann das saure Permanganatreagens auch verwendet werden, um nachzuweisen, ob die Aminosäuren Cys–SH, Cys–S–S–Cys, Met, Trp, Tyr in einem Protein vorhanden sind, da sie auch im Peptidverband positiv reagieren. Peptide, die keine dieser Aminosäuren enthalten, zeigen keine Reaktion.

Das Reagens kann nicht eingesetzt werden, wenn das Laufmittel selbst Permanganat reduziert und nicht leicht flüchtig ist.

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