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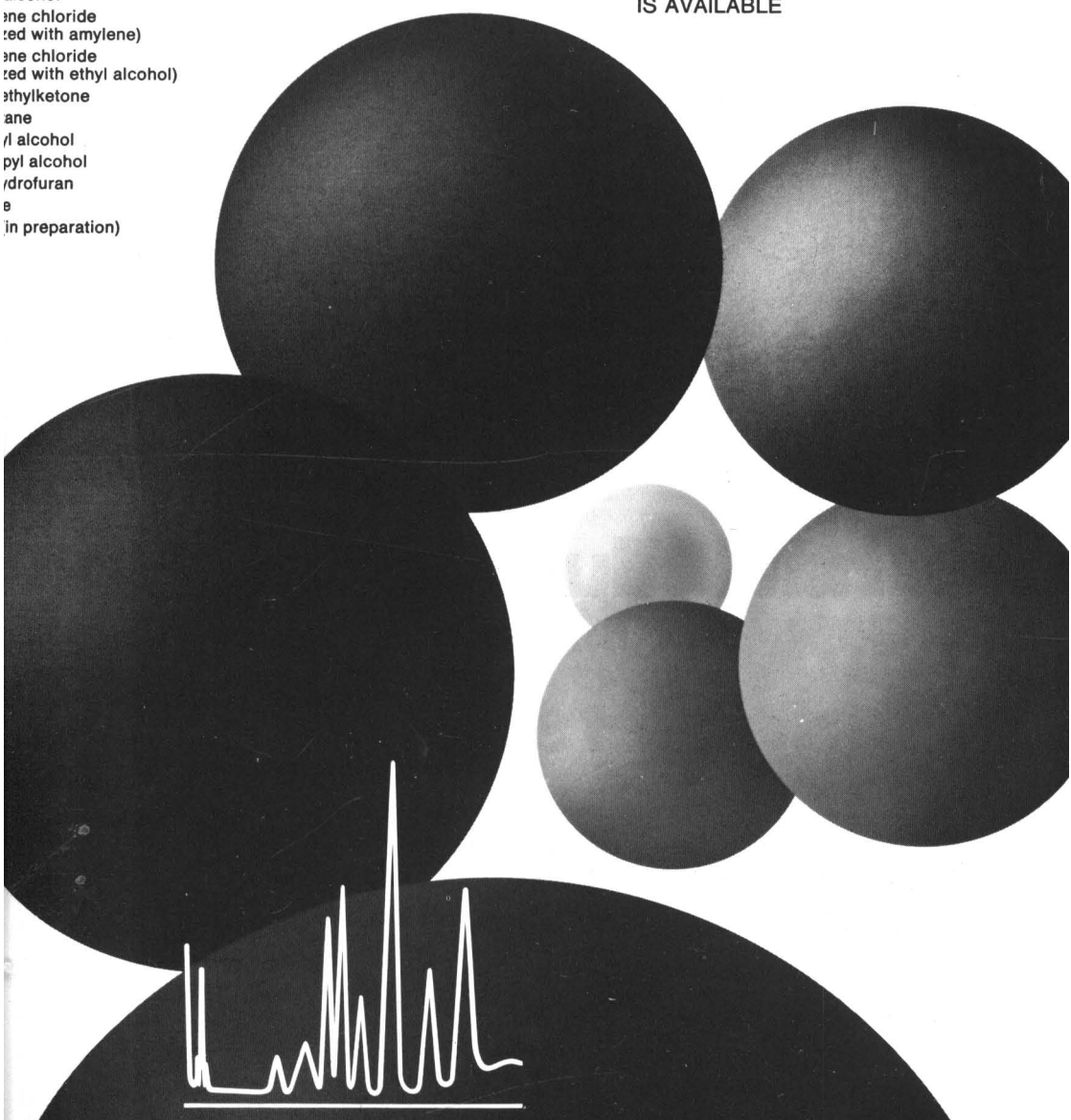

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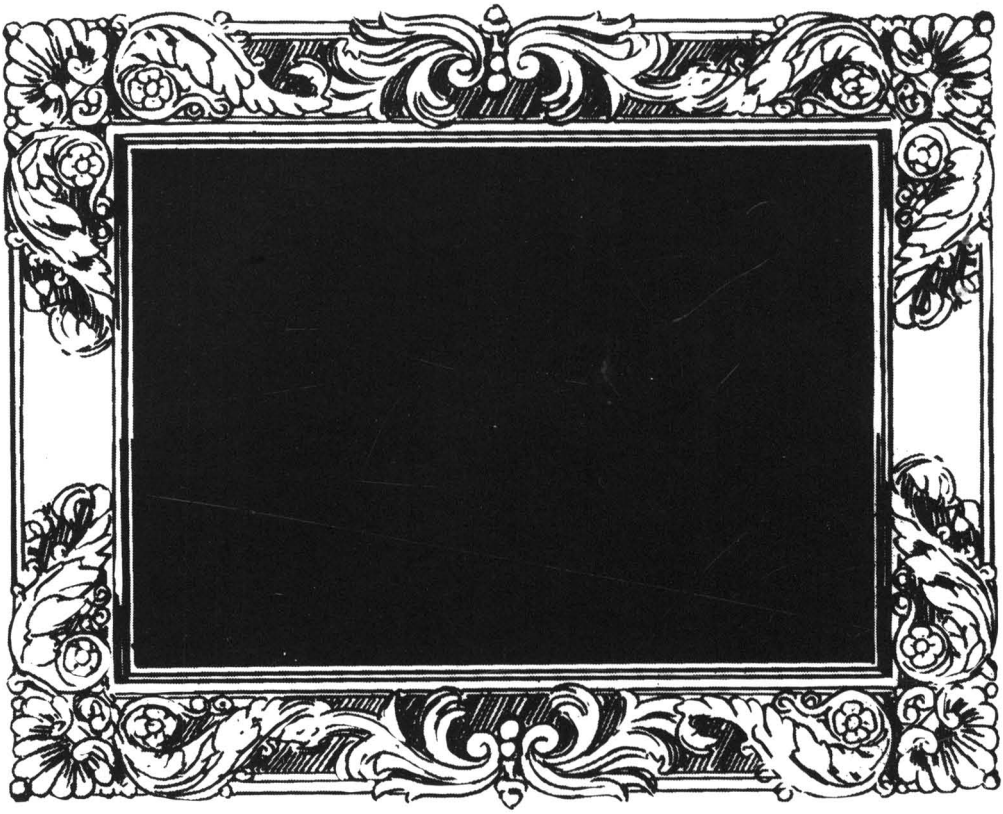

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CONTINUOUS FLOW ELECTROPHORESIS. THE CRESCENT PHENOMENA REVISITED

I. ISOTHERMAL EFFECTS

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(Received February 6th, 1980)

SUMMARY

Asymptotic solutions are derived for the partial differential equations (PDEs) governing solute behavior in the continuous flow electrophoresis device under isothermal operating conditions. In the limit, $D = 0$, analytical solutions are derived for the solute crescent shape and the concentration profile. In the limit, $((d/L)Pe_0)^{1/2} \gg 1$, an approximate solution to the PDE is found. These solutions are then used to predict the net dispersion of the concentration profile as a function of the fluid velocity in the chamber, the electrophoretic velocity of the solute and the electroosmotic flow at the chamber walls. The effects of diffusion on the net dispersion of the solute is also discussed for these limiting cases.

INTRODUCTION

In the current generation of continuous flow electrophoresis (CFE) devices a curtain of fluid is passed between two plates. An electric field is then established perpendicular to the direction of fluid motion and parallel to the plates. This allows a steady inlet stream of solute, introduced into the fluid curtain, to be fractionated according to its component electrophoretic mobilities. In this manner the inlet solute stream is continuously split into primary components and each component is collected separately at the outlet of the device.

Theoretical analysis of the concentration profiles in the CFE device has advanced in two main themes. The first of these, used by Strickler and Sachs[1], considers the non-diffusive limit of solute transport. The authors used their analysis to demonstrate the transport of solute in the electric field and, in particular, to elucidate the effects of electroosmotic flow at the chamber walls. Their work was qualitative in nature since it considered only the displacement of the solute stream and not the concentration profile of the exit stream. Their basic ideas have since been extended to predict solute concentration profiles using numerical procedures[2,3].

The second theme includes the effects of diffusion on the concentration profile[4]. This model is based on the usual equation of convection with diffusion and

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includes electrophoretic migration orthogonal to the axis of convection. Although in principle this linear differential equation has an analytical solution, the fo m of the solution is very complicated. Reis *et al.*[4] propose an approximate solution based on the analysis of Gill and Sankarasubramanian[5] which considers the effects of diffusion along the three axes but ignores the effects of electroosmosis on the solute concentration profile.

While it is important to consider the effects of diffusion in the CFE analysis, the magnitude of the diffusive effect as compared with the electroosmotic effect is usually very small under normal operating conditions. In this paper two methods of calculating the concentration profiles in the CFE are described. The first is an analytical method for calculating the concentration profiles in the non-diffusive limit. The phenomenon of crescent formation is discussed in some detail along with general results from the analytical analysis. The second method is an approximate solution to the convection-diffusion equation which includes the effects of osmotic migration. This solution is compared with the solution of Reis *et al.*[4] in the limit as electroosmosis becomes unimportant and to the analytical solution in the limit as diffusion becomes unimportant.

THEORETICAL

Convective dispersion in the CFE

In the absence of diffusion solute particles are assumed to follow the fluid and electrokinetic motions in their two respective axes. For the purposes of this study the left handed coordinate system of Saville and Ostrach[5] is used (see Fig. 1). The fluid

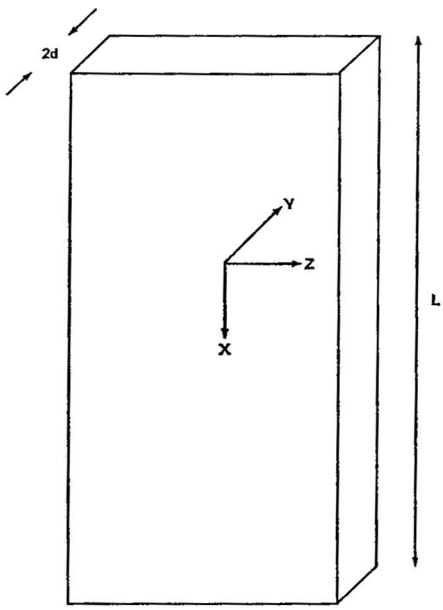

Fig. 1. Definitional sketch for the CFE device.

flow is in the positive x direction with maximum velocity V_M. The electrophoretic velocity is in the positive z direction as is the electroosmotic flow. Then the formulas for the velocities in the x and z directions are

$$V_x = i_x V_M (1 - y^2/d^2) \tag{1}$$

$$V_z = i_z \{V_E - 3/2 V_E (1 - y^2/d^2) + U\} \tag{2}$$

where V_M is the maximum velocity in the x direction, V_E is the electroosmotic wall velocity and U is the solute electrophoretic velocity. Since the particles do not deviate from their characteristic fluid stream lines, the displacement of any particle along the z axis is determined by its net electrokinetic velocity multiplied by the solute holdup time,

$$\Delta = [U + V_E - \tfrac{3}{2} V_E (1 - y^2/d^2)] \cdot [L/V_M (1 - y^2/d^2)] \tag{3}$$

If a band of solute having thickness, 2δ, and width, γ, is continuously fed into the CFE at $x = 0$, then, as the solute is displaced along the z axis the forward and rearward points of the band itself deforms in the velocity field. The solute band may take on three distinct shapes depending on the conditions of operation of the CFE.

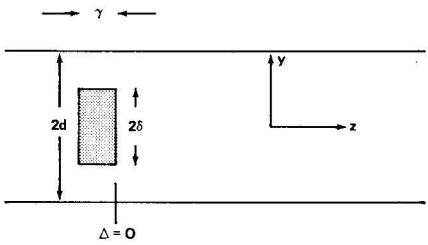

Fig. 2. Definitional sketch for the sample inlet of the CFE device.

(1) If $V_E = -U$ then the profile always appears as in Fig. 2 except that the band is displaced by $\Delta = 3/2\ UL/V_M$.

(2) If $V_E \neq -U$ and $\dfrac{V_M \gamma}{L} > \left| \dfrac{(U + V_E)(\delta/d)^2}{[1 - (\delta/d)^2]} \right|$ then

the cresent is "blunt" and the tail region has a constant concentration profile (see Fig. 3).

(3) If $V_E \neq -U$ and $\dfrac{V_M \gamma}{L} < \left| \dfrac{(U + V_E)(\delta/d)^2}{[(1 - (\delta/d)^2]} \right|$ then

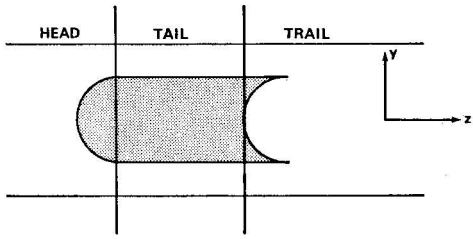

Fig. 3. Sketch of the "blunt" crescent showing the head, tail and trail regions.

the crescent is "developed" (see Fig. 4).

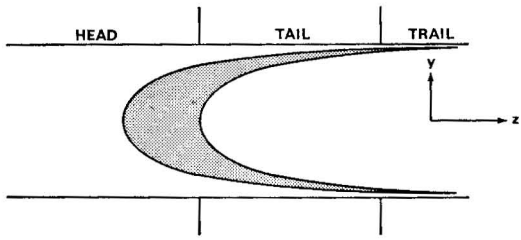

Fig. 4. Sketch of the developed crescent showing the head, tail and trail regions.

The crescent formation is a natural artifact of band deformation due to the orthogonal flows in the CFE. If the solute has formed a crescent as viewed in the y–z plane, then an analysis of the solute concentration (averaged over y) must be split into three parts. These parts are herein termed the crescent head, tail and trail. This is necessary because, when the solute input is rectangular in shape, integration across the y domain, $y\varepsilon(-1,1)$, is performed differently in each of the three regions. In the head, which occupies the region,

$$z\varepsilon\left(\frac{U+V_E}{V_M}L-\gamma,\ \frac{U+V_E}{V_M}L\right),$$

integration is performed from the centerline to the outer parabola. In the tail, which occupies the intermediate area between the head and the trail, integration is performed from the inner parabola to the outer parabola. And, in the trail, which occupies the region,

$$z\varepsilon\left(\left[\frac{U+V_E}{V_M(1-\delta^2/d^2)}-3/2V_E\right]L-\gamma,\ \left[\frac{}{V_M(1-\delta^2/d^2)}-3/2V_E\right]L\right),$$

integration must be performed from the inner parabola to δ, the outer boundary of the solute inlet.

The boundaries of the inner and outer parabolas are determined from the formulas for the displacement, Δ. Solving eqn. 3 for y yields

$$y=\pm\left\{\frac{\frac{\Delta}{L}+\frac{\frac{1}{2}V_E-U}{V_M}}{\frac{\Delta}{L}+\frac{\frac{3}{2}V_E}{V_M}}\right\}^{1/2} \tag{4}$$

where the negative root is discarded. The parabola with its origin at $\Delta-\gamma$ is given by the equation

$$y'=+\left\{\frac{\frac{\Delta+\gamma}{L}+(\frac{1}{2}V_E-U)/V_M}{\frac{\Delta+\gamma}{L}+\frac{3}{2}V_E/V_M}\right\}^{1/2} \tag{5}$$

Having determined the shape and the boundary of the solute profile, it is now possible to calculate the concentration of the solute in the fluid phase. There are two definitions of the concentration that have significance for the CFE. One is the concentration in the plane of viewing; that is, looking along the y axis into the x–z plane of the CFE as one might do with a photo-scanning device (see Krumrine[2]). The other is the solute flux through the plane of collection. This is the concentration which would be collected at each point along the z axis at the outlet of the CFE.

The concentration in the plane of viewing is defined as

$$\bar{C}(L, z) = {}_0\int^{d} C(L, y, z)\, dy / {}_0\int^{d} dy \tag{6}$$

The concentration in the plane of collection is defined as

$$\hat{C}(L, z) = {}_0\int^{d} N_x(L, y, z)\, dy / {}_0\int^{d} V_x\, dy \tag{7}$$

here

$$N_x(L, y, z) = V_x C(L, y, z) - D \frac{\delta C}{\delta x}(L, y, z) \tag{8}$$

The analytical solutions are useful in predicting solute behavior in the CFE without recourse to numerical methods. These solutions are limited because they only represent one geometry for the solute input, but they are still worth using as a first approach to column design and, as will be seen later in this paper, as an example of the limiting behavior in the CFE as diffusion becomes negligible.

For the case given above where the cresent is developed and the tail proceeds the head in the positive direction (*i.e.*, $V_E > -U$), these concentrations are

(1) In the head region,

$$z\varepsilon\left(\frac{U + V_E}{V_M} L - \gamma, \frac{U + V_E}{V_M} L\right)$$

$$\frac{\bar{C}}{C_0} = \left\{ \frac{(\Delta + \gamma)\frac{V_M}{L} + \frac{1}{2}V_E - U}{(\Delta + \gamma)\frac{V_M}{L} + \frac{3}{2}V_E} \right\}^{1/2} \tag{9}$$

$$\frac{\hat{C}}{C_0} = \left\{ \frac{(\Delta + \gamma)\frac{V_M}{L} + \frac{1}{2}V_E - U}{(\Delta + \gamma)\frac{V_M}{L} + \frac{3}{2}V_E} \right\}^{1/2} \left\{ \frac{(\Delta + \gamma)\frac{V_M}{L} + 2V_E + \frac{U}{2}}{(\Delta + \gamma)\frac{V_M}{L} + \frac{3}{2}V_E} \right\} \tag{10}$$

(2) In the tail region

$$\frac{\bar{C}}{C} = \left\{ \frac{(\Delta + \gamma)\frac{V_M}{L} + \frac{1}{2}V_E - U}{(\Delta + \gamma)\frac{V_M}{L} + \frac{3}{2}V_E} \right\}^{1/2} - \left\{ \frac{\Delta \frac{V_M}{L} + \frac{1}{2}V_E - U}{\Delta \frac{V_M}{L} + \frac{3}{2}V_E} \right\}^{1/2} \tag{11}$$

$$\frac{\hat{C}}{C_0} = \left\{\frac{(\Delta+\gamma)\frac{V_M}{L} + \frac{1}{2}V_E - U}{(\Delta+\gamma)\frac{V_M}{L} + \frac{3}{2}V_E}\right\}^{1/2} \left\{\frac{(\Delta+\gamma)\frac{V_M}{L} + \frac{1}{2}V_E + \frac{1}{2}U}{(\Delta+\gamma)\frac{V_M}{L} + \frac{3}{2}V_E}\right\} -$$

$$- \left\{\frac{\frac{V_M}{L} + \frac{1}{2}V_E - U}{\Delta\frac{V_M}{L} + \frac{3}{2}V_E}\right\}^{1/2} \left\{\frac{\Delta\frac{V_M}{L} + 2V_E + \frac{1}{2}U}{\Delta\frac{V_M}{L} + \frac{3}{2}V_E}\right\} \tag{12}$$

(3) And in the trail region,

$$z\varepsilon\left(\left(\frac{U+V_E}{V_M\left(1-\frac{\delta^2}{d^2}\right)} - \frac{3}{2}V_E\right)L - \gamma, \left(\frac{U+V_E}{V_M\left(1-\frac{\delta^2}{d^2}\right)} - \frac{3}{2}V_E\right)L\right)$$

$$\frac{\bar{C}}{C_0} = \frac{\delta}{d} - \left\{\frac{\Delta\frac{V_M}{L} + \frac{1}{2}V_E - U}{\Delta\frac{V_M}{L} + \frac{3}{2}V_E}\right\}^{1/2} \tag{13}$$

$$\frac{\hat{C}}{\bar{C}_0} = \frac{1}{3}\frac{\delta}{d}\left(3 - \frac{\delta^2}{d^2}\right) - \left\{\frac{\Delta\frac{V_M}{L} + \frac{1}{2}V_E - U}{\Delta\frac{V_M}{L} + \frac{3}{2}V_E}\right\}^{1/2} \left\{\frac{\Delta\frac{V_M}{L} + 2V_E = \frac{1}{2}U}{\Delta\frac{V_M}{L} + \frac{3}{2}V_E}\right\} \tag{14}$$

The effects of diffusion in the CFE

When diffusion of solute is included in the analysis of the concentration profiles in the CFE, the system is then described by the usual equation of diffusion with convection and electrophoretic migration. The general form of this differential equation is

$$V_x(y)\frac{\delta C}{\delta x} + (U + V_E(y))\frac{\delta C}{\delta z} = D\left(\frac{\delta^2 C}{\delta x^2} + \frac{\delta^2 C}{\delta y^2} + \frac{\delta^2 C}{\delta z^2}\right) \tag{15}$$

with the boundary conditions that

$y = \pm d$	$\delta C/\delta y = 0$
$z = \pm\infty$	$C = 0$
$x = 0$	$C = C_0\delta(z)$
$x = \infty$	C is finite

(16)

Using the dimensionless parameters

$x^* = x/d$	$\mathrm{Pe}(y^*) = V_x(y^*)d/D$
$y^* = y/d$	$\mathrm{El}(y^*) = [U + V_z(y^*)]\,d/D$
$z^* = z/d$	$C^* = C/C_0$

which then gives the dimensionless form of the equation

$$\mathrm{Pe}(y^*)\frac{\delta C^*}{\delta x^*} + \mathrm{El}(y^*)\frac{\delta C^*}{\delta z^*} = \frac{\delta^2 C^*}{\delta x^{*2}} + \frac{\delta^2 C^*}{\delta y^{*2}} + \frac{\delta^2 C^*}{\delta z^{*2}} \tag{17}$$

with boundary conditions that

$$\begin{array}{ll} y^* = \pm 1.0 & \delta C^*/\delta y^* = 0 \\ z^* = \pm \infty & C^* = 0 \\ x^* = 0 & C^* = \delta(y^*) \\ x^* = +\infty & C^* \text{ is finite} \end{array} \tag{18}$$

In order to simplify the mathematics of this problem it is desired to eliminate the term describing diffusion along the y axis. The explanation for doing this is intuitive in nature and is given immediately below where λy is the mean diffusional displacement along the y axis.

$$\frac{\lambda_y}{\mathrm{d}} = \frac{(2\mathrm{D}t)^{1/2}}{\mathrm{d}} = \left(\frac{(L/\mathrm{d})}{\mathrm{Pe}(y^*)}\right)^{1/2} \ll 1.0 \tag{19}$$

When this criterion is met, diffusional spreading along the y axis of the chamber is very small compared to the thickness, d, of the chamber. Dispersional spreading along the z^* axis due to the movement of solute from the center of the chamber becomes small compared to the dispersion due to convective influences, although it probably remains slightly more important than diffusion along the other two axes. The net result of this assumption is that the concentration in the vicinity of the peak is slightly overestimated with the leading edge of the curve somewhat overextended, the tail behind the peak is underestimated and the farthest edge of the tail is overestimated since the solute would diffuse toward the center of the chamber as well as along the x and z axes and, sampling the higher velocities, it would leave the chamber sooner. Thus, concentration profiles generated from this approximate solution give a conservative overestimate of the diffusive effect on dispersion in the CFE.

It is instructive to point out that in the limit of no diffusion we have already seen that the solute concentration profile peak exits the CFE chamber at $z = (\mathrm{Pe}_0/\mathrm{El}_0)x$. In the limit as diffusion dominates convective transport the solute is able to frequently sample all positions on the y axis and so the peak will exit the column at $z \cong \frac{1}{2}(\mathrm{Pe}_0/\mathrm{El}_0)x$ since each particle travels through the chamber at the average fluid velocity. This means that as operating conditions go from low diffusion to high diffusion effects there is a shift of 50% in the displacement of the peak from the origin. This will be important later in discussing the results of Reis.

The equation to be solved then is

$$\frac{\delta^2 C^*}{\delta x^{*2}} + \frac{\delta^2 C^*}{\delta z^{*2}} = \mathrm{Pe}(y^*)\frac{\delta C^*}{\delta x^*} + \mathrm{El}(y^*)\frac{\delta C^*}{\delta z^*}. \tag{20}$$

Using the transform

$$C^*(x^*,y^*,z^*) = \mathrm{f}(x^*,y^*,z^*)\exp\left(\mathrm{Pe}(y^*)x^* + \mathrm{El}(y^*)z^*\right)/2 \tag{21}$$

reduces the differential equation to

$$\frac{\delta^2 \mathrm{f}}{\delta z^{*2}} + \frac{\delta^2 \mathrm{f}}{\delta x^{*2}} = \left[\frac{\mathrm{Pe}^2(y^*) + \mathrm{El}^2(y^*)}{4}\right]\mathrm{f} \tag{22}$$

This equation automatically satisfies the boundary conditions on y^*. The conditions on x^* and z^* are

$$\begin{aligned} x^* &= \pm\infty & f &= 0 \\ z^* &= 0 & f &= \delta(y^*) \\ z^* &= \infty & f &= 0 \end{aligned} \tag{23}$$

Because the domain of x is semi-infinite the fourier sine transform may be used where

$$F_x(f) = \left(\frac{2}{\pi}\right)^{1/2} \int_0^\infty f(x) \sin(sx)\, dx \tag{24}$$

$$f(x) = \left(\frac{2}{\pi}\right)^{1/2} \int_0^\infty F_x(f) \sin(sx)\, ds \tag{25}$$

and the fourier exponential transform is used in z.

$$F_e(f) = {}_{-\infty}\int^{+\infty} f(y) \exp + (i\alpha y)\, dy \tag{26}$$

$$f(y) = \frac{1}{2\pi} \int_{-\infty}^{+\infty} F_e(f) \exp - (i\alpha y)\, d\alpha \tag{27}$$

which yields the double transform

$$F_s(Fe(f)) = s/(\alpha^2 + s^2 + \gamma^2) \tag{28}$$

where $\gamma^2 = (Pe^2(y^*) + El^2(y^*))/4$ and $K_1(z)$ is a modified Bessel function of the second kind of order one.

The solution for the concentration profile in the CFE is then

$$C^*(x^*, y^*, z^*) = \frac{(2\pi)^{-1/2}\gamma x^*}{(x^{*2} + z^{*2})^{1/2}} K_1(\gamma(x^{*2} + z^{*2})^{1/2}) \exp\left(\frac{(Pe\, x^* + El\, z^*)}{2}\right) \tag{29}$$

Calculations of the concentration in the plane of viewing and concentration through the plane of collection are generated from formulas 6 and 7, respectively. The integrations are done numerically and the results are presented in the next section.

RESULTS

The solution generated in this manner is approximate and so it is helpful to compare it with other extant solutions. For this purpose the functions generated by Reis *et al.*[4] are shown here as well as the analytic solutions generated earlier in this paper. The results of Reis *et al.* are for the special case when the CFE chamber walls do not generate an osmotic flow. The form of that solution gives the concentration in the plane of collection. Because both of these approximate solutions use a Dirac function for the solute input they cannot be directly compared to the analytical solution in the non-diffusive limit. For this reason the input width, and concentration of the analytical solution are modified so that the height and width of the output correspond roughly to the diffusive spreading if there were no electric field.

In Fig. 5 a comparison is made of the solution of Reis *et al.*[4] and the solution given in this paper at Peclet numbers of 20 and 100 when there is no imposed electric field. Agreement between these results is good although the Reis solution is consistently higher over the z axis. This may be due to the fact that Reis' solution, as is

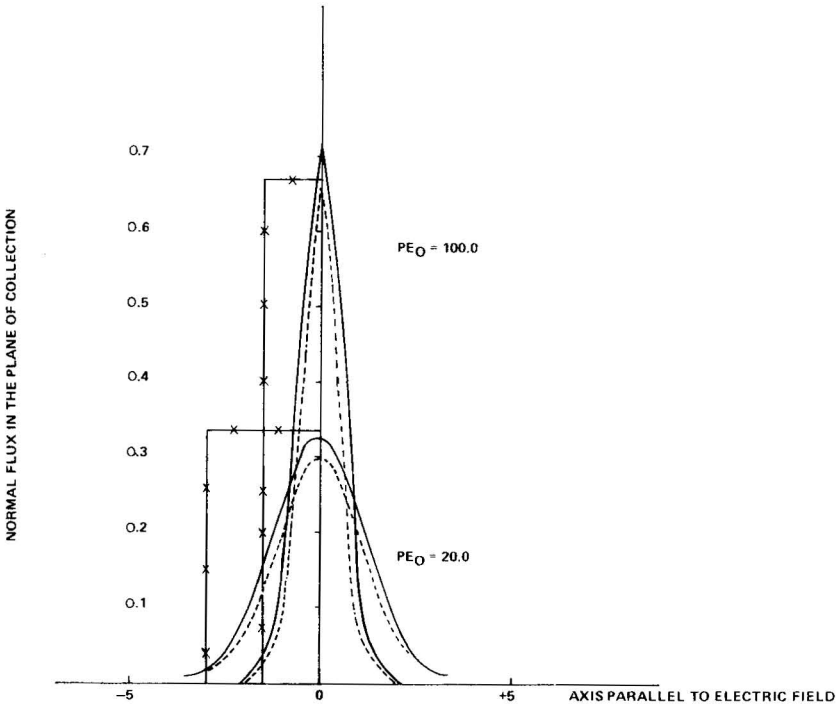

Fig. 5. A comparison of Reis' solution to the solutions derived in this paper when there is no applied electric field. —*—, Analytical solution ($D = 0$); ——, Reis' solution; – – –, this work. $L/d = 10.0$; $U/V_M = 0.0$; $V_E/V_M = 0.0$.

stated in his paper, is not normalized whereas the solution generated in this paper is normalized. Figs. 6 and 7 are a comparison, at Peclet numbers of 20 and 100, of the two solutions when an electrophoretic velocity is included in the solution. In Fig. 6 it is immediately apparent that the peak locations differ between the two solutions. Fig. 7 demonstrates that an increase in the Peclet number has opposite effects on these two solutions. Reis' solution predicts that increasing the Peclet number leads to increased dispersion while this work shows a decrease in dispersion.

This peculiar behavior of Reis' solution was predicted in his paper[4] and, in fact, further calculations show that his solution actually goes through a minimum in dispersion for $0 < \mathrm{Pe}_0 < 100$ when $L = 10$. No explanation for this behavior is offered in his paper and none is given here (Fig. 8).

Figs. 9 and 10 are a comparison of Reis' solution, the solution from this paper and the analytical solution in the limit of zero diffusion for $\mathrm{Pe} = 20$, 100 and $L = 100$. The last two solutions show very good agreement in the position of their peaks and

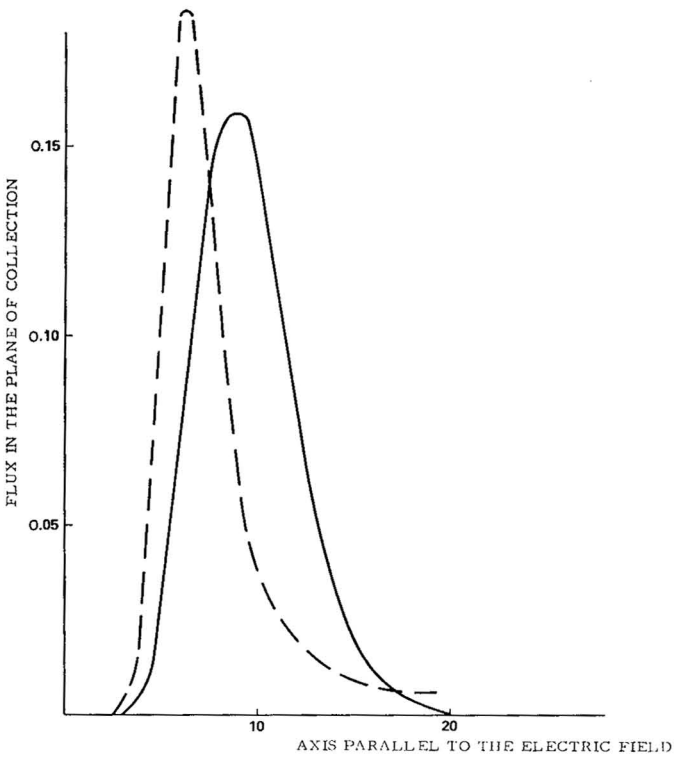

Fig. 6. A comparison of Reis' solution with the solution derived in this paper at a Peclet number of 20 with no electroosmotic flow. ——, Reis' solution; – – –, this work. $U/V_M = 0.6$; $L/d = 10.0$; $Pe_0 = 20.0$; $V_E/V_M = 0.0$.

in their respective trailing dispersions. The third solution is displaced from the first two by nearly 50% and otherwise has no resemblence to the analytical result.

It is apparent from the graphical work presented here that Reis' solution differs substantially from the solutions presented in this paper. Reis' solution does not reduce to the analytic solution in the limit as $(Pe_0 d/L)^{\frac{1}{2}} \to \infty$ as do the other solutions. There are two important points which suggest a reason why this happens.

In the first place, the elution peak arrives at a position on the z axis associated with the average fluid velocity rather than the maximum fluid velocity. In the limit as $(Pe_0 d/L)^{\frac{1}{2}}$ is large the latter would be true while for small $(Pe_0 d/L)^{\frac{1}{2}}$ the former would be expected.

Secondly, Reis *et al.* assert that the parameter

$$\alpha = \langle CV \rangle / \langle C \rangle \langle V \rangle = \hat{C}/\bar{C} \tag{30}$$

has a value that is never very different from 1.0. This would be expected at low values of $(Pe_0 d/L)^{\frac{1}{2}}$ since the solute would manage to frequently sample all positions on the y axis, but as may be seen from the zero diffusion results

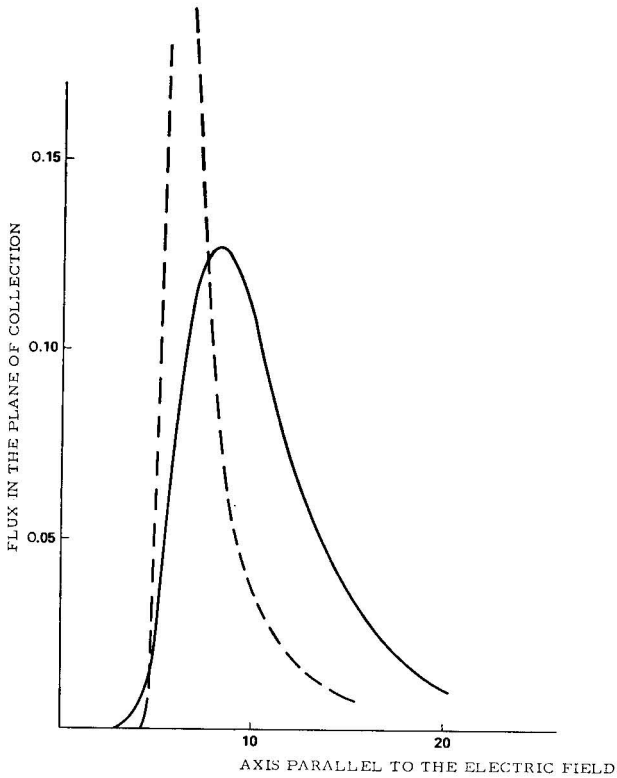

Fig. 7. A comparison of Reis' solution with the solution derived in this paper when the Peclet number is 100 and there is no electroosmotic flow. ——, Reis' solution; – – –, this work. $U/V_M = 0.6$; $L/d = 10.0$; $Pe_0 = 100.0$; $V_E/V_M = 0.0$.

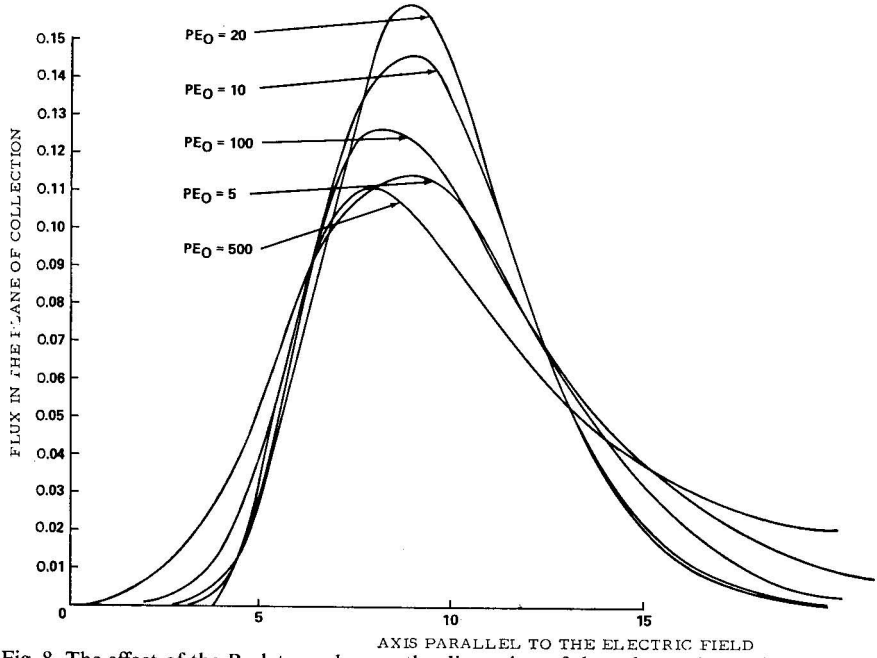

Fig. 8. The effect of the Peclet number on the dispersion of the solute using Reis' solution. $U/V_M = 0.6$; $L/d = 10.0$; $V_E/V_M = 0.0$.

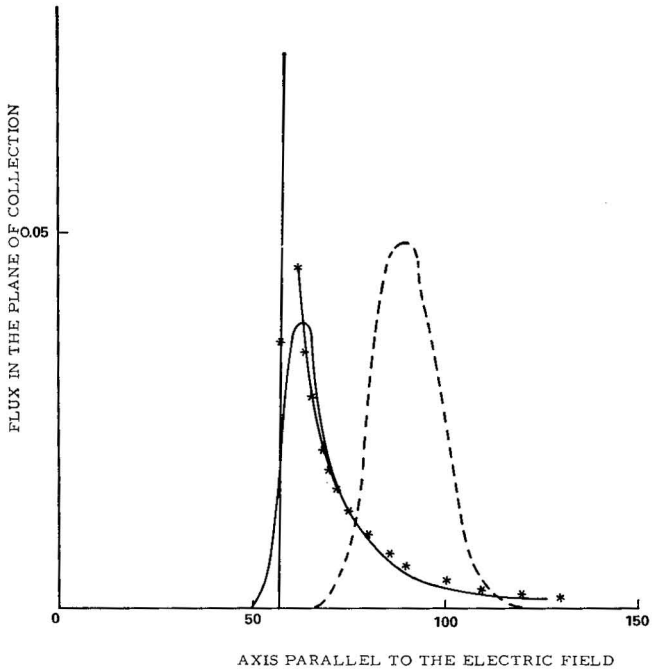

Fig. 9. A comparison of Reis' solution with the solutions derived in this paper when the non-dimensional column length is 100 and the Peclet number is 20. —*—, Analytical solution; – – –, Reis' solution; ——, this work. $U/V_M = 0.6$; $L/d = 100.0$; $Pe_0 = 20.0$; $V_E/V_M = 0.0$.

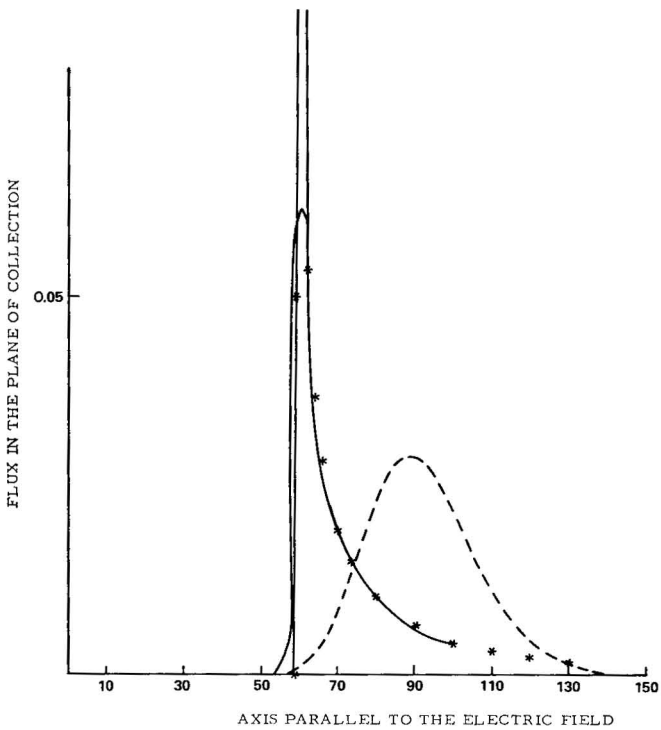

Fig. 10. A comparison of Reis' solution with the solutions derived in this paper when the non-dimensional column length is 100 and the Peclet number is 100. —*—, Analytical solution; – – –, Reis' solution; ——, this work. $U/V_M = 0.6$; $L/d = 100$; $Pe_0 = 100.0$; $V_E/V_M = 0.0$.

$$\frac{\hat{C}}{\bar{C}} = \frac{\frac{3}{2}U + \gamma\frac{V_M}{L}}{U + \gamma\frac{V_M}{L}} \tag{31}$$

at the peak of the elution curve. The maximum value of α is 1.5 when γ is small. These two points together suggest that the solution of Reis *et al.* is only valid at low values of $(Pe_0 d/L)^{\ddagger}$.

Fig. 11 shows the effect of increasing the Peclet number on the dispersion of the concentration profile. From this figure it is apparent that there is no minimum in the dispersion of the solute with increasing Peclet number. Also, a very slight shift is noticeable in the position of concentration peak.

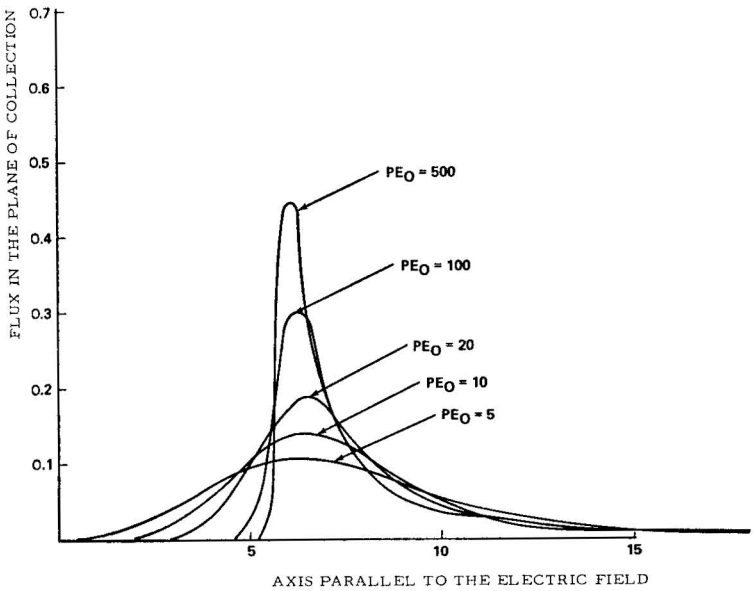

Fig. 11. The effect of the Peclet number on dispersion in the approximate solution derived in this paper. $U/V_M = 0.6$; $L/d = 10.0$; $V_E/V_M = 0.0$.

The profiles shown in Fig. 12 include the effect of an osmotic wall velocity. They clearly show the beneficial effects of operating the CFE such that $U = -V_E$. Finally, Fig. 9 is a graph of the crescents formed by the profiles in Fig. 13. It is the crescent formation phenomena which contribute the majority of the dispersion to the solute concentration profile. Notice that when $U = -V_E$ the crescent shape is not formed but diffusive influences cause the solute to assume an hourglass configuration.

CONCLUSIONS

The results of the latter part of this paper must be interpreted carefully and only in the light of the assumptions used in the solution of the problem. The heuristic

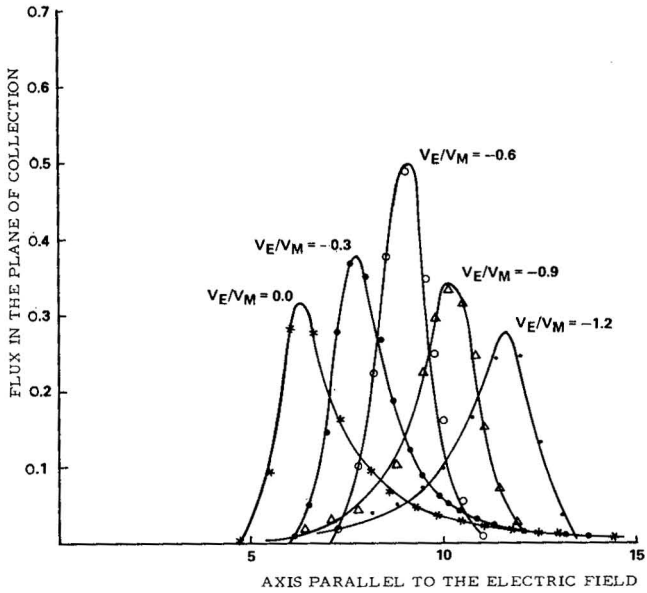

Fig. 12. The effect of electroosmotic velocity on dispersion using the approximate solution derived in this paper. $L/d = 10.0$; $Pe_0 = 100.0$; $U/V_M = 0.6$.

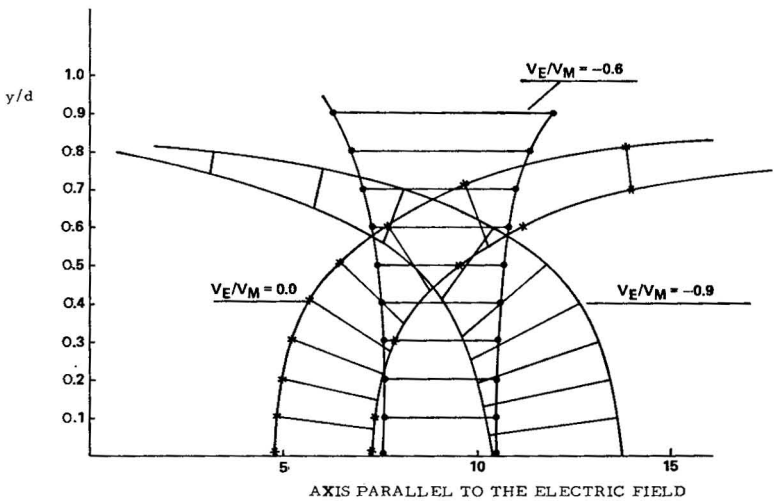

Fig. 13. The effect of the electroosmotic velocity on the formation of crescent when the effect of diffusion is included. Notice that the crescent in the center, $V_E/V_M = -U/V_M$, has an hourglass shape rather than a typical crescent shape. $U/V_M = 0.6$; $Pe_0 = 100.0$; $L/d = 10.0$.

application of mathematic approximations for simplification's sake can only be justified, in this case, by the accuracy of these results when they are studied in their asymptotic limit, $(Pe_0 d/L)^{\frac{1}{2}} \to \infty$. From these results we are able then to extract some useful information.

In the first place these equations predict a phenomenon as yet undetected in operating devices. This is the formation of an hourglass configuration of solute in the chamber slit. Again it is important that the results here only slightly overestimate the diffusive effect and so this phenomenon is not just an artifact of the calculation.

Secondly, the results point out the shortcomings of the earlier work done by Reis *et al.* While it may be that the error in their analysis is due simply to truncation of their analysis, the solution presented in their paper is clearly inapplicable to CFE operation at high values of $(Pe_0 d/L)^{\frac{1}{2}}$. In this limit the approximate solution presented here agrees very well with the analytical solutions presented in the first part of the paper. These solutions adequately describe the synergetic effects of the parabolic fluid and osmotic velocities on the dispersion of solute in the CFE chamber.

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PERFLUOROALKANOIC ACIDS AS LIPOPHILIC ION-PAIRING REAGENTS IN REVERSED-PHASE LIQUID CHROMATOGRAPHY OF PEPTIDES INCLUDING SECRETIN

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SUMMARY

The influence of various perfluoroalkanoic acids, as lipophilic ion-pairing reagents, on the retention and selectivity of underivatized hexa- and heptacosapeptides was studied on octadecylsilyl-silica. The retention, and in several cases the selectivity, increases in the series from trifluoroacetic acid to perfluorodecanoic acid. With perfluorooctanoic and perfluorodecanoic acid it was possible for the first time to follow directly the aspartyl-glycyl($\alpha \rightarrow \beta$)-rearrangement in the gastrointestinal 27-peptide hormone secretin.

INTRODUCTION

High-performance liquid chromatography (HPLC) is used to an increasing extent in peptide chemistry. It can be employed to follow reactions, in the purification of products and to check on purity, especially by the application of reversed-phase chromatography and of ion-pairing reagents[1–3].

The analysis and purification of unprotected peptides can be effected by adding trifluoroacetic acid to the eluent[4,5]. However, the selectivity between closely related unprotected peptides on octadecylsilyl-silica was less than with the corresponding protected peptides[6].

The addition of alkyl sulphonates and alkyl sulphates to the eluent has been described for the separation of many basic compounds[7–9]. With sulph(on)ates UV detection is not possible below 230 nm. Horváth *et al.*[9] have described the use of perfluorooctanoic acid and perfluorodecanoic acid for the separation of catecholamines, but they did not obtain reproducible results.

We have investigated the change of selectivity between unprotected peptides as a function of the lipophilicity of the ion-pairing reagent.

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TABLE I

THE k' VALUES OF SECRETIN AND ANALOGUES

$k' = (V_{peptide} - V_{water})/V_{water}$. Standard abbreviations are used for amino acids[14].

*Ion-pairing reagent**	*n*											
	0		*1*		*2*		*5*		*6*		*8*	
	5 mM	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*
Secretin (S)	1.3	2.2	2.7	5.7	8.5	19.0	1.9	5.4	5.8	15.6	4.8	12.5
[Aspartoyl^3]-S	1.2	2.2	2.8	5.7	9.2	19.9	2.2	6.4	6.9	18.6	5.9	15.1
[β-Asp^3]-S	1.3	2.2	2.7	5.2	7.9	16.5	1.7	4.7	5.0	13.4	4.0	10.7
[Ala^1]-S	1.6	2.9	2.7	5.8	7.7	15.7	1.2	3.0	3.0	6.9	2.0	5.0
[desamino-His^1]-S	1.6	2.8	2.9	6.0	8.0	15.6	1.2	3.0	3.0	7.1	2.0	4.2
Eluent, methanol–water		65:35		67:33		67:33		79:21		79:21		85:15

* Perfluoroalkanoic acids, $CF_3(CF_2)_nCOOH$, were used.

TABLE II

THE k' VALUES OF HEXAPEPTIDES

Details and footnote as in Table I.

Ion-pairing reagent	*n*											
	0		*1*		*2*		*5*		*6*		*8*	
	5 mM	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*	*5 Mm*	*10 mM*	*5 mM*	*10 mM*	*5 mM*	*10 mM*
S(1–6)-NH_2	4.0	5.5	6.4	8.9	3.0	3.7	3.2	4.6	3.2	4.3	5.8	9.5
[Aspartoyl^3]-S(1–6)-NH_2	3.9	5.7	6.6	9.2	3.2	3.9	3.5	4.7	3.3	4.6	6.0	9.6
[β-Asp^3]-S(1–6)-NH_2	3.4	4.8	5.3	7.7	2.7	3.4	3.0	4.1	3.0	4.0	5.4	8.5
S(13–18)	1.3	2.2	3.9	7.2	3.6	5.0	8.4	14.5	9.0	15.3	25.7	32.2
[β-Asp^{15}]-S(13–18)	0.9	1.5	2.9	5.5	3.1	4.5	7.9	13.9	8.5	14.9	24.6	32.1
Eluent, methanol–water		13:87		20:80		40:60		60:40		65:35		70:30

EXPERIMENTAL

Chemicals and materials

All peptides were synthesized in this laboratory. Amino acid residues are of the L configuration. The synthetic segments of porcine secretin are designated S(*n–m*), in which *n* and *m* are the sequence numbers of the corresponding amino acid residues in secretin, if the histidine residue occupies position 1. The following peptides were used: porcine secretin (S) (Fig. 1), [desamino-His[1]]-S, [aspartoyl[3]]-S, [Ala[1]]-S, [β-Asp[3]]-S, S(1–6)-NH_2, [aspartoyl[3]]-S(1–6)-NH_2, [β-Asp[3]]-S(1–6)-NH_2, S(13–18), [β-Asp[15]]-S(13–18).

H - His - Ser - Asp - Gly - Thr - Phe - Thr - Ser - Glu - Leu - Ser - Arg - Leu - Arg -
1 2 3 4 5 6 7 8 9 10 11 12 13 14

- Asp - Ser - Ala - Arg - Leu - Gln - Arg - Leu - Leu - Gln - Gly - Leu - Val - NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27

Fig. 1. The sequence of porcine secretin.

Reagent-grade methanol was used and mixed on a volume-to-volume basis with freshly distilled water. The ion-pairing reagents used were: perfluorodecanoic acid (PCR Research Chemicals, Gainesville, FL, U.S.A.), perfluorooctanoic acid and perfluoroheptanoic acid (Riedel-de Haen, Hannover, G.F.R.), heptafluorobutyric acid and trifluoroacetic acid (Aldrich, Milwaukee, WI, U.S.A.) and pentafluoropropionic acid (E. Merck, Darmstadt, G.F.R.).

Apparatus

A Waters Model 6000 A pump with a Model U6K injector was used in combination with a Pye Unicam LC 3 or a Schoeffel Spectroflow Monitor SF 770 for variable-wavelength UV detection. The column (15 × 0.4 cm I.D.) was packed[10] with Nucleosil C_{18}, 7 μm (Macherey, Nagel & Co., Düren, G.F.R.).

Procedures

The peptides were dissolved in the eluent (1 mg/ml). The eluents were degassed by careful filtration under reduced pressure, after which the alkanoic acid was added. The observed pH of the eluents varied between 2.1 and 2.5. The column was kept at ambient temperature. After use it was flushed, via a gradient, with methanol and stored in this solvent. The wavelength of the UV detection was set between 205 and 225 nm, depending on the alkanoic acid, its concentration and the UV detector.

RESULTS AND DISCUSSION

The use of perfluoroalkanoic acids in the present concentration range makes UV detection possible at 200 nm up to 220 nm, depending on the acid used, since peptides absorb fairly strongly at these wavelengths.

The influence of the perfluoroalkanoic acids on the capacity factors of secretin, partial sequences and their analogues is summarized in Tables I and II. The retention greatly increases with increasing chain length of the counter-ion, so that the percentage of methanol in the eluent in the series of trifluoroacetic acid to perfluorodecanoic acid

had to be raised in order to get usable retention times. Increase of the concentration of the counter-ion causes an increase of the retention. The influence of the chain length of the counter-ion on the retention is much greater with the 6- than with the 27-peptides.

The separation between secretin and its analogues was improved by increasing the chain length of the ion-pairing reagent (Fig. 2). The change of the methanol percentage is probably not the primary cause for the selectivity change, because with pentafluoropropionic acid and heptafluorobutyric acid a change of selectivity occurred in the same solvent system (Table I, Fig. 2). Both [Ala^1]-S and [desamino-His^1]-S have one basic group less than secretin, which accounts for their retention becoming less than that of secretin when the chain length of the counter-ion increases (Table I, Fig. 2). The same behaviour is found with S(13–18) and S(1–6)-NH_2, containing one basic group less (Table II, Fig. 3). The concentration of the counter-ion has little influence on the selectivity.

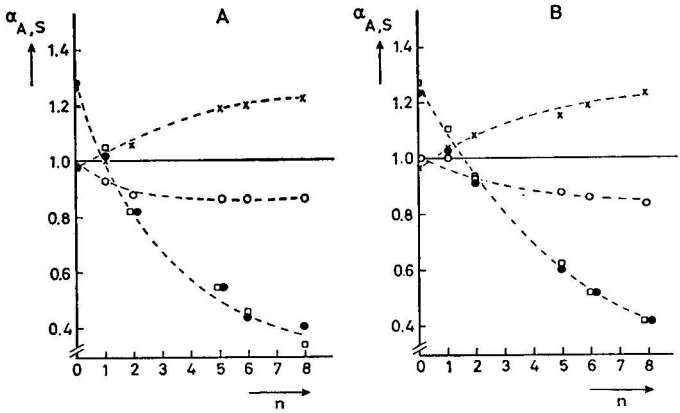

Fig. 2. The selectivity, $\alpha_{A,S} = k'$ (analogue)/k' (secretin), between secretin and some analogues with different counter-ions, $CF_3(CF_2)_nCOOH$, on a Nucleosil C_{18} column. Eluent: methanol–water (see Table I). Concentration of counter-ion: 0.01 *M* (A) and 0.005 *M* (B). ×, [Aspartoyl^3]-S; ○, [β-Asp^3]-S; ●, [Ala^1]-S; □, [desamino-His^1]-S.

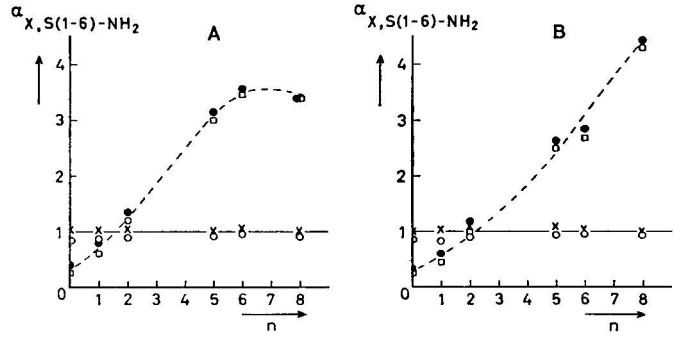

Fig. 3. The selectivity, $\alpha_{A,S(1-6)\text{-}NH2} = k'$ (analogue)/k' (S(1-6)-NH_2), between S(1–6)-NH_2 and some hexapeptides with different counter-ions, $CF_3(CF_2)_nCOOH$, on a Nucleosil C_{18} column. Eluent: methanol–water (see Table II). Concentration of counter-ion: 0.01 *M* (A) and 0.005 *M* (B). ●, S(13–18); □, [β-Asp^{15}]-S(13–18); ×, [aspartoyl^3]-S(1–6)-NH_2; ○, [β-Asp^3]-S(1–6)-NH_2.

Two mechanisms account for the effects of the addition of lipophilic ions to the eluent[11]. In the case of "dynamic ion exchange" the lipophilic ion is strongly adsorbed on the stationary phase (*e.g.*, octadecylsilyl-silica), which takes on the properties of an ion exchanger[12,13]. Thus, separation is mainly based on the number and strength of the ionic groups of the solute. With "ion-pair chromatography" the ionized solute and a lipophilic counter-ion form a neutral pair in the eluent, which can be adsorbed on the stationary phase[9]. The number of basic groups and the lipophilicity of the solute and the counter-ion determine the retention. In order to get an impression of the mechanism, the capacity factors of the perfluoroalkanoic acids were determined in the solvent systems used for the separations, but without the acid. For the eluents used with the heptacosapeptides (Table I), the capacity factors (k') varied from 0.2 (trifluoroacetic acid) to 0.6 (perfluorodecanoic acid). The eluents used with the hexapeptides (Table II) gave higher values, 0.2 (trifluoroacetic acid) to 2.1 (perfluorodecanoic acid). This indicates that the adsorption of the perfluoroalkanoic acids on octadecylsilyl-silica is low for the eluents used for the heptacosapeptides and low to moderate for the eluents used for the hexapeptides.

We conclude that mainly ion-pair formation in the eluent takes place. However, with perfluoroheptanoic acid, perfluorooctanoic acid and perfluorodecanoic acid in the eluents used for the hexapeptides a dynamic ion-exchange mechanism cannot be excluded. The increase of the selectivity for the separation of secretin, [aspartoyl[3]]-S and [β-Asp[3]]-S can be best explained by the formation of an ion-pair, resulting in shielding of the hydrophilic parts of the molecule. Changes in the con-

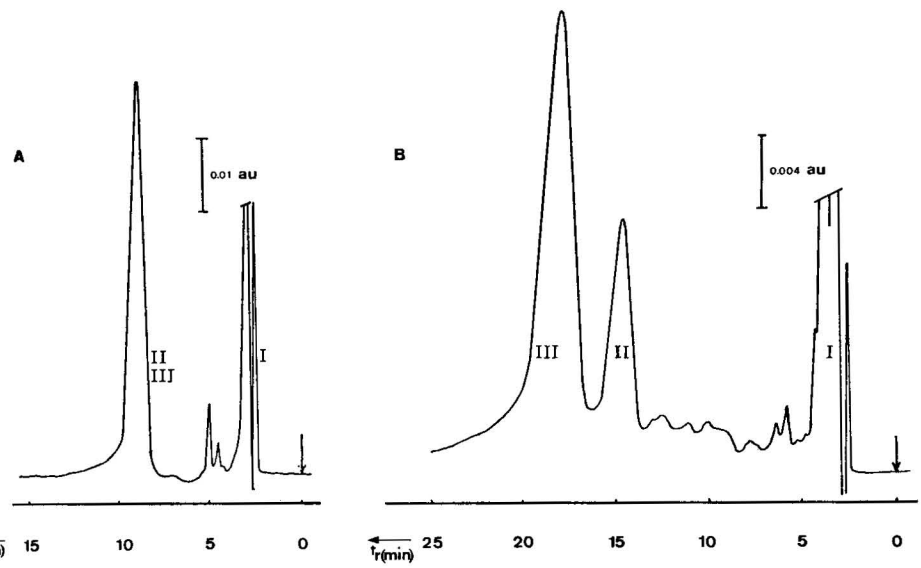

Fig. 4. The separation of products formed by rearrangement of synthetic porcine secretin. Secretin was dissolved (c = 0.5 mg/ml) in 0.1 M ammonium acetate (adjusted to pH 7.5). After storage at 50°C during 65 h, 10 μl (A) or 40 μl (B) were injected. Column: Polygosil C_{18} (10 μm), 30 × 0.4 cm I.D. Eluents: A, methanol–water–trifluoroacetic acid (65:35:0.1); B, methanol–water (82:18) with 0.005 M perfluorooctanoic acid. UV detection: A, 205 nm; B, 215 nm. Flow-rate: 1 ml/min. Peaks: I = solvent; II = [β-Asp[3]]-secretin; III = secretin.

formation and/or configuration of the hydrophobic part of the molecule can thus influence the retention, as in protected peptides, where relative small changes affect the retention.

Our research on a series of perfluoroalkanoic acids has appreciably extended the analysis of underivatized peptides. The gastrointestinal 27-peptide hormone secretin (Fig. 1) and some closely related derivatives can now be separated.

It appeared to be possible to follow directly the aspartyl-glycyl($\alpha \rightarrow \beta$)-rearrangement in secretin with perfluorooctanoic acid or perfluorodecanoic acid in the eluent (Fig. 4). Details of this rearrangement will be published elsewhere.

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REVERSED-PHASE AND SOAP THIN-LAYER CHROMATOGRAPHY OF PEPTIDES

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SUMMARY

The chromatographic behaviour of 38 peptides on layers of silanized silica gel alone and impregnated with anionic and cationic detergents has been investigated. On the basis of the comparison with the amino acid constituents, it has been possible to predict the sequence of the affinities of the peptides with the different solvents. Many separations have been carried out on layers impregnated with 4% dodecylbenzenesulphonic acid solution.

INTRODUCTION

With reversed-phase chromatography on thin layers of silanized silica gel impregnated with detergents (soap TLC), it has been possible to perform many separations of amino acids that can be effected only with difficulty or not at all on ion-exchange thin layers[1]. The type and concentration of the detergent on the silanized silica gel determines the separations that can be achieved. The retention of amino acids seems to be controlled by their interactions with both the hydrophobic side-chain and the functional group (ion-exchange) of the detergent.

In this work we investigated whether soap TLC can also be used for the separation of peptides. Interesting results have recently been achieved with reversed-phase chromatography on C_{18}-bonded silica columns[2] and on μBondapak alkylphenyl columns in the presence of ion-pairing reagents in the eluent[3–5].

EXPERIMENTAL

The compounds were dissolved in water–methanol (1:1). The amount of substance deposited on the layer was between 1 and 2 μg. The peptides were detected by spraying the wet layers with a solution of 1% ninhydrin in pyridine–glacial acetic acid (5:1) and then heating the layers at 100°C for 5 min.

The layers (thickness 300 μm) were prepared with a Chemetron automatic apparatus by mixing 20 g of silanized silica gel 60 HF (C_2) (Merck, Darmstadt, G.F.R.) in 50 ml of 95% ethanol with a known concentration of detergent. The detergent concentrations reported in the text refer to the alcoholic solution in which

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the silanized silica gel was suspended. The detergents used were triethanolamine dodecylbenzensulphonate (DBS), sodium dioctylsulphosuccinate (Na-DSS) (Serva, Heidelberg, G.F.R.), dodecylbenzensulphonic acid (H-DBS) (ICN Pharmaceutical, Plainview, NY, U.S.A.) and N-dodecylpyridinium chloride (N-DPC) (Merck). All measurements were carried out at 25°C. The migration distance was 11 cm unless otherwise stated.

The amino acids used were glycine (Gly), alanine (Ala), serine (Ser), isoleucine (Ile), leucine (Leu), proline (Pro), tyrosine (Tyr), phenylalanine (Phe), valine (Val), histidine (His), aspartic acid (Asp), arginine (Arg), tryptophan (Trp) and methionine (Met).

RESULTS AND DISCUSSION

Table I reports the chromatographic characteristics of 38 peptides on layers of silanized silica gel alone and impregnated with DBS, Na-DSS and H-DBS, eluting with an aqueous–organic mixture containing 5.7% acetic acid and 30% methanol (apparent pH = 2.75).

On layers of silanized silica gel alone the peptides with hydrophilic or basic amino acids (glycine, alanine, serine, arginine, histidine and aspartic acid) are not retained by the stationary phase and run with the solvent front. The peptides with one or more hydrophobic amino acid residues (valine, leucine, isoleucine, tyrosine and phenylalanine) are more retained. The strongest retention is observed with the pentapeptide, which is mainly formed by hydrophobic amino acids and therefore it can be easily separated from all others, which is different to the results observed on impregnated layers. The chromatographic behaviour of the peptides on this layer is similar to that observed on alkylphenyl and C_{18}-bonded silica columns eluting with acidic solutions[2,4].

On layers impregnated with 4% DBS a general increase in the retention of peptides, especially of those containing hydrophobic and basic amino acids, is observed. Some peptides (Gly–Pro and Ala–Pro), however, give rise to elongated spots and the detection of the peptides that are only slightly retained (Asp–Gly and Asp–Ala) is difficult owing to the violet colour which appears in the neighbourhood of the solvent front[1].

In the presence of 4% Na-DSS, although an increase in the retention with respect to DBS is observed, a change in the sequence of the R_F values for the different peptides is not achieved. On this layer several peptides give rise to non-compact spots (Gly–Gly, Gly–Ala, Ala–Gly) and others (Gly–Ser and Ala–Ser) give rise to a second spot with $R_F = 0.92$, which can probably be ascribed to the amino acid constituents that are formed on the layer by hydrolysis of the peptides. This last occurrence is probably related to the acid–base characteristics of this detergent, which is formed from the sodium salt of a weak acid.

On layers impregnated with 4% H-DBS, all peptides are strongly retained and the spots are very compact. For these reasons the study has been focused on layers impregnated with this detergent.

Table I also shows the influence of the H-DBS concentration on the chromatographic behaviour of the peptides. As the percentage of detergent is increased a sharp increase in the retention of all compounds, and in some instances even a change in the affinity sequence, is observed. The reversal of the sequence of the R_F values

TABLE I

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL ALONE AND IMPREGNATED WITH DETERGENTS

Eluent: water–methanol–acetic acid (64.3:30:5.7).

Peptide	*Layer*					
	SiO_2	*SiO_2 + 4% DBS*	*SiO_2 + 4% Na-DSS*	*SiO_2 + 1% H-DBS*	*SiO_2 + 2% H-DBS*	*SiO_2 + 4% H-DBS*
Gly–Gly	0.96	0.76	0.60	0.88	0.68	0.44
Gly–Ala	0.96	0.68	0.55	0.85	0.62	0.39
Gly–Ser	0.96	0.81	0.71	0.93	0.75	0.53
Gly–Ile	0.83	0.22	0.14	0.38	0.22	0.09
Gly–Leu	0.81	0.20	0.12	0.32	0.20	0.08
Gly–Pro	0.96	e.s.*	0.51	0.75	0.56	0.28
Gly–Tyr	0.90	0.44	0.33	0.59	0.44	0.22
Gly–Phe	0.72	0.18	0.12	0.29	0.18	0.07
Ala–Ala	0.95	0.60	0.48	0.74	0.58	0.36
Ala–Gly	0.96	0.74	0.60	0.87	0.67	0.43
Ala–Ser	0.96	0.77	0.70	0.92	0.71	0.50
Ala–Val	0.90	0.37	0.25	0.52	0.37	0.20
Ala–Ile	0.77	0.21	0.14	0.33	0.21	0.09
Ala–Pro	0.92	0.48**	0.43	0.67	0.49	0.24
Ala–Tyr	0.90	0.40	0.30	0.54	0.41	0.20
Ala–Hys	0.96	0.16	0.06	0.38	0.12	0.02
Asp–Gly	0.96	n.d.***	0.92	0.95	0 78	0 58
Asp–Ala	0.96	n.d.	0.78	0.88	0.68	0.49
Phe–Gly	0.76	0.19	0.13	0.32	0.19	0.08
Phe–Ala	0.75	0.17	0.12	0.26	0.15	0.07
Arg–Gly	0.96	0.06	0.03	0.21	0.05	0.02
Arg–Asp	0.96	0.11	0.05	0.29	0.09	0.03
Ile–Gly	0.90	0.30	0.24	0.47	0.35	0.14
Leu–Leu	0.55	0.06	0.05	0.09	0.05	0.03
Leu–Val	0.71	0.13	0.11	0.20	0.12	0.07
Leu–Tyr	0.72	0.16	0.14	0.24	0.15	0.08
Gly–Gly–Gly	0.96	0.75	0.56	0.83	0.66	0.42
Gly–Gly–Ala	0.96	0.64	0.49	0.78	0.59	0.38
Gly–Ala–Gly	0.96	0.67	0.49	0.78	0.59	0.38
Gly–Ala–Ala	0.96	0.60	0.46	0.74	0.56	0.36
Ala–Ala–Ala	0.96	0.61	0.51	0.74	0.57	0.38
Gly–Gly–Phe	0.76	0.24	0.16	0.29	0.17	0.07
Gly–Leu–Tyr	0.63	0.13	0.12	0.17	0.12	0.06
Leu–Gly–Phe	0.47	0.05	0.04	0.09	0.05	0.02
Gly–Gly–Gly–Gly	0.96	0.75	0.56	0.83	0.64	0.39
Ala–Ala–Ala–Ala	0.96	0.63	0.55	0.75	0.59	0.41
Leu–Trp–Met–Arg	0.49	0.00	0.00	0.00	0.00	0.00
Leu–Trp–Met–Arg–Phe	0.11	0.00	0.00	0.00	0.00	0.00

* e.s. = elongated spot.

** Tailing.

*** n.d. = not determined.

for tetra-Gly and tetra-Ala on changing from 1% to 4% H-DBS on the layer should be noted.

Influence of eluent acidity

As with amino acids, the chromatographic behaviour of peptides is consider-

ably affected both by the apparent pH of the eluent and by the counter ion (Na^+) concentration.

On the basis of the acid–base characteristics of these compounds (Table II), species with different charges may exist in solution depending on the apparent pH of

TABLE II

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL IMPREGNATED WITH 4% H-DBS

Eluents: (1) 0.1 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 1.25); (2) 0.05 *M* HCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 1.55); (3) 0.1 *M* NaCl + 1 *M* CH_3COOH in 30% CH_3OH (pH 2.75); (4) 0.1 *M* NaCl + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 3.30); (5) 0.1 *M* CH_3COONa + 0.1 *M* CH_3COOH in 30% CH_3OH (pH 5.10); (6) 1 *M* CH_3COONa in 30% CH_3OH (pH 8.15).

Peptide	*Eluent*						$pK_{(-COOH)}$[6]	$pK_{(-NH^+)}$[6]
	1	*2*	*3*	*4*	*5*	*6*		
Gly–Gly	0.68	0.60	0.70	0.70	0.69	0.83	3.22	8.17
Gly–Ala	0.60	0.55	0.64	0.64	0.71	0.85	3.17	8.23
Gly–Ser	0.73	0.70	0.75	0.76	0.75	0.85	2.92	8.10
Gly–Ile	0.17	0.15	0.19	0.13	0.32	0.67	—	—
Gly–Leu	0.14	0.12	0.16	0.11	0.28	0.62	3.18	8.29
Gly–Pro	0.42	0.36	0.49	0.49	0.59	0.79	2.81	8.65
Gly–Tyr	0.31	0.30	0.40	0.30	0.50	0 78	2.93	8.45
Gly–Phe	0.12	0.08	0.12	0.11	0.24	0.52	3.12	8.17
Ala–Ala	0.56	0.52	0.62	0.62	0.70	0.83	3.30	8.14
Ala–Gly	0.66	0.59	0.69	0.69	0.73	0.85	3.17	8.18
Ala–Ser	0.72	0.68	0.74	0.74	0.73	0.85	—	—
Ala–Val	e.s.*	0.27	0.36	0.34	0.52	0.78	—	—
Ala–Ile	0.15	0.13	0.19	0.16	0.30	0.71	—	—
Ala–Pro	0.37	0.32	0.43	0.43	0.55	0.78	3.04	8.38
Ala–Tyr	0.31	0.30	0.39	0.32	0.46	0.78	—	—
Ala–His	0.10	0.04	0.15	0.14	0.15	0.76	2.65	6.83, 9.51
Asp–Gly	0.74	0.71	0.75	0.77	0.77	0.87	2.10, 4.53	9.07
Asp–Ala	0.62	0.61	0.68	0.65	0.67	0.84	—	—
Phe–Gly	0.13	0.11	0.13	0.09	0.15	0.37	—	—
Phe–Ala	0.12	0.10	0.15	0.08	0.18	0.49	—	—
Arg–Gly	0.06	0.03	0.06	0.04	0.05	0.56	—	—
Arg–Asp	0.08	0.04	0.09	0.07	0.11	0.69	—	—
Ile–Gly	0.23	0.21	0.28	0.20	0.31	0.56	—	—
Leu–Leu	0.08	0.04	0.06	0.03	0.07	0.31	—	—
Leu–Val	0.11	0.09	0.13	0.08	0.21	0.56	—	—
Leu–Tyr	0.12	0.11	0.16	0.09	0.21	0.55	2.87	8.36
Gly–Gly–Gly	0.66	0.58	0.67	0.66	0.62	0.82	3.28	8.00
Gly–Gly–Ala	0.59	0.52	0.63	0.61	0.64	0.82	—	—
Gly–Ala–Gly	0.62	0.54	0.64	0.62	0.61	0.82	—	—
Gly–Ala–Ala	0.59	0.51	0.62	0.59	0.63	0.82	3.38	8.10
Ala–Ala–Ala	0.60	0.54	0.63	0.61	0.69	0.82	3.39	8.03
Gly–Gly–Phe	0.13	0.10	0.13	0.09	0.22	0.55	—	—
Gly–Leu–Tyr	0.09	0.08	0.10	0.06	0.14	0.40	—	—
Leu–Gly–Phe	0.04	0.03	0.03	0.02	0.04	0.20	—	—
Gly–Gly–Gly–Gly	0.62	0.56	0.67	0.65	0.59	0.78	—	—
Ala–Ala–Ala–Ala	0.62	0.57	0.69	0 66	0 63	0.80	3.42	7.94
Leu–Trp–Met–Arg	0.00	0.00	0.00	0.00	0.00	0.00	—	—
Leu–Trp–Met–Arg–Phe	0.00	0.00	0.00	0.00	0.00	0.00	—	—

* e.s. = elongated spot.

the eluent. On the other hand, as the peptides are generally hydrolysed with strongly alkaline or acidic eluents, giving rise to several spots on the layer or to elongated spots, the study was restricted to the pH range 1.25–8.15 (Table II). In this pH range the peptides change from the cationic to the zwitterionic form and, even if not completely, to the anionic form.

In the pH range in which the cationic form prevails (columns 1 and 2), an increase in the R_F values as the pH decreases is observed, as H^+ acts as a counter ion. On plotting the R_M values of the peptides as a function of the apparent pH of the eluent in the pH range 1.25–2.25, straight lines are obtained for most compounds. The $\Delta R_M/\Delta$pH slopes are between 0.3 and 0.5. These values are much lower than the theoretical values and are even lower than those obtained with amino acids[1]. For such peptides, however, the retention seems to be affected by an ion-exchange process.

The zwitterionic form of the peptides, which prevails at pH 5.1 (column 5), exhibits a smaller retention than the corresponding cationic form (see columns 3 and 4), as observed with amino acids[1]. As the apparent pH of the eluent is increased (column 6), a further decrease in retention by the layer is observed, in accordance with the prevalence of the anionic form of most peptides at such pH values.

As regards the behaviour of the single peptides, it is interesting that for those formed by hydrophilic amino acids an increase in pH results in a levelling of the R_F values, while for the others an increase in the resolving power is observed.

The affinity sequence of the different peptides can be predicted on the basis of the R_F values of the amino acid residues. In fact, the sequence of the oligomers Gly–Gly, Ala–Ala and Leu–Leu is identical with that of the corresponding amino acids under the same experimental conditions[1], apart from a sharper retention of the peptides. As the number of the amino acid residues is increased, such agreement no longer applies, as tetra-Ala, for instance, is less retained than tetra-Gly even in acidic solutions.

With dipeptides formed by two different amino acids, referring to the oligomer (*i.e.*, Gly–Gly or Ala–Ala) and replacing the final residue with other amino acids, the retention of the resulting peptides is correlated with that of the substituent amino acid. For instance, the sequence of R_F values Ala–Ser > Ala–Gly > Ala–Ala > Ala–Pro > Ala–Val > Ala–Ile > Ala–His, which is observed in acidic solutions, is identical with that found for the different amino acids (Ser > Gly > Ala, etc.) with the same eluents[1]. The replacement of the starting residue in the oligomer dipeptides generally does not affect the above sequence, but it results in a smaller change in the R_F values.

Influence of organic solvent concentration

The study of the influence of the organic solvent concentration on the retention of the peptides was performed keeping the acetic acid and sodium chloride concentrations constant at 1 and 0.1 *M*, respectively. As the percentage of methanol in the solution is increased, a decrease in retention is observed for most compounds, together with a reduction in the resolving power of the layer. Such a trend is similar to that observed for amino acids[1].

Use of water–organic solvent mixtures as eluents

We studied water–methanol, water–ethanol and water–acetic acid mixtures, which with amino acids yielded good results from an analytical standpoint. With peptides, the use of these eluents, especially water–acetic acid, gave useful results, particularly for dipeptides with low molecular weights, which are more clearly differentiated than with eluents used previously.

Table III gives the R_F values of peptides on silanized silica gel impregnated

TABLE III

R_F VALUES OF PEPTIDES ON THIN LAYERS OF SILANIZED SILICA GEL ALONE AND IMPREGNATED WITH ANIONIC OR CATIONIC DETERGENTS

Eluents: (1) water–acetic acid (7:3); (2) water–acetic acid (1:1); (3) 0.1 *M* CH_3COOH + 0.1 *M* CH_3COONa in 30% CH_3OH; (4) 1 *M* CH_3COOH in 30% CH_3OH.

Peptide	*SiO_2 + 4% H-DBS*		*SiO_2, eluent 3*	*SiO_2 + 4% N-DPC*	
	Eluent 1	*Eluent 2*		*Eluent 4*	*Eluent 3*
Gly–Gly	0.46	0.68	0.96	0.97	0.96
Gly–Ala	0.38	0.66	0.96	0.97	0.96
Gly–Ser	0.54	0.75	0.96	0.97	0.96
Gly–Ile	0.12	0.44	0.93	0.93	0.87
Gly–Leu	0.10	0.40	0.90	0.90	0.86
Gly–Pro	0.27	0.58	0.96	0.97	0.95
Gly–Tyr	0.27	0.69	0.94	0.84	0.77
Gly–Phe	0.10	0.41	0.78	0.81	0.69
Ala–Ala	0.35	0.64	0.96	0.97	0.96
Ala–Gly	0.43	0.70	0.96	0.97	0.96
Ala–Ser	0.51	0.72	0.96	0.97	0.96
Ala–Val	0.19	0.52	0.96	0.97	0.96
Ala–Ile	0.10	0.41	0.93	0.94	0.87
Ala–Pro	0.24	0.54	0.96	0.95	0.95
Ala–Tyr	0.26	0.62	0.96	0.88	0.81
Ala–His	0.03	0.18	0.96	0.97	0.96
Asp–Gly	0.58	0.79	0.96	0.94	0.88
Asp–Ala	0.46	0.71	0.96	0.94	0.89
Phe–Gly	0.10	0.42	0.79	0.86	0.74
Phe–Ala	0.08	0.41	0.81	0.89	0.81
Arg–Gly	0.02	0.13	0.96	0.97	0.96
Arg–Asp	0.02	0.18	0.96	0.97	0.96
Ile–Gly	0.15	0.48	0.94	0.96	0.95
Leu–Leu	0.03	0.26	0.57	0.80	0.71
Leu–Val	0.06	0.35	0.82	0.92	0.85
Leu–Tyr	0.10	0.49	0.74	0.79	0.69
Gly–Gly–Gly	0.39	0.67	0.96	0.97	0.96
Gly–Gly–Ala	0.34	0.67	0.96	0.97	0.96
Gly–Ala–Gly	0.35	0.67	0.96	0.97	0.96
Gly–Ala–Ala	0.34	0.67	0.96	0.97	0.96
Ala–Ala–Ala	0.36	0.68	0.96	0.97	0.96
Gly–Gly–Phe	0.07	0.43	0.80	0.78	0.66
Gly–Leu–Tyr	0.06	0.48	0.62	0.71	0.45
Leu–Gly–Phe	0.02	0.25	0.47	0.70	0.46
Gly–Gly–Gly–Gly	0.39	0.68	0.96	0.97	0.96
Ala–Ala–Ala–Ala	0.36	0.73	0.96	0.97	0.96
Leu–Trp–Met–Arg	0.02	0.05	0.32	0.79	0.58
Leu–Trp–Met–Arg–Phe	0.00	0.03	0.07	0.51	0.19

with 4% H-DBS eluting with mixtures containing 30% and 50% acetic acid; in both instances very compact spots are obtained. The former eluent is more suitable for the separation of hydrophilic dipeptides such as Gly–Gly, Gly–Ser and Ala–Ala, and the latter for the separation of hydrophobic dipeptides such as Leu–Leu, Leu–Val and Leu–Tyr.

Layers impregnated with cationic detergents

Table III gives the R_F values of peptides on layers of silanized silica gel impregnated with 4% N-DPC eluting with 1 *M* acetic acid in 30% methanol (apparent pH = 2.75) and with 0.1 *M* acetate buffer in 30% methanol (apparent pH = 5.10), and also results obtained on silanized silica gel with the eluent at pH 5.10.

On layers impregnated with N-DPC, the retention of the peptides eluting with the solution at pH = 2.75 is smaller than that observed in silanized silica gel alone, which is different to the results observed on layers impregnated with anionic detergents under the same elution conditions. Such behaviour can be ascribed to the repulsive forces between the positive charge of the peptide and that of the quaternary nitrogen atom of the cationic detergent. From an analytical standpoint, therefore, the use of these layers for the separation of peptides with hydrophilic amino acid residues and, generally, of those with low molecular weights does not offer any advantage over silanized silica gel alone, as such compounds are less well differentiated. For peptides with higher molecular weights and formed by hydrophobic amino acids, the smaller retention may offer the best separation conditions.

With the eluent at pH 5.10, an increase in retention is observed for those peptides formed by one or more hydrophobic amino acids and by aspartic acid. At this pH the peptides are prevalently in the zwitterionic form, which causes a decrease in the detergent–peptide repulsive forces and gives rise to an anion-exchange process with the counter ion of N-DPC.

With respect to the layers of silanized silica gel alone, where the above interactions are not possible, the peptides generally exhibit a stronger retention. However, the dipeptide Leu–Leu, the tetrapeptide Leu–Trp–Met–Arg and the pentapeptide are less retained under these elution conditions.

The use of layers impregnated with cationic detergents therefore seems very interesting for large peptides with one or more hydrophobic amino acid residues. A study of this subject is now being undertaken.

Analytical applications

Of the separations that are possible on the basis of the chromatographic data on layers impregnated with anionic detergents, we carried out some of those which are more interesting from an analytical standpoint.

Fig. 1 shows the separation of six dipeptides containing glycine as starting residue and that of Asp–Gly from Asp–Ala on layers of silanized silica gel impregnated with 4% H-DBS, eluting with 1 *M* acetic acid in 30% methanol. Under such elution conditions Gly–Gly has also been separated from Ala–Ala. The separation of these two peptides has also been performed by eluting with water–acetic acid (7:3).

Fig. 2 shows the separation of seven dipeptides containing alanine as starting

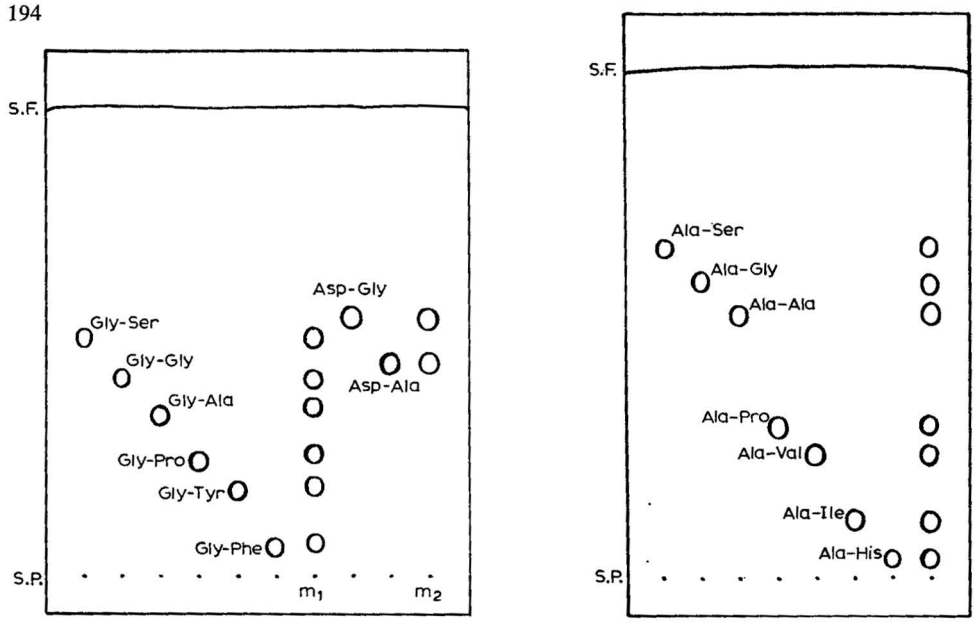

Fig. 1. Thin-layer chromatogram of peptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 12.5 cm. Eluent: water–methanol–acetic acid (64.3:30:5.7). m_1 = mixture of six peptides; m_2 = mixture of Asp–Gly and Asp–Ala. S.P. = starting point; S.F. = solvent front.

Fig. 2. Thin-layer chromatogram of peptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 13.5 cm. Eluent: 0.05 *M* HCl + 1 *M* CH_3COOH in 30% methanol (pH = 1.55). S.P. = starting point; S.F. = solvent front.

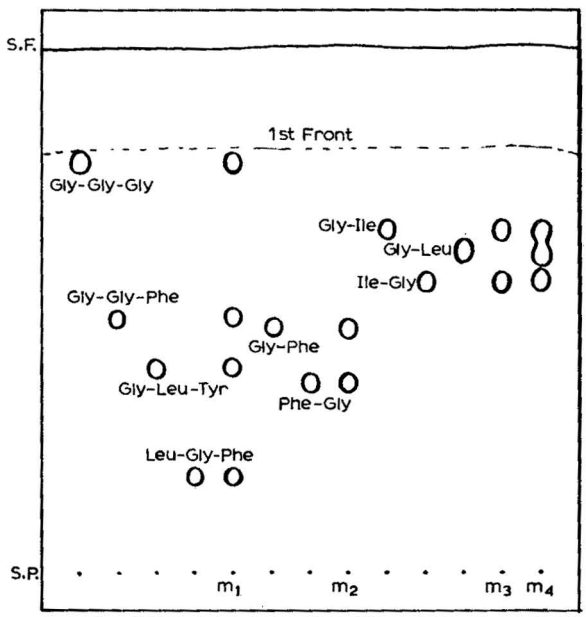

Fig. 3. Thin-layer chromatogram of tripeptides and pairs of isomeric dipeptides on silanized silica gel impregnated with 4% H-DBS solution. Migration distance = 14 cm. Eluent: 1 *M* sodium acetate solution in water–methanol (7:3). m_1 = mixture of tripeptides; m_2 = mixture of Gly–Phe and Phe–Gly; m_3 = mixture of Gly–Ile and Ile–Gly; m_4 = mixture of the three isomeric peptides. S.P. = starting point; S.F. = solvent front.

residue, eluting with 0.05 *M* hydrochloric acid + 1 *M* acetic acid in 30% methanol (apparent pH = 1.55). Such a separation cannot be effected on layers of silanized silica gel alone, which is more suitable for the separation of hydrophobic polypeptides. In this connection it should be noted that the separation of the peptides Gly–Gly–Gly, Gly–Gly–Phe, Gly–Leu–Trp, Leu–Gly–Phe, Leu–Trp–Met–Arg and Leu–Trp–Met–Arg–Phe) can be effected only on silanized silica gel alone, with 0.1 *M* acetate buffer in 30% methanol as eluent.

In Fig. 3 are shown the separations of the four above-mentioned tripeptides and of pairs of isomeric dipeptides on layers impregnated with 4% H-DBS, eluting with 1 *M* sodium acetate in 30% methanol. It should be noted that among the isomeric pairs of dipeptides the only separation not completely achieved is Gly–Ile/Gly–Leu. Similar behaviour is observed on the same layers in the separation of Gly–Ala from Ala–Gly, eluting with 1 *M* acetic acid in 30% methanol or with water–acetic acid (7:3).

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INTERACTIONS OF INSOLUBILIZED LECTINS WITH MEMBRANE GLYCOPROTEINS IN PRESENCE OF DETERGENTS

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SUMMARY

The effects of several detergents commonly used to solubilize membrane glycoproteins have been investigated on the binding of hepatoma cell surface [^{3}H]-galactoglycoproteins to, and their elution from, concanavalin A or *Ricinus communis* lectins conjugated to Sepharose 4B. The optimum conditions (pH, ionic strength) in the presence of ionic [sodium deoxycholate (DOC) and sodium dodecyl sulphate (SDS)] and non-ionic detergents (Triton X-100) at a constant concentration were determined in order to ascertain which would yield the better efficiency. The effects of different detergent concentrations on binding and elution were then studied. The range of concentrations for each detergent to be used without modifying efficiency was determined. Triton X-100 and DOC (0.1–1%) did not change the efficiency on Ricinus lectin–Sepharose, whereas SDS, at a concentration greater than 0.05%, caused a dramatic decrease in efficiency. On concanavalin A–Sepharose, by contrast, the non-ionic detergent had no effect on the efficiency at all the concentrations tested (0.1–1%), while concentrations of more than 0.5% DOC and 0.1% SDS significantly decreased both binding and elution.

INTRODUCTION

Affinity chromatography using columns of insolubilized lectins is an effective procedure for the fractionation and isolation of glycoproteins and glycopeptides[1–3]. Since membrane glycoproteins are insoluble in neutral aqueous solutions, buffers containing ionic or non-ionic detergents must be used[4]. However, these detergents may either modify the native structure of the insolubilized lectins and/or change the interactions between the lectin and the membrane glycoproteins. Therefore, before using columns of insolubilized lectins to isolate glycoproteins, their fixation and elution should be studied under various experimental conditions (pH, ionic strength, detergent concentration) in order to determine the optimum procedure. Such studies have been done on concanavalin A and Ricinus lectin conjugated to Sepharose 4B using three ionic and non-ionic detergents and membrane glycoproteins from hepatoma cell surfaces. The optimum conditions were then applied to the fractionation of

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these glycoproteins in order to isolate the receptors of these lectins implicated in cell regulation[5] and in their toxic effect[6].

MATERIALS AND METHODS

Concanavalin A–Sepharose was obtained from Pharmacia, Uppsala, Sweden. *Ricinus communis* lectin (molecular weight: 120,000) was purified according to Nicolson and Blaustein[7] and was covalently conjugated to CNBr-activated Sepharose 4B (Pharmacia) as directed by the manufactures.

The detergents used were octylphenoxypolyethoxy ethanol (Triton X-100) from Sigma (St. Louis, MO, U.S.A.), sodium deoxycholate (DOC) from E. Merck (Darmstadt, G.F.R.) and sodium dodecyl sulphate (SDS) from Touzard and Matignon (Vitry-sur-Seine, France).

The labelling of the cell surface glycoproteins, using galactose oxidase and sodium [^{3}H] borohydride, has been described previously[8], as has the [^{3}H]galactoglycoprotein release from cells and their initial fractionation[9]. The specific radioactivity of [^{3}H]galactoglycoprotein was $0.18 \cdot 10^6$ dpm/mg of the protein.

Binding of [^{3}H]galactoglycoproteins to concanavalin A and Ricinus communis beads

The beads and the [^{3}H]galactoglycoproteins were equilibrated in 0.02 *M* Tris–HCl, pH 7 or 7.8, containing various concentrations of sodium chloride and the detergents. The binding studies were done in centrifuge tubes. The beads (500 μg of insolubilized concanavalin A or *Ricinus communis* lectin) were incubated routinely with [^{3}H]galactoglycoproteins (10,000 cpm) for 2 h at room temperature with gentle shaking. They were then centrifuged and washed five times with the buffer. To determine the radioactivity of each supernatant, an aliquot of each was added to 10 ml scintillation fluid (PCS, Amersham-Searle, Arlington Heights, IL, U.S.A.) and counted in a liquid scintillation spectrometer (Intertechnique SL 300). The efficiency of [^{3}H]galactoglycoprotein binding to the lectin–Sepharose was estimated by:

$$\frac{\text{cpm of } ^3\text{H added} - \text{cpm of } ^3\text{H unbound}}{\text{cpm of } ^3\text{H added}} \times 100\%$$

Elution of bound [^{3}H]galactoglycoprotein

Elution was carried out with the same buffer as that used for fixation, but containing 0.2 *M* α-methylglucoside (Sigma) for concanavalin A–Sepharose and 0.1 *M* lactose (E. Merck) for *Ricinus communis* lectin–Sepharose. The procedure was the same as that for fixation. The efficiency of [^{3}H]glycoprotein elution was estimated by:

$$\frac{\text{cpm of } ^3\text{H eluted}}{\text{cpm of } ^3\text{H added} - \text{cpm of } ^3\text{H unbound}} \times 100\%$$

The results were averages from four separate experiments and a new preparation of the glycoprotein fraction was used for each experiment.

RESULTS

Effects of pH and ionic strength on [^{3}H]galactoglycoprotein binding at constant detergent concentration

The detergent concentrations used in these experiments were 0.25% for DOC and Triton X-100 and 0.05% for SDS.

The efficiency of [^{3}H]galactoglycoprotein binding to concanavalin A beads at pH 7 was similar irrespective of the detergent present and was unaffected by ionic strength. By contrast, at pH 7.8, the presence of saline (0.25 *M* NaCl) increased the efficiency to that at pH 7 and in the absence of NaCl. This was also the case with DOC and Triton X-100, but not with SDS (Fig. 1a,b). The optimum conditions for binding were obtained using either a Tris–HCl buffer at pH 7 (with no additional salt) or a Tris–HCl buffer at pH 7.8 (0.25 *M* NaCl) and DOC or Triton X-100. Only the Tris–HCl buffer pH 7 gave optimum conditions for binding when SDS was used (Fig. 1a, b). Under these conditions, 40% of the [^{3}H]galactoglycoprotein was bound to the concanavalin A beads.

The efficiency of [^{3}H]galactoglycoprotein binding to *Ricinus communis* beads

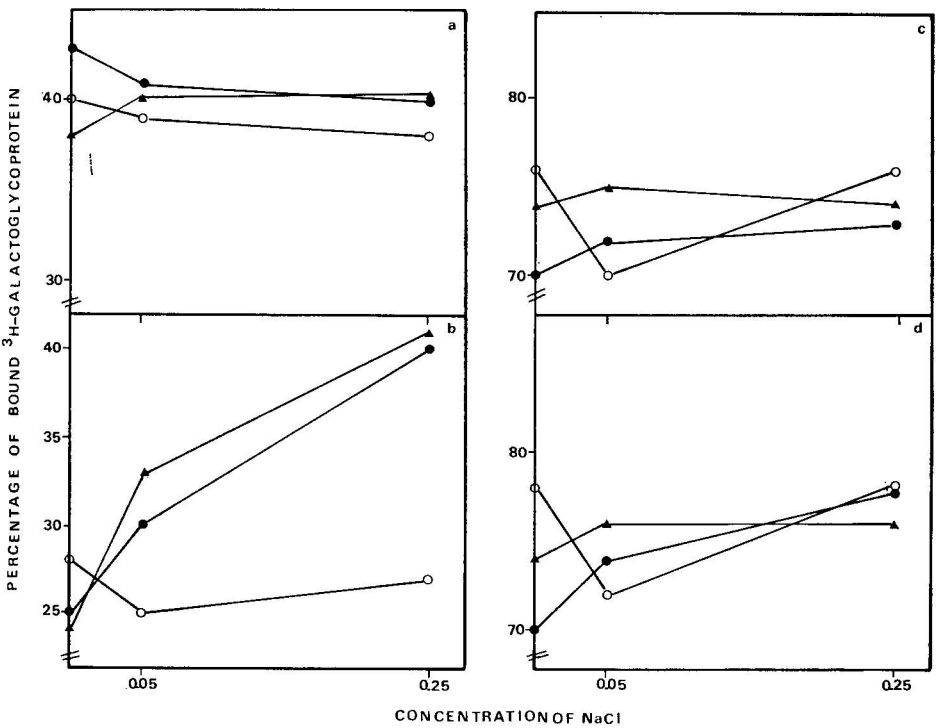

Fig. 1. Effects of pH and ionic strength on efficiency of [^{3}H]galactoglycoprotein binding to concanavalin A beads (left) and Ricinus lectin beads (right) in the presence of a constant concentration of detergent. The binding was performed using 0.02 *M* Tris–HCl buffer at pH 7 (a, c) or pH 7.8 (b, d), containing different concentrations of sodium chloride and DOC (0.25%) (●), Triton X-100 (0.25%) (▲), or SDS (0.05%) (○). Each point in this and other figures represents the average value obtained from four separate experiments.

was similar to the above at both pH 7 and pH 7.8 and unaffected by saline concentration whichever the detergent used. 70–75% of the [^{3}H]galactoglycoproteins were bound (Fig. 1c, d).

Effects of pH and ionic strength on the elution of bound [^{3}H]galactoglycoprotein

The elution of [^{3}H]galactoglycoproteins bound to concanavalin A beads was increased at pH 7.8 in the absence of NaCl and in the presence of either DOC or Triton X-100 (Fig. 2a, b). Using SDS, optimum elution was obtained with a buffer at pH 7 and in the absence of NaCl, or at pH 7.8 with 0.25 *M* NaCl (Fig. 2a,b). 50% of the bound [^{3}H]galactoglycoprotein was eluted using DOC and Triton X-100 and 60% with SDS (Fig. 2a, b). With each detergent, the elution of the [^{3}H]galactoglycoproteins bound to *Ricinus communis* beads was increased at pH 7.8 and with no NaCl. The optimum efficiency of elution was unaffected by the saline concentration in the buffer. 70% of the [^{3}H]galactoglycoproteins were eluted (Fig. 2c, d).

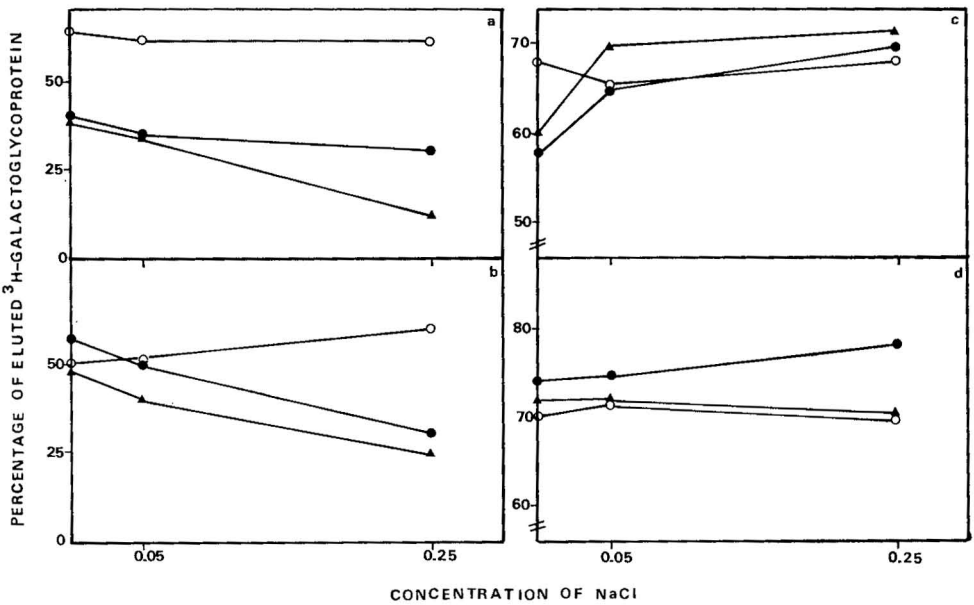

Fig. 2. Effects of pH and ionic strength on [^{3}H]galactoglycoprotein elution efficiencies from concanavalin A beads (left) and Ricinus beads (right) in the presence of a constant concentration of detergent. Elution was performed using 0.02 *M* Tris–HCl buffer at pH 7 (a, c) or pH 7.8 (b, d), containing either 0.2 *M* α-methylglucoside (for concanavalin A) or 0.1 *M* lactose (for Ricinus lectin) and different concentrations of sodium chloride and detergent (see Fig. 1).

Specificity of the interaction between [^{3}H]galactoglycoproteins and the lectin beads

The [^{3}H]galactoglycoproteins were incubated with the beads using the optimum buffer conditions determined above and, in addition, either 0.2 *M* α-methylglucoside (concanavalin A beads) or 0.1 *M* lactose (Ricinus beads) was added to the buffer. The binding of [^{3}H]galactoglycoprotein to the concanavalin A or Ricinus beads was either 70% or 90% inhibited by the saccharide inhibitor of lectin.

Effects of different detergent concentrations on the binding and elution steps

For these experiments, the optimum conditions determined were used. The [^{3}H]galactoglycoprotein fractions were soluble at all of the detergent concentrations studied, *i.e.*, in the presence of detergent concentrations greater than 0.02% of SDS or 0.1% of Triton X-100 or DOC.

On concanavalin A beads, the efficiency of binding and elution was unaffected by the concentration of Triton X-100. By contrast, concentrations greater than 0.1% SDS or 0.5% DOC caused a marked decrease in the binding and elution efficiencies (Fig. 3).

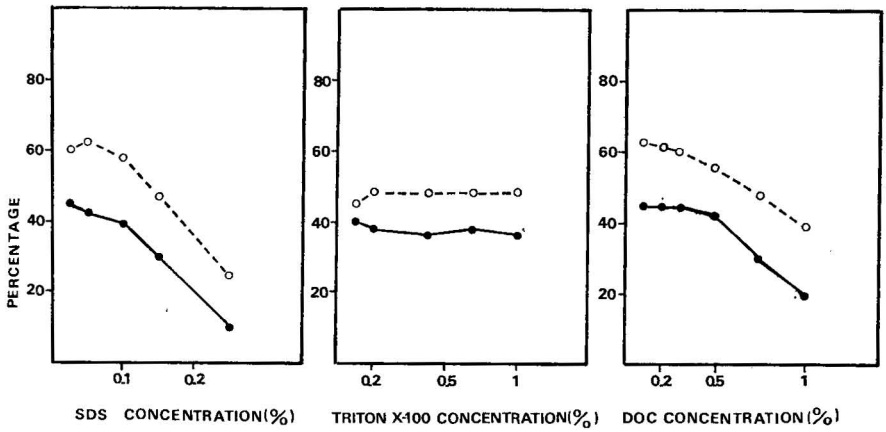

Fig. 3. Effect of different detergent concentrations on the binding and elution steps for [^{3}H]-galactoglycoproteins and concanavalin A beads. In the presence of SDS, fixation was performed using 0.02 *M* Tris–HCl pH 7 and elution using the same buffer containing 0.2 *M* α-methylglucoside. In the presence of Triton X-100 and DOC, fixation was performed using 0.02 *M* Tris–HCl pH 7 and elution using 0.02 *M* Tris–HCl pH 7.8 containing 0.2 *M* α-methylglucoside. The percentages of bound (●) and eluted (○) [^{3}H]galactoglycoprotein are shown.

On *Ricinus communis* beads, the efficiencies of binding and elution were unaffected by the concentration of Triton X-100 or DOC, but in the presence of SDS these efficiencies were dramatically decreased at concentrations greater than 0.05% (Fig. 4).

DISCUSSION

We have determined the optimum conditions for lectin affinity chromatography in the presence of different detergents commonly used to solubilize membrane constituents. In such procedures, the detergent and particularly its concentration has to be chosen so as to maintain the solubility of the membrane constituents and the eluted products, and to allow the specific interactions between the glycoproteins and the conjugated lectin to take place without modifying their native structure.

Few studies have been carried out on either the effects of different types of detergent or the binding and elution steps of these methods. The fixation of solubilized erythrocyte membrane constituents has been studied using conjugated

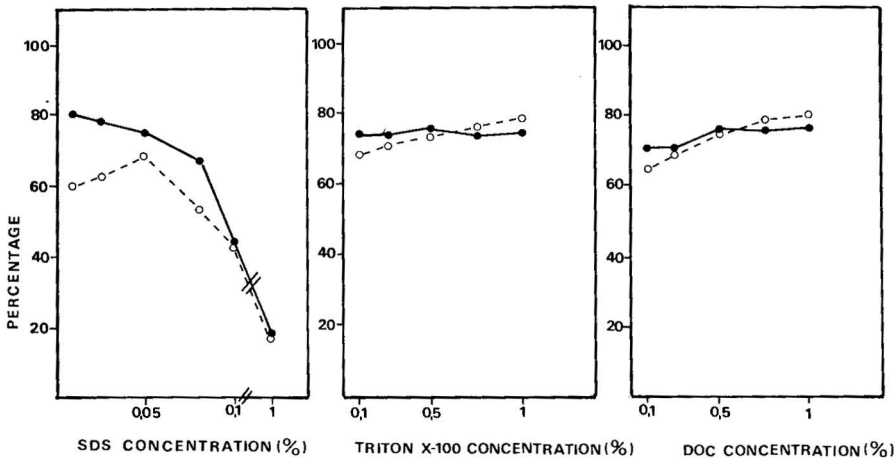

Fig. 4. Effects of different detergent concentrations on the binding and elution steps for [^{3}H]galactoglycoprotein and Ricinus lectin beads. For each detergent, fixation was performed using 0.02 *M* Tris–HCl pH 7.8 and elution using the same buffer containing 0.1 *M* lactose. Other details as in Fig. 3.

wheat germ agglutinin in order to purify the glycophorin[10], and another study has been reported[11] using the hydrophobic seroglycoprotein, fetuin, which had previously been purified. Since both solubilization and affinity chromatography may be modified by the presence of other membrane hydrophobic constituents such as lipids, membrane glycoproteins could prove to be a more reliable model.

Our data indicate that at a constant detergent concentration the buffer pH is of primary importance. However, Lotan *et al.*[11], using conjugated concanavalin A, found that the maximum efficiencies of binding and elution were affected by ionic strength. In our experience, the choice of buffer pH depends on both the conjugated lectin and the detergent. The maximum recovery of bound product may require a change of pH between fixation and elution. Thus, in the presence of DOC or Triton X-100, the fixation on conjugated concanavalin A increased at pH 7 while the elution was facilitated at pH 7.8.

If the solubilization of the membrane constituents and eluted products requires high concentrations of detergent, a non-ionic detergent (*e.g.*, Triton X-100) is more appropriate than an ionic detergent. The effects on the insolubilized lectin have been shown to be negligible at all the concentrations studied. Similar results have been reported by Lotan *et al.*[11]. The most likely explanation is that the non-covalent bound of the protein structure are not broken by Triton X-100[4].

Ionic detergents such as SDS or DOC may be used without affecting the insolubilized lectins or the affinity, but the range of concentrations applicable is limited and depends on the lectins involved. For example, sodium deoxycholate did not change the efficiency on insolubilized Ricinus beads, but a concentration greater than 0.5% caused a marked decrease of fixation and elution on insolubilized concanavalin A. Our results have shown that both ionic and non-ionic detergents can be used. Identical results have been obtained using affinity chromatography under the optimum conditions determined above. During filtration, insolubility could

be a complication owing to the high concentration of protein in the filtered product. This results in excessive fixation and a low elution efficiency, but can be avoided if the product is used in solution at very low concentration.

Good specificity of [^{3}H]galactoglycoprotein binding was obtained in our experiments as shown by the inhibition of the binding to the lectin beads in the presence of a saccharide inhibitor. The differences in [^{3}H]galactoglycoprotein binding efficiencies between insolubilized concanavalin A and the Ricinus lectin could be explained in terms of the direct accessibility of unmasked galactosyl residues to the insolubilized Ricinus lectin. This would also agree with the number of receptor sites available for each lectin as determined previously[5,6].

While simple sugar elution resulted in the release of almost all the glycoprotein that was initially bound to the Ricinus lectin, only 50% of the material bound to concanavalin A was found to be released under optimum conditions. This result is in agreement with the findings of Nachbar *et al.*[12] and would suggest that some hydrophobic interaction takes place between the insolubilized lectin and the glycoproteins. Our results also indicate that the same receptor molecules bind different lectins. If each lectin receptor bound only one distinct class of glycoprotein, the sum of the percentages of [^{3}H]glycoprotein bound to concanavalin A and Ricinus lectin would not exceed 100. The fact that addition of the percentages yields a sum greater than 100 suggests that these two lectins share the same receptors.

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RETENTION BEHAVIOUR OF *o*-, *m*- AND *p*-ISOMERS OF BENZENE DERIVATIVES ON A SILICA GEL HYDROXYLATED SURFACE IN LIQUID CHROMATOGRAPHY

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SUMMARY

The retention order of *o*-, *m*- and *p*-isomers of polar substituted benzenes has been investigated on columns packed with silica gel with a hydroxylated surface, the eluent being *n*-hexane with different polar additions. The elution order is determined by the nature and position of the substituent groups, their influence on the electron density distribution in the benzene ring, the possibility of the formation of intra-molecular and inter-molecular hydrogen bonds and the orientation of adsorbate molecules relative to the adsorbent surface.

INTRODUCTION

In gas-adsorption chromatography (GAC) on polar adsorbents the additional non-specific inter-molecular interaction of the hydrocarbon parts of molecule with the adsorbent considerably diminishes the difference in total adsorption energy of *o*-, *m*- and *p*-isomers of aromatic compounds. Therefore, the selectivity of such well known polar adsorbents as porous silica with a hydroxylated surface towards these isomers in GAC is usually lower than in liquid-adsorption chromatography (LAC). Adequate separation of these isomers in GAC is obtained only upon a further increase in the adsorbent specificity, for example when using such ion adsorbents as $BaSO_4$ (ref. 1). When using non-polar or weakly polar eluents, LAC provides the separation of *o*-, *m*- and *p*-isomers of aromatic compounds even on such a weakly specific adsorbent as hydroxylated silica. The higher selectivity towards these isomers in LAC compared with GAC is caused by the fact that LAC separation is much more influenced by differences in specific inter-molecular interactions between polar groups of the molecule and polar groups or ions of the adsorbent[2–4]. The non-specific inter-molecular interaction of an adsorbent with methyl and methylene groups of benzene derivatives is not as important as in GAC, because the molecules of the

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eluent (saturated hydrocarbons) also contain these groups. However, inter-molecular interactions of methyl and methylene groups of benzene derivatives with similar groups of eluent molecules are of considerable importance. It has been shown[5] that with mono-*n*-alkylbenzenes, beginning with ethylbenzene, the increase in the length of the *n*-alkyl substituent leads to a decrease in the retention on the hydroxylated surface of silica gel owing to the inter-molecular interaction with the eluent (*n*-hexane).

The LAC separation of isomers of substituted benzenes with polar groups in *o*-, *m*- and *p*-positions on silica gel, eluted with saturated hydrocarbons containing some polar additions, is ensured by the different abilities of the polar substituents to form hydrogen bonds with silanol surface groups, the electron density distribution in the benzene nucleus of these molecules and their orientation relative to the adsorbent surface.

LAC has been used successfully for the separation of *o*-, *m*- and *p*-isomers of alkylphenols and chlorophenols[6,7], methyl- and chloroanilines[8,9] and other isomers[10,11] the aim being to maximize adsorbent selectivity; the adsorbent was impregnated with cadmium and silver salts.

In this work we studied the regularities of the order of retention of benzene derivatives with different substituents in the *o*-, *m*- and *p*-positions on the hydroxylated surface of silica gel when eluting with weakly polar eluents.

EXPERIMENTAL

A TSVET-304 liquid chromatograph equipped with a UV detector (254 nm) was used at room temperature for the measurements. Stainless-steel columns (length 20 cm, I.D. 4 mm) were packed with hydroxylated silica gel C-3 (with a specific surface area of 260 m^2/g and a particle size of 10–15 μm) by the slurry packing method from chloroform. *n*-Hexane–chloroform–isopropanol was used as the eluent. The retention of *o*-, *m*- and *p*-isomers of benzene derivatives containing CH_3, *tert*.-C_4H_9, Cl, Br, OH, $COOCH_3$, NH_2 and NO_2 substituents was investigated.

RESULTS AND DISCUSSION

At a small surface coverage adsorption from non-polar or weakly polar eluents on silica gel with a hydroxylated surface, and the corresponding retention volumes, are determined mainly by specific intermolecular interactions and, if it is possible, by hydrogen bonding between polar groups of the adsorbed molecules and surface silanol groups[4,12]. When separating alkyl-substituted *o*-, *m*- and *p*-isomers of aromatic hydrocarbons (+R substituents) the order of elution of these isomers is influenced by the change in the electron density distribution in the benzene ring[4].

In the separation of *o*-, *m*- and *p*-isomers with polar +R and −R substituents three basic cases may be observed. In the first case, both polar substituents are similar type, in the *o*-position, they do not form strong intra-molecular bonds, or intra-molecular hydrogen bonds in particular (the polar substituents may be different, but it is important that they do not exhibit strong intra-molecular interactions in the *o*-position). In this case the dipole moment increases in the order $p < m < o$. The same order would be expected in the retention of these isomers.

In the second case, both substituents are also polar, identical or not identical and in the *o*-position strong, intra-molecular interactions come into play. Therefore,

the *o*-isomers of such compounds should be retained less than the *m*- and *p*-isomers. Their dipole moments depend on the electron-donating or electron-accepting character of the substituent groups which influence the electron density in the benzene ring. For most of the investigated compounds falling into this group, the dipole moments follow the order $o < m < p$. However, in addition to the electron density distribution, the orientation of the molecule relative to a particular surface and also the influence of the eluent must be considered.

In the third case, one substituent is polar and the other is non-polar (alkyl group). The contribution of the non-polar substituent itself to the retention volume in LC is negligible, but a +R substituent increases the electron density in the benzene ring. However, this effect is smaller than the influence of strong inter-molecular interactions between the substituent polar group of the molecule and surface silanol groups. Therefore, an increase in retention may be expected in the sequence $o < m < p$, *i.e.*, in the order of decrease in the shielding of polar group with alkyl substituent.

Retention volumes of isomers of 16 benzene derivatives were measured in order to establish the retention regularities of such *o*-, *m*- and *p*-isomers on silica gel with a hydroxylated surface (Table I). An increase in the capacity ratio, K_e, for silica gel columns with increase in dipole moment is observed for most of the benzene derivatives investigated. With dioxybenzenes and toluidines a decrease in K_e is observed with the increase in dipole moment, corresponding to the elution order $p < m < o$. It should be noted, however, that the molecular dipole moment does not represent the total capacity of the molecule to engage in specific interactions with the adsorbent.

When the substituent polar groups do not form intra-molecular bonds, the isomers are generally retained in the order $p < m < o$. This order corresponds to the increase in the total dipole moment of the molecule and to the probability that both polar groups interact with the hydroxyl groups of the silica gel surface.

Fig. 1 shows the chromatograms of *o*-, *m*- and *p*-isomers of dimethyl phthalate, methyl nitrobenzoate and xylene. All of these isomers are retained in the order $p < m < o$ because in this instance no strong intra-molecular bonds are formed between the substituent groups. Therefore, *o*-isomers are the most retained as their dipole moments are the highest and they can take the most advantageous position relative to the hydroxyl groups on the silica gel surface.

Fig. 2 shows the chromatograms of nitroaniline, nitrophenol, bromophenol and dihydroxybenzene isomers. The order of retention of these compounds is $p < m < p$, which it is opposite to that of the isomers of the first group. In this instance *o*-isomers are the least retained as the inter-molecular interaction of their polar groups with the silanol groups of the adsorbent surface are weakened by intra-molecular hydrogen bonds between closely positioned substituent groups ($NH_2 \ldots NO_2$; $OH \ldots NO_2$; $OH \ldots Br$; $OH \ldots OH$).

Fig. 3 shows chromatograms of isomers of *tert.*-butylphenol, nitrotoluene and cresol. The substituent groups in the molecules of these compounds do not form strong intra-molecular bonds. However, the retention order $o < m < p$ in this instance is the same as for molecules in which both substituent groups are polar. This retention order is explained by the fact that in these instances the polar group is shielded by methyl or *tert.*-butyl groups, the effect being most notable when two *tert.*-butyl groups are in the *o*-position relative to the hydroxyl group.

TABLE I

VALUES OF CAPACITY RATIO, K_e, AND SELECTIVITY COEFFICIENT, α, OF BENZENE DERIVATIVES ON SILICA GEL WITH A HYDROXYLATED SURFACE

Eluents: (1) *n*-hexane; (2) *n*-hexane–chloroform (90:10); (3) *n*-hexane–chloroform–isopropanol (78:20:2); (4) *n*-hexane–chloroform–isopropanol (75:20:5); Dipole moments[13] (μ) are also given.

Compound	$\mu_{(D)}$			*Eluent*	K_e			α					
	o-	*m-*	*p-*		*o-*	*m-*	*p-*	*m/p*	*o/m*	*o/p*	*m/o*	*p/m*	*p/o*
Bromonitrobenzenes	4.0		2.45	1	9.3	—	6.7			1.4			
Dimethyl phthalates	2.8	2.46	2.2	2	3.3	1.9	1.3	1.8	1.5	2.6			
Methyl nitrobenzoates				2	3.6	2.5	2.1	1.2	1.5	1.8			
Dinitrobenzenes	6.05	3.81	0.32	3	—	0.7	0.3	2.3					
Xylenes	0.58	0.37	0.12	1	2.25	2.09	1.92	1.09	1.08	1.17			
Bromophenols	1.36	—	2.8	3	1.1	5.1	7.5				4.7	1.3	5.9
Chlorophenols	1.43	2.17	2.68	3	0.95	—	7.5				—	—	6-8
Nitrophenols	3.11	3.9	5.05	4	0.3	0.9	1.4				2.7	1.5	4.1
Nitroanilines	4.06	4.91	6.32	4	0.95	2.7	5.4				3.0	1.9	5.7
Dihydroxybenzenes	2.58	1.53	0	4	2.5	3.3	4.0				1.3	1.24	1.64
Chloroanilines	1.84	2.91	3.0	3	0.6	—	3.4				—	—	5.9
Aminophenols				4	4.6	13.5	15.1				3.6	1.2	4.4
Nitrotoluenes	3.66	4.14	4.42	1	7.5	8.9	11.1				1.2	1.3	1.5
tert.-Butylphenols	—	—	1.65	4	1.7	—	7.1				—	—	4.0
Cresols	1.44	1.6	1.64	3	0.3	1.8	1.9				5.9	1.07	6.3
Toluidines	1.58	1.44	1.31	3	1.4	1.8	2.05				1.3	1.14	1.5

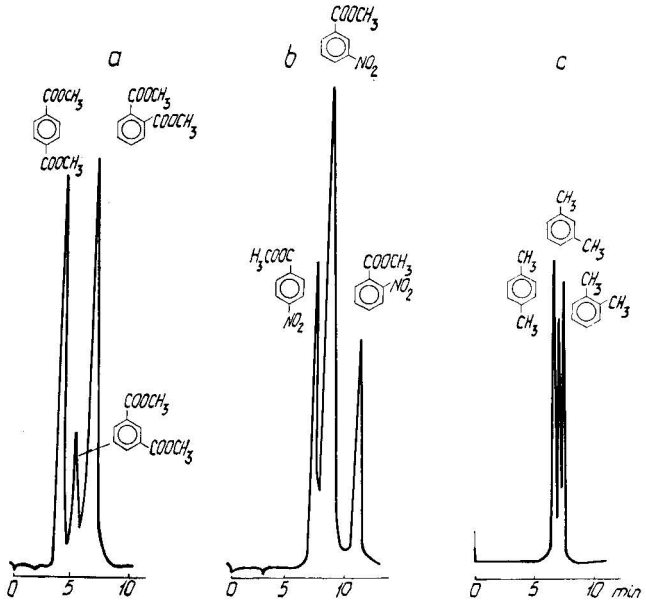

Fig. 1. Chromatogram of isomers (elution order $p < m < o$) on silica gel with a hydroxylated surface. (a) and (b) column 20 cm × 4 mm I.D., silica gel C-3, particle size 10–15 μm, eluent *n*-hexane–chloroform (90:10), flow-rate 1.2 cm^3/min; (c) column 30 cm × 4 mm I.D., silica gel KCC-4, particle size 5–8 μm, eluent *n*-hexane, flow-rate 3.7 cm^3/min [data in (c) from ref. 5].

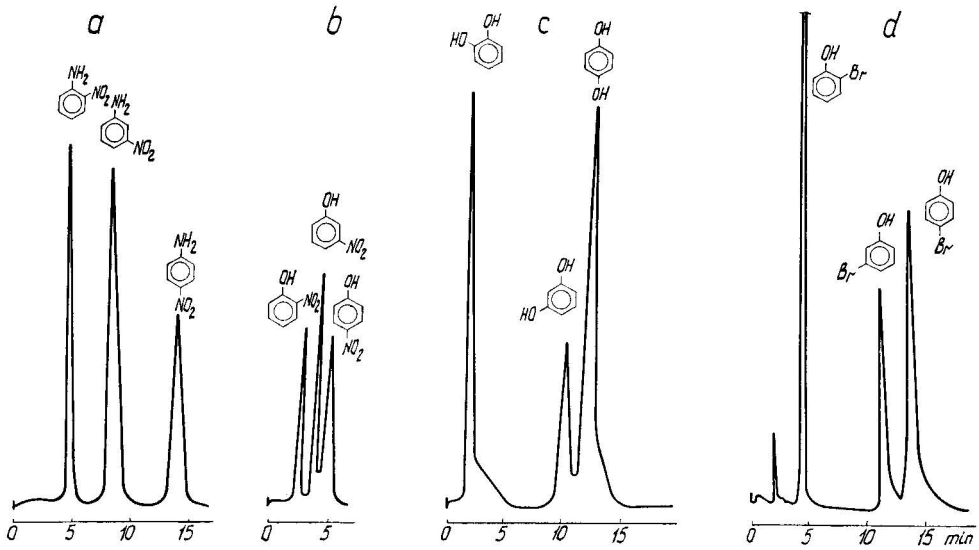

Fig. 2. Chromatogram of isomers (elution order $o < m < p$) on silica gel C-3 with a hydroxylated surface, particle size 10–15 μm, column 20 cm × 4 mm I.D. (a), (b) and (c) eluent *n*-hexane–chloroform–isopropanol (75:20:5), flow-rate 1.2 cm^3/min; (d) eluent *n*-hexane–chloroform–isopropanol (78:20:2), flow-rate 1.5 cm^3/min.

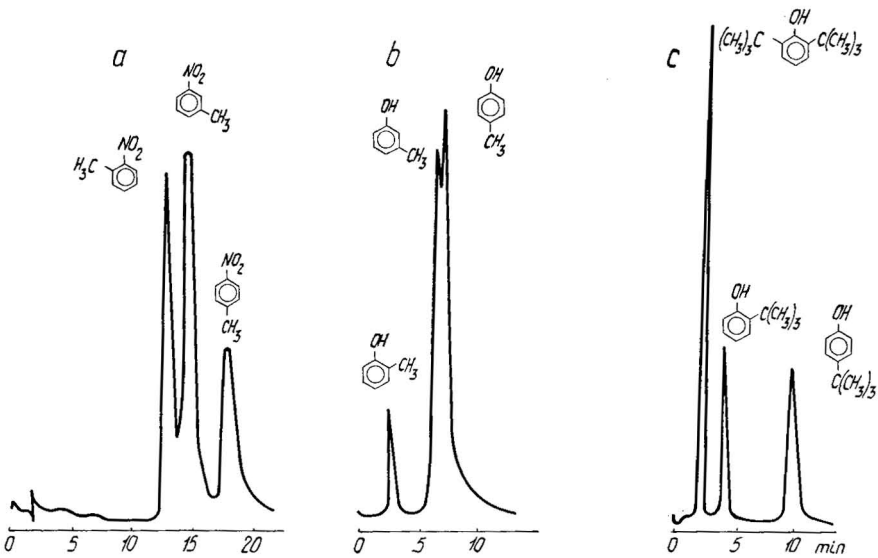

Fig. 3. Chromatogram of isomers (elution order $o < m < p$) on silica gel C-3 with a hydroxylated surface, particle size 10–15 μm, column 20 cm × 4 mm I.D. (a) Eluent *n*-hexane, flow-rate 2 cm^3/min; (b) eluent *n*-hexane–chloroform–isopropanol (78:20:2), flow-rate 1 cm^3/min; (c) eluent *n*-hexane–chloroform–isopropanol (75:20:5), flow-rate 2 cm^3/min.

Hence it has been shown that the order of retention of *o*-, *m*- and *p*-isomers with different functional groups is influenced by several factors such as the nature of the substituents, their probable intra-molecular interaction in the *o*-position, their influence on the electron density distribution in the benzene ring (+R and −R substituents) and steric hindrance (caused by non-polar substituents in the *o*-position) to the formation of strong specific inter-molecular interactions between a polar substituent of the molecule and the silanol group of the adsorbent surface. The regularities obtained for the order of retention of *o*-, *m*- and *p*-isomers help in predicting the retention order for isomers of many similar compounds, taking into account the character of the substituent groups, their mutual influence (possibility of intra-molecular bond formation) and their specific inter-molecular interaction with the polar adsorbent with a favourable orientation.

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SURVEY OF SELECTED HYDRIDES AS DOPING AGENTS FOR A HYDROGEN-ATMOSPHERE FLAME-IONIZATION DETECTOR

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SUMMARY

The hydrogen-atmosphere flame-ionization detector for gas chromatography exhibits a selective and enhanced response for metal containing compounds when its atmosphere is doped with small amounts of silane. In this study, response characteristics of the flame were investigated for a variety of organic compounds when the hydrogen atmosphere was doped with small amounts of methane, silane, germane, or phosphine. Responses of pure hydrocarbons and compounds containing F, Cl, O, S, N, P, As, Sb, Si, or Ge were either unaffected by the addition of doping agents, or their variations were not considered analytically significant. As expected, compounds of Fe, Sn and Pb exhibited enhanced responses with silane doping. Mo and W compounds showed increased ionization with methane. Several compounds increased response with the introduction of germane, but noise also increased such that no gain in signal-to-noise ratio was obtained.

Phosphine proved to be the doping agent with the most potential. Response intensities for compounds containing Fe, Sn, Pb, Mo and Sb appeared analytically useful, but more significant, was the fact that the peaks were negative. Thus, it appears that a potential exists for the development of a phosphine-doped detector in which compounds not containing elements of interest would produce deminutive positive peaks while metal containing compounds would respond with enhanced negative peaks. Further studies on this mode of operation are recommended.

INTRODUCTION

When properly optimized, a hydrogen-atmosphere flame-ionization detector (HAFID), constructed from a commercial flame-ionization detector (FID), is capable of detecting sub-picogram quantities of certain metal compounds with selectivities against hydrocarbons greater than 10^5 (ref. 1). Direct gas chromatographic (GC) determinations of antiknock agents, tetraethyllead and methylcyclopentadieneylmanganese, in gasolines have recently been demonstrated[2].

Operation of an HAFID differs from that of an FID in that the oxident (air, enriched with oxygen) is introduced to the flame with carrier gas while the fuel (hydrogen, doped with small amounts of silane) is brought directly into the detector

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to produce a reducing atmosphere. While position, potential and polarity of the collector electrode, flow-rates of detector gases, and general geometry of the detector housing have been shown to affect response[1,3,4], enhancement of metal ionization by addition of silane to the hydrogen atmosphere is the detector's most mechanistically interesting requisite.

Silane may be involved with charge transfer processes that enable ions produced from burning metal compounds to be more efficiently collected by the electrode[1], but its function is not well understood. Since silane is the only doping agent that has ever been investigated, a survey of several hydrides as dopants for the hydrogen atmosphere was undertaken to compare their effect on response. This paper reports the results of that survey.

EXPERIMENTAL

Conditions

An FID on a Hewlett-Packard 5830A gas chromatograph was converted to an HAFID as described in ref. 2. This detector was maintained at 250°C throughout this study with gas flows of 1600 ml/min for hydrogen, 120 ml/min for air, 150 ml/min for oxygen and 20 ml/min for the helium carrier gas. A 6 ft. × 1/4 in. O.D. (2 mm I.D.) borosilicate column packed with 80–100 mesh Ultra-bond 20 M (RFR, Hope, RI, U.S.A.) was used with oven temperatures selected for individual test compounds to achieve practical retention times. Once an operating temperature was established, each compound was chromatographed at that temperature throughout the study. The injection port was maintained at 225°C.

Procedures

Test compounds used in this study are listed below along with their supply sources. Fluorobenzene, chlorobenzene, dipropylsulfide and amyl ether (Eastman Organic Chemicals, San Francisco, CA, U.S.A.); hexacarbonyltungsten, hexacarbonylmolybdenum and tetrabutylgermane (Alfa Division, Ventron, Danvers, MA, U.S.A.); ethyl benzene and pyridine (Fisher Scientific, Santa Clara, CA, U.S.A.); tetraethylsilane (Pfaltz & Bauer, Stanford, CT, U.S.A.); dodecane (Alltech, Arlington Heights, IL, U.S.A.); tetraethyllead (ICN Pharmaceuticals, Plainview, NY, U.S.A.); ferrocene, tetrabutyltin, triphenylantimony and triphenylarsine (Aldrich, Milwaukee, WI, U.S.A.); nitrobenzene and tributylphosphate (J. T. Baker, Hayward, CA, U.S.A.); and aniline (Mallinkrodt, St. Louis, MO, U.S.A.).

Each compound was selected for its structure or type of heteroatom it contained. Individual solutions in "glass-distilled" hexane (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were prepared at concentrations ranging from $1 \cdot 10^{-10}$ g/μl to $1 \cdot 10^{-6}$ g/μl in decade steps. A 0.5-μl volume of solution was injected at the concentration required to produce a measurable peak at $1 \cdot 10^{-10}$ A f.s. These signals, which were substantially above noise level for all experimental conditions, allowed comparison of concentrations with approximately equal responses. Reproducibility was always better than 10%.

Methane, silane, germane, and phosphine, obtained from Airco Specialty Gases (Santa Clara, CA, U.S.A.) as 1% CH_4 in hydrogen, 100 ppm SiH_4 in hydrogen, 1% GeH_4 in hydrogen and 1% PH_3 in hydrogen, were investigated as doping agents.

Each hydride was added to the hydrogen atmosphere at a mixing ratio of approximately 10 or 50 ppm. The condition in which no doping agent was added to the hydrogen atmosphere was also investigated.

Responses of the different test compounds for each doping condition were compared by calculating the ionization ratio (ε), the number of ions responding/number of molecules of test compound injected. These ratios were calculated from the relation

$$\varepsilon = R/MF$$

where R is response in Coulombs, M is moles of test compound injected and F is Faraday's constant. Results are reported as $-\log \varepsilon$ or, by analogy with p-functions as $p\varepsilon$.

To insure against errors from unwarrented contaminations, responses for each test compound were established in a non-doped system prior to the addition of doping agents. Moreover, responses using the lower mixing ratio (10 ppm) were always obtained before those using the higher mixing ratio (50 ppm). Between the addition of each doping agent, the detector was cleaned by washing with HCl, rinsing with distilled water and acetone, and baking at 300°C overnight. Non-doped responses were confirmed with ferrocene and dodecane to insure that effects of previous doping agents had been eliminated before experiments with the next doping agent were begun.

RESULTS AND DISCUSSION

Table I lists results obtained for flame ionization of each of nineteen test compounds under nine doping conditions. Data are reported as the negative log of the ionization ratio ($p\varepsilon$). A parenthetically enclosed minus sign following a $p\varepsilon$ value denotes that the chromatographic peak was observed as a decrease in the flame's background current (*i.e.*, as a negative peak).

An arbitrary $p\varepsilon$ value of 5.00 was chosen as that below which a response for a given compound was indicative of analytical utility. The basis for selection of this cut-off value was its comparison with normal FID values. Commercial FIDs commonly respond with a sensitivity of 0.015 C/g of carbon, producing approximately one ion for each 500,000 carbon atoms introduced into the flame. For dodecane in the FID, a $p\varepsilon$ value would be on the order of 4.6. Values of 5.00 indicate that test compounds have only slightly less sensitivity in the HAFID than a hydrocarbon has in an FID. Since in this study only two dopant concentrations were selected, it is probable that optimal operating conditions for the HAFID were not achieved and ionization efficiencies can be improved over those reported here.

Table II ranks test compounds whose responses qualified as analytically useful. The lowest $p\varepsilon$ value observed was 2.04, obtained for ferrocene when hydrogen was doped with 50 ppm of silane. The first two peaks in Fig. 1 demonstrate that the ferrocene response is increased at least 1000 times by the addition of silane since 50 pg of the compound gave a slightly larger peak when the detector was doped with silane than did 50 ng of ferrocene without doping. Both germane- and methane-doping enhanced ferrocene response less than silane-doping.

As expected from earlier studies, tetrabutyltin and tetraethyllead also responded

TABLE I

SUMMARY OF pε VALUES

Compound	*pε*								
	No doping gas	*CH_4 (10 ppm)*	*CH_4 (50 ppm)*	*SiH_4 (10 ppm)*	*SiH_4 (50 ppm)*	*PH_3 (10 ppm)*	*PH_3 (50 ppm)*	*GeH_4 (10 ppm)*	*GeH_4 (50 ppm)*
Fluorobenzene	6.45	6.29	6.60	6.40	6.40	6.10	5.99	6.76	7.08
Hexacarbonyltungsten	4.97	4.94	4.84	5.05	4.68	6.07 (−)	5.63 (−)	5.02	5.52
Hexacarbonylmolybdenum	4.12	4.05	3.80	4.16	4.02	4.30 (−)	4.10 (−)	4.88	5.77
Dipropylsulfide	6.86	6.69	6.85	6.77	6.26	7.12	6.49	6.63	7.24
Ethyl benzene	6.56	6.56	6.68	6.45	6.51	6.20	6.16	6.73	7.40
Tetraethylsilane	6.71	6.49	6.78	6.49	6.73	6.18	6.27	6.72	7.41 (−)
Pyridine	6.48	6.39	6.73	6.36	6.32	6.15	6.00	6.59	7.31
Chlorobenzene	6.35	6.33	6.54	6.80	6.58	6.00	5.86	6.59	6.95
Amyl ether	6.72	6.66	6.95	6.66	6.70	6.20	6.10	6.71	7.70
Dodecane	6.60	6.56	6.89	6.48	6.56	6.04	5.89	6.69	6.62
Tetraethyllead	6.44	6.28	6.15	5.48	4.47	5.64 (−)	4.56 (−)	3.80	5.47
Ferrocene	5.30	5.25	4.88	3.79	2.04	3.76 (−)	3.46 (−)	4.20	5.30
Nitrobenzene	6.34	6.33	6.71	6.29	6.27	5.86	5.87	6.30	6.35
Aniline	6.82	6.49	6.80	6.56	6.50	6.19	6.03	6.51	6.70
Tetrabutylgermane	6.36	6.22	6.59	5.32	5.02	5.02	5.30 (−)	6.11	6.64
Tetrabutyltin	5.56	5.46	5.63	4.59	3.69	4.82 (−)	4.26 (−)	3.65	5.14
Tributylphosphate	6.63	6.57	6.72	6.59	5.22 (−)	6.09	6.10	5.56	6.45
Triphenylantimony	6.54	6.48	6.72	5.72	5.30	5.92 (−)	4.62 (−)	6.21	6.41
Triphenylarsine	6.57	6.48	6.70	6.47	6.02	6.16	5.96	5.81	6.02

TABLE II
RESPONSES INDICATING ANALYTICAL POTENTIAL

Compound	*Response*	*Condition*
Ferrocene	2.04	SiH_4 (50 ppm)
	3.46 (–)	PH_3 (50 ppm)
	3.76 (–)	PH_3 (10 ppm)
	3.79	SiH_4 (10 ppm)
	4.20	GeH_4 (10 ppm)
	4.88	CH_4 (50 ppm)
Tetrabutyltin	3.65	GeH_4 (10 ppm)
	3.69	SiH_4 (50 ppm)
	4.26 (–)	PH_3 (50 ppm)
	4.59	SiH_4 (10 ppm)
	4.82 (–)	PH_3 (10 ppm)
Tetraethyllead	3.80	GeH_4 (10 ppm)
	4.47	SiH_4 (50 ppm)
	4.56 (–)	PH_3 (50 ppm)
Hexacarbonylmolybdenum	3.80	CH_4 (50 ppm)
	4.02	SiH_4 (50 ppm)
	4.05	CH_4 (10 ppm)
	4.10 (–)	PH_3 (50 ppm)
	4.12	No doping
	4.16	SiH_4 (10 ppm)
	4.30 (–)	PH_3 (10 ppm)
	4.88	GeH_4 (10 ppm)
Triphenylantimony	4.62 (–)	PH_3 (50 ppm)
Hexacarbonyltungsten	4.68	SiH_4 (50 ppm)
	4.84	CH_4 (50 ppm)
	4.94	CH_4 (10 ppm)
	4.97	No doping

strongly when silane was introduced as a doping gas, but surprisingly, these compounds responded best with 10 ppm of germane. Addition of germane, however, dramatically increased noise, as can be seen in Figs. 2 and 3. Thus, signal-to-noise ratios (the primary criteria for judging analytical utility) decreased when compared to the silane doped system. Although the origin of this noise is unclear, a heavy brown deposit which coated the inside of the detector housing and the collecting electrode during germane-doping may indicate that the noise was caused by formation of particles such as Ge_3N_4 in the flame. An insulating layer of this material on the collecting electrode apparently reduced the effective electrical field and when germane-doping was increased from 10 to 50 ppm, background current and sensitivity decreased as is shown in Tables I and III. Doping with germane is not analytically useful unless these problems can be eliminated.

Hexacarbonylmolybdenum and hexacarbonyltungsten produced their most sensitive responses with methane-doping. They also exhibited the most sensitive responses of any of the test compounds when no doping agents were added to the detector. Thus, it may be possible to use the HAFID for metal carbonyls without doping the hydrogen-atmosphere.

Phosphine proved the most interesting of doping agents investigated since, when added to the hydrogen-atmosphere at the 50-ppm level, strong *negative* responses for ferrocene, tetrabutyltin and tetraethyllead were observed, as is shown in Figs. 1–3.

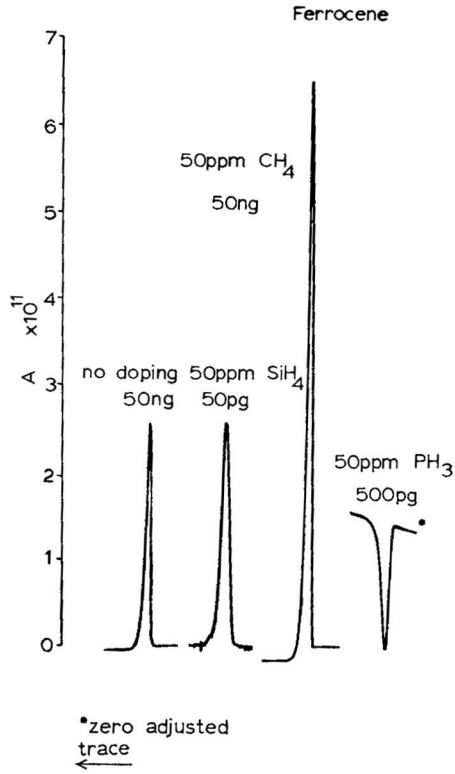

Fig. 1. Selected chromatographic peaks of ferrocene. (In all figures, recorder pen traced from right to left.)

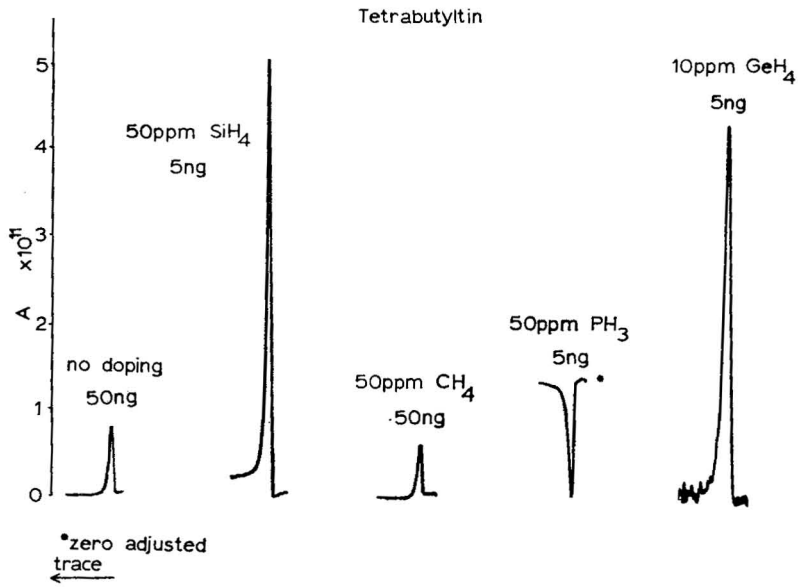

Fig. 2. Selected chromatographic peaks of tetrabutyltin.

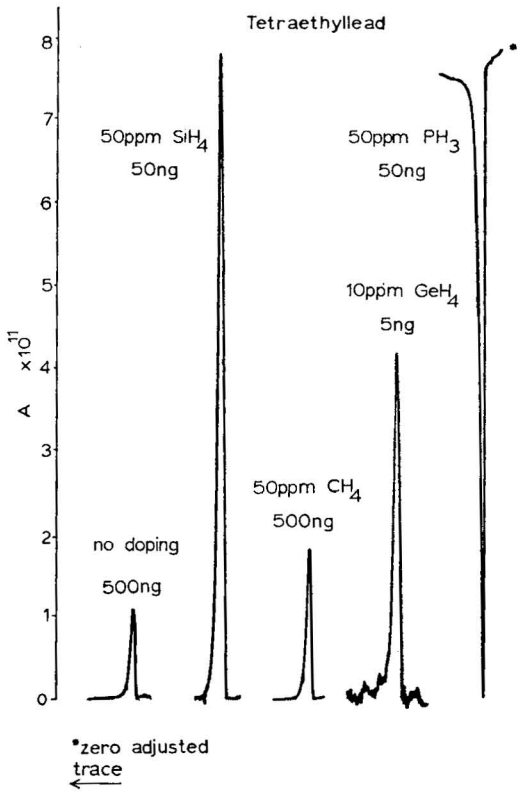

Fig. 3. Selected chromatographic peaks of tetraethyllead.

TABLE III
HAFID BACKGROUND CURRENT

Condition	*Background current (nA)*
No doping	0.27
CH_4 (10 ppm)	0.35
CH_4 (50 ppm)	0.40
SiH_4 (10 ppm)	0.32
SiH_4 (50 ppm)	0.36
PH_3 (10 ppm)	0.68
PH_3 (50 ppm)	0.82
GeH_4 (10 ppm)	0.41
GeH_4 (50 ppm)	0.17

Hexacarbonyltungsten, hexacarbonylmolybdenum, tetrabutylgermane and triphenylantimony also responded negatively with phosphine, while all other compounds exhibited positive peaks.

Three other test compounds, tetraethylsilane, tetrabutylgermane and tributylphosphate, showed interesting results as illustrated in Figs. 4–6. Note that each of these compounds contains a heteroelement which is contained in one of the doping hydrides. Detector responses for tetraethylsilane with silane-doping, for tetrabutyl-

germane with germane-doping, and for tributylphosphate with phosphine-doping were not significantly different from those obtained without doping. Responses for tetraethylsilane and tetrabutylgermane, Figs. 4 and 5, were unique in that they exhibited peak tailing with 10 ppm phosphine. Since this phenomenon did not occur when these compounds were detected under other doping conditions, tailing cannot be attributed to chromatography, but must be due to residual ionization in the detector after the peak has passed through the flame. When the higher amount of phosphine was introduced, the peak for tetrabutylgermane was nearly symmetrical

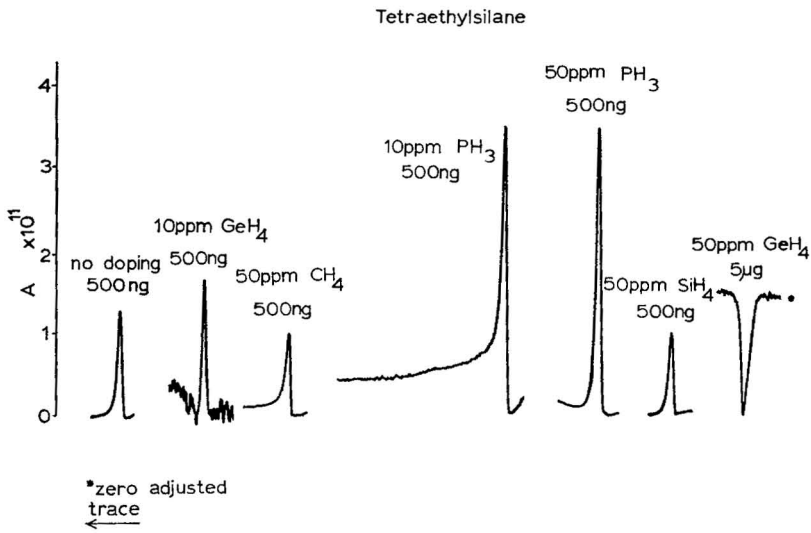

Fig. 4. Selected chromatographic peaks of tetraethylsilane.

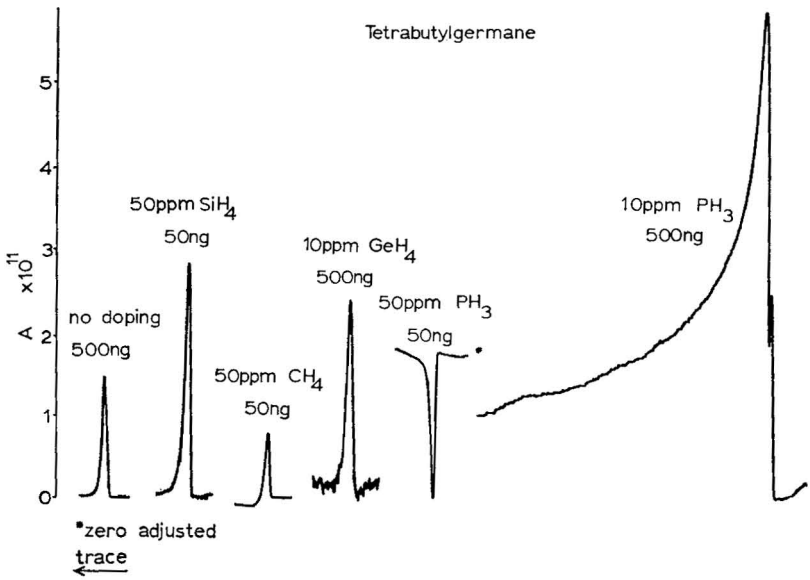

Fig. 5. Selected chromatographic peaks of tetrabutylgermane.

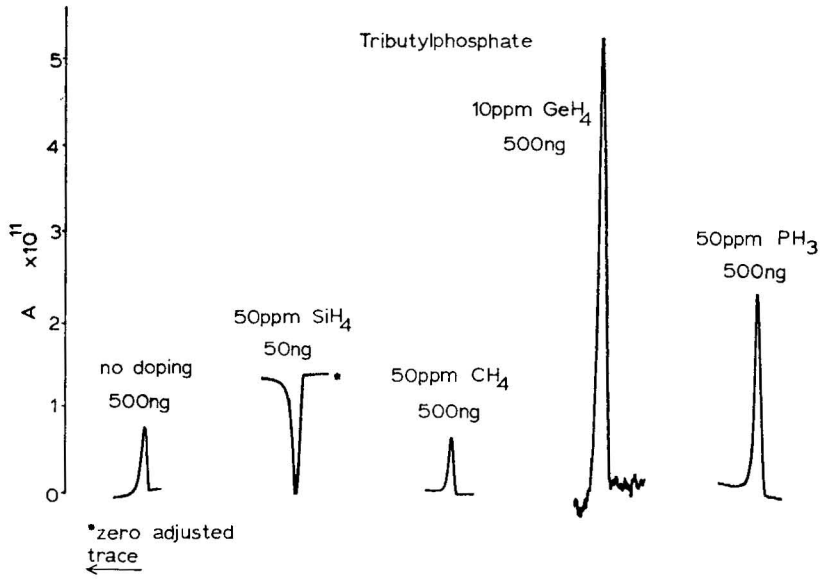

Fig. 6. Selected chromatographic peaks of tributylphosphate.

but inverted. Peak inversions as a function of doping concentration have been observed before[1] but their mechanism has not been explained. Other peak inversions in this study were also seen: the tributylphosphate peak inverted when silane was increased from 10 to 50 ppm as is shown in Fig. 6 while the tetraethylsilane peak inverted when germane was increased from 10 to 50 ppm as is shown in Fig. 4.

The occurrence of negative peaks raises the intriguing question of how responses in the HAFID are obtained. Negative responses, of course, result from a reduction in the background current of the flame. From Table III, which compares background currents at each operating condition, the background current produced with phosphine is seen to be more than three times that of the non-doped system and at least twice that of any of the other doped systems. It may be that formation of pH_4^+ in the detector is responsible for this current which can be readily neutralized when certain anions are produced during combustion of GC eluents. This explanation is, of course, speculative and incomplete. It does not explain why some compounds produce negative responses while others produce positive ones, nor does it explain peak inversions. Currently, work is underway to identify ions in a silane-doped, a phosphine-doped and a non-doped HAFID. Until ion identities are known, it is difficult to discuss mechanisms of these detectors.

In general, the remainder of the test compounds (dodecane, ethyl benzene, amyl ether, aniline, nitrobenzene, pyridine, chlorobenzene, fluorobenzene, dipropylsulfide and triphenylarsine) responded with reduced sensitivity and remarkable stability regardless of changes in doping conditions. Dodecane had a $p\varepsilon$ value of around 6.6, indicating that the HAFID response is about two orders of magnitude less than that of the FID for hydrocarbon compounds. This reduction in sensitivity can be attributed to oxidation to CO and CO_2 in the oxygen rich pre-combustion zone of the HAFID[5] and to the relative locations of the collecting electrodes in the HAFID and the FID.

Fig. 7 shows typical responses for dodecane where all were similar in magnitude except that with 50 ppm PH_3 which was enhanced several times over the non-doping condition. This enhancement is not sufficient to reduce utility of PH_3 as a doping agent since dodecane response increased less than an order of magnitude.

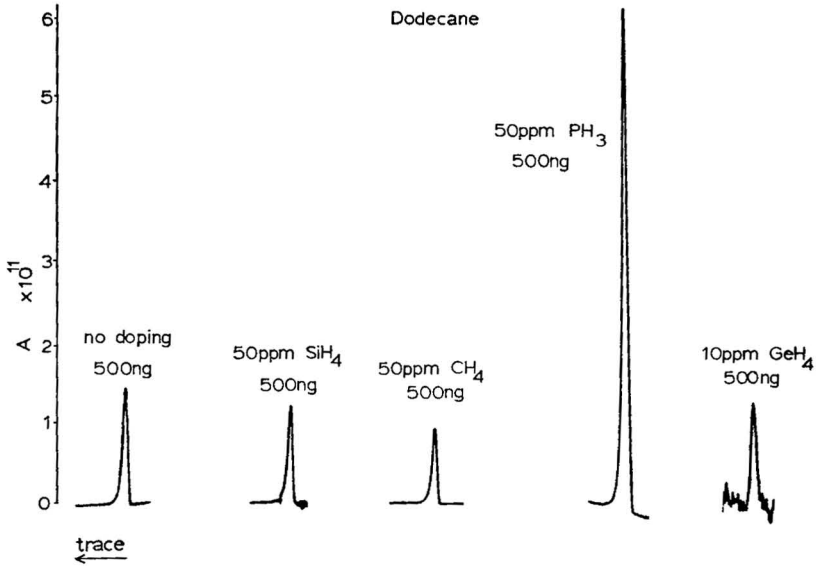

Fig. 7. Selected chromatographic peaks of dodecane.

The detector's ability to discriminate against compounds containing C, H, O, N, P, or halides (those that make up the bulk of complex organic matrixes where traces of organometallics may be contained) under a variety of doping conditions is indicative of its potential as a selective analytical tool for the analyses of metal containing compounds by GC. Although application of a phosphine-doped HAFID must be approached with caution, due to the high toxicity of this doping gas, it appears promising since response is not only sensitive and selective for metal compounds but is also specific for them by the virtue of inverted peaks.

ACKNOWLEDGEMENTS

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NEW CELLULOSE GEL FOR CHROMATOGRAPHY

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SUMMARY

Cellulose gel particles of spherical or irregular shape were prepared by dispersing cellulose–aqueous calcium thiocyanate solution or gel in organic solvents followed by regeneration of cellulose with methanol. These gel particles were tested as column packings for gel chromatography. The gel showed high porosity and sufficient rigidity to be used for gel filtration of large solutes. The pore size of the gel ranged from about a hundred to several thousand Ångströms, depending on the concentration and the molecular weight of the starting cellulose materials. In comparison with conventional macroporous gels such as agarose or cross-linked dextran gels, the cellulose gel has similar low adsorptivity and chemical stability, higher thermal resistance and remarkably improved mechanical strength.

INTRODUCTION

The use of gel substances as column packing in aqueous chromatography, such as gel filtration, ion-exchange or affinity chromatography, is becoming more important as a separation technique in industrial processes as well as in laboratories. Column packing materials for these chromatographies have conventionally been made of cross-linked dextran, agarose or polyacrylamide. However, these products are generally too expensive to be used in industrial applications since they are made from rather uncommon natural substances or they require carefully controlled processes for preparation.

On the other hand, cellulose, the most abundant natural material, has mainly been used in the form of a so-called "microcrystalline" powder for partition or ion-exchange chromatography. Since this material is a dense and very fine fibrous powder of irregular shape, it cannot accommodate large solutes and the packed gel bed suffers from clogging or contraction. Several attempts to prepare modified cellulose packing have been reported. In one method, cellulose powder is reinforced by graft cross-linking[1]; in another, cellulose is dissolved in an aqueous solution such as viscose[2] or Schweizer's solution[3], then dispersed in a non-polar liquid to generate spherical gel particles. Although several products prepared by these procedures are now available, the potential of cellulose as a chromatographic material has not been fully developed.

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In this study a new procedure of cellulose gel preparation is developed which is similar to that of the second method above except for the use of a concentrated aqueous solution of calcium thiocyanate as solvent. The dissolution behaviour of cellulose in this salt solution and the porous structure of the regenerated cellulose gel have been reported[4]. Here, the cellulose gel was prepared in granular or bead form and its performance for gel filtration was examined and compared with conventional gel packings.

EXPERIMENTAL

Preparation of cellulose gel particles

Material. Two kinds of cellulose material of different degrees of polymerization (DP) were used as starting materials: (a) Whatman CF-1 cellulose powder, DP 180; (b) Cotton linter cellulose, DP 1620. Characterization of the starting and the regenerated cellulose has been reported previously[4].

Dissolution of cellulose. Dry cellulose material was weighed and dissolved in an aqueous solution of 59% (w/w) calcium thiocyanate (Wako, Osaka, Japan) at 120–140°C in the absence of air. The solution obtained formed a gel when cooled below about 80°C.

Dispersion of cellulose solution. Three methods were examined for preparation of cellulose gel particles.

(a) The salt–cellulose solution was cooled to room temperature to form a salt–cellulose gel. This gel was dispersed in methanol with a laboratory blender. Since methanol dissolves the salt, dispersion and regeneration take place simultaneously in this procedure.

(b) The salt–cellulose gel was dispersed in 3–4 times its volume of a non-polar liquid containing a small amount of dispersion aid (sorbitan monooleate) at room temperature. The suspension was poured into a large volume of methanol.

(c) The same procedure as (b) was carried out at a high temperature at which the salt–cellulose solution remained liquid. The suspension was poured immediately into cold methanol.

In the last two methods, *o*-dichlorobenzene was chosen as the dispersion medium because of its high boiling point and low flammability. A conventional laboratory blender was used for dispersion. The cellulose gel particles obtained by these methods were collected on filter-paper and thoroughly washed with methanol and then with tap-water. Finally, the particles were fractionated by wet-sieving.

Gel filtration chromatography

The gel particles suspended in deionized water were packed in a glass column (500 × 10 mm with plungers; Pharmacia) to form a *ca.* 30-cm long gel bed. Approximately 0.2% solutions of standard solutes were separately injected into the column through a switching valve connected to a sampling port. The flow-rate was adjusted to 0.15–0.30 ml/min and kept constant within ±5% by a peristaltic pump fitted at the drain. Elution of solutes was monitored by a refractometer (Waters R-403). For comparison, the same test was carried out on two kinds of commercial cellulose packings (Whatman DE-52 and Pharmacia DEAE-Sephacel) and an agarose gel reinforced by cross-linking (Sepharose CL-2B, Pharmacia).

Table I lists the probe solutes used and their molecular diameters in water. The latter values were calculated from the diffusion coefficients or limiting viscosity numbers according to the well-known relations

$$R_d = \frac{kT}{6\pi\eta_w D} \tag{1}$$

$$R_v = 0.54\,(M[\eta])^{1/3} \tag{2}$$

where R_d and R_v are the radii of hydrodynamically equivalent spheres based on diffusion and viscosity respectively, k is the Boltzmann constant, T is the absolute temperature, η_w is the viscosity of water and D and $[\eta]$ are respectively the diffusion coefficient and the limiting viscosity number of the solute in water. The values of D, and $[\eta]$ were obtained from reported data and from the molecular weight of dextran, poly(ethylene oxide) and oligosaccharides[5].

TABLE I

MOLECULAR WEIGHTS AND SIZES OF STANDARD SOLUTES

Polymer	*Molecular weight, M_w**	*M_w* / M_n*	*Molecular diameter (Å)*	*Manufacturer*
Dextran				
T-2000	$2\cdot10^6$	—	580	Pharmacia (Uppsala, Sweden)
T-500	$5.11\cdot10^5$	2.67	330	
T-70	$6.85\cdot10^4$	1.70	130	
T-40	$3.95\cdot10^4$	1.34	100	
T-10	$9.4\cdot10^3$	1.71	50	
Poly(ethylene oxide)				
RE-7	$1.52\cdot10^6$	1.12	1140	Toyo Soda (Tokyo, Japan)
RE-6	$8.76\cdot10^5$	1.10	810	
RE-5	$3.48\cdot10^5$	1.05	460	
RE-4	$1.76\cdot10^5$	1.04	310	
Uniform Latex**				
0.5 μm	—	—	5000	Dow Chemical (Midland, MI, U.S.A.)
0.087 μm	—	—	870	
Raffinose	504	1.00	12	
Glucose	180	1.00	8	

* The values for Dextran T fractions and standard poly(ethylene oxide) were as given by the manufacturers.

** Used for void volume determination of large-pore gels.

RESULTS AND DISCUSSION

Table II gives the preparation conditions of the cellulose gel particles. It has been shown that cellulose does not significantly decompose under these conditions except for chain scission in long molecules[4]. Since spherical shape is desirable for chromatography packings, dispersion method (c) was mainly adopted. However, CF-1:9% gel*, the highest concentration grade prepared here, failed to form spherical particles because of its tendency to re-aggregate after dispersion at high temperatures.

* The cellulose gels are designated as: (cellulose material): (initial concentration (%, w/w)).

TABLE II

PREPARATION CONDITIONS OF CELLULOSE GEL PARTICLES

o-DCB = *o*-Dichlorobenzene.

Cellulose material	*Initial concentration of cellulose (%, w/w)*	*Dissolving condition*		*Dispersing condition*		*Particle shape*
		Temperature (°C)	*Heating time (min)*	*Liquid*	*Temperature (°C)*	
Whatman CF-1 cellulose powder	3.0	120–140	60	*o*-DCB	100–120	Spherical
	6.0	130–150	65	*o*-DCB	100–120	Spherical
				Methanol	25	Irregular
	9.0	130–150	80	*o*-DCB	25	Irregular
Cotton linter	1.0	120–140	30			
	1.5	120–140	30	*o*-DCB	100–120	Spherical
	3.0	140–160	60			

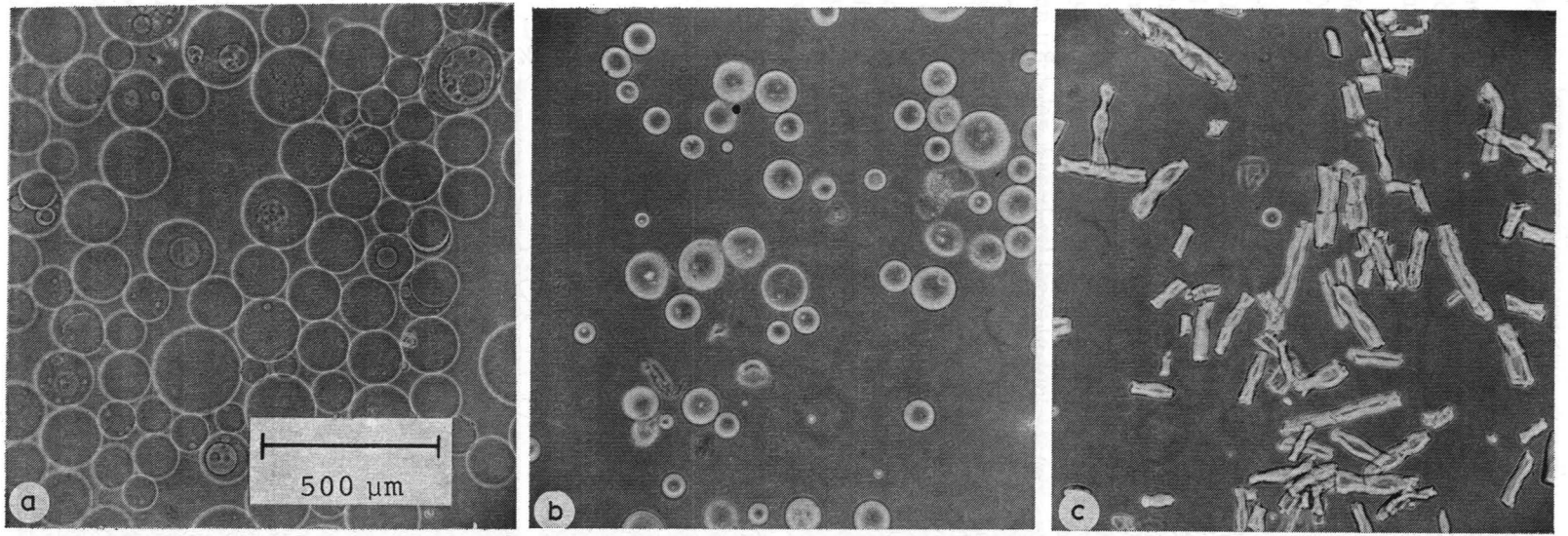

Fig. 1. Phase-contrast photomicrographs of commercial gel packings. (a) Sepharose CL-2B, (b) DEAE-Sephacel and (c) DE-52. Magnification as shown in (a) for all parts of Figs. 1 and 2.

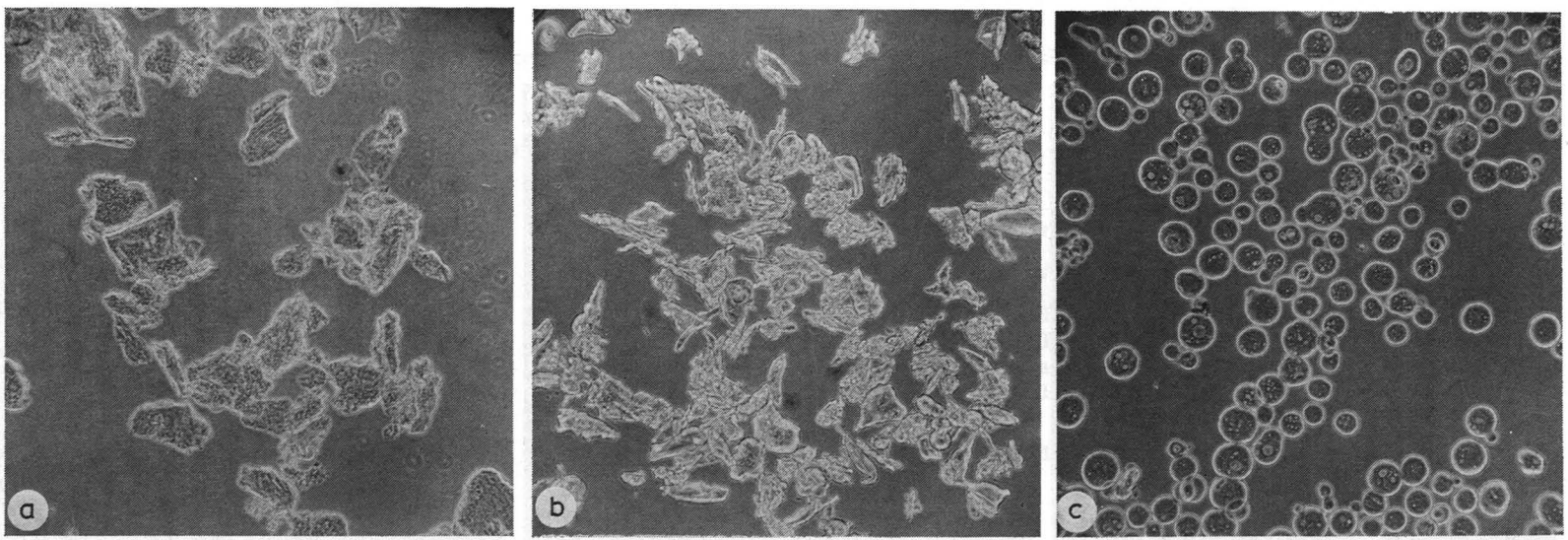

Fig. 2. Phase-contrast photomicrographs of CF-1: 6% cellulose gel particles. Prepared by dispersing in (a) cold methanol, (b) cold o-dichlorobenzene and (c) hot *o*-dichlorobenzene.

Figs. 1 and 2 show phase-contrast photomicrographs of the cellulose gel particles obtained, together with those of commercial gel particles examined. The gel particles obtained by dispersing the salt–cellulose gel in methanol or in cold *o*-dichlorobenzene are of irregular shape as expected (Fig. 2a and b). On the other hand, the particles obtained by dispersing the cellulose solution in hot *o*-dichlorobenzene (Fig. 2c) were nearly spherical, similar to DEAE-Sephacel or Sepharose CL-2B (Fig. 1).

Rigidity of the cellulose gel particles depends on both cellulose concentration and DP of the cellulose material. In general, increase in concentration and DP of the cellulose material results in higher rigidity of the gel obtained. The packed gel bed of the spherical cellulose particles showed sufficient mechanical stability under flow at cellulose concentrations greater than 3% for CF-1 and 1% for cotton linter. The packed gel bed of CF-1:6% or cotton linter:3% gel was markedly more stable to eluent flow than Sepharose CL-2B, which has similar pore and particle sizes to these gels. The bed of an irregular-shaped gel tends to contract and clog more readily than that of a spherical gel of the same grade.

Figs. 3–6 show the chromatograms obtained. Table III summarizes the values of capacity ratio and height equivalent to a theoretical plate. The irregular-shaped gels show lower capacity ratios and poorer resolution than the spherical gels. The latter show as good performance as the commercial agarose or dextran gels. The resolution could be improved by preparing smaller and more uniform particles. Since a gel of high cellulose concentration is quite rigid, it is potentially useful for high-pressure chromatography.

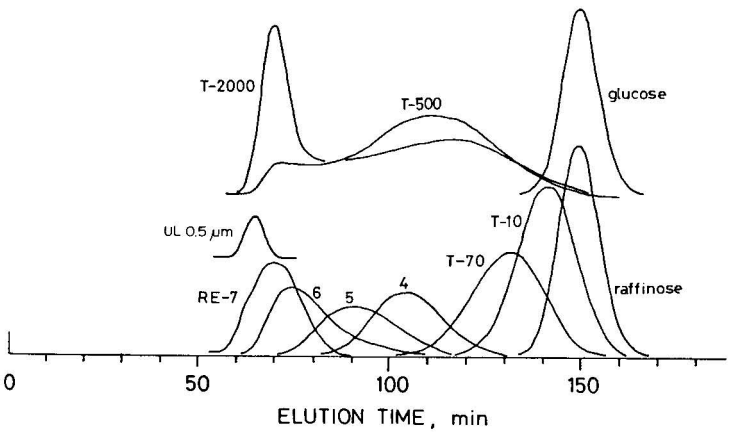

Fig. 3. Gel filtration chromatogram of standard polymers on CF-1: 6% cellulose gel (74–210 μm fraction of spherical beads). Column dimensions: 29.5 × 1.0 cm. Sample volume: 0.5 ml. Flow-rate: 0.162 ml/min.

Figs. 7 and 8 show the calibration curves determined from the chromatograms. Elution times for symmetrical curves were determined from peak positions, for unsymmetrical curves from the position of the vertical line dividing the area under the curve into two parts of equal area. The results were plotted against molecular diameter of the solutes instead of molecular weight so that a universal single curve can be drawn for the different series of polymers. This representation seems pertinent

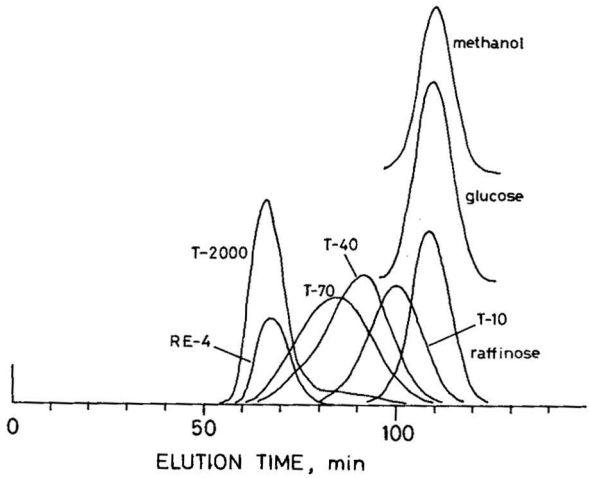

Fig. 4. Gel filtration chromatogram on CF-1: 9% cellulose gel (105–210 μm fraction of irregular-shaped particles). Column: 31.2 × 1.0 cm. Flow-rate: 0.242 ml/min.

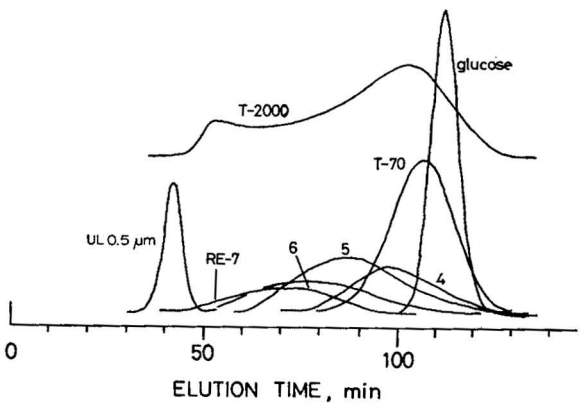

Fig. 5. Gel filtration chromatogram on cotton linter: 1.0% gel (44–210 μm fraction of spherical beads). Column: 29.6 × 1.0 cm. Flow-rate: 0.224 ml/min.

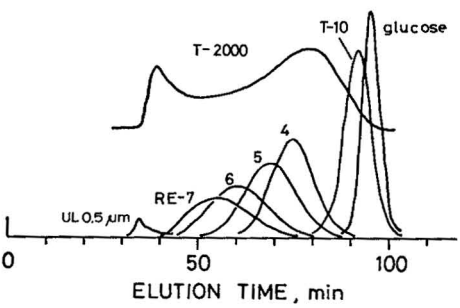

Fig. 6. Gel filtration chromatogram on cotton linter: 1.5% gel (44–149 μm fraction of spherical beads). Column: 25.5 × 1.0 cm. Flow-rate: 0.229 ml/min.

TABLE III

GEL FILTRATION PERFORMANCES OF CELLULOSE AND OTHER GEL PACKINGS

HETP = Height equivalent to a theoretical plate; V_t = internal volume of gel; V_0 = void volume.

Gel	*Shape and size (μm)**	*HETP (mm)*	*Flow-rate (ml/min)*	*Capacity ratio, V_t/V_0*
CF-1: 3%	Spherical, 74–210	0.69	0.164	1.86
6%	Irregular, 149–297	1.13	0.169	0.69
6%	Spherical, 74–210	0.30	0.162	1.27
9%	Irregular, 105–210	0.46	0.242	0.82
Cotton linter: 1.0%	Spherical, 44–210	0.31	0.224	1.22
1.5%	Spherical, 44–149	0.12	0.229	1.94
3.0%	Spherical, 105–210	0.23	0.227	1.00
DEAE-Sephacel	Spherical, 40–130**	0.44	0.157	1.94
DE-52	Fibrous, 30–60 × 100–300**	1.04	0.157	1.00
Sepharose CL-2B	Spherical, 60–250**	0.23	0.155	1.78

* Nominal value from sieve opening.

** Obtained by microscopic observation. For DE-52, width × length.

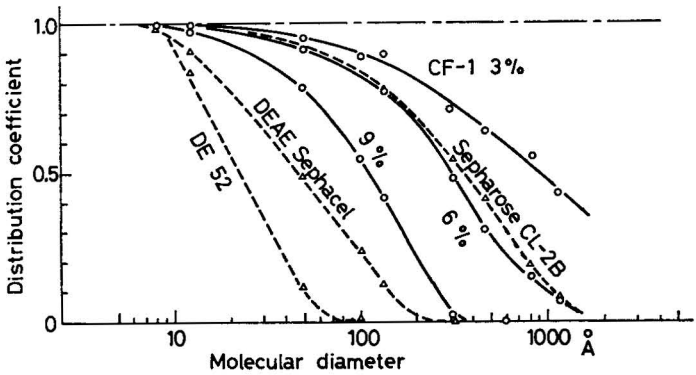

Fig. 7. Calibration curves for CF-1 cellulose gels and commercial cellulose and agarose gels.

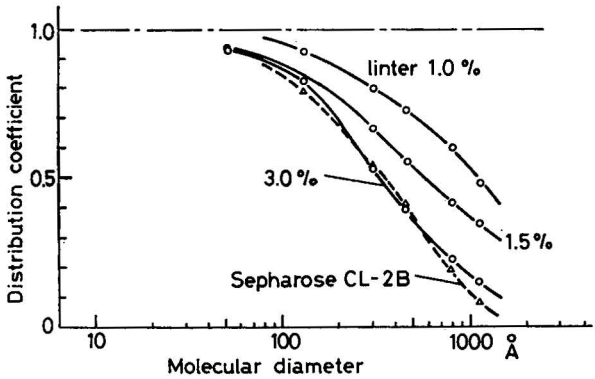

Fig. 8. Calibration curves for cotton linter cellulose gels.

since the curves can be drawn smoothly on the plots for dextran and poly(ethylene oxide) fractions. (In Figs. 7 and 8 the points at above 300 Å correspond to the poly (ethylene oxide) fractions; the others to the dextran fractions.

These curves can be regarded as normalized pore size distributions of the gels provided that partition equilibrium is established instantaneously between the inside and outside of the gel particles. The calibration curve of the cellulose gel changes systematically with cellulose concentration. The mean pore size increases with decrease in the cellulose concentration. It also depends on the kind of cellulose material or its DP. Cotton linter gave a smaller pore size than CF-1 at the same cellulose concentration. This is probably due to the fact that longer cellulose molecules cause greater shrinking of the gel structure when cellulose is regenerated, and is in agreement with previous measurements of the water content of the gels[4].

CF-1:9% gel, which has the smallest pore size, nevertheless has larger pores than the commercial cellulose packings tested. Although the pore size of the cellulose gel would be further reduced on increasing the initial cellulose concentration, it was difficult to dissolve large amounts of cellulose because of the high viscosity of the solution. The upper limit of cellulose concentration was about 10% for CF-1 and 5% for cotton linter under the present dissolution conditions.

The gels of low cellulose concentration have very large pores. CF-1:6% and cotton linter:3% gels gave calibration curves almost equal to that of Sepharose CL-2B, which has one of the largest pore sizes so far available. Gels having the lowest cellulose concentrations, which showed wider fractionation ranges for large molecules, will be advantageous for separation of ultra-high-molecular-weight solutes such as native dextran, viruses or DNA.

No adsorption of solutes on the cellulose gel was detected except for a weak aromatic sorption effect as in the case of Sephadex. The ion-exchange capacity of CF-1:6% gel was estimated by pH-metric titration to be less than 10^{-5} equiv./g dry cellulose, which is of the same order as that of Sephadex.

This cellulose gel probably consists of semi-crystalline cellulose microfibrils instead of single molecular chains or aggregates of several molecular chains as in dextran or agarose gels, thus explaining the enhanced rigidity of the gel. For the same reason, the imbided water can be replaced by any polar or non-polar liquid without significant contraction of the gel. In addition, the gel is thermally stable and contains densely distributed hydroxyl groups. These features are highly advantageous for chemical modification of the gel for the purposes of ion-exchange or affinity chromatography.

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CHROM. 12,790

DETERMINATION OF RESIDUES OF ANABOLIC DRUGS IN MEAT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The residues in meat of seven estrogenic drugs used in anabolic preparations for animal production were analysed as trimethylsilyl ethers by electron-impact gas chromatography–mass spectrometry following a simple clean-up procedure. The compounds under investigation were: 17β-estradiol, diethylstilbestrol, hexestrol, dienestrol, stilbestrol, ethynylestradiol and zeranol. The method includes extractive homogenization of 10 g of meat in tetrahydrofuran, followed by liquid–liquid partition between acetonitrile and hexane and finally a chromatographic purification step on a small silica gel column. Gas chromatography was carried out on a 10-m glass capillary column coated with SE-54 using a temperature program from 100 to 250°C. The capillary column was connected to the ion source by an all-glass open-split interface with a scavenger gas-line. Detection of anabolic residues was performed with selected ion monitoring on intensive ions in the mass region above *m/e* 400, resulting in a detection limit of 1–5 ppb (10^9). Quantitative determinations were performed using dodecyl gallate as an internal standard applying the signal ratio of the drug and the standard.

INTRODUCTION

In many countries anabolic drugs are used to improve the growth rate and the feed conversion of animals in livestock breeding. The use of drugs in meat production involves a possible health risk if residues remain in the meat. Therefore, sensitive methods of residue analysis are necessary for the surveillance of food from animal produce in the market.

Biological methods based on morphological alterations in the sexual organs of test animals are now of minor importance. Chromatographic methods, including thin-layer chromatography, gas chromatography and high-performance liquid chromatography, were developed for the residue analysis of single compounds as well as for groups of anabolic drugs. Reviews covering the chromatographic methods until 1976 and 1978 were given by Ryan[1] and Günther[2].

In the last two years numerous modifications and improvements of existing

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procedures as well as the development of new methods have been published, indicating the widespread interest in this field[3–16]. A continuing urgent need for reliable methods for anabolic drug residue analysis is evident. Competitive protein binding methods are widely used because of their high sensitivity and simple handling. The most prominent type is the radioimmunoassay, which combines high specificity with high sensitivity. Detection methods are now available for most of the anabolic drugs used in animal production[17–22]. It must be considered as a drawback of the radioimmunoassay that specific antibodies and radiolabelled test compounds of high specific radioactivity are necessary for each hormone, drug and their metabolites.

A competitive protein binding method using the natural estrogen receptor protein from bovine uterus cytosol was developed as a screening procedure for estrogenic components in anabolic drugs[23,24]. This assay has to be combined with specific methods for qualitative and quantitative determination of the various compounds in question.

The most promising method to meet this goal is gas chromatography–mass spectrometry (GC–MS). GC–MS has been successfully used for many years in clinical chemistry for the analysis of steroid hormones and their metabolites in urine and blood samples, as well as for doping controls in the urine of athletes[25–30].

The application of GC–MS for residue analysis in meat has been reported only in a few cases. Day *et al.*[31] described the confirmation of diethylstibestrol (DES) residues in beef liver as the bisdichloroacetate with GC–MS using the GC method of Donoho *et al.*[32]. Höllerer and Jahr determined estrogenic anabolics in bovine liver with GC–MS using the trimethylsilyl ethers of these drugs. The detection limit for the stilbene derivatives investigated by them in meat samples was reported as 4–40 ppb[33].

In this paper we report the determination of residues of anabolic drugs in meat at the ppb level. The method includes a simple clean-up, the formation of trimethylsilyl ethers and the detection and identification of the derivatives with electron-impact GC–MS using chromatographic separation with glass capillary columns.

EXPERIMENTAL

Instrumentation

A Finnigan Model 4000 quadrupole mass spectrometer with an integrated data system Finnigan/Incos 2300 was used for all measurements. The ion source was a combined type for electron impact and chemical ionization. The mass spectrometer was connected to a Finnigan gas chromatograph Model 9610 via an all-glass open-split interface of our own design[34]. Gas chromatographic analyses were carried out on wall-coated glass capillary columns in the Finnigan Model 9610 gas chromatograph or in a Carlo Erba gas chromatograph Model 2101 equipped with a flame ionization detector.

Materials

Zeranol was extracted and purified from the pharmaceutical Ralgro (TAD, Cuxhaven, G.F.R.)[35]. Hexestrol, dienestrol, and diethylstilbestrol were supplied by ICN Pharmaceuticals (Plainview, NY, U.S.A.). 17β-Estradiol and ethynylestradiol (Merck, Darmstadt, G.F.R.) were chromatographically pure. The silylating agents

trimethylchlorosilane (TMCS) and N,O-bis(trimethylsilyl)acetamide (BSA) were purchased from Fluka (Buchs, Switzerland). Silica gel 60 (70–230 mesh) for column chromatography (Art. 7734) and all other chemicals were products of Merck. WCOT-glass capillary columns with an inner diameter of 0.3 mm coated with SE-54 were used at 10-m length. The columns were supplied by Jaeggi (Trogen, Switzerland) or were prepared in our laboratory according to the barium carbonate procedure of Grob[36–38].

An Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, G.F.R.) was used to homogenize the meat and for the following solvent extraction.

METHODS

Clean-up procedure

10 g of minced meat were mixed with 25 ml of tetrahydrofuran in a 100-ml centrifuge tube and homogenized with an Ultra-Turrax homogenizer. After cleaning the homogenizer with another 25 ml of tetrahydrofuran the homogenate was centrifuged at *ca.* 1800 *g*. Then the supernatant was decanted through a plug of glass wool. The residue in the centrifuge tube was again homogenized with 25 ml of tetrahydrofuran, cleansed and centrifuged as in the first step. The supernatant solutions were combined and evaporated under a vacuum at 40°C nearly to dryness. The remaining water was vaporized by means of added ethanol. The dry residue was dissolved in 25 ml of acetonitrile and twice extracted with 25 ml of hexane. The combined hexane phases were re-extracted with 25 ml of acetonitrile, and the acetonitrile phase was again evaporated to dryness. The residue was dissolved in a small volume of benzene and fractionated on a silica gel column (200 × 15 mm I.D.). The column was prepared from a slurry of 7 g of silica gel 60 in benzene. The silica gel 60 was first deactivated by the addition of 2% (w/w) water. After the addition of the meat extract dissolved in benzene the column was washed with 50 ml of benzene. Then the estrogenic compounds were eluted with 50 ml of ethyl acetate.

Derivatization

The concentrated ethyl acetate fraction was transferred into a derivatization tube. It was evaporated to dryness with a nitrogen stream at 70°C. The residue was mixed with 0.1 ml of the silylating reagent consisting of BSA with 10% TMCS. The derivatization tube was closed with a teflon coated septum and held for 1 h at 70°C. After the tube was cooled to room temperature, 0.9 ml of hexane was added.

Gas chromatography

All gas chromatograms were run on 10-m wall-coated open tubular glass capillaries coated with the non-polar silicon phase SE-54. Helium was used as carrier gas at a flow-rate of 2.5 ml/min at room temperature. Injection port and detector were held at 230 and 260°C. A sample volume of 1 μl was splitless injected into the 'cold' column at 90°C, according to the method of Grob[39]. Just 40 sec after the injection, the split of the carrier gas was reopened and a flow-rate of *ca.* 20 ml/min was used as a septum purge. After 1 min a linear temperature program was started with a heating rate of 20°C/min to a final temperature of 250°C, which was held for 5 min.

GC–MS

The design and the operation of the all-glass open-split interface in residue analysis were described in detail elsewhere[34]. The temperature of the interface was 250°C, and the ion source was held at 180°C. Electron-impact ionization was performed at 70 eV with an ion current of 300 μA.

The GC–MS system was operated mainly in the following two modes: cyclic scan and multiple ion monitoring. In the cyclic scan mode the chromatographic run was continuously scanned with a scan time of 0.5 or 1.0 sec for the mass range from 50 to 600 *m/e*. After the run was finished, the crude data were processed using the various computer programs for data enhancement, library search or mass chromatogram reconstruction. Multiple ion monitoring was performed with a maximum of 20 selected masses in a cycling time of 0.9 sec. But mainly 6–10 selected ions were scanned with a cycling time of 0.5 sec, with longer sampling intervals on the ions with lower intensities.

When meat samples were analysed, the ion source was protected from most of the contaminating substances from the biological matrix by venting them using a scavenger gas-line installed in the open-split connection. Only the fractions from the gas chromatographic eluate of analytical interest were transferred to the ion source. Details of the procedure are given in a separate paper[34].

RESULTS AND DISCUSSION

Fig. 1 reveals the problems with which the analyst is confronted in GC analysis of anabolic drug residues in samples from meat. After undergoing the described clean-up procedure, an extract from meat was trimethylsilylated and separated on a 10-m SE-54 glass capillary column using a flame ionization detector. The chromatogram in Fig. 1A shows a great number of substances extracted from the biological matrix. Under the same chromatographic conditions a mixture of trimethylsilylated anabolic drugs and an internal standard was separated and recorded. The resulting chromatogram is shown in Fig. 1B. The concentration of the test substances in Fig. 1B is 10 ng in the injection volume of 1 μl. Fig. 1C shows the chromatogram of the same meat sample as in Fig. 1A, but mixed with the anabolic drugs and an internal standard at a concentration of 1 ppm. This is the same amount of substance as used for the chromatogram in Fig. 1B.

From these experiments it is evident that the combination of a sample clean-up with a non-specific gas chromatographic detector is not suitable for the determination of anabolic drug residues in meat at the ppb level. All experiments to develop a reliable multiresidue method for the estrogenic anabolics at the ppb level on the basis of gas chromatography with halogenated derivatives failed in our laboratory although various procedures for single compounds have been reported[1,2,9,32]. All these methods require laborious clean-up, which results in considerable losses of the residue compound to be detected. Therefore, we relinquished our attempts to develop a GC–ECD method as a general screening for meat samples prior to GC–MS. For this purpose we have for the past three years applied a fast biochemical assay of high sensitivity using the natural estrogen receptor from bovine uterus[23].

Highest sensitivity in GC–MS analysis can be attained with selected ion monitoring. For the successful application of this method in biological samples an im-

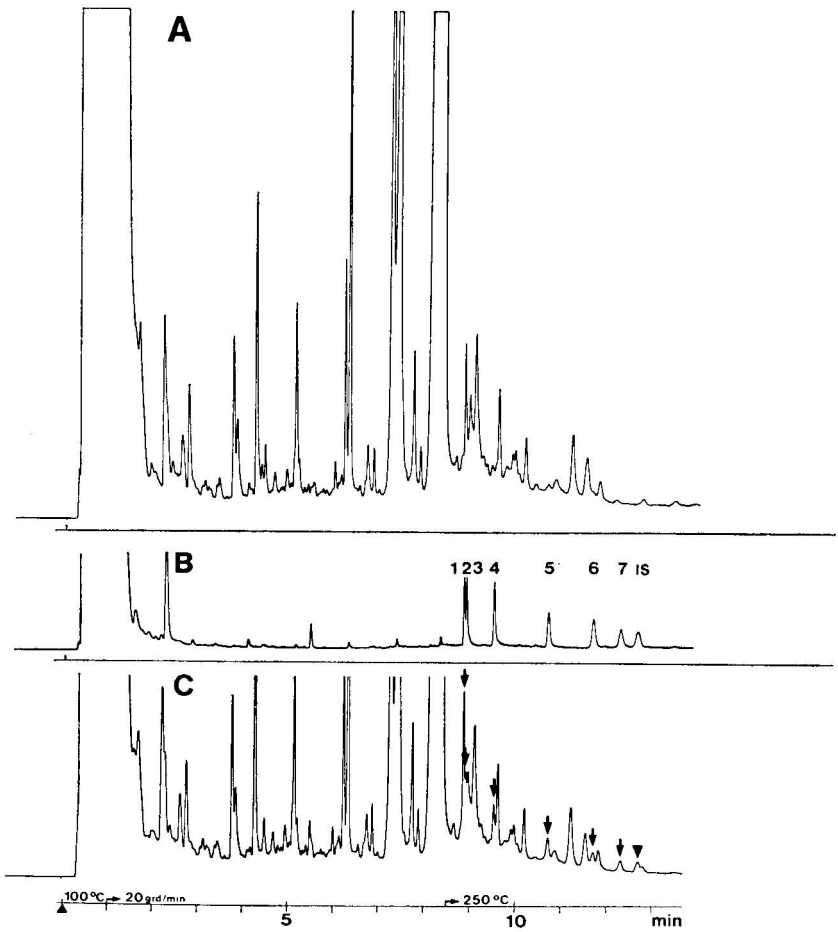

Fig. 1. Capillary gas chromatogram of a meat sample after clean-up and trimethylsilylation using a WCOT glass column of 10-m length coated with SE-54 (0.3 mm I.D.). Detector: FID. A, Meat sample free of anabolic residues. B, Standard mixture of 10 ng of hexestrol (1), diethylstilbestrol (2), dienestrol (3), stilbestrol (4), 17β-estradiol (5), ethynylestradiol (6), zeranol (7) and the internal standard dodecyl gallate (IS). C, Meat sample as in A but the standard mixture from B added. The injected amount of test substances is again 10 ng, equivalent to 1 ppm in the meat. All chromatograms at the same detector sensitivity. For experimental conditions see text.

portant prerequesite is a mass fragmentation pattern of the substances to be determined, which exhibits high ion intensities in the higher mass range. Trimethylsilyl ethers offer a good compromise of suitable mass spectral characteristics and the important gas chromatographic properties of volatility and stability. Table I lists the ions suitable for selected ion monitoring of the trimethylsilyl ethers of the analysed estrogenic anabolics and the internal standard dodecyl gallate. With the exception of the hexestrol derivative, the most intense ions from all compounds are found above *m/e* 400. The trimethylsilyl ether of hexestrol is fragmented nearly exclusively to a single stable ion, which results from cleavage of the central C—C bond and thus occurs at a very high absolute intensity. This can be seen in Fig. 2, where a plot of

TABLE I

IONS SUITABLE FOR SELECTED ION MONITORING OF THE TRIMETHYLSILYL ETHERS OF THE ANALYSED ANABOLIC DRUGS

Substance	*Symbol*	*Ions*		
		*M**	*M − 15*	*Other ions*
Ethynylestradiol	ETO	440(40)	425(100)	300(43) 285(62)
Diethylstilbestrol	DES	412(100)	397(15)	383(16)
Dienestrol	DIE	410(90)	395(51)	381(15)
Hexestrol	HEX	414(1)	399(2)	207(100)
17β-Estradiol	OST	416(100)	401(8)	285(67)
Zeranol	ZER	538(13)	523(13)	433(70) 335(27) 307(37)
Dodecyl gallate**	IS	554(100)	539(4)	281(42) 369(15)

* Molecular ion (relative abundance).
** Internal standard.

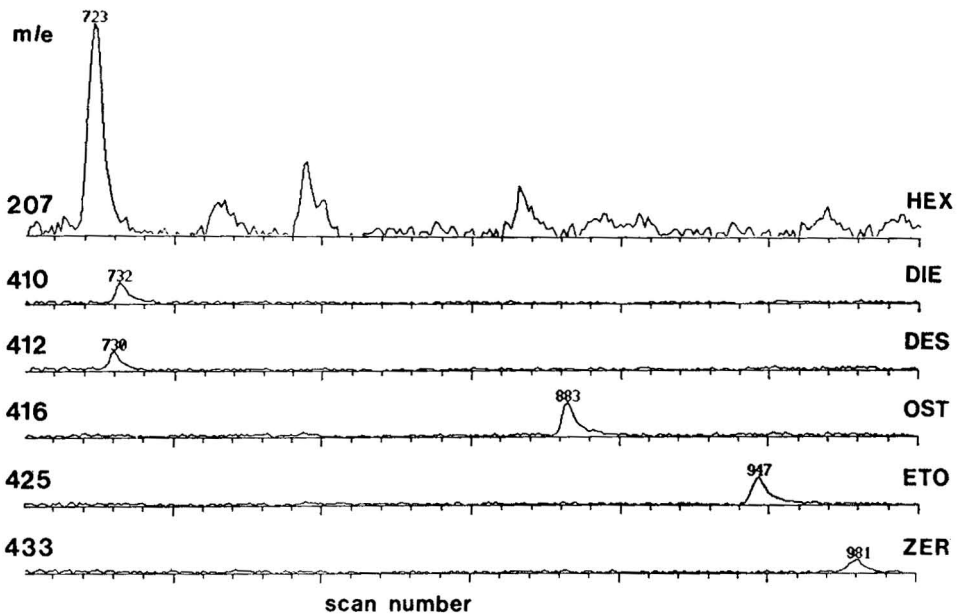

Fig. 2. Selected ion monitoring of anabolic drugs at the subnanogram level. Injected amounts: 50 pg of hexestrol (HEX), 100 pg of dienestrol (DIE), 50 pg of diethylstilbestrol (DES), 150 pg of 17β-estradiol (OST), 750 pg of ethynylestradiol (ETO) and 500 pg of zeranol (ZER), as trimethylsilyl ethers.

the selected ions from all estrogen derivatives is shown. Hexestrol and dienestrol were in this experiment at the lowest concentration levels. It is obvious that the detection limit depends not only on the absolute ion intensity but also on the background. The chosen substance concentrations in Fig. 2 correspond with ppb levels in meat.

Fig. 3 shows the detection limit in meat samples for the three compounds of the stilbene type. These compounds are of particular importance because they exhibit the greatest health risk from the estrogenic anabolics discussed here. In the upper

plot (Fig. 3A) a meat sample free of stilbene derivatives is shown. At *m/e* 207 there is a relatively high background demonstrating a great number of compounds in the biological matrix which form this ion if electron-impact ionization is used. Fragments with *m/e* 410 are rare, whereas a number of substances yield ions with *m/e* 412. The mass fragmentogram at *m/e* 412 emphasizes the importance of a high separation power of the chromatographic system, which makes it possible to differentiate the DES residue from substances of similar chromatographic properties forming fragments of *m/e* 412. The addition of 2 ppb of the stilbene derivatives to the meat sample resulted in additional peaks in the corresponding mass chromatograms at the expected retention times (Fig. 3B). This typical experiment leads to the conclusion that the analysed meat sample is free of the three stilbene derivatives tested for at the level of 1 ppb. In this case 1 ppb can be considered as the detection limit for all three stilbenes.

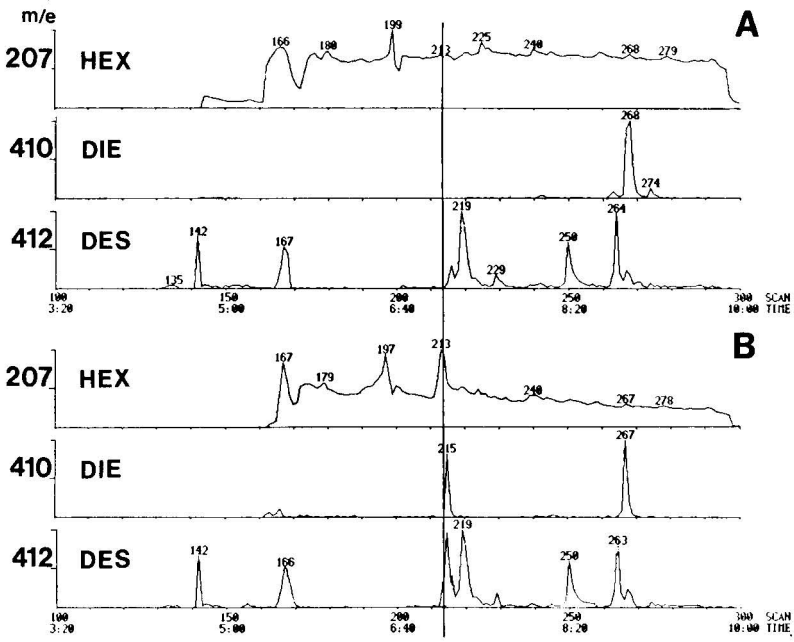

Fig. 3. Selected ion monitoring of estrogenic stilbenes in meat near the detection limit. A: Meat free of anabolic residues. B: The sample from A with an addition of 2 ppb hexestrol (HEX), dienestrol (DIE) and diethylstilbestrol (DES).

Compared with the detection of the synthetic stilbene derivatives the determination of zeranol residues is more complicated. Zeranol is a commercial drug produced by hydrogenation and reduction from the naturally occurring mycotoxin zearalenone. In the animal, zeranol is partially metabolized into zearalanone. These three substances may occur together and are isolated from meat using the described clean-up procedure. After trimethylsilylation they can be separated by gas chromatography and detected with selected ion monitoring (Fig. 4). The mass spectra of the trimethylsilyl ethers are shown in Fig. 5. The fragments in the lower and middle mass

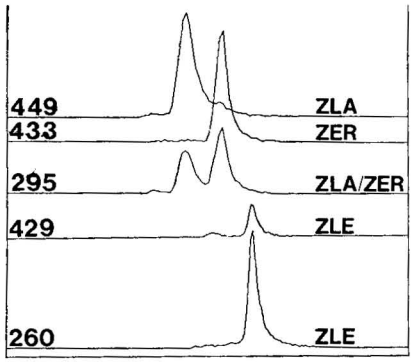

Fig. 4. Selected ion monitoring of the trimethylsilylethers of the zeranol group. Meat sample with the addition of 50 ppb of zeranol (ZER), zearalanone (ZLA) and zearalenone (ZLE).

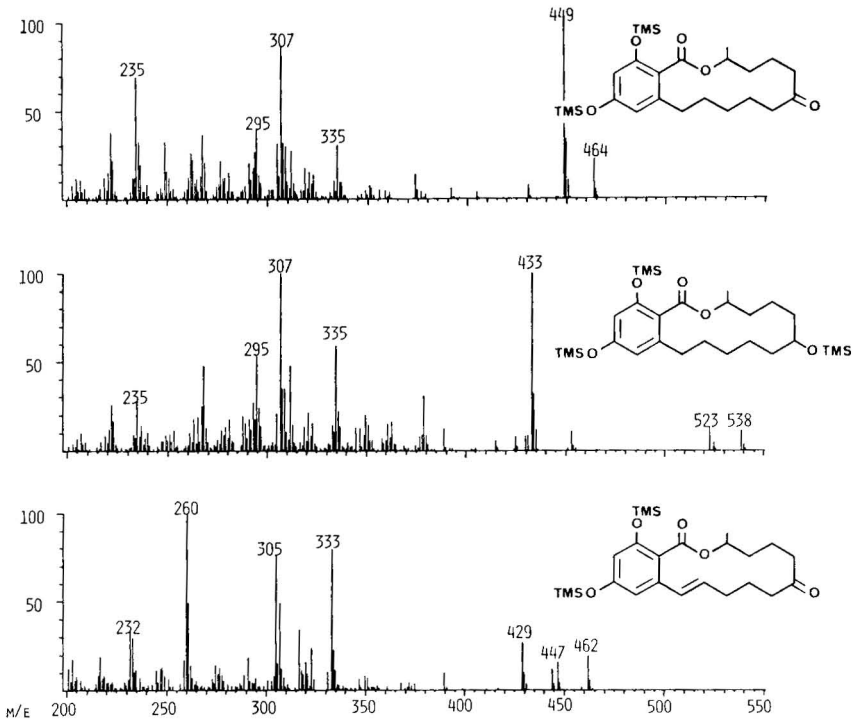

Fig. 5. Mass spectra of the trimethylsilyl ethers of the zeranol group. Electron-impact ionization under GC–MS conditions (70 eV).

regions of zeranol and zearalanone are nearly identical because of their structural similarity. Fragments from the mass spectrum of zearalenone can interfere with those from zeranol and zearalanone as isotopic peaks or by the addition of a proton. With appropriate selection of fragment ions the unequivocal detection of the three compounds can be achieved, as is demonstrated in Fig. 4.

A complex problem in residue analysis at trace level in biological samples with GC–MS is quantitation. The best method for quantitative determination of a single substance is the isotopic dilution method. At the beginning of the analysis a certain amount of the deuterated compound is added as a substance-specific internal standard. Quantitation is performed using the signal ratio of the same fragments which differ in their masses because of the incorporated deuterium[40]. This method is widely used as a reference method for steroid analysis in clinical chemistry[41,42]. The prerequisite for this method is that the deuterated drug or metabolite with high isotopic purity is at one's disposal. Deuterated anabolic drugs are not yet commercially available. On the other hand, the isotopic dilution method is obviously not of great value in multiresidue analysis. Therefore, an internal standard was chosen exhibiting good mass spectral characteristics combined with a suitable retention time in our chromatographic system. Dodecyl gallate served best for this purpose. The molecular ion at *m/e* 554 is the most intense ion in the mass spectrum (Table I) and the compound is eluted immediately after the last anabolic drug (see Fig. 1B).

Fig. 6 shows a plot of the signal ratio of the selected ions of the drugs and the internal standard as a function of drug concentration. With these two stilbene derivatives a linear calibration curve was found. Fig. 6 again indicates the high intensity of the *m/e* 207 ion from TMS-hexestrol in comparison with the intensity of typical fragments from the other drugs. Depending on the signal to noise ratio of the individual compounds in biological samples, quantitative determinations can be achieved starting at the level of 10–20 ppb.

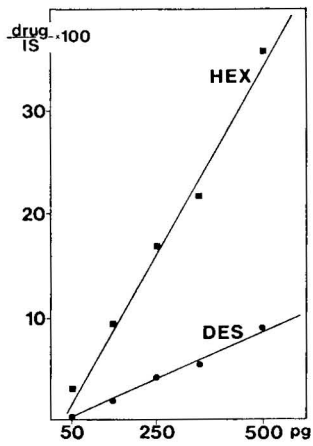

Fig. 6. Quantitative determination of estrogenic anabolics using selected ion monitoring and internal standard. Calibration plot of the signal ratio of drug to internal standard. Internal standard: 10 ng of dodecyl gallate. Amounts of hexestrol (HEX) and diethylstilbestrol (DES) are indicated on the abscissa.

The GC–MS method using the trimethylsilyl ethers of estrogenic anabolic compounds is suitable for the detection and identification of residues in meat at the lower ppb level. The proof of absence of the stilbene derivatives, which are considered as a risk to human health, can be achieved down to 1 ppb or even less, if single ion

monitoring is used. For unequivocal identification, in addition to reproducible retention times on a capillary column and a signal at a substance-specific ion, the simultaneous registration of two or three masses is necessary.

Trimethylsilyl ethers are the derivatives mostly used in GC–MS analysis of steroids in biological samples with electron-impact ionization. These derivatives were found to be suitable for some estrogenic anabolics by Höllerer and Jahr[33]. Our attempts to develop a screening procedure based on ECD–GC with halogenated acyl derivatives included a systematic study of the gas chromatographic properties of a series of derivatives. We found that heptafluorobutyrates were the most suitable. However, zeranol formed a critical derivative, as was the case with other acylating agents. It is very sensitive during chromatography and is easily destroyed if any active spots arise in the system. Heptafluorobutyrates do not exhibit better mass spectral characteristics than the trimethylsilyl ethers, so that the better chromatographic stability of the latter makes them the derivatives of choice.

In recent years other alkylsilyl ethers have been proposed for the GC–MS analysis of steroids and phenolic compounds. The most promising derivatives are the *tert.*-butyldimethylsilyl ethers, which demonstrate superior mass spectral fragmentation characteristics with electron-impact ionization[43–46]. A disadvantage of these derivatives is their low volatility, which means that higher temperatures are required. This is of particular significance with compounds containing three hydroxyl groups, such as zeranol. Although the *tert.*-butyldimethylsilyl ethers can be of importance for the mass spectral identification of some individual estrogens, the trimethylsilyl ethers are more suitable for routine trace analysis in meat.

Quantitation with GC–MS is frequently better achieved using chemical ionization (CI). Our investigations of various derivatives, including the trimethylsilyl ethers and heptafluorobutyrates, were performed with methane and isobutane as reactant gases. Under various experimental conditions we always found that with CI the total ionization of the compounds was less compared with electron-impact ionization. This means a higher detection limit for the anabolics and therefore CI is less suitable for trace analysis.

In our laboratory we start the routine analysis of anabolics in meat with a competitive protein binding method in which the estrogen receptor isolated from the cytosol of bovine uterus cells is used as a highly specific and sensitive reagent for estrogenic compounds[23]. The combination of this time-saving biochemical screening procedure with the GC–MS method enables us to focus our attention on estrogen positive samples. Moreover, the agreement of biochemical and MS data makes the trace analysis of estrogenic anabolic compounds in meat highly reliable at the ppb level.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DENATONIUM BENZOATE IN RAPESEED OIL

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SUMMARY

A method for the determination of denatonium benzoate [benzyldiethyl(2,6-xylylcarbamoylmethyl)ammonium benzoate] in rapeseed oil has been developed which utilizes a simple extraction and concentration technique followed by reversed-phase high-performance liquid chromatography. The column eluent was monitored at 210 nm and peak area data was generated by a computing integrator. Detection was possible below 5 ng and calibration curves were linear to 100 ng or more.

INTRODUCTION

The rate of duty assessed on rapeseed oil imported into the U.S.A. varies considerably depending upon its intended use and whether or not it has been rendered unfit for use as a food. In rendering vegetable oils, such as rapeseed oil, unfit for use as food, a denaturant is commonly added. One such substance, specified in government regulations as acceptable for use in the denaturing of vegetable oils, is denatonium benzoate at a level of not less than 2 ppm.

A number of methods have been proposed for the quantitation of denatonium benzoate. The older of these generally involve colorimetric reactions[1,2] or thin-layer chromatography[3], methods which suffer from a lack of specificity or quantitative accuracy and may be extremely time-consuming. A more recent method has appeared which utilizes high-performance liquid chromatography (HPLC) to determine denatonium benzoate in alcoholic toilet preparations[4]. However, in this method, which utilizes a silica gel column, denatonium benzoate is not completely resolved from the solvent "front" and appears simply as a shoulder on a tailing edge. To improve the chromatography, it was decided to investigate the use of chemically bonded phases. Good results have been achieved with these phases in the separation of other quaternary ammonium salts[5,6]. The HPLC procedure selected is reversed-phase consisting of separation on a chemically bonded cyano-type polar phase on microparticulate silica with a mobile phase of acetonitrile–water (60:40) containing 0.01 *M* sodium chloride. The presence of an ionic specie in the mobile phase proved essential and the system afforded baseline separation of denatonium benzoate from the solvent "front". The separation of denatonium benzoate from a viscous, oily matrix presented

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more formidable problems than those encountered by others[4] in its separation from alcoholic preparations. Advantage was taken of its unusual solubility characteristics in developing a one-step extraction procedure in which an aliquot of rapeseed oil is diluted with petroleum ether and extracted with methanol–water (1:1). Recovery problems encountered in developing a suitable extraction procedure were overcome by running rapeseed oil standards spiked with known concentrations of denatonium benzoate in parallel with the samples. Results are calculated from peak areas generated by a computing integrator.

EXPERIMENTAL

Apparatus

The liquid chromatograph was a Waters Assoc. (Milford, MA, U.S.A.) Model 201 equipped with a 6000A pump, U6K universal injector, and a Perkin-Elmer (Coleman) Model LC-55 variable-wavelength UV detector. The chromatograph was attached to a Fisher Recordall Series 5000 (10 mV full scale) recorder and to a Perkin-Elmer M-1 computing integrator. The chromatographic column, 25 cm × 5 mm I.D., was packed in the laboratory with the aid of an HPLC slurry packing unit with Chromosorb LC-8 (obtained from Supelco, Bellefonte, PA, U.S.A.), 10 μm particle size, a chemically bonded cyano-type polar phase on microparticulate silica. A guard column, 7 cm × 2 mm I.D., containing C_{18}/Corasil 37–50 μm (Waters Assoc.), was placed immediately ahead of the chromatographic column.

Chemicals

The methanol, acetonitrile and water were each HPLC grade. The denatonium benzoate (Macfarlan Smith, Edinburgh, Great Britain) was obtained from J. H. Walker (Larchmont, NY, U.S.A.) and the rapeseed oil was of a quality typical of that imported into the U.S.A. All other chemicals were reagent grade.

Procedure for extraction of denatonium benzoate from rapeseed oil

Pipet 5.0 ml of rapeseed oil into a small separatory funnel, add 5 ml of petroleum ether and mix. Add 5.0 ml of methanol–water (1:1) and shake gently for 30 sec. Allow the layers to separate, then draw off the lower aqueous layer into a 10-ml conical centrifuge tube. Place the tube in a beaker of warm water on a hot plate (water temperature about 50°C) and apply a gentle stream of air to the top of the tube. Evaporate to dryness, washing the walls of the tube twice with a small portion of methanol as dryness is approached to concentrate the sample in the tip of the tube. Dissolve the residue in 100 μl of mobile phase (acetonitrile–water, 60:40, containing 0.01 *M* sodium chloride).

Procedure for preparation of standards

Methanol. A stock solution containing 100 ppm of denatonium benzoate in methanol was prepared. From this stock solution, standard solutions containing 0.5, 1.0, 2.0, 5.0 and 10.0 ppm denatonium benzoate in methanol were prepared. A 5.0-ml aliquot of each was evaporated to dryness in a 10-ml conical centrifuge tube as described above. Each residue was dissolved in 100 μl of mobile phase. This series of standard solutions was used to prepare a standard curve.

Spiked rapeseed oil. A stock solution containing 100 ppm of denatonium benzoate in rapeseed oil (predetermined to be blank at 210 nm by the extraction procedure described above) was prepared. Dissolution of the solid in the oil was accomplished by shaking then sonication. Working standard solutions containing 0.5, 1.0 and 2.0 ppm denatonium benzoate were prepared from the stock solution by appropriate dilution with additional rapeseed oil (available from a number of domestic suppliers). Aliquots of these standards were extracted and run in parallel with denatured rapeseed oil samples.

Procedure for liquid chromatography

The mobile phase consisted of an acetonitrile–water (60:40) mixture containing 0.01 *M* sodium chloride (sonicated under vacuum prior to use). At the start of a run, the column was equilibrated with mobile phase. The flow-rate was 1.2 ml/min. The sample injection volume was 10 μl and the column eluent was monitored in the ultraviolet at 210 nm. Denatonium benzoate eluted in about 6.7 min. At the end of each run, the column was washed with water for approximately 30 min to remove all salt.

RESULTS AND DISCUSSION

Fig. 1 shows a standard curve obtained from a series of denatonium benzoate standards prepared in methanol. This curve clearly demonstrates the linearity of the chromatographic procedure over the range of 0.5 to 10.0 ppm. Each concentration level was run in triplicate.

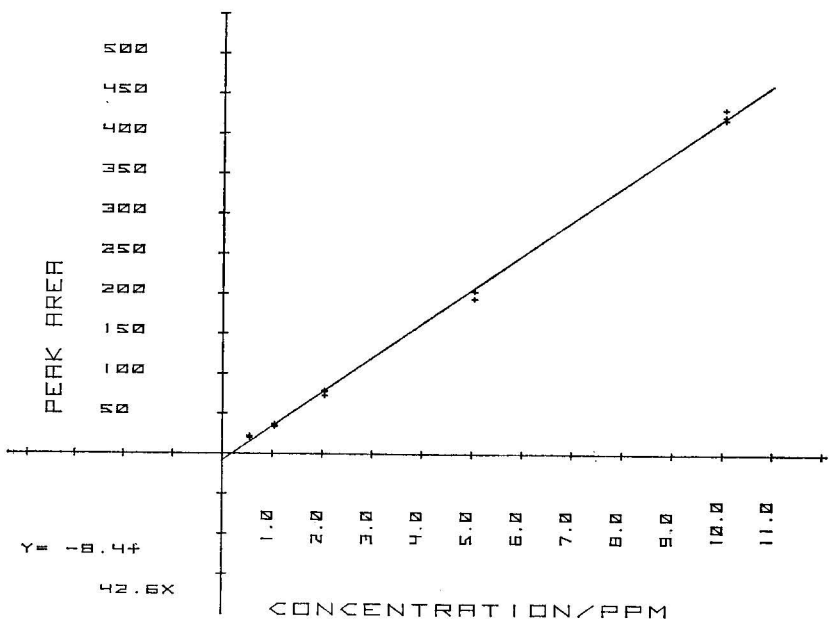

Fig. 1. Typical calibration curve obtained from denatonium benzoate standards prepared in methanol.

The results shown in Table I were obtained by comparing peak areas of rapeseed oil samples spiked with 0.5, 1.0 and 2.0 ppm denatonium benzoate with a standard curve. Though the percent recovery showed consistency, it was low.

TABLE I

RECOVERY OF DENATONIUM BENZOATE FROM SPIKED RAPESEED OIL

ppm added	*Recovery (%)*	*ppm added*	*Recovery (%)*
0 (blank)	0	2.0	68
0.5	68	2.0	67
0.5	71	2.0	80
1.0	72	2.0	72
1.0	75	2.0	72
2.0	75	2.0	80
2.0	67		

Separation

Considerable effort was expended in attempting to improve the extraction procedure. Denatonium benzoate is a quaternary amine whose structure, seen in Fig. 2, is more complex than most. Its solubility in water is 4.5 % (w/v) yet it is 15 times more soluble in methanol, 7 times more soluble in chloroform and practically insoluble in ether. While attempts were made to utilize this solubility data to advantage, no extracting solvent combination tried performed better than the one ultimately selected. Slight improvement in the percent recovery was observed when the volume of the extractant was doubled or the volume or proportion of petroleum ether was increased but none of these improvements was large enough to warrent adopting a more lengthy procedure.

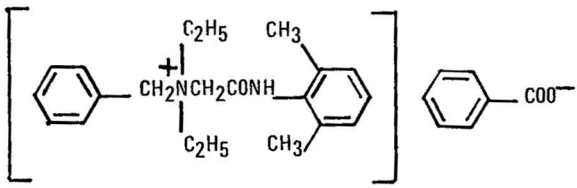

Fig. 2. Structural formula of denatonium benzoate.

In the course of this investigation, similar extractions were performed on corn oil and mineral oil spiked with comparable levels of denatonium benzoate. Following chromatography of the extracts, comparison of peak areas of these samples with the standard curve yielded even lower recoveries than for rapeseed oil suggesting that the ease of separation is matrix dependent. It is evident that an equilibrium exists between the dissociated and undissociated forms of denatonium benzoate and that these forms are not extracted with the same efficiency. To compensate for the low recovery, it was decided that comparison of peak areas of spiked rapeseed oil standards with those of denatured samples, extracted and run in parallel, offered the best solution. Commercial samples of denatured rapeseed oil gave results in the expected range. The use of a fixed loop injector in place of the U6K universal injector, where sample

volume is dependent upon the amount measured in a syringe, is recommended since it would eliminate the necessity of repetitive injections.

Because sample extracts required concentration for detection, the possibility that the residues resulting from evaporation to dryness were redissolving slowly or with difficulty was examined. Sample tubes were stoppered and allowed to stand 2–3 h following the addition of the 100 μl of mobile phase before injection into the liquid chromatograph or were mixed on a vortex mixer or in a sonicator instead of the usual practice of gentle manual mixing followed by a wait of approximately 15 min. While no increase in peak area or peak height was found as the result of these additional manipulations, it was observed that the chromatographic solutions remained stable for at least 5 days when properly stoppered.

Chromatography

A C_{18} column having octadecyl groups chemically bonded to microparticulate silica was initially tested using methanol–water containing pentanesulfonic acid as the mobile phase. This chromatography resulted in assymetrical peaks and minimal separation of denatonium benzoate from the solvent "front". In addition, the apparent efficiency of this column was greatly reduced from what previous experience indicated it should be. Modifying the mobile phase by changing its composition or pH or employing a different ion-pairing agent yielded no improvement. An amine column and a diol column (amine or diol groups chemically bonded to microparticulate silica) were each tested with a wide range of mobile phase compositions with similar results. The column ultimately selected was a cyano column whose packing consisted of cyano groups chemically bonded to microparticulate silica. We are aware of few publications describing applications of this bonded phase.

One advantage in using bonded phases is the range of polarity that they offer. With the cyano column it was observed that, in the absence of an ionic specie in the mobile phase, denatonium benzoate was retained by the stationary phase whereas the opposite was true of the C_{18} column. It was found that ionic strength was, in fact, the critical factor affecting separation. Several common salts were tried (*e.g.* potassium chloride, potassium perchlorate, sodium phosphate and sodium chloride), and sodium chloride (0.01 *M*) was selected because of its low cost and ready availability. As expected in reversed phase, increasing the proportion of water in the mobile phase increased the retention time. The 60:40 ratio of acetonitrile–water afforded complete baseline separation of denatonium benzoate from the solvent "front" while keeping run time to a minimum. Examples of typical HPLC curves are shown in Fig. 3.

In this system cetyltrimethyl ammonium bromide (cetrimide) eluted with the solvent "front" while cetylpyridinium bromide was strongly retained. The fact that, of the compounds tested, this system appeared uniquely suited only for denatonium benzoate led to abandoning a search for an internal standard. Denatonium benzoate gave maximum absorbance at 210 nm in our spectrophotometer.

Englehardt[7], in discussing chemically modified stationary phases, notes that since it is impossible to react all the surface hydroxyl (silanol) groups on the silica, the selectivity of the stationary phase is also affected by the remaining ones. Pryde and Gilbert[8] note that the mechanism of reversed-phase chromatography has not been satisfactorily worked out as yet. These authors state that retention on chemically modified supports must be by a mixture of adsorption and partition. Kirkland and

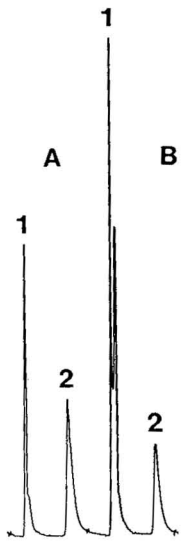

Fig. 3. Liquid chromatograms of (A) standard and (B) sample. 1 = Solvent "front"; 2 = denatonium benzoate.

De Stefano[9] state, too, that the chemically-bonded cyano phase selectively retains compounds which can readily hydrogen bond. It is possible that the chloride ion in this system serves to block those silanol sites not chemically bonded which might otherwise interact with the solute impeding its progress through the column. Salting out effects are also a possibility since it was our observation that the anion used in the mobile phase mattered little providing it was available in sufficient quantity.

We were unable to suppress completely the dissociation of the denatonium benzoate. The first peak to elute (at the solvent "front") generally contained a shoulder and sometimes appeared as two overlapping peaks. These are believed to be denatonium chloride and benzoic acid. The latter eluting peak would appear to be denatonium benzoate in undissociated form. Lowering the pH of the mobile phase to 4.0 with hydrochloric acid resulted in a 30 % increase in the area of the first peak. Buffering at pH 7.0 offered no advantage over a system containing the salt alone. Experiments conducted with buffered mobile phases in the pH 4.0 to 8.0 range, the normal working range for liquid chromatographic systems, showed that it was impossible, within those confines, to convert denatonium benzoate entirely to one form or the other. The linearity observed in the standard curve when the area of the second peak (denatonium benzoate) was plotted against concentration indicated that the procedure outlined in this paper was quantitative.

CONCLUSIONS

The method presented in this paper offers a means for the rapid separation and quantitative determination of denatonium benzoate in rapeseed oil. The chromatographic system described is specific, sensitive, and affords complete separation of the compound from the solvent "front". The utility of the cyano bonded phase for separating this type of compound is clearly demonstrated.

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AMINOGLYCOSIDE ANTIBIOTICS: THIN-LAYER CHROMATOGRAPHY, BIOAUTOGRAPHIC DETECTION AND QUANTITATIVE ASSAY

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SUMMARY

A sensitive method is described for the identification of aminoglycoside antibiotics, for the detection of agents exhibiting biological activity in the course of fermentation, isolation and purification, and for following their chemical conversion. In the multi-component nebramycin complex the ratio of the factors can be established by the quantitative assay described.

INTRODUCTION

The separation and identification of biologically active compounds, e.g., antibiotics, are of increasing importance, and high-resolution thin-layer chromatography (TLC) is a simple, quick and selective method. In antibiotic research, microbiological detection is also of great importance, and different techniques are known for ensuring the diffusion of the test material into the culture medium layer containing agar and inoculated with the test organism, *e.g.*, (a) by spraying the warm, liquid, inoculated medium onto the chromatogram[1–4] (b) by tightly pressing the agar layer (previously prepared) on alumina[5] or glass plates[6,7] used directly for the chromatogram, (c) by inserting wet filter-paper between the chromatogram and the agar layer[8–10] and (d) by forming a collodion film from the chromatogram and placing it on the agar plate inoculated with the test organism[11]. Layers containing glass-fibre[12] and other layers[13,14] that exhibit significantly low adsorption towards the investigated substances are also employed.

To improve the detection of growing or fully developed microorganisms, specific dyes are used[1,2,15].

The separation of aminoglycoside antibiotics[16,17], especially of the individual factors of an antibiotic complex, is very difficult because they have only slight structural differences. The method described in this paper for the chromatography and bioautographic detection of different aminoglycoside antibiotics and components of the nebramycin complex[18] is suitable for the qualitative control of the enriched material and also for following their chromatographic resolution and purification. The advantage of this method is that it can be used for both qualitative and quantitative control of the ratio of the different components during the course of fermentation, directly from the fermentation broth.

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EXPERIMENTAL

Materials and methods

All reagents and solvent were of analytical-reagent grade.

Thin-layer plates (20 × 20 cm) were prepared from silica gel G (according to Stahl; Reanal, Budapest, Hungary). Also, 20 × 2 × 0.3 and 16 × 2 × 0.3 cm glass plates were used for framing the chromatograms after development in Desaga glass chambers. Standard solutions for spotting were prepared by dissolving each substance in water (100 μg/ml) (with the nebramycin complex the concentration of the individual components 2, 4 and 5′ should not be less than 20%).

The two developing solvents employed were (A) methyl ethyl ketone (MEK) –96% ethanol–25% ammonia solution (1:1:1) and (B) chloroform–methanol–25% ammonia solution (1:7:4). Both solvent systems were mixed freshly before use.

For staining the bioautograms, 0.5-1% tetrazolium blue (TB) (2,2′,5,5′-tetraphenyl-3,3′-dimethoxy-4,4′-biphenyleneditetrazolium chloride) (Reanal) solution and 0.02% tetrazolium violet (INT) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride] solution (Fluka, Buchs, Switzerland) were employed.

The microbiological assay was prepared with *Bacillus subtilis* ATCC 6633 as test organism inoculated into the culture medium, containing 1 l of bouillon (1 kg of minced beafheart, boiled for 2 h with 2 l of tap water and subsequently filtered), 5 g of peptone and 14 g of agar-agar (fibrous). The pH was adjusted to 8.4 with sodium hydroxide solution before sterilization.

Procedure

Silica gel plates with a 0.25-mm thick layer were prepared with a Camag or Desaga TLC plate coater, dried at room temperature for 1–2 days and used without further pre-treatment. Leaving a 2.5-cm frame, lines were drawn before use to ensure separate 1.5-cm wide tracks.

The amount of sample to be applied was chosen according to the properties of the substance being investigated. For nebramycin a solution containing 0.1–0.5 μg of test substance was spotted with a micropipette. Occasionally a larger amount, 1–5 μg, of the substance must be spotted and chromatographed when the aim of the analysis is the detection of impurities or the proving of purity. On each plate a standard solution was also spotted on the track in addition to the sample. For the separation of the nebramycin complex and of the different aminoglycoside antibiotics solvent systems A and B, respectively, were applied. The plates were developed for a distance of 15 cm at room temperature and air-dried.

For the microbiological detection the agar medium was melted, then cooled to 50°C and 1/10th volume of an inoculum of the test organism was added (viable count $0.9 \cdot 10^7$). A 25-ml volume of this medium, inoculated with *Bacillus subtilis*, was cautiously poured on to glass-framed plates (fixed by adhesive tape) in order to ensure a cover of uniform thickness on the silica gel surface. Then it was covered with a 20 × 20 cm glass chromatographic plate and incubated for 10–16 h at 37°C.

Quantitative assay was performed by using a calibration graph (for a good evaluation, identical volumes of solutions with different concentrations from the standard and test solution must be spotted).

RESULTS AND DISCUSSION

Figs. 1 and 2 demonstrate that the major nebramycin components show readily discernible, completely resolved inhibition spots, even if less than 0.5 μg was applied. This assay can also be used directly for fermentation broths. By measuring samples obtained at the required intervals, the formation of individual components and their approximate ratios can be followed. For more exact assays, identical amounts from different concentrations of the same sample are spotted, which permits also the detection of minor factors.

Fig. 1. Bioautogram of a thin-layer chromatogram of nebramycin components. Left to right: nebramycin 2, 4, 5′, 5, 6, complex. Developing solvent: A. Test organism: *Bacillus subtilis*. Detection: INT.

In Fig. 3, a bioautogram prepared for the determination of apramycin and carbamoyl-tobramycin formed in the fermentation broth, and of tobramycin transformed by basic hydrolysis, is illustrated.

For quantitative assays a calibration graph is constructed from the inhibition spots of standard materials. The areas of sample spots applied to the same plate are measured and compared with the corresponding values on the calibration graph. A graph of area of the inhibition spots against the logarithm of the concentration of the substances applied gives a straight line; 0.1–0.3 μg amounts are suitable for constructing the calibration graph. Individual calibration graphs have to be prepared for each component because of their different specific activities. Uniform thickness of the silica gel and agar layers is essential.

The method described is suitable for the determination of the components of the nebramycin complex, as their desorption from the thin-layer plate (silica gel G) is sufficient for quantitative assay.

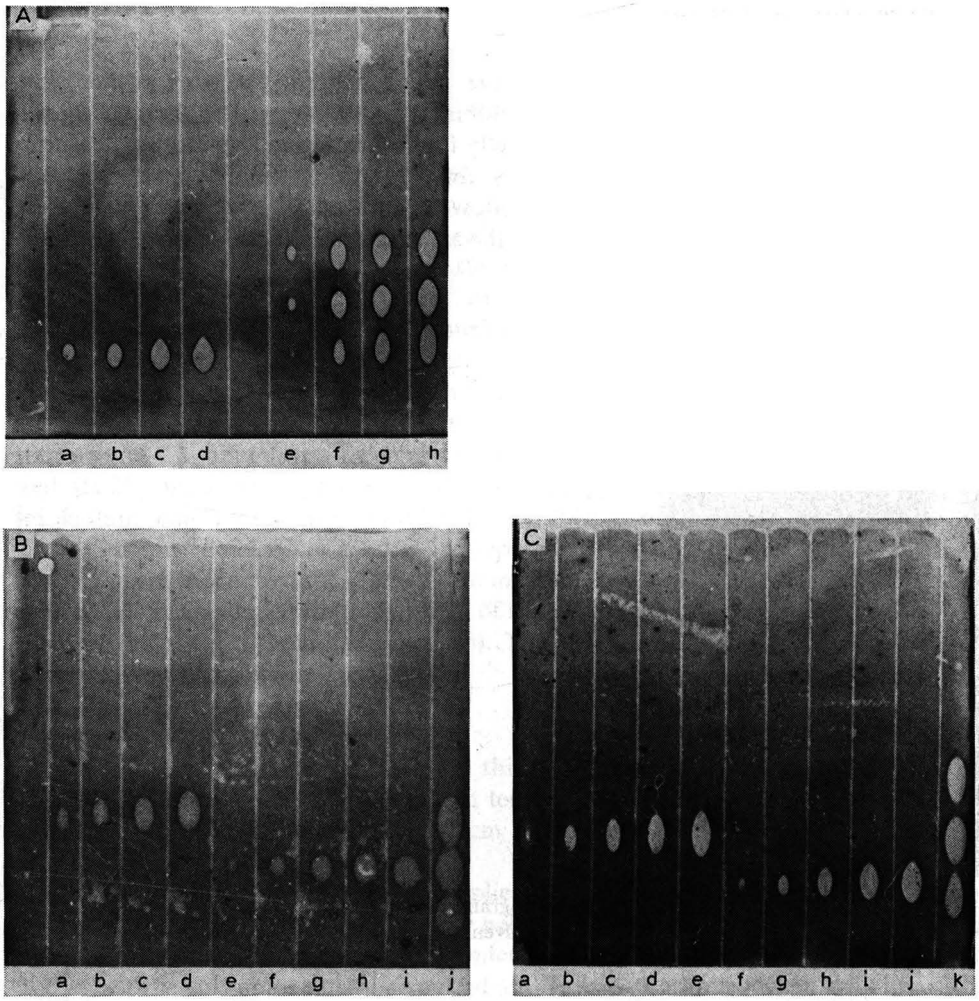

Fig. 2. (A) Bioautogram of various amounts of nebramycin components. Experimental conditions as in Fig. 1. (a)–(d) Nebramycin 2 (apramycin): 0.2, 0.3, 0.4, 0.5 μg; (e)–(h) nebramycin complex with the following concentrations of 2, 4 and 5′: (e) 0.1, 0.05, 0.05 μg; (f) 0.2, 0.1, 0.1 μg; (g) 0.3, 0.2, 0.2 μg; (h): 0.4, 0.3, 0.3 μg. (B) (a)–(d) nebramycin 5′ (carbamoyl-tobramycin): 0.05, 0.1, 0.2, 0.3 μg; (e)–(i) nebramycin 4 (carbamoyl-kanamycin B): 0.05, 0.1, 0.2, 0.3, 0.4 μg; (j) nebramycin complex. (C) (a)–(e) nebramycin 6 (tobramycin): 0.05, 0.1, 0.2, 0.25, 0.3 μg; (f)–(j) nebramycin 5 (kanamycin B): 0.05, 0.1, 0.2, 0.25, 0.3 μg; (k) nebramycin complex.

Fig. 4 shows a chromatogram developed with solvent B. The method can be applied to the separation, detection and identification of various aminoglycoside antibiotics. The R_F values of substances in solvent B are given in Table I.

For the measurement of the specific activities of individual substances the corresponding microorganism is inoculated into the medium and poured on to the plate. In addition to *Bacillus subtilis*, *Sarcina lutea* (Hussey) and *Mycobacterium phlei* were also applied.

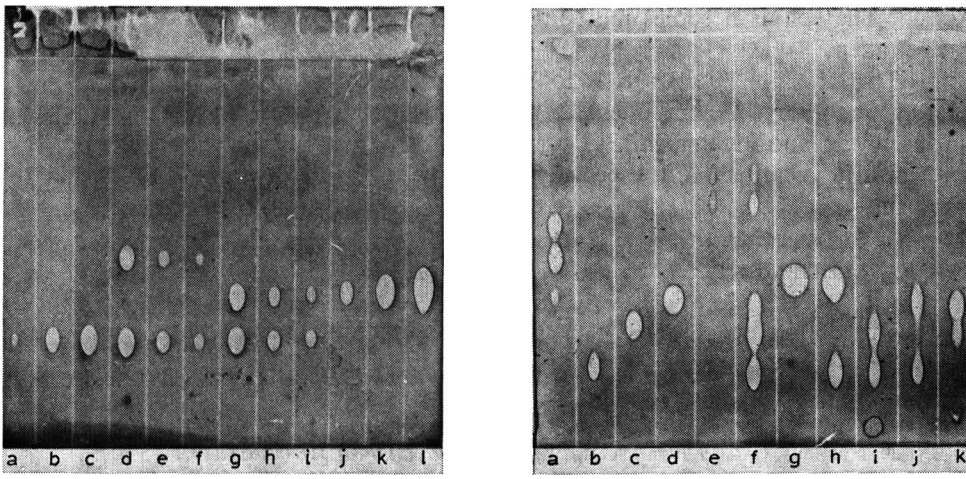

Fig. 3. Bioautogram of a fermentation broth in the original state and after basic hydrolysis (semi-quantitative determination). Experimental conditions as in Fig. 1. (a)–(c) Apramycin in different concentrations; (d)–(f) fermentation broth, serial dilution; (g)–(i) fermentation broth, after hydrolysis, serial dilution; (j)–(l) tobramycin, in different concentrations.

Fig. 4. Thin-layer chromatogram of some aminoglycoside antibiotics. Developing solvent: B. Detection: bioautography. Test organism: *Bacillus subtilis*. (a) Nebramycin complex, 0.4 μg; (b) neomycin (N), 0.1 μg; (c) paromomycin (P), 0.2 μg; (d) kanamycin (K), 0.3 μg; (e) gentamycin (G), 0.3 μg; (f) N + P + K + G, 0.15 + 0.1 + 0.1 + 0.3 μg; (g) neamine, 0.5 μg; (h) N + neamine, 0.15 + 0.5 μg; (i) N + P, 0.1 + 0.1 μg; (j) N + K, 0.1 + 0.1 μg; (k) P + K, 0.1 + 0.1 μg.

TABLE I

R_F VALUES OF AMINOGLYCOSIDE ANTIBIOTICS IN SOLVENT SYSTEM B

Compound	$R_F \times 100$
Neomycin	12
Paromomycin	23
Kanamycin A	30
Nebramycin (2, 5; 4, 6; 5′)	32; 42; 51
Gentamicin (C_{1a}; C_1, C_2)	56; 63
Sisomicin	60
Neamine	37
Amikacin	6

Investigations can be carried out with clone colonies of unidentified micro-organisms producing active compounds, grown on a solid medium. A small disc of the single colony is cut out and placed on the silica gel chromatographic plate for 5–15 min. The biologically active substances will diffuse into the silica gel and then the investigation can be performed following the method described.

The visibility of the inhibition zones on the bioautogram can be improved by spreading a 0.5–1.0% TB solution on the plate after incubation for 12–16 h. Approximately 60 min later the rim of the spots becomes pronounced. Staining the plates with a solution of INT gives colourless spots on a deep red or wine-coloured back-

ground. The colour becomes most intense within 5-10 min, and good photographs can be taken.

The detection limits are as follows: nebramycin components 4, 5, 5′ and 6, 0.05 μg; nebramycin component 2, 0.1 μg; gentamicin, 0.2 μg; and neomycin, paromomycin and kanamycin, 0.1 μg. The sensitivity can be increased by applying thinner silica gel layers and, especially, thinner agar layers or more sensitive micro-organisms.

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BRAIN GANGLIOSIDES: AN IMPROVED SIMPLE METHOD FOR THEIR EXTRACTION AND IDENTIFICATION

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SUMMARY

Total ganglioside extracts prepared from brain tissue were concentrated either by dialysis against Carbowax or by employing Millipore filter cones. Thin-layer chromatography was then carried out using silica gel plates. After location of the various fractions quantitation was effected by direct densitometry.

The methods that have been adopted are rapid and suitable for the study of brain gangliosides in post mortem and biopsy material in a clinical chemistry laboratory.

INTRODUCTION

Gangliosides serve as surface membrane receptors and play a significant role in maintaining the internal environment of the body. They are a class of negatively charged glycosphingolipids the molecules of which contain both hydrophilic and hydrophobic regions. The hydrophilic moiety consists of a carbohydrate portion to which one or more sialic acid residues are attached and the hydrophobic moiety is ceramide. Details of results of structural analysis studies are given in a review by Svennerholm[1] and are summarised in Table I.

The functional role of the gangliosides is still speculative but they have been implicated in several neurological mechanisms[2–5]. Genetically determined attenuation of one or more of the enzymes involved in ganglioside metabolism leads to severe neurological dysfunction in affected patients and is usually accompanied by ganglioside accumulation[6] such that both concentration and pattern of human brain gangliosides differs markedly from the normal.

One of the obstacles in the clinico-chemical study of the gangliosides has been the lack of a simple and reliable method for the isolation of gangliosides from tissue extracts. Difficulties have arisen primarily because of the formation of easily contaminated micelles in aqueous and organic solvents and inefficient extraction and concentration procedures. Most methods are based upon that of Folch *et al.*[7] whereby total

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TABLE I

STRUCTURE AND NOMENCLATURE OF BRAIN GANGLIOSIDES

The structures and nomenclature shown are based on the work of Svennerholm[1]. This nomenclature is used throughout the text with the addition of GQ 1, (a ganglioside similar in structure to GT 3 but having four sialic acid residues) and G7 of unknown structure; the nomenclature adopted by Zanetta *et al.*[12].

Compound	*Structure*	*Symbol*
Monosialoganglioside	Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2	GM3
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2	GM2
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 3 ↑ ↑ Gal 1 NANA 2	GM1
Disialoganglioside	Gal 1 → 4 Gluc → Cer 3 ↑ NANA 2 → 8 NANA 2	GD3
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 ↑ NANA 2 → 8 NANA 2	GD2
	GalNac 1 → 4 Gal 1 → 4 Gluc 1 → Cer 3 3 ↑ ↑ Gal 1 NANA 2 → 8 NANA 2	GD1b
	GalNac 1 → 4 Gal 1 → 4 Gluc → Cer 3 3 ↑ ↑ Gal 1 NANA 2 3 ↑ NANA 2	GD1a
Trisialoganglioside	GalNac 1 → 4 Gal 1 → 4 Gluc → Cer 3 3 ↑ ↑ Gal 1 NANA 2 → 8 NANA 2 3 ↑ NANA 2	GT1

lipids are extracted in chloroform–methanol mixture and the gangliosides partitioned into an aqueous phase which is then dialysed. Numerous modifications have been applied to increase total yield or to reduce contamination by non-gangliosidic material one of the most successful of which appears to be that of Suzuki[8]. The final dialysis can be avoided using the technique of Carter and Kanfer[9] whereby water insoluble calcium complexes of the gangliosides are formed.

The simplest and most effective procedures for the separation of lipophilic mixtures are systems relying upon adsorption chromatography. As a consequence silica gel has proved to be the chromatographic support of choice and both thin-layer (TLC) and column chromatography have been utilised. Several solvent systems have been formulated including chloroform–methanol–water mixtures[10] and more recently tetrahydrofuran–water[11] and methyl acetate–isopropanol–water[12] which achieve good resolution with rapid separation time. Harth *et al.*[13] have applied total lipid extracts directly to silica gel plates and have obtained ganglioside separation with some purification by using three solvent systems successively, fractionation is however time consuming.

After chromatograms have been developed the gangliosides may be visualised using orcinol[14], resorcinol[15] or Ehrlich's reagent[16] which detect the sialic acid residues. Such procedures afford a means of ready qualitative assessment and quantitative evaluation by direct-scanning densitometry[17,18].

We describe here our own improved method based on selective combination of some of these techniques, for the isolation and concentration of brain gangliosides and their identification.

EXPERIMENTAL

Extraction procedures

All chemicals and reagents (British Drug Houses, Poole, Great Britain) were of analytical grade purity. Solvents were used without further purification. Homogenisation of samples was achieved with a Polytron homogeniser (Northern Media Supply, Brough, Great Britain), and extracted samples were applied to chromatograms with Hamilton syringes. Visking cellophane dialysis tubing (8/32) was obtained from Medicell International (London, Great Britain) and filter cones were obtained from Amicon (High Wycombe, Great Britain).

A representative sample of brain tissue was weighed and chopped into small pieces. Portions of approximately 1 g in weight were homogenised in nineteen volumes of chloroform–methanol (2:1, v/v) for 1 min. The homogenates were combined and rehomogenised for a further 2 min. After centrifugation, the supernatant was removed, four volumes of 0.1 *M* potassium chloride was added to it and the resultant homogenised for 1 min. The upper phase was then removed and the lower phase washed twice with ten volumes of theoretical upper phase containing potassium chloride (*i.e.* chloroform–methanol–0.1 *M* potassium chloride; 3:48:47). All the upper phases were pooled and the residual chloroform driven off under a stream of nitrogen. The final extract was then concentrated by sealing the resultant solution in Visking dialysis tubing and subjecting it to overnight dialysis against 20% Carbowax at 4°C. The concentrate was freeze dried and after weighing, was redissolved in 1 ml of distilled water prior to application to the chromatography plate. Alternatively, the sample was concentrated by centrifugation in a Millipore centriflow membrane cone RF25. About 7 ml of the extract was placed in the cone and concentrated to approximately 1 ml. Further additions were made to the cone and the procedure repeated until the whole aqueous phase had been concentrated. The inside of the cone was washed with this residual fluid to recover any material that might have adhered to the surface. This filtrate was freeze dried and after weighing the residue was dissolved in 1 ml of distilled

water and submitted to TLC. When knowledge of this weight was not required the filtrate was applied directly.

Chromatography procedure

Merck silica gel G 60, a medium porosity gel containing 13% calcium sulphate and having a mean pore diameter of 60 Å, was used for the preparation of thin-layer plates for chromatography. Precoated plates were also purchased from E. Merck (Darmstadt, G.F.R.) both glass backed, 5 × 20 cm, and aluminium foil-backed 20 × 20 cm, each 250 μm thick.

Chromatography was done in Shandon Universal TLC Chromotanks (Shandon, London, Great Britain) and plates were dried with a domestic hairdryer and sprayed with location reagents using Shandon spray apparatus (Shandon). Activation of plates was achieved in a hot-air oven which was also used for complete location of chromatographed material. Scanning of thin-layer plates was achieved on a Chromoscan 200 with a scan 201 (Joyce, Loebl & Co., Burlington, MA, U.S.A.). The weighing of samples of portions of chromatographic scans less than 1 mg in total weight was done on a Sauter five place microbalance.

Samples were applied to the plates as a band (1.8 cm from the lower edge of the plate) thereby making visualization easier and reducing the amount of tailing that might occur during development. The band, normally spread over 1.5 cm, was applied in the form of a series of tiny confluent drops. A hairdryer was used to evaporate off the sample solvent when necessary. The total volume applied to the plate was dependant upon the weight of the lyophilate dissolved. A 200-μg amount of ganglioside in solution was applied to either pre-prepared commercial plates or glass backed plates prepared by us. The solvent mixtures were prepared freshly for each chromatographic run and the chromotography tanks were lined with Whatman 3 MM paper which dipped into the solvent. Plates were chromatographed in either chloroform–methanol–1.4 *M* ammonium hydroxide (55:40:10) or tetrahyfrofuran–0.05 *M* potassium chloride in various combinations. The chromatogram was allowed to develop until the solvent front was within 2 cm of the upper edge of the plate. Excess solvent was removed by the use of a hairdryer in a fume cupboard followed by final complete removal by placing the plate in an oven at 120°C for 3 min. The location reagent consisted of a solution containing 200 mg of resorcinol in 100 ml of 4 *M* hydrochloric acid with 2.5 ml of 0.1 *M* copper sulphate added. (The solution is prepared at least 4 h before use but may be kept in a dark brown bottle at 4°C for about 4 weeks). The dried plates were sprayed lightly and covered carefully with a clean plate before being placed in an oven 120°C for about 20 min. A blue colour against a white background reveals the presence of sialic acid-containing compounds. Quantitation of gangliosides was achieved by placing the plate in a Chromoscan 200 which was adjusted to zero absorbance on an unstained portion of the plate area before scanning the stained gangliosides automatically. A red filter (610 to 750 nm), which was complementary to the blue colour of the localised gangliosides, was used and the 1:4 cam on the instrument was used to scale expand the reading which was recorded simultaneously in the form of a line graph so that for each band a peak was obtained. Peak areas were calculated by cutting out the peak from the paper trace and weighing the paper on a suitable five place microbalance.

RESULTS AND DISCUSSION

Thin-layer chromatographic investigation[19] of several published modifications of the Folch[7] extraction procedure showed that they were incomplete, traces of the smaller-molecular-weight components GM3 and G7 being present in the organic residues after extraction. At the present time this is not important since the relative concentrations of these fractions do not appear to have any primary diagnostic significance. This may change in the future when it will be necessary to modify existing methods.

When a general lipid location reagent was applied to developed chromatograms of the purified extracts it was apparent that the degree of purity achieved was roughly proportional to the sophistication of the extraction method. The compromise technique described here was selected on the basis of degree of contamination and the time involved.

The two modifications for the concentration of ganglioside extracts seem to be successful. The cone method is rapid and would appear to have a place in the hospital laboratory repertoire where a result is required on a very small sample. Routinely the method can also be used prior to lyophylisation. A minor disadvange is that several seedings have to be made to ensure optimal results. Chromatography of the reconstituted filtrate from the cone shows an absence of gangliosides, however the presence of sialic acid in the filtate demonstrated by the method of Warren[20] indicates the possibility of some breakdown during the procedure but if this is the case then such losses are non-selective since the relative percentage of each ganglioside remains unchanged within the tolerances that can be applied. Dialysis against Carbowax produced similar relative proportions of ganglioside fractions (Table II) and was selected for the standard procedure not only on the grounds of cost but because dialysis takes place overnight and therefore no working time is lost.

It was considered that the time taken and the degree of separation achieved using the classical chloroform–methanol–water system for chromatography did not warrant its routine use consequently the solvent of Eberlein and Gercken[11] was examined. Variations in relative proportions of tetrahydrofuran–0.05 *M* potassium chloride significantly altered the separation of the gangliosides (Fig. 1) and it was confirmed that the original solvent system suggested was in fact optimal but that a shorter development time was feasible. If the relative proportion of tetrahydrofuran to aqueous potassium chloride was maintained at 5:1 then concentration changes over the range 0.025–0.1 *M* potassium chloride were without signicance.

A comparison of different types of thin-layer plate showed that the resolution on commercial glass backed plates was better than that achieved on laboratory prepared plates. However foil backed commercial plates showed a lowering in relative R_F values and a tendency for the coating to lift during the location process. Furthermore, the densities of the resultant bands were so reduced that only half the sensitivity was realised. Van den Eijnden[10] observed that poor results were obtained when purified gangliosides were separated on precoated commercial plates, the addition of potassium chloride and calcium chloride to the chromatographic solvent markedly improving resolution. Consequently a number of other compounds were employed in place of 0.05 *M* potassium chloride within the tetrahydrofuran system, these included hydrochloric acid, sodium chloride, acetic acid and sodium hydroxide (all at 0.05 *M*

TABLE II

COMPARISON OF THE RELATIVE PROPORTION OF GANGLIOSIDES FOUND USING TWO DIFFERENT METHODS FOR SAMPLE CONCENTRATION

Gangliosides are identified from left to right in order of increasing mobility in tetrahydrofuran–water with added potassium chloride (see text).

Sample	*Method*	*Relative % of each fraction*									
		GQ1	*GT1*	*GD1b*	*GD2*	*GD1a*	*GD3*	*GM1*	GM_2	GM_3	G_7
1	Cone	1.94	14.62	5.93	—	54.77	4.90	15.50	—	1.72	0.62
	Carbowax	1.52	15.87	6.87	—	53.02	4.51	16.05	—	1.86	0.30
2	Cone	trace	1.08	5.42	—	24.12	7.26	53.87	—	5.12	3.23
	Carbowax	trace	1.23	4.93	—	22.18	6.42	54.72	—	7.12	3.40

concentration) and calcium chloride, calcium acetate, calcium sulphate and sodium sulphate (all at 0.025 *M* concentration). Results indicated that it is the chloride ion only that has a significant affect, other ions and changes in pH appeared to be insignificant. It is probable that the chloride ion is bound by electrostatic attraction to the sialic acid residues of the gangliosides thereby reducing the strong interaction of dissociated sialic acid and the silica residues of the plate. Thus band broadening and other specific interactions between gangliosides are decreased, this being reflected in changes in R_F values and better resolution (Fig. 2).

Attempts at quantitation of the gangliosides using resorcinol location followed by densitometric scanning showed that the scan area of the peaks obtained were linearly proportional to the amount of ganglioside present when varying amounts of between 50 and 350 μg of a total ganglioside extract were seeded. Over the range 100–

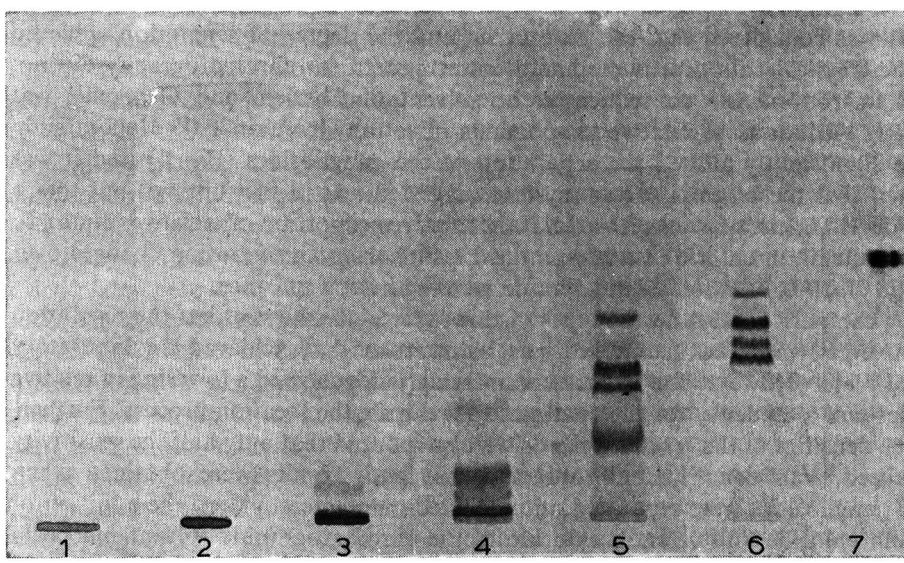

Fig. 1. The effect of alteration of the relative volume of aqueous potassium chloride to tetrahydrofuran on TLC separation of brain gangliosides. The relative volume of potassium chloride to five volumes of tetrahydrofuran was: 1(0), 2(0.4), 3(0.6), 4(0.8), 5(1.0), 6(1.2), 7(2.0).

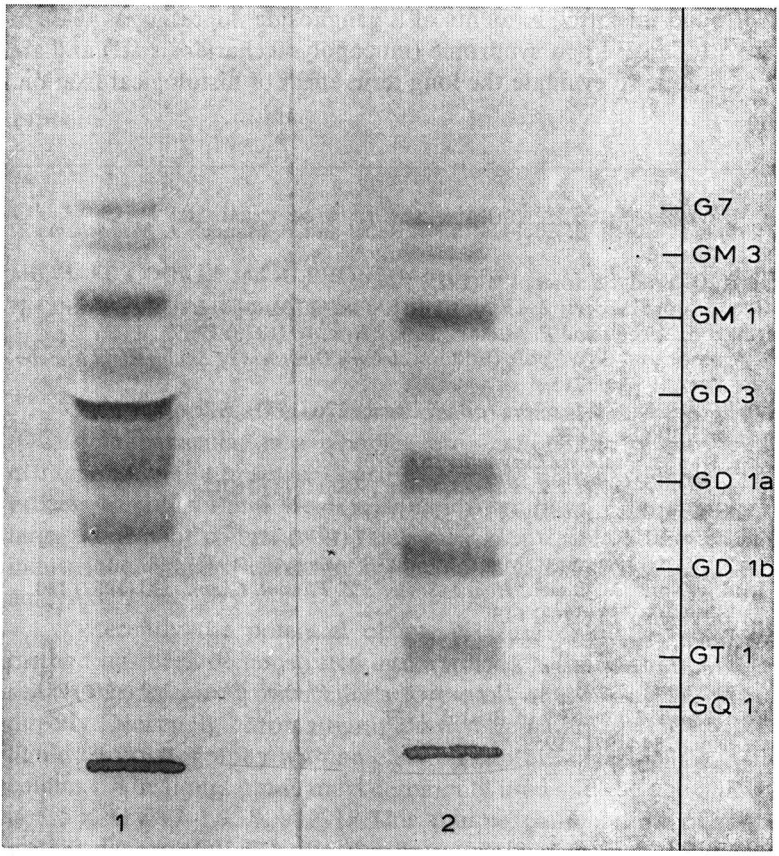

Fig. 2. The effect of chloride ion on the TLC of brain gangliosides. 1 = Tetrahydrofuran with water (5:1); 2 = tetrahydrofuran with 0.05 *M* potassium chloride (5:1).

300 μg there was little difference in calculated relative percentages of each fraction except when this quantity was very low and therefore the scan figure subject to error. A maximum scan error of less than 5% was obtained for all fractions other than GM3 (7%) and G7 (31%), however, both of these constitute extremely small fractions within the total (2% and 0.3% respectively) and the results obtained are diagnostically useful for the major gangliosides. A problem associated with resorcinol location is that the keeping properties are poor and fading occurs within 3 days, the background taking on a pink colouration making rescanning impracticable.

The overall thin-layer procedure using the optimal tetrahydrofuran–potassium chloride solvent appears to be more efficient under the conditions used than at first reported since a further fraction is identified as lying between the seeding line and GT1. Because this band and certain subfractions of other bands appear to be the same as those isolated by Zanetta *et al.*[12] using a different solvent system it was decided to adopt their nomenclature.

We have successfully used the method described for the identification of GM1 gangliosides in human and cat brain affected by GM1 gangliosidosis and the demonstration of GM2 ganglioside in patients with Tay Sachs disease.

We have also found increased amounts of a ganglioside migrating as GD2 in brain from patients with San Filippo syndrome (mucopolysaccharidosis III) and are currently using the technique to evaluate the long term effect of histological fixation.

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Note

OV-17–QF-1 capillary column for organochlorine pesticide analysis

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The determination of pesticides in general and of organochlorine pesticides (OCPs) in particular is a complex problem. Difficulties arise because the normal extraction method of solvent partitioning and Florisil column "clean-up" is a difficult process to control and yields a complex sample[1,2]. The complexity arises from the large number of compounds which may be present. The ubiquitous distribution of industrial chemicals such as polychlorinated biphenyls (PCBs) further complicates the analysis[3–6].

Recently the potential of cyclic steam extraction as a sample preparation method has been demonstrated and applied[6–9]. Extracts obtained in this manner are sufficiently free from interferents to permit analysis by gas chromatography (GC) directly. Normally chromatographic determination of OCPs is performed by gas–liquid chromatography with an electron-capture detector (GLC–ECD) using packed columns. The liquid phase most commonly used and the one officially recommended[10] is 1.5% OV-17–1.95% QF-1. This unique combination of a partially-phenylated methyl silicone (OV-17), and a trifluoromethyl-substituted methyl silicone (QF-1) is, when correctly prepared, capable of giving a reasonable separation for many OCPs. Difficulties arise, however, when, for example *p,p'*-DDE and dieldrin are present in the same sample. Likewise the presence of minor components is seldom revealed when packed columns are used. For example commercial grade *p,p'*-DDT contains a significant quantity of *o,p'*-DDT and dehydrochlorination (the natural environmental degradation reaction) should therefore yield *o,p'*-DDE. *o,p'*-DDE is seldom identified in chromatograms obtained on packed columns presumably because it co-elutes with another major component (such as aldrin). The obvious solution is to apply capillary GC to the separation of organochlorine compounds. The technology of capillary GC has been the subject of considerable research interest in recent years. Much theoretical consideration has been given to the preparation of the surface prior to coating, to deactivation of the surface prior to coating, to the method of coating the column and to assessment of column performance by means of standard compounds. In our work we have adopted two criteria for assessing column performance. These are: (1) does the column perform the desired separation and (2) does the useful column lifetime justify the time spent in preparation? We now report the simple preparation of a mixed phase (2% OV-17–1.5% QF-1) capillary column which gives superior performance to either OV-17 or SE-30 with respect to organochlorine pesticides.

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EXPERIMENTAL

Reagents

All solvents were distilled before use. Standard pesticide compounds were obtained from the National Physical Laboratory, Teddington, Great Britain and were used as received. OV-17 and QF-1 were purchased from Jones Chromatography (Llanbradach, Great Britain). The preparation of the mixed phase column is described in detail below.

Equipment

A Fractovap 2151 Series gas chromatograph (Carlo Erba, Milan, Italy), fitted with a splitless injection system and flame-ionisation detector was used. The hydrogen carrier gas flow-rate was 2 ml min^{-1}. An injection port temperature of 250°C and a temperature programme of 60°C hold 2 min, then 5°C min^{-1} to 200°C, then 6°C min^{-1} to 225°C, hold 3 min were employed unless other wisestated. Amplifier sensitivities were $\times 32$ for Fig. 1 and $\times 16$ for Fig. 2. PTFE-faced septa were used for *ca.* ten injections each before they began to break up and deposit small particles in the injection port

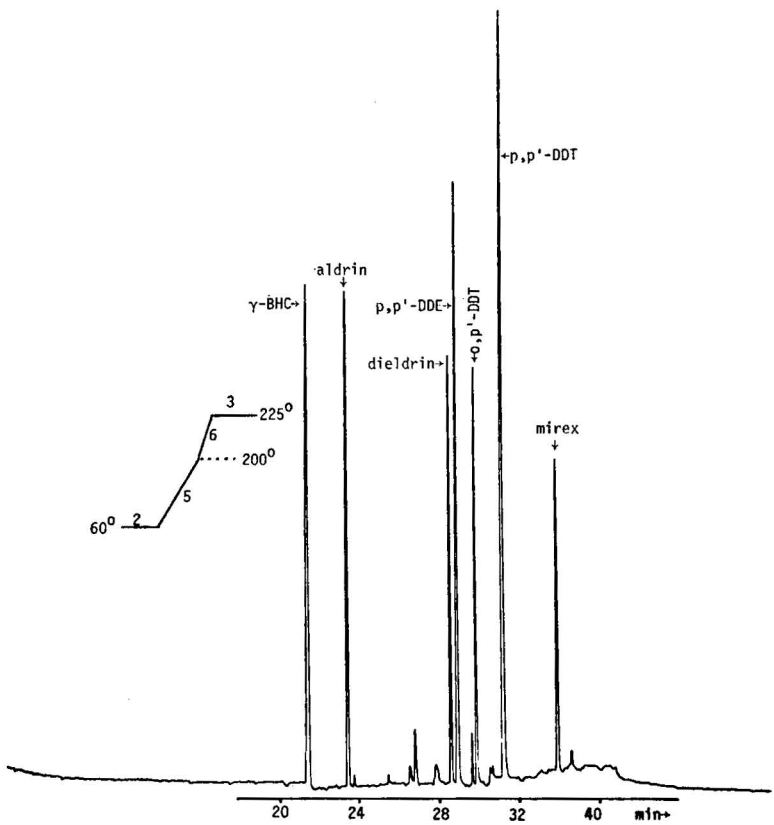

Fig. 1. The mixture of organochlorine pesticides chromatographed on an OV-17–QF-1 capillary column.

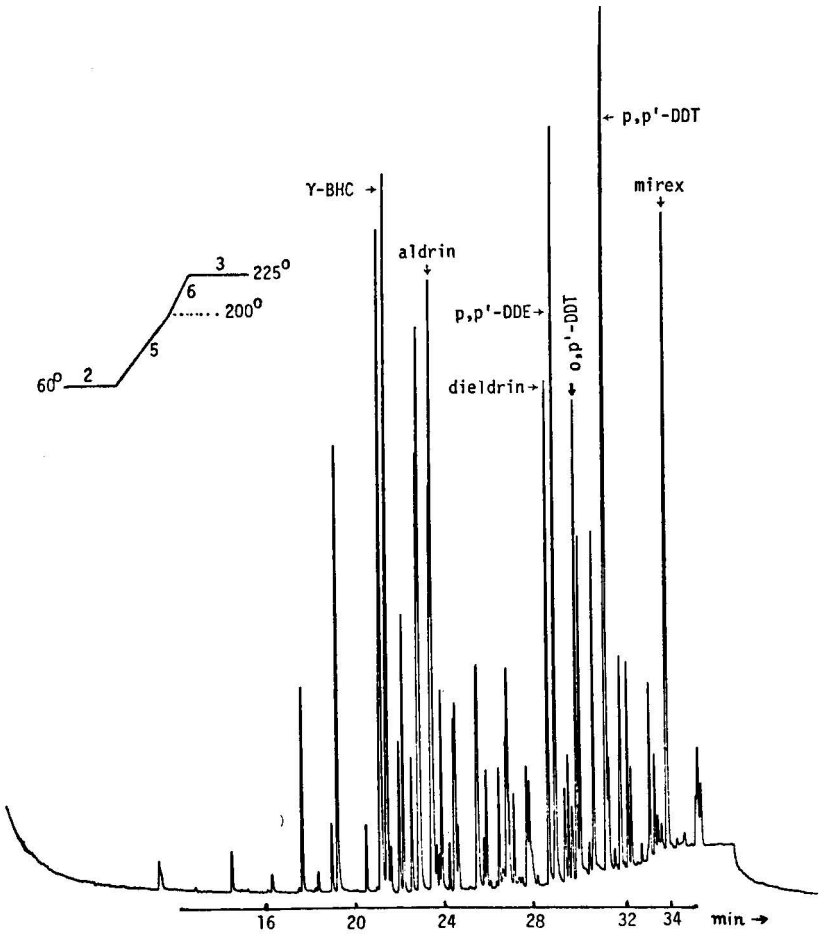

Fig. 2. The mixture of organochlorine pesticides, with Aroclor 1242 and Aroclor 1260 added, chromatographed on an OV-17–QF-1 capillary column.

liner. Septa were replaced and the glass liner cleaned, regularly. Glass capillary columns were drawn on a Hewlett-Packard Model 1045 A drawing machine.

RESULTS AND DISCUSSION

Column preparation

Columns were drawn from soda glass tubing. Before drawing the inside of the tube was thoroughly washed with freshly prepared chromic acid. The intention was to remove traces of organic material adsorbed on the glass surface but the process may also be beneficial in leaching out unwanted metal ions. After acid washing the tube was cleaned with water, then acetone and finally dried by passage of nitrogen. Capillaries were drawn to give a length of 20 m and an internal diameter of 0.3 mm (nominal). The column was then filled to *ca.* 90% capacity with HCl gas (from a

cylinder, nominal purity 99%), sealed, and heated at 350°C for a minimum of 3 h (refs. 11 and 12).

The etched column was filled to *ca.* 20% with a "plug" of Carbowax 20 M (0.1%), in dichloromethane and this "plug" was pulled through the column by application of a vacuum. The rate of travel of this deactivating solution through the column was carefully regulated by means of a needle valve to one turn per minute. After passage of this solution the column was installed in the gas chromatograph, a flow-rate (2 ml min^{-1}) of hydrogen established, and the following temperature programme performed: 50°C hold 30 min; increase 1°C min^{-1} to 280°C; hold 60 min. The whole coating and conditioning procedure was then repeated. The relevance of hydrogen as a carrier gas to this procedure is unknown but it may serve to reduce the possibility of oxidative degradation of the liquid phase (note: if the column is not to be coated immediately after the Carbowax 20 M deactivation treatment then the ends should be sealed until use).

The deactivated capillary was then coated with a solution of 2% OV-17–1.5% QF-1 in chloroform. The column was filled to approximately 20% and the plug pulled through again using vacuum controlled by a needle valve to give a flow-rate of 1 turn min^{-1}. A buffer column of about 10 m was placed between the analytical column and the needle valve to ensure a constant coating velocity as the coating solution neared the end of the analytical column. The column was placed in the gas chromatograph and the temperature was slowly raised, (1°C min^{-1}), to 230°C and maintained there for about 12 h. The maximum column temperature employed subsequently was 225°C.

Separation of organochlorine compounds

A solution (hexane) containing the nominally "pure" standard organochlorine pesticides, γ-BHC, aldrin, dieldrin, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT and mirex was prepared. These were selected as typical compounds representative of the full range of OCPs. Chromatograms were obtained using a standard injection size (0.6 μl). A typical chromatogram is shown in Fig. 1. Peak shape and resolution are excellent as illustrated by the resolution to baseline of the dieldrin/*p,p'*-DDE pair. The elution of dieldrin before *p,p'*-DDE is the reverse of that which is usually observed[13]. All peaks were identified by co-injection.

Environmental extracts containing OCP residues are often contaminated by PCB[7–9]. The performance of this mixed phase column with respect to PCB residues is displayed in Fig. 2.

To the standard mixture of OCPs were added the PCB mixtures Aroclor 1242 and Aroclor 1260. Aroclor 1242 was found to elute completely before *p,p'*-DDE and *ca.* 95% of the components of the Aroclor 1260 eluted later than *p,p'*-DDE. Incidentally the separation of Aroclor 1242 on this column was found to be superior to that obtained on a single phase OV-17 column[14]. From the chromatogram (Fig. 2) it can be seen that one component of Aroclor 1242 elutes on the leading edge of the aldrin peak. A comparison of the ratio of peak heights of aldrin and γ-BHC in the presence and absence of Aroclor 1242 reveals no difference. Thus the aldrin response is not perturbed by the proximity of this component. Quantitation by peak height should thus be possible. All the other organochlorine pesticides are well resolved from PCB compounds. Mirex elutes at the end of the chromatographic run at a temperature of 220°C. The sample preparation procedure which requires column chromatographic

separation of OCP's from PCBs prior to determination on packed columns would thus appear unnecessary if a mixed phase (OV-17–QF-1) capillary column were to be used for the determination.

CONCLUSION

A mixed phase (OV-17–QF-1) glass capillary column has been prepared by a simple coating procedure which provides excellent separation of organochlorine compounds. In spite of the simple preparation procedure, columns appear stable over long periods of use ($>$ 3 months) and give excellent peak shapes for these mildly polar compounds. Quantitation should therefore be routinely achievable by peak height measurement. To demonstrate this possibility known weights of γ-BHC (from 5 to 40 ng) were injected and a calibration curve based on peak height was constructed. The regression equation for the line was $y = 3.5x - 4.67$, with a correlation coefficient of 0.9994.

Substitution of OV-210 for QF-1 would allow a higher temperature limit to be employed should this prove necessary.

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Note

Surface ion exchange and adsorption of some dyes on α-$Zr(HPO_4)_2 \cdot H_2O$ micro-crystals

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Electrical conductance[1] and membrane potential measurements[2] have shown that, at temperatures below 60°C, the ionic transport properties of α-zirconium phosphate [α-$Zr(HPO_4)_2 \cdot H_2O$] depend on the number and nature of counter ions present on the surface of the micro-crystals. Further, α-zirconium phosphate, both as the salt and as some organic derivatives, has already been employed in processes involving the surface such as catalysis[3] and chromatography[4–6]. It was therefore of interest to investigate the chemical and physical properties of the surface of the α-zirconium phosphate micro-crystals. In previous papers[7,8] we have reported the determination of the surface ion-exchange capacity by the Cs^+ ion-exchange procedure, and ion exchange with alkali metal, alkaline earth metal and some transition metal ions was also investigated.

This paper reports a study on the uptake of large organic cations on the surface of α-zirconium phosphate micro-crystals. In particular, methylene blue and crystal violet were employed and the H^+–organic cation exchange occurring at the surface of the micro-crystals was examined. The adsorption of alizarin on micro-crystals, whose surface was first loaded with a known amount of methylene blue or crystal violet, was also investigated.

EXPERIMENTAL

Chemicals

All reagents used were Carlo Erba (Milan, Italy) R.P.E. products, except $ZrOCl_2 \cdot 8H_2O$, which was a Merck (Darmstadt, G.F.R.) pro analysi product.

Ion-exchange material

α-$Zr(HPO_4)_2 \cdot H_2O$ was prepared by slow decomposition of zirconium fluoro complexes in the presence of phosphoric acid[9]. The crystals were washed with distilled water to pH 4.5 and stored over P_4O_{10}. The total amount of the surface exchangeable protons was obtained by the procedure described previously[7]. The surface ion-exchange capacity of the micro-crystals employed was 2.9 ± 0.1 μequiv. per gram of exchanger.

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Methylene blue and crystal violet surface ion exchange

Uptake. A 1-g amount of α-$Zr(HPO_4)_2 \cdot H_2O$ was equilibrated with a $1 \cdot 10^{-4}$ *M* solution of methylene blue or crystal violet at 20 ± 1°C for 24 h. The solutions were analysed for their Cl^-, H_3O^+, methylene blue and crystal violet contents.

Release. A 10-g amount of α-$Zr(HPO_4)_2 \cdot H_2O$ was equilibrated, with stirring, with 500 ml of a $1 \cdot 10^{-4}$ *M* solution of methylene blue or crystal violet, then the solid was washed three times with 250 ml of acetone and dried in air. A 1-g amount of this material was equilibrated, with stirring, with 25 ml of hydrochloric acid of suitable concentration at 20 ± 1°C for 24 h. The solutions were analysed for their methylene blue or crystal violet content.

Alizarin adsorption

Some samples (2 g) of α-$Zr(HPO_4)_2 \cdot H_2O$, whose surface was loaded with a known amount of methylene blue or crystal violet, were equilibrated with 15 ml of $1 \cdot 10^{-4}$ *M* alizarin in acetone solution at 20 ± 1°C for 24 h. The solutions were analysed for their alizarin contents.

Analytical methods used

pH and chloride ion concentration were measured with a Beckman Research pH meter. Silver–silver chloride electrodes were prepared according to Brown's method[10]. Methylene blue, crystal violet and alizarin concentrations were determined with a Beckman 25 spectrophotometer at 664, 591 and 426 nm, respectively. The absorbances of methylene blue and crystal violet solutions were determined at constant pH (4.5).

RESULTS AND DISCUSSION

Methylene blue and crystal violet ion exchange

α-$Zr(HPO_4)_2 \cdot H_2O$ has a layered structure such that, under suitable conditions (room temperature and pH $\leqslant 4$), only its surface protons can be replaced by cations having ionic radii greater than 1.32 Å[11,12]. Hence it was interesting to study the interaction between the α-zirconium phosphate surface and large organic cations; for this purpose methylene blue and crystal violet, whose structural formulae are shown in Fig. 1, were used. Fig. 2 reports the uptake of methylene blue and crystal violet from $1 \cdot 10^{-4}$ *M* solutions; the added dye was completely taken up by the surface of the ion exchanger up to a level of 1.5 μequiv./g; this value is about 50% of the surface ion-exchange capacity (2.9 μequiv./gram), determined by caesium ion uptake. When further dye was added, the slopes of the uptake curves decreased and vanished above 3 μequiv./g.

The dye uptakes corresponding to the plateau is 2 μequiv./g for methylene blue and 1.8 μequiv./gram for crystal violet; these values are about 70% and 60% of the total surface ion-exchange capacities, respectively. The strong affinity of the two dyes for the α-zirconium phosphate surface seems to indicate that the surface protons are exchanged by the cationic dyes. In agreement with this hypothesis, the uptake occurs at various pH values, but pH measurements do not often permit a careful discrimination between exchange and adsorption. However, it can be pointed out that proton exchange does not alter the chloride ion concentration, whereas adsorption necessarily involves ion pairs (organic cation + chloride ion) because of the electroneutrality of

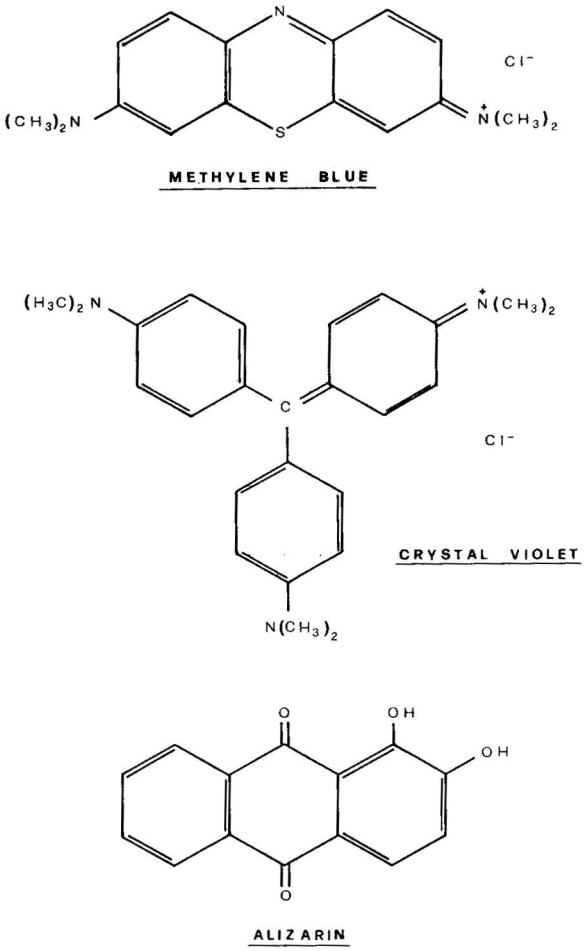

Fig. 1. Structural formulae of methylene blue, crystal violet and alizarin.

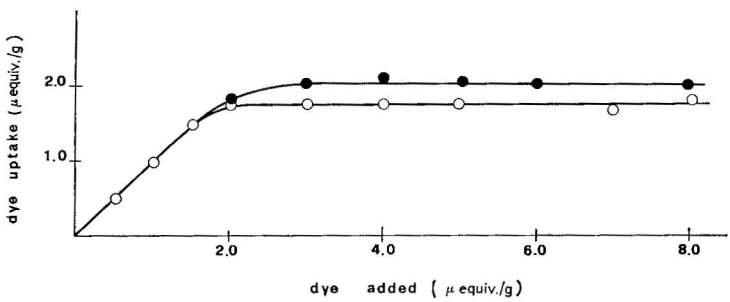

Fig. 2. Methylene blue (●) and crystal violet (○) uptake on the surface of α-Zr $(HPO_4)_2 \cdot H_2O$ micro-crystals, from $1 \cdot 10^{-4}$ *M* solutions of dye at 20°C.

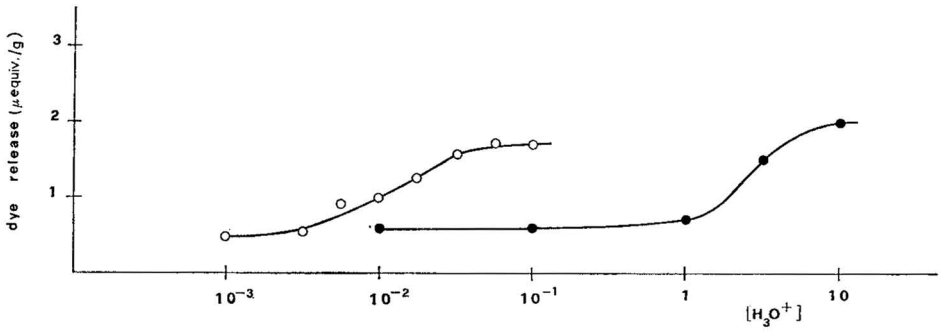

Fig. 3. Methylene blue (●) and crystal violet (○) release for samples whose surface was saturated by the dye, as a function of H_3O^+ concentration. Conditions: 1 g of exchanger equilibrated with 25 ml of HCl of suitable concentration.

the exchanger. Potentiometric determinations with the silver–silver chloride electrode have shown that the chloride concentration is constant and equal to $1 \cdot 10^{-4}$ *M* for all the uptake curve points, so confirming the ion-exchange hypothesis.

Therefore, washing of the micro-crystals with an aprotic solvent should not alter the amount of dye present on the surface and it should be possible to elute all of the dye with an eletrolyte solution of suitable concentration. To confirm this experimentally, a large amount of α-zirconium phosphate was equilibrated with an aqueous solution of dye, in order to saturate the micro-crystal surfaces with methylene blue or crystal violet. After washing with acetone (for further details, see under Experimental) some samples of this material (1 g) were equilibrated with 25 ml of hydrochloric acid of suitable concentration. The results are shown in Fig. 3: in both instances the maximal release value is equal to that of the maximal uptake. Further, it is clear that the affinity of methylene blue for the zirconium phosphate surface is greater than that of crystal violet, probably because of its smaller steric hindrance, higher charge density on the nitrogen atoms and its ability to make dimers on the micro-crystal surfaces[13] and in solution. Finally, let us consider the possible positions of these two organic cations on the zirconium phosphate surface. Methylene blue and crystal violet are planar cations: if they were lying parallel to the surface (Fig. 4), they would be able to cover three or four fixed charges, so that dye uptake would not be greater than 25 or 33 % of the surface ion-exchange capacity. As the ion-exchange percentages are greater than these values, it seems to indicate that the dye molecules are lying perpendicularly or obliquely to the surface plane.

Alizarin adsorption

Alizarin is a neutral molecule whose structure is similar to that of methylene blue (Fig. 1). Preliminary experiments have shown that alizarin adsorption on α-$Zr(HPO_4)_2 \cdot H_2O$ micro-crystals is negligible (about 1 % of the surface ion-exchange capacity). Thus, it was interesting to investigate if alizarin adsorption was increased by the presence of organic cations on the zirconium phosphate surface. For this purpose, 2 g of α-$Zr(HPO_4)_2 \cdot H_2O$, whose surface was previously loaded with a known amount of methylene blue or crystal violet, were equilibrated with 15 ml of $1 \cdot 10^{-4}$ *M* alizarin in acetone solution. Fig. 5 shows the results of these experiments. Alizarin

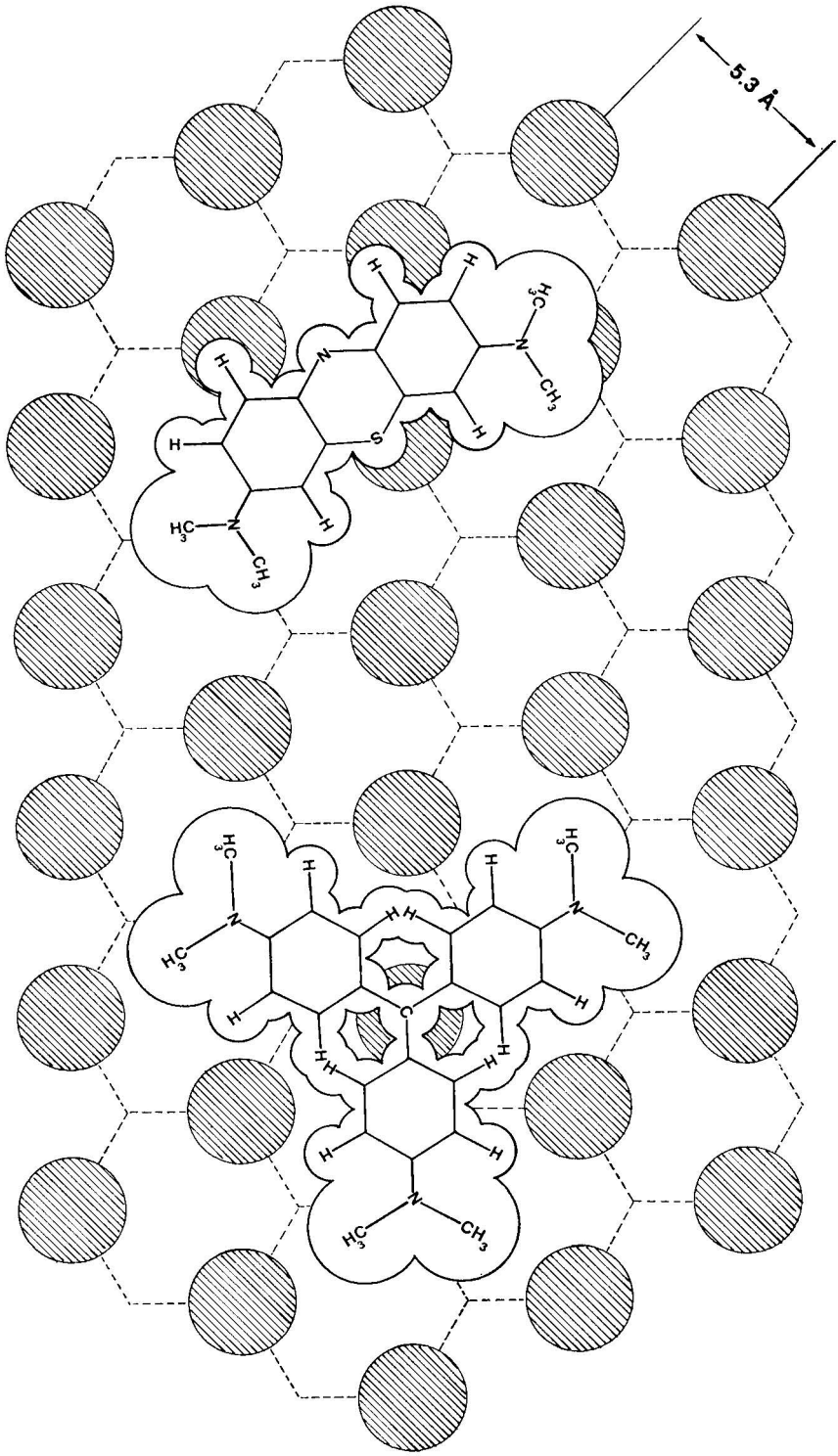

Fig. 4. Possible positions of methylene blue and crystal violet lying parallel to the α-zirconium phosphate surface. Circles represent surface fixed charges.

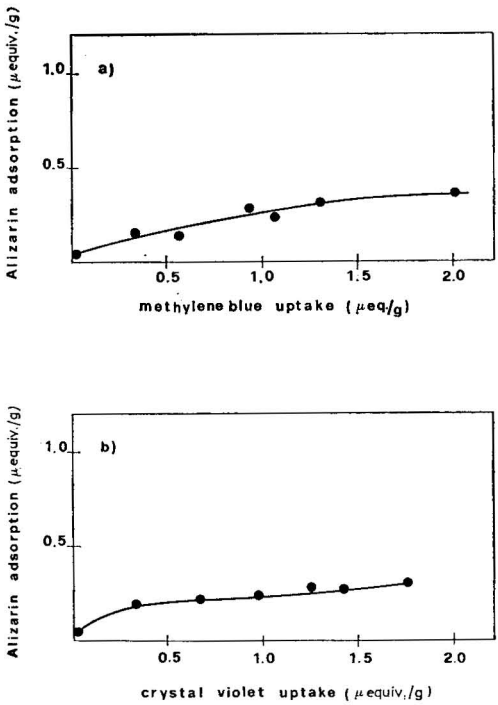

Fig. 5. Alizarin adsorption as a function of (a) methylene blue and (b) crystal violet uptake on the surface of α-Zr $(HPO_4)_2 \cdot H_2O$ micro-crystals. Conditions: 2 g of α-Zr $(HPO_4)_2 \cdot H_2O$ equilibrated with 15 ml of a $1 \cdot 10^{-4}$ *M* alizarin in acetone solution at 20°C.

adsorption increases with dye uptake from 0.04 to 0.3–0.4 μequiv./g; further, the slopes of the adsorption curves decrease with increasing dye uptake, showing that the interaction of alizarin with each organic cation is greater when the alizarin adsorption is smaller.

CONCLUSION

Large monovalent, organic cations, such as methylene blue and crystal violet, are able to exchange reversibily the protons present on the surface of α-$Zr(HPO_4)_2 \cdot H_2O$ micro-crystals. For conversions of less than 50% their affinity for surface fixed charges is greater than that for protons, probably owing to their smaller hydration energy. Further, surface replacement of protons with methylene blue or crystal violet increases alizarin adsorption from acetone solutions. From a practical point of view, these results show that it is possible to change reversibily the adsorption properties of α-$Zr(HPO_4)_2 \cdot H_2O$ micro-crystals, replacing surface protons by different organic cations, and so to employ the same material for different kinds of separations. Other than as a solid support in gas chromatography[4–6], the utilization of α-zirconium phosphate micro-crystals (with their surface hydrogens or in a suitable organic cationic form), in thin-layer or high-performance liquid chromatographic separations can thus be envisaged.

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Note

Human oxymyoglobin: isolation and characterization

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(First received January 22nd, 1980; revised manuscript received February 26th, 1980)

In red muscles such as the cardiac and the skeletal, myoglobin plays an essential role in maintaining aerobic metabolism, both as an oxygen store and by facilitating oxygen diffusion[1–3]. When these muscles suffer from ischemia or other injuries causing cell destruction, the soluble myoglobin will be cleared into blood with myoglobinemia and even into urine with myoglobinuria. The very sensitive quantitation of myoglobin in serum and urine can therefore provide an important new diagnostic test for acute myocardial infarction as well as for other muscular diseases, such as crush syndrome, progressive muscular dystrophy and polymyositis.

In response to the recent increasing need for highly purified human myoglobin for radioimmunoassay[4] and enzyme immunoassay[5], this communication deals with the isolation and characterization of oxymyoglobin from human muscle.

In contrast to the classical preparations of myoglobin in the met-form[6–10], modern procedures for isolating oxymyoglobin directly from muscle tissues all stem from the method of Shikama and co-workers[11]. This has been improved with some refinements and controls using bovine heart muscle, a more readily available source[12–16].

In work described in this report, essentially the same procedure was successfully applied for isolating oxymyoglobin from human muscle for the first time.

EXPERIMENTAL

Myoglobin was extracted overnight at pH 8.0 from the minced, partially thawed muscle (1 kg) of human psoas with 1.5 volumes of cold distilled water. The muscle was obtained at autopsy from adult patients who died of non-muscular disorders and was stored at −5°C by the First Department of Internal Medicine, Tohoku University School of Medicine, Sendai. All procedures were carried out at low temperature (0–4°C) as far as possible. The insoluble material was removed by centrifugation at 3000 *g* for 10 min, and the supernatant was decanted through a doubled gauze cloth to remove fatty substances. This extract was then fractionated with ammonium sulphate between 60 and 100% saturation at pH 7.0 in the presence of $5 \cdot 10^{-4}$ *M* EDTA. The precipitate was centrifuged at 30,000 *g* for 15 min and dissolved in a minimum volume of 5 m*M* Tris–HCl buffer (pH 8.4). The solution was then dialyzed against the same buffer containing $5 \cdot 10^{-4}$ *M* EDTA. The crude myoglobin solution (*ca.* 500 ml), which still contained a large amount of hemoglobin, was applied to four Sephadex G-50 columns (Pharmacia, Uppsala, Sweden; fine, 90 × 5 cm I.D.) equil-

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ibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The column was eluted with the same buffer to separate myoglobin completely from hemoglobin, and the effluent myoglobin solution (*ca.* 500 ml) was dialyzed against 5 m*M* Tris–HCl buffer pH 8.4 containing $5 \cdot 10^{-4}$ *M* EDTA. At this stage about 60% of the myoglobin was in the oxy-form.

The dialyzed myoglobin solution was applied to a DEAE–cellulose column (Whatman DE-32, 15 × 4 cm I.D.), which had been equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The column was washed with a large volume of 15 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 60 ml/h, until the major brown band of metmyoglobin was eluted completely. The major oxymyoglobin component was then eluted with 30 m*M* Tris–HCl buffer (pH 8.0). When concentration was required, the effluent oxymyoglobin solution was dialyzed and applied to a short DEAE–cellulose column (2 × 4 cm I.D.) which had been equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The myoglobin was then eluted with 50 m*M* Tris–HCl buffer (pH 8.0). The yield of the native oxymyoglobin was *ca.* 0.54 g from 1 kg of human psoas muscle, whose total myoglobin content was *ca.* 0.9 g or 0.056 mmole per kg of wet weight.

RESULTS AND DISCUSSION

In this procedure the essential step was the chromatographic separation of oxymyoglobin (MbO_2) from metmyoglobin (metMb) in the hemoglobin-free extract on a DEAE–cellulose column. A typical elution profile of human myoglobin on DEAE–cellulose is shown in Fig. 1. This clearly shows that the absorbance ratio of 582 nm/592 nm, used for identification[12], changed from 1.0 for the first major peak

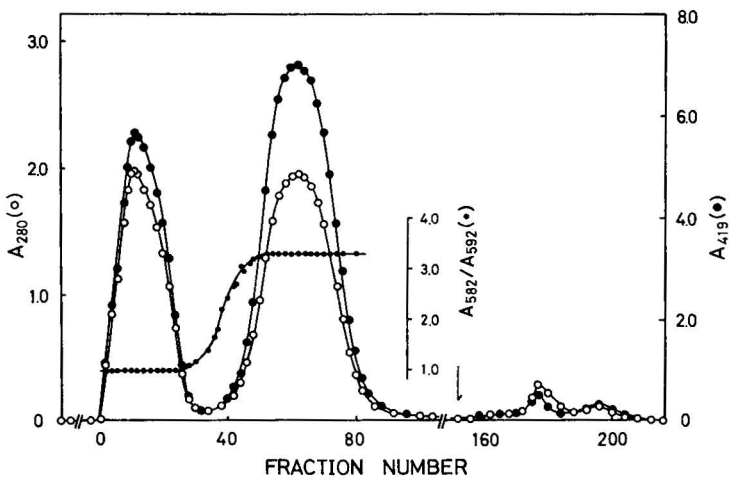

Fig. 1. DEAE–cellulose chromatography of human myoglobin. Myoglobin (50 ml, 270 mg) was applied to a DEAE–cellulose column (20 × 3 cm I.D.), equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The major fractions were eluted with 15 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 30 ml/h. At the point indicated by an arrow, the buffer was changed to 50 m*M* Tris–HCl (pH 8.0) for elution of the minor components. The protein and the heme protein levels were monitored by the absorbances at 280 nm (○) and at 419 nm (●), respectively. MbO_2 and metMb were identified by the absorbance ratio of 582 nm/592 nm (•). Fraction size: 7 ml.

to 3.3 for the second, indicating the presence of metMb and MbO_2, respectively, with a satisfactory separation. It should be noted that two faint coloured bands remained in the column even after the complete elution of the major fractions. These minor components were readily eluted by changing the buffer to 50 m*M* Tris–HCl (pH 8.0), and were identified as the met-form for the former peak and the oxy-form for the latter. The content of the minor components was less than 8% of the total myoglobin obtained, and no difference was observed in the visible spectrum between the major and the minor.

Heterogeneity in human metmyoglobin preparations has been previously reported. Rossi-Fanelli and Antonini first observed the presence of three components in paper electrophoresis[7]. Perkoff *et al.*[9] found four fractions on DEAE–cellulose chromatography, but only two components were demonstrable in starch gel electrophoresis when the metmyoglobin used was converted into cyanmetmyoglobin. The major component accounted for 75–80% of the total myoglobin, and the remaining minor one(s) appeared to differ in the primary structure. Boesken *et al.*[10] also found three bands in polyacrylamide gel electrophoresis. From the presence of only one band in the crude muscle extract, however, they concluded that alteration in surface charge might occur after purification.

To examine the purity of the major products of our primary concern, therefore, the MbO_2 and metMb were subjected to disc electrophoresis on 8% polyacrylamide gel in 0.3 *M* Tris–HCl buffer (pH 8.9) as well as in 0.1% SDS plus 0.1 *M* Tris–Bicine buffer (pH 8.3). They showed a single band in both gels.

The spectroscopic properties of human major MbO_2 are compared in Table I with those of native MbO_2 isolated from other species. For sperm whale myoglobin, the polymorphic forms were first developed on a DEAE–Sephadex column (Pharmacia, A-25) with 15 m*M* Tris–HCl buffer (pH 8.7). The major fraction was then applied to a CM–cellulose column (Whatman, CM-32) equilibrated with 5 m*M* Tris–HCl buffer (pH 7.0) to separate the MbO_2 completely from the metMb with 10 m*M* Tris–HCl buffer (pH 7.5).

TABLE I

ABSORPTION MAXIMA, EXTINCTION COEFFICIENTS AND CHARACTERISTIC EXTINCTION RATIOS OF MAJOR OXYMYOGLOBINS AT pH 8.0*

Source (ref.)	*Absorption maximum (nm) (extinction coefficient (mM^{-1} cm^{-1}))*				α/β	γ/UV
	α	β	γ	*UV*		
Human	582 (15.4)	544 (14.4)	418 (133)	280 (36.9)	1.07	3.60
Horse (11)	582 (15.3)	544 (14.3)	418 (133)	281 (36.3)	1.07	3.66
Bovine (13)	581 (15.5)	544 (14.5)	418 (134)	280 (36.4)	1.07	3.68
Sperm whale	581 (15.4)	543 (14.3)	418 (129)	280 (36.6)	1.08	3.52

* The concentration of myoglobin was determined after conversion into cyanmetmyoglobin using the extinction coefficient of 11.3 mM^{-1} cm^{-1} at 540 nm on the basis of mol.wt. 17,000 by Drabkin[17], except that for sperm whale myoglobin the value of 10.7 mM^{-1} cm^{-1} obtained by Hanania *et al.* was used[18].

The extinction ratio of α- to β-maximum (α/β) can provide a most sensitive and useful criterion for estimating the extent of contamination of metMb in the preparations of MbO_2. The values of 1.07–1.08 are the highest ratios obtained so far for native MbO_2 preparations. While this drops to less than 1.00, one must recognize that such a preparation contains more than 30% of metMb at pH 7. This is mainly due to the rapid autoxidation of MbO_2 to metMb, the mechanistic details of which have been extensively studied[11,13–16]. Therefore, the rate of autoxidation of human MbO_2 was measured according to our standard procedure[16], and the observed first-order rate constant, k_{obs}, was determined as follows: $0.83 \cdot 10^{-2}$ h^{-1} for human MbO_2, $0.72 \cdot 10^{-2}$ h^{-1} for bovine, and $0.50 \cdot 10^{-2}$ h^{-1} for sperm whale in 0.1 *M* phosphate buffer, pH 7.2 at 25°C. Although human MbO_2 is oxidized more easily to metMb with a half-life period of 83.5 h at pH 7.2 and 25°C, it is sufficiently stable for many purposes if stored and handled at low temperature (0–4°C) as far as possible, because there is a marked effect of temperature on the autoxidation rate[13].

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Note

UV-induced silica gel GF photoluminescence studied by liquid scintillation spectrophotometry

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The techniques and problems in thin-layer radiochromatographic analyses were described in a recent monograph devoted to radiochromatography[1]. One problem which is not discussed in that reference is the possibility of spontaneous light emissions which can occur during liquid scintillation counting of 254 nm UV-irradiated silica gel GF thin-layer zones. This potential problem is apparently recognized by many radiochromatographers[2], but literature which describe thin-layer zonal analysis techniques[3–6] make no specific mention of this UV-induced photoluminescent effect. Based on some preliminary findings[7] and literature which describe the measurement of spontaneous light emissions by liquid scintillation counting[8–10] we investigated the nature of 254 nm UV-induced silica gel GF photoluminescence. The present report describes the results of our findings.

EXPERIMENTAL

Materials

Pre-coated silica gel G and silica gel GF thin-layer plates (thickness 0.25 mm) were purchased from Analtech (Newark, DE, U.S.A.) and used without modification. E. Merck silica gel GF, Type 60, was purchased from MCB Reagents (Cincinnati, OH, U.S.A.) and applied onto glass plates (thickness 0.25 mm) as an aqueous slurry with a Desaga applicator. A sample of the Analtech manganese-activated zinc silicate powder was generously provided by Dr. Herman Felton, Analtech. Woelm fluorescent green indicator was purchased from ICN Pharmaceuticals, Life Science Group (Cleveland, OH, U.S.A.). The scintillator solutions employed in these studies included Scintiverse (Fisher Scientific, Fairlawn, NJ, U.S.A.), Bray's solution[11], and a toluene cocktail made with 90 mg/l PPO and 4.50 g/l POPOP. All solvents and reagents used in these experiments were purchased from Fisher Scientific, except naphthalene, which was purchased from New England Nuclear Corporation (Boston, MA, U.S.A.).

Instrumentation

Ambient temperature liquid scintillation counting was performed with a

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Beckman LS 100-C instrument operated with a gain setting of 339 mV. Channel 1 had a present discriminator setting to monitor tritium, channel 2 had preset discriminator settings to monitor tritium–carbon-14 or carbon-14 and channel 3 was preset to monitor tritium through phosphorus-32 β-energy levels. The instrument was operated in near darkness within a temperature range of 24.5–26.5°C. Instrumental efficiency for carbon-14 was $> 95\%$ in channel 2, and $> 46\%$ for tritium in channel 1. Samples were counted for 5, 10 or 20 min with a preset error of 2% and the instrument was operated in the continuous cycle mode for up to 4 days. Discriminator settings for the various energy levels in the Beckman LS 100-C were established by counting UV-irradiated silica gel GF samples in a Beckman 7500 liquid scintillation counter. Discriminator settings for the Beckman 7500 instrument were 0–377 for tritium and 377–700 for carbon-14.

Subambient photoluminescent effects were recorded with a Packard Model 3225 liquid scintillation counter operated at 15–18°C with gain settings of 500 for tritium and 100 for carbon-14. The instrument was operated with discriminator settings of 0–300 for the tritium channel (channel 1) and 300–1000 for the carbon-14 channel (channel 2). Samples were counted for 5, 10 or 20 min in the cycle repeat mode for up to fourteen cycles per sample. Counting efficiency for tritium in this instrument was $> 42\%$ in channel 1 and $> 90\%$ for carbon-14 in channel 2.

Sample preparation

Sampling of the Analtech and Merck silica gel GF thin-layer plates has been described elsewhere[7]. Sampling of the manganese-activated zinc silicate powder was carried out under yellow lights and consisted of the following: (a) 1 g of the respective indicators were mixed with 99 g of Analtech silica gel G in a rotary mixer. The samples were divided into 80 ± 10 mg aliquots, transferred to glassine paper, then irradiated for 10 sec with a Mineralight Model UVS-11 short-wave UV lamp (Ultraviolet Products, San Gabriel, CA, U.S.A.) positioned 12 cm above the silica gel surface; (b) 1.5 ± 0.4 mg aliquots of the Analtech and Woelm zinc silicate indicators were placed on glassine paper and irradiated for 10 sec with the 254 nm UV lamp. Non-irradiated silica gel G, silica gel GF, zinc silicate indicator samples and solvents served as controls in procedure (a) and (b). Following UV irradiation, samples were added to glass scintillation vials along with 10 ml of the respective scintillator solutions. Both the control and UV-irradiated samples were mechanically shaken for 5 min prior to liquid scintillation analyses, and all experiments were performed in either duplicate or triplicate.

RESULTS AND DISCUSSION

Photoluminescent effects in samples of 254 nm UV-irradiated Analtech and Merck silica gel GF thin-layer powder and Analtech and Woelm manganese-activated zinc silicate indicators were studied by liquid scintillation spectrophotometry at 26°C and 18°C. The gel and indicator samples were added to organic scintillators composed of xylene, toluene or dioxan and UV-induced spontaneous light emissions were recorded with scintillation spectrophotometer discriminator settings corresponding to tritium, tritium–carbon-14, carbon-14 or tritium–phosphorus-32 β-energy levels. The intensity of the photoluminescent decay curves recorded

for the various UV-irradiated gels and indicators ranged from $> 10^5$ cpm at 26°C for the Analtech silica gel GF powder to > 700 cpm at 18°C for the Merck silica gel GF and Woelm zinc silicate powders. The duration of the UV-induced photoluminescent effects ranged from 86 h for the Analtech silica gel GF at 18°C, to 12 h for the Merck and Woelm materials. These results are summarized in Tables I and II. Tables I and II also show that 254 nm UV-induced silica gel GF photoluminescence should not interfere with liquid scintillation counting of carbon-14, provided that the counting channels have discriminator settings of > 300.

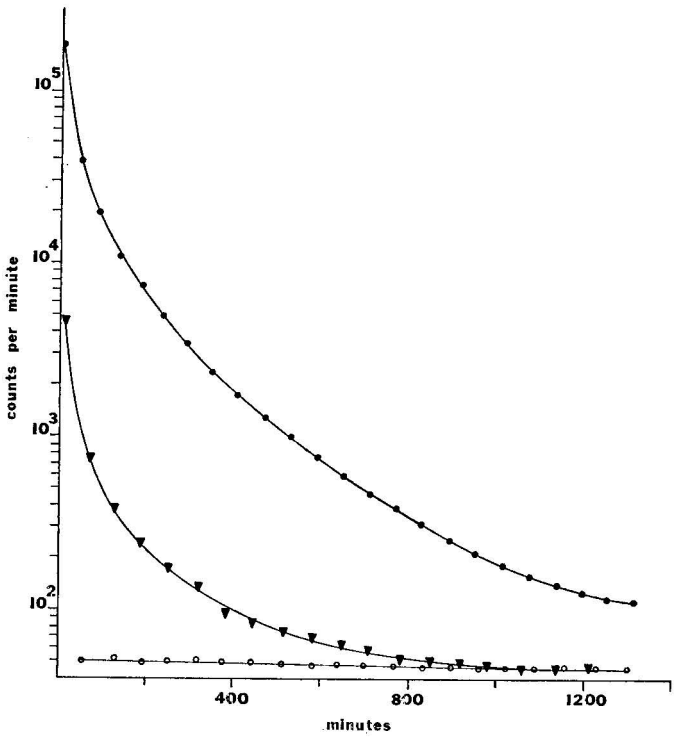

Fig. 1. Duration and intensity of 254 nm UV-induced photoluminescence from Analtech and Woelm manganese-activated zinc silicate indicators in toluene. ●, Analtech zinc silicate indicator; ▼, Woelm zinc silicate indicator; ○, background counts per minute. Similar results were obtained with xylenes and dioxan solvents.

One interesting result was recorded at 26°C. Discriminator settings of 0–1000 were used to monitor photoluminescence decay processes of UV-irradiated Analtech and Woelm zinc silicate samples in xylenes, dioxan or toluene which did not contain added organic scintillators. These results are shown in Fig. 1. It is evident from those decay curves that Analtech zinc silicate powder photoluminescences with more intensity and with longer duration than corresponding amounts of Woelm zinc silicate powder. These results may prove to be useful in some types of radio-luminescence analyses[12,13] which employ silica gel GF.

TABLE I

INTENSITY AND DURATION OF AMBIENT TEMPERATURE PHOTOLUMINESCENCE RECORDED FOR 254 nm UV-IRRADIATED SILICA GEL GF AND ZINC SILICATE SAMPLES

Scintillator mixtures	*Counting channel*	*Analtech silica gel GF*		*Analtech silica gel G and Analtech indicator*		*Merck silica gel GF*		*Analtech silica gel G and Woelm indicator*	
		*cpm**	*hours***	*cpm**	*hours***	*cpm**	*hours***	*cpm**	*hours***
Scintiverse	1	>10,000	60	>1000	36	>4000	10	500	12
	1–2	>10,000	60	>1000	36	>4000	10	500	12
	1–3	>10,000	60	>1000	36	>4000	10	500	12
	2	***				***		***	
Bray's solution	1	>10,000	60	>1000	36	>4000	10	>500	12
	1–2	>10,000	60	>1000	36	>4000	10	>500	12
	1–3	>10,000	60	>1000	36	>4000	10	>500	12
	2	***				***		***	
Toluene solution	1	>10,000	60	>1000	36	>500	10	500	12
	1–2	>10,000	60	>1000	36	>500	10	500	12
	1–3	>10,000	60	>1000	36	>500	10	500	12
	2	***				***		***	

* Data represent the lowest cpm recorded from either duplicate or triplicate samples approximately 2 h after sample irradiation.

** Represents the minimum number of hours required to reach background levels of 20–60 cpm.

*** Photoluminescence was not detected.

TABLE II

INTENSITY AND DURATION OF SUBAMBIENT TEMPERATURE PHOTOLUMINESCENCE FOR 254 nm UV-IRRADIATED SILICA GEL GF AND MANGANESE-ACTIVATED ZINC SILICATE SAMPLES

Solvent	*Counting channel*	*Analtech silica gel GF*		*Analtech silica gel G and Analtech indicator*		*Merck silica gel GF*		*Analtech silica gel G and Woelm indicator*	
		*cpm**	*hours***	*cpm**	*hours***	*cpm**	*hours***	*cpm**	*hours***
Scintiverse	1	>3000	>86	<1000	48	<1000	11	>700	12
	1–2	>3000	>86	<1000	48	<1000	11	>700	12
	2	***		***		***		***	
Bray's solution	1	>3000	>86	<1000	48	<1000	11	>700	12
	1–2	>3000	>86	<1000	48	<1000	11	>700	12
	2	***		***		***		***	12
Toluene solution	1	>3000	>86	<1000	48	<1000	11	>700	12
	1–2	>3000	>86	<1000	48	<1000	11	>700	12
	2	***		***		***		***	

*–*** As in Table I.

ACKNOWLEDGEMENTS

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Note

Nucleic acid bases and derivatives: detection by Dns derivatization thin-layer chromatography

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During our studies on the chemistry of abnormal inclusions in the brain, we needed a sensitive and convenient method to analyze for CMP and related compounds. Such compounds may undergo a variety of chemical reactions[1]. Therefore we investigated the reaction of dimethylaminonaphthalene-5-sulfonyl chloride (Dns-Cl) towards nucleic acid bases and their derivatives. The reaction conditions are presented here, and thin-layer chromatography (TLC) of the fluorescent products is also described. This is apparently the first report on the detection of such molecules by the sensitive Dns derivatization method.

EXPERIMENTAL

Dns derivatization

The Dns–Cl reaction was adapted from that described by Gray[2] for amino acids and peptides. Nucleic acid bases and derivatives were purchased from Sigma (St. Louis, MO, U.S.A.) and Dns-Cl was from Pierce (Rockford, IL, U.S.A.). The stoichiometry of Dns-Cl to amino groups was modeled after the optimal conditions described by Airhart *et al.*[3] for amino acids. We used a different buffer and a higher pH than most investigators have reported for the Dns-Cl reaction. The following reaction conditions were used to prepare our Dns standards. Buffer was made with deionized water which had been boiled and flushed with nitrogen gas to remove any traces of ammonia, which if present, gives rise to Dns-amide. Each compound (0.5 mg) was dissolved in 1.25 ml of untritated 10 m*M* Na_3PO_4, pH 11.7. Then 1.25 ml of Dns-Cl in acetone (1.0 mg/ml) was added and the reaction was carried out overnight at 25°C in the dark. Subsequent steps were also protected from light[4].

TLC

An appropriate aliquot of the reaction mixture was spotted on a low-fluorescence sheet[5] of Polyamide A 1700 (Pierce), 15 cm in height. For most compounds, the amount spotted was 0.25–1.0 μl, or 50–200 ng of the individual compound. Chromatograms were subjected to ascending chromatography in various solvents. After

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the solvent had migrated nearly to the top, the sheet was air-dried. Dns spots were visualized under UV light.

RESULTS AND DISCUSSION

Dns derivatization

Some nucleic acid bases and their derivatives —especially the cytosine series— were not adequately derivatizated when sodium bicarbonate, pH 8.5 (ref. 2) was employed as the buffer. We checked the effect of the basicity on the reaction, between pH 7–13. The best results were obtained between pH 11–12.2. We then routinely used untitrated trisodium phosphate, pH 11.7.

The concentrations of reactive groups and Dns-Cl were kept at about 500 pmoles/μl and 1840 pmoles/μl, respectively, as recommended for amino acids[3,6]. However, we found that those values could be increased up to four- and two-fold, respectively, with no apparent loss in the efficiency of Dns derivatization of the nucleic acid bases and their derivatives.

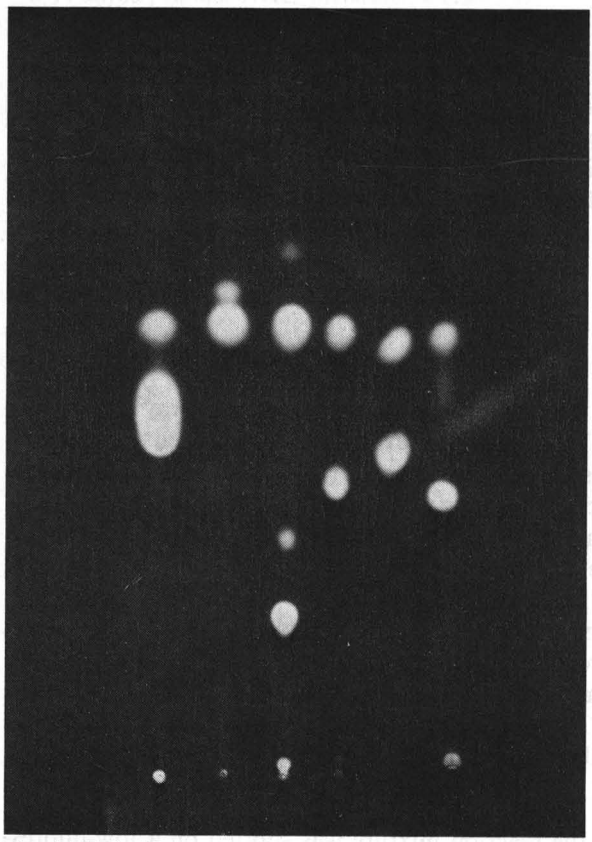

Fig. 1. TLC of Dns compounds on a sheet of Polyamide A 1700. From left to right: Dns–adenine, –cytosine, –guanine, –thymine, –uracil and –xanthine. The solvent was 6% HCOOH. The spot in common to each lane is hydrolyzed reagent (Dns-OH), a side-product always present in Dns derivatizated samples.

TLC

Fig. 1 shows the results of chromatography of the Dns derivatized nucleic acid bases adenine, cytosine, guanine, thymine, uracil and xanthine. About 200 ng of each of the compounds were spotted. These amounts were readily visualized as their Dns derivatives under UV light. Spots were still faintly detected when the amounts were 4–20 fold less.

Dns derivatization method was more sensitive for some of the compounds than for others. Minimal amounts required for visualization were: adenine and its derivatives (adenosine, AMP, and so forth), 10–20 ng; cytosine and its derivatives, 50–100 ng; others (listed in Table I), 30–60 ng. For a comparison of sensitivities based on molar amounts, 10 ng of adenine = 74 pmoles while 50 ng of cytosine = 450 pmoles.

Additional spots in the guanine sample (in the third lane, Fig. 1) were considerably weaker in intensity than the main spot presumed to Dns-guanine itself. The extra compounds were present in different batches of guanine; they are probably contaminants of the guanine standard.

Table I gives the migration data for chromatography of the Dns derivatizated

TABLE I

R_F AND R_D VALUES FOR Dns-NUCLEIC ACID BASES AND DERIVATIVES ON POLYAMIDE A 1700 SHEETS

Solvent systems: A, 6% formic acid; B, ethyl acetate–ethanol–ammonium hydroxide (20:2.5:1); C, ethyl acetate–ethanol–ammonium hydroxide (20:5:1), = solvent F of Metrione[13], for Dns-amino acids.

R_D = the distance of migration of the compound of interest, divided by the distance of migration of Dns-OH in that sample.

Dns-compound	R_F			R_D		
	A	*B*	*C*	*A*	*B*	*C*
Adenine	0.54	0.93	0.96	0.80	11.4	7.0
Cytosine	0.66	0.83	0.92	1.07	10.1	6.2
5-Methylcytosine*	—	0.88	—	—	9.0	—
Guanine	0.21	0.57	0.83	0.35	6.4	5.0
Thymine	0.40	0.91	0.94	0.66	11.4	6.8
Uracil	0.43	0.75	0.90	0.72	9.5	6.7
Xanthine	0.37	0.11	0.73	0.62	1.4	4.9
Hypoxanthine	0.36	0.50	0.82	0.61	5.6	6.0
Adenosine	0.78	0.92	0.16	1.25	7.7	0.47
Cytidine	0.89	0.87	0.09	1.43	6.9	0.26
Guanosine	0.76	0.91	0.39	1.25	9.6	1.15
Thymidine	0.45	0.69	0.13	0.72	6.7	0.37
Uridine	0.66	0.57	0.26	1.08	5.3	0.77
AMP	0.77	0.00	0.00	1.24	0.00	0.00
CMP	0.87	0.00	0.00	1.38	0.00	0.00
GMP	0.74	0.00	0.00	1.12	0.00	0.00
TMP	0.34	0.00	0.00	0.53	0.00	0.00
UMP	0.69	0.00	0.00	1.11	0.00	0.00
2′-dAMP	0.71	0.00	0.00	1.23	0.00	0.00
2′,3′-cAMP	0.69	0.20	0.36	1.13	1.65	3.5
3′,5′-cAMP	0.65	0.18	0.27	1.10	1.67	3.1
NAD	0.80	0.01	0.00	1.43	0.04	0.00

* A Dns spot was found for this compound only in solvent B.

compounds tested. Besides the conventional R_F value, we present R_D as defined in Table I. Comparisons made between different chromatographic runs per solvent, showed the R_D value to be highly reproducible — more so than was the R_F value.

Other results

We checked to see whether the presence of a biological tissue (brain) would interfere with Dns derivatization. Portions of normal rabbit substantia nigra were homogenized in the Dns derivatization buffer in the presence of several compounds (adenine, thymine or CMP). The weight ratios of wet brain to the solid compound were 10:1 and 100:1. Dns derivatization and TLC were carried out as described in Experimental. No interference was found in the analysis for those compounds tested. Only several additional faint spots appeared from the tissue tested at the above larger ratio.

Stability of the Dns compounds was investigated with respect to incubation in the reaction mixture, and mild acid hydrolysis. Dns derivatization at pH 11.7 for 8, 16, 24, or 48 h gave the same spot intensities. This contrasts with Dns–hexosamine spots which were markedly diminished in their intensities after 24 h, compared to several hours of Dns derivatization[7]. The Dns–nucleic acid bases and derivatives were also dried and hydrolyzed in 2 *N* HCl at 70°C for 16 h. Five Dns derivatized species were destroyed: adenine, guanine, xanthine, thymidine, and TMP. The others (see Table I) were unaffected by the acid treatment.

While the Dns method worked for those compounds listed, little success was obtained with the di- and triphosphates *e.g.* ADP and ATP. Perhaps steric hindrance is presented by the extra phosphate atoms. For example, rotary dispersion studies have indicated that in aqueous solution the pyrophosphate chain of ATP may be folded back to give bonding between the β- and γ-phosphates and the adenine amino group[8].

The present Dns method can be useful for compounds such as those in Table I, based on its simplicity and sensitivity. Some workers may also wish to prepare fluorescent derivatives (Dns) of the nucleic acid base moiety, for a variety of studies.

Other methods of analysis should also be mentioned for comparison. Post-labelling of nucleosides with [^{3}H]borohydride can detect about 25 ng of individual nucleosides[9]. Ethenylation and gas chromatography have been used for nucleosides and nucleotides of cytosine in the 500–4000 ng range[10]. High-performance liquid chromatography of nucleosides was quantitative down to 0.1 nmoles or about 25 ng[11]. Gas chromatography–mass spectrometry was used to detect as little as 1.6 pmoles (200 pg) of 5-methylcytosine[12].

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Note

Gas chromatographic separation of chiral 2-hydroxy acids and 2-alkyl-substituted carboxylic acids

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Gas chromatographic methods are preferentially applied for the determination of the configuration of optically active compounds, especially when only small and impure samples are available. Besides the biological relevance of a certain configuration, the quantitative measurement of enantiomer composition in asymmetric syntheses has increasing importance.

Direct enantiomer resolution on chiral stationary phases has been used as the most elegant method for stereochemical assignments in the field of amino acids and amines[1–4], based on the fundamental investigations of Gil-Av and co-workers. Only recently the enantiomers of 2-hydroxy acids could be separated on special chiral stationary phases as O-trifluoroacetyl-hydroxy acid isopropyl esters[5].

Alternatively, chiral compounds may be separated as diastereomeric derivatives on achiral stationary phases. As in amino acids two functional groups are available in hydroxy acids for introducing a chiral substituent. Both techniques have been used before[6–10]. For amino acids (+)-3-methylbutan-2-ol proved to be an excellent reagent to form diastereomeric esters, which could be very well resolved as N-pentafluoropropionyl derivatives.

In this paper we report on the separation of the (+)-3-methyl-2-butyl esters of O-trifluoroacetylated (O-TFA) or O-trimethylsilylated (O-TMS) 2-hydroxy acids and of branched carboxylic acids.

EXPERIMENTAL

Materials

(+)-3-Methylbutan-2-ol was prepared, as described by Halpern and Westley[12], by esterification of L-valine with racemic 3-methylbutan-2-ol and fractional crystallization of the *p*-toluene sulphonic acid salt. After two crystallizations and alkaline hydrolysis of the ester, (+)-3-methylbutan-2-ol was obtained in 98.5% optical purity and 35% overall yield.

Esterification of 2-hydroxy acids and 2-alkyl(aryl)-carboxylic acids

Amounts of 100 μg of the acid were heated for 2 h at 80°C with 50 μl of (+)-3-methylbutan-2-ol–HCl gas (7 *N*) in a screw cap vial with a PTFE lining in the cap. Excess reagent was removed with a current of dry N_2.

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O-Trifluoroacetylation

To the residue of (+)-3-methyl-2-butyl esters, 200 μl of CH_2Cl_2 and 50 μl of trifluoroacetic anhydride were added and kept at room temperature for 30 min. After removal of the excess reagent with nitrogen, the residue was dissolved in 200 μl of CH_2Cl_2 and investigated by gas chromatography.

O-Trimethylsilylation

Alternatively 50 μl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA, Macherey, Nagel & Co, Düren, G.F.R.) were added to the esters and kept at room temperature for 1 h.

Gas chromatography

The preparation of glass capillaries has been described in a previous publication[13]. The SE-30 capillary was purchased from Franzen Analysentechnik (Bremen, G.F.R.). Gas chromatographic investigations were run on a Carlo Erba Model 2101 gas chromatograph with inlet split system (split ratio 30:1), hydrogen as carrier gas, and flame ionization detector.

RESULTS AND DISCUSSION

Previous investigations of diastereoisomeric 2-hydroxy acid derivatives in most cases were performed on long steel capillaries or on packed columns with peak shapes,

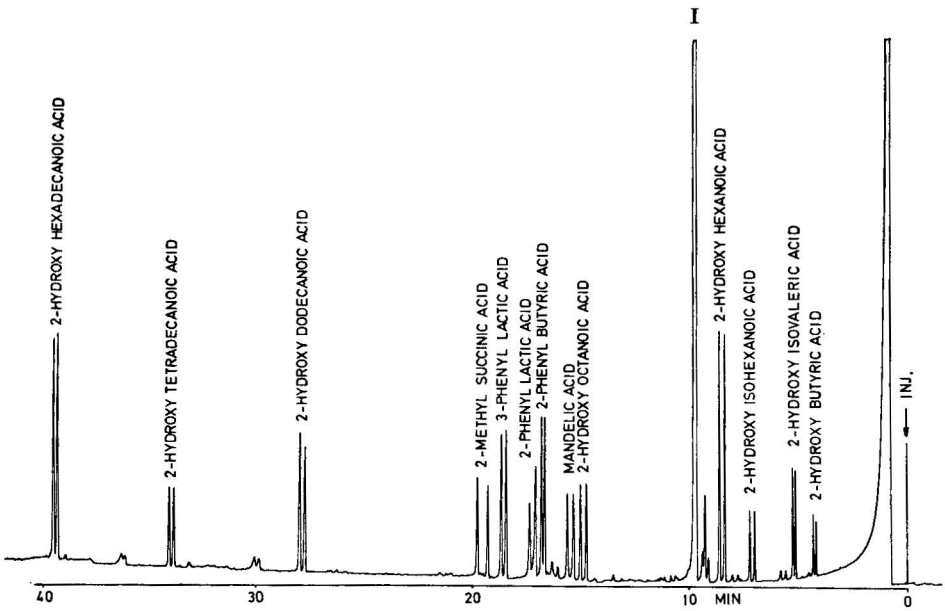

Fig. 1. Gas chromatogram of DL-2-O-TFA-carboxylic acid (+)-3-methyl-2-butyl esters and DL-2-alkyl(aryl)-carboxylic acid (+)-3-methyl-2-butyl esters on a 25-m glass capillary column (SE-30, 0.3 mm I.D.). I = N-TFA-L-valine-O-(+)-3-methyl-2-butyl ester (internal standard). Deactivation, Carbowax 20M; column temperature, 80°C; temperature programme, 3°C/min to 240°C; carrier gas, 0.7 bar hydrogen; split ratio, 1:30.

TABLE I

SEPARATION FACTORS (α) FOR 2-O-TFA- AND 2-O-TMS-CARBOXYLIC ACID (+)-3-METHYL-2-BUTYL ESTERS ON 25-m GLASS CAPILLARY A (SE-30) AND B (OV-17)

Compound	*α-Value O-TFA derivative*		*Temperature (°C)*		*α-Value O-TMS derivative*		*Temperature (°C)*	
	Column A	*Column B*	*Column A*	*Column B*	*Column A*	*Column B*	*Column A*	*Column B*
Lactic acid	1.015	1.019	100	100	1.047	1.033	100	100
2-OH-Butyric acid	1.032	1.036	100	100	1.042	1.044	100	100
2-OH-Isovaleric acid	1.029	1.032	100	100	1.034	1.039	100	100
2-OH-Isohexanoic acid	1.050	1.046	100	100	1.037	1.036	100	100
2-OH-Hexanoic acid	1.045	1.053	100	100	1.041	1.045	100	100
2-OH-Octanoic acid	1.035	1.034	130	130	1.032	1.032	130	130
2-OH-Dodecanoic acid	1.023	1.022	190	190	1.017	1.019	190	190
2-OH-Tetradecanoic acid	1.026	1.025	190	190	1.019	1.020	190	190
2-OH-Hexadecanoic acid	1.024	1.028	190	190	1.018	1.022	190	190
Malic acid	1.021	1.029	140	140	1.016	10.17	140	140
2-Phenyllactic acid	1.032	1.037	140	140	1.007	1.032	140	150
3-Phenyllactic acid	1.029	1.029	140	140	1.030	1.033	140	150
Tropaic acid	1.013	1.026	140	140	1.016	1.025	140	150
Mandelic acid	1.032	1.040	140	140	1.010	1.015	140	140
4-OH-3-Methoxymandelic acid	1.023	1.026	200	200	1.033	1.015	200	200
3-OH-4-Methoxymandelic acid	1.023	1.002	200	200	1.030	1.011	200	200
4-OH-Mandelic acid	1.011	1.033	140	140	not separated	1.019	—	200

analysis times and sensitivities not comparable with modern standards. The excellent results with amino acid derivatives[11] encouraged us to apply the same technique to 2-hydroxy and other chiral carboxylic acids. Although (+)-3-methyl-2-butyl esters of 2-hydroxy acids with free hydroxy groups are sufficiently volatile for gas chromatography, the peak shapes of acylated or silylated derivatives are better. Both types of derivative are sufficiently stable over several days. The O-TFA derivatives (Fig. 1) are more volatile and show slightly larger separation factors (α) than the O-TMS derivatives (Fig. 2). The results are summarized in Tables I and II. The order of elution of diastereoisomers was proved as far as pure enantiomers were available. In these cases (lactic acid, malic acid, mandelic acid) the L-enantiomers have the longer retention time.

The derivatives of 3-hydroxybutyric acid could not be separated. It seems to be necessary that the carboxy group, which is esterified with the chiral alcohol, is directly attached to the asymmetric center. Similar results have been obtained by Rose and co-workers[14].

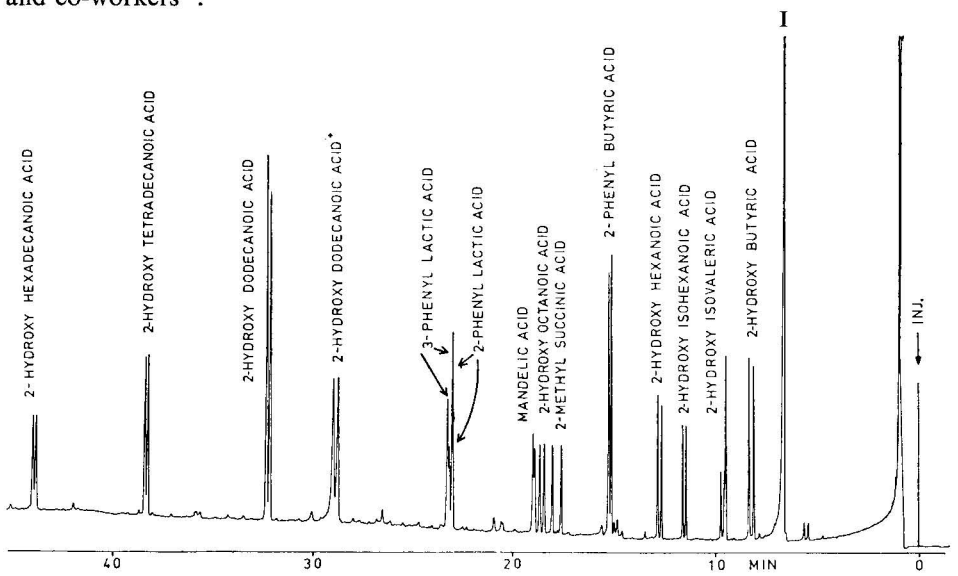

Fig. 2. Gas chromatogram of DL-2-O-TMS-carboxylic acid (+)-3-methyl-2-butyl esters. Same conditions as in Fig. 1. (* = 2-hydroxydodecanoic acid with underivatized hydroxy group).

TABLE II

SEPARATION FACTORS (α) FOR 2-ALKYL(ARYL)-CARBOXYLIC ACID (+)-3-METHYL-2-BUTYL ESTERS ON 25-m GLASS CAPILLARY A (SE-30) AND B (OV-17)

Compound	*α-Value*		*Temperature (°C)*	
	Column A	*Column B*	*Column A*	*Column B*
2-Methylsuccinic acid	1.061	1.057	140	140
2-Phenylsuccinic acid	1.032	1.022	190	190
2-Phenylbutyric acid*	1.027	1.027	100	120

* Enantiomeric 2-phenylbutyric acid amides have been separated on optically active stationary phases by Weinstein and co-workers[3].

Although all methods based on the formation of diastereoisomers are inaccurate principally because of the different reaction kinetics in the formation of the diastereomeric derivatives and because of the lack of enantiomer reagents with 100% optical purity[15], the described procedure can be recommended for the assignment of the configuration of carboxylic acids with an asymmetric centre at C-2.

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Note

Use of the image analyser Optomax for the quantitative evaluation of antibiotics separated by gel electrophoresis and by thin-layer chromatography

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Many antibiotics in current use are complex mixtures of biologically active components and possibly some degradation products. The composition of the same antibiotic produced by different manufacturers can vary greatly, *e.g.* neomycin[1]. A knowledge of an antibiotic's composition is essential when considering the replacement of a microbiological assay by a chemical or physical assay. Both gel electrophoresis and thin-layer chromatography (TLC) have proved invaluable in demonstrating the heterogeneous nature of many antibiotics. Quantitative *in situ* bioautographic determinations have been described for biologically active components separated by gel electrophoresis[2] and by TLC[3] and several quantitative densitometric determinations of antibiotic complexes have been reported[4–6].

The Optomax image analyser has been used in the Division of Antibiotics for measuring the area of zones of inhibition of growth in antibiotic diffusion assays. This report describes the use of the Optomax for the determination of the amount of benzylpenicillin in carbenicillin by an *in situ* microbiological assay after the electrophoretic separation of the antibiotics in an agar gel. The content of kanamycin B in kanamycin was estimated using the Optomax to measure the areas of zones produced by the ninhydrin reaction after separation by TLC. The relative composition of samples of polymyxin B have been determined using biological assay and colorimetric assay after separation of the components by TLC.

EXPERIMENTAL

The Optomax (Micro Measurements, Saffron Walden, Great Britain) is a modular image analysis system which utilises television scanning techniques to make measurements on any image which can be received by a television camera. The basic system measures areas on features in the image which can be differentiated from the background by sufficient "grey level" difference (*i.e.* contrast).

The television scanner had a Vivitar 135-mm lens fitted with a gelatin filter (Spectrum Red, 608, Ilford, Great Britain) to improve the image contrast. The objects were illuminated from below by four small fluorescent tubes (Gro-lux, Sylvania, Great Britain), dark ground illumination was used for the measurement of zones of inhibition of growth. For the measurement of the ninhydrin zones, direct transmitted light was used; a clear precoated silica gel plate was placed in the base below the light source to ensure a uniformly illuminated background.

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Benzylpenicillin sodium B.C.R.S.* and a sample of carbenicillin sodium were used for the determination of the benzylpenicillin in carbenicillin. Details of the method used are described in the British Pharmacopoeia[7]. The zones of inhibition of growth were measured using the Optomax. The conventional statistical method of parallel line assay for two + two dose assay was used for the analysis of the results. The area of the zone of inhibition of growth was taken as the response metameter and was analysed in relation to the logarithm of the dose.

Kanamycin B was separated from kanamycin by TLC[8] using precoated silica gel G plates, layer thickness 0.25 mm (E. Merck, Darmstadt, G.F.R.; Art. 5721). The plates were heat activated at 100°C for 60 min; solutions of 1 μl were applied to the plates, which were then developed in an equilibrated chromatography tank containing an aqueous solution of 10% w/v KH_2PO_4. The plates were chromatographed over a 15-cm path, removed, air-dried and dipped into ninhydrin reagent (300 mg ninhydrin in 5 ml 2,4,6-trimethylpyridine and 95 ml ethanol), air-dried, then heated at 100°C for 10 min. The dark blue zones were measured with the Optomax using direct transmitted light without dark ground illumination. To estimate the content of kanamycin B in a sample of kanamycin, three duplicate doses of the standard (the International Reference Preparation of Kanamycin B) 1.5, 3.0, 6.0 μg, were placed on the chromatographic plate, together with duplicate doses of 100 μg of the samples. The amount of kanamycin B in the samples was calculated from the standard curve.

The TLC separation of polymyxin B was as previously described[9]. For the colorimetric assay the developed plates were treated with ninhydrin reagent and measured as for kanamycin B. Biological activity was estimated using *Bordetella bronchiseptica* (NCTC 8344). After incubation the seeded agar was floated off the chromatographic plate and transferred to a clean glass plate; excess water was drained off and the areas of inhibition of growth were measured with the Optomax. The results of both assays were expressed as percentages of the two main components: polymyxin B_1 and B_2 in the total $B_1 + B_2$.

Because the purified individual components were unavailable, no calibration curves could be obtained for response metameter against known amounts of each component. Regression coefficients (b) for polymyxin B_1 and B_2 were obtained by measuring the response metameters of the two components after separation for several concentrations of a sample of polymyxin B. The regression coefficients of the two components were almost identical and the means were used for each assay (b = 12853 biological assay and b = 3018 ninhydrin response).

The relative concentrations of the two main components were calculated from the following:

$$\log \frac{\text{concn. } B_1}{\text{concn. } B_2} = \frac{\text{area } B_1 - \text{area } B_2}{b} = \frac{4341-2503}{3018} = 0.609$$

$$\frac{\text{concn. } B_1}{\text{concn. } B_2} = 4.064$$

Relative concentrations; polymyxin B_1 = 80.26 and polymyxin B_2 = 19.74.

* B.C.R.S. = British Chemical Reference Substance.

RESULTS AND DISCUSSION

The reproducibility of the Optomax for measuring the zones of inhibition of growth produced by benzylpenicillin and the coloured zones resulting from the reaction of kanamycin B with ninhydrin is demonstrated in Table I. The variability associated with the application of the solutions is shown in Table II. Where the area measured was small, the variability was greatest, as shown with kanamycin B. In both assay methods the coefficients of variation were greater for the repeated application of the solutions than for the repeated measurement of a single zone. Variation in the application of the solutions was a greater source of error than instrumental parameters in the quantification of biological response of colorimetric response.

TABLE I

REPRODUCIBILITY AS A FUNCTION OF MEASUREMENT

Zones of inhibition of growth produced by benzylpenicillin in an agar-gel after electrophoresis and zones produced by kanamycin B after TLC and visualisation with ninhydrin reagent, each single zone measured ten times.

Substance	*Amount (μg)*	*Mean area (arbitrary units)*	*Coefficient of variation (%)*
Benzylpenicillin	0.025	22730	0.15
	0.05	27798	0.16
	0.1	34117	0.11
Kanamycin B	1.0	1051.6	2.05
	2.0	1720.3	1.58
	4.0	2274.7	0.84
	8.0	3554.5	0.58

TABLE II

REPRODUCIBILITY AS A FUNCTION OF APPLICATION SOLUTIONS

Zones of inhibition of growth produced by benzylpenicillin, 5 μl volumes, in an agar-gel after electrophoresis and zones produced by kanamycin B, 1 μl volumes, after TLC and visualisation with ninhydrin reagent, ten replicate zones each measured once.

Substance	*Amount (μg)*	*Mean area (arbitrary units)*	*Coefficient of variation (%)*
Benzylpenicillin	0.0125	6009	2.10
	0.025	10415	0.99
	0.05	14924	1.43
	0.1	20067	1.12
Kanamycin B	1.0	1042.1	7.00
	2.0	1964.8	5.24
	4.0	2546.2	4.53
	8.0	4181.1	6.24

There was a linear relationship between the area of the zone of inhibition of growth and the logarithm of the dose of benzylpenicillin over the range 0.0125–0.1 μg. Similarly, a direct relationship was shown for the colorimetric response and the logarithm of the dose of kanamycin B over the range 0.5–8.0 μg. Correlation co-

efficients from separate experiments ranged from 0.9919 to 0.9999 for benzylpenicillin and 0.9919 to 1.000 for kanamycin B, confirming that the logarithm of the dose–response relationships were linear. The actual response recorded for the same dose varied from experiment to experiment, as indicated in Tables I and II. Therefore, the estimation of unknown quantities must be made by comparison with the known concentration of a standard or reference substance included in the same experiment. This is the basis of most biological assays and therefore the experimental design included standards for the biological assay of benzylpenicillin in carbenicillin. For the estimation of kanamycin B in kanamycin, reference was made to the calibration graph obtained with known concentrations of the reference substance included in each experiment.

The benzylpenicillin content of a sample of carbenicillin was estimated on three separate occasions. The precision of each estimate was within $\pm$ 5% and there was good agreement between the three independent estimates (Table III). The weighted mean estimate was 36.67 mg of benzylpenicillin sodium per 1000 mg of carbenicillin. Even after an electrophoretic separation the Optomax can be used with confidence to measure the zones of inhibition of growth produced by benzylpenicillin.

TABLE III

ESTIMATE OF THE BENZYLPENICILLIN (mg/1000 mg) CONTENT IN A SAMPLE OF CARBENICILLIN DETERMINED BY BIOASSAY AFTER ELECTROPHORETIC SEPARATION

Experiment	*Benzylpenicillin (mg/1000 mg)*	*Confidence limits (%) (P = 0.95)*
1	35.23	$\pm$ 3.9
2	38.51	$\pm$ 3.6
3	36.36	$\pm$ 4.2

To determine the accuracy of estimating the amount of kanamycin B in kanamycin, known amounts of kanamycin B were added to a sample of kanamycin containing no detectable kanamycin B; the estimates and the percentage recovery are shown in Table IV. Quantification of kanamycin B with the Optomax was found to be simple and accurate, after chromatographic separation.

Estimates of kanamycin B in samples of kanamycin, obtained using the current chromatographic system and measuring the areas with the Optomax, were compared with previous results obtained by TLC and visually matching the zones against known standards. The results are shown in Table V. The agreement between the two estimates of kanamycin B after TLC was very good.

The relative proportions of polymyxin B_1 and B_2 were determined in samples of polymyxin after TLC separation on the basis of antimicrobial activity and ninhydrin reactivity, (Table VI). If, on the basis of the known composition of the two polymyxin components, it is assumed that they have identical ninhydrin reactivity, then the differences in the results obtained biologically indicate that polymyxin B_2 is about 3.5 times as active against the test organism as polymyxin B_1. The correlation between the biological and the chemical assays for the estimates of polymyxin B_1 and B_2 was very good—correlation coefficients $r = 0.93$ were obtained for both components when

TABLE IV

ESTIMATION OF KANAMYCIN B IN SAMPLES OF KANAMYCIN A TO WHICH KNOWN AMOUNTS OF KANAMYCIN B HAD BEEN ADDED

The results are the mean of duplicate determinations calculated from a three point logarithm dose-response graph (μg kanamycin B per 100 μg kanamycin A).

Kanamycin B		*Recovery (%)*
Amount added (μg)	*Amount estimated (μg)*	
1.0	1.018	101.84
1.5	1.477	98.50
2.0	2.007	100.35
2.5	2.544	101.74
3.0	3.039	101.32
4.0	4.204	105.09
5.0	4.963	99.26
6.0	5.926	98.76

TABLE V

ESTIMATION OF KANAMYCIN B (AS A PERCENTAGE) IN SAMPLES OF KANAMYCIN

Determinations were made after TLC using the Optomax and by visual matching.

Sample	*Kanamycin B (%)*	
	Optomax	*Matching*
1	2.3	3
2	4.2	5
3	0.0	None detected
4	2.4	3
5	3.2	4

TABLE VI

THE RELATION PROPORTIONS OF POLYMYXINS B_1 AND B_2 IN SAMPLES OF POLYMYXIN B

The proportions were determined by biological and colorimetric assay after TLC.

Sample	*Bioassay*		*Colorimetric assay*	
	B_1	B_2	B_1	B_2
A	53.39	46.59	80.26	19.74
B	54.59	45.41	84.24	15.76
C	45.78	54.21	63.80	36.20
D	51.51	48.49	82.92	17.08
E	54.76	45.24	84.95	15.05
F	55.45	44.55	83.00	17.00
G	50.35	49.65	72.05	27.95

comparing the two methods. The incomplete separation of polymyxin and the lack of sensitivity for the minor components meant that only the two major components could be quantified. Nevertheless, a method which enables the antimicrobial activities of constituents of complex antibiotic mixtures to be compared is extremely useful.

The Optomax has already proved to be satisfactory for the measurement of areas of inhibition of growth obtained in a microbiological assay. It followed that it could be used for the measurement of areas of inhibition of growth produced by antibiotics after separation by either gel electrophoresis or TLC. The ability of the Optomax to measure areas of any shape make it particularly useful, as separation techniques often produce distorted zones.

The Optomax purchased by The National Institute for Biological Standards and Control was intended to be used for measuring areas of inhibition of growth. Thus, the results of the estimation of kanamycin B were very encouraging; the use of the Optomax to measure areas produced by reaction with ninhydrin on chromatographic plates is a valuable bonus. The examination of polymyxin B demonstrates its application to quantification of the chemical composition and antimicrobial activity of a heterogeneous antibiotic complex, a facility of value in laboratories engaged in the correlation of biological activity and chemical composition.

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Note

Separation and determination of the degradation products of tributyl phosphate by high-speed analytical isotachophoresis

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Tributyl phosphate (TBP) is an excellent solvent for the extraction of heavy metals from aqueous phases[1,2] and has particular significance at present for the regeneration of fuel elements in the nuclear energy industry[3,4]. The extraction yield is, however, reduced by hydrolytic[1] or radiolytic[5] degradation of TBP into dibutyl phosphate (DBP), monobutyl phosphate (MBP), orthophosphate (P) and butanol. DBP and MBP form with the extracted metals complex compounds that remain dissolved in the aqueous phase or form precipitates and thus decrease the extraction yield[6–8] substantially.

Analysis of the degradation products is not easy. All of the acidic components present interfere in potentiometric alkalimetry. Paper[9] and thin-layer[10] chromatography provide poor sensitivity of detection. The determination of the degradation products by gas chromatography is very laborious, as it requires preliminary preparation of methyl[11] or trimethylsilyl[12] derivatives. The above circumstances on the one hand and the successful results of using high-speed analytical isotachophoresis in the analysis of mixtures of different types of phosphates[13] on the other led us to apply this method to the determination of the degradation products of TBP.

EXPERIMENTAL

β-Alanine and histidine were obtained from Loba Chemie (Vienna, Austria), imidazole from Koch-Light (Colnbrook, Great Britain), Mowiol 8-88 [poly(vinyl alcohol)] from Hoechst (Frankfurt, G.F.R.) and morpholinoethanesulphonic acid (MES) from Sigma (St. Louis, MO, U.S.A.). MBP and DBP were obtained as a 53:47 (w/w) mixture from the Institute for Ore Research (Prague, Czechoslovakia). All other chemicals were supplied by Lachema (Brno Czechoslovakia).

Experiments were carried out at room temperature in the equipment for high-speed analytical isotachophoresis (dimensions of the capillary, 0.2 × 1 × 200 mm). Zones were detected by measuring the potential by means of two platinum contacts 0.05 mm distant from one another in the longitudinal direction; a power supply with a stabilised current up to 400 μA and with a maximal voltage of 16 kV was used. A detailed description can be found elsewhere[14,15]. A Perkin-Elmer Model 196 line recorder was used.

Standard aqueous solutions of DBP, MBP and P were prepared by the dissolution of the 53:47 (w/w) mixture of MBP and DBP in 0.005 *M* $NH_4H_2PO_4$.

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Extraction yields of the degradation products were investigated as follows. A 10-ml volume of TBP was enriched with 20 μl of the MBP–DBP mixture, the mixture thus obtained was agitated vigorously for 5 min, then 10 ml of 0.5 *M* tris-(hydroxymethyl)aminoethane (Tris) solution were added and the mixture was stirred on an electromagnetic stirrer for a certain period in order to extract DBP and MBP into the aqueous phase. After phase separation, 3-μl samples of the aqueous phase were analysed for the contents of DBP and MBP. DBP and MBP contents were also determined in unenriched TBP with 6-μl samples taken from the aqueous phase. Quantitation of the extraction was followed by comparing the preceding results with the analyses of a standard prepared by dissolving 20 μl of the of MBP–DBP mixture directly in 10 ml of 0.5 *M* Tris solution.

RESULTS AND DISCUSSION

Successful analysis of DBP, MBP and P by isotachophoresis requires that a suitable leading anion is used and that the pH of the leading electrolyte should be selected such that there are sufficient differences between the effective mobilities of the components under analysis. As the components under investigation are medium-strength acids, the most suitable pH for the separation will probably lie in the range from slightly acidic to neutral. Five electrolyte systems were tested in this range, and their characteristics and the values of the driving electric current are presented in Table I. In all instances 0.01 *M* Cl^- served as the leading anion.

TABLE I

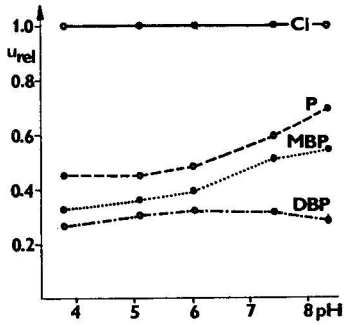
CHARACTERISTICS OF OPERATIONAL ELECTROLYTE SYSTEMS AND VALUES OF THE DRIVING CURRENT USED

pH	*Leading electrolyte*	*Terminating electrolyte*	*Driving current (μA)*
3.8	0.010 *M* HCl + β-alanine	0.010 *M* glutamic acid	160
5.1	0.10 *M* HCl + hexamethylene-tetramine	0.010 *M* morpholinoethane-sulphonic acid	80
6.0	0.010 *M* HCl + imidazole	0.010 *M* diethyl barbiturate, Na salt	190
7.4	0.010 *M* HCl + Tris	0.010 *M* glycine + $Ba(OH)_2$	50

The dependence of the relative effective mobilities of MBP, DBP and P on the pH of the leading electrolyte, determined from the heights of the steps in the record obtained on detection with a gradient detector (cf. ref. 16), is shown in Fig. 1.

The components under investigation obviously differ in their mobilities over the entire range studied and can be separated with success.

The most rapid separation was obtained at pH 6 in the system with histidine + HCl as the leading and morpholinoethanesulphonic acid as the terminating electrolyte; the record of this separation is presented in Fig. 2. For quantitation, the dependence of the step length in the record on the amount injected in the range *ca.* $8 \cdot 10^{-10}$–$33 \cdot 10^{-10}$ mole was measured. The volume of the standard solutions injected was 1–6 μl.



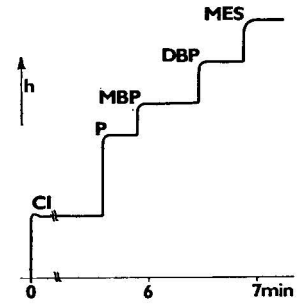


Fig. 1. Dependence of the relative effective mobilities on pH of the leading electrolyte. Chloride served as a reference substance, its $\mu_{rel} = 1.0$.

Fig. 2. Isotachophoregram of a model mixture of DBP, MBP and P. A 3-μl volume of a mixture of 5.0 m*M* P, 8.1 m*M* MBP and 5.4 m*M* DBP was analysed. The leading electrolyte was 20 m*M* histidine + 10 m*M* HCl, pH 6.0, and the terminating electrolyte was 10 m*M* morpholinoethane sulphonic acid (MES). The driving current was 190 μm and the chart speed 40 mm/min.

The dependences were linear, with linear correlation coefficients of 0.9996, 0.9987 and 0.9999 and standard deviations of the regression line of $2.8 \cdot 10^{-10}$, $3.2 \cdot 10^{-10}$ and $2.3 \cdot 10^{-10}$ mole for P, MBP and DBP, respectively. The relative standard deviations for the mean of the calibration range were 1.7, 1.9 and 1.4%, respectively.

In order to establish the possibilities of determining the degradation products in technical TBP, experiments were carried out in which TBP enriched with standard additions of MBP and DBP was extracted with a known volume of 0.5 *M* sodium hydroxide, 1 *M* sodium carbonate or 0.5 *M* Tris solution and the aqueous phase was subjected to analysis. On the basis of these experiments, 0.5 *M* Tris solution was selected as a suitable extractant. It was found that MBP and DBP in the concentration range from $8 \cdot 10^{-10}$ to $350 \cdot 10^{-10}$ mole dissolved in 10 ml of TBP are extracted quantitatively (more than 99%) into the aqueous phase by 10 ml of 0.5 *M* Tris solution in 5 min.

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Book Review

Modern size-exclusion liquid chromatography: Practice of gel permeation and gel filtration chromatography, by W. W. Yau, J. J. Kirkland and D. D. Bly, Wiley-Interscience, New York, Chichester, Brisbane, Toronto, 1979, XVII + 476 pp., price £ 15.00, ISBN 0-471-03387-1

The book *Modern size-exclusion liquid chromatography* is not just another book in the rapidly expanding literature on liquid chromatography. It is the first comprehensive treatise on size-exclusion chromatography (SEC), and as such fills an important gap in the existing literature.

The authors succeed in distilling into slightly less than 500 pages the essence of twenty years of SEC research and practice. The book presents a totally integrated, critical, in-depth treatment of the subject. It reviews its history, but focusses on the modern aspects of the technique. SEC is viewed in the context of the whole field of liquid chromatography, as well as in its relationship to other techniques of polymer molecular characterization. The treatment of the methods of gel permeation and gel filtration chromatography have been integrated. The book combines theory of retention, resolution and column packing design with practical know-how of equipment, operating variables, laboratory techniques and data handling. Special techniques, such as preparative and small-molecule separations and recycle SEC are treated as well.

One chapter deals with the SEC application to synthetic polymers. It does not attempt to give a comprehensive account of the reported application literature but is oriented towards illustrating the value and types of information available from SEC. The treatment of gel filtration is less successful. Its coverage in one chapter is too short, but this detracts little from the overall value of the book. All the important references are included, and the various chapters abound in illustrations from the general SEC literature. With its scope and depth, the book is destined to become the "bible" of size-exclusion chromatography for years to come.

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Book Review

Recent developments in mass spectrometry in biochemistry and medicine, Vol. 2, edited by A. Frigerio, Plenum, New York, London, 1979, X + 492 pp., price US$ 45.00, ISBN 0-306-40294-7

According to its cover, this book "gathers in one convenient source contributions from a variety of spectroscopic specialties. The authors present a comprehensive overview of the field . . .". Well, not quite.

The book does contain, on almost 500 type-written pages, some 33 research papers that were presented at the *Fifth International Symposium on Mass Spectrometry in Biochemistry and Medicine, Rimini, Italy, June 1978*. There are only two "overviews": An introductory one by Benakis on mass spectrometry in drug metabolism, and a more specific one by Brandenberger on anion mass spectrometry.

The latter also provides the only light touch —intended, I'm sure— through an appeal with exchangeable adjectives: "Twelve positive aspects of negative ion mass spectrometry". The remaining contributions are for the most solid, well-documented research reports that could have appeared in any reputable journal. Their tongue is English, though frequently of the Continental variety. Their meaning, however, is always clear, and there are amazingly few typing errors.

Mass spectrometry is the red thread of the book: The editor uses it to string up a motley row of papers in order of their biological content. It is this content to which "Recent Developments" in the title must refer —because mass spectrometry itself serves merely as a tool in all but a few investigations.

The first third of the book contains mostly law-induced studies of the metabolism of drugs: dibenzo[*c*,*f*]-[1,2]diazepine, propildazine, sydnocarb, 1-(2-nitro-3-methyl-phenoxy)3-*tert*.-butylaminopropan-2-ol, suloctidil, thiopropamine, (2-ethyl-2, 3-dihydro-5-benzofuranyl) acetic acid, niclosamide, tetrahydrocannabinol, and 1-methyl-3-hydroxy-5-phenyl-7-chloro-2H-1,4-benzodiazepin-2-one. Throughout the book one finds analyses: Of vincamine, phosphonoacetic acid, cyanide, thiocyanate, amino acids, and cadaverine in blood or plasma; of polyamines, steroids, organic acids and amino acids in urine; of biogenic amines in brain; of bile acids, prostaglandins, respiratory gases as well as of "metabolic profiles". Some of the analytical approaches are quite sophisticated and most are, as they should be, mission-oriented: These relate, for instance, to the synthesis of antibiotics, to poisoning, to schizophrenia, to virilism and pregnancy, to respiration and, prominently, to cancer.

It is not unusual to have such symposium papers bound in a book rather than publishing them in the appropriate journals. Whether this is a wise decision is open to debate. One may laud the interdisciplinary nature of the book, or one may decry the burden its eclectic nature puts on libraries and information retrieval. User preference, I would hope, will finally clarify the present confused situation.

Champfèr (Switzerland) WALTER A. AUE

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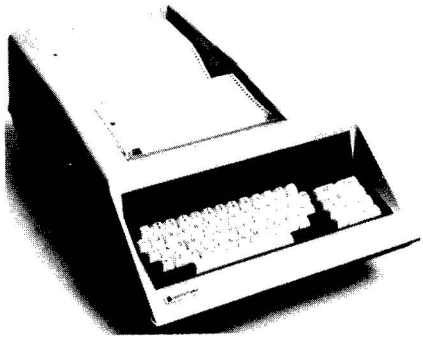
APPARATUS

N-1456

SP 4100 WITHOUT BASIC

The Spectra-Physics SP 4100 computing integrator is now available in two versions: with and without BASIC programmability. The new version without BASIC provides the preprogrammed features of the original SP 4100 at a lower price. An SP 4100 without BASIC can be easily upgraded to BASIC capability. A simple printed circuit card exchange is all that is required. With BASIC the SP 4100 provides the chromatographer with the capability to make custom report calculations and formatting of results.



N-1457

COLUMNS FOR CARBOHYDRATE ANALYSIS

From the Hamilton Company are available two new pre-packed stainless-steel columns for partition chromatography of carbohydrates. The columns are packed with the new HC-40 (4.0% cross-linked) and HC-75 (7.5% cross-linked) DVB–polystyrene sulfonate resins and are calcium loaded. The particle size is said to be precisely controlled (10–15 μm). The flow-rate in columns 300 mm × 8 mm run at 80°C with water eluant (sample size 12 μl) is typically 0.6 ml/min.

N-1463

GEL CASTING SYSTEM

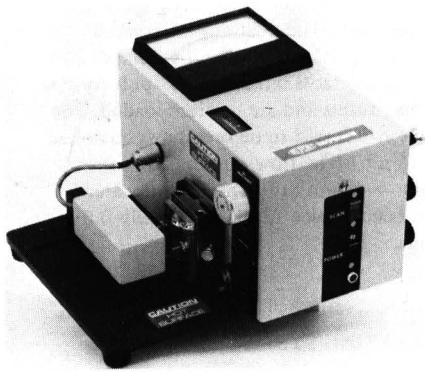
A device simplifying the casting of thin-layer polyacrylamide gels for analytical electrofocusing is available now from Bio-Rad Laboratories. The gels are cast by pipetting monomer/ampholyte mixture under one glass plate that rests on precision spacer rails in the acrylic CTL (Capillary Thin Layer) casting tray. Surface tension retains the gel solution under the plate, and the inhibitory effect of the acrylic is said to ensure that the polymerized gel sticks to the plate but not to the tray. Either light or chemical polymerization can be used.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1464

IR ABSORPTION HPLC DETECTOR

The new Du Pont infrared detector for liquid chromatography is equipped with a thermostatted measuring cell. The measuring cell has NaCl or CaF_2 windows. Every suitable wavelength in the range between 2.5 and 14.5 μm can be chosen. A built-in motor provides scanning facilities in the same range. The IR detector has a resolution of 0.12 μm at 5 μm. The measuring cell has an optical path length of 1.0 or 0.2 mm. The detector is said to have a high baseline stability especially at high temperatures, and temperature fluctuations are said to have no effect on the measurements. As examples of applications the analyses of copolymers and glycerides are given.



N-1467

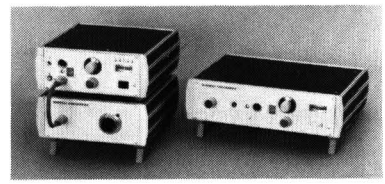
LABORATORY AND PROCESS GAS CHROMATOGRAPHS

In the catalog MP 44 Siemens AG gives a complete description of their laboratory gas chromatography program. The catalog contains not only information on the gas chromatographs L 350 and L 402, but also describes the Siemens detector program and all options available on the Siemens GC instruments. In a brochure the Siemens process gas chromatograph P 101 is fully described. This instrument for use in the process industry is equipped with microprocessor electronics which make it easy to handle even for not very skilled operators. The instrument can be operated as a stand alone unit and with several other instruments of the same type, by remote control.

N-1465

MICRO DIFFERENTIAL REFRACTOMETER

Winopal Research have introduced their Lamidur 80 micro differential refractometer. The Lamidur 80 is a differential refractometer working on the light transmission principle, and according to the manufacturer universally due to the possible exchange of the cells being used for various purposes. The cell is made of quartz and divided under 45°. The cell has a dead volume of 8 μl and can resist pressures up to 90 bar.



N-1471

AUTOMATED HPLC SYSTEM

An automated HPLC system was introduced recently by the Schoeffel Instrument Division of Kratos, Inc. The system employs the KLIC 1 Interactive Controller to control all the system elements including the series 250 chromatograph and detector. The KLIC 1 has a 1000-step programmable memory and is capable of controlling gradient, flow, wavelength, and injection programming. Additionally the KLIC 1 can observe and maintain the proper action of external system elements such as fraction collectors, autoinjectors, and pumps. The two-way interactive feedback control permits automatic in-process servicing of malfunctioning components. The control can, for instance, sense a bubble in the pump head and automatically step-up the flowrate to flush it out.

N-1468

DENSITOMETER

The microprocessor controlled Model CDS-200 clinical densitometer from Beckman Instruments offers three operational modes (transmittance, reflectance and fluorescence) to scan and evaluate electrophoresis and other separation methods. The instrument scans opaque media such as paper, TLC plates and uncleared cellulose acetate membranes. The built-in microcomputer controls the scanning, the computation of the results and the printing for data for up to 65 fractions. Unwanted fractions can be eliminated from the calculations by touching a button. Automatic and manual zero adjust, peak height setting and fraction selection enable the operator to choose the optimal method of determining the appropriate chart section.

N-1469

SWEEP CO-DISTILLER

A technical brochure describes the Kontes Sweep Co-Distiller which is designed for rapid cleanup of samples in pesticide residue, fat and oil, and other heterogeneous material prior to analysis. Samples of 2 grams or less with residue levels as low as 0.025 ppm are said to be cleaned up easily in about 20 minutes. The brochure contains information on run time, capacity, preparation and operation.

CHEMICALS

N-1458

MERCK CATALOG

From E. Merck is available the new 1980 general catalog. The book gives extended information on the Merck products for many different purposes. The yellow pages numbered 35 to 52 contain information on the Merck products for all types of chromatography and amino acid analysis.

N-1461

TRIGLYCERIDE ANALYSIS BY HPLC

The Supelcosil®-HPLC columns are reversed-phase columns. Eluted with non-aqueous mobile phase these columns make it possible to analyze many materials previously difficult to separate. These columns are said to be suited, for example, for the analysis of extremely complex triglyceride mixtures, without the use of argentation chromatography.

N-1462

HYPERSPHERES

Shandon have introduced two new Hyperspheres column packings. The first, Cyano Propyl Silyl (CPS) Hypersil has been developed for normal-phase gas chromatography of polar compounds, and operates with an aqueous or a non-aqueous mobile phase. In normal phase it complements the use of Shandon's APS Hypersil and silica for applications such as carbohydrates, alcohols, dyes and vitamins. In reversed phase CPS Hypersil is complementary to other reversed-phase Hyperspheres and is also applicable to ion-pair chromatography.

MOS (di Methyl Octyl Silyl) Hypersil is a C8 reversed-phase bonded material optimised for the separation of compounds of moderate polarity.

N-1473

PURE WATER

Traces of organic materials in water can disturb HPLC analyses. For the purification of water to overcome these problems Hydro Service & Supplies, Inc., introduces a purification column for the polishing of distilled or deionized water to produce water that is virtually free of organic substances. The UV absorbance of the purified water is typically less than 0.005 units at 0.02 a.u.f.s. sensitivity and 254 nm when analyzed by trace enrichment methods.

PROCEDURES

N-1459

KONTES QUANT NOTES, VOL. 5, NO. 2

Kontes Quant Notes is a periodical review of thin-layer chromatography published by Kontes. The editor is Prof. Joseph Sherma from Lafayette College, Easton, PA, U.S.A. The first article by Dr. Felton from Analtech, Inc. is on reversed-phase TLC. Following are the abstracts of 16 articles on applications of TLC and HPTLC especially in the biochemical and clinical fields. The issue also gives a summary of TLC symposia to be held in the first 6 months of 1980.

NEW BOOKS

Solving problems in analytical chemistry, by S. Brewer, Wiley, Chichester, New York, 1980, *ca.* 512 pp., price *ca.* US$ 11.90, £ 5.45, ISBN 0-471-04098-3.

Experimental organic chemistry, by M.P. Doyle and W.S. Mungall, Wiley, Chichester, New York, 1980, *ca.* 352 pp., price *ca.* US$ 19.15, £ 9.60, ISBN 0-471-03383-9.

The organic chemistry of drug synthesis, Vol. 2, by D. Lednicer and L.A. Mitscher, Wiley, Chichester, New York, 1980, *ca.* 350 pp., price *ca.* US$ 26.60, £ 12.20, ISBN 0-471-04392-3.

Treatise on analytical chemistry, Vol. 16, Functional groups, Part 2, Analytical chemistry of inorganic and organic compounds, edited by I.M. Kolthoff and P.J. Elving, Wiley, Chichester, New York, 1980, *ca.* 576 pp., price *ca.* US$ 56.50, £ 25.90, ISBN 0-471-05857-2.

Experimental methods in polymer chemistry: Physical principles and applications, by J.F. Rabek, Wiley, Chichester, New York, 1980, *ca.* 912 pp., price *ca.* US$ 110.00, £ 40.00, ISBN 0-471-27604-9.

Electrofocus '78 – A presentation of papers on electrofocusing, edited by H. Haglund, J.G. Westerfield and J.T. Ball, Jr., Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, 200 pp., price Dfl. 61.50, US$ 30.00, ISBN 0-444-00375-4.

Atmospheric pollution 1980 (Proc. 14th Int. Colloq., Paris, May 5–8, 1980), edited by M.M. Benarie, Elsevier, Amsterdam, Oxford, New York, 1980, XVI + 434 pp., price Dfl. 150.00, US$ 73.25, ISBN 0-444-41889-X.

Trace chemistry of aqueous solutions – General chemistry and radiochemistry, by P. Benes and V. Majer, Elsevier, Amsterdam, Oxford, New York, 1980, 252 pp., price Dfl. 115.00, US$ 56.00, ISBN 0-444-99798-9.

MEETING

4th INTERNATIONAL SYMPOSIUM ON AFFINITY CHROMATOGRAPHY AND RELATED TECHNIQUES

The 4th International Symposium on Affinity Chromatography and Related Techniques – Theoretical Aspects, Industrial and Biomedical Applications will be held from June 22–26, 1981 in the University of Nijmegen, The Netherlands. The scope of the meeting will cover the following topics:

Theoretical Aspects – Ligand/ligate interaction in homogeneous and heterogeneous systems. General theory of electrostatic, hydrophobic and charge-transfer interaction. Theoretical analyses of affinity separations. Matrix structure. Column/batch procedures.

Polymeric Matrices and Ligand Immobilization – Natural and synthetic polymers. Immobilization of ligand molecules.

Applications – Isolation and purification.

The proceedings of the symposium will be published by Elsevier Scientific Publishing Company in the Analytical Chemistry Symposia Series.

Plenary lectures will be presented by invited speakers. Participants wishing to present a paper and/or poster should address themselves for detailed information to the organizing committee at the following address: Secretariat, Department of Organic Chemistry/Faculty of Sciences, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands.

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PUBLICATION SCHEDULE FOR 1980

ournal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical \pplications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2 195/3	196/1 196/2 196/3	197/1 197/2 198/1	The publication schedule for further issues will be published later.		
Chromatographic Reviews			184/1	184/2					184/3				
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1	183/2	183/3			

INFORMATION FOR AUTHORS

Detailed *Instructions to Authors* were published in Vol. 193, No. 3, pp. 529–532. A free reprint can be obtained ıy application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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