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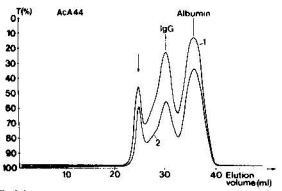
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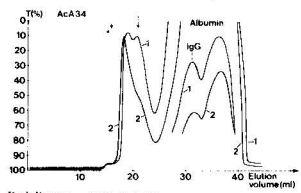
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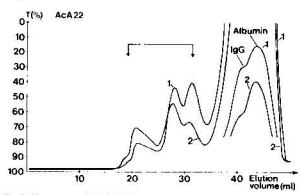
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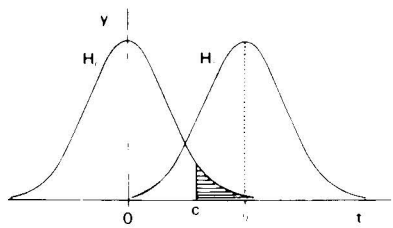
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Physical Sciences Data, Vol. 2

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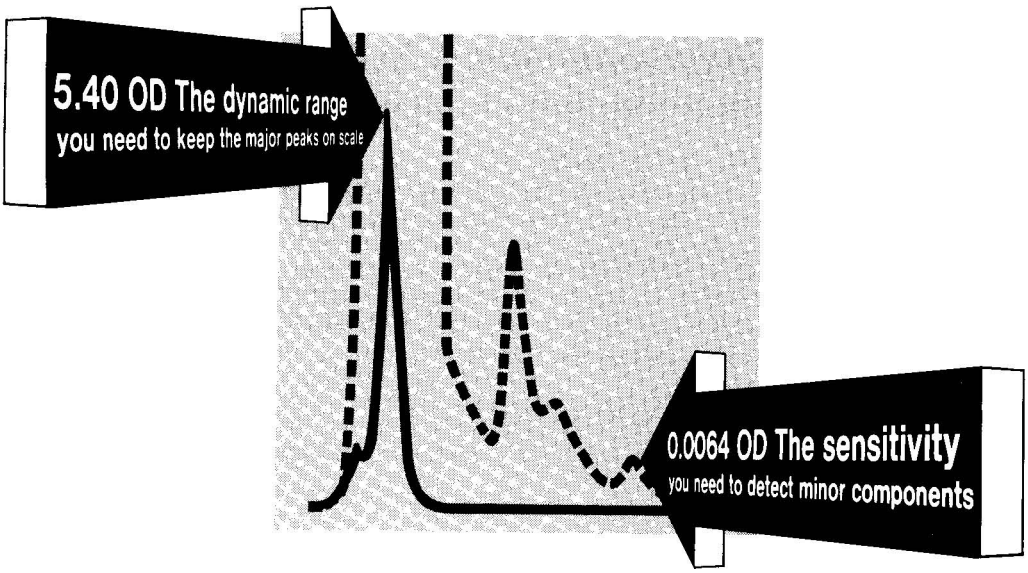
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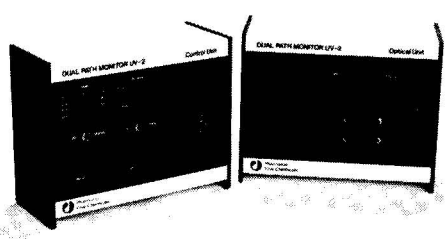
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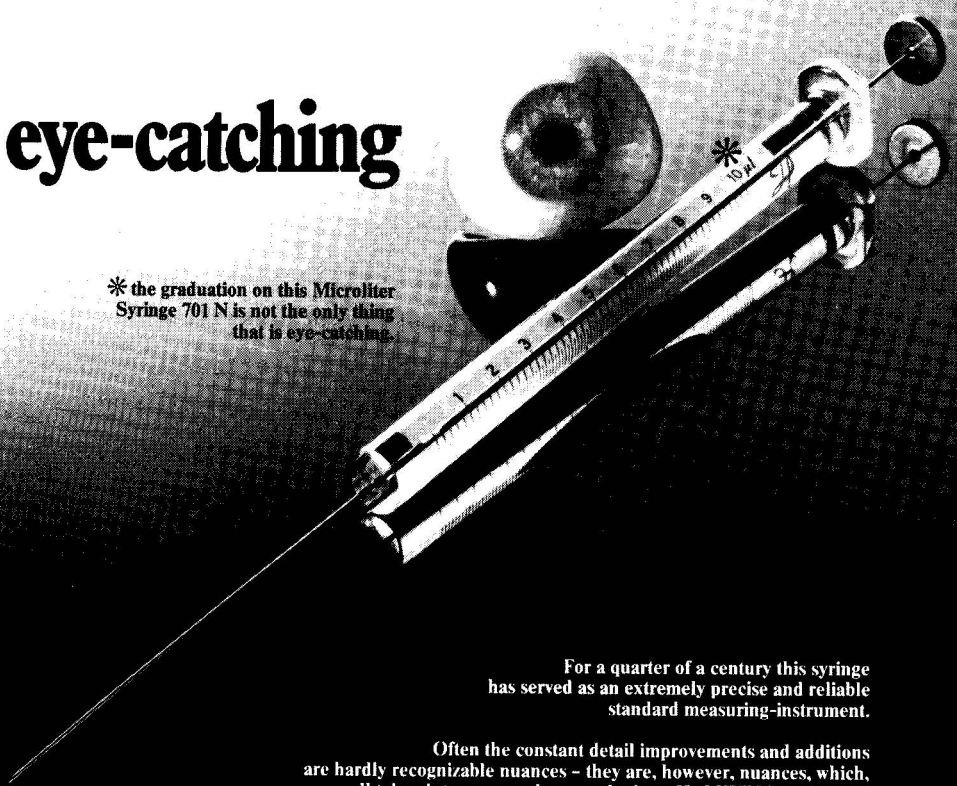
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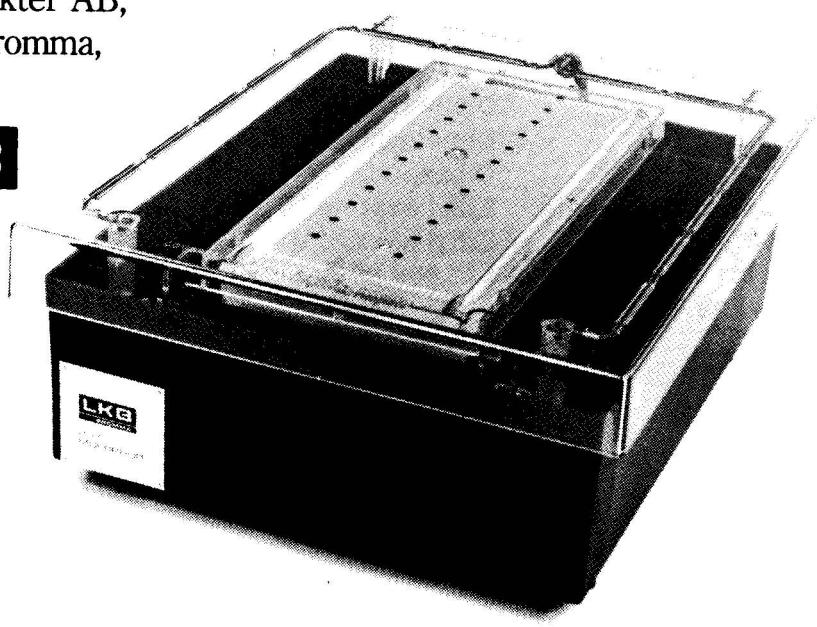
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DEPENDENCE OF THE CAPACITY RATIO ON THE COMPOSITION OF THE BINARY MOBILE PHASE IN LIQUID–SOLID ADSORPTION CHROMATOGRAPHY

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SUMMARY

The dependence of the capacity ratio on the composition of the binary mobile phase is discussed in terms of Snyder's treatment. The relationships obtained for the capacity ratio have been tested on experimental data. The derivation of the relationship for the capacity ratio discussed by Jandera and Churacek has been examined.

INTRODUCTION

Increasing interest in gradient elution chromatography as a rapid method of analysis has made desirable the eluadation of the theoretical basis of the process of liquid–solid adsorption chromatography. The basic problems involved are optimization of the time of analysis and the resolution. One of the main parameters that determines the process of elution is the capacity ratio. Although in isocratic elution at an infinitely low concentration of the sample the capacity ratio remains constant for a given chromatographic system, in gradient elution it is necessary to know the relationship between the capacity ratio, k_{AB}, and the composition of the mobile phase A–B.

It follows from many papers[1–8] that three types of relationships describing the dependence between the capacity ratio and the concentration of the component that is the more efficient eluent in a binary mobile phase can be distinguished:

$$\log k'_{AB} = a + b \log x_B \tag{1a}$$

$$\log k'_{AB} = a + b\, x_B \tag{1b}$$

$$1/k'_{AB} = a + b\, x_B \tag{1c}$$

where x_B is the molar fraction (or concentration) of the more efficient eluent B in the binary mobile phase A–B and a and b are constants. Numerous studies[5,7,9] have

shown that these three relationships can be used to characterize a chromatographic system.

DISCUSSION OF THE JANDERA–CHURACEK DERIVATION OF THE RELATIONSHIP BETWEEN LOG k'_{AB} AND LOG x_B

Jandera and Churacek[7] derived eqns. 1a and 1b; the former was obtained on the basis of Snyder's theory of liquid–solid adsorption chromatography[10]. Their derivation of eqn. 1a requires further comment. According to their derivation, the constants a and b are expressed by $a = \log k'_B$ and $b = -n = -\omega_S/\omega_B$, where k'_B is the capacity ratio for a sample compound S in pure solvent B and ω_S and ω_B are the molecular areas occupied by molecules of sample S and solvent B, respectively.

Let us discuss the constants a and b according to Snyder's treatment[10]. Snyder obtained the following expression:

$$\varepsilon_{AB} = \varepsilon_A + (\alpha\omega)^{-1} \log [x_B \, 10^{-\alpha\omega(\varepsilon_A - \varepsilon_B)} + 1 - x_B] \tag{2}$$

for the strength ε_{AB} of a binary solvent A–B, assuming the following:

(a) an expression for equilibrium constant, K, defining the following reaction:

$$B_{(\text{liquid phase})} + A_{(\text{solid phase})} \rightleftharpoons B_{(\text{solid phase})} + A_{(\text{liquid phase})}$$

$$K = 10^{\alpha\omega(\varepsilon_B - \varepsilon_A)} = \frac{y_B (1 - x_B)}{(1 - y_B) x_B} \tag{3}$$

(b) a relationship between the capacity ratios k'_{AB} and k'_B:

$$\log (k'_{AB}/k'_A) = \alpha \omega (\varepsilon_A - \varepsilon_{AB}) \tag{4a}$$

$$\log (k'_B/k'_A) = \alpha \omega (\varepsilon_A - \varepsilon_B) \tag{4b}$$

(c) additivity of the capacity ratios k'_A and k'_B:

$$k'_{AB} = y_B k'_B + (1 - y_B) k'_A \tag{5}$$

(d) equality of the molecular areas occupied by molecules of adsorbed samples, solvents A and B:

$$\omega_A = \omega_B = \omega_S = \omega \tag{6}$$

In the above equations, k'_A denotes the capacity ratio for a sample compound in pure solvent A; α is the surface activity function; ε_A and ε_B denote the strengths of the solvents A and B, respectively; and y_B is the molar fraction of solvent B in the surface phase. Jandera and Churacek, again introducing eqn. 2 into eqn. 4a but this time omitting Snyder's assumption about the equality of the molecular areas of

the sample compound S and the more efficient eluent B, as well as neglecting the term $1 - x_B$ in eqn. 2, obtained eqn. 1a. A direct consequence of neglecting the term $1 - x_B$ is an irregular behaviour of eqn. 1a in the region of infinitely low concentrations of x_B. In real systems, it would be expected that k'_{AB} will tend towards k'_A at $x_B \to 0$, whereas eqn. 1a gives $k'_{AB} \to \infty$ within this limit. Eqn. 1a also suggests that k_{AB} for any sample component chromatographed does not depend on the properties of the less efficient eluent A in the binary solvent mixture A–B. Hence, it can be concluded that for a less efficient eluent in the binary mobile phase, k'_{AB} will be constant at a given concentration of the more efficient eluent B. However, numerous experimental data show that k'_{AB} at a given concentration x_B is dependent on the properties of all components of the mobile phase. The treatment of Jandera and Churacek resulted in a value of $n \neq 1$, which is due to the incorrect application of eqn. 6. Although the values of n in eqn. 1a may be different from unity, which is often confirmed experimentally, the correct application of Snyder's assumption leads to the value $n = 1$.

DERIVATION OF THE DEPENDENCE OF THE CAPACITY RATIO IN TERMS OF SNYDER'S THEORY

In the light of the above discussion, it seems appropiate to derive a relationship between the capacity ratio, k'_{AB}, and the composition of the binary mobile phase in which the parameters depend on the properties of both the solvents of the mobile phase and the sample being chromatographed. Moreover, this relationship should be correct for the whole range of concentrations x_B. Such a relationship can be obtained by using Snyder's assumptions (a)–(d) above. Combining eqn. 4b with eqn. 3, we obtain

$$k'_A/k'_B = \frac{y_B (1 - x_B)}{(1 - y_B) x_B} \tag{7}$$

Then, elimination of the variable y_B from eqns. 5 and 7 leads to

$$\frac{1}{k'_{AB}} = \frac{1}{k'_A} + \left(\frac{1}{k'_B} - \frac{1}{k'_A}\right) x_B \tag{8}$$

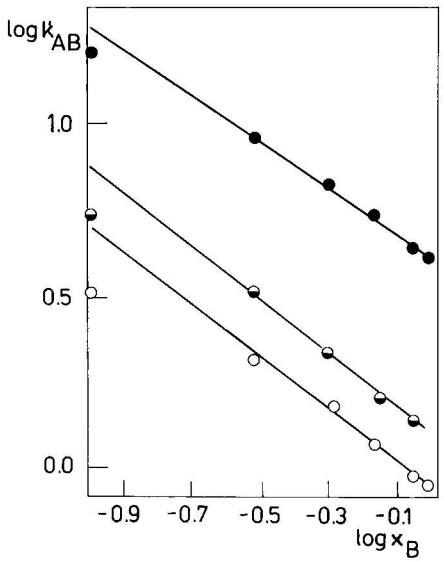
This equation is related to the empirical relationship in eqn. 1c. Of course, eqn. 8, assuming $1 - x_B \ll K x_B$, gives

$$\log k'_{AB} = \log k'_B - \log x_B \tag{9}$$

which corresponds to the special case of the empirical relationship in eqn. 1a, *i.e.*,

$$\log k'_{AB} = a - \log x_B \tag{10}$$

with $-b = n = 1$. It can easily be seen that eqn. 8 predicts correctly the values of k'_{AB} at the limiting concentrations of x_B ($x_B = 0$ and $x_B = 1$) and is a direct consequence of Snyder's theory[10], *i.e.*, this equation is obtained from Snyder's theory



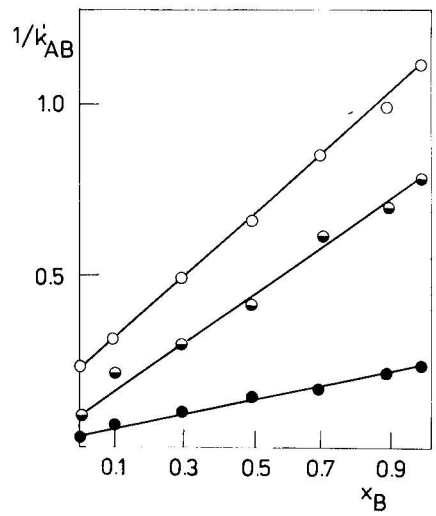


Fig. 1. Linear dependences plotted according to eqn. 1a. (●) A = C_6H_6, B = $C_6H_{12}O_6$, S = 2-amino-3-methylpyridine, stationary phase = alumina; (○) A = CCl_4, B = $CHCl_3$, S = 2,6-dimethylquinoline, stationary phase = alumina; (◒) A = CCl_4, B = $CHCl_3$, S = quinoline, stationary phase = silica gel.

Fig. 2. Linear dependences plotted according to eqn. 8. Symbols as in Fig. 1.

without any further simplifications. Eqn. 8 has also been discussed by Soczewiński[9] and Scott and Kucera[8] by using other theoretical approaches.

In Figs. 1 and 2 the agreement of eqns. 1a and 8 with the experimental data obtained by Ościk and Chojnacka[11] and Rózyło[12] is compared. It can be seen that both equations describe the experimental data at higher values of x_B, but at low concentrations of solvent B eqn. 8 gives considerably better results. These results confirm the theoretical discussion concerning the regions of applicability of eqns. 1a and 8. It is interesting, however, that eqn. 1a agrees very well with the experimental data at higher values of x_B for adsorption systems with $n \neq 1$. This agreement is not covered by the Snyder's treatment based upon assumptions (a)–(d); however, if A and B have very different molecular sizes, changes must be made to assumptions (a) and (d), which leads to a more general relationship for k'_{AB}.

A more generalized form of reaction in (a) can be written as

$$B_{(\text{liquid phase})} + mA_{(\text{solid phase})} \rightleftharpoons B_{(\text{solid phase})} + mA_{(\text{liquid phase})}$$

where $m = \omega_B/\omega_A$. This leads to the following expression for the equilibrium constant:

$$K = \frac{(1 - x_B)^m y_B}{x_B (1 - y_B)^m} \tag{11}$$

when $m \neq 1$, eqn. 8 cannot be solved analytically with respect to y_B, and only numerical solutions are available which are not convenient for gradient optimization.

However, in the special case when y_B approaches unity and K is sufficiently large (a large difference in concentration between the two components of the mobile phase), from eqns. 8 and 5 the following expression can be obtained:

$$\log k'_{AB} = \log \left(k'_A / K^{\frac{1}{m}}\right) - \frac{1}{m} \log x_B \tag{12}$$

for high x_B values. This equation is formally analogous to eqn. 1a where $-1/m = b \neq -1$ (or $n \neq 1$). Thus, in conclusion, it can be stated that on the basis of Snyder's theory, full mathematical justification has been found for eqn. 8, and this theory leads to the approximate eqns. 9 ($b = -1$) and 12 ($b \neq -1$).

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CHROM. 10,948

THEORY OF CHROMATOGRAPHY ON HYDROXYAPATITE COLUMNS WITH SMALL LOADS*

V. DETERMINATION OF THE ADSORPTION ENERGY OF THE ε-AMINO GROUP OF POLY-L-LYSINE AND THE MANNER OF ADSORPTION OF THE MOLECULE

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(First received December 15th, 1977; revised manuscript received February 10th, 1978)

SUMMARY

In an earlier paper, the energy of adsorption of nucleoside phosphates on to a C site of hydroxyapatite was estimated. In this paper, the theory of chromatography developed for rigid molecules in earlier papers is extended to the case of flexible molecules, and the experimental chromatogram obtained by Bernardi for a low-molecular-weight poly-L-lysine is analysed by using a method similar to that applied to nucleoside phosphates. It is estimated that the energy of adsorption on to a P site for an ε-amino group of the side-chain of poly-L-lysine is 2–2.2 kcal/mole. It is probable that the feature of interaction among competing or sodium ions near the crystal surface of hydroxyapatite is different from that which occurs in the usual bulk solution. On the basis of a crystallographic study, it can also be deduced that a P site is constructed with six oxygen ions belonging to three crystal phosphates and that P sites are arranged hexagonally on the $(\vec{a}, \vec{b})$ crystal surface of hydroxyapatite with a minimal distance of 9.42 Å. The manner of the adsorption of poly-L-lysine on an array of P sites is discussed by using space-filling models for both poly-L-lysine and the crystal surface, the latter being constructed on the surface of the plasticine, which is compared with the results obtained from the analysis of the chromatographic data. The chromatographic data for lysozyme and cytochrome *c* discussed in an earlier paper are again discussed on the basis of several parameters obtained in this paper.

INTRODUCTION

Bernardi[1,2] and Bernardi and Kawasaki[3] concluded that the principal mechanisms of the chromatography of nucleoside phosphates (or nucleic acids) and acidic

* We have omitted the term "rigid molecules" from the title, as the theory has now been extended to the case of molecules with any flexible structures.

polypeptides must be the specific competition of the phosphate and carboxyl groups of these molecules with phosphate ions of the eluent for common adsorbing sites (called C sites[4–7]) of hydroxyapatite (HA)*. In fact, the elution of these acidic molecules is carried out only by phosphate ions and is hardly influenced by cations or chloride ions in the buffer[1–3]. Bernardi and co-workers[8,9] also concluded, on the basis of the chromatographic data for a number of synthetic polypeptides and proteins with different isoelectric points, that there is a second type of adsorbing site on the surface of HA. These sites (called P sites[4–6]) must adsorb the basic groups of basic polypeptides and basic proteins, and the elution of basic molecules from the HA columns must be due to specific competition of these molecules with cations of the buffer which are also adsorbed on to P sites, because the elution of basic molecules seems to be carried out only by cations (see refs. 8 and 9 and Appendix I in ref. 10)**.

The fact that the elution of acidic and basic molecules is hardly influenced by cations and anions, respectively, of the same buffer (see above) suggests that C and P sites exist in different domains on the crystal surface. The fact that the adsorption capacity of HA for basic proteins is much larger than that for acidic DNA (see Appendix I in ref. 4) also suggests that C and P site domains are separated and that the area of the P site domain is much larger than that of the C site domain. HA crystals prepared for our chromatography have blade-like shapes (see Appendix II in ref. 10)***. When a typical basic protein, cytochrome *c*, is adsorbed on HA, the flat surfaces of the crystals, which represent the largest part of the total crystal surfaces, are stained; this indicates that the adsorption of cytochrome *c* and other basic molecules must occur mainly on flat crystal surfaces, and the adsorption of acidic molecules on side faces that occupy only a small proportion of the total crystal surfaces (see Appendix II in ref. 10 and Appendix I in ref. 4). On the other hand, from the measurement of the angles made by two different side faces of the crystals, it is highly probable that $(\vec{a}, \vec{b})$ planes of unit crystal cells[11–13] appear on flat crystal surfaces and that both $(\vec{a}, \vec{c})$ and $(\vec{b}, \vec{c})$ planes[11–13] appear on some of the side faces (see Appendix II in ref. 10).

Taves and Reedy[14] showed, on the basis of a crystallographic study, that both phosphate ions and phosphate groups of the polyphosphate ion (as a part of the

* To be precise, it should be stated that the mechanism of chromatography is a competition between acid molecules themselves and phosphate ions of the eluent, because the possibility that some crystal sites under an adsorbed molecule are covered by the molecule but are not reacting with phosphate or carboxyl groups of the molecule should be taken into account. The adsorption of phosphate ions of the eluent on to some crystal sites under the adsorbed molecule may be blocked owing to the presence of the molecule, even if they are not occupied by or reacting with acidic groups of the molecule. This is the reason why the value of the parameter ξ can be less than unity (see later).

** It is difficult to prove directly that anions do not interfere in the elution of basic polypeptide, as it is impossible to perform the experiment by eliminating the anions from the system. However, this is highly probable from the experiment with proteins with different isoelectric points (see Appendix I in ref. 10 and refs. 8 and 9). In contrast to the case with basic molecules, the proof that the elution of acidic molecules is carried out only by phosphate ions of the buffer is much easier and clearer, because chloride ions, although anions, do not interfere in the elution of acidic molecules; it can be shown that elution phosphate molarities of acidic molecules are hardly influenced by the coexistence of KCl and NaCl, except under some extreme experimental conditions[1–3].

*** All chromatographic experiments cited in this paper were carried out by using HA crystals prepared by the method of Bernardi[2], microphotographs of which are shown in Appendix II in ref. 10.

nucleoside phosphate) must be adsorbed on hydroxyl positions devoid of hydroxyl ions existing on the surface of HA. It is assumed that these positions correspond to chromatographic C sites (see above). In Parts I–III of this series[4–6] a theory of chromatography on HA columns with small loads was developed on the basis of the competition model (see above), and in Part IV[7] the experimental chromatogram obtained by Bernardi[15] for a mixture of AMP, ADP, ATP and adenosine tetraphosphate was analysed by using the theory developed earlier[4–6] and on the basis of the model of Taves and Reedy[14] for the adsorption of both phosphate and polyphosphate ions on the crystal surface (see above). By means of this analysis, it was estimated that the adsorption energy on to a C site for a univalent phosphate group on the polyphosphate chain of nucleoside phosphates is 0.9–1 kcal/mole, and that the adenosine group of these molecules covers at most only one crystal site; arguing in the other direction, these reasonable conclusions strongly support the idea of the one-to-one correspondence between a hydroxyl position and a chromatographic C site, and the model of Taves and Reedy[7]. Further, this idea is compatible with the conclusion obtained from the adsorption experiment that acidic molecules must be adsorbed on side faces of the HA crystal that correspond to both $(\vec{a}, \vec{c})$ and $(\vec{b}, \vec{c})$ planes (see above), because, according to the Taves and Reedy[14] model, hydroxyl positions devoid of hydroxyl ions must appear on crystal surfaces corresponding to these planes.

In this work, a chromatogram obtained by Bernardi[16] for a low-molecular-weight poly-L-lysine (see Fig. 1) has been analysed with a method similar to that applied in Part IV[7] for the analysis of the chromatogram of the nucleoside phosphates mixture. It is shown that the theory developed in earlier papers[4–7] for molecules with rigid structures is valid for molecules with any flexible structure only if another physical interpretation is given to the parameter σ. It is estimated that the adsorption energy on to a P site for an ε-amino group on the side-chain of poly-L-lysine is 2–2.2 kcal/mole. The dependence of the activity of competing or sodium ions on the molarity is also discussed; this is different from that observed for the sodium salt in the usual aqueous solution, and it can be suggested that the feature of interactions among sodium ions near the HA surface is different from that which occurs in the usual solution. It can be considered that poly-L-lysine molecules have highly stretched conformations under the experimental conditions (see Appendix I). In Appendix I, the adsorption of poly-L-lysine on the HA surface is discussed. In Appendix II, the HA surface structure, parallel to $(\vec{a}, \vec{b})$ planes of unit cells (see above), is explored on the basis of crystallographic data. It is probable that a P site is constructed with six oxygen ions (three O_{II} and three O_{III} ions[11–13]) belonging to three crystal phosphates and that P sites are arranged hexagonally on the crystal surface [corresponding to $(\vec{a}, \vec{b})$ planes] with a minimum distance of 9.42 Å. In Appendix III, the configuration of poly-L-lysine adsorbed on an array of P sites is again discussed by using space-filling molecular models for both poly-L-lysine and the crystal surface, the latter being constructed on the surface of plasticine. In Appendix IV, the chromatographic data for lysozyme and cytochrome *c* discussed earlier[4] are again discussed on the basis of several results presented in this paper.

THEORETICAL

(A) Extension of the theory of chromatography for rigid molecules to the general case of flexible molecules

The theory of chromatography for rigid molecules developed in earlier papers[4–7] can be extended to the general case of flexible molecules. According to the previous theory[4–7], the ratio, B, of the number of molecules in the mobile phase to the total number of molecules in a column section (which is the fundamental factor determining the position of a chromatographic peak) depends upon the factor $\beta\sigma$ (see eqns. 1 and 2 in ref. 4*) where β is a parameter concerning the properties of the column assuming the activity coefficient for the sample molecule in the column interstices is constant. This is a reasonable assumption, as the concentration of the sample molecule in the column interstices is usually very low, which means that the value of the activity coefficient is virtually unity. Eqn. A1 in Appendix I in ref. 4 shows that β is proportional to the coordination number, z, of adsorption sites on the crystal surface of HA; the parameter B therefore depends on the product of σ and z. Provided that the molecule is rigid and that it is adsorbed in the energetically most stable manner on the surface of HA, the product σz would represent the number of possible orientations plus configurations of the molecule on the surface of HA. Therefore, if there is only one energetically most stable configuration, σ is the symmetry factor of the molecule, the value of which can lie between $1/z$ and 1, and is unity for the usual asymmetric molecule. If there are n energetically most stable configurations of the molecule, the value of σ should be n times as large. It can also be considered that the factor $-x\varepsilon - kT\ln(z\sigma)$, the value of which is different from that of the factor $-\ln q$ in eqn. 1 in ref. 4 by a constant value, and where $x\varepsilon$** represents the adsorption energy per molecule, represents a type of free energy of adsorption per molecule. In general, the configuration of the molecule on the crystal surface would follow a Boltzmann distribution and the molecule would be adsorbed virtually in the energetically most stable configuration only if the value of the adsorption energy, ε, per adsorption group of the molecule is large enough. Therefore, the general physical meaning of the factor $z\sigma$ for the rigid molecule would be the approximate number of possible orientations plus configurations of the molecule on the crystal surface, provided that the value of the factor $-x\varepsilon - kT\ln(z\sigma)$ is close to the minimum. More precisely, values of both σ and $-x\varepsilon - kT\ln(z\sigma)$ must be relevant to the water structure near the crystal surface. It is easy to understand that the theory developed in earlier papers[4–7] can be extended to the general case of flexible molecules if, in this instance, a physical meaning of the approximate number of the possible orientations and configurations plus possible stereochemical conformations of the molecule is assigned to the parameter $z\sigma$; as above, the factor $-x\varepsilon - kT\ln(z\sigma)$ should be close to the minimum.

(B) Interpretation of the experimental chromatogram of poly-L-lysine

It can be seen in Fig. 1 that the chromatogram of a low-molecular-weight poly-L-lysine hydrobromide (for details of the molecule, see the legend of Fig. 1)

* In some instances in ref. 4, the symbol β_3 was used instead of β.

** In ref. 4, the symbol ε_3 was used instead of ε.

contains a sharp peak (peak 1) that appears before the molarity gradient of sodium ions begins and a series of peaks (peaks 2–9) that are eluted at different sodium molarities. The intervals between the latter peaks are roughly constant, and the heights of peaks decrease gradually on the right-hand side of the chromatogram. On the left-hand side, they begin to decrease but stop abruptly.

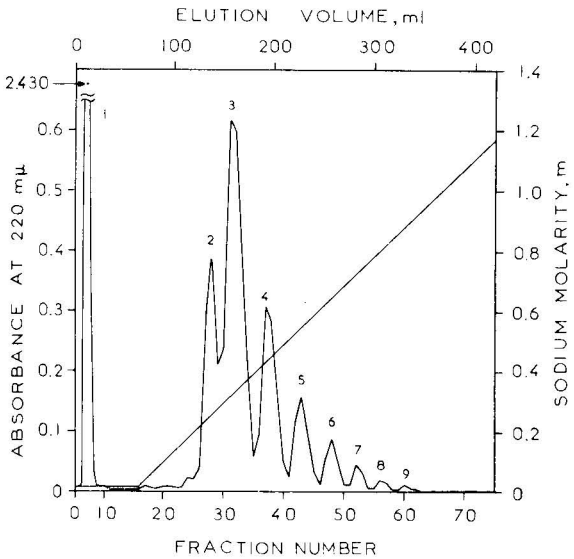


Fig. 1. Chromatography, at room temperature, of a poly-L-lysine·HBr sample [Miles-Yeda, code No. 8120A (new code No. 71-120A) lot No. LY125] with molecular weights between 1500 and 8000, *i.e.*, with degrees of polymerization of 7–38. Fifty-nine A_{220} units, or 20 mg as estimated by using Fig. 10.1 in ref. 17, of the sample in 4 ml of 0.01 *M* sodium phosphate buffer of pH 6.8 (NaP) were loaded at zero volume on a 22 × 1 cm HA column. After rinsing the column with 30 ml of 0.01 *M* NaP, elution was carried out with a linear molarity gradient of NaP constituted by three 150-ml solutions of 0.01, 0.5 and 1 *M* NaP. The yields were 23% and 50%, respectively, for peak 1, which is not retained on the column, and for the other part of the chromatogram including peaks 2–9 that elutes after the molarity gradient begins, with an overall recovery of 73%. The number of molecules involved in the latter part of the chromatogram, which can be estimated to be equivalent to 10 mg (see above), would be small enough (for a 22 × 1 cm column) for the effect of mutual interactions among molecules adsorbed on HA or the mutual displacement of molecules[18] to be virtually negligible (*cf.*, Fig. 3 in ref. 19, from which it can be estimated that the maximum possible displacement of the elution molarity is of the order of 0.02 *M*). Some other information involved in the figure is given in Table I. [This figure was reproduced with slight modifications from Fig. 5a in ref. 16, where it is mentioned that potassium buffer was used (instead of sodium buffer) and that the maximum height of peak 1 is 2.067 (instead of 2.430), which are misprints. Some information not given in ref. 16 but only in this paper was obtained directly from the original data with the permission of Prof. G. Bernardi].

It can be shown theoretically that, with sufficiently small molecules, the elution molarity is governed mainly by the parameter x rather than by the parameter $\xi\,(= x/x')$*, where x is the number of adsorption groups per molecule that react with sites of HA and x' is the number of sites of HA where competing ions cannot be adsorbed owing to the presence of an adsorbed molecule; this means that peaks

* With large molecules, the elution molarity is governed by the parameter ξ.

that appear at high sodium molarities involve molecules with high x values or high molecular weights. As it can be considered that poly-L-lysine is adsorbed on P crystal sites by using ε-amino residues of side-chains (for details, see Appendix I and Discussion), the discrete distribution of peaks (Fig. 1) must be a reflection of a stepwise variation in numbers of ε-amino groups per molecule that react with P sites. It can also be assumed that the gradual decrease in the heights of the peaks on the right-hand side of the chromatogram (as in a binominal distribution) reflects a statistical property concerning the distribution of the polypeptide chain lengths of the poly-L-lysine sample. A similar consideration was made in earlier papers[10,20] for the interpretation of the chromatogram of tropocollagen. The reason why the distribution of the peaks that appear in the sodium gradient stops abruptly on the left-hand side (see Fig. 1) can be understood, for poly-L-lysine, by assuming that for small components the total energy per molecule of the interaction with P sites is too small to be retained on the column, and that these molecules are involved in peak 1, which appears before the sodium gradient begins (see Fig. 1). The possibility that the asymmetry of the distribution of peaks 2–9 is due to mutual interactions among macromolecules on the crystal surface, as considered in earlier papers (*e.g.*, refs. 10, 18, 20 and 21) can almost be excluded, as the load of the sample is small (see the legend of Fig. 1).

Now, the fact that the width of peak 1 is very narrow (see Fig. 1) indicates that the value of x for molecules involved in this peak is almost constant. The situation is the same as for peaks 2–9 (see above). The fact that the height or the area of peak 1 is (much) larger than that of peak 2, and that the total chromatogram including peak 1 has two maxima (see Fig. 1), suggests that the value of x for molecules involved in peak 1 is the minimum possible value that can be realized, because the statistical distribution must have only one maximum unless there is a reason for the distribution to be limited*. It follows from this that the number, x, of ε-amino groups per molecule that react with P sites must be (approximately; see Discussion) 1, 2, ..., 9 for molecules involved in peaks 1, 2, ..., 9, respectively, because the minimum possible value of x is (approximately; see Discussion) unity. Further, if the value of x for molecules involved in peak 1 is equal to or greater than about 2, it is difficult to explain why only peak 1 elutes before the sodium gradient begins; in order for this to occur, the ratio of the adsorption energy (or the value of x) for a molecule involved in peak 2 to the energy (or the value of x) for a molecule in peak 1 must be much greater than the ratios of the energies (or values of x) between molecules involved in the other pairs of successive peaks (for details of the argument for values of x, see Discussion).

Finally, it is reasonable to assume that all molecules of poly-L-lysine have almost identical values of the parameter ξ; this means that the value of x or the adsorption energy per molecule increases linearly with the increase in the value of x' or the dimensions of the molecule.

* It can be shown that the fraction number that corresponds to the centre of gravity of the total chromatogram including peak 1, *i.e.*, the average fraction number between 30 and 31, is almost identical with the fraction number that corresponds to the maximum height of the chromatogram eluting in the sodium gradient, *i.e.*, 30; this also suggests that the poly-L-lysine sample is a statistical assembly of components with different chain lengths and that components with minimum possible values of x are involved in peak 1.

(C) Methods for the estimation of the value of the adsorption energy for an ε-amino group of poly-L-lysine and other experimental parameters

In Part IV[7], the value of the adsorption energy on to a C crystal site for a univalent phosphate group on the polyphosphate chain of nucleoside phosphates, the value of the proportionality constant, φ', between the parameter Λ (see ref. 7) and the molarity, m, relating to phosphate ions of the eluent etc. were estimated from the chromatogram for a mixture of AMP, ADP, ATP and adenosine tetraphosphate (Fig. 3 in ref. 7). We can find the following parallels between the chromatography of nucleoside phosphates (Fig. 3 in ref. 7) and that of poly-L-lysine (Fig. 1): firstly, whereas with nucleoside phosphates there was a difference of unity in numbers of univalent phosphate groups adsorbed on C sites between molecules involved in successive chromatographic peaks (*i.e.*, between AMP and ADP, between ADP and ATP, and between ATP and adenosine tetraphosphate; see Table I in ref. 7), with poly-L-lysine there is also a difference of unity (not always precisely unity; see Discussion) in the numbers of ε-amino groups adsorbed on P sites between molecules involved in successive peaks in Fig. 1 (see Section *B*). Secondly, whereas with nucleoside phosphates there was a difference of unity in numbers of C sites on which phosphate ions cannot be adsorbed owing to the presence of an adsorbed molecule between nucleoside phosphates involved in successive peaks (see Table I in ref. 7), with poly-L-lysine there is a difference of almost $1/\xi$ in the numbers of P sites on which sodium ions cannot be adsorbed owing to the presence of a molecule between components involved in successive peaks, because any molecules of poly-L-lysine must have almost identical values of the parameter ξ (see Section *B*). From these parallels it can be understood that the value of the adsorption energy, ε, for an ε-amino group of poly-L-lysine on to a P crystal site, the value of the parameter φ' for sodium ions, etc., can be estimated from Fig. 1 if the value of the parameter ξ is given, and if the parameter Λ and the molarity m relating to sodium ions in the eluent are proportional to each other.

The assumption of the proportionality between Λ and m (see above) is unreasonable, however, with poly-L-lysine, because molecules elute over a range of high sodium molarities (0.25–0.9 *M*) (see Fig. 1), in contrast to the case with nucleoside phosphates, which elute at phosphate molarities lower than 0.3 *M* (see Fig. 3 in ref. 7). Nevertheless, it is possible to calculate apparent values of the parameters ε, φ', etc., from any pair of successive peaks in Fig. 1* if the parameter φ' in the term $\varphi' m_{in}$ in eqn. 1 in ref. 7 is replaced with the ratio, φ'_{in}, of Λ to m when m is equal to its initial value of m_{in} (= 0.015 *M*) (see Table I; for the method of evaluation of the parameter φ'_{in}, see below). As Λ and m must be proportional to each other when m is close to zero, if both parameters φ'_{in} and ξ are given, and if apparent values of ε, φ', etc., obtained from different pairs of peaks that appear at different (average) sodium molarities are extrapolated to zero molarity, then the true value of the parameter ε, the value of the parameter φ' at $m = 0$ (called φ), etc., can be obtained.

Now, provided that only the value of ξ is given, the value of φ'_{in} can be estimated as follows. For a hypothetical value of φ'_{in}, apparent values of φ' are cal-

* With nucleoside phosphates, values of the corresponding parameters were calculated from the pair of chromatographic peaks of ATP and adenosine tetraphosphate[7].

TABLE I

DATA INVOLVED IN FIG. 1.

Parameter	*Symbol**	*Value*	*Notes***
Column length	L	22 cm	
Column diameter	$\varnothing$	1 cm	
Slope of sodium molarity gradient (molarity per elution volume)	grad	0.0033 M/ml	1
Interstitial volume per unit length of column	v	0.628 ml/cm	2
Total interstitial volume of column	V_T	13.816 ml	
Slope of sodium molarity gradient (molarity per column length)	g	0.00207 M/cm	2
Product of g and L	s	0.0456 M	
Molarity of sodium ions before the gradient begins	m_{in}	0.015 M	3
Volume of solvent eluted from the sample load until the gradient begins	V'	66 ml	
Temperature	T	Room temperature	4
Elution volume at the maximum height of the peak 1		12 ml	
Elution sodium molarity at the maximum height			
of peak 2		0.24_8 M	
of peak 3		0.30_8 M	
of peak 4		0.42_5 M	
of peak 5		0.54_5 M	
of peak 6		0.64_2 M	
of peak 7		0.72_0 M	
of peak 8		0.80_0 M	
of peak 9		0.87_7 M	

* Some of the symbols do not appear in the text, but they are shown here in order to facilitate comparison of this paper with Part IV[7].

** Notes: (1) Note that the gradient is not expressed in terms of the phosphate molarity but of the sodium molarity, because poly-L-lysine competes with sodium ions of the buffer (see text). (2) See Table II in ref. 7. (3) This is the approximate value in elution volumes immediately before the sodium gradient begins; *cf.*, ref. 7. (4) If necessary for the calculation, this is assumed to be 25 °C.

culated from different pairs of peaks appearing at different sodium molarities. These values of φ' are extrapolated to $m = m_{in}$. The extrapolated value of φ', $\varphi'_{(m=m_{in})}$, must, in general, be different from the initially assumed value of φ'_{in}. Calculations are repeated until the value of φ'_{in} that produces a value of $\varphi'_{(m=m_{in})}$ identical with itself is found, which must be the true value of φ'_{in}. Actually, the value of φ'_{in} is virtually equal to the value of φ (see above), because the value of m_{in} (0.015 M) is close to zero (see Results of Calculations).

As values of ε, φ'_{in} ($\approx \varphi$), etc., were calculated when the value of ξ was given, it is possible to determine the empirical formula of the function $\varphi'(m)$ (which fulfils the relationship $\varphi' = \varphi'_{in}$ when $m = m_{in}$ for any given value of ξ) in such a way that the positions of peaks 2–9 of poly-L-lysine calculated by using this function would be equal to those obtained experimentally. It will be shown that, for any reasonable value of ξ, Λ is proportional to m (*i.e.*, φ' is constant) when m is small enough.

Now, we must estimate the value of ξ. It can be considered that the value of $\varphi' e^{-\varepsilon'/kT}$, where ε' is the adsorption energy of a competing ion on to a crystal

site, is independent of the type of competing ions when the molarity, m, of the ions is low enough, because $\varphi' e^{-\varepsilon'/kT}$ is proportional to the activity coefficient of the ions*. Both the (average) value of φ', when m is small, for phosphate ions of the buffer and the value of the adsorption energy for a univalent phosphate group on the polyphosphate chain of nucleoside phosphates are known (see above). In order to estimate the value of ξ, the following assumptions must be made: (1) the adsorption energy for a univalent phosphate ion of the buffer (the phosphate buffer at pH 6.8 used in the experiment is an equimolar mixture of univalent and divalent phosphate ions) is equal to the energy for a univalent phosphate group on the polyphosphate chain of the nucleoside phosphate; (2) the adsorption energy for a divalent phosphate ion of the buffer is twice as large as the energy for a univalent ion; (3) the proportion of univalent and divalent ions adsorbed on the crystal surface follows the Boltzmann distribution; (4) the (average) value of $\varphi' e^{-\varepsilon'/kT}$ calculated for phosphate ions on the basis of assumptions (1)–(3) is virtually equal to the limiting value when m tends to zero; and (5) the adsorption energy for a sodium ion is virtually equal to the energy for an ε-amino group of poly-L-lysine (because both the sodium ion and the ε-amino group have a univalent charge). The value of the parameter ξ can now be estimated in such a way that the value of $\varphi e^{-\varepsilon'/kT}$ [*i.e.*, $e^{-\varepsilon'/kT} \lim_{m \to 0} \varphi'(m)$] for sodium ions obtained by using assumption (5) and the hypothetical value of ξ be equal to the (average) value of $\varphi' e^{-\varepsilon'/kT}$ obtained for phosphate ions. Hence, values of parameters ε, φ ($\approx \varphi'_{in}$), etc., and the empirical formula of the function $\varphi'(m)$ can finally be determined. (For details of the method, see Results of Calculations).

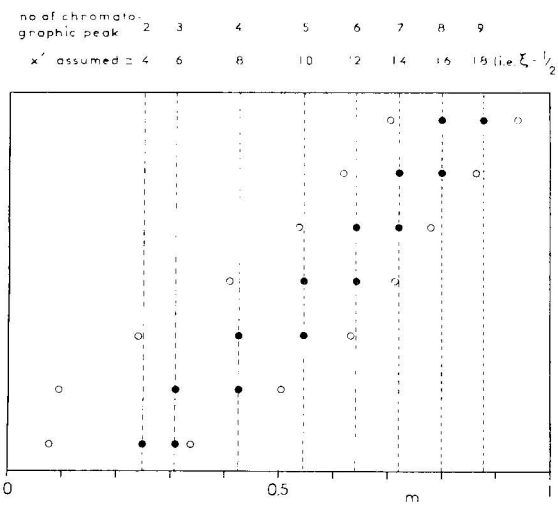
RESULTS OF CALCULATIONS

Fig. 2 illustrates an example of calculations of apparent values of φ', ε, $\beta\sigma e^{\varepsilon_0/kT}$, ε_0 and $\beta\sigma$ (for the definition of ε_0 and methods of calculations of ε_0 and $\beta\sigma$, see below) obtained on the basis of elution molarities of seven different pairs of successive peaks in Fig. 1 (*i.e.*, peaks 2 and 3, peaks 3 and 4, ..., peaks 8 and 9), and by assuming $\xi = 1/2$ and $\varphi'_{in} = 10$. In Fig. 2, the abscissa is the molarity of sodium ions and dotted vertical lines indicate the positions of the maximum heights of peaks 2–9 in Fig. 1. As $\xi = 1/2$ and $\xi = x/x'$, the values of x' for molecules involved in peaks 2–9 must be equal to 4, 6, ..., 18, respectively; pairs of closed circles on different levels indicate pairs of peaks chosen as the basis of calculations of apparent values of the parameters. From a pair of peaks, two apparent values of φ' and two apparent values of ε (or the adsorption energy for an ε-amino group as the difference in the apparent adsorption energies between molecules involved in successive peaks) can be calculated, at the same time with apparent elution molarities for the right- and the left-hand neighbour peaks of the pair chosen as the basis of the calculation, which are shown by open circles in Fig. 2**. In fact, if the value of

* $\varphi' = \Lambda/m$ and $\Lambda = \lambda e^{\varepsilon'/kT}$, where λ is the absolute activity of competing ions. Therefore, we have $\varphi' e^{-\varepsilon'/kT} = \lambda/m$, λ/m being proportional to the activity coefficient[4].

** In ref. 7, φ', ε and $\beta\sigma e^{\varepsilon_0/kT}$ were calculated at the same time only with the elution molarity for the left-hand neighbour peak of the basic pair, *i.e.*, they were calculated at the same time with the theoretical elution molarity for the peak of ADP by choosing peaks of ATP and adenosine tetraphosphate as the basic pair. The fact that these parameters can also be calculated at the same time with the elution molarity for the right-hand neighbour peak is proved in the Appendix in ref. 7.

φ' is independent of the sodium molarity and if values of both ξ and φ'_{in} are correctly assumed, then open circles (see above) must be superposed on the dotted vertical lines, and values of both φ' and ε calculated at the same time with theoretical elution molarities of the right- and the left-hand peaks of the basic pair must be equal to each other; this means that values of both φ' and ε calculated from any basic pairs of peaks must also be equal to one another. In parentheses above and below the other values in the tabular matter to the right side of Fig. 2 are given values of φ' and ε obtained concomitantly with positions of open circles on the right- and the left-hand sides of the pair of closed circles, respectively; the values between those in parentheses are the means of the values of φ' and ε given in parentheses. The fact that the values of both φ' and ε depend not only on whether the molarity for the right- or the left-hand peak is calculated at the same time but also on which pair of the peaks is chosen as the basis of the calculation would indicate that the value of φ' depends on the sodium molarity or/and that the hypothetical value of ξ is incorrect (see above)*. It can be considered, however, that the dependences of apparent values of φ' and ε on the method of the calculation are mainly due to the dependence of the value of φ' on the sodium molarity because, for any reasonable hypothetical values of ξ, values of both φ' and ε depend in similar ways on the method of calculation.



φ'	ε (kcal/mole)	$\beta\sigma e^{\varepsilon_0/kT}$	ε_0 (kcal/mole)	$\beta\sigma$
(0.67)	(0.813)			
0.72	0.855	935.7	5.982	0.0386
(0.76)	(0.896)			
(0.80)	(0.864)			
0.865	0.916	515.8	5.496	0.0483
(0.93)	(0.968)			
(0.98)	(0.898)			
1.07	0.958	282.2	4.791	0.0869
(1.16)	(1.018)			
(1.23)	(1.035)			
1.38	1.122	105.0	4.488	0.0539
(1.53)	(1.209)			
(1.66)	(1.223)			
1.97	1.367	29.96	4.101	0.0295
(2.28)	(1.511)			
(2.63)	(1.376)			
3.56	1.642	9.969	3.283	0.0391
(4.49)	(1.907)			
(5.09)	(1.249)			
6.91	1.508	7.550	1.508	0.5920
(8.82)	(1.767)			

Fig. 2. Example of calculations of apparent values of φ', ε, $\beta\sigma e^{\varepsilon_0/kT}$, ε_0 and $\beta\sigma$ obtained on the basis of the elution molarities of seven different pairs of successive peaks in Fig. 1 (*i.e.*, pairs of peaks 2 and 3, peaks 3 and 4, ..., peaks 8 and 9), and by assuming $\xi = 1/2$ and $\varphi'_{in} = 10$.

On the right of Fig. 2 are tabulated values of $\beta\sigma e^{\varepsilon_0/kT}$ calculated from the seven pairs of chromatographic peaks in Fig. 1 by using a method similar to that in ref. 7, where ε_0 is the apparent adsorption energy for molecules involved in the left-hand neighbour peak of the pair chosen as the basis of the calculation; apparent

* The value of φ'_{in} chosen here is that which should be true provided the hypothetical value of ξ is true (see Theoretical, Section *C*, and below).

values of both ε_0 and $\beta\sigma$ calculated by assuming $\varepsilon_0 = x\varepsilon$* are also tabulated, where ε is the mean value shown between the values in parentheses in the second column. It can be seen from the table in Fig. 2 that the values of all of the parameters φ', ε, $\beta\sigma e^{\varepsilon_0/kT}$ and ε_0 change monotonously from top to bottom in the respective columns except for the value of ε calculated from the pair of peaks 2 and 3. It can also be seen that the value of $\beta\sigma$ is always of the order of 10^{-2} when the basic pairs of peaks for the calculation are chosen from peaks 3–9; it is exceptionally high when the pair of peaks 2 and 3 is chosen as the basis of the calculation, however. It can be considered that this exceptionally high value of $\beta\sigma$ is related to the fact that the interval between peaks 2 and 3 is very narrow or that the elution molarity of peak 2 is too high (for an explanation of the elution molarity of peak 2, see Discussion).

The points in Fig. 3 are logarithms of mean values of φ' shown between the values given in parentheses in the first column of the table in Fig. 2 *versus* mean elution molarities for pairs of peaks used as the basis of the calculations of corresponding φ' values; the value of φ' calculated from peaks 2 and 3 is not plotted, as the position of peak 2 is exceptional (see above). The intermediate straight line in Fig. 3 is the regression line for $\ln\varphi'$ on m obtained by the least-squares method for the points in the same figure; the upper and the lower broken lines are regression lines for corresponding points (not shown) obtained by assuming that $\varphi'_{in} = 14$ and $\varphi'_{in} = 6$.

The three points in Fig. 4 are the exponents of the ordinate values of the three regression lines at $m = m_{in}$ (= 0.015 M) in Fig. 3 [denoted by $\varphi'_{(m=m_{in})}$] *versus*

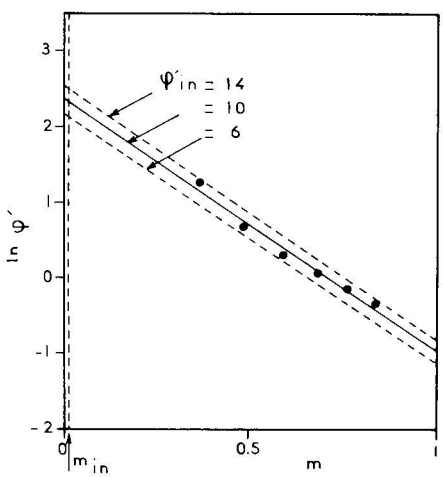


Fig. 3. Points: plots of logarithms of mean values of φ' shown between the upper and lower values in parentheses in the first column of the table in Fig. 2 *versus* mean elution molarities of pairs of peaks used as the basis of calculations of corresponding φ' values. The values of φ' calculated from peaks 2 and 3 are not plotted, as the position of peak 2 is exceptional. The intermediate line is the regression line for these points; the upper and the lower broken lines are regression lines for corresponding points (not shown) obtained by assuming $\varphi'_{in} = 14$ and $\varphi'_{in} = 6$ instead of $\varphi'_{in} = 10$.

* It is also assumed that values of x involved in peaks 2, 3, ..., 9 are exactly equal to 2, 3, ..., 9, respectively. For the validity of this assumption, see Discussion.

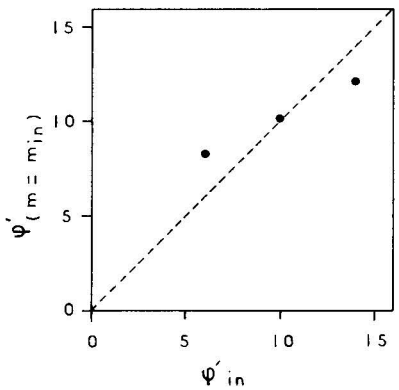


Fig. 4. Points: plots of the exponents of ordinate values of the three regression lines at $m = m_{in}$ ($= 0.015\ M$) in Fig. 3 [denoted by $\varphi'_{(m=m_{in})}$] *versus* the corresponding φ'_{in} values. It can be seen that the value of $\varphi'_{(m=m_{in})}$ coincides with the value of φ'_{in} when $\varphi'_{in} \approx 10$.

the corresponding φ'_{in} values. It can be seen from Fig. 4 that the value of $\varphi'_{(m=m_{in})}$ coincides with that of φ'_{in} when $\varphi'_{in} \approx 10$, which means that the value of φ'_{in} should be 10 if $\xi = 1/2$ (see Theoretical, Section *C*).

The points and the crosses in Fig. 5 are apparent values of ε (mean values shown between the values given in parentheses in Fig. 2) and $\beta\sigma$ in the table in Fig. 2 *versus* the mean elution molarities of pairs of peaks used as the basis of the calculations of ε and $\beta\sigma$, where $\xi = 1/2$ and $\varphi'_{in} = 10$; values of ε and $\beta\sigma$ calculated from the pair of peaks 2 and 3 are not plotted, as in Fig. 3. The oblique straight line in

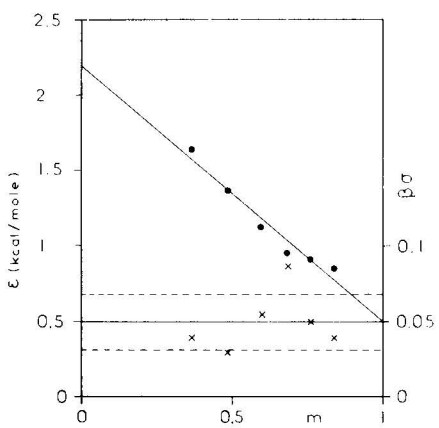


Fig. 5. Symbols: plots of apparent values of (●) ε (mean values shown between the upper and lower values in parentheses) and (×) $\beta\sigma$ in the table in Fig. 2 *versus* mean elution molarities of pairs of chromatographic peaks used as the basis of calculations of corresponding ε and $\beta\sigma$, respectively. Values of ε and $\beta\sigma$ calculated from the pair of chromatographic peaks 2 and 3 are not plotted, as the position of peak 2 is exceptional. The oblique straight line is the regression line for the points, from which it can be estimated that the value of ε extrapolated to $m = 0$ is 2.2 kcal/mole, which must be the true value (provided $\xi = {}^1/_2$); the horizontal continuous and two broken lines indicate the mean value and the range of the standard deviation of the apparent values of $\beta\sigma$, respectively. It is assumed that the mean value is roughly equal to the true value of $\beta\sigma$.

Fig. 5 is the regression line for apparent values of ε on m obtained by the least-squares method for the points in the same figure, from which it can be estimated that the value of ε extrapolated to $m = 0$ is 2.2 kcal/mole, which must be the true value (provided $\xi = 1/2$); the horizontal continuous line and the two broken lines indicate the mean value and the range of the standard deviation of the apparent values of $\beta\sigma$, respectively, and we assume simply that the mean value is about equal to the true value of $\beta\sigma$, which is a rough approximation. However, the considerable random variation of the apparent values of $\beta\sigma$ around the mean value in Fig. 5 suggests that the chromatography does not depend substantially on the value of $\beta\sigma$, which can be verified (see below).

Now, it is possible to calculate theoretical positions of the peaks in Fig. 1 by using values of parameters φ'_{in}, ε and $\beta\sigma$ estimated above with the assumption that $\xi = 1/2$, and also by assuming several empirical equations for the function $\varphi'(m)$, which should fulfil a condition such that $\varphi' = \varphi'_{in}$ when $m = m_{in}$. As the function $\varphi'(m)$ should be related to the activity coefficient of competing ions (see below), a simple assumption for this function may be that

$$\varphi'(m) = \varphi\gamma' \tag{1}$$

where

$$\gamma' = e^{-\alpha R(m)} \tag{2}$$

and α is a constant. If the dependence of φ' on m is essentially due to the dependence of the activity of competing ions on the molarity in the column interstices far enough from HA surfaces for it not to be influenced by HA surfaces, then the parameter γ' in eqn. 1 should be equal to the activity coefficient, γ, for the ions in the usual aqueous solution (see Discussion). The curves in Fig. 6 represent theoretical elution molarities for peaks 2–9 in Fig. 1 as functions of the parameter α in eqn. 2 calculated by assuming 1/2 for the value of ξ and three different equations for the function $R(m)$:

$$R(m) = \sqrt{m} \tag{3a}$$

$$R(m) = m \tag{3b}$$

or

$$R(m) = m^2 \tag{3c}$$

The broken vertical lines in Fig. 6 represent the positions of the maximum heights of peaks 2–9 in Fig. 1. It can be shown by using the same model that the adsorption energy for molecules involved in peak 1 is too small for them to be retained on the column and that these molecules elute at a volume of 34.9 ml, which explains qualitatively the fact that it is only peak 1 that elutes before the gradient of sodium ions begins (see Fig. 1). The theoretical elution volume of peak 1 is larger than the experimental value of 12 ml (see Table I), however, and this will be reconsidered in the Discussion. It can be seen from Fig. 6 that a best fit between the theoretical and experimental elution molarities for molecules retained on the column is obtained only when $R(m) = m$ and when $\alpha = 0.9$, except for the position of peak 2. In the above, we assumed, for the value of $\beta\sigma$, the mean value 0.049 of the apparent values

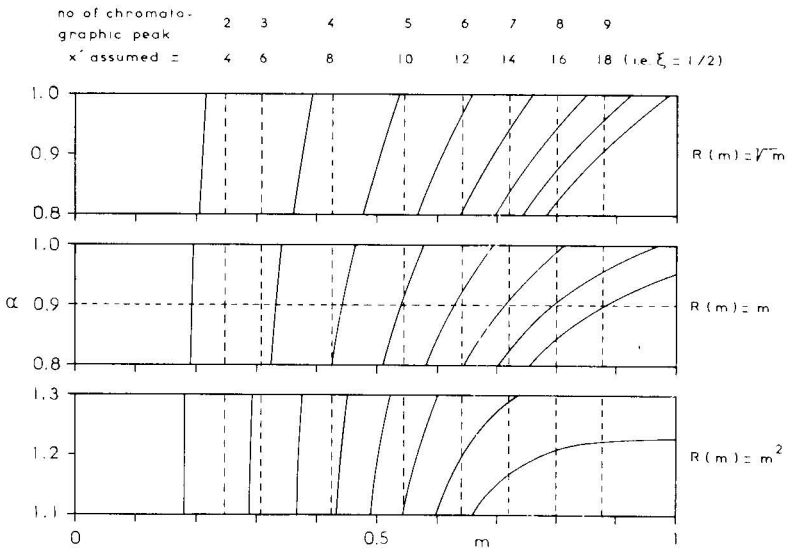


Fig. 6. Theoretical elution sodium molarities for peaks 2–9 in Fig. 1 (abscissa) as functions of the parameter α in eqn. 2 (ordinate) calculated by assuming $\xi = 1/2$ and three different equations for the function $R(m)$. The vertical broken lines are positions of the maximum heights of peaks 2–9 in Fig. 1. It can be seen that the best fit between the theoretical and experimental values is obtained only when $R(m) = m$ and $\alpha = 0.9$, except for the position of peak 2.

of this parameter in Fig. 5. Similar results are obtained if both extreme values (0.031 and 0.068) of the range of the standard deviation of the apparent values (Fig. 5) are assumed for $\beta\sigma$: it is only peak 1 that elutes before the sodium gradient begins and it appears at elution volumes of 27.0 and 42.3 ml if $\beta\sigma = 0.031$ and 0.068, respectively; it is only when $R(m) = m$ and $\alpha = 0.96$ and 0.86 that best fits between the theoretical and experimental results are obtained with respect to the elution molarities of peaks 3–9 if $\beta\sigma = 0.031$ and 0.068, respectively, the interval between peaks 2 and 3 calculated theoretically always being larger than the empirical value.

If different values of 2/3 and 1/3 are assumed for ξ, different values are obtained for φ'_{in}, ε and $\beta\sigma$ (see Table II), but it is also when $R(m) = m$ that the best fits between theoretical and experimental results are obtained with respect to the elution molarities of peaks 3–9; in these instances, also, the interval between peaks 2

TABLE II

VALUES OF ε, $\beta\sigma$, φ'_{in} ($\approx \varphi$) AND α ESTIMATED FOR SEVERAL HYPOTHETICAL VALUES OF ξ

ξ	ε (kcal/mole)	$\beta\sigma$	φ'_{in} ($\approx \varphi$)	α^*
1	$\gtrsim$2.2		$\gtrsim$50	
2/3	2.03	0.070 ± 0.022	15	0.87 (0.82, 0.91)
1/2	2.20	0.049 ± 0.018	10	0.90 (0.86, 0.96)
1/3	2.63	0.024 ± 0.012	7	0.97 (0.94, 1.03)

* The left- and the right-hand values in parentheses are those derived by assuming the upper and the lower limits, respectively, of $\beta\sigma$ in the third column.

and 3 calculated theoretically is larger than the experimental value; it is only peak 1 that elutes before the sodium gradient begins and the theoretical elution volumes for peak 1 are larger than the experimental value, being 28.7–42.7 ml if $\xi = 2/3$ and 24.3–45.1 ml if $\xi = 1/3$. Fig. 7 is the corresponding figure to Fig. 6 for the case when $\xi = 2/3$. If it is assumed that $\xi = 1$, it is difficult to estimate the exact value of φ'_{in}. In this instance, in plots corresponding to those in Fig. 4, the points approach so closely to the straight line $\varphi'_{(m=m_{in})} = \varphi'_{in}$ that the points and the straight line are almost superposed when $\varphi'_{in} \gtrsim 50$, from which it can be estimated (*cf.*, Fig. 5) that $\varepsilon \gtrsim 2.2$ kcal/mole, because the value of ε increases monotonously with increase in φ'_{in}. Table II summarizes the results of the calculations obtained so far.

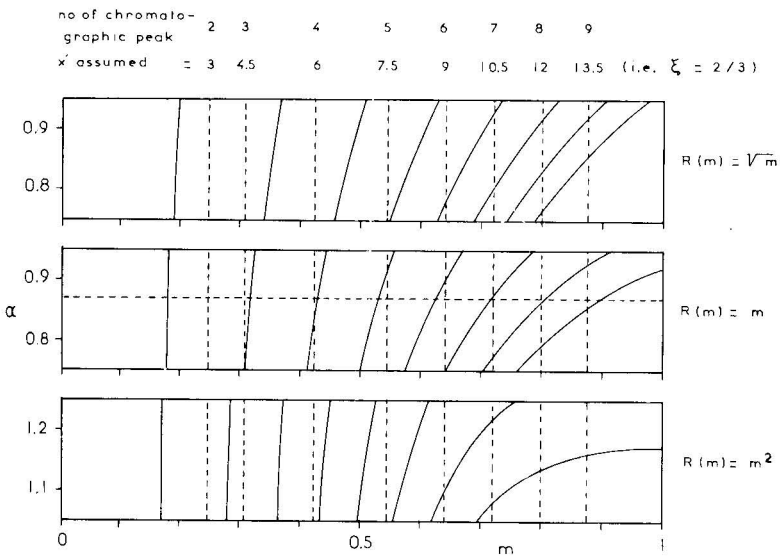


Fig. 7. As Fig. 6, but assuming $\xi = 2/3$. In this instance also it is only when $R(m) = m$ but $\alpha = 0.87$ that the best fit between the theoretical and experimental values is obtained, except for the position of the peak 2.

In Fig. 8, the parameter γ' (see eqns. 2 and 3b) for three different hypothetical values of ξ (2/3, 1/2 and 1/3) and optimal α values (see Table II) are plotted as functions of the molarity of sodium ions in the eluent. In Fig. 8, mean activity coefficients (γ) for both sodium chloride and potassium chloride measured in the usual aqueous solutions (cited from ref. 22) are plotted as functions of the molarities of these salts. It can be suggested that the different behaviours of γ' and γ are mainly due to different (average) features of the interactions of the ions between the interstices of the HA column and the usual bulk solution (see Discussion).

Fig. 9 illustrates the relationships between Λ ($= \varphi' m$; see ref. 4) and m calculated by using the functions $\gamma'(m)$ shown in Fig. 8 for three different values of ξ. It can be seen from Fig. 9 that there are almost linear relationships between Λ and m, for any values of ξ, if the sodium concentration is lower than *ca.* 10^{-1} *M*.

We discussed above possible values of φ'_{in}, ε and $\beta\sigma$ and possible equations for $\varphi'(m)$ for some hypothetical values of ξ. We must now estimate the value of

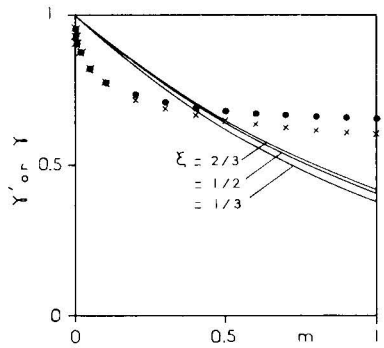


Fig. 8. Curves: plots of γ' (eqn. 2) as functions of the sodium molarity for three different hypothetical values of ξ and best α values (see Table II). Symbols: plots of activity coefficients (γ) of (●) NaCl and (×) KCl at 25 °C as functions of salt molarities. It can be suggested that the different behaviours of γ' and γ are mainly due to different (average) features of interactions of ions between the interstices of the HA column and the usual bulk solution.

ξ. Table IV in ref. 7 summarizes values of the parameter φ' for phosphate ions of the buffer (pH 6.8) and the adsorption energy for one of the univalent phosphate groups on the phosphate chain of nucleoside phosphates estimated from the experimental chromatogram for a mixture of AMP, ADP, ATP and adenosine tetraphosphate. By using the value of the adsorption energy for the phosphate group estimated in ref. 7 and assumptions (1)–(3) under Theoretical (Section *C*), the average value of the adsorption energy for the phosphate ion of the buffer can be estimated to be 1.70–1.86 kcal/mole and, by using this value, assumption (4) under Theoretical (Section *C*) and the value of φ' estimated in ref. 7, the limiting value of $\varphi' e^{-\varepsilon'/kT}$ when m tends to zero [see Theoretical (Section *C*); denoted by Γ^0] is estimated to be 0.26–0.29 M^{-1}. On the other hand, Γ^0 can be evaluated by using assumption (5)

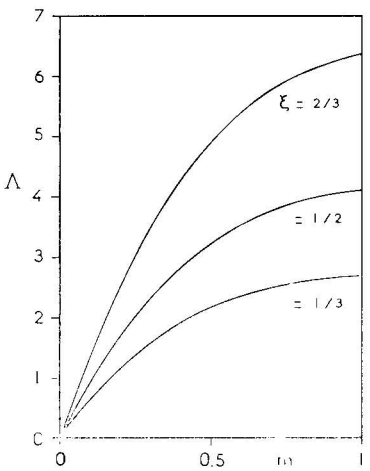


Fig. 9. Λ as functions of m calculated by using functions γ' (m) shown in Fig. 8 for three different hypothetical values of ξ. It can be seen that there are almost linear relationships between Λ and m, for any value of ξ, if the sodium molarity (m) is lower than about 10^{-1} M.

under Theoretical (Section *C*) and values of ε and φ'_{in} ($\approx \varphi$) estimated for some different hypothetical values of ξ (Table II), the results of which are shown in the second column in Table III. It can be seen in Table III that the value of I'^0 estimated for the phosphate ion (see above) is closest to the value for the sodium ion obtained by assuming that $\xi = 1/2$. It is also close to the value for the sodium ion obtained by assuming that $\xi = 2/3$.

TABLE III

VALUES OF I'^0, I', β AND σ ESTIMATED FOR SEVERAL HYPOTHETICAL VALUES OF ξ

ξ	I'^0 (M^{-1})	I' (unit^3)	β	σ^*
1	~1.2	~3.0	~0.12	
2/3	0.49	1.21	$4.79 \cdot 10^{-2}$	1.0–1.9
1/2	0.25	0.62	$2.46 \cdot 10^{-2}$	1.3–2.7
1/3	0.08	0.20	$0.79 \cdot 10^{-2}$	1.5–4.6

* The left- and the right-hand values are those derived by assuming the lower and the upper values of $\beta\sigma$ in the third column in Table II, respectively.

Finally, we estimate the value of the parameter β, which enables us to estimate, by using the value of $\beta\sigma$ shown in Table II, the value of σ that is relevant to both the geometrical configuration and the stereochemical conformation of the molecule on the crystal surface [see Theoretical (Section *A*)]. The parameter β is defined, for small loads, by eqn. A1 in Appendix I in ref. 4 or by

$$\beta = I'z \cdot \frac{\delta A}{\delta V} \tag{4}$$

where z is the coordination number of P crystal sites which must be equal to 6 (see Appendix II); $\delta A/\delta V$ is the ratio of the effective surface of HA where there are P sites to the interstitial volume in the column section, which is estimated to be 6.6×10^{-3} unit^{-1}, "unit" meaning the unit length (8.77 Å) defined such that the area of the elementary surface of HA that contains a single P site would be equal to unity if it is measured in terms of this unit (see Appendix I in ref. 4); I' is the ratio of the absolute activity to the concentration (number/volume) of macromolecules, which must be identical with I'^0, because the actual concentration of macromolecules in the interstices of the column is very low and the value of I' or I'^0 is independent of the type of molecules or ions. It should be noted, however, that I' has dimensions of volume and that, in eqn. 4, the volume should be expressed in terms of the unit of (8.77 Å)^3 (see above). As 1 M corresponds to 0.406 [number/(8.77 Å)] (see Appendix I in ref. 4), by using 0.26–0.29 M for the value of I'^0 obtained for phosphate ions (see above), the value of I' in eqn. 4 can be estimated to be 0.64–0.71 unit^3. Now, by substituting values of I', z and $\delta A/\delta V$ in eqn. 4, β is estimated to be 0.025–0.028. The parameter β appears to have dimensions of area, because I' and $\delta A/\delta V$ have dimensions of volume and reciprocal length, respectively, and z is a dimensionless quantity. It should be considered in eqn. 4, however, that $\delta A/\delta V$ has dimensions of reciprocal volume, because the physical meaning of $\delta V/\delta A$ is the interstitial volume measured in units such that the area of the elementary surface of HA that

contains a single P site would be equal to unity, which means that β is a dimensionless quantity. In the third column in Table III, values of I' calculated from values of I'^0 for sodium ions obtained by assuming that $\xi = 1$, 2/3, 1/2 and 1/3 are shown; in the last two columns, values of β and σ calculated by using values of I' in the third column and values of $\beta\sigma$ in the third column in Table II are shown for $\xi = 2/3$, 1/2 and 1/3, respectively. It can be seen from Table III that values of β calculated by assuming $\xi = 2/3$ and $\xi = 1/2$ are close to the value of 0.025–0.028 calculated by using the value of I'^0 obtained for phosphate ions (see above), which is a natural conclusion, because this value of I'^0 is close to the corresponding values for sodium ions obtained by assuming $\xi = 2/3$ and $\xi = 1/2$ (see above). It can also be seen from Table III that the values of σ are always slightly larger than unity; this suggests that the number of possible stereochemical conformations of the poly-L-lysine molecule on the crystal surface is close to unity (see Discussion).

DISCUSSION

In Appendix I, the result of hydrodynamic studies on poly-L-lysine solutions carried out by Daniel and Alexandrowicz[23] are mentioned, which shows that low-molecular-weight poly-L-lysine molecules such as those used to obtain Fig. 1 have highly stretched conformations. It is probable that molecules of poly-L-lysine are adsorbed on arrays of P sites on crystal surfaces by conserving extended molecular conformations (see Appendix I). Further, it can be considered (see Appendix I) that, along the highly stretched chain of poly-L-lysine, there are two arrays of ε-amino groups of the side-chains arranged with a minimum distance of 7.23 Å, and that every four ε-amino groups on one of the two arrays, *i.e.*, every eight ε-amino groups of the whole molecule, react with every three P sites on an array of P sites arranged with a minimum interdistance of 9.42 Å on the HA surface; this model is compatible with the estimation that the molecular weights of the sample range between 1500 and 8000 daltons (see the legend of Fig. 1 and Appendix I). This model would also be compatible with the value of σ being slightly larger than unity as estimated in Results of Calculations (see Table III); according to this model, the number of possible stereochemical conformations of the molecule on the crystal surface must virtually be equal to unity, and the number of possible geometrical configurations on the crystal surface for the $(8n + m)$-mer molecule, where $n = 0, 1, 2, \ldots$ and $m = 1, 2, \ldots, 8$, can be estimated to be m, which must virtually be equal to σ [see Theoretical (Section *A*)]. As the position of the peak is influenced only slightly by the value of $\beta\sigma$ or σ (see Results of Calculations), molecules with different values of σ would appear in the same peak if they have the same value of x. It can be estimated that the average value of σ is 4.5, which is slightly larger than the values estimated in Table III; this discrepancy might be due to a change in the water structure near the crystal surface that would occur when charged molecules are adsorbed on the crystal surface [*cf.*, Theoretical (Section *A*)] and/or the fact that the estimation of the value of $\delta A/\delta V$ (see Results of Calculations) is not sufficiently precise.

For this type of adsorption of the molecule there are, logically, at least three possible values for the parameter ξ, *viz.*, 1, 1/2 and 1/3. $\xi = 1$ corresponds to the case when, of three P sites under the adsorbed molecule, two sites except the one reacting with the ε-amino group (see above) can react freely with sodium ions of

the buffer. $\xi = 1/2$ corresponds to the case when one of the two crystal sites that are not reacting with the ε-amino group but are under the adsorbed molecule cannot interact with the sodium ion of the buffer being blocked by the presence of the molecule. Finally, $\xi = 1/3$ corresponds to the case when neither of these two P sites can adsorb sodium ions. In Appendix II, the distribution and the structure of P sites on the crystal surface of HA are explored on the basis of crystallographic data, according to which it is probable that P sites are arranged hexagonally with a minimum distance of 9.42 Å (between centres of sites) on the $(\vec{a}, \vec{b})$ surface of HA and that a P site is constructed with six oxygen ions (belonging to three crystal phosphates) arranged in two triangular arrays parallel to the crystal surface. In Appendix III, the adsorption of the stretched polypeptide chain of poly-L-lysine on an array of P sites is studied by using space-filling molecular models for both poly-L-lysine and the crystal surface, the latter being constructed on the surface of the plasticine. This study suggests that the value of ξ is of the order of 1/2. In Appendix IV, the chromatographic data for lysozyme discussed in ref. 4 are again discussed on the basis of several results obtained in Results of Calculations, which suggests that the value of φ is greater than about 7; this means that the value of ξ for poly-L-lysine is greater than about 1/3 (see Table II). Under Results of Calculations, it was shown, on the basis of assumptions (1)–(5) in Theoretical (Section *C*), that the most probable value of ξ is 1/2, but that the value 2/3 is also probable. It follows from these estimations that the most probable value for the adsorption energy, ε, for an ε-amino group of poly-L-lysine on to a P crystal site is 2–2.2 kcal/mole (see Table II).

Under Results of Calculations, all calculations were carried out by assuming that the number, x, of ε-amino groups of a poly-L-lysine molecule that can react with P sites is unique or that the molecule is adsorbed in the energetically most stable configuration (or configurations). More precisely, it should be considered that the geometrical configuration of the molecule on the crystal surface follows a Boltzmann distribution and that x represents the average number of adsorption groups that react with crystal sites. However, the value of 2–2.2 kcal/mole for the adsorption energy, ε, obtained on the basis of the assumption of a unique value of x (see above) justifies this assumption, because values 2–2.2 kcal/mole for ε correspond to values of 29–41, respectively, for the Boltzmann factor, $e^{\varepsilon/kT}$, at 25°C. This means that the probability that the molecule is adsorbed in the energetically most stable configuration (or configurations) by using the maximum possible number of ε-amino groups is approximately 29–41 times as high as the probability that it is adsorbed in different configurations, assuming that possible numbers of any geometrical configurations on the crystal surface with identical adsorption energies are about equal to one another. Hence, the molecule must virtually be adsorbed in the energetically most stable configuration (or configurations) (but see below).

It was shown (Results of Calculations) that the apparent value of $\beta\sigma$ calculated from the pair of peaks 2 and 3 in Fig. 1 is exceptionally large (see the table in Fig. 2); this is due to the fact that the interval between peaks 2 and 3 is very narrow or that the elution molarity of peak 2 is too high, which is evident from Figs. 6 and 7. The possibility that this is experimentally fortuitous can almost be discounted, because other similar experiments have shown the same tendencies (Fig. 5b in ref. 16, Fig. 1a in ref. 8 and unpublished work by Prof. G. Bernardi). A reasonable explanation for this phenomenon can be obtained by assuming that molecules with

relatively short peptide chains (presumably with 9–16 amino residues; *cf.*, Appendices I and III) involved in peak 2 can be adsorbed on the crystal surface by using not only two ε-amino groups but also more than two at the expense of the deformation of the stretched conformation of the molecule. If this is true, one cannot exclude the possibility that one of the groups that react with P sites in a deformed molecular conformation is the α-amino group of the N-terminal residue. As it can be considered (see Appendix I) that the stretched conformation of the molecule is mainly due to the energetical factor, *i.e.*, the repulsive interactions among charged ε-amino groups on the side-chains of the molecule, the apparent average value of the adsorption energy per amino group must decrease when there is a deformation of the molecular conformation. The decrease in the adsorption energy per amino group may also be possible if contacts of some amino groups to P sites are made incompletely in this molecular conformation. These ideas may explain qualitatively the reason why chromatographic peak 2 is displaced only slightly to a higher sodium molarity. In Fig. 1, there appears to be a small shoulder on the left-hand side of peak 2, the position of which corresponds approximately to that realized provided that molecules are adsorbed by using only two ε-amino groups (see Figs. 6 and 7). This might suggest that some molecules (perhaps the smallest ones) among those involving presumably 9–16 amino residues are adsorbed by using two amino groups only. However, one cannot exclude the possibility that the occurrence of this small shoulder is experimentally fortuitous.

It was also shown (Results of Calculations) that the experimental elution volume for peak 1 is smaller than the theoretical value. A similar result was obtained in the chromatography of the mixture of nucleoside phosphates[7]. A qualitative explanation for these phenomena can be obtained by assuming that the conformation or the adsorption of the molecule on the crystal surface is influenced considerably by thermal motions of the molecule when it is fixed on the crystal surface with small energies and that the average energy of fixation per molecule becomes even smaller. This argument should be distinguished from that applied to the manner of adsorption of relatively large molecules (presumably with more than 16 amino residues) involved in peaks 3–9, which were used for estimations of the values of ε, φ, σ, etc.; in this instance both the conformation and the adsorption energy of the molecule on the crystal surface can be considered to be virtually unique, because the loss of energy per molecule by the deformation of the molecular conformation must be large, and one manner of adsorption of the molecule on the HA surface must be energetically much more stable than the others (see above).

Finally, we have seen in Fig. 8 that the dependence of γ' on the molarity of sodium ions in the interstices of the column is different from that of the activity coefficient, γ, of the sodium salt in the usual aqueous solution. It can be suggested that this difference is due at least partially to the different features of the interactions among sodium ions near the HA surface from that which occurs in the usual aqueous solution*. It is not likely that the different behaviour of γ' and γ is due to interactions

* It might be possible that different behaviour of γ and γ' results from the fact that γ is the mean activity coefficient of the salt whereas γ' is concerned only with sodium ions. It is known, however, that the tendencies of the dependence of γ on the ionic strength of the solution are similar between different salts, *i.e.*, γ always decreases almost exponentially with increase in the square root of the ionic strength when the ionic strength is low enough (Debye–Hückel theory), while it can be seen in Fig. 8 that γ' decreases exponentially with increase in the ionic strength itself; this suggests that the feature of interactions among ions near the crystal surface is fundamentally different from that in the usual aqueous solution.

between sodium ions completely fixed on P sites, because 9.42 Å for the minimum distance between P sites (see Appendix II) seems to be too large for sodium ions with an ionic radius of about 1.0 Å (see ref. 24) to interact strongly enough with one another. It should be added that the exchanges of several types of cations and anions at the crystal surface of HA were studied on a kinetic basis by Pak and Bartter[25]; they suggested that these ions are substituted for one another at the crystal surface, including the hydration shell that is in contact with the surface, in such ways that the charge balance at the crystal surface, including the hydration shell, is conserved.

APPENDIX I

It is shown, by using both optical and hydrodynamic methods, that poly-L-lysine in aqueous solution undergoes a transition from a random coil to a helical conformation with an increase in pH at a pH of about 10, which is the approximate pK_a value of the ε-amino group[26,27]. This indicates that the random coil and the helical conformations are realized when side-chains of the molecule are positively charged and neutral, respectively[26], and that the molecule is in both charged and random-coil states under the chromatography conditions at pH 6.8. Several hydrodynamic properties of charged poly-L-lysine hydrochloride in aqueous solution both in the absence of the salt and in the presence of 0.01–1 *M* sodium chloride were investigated in detail by Daniel and Alexandrowicz[23]. They concluded that the effect of the ionic strength on the conformation of the molecule is small and that the frictional coefficient of the macromolecule does not depend, or depends only slightly, on the ionic strength of the solution, especially when molecules have small chain lengths; although the conformation of the molecule is called a random coil, the peptide chain of a rather small molecular weight poly-L-lysine hydrochloride (with a degree of polymerization of 50–70) is sufficiently extended in both the absence and the presence of the salt to make its frictional coefficient approximately equal to that calculated from the equation for a corresponding cylinder with a length of the repeating unit and a radius of the unit of 3.64 and about 6 Å, respectively[23]. Further, by interpreting the experimental results for the frictional coefficient with the Kuhn–Kuhn–Silberberg equation[28], values of a statistical element length of 44 Å (or 12 monomer units) and a hydrodynamic diameter of about 8 Å were obtained[23]. It is probable that the poly-L-lysine hydrobromide sample with a degree of polymerization of 7–38 (see the legend of Fig. 1) used in the chromatography that resulted in Fig. 1 is highly stretched and that it is adsorbed on an array of P sites of HA conserving this molecular conformation (see below); this would be compatible with the value of σ being slightly larger than unity (see Table III), estimated from the chromatography (see Discussion).

The fact that the polypeptide chain of poly-L-lysine is highly stretched suggests that the region on the "molecular surface" in which a charged ε-amino group can move without a considerable loss of energy is small, because the stretching of the polypeptide chain itself must be due to mutual electrostatic repulsions among ε-amino groups themselves; this would mean that a close fit of the distance between ε-amino groups with that between P sites of HA is neecssary in order for the molecule to be fixed stably on the HA surface. According to the Corey and Pauling model[29],

a fully extended polypeptide chain has a periodicity of 7.23 Å (we denote this periodicity by r; Table AI) in the axial direction of the molecule and two side-chains orienting in opposite directions are involved in each repeating unit (*cf.*, Fig. A6). It is probable that the stretched polypeptide chain of poly-L-lysine, along both sides of which are arranged ε-amino groups of the side-chains with a minimum interval of 7.23 Å (presumably with only slight fluctuations; see above) is adsorbed by using one of these two arrays of ε-amino groups on one of the arrays of P sites on the crystal surface (*cf.*, Fig. A6); it is improbable that the molecule is adsorbed on the crystal surface by using both arrays of ε-amino groups at the same time because, for this manner of adsorption, a considerable deformation of the molecular conformation is necessary and there is no reason why the (free) energy can be increased by this manner of adsorption (see below).

TABLE AI

RELATIONSHIPS BETWEEN THE PERIODICITY, r, FOR ε-AMINO GROUPS ARRANGED ON ONE OF THE TWO ARRAYS OF THESE GROUPS ALONG THE FULLY EXTENDED POLYPEPTIDE CHAIN OF POLY-L-LYSINE AND PERIODICITIES q_1, q_2, q_3 AND q_4 FOR P SITES ON THE CRYSTAL SURFACE OF HA

(a) v	rv (Å)	v	rv (Å)
1	7.23	5	36.15
2	14.46	6	43.38
3	21.69	7	50.61
4	28.92	8	57.84

(b) n	q_1n (Å)	v	$rv - q_1n$ (Å)
1	9.42	1	−2.19
2	18.84	3	+2.85
3	28.26	4	+0.66
4	37.68	5	−1.53
5	47.10	7	+3.51
6	56.52	8	+1.32

(c) n	q_2n (Å)	v	$rv - q_2n$ (Å)
1	16.32	2	−1.86
2	32.64	5	+3.51
3	48.96	7	+1.65

(d) n	q_3n (Å)	v	$rv - q_3n$ (Å)
1	24.93	3	−3.24
2	49.86	7	+0.75

(e) n	q_4n (Å)	v	$rv - q_4n$ (Å)
1	33.98	5	+2.17

In Appendix II, the distribution and the structure of P sites on the crystal surface of HA are explored on the basis of crystallographic data, from which it is probable that P sites are arranged hexagonally on the crystal surface with a minimum distance of 9.42 Å. Fig. A1 illustrates schematically P sites arranged on the crystal surface and four different arrays of P sites with minimum intervals q_1 (= 9.42 Å), q_2 (= 16.32 Å), q_3 (= 24.93 Å) and q_4 (= 33.98 Å)*. Table AI shows how the distances between ε-amino groups on one of the sides of the fully extended polypeptide chain (see above) match those between P sites on the surface of HA: in part (a) are given the values of the minimum distance between ε-amino groups and multiples of this value; in the second columns in parts (b), (c), (d) and (e) are given values of q_1, q_2, q_3 and q_4 (see Fig. A1) and their multiples, and in the last column in parts (b), (c), (d) and (e) are given the minimum possible differences between intervals of ε-amino groups [part (a)] and those of P sites. It can be seen from Table AI that the smallest difference of 0.66 Å between the interval of ε-amino groups and that of P sites occurs when poly-L-lysine is adsorbed on the array of P sites with a minimum distance of q_1 or 9.42 Å and when $v = 4$ and $n = 3$, *i.e.*, when every four ε-amino groups on one of the two arrays of these groups along the polypeptide chain react with every three P sites on the array of the minimum distance between P sites on the crystal surface [line 3 in part (b) of Table AI]. It should be noted that the slight difference between the interval of ε-amino groups and that of crystal sites is due to

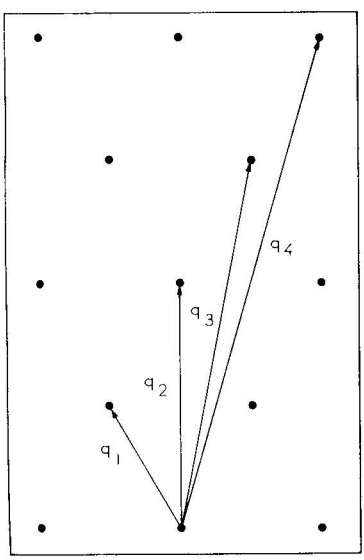


Fig. A1. Schematic representations of P sites arranged on the $(\vec{a}, \vec{b})$ surface of HA and four different arrays of P sites with minimum intervals q_1 (= 9.42 Å), q_2 (= 16.32 Å), q_3 (= 24.93 Å) and q_4 (= 33.98 Å).

* In ref. 4, it is mentioned that $|\vec{a}| \equiv q_1 = 9.432$ Å, which is cited from ref. 11. In this paper, we use the value 9.42 Å for $|\vec{a}|$ obtained on the basis of a more precise crystallographic study of HA in ref. 12.

the fact that the former is slightly larger than the latter, which must be a better condition for the adsorption of the molecule on to the crystal surface than if the former interval was smaller than the latter, because the fully stretched molecule can contract but cannot extend any more. Further, Table AI shows (1) that, in order for a larger proportion than one quarter of the ε-amino groups on one of the two arrays of these groups along the polypeptide chain to interact with P sites, *i.e.*, in order for the value of v to be smaller than 4, the difference between the interval of ε-amino groups and that of P sites has to be much larger than 0.66 Å or it has to be 1.86–3.24 Å, and (2) that, in order for the difference between the interval of adsorption groups and crystal sites to be smaller than 0.66 Å, the value of v has to be greater than 5, *i.e.*, the proportion of ε-amino groups that can react with P sites has to be less than one fifth of those existing on one of the two arrays of the side-chains of the polypeptide chain, because the adsorption of the molecule has to occur on the array of P sites with a minimum distance larger than q_4 or 33.98 Å. This indicates that the manner of adsorption of poly-L-lysine such that every four ε-amino groups on one of the two arrays of the side-chains of the polypeptide chain interact with every three P sites arranged on an array of P sites with a minimum distance of 9.42 Å is energetically the most stable, assuming (a) that there is virtually no increase in the energy of interaction between an ε-amino group and a P site if the difference between the interval of ε-amino groups and that of P sites decreases further from 0.66 Å, but (b) that there is a considerable loss of energy if the difference between the two intervals exceeds 1.86 Å, which are reasonable assumptions.

This model for the manner of adsorption of poly-L-lysine on the surface of HA is strongly supported by the following result of a simple calculation. As the degree of polymerization of the poly-L-lysine sample used to obtain the chromatogram in Fig. 1 is estimated to range between 7 and 38 (see the legend of Fig. 1), by assuming that every eight ε-amino groups of the whole molecule interact with P sites, the numbers of ε-amino groups per molecule that interact with P sites or the value of x can be estimated to range between 7/8 and 38/8, *i.e.*, between about 1 and 5; these x values correspond, according to the interpretation of the chromatogram made under Theoretical (Section *C*), to the x values for molecules involved in peaks 1–5, which occupy most of the chromatogram in Fig. 1.

Finally, we see no reason why the (free) energy can be increased provided that molecules are adsorbed on the crystal surfaces by taking conformations different to a stretched conformation because, firstly, deformation of the molecular conformation from the stretched state must accompany a decrease in energy because of an increase in repulsive interactions among charged ε-amino groups (see above), and secondly, the range of the values of x estimated from the experimental chromatogram can be explained by assuming the stretched molecular conformation which should be the most stable (see above, but see also Discussion for the manners of adsorption of molecules with very short peptide chains presumably with less than 17 amino residues).

APPENDIX II

It would be reasonable, in general, to consider that mechanisms of the adsorption of both free ions and adsorption groups of macromolecules on to crystal

surfaces resemble the first step of the epitaxial growth of the crystal, where epitaxy consists in the growth of one crystal, in one or more particular orientations, on a substrate of another, with a near geometrical fit between the respective networks that are in contact[30]. In the Introduction, it was mentioned that both free cations of the buffer and basic groups of basic macromolecules must be adsorbed on the flat crystal surface of HA which is parallel to $(\vec{a}, \vec{b})$ planes of unit cells. It is probable that both cations and basic groups are adsorbed, if not exactly, on positions of the crystal surface where calcium ions should be coordinated, provided that the crystal structure extends over the actual surface. This idea is supported by the fact that, at least in the interior of the crystal structure, Ca^{2+} ions can be partially replaced with ions such as Na^+, K^+ and Pb^{2+} (ref. 31). It is known that there are two types of calcium ions, called Ca_I and Ca_{II} ions, in the interior of the HA structure, classified according to the environments in which the ions are arranged*, which are shown in Figs. A2 and A3, respectively. Therefore, there are two types of possible positions of P sites on the crystal surface of HA.

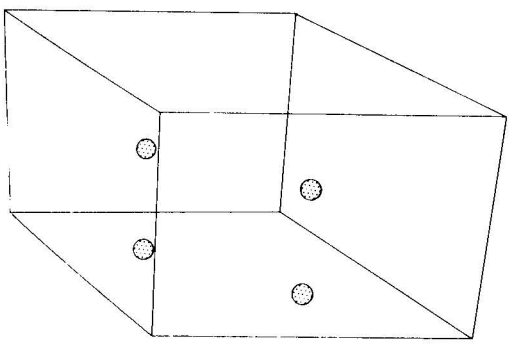


Fig. A2. Perspective view of Ca_I ions. The outline of the unit cell is also shown, where $\vec{a}$ is horizontally directed to the right in the plane of the paper, $\vec{b}$ is directed into the paper and $\vec{c}$ is vertical and upwards. The ions are shown at a small size in comparison with the size of the unit cell. It can be seen that there are two columns of Ca_I ions per unit cell along two three-fold rotational symmetry axes, and that each column is constituted of two Ca_I ions occurring at $z = 0.00$ and $z = 0.50$ (nearly but not precisely at $z = 0$ and $z = 1/2$, respectively). (Reproduced from ref. 13 with the permission of Prof. R. A. Young).

We examine first the possibility that a Ca_{II} position (devoid of calcium ions) on the crystal surface corresponds to a P site. As can be seen in Fig. A3, there are a pair of triangular arrays of Ca_{II} ions per unit cell centred on each edge (6_3 axis) of the cell, which lie on two mirror planes [parallel to the $(\vec{a}, \vec{b})$ plane] being at levels of $z = 1/4$ and $z = 3/4$, and they are inserted by two parallel triangles of oxygen ions (O_{III} ions; not shown in the figure) being at $z = 0.07$ and $z = 0.43$ and at $z = 0.57$ and $z = 0.93$. As six O_{III} ions inserting one of the Ca_{II} triangles belong to three crystal phosphates, the phosphorus atoms of which are at the same level as that of the Ca_{II} triangle itself, it is impossible for Ca_{II} ions to be exposed to the medium at the crystal surface parallel to $(\vec{a}, \vec{b})$ planes of unit cells. Therefore, it is also im-

* For details of the crystallography of HA, see refs. 11–13.

possible for "Ca_{II} positions devoid of calcium ions", if they exist, to be exposed to the medium at the $(\vec{a}, \vec{b})$ crystal surface. However, as Ca_{II} triangles are smaller than O_{III} triangles and as they are both centred on the same 6_3 axes of the unit cells (see Fig. A3), it might be possible that, although Ca_{II} positions are buried in "holes" made by O_{III} triangles at the crystal surface, they are partially exposed to the medium. Therefore, if they are devoid of calcium ions, they might constitute adsorbing sites; this is improbable, however, because, there appears to be no reason why only Ca_{II} positions at the crystal surface that are coordinated by six oxygen ions in the same manner as for Ca_{II} positions in the interior of the crystal should be devoid of calcium ions. Even if only Ca_{II} positions at the crystal surface are devoid of calcium ions (although this is an improbable assumption), it is improbable that they correspond to P sites for the following reason: if Ca_{II} positions on the crystal surface correspond to P sites, there must be three P sites per unit crystal cell or parallelogram made by vectors $\vec{a}$ and $\vec{b}$ where $|\vec{a}| = |\vec{b}| = 9.42$ Å, from which it can be estimated that the number of P sites that are covered by a molecule of lysozyme adsorbed on the crystal surface is of the order of 30, assuming that lysozyme is represented by a prolate spheroid of dimensions $45 \times 30 \times 30$ Å, that the adsorption is such that the maximum molecular surface is brought into contact with the crystal and that most of the sites under the adsorbed molecule are covered[4]. On the other hand, the most probable numbers of P sites covered by an adsorbed lysozyme are estimated to be 4–8 or even 6–7 from the analysis of the chromatographic behaviour of lysozyme (see Appendix IV), which could be explained only by assuming that there is only one P site per parallelogram of vectors $\vec{a}$ and $\vec{b}$*.

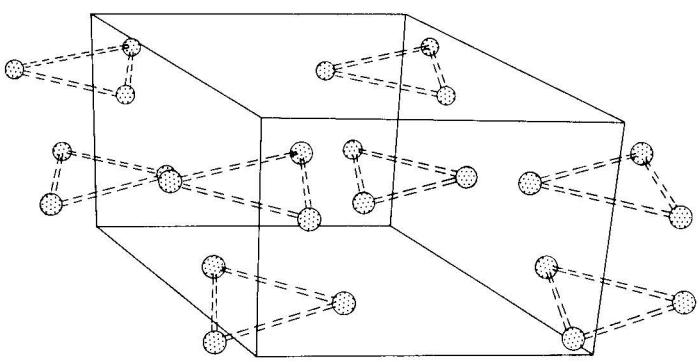


Fig. A3. As Fig. A2, but for Ca_{II} ions. It can be seen that there are a pair of triangular arrays of Ca_{II} ions centred on each edge (6_3 axis) of the unit cell, or that there are a pair of triangular arrays of Ca_{II} ions per unit cell, which lie on two mirror planes [parallel to the $(\vec{a}, \vec{b})$ plane] being at levels of $z = 1/4$ and $z = 3/4$, respectively. (Reproduced from ref. 13 with the permission of Prof. R. A. Young).

* It may be possible that they are only some of the sites under the adsorbed molecule that are covered by the molecule, as with poly-L-lysine (see Appendix III). Even taking into account this possibility, the assumption that there is only one P site per parallelogram of vectors $\vec{a}$ and $\vec{b}$ seems to be most reasonable in order to explain the very small number of P sites covered by an adsorbed lysozyme estimated experimentally. In fact, this number would suggest that some of P sites (arranged at every 9.42 Å) under the adsorbed molecule are not covered by the molecule.

Now, we examine the alternative possibility that it is a Ca_I position (devoid of calcium ions) that corresponds to a P site. As can be seen in Fig. A2, there are two columns of Ca_I ions per unit cell along two three-fold rotational symmetric axes, and each column consists of two Ca_I ions occurring at $z = 0.00$ and $z = 0.50$ (nearly but not precisely, at $z = 0$ and $z = 1/2$, respectively). Each Ca_I ion is coordinated by nine oxygen ions in three triangular arrays parallel to the $(\vec{a}, \vec{b})$ plane, centred on the Ca_I column itself, and one above (along positive $\vec{c}$), one below and one at nearly the same level (*cf.*, Figs. A4 and A5). Fig. A4 illustrates schematically a projected view of a column of unit crystal cells on an $(\vec{a}, \vec{c})$ or a $(\vec{b}, \vec{c})$ plane, in which the dots and thick horizontal lines represent Ca_I ions and oxygen triangles, respectively; thick lines at $z = 0.07$, $z = 0.43$, etc., which represent triangles of O_{III} ions, are drawn long in order to emphasize that these triangles are larger than the other two types of triangles of O_I and O_{II} ions which are represented by thick lines inserted between two dots of Ca_I ions and those inserted between two thick lines of O_{III} ions, respectively. Projected views, on an $(\vec{a}, \vec{b})$ plane, of three triangles of O_I, O_{II} and O_{III} ions at $z = 3/4$, $z = 3/4$ and $z = 0.93$ are drawn in Fig. A5, from which it can be understood that three successive O_{III}, O_{II} and O_{III} triangles (the levels of which are relatively close to one another; see Fig. A4) belong to three crystal phosphates, the phosphorus atoms of which are at the same level as that of the O_{II} triangle itself of this series; this excludes the possibility that a plane inserted between O_{II} and O_{III}

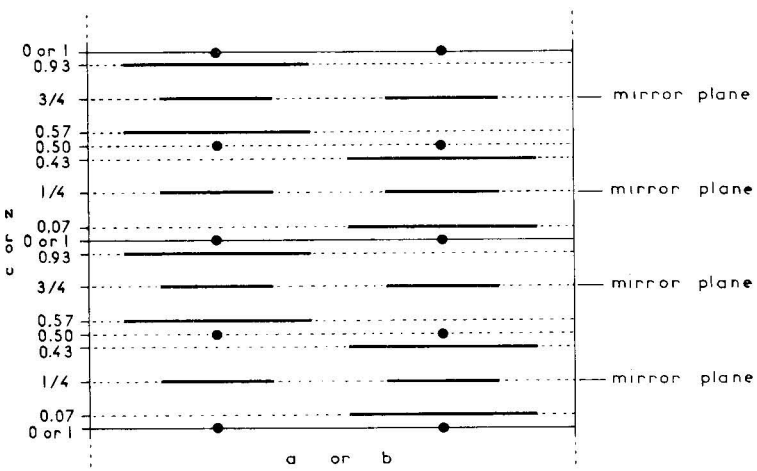


Fig. A4. Schematic representation of a projected view of a column of HA unit cells on an $(\vec{a}, \vec{c})$ or a $(\vec{b}, \vec{c})$ plane. The dots and thick horizontal lines represent Ca_I ions and oxygen triangles, respectively. [The other components of the crystal: phosphorus at mirror planes, Ca_{II} ions at mirror planes near 6_3 axes (see Fig. A3) and hydroxyl ions near mirror planes at 6_3 axes are not shown). Thick lines at $z = 0.07$, $z = 0.43$, etc., which represent triangles of O_{III} ions, are drawn long in order to emphasize that these triangles are larger than the other two types of triangles of O_I and O_{II} ions, which are represented by thick lines inserted between two dots of Ca_I ions and those inserted between two thick lines of O_{III} ions, respectively. Three successive O_{III}, O_{II} and O_{III} triangles, the levels of which are relatively close to one another, belong to three crystal phosphates, the phosphorus atoms of which are at the same level as that of the O_{II} triangle itself of this series; this excludes the possibility that a plane inserted between O_{II} and O_{III} triangles appears on the crystal surface.

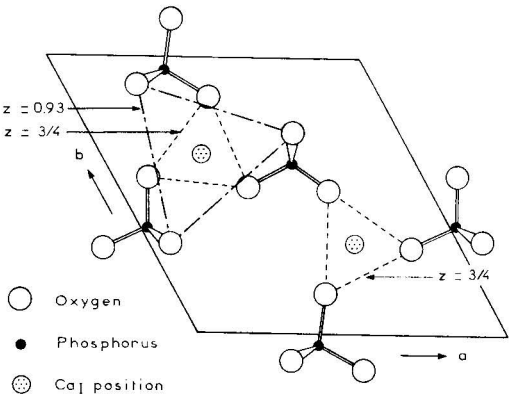


Fig. A5. Deduced structure of the $(\vec{a}, \vec{b})$ surface of HA. Crystal phosphates and two Ca_I positions appearing on the $(\vec{a}, \vec{b})$ surface of HA are shown at a small size in comparison with the outlined unit cell. It can be considered that both the cation of the buffer and the positively charged group of the macromolecule are adsorbed mainly at or near the left-hand Ca_I position coordinated by three O_{II} and three O_{III} ions that constitute trianglular arrays at $z = 3/4$ and $z = 0.93$, respectively, and that this position corresponds to a P site. It is probable that the cation and the positively charged group of the macromolecule are also adsorbed weakly at or near the right-hand Ca_I position coordinated by three O_I ions at $z = 3/4$. (Reproduced with modifications from Fig. 2c in ref. 13 with the permission of Prof. R. A. Young).

triangles appears on the crystal surface. Thus, it must be a plane between two O_{III} triangles centered on neighbouring Ca_I columns or the level of Ca_I positions that appears on the crystal surface (see Fig. A4). Therefore, Ca_I positions exposed to the medium at the crystal surface would be expected to adsorb both cations of the buffer and positively charged groups of macromolecules, if they are devoid of calcium ions; the latter condition is probable because two types of Ca_I positions at the crystal surface are coordinated by only six oxygen ions (in two triangular arrays) and by only three (in a single triangular array) (see Figs. A4 and A5); in contrast to a Ca_I position in the interior of the crystal, which is coordinated by nine oxygen ions in three triangular arrays (see Fig. A4), and whether or not Ca_I positions at the crystal surface are occupied by calcium ions does not seem to be important for the cooperativity of the crystal structure as a whole.

It is probable that Ca_I positions coordinated by six oxygen ions adsorb both free cations and adsorption groups of macromolecules with much higher probabilities than those coordinated by three oxygen ions because, firstly, the probability that both free ions and adsorption groups of macromolecules are adsorbed on to Ca_I positions coordinated by six oxygen ions must be higher than the probability that they are adsorbed on posititions coordinated by three oxygen ions, for energetic reasons. Secondly, the chromatographic results with lysozyme could be explained most satisfactorily by assuming that there is only one adsorbing site per parallelogram made by vectors $\vec{a}$ and $\vec{b}$ (see above) and this is possible only when one of the two types of Ca_I positions is a practical adsorbing site. Thirdly, it was shown in ref. 14 that the HPO_4^{2-} ion adsorbed on a hydroxyl position (C site) of HA should have three appropriate calcium to oxygen distances of 2.39–2.63 Å and a hydrogen bond length of 2.46 Å and, in Part IV[7], that the energy of the adsorption for a univalent

phosphate group on the polyphosphate chain of nucleoside phosphates (which should also be adsorbed on to C sites) should be 0.9–1 kcal/mole (see Introduction)*. Under Results of Calculations, the adsorption energy for an ε-amino group of poly-L-lysine on to a P site was estimated to be 2–2.2 kcal/mole, in spite of the fact that this adsorption group has only a single charge, which seems to be possible only when the adsorption group is coordinated by six oxygen ions. Fourthly, it is improbable that Ca_I positions coordinated by six oxygen ions are almost always occupied by calcium ions and that only those coordinated by three oxygen ions are available for the adsorption of both cations of the buffer and adsorption groups of macromolecules because, even if the energy for the occupation of a Ca_I position by a calcium ion is higher than that for occupation by an adsorption group of the macromolecule, the energy per molecule with several active adsorption groups would exceed the energy per calcium ion, and Ca_I positions would be occupied by adsorption groups of macromolecules rather than calcium ions. Similarly, if the concentration of competing cations in solution is much higher than that of calcium ions (which is the usual practice), Ca_I positions must be occupied by competing cations rather than calcium ions. Finally, the possibility that one of the two types of Ca_I positions is occupied only by competing cations and that the others are occupied only by adsorption groups of macromolecules can almost be excluded from the study of the adsorption of poly-L-lysine on the crystal surface performed by using space-filling models (see Appendix III).

Hence, it is probable that a Ca_I position coordinated by six oxygen ions (three O_{II} and three O_{III} ions) corresponds to a P site, the detailed structure of which is shown in both Figs. A5 and A6.

APPENDIX III

In Appendix II, it was mentioned that P sites must be arranged hexagonally with a minimum distance of 9.42 Å on the $(\vec{a}, \vec{b})$ surfaces of HA and that a P site must be constructed with six oxygen ions (three O_{II} and three O_{III} ions) belonging to three crystal phosphates. In Appendix I, it was mentioned that the polypeptide chain of poly-L-lysine must be highly stretched under the experimental conditions

* It can be considered that a C site is constructed with two Ca_{II} ions (at the same z surrounding the same 6_3 axis; *cf.*, Fig. A3) or two Ca_{II} ions plus an oxygen ion of the crystal phosphate when the adsorbed phosphate or the phosphate group of the adsorbed polyphosphate contains a hydrogen atom. In Fig. 1 in ref. 14 (or Fig. 1 in ref. 7), the manner of fixation of an HPO_4^{2-} ion on to a hydroxyl position (a C site) of the HA part of octacalcium phosphate is shown, where it can be seen that an oxygen (No. 35) of the phosphate ion interacts with two Ca_{II} ions (Nos. 6 and 8) of HA with distances of 2.63 and 2.37 Å. respectively; another oxygen (No. 38) of the phosphate ion interacts with Ca_{II} ion (No. 6) of HA with a distance of 2.39 Å, and another oxygen (No. 36) of the phosphate ion interacts with an O_{III} ion (No. 20) of HA through the hydrogen atom of the phosphate ion constituting a hydrogen bond length of 2.46 Å. It should be noted that, in Fig. 1 in ref. 14, atomic positions in the HA cell are the mirror image of those in Fig. A5 or that, in the former figure, atomic positions are projected from under the plane of the paper of the latter figure. It should also be noted that coordinates of the atoms determined for the HA part of octacalcium phosphate[32] are slightly different from those determined very precisely for the HA crystal[12]; therefore, the distances between the oxygen and hydrogen atoms of the phosphate ion and some crystal ions estimated above would be slightly different in adsorption of the phosphate ion on to the HA crystal surface in the chromatographic experiment.

and that it must be adsorbed on the surface of HA in such a manner that every four ε-amino groups on one of the two arrays of these groups along the stretched polypeptide chain, where the minimum distance between these groups is 7.23 Å, must react with every three P sites on an array of P sites arranged with a minimum distance of 9.42 Å on the crystal surface. For this type of adsorption, there are, logically, at least three possible values of the parameter ξ, *viz.*, 1, 1/2 and 1/3 (see Discussion).

Fig. A6a shows a photograph of space-filling models of poly-L-lysine undecamer and two sodium ions adsorbed on the model surface of HA, which was constructed on the surface of the plasticine also by using space-filling models of phosphate ions. Parallelograms on the surface of the plasticine show outlines of crystal unit cells projected on an $(\vec{a}, \vec{b})$ plane. On the left-hand side of the photograph, a free P site constructed with three O_{II} and three O_{III} ions and a presumably weak adsorbing site constructed with three O_I ions can be seen (see Appendix II and Fig. A5). It can also be seen that a molecule of poly-L-lysine that is extended almost completely not only in the axial direction, the N-terminal being on the left-hand side, but also in the direction perpendicular to it*, is adsorbed by using two ε-amino groups of the side-chains belonging to the second amino residues from the N- and the C-terminal residues of the peptide chain on to every three P sites. For the conformation of the ε-amino group reacting with a P site, it is assumed that the plane defined by the three hydrogens of the tetrahedron of the charged or the protonated primary amine is parallel to the triangular arrays of both O_{II} and O_{III} ions of crystal phosphates and that each hydrogen of the primary amine is superposed on each O_{II} ion and inserted by two O_{III} ions; in this manner of adsorption, each hydrogen of the ε-amino group can be kept in contact with one O_{II} and two O_{III} ions with reasonable distances of about 2.5 Å (see Fig. A6a). One could not, however, preclude the possibility that the triangle of the three hydrogens of the protonated primary amine is superposed on the triangle of the O_{II} ions with a displacement of 60° to each other. It should be noted in Fig. A6 that the molecule is brought into contact with the HA surface through the array of five ε-amino groups and not through the opposite array of six ε-amino groups, which is only for the sake of convenience. It is evident that, if the molecule is adsorbed through this array, it can be adsorbed in two different configurations; therefore, there is a total of three possible geometrical configurations on the HA surface for the stretched undecamer molecule (*cf.*, Discussion).

Possible conformations for three ε-amino groups of the molecule that are in contact with the crystal surface but are not superposed on P sites (see Fig. A6a) appear to be limited, being blocked by the existences of protruding O_{III} ions on the crystal surface, which can be understood from Fig. A6b, where three intermediate side-chains in contact with the HA surface of the model molecule in Fig. A6a are shown from the rear. It can be seen in Fig. A6b that a hydrogen of the ε-amino

* This is an assumption, but it is reasonable because the average distance among charged ε-amino groups is maximal in this conformation. Under Discussion, it was suggested that poly-L-lysine with 9–16 amino residues is adsorbed on the crystal surface not only in the stretched conformation (in the axial direction) but also in a deformed conformation or conformations by using more than two ε-amino groups. Here, we consider only the stretched conformation for the undecamer model of poly-L-lysine, however, because our purpose is to understand the manner of adsorption of molecules with higher molecular weights by using this model.

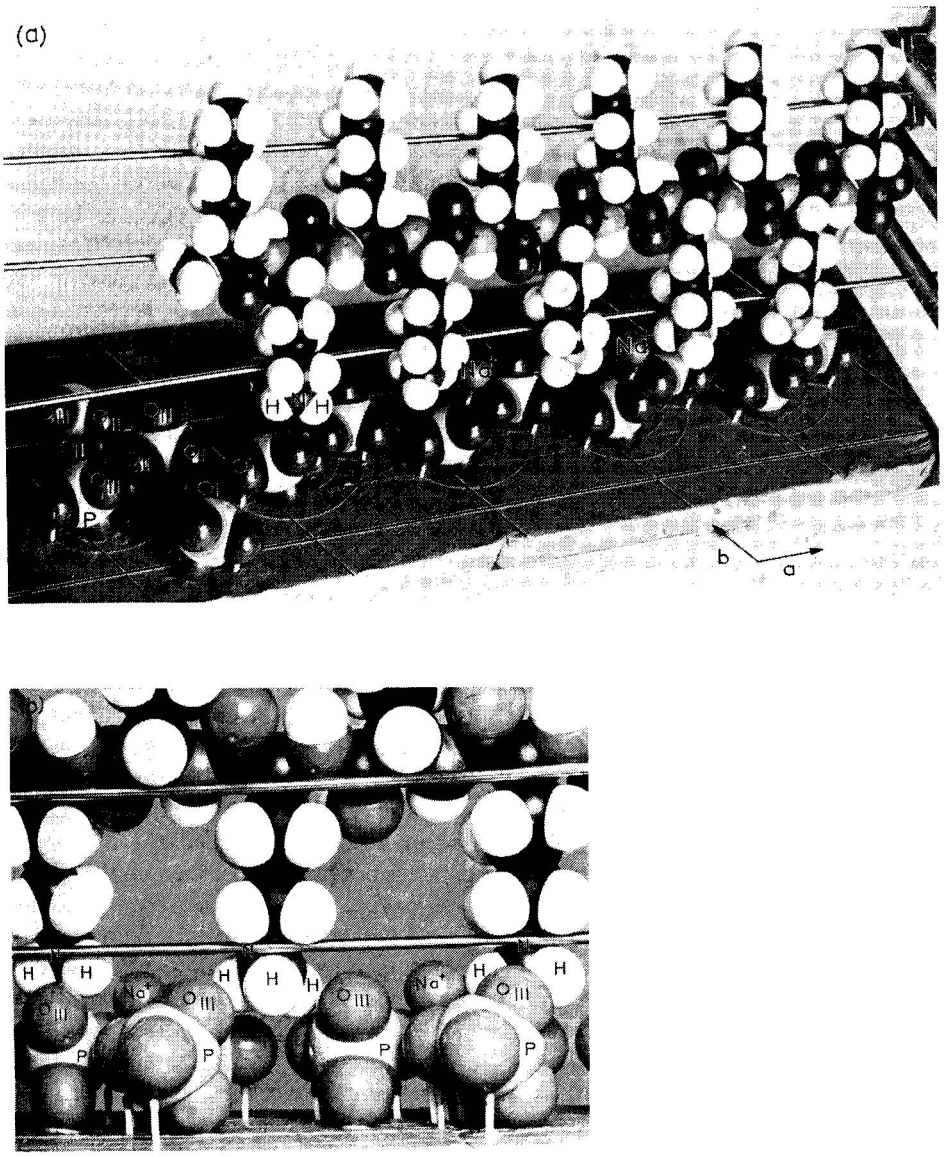


Fig. A6. (a) Photograph of space-filling models of a poly-L-lysine undecamer and two sodium ions adsorbed on the model surface of HA which is constructed on the surface of the plasticine also by using space-filling models of phosphates. Parallelograms on the surface on the plasticine show outlines of crystal unit cells projected on an $(\vec{a}, \vec{b})$ plane. On the left-hand side, a free P site constructed with three O_{II} and three O_{III} ions and a presumably weak adsorbing site constructed with three O_I ions can be seen (*cf.*, Fig. A5). It can also be seen that a molecule of poly-L-lysine which is almost completely extended not only in the axial direction, the N-terminal being on the left-hand side, but also in the direction perpendicular to it is adsorbed by using two ε-amino groups of the side-chains belonging to the second amino residues from the N- and C-terminal ones of the peptide chain, respectively, onto every three P sites. (b) Photograph of three intermediate side-chains in contact with the HA surface but not superposed on P sites of the model molecule shown in (a), taken from the rear side, and two sodium ions adsorbed on P sites.

group of the right-hand side-chain intrudes slightly into the triangular array of O_{III} ions of crystal phosphates in order to avoid protruding O_{III} ions on the crystal surface, while the left-hand side-chain is slightly raised in order to avoid another O_{III} ion; the conformation of the intermediate ε-amino group seems to be less limited. In spite of the presences of these side-chains of the molecule, adsorption of competing or sodium ions on to both of Ca_I positions (see Appendix II), which are under the adsorbed molecule but are not superposed by ε-amino groups, seems to be possible (see Figs. A6a and b) and the value of ξ seems to be close to unity. However, the position of the hydrogen of the right-hand ε-amino group in Fig. A6b or the ε-amino group of the left-hand side-chain of the intermediate three side-chains in contact with the HA surface in Fig. A6a is so close to the Ca_I position of the crystal that it might prevent the adsorption of the sodium ion on to this Ca_I position, whereas it does seems unlikely that the adsorption of the sodium ion on to the left-hand Ca_I position in Fig. A7b is prevented; this would mean that the value of ξ is close to 1/2. Further, it was shown by Bernardi (unpublished work) that an almost identical chromatogram for the low-molecular-weight poly-L-lysine sample can be obtained by using a K^+ system instead of the Na^+ system, which would mean that potassium ions are adsorbed on the crystal surface in a similar manner to sodium ions in relation to the position of the adsorbed macromolecule. As the ionic radius of the potassium ion (1.3 Å) is larger than that of the sodium ion (1.0 Å)[24], it might be very difficult for the potassium ion to be adsorbed on to the right-hand Ca_I position in Fig. A6b. One cannot, however, exclude the possibility such that adsorption of sodium or potassium ions on to both or one of the Ca_I positions is partially hindered by side-chains of poly-L-lysine, and/or that ε-amino groups of the molecule interact slightly with the surface of HA even if they are not superposed completely on P sites, which would contribute slightly to the (apparent) value of ξ. Taking into account all of these factors, the probable value of ξ seems to be of the order of 1/2.

Finally, the possibility that one of the two types of Ca_I positions on the crystal surface that are coordinated by six and three oxygen ions, respectively, react with adsorption groups of macromolecules and that the other Ca_I positions react only with competing ions (which was considered in Appendix II) can almost be excludep by examining Fig. A6a; it is evident that all Ca_I positions coordinated by only three O_I ions are acceptable to sodium ions, even if the maximum number of Ca_I positions coordinated by three O_{II} and three O_{III} ions are occupied by macromolecules and *vice versa*, which means that there is no competition between macromolecules and sodium ions for adsorption on to the crystal surface if they are adsorbed on to different types of Ca_I positions.

APPENDIX IV

In an earlier paper[4], chromatographic data for lysozyme and cytochrome *c* were plotted on the $[m_{elu(K^+)}, \ln s_{(K^+)}]$ plane, and values of parameters such as x' and $\ln q$ were estimated for these basic molecules on the basis of the theory developed in the same paper[4] and by assuming, in most calculations, that the activity coefficient of K^+ ions in the interstices of the column is equal to the mean activity coefficient of potassium chloride measured in the usual aqueous solution. Under Results of Calculations, it was shown, however, that the dependence of the activity of sodium

ions on the concentration in the column interstices is different from that measured for sodium chloride in the usual aqueous solution, probably owing to particular interactions among sodium ions occurring near the crystal surface of HA (see Discussion); the "activity coefficient", γ', of sodium ions in the column interstices varies less with an increase in the cation concentration, m, than does the activity coefficient, γ, of sodium chloride or potassium chloride in the usual aqueous solution in the range of small m values (see Fig. 8), and as the result of the mild dependence of γ' on m, the parameter A increases almost linearly with an increase in m when m is small (see Fig. 9). Here, by assuming that the dependence of A on m for potassium ions

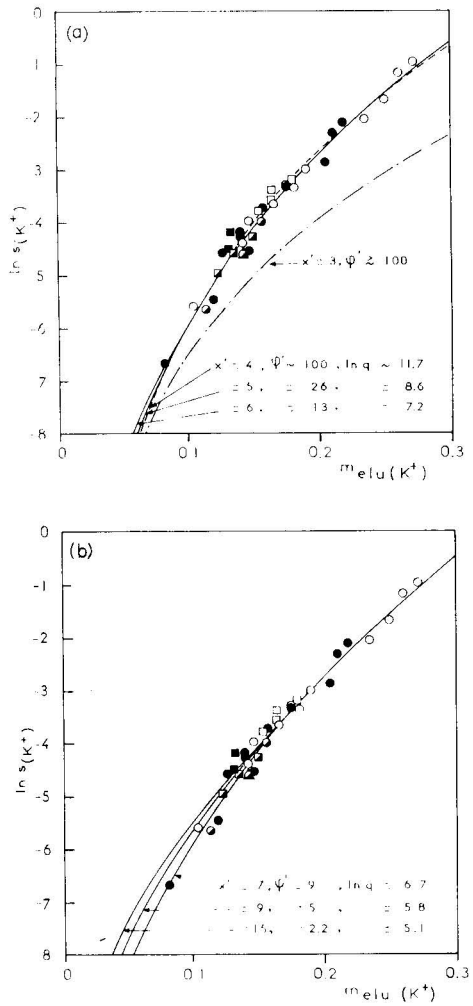


Fig. A7. (a) Points: experimental plots of ln $s_{(K^+)}$ *versus* $m_{elu(K^+)}$ for lysozyme reproduced from Fig. 2b, c or e in ref. 4. The curves are theoretical, obtained by giving values of 3–6 to x'. It can be seen that good fits with the experimental results can be obtained only when $x' \geqslant 4$. (b) As (a), but the theoretical curves were obtained by giving values of 7–15 to x'. It can be seen that good fits with the experimental values can be obtained only when $x' = 7$.

is virtually the same as that for sodium ions*, chromatographic data for lysozyme and cytochrome *c* shown in Fig. 2b, d and e and Fig. 4 in ref. 4, respectively, are again analysed on the basis of eqn. 15 in ref. 4, and more precise values of x', $\ln q$, etc., for these molecules are estimated.

The points in Fig. A7a and b are experimental plots of $\ln s_{(K^+)}$ *versus* $m_{elu(K^+)}$ (for definitions of these parameters, see ref. 4) for lysozyme reproduced from Fig. 2b, d or e in ref. 4; the curves are theoretical, calculated by using eqn. 15 in ref. 4, assuming a linear relationship between Λ and m (see above) and giving values of 3–7, 9 and 15 for x' and values that give the best fits with the experimental results for φ' and $\ln q$ (except when $x' = 3$; see below)**. It can be seen in Fig. A7a and b that, when $x' \geqslant 9$, the theoretical curves are less curved than the experimental result; when $4 \leqslant x' \leqslant 7$, good fits between the theoretical and experimental results are obtained, and when $x' = 3$ the average slope of the theoretical curve is almost constant and is always less than that of the experimental result provided that $\varphi' \gtrsim 100$. If $\varphi' \lesssim 100$, the average slope of the curve becomes even smaller, and no good fits with the experimental values can be obtained when $x' = 3$. In the first three columns in Table AII are summarized the best values of x', φ' and $\ln q$; it should be noted that best values (10–15) for φ obtained from the experiment with poly-L-lysine (see Results of Calculations and Discussion) are involved in the region of the best values of φ' obtained from a different type of experiment for lysozyme. In columns 6, 8 and 9 in Table AII are given values of $x\varepsilon/kT$, x and ξ for lysozyme estimated for two most reasonable values of φ' (13 and 9), which are slightly smaller than best values (15 and 10) for φ (see above). In fact, by taking into account the fact that the linear dependence of Λ on the molarity, m, becomes slightly invalid with increase in m in the range of the experiment with lysozyme, *i.e.*, in the range $0 \lesssim m \lesssim 0.3$

TABLE AII

PARAMETERS ESTIMATED FOR LYSOZYME

x'	φ'	$\ln q$	β (assumed)	σ (assumed)	$x\varepsilon/kT$	ε (assumed) (kcal/mole)	x	ξ
4	~100	~11.7						
5	26 ± 2	8.6						
6	13 ± 2	7.2	$4.79 \cdot 10^{-2}$	1	10.2	2.03	3.0	0.50
				2	9.6		2.8	0.47
				4	8.9		2.6	0.43
				10	7.9		2.3	0.38
7	9 ± 1	6.7	$2.46 \cdot 10^{-2}$	1	10.4	2.20	2.8	0.40
				2	9.7		2.6	0.37
				4	9.0		2.4	0.34
				10	8.1		2.2	0.31
8	7 ± 1	6.5						

* This is a reasonable assumption, because almost identical chromatograms for the low-molecular-weight poly-L-lysine sample are obtained by using Na^+ and K^+ systems (Bernardi, unpublished data; see Appendix III).

** It can be understood from eqn. 15 in ref. 4 that the shape of the curve depends on x' and φ' and that the curve moves vertically when $\ln q$ changes.

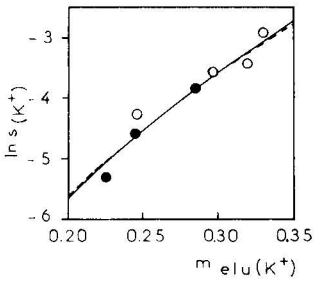


Fig. A8. Points: experimental plots of ln $s_{(K+)}$ *versus* $m_{elu(K+)}$ for cytochrome *c* reproduced from Fig. 4 in ref. 4. The continuous and broken curves are theoretical, obtained by giving best values of 9 and 13 to φ', respectively.

(compare Fig. A7a and b with Fig. 9), the values of 13 and 9 for φ' obtained from the experiment with lysozyme would almost correspond to best values of 15 and 10 for φ obtained from the experiment with poly-L-lysine. Further, it was assumed that the value of ε for lysozyme is equal to that obtained for poly-L-lysine (see column 7 in Table AII; *cf.*, Tables II and III); for σ, several values between 1 and 10 were assumed. As lysozyme is a rigid molecule and as the value of ε can be considered to be large enough for the molecule to be adsorbed virtually in the energetically most stable configuration (or configurations) (see Discussion), the value of σ would represent the number of energetically most stable configurations of the molecule on the crystal surface [see Theoretical (Section *A*]. It can be seen in Table AII that the theoretical values of both x and ξ are not much influenced by the value of σ, however.

The points in Fig. A8 are experimental plots (corresponding to those in Fig. A7) for cytochrome *c* reproduced from Fig. 4 in ref. 4; two curves are theoretical, calculated by giving the best two values 13 and 9 (see above) to the parameter φ' and values that give the best fits with the experimental results to parameters x' and $\ln q$, which are given in columns 1 and 3 in Table AIII, respectively. In columns 6, 8 and 9 in Table AIII are given values of $x\varepsilon/kT$, x and ξ for cytochrome *c* estimated for the two most reasonable values of φ' and for several hypothetical values of σ. In this instance also, theoretical values of both x and ξ are not much influenced by the value of σ.

TABLE AIII

PARAMETERS ESTIMATED FOR CYTOCHROME *c*

x'	φ'	$\ln q$	β *(assumed)*	σ *(assumed)*	$x\varepsilon/kT$	ε *(assumed) (kcal/mole)*	x	ξ
5.5 ± 0.2	13	9.5	$4.79 \cdot 10^{-2}$	1	12.5	2.03	3.6	0.65
				2	11.9		3.5	0.64
				4	11.2		3.3	0.60
				10	10.2		3.0	0.55
6.4 ± 0.2	9	9.1	$2.46 \cdot 10^{-2}$	1	12.8	2.20	3.4	0.53
				2	12.1		3.3	0.52
				4	11.4		3.1	0.48
				10	10.5		2.8	0.44

Finally, it should be recalled that theoretical curves in Figs. A7 and A8 were calculated by taking into account the fact that the R_F or the B value[4] of the molecules before the molarity gradient of potassium ions begins (*i.e.*, when the potassium concentration is 0.0015 M) is virtually zero, which was verified experimentally. This can be verified also from Tables AII and AIII: by using values in Tables AII and AIII and eqn. 1 in ref. 4, it can be shown that the B values of the molecules at a potassium concentration of 0.0015 M are close to zero, *i.e.*, $0.8 \cdot 10^{-3}$–$1.4 \cdot 10^{-3}$ for lysozyme and $0.8 \cdot 10^{-4}$–$1.2 \cdot 10^{-4}$ for cytochrome *c*.

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STUDIES ON MICRO HIGH-PERFORMANCE LIQUID CROMATOGRAPHY

V. DESIGN OF A MICROSCALE LIQUID CHROMATOGRAPH AND ITS APPLICATION TO CATION-EXCHANGE SEPARATION OF ALKALI METALS

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SUMMARY

A microscale liquid chromatograph has been designed and optimized by estimating band broadenings. The high-performance cation-exchange resin, Hitachi No. 2610, was packed into the column, and applied to the separation of alkali metals by radioactivity detection. Four alkali metals were separated within 26 min, using only 208 μl of 0.7 *N* hydrochloric acid as carrier solution.

INTRODUCTION

The efficiency of a chromatographic column is evaluated by the Plate Theory, especially in terms of plate height (HETP). Plate height is affected by various operational parameters, so that it is usually measured under fixed conditions. To evaluate the effect of column diameter (d_c) on the efficiency, plots of the HETP against d_c have been made[1,2]. Knox and Parcher[1] have shown, in their investigation of the effect on peak dispersion of the ratio of column diameter to particle diameter, that the HETP tended to decrease with decreasing column diameter. However, Wolf[2] found that the HETP *increased* with decreasing column diameter. In neither investigation, however, was the microcolumn practically examined, and the contradictory results give the impression that a microcolumn cannot be used to obtain effective separations.

Recently, we have designed microcolumns of less than 0.5 mm diameter and studied practical separations and the effect of column diameter on efficiency. We found that the microcolumns gave satisfactory separations and plate heights similar to those obtained using conventional columns. The microscale liquid chromatograph was successfully applied to various chromatographic separations in our laboratory[3–7]. This paper reports the design of the microscale liquid chromatograph and its application to cation-exchange separation of alkali metals.

DESIGN OF THE MICROSCALE LIQUID CHROMATOGRAPH

Basic equations

In contrast to the ordinary liquid chromatograph, extra-column band broadening is a serious problem when using a microcolumn. Therefore, it is important to design the microscale column by evaluating and minimizing the broadening contributions due to injection, column, connecting tube and detection cell.

The band broadening registered on the recorder is given by[8–11] $\sigma^2 = \sigma_i^2 + \sigma_c^2 + \sigma_t^2 + \sigma_d^2$ where the sigma terms express the broadening due to injection (σ_i), column (σ_c), connecting tube (σ_t) and detection cell (σ_d). When a flow-through cell is used, the broadening also depends on the time taken for the solute band to pass through the cell. Therefore, the total band broadening is given by eqn. 1, expressed in terms of volume:

$$Vw = \sqrt{(Vw_i^2 + Vw_c^2 + Vw_e^2)} + V_d \tag{1}$$

where Vw_i and Vw_c are the broadening contributions due to injection and column, respectively, and V_d is the volume of the detection cell. Vw_e is given by

$$Vw_e^2 = Vw_{t_1}^2 + Vw_{q_1}^2 + Vw_{q_2}^2 + Vw_{t_2}^2 + Vw_d^2 \tag{2}$$

where Vw_{t_1} and Vw_{t_2} are the broadenings due to the connecting tubes between injection and column and between column and detection cell, respectively, Vw_{q_1} and Vw_{q_2} are those due to the quartz wool plugs, and Vw_d is that due to the detection cell.

The band broadening in the column is given by eqn. 5, which is obtained from the Plate Theory (eqns. 3 and 4):

$$N = 16\left(\frac{V_r}{Vw_c}\right)^2 \tag{3}$$

$$k' = \frac{V_r - V_0}{V_0} \tag{4}$$

thus

$$Vw_c = \frac{4\,V_0\,(1 + k')}{\sqrt{N}} \tag{5}$$

where N is the theoretical plate number, V_r is the retention volume, V_0 is the volume of mobile phase in the column, and k' is the capacity ratio.

The band broadening in the connecting tube (empty) is given by eqn. 6 when $Q/DL \ll 1$:[12]

$$Vw_t = \sqrt{\left(\frac{0.212\,S\,Q\,V_t}{D}\right)} \tag{6}$$

where L, S and V_t are the length, cross-section and volume of the connecting tube, respectively, Q is the volumetric flow-rate, and D is the diffusion coefficient; eqn. 6

shows that the broadening due to the tube decreases with decreasing tube diameter. In the present work, a very short tube was used, so the condition $Q/DL \ll 1$ was not satisfied. In such cases, the actual broadening may be more or less than the calculated value. It is considered, however, that any such error would have little bearing on the design, since the broadening contribution due to the short tube would be much smaller than that due to the column.

The detection cell is also an empty tube, so that the contribution of Vw_d in eqn. 2 can be estimated from eqn. 6. However, the full volume of the cell cannot affect Vw_d; took half the volume as the effective figure.

The band broadening due to the quartz wool plug was investigated experimentally and found to be given approximately by

$$Vw_q \approx 0.5\ Vw'_t \approx \sqrt{\left(\frac{0.212\ S\ Q\ V_q}{4D}\right)} \tag{7}$$

where V_q is the volume of the plug.

The effect of Vw_i on Vw in eqn. 1 was studied and the following approximation was obtained:

$$Vw_i^2 \approx 2.43\ V_i^2 \tag{8}$$

where V_i is the injection volume of sample. Eqn. 8 was derived according to the following investigations where the band broadenings were examined by changing the injection volume.

Experiment 1. Sampling tube, 0.3 mm I.D.; connecting tube between injection and detection cell, 220 × 0.5 mm I.D.; detection cell, 0.73 μl; sample, methyl violet; carrier, ethyl alcohol; flow-rate, 25/3 μl/min; detection, UV detector (254 nm).

The results obtained are shown in Fig. 1. The smallest band broading recorded was 15.34 μl, when $V_i = 0.2$ μl. Under these conditions, $Vw_e^2 = (15.34-0.73)^2$; hence from eqn. 1, $Vw_i^2 = 2.2$–$2.6\ V_i^2$. However, some of the injection volumes in this experiment were considerably larger than those used in practice. Therefore, a second experiment was performed on a practical scale.

Experiment 2. Sampling tube, 0.39 mm I.D.; column, 121 × 0.5 mm I.D., packed with Permaphase ODS; quartz wool plugs, 0.5 mm ⌀ × 0.3 mm; connecting tubes between column and detection cell, 12 × 0.39 mm I.D. and 85 × 0.15 mm I.D.;

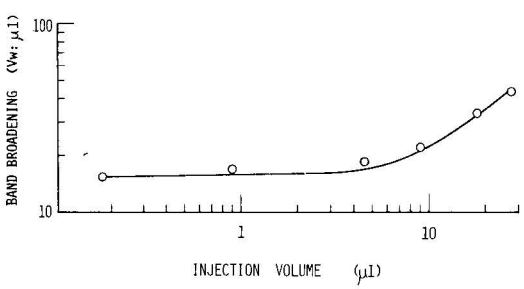


Fig. 1. Band broadening as a function of injection volume. The points are experimental values, and the line represents theoretical values.

detection cell, 0.09 μl; sample, benzene; carrier, ethyl alcohol; flow-rate, 25/3 μl/min; detection, UV detector (254 nm).

The results obtained are shown in Fig. 2. The data were treated as in experiment 1, and we obtained $Vw_c^2 + Vw_e^2 = (2.06)^2$ and $Vw_i^2 = 2.3\text{–}2.5\ V_i^2$. As expected from eqn. 1, the factor 2.3–2.5 increases with increasing V_i, so we applied the result obtained from the 1.87-μl injection to determine the factor exactly and arrived at eqn. 8. Based on this conclusion, the theoretical band broadenings for experiments 1 and 2 were calculated, and are shown by the continuous lines in Figs. 1 and 2, from which it can be seen that the experimental values are in good agreement with the theoretical ones; the errors are smaller than the reproducibilities on the practical scale experiment, as shown in Fig. 2.

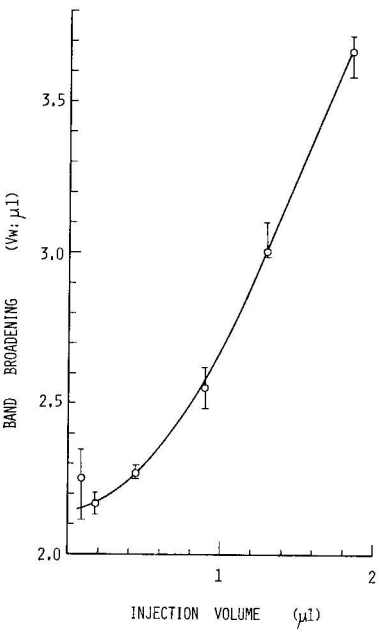


Fig. 2. Band broadening as a function of injection volume. The points and bars show the reproducibility and average of experimental values in four runs, and the line represents theoretical values.

Typical examples

For analytical purposes, it may be essential that the total extra-column broadening contributions are less than 10%; *i.e.* $Vw/Vw_c = 1.1$. We should thus have $V_i/Vw_c = 0.15$, $Vw_e/Vw_c = 0.3$ and $V_d/Vw_c = 0.03$. Let us now consider the following typical examples of how the injection volume, detection cell and connecting tube parameters are determined using these "ideal" figures.

Consider the following experimental conditions: HETP = 0.1 mm; $D = 1.0 \times 10^{-5}$ cm^2/sec; $Q = 8$ μl/min; column, 100×0.5 mm I.D.; quartz wool plugs, 0.5 mm $\varnothing \times 0.5$ mm; packing ratio, 60:40 (particles:vacancy).

The injection volume, detection cell and connecting tube parameters are estimated as follows:

For $k' = 1$ solute: injection volume, 0.3 μl; detection cell, 1.9×0.2 mm I.D. (0.06 μl); connecting tube between column and detection cell, 1.5×0.2 mm I.D.

For $k' = 3$ solute: injection volume, 0.6 μl; detection cell, 1.7×0.3 mm I.D. (0.12 μl); connecting tube, 6.4×0.3 mm I.D.

In order to achieve this instrumentation, it is necessary to (1) inject the sample by the on-column method, (2) detect the separated solutes by the on-cell method, (3) reduce the diameter of the connecting tube as much as is practicable. The lengths of connecting tubes between injection and column and between column and detection cell can be reduced to zero and 1.5 mm, respectively, and the diameter to 0.1 mm.

Microscale liquid chromatograph for the separation of alkali metals

The microscale system used in the present work is specified in Table I. It was designed for the $k' = 9.3$ solute, namely the Na^+ ion, which has the smallest k' value of the alkali-metal ions in 0.7-*N* HCl. Some basic parameters, such as HETP, V_0 and k', were preliminarily determined; when such parameters are available in the literature on ordinary-scale liquid chromatography, they may be used to design a microscale column, at least to a first approximation.

TABLE I

THE MICROSCALE LIQUID CHROMATOGRAPH DESIGNED FOR CATION-EXCHANGE SEPARATION OF ALKALI METALS

Basic parameters: $H = 0.12$ mm; $V_0 = 5.7$ μl (packing ratio: 60/40); k' (Na^+ in 0.7-*N* HCl) = 9.3; $Q = 8$ μl/min; $D = 1.35 \times 10^{-5}$ cm^2/sec (value in infinite dilution)[13].

		Broadening contribution
Injection volume of sample	0.5 μl*	0.6%
Connecting tube between injection and column	zero	—
Quartz wool plug	0.5 mm ∅ × 0.5 mm	0.1
Column	72 × 0.5 mm I.D.	94.1
Quartz wool plug	0.3 mm ∅ × 0.5 mm	0.01
Connecting tube between column and detection cell	20 × 0.25 mm I.D.	1.0
Detection cell	8 × 0.25 mm I.D., 0.4 μl	4.2**

* Designed as $Vw/Vw_c = 1.05$ ($V_i/Vw_c = 0.05$, $Vw_e/Vw_c = 0.12$, $V_d/Vw_c = 0.04$); thus the injection volume can be increased to 2 μl ($Vw/Vw_c = 1.1$, $V_i/Vw_c = 0.21$) if necessary.

** $V_d = 4.0\%$, $Vw_d = 0.2\%$.

In our system, the longer connecting tube between the column and the detection cell was used because when radioactivity is applied during detection, the cell must be shielded with an acrylic acid resin plate of 10 mm thickness; the 20-mm connecting tube gave more effective shielding than the 10-mm tube[7].

EXPERIMENTAL

Apparatus

The JRR-4 reactor at the Japan Atomic Energy Research Institute was used to activate the alkali metals. It has a thermal-neutron flux of 1.8×10^{13} n/cm^2/sec at the irradiation position.

An end-window type GM-counting tube connected to a scaler was used to record the radioactivity.

A micro-feeder (Model MF-2, from Azumadenkikogyo, Tokyo, Japan) was used to drive the carrier and sample solutions through the ion-exchange bed and detection cell. The micro-feeder consists of a small synchronous motor, gears and screws, by which the flow-rate can be changed stepwise. By combining a 250 μl syringe and a 0.31 mm I.D. sampling tube with the micro-feeder, 0.1–2.0 μl of sample could be collected with an accuracy of $\pm$0.01 μl.

Reagents

Ion-exchange resins. TSK LS-212 (obtained from Toyo Soda, Tokyo, Japan), Hitachi No. 2610 (Hitachi, Tokyo, Japan), Shodex HC-125s, Shodex HP-175 (Showa Denko, Tokyo, Japan) and Dowex 50W-X8 (200–400 mesh; Dow Chem., Midland, Mich., U.S.A.) cation-exchange resins were used. The particle sizes are *ca.* 6, 14, 12.5, 17.5 and 70 μm in diameter, respectively, and the ion-exchange capacities *ca.* 4 mequiv./g. Each of these ion exchangers has the sulphonic acid ionic group in styrene–divinyl benzene matrix, and are porous polymer particles except for Dowex 50W-X8. They were conditioned with carrier solution before use.

Radioactive alkali-metal solutions. Sodium and potassium nitrates and rubidium and caesium sulphates were wrapped in aluminium foil, irradiated in a reactor for 1 h, and then dissolved in small amounts of water. The sample solution containing the four alkali metals was prepared by mixing the solutions. The following radioactive nuclides were produced: ^{24}Na ($T_{\frac{1}{2}}$, 15 h, $\beta_{max.}$, 1.39 MeV); ^{42}K (12.5 h, 3.52 MeV); ^{86}Rb (18.7 day, 1.78 MeV); and ^{134}Cs (2.1 yr, 0.66 MeV)[14].

Radioactive ^{131}I solution. Carrier-free $^{131}I^-$ solution was obtained from the Japan Radioisotopes Assoc., and diluted to 29 μCi/μg-I and 0.25 μg-I/μl by adding potassium iodide and water; the solution was used to measure the volume of mobile phase in the column.

All the reagents used were of analytical grade, and deionized water was used throughout.

Procedure

0.5 mm I.D. (1.5 mm O.D.) PTFE tubing was used for the column material, in which the high-performance cation-exchange resin, Hitachi No. 2610, was loaded to 72 mm in length by the slurry-packing technique[3]. Each end of the column was sealed with quartz wool. The top 2 mm of the column was left unpacked for use in the sample injection and carrier solution connections.

A 1300 $\times$ 0.5 mm I.D. PTFE tube was connected to a 250-μl syringe on the micro-feeder unit, and a glass capillary sampling tube, 15 $\times$ 0.31 mm I.D. (0.51 mm O.D.), was linked to the other end of the PTFE tube. Benzene was drawn through to protect the metallic parts of the syringe from corrosion, then 0.7 *N* HCl carrier solution. Finally, 0.5 μl of sample solution was drawn into the sampling tube, which was then inserted into the ion-exchange column inlet. The sample and carrier solutions were driven through the ion-exchange bed at a flow-rate of 8 μl/min.

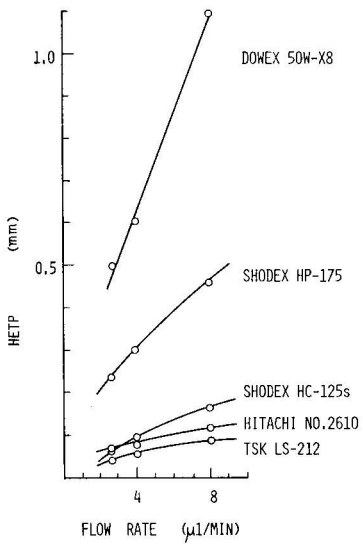
The separated alkali-metal ions were driven into the detection cell through the connecting tube. The connecting tube and detection cell were made by elongating the end of the ion-exchange column to 0.25 mm I.D. $\times$ 20 mm and 4 mm, respectively,by

heating with a gas burner. The detection cell was placed close to the GM-tube window through a 0.7 mm ∅ hole bored through the acrylic acid resin shield, and the eluent was collected on filter papers through another hole[7]; hence fractionation is possible, if necessary. The measurement of radioactivity was followed by reading the integrated counts on the scaler every 15 sec. Therefore, the differential elution profile curve could be obtained by subtraction.

RESULTS AND DISCUSSION

Selection of ion exchanger

In order to compare the ion exchangers, the plate heights obtainable were measured and compared at various flow-rates, using radioactive ^{137}Cs solution, 3-*N* HCl as carrier, and 55–70 × 0.5 mm I.D. columns. The results are shown in Fig. 3. As expected, Dowex 50W-X8 gave high plate heights at fast flow-rates, so that it cannot be used in high-performance chromatography. Similarly, Shodex HP-175 is somewhat inadequate, but TSK LS-212, Hitachi No. 2610 and Shodex HC-125-s gave satisfactory plate heights. In the present work, all the subsequent investigations were carried out with the Hitachi No. 2610.



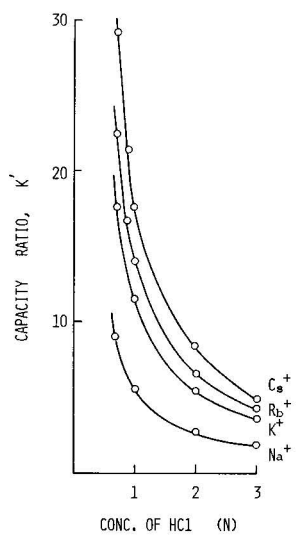


Fig. 3. The height equivalent to a theoretical plate, HETP, at various flow-rates.

Fig. 4. Capacity ratios of alkali-metal ions in hydrochloric acid media.

Separation of alkali-metal ions

The capacity ratios, k', of alkali metals in various concentrations of hydrochloric acid were measured and calculated from eqn. 2. The results are shown in Fig. 4. In this case, the volume of mobile phase in the column, V_0, was measured using the radioactive ^{131}I solution, and the value of 5.7 μl for the 72 × 0.5 mm I.D. column was obtained. Thus, the packing ratio was estimated to be *ca.* 60:40.

The chromatographic separation of alkali-metal ions was carried out with 0.7 *N* HCl as carrier solution. The elution profile curve is shown in Fig. 5, from which it can be seen that the separation of four alkali-metal ions was satisfactory. The resolutions were estimated to be *ca.*: Na/K, 4.7; K/Rb, 1.2; Rb/Cs, 1.4.

When the 80 × 0.5 mm I.D. column was used at a flow-rate of 16 μl/min, the separation time required was reduced to 15 min, and the resolutions were about: Na/K, 4.0; K/Rb, 1.0; Rb/Cs, 1.3.

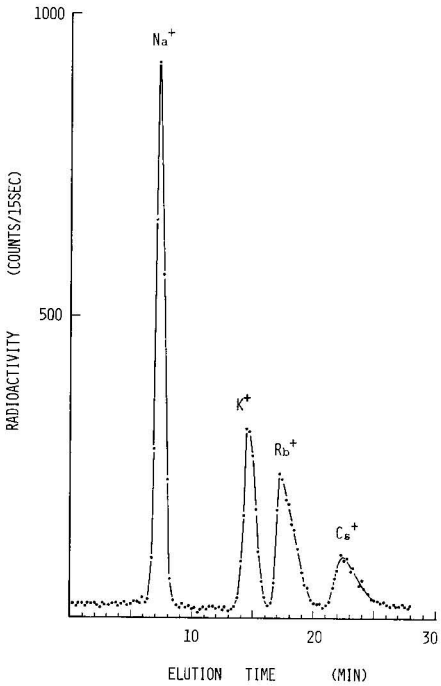


Fig. 5. Elution profile curve for cation-exchange separation of alkali-metal ions: sodium, 0.07 μg; potassium, 0.2 μg; rubidium, 3.5 μg; caesium, 6.5 μg.

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SEPARATION OF EPIMERS OF BUDESONIDE AND RELATED CORTICOSTEROIDS BY REVERSED BONDED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

Optimal conditions for the separation of the epimers of homologous 16α,17α-acetals of 6α,9α-difluoro-, 9α-fluoro- or non-fluorinated 16α-hydroxyprednisolone on μBondapak C_{18} have been evaluated. The separation factor increases strongly with increasing alkyl chain length at the asymmetrical carbon, C-22, the position where configurational differences of the molecules are located, and weakly with fluorine substitution at 6α and 9α positions. Acetate esterification of the C-21 hydroxy group does not influence the separation factor. Of the different types of organic modifier added to water in the eluents, ethanol gives the best results. Both the capacity and the separation factors increase with a decrease in the concentration of the organic modifier in the mobile phase, resulting in large improvements of separation of epimers. The retention mechanisms are discussed on the basis of molecular structures and current models of the chromatographic system.

INTRODUCTION

A great number of 16α,17α-acetals of 6α,9α-difluoro- (I), 9α-fluoro- (II) and non-fluorinated (III) 16α-hydroxyprednisolone have been synthesized and tested for anti-inflammatory and systemic activities by Brattsand *et al.*[1,2]. It has been reported[3] that the anti-inflammatory and corticoid activities of the 16α,17α-acetonide of triamcinolone are ten times greater than that of its precursor triamcinolone (II). The same enhancement was observed for the I, II and III 16α,17α-acetals[4].

The acetals were prepared by treating I, II or III with aldehydes, R–CHO, where R represents straight hydrocarbon chains with 1–9 carbon atoms. Accordingly, a new centre of chirality (C-22) was introduced into the molecule and two epimers were formed[2]. In 1969 Thalén[5] developed a method for separation and isolation of these epimers on a preparative scale by column chromatography on Sephadex LH-20, which made it possible to investigate the physiological characteristics of the separate epimers.

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At the same time, Thalén[5] found that the separation of the epimers increased in his chromatographic system with increasing chain length of the alkyl substituent at C-22. He also found that the separation factor for the epimers of 16α,17α-acetals with the same alkyl group at C-22 increased in the order I > II > III, *i.e.* with increasing fluoro substitution.

Structure elucidation of the epimers as well as of the epimeric mixtures has also been performed by Thalén and co-workers[1,2,5]. One of the acetals, budesonide, which is *ca.* 1:1 epimeric mixture of 16α,17α-(22*S*)- and 16α,17α-(22*R*)-propylmethylenedioxypregna-1,4-diene-11β,21-diol-3,20-dione, is now under clinical evaluation. The crystal and molecular structures of these epimers have been analysed by Albertsson *et al.*[6].

During the evaluation of budesonide there was need for an analysis system with high speed, linearity and sensitivity. A high separation efficiency is required as well, as in many cases the two epimers must be separated and individually determined. Preliminary investigations indicated that high-performance liquid chromatography (HPLC) system could fulfil most of the demands, whereas other chromatographic systems often used for corticosteroids, such as thin-layer chromatography (TLC), paper chromatography (PC) or gas–liquid chromatography (GLC) are inadequate with respect to speed, linearity and separation efficiency (TLC, PC) or are complicated by slow derivatization processes involving side-reactions and high-temperature operation (GLC).

As the use of GLC in the study of corticosteroids is limited, HPLC has become a very important tool for the separation and determination of these compounds, particularly in the pharmaceutical industry[7] and in clinical chemistry[8]. Adsorption chromatography on silica[9,10] or alumina[11], straight- and, especially, reversed bonded-phase chromatography[12–22] and liquid–liquid partition chromatography[22–28] have been used. A prediction of the relative merits of these different modes of chromatography is difficult, as systematic comparisons are often lacking in the literature and there are uncertainties concerning the mechanisms in adsorption and bonded-phase chromatography. However, with respect to the corticosteroids studied in this work, the retention mechanism of the reversed bonded-phase system was shown to be completely different from those operative in adsorption chromatography on silica[29]. Although the straight-phase mode was shown to be more effective for the separation in some cases, the reversed-phase mode was more selective for most of the compounds. The present work is thus devoted to studies of the latter system.

Retention mechanisms in reversed-phase chromatography, using a chemically bonded stationary phase of octadecylsilane, have been considered by several authors[14,30–41]. Karch *et al.*[34] pointed out similarities in the retention behaviour between an LC system and a GC system, based on octadecylsilane and graphitized carbon black as stationary phases, respectively. From this, they suggested an interaction of the solute with the non-polar stationary phase by dispersion forces as the main mechanism of retention. Locke[36], on the other hand, suggested that interactions between the solute and the octadecylsilane phase are weak and non-selective, and therefore the solute properties in the mobile phase are important for selectivity. Horváth *et al.*[40] and Karger *et al.*[39] used a solvophobic concept for the solute interactions in the chromatographic process, and Horváth[40] developed the concept in a thermodynamic quantitative way. On the other hand, Westerlund and Theodorsen[41] con-

sidered the influence of partition effects, as they found that a thin layer of methanol is spontaneously adsorbed on to the support (0.1 ml/g) when methanol–aqueous phosphate buffer mixtures, containing quaternary ammonium compounds, were used as a mobile phases.

Owing to the favourable properties of the C_{18} system in separating corticosteroid derivatives[29], the present work deals with various ways to obtain optimal conditions within this system. In addition the retention behaviour is discussed on the basis of current theories and the molecular structures of the solutes.

EXPERIMENTAL

Liquid chromatography

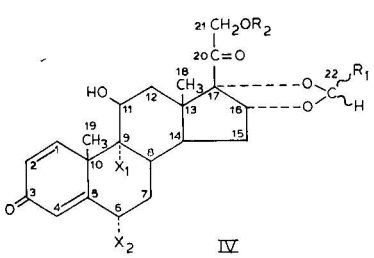
The delivery system consisted of a double-piston reciprocating constant flow pump Model M-6000 (Waters Assoc., Milford, Mass., U.S.A.). A flow-rate of 1.0 ml/min was used in all experiments, except when the HETP dependence on the flow-rate was studied. The column (30 cm × 4 mm I.D.) was packed with a microparticulate (10 ± 2 μm) octadecylsilane bonded phase (μBondapak C_{18}, Waters Assoc.). Further details concerning the packing material are given in ref. 29. A volume of 5 μl of solutions of the steroids, dissolved in ethanol or in the eluent at total concentrations of *ca.* 2 μg/μl, were injected into a U6K universal injector (Waters Assoc.). A UV absorption detector, Model 400 (Waters Assoc.), which monitors light absorption at 254 nm, was used for detection. The 12-μl flow cell has a conical shape that minimizes deflections in UV absorbance caused by changes in refractive index of the eluent.

Chemicals

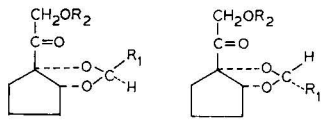
Eluents were prepared from glass-distilled water and various amounts of methanol, ethanol, 1-propanol, acetonitrile or tetrahydrofuran. The organic solvents were either of spectroscopic or pro analysi grade and they were used as received.

Corticosteroids investigated

The corticosteroids studied are described by the structural formula IV, in which $X_1 = X_2 = F$; $X_1 = F$, $X_2 = H$; and $X_1 = X_2 = H$ for the 6α,9α-difluoro, 9α-fluoro, and non-fluorinated derivatives respectively. For each of the acetals a pair of epimers, A and B, is defined by the configuration at C-22. By varying the alkyl chain length, R_1, at C-22 and by preparing esters, $R_{2,1}$ at the 21-hydroxyl, a great number of corticosteroids were synthezised and structural evidence was given by



IV



Epimer A Epimer B

nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS)[1,2,5]. The derivatives studied here are summarized in Table I.

The UV absorptivities of the corticosteroids should be similar to those of the epimers of budesonide, which in ethanol were $1.5 \cdot 10^4$ and $1.2 \cdot 10^4$ at 242 ($\lambda_{max.}$) and 254 nm, respectively, as the structure of ring A is common to all substances. At 254 nm this corresponds to a detection limit of *ca.* 10 ng at reasonable values of the capacity factor.

TABLE I

FUNCTIONAL GROUPS OF THE 16α-HYDROXYPREDNISOLONE ACETAL DERIVATIVES

Structural formulae of the compounds are given in the text.

Compounds	R_1	R_2
Acetals of 16α-hydroxyprednisolone	CH_3	H
	CH_2CH_3	H, CH_3CO
	$(CH_2)_2CH_3$	H, CH_3CO
	$(CH_2)_4CH_3$	H, CH_3CO
Acetals of 9α-fluoro-16α-hydroxyprednisolone	CH_3	H
	CH_2CH_3	H
	$(CH_2)_2CH_3$	H
	$(CH_2)_3CH_3$	H
Acetals of 6α,9α-difluoro-16α-hydroxyprednisolone	CH_3	H
	$(CH_2)_2CH_3$	H
	$(CH_2)_4CH_3$	H

Theory

The resolution, R_s, between two peaks 1 and 2 is a function of the number of theoretical plates, N, the separation factor, α, and the capacity factor, k', according to

$$R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha}\sqrt{N}\frac{k_2'}{1 + k_2'\left(\frac{\alpha + 1}{2\alpha}\right)} \quad (1)$$

For $\alpha \approx 1$, the equation is approximated by

$$R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha}\sqrt{N}\frac{k_2'}{1 + k_2'} \quad (2)$$

which is the relationship generally given in the literature for the resolution of closely spaced peaks. By introducing the number of effective plates in the column, N_{eff}, the resolution eqn. 2 is reduced to:

$$R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha} N_{eff}^{1/2} \quad (3)$$

as

$$N_{eff} = N\left(\frac{k_2'}{k_2' + 1}\right)^2 \quad (4)$$

RESULTS AND DISCUSSION

Optimization of N, k' and u

The dependence of the square root of the effective number of theoretical plates on k' for some of the 16α-hydroxyprednisolone derivatives is shown in Fig. 1. The continuous line was calculated from eqn. 4 for $N = 2100$. The close fit of experimental values to the curve indicates that N is independent of k'. As band broadening arising from slow mass transfer in the stationary phase, diffusion in a stagnant mobile phase and extra-column effects would be a function of k'[42–46], the result in Fig. 1 indicates that the band broadening originates mostly from combined mechanisms of flow and diffusion in the moving mobile phase[44,45]. These effects have been discussed in detail by Giddings[43] and by Knox and Saleem[46]. However, it should be pointed out that compared with the steroid derivatives, a larger plate number is obtained for smaller molecules, *e.g.* for benzene $N = 3400$ in the system used in Fig. 1. If band broadening originates from processes in the moving mobile phase, a reduced plate number with increasing molecule size is probably due to a slower diffusion for the larger molecules.

For the μBondapak C_{18} support the dependence of N on the linear flow-rate, u, is shown in Fig. 2. The shape of the curve is similar to that obtained by Karch

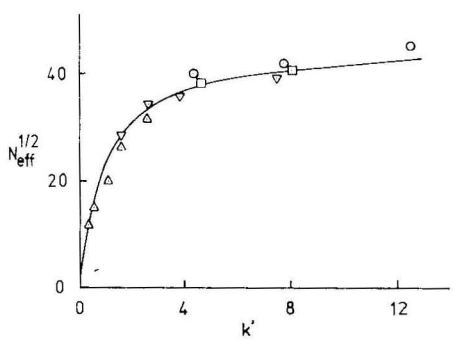


Fig. 1. Square root of N_{eff} plotted against k'. Support: μBondapak C_{18}. Mobile phases: ethanol–water, varied between 40:60 and 60:40 (v/v). Flow-rate: 1.0 ml/min. The alkyl chain at the chiral centre C-22 of the 16α-hydroprednisolone acetal derivatives was: △, CH_3 (epimer A and B); ⊡, $(CH_2)_2CH_3$ (epimer B); ▽, $(CH_2)_4CH_3$ (epimer B); ○, $(CH_2)_6CH_3$ (epimer A).

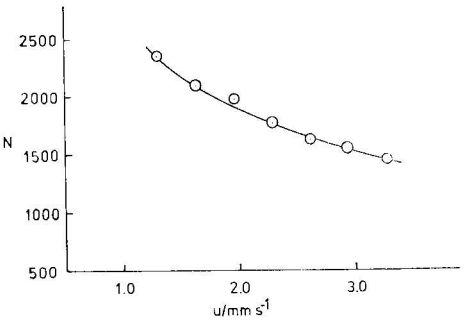


Fig. 2. Plate number plotted against linear flow-rate. Support: μBondapak C_{18}. Mobile phase: ethanol–water (42:58). Solute: epimer A of budesonide.

et al.[34] when *n*-pentanol, phenol and ethanol were chromatographed with water–methanol (1:1, v/v) as mobile phase on LiChrosorb SI-100, C_{18}, 10-μm particles. The column efficiency of the μBondapak C_{18} column for benzene (H = 88 μm, u = 1.7 mm/sec) is comparable with the LiChrosorb SI-100 column (H = 86 μm, u = 1.66 mm/sec) for *n*-pentanol[34]. As in our work they obtained a decrease in H with a smaller solute size, since a reduction of *ca.* 25% was obtained in going from *n*-pentanol to ethanol.

When the flow velocity is decreased, the plate number increases according to Fig. 2 and so does the effective plate number. A reduced flow-rate will lead to unrealistic long retention times, but this can be compensated for by increasing the strength of the eluent. However, if we select a definite retention time of the solute, k' decreases when flow decreases and, due to this, the effective plate number is reduced (see eqn. 4). Thus, for each retention time there is an optimal flow range with respect to the effective plate number, as shown in Fig. 3. The figure shows that the optimal range moves from high flow velocities to low when the retention time is increased. The efficiency increases with increasing analysis time.

Retention behaviour and optimization of α

For a reversed-phase packing material consisting of octadecylsilane groups attached to a silica surface the retention behaviour can be controlled by varying the concentration of an organic modifier in water. As shown in Fig. 4, the retention of the 16α,17α-acetals of 16α-hydroxyprednisolone increases with a decrease in the concentration of ethanol in water. For individual epimers, log k' increases linearly with the concentration of water, and this relationship has been found earlier for other compounds[14,26,39,41]. Preliminary studies on a few of the steroids in the ethanol–water eluents have indicated a linear relationship between log solubility and the proportion of water, and also that the solubilities of the homologous steroids decrease with the alkyl chain length at C-22 at any given ethanol–water ratio. This is in agreement with the model of Locke[38], but more solubility data are required for a quantitative evaluation. Locke suggests that similar types of solutes are separated on the basis of their relative solubilities, as solute adsorption on to the stationary phase involves only weak dispersive forces of interaction[38]. The result also agrees with those of Horváth *et al.*[40], who state that the relative retention is governed by solvophobic

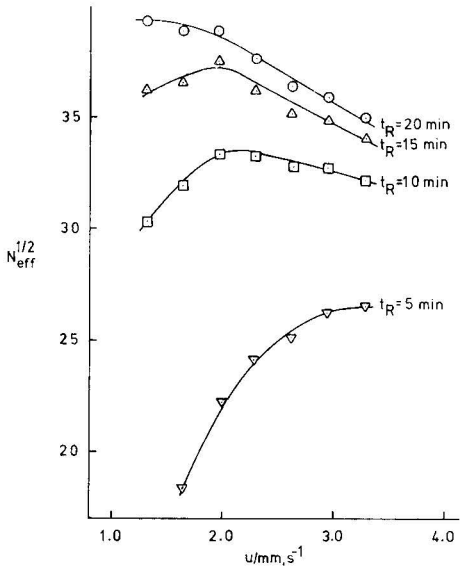


Fig. 3. Square root of N_{eff} plotted against flow-rate for various retention times of the solute. Support: μBondapak C_{18}. Solute: epimer A of budesonide. Mobile phases: ethanol–water, varied from 40:60 to 60:40 (v/v). N_{eff} values were calculated by use of Fig. 2 and eqn. 4.

forces acting on the solutes in the mobile phase. Actually, for given solutes, they predicted a linear dependence of log k' on the surface tension of the eluent. Within the mobile phase compositions reported in Fig. 4, the surface tension is a linear function of the ethanol concentration.

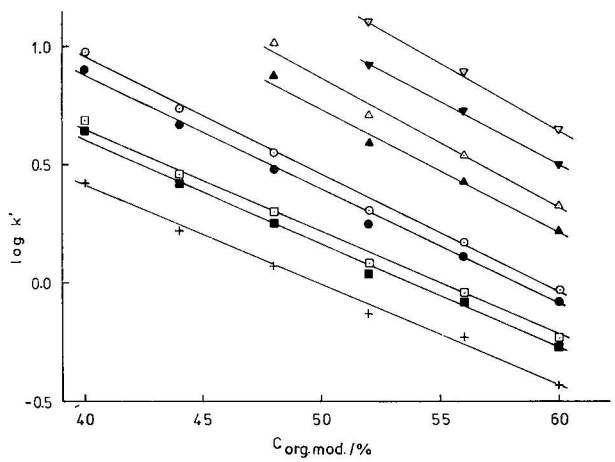


Fig. 4. Log k' plotted against concentration of ethanol in aqueous eluents (v/v). Support and flow-rate: see Fig. 1. Solutes: 16α-hydroxyprednisolone acetals with alkyl chains at the chiral centre C-22: +, CH_3 (epimer A and B); ■ □, CH_2CH_3 (epimer B and A, respectively); ● ○, $(CH_2)_2CH_3$ (epimer B and A, respectively); ▲ △, $(CH_2)_4CH_3$ (epimer B and A, respectively); ▼ ▽, $(CH_2)_6CH_3$ (epimer B and A, respectively).

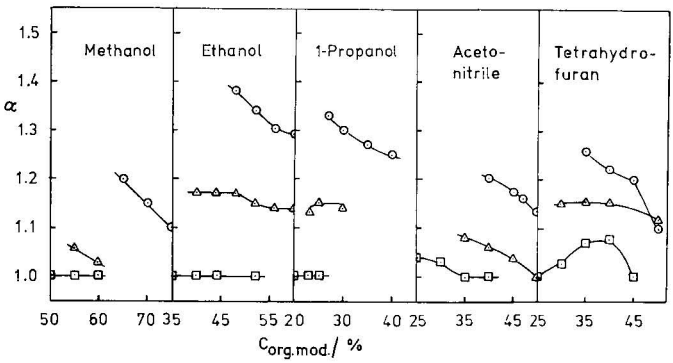


Fig. 5. Epimer separation factors plotted against the concentration of organic modifiers in aqueous mobile phases (v/v). Support and flow-rate: see Fig. 1. Solutes: as in Fig. 4 with alkyl chains at C-22: ⊡, CH_3; △, $(CH_2)_2CH_3$; ○, $(CH_2)_4CH_3$.

Separation factors obtained for the epimers of three different homologous steroids are plotted against the concentration of organic modifier in water in Fig. 5. It is seen that the levels in the values of the separation factors are different when various organic modifiers are used. This is in contradiction to the findings of Karger *et al.*[39], who found that the selectivity is roughly independent of the type of organic modifier when they separated *n*-alcohols on a chemically bonded octadecylsilane stationary phase. However, in accordance with our observations, they found that α increases with a decrease in the concentration of the organic modifier. In Fig. 5 this

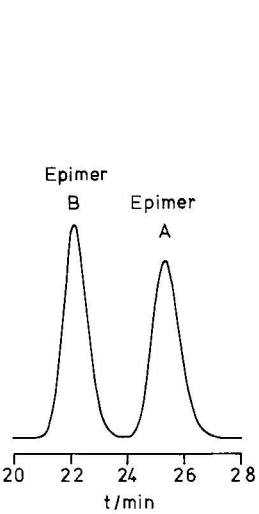


Fig. 6. Optimal separation of the epimers of budesonide. Support and flow-rate: see Fig. 1. Mobile phase: ethanol–water (40:60).

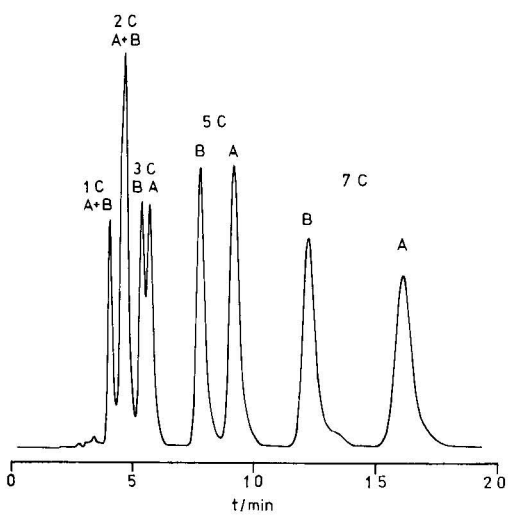


Fig. 7. Separation of 16α-hydroxyprednisolone acetal derivatives. Support and flow-rate: See Fig. 1. Mobile phase: ethanol–water (60:40). Solutes: the epimers and the number of carbons atoms in the alkyl chains at C-22 in the acetals are given in the figure.

effect is more pronounced the longer the hydrocarbon chain bonded to the asymmetric carbon at position C-22.

In the present work the selection of type and concentration of modifier is critical, as under optimal conditions a proper separation of the epimers of budesonide according to eqn. 3 ($R_s \geqslant 1.5$) is obtained when $\alpha \geqslant 1.16$. This α value is exceeded only in the ethanol–water system when the proportion of water is larger than 52% (see Fig. 5). An optimal separation of the epimers of budesonide is shown in Fig. 6.

The effect of increasing the proportion of water in the eluent is thus to improve the separation of epimers owing to the effect on k' (see eqn. 2), as well as to increases in the separation factor (see Fig. 5). This is demonstrated in Figs. 7 and 8, *e.g.* the resolution of the epimers of the hexylidenedioxy homologue, $R_1 = (CH_2)_4CH_3$, is increased from 1.8 to 3.3 when the ethanol concentration is decreased from 60 to 48% (v/v). The figures also show the drastic increase in separation of epimers with alkyl chain length and that epimer B is eluted before epimer A.

Solute structures and retention

The molecular structures of the epimers A and B of budesonide in the solid crystalline state are shown in Fig. 9. A more detailed description of the structures is given in ref. 6. In both epimers the propyl chain at C-22 is disordered and in solution the chain probably rotates freely. In both epimers the chain extends α-axially with respect to the steroid nucleus. In epimer B the E-ring is in the half-chair conformation (conformation I) whereas in epimer A C-22 has been displaced towards the steroid nucleus (conformation II). The molecular structures of the two epimers are so close that they crystallize together in the same solid solution[6], and if the structures in Fig. 9 were unique even in the chromatographic system, a separation would not be likely. Let us, therefore, consider the possibility of formation of both con-

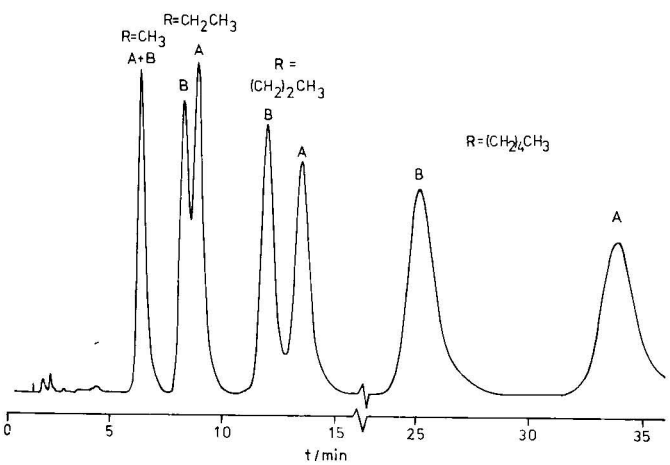


Fig. 8. Separation of 16α-hydroxyprednisolone acetal derivatives. Mobile phase: ethanol–water (48:52). For other conditions see Fig. 7.

formations of ring E for both epimers, *i.e.*:

$$\text{Epimer } A_I \rightleftarrows \text{Epimer } A_{II} \tag{5}$$

$$\text{Epimer } B_I \rightleftarrows \text{Epimer } B_{II} \tag{6}$$

where I and II denote the two conformations of ring E and where epimers A_{II} and B_I are those found in the solid state[6] and shown in Fig. 9.

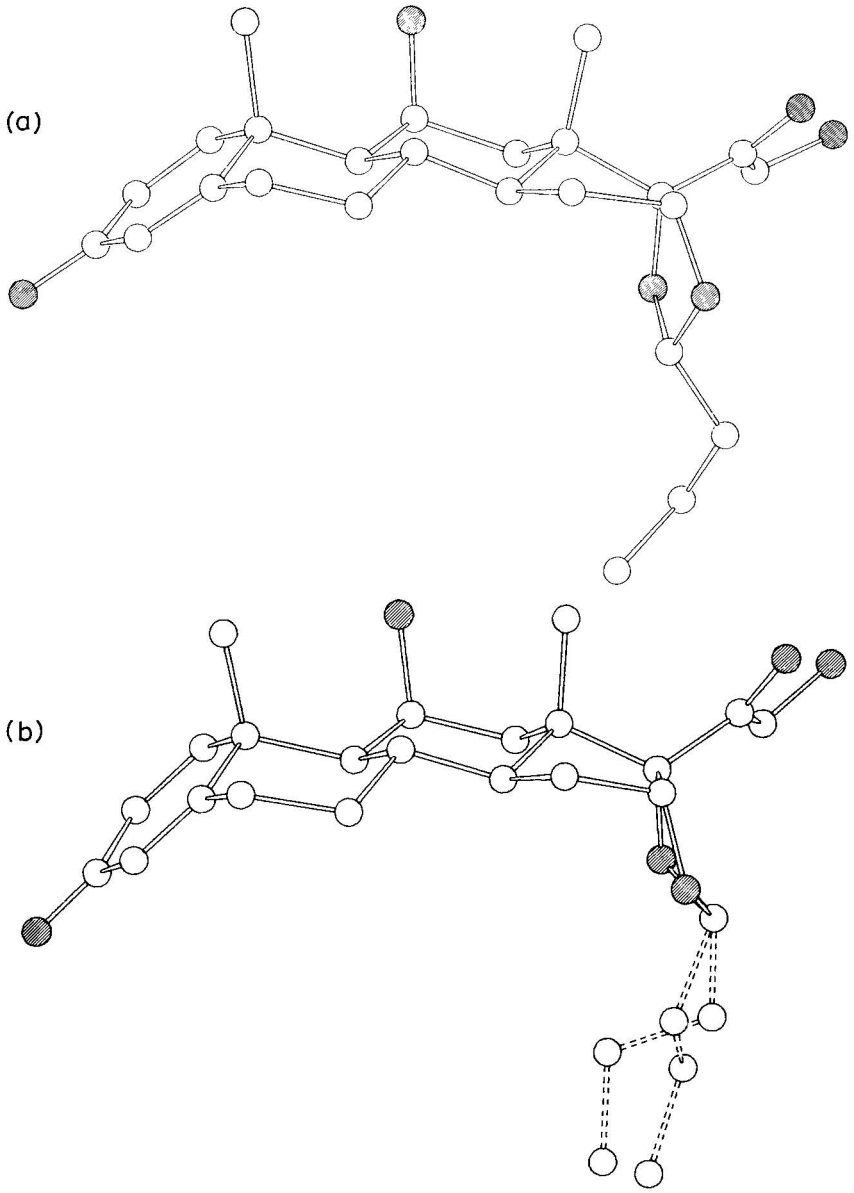


Fig. 9. Molecular structures of epimers A (a) and B (b) of budesonide. Hydrogen atoms are not shown. ○, Carbon atoms; ◍, oxygen atoms.

In epimer B_{II} the propyl chain at the chiral centre C-22 would be directed towards the steroid nucleus and the distance between the two molecular parts would probably be too close. It seems therefore likely that a flipping over from B_I to B_{II} would require a high activation energy and probably equilibrium 6 is shifted to the left even in a polar solvent such as water. In epimer A, however, there is no steric hindrance for a changeover from A_{II} to A_I. In the latter the propyl chain will extend along the main molecular direction, which favours the lipophilic character of this molecule, compared to epimer A_{II} and epimer B_I, which are more bulky. Therefore, the polarity of the surrounding media might discriminate between the two conformations A_I and A_{II} and the equilibrium 5 might be shifted to the left when the lipophilic character is increased. The extension of the alkyl chain along the molecule in epimer A_I favours a close contact between the lipophilic α-face of the steroid and the C_{18}-chain. In epimer B_I the contact area in the complex should be smaller, owing to the curved form of the molecule, (see Fig. 9). This would explain the larger retention for epimer A compared with epimer B (see Figs. 7 and 8). There is some analogy between the structural effect discussed and the effect of alkyl chain branching on retention for isomeric alcohols in reversed-phase systems. Alcohol isomers were studied by Karch *et al.*[34] and by Callmer *et al.*[47]. For instance, on μBondapak C_{18} with methanol–water (5:95) as mobile phase, the elution order for the isomeric alcohols was: tertiary, secondary, normal[47].

Horváth *et al.*[40] stated that the chromatographic process for a chemically bonded reversed phase is governed by solvophobic interactions in which the repulsion between the polar solvent molecules and the non-polar parts of the solute is of importance. They derived an expression for the capacity factor, k', which for closely related substances could be simplified to:

$$\ln k' = A'' + \frac{N}{RT} \Delta A \gamma \tag{7}$$

where A'' expresses the sum of different electrostatic effects and Van der Waals interactions between the solute and the C_{18}-ligand, γ is the surface tension of the eluent and N, R and T have their usual meanings. The contact area between the C_{18}-chain and the solute is designated by ΔA, which is a fraction of the hydrocarbon surface area (HCSA). For a homologous series, we therefore expect a linear relationship between $\log k'$ and the number of carbons in the alkyl chain at C-22, and this is shown in Fig. 10 for the 16α-hydroxyprednisolone derivatives in various eluents. It is seen that the slopes of the lines are different for the two epimers and that the slopes in-increase with increasing proportion of water in the eluent. Derivatization of eqn. 7 with respect to ΔA and insertion of the numerical constants yields

$$\frac{\mathrm{d} \log k'}{\mathrm{d}\Delta A} = \frac{\gamma}{940.7} \tag{8}$$

Derivative values calculated from eqn. 8 as well as the slopes obtained from Fig. 10, using the HCSA of 18.1 Å^2 for one methylene group[48], are given in Table II for the two epimers at different compositions of the eluent. Comparisons of the values indicate that for epimer A the contact area per methylene group varies from *ca.* 34

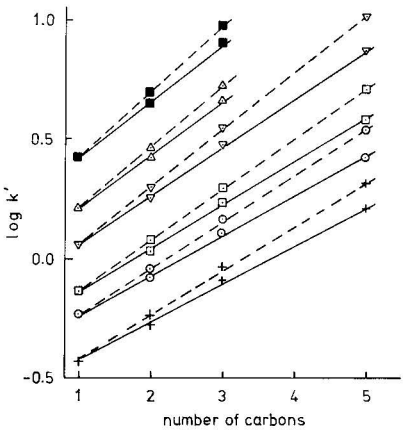


Fig. 10. Log k' plotted against the number of carbon atoms in the alkyl chain at chiral centre C-22 of epimers A (– – –) and B (———) of 16α-hydroxyprednisolone acetals. Support and flow-rate: See Fig. 1. Mobile phases: ethanol–water, (+) 60:40, (○) 56:44, (□) 52:48, (▽) 48:52, (△) 44:56, (■) 40:60).

TABLE II

DERIVATIVE VALUES CALCULATED FROM EQN. 8 COMPARED WITH SLOPES OF THE LINES FOR EPIMERS A AND B ESTIMATED FROM FIG. 10

Mobile phase, ethanol–water (v/v)	*d log k'/dΔA (Å^{-2})*	*Slope*, epimer A (Å^{-2})*	*Slope*, epimer B (Å^{-2})*
60:40	0.0292	0.0100	0.0086
56:44	0.0302	0.0109	0.0093
52:48	0.0311	0.0117	0.0100
48:52	0.0321	0.0132	0.0113
44:56	0.0330	0.0144	0.0122
40:60	0.0339	0.0156	0.0130

* A HCSA value of 18.1 Å^2 for one methylene group[48] was used in the calculations.

to 45% of the HCSA when the ethanol concentration is changed from 60 to 40%. Corresponding variation of the contact area of epimer B is 29.5 to 38.5% of the HCSA value. The higher values for epimer A are plausible if, as discussed above, there is a flipping over from epimer A_{II} in the mobile phase to epimer A_I, representing a larger area, in the stationary phase. The increase in the percentage contact areas for both epimers with decrease in the ethanol concentration may be a consequence of a diminishing HCSA, *e.g.* a folding of the alkyl chain, when the hydrophobic forces increase.

In Fig. 11, log k' is plotted against the number of carbons in the alkyl chain at C-22 for the 16α-hydroxyprednisolone and its 9α-fluoro and 6α,9α-difluoro derivatives. The increasing differences in retention with increasing alkyl chain length, resulting in various slopes for the lines of the two epimers, is clearly seen for all

derivatives. However, the slope of the line for each of the epimers A and B is constant irrespective of the substitutions of hydrogen for fluorine at 6α and 9α positions and also of the acetate esterification of the 21-hydroxy group. Introduction of fluorine in 6α and 6α,9α-positions might reduce the total HCSA, which results in smaller values of log k'. The figure also shows that the substitutions influence the two epimers slightly differently so that the separation factors are increased by 0.02 and 0.04 log units for the separation of 9α and 6α,9α derivatives, respectively. As the alkyl chain in none of the conformations of epimers A or B will sterically interfere with the acetate ester group, esterification at the C-21 hydroxyl does not influence the separation factors of epimers. However, a constant contribution to the lipophilic character of all epimers results in larger log k' values for the esters (see Fig. 11). The figure also shows that acetonides that correspond to the non-fluorinated, 9α-fluorinated and the 6α,9α-difluorinated 16α-hydroxyprednisolone derivatives fit into the straight lines of epimer A if a constant carbon number of 1.4 is taken. This would indicate that the acetonides conformationally behave more like epimer A than epimer B in the chromatographic system.

ACKNOWLEDGEMENTS

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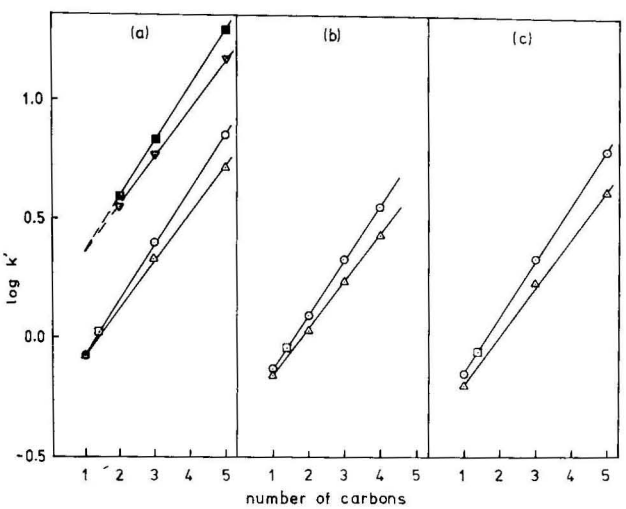


Fig. 11. Log k' plotted against the number of carbon atoms in the alkyl chain at C-22 of steroidal acetals and acetonides. Mobile phase: ethanol–water (50:50). Support and flow-rate: See Fig. 1. Solutes: (a) non-fluorinated, (b) 9α-fluoro- and (c) 6α,9α-difluoro-16α-hydroxyprednisolone acetals (△, ○, ▽, ■) or (⊡) acetonides. (△), (▽) and (○) (■) refer to epimers B and A, respectively, and (▽) (■) are 21-acetate esters.

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SEPARATION OF EPIMERS OF BUDESONIDE AND RELATED CORTICOSTEROIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A COMPARISON BETWEEN STRAIGHT- AND REVERSED-PHASE SYSTEMS

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SUMMARY

Liquid chromatographic conditions for epimer separations of homologous 16α,17α-acetals of 16α-hydroxyprednisolone were investigated in straight-phase systems (on μBondapak NH_2, μBondapak CN and μPorasil supports) and reversed-phase systems (on μBondapak C_{18}, μBondapak alkylphenyl and μBondapak CN supports). In the straight-phase systems the separation is practically independent of the alkyl chain length at the centre of chirality (C-22), whereas in the reversed-phase systems the separation is strongly influenced by the chain length. Long-chain ($\geqslant C_3$) epimers are preferentially separated by the latter systems, but straight-phase systems must be applied for the separation of short-chain epimer pairs. For separation of homologous steroids the reversed-phase systems are superior. Optimal separations of epimers were obtained using systems based on the μBondapak C_{18} and μBondapak NH_2 supports.

INTRODUCTION

A previous paper[1] has detailed the liquid chromatographic behaviour of a number of newly synthesized corticosteroids[2,3] with high anti-inflammatory potency. The investigation was carried out using a reversed-phase system based on the μ-Bondapak C_{18} packing material. The compounds studied were 16α,17α-acetals of 16α-hydroxyprednisolone with various alkyl chain lengths at C-22. The influence of fluorine substitution at the 6α and 9α positions and the esterification of the 21-hydroxyl group on retention were investigated.

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The acetalization of the 16α- and 17α-hydroxyl groups in the steroids studied leads to the introduction of a new chiral centre (C-22) and thus to the formation of two epimers[2,3]. The work aimed at evaluating the optimal chromatographic conditions for the separation of these epimers in order to facilitate quantitative determinations. It was shown that the choice of organic modifier in water was critical for the separations[1]. It was also found[1] that the reversed-phase system is highly selective with respect to the length of the hydrocarbon chain at C-22. The separation factor for epimers increased linearly from 1.0 for methyl to *ca.* 1.6 for heptyl substitution at the chiral centre. This means that a reversed-phase system is not sufficient for the complete analytical separation of all the epimers of interest.

The present work deals with different modes of high-performance liquid chromatography (HPLC), involving three reversed-phase and three straight-phase systems. As solute–stationary phase as well as solute–mobile phase interactions differ in different modes of chromatography, the use of various systems might give complementary results as shown, *e.g.* for digitalis glycosides[4] and alkylphenols[5]. The selectivity in straight-phase adsorption chromatography has been discussed by Snyder[6,7], Soczewinski[8,9] and Scott and Kucera[10], and the retention mechanisms of reversed phases have been considered by several authors, as discussed in ref. 1.

EXPERIMENTAL

Equipment

The liquid chromatograph was from Waters Assoc. (Milford, Mass., U.S.A.), involving a M 6000A pump, a U6K injector system and a M 440 UV detector (254 nm).

Column and packing materials

Prepacked columns (300 mm × 3.9 mm I.D.) were received from Waters Assoc., and the supports investigated were μPorasil, μBondapak NH_2, μBondapak CN (used both in straight- and reversed-phase modes), μBondapak C_{18} and μBondapak alkylphenyl. These supports consist of 10 $\pm$ 2 μm irregular particles. Further details concerning the packing materials were kindly supplied by Dr. D. Vivilecchia[11], and these are given in Table I. The μBondapak alkylphenyl material is newly introduced and only one column was available and tested. In other cases at least two columns were investigated. The V_0 values of the columns were in the range 2.5–3.0 ml, which is in good agreement with a total column porosity of *ca.* 0.8.

TABLE I

SUPPORTS USED IN THE PRESENT WORK

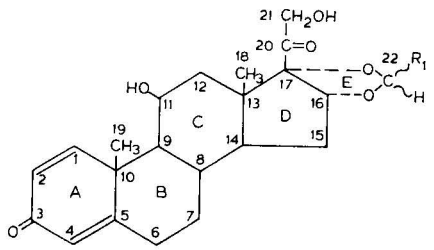
Support	*Surface area (m^2)*	*Surface coverage of functional group (μmol/m^2)*	*Packing weight per column (g)*
μPorasil	320	8	1.7
μBondapak C_{18}	320	1.1	1.9
μBondapak NH_2	320	3.6	1.8
μBondapak CN	320	3.5	1.8

Chromatographic conditions

5–10 μl samples (2 mg/ml), dissolved either in ethanol or in the mobile phase, were injected. The flow-rate in all experiments was 1.0 ml/min, which corresponds to a linear velocity of *ca.* 1.8 mm/sec. Eluents for the straight-phase systems were made from mixtures of hexane (pro analysi) and ethanol (spectroscopic grade), and the mobile phases of the reversed-phase systems were prepared from glass-distilled water and ethanol. All experiments were performed at ambient temperature.

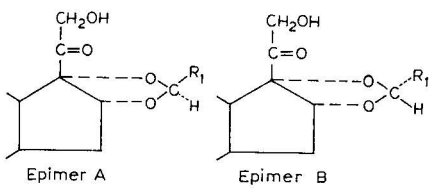
Samples

The corticosteroids investigated are described by the structural formula I (R_1 = CH_3, CH_2CH_3, $(CH_2)_2CH_3$ or $(CH_2)_4CH_3$).



I

For each of the acetals a pair of epimers, A and B, is defined by the configuration at C-22. The molecular structures of the epimers of budesonide, R_1 = $(CH_2)_2CH_3$, have been determined by Albertsson *et al.*[12], and have also been discussed briefly in ref. 1.



RESULTS AND DISCUSSION

Plate numbers of straight-phase adsorption and bonded-phase systems

Typical plate numbers for the corticosteroids in the straight- and reversed-phase (ODS) systems were 4300 and 2150, respectively, which corresponds to HETP values of 70 and 140 μm. The plate number of the straight-phase systems is thus a factor two higher than that of the ODS system. For the reversed-phase system it was suggested that the relatively low plate number might originate from combined processes in the mobile phase in which the diffusion of the solute plays an important role[1]. The sample diffusion coefficient is inversely related to the viscosity of the eluent according to the Wilke–Chang equation[13,14]. The differences in efficiency of the re-

versed and straight phases would then partly be a consequence of higher viscosity of ethanol–water mixtures compared with hexane–ethanol mixtures.

Selectivity of straight-phase systems

The selectivities of the straight-phase systems investigated are reflected by the curves in Fig. 1, where log k' is plotted against the number of carbon atoms in the alkyl chain attached to the centre of chirality. The increasing lipophilic character of the molecules, which follows the addition of methylene groups to the alkyl chain, causes a decrease in the capacity factor. The effect, however, levels out as the alkyl chain length increases, and the selectivity with respect to homologues is very low compared with the reversed-phase systems. In straight-phase chromatography interactions between polar groups of the solute and the stationary phase are the main contribution to retention. However, as the polar groups are identical for all the steroids studied, the selectivity may, to a large extent, be governed by weak dispersive solute–solvent interactions in the mobile phase. An influence of solute–solvent interactions on the selectivity is plausible, because the resolution was diminished by adding chloroform, in which the steroids are highly soluble. Alterations of the hexane–ethanol proportions had very little effect on the selectivity.

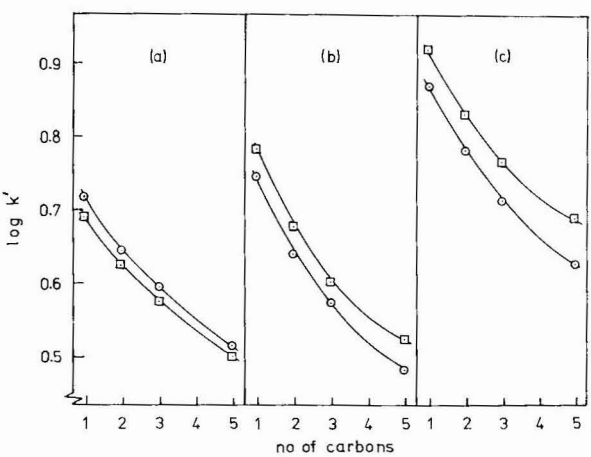


Fig. 1. Log k' plotted against the number of carbons attached to the centre of chirality (C-22) of 16α-hydroxyprednisolone derivatives. Support: (a) μBondapak CN, (b) μPorasil, (c) μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/min. Solute: (○) epimer A and (□) epimer B of 16α-hydroxyprednisolone derivatives.

The separation of epimers on different straight-phase supports increased in the order CN, SiOH, NH_2 (Fig. 1). The order corresponds to increasing polarity of the packing materials. This suggests that solute–stationary phase interactions contribute to the separations, as the same mobile phase was used in all experiments. The trend even causes the elution order of the epimers to change in the isopropyl-cyano system, as for this system epimer B is eluted before epimer A. It is noteworthy that for all the straight-phase systems the separation of epimers is mainly independent of the alkyl chain length at the chiral centre C-22, which is further demonstrated

by Figs. 2 and 3. This is logical, owing to the low selectivity of the systems with respect to the number of methylene groups in the alkyl chain, and it is also compatible with the findings of Scott *et al.*[15]. They prepared diastereomers by reaction between optically pure α-methyl-*p*-nitrobenzylamine and acylic isoprenoid acids with alkyl chains of various lengths. It was suggested that the distance between the chiral centre of the acid and the polar amide group of the derivatives was a determining factor for the degree of separation of the diastereomers in adsorption chromatography systems. For the 16α-hydroxyprednisolone derivatives studied here, the distance between polar groups, *e.g.* carbonyl at C-20, hydroxyl at C-21, oxygens at C-16, C-17 and the centre of chirality at C-22, is approximately independent of the alkyl chain at C-22. Consequently the length of the latter has only a marginal effect on the separation of the epimers. Chromatograms illustrating optimal separations of typical 16α-hydroxyprednisolone acetals in the NH_2 system are shown in Figs. 2 and 3.

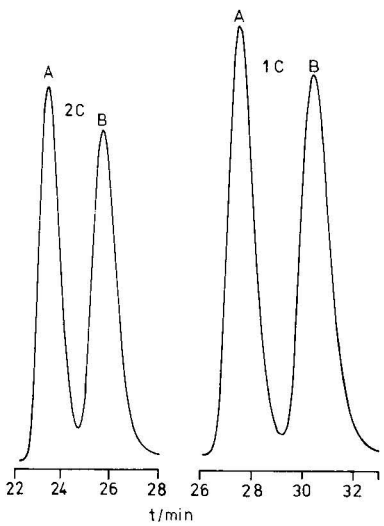


Fig. 2. Separation of 16α-hydroxyprednisolone acetals. Support: μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/min. Solute: number of carbons of the alkyl chain at C-22 and epimers are given in the figure.

The relative elution order of the epimers suggests that epimer B is more polar than epimer A. This has been proposed from a consideration of the molecular structures of the epimers of budesonide[1]. In epimer A the alkyl chain may be directed either equatorially or axially whereas in epimer B, for steric reasons, it is supposed to be directed only axially with respect to the steroid nucleus. When the chain is oriented axially in the two epimers their structures are very close. These structures are more bulky and probably more polar than epimer A with the chain directed equatorially. Moreover, in the equatorial conformation of epimer A the alkyl chain may sterically interfere with the polar groups at C-16, C-17, C-20 and C-21 and thus slightly shield the polar functional groups. This effect might decrease the polarity of epimer A compared with epimer B and the effect might be more pronounced with

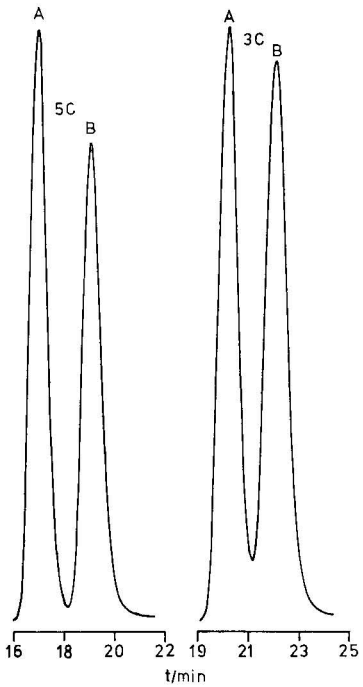


Fig. 3. Separation of 16α-hydroxyprednisolone acetals. Support: μBondapak NH_2. Mobile phase: hexane–ethanol (92.5:7.5). Flow-rate: 1.0 ml/nim. Solute: number of carbons of the alkyl chain at C-22 and epimers are given in the figure.

increasing chain length. This would even, to some extent, enhance the separation of epimers with long chain length, as was found in the silica and amino systems (Fig. 1).

Selectivity of reverse bonded-phase systems

The reversed-phase systems exhibit a more systematic retention behaviour than the straight-phase systems (Fig. 4). Fig. 4 shows that log k' varies linearly with the carbon number of the alkyl chain and that epimer B is eluted before epimer A for all the packing materials. However, the slopes of the lines representing the two epimers are different, *i.e.* the separation of epimers increases strongly with the carbon number in the substituent at C-22. It is also notable that for the two epimers with a methyl group at C-22 there is a slight separation in the alkylphenyl system, whereas negligible and no separation is obtained in the isopropylcyano and ODS systems, respectively. Chromatograms illustrating these features are shown in Figs. 5 and 6. According to Horváth *et al.*[16] the retention in a reversed-phase system is determined by solvophobic interactions between the polar mobile solvent and the non-polar parts of the solute. This gives rise to a high selectivity of the systems with respect to the number of methylene groups in the alkyl chain of the 16α-hydroxyprednisolone acetals compared with the straight-phase adsorption systems, as discussed above. Horváth *et al.* derived an expression for the capacity factor, k', saying that for closely related sub-

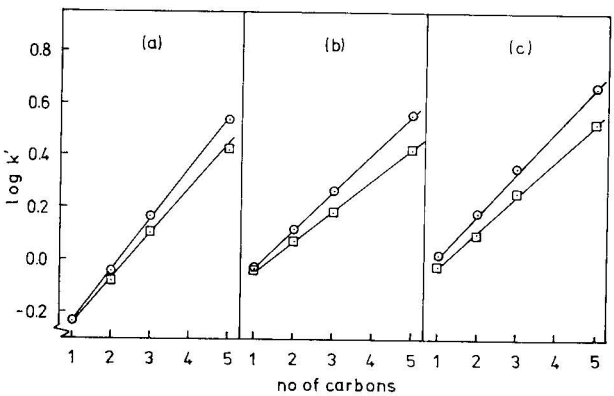


Fig. 4. Log k' plotted against the number of carbons at C-22 of the 16α-hydroxyprednisolone acetals. Support: (a) μBondapak C_{18}, (b) μBondapak CN, (c) μBondapak alkylphenyl. Mobile phase: (a) water–ethanol (44:56); (b) water–ethanol (60:40); (c) water–ethanol (45:55). Solute and flow-rate: see Fig. 1.

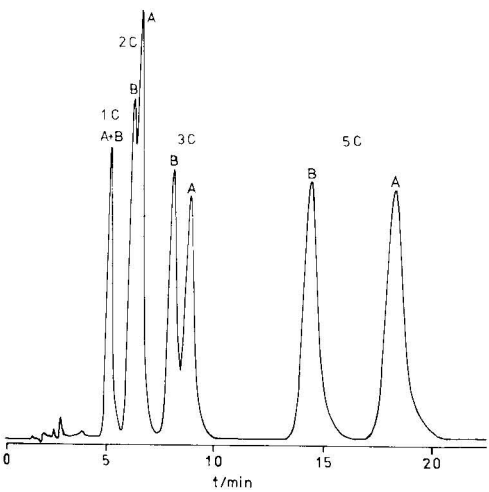


Fig. 5. Chromatogram of 16α-hydroxyprednisolone derivatives. Support: μBondapak C_{18}. Mobile phase: water–ethanol (48:52). Solute and flow-rate: see Fig. 2.

stances, $\log k'$ is linearly related to the contact area, ΔA, between the solute and the C_{18} ligand of an ODS support. Derivation of the approximated Horváth equation with respect to the contact area gives

$$\frac{\mathrm{d}\log k'}{\mathrm{d}\Delta A} = \frac{\gamma}{940.7} \tag{1}$$

where γ is the surface tension of the eluent. Eqn. 1 reflects a change in retention with a change in contact area when a methylene group is added to the alkyl chain. By comparing the slopes obtained from eqn. 1 with experimental slopes of lines like those in Fig. 4, using a hydrocarbon surface area (HCSA) for one methylene group

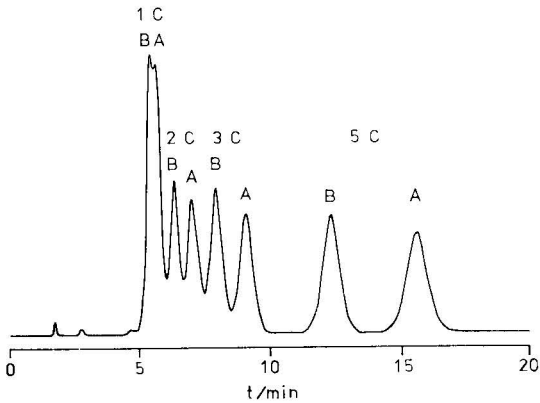


Fig. 6. Chromatogram of 16α-hydroxyprednisolone derivatives. Support: μBondapak alkylphenyl. Mobile phase: water–ethanol (45:55). Solute and flow-rate: see Fig. 2.

of 18.1 Å^2, the relative contact areas as a percentage of HCSA can be obtained[1]. Values are plotted against the concentration of ethanol in the eluents for the different packing materials (Fig. 7). It is seen that there are variations in the values with the eluent composition, and this is probably due to solvation effects[1]. However, the different levels of the curves indicate, in terms of the Horváth model, that the contact areas in the stationary phase complexes decrease in the order ODS > alkylphenyl > isopropylcyano. This also means that the selectivity increases with increasing lipophilic character of the stationary phase. The larger slopes in the plots of carbon number against log k' (Fig. 4) for epimer A compared with B indicate a closer contact between the alkyl chain of epimer A and the stationary bonded phase. This should occur if there is a flipping over from one conformation in the mobile phase (alkyl chain at C-22 directed axially) to another representing a larger HCSA, in the stationary phase (alkyl chain directed equatorially).

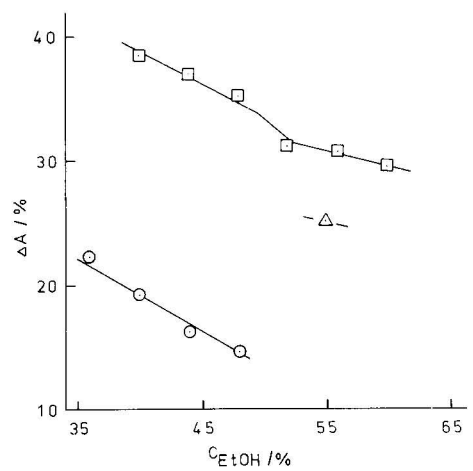


Fig. 7. Relative contact areas in percentage of HCSA for a methylene group of the alkyl chain at C-22 plotted against the concentration of ethanol in aqueous eluents. Support: (□) μBondapak C_{18}, (△) μBondapak alkylphenyl, (○) μBondapak CN.

Resolution

For closely related substances, *e.g.* for the separation of epimers of corticosteroids, the resolution equation is approximated by[1]

$$R_s = \frac{1}{4}\frac{\alpha - 1}{\alpha}\sqrt{N}\frac{k'}{1 + k'} \qquad (2)$$

In Fig. 8 the resolution of epimers is plotted against the carbon number of the alkyl chain at C-22 of the 16α-hydroxyprednisolone acetals. The plots are for $k' = 6$. Eqn. 2 shows that an increase of a factor two in N, which was obtained by changing the system from reversed to straight phase, only marginally influences R_s. Baseline separation for all epimer pairs ($R_s \geq 1.5$) is obtained only on the amino system. However, equally good or better separation is obtained in the C_{18}-system for alkyl chains of $\geq C_3$, because the separation factor increases strongly with increasing alkyl chain length. In the straight-phase systems the separation factors and resolutions are roughly independent of the alkyl chain length. In these systems the resolution of the epimers increases with the polarity of the adsorption phase, *i.e.* in the order CN, SiOH, NH_2. Comparison of Figs. 2 and 3 with Fig. 5 reveals that for homologues the reversed-phase system is superior to the straight-phase system. The selectivity increases with increasing lipophilic character of the stationary bonded phase, *i.e.* in the order isopropylcyano < alkylphenyl < ODS, which corresponds to an increase in the bristle length, as shown by Karch *et al.*[18] for *n*-alkanes.

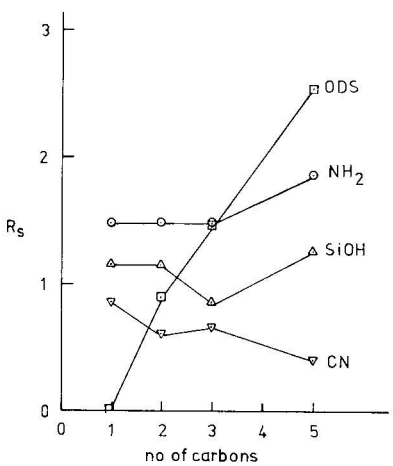


Fig. 8. The resolution of epimers of 16α-hydroxyprednisolone acetals plotted against the number of carbons at the centre of chirality (C-22). Supports are given in the figure, and $k' = 6$ was used in all cases.

The results illustrate that the HCSA of the solute is most important for selectivity of the reversed-phase systems, whereas the polar groups of the packing material and the solute play important roles in the straight-phase systems. Although, among the systems studied, the one based on ODS is best fitted for most separations, at least two systems must be used to obtain optimal resolutions of all solutes studied in the present investigation.

ACKNOWLEDGEMENT

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DETERMINATION OF THE COMPOSITION AND MOLECULAR-WEIGHT DISTRIBUTION OF A POLY(VINYL CHLORIDE–VINYL ACETATE) COPOLYMER BY GEL-PERMEATION CHROMATOGRAPHY AND INFRARED SPECTROSCOPY

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SUMMARY

A combination of infrared (IR) analysis with gel-permeation chromatography (GPC) has permitted the determination of both the molecular-weight distribution and the composition of a vinyl chloride–vinyl acetate copolymer as a function of molecular weight. A sample of the copolymer was fractionated by repeated injection into an analytical scale GPC. Copolymer films made from the fractions were then subjected to IR analysis for the determination of the vinyl acetate content. The latter was high at low molecular weights and decreased with increasing molecular weight. Q factors for the copolymer at each increment of elution volume have been calculated from the Mark–Houwink equations for polyvinyl chloride ,polyvinyl acetate and polystyrene and from the vinyl acetate content of the copolymer. Molecular-weight averages of the copolymer have been calculated using a polystyrene calibration curve and the Q factors for the copolymer which are a function of elution volume and vinyl acetate content. The values calculated by this method are in good agreement with those measured by other workers.

INTRODUCTION

It is evident that the physical properties of copolymers are dependent on their composition and molecular weight. Several studies have been made of the determination of the copolymer composition as a function of molecular weight. In one study[1], fractional precipitation followed by IR analysis and viscosity measurement of the fractions was employed for a poly(vinyl chloride–vinyl acetate) copolymer [P(VC–VAc)] while another study[2] reported the use of gel-permeation chromatography (GPC) in combination with UV and differential refractive index (RI) detectors for a styrene–butadiene copolymer. The relationship between copolymer composition and molecular weight for a poly(styrene–vinyl stearate) copolymer has been investigated by preparative GPC and IR analysis of the fractions[3], and by the rapid stop-and-go GPC–IR technique[4].

Fractionation depends upon solubility parameters which are a function of both

composition and size, so that complete separations are not obtained. GPC should be useful in this context since its primary function is to separate polymer molecules of different size, independently of polymer composition. Several problems arise when the GPC technique is applied to P(VC–VAc) for the measurement of composition as a function of molecular weight. Vinyl chloride and vinyl acetate (VAc) are insensitive to a UV detector at *ca.* 254 nm where the usual solvent tetrahydrofuran (THF) has minimal absorbance. No adequate solvent has been found for an IR detector used in series with a RI detector. However, the dependence of the composition of P(VC–VAc) on molecular weight has been determined approximately[1], and a knowledge of the relationship between copolymer compositions and molecular weight would be very important for the precise measurement of molecular-weight averages by GPC.

In a recent paper by Janča and Kolínský[5], molecular weights and compositions of P(VC–VAc) copolymer fractions obtained by GPC and fractional precipitation were determined. In the range of VAc contents between 10 and 13%, a single Mark–Houwink equation for P(VC–VAc) was obtained, which can be used as a universal calibration method for the P(VC–VAc) copolymer. However, it is doubtful whether this equation is still effective outside of the given composition range.

The measurement of the composition of P(VC–VAc) as a function of molecular weight is described in this paper. P(VC–VAc) was fractionated with analytical GPC and large fractions were collected by repeated injection. Polymer films were made from the fractions by evaporating off the solvent, and the composition of the films was determined by IR analysis. A new and simple method for the calculation of molecular-weight averages of the entire copolymer, using a combination of universal calibration and the Q-factor method, is discussed.

EXPERIMENTAL

Apparatus

A Nihonbunko (JASCO) Trirotar high-performance liquid chromatograph with a differential refractometer detector (Waters Assoc., Model R-401) was used for GPC measurements. Two Shodex A 80M high-performance GPC columns (50 cm × 8 mm I.D.) packed with a mixture of polystyrene gels of nominal porosity 10^3, 10^4, 10^5 and 10^6 Å were used. A Nihonbunko Model IR-G IR spectrometer was used for the determination of the P(VC–VAc) copolymer composition. Ultrasonic treatment was carried out in a 150-W 26-kHz (nominal) laboratory ultrasonic cleaner (Kokusai Electric Co.).

Samples

P(VC–VAc) copolymer provided by Mitsui Chemical Co. (Tokyo, Japan) was used. The product was synthesized by suspension polymerization and contained 11.6 wt. % VAc. Several different copolymers having different VAc contents between 3 and 14 wt. % were used to calibrate the IR analysis. Poly(vinyl chloride) (PVC) samples used for the examination of molecular aggregation were PVC[1] obtained by suspension polymerization. Polystyrene standards for a calibration curve were obtained from Pressure Chemical Co. (Pittsburgh, Pa., U.S.A.), and poly(vinyl acetate) (PVAc) and PVC for the measurement of Q factors were purchased from ArRo Labs. (Joliet, Ill., U.S.A.).

GPC measurements

The solvent was THF, containing 0.025% butylated hydroxytoluene (BHT) as an antioxidant, at a flow-rate of 1.5 ml/min, and the temperature was ambient. A six-port injection valve permitted sample injections of constant volume by using a 0.25-ml loop. Sample concentrations were 0.05% (w/v) for the construction of a calibration curve and 0.1% (w/v) for the calculation of molecular-weight averages. PVC and P(VC–VAc) were ultrasonically treated at room temperature for 20 min after dissolving in THF. All the sample solutions were filtered through a Millipore PTFE membrane filter (1 μm) before injection into the gel permeation chromatograph.

VAc content of P(VC–VAc)

P(VC–VAc) copolymer containing 11.6 wt. % VAc (8.7 mol %) was dissolved in THF to give a 0.5% (w/v) solution, and then ultrasonically treated for 20 min. The volume injected into the gel permeation chromatograph was 0.5 ml and six fractions were collected over the elution range of the copolymer. The volume taken for fraction 1 was 2.0 ml, for each of fraction 2–5 was 1.5 ml and for fraction 6 was 2.5 ml (see Fig. 2). This fractionation was repeated ten times and the identical fractions from the individual injections were combined. The chromatogram was corrected for concentration effects[6].

Polymer films from the combined fractions were obtained by dropping the solutions on to a PTFE sheet and evaporating the solvent under a 250-W IR lamp. BHT added in the THF was extracted from the films by immersing them in methanol overnight. For the construction of the IR calibration curve, copolymer films of known VAc content were prepared from 10 ml of 0.1% solutions. The VAc contents in copolymer films were determined from the ratio of the intensity of the carbonyl absorbance at 1744 cm^{-1} to that of the methylene absorbance at 1425 cm^{-1} (ref. 7). The preparation of polymer films and the measurement of their composition were done in triplicate.

Molecular aggregation in PVC

Solutions of 0.1% (w/v) PVC in THF were prepared and exposed to ultrasonic irradiation for 3–40 min. Also, other PVC solutions in cyclohexanone were heated without the ultrasonic treatment at 130° for 5–60 min, and then, after cooling, a 0.5-ml portion was injected into the gel permeation chromatograph.

RESULTS AND DISCUSSION

VAc content of P(VC–VAc) fractions

The compositions of fractions of P(VC–VAc) as determined by IR analysis are summarized in Table I. The calibration curve for the determination of the VAc contents was expressed as

$$\text{VAc wt. \%} = 6.4\,(D_{1744}/D_{1420}) \qquad (1)$$

where D_{1744} and D_{1425} are the absorbances at 1744 cm^{-1} and 1425 cm^{-1}. This equation is valid for the copolymer in the range of 2–15 wt. % VAc.

The average weight percent of VAc for the whole copolymer as calculated from the VAc content and the weight percent of each fraction was 11.57. This is in agreement with the analysis of the unfractionated sample. The weight percent of

TABLE I

VINYL ACETATE CONTENT OF P(VC–VAc) FRACTIONS

Wt.% of VAc in unfractionated sample, 11.6. mol.% of VAc in unfractionated sample, 8.7. wt.% of VAc in the whole copolymer, calculated from each fraction, 11.57.

Fraction No.	*VAc (wt. %)*				*VAc (mol %), mean*	*Weight per cent fraction*
	Run			*Mean*		
	1	*2*	*3*			
1	5.9	6.3	6.9	6.4	4.7	7.1
2	10.0	10.0	10.6	10.2	7.6	24.4
3	11.3	11.0	11.9	11.4	8.6	31.8
4	14.0	12.4	13.1	13.2	10.0	22.6
5	14.5	14.2	14.0	14.2	10.7	10.3
6	14.6	15.0	14.2	14.6	11.1	3.8

each fraction was calculated from the area of the chromatogram (see Fig. 1). A correction was made for the difference in response factors between PVC and PVAc. A tendency for the VAc content to increase from the first fraction to the last, *i.e.*, with decreasing molecular weight of the copolymer, can be observed (Table I). Similar results have been observed by Takeuchi and Mori[1] and Janča and Kolínský[5].

Determination of Q factors for PVC and PVAc

The measurement of polymer molecular-weight averages by GPC requires the construction of a calibration curve. A series of relatively monodisperse polystyrenes is generally used for the calibration of GPC systems. However, the calibration curve thus obtained cannot be applied directly to polymers other than polystyrene. The universal calibration method must be used in these cases[8]. In practive, knowing the Mark–Houwink equations for polystyrene and the polymer in question, the standard polystyrene curve can be transformed for the polymer in question.

A less accurate, but simpler procedure for the calculation of molecular-weight averages is to use the Q factor. This factor is obtained by dividing the molecular weight of the monomer unit by its extended chain length calculated from bond lengths and angles. Molecular-weight averages of the given polymer are obtained by calculating extended chain-length averages using a polystyrene calibration curve of log (extended chain length) *versus* elution volume. These values are then multiplied by the Q factor of the polymer. This procedure would be accurate if both the ratios of the extended chain length and the actual chain length in solution for polystyrene and the given polymer are the same or vary in the same proportion. Since the calculated Q factors of some polymers are usually inaccurate, it is preferable to first determine the Q factors experimentally in the same solvent at the same temperature, using fractions of the given polymer[9]. Although the use of Q factors for GPC molecular-weight calculations is not an accurate approach, it is still valuable for the calculation of copolymer molecular-weight averages because copolymer composition varies somewhat as a function of the copolymer molecular weight.

The curve of log M *versus* elution volume for a polymer B can be obtained from that for a polymer A using[10]

$$\log M_B = \frac{1}{1 + a_B} \cdot \log \frac{K_A \Phi_B}{K_B \Phi_A} + \frac{1 + a_A}{1 + a_B} \cdot \log M_A \qquad (2)$$

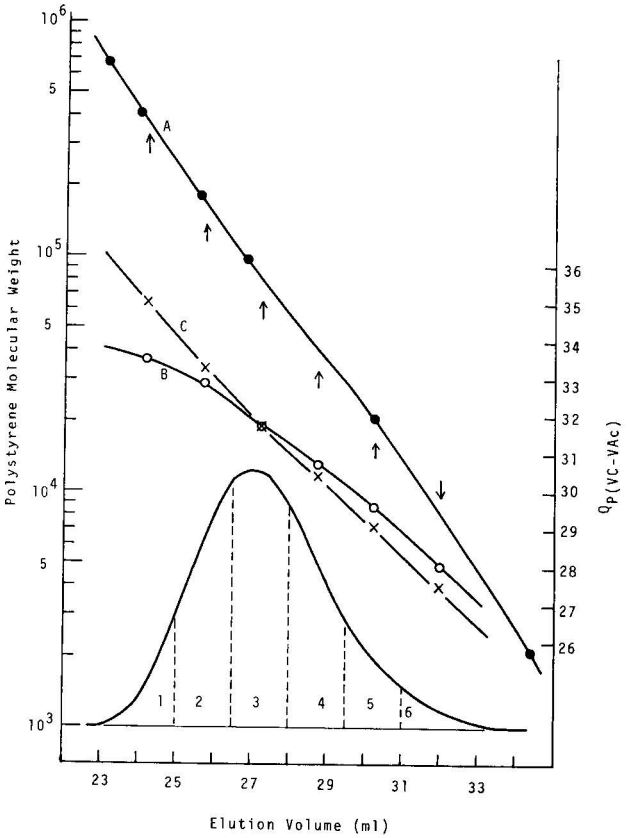


Fig. 1. Gel permeation chromatogram and Q factor for P(VC–VAc). A = polystyrene calibration curve; B = relation between Q factor and elution volume at different VAc contents; C = relation between Q factor and elution volume at constant VAc content.

where a and K refer to the Mark–Houwink coefficients for each polymer in the solvent used for GPC and at the experimental temperature; the Φ terms are obtained from

$$\Phi = \Phi_0(1 - 2.6\varepsilon + 2.86\varepsilon^2) \tag{3}$$

$$\varepsilon = (2a - 1)/3 \tag{4}$$

Φ_0 is a constant which cancels out. A refers to the standard polystyrene and B to the polymer in question. Taking polymer B as PVC or PVAc in eqn. 2, the molecular weights of PVC or PVAc molecules (M_{PVC}, M_{PVAc}) eluted at the same volume as a polystyrene molecule having molecular weight M_{PSt} can be calculated. Assuming that the Q factor of polystyrene is constant over the whole molecular-weight range and its value is 41.3, the Q factors of PVC and PVAc can be obtained from

$$Q_{PVC} = \frac{M_{PVC} \times 41.3}{M_{PSt}} \tag{5}$$

$$Q_{PVAc} = \frac{M_{PVAc} \times 41.3}{M_{PSt}} \tag{6}$$

Using several pairs of Mark–Houwink coefficients for PVC and PSt in THF at 25° from the literature, various series of Q factors for PVC were calculated. Some results are listed in Table II. As can be seen from Table II, the Q factors for PVC and PVAc increase or decrease with increasing molecular weight, unless the Mark–Houwink exponents of both PSt and PVC or PVAc are equal. According to the specifications for PVC provided by ArRo Labs., the Q factors for PVC vary with molecular weight and are 25.4 at molecular weight 2.5×10^4 and 28.0 at molecular weight 1.3×10^5, assuming the Q factors for PSt to be constant. (This assumption does not affect the values of calculated molecular-weight averages for PVC and PVAc, providing polystyrene is used as the primary standard.)

TABLE II

SEVERAL SERIES OF Q FACTORS FOR PVC (SERIES I–VI) AND PVAc (SERIES VII–IX)

Molecular weight of PSt (M_A)	*Series* *I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*
2100	27.3	27.7	25.2	29.7	27.1	23.9	36.5	41.1	42.4
20,400	25.8	29.1	25.2	27.5	25.4	27.0	41.6	50.8	48.3
97,200	24.8	30.0	25.2	26.1	24.2	29.4	45.6	58.8	52.8
180,000	24.4	30.4	25.2	25.5	23.8	30.5	47.3	62.3	54.7
411,000	23.9	30.9	25.2	24.8	23.2	31.9	49.6	67.3	57.3
670,000	23.6	31.2	25.2	24.4	22.8	32.7	51.0	70.4	58.9
1,800,000	23.0	31.8	25.2	23.6	22.2	34.5	54.0	77.3	62.4
PSt									
$K \times 10^4$	1.17	1.91	0.682	1.60	1.17	0.86	1.17	0.86	1.91
a	0.725	0.682	0.766	0.706	0.717	0.74	0.725	0.74	0.682
PVC (or PVAc)									
$K \times 10^4$	1.60	5.01	1.63	1.63	1.50	5.01	3.5	4.28	4.28
a	0.75	0.649	0.766	0.766	0.77	0.649	0.63	0.591	0.591
Ref.	11	12	13, 14	14	15	12, 16	11	16, 17	12, 17

Series II and VI in Table II were assumed to provide effective Q factors for PVC. Molecular-weight averages for standard PVC and PVAc were calculated using eqn. 2 and Mark–Houwink coefficients from series II, VI, VIII and IX in Table II. The results are shown in Table III. The Q factors in series VI and VIII are believed to be better than the other values, when compared to these values. Several other combinations of Mark–Houwink coefficients for PSt and PVC from ref. 18 were evaluated, but the results were not satisfactory. Substituting the coefficients from series VI and VIII into eqn. 2 yields the following equations from which the molecular weights of PVC and PVAc corresponding to that of PSt appearing at the same elution volume may be calculated

$$\log M_{PVC} = -0.4224 + 1.0552 \log M_{PSt} \tag{7}$$

$$\log M_{PVAc} = -0.3137 + 1.0937 \log M_{PSt} \tag{8}$$

TABLE III

MOLECULAR-WEIGHT AVERAGES FOR STANDARD PVC AND PVAc OBTAINED BY GPC

Standard values were obtained from ArRo Labs. $\bar{M}_w$ was obtained from light-scattering data using THF as solvent at 25°. $\bar{M}_n$ was obtained using membrane osmometry, with THF as solvent at 25°. Calculated Q factors are: 24.8 for PVC and 34.1 for PVAc.

Polymer	*Standard value*	*Using calculated Q factor*	*Using series II and IX*	*Using series VI and VIII*
PVC	$\bar{M}_w \times 10^{-4} = 13.2$	10.6	13.0 ± 0.2	12.8 ± 0.2
	$\bar{M}_n \times 10^{-4} = 5.40$	5.0	6.0 ± 0.2	5.7 ± 0.2
	$\bar{M}_w/\bar{M}_n = 2.44$	2.12	2.2	2.2
PVAc	$\bar{M}_w \times 10^{-4} = 33.1$	15.7	25.3 ± 0.2	29.5 ± 0.2
	$\bar{M}_n \times 10^{-4} = 8.30$	5.6	8.2 ± 0.4	8.8 ± 0.4
	$\bar{M}_w/\bar{M}_n = 3.99$	2.80	3.1	3.4

Q factors corresponding to these molecular weights are

$$Q_{PVC} = 15.62\, M_{Pst}^{0.0552} \tag{9}$$

$$Q_{PVAc} = 20.06\, M_{Pst}^{0.0937} \tag{10}$$

Calculation of molecular-weight averages of P(VC–VAc)

Systematic studies of the molecular-weight distribution of P(VC–VAc) by GPC and viscosity measurement were reported[5,19] and Mark–Houwink equations were proposed. GPC was used to fractionate P(VC–VAc). Number-average[19] and weight-average[5] molecular weights of fractions were determined in conjunction with their intrinsic viscosity. However, Chen and Blanchard[19] did not mention the variation of composition of the fractions and Janča and Kolínský[5] obtained an equation valid only for a limited VAc range. It is not practical to calculate molecular-weight averages for P(VC–VAc) copolymer using a universal calibration curve obtained with a single Mark–Houwink equation[5]. One must take into account not only the overall average composition but also heterogeneities in composition of the copolymer.

I propose an improved method for the calculation of molecular-weight averages of copolymers having heterogeneities in composition. This method can be applied to any copolymers if Mark–Houwink equations for the homopolymer components of the copolymer and for a standard polymer such as polystyrene in the solvent used in GPC and at the experimental temperature are known. Construction of a calibration curve for each homopolymer is not necessary. It is assumed that the chain length of P(VC–VAc) in solution is the sum of the VC and VAc parts of the copolymer. The chain length of both parts must be governed by the overall chain length of the copolymer. The Q factor for P(VC–VAc) at the given elution volume is then calculated using

$$Q_{P(VC-VAc)} = m_{PVC}\, Q_{PVC} + m_{PVAc}\, Q_{PVAc} \tag{11}$$

where m_{PVC} and m_{PVAc} are the molar fractions of VC and VAc in P(VC–VAc) and Q_{PVC} and Q_{PVAc} are the Q factors for PVC and PVAc at the same elution volume as that of P(VC–VAc) and are obtained from eqns. 9 and 10. The mol. % of VC in copolymer is the difference.

The Q factors of P(VC–VAc) are calculated as follows. (a) The chromatogram of P(VC–VAc) and a polystyrene calibration curve of molecular weight *versus* elution volume are plotted as in Fig. 1. The areas defined by the dashed lines represent each fraction. (b) The polystyrene molecular weight of each fraction is determined from the average elution volume of the fraction using the polystyrene calibration curve (Fig. 1, arrows). (c) Substituting these polystyrene molecular weights into eqns. 9 and 10 yields Q factors for PVC and PVAc at the average elution volume of each fraction of P(VC–VAc). (d) These Q factors and molar fractions in Table I are substituted into eqn. 11 and Q factors for P(VC–VAc) at each average elution volume are obtained. (e) Plotting the Q factors for P(VC–VAc) and the average elution volume and connecting each point yields a calibration curve giving the Q factor of P(VC–VAc) at the elution volumes. (f) The molecular weight of P(VC–VAc) at any elution volume can be obtained by multiplying the polystyrene extended chain length at the elution volume by $Q_{P(VC-VAc)}$. Molecular-weight averages for P(VC–VAc) are calculated by the usual procedure.

The calculated values of the molecular-weight average for P(VC–VAc) are shown in Table IV. For comparison, three other molecular-weight averages are listed. These were calculated from the molecular weights of polystyrene, from the Q factor (= 25.6) obtained from the molecular weight, bond length and angle of the monomer unit and from Q factors obtained assuming a constant VAc content.

TABLE IV

MOLECULAR-WEIGHT AVERAGES FOR P(VC–VAc) CALCULATED BY USING SEVERAL METHODS

Method	$\bar{M}_w \times 10^{-4}$	$\bar{M}_n \times 10^{-4}$	$\bar{M}_w/\bar{M}_n$
Method as in the text	7.87	3.66	2.2
Response correction	7.78	3.61	2.2
At constant VAc content	8.03	3.64	2.2
At $Q = 25.6$	6.31	3.10	2.0
As polystyrene	10.20	5.00	2.0
Using literature value*	8.40	4.03	2.1

* $[\eta] = 6.72 \times 10^{-4} M_w^{0.611}$ for P(VC–VAc) (ref. 5); $[\eta] = 0.86 \times 10^{-4} M_w^{0.74}$ for PSt (ref. 16).

When a differential refractometer is used as a detector, its response is proportional to the differential refractive index of the solute and the solvent and to the concentration of the solute. The specific refractive index increment ($\partial n/\partial c$) of PVC (= 0.115) is twice that of PVAc (= 0.058) (ref. 18). The system was calibrated by injecting various amounts of PVC and PVAc. The areas under the chromatograms were determined by graphical integration and used to calculate the response-factor ratio. The results were close to the above values. The chromatogram of P(VC–VAc) must be corrected by multiplying the response of the VAc unit by 2 using eqn. 12:

$$(H_{corr.})_i = (H_{uncorr.})_i \times (1 + W_{VAc,i}) \tag{12}$$

where $(H_{corr.})_i$ is the corrected height of each increment of the elution volume in question, $(H_{uncorr.})_i$ is the uncorrected height of each increment of the elution volume and $W_{VAc,i}$ is the weight fraction of VAc content at the elution volume. Molecular-weight averages calculated from the corrected chromatogram are shown in Table IV.

The weight-average molecular weight of P(VC–VAc) determined using light scattering was 7.7×10^4 and is in good agreement with the experimental values in Table IV. The molecular-weight averages calculated assuming a constant VAc content and that calculated using the Mark–Houwink equation obtained by Janča and Kolínský[5] are slightly higher. Large differences were not observed between these values since the VAc content is not high. Fig. 2 shows a normalized molecular-weight distribution of P(VC–VAc) and the VAc composition as a function of molecular weight.

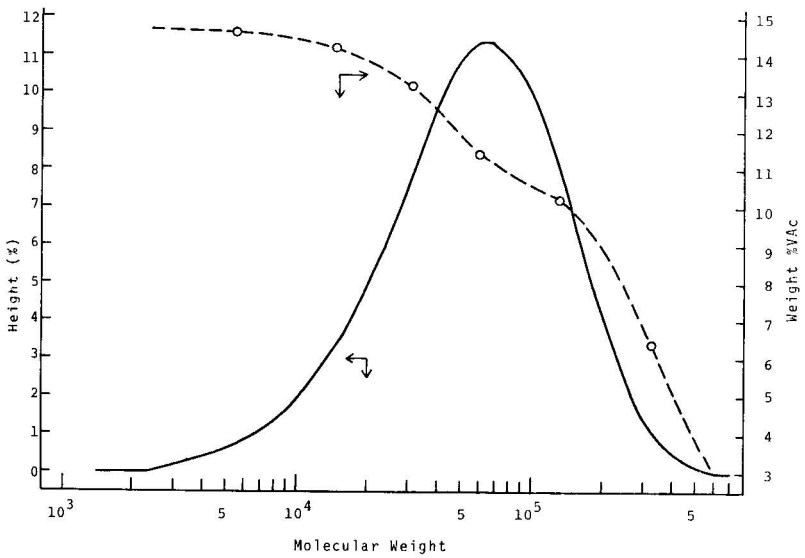


Fig. 2. The composition (– – –) and molecular-weight distribution (———) of P(VC–VAc).

The problem of molecular aggregation in PVC

The presence of molecular aggregates in PVC complicates the molecular-weight determinations. Several attempts have been made to eliminate the aggregates[12,20,21]. The dispersal of aggregates by ultrasonic treatment of a PVC solution in THF for periods as brief as 15 min has been reported[12]. Simultaneous degradation of the PVC molecules appeared to be prevented by the addition of a small amount of a non-ionic surfactant to the solutions. Heating PVC at 90° in THF for 2 h was also found to dissociate the aggregates[21].

In the present study the effects of ultrasonic and heat treatments were investigated. Molecular-weight averages were changed appreciably by these treatments (decreases by *ca.* 12–15%), but almost identical values were obtained after ultrasonic treatment for 5 min and heat treatment for 10 min. These experiments support the conclusion that dispersion of PVC molecules by these treatments is effective, and

that molecular degradation is essentially absent during these treatments. Similar results have been obtained when a non-ionic surfactant was added to the PVC solution. This implies that the non-ionic surfactant may not be necessary. Heating at a temperature of 95° was not effective in dispersing the aggregates even after heating for 60 min. Boiling of THF solutions had little dispersive effect.

After ultrasonic irradiation the high-molecular-weight tail disappeared, the entire chromatogram moved toward lower molecular weights and the height of the peak maximum increased. The low-molecular-weight portion was unchanged by ultrasonic treatment. Membrane filters of 1-μm pore diameter were blocked by an ultrasonically untreated THF solution of P(VC–VAc).

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GASEOUS DEGRADATION PRODUCTS FROM THE PYROLYSIS OF INSULATING MATERIALS USED IN LARGE ELECTRICITY GENERATORS

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SUMMARY

Samples of insulating materials, typical of those used in large generators were pyrolysed at 700° in a hydrogen atmosphere and the products analysed by combined gas chromatography–mass spectrometry.

It is shown that the identification of a wide range of compounds is possible and that characteristic groupings and single compounds are produced from particular insulating materials.

Monitoring the generator cooling hydrogen for these products provides early warning that specific insulating materials within the generator are starting to degrade.

INTRODUCTION

Large electricity generators contain a number of organic insulating materials which may degrade to give gaseous products, either by ageing or because of electrical faults which produce hot spots or arcing. The value of gas analysis in the detection of faults in oil-filled transformers is well established[1]. Similar techniques can be applied to generators. By regular analysis of the hydrogen used for cooling the generator, impurities known to result from the degradation of a particular insulating material or from a specific degradation process can be identified. This may provide a valuable early warning of a developing fault. The method used for analyzing the cooling hydrogen for a wide range of possible insulation degradation products has been reported elsewhere[2].

Products which are characteristic of the degradation of a range of insulating materials at temperatures between 160 and 280° and for periods from 2 to 28 days have been reported[3]. This paper describes the results of degrading the same insulating materials for 10 sec at 700°. A temperature of 700° was considered to be realistic and consistent with typical generator fault temperatures and the need to produce sufficient amounts of degradation product to allow identification.

EXPERIMENTAL

The following insulating materials were studied in this work: epoxy-resin bonded mica; copal-resin bonded mica; high-temperature-resistant nylon paper;

phenolic-resin impregnated wood; press paper; asphalt-based core-plate varnish; lignin sulphonate-based core-plate varnish.

Small pieces of the insulating material were inserted into a disposable silica silica tube (25×2 mm I.D.) and held in place with ceramic wool. The silica tube, which was weighed before and after pyrolysis to determine the weight of material lost, was fitted in the coil probe of a Chemical Data Systems Pyroprobe 100 pyrolyzer. This in turn was fitted into the injector block of a Perkin-Elmer Model F30 gas chromatograph which was linked by a silicone-rubber membrane interface to a VG Micromass 12 mass spectrometer. The signal from the flame ionization detector of the gas chromatograph was recorded on a potentiometric recorder and the peak areas were measured using an Infotronics CRS 204 integrator. The degradation products were separated using Porapak Q and Tenax GC columns. Hydrogen was used as the carrier gas in both cases so that pyrolysis could occur in conditions as similar as possible to those occurring in a generator.

RESULTS

It is of value to determine the relative amounts of the degradation products found, in order to assess their potential as characteristic products. However, it is impractical to do this accurately, due to the difficulties involved in calibrating the chromatograph and the mass spectrometer for all the compounds present. Consequently the results are only semi-quantitative. Table 1 gives the relative amounts of degradation product as a convenient series of ranges, n, derived from the number of integrator counts, I, using the relationship $I \times 10^{-6} = 2^{n-1}$ *i.e.* $n = 1$ defines the range $0 < I \leqslant 10^6$, $n = 2$ defines the range $10^6 < I \leqslant 2 \times 10^6$ etc. The values of n range from 1 to 10. The integrator counts for unresolved peaks were apportioned using a method based on the relative size of the largest peaks in the mass spectrum of each compound.

DISCUSSION

Epoxy-resin bonded mica

Details of the manufacture and chemical nature of this material have been reported by Parriss[4]. Products resulting from the pyrolysis of various types of epoxy resin have been reported by Lee[5,6] who pyrolysed two resins at 350 and 450°; by Gac *et al.*[7] (450 to 800°); Bishop and Smith[8] (400 to 700°); Weigand and Hanna[9] (600°); Sakamoto *et al.*[10] (up to 290°); Andreev *et al.*[11] (200°) and Iglauer[12] (1000°). Their results are generally similar to the results of this work although in most cases only a small number of products were identified.

Bishop and Smith[8] pyrolysed two resins for 30 sec at 400, 500, 600 and 700°. In both cases the effect of increasing the pyrolysis temperature was to increase the amounts of pyrolysis products produced allowing a larger number of products to be identified. No compounds which were identified at a lower pyrolysis temperature were absent at a higher temperature. This would indicate that the main effect of increasing the pyrolysis temperature over this range is to increase the amount but not change the types of product produced.

Lee[5,6] and Bishop and Smith[8] give degradation schemes which explain the

source of some of the products found. The low-molecular-weight hydrocarbons derive from the carbon chains in the molecule; the alkyl chlorides from residual epichlorohydrin used in epoxy manufacture; acraldehyde, acetaldehyde and acetone derive from the chains between the benzene rings. The other oxygen-containing alkyl compounds also probably derive from this source. Butanone is used as a solvent for the resin. Benzene and benzene substituted with hydroxyl and alkyl groups originate from the benzene rings and side chains in the polymer.

Copal-resin bonded mica

This material is manufactured from a processed blend of esterified natural copal resin, hydrocarbon resin and processed unsaturated oils (linseed, tung). The solvent is white spirit. The final product will consequently have a highly complex and indeterminate structure. The degradation products comprise primarily aromatic hydrocarbons, alkanes and alkenes. Aldehydes are of importance in this case, since three out of a total of five aldehydes found were not detected amongst the degradation products of the other materials studied. Small amounts of ethanol, acetone and cresol were also detected.

Nylon paper

This material has the general formula:

$$—(–Ar\text{-}CO\text{-}NH\text{-}Ar\text{-}NH\text{-}CO–)_n—$$

where Ar represents a benzene ring with *meta* linkage. Iglauer[12] has reported the presence of benzene, aniline and toluene resulting from the pyrolysis of nylon paper at 1000°. In this work the major products were aniline, N,N-dimethyl acetamide, benzene and cyclohexene. Only a small amount of toluene was detected. Other products included small amounts of phenol, alkenes, 1,4-dioxan and acetic acid.

Phenolic-resin impregnated wood

This material is made by impregnating beechwood with a modified phenolic resin[13] which is cured by heat and pressure. The degradation products might be expected to be a mixture of those for the resin and the wood. Aromatic hydrocarbons and phenols have been shown to be typical of phenolic resin degradation at 700–750° by Zulaica and Guiochon[14]. Jackson and Conley[15] have studied the oxidative degradation of phenolic resins between 300 and 1000° and found little variation in the type of degradation product over a wide temperature range.

Goos and Reiter[16] have shown that oxygen-containing compounds such as alcohols, acids, aldehydes, ketones, esters and furans are all typical of wood degradation. In this work a large number of typical compounds from both resin and wood were found in the pyrolysis products.

Press paper

This material comprises some 99.5% cellulose. Wodley[17] has analyzed the degradation products of α-cellulose, pyrolyzed at 330° in nitrogen. His results are similar to this work in which a wide range of carbonyl compounds were found amongst the degradation products, together with alcohols and furans. A number of

TABLE I

DEGRADATION PRODUCTS

This table gives the relative amounts of degradation product as a convenient series of ranges, n, derived from the number of integrator counts I, using the relationship $I \times 10^{-6} = 2^{n-1}$, *i.e.* $n = 1$ defines the range $0 < I \leqslant 10^6$, $n = 2$ defines the range $10^6 < I \leqslant 2 \cdot 10^6$, etc. The compounds which have been underlined in the table uniquely or predominantly derive from one particular insulating material.

Degradation products		*Epoxy-resin bonded mica, Type 1*	*Epoxy-resin bonded mica, Type 2*	*Copal-resin bonded mica*	*Nylon paper*	*Phenolic-resin impregnated wood*	*Press paper*	*Asphalt-based core-plate varnish*	*Lignin sulphonate-based core-plate varnish*
Name	*Formula*								
Methane	CH_4	5	7	3	1	4	3	7	8
Ethylene	C_2H_4	4	5	3	3	1	3	6	4
Ethane	C_2H_6	3	5	2	1	2	2	7	5
Hydrogen cyanide	HCN					1			3
Hydrogen sulphide	H_2S								1
Propylene	C_3H_6	7	9	3	1	1	3	8	4
Propane	C_3H_8			3	1	1	3	7	3
Formaldehyde	HCHO						3		
Methyl chloride	CH_3Cl	4	5		1	1			4
Dimethyl ether	CH_3OCH_3	1	4						4
Sulphur dioxide	SO_2								7
Methanol	CH_3OH	3	2			4	1	1	6
Acetaldehyde	CH_3CHO	7	8	3	1	4	4	7	3
Dimethyl disulphide	$(CH_3S)_2$								2
Butene	C_4H_8	1	2	4	1	2	3	6	3
Butane	C_4H_{10}		1	3	1	1	2	5	3
1,3-Butadiene	C_4H_6			4					
Ethyl chloride	C_2H_5Cl	3	5						
Ethanol	C_2H_5OH	3	5	1	1	1			
Methyl cyanide	CH_3CN				1	2		3	2
Propionaldehyde	C_2H_5CHO			4					
Furan	C_4H_4O			1		2	4	1	3

Acraldehyde	**CH_2CHCHO**	7	10	4		2	4	8	1
Pentene	C_5H_{10}			7	1			5	
Pentane	C_5H_{12}							5	2
Pentadiene	C_5H_8			3			1	6	1
Acetone	CH_3COCH_3	6	10	4		4	4	6	3
Propan-2-ol	$CH_3CH(OH)CH_3$					1			
Pyruvaldehyde	CH_3COCHO						5		
Cyclopentadiene	C_5H_6					1	1	3	
Allyl alcohol	CH_2CHCH_2OH	6	8						
Acetic acid	CH_3COOH	6	4		3	6	4	7	4
Methyl pyrrole	$C_4H_4NHCH_3$								4
3-Butenaldehyde	C_3H_5CHO						3		
Hexane	C_6H_{14}							7	
Carbon disulphide	CS_2								2
Butanone	$CH_3COC_2H_5$	8	8						
Hexene	C_6H_{12}			6	1			7	
Nitrobenzene	$C_6H_5NO_2$								2
Ethyl cyanide	C_2H_5CN					1			1
Methyl furan	$C_4H_3OCH_3$					3	5		5
Dimethyl sulphide	$(CH_3)_2S$								1
Vinyl acetate	$CH_3COOCHCH_2$					5			
Biacetyl	$C_4H_6O_2$					4	6		
Methyl formate	$HCOOCH_3$					1	5		
Methyl acetate	CH_3COOCH_3					4	4		2

(Continued on p. 90)

TABLE I *(continued)*

Degradation products		*Epoxy-resin bonded mica, Type 1*	*Epoxy-resin bonded mica, Type 2*	*Copal-resin bonded mica*	*Nylon paper*	*Phenolic-resin impregnated wood*	*Press paper*	*Asphalt-based core-plate varnish*	*Lignin sulphonate-based core-plate varnish*
Name	*Formula*								
Methyl acrylate	$CH_2CHCOOCH_3$					3			
Methyl propionate	$C_2H_5COOCH_3$					3			
Methyl cyclopentadiene	$C_5H_5CH_3$			4		1	1	6	
Cyclohexene	C_6H_{10}			5	5		2	3	
Crotonaldehyde	$CH_3CHCHCHO$						3		
Propionic acid	C_2H_5COOH						3		
Benzene	C_6H_6	5		3	3	1	3	5	
Acetol	CH_3COCH_2OH		8			6	6		
Methyl isopropenyl ketone	$CH_3COC(CH_2)(CH_3)$						3		
Heptane	C_7H_{16}			2		1		6	
Dimethyl furan	$C_4H_2O(CH_3)_2$	5	5			1	3		3
1,4-Dioxan	$C_4H_8O_2$				1				
Heptene	C_7H_{14}			4	1			6	
n-Valeraldehyde	C_4H_9CHO			2					
2-Methoxyethyl acetate	$CH_3COOCH_2CH_2OCH_3$		6			4			
Pyrrole	C_4H_5N								3
Methylcyclohexane	$C_6H_{11}\cdot CH_3$			1				4	
Dihydropyran	C_5H_8O			4					
Octane	C_8H_{18}						1		

Toluene	$C_6H_5CH_3$	5	6	5	1	1	4	7	3
Octene	C_8H_{16}			2	1			7	1
Ester	R_1COOR_2					4			
Acetic anhydride	$(CH_3CO)_2O$					4			
Ester	R_1COOR_2					1			
Allyl acrylate	$CH_2CHCOOCH_2CHCH_2$		6						
Cyclohexanone	$C_6H_{10}O$							6	
Hexanaldehyde	$C_5H_{11}CHO$			2					
Ester	R_1COOR_2					5			
Ethyl cyclohexene	$C_6H_9 \cdot C_2H_5$			1				5	
Dimethyl pyrazole	$C_3H_2N_2(CH_3)_2$					1			
Dimethyl pyrrole	$C_4H_2NH(CH_3)_2$								3
Furaldehyde	C_4H_3OCHO					5	6		
Nonane	C_9H_{20}							6	
Furfuryl alcohol	$C_4H_3OCH_2OH$					2			
Nonene	C_9H_{18}			4	1			5	
Xylene	$C_6H_4(CH_3)_2$	8	5	4			2	7	2
Dimethyl pyrrole	$C_4H_2NH(CH_3)_2$								2
Xylene	$C_6H_4(CH_3)_2$		5	1				6	
N,N-Dimethylacetamide	$CH_3CON(CH_3)_2$				5				
Hydroquinone	$C_6H_4O_2$						2		

(Continued on p. 92)

TABLE I *(continued)*

Degradation products		*Epoxy-resin bonded mica, Type 1*	*Epoxy-resin bonded mica, Type 2*	*Copal-resin bonded mica*	*Nylon paper*	*Phenolic-resin impregnated wood*	*Press paper*	*Asphalt-based core-plate varnish*	*Lignin sulphonate-based core-plate varnish*
Name	*Formula*								
Styrene	$C_6H_5CHCH_2$			4			5	6	2
Dimethyl pyrrole	$C_4H_2NH(CH_3)_2$								2
Furyl methyl ketone	$C_4H_3OCOCH_3$						4		1
Hexahydroindene	C_9H_{16}			2					
Decane	$C_{10}H_{22}$							6	
Decene	$C_{10}H_{20}$			3	1			5	
Ester	R_1COOR_2					6			
Methylethyl benzene	$C_6H_4C_2H_5CH_3$			5			4	5	3
Methylethyl benzene	$C_6H_4C_2H_5CH_3$			4				5	
Trimethyl furan	$C_4HO(CH_3)_3$								2
Phenol	C_6H_5OH	7	9		1	6	3	6	4
Methyl furaldehyde	$C_4H_2O(CHO)(CH_3)$						4		
Benzofuran	$C_4H_3OC_6H_5$						4		1
Indan	C_9H_{10}							5	3
Methyl ethyl benzene	$C_6H_4C_2H_5CH_3$							6	
Aniline	$C_6H_5NH_2$				5				
Undecane	$C_{11}H_{24}$							6	
Undecene	$C_{11}H_{22}$			3				5	
Methyl styrene	C_9H_{10}			4					

Methyl styrene	C_9H_{10}			4						
Hydroxy methyl cyclopentenone	$C_5H_6O(CH_3)(OH)$						3			
Indene	C_9H_8			5			6	6	2	
Cresol	$C_6H_4(OH)(CH_3)$		8	2	6			5	4	
Dimethyl styrene	$C_{10}H_{12}$								2	
Propyl benzene	$C_6H_5C_3H_7$			4						
Dodecane	$C_{12}H_{26}$							6		
Dodecene	$C_{12}H_{24}$							6		
Methyl benzofuran	$C_4H_2O(C_6H_5)(CH_3)$								1	
Cresol	$C_6H_4(OH)(CH_3)$	6	9	1		8			2	
Cyclohexenyl methyl ketone	$C_6H_9COCH_3$					6				
Cinnamaldehyde	$C_6H_5CHCHCHO$	6								
Dimethyl phenol	$C_6H_3(CH_3)_2(OH)$		7			8			3	
Butenyl benzene	$C_6H_5C_4H_7$							6		
Pentyl benzene	$C_6H_5C_5H_{11}$							6		
Benzyl alcohol	$C_6H_5CH_2OH$					6				
Organosulphur									5	
Tridecane	$C_{13}H_{28}$							6		
Tridecene	$C_{13}H_{26}$							6		
Methyl indene	$C_9H_7CH_3$						5	5		
Organo-sulphur									4	

(Continued on p. 94)

TABLE I *(continued)*

Degradations products		*Epoxy-resin bonded mica, Type 1*	*Epoxy-resin bonded mica, Type 2*	*Copal-resin bonded mica*	*Nylon paper*	*Phenolic-resin impregnated wood*	*Press paper*	*Asphalt-based core-plate varnish*	*Lignin sulphonate-based core-plate varnish*
Name	*Formula*								
Trimethyl phenol	$C_6H_2(OH)(CH_3)_3$					8			
Organo-sulphur									4
Tetradecene	$C_{14}H_{28}$							8	
Trimethyl phenol	$C_6H_2(OH)(CH_3)_3$					6			
Trimethyl phenol	$C_6H_2(OH)(CH_3)_3$					8			
Pentadecane	$C_{15}H_{32}$							8	
Pentadecene	$C_{15}H_{30}$							5	
Dimethoxy benzene	$C_6H_4(OCH_3)_2$						5		4
Dimethoxy benzene	$C_6H_4(OCH_3)_2$								6
Organo-sulphur									5
Hexadecane	$C_{16}H_{34}$						8		
Thymol	$(CH_3)C_6H_3CH(CH_3)_2(OH)$					7			
Heptadecane	$C_{17}H_{36}$							9	

aromatic compounds were also found. The presence of aromatic compounds was also reported by Wodley who deduced that they resulted from reactions between the initial products formed by breaking the cellulose molecule.

Asphalt-based core-plate varnish

This material is made by heating natural asphalt with linseed oil and other ingredients and dissolving the product in a mixture of solvents. The resulting varnish has a complex structure. The degradation products are primarily alkanes and alkenes with some aromatic hydrocarbons. Other products included some oxygen-containing compounds such as acraldehyde, acetic acid, acetone and cyclohexanone.

Lignin sulphonate-based core-plate varnish

This varnish is made from ammonium lignin sulphonate, a range of monosaccharides, furaldehyde, phosphoric acid and detergent (alkyl and aryl sulphonates). A wide range of compounds was identified in the degradation products. A number of organo-sulphur compounds were present, deriving from the sulphonate groups. Sulphur dioxide was also found. The nitrogen in compounds such as hydrogen cyanide, methyl and ethyl cyanides, nitrobenzene and pyrroles, present in the degradation products, probably derives from the ammonium ion in the raw materials. Other compounds present included furans, derived from the furaldehyde or the monosaccharides, hydrocarbons and methanol.

CONCLUSIONS

It is clear from the above discussion of the results that certain groups of compounds result from the degradation of particular insulating materials in hydrogen at 700°. It is also possible to pick out certain compounds which uniquely or predominantly derive from one particular insulating material. These compounds have been underlined in Table I and are listed as follows. Epoxy-resin: alkyl chloride, dimethyl ether, ethanol, allyl alcohol, butanone, allyl acrylate. Copal-resin: 1,3-butadiene, alkyl aldehydes, dihydropyran, methyl styrene, propyl benzene. Nylon paper: N,N-dimethyl acetamide, aniline. Phenolic-resin-impregnated wood: esters, acetic anhydride, benzyl alcohol, trimethyl phenol, thymol. Press-paper: pyruvaldehyde, butenaldehyde, crotonaldehyde, propionic acid, higher ketones, methyl furaldehyde, benzofuran. Asphalt-based core-plate varnish: alkanes, alkyl cyclohexane, cyclohexanone. Lignin-sulphonate-based core-plate varnish: sulphur dioxide, organosulphur compounds, pyrroles, carbon disulphide, nitrobenzene.

Monitoring the generator cooling hydrogen for these products may provide a warning of a developing fault since, although these results were obtained at 700°, the results of other workers[8,16] suggest that the same products are formed over a wide temperature range, with the amount of product increasing with increasing temperature.

ACKNOWLEDGEMENT

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COMPARATIVE STUDY OF THE GAS–LIQUID CHROMATOGRAPHIC BEHAVIOUR OF THE PENTAFLUOROBENZYL ESTERS AND THE METHYL ESTERS OF TEN CHLOROPHENOXYALKYL ACIDS

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SUMMARY

The gas–liquid chromatographic (GLC) behaviour of the pentafluorobenzyl and methyl esters of ten structurally related chlorophenoxyalkyl acids was studied by comparing their retention indices on nine frequently used liquid phases with increasing McReynolds constants. The resolution of each liquid phase is different for the pentafluorobenzyl and the methyl ester derivatives. However, using an apolar and a polar phase, a graph of the retention index (I) on the apolar phase against the difference in retention indices (ΔI) between the polar and the non-polar phase, shows a good correlation between structure and retention in gas–liquid chromatography. The graphs constructed for the pentafluorobenzyl and for the methyl esters have the same structure–retention correlations.

INTRODUCTION

The separation and/or detection of chlorophenoxyalkyl acids, as the free acids or after derivatization under a variety of experimental conditions, has been achieved using spectrophotometry[1–4], UV spectrophotometry[5–7], paper- and thin-layer chromatography[8–14] and gas–liquid chromatography (GLC), combined with microcoulometric, flame-ionization or electron-capture detection, in herbicide formulations[15], water[16,17], plant tissues, crops and wheat[18–25], food[26–33], soil[16,21,25,34,35] and animal and human biological samples[36–38]. In most of the GLC methods, the chlorophenoxyalkyl acids are determined as their methyl esters after direct derivatization[39–41] or after transesterification[42,43]. Another derivatization reagent for carboxylic acids is pentafluorobenzyl bromide, which has been applied in chlorophenoxyalkyl acid analysis[44–46]. It is an interesting reagent because of the increased electron-capture sensitivity of the derivatives formed[47,48]. The fact that the pentafluorobenzyl ester derivatives of ten chlorophenoxyalkyl acids are completely separated by GLC on DC-200, unlike their methyl esters, stimulated us to make a comparative study of the GLC behaviour of both series of derivatives on different liquid phases with increasing McReynolds constants.

EXPERIMENTAL

Chlorophenoxyalkyl acids

The ten chlorophenoxyalkyl acids examined are shown in Fig. 1. They all are systemic herbicides, except *p*-chlorophenoxyisobutyric acid (CPIB) or clofibrinic acid, the active metabolite of clofibrate, a hypolipidemic drug. CPIB has the same UV spectrum as MCPP and for this reason it was included in this study.

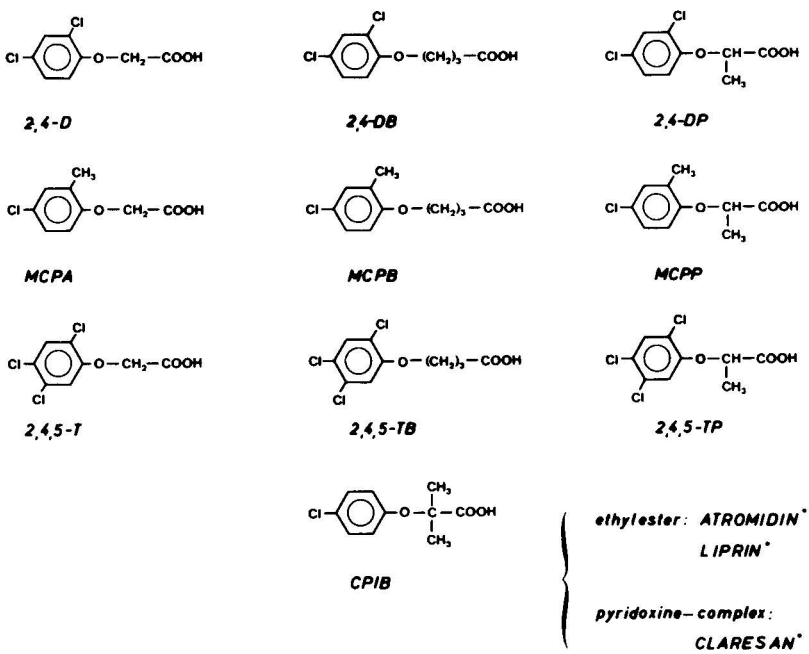


Fig. 1. The chlorophenoxyalkyl acids examined.

Reagents

Analytical-reagent grade chlorophenoxyalkyl acids (Riedel-de-Haën, Seelze-Hannover, G.F.R.) and pure *p*-chlorophenoxyisobutyric acid (Triosol, Laboratoires de Recherches Biologiques, Maisières, Belgium) were used as standards. The other reagents and solvents used for this study were: p.a. anhydrous potassium carbonate (J. T. Baker, Deventer, The Netherlands), 1% solution of p.a. pentafluorobenzyl bromide (PFB-Br, Pierce, Rockford, Ill., U.S.A.) in acetone, diazomethane solution in diethyl ether prepared from N,N'-dimethyl-N,N'-dinitrosoterephthalamide, 70% in mineral oil (Aldrich, Beerse, Belgium), p.a. ethyl acetate and p.a. *n*-hexane (Merck, Darmstadt, G.F.R.).

Standard solutions

All standard chlorophenoxyalkyl acid solutions were prepared at a concentration of 5 μg/μl in acetone.

n-Hydrocarbon solutions

For determinations of retention indices, 1 μg/μl *n*-hydrocarbon solutions in *n*-hexane were prepared.

Derivatization methods

With pentafluorobenzylbromide. Anhydrous potassium carbonate (10 mg) and 0.5 ml PFB-Br solution were added to 0.1 ml of a standard solution of chlorophenoxyalkyl acid in a glass-stoppered test-tube, which was heated for 90 min in a water-bath at 60°. The acetone was evaporated under a gentle stream of nitrogen; 1 ml of distilled water and 1 ml of ethyl acetate were added to the residue and the mixture was shaken for 1 min. The phases were allowed to separate and 0.5 μl of the ethyl acetate phase was injected into the chromatograph.

With diazomethane (CH_2N_2). A 0.1-ml volume of a standard chlorophenoxyalkyl acid solution was evaporated to dryness under nitrogen, and 0.5 ml of diazomethane solution in diethyl ether was added. The mixture was shaken for 2 min and the diethyl ether evaporated under nitrogen; 1 ml of ethyl acetate was added and 0.5 ml was injected into the chromatograph. After measurement of the retention time of each standard, a mixture of the ten standard chlorophenoxyalkyl acid derivatives was injected under the same GLC conditions.

Apparatus

A Varian 1800 gas chromatograph equipped with a flame-ionization detector was used. The chromatograms were recorded on a Varian Aerograph Model 20 1-mV recorder. The retention times used for retention index calculations were measured with an HP 3380 A electronic integrator.

GLC conditions

Silanized Pyrex glass columns of length 1.80 m, I.D. 2 mm and O.D. 1.8 in. were used.

The liquid phases examined were as follows:

(1) 5% DC-200 on Varaport 30 (100–120 mesh);
(2) 3% DC-11 on Chromosorb W AW DMCS (100–120 mesh);
(3) 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh);
(4) 3% QF-1 on Varaport 30 (100–120 mesh);
(5) 3% OV-225 on Gas-Chrom Q (100–120 mesh);
(6) 3% XE-60 on Chromosorb W AW DMCS (100–120 mesh);
(7) 2.5% NPGA on Varaport 30 (100–120 mesh);
(8) 3% FFAP on Varaport 30 (100–120 mesh);
(9) 3% OV-275 on Chromosorb W (100–120 mesh).

The columns were conditioned for 24 h at 230° by passing a slow flow of nitrogen (10 ml/min) through them.

The conditions for the GLC of the PFB esters were: injector and detector temperature, 250°; column temperature, 210°. The conditions for the GLC of the methyl esters were: injector and detector temperature, 195°; column temperature, 165° for DC-200 and QF-1 and 175° for DC-11, OV-17, XE-60, OV-225, NPGA, FFAP and OV-275. The carrier gas was nitrogen at a flow-rate of 30–35 ml/min. The air flow-rate was 300 ml/min and the hydrogen flow-rate 30 ml/min.

Calculations

For calculating the retention indices, four or five *n*-hydrocarbons were chromatographed simultaneously with the derivatives on each column.

The retention indices of the PFB ester and the methyl ester derivatives were calculated both with the Kováts equation and by linear regression, plotting logarithm of the retention time on the ordinate and the carbon number of the *n*-hydrocarbons used $\times 100$ on the abscissa.

RESULTS AND DISCUSSION

The McReynolds constants of the liquid phases used are given in Table I[49]; they characterize the polarity of the liquid phases[50,51]. The retention times and the retention indices on the different liquid phases, calculated as mentioned above, are

TABLE I

GLC PHASES EXAMINED AND THEIR McREYNOLDS CONSTANTS

Liquid phase	*McReynolds constants*				
	X	*Y*	*Z*	*U*	*S*
DC-200	16	57	45	66	43
DC-11	17	86	48	69	56
OV-17	119	158	162	243	202
QF-1	144	233	355	463	305
OV-225	228	369	338	492	386
XE-60	204	381	340	493	367
NPGA	234	425	312	462	438
FFAP	340	580	397	602	627
OV-275	629	872	763	1106	849

TABLE II

RETENTION TIMES AND RETENTION INDICES OF PFB ESTERS, CALCULATED WITH LINEAR REGRESSION AND WITH KOVÁTS EQUATION ON THE NINE GLC–LIQUID PHASES EXAMINED

PFB ester	*DC-200*			*DC-11*			*OV-17*			*QF-1*		
	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}
CPIB	3′20″	1933	1934	2′32″	1951	1953	6′17″	2160	2161	3′01″	2571	2573
MCPP	3′46″	1974	1977	2′51″	1994	1997	7′46″	2225	2229	3′28″	2626	2634
MCPA	4′16″	2015	2020	3′10″	2035	2037	10′14″	2310	2315	4′22″	2721	2731
2,4-DP	4′34″	2038	2043	3′22″	2059	2060	10′14″	2310	2315	4′22″	2721	2731
2,4-D	5′14″	2083	2089	3′56″	2117	2118	13′46″	2402	2408	5′46″	2837	2847
2,4,5-TP	6′51″	2173	2179	4′47″	2192	2191	16′22″	2455	2459	6′15″	2870	2880
2,4,5-T	7′57″	2222	2228	5′25″	2239	2239	22′55″	2559	2560	8′34″	3001	3008
MCPB	8′50″	2257	2262	5′52″	2270	2271	22′55″	2559	2560	8′34″	3001	3008
2,4-DB	10′39″	2319	2323	6′51″	2328	2332	30′27″	2647	2646	10′49″	3098	3103
2,4,5-TB	17′18″	2481	2480	10′27″	2489	2492	53′21″	2819	2815	17′05″	3288	3290

* Calculated with linear regression.

** Calculated with Kováts equation.

tabulated for both series of derivatives in Tables II and III. The liquid phases DC-200 and DC-11 have McReynolds constants in the same range, and have identical separation patterns. This is also true for OV-225 and XE-60. However, we found for both the PFB ester and the methyl ester derivatives different orders of elution on liquid phases with different polarities. This is illustrated by the chromatograms in Figs. 2 and 3. From these chromatograms, it was concluded that both the PFB esters and the methyl esters of MCPA, 2,4-D and 2,4,5-T change positions with other compounds in the elution sequence. Tables IV and V give their elution positions on the different liquid phases used with respect to the other derivatives. Another important fact is that on all liquid phases, CPIB as either the PFB ester or the methyl ester is always the first eluting compound, completely separated from the herbicides.

The methyl esters and the PFB esters of the ten chlorophenoxyalkyl acids have been synthesized in our laboratory and their mass spectra recorded[52,53]. We were able to confirm by gas chromatography–mass spectrometry the structures of the methyl and PFB esters of the ten compounds.

A plot of retention indices against a specific phase polarity constant from the McReynolds system for a series of GLC phases gives complete information about the GLC behaviour of a series of compounds. Such graphs constructed for the PFB and methyl ester derivatives are given in Figs. 4 and 5, respectively. It should be noted that the GLC behaviour of both series of derivatives is not determined exclusively by the selected phase polarity constant, representing a specific type of solvent–solute interaction.

In both Figs. 4 and 5 the retention indices of the chlorophenoxyacetic acid derivatives increase faster on more polar phases in comparison with the other derivatives. This means that the chlorophenoxyacetic acid esters are more polar than their 2-propionic and butyric acid analogues. This also explains why changes in the elution order occur on some GLC phases, as summarized above.

XE-60			*OV-225*			*NPGA*			*FFAP*			*OV-275*		
R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}
3′48″	2544	2546	3′31″	2560	2563	3′00″	2554	2555	8′25″	2525	2525	2′48″	2739	2745
4′35″	2619	2626	4′26″	2642	2647	3′52″	2646	2651	11′21″	2620	2619	3′22″	2806	2818
6′21″	2748	2759	6′19″	2767	2771	6′09″	2814	2820	21′08″	2819	2820	5′36″	2991	3002
6′21″	2748	2759	6′19″	2767	2771	5′27″	2770	2776	17′24″	2757	2758	4′49″	2937	2948
9′22″	2903	2909	9′39″	2917	2919	9′10″	2959	2963	35′05″	2980	2980	9′10″	3170	3181
9′22″	2903	2909	9′39″	2917	2919	8′37″	2937	2942	27′26″	2902	2902	6′33″	3048	3059
14′36″	3079	3078	15′34″	3085	3085	15′24″	3147	3149	59′13″	3148	3147	12′58″	3296	3302
12′33″	3019	3021	13′28″	3034	3034	12′11″	3062	3065	43′03″	3046	3046	9′10″	3170	2181
16′55″	3138	3134	18′27″	3145	3144	16′57″	3181	3183	65′09″	3178	3177	12′58″	3296	3302
28′57″	3352	3347	32′24″	3345	3343	30′29″	3393	3391	117′29″	3366	3366	20′23″	3460	3459

TABLE III

RETENTION TIMES AND RETENTION INDICES OF METHYL ESTERS, CALCULATED WITH LINEAR REGRESSION AND WITH KOVÁTS EQUATION ON THE NINE GLC LIQUID PHASES EXAMINED

Methyl ester	*DC-200*			*DC-11*			*OV-17*			*QF-1*		
	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}
CP1B	2′55″	1474	1473	1′53″	1499	1498	5′30″	1708	1706	2′07″	1886	1886
MCPP	3′27″	1518	1519	2′15″	1544	1546	7′09″	1770	1769	2′29″	1937	1944
MCPA	3′30″	1522	1523	2′16″	1548	1549	8′31″	1812	1812	2′51″	1982	1993
2,4-DP	4′16″	1574	1576	2′30″	1595	1597	9′48″	1846	1846	3′13″	2022	2033
2,4-D	4′33″	1591	1593	2′37″	1613	1615	12′12″	1899	1899	3′57″	2089	2097
2,4,5-TP	7′13″	1712	1716	3′46″	1734	1738	17′36″	1986	1988	11′58″	2163	2169
2,4,5-T	7′52″	1736	1736	4′03″	1752	1753	22′48″	2047	2049	6′15″	2238	2241
MCPB	7′54″	1740	1740	4′05″	1756	1756	20′31″	2022	2024	6′06″	2231	2233
2,4-DB	9′55″	1795	1799	4′59″	1811	1813	29′01″	2105	2105	7′57″	2316	2316
2,4,5-TB	18′18″	1956	1955	8′41″	1964	1962	57′09″	2267	2266	13′34″	2491	2481

* Calculated with linear regression.
** Calculated with Kováts equation.

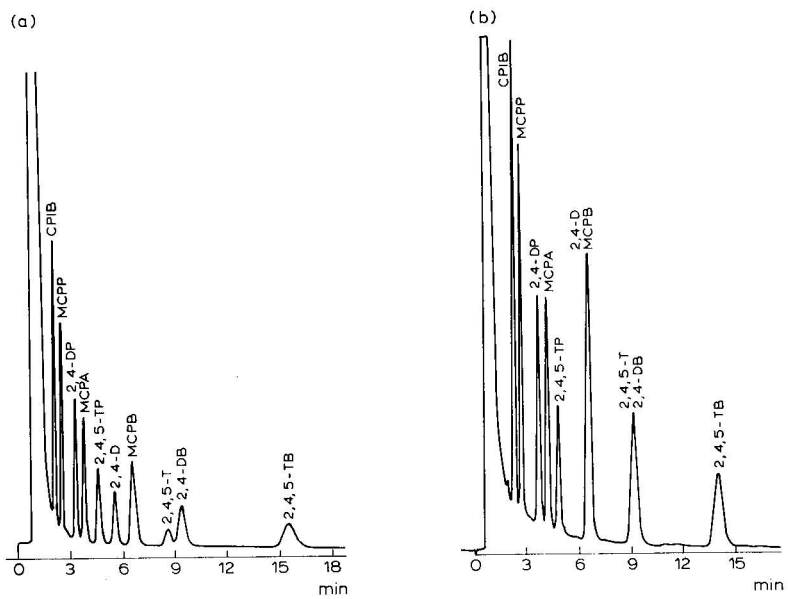


Fig. 2.

XE-60			*OV-225*			*NPGA*			*FFAP*			*OV-275*		
R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}	R_t	I^*	I^{**}
3'39"	1906	1903	3'10"	1953	1955	2'55"	1945	1948	7'52"	2026	2025	2'37"	2146	2152
4'31"	1975	1984	4'11"	2031	2035	4'00"	2031	2037	11'25"	2115	2115	3'18"	2213	2226
5'38"	2045	2057	5'21"	2108	2113	5'54"	2136	2143	19'25"	2241	2242	5'16"	2348	2360
6'10"	2074	2087	6'06"	2136	2141	5'54"	2136	2143	19'25"	2241	2242	5'00"	2332	2345
8'27"	2174	2189	8'43"	2236	2241	9'36"	2265	2270	37'07"	2396	2395	9'22"	2513	2520
9'50"	2222	2234	10'21"	2283	2287	10'37"	2295	2299	33'30"	2372	2371	7'09"	2435	2445
14'21"	2343	2348	15'44"	2400	2402	19'00"	2453	2453	71'13"	2552	2551	14'43"	2643	2643
11'41"	2277	2287	12'22"	2333	2336	12'56"	2349	2352	41'12"	2421	2420	9'00"	2501	2508
11'54"	2390	2393	18'17"	2442	2443	19'33"	2460	2460	87'18"	2552	2551	13'55"	2627	2628
30'31"	2580	2570	34'53"	2623	2620	40'34"	2668	2655	143'57"	2720	2720	23'25"	2776	2769

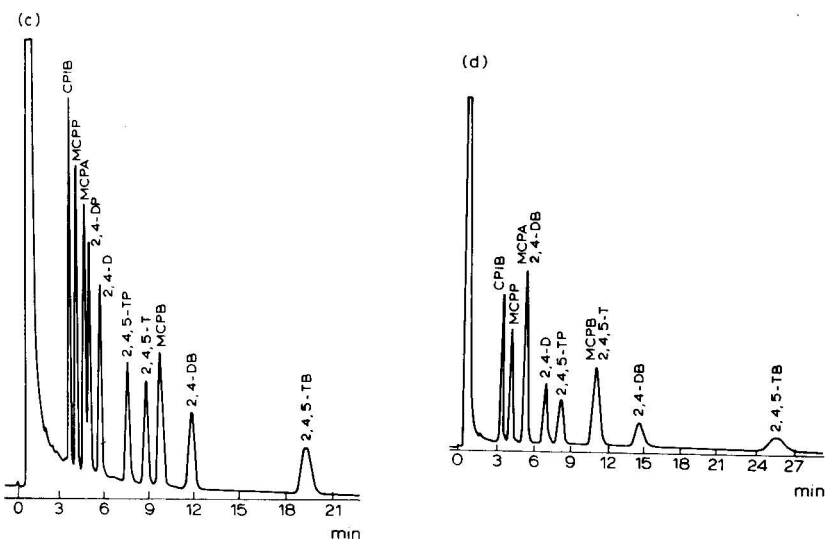


Fig. 2. Chromatograms of PFB esters. (a) 1% FFAP on Varaport 30 (100–120 mesh); (b) 3% OV-275 on Chromosorb W (100–120 mesh); (c) 5% DC-200 on Varaport 30 (100–120 mesh); (d) 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh).

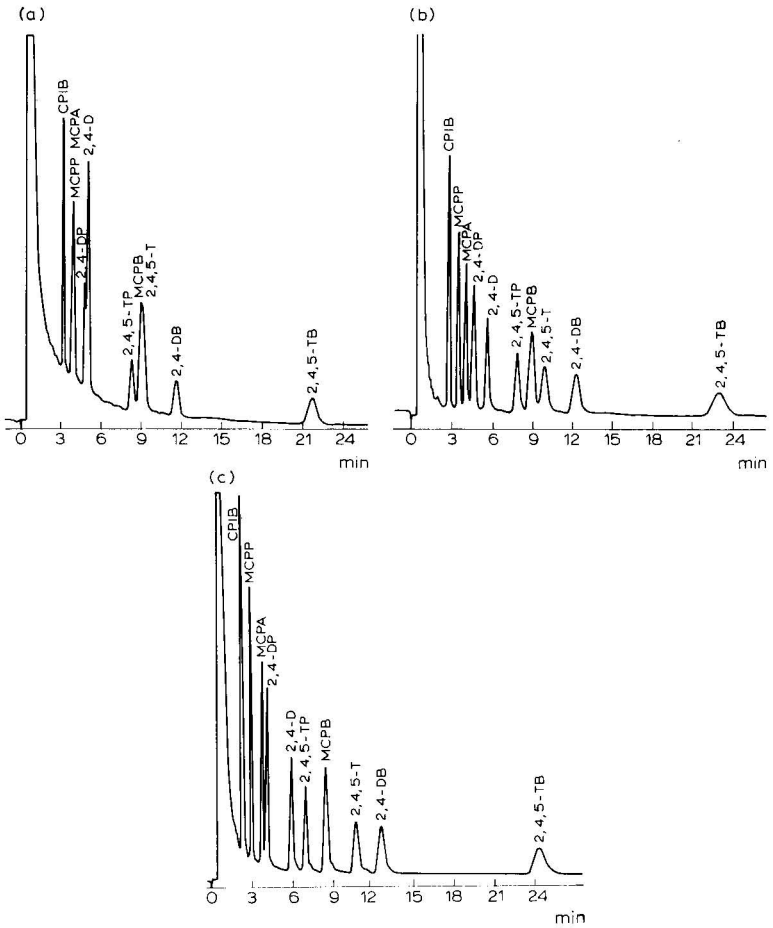


Fig. 3.

In some respects the PFB esters and the methyl esters have different GLC properties.

On all of the liquid phases examined, the MCPP and MCPA PFB esters were completely separated, unlike their methyl esters. The ten PFB ester derivatives were completely separated on the apolar phases DC-200 and DC-11, and on the polar phase FFAP with a different elution order. The methyl ester derivatives, however, were completely separated with the same elution order on the semi-polar phases OV-17, XE-60 and OV-225. On DC-200, DC-11 and FFAP they were not completely separated. All of these results contribute to the GLC identification of these ten structurally related chlorophenoxyalkyl acids.

In studies of correlations between structure and retention in GLC, the advantages of the retention index are accentuated by the fact that changes in logarithmic retention parameters may be regarded as proportional to the corresponding differences in the magnitude of the intermolecular forces between the solute and the stationary phase. Graphs with retention indices on two stationary phases along the

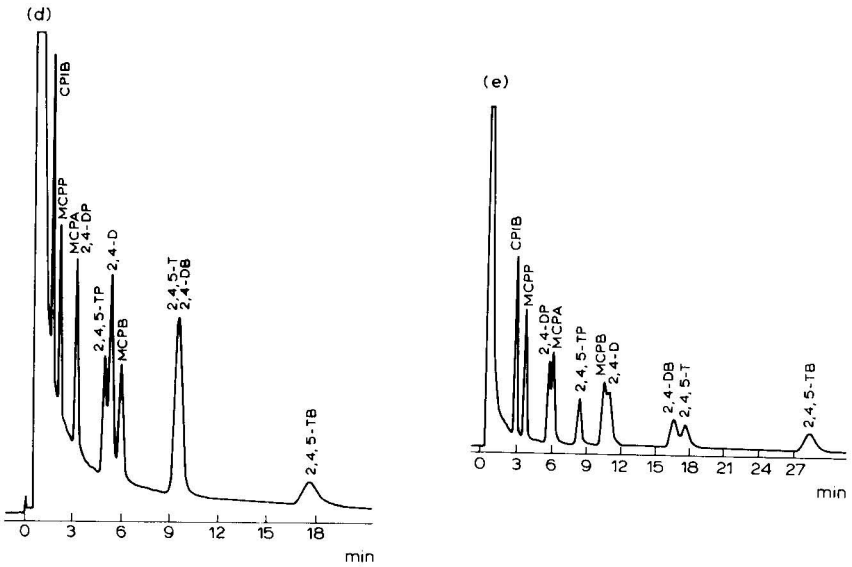


Fig. 3. Chromatograms of methyl esters. (a) 5% DC-200 on Diatoport S (100–120 mesh); (b) 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh); (c) 3% XE-60 on Chromosorb W (100–120 mesh); (d) 1% FFAP on Varaport 30 (100–120 mesh); (e) 3% OV-275 on Chromosorb W (100–120 mesh).

TABLE IV

POSITIONS OF CHLOROPHENOXYACETIC ACID PFB ESTERS ON THE DIFFERENT LIQUID PHASES WITH RESPECT TO OTHER DERIVATIVES

MCPA-PFB eluting before 2,4-DP-PFB	*MCPA-PFB eluting together with 2,4-DP-PFB*	*MCPA-PFB eluting after 2,4-DP-PFB*
Phases: DC-200 DC-11	Phases: OV-17 QF-1 OV-225 XE-60	Phases: NPGA FFAP OV-275
2,4-D-PFB eluting before 2,4,5-TP-PFB	*2,4-D-PFB eluting together with 2,4,5-TP-PFB*	*2,4-D-PFB eluting after 2,4,5-TP-PFB*
Phases: DC-200 DC-11 OV-17 QF-1	Phases: OV-225 XE-60	Phases: NPGA FFAP OV-275
2,4,5-T-PFB eluting before MCPB-PFB	*2,4,5-T-PFB eluting together with MCPB-PFB*	*2,4,5-T-PFB eluting after MCPB-PFB*
Phases: DC-200 DC-11	Phases: OV-17 QF-1	Phases: OV-225 XE-60 NPGA FFAP OV-275
2,4,5-T-PFB eluting together with 2,4-DB-PFB	*2,4-D-PFB eluting together with MCPB-PFB*	
Phase: OV-275	Phase OV-275	

TABLE V

POSITIONS OF CHLOROPHENOXYACETIC ACID METHYL ESTERS ON THE DIFFERENT LIQUID PHASES WITH RESPECT TO OTHER DERIVATIVES

MCPA-CH_3 eluting together with MCPP-CH_3	*MCPA-CH_3 eluting after MCPP-CH_3 and before 2,4-DP-CH_3*	*MCPA-CH_3 eluting after MCPP-CH_3 and together with 2,4-DP-CH_3*	*MCPA-CH_3 eluting after MCPP-CH_3 and after 2,4-DP-CH_3*
Phases: DC-200 DC-11	Phases: OV-17 QF-1 XE-60 OV-225	Phases: NPGA FFAP	Phases: OV-275
2,4-D-CH_3 eluting together with 2,4-DP-CH_3	*2,4-D-CH_3 eluting after 2,4-DP-CH_3 and before 2,4,5-TP-CH_3*	*2,4-D-CH_3 eluting after 2,4-DP-CH_3 and togther with 2,4,5-TP-CH_3*	*2,4-D-CH_3 eluting after 2,4-DP-CH_3 and after 2,4,5-TP-CH_3*
Phases: DC-200 DC-11	Phases: OV-17 QF-1 XE-60 OV-225 NPGA	Phase: FFAP	Phase: OV-275
2,4,5-T-CH_3 eluting together with MCPA-CH_3 and before 2,4-DB-CH_3	*2,4,5-T-CH_3 eluting after MCPB-CH_3 and before 2,4-DB-CH_3*	*2,4,5-T-CH_3 eluting after MCPB-CH_3 and together with 2,4-DB-CH_3*	*2,4,5-T-CH_3 eluting after MCPB-CH_3 and after 2,4-DB-CH_3*
Phases: DC-200 DC-11 QF-1	Phases: OV-17 OV-225 XE-60 NPGA	Phase: FFAP	Phase: OV-275

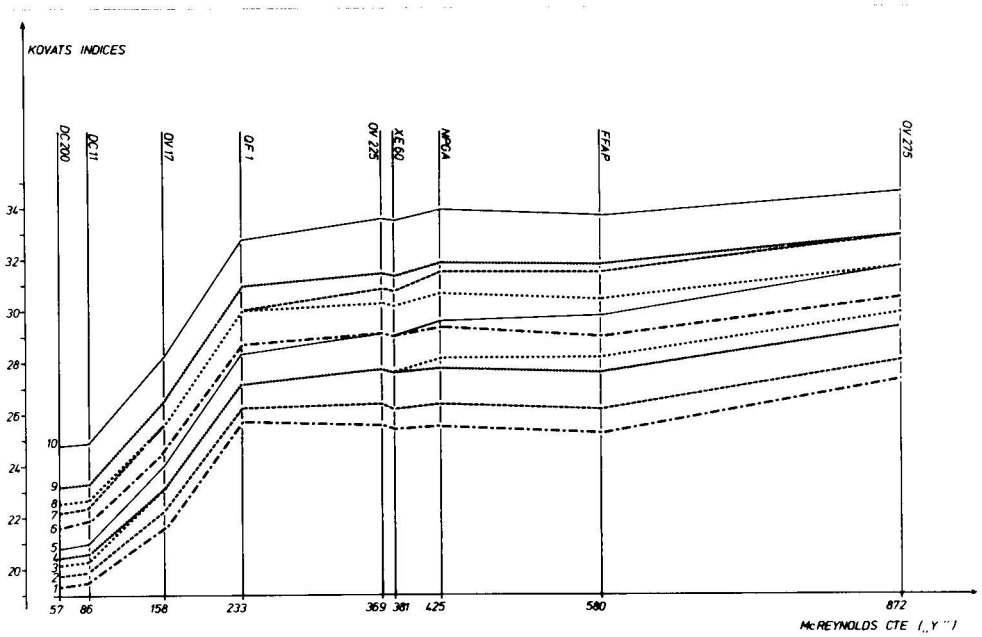


Fig. 4. Retention indices *versus* a McReynolds phase polarity constant of PFB esters for the nine liquid phases examined. 1 = CPIB; 2 = MCPP; 3 = MCPA; 4 = 2,4-DP; 5 = 2,4-D; 6 = 2,4,5-TP; 7 = 2,4,5-T; 8 = MCPB; 9 = 2,4-DB; 10 = 2,4,5-TB.

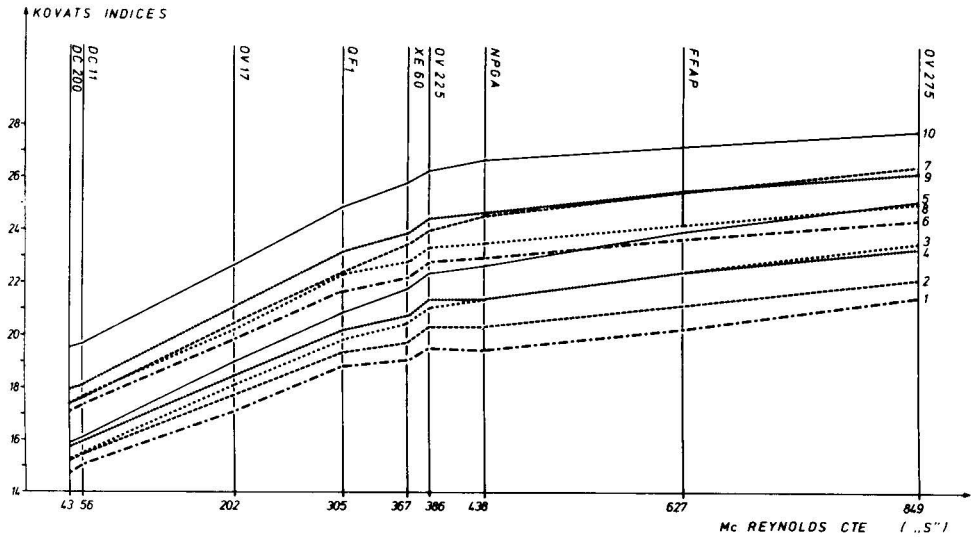
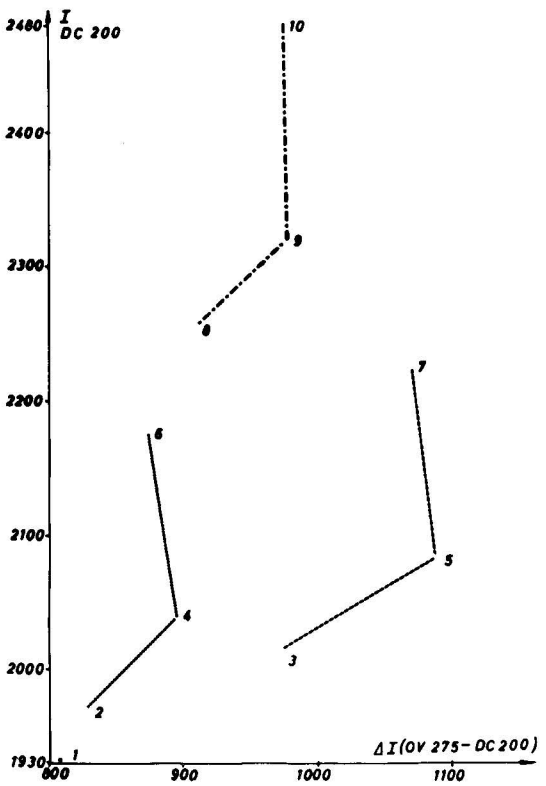


Fig. 5. Retention indices *versus* a McReynolds phase polarity constant of methyl esters for the nine liquid phases examined. 1 = CPIB; 2 = MCPP; 3 = MCPA; 4 = 2,4-DP; 5 = 2,4-D; 6 = 2,4,5-TP; 7 = 2,4,5-T; 8 = MCPB; 9 = 2,4-DB; 10 = 2,4,5-TB.

two axes are often recommended as a means for illustrating relationships between structure and retention and for extracting structural information from retention data[54]. The most fruitful results are normally obtained when one non-polar and one polar stationary phase are used. However, calculating the correlation coefficient between the retention indices on DC-200 and on OV-275, according to Moffat *et al.*[55], we obtain high values for both the PFB and the methyl esters. The reason is that the changes in the elution order of some derivatives after GLC on both stationary phases has no influence on the calculation of the correlation coefficient. More direct information is obtained when the retention index on the polar phase is replaced by the difference in retention indices, ΔI, between the polar and the non-polar phase. This concept, introduced by Kováts[56], represents the magnitude of polar and specific interactions, whereas the retention index on the non-polar phase represents the magnitude of non-polar and dispersion interactions between the compound and the stationary phase. As the integral retention index on the polar phase corresponds to the sum of polar and non-polar interactions, it can be argued that the *I versus* ΔI method gives the more direct characterization of a compound.

Figs. 6 and 7 show *I* (DC-200) *versus* ΔI (OV-275 – DC-200) graphs for the PFB and the methyl esters, respectively. The relative positions of the ten compounds in both figures are the same, independent of the derivatization method.

The basis of the retention index system is the constant increment in the retention index between members of a homologues series. The addition of structural units other than the methylene group may cause similar characteristic retention index increments[57]. Hence, some qualitative analytical information can be obtained from the retention data and *I versus* ΔI graphs. The position of an unknown compound on the *I versus* ΔI graph indicates the type of structure, and permits the exclusion of certain structural possibilities.



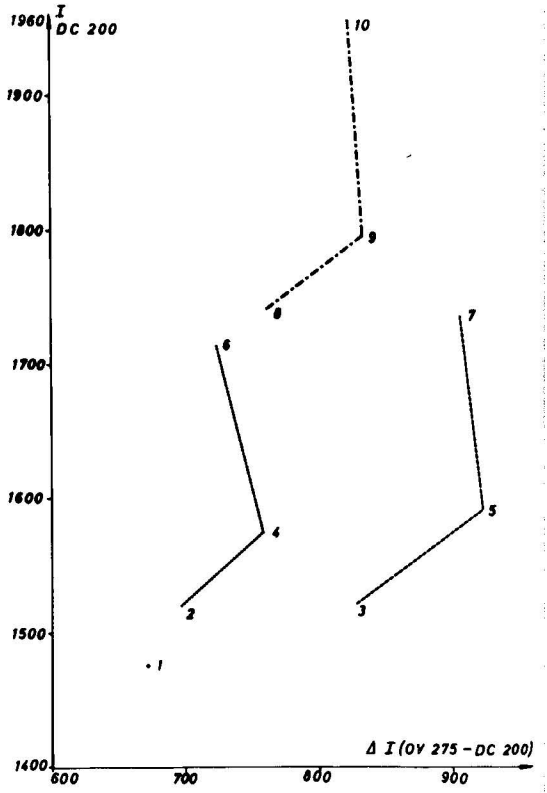


Fig. 6. *I versus ΔI* for PFB esters. 1 = CPIB; 2 = MCPP; 3 = MCPA; 4 = 2,4-DP; 5 = 2,4-D; 6 = 2,4,5-TP; 7 = 2,4,5-T; 8 = MCPB; 9 = 2,4-DB; 10 = 2,4,5-TB.

Fig. 7. *I versus ΔI* for methyl esters. 1 = CPIB; 2 = MCPP; 3 = MCPA; 4 = 2,4-DP; 5 = 2,4-D; 6 = 2,4,5-TP; 7 = 2,4,5-T; 8 = MCPB; 9 = 2,4-DB; 10 = 2,4,5-TB.

The approximate retention index data for a particular structurally related compound can be calculated. Structural units can be defined that correspond to characteristic changes in *I* and *ΔI*. Hence we find that the methylchloro-, dichloro- and trichloro-derivatives of the acetic, 2-propionic and butyric compounds have the same positions relative to each other in both of the *I versus ΔI* graphs. For both series of derivatives, the graphs were constructed using the apolar DC-200 phase and the polar OV-275 phase. On both phases the separation of the methyl ester derivatives is unsatisfactory, but on the *I versus ΔI* graph each methyl ester has a particular position and unambiguous identification is possible.

We conclude that using *I versus ΔI* graphs, a new derivatization technique will not contribute to the better identification of a series of structurally related compounds. However, the resolution for a series of compounds on some GLC phases can be increased so that a better separation pattern is obtained. A specific derivatization can also increase the sensitivity of the derivatives formed when a specific detector is used.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CAROTENOIDS

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SUMMARY

The potential of high-pressure liquid chromatography (HPLC) for separation of carotenoids on silica has been studied with selected model carotenoids (carotenes, diols, *cis–trans* isomers and diastereoisomers) in comparison with conventional thin-layer chromatography and circular paper techniques. Fast and efficient separation on the analytical scale was obtained, including that of epimers not previously separated. The advantage of HPLC coupled with a UV–VIS scanning spectrophotometer for the analysis of *cis–trans* isomerization mixtures of carotenoids is pointed out.

INTRODUCTION

Traditionally, carotenoids have been separated by column chromatography[1,2] and more recently, by thin-layer chromatography (TLC)[3]. Various types of paper chromatography have also been used[4,5], and gas chromatography (GC) has been applied to perhydrocarotenes[6].

Despite the application of high-pressure liquid chromatography (HPLC) to other types of compounds, few reports on HPLC of carotenoids have been published. Stewart and Wheaton[7] separated carotenes on magnesium oxide and xanthophylls on zinc carbonate; separation of double-bond isomers and *cis–trans* isomers was achieved, but retention times were long. More recently, diastereoisomeric furanoxides have been separated on magnesium oxide columns[8], and, in the apo-series, *cis–trans* isomers of retinal have been separated on Zorbax SIL[9]. Hajlbrahim *et al.*[10] have recently achieved good separation of components of algal extracts on Partisil (5-μm silica) columns. Recently, Stewart[21] employed HPLC on silica for separation of citrus carotenoids.

We have investigated the scope and limitations of analytical HPLC on silica for the separation of carotenes (Group 1), diols (Group 2), *cis–trans* isomers (Group 3) and diastereoisomers (Group 4) in comparison with common TLC and paper chromatographic systems.

MATERIALS AND METHODS

Samples

The samples used were authentic compounds from our carotenoid collection and were members of four groups, *viz.*:

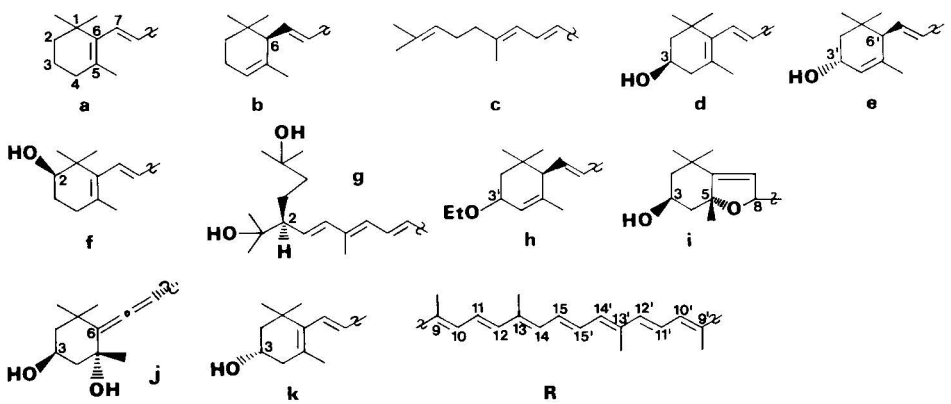
Group 1 (carotenes): 6′*R*-β,ε-carotene (1 in Scheme 1); β,β-carotene (2); β,ψ-carotene (3) and lycopene (4).

Group 2 (diols): 3*R*,3′*R*,6′*S*-lutein (5); 3*R*,3′*R*-zeaxanthin (6) and 2*R*,2′*R*-β,β-carotene-2,2′-diol (7).

Group 3 (*cis–trans* isomers): 3*R*,3′*R*,6′*S*-lutein (5) (stereoisomeric set) and 2*S*,2′*S*-bacterioruberin (8) (stereoisomeric set).

Group 4 (diastereoisomers): 3*R*,3′*R*,*S*,6′*S*-lutein 3′-ethyl ethers (9); 3*R*,5*R*,8*R*,*S*,3′*R*,5′*R*,8′*R*,*S*-auroxanthins (10); 3*S*,5′*R*,6′*R*,3′*R*,5′*R*,8′*R*,*S*-neochromes (11) and 3*R*,3′*R*-zeaxanthin (6) plus 3*R*,3′*S*-zeaxanthin (12).

Cis–trans isomerization of double bonds in the polyene chain was effected by iodine-catalyzed isomerization in benzene in daylight[11]. The 3′-epimers of compound 9 were prepared by treatment of lutein (5) with acidified ethanol, occurring by S_N1-mechanism[12]. The 8,8′-diastereoisomers of auroxanthin (10) and the 8′-epimers of neochrome (11) were obtained by treatment of violaxanthin and neoxanthin, respectively, with acidified chloroform[13,14]. Epizeaxanthin (12) was obtained, by double-bond migration, from lutein (5) (see ref. 15).



1	a–R–b	5	d–R–e	9	d–R–h
2	a–R–a	6	d–R–d	10	i–R–i
3	a–R–c	7	f–R–f	11	j–R–i
4	c–R–c	8	g–R–g	12	d–R–k

Equipment

A DuPont 830 liquid chromatograph equipped with gradient-elution acessories and thermostat-regulated oven was used in conjunction with a Varian Series 634 double-beam spectrometer as detector, which was set monochromatically at 400–490 nm, depending on the wavelength of max. absorption for the sample. The spectrometer was equipped with an 8-μl flow-through cell and, during separation, absorption spectra in the range 350–600 nm were recorded for the components of each HPLC peak.

RESULTS AND DISCUSSION

Optimum conditions

Columns of silica (various brands; particle size 5–10 μm), dry- or slurry-packed and commercial columns were tested. Optimum performance was obtained with a commercial Spherisorb (5-μm) column (4.6 × 250 mm) operated at 300 p.s.i. by gradient elution (0 to 40% of acetone in hexane containing 0.1% of methanol) at 1.25 ml/min (see ref. 16). For this column, the number of theoretical plates was 4350 (for toluene) and 6250 (for nitrobenzene). Resolution factors (R_s) for the carotenoids were calculated[17]; these varied from 0.86 (for 1 and 3) to 2.16 for the 8′-epimers of 11). Retention times (see Table I) ranged from 4–26 min.

Group 1 (carotenes)

As can be seen from Table I, aliphatic (4) monocyclic (3) and bicyclic (1 and 2) carotenes were separated in hexane containing 0.1% of methanol; no separation of the double-bond positional isomers β,ε-carotene (1) and β,β-carotene (2) was achieved on the silica column. The HPLC separation compared well with that obtained by TLC on silica plates, but was inferior to that obtained on special plates[18].

Group 2 (diols)

The 3,3′-diols lutein (5) and zeaxanthin (6) [corresponding to β,ε-carotene (1) and β,β-carotene (2)] were separated from eachother, and from the 2,2′-diol (7), with a mobile phase of up to 30% of acetone in hexane (see Table I). The separations compared favourably with those obtained by TLC and by circular development on silica-filled paper[5] (separations on the latter commercial paper are now inferior to those attained when the paper was first introduced[5]).

Group 3 (cis–trans isomers)

The stereomutation mixture of lutein (5) gave three zones on TLC and circular paper chromatography and five peaks by HPLC (see Table I) (the designations neo-U, neo-V, etc., denote lower R_F value, and neo-A, neo-B, etc., higher R_F value, than the all-*trans*-isomer[11]).

Stereochemical assignment of the *cis*-isomers has recently been discussed[19]. Four sterically unhindered mono-*cis*-isomers of lutein (5) are possible, and our results indicate better chromatographic resolution by HPLC than was previously obtained (two major *cis*-isomers). However, isolation of the individual isomers for further characterization is required.

Bacterioruberin (8), after iodine-catalyzed stereomutation, yielded five peaks by HPLC (see Fig. 1). Identification of neo-U as di-*cis*, neo-A as 5-mono-*cis*, neo-B as 9-mono-*cis* and neo-C as 13-mono-*cis* is based on shifts in λ_{max}, and relative *cis*-peak intensities[11], excluding the formation of sterically hindered and central *cis*-bonds. The separation of the mono-*cis*-isomers is superior to that previously attained on silica-filled paper[20].

The possibility of scanning the electronic spectra during the HPLC development of such labile stereoisomers is a great advantage. In this context, HPLC of carotenoids is superior to conventional TLC or paper chromatography, both of which

TABLE I

SEPARATIONS OF CAROTENOIDS

Carotenoid	*HPLC*[a]			*TLC (R_C)*		*Circular paper (R_F)*[d]
	t_R (min)	*Eluent*[b]	*Gradient rate (%/min)*	*Silica*	*Special plates*[c]	
Group 1						
β,ε-Carotene (1)	4.2	0	0	0.71[f]	0.69[g]	0.70[e]
β,β-Carotene (2)	4.2			0.71	0.57	0.70
β,ψ-Carotene (3)	4.6			0.64	0.46	0.55
Lycopene (4)	5.0			0.57	0.03	0.35
Group 2						
2,2′-Diol (7)	8.0	0–30	10	0.67[k]	0.52[k]	0.85
Lutein (5)	9.5			0.61	0.48	0.77
Zeaxanthin (6)	9.7			0.61	0.29	0.63
Group 3						
Lutein (5)						
Neo B	12.4					
Neo A	13.1	0–40	3			
All-*trans*	14			0.70[l]		0.70[h]
Neo U	14.8			0.62		0.55
Neo V	15.4			0.52		0.40
Bacterioruberin (8)		20–60	3			
Neo A	14.1			0.61[l]		0.55[j]
All-*trans*	14.6			0.41		0.50
Neo U	15.1					0.40
Neo V	16.0					
Neo W	16.3					
Group 4						
Lutein 3′-ether (9)		0–30	1	0.54[k]		0.57[g]
Epimer 1	17.5					
Epimer 2	17.9					
Auroxanthin (10)		0–40	1			
Epimer 1	25.5			0.20[k]		0.70[i]
Epimer 2	25.9					
Neochrome (11)		0–40	3			
Epimer 1	17.5			0.63[l]		0.80[j]
Epimer 2	17.8			0.60		0.70
Mono-*cis*	18.4					
Zeaxanthins (6 + 12)	13.6	0–40	1			0.63[h]

[a] Pressure, 300 p.s.i.; flow-rate, 1.4 ml/min.
[b] Percentage of acetone in hexane containing 0.1% of methanol.
[c] See ref. 18.
[d] S. & S. No. 287 (silica-filled) (see ref. 5).
[e] S. & S. No. 288 (alumina-filled); mobile phase, hexane.
[f] Mobile phase: acetone–hexane (2:98).
[g] Mobile phase: acetone–hexane (5:95).
[h] Mobile phase: acetone–hexane (10:90).
[i] Mobile phase: acetone–hexane (20:80).
[j] Mobile phase: acetone–hexane (30:70).
[k] Mobile phase: acetone–hexane (40:60).
[l] Mobile phase: acetone–hexane (60:40).

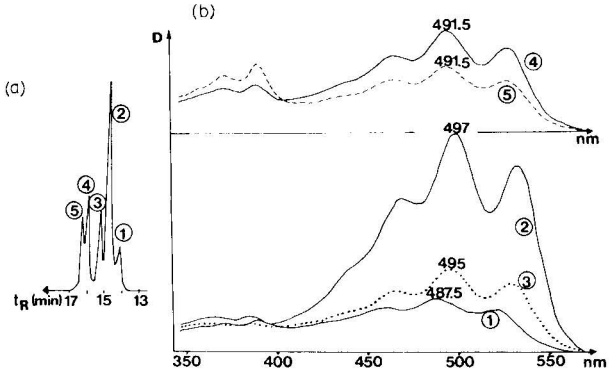


Fig. 1. HPLC separation of bacterioruberin (8) (stereoisomeric set) on Spherisorb (5 μm) at 300 p.s.i., with acetone–hexane 20–60% (gradient-rate, 3%/min) at 1.4 ml/min as mobile phase and the detector set at 490 nm. (a) Chromatogram; (b) electronic spectra of components. Peak and spectra numbers: 1 = neo-A, di-*cis*; 2 = all-*trans*; 3 = neo-U, 5-*cis*; 4 = neo-V, 9-*cis*; 5 = neo-W, 13-*cis*.

require elution and concentration procedures (with the inevitable partial reversible isomerization of the individual isomers) before spectra can be recorded.

Group 4 (diastereoisomers)

The two 3′-epimeric lutein ethyl ethers (9) have been separated for the first time by HPLC (see Fig. 2 and Table I). Of the expected three 8(8′)-diastereomers of auroxanthin (10), two were separated by HPLC and none by the other methods tested. Neochrome (11) was shown by HPLC to contain the two 8′-epimers and a mono-*cis*-isomer formed during acid treatment. The electronic spectra permitted reliable distinction between epimeric or *cis–trans* relationships during HPLC; separation into two zones only was obtained by TLC or circular development on paper. Finally, separation of natural zeaxanthin (6) and epizeaxanthin (12) was not achieved in any system.

The results indicate that separation of diastereoisomers in the HPLC system tested requires the presence of two chiral centres in the same end-group, with epimeric relationship for one of these centres. This could explain the separation of mono-ols (9, end-group h) and triols (11, end-group i) and failure to separate diols (6 and 12,

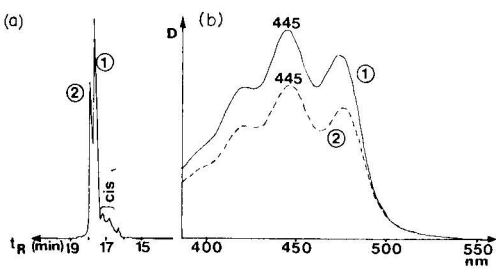


Fig. 2. HPLC separation of 3′-epimers of lutein 3′-ethyl ether (9), with acetone–hexane 0–40% (gradient-rate, 1%/min) at 1.4 ml/min as mobile phase and the detector set at 445 nm; other conditions as in Fig. 1. (a) Chromatogram; (b) electronic spectra of 3′-epimers.

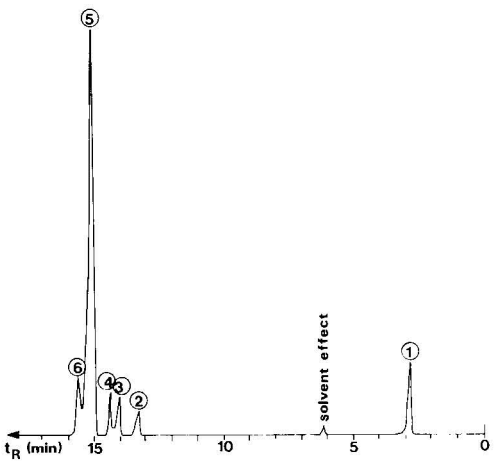


Fig. 3. HPLC separation of crude acetone extract of *Ochromonas* sp. Conditions as in Fig. 2, except that the gradient-rate was 3%/min and the detector was set at 450 nm. Peaks: 1 = β,β-carotene (2); 2 = zeaxanthin (6) plus chlorophyll; 3 and 4 = neochromes (11); 5 = fucoxanthin; 6 = neoxanthin.

end-groups d and k). However, the poor separation of the auroxanthin diastereoisomers (10, diol with two end-groups i) was unexpected.

Application of the method

Separation of a crude acetone extract of an *Ochromonas* sp. during 16 min (excluding the time for recording the spectra) is shown in Fig. 3, demonstrating separation of β,β-carotene (2), zeaxanthin (6), the neochromes (11), fucoxanthin and neoxanthin; in this instance, the detector was set at 450 nm. The peak areas do not reflect the exact concentrations of the individual carotenoids, owing to the different $\lambda_{max.}$ and extinction coefficients at 450 nm. The analytical advantages are obvious. For unknown mixtures, tentative identification based on electronic spectra and co-chromatography with authentic carotenoids can be made. The amount of an unknown mixture required is determined by the amount needed to record good quality electronic spectra for each component during the HPLC run (in this instance estimated to be *ca.* 0.2 μg of coloured carotenoid for adequate detection). However, for certain identification, mass spectra need to be obtained also.

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CHROM. 11,132

STUDIES OF CHROMATOGRAPHIC PACKINGS COMPRISING POROUS POLYMERS

I. SORPTION PROPERTIES OF POROUS STYRENE POLYMERS LATTICED BY DI(METHACRYLOYLOXYMETHYL)NAPHTHALENES

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SUMMARY

The adsorption properties of porous styrene polymers prepared with di(methacryloyloxymethyl)naphthalenes as the latticing medium have been studied with a view to their utility in the preparation of chromatographic packings for gas and liquid chromatography. Nitrogen adsorption and desorption isotherms have been constructed to calculate the specific surface area, pore-size distribution, mean pore radius and the *t* curve for different samples of these porous polymers. The surface properties of the samples investigated have been analyzed with respect to the synthesis conditions.

INTRODUCTION

A large number of papers have been published on the problem of obtaining good quality chromatographic packings. Current studies include the improvement of the preparation of already known chromatographic packings and the development of new packings. Great possibilities for the preparation of new chromatographic packings exist with chemically bonded phases[1–4] and the polymerization of organic compounds[5,6]. The application of porous polymers as chromatographic packings shows much promise. In this case there are unlimited possibilities for the synthesis of new kinds of packings (of defined porous structure and chemical nature of the surface). The majority of the known porous polymer packings were prepared on the basis of styrene with divinylbenzene[5,6]; this last compound influences the formation of porous structures in the packing, and is termed the latticing medium.

In this paper, particular consideration is given to the synthesis of porous polymers from styrene and the new latticing medium, a mixture of 1,4- and 1,5-di(methacryloyloxymethyl)naphthalenes. The specific surface area, pore-size distribution and *t* curves are calculated to characterize the surface properties of the prepared samples. These physicochemical studies are useful in the determination of the optimum conditions for the synthesis of such porous polymers, which may be used

as chromatographic packings for gas chromatography (GC) and liquid chromatography (LC).

EXPERIMENTAL

Reagents

Materials used for the synthesis of the porous polymers were as follows: styrene (chemically pure; Polskie Odczynniki Chemiczne, Gliwice, Poland), mixture of the isomers 1,4- and 1,5-di(methacryloyloxymethyl)naphthalenes having a fusion temperature of 110–113 °C (obtained from the reaction of a dichloromethyl derivative of naphthalene[7] with potassium methacrylate according to ref. 8; methacrylic acid was obtained from Reachim, Moscow, U.S.S.R.), vinyl polyalcohol (stabilizer), benzil superoxide (initiator), toluene (obtained from Polskie Odczynniki Chemiczene) and *n*-dodecane (Reachim).

Synthesis of the porous polymer

In a 1-l three-necked flask supplied with a mixer, back-coder and thermometer, 7 g of vinyl polyalcohol were dissolved in 350 ml of water and heated for 2 h at 80 °C. Then a freshly prepared mixture of styrene, a mixture of 1,4- and 1,5-di(methacryloyloxymethyl)naphthalenes, benzil superoxide, toluene and *n*-dodecane (in the proportions given in Table I) was poured slowly into the flask. The resulting mixture was heated under reflux at 80–85 °C for 24 h. The polymer formed was filtered off under reduced pressure and washed several times with hot water (1 l) and then with cold water (200 ml). To remove the stabilizer, suspension, solvents and unlatticed low-molecular-weight polymer, the product was repeatedly extracted with boiling benzene (5 × 100 ml), boiling methanol (5 × 100 ml), boiling acetone (5 × 100 ml) and then again with benzene (2 × 100 ml). The polymer was dried for 2 h at 50–60 °C under a pressure of 4 mmHg and then for 2 h at 150 °C under atmospheric pressure. The above synthesis was very similar to that described in ref. 9.

Measurement of adsorption and desorption isotherms

Nitrogen adsorption and desorption measurements were performed, at 78 °K,

TABLE I

COMPOSITION OF REACTION MIXTURES USED IN THE SYNTHESIS OF POROUS POLYMERS

Samples	*A*	*B*	*C*	*D*	*E*	*F*
Styrene (*a*), g	30	20	20	9	9	9
Mixture of 1,4- and 1,5-di(methacryloyloxymethyl)naphthalenes (*b*), g	10	10	10	10	10	10
Toluene (*c*1), g	34.7	34.7	26	34.7	34.7	52
n-Dodecane (*c*2), g	15	15	11.3	7.5	9	7.5
Weight ratio [$b/(a + b)$], %	25	33.3	33.3	52.6	52.6	52.6
Volume ratio [$(c1 + c2)/(a + b + c1 + c2)$], %	59.2	66.5	59.8	73.5	74.3	79.5
Volume ratio [$(a + b)/(c1 + c2)$], %	68.8	50.3	67.1	36	34.6	25.7
Weight ratio [$(a + b)/(c1 + c2)$], %	80.5	60.4	80.4	45	43.5	31.9

TABLE II

SORPTION PARAMETERS FOR SAMPLES OF POROUS POLYMERS

S = Specific surface area; C = BET constant; D = standard deviation for BET plot; R_m = pore radius at maximum pore-size distribution; (R_1, R_2) = interval of R for which the pore size-distribution is $>5 \cdot 10^{-4}$.

Sample	$S\ (m^2/g)$	C	$D \cdot 10^4$	$R_m\ (Å)$	(R_1, R_2)	$R_2 - R_1$
A	17	220	7.64	120	90–210	120
B	54	82	1.13	260	100–370	270
C	54	82	0.80	180	70–360	290
D	67	71	0.86	140	40–230	190
E	87	76	0.64	160	50–370	320
F	73	152	1.85	140	30–290	260

on the porous polymers by the volumetric method using a Sorptomatic 1806 instrument (Carlo Erba, Milan, Italy). The samples were first vacuum degassed (below 0.005 mmHg) for 2 h at 150 °C. The surface area was calculated by the BET method, using 16.2 Å^2 as the area of coverage per nitrogen molecule. The adsorption and desorption isotherms, and the pore-size distributions, were calculated from the Sorptomatic data according to Barrett *et al.*[10] and Wheeler[11].

The measurement of the specific surface area of sample B was carried in two ways: in the first case the sample was degassed for 2 h at 150 °C; in the other case the sample was degassed for 4 h at 150 °C. The difference between the measurements of the specific surface area was less than 2 m^2/g, *i.e.*, $< 4\%$.

RESULTS AND DISCUSSION

In Table II, the specific surface area, S, BET constant, C, the standard deviation for the BET plot, D, pore radius at maximum pore-size distribution, R_m, and the

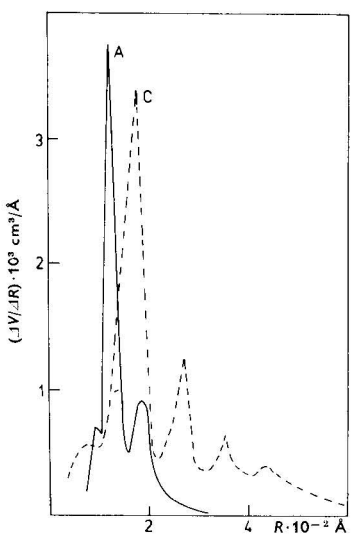


Fig. 1. Pore-size distributions characterizing the samples A (solid line) and C (dashed line).

interval of R for which the pore-size distribution is $> 5 \cdot 10^{-4}$ cm^3/Å are summarized for all the investigated samples. Fig. 1 shows the pore-size distributions for sample A (solid line) and C (dashed line). The pore-size distributions for other samples are very similar to those shown in Fig. 1. (The distribution for sample D is similar to that of A; however, the distributions for samples B, E and F are similar to that of C.) All the functions have one distinct maximum and may be approximated by a quasi-gaussian function. The small peaks are probably associated with the method of differentiation of the experimental points.

From Tables I and II certain conclusions can be drawn which might be helpful in the preparation of porous polymers for chromatography. It appears that the surface area of the samples investigated (solid line in Fig. 2) increases with increasing percentage content of the latticing medium [expression $b/(a+b)$ in Table I]. The dependence of the radius R_m on $b/(a+b)$ is more complex (dashed line in Fig. 2). In the region of higher values of $b/(a+b)$ an increase in the surface area S and decrease in R_m is observed; in this case the increase in surface area may be due to a decrease in pore size. In this region a change in the percentage content of the latticing medium does not result in considerable changes in the porous structure of the polymer, but it does affect the pore sizes. It is more difficult to explain correlations of this type in the region of lower values of $b/(a+b)$. In this case a change of the structure of the porous lattice is possible [with increasing $b/(a+b)$, S and R_m both decrease].

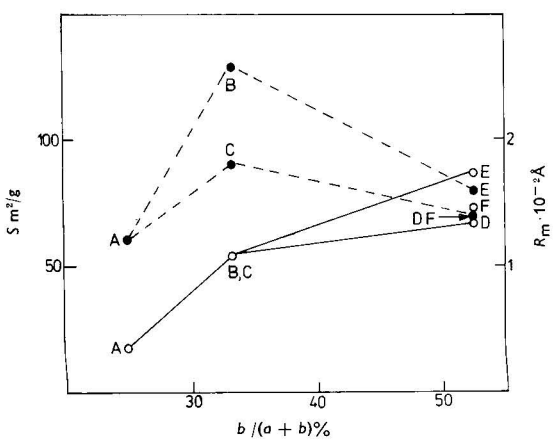


Fig. 2. Dependences of the specific surface area S (solid line with white circles) and the pore radius R_m (dashed line with black circles) on the weight ratio $b/(a+b)$.

Fig. 3 shows the dependence of S and R_m on the weight percentage, $(a+b)/(c1+c2)$. For the first three points (samples D, E and F) the expression $b/(a+b) = 0.526$, whereas for other points B and C the expression $b/(a+b) = 0.333$. These results indicate that a change in the quantity of solvents $(c1+c2)$ does not have a significant effect on the value of the specific surface area; however, it effects the formation of the porous structure.

Packings for adsorption chromatography should be characterized by a homogeneous porous structure, which does not contain micropores. The presence ofmicropores, causes a deterioration in the quality of chromatographic packings[12]. The

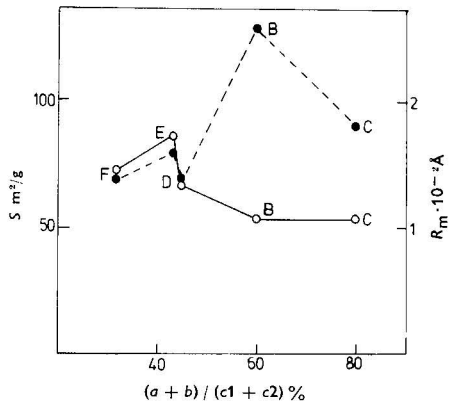


Fig. 3. Dependences of the specific surface area S (solid line with white circles) and the pore radius R_m (dashed line with black circles) on the weight ratio $(a + b)/(c1 + c2)$.

studies carried out here indicate that the porous polymer samples tested do not possess a micropore structure. Fig. 4 shows t curves[13] for samples B, C, D, E and F, plotted with respect to sample A, which may be considered as nonporous (specific surface area 17 m^2/g). The calculated t curves are straight lines passing through the origin, which is characteristic for adsorbents without micropores.

At the present stage of the experiments on porous polymers prepared from styrene and di(methacryloyloxymethyl)naphthalenes, the following conclusions can be drawn: (i) the values of the specific surface area of the prepared samples are > 50 m^2/g, when the percentage content of the latticing medium, $b/(a+b)$, is in the interval 30–50%; (ii) the dependence of the porous structure of these packings on the

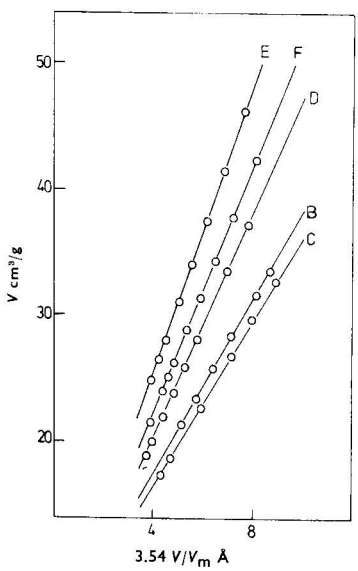


Fig. 4. Dependence of the amount adsorbed, V, on the thickness of the film calculated for samples B, C, D, E and F. The thickness of nitrogen film, equal to 3.54 V/V_m (where V_m is the monolayer capacity), was calculated by using the adsorption data for sample A.

synthesis conditions is complex and requires further study; (iii) these porous polymers have a relatively homogeneous porous structure, and there is no indication of the existence of micropores.

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CHROM. 11,006

COMPARISON OF HEADSPACE-GAS AND LIQUID-EXTRACTION DETERMINATIONS OF HYDROCARBONS IN WATER BY THE STANDARD-ADDITION METHOD

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SUMMARY

The methods of headspace-gas and liquid-extraction analysis, comprising the gas chromatography of samples of the gaseous or liquid-extract phases withdrawn from closed equilibrated systems and involving the standard-addition quantitation, have been examined and compared for the determination of trace amounts of hydrocarbons in water. The liquid-extraction method is more accurate but it yields chromatograms with an interfering background due to the liquid extractant. The sensitivity of determination of volatile hydrocarbons in water is roughly the same for each method, concentrations amenable to reliable determination amounting to tens of μg/l on a packed column with a flame-ionization detector.

INTRODUCTION

Headspace-gas analysis, in the variant comprising the analysis of samples taken from the gas present over a liquid in a closed equilibrated gas–liquid system, has been applied by several workers[1,2] to the determination of hydrocarbons and/or halogenated hydrocarbons in water. Analogous procedures involving liquid–liquid rather than gas–liquid equilibration have also been described[3]. Recently, the standard-addition method has been examined as a means of quantitation in headspace-gas analysis of hydrocarbons. The aim of the present paper is to show the possibility of using the standard-addition method for quantitative analysis of liquid–liquid and/or ternary gas–liquid–liquid systems and to compare the headspace-gas–standard-addition method with an analogous procedure involving a single-step liquid extraction and analysis of a sample of the extract. In both cases, systems with water containing trace amounts of several hydrocarbons were chosen as models.

EXPERIMENTAL

The physical arrangement of the system for headspace-gas analysis was the same as that described previously[4]. A 50-ml volume of distilled water was pipetted into a 100-ml flask whereupon the latter was closed with a special ground-glass stopper

and placed in a water-bath, where it was kept at 40° for 20 min while agitating the contents by means of a magnetic stirrer; the volume of the gaseous phase in the stoppered flask was 45 ml. 3.5 μl of a solution containing 0.5769, 1.2156, 1.2615, 1.6208 and 2.0349 μg/μl of hexane, benzene, 2,4-dimethylhexane, octane and 1-nonene, respectively, in acetone were then placed in the flask by use of a 7005 N (5-μl) Hamilton syringe (Hamilton, Micromesure BV, The Hague, The Netherlands).

After allowing 20 min for equilibration, a 1-ml sample of the gaseous phase was withdrawn slowly (*ca.* 0.5 min) from the system and analyzed by gas chromatography (first analysis). Then another 3.5-μl charge of the above solution of hydrocarbons was placed in the flask and, after 20 min, again a 1-ml sample of the gaseous phase was withdrawn and analyzed (second analysis). From the amounts of standards added after completion of the first analysis and the chromatograms obtained in both analytical steps it is possible to calculate the initial amounts of the solutes in the system. The headspace-gas samples were taken by use of a 1-ml gas-tight syringe (Chirana, Brno, Czechoslovakia) kept at *ca.* 60°.

The system designed for analysis by the liquid-extraction method was contained in a 20-ml glass ampoule equipped with a short capillary-tube to which an inlet-port head was attached. The inlet-port head was the same as that employed in the arrangement for headspace-gas analysis[4]. Analytical-grade carbon disulphide was employed as the extractant. Volumes of 2 ml CS_2 and 5 ml of distilled water were injected into the ampoule by use of syringes, and the ampoule was closed by the inlet-port head; a schematic representation of the system used in the liquid-extraction analyses is shown in Fig. 1. A 3.5-μl volume of a solution containing 8.241, 17.366, 18.022, 23.154 and 29.070 μg/μl of hexane, benzene, 2,4-dimethylhexane, octane and 1-nonene, respectively, in acetone was then injected into the system using a 5-μl Hamilton syringe and the contents of the ampoule were vigorously shaken for some time in order to promote equilibration. The ampoule was placed in a water-bath at 22° and revolved there for 20 min at 10 rpm about its shorter axis, the latter being positioned horizontally. The ampoule was then removed from the bath, positioned up-side down and a 1-μl charge of the CS_2 phase was withdrawn by a 7001 N (1-μl) Hamilton syringe and injected into the gas chromatograph (first analysis). Another 3.5-μl charge of the standard hydrocarbon solution was placed in the ampoule, the procedure was repeated and a 1-μl sample of the extract was withdrawn and analyzed (second analysis). As with the headspace-gas method, the chromatograms obtained in the two

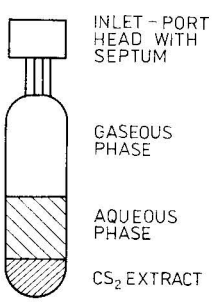


Fig. 1. Representation of the system employed in the liquid-extraction method.

analyses and the knowledge of the amounts of the standards added after completion of the first analytical step enable the initial amounts of the solutes in the system to be calculated.

The standard solutes as well as the acetone and carbon disulphide were analytical grade chemicals from Fluka, Buchs, Switzerland (hexane), Lachema, Brno, Czechoslovakia (benzene, 2,4-dimethylhexane and acetone), VEB Laborchemie, Apolda, G.D.R. (octane), Polyscience, Evanston, Ill., U.S.A. (1-nonene) and Riedel-De Haën, Seelze-Hannover, G.F.R. (CS_2).

With both methods, the chromatographic analyses were carried out on a Hewlett-Packard 402 gas chromatograph (Avondale, Pa., U.S.A.). Conditions: FID, sensitivity attenuation 1/16; glass column (180 cm × 3 mm I.D.) packed with 8.14 g of 10% (w/w) Apiezon K on Chromaton N (0.2–0.25 mm); column temperature, 80°; nitrogen carrier gas; flow-rates of 26, 29.4 and 200 ml/min, for nitrogen, hydrogen and air, respectively. The Chromaton N and Apiezon K were products of Lachema and AEI (Manchester, Great Britain), respectively.

CALCULATION OF RESULTS

A mass balance of solute in the system leads to the following relations[5] for the headspace-gas and liquid-extraction methods of analysis, respectively:

$$W_i = W_{iG}\left(1 + K_{LG} \cdot \frac{V_L}{V_G}\right) = \frac{W_s - w_i}{(A_i' v_G / A_i v_G') - 1} \tag{1}$$

$$W_i = W_{ie}\left(1 + \frac{V_p}{K_{ep} V_e}\right) = \frac{W_s}{(A_i' v_e / A_i v_e') - 1} \tag{2}$$

where W_i is the initial weight of solute i in either system (amount to be determined), W_{iG} is the weight of the solute in the gaseous phase, V_L and V_G are the volumes of the aqueous and the gaseous phase and K_{LG} is the distribution constant of the solute, defined as the ratio of solute concentrations in the liquid and the gaseous phase, W_{ie} is the weight of the solute in the liquid extract, V_p and V_e are the volumes of the parent liquid and the extract, K_{ep} is the distribution constant of solute, defined as the ratio of its concentrations in the extract and parent liquid, W_s is the weight of standard added to the system, w_i is the weight of the solute taken out of the system in sampling the gaseous phase for the first analysis, A_i and A_i' are the solute peak areas in the chromatograms obtained in the first and in the second analyses, v_G and v_G' are the volumes of the gaseous phase and v_e and v_e' are the volumes of the extract used in the gas chromatograph in the first and in the second analyses, respectively.

It is possible to employ peak heights instead of peak areas with the standard-addition method; *i.e.*, the ratios A_i'/A_i can be replaced by the corresponding peak-height ratios, h_i'/h_i, in both the above relations. Further, if $v_G = v_G'$ and $v_e = v_e'$, in the present case, the expressions are simplified accordingly; all the results presented in this work have been calculated from peak heights.

The expression $1 + (V_p/K_{ep}V_e)$ in eqn.2 (a system factor) applies to a system comprising only the parent liquid and the liquid extract. However, the system used in

this work comprised also the gaseous phase, so that the situation has to be expressed by

$$W_i = W_{ie}\left(1 + \frac{V_p}{K_{ep}V_e} + \frac{V_{Ge}}{K_{eG}V_e}\right) \quad (3)$$

In this case, V_{Ge} and K_{eG} are the volume of the gaseous phase in the ternary parent liquid–extract–gas system and the distribution constant of the solute, defined by the ratio of solute concentrations in the extract and the gaseous phase. The last expression in relation 2 remains unaltered, *i.e.*, the calculation of the results is independent of the form of the system factor[5].

RESULTS AND DISCUSSION

In all the measurements the values of W_s were equal to those of W_i, and the values of w_i were determined by external calibration. Table I shows the results of the headspace-gas analysis, while the results of liquid-extraction analysis are given in Table II. In both kinds of analysis, chromatograms of comparable sizes were recorded at a fixed detector-sensitivity setting. It is apparent from the data in Tables I and II that the headspace-gas method of analysis is less accurate than its liquid-extraction analogue. On the other hand, a direct comparison of the data suggests that the headspace-gas method is more sensitive. Supposing the entire amounts of solutes (values

TABLE I

RESULTS OBTAINED BY THE HEADSPACE-GAS–STANDARD-ADDITION METHOD
$S/\sqrt{n}$ = Standard deviation of the average, n = number of determinations (9), $t_{exptl.}$ = experimental Student coefficient; t_{crit} = 2.26.

Solute	W_i (μg)		*Error*		$S/\sqrt{n}$	$t_{exptl.}$
	Given	*Found*	μg	%		
Hexane	2.02	1.88	0.14	6.9	0.08	1.75
Benzene	4.25	4.23	0.02	0.5	0.20	0.10
2,4-Dimethylhexane	4.42	4.57	0.15	3.4	0.25	0.60
Octane	5.67	6.43	0.76	13.4	0.43	1.77
1-Nonene	7.12	8.36	1.24	17.4	0.63	1.97

TABLE II

RESULTS OBTAINED BY THE LIQUID-EXTRACTION–STANDARD-ADDITION METHOD
For the meaning of symbols see Table I; n = 10, t_{crit} = 2.22.

Solute	W_i (μg)		*Error*		$S/\sqrt{n}$	$t_{exptl.}$
	Given	*Found*	μg	%		
Benzene	60.8	57.2	3.6	5.9	2.8	1.28
2,4-Dimethylhexane	63.1	60.5	2.6	4.1	2.4	1.08
Octane	81.0	80.5	0.5	0.6	3.6	0.14
1-Nonene	101.7	97.7	4.0	3.9	5.0	0.80

of W_i) had originally been present in the aqueous phase only, as would be the case in an actual analysis of water, it is possible to infer from the data in Tables I and II that concentrations of tenths and tens of μg/ml of hydrocarbons in water were determined by the headspace-gas and liquid-extraction standard-addition methods, respectively. This sensitivity is still insufficient for many applications in modern water-pollution control; often it is necessary to determine hydrocarbons in water in concentrations of tenths of μg/l or less. This sensitivity could theoretically be attained by employing capillary gas chromatography with unsplit sample injection[6] and utilizing the reserve (about an order of magnitude) in detector sensitivity.

In order to make an unequivocal comparison of the sensitivities of the headspace-gas and liquid-extraction methods, it is necessary to compare situations in which the same amounts of water containing the given contents of hydrocarbons to be determined are analyzed, employing a reasonably small amount of the extractant and injecting into the gas chromatograph the maximum admissible charges of the headspace gas and the extract, respectively. In such a comparison, the measure of the sensitivity of analysis is the solute weight in the charge introduced into the gas chromatograph under the above conditions. It follows from eqns. 1 and 2 that

$$w_{iG} = \frac{W_i v_G}{V_G + K_{LG} V_L} \tag{4}$$

and

$$w_{ie} = \frac{W_i v_e}{V_e + (V_p / K_{ep})} \tag{5}$$

Owing to the low solubility of hydrocarbons in water, except for aromatics, the values of K_{LG} are[1,7] of the order of 10^{-2}. On the other hand, since $K_{ep} = K_{eG}/K_{LG}$, where K_{eG} is the constant of solute distribution between the extract phase and a gas, the values of K_{ep} of hydrocarbons in a nonpolar extractant–water system will be very large. This implies that $K_{LG} V_L \ll V_G$ and $(V_p/K_{ep}) \ll V_e$ even if $V_e \ll V_p$ and $V_L = V_G$ with such systems. Hence, if $V_L = V_p = 50$ ml, $W_i = 1$ μg, $V_G = 50$ ml, $V_e = 100$ μl, $v_G = 5$ ml and $v_e = 10$ μl, calculations by eqns. 4 and 5 with the terms $K_{LG} V_L$ and V_p/K_{ep} being neglected yield $w_{iG} = w_{ie} = 0.1$ μg. It follows from this rough estimate that the sensitivities attainable by either method are approximately the same. However, this situation can be substantially altered by employing different extractants and/or condensing the extract.

A great advantage of the headspace-gas method over the liquid-extraction method is that it is not necessary to introduce into the system any substance that might interfere with the analysis; with the liquid-extraction method the background chromatogram of the extractant may be a source of serious difficulties. This situation is apparent from the chromatograms in Fig. 2 and 3. While the chromatogram of a headspace-gas sample is free from artifact peaks, in the chromatogram of a sample of the liquid extract the peak of hexane is completely obscured by the background response of carbon disulphide. In addition, the peak of benzene had to be corrected for a blank value due to an impurity present in the carbon disulphide. With larger samples of the extract and lower concentrations of the solutes the situation would obviously be worsened accordingly.

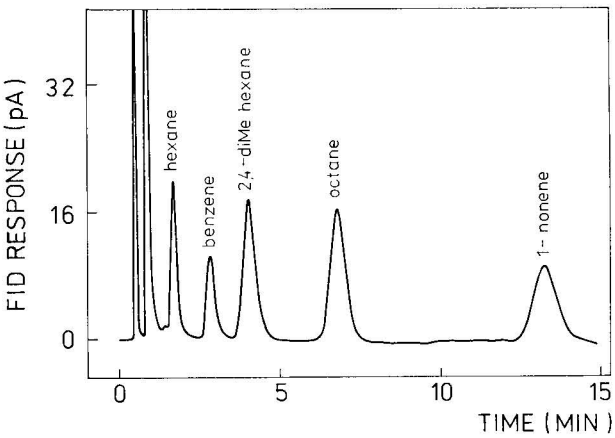


Fig. 2. Chromatogram of a 1-ml sample of the headspace gas. FID, sensitivity attenuation $^1/_{16}$.

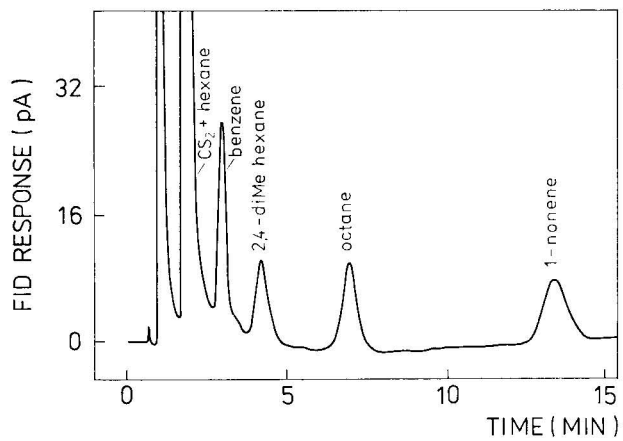


Fig. 3. Chromatogram of a 1-μl sample of the CS_2 extract. Details as in Fig. 2.

CONCLUSIONS

The standard-addition method is a suitable means of quantitation in trace analysis of hydrocarbons in water by both the headspace-gas technique and its analogue involving liquid extraction and analysis of the extract. When sampling the headspace gas and the liquid extract by use of conventional syringes and injecting the samples directly into the gas chromatograph, the accuracy of the results obtained by the headspace-gas method is somewhat lower. If a nonpolar extractant is used with the latter method, both methods render approximately the same sensitivity of analysis. Employing a packed column and a FID, both the methods provide a reliable determination of tens of μg/l of volatile hydrocarbons in water. In contrast to the headspace-gas method, in the liquid-extraction method the chromatograms may be impaired by the interfering background of the extractant.

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ASSAY OF PEMOLINE IN HUMAN PLASMA, SALIVA AND URINE BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION

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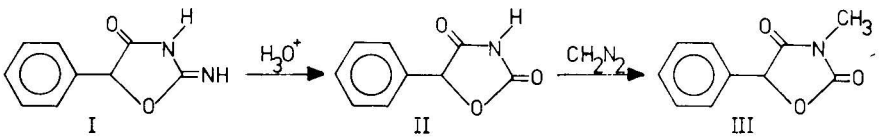
SUMMARY

A simple gas chromatographic assay of the psycho-stimulant pemoline in human urine, plasma and saliva has been developed. Instead of direct extraction of the drug from urine, plasma and saliva, it is hydrolyzed to 5-phenyl-2,4-oxazolidinedione with 1 *N* hydrochloric acid. After extraction this compound is methylated with diazomethane and determined by gas–liquid chromatography using a capillary SCOT column with a mixed stationary phase, a solid injection system and a nitrogen-selective detector. 5-Phenyl-2,4-oxazolidinedione, which was also found to be a metabolite of pemoline, could be determined quantitatively in human urine.

INTRODUCTION

Pemoline (2-imino-5-phenyl-4-oxazolidinone, Fig. 1, I) is a stimulant of the central nervous system and is the major active constituent in many drug preparations. Because of its low lipophilicity and its instability under acidic and basic conditions, it is difficult to extract pemoline from biological fluids. As a consequence, methods such as thin-layer chromatography (TLC)[1] and high-performance liquid chromatography[2], which employ direct extraction of pemoline from human urine, lack the sensitivity and selectivity required for quantitative determination. Gas–liquid chromatography (GLC) cannot be applied because pemoline is not eluted unchanged and derivatization yields various compounds. For these reasons several authors[3–6] have recently described methods where pemoline is hydrolyzed by acidic treatment to 5-phenyl-2,4-oxazolidinedione (Fig. 1, II) prior to extraction. This compound is easily extracted and determined by GLC. However, all these methods neglect the occurrence

of compound II as a metabolite of pemoline. In addition, they are not practical and/or sufficiently sensitive for the assay of pemoline in plasma and saliva.



I II III

Fig. 1. Derivatization scheme of pemoline (I). First, acidic hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione (II), followed by methylation to N-methyl-5-phenyl-2,4-oxazolidinedione (III).

In this paper an elaboration of our previously published method[5] is reported for human plasma, saliva and urine. The quantitative determination of the oxazolidinedione metabolite in urine is also described.

MATERIALS AND METHODS

Materials

Pemoline and fensuximide (1-methyl-3-phenyl-2,5-pyrrolidinedione, internal standard) were kindly supplied by Chemische Industrie (Katwijk, The Netherlands). 5-Phenyl-2,4-oxazolidinedione was synthesized by acidic hydrolysis of pemoline according to the method of Traube and Asher[7]. Other reagents were distilled dichloromethane (J. T. Baker, Phillipsburg, N.J., U.S.A.), distilled *n*-pentane (J. T. Baker) and concentrated hydrochloric acid.

Apparatus

A Philips GCV gas chromatograph, equipped with a nitrogen-selective detector (rubidium chloride, Model 795014) was used. The capillary SCOT-column (8 m × 0.5 mm I.D., Duran 50 glass) was prepared according to Cramers *et al.*[8], with slight modifications to the stationary phase and solvents. In the first coating step a siliceous support Cap-O-Sil, deactivated with benzyltriphenylphosphonium chloride (BTPPC) and suspended in the polar stationary phase PPE-21 (poly-*m*-phenyl ether ether high polymer, Chrompack, Middelburg, The Netherlands), was used. In the second coating step an additional, mixed stationary phase was coated dynamically on the surface layer of Cab-O-Sil. The mixed stationary phase consisted of 1.6% (w/w) PPE-21 and 2.6% (w/w) OV-17 (Chrompack) in toluene. The solid injection system was a modified pyrolysis sluice system (Becker, Model 767), which has been described for the determination of antiepileptic drugs[9].

The temperatures of the injection port, detector and column were 250, 260 and 190°, respectively. The flow-rates of air, hydrogen and the carrier gas (helium) through the column were 300, 35 and 5 ml/min, respectively; at the end of the column an additional flow of nitrogen was used to ensure a total flow-rate of 70–80 ml/min which is required for optimal performance of the nitrogen-selective detector.

An LKB 2091–2130 gas chromatograph–mass spectrometer–computer system was used for the unambiguous identification of the compounds eluted from the gas

chromatograph. The column, injection system and other gas chromatographic conditions were as specified before; the ion source temperature was 200°, the separator temperature 210°, electron energy 70 eV, accelerating voltage 3.5 kV and trap current 50 μA.

Hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione

Hydrolysis of pemoline in human urine to the oxazolidinedione II is rapid and essentially complete in 15 min at 80° in 1 *N* hydrochloric acid[5]. Under the same conditions, complete conversion of pemoline is achieved in human plasma and saliva; this was verified by adding known amounts of the oxazolidinedione II to plasma and saliva and comparing the results with those obtained by adding equivalent amounts of pemoline to similar samples.

Assay of urine samples

Extraction procedure for metabolite II. To 2.0 ml of urine in a centrifuge-tube was added 0.2 ml of concentrated hydrochloric acid. The mixture was immediately extracted once with 5 ml of dichloromethane–*n*-pentane (1:1) on a Cenco Whirlmixer for 15 sec, followed by centrifuging for 3 min at 3000 rpm (600 *g*). The upper organic layer was transferred to a conical evaporation-tube and 0.10 ml of ethanol containing 20.0 μg fensuximide was added as external standard. The solvent was then evaporated to dryness at 35° in a stream of dry air, and the residue was dissolved in 0.5 ml of a freshly prepared (from N-nitrosomethylurea), distilled, ethereal diazomethane solution. After at least 15 min, 5–10 μl of this solution were placed on the needle of the solid injection system, and, following evaporation of the solvent, injected into the gas chromatograph.

Extraction procedure for the hydrolysis product of pemoline. After the "oxazolidinedione metabolite" pre-extraction described above, the centrifuge-tubes containing the acidic urinary layer were heated in a water-bath at 80° for 15 min. After cooling to room temperature, 0.10 ml of ethanol containing 20.0 μg of fensuximide as internal standard was added and the mixture was extracted and assayed as described for the metabolite II in urine.

Assay of plasma samples

To 3.0 ml of human plasma in a centrifuge-tube were added 0.3 ml of concentrated hydrochloric acid and 5 ml of dichloromethane. The mixture was stirred immediately on a Cenco Whirlmixer for 15 sec, followed by centrifuging for 3 min at 3000 rpm (600 *g*). A 2.0-ml volume of the upper plasma layer was pipetted into another centrifuge-tube, 0.2 ml of concentrated hydrochloric acid were added and the mixture was hydrolyzed at 80° for 15 min. After cooling to room temperature and addition of 0.10 ml of ethanol containing 2.0 μg of fensuximide as internal standard, the mixture was extracted once with 5 ml of dichloromethane on a Cenco Whirlmixer for 10 sec and centrifuged for 3 min at 3000 rpm (600 *g*). The lower organic layer was transferred to a conical evaporation-tube and the solvent was evaporated to dryness at 35° in a stream of dry air. The residue was dissolved in 0.5 ml of a freshly prepared (from N-nitrosomethylurea), distilled, ethereal diazomethane solution. After at least 15 min the ethereal solution was evaporated again and the residue was dissolved in 0.05 ml of absolute ethanol. A 10–15-μl of this solution were placed on the needle of

the solid injection system, and, after evaporation of the solvent, injected into the gas chromatograph.

Assay of saliva samples

To 2.0 ml of saliva in a centrifuge-tube were added 0.2 ml of concentrated hydrochloric acid. After hydrolysis on a water-bath at 80° for 15 min, the extraction was completed as described for the plasma samples.

Preparation of calibration graphs and determination of extraction yields

The concentrations of the "oxazolidinedione metabolite" in urine and of pemoline in urine, plasma and saliva were calculated with the aid of calibration graphs, which were prepared by adding known amounts of the oxazolidinedione II to 2.0 ml of urine and known amounts of pemoline to 2.0 ml of urine, 3.0 ml of plasma and 2.0 ml of saliva, respectively. The samples were analyzed by the procedures described above. The ratio of the peak height of the resulting methylated oxazolidinedione (Fig. 1, III) to that of the internal standard was plotted against the known concentrations of the oxazolidinedione II and pemoline, respectively.

For the determination of the extraction yields from urine, plasma and saliva, the same procedures were followed, except that fensuximide was used as an external standard. The ratios found were compared with the ratios for standard amounts of the methylated oxazolidinedione III.

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

The relatively great sensitivity and selectivity of the nitrogen-selective detector and the use of a capillary SCOT-column facilitate the determination of low concentrations of pemoline in plasma and saliva. A prerequisite is the acidic hydrolysis to the oxazolidinedione II prior to extraction and methylation by diazomethane. This results in a short retention time, a better chromatographic behaviour and a detection limit of 1 ng per single injection with a signal-to-noise ratio of 10:1.

In Fig. 2 typical gas chromatograms are shown of a blank urine extract and a urine extract obtained from a human volunteer, having previously received 50 mg of pemoline. The lowest detectable concentration of pemoline in urine is *ca.* 100 ng/ml, which is about five times lower than obtainable by the method previously described[5]. This higher sensitivity is a direct consequence of the use of a capillary SCOT-column with a total number of 10,000 theoretical plates (calculated for the methylated oxazolidinedione III), compared with a micropacked column which has a considerably lower total number of theoretical plates ($N = 1000$ for the same compound).

In Figs. 3 and 4 gas chromatograms of extracts of plasma are shown, containing 0.61 μg pemoline per ml, and of saliva, containing 0.64 μg pemoline per ml, respectively, obtained after oral administration of 50 mg of pemoline to a human volunteer; gas chromatograms of blank plasma and saliva extracts from the same volunteer are also shown. There is no interference from endogenous urine, plasma or saliva constituents. The retention times of the compounds to be measured are short and the total time for one GC run is 5 min.

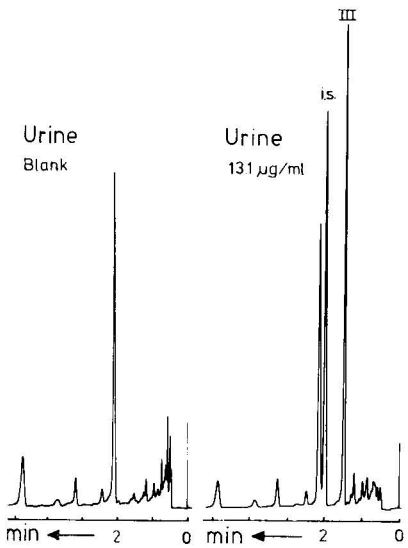


Fig. 2. Gas chromatogram of a 2.0 ml blank extract of human urine and of human urine containing 13.1 µg/ml pemoline.

Extraction procedure and precision

Because of the high lipophilicity of the oxazolidinedione II (a weak acid with $pK_a = 5.5$) only short extraction times (15 sec for urine and 10 sec for plasma and saliva) are needed, immediately after the acidic hydrolysis of pemoline at pH 0. For urine a short "oxazolidinedione metabolite" pre-extraction was necessary. In plasma

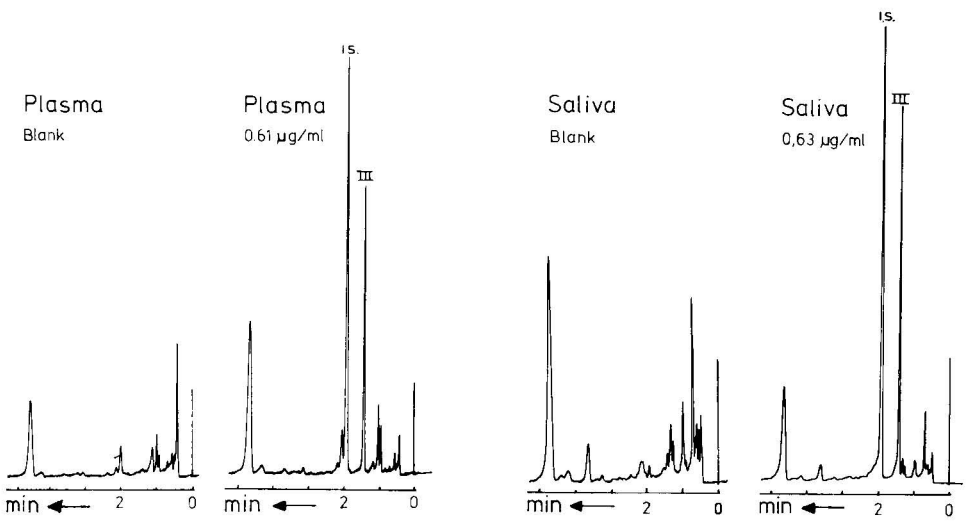


Fig. 3. Gas chromatogram of a 2.0 ml blank extract of deproteinized human plasma and of an extract of plasma containing 0.61 µg/ml pemoline.

Fig. 4. Gas chromatogram of a 2.0 ml blank extract of human saliva and of saliva containing 0.63 µg/ml pemoline.

a short pre-extraction with dichloromethane resulted in a lower detectable concentration of pemoline.

According to the standard curves (Fig. 5), there is a linear relation between the ratio of the peak height of the methylated oxazolidinedione III to that of fensuximide and the concentration of pemoline between 0.05 and 1.0 μg/ml of plasma and saliva. For urine samples, good linearity was obtained for concentrations of pemoline and the "oxazolidinedione metabolite" between 100 ng and 25 μg/ml.

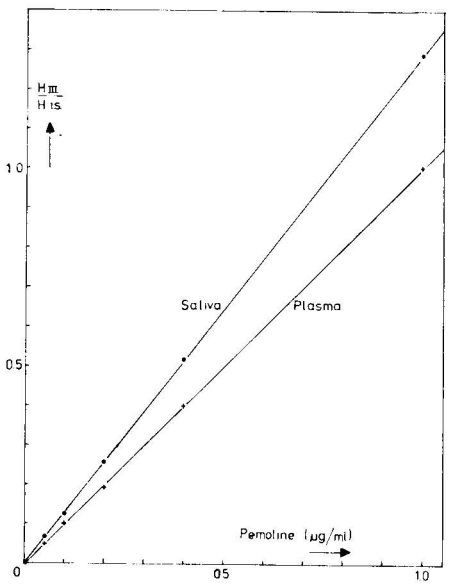


Fig. 5. Sta ndard calibration graphs for pemoline added to plasma and saliva, carried through the analytical procedure.

The reliability and accuracy of the assay procedure was assessed with the aid of five calibration curves each for urine, plasma and saliva. The corresponding correlation coefficients were in all cases better than 0.999, clearly establishing the linearity of the calibration curves. In addition, the mean recoveries were determined for the same concentration range as used to construct the calibration curves, using fensuximide as an external standard. The extraction yield of 67% for urine with a standard deviation at each concentration of $\leqslant 3\%$ ($n = 5$) is only slightly higher than the results obtained previously[5], due to the three times longer extraction time (15 sec) and the "oxazolidinedione metabolite" pre-extraction. For plasma and saliva, extraction times of 10 sec gave satisfactory and reproducible extraction yields of 82 and 100% with a standard deviation at each concentration of $\leqslant 3$ and $\leqslant 2\%$ ($n = 5$), respectively.

Identification of the extraction products and the "oxazolidinedione metabolite"

The analysis of urine, plasma and saliva samples by means of a GC–MS–computer system showed that the methylated oxazolidinedione III and fensuximide are eluted from the gas chromatograph without chemical alteration under the con-

ditions applied. Fig. 6 shows a reconstructed mass chromatogram for three specific *m/e* values obtained from a saliva extract. The retention times, at which the characteristic masses [*m/e* 191 (100%) and *m/e* 105 (90%) for the methylated oxazolidinedione III, and *m/e* 189 (20%) and *m/e* 105 (8%) for fensuximide[5]] appear simultaneously, are equal to the values for authentic methylated oxazolidinedione III and fensuximide. Furthermore, the peak having a retention time of 2.1 min (Fig. 2) appeared to correspond to methylated hippuric acid[5], and that having a retention time of 4.8 min to that of caffeine[5] (Figs. 2–4).

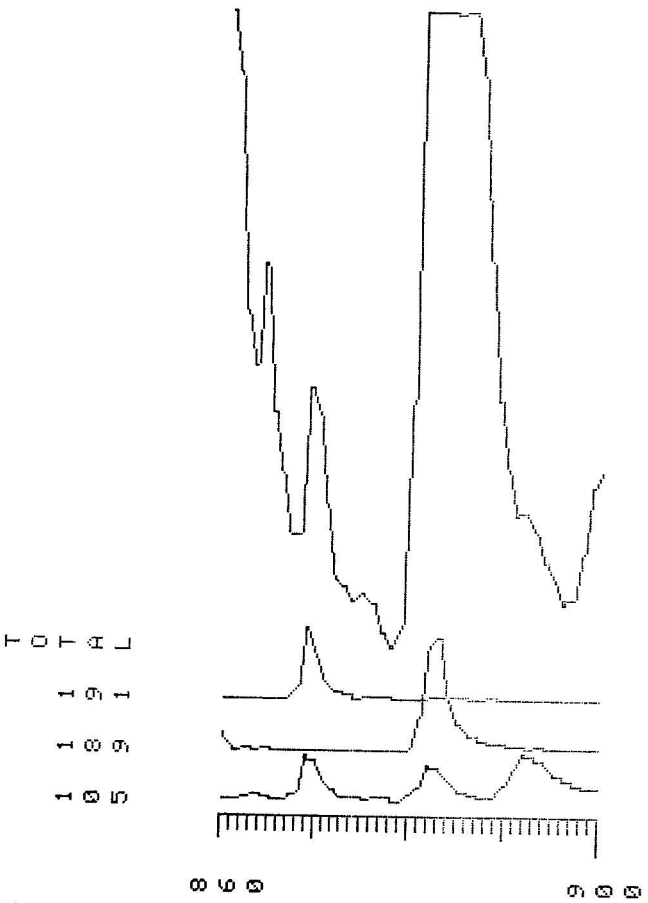


Fig. 6. Total ion current and mass chromatogram of three selected *m/e* values, characteristic of the methylated oxazolidinedione III and fensuximide: *m/e* 191 and *m/e* 105 for III, and *m/e* 189 and *m/e* 105 for fensuximide.

To investigate whether the "oxazolidinedione metabolite" is a real metabolite or an artefact of the acidic extraction procedure the following experiment was performed. Urine, plasma and saliva, spiked with 25 μg/ml pemoline, were extracted as described before except that fensuximide was used as external standard and except acidic hydrolysis. Since the peak height ratio found with GLC–AFID and GLC–MS did not significantly exceed that of the blanks, it must be concluded that the "ozazoli-

dinedione metabolite" which is extracted prior to acidic hydrolysis is indeed a real metabolite. This metabolite was identified only in the urine of human volunteers after oral administration of 50 mg of pemoline.

CONCLUSIONS

The assay described for the quantitative determination of pemoline in human urine, plasma and saliva is rapid and relatively simple. It takes account of the occurrence of 5-phenyl-2,4-oxazolidinedione itself as a metabolite of pemoline, while mandelic acid, another metabolite of pemoline, or other endogenous substances do not interfere with the method. The method has been successfully applied to the study of pharmacokinetics of pemoline and its "oxazolidinedione metabolite" in human urine, plasma and saliva[10].

The SCOT-columns (with mixed stationary phases) are not yet in common use but they appear to be preferable to packed and micropacked columns when it is required to determine low concentrations of drugs or metabolites in biological fluids for pharmacokinetic studies.

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PURIFICATION OF ACETYLCHOLINESTERASE FROM PIG CEREBRAL CORTEX BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Acetylcholinesterase from pig cerebral cortex was solubilised with 1% (w/v) Triton X-100 and purified by affinity chromatography. Three different ligands were investigated and details are given for their preparation. The elution profile depended on the presence of Triton X-100, the ionic strength and the inhibitor used to remove the enzyme from the column as well as the nature of the affinity material.

The most efficient purification was obtained when the enzyme was eluted from a column containing the acetylcholinesterase inhibitor [1-methyl-9-(N$^{\beta}$-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide covalently linked to Sepharose 4B. A recovery of 44% of the applied enzyme was eluted from the column with a specific activity of 148 μmoles min^{-1} mg^{-1} and a purification of 900-fold.

INTRODUCTION

Acetylcholinesterase (AChE; acetylcholine hydrolase, EC 3.1.1.7) is present in the central nervous system of all vertebrates[1] and reports from several groups of workers have shown that the brain enzyme exists in a number of molecular forms[2–4]. Work in our own laboratory has concentrated on pig cerebral cortex as the source of AChE and the effect of a number of methods of solubilisation has been studied together with the multiple enzymic forms of the resultant preparations[5]. This initial investigation was extended to a study of the naturally soluble and membrane forms of the enzyme[6] and the multiple molecular forms of isolated subcellular fractions[7]. In the above work, relatively crude preparations of the enzyme were used and in this paper we present the results obtained during attempts to purify the enzyme by affinity chromatography using several affinity materials.

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MATERIALS AND METHODS

Materials

Fresh pig brains were kindly provided by Walls Slaughterhouse (Acton, Great Britain) and the Co-operative Society Slaughterhouse (Woolwich, London, Great Britain).

5,5′-Dithiobis-(2-nitrobenzoate) (DTNB) and acetylthiocholine iodide (ATChI) were obtained from Sigma (London, Great Britain) and Triton X-100 from BDH (Poole, Great Britain).

Materials for the three affinity columns were obtained from the following sources. Sepharose was bought from Pharmacia (London, Great Britain), N-benzyloxycarbonyl-ε-aminocaproic acid and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride were purchased from Aldrich (Wembley, Great Britain). Triethylamine, *p*-dimethylaminoaniline and phenol (redistilled before use) were supplied by Fisons (Loughborough, Great Britain). Anhydrous HBr in glacial acetic acid, isobutylchloroformate, iodomethane, 9-chloracridine and aminopyridine were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

All other reagents used were analytical grade and all solutions were prepared in glass distilled water.

Preparation of the enzyme

Porcine brains were obtained from the slaughterhouse within 20 min of death, transported to the laboratory in crushed ice, then stored frozen until required. After thawing at 4°, the membranes and blood vessels were removed from the surface of the brains and the cortex excised. The AChE was then solubilised using the method of Ho and Ellman[2] with slight modifications. The brain cortex was finely chopped and a 20% (w/v) suspension prepared in sodium phosphate buffer (0.03 *M*, pH 7.0) by homogenising for 5 min at 4° in a Waring blender. The suspension was then centrifuged at 100,000 *g* for 1 h and the supernatant decanted. The AChE in this fraction was referred to as the "naturally soluble" enzyme. The pellet was resuspended in the buffer solution to give a protein concentration of 8 mg/ml and Triton X-100 added to a final concentration of 1% (w/v). The mixture was stirred for 10 min at room temperature and centrifuged at 100,000 *g* for 1 h. The supernatant was taken as the "Triton solubilised enzyme".

Assay of acetylcholinesterase

AChE activity was determined at 30° and pH 8.0 by a colorimetric method using acetylthiocholine as substrate[8]. For routine assay, 50 μl of enzyme was mixed with 3 ml of sodium phosphate buffer (0.1 *M*, pH 8) and incubated at 30° for 10 min. After this time 100 μl of DTNB (0.01 *M*) was added to the mixture followed by 20 μl of ATChI (158.5 m*M*) to give a final substrate concentration of 1 m*M*. The hydrolysis of acetylthiocholine produces thiocholine which reacts with DTNB to give 5-thio-2-nitrobenzoate, a yellow chromophore that absorbs at 412 nm. The enzyme activity was then measured by following the increase in absorbance at 412 nm in a Perkin-Elmer SP124 double beam spectrophotometer. The activity was determined in I.U. from the initial slope of the curve by calculating the extinction change (ΔE) with time (min) and allowing for the molar extinction coefficient of the chromo-

phore ($1.36 \cdot 10^4$ l mole^{-1} cm^{-1}), the volume of the enzyme (0.05 ml), the total volume of the reaction mixture (3.17 ml) and a factor of 1000 to obtain μmoles then:

$$\text{AChE activity} \quad (\Delta E \cdot 1000 \cdot 3.17)/(\text{min} \cdot 1.36 \cdot 10^4 \cdot 0.05)$$
$$= (\Delta E/\text{min}) \cdot 4.66 \ \mu\text{moles min}^{-1}\ \text{ml}^{-1}$$

Estimation of protein

Protein was determined by the method of Lowry *et al.*[9] using crystalline bovine serum albumin as standard. However if Triton X-100 was present, a gelatinous precipitate was formed, but this interference was overcome by centrifuging the precipitate (1000 *g* for 5 min) and incorporating Triton X-100 in the reagent blank and standard[10,11].

Affinity column I (MAP-agarose)

Preparation of the ligand N-methyl-3-aminopyridinium iodide. Aminopyridine (53 mmoles; 5 g) was dissolved in 75 ml of acetone, added to iodomethane (292 mmoles; 15 ml) and stirred for 18 h. The precipitate was then filtered and washed with acetone.

N-methyl-3-aminopyridinium iodide (MAP) was prepared in 11.2 g (95%) yield; m.p. (found): 120–122°; m.p. (literature) 123°.

Preparation of MAP-agarose. The affinity material was built up stepwise by a method based on the procedures used by Berman and Young[12] and Goodkin and Howard[13]. Cyanogen bromide (30 g) was added with stirring to 100 ml of washed Sepharose 2B suspended in 100 ml of water. The temperature was kept below 20° with crushed ice and the pH at 11.0 with 4 *M* NaOH. After 12 min the suspension was filtered rapidly under suction with 1 l of sodium borate buffer (0.1 *M*, pH 9.5) at 4°. Diaminobutane (198 mmoles; 17.4 g) in 100 ml of sodium borate buffer (0.1 *M*, pH 9.5) was immediately added and the mixture shaken gently at 4° overnight. The excess amine was removed by filtration on a sintered glass funnel and the washed Sepharose dispersed in 100 ml of water. Succinic anhydride (100 mmoles; 10 g) dissolved in 105 ml water at 4° was added to the Sepharose and the pH raised to 6.0 with 4 *M* NaOH. The pH was carefully monitored and after it had stabilised the suspension was left for 5 h at 4°.

N-Methyl-3-aminopyridinium iodide (0.6 mmoles; 0.13 g) was added to 6 ml of the succinylated resin in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (4.5 mmoles; 0.86 g), shaken gently overnight at 4° and thoroughly washed before use with elution buffer.

Affinity column II (ε-aminocaproyl-PTA-agarose)

Preparation of ligand [N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide hydrobromide. The method was based on that of Dudai and Silman[14] with some modifications.

N-Benzyloxycarbonyl-ε-aminocaproic acid (60 mmoles; 15.9 g) was dissolved in 200 ml of ethyl acetate with vigorous stirring and cooled in an ice bath. Triethylamine (60 mmoles; 6.1 g) and isobutylchloroformate (60 mmoles; 8.2 g) were added with stirring and the mixture left to stand for 20 min. The solution was then vig-

orously stirred and 60 mmoles (8.2 g) of *p*-dimethylaminoaniline dissolved in 50 ml of ice cold ethyl acetate was added dropwise and the solution left at room temperature for a further 5 h. The resulting precipitate was filtered, washed successively with water, ethyl acetate and light petroleum (b.p. 40–60°) and dried by suction. This crude material was then treated with activated charcoal in hot methanol and recrystallised from hot methanol.

N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-N,N′-dimethyl-*p*-phenylenediamine was prepared in 8.23 g (36%) yield; m.p. (found) 110–111°; m.p. (literature) 110–111°.

The above compound (22.1 mmoles; 8.23 g) was suspended in 40 ml of methanol with 110.5 mmoles (5.26 g) of iodomethane and refluxed for 3 h. The product was taken to dryness under reduced pressure, triturated with 200 ml of diethyl ether and recrystallised from 100 ml of absolute ethanol.

[N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-*p*-aminophenyl] trimethyl ammonium iodide was prepared in 7.64 g (71%) yield; m.p. (found) 142.5–144°; m.p. (literature) 143–145°.

This compound (10 mmoles; 5.25 g) was dissolved in 10 ml of glacial acetic acid + HBr, left at 25° for 40 min, then precipitated with 400 ml of dry diethyl ether. The solidification was completed by trituration with five batches of dry diethyl ether. The solid was dried for 48 h in a vacuum desiccator over pellets of NaOH, then finally recrystallised from 200 ml of absolute ethanol.

[N-(ε-Aminocaproyl)-*p*-aminophenyl] trimethyl ammonium bromide hydrobromide was prepared in 2.95 g (69%) yield; m.p. (found) 190–192°; m.p. (literature) 191–194°.

For convenience this compound is referred to as ε-aminocaproyl-PTA ,using the convention of Dudai and Silman[14].

Preparation of ε-aminocaproyl-PTA-agarose. The material for the affinity column was prepared by a method based on that of Axen *et al.*[15] as modified by Blumberg *et al.*[16]. Cyanogen bromide (7 g) was stirred with 70 ml of water for 10 min during which time most of it had dissolved. A slurry of Sepharose 4B was washed with water and 70 ml of this was added to the stirred cyanogen bromide. The pH was immediately adjusted to 11.0 with 6 *M* NaOH and the mixture cooled below 20° with crushed ice for 8 min. The activated gel was then rapidly washed with 1 l of ice cold water on a Buchner funnel and the wet gel quickly added to a solution of ε-aminocaproyl-PTA (117 μmoles; 50 mg) in 36 ml of 0.5 *M* $NaHCO_3$. The mixture was shaken gently for 16 h in the cold room, filtered and washed thoroughly with 0.1 *M* $NaHCO_3$ and water. The amount of ligand coupled was found by estimating spectrophotometrically the quantity of ligand remaining in the washings. (At pH 9.8, ε-aminocaproyl-PTA, $\lambda_{max.}$ = 245 nm, E = 15,500 l $mole^{-1}$ cm^{-1})[14].

Approximately 1 μmole of ligand was coupled per ml of Sepharose. The affinity column was washed extensively with the elution buffer before use.

Affinity column III (MAC-agarose)

Preparation of ligand [1-methyl-9-(N$^{\beta}$-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide. The method of preparation was based on that of Dudai and Silman[14] with some amendments advised by Silman (personal communication). Phenol (1.28 moles; 120 g) was heated at 70° and 93.6 mmoles (20 g) of 9-chloracridine added to the melt. After all the solid had dissolved, 100 ml of 1,2-

propylenediamine was added to the vigorously stirred mixture and the temperature raised to 120°. It was critical that this temperature was not exceeded. After 30 min, the 9-(β-aminopropylamino) acridine was precipitated by pouring the reaction mixture with rapid stirring into 1600 ml of 0.75 *M* NaOH. This was left overnight to complete the solidification, filtered and washed successively with 2 *M* NaOH and water. The crude product was dried in vacuo, refluxed in 1100 ml of benzene and filtered. The insoluble residue was discarded and the filtrate concentrated to 200 ml, mixed with 50 ml of petroleum and left in the cold room overnight. The crystalline product was filtered and washed with benzene.

9-(β-Aminopropylamino) acridine was prepared in 9.5 g (40%) yield; m.p. (found) 131–139°; m.p. (literature) 131–133°.

N-Benzyloxycarbonyl-ε-aminocaproic acid (30 mmoles; 7.9 g) was dissolved in 150 ml dry ethyl acetate in a salt ice bath at −10° and stirred vigorously. Triethylamine (30 mmoles; 3.05 g) followed by isobutylchloroformate (30 mmoles; 4.1 g) were added to the mixture which continued to be stirred for a further 20 min at −10°. The precipitated triethylamine hydrochloride was washed with 20 ml of dry ethyl acetate and the combined filtrate and washings returned to the salt ice bath when a solution of 9-(β-aminopropylamino) acridine (25 mmoles; 6.3 g) in 100 ml of dimethylformamide was added. The mixture was left for a further 10 min at −10° and then 12 h at room temperature. Thin-layer chromatography (TLC) on silica gel in glacial acetic acid indicated that the reaction had gone to completion. The mixture was evaporated to dryness under reduced pressure, the residue dissolved in 50 ml of absolute methanol and 8 ml of iodomethane were added to the solution which was then refluxed for 4 h and left overnight at room temperature. TLC on silica gel in ethyl acetate was used to check the reaction to be gone to completion. The solution was evaporated to dryness under reduced pressure, extracted twice with 100-ml aliquots of dry ethyl acetate and the product recrystallised from 80 ml of 2-propanol. The product was finally filtered and washed with ice cold 2-propanol followed by ice cold diethyl ether.

[N-(N-Benzyloxycarbonyl-ε-aminocaproyl)-β-aminopropylamino) acridinium iodide was prepared 7.2 g yield; m.p. (found) 155–157°; m.p. (literature) 156–157°.

The above quaternary compound (6.4 mmoles; 4.1 g) was dissolved in 40 ml of anhydrous glacial acetic acid and 80 ml of anhydrous HBr in glacial acetic acid were added with stirring. This solution was left to stand at room temperature for 30 min and the product precipitated with dry diethyl ether. The precipitate was triturated with five batches of diethyl ether, filtered, washed with diethyl ether and stored in a vacuum desiccator over dry NaOH pellets for 24 h. The product was finally recrystallized from absolute ethanol.

[1-Methyl-9-(N$^{\beta}$-ε-aminocaproyl)-β-aminopropyl amino] acridinium bromide hydrobromide was prepared in 2.5 g (72%) yield; m.p. (found) 236–240°; m.p. (literature) > 240° with decomposition. For convenience, this compound is referred to as MAC[14].

Preparation of MAC-agarose. The ligand was coupled to the Sepharose 4B in the same way as described for the preparation of the ε-aminocaproyl-PTA-agarose. The ligand MAC (0.09 mmoles; 50 mg) was mixed with 70 ml of the cyanogen bromide activated Sepharose 4B. The washings were assayed spectrophotometrically when 0.5–1.0 μmole of MAC was found to be coupled per ml of Sepharose. (At pH

8.3, $\varepsilon = 7{,}880$ l mole^{-1} cm^{-1} at 393 nm, $\varepsilon = 12{,}050$ l mole^{-1} cm^{-1} at 410 nm and $\varepsilon = 10{,}150$ l mole^{-1} cm^{-1} at 431 nm)[14].

Conditions for use of affinity columns

All operations were carried out at 4°.

Binding. The columns used had a bed volume of 12 ml and the enzyme was run through the affinity material at a rate of 30–40 ml/h.

Elution. The standard elution buffer was 0.03 *M* sodium phosphate, pH 7.0 containing 1% (w/v) Triton X-100 and all solutions used for elution were prepared in this medium. The column was first washed with 5–10 column volumes of buffer until the eluate gave a zero reading of protein. The elution was then continued with 5 column volumes of the standard buffer containing the competitive inhibitor edrophonium chloride (10 m*M*) or decamethonium bromide (10 m*M*) and 2–5-ml fractions were collected. Finally, the column was washed with five column volumes of buffer containing 1 *M* NaCl.

Washing. The column was prepared for further use by washing with 6 *M* guanidine hydrochloride and 50 column volumes of elution buffer.

Enzyme assay in fractions. The enzyme was assayed by the method previously described after dialysis for 72 h against three changes of 2 l of elution buffer.

RESULTS

Column I (MAP-agarose)

After equilibration of the column, 1 ml of the enzyme which contained approximately 25 mg of protein was applied to the column. The standard elution procedure was carried out with edrophonium chloride (10 m*M*) as inhibitor. The elution profile obtained (Fig. 1) showed two peaks of activity, one emerging from the column after edrophonium chloride and the other following elution with high salt concentration (1 *M* NaCl). The enzyme eluted in the edrophonium chloride peak gave only a 5-fold purification and contained 35% of the total AChE activity applied to the column. If Triton X-100 was excluded from the elution media, the AChE was quantitatively adsorbed onto the column and could not be removed by the inhibitor or high salt solution until the detergent was reintroduced into the buffer.

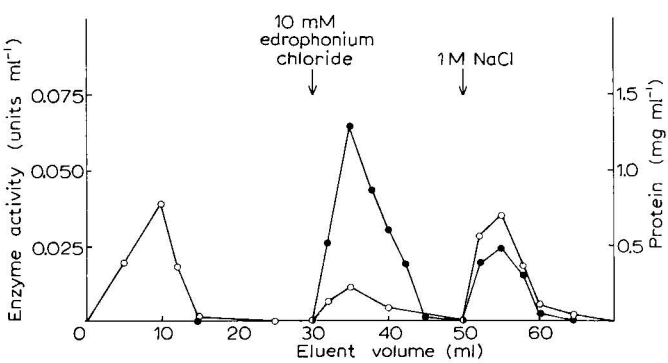


Fig. 1. Fig. 1. Elution of acetylcholinestetase activity (●) and protein (○) from an MAP-agarose column.

Column II (ε-aminocaproyl-PTA-agarose)

A much greater volume of the enzyme was used in this column (150–200 ml) which was washed with buffer until protein could no longer be detected in the eluate. The column was then eluted with buffer containing edrophonium chloride followed by high salt concentration as previously described. Rather surprisingly, only 0.4% of the AChE activity appeared in the inhibitor peak with no significant purification. Increasing the concentration of inhibitor 5-fold to 50 m*M* made no difference to the yield or degree of purification of the enzyme. In view of this, a different inhibitor, namely, decamethonium bromide was used to remove the AChE from the column and this proved to be more effective. The protocol was the same as that described above except that decamethonium bromide (10 m*M*) was substituted for the edrophonium chloride. With this modification, there was a significant improvement in the elution with 35% of the AChE activity appearing in the inhibitor peak and a 16-fold purification (Fig. 2). As with the previous column, exclusion of Triton X-100 from the elution buffer led to strong retention of the enzyme to the column with none appearing in the collected fractions.

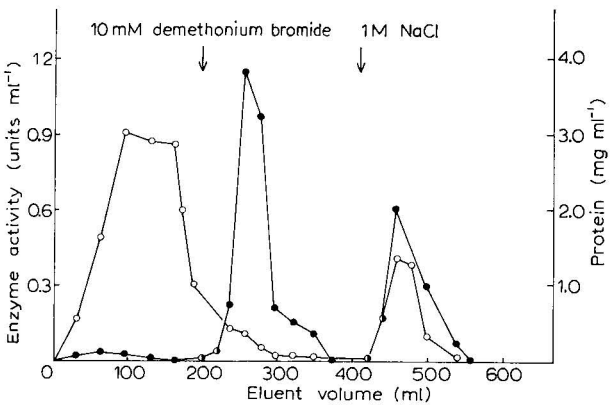


Fig. 2. Elution profile of acetylcholinesterase activity (●) and protein (○) from an ε-aminocaproyl-PTA affinity column.

Column III (MAC-agarose)

As with column II, large volumes of the enzyme could be applied without any loss in the collected fractions. The elution procedure was the same as for column II with a wash of successively decamethonium bromide and 1 *M* NaCl.

A total of 44% of the AChE activity was eluted in the decamethonium peak which had a specific activity of 148 μmoles min^{-1} mg^{-1}, a purification of 900-fold (Fig. 3). When this purified enzyme was rechromatographed on another MAC column and eluted with decamethonium bromide, the specific activity was at least doubled although the protein content could not be determined accurately due to its low level.

A summary of the results obtained for all three columns is shown in Table I.

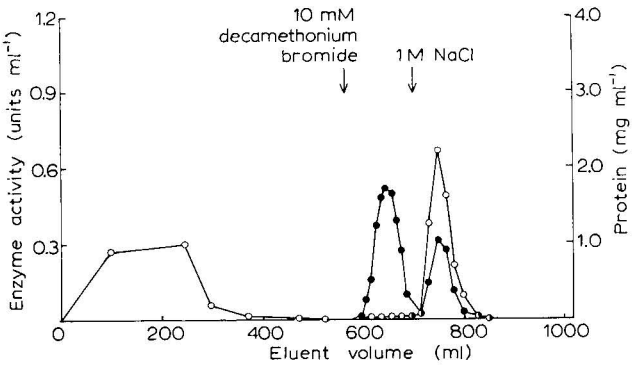


Fig. 3. Purification of acetylcholinesterase by affinity chromatography on MAC-agarose column. Acetylcholinesterase (●), protein (○).

TABLE I

AFFINITY CHROMATOGRAPHY OF ACETYLCHOLINESTERASE ON THREE DIFFERENT MATERIALS

*Affinity material**		*Protein (mg)*	*Enzyme activity (μmoles min⁻¹)*	*Specific activity (μmoles min⁻¹mg⁻¹)*	*Yield (%)*	*Purification (times)*
MAP-agarose	Total applied	25	1.671	0.067		
	Recovered in peak	1.75	0.585	0.336	35	5
ε-Aminocaproyl-PTA-agarose	Total applied	955	150	0.157	—	—
	Recovered in peak	21	52	2.476	35	16
MAC-agarose	Total applied	713	117	0.164	—	—
	Recovered in peak	0.349	51.5	147.6	44	900

* See Fig. 4 for the formulae.

DISCUSSION

The first affinity column to be prepared was MAP-agarose as this had been used by Goodkin and Howard[13] to purify the AChE of rat brain synaptosomal membranes. The attraction of this method was that the rat brain enzyme had been solubilised with Triton X-100 and so a procedure was available which involved the presence of detergent in the elution medium. The results show that the column was effective in binding the AChE and other proteins and since detergent was present in the elution medium, it was unlikely that the enzyme was being retarded by hydrophobic interactions on the apolar side chain. Elution with edrophonium chloride removed 35% of the AChE activity from the column but with an overall purification of only 6-fold. This compared unfavourably with the results of Goodkin and Howard[13] in which they succeeded in purifying the rat brain enzyme by 100- to 150-fold. The reason for this difference possibly lies in the different properties of the rat brain and pig brain acetylcholinesterases. In view of the disappointing results obtained in the purification of AChE, another column was prepared. The phenyltrimethylammonium ligand was selected as this has been shown to be a good competitive inhibitor of AChE especially when bound to an ε-aminocaproyl side-arm. Dudai *et al.*[17] have shown this moiety to have an inhibitor constant (K_i) of 6 μM for AChE.

The ε-aminocaproyl-PTA-agarose was very efficient in binding the pig brain AChE as shown by the fact that large volumes of the enzyme could be applied without being eluted by the buffer. The largest volume used was 300 ml of extract containing about 150 units of AChE activity. Even with this load very little of the enzyme appeared in the eluate although contaminating protein was washed off the column. The only factor limiting the volume of extract applied to the column was getting sufficient starting material. However the initial experiments with this column were disappointing and only a trace of the enzyme activity (0.4%) was found in the crude edrophonium chloride wash. Increasing the inhibitor concentration to 50 m*M* made no difference. This may have been due to the fact that edrophonium chloride has only one positively charged nitrogen atom which would mean that if the enzyme was binding to the quaternary atom on the column by way of one of its peripheral anionic sites as well as by the active centre anionic site then the edrophonium chloride would not be very effective. For this reason, another competitive inhibitor, decamethonium bromide, was used in an attempt to elute the AChE. This molecule was chosen because it has two positive centres separated by a decamethylene bridge which has just the correct dimensions to span the distance between the active anionic and peripheral anionic sites. Furthermore, several other groups seem to have preferred to use this inhibitor in the affinity chromatography of AChE[17].

Decamethonium bromide was better than edrophonium chloride at removing the acetylcholinesterase from the column and a recovery of 35% was obtained. Again the purification was small, being only 16-fold, which compared unfavourably with the 700-fold, obtained by Chan *et al.*[18]. Another affinity column (MAC-agarose) was therefore prepared to try to improve purification.

The MAC-agarose was highly successful at binding the AChE and up to 250 ml of the crude extract could be run through the column without any of then enzyme being lost in the eluate. The yield in the decamethonium peak was the best obtained of the methods so far tried at 44% and the 900-fold purification with a specific activity of 148 μmoles min^{-1} mg^{-1} was also satisfactory. As with the previous two columns, a further 13% of enzyme activity was obtained with the salt wash so this compares favourably with the results of Dudai *et al.*[17], who eluted 50% of the electric eel AChE from the same column in the presence of 1 *M* NaCl. The preliminary experiments with this column did include 1 *M* NaCl in all the eluting media as carried out by Dudai *et al.*[17], but only very small amounts of the enzyme were retarded whereas the above group had great success in binding the eel AChE to the column at high ionic strength. The difference between these two results may have been due to the enzyme originating from separate species or to the fact that Dudai's group had no Triton X-100 in their preparation whereas this detergent was present at all stages of our preparation. However the column was very successful with the pig brain AChE where the enzyme was eluted with decamethonium bromide at low ionic strength. When this enzyme was rechromatographed on the column, the specific activity was at least doubled giving a usefully purified preparation. The area of uncertainty arose from the problem of measuring the protein content with any great accuracy because of its low level.

A second peak of activity following the inhibitor peak was obtained for all three columns when they were washed with 1 *M* NaCl. The ligands were chemically diverse (Fig. 4) and yet from 12–14% of the activity was consistently removed at

high ionic strength. This suggests that the enzyme, eluted with 1 *M* NaCl, may have been binding to the agarose matrix as this was the one feature common to all three columns. The binding of this minor peak of enzyme activity would appear to be electrostatic since a high salt concentration was required to remove it from the column. Electrophoresis of this peak on a gradient of polyacrylamide showed the same range of molecular forms as the purified enzyme obtained from the main peak and the crude enzyme extract[5]. This second peak does not therefore appear to be a different molecular form of pig brain AChE and as mentioned above probably represents a degree of non-specific binding of the enzyme to the agarose matrix. Little or no purification was obtained in this second peak which was therefore not used in subsequent work.

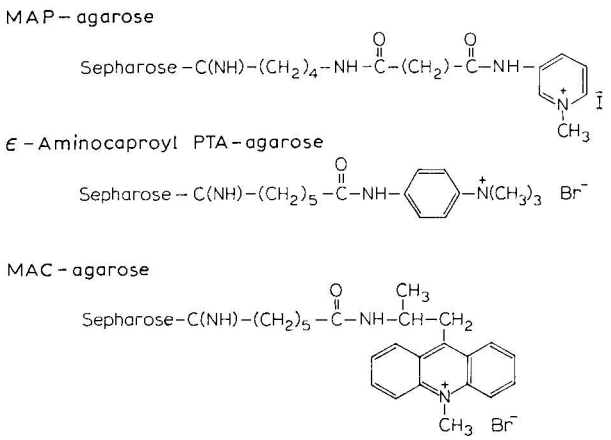


Fig. 4. The ligands used during the purification of acetylcholinesterase by affinity chromatography.

The other result common to all three columns was the fact that if Triton X-100 was excluded from the elution media then AChE was quantitatively bound to the column and could not be removed by changing the concentration of the inhibitor or salt. It seems likely that the detergent-depleted enzyme was binding hydrophobically to the apolar side chains linking the agarose and the ligand. However if only 0.1% (w/v) Triton X-100 was reintroduced into the decamethonium solution the enzyme could be removed and this was the method used for preparing a low-detergent sample of AChE.

The results with these columns clearly show that a published method for purifying an enzyme from one source by affinity chromatography cannot necessarily be applied to the same enzyme from another source. Some ligands that had been successfully used for AChE were found to be unsatisfactory for the pig brain enzyme. However MAC-agarose proved to be highly efficient with a satisfactory yield and a good degree of purification. This material has thus been used in further studies.

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AFFINITY CHROMATOGRAPHY OF AN S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASE USING IMMOBILIZED S-ADENOSYLHOMOCYSTEINE

PURIFICATION OF THE INDOLETHYLAMINE N-METHYLTRANSFERASES OF *PHALARIS TUBEROSA*

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SUMMARY

In the cases that have been studied so far, S-adenosylhomocysteine (SAH) is a powerful inhibitor of S-adenosylmethionine (SAM) binding to SAM-dependent methyltransferases. We deduced, from the available data on the binding of SAM and SAH analogues to SAM dependent methyltransferases, that linkage of SAH through the carboxyl group to an immobilized support would lead to a more general affinity adsorbent for SAM-dependent methyltransferases than linkage through other functional groups. This paper describes the synthesis of this affinity adsorbent and its use to purify the two indolethylamine N-methyltransferases of *Phalaris tuberosa*.

INTRODUCTION

Phalaris tuberosa, a pasture grass of southeast Australia, contains two indolethylamine N-methyltransferases. The primary indolethylamine N-methyltransferase (PIM) catalyzes the N-methylation of the primary amines

$$\text{Indolethylamine} + \text{SAM} \rightarrow \text{N-methylindolethylamine} + \text{SAH}$$

where the indolethylamine is tryptamine (T), or its 5-hydroxy (5-OHT) (serotonin), or its 5-methoxy (5-MeOT) derivative. The corresponding products are N-methyltryptamine (MT), or the 5-hydroxy (5-OHMT) or the 5-methoxy (5-MeOMT) derivative.

The secondary indolethylamine N-methyltransferase (SIM) catalyzes the N-methylation of the above N-methyltryptamines to the corresponding N,N-dimethyltryptamines [DMT, 5-OHDMT (bufotenin) and 5-MeODMT]. DMT and 5-Me-

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ODMT are the physiological products of this pathway in *P. tuberosa*, from tryptophan, although the ratio of DMT to 5-MeODMT varies with the particular strain; 5-OHDMT is present, in lower concentrations[1,2].

The enzymes were not separated by preparative chromatographic techniques (gel filtration, CM- and DEAE-Sephadex ion-exchange, or hydroxyapatite) and the presence of two enzymes, in the 80-fold purified preparation, was deduced from the behavior of the enzyme activities towards various environmental effects (sodium chloride, urea, temperature, inactivation with time, pH optima and activity in solutions of various buffers, cations and anions). The PIM activities always correlated together and the SIM activities always correlated together[1,2].

Initial velocity and product inhibition studies showed that the mechanism for SIM was rapid equilibrium random bi-bi with formation of one dead-end complex[1,3] (not ordered as in ref. 2). The mechanism for PIM is likely to be the same due to the many other similarities of the enzymes[1–3], thus the enzymes will bind to the reactants independently and will bind to a suitable immobilized form of any of these four compounds.

Preliminary details of the affinity adsorbent described here have been reported before[3]. This paper is a full description of the affinity purification. Further details[1,2] will be described in subsequent papers.

MATERIALS AND METHODS

Buffers

All buffers contained ethylenediaminetetracetic acid (EDTA) (1 m*M*), mercaptoethanol (10 m*M*) and are 50 m*M* with respect to the buffering ion. The washing and eluting buffers for affinity chromatography contained bovine serum albumin (BSA) (250 μg/ml) and sodium chloride (150 m*M*). The fraction collecting tubes contained 0.2 vol. of 1 *M* Tris–HCl, pH 8.50 to restore the pH of the eluate to the original pH.

Enzyme preparation

This is described in ref. 1 and will be the subject of a future paper. Briefly, the shoots of ten-days old seedlings of *P. tuberosa* were homogenized, centrifuged (1 h at 140,000 *g*), the supernatant fractionated by ammonium sulphate, and the 50–60% saturation fraction chromatographed on DEAE-Sephadex. This gave an 80-fold purified preparation with 40% recovery of PIM and 30% recovery of SIM.

Enzyme assay

This is a modification of a previously described method[4]. The assay mixture containing: 80 μl enzyme in assay buffer (50 m*M* Tris–HCl, pH 8.50), 10 μl indolethylamine (100 nmole, final concentration 1 m*M*) and 10 μl [methyl-^{14}C] SAM (20 nmole, 10 pCi, final concentration = 200 μ*M*) (total volume 100 μl) was incubated at 25° for 1 h in a capped scintillation vial. The reaction was terminated by addition of 1 *M* boric acid–sodium carbonate, pH 10.0 (200 μl) and then at convenience water (2 ml) was added (this lowers the blank level for the assay). Only the desired enzyme products were produced by the 80-fold purified preparation and these were extracted directly into scintillant [toluene, 10 ml containing 2,5-diphenyloxazole (4 g/l) and

1,4-di(2-(5-phenyloxazolyl))benzene (100 mg/l)], by shaking, after addition of the scintillant to the reaction mixture. The mixture could then be counted directly (boiled enzyme blank, 100 ± 30 pmole/h) or after decanting the scintillant following freezing of the mixture (boiled enzyme blank, 4 ± 2 pmole/h).

The presence of both enzymes in the enzyme preparation means that the assay for PIM will be systematically high because of the further methylation of the PIM reaction product by SIM. This led to a 30% overestimation of PIM activity and precluded kinetic analysis of this enzyme. However, since the ratio of PIM/SIM did not vary much in different enzyme preparations, this did not lead to any complication in estimating the presence of PIM activity.

Materials

Materials were purchased as follows: [methyl-^{14}C]-SAM, 0.5 mCi/mmole, CFA 428 from The Radiochemical Centre (Amersham, Great Britain); Agarose A-0.5 m from Bio-Rad Labs. (Richmond, Calif., U.S.A.); Sepharose 4B from Pharmacia (Uppsala, Sweden); tryptamine from BDH (Poole, Great Britain); other tryptamines were synthesized[5]; SAH, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide from Sigma (St. Louis, Mo., U.S.A.); cyanogen bromide, ethylenediamine, diaminodipropylamine from Mallinkrodt (St. Louis, Mo., U.S.A.) and *Phalaris tuberosa* seeds (commercial cultivar) from Rumseys Seeds (Sydney, Australia).

The activation and coupling were done by standard methods[6]. After cyanogen bromide activation, SAH was coupled directly to the gel through the N^6-group, by keeping the pH low (pH 7), this condition preferring coupling through the less basic N^6-group rather than the homocysteine amino group. Otherwise ethylenediamine or diaminodipropylamine were coupled to the activated gel, followed by carbodiimide coupling of the SAH. Gels (10 ml) were used, with side chain concentrations of 5–10 m*M* (as judged by titration), and SAH concentrations in the coupling mixture of 10–20 mg/10 ml column.

The proposed structure of the affinity column is shown in Fig. 1.

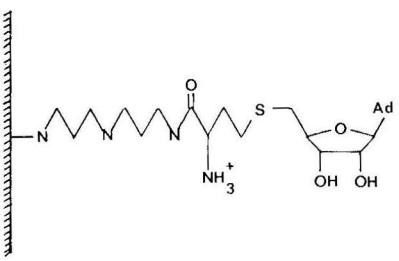


Fig. 1. The structure of the SAH affinity adsorbent.

RESULTS

Design of the affinity adsorbent

Earlier work has shown that SAH is a general inhibitor of SAM dependent methyltransferases, causing non-linearity in the time course of reaction[7], inhibiting tRNA[8,9] phenylethylamine, catechol and acetylserotonin[10] imidazole, acetylserotonin and homocysteine[11] catechol[12] and fatty acid carboxyl[13] methyltransferases.

Kinetic parameters and competition types have been determined for many enzymes and in all cases SAH was a strong inhibitor: tyramine and N-methyltyramine[14] phenylethylamine[10] catechol[15,16] histamine[17], N^2-guanine[18], protein II[19] arginine[20], lysine[21] indolethylamine (rabbit)[22], coniine[21], isoflavone[24] protein I[25], virus mRNA[26] and caffeic acid[27,28] methyltransferases.

SAM and SAH are co-factors for the bacterial DNA restriction modification enzymes and are allosteric effectors for some of the restriction endonucleases. Enzymically these methyltransferases differ from the other enzymes described here, through their low turnover number (in the order of minutes per reaction) and low K_m's[29–36].

SAH binds strongly to SIM[1,3] (competitive with respect to SAM binding, $K_i = 4\ \mu M$). PIM and SIM are not inhibited by adenosine (1 mM) or homocysteine (1 mM). We decided to try an affinity adsorbent containing an immobilized form of SAH. This adsorbent we hoped would also be of general use for purifying other SAM dependent methyltransferases. SAM was not tried as an affinity ligand because of its greater chemical and enzymic lability.

We then had to choose a suitable way of immobilizing SAH. There are three groups on SAH through which suitable linkages can be formed to an agarose gel. They are the 6-amino and the homocysteine amino and carboxyl groups. The nature of the SAM–SAH binding site has been investigated for three methyltransferases[11] using SAM analogues with one functional group missing. The homocysteine amino was required for binding to all methyltransferases, the acid group for only one enzyme and the purine 6-amino was not required for binding to any enzyme. Work on another enzyme showed that changing the base of SAH to guanine or cytosine produced a compound that was a poor inhibitor of the enzyme[37]. S-adenosylhomocysteamine (acid group removed) was still a good inhibitor.

The analogue with the ribose ring O replaced by $-CH_2-$ was inactive with catechol O-methyltransferase[16]. The 2-fluoro derivative was a poorer inhibitor of the enzyme than SAH ($K_i = 900\ \mu M$, as against 50 μM). The 6-hydroxy derivative was even a poorer ($K_i = 10$ mM) inhibitor.

An extensive study of SAH derivatives with five different methyltransferases[38] with modifications in the amino acid residue, the bases and the ribose residue, showed that the only derivatives which bound were the 3-deaza (tubercidinyl derivative), N^6-methyl-3-deaza, 3′-deoxy and arabinose analogues.

With N^2-guanine (in tRNA) methyltransferase[18] there was no inhibition when the 6-amino group of SAH was replaced by hydrogen or hydroxy. Monoalkylation, to 6-methylamino produced little change in inhibition, but dialkylation, to the 6-dimethylamino, destroyed inhibition[18,39]. The amino acid functions were not too important for this enzyme, D-SAH binding almost was well as L-SAH and S-adenosylthioenthanol, -propanediol and -propanoic acid still binding the enzyme. The amino group was more important than the acid group for binding as shown by the relative strength of binding of S-adenosylcysteamine, -ethanolamine and -propionic acids.

S-Tubercidinylhomocysteine (3-deaza-SAH) was a good inhibitor of catechol O-methyltransferase[40]. Changing the 5′-O to an amine or amide produced poor inhibitors[41].

Thus the acid function is the least stringently required functional group, of the three, for binding of SAH to most enzymes. Some enzymes seem to require the

correct hydrogen bonding at the N^6-site and others do not. The variability for the homocysteine amino group binding is even greater still. Thus for an affinity adsorbent, for general application, the preferred linkage of SAH to a gel would be through the acid group.

Non-enzymic hydrolysis of the glycosidic bond in SAH would be expected with time. The immobilized SAH would also be susceptible to nuclease or glycosidase activity in enzyme preparations. In fact, the binding of the enzymes to the gel decreased with time (the gels were unusable after about a month), but the reasons for this was not investigated.

Behaviour of the enzymes on the affinity adsorbents

Three gels were made, the first with SAH linked directly to the gel via the 6-amino group, the second with the acid function linked via an ethylenediamine side arm and the third with the acid function linked via a diaminodipropylamine side arm. The same concentration of SAH was used in these different gels.

All column buffers had high (150 m*M*) concentrations of sodium chloride to minimize ion-exchange effects. No differences were found in the chromatographic behaviour of the columns if A-0.5 m Agarose or Sepharose 4B were used as gel materials.

The enzymes were not retained by the first two gels under any conditions. The enzymes were retained by the third gel when applied in the optimal (assay) buffer (pH 8.5). Activity was eluted by decreasing the pH of the eluting buffers (pH 6.0, sodium maleate), but not by increasing the pH (pH 10.5, sodium carbonate). Under these conditions, irreproducible and very low recoveries of PIM (virtually undetectable) and SIM occurred, followed by a rapid loss of activity (within hours) after chromatography.

We assumed that in the more highly purified state, the enzymes were dena-

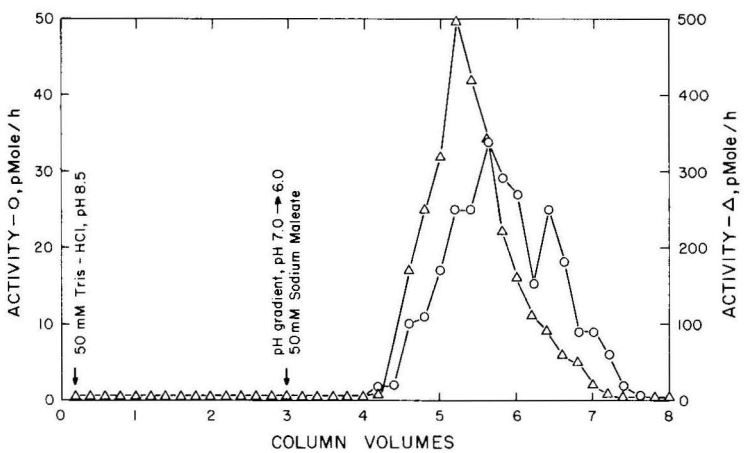


Fig. 2. Elution profile for the chromatography of the methyltransferases (200 μg total protein). ○, PIM, recovery 60–70%, substrate-tryptamine; △, SIM, recovery 125–150%, substrate-N-methyltryptamine; column, 1.2 × 10 cm; elution rate, 25 ml/h; eluting buffers, after washing with the initial buffer (pH 8.5, Tris–HCl) the enzymes were eluted with a linear gradient of sodium maleate (pH 7.0), start buffer, and sodium maleate (pH 6.0), finish buffer.

turing more quickly. Good recovery of the enzymes occurred when BSA was added to washing and eluting buffers and the eluates were returned to the assay pH and buffer. Under these conditions, 30% of PIM and 80% of PIM activity recovered from the columns, was present three days after elution. This is still less stable than the enzyme preparation before chromatography. No enzyme activity was eluted with up to 15 column volumes of initial buffer under these conditions. For a typical elution profile see Fig. 2.

DISCUSSION

The enzymes only bind to the gel with the long side arm (*i.e.*, when the ligand is "free"[6]) and not to the otherwise identical gel with the short side arm. This is consistent with the mechanism of retention being an affinity effect.

Due to the presence of protein in the eluting buffers, no estimate of the purification of the procedure is possible. However, the affinity adsorption of the enzymes followed by the sudden increase in lability of the enzymes on elution would indicate that a purification had been effected. The increase in activity of SIM following elution (150% recovery) also indicates that an inhibitor is being removed. The two enzymes were not separated by this procedure. This would indicate that the kinetic constants for the binding of PIM and SIM to SAH are similar. The elution profiles however are sufficiently different to show that they are two different enzymes (without a pH gradient the elution profiles were identical).

The enzymes were eluted under mild conditions (pH change). The enzymes require a high pH for optimal activity and are eluted from the affinity column by decreasing the pH but not by increasing the pH. This indicates the possibility that an uncharged amine group is required at the SAM–SAH-enzyme site. A possible contender for this group would be the homocysteine amine, particularly since this amine is required for bonding to many methyltransferases. If this is so, a decrease of pH of the eluting buffer by x pH units will lead to a decrease in the binding affinity of the SAH-enzyme complex by a factor of 10^x, thus leading to elution of methyltransferases with K_i's for SAH greater than 10^{-8} M, from affinity gels containing immobilized SAH at a concentration of 10^{-3} M, with a pH change of less than 5 units. If however, the pH optimum for a methyltransferase was low (around pH 5–7), the enzyme would probably require the protonated form of the amine (or a carboxy group) for binding, and the enzyme would be eluted by an increase in pH. Due to the ionic nature of SAH and hence its interactions with an enzyme, it seems likely that most enzymes would be eluted from an SAH gel by only a change in pH.

After we had communicated to Professor S. A. Brown, our approach of immobilizing SAH via its carboxyl group and a spacer arm, he and Dr. S. K. Sharma devised another affinity system based on this principle for the purification of furanocoumarin O-methyltransferase from *Ruta graveolens* L.[42].

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NON-AQUEOUS REVERSED-PHASE LIQUID CHROMATOGRAPHY

A NEGLECTED APPROACH TO THE ANALYSIS OF LOW POLARITY SAMPLES

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SUMMARY

It has been found that many hydrophobic substances such as hydrocarbons and glyceride oils may not completely elute from a reversed-phase liquid chromatographic column when using semi-aqueous mobile phases containing methanol or acetonitrile. Addition of lower-polarity solvents such as methylene chloride or tetrahydrofuran to the carrier causes rapid elution of these non-polar substances. By using a highly retentive reversed-phase packing, Zorbax ODS, it has been demonstrated that non-aqueous mobile phases provide an effective way of separating water-insoluble, non-polar substances. The enhanced solubility of compounds in these non-aqueous solvents greatly facilitates the detection of compounds which have poor UV absorbance/fluorescence characteristics.

INTRODUCTION

Over the last few years, the so-called reversed-phase method of separating chemical species has enjoyed ever-increasing popularity. One of the most significant of the early developments in this field was the work described by Locke[1]. In 1968, he reported retention data for hydrocarbon samples on a chromatographic system comprising squalane as stationary phase and pure acetonitrile as the carrier liquid. Schmit *et al.*[2] in 1971 described the chromatographic behavior of a packing which possessed a chemically bonded hydrophobic stationary phase. This chromatographic packing, known as Permaphase ODS, was the first of many products of different manufacture which shared a similar octadecylsilane-type of bonded stationary phase. Chromatographic packings of the type described have been widely used for the separation of organic chemical mixtures.

Almost invariably the mobile phase used in reversed-phase work is prepared from a water–organic solvent mixture where the concentration of the organic component determines the rate of elution of a given compound from the chromatographic

column. Organic solvents such as methanol and acetonitrile have been widely used in admixtures with water to affect the separation of samples which are generally soluble in organic, but insoluble in aqueous solvents. In recent years application of the reversed-phase method has been extended by the use of solvents such as tetrahydrofuran and acetic acid[3] and dioxane[4] in aqueous admixtures. The scope of the reversed-phase method has also been broadened to include samples which are quite ionic in character; this is achieved by adding a complementary organic counter-ion to the mobile phase. This last-mentioned approach may be considered as a form of ion-pair chromatography, similar in principle to the work developed by Eksborg *et al.*[5], or alternatively, as a dynamically loaded ion-exchange system.

At the other end of the polarity scale from ionic species, lie many compounds which are very hydrophobic in character, for example, hydrocarbons, fats, vegetable and mineral oils. These substances, by their very nature, are insoluble in water and generally have negligible solubility in solvents such as methanol and acetonitrile. There have been many reports in the literature of the liquid chromatographic separation of low-polarity substances using hydrophobic chromatographic packings. Much of this literature relates to the use of porous polymer beads, especially of the styrene–divinylbenzene type, as the chromatographic support. Generally, the studies described in these reports fall into two separate approaches. Pokorny *et al.*[6] have utilized gel-permeation chromatography to affect the separation of low-molecular-weight solutes: tetrahydrofuran is the most commonly used carrier and sample components elute in order of decreasing molecular size. Conversely, studies such as those by Lawrence[7], Chu and Pietrzyk[8] and Janák *et al.*[9] have relied on semi-aqueous mobile phases to enhance solute–support interactions. Unfortunately, this approach suffers from two limitations. Firstly, the solubility of low polarity compounds in semi-aqueous solvents is generally very low. Secondly, porous polymer beads of the styrene–divinylbenzene type normally used have poor mass transfer characteristics which results in broad eluting peaks.

In the present study, the behavior of a range of low-polarity compounds has been examined on an efficient, modern reversed-phase chromatographic packing which is known to offer good mass transfer characteristics. The principal objective has been to assess the analytical utility of columns containing reversed-phase chromatographic packings when the mobile phase is prepared from solvents other than water, *i.e.*, solvents in which low-polarity compounds have an appreciable solubility. Very little has been reported in the literature relating to elution chromatography performed in this manner since the work of Martinů and Janák[10]. These authors described the separation of several simple aromatic compounds on Porapak T, a commercially available porous polymer type of chromatographic support, with hexane as the carrier solvent.

The objective throughout this study has been to demonstrate the feasibility of working with non-aqueous solvents in reversed-phase chromatography.

EXPERIMENTAL

Apparatus

Various high-performance liquid chromatographs were used during the course of this study. The principal components comprised the following units: (1) Model 830

liquid chromatograph, equipped with a thermostatically controlled column compartment, septumless injector and 254-nm ultraviolet absorbance detector (cell volume 8 mm^3, maximum operating sensitivity of 0.01 a.u.f.s.); (2) Model 833 flow controller; (3) Model 837 spectrophotometer (cell volume 8 mm^3, wavelength range 195 to 650 nm); (4) infrared detector (cell volume 30 mm^3, optical pathlength 0.23 mm); (5) twin pen strip chart recorder (1 mV span, Hewlett-Packard Model 7130A). All of these units are available from Du Pont Instruments.

Chromatographic columns

All columns were fabricated from precision stainless-steel tubing 250 × 4.6 mm I.D. End fittings were conventional Swagelok reducing units, bored out to eliminate dead volume in the column end fittings. The chromatographic packing material used throughout this study was Zorbax ODS (Du Pont, Wilmington, Del., U.S.A.). This material has a linear aliphatic octadecyl hydrocarbon monomolecular layer chemically bonded to 6-μm diameter porous silica microspheres. The packing was held in the column with Hastalloy frits. The porosity of the inlet and outlet frits was 5 μm and 2 μm, respectively.

Solvents

Liquid chromatography grade solvents were used throughout this work. Acetonitrile, methanol, methylene chloride and tetrahydrofuran were purchased from either Rathburn Chemicals (Walkerburn, Great Britain) or Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.).

Samples

Most of the compounds used in this study were obtained from other departments within the Du Pont Company. Exceptions are the triglycerides and aliphatic hydrocarbons which were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and Chem Service (West Chester, Pa., U.S.A.). The sample of cod liver oil was supplied by an undisclosed donor, but was identical with commercially available products.

Methodology

Studies concentrated on solutes which were known from previous experiments to be retained relative to the "solvent front" on Zorbax ODS chromatographic packing when either pure methanol or pure acetonitrile was used as the mobile phase. In these cases, the influence of a less polar solvent added to the mobile phase was studied. Solvents that were favored in this study were methylene chloride and tetrahydrofuran as these offer excellent solubilizing power for non-polar samples such as fats and oils.

The influence of mobile phase composition on sample retention was investigated by introducing dilute solutions of the samples into the chromatograph and noting the elution behavior at each of several fixed compositions of mobile phase. Typical tests were performed with 10%, 20%, 40%, 60% and 80% of the less polar solvent added to the acetonitrile. Samples were dissolved in either the mobile phase or methylene chloride at the 1% (w/v) level, when using an ultraviolet detector or at the 10% (w/v) level, when using refractometric or infrared detectors. Injection volumes ranged from 10 to 50 mm^3 depending on the operating conditions.

Several different chemical sample mixtures were studied which were considered to be representative of low-polarity compounds. The chromatograms obtained are described in the next section.

RESULTS AND DISCUSSION

Influence of mobile phase composition

Saturated triglycerides can be considered as typical samples which have low solubility in semi-aqueous solvents and tend to be retained strongly on reversed-phase chromatographic packings. The test mixture was selected to represent liquid solutes which mix readily with the mobile phase and solid solutes which possess different solubility characteristics in the mobile phase solvents, *i.e.*, tricaprylin, trilaurin and tripalmitin. Fig. 1 shows the influence on retention of the addition of tetrahydrofuran to an acetonitrile mobile phase. The chromatograms clearly demonstrate that the retention of these glyceride samples is decreased by increasing the proportion of tetrahydrofuran in acetonitrile through the range 10–60% (v/v). Tetrahydrofuran concentrations greater than 60% cause the glycerides to elute close to the solvent front. Progressively decreasing the concentration of this solvent, particularly below 20% by volume tetrahydrofuran, leads to distorted peaks with a characteristic leading edge. This is believed to be due to the low solubility of these solutes in the mobile phase.

Fig. 2 shows a similar examination of a group of hydrocarbons, specifically: decane, decene, dodecane, and dodecene. In this study, methylene chloride is used as the modifying solvent and the infrared detector is operated at 3.4 μm, the wavelength

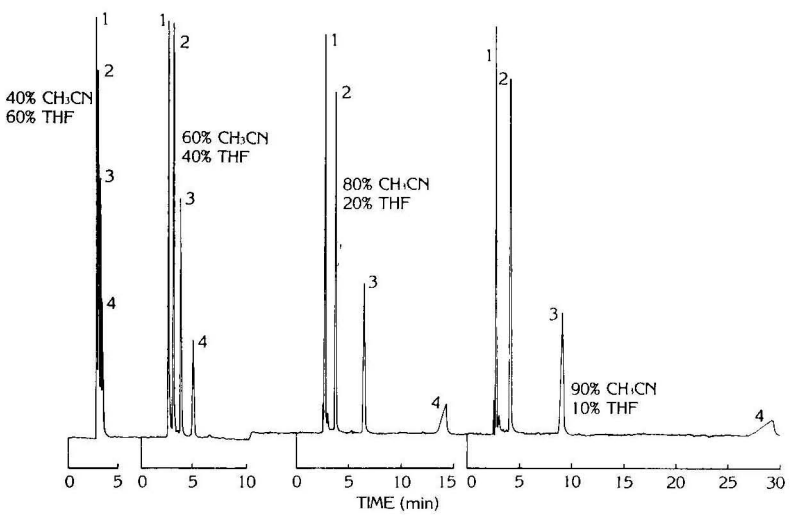


Fig. 1. Influence of mobile phase composition on elution of saturated triglycerides. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 mm × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary: tetrahydrofuran; flow-rate: 0.6 ml/min; pressure: 21 bar; temperature: 40°; detector: infrared, 5.75 μm. Peak identity: (1) tributyrin; (2) tricaprylin; (3) trilaurin; (4) tripalmitin.

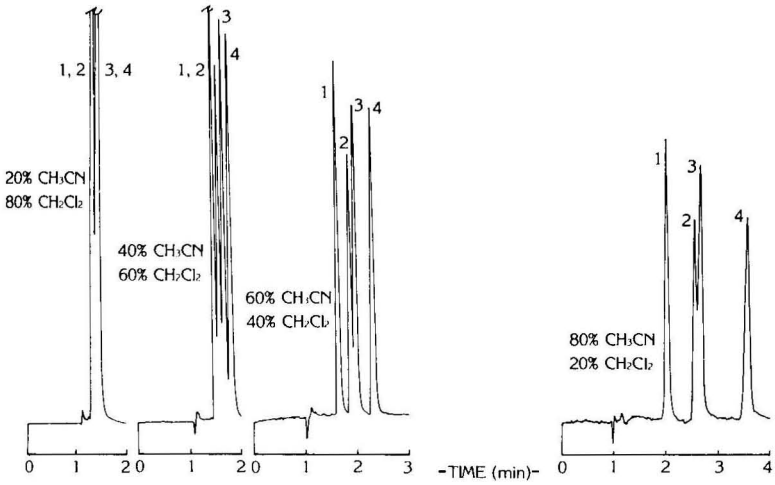


Fig. 2. Influence of mobile phase composition on elution of aliphatic hydrocarbons from Zorbax ODS. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary, methylene chloride; proportions: see figure; flow-rate: 1 ml/min; temperature: 24°; detector: infrared, 3.4 μm. Peak identity: (1) decene; (2) dodecene; (3) decane; (4) dodecane.

corresponding to methylene absorption rather than 5.75 μm (carbonyl absorption). Addition of 20–40% (v/v) of methylene chloride caused the sample components to elute within k' 1 to 10, the generally accepted practical working range of capacity factors. Any further increase in the methylene chloride concentration leads to rapid elution of all components, although even under these conditions a certain degree of resolution is obtained between the sample components.

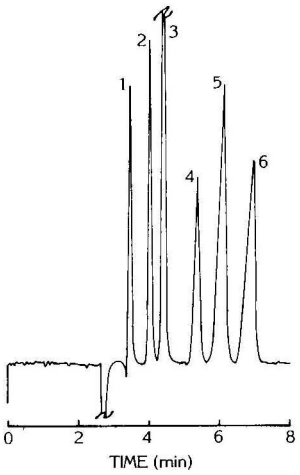
Application of the method to the separation of hydrocarbons

Aliphatic compounds. Illustration of the potential separating power of this non-aqueous method is made by a high-speed separation of various aliphatic hydrocarbon mixtures.

Fig. 3 illustrates the isocratic separation of a number of saturated hydrocarbons by liquid chromatography. This chromatogram clearly indicates the principle of rapid liquid-phase separations of samples which would normally be studied by gas chromatography. Unsaturated hydrocarbons have been found to behave in a similar manner to alkanes. Fig. 4, for instance demonstrates the separation of homologs of linear alkenes of importance in the petroleum industry; again, high-speed, high-resolution separations are achieved.

An example with important significance to many industrial processes is shown in Fig. 5 where hydrocarbons with the same carbon number are resolved according to their chemical class: aromatic, olefinic, aliphatic. The figure illustrates the rapid separation of butylbenzene and decene from decane.

Aromatic hydrocarbons. Compounds which are aromatic in character can be readily detected by ultraviolet absorbance measurements. In this situation it is possible to change the composition of the mobile phase, *i.e.*, use gradient elution, to



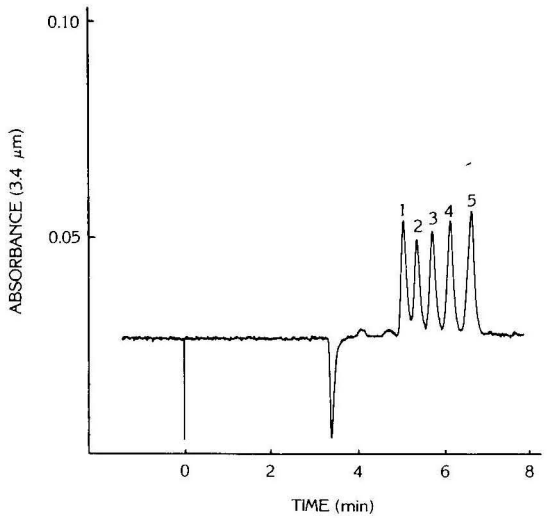


Fig. 3. Separation of saturated hydrocarbons using non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: methylene chloride–acetonitrile (1:4); flow-rate: 1.0 ml/min; pressure: 20.5 bar; temperature: 40°; detector: IR, 3.4 μm, 0.1 a.u.f.s. Peak identity: (1) hexane; (2) decane; (3) dodecane; (4) hexadecane; (5) octadecane; (6) eicosane.

Fig. 4. Separation of alkene homologs by non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 mm × 4.6 mm; mobile phase: tetrahydrofuran–acetonitrile (1:9); flow-rate: 0.75 ml/min; temperature: 27°; detector: IR (3.4 μm). Peak identity: (1) *n*-decene-1; (2) *n*-undecene-1; (3) *n*-dodecene-1; (4) *n*-tridecene-1; (5) *n*-tetradecene-1.

increase progressively the speed of elution of the sample constituents. The effect of an acetonitrile to methylene chloride gradient on the elution of components in a synthetic mixture of aromatic hydrocarbons is illustrated in Fig. 6. In this example the methylene chloride concentration was increased from zero to about 30% (v/v) during the analysis. The order of elution is comparable to that achieved with semi-aqueous mobile phases, however, under the latter conditions, solutes such as coronene and decacyclene are retained very strongly on the column unless the column is operated at a temperature above 70°.

Aromatic polymers. In a manner analogous to the separation of polyaromatics, it is possible to fractionate some low-molecular-weight polymers by the non-aqueous method. Application to the separation of oligomers in a commercial sample of polystyrene, molecular weight 800, is illustrated in Fig. 7. The order of elution is in increasing molecular size, opposite to that observed in size-exclusion chromatography. The selectivity in this non-aqueous reversed-phase method is much greater than with separations based simply on steric considerations.

Application of the method to oil-soluble vitamins

Carotenoids. A sample of commercial β-carotene was examined using a gradient elution system from acetonitrile to methylene chloride. Fig. 8 illustrates the results obtained when the separation was monitored at a wavelength of 497 nm. The

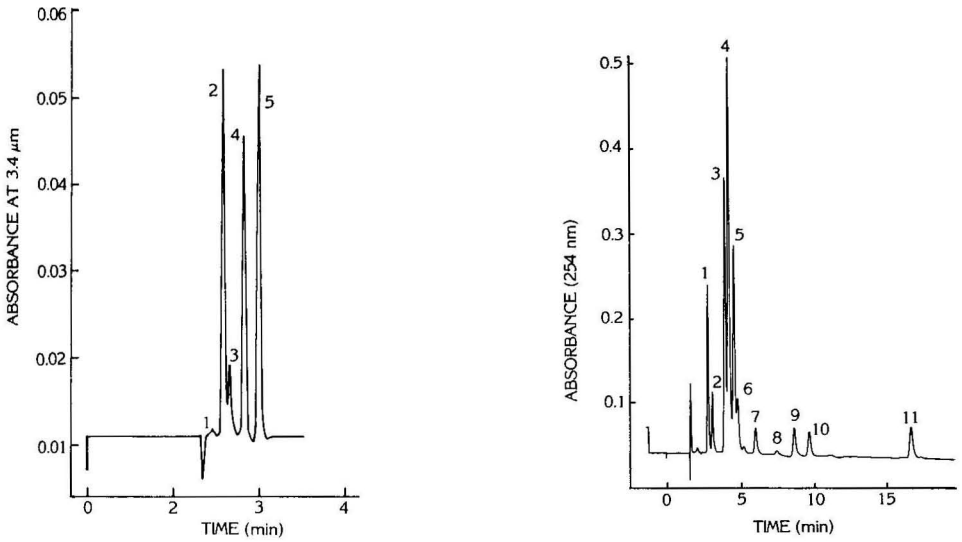


Fig. 5. Separation of hydrocarbons with same carbon number: aromatic, olefinic, aliphatic. Operating conditions: see Fig. 3. Peak identity: (1) impurity from butylbenzene; (2) butylbenzene; (3) impurity from decene; (4) decene; (5) decane.

Fig. 6. Separation of polynuclear aromatic hydrocarbons by non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary, methylene chloride; program: linear 0–30% methylene chloride in 15 min; flow-rate: 2 ml/min; temperature: 24°; detector: UV (254 nm). Peak identity: (1) fluoranthene; (2) pyrene; (3) benzo[*mno*]fluoranthene; (4) benzo[*e*]pyrene; (5) benzo[*a*]pyrene; (6) 3,4-benzotetraphene; (7) benzo[*ghi*]perylene; (8) benzo[*rst*]pentaphene; (9) coronene; (10) benzo[*a*]naphtho[8,1,2-*c*,*d*,*e*]naphthacene; (11) decacyclene.

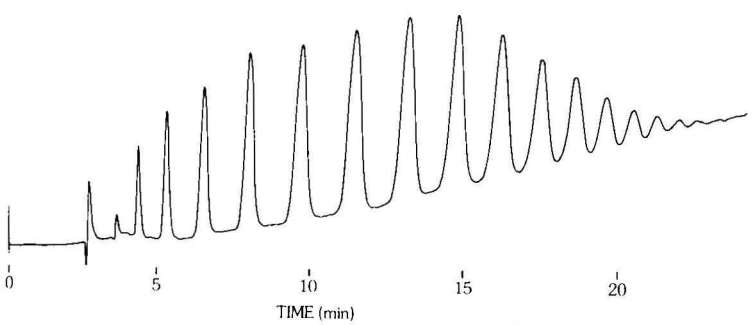


Fig. 7. Separation of polystyrene 800 oligomers by non-aqueous reversed-phase. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 15 cm × 4.6 mm I.D.; mobile phase: primary, methanol, secondary: tetrahydrofuran; program: linear (1.4%/min); flow-rate: 1.5 ml/min; temperature: ambient; detector: UV (254 nm).

upper trace represents the detector output signal amplified by a factor of ten over the lower trace. Minor components can be readily detected in the β-carotene. These trace components are almost certainly related to carotenoids since the operating wavelength selected to monitor the separation, 497 nm, would be insensitive to most non-carotenoid solutes likely to coexist in this sample.

Vitamin D_3 in fish liver oil. Samples of commercial cod liver oil were examined to establish the feasibility of direct determination of vitamin D_3 and A palmitate without any sample pre-treatment.

Fig. 9 contains reproductions of the chromatograms obtained when cod liver oil samples containing 85 and 285 I.U. of vitamin D_3 were examined by the reversed-phase method. The methylene chloride portion of the mobile phase greatly increased the solubility of the oil. This fact was particularly important as, in order to detect the low levels of vitamin, it was necessary to inject a sample prepared by mixing the cod liver oil with an equal volume of methylene chloride, *i.e.*, a 50% (v/v) sample concentration.

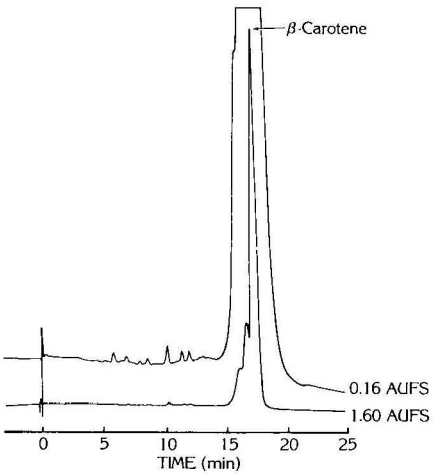


Fig. 8. Separation of minor components in β-carotene using non-aqueous reversed-phase chromatography. Operating conditions: instrument: Du Pont high-performance liquid chromatograph; column: Zorbax ODS, 250 × 4.6 mm I.D.; mobile phase: primary, acetonitrile, secondary: methylene chloride; program: linear addition of methylene chloride at 2%/min; flow-rate: 1 ml/min; temperature: 24°; detector: visible absorbance, 497 nm.

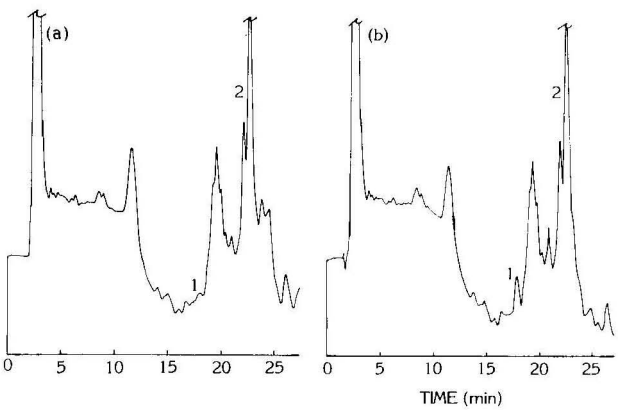


Fig. 9. Detection of vitamin D_3 in cod liver oil using non-aqueous reversed-phase chromatography. (a) Native cod liver oil, (b) Native cod liver oil, approx. 85 I.U. D_3/g. spiked to give 285 I.U. D_3/g. Operating conditions: see Fig. 8, except detector: UV, 254 nm. Peak identity: (1) vitamin D_3; (2) vitamin A palmitate.

CONCLUSIONS

The analytical utility of performing reversed-phase separations on modern, highly efficient bonded packings with non-aqueous mobile phase has been demonstrated. In this configuration, a mechanism of separation comparable to the earlier use of paraffin oil-impregnated cellulose layers can be envisaged. However, the use of chemically bonded chromatographic packings considerably increases the reproducibility and stability of the separation system.

Chromatographic systems using a totally organic mobile phase offer several attractions for the analysis of hydrophobic samples relative to semi-aqueous systems.

Firstly, many non-polar substances are insoluble in water and only sparingly soluble in methanol and acetonitrile, the most popular organic solvents used in reversed-phase work. Tetrahydrofuran and especially chlorinated solvents are exceedingly good solvents for hydrophobic substances; thus sample solubility is not a problem. In some instances a concentration approaching 20% (w/v) of the sample in the mobile phase is possible. Under these circumstances no precipitation of the sample on the column is observed. The high solubility of the sample in the carrier liquid is beneficial in that relatively large samples may be analyzed, aiding detection when using the less sensitive differential refractive index and infrared monitors. A high sample load also improves the limits of detection of minor components and makes semi-preparative chromatography more attractive.

The types of samples studied in the present work, namely hydrocarbons and fat-soluble vitamins, have been analyzed previously to some extent using semi-aqueous mobile phases. The observations in this work suggest that in many instances incomplete elution of the sample would have occurred unless the separations were carried out at elevated temperatures. It is evident, in retrospect, that the incomplete elution of sample components when using methanol or acetonitrile has probably contributed significantly to the steady deterioration of some chromatographic columns in use. Clearly, even if the component of interest is eluted from the column, as in the case of the analysis of vitamins A and D, the matrix, which is invariably a glyceride based oil, is still retained. Any retained constituent, if not flushed from the system routinely, will result in premature failure of the column. In spite of the relatively large samples frequently injected, *e.g.*, approximately 10 mg, during this study, none of the four columns used showed any signs of deterioration. The columns were in use intermittently over a period of eight months.

The analysis of hydrocarbons by this reversed-phase method is particularly interesting in that by adjusting the sample concentration and choosing the appropriate detector it is possible to monitor different hydrocarbon types. A fluorescence detector would observe only the heavier polynuclear aromatic substances. An ultraviolet detector with variable wavelength control could be used to monitor selectively various polyunsaturated and aromatic constitutents. An overall qualitative picture of the components present in the sample is probably the best obtained by using a refractive index or an infrared absorbance detector. It is clearly recognized that several of the solutes selected to demonstrate the non-aqueous approach to reversed-phase chromatography can be analyzed either by gas–liquid chromatography and/or more conventional reversed-phase methods. This use of well characterized samples should clearly indicate the type of results obtained, *i.e.*, a separation of homologs rather

than a class or type separation. Samples with a given carbon content, as shown in Fig. 5, are resolved, suggesting that compounds belonging to different hydrocarbon types do not elute at exactly the same time. Other experimental tests, not detailed in this report, suggest that higher-molecular-weight alkanes, *e.g.*, the kerosine range, can also be fractionated to some extent by using a mobile phase containing an increased concentration of methylene chloride.

Evidence to substantiate a reversed-phase mechanism can be drawn from the observation that a solvent of lower polarity, *e.g.*, methylene chloride, will accelerate elution of components whereas a more polar solvent, *e.g.*, acetonitrile holds the sample components on the column. If adsorption on non-chemically bonded sites of the support contributed to the separation mechanism, the opposite effect of solvent polarity would be expected.

ACKNOWLEDGEMENTS

I would like to thank Dr. S. D. Abbott of Du Pont de Nemours, Wilmington, for providing the excellent chromatogram of the separation of polystyrene oligomers which is reproduced as Fig. 7 of this paper. The management of the Du Pont Company must also be thanked for granting their permission to publish these data.

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THE DEVELOPMENT OF BRITISH PHARMACOPOEIA MONOGRAPHS FOR IDOXURIDINE AND IDOXURIDINE EYE DROPS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY FOR ASSAY AND FOR CONTROLLING RELATED IMPURITIES

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SUMMARY

The monograph published in the 1973 edition of the British Pharmacopoeia (BP) for idoxuridine required revision because it contained a non-specific assay and no tests for related impurities. It was also necessary to prepare a new monograph for idoxuridine eye drops. The Japanese Pharmacopoeia contains a thin-layer chromatographic test for impurities but this was not considered ideal. Improved thin-layer chromatographic tests were sought but when this was unsuccessful, methods using high-pressure liquid chromatography were examined. A system using reversed-phase chromatography was selected for inclusion in BP Addendum 1977 since it provided a specific assay method and limit test for related impurities which could be applied to both the drug substance and the eye drops.

INTRODUCTION

Idoxuridine (5-iodo-2′-deoxyuridine) has specific anti-viral properties inhibiting the formation of DNA in virus infected cells and thus preventing replication in species such as Adenovirus, Cytomegalovirus, Herpes simplex and Vaccinia[1]. Accordingly it is used in the form of eye drops and eye ointment for the treatment of virus-induced conditions such as herpetic keratitis.

A monograph for idoxuridine was first published in the British Pharmacopoeia (BP) 1973 edition[2], but it was necessary to improve this because the assay used was non-specific being based on the determination of iodine and no control of possible related impurities was applied. In addition it became necessary to develop a monograph for idoxuridine eye drops.

Monographs for idoxuridine are also included in the United States Pharmacopeia (USP)[3] and Japanese Pharmacopoeia[4]. The assay in the USP monograph like that in BP1973 is non-specific, in this case being a non-aqueous titration with sodium methoxide and again no specific test for related impurities is included. The assay in the Japanese Pharmacopoeia monograph is also a sodium methoxide titration but in

this case a test to control impurities by thin-layer chromatography (TLC) is included. This test involves two-dimensional chromatography of a 50-μg sample of idoxuridine on a fluorescent chromatoplate and the requirement is that no secondary spots shall be visible when inspected under ultraviolet radiation at 254 nm. This test was considered unsatisfactory for the following reasons:

(i) It is undesirable to stipulate that no secondary spots should be visible since the result becomes dependant on such factors as the eyesight of the analyst or the nature of the equipment used in which the assessment is carried out. In BP monographs a standard is usually applied to the chromatoplate. This may be a sample of the impurity in question at the maximum permitted level or a dilution of the material under examination with the requirement that the intensity of any secondary spots in the sample are no greater than the standard.

(ii) It is considered undesirable to use two-dimensional chromatography for a test of this nature. This mode of separation inevitably results in greater diffusion of spots leading to loss of sensitivity. Two-dimensional separations also suffer from the disadvantage that standards cannot be satisfactorily applied to the same plate as the sample under test.

(iii) TLC work carried out at the BP laboratory indicated that for one of the likely impurities, 5-iodouracil, the minimum level that could be assessed using 254-nm radiation after a one-dimensional separation was 1 μg, which would correspond to a 2% level in the Japanese Pharmacopoeia test. In the BP monographs it was desired to control impurities at 0.5% levels.

The degradation pathways of idoxuridine have been studied by Ravin *et al.*[5] and Simpson and Zappala[6], and the main routes are illustrated in Fig. 1. They found that the principal effect of light-induced degradation was loss of iodine leading to

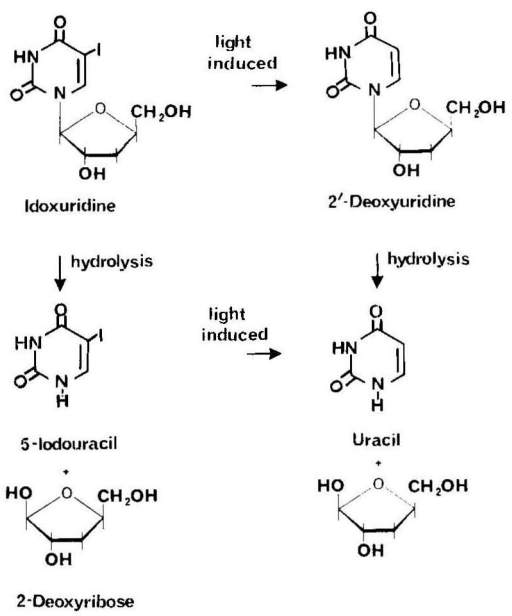


Fig. 1. Degradation pathways of idoxuridine.

2'-deoxyuridine although intense ultraviolet irradiation gave rise to some unknown product which they thought was probably due to breakdown of the pyrimidine ring. In aqueous solution, the principal products of heat-induced degradation were 5-iodouracil and 2-deoxyribose, only low levels of 2'-deoxyuridine being reported by this route. This result was found at any pH, but the rate of degradation was far more rapid under basic conditions. The generation of uracil by either route was extremely slow. Degradation studies were monitored by paper chromatography and under the conditions used, uracil was only detected in samples that had decomposed to an extent of about 50%.

These workers devised a method of separating idoxuridine from its impurities by partition chromatography on a Celite column impregnated with 0.1 *M* hydrochloric acid and monitoring the eluate spectrophotometrically at 292 nm. This method forms the basis of the assays for the Idoxuridine Ophthalmic Solution and Eye Ointment monographs in the USP[3]. This was not considered satisfactory in the present study for the following reasons:

(i) The method was not considered sufficiently robust for a pharmacopoeial test since variations in the nature of columns prepared in different laboratories and between different batches of Celite would be expected to create difficulties in obtaining repeatability between laboratories. These difficulties may well be overcome by those with experience of this procedure but pharmacopoeial tests are often required for use on a "one off" basis and are designed accordingly.

(ii) Although this represents an improvement on the monograph in BP1973 since the assay is specific for idoxuridine, it is doubtful whether the precision of such a procedure would allow an analyst to discriminate against samples containing contaminants above the 0.5% level.

What was needed was a specific test for these impurities such as a TLC limit test. If this were unsuccessful high-pressure liquid chromatography (HPLC) would be investigated with a view to providing both specific assays for the drug substance and the eye drops and also specific tests for impurities in the two monographs.

EXPERIMENTAL

Thin-layer chromatography

TLC was carried out on 20 × 20 cm glass-backed chromatoplates either hand prepared with a 250-μm layer of silica gel Type 60 GF or HF (Merck, Darmstadt, G.F.R.) or were precoated silica gel 60 F_{254} (Merck). Spots were located by viewing under shortwave ultraviolet radiation (254 nm).

High-pressure liquid chromatography

A Waters Assoc. (Milford, Mass., U.S.A.) ALC 202 chromatograph fitted with an M-6000A solvent delivery system, a U 6K injector and a 440 ultraviolet detector operating at 254 nm were used. Normal phase chromatography was carried out on a 30 cm × 4 mm I.D. column packed with 10-μm silica particles (μPorasil, Waters Assoc.). Reversed-phase work was carried out on a 30 cm × 4 mm I.D. column packed with 10-μm silica particles modified with chemically bonded octadecasilyl groups (μBondapak C_{18}, Waters Assoc.).

Materials

Samples of idoxuridine and Kerecid eye drops containing 0.1 % (w/v) idoxuridine and 0.002% (w/v) sodium thiomersalate preservative were kindly provided by Smith Kline & French (Welwyn Garden City, Great Britain). Samples of 5-iodouracil, 2'-deoxyuridine and uracil were obtained from Sigma (St. Louis, Mo., U.S.A.). All organic solvents were AnalaR grade obtained from BDH (Poole, Great Britain) and were used without any further treatment. Mobile phases for reversed-phase work were prepared using glass distilled water and were de-aerated by stirring for about 15 min under reduced pressure.

RESULTS AND DISCUSSION

Thin-layer chromatography

Several different systems were investigated of which one showing promise used hand-prepared chromatoplates and chloroform–methanol–acetic acid (8:1:1) as the mobile phase. TLC results are tabulated in Table I and illustrated in Fig. 2.

TABLE I

R_F VALUES OF IDOXURIDINE AND IMPURITIES IN SOME TLC SYSTEMS

Mobile phase	*5-Iodouracil*	*Uracil*	*Idoxuridine*	*2'-Deoxyuridine*
Chloroform–methanol–acetic acid (8:1:1)	0.64	0.39	0.30	0.16
Isopropanol–conc. ammonia (9:1), unsaturated	0.42	0.35	0.15	0.20
Isopropanol–conc. ammonia (9:1), saturated	0.30	0.27	0.09	0.16

The minimum loading that could be assessed by the quenching of 254-nm radiation was about 1 μg for 5-iodouracil and 0.5 μg for 2'-deoxyuridine so a loading of 200 μg idoxuridine would be required to control impurities at the 0.5% level. Compact spots for idoxuridine could only be achieved in this system when loadings no greater than 25 μg were applied to the chromatoplate. Tailing spots were obtained with higher levels and since the 2'-deoxyuridine spot ran behind the principal spot, the tailing interfered with the assessment of this impurity.

This difficulty was partially overcome by an alternative system using precoated chromatoplates and 2-propanol–concentrated ammonia (9:1) as the mobile phase under unsaturated tank conditions[7]. As can be seen from Table I and Fig. 2 the change from the acidic to the basic system did not affect the order of retention of 5-iodouracil and uracil, but that of idoxuridine and 2'-deoxyuridine was reversed so that the impurity was no longer in the tail of the principal component. It was also found that this system gave rise to far less tailing than was experienced with the acidic system and 200 μg of idoxuridine could readily be chromatographed.

This system suffered from two disadvantages:

(1) The requirement that an unsaturated system be used. For official methods that are designed to be used in a number of different laboratories it was considered that greater reproducibility of results is likely to be achieved under saturated conditions. With unsaturated systems the composition of the atmosphere within the tank

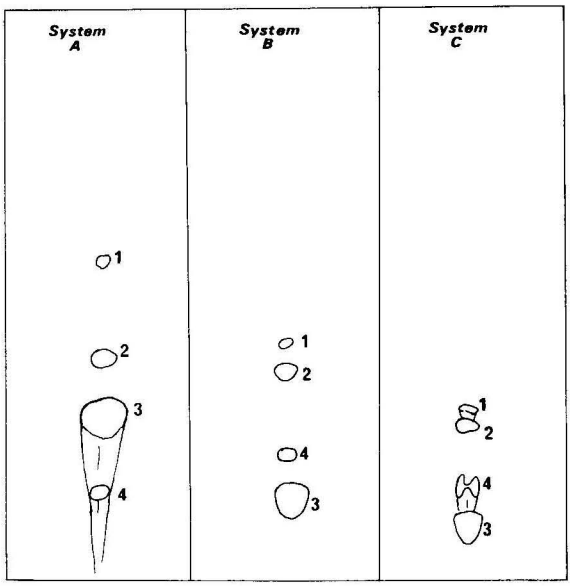


Fig. 2. Thin-layer chromatograms of idoxuridine and its impurities. System A: chromatoplate, silica gel 60 HF; mobile phase, chloroform–methanol–acetic acid (8:1:1). Sample: 10 μl of a solution in methanol containing 0.01% each of 5-iodouracil (spot 1), uracil (spot 2) and 2′-deoxyuridine (spot 4) and 1.0% of idoxuridine (spot 3). System B: chromatoplate, silica gel 60 F254 precoated plate; mobile phase, isopropanol–conc. ammonia (9:1) under unsaturated tank conditions. Sample: 4 μl of a solution in methanol–conc. ammonia (5:1) containing 0.025% each of 5-iodouracil (spot 1), uracil (spot 2), 2′-deoxyuridine (spot 4) and 5% of idoxuridine (spot 3). System C: same as System B but under saturated tank conditions.

is likely to be subject to greater variation from laboratory to laboratory which could result in variations in the degree of separation achieved.

This method was attempted under saturated conditions and as is evident from Table I and Fig. 2 the retention of components were reduced and the separation was poorer. Apart from this, the 2′-deoxyuridine no longer ran as a compact spot but instead an "H" shaped spot which tailed into the principal spot resulted.

It would appear from this that under saturated conditions the activity of the silica in this separation was increased. A possible explanation is that under unsaturated conditions separations were obtained that were better than those achieved in acidic systems because of the greater solubility of idoxuridine in aqueous or alcoholic bases. Under saturated conditions, however, the silica surface was modified by the ammonia in the system and it too was rendered basic leading to a greater affinity of the components for the standing phase.

(2) The minimum visible level of 5-iodouracil was about 1 μg and so to control this impurity at the 0.5% level it was necessary to chromatograph 200 μg of idoxuridine. For the drug substance this presented no difficulty since a 5% solution could be prepared in methanol–concentrated ammonia (5:1) requiring 4-μl applications to the chromatoplate. The eye drops being a 0.1% solution would require either a 200-μl application or a preliminary concentration of the solution, a process which in itself could lead to some degradation.

For these reasons it was considered that there was adequate justification in applying HPLC to this analysis.

High-pressure liquid chromatography

Following on from the work done on TLC, preliminary HPLC work was carried out on a silica column using a mobile phase composed of chlorinated hydrocarbon and methanol. Chloroform was not used because of the ethanol stabiliser which often varies from batch to batch. Dichloromethane was found to be a suitable alternative. Good separations were obtained on the μPorasil column with dichloromethane–methanol (95:5) as the mobile phase at a flow-rate of 2.0 ml per minute. Under these conditions, capacity ratios (k' values) as shown in Table II were obtained and a typical chromatogram is shown in Fig. 3.

TABLE II

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING SILICA COLUMN

Component	*k' value*
5-Iodouracil	0.57
Idoxuridine	2.14
Uracil	3.76
Unknown	4.38
2′-Deoxyuridine	6.14

Although this system seemed promising for testing the drug material, it was not satisfactory for the eye drops. Since this was an aqueous solution, it could not be injected directly onto the column. The alternative was a preliminary treatment such as evaporation to dryness and dissolution of the residue in a non-aqueous solvent. This too was undesirable since it added to the analysis time and these was some risk of degradation during the evaporation stage. What was required to avoid this difficulty was a system using a chemically modified silica such as one using reversed-phase chromatography.

This was satisfied using the μBondapak C_{18} column on which an aqueous methanolic mobile phase was suggested to us[8]. Good results were obtained using water–methanol (87:13) at a flow-rate of 1.7 ml/min and under these conditions, the k' values given in Table III resulted and a typical chromatogram is shown in Fig. 4.

It is of interest to note that the use of a reversed-phase system did not result in a reversal of the order of elution of all peaks. The components of this system could be considered in two different groups. Uracil and 5-iodouracil are pyrimidines, idoxuridine and 2′-deoxyuridine are nucleosides. It is probable that the various factors leading to separation contribute to different extents within these two groups. As a result the order of elution of uracil and 5-iodouracil was reversed on the two columns as also was the order of elution for idoxuridine and 2′-deoxyuridine. This effect led to a further advantage of the reversed-phase system. It was hoped to use this system for controlling the level of impurities in idoxuridine and for this purpose it was proposed to use sensitivities that would lead to off-scale idoxuridine peaks. In the silica system,

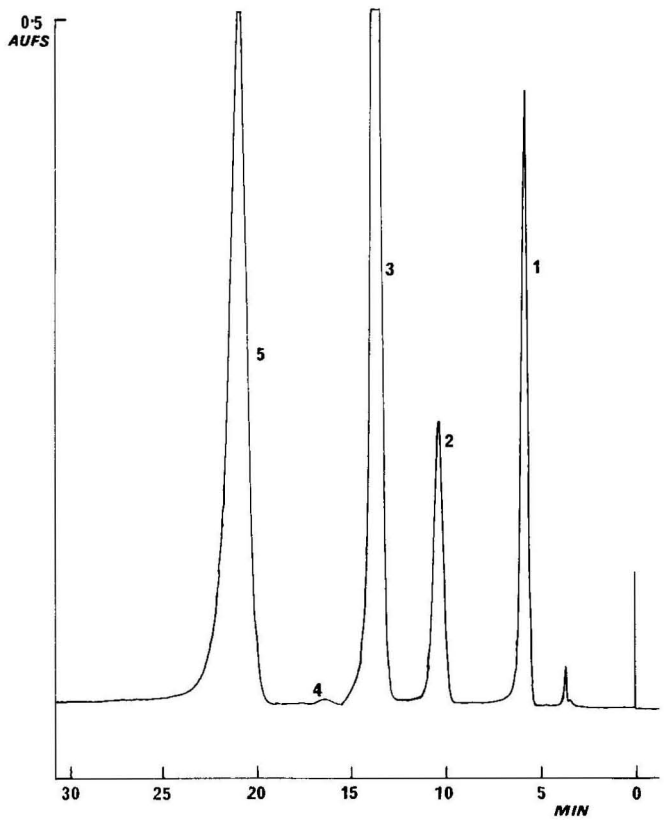


Fig. 3. Liquid chromatogram of idoxuridine and its impurities. Column: 30 cm × 4 mm I.D. μPorasil. Eluent dichloromethane–methanol (95:5). Flow-rate: 2 ml/min. Sample: 4 μl of a solution in methanol containing 0.7% each of 5-iodouracil (peak 1), idoxuridine (peak 2), uracil (peak 3), 2′-deoxyuridine (peak 5). Peak 4 is an unknown impurity.

the 2′-deoxyuridine would have eluted in the tail of this principal peak which would have adversely affected the accuracy of the test. In the reversed-phase system, all the impurity peaks eluted before the principal peak.

Assay

The system was first examined for its suitability as an assay method both for the drug substance and the eye drops. For this assay it had been suggested to us that sulphathiazole be used as the internal standard[8]. In this system sulphathiazole eluted just after idoxuridine as indicated in Table III. The sample of eye drops available for examination contained thiomersalate preservative, but it is believed that benzalkonium chloride may also be used. To test the method it was necessary to examine the linearity, the precision and whether preservatives would interfere.

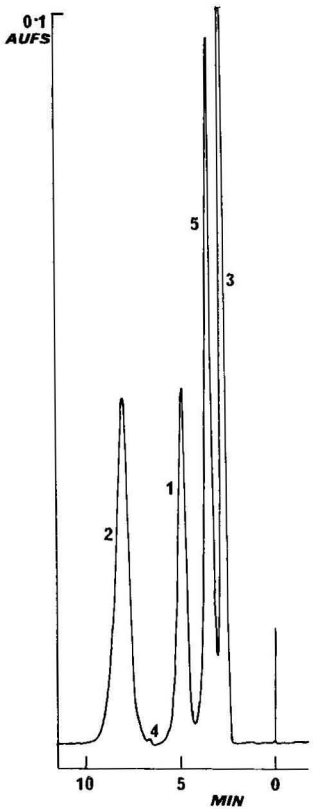
Linearity

For eye drops, pharmacopoeial monographs typically allow assay limits of 90 to 110% of the declared content. To include the region of interest, the linearity

TABLE III

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING REVERSED-PHASE SYSTEM WITH WATER–METHANOL (87:13) AS MOBILE PHASE

Component	*k' value*
Uracil	1.90
2'-deoxyuridine	2.76
5-Iodouracil	4.52
Unknown	5.80
Idoxuridine	7.90
Sulphathiazole	10.60



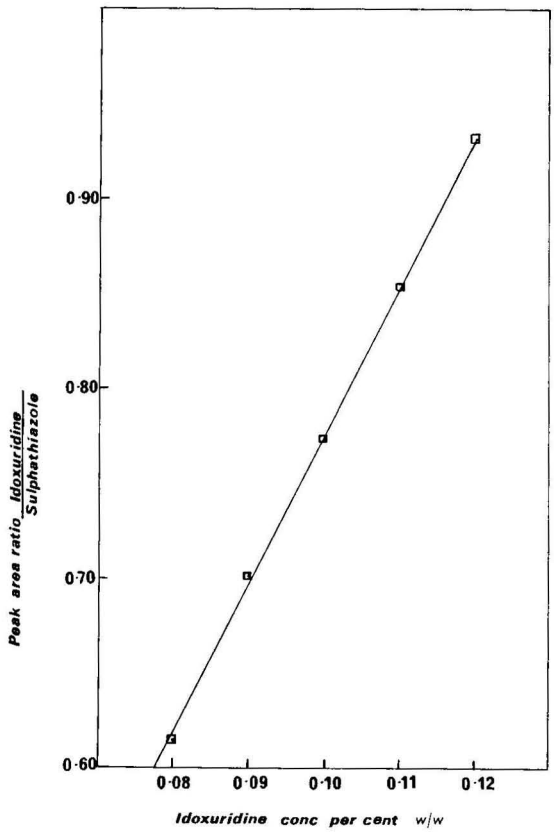


Fig. 4. Liquid chromatogram of idoxuridine and its impurities. Column: 30 cm × 4 mm I.D. μBondapak C_{18}. Eluent: water–methanol (87:13). Flow-rate: 1.7 ml/min. Sample: 10 μl of a solution in water containing 0.034% of 5-iodouracil (peak 1), 0.080% of idoxuridine (peak 2), 0.006% uracil (peak 3), 0.013% 2'-deoxyuridine (peak 5). Peak 4 is an unknown impurity.

Fig. 5. Plot of ratio idoxuridine peak area/sulphathiazole peak area *versus* idoxuridine concentration to demonstrate linearity of assay.

was tested from 80 to 120%. Solutions were prepared containing 0.08, 0.09, 0.10, 0.11 and 0.12% idoxuridine in water. To 15-ml aliquots, were added 2 ml of a 0.12% solution of sulphathiazole in 10% aqueous ethanol the volumes were then made up to 20 ml with water. Aliquots (15 μl) of each solution were injected in duplicate and

TABLE IV

PEAK AREA RATIOS IDOXURIDINE/SULPHATHIAZOLE FOR A SERIES OF IDOXURIDINE CONCENTRATIONS

Concentration of idoxuridine (%)	*Ratio of peak areas idoxuridine/sulphathiazole*	*Mean*
0.08	0.6163	0.6146
	0.6128	
0.09	0.7064	0.7010
	0.6956	
0.10	0.7736	0.7740
	0.7745	
0.11	0.8528	0.8537
	0.8546	
0.12	0.9305	0.9310
	0.9315	

the areas of the peaks due to idoxuridine and sulphathiazole determined. The results are shown in Table IV and the linearity is demonstrated in Fig. 5.

Precision

To test the precision of the assay a 0.10% solution of idoxuridine was prepared in water. To a 15-ml aliquot was added 2 ml of a 0.12% solution of sulphathiazole in 10% aqueous ethanol and the volume was made up to 20 ml with water. Six replicate aliquots of 15 μl were analysed and the areas of the peaks due to idoxuridine and sulphathiazole determined. The mean and coefficient of variance were determined as shown in Table V.

TABLE V

DETERMINATION OF PRECISION OF ASSAY USING ALIQUOTS OF A 0.1% SOLUTION OF IDOXURIDINE

Sample	*Ratio of peak areas idoxuridine/sulphathiazole*
1	0.7262
2	0.7249
3	0.7307
4	0.7232
5	0.7197
6	0.7201
Mean	0.7242
Standard deviation	0.0041
Coefficient of variance	0.57

Interference by preservatives

The sample of eye drops available for examination contained 0.002% thiomersalate as a preservative. It was found that if an aqueous solution of sodium thiomersalate was injected into the chromatograph under the conditions of the assay, it eluted just after the inert peak and would therefore cause no interference. When formulated into the eye drops, over a period of time, thiomersalate degrades giving

rise to a series of slightly more retained peaks; however, these still give rise to no interference and Fig. 6 shows a typical separation of a sample of eye drops.

If benzalkonium chloride is used as a preservative, under the assay conditions it is retained on the column and the levels normally present when used as a preservative would not be expected to lead to any interference; however, to be sure of this, after a few analyses, any benzalkonium present may be washed off the column with methanol.

Assay method

The following procedure is used to assay idoxuridine or idoxuridine eye drops. Prepare a 0.1 % solution of the sample in water. To a 15-ml aliquot of the sample add 2 ml of a solution of sulphathiazole prepared by dissolving 120 mg of the sulphonamide in 10 ml of ethanol and diluting to 100 ml with water. Dilute this solution to 20 ml with water. Aliquots of this solution and a similarly prepared standard are injected into the chromatograph and results may be determined by measuring peak areas or peak heights. The time taken for an analysis is about sixteen minutes.

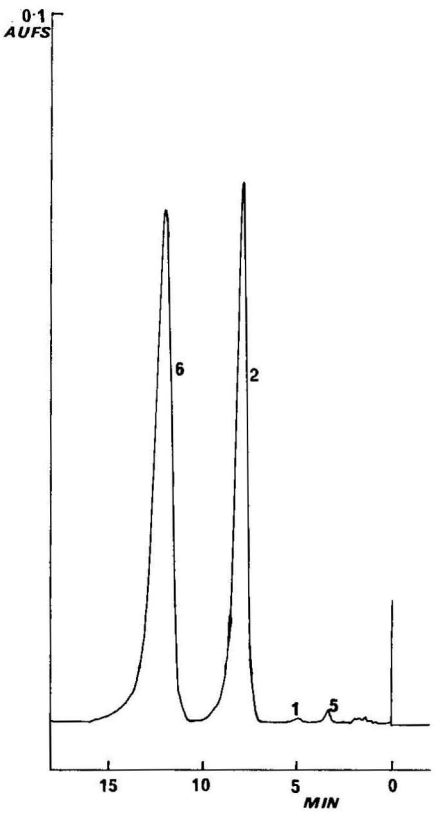


Fig. 6. Liquid chromatogram of idoxuridine eye drops. Conditions as in Fig. 4. Sample: 10 μl of a solution in water containing 0.075% idoxuridine (peak 2) and 0.012% sulphathiazole (peak 6). Small peaks due to 5-iodouracil (peak 1) and 2′-deoxyuridine (peak 5) are also visible.

Determination of impurities

The assay described above may be regarded as specific for idoxuridine since related impurities do not contribute to the figures obtained in the determination. It is currently popular to refer to an assay of this type as being "stability indicating". It is apparently considered that degradation of the material of interest will lead to a reduction of its peak size and this reduction may then be used to monitor the formation of degradation products and therefore the stability of the material under investigation. This approach was considered unsatisfactory for our purposes because:

(1) We proposed to limit impurities at the 0.5% level. It was shown earlier that the coefficient of variance for the assay was 0.57. The assay therefore did not have sufficient precision to detect 0.5% levels of degradation with a sufficiently high confidence level.

(2) Pharmacopoeial assay standards for formulations of the type under consideration customarily allow ranges of 90.0 to 110.0% of the stated amount. The intention here is to allow for batch to batch variations that are likely during production and not for the subsequent degradation of the material. If this were the case, a batch could be prepared assaying at 0.11% idoxuridine and after a prolonged period of storage the assay figure could fall to 0.09%. On both occasions the material would satisfy the requirement in spite of about 18% degradation having occurred. This is, of course, unacceptable and for these reasons tests that specifically measure the levels of degradation products are preferred.

In this case, the impurities of interest were 5-iodouracil, 2'-deoxyuridine and uracil. As was mentioned earlier, it had been demonstrated that compared with the former materials, uracil is generated only very slowly being a secondary product of degradation[6]. It was felt that before any significant amounts of uracil were formed, a sample would contain well over the allowed level of the more readily formed 5-iodouracil or 2'-deoxyuridine. For this reason it was considered adequate to limit the levels of these two contaminants.

As is apparent from Table III and the chromatogram shown in Fig. 4, the chromatographic conditions used in the assay provided an adequate separation of these impurities. The difficulty here was the internal standard. The intention was to inject larger quantities of idoxuridine and to use higher sensitivities in order to produce appreciable responses from low levels of the impurities of interest. The internal standard would be expected to produce a comparable response but the idoxuridine peak would be well off scale. It was undesirable for the internal standard

TABLE VI

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING REVERSED-PHASE SYSTEM WITH WATER–METHANOL (96:4) AS MOBILE PHASE

Component	*k' value*
Uracil	2.64
Sulphanilamide	4.25
2'-Deoxyuridine	5.66
5-Iodouracil	8.36
Unknown	14.23
Idoxuridine	20.82

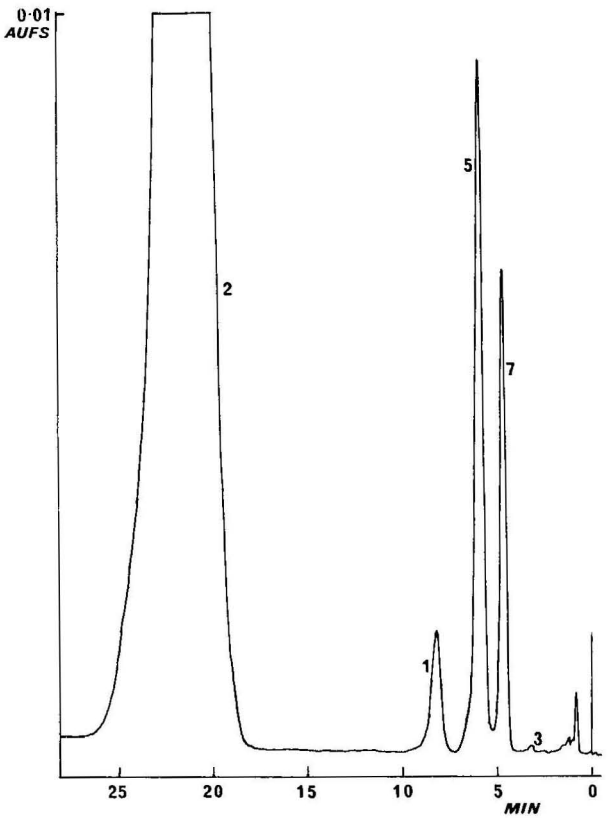


Fig. 7. Liquid chromatogram of idoxuridine. Column: 30 cm × 4 mm I.D. μBondapak C_{18}. Eluent: water–methanol (96:4). Flow-rate: 1.7 ml/min. Sample: 15 μl of a solution in water containing 0.08% idoxuridine (peak 2), 0.0004% each of 5-iodouracil (peak 1) and 2′-deoxyuridine (peak 5), 0.0001% of sulphanilamide (peak 7). A trace of uracil is also visible (peak 3).

to elute in the tail of the principal peak but ideally it would elute close to the peaks of interest in this test. For this reason sulphathiazole was considered unsatisfactory and of the alternative materials examined, sulphanilamide gave the most promising results, eluting just before the 2′-deoxyuridine. For optimum resolution the solvent was weakened to water–methanol (96:4) and under these conditions the k' values for idoxuridine and its impurities were as shown in Table VI.

Figs. 7 and 8 show chromatograms of idoxuridine and idoxuridine eye drops respectively containing 0.5 and 0.4% levels of 5-iodouracil and 2′-deoxyuridine. This method was tested by preparing a 0.1% solution of idoxuridine. To 16 ml were added 2 ml of a 0.001% aqueous solution of sulphanilamide and the solution was diluted to 20 ml. Similar solutions were prepared but also made to contain 0.0002, 0.0004 and 0.0008% of 5-iodouracil and 2′-deoxyuridine which was equivalent to 0.25, 0.50 and 1.00% levels of these impurities in the idoxuridine. Aliquots (25 μl) of these solutions were chromatographed in duplicate and from the areas of the peaks due to the internal standard and impurities the recovery of impurities was determined. The results are given in Table VII. For the purposes of a limit test for these impurities these results were considered to be quite satisfactory.

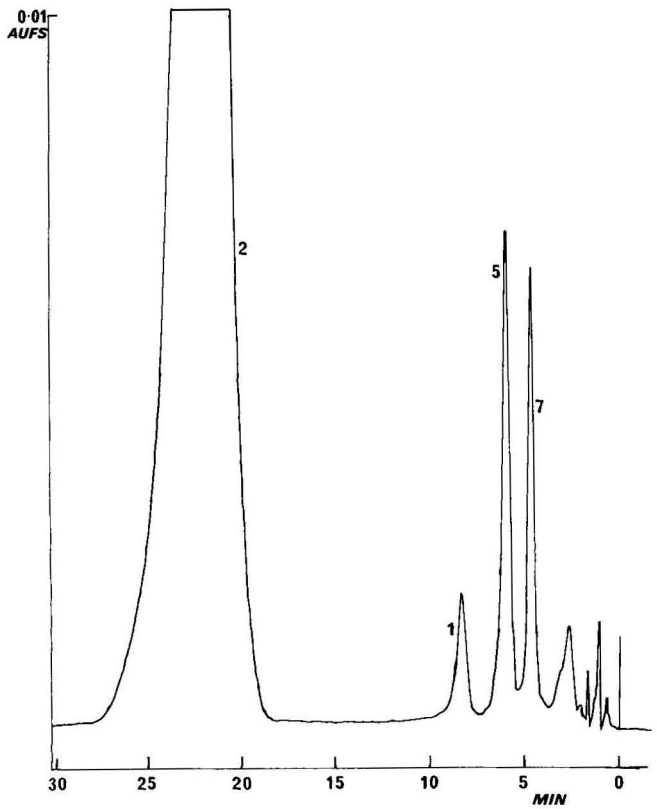


Fig. 8. Liquid chromatogram of idoxuridine eye drops. Conditions as in Fig. 7. Sample as in Fig. 7 except concentrations of 5-iodouracil and 2-deoxyuridine are 0.0003%. Some peaks due to thiomersalate and its degradation products appear before the sulphanilamide peak. Peak identities are as in Fig. 7.

TABLE VII

DETERMINATION OF ACCURACY–RECOVERY OF 5-IODOURACIL AND 2′-DEOXYURIDINE FROM SAMPLES OF IDOXURIDINE SPIKED WITH THE IMPURITIES

Level of impurity added (%)	*5-Iodouracil*		*2′-Deoxyuridine*	
	Found (%)	*Recovery (%)*	*Found (%)*	*Recovery (%)*
0	0.06	—	0.08	—
0.25	0.28	88.0	0.32	96.0
0.50	0.56	100.0	0.60	104.0
1.00	1.04	98.0	1.10	102.0

Method for impurity test

The procedure for the impurity test is as follows. Prepare a 0.1% solution of the sample in water. To 16 ml add 2 ml of a 0.001% aqueous solution of sulphanilamide and dilute to 20 ml with water. A standard solution is prepared containing 0.0004% of 5-iodouracil and 2′-deoxyuridine and 0.0001% of sulphanilamide.

Aliquots of the sample and standard are injected into the chromatograph and the levels of impurities determined by measuring peak areas or peak heights. The time taken for an analysis is about 25 min.

CONCLUSION

An HPLC system using reversed-phase chromatography has been successfully developed and incorporated into BP monographs for idoxuridine and idoxuridine eye drops[9]. This method provides the monographs both with specific assay methods and with tests for controlling the levels of impurities that may arise either during manufacture or as a result of degradation.

ACKNOWLEDGEMENTS

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ELEVATED COLUMN TEMPERATURE FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ERYTHROMYCIN AND ERYTHROMYCIN ETHYLSUCCINATE

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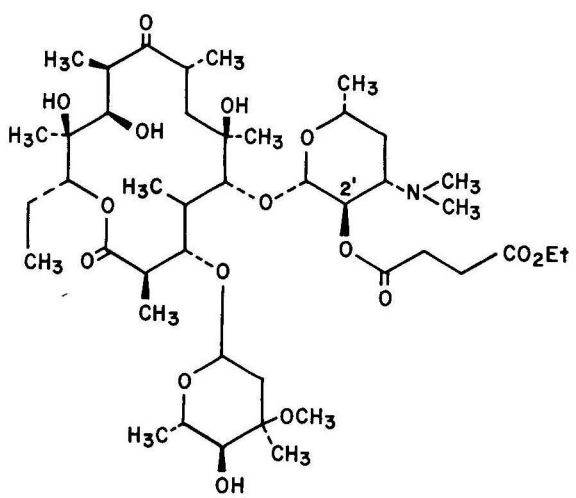
SUMMARY

An elevated column temperature has been found to be an ideal tool not only in shortening chromatographic analysis time, but to increase resolution of erythromycin ethylsuccinate peaks and drastically decrease the height equivalent for a theoretical plate (HETP). When the logarithms of HETP were plotted against the column temperature, a linear relationship was obtained with a correlation coefficient of 0.998.

The high-performance liquid chromatographic method thus developed can separate erythromycin, erythromycin ethylsuccinate and its degradation compounds with a relative standard deviation of 1.4% for the assay of erythromycin ethylsuccinate.

INTRODUCTION

Erythromycin ethylsuccinate is a pro-drug for pediatric use. The erythromycin ethylsuccinate (EES) is esterified at the 2′ position of the desosamine moiety of erythromycin (E) with ethylsuccinyl chloride to stabilize erythromycin in gastric fluid



Erythromycin Ethylsuccinate
Emp. Formula: $C_{43}H_{75}O_{16}N$
Formula Weight: 862.04
Monohydrate, M.W.: 880.01

and to improve taste for oral, pediatric use. The esters of erythromycin, *e.g.* EES, erythromycin propionate, etc., are microbiologically inactive and must be hydrolyzed *in vivo* or by an assay microorganism during incubation to exhibit biological activity[1]. In addition to erythromycin A ethylsuccinate, (EES-A), a commercial preparation of EES may contain erythromycin A (E-A), erythromycin B ethylsuccinate (EES-B), erythromycin C ethylsuccinate (EES-C), anhydroerythromycin ethylsuccinate, 8,9-anhydro-6,9-hemiketal erythromycin ethylsuccinate, etc. The microbiological assay method as accepted by FDA[2] is incapable of differentiating EES-A from E-A or any other impurities, thus, is not an ideal stability nor efficacy-indicating assay method. This paper reports the development of a high-performance liquid chromatographic (HPLC) method which is capable of making that distinction.

EXPERIMENTAL

Analytical instruments

A modular liquid chromatograph (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) equipped with a high-pressure mini-pump and a variable-wavelength detector (Spectro Monitor I) at 215 nm was used. The attenuation of the detector was either 0.04 or 0.08 a.u.f.s. depending on sample concentration. A sample of EES was injected through a Rheodyne injector (Model 70-10, Berkeley, Calif., U.S.A.) with a 100-μl fixed loop. A reversed-phase column [either μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) 300 $\times$ 3.0 mm I.D. or LiChrosorb RP-18 5 μm (Brownlee Lab., Santa Clara, Calif., U.S.A.) 250 $\times$ 4.6 mm I.D.] with a pre-column of 51 $\times$ 2.1 mm I.D. stainless steel, packed with μBondapak C_{18} was used.

The mobile phase was pumped at a flow-rate of 0.9 ml/min (1100 p.s.i.). The column temperature was maintained at 70° $\pm$ 0.1° by a rubber water jacket connected to a Lauda K-2/R controlled-temperature circulating water bath (Brinkmann, Lauda, G.F.R.). Both the pre-column and the analytical column were placed inside the water jacket. The columns were mounted vertically with the inlet side up to avoid possible channeling due to dissolution of silica gel under the chromatographic conditions used. Under continuous usage, a few millimeter void may be noted at the inlet side of the pre-column in two to three weeks. However, the analytical column will not be affected. The pre-column may need replacement when the total column pressure suddenly exceeds over 4000 p.s.i. The analytical column was stable over months of continuous use.

Mobile phase

The composition of the mobile phase used was as follows: acetonitrile–0.2 *M* ammonium acetate–water (60–57:10:40–33), pH 7.0, for μBondapak C_{18} and (65–60:10:25–30), pH 7.0, for LiChrosorb RP-18 packing. The acetonitrile used was UV grade, distilled-in-glass obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The 0.2 *M* ammonium acetate was prepared by weighing 15.5 g of reagent grade ammonium acetate into a 1-l measuring cylinder and adding water to the volume. The amount of the solvent in the mobile phase may have to be modified to obtain the maximum performance of the column.

The mobile phase was filtered through a Fluoropore filter (FHLP04700; Millipore, Bedford, Mass., U.S.A.) and degassed under vacuum with sonication prior

to use. After the end of analysis, the column was throughly rinsed with methanol and stored in methanol until used.

EES reference standard solution

U.S.P. EES reference standard powder (5–6 mg) was accurately weighed into a 10-ml volumetric flask. Just prior to analysis, the mobile phase was added to volume and sonicated to facilitate dissolution. For routine analytical laboratory work, about 60 mg of EES should be weighed into a 100-ml volumetric flask for convenience.

For the analysis of EES in formulation, the 10 ml of the reference standard solution must be washed with 20 ml of heptane for 2 min prior to analysis. This simulated sample preparation step is needed since acetonitrile is slightly miscible in heptane thereby affecting quantification.

Erythromycin reference standard solution

Pharmacopoeia (U.S.P.) erythromycin reference standard (8–9 mg) was accurately weighed into a 10-ml volumetric flask. Just prior to the analysis, the mobile phase was added to volume and sonicated to facilitate dissolution.

For the analysis of the EES formulation, the 10 ml of the reference standard solution must be washed with 20 ml of heptane for 2 min prior to analysis. This simulated sample preparation step is needed since acetonitrile is slightly miscible in heptane thereby affecting quantification.

Sample preparation

EES bulk powder. The powder (5–6 mg) was accurately weighed into a 10-ml volumetric flask. Just prior to the analysis, the mobile phase was added to volume and sonicated to facilitate dissolution. For routine laboratory work, about 60 mg of EES powder should be weighed into a 100-ml volumetric flask for convenience. Just prior to the analysis, the mobile phase is added to volume and sonicated to facilitate dissolution.

EES formulation: 200 mg and 400 mg per 5 ml. Shake vigorously and take approximately a 3-ml sample using a disposable syringe or a pipette. Weigh the filled syringe before and after the sample has been transferred into a 250-ml volumetric flask to obtain the weight of sample by difference. When a pipette is used to quantitatively transfer the sample, thoroughly rinse the pipette with the mobile phase; no weighing or correction with the use of specific gravity of the sample is needed. Just prior to the analysis, the mobile phase is added to volume and sonicated to facilitate dissolution.

Pipette 10 ml of the diluted sample into a 10-dram vial and shake vigorously with 20 ml of heptane for 2 min to remove interfering compounds. Centrifuge the vial for 1 min at 1500 *g*. Remove the top heptane layer by aspiration and inject the bottom aqueous layer.

Calculation

The following equations were used to calculate the quantity of EES in powder:

$$\text{EES-A } (\mu\text{g/mg}) = \frac{A}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \qquad (1)$$

$$\text{EES-B } (\mu\text{g/mg}) = \frac{B}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \quad (2)$$

$$\text{EES-C } (\mu\text{g/mg}) = \frac{C}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \quad (3)$$

$$\text{E-A } (\mu\text{g/mg}) = \frac{E}{Et} \times \frac{Wt_2}{W} \times F_2 \quad (4)$$

where A, B and C are the peak areas of EES-A, -B and -C, respectively, in a sample; E is the peak height of E-A in a sample; At the peak area of EES-A in the U.S.P. EES reference standard; Et the peak height of E-A in the U.S.P. E reference standard; W the weight of a sample; Wt_1 and Wt_2 the weights of the U.S.P. EES and U.S.P. E reference standards, respectively; F_1 the purity of the U.S.P. EES reference standard; and F_2 the assigned potency of the U.S.P. E reference standard (990 μg/mg for the Issue H). For the calculation of EES and E-A contents in the formula, multiply the eqns. 1–4 with a suitable dilution factor to obtain the quantity in a 5-ml dosage unit. When the E-Mycin Liquid® is weighed instead of measured by volume, the specific gravity of the E-Mycin Liquid must be a factor in eqns. 1–4 to correct for volume–weight difference.

RESULTS AND DISCUSSION

Establishment of chromatographic condition

Column temperature was found to be an ideal tool not only in shortening chromatographic analysis time but to increase resolution of peaks and to decrease drastically the height equivalent for a theoretical plate (HETP) of EES. The relationship between HETP and column temperature was linear when the logarithms of HETP were plotted against the column temperature (Fig. 1), $\ln y = -0.023\,x - 0.462$ with a correlation coefficient of 0.998. Fig. 2A and B dramatically illustrate the improvements of chromatography by the increase in column temperature from 23° to 70°.

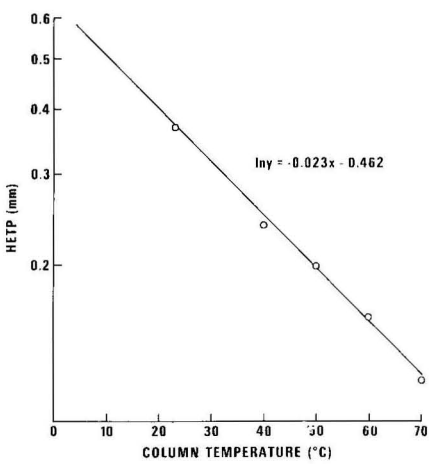


Fig. 1. Effect of column temperature on the HETP using μBondapak C_{18} for the analysis of EES

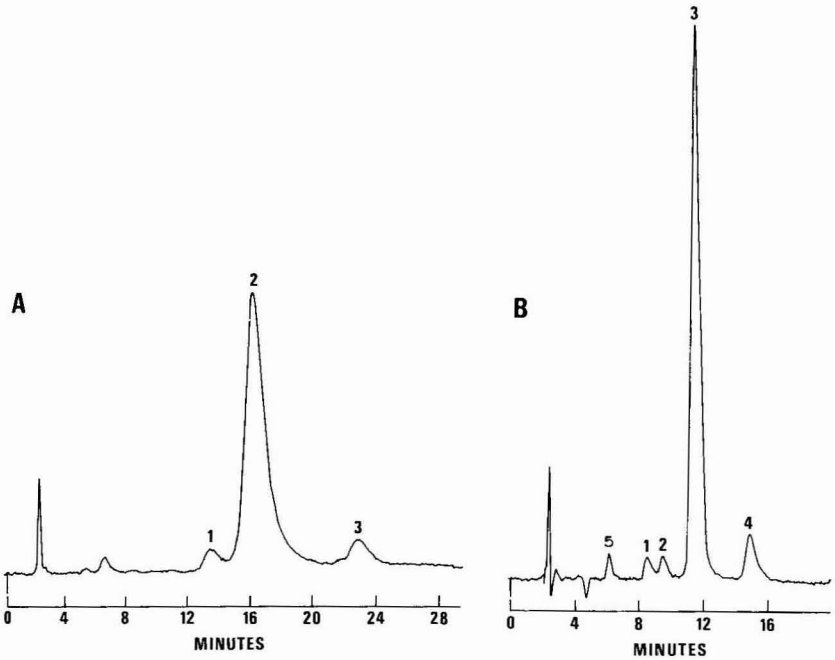


Fig. 2. (A) Effect of column temperature on the chromatographic performance at 25°. Peaks: 1 = E-A and EES-C; 2 = EES-A; 3 = EES-B. (B) Effect of column temperature on the chromatographic performance at 70°. Peaks: 1 = EES-C_1; 2 = EES-C_2; 3 = EES-A; 4 = EES-B, 5 = E-A.

Although Karger *et al.*[3] observed that increase in column temperature (from 25 to 60) caused a small decrease in the hydrophobic selectivity of alcohols on the reversed-phase column, many investigators[4–8] reported significant decrease in HETP by increase in column temperature.

Increase in temperature has been shown to reduce viscosity of a mobile phase, and increase in the mass transfer or diffusion of the solute to the stationary phase[8,9]. Giddings has shown the effects of viscosity of moving phase and diffusion coefficient on theoretical plate heights in the following equation[9]:

$$H = \frac{q\,R\,(1 - R)\,d^2\,u}{D_s}$$

where H is the plate height; d the viscosity of the moving phase; D_s the diffusion coefficient of the sample to the stationary phase; q the constant dependent on the geometry of the stationary phase; R the fraction of the sample in the mobile phase; and u the average mobile phase velocity.

The column temperature of 70° was selected for the analysis of EES, since a temperature above 70° is too close to the boiling point of acetonitrile and any bubble which may form in the mobile phase would adversely affect the efficiency of the column and stability of a UV monitor.

Effects of pH on the elution volume of E-A and EES

The pH of the mobile phase was shown to affect greatly the elution volume and separation between erythromycins A, B, and C[10]. However, pH of the mobile phase has been shown to have little effect on the elution volume of EES (Fig. 3). These phenomena are partly attributable to the difference in the pK values of E-A and EES. Therefore, the pH of the mobile phase has been used as a factor to effect separation between E-A and EES-C peaks.

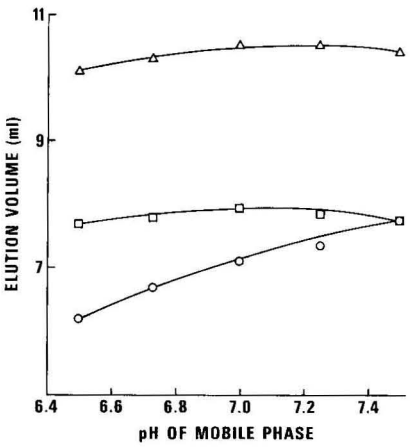


Fig. 3. The effect of pH of the mobile phase on the elution volumes of (△) EES-A, (□) EES-C, and (○) E-A using a μBondapak C_{18} column. Mobile phase, acetonitrile–0.2 M ammonium acetate–water (58:10:32).

Effects of particle size of the reversed-phase column support

A 5-μm silica gel column support (LiChrosorb RP-18) and a 10-μm silica gel support (μBondapak C_{18}) were used to examine the effects of particle size on chromatography of EES.

Use of smaller particle-size support contributed to about 50% increase in theoretical plates (from 11,000 per m for 10-μm particle size to 16,000 per m for 5-μm particle size). This increase in theoretical plates which resulted in increase in peak height obtained by use of the 5-μm particle-size support has been considered as an added advantage to detect and to quantify minor impurities and degradation compounds. The K' value also increased when 5-μm particle size was used. Therefore, increase in acetonitrile concentration in the mobile phase, from about 60 to 65%, is necessary to shorten the chromatographic time.

Since an elevated column temperature is used for the analysis, only a slight increase in column pressure has been experienced (600 and 1000 p.s.i. for 10-μm and 5-μm particle-size column, respectively).

Identification of impurities and degradation compounds

As may be seen in Fig. 2B, commercial preparations of EES contain various amounts of impurities and degradation compounds. In order to identify the major impurities and degradation compounds, a small-scale preparative LC instrument was

constructed using a 122 cm × 7 mm I.D. stainless-steel column packed with μBondapak C_{18}–Porasil B. The column was jacketed to maintain a temperature of 70°.

About 100 mg of EES bulk powder was dissolved in 1 ml of methanol and injected on to the pre-column via a 100-ml loop injector. The column effluents corresponding to those peaks of interest were collected into 50-ml round-bottom flasks immersed in a methanol-dry ice bath. The procedure was repeated for 10 times. The effluents thus collected were freeze-dryed overnight. Immediate freezing of column effluents was found to be essential to minimize degradation of the compounds collected.

A few milligram quantities of powder thus obtained were analyzed by mass spectrometry using the electron impact ionization mode. Identification of the peaks was also made by matching the elution volume (LC) and R_F values (thin-layer chromatography) of the peaks with authentic compounds synthesized by reacting erythromycin B, erythromycin C, and anhydroerythromycin A with ethylsuccinyl chloride. Anhydroerythromycin A ethylsuccinate and 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate were also prepared using the methods as outlined by Stephens and Conine[11].

The mass spectra of the column effluents are shown in Fig. 4A–D. The examination of the mass spectrum (Fig. 4A) corresponding to the major peak, erythromycin A ethylsuccinate, indicated that the molecular ion observed at *m/e* 862 was weak, presumably due to low volatility and ready fragmentation of the glucosidic bonds of the sugars. Other weak ions were found at *m/e* 844 (M−H_2O),776$C_{43}H_{73}NO_{15}$ (M−$CH_3CH_2CHCCH_3OH$ of erythronolide), 716 $C_{37}H_{65}NO_{12}$ (M−ethylsuccinyl), 685 $C_{35}H_{59}NO_{12}$ (M−cladinose), 558 $C_{29}H_{50}O_{10}$ (M−desosamine), 383 $C_{21}H_{34}O_6$ (M−cladinose−desosamine). Much stronger peaks were observed at *m/e* 304 $C_{14}H_{25}NO_6$, and 286 $C_{14}H_{24}NO_5$ (desosaminyl ethylsuccinate) and 158 $C_8H_{16}NO_2$ (desosaminyl). The fragmentation pattern observed was similar to that of erythromycin reported by Rinehart *et al.*[12].

The mass spectra of column effluents corresponding to erythromycin C ethylsuccinate, anhydroerythromycin ethylsuccinate, 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate peaks showed similar pattern of fragmentation (Fig. 4B–D). The mass spectrum of erythromycin C ethylsuccinate failed to yield the parent ion, but the *m/e* 829 (M−H_2O) peak was strong. Weak peaks were observed at *m/e* 685 $C_{35}H_{59}NO_{12}$ (M−cladinose), 382 $C_{21}M_{34}O_6$ (erythronolide), and 303 $C_{14}H_{25}NO_6$ (desosaminyl). Stronger peaks were observed at *m/e* 287 $C_{14}H_{25}NO_5$ (desosaminyl ethylsuccinate), 158 $C_8H_{16}NO_2$ (desosaminyl), and 146 $C_7H_{14}O_3$ (cladinose C).

The mass spectra of anhydroerthromycin ethylsuccinate yielded *m/e* of 844 (M), 685 $C_{35}H_{57}NO_{12}$ (M−cladinose), 365 $C_{21}H_{32}O_5$ (anhydroerythronolide), 303 $C_{14}H_{25}NO_6$ (desosaminyl ethylsuccinate), 287 $C_{14}H_{25}NO_5$ (desosaminyl ethylsuccinate). The mass spectra of 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate was similar to that of anhydroerythromycin ethylsuccinate.

Stability of erythromycin ethylsuccinate at various pH values

In order to investigate the stability of EES in various pH solutions, EES was dissolved in 10% methanolic 0.02 *M* phosphate buffer solution and the pH was adjusted from 2.0 to 8.0. The solution thus prepared was placed in a 37° water bath and analyzed periodically by LC.

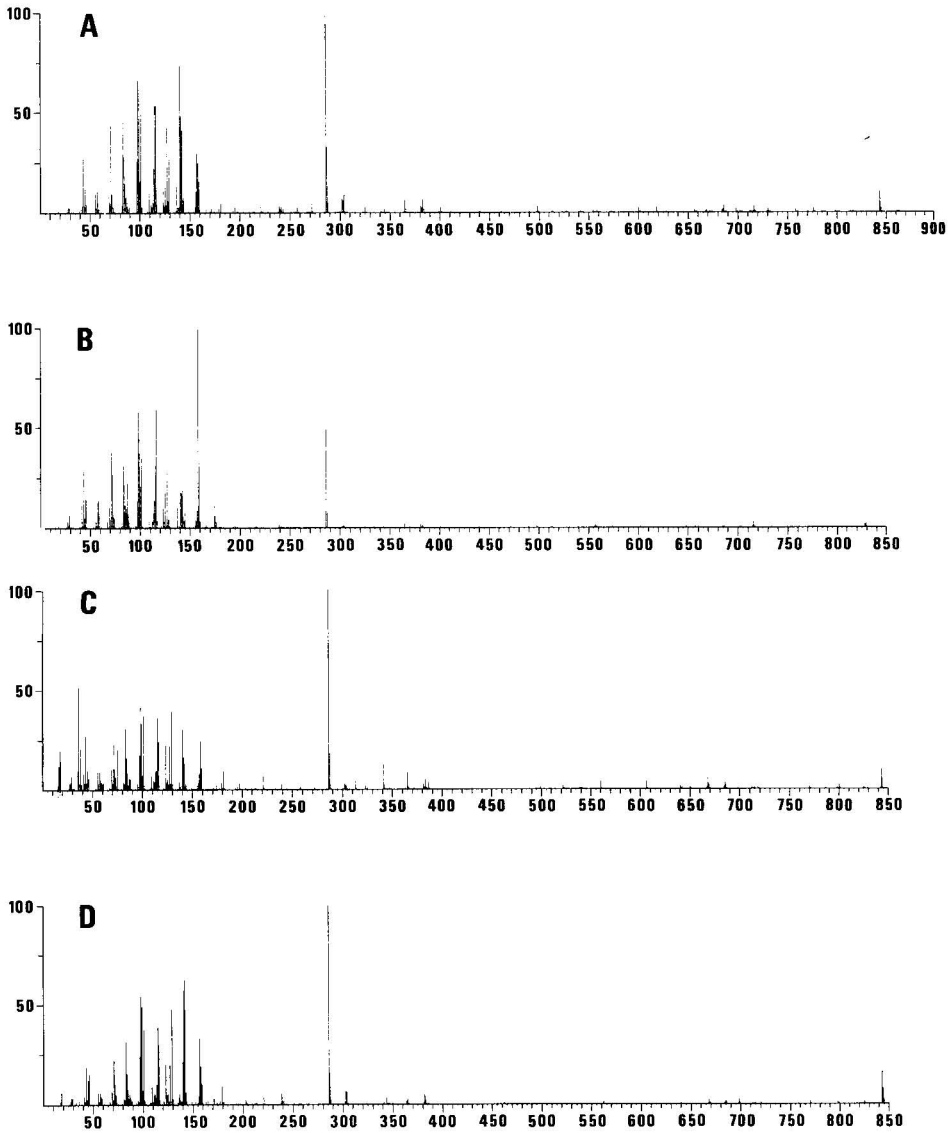


Fig. 4. Electron impact mass spectra. (A) EES-A, (B) EES-C, (C) anhydroerythromycin ethylsuccinate, (D) 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate.

Contrary to popular belief, EES was most stable at pH 5.0 (Table I). At high pH the EES readily hydrolyzes to form E-A and at low pH the EES rapidly anhydrates to form anhydroerythromycin ethylsuccinate and 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate. A typical chromatogram indicating separation of degradating compounds is shown in Fig. 5.

Precision of the HPLC method

Precision of the HPLC method for quantification of EES-A in EES bulk drug

TABLE I

STABILITY OF EES AT VARIOUS pHs

6.0 mg EES per 10 ml of 10% methanol in 0.02 *M* phosphate buffer at 37°.

pH	*Time*	*EES (%)*	*E (%)*	*Anhydro EES*	*EES hemiketal*
8.0	0	100	0	0	0
	17 min	52.8	24.2	0	0
	32 min	46.1	25.4	0	0
	46 min	43.0	34.0	0	0
	1 h 33 min	33.9	41.7	0	0
	2 h 31 min	19.5	60.6	0	0
7.0	5 min	86.3	13.7	0	0
	25 min	43.3	43.1	0	0
	38 min	27.9	51.8	0	0
	52 min	18.6	58.5	0	0
	1 h 05 min	13.5	60.6	0	0
	1 h 25 min	9.4	64.5	0	0
6.0	5 min	97.0	3.0	0	0
	20 min	88.9	7.7	0	0
	39 min	80.3	13.8	0	0
	1 h 22 min	62.2	25.1	0	0
	2 h 48 min	27.6	44.8	0	0
5.0	5 min	98.7	0	1.3	0
	21 min	97.2	0	2.6	0
	44 min	93.4	0	5.2	2.0
	2 h 41 min	74.7	0	16.7	9.5
	5 h 31 min	54.5	0	27.9	16.0
4.0	5 min	100	0	0	0
	22 min	73.3	0	15.5	7.0
	50 min	57.2	0	27.7	15.2
	1 h 15 min	50.5	0	32.1	17.1
	3 h 15 min	24.1	0	54.5	27.9
	6 h 45 min	10.1	0	68.5	23.0
3.0	5 min	44.5	0	40.0	16.8
	34 min	0	0	73.7	12.0
	1 h 03 min	0	0	76.8	8.3
	2 h 35 min	0	0	77.7	7.6
2.0	5 min	5.6	0	57.6	11.7
	1 h 29 min	0	0	62.8	7.0

was determined by analyzing 6 individually weighed and prepared EES powders. The relative standard deviation of the method is 1.4% (Table II).

Quantitative determination

Since molar absorptivity of E-A is approximately 2.4 times less than that of EES (Table III), the U.S.P. erythromycin reference standard must be assayed each time to precisely quantify E-A content in EES.

Analysis of bulk powder

Table IV lists the results of HPLC analysis comparing the quality of EES

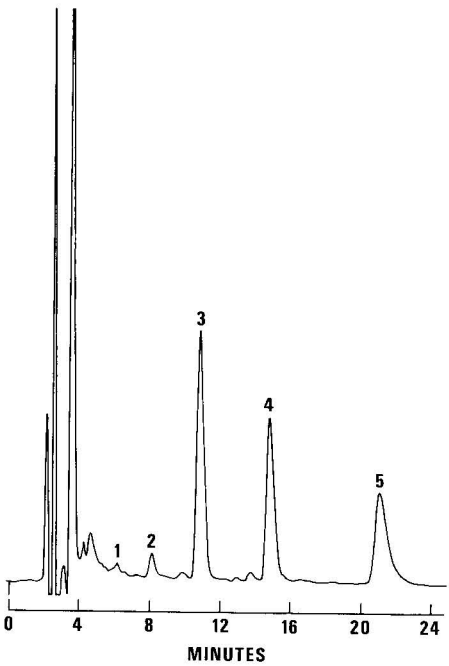


Fig. 5. Chromatogram showing separation of compounds of erythromycin ethylsuccinate degraded by exposure in simulated gastric fluid. Peaks: 1 = E-A; 2 = EES-C, 3 = EES-A, 4 = anhydro-erythromycin ethylsuccinate, 5 = 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate.

TABLE II

PRECISION OF HPLC ASSAY FOR EES POWDER

Weight of EES (mg per 10 ml)	*Height of EES peak*	*Ratio of peak height–weight of sample*
15.075	48.40	3.21
16.085	51.85	3.22
16.050	52.50	3.27
15.430	50.45	3.27
14.596	47.85	3.28
15.920	53.25	3.34
	Relative standard deviation	1.43%

TABLE III

RELATIVE MOLAR ABSORPTIVITY OF E-A AND EES AT 215 nm

Assay day	*Weight of U.S.P. E-A corrected for purity*	*Area of erythromycin peak*	*Area–weight ratio*	*Weight of U.S.P. EES corrected for purity*	*Area of EES-A peak*	*Weight–area ratio*	*Relative molar absorptivity*
1	8.999	817898	90888	5.583	1146439	205345	2.26
2	9.227	806792	87438	5.775	1299246	224978	2.57
3	9.092	866058	95255	5.255	1232139	234469	2.46
						Average	2.43

TABLE IV

HPLC ANALYSIS OF EES BULK POWDER

Supplier	*Sample*	*E-A (μg per mg EES)*	*EES-A (μg per mg EES)*	*EES-B (μg per mg EES)*	*EES-C (μg per mg EES)*	*Anhydro EES-A (μg per mg EES)*	*Hemiketal (μg per mg EES)*
Company A	1	26	915	—	—	—	—
	2	36	910	—	—	—	—
Company B	1	73	769	74	43	—	—
	2	29	780	138	38	—	—
	3	26	779	134	26	—	—
	4	27	805	99	22	—	—
Company C	1	50	857	—	48	—	—
	2	55	817	—	58	—	—
	3	30	831	—	62	—	—
	4	34	844	—	115	6	9

bulk drugs purchased from various suppliers. All the EES powder analyzed contained a varying degree of E-A. In addition, EES powder from company B contained a substantial quantity of EES-B and EES-C and EES powder from company C contained EES-C but no detectable EES-B. One lot of EES from company C contained a trace quantity of anhydro EES and hemiketal EES. A typical chromatogram of EES from company B is shown in Fig. 6.

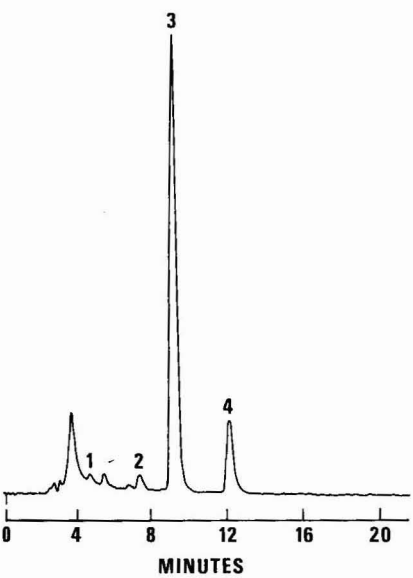


Fig. 6. A typical chromatogram of erythromycin ethylsuccinate manufactured by company B. Peaks: 1 = E-A; 2 = EES-C; 3 = EES-A; 4 = EES-B.

Analysis of EES formulation

When the placebo of the EES formulation was injected on the HPLC system after a simple dilution in a mobile phase, a small peak with the identical retention volume as EES-A was detected. However, the sample preparation procedure developed which include a 2-min heptane extraction effectively eliminated the interfering peak.

The precision of the HPLC method for quantification of EES-A in the formulation was determined by analyzing 6 individually weighed samples of both the 200 mg per 5 ml and 400 mg per 5 ml dosage forms. The relative standard deviations of the assay method are 1.32 and 0.67%, respectively.

Recovery of EES-A from E-Mycin Liquid was investigated by quantitatively adding EES into a placebo of both 200 mg per 5 ml and 400 mg per 5 ml dosage forms at 80, 100 and 120% of the production target. The average recovery for the 200 mg per 5 ml formulation was 99.3% and that for the 400 mg per 5 ml formulation was 98.9%, respectively.

Since the HPLC method is capable of separating and quantifying erythromycin, erythromycin ethylsuccinate and their degradation compounds, the method is of value as a stability indicating assay method.

ACKNOWLEDGEMENTS

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PRÄPARATIVE ISOLIERUNG VON DESOXIRIBOADENYLSÄUREN AUS HYDROLYSATEN OXIDIERTER HERINGSSPERMEN-DNA

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SUMMARY

Preparative isolation of deoxyriboadenylic acids from hydrolysates of oxidized herring sperm DNA

The preparative-scale chemical degradation of DNA from herring sperm to mixtures of purine oligonucleotides or deoxyriboadenylic acids is described. The deoxyriboadenylic acid mixture was separated into six fractions according to increasing ionic charge by column chromatography on QAE-Sephadex A-25. Of the first fraction, 75% was deoxyriboadenosine monophosphate; of the second, 99% consisted of a mixture of $p(dA)_2$ and $(dA)_2p$, while of the third 93% was pdAp. The remaining fractions contained more or less complex mixtures of longer-chain oligonucleotides which could be further separated by subsequent re-chromatography on QAE-Sephadex at pH 9.6. By application of paper chromatography to the fractions obtained from column chromatography, the pure nucleotide phosphates pdAp, $p(dA)_2p$, $p(dA)_3p$, $p(dA)_4p$ and the mixtures of sequence isomers $p(dA)_2$, $(dA)_2p$; $p(dA)_3$, $(dA)_3p$; and $p(dA)_6$, $(dA)_6p$ could be isolated preparatively. The nucleotide phosphates were converted to $(dA)_2$, $(dA)_3$ or $(dA)_4$ and the mixtures of sequence isomers to $(dA)_2$, $(dA)_3$ or $(dA)_6$ by treatment with alkaline phosphatase. By this means dephosphorylated, paper chromatographically pure oligodeoxyriboadenylic acid may be obtained in a preparative scale from the hydrolysates. The structures of the nucleotides thus isolated were deduced from the absorbtion characteristics, the R_F values and the results of enzymatic degradation.

EINLEITUNG

Oligomere der Desoxiriboadenylsäure sind im präparativen Massstab bisher nur durch die sehr aufwendige chemische Polykondensation von Desoxiriboadenosinmonophosphat erhältlich[1]. Die chemische Polykondensation ist so kompliziert, dass sie nur von Spezialisten erfolgreich praktiziert werden kann. Ausserdem treten während der Kondensation Nebenreaktionen auf, durch die auf Kosten der gewünschten linearen Oligonucleotide zyklische Verbindungen, Pyrophosphatderivate und verschiedene andere Reaktionsprodukte gebildet werden. Hinzu kommt, dass bei der chemischen Polykondensation bevorzugt die kurzen Oligomeren mit bis zu vier

Monomereinheiten gebildet werden, während längere Fragmente in nur sehr geringen Ausbeuten entstehen. Definierte lineare Oligodesoxiriboadenylsäuren sind aus dem Kondensationsansatz erst nach mehreren aufwendigen Trennschritten in papierchromatographisch reiner Form erhältlich.

Bei der Suche nach einfachen Wegen zur Darstellung von Desoxiriboadenylsäuren fanden wir, dass Jones *et al.*[2-4] zur Ermittlung von Adenylsäurefragmenten Desoxiribonucleinsäuren (DNA) chemisch im analytischen Massstab zu Oligodesoxiriboadenylsäuren auf folgendem Weg abgebaut haben. Zunächst wurden die Nucleobasen der DNA (3 g Ansatz) bis auf Adenin mit alkalischer $KMnO_4$-Lösung oxidativ zu Ureidogruppen abgebaut, wobei die Polymerhauptkette erhalten blieb. Die Ureidogruppen in dieser sogenannten "oxidierten DNA" wurden anschliessend durch Hydrazinolyse in Hydrazone überführt, aus denen dann mit Benzaldehyd die Aldehydfunktionen freigesetzt wurden. Die so vorbehandelte DNA wurde im Alkalischen hydrolysiert und das neutralisierte Hydrolysat (40 mg Ansatz) an einer DEAE-Cellulose-Säule in mehrere Fraktionen aufgetrennt. Papierchromatographisch und aus dem Phosphat–Adeninverhältnis konnten die Autoren in den verschiedenen Fraktionen Oligomere der Adenylsäure mit bis zu drei Monomereinheiten nachweisen, während sie die Anwesenheit höherer Fragmente vermuteten.

Wir haben das Verfahren, das Jones *et al.* als analytische Methode konzipiert hatten, so modifiziert, dass sich in einem Ansatz der oxidative Abbau mit 30 g DNA und die Fraktionierung des Hydrolysats im 5 g Ansatz praktikabel durchführen lässt. Damit haben wir einen neuen Weg erschlossen, auf dem Desoxiriboadenylsäuren in präparativen Mengen in chromatographisch reiner Form sehr einfach zugänglich sind, wie wir in der vorliegenden Arbeit zeigen werden.

EXPERIMENTELLER TEIL

Reagenzien

Hydraziniumhydroxid zur Synthese (Merck-Schuchardt, München, B.R.D.); Kaliumpermanganat p.A.; die übrigen Chemikalien wurden in "chemisch reiner" Form verwendet; DNA, pdA, dA (PWA Waldhof, Mannheim, B.R.D.); die übrigen Referenznucleotide wurden in unserem Labor synthetisiert[5,6]; QAE-Sephadex A-25 (Pharmacia, Uppsala, Schweden); Chromatographiepapier 2316 (Schleicher & Schüll, Dassel, B.R.D.); DEAE-Cellulose (Whatman DE-23; W. & R. Balston, Maidstone, Grossbritanien); Enzyme (Boehringer, Mannheim, B.R.D.); Membranen (Amicon, Lexington, Mass., V.S.).

Pufferlösungen

1 *M* Tris–HCl-Puffer (pH 7.5): 121 g (1 *M*) Tris-(hydroxymethyl)aminomethan werden in 900 ml Wasser gelöst, mit 50 ml konz. HCl versetzt, dann mit HCl auf pH 7.5 eingestellt und schliesslich mit Wasser auf 1000 ml aufgefüllt.

1 *M* $NaHCO_3$–Na_2CO_3-Puffer (pH 9.6): 65.3 g (0.78 *M*) $NaHCO_3$ und 23.5 g (0.22 *M*) wasserfreies Na_2CO_3 werden mit Wasser auf 1000 ml aufgefüllt.

Laufmittelsysteme

A: Äthanol–1 *M* NH_4Ac (7:3) pH 7.5; B: *n*-Propanol–Konz. Ammoniak–Wasser (55:10:35); C: Isobuttersäure–konz. Ammoniak–Wasser (66:1:33).

Depyrimidinierung und hydrolytische Spaltung der DNA aus Heringsspermen

Käufliche Heringsspermen-DNA (40 g) werden in 160 ml Hydraziniumhydroxid gelöst und 4 h bei 60° im Trockenschrank belassen. Anschliessend wird die hierbei entstandene, braune Reaktionslösung am Rotationsverdampfer zur Trockene eingeengt, der Rückstand in Wasser gelöst und wieder zur Trockene gebracht. Den Rückstand versetzt man mit 1.5 l einer 1.2 *M* KOH Lösung und erhitzt den Ansatz 1 h auf dem siedenden Wasserbad. Anschliessend wird die Lösung im Eisbad auf *ca.* 5° abgekühlt und mit *ca.* 25%iger Perchlorsäure neutralisiert. Nach *ca.* 12 stündigem Stehenlassen im Kühlschrank wird der ausgefallene Niederschlag auf der Nutsche abgesaugt und mehrmals mit kaltem Wasser nachgewassen. Filtrat und Waschwasser werden vereinigt, mit Wasser auf 5 l aufgefüllt und säulenchromatographisch fraktioniert.

Säulenchromatographische Trennung des depyrimidinierten DNA-Hydrolysates an einer DEAE-Cellulose-Säule bei pH 7.5

Das in 5 l Wasser gelöste DNA-Hydrolysat wird auf eine DEAE-Cellulose-Säule mit *ca.* 1 l/h gepumpt. Die Säulenfüllung beträgt 50 × 7 cm. Nach dem Auftragen der Lösung wird die Säule bei gleichem Durchfluss zunächst mit 5 l Wasser bei Raumtemperatur eluiert. Hierbei sinkt die bei 260 nm gemessene Absorption des Eluats unter 1 A_{260}-Einheit. Das Eluat wird verworfen. Anschliessend eluiert man die Säule mit *ca.* 12–15 l einer 0.1 *M* NaCl-Lösung, die mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert ist, bis die Absorption des Eluats unter 4 A_{260}-Einheiten sinkt. Im dritten und letzten Schritt werden die noch adsorbierten Purinnucleotide mit 1 *M* NaCl-Lösung von der Säule eluiert. Die mit 0.1 *M* NaCl–0.05 *M* Tris–HCl eluierten Purinnucleotide werden vereinigt und am Rotationsverdampfer soweit konzentriert, bis Salz ausfällt. Das Konzentrat wird vom Salz befreit und anschliessend an einer UM 2 Membran so lange ultrafiltriert, bis das Eluat mit $AgNO_3$ keine Reaktion zeigt. Das salzfreie Retentat wird lyophilisiert und man erhält *ca.* 7 g niedermolekulare Purinnucleotide als gelbliches Pulver. Die mit 1 *M* NaCl eluierten höhermolekularen Purinnucleotide werden analog aufgearbeitet und ergeben *ca.* 5 g eines hellbraunen Pulvers, das in der folgenden Oxidationsreaktion eingesetzt wird.

Oxidation der Heringsspermen-DNA und der höhermolekularen Purinoligonucleotide

Käufliche Heringsspermen-DNA (Reaktionsansatz 1–4, Tabelle I) oder die mit 1 *M* NaCl eluierten höhermolekularen Purinoligonucleotide (Reaktionsansatz 5, Tabelle I) werden gemeinsam mit $NaHCO_3$ in 1 l Wasser gelöst und auf 37° erwärmt. Die Lösung wird mit 1 l einer ebenfalls auf 37° erwärmten $KMnO_4$-Lösung versetzt. Hierbei stellt sich der pH-Wert der Lösung auf *ca.* 8 ein (Reaktionsansatz 1 und 5). Die Reaktionslösungen der Ansätze 2, 3 und 4 werden dagegen mit 2 *N* NaOH exakt auf pH 9 eingestellt. Man belässt die Ansätze 1, 2, 3 und 5 für 19 h bei 37°, während Ansatz 4 nur 3 h bei dieser Temperatur verbleibt. Anschliessend werden die violett gefärbten Lösungen der Ansätze 1, 2 und 5 durch Zugabe von festem $NaHSO_3$ entfärbt. Der ausgefallene Niederschlag wird abfiltriert und mit Wasser nachgewaschen. Das hellgelbe Filtrat wird mit dem Waschwasser vereinigt und an einer UM-10 Membran durch Ultrafiltration entsalzt. Nach der Gefriertrocknung der entsalzten Retentate werden die "oxidierte DNA" und die "oxidierten Purinnucleotide" in der folgenden Reaktion weiterbehandelt.

TABELLE I

BEDINGUNGEN ZUR OXIDATION DER HERINGSSPERMEN-DNA UND DEREN ANSCHLIESSENDE ALKALISCHE HYDROLYSE ZU DESOXIRIBOADENYLSÄUREN

Oxidation (bei 37°)								*Alkalische Hydrolyse (1 h bei 100°)*			
Reaktionsansatz Nr.	*DNA** (g)	$KMnO_4$* (g)	$NaHCO_3$* (g)	$NaHSO_3$ (g)	*pH-Wert*	*Zeit* (h)	*"Oxidierte DNA"* (g)	*KOH* (l)	(M)	*Ausbeute* (g)	(%)
1	10	20	46.6	8.1	*ca.* 8	19	7	0.5	0.3	3.0	30
2	30	60	46.6	6.8	9.0	19	24	1.0	1.0	9.5	32
3	30	30	46.6	–	9.0	19	26	1.0	1.0	9.0	30
4	30	30	46.6	–	9.0	3	25	1.0	1.0	8.5	28
5	9***	9	42.0	8.0	*ca.* 8	19	7**	0.5	0.3	5.5	61

* Gemeinsam in 2 l Wasser gelöst.
** "Oxidierte Purinoligonucleotide".
*** Purinoligonucleotide.

Hydrazinolyse und alkalische Hydrolyse der "oxidierten DNA" und "oxidierten Purinoligonucleotide"

"Oxidierte DNA" bzw. "oxidierte Purinoligonucleotide" (7–26 g) werden in 40–150 ml Hydraziniumhydroxid gelöst und 4 h bei 60° belassen. Anschliessend engt man die Lösung am Rotationsverdampfer auf die Hälfte ein und lässt sie langsam zum 5-fachen Volumenüberschuss an frisch destilliertem Benzaldehyd unter Rühren zutropfen. Da die Reaktion exotherm verläuft, werden die grösseren Ansätze unter Kühlung durchgeführt. Während des Zutropfens der Lösung wird der Benzaldehyd zunächst milchig trübe, später gelb und gegen Ende der *ca.* 20-min Reaktion fallen gelbe Nadeln aus, die einen Kristallbrei bilden. Durch Zugabe von drei Teilen Äther und einem Teil Wasser wird der Kristallbrei gelöst. Die wässrige Phase wird abgetrennt, anschliessend noch 4–5 mal mit Äther ausgeschüttelt und zum Schluss am Rotationsverdampfer zur Trockene gebracht. Die Rückstände der Reaktionsansätze 1 und 5 werden mit je 500 ml 0.3 *M* KOH gelöst, während die Reaktionsprodukte aus den Ansätzen 2, 3 und 4 in jeweils 1 l 1 *M* KOH aufgenommen werden (vgl. Tabelle I). Die alkalische Hydrolyse wird 1 h bei 100° durchgeführt. Dann wird die Lösung im Eisbad auf *ca.* 5° gekühlt und mit ca. 25%iger Perchlorsäure neutralisiert. Nach der Neutralisation lässt man den Ansatz *ca.* 12 h im Kühlschrank stehen, saugt den Niederschlag ab und wäscht ihn mit kaltem Wasser nach. Das klare Filtrat wird mit dem Waschwasser vereinigt, an einer UM-2 Membran entsalzt und anschliessend lyophilisiert.

Isolierung von Desoxiriboadenylsäuren

(1) Säulenchromatographische Trennung der DNA- und Purinoligonucleotidhydrolysate an QAE-Sephadex A-25 bei pH 7.5 (vgl. Fig. 1). *Ca.* 5 g (60,000–70,000 A_{260}-Einheiten) des DNA-Hydrolysates oder der hydrolysierten Purinoligonucleotide werden in 40 ml einer 0.025 *M* NaCl-Lösung, die mit 0.025 *M* Tris–HCl auf pH 7.5 gepuffert ist, auf eine QAE-Sephadex A-25 Säule gepumpt. Die Säulenfüllung beträgt 70 × 3 cm und ist mit 0.05 *M* NaCl–0.05 *M* Tris–HCl (pH 7.5) äquilibriert. Der Durchfluss wird mit einer Schlauchpumpe auf *ca.* 700 ml/h eingestellt. Nach dem

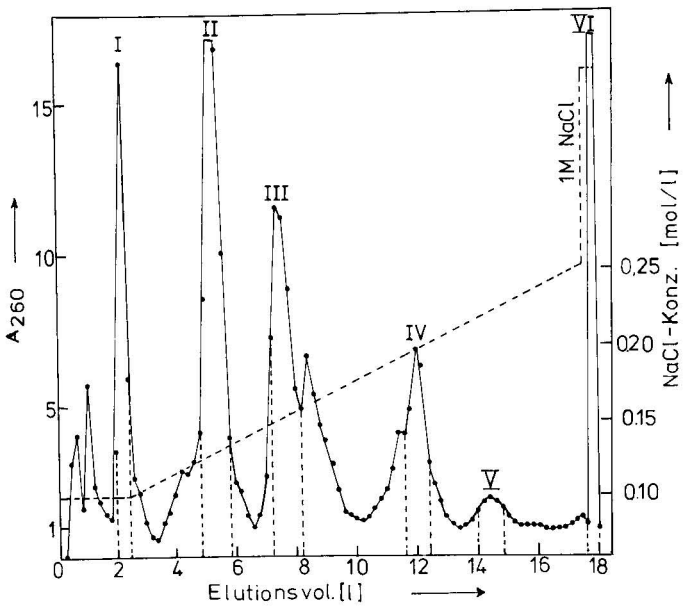


Fig. 1. Elutionsprofil der säulenchromatographischen Trennung von 5 g des "oxidierten DNA"-Hydrolysates aus Reaktionsansatz 4 (vgl. Tabelle I und II) an QAE-Sephadex A-25. Säulenfüllung: 70 × 3 cm; Durchfluss: 700 ml/h. Die Elution erfolgt bei 20° im steigenden NaCl-Gradienten, der mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert ist. Abschliessend wird die Säule mit 1 *M* NaCl eluiert. Die Produkte innerhalb der senkrechten Strichelung werden aufgearbeitet und rechromatographiert (vgl. Fig. 2 und 3).

Auftragen der Probe wird die Säule zunächst bei 20° mit 2.5 l 0.1 *M* NaCl eluiert. Fraktionen zu *ca.* 20 ml werden gesammelt. Die Absorption jeder 10. Fraktion wird spektralphotometrisch bei 250, 260 und 280 nm gemessen. Die graphische Auftragung der bei 260 nm gemessenen Absorptionswerte gegen das Elutionsvolumen führt zu dem in Fig. 1 abgebildeten Elutionsprofil. Bei den Elutionsprofilen der Fig. 2 und 3 wird entsprechend verfahren.

Die Fraktionen von Peak I werden innerhalb der senkrechten Strichelung vereinigt und an einer UM-05 Membran entsalzt. Anschliessend wird die Säule im linear steigenden NaCl-Gradienten eluiert, der ebenfalls mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert ist. Der Gradient beginnt mit 5 l 0.1 *M* NaCl im Mischgefäss und 5 l 0.2 *M* NaCl im Vorratsgefäss und wird dann mit 2.5 l 0.2 *M* NaCl im Mischgefäss und 2.5 l 0.25 *M* NaCl im Vorratsgefäss fortgesetzt. Nach dem NaCl-Gradienten eluiert man die Säule mit 0.5 l 1 *M* NaCl. Die Fraktionen von Peak II–VI werden innerhalb der senkrechten Strichelung vereinigt. Zur Entsalzung werden die Fraktionen aus Peak II und III an einer UM-2 Membran, die Oligonucleotide aus Peak IV–VI an einer UM-10 Membran ultrafiltriert und anschliessend lyophilisiert. Die A_{260}-Einheiten der vereinigten Peakfraktionen sind gemeinsam mit den Ausbeuten an Lyophilisat in Tabelle II zusammengestellt. Produkte gleicher Peaknummer aus den Reaktionsansätzen werden vereinigt und wie unter (3) beschrieben bei pH 9.6 rechromatographiert.

(2) Rechromatographie des Lyophilisats von Peak VI der Fig. 1 an QAE-

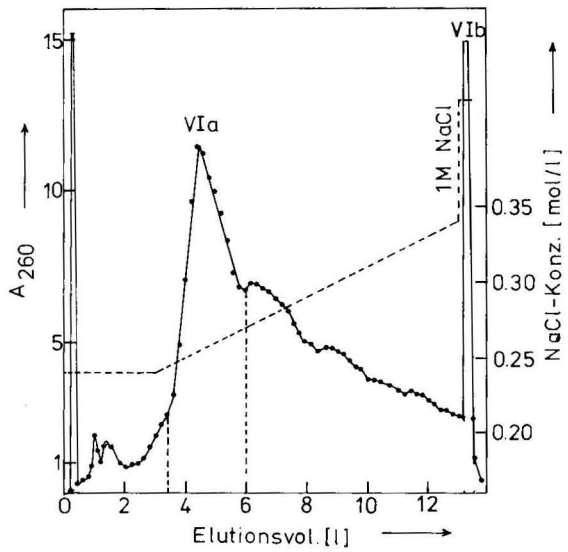


Fig. 2. Elutionsprofil der säulenchromatographischen Nachtrennung von 5 g der in Peak VI (vgl. Fig. 1) eluierten DNA-Fragmente an QAE-Sephadex A-25. Säulenfüllung: 70 × 3 cm; Durchfluss: 700 ml/h. Die Elution erfolgt bei 20° im steigenden NaCl-Gradienten, der mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert ist (vgl. Tabelle II). Abschliessend wird die Säule mit 1 *M* NaCl eluiert. Die Produkte innerhalb der senkrechten Strichelung von Peak VIa werden aufgearbeitet und rechromatographiert (vgl. Fig. 3).

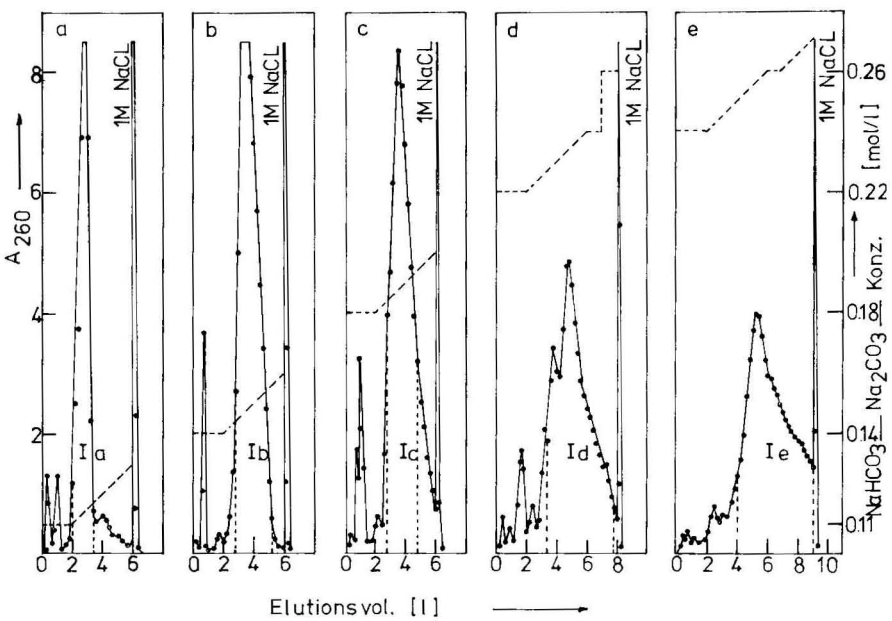


Fig. 3. a–e: Elutionsprofile der säulenchromatographischen Nachtrennung von *ca.* 1 g der in den Peaks II–V und VIa (vgl. Fig. 1 und 2) eluierten Desoxiriboadenylsäuren an QAE-Sephadex A-25. Säulenfüllung: 45 × 2 cm; Durchfluss: 600 ml/h. Die Elution erfolgt bei 20° im steigenden $NaHCO_3$–Na_2CO_3-Gradienten bei pH 9.6 (vgl. Tabelle III). Abschliessend wird die Säule mit 1 M NaCl eluiert. Die Produkte innerhalb der senkrechten Strichelung werden aufgearbeitet. Die Charakterisierung der in den Peaks Ia–e eluierten Oligoadenylsäuren ist in Tabelle IV–VII zusammengefasst.

TABELLE II

BEDINGUNGEN UND ERGEBNISSE DER SÄULENCHROMATOGRAPHISCHEN TRENNUNG VON OXIDIERTEN DNA-HYDROLYSATEN AN QAE-SEPHADEX A-25 (VGL. FIG. 1 UND 2)

Ca. 5 g der DNA-Hydrolysate aus den Reaktionsansätzen 1–5 (vgl. Tabelle I) oder 5 g der in Peak VI eluierten Oligoadenylsäuren werden bei 20° chromatographiert bzw. rechromatographiert. Die Elution erfolgt im NaCl-Gradienten, der mit 0.05 *M* Tris–HCl auf pH 7.5 gepuffert ist. Säulenfüllung: 70 × 3 cm, Durchfluss *ca.* 700 ml/h.

		Peak Nr. (Fig. 1) und Elution bei NaCl (M)						*Zwischen-peak-fraktionen*	*Isolierte Gesamt-menge*	
		I	*II*	*III*	*IV*	*V*	*VI*			
		0.10	*0.12–0.13*	*0.14–0.16*	*0.19–0.20*	*0.21–0.23*	*1.00*		*g*	*%*
DNA-Hydrolysat aus Reaktionsansatz 1										
A_{260}-Einheiten		2800	8200	10,400	8700	5800	8900	15,300		
	(%)	4.6	13.6	17.3	14.5	9.6	14.8	25.6		
Isolierte Menge	(g)		0.58	0.60	0.58	0.35	0.50	–	2.61	52.2
	(%)		11.6	12.0	11.6	7.0	10.0	–		
DNA-Hydrolysat aus Reaktionsansatz 2										
A_{260}-Einheiten		2700	6500	14,600	8800	3500	9400	21,000		
	(%)	4.0	9.8	21.9	13.2	5.3	14.1	31.6		
Isolierte Menge	(g)		0.37	0.85	0.47	0.20	0.55	–	2.44	48.8
	(%)	–	7.4	17.0	9.4	4.0	11.0	–		
DNA-Hydrolysat aus Reaktionsansatz 3										
A_{260}-Einheiten		3700	11,100	7600	6800	2800	8300	20,200		
	(%)	6.1	18.4	12.6	11.2	4.6	13.7	33.4		
Isolierte Menge	(g)		0.62	0.41	0.37	0.18	0.48	–	2.06	41.2
	(%)		12.4	8.2	7.4	3.6	9.6	–		
DNA-Hydrolysat aus Reaktionsansatz 4										
A_{260}-Einheiten		4800	12,400	9600	4100	1500	15,200	21,100		
	(%)	7.0	18.0	14.0	6.0	2.2	22.1	30.7		
Isolierte Menge	(g)		0.58	0.54	0.26	0.10	0.87	–	2.35	47.0
	(%)		11.6	10.8	5.2	2.0	17.4	–		
DNA-Hydrolysat aus Reaktionsansatz 5										
A_{260}-Einheiten		6000	3900	7800	7000	4800	11,300	21,500		
	(%)	9.6	6.3	12.5	11.2	7.7	18.1	34.5		
Isolierte Menge	(g)	–	0.23	0.43	0.40	0.27	0.65	–	1.98	39.6
	(%)	–	4.6	8.6	8.0	5.4	13.0	–		
Rechromatographie der Oligoadenylsäuren aus Peak VI										
Peak Nr. (Fig. 2)				VIa			VIb			
Elution bei NaCl (*M*)				0.24–0.27			1.00			
A_{260}-Einheiten				22,000			22,300	25,900		
	(%)			31.3			31.8	36.9		
Isolierte Menge	(g)			1.4			–	–	1.40	28.0
	(%)			28.0			–	–		

Sephadex A-25 bei pH 7.5 (vgl. Fig. 2). 5 g (*ca.* 71,000 A_{260}-Einheiten) des Lyophilisats von Peak VI (Fig. 1) werden in 40 ml 0.12 *M* NaCl–0.05 *M* Tris–HCl (pH 7.5) auf eine QAE-Sephadex A-25 Säule aufgepumpt. Die Säulenfüllung beträgt 70 × 3 cm. Nach dem Auftragen der Probe wird die Säule unter den in (1) beschriebenen Bedingungen zunächst mit 3 l 0.24 *M* NaCl–0.05 *M* Tris–HCl (pH 7.5) gewaschen. Anschliessend lässt man 10 l eines linearen NaCl-Gradienten folgen, der mit 0.05 *M* Tris–HCl auf

pH 7.5 gepuffert ist. 5 l 0.24 *M* NaCl befinden sich im Mischgefäss, während 5 l 0.34 *M* NaCl im Vorratsgefäss enthalten sind. Nach dem Gradienten eluiert man die Säule abschliessend mit 0.5 l 1 *M* NaCl. Der Elutionsverlauf ist in Fig. 2 dargestellt. Die Fraktionen von Peak VI a werden innerhalb der senkrechten Strichelung vereinigt, an einer UM-10 Membran entsalzt und ergeben 1.4 g eines bräunlichen Lyophilisats, das unter (3) beschrieben, bei pH 9.6 nachgetrennt wird.

(3) Rechromatographie der Oligodesoxiriboadenylsäuren aus Peak II–V und VIa der Fig. 1 und 2 an QAE-Sephadex A-25 bei pH 9.6 (vgl. Fig. 3a–e). Jeweils *ca.* 1 g (*ca.* 11,000–16,000 A_{260}-Einheiten) der Lyophilisate von Peak II–V und VIa (Fig. 1 und 2) werden in 10 ml $NaHCO_3$–Na_2CO_3-Puffer auf eine QAE-Sephadex A-25 Säule aufgetragen. Die Säulenfüllung beträgt 45 × 2 cm. Die säulenchromatographischen Trennungen werden unter den in Tabelle III aufgeführten Bedingungen wie folgt durchgeführt. Nach dem Auftragen der Probe wäscht man die Säule bei einem Durchfluss von *ca.* 600 ml/h zunächst mit 2 l Pufferlösung und lässt dann 4 l eines linearen $NaHCO_3$–Na_2CO_3-Gradienten (pH 9.6) folgen. Nach dem Gradienten wird die Säule in den Trennungen a–c abschliessend mit *ca.* 0.5 l 1 *M* NaCl eluiert. Die Rechromatographie der Lyophilisate von Peak V (Fig. 1) bzw. von Peak VIa (Fig. 2) wird nach dem Gradienten mit 1 l 0.24 *M* bzw. 1 l 0.26 *M* $NaHCO_3$–Na_2CO_3 fortgesetzt. Die Rechromatographie wird mit 0.5 l 1 *M* NaCl beendet, während in der Trennung e ein zweiter Gradient angefügt wird: 1 l 0.26 *M* $NaHCO_3$–Na_2CO_3 im Mischgefäss; 1 l 0.27 *M* $NaHCO_3$–Na_2CO_3 im Vorratsgefäss. Nach dem zweiten Gradienten wird die Säule abschliessend mit 0.5 l 1 *M* NaCl eluiert. Die Fraktionen innerhalb der senkrechten Strichelung (Fig. 3a–e) werden vereinigt und wie unter (1) beschrieben aufgearbeitet. Die Ergebnisse der Rechromatographie sind in Tabelle IV zusammengefasst.

TABELLE III

BEDINGUNGEN ZUR RECHROMATOGRAPHIE VON DESOXIRIBOADENYLSÄUREN AN QAE-SEPHADEX A-25 (VGL. FIG. 3)

Ca. 1 g der Lyophilisate aus Peak II–V und VIa (vgl. Tabelle II) werden bei 20° chromatographiert. Die Elution erfolgt im $NaHCO_3$–Na_2CO_3-Gradienten bei pH 9.6. Säulenfüllung: 45 × 2 cm; Durchfluss: *ca.* 600 ml/h.

Aufgetragene Lyophilisate				*Konzentration des $NaHCO_3$–Na_2CO_3-Puffers (mM)*			*Peak I (Fig. 3)*
Peak Nr.	*Fig.*	*A_{260}-Einheiten*	*Gelöst in 10 ml $NaHCO_3$–Na_2CO_3 (mM)*	*Waschlösung 2 l*	*Gradient 2 l → 2 l*	*Elution erfolgt von–bis*	
II	1	10,700	55	110	110 → 130	110–117	a
III	1	15,300	70	140	140 → 160	142–155	b
IV	1	14,900	90	180	180 → 210	183–194	c
V	1	16,300	110	220	(1) 220 → 240	227–240	d
					(2) 1 l 240		
VIa	2	13,100	120	240	(1) 240 → 260		
					(2) 1 l 260	250–270	e
					(3) 1 l 260		
					→ 1 l 270		

TABELLE IV

ERGEBNISSE DER RECHROMATOGRAPHIE VON DESOXIRIBOADENYLSÄUREN AUS PEAK II–V UND VIa (VGL. TABELLE II, FIG. 3) AN QAE-SEPHADEX A-25 BEI pH 9.6

Peak (Fig. 3)	*A_{260}-Einheiten*		*Bezeichnung*	*Peakanteil (%)**
	Isoliert	*Anteil der Mischung (%)*		
Ia	6700	62.6	$p(dA)_2$	77
			$(dA)_2p$	22
			Unbekannt	1
Ib	13,000	85.0	pdAp	93
			$p(dA)_3$, $(dA)_3p$***	3
			Unbekannt	4
Ic	9700	65.1	$p(dA)_2p$	42
			$p(dA)_2pR$**	40
			Unbekannt	18
Id	11,400	69.7	$p(dA)_3p$	29
			$p(dA)_2pRp$**	32
			$p(dA)_3pR$**	26
			$p(dA)_2pR$**	3
			Unbekannt	10
Ie	7700	58.7	$p(dA)_4p$	16
			$p(dA)_6$, $(dA)_6p$***	6
			Unbekannt	78

* Papierchromatographisch aus A_{260}-Einheiten ermittelt.
** Hypothetische Struktur mit unbekanntem Rest R.
*** Mischung aus Sequenzisomeren.

ERGEBNISSE

Als Ausgangsmaterial verwenden wir DNA aus Heringsspermen, die als technisches Produkt preisgünstig im Handel angeboten wird und ohne zusätzliche Reinigung für unsere Zwecke verwendet werden kann.

Da nach der von Jones *et al.*[2] angegebenen Vorschrift 30 g DNA in einem Ansatz nicht praktikabel zu oxidieren sind, wird zur Ermittlung der optimalen Reaktionsbedingungen, die DNA unter verschiedenen Bedingungen oxidiert. Die Oxidation wird in wässriger $NaHCO_3$-Lösung bei 37° mit unterschiedlichen Mengen an $KMnO_4$, bei verschiedenen Konzentrationen und während unterschiedlich langer Reaktionszeiten entweder bei pH 8–9 oder exakt bei pH 9 durchgeführt. Die Bedingungen und Ausbeuten sind für die einzelnen Reaktionsansätze in Tabelle I zusammengefasst.

In den Ansätzen 1 und 2 behält die, durch $KMnO_4$ violett gefärbte Reaktionslösung, ihre Farbe während der gesamten Reaktionszeit. Der $KMnO_4$-Überschuss wird nach der Oxidation mit $NaHSO_3$ reduziert. Dagegen entfärben sich die Reaktionslösung der Ansätze 3 und 4 im Verlauf der Reaktion, so dass unter diesen Bedingungen das eingesetzte $KMnO_4$ vollständig zur Oxidation verbraucht wird. Nach der Oxidation wird der ausgefallene Braunstein abfiltriert, das Filtrat neutralisiert und zur Entfernung von Salz und niedermolekularen Fragmenten an einer UM-10 Membran ultrafiltriert. Das salzfreie Retentat wird lyophilisiert und man erhält die "oxidierte DNA" als gelbliches Pulver. Die Ausbeute an "oxidierter DNA" liegt, bezogen

auf die Menge der eingesetzten DNA, bei durchschnittlich 82%. Aus den Ausbeuten der Reaktionsansätze 1–4 (vgl. Tabelle I) folgt, dass die Oxidation weitgehend konzentrationsunabhängig ist, bei pH 8–9 abläuft und bereits nach 3 h beendet ist.

Nach der Oxidation werden die Ureidogruppen der "oxidierten DNA", wie von Jones und Walker[4] beschrieben, durch Hydrazinolyse und Benzaldehydbehandlung in Aldehydgruppen überführt. Dann wird die DNA unter den in Tabelle I angegebenen Bedingungen mit überschüssiger KOH in Oligodesoxiriboadenylsäurefragmente gespalten. Die von uns verwendeten, unterschiedlichen KOH-Konzentrationen wirken sich nicht signifikant auf die Ausbeute an Hydrolyseprodukten aus, so dass die alkalische Hydrolyse der "oxidierten DNA" nicht an exakte Konzentrationsbedingungen gebunden ist. Die Hydrolysate werden an einer UM-2 Membran durch Ultrafiltration von Salz und zum Teil auch von niedermolekularen Verbindungen befreit und dann lyophilisiert. Die durchschnittliche Ausbeute an DNA-Hydrolysat liegt bei 30% bezogen auf die Menge der eingesetzten Heringsspermen-DNA.

Zur Überprüfung der Oxidationsreaktion werden im Reaktionsansatz 5 (vgl. Tabelle I) Purinoligonucleotide anstelle der DNA eingesetzt. Purinoligonucleotide erhält man durch Depyrimidinierung nach der von Türler und Chargaff[7] beschriebenen Methode, die wir mit entsprechender Änderung im 40 g Ansatz durchführen. Die depyrimidinierte DNA wird alkalisch hydrolysiert und das neutralisierte Hydrolysat säulenchromatographisch in nieder- und höhermolekulare Purinoligonucleotide getrennt. Die isolierten höhermolekularen Fragmente werden mit einem Überschuss an $KMnO_4$ 19 h oxidiert und anschliessend wie "oxidierte DNA" unter den in Tabelle I angegebenen Bedingungen weiterbehandelt. Die Ausbeute an Lyophilisat beträgt, bezogen auf die eingesetzte Purinoligonucleotidmenge, 61% und entspricht der Ausbeute, die beim Abbau der Heringsspermen-DNA erhalten wird. Die gute Übereinstimmung zwischen den Ergebnissen des DNA- und Purinoligonucleotidabbaus beweist, dass nur die Pyrimidinbasen und Guanin, nicht aber Adenin oxidativ zerstört werden.

Zur Gewinnung der Oligodesoxiriboadenylsäuren aus den Hydrolysaten verwenden wir die zur Isolierung synthetischer Oligonucleotide bewährten Trennmethoden. Das Hydrolysat wird daher zunächst an dem Anionenaustauscher QAE-Sephadex A-25 im steigenden Salzgradienten säulenchromatographisch in Komponenten steigender Phosphatladung fraktioniert. Jeweils 5 g eines Lyophilisats (vgl. Tabelle II) werden an einer Säulenfüllung von 70 × 3 cm unter identischen Bedingungen zunächst bei pH 7.5 im NaCl-Gradienten chromatographiert. Der Elutionsverlauf wird spektralphotometrisch bei 250, 260 und 280 nm verfolgt. Die gemessenen Absorptionswerte bei 260 nm werden gegen das Elutionsvolumen aufgetragen und führen zu dem in Fig. 1 abgebildeten Elutionsprofil. In Fig. 1 ist stellvertretend für die übrigen Trennungen das Elutionsprofil der aus dem Reaktionsansatz 4 erhaltenen Hydrolysate abgebildet, da die Elutionsprofile aller 5 Hydrolysate grundsätzlich untereinander übereinstimmen.

Nach dem Auftragen der Probelösung eluiert man die Säule zuerst mit 0.1 *M* NaCl. Bei dieser Salzstärke werden 2 kleine Peaks und später 1 Hauptpeak eluiert. Im darauf folgenden, linear von 0.1 *M* bis 0.25 *M* NaCl steigenden Gradienten verlassen nacheinander weitere 4 Hauptpeaks die Säule. Bei der abschliessenden Elution mit 1 *M* NaCl wird der 6. Hauptpeak erhalten. Die deutlich von einander abgetrennten Peaks treten in allen 5 Trennungen an identischen Positionen aber mit unterscheid-

lichen Höhen auf. Die in Tabelle II aufgeführten Daten lassen keinen direkten Zusammenhang zwischen den Reaktionsbedingungen und der Veränderung eines bestimmten Peaks erkennen.

Die gemessenen Absorptionsverhältnisse der Peakfraktionen, die für 280/260 zwischen 0.19 und 0.45 liegen, deuten bereits darauf hin, dass in den verschiedenen Peaks als Hauptprodukte Oligoadenylsäuren enthalten sind, da die berechneten Absorptionsverhältnisse von 280/260 für Adenylsäuren mit 0.19–0.25 angegeben werden[8]. Bei Anwesenheit von Oligomeren der Thymidyl-, Cytidyl- und Guanylsäure sollten die Absorptionsverhältnisse der Peakfraktionen für 280/260 bei 0.73, 0.98 bzw. 0.67 liegen. Fraktionen im An- und Abstieg der Peaks, die 280/260 Verhältnisse von 0.30 und höher aufweisen, werden verworfen, so dass nur die Fraktion innerhalb der senkrechten Strichelung vereinigt werden. Die vereinigten Fraktionen werden konzentriert, durch Ultrafiltration entsalzt und lyophilisiert. Nach der säulenchromatographischen Trennung und Aufarbeitung verbleiben vom ursprünglichen Hydrolysat noch *ca.* 40–50%.

Die papierchromatographische Untersuchung der isolierten Peakfraktionen aller 5 Hydrolysate zeigt, dass in sich entsprechenden Peaks grundsätzlich die gleichen Hauptprodukte eluiert werden, so dass wir die Lyophilisate gleicher Peaknummer vereinigen. Die papierchromatographische Untersuchung im Laufmittelsystem A und B zeigt, dass Peak I als Hauptprodukt eine im R_F-Wert mit gekauftem pdA identische Substanz enthält. Die Lyophilisate von Peak II–V erweisen sich im Papierchromatogramm als komplex zusammengesetzte Mischungen, deren Komponenten langsamer als pdA wandern und sich somit wie Oligomere der Desoxiriboadenylsäure verhalten Das Lyophilisat von Peak VI ist so komplex zusammengesetzt, dass es papierchromatographisch nicht aufgetrennt und daher bei pH 7.5 an QAE-Sephadex rechromatographiert wird. Die Nachtrennung führt zu einem geringfügig aufgelösten Elutionsprofil, das in Fig. 2 abgebildet ist. Die Fraktionen aus Peak VIa, die *ca.* 30% der ursprünglichen Mischung enthalten, werden entsalzt und lyophilisiert, während die übrigen Fraktionen nicht aufgearbeitet werden.

Im folgenden zweiten Trennschritt werden die Lyophilisate von Peak II–V und VIa (Fig. 1 und 2) säulenchromatographisch an QAE-Sephadex bei pH 9.6 nachgetrennt. Jeweils *ca.* 1 g der 5 verschiedenen Lyophilisate wird an einer Säulenfüllung von 45 × 2 cm unter den in Tabelle III angegebenen Bedingungen im steigenden $NaHCO_3$–Na_2CO_3 Gradienten fraktioniert. In Fig. 3a–e sind die Elutionsprofile abgebildet, die bei der Rechromatographie der Lyophilisate erhalten werden. Allen 5 Elutionsprofilen ist gemeinsam, dass nach mehreren kleinen Peaks immer zwei Hauptpeaks folgen, von denen der erste im steigenden $NaHCO_3$–Na_2CO_3 Gradienten, der zweite im 1 *M* NaCl die Säule verlässt. Die beiden Hauptpeaks, die in Fig. 3a und b noch deutlich voneinander getrennt sind, überlappen sich in Fig. 3c–e in zunehmendem Masse. Aus den Absorptionsverhältnissen von 280/260 sieht man, dass bei allen 5 Trennungen immer der erste Hauptpeak (280/260 < 0.30) als Hauptprodukt Oligoadenylsäuren enthält, während im zweiten Hauptpeak (280/260 < 0.50) die Anwesenheit von Oligoadenylsäuren weitgehend ausgeschlossen werden kann. Die Fraktionen des zweiten Hauptpeaks werden verworfen. Die Fraktionen von Peak Ia–e die zwischen 59–85% der jeweils aufgetragenen Lyophilisate enthalten (vgl. Tabelle IV), werden vereinigt, entsalzt, lyophilisiert und anschliessend wiederum papierchromatographisch im Laufmittelsystem A und B untersucht. Hierbei zeigt sich, dass in

den verschiedenen Lyophilisaten immer noch eine unterschiedliche Anzahl von Komponenten enthalten ist, die im Papierchromatogramm in mehr oder weniger gut aufgetrennten Flecken wandern.

Alle im Papierchromatogramm auftretenden Flecken eines Lyophilisats werden ausgeschnitten und eluiert. Im Eluat der einzelnen Flecken bestimmt man spektralphotometrisch die A_{260}-Einheiten und errechnet daraus die prozentuale Zusammensetzung des betreffenden Lyophilisats. Die eluierten Hauptprodukte werden konzentriert und im Laufmittelsystem C rechromatographiert. Wandert ein DNA-Fragment in 2 bzw. 3 unterschiedlichen Laufmittelsystemen auf dem Papier als ein einheitlicher Fleck, so wird die Substanz üblicherweise mit "chromatographisch rein" bezeichnet. Da auf einem normalen Papierbogen (60 × 58 cm) bis zu 800 A_{260}-Einheiten chromatographiert werden können, sind auf diesem Weg praktisch alle Komponenten eines Lyophilisats in präparativen Mengen chromatographisch rein zu isolieren.

Die Struktur der isolierten chromatographisch reinen Nucleotide wird mit den Methoden ermittelt, mit denen bekanntlich seit Jahren die Strukturen synthetischer Oligonucleotide eindeutig bewiesen werden. Zur Strukturbestimmung werden Absorptionsverhältnisse, R_F-Werte und Ergebnisse des enzymatischen Abbaus herangezogen.

Aus den gemessenen Absorptionsverhältnissen von 250/260 und 280/260 (vgl. Tabelle V), die mit den in der Literatur[8] für Riboadenylsäuren angegebenen Werte sehr gut übereinstimmen, folgt, dass die isolierten DNA-Fragmente Oligomere der Adenylsäuren sind und dass die Anwesenheit von Thymidyl-, Guanyl- und Cytidylsäuren auszuschliessen ist.

Aus dem säulenchromatographischen Verhalten, den R_F-Werten und, soweit vorhanden, mit Hilfe gekaufter und/oder synthetischer Vergleichssubstanzen[5,6], die wir in einem anderen Zusammenhang hergestellt haben, wird die Kettenlänge der isolierten Oligoadenylsäuren bestimmt.

Die so ermittelte Struktur der Oligoadenylsäuren wird abschliessend durch enzymatischen Abbau mit alkalischer Phosphatase und Phophodiesterase aus Schlangengift überprüft und bestätigt[9–11]. Die Phosphatase entfernt aus Oligonucleotiden endständige Phosphatgruppen ohne die Polymerketten anzugreifen. Die Entfernung terminaler Phosphatgruppen äussert sich im deutlichen Anstieg des R_F-Wertes. Oligonucleotide gleicher Sequenz, aber unterschiedlicher Anzahl terminaler Phosphatgruppen, sind papierchromatographisch aufgrund ihrer sehr unterschiedlichen R_F-Werte eindeutig zu unterscheiden. Nach dem Phosphataseabbau wird papierchromatographisch einerseits die Vollständigkeit des Abbaus und andererseits nochmals die Reinheit der Produkte überprüft. Aus dem Vergleich der R_F-Werte vor und nach dem Abbau lässt sich mit Sicherheit entscheiden, ob das ursprüngliche Nucleotid 1 und 2 oder keine terminalen Phosphatgruppen aufweist. Die dephosphorylierten Oligonucleotide werden vom Papier eluiert und zur Bestimmung der Kettenlänge mit Phosphodiesterase aus Schlangengift hydrolysiert. Phosphodiesterase aus Schlangengift baut bekanntlich als Exonuclease lineare Oligonucleotide vom 3'-Ende schrittweise ab und setzt dabei aus dephosphorylierten Oligonucleotiden 5'-Mononucleotide und den 5'-terminalen Baustein als Mononucleosid frei[9]. Bei der enzymatischen Hydrolyse der dephosphorylierten Oligodesoxiriboadenylsäuren mit Phosphodiesterase bilden sich somit dA und pdA. Oligodesoxiriboadenylsäuren mit nur einer ter-

TABELLE V

ABSORPTIONSVERHÄLTNISSE DER ISOLIERTEN, CHROMATOGRAPHISCH REINEN DESOXIRIBOADENYLSÄUREN

Adenylsäuren Bezeichnung	*Absorptionsverhältnisse*	
	250/260	*280/260*
$p(dA)_2$, $(dA)_2p$*	0.83 (0.83)***	0.22 (0.19)***
$p(dA)_3$, $(dA)_3p$*	0.83 (0.85)§	0.23 (0.24)§
$p(dA)_6$, $(dA)_6p$*	0.86	0.30
pdAp	0.79	0.14
$p(dA)_2p$	0.84	0.23
$p(dA)_3p$	0.83	0.23
$p(dA)_4p$	0.85 (0.85)§§	0.25 (0.25)§§
$(dA)_2$	0.83 (0.83)***	0.19 (0.19)***
$(dA)_3$	0.84	0.23
$(dA)_4$	0.84	0.24
$(dA)_6$	0.86	0.30
$p(dA)_2pR$**	0.79	0.15
$p(dA)_2pRp$**	0.81	0.16
$p(dA)_3pR$**	0.84	0.25
$(dA)_2pR$**	0.78	0.14
$(dA)_3pR$**	0.84	0.20

* Mischung aus Sequenzisomeren.
** Hypothetische Struktur mit unbekanntem Rest R.
*** Berechnet für $p(A)_2$.
§ Berechnet für $p(A)_3$.
§§ Berechnet für $p(A)_4$.

minalen 5′-Phosphatgruppe werden dagegen ausschliesslich zu pdA abgebaut. Bei der Spaltung von Oligodesoxiriboadenylsäuren mit nur einer terminalen 3′-Phosphatgruppe werden aus dem Dinucleotid dA und pdAp, aus den höheren Fragmenten dA, pdA und pdAp gebildet. Der Abbau von Oligodesoxiriboadenylsäurephosphaten $p(dA)_np$ führt auf diesem Weg zu pdA und pdAp. Eine 3′-Phosphatendgruppe erschwert den enzymatischen Abbau mit Phosphodiesterase, so dass kurze Oligonucleotide nur sehr langsam und längere nicht mehr abgebaut werden. Aus diesem Grund sind zur enzymatischen Hydrolyse der 3′-Nucleotide bzw. Nucleotidphosphate höhere Enzymkonzentrationen und längere Reaktionszeiten als bei 5′-Nucleotiden bzw. Oligomeren ohne terminale Phosphatgruppen erforderlich.

Nach dem enzymatischen Abbau wird das Hydrolysat papierchromatographisch getrennt und die hierbei auftretenden Spaltprodukte dA und pdA mit Hilfe gekaufter Referenzsubstanzen identifiziert. Auftretendes pdAp wird mit Phosphatase abgebaut und dann als dA nachgewiesen. Aus der Art der Spaltprodukte kann man feststellen, ob die Oligodesoxiriboadenylsäuren am 5′ oder 3′ bzw. an beiden Enden Phosphatgruppen tragen. Zur Ermittlung der Kettenlänge werden die einzelnen Spaltprodukte quantitativ vom Papier eluiert. Aus dem Verhältnis der im Eluat gemessenen A_{260}-Einheiten von dA und pdA lässt sich bspw. die ursprüngliche Kettenlänge einer dephosphorylierten Oligoadenylsäure ermitteln.

Die Charakterisierung und Identifizierung der Lyophilisate von Peak Ia–e, die in Tabelle V–VII zusammengefasst sind, führen im einzelnen zu folgenden Ergebnissen.

TABELLE VI

PAPIERCHROMATOGRAPHISCHE R_F-WERTE DER ISOLIERTEN DESOXIRIBOADENYLSÄUREN VOR UND NACH DEM ABBAU MIT ALKALISCHER PHOSPHATASE

A,B,C: siehe *Laufmittelsysteme*.

Adenylsäuren Bezeichnung	*R_F-Werte absteigend im Laufmittelsystem*				
	A			*B*	*C*
	Relativ zu		*Nach Phosphataseabbau*	*Relativ zu pdA*	*Relativ zu pdA*
	pdA	*p(dA)₂*	*Relativ zu pdA*		
$p(dA)_2$, $(dA)_2p$*	0.38		2.00	0.84	0.98
pdAp	0.16		3.73	0.66	0.73
$p(dA)_3$, $(dA)_3p$*	0.16		1.07	0.66	0.98
$p(dA)_2p$		0.16	2.00	0.50	0.78
$p(dA)_2pR$**		0.76	1.39	0.77	
$p(dA)_2pRp$**		0.22	1.39	0.54	
$p(dA)_3p$		0.06	1.07	0.38	0.82
$p(dA)_3pR$**		0.39	0.84	0.67	
$p(dA)_4p$			0.90***	0.25	0.74
$p(dA)_6$, $(dA)_6p$*			0.49***	0.25	0.53

* Mischung aus Sequenzisomeren.
** Hypothetische Struktur mit unbekanntem Rest R.
*** Im System B.

TABELLE VII

ENZYMATISCHE HYDROLYSE DER DESOXIRIBOADENYLSÄUREN MIT PHOSPHODIESTERASE AUS SCHLANGENGIFT

Adenylsäuren Bezeichnung	*Spaltprodukte*	*Verhältnisse der Spaltprodukte*	
		Erwartet	*Gefunden*
$p(dA)_2$, $(dA)_2p$*	pdA, pdAp, dA		
$p(dA)_3$, $(dA)_3p$*	pdA, pdAp, dA		
$p(dA)_6$, $(dA)_6p$*,**	pdA		
$p(dA)_2p$	pdA, pdAp	1:1	1.00:1.28
$p(dA)_3p$	pdA, pdAp	2:1	2.30:1.00
$(dA)_2$	dA, pdA	1:1	1.14:1.00
$(dA)_3$	dA, pdA	1:2	1.00:1.82
$(dA)_4$	dA, pdA	1:3	1.00:3.20
$(dA)_6$	dA, pdA	1:5	1.00:4.87
$p(dA)_2pR$***	keine		
$p(dA)_3pR$***	keine		
$(dA)_2pR$***	keine		
$(dA)_3pR$***	keine		

* Mischung der Sequenzisomeren.
** Wird nicht gespalten.
*** Hypothetische Struktur mit unbekanntem Rest R.

Peak I (Fig. 1) enthält zu 75% Desoxiriboadenosinmonophosphat, das mit pdA in den Absorptionsverhältnissen und R_F-Werten übereinstimmt. Durch Phosphatasebehandlung lässt sich das Nucleotid vollständig abbauen. Das Abbauprodukt wird im Laufmittelsystem A eindeutig als dA identifiziert.

Das Lyophilisat von Peak Ia enthält zu 99% eine Mischung der sequenzisomeren Dinucleotide $p(dA)_2$ und $(dA)_2p$, die in den Laufmittelsystemen A, B, C als ein Fleck wandern, der im R_F-Wert mit dem von synthetischem $p(dA)_2$ identisch ist. Die Absorptionsverhältnisse der vom Papier eluierten Dinucleotidmischungen stimmen sowohl mit den berechneten Werten als auch mit denen des synthetischen Dinucleotids überein. Nach Phosphatasebehandlung wandert das vollständig abgebaute Produkt im Laufmittelsystem A wiederum als einheitlicher Fleck, der im R_F-Wert mit dem von synthetischem $(dA)_2$ identisch ist. Beim Abbau des Dinucleosidphosphats mit Phosphodiesterase erhält man dA: pdA im Verhältnis 1.14:1. Den eindeutigen Beweis für die Anwesenheit beider Sequenzisomeren im einheitlich wandernden Fleck liefert der direkte Abbau mit Phosphodiesterase aus Schlangengift. Bei der enzymatischen Hydrolyse des aus dem Papier eluierten Substanzflecken mit Phosphodiesterase entstehen ausser pdA auch dA und pdAp. Die Bildung von dA kann nicht auf eine möglicherweise in der Phosphodiesterase enthaltene Phosphatase zurückgeführt werden, da im Kontrollversuch aus gekauftem pdA kein dA nachweisbar gebildet wird. Aus den Verhältnissen der A_{260}-Einheiten von pdA:dA + pdAp ergibt sich, dass im Lyophilisat von Peak Ia etwa 77% $p(dA)_2$, das zu pdA abgebaut wird, neben etwa 12% $(dA)_2p$, aus dem dA und pdAp gebildet werden, enthalten sind.

Das Lyophilisat von Peak Ib setzt sich zu 93% aus pdAp, zu 4% aus nicht näher untersuchten Verbindungen und zu 3% aus einer Mischung der sequenzisomeren Trinucleotide $p(dA)_3$ und $(dA)_3p$ zusammen. pdAp wird papierchromatographisch im Laufmittelsystem C von den Trinucleotiden und Nebenprodukten befreit und ist auf diesem Weg in chromatographisch reiner Form erhältlich. Durch Behandlung mit Phosphatase lässt sich pdAp vollständig abbauen. Das Abbauprodukt wandert im Laufmittelsystem A als ein Fleck, dessen R_F-Wert und Absorptionsverhältnisse mit dem Referenznucleosid dA identisch sind. Bei der Behandlung von pdAp mit Phosphodiesterase können keine Spaltprodukte nachgewiesen werden. Dieser Befund ist ein zusätzlicher Beweis, dass die verwendete Phosphodiesterase keine Phosphatase enthält. Die Mischung der sequenzisomeren Trinucleotide wird zwar papierchromatographisch nicht aufgetrennt, kann aber im Laufmittelsystem C von den übrigen Verbindungen des Lyophilisats vollständig befreit werden. Bei der Behandlung mit Phosphatase werden die Trinucleotide vollständig abgebaut. Das Abbauprodukt stimmt sowohl in den Absorptionsverhältnissen als auch im R_F-Wert mit synthetischem $(dA)_3$ überein und wird von Phosphodiesterase zu dA und pdA im Verhältnis 1:1.82 gespalten. Die Anwesenheit beider Sequenzisomeren wird dadurch bewiesen, dass beim direkten Abbau, der vom Papier eluierten Trinucleotidmischung, mit Phosphodiesterase als Spaltprodukte pdA, dA und pdAp auftreten. Aus den Verhältnissen der A_{260}-Einheiten von pdA:dA:pdAp errechnet sich, dass die Mischung *ca.* 92% $p(dA)_3$ neben *ca.* 8% $(dA)_3p$ enthält.

Das Lyophilisat in Peak Ic trennt sich im Papierchromatogramm in mehrere Neben- und 2 Hauptflecken auf, die jeweils 42% bzw. 40% der aufgetragenen A_{260}-Einheiten enthalten. Der langsam wandernde Hauptflecke erweist sich in den Laufmittelsystemen A, B und C als einheitlich und wird auf folgendem Weg eindeutig als $p(dA)_2p$ identifiziert. Nach der Behandlung mit Phosphatase ist das Abbauprodukt papierchromatographisch einheitlich, im R_F-Wert und in den Absorptionsverhältnissen mit synthetischem $(dA)_2$ identisch und wird von Phosphodiesterase zu dA und pdA im Verhältnis 1.14:1.00 gespalten. Beim direkten Abbau mit Phosphodiesterase erhält

man pdA und pdAp. Der schneller wandernde Hauptfleck, der in den Laufmittelsystemen A und B einheitlich wandert, enthält vermutlich aus folgenden Gründen ein unvollständig hydrolysiertes Fragment der Oligodesoxiriboadenylsäure. Beim Abbau mit Phosphatase steigt der R_F-Wert so an, dass vermutlich nur eine terminale Phosphatgruppe abgespalten wird. Das dephosphorylierte Produkt kann aber anschliessend nicht mit Phosphodiesterase hydrolysiert werden. Wir nehmen daher an, dass mit Phosphatase die 5′-Phosphatgruppe entfernt wird, während ein unbekannter Rest R das 3′-Ende vor dem Angriff der Phosphodiesterase schützt. Aufgrund der Absorptionsverhältnisse und der säulen- und papierchromatographischen Daten weist das 2. Hauptprodukt aus Peak Ic vermutlich die Struktur $p(dA)_2pR$ oder $(dA)_2$-pRp auf.

Im Lyophilisat von Peak Id findet man papierchromatographisch 3 Haupt- und mehrere Nebenprodukte. Auf die Hauptprodukte entfallen im einzelnen jeweils 25–30% und zusammen etwa 87% der A_{260}-Einheiten des Lyophilisats, so dass sich die restlichen 13% auf mehrere, nicht näher identifizierte Nebenprodukte verteilen. Die Hauptprodukte werden vom Papier eluiert, in den Laufmittelsystemen B und C rechromatographiert und dann papierchromatographisch rein erhalten. Die Identifizierung der isolierten Hauptprodukte führt zu folgenden Ergebnissen. Die Absorptionsverhältnisse zeigen, dass die 3 Hauptprodukte aus Oligoadenylsäuren aufgebaut sind. Das Hauptprodukt mit dem niedrigsten R_F-Wert weist aus folgenden Gründen eindeutig die Struktur $p(dA)_3p$ auf. Beim Abbau mit Phosphodiesterase erhält man pdA:pdAp im Verhältnis 2.30:1.00. Beim Abbau mit Phosphatase verliert die Verbindung zwei Phosphatendgruppen. Das dephosphorylierte Produkt ist papierchromatographisch rein, im R_F-Wert und in den Absorptionsverhältnissen mit synthetischem $(dA)_3$ identisch und wird von Phosphodiesterase zu dA und pdA im Verhältnis 1.00:1.82 hydrolysiert. Die beiden schneller wandernden Hauptprodukte werden ebenfalls mit Phosphatase abgebaut. Aus dem unterschiedlichen Anstieg der R_F-Werte nach Phosphataseabbau folgt, dass die eine Oligodesoxiriboadenylsäure, deren R_F-Wert von 0.39 auf 0.84 steigt, nur eine terminale Phosphatgruppe verliert. Der R_F-Wert der anderen Oligodesoxiriboadenylsäure steigt von 0.22 auf 1.39, so dass hier zwei terminale Phosphatgruppen entfernt werden. Die abgebaute Verbindung ist in ihrem R_F-Wert mit dem Abbauprodukt des zweiten Hauptprodukts (R_F = 1.39) aus Peak Ic identisch. Beide Oligodesoxiriboadenylsäuren aus Peak Id sind nach der Phosphatasebehandlung mit Phosphodiesterase nicht spaltbar, so dass vermutlich das 3′-Ende wiederum mit einem unbekannten Rest R blockiert ist. Wir nehmen daher an, dass die Oligodesoxiriboadenylsäure mit dem R_F-Wert von 0.39 die Struktur $p(dA)_3pR$ oder $(dA)_3pRp$, die andere die Struktur $p(dA)_2pRp$ aufweist. Mit dieser Annahme lassen sich sowohl die chromatographischen Eigenschaften als auch die Ergebnisse des enzymatischen Abbaus plausibel erklären.

Das Lyophilisat von Peak Ie wandert im System B zu 80% als Bande, während die restlichen 20% einen langsamer wandernden Flecken bilden, der zur weiteren Identifizierung eluiert und im Laufmittelsystem C nachgereinigt wird. Bei der Nachtrennung erhält man zwei Flecken. Der schneller wandernde Fleck wird aus folgenden Gründen als $p(dA)_4p$ identifiziert. Mit Phosphatase wird der eluierte Fleck vollständig abgebaut. Das Abbauprodukt wandert im Laufmittelsystem B als einheitlicher Fleck, der im R_F-Wert und in den Absorptionsverhältnissen mit synthetischem $(dA)_4$ identisch ist. Mit Phosphodiesterase wird das Abbauprodukt in dA und p(dA) im Ver-

hältnis 1:3.2 gespalten. Im zweiten, langsamer wandernden Flecken werden die Sequenzisomeren $p(dA)_6$ und $(dA)_6p$ nachgewiesen. Aus dem eluierten Fleck erhält man nach Phosphataseabbau ein chromatographisch reines Produkt, dass im R_F-Wert mit synthetischem $(dA)_6$ identisch ist und durch Phosphodiesterase vollständig zu dA und pdA im Verhältnis 1:4.87 hydrolysiert wird. Die Anwesenheit beider Sequenzisomeren beweist der direkte Abbau mit Phosphodiesterase. Hierbei werden nur etwa 60% hydrolysiert, während die restlichen 40% nicht gespalten werden. Das nicht abgebaute Produkt lässt sich durch Phosphatase in $(dA)_6$ überführen. Aus den Verhältnissen der A_{260}-Einheiten folgt, dass sich die Mischung zu etwa 60% aus $p(dA)_6$ und zu 40% aus $(dA)_6p$ zusammensetzt.

DISKUSSION

Unsere Ergebnisse bestätigen grundsätzlich die Angaben von Jones *et al.*, zeigen aber darüber hinaus, dass nicht nur DNA, sondern auch Purinoligonucleotide durch Oxidation mit $KMnO_4$ und anschliessender alkalischer Hydrolyse im präparativen Massstab gezielt zu Oligodesoxiriboadenylsäuren abgebaut werden können. Aus der durchschnittlichen Ausbeute an Adenylsäuren, die 30% bezogen auf die eingesetzte DNA Menge, bzw. 61% bezogen auf die Purinoligonucleotidmenge beträgt, lässt sich abschätzen, dass die Heringsspermen-DNA je zu 30% aus Adenyl- und Thymidyl- und je zu 20% aus Guanyl- und Cytidylsäuren aufgebaut ist und somit ein Molverhältnis von dA + dT/dG + dC von *ca.* 1.50 erwarten lässt. Der Wert steht in gutem Einklang mit den in der Literatur für Heringsspermen-DNA angegebenen Molverhältnis von 1.27[12]. Für die präparative säulenchromatographische Auftrennung der Hydrolysate, die wir im 5 g Ansatz durchführen, eignet sich unserer Erfahrung nach QAE-Sephadex besser als die von Jones *et al.* für die Auftrennung von 40 mg Hydrolysat verwendete DEAE-Cellulose. QAE-Sephadex ermöglicht als Perlpolymerisat eine homogene Säulenpackung mit hohem Durchfluss und weist ausserdem eine höhere Trennkapazität als DEAE-Cellulose auf. Bei der säulenchromatographischen Fraktionierung der Hydrolysate erhalten wir etwa das von Jones *et al.* beschriebene Elutionsprofil. In den verschiedenen Fraktionen finden wir aber nicht nur die von den Autoren qualitativ nachgewiesenen oder vermuteten Oligodesoxiriboadenylsäuren, die wir in chromatographisch reiner Form präparativ isolieren, sondern noch weitere DNA-Fragmente, die von den Autoren nicht erwähnt oder nicht gefunden werden. Hierzu zählen das Desoxiriboadenosinmonophosphat, die verschiedenen sequenzisomeren Oligodesoxiriboadenylsäuren und die vermutlich unvollständig abgebauten, am 3'-Ende mit einem unbekannten Rest R blockierten Fragmente, der hypothetischen Strukturen $p(dA)_2pRp$, $p(dA)_2pR$, $(dA)_2pRp$, $(dA)_3pRp$, $p(dA)_3pR$. Der mit R bezeichnete Rest ist möglicherweise die ungesättigte Aldehydgruppe, die nach den Untersuchungen von Jones *et al.*[13] im Verlauf des DNA-Abbaus zwar auftritt, aber unter alkalischen Bedingungen hydrolytisch sehr schnell abgespalten werden sollte.

Die Anwesenheit unterschiedlich hydrolysierter DNA-Fragmente kompliziert die präparative Isolierung chromatographisch reiner Oligodesoxiriboadenylsäuren aus dem DNA-Hydrolysat erheblich. Nach zwei säulenchromatographischen Trennungen und einem anschliessenden papierchromatographischen Trennschritt sind bisher nur Fragmente mit bis zu sechs Monomereinheiten in chromatographisch reiner Form

aus den Hydrolysaten zu gewinnen. Zur Zeit versuchen wir mit anderen Trennmethoden auch die höhermolekularen Oligodesoxiriboadenylsäurefragmente zu isolieren, die mit Sicherheit in den Hydrolysaten enthalten sind. In Tabelle VIII sind die Desoxiriboadenylsäuren zusammengestellt, die wir bisher in chromatographisch reiner Form, in präparativen Mengen aus den DNA-Hydrolysaten erhalten. Die Mengenangaben sind aus den A_{260}-Einheiten der papierchromatographisch isolierten Nucleotide hochgerechnet und somit nur als Orientierungsdaten anzusehen, die nicht die tatsächliche Verteilung der Adenylsäuresegmente einer Heringsspermen-DNA wiedergeben. Die in der Tabelle aufgeführten Oligodesoxiriboadenylsäuren sind durch präparative Isolierung aus DNA Hydrolysaten wesentlich einfacher als durch chemische Synthese zugänglich.

TABELLE VIII

HOCHGERECHNETE MENGE DER DESOXIRIBOADENYLSÄUREN, DIE AUS 100 g HERINGSSPERMEN-DNA NACH OXIDATIVEM ABBAU, ALKALISCHER HYDROLYSE UND DREI CHROMATOGRAPHISCHEN TRENNSCHRITTEN IN CHROMATOGRAPHISCH REINER FORM DIREKT (I) ODER NACH PHOSPHATASEABBAU (II) ZUGÄNGLICHT SIND

Bezeichnung der Adenylsäuren		*Mengen (mg)*
I	*II*	
pdAp		2600
$p(dA)_2$, $(dA)_2p$*		1700
$p(dA)_2p$		700
	$(dA)_2$	2400
$p(dA)_2pR$**		650
	$(dA)_2pR$**	850
$p(dA)_3$, $(dA)_3p$*		<100
$p(dA)_3p$		250
	$(dA)_3$	350
$p(dA)_3pR$**		200
	$(dA)_3pR$**	200
$p(dA)_4p$		<100
	$(dA)_4$	<100
$p(dA)_6$, $(dA)_6p$*		$<$ 50
	$(dA)_6$	$<$ 50

* Mischung aus Sequenzisomeren.
** Hypothetische Struktur mit unbekanntem Rest R.

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ZUSAMMENFASSUNG

Der chemische Abbau von DNA aus Heringsspermen zu Purinoligonucleotid- oder Desoxiriboadenylsäuregemischen wird im präparativen Massstab beschrieben.

Die Desoxiriboadenylsäuregemische werden an QAE-Sephadex A-25 bei pH

7.5 säulenchromatographisch in sechs Hauptfraktionen getrennt. Die 1. Fraktion erhält zu 75% Desoxiriboadenosinmonophosphat, die 2. zu 99% eine Mischung aus $p(dA)_2$, $(dA)_2p$ und die 3. zu 93% pdAp. In den darauf folgenden Fraktionen werden mehr oder weniger komplex zusammengesetzte Mischungen längerkettiger Oligonucleotide eluiert, die durch Rechromatographie an QAE-Sephadex bei pH 9.6 weiter aufgetrennt werden. Aus den vorgetrennten Fraktionen werden papierchromatographisch die Nucleotidphosphate pdAp, $p(dA)_2p$, $p(dA)_3p$, $p(dA)_4p$ sowie die Mischungen der Sequenzisomeren $p(dA)_2$, $(dA)_2p$; $p(dA)_3$, $(dA)_3p$ und $p(dA)_6$, $(dA)_6p$ in präparativen Mengen chromatographisch rein isoliert.

Durch Behandlung mit alkalischer Phosphatase werden die Nucleotidphosphate in $(dA)_2$, $(dA)_3$ oder $(dA)_4$ und die Mischungen der Sequenzisomeren in $(dA)_2$, $(dA)_3$ oder $(dA)_6$ überführt. Auf diesem Weg sind auch dephosphorylierte Oligodesoxiriboadenylsäuren in papierchromatographisch reiner Form in präparativen Mengen aus den Hydrolysaten erhältlich.

Die Struktur aller isolierten Desoxiriboadenylsäuren wird aus Absorptionsverhältnissen, R_F-Werten und durch enzymatischen Abbau bewiesen.

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QUANTITATIVE ANALYSE VON FLAVONOIDEN AUF DÜNNSCHICHT-PLATTEN

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SUMMARY

Quantitative analysis of flavonoids on thin-layer plates

Quantitative *in situ* analysis of flavonones, flavonols and flavonolglycosides was studied on thin-layer plates. Absorption and quenching were not satisfactory. Fluorometry was most effective after spraying the flavonoles, flavonones and flavonolglycosides with $Al_2(SO_4)_3$ on thin-layer plates coated with cellulose powder. Flavonoles could be determined also without spraying. The wavelengths of excitation and fluorescence maxima of these substances were determined and the intensity of fluorescence in relation to the number and the position of the hydroxylgroups in the molecules are discussed. The quantitative determination of 0.01 μg for flavonols and of 0.05 μg for flavonones and flavonolglycosides is possible.

EINLEITUNG

Die zunehmenden Erkenntnisse über die Bedeutung der Polyphenole als wichtige Pflanzenbestandteile[1] erfordern neben den qualitativen Trennungen auch mehr quantitative Analysen dieser Verbindungen. Obwohl neuerdings mit Erfolg verschiedene Polyphenole gaschromatographisch getrennt und analysiert wurden[2–5], überwiegen die einfacheren dünnschichtchromatographischen (DC) Trennungen bei weitem. Wenig quantitative Direktanalysen auf DC-Platten wurden jedoch bisher ausgearbeitet[6–8]. Im Rahmen dieser Arbeiten sollten die verschiedenen Möglichkeiten der quantitativen Direktbestimmungen von Polyphenolen auf ihre praktische Anwendbarkeit überprüft werden. Als Kriterien für die Brauchbarkeit einer Methode wurden Fehlerbreite, Empfindlichkeit und Störungsanfälligkeit herangezogen. Ausserdem sollten möglichst viele verschiedenartige Polyphenole auf einer DC-Platte bestimmt werden können. An dieser Stelle wird über die Untersuchungen zur Analyse von Flavonen, Flavonolen und Flavonolglycosiden berichtet.

METHODEN UND GERÄTE

Als Reinsubstanzen wurden die Flavone Chrysin und Apigenin, die Flavonole Kämpferol, Kämpferid, Quercetin und Myricetin und die Flavonolglycoside Quercitrin

und Rutin verwendet. Die Substanzen wurden von der Fa. Roth (Karlsruhe, B.R.D.) bezogen. Ferner wurden Flavonoide aus Blättern von Prunus- und Vaccinium-Arten untersucht.

Folgende DC-Fertigplatten wurden in die Untersuchungen einbezogen: Kieselgel 60, Cellulose F (mit Fluoreszenzindikator) und Cellulose (ohne Fluoreszenzindikator) von Merck (Darmstadt, B.R.D.), MN Cel 400-10 von Macherey, Nagel & Co. (Düren, B.R.D.) und SS Cellulose G 1440 von Schleicher & Schüll (Dassel, B.R.D.)

Zum Auftragen der Substanzen wurden kalibrierte Wegwerfkapillaren von Camag verwendet. Die chromatographischen Trennungen der Polyphenole erfolgten fast ausschliesslich mit Butanol–Bisessig–Wasser (BAW) (5:4:1, v/v; obere Schicht) und Ameisensäure (5%).

Soweit erforderlich erfolgte das Besprühen der Platten zweimal hintereinander nach Zwischentrocknung mit einem Unispray von Camag. Als Sprühlösungen wurden verwendet Naturstoffreagenz A nach Neu[9] in 1%iger methanolischer Lösung und eine 4%ige wässrige Aluminiumsulfatlösung nach Van Buren *et al.*[7].

Die photometrischen Analysen der Polyphenole auf den DC-Platten wurden mit einem Farrand Vis-UV-2-Chromatogram Analyser durchgeführt. Durch die Ausrüstung dieses Scanners mit zwei Gittermonochromatoren, einem Anregungs- und einem Analyser-Monochromator, und zwei Photomultipliern als Proben und Referenzsignalempfänger kann man zwischen 200 und 720 nm Fluoreszenzen und Quenching bei jeder beliebigen Anregungs- und Fluoreszenzwellenlänge und die Absorption bei jeder Wellenlänge im Zweistrahlverfahren als Differenzsignal zwischen Substanz und Untergrund messen. Auch die Aufnahme von Absorptions-, Anregungs- und Fluoreszenzspektren der zu untersuchenden Verbindungen ist möglich, indem man hintereinander auf dem Plattenuntergrund und auf der Substanz + Plattenuntergrund den gewünschten Spektralbereich automatisch durchfährt. Das Differenzspektrum aus beiden so erhaltenen Spektren ergibt das der reinen Substanz (vgl. Fig. 1, 3–5).

Die quantitativen Analysen wurden meist an eindimensional getrennten Proben

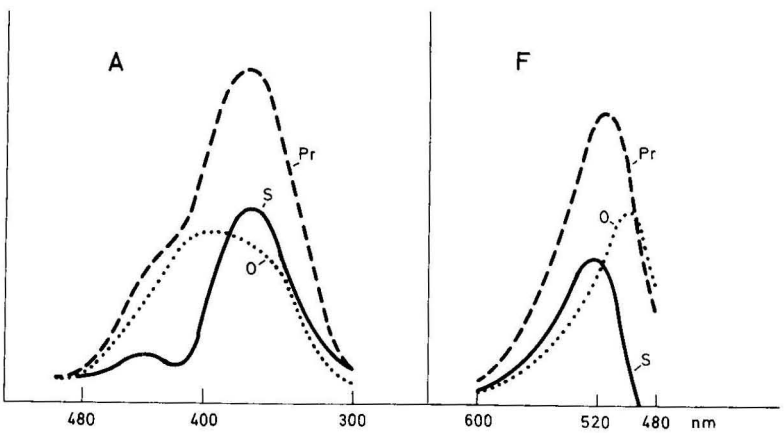


Fig. 1. Quercetin (ohne Besprühen). O, Spektren der Cellulose allein; Pr, Spektren der Probe + Celluloseuntergrund; S, durch Subtraktion gewonnenes Spektrum der Substanz allein. (A), Anregungsspektren, Fluoreszenzwellenlänge konstant, 520 nm. (F) Fluoreszenzspektren, Anregungswellenlänge konstant, 370 nm.

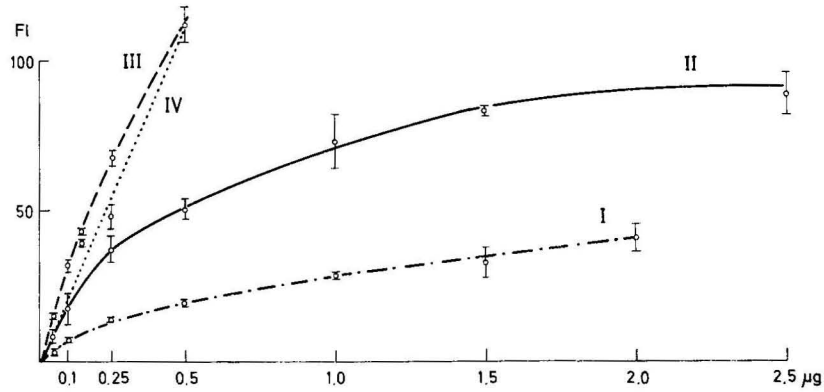


Fig. 2. Quercetin-Eichkurven. I, Absorptionsmessungen; II, Fluoreszenzmessungen (ohne Besprühen); III, Fluoreszenzmessungen nach Besprühen mit Aluminiumsulfat; IV, Fluoreszenzmessungen nach Besprühen mit Naturstoff Reagenz A.

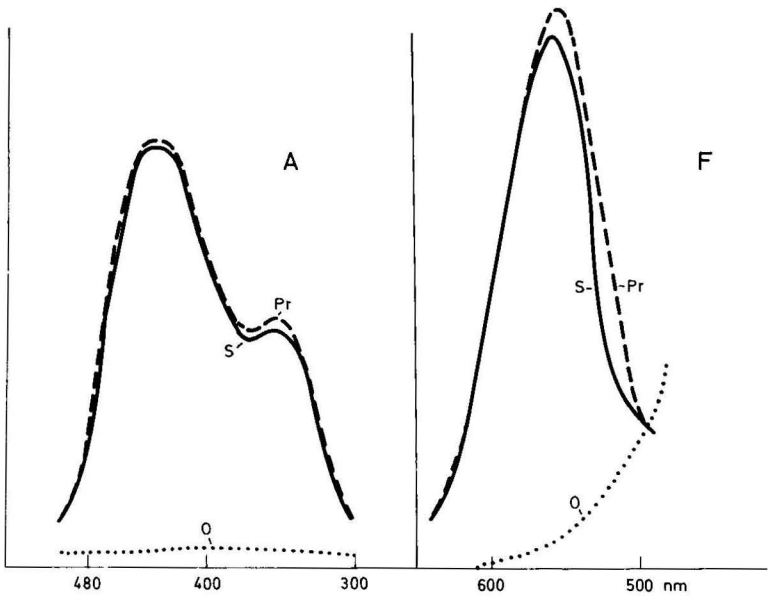


Fig. 3. Quercetin + Naturstoff Reagenz A. A, F, O, Pr und S vergl. Fig. 1. Fluoreszenz bei 560 nm, Anregung bei 370 nm.

vorgenommen. Soweit zweidimensionale Entwicklungen analysiert werden mussten, wurde die Platte auf dem mobilen Chromatogrammträger des Scanners so ausgerichtet, dass die zu messenden Flecken genau in der Fortbewegungsrichtung des Scannertisches lagen. Sind mehrere Substanzen in verschiedenen Richtungen auf einer Platte verteilt, werden jeweils Neujustierungen notwendig.

ERGEBNISSE UND DISKUSSION

DC-Platten

Die im methodischen Teil angeführten Flavonoide wurden verwendet, um

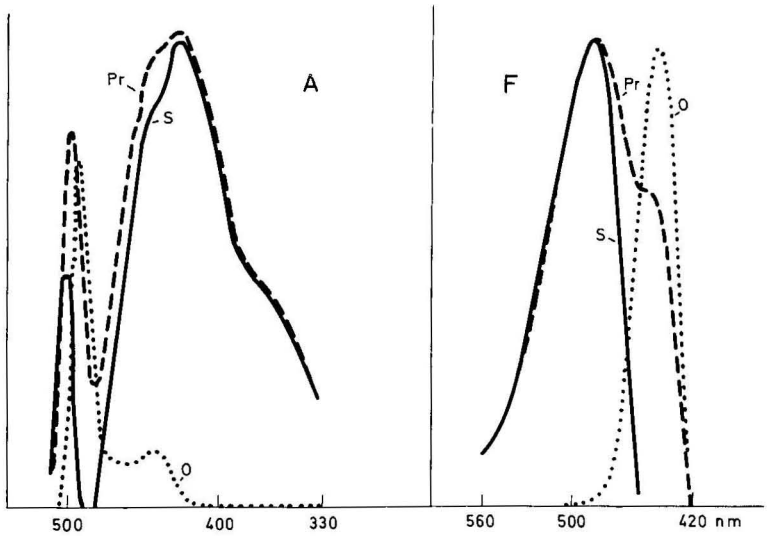


Fig. 4. Quercetin + Aluminiumsulfat. A, F, O, Pr und S vergl. Fig. 1. Fluoreszenz bei 485 nm, Anregung bei 420 nm.

nach Möglichkeit einen Einblick zu gewinnen, inwieweit Zahl und Stellung der Hydroxylgruppen und eine Glycosidierung in 3-Stellung das optische Verhalten dieser Verbindungen auf DC-Platten beeinflussen. Ein visueller Vergleich der sowohl auf Cellulose als auch auf Kieselgel aufgetragenen Flavonoide zeigte bereits, dass alle auf Kieselgel aufgetragenen Substanzen (je 0.5 μg) unter dem langwelligen UV-Licht nur schwach zu erkennen waren und nicht fluoreszierten. Dagegen erschienen auf Cellulose die Flavonolglycoside als deutlich dunkel blauviolette Flecken und die Flavonole fluoreszierten gelb. Nach dem Besprühen beider Platten-Arten mit Aluminiumsulfat wurden die in Tabelle I zusammengestellten Farben der einzelnen Flavonoide gefunden. Man sieht, dass auf Cellulose im nahen UV-Licht alle Flavonoide fluoreszieren, während auf Kieselgel nur die Aglycone schwache Fluoreszenz zeigen. Da zudem auf Cellulose für die in Frage kommenden Polyphenole gute qualitative Trennungsergebnisse vorlagen[10–13], erhielten diese Schichten später den Vorzug.

TABELLE I

FARBEN VON FLAVONOIDEN AUF CELLULOSE- UND KIESELGEL-PLATTEN NACH DEM BESPRÜHEN MIT ALUMINIUMSULFAT

l. = leicht; i. = intensiv; fl. = fluoreszierend.

Substanz (je 0.5 µg)	*Cellulose*		*Kieselgel*	
	Im sichtbaren Licht	*Unter UV-Licht*	*Im sichtbaren Licht*	*Unter UV-Licht*
Apigenin	l. gelb	gelb-grün, fl.	l. gelb	gelb
Kämpferid	l. gelb	l. grün, fl.		—
Kämpferol	gelb-grün	i. grün, fl.	gelb	grün, fl.
Quercetin	gelb-grün	i. grün, fl.	gelb-braun	gelb-grün, fl.
Myricetin	gelb-grün	i. grün, fl.	gelb-braun	gelb, fl.
Quercitrin	l. gelb	gelb-grün, fl.	l. gelb	l. gelb
Rutin	l. gelb	gelb-grün, fl.	l. gelb	l. gelb

Die Cellulose-Platten verschiedener Herkunft wiesen zwar keine prinzipiellen, doch teilweise graduelle Unterschiede auf. So zeigte sich, dass die Absorption und Fluoreszenz der Cellulose innerhalb einer Platte in einigen Fällen bis zu 100% differierten. Schwankungen dieser Grössenordnungen wirken sich besonders bei Fluoreszenzmessungen auf die Genauigkeit der Ergebnisse aus, wenn das Fluoreszenzmaximum der Substanz von den Ausläufern der Eigenfluoreszenzbande der Cellulose stark unterlagert wird (vgl. Fig. 1). Als relativ konstant erwiesen sich bisher die Cellulose G 1440-Platten.

Absorptionsmessungen

Von allen angegebenen Flavonoid-Vergleichsubstanzen wurden die Absorptionsspektren sowohl auf Cellulose- als auch auf Kieselgel-Platten gemessen. Die ersten Absorptionsmaxima der Flavonoide auf Cellulose-Platten sind in Tabelle II aufgeführt. Zum Vergleich sind auch die von Mabry *et al.*[14] in Methanollösung bestimmten Absorptionsmaxima angegeben. Man sieht, dass bei allen Flavonoiden das Maximum auf Cellulose gegenüber dem in methanolischer Lösung bathochrom verschoben ist. Die Verschiebung ist um so stärker, je geringer die Zahl der Hydroxylgruppen ist. Wie man aus der sehr flachverlaufenden Eichkurve des Quercetins in Fig. 2 (Kurve I) sieht, ist die Empfindlichkeit der Methode jedoch gering. Auch die Unterschiede in der Empfindlichkeit einzelner Flavonoide sind nicht gross, da sich die Extinktions-

TABELLE II

ABSORPTIONS-, ANREGUNGS- UND FLUORESZENZ-MAXIMA VON FLAVONOIDEN AUF CELLULOSE-PLATTEN

AS = Aluminiumsulfat; RNA = Naturstoff Reagenz A; S = Schulter.

Substanz	*Absorptionsmax. (nm)*	*Absorptionsmax. in Methanollösung (lit. 14)*	*Anregungsmax. (nm)*	*Fluoreszenzmax. (nm)*
Chrysin	335	313	—	—
+ AS		380	—	—
Apigenin	350	336	—	—
+ AS		384	390	500
Kämpferid	370	367		560
+ AS		423	*	*
+ NRA			375 (420)	480 (560)
Kämpferol	400	367	367	530
+ AS		424	(360) 420	475
+ NRA			(380) 425	510
Quercetin	380	370	368 (440)	524
+ AS		458	(366S) 422 (440S)	488
+ NRA			(365) 435	558
Myricetin	375	374	375 (450)	530
+ AS		450	(370S) 420	485
+ NRA			(358) 450	550
Quercitrin	368	350	—	—
+ AS		430	400 (428)	495
+ NRA			(350) 440	525
Rutin	375	359	—	—
+ AS		433	405	500

* Nicht gemessen.

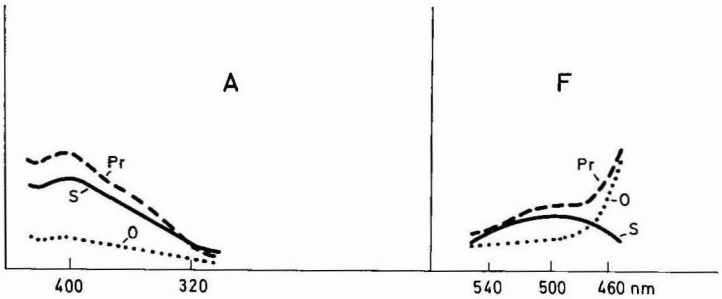


Fig. 5. Quercetin + Aluminiumsulfat. A, F, O, Pr und S vergl. Fig. 1. Fluoreszenz bei 490 nm, Anregung bei 400 nm.

module dieser Verbindungen nicht wesentlich unterscheiden. Für quantitative Analysen dieser Stoffklasse sind daher direkte Absorptionsmessungen schlecht geeignet.

Quenching

Beim Quenching wird bekanntlich die Fluoreszenz des der Platte zugesetzten Leuchtstoffes durch die Substanzen, entsprechend ihren Konzentrationen, mehr oder weniger stark gelöscht. Das Anregungsmaximum der verwendeten Cellulose F-Platten wurde bei 272 nm und das Fluoreszenzmaximum bei 522 nm festgestellt. Leider lässt die Homogenität der Fluoreszenzintensität innerhalb der Platten sehr zu wünschen übrig. Die Nachweisgrenze der einzelnen Flavonoide steigt dadurch auf $\geqslant 0.1$ μg an und eine quantitative Analyse ist erst $\geqslant 0.5$ μg möglich. Die Schwankungsbreiten der Einzelwerte sind noch grösser als bei entsprechenden Absorptionsmessungen. Wie eingehende Messungen zeigten, liegen in diesen Wellenlängenbereichen jedoch keine Beeinflussungen der Ergebnisse durch Eigenfluoreszenz der Flavonoide vor. Die von Pollak[15] für Quenching beschriebenen Schwierigkeiten haben sich bestätigt. Für weitere quantitative Analysen der Flavonoide wurde Quenching nicht mehr berücksichtigt.

Fluoreszenzmessungen

Direkte fluorimetrische Methode. Die Flavonole zeigen, im Gegensatz zu den Flavonolglycosiden und Flavonen, im langwelligen UV-Licht auf Cellulose auch ohne Besprühen mit Reagenzien leichte Gelbfluoreszenz. Durch die Aufnahme der Anregungs- und Fluoreszenzspektren —in Fig. 1 stehen stellvertretend die des Quercetins— wurden die in Tabelle II angegebenen Anregungs- und Fluoreszenzmaxima ermittelt. Aus der Fig. 1 geht hervor, dass das Quercetin bei 368 nm ein intensives und bei 440 nm ein kleines Anregungsmaximum hat. Ein kleines Fluoreszenzmaximum liegt bei 524 nm. Zwar ist bei 430 nm ein weit grösseres, nicht mehr eingezeichnetes Maximum vorhanden, die Substanzfluoreszenz bei einer aufgetragenen Menge von 0.5 μg beträgt hier jedoch nur *ca.* 6% der sehr starken Eigenfluoreszenz der Cellulose. Da bereits kleine Änderungen des Plattenuntergrundes den Fehler der Quercetinbestimmung um ein Vielfaches verstärken, ist dieser Fluoreszenzbereich für quantitative Analysen nicht geeignet. Dem Fluoreszenzmaximum des Quercetins bei 524 nm sind nur noch *ca.* 30% Cellulose-Eigenfluoreszenz unterlagert. Die Basislinie bleibt trotz nötiger grösserer optischer und elektronischer Verstärkung ruhiger und dadurch wird

die Empfindlichkeit grösser und die Nachweisgrenze der Verbindung niedriger als vergleichsweise bei 430 nm. Maximale Anregung und Fluoreszenz ist also nicht in jedem Falle mit optimaler Nachweismöglichkeit identisch. In Fig. 2 (Kurve II) ist als Beispiel die Eichkurve des Quercetins bei Anregung 368 nm und Fluoreszenz 524 nm wiedergegeben. Wie man sieht, bleibt die Fluoreszenz bei Substanzmengen grösser als 1.5 μg nahezu konstant. Das optische Verhalten der übrigen untersuchten Flavonole ist, mit Ausnahme kleiner Verschiebungen der Maxima (vgl. Tabelle II), dem des Quercetins sehr ähnlich.

Bei Flavononen und Flavonolglycosiden konnte unter diesen Bedingungen keine Fluoreszenz nachgewiesen werden. Auch für Flavonole ist diese Methode zur quantitativen Analyse nur mit Einschränkungen zu empfehlen.

Fluoreszenzmessungen nach dem Besprühen mit Naturstoffreagenz A nach Neu. Unmittelbar nach dem Besprühen mit Naturstoffreagenz A (Lit. 9) und Trocknen der Platte fluoreszieren auch die Flavone und Flavonolglycoside deutlich gelb. Die Fluoreszenz der Flavonole verstärkt sich erheblich. In der Fig. 3 sieht man am Beispiel des Quercetins, dass die Anregungs- und Fluoreszenzmaxima von der Eigenfluoreszenz der Cellulose-Platte praktisch nicht mehr beeinflusst werden. Die in Tabelle II zusammengestellten Anregungs- und Fluoreszenzmaxima aller untersuchter Flavonoide liegen in den gleichen Wellenlängenbereichen. Der hohen Empfindlichkeit dieser Methode, zu ersehen aus der Eichkurve des Quercetins in Fig. 2 (Kurve IV) steht leider die geringe Beständigkeit der Fluoreszenz entgegen. So haben zeitabhängige Intensitätsmessungen ergeben, dass die Fluoreszenz innerhalb von 7 h unregelmässig bis zur Hälfte absinken kann. Dadurch ist auch diese Methode für Serienmessungen schlecht geeignet.

Fluoreszenzmessungen nach dem Besprühen mit Aluminiumsulfat. Die vollen Fluoreszenzintensitäten der mit Aluminiumsulfat behandelten Flavonoide wird erst nach einigem Stehen der behandelten Platten, am besten über Nacht, erreicht, ist aber dann lange Zeit beständig. Stellvertretend für die aufgenommenen Anregungs- und Fluoreszenzspektren auch der übrigen Verbindungen sind in Fig. 4 die des Quercetins und in Fig. 5 die des Quercitrins wiedergegeben. Die entsprechenden Anregungs- und Fluoreszenzmaxima sind in Tabelle II zu finden. Die Hauptanregungsbanden der mit Aluminiumsalz behandelten Flavonole sind bereits als schwache Vorbanden ohne Besprühen vorhanden, während umgekehrt die ursprünglichen Hauptbanden nach der Behandlung zu untergeordneten Nebenbanden oder Bandenschultern reduziert werden (vgl. Fig. 1 und 4). Ähnliche Erscheinungen waren auch nach der Behandlung der Flavonole mit Naturstoffreagenz A zu beobachten. Die Anregungsmaxima der mit Aluminiumsalz besprühten Glycone liegen im Schnitt *ca.* 20 nm weiter im kürzeren Wellenlängenbereich als die der Aglycone. Die Fluoreszenzmaxima der mit Aluminiumsalz behandelten Verbindungen liegen zwischen 480 und 500 nm und sind gegenüber denen der nicht bzw. mit Naturstoffreagenz A besprühten Flavonolen 30–40 nm hypsochrom verschoben. Durch die langwellige Verschiebung der Anregungsmaxima nach der Behandlung der Flavonoide mit Aluminiumsulfat wird die Fluoreszenzanregung der Cellulose und damit die Untergrundfluoreszenz der Platten stark unterdrückt. Damit wird wiederum die Empfindlichkeit und die Nachweisgrenze der Methode stark erhöht. Wie die in Fig. 2 dargestellte Eichkurve III für Quercetin zeigt, ist die Fluoreszenz bis 0.5 μg nahezu linear der Substanzkonzentration. Bei höheren Konzentrationen neigt sich auch diese Kurve langsam zur

x-Achse. Der durchschnittliche Fehler beträgt beim Nachweis von 0.05 μg Quercetin $\pm 3.0\%$. Bei entsprechender optischer und elektronischer Verstärkung des Signals ist eine quantitative Analyse bis 0.01 μg, ein qualitativer Nachweis bis 0.005 μg möglich. Die übrigen Flavonole verhalten sich ähnlich. Die Nachweisgrenze der Flavonolglycoside und Flavone liegen um *ca.* Faktor 5 höher.

Mit dieser Methode wurden auch Reihenuntersuchungen von Flavonolglycosiden aus *Prunus avium* L., *P. cerasus* L., und *Vaccinium vitis idea* L. bestimmt. Die Anregung erfolgte bei 400 nm und die Fluoreszenz wurde bei 490 nm gemessen. Der mittlere Fehler, einschliesslich des Auftragfehlers, betrug bei den *Prunus*-Arten $\pm 2.3\%$ und bei *V. vitis idea* $\pm 3.1\%$. Durch eine verbesserte, automatische Auftragemethode lässt sich dieser Fehler sicher noch senken.

Fluoreszenz der Flavonoide in Abhängigkeit von Zahl und Stellung der Hydroxylgruppen

Zahl und Stellung der Hydroxylgruppen zueinander und zur Ketogruppe im heterocyclischen Ring beeinflussen bei Zusatz von Aluminiumsalzen zu Flavonoid-Lösungen vor allem die bathochromen Verschiebungen der Absorptionsmaxima[14,16]. Durch Besprühen der Substanzen auf Celluloseplatten mit Aluminiumsulfat werden dagegen vor allem die Fluoreszenzintensitäten unterschiedlich verändert. Um quantitative Vergleiche anstellen zu können, wurde mit 0.5 μg Myricitin auf Celluloseplatten bei einer Anregungswellenlänge von 420 nm und einer Fluoreszenzwellenlänge von 490 nm der Scanner-Abgleich vorgenommen und alle Flavonoide in derselben Menge und bei den gleichen Wellenlängen in Relation gemessen. In Tabelle III sind die erhaltenen Intensitäten als Flächenintegrale angegeben. Diese Werte wurden mit den angeführten Molekulargewichten linear auf Molekularbasis umgerechnet. Da nicht bei allen Flavonoiden die Anregungs- und Fluoreszenzmaxima genau bei 420

TABELLE III

RELATIVE FLUORESZENZ-INTENSITÄT VON FLAVONOIDEN AUF CELLULOSE-DC-PLATTEN NACH DEM BESPRÜHEN MIT $Al_2(SO_4)_3$

Anregung bei 420 nm, Fluoreszenz bei 490 nm.

Substanz	*Flächenintegral pro 0.5 µg Substanz (cm²)*	*Molekular-gewicht*	*Flächenintegral pro 10^{-9} Mol (cm²)**	
			Nicht korrigiert	*Korrigiert*
Apigenin (4′,5,7-Trihydroxyflavon)	33.5 (300 V)	270.23	6.04 (100 V)	11.0
Kämpferid (3,5,7-Trihydroxy-4′-methoxyflavon)	58.0 (300 V)	300.27	11.61 (100 V)	
Kämpferol (3,4′,5,7-Tetrahydroxyflavon)	117.5 (100 V)	286.24	67.27 (100 V)	
Quercetin (3,3′,4′,5,7-Pentahydroxyflavon)	106.0 (100 V)	302.24	64.08 (100 V)	
Myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavon)	101.0 (100 V)	318.24	64.28 (100 V)	
Quercitrin (Quercitin-3-rhamnosid)	24.5 (300 V)	482.42	7.88 (100 V)	8.7
Rutin (Quercitin-3-rutinosid)	24.0 (300 V)	664.58	10.63 (100 V)	11.9

* Die linearproportionale elektronische Verstärkung ist in Klammern angegeben.

nm bzw. 490 nm liegen, war es teilweise notwendig auf die Fluoreszenzintensitäten an den Maxima mit Hilfe der aufgenommenen Spektren zu korrigieren. Bei Flavonolen ist der ohne Korrektur entstehende Fehler vernachlässigbar klein. Bei Flavonolglycosiden beträgt er *ca.* 10% und bei Apigenin sogar *ca.* 80%.

Zusammenfassend lässt sich über die Beeinflussung der Fluoreszenz von Flavonoiden durch Aluminiumsulfat folgendes sagen: Fehlen Hydroxylgruppen sowohl in 3- als auch in 4'-Stellung (Chrysin), so ist keine Fluoreszenz feststellbar. Durch eine OH-Gruppe in 3- oder 4'-Stellung scheint die Fluoreszenzintensität gleich stark beeinflusst zu werden. Die Methoxylgruppe in 4'-Stellung (Kämpferid) trägt anscheinend, im Gegensatz zur freien Hydroxylgruppe (Kämpferol), wenig zur Steigerung der Intensität bei. Zusätzliche Hydroxylgruppen in 3'- und 5'-Stellung zur 4'-OH-Gruppe bringen weder stärkere spektrale Verschiebungen noch Fluoreszenzsteigerungen. Durch Glycosidierung der 3-Hydroxylgruppe werden die Hauptanregungsmaxima nach kürzeren und die Fluoreszenzmaxima nach längeren Wellenlängen verschoben und die Fluoreszenzintensität stark vermindert. Die Daten kommen denen des Flavonons Apigenin nahe, das keine 3-Hydroxylgruppe besitzt. Die Glycosidierung der 3-OH-Gruppe wirkt sich ähnlich fluoreszenzmindernd aus wie eine Veresterung der 4'-Hydroxylgruppe in Kämpferid.

ZUSAMMENFASSUNG

Die direkte quantitative Analyse von Flavonen, Flavonolen und Flavonolglycosiden auf Dünnschichtplatten wurde untersucht. Absorptionsmessungen und Quenching erwiesen sich als nicht befriedigend. Am besten eignen sich Fluoreszenzmessungen auf Celluloseplatten, die bei Flavonolen bereits ohne weitere Behandlungen, bei den meisten Flavononen und Flavonolglycosiden am besten nach besprühen mit $Al_2(SO_4)_3$ vorgenommen werden können. Die Wellenlänge der Anregungs- und Fluoreszenzmaxima wurden bestimmt und die Fluoreszenzintensitäten in Abhängigkeit von der Zahl und Stellung der Hydroxylgruppen im Molekül diskutiert. Der quantitative Nachweis ist bei Flavonolen bis 0.01 μg, bei Flavonen und Flavonolglycosiden bis 0.05 μg möglich.

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THIN-LAYER GEL CHROMATOGRAPHY OF PROTEINS IN MILD AND DENATURING DETERGENTS

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SUMMARY

Thin-layer gel chromatography of proteins in the presence of the mild detergents, sodium deoxycholate and Triton X-100, or in the presence of a denaturing detergent, sodium dodecyl sulfate, is described in terms of application to (glyco)-proteins of membrane origin. In these detergent systems, a nearly linear relationship was observed between the value of the distribution coefficient of a protein and the protein's equivalent hydrodynamic radius. The fractionation properties of the gel employed in this investigation appeared to be different in Triton X-100 than in the other two detergent systems. The fixation and replica-staining of glycoproteins separated in the presence of detergent is also discussed.

INTRODUCTION

Thin-layer gel chromatography (TLG)[1,2] has found ready application for the rapid, inexpensive separation and/or size estimation of microgram quantities of water-soluble proteins[3–5]. Recently, this method was adapted for the molecular weight estimations of polypeptide chains in random coil-producing solvents[6].

The study of membrane proteins and glycoproteins is complicated by the fact that most integral membrane proteins are insoluble in dilute, aqueous buffers and must be solubilized by the presence of a detergent in the aqueous solvent. Additionally, microgram quantities of material are routinely encountered in membrane protein research, and these protein quantities are eminently suited to TLG applications. Before its possible application to any membrane protein systems, TLG of some common hydrophilic proteins in mild or denaturing detergents was examined in our laboratory to document the specific method and to determine the hydrodynamic sizes to which the method might be applicable. The results of this investigation are reported herein.

EXPERIMENTAL

Materials

Sephadex G-200 with a particle size of 10–40 μm was obtained from Sigma (St. Louis, Mo., U.S.A.).

α_1-acid glycoprotein (human glycoprotein fraction VI), human globulin fraction II (IgG), and ovalbumin were obtained from Miles Labs (Elkhart, Ind., U.S.A.). Chymotrypsinogen-A and ovomucoid were obtained from Worthington Biochemicals (Freehold, N.J., U.S.A.). Dinitrophenyl-bovine serum albumin (DNP-BSA) was prepared by the method of Little and Eisen[7]. The nominal DNP-content of the preparation as estimated spectrophotometrically was 16.5 moles/mole of BSA. Myosin was prepared by the method of Perry[8].

Sodium dodecyl sulfate (SDS), specially pure, was obtained from BDH (Poole, Great Britain); all other chemicals were reagent grade or the best commercially available.

Methods

Three solvent systems were employed; each contained a detergent with properties rather distinct from the other two. These solvent systems represented a denaturing detergent solvent system[9], SDS, a non-ionic, mild detergent solvent system[10], Triton X-100, and a mild anionic detergent solvent system[11], sodium deoxycholate (DOC). The compositions of the three solvent systems employed are given in Table I.

TABLE I

DETERGENT SOLVENT SYSTEMS

Detergent	*Running buffer*	*Sample preparation buffers*
SDS	0.04 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.1% SDS	0.04 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 10.0% SDS 0.13 *M* β-Mercaptoethanol
Triton X-100	0.025 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.05% Triton X-100	0.025 *M* Sodium phosphate, pH 7.2 0.02% NaN_3 0.1% Triton X-100
Deoxycholate	0.025 *M* Carbonate, pH 9.7 (I = 0.1) 0.02% NaN_3 0.01 *M* DOC	0.025 *M* Sodium carbonate, pH 9.7 0.02% NaN_3 0.02 *M* Sodium deoxycholate

TLG was performed in a gel bed 0.8 mm thick which was spread on a 20 × 30 cm heavy glass plate. The gel filtration slurry was prepared by the addition of 6 g of Sephadex G-200 to 150 ml of the appropriate detergent solvent system. This was sufficient Sephadex slurry to prepare two plates. Upon addition of the Sephadex to the solvent, the flask was gently swirled or vortexed for a length of time sufficient to insure complete mixing. The Sephadex gel was then allowed to hydrate for at least 24 h before pouring the plates. A swelling time of 72 h, as recommended by the manufacturer, produced no improvement in the protein zone resolution. One side of a glass plate was marked with a line which was 3.5 cm from a 20-cm edge and parallel to it; this line served as a guide for sample application. Routinely, seven or eight samples were applied at equally spaced intervals along the line.

Immediately before pouring a plate, the Sephadex slurry was gently vortexed and deaerated to remove or bring to the top of the slurry any recalcitrant air bubbles;

these bubbles were then removed by gentle aspiration. Once all bubbles were removed, the slurry was gently swirled to produce an even suspension, and about 60 ml of the slurry were poured across the same end of the plate as the inscribed line but on the opposite side. The gel bed was spread to a thickness of 0.8 mm with a commercial TLG-spreader; in our hands, the TLG-spreader purchased from Pharmacia (Piscataway, N.J., U.S.A.) yielded the best results. The spreader was placed parallel to the narrow edge of the plate and moved slowly towards the opposite end, pushing a ridge of Sephadex before it. A second line of Sephadex could be added to the plate if more were needed. However, if the spreader was stopped and restarted, a slight discontinuity in the gel was apparent at that point. Bubbles which were occasionally present and which adhered to the trailing edge of the spreader produced thinning or channels in the gel bed. This situation resulted in a discontinuity of solvent flow rates across the width of the gel bed.

Experiments were performed in a Plexi-glass chamber (40 × 28 × 18 cm) similar to that described by Morris[3]. Whatman No. 3MM chromatography paper (22 × 7 cm) soaked in the appropriate solvent served as the wicks between the gel bed and the buffer reservoirs. The eluent was allowed to percolate through the gel overnight to equilibrate the plate prior to sample application. A plate incline of 15 degrees was routinely employed and produced an eluent flow-rate sufficient to move the void volume marker about three-fourths the length of the plate in 5 h.

Protein sample solutions contained about 10 mg/ml of each protein species in the detergent system which was under investigation. When the SDS solvent system was employed, the SDS–protein complexes were formed by heating the proteins in the sample preparation buffer at 100° for 10 min[12]. Myosin was employed as the void volume marker and dinitrophenyl glycine (DNP-Gly) as the internal volume marker in the SDS system. DNP-BSA or BSA could equally serve as void volume markers in SDS because each possessed the same TLG mobility as myosin. DNP-BSA and DNP-Gly served as void volume and internal volume markers, respectively, in the DOC and Triton X-100 systems. Although the hydrodynamic radius of native BSA is sufficiently small to be partially included in the G-200 pore volume, DNP-BSA behaved hydrodynamically as though it were much larger (see the Results and discussion section for an explanation) and migrated at the same rate in G-200 by TLG as myosin.

A sample volume of up to 5 μl could be applied to the gel bed without noticeable zone spreading. We routinely applied 2.5 μl loads (25 μg of each protein species in the sample), as this volume yielded the best resolution in our hands. The experimental run was stopped when the void volume marker was approximately 3 cm from the distal end of the plate.

Proteins and/or glycoproteins were detected by using the technique of replica-staining[5] on Whatman No. 3MM chromatography paper. The plate was removed from the chromatography chamber and the Whatman No. 3MM paper, cut to the dimensions of the gel bed, was carefully layered (rolled) onto the surface of the gel bed and equilibrated for 5 min. The paper was carefully removed from the gel surface and dried for 30 min at 80°. At this point, the DNP-Gly and DNP-BSA zones were visible and were circled in pencil for reference. Staining for protein was performed in Coomassie Brilliant Blue (0.25% in methanol–acetic acid, 9:1, v/v) for 5 min, the replica was rinsed in distilled water and destained in methanol–acetic acid–water

(5:1:5, v/v) for 15 min. The paper was dried and the protein migration distances measured.

Fixation of glycoproteins prior to glycoprotein-specific staining was accomplished by a slightly different fixation procedure. The Whatman paper was carefully removed from the gel surface and was washed with three successive changes of phosphotungstic acid (10%, w/v in 2 *N* hydrochloric acid). This treatment not only fixed glycoproteins some of which are soluble in trichloroacetic acid[13], but also removed the Sephadex beads and detergent from the chromatogram which significantly reduced its background color after glycoprotein staining. The chromatogram was continuously agitated in the third phosphotungstic acid wash for 5 min or until the Sephadex beads no longer adhered to the paper and the wash solution lost its "soapy" appearance. The phosphotungstic acid fixative was removed from the replica by agitation in three successive washes, the first two of which were distilled/deionized water, and the last 95% ethanol. The chromatogram was then air dried. Periodate oxidation–fuschin Schiff base staining, a glycoprotein-specific stain, was performed according to the procedure of McGuckin and McKenzie[14]. Steps 1, 3 and 7 from that procedure were performed in the dark.

Analyses of the data

TLG migration distances of the proteins were expressed either (a) relative to the migration distance of one of the proteins[3,5], (b) relative to the migration distance of the void volume marker[6], (c) as the distribution coefficient, K_t, defined by Klaus *et al.*[6], or (d) as the TLG distribution coefficient, K_d, defined by Ackers[15].

The molecular weights and equivalent hydrodynamic radii (R_e) of the reduced polypeptides complexed with SDS and chromatographed in the presence of SDS were taken from refs. 12 and 16. The R_e values for most of the native proteins in each of the two mild detergent systems were calculated from literature values for the intrinsic viscosity or the sedimentation coefficient of the native protein in dilute buffer[12,16].

The equivalent hydrodynamic radii of BSA and IgG in each of the mild detergents were experimentally estimated from sedimentation velocity measurements. BSA exhibited an $s^0_{20,w}$ of 4.3 S in each of the mild detergent solvent systems as well as in dilute buffer. In Triton X-100, however, the sedimentation coefficient exhibited a slight positive dependence on BSA concentration; this suggests a rapidly associating–dissociating system. IgG exhibited an $s^0_{20,w}$ of 6.9 S in the DOC solvent system and $s^0_{20,w}$ of 7.2 S $\pm$ 0.1 S in the Triton X-100 solvent system. These sedimentation coefficients yielded values for R_e of 50 Å and 48 Å, respectively.

RESULTS AND DISCUSSION

The fractionation properties of the chromatographic support medium, G-200, was compared among the three solvent systems. This was made possible by expressing the sizes of the proteins and polypeptides utilized in the investigation in terms of an equivalent hydrodynamic radius[9]. The equivalent hydrodynamic radius, R_e, takes into account the intrinsic native conformational and the detergent-induced conformational differences among the proteins and thus permits a comparison among solvents[9]. The equivalent hydrodynamic radius of a protein (polypeptide) is plotted

versus the error function compliment of the protein's K_d value; this is known as the Ackers equation[17]. That is,

$$R_e = A + B \operatorname{erf}^{-1}(1 - K_d),$$

where A and B are constants for a specific gel chromatography support medium in a given solvent system. Differences in the properties of a support medium between two different solvent systems are manifested by a change in the slope and/or intercept of the data plotted according to the Ackers equation[9]. Protein migration rates in the three detergent solvent systems, plotted according to the aforementioned equation, are presented in Fig. 1.

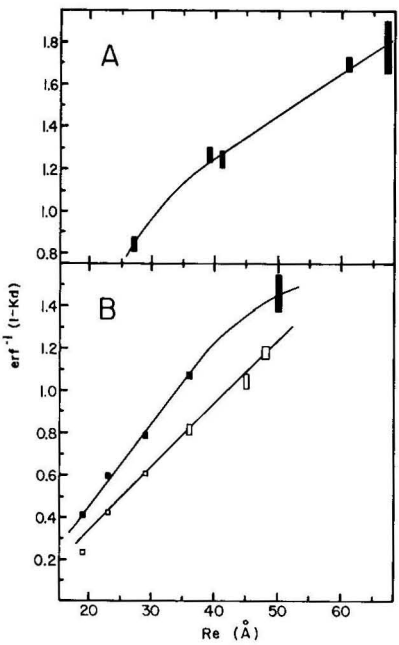


Fig. 1. Thin-layer gel chromatography in the presence of detergents. Data are presented according to the relationship given by Ackers[17]. A, Reduced polypeptides in the presence of SDS. The polypeptides and their respective hydrodynamic radii are: IgG heavy chain, 67 Å; ovalbumin, 61 Å; chymotrypsinigen A, 41 Å; IgG light chain, 39 Å and lysozyme, 27 Å. B, Proteins in the presence of Triton X-100 (□) or in the presence of sodium deoxycholate (■). The proteins and their respective hydrodynamic radii are: IgG, 50 Å in DOC and 48 Å in Triton X-100; aldolase, 45 Å; BSA, 36 Å; ovalbumin, 29 Å; chymotrypsinogen A, 23 Å and ribonuclease A, 19 Å.

A comparison of the data among the three detergent systems strongly suggests that the size discriminatory properties of G-200 do not remain constant among the systems. The data for polypeptides in the SDS and DOC systems would appear to all lie on the same biphasic curve; the solutes of $R_e < \approx 30$ Å form a linear segment of slope greater than that linear segment formed by solutes of $R_e > 30$ Å. The Triton X-100 system exhibits fractionation properties which are distinctly different from other

two detergent systems. A solute of given R_e will be retarded to a greater extent by the G-200 in the Triton X-100 system than in the other two detergent systems which we examined. For example, K_d for a solute of $R_e = 40$ Å is 0.18 in Triton X-100 and 0.08 in DOC or SDS. These results are somewhat puzzling in view of the fact that column gel chromatography has suggested that agarose gels possess fractionation properties which remain identical in dilute buffers, 6 *M* guanidinium chloride, and a number of detergent systems[9,16,18]. It was, however, recently inferred that the fractionation characteristics of gel chromatographic support media may be affected by the presence of some detergents[18]. Thus, it is to be expected that K_d for a given polypeptide on a given gel chromatographic support medium may vary from one detergent system to the next. This occurs because the detergent affects either the protein's conformation or the chromatographic support medium's fractionation properties, or both.

The data of Fig. 1 also demonstrate the fractionation range of the G-200 in the detergent systems. As can be seen, the upper limit of the fractionation range is an R_e of about 70 Å; this corresponds to a polypeptide of molecular weight 50,000 complexed with SDS in the usual fashion or a globular protein of about 500,000 molecular weight*. This limit may be compared to an upper limit for R_e of about 85 Å inferred from the data for Sephadex G-200 in 6 *M* Gdm·Cl[6]. Resolution of components in each of the three detergent solvent systems was comparable to the resolution reported for TLG in dilute buffers[3,5]; components with equivalent hydrodynamic radii of 65, 40, 30, 23 and 19 Å could be resolved from one another when initially applied to the gel as a mixture.

As expected from previous observations by column gel chromatography of reduced polypeptides in SDS[9,19], plots of the logarithm of polypeptide molecular weight *versus* any one of a number of functions of TLG migration distance (see Experimental) were relatively linear. Thus, though perhaps not quite as precise, TLG in SDS may substitute for column gel chromatography in SDS as an empirical molecular weight estimation method when only microgram quantities of protein are available. The apparent molecular weight given by TLG in SDS for a polypeptide of unknown molecular weight was within two percent of the value estimated by column gel chromatography in SDS and within ten percent of the molecular weight estimated by sedimentation equilibrium.

In our hands, the procedural aspects, the sensitivity, and the resolution of TLG in the presence of detergent was quite similar to that in dilute buffers. With the replica-staining technique which we employed for zone detection on TLG, about 10 μg of protein in the 2.5 μl sample application volume were required for a well-defined spot. About 25 μg appeared to be the optimal quantity of protein, and it became increasingly more difficult to measure precisely migration distances with sample quantities in excess of this amount. For the detection of glycoprotein-associated carbohydrate, about 12 μg of carbohydrate represented the lower limit of detection. Additionally, as much as 100 μg of a carbohydrate-free protein gave no color

* Because of the asymmetric nature of many proteins and of the propensity of integral membrane proteins to bind large amounts of detergent, it is actually meaningless to talk about molecular weights in this context. The values referred to above with reference to globular proteins are intended to present the reader with a more common reference to visualize the hydrodynamic radii which are mentioned.

development by the staining procedure we employed. Thus, for the routine sample loads of 25 to 50 μg of material, a glycoprotein with a typical oligosaccharide structure would have to possess at least 25% carbohydrate to be visualized by the detection procedure employed. In practice, the carbohydrate detection limits depend on the nature of the oligosaccharide side chains attached to the polypeptide since the chemical nature of the sugar constituents and the degree of chain branching will ultimately determine the color yield with the Schiff base reagent[20]. Obviously, the sensitivity of detection for either proteins or glycoproteins can be increased by employing radioactive labeling, either *in vivo* or *in vitro*[20,21].

It is possible to utilize DNP-BSA as a TLG void volume marker in the presence of the mild detergents only because the derivatized protein apparently undergoes a partial unfolding and exhibits a rapid association–dissociation in dilute buffer or in the mild detergent solvent systems. In the analytical ultracentrifuge, DNP-BSA appears as a polydisperse system with species of sedimentation coefficients from 3 S to 8 S; the weight-average sedimentation coefficient in the DOC solvent system is 7.6 S. Native BSA in DOC exhibites a single sedimenting boundary of 4.3 S. Since both unfolding and aggregation increase the hydrodynamic radius, a combination of the two effects is apparently sufficient to produce DNP-BSA in a molecular state whose average hydrodynamic dimensions precluded its entrance into any of the pore volume of G-200, *i.e.*, $R_e > \approx 70$ Å. Of course, in the typical SDS complex, the reduced polypeptide chain of DNP-BSA (or BSA) of $R_e = 85$ Å is also excluded from G-200. As a void volume marker for TLG, DNP-BSA offers advantages over Blue Dextran or large, non-chromophoric proteins. Like Blue Dextran, it is visible at all times during the run, yet, like other large proteins, it migrates as a discrete zone on TLG rather than exhibiting irregular zone spreading as does Blue Dextran[6].

It should be mentioned at this point that we have intentionally not emphasized the use of TLG in SDS as an empirical means for the estimation of polypeptide chain molecular weights or the use of TLG in mild detergents to estimate empirically equivalent hydrodynamic radii. Obviously, TLG may serve these purposes, but its application in such endeavors should be done only with the realization of the many potential pitfalls associated with these types of empirical measurements[12,18]. In these detergent systems, the internal volume marker, DNP-Gly, is slightly retarded in its migration by weak interactions with the gel matrix. This slight retardation of the internal volume marker yields absolute K_d values which are slightly smaller than if a totally inert volume marker, such as NaCl, had been employed. Though the absolute K_d values are slightly affected, there is no effect on the relation between R_e and $\text{erf}^{-1}(1 - K_d)$.

In conclusion, TLG in the presence of detergents is a simple, inexpensive method for separating microgram quantities of (glyco)proteins which are inherently insoluble in dilute buffers. This, of course, suggests that its greatest utility may lie with the separation, according to size, of the small quantities of detergent soluble proteins frequently attending membrane research.

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ISOELECTRIC FOCUSING OF THE PHOSPHOPROTEIN OF RAT-INCISOR DENTIN IN AMPHOLINE AND ACID pH GRADIENTS

EVIDENCE FOR CARRIER AMPHOLYTE–PROTEIN COMPLEXES

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SUMMARY

Rat-incisor phosphoprotein (RIP) has been subjected to isoelectric focusing at 4° in (a) an Ampholine pH gradient of 2.5–4 and (b) an acid pH gradient created by electrolysis of a system of acids and acidic ampholytes and covering the pH range 0.5–3.5. In the Ampholine gradient, the RIP unexpectedly formed several adjacent and strongly opalescent bands in the pH range 2.5–3.1. These bands, which migrated slowly toward the anode on prolonged focusing, are interpreted as being the result of an interaction between the amino groups of the Ampholine and the numerous phosphate groups of the protein. In the acid pH gradient, the RIP focused into one narrow zone corresponding to an isoelectric pH of 1.1 at 4°. This value is consistent with the amino-acid composition and the phosphate content of the protein.

INTRODUCTION

Besides collagen, preparations of decalcified dentin of different species have proved to contain a class of proteins rich in aspartic acid, serine and organically bound phosphate[1–5]. These phosphoproteins are of special interest to workers in the field of calcified tissues, as it has been postulated[1,6] that they play an essential role in the mechanism by which hydroxyapatite crystals are deposited within the organic matrix.

However, from a methodological point of view, the dentin phosphoproteins are also very interesting to those working in the field of isoelectric focusing (IEF). Because of their high content of strongly acid phosphate groups, but low content of basic groups, these proteins would be expected to have isoelectric points too low

to permit focusing within the usual carrier-ampholyte pH gradients (which cover pH values down to about 2.5). For the phosphoprotein of rat incisors (RIP), for instance, a rough estimate, made by means of the Linderstrøm–Lang equation[7,8] and based on the amino acid and phosphate contents[5], indicates a p*I* value of *ca.* 1. Running the RIP in a pH gradient established from commercial carrier ampholytes would thus be of little value, as the protein should focus near the anode, where accurate evaluation of its p*I* is impossible owing to the steepness of the pH course.

Surprisingly, this conclusion has proved to be erroneous. If the RIP is subjected to IEF in an Ampholine pH gradient of 2.5–4 created in the 1.5-ml column described earlier[8,9], the final 280-nm scan shows a complicated pattern with several adjacent peaks in the pH region 2.5–3.1.

On the other hand, the RIP focuses into one narrow zone in the expected pH region if the Ampholine pH gradient is exchanged for one created by electrolysis of a system of acids and acidic ampholytes and covering the pH range 0.5–3.5.

EXPERIMENTAL

Isolation and purification of RIP

Incisors dissected from Sprague–Dawley rats were freed from adhering soft tissue, dried and disaggregated. RIP was isolated either by demineralization of the teeth in 0.5 *M* acetic acid and subsequent extraction with 1 *M* NaCl in 50 m*M* Tris–hydrochloric acid buffer[2] (henceforth referred to as HAc-RIP) or by simultaneous demineralization and extraction with neutral 0.5 *M* EDTA[10] (EDTA-RIP). After dialysis against distilled water and freeze-drying, the crude RIP preparations were purified by molecular-sieve chromatography on Sepharose 4B, followed by ion-exchange chromatography on DEAE-cellulose[5].

IEF in Ampholine pH gradient

The 1.5-ml column described earlier[8] was filled with the following system of aqueous solutions: 0.30 ml of anode solution (containing 0.1 μl of concentrated sulphuric acid per ml of water), 0.20 ml of top solution (1 % of Ampholine pH 2.5–4), 0.70 ml of intermediate solution (1 % of Ampholine pH 2.5–4, 120 g/l of sucrose and 30–60 μg of RIP), 0.30 ml of bottom solution (1 % of Ampholine pH 2.5–4 and 200 g/l of sucrose) and 0.23 ml of cathode solution (0.75 % of Ampholine pH 2.5–4, 0.25 % of Ampholine pH 5–7 and 200 g/l of sucrose). Formation of the density gradient, scanning of the column during the run, fractionation of the column contents after completion of the run and subsequent evaluation of the pH course were carried out as described previously[8,9,11].

IEF in acid pH gradient

Ampholine-free pH gradients covering the approximate pH range 0.5–3.5 were created in an LKB 110-ml column (Type 8100-1) by the method developed by Jonsson and Pettersson[12,13]. The dense and less-dense solutions used for the formation of the density gradient were composed as follows. In addition to the sample (8–20 mg of RIP), the dense solution consisted of 0.30 ml of concentrated sulphuric acid ($pK_1 \approx -3$, pK_2 1.99 at 25°) and 30 g of sorbitol in 60 ml of water. The less-dense solution contained 0.30 g of citric acid (pK_1 3.08), 0.30 ml of concentrated formic acid (p*K*

3.75), 0.20 ml of concentrated acetic acid (pK 4.75), 0.10 g of aspartic acid (pI 2.98 and 0.15 g of glutamic acid (pI 3.22) in 55 ml of water.

The anode was at the bottom of the column, 0.085 M sulphuric acid being used as the anode solution and 0.025 M sodium hydroxide as the cathode solution. The bottom electrode solution also contained 530 g/l of sorbitol.

The procedure used for filling the column differed from that recommended by the manufacturer. The bottom electrode solution (*ca.* 11 ml) was slowly pumped into the column *after* the density gradient had been introduced through the top nipple in the usual way. This modification minimized the risk of a skew distribution of the bottom electrolyte, with the ensuing formation of a slanting protein zone on focusing[14].

Before layering 5 ml of cathode solution on top of the density gradient, the upper 10 ml of the latter were exchanged for 10 ml of a solution of histidine (0.45 g of histidine per 15 ml of water). As demonstrated by Pettersson[13], histidine (pI 7.47) prevents quenching of the current caused by the formation of an electrolyte-free region between the weakest acid and the cathode base.

The column was thermostatically controlled at 4.0°, and constant power of *ca.* 0.8 W was applied. As the focusing was slow in an acid pH gradient (due to the high conductance), electrolysis was continued for *ca.* 160 h.

After the runs were finished, the column contents were fractionated into 2-ml fractions, with one exception. When focusing samples of HAc-RIP and EDTA-RIP simultaneously, that part of the column contents expected to contain protein was divided into 0.7-ml fractions in order to increase the resolution of the protein assay (see below).

Evaluation of acid pH gradient

The pH values of the 2-ml fractions were measured at 4.0 and 25.0° by means of the equipment described elsewhere[15]; those of the 0.7-ml fractions were measured at 25.0° as described by Fredriksson[11]. The electrodes were standardized with commercial buffers (Radiometer S1510 and S1316; Merck, pH 1.00).

Protein assay

As RIP has a low absorptivity at 280 nm[5] and, moreover, the sorbitol is partly degraded into strongly UV-absorbing substances in the acid pH gradient[13], the RIP content of the fractions was monitored by two other techniques.

(*i*) After dialysis of 100 μl of each fraction against distilled water, the relative phosphate content was determined by a modified molybdate assay[5,10].

(*ii*) Like other polyanionic biopolymers, the RIP is precipitated by long-chain aliphatic ammonium salts such as cetylpyridinium chloride (CPC). Thus, the relative amount of RIP in each fraction could be estimated simply by adding CPC and measuring the resulting turbidity at 550 nm. A good correlation between the phosphate and CPC assays was obtained (*cf.* Fig. 2a).

Normally, 300 μl of a 1% (w/v) solution of CPC (Sigma, St. Louis, Mo., U.S.A.) were added to 100 μl of each fraction. In the experiment reported in Fig. 2c, the ratio of CPC and fraction volumes was reversed because the loading of RIP was smaller by a factor of 3.

RESULTS AND DISCUSSION

As mentioned in the introduction, the IEF of RIP in an Ampholine pH gradient 2.5–4 gave a surprising result (Fig. 1). Instead of accumulating close to the anode as expected, the protein spread over a wide pH range (2.5–3.1), showing several peaks that were not clearly separated from each other.

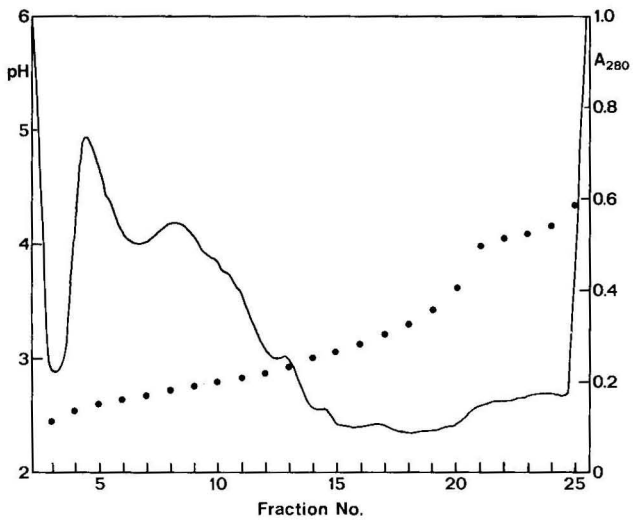


Fig. 1. Scan of 1.5-ml column[8,9] at 280 nm (solid curve) obtained after density-gradient IEF at 4° of 42 μg of HAc-RIP in 1% of Ampholine pH 2.5–4 for 105 min at an average field strength of 20 V/cm. Superimposed are pH values at 25° of 60-μl fractions of the column contents[11].

On visual inspection, the corresponding part of the column contents was found to be strongly opalescent, and with higher loadings of RIP, a precipitate was also observed*. Moreover, repeated scanning of the column revealed a slow migration and redistribution of the sample towards the anode on prolonged focusing.

These observations show that the peaks and shoulders in Fig. 1 do not represent different proteins, but are due to the formation of Ampholine–protein complexes, probably through interaction between the amino groups of the Ampholine and the numerous phosphate groups of the RIP. In fact, a recent paper by Righetti *et al.*[16] on the IEF of acidic dyes in polyacrylamide gel supports this assumption. By altering the ratio of dye to carrier ampholyte, these workers obtained evidence for the formation of strong complexes between the Ampholine and some dyes containing sulphonate groups.

Similar experiments with RIP proved unsuccessful, however, because small amounts of this protein could not be localized by staining, and larger amounts were precipitated. The affinity of Ampholine for the RIP is so high that cationic dyes such as toluidine blue and alcian blue, which bind to the protein in ordinary acidic buffers, are displaced by the carrier ampholytes. This was evident from a spectro-

* A certain opalescence was observed when the protein was added to the initial solution of Ampholine. On the other hand, an equally concentrated solution of RIP in water was perfectly clear.

photometric competitive-binding study carried out essentially as described by Righetti and Gianazza for heparin[17].

Indirect evidence of the formation of complexes between RIP and Ampholine was obtained when the Ampholine pH gradient was exchanged for an acid pH gradient created by electrolysis of a mixture of acids and acidic ampholytes. IEF of a sample of HAc- or EDTA-RIP in the latter type of pH gradient (see Figs. 2a and 2b) gave rise to only one peak in the absorbance curve at 280 nm (shown in Fig. 2a only*).

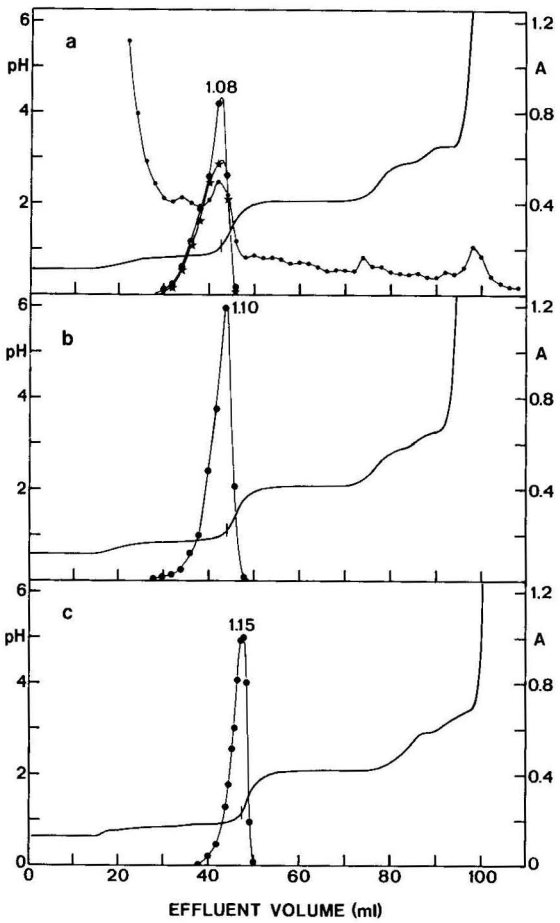


Fig. 2. Distribution of RIP obtained in an LKB 110-ml column after density-gradient IEF at 4° in a pH gradient created by electrolysis of a system of acids and acidic ampholytes[12,13]. Time of focusing 160 h; power *ca.* 0.8 W. The resulting pH course as measured at 25° (see ref. 15) is shown by the solid curve. The RIP content of 2- or 0.7-ml fractions of column effluent was monitored by means of the UV absorbance at 280 nm (•), a colorimetric phosphate assay[5,10] at 700 nm (★), and/or CPC-turbidity measurements at 550 nm (●). The protein samples consisted of: (a) 20 mg of HAc-RIP; (b), 20 mg of EDTA-RIP and (c), 4 mg each of HAc-RIP and EDTA-RIP. The figures at the peaks are the p*I* values of the RIP samples.

* The rest of the peaks in the UV absorbance curve were present also in a run without protein.

The assays made on the fractionated effluent from the column confirmed that the corresponding zone contained RIP. Moreover, comparison of the absorbance and pH curves revealed that the protein had focused in the expected region.

In a previous communication[5], samples of RIP prepared by the acetic acid–sodium chloride and EDTA extraction procedures were compared. The two preparations displayed a number of common properties, *e.g.*, a molecular weight of 30,000 ± 2000, similar chromatographic behaviour, and identical electrophoretic mobilities in Tris–hydrochloric acid buffer (pH 8.4). On the other hand, the amino acid analyses indicated a 15% lower content of serine in EDTA-RIP. It was concluded, however, that with regard to the inherent uncertainty in the methods of analysis employed, no definite difference in composition between HAc- and EDTA-RIP could be established.

The results of the IEF runs seem to support this conclusion. EDTA-RIP (Fig. 2b) focuses at the same pH as HAc-RIP (Fig. 2a). If equal amounts of HAc-RIP and EDTA-RIP are focused simultaneously, a single non-broadened peak is obtained (Fig. 2c).

It should be remembered, however, that a pH gradient shaped as in Fig. 2 is not ideal for the disclosure of small variations in the amino acid composition of RIP preparations. Unfortunately, the p*I* of RIP is located in a region where the pH course shows a steep transition from one shallow part to another. The resolving power in this part of the pH gradient is probably not better than 0.1 unit of pH.

As demonstrated by Fredriksson[18], the pH value assigned to the concentration maximum of a focused protein will not necessarily be the true p*I* of the protein if the temperatures of focusing and pH measurement do not coincide. In an Ampholine pH gradient, for instance, the p*I* value evaluated from an IEF run at 4° followed by pH measurements at 25° may differ by as much as 0.2 unit of pH from the true value at 25°.

In order to investigate the influence of the temperature of pH measurement on the acid pH gradient, two runs with HAc-RIP were carried out at 4°, the pH course being mapped at 4° in one run and at 25° in the other; the resulting p*I* values for RIP were 1.10 and 1.08, respectively. As the disparity between these values is less than the experimental uncertainty, the pH courses of the subsequent runs were evaluated at the more convenient temperature of 25°.

With a knowledge of the isoelectric pH and the amino acid composition of RIP, and by assuming that the isoelectric pH coincides with the isoionic pH, *i.e.*, binding of anions to the protein is negligible, it is possible to estimate the intrinsic dissociation constant (K_{int}) of the phosphate groups by reversing the calculation mentioned in the introduction. From the data of Jontell *et al.*[5], the number of ionic groups per molecule of HAc-RIP can be calculated as 99 β,γ-carboxy-groups, 1 α-carboxy-group, 63 phosphate groups and 6 basic groups. If the pK_{int} values for the carboxy- and the basic groups are assumed to be normal (*i.e.*, 4.6, 3.8 and >6.5, respectively[19]), the isoelectric condition of zero net charge on the protein molecule requires the average degree of dissociation, x_i (see ref. 8), to be 6/63 for the phosphate groups at pH 1.1. Insertion of these figures into the Linderstrøm-Lang equation[8] gives

$$pK_{int} = 1.1 - \log \frac{6/63}{1 - 6/63} = 2.1$$

For EDTA-RIP, which has 66 phosphate and 9 basic groups according to the phosphate and amino acid analyses[5], analogous reasoning gives a pK_{int} value of 1.9.

Unfortunately, the pK_{int} values obtained are difficult to compare with literature data. As, to our knowledge, no experimental values have hitherto been reported for the pK_1 of orthophosphate bound in monoester linkage to a protein, we are reduced to making a comparison with small model compounds resembling protein-linked phosphate. According to Tanford[19], glycerol 2-phosphate, with a pK_1 of 1.3 at 25°, is a relevant molecule. For a number of small peptides, which (like RIP) contained phosphoserine, Fölsch and Österberg[20] obtained $pK_1 \leqslant 1$ (and pK_2 5.4–6.0) for the phosphate group. In view of these data, our values for the phosphate groups of RIP are much too high.

In itself, the discrepancy is not surprising, as we have applied the Linderstrøm-Lang equation to a protein that is probably non-globular and has its charge unevenly distributed over the surface. It is interesting to note, however, that Lee *et al.*[21], in a recent nuclear magnetic resonance study of the phosphoprotein of bovine dentin measured an average pK_2 of 6.8 for the phosphate groups. As this value is considerably higher than the pK_2 values obtained by Fölsch and Österberg, and at the same time not far from the pK_2 of orthophosphoric acid (7.2 at 25°), it may be that the phosphate groups of RIP in fact have an average pK_1 close to that of orthophosphoric acid (*i.e.*, 2.1 at 25°).

The experiments reported in this paper have opened an important field of application for acid pH gradients covering pH values below 3. So far, phosphoproteins are known to be present in the dentin of six mammalian species (rat, guinea pig, rabbit, dog, ox and man[4]. Phosphoproteins have also been isolated from egg yolk[22] and pig brain[23], and it is likely that other strongly acid phosphoproteins will be found in future.

In this situation, it is unsatisfactory that the design of an acid pH gradient of appropriate range and slope is still mostly a matter of trial and error. We have therefore initiated a systematic investigation of the formation of such pH gradients.

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ISOELECTRIC FOCUSING OF PEPTIDES

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SUMMARY

Analytical isoelectric focusing of peptides in 0.7-mm thick gel slabs, in presence of 8 *M* urea, is made possible by staining the gel directly in Coomassie Brilliant Blue G-250 dissolved in 1 *N* H_2SO_4–12% trichloroacetic acid solution. The carrier ampholytes are soluble in this solvent whereas the peptides form a macromolecular aggregate with the dye, thus being trapped and precipitated within the gel matrix. The minimum critical peptide length for precipitation and staining is of the order of 15 amino acids. Below this length, only some basic, lysine-containing peptides are able to form faint, stained precipitates. About 20 μg peptide per an average band contained in a 3.5-mm^3 gel volume can be detected by this technique. It is hypothesized that the dye cross-links different peptide chains by binding to basic residues *via* its SO_3^- groups.

INTRODUCTION

In theory, isoelectric focusing (IEF) should be adaptable to the separation of any amphoteric compound, including substances of low molecular weight, such as peptides, and even free amino acids. In practice, this is often a difficult proposition as the carrier ampholytes used in IEF, being themselves polyamino-polycarboxylic acids, exhibit many of the properties typical of proteins and peptides, including similar behaviour on ion-exchange chromatography and on molecular sieves and similar reactivities with common protein stains. Thus, it is often difficult not only to detect the focused peptides, but also to separate them, after focusing, from contaminant carrier ampholytes. In the case of free amino acids, the situation is even worse as all mono-amino-monocarboxylamino acids are practically isoelectric over the pH range 4–8 (ref. 1), and are thus found as several peaks spread over most of the separation column[2,3]. Only recently, by performing IEF in presence of 0.1-*M* NaCl, has it been possible to condense them at their theoretical p*I*'s, in single peaks of rather broad distribution[4].

Examples of the separation of peptides are very scarce in the literature. This

is because only peptides containing aromatic residues can be easily distinguished from ampholytes by UV absorbancy. Thus Catsimpoolas[5], by *in situ* analytical scanning IEF, has been able to show separations of chromophoric (UV absorbing) amino acid derivatives and dipeptides, such as L-lysyl-L-tyrosine and L-hystidyl-L-tyrosine. Kopwillem *et al.*[6] used gel IEF and analytical isotachophoresis to analyse peptides in the amino acid sequence 125–156 from human growth hormone (HGH)[7], synthesized by the Merrifield technique[8]. In order to detect the various bands, the peptide was synthesized with a dinitrophenyl-His residue, so that the focused bands were visible as bright yellow zones under UV light. In the case of peptide antibiotics of low solubility in water, the separation has been performed by IEF in gels containing 50% dimethyl sulphoxide (DMSO)[9]. After focusing, the gel is extruded into distilled water: the DMSO is rapidly leached out and the focused antibiotic zones precipitate at their p*I* values as opalescent bands that can be quantitated by a scan at 600 nm and recovered fully active. Clearly, these examples are only of limited applicability to very particular cases. Recently, Noble *et al.*[10] have applied IEF to the analysis and purification of the amino terminal 54-residue fragment of HGH; however, this instance too is not representative for the separation of peptides as the long fragment behaves as a protein and can thus be fixed in trichloroacetic acid (TCA) and stained by the usual techniques[11].

In the present report, we describe a new method for the separation and detection of peptides with lengths ranging from 8 to 54 amino acids.

MATERIALS AND METHODS

Acrylamide, N,N′-methylenebisacrylamide (Bis), ammonium persulphate and tetramethylethylenediamine (TEMED) were from Bio-Rad Labs., Richmond, Calif., U.S.A. Coomassie Brilliant Blue G-250 (xylene brilliant cyanin G) was purchased from Serva, Heidelberg, G.F.R. The peptides used were mostly sequences of the HGH molecule, or of the ovine growth hormone (OGH), synthesized by the Merrifield technique[8] or by conventional methods. Their lengths, molecular weights (MW) and amino acid sequences are listed in Table I. The peptides were dissolved in 8 *M* urea (ultrapure, from Mann Labs., New York, N.Y., U.S.A.) and 10% 2-mercaptoethanol (Serva) at a concentration of 10 mg/ml.

Isoelectric focusing

IEF was performed in gel slabs, in the LKB Multiphor 2117 cell, using an LKB constant wattage power supply. However, instead of using the usual 2-mm thick slab, we cast a 0.7-mm thick gel slab by using a home-made rubber gasket cut out of a 0.7-mm rubber sheet. The gel contained 7% acrylamide (the ratio of acrylamide to Bis being 25:1), 2% Ampholine (pH 3.5–10) and 8 *M* urea. 1 *M* NaOH and 1 *M* H_3PO_4 were used as catholyte and anolyte, respectively. The gel was cooled at 10° with a Lauda thermostat. After pre-focusing for 1 h at 10 W, the samples (10–15 μl) were loaded soaked in filter paper strips at the anodic side. After focusing for 1 h, all the filter paper strips were removed from the gel surface and focusing continued for an additional 2 h (total running time: 4 h). The voltage, at equilibrium, was usually 1000 V. pH measurements were made as previously described[16]. No p*I* corrections were made for the presence of urea, as suggested by Ui[17].

TABLE I

PEPTIDES USED IN THIS STUDY

Peptides *	*Number of residues*	*Sequence*	*MW*
HGH 139–146[12,**]	8	Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe	1048
OGH 125–133[13,**]	9	Arg-Glu-Leu-Glu-Asp-Val-Thr-Pro-Arg	1114
HGH 35– 44[**,***]	10	Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe	1302
HGH 33– 44[**,***]	12	Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe	1502
HGH 31– 44[**,***]	14	Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr--Ser-Phe	1778
		Dnp Dnp	
HGH 15– 36[§,§§]	22	Leu-Arg-Ala-His-Arg-Leu-His-Gln-Leu-Ala-Phe--Asp-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile	3082
HGH 1– 24[14,§,§§§]	24	Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp--Asn-Ala-Met-Leu-Arg-Ile-Ser-Leu-Leu-Leu-Ile--Gln-Ser	2759
HGH 111–134[§,§§]	24	Tyr-Asp-Leu-Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile--Gln-Thr-Leu-Met-Gly-Arg-Leu-Glu-Asp-Gly--Ser-Pro-Arg	2749
HGH 166–191[15,§] (oxidized with O_2)	26	Phe-Arg-Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe--Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly--Ser-Cys-Gly-Phe	3049
HGH 25– 51[14,§,§§§]	27	Trp-Leu-Glu-Pro-Val-Glu-Phe-Ala-His-Arg-Leu--His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu--Phe-Glu-Glu-Ala-Tyr-Ile	3382
HGH 157–191[14,§] (tris-*S*-carbamido-methylated)	35	Leu-Lys-Ans-Tyr-Gly-Leu-Leu-Tyr-Cys-Phe-Arg--Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-Leu--Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser--Cys-Gly-Phe	4287
HGH 1– 36[15,§]	36	Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp--Asn-Ala-Met-Leu-Arg-Ala-His-Arg-Leu-His-Gln--Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Glu--Glu-Ala-Tyr-Ile	4354
HGH 96–134[15,§]	39	Val-Phe-Ala-Asn-Ser-Leu-Val-Tyr-Gly-Ala-Ser--Asn-Ser-Asp-Val-Tyr-Asp-Leu-Leu-Lys-Asp-Leu--Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met-Gly-Arg--Leu-Glu-Asp-Gly-Ser-Pro-Arg	4273
HGH 115–156[§,§§]	42	Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu-Met--Gly-Arg-Leu-Glu-Asp-Gly-Ser-Pro-Arg-Thr-Gly--Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe-Asp--Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu	4757
HGH 103–156[§,§§]	54	Tyr-Gly-Ala-Ser-Asn-Ser-Asp-Val-Tyr-Asp-Leu--Leu-Lys-Asp-Leu-Glu-Glu-Gly-Ile-Gln-Thr-Leu--Met-Gly-Arg-Leu-Glu-Asp-Gly-Ser-Pro-Arg--Thr-Gly-Gln-Ile-Phe-Lys-Gln-Thr-Tyr-Ser-Lys--Phe-Asp-Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu	6055

* All peptides are crude products of reaction.
** Synthetized by conventional methods.
*** Manuscript in preparation.
§ Synthetized by solid-phase method.
§§ Unpublished results.
§§§ Synthetized before the HGH primary structure revision.

Staining

We used the method described by Blakesley and Boezi[18] for disc gel electrophoresis. To a 0.2% (w/v) aqueous solution of Coomassie B.B. G-250 an equal volume of 2 *N* H_2SO_4 is added. After stirring for 3 h, the precipitate is filtered through a Whatman No. 1 paper. To the clear brown filtrate 1/9 volume of 10 *N* KOH is added, producing a purple solution into which 100% (w/v) TCA is poured to give a final concentration of 12% (w/v). The resulting clear greenish light-blue solution is then ready for use. After focusing, the gel is bathed directly in this staining solution, and shaken for at least 3 h. It is then transferred into distilled water, where the blue colour of the bands is intensified. No background staining is observed in the gel, as the carrier ampholytes are soluble in this solution, and only the precipitated peptides slowly absorb the dye from the solution. One litre of this staining mixture can be used at least ten times with our thin gels (30 ml of gel solution are used to polymerize a gel slab).

RESULTS

Fig. 1 shows the focusing pattern, in the pH range 3.5–10, of the 15 peptides investigated. These peptides are representative of practically any amino acid composition, as there are acidic (samples 1, 3 and 6), close to neutral (sample 2) and basic (samples 4, 5 and 7) components, covering essentially any possible p*I* range. The first four peptides used (octa-, nona-, deca- and dodeca-peptides) are not precipitated in the H_2SO_4–TCA mixture, nor can they fix the stain, except for the octapeptide HGH 139–146, which is seen as a very faint band against the cathodic filter paper strip (pH *ca.* 12; not shown). This peptide contains two Lys and no acidic residues (except for the carboxyl terminus) and it ceases migrating at this very high pH because of partial deprotonation of the two Lys groups. Oddly, the nonapeptide, which also contains two basic residues (Arg, but also three acidic amino acids, two Glu and one Asp) does not precipitate nor pick up any stain. Starting from the tetradecapeptide, all the peptides analysed are fixed and stained in this stain mixture. The tetradecapeptide exhibits a single, homogeneous band with an uncorrected p*I* of 4.7. This p*I*, upon correction for the urea presence, as recommended by Ui[17], is in excellent agreement with the theoretical p*I* calculated with the Linderstrøm–Lang[19] equation, on the basis of its known amino acid composition (one carboxyl terminus, three Glu, two Lys and one amino terminus). The same applies to all other peptides investigated, which rules out any spurious band formation due to carrier ampholyte–peptide interaction. This has also been confirmed by excising and rerunning single, homogeneous bands in the focusing pattern. They run through to their original p*I* without splitting into several bands (not shown). This is a clear indication of absence of artefacts[20].

Fig. 2A shows the pattern obtained by focusing the same peptides and then fixing the gel in 10% TCA. It can be seen that only the major bands can be distinguished and much of the fine heterogeneity is lost. Moreover, all peptides shorter than 24 amino acids are lost, as they are soluble in 10% TCA. It is customary, in IEF, to fix first the protein pattern in 10% TCA, in order to leach out the carrier ampholytes, then to remove the excess of TCA by washing the gel three times in water and then to stain the protein bands in Coomassie B.B. R-250 dissolved in alcohol–acetic acid[11]. When this is done, quite a few of the peptide bands, already precipitated

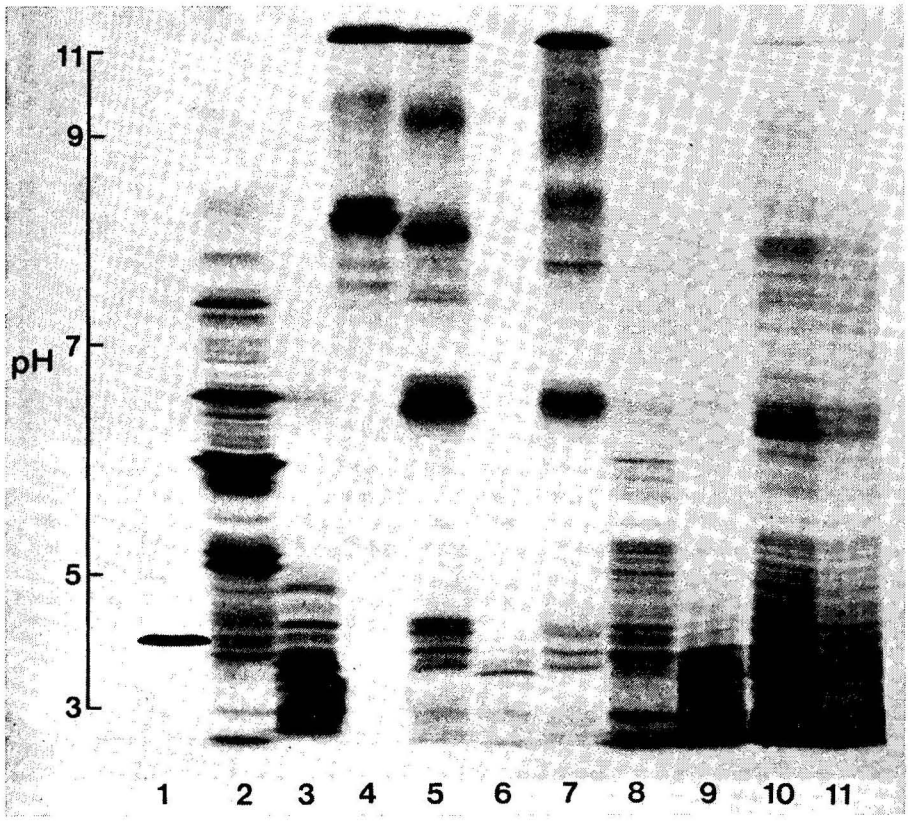


Fig. 1. IEF of peptides in the length range 8–54 amino acids. The gel slab was 0.7 mm thick and contained 7% acrylamide, 2% Ampholine pH 3.5–10 and 8 *M* urea. 10–15 μl of sample (10 mg/ml) were applied in filter paper strips at the anode after 1 h prefocusing. Total running time: 4 h at 10 W (1000 V at equilibrium). The gel was then dipped in the staining solution of Blakesley and Boezi[18]. Samples: 1 = HGH 31–44; 2 = HGH 15–36; 3 = HGH 111–134; 4 = HGH 1–24; 5 = HGH 166–191; 6 = HGH 25–51; 7 = HGH 157–191; 8 = HGH 1–36; 9 = HGH 96–134; 10 = HGH 115–156 and 11 = HGH 103–156. The octa, nona-, deca- and dodeca-peptides were neither fixed in the gel nor stained.

in 10% TCA, are lost in subsequent washings, as shown in Fig. 2B, so that the peptide patterns thus obtained are totally unreliable.

By running a series of standard dilutions of the tetradecapeptide, we have seen that by this method *ca.* 20 μg peptide per band (in *ca.* 3.5 mm^3 of gel volume, assuming an average band 10 mm long, 0.7 mm thick and 0.5 mm wide) can be detected (Fig. 3). This detection limit is much lower than the 0.2 μg detectable protein limit in a gel when stained with Coomassie B.B. R-250 in solution[21]. On the other hand, it must be emphasized that in the present method there is no need for background destaining and also that, in the latter method, because the gel is often destained at high temperatures, there is a great risk of the protein zones losing their stain even before the background is destained[22]. Moreover, this detection limit applies to the most difficult cases, *i.e.* to short peptides, which have a greater tendency to be solubilized and a lower dye affinity.

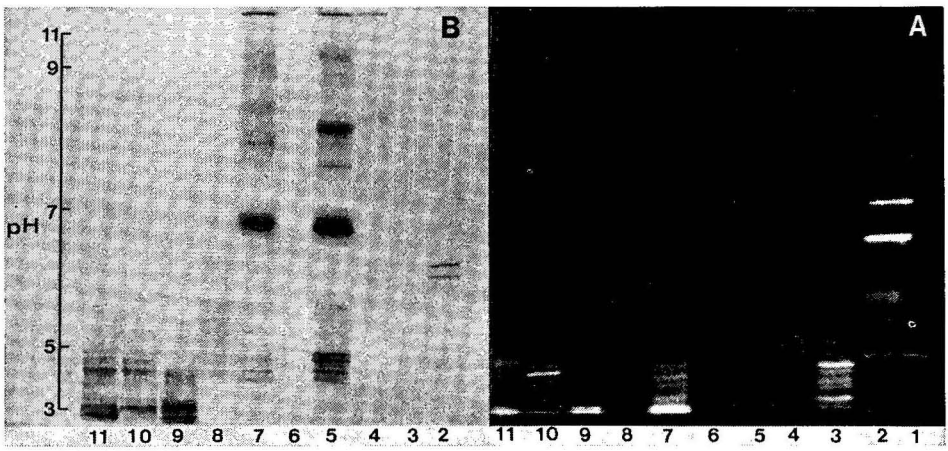


Fig. 2. IEF of peptides followed by conventional staining. IEF conditions and samples as in Fig. 1. At the end of focusing, the gel was bathed in 10% TCA and photographed (side A). After fixing the peptides, the excess TCA was washed with three rinses in water and the gel was then stained by conventional methods (Coomassie B.B. R-250 dissolved in alcohol–acid, side B). Note the total loss of samples 1, 3 and 6 and the heavy loss of material in all other samples, including high MW peptides. Since the same amount of material has been applied, the stain intensity here should be compared with the stained pattern in Fig. 1.

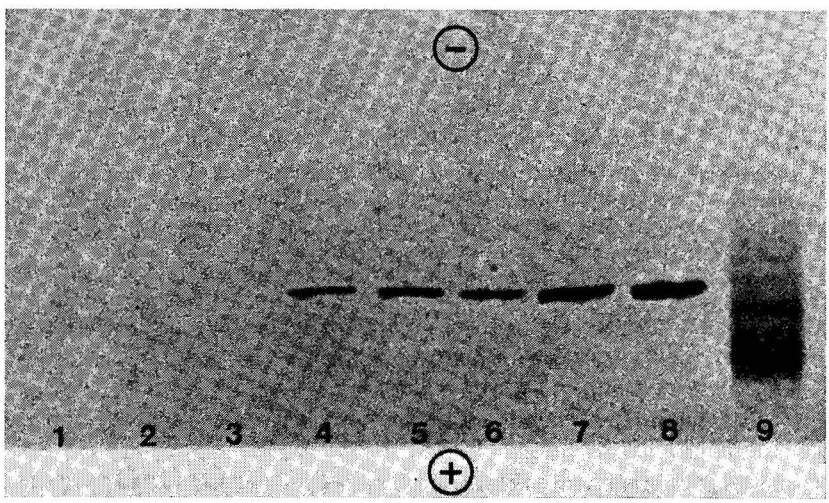


Fig. 3. Sensitivity of the staining method. Samples 1–8 refer to the tetradecapeptide HGH 31–44, in different loads. 1 = 1 μg; 2 = 5 μg; 3 = 10 μg; 4 = 20 μg; 5 = 30 μg; 6 = 40 μg; 7 = 70 μg; 8 = 100 μg; 9 = 100 μg of HGH 157–191. In the case of HGH 31–44, the minimum critical concentration for staining appears to be of the order of 20 μg.

DISCUSSION

For a successful fractionation of peptides by IEF, the following general experimental rules apply.

(1) Focusing should always be performed in the presence of urea or other appropriate dissociating agents (such as DMSO, dimethylformamide or tetramethyl-

urea[23]) in order to prevent aggregation of peptides and also any possible carrier ampholyte–peptide interaction, which would then generate an artefactual heterogeneity, because the peptide, existing in solution mostly as a random coil, has all its possible binding sites exposed to the solvent.

(2) IEF should be performed in thin gel layers. We have found a 0.7-mm thickness highly satisfactory because it is thin enough to allow for rapid fixing in the H_2SO_4–TCA solution, quick dye absorption and rapid leaching out of urea and carrier ampholytes, thus preventing diffusion of small peptides, and thick enough to allow for proper gel handling. Moreover, there is a considerable saving of expensive Ampholine and acrylamide and a much easier drying of the gel slab for permanent storage.

(3) The sample, when soaked in filter paper, should always be applied at the anodic side. As paper always contains a few carboxyl groups, they are fully ionized at basic pH but completely protonated at this low pH, thus preventing any absorption of the samples, especially if basic, on the filter paper. When applying the sample at the cathode, we have noticed strong absorption of basic peptides (samples 4, 5 and 6 in Fig. 1) with consequent loss of several bands from the stained pattern, which could only be abolished when the sample was applied in a liquid form in pockets pre-cast in 2-mm thick gels (not shown). In addition, the low pH at the anode will prevent possible carbamylation of the protein basic groups, in case traces of cyanate are present in the sample. The 1-h pre-run of the gel, prior to sample application, will also discharge at the anode any harmful persulphate and traces of cyanate in the gel.

(4) One drawback of the present method is that not all peptides are solubilized in the 8 *M* urea–10% 2-ME mixture. Two of the 15 peptides are poorly soluble in this solvent, so that only a few components (the most acidic, see samples 6 and 9, in Fig. 1) out of a very heterogeneous mixture can be focused and detected. Unfortunately, neutral detergents, which would often help solubilizing hydrophobic compounds, cannot be used (see below).

(5) Detergents (such as Nonidet P-40 or Triton X-100) should not be used in the present method. They bind to carrier ampholytes, forming complexes that are insoluble in 10% TCA (the gel becomes milky) but soluble in hot (60–70°) water. However, with the present staining technique, a tertiary complex detergent–dye–carrier ampholyte is formed, which cannot be solubilized without disaggregating too the dye–peptide complex.

From a theoretical point of view, we should like to stress the following points.

(1) The minimum critical peptide length for fixation in the gel matrix and dye binding appears to be around 15 amino acids. Insolubilization of peptide by dye binding is a function of both size and amino acid composition. Above this length, the size plays the most important role, whereas below it the peptide composition is critical.

(2) Dye binding is favoured by the presence of basic amino acids (Arg, Lys and His) and by a relatively high content of hydrophobic amino acids on the peptide. Besides binding to basic groups, the dye can interact with hydrophobic residues, particularly if they are close together. As a corollary, a relatively high content of hydrophilic (especially acidic) amino acids does not favor dye binding.

(3) Our data suggest that dye binding is stronger with Lys than with Arg (possibly for steric reasons). The octapeptide, which contains two Lys residues, binds

the dye (even if very poorly) and forms a faint precipitate. The nonapeptide, which contains two Arg residues instead of Lys, does not seem to bind the dye or at least forms soluble complexes, which are eluted from the gel.

(4) We hypothesize that the peptide is insolubilized because the dye binds two chains simultaneously (via its SO_3^- groups), thus acting as a bridge or a cross-link between chains and forming a macromolecular aggregate that is trapped within the random meshwork of fibres of the gel (for the dye formula, see Diezel *et al.*[24]). If this is so, a peptide has to have at least one basic residue, so that the dye can act as a cross-link over several chains, utilizing the amino terminus plus the basic residue. A chain with no basic residues would only have the amino terminus as a binding site, so that the dye could only link two chains at a time, thus forming a soluble complex that would escape detection (see the model in Fig. 4). It must also be emphasized that multiple binding sites on the polypeptide will also increase the stability of the complex through stacking of the aromatic rings of the dye molecules.

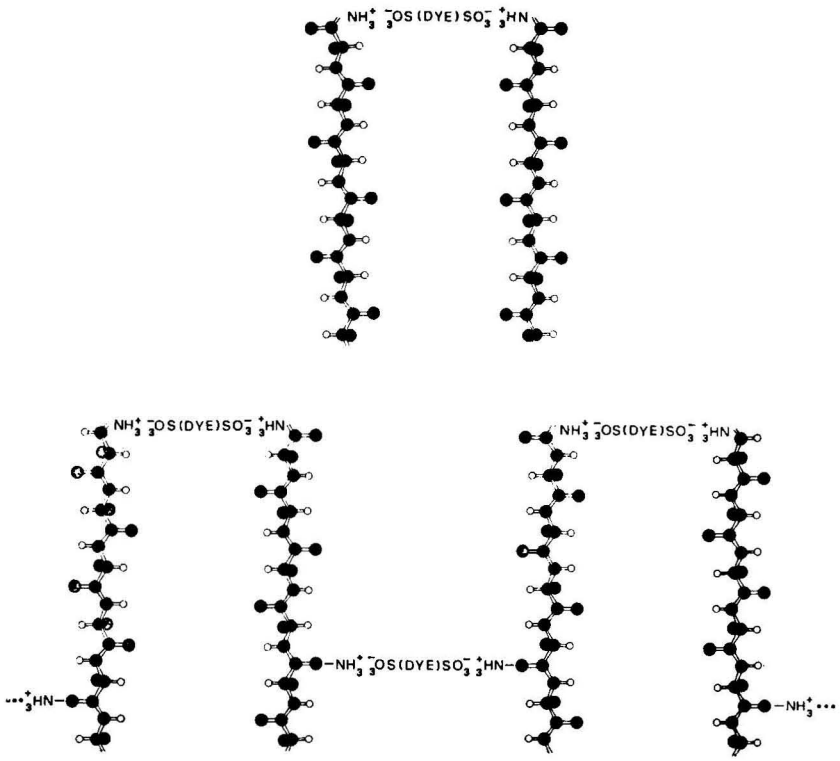


Fig. 4. Proposed dye–peptide binding model. A peptide with no basic residues (upper part), will only bind the dye via its NH_2 terminus, thus forming a dimeric complex that readily splits because it is extremely unstable in H_2O. The lower part shows a peptide with an additional binding site in the chain (an ε-NH_2 group of Lys). In this case, a macromolecular aggregate is formed, that is trapped in the gel matrix and is insoluble in water. This model should be seen not as linear, as depicted, but as a three-dimensional array of chains. The aggregate is further stabilized by stacking among aromatic rings of different dye molecules, made possible by the ionic interactions of the two sulphate groups of the dye with the peptide amino groups.

We feel that, with the present method, it is possible to analyse by IEF peptides in the length range 15–50 amino acids, the range in which most of the biologically active peptides are located.

ACKNOWLEDGEMENT

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USEFUL MODIFICATION OF PAULY'S REAGENT

DETECTION OF LOW-MOLECULAR-WEIGHT HISTIDINE-CONTAINING PEPTIDES BY ISOELECTRIC FOCUSING

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SUMMARY

An improved method for the detection of low-molecular-weight (<10,000 daltons) histidine-containing peptides on isoelectric focusing is described.

The major advantage of the method is that the staining mixture (a modification of Pauly's reagent) does not react with ampholytes of similar size and pI, permitting accurate determination of the isoelectric points of these peptides.

INTRODUCTION

The high-resolution technique of isoelectric focusing, which utilizes electrophoresis in a pH gradient to separate proteins according to their isoelectric points, is used extensively[1,2]. It has been applied mainly to the separation and characterization of peptides of high molecular weight[2]. Difficulties of detection, peptide loss and the rapid diffusion during conventional staining, as well as the difficulties encountered in distinguishing carrier ampholytes of similar size and pI, have prevented the extension of the technique to the analytical resolution of low-molecular-weight peptides.

We describe here a versatile method for the detection of low-molecular-weight histidine-containing peptides, using a modification of Pauly's[3] staining method.

MATERIAL AND METHODS

The carrier ampholyte solution, pH range 3.5–10, was obtained from LKB (Bromma, Sweden). The calibrated mixture of pH markers, acrylamide and N,N'-methylene bisacrylamide were purchased from Serva (Heidelberg, G.F.R.). Ammonium persulphate was obtained from Fluka (Buchs, Switzerland).

Apparatus

The Flat-sheet Gel Electrophoresis Cell 82100* and the stabilized Power supply 67502 (Camag, Muttenz, Switzerland) were used.

Preparation of the gel

The gel was prepared form the following refrigerated stock solutions: A, 30 g of acrylamide and 1 g of N,N′-methylenebisacrylamide dissolved in 100 ml water; B, 8 *M* urea; C, 1% ammonium persulphate in water.

To prepare the gel, 13.75 ml of stock solution A, 36.50 ml of stock solution B, 3.3 ml of stock solution C and 2.0 ml of LKB ampholines (pH range 3.5–10) were mixed.

Gel polymerization

The mixture was filled into the apparatus by means of a capillary and polymerized within *ca.* 30 min.

Sample application

Samples were easily applied with the aid of small strips of Whatmann No. 3MM filter paper (5 mm × 10 mm). A 50-μg amount of the peptides listed in Table I, dissolved in 10 μl of water, were applied using a 10-μl Hamilton dispenser. The peptides were synthesized in our laboratories.

TABLE I

PEPTIDES USED IN THIS INVESTIGATION

Synthetic peptide	*Spot No.*	*Molecular weight*
p-Glu-His-NH_2	1	265
p-Glu-His-OCH_3	2	280
p-Glu-His-Pro-NH_2HOAc	3	362
H-Asn-Gln-His-OH	4	397
p-Glu-His-Pro-OHHCl	5	409
Val^5-angiotensin II	6	1032
Human calcitonin	7	3318
Porcine ACTH (1–39)	8	4567
Human insulin	9	5808

Electrofocusing conditions

The anodic and cathodic chambers were filled with 10% phosphoric acid and 1 *N* NaOH, respectively. Focusing was performed at a constant temperature of 10°, maintained by circulating cooling water. The voltage was steadily increased in such a way that the current never exceeded 25 mA (Fig. 1). At the beginning of the operation the potential difference between the two electrodes was 80 V, at the end it was 500 V (Fig. 1).

* This horizontal flat-sheet gel electrophoresis cell (System CIBA-GEIGY) was developed in collaboration with Camag.

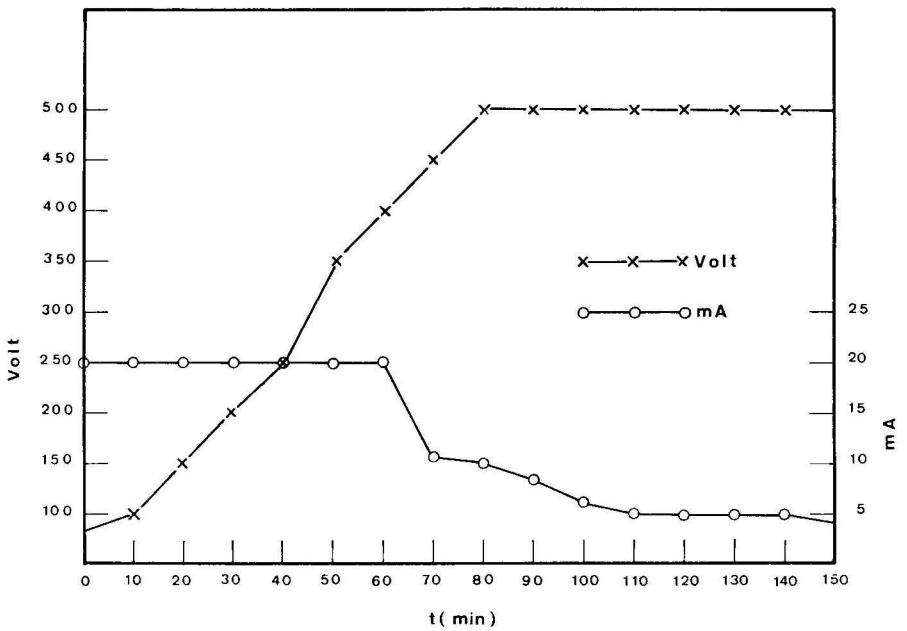


Fig. 1. Record of voltage and current during focusing in polyacrylamide gel (pH range 3.5–10).

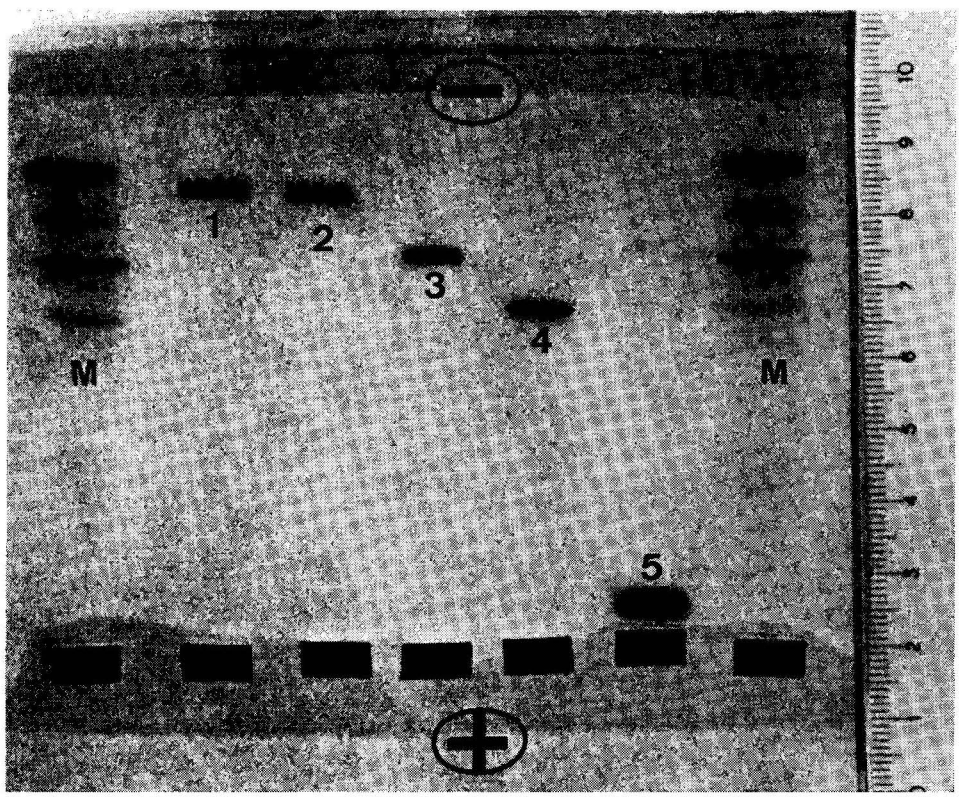


Fig. 2. Isoelectric focusing of peptides listed in Table I with MW $<$ 1000. M = pH markers (whale myoglobin, horse myoglobin, β-lactoglobulin A and B).

Staining procedure

After removal from the apparatus, the gel was stained with the following modified Pauly's reagent[3]:

1 part stock solution I (0.4 *M* sulphanilic acid in water);
1 part stock solution II (0.4 *M* sodium nitrite in water);
1 part stock solution III (2.0 *N* HCl);
7 parts water;
10 parts stock solution IV (2.0 *N* sodium carbonate).

Stock solutions were kept at 4°.

Red peptide bands were observed following immersion of the gel in this staining solution.

Another procedure was used for histidine-containing peptides of higher molecular weight (MW $\geqslant$ 3000) and histidine-containing proteins. The peptides were first fixed by immersion of the gel in 15% trichloroacetic acid for *ca.* 3 h followed by rinsing with 2 *N* Na_2CO_3 and staining in the usual manner.

RESULTS AND DISCUSSION

Figs. 2 and 3 show the results of applying this staining technique to low

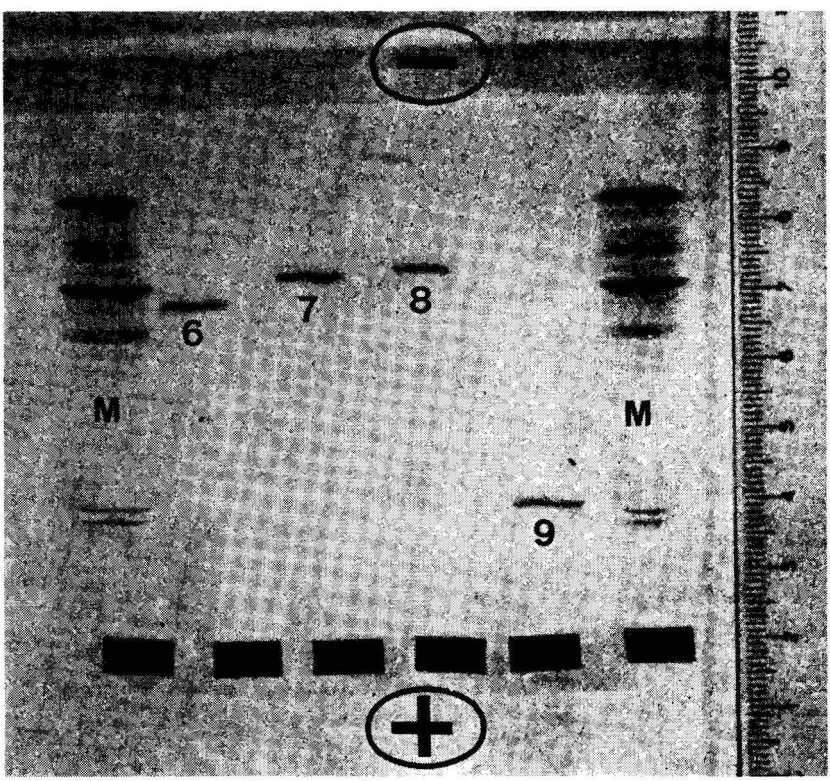


Fig. 3. Isoelectric focusing of peptides listed in Table I with MW > 1000. M = pH markers (whale myoglobin, horse myoglobin, β-lactoglobulin A and B).

molecular weight, histidine-containing peptides, and demonstrate that the method gives a satisfactory staining intensity permitting accurate determination of the isoelectric points (p*I*).

The observation that the staining mixture does not react with ampholines is of particular interest.

In order to determine the p*I* values, small pieces of gel can be placed in a test tube and, after diffusion into 0.5 ml of distilled water, the pH is determined using a microelectrode. As an alternative to pH measurements along the length of the gel, a calibrated mixture of pH markers may be used[4]. Fig. 4 shows the pH range curve and the isoelectric points of the pH markers.

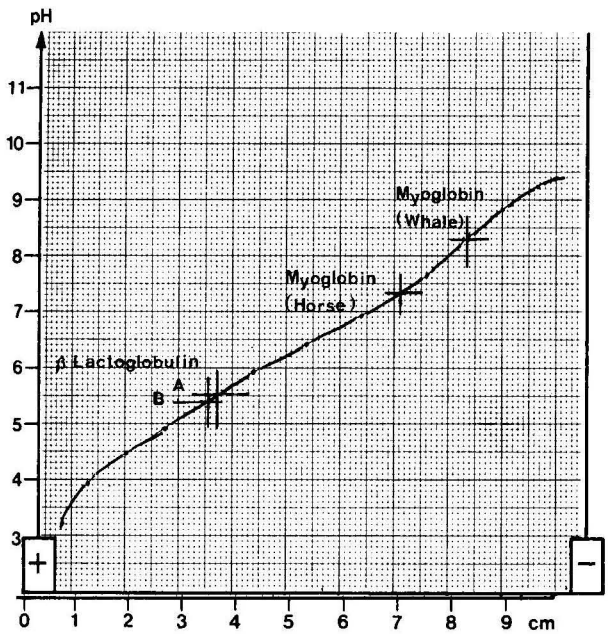


Fig. 4. pH range curve of the higher molecular weight (MW > 1000) peptides and isoelectric points of pH markers.

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CHROM. 10,973

KONTINUIERLICHE TRÄGERFREIE ISOELEKTRISCHE FOKUSSIERUNG

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SUMMARY

Continuous free-flow isoelectric focusing

Investigations of continuous isoelectric focusing are carried out in a modified apparatus for free-flow electrophoresis. This procedure enables amphoteric biochemical substances to be separated without damage. The optimum conditions for separation are derived from the influences of various parameters on the separation result. Individual proteins and their mixtures are used as test substances.

EINLEITUNG

Die isoelektrische Fokussierung gestattet bei hoher Auflösung die schnelle Trennung amphoterer biochemischer Substanzen wie Peptide, Proteine, Enzyme und Hormone[1–4]. Im analytischen, aber auch im präparativen Bereich werden die Trennungen meist in Polyacrylamidgel oder in granulierten Gelen durchgeführt. Bei präparativen Fokussierungen in Säulenapparaturen erfolgt die Stabilisierung des pH-Gradienten durch Dichtegradienten.

Über kontinuierliche Verfahren zur isoelektrischen Fokussierung ist wenig bekannt. So dienen Versuche in Eigenbauapparaturen dazu, die Technik der trägerfreien Durchflusselektrophorese auch für eine Fokussierung auszunutzen[5–7]. Um Störungen durch Thermokonvektion bei geringen Durchflussgeschwindigkeiten zu umgehen, wird von anderen Autoren eine Stabilisierung entweder durch Sephadex-Gel[8] oder durch Dichtegradienten[9] vorgeschlagen. Diese Verfahren haben den Nachteil sehr langer Verweilzeiten.

In einer analytischen Trennapparatur zur trägerfreien Durchflusselektrophorese werden Verweilzeiten von 30 min durch mehrmalige Eingabe der Fraktionen (recycling)[10] erhalten. Der Vorteil der kontinuierlichen Arbeitstechnik geht dabei jedoch verloren.

Ausgehend von Erfahrungen mit der schnellen Gleichgewichtseinstellung bei der trägerfreien kontinuierlichen Trennung und Anreicherung von Metallionen wird die hierbei verwendete und bereits beschriebene Trennapparatur[11] für die isoelektrische Fokussierung umgerüstet. Die Ergebnisse der grundlegenden Untersuchungen über den Einfluss verschiedener Parameter wie Verweilzeit und Feldstärke auf die

Ausbildung und Stabilität des pH-Gradienten liefern die optimalen Trennbedingungen. Trennkapazität, Trennschärfe sowie Reproduzierbarkeit werden anhand einiger Proteintrennungen untersucht.

EXPERIMENTELLES

Chemikalien, Geräte und Versuchsanordnung

Die Trägerampholyte "Servalyt" und die Testproteine sind bezogen von Serva (Heidelberg, B.R.D.). Bei den Chemikalien für die Elektrodenpuffer handelt es sich um p.a.-Substanzen von Merck (Darmstadt, B.R.D.).

Die Elektrophoreseapparatur setzt sich zusammen aus der Eigenbaukammer, dem hochstabilisierten Netzgerät von Knott Elektronic (München, B.R.D.) und der Kühlbox 2000 von Serva. Die Zufuhr des Probengemisches erfolgt über eine Schlauchpumpe Varioperpex von LKB (Bromma, Schweden). Zur Extinktionsmessung dient das Durchflussphotometer PM 2 D von Zeiss (Oberkochen, B.R.D.). Die pH- und Leitfähigkeitsmessungen erfolgen mit dem WTW Digitalmeter DIGI 610 (Weilheim, B.R.D.).

Fig. 1 zeigt die schematische Darstellung der Versuchsanordnung.

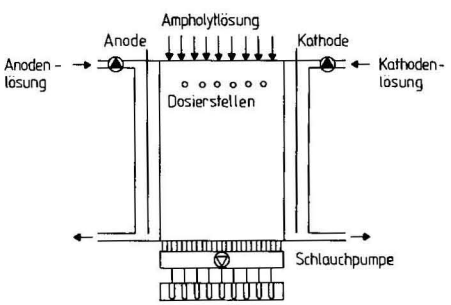


Fig. 1. Schematische Darstellung der Versuchsanordnung zur trägerfreien isoelektrischen Fokussierung.

Die eigentliche Trennkammer besteht aus einem zweiteiligen Plexiglasblock mit Trennspalt, Kühlkammern, Elektrodenräumen und Zu- bzw. Abflussbohrungen. Der Trennspalt wird durch zwei Glasplatten gebildet, die durch dünne PVC-Streifen auf einem Abstand von 0.4 mm gehalten werden. Die Zufuhr der Trägerampholytlösung erfolgt über neun PVC-Schläuche. Die Analysenlösung wird mittels einer regelbaren Schlauchpumpe durch spezielle Bohrungen (Dosierstellen) in den Trennspalt zugeführt. Am Ende der Trennstrecke entnimmt man die Flüssigkeit durch 87 Spaghettischläuche. Eine Schlauchpumpe mit elektronischem Regelteil sorgt für den kontinuierlichen Durchfluss der Lösung. An den Längsseiten befinden sich die beiden Elektrodenräume. Trennspalt und Elektrodenräume stehen über Ionenaustauschermembranen im elektrischen Kontakt. Als Elektrodenlösung dienen auf der Anodenseite 0.4 *M* H_3PO_4 und auf der Kathodenseite 0.6 *M* NaOH. Die Elektrodenlösungen werden kontinuierlich durch Zupumpen erneuert.

Die gesamte Versuchsanordnung befindet sich in einer Kühlbox, die das Arbeiten im Temperaturbereich von -10 bis $+10°$ gestattet. Hierdurch werden Träger-

ampholyt- und Analysenlösung vorgekühlt und die Fraktionen auf der Trenntemperatur gehalten.

Die Bestimmung der getrennten Substanzen erfolgt durch Messung der Extinktion bei 280 nm im Durchflussphotometer und Ermittlung des p*I*-Wertes. Zusätzlich werden noch pH und Leitfähigkeit der einzelnen Fraktionen gemessen.

ERGEBNISSE

Ausbildung des pH-Gradienten

Die vollständige Ausbildung des pH-Gradienten muss gewährleistet sein. Sie hängt von der Feldstärke und der Verweilzeit ab. Bei der vorgegebenen Versuchsanordnung kann die Feldstärke zwischen 0 und 180 V/cm variiert werden. Die Durchflussgeschwindigkeit ist von 0 bis 800 cm^3/h kontinuierlich regelbar. Hieraus lassen sich bei bekanntem Trennspaltvolumen die entsprechenden Verweilzeiten berechnen.

Für jede angelegte Feldstärke muss die für die Ausbildung des pH-Gradienten notwendige Verweilzeit ermittelt werden. Die optimale Feldstärke beträgt bei der gewählten Versuchsanordnung 110 V/cm. Fig. 2 zeigt die Ausbildung des pH-Gradienten bei schrittweiser Änderung der Verweilzeit und damit der Durchflussgeschwindigkeit. Das Leitfähigkeitsprofil zeigt, dass bei einer Verweilzeit von 20 min die vollständige Ausbildung des pH-Gradienten erfolgt ist. Eine weitere Erhöhung der Verweilzeit führt zu keiner Verbesserung des pH-Gradienten.

Verwendet wird 1%ige Trägerampholytlösung. Versuche mit geringeren Konzentrationen ergeben deutliche Verschlechterungen in der Trennkapazität. Fokussierungen mit höheren Trägerampholytkonzentrationen erfordern höhere Feldstärken bzw. geringere Durchflussgeschwindigkeiten und führen aufgrund der höheren Leitfähigkeit zu einer Zunahme der Jouleschen Wärme und damit zu Kühlproblemen.

Fokussierungen von Einzelproteinen und Proteingemischen

Untersucht werden die Trennkapazität, das Auflösungsvermögen und der Einfluss der Zugabestelle.

Mit Trennkapazität wird die maximal fokussierbare Proteinmenge bezeichnet. Sie ist von der eingesetzten Substanz abhängig. Der Durchschnittswert beträgt bei 1%iger Trägerampholytlösung 1 mg/h für das Einzelprotein. Bei grösseren Durchsätzen treten Abweichungen von der ursprünglichen Form des pH-Gradienten auf. Für einwandfreie Trennungen nahe beieinander gelegener Zonen ist die Trennkapazität oft geringer, wie das Beispiel der Fokussierung von Pferdemyoglobin (Fig.3) zeigt.

Allgemein hängt das Auflösungsvermögen der isoelektrischen Fokussierung von der Feldstärke, aber auch von der Steilheit des pH-Gradienten ab und entspricht der p*I*-Wertdifferenz zweier noch getrennter Substanzen[12]:

$$pI = 3\sqrt{\frac{D\,\frac{dpH}{dx}}{-E\,\frac{dn}{dpH}}}$$

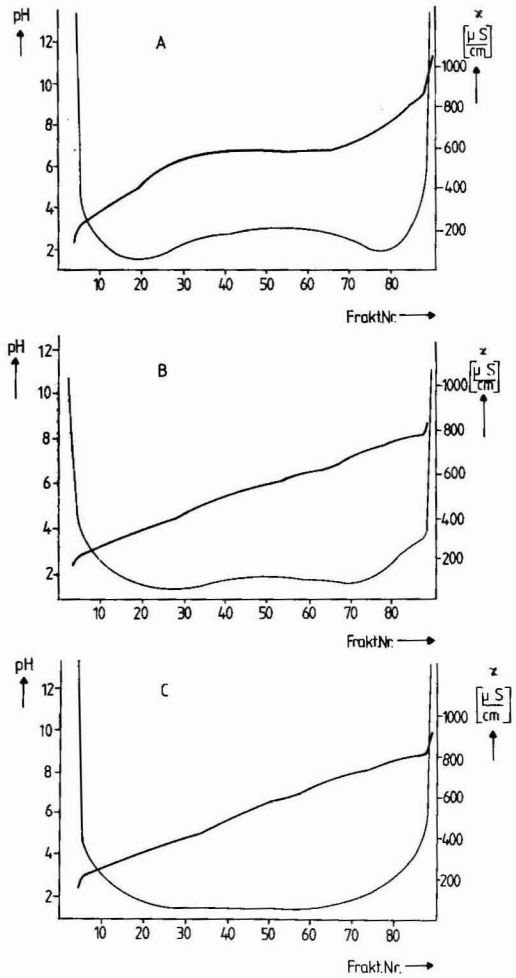
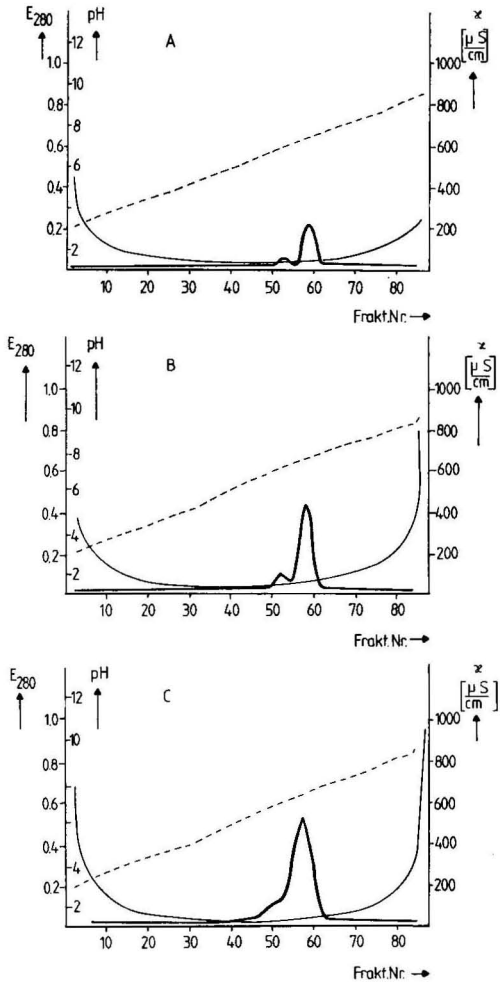


Fig. 2. Abhängigkeit des pH-Gradienten und des Leitfähigkeitsprofils von der Verweilzeit bzw. Durchflussgeschwindigkeit der Trägerampholytlösung bei einer Feldstärke von 110 V/cm. Untere Kurve: Verlauf der Leitfähigkeit, obere Kurve: Verlauf des pH-Wertes. A, Durchflussgeschwindigkeit: 120 cm^3/h, Verweilzeit: 10 min; B, Durchflussgeschwindigkeit: 70 cm^3/h, Verweilzeit: 17 min; C, Durchflussgeschwindigkeit: 50 cm^3/h, Verweilzeit: 20 min.

wo D = Diffusionskoeffizient des Proteins, dpH/dx = Anstieg des pH-Gradienten, E = Feldstärke, dn/dpH = Änderung der Beweglichkeit des Proteins mit dem pH-Wert und D und dn/dpH = Konstanten des Proteins.

Im flachen pH-Gradienten sind die Zonen im Vergleich zur Fokussierung im ausgedehnten pH-Bereich zwar breiter, die Trennungen als solche aber besser (Fig. 4). Das Bandenverhältnis ist in beiden Fällen gleich.

Ein wesentlicher Einfluss der Zugabestelle auf die Fokussierung tritt bei den gewählten Trennbedingungen nicht auf. Auch bei Zugabe im Anoden- oder Kathodenbereich werden, wie das Beispiel der Fokussierung von Rinderserumalbumin (Fig.



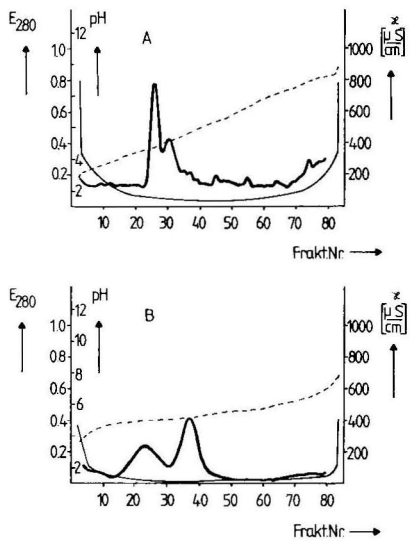


Fig. 3. Einfluss der Trennkapazität auf die Trennschärfe bei der Fokussierung von Pferdemyoglobin. Feldstärke: 110 V/cm, Verweilzeit: 25 min. A, 0.2 mg Protein/h; B, 0.4 mg Protein/h; C, 0.8 mg Protein/h.

Fig. 4. Einfluss der Steilheit des pH-Gradienten auf das Auflösungsvermögen bei der isoelektrischen Fokussierung. Trennung von Rinderserumalbumin und β-Lactoglobulin. Feldstärke: 110 V/cm, Verweilzeit: 25 min. A, pH-Gradient von 2–11; B, pH-Gradient von 4–6.

5) zeigt, übereinstimmende Ergebnisse erhalten. Ist die Zugabestelle sehr weit vom Fokussierungsbereich des Proteins entfernt, sind die erhaltenen Zonen lediglich etwas breiter. Bei Mehrkomponentengemischen (Fig. 6), die mit hoher Auflösung getrennt werden sollen, reicht unter Umständen eine Trennoperation nicht aus. In diesen Fällen müssen entsprechende Fraktionen entweder im selben oder besser im eingeengten pH-Bereich nochmals getrennt werden.

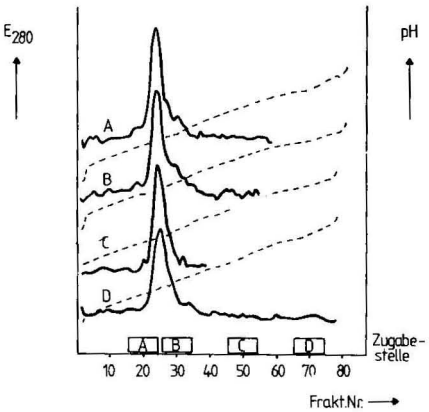


Fig. 5. Abhängigkeit der Fokussierung von der Zugabestelle beim Rinderserumalbumin. Feldstärke: 110 V/cm, Verweilzeit: 25 min.

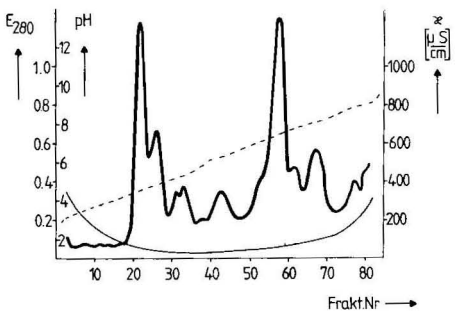


Fig. 6. Isoelektrische Fokussierung eines Proteintestgemisches. (Ferritin, Rinderserumalbumin, β-Lactoglobulin, Conalbumin, Pferdemyoglobin, Walmyoglobin, Ribonuclease, Cytochrom *c*). Feldstärke: 110 V/cm, Verweilzeit: 25 min.

ZUSAMMENFASSUNG

In einer umgerüsteten Apparatur zur trägerfreien Durchflusselektrophorese werden Untersuchungen zur kontinuierlichen isoelektrischen Fokussierung durchgeführt. Dieses Verfahren erlaubt die schonende präparative Trennung amphoterer biochemischer Substanzen. Aus den Einflüssen der verschiedenen Parameter auf das Trennergebnis ergeben sich die optimalen Trennbedingungen. Als Testsubstanzen dienen Einzelproteine und Proteingemische. Bei Verweilzeiten von 20–30 min werden reproduzierbare Trennergebnisse erhalten, damit wird die kontinuierliche trägerfreie isoelektrische Fokussierung für präparative Trennungen interessant.

DANK

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CHROM. 11,133

GAS–LIQUID CHROMATOGRAPHY OF THIOLS USING FLAME-PHOTOMETRIC DETECTION AND A DEACTIVATED TRANSFER LINE

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SUMMARY

2-Mercaptobenzothiazole and benzotriazole have been assessed as reagents for the deactivation of the transfer line between a glass SCOT-capillary column and a flame-photometric detector operated in the sulphur mode. 2-Mercaptobenzothiazole was only moderately effective but coating with benzotriazole gave excellent results and proved suitable for both isothermal and temperature programmed analysis. The minimum detectable level achieved for butanethiol was $1.0 \cdot 10^{-10}$ g when a benzotriazole-treated line was used.

INTRODUCTION

The major problem encountered in the gas chromatography of trace amounts of sulphur compounds is the loss which occurs in the analytical system. This loss, which is particularly acute in the case of thiols is caused by adsorption onto, or reaction with, components of the column[1–7]. A similar loss occurs in the transfer line to the detector[1,8–10].

Losses in the column may be reduced by careful choice of column material[1,3,6,7,11], support[1,3,4], and stationary phase[1,3] and then minimised by conditioning the system with repeated injections of the sample[4], or other suitable sulphur compounds[3,4,6]. In a few cases losses can also be diminished by use of carrier gas continuously doped with hydrogen sulphide or other sulphur compound[3].

Loss in the transfer line may be reduced by keeping this as short as possible or eliminated by fitting the column outlet into the base of the detector[1,11,12]. This is not always feasible, however, and the United Analysts flame-photometric detector (FPD) incorporates a considerable length of metal capillary between the column outlet and the detector base. In some other applications, the minimum length of the transfer line may also be appreciable. For example, if the column effluent is split between two detectors, it is exposed to a considerable area of active sites within pipework and fittings where losses may occur. In addition, in studies on food volatiles a transfer line to an odour port in the wall of the chromatograph oven, where the aroma of the eluted compounds may be assessed, represents a further example of

a moderately long line from which it is necessary to eliminate losses of sulphur compounds.

Since acceptable columns, in particular capillary columns employing polar phases, can fairly readily be prepared, we have used organic compounds which function as corrosion inhibitors to deactivate stainless-steel transfer lines. Whilst this study was in progress, Pearson[11] and Pearson and Hines[13] reported that the treatment of stainless steel with the water-soluble silanizing agent, Siliclad, markedly reduces loss of thiols, although he gave no figures to indicate the efficiency and permanency of the treatment.

EXPERIMENTAL

Reagents

Butanethiol and 2-mercaptobenzothiazole (2-MBT) were obtained from Aldrich (Gillingham, Great Britain), benzotriazole (BTA) from ABM (Stockport, Great Britain), hexamethyldisilazane (HMDS), Carbowax 20M and Atpet 80 from Phase Separations (Queensferry, Great Britain), Silanox 101 (particle size 6–10 μm) from Cabot (Boston, Mass., U.S.A.) and other reagents from BDH (Poole, Great Britain).

Chromatographic instrumentation

The instrument used for gas chromatography was a hybrid composed of the following. Analyser oven and flame-ionization detector (FID) amplifier, Pye series 104 (Pye Unicam, Cambridge, Great Britain); oven temperature and temperature programme modules (Beckman, Fullerton, Calif., U.S.A.), modified in the workshop of the Electrical and Electronic Engineering Department, University of Leeds; FID, made in the workshop of the Procter Department and heated by conduction from the analyser oven; FPD, United Analysts (East Boldon, Great Britain) heated by conduction from the analyser oven and used with hydrogen and oxygen flow-rates of 100 ml/min and 6 ml/min, respectively.

Preparation of SCOT column

Coiled glass capillary tubing (50 m $\times$ 0.5 mm I.D.) was prepared, using an apparatus of the Desty type[14] arranged for drawing and coiling capillary columns, from a length of Pyrex glass tubing (7 mm O.D. $\times$ 4 mm I.D.) which had previously been cleaned using detergent (Decon 75) solution. After annealing at 450° for 3 h and cooling, the capillary was connected to a glass pressure reservoir and dichloromethane (5 ml) passed through it by application of nitrogen pressure. Just before the last of the solvent had left the reservoir, a suspension of Silanox 101 (0.4 g) in dichloromethane (10 ml) containing Carbowax 20M (0.5 g) and Atpet (0.02 g) was added to the reservoir and allowed to flow slowly (*ca.* 10 cm/min) through the column by judicious adjustment of the nitrogen pressure. It was necessary to subject the suspension to ultrasonic vibration for 10 min to ensure adequate dispersion of the particles; it was also essential to ensure that no air bubbles were trapped in the coating suspension and that the movement of the suspension through the coiled tubing did not cease, otherwise blockage would almost certainly occur[15]. After the suspension had left the column, the latter was dried using a stream of nitrogen and conditioned at 180° until its performance was satisfactory.

Chromatography

The column was attached by means of heat-shrinkable PTFE tubing to a glass injection block as described by Cronin[16] and was used with nitrogen as carrier gas at a flow-rate of 4 ml/min. Chromatography was carried out isothermally at 93°, or with temperature programming from 60 to 180° at 2°/min. An injection temperature of 175° was used. The effluent was usually split between the FID and FPD in the ratio 1:1 by means of a stainless-steel tee coupling and stainless-steel capillary tubes (1.6 mm O.D. × 0.3 mm I.D.), although this communication refers only to the response observed on the FPD. On occasion, however, the total column effluent was diverted to the FPD by substituting a solid plug for the FID transfer line at the tee. The FPD line also possessed one section of greater diameter; the precise configuration of the links to the detector will be described later, together with the characteristics of the United Analysts detector.

Deactivation of the transfer line

With hydrogen sulphide. The line was removed from the analyser oven and a stream of hydrogen sulphide (generated from ferrous sulphide and hydrochloric acid) passed through it for 7 h. After heating at 150° in a stream of nitrogen the line was reconnected.

With azoles. The line and effluent splitter was removed from the analyser oven and connected to a reservoir. A solution (1 % in acetone, 25 ml) of 2-MBT or BTA was allowed to flow under gravity so that the last portion of the solution remained in the line. After 1 h the liquid was replaced using fresh solution (25 ml) and again left to stand for 1 h. Replacement of the liquid was carried out three times more before the tube was rinsed with acetone (10 ml) and water (30 ml), conditioned by heating to 150° in a slow stream of nitrogen for 5 h and reconnected. The splitter was similarly treated by filling with the azole solution, which was changed hourly, and allowing to stand for 5 h before washing and conditioning as for the tube.

RESULTS AND DISCUSSION

When small quantities of butanethiol (4.0 ng) were chromatographed using an untreated transfer line, none of the thiol reached the detector. Initially, attempts were made to deactivate the transfer line by passing octanethiol vapour through it and, although some improvement was noted, it was slight, short-lived and inconsistent. Thus, an attempt was made to eliminate active adsorption sites by treatment with the more reactive hydrogen sulphide. Continuous doping of column and transfer line to eliminate losses has been used by Perry and Carter[3]. A similar technique is not suited to the needs of this laboratory, where gas chromatographic effluents from complex mixtures of food volatiles are assessed sensorially, since, clearly, this cannot be carried out in the presence of hydrogen sulphide. The disturbance of the signal from a doped background, which is known to occur when compounds which do not contain sulphur are eluted[17,18], would also cause serious problems during the chromatography of aroma concentrates containing many such compounds. Accordingly, the transfer line was treated with hydrogen sulphide for a period of 7 h and reconnected in the apparatus. Fifteen aliquots (0.5 μl; 4.0 ng) of a freshly prepared solution of butanethiol (0.001 %, v/v, in toluene) were chromatographed on a Car-

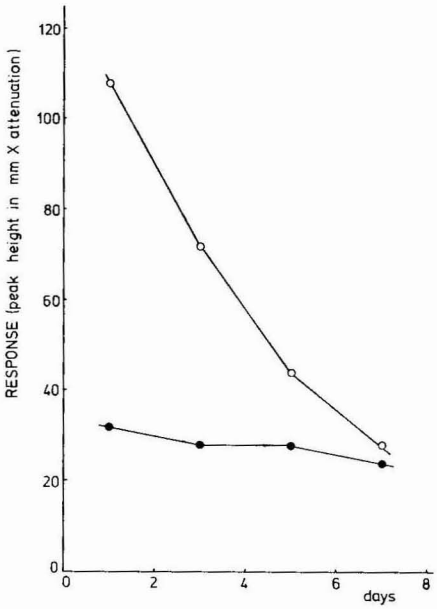


Fig. 1. Response of FPD to 4.0-ng injections of butanethiol after treatment of transfer line with hydrogen sulphide. ●, First treatment; ○, second treatment.

bowax 20M SCOT-capillary column. (The butanethiol sample available contained 5% dibutyl disulphide; thus 0.5 μl of a 0.001% (v/v) solution contained 4.0 ng, not 4.2 ng as would be obtained from pure butanethiol).

The first five injections were regarded as conditioning the column, the injection block, etc. The detector response from the next ten were noted, extreme values were rejected and the average of the eight remaining was calculated. The experiment was repeated on alternate days during 1 week using the same solution and a decrease of response with time observed (Fig. 1). Treatment of the transfer line with hydrogen sulphide was repeated and the detector response measured using a freshly prepared solution of butanethiol. The results (Fig. 1) showed an improvement over those from the initial hydrogen sulphide treatment but the response was still low and decreased with time. A part of the decrease was due to loss of thiol when the dilute solution was stored (see below) but, since hydrogen sulphide treatment was only partially effective, the experiment was not repeated using a fresh solution on each occasion.

Since treatment with hydrogen sulphide had proved only partially effective, deactivation using organic compounds which inhibit corrosion of metals was considered. The most useful corrosion inhibitors consist of a hydrocarbon residue attached to a polar or ionizable group and usually contain sulphur, nitrogen or oxygen atoms. Benzothiazole, BTA, 2-MBT, mercaptobenzimidazole and mercaptobenzoxazole are particularly effective inhibitors of corrosion in copper, copper alloys, iron and steel[19–22]. These compounds exhibit their protective effect through a layer adsorbed onto the metal surface[21–24], and, at least in the case of BTA and copper, chemisorption is involved[21,23,24]. No studies of the corrosion inhibition of stainless steel by these particular compounds have been reported although inhibition by

amines[25,26], quinoline[25,27], dibenzylsulphoxide[25,28], phenylthiourea[25] and a number of proprietary preparations[29] is known. However, since stainless steel clearly contains active adsorption sites, it appeared reasonable to anticipate that it might form stable complexes with high-boiling inhibitors of the azole-type with a consequent reduction in the number of sites available to accept thiols.

It is considered that reaction of azole-type corrosion inhibitors occurs, at least in the case of copper, through the nitrogen atom[30]. However, the compound, 2-MBT, first chosen also contained a thiol group in order to increase the possibility of its reaction with the stainless-steel surface.

After coating the transfer line and the effluent splitter with 2-MBT, fifteen aliquots (0.5 μl; 4.0 ng) of a freshly prepared butanethiol solution (0.001 %, v/v, in toluene) were chromatographed and the average response calculated as above. As indicated in Fig. 2, the FPD response was considerably greater than when the line was treated with hydrogen sulphide, but it decreased by approximately 25 % during the course of one week. This decrease was largely due to loss of the thiol from the dilute solution during storage since the response was largely restored when a freshly prepared solution was chromatographed at the beginning of the second 7-day period*.

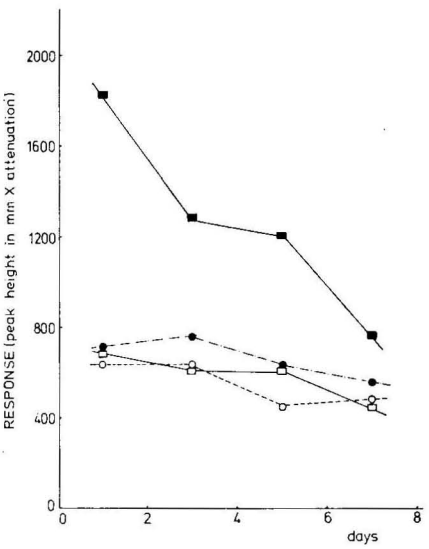


Fig. 2. Response of FPD to 4.0-ng injections of butanethiol after treatment of transfer line with 2-MBT. ●–·–·–●, First week of first coating; ○---○, second week of first coating; □—□, third week of first coating; ■—■, first week of second coating.

During the second 7-day period and in all subsequent experiments, either a freshly prepared solution was used each day or a correction made for the loss of butanethiol which occurred upon storage (solutions were stored for not more than one week, by which time the response had fallen by about 17 %, corresponding to a butanethiol loss of approximately 8 %). Loss of thiols has been encountered in gas mixtures by

* The 7-day periods were not quite consecutive but were separated by 2–3 days.

several authors[31–33] but not, apparently, in solution. The loss was mainly due to oxidation giving dibutyl disulphide, although this compound was not detected when chromatography was carried out at 93° because it was present at less than its minimum detectable level (MDL) at that temperature[34]. However, when a solution which had been stored for one week was examined under other conditions, it was found that dibutyl disulphide equivalent to approximately 6% of the initial thiol concentration had formed during storage[34].

A real loss of response was, however, observed during the second and third 7-day periods (Fig. 2), suggesting that some of the 2-mercaptobenzothiazole was bleeding from the transfer line and reducing its efficiency. Confirmation that bleeding was occurring was given by the presence of a small negative peak for the solvent (toluene) during the first week. The negative peak was, on average, twice as large during the second 7-day period and larger again by a factor of *ca.* 2.5 during the third. Small negative peaks for the solvent were also noted when the transfer line was treated with hydrogen sulphide. The appearance of a negative peak for a hydrocarbon on a steady base line when using a FPD is diagnostic of a bleed containing sulphur. It results from the partial quenching, by the hydrocarbon, of the steady emission from the sulphur[7,17,18,32].

A further consequence of the presence of a bleed containing sulphur is that the response of the FPD to eluted sulphur compounds is enhanced; in fact, deliberate introduction of a steady sulphur bleed can be used as a means of increasing the sensitivity of the detector[17,18]. This is because the signal from the FPD is proportional, not to the amount of sulphur passing through it, but to the *n*th power of that amount (when *n* is usually 1.5–2.0 and theoretically should be 2). Thus, not only is the response enhanced, but small amounts of sulphur are overemphasised relative to larger ones and so the values recorded for butanethiol, particularly at the end of the second and subsequent 7-day periods, are probably somewhat high*; the same reason may account for the increase in response between the end of the second week and the beginning of the third.

The transfer line was again treated with 2-MBT and the behaviour of a freshly prepared butanethiol solution examined as above. The initial response (Fig. 2) was considerably higher than after the initial coating but fell off very sharply during 7 days to a value little higher than that obtained after the first coating, thus indicating the second coating to be less stable than the first. The negative solvent peak was of a size similar to that observed during the third 7-day period following the initial coating; thus the FPD response is likely to be erroneously high by a similar amount.

The bleed indirectly observed at 93° indicated that the coating was unlikely to be suitable for temperature programmed studies and this was confirmed by injecting pentane (1 μl) onto the column and raising the oven temperature from 65° to 150° at 2°/min when a rapidly rising base line was observed. After 3 repetitions,

* It has been stated[3] that when a background due to sulphur is present, the response to samples is then approximately proportional to their concentration, rather than to the square of their concentration. This is only true when the sample response in the absence of background is less than 10–20% of the background response[17]; when the background response is low, *e.g.* of the same order as that given by the smaller samples entering the detector, the effect is to enhance (on the basis of response proportional to the square of the concentration) low values relative to higher ones.

the FPD response to 4.0-ng injections of butanethiol was only 4–5% of that observed prior to carrying out temperature programming.

With weekly recoating, 2-MBT deactivation of the transfer line would be useful for isothermal studies at or below 93°, although not without disadvantages. However, it is clearly unsuited to the temperature-programmed studies necessary in flavour analysis. Attention was thus turned to BTA in the hope that this would be more resistant to removal due to bleeding and, since it is sulphur-free, any slight bleeding which might occur would not affect the response given by the FPD.

The transfer line and associated couplings were well washed using acetone, dried and internally coated with BTA to give line 1_{BTA}. The efficiency of deactivation of 1_{BTA} was assessed as described for 2-MBT and found to be considerably higher (Fig. 3); response to the standard injection fell slightly throughout the first 7-day period but remained approximately constant during the second 7-day period.

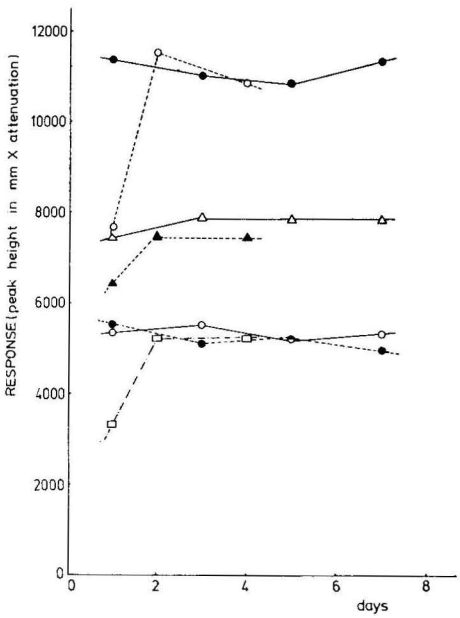


Fig. 3. Response of FPD to 4.0-ng injections of butanethiol after treatment of transfer line with BTA. ●---●, Line 1_{BTA}, first week of first coating; ○—○, line 1_{BTA}, second week of first coating; □-·-·-□, line 1_{BTA}, first week after carrying out temperature programme sequence; △—△, line 1_{BTA}, first week of second coating; ▲---▲, line 1_{BTA}, first week after carrying out temperature programme sequence subsequent to second coating; ●—●, line 2_{BTA}, first week after coating; ○---○, line 2_{BTA}, first week after carrying out temperature programme sequence.

Suitability of the BTA coating in conjunction with temperature programming was assessed by carrying out four temperature programmed analyses (65–150° at 2°/min) using pentane (1 μl). The response to the standard butanethiol injection, determined immediately after programming, had fallen markedly (by 36%) but by the second day the coating had recovered almost all of its efficiency (Fig. 3). The loss of efficiency on temperature programming and its recovery following repeated injec-

tion of butanethiol (*i.e.* by day 2) may, in fact, be largely due to the uncovering, and recovering, of active sites within the column rather than the transfer line. After recoating with BTA the situation was even more satisfactory, a stable coating being obtained which afforded a higher response to the butanethiol solution (Fig. 3) and one which improved somewhat between days 1 and 3 and then remained constant.

Immediately after again performing the temperature programming sequence the response to butanethiol had fallen by 12%, but virtually all of this had been regained by the second day after ending the sequence (Fig. 3). At this time the MDL for butanethiol was $2.0 \cdot 10^{-10}$ g ($1.2 \cdot 10^{-11}$ g sulphur/sec) as compared with a value of $8.0 \cdot 10^{-9}$ g ($2.4 \cdot 10^{-10}$ g sulphur/sec) using the uncoated line.

The BTA-coated transfer line 1_{BTA} was used in conjunction with temperature-programmed gas chromatography of food volatiles for 6 months and was recoated once during that time. The performance of the line was still satisfactory at the end of this period, the MDL of butanethiol then still being $2.0 \cdot 10^{-10}$ g. This value relates to the system in which the column effluent was equally divided between FPD and FID. When the total effluent was directed to the FPD, the MDL became $1.0 \cdot 10^{-10}$ g ($5.9 \cdot 10^{-12}$ g sulphur/sec). The precise halving of the MDL when all the column effluent passed through the transfer line indicates that any losses which occur in the injection block and column must be small.

The BTA-deactivated line, 1_{BTA}, had previously been coated with 2-MBT and cleaned with acetone before treatment with BTA. If any chemisorption of 2-MBT to the line had occurred, this would not have been reversed during the cleaning procedure and the line would therefore have been deactivated partly by 2-MBT and partly by BTA.

In order to obtain a line known to be entirely deactivated by BTA, a new transfer line and associated couplings were thoroughly cleaned using, successively, dichloromethane, concentrated nitric acid, water and acetone, dried and coated to give line 2_{BTA}. The efficiency of deactivation of 2_{BTA} was assessed using butanethiol as above when the response obtained (Fig. 3) was 52% higher than that for line 1_{BTA}. After four temperature programmed runs had been performed, the efficiency had fallen somewhat, but had been fully recovered by the second day.

Although the response to a 4.0 ng injection of butanethiol was considerably enhanced using the line 2_{BTA}, the MDL of the thiol was the same ($2.0 \cdot 10^{-9}$ g when the effluent was divided between the two detectors and $1.0 \cdot 10^{-9}$ g when the total effluent was directed to the FPD) as when line 1_{BTA} was used. The reason for the failure of an increased response to be matched by an improvement in the MDL is not obvious. However, it does indicate the probability that loss in the transfer line is no longer the limiting factor and that improvements would have to be made elsewhere, probably within the detector itself, to obtain greater sensitivity. It may be that the presence of 2-MBT thiol groups in the line 1_{BTA} may have caused a very slight increase in the butanethiol peak width which did not occur when line 2_{BTA} was used, although any such difference was too small to be detected by measurement. In contrast, the small peak obtained at the MDL is, by its very nature, relatively broad and any effect due to the presence of 2-MBT in the transfer line likely to be negligible.

ACKNOWLEDGEMENTS

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GAS–LIQUID–SOLID CHROMATOGRAPHIC DETERMINATION OF PHENOLS IN AIR USING TENAX-GC AND ALKALINE PRECOLUMNS

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SUMMARY

A simple and rapid gas–liquid–solid chromatographic method for the determination of trace concentrations (at ppb* levels) of 11 phenols in air has been developed, using a Tenax-GC precolumn and a Tenax-GC plus alkaline precolumn. The phenols were identified and quantitated from the differences between the chromatogram obtained using the Tenax-GC precolumn and that obtained using the Tenax-GC plus alkaline precolumn. Complete separation was achieved within *ca.* 7 min. No evidence was found for the interference of certain acidic compounds, such as lower fatty acids (C_2–C_7) and mercaptans (C_5–C_7). The method was applied to specimens of ambient air near a phenolic resin factory, and of urban air in the Nagoya area.

INTRODUCTION

Phenols and their derivatives are widely used as antiseptics, disinfectants and pesticides, and in many other chemical applications. Human industrial exposure has been generally limited to accidental contact of phenols with the skin or to inhalation of vapour. Qualitative and quantitative analysis of phenols is a common problem in odour pollution analysis, because these compounds have odour threshold values at ppm or ppb* levels in air[1,2].

Gas chromatography (GC) is widely used for the analysis of mixtures of phenol, cresols and xylenols in automobile exhausts[3,4], cigarette smoke[5–8], waste gas[9], liquid swine manure[10], phenolic resins[11–13] and water[14–17]. However, these analyses were primarily of relatively high concentrations. There have been few reports of the analysis of the lower concentrations of phenols that occur in air.

The complete separation of phenol and *o*-, *m*- and *p*-cresols on GC columns is relatively difficult if packed columns are used. Various types of liquid phase have been proposed for the GC of phenolic isomers[18], but these techniques are not suitable

* Throughout this article, the American billion (10^9) is meant.

for the routine analysis of large numbers of samples, either because long analysis times are necessary to effect complete separation of phenolic isomers, or because the column life is short owing to thermal decomposition.

Recently, DiCorcia[19] reported good separation of phenols using gas–liquid–solid chromatography (GLSC), with a column packed with 0.1–0.5% FFAP on Sterling FT-G (graphitized carbon black). The effectiveness of this packing material is seen from the fact that cresols are separated in an analysis time of only 3 min, and the fractionation of a mixture of more than 20 phenols is achieved by the use of a single column, with an elution time of not more than 45 min.

This paper describes a simple and rapid GLSC method that uses as the column packing material 0.1% SP-1000 on Carbopack C (80–100 mesh). The phenols in air were concentrated on a Tenax-GC precolumn at room temperature, and analysed by GC with a flame ionization detector (FID). The phenols were identified and quantitated from the difference between the chromatogram obtained using the Tenax-GC precolumn and that obtained using the Tenax-GC plus alkaline precolumn. The method was applied to specimens of ambient air near a phenolic resin factory, and of urban air in the Nagoya area.

EXPERIMENTAL

Reagents

Eleven phenols and solvent ethanol were obtained from Wako (Osaka, Japan), or Tokyo Kasei Kogyo (Tokyo, Japan). All reagents were guaranteed- or reagent-grade chemicals. The purity of each phenol was checked by GC. The procedure for the preparation of the standard phenolic solution was as follows: 0.095–0.45 mole of each phenol was dissolved in 10 ml of distilled water or ethanol, and 0.2 ml of each solution were mixed together.

GLSC conditions

The gas chromatograph used was a Shimadzu Model GC $5AP_5F$, equipped with a FID and a digital integrator (Shimadzu Model ITG-2A) for the determination of the relative retention times and the calibration curves. The latter were plotted as peak height or peak area (as the counts of the digital integrator) against quantity (ng) of the phenols. The glass analytical column (1.75 m × 3 mm I.D.) was packed with 0.1% SP-1000 on Carbopack C (80–100 mesh), obtained from Gasukuro Kogyo, Tokyo, Japan. The optimum chromatographic conditions for FID were as follows: column temperature; 205–220°; injection port and detector temperature, 250°; carrier gas (nitrogen) flow-rate, 37–55 ml/min; hydrogen and air flow-rates, 50 ml/min and 1.0 l/min, respectively. The glass precolumn[20] (18 cm × 4 mm I.D.) was packed with Tenax-GC (60–80 mesh). Air samples were preconditioned at 250° for 10 h with a constant flow-rate of nitrogen (50 ml/min), and then concentrated at room temperature before being injected into the chromatograph at a temperature of 250° for 35 sec. The concentration velocity of specimen air samples was 0.22–0.33 l/min. The Tenax-GC plus alkaline precolumn contained 2% KOH on glass beads (30–60 mesh; 7 cm × 4 mm I.D.). With the latter precolumn the peaks of phenols disappeared completely in the chromatograms, enabling the phenols to be identified by comparison with the chromatograms from the ordinary Tenax-GC precolumn.

TABLE I

RELATIVE RETENTION TIMES OF 11 PHENOLS AND 10 LOWER FATTY ACIDS USING THE DIRECT INJECTION METHOD (PHENOL = 1.00)

0.1% SP-1000 on Carbopack C (80–100 mesh), 1.75 m, 205°, N_2 55 ml/min.

Compound	*RtRd*	*Compound*	*RtRd*
Phenol	1.00	Acetic acid	0.21
o-Cresol	2.31	Propionic acid	0.31
m-Cresol	2.70	*iso*-Butyric acid	0.37
p-Cresol	2.90	*n*-Butyric acid	0.41
p-Ethylphenol	3.90	*iso*-Valeric acid	0.61
o-Ethylphenol	5.06	*n*-Valeric acid	0.71
2,6-Xylenol	5.85	*iso*-Caproic acid	1.20
2,5-Xylenol	7.38	*n*-Caproic acid	1.43
3,5-Xylenol	8.00	*n*-Heptanoic acid	3.20
2,3-Xylenol	8.04	*n*-Octanoic acid	7.49
3,4-Xylenol	9.47		

RESULTS AND DISCUSSION

Table I shows the relative retention times (*RtRd*) of 11 phenols, and of 10 lower fatty acids (C_2–C_8) that are occasionally found with the phenols in polluted air, as determined by the direct injection method on the SP-1000 column at a column temperature of 205°. The retention time of phenol was defined as unity.

Table II shows the relative retention times (*RtRp*) of 11 phenols as determined by the Tenax-GC precolumn injection method on the SP-1000 column at a column temperature of 220°. The retention time of phenol was again defined as unity.

Fig. 1 shows a plot of *RtRp* against *RtRd* for the 11 phenols. The plot is linear, and thus it is possible to estimate qualitatively *RtRp* values from *RtRd* values. The data in Table I confirm that the analysis of the phenols under these conditions is not interfered with by the fatty acids, because there is no overlap between phenol peaks and fatty acid peaks.

TABLE II

RELATIVE RETENTION TIMES OF 11 PHENOLS USING THE TENAX-GC PRECOLUMN INJECTION METHOD (PHENOL = 1.00)

0.1% SP-1000 on Carbopack C (80–100 mesh), 1.75 m, 220°, N_2 37 ml/min.

Compound	*RtRp*
Phenol	1.00
o-Cresol	1.82
m-Cresol	2.03
p-Cresol	2.14
p-Ethylphenol	2.74
o-Ethylphenol	3.37
2,6-Xylenol	3.92
2,5-Xylenol	4.71
3,5-Xylenol	5.05
2,3-Xylenol	5.03
3,4-Xylenol	5.86

Fig. 2 shows the retention times of phenols, lower fatty acids, aromatic hydrocarbons, aliphatic hydrocarbons (saturated) and aliphatic hydrocarbons (unsaturated), alcohols, mercaptans, halides and other compounds as determined by the direct injection method on a SP-1000 column at a column temperature of 210° and nitrogen carrier flow-rate of 50 ml/min. Phenol overlaps with 2,3,4-trimethylpentane and hexachloroethane; *o*-cresol overlaps with styrene; *m*-cresol overlaps with *o*-, *m*- and *p*-xylene, and *iso*-amyl propionate; 2,6-xylenol overlaps with *m*-tolualdehyde; 2,3- and

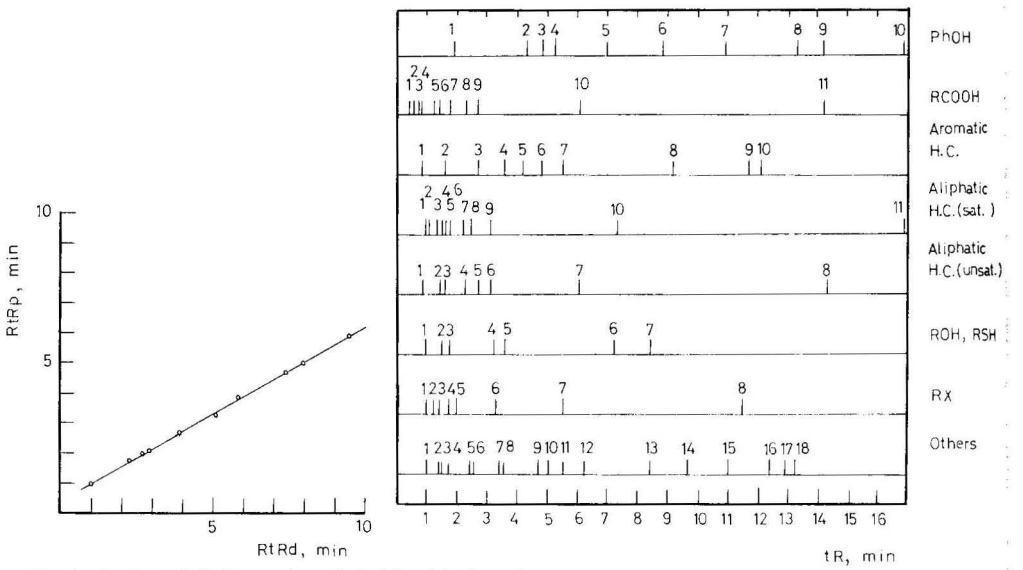


Fig. 1. A plot of *RtRp* against *RtRd* for 11 phenols.

Fig. 2. Retention times data. *PhOH*: 1 = phenol; 2 = *o*-cresol; 3 = *m*-cresol; 4 = *p*-cresol; 5 = *p*-ethylphenol; 6 = *o*-ethylphenol; 7 = 2,6-xylenol; 8 = 2,5-xylenol; 9 = 2,3- + 3,5-xylenols; 10 = 3,4-xylenol. *RCOOH*: 1 = acetic acid; 2 = propionic acid; 3 = *iso*-butyric acid; 4 = *n*-butyric acid; 5 = *iso*-valeric acid; 6 = *n*-valeric acid; 7 = diethylacetic acid; 8 = *iso*-caproic acid; 9 = *n*-caproic acid; 10 = *n*-heptanoic acid; 11 = *n*-octanoic acid. *Aromatic hydrocarbons:* 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = *iso*-propylbenzene; 5 = styrene; 6 = *o*-, *m*- and *p*-xylene; 7 = *n*-propylbenzene; 8 = *p*-cymene; 9 = *n*-butylbenzene; 10 = mesitylene. *Aliphatic hydrocarbons (saturated):* 1 = *n*-hexane; 2 = methylcyclohexane; 3 = 3-methylhexane; 4 = 2,2,4-trimethylpentane; 5 = *n*-heptane; 6 = 2,3,4-trimethylpentane; 7 = 2,4- + 2,5-dimethylhexane; 8 = 3-methylheptane; 9 = *n*-octane; 10 = *n*-nonane; 11 = *n*-decane. *Aliphatic hydrocarbons (unsaturated):* 1 = hex-1-ene; 2 = hept-1-ene + hept-2-ene (*cis*, *trans*) + hept-3-ene + 2,4,4-trimethylpent-2-ene; 3 = 2,4,4-trimethylpent-1-ene; 4 = 2-ethylhex-1-ene; 5 = oct-1-ene; 6 = oct-2-ene; 7 = non-1-ene + non-4-ene; 8 = dec-1-ene. *ROH, RSH*: 1 = pentan-1-ol; 2 = *n*-amyl mercaptan; 3 = hexan-1-ol; 4 = *n*-hexyl mercaptan; 5 = heptan-1-ol; 6 = *n*-heptyl mercaptan; 7 = octan-1-ol. *RX*: 1 = *iso*-amyl chloride; 2 = *n*-amyl chloride; 3 = 1,1,2,2-tetra-chloroethane + tetrachloroethylene; 4 = *p*-dibromobenzene; 5 = hexachloroethane; 6 = benzyl chloride; 7 = *o*-dichlorobenzene; 8 = *n*-octyl chloride. *Others*: 1 = cyclohexanone; 2 = *n*-capronaldehyde + hexan-2-one; 3 = *n*-butyl acetate + *iso*-amyl formate; 4 = *n*-amyl formate; 5 = *iso*-amylacetate + *n*-butyl propionate; 6 = heptan-2-one; 7 = *n*-hexyl formate; 8 = benzaldehyde; 9 = *iso*-amyl propionate; 10 = *iso*-octyl acetate; 11 = octan-2-one; 12 = *n*-hexyl acetate; 13 = acetophenone; 14 = *o*-tolualdehyde; 15 = *m*-tolualdehyde; 16 = nonan-2-one + *n*-octyl acetate; 17 = *p*-tolualdehyde; 18 = n-hexyl propionate. Conditions: 0.1% SP-1000 on Carbopack C (80–100 mesh), 1.75 m, 210°, N_2 50 ml/min, direct injection method.

3,5-xylenol overlap with *n*-octanic acid and dec-1-ene; and 3,4-xylenol overlaps with *n*-decane. These compounds, except for *n*-octanoic acid, passed through the alkaline precolumn, but phenols were completely trapped. Therefore, the phenols in the sample air can be identified and quantitated from the differences between the chromatograms obtained using the Tenax-GC precolumn and that obtained using the Tenax-GC plus alkaline precolumn.

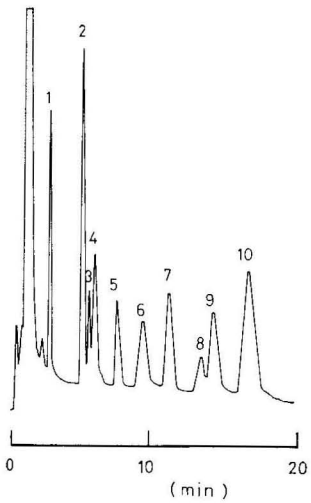


Fig. 3. Typical chromatogram of 11 phenols obtained with Tenax-GC precolumn. Peaks: 1 = phenol 68 ng; 2 = *o*-cresol 180 ng; 3 = *m*-cresol 38 ng; 4 = *p*-cresol 64 ng; 5 = *p*-ethylphenol 52 ng; 6 = *o*-ethylphenol 56 ng; 7 = 2,6-xylenol 96 ng; 8 = 2,5-xylenol 36 ng; 9 = 2,3- + 3,5-xylenol (36 + 64 ng); 10 = 3,4-xylenol 180 ng. Conditions: 0.1% SP-1000 on Carbopack C (80–100 mesh), 1.75 m × 3 mm I.D., 220°, N_2 37 ml/min; FID: range 2 (× 0.01 V), sensitivity 10^2 (× MΩ).

Fig. 3 shows a typical chromatogram of the 11 phenols obtained using the Tenax-GC precolumn injection method. Complete separation occurred within 18 min with no tailing, except for 2,3- and 3,5-xylenols, the peaks of which overlapped. It is particularly noteworthy that *o*-, *m*- and *p*-cresols were completely separated within *ca.* 7 min, with no tailing.

Under these GC conditions, the mixture of 11 phenols gives 10 peaks, and the components present in lower concentrations can be detected by normal FID. This GC column has a separating power better than that of some glass capillary columns. Because capillary columns are necessary, in general the splitter holding for a high resolving power which is low produced the lower sensitivity to lower concentration compounds[18].

Table III shows that the values of the retention times and the peak areas of nine phenols determined by the Tenax-GC precolumn injection method show good uniformity and reproducibility.

The FID response produced a linear relationship in the range 2–3000 ng for four representative phenols (phenol and *o*-, *m*- and *p*-cresols), and the minimum detectable amount at twice the noise level was *ca.* 1 ng for all four phenols, using the

TABLE III

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS OF NINE PHENOLS USING THE TENAX-GC PRECOLUMN INJECTION METHOD

$n = 6$; C.V. = coefficient of variation. For chromatographic conditions see Experimental. N_2 55 ml/min; column temperature 205°; FID range 4 (× 0.01 V), sensitivity 10^2 (× MΩ); Tenax-GC precolumn 18 cm × 4 mm I.D. (60–80 mesh).

Compound	*Amount (ng)*	*Retention time (min)*	*C.V. (%)*	*Peak area**	*C.V. (%)*
Phenol	170	2.70 ± 0.026	0.96	267 ± 18.3	6.85
o-Cresol	450	5.17 ± 0.035	0.68	545 ± 28.2	5.17
m-Cresol	94	5.86 ± 0.035	0.60	144 ± 8.52	5.92
p-Cresol	160	6.22 ± 0.039	0.63	224 ± 23.1	10.31
p-Ethylphenol	130	8.05 ± 0.046	0.57	200 ± 8.89	4.45
o-Ethylphenol	140	10.15 ± 0.052	0.51	158 ± 11.6	7.34
2,6-Xylenol	240	11.75 ± 0.055	0.47	328 ± 18.5	5.64
2,5-Xylenol	91	15.65 ± 0.067	0.43	280 ± 19.1	6.82
3,4-Xylenol	450	18.47 ± 0.085	0.46	667 ± 39.9	5.98

* Expressed as the counts of a digital integrator.

Tenax-GC precolumn injection method. Fig. 4 shows the typical calibration curves for phenol and *o*-, *m*- and *p*-cresols.

Fig. 5(a) shows a typical chromatogram of phenol (peak 1) and *o*-cresol (peak 2) in the ambient air of a phenolic resin factory. The volume collected was 0.3 l, and the sample was trapped directly into the Tenax-GC precolumn. Fig. 5(b) shows a typical chromatogram of the air collected on the Tenax-GC precolumn, but passed through the alkaline precolumn. In this chromatogram the phenol peak has completely disappeared, but the *o*-cresol peak is still present, confirming the identification of

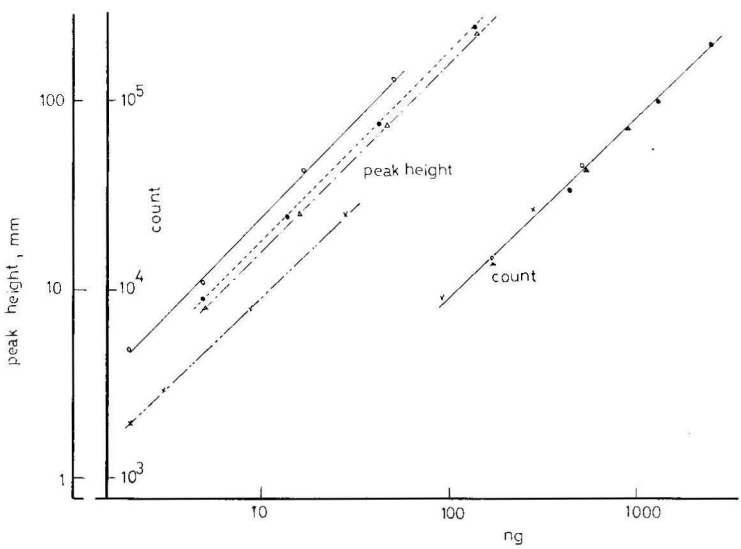


Fig. 4. Typical calibration curves for phenol (○), *o*-cresol (●), *m*-cresol (×) and *p*-cresol (△) using Tenax-GC precolumn. FID: range 1 (× 0.01 V), sensitivity 10^2 (× MΩ).

phenol and *o*-cresol made from the chromatogram in Fig. 5(a). Fig. 5(c) shows a typical chromatogram of small amounts of the standard 11 phenols. The concentrations of phenol and *o*-cresol detected were calculated to be 50 and 40 ppb, respectively.

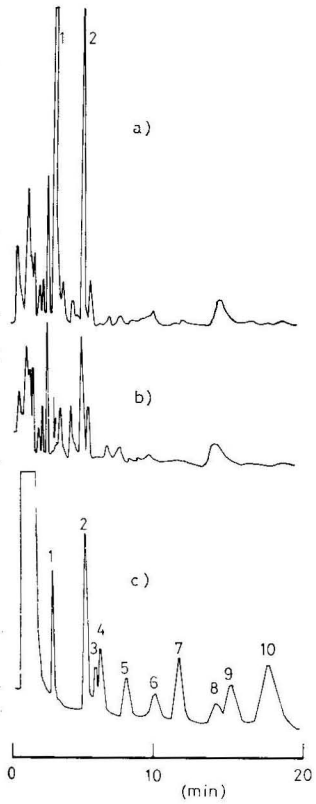


Fig. 5. Typical chromatograms of phenols in the ambient air near a phenolic resin factory, using (a) Tenax-GC precolumn only, (b) Tenax-GC plus alkaline precolumn, and (c) the standard 11 phenols with Tenax-GC precolumn injection. (a) Collected volume added to the Tenax-GC precolumn: 0.3 litre. Peaks: 1 = phenol (50 ppb); 2 = *o*-cresol (40 ppb). (b) Collected volume added to the Tenax-GC plus alkaline precolumn: 0.3 l. (c) Standard 11 phenols with Tenax-GC precolumn injection. Peaks: 1 = phenol 17 ng; 2 = *o*-cresol 45 ng; 3 = *m*-cresol 9 ng; 4 = *p*-cresol 16 ng; 5 = *p*-ethylphenol 13 ng; 6 = *o*-ethylphenol 14 ng; 7 = 2,6-xylenol 24 ng; 8 = 2,5-xylenol 9 ng; 9 = 2,3- + 3,5-xylenols (9 + 16 ng); 10 = 3,4-xylenol 45 ng. The GC conditions are as given in the caption to Fig. 3.

The method was also applied to the determination of phenol in samples of urban air from the Nagoya area. The volume concentrated was 5 l. The range and average concentration of phenols detected in 39 such samples, collected between August 3rd and 20th, 1977, were 0.05–2.3 ppb and 0.44 ppb, respectively.

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CHROM. 10,999

DETECTION OF GOITRIN AND ITS HEPTAFLUOROBUTYRYL DERIVATIVE BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE, ELECTROLYTIC CONDUCTIVITY AND SULFUR DETECTORS

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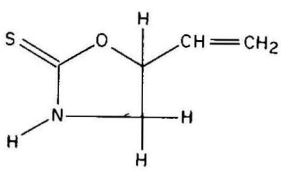
(First received June 21st, 1977; revised manuscript received March 7th, 1978)

SUMMARY

The separation of goitrin by two gas–liquid chromatographic columns, the response of different detectors and the use of the heptafluorobutyric (HFB) anhydride derivative of goitrin to improve sensitivity was investigated. The non-polar 3% OV-1 and the intermediate 4% SE-30/6% SP-2401 on 80–100 mesh Chromosorb W HP gave comparable results and were considered interchangeable. The sensitivities of the electron capture, sulfur 394 nm emission, chlorine and nitrogen Coulson electrolytic conductivity detectors were inadequate for goitrin *per se*. Chromatography and sensitivity of all detectors to goitrin were greatly improved by using by using the HFB derivative. It was possible to detect 1 to 60 ng of goitrin-HFB in standard solutions. Application of the technique to goitrin-spiked (2 ppm) milk, cleaned up by high-performance liquid chromatography, encountered no problems and was considered satisfactory for all four detectors.

INTRODUCTION

Goitrin, 1,5-vinyloxazolidine-2-thione (Fig. 1), is the aglycone hydrolysis product of progoitrin, a glucosinolate found in rape, turnip and cabbate[1–4]. Goitrin is similar to thiouracil in antithyroid activity[1]. The presence of this compound in foods for human consumption is of some concern to health authorities since residues may occur in milk and other products from animals fed with rapeseed meal protein supplements[5–7].



GOITRIN

Fig. 1. Chemical structure of goitrin.

Analytical procedures for glucosinolates have been reported that are based on ultraviolet (UV) measurements[1,3,4], infrared (IR)[8] and gas–liquid chromatography (GLC) with flame ionization detection[6,10–13]. The latter technique is preferred as it separates and detects various glucosinolates or their hydrolytic products. However, these methods are not adequate for milk as they do not have the necessary sensitivity and specificity for regulatory purposes. There is a need to improve the method.

Recently, we undertook to investigate the response of other detectors such as the ^{63}Ni electron-capture (ECD), Coulson electrolytic conductivity (CECD, nitrogen and halogen modes) and Melpar flame photometric emission (MFPD, sulfur, 394 nm) for goitrin and its haptafluorobutyryl (HFB) derivative. This report describes our results which includes the application of the HFB derivatization technique to milk samples spiked at 2.0 ppm with goitrin.

EXPERIMENTAL

A goitrin standard was prepared from a crude extract of rapeseed meal by thin-layer chromatographic (TLC) isolation techniques. Identity and an indication of purity were established using UV, IR, high-resolution mass spectrometry and nuclear magnetic resonance procedures. A second portion of purified goitrin was obtained from Lancaster Synthesis (Lancaster, Great Britain). Goitrin from both sources were identical as based on the above-mentioned tests. Stock solutions were prepared in methylene chloride and aliquots were serially diluted with the same solvent to the desired concentrations.

All organic solvents were purchased as glass-distilled residue-free grade.

Trimethylamine (anhydrous, Eastman-Kodak, Rochester, N.Y., U.S.A.) in glass ampoules was cooled in ice water and added with stirring to cool, tared benzene to produce a 0.5-*M* solution.

Heptafluorobutyric anhydride (HFBA, PCR Research Chemicals, Gainville, Fla., U.S.A.) was used as received from the supplier.

Gas–liquid chromatography

Two columns were selected for evaluation in resolving goitrin; a non-polar, 3% OV-1, and an intermediate one consisting of 4% SE-30/6% SP-2401, each coated on 80–100 mesh Chromosorb W HP. The stationary phases were packed in approximately 110 cm × 4 mm I.D. borosilicate glass columns and conditioned at temperatures ten degrees below the specified operating maximum of their liquid phases until a consistent response was obtained.

The CECD, MFPD and ECD were fitted to a Tracor Microtek MT220 gas chromatograph. Operating conditions for the CECD in the nitrogen or halogen mode were: pyrolysis furnace, 800°; transfer unit, 235°; d.c. bridge potential, 30 V; hydrogen flow-rate 37 ml/min. The ECD temperature was 280°, while the nitrogen purge gas flow-rate was 42 ml/min. The MFPD block temperature was 175° and the flame gases were: hydrogen (200 ml/min) and air (200 ml/min). The CECD and MFPD used helium as a carrier gas (flow-rate, 40 ml/min) while the ECD system used nitrogen (flow-rate, 40 ml/min). Inlet and column temperatures for all systems were 200 and 149°, respectively.

Derivative preparation[14]

Aliquots containing 20–100 μg of goitrin in methylene chloride were transferred to 15-ml glass stoppered or PTFE-lined screw-cap centrifuge tubes. The solutions were taken to dryness by evaporation at room temperature, with the assistance of a gentle stream of nitrogen gas. To each tube was added 1 ml benzene, 20 μl HFBA, followed by 0.1 ml of a 0.5-*M* TMA solution, and thorough mixing. The reaction was allowed to proceed for 30 min at room temperature. At the end of this time 12 ml distilled water were added to each tube, and the mixture thoroughly shaken for 1 min. The water phase was separated from the benzene by centrifuging for 5 min at 500 *g*, then discarded. Aliquots of the washed benzene phases were then analyzed under the various GLC conditions reported.

Analysis of milk samples

Homogenized milk samples were extracted according to Benns *et al.*[15] for analysis by high-pressure liquid chromatography (HPLC). Briefly, the milk was extracted with ether, which was evaporated to dryness. Warm water was added to the residue which dissolved the goitrin. This was extracted with hexane to remove the lipid then finally extracted with ether again for evaporation and analysis by HPLC. An aliquot of the final extract equivalent to 25 g of milk was spiked with 50 μg goitrin and taken to dryness, then dissolved in 1 ml of benzene. The solution was treated exactly as described above for the derivatization, then analysed by GLC with the four detectors. Recoveries through this extraction method have been shown to be $>75\%$ at the 2.0 ppm level of spiking[15].

RESULTS AND DISCUSSION

The direct GLC of goitrin was found to be unsuitable for application to its dermination in milk samples at low ppm levels. The sensitivities of the various detectors to goitrin itself were very poor and could not be used for residue purposes. Electron capture detection proved to be the most sensitive but still required 200–300 ng to produce a 10-cm peak (2.5 min retention time) at $16 \cdot 10^{-2}$ sensitivity setting. The other detectors required microgram quantities for a response.

To improve chromatography and increase detector sensitivity, we sought to prepare a suitable derivative. The HFB derivative proved to be very satisfactory providing much superior response to all detectors evaluated. Fig. 2 illustrates the reaction. The HFB group attaches to the nitrogen atom of the goitrin molecule. Some chromatographic results are shown in Fig. 3 for the four detectors studied. Both OV-1 and SE-30/SP-2401 column packings gave good resolution and were considered interchangeable. Two peaks were present in all chromatograms except those obtained with the MFPD which showed only one derivative peak. This peak corresponded to the latter peak of the other chromatograms and thus was used for quantitative measurements in the milk samples. Although the absolute yield of product was not calculated, the observed sensitivities indicate a high conversion the derivative. The yield wss very consistent producing linear results over a range of 10–100 μg reacted.

Fig. 4 shows some response plots of HFB-goitrin obtained with the four detectors using the mixed column for chromatography. It can be seen that the ECD was the most sensitive to the derivative. The CECD in the halogen mode was the

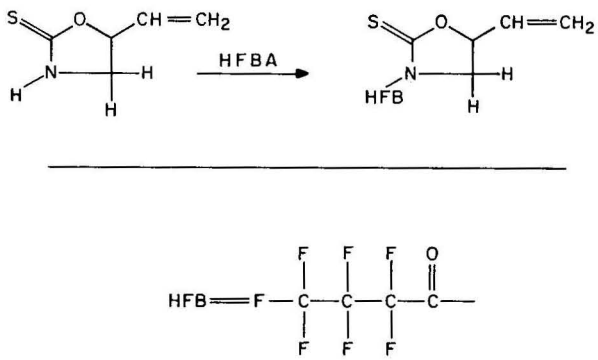


Fig. 2. HFBA reaction with goitrin.

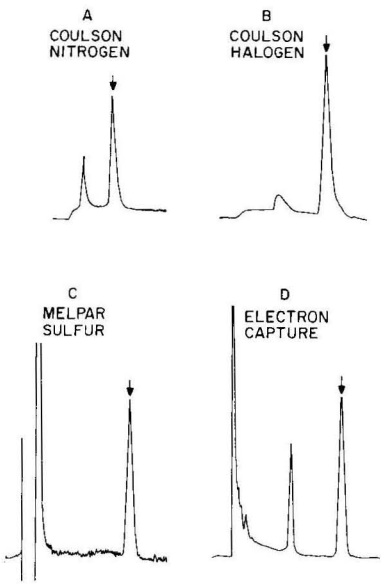


Fig. 3. Detection of HFB-goitrin by A, CECD, nitrogen mode; 130 ng injected; 1 × attenuation; t_R = 6.5 min; B, CECD, halogen mode; 38 ng injected; 1 × attenuation; t_R = 6.8 min; C, MFPD, sulfur; 45 ng injected; 2 × attenuation·10^{-3}; t_R = 6.9 min; D, ECD, 0.6 ng injected; 8 × attenuation·10^{-2}; t_R = 6.9 min. All chromatograms obtained on the 4% SE-30/6% SP-2401 column.

second most sensitive. This is attributed to the large number of fluorine atoms in the HFB derivatives. The nitrogen detector was the least sensitive of the four.

Application of this derivatization technique to spiked milk extracts proved to be very satisfactory. A goitrin concentration of 2 ppm was easily detected in the milk samples. No problems were encountered in carrying out the reaction in the presence of milk co-extractives. Fig. 5 shows some representative chromatograms of spiked milk samples, obtained with the four detectors studied. All detectors proved to be satisfactory for the analysis, although the ECD was sensitive enough to keep the quantity of sample injected to less than one-tenth that of the other detectors. This

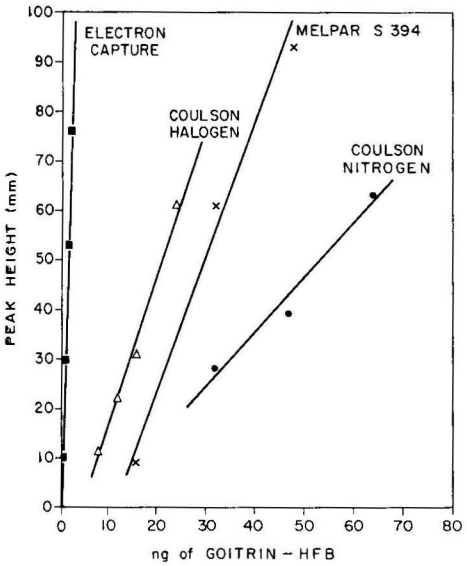


Fig. 4. Response plots for HFB-goitrin obtained with the various detectors used at sensitivity setting described in Fig. 3.

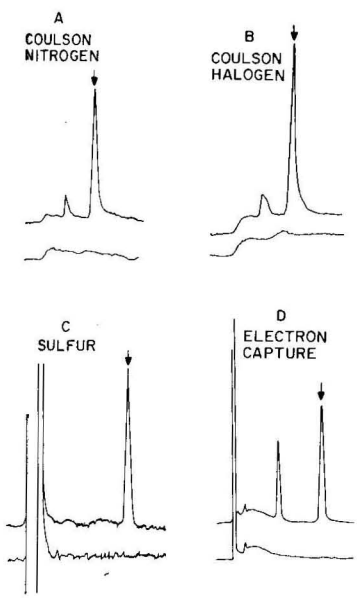


Fig. 5. Chromatograms of homogenized milk extracts containing 2.0 ppm goitrin. A, CECD, nitrogen mode, 62.5 mg injected; B, CECD, halogen mode, 39 mg injected; C, MPFD, sulfur; 32.5 mg injected; D, ECD, 0.29 mg injected. Top chromatogram, spiked milk; bottom chromatogram, blank milk.

may be of some importance if goitrin analysis is carried out on a routine basis, since column contamination would be much reduced with smaller quantities injected.

CONCLUSION

The GLC analysis of goitrin as a HFB derivative shows much promise for application to ppm or lower levels in foods. The various sensitivities and selectivities of the detectors to the derivative should prove to be very useful in development of an analytical method.

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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF β-DIKETONE-CONTAINING PLANT WAXES

USE OF TRIMETHYLSILYL ETHERS*

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(First received October 21st, 1977; revised manuscript received March 20th, 1978)

SUMMARY

A number of β-diketones and β-diketone-containing waxes have been trimethylsilylated and analyzed by gas chromatography–mass spectrometry. The trimethylsilyl enol ethers of β-diketones and hydroxy and oxo β-diketones are completely separated by gas chromatography. Mass spectrometry of these derivatives gives simpler fragmentation patterns than those obtained with untreated β-diketones so that structure determination of β-diketones and oxygenated β-diketones, including structural isomers, is considerably facilitated. Trimethylsilyl derivatives of free acids and alcohols formed at the same time are separated from each other and from hydrocarbons by gas chromatography and are also identified by mass spectrometry.

INTRODUCTION

Analysis of unfractionated waxes from many grass species by gas–liquid chromatography (GLC) shows the presence of β-diketones and hydroxy β-diketones[1–3], but when oxo β-diketones are also present[4] they cannot be distinguished from hydroxy β-diketones. Also when the hydroxy β-diketones are mixtures of isomers[5–7] they cannot be identified by GLC alone. Mass spectrometry (MS) of β-diketones[8,9] and gas chromatography (GC)–MS of β-diketone containing whole waxes[10] has shown that these compounds give characteristic spectra and that the positions of the β-diketone grouping and of hydroxyl substituents can be established[1,9,10]. However MS of β-diketones gives a relatively large number of fragments due to the many possible α-cleavages and also to McLafferty rearrangements[9] which is a disadvantage when mixtures of isomers are present.

We have now found that β-diketones and oxygenated β-diketones can be largely converted to trimethylsilyl (TMS) enol ethers and that the derivatives are better resolved and more conveniently identified by GC–MS analysis than in previous analytical procedures.

* Issued as NRCC No. 16679.

EXPERIMENTAL

Materials

Long-chain acids, alcohols and esters were previously[1] prepared synthetic compounds. β-Diketones and hydroxy and oxo β-diketones were previously isolated from the grass waxes analyzed earlier[4,5]. Mixtures containing β-diketones and natural waxes were trimethylsilylated as follows: wax (5 mg) was dissolved in 0.3 ml toluene–pyridine (5:1) and 25 μl hexamethyldisilazane; 12 μl trimethylchlorosilane were added and the mixture was kept at room temperature overnight[11].

GC–MS conditions

A Finnigan Model 3300 GC–MS system with an Incos Model 2000 data system was used. The transfer lines and the jet separator were maintained at 275° and the GC injector was kept at 300° and the ionization voltage was 70 eV. The glass column was 1.7 m × 2 mm I.D. and was packed with 1.5% Dexsil 300 on 80–100 mesh acid washed and silanized Chromosorb W. The initial helium pressure was 25 p.s.i. and rose to 40 p.s.i. during programming; temperature was programmed from 175° to 380° at 4°/min. For the first 60 sec after sample injection column effluent was diverted through the GC vacuum divert valve into a mechanical pump to avoid contamination of the ion source.

RESULTS AND DISCUSSION

The reconstructed gas chromatogram (RGC) obtained when a trimethylsilylated mixture of approximately equal amounts of octacosanol, hentriacontane-14,16-dione, 25-hydroxyhentriacontane-14,16-dione, 25-oxohentriacontane-14,16-dione, and docosyl docosanoate was examined by GC–MS is shown in Fig. 1. Some

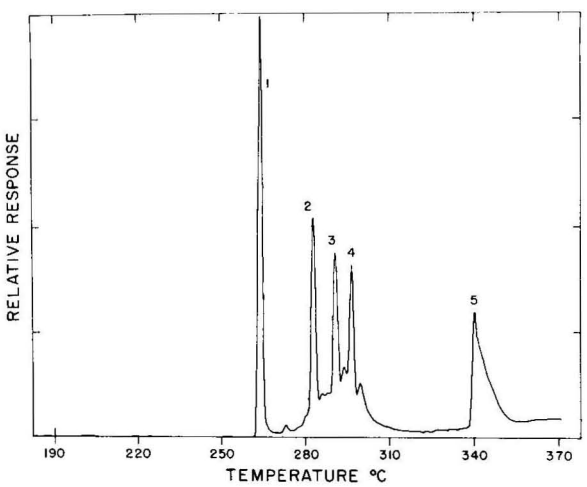


Fig. 1. Reconstructed gas chromatogram from GC–MS analysis of trimethylsilylated mixture of approximately equal amounts of octacosanol (1), hentriacontane-14,16-dione (2), 25-hydroxyhentriacontane-14,16-dione (3), 25-oxohentriacontane-14,16-dione (4) and docosyl docosanoate (5).

decomposition of the β-diketone derivatives occurred during GC but they mainly appeared as TMS ethers of the enol forms. The TMS ether of the hydroxy β-diketone (peak 3) eluted earlier than, and well separated from the oxo β-diketone derivative (peak 4). The intensities of the peaks were not proportional to the amounts of the components and the response of the alcohol TMS ether is particularly large.

Fragment ions produced from the above three β-diketone TMS derivatives and from some other oxygenated β-diketone wax components (mentioned below) were 1. hentriacontane-14,16-dione *m/e* (rel. int.).: 536 M^+ (2), 521 M − 15 (8), 446 (6), 353 (37), 325 (36), 250 (6), 185 (12), 172 (12), 169 (12), 157 (16), 75 (18), 73 (100); 2. 25-hydroxyhentriacontane-14,16-dione: 624 M^+ (1), 609 M − 15 (5), 539 (22), 441 (5), 351 (7), 325 (16), 187 (24), 169 (9), 157 (6), 75 (28), 73 (100); 3. mixture of 8- and 9-hydroxyhentriacontane-14,16-diones: 624 M^+ (0.1), 609 M − 15 (1), 525 (0.2), 511 (0.1), 413 (2), 353 (18), 215 (4), 201 (6), 157 (7), 75 (23), 73 (100); 4. 25-oxohentriacontane-14,16-dione: 550 M^+ (1), 535 (5), 465 (1), 367 (19), 325 (29), 185 (10), 183 (5), 169 (12), 157 (12), 113 (19), 75 (20), 73 (100); 5. 10-oxohentriacontane-14,16-dione: 550 M^+ (1), 535 M − 15 (4), 423 (1), 353 (25), 339 (8), 255 (8), 249 (9), 225 (16), 222 (9), 197 (18), 185 (19), 183 (6), 169 (17), 155 (11), 75 (21), 73 (98), 43 (100).

Fig. 2 shows the probable mass fragmentation patterns of these derivatives, the TMS group is shown as attached to only one of the two equally likely positions.

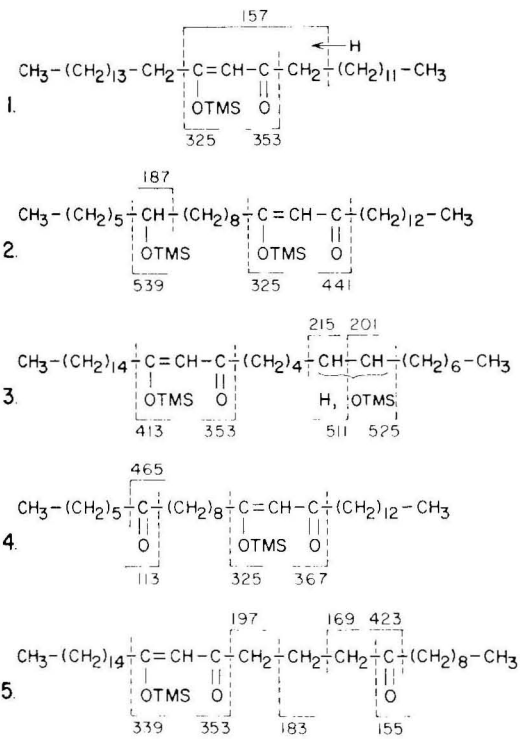


Fig. 2. Mass fragmentation of TMS enol ethers of β-diketones. 1 = Hentriacontane-14,16-dione, 2 = 25-hydroxyhentriacontane-14,16-dione, 3 = mixture of 8- and 9-hydroxyhentriacontane-14,16-diones, 4 = 25-oxohentriacontane-14,16-dione and 5 = 10-oxohentriacontane-14,16-dione.

The unsubstituted β-diketone derivative (Fig. 2.1) undergoes cleavage on both sides of the oxygenated carbons giving only two principal ions *m/e* 325 and 353. The MS of the underivatized β-diketone, on the other hand, shows 6–8 major α-cleavage ions[9]. The fairly prominent ion *m/e* 157 may arise as shown from one McLafferty rearrangement, it is analogous to the ion *m/e* 100 in MS of the β-diketone which arises from two McLafferty rearrangements[9]. This ion also appears in most of the other spectra.

The hydroxy β-diketones form di-TMS derivatives. When the hydroxyl group is at C-25 (Fig. 2.2) the ion *m/e* 353 is replaced by a much less intense ion *m/e* 441 showing that the hydroxyl is attached to the C_{15}-side of the molecule. Location of the hydroxyl group is shown by the expected α-cleavage[12] giving intense ions *m/e* 187 and 539. When hydroxyl groups are at C-8 or C-9 (Fig. 2.3), as they are in the mixed hydroxy β-diketones from spring wheat wax[5], the ion *m/e* 353 shows that the hydroxyls are attached to the C_{13}-side of the molecule. In MS of these isomers, presumably because the hydroxyl is relatively close to the β-diketone group, the α-cleavage fragments *m/e* 525 and 511 are extremely weak and only ions *m/e* 201 and 215 indicate the point of substitution. When the hydroxyl is at C_5, however, α-cleavage fragments are more prominent again (5-hydroxy β-diketone was isolated from wax of another grass species; A. P. Tulloch, unpublished results).

The β-diketone with an oxo group at C-25 also forms a TMS derivative (Fig. 2.4) which gives prominent ions *m/e* 325 and 367 showing that the carbonyl is on the C_{15}-side of the β-diketone. One ion, *m/e* 465, formed by α-cleavage at the carbonyl group is very weak but the other, *m/e* 113, is strong and shows the position of the group in the chain. Considerably more fragments are produced on GC–MS of the isomeric 10-oxo β-diketone derivative (Fig. 2.5) probably because the oxo and β-diketone groups are quite close. There is a strong ion at *m/e* 353 but the corresponding ion, *m/e* 339, is weaker. The position of the oxo group in the C_{13}-chain is indicated by the α-cleavage ion *m/e* 155, however, there is a stronger ion at *m/e* 169 which might be taken to indicate oxygenation at C_{11}. There are also strong ions at *m/e* 185 and 197 and at *m/e* 255, 249, 225 and 222. The latter ions are probably due to decomposition on the column (which also occurs with the underivatized β-diketone[4]) since they are stronger in scans taken before the main peak. GC–MS identification of this oxo β-diketone as the TMS derivative would be unreliable but additional GC–MS of the parent compound would give useful results since the same misleading ions do not occur in both spectra[4].

The results of GC–MS analysis of two trimethylsilylated plant waxes are shown in Fig. 3, the upper RGC was obtained with wax from *Agropyron intermedium*[4] and the lower RGC from wax of spring wheat (Selkirk variety)[5]. In Fig. 3.1 peaks 7, 8 and 10 are due to TMS derivatives of the same β-diketone discussed above, the 25-hydroxy β-diketone and the 25-oxo β-diketone (together with a minor amount of the 10-oxo derivative), respectively; peak 9 is due to decomposition products of oxo β-diketones and peak 11 to unreacted oxo β-diketone. In Fig. 3.2 peak 11 is due to the same β-diketone and peak 12 to a mixture of 8- and 9-hydroxy β-diketones as TMS derivatives. These results agree with those obtained previously[4,5] in which the β-diketones were isolated by silicic acid column chromatography and their structures determined by examination of the products of alkali degradation.

Free acids and free alcohols in the waxes were also converted to TMS derivatives and identified by GC–MS. Thus C_{24} and C_{26} alcohol TMS ethers appear

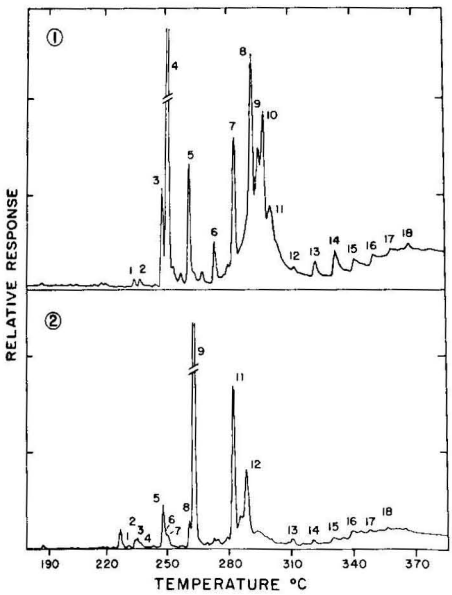


Fig. 3. Reconstructed gas chromatograms from GC–MS analysis of trimethylsilylated waxes. (1) Wax from *Agropyron intermedium*. Peaks 1, 3, 5 and 6 are C_{27}–C_{33} hydrocarbons; peaks 2 and 4 are C_{24} and C_{26} alcohol TMS ethers; peak 7 is hentriacontane-14-16-dione; peak 8 is 25-hydroxyhentriacontane-14,16-dione; peak 10 is a mixture of 25-oxo and 10-oxohentriacontane-14,16-diones all as TMS enol ethers; peak 9 is decomposition productions; peak 11 is unreacted oxo β-diketone; peaks 12–18 are C_{38}–C_{50} esters. (2) Wax from Selkirk variety of spring wheat. Peak 1 is C_{22} acid TMS ester; peaks 3 and 6 are TMS esters of *trans* 2,3 unsaturated C_{22} and C_{24} acids; peaks 2, 5 and 8 are C_{27}–C_{31} hydrocarbons; peaks 4, 7, 9 and 10 are TMS ethers of C_{24}–C_{30} alcohols; peak 11 is hentriacontane-14,16-dione; peak 12 is a mixture of 8- and 9-hydroxyhentriacontane-14,16-diones, all as TMS enol ethers; peaks 13–18 are C_{38}–C_{48} esters.

as peaks 2 and 4 in Fig. 3.1 and C_{24} to C_{30} TMS ethers appear as peaks 4, 7, 9 and 10 in Fig. 3.2. The TMS ethers were identified by the strong M − 15 ion[12]. Free acids were not present in the wax from *A. intermedium*[4] but in Fig. 3.2, peak 1 is the TMS ester of saturated C_{22} acid and peak 3 and 6 are due to TMS esters of *trans* 2-docosenoic and *trans* 2-tetracosenoic acids. The TMS ester of the saturated acid was identified by characteristic ions at *m/e* 117, 129, 132, 145 and 201 (ref. 13) together with the molecular ion and the intense M − 15 ion. The presence of ions *m/e* 129, 132 and 145 and of a parent ion distinguish TMS esters of saturated fatty acids from TMS ethers of 2-alkanols, which also give a strong ion at *m/e* 117[12]. TMS esters of the *trans* 2,3-unsaturated acids were characterized by ions at *m/e* 117, 129, 143 and 155 and by the molecular ion and the intense M − 15 ion. These acids are found in waxes of certain *Triticum* species and have characteristic GLC emergence times[2,5] but the GC–MS results confirm the chain length.

The hydrocarbon compositions of the waxes illustrated in Fig. 3 were known so that peaks 1, 3, 5 and 6 in Fig. 3.1 can be assigned to C_{27} to C_{33} odd-carbon hydrocarbons and peaks 2, 5 and 8 in Fig. 3.2 to C_{27} to C_{31} hydrocarbons. The MS of the hydrocarbons lacked parent ions so that the chain length could not be determined.

Thus the order of emergence on GLC analysis of these other wax components is C_n hydrocarbon, C_{n-3} alcohol TMS ether and C_{n-3} acid TMS ester, all three components being completely resolved. When *trans* 2,3-unsaturated acids are present their TMS esters appear between the peaks due to hydrocarbon and alcohol (with 2 more carbons than the unsaturated acid) TMS ether and partly overlap them. Previously whole waxes were treated with diazomethane to convert free acids to methyl esters and then acetylated to convert alcohols to acetates and analyzed by GLC with Dexsil 300 as liquid phase. The order of elution was C_n hydrocarbon, C_{n-3} acid methyl ester and C_{n-3} alcohol acetate[1,2,14].

Long-chain ester peaks also appeared in the RGC (Figs. 1 and 3) but since the jet separator was not heated above 275° the peaks were broader than they are in normal GLC analysis[1,14]. Esters could, however, be identified by the M^+ ion and some of the parent acids were indicated by $RCO_2H_2^+$ fragments.

Thus most components of plant waxes are resolved by GC analysis of the trimethylsilylated whole wax and almost all are identifiable from MS. The procedure is shorter and more convenient, from a qualitative point of view, than previous GLC procedures[1,2] which involve diazomethane treatment and acetylation. Also β-diketone and substituted β-diketones can be identified without preliminary separation and chemical degradation. At present the procedure is less satisfactory as a quantitative method since acids and alcohols, as TMS derivatives, give much larger responses than the β-diketone derivatives, though use of internal standards could give useful results. Quantitative analysis of waxes containing appreciable amounts of long-chain esters would also be quite difficult by this method.

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CHROM. 11,041

GAS CHROMATOGRAPHIC DETERMINATION OF NALIDIXIC ACID IN TABLETS

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SUMMARY

A gas chromatographic method has been established for the quantitative analysis of nalidixic acid. The method is based on the derivatization of nalidixic acid with diazomethane, and 5-α-cholestane is used as an internal standard. The sample is chromatographed on a glass column packed with 1% OV-1 on Chromosorb W. Quantitation is achieved by measuring peak-height ratios. The improved simplicity, specificity and accuracy of the method has been demonstrated for the quantitation of nalidixic acid in tablets.

INTRODUCTION

A variety of methods has been developed for the analysis of nalidixic acid in pharmaceutical products and biological specimens: among them, the chemical methods[1–3] centred on acid-base neutralization, the polarographic analysis based on the reduction of nalidixic acid to the corresponding 4-hydroxy derivative[4], and the fluorophotometric methods[5–7] the UV spectrophotometric methods[8–10], the microbiological assay[11], and paper and thin-layer chromatographic detections[2,12]. Although high-performance liquid chromatographic methods have been developed for nalidixic acid quantitation[13,14], no gas chromatographic (GC) method has been established yet.

This report describes a GC method for the determination of nalidixic acid in tablets. The results indicate that the method is simple, specific and accurate.

EXPERIMENTAL

GC conditions

A Varian 1740 gas chromatograph equipped with a dual flame-ionization detector was used. The column was a 1.83 m × 2 mm I.D. coiled glass tube packed with 1% OV-1 on Chromosorb W AW DMCS (80–100 mesh). The injection port and

detector were kept at 280°. The column temperature was set initially at 190° and programmed at 10°/min to 280°. Nitrogen was used as the carrier gas at a flow-rate of 41 ml/min. A PTFE-faced septum (Varian Aerograph) was used in the injection port. An injection of 10 μl of derivatized sample solution divided into several portions was made before each day's run in order to minimize column loss; otherwise lower peak-height ratios in the initial chromatographic analysis were encountered, probably due to column adsorption.

Chemicals and reagents

Nalidixic acid was a gift from Daiichi Seiyaku (Tokyo, Japan), 7-hydroxymethylnalidixic acid and 3,7-dicarboxynalidixic acid were generously supplied by the Sterling-Winthrop Research Institute (Rensselaer, N.Y., U.S.A.), 5-α-cholestane of guaranteed grade was purchased from Nakarai Chemical (Kyoto, Japan), 1% OV-1 on Chromosorb W AW DMCS (80–100 mesh) was the product of Antek Instrument (Houston, Texas, U.S.A.), and N,O-bis(trimethylsilyl)acetamide (BSA), trimethylsilylchlorosilane (TMCS), N-trimethylsilylimidazole (TMSI), and *p*-tolylsulphonylmethylnitrosamide (E. Merck, Darmstadt, G.R.) were used without further treatment. Chloroform and other reagents were of analytical grade.

Solutions of the internal and reference standard were prepared by accurately weighing *ca.* 80 mg of 5-α-cholestane or *ca.* 50 mg of nalidixic acid respectively, into a 25-ml volumetric flask and dissolving and diluting to volume with chloroform.

Analytical calibration

Eight samples containing the reference standard over the range of 0.3–2.5 ml were pipetted into a series of 10-ml vials, 0.5 ml of the internal standard solution were added and mixed well. Each sample was evaporated to dryness on a water-bath. The residue was treated with diazomethane as described under *Derivatization procedure*. A calibration graph was constructed by plotting the weight ratios of nalidixic acid to 5-α-cholestane against their peak-height ratios.

Sample preparation

Each tablet was accurately weighed and a suitable quantity of the finely pulverized tablet mass equivalent to *ca.* 50 mg of nalidixic acid was accurately transferred to a 25-ml volumetric flask. The volume was made up with chloroform and the mixture was stirred for 30 min by means of a magnetic stirrer. About 10 ml of the suspension were then transferred to a glass-stoppered centrifuge-tube and centrifuged at 1000 *g* for 10 min. A 1.5-ml aliquot of the supernatant was pipetted into a 10-ml vial containing 0.5 ml of the internal standard solution, the solution was mixed well and evaporated to dryness on a water-bath. The residue was derivatized as described below.

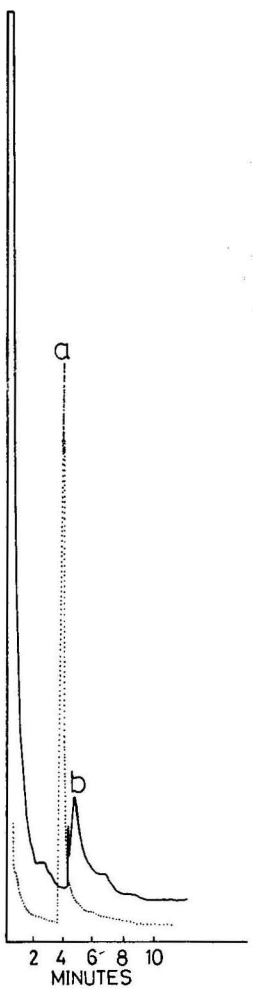
Derivatization procedure

Each residue was dissolved in 0.5 ml of chloroform, then a suitable amount of diazomethane–diethylether solution, prepared according to the method of De Boer and Backer[15] using ethanol as the reaction mixture instead of Carbitol, was added untill the solution appeared persistently yellow. The solution was allowed to stand for 30 min at room temperature and then evaporated to dryness on a water-bath. The

residue obtained was dissolved in 0.5 ml of chloroform and GC determination was performed by injection of 0.4–1.0 μl of the chloroform solution.

RESULTS AND DISCUSSION

Derivatization of nalidixic acid with silylating agents has been examined at room temperature. The mixtures of silylating agents employed were pyridine–BSA (0.5 + 0.2 ml), pyridine–BSA–TMCS (0.25 + 0.1 + 0.1 ml), pyridine–TMSI–TMCS (0.25 + 0.1 + 0.1 ml) and pyridine–BSA–TMSI–TMCS (0.25 + 0.1 + 0.1 + 0.1 ml) under the same chromatographic conditions. The chromatographic properties of



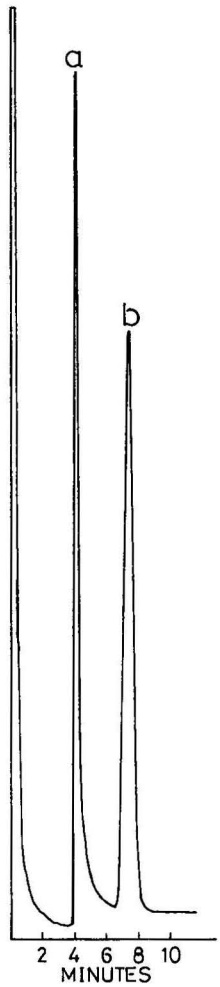


Fig. 1. Composite gas chromatogram of (a) nalidixic acid treated with diazomethane and (b) nalidixic acid without derivatization.

Fig. 2. Gas chromatogram obtained from the analysis of powdered tablet material: (a) nalidixic acid methyl ester, (b) 5-α-cholestane (internal standard).

nalidixic acid derivatized with these silylating systems were not satisfactory, resulting in peak broadening and lower peak-height ratios as compared to that derivatized with diazomethane. Direct GC analysis of nalidixic acid without derivatization was also made under the same chromatographic conditions. The results appeared as splitting and tailing peaks of poor response accompanied by a minor shoulder on the tailing of the solvent peak, possibly due to a degradation product of nalidixic acid obtained at the relatively high injection temperature. This is in complete contrast to the reproducible sharp peak of diazomethane-treated nalidixic acid (Fig. 1). Peak a in Fig. 1 was further analyzed by use of a JEOL-01SG-2 mass spectrometer under the conditions of separator temperature 200°, ionization-source temperature 300°, ionization energy 75 eV and acceleration energy 8 kV. The spectrum obtained exhibited a molecular ion peak at $m/e = 246$, indicating the formation of nalidixic acid methyl ester. Fig. 2 shows a typical gas chromatogram obtained for a tablet sample after derivatizing with diazomethane. Both nalidixic acid and the internal standard gave well resolved, sharp and symmetric peaks. The retention time was relatively short, 4.05 min for nalidixic acid and 7.37 min for 5-α-cholestane (internal standard).

The linearity of the system was demonstrated by analyzing eight different amounts of nalidixic acid over the range of 0.62–5.18 mg. A linear regression equation ($y = 0.8544x - 0.1861$) was obtained with a correlation coefficient of 0.9985.

To determine the recovery of nalidixic acid, samples were prepared containing six different levels of known amounts of nalidixic acid. The analytical results are presented in Table I. The overall recovery for six samples was 101.0 ± 0.73%. The small standard deviation demonstrates that nalidixic acid can be measured with high precision.

TABLE I

ASSAY DATA FOR KNOWN AMOUNTS OF NALIDIXIC ACID

Amount (mg)		*Recovery (%)*
Known	*Found*	
1.65	1.65	100.0
2.06	2.07	100.5
3.09	3.11	100.6
3.69	3.75	101.6
4.10	4.19	102.2
4.72	4.76	100.8
	Mean recovery (%)	101.0
	S.D. (%)	0.73

Nalidixic acid tablets obtained from two different commercial sources were analyzed and the results are shown in Table II. The present method is feasible for the quantitation of nalidixic acid in tablets and is simple, specific and accurate.

The proposed method was also used to detect a sample mixture of nalidixic acid and two of its metabolites, 3,7-dicarboxynalidixic acid and 7-hydroxymethylnalidixic acid. A simultaneous resolution was attainable as presented in Fig. 3. The

chromatographic property of 7-hydroxymethylnalidixic acid was poor, probably due to the polar function of the hydroxyl group, and can be highly improved by further treatment with BSA. The applicability of this partially modified method of determining nalidixic acid and its biotransformation products is under investigation.

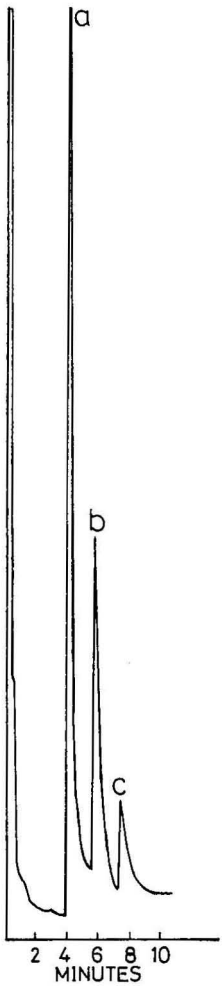


Fig. 3. Gas chromatogram of (a) nalidixic acid, (b) 3,7-dicarboxynalidixic acid and (c) 7-hydroxymethylnalidixic acid, after derivatizing with diazomethane.

ACKNOWLEDGEMENTS

We are indebted to Daiichi Seiyaku for the gift of nalidixic acid and to Dr. F. C. Nachod of the Sterling-Winthrop Research Institute for generously supplying 7-hydroxymethylnalidixic acid and 3,7-dicarboxynalidixic acid. Thanks are also due to Dr. L. C. Lin of the National Taiwan University and Professor C. Y. Hsü for their continuous encouragement.

TABLE II

ASSAY RESULTS FOR NALIDIXIC ACID TABLETS (500 mg PER TABLET) OBTAINED FROM A COMMERCIAL SOURCE

Tablet number	*Amount found (mg per tablet)*	*Percentage of claim*
T1	516.1	103.2
T2	523.5	104.7
T3	534.9	107.0
T4	527.8	105.6
T5	509.9	102.0
T6	524.2	104.8
	Mean recovery (%)	104.6
	S.D. (%)	1.61
W1	512.6	102.5
W2	530.8	106.2
W3	533.2	106.6
W4	530.1	106.0
W5	514.0	102.8
W6	526.0	105.2
	Mean recovery (%)	104.9
	S.D. (%)	1.64

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CHROM. 10,966

IDENTIFICATION OF ABSCISIC ACID IN SHOOTS OF *PICEA ABIES* AND *PINUS SYLVESTRIS* BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

A VERSATILE METHOD FOR CLEAN-UP AND QUANTIFICATION

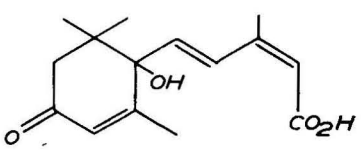
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SUMMARY

cis-trans-Abscisic acid has been identified by gas chromatography–mass spectrometry in the current year's shoots of Scotch pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). Clean-up was afforded on a combined Celite–poly-N-vinylpyrrolidone–Sephadex LH-20 column. For quantitative determinations, the extracts were submitted to gas chromatography with an electron-capture detector; in this way 1-g samples of vegetative tissues of spruce and pine could be analysed for *cis-trans*-abscisic acid (as the methyl ester) with a detection limit of 1 ng/g fresh weight.

INTRODUCTION

Abscisic acid (ABA), an endogenous plant growth hormone[1], has been reported to occur in a number of species at a concentration of *ca.* 100 ng/g on a fresh-weight basis; however, only a few determinations of ABA have been made in conifers.



cis-trans Abscisic acid

Investigations of plant hormones have usually involved separate bioassays for the detection and measurement of each hormone. Such assays are of limited reliability,

both qualitatively and quantitatively, which restricts the scope of investigations that can be undertaken. Thus, chemical methods have been designed that separate the hormone from interfering compounds and quantitatively measure the amount present.

In 1969 Bonnet-Masimbert identified ABA in dormant buds of *Pinus sylvestris* by gas chromatography (GC), using the retention time on two different columns[2]. In 1972, Jenkins and Shepherd[3] identified ABA in stems of *Pinus radiata*, and Little *et al.*[4] in dormant buds of *Abies balsamea* by combined GC–mass spectrometry (MS). These reports deal only with the identification of ABA, and no quantitative method has been suggested for ABA in conifers.

In view of the low levels of growth regulators in vegetative tissues, large amounts of tissue need to be extracted, and removal of the associated impurities is both tedious and time-consuming. In 1970, Seeley and Powell demonstrated that the methyl ester of ABA has electron-capturing properties of a very high order[5]. With suitable clean-up procedures, GC with an electron-capture detector (ECD) would offer a simple technique of routine analysis for ABA in a minimal amount of vegetative tissue.

This study was undertaken in an effort to identify ABA in shoots of *Picea abies* and *Pinus sylvestris* by GC and to design an analytical method for its quantification on a routine basis.

EXPERIMENTAL

Materials

Shoots of pine and spruce were collected in Northern Sweden in September and June, respectively. The shoots were wrapped in aluminium foil, immediately frozen and stored at −20°.

Reagents

Methanol (redistilled), diethyl ether (p.a.), ethyl acetate (p.a.), poly-N-vinylpyrrolidone (PVP, Polyclar AT powder, GAF Corp., New York, N.Y., U.S.A.), Celite (80–120 mesh) for gas chromatography (BDH, Poole, Great Britain), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), (±)*cis-trans*-ABA (Sigma, St. Louis, Mo., U.S.A.), (±)*cis-trans*-[2-^{14}C]-ABA (New England Nuclear, Boston, Mass., U.S.A.) and scintillation liquid (Insta-Gel; Packard, Downers Grove, Ill., U.S.A.) were used.

(±)*trans-trans*-ABA was prepared by illumination of a methanolic solution of *cis-trans*-ABA with UV radiation[4]. The identity of *trans-trans*-ABA was confirmed by GC–MS of its methyl ester.

Scintillation spectrometer

A Packard Tri-Carb liquid scintillation spectrometer, Model 3375, was used. All counts were corrected for background and for quenching.

Gas chromatographic system

A Pye Unicam GCV gas chromatograph equipped with an FID and an ECD was used. It was fitted with a glass column (1.8 m × 0.4 cm I.D.) packed with 1% OV-17 on Chromosorb W, AW DMCS (100–120 mesh). The operating conditions

were: injector temperature, 220°; column temperature, 200°; detector temperature, 250°; carrier gas, nitrogen at a flow-rate of 40 ml/min.

An SF-96 glass capillary column (34 m × 0.29 mm I.D.) with 122, 100 theoretical plates at $k = 5.93$ for *n*-tetradecane at 120° was used. The column was operated at 200°, with a nitrogen carrier-gas pressure of 0.6 atm resulting in average linear velocities of 35 cm/sec. The column was mounted in the gas chromatograph mentioned above with an inlet splitter (split ratio 1:20) and with nitrogen (40 ml/min) as make-up gas to the FID. A vaporizer temperature of 275° was used, and 5 μl was injected.

Gas chromatographic–mass spectrometric system

An LKB 9000 mass spectrometer equipped with a Pye Unicam Model 84 gas chromatograph was used. The operation conditions were as indicated above, except for the carrier gas, which was helium at a flow-rate of 25 ml/min. The temperature of the connection between the gas chromatograph and the mass spectrometer was 250°. The operating conditions for the mass spectrometer were: separator temperature 250°; ion-source temperature, 270°; electron energy, 70 eV. The most abundant ion in the spectrum (*m/e* 190) was monitored for the single-ion detection.

Extraction procedure

Extracts for identification of ABA. Shoot tissue (100 g fresh weight) and [^{14}C]-ABA were homogenized (Ultra-Turrax) with cold methanol (2 × 500 ml) at −18° for 4 h. The extract was filtered and evaporated to 500 ml, then filtered through 10 g of PVP in a 5.5-cm Büchner funnel; the filtrate was concentrated to 15 ml. The extract now consisted of an aqueous solution with pH 3.3, and this was chromatographed on two Sephadex LH-20 columns in series. The first column (8 cm × 4 cm I.D.) was eluted with 0.05 *M* phosphate buffer of pH 3.0, the fractions being collected and scintillation counted. The scintillation showed the radioactivity to be eluted between 215 and 390 ml. This fraction was extracted with diethyl ether (2 × 200 ml), the extract was evaporated to dryness, and the residue was dissolved in 1 ml of methanol (part of this crude extract did not dissolve in this portion of methanol, but scintillation showed no activity in the insoluble residue). The methanol solution was applied to a second LH-20 column (60 cm × 0.9 cm I.D.) and eluted with methanol. The radioactive fraction was eluted between 35 and 39 ml. This fraction was evaporated to dryness, the residue was dissolved in 2 ml of diethyl ether plus a few drops of methanol and this solution was methylated with diazomethane in diethyl ether for 30 min. The mixture was then evaporated to dryness in a stream of nitrogen, and the residue was dissolved in 100 μl of ethyl acetate for analysis.

Smaller extracts for quantification of ABA. Shoot tissue (10 g fresh weight) and [^{14}C]ABA were homogenized with cold methanol (2 × 100 ml) at −18° for 4 h. The extract was filtered, and 10% of the solution was evaporated to 2 ml; this corresponded to material from 1 g of shoots (fresh weight). The methanol extract was chromatographed on a combined Celite–PVP–Sephadex LH-20 column with 0.05 *M* phosphate buffer of pH 3.0 as eluent. The column (28 cm × 1.1 cm I.D.) consisted of an upper layer of 1.0 cm of Celite, a middle layer of 2.5 cm of PVP and a bottom layer of 24.0 cm of Sephadex LH-20. In packing this column, the Sephadex LH-20 layer was first eluted for 30 min before the PVP and Celite were introduced. When

the fractions were collected and counted, the scintillation showed the activity to be between 50 and 70 ml. This fraction was extracted with diethyl ether (2 × 20 ml) and evaporated to small volume. After methylation as described above and evaporation to dryness, the residue was dissolved in 100 μl of ethyl acetate for analysis.

Evaporation of larger volumes of solvents was performed on an evaporator with a water-bath at 50°; smaller volumes were evaporated in a stream of nitrogen.

RESULTS AND DISCUSSION

This work was done on the current year's shoots of Scotch pine (*Pinus sylvestris*) and on elongating shoots of Norway spruce (*Picea abies*). Scintillation of added [^{14}C]-ABA was performed to monitor the ABA fraction during the clean-up procedures and to calculate the recovery. Two different clean-up procedures were developed in order to meet the requirements for ABA in the final extracts and to minimize the amount of interfering components in the final analyses.

Identification of ABA as its methyl ester was performed by GC–MS in a system equipped with an ordinary packed column. Two extracts from pine and two from spruce were used for identifications; [^{14}C]ABA was not added to these extracts so

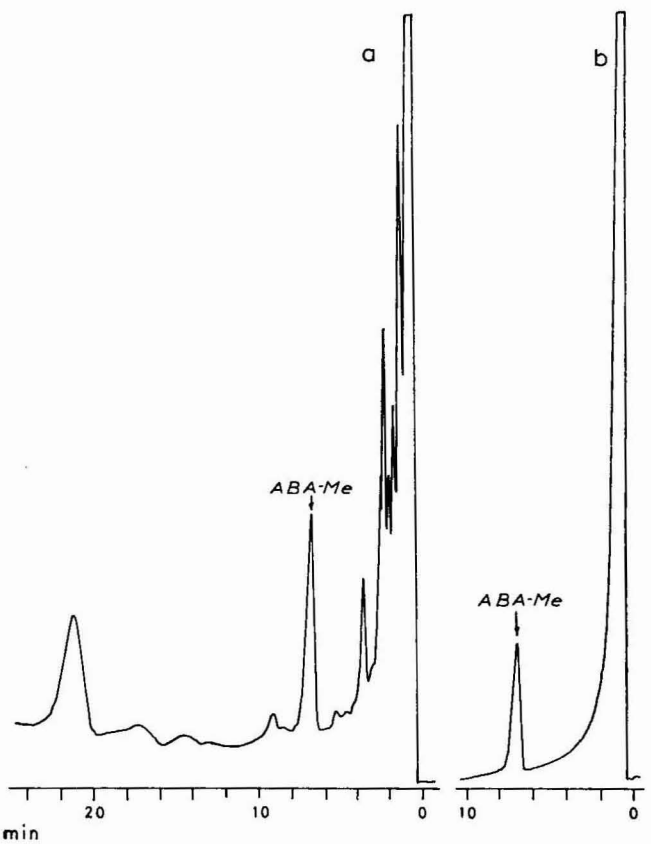


Fig. 1. Gas chromatogram (FID) of: (a) 100-g spruce extract; (b) ABA standard.

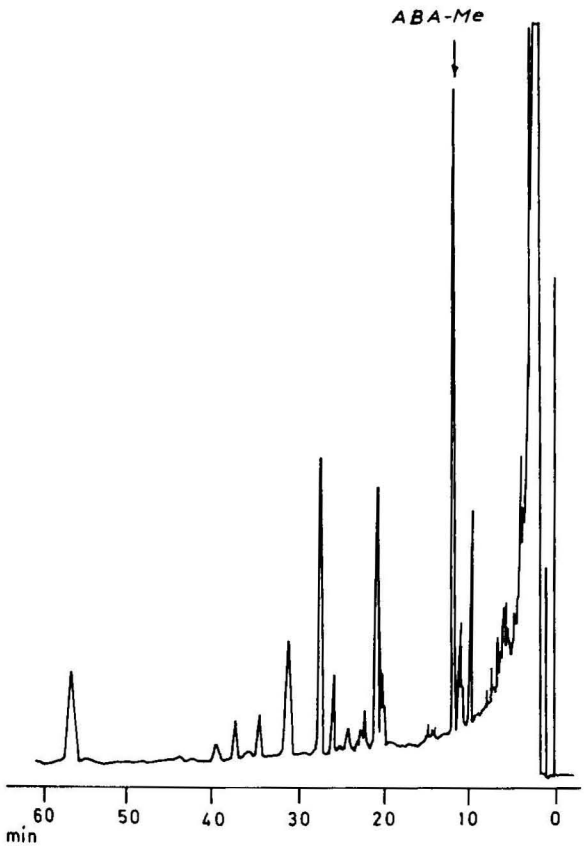


Fig. 2. The same extract as in Fig. 1 analysed on a 34-m SF-96 glass capillary column (FID).

as to avoid misleading results. Complete mass spectra were obtained and shown to be identical with those of an authentic sample and with spectra already published[3,6,7].

Under the conditions used, the isomeric esters *trans-trans*-ABA and *cis-trans*-ABA were well separated on the GC column. The retention time for *trans-trans*-ABA on a 1% OV-17 column operated at 200° was 9 min; that for *cis-trans*-ABA was 7 min. Only *cis-trans*-ABA was detected in the extracts, and, under the conditions used, no tendency on the part of *cis-trans*-ABA to isomerize into *trans-trans*-ABA was observed. Nevertheless, direct sunlight was avoided, and dim lighting and cold storage were used in the process when feasible.

The identity of *cis-trans*-ABA was further confirmed by GC on a capillary column with SE-30 as stationary phase.

For quantitative analyses of ABA in shoots, 1 g of fresh-weight tissue was used, and clean-up was performed on a combined Celite–poly-N-vinylpyrrolidone (PVP)–Sephadex LH-20 column. In this way, laborious and time-consuming handling of large volumes of solvents could be avoided. The elution volumes for the ABA fraction were shown to be relatively constant, and no [^{14}C]ABA was added to the extracts in the final determinations. By scintillation on added [^{14}C]ABA, the recovery for this clean-up procedure was found to be 85%. After methylation with diazo-

methane[8], the extracts were first subjected to GC with a conventional column and flame ionization detector (FID) in order to obtain some idea of the contents of the extract; the detection limit of ABA methyl ester under these conditions was 5 ng. The extract was then diluted at least 1000-fold and injected on to the same column attached to an ECD with a detection limit of *ca.* 5 pg of ABA methyl ester.

It is well known that an ECD is easily contaminated or overloaded, with consequent severe analytical problems; however, it offers a great advantage in this analysis, as ABA methyl ester has a very low detection limit. The FID was first used as a safety precaution so as to avoid contamination problems.

Despite the clean-up procedures, many components still remain in the final extract (see Figs. 1 and 2). However, as can be seen in Figs. 3 and 4, after further dilution, most of these components disappear on the ECD chromatogram, making this method well suited to trace analysis for ABA in small amounts of conifer tissue.

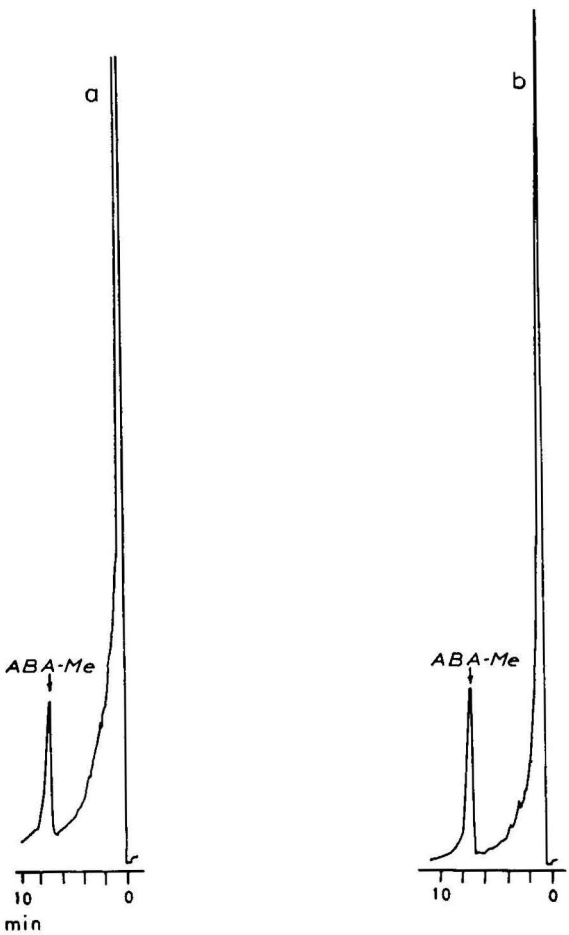


Fig. 3. Gas chromatogram (ECD) of spruce extract. (a) From Fig. 1, diluted 1000 times; (b) 100 pg of reference standard.

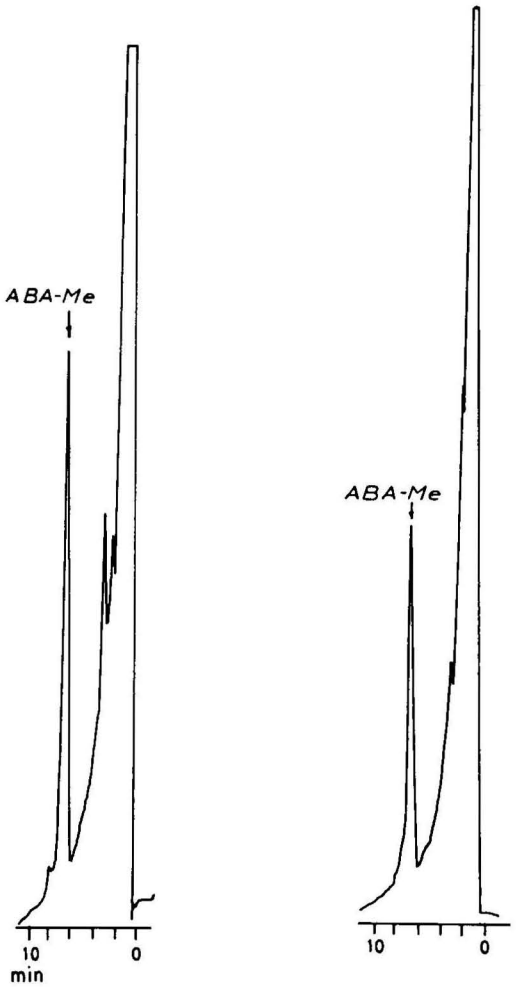


Fig. 4. Gas chromatogram (ECD) of spruce extract from: (a) 1 g of shoot tissue; (b) 50 pg of ABA standard.

By this method, the amounts of *cis-trans*-ABA in the extracts were 170 ± 10 ng/g for pine and 320 ± 10 ng/g for spruce (fresh-weight basis).

The quantification of ABA on a conventional column agreed well with that on the capillary column, and this was further demonstrated by MS with single-ion detection on the main fragment (*m/e* 190)[9,10]. However, the amount of ABA ester in the extracts as determined by this method was about 10% higher than that found in the determinations using ECD. This might be explained by contribution to the ion at *m/e* 190 from interfering natural products in the extracts with the same retention time as ABA ester on a conventional column.

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RAPID MICRO-METHOD FOR THE MEASUREMENT OF PHENOBARBITONE, PRIMIDONE AND PHENYTOIN IN BLOOD PLASMA OR SERUM BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid gas–liquid chromatographic method has been developed for the analysis of phenobarbitone, primidone and phenytoin in small (50 μl) volumes of either blood plasma or serum. Neither solvent transfer nor evaporation are required in the extraction, which takes less than 3 min to complete, and a quantitative analysis may be performed, in duplicate, within 20 min. Sources of interference in the assay are minimal, and prior treatment of the column with γ-glycidoxypropyltrimethoxysilane facilitates the measurement of as little as 10 ng of underivatized drug "on-column" using a flame-ionisation detector. The method has proved valuable when used for the detection and measurement of these three compounds at concentrations of 2 mg/l or greater.

INTRODUCTION

The measurement of the plasma or serum concentrations of phenobarbitone, primidone and phenytoin may be of importance both in the treatment of epilepsy and in the diagnosis of acute poisoning. Where samples which may contain more than one of these drugs are to be analysed, either a gas–liquid (GLC) or a liquid chromatographic procedure is preferable since both qualitative and quantitative information can be obtained from a single step. However, many of the methods currently available require relatively large (1 ml) sample volumes[1–3] and involve tedious extract purification and concentration procedures[1–6]. Moreover, derivatization prior to GLC has been advocated[2,3,6] while, in addition to flame-ionisation (FID), nitrogen-selective detection has been employed[4,6].

The method described here is an extension of that described for the analysis of barbiturates and some other drugs in plasma specimens obtained from poisoned patients[7]. A small (50 μl) volume of sample was extracted under acidic conditions with an equal volume of chloroform containing an internal standard, and 3–5-μl portions

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of this extract were analysed by GLC–FID using two independent column systems. However, the analysis, again by GLC–FID, of a portion of this same extract on a third column has been found to facilitate the simultaneous identification and measurement of phenobarbitone, primidone and phenytoin at the concentrations attained not only after overdosage but also during therapy.

EXPERIMENTAL

Chemicals and reagents

Phenobarbitone, primidone and phenytoin (micronised) were obtained from May and Baker (Dagenham, Great Britain), I.C.I. (Macclesfield, Great Britain) and Parke Davis & Co. (Pontypool, Great Britain), respectively. γ-Glycidoxypropyltrimethoxysilane (A-187) was supplied by Union Carbide U.K. (Southampton, Great Britain). The internal standard, tetraphenylethylene (TPE), was obtained from Koch-Light Labs. (Colnbrook, Great Britain) and was used as a 10 mg/l solution in chloroform (analytical-reagent grade). Sodium dihydrogen orthophosphate (analytical-reagent grade) was used as a 4 mole/l aqueous solution ("phosphate buffer").

Gas–liquid chromatography

A Pye 104 Model 24 dual-column gas chromatograph fitted with FIDs was used, and integration of peak areas was performed by a Hewlett-Packard 3352 data system. The column and detector oven temperatures were 260 and 300°, respectively; injection port heaters were not employed. The carrier gas (argon) flow-rate was 60 ml/min and the hydrogen and oxygen[7] inlet pressures were 15 and 10 p.s.i., respectively, giving flow-rates of approximately 45 and 200 ml/min.

The column, a coiled glass tube (1.5 m × 4 mm I.D.) was immersed in a solution of 5% dichlorodimethylsilane in toluene for 1 h. It was then washed with methanol, dried at 100° and subsequently packed with 2% (w/w) SP-525 (Chromatography Services, Merseyside, Great Britain) on Varaport 30 (80–100 mesh). The column packing was prepared using the rotating evaporator technique, and phosphoric acid-treated glass wool (Chromatography Services) was used to plug the injection-end of the column. The packed column was conditioned at 275° with an argon flow-rate of 60 ml/min for 16 h prior to the injection of from 10 to 20 μl of A-187. Thereafter, occasional injections of 5 to 10 μl of this compound were performed to maintain the column in the deactivated form.

The chromatography on this system of a solution containing phenobarbitone, primidone and phenytoin together with TPE is illustrated in Fig. 1. The retention times of these compounds and some additional drugs on this same system are given in Table I.

Extraction procedure

Sample (50 μl), phosphate buffer (5 μl) and internal standard solution (50 μl) were added[7] to a clean Dreyer tube (Poulten, Selfe and Lee, Wickford, Great Britain). The contents of the tube were mixed thoroughly on a vortex mixer for 30 sec and the tube was centrifuged for 2 min at 9950 *g* in an Eppendorf centrifuge 5412 (Anderman & Co., East Molesey, Great Britain) which was modified to accept Dreyer tubes by slight drilling-out of the 0.4-ml test tube centrifuge adaptors. Subsequently, a 3–5-μl

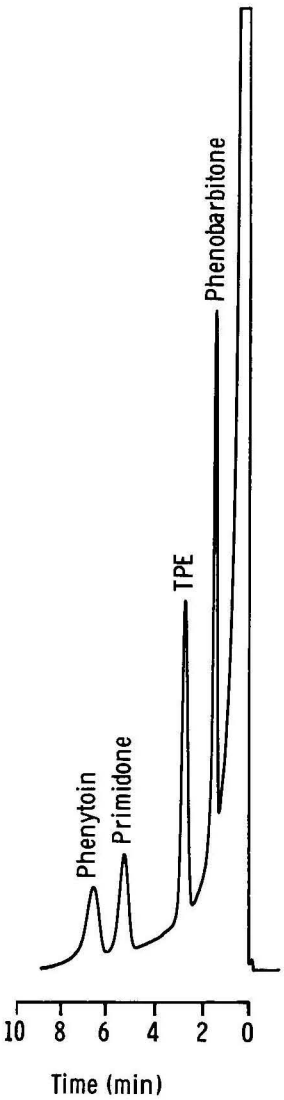


Fig. 1. Analysis of a chloroform solution containing phenobarbitone (20 mg/l), primidone, phenytoin and TPE (all 10 mg/l) on the SP-525 column system; 4-μl injection.

portion of the chloroform phase was obtained[7] and injected onto the column of the gas chromatograph using a syringe fitted with an 11.5-cm needle. The extraction was performed in duplicate and the mean result taken. If the difference between the duplicates was greater than approximately 10%, both the extractions and the analysis were repeated.

Instrument calibration and calculation of results

Standard solutions containing each of the drugs under study at a range of concentrations were prepared in chloroform by dilution of an ethanolic solution of

TABLE I

RETENTION TIMES OF PHENOBARBITONE, PRIMIDONE, PHENYTOIN AND SOME OTHER COMPOUNDS ON THE SP-525 SYSTEM

Compound	*Retention time*	
	min	*Relative to TPE*
Pheneturide	0.48	0.17
Barbitone	0.51	0.18
Allobarbitone	0.60	0.21
Allylbarbitone	0.60	0.21
Amylobarbitone	0.60	0.21
Butobarbitone	0.60	0.21
Pentobarbitone	0.63	0.22
Phenacetin	0.66	0.23
Quinalbarbitone	0.71	0.25
Meprobamate	0.80	0.28
Hexobarbitone	0.88	0.31
Methoin	0.94	0.33
Glutethimide	1.03	0.36
Propylphenazone	1.08	0.38
Ethotoin	1.11	0.39
Caffeine	1.31	0.46
Cyclobarbitone	1.40	0.49
Phenazone	1.45	0.51
Phenobarbitone	1.58	0.55
Heptabarbitone	1.65	0.58
Iminostilbene	1.71	0.60
Methaqualone	2.34	0.82
TPE	2.85	1.00
Phenylbutazone	3.16	1.11
Feprazone	3.62	1.27
Azapropazone	4.53	1.59
Primidone	5.40	1.89
Carbamazepine	5.87	2.06
Phenytoin	6.71	2.35
Chlordiazepoxide	8.21	2.88
Cholesterol*	(13.4)	(4.70)

* This compound was not eluted from some of the column systems used (see text).

phenobarbitone (2.0 g/l), primidone and phenytoin (both 1.0 g/l). The range of concentrations obtained is given in Table II. Each standard also contained TPE (10 mg/l), obtained by dilution of a solution (1.0 g/l) of this compound in chloroform. These standard solutions were stable for at least one year if stored at ambient temperature and in the absence of light.

The ratio of the peak area of drug to the peak area of TPE, when plotted against drug concentration, was linear for all three drugs over the stated concentration ranges. Details of the calibration graphs normally obtained are given in Table II. The drug concentration in the extract was ascertained from the appropriate graph, and the drug concentration in the sample was calculated by use of the recovery data presented in Table III.

TABLE II

DRUG STANDARD SOLUTIONS AND CALIBRATION DETAILS

Drug	*Regression coefficient (l/mg)*	*Standard error of coefficient*	*Intercept on y-axis*	*Standard solutions of drug available (mg/l)* *											
				5	*10*	*20*	*30*	*40*	*50*	*60*	*75*	*80*	*100*	*150*	*200*
Phenobarbitone	0.053	0.001	−0.040		×	×		×		×		×	×	×	×
Primidone	0.058	0.001	−0.033	×	×	×	×	×	×		×		×		
Phenytoin	0.065	0.001	−0.098	×	×	×	×	×	×		×		×		

* Each standard also contained TPE (10 mg/l).

TABLE III

RECOVERY OF ADDED DRUG FROM HEPARINISED BOVINE PLASMA ($n = 25$ IN EACH CASE)

Compound	*Percentage recovery (mean ± S.D.)*	*Recovery factor*
Phenobarbitone	75.8 ± 3.0	1.32
Primidone	42.2 ± 1.8	2.37
Phenytoin	86.2 ± 2.5	1.16

RESULTS AND DISCUSSION

Column deactivation and stability

Both phenobarbitone and phenytoin gave rise to positively skewed peaks if the column was not deactivated with A-187[8,9]. Such treatment was only required infrequently (at most, weekly with a relatively new column, and monthly thereafter), although if the column was maintained at approximately 160° with argon flow when not in use, this minimised the need for further treatment. However, disturbance of the column packing (*e.g.* by too-rapid changing of septa) necessitated additional deactivation especially with a relatively new column. These SP-525 columns were useable for at least six months, a stability similar to that claimed[4] for the relatively polar phase WG-11. Argon was here preferred to nitrogen as the carrier gas since it was available in an oxygen-free form, and thus oxidation of the stationary phase was minimised.

Recovery studies

Standard solutions containing phenobarbitone (20–100 mg/l, in increments of 20 mg/l), primidone and phenytoin (both 10–50 mg/l, in increments of 10 mg/l) were prepared in heparinised bovine plasma[7]. The mean percentage recoveries calculated from quintuplicate analyses of each solution are given in Table III. Each recovery was uniform over the range studied, and confirmation of the validity of these recovery factors when applied to specimens from other sources was obtained. Analyses of commercially available "quality control" specimens: "Wellcomtrol" (Wellcome Reagents, Beckenham, Great Britain) and "Biotrol-Therapeutique I and II" (Laboratories

TABLE IV

RESULTS OBTAINED FROM REPLICATE ANALYSES (n = 20) OF "QUALITY CONTROL" SPECIMENS: WELLCOMTROL

Compound	*Results from reference laboratories (mg/l)*		*Results from the present method (mg/l)*	
	Mean	*Range*	*Mean*	*S.D.*
Phenobarbitone	21.6	12.8–33.5	22.1	0.8
Primidone	12.6	7.6–19.6	12.6	0.6
Phenytoin	15.3	10.0–21.3	15.9	0.7

TABLE V

RESULTS OBTAINED FROM REPLICATE ANALYSES (n = 20) OF "QUALITY CONTROL" SPECIMENS: BIOTROL-THERAPEUTIQUE

Solution No.	*Compound*	*Intended concentration (mg/l)*	*Means of results from reference laboratories (mg/l)*		*Results from the present method (mg/l)*	
			GLC	*Immunoassay*	*Mean*	*S.D.*
I	Phenobarbitone	10	10	11.5	10.0	0.5
	Phenytoin	10	10.2	11.2	10.7	0.4
II	Phenobarbitone	50	51	52	50.3	1.9
	Phenytoin	50	52.2	46	52.0	2.8

Biotrol, Paris, France) were performed, and the results of replicate analyses (n=20) of each solution are summarised, together with the reference data supplied, in Tables IV and V.

Assay reproducibility

The good reproducibility attainable is shown by the results presented in Tables III and VI. Similar coefficients of variation (5.4% or less) were obtained in the analyses summarised in Tables IV and V. These results are comparable to those given by the procedure of Soldin and Hill[10], which also employs the rapid admixture of a small volume of sample and an equal volume of an internal standard solution, followed by the chromatographic analysis of a portion of the resulting mixture. On the infrequent occasions where a difference between duplicates greater than approximately 10% was obtained with the present method, the analysis was normally repeated. Such relatively large variations were attributed to large errors in the sample volume measurement.

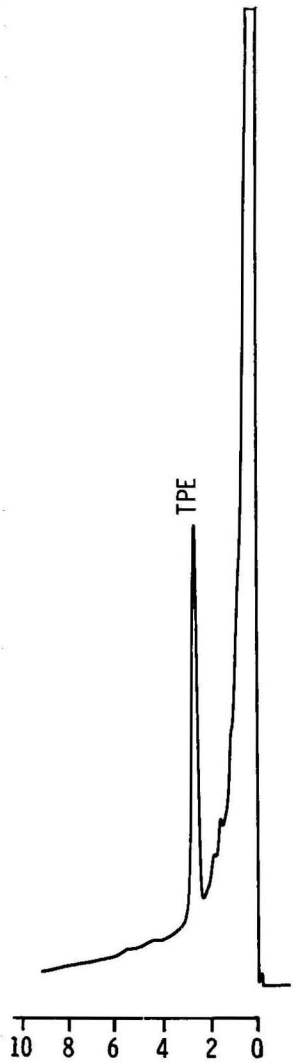
TABLE VI

COEFFICIENTS OF VARIATION (C.V.) ASSESSED FROM THE DIFFERENCE BETWEEN DUPLICATE ANALYSES

Compound	*n*	*Range (mg/l)*	*Mean (mg/l)*	*C.V. (%)*
Phenobarbitone	53	2.2–43.8	18.24	5.7
Primidone	28	2.5–11.9	7.33	4.7
Phenytoin	99	2.1–35.0	8.67	6.3

Specificity

Interference from endogenous sample constituents was minimal. An example of the chromatogram normally obtained on analysis of a drug-free human plasma specimen is given in Fig. 2; analogous extracts performed without the addition of TPE did not reveal compounds which could elute with this standard. The analysis of an extract of plasma obtained from a patient treated with primidone and phenytoin is illustrated in Fig. 3.



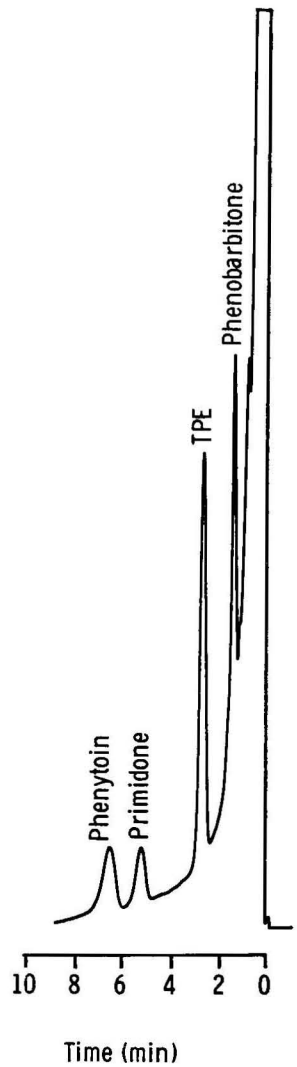


Fig. 2. Analysis of an extract of drug-free human plasma; 5-μl injection. The TPE concentration was 10 mg/l.

Fig. 3. Analysis of an extract of plasma obtained from a patient treated with primidone (750 mg/day) and phenytoin (400 mg/day); 5-μl injection. The TPE concentration was 10 mg/l, and the plasma concentrations of phenobarbitone, primidone and phenytoin were found to be 15.8, 10.0 and 8.9 mg/l, respectively.

The retention times on the SP-525 system of a number of commonly prescribed drugs are given together with those of some other compounds in Table I. Only phenylbutazone has been observed to interfere in the assay, and the presence of this compound would necessitate the choice of an alternative standard if a quantitative result was required. Cholesterol eluted from some of the columns used as a sharp, symmetrical peak, but was not observed to elute from others. This compound did not interfere in the analyses (Table I), but its presence did reduce the rate at which these analyses could be performed. The selectivity given by both the extraction procedure and the relatively high-temperature GLC column system represents an advantage of the method over liquid chromatographic procedures such as that of Soldin and Hill[10] in that interference from relatively polar compounds such as 5-(*p*-hydroxyphenyl)-5-phenylhydantoin and gentamicin did not occur.

Limits of sensitivity

The limit of sensitivity towards both phenobarbitone and phenytoin is influenced by the adsorbtion which occurs on-column, leading to intercepts on the calibration graphs corresponding to approximately 0.8 and 1.5 mg/l, respectively (Table II). On the other hand, the relatively poor recovery of primidone becomes a limiting factor even though the relevant intercept corresponded to only approximately 0.6 mg/l. Thus, the detection limit of each drug was taken to be approximately 2 mg/l, although the measurement of such low concentrations was not as accurate as those performed at higher values. However, the results presented in the following section show that these limits did not restrict the application of this method to the analysis of the drugs under study at the concentrations achieved during therapy. (Note: smaller (20 μl) or larger sample volumes may be used in the assay without affecting the limits of sensitivity provided that the other reagents are used in proportion.)

Comparison with the results obtained using a second GLC assay

The method was evaluated by comparison to the results given by a second GLC assay[1] in the analysis of 131 specimens of plasma or serum obtained from patients treated with one or more of the compounds under study. The results of the comparison are summarised in Table VII, although qualitative differences in the

TABLE VII

SUMMARY OF THE QUANTITATIVE RESULTS OF THE COMPARISON BETWEEN THE PRESENT METHOD AND A SECOND GLC ASSAY

A = Method of Toseland *et al.*[1], B = present method.

Compound	*n*	*r*	*Drug concentration (mg/l)*				*Regression parameters (A on B)*
			A		*B*		
			Mean	*S.D.*	*Mean*	*S.D.*	
Phenobarbitone	65	0.87	19.68	13.30	17.21	11.14	A = 1.04 B + 1.79
Primidone	23	0.86	7.96	3.83	7.81	3.04	A = 1.08 B − 0.49
Phenytoin	99	0.92	9.54	8.25	9.20	6.56	A = 1.16 B − 1.15

results given by each method were observed in 19 cases. A compound with the retention time of phenobarbitone (4.1 and 6.2. mg/l) was detected in two specimens by the present method although neither of these patients were thought to be prescribed this drug. In contrast, phenobarbitone at concentrations of 3 mg/l or less was found in four other specimens by the second GLC assay. This drug was known to be prescribed to one of these latter patients, and was thought to be prescribed to two others. Primidone (approximately 3 mg/l) was detected by both methods in the fourth specimen, although this compound was not initially thought to be prescribed to this patient. A compound corresponding to primidone at concentrations between 2.5 and 7.6 mg/l was detected in three specimens by the present method alone; primidone (750 mg/day) was prescribed to two of these patients. Finally, phenytoin was detected at concentrations of 3.1 mg/l or less in 11 specimens by the method described here; all of these patients were either known or thought to be prescribed this drug. These differences in the detection of phenobarbitone and phenytoin occurred near the limits of sensitivity of both assays and may represent interference in those cases where the "detected" drugs were not prescribed to the patients in question. However, the failure of the second GLC assay to detect primidone in samples from patients treated with this drug may have arisen from the use of insufficient solid ammonium sulphate to ensure an acceptable recovery of primidone in this method.

Further applications of the method

The SP-525 column may be used in the rapid analysis of some other compounds (notably hexobarbitone, cyclobarbitone, heptabarbitone and methaqualone) which could be analysed previously[7] together with phenobarbitone on a Poly A 103 system at 230°. In addition, this SP-525 system is used at the same temperature as the OV-7 column employed in our laboratory for plasma benzodiazepine analyses[11] and thus both systems may be operated in the same chromatograph. The present method has also proved useful in confirming the presence of chlordiazepoxide in overdose since this compound is extracted under the conditions of this assay and elutes as a symmetrical peak (Table I). (Note: the use of the Eppendorf centrifuge 5412 instead of relatively low-speed instruments minimises both the time required for extract preparation and the frequency of emulsion formation in these direct-extract analyses).

CONCLUSIONS

The method described here has proved to be a valuable addition to the techniques available for our 24-h emergency toxicology service. The SP-525 column system is stable for a relatively long period and not only permits the detection and measurement of phenobarbitone, primidone and phenytoin by means of the same extract used for assay of other barbiturates, but also obviates the requirement for a column system for use in phenobarbitone analyses only. In addition, the technique shows advantages of minimal sample and reagent requirement, speed of assay, and good accuracy and reproducibility when used for the analysis of these compounds at the concentrations achieved during therapy.

ACKNOWLEDGEMENTS

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QUANTITATIVE DETERMINATION OF 1,3-BIS(TETRAHYDRO-2- FURANYL)-5-FLUORO-2,4-PYRIMIDINEDIONE AND ITS METABOLITES IN PLASMA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

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SUMMARY

1,3-Bis(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione has been developed clinically as an antitumor agent. A high-pressure liquid chromatographic method was developed with which it could be measured in plasma with a sensitivity of 0.050 μg/ml. Two of its metabolites, 1-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione and 3-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione, could be determined at the same time with a sensitivity of 0.025 μg/ml.

A gas chromatographic–mass fragmentographic method was developed for the specific determination of the third metabolite, 5-fluoro-2,4-pyrimidinedione, as its silylated derivative with a sensitivity of 0.001 μg/ml.

The precision and sensitivity of the assay appear to be satisfactory for determination of the plasma level of the drug.

INTRODUCTION

1,3-Bis(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [1,3-bis(tetrahydro-2-furanyl)-5-fluorouracil, FD-1] is a new derivative of 5-fluoro-2,4-pyrimidinedione (5-fluorouracil, 5-FU) that has been developed as an antitumor agent.

The compound FD-1 has stronger antitumor activity on experimental tumors and lower toxicity than the compound named Ftorafur or FT-207, 1-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [1-(tetrahydro-2-furanyl)-5-fluorouracil, 1-FT]. Moreover, after oral administration to mice the concentrations of 5-FU in the blood and tissues are maintained at higher levels than those of 1-FT[1].

FD-1 is degraded to 3-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [3-(tetrahydro-2-furanyl)-5-fluorouracil, 3-FT] by an enzyme in liver microsomes and FD-1 also changes to 1-FT spontaneously. The 1-FT formed spontaneously is converted to the active substance 5-FU enzymatically, and the 3-FT formed enzymatically changes to 5-FU spontaneously[2] (Fig. 1).

A method for assay of FD-1 and its metabolites, 1-FT, 3-FT and 5-FU, has been reported in which FD-1, 1-FT and 3-FT are determined by thin-layer chromato-

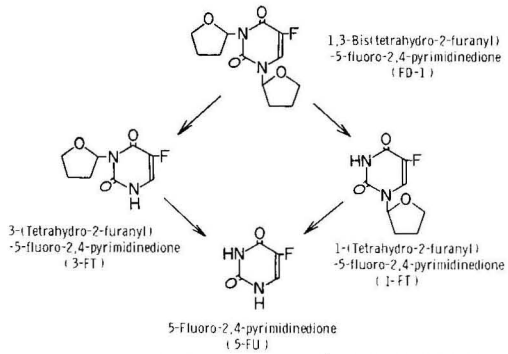


Fig. 1. The metabolic pathway of FD-1 to 5-FU.

graphy (TLC) and 5-FU is determined by microbiological assay using a sensitive bacterial strain[2].

Several methods are available for measuring 1-FT and 5-FU in plasma after administration of 1-FT: bioassay to determine 1-FT and 5-FU separately[3,4], high-pressure liquid chromatography (HPLC) with a reversed-phase chromatographic system for simultaneous determination of 1-FT and 5-FU[5], and a method using a combination of gas–liquid chromatography (GLC) for determination of 1-FT and gas chromatography–mass fragmentography (GC–MF) for determination of 5-FU[6,7]. These methods, however, are not suitable for determination of FD-1, 1-FT, 3-FT and 5-FU in plasma after the administration of FD-1, because the physico-chemical properties of FD-1 and 3-FT were not especially fitted for these analytical procedures. Therefore, in this study FD-1, 1-FT and 3-FT were extracted separately from 5-FU. The first three compounds were then determined by HPLC using an adsorption chromatographic system, while 5-FU was measured by GC–MF. Both the precision and sensitivity of this simple and rapid assay method were satisfactory.

EXPERIMENTAL

Materials

FD-1, 1-FT and 3-FT were synthesized and purified at the Research Laboratory, Taiho Pharmaceutical Co., Tokushima, Japan[8]. 5-FU was obtained from Sigma, St. Louis, Mo., U.S.A. N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce, Rockford, Ill., U.S.A. The other chemicals used were obtained from Wako Pure Chemicals, Osaka, Japan. Chloroform, ethyl acetate, ethanol and methanol were liquid chromatographic grade materials. 1,2-Dichloroethane and pyridine were dehydrated and purified by distillation before use.

Instruments

A Shimadzu-Du Pont Model LC-1 liquid chromatograph (Kyoto, Japan) equipped with a high-pressure injection valve (Model 513A, Gaschro Kogyo Co., Tokyo, Japan) was used.

A Du Pont Zorbax SIL chromatographic column (25 cm × 6.2 mm I.D.) was used for the separation, the mobile phase being 1,2-dichloroethane–ethanol (24:1) and the flow-rate being 1.5 ml/min. The column was maintained at room temperature.

A Model UVD-1 instrument was used for UV detection at 254 nm and the attenuator was set at 0.04 a.u.f.s. Peak areas were determined with a Shimadzu Chromatopac Model 1A apparatus.

A JEOL Model JMS-01SG-2 mass spectrometer with an electron-impact source (Tokyo, Japan) connected with a JEOL Model JGC-2K gas chromatograph was used.

The coiled glass column (2 m × 2 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80–100 mesh) (Gaschro Kogyo Co.) and was conditioned at 280° for 3 days. The injector, column and detector temperatures were 210°, 175° and 200°, respectively. The carrier gas was helium and the flow-rate was 40 ml/min.

The mass spectrometer was used under the following conditions: 75 eV ionization energy, 200 μA ionization current, 10 kV acceleration voltage and 3.0 kV ion multiplier voltage. The peaks of molecular ions and basic ions of the silylated derivatives of 5-FU and thymine (internal standard), of *m/e* 274 and 259 for the silylated derivative of 5-FU, M^+ and $M-CH_3$, and *m/e* 270 and 255 for the silylated derivative of thymine, M^+ and $M-CH_3$ were selected for mass fragmentographic analysis.

Extraction procedure

The plasma was separated from the blood, and stored frozen before the analysis.

The plasma (1 ml) obtained after administration of FD-1 was diluted with 1 ml of physiological saline, adjusted to be pH 2.0 with 5 *N* HCl and shaken vigorously with 20 ml of chloroform for 10 min. This extraction was repeated once more. The chloroform layer containing FD-1, 1-FT and 3-FT was concentrated at a temperature below 25°, and then transferred to a test-tube (10 ml capacity) by washing with chloform, and dried under a gentle stream of nitrogen. The residue was dissolved in 100 μl of 1,2-dichloroethane and 20 μl of this solution was injected into the liquid chromatograph.

The aqueous layer containing 5-FU separated from the chloroform layer was neutralized, mixed with 0.2 ml of 0.5 *M* NaH_2PO_4 solution and centrifuged at 1670 *g* for 10 min to remove proteins. The supernatant was extracted with 30 ml of ethyl acetate by vigorous shaking for 15 min. The ethyl acetate layer was concentrated at 40°, transferred to a reaction vial (1 ml capacity) by washing with methanol, and dried under a gentle stream of nitrogen at 40°. Methanol solution supplemented with 0.05 μg of thymine as an internal standard was added to the residue, and the mixture was dried under the same conditions as before. The dried residue was stored overnight over phosphorus pentoxide under reduced pressure. A freshly prepared solution of 100 μl pyridine containing 20% BSTFA was added to the residue, and the solution was kept at 60° for 10 min to allow silylation. Then 2–3 μl of this solution was injected into the GC–MF apparatus.

Calibration curves

Calibration curves for the determinations of FD-1, 1-FT, 3-FT and 5-FU were prepared by adding known amounts of each of these compounds to 1 ml of normal plasma and assaying the mixtures by the same analytical procedure.

The calibration curves for the determinations of FD-1, 1-FT and 3-FT by HPLC were prepared by plotting the peak areas against the concentrations of these compounds. The calibration curve for determination of 5-FU by GC–MF was prepared by plotting the ratio of the peak height of the silylated derivative of 5-FU to that of the silylated derivative of thymine as an internal standard, against concentration. As shown in Figs. 2 and 3, all these calibration curves were linear.

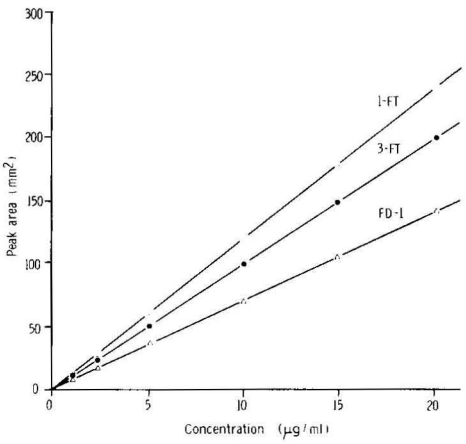


Fig. 2. Calibration curves of FD-1, 1-FT and 3-FT extracted from plasma.

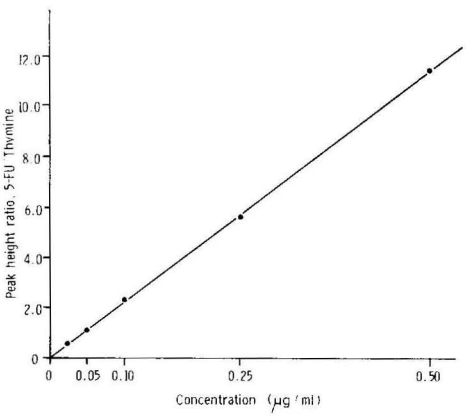


Fig. 3. Calibration curve of 5-FU extracted from plasma. Ratios of the peak height of 5-FU to that of thymine (internal standard) as their silylated derivatives are plotted against concentration.

RESULTS AND DISCUSSION

The concentration of 5-FU in clinical blood samples was found to be much lower than the concentrations of the other three compounds, FD-1, 1-FT and 3-FT. Therefore, it was difficult to establish a sensitive assay procedure for determining all four compounds simultaneously. Thus methods for assay of the three compounds FD-1, 1-FT and 3-FT, and of 5-FU separately were investigated.

GLC and GC–MF are not suitable for simultaneous determinations of FD-1, 1-FT and 3-FT, because 3-FT and more especially, FD-1 tend to decompose on heating and also during processes such as on-column methylation and silylation. The separate determinations of these three compounds by bioassay is also difficult, involving complicated procedures. Therefore, HPLC was adopted because it does not require the preparation of derivatives and can be performed at room temperature.

Assay of 5-FU by HPLC using a reversed-phase chromatographic and adsorption chromatographic system was found not to be sensitive enough for measuring the low concentrations of 5-FU in blood samples. Therefore, GC–MF in association with derivative preparation was used to obtain a high sensitivity.

In a previous study[2], it was found that FD-1, 1-FT and 3-FT could be extracted from aqueous solution with an organic solvent, while 5-FU remained in the aqueous layer. Therefore, in this study extraction with chloroform from an aqueous solution acidified with HCl was employed for quantitative separation of FD-1, 1-FT and 3-FT from 5-FU.

HPLC was not satisfactory for separative determinations of FD-1, 1-FT and 3-FT when a reversed-phase chromatographic column was used, because the detection sensitivity was low for FD-1, which is highly lipophilic, and because the lipophilic substances that were extracted from the plasma with chloroform influenced the reproducibility of the assay. Therefore, a column from the adsorption chromatography series Zorbax SIL was used. A mobile phase consisting of a mixture of an alcohol, such as methanol, ethanol or isopropanol, and a non-polar solvent, such as chloroform, dichloromethane, 1,2-dichloroethane or *n*-hexane, was found to be suitable for separation of standard FD-1, 1-FT and 3-FT.

The separation of FD-1, 1-FT and 3-FT from other plasma components extracted with chloroform was then investigated. The best and most reproducible separation was obtained using 1,2-dichloroethane–ethanol (24:1) as the mobile phase. A detector with a fixed wavelength (254 nm) was used routinely. The use of a wavelength of 270 nm with a variable-wavelength detector (Model SPD-1, Shimadzu), however, gave the same results as those obtained at 254 nm.

Known amounts of FD-1, 1-FT and 3-FT were added to the plasma of a Beagle dog, and the plasma was then diluted with physiological saline and adjusted to pH 2.0. Then the compounds were extracted twice with 10 volumes of chloroform. The HPLC separation of these compounds is shown in Fig. 4. The retention times for FD-1, 1-FT and 3-FT were 4.5 min, 6.9 min and 10.5 min, respectively. The recoveries from plasma are shown in Table I; quantitative values were obtained.

The detection limits of this HPLC method were 0.050 μg per ml plasma for FD-1 and 0.025 μg per ml plasma for 1-FT and 3-FT.

Decomposition of FD-1 to 1-FT during extraction with chloroform amounted to 0.04%. No noticeable decomposition of FD-1 to 3-FT was observed, and thus the observed values for these compounds were not affected by decomposition. Moreover, no decomposition of FD-1, 1-FT or 3-FT inside the adsorption chromatographic column was observed.

The method for assay of 5-FU by GC–MF was investigated on the basis of previous reports[6,7,9–16] on its assay in plasma after administration of 5-FU or 1-FT.

Various conditions were examined for separation of 5-FU following administration of FD-1 and for its extraction from the aqueous layer with a solvent or by

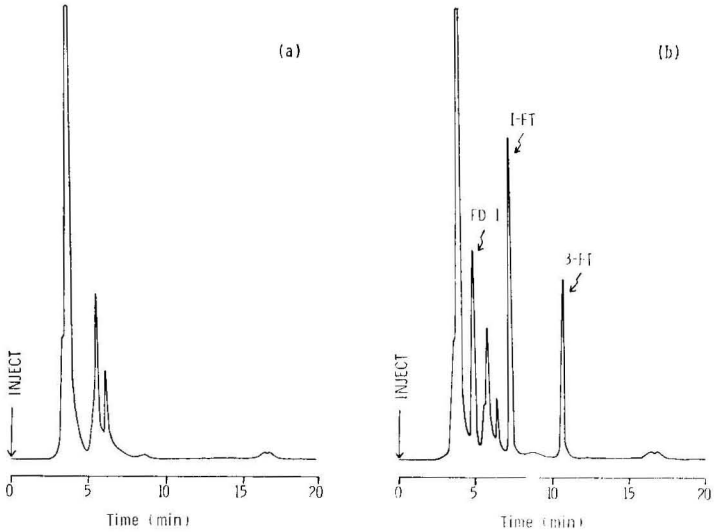


Fig. 4. Liquid chromatograms showing the separation of (a) control and (b) FD-1, 1-FT and 3-FT extracted from dog plasma.

TABLE I

RECOVERIES ON EXTRACTION OF FD-1, 1-FT AND 3-FT FROM SALINE AND PLASMA
Each value is the mean of three determinations by HPLC.

Compound	*Added (μg/ml)*	*Recovery from saline (%)*	*Recovery from plasma (%)*
FD-1	25.0	100.1	100.0
	5.0	101.4	100.3
	1.0	100.3	100.3
	0.5	101.3	101.0
	0.1	99.9	100.2
	Mean ± S.D. (%)	100.6 ± 1.1	100.4 ± 1.3
1-FT	25.0	100.1	100.3
	5.0	101.7	101.4
	1.0	100.5	100.3
	0.5	100.8	100.4
	0.1	100.3	100.2
	Mean ± S.D. (%)	100.7 ± 1.5	100.3 ± 1.4
3-FT	25.0	99.9	99.9
	5.0	101.0	100.2
	1.0	100.7	100.4
	0.5	100.6	100.5
	0.1	100.2	100.3
	Mean ± S.D. (%)	100.5 ± 1.4	100.3 ± 1.2

column chromatography, subsequent to chloroform extraction to separate FD-1, 1-FT and 3-FT. The following procedure was found to be the simplest and most rapid and to be the most reliable with the highest recovery. The aqueous layer containing 5-FU was neutralized and adjusted to pH 6.0 with 0.5 *M* NaH_2PO_4 solution. Then the

mixture was deproteinized and extracted with ethyl acetate. To obtain a derivative of 5-FU suitable for GC–MF, the procedures of silylation with BSTFA and on-column methylation with methanol containing 2% phenyltrimethylammonium hydroxide were compared, since both reactions are known to be quantitative. The silylation procedure was found to result in a higher detection sensitivity and a better separation of 5-FU from plasma components. Thymine was chosen as an internal standard for multiple ion detection. It can be silylated in the same way as 5-FU, and the molecular ion peak, basic ion peak, *m/e* 270 and *m/e* 255 in the mass spectrum of its silylated derivative are smaller by a mass number of 4 than those of the silylated derivative of 5-FU.

The pyridine solution containing 20% BSTFA used for silylation was prepared just before use. As the silylated derivative is easily hydrolyzed, the residue obtained after extraction with ethyl acetate was dried overnight over phosphorus pentoxide.

The separation patterns of dog plasma with known amounts of added 5-FU obtained by GC–MF are shown in Fig. 5. The retention time of the silylated derivative of 5-FU was 2.2 min and that for the internal standard, thymine, was 3.2 min. The overall recoveries of added 5-FU were 86.1 ± 3.7%, as shown in Table II.

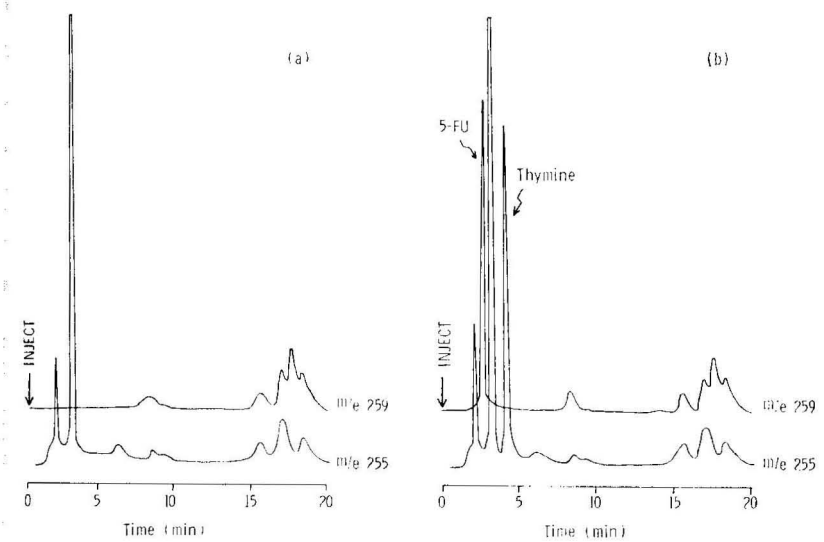


Fig. 5. Mass fragmentograms showing the separation of (a) control and (b) 5-FU extracted from dog plasma with thymine as an internal standard. Results are for the silylated derivatives.

The detection limit for 5-FU in this GC–MF method was 0.001 μg per ml plasma, which was sufficiently high. The detection limit of 5-FU itself in the adsorption chromatographic HPLC method as described before was also investigated using various concentrations of ethanol in the mobile phase and various flow-rates. The detection limit was found to be 0.100 μg per ml plasma.

As the concentrations of FD-1, 1-FT and 3-FT in plasma were much higher than that of 5-FU, the decompositions of these three compounds during the analytical procedure might affect the determination of 5-FU. Therefore, the decompositions of

TABLE II

RECOVERIES ON EXTRACTION OF 5-FU FROM SALINE AND PLASMA

Each value is the mean of three determinations by GC–MF.

Added (μg/ml)	*Recovery from saline (%)*	*Recovery from plasma (%)*
1.000	90.7	83.7
0.500	91.3	84.8
0.250	93.5	87.7
0.100	89.6	85.5
0.050	90.0	82.9
0.025	90.4	88.5
0.010	93.1	89.9
0.005	92.4	86.1
Mean ± S.D. (%)	91.4 ± 3.2	86.1 ± 3.7

these three compounds to 5-FU in dog plasma were examined. As shown in Table III, no decomposition of FD-1 was observed and the decompositions of 1-FT and 3-FT were too small to influence the assay of 5-FU.

This assay method was then applied to human and rat plasma to which FD-1 and its metabolites, 1-FT, 3-FT and 5-FU, were added. The results obtained for the separations, recoveries and detection limits were in good agreement with those obtained with dog plasma. This method can also be applied to other biological fluids, such as urine, bile, lymph, cerebrospinal fluid and ascites fluid.

TABLE III

DECOMPOSITIONS TO 5-FU OF FD-1, 1-FT AND 3-FT DURING THE ENTIRE ANALYTICAL PROCEDURE

Each value is the mean of three determinations by GC–MF.

Compound	*Added (μg/ml)*	*5-FU detected (μg/ml)*
FD-1	0.5	0
	1.0	0
	5.0	0
	25.0	0
1-FT	0.5	<0.001
	1.0	<0.001
	5.0	0.0022
	25.0	0.0017
3-FT	0.5	<0.001
	1.0	0.0011
	5.0	0.0010
	25.0	0.0021

The concentrations of FD-1, 1-FT, 3-FT and 5-FU in the plasma after administration of FD-1, determined by the present method, were compared with those determined by TLC and bioassay which had been reported previously[2]. Furthermore the concentrations of 1-FT and 5-FU in the plasma after administration of 1-FT determined by the present method and by bioassay were compared; the concentrations of FD-1, 1-FT and 3-FT after oral administration of FD-1 to rats and man determined

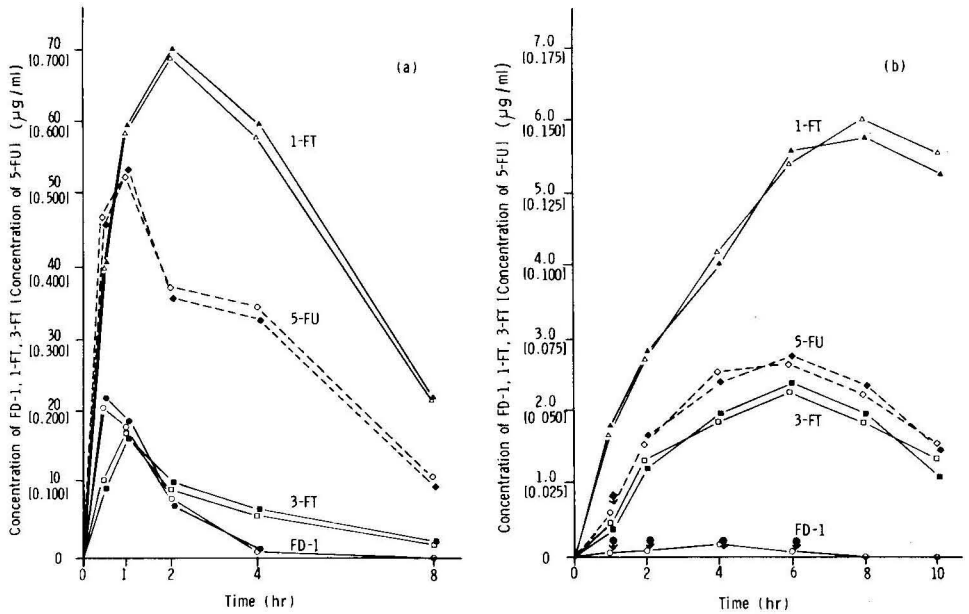


Fig. 6. Comparison of plasma levels of FD-1, 1-FT, 3-FT and 5-FU determined by HPLC (○, △, □), TLC (●, ▲, ■), GC–MF (◇) and bioassay (◆). The single oral doses given were (a) 135 mg (0.5 mmole) FD-1 per kg to rats and (b) 400 mg FD-1 per person to man (7.3 mg per kg). The arrowed symbols indicate the detection limit.

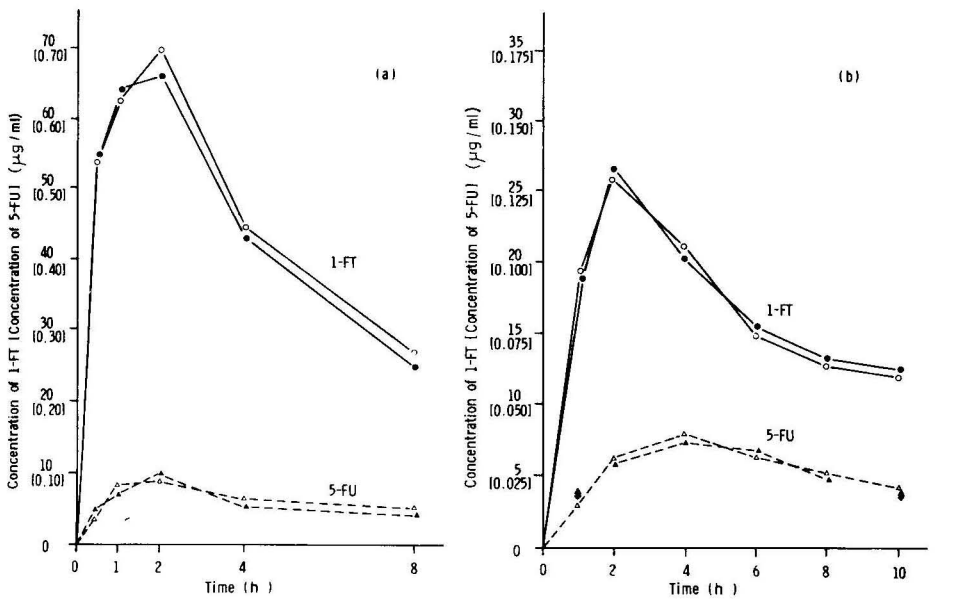


Fig. 7. Comparison of plasma levels of 1-FT and 5-FU determined by HPLC (○), GC–MF (△) and bioassay (●, ▲). The single oral doses given were (a) 100 mg (0.5 mmole) 1-FT per kg to rats and (b) 800 mg 1-FT per person to man (14.5 mg per kg). The arrowed symbol indicates the detection limit.

TABLE IV

COMPARISON OF SENSITIVITIES AND PRECISIONS OF SEVERAL METHODS FOR ASSAY OF FD-1, 1-FT, 3-FT AND 5-FU

Compound	*Sensitivity as plasma level (μg/ml)*			
	HPLC	*GC–MF*	*TLC*	*Bioassay*
FD-1	0.050	—	0.200	—
1-FT	0.025	—	0.200	2.5*
3-FT	0.025	—	0.200	—
5-FU	—	0.001	—	0.020
Precision (S.D.)	±1.3	± 3.7	± 4.1	± 10.7

* Determined after administration of 1-FT.

by HPLC and by TLC were compared, and the concentrations of 5-FU determined by GC–MF and by bioassay were compared; the concentrations of 1-FT after oral administration of 1-FT determined by HPLC and by bioassay were compared, and the concentrations of 5-FU determined by GC–MF and by bioassay were compared. The results obtained are shown in Figs. 6 and 7.

The results obtained by the present method utilizing HPLC and GC–MF were in good agreement with those obtained by TLC or by bioassay. As shown in Table IV, the method is precise and has higher sensitivity than TLC or bioassay, and in addition it is rapid and simple. Thus it should be useful for basic and clinico-pharmacological investigations on the compounds FD-1 and 1-FT.

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CELLULAR UPTAKE AND METABOLISM OF DAUNORUBICIN AS DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO L1210 CELLS

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(Received February 2nd, 1978)

SUMMARY

The metabolism of daunorubicin (DNR) to daunorubicinol and daunomycinone in murine leukaemia cells has been examined by means of high-performance liquid chromatography. A rapid and efficient extraction method has been developed that permits the recovery of the drug and its metabolites from cell homogenates. By means of high-performance liquid chromatography daunorubicinol and daunomycinone have been separated from DNR and the intracellular concentration of the compounds determined. The method developed is very rapid and sensitive, and amounts as small as 30 pg of DNR can be detected. The results indicate that the aldo-keto reductase is not very active in L1210 cells in culture, the main intracellular product found being DNR.

INTRODUCTION

Since daunorubicin (DNR) is used as an antitumour agent in the treatment of acute leukaemias and malignant lymphomas, the quantitative determination of the drug and its metabolites in tissues and cells is becoming more important both clinically and experimentally. Previous methods required a purification step and were time consuming. Langone *et al.*[1] recently suggested the use of high-performance liquid chromatography (HPLC) for the determination of doxorubicin (DOX, 14-dihydrodaunorubicin) and its metabolites in urine and Hulhoven and Desager[2] used HPLC to determine DNR and its main metabolite daunorubicinol (DOL, 13-dihydro-DNR) in plasma.

We describe here a method for the extraction of DNR and its metabolites from cell homogenates and their quantitative determination by HPLC down to a concentration of 30 pg (1.5 ng/ml).

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MATERIALS AND METHODS

DNR, DOL and DOX hydrochloride were generously supplied by Dr. Maral (Rhône-Poulenc, Paris, France). The solvents were of analytical grade (Merck, Darmstadt, G.F.R.) and were used without purification. Phosphate-buffered saline (PBS) at pH 7.4 has the following composition: NaCl, 140 m*M*; KCl, 3 m*M*; KH_2PO_4, 8 m*M*; Na_2HPO_4 8 m*M* in deionized water.

Drug-uptake experiments

L1210 leukaemia cells were cultivated in suspension[3] using RPMI 1640 medium (Eurobio, Paris, France), supplemented by 10% fetal calf serum[4]. $3 \cdot 10^5$ L1210 cells were incubated at 37° for 24 h in 2 ml of medium before addition of DNR at a concentration of 1 μg/ml. After various times the cells were centrifuged and washed with PBS.

Extraction procedure

Cells were suspended in 2 ml of PBS and sonicated at 50 W for 20 sec with a Branson B-12 sonicator (Branson, Conn., U.S.A.). A 0.1-ml sample of the internal standard (DOX at 10 μg/ml) and 1.8 ml of the extraction mixture (chloroform–methanol 4:1) were then added to 0.1 ml of the cell homogenate. The sonication tubes were stoppered with silicone elastomer corks and the contents sonicated again for 15 sec. 20 μl of the lower organic layer were then injected directly into the chromatograph using a 100-μl Hamilton syringe.

High-performance liquid chromatograph

A Hewlett-Packard Model 1084 high-performance liquid chromatograph was used with a six-port injection valve (Rheodyne, Model 7120 with a loop of 20 μl). The stationary phase consisted of 7-μm silica-gel particles prepacked into a 250 × 3 mm stainless-steel column (Hibar, LiChrosorb Si 60; Merck). The mobile phase was chloroform–methanol–glacial acetic acid–water (720:210:35:30) at a flow-rate of 1.0 ml/min.

A Gilson Fl-IA/B fluorimeter (Gilson, Middleton, Wisc., U.S.A.) was connected in series with the Hewlett-Packard detector (254 nm wavelength). Excitation and emission wavelengths of 480 nm and 560 nm were selected by use of the corresponding interference filters. The detection and integration of the peaks were performed by the built-in Model 1080 software integrator of the chromatograph. Drug concentrations were calculated by taking into account their specific fluorescence (see Fig. 1).

RESULTS AND DISCUSSION

A limit of sensitivity of 10 ng/ml DNR could be obtained by using the built-in UV detector at maximal amplification (10^{-4} absorbance units per cm). An increased selectivity and also a greater sensitivity were obtained by using the Model Fl-IA/B fluorimeter connected in series with the Hewlett-Packard detector. The lowest amount of DNR detectable is 1.5 ng/ml. This amount is defined by the height of the DNR peak being three times that of the noise level.

Calibration graphs for an equimolar mixture of DNR, DOL and DOX are

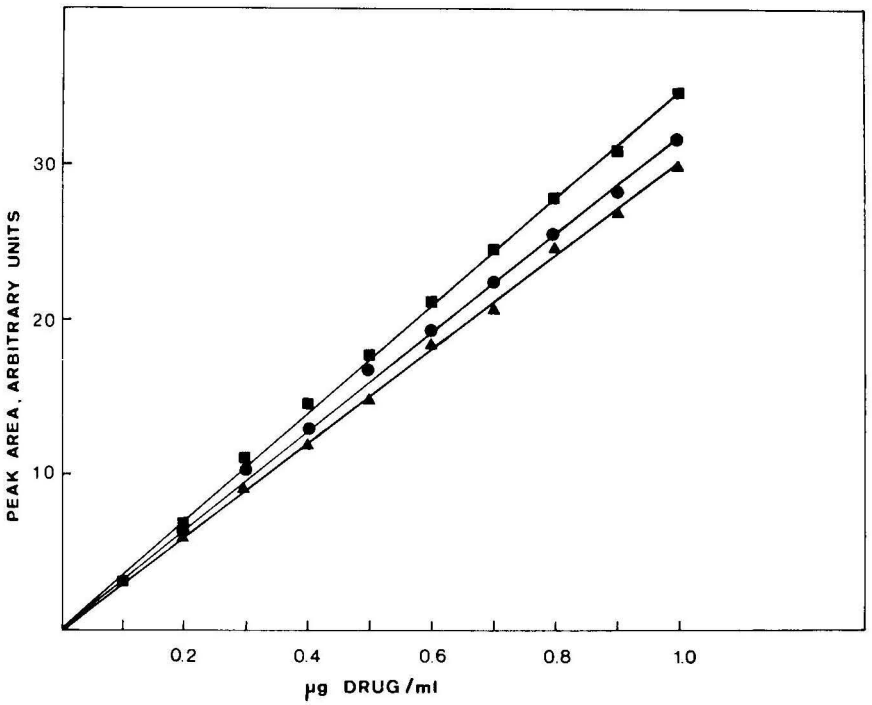


Fig. 1. Calibration curves of DNR (■), DOL (●) and DOX (▲) as measured by HPLC. The integrated peak areas given were obtained from the integrator of the fluorimeter. The flow-rate was set at 1 ml/min.

rectilinear in the range 10–1000 ng/ml (Fig. 1). The reproducibility of the retention times and the accuracy of the integrated surfaces are excellent, as shown in Table I.

The elution system of chloroform–methanol–acetic acid–water was chosen in order to separate DNR from its metabolites in less than 10 min. Fig. 2 shows the separation of four compounds related to DNR. It can be seen that the peaks are sharp and well resolved. From the ratio of retention time (t_R) and baseline bandwidth (w)

TABLE I

REPRODUCIBILITY OF THE RETENTION TIMES AND PEAK AREAS OBTAINED BY HPLC OF DNR, DOL AND DOX IN AN EQUIMOLAR MIXTURE (0.18 nM/ml)

Twelve different samples of the equimolar mixture were injected into the liquid chromatograph. The flow-rate was set at 3.0 ml/min. The retention times and areas given for each run by the 1080 software integrator were averaged and the standard deviations calculated.

Sample constituents	*Retention time (min)*		*Peak area (integrated counts × 10^3)*	
DNR	2.38	± 0.01	25.36	± 0.72
DOL	3.03	± 0.01	24.97	± 0.98
DOX	3.30	± 0.02	21.64	± 0.95

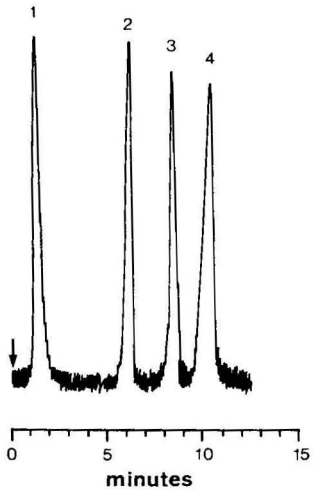


Fig. 2. Chromatogram of a sample mixture of daunomycinone (1), DNR (2), DOL (3) and DOX (4).

we have calculated, using eqn. 1 (ref. 5), an efficiency of 3100 theoretical plates (N) for our 25-cm column.

$$N = 16(t_R/w)^2 \quad (1)$$

The retention times for the compounds reflected their hydrophilic character since the most apolar compound daunomycinone (the aglycone of DNR), is eluted first (t_R = 1.4 min) while the more polar hydroxyl derivatives DOL (t_R = 8.7 min) and DOX (t_R = 10.1 min) are eluted after DNR (t_R = 6.4 min).

We have used this method to determine the uptake and metabolism of DNR by leukaemia L1210 cells grown in culture. As shown in Fig. 3, the recovery of DNR

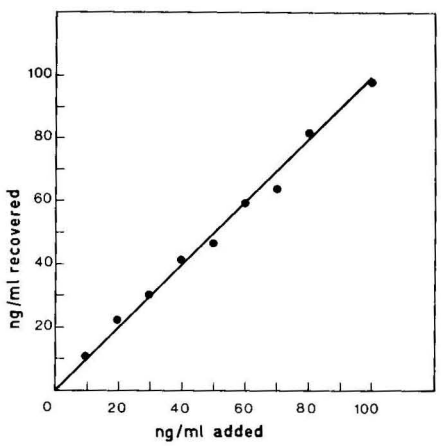


Fig. 3. Recovery of DNR from an L1210 cell homogenate. DNR was added to $3 \cdot 10^5$ L1210 cells in 1 ml of RPMI medium in order to obtain drug concentrations of 10–100 ng/ml. Each tube was sonicated for 20 sec at 50 W. To 0.1 ml of L1210 cell homogenate was added 0.1 ml of DOX at 10 μg/ml borate buffer at pH 9.8 and the drugs were extracted with 1.8 ml of chloroform–methanol (4:1). 20 μl of the organic phase were injected into the chromatograph.

from L1210 cell extracts is close to 100%. The same extraction procedure gave complete recovery of DNR from mouse whole blood[6]. The time course of the uptake and metabolism of DNR by L1210 cells, as determined by our methods, is illustrated in Fig. 4. The rate of uptake of DNR is linear up to 150 min and the accumulation reaches a plateau level of 3.0 μg/mg of cell protein after 5 h. This result indicates that DNR is accumulated about 600 times by L1210 cells if we assume by comparison with cultivated fibroblasts[7] that 1 mg of cell protein corresponds to a volume of 5 μl. The transformation of DNR into DOL proceeds very slowly, only 3% of DNR being metabolized after 22 h. Significant amounts (up to 10% of the total fluorescence) of daunomycinone were also detected after 22 h of incubation of the cells in presence of the drug.

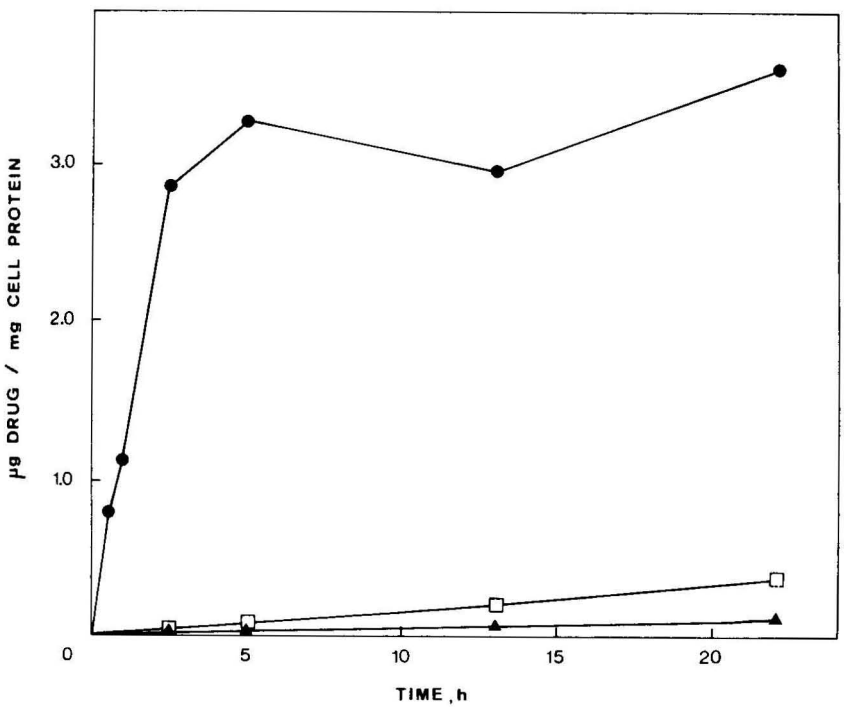


Fig. 4. Intracellular uptake and metabolism by L1210 leukaemia cells *in vitro*. $3 \cdot 10^5$ L1210 cells in 2 ml of RPMI medium supplemented with 10% calf serum were incubated in the presence of DNR at 1 μg/ml. Samples were taken at intervals and to 0.1 ml of cell homogenate were added 0.1 ml of DOX (internal standard) at 10 μg/ml of borate buffer (pH 9.8), and 1.8 ml of chloroform–methanol (80:20). After sonication, 20 μl of the lower organic layer were injected into the chromatograph. ●, DNR; □, daunorubicin aglycone; ▲, DOL.

The method described allows a rapid determination of DNR and its fluorescent metabolites in cells since it takes, at the most, 15 min to obtain the chromatogram subsequent to harvesting of the cells. The sensitivity is very high since it is possible to detect drug concentrations down to 1.5 ng/ml. DNR is easily distinguished from its main metabolites since the chromatogram peaks are sharp and well resolved.

However, our method does not allow the separation of very apolar derivatives, like the aglycones of DNR described by Takanashi and Bachur[8]. Moreover, polar anthracyclines like DOX interact strongly with the silica gel, thus precluding the use of our method for rapid separation of DOX and its polar derivatives.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

V. SEPARATION OF THYROIDAL IODO-AMINO ACIDS BY HYDROPHILIC ION-PAIRED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of thyroidal iodoamino acids has been carried out by high-performance liquid chromatography in phase systems consisting of chemically bonded C_{18}-hydrophobic supports as the stationary phase and water–organic solvent mixtures containing phosphoric acid or other ion-pairing reagents as the mobile phase. Under conditions of hydrophilic ion-pair formation, excellent resolution of the iodoamino acids is observed. This method permits the rapid separation and, hence, analysis of mixtures containing thyroxine, 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine and related compounds in *ca.* 30 min with sensitivity, using a UV monitor at 210 nm, at the 1–10-pmole level.

INTRODUCTION

During the past few years, much attention has been focused on new methods for the quantitative determination of drugs, drug metabolites and endogenous compounds present in biological media. The application of high-pressure (performance) liquid chromatographic (HPLC) techniques has proved particularly useful for the separation and analysis of those biogenic compounds which exist in aqueous solution only in ionized states, *e.g.*, quarternary ammonium compounds or as amphoteric substances. Early studies using reversed-phase liquid–liquid chromatography were limited in their application due to the low efficiency and stability of the columns. However, recent developments in chromatographic methodology have made available highly efficient reversed-phase columns with chemically bonded stationary phases

coupled to silica micro-particles clearly proving the more versatile type of column support. Ion-pair partition combined with reversed-phase liquid–liquid chromatography has been found to give separation systems of high efficiency and selectivity for a large variety of compounds. One obvious application of these methods is the analysis of the thyroidal iodoamino acids both in the pharmaceutical and clinical situation. Karger *et al.*[1] have reported an ion partion liquid–liquid separation of thyroxine (T_4) and triiodothyronine (T_3) using perchlorate ion pairs with a mobile phase consisting of methylene chloride–butanol (17:3). A variety of other procedures has been described[2–6] for the separation of mixtures of these iodo compounds by thin-layer (TLC) and paper chromatography, Sephadex gel or controlled-pore glass exclusion chromatography, ion-exchange column chromatography and gas–liquid chromatography (GLC). Many of these earlier methods have special requirements in terms of derivatization of the sample or require large amounts of material.

Recently we reported[7,8] the use of phosphoric acid and other ion-pairing reagents in reversed-phase HPLC for the analysis of underivatized peptides. One advantage of this rapid procedure was that aqueous samples can be applied directly to a chromatographic support and subsequently eluted with an aqueous mobile phase. We illustrated the selectivity of this method by the resolution of small peptides differing only in one amino acid residue. Utilising a similar approach we now report in this paper a new method for the rapid chromatographic analysis of the thyroidal iodoamino acids by reversed-phase partition HPLC.

EXPERIMENTAL

Chromatographic equipment

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of two M-6000A solvent-delivery pumps, a U6K universal liquid chromatograph injector and a M-660 solvent programmer, coupled either to a Series 440 UV absorbance detector and a Rikadenki double channel recorder or to a Cecil 515 or Cecil 212 variable-wavelength UV monitor with 10-μl or 8-μl flow-through cells, respectively, and a Linear Instruments Corp. double-channel chart recorder. Unless otherwise indicated the detector operated at a wavelength of 254 nm.

The Bondapak C_{18}-Corasil (37–50 μm) and μBondapak C_{18} (10 μm) columns, which had normal column dimensions of 61 cm $\times$ 2 mm I.D. and 30 cm $\times$ 4 mm I.D., resp., were purchased from Waters Assoc.

Samples injections were made with Pressure-Lok liquid syringes (0–10 μl, 0–25 μl) Series B110 from Precision Sampling. Solvents were filtered using a Pyrex filter holder (Millipore) while iodoamino acid samples were filtered using a Swinney Filter assembly (Millipore) with AP2500 filters.

Reagents

All solvents were AnalaR grade. The methanol was supplied by Mallinckrodt (St. Louis, Mo., U.S.A.) or Fisher Scientific (Pittsburgh, Pa., U.S.A.) and distilled from 0.1% metaphenylene diamine. The acetonitrile, supplied by Fisher Scientific, was further purified by the method of Walter and Ramaley[9]. Orthophosphoric acid was from May and Baker (Dagenham, Great Britain), potassium dihydrogen phosphate from BDH (Poole, Great Britain) and the ion-pairing chromatographic

reagents, PIC Reagent A and PIC Reagent B-5 and B-7 were from Waters Assoc. Water was de-ionised and glass distilled. The iodoamino acids were either obtained from Sigma (St. Louis, Mo., U.S.A.) or prepared by well established literature methods; rT_3 was a generous gift from Dr. Steinmaus, Henning, Berlin, G.F.R. Stock solutions of thyroxine (T-4), 3,3′,5-triiodothyronine (T-3), 3,3′,5′-triiodothyronine (rT_3), 3,5-diodothyronine (T-2), 3,5-diiodotyrosine (DIT), 3-iodotyrosine (MIT), thyronine (T_0) and tyrosine (Tyr) were prepared by dissolving the compounds in 1% methanolic NH_4OH (methanol–concentrated ammonium hydroxide, 99:1) at a concentration of *ca.* 10 mg/ml.

Methods

A flow-rate of 2 ml/min was generally used, which was maintained at a pressure of *ca.* 1500 p.s.i. for the Bondapak C_{18}-Corasil and 2200 p.s.i. for the μBondapak C_{18} column. The solvent reservoirs, pre-column delivery systems and columns were maintained at a temperature of *ca.* 20°. Samples of the iodoamino acids were prepared from the stock solutions by serial dilution using Eppendorf micro-syringes and diluted with the first eluting solvent. Sample size varied between 10 ng and 10 μg of material injected in volumes 1–25 μl. Detection was at a fixed wavelength of 254 nm using the Series 440 detector or in the range of 190–280 nm, depending on the nature of the sample and mobile phase, using the variable-wavelength detectors. All bulk solvents were degassed separately; water and 100 m*M* KH_2PO_4 for at least 30 min, organic solvents for 1.5 min. The solvents were mixed to the required volumes, degassed for 1.5 min and equilibrated at room temperature. Orthophosphoric acid was added, where indicated, to the aqueous solvents at a concentration of 0.1%. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents or re-equilibrated with the starting solvent after a gradient run for at least 30 min. The mobile-phase solutions were routinely filtered using 0.5 μm Millipore filters.

RESULTS AND DISCUSSION

In contrast to adsorption or polar-phase ion-pair partition chromatography where the order of elution follows the increase in polarity, in the reversed-phase mode for a given mobile phase composition the more polar compounds elute earlier. In preliminary experiments using C_{18}-Corasil and μBondapak C_{18} columns and mobile phases of water–methanol (5–75%) or water–acetonitrile (5–50%), good separations of Tyr, MIT and DIT could be achieved, although these elution systems did not resolve adequately mixtures of the higher thyronine analogues due to their long retention times and poor peak shapes. Previously, we had observed[7,8] with underivatized peptides that the addition of sodium or potassium dihydrogen phosphate (up to 100 m*M*) or orthophosphoric acid (0.1%) to the mobile phase dramatically reduced retention times of non-polar compounds, increased resolution and allowed the use of eluants which contain significantly lower concentrations of organic solvents. Shown in Fig. 1 is the separation of MIT and DIT on a C_{18}-Corasil column eluted with 5% methanol–100 m*M* NaH_2PO_4. Under these conditions Tyr emerges at the solvent front, whilst the thyronine analogues T_3 and T_4, again have very long retention times and consequently are poorly resolved. However these materials can be completely resolved by increasing the methanol concentration to 50% (Fig. 2). This order

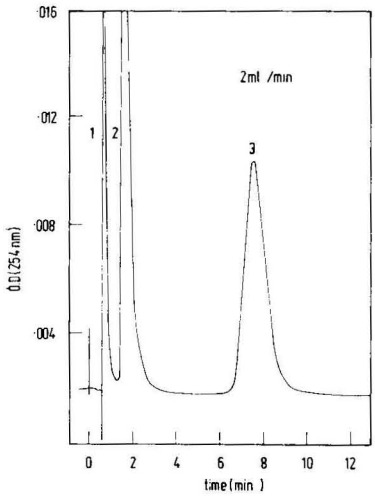


Fig. 1. Separation of tyrosine (1), 3-iodotyrosine (2) and 3,5-diiodotyrosine (3) on a C_{18}-Corasil column with a 5% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4 mobile phase; 2 ml/min; injection volume 10 μl, 20 μg each.

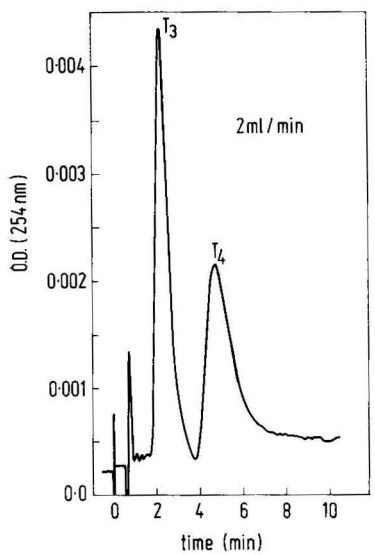


Fig. 2. Separation of the thyroid hormones R_3 and T_4 on a C_{18}-Corasil column using a mobile phase containing 50% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4. Other conditions as for Fig. 1.

of elution is in accordance with the anticipated increasing hydrophobicity of the solvated iodoamino acids taking into account the known hydrophobic nature of the iodo-group[10]. Furthermore, this observed order parallels earlier reports[1,5] of the elution order of T_3 and T_4 on controlled-pore glass and silica particles coated with a stationary phase of 0.2 *M* $HClO_4$–0.8 *M* $NaClO_4$. It is interesting to note that T_0 and

DIT are poorly separated under these conditions. Further studies rapidly confirmed that the remaining thyroidal amino acids also segregated into the two loose chromatographic groupings: tyrosine and its iodo derivatives and thyronine and its iodo analogues, on chemically bonded reversed-phase columns. Although it is possible to carry out a two-stage elution to achieve the complete separation of the compounds MIT, DIT, T_0, T_1, T_2, T_3, rT_3 and T_4 on C_{18}-Corasil columns, two alternatives were examined. Firstly, continuous gradients can now be readily achieved under programmed solvent-delivery conditions. A chromatographic profile of the thyroid hormones under conditions of gradient elution using the elution system 100 m*M* NaH_2PO_4–0.1% H_3PO_4 with a 5–80% methanol gradient is shown in Fig. 3. As can be seen, this method permits the resolution of iodo-amino acids with widely differing polarities. However, due to the change in the methanol concentration during the gradient, the baseline optical density drifts with inherent loss of sensitivity. These difficulties can however be overcome by using reversed-phase packings of smaller particle diameter *e.g.*, 10 μm for the fully porous μBondapak C_{18} supports compared to 37–50 μm for the pellicular C_{18}-Corasil. As can be seen in Fig. 4, the complete separation of many of the thyroidal amino acids can be achieved on μBondapak C_{18} columns by an isocratic elution with methanol–water–H_3PO_4 (50:50:0.1) in less than 30 min. Similar elution profiles were observed using acetonitrile (20%) instead of methanol.

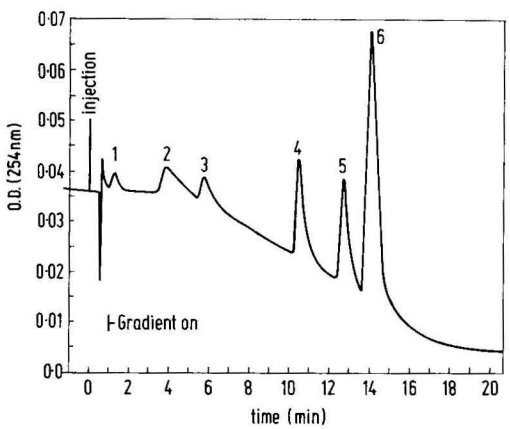


Fig. 3. Gradient elution of iodo-amino acids on a C_{18}-Corasil column using a 5–80% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4 gradient; 2 ml/min; injection volume 10 μl, 1 μg each spiked with thyroxine; 1 = MIT, 2 = DIT, 3 = T_0, 4 = T_2, 5 = T_3 and 6 = T_4. The gradient was generated using programme 5 on the M-660 solvent programmer.

It is noteworthy that the two triiodothyronine isomers, T_3 and rT_3 and thyroxine are completely resolved under these conditions (Fig. 5). The simplicity of the present method has considerable advantages over the other existing methods which usually require prior derivatization[2] or lengthy TLC or GLC techniques for the chromatographic separation and analysis of these closely related compounds. In view of their high extinction coefficients, reproducible analysis of the thyroid hormones could be carried out at *ca.* a 10-pmole level at 254 nm while further improve-

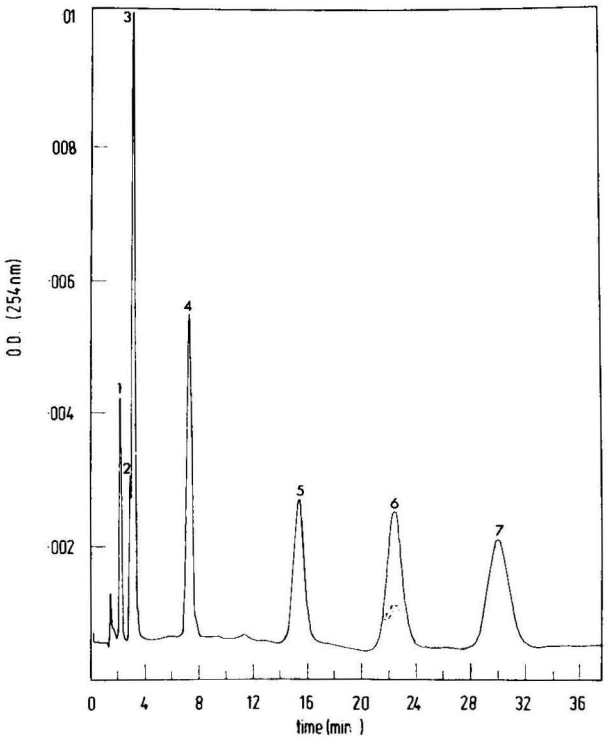


Fig. 4. Isocratic elution of iodo-amino acids using 50% methanol–water–0.1% H_3PO_4; 2 ml/min, on a μBondapak C_{18} column; injection volume 10 μl, 0.2 μg each; 1 = MIT, 2 = DIT, 3 = T_0, 4 = T_2, 5 = T_3, 6 = rT_3 and 7 = T_4.

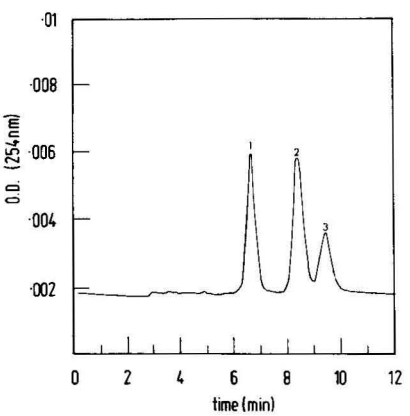


Fig. 5. Separation of the thyroid hormones T_3, rT_3 and T_4 using 62.5% methanol–water–0.1% H_3PO_4, 1 ml/min, on a μBondapak C_{18} column.

ment in sensitivity (*ca.* a factor of 10 depending on the iodoamino acid) can be achieved by using variable-wavelength monitors operating at 210 nm under the elution conditions.

As is evident from Figs. 4 and 6, the compounds DIT and T_0 are poorly

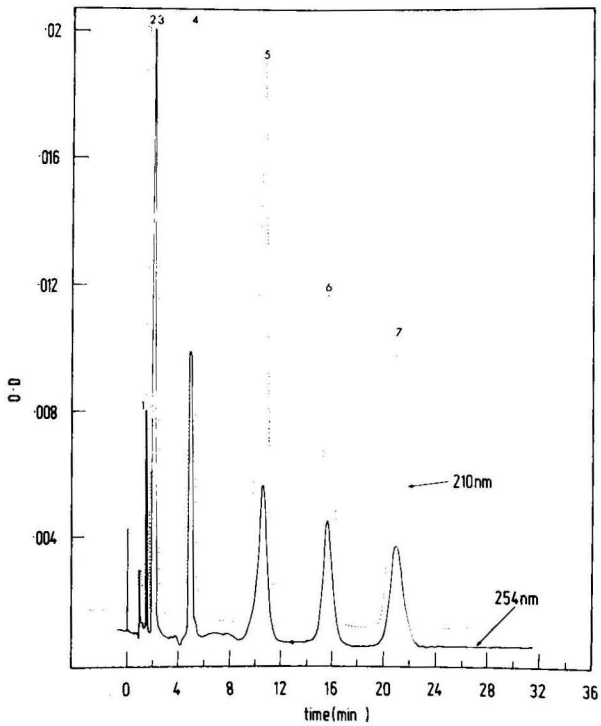


Fig. 6. Comparative elution profiles of the iodo-amino acids detected at 210 and 254 nm, chromatographic conditions as given in Fig. 4, injection volume 20 μl.

resolved on a μBondapak column using the elution system methanol–water–H_3PO_4 (50:50:0.1). However adequate separation of these two compounds can be achieved (at the expense of longer retention times for the less polar iodothyronines) using a lower methanol concentration or acetonitrile–water–H_3PO_4 (15:85:0.1). Alternatively, purified hydrophobic ion-pairing reagents, *e.g.*, the PIC reagents, can be used to improve the resolution of DIT and T_0 although the PIC reagents themselves

TABLE I

RETENTION TIMES OF 3,5-DIIODOTYROSINE (DIT) AND THYRONINE (T_0)

Eluents	*Retention time (min)*	
	3,5-Diiodotyrosine	*Thyronine*
Acetonitrile–water (15:85) + H_3PO_4*	5.6	6.7
Methanol–water (40:60) + H_3PO_4*	1.4	1.6
Methanol–Pic A (40:60)*	3.1	4.2
Methanol–Pic A (40:60) + H_3PO_4*	3.1	3.5
Methanol–Pic A (40:60) + Pic B_7*	3.2	3.4
Methanol–water (40:60) + H_3PO_4**	2.8	3.7
Methanol–water (50:50) + H_3PO_4**	2.4	2.7

* 2 ml/min, on C_{18}-Corasil column.
** 2 ml/min, on μBondapak column.

do not appear to have as significant an effect on the resolution of these two compounds as has been observed with mixtures of other amphoteric molecules (Table I).

In conclusion, a set of conditions which permit the rapid analysis and separation of the thyroid hormones and related compounds is reported. The method, which employs underivatized samples, is amenable to direct quantitation and is rapid. It is logical to expect that application of this, and similar, HPLC approaches to the rapid detection of thyroid hormones and their metabolites will be of considerable value for the routine pharmaceutical analysis and for both *in vitro* and *in vivo* studies involving these compounds.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Nachtmann *et al.*[11] have recently reported a catalytic detection system for post-chromatographic analysis of iodine-substituted molecules. This elegant method, based on the Sandell–Kolthoff reaction, permits trace determinations of iodinated molecules with the limit of detection for tetraiodothyronine below the 1-ng level. Associated with an efficient chromatographic separation, this new detection method should have sufficient sensitivity for the sequential determination of the thyroid hormones and their metabolic products in plasma and tissues. The ability to rapidly carry at these analyses contrasts with other methods presently used including those based on radio immunoassay techniques.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF HYDROXYCINNAMIC ACID–TARTARIC ACID ESTERS AND THEIR GLUCOSE ESTERS IN *VITIS VINIFERA**

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SUMMARY

A high-pressure liquid chromatographic technique has been developed to separate and quantify the amount of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyl-tartaric acid in grapes. The method features direct analysis of the grape juice with minimum sample preparation. The identity of the hydroxycinnamic acid–tartaric acid esters has been established through hydrolysis of the esters followed by gas–liquid chromatography and high-pressure liquid chromatography. The absence of chlorogenic and neochlorogenic acids often reported in grapes has been substantiated. Evidence will be presented for the occurrence of an ester of caffeic acid–glucose–tartaric acid.

INTRODUCTION

Esters of the hydroxycinnamic acids are widely distributed in the plant world, especially in fruits [1–3]. Numerous researchers[1,4–7] have identified chlorogenic acid (3-caffeoylquinic acid), its various positional isomers, and the analogous *p*-coumaroyl- and feruloylquinic acids[8] in *Vitis vinifera* grapes. Hydroxycinnamic acid–tartaric acid esters have been shown in grape skins[9], whole grapes[10], spinach chloroplasts[11–14], and the leaves of members of the Compositae[2,3,15–18].

As late as 1976 Singleton and Noble[19] noted that whether the esters of quinic acid, tartaric acid, or both are present in *V. vinifera* grapes is still unresolved. Recently, Singleton has shown conclusively the presence of hydroxycinnamic acid–tartaric acid esters in *V. vinifera*[20]. Glucose esters and glycosides of the hydroxycinnamic acids have been identified in numerous plants by Harborne and Corner[21], but have not been shown to be present in grapes.

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One of the major problems involved in separating and identifying hydroxycinnamic acid esters is their similarity in chemical characteristics. The quinic acid, tartaric acid, and glucose esters of a single hydroxycinnamic acid show similar ultraviolet (UV) absorption spectra with maxima for each ester typically varying by 3–5 nm from each other[22,23]. Many of the common paper chromatography solvents can not adequately separate the different esters of a single hydroxycinnamic acid[22].

High-pressure liquid chromatography (HPLC) using reversed-phase packings has been shown to completely separate the various hydroxycinnamic acids[24]. Court[25] separated the various positional isomers of chlorogenic acid on a reversed-phase packing. 3-Caffeoylquinic acid and 3-feruloylquinic acid are cleanly separated by HPLC and isochlorogenic acid, composed of three isomers of dicaffeoylquinic acid, yields three distinct peaks when analyzed by HPLC[16]. The sensitivity of HPLC for phenolic compounds is extremely good, easily detecting as little as 5 ng of the hydroxycinnamic acids.

In this study, we set out to establish the identity of the major hydroxycinnamic acid esters present in *V. vinifera* grapes, and to quantify the amounts present in various varieties.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) high-pressure liquid chromatograph consisting of two Model 6000A chromatography pumps, a Model 660 solvent programmer, and a U6K injector was used. A Micromeritics Chromonitor 785 (Norcross, Ga., U.S.A.) was used to measure the optical density of the eluates. A Houston Instruments Omniscribe strip chart recorder (Austin, Texas, U.S.A.) provided a visual display while an Autolab Minigrator (Spectra-Physics, Santa Clara, Calif., U.S.A.) was used to integrate the peak areas. A 25 mm × 4.6 mm I.D. Zorbax ODS chromatography column (Du Pont Instruments, Wilmington, Del., U.S.A.) equipped with a 5 cm × 4.6 mm I.D. pre-column packed with 10-μm μBondaPak ODS (Waters Assoc.) was used for the chromatographic separations.

Preparative column chromatography was done on a 50 cm × 2.4 cm glass column packed with Polyamid CC-6 (Brinkmann Instruments, Westbury, N.Y., U.S.A.). Fractions were collected on an automatic fraction collector (Instrumentation Specialties Company, Lincoln, Nebr., U.S.A.).

Gas–liquid chromatography (GLC) was performed on a Varian Aerograph Model 1740-1 gas chromatograph (Palo Alto, Calif., U.S.A.) equipped with dual flame ionization detectors. A 6 ft. × 2 mm I.D. stainless-steel column packed with 3% SE-30 on Chromosorb W (70–80 mesh) was used for the separation of silylated sugars and tartaric acid.

Spectrophotometric measurements were made with a Beckman DU modified with an optical density converter, light source stabilizer, and cuvette positioner (Gilford Instrument Labs, Oberlin, Ohio, U.S.A.). A Buchler rotary evaporator (Fort Lee, N.J., U.S.A.) was used for concentration of samples. All samples and HPLC solvents were filtered through a 0.45-μm Millipore cellulose acetate or PTFE membrane filter (Bedford, Mass., U.S.A.) prior to use.

Reagents

Reagent-grade methanol, acetonitrile, pyridine, phosphoric acid, glutamic acid, tartaric acid, chlorogenic acid, glucose, and xylose were obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). Trimethylchlorosilane (TMS), hexamethyldisilazane (HMDS), and N,O-bis(trimethylsilyl)-acetamide (BSA) were purchased from Pierce Chemical Company (Rockford, Ill., U.S.A.). Dr. Joseph Corse (Western Regional Research Lab., USDA, Albany, Calif., U.S.A.) kindly furnished samples of 3-caffeoylquinic acid (chlorogenic acid), 5-caffeoylquinic acid (neochlorogenic acid), and 3-feruloylquinic acid.

All organic solvents used for HPLC were re-distilled prior to use. All water was distilled and stored in glass.

Procedure for isolation and characterization

Grapes (*V. vinifera*) held frozen at −40° were quickly defrosted in a microwave oven. Sulfur dioxide (1000 ppm), as sodium bisulfite, was added. The grapes were crushed and forcefully squeezed by hand through cheesecloth. The pulp and seeds were discarded and the juice (200–400 ml) was extracted three times using the ethanol–ammonium sulfate technique of Singleton[27] to remove sugars and organic acids and concentrate the phenolic compounds. Ethanol was removed from the combined extracts on a rotary vacuum evaporator. The extraction process was repeated. The combined extracts were evaporated to a volume of 2–10 ml and filtered through a 0.45-μm Millipore filter.

The unknowns were then separated from the extract by preparative HPLC methods using conditions similar to analytical conditions or by column chromatography on Polyamid CC-6. Column chromatography on Polyamid CC-6, due to its different selectivity from C_{18}, is preferred since it removes compounds which might otherwise interfere with HPLC separations.

A 50 cm × 2.4 cm glass column was packed with Polyamid CC-6 equilibrated to 2% formic acid. The extract was applied to the top of the column. Any sugars, salts, or organic acids present were eluted off the column with 250 ml of 2% formic acid. The column was then eluted with 1500 ml methanol–formic acid–water linear gradient progressing from 25:2:73 to 50:2:48 followed by an additional 500 ml of methanol–formic acid–water (50:2:48). Fractions of 10 ml were collected. The elution order of the esters from Polyamid CC-6 is the opposite of that for C_{18} reversed-phase packing. The fractions were analyzed by HPLC for the occurrence of the various esters. The appropriate fractions were pooled and evaporated to 2–5 ml on a rotary vacuum evaporator.

Material obtained in this manner was then further purified by HPLC. An aliquot of sample to be purified was injected on to the Zorbax ODS column and eluted with 6–10% acetonitrile in distilled water adjusted to pH 2.6 with phosphoric acid. In all cases the distilled water was adjusted to the specific pH prior to the addition of the acetonitrile. The appropriate fractions were collected and pooled. Further purification was performed as necessary.

Characterization

Once the purified ester had been obtained, the UV absorption spectrum of the compound was determined and the components of the ester were identified through

hydrolysis and GLC and HPLC analyses of the hydrolysate. In order to obtain a UV spectrum of the purified material, it was first necessary to remove any traces of mineral acid introduced in the eluting solvent during purification of the ester. This was most easily done by injecting an aliquot of pure ester into the chromatograph and eluting with distilled water to which no acid had been added. Any acid present in the sample elutes in the solvent front followed by the ester which elutes as a broad peak, probably due to ionization of the tartaric acid. This material can then be evaporated to dryness and dissolved in 100% ethanol for spectral measurements. Failure to remove mineral acid prior to suspension in 100% ethanol results in major spectral changes.

The ester was then hydrolyzed for either 4 h in 2 *N* NaOH at room temperature or 6 h in 2 *N* HCl in a boiling water bath. A small aliquot of hydrolysate was adjusted to pH 2–4 and analyzed by HPLC using the Zorbax ODS column with 2.5 ml/min of 10–20% acetonitrile in distilled water, pH 2.6. This confirmed the hydrolysis of the ester and the identity of the hydroxycinnamic acid moiety. The retention time of the hydrolysate peak was compared to that of known standards. Alkaline hydrolysates were then treated by the addition of Dowex 50 (hydrogen form), filtered, and evaporated to dryness. Acid hydrolysates were directly evaporated to dryness.

Hydrolysates were prepared for paper chromatography by the addition of a small amount of methanol–water (1:1). Whatman No. 1 paper was used with butanol–acetic acid–water (5:1:4) ascending for tartaric acid analysis along with standards of quinic, citric, malic, tartaric, and caffeic acids. The chromatogram was first visualized under UV light to detect hydroxycinnamic acids and then sprayed with a 1% solution of sodium metavanadate in water. Ethyl acetate–pyridine–water (12:5:4) was used, ascending, for glucose analysis with standards of glucose, galactose, fructose, xylose, arabinose, and rhamnose. The chromatogram was sprayed with *p*-anisidine–phthalic acid[28] and heated for 2–5 min at 100°.

For GLC, the same hydrolysis procedure was used. The concentration of the ester solution to be hydrolyzed was first determined as will be outlined later. An internal standard, glutamic acid for tartaric acid esters and xylose for glucose esters, was then added to the hydrolysate in an amount equimolar to the hydroxycinnamic acid present. The ester plus internal standard was then hydrolyzed and dried as just discussed. The dry hydrolysate was then silylated with either 0.3 ml BSA or 0.3 ml HMDS–TMCS (2:1) in 0.2 ml of dry pyridine. Samples were heated for 10 min at 80° to complete the silylation reaction. The same procedure was followed for an equimolar standard of either tartaric acid or glucose and the appropriate internal standard.

An amount of 1–2 μl of sample was injected into the gas chromatograph using essentially the same conditions as used by Johnson and Nagel[29]. The retention times of peaks from the hydrolyzed ester sample were compared with those of the standards. The ratio of the peak height or area of the hydrolysate peaks to that of the internal standard was established. A similar ratio was established for the standard solution containing equimolar amounts of glucose or tartaric acid and internal standard. Since the internal standard had been added in an equimolar amount to the ester prior to hydrolysis, a ratio for the two peaks equal to that of the equimolar standard would indicate a 1:1 ratio of hydroxycinnamic acid to glucose or tartaric acid.

The ratio of tartaric acid to monocaffeoyltartaric acid was also determined by hydrolyzing a known amount of ester and then performing a metavanadate test for tartaric acid[30]. The amount of tartaric acid found was then compared to the amount

of tartaric acid that would have been present if the hydroxycinnamic acid to tartaric acid ratio was 1:1. Similarly, the phenol–sulfuric acid assay[31] was used to quantify the amount of glucose in the ester containing caffeic acid, tartaric acid and glucose. Standards of caffeic acid and tartaric acid were also run to determine if either would interfere with the test.

Standard curves

The concentration of the various hydroxycinnamic acid–tartaric acid esters was determined from purified solutions of the esters in 100% ethanol. The optical density of each solution was measured at $\lambda_{max.}$ and compared to the molar absorptivity of the corresponding free hydroxycinnamic acid. For the quinic acid esters of the hydroxycinnamic acids, the molar absorptivity is essentially the same as that of the corresponding free acids[1,32,33] and the same is assumed to be true for esters of glucose and tartaric acid. Varying amounts of each standardized ester solution were then injected into the liquid chromatograph and eluted with 6–10% acetonitrile in water, pH 2.6. The area under the peak was measured by the integrator using a detection wavelength of 320 nm. Three injections were made for each quantity of ester injected and the results averaged. The area was plotted against the amount injected.

Grape sample analysis

Grapes (*V. vinifera*) were obtained from the Irrigated Agriculture Research and Experiment Station, Prosser, Wash., U.S.A. White Riesling grapes were sampled twice during the growing season while other varieties were harvested at a random date. The grapes were transported to Pullman, Wash. the same day and placed in a blast freezer at −40°. They were stored in glass jars until analysis.

For analysis, 1000 ppm of sulfur dioxide as sodium bisulfite was added to 100 g of de-stemmed grapes. The sample was quickly defrosted by microwave oven, immediately crushed, and forcefully squeezed through cheesecloth. The juice yield was recorded. The juice was centrifuged, filtered through a 0.45-μm Millipore cellulose acetate filter, and stored refrigerated under nitrogen prior to analysis.

An aliquot of sample, usually 50–100 μl, was injected into the chromatograph. For White Riesling grapes, the following solvent elution pattern was used: (1) 2.5 ml/min of 6% acetonitrile in distilled water, pH 2.6 for 15 min followed by (2) a linear gradient from 6% to 16% acetonitrile in water, pH 2.6, in 15 min at 2.5 ml/min, (3) column flush with 1.5 ml acetonitrile, and (4) equilibration for 5 min with the solvent used in (1). For other varieties, an isocratic elution using 6% acetonitrile in distilled water, pH 2.6, gave comparable results. The first system is useful when analyzing for caffeic acid and chlorogenic acid in addition to hydroxycinnamic acid–tartaric acid esters. Using isocratic elution excessive peak spreading occurs for any peak eluting after monoferuloyltartaric acid.

The area under each peak was determined at 320 nm by the integrator. The average of duplicate or triplicate analyses of each sample was determined.

In the case of White Riesling grapes, the elution times of the various peaks were compared to those for chlorogenic and neochlorogenic acid. Also, samples of these standards were simultaneously injected along with grape samples.

RESULTS

Fig. 1 shows a typical chromatogram of White Riesling grape juice using the 6–16% acetonitrile program detailed above. Peaks 1–5 correspond to esters that were purified as described previously. Peak 6 was identified by the use of a commercially available standard. The UV absorption maxima of peaks 1–5 in 100% ethanol are shown in Table I along with those of reference compounds. The esters which will be shown to be hydroxycinnamic acid–tartaric acid esters have, as a group, absorption maxima slightly higher in wavelength than their corresponding quinic acid esters which, in turn, are slightly higher than the corresponding free hydroxycinnamic acids.

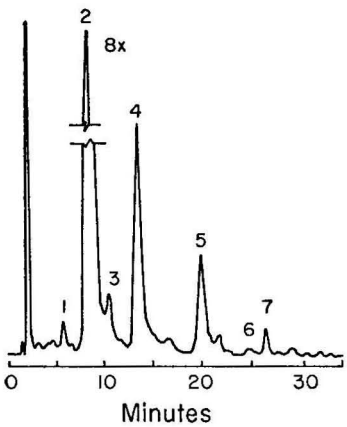


Fig. 1. Elution pattern of a typical White Riesling juice sample. Peaks: 1, caffeic acid–tartaric acid–glucose ester; 2, monocaffeoyltartaric acid; 3, *p*-coumaric acid–tartaric acid–glucose ester; 4, mono-*p*-coumaroyltartaric acid; 5, monoferuloyltartaric acid; 6, caffeic acid; 7, unknown.

TABLE I

ABSORPTION MAXIMA OF CINNAMIC ACID DERIVATIVES

Peak No.	*Compound*	*Absorption maximum (nm)*
1	Caffeic acid–tartaric acid–glucose	330
2	Monocaffeoyltartaric acid	333
3	*p*-Coumaric acid–tartaric acid–glucose	318
4	Mono-*p*-coumaroyltartaric acid	312
5	Monoferuloyltartaric acid	327
	Chlorogenic acid	328*
	p-Coumaroylquinic acid	315*
	Feruloylquinic acid	325*
	Caffeic acid	324
	p-Coumaric acid	310
	Ferulic acid	322

* From ref. 22.

Peak 2 yields caffeic acid upon alkaline hydrolysis as determined by both paper chromatography and HPLC of the hydrolysate. Paper chromatography of the hydrolysate followed by spraying with 1% metavanadate revealed a diffuse violet-orange spot. A tartaric acid standard yielded an identical color reaction while standards of malic, quinic, and citric acids yielded clear spots on the yellow background. Quantitative analysis of the hydrolysate by the metavanadate technique yielded an average of 0.87 tartaric acid residues per caffeic acid. Quantitative GLC yielded 0.91 (peak height analysis) and 0.69 (peak area calculated by integrator) tartaric acid residues per caffeic acid.

Peak 4 yielded *p*-coumaric acid upon alkaline hydrolysis as determined by HPLC. Silylation of the hydrolysate followed by GLC revealed 0.92 tartaric acid residues per *p*-coumaric acid based on peak height analysis.

Peak 5 yielded ferulic acid upon alkaline hydrolysis as determined by HPLC. Ratios of 0.73 and 0.88 tartaric acid residues per ferulic acid were found based on peak areas and peak height analysis, respectively. Based on the above results peaks 2, 4, and 5 are respectively assigned the identities of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyltartaric acids.

Peak 1 yielded, under both acid and alkaline hydrolysis conditions, caffeic acid as shown by HPLC. Paper chromatography of an alkaline hydrolysate indicated glucose, but was not definitive. Analysis of the same hydrolysate by GLC showed significant amounts of glucose. Using the phenol–sulfuric acid assay with glucose as a standard, the hydrolysate yielded 1.15 glucose residues per caffeic acid. GLC analysis of acid hydrolysates yielded 1.26 glucose and 1.77 tartaric acid per caffeic acid based on peak area.

Peak 3 yielded *p*-coumaric acid as determined by HPLC. Acid hydrolysates rendered an average of 0.78 glucose and 1.26 tartaric acid residues per *p*-coumaric acid based on the peak area of GLC analysis.

Based on the results it is postulated that peak 1 is composed of caffeic acid, glucose and tartaric acid and peak 3 is composed of *p*-coumaric acid, glucose and tartaric acid in equimolar amounts.

In each case the tri-ester elutes just prior to its corresponding tartaric acid ester. This would imply that a ferulic acid–tartaric acid–glucose ester should elute just prior to the monoferuloyltartaric acid. A very small, poorly resolved peak has been observed in the chromatogram just prior to monoferuloyltartaric acid. Its response to changes in either the pH of the eluting solvent or the UV detection wave length parallel those of monoferuloyltartaric acid. While it is anticipated that this peak would correspond to an ester of ferulic acid, tartaric acid, and glucose, it is a minor peak requiring great concentration before meaningful amounts can be isolated.

The effect of pH of the eluting solvent on relative retention was examined. A pH shift in the eluting solvent from 2.3 to 3.4 drastically alters the retention time of the hydroxycinnamic acid–tartaric acid esters. At a flow-rate of 3.0 ml/min using 4% aqueous acetonitrile, monocaffeoyltartaric acid elutes in 735 sec at pH 2.3, 510 sec at pH 2.6, 380 sec at pH 2.7 and 210 sec at pH 3.4. The first pK_a of tartaric acid is 2.98 while the pK_a values of the hydroxycinnamic acids are in the range of 4.5. The shift in retention time attributable to the change in pH is most likely linked to the percent ionization of the tartaric acid. The higher pH yields a more polar compound which is retained less by the reversed-phase packing material. The opposite is true

at lower pH values. While this pH effect necessitates careful preparation of solvents it can also be used to optimize HPLC separations where two compounds can not be adequately resolved and only one is pH sensitive. By simply shifting the pH slightly the peaks may be resolved. This method was used to optimize the HPLC conditions used for grape analysis and has also been a useful tool in preparative HPLC.

On the evidence available it is possible to speculate on the type of linkages found in the tri-ester. The absorption maxima of the compounds are very close to that of the glucose, tartaric acid, and quinic acid esters of the hydroxycinnamic acids and not similar to the maxima quoted by either Harborne and Corner[21] or Steck[22] for the corresponding glycosides. Alkaline hydrolysis of peak 1 yields caffeic acid and glucose. The above results suggest the hydroxycinnamic acid is bound by an ester linkage. Since the compounds are pH sensitive and respond in an identical manner to the corresponding tartaric acid ester, at least one of the carboxylic acid groups on the tartaric acid must be free. It might be hypothesized that both carboxylic acid groups are free since the pH response is similar to that of the tartaric acid esters which have two ionizable carboxylic acid groups[10,23].

Fig. 2 shows a chromatogram of some standard compounds using the same eluting conditions as in Fig. 1. Both chlorogenic and neochlorogenic acids have been reported in grapes[1,4,6,8]. While it appears that neochlorogenic and chlorogenic acids could be peaks 3 and 7, respectively, simultaneous injection of neochlorogenic and chlorogenic acids along with juice samples into the liquid chromatograph showed neochlorogenic acid eluting between peaks 2 and 3 and chlorogenic acid eluting between peaks 6 and 7. There is no evidence for the occurrence of either chlorogenic or neochlorogenic acid in any *V.vinifera* grape analyzed. It is unlikely, based on the above, that 4-caffeoylquinic acid occurs in *V. vinifera.*

Peak 6, based on simultaneous injections with a standard, was caffeic acid. The relatively insignificant amount of free caffeic acid present confirms firstly that the

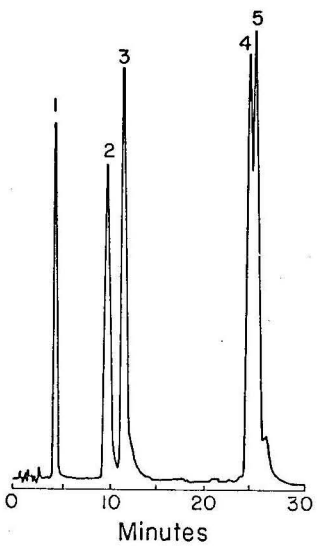


Fig. 2. Elution pattern of various standard phenolic acids. Peaks: 1, gallic acid; 2, neochlorogenic acid; 3, gentisic acid; 4, caffeic acid; 5, chlorogenic acid.

major amount of caffeic acid is found in bound forms, and secondly that very little, if any, hydrolysis of caffeic acid-containing compounds occurred during sample preparation and analysis. It should be noted that caffeic acid elutes prior to chlorogenic acid in our elution program. This is the opposite of other investigations[25,34] using C_{18} reversed-phase packings.

Simultaneous injection also showed that the relatively broad peak eluting after monoferuloyltartaric acid was a mixture of *d,l*-catechin. This peak is not observed in Fig. 1 because the low molar absorptivity of catechin yields a relatively small peak, and at 320 nm the absorptivity of catechin is only 7% of that at maximum. Thus, the catechin simply does not show. The elution order of catechin relative to monoferuloyltartaric acid can be changed by adjusting the solvent pH as discussed.

One of the major aspects of this analytical technique is the minimum amount of sample preparation necessary. Because this is a direct sampling technique, there is no need for internal standards or standard curves related to sample preparation. Standard curves related to chromatographic parameters were all linear and passed through the origin. The percent error on repeated injections of the same sample typically runs 1% for caffeoyltartaric acid, 2% for *p*-coumaroyltartaric acid, and as high as 5% for feruloyltartaric acid. The decrease in reproducibility of the monoferuloyltartaric acid is attributable to the small amount of this compound present in the grape juice, slight increases in peak spreading, and some variation in the resolution of minor peaks in this area of the program. In the latter case, the integrator used to analyze the peak areas readily detects these small changes in resolution.

Table II shows the range of variation for the three tartaric acid esters among a few *V. vinifera* varieties. Two sets of values are also given for mature and immature White Riesling grapes. There is considerable variation in the quantity of hydroxycinnamic acid–tartaric acid ester both on a varietal basis and during the course of maturation.

TABLE II

CONCENTRATION OF THE TARTRATE ESTERS IN THE JUICE OF DIFFERENT GRAPE VARIETIES

Variety	*°Brix*	*Caffeoyl-tartaric (ppm)*	*p-Coumaroyl-tartaric (ppm)*	*Feruloyl-tartaric (ppm)*
Semillon	23.3	74.8	8.3	2.9
Chardonnay	22.5	99.4	19.8	3.1
White Riesling (immature)	7.2	392.8	63.4	30.8
White Riesling	21.8	198.2	20.9	15.9
Cabernet Sauvignon	21.7	70.9	16.7	2.4
Pinot Noir	22.8	211.4	27.5	6.3

Additional detail and more varieties of *V. vinifera* along with detailed data regarding the change in hydroxycinnamic acid–tartaric acid ester content of White Riesling grapes during maturution will be published elsewhere[35].

Sufficient monocaffeoyltartaric acid was available for a taste evaluation. At 80 ppm in distilled water a bitter, astringent, drying taste was present. Since all grapes

sampled showed monocaffeoyltartaric acid concentrations at or considerably above this amount, the compound must contribute to the astringency of grapes and wine.

CONCLUSION

HPLC techniques have been developed for analysis of the hydroxycinnamic acid–tartaric acid esters that is quantitative and requires a minimum of sample preparation. The presence of monocaffeoyl-, mono-*p*-coumaroyl-, and monoferuloyltartaric acid in the juice of *V. vinifera* has been substantiated. There is evidence for esters of caffeic acid, tartaric acid and glucose, and *p*-coumaric acid, tartaric acid and glucose. Neither chlorogenic nor neochlorogenic acid occurs in *V. vinifera*.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TETRAPYRROLE PIGMENTS

PHEOPHYTINS ESTERIFIED WITH DIFFERENT DITERPENE ALCOHOLS, ISOMERIC BILIVERDINS AND SYNTHETIC BILINS

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SUMMARY

Complex mixtures of pheophytins esterified with different C-20 diterpene alcohols have been separated by high-performance liquid chromatography in a reversed-phase system. The isomers of some monomeric and dimeric biliverdins have also been studied. Analytical and semi-preparative work is reported.

INTRODUCTION

Because of its speed, ease of quantitation and the wide variety of adsorbents, high-performance liquid chromatography (HPLC) has become increasingly important in the separation of non-volatile compounds. We wish to report the application of HPLC to the separation of tetrapyrrole pigments encountered in studies on the biosynthesis[1] and reactivity[2] of photoactive plant tetrapyrrole pigments. The HPLC of complex mixtures of plant pigments involving an elaborate program on a single column has been reported recently[3]; this paper focuses on the analysis of mixtures of compounds very similar to each other, *e.g.*, of pheophytins esterified with different diterpene alcohols, and of isomers of mono- and dimeric bile-pigments. From a similar point of view, Evans *et al.*[4] have investigated the separation of isomeric porphyrins.

EQUIPMENT AND METHODS

Liquid chromatography

HPLC was carried out with a system consisting of two Waters Model 6000 A pumps, an injector (Model U6K) and an LDC Model 1202 variable-wavelength double-beam detector fitted with a 8-μl flow-through cell (10 mm path-length). For maximum sensitivity, the detector was set to the maxima of the pigment's main absorption bands; for positive identification, the monitor was also set to other characteristic wavelengths, *e.g.*, to 667 nm for pheophytins of the *a* series. Runs were

made at room temperature with the solvent compositions given in the text or in the legends to the figures.

Gas chromatography of pheophytin-esterifying alcohols

Gas chromatography (GC) of the pheophytin-esterifying alcohols was carried out with a GI gas chromatograph (Carlo Erba, Milan, Italy), equipped with a WG 11 glass capillary (25 m × 0.3 mm I.D.) (Werner Günther, Düsseldorf, G.F.R.). The carrier gas was hydrogen (4 ml/min), the injection was splitless at 160° oven temperature and the temperature was raised in 2 min to 180°. Under these conditions, retention times for the alcohols were 10.5 min (P)*, 14.5 min (THGG), 17.5 min (DHGG) and 22.5 min (GG). The sensitivity was 0.06 nmole of alcohol in 2 μl of hexane injected.

Both the gas and the liquid chromatograph were connected to an Autolab system IV integrator (Spectra-Physics, Darmstadt, G.F.R.).

Pigments and reagents

Pheophytins. 7 days old aetiolated oat seedlings (*Avena sativa* L.; Bayerische Futter und Saatbau) were harvested, and the leaves were irradiated with white fluorescent light (2000 lux) for 1 min. After a dark period (*ca.* 20 min at 23–24°), the mixture of esterified pheophytins was isolated by standard procedures as described earlier[1].

Alcohols. The esterifying alcohols were obtained by the usual method of saponification of the pheophytins[1].

Bile pigments. Biliverdins (IXα–IXδ) were obtained by oxidative degradation of hemin (Sigma)[5], the octaethylbilins by cleavage of octaethylchlorin[6]. The dimers 1–4 were obtained photochemically[7]. All solvents were distilled before use.

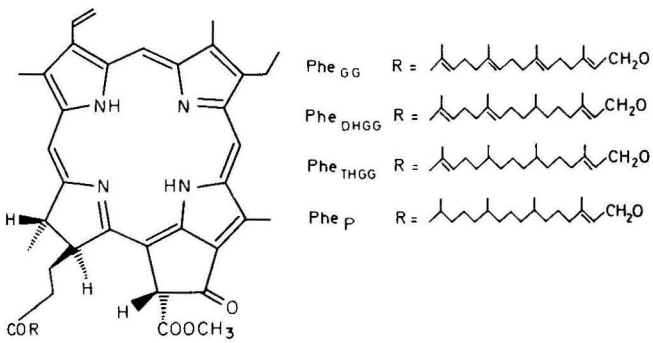
RESULTS AND DISCUSSION

Pheophytins

During our research on the last steps of chlorophyll biosynthesis, we have recently detected a series of intermediates between Chlid and Chl *a* esterified with different diterpene alcohols[1]. By analysis of the kinetics by which these intermediates are formed, it was possible to formulate a sequence in which Chlid is first esterified with GG and then hydrogenated via Chl_{DHGG} and Chl_{THGG} to Chl_P. The quantitative analysis of these pigments was based on an indirect method (demetallation, thin-layer chromatography (TLC) on silica, and saponification of the resulting mixture of pheophytins; the alcohol mixture thus obtained was then analysed by GC[1]). To avoid this indirect and time-consuming method, we attempted to separate the esterified pigments at the pheophytin stage. Partial resolution was possible by TLC on silica previously buffered and impregnated with silver nitrate. By this means, Phe_P and Phe_{THGG} could be separated from Ph_{GG} and Phe_{DHGG}, but separation was not

* Abbreviations: Chlid = chlorophyllid *a*; Chl *a* = Chlorophyll *a*; GG = geranylgeraniol; DHGG = dihydrogeranylgeraniol; THGG = tetrahydrogeranylgeraniol; P = Phytol; Chl_P, Chl_{THGG}, Chl_{DHGG} and Chl_{GG} = chlorophyllid *a* esterified with the respective alcohols; Phe *a* = pheophytin *a*; Phe_P, Phe_{THGG}, Phe_{DHGG} and Phe_{GG} = Pheophorbide *a* esterified with the respective alcohols.

complete, and elution of the pigments from the TLC plates gave very low yields, owing to partial formation of silver complexes[8].



Recent reports[3,4] on the separation of plant pigments prompted us to apply HPLC. As pheophytins esterified with GG, DHGG, THGG and P differ only by the number of double bonds in the hydrophobic (alcohol) part of the molecule[8,9], reversed-phase chromatography should be most suitable for this approach.

A typical chromatogram for analytical separation of the esterified mixture of pheophytins on a microparticulate C_{18} bonded reversed-phase column is shown in Fig. 1. The chromatogram has five peaks, in contrast to the GLC analysis of the saponified mixture of pheophytins, which yields only the four isoprenoid alcohols, P, THGG, DHGG and GG (first column of Table I). To obtain sufficient material for identification of the peaks, the HPLC column was overloaded (80–100 μg of Phe

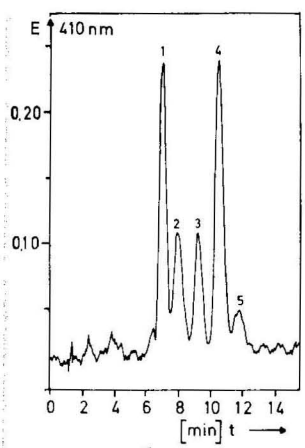


Fig. 1. HPLC of a mixture of Phe_{GG}, Phe_{DHGG}, Phe_{THGG} and Phe_P on a stainless-steel column (4 × 300 mm) packed with μBondapak C_{18} (Waters), with methanol–acetone (90:10) v/v) as eluent; flow-rate, 1.5 ml/min at 22°. For maximum sensitivity, the detector was set at 410 nm (Soret band; sensitivity 0.1 nmole of pigment/2 ml). A similar pattern was obtained at the characteristic wavelength of 667 nm, but the sensitivity was poorer by a factor of *ca.* 3 (decreased ε, increased signal-to-noise ratio).

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF THE ESTERIFYING ALCOHOLS

All values are expressed in nmoles. The compositions shown are those of the pheophytin mixture (column 1) and the five fractions obtained from micropreparative HPLC (for conditions, see legend to Fig. 1).

Alcohol	*Phe mixture (614)**	*Fraction 1 (161)**	*Fraction 2 (240)**	*Fraction 3 (67.9)**	*Fraction 4 (281)**	*Fraction 5 (67)**
GG	247	159	89	2.8	–	–
DHGG	95	–	150	3.9	–	–
THGG	125	–	–	37.4	52	9
P	151	–	–	–	270	60

* Determined spectrophotometrically.

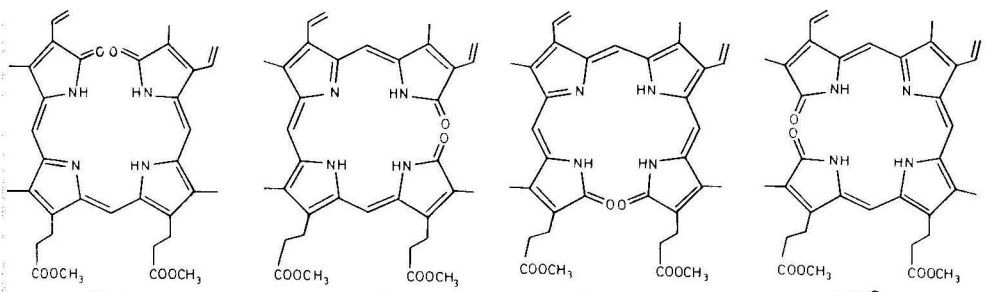
per injection), as indicated by a marked decrease in resolution, and each peak was collected. The five crude fractions (1–5) thus obtained were then re-examined by HPLC. According to this analysis, the first fraction obtained was pure, and fractions 2–5 were highly enriched. Both fractions 4 and 5 were contaminated with material from peak 3, although they were relatively well separated from the other pheophytins. Fraction 2 still contained material from peak 1, and fraction 3 of material from peaks 1 and 2. All five fractions have been identified as pheophorbide esters of the *a*-series by UV–VIS spectroscopy. As the extinction coefficients of pheophorbides esterified with different alcohols can be considered as identical, the total amount of pigment in each fraction (see column headings of Table I) was determined spectrophotometrically by using the known extinction coefficient[10] for Phe *a*.

To identify the respective esterifying alcohols, each fraction was saponified, and the alcohols were analysed quantitatively by GLC (Table I). Of the four alcohols, fraction 1 contains only GG. Thus, peak 1 of the HPLC corresponds to Phe_{GG}. In both fractions 4 and 5, phytol was detected as the major alcohol besides THGG. To analyse these fractions further, pheophytin *a* was isolated from green leaves and submitted to HPLC under similar conditions. The chromatogram showed a large peak 4 and a small peak 5; after saponification, phytol was the only alcohol detectable in the material from either peak. Thus, both peaks 4 and 5 are pheophytins esterified with phytol. Based on the relative amounts, peak 4 corresponds to Phe_P and peak 5 then is presumably the C_{10} epimer Phe_P *a'* (refs. 11 and 12). If there exists an *a'* series for pheophytins esterified with other alcohols, one would always expect one large and a second smaller peak for each Phe_{GG}, Phe_{DHGG}, Phe_{THGG} and Phe_P; the results in Table I lend support to this assumption.

With the identification of the pheophytin first eluted as Phe_{GG}, and the last two peaks as Phe_P, the HPLC mobility apparently decreases with decreasing number of double-bonds. A similar dependence on the number of double-bonds has been found for *n*-C_{18} acids[13,14]. Thus, one would expect peak 2 to be Phe_{DHGG}, and peak 3 to be Phe_{THGG}. The GLC of the alcohols after saponification of the two peaks confirmed this assumption, although separation of the pheophytins was not optimum. According to these results, it is possible to separate the pheophorbide esters by HPLC according to their esterifying alcohols. Currently, this method is optimized for quantitative analysis, and to further investigate the problem of the *a'*-series pigments.

Biliverdin dimethyl esters

Biliverdins are the first stable products of enzymic and non-enzymic cleavage of the tetrapyrrole macrocycle in iron porphyrins; they are also obtainable by chemical oxidation of bilirubins[15]. In both cases, the "genuine" biliverdin IXα is accompanied by other isomers. The separation of these mixtures, which is also of potential medical importance, has been studied by several authors[5,15,16]. Analytical separation of the four type-IX biliverdin dimethyl esters (α–δ) have been reported by both one-[15] and two-dimensional[5] TLC on silica, but preparative separation was incomplete, especially for the IXβ and IXδ isomers[5]. By HPLC, the isomeric IX esters (α–δ) obtained from the non-enzymic cleavage of haemin[5] can be separated analytically both on silica and on a C_{18} reversed-phase column, and preparative separation is possible on silica in milligram amounts per run. For analytical separation, gradient elution is unnecessary. The identification of the four isomers was carried out by co-chromatography with authentic samples by O'Carra's method[15]. On silica, the decreasing mobility (IXγ > IXβ > IXδ > IXα) corresponds to the sequence observed on TLC with mixtures of *n*-heptane–methyl ethyl ketone–acetic acid[5] or of carbon tetrachloride–acetone as mobile phase. The latter mixture, which is commonly used for the separation of cyclic tetrapyrroles, gave complete separation of the four isomers on HPTLC plates; however, this mixture should never be used in HPLC, as it is corrosive to stainless steel. Replacement of either of the solvent components decreased the separation of IXβ and IXδ considerably (due to peak broadening), but the resolution could be restored by addition of small amounts of pyridine, an effect common with many of the bile pigments studied; this effect is most pronounced with bilins having high p*K* values [*e.g.*, dihydrobilin (DHB)] and is therefore probably due to deprotonation of bilin cations present in equilibrium on the acidic silica surface. The best solvent system with regard to separation and freedom from corrosion is toluene–acetone–pyridine (90:10:1) (Table II).



On the C_{18} reversed-phase, the four isomers were separated by water–methanol (22:78) (Fig. 2). The order of elution was inverted for the isomers IXα and IXγ, but the "inner pair" of isomers (IXβ and IXδ) were eluted in the same order as from silica. For analytical purposes, this method is preferable to the use of silica because it gives better resolution and because the column is less contaminated by (polar) by-products. With the detector set at 335 nm, the detection limit with the equipment used is 2.5 pmoles with a signal-to-noise ratio of 10. As most bilins show a broad

TABLE II

SEPARATION OF ISOMERIC BILIVERDIN DIMETHYL ESTERS

These results were obtained on a micropreparative silica column [7 × 600 mm; 37–75 μm Bondapak packing (Waters)], with a linear gradient (40 min) from 100% to 50% toluene–pyridine (99:1, v/v), the remaining eluent being toluene–acetone–pyridine (70:30:1, v/v) (6 ml/min). For analysis on reversed-phase microparticulate column, see Fig. 2. The values are expressed as a percentage of total peak area.

Fraction of preparative separation	*Isomer*			
	*IXα (11.1 min)**	*IXβ (13.3 min)**	*IXδ (14.5 min)**	*IXγ (18.8 min)**
1	—	—	—	>97
2	4	96	—	—
3	—	7	93	—
4	>97	—	—	—

* Retention time on analytical column.

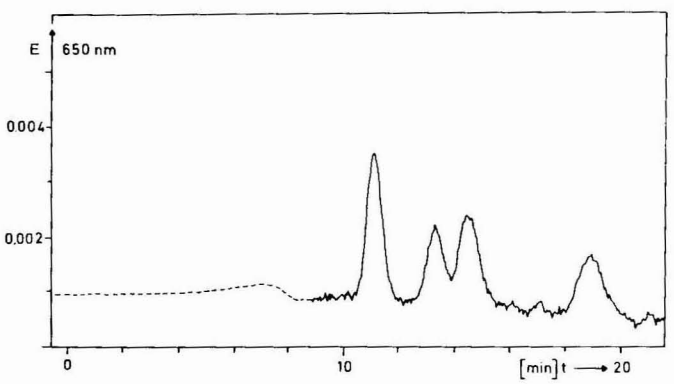


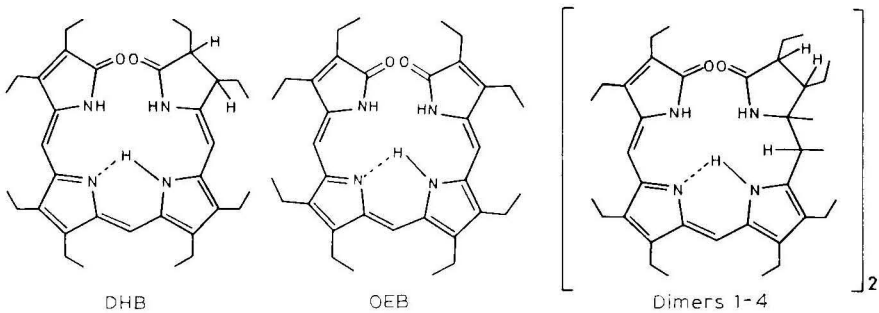
Fig. 2. HPLC of a mixture of biliverdins IXα, IXβ, IXγ and IXδ. Column as in Fig. 1, but with water–methanol (22:78) as eluent (1.5 ml/min). Elution order: IXα, IXβ, IXδ, IXγ.

absorption band in the range 300 to 350 nm, detection at 645 nm is more suitable for the selective detection of the verdins, although there is concomitant loss of sensitivity by a factor of 5–10 (decreased ε, increased signal-to-noise ratio). As the absorption spectra of the isomers IXα, IXβ, IXγ and IXδ are very similar, the relative peak areas were taken as being proportional to the isomer distribution.

The semi-preparative separation of the biliverdins in amounts of 1–5 mg was possible on preparative silica by gradient elution with a 50-min cycle time (Table II). Preparative separation on the C_{18} reversed-phase column was less satisfactory, owing to partial irreversible absorption of the bilins. It appears that reversed-phase separations are generally more difficult to scale-up. One problem is irreversible adsorption on free OH-groups, although this can be reduced efficiently by pre-treating the column material with silylating agents (10% of BSTFA in absolute tetrahydrofuran; 2 h; 70°). However, even after this treatment, the selectivity of the preparative (37–75 μm) material differs from that of the microparticulate phase (10 μm). Similar difficulties have also been encountered with the pheophytins.

Synthetic bilins

2,3-Dihydro-octaethylbilindione (DHB), which has been prepared to study the reactivity of the chromophore characteristic for plant bile pigments, undergoes light-induced dimerization in the presence of one-electron oxidants[7]. The reaction mixtures containing mainly DHB, the fully unsaturated octaethylbilindione (OEB) and the dimers 1–4 (see formula) can be analysed on both silica and on reversed-phase microparticulate packings.



On silica, the best solvent system is again toluene–acetone containing small amounts of pyridine to prevent spreading. Independently of the pyridine concentration, the relative elution order of the monomers DHB and OEB, as well as that of dimers 1–4, is similar to that on silica HPTLC plates [carbon tetrachloride–acetone (9:1)]. However, although the monomers precede the dimers on plates, they are retarded by the pyridine necessary during HPLC and overlap with the dimers (Table III). Nevertheless, complete analysis is possible by making two consecutive developments with different detector wavelengths, *e.g.*, 610 nm (selective for DHB) and 570 nm (for the dimers). On reversed-phase columns, the most pronounced effect is a distinct separation of the fast-migrating monomers from the slow-migrating dimers (Table 3), which persists after changing the eluent composition and indicates a pronounced sieving effect. As the discrimination between dimers 1–4 is less pronounced (Table III), however, silica is the superior stationary phase.

TABLE III

RETENTION TIMES OF SYNTHETIC MONO- AND DIMERIC BILINS

Values are for HPLC on 10-μm microparticulate columns (4 × 300 mm). Conditions on silica: solvent A, toluene–pyridine (98.5:1.5, v/v); solvent B, toluene–acetone (9:1, v/v); ratio A:B = 9:1, increased to 1:1 after elution of dimer 3. Flow-rate 2.5 ml/min. Conditions on reversed-phase column: methanol as eluent (1.5 ml/min).

Column	*Retention time (min)*					
	DHB	*OEB*	*Dimer 1*	*Dimer 2*	*Dimer 3*	*Dimer 4*
Silica	3.3	4.8	2.9	3.3	6.5	10.0
C_{18} reversed-phase	3.6	3.1	9.9	7.4	9.6	8.2

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REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF SOME TRYPTAMINE DERIVATIVES

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SUMMARY

A quantitative reversed-phase high-pressure liquid chromatographic method is described for tryptamine, bufotenine, N-methyltryptamine and N,N-dimethyltryptamine. All compounds were eluted with base-line separation within 15 min. The method is used to determine the tryptamine level in a leaf extract of *Acacia podalyriaefolia* A. Cunn. Satisfactory recoveries of tryptamine from plant material, and N,N-dimethyltryptamine from urine were obtained.

INTRODUCTION

Tryptophan-derived indole alkylamines occur widely in the plant family Leguminosae, especially in the genera *Acacia*, *Desmodium*, *Lespedeza*, *Mimosa*, *Piptadenia* and *Prosopsis*[1,2]. The N,N-dimethylated compounds, N,N-dimethyltryptamine, 5-methoxy-N,N-dimethyltryptamine and 5-hydroxy-N,N-dimethyltryptamine (bufotenine) are of particular interest, since they are considered to be hallucinogenic[3]. Tryptamine derivatives occur as normal constituents of blood and urine[4], and in the brain[5], and tryptamine is a putrefactive product in decaying human tissue[6]. N,N-dimethyltryptamine has been implicated as an abnormal metabolite in body fluids of schizophrenic patients[7,8].

Gas–liquid chromatography (GLC) has been widely used for the analysis of tryptamines, though not without difficulty. When the compounds are chromatographed without prior derivatization the primary amines tail severely on common stationary phases[9], and inadequate resolution is obtained between tryptamine, N-methyltryptamine and N,N-dimethyltryptamine[10]. The reactions of tryptamine with pentafluoropropionic anhydride[11] and 2-bromopropane-sodium hydride[12] lead to several products, although single, symmetrical peaks are reported by the formation of acetate[13], enamine (Schiff's base)[14] and isothiocyanate[3] derivatives. When the secondary amine N-methyltryptamine is silylated, silyl ethers of both the indole and alkyl –NH groups are formed[3]. More recent work, however, has demonstrated the applicability of GLC to the analysis of low levels of endogenous indole alkylamines using derivatives detected by electron capture[15,16].

A convenient method for the assay of selected tryptamines, avoiding the need for derivatization, is described in this communication. A primary, secondary and two tertiary indole alkylamines are rapidly resolved by isocratic elution using a reversed-phase high-pressure liquid chromatographic (HPLC) system. The method is used to quantitate the tryptamine level in an extract of *Acacia podalyriaefolia* leaves, and is currently being used to screen other species of the Leguminosae for the occurrence of tryptamine bases.

EXPERIMENTAL

HPLC separations were carried out using a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 liquid chromatograph, equipped with a Beckman 25 variable-wavelength UV spectrophotometer and recorder. Separations were conducted on a Waters Assoc. 30×4 mm I.D. μBondapak C_{18} column. Thin-layer chromatography (TLC) was performed on glass-backed pre-coated silica gel GF_{254} plates (20×20 cm, 0.25 mm thickness) (Merck, Darmstadt, G.F.R.). The low resolution mass spectrum was obtained by means of the Hitachi Perkin-Elmer Model RMU-6D spectrophotometer.

Chemicals

Tryptamine hydrochloride, bufotenine monooxalate hydrate and N-methyltryptamine were obtained from Sigma, St. Louis, Mo., U.S.A. Bufotenine was generously donated by Dr. J.-E. Lindgren, Swedish Medical Research Council, Karolinska Institutet, Stockholm, Sweden, and N,N-dimethyltryptamine by Dr. S. Khalil, College of Pharmacy, North Dakota State University, Fargo, N.D., U.S.A. All compounds were checked for purity by TLC. Solvents used for HPLC were of reagent grade and redistilled in glass.

Plant material

An 80% ethanolic extract of *Acacia podalyriaefolia* A. Cunn., collected in Australia, was supplied by the Developmental Therapeutics Program (Natural Products Branch) of the National Cancer Institute, formerly the Cancer Chemotherapy National Service Center, Bethesda, Md., U.S.A.

High-pressure liquid chromatography

Standard solutions of tryptamine hydrochloride, bufotenine monooxalate hydrate, N-methyltryptamine and N,N-dimethyltryptamine were dissolved in methanol–0.1 *M* ammonium carbonate (1:1), since this resulted in better resolution than when the eluting solvent was used to dissolve the compounds. These solutions were mixed and subjected to HPLC under the following operating conditions: eluting solvent, 1,4-dioxane–0.1 *M* ammonium carbonate (4:5); flow-rate, 1 ml/min at ambient temperature; wavelength of UV detector, 280 nm; 0.5 absorbance units full scale (a.u.f.s.) and recorder chart speed, 0.2 in./min.

Beer's law curves were obtained from triplicate 20 μl injections of four concentrations for each compound. The weights (in 20 μl) of the compounds applied to the column were: tryptamine (base equivalent) 0.99, 1.98, 2.97, 3.98 μg, bufotenine (base equivalent) 2.12, 4.24, 6.36, 8.48 μg, N-methyltryptamine, 2.43, 4.87, 7.29, 9.73

μg, N,N-dimethyltryptamine, 8.11, 16.21, 24.33, 32.43 μg. For tryptamine and bufotenine, peak heights were measured, while the peak areas of N-methyltryptamine and N,N-dimethyltryptamine were determined by measuring the product of height by width at half peak height.

The limit of detection for each compound was established by diluting a solution containing a known amount of the indole alkylamines, until the signal-to-noise ratio at the most sensitive UV setting (0.1 a.u.f.s.) was about 3:1.

Extraction of plant material

A dried 80% ethanolic extract of *Acacia podalyriaefolia* was subjected to a modification of a previously published extraction scheme[17]. About 2 g of accurately weighed plant material was thoroughly moistened with 28% NH_4OH solution and dried on a steambath. On cooling, the sample was placed in a 100-ml boiling flask fitted with a condenser, 20 ml of chloroform were added, and the mixture refluxed on a steam bath for 30 min. The refluxed mixture was cooled to room temperature and filtered. The residue was returned to the flask, an additional 15 ml of chloroform added, and the 30-min reflux period repeated. After cooling and filtering, the filtrates were combined, and partitioned with 10 ml of a 1% w/v HCl solution. Alkaloids were shown to be absent from the chloroform extract by TLC, and it was discarded. The acidic aqueous layer was adjusted to pH 8.5 with 28% NH_4OH, and the free base extracted into 3×10 ml chloroform. Chloroform was removed under reduced pressure, and the residue taken up in 6.0 ml methanol–0.1 *M* ammonium carbonate (1:1). Triplicate 20-μl injections were applied to the chromatographic column.

Although tryptamine has been isolated previously from *Acacia podalyriaefolia* leaves[18], the identity of the base obtained in this extraction procedure was checked by mass-spectral analysis after purification by preparative TLC in methanol–28% ammonia (131:2).

Recovery experiments

Tryptamine HCl (1.60 mg) was added to *Acacia podalyriaefolia* residue, from which all previous traces of tryptamine had been extracted, and the residue was re-extracted as described previously. The final residue was diluted to 8.0 ml with methanol–0.1 *M* ammonium carbonate (1:1), and the overall percentage recovery of tryptamine was obtained by analysis of peak heights from triplicate 20-μl injections of this solution.

The recovery of N,N-dimethyltryptamine in urine was investigated by dissolving 2.60 mg of the compound in 1.5 ml urine, and diluting with an equal volume of acetonitrile. Three 20-μl portions of the supernatant were directly injected onto the HPLC column, and the percentage recovery calculated from the mean peak area.

RESULTS AND DISCUSSION

The retention times of tryptamine, bufotenine, N-methyltryptamine and N,N-dimethyltryptamine are shown in Table I; Fig. 1 is a chromatogram showing base-line separation between the compounds within a short overall elution time. Attempts to remove the solvent impurity peak shown in Fig. 1 by double redistillation of solvents, and by using superior grades of solvents, were unsuccessful. However, this peak is

TABLE I

HPLC CHARACTERISTICS OF TRYPTAMINES

Compound	*Retention time (min)*	*Slope***	*Y-Axis intercept****	*Correlation coefficient****	*Lowest concentration detected (μg/ml)*§
Tryptamine*	5.1	5.14	+0.50	0.997	2.0
Bufotenine*	6.9	2.74	+0.56	0.996	3.8
N-Methyltryptamine**	8.7	0.85	−0.37	0.995	4.9
N,N-Dimethyltryptamine**	13.2	0.63	−0.85	0.994	11.9

* Peak height determination.
** Peak area determination.
*** UV detector 0.5 a.u.f.s. (within weight ranges in text).
§ UV detector 0.1 a.u.f.s.

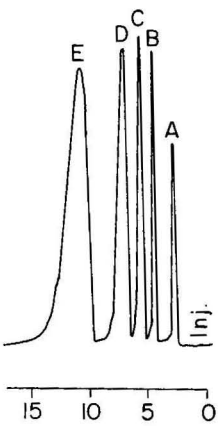
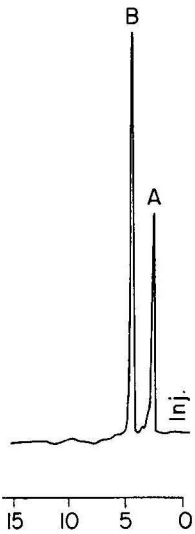


Fig. 1. Liquid chromatogram of standard tryptamines. For operating conditions, see text. A = Solvent impurity; B = tryptamine; C = bufotenine; D = N-methyltryptamine; E = N,N-dimethyltryptamine.

well removed from those of the four tryptamines assayed. Table I lists the slopes, *y*-axis (peak height/area) intercepts, and correlation coefficients of linear regression lines calculated for each compound from the points used to determine Beer's law standard curves. The detector response was linear at 0.5 a.u.f.s. for all the indole alkylamines, within the weight ranges listed in the experimental section of this paper.

Tryptamine was confirmed as a constituent of *Acacia podalyriaefolia* leaves, by comparison of its mass spectrum with published data[19], and was calculated to comprise 0.06% (w/w) of the plant extract by this HPLC assay. Fig. 2 shows a typical chromatogram of tryptamine from this plant source.

Of the three UV absorption bands that occur in the indole chromophore[20], the band at 280 nm, with a relatively low absorption intensity, was used to increase the selectivity of this HPLC assay for tryptamines in the presence of other plant constituents. The sensitivity of this method (detection limits for each compound are shown in Table I) does not approach those of other methods designed to estimate the very low levels of endogenous indole alkylamines[15,16].



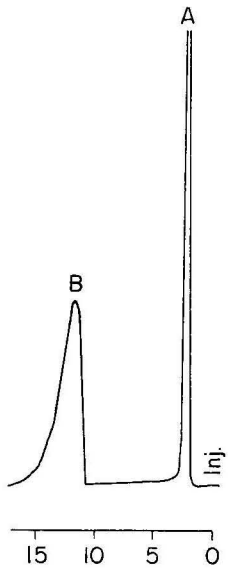


Fig. 2. Liquid chromatogram of *Acacia podalyriaefolia* leaf extract. A = Solvent impurity; B = tryptamine.

Fig. 3. Liquid chromatogram of N,N-dimethyltryptamine in urine. A = Urine impurity; B = N,N-dimethyltryptamine.

The recovery of tryptamine, introduced as a weighed amount of the hydrochloride salt, from the extraction and HPLC procedures was 95.3%. Accordingly, this method is suitable for the further investigation of tryptamines occurring as possible hallucinogenic constituents of plants, and could be also used to determine the normal levels of these bases in foodstuffs. It is unlikely that bufotenine would be quantitatively extracted by the method described in this study, due to the formation of a polar phenolate anion at basic pH[15]. However, if plant material containing bufotenine is purified by methods other than an acid-base shake out, such as by ion-exchange, this assay would be applicable to quantitate bufotenine levels.

N,N-Dimethyltryptamine was almost quantitatively recovered from urine (100.4%) by direct injection of urine diluted with acetonitrile. A typical chromatogram showing the resolution from urine impurities is shown in Fig. 3. Although the concentration of N,N-dimethyltryptamine used in this recovery experiment is far higher than endogenous levels in urine[4], the method might be of use in estimating this amine in urine after preliminary extraction and concentration procedures. It would be of interest to investigate the separation of N,N-dimethyltryptamine from its metabolites[21] using this HPLC system, although this is outside the scope of the present work.

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CHROMATOGRAPHIC ANALYSIS OF PARAFFINIC WAXES

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SUMMARY

Gel permeation and gas chromatographic analyses of crude paraffinic waxes are reported. Molecular weights ($\bar{M}_n$ and $\bar{M}_w$) and molecular-weight distributions of crude paraffinic waxes containing different amounts of oil have been determined using a gel permeation chromatographic calibration curve for *n*-paraffins. A fraction consisting mostly of *n*-C_{18}–C_{30} yields a distribution curve that peaks at 27.6 counts with a $\bar{M}_w/\bar{M}_n$ ratio of 1.02 using four 4 ft. × 3/8 in. Styragel columns having pore volumes of 500, 500, 100 and 100 Å. A fraction consisting of mostly oil (m.p. <23°) yields a distribution curve that peaks at 27.9 counts, with a $\bar{M}_w/\bar{M}_n$ ratio of 1.09. It is shown that gel permeation chromatography gives reliable $\bar{M}_n$ and $\bar{M}_w$ values for crude paraffins having $\bar{M}_w/\bar{M}_n < 1.05$ or containing <30 wt. % oil, using a calibration curve for *n*-paraffins. Gas and gel permeation chromatograms of the fractions are presented and are found to complement each other well.

INTRODUCTION

Paraffinic waxes having a low oil content (<1%, w/w) are useful materials in the coatings and plastic-processing industries and as starting materials for chlorinated paraffins and other products. These waxes are obtained from a petroleum fraction by various techniques and are *n*-alkanes with chain lengths ranging from 16 to 50 carbon atoms.

It is known that crude oils from eastern Venezuela[1] such as Guere and Anaco have a wax content that may be as high as 20% by weight. In this paper we report our attempts to characterize paraffinic waxes obtained locally by gas–liquid and gel-permeation chromatography. Our interest lies mainly in analyzing crude paraffinic waxes in order to select sources capable of yielding *n*-paraffins suitable for given applications. We were especially interested in identifying a source having a high content of *n*-paraffins in the C_{16}–C_{26} range, since this fraction is a useful starting material for chlorinated paraffins, as was shown by Rotenberg[2].

Crude paraffinic waxes are usually characterized by their oil content and their

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melting point range. A typical sample from a refinery may contain 30% (w/w) oil and a melting-point range as wide as 10°. The crude waxes form a two-phase system upon standing and often the melting-point ranges depend on the sampling techniques used. If the crude waxes are fractionated, the molecular weights of the fractions can be estimated from their melting points. Molecular-weight distributions, however, must be obtained either by gas or gel chromatography.

In our laboratory, gas chromatography (GC) is used routinely to analyze *n*-paraffins up to a chain length of 40 carbon atoms; however, we sought a method that would give us more information on all of our crude samples as well as the chlorinated products. The temperatures required to analyze *n*-paraffins having chain lengths above C_{40} are almost at the upper useful limit of routine GC. Gel permeation chromatography (GPC), on the other hand, has been used extensively to characterize small and large molecules[3–5]. The elution characteristics of solvents, as well as asphalts and crude oils, have also been studied[6]. In GPC, the large molecules exit from the columns first and the small molecules exit last. Thus, GPC permits the routine analysis of high-molecular-weight materials. The analysis of temperature-sensitive compounds is also facilitated by GPC.

EXPERIMENTAL

Four wax samples (A, B, C and D) containing 30% (w/w) oil were obtained from an eastern Venezuelan refinery for analysis. The waxes were melted and stirred well before obtaining the samples used in the analyses. Solutions containing 0.5 g of wax per 100 ml of tetrahydrofuran were used for GPC analyses. Benzene was used as the solvent for GC analyses. Sample D was fractionated by the sweating technique using a 500-ml büchner funnel and a procedure resembling the industrial process. A 200-g sample of the wax was placed in the funnel, which was then placed in an oven at 50 ± 1° for 2 days. The material that did not melt at this temperature was labelled fraction 1 and the material that did melt was cooled, placed in the filter and kept in the oven at 40 ± 1° for 2 days. This procedure was repeated and fractions 2, 3 and 4 were collected at 40, 30 and 23°, respectively. The fifth fraction consisted of the oil that had seeped through the filter at 23°.

GC analyses were carried out in a Packard–Becker Model 420 gas chromatograph equipped with a thermal conductivity detector and a 5 ft. × 1/8 in. column packed with 6% (w/w) SE-30 on Ultraport at 240°. Retention times of *n*-paraffins were determined using analytical standards purchased from PolyScience Corp. (Evanston, Ill., U.S.A.; Kit 26 A). A plot of log retention time of the peaks *versus* carbon number gave a straight line. Peak areas were obtained by triangulation and planimetry.

GPC analyses were carried out using a Waters Model 200 instrument equipped with four 4 ft. × 3/8 in. columns having pore volumes of 500, 500, 100 and 100 Å. Using tetrahydrofuran as a solvent, the columns yielded 640 plates per ft. for dodecane at 30°. A typical gel permeation chromatogram for a four-component mixture is shown in Fig. 1. In this and in all the gel permeation chromatograms, the abscissa gives the elution volume in counts (1 count = 4.69 ml) and the ordinate gives the response of the differential refractometer. A calibration curve was obtained by determining the elution volumes of six *n*-paraffins purchased from Humphrey Chemical Co. (Chicago, Ill., U.S.A.). The best plot gave the following relation which was used

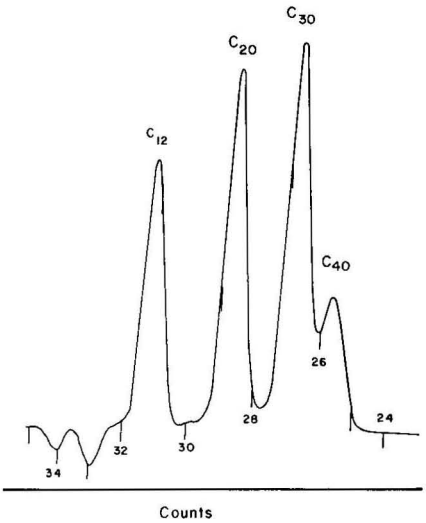


Fig. 1. GPC trace for a mixture of *n*-alkanes, showing the response of the differential refractometer. One count is equal to 4.69 ml.

in all the calculations: $\log \bar{M} = -0.0971(\text{counts}) + 5.215$. Good agreement was obtained between $\bar{M}_n$ and $\bar{M}_w$ values obtained by GPC using the procedure outlined in the Water's GPC Manual and the theoretical values of known mixtures. Utilization of Polyscience calibration mixtures consisting of 10% (w/w) C_{18}, 25% (w/w) C_{19}, 30% (w/w) C_{21} and 35% (w/w) C_{22} ($\bar{M}_n = 288$) yielded an average $\bar{M}_n$ of 298, which is within 3.5% of the theoretical value.

TABLE I

GPC ANALYSIS OF CRUDE PARAFFINIC WAXES (30 WT.% OIL)

$\bar{M}_n$ and $\bar{M}_w$ values were calculated using the calibration curve for *n*-paraffins.

Sample	*Counts at GPC peak*	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	*Av. chain length*
A	24.2	664	685	1.03	C_{47}
B	26.2	490	508	1.04	C_{35}
C	26.8	417	242	1.02	C_{30}
D	27.6	353	359	1.02	C_{25}

TABLE II

GPC ANALYSIS OF THE FRACTIONS OBTAINED FROM SAMPLE D

The oil content was estimated from the gas chromatogram.

Fraction	*M.p. range (°C)*	*Oil content (%, w/w)*	*GPC peak*	*Av. chain length*	*Yield (%, w/w)*
1	51–52	10	27.6	C_{25}	55
2	41–43	31	27.6	C_{25}	22
3	31–33	54	27.6	C_{25}	10
4	23–27	66	27.6	C_{25}	4
5	<23	87	27.9	C_{23}	8

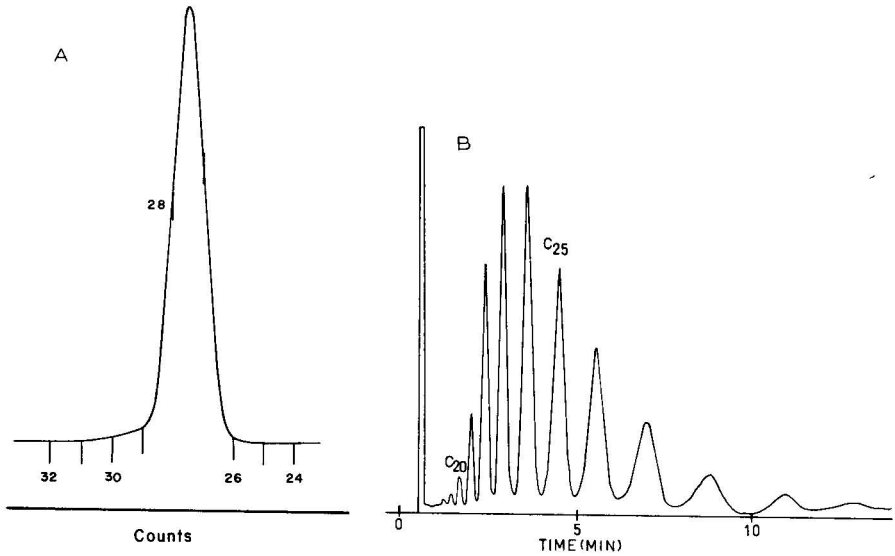


Fig. 2. Gel permeation (A) and gas (B) chromatograms for fraction 1. See Table II.

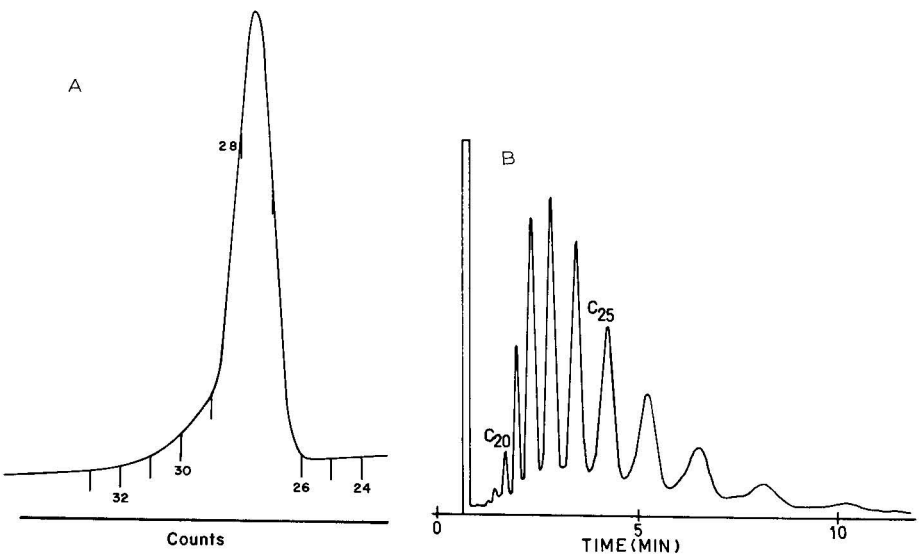


Fig. 3. Gel permeation (A) and gas (B) chromatograms for fraction 2.

RESULTS AND DISCUSSION

All of the samples obtained were analyzed by GPC, but only samples C and D yielded useful GC chromatograms. Table I shows the results of the GPC analyses. The calibration curve obtained with the *n*-paraffins was used to estimate the molecular-weight averages of these samples, although it was known that the waxes contained 30% (w/w) oil according to refinery specifications.

In order to evaluate the effect the oil had on our estimates of molecular weights

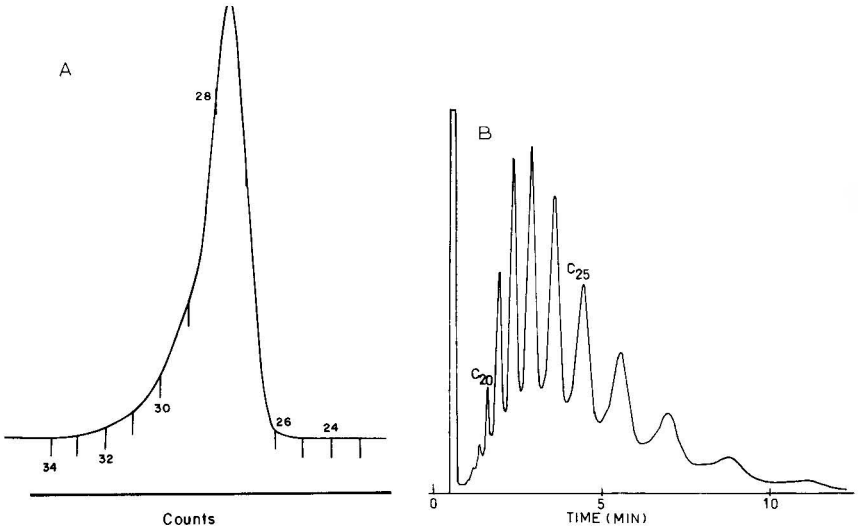


Fig. 4. Gel permeation (A) and gas (B) chromatograms for fraction 3.

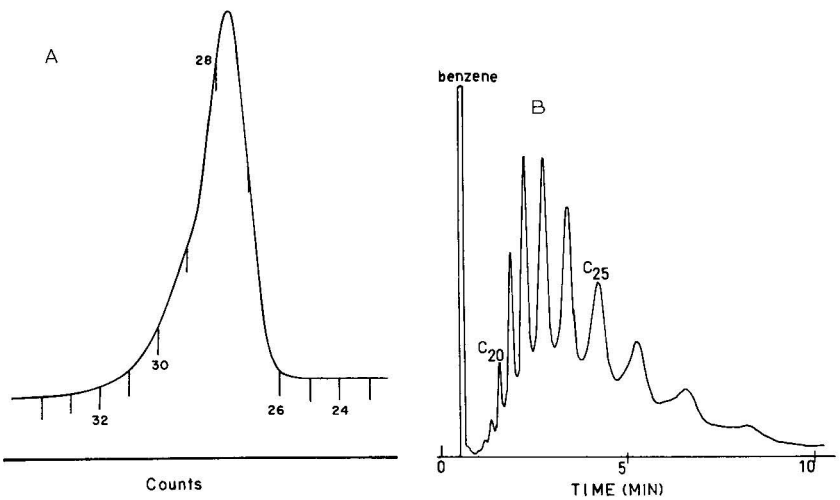


Fig. 5. Gel permeation (A) and gas (B) chromatograms for fraction 4.

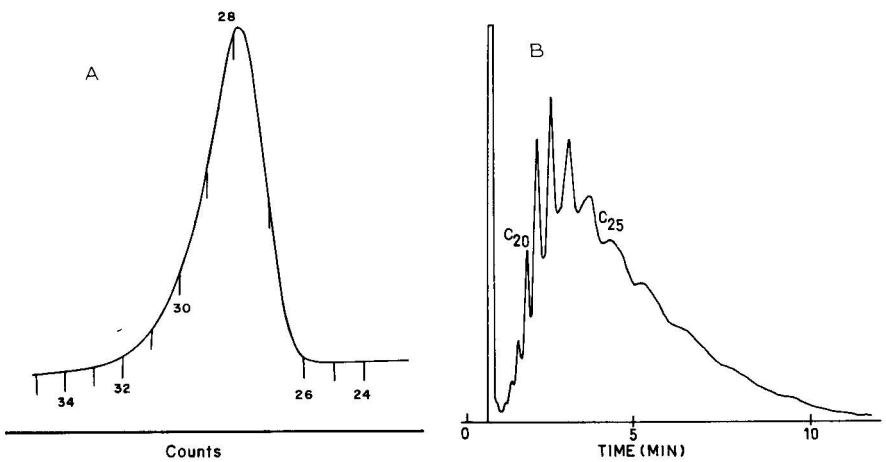


Fig. 6. Gel permeation (A) and gas (B) chromatograms for fraction 5.

and on molecular-weight distributions, sample D was fractionated following the procedure outlined previously in the experimental section. Both Hendrickson and Moore[7] and Schulz[8] have shown that there is no simple relation between molar volume and/or an effective carbon chain length with the elution volume for branched hydrocarbons. Schulz measured the elution volumes of 32 branched alkanes (C_7–C_{11}) and found that the elution behaviour of these compounds correlates well with a function that takes into account both molar volume and carbon number for a homologous series, for example the 5-methylalkanes. Since paraffinic waxes are complex mixtures of *n*-alkanes and branched alkanes (oil), the best that could be expected was approximate $\bar{M}_n$ and $\bar{M}_w$ values.

Table II summarizes the data obtained for the fractionated samples. Figs. 2A–6A show the gel permeation chromatograms and 2B–6B show the gas chromatograms for the five fractions.

An inspection of Figs. 2A–6A reveals that as the oil content increases the base of the gel permeation chromatogram increases, while the peak remains at the same elution volume up to an oil content of *ca.* 66%. Fig. 6A shows that the elution volume measured at the GPC peak changes for the fraction that is mostly oil (m.p. $<23°$). Figs. 2B–6B show that as the oil content increases in a fraction a broad peak appears, underneath the peaks representing the *n*-paraffins, and grows until the *n*-paraffin peaks almost disappear. The oil content of each fraction was estimated by outlining the broad peak and dividing its area by the total area. A sample with <1% oil gives a gas chromatogram with a very straight baseline[9].

Table III summarizes the GPC data. Perhaps the most important information obtained is the $\bar{M}_w/\bar{M}_n$ value which gives an idea of the broadness of the distribution. The fraction containing the most oil has the highest M_w/M_n value. As the melting point of the fraction decreases, both the GPC and GC data show that the distribution of *n*-alkanes is shifted to lower carbon numbers, as expected. If the $\bar{M}_n$ and $\bar{M}_w$ values of sample D, fraction 1 and fraction 2 are compared, the $\bar{M}_n$ values differ by at most 8%, although the fractions contain 10–30 wt. % oil.

TABLE III

MOLECULAR-WEIGHT DISTRIBUTION OF THE FRACTIONS

$\bar{M}_n$ and $\bar{M}_w$ were estimated using the calibration curve for *n*-paraffins. The oil content was estimated from the gas chromatogram.

Fraction	$\bar{M}_n$	$\bar{M}_w$	$\bar{M}_w/\bar{M}_n$	*Oil content (%, w/w)*
1	340	346	1.02	10
2	329	346	1.05	31
3	293	314	1.07	54
4	293	319	1.09	66
5	266	290	1.09	87
Sample D	353	359	1.02	30

An $\bar{M}_n$ value for the fraction containing the least oil (fraction 1) was also obtained by GC. This was done by considering that the area under a given peak divided by the total area is proportional to the concentration of the component responsible for the peak. Peak areas were obtained by triangulation and an $\bar{M}_n$

value of 346 was obtained for fraction 1. This value is in good agreement with the $\bar{M}_n$ value obtained from GPC data calculated using the calibration curve for *n*-paraffins.

CONCLUSIONS

The results of this study show that GPC values of $\bar{M}_n$, $\bar{M}_w$ and $\bar{M}_w/\bar{M}_n$ of crude paraffinic waxes are useful in characterizing these samples although calibration curves for the complex mixtures may not be available. Crude paraffinic waxes containing $>30\%$ (w/w) oil show $\bar{M}_w/\bar{M}_n$ values of >1.05. A sample having a melting point below 23° and an estimated oil content of 87% shows a $\bar{M}_w/\bar{M}_n$ ratio of 1.09. As the melting point of the fraction decreased both GPC and GC show that the distribution changes to lower carbon numbers. Apparently, the oil sample has a distribution curve with a peak at 27.9 counts and an $\bar{M}_w/\bar{M}_n$ value of 1.09, while the main *n*-paraffinic fraction is a distribution of C_{18}–C_{30} with a peak at 27.6 counts and an $\bar{M}_w/\bar{M}_n$ ratio of 1.02. The distribution curve of the *n*-paraffins is apparently affected only slightly when the oil is present in amounts up to 30%. GC data complement very nicely the information obtained by GPC for paraffinic waxes having an average chain length of C_{25}, and one would expect the same for fractions having greater chain lengths.

ACKNOWLEDGEMENT

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CHROM. 10,996

ION-PAIR LIQUID CHROMATOGRAPHY OF AMITRIPTYLINE AND METABOLITES IN PLASMA

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SUMMARY

A method is described for the determination of amitriptyline and nortriptyline in the plasma of patients during treatment. The method is based on liquid chromatography of the amines as ion-pairs with perchlorate. The separation column is packed with silica gel, and a mixture of diisopropyl ether, dichloromethane, methanol and water containing the counter ion is used as eluent. High efficacy and stability of the system is achieved. The separation of metabolites is demonstrated. The *cis*- and *trans*-10-hydroxy isomers of amitriptyline and nortriptyline are easily resolved with this chromatographic system.

INTRODUCTION

Amitriptyline and its N-demethylated metabolite (nortriptyline) are two important tricyclic antidepressant drugs in widespread clinical use. Studies have shown that the therapeutic as well as the adverse effects of these drugs are related to the plasma concentrations achieved during treatment[1–8] and several liquid chromatographic methods for the determination of tricyclic antidepressant drugs have been described[9–12].

Ion-pair partition chromatography is a highly suitable technique for the measurement of these drugs[13–15] and the present study is a modification of this technique that offers advantages with respect to stability and reproducibility compared with an earlier system[15]. By using this method, therapeutic plasma concentrations of amitriptyline and nortriptyline have been measured; the separation of the major metabolites of the drugs is also described.

EXPERIMENTAL

Apparatus

The liquid chromatograph incorporated an LDC 711-26 solvent delivering

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system. The detector unit was an Altex Model 153 analytical UV detector operated at 254 nm. The injector was a Rheodyne Model 70-10 sample injection valve (Altex Scientific, Berkeley, Calif., U.S.A.). The volume of the injection loop was 70 μl.

Chromatographic system

The separation column (100 or 125 mm $\times$ 4.6 mm I.D.) was of stainless steel and was packed with Partisil 5 (H. Reeve Angel, Clifton, N.J., U.S.A.) by the balanced-density slurry technique[16]. Equal volumes of tetrabromoethane, carbon tetrachloride and dioxan were used as suspending medium for the particles. The mobile phase consisted of perchloric acid, sodium perchlorate, methanol, dichloromethane and diisopropyl ether and was degassed in an ultrasonic bath for 45 min before use.

Reagents and chemicals

Diisopropyl ether was of synthesis grade (E. Merck, Darmstadt, G.F.R.; stabilized with 2,6-di-*tert.*-butyl-4-methylphenol); all other chemicals and solvents were of analytical grade. The internal standard, 2-(dibenz[*b*,^{5}]azepin-5-yl)-N-methyl ethylamine (Fig. 1), was obtained from Ciba Geigy (Basel, Switzerland).

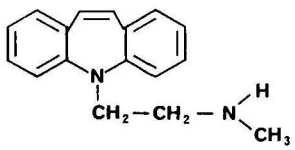


Fig. 1. Structure of internal standard.

Analytical procedure

Plasma samples (1 ml) were extracted by methods previously described[15,17]. Briefly, 50 μl of the internal standard solution (2.74 μM) and 1 ml of 2 M carbonate buffer (pH 10.5) were added to the samples and extraction of the drugs into *n*-hexane (5 ml) was achieved by gentle shaking to avoid the formation of an emulsion. The compounds were back-extracted into 1 ml of 0.25 N sulphuric acid and, after this solution had been made alkaline with 0.2 ml of 2.5 M sodium hydroxide, they were finally extracted into 0.1 ml of diisopropyl ether; this extract was used for the liquid chromatography.

RESULTS AND DISCUSSION

Chromatography

In previous ion-pair partitition chromatographic systems for tricyclic anti-depressant drugs[13–15], the stationary aqueous phase was pre-coated on the support. In the present system, a mobile phase that is under-saturated with the aqueous phase containing the counter ion is used. This technique gave highly stable and reproducible columns[18], without the need for thermostatic control of the chromatographic system that is essential with pre-coated columns.

Originally, a mixture of methanol and dichloromethane was used as the organic solvent for the mobile phase; however, the presence of diisopropyl ether was found to be necessary to retain the compounds and for good column performance.

It is our hypothesis that the polar silica gel support partly adsorbs the more polar components of the mobile phase. Support for this theory was obtained by measurements of the interstitial volume (V_m) of the column with tetrachloroethylene as the marker during equilibration of a column. The results are illustrated in Fig. 2. The intercept value, 0.74, was obtained with dry dichloromethane as mobile phase and probably represents the total porosity (ε_t) of the column[18]. The support availability for the mobile phase, represented by ε_m which is equal to V_m/V_0, where V_0 is the volume of the empty column), decreases to an equilibrium value of 0.68 or 0.62 after the passage of about 12 or 90 columns volumes, respectively, indicating that adsorption of a stationary phase occurs. It can be calculated from $\varepsilon_t = \varepsilon_m + \varepsilon_s$ (where ε_s is given by V_s/V_0 is the volume of the stationary phase) that this corresponds to the presence of 0.12 or 0.21 ml of stationary phase, which is about one-fifth and one-third, respectively, of the volume for a pre-coated ion-pair partition system. The adsorbed phase probably consists of a mixture of the aqueous phase and methanol.

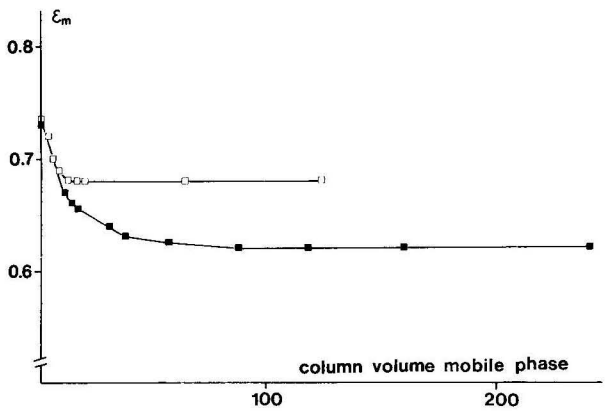


Fig. 2. Curves of porosity *vs.* number of column volumes of mobile phase. Column, 125 × 4.6 mm I.D. Mobile phase, 0.01 *M* perchloric acid + 0.09 *M* sodium perchlorate–methanol–dichloromethane–diisopropyl ether; □, 0.9:10:12:30; ■, 0.9:5.2:12:30. Flow-rate, 1.0 ml/min.

In straight-phase ion-pair chromatography the capacity factor (k') is given by

$$k' = \frac{V_s}{V_m \cdot E^*_{HAX} \cdot (X)} \tag{1}$$

where E^*_{HAX} is the conditional ion-pair extraction constant and (X) is the concentration of counter-ion in the stationary phase. The capacity factors decrease with increasing concentration of perchlorate ions in accordance with eqn. 1 as illustrated in Fig. 3. However, the relationship is not rectilinear, partly because of the varying ionic strength and also because of the influence of other retention mechanisms. The possibility of regulating the capacity factors by adding methanol to the mobile phase is demonstrated in Fig. 4. The main features are that the capacity factors decrease as the methanol concentration increases above 11.8% (for the mobile phase studied). Some selectivity changes, especially between amitriptyline and the 10-hydroxy

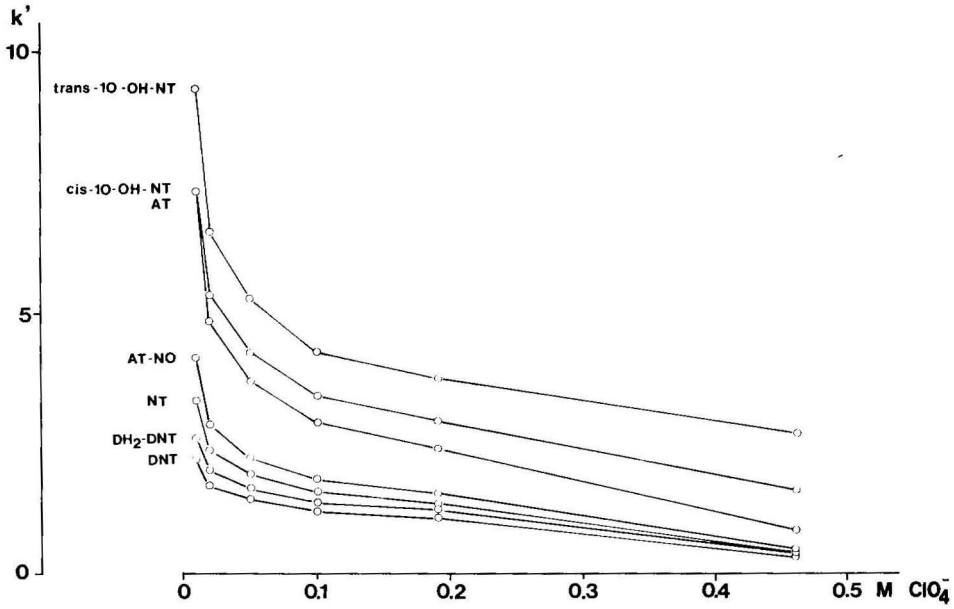


Fig. 3. Influence of counter-ion concentration on capacity factor (k'). Column, as in Fig. 1. Mobile phase, perchloric acid + sodium perchlorate–methanol–dichloromethane–diisopropyl ether (0.9:5.2:12:30). Flow-rate, 1.1 ml/min. For abbreviations see Fig. 7.

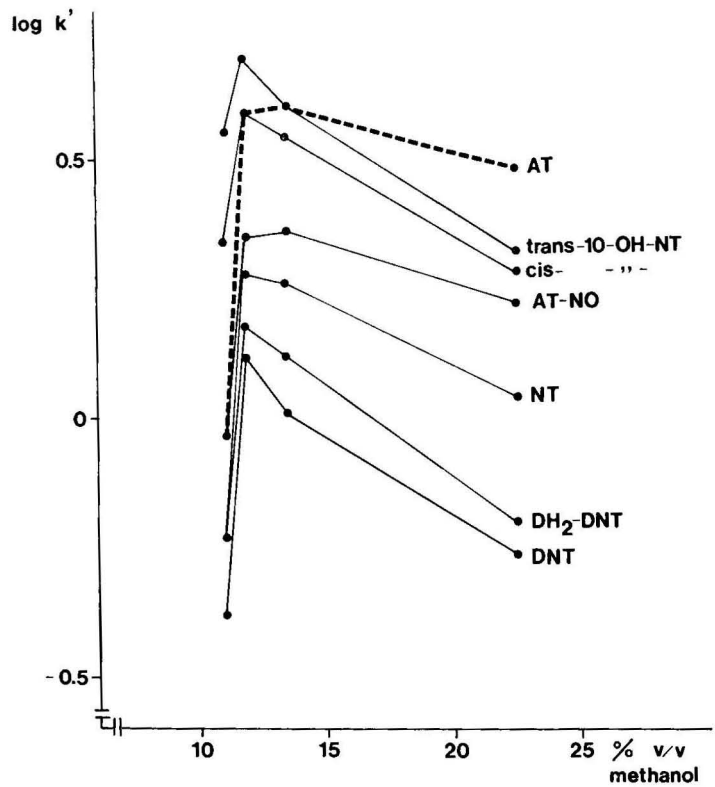


Fig. 4. Influence of methanol concentration on capacity factor (k'). Column, as in Fig. 1. Mobile phase, 0.01 M perchloric acid + 0.09 M sodium perchlorate–dichloromethane–diisopropyl ether (0.9:8:30)–methanol. Flow-rate, 1.1 ml/min. For abbreviations see Fig. 7.

metabolites of nortriptyline, can be seen. With decreasing methanol concentration below 11.8%, the mobile phase will approach saturation with respect to water and counter ion. The column efficiency with the system, calculated as the height of a theoretical plate, is between 0.02 and 0.04 mm.

Bioanalysis

The system has been successfully applied to the measurement of plasma concentrations of amitriptyline and nortriptyline in patients receiving therapeutic doses of these drugs. Typical traces obtained from the analysis of 1-ml plasma samples (a) containing no drug and (b) obtained from a patient receiving amitriptyline are shown in Fig. 5. Chlorimipramine and demethylchlorimpiramine have also been measured by using this chromatographic system and the extraction procedure described earlier[15].

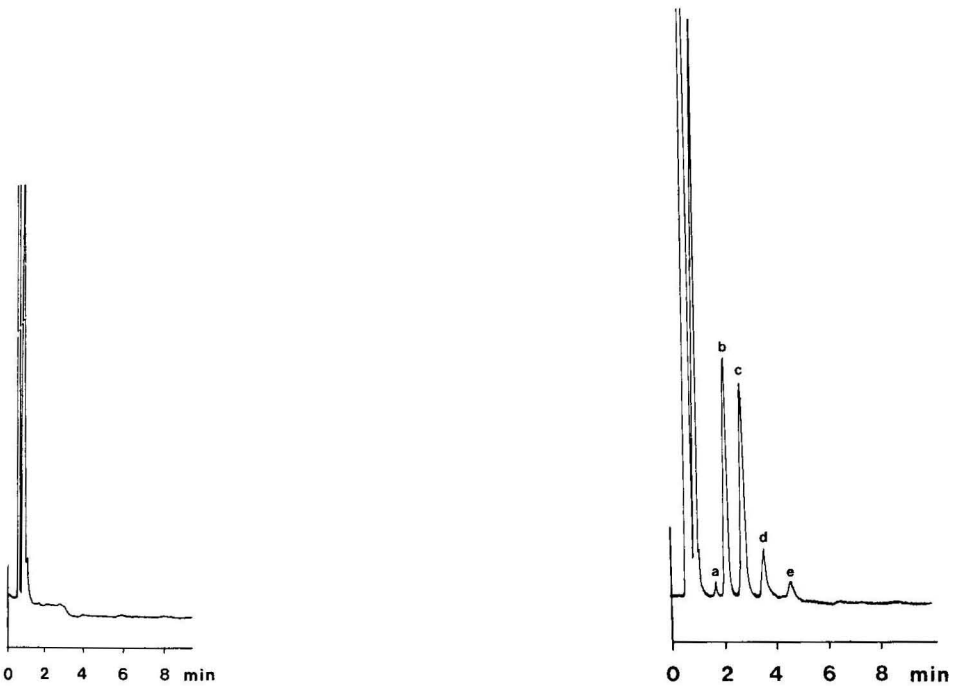


Fig. 5. Chromatograms from plasma samples. Column, as in Fig. 1. Mobile phase, 0.1 *M* perchloric acid–methanol–dichloromethane–diisopropyl ether (0.9:5.2:10:30). Flow-rate, 1.9 ml/min. (a) Blank human plasma, (b) plasma of a patient receiving amitriptyline. Peaks: a = demethylnortriptyline, b = nortriptyline (462 n*M*), c = internal standard (135 n*M*), d = amitriptyline (226 n*M*), e = *trans*-10-hydroxynortriptyline.

The reproducibility of the method determined from measurements on 38 duplicate plasma samples and calculated as the coefficient of variation was 10.1% for amitriptyline and 5.2% for nortriptyline; the concentration range was 90–900 n*M*.

A comparison between the proposed technique and a mass fragmentographic method[19] was carried out for the analyses of plasma nortriptyline in a group of 10

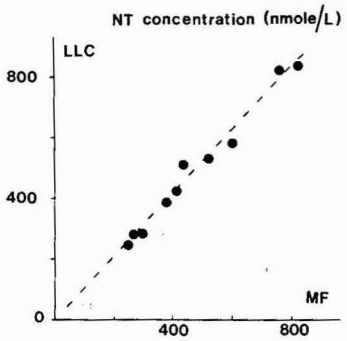


Fig. 6. Comparison of results obtained by mass fragmentography (abscissa) and by liquid chromatography (ordinate) for nortriptyline in plasma. Line of best fit: $y = 1.04x - 8.60$ ($n = 10$, $r = 0.99$).

patients; agreement between the results obtained by the two methods was close ($r = 0.99$; Fig. 6).

The high resolving capacity of the system is demonstrated in the separation of amitriptyline and nortriptyline from their respective metabolites (Fig. 7). It was possible to resolve the stereoisomeric forms of 10-hydroxyamitriptyline and 10-hydroxynortriptyline. The stereoisomers of 10-hydroxynortriptyline saved from an earlier study in this laboratory[20] gave retention times of 4.5 and 6.0 min, respectively. The absolute configuration of *trans*-10-hydroxynortriptyline has now been established[21], and it has the same retention time as isomer I, showing that the isomers I and II reported by Bertilsson and Alexanderson[20] represent the *trans*- and *cis*-forms, respectively. For the stereoisomers of 10-hydroxyamitriptyline (Fig. 7), it is possible to argue that isomers I and II represent the *trans*- and the *cis*-forms, respectively, by analogy with the order obtained with 10-hydroxynortriptyline.

Using this chromatographic system, quantitation of the metabolites of amitriptyline is possible. The clinical application of this study will be reported in a later publication.

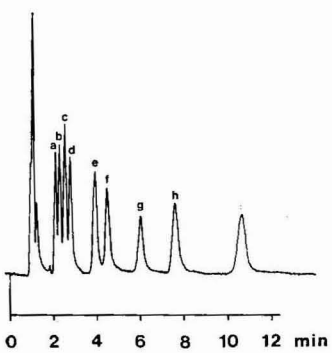


Fig. 7. Separation of amitriptyline and its metabolites. Column, as in Fig. 1. Mobile phase, 0.1 *M* perchloric acid + 0.9 *M* sodium perchlorate–methanol–dichloromethane–diisopropyl ether (0.85:5.1:5:30). Flow-rate, 1.1 ml/min. Peaks: a = demethylnortriptyline (DNT), b = 10,11-dehydrodemethylnortriptyline (DH_2-DNT), c = nortriptyline (NT), d = amitriptyline-N-oxide (AT-NO), e = amitriptyline (AT), f = *cis*-10-hydroxynortriptyline (*cis*-10-OH-NT), g = *trans*-10-hydroxynortriptyline (*trans*-10-OH-NT), h = 10-hydroxyamitriptyline II and i = 10-hydroxyamitriptyline I.

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Note

Preparation of a column with octanol-like properties for high-performance liquid chromatography

Direct measurements of partition coefficients in an octanol–water system

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(Received March 25th, 1978)

The hydrophobic character of chemicals, which is expressed in terms of the partition coefficient, P_{oct}, between *n*-octanol and water, is one of the most important parameters in the analysis of quantitative structure–activity relationships (QSAR)[1,2]. However, there is no convenient method available to determine the P_{oct} value accurately; the flask-shaking method usually adopted is time-consuming, involves troublesome quantitative measurements and requires high purity of the chemicals and of *n*-octanol.

There have recently been attempts using high-performance liquid chromatography (HPLC) to determine the hydrophobicities (P_{HPLC}) of chemicals. If this method could be perfected, it would be very useful for avoiding the disadvantages of the flask-shaking method. In most previous studies on HPLC, ODS[3,4] or persilanized ODS[5] has been used as the stationary phase. However, it would be preferable to use an octanol-coated column to obtain hydrophobicities directly reflecting P_{oct}. Mirrlees *et al.*[6] prepared a column by coating *n*-octanol on Kieselguhr that had previously been fractionated and silanized, and eluting excess *n*-octanol with the mobile phase (*in situ* coating method). Using this column they obtained good correlations between the P_{HPLC} values of miscellaneous compounds and their P_{oct} values. However, their method of preparing the column seems rather tedious. Moreover, Henry *et al.*[7] did not obtain good correlations between P_{HPLC} and P_{oct} values for sulphonamides, using Corasil II coated with 1% octanol as the stationary phase.

Thus in order to establish a practical method of using octanol in HPLC, it is necessary to find suitable experimental conditions, including a simple method for preparation of the column. This paper reports a convenient method for preparing an octanol-coated column that has high stability and gives good results.

HPLC PROCEDURE

All chemicals were obtained from commercial sources and were used without further purification.

HPLC was performed on a JASCO high-performance micro liquid chromatograph, Model Familic-100, because this apparatus is designed so that the length of

the column can be easily varied. The supporting medium was packed in a PTFE tube, 0.5 mm I.D., connected to a UV-detector, JASCO UVIDEC-100. Retention times were measured from the chromatographic peaks, recorded on a Hitachi QPD-54 recorder.

If the adsorption of solutes on solid supports does not affect the retention time and the properties of the eluent are the same in the pores of the support and in the mobile phase, the retention time, t_R, should be linearly dependent on the partition coefficient of the solute between the stationary and mobile phases, P_{HPLC}, as shown in eqn. 1 (ref. 8):

$$t_R = t_0 + t_0 q P_{HPLC} \quad (1)$$

where t_0 is the retention time of the non-retained compound and q is the volume ratio of the stationary and mobile phases (V_s/V_m). Then the capacity factor, k', is expressed by

$$\log k' = \log[(t_R - t_0)/t_0] = \log P_{HPLC} + \log q \quad (2)$$

When adsorption chromatography of methanol, benzyl alcohol and nitrobenzene was performed using Corasil I, the t_R values were almost the same: t_R(methanol):t_R(benzyl alcohol):t_R(nitrobenzene) = 1.0:1.0:1.2; however, on silica gel (WC-01, JASCO) the t_R values were very different: t_R(methanol):t_R(benzyl alcohol):t_R(nitrobenzene) = 1.0:1.3:3.5. Thus, we used Corasil I as the solid support.

To prepare the column, Corasil I was heated at 110° overnight and then, while still hot, mixed with *n*-octanol. The slurry was packed in a PTFE tube, and excess *n*-octanol removed by elution with the mobile phase (HCl–KCl buffer, pH 2.0, saturated with *n*-octanol) until a stable baseline was obtained on the recorder. This took about 10 min at a flow-rate of 16 μl/min. Then 0.1 μl of a solution of the test compound in water or 10% methanol was loaded on the column and eluted with the mobile phase. Methanol ($\log P_{oct} = -0.82$) or benzenesulphonic acid ($\log P_{oct} = -2.25$) was used as a non-retained marker compound. At a flow-rate of 16 μl/min, this column was stable for more than 60 h of use, giving consistent t_R values.

The t_R of *p*-cyanophenol was measured at various flow-rates of the mobile phase and k' was calculated from t_R. Fig. 1 shows the changes of $\log k'$ and $\log t_c$ with flow-rate ($t_c = t_R - t_0$). Values of $\log k'$ were almost constant at flow-rates of up to 20 μl/min, but gradually decreased with increasing flow-rates above 20 μl/min, whereas $\log t_c$ decreased continuously with increasing flow-rates. These results indicate that, at flow-rates of less than 20 μl/min, t_c is affected by a small fluctuation of the flow-rate, but k' is not. Thus the hydrophobic property determined by HPLC should be represented by the k' value. In this study, we measured k' values at a flow-rate of 16 μl/min.

The reproducibility of measurements was examined using benzyl alcohol and *p*-cyanophenol. The standard errors of $\log k'$ in ten runs with a 14-cm column were 0.003 for benzyl alcohol and 0.005 for *p*-cyanophenol, indicating that $\log k'$ can be determined with high reproducibility.

When we examined the *in situ* coating method using Corasil I as a support, as described by Mirrlees *et al.*[6], we did not obtain satisfactory results, *n*-octanol be-

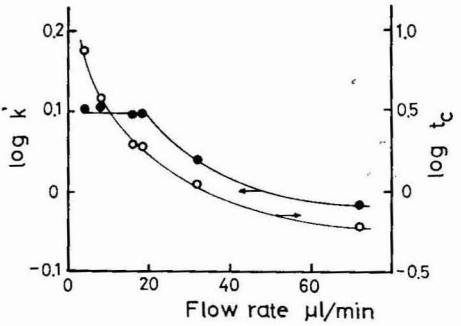


Fig. 1. Effect of the flow-rate of the mobile phase on log k' (●) and log t_c (○) values of *p*-cyanophenol. Mobile phase, 0.2 *N* HCl–KCl buffer, pH 2.0; column length, 14 cm.

coming dislodged and t_R decreasing gradually during the performance of the chromatography.

RELATIONSHIP BETWEEN P_{HPLC} AND P_{oct}

We measured the t_R values of miscellaneous compounds, such as benzene, benzoic acids and phenols, using column lengths of 4 cm and 14 cm; k' was expressed as the mean value of four replicate runs. The linear relationship between log k' and log P_{oct} with 4-cm and 14-cm columns is shown in Fig. 2, and eqns. 3 and 4 were derived.

With a 4-cm column

$$\log k' = 0.960 \log P_{oct} - 1.551 \; (n = 9, r = 0.992, s = 0.023) \quad (3)$$

With a 14-cm column

$$\log k' = 0.965 \log P_{oct} - 1.456 \; (n = 10, r = 0.996, s = 0.024) \quad (4)$$

In eqns. 3 and 4, n is the number of compounds, r is the correlation coefficient and s is the standard error of estimate of log k' on log P_{oct}. With both columns the slope of the straight line is almost unity and a very good correlation is obtained.

Use of a shorter column for compounds with high log P_{oct} values makes it easier to obtain accurate t_R values, because the peak is sharper and the operational time is shorter: for example, with a 14-cm column, the t_R of salicylic acid (log P_{oct} = 2.26) was 23.1 min, but the peak of the more hydrophobic *p*-chlorophenol (log P_{oct} = 2.39) was so broad that t_R could not be determined; with a 4-cm column, however, the t_R of salicylic acid was 11.5 min and the peak of *p*-chlorophenol was sharp enough to obtain a reproducible t_R value of 16.6 min.

The partition coefficients determined in two partitioning systems, P_1 and P_2, can be represented by eqn. 5, if the hydrogen-bonding properties of the two organic solvents are similar[9].

$$\log P_1 = m(\log P_2) + b \quad (5)$$

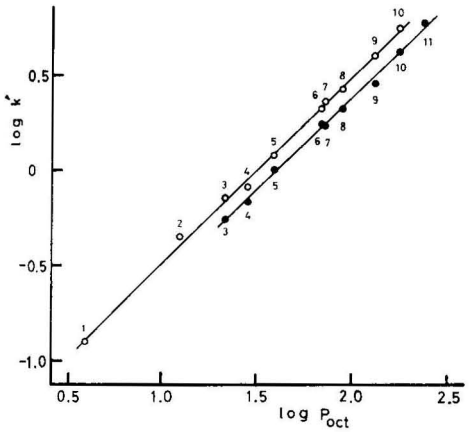


Fig. 2. Plots of log k' against log P_{oct} with different column lengths. 1 = Hydroquinone; 2 = benzyl alcohol; 3 = *p*-methoxyphenol; 4 = phenol; 5 = *p*-cyanophenol; 6 = nitrobenzene; 7 = benzoic acid; 8 = methylparaben; 9 = benzene; 10 = salicylic acid; 11 = *p*-chlorophenol. log P_{oct} values are taken from ref. 9. Mobile phase; 0.2 *N* HCl–KCl buffer, pH 2.0. Column length: ●, 4 cm; ○, 14 cm. Flow-rate, 16 μl/min.

Denoting P_1 as P_{HPLC}, and P_2 as P_{oct}, eqn. 6 is derived by a combination of eqns. 2 and 5.

$$\log k' = m(\log P_{oct}) + \log q + b \tag{6}$$

The value of the slope, m, in eqn. 6 should be unity, as obtained in this work, if the t_R is governed by the partition between mobile phase and *n*-octanol coated on the support. Thus the value of m is a good measure of whether the stationary phase is octanol-like. Henry *et al.*[7] obtained an m value of 0.72 for sulphonamides with a column of Corasil II coated with 1% octanol, which suggests that there is a direct interaction of silanol groups on the support with solutes in the mobile phase.

Eqn. 5 was reported not to hold for compounds capable of forming hydrogen bonds if the properties of the two organic solvents with respect to hydrogen-bond formation are different[10]. In the case of an ODS column, good linearity between log P_{oct} and log P_{HPLC}, with a slope close to unity was reported for a series of penicillins[4]. However, phenols and monosubstituted benzenes did not give a linear relationship, but two straight lines with different slopes of less than 1 and different intercepts: one for compounds that did not form hydrogen bonds and the other for amphiprotic compounds[11].

It should be noted that all the data-points for compounds with various hydrogen-bonding abilities fall on the same straight line in Fig. 2 with either column length. This also indicates that the properties of the stationary phase used in this study were very similar to those of *n*-octanol, and that the k' value represented P_{oct} well.

It is concluded that P_{oct} values of wide variety of compounds can be determined easily and accurately by the method described in this paper.

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Note

Gas chromatographic study of temperature-programmed desorption of hydrogen on platinum–alumina*

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Temperature-programmed desorption (TPD) or flash desorption is one of the most important methods in studies of the active centres of catalysts, adsorption, surface heterogeneity and catalytic reactions[1–3]. Amenomiya and Cvetanovic[4] suggested an adaptation of the TPD method, which consists in heating the catalyst with the gas adsorbed irreversibly at the lowest temperature of study at a programmed heating rate and measuring the gas desorbed continuously into the stream of an inert carrier gas by a gas detector. Their apparatus consisted of a static volumetric system for adsorption measurements and a flow system for TPD of the gases adsorbed irreversibly on the catalyst under reduced pressure. The catalyst was packed in a shallow-bed reactor. Komers *et al.*[5] extended the TPD technique to a metal catalyst with a small surface area of exposed metal and studied chemisorption and surface reactions of ethylene on platinum–silica.

In this study, a gas chromatographic technique with a catalyst packed in a long chromatographic column as a desorption reactor for TPD studies is described. The kinetic parameters of the desorption of hydrogen on a platinum–alumina catalyst have been determined by measuring the shift in the characteristic desorption temperature as a function of the linear heating rate.

EXPERIMENTAL

The TPD chromatograms for hydrogen on Engelhard RD-150 platinum–alumina reforming catalyst [containing 0.6% (w/w) of platinum and having a particle density of 1.35 g/cm^3, a pore volume of 0.37 cm^3/g and a porosity of 0.5] were obtained by measuring the hydrogen desorbed continuously in a stream of nitrogen with a thermal conductivity detector (TCD) using a Perkin-Elmer 900 gas chromatograph.

Two identical stainless-steel chromatographic columns (3 mm O.D. and 2 min I.D.) (columns A and B) with a bed length of 90 cm, each packed with the same amount (2.53 g) of the platinum–alumina catalyst (heated in air at 500 °C for 4 h before used) in the form of particles of size 177–250 μm, were prepared and fitted to the chromatographic oven which was used for the linear temperature-programmed heating.

* NCL Communication No. 2202.

The catalyst in both columns was reduced with hydrogen at 400 °C for 4 h. The hydrogen was then replaced with nitrogen (purified by passage over heated copper and molecular sieve to remove trace amounts of oxygen and moisture) and the catalyst was heated at 400 °C in the flow of nitrogen for 2 h. The dispersion of platinum in the catalyst was measured by the gas chromatographic pulse technique[6] based on the titration of chemisorbed oxygen with hydrogen, and was found to be $42 \pm 3\%$.

The catalyst in column A (connected to the sensitive section of the TCD) was used for the desorption studies. Column B (connected to the reference section of the TCD) was used as a dummy column. In order to check the stability of the baseline, several trial runs were carried out on the clean catalyst without desorption for each of the heating rates used in the study for the programmed heating range of 100–400 °C. Even though both columns were packed with the same amount of catalyst, there was a shift in the baseline, probably owing to unequal packing. Hence, the nitrogen flow-rate in the dummy column (B) was adjusted by trial and error until a steady baseline without a shift was obtained for the entire programmable temperature range at a fixed carrier gas flow-rate (170 ml/min) through column A.

The initial temperature chosen for the TPD run was 100 °C. Before starting the TPD, the catalyst in column A was saturated with hydrogen at this temperature by passing over it a number of pulses of hydrogen of known volume (0.25 ml) using a gas sampling valve. The amount of hydrogen irreversibly chemisorbed (or the initial surface coverage) was determined from these pulse experiments by a method described elsewhere[7]. The initial surface coverage was 0.063 ± 0.005 ml of hydrogen (at N.T.P.) per gram of catalyst for all of the TPD runs. This value does not include the residual hydrogen on the catalyst surface after desorption at 400 °C for 2 h.

The TPD chromatograms were obtained at six different heating rates in the range 4–24 °C/min and at a nitrogen flow-rate of 170 ml/min measured at room temperature (or 115–200 cm/sec, corrected for temperature variations).

The basic requirement of TPD is that the surface temperature of the adsorbent should be uniform throughout the bed at any instant of the heating period. This requirement can be met only if the heat transfer rate is very high. In this study, high heat transfer rates were expected because of the use of the well mixed (due to the rapid circulation of hot air) chromatographic oven, the long bed desorption reactor (with a small diameter) and the high gas flow-rate through the catalyst column. Another requirement for TPD is that the desorption process should be free from mass transfer limitations. Diffusional resistances to desorption, such as intraparticle diffusion and external film diffusion, were also eliminated by the use of small catalyst particles and a high linear carrier gas velocity, respectively.

RESULTS AND DISCUSSION

The TPD chromatograms for hydrogen on the platinum–alumina catalyst at different heating rates are shown in Fig. 1. The desorption appears to be first order because the chromatograms are asymmetric. Further, there is only one peak for the desorption, which indicates the involvement of only one type of active centre in the desorption process.

The characteristic desorption temperatures (T_m) at which the desorption rate is

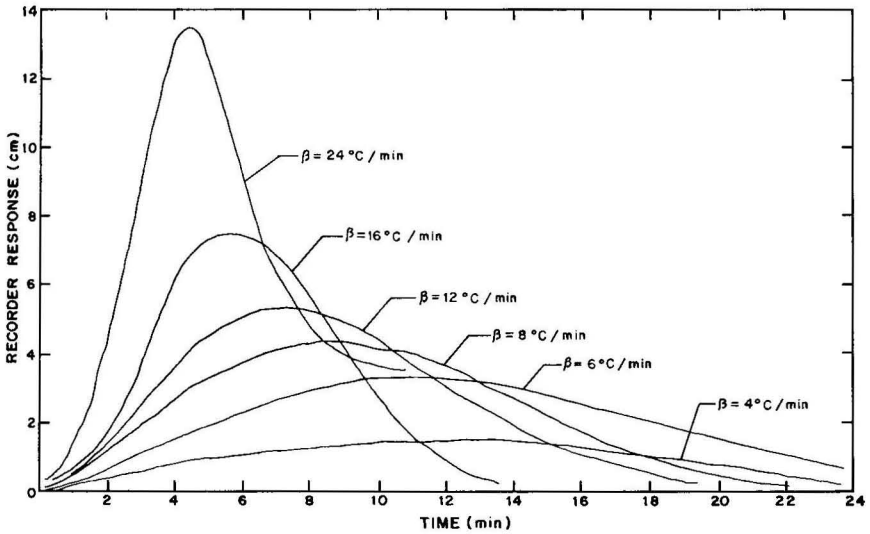


Fig. 1. TPD chromatograms for hydrogen of platinum–alumina. Carrier gas, nitrogen.

maximal for different heating rates are listed in Table I. It can be seen that there was a shift in T_m from 424 to 481 °K when the heating rate (β) was increased from 4 to 24 °C/min.

The activation energy for desorption was obtained from these data by using the relationships developed by Redhead[8], Carter[9] and Amenomiya and Cvetanovic[4] for the first-order desorption process when readsorption does not occur and assuming that the activation energy for desorption is independent of the surface coverage:

$$(k_d)_m = \frac{\beta E_d}{R T_m^2} = A \exp(-E_d/R T_m) \tag{1}$$

or

$$\log(T_m^2/\beta) = \frac{E_d}{2.303 R}\left(\frac{1}{T_m}\right) + \log\left(\frac{E_d}{AR}\right) \tag{2}$$

TABLE I

RESULTS OF TPD OF HYDROGEN ON PLATINUM–ALUMINA

Heating rate, β (°C/min)	*Temperature of peak maximum, T_m (°K)*	*Desorption rate constant at T_m, k_d (sec^{-1})*
4	424	$1.98 \cdot 10^{-3}$
6	437	$2.80 \cdot 10^{-3}$
8	443	$3.63 \cdot 10^{-3}$
12	459	$5.08 \cdot 10^{-3}$
16	469	$6.49 \cdot 10^{-3}$
24	481	$9.25 \cdot 10^{-3}$
Activation energy of desorption (E_d)		10.7 kcal/mole*
		10.5 kcal/mole**
Pre-exponential factor (A)		602 sec^{-1}

* Obtained from eqn. 2.
** Obtained from eqn. 3.

where $(k_d)_m$ is the desorption rate constant at T_m, T_m is the temperature corresponding to the maximum desorption rate, E_d is the activation energy for desorption, β is the linear heating rate, A is the pre-exponential factor and R is the gas constant. The plot of log (T_m^2/β) against $1/T_m$ was a straight line (Fig. 2). The activation energy (E_d) and the pre-exponential factor (A) were obtained from the slope of the linear plot and by substituting the value of E_d in eqn. 1, respectively. The values of k_d, E_d and A are listed in Table I.

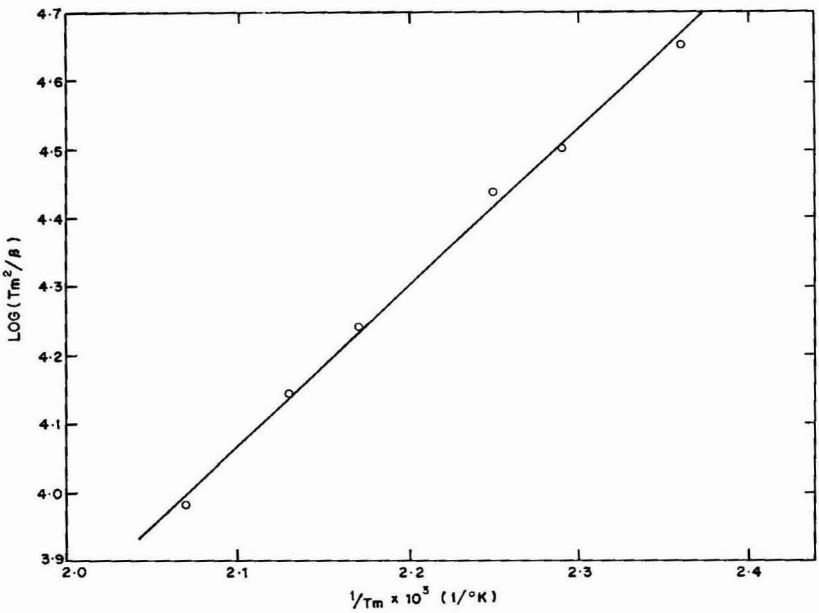


Fig. 2. Graph of log (T_m^2/β) versus $1/T_m$.

The activation energy can also be obtained[8] by plotting log β against log T_m and using the equation

$$\left(\frac{E_d}{R\,T_m}\right) + 2 = \mathrm{d}(\log \beta)/\mathrm{d}(\log T_m) \tag{3}$$

The values of E_d obtained by means of eqns. 2 and 3 are very similar (Table I).

It should be noted that the values of E_d and A given in Table I are valid for first-order desorption when no readsorption occurs. In this work, as the initial surface coverage is very small and the desorption is carried out in the stream of inert gas flowing over the catalyst at a very high rate, the concentration of the desorbed gas is extremely small and the assumption that readsorption does not take place during the TPD is valid.

The value of E_d (10.7 kcal/mole) obtained in this work is very close to the value of ΔH (10 kcal/mole) for the adsorption of hydrogen on platinum–alumina [0.5% (w/w) of platinum] determined by Gruber and Hausen[10] by gas chromatography from the temperature dependence of the retention volume at low surface

coverage. Tsuchiya *et al.*[11] investigated the chemisorption of hydrogen on a platinum black catalyst over the temperature range −196–400 °C by TPD and observed four different states (α, β, γ and δ) of chemisorbed hydrogen with different activation energies. Only one state of chemisorbed hydrogen on alumina-supported platinum was observed from the present TPD studies in the temperature range 100–400 °C, because the preadsorption temperature and the catalyst pre-treatment before starting the TPD runs were different in the two studies. However, a comparison between these two studies shows that the hydrogen chemisorbed in the present investigation corresponds to the γ-hydrogen of the previous study[11], which was also found to follow a first-order desorption process.

Gas chromatography with a catalyst packed in a long column with a small diameter offers many advantages for TPD studies, such as the elimination of mass transfer and heat transfer resistances for achieving a uniform temperature throughout the catalyst bed at any instant of the heating period, and the applications of the well established gas chromatographic methods[7,12] for determining the initial surface coverages (*i.e.*, irreversible or reversible adsorption) and the physico-chemical surface properties of the catalyst under study. Thus, the requirement of an additional static volumetric system with a high-vacuum arrangement for measuring adsorption in TPD studies is eliminated. The problem of a shift in the baseline during temperature programming can be avoided by using a flame-ionization detector, which is not sensitive to small changes in the flow-rate, for studying the TPD of hydrocarbons or other detectable adsorbates. Further, this technique can be extended to study the TPD of reversibly adsorbed species.

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Note

Preparation and properties of beef heart mitochondrial ATPase covalently bound to Sepharose 4B*

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The most significant recent advancements in the understanding of the process of ATP synthesis have occurred in the realm of the molecular mechanism of the mitochondrial ATPase. Recent reviews[1–3] emphasize the importance of these results in obtaining a complete understanding of the process of ATP synthesis. The mitochondrial ATPase has been shown to contain an essential arginine[4] and tyrosine[5] and a sulfhydryl group is necessary for anion activation[6]. A number of studies concerning the kinetic and regulatory properties of the enzyme have appeared[7–9] as well as work on the nucleotide[10,11] and phosphate[12] binding properties.

Recently, the role of the loosely associated proteins that make up the "ATP synthase complex" has come under serious scrutiny. The role of these proteins in regulation or membrane attachment is currently being rigorously studied[1–3], but the lack of rapid isolation techniques for these complex proteins has been a severely limiting step.

The preparation of an insolubilized mitochondrial ATPase preparation that maintains all of the kinetic properties of the soluble enzyme is described here. In addition, this preparation is stable under conditions which will enable it to be used as an "affinity probe" for the proteins that can normally associate with the ATPase molecule.

MATERIALS AND METHODS

Sepharose 4B (200 ml packed gel) was thoroughly washed with distilled water and allowed to react with 60 g CNBr (300 mg/ml packed gel) at 20° according to the method of Cuatracasas[13]. After the Sepharose had been activated, it was washed with water and 100 ml of the activated product was immediately mixed with an ice cold

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solution (100 ml) of 1,7-diamino heptane (0.1 *M*) adjusted to pH 10.0 with 6 *N* HCl. The remainder of the activated Sepharose 4B was mixed with an ice cold solution (100 ml) of 0.1 *M* ethylenediamine adjusted to pH 10.0 with 6 *N* HCl. These solutions were incubated at 4° with gentle stirring for 24 h. The resins were washed with water, incubated in 0.1 *N* NaOH for 15 min at room temperature, then washed to neutrality with water. The presence of primary amino groups bound to the resin was tested by allowing a small portion of resin to react with an alkaline solution of trinitrobenzene sulfonate[13]. The appearance of an orange color, associated with the resin bead, confirmed the synthesis of amino heptyl and amino ethyl Sepharose 4B.

The succinyl derivative of each resin was prepared by allowing the resin to react with a solution of succinic anhydride at pH 6.0 (ref. 13). The N-hydroxysuccinimide mixed anhydride of the succinylated resin was prepared exactly according to Cuatracasas and Parikh[14]. F_1·ATPase was attached to the activated resin[14] by incubating 20 ml solution containing 6.0 mg/ml in 0.1 *M* phosphate pH 7.5, with 20 ml of packed activated resin at room temperature for 2 h. The resulting resin was washed with a large volume of 0.1 *M* Tris–glycine buffer pH 7.5 at room temperature and, subsequently, with a large volume of a buffer containing 0.1 *M* sucrose and 50 m*M* Tris–Cl pH 8.0. The resin was used without further treatment.

ATPase activity was assayed by modified measurement of phosphate release. The washed resin was mixed with about 4 ml of buffer (0.1 *M* sucrose, 50 m*M* Tris–Cl, pH 8.0) for each ml of resin, and suspended using gentle stirring. A small volume of the suspension was rapidly pipetted to a reaction tube equilibrated at 30°. After 15 min of incubation at 30°, the reaction was terminated by addition of cold perchloric acid to a final concentration of 3%. The resultant slurry was filtered and washed with a small volume of water, and the combined filtrate and wash were assayed for inorganic phosphate by the procedure of Sumner[15]. In all cases, equivalent blanks were prepared by adding the perchloric acid prior to addition of resin-bound ATPase.

The ATPase used in these studies was prepared as described by Knowles and Penefsky[16] and protein was determined by a biuret procedure[17]. All chemicals were from common commercial suppliers and were of the highest purity available.

RESULTS

Kinetic properties of enzymes are sensitive probes of structure and stability. Consequently, we used the known kinetic properties of the mitochondrial ATPase to monitor the effect of immobilization on the ATPase molecule. When ATP is used as the substrate, a double reciprocal plot shows that in the absence of activating anions, negative cooperativity is observed, a property of the soluble enzyme under similar assay conditions[18]. Likewise when a saturating amount of an activating anion, such as bicarbonate, is added to the assay medium, the double reciprocal plot becomes linear, and the initial velocity of ATP hydrolysis is more rapid at any given substrate concentration (Fig. 1).

A distinctive feature of the mitochondrial ATPase is that with substrates other than ATP, the observed kinetics yield linear double reciprocal plots[18]. Fig. 2 clearly shows that insolubilized ATPase maintains this property. The double reciprocal plot of ITP hydrolysis rates by the insolubilized ATPase shows a K_m value of approximately 4.5 m*M* and a $V_{max.}$ of 200 nmoles/min/ml gel.

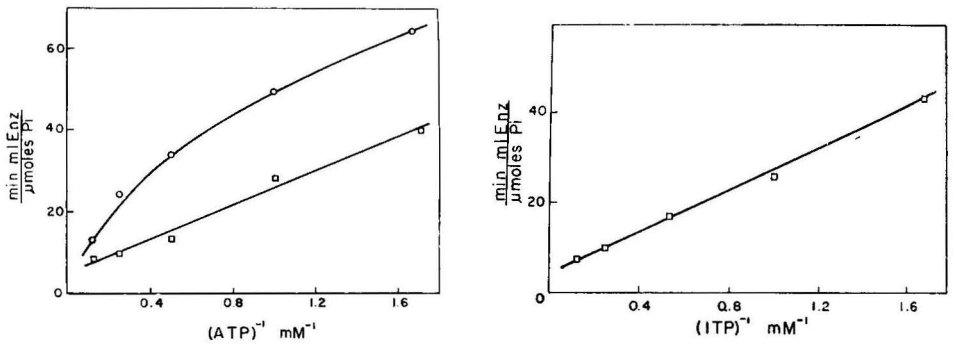


Fig. 1. A double reciprocal plot of the hydrolysis of ATP by immobilized ATPase in the absence (circles) and presence (squares) of 20 m*M* bicarbonate.

Fig. 2. A double reciprocal plot of the hydrolysis of ITP by immobilized ATPase.

It has been recently shown[19] that the substrate specificity of the mitochondrial ATPase is dependent upon the compositions of the assay medium. In purely aqueous medium, the ATPase shows negative cooperativity with respect of ATP concentration, but ITP hydrolysis exhibits no apparent cooperativity. If the medium becomes less polar (*i.e.*, 20% methanol), ATP hydrolysis becomes non-cooperative (and therefore shows enhanced hydrolytic rates) while ITP hydrolysis is inhibited. The data in Table I show that the same phenomenon occurs with the insolubilized mitochondrial ATPase. The insolubilized ATPase shows enhanced ATP hydrolyzing activity in 20% methanol as compared to the aqueous control, while ITP hydrolysis activity is inhibited in the methanolic medium.

TABLE I

EFFECT OF 20% METHANOL ON THE RATES OF IMMOBILIZED ATPASE CATALYZED ATP OR ITP HYDROLYSIS

Substrate	*Concentration of methanol (%)*	*V (nmoles/min/ml)*
ATP (8 m*M*)	0	81
ATP (8 m*M*)	20	126
ATP (1 m*M*)	0	20
ATP (1 m*M*)	20	47
ITP (8 m*M*)	0	130
ITP (8 m*M*)	20	68
ITP (1 m*M*)	0	37
ITP (1 m*M*)	20	20

A feature that distinguishes the soluble and immobilized forms of ATPase is its stability to cold inactivation. In an aqueous buffer system, the immobilized ATPase is more stable at 0° than the soluble enzyme (Fig. 3). In a medium containing 70% glycerol, the enhanced stability of the immobilized ATPase is more dramatic (Fig. 3). Immobilized ATPase was therefore stored routinely in 70% glycerol at 0–4° for 2 to 3 weeks without noticeable loss in activity.

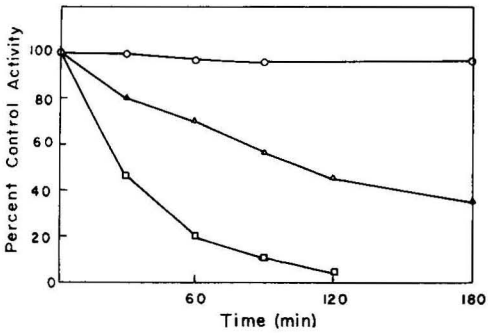


Fig. 3. The effect of storage at 4° on the activity of soluble and immobilized ATPase. The squares represent the activity of soluble ATPase in a completely aqueous medium, the triangles represent the immobilized ATPase in a completely aqueous medium and the circles represent the immobilized ATPase in 70% glycerol.

DISCUSSION

Recently Swanljung and Frigeri[20] demonstrated the feasibility of using the immobilized ATPase inhibitor protein of Pullman and Munroy[21] as an affinity chromatography tool for ATPase isolation. This technique has potential use in problems requiring rapid ATPase isolation, especially from small tissue sources. Conversely, the preparation of a fully functional immobilized ATPase may play a key role in the isolation of the other proteins of the mitochondrial ATP synthetase complex, and in the subsequent elucidation of the nature of the complex itself.

The data presented in this communication clearly demonstrate that under the proper conditions, the mitochondrial ATPase can be covalently linked to Sepharose 4B via a spacer. This preparation is shown to possess all the kinetic properties of the soluble ATPase except that it is more stable in glycerol at low temperatures. Studies are underway to employ the immobilized ATPase as an affinity column for the associated mitochondrial proteins of the ATP synthetase complex.

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Note

Use of the alkali-flame ionization detector for the determination of methomyl residues in plant materials*

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The rubidium sulfate alkali-flame ionization detector (Rb_2SO_4-AFID) is selectively sensitive to nitrogen-containing compounds such as the insecticide methomyl (S-methyl-N-[(methylcarbamoyl)-oxy] thioacetimidate). Methomyl may be quantitatively detected in the nanogram range by gas chromatography and the procedure was used to obtain methomyl residue data in a variety of vegetable crops.

Some investigators have hydrolyzed methomyl to the corresponding oxime (methyl N-hydroxythioacetimidate) with subsequent analysis by either a microcoulometric detector in the sulfur mode or a flame photometric detector with a 394 nm sulfur interference filter and temperature programmed[1–3]. Lee *et al.*[4] used the flame photometric detector with the sulfur filter, under isothermal conditions, but they modified the procedure of Pease and Kirkland[1] by eliminating interfering sulfur compounds in rape plants and seeds prior to analysis. Williams[5] demonstrated that methomyl could be analyzed directly on a microcoulometer gas chromatograph in the nitrogen specific mode. Reeves and Woodham[6] used the flame photometric detector in the isothermal mode and they analyzed for the parent compound in tobacco and environmental samples.

The method of Reeves and Woodham[6] performed satisfactorily for most vegetable crops. However, interference from sulfur compounds was noted in the analysis of Chinese radish greens and roots. The solution to this problem was the use of the rubidium sulfate detector. Reproducible peak heights were obtained on the chromatogram with repeated injections of a standard methomyl solution; intervals of 30 to 40 min after a sample injection may be necessary to retain the original detector sensitivity.

MATERIALS AND METHODS

A Varian Aerograph Model 1400 gas chromatograph originally equipped with a hydrogen flame ionization detector was converted to Rb_2SO_4-AFID operation with a conversion kit available from the manufacturer. The following gas chromatographic conditions were used: 6 ft. (1.82 m) × 2 mm I.D. borosilicate glass column containing

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5% Carbowax 20M on high-performance grade Chromosorb W (80–100 mesh); column temperature 160°, injection port temperature 220°, detector temperature 260°; flow-rates were nitrogen 25 ml/min, hydrogen 35 ml/min and air 220 ml/min. The flow-rates for this detector are critical and must be optimized according to the manufacturer's instructions. A methomyl reference standard was furnished by E. I. du Pont de Nemours (Wilmington, Del., U.S.A.). Standard solutions of methomyl in ethyl acetate contained 1.25–10.0 ng/μl.

Methomyl was extracted from crop samples using the procedure of Fung[3]. The ethyl acetate extract was evaporated to dryness by vacuum evaporation and purified by the coagulation and florisil column cleanup procedure of Reeves and Woodham[6]. The residue was transferred with ethyl acetate to a 5-ml centrifuge tube, the volume was adjusted to 0.5 ml, and a 2-μl aliquot was injected into the gas chromatograph. The amount of methomyl in the aliquot was determined from a calibration curve prepared on the day of analysis by injections of 2.5, 5, 10 and 20 ng of methomyl.

Representative gas chromatograms in Fig. 1 illustrate the results of the analysis of methomyl residues from 50-g Chinese radish samples. Recovery of methomyl from untreated control samples fortified with methomyl at the 0.1 ppm level averaged 72%. The limit of detectability was 0.02 ppm.

The method has also been applied to leafy vegetables and cucurbits; chromatograms of these samples exhibited some crop background peaks, but these peaks did not interfere with the resolution of methomyl.

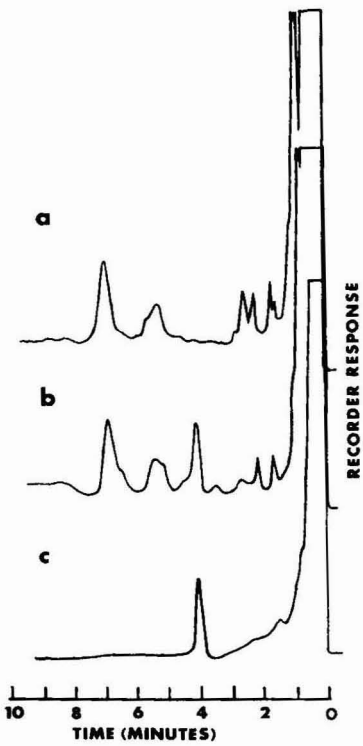


Fig. 1. Chromatograms of (a) untreated Chinese radish; (b) untreated Chinese radish fortified with 0.1 ppm methomyl; (c) methomyl standard, 10 ng.

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Note

Gas chromatographic analysis of linear polyamides and copolyamides

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While the acidic hydrolysis of certain polyamides has been the subject of several reports[1–4] of satisfactory analytical procedures for the constituent diamine, prolonged reaction periods or elevated pressures are necessary in order to carry the sluggish reaction stowards completion.

Anton[1] effected the hydrolysis of nylon samples resulting from diamine-dicarboxylic acids by refluxing for 24 h with 6 *N* hydrocholic acid at atmospheric pressure or for 4 h at 40 p.s.i. The hydrolysate was divided and the diamines were extracted with *n*-butanol from alkaline solution for examination. The methyl esters of the dicarboxylic acids were prepared with methanolic boron trifluoride and chromatographed. Nylon 6, yielding ω-aminocaproic acid, was not resolved whereas with the column conditions used nylon 6,10 and nylon 6,T could not be identified due to coincident peaks of dimethyl sebacate and dimethyl terephthalate.

Mori *et al.*[2] employed the same acid hydrolysis but at 130° with an additional derivative step to allow separation fo the products. Nylon 6 required 2 h, nylon 6,6 4 h and nylon 11 and 12 8 h of digestion to effect cleavage. The hydrolysis products were esterified with hydrochloric acid and methanol and then subjected to reaction with trifluoroacetyl anhydride.

The same workers[3] later reported a modified procedure involving a single derivative step. The method involved trimethylsilation of the diamine hydrochlorides, the dibasic acids and the ω-aminoalkanoic acid hydrochlorides from the hydrolysate. The dried products were directly silylated with bis(trimethylsilyl)acetamine (BSA) and chromatographed. The effect of hydrochloric acid on the TMS derivative formation was eliminated by the addition of triethylamine to the reaction products. An essentially identical procedure using hydrochloric acid and TMS derivative formation was reported at about the same time by Tengler[4].

The degradation of polyamides and polyimides using hydrazine has been reported by Mlejnek and Cveckova[5] and earlier with polyimides by Dine-Hart and Wright[6,7], but the procedure, which involves a prolonged (8-h) pre-treatment with hydrochloric acid, is of little utility as it is less attractive than the method of Anton[1].

Frankoski and Siggia first applied alkali fusion to esters that are difficult to saponify[8] and later to amides, anilides and ureas[9]. The fusion involves heating solid

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alkali with the sample in molar ratios of 50:1 or greater at 360° for 0.5 h for nylon 6,6 and 6,10, after which the liberated diamine is determined by gas chromatography. A review by Whitlock and Siggia[10] discusses fusion reaction gas chromatography generally and indicates briefly the analysis of the diamines from polyimides, poly (amideimides) and aromatic polyamides.

Elimination of the prolonged digestion is an obvious advantage of alkali fusion although only the amino component is determined. In the work of Siggia and co-workers[8–10] a reaction chamber constructed from a pyrolyser is used, which prevents the use of the gas chromatograph during the fusion.

This paper reports a rapid method for the analysis of diacid-diamine and ω-aminoalkanoic acid polyamides by the use of alkali fusion with analysis of the diamine and the methyl esters of the dicarboxylic or the ω-aminoalkanoic acids in about 45 min.

EXPERIMENTAL

Gas chromatography

The analyses were performed on a Hewlett-Packard Model 5750B research chromatograph fitted with column splitters and multi-columns in parallel with flame-ionization detection. Signals were recorded on a multi-channel recorder or an on-line computer processor.

The stainless-steel columns (2 m $\times$ 6.35 mm I.D. were maintained isothermally at 200° and were packed with 5% diethylene glycol adipate on Chromosorb W AW DMCS for the esters and 1% Apiezon L on 60–80-mesh glass beads for the diamines.

Alkali fusion

The fusion was carried out using 0.1 g of the polymer with 1 g of the potassium hydroxide–sodium acetate reagent prepared according to the work of Frankoski and Siggia[8,9]. The reaction was carried out in 9 mm O.D. borosilicate glass tubes that were sealed under reduced pressure. Four identical tubes could be prepared and heated in a cylindrical block of stainless steel containing four holes of appropriate diameter. The block was heated with resistance heaters, the input being suitably regulated to give a temperature of 300°. After the reaction, the tubes were removed from the heater, allowed to attain room temperature and then refrigerated to 0° before opening to prevent loss of the volatile amine.

On cooling, water was added to the reaction product and the diamine extracted with two 30-ml portions of *n*-butanol. For the diamine analyses the volume was reduced to 5 ml and 1 μl was taken for chromatography. For the dicarboxylic acid analysis the aqueous solution was acidified with dilute hydrocholic acid and evaporated to dryness. The free acids were mixed with 10 ml of boron trifluoride–methanol reagent and heated to boiling, while aromatic diacids required refluxing for 1 h. The resulting solution of the diesters was transferred to a separating funnel containing 30 ml of water and then extracted twice with toluene. The toluene was dried over anhydrous magnesium sulphate and the volume reduced to approximately 5 ml and transferred into a volumetric flask prior to gas chromatography.

RESULTS AND DISCUSSION

Analyses were carried out on four polyamide samples produced by diamine-dicarboxylic acid condensation. The samples of nylon 6,6, 6,9, 6,10 and 6,12, which are condensation products of 1,6-diaminohexane with hexanedioc acid (adipic acid), nonanedioc acid (azelaic acid), decanedioic acid (sebacic acid) and dodecanedioic acid, respectively, were examined after fusion for 30 min.

Essentially quantitative conversions were achieved; the yields of polymer based on dimethyl ester and diamine recoveries for a series of analyses are shown in Tables I and II, respectively, together with the errors involved. The values each refer to the starting polymer calculated to 0.05 g. The chromatogram of methyl esters and of the recovery from nylon 6,9 is shown in Fig. 1, while Fig. 2 shows the separation of the 1,6-diaminohexane from nylon 6,6.

TABLE I

QUANTITATIVE DETERMINATION OF METHYL ESTERS OF DICARBOXYLIC ESTERS

Nylon	*Weight of polymer (g)*					*Error (%)*
	1	*2*	*3*	*4*	*Average*	
6,6	0.0487	0.0490	0.0485	0.0498	0.0490	−2
6,9	0.0491	0.0495	0.0496	0.0498	0.0495	−1
6,10	0.0485	0.0491	0.0487	0.0487	0.0488	−2.4
6,12	0.0480	0.0490	0.0487	0.0487	0.0486	−2.8

TABLE II

QUANTITATIVE DETERMINATION OF 1,6-HEXANEDIAMINE IN NYLON SAMPLES

Nylon	*Weight of polymer (g)*					*Error (%)*
	1	*2*	*3*	*4*	*Average*	
6,6	0.0488	0.0490	0.0489	0.0491	0.04895	2.1
6,9	0.0490	0.0492	0.0492	0.0490	0.0491	1.87
6,10	0.0491	0.0493	0.0485	0.0487	0.0489	2.21
6,12	0.0490	0.0489	0.0493	0.0490	0.04906	1.88

Analyses of copolyamides of nylons 6,6/6,10 and 6,9/6,12 were examined and compositions on the basis of methyl esters are shown in Table III.

Nylon 6, T, the condensation product of 1,6-diaminohexane and terephthalic acid, was readily cleaved, but the reaction was incomplete after 0.5 h of heating and 1.0 h was employed, after which time increased yields of the components were not obtained. The retention times of dimethyl terephthalate and dimethyl sebacate are very similar on the column used but are experienced together in polyesters and are capable of separation, as demonstrated by Jankowski and Garner[11], who separated the aliphatic diester and the three isomeric phthalate esters with a Ucon 50HB 280X column. The aromatic character is not common with the other simple nylons and would usually be obvious from the infrared spectrum and possibly from the reduced rate of the fusion reaction.

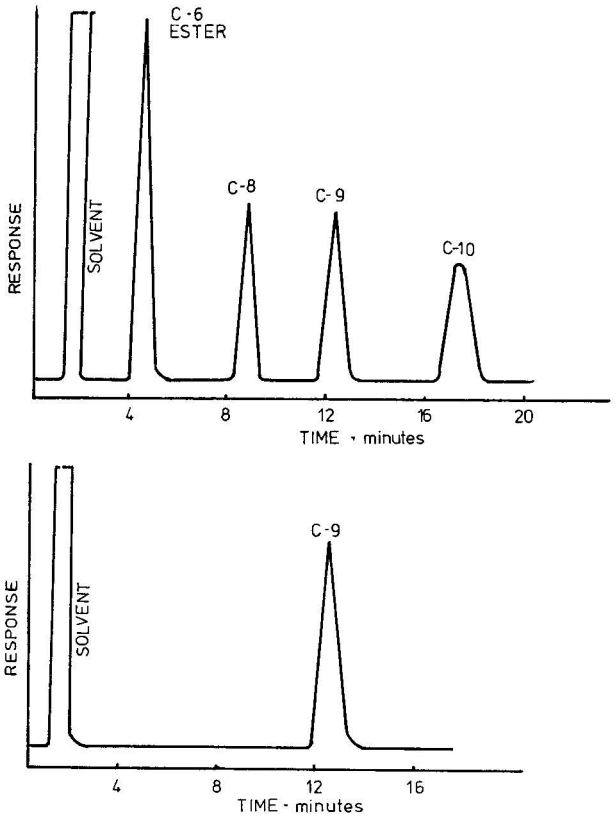


Fig. 1. Chromatograms showing separation of the methyl esters of aliphatic dicarboxylic acids and dimethyl nonanedienoate recovered from nylon 6,9.

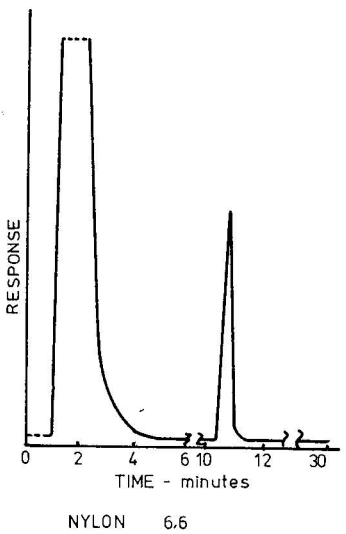


Fig. 2. Chromatogram showing 1,6-diaminohexane recovered from nylon 6,6.

TABLE III

ANALYSIS OF COPOLYAMIDES

Copolyamide	*Composition (%)*			*Error (%)*
	Actual	*Analysis*	*Average*	
Nylon 6,6/6,10	60:40	61.4:38.6; 61.2:38.8	61.3:38.7	2.16
Nylon 6,9/6,12	60:40	60.2:39.8; 61.1:38.9	60.66:39.34	1.1

Analyses of ω-aminoalkanoic acids as the methyl esters were carried out on nylon 6 (ω-aminocaproic acid), nylon 11 (ω-amino-*n*-undecanoic acid) and nylon 12 (ω-amino-*n*-dodecanoic acid) with the results as shown in Table IV.

The polyamide samples examined indicate an error in recovery of about 2% with diamine-dicarboxylic acid, ω-aminoalkanoic acid types and with several copolyamides. The losses are believed to be associated primarily with the liquid–liquid extractions that are required. Preliminary examination of products with amines and acids of high functionality and of greater molecular weight has indicated the successful use of alkali fusion, the acids being examined as the esters, while chromatography of the amines is facilitated by derivative formation, as has been used in earlier work on simpler products and in recent work by Schlueter and Siggia[12].

TABLE IV

QUANTITATIVE DETERMINATION OF METHYL ω-AMINOALKANOATES

Nylon	*Weight of polymer (g)*					*Error (%)*
	1	*2*	*3*	*4*	*Average*	
6	0.0491	0.0488	0.0490	0.0495	0.0491	1.8
11	0.048	0.0492	0.0493	0.0491	0.0489	2.2
12	0.0492	0.0489	0.0491	0.0488	0.0490	2.0

The determination is conducted without restricting the use of the chromatograph or requiring the cumbersome reactor of Siggia and co-workers. The procedure is rapid and specific and allows a complete analysis, as reported by Anton[1] in 4–24 h, to be achieved in essentially the same time as the diamine analysis of Siggia and co-workers, *i.e.*, 1–2 h, for both diamine-dicarboxylic acid and ω-aminoalkanoic acid types of polyamides.

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Note

Separation of benzyl compounds by gas–liquid chromatography

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(First received April 25th, 1977; revised manuscript received April 4th, 1978)

The gas chromatography of benzyl compounds has not been studied quantitatively, although some have been included in studies of various aromatic compounds[1–3]. Hishta and Bonstein[4] separated mono-, di- and tribenzylamines using a Carbowax 1500 column. The present investigation was concerned with the quantitative separation of benzyl compounds on nine different liquid stationary phases, and a general equation relating the specific retention volumes with the reciprocal of the column temperature was derived.

EXPERIMENTAL

Apparatus

A Beckman GC-45 gas chromatograph equipped with a flame-ionization detector was used, together with a Beckman 10 recorder with a 1-mV capacity. Nitrogen was used as the carrier gas at an optimal flow-rate of 30 ml/min at N.T.P. Stainless-steel columns (6 ft. $\times \frac{1}{4}$ in. O.D.) were used. Each column was packed with 20% (w/w) of stationary phase on 80–100-mesh Chromosorb W. The column temperature was controlled to within $\pm 0.08°$.

Materials

All of the benzyl compounds were obtained from Fluka (Buchs, Switzerland) in the purest available grades. The purities were checked.

Sampling

A mixture containing 0.25 g of each benzyl compound was prepared. The sample sizes ranged from 0.2 to 0.4 μl and injections were made with a 1-μl Hamilton syringe. Each injection for an individual compound or mixture was repeated twice and the results were reproducible to within $\pm 1\%$.

RESULTS AND DISCUSSION

The retention data for benzyl compounds at different column temperatures (90–180°) were expressed in the form of specific retention volumes[5,6], V_g^0, and these values were further processed by linear regression so as to obtain the constants a and b of the equation

$$\log V_g^0 = (a/T) - b \qquad (1)$$

TABLE I

CONSTANTS a AND b IN EQN. 1 FOR BENZYL COMPOUNDS ON POLYSILOXANE LIQUID STATIONARY PHASES

Compound	*OV-1*		*OV-3*		*OV-11*		*OV-25*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Benzyl chloride	1949.92	3.248	2249.23	3.793	2278.47	3.724	2306.99	3.944
Benzylamine	2117.23	3.653	2249.23	3.793	2450.06	4.143	2434.36	4.162
Benzyl alcohol	2117.23	3.653	2353.13	4.015	2404.53	3.969	2434.36	4.162
Benzyl acetate	2281.27	3.863	2573.58	4.339	2645.48	4.325	2706.70	4.613
Benzyl methyl ketone	2281.27	3.863	2580.40	4.303	2759.69	4.561	2807.29	4.825
Benzyl cyanide	2453.83	4.229	2711.73	4.623	2730.27	4.437	2817.84	4.775
Dibenzyl ether	2802.05	4.067	3064.03	4.396	3383.31	4.896	3345.13	4.975
Dibenzylamine	2856.07	4.077	3176.45	4.517	3364.47	4.712	3313.39	4.766
N-Benzylaniline	2917.45	4.204	3176.45	4.517	3433.52	4.824	3439.17	4.979
Benzyl phenyl ketone	2984.69	4.291	3186.33	4.513	3459.83	4.841	3473.02	4.999
Benzyl benzoate	2984.69	4.291	3229.21	4.554	3548.39	5.012	3473.39	5.000
Benzyl salicylate	3142.49	4.470	3335.38	4.610	3383.09	4.464	3415.37	4.717

where T is the absolute temperature. The results are given in Tables I and II. These values of a and b allowed a satisfactory pre-calculation of the specific retention volumes. Further, the heats of solution, ΔH, were calculated from the expression[7]

$$a = d \log V_g^0 / d\left(\frac{1}{T}\right) = -\Delta H / 2.303\ R \qquad (2)$$

where R is the gas constant. The values of ΔH obtained are given in Table III. The values of ΔH on polysiloxanes and polyester liquid stationary phases do not differ appreciably with increases in the percentage of phenyl groups in the former and the

TABLE II

CONSTANTS a AND b IN EQN. 1 FOR BENZYL COMPOUNDS ON POLYESTER AND CARBOWAX 6000 LIQUID STATIONARY PHASES

Compound	*DEGS*		*DEGGLU*		*DEGA*		*EGTCP*		*Carbowax 6000*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
Benzyl chloride	2204.39	4.046	9313.29	4.040	2279.82	4.002	1931.84	2.726	1240.01	2.125
Benzylamine	2890.75	5.105	2876.27	4.983	2901.94	5.103	2606.35	3.980	1943.95	3.572
Benzyl alcohol	2850.43	5.204	2800.93	4.756	2761.00	4.718	2606.35	3.980	2158.35	4.113
Benzyl acetate	2769.07	4.960	2949.19	4.980	2976.37	5.112	2606.35	3.980	2527.61	4.900
Benzyl methyl ketone	2883.01	4.918	2956.72	4.835	2966.65	4.935	2832.99	4.247	2867.00	5.659
Benzyl cyanide										
Dibenzyl ether	3006.22	4.633	3222.80	4.822	3245.89	4.916	4860.28	8.179	2866.71	5.113
Dibenzylamine	3369.41	4.966	3521.51	5.127	3453.37	5.016	5344.62	8.882	3389.76	5.991
N-Benzylaniline	3369.41	4.966	3367.04	4.551	3614.06	5.156			3574.61	6.315
Benzyl phenyl ketone	3315.06	4.959							3574.61	6.315
Benzyl benzoate	3410.08	4.942							3786.29	6.717
Benzyl salicylate										

TABLE III

HEATS OF SOLUTION ($-\Delta H$) OF BENZYL COMPOUNDS (kcal/mole) IN DIFFERENT LIQUID STATIONARY PHASES

Compound	*OV-1*	*OV-3*	*OV-11*	*OV-25*	*DEGS*	*DEGGLU*	*DEGA*	*EGTCP*	*Carbowax 6000*
Benzyl chloride	10.052	10.256	10.390	10.520	10.052	10.549	10.396	8.809	5.654
Benzylamine	13.182	10.256	11.172	11.101	13.182	13.116	13.233	11.885	8.864
Benzyl alcohol	12.998	10.730	10.965	11.101	12.998	12.772	12.590	11.885	9.842
Benzyl acetate	12.627	11.736	12.063	12.343	12.343	13.448	13.572	11.885	11.526
Benzyl methyl ketone	13.147	11.767	12.584	12.801	13.147	13.483	13.528	12.918	13.074
Benzyl cyanide		12.365	12.450	12.849					
Dibenzyl ether	13.708	13.972	15.428	15.254	13.708	14.696	14.801	22.163	13.072
Dibenzylamine	15.365	14.485	15.342	15.109	15.365	16.058	15.747	24.371	15.457
N-Benzylaniline	15.365	14.485	15.657	15.683	15.365	15.354	16.480		16.300
Benzyl phenyl ketone	15.117	14.530	15.777	15.837	15.117				16.300
Benzyl benzoate	15.550	14.725	16.181	15.839	15.550				17.266
Benzyl salicylate		15.209	15.427	15.574					

number of CH_2 groups in the latter, but there are appreciable variations in the ΔH values of benzyl compounds that contain two phenyl rings. On Carbowax 6000, the variation in ΔH values ranged between 5 and 17 kcal/mole, depending on the structure of the benzyl molecules and whether these molecules are able to reduce the internal association of this liquid phase[7].

The results in Tables I and II indicate that the best separation was achieved with OV-11 and DEGGLU as liquid stationary phases.

ACKNOWLEDGEMENTS

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Note

Comparison of Bentone and liquid-crystal stationary phases for the gas chromatographic separation of isomers

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(Received March 22nd, 1978)

The usual stationary phases employed in gas–liquid chromatography (GLC) discriminate between samples on the basis of their boiling points (non-polar phases) or on differences in the degree of solute–stationary phase interactions (polar phases). It is therefore very difficult to separate positional isomers having similar boiling points and only very small differences in polarity. Bentone is a selective stationary phase owing its selectivity to the layered structure of the clay, molecules having structures which can easily be accommodated in the layers being readily absorbed. Elution of sample materials is difficult from untreated Bentones and it is customary to sacrifice some selectivity for ease of elution, swelling the clay by the incorporation of a conventional stationary phase.

Liquid crystals are substances which are intermediate between crystalline solids and isotropic liquids and possess sharp transition temperatures between mesophases. Linear molecules such as 4,4′-azoxyanisole will pass from a solid into a nematic mesophase where the ordered structure is retained in the liquid state. They will therefore behave towards solutes in a manner similar to Bentone 34, retaining linear molecules rather than the non-linear isomers. Liquid crystals can therefore be of great use for the separation of benzene positional isomers and for other materials differing mainly in molecular geometry. Kelker and Von Schivizhoffen[1] have extensively reviewed the application of liquid crystals to gas chromatography. Relatively strong deviations of activity coefficients from unity for *p*-substituted compounds on *p*-substituted mesophases have been observed leading to excellent selectivity for these isomers. Limitations of liquid-crystal phase selectivity lie in the fact that the anisotropy of the phase is at a maximum just above the crystal–nematic mesophase transition and decreases, with consequent decrease in isomer selectivity, with increasing temperature.

In order to compare the behaviour of this class of selective stationary phases the readily available liquid-crystal materials 4,4′-azoxyanisole (crystal–nematic 118°), 4,4′-azoxyphenetole (138°) and the eutectic mixture consisting of 57.5% of the former and 42.5% of the latter compound (98°) will be used as stationary phase 2° above their melting points. Comparison with a strictly non-polar conventional phase, Apiezon MH[2], and with a selective Bentone 34–Apiezone MH phase will be made in

terms of the Kovats retention indices[3] of the isomers of xylene, chlorotoluene and methylanisole in order to determine whether the disadvantage of a very narrow temperature range is more than offset by improved resolution by liquid crystals over Bentone phases.

EXPERIMENTAL

The GLC apparatus used was a Pye Series 104 gas chromatograph, dual column, employing katharometer detectors. All retention times were measured using a stop-watch, dead times were obtained by injection of air samples. The columns were 2 m × ¼ in. O.D. copper packed with 15% coatings of stationary phase on Chromosorb W AW DMCS (80–100 mesh) support. The five stationary phases employed were (i) Apiezon MH, (ii) Bentone 34–Apiezon MH (1:1), (iii) 4,4′-azoxyanisole, (iv) 4,4′-azoxyphenetole and (v) the eutectic mixture azoxyanisole–azoxyphenetole (57.5:42.5). The carrier gas was hydrogen with a flow-rate of 50 ml/min. Column temperatures in the range 100–160° were used with Apiezon MH and Bentone columns. Single column temperatures of 140, 120 and 100° were used for determinations of retention-index values of disubstituted benzenes on 4,4′-azoxyphenetole, 4,4′-azoxyanisole and the eutectic mixture, respectively.

RESULTS AND DISCUSSION

Differences in the Kovats retention index, I, are a measure of the separating power of a stationary phase in that if two substances have identical I values at a certain temperature, it will be impossible to separate them at that temperature, using the stationary phase, independent of the length of the column. The xylene results in Table I for Apiezon MH for instance, reveal that this stationary phase will not separate *m*- and *p*-isomers which will be eluted together before the *o*-isomer at any column temperature in the range 100–140°. This is a classical separation problem and one of the first to which Bentone 34 was applied as a selective stationary phase[4–7]. In a more

TABLE I

RETENTION INDICES, I, OF XYLENE, CHLOROTOLUENE AND METHYLANISOLE ISOMERS

Stationary phase	*Temperature (°C)*	*I*								
		Xylene			*Chlorotoluene*			*Methylanisole*		
		o	*m*	*p*	*o*	*m*	*p*	*o*	*m*	*p*
Apiezon MH	100	911	888	886	983	985	986	1007	1022	1023
	120	917	893	891	987	989	990	1010	1024	1025
	140	923	898	896	991	993	994	1014	1027	1028
Bentone 34 / Apiezon MH	100	966	951	935	1073	1074	1070	1157	1200	1163
	120	974	958	943	1077	1078	1074	1163	1216	1168
	140	982	965	952	1080	1081	1077	1169	1235	1173
Liquid crystal eutectic	100	1107	1075	1085	1181	1205	1225	1228	1292	1316
4,4′-Azoxyanisole	120	1140	1106	1107	1227	1251	1263	1313	1316	1370
4,4′-Azoxyphenetole	140	1086	1032	1053	1166	1166	1183	1243	1297	1308

recent study of this separation problem the best xylene separations were claimed on a column containing 7.5% Bentone 34 and 20% squalane[8]. Although a conventional stationary phase is necessary to swell the Bentone 34 it is known that some selectivity is lost, the magnitude of the loss being related to the amount of conventional phase. In this work the Bentone column contained 7.5% of this material swollen with an equal weight of Apiezone MH paraffinic grease so the selectivity could be expected to be high. The results in Table I show that a sufficiently long column packed with this material will resolve the xylene isomers, their elution order being *p*, *m*, *o*. The results on the liquid-crystal phases are quite different: *p*-azoxyphenetole will separate *m*- and *p*-isomers quite well whereas *p*-azoxyanisole gives no separation. The behaviour of the eutectic mixture is intermediate as might be expected. Ignoring temperature differences, the index values for the *m*- and *p*-xylenes on the eutectic are 1075 and 1084, respectively, calculated on the eutectic composition. This is very good agreement with the 1075 and 1085 values determined experimentally at 100°, the results showing that the eutectic mixture is a suitable stationary phase for this separation. The order of elution now being *m*, *p*, *o*. Kelker[9] has described an excellent separation of benzene, toluene, ethylbenzene and the xylene isomers on 4,4′-azoxyphenetole.

Table I also shows the relative performances of the stationary phases for the separation of chlorotoluenes. Whereas Apiezon MH is quite unable to separate the *m*- and *p*-isomers (both have b.p. 162°) and a separation of the *o*-isomer is virtually impossible, the Bentone-column results indicate that a *m*/*p* separation would be extremely difficult and an *o*/*m* separation impossible on this phase. In this case liquid-crystal phases prove to be the solution to this difficult problem. Although 4,4′-azoxyphenetole will not resolve *o*- and *m*-chlorotoluenes, both 4,4′-azoxyanisole and the eutectic mixture are capable of complete isomer resolution, the elution order being *o*, *m*, *p*. Chiavari[10] has previously reported the use of 2,6-naphthylene-bis(*p*-alkoxybenzoate) as a liquid-crystal phase suitable for the separation of *m*- and *p*-chlorotoluenes.

In Table I the methyl anisole data shows that the *o*-isomer will be eluted first and the *m*- and *p*-isomers will not be resolved by the Apiezon MH phase. Great difficulty in separating the *o*- and *p*-isomers on Bentone would be experienced with these isomers being eluted before the *m*-isomer. The behaviour of methylanisoles with liquid-crystal phases is quite different, the order of elution being *o*, *m*, *p*, with a relatively simple separation of the *o*-isomer and a more difficult separation of the *m*/*p* pair. Chiavari also investigated the behaviour of methylanisoles and decided that the 2,6-naphthylene-bis(*p*-alkoxybenzoate) phase was suitable for this isomer separation.

It would seem then that in terms of separating power, as measured by the differences in retention index values, liquid crystal phases such as those investigated here are marginally superior to Bentone phases. Their limitation is given by restriction to working temperatures a few degrees above their melting points where the anisotropy and consequently the selective retention of isomers is at a maximum. Whereas Bentone 34 will function from ambient temperatures to 180°, a range of liquid crystals of varying crystal–nematic mesophase transition temperatures are required to span the range. Such a range of materials are available, the eutectic mixture and the two azoxyethers described here span the range 100–140°. The 4-methoxycinnamic ester

of 4-hydroxy-4′-methoxy-azobenzene, with a transition temperature of 163° could be used at a column temperature of 165° and the terephthaldialdehyde-bis(4-ethoxyphenylimine) transition temperature of 196° would be useful at 200°, thereby spanning the general column-temperature working range in GLC.

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Note

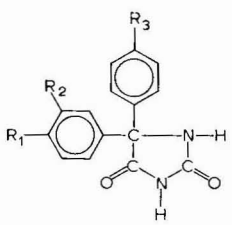
Identification and estimation of phenytoin and its major metabolite in rat brain following its administration by gas–liquid chromatography and gas–liquid chromatography–mass spectrometry

K. K. MIDHA*, C. CHARETTE, H. S. BUTTAR and I. DUPUIS

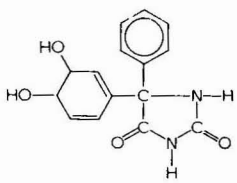
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Phenytoin (I, DPH), a commonly prescribed anticonvulsant drug[1], undergoes biotransformation to several metabolites (II–VII) in various species. These meta-



I–VI, VIII



VII

Compound	R_1	R_2	R_3	*Name*
I	H	H	H	5,5-(diphenyl)-hydantoin
II	OH	H	H	5-(*p*-hydroxyphenyl)-5-phenylhydantoin
III	H	OH	H	5-(*m*-hydroxyphenyl)-5-phenylhydantoin
IV	OH	OH	H	5-(3,4-dihydroxyphenyl)-5-phenylhydantoin
V	OCH_3	OH	H	5-(4-methoxy-3-hydroxyphenyl)-5-phenylhydantoin
VI	OH	H	OH	5-5-bis-(4-hydroxyphenyl)-5-hydantoin
VII				5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin
VIII	CH_3	H	H	5-(*p*-methylphenyl)-5-phenylhydantoin

bolites have been identified in urine, plasma, bile and liver[2–8]. The presence of I in brain has been reported as an evidence for its central anticonvulsant action[9]. In this communication, we report identification and quantitation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (II, *p*-HPPH) and I in rat brain following intravenous administration of I by gas–liquid chromatography (GLC) and GLC–mass spectrometry (MS).

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EXPERIMENTAL

Instrumentation

For GLC–MS analysis, a Hewlett Packard 5985 GC–MS data system was used under the following conditions: 70 eV as the ionization beam energy and the source was maintained at 150°. The chromatographic column was a coiled-glass tubing 1.22 m × 2 mm I.D. packed with 3% OV-7 on Chromosorb W (80–100 mesh; Chromatographic Specialties, Brockville, Canada). The injection port, the column and the interphase temperatures were 300, 215 and 250°, respectively. The carrier gas was helium with a 26 ml/min flow-rate. Aliquots (2–4 μl) were chromatographed.

For GLC analysis a Tracor 550 gas chromatograph (Canlab, Montreal, Canada) with a flame-ionization detector was used. The 1.83 m × 4 mm I.D. coiled-glass column was packed with 5% OV-7 on Chromosorb W (100–120 mesh; Chromatographic Specialties). The column temperature was 240°, the injection port and the detector temperatures were 310°, and the carrier gas was nitrogen at a flow-rate of 50 ml/min. Hydrogen and air flow-rates were were adjusted for optimal sensitivity.

Standards and reagents

Stock solutions containing 400 μg/ml of I were prepared by dissolving appropriate amounts of the sodium salt (Parke, Davis and Co., Brockville, Canada) in distilled water. Stock solutions containing 100 μg/ml of the metabolite II (Aldrich, Milwaukee, Wisc., U.S.A.) were prepared by dissolving the appropriate amount in 0.01 *N* NaOH. Appropriate dilutions of the drug (20–100 μg/ml) and the metabolite (2–24 μg/ml) were prepared as required.

Aqueous solutions (400 μg/ml) of the internal standard VIII, 5-(*p*-methylphenyl)-5-phenylhydantoin (Aldrich) were prepared by dissolving an appropriate amount of the internal standard in 0.01 *N* NaOH and diluting to the appropriate concentration with distilled water before use. Methanolic trimethylanilinium hydroxide was synthesized according to the method of Barrett[10].

Diethylether (Mallinckrodt, Montreal, Canada) was glass-distilled prior to use. All the other chemicals were of analytical grade.

Animals

Female Wistar rats (184 ± 10 g) were treated with Dilantin (100 mg/kg; i.v.) and killed 5 h after treatment. The brains were immediately removed; a 10% homogenate solution was made with citrate buffer (pH 7.3) and then stored at −20° until assay.

Extraction procedure

A modified literature procedure[11] was employed. To 4-ml samples of rat brain homogenate in screw-capped centrifuge tubes (20 ml) were added 1 ml of the internal standard (52 μg/ml), 1 ml distilled water (for calibration curves, the distilled water contained I and II) and 3 ml of 1 *N* HCl. The samples were then extracted twice with 7-ml portions of diethyl ether by shaking (Roto-Rack, Fisher Scientific, Montreal, Canada) for 10 min at 30 rpm followed by centrifugation at 600 *g* for 10 min.

The organic extracts were transferred into a tube (20 ml) containing 5 ml of 0.2 *M* phosphate buffer pH 11.2. The tubes were mixed for 10 min followed by cen-

trifugation for 10 min, after which the organic extract was discarded. A 4-ml portion of the aqueous phosphate solution was transferred to another tube (20 ml), acidified with 2 ml of 2 *N* HCl and extracted twice with 5-ml portions of diethyl ether (mixed 10 min, centrifuged 10 min).

Four ml of the first extract and 5 ml of the second extract were transferred into an evaporating tube (Canlab) and the combined ether extracts were evaporated to dryness at 40° under a stream of dry nitrogen. The dried extracts were dissolved by mixing (Vortex Genie Mixer, Fisher Scientific) with 100 μl of methanolic trimethylanilinium hydroxide (0.2 *M*) and aliquots (2–4 μl) were injected into the GLC–MS system or the GLC.

Calibration curves

Peak height ratios were calculated by dividing the height of the peak from the drug or the metabolite by the height of the internal standard. Calibration curves were assembled from the results of spiked control brain homogenate by plotting the peak height ratios against the concentrations of the drug or the metabolite. The recovery of I and II from brain homogenate was similar to that reported earlier from this laboratory[11].

RESULTS AND DISCUSSION

Identification of phenytoin and its possible metabolites in rat brain was carried out by GLC–MS using the selected-ion-monitoring technique. It has been demonstrated[8,11] that flash-heater methylation with trimethylanilinium hydroxide converts compounds I–V into their respective di-, tri- or tetramethylated derivatives which are separated by GLC. These derivatives give molecular ions when subjected to MS analysis in the electron impact mode. Therefore, in the present study, aliquots of extracts of brain homogenates in trimethylanilinium hydroxide were injected and ions at *m/e* 280, 310 and 340 were monitored for the presence of I, II, III, IV and V.

Fig. 1 shows the presence of signals only at *m/e* 280 and 310 but not at *m/e* 340. The peak at *m/e* 280 had the same retention time as the methylated synthetic sample of I, and the peak at *m/e* 310 had the same retention time as the methylated synthetic sample of II (whereas methylated III had a shorter retention time).

The complete mass spectra (Fig. 2) of peaks eluting at retention times 3.16 and 7.9 min, respectively were identical to the mass spectra of methylated synthetic I and II, respectively. This established the presence of I and II in rat brain following the administration of I to rats. Quantitative estimation of I and II in rat brain was accomplished by GLC.

Blank and spiked rat brain homogenates were extracted and injected into the GLC as described previously. The typical chromatogram obtained by processing control blank brain homogenate showed extraneous peaks at 1.7 and 3.9 min, respectively. However, these peaks did not interfere with peaks due to the flash-heater methylated derivatives of I (retention time, t_R, 4.7 min), internal standard VIII (t_R 6.2 min) and II (t_R 10.0 min). The amounts of I and II in unknown samples were estimated from calibration curves assembled from the results of spiked control brain homogenates.

In the six female Wistar rats examined, the concentration of phenytoin (I) and

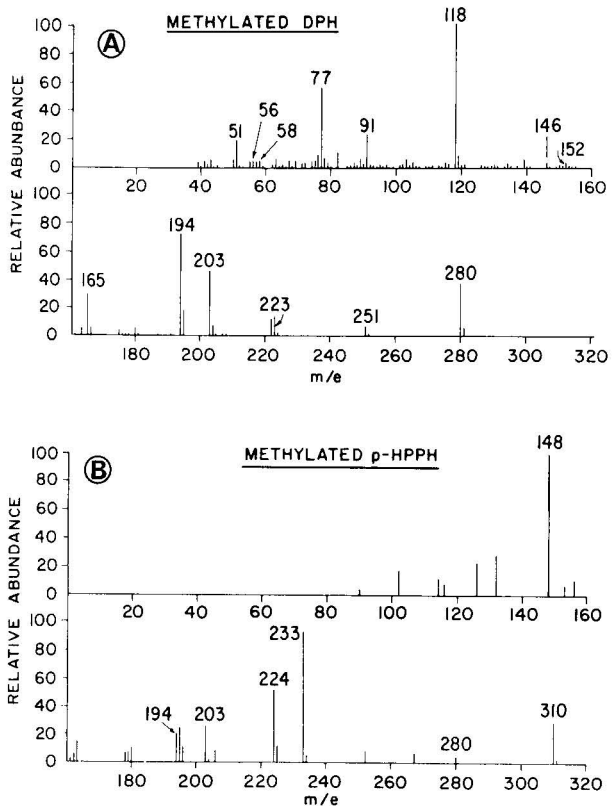


Fig. 1. Chromatogram (A) of a trace from selected-ion-monitoring technique and (B) of total ion from flash-heater methylated extract of rat brain homogenate injected into GC–MS.

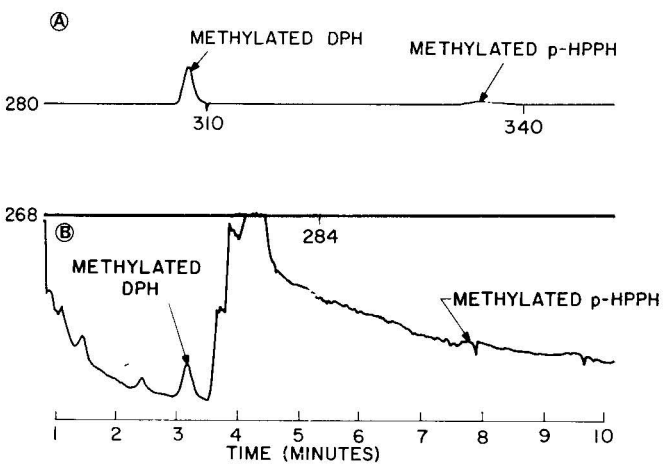


Fig. 2. Mass spectra of flash-heater methylated phenytoin I (A) and metabolite II (B) from rat brain homogenate extract.

TABLE I

CONCENTRATIONS OF PHENYTOIN I AND ITS METABOLITE 5-(*p*-HYDROXYPHENYL)-5-PHENYLHYDANTOIN II IN RAT BRAIN FOLLOWING INTRAVENOUS ADMINISTRATION OF DILANTIN 100 mg/kg

Animal	*Injected (mg)*	*Amount of I in brain (μg/g)*	*Amount of II in brain (μg/g)*
1	20.25	27.28	0.85
2	18.25	26.49	0.72
3	18.25	32.88	0.75
4	17.25	28.83	0.75
5	18.00	29.95	0.90
6	18.50	29.02	1.00
Mean	18.42	29.08	0.83
S.D.	± 1.00	± 2.25	± 0.11
Coefficient of variation (%)	± 5.41	± 7.72	± 13.10

the metabolite II in the brain were estimated to 29.08 ± 2.25 μg/g and 0.83 ± 0.11 μg/g (Table I) at 5 h following the administration of Dilantin at 100 mg/kg.

In summary, the presence of phenytoin and its metabolite II, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, in rat brain following administration of phenytoin has been demonstrated by GLC–MS using selected-ion-monitoring technique. A GLC procedure is described for estimation of phenytoin and its metabolite II in rat brain.

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Note

Improved assay for the rapid determination of bis(*p*-chlorophenyl)acetic acid in human urine

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For the past decade, the potential health hazard of persistent chlorinated hydrocarbon insecticides to human beings has been a subject of primary interest[1]. The trichlorobis(*p*-chlorophenyl)ethane family of compounds, certainly the most widely used insecticides since World War II, constitute a serious problem, not only for workers exposed to them in factories but also for the general population.

The analysis of residues of these chlorinated insecticides and the quantitative measurement of their main metabolites have stimulated the development of sensitive and accurate analytical methods. According to many authors[2–4], the urinary excretion of bis(*p*-chlorophenyl)acetic acid (DDA), a relatively water-soluble metabolite, could be used as an indicator of exposure to DDT. In comparison to the conventional methods for measuring DDT and other metabolites in fatty tissues or blood samples, the great advantage of systematic monitoring of DDA in human urine becomes obvious.

In previous papers, DDA excretion levels were estimated either by a tedious spectrophotometric procedure after ion-exchange purification[5] or by a gas–liquid chromatographic (GLC) separation after methylation of the carboxylic function with 10% boron trifluoride in nanograde methanol[6]. The latter method could be applied with the Coulson conductivity detector or with an electron-capture detector (ECD). This procedure requires a time-consuming microflorisil clean-up step[7].

This paper reports modifications of the GLC method which improve its rapidity, accuracy and selectivity for the detection of DDA in human urine.

EXPERIMENTAL

Chemicals

All the chemicals and solvents were of analytical grade supplied by Carlo-Erba

(Milan, Italy) and were used without further purification. Florisil for column chromatography was purchased from E. Merck (Darmstadt, G.F.R.), DDA and Diazald 99% from Aldrich-Europe (Beerse, Belgium). Diazomethane in diethyl ether was prepared monthly following the usual technique[8]. Boron trifluoride in propanol (14%, w/v) was obtained from Applied Science Labs. (Mornard, Belgium). The different esterification procedures were performed according to the suppliers' technical notes[9,10].

Apparatus

All the glassware was cleaned in acids and rinsed with distilled water and solvents prior to a final check for sample contamination.

A Tracor Model 550 gas chromatograph equipped with a 10-mCi ^{63}Ni pulse-frequency-modulated ECD was used. The pulse mode was selected at 55 V (pulse width, 3 μsec; pulse rate, 270 μsec). The instrument was operated with a glass column (Pyrex 6 ft. $\times$ 0.25 in. O.D.) packed with 1% QF-1 on Gas-Chrom Q (100–120 mesh). The flow-rates of both the carrier and make-up gas (argon–methane, 90:10) were regulated at 30 ml/min. The temperatures of the injector block, column oven and detector were 250°, 190° and 290° respectively. Gas chromatographic–mass spectrometric (GC–MS) analysis was performed on a LKB 9000 S instrument. The chromatographic separation conditions were identical to those previously described. The temperatures of the separator and ion source were 260° and 270°, respectively. The mass spectra were recorded with 70-eV incident electrons and a 60-μA trap current.

Procedure

Urine samples (5–50 ml depending on the degree of DDT exposure) were mechanically extracted for 10 min in test-tubes or funnels with an equal volume of a 2% acetic acid solution in hexane. After centrifugation at 1155 *g* for 5 min, the upper organic phase was transferred to a conical tube. The remaining aqueous phase was re-extracted with the same quantity of solvent mixture.

The combined organic phases were carefully dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo* at 50° to eliminate the remaining traces of water. Methylation of the residue was performed at room temperature with 500 μl of an ethereal solution of diazomethane. After 15 min, the resulting solution was evaporated to dryness under a nitrogen stream. The residue was finally dissolved in an appropriate volume of hexane containing 0.1 μg/ml DDA propyl ester as internal standard. An aliquot (1–5 μl) was injected into the gas chromatograph and the integrations of both the DDA methyl and propyl ester peaks were used for the quantitative measurement. Standard calibration curves were prepared by adding known amounts of DDA to human urine free from DDA (Fig. 1).

RESULTS AND DISCUSSION

Several derivatization and isolation techniques were applied to the same spiked urine specimens in order to eventually bypass the tedious and time-consuming clean-up procedure which is recommended for the DDA determination by electron-capture gas chromatography[7].

Figs. 2 and 3 show different chromatograms recorded under optimal conditions.

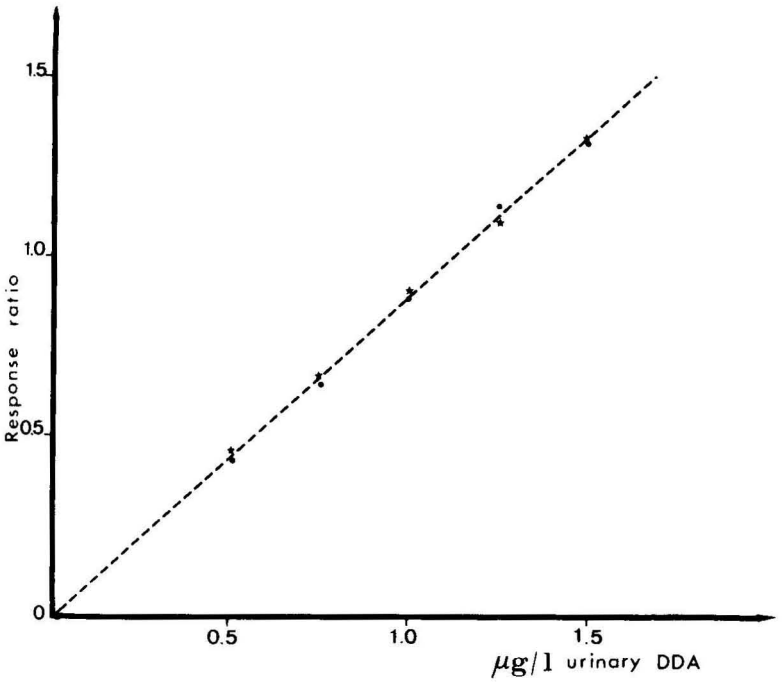


Fig. 1. Standard calibration curve for DDA determination in urine (★) and water (●) samples using 0.1 μg/ml of DDA propyl ester as internal standard. Attenuation: $4 \cdot 10^{-10}$ A. Full scale 1 mV recorder.

The former was obtained on a sample derivatized with boron trifluoride–methanol (A = crude extract; B = after a microflorisil clean-up procedure), the latter after diazomethane derivatization. From these figures, the following observations can be made. (1) After treatment with boron trifluoride–methanol, it is clear that the microflorisil column purification is required in order to avoid the elution of many interfering peaks which result in a low efficiency of chromatographic separation. In addition, the presence of several compounds having low volatility greatly increases the delay between analyses. (2) In contrast, the diazomethane esterification leads to cleaner chromatographic traces where eventual interferences from endogenous or exogenous compounds are significantly reduced.

In conclusion, the diazomethane methylation technique constitutes an important improvement of the methodology which, in practice, obviates the need for the clean-up step. Consequently, as described, our procedure is not only simpler but also enables the achievement of clear chromatographic traces thus reducing the risk of ECD contamination.

Precision. The variation coefficients of 10 urine samples containing 10, 50 and 100 μg/l were 10, 8 and 6%, respectively.

Accuracy. The accuracy of the assay was examined on spiked urines. From the data presented in Table I, a 0.998 correlation coefficient was obtained demonstrating the high accuracy of the method.

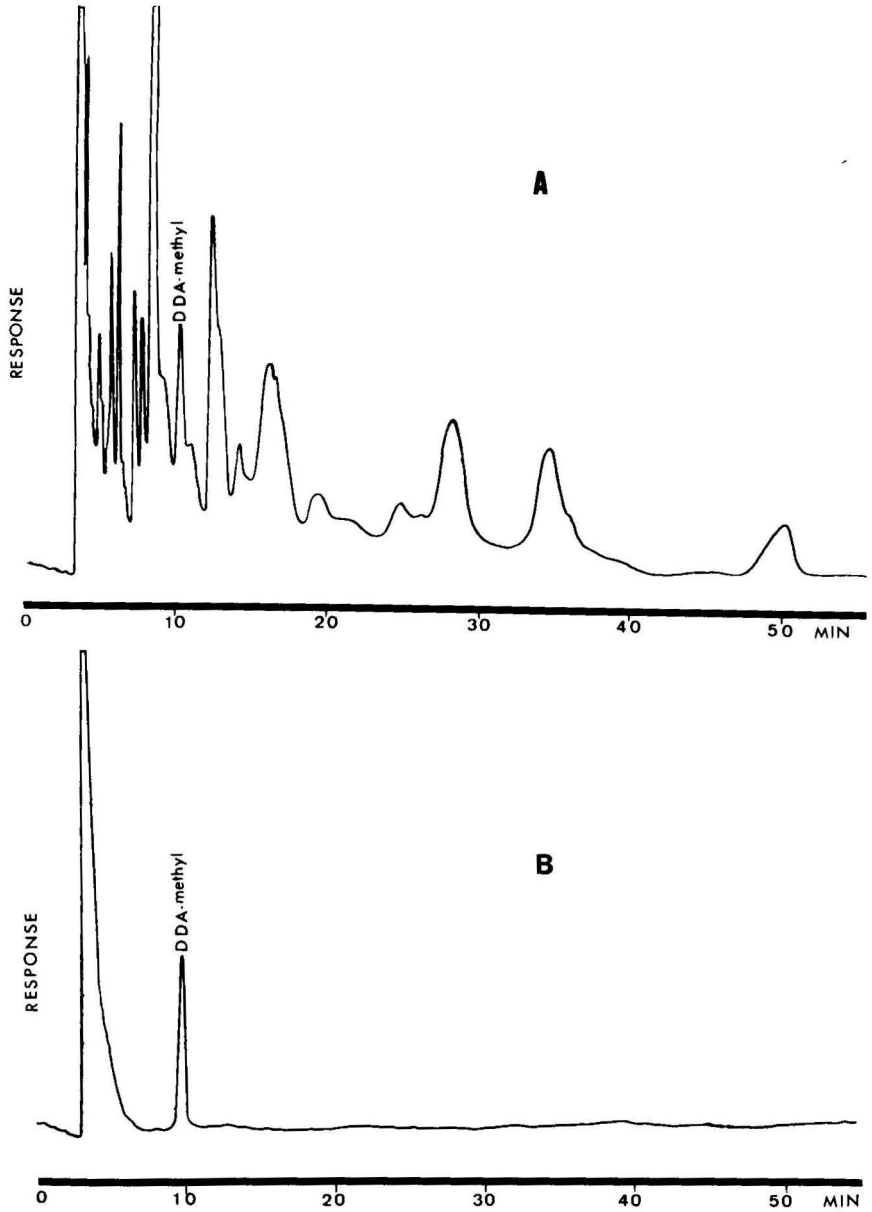


Fig. 2. GC separation of urinary extract of DDA methylated with boron trifluoride–methanol before (A) and after (B) microflorisil clean-up. For GLC conditions, see Experimental, except that the oven temperature was 160°.

Specificity. The specificity of our technique was assessed by GC–MS analysis of different urinary extracts obtained from people exposed to insecticides. Fig. 4 shows the mass spectrum recorded at the top of the GC peak corresponding to DDA methyl ester. It is absolutely identical to the spectrum obtained from the reference compound.

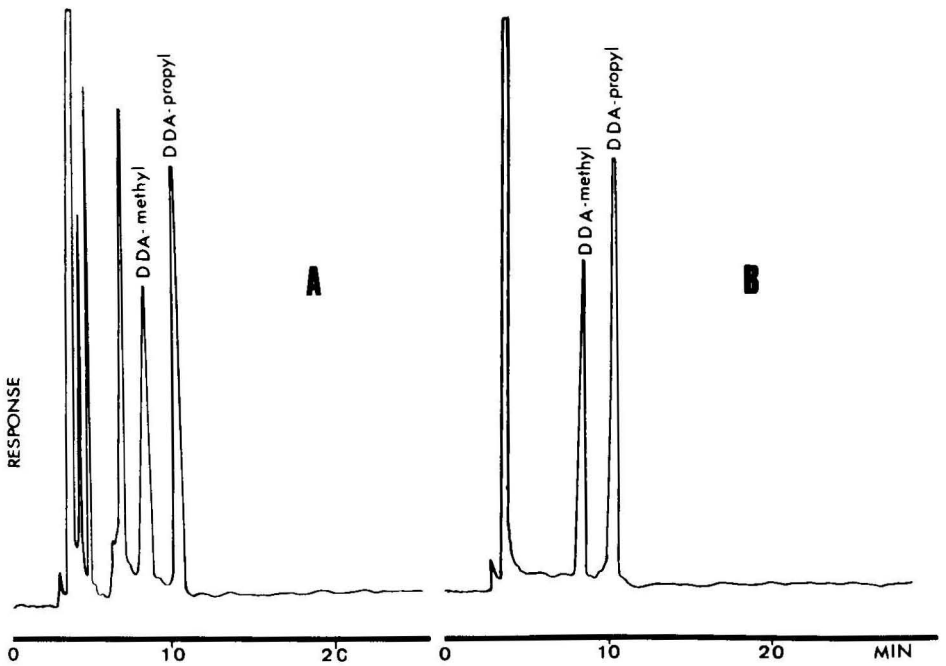


Fig. 3. Gas chromatograms of a urinary extract of DDA methylated with diazomethane before (A) and after (B) microflorisil clean-up. For GLC conditions, see Experimental.

TABLE I

ACCURACY OF THE DETERMINATION OF DDA

Sample	*Amount of DDA (μg/l)*	
	Added	*Found*
A	20	19
B	10	8
C	50	51
D	100	95
E	15	17
F	25	24
G	35	37
H	5	6

Rapidity. Since only a single extraction procedure is required in order to obtain a clear chromatographic trace with short retention times for DDA esters, a great number of samples (about 20–30) can be analyzed by one operator within a normal working day.

Sensitivity. The extraction yields of *ca.* 95% make this method very sensitive and totally suitable for the routine examination of human urine samples. The absolute detection limit is *ca.* 0.05 ng.

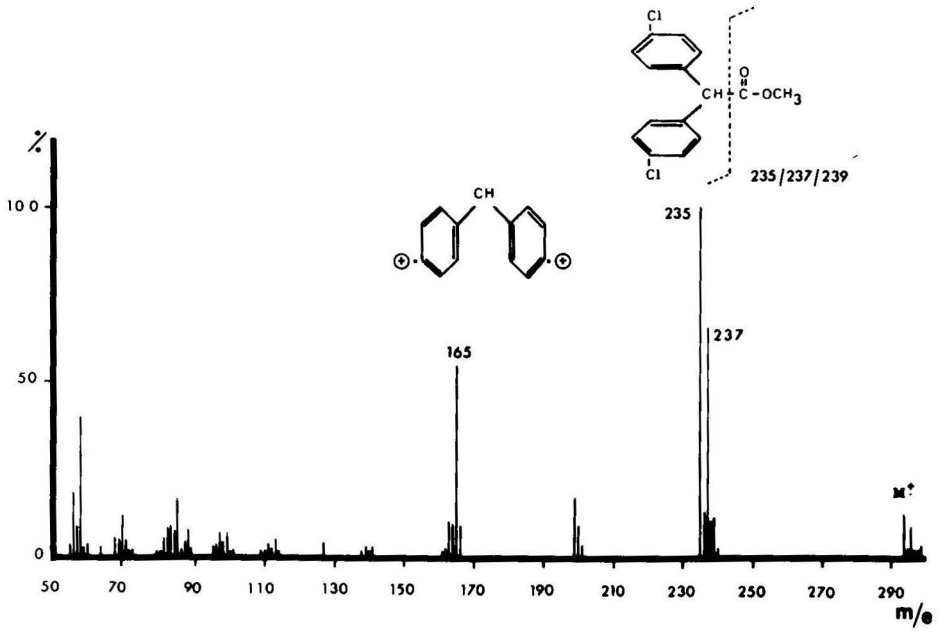


Fig. 4. Mass spectrum recorded at the top of the GLC peak corresponding to the DDA methylation.

Under our experimental conditions, the use of a suitable diazomethane methylation procedure in addition to an appropriate internal standard has yielded a simple, specific, rapid and precise technique for DDA determination in human urine. The main advantage of this method is the rapidity which allows at least 10 times as many analyses to be performed in comparison with the previous method of Crammer *et al.*[6].

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CHROM. 11,128

Note

Affinity chromatography on immobilized S-adenosyl-L-homocysteine

Purification of a furanocoumarin O-methyltransferase from cell cultures of *Ruta graveolens* L.

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(Received December 16th, 1977)

To a large extent, the present interest in affinity chromatography for enzyme purification originates from the paper of Cuatrecasas *et al.*[1]. This study, in which the name affinity chromatography was used for the first time, stimulated an extensive use of this method in the isolation of enzymes, their inhibitors, antibodies, antigens, nucleic acids and a great number of other products, as evidenced by many literature references. The ever increasing number of commercially available insoluble affinants is the best evidence of the rapid development and important role of this method[2].

Agarose, a polydextran carrier, is the most common support in affinity chromatography to date. Cuatrecasas[3,4] has reported the preparation of a great number of agarose derivatives and various groups can therefore be employed for binding. These derivatives are available now as commercial products and include, for example, AH-Sepharose 4B (agarose with covalently bound 1,6-diaminohexane) which binds compounds through their carboxyl groups via a soluble carbodiimide. In this communication we wish to report an affinity system in which S-adenosyl-L-homocysteine was coupled to AH-Sepharose 4B by the carbodiimide coupling procedure (Fig. 1). The potential usefulness of this affinity system is illustrated by results of its application to the purification of an O-methyltransferase which catalyses the transfer of a methyl group from S-adenosyl-L-methionine to a phenolic furanocoumarin in *Ruta graveolens*[5]. Mack and Slaytar[6] have recently used a similar affinity system for the purification of an indole ethylamine N-methyltransferase from *Phaoaris tuberosa*.

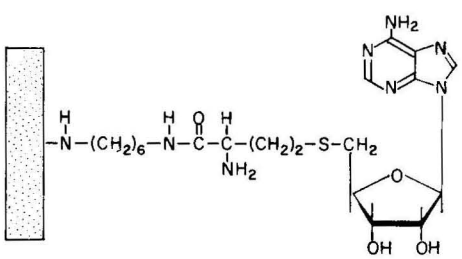


Fig. 1. Structure of S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B. The shaded rectangular area to the left represents the Sepharose matrix.

MATERIALS AND METHODS

Materials

These were obtained as follows: AH-Sepharose 4B from Pharmacia (Uppsala, Sweden); S-adenosyl-L-homocysteine from Sigma (St. Louis, Mo., U.S.A.); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl from BioRad Labs. (Richmond, Calif., U.S.A.); [$^{14}CH_3$]S-adenosyl-L-methionine (Amersham, Chicago, Ill., U.S.A.) had a specific activity of 58 mCi/mmole.

Methods

Enzyme isolation. Cells from cultures of *R. graveolens*, maintained as previously described[7], were harvested 6 days from transfer, at which time the O-methyltransferase activity was near its maximum[5]. A crude extract of the enzyme was prepared[5] and filtered through a G-25 Sephadex column (26 × 1.4 cm) equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.5) at 4°. The Sephadex eluate was used in the experiments reported here.

Activity assay. Fractions emerging from the chromatographic columns were monitored for O-methyltransferase activity against bergaptol (5-hydroxypsoralen) as the substrate, with [$^{14}CH_3$]S-adenosyl-L-methionine as the methyl donor. Enzyme activity was expressed as nmoles of [$^{14}CH_3$]bergapten (5-methoxypsoralen) formed per hour at 30°, under standard assay conditions[5].

Protein determination. Protein concentration was determined by the newly developed procedure of Bradford[8,9]. Bovine γ-globulin was used as the protein standard.

Preparation of the affinity matrix: carbodiimide coupling of S-adenosyl-L-homocysteine to AH-Sepharose 4B. All operations were performed at room temperature unless otherwise stated. Freeze-dried AH-Sepharose 4B (2 g) was swelled in an excess of 0.5 *M* sodium chloride solution (50 ml) and allowed to stand for 15 min after gentle stirring. The gel was washed with 0.5 *M* sodium chloride solution (400 ml) in order to remove the lactose and dextran present originally in the Sepharose 4B powder. Sodium chloride was then removed by washing the gel with 100 ml of deionized water (previously adjusted to pH 4.6). The ligand, S-adenosyl-L-homocysteine, was dissolved in 8 ml of deionized water (pH 4.6) to give a final concentration of 3.85 mg/ml, and added to the gel. The pH was 5.6 after mixing, and was adjusted to 4.6 by addition of dilute HCl. Solid carbodiimide powder (230 mg) was added, in small batches at regular intervals, to the slurry to give a final concentration of 0.1 *M*. The pH was maintained at 4.6–6.0 for 1 h, and the reaction was allowed to proceed for another 24 h. The gel was finally washed with deionized water (pH 4.6) and stored at that pH at 4°. Before use, the gel was equilibrated with 0.05 *M* Tris–HCl buffer, (pH 7.5) at 4° and packed into a column.

RESULTS AND DISCUSSION

The ligand, S-adenosyl-L-homocysteine, was selected as an ideal derivative for coupling to AH-Sepharose 4B for the purification of O-methyltransferase, since it has a strong inhibitory effect in S-adenosyl-L-methionine-homocysteine methyltransferase systems, including the O-methyltransferase reactions[10,11]. When the Sephadex G-25 eluate containing the crude O-methyltransferase from *R. graveolens* cell cultures

was applied to the ligand-bound AH-Sepharose 4B column at the optimal pH of the enzyme (Tris–HCl buffer, pH 7.5) and the affinity column developed at this pH, a protein peak containing no methyltransferase activity appeared between 1 and 2.5 elution volumes (Fig. 2). No O-methyltransferase appeared in the eluate even after more than 4 elution volumes of buffer had passed through the column. It appeared, therefore, that inactive protein passed through the column unretarded, but that the O-methyltransferase was retarded owing to its interaction with the S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B. As shown also in Fig. 2, the O-methyltransferase began to be eluted about one elution volume after the developing solvent was changed to 0.1 *M* acetic acid (pH 3). No additional protein was eluted when the column was washed with excess of 0.1 *M* acetic acid (pH 3). The specific activity of the enzyme was constant across its elution profile, showing that the enzyme was free of any inactive protein contaminants. This chromatographic procedure resulted in a 50-fold increase in the specific activity of the O-methyltransferase.

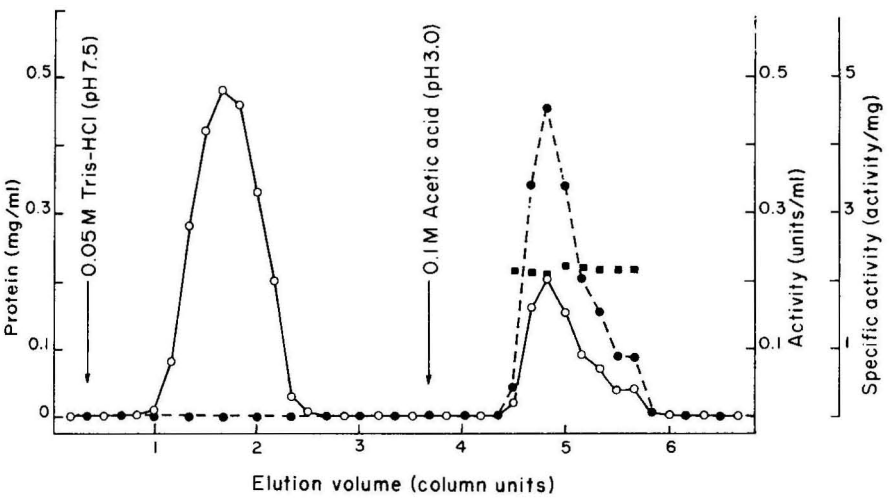


Fig. 2. Affinity chromatography of O-methyltransferase on a column (15 × 0.7 cm) of AH-Sepharose 4B coupled with S-adenosyl-L-homocysteine. Elution volume (in column volume units) was measured from the time of application of the sample, and the flow-rate was 28 ml/h. ○—○, Protein; ●---●, O-methyltransferase activity; ■, specific activity. Since O-methyltransferase from *R. graveolens* cell cultures is believed to be unstable at low pH[5], fractions eluted in 0.1 *M* acetic acid were collected in test tubes containing 0.5 ml of 0.05 *M* Tris–HCl buffer (pH 7.5) in order to raise the pH immediately.

In the preparation of sorbents for affinity chromatography, the introduction of functional groups into the agarose gel carrier is usually by treatment with cyanogen bromide followed by reaction with a bifunctional alkylamine. Recently it has been observed that coupling of aliphatic amines to agarose by the cyanogen bromide reaction yields isourea linkages which are positively charged at pH 7 (ref. 12). The presence of these charged sites in affinity gels causes a significant non-specific adsorption of proteins[12,13]. In order to test for non-specific adsorption in our "affinity gel" due to ionic binding, the same G-25 Sephadex eluate containing the O-methyltransferase was chromatographed on a "control gel". This gel was composed of

Sepharose 4B substituted with the spacer-arm assembly, and bearing no bio-specific ligand (S-adenosyl-L-homocysteine). As shown in Fig. 3, the adsorption–elution behaviour of the O-methyltransferase on this "control gel" is entirely different from its behaviour on the "affinity gel" (see Fig. 2). The active O-methyltransferase was found to be unretarded and eluted in 0.05 *M* Tris–HCl buffer (pH 7.5). The specific activity varied across the elution profile (Fig. 3) indicating the presence of other inert proteins. These results demonstrate clearly, again, that the process involved in the separation is related to the functional ability of the enzymatic binding site for the specific ligand (S-adenosyl-L-homocysteine) attached to AH-Sepharose 4B.

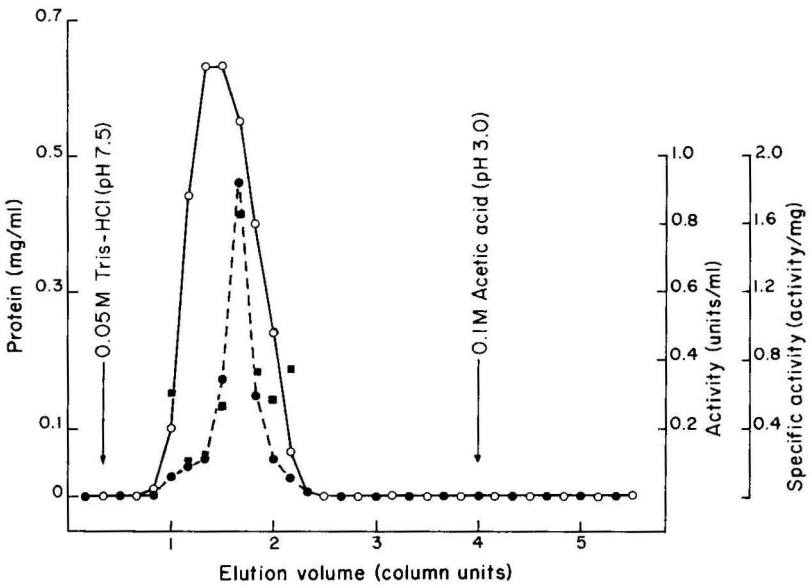


Fig. 3. Chromatography of O-methyltransferase on a ligandless "control gel", *i.e.*, matrix-spacer arm assembly bearing no bio-specific ligand (S-adenosyl-L-homocysteine). The size of the column was 13 × 0.7 cm and the flow-rate was 22 ml/h. Elution volume was measured from the time of application of the sample. ○—○, Protein; ●---●, O-methyltransferase activity; ■, specific activity.

All experiments were carried out using a buffer, 0.05 *M* Tris–HCl (pH 7.5), as the column eluent. The columns were washed with about ten elution volumes of buffer between chromatography of successive samples and were re-usable within two weeks of preparation. Chemical stability of our "affinity gel" under operating conditions seems likely to be a problem, however, because the adsorbent lost its characteristic binding ability after two weeks in operation at pH 7.5 and 4°. This could have been due to exposure of the bio-specific ligand to the action of degradative enzymes presumed to be present in the crude extracts of *R. graveolens* cell cultures.

Finally, the significance of affinity chromatography using S-adenosyl-L-homocysteine coupled to AH-Sepharose 4B as described above could go well beyond the purification of O-methyltransferases. This follows because many multi-substrate enzymes employ, as one of their substrates, a ligand common to many other enzymes (for example: ATP, NAD^+). It has been suggested that immobilized forms of such substrates, or their analogues, would constitute "general ligands" whose wide spe-

cificity would make them useful in the purification of a wide range of enzymes[14–16]. The ligand, S-adenosyl-L-homocysteine, may therefore be another example of a "general ligand" for methyltransferases, as is NAD^+ among dehydrogenases. If this is so, it should be possible to apply our affinity system also for the purification of other methyltransferases, such as N-methyltransferases.

ACKNOWLEDGEMENTS

This research was supported by operating grant No. A2487 of the National Research Council of Canada. We are particularly grateful to Dr. Joseph Mack, formerly of the University of California, Davis, for helpful suggestions. Mr. Pheeroz Virani provided competent technical assistance.

NOTE ADDED IN PROOF

While this paper was in press, Kim *et al.*[17] reported the use of essentially the same affinity system described above for the purification of an S-adenosylmethionine: protein carboxyl O-methyltransferase from calf brain.

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Note

Quantitative high-performance liquid chromatographic analysis of 12-oxododeca-4,8-dienoic acid and its by-products in the nylon 12 process

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(First received February 8th, 1978; revised manuscript received March 31st, 1978)

In the first step of the nylon 12 process according to Snia Viscosa patents[1–3], 12-oxododeca-4,8-dienoic acid is obtained by ozonolysis of 1,5,9 (*cis*, *trans*, *trans*)-cyclododecatriene followed by transposition of the monozonide, using a blend of acetic acid and vaseline oil as the solvent (Fig. 1).

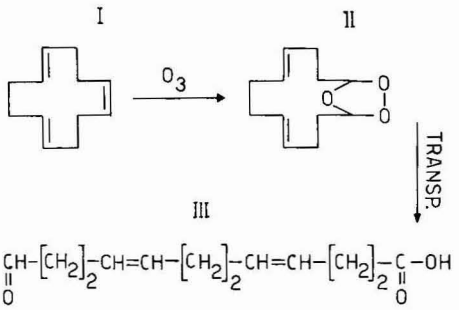


Fig. 1. Reaction scheme. Compound I = 1,5,9(*cis*,*trans*,*trans*)-cyclododecatriene; II = 1,5,9-cyclododecatriene monozonide; III = 12-oxododeca-4,8-dienoic acid.

In this paper, we describe a method for the quantitative determination of the reagents and the main products by high-performance liquid chromatography (HPLC). Gas–liquid chromatography is not suitable as some compounds are high boiling and thermally unstable.

EXPERIMENTAL AND RESULTS

Reagents and standards

The reagents used were dioxane (R.P.), *n*-hexane (R.P.) and acetic acid (R.P.) (Carlo Erba, Milan, Italy). The following standards were double distilled or recrystallized (purity 99%): 1,5,9(*cis*, *trans*, *trans*)-cyclododecatriene, 12-oxododeca-4,8-dienoic acid, vaseline oil, dodeca-4,8-diene-1,12-dioic acid, dodeca-4,8-diene-1,12-dial and benzophenone (internal standard).

Apparatus

A Hewlett-Packard Model 1010-B liquid chromatograph was used, equipped with a differential refractometric detector (Hewlett-Packard Model 1031) connected with a stainless-steel column (50 × 0.3 cm) packed in our laboratory with LiChrosorb Si-60, particle size 10 μm (Merck, Darmstadt, G.F.R.) by the slurry-packing technique[4,5]. The injector was a Rheodyne Model 7120.

Method

The mobile phase was dioxane–*n*-hexane–acetic acid (74.6:25:0.4). The flow-rate was 1 ml/min, the column temperature 48° and the pressure 25 atm. The presence of acetic acid is necessary in order to prevent tailing of the peaks of acidic compounds.

Samples obtained from different process steps were weighed with the internal standard and dissolved in a solvent with the same composition of the mobile phase. The injector loop volume was 20 μl. The maximum detector sensitivity utilized was × 8 and the solution concentration was about 4%.

The five compounds and the internal standard were completely separated, as can be seen from the chromatogram of a standard mixture (Fig. 2). Retention times are given in Table I.

Calibration graphs were obtained by plotting the ratio of the peak heights against the amounts of the compounds (Fig. 3).

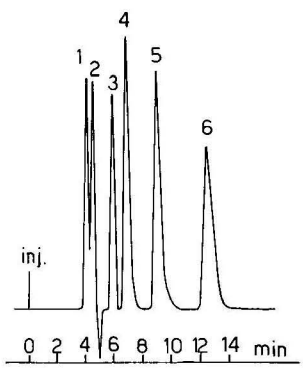


Fig. 2. Chromatogram of standard mixture. Peaks as in Table I.

TABLE I

RETENTION TIMES OF COMPOUNDS EXAMINED

*No.**	*Compound*	*Retention time (min)*
1	Vaseline oil	4.0
2	1,5,9(*cis,trans,trans*)-Cyclododecatriene	4.4
3	Benzophenone (internal standard)	6.0
4	Dodeca-4,8-diene-1,12-dial	6.8
5	12-Oxododeca-4,8-dienoic acid	9.5
6	Dodeca-4,8-diene-1,12-dioic acid	12.8

* Peak Nos. in Fig. 1.

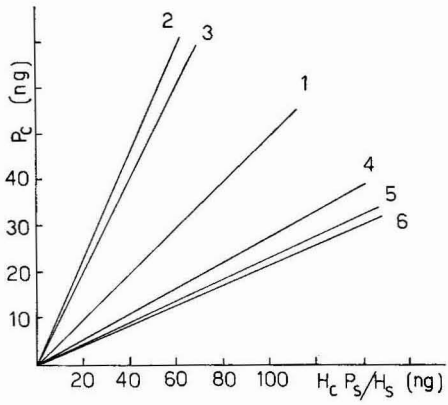


Fig. 3. Calibration graphs for the compounds listed in Table I. H_c = peak height of compound (mm); H_s = peak height of standard (mm); P_c = amount of compound (ng); P_s = amount of standard (ng).

Sensitivity and error

The minimum detectable amount when using this method is between 10 ng [1,5,9(*cis*, *trans*, *trans*)-cyclodocatriene] and 50 ng (12-oxododeca-4,8-dienoic acid). The standard deviation is between 0.5% and 1.5% for the same two compounds, respectively.

Some of the samples contained 10% of water which was not completely mixed with the eluent, but this had no effect on the calibration graphs. Variations in the flow-rate, if less than 2%, do not change the calibration graphs to an appreciable extent.

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CHROM. 11,091

Note

Rapid preparative separation of amino acids with the chromatofuge

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(Received April 18th, 1978)

Ion-exchange chromatography is the preferred analytical method for resolving mixtures of amino acids[1]. Applications of this method to preparative work are hampered by high cost, low flow-rates, or the need for high pressures. In the chromatofuge[2], centrifugal force replaces pressure and effects rapid fractionation of relatively large amounts of material by radial migration through a hollow column rotating around its axis. A larger version of the original chromatofuge was constructed and its applicability to the isolation of amino acids on a preparative scale was tested.

EXPERIMENTAL

The present version of the chromatofuge is basically a modified basket centrifuge. It is schematically represented by Fig. 1. An International centrifuge, size 2 (International Equipment, Boston, Mass., U.S.A.)* was fitted with a modified 3-l perforated 4 × 11 in. diameter cylindrical basket (G), and with a custom-made collector (K), designed to drain without appreciable hold-up into an outlet tube (L). The 2-in. top ledge of the basket was widened to 3.5 in. in order to accommodate a sorbent layer 3.2 in. in thickness. The delivery tube (F), made of 0.25-in. I.D. pipe, had a nozzle (Floodjet Nozzle 1/8 KSS 2.5; Spraying Systems, Wheaton, Ill., U.S.A.) attached to its end, which produced, in operation, a vertical sheet of liquid.

The ion-exchange resin (Dow Chem., Midland, Mich., U.S.A.; 4.5 kg of Dowex 50W-X8, finer than 400 mesh) was suspended in an equal weight of water. After standing at room temperature for 150 min, the water was decanted and replaced by an equal volume of fresh water. After 30 min, the water and fines were decanted and replaced by an equal volume of 0.1 *N* NaOH. After another 30 min, the NaOH solution and fines were decanted, and the resin was washed with three portions of water. It was allowed to stand in the last water wash overnight and was subsequently washed twice with 0.2 *N* HCl and three times with water, being kept in the last water wash overnight. Next, the resin was treated twice with an equal volume of 0.2 *N*

* Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product named to the exclusion of others which may also be suitable.

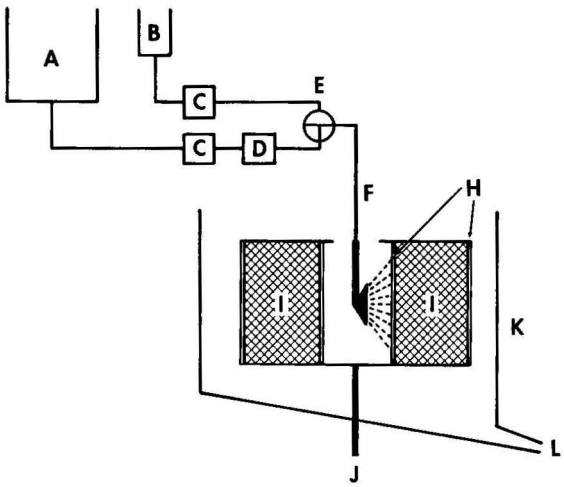


Fig. 1. Schematic representation of the chromatofuge. A = Eluent reservoir; B = sample reservoir; C = pump; D = flow-meter; E = 3-way valve; F = delivery tube; G = centrifuge basket; H = felt lining; I = ion exchanger; J = drive shaft; K = collector; L = effluent outlet. All pipes are made of 1/4-in. stainless-steel tubing.

NaOH and washed three times with water. Finally, a slurry of resin with an equal weight of water was prepared for packing the basket.

The delivery tube was taken out of the chromatofuge and the basket was lined with a previously moistened strip of polypropylene F felt (Pacific States Felt Mfg., San Francisco, Calif., U.S.A.) (H), 4 in. wide. Then, with the centrifuge spinning at 1000 rpm (200 *g*), the slurry was slowly poured into the basket, forming a uniform column with a hollow core. The centrifuge was stopped and the inner wall of the hollow cylinder was lined with another moistened 4-in. strip of felt to protect it from spray damage. With the delivery tube placed in position, the chromatofuge was now ready for operation.

The amino acid samples were dissolved in 250 ml of a buffer solution, prepared by making 19.6 g sodium citrate·$2H_2O$ and 16.5 ml conc. HCl to 1 l with water (pH 2.2, 0.2 *N* Na^+ conc.). For elution, a buffer, prepared by making 3432.5 g sodium citrate·$2H_2O$, 655 ml conc. HCl and 5 ml octanoic acid to 100 l with water (pH 5.29, 0.35 *N* Na^+) was used.

The eluent was transferred to Reservoir A (Fig. 1), which had a capacity over 50 l, and the sample was introduced into Reservoir B. While the centrifuge was running at 1000 rpm (200 *g*), the resin was first flushed with 10 l of eluent by turning the 3-way valve (Three-Way Ball Valve SS 44 X S6; Whitey, Oakland, Calif., U.S.A.) (Fig. 1) to connect the delivery tube with Reservoir A through a metering pump (All-Chem Rotary Pump, Model PP2; ECO Pump, South Plainfield, N.J., U.S.A.; driven with a Master Motor, Style 208270, with Master Speed Ranger, Style 3035850, Size VD 15, Master Electric, Dayton, Ohio, U.S.A.) and flow-meter (Precision Bore Flowrator Tube No. 4-19-10/77; Fischer and Porter, Hatboro, Penn., U.S.A.). The valve was switched to Reservoir B, effecting sample delivery through a metering pump (All-Chem Rotary Pump, Model PP1, ECO Pump; driven with a Master Motor,

Style 209470, with Master Speed Ranger, Style 3035850, Size VD 15, Master Electric) within 30 sec. Switching back to Reservoir A, eluent was then delivered to the column at a flow-rate of 1 l/min. The effluent from the chromatofuge was collected in 500-ml fractions for subsequent analysis.

For qualitative analysis the thin-layer chromatographic method of Von Arx and Neher[3] was used. Samples of effluent were spotted on Eastman Chromagram Sheets (Sheet 13255; Eastman-Kodak, Rochester, N.Y., U.S.A.), precoated with a 160-μm layer of microcrystalline cellulose. The sheets were developed with *n*-butanol–acetone–diethylamine–water (10:10:2:5). The amino acids were detected by spraying with 0.2% ninhydrin in acetone, followed by heating the sheets over a steam bath.

For quantitative analysis a Technicon Autoanalyzer (Technicon Instruments, Tarrytown, N.J., U.S.A.) was used according to the method of Quinn *et al.*[4]. The details of operation are evident from the left side of Fig. 2.

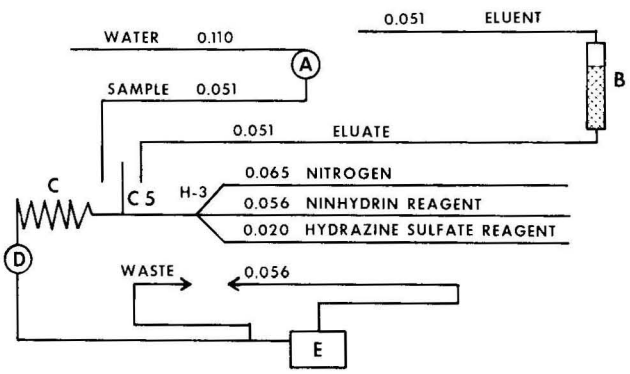


Fig. 2. AutoAnalyzer connections for amino acid analysis of samples of the fractions from the chromatofuge (left) or of the eluate from the ion-exchange column (right). A = Sampler, furnishing 40 samples/h; B = ion-exchange column; C = double heating coil; D = heating bath at 95°, containing a 40-ft. glass coil; E = colorimeter at 570 nm; F = recorder. The figures denote the internal diameter of plastic tubing, in mm.

For comparison, the same eluent was used in a Dowex 50W-X8 column (8 × 0.9 cm I.D.) to separate the same amino acid mixtures as the chromatofuge. The column was always loaded with 0.25 ml of sample in the sample buffer (see above) and eluted with the eluent buffer (see above) at a flow-rate of 1 ml/min. The column effluent was monitored by replacing the sampler (see left side of Fig. 2) with a 0.051-mm tube, connected to the column outlet (see right side of Fig. 2).

RESULTS AND DISCUSSION

The selection of amino acids for this feasibility study was based partly on our ability to separate them by analogous conventional column chromatography and partly on the practical significance of the isolation of the nutritionally important ones. The purification of amino acids, whether they are obtained by synthesis or isolation from natural products, is a major factor in their cost and availability. We have tested

the performance of our chromatofuge by comparison with the short-column analytical separation of basic amino acids reported by Spackman *et al.*[5].

Fig. 3a represents the chromatogram we obtained with the conventional ion-exchange column. With 50 μg of each of the five amino acids shown, the results were similar to those in ref. 5, except for the arginine peak, which was somewhat broader in our system. Because the elution of arginine was expected to require large amounts of eluent, this amino acid was omitted from the chromatofuge tests. Fig. 3b represents the chromatogram obtained with up to 5 g of each of the remaining four amino acids. The less complete separation with the chromatofuge compared to the conventional column reflects the much greater sample load and the much more rapid flow-rate. The flow-rate was arbitrarily selected to determine the resolving efficiency of the chromatofuge at different sample loads. The flow-rate through the ion-exchanger is controlled by the speed of rotation rather than the eluent feed rate. The efficiency of the chromatofuge will be constant at constant speed of rotation as long as the eluent is not fed at a rate that causes flooding of the basket. More rapid separation could have been accomplished by increasing the speed of rotation. While the separations are not as complete as in conventional column chromatography, they are adequate for preparative purposes. Greater purification and concentration is attainable by rechromatographing the effluent in the chromatofuge.

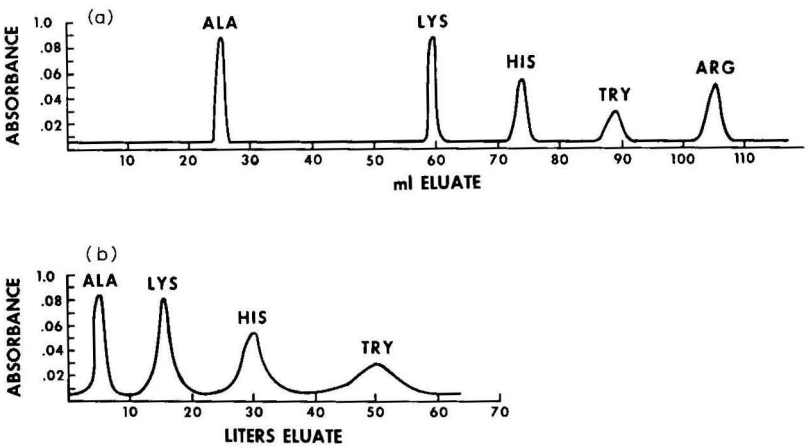


Fig. 3. Chromatogram of amino acids from a conventional column (a) and from the chromatofuge (b). ALA = Alanine; LYS = lysine; HIS = histidine; TRY = tryptophan; ARG = arginine.

The recovery of amino acids from the chromatofuge was just as good as that from conventional chromatography (Table I). Moreover, it was independent of sample load up to 5 g per amino acid.

Our results indicate that rapid and efficient separations by ion-exchange are feasible with the aid of the chromatofuge. Relatively large quantities of amino acids can thus be prepared in pure form without recourse to expensive high-pressure equipment. Further work will be required to delineate optimal conditions of rotational and pumping speeds as well as basket and spray configurations.

TABLE I

PERCENT RECOVERY OF AMINO ACIDS FROM 50 μg OF EACH AMINO ACID, SEPARATED BY A CONVENTIONAL ION-EXCHANGE COLUMN, AND 1, 2, OR 5 g OF EACH AMINO ACID, SEPARATED BY THE CHROMATOFUGE

Amino acid	*Recovery (%)*			
	Amount of each amino acid			
	50 μg	*1 g*	*2 g*	*5 g*
Alanine	93	94	91	92
Lysine	91	90	91	91
Histidine	90	89	87	91
Tryptophan	85	83	84	85

ACKNOWLEDGEMENT

The authors gratefully acknowledge the technical assistance of J. T. Ono.

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Note

Chromatographic separation of tryptophan metabolites on a polyamide column

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(First received November 23rd, 1977; revised manuscript received April 13th, 1978)

Different chromatographic methods for the separation of tryptophan metabolites from different biological materials have recently been reviewed[1]. During the last decade, different column chromatographic techniques using sialic acid[2], Amberlite IR-120[3], Sephadex G-25[4], Sephadex G-10[5], Dowex-1[6], and DEAE cellulose[7] have been described. These methods have the disadvantage of being limited in application to either a small number of tryptophan metabolites or a specific group of indole derivatives[2–8]. However, a method for the separation of tryptophan and 15 metabolites by ion-exchange chromatography using Sephadex QAE-A 25 and carboxymethylcellulose in a continuous system has been reported[9].

In this communication a method is described for the separation, identification and quantitative determination of tryptophan metabolites by chromatography on polyamide columns. It has been applied successfully for the separation of tryptophan metabolites in *P. notatum* culture media.

MATERIALS AND METHODS

The indole derivatives were purchased from Sigma (St. Louis, Mo. U.S.A.) and polyamide resin from Wölm (Eschwege, G.F.R.). Formylanthranilic acid was prepared from anthranilic acid and 98% formic acid according to the method described by Zentmyer and Wagner[10].

Samples and chromatographic procedures

Polyamide was washed and equilibrated with 20% ethanol before packing on to the column (30 × 0.7 cm I.D.). 10-ml amounts of standard solutions containing 4 or 5 compounds dissolved in 20% ethanol were applied at the top of the column. Unless otherwise stated the concentration of each compound was 0.1 mg/ml. The adsorbed materials were eluted with ethanol in a stepwise gradient ranging from 20 to 95% ethanol prepared in distilled water. The absorbances of the eluates were recorded at 280, 300, 330 and 360 nm.

Analysis of the column effluent

Eluates from the column were concentrated under vacuum and subjected to paper chromatographic analysis using Whatman No. 1 paper sheets. Spotted chroma-

tograms were developed using two solvent systems; propan-2-ol–ammonia–water (200:10:20) (I) and 20% KCl (II). The air-dried chromatograms were examined in UV light and spots were visualized by spraying the chromatograms with Ehrlich or sulphanilic acid spray reagents[11,12]. The extinction coefficients at 330, 280 and 300 nm were used for the quantitative determination of anthranilic, formylanthranilic and indole-3-acetic acids, respectively. Tryptophan was determined according to the method of Concon[13].

Maintainance, subculturing, and growth medium of P. notatum

Cultures were grown and maintained on Czapek Dox's medium as previously described[14]. The basal medium is supplemented with 0.37% tryptophan.

RESULTS AND DISCUSSION

The behaviour of the tryptophan metabolites considered and their eluants is shown in Table I. The tested standards were eluted in the following order; tryptamine hydrochloride, tryptophan, and kynurenine sulphate (20% ethanol); indole-3-aldehyde (early fraction of 40% ethanol); anthranilic acid (late fraction of 40% ethanol), indole-3-acetic acid (60% ethanol); indole-3-acrylic acid, formylanthranilic acid (80% ethanol); and indole-2-carboxylic acid (95% ethanol). The unresolved compounds can be easily separated and identified by paper chromatography using the solvent systems I and II (Table I). The data listed in Table I show that neutral compounds and those of low polarity tend to be eluted at lower concentrations of ethanol.

TABLE I

ELUTION OF TRYPTOPHAN METABOLITES ON A POLYAMIDE COLUMN

Compounds	*Ethanol (%)*					*1% Formic acid in ethanol*	R_F		*UV*	*Spray reagents*	
	20	*40*	*60*	*80*	*90*		*I*	*II*		*Ehrlich*	*Sulphanilic*
L-Tryptophan	+						20	49	–	Purple	–
Tryptamine HCl	+						73	43	–	Red-purple	–
L-Kynurenine sulphate	+						20	64	blue	Orange	–
Indole-3-aldehyde		+					92	43	–	Pink turning purple	–
Anthranilic acid		+					37	71	dark blue	Yellow turning orange	Yellow
Indole-3-acetic acid			+				33	65	–	Pink turning grey turning brown	–
Indole-3-acrylic acid				+			30	14	–	Brown	
Formylanthranilic acid				+			50	70	–	Orange after a time	
Indole-2-carboxylic acid					+		43	34		Pink, fading	Orange
2,3-Dihydroxy-benzoic acid						+	37	57		Negative	Pink

The methods available for colorimetric determination of tryptophan metabolites in fermentation media with or without paper chromatography lack specificity[15–17]. Also, interference by phenolic compounds present in culture media with the nitrite. Ehrlich and Salkowski reagents on the chromatograms of partially purified fractions, is well known[13,18].

The present method is used for the isolation and identification of *P. notatum* tryptophan metabolites. Four day culture filtrate was extracted with three successive portions of ethyl acetate, after acidification with tartaric acid. The extracted metabolites were separated into acidic and non-acidic fraction according to the method of Larsen[19]. The elution profile for the acidic fraction is shown in Fig. 1. The elution sequence was established by paper chromatography as described above. Although the elution sequence is reproducible, a shift in the position of anthranilic acid and indoleacetic acid peak was observed. This may be due to overloading of the column by the presence of phenolic acids *e.g.* 2,3-dihydroxybenzoic acid in this fraction. However, it was eluted by 1% formic acid in ethanol (Fig. 1). Paper chromatographic analysis of the neutral fraction revealed the presence of catechol, but no other tryptophan metabolites.

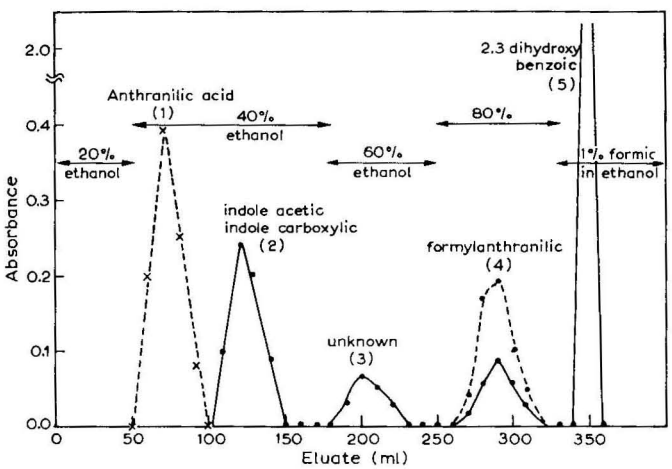
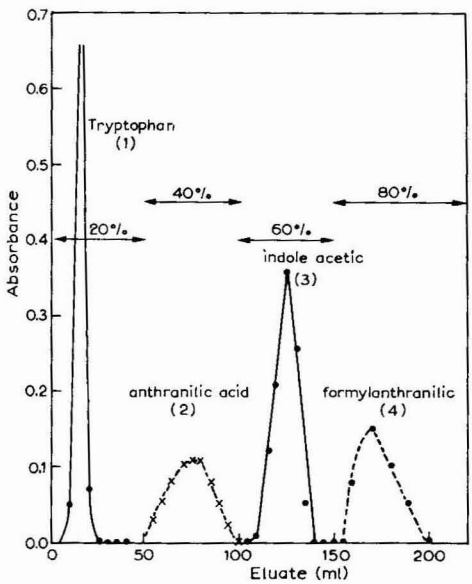


Fig. 1. Separation of tryptophan metabolites in the acidic fraction from *P. notatum* culture filtrate on a polyamide column (0.7 × 30 cm). Absorbance at: ●—● 280 nm; ●---● 300 nm; ×---× 330 nm.

For microscale quantitative determinations a mixture (50–150 μg) of tryptophan, anthranilic acid, indole-3-acetic acid and formylanthranilic acid in 7.5 ml of 20% ethanol was applied to a polyamide column (21 × 0.5 cm I.D.). A typical elution profile for four standard materials encountered in *P. notatum* culture filtrate is shown in Fig. 2. The extinction coefficients were used for quantitative determination of the microgram amounts of standard. It is obvious from the quantitative recoveries that no irreversible adsorption takes place on the column. (Fig. 3 and Table II). The method is reproducible (Fig. 3) and could be useful for the determination and qualitative identification of tryptophan metabolites in fermentation media and possibly in other biological fluids.



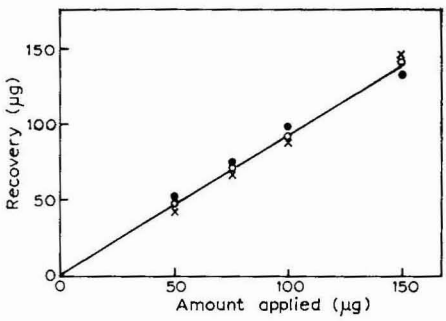


Fig. 2. Typical elution profile for standard mixture of L-tryptophan, anthranilic acid, indole-3-acetic acid and formylanthranilic acid (150 µg each) on a polyamide column (0.5 × 10 cm). Absorbance at: ●—● 280 nm; ×---× 330 nm; ●---● 300 nm.

Fig. 3. Peak area as function of amount of sample applied to the polyamide column. ×, Tryptophan; ○, anthranilic acid; ●, formylanthranilic acid.

TABLE II

RECOVERY OF TRYPTOPHAN, ANTHRANILIC ACID AND FORMYLANTHRANILIC ACID

Concentration (µg)	*Compounds*		
	Tryptophan	*Anthranilic acid*	*Formylanthranilic acid*
50	40.6 (35.8–46.0) SE ± 2.7	47.66 (45.0–50.0) SE ± 1.06	47.23 (46.0–48.2) SE ± 0.77
75	63.4 (62.1–65.0) SE ± 0.83	71.06 (70.0–73.2) SE ± 1.06	70.4 (69.3–71.9) SE ± 0.77
100	89.06 (85.6–91.6) SE ± 1.03	91.2 (81.2–97.6) SE ± 5.08	89.3 (87.7–90.0) SE ± 3.7
150	133.3 (126.0–144.0) SE ± 3.07	136.9 (130.0–142.8) SE ± 3.73	134.5 (130.5–146.0) SE ± 2.8

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Note

New solvent mixtures for thin-layer chromatography of aflatoxin M_1

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First received August 31st, 1977; revised manuscript received April 17th, 1978)

The identification of aflatoxin M_1 is generally accomplished by thin-layer chromatography (TLC) with detection under UV light. Separation of aflatoxin M_1 from interfering substances on chromatoplates depends on the solvent mixture used for development. The following solvent mixtures are most often used: (1) chloroform–*n*-propanol[3]; (2) chloroform–acetone–*n*-propanol[3]; (3) chloroform–acetone–*n*-amyl alcohol[4]; (4) chloroform–acetone–isopropanol[1]; and (5) chloroform–acetone[5]. In milk and milk products, the separation of aflatoxin M_1 from interfering substances using these solvents is not satisfactory. Some blue interferring spots have the same R_F as aflatoxin M_1. Therefore it was decided to construct a new solvent mixture to improve the detection of aflatoxin M_1.

EXPERIMENTAL

The milk extract was prepared from 42 samples of powdered milk according to methods described previously[1,5–8]. All of the extracts were collected in one vial and kept as the stock solution.

Radial chromatography[9] (as shown in Fig. 1) was used to select an appropriate developing solvent. The following six spots were applied on chromatoplates (for each solvent) by use of 20 μl of liquid for each spot: two spots of aflatoxin M_1 standard; two spots of milk extract; and two spots of milk extract containing the internal standard. To one spot of each pair was applied 1 ml of one of 20 different solvents (Table I). The other spots were left as controls. These experiments showed which solvents were the best developers of the investigated substances, as well as for the separation of interfering substances from aflatoxin M_1. On this basis 20 different solvent mixtures were used for the separation of aflatoxin M_1 on chromatoplates coated with silica gel GHR. The five most promising mixtures are shown in Table II and Fig. 2.

RESULTS AND DISCUSSION

The results are presented in Table I and Fig. 1. Among the 20 different solvents,

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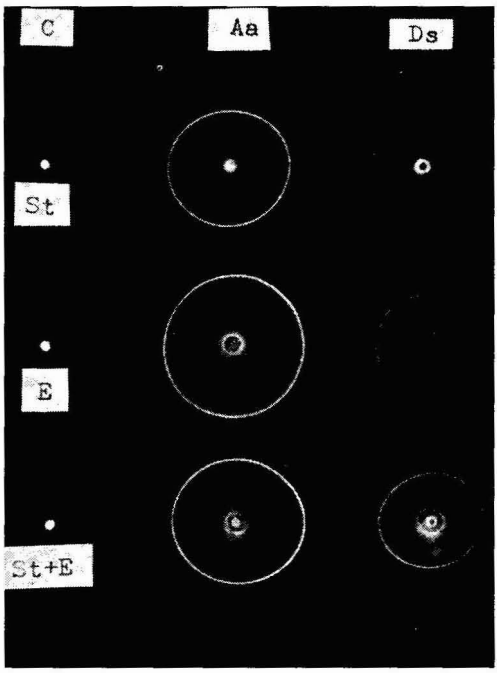


Fig. 1. Selection of solvent for the resolution of aflatoxin M_1 from milk extract. St = aflatoxin M_1 internal standard; E = milk extract; St + E = milk extract with aflatoxin M_1 internal standard; C = control (three undeveloped spots of milk extract, aflatoxin M_1 and milk extract plus aflatoxin M_1; Aa = acetic acid (three developed spots of milk extract, aflatoxin M_1 and milk extract plus aflatoxin M_1); Ds = developing solvent No. 2 in Table II (three developed spots of milk extracts, aflatoxin M_1 and milk extract plus aflatoxin M_1).

TABLE I

SELECTION OF SOLVENTS FOR THE SEPARATION OF MILK EXTRACTS FROM THE AFLATOXIN M_1 STANDARD

— = Immobile (start line); ± = weakly mobile; + + = intermediate mobile; + ± + = strongly mobile (front line).

No.	*Solvent*	*Dielectric constant**	*Milk extract*	*Aflatoxin M_1 standard*
1	*n*-Hexane	1.9	—	—
2	*n*-Heptane	1.9	—	—
3	Light petroleum (b.p. 60–80°)	2.0	—	—
4	Carbon tetrachloride	2.2	—	—
5	Dioxan	2.2	—	—
6	Benzene	2.3	—	—
7	Toluene	2.4	—	—
8	Diethyl ether	4.3	+ ±	±
9	Chloroform	4.8	+	+
10	Ethyl acetate	6.0	+ + ±	+ ±
11	Acetic acid	6.1	± +	—
12	Pyridine	12.3	+ ±	+ +
13	*n*-Amyl alcohol	13.9	±	±
14	Isopropanol	18.3	±	+
15	*n*-Propanol	20.1	+ +	+ +
16	Acetone	20.7	+ +	+ + +
17	Ethanol	24.3	± +	+ + +
18	Methanol	32.6	+ + +	± + +
19	Acetonitrile	37.0	+ +	± + +
20	Water	78.5	±	+

* Data from ref. 9.

TABLE II

DEVELOPING SOLVENTS FOR TLC OF MILK EXTRACTS AND AFLATOXIN B_1 AND M_1 STANDARDS

No.	*Solvent*	*Proportion*	*Resolution of aflatoxins B_1 and M_1*	*Separation of aflatoxins from fluorescent substances*	
				B_1	M_1
1	*n*-Hexane	10			
	Light petroleum	10			
	Benzene	10			
	Chloroform	40	+	+	−
	Acetone	10			
	Acetonitrile	10			
	Acetic acid	10			
2	*n*-Hexane	10			
	Light petroleum	10			
	Benzene	10			
	Chloroform	20	+	+	+
	Acetone	10			
	Acetonitrile	10			
	Acetic acid	30			
3	Chloroform	85			
	Acetone	10	+	+	+
	n-Propanol	5			
4	*n*-Hexane	10			
	Light petroleum	10			
	Benzene	10	+	−	+
	Chloroform	55			
	Acetone	10			
	n-Propanol	5			
5	*n*-Hexane	10			
	Light petroleum	10			
	Benzene	10	+	+	+
	Chloroform	55			
	Acetone	10			
	n-Amyl alcohol	5			

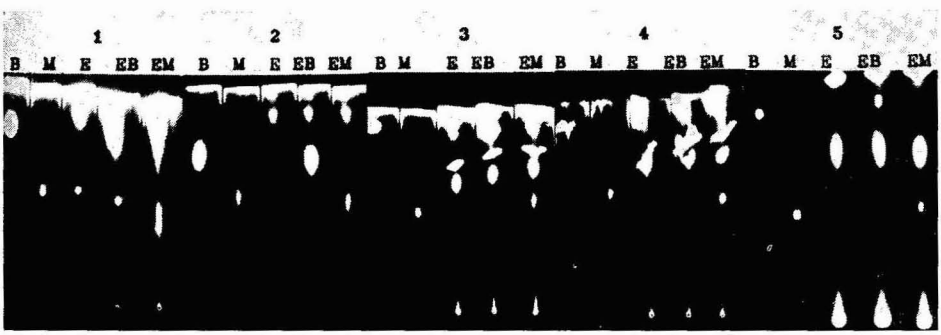


Fig. 2. Chromatoplates with aflatoxin B_1, M_1 and milk extract developed in different developing solvents. B = Aflatoxin B_1; M = aflatoxin M_1; E = milk extract; EB = milk extract with aflatoxin B_1 internal standard; EM = milk extract with aflatoxin M_1 internal standard; 1–5 = number of solvent in Table II.

seven separate aflatoxin M_1 from interfering substances only slightly (diethyl ether, ethyl acetate, water, dioxane, acetone, ethanol and acetonitrile) and one (acetic acid) significantly well. Four solvents (diethyl ether, ethyl acetate, acetic acid and water) developed the interfering substances and four (dioxan, acetone, ethanol and acetonitrile) were good developers for aflatoxin M_1. On the basis of polarity, the solvents might be divided into two groups: (a) weakly polar (diethyl ether, ethyl acetate and acetic acid) and (b) intermediate polar (acetone, ethanol and acetonitrile). Their dielectric constants are 4.3, 6.0 and 6.1 (a) and 20.7, 24.3 and 37.5 (b), respectively.

The HEBCA[2] solvent mixture was used as a base for the experiments because it had been established for the separation of aflatoxins in food extracts. In this mixture, hexane (H) petroleum ether (E) and benzene (B) were used for the separation of lipids, and chloroform (C) with acetone (A) for the development and resolution of aflatoxins.

In the milk extracts, some interfering substances had the same R_F value and blue colour as aflatoxin M_1 when the HEBCA mixture was used for the development of TLC plates. Also, aflatoxin M_1 was too close to the start line. Therefore, two additional solvents, acetic acid and acetonitrile, were used to improve the separation of aflatoxin M_1 from the other substances. The new developing solvent mixture $HEBCAA_cA_a$ (No. 2 in Table II) had the following composition: hexane–light-petroleum (b.p. 60–80°)–benzene–chloroform–acetone–acetonitrile–acetic acid (1:1:1:2:1:1:3). The mixture was estimated as the best because it changed the colour of interfering substances from blue to pale green as well as improving the resolution and separation of aflatoxin M_1 significantly.

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Note

A specific reaction for chenodeoxycholic acid

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(First received May 17th, 1977; revised manuscript received March 6th, 1978)

Thin-layer chromatography (TLC) and paper chromatography of bile acids are often used in clinical laboratories, although for quantitative and qualitative analysis gas chromatography is the method of choice[1–3]. For the detection of bile acids in TLC, a phosphomolybdic acid reagent is often used[4–6].

Many specific reactions of bile acids have been described[7,8], the most specific for chenodeoxycholic acid being a reaction with ammonium ceric sulphate reagent[8].

A reaction specific for chenodeoxycholic acid in the test-tube was first described by Wieland and Reverey[9]. Chenodeoxycholic acid gives a cherry-red colour with acetic anhydride and sulphuric acid (the Liebermann–Burchard reagent). The same reagent was later used by Anthony and Beher[7], but the colour given by chenodeoxycholic acid as described by these authors was not red and not useful in clearly differentiating between chenodeoxycholic and deoxycholic acids[8]. Here, we report our experiences in differentiating between the bile acids mentioned above.

METHODS AND RESULTS

In TLC different solvent systems[4–6] and pre-coated plates [*e.g.*, Silufol (Kavalier) and DC Fertigplatten Kieselgel ohne Fluoreszensindikator (Merck)] were used. The detection reagent was the same as reported previously[7,8] (acetic anhydride containing 10% of sulphuric acid), and the plates were dried at room temperature before being sprayed.

The colour given by chenodeoxycholic acid was deep red; deoxycholic acid and cholic acid both gave yellow spots, and lithocholic acid gave no colour.

The red colour of chenodeoxycholic acid was stable for approximately 30 min, and the reaction was specific for both the free and the conjugated acid. The smallest amount that could be visually detected was 2 μg.

We have used this method many times in analyses of samples of chenodeoxycholic acid and in analysing human and rat bile for bile acids. The specific red colour of chenodeoxycholic acid observed by us was different from that described by Anthony and Beher[7] and by Sundaram and Sodni[8]; the difference is probably due to different experimental conditions, especially in temperature during the reaction.

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Note

Dosage de toxines d'*Aspergillus fumigatus* en chromatographie sur couche mince

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(Reçu le 6 mars 1978; manuscrit modifié reçu le 7 avril 1978)

Des substances toxiques de différents types ont été décrites chez *Aspergillus fumigatus*[1–3]. Récemment, nous avons mis en évidence un nouveau groupe de quatre composés, les fumitoxines A, B, C et D[4]. La structure exacte de ces toxines n'a pas encore été établie mais plusieurs constantes physiques et quelques caractéristiques chimiques sont connues[5]. Les seules possibilités d'évaluation quantitative dans les extraits bruts dont nous disposions étaient des dosages biologiques (arrêt du développement de l'embryon de poulet, inhibition de cultures cellulaires, effet létal pour *Artemia salina* L.). Mais ces méthodes ne permettent pas de distinguer les toxines les unes des autres.

En utilisant des propriétés d'absorption dans l'ultraviolet (UV) et la possibilité de séparation des fumitoxines par chromatographie sur couche mince (CCM), nous nous sommes attachés à mettre au point une méthode de dosage par photodensitométrie.

MATÉRIEL ET MÉTHODES

Fumitoxines

Les substances cristallisées ont été préparées au laboratoire à partir d'extraits organiques de cultures *in vitro* d'*Aspergillus fumigatus* Fres.[5].

Chromatographie sur couche mince

Tous les essais ont été effectués sur couches minces de gel de silice de 0.25 mm d'épaisseur (plaques finies Merck, en verre). En chromatographie d'adsorption, les plaques sont préalablement activées à 110° durant 40 min. En chromatographie de partage, l'eau étant la phase stationnaire, elles sont hydratées avant l'emploi. Les fumitoxines sont déposées en solution chloroformique sous volume de 2 μl. Après le développement, les plaques sont séchées sous courant d'azote. Les composés absorbant dans l'UV sont repérés en plaçant sous le chromatogramme une couche mince de cellulose qui, en "brillant" sous UV de grande longueur d'onde (lampe Philips HPW 125) fait apparaitre les substances absorbantes sous la forme de taches sombres, sur fond blanc-bleu.

Spectrophotométrie

Les spectres d'absorption dans l'UV des fumitoxines cristallisées sont effectués en solution éthanolique (20 μg/ml), dans des cuves en quartz, sur un spectrophotomètre D. B. Beckman. Les mesures photodensitométriques sont réalisées par transparence sur un photomètre-intégrateur Vernon PHI-5 (Vernon, Paris, France) équipé d'un bloc d'alimentation et d'une lampe-source "Mercure" haute pression en utilisant la bande d'émission à 365 nm. Les lectures sont effectuées en utilisant l'étalon interne de densité de l'appareil pour 0.2 ou 1 de densité optique à pleine échelle.

Essais sur extraits de produit végétal

Pour simuler un dosage à partir d'extraits de produits naturels contaminés, 50 g de drêche de betterave sont soumis à une extraction chloroformique (63°, 2 × 1 h, 4.5 ml de chloroforme par gramme de drêche sèche). Après élimination des lipides par l'hexane et concentration, l'extrait est additionné d'une solution chloroformique de fumitoxine A (concentrations allant de $1 \cdot 10^{-5}$ à $1 \cdot 10^{-7}$).

RÉSULTATS ET DISCUSSION

En CCM, la séparation des quatre fumitoxines par adsorption est très délicate et peu reproductible. Elle est plus aisée et plus constante en chromatographie de partage (Tableau I). Les phases acides accélèrent la migration mais donnent des séparations médiocres. Les phases basiques détruisent totalement les toxines étudiées. L'utilisation de l'eau comme phase stationnaire permet une bonne séparation et des valeurs de R_F

TABLEAU I

CARACTÉRISTIQUES CHROMATOGRAPHIQUES ET PHOTOMÉTRIQUES DES FUMITOXINES

Systèmes utilisés: (1) méthanol–chloroforme (2:98); (2) éther éthylique; (3) benzène–acétate d'éthyle (30:70); (4) benzène–acétate d'éthyle (30:70) saturé d'eau; (5) toluène–acétate d'éthyle (50:50) saturé d'acide acétique 1 *N*.

Toxine	R_F					*Constantes spectrophotométriques solns. de 20 µg/ml*	
	Adsorption			*Partition*		$\lambda_{max.}$	*Densité optique*
	1	*2*	*3*	*4*	*5*		
Fumitoxine A	0.26	0.08	0.09	0.33	0.47	320	0.540
						334	0.810
						348	0.740
Fumitoxine B	0.37	0.15	0.16	0.43	0.49	320	0.620
						334	0.750
						348	0.660
Fumitoxine C	0.27	0.13	0.12	0.39	0.49	320	0.670
						334	0.840
						348	0.780
Fumitoxine D	0.23	0.01	0.05	0.21	0.43	320	0.710
						334	0.760
						348	0.640

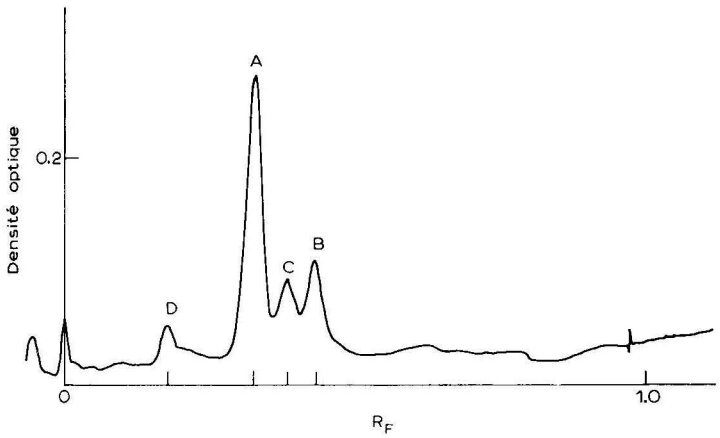


Fig. 1. Courbe photodensitométrique du chromatogramme d'une séparation sur couche mince des fumitoxines A, B, C et D dans un extrait de culture d'*A. fumigatus*. Plaque de gel de silice 60 (Merck) de 0.25 mm d'épaisseur, développée en benzène–acétate d'éthyle (30:70) saturé d'eau. Photomètre intégrateur enregistreur PHI-5 Vernon, source "Mercure" haute pression, bande d'émission à 365 nm.

très reproductibles (écart type: $\pm$ 0.02 pour 8 essais). Le système choisi pour les mesures photodensitométriques est un mélange benzène–acétate d'éthyle 30:70 (v/v) saturé d'eau (Fig. 1). Dans les extraits bruts, la séparation peut être sensiblement améliorée par la technique du développement multiple (2 à 3 développements).

Les spectres d'absorption dans l'UV des fumitoxines en solution éthanolique présentent les trois mêmes $\lambda_{max.}$; pour chacun de ceux-ci, les densités optiques sont très proches (Tableau I). L'essentiel de l'absorption se fait dans l'UV de grande longueur d'onde (330–350 nm). Ceci rend possible des mesures photodensitométriques par transparence, au travers de couches minces dont le support, en verre, n'arrête que

TABLEAU II

RELATION ENTRE LES QUANTITÉS DE FUMITOXINE A DÉPOSÉE ET L'ABSORPTION DANS L'UV EN CCM

Quantité de toxine déposée (µg)	*Lecture*		*Erreur (%)*	*Nombre d'essais*
	Intégration (coups)	*Surface des pics (mm²)*		
20	370.5	1482	3.2	11
15	334.7	1320	2.8	9
10	321.7	1161	3.3	9
5	181.3	623	3.4	9
2	73.2	292	4.2	11
1	38.4	153	4.6	9
0.8	33.5	134	6.5	11
0.4	20.7	83	6.2	6
0.2	13.7	55	8.4	6
0.1	6.7	27	9.7	11
0.05	–	14	32	6
0.027	limite de détection		–	–

TABLEAU III

DOSAGE DE LA FUMITOXINE A DANS UN EXTRAIT CONTAMINÉ DE DRECHE DE BETTERAVE

Concn. de fumitoxine incorporée (10^{-6})	*Concn. mesurée (10^{-6})*	*Écart type pour 6 essais*	*Erreur maximum (%)*	*Volume d'échantillon déposé (µg)**
10	9.83	0.05	2.2	10
5	4.82	0.05	4.8	10
1	1.03	0.02	5.0	10
0.5	0.52	0.03	10.0	20
0.1	0.12	0.02	44.0	30
0.05	non dosable	—	—	(50)

* L'extrait dégraissé de 50 g de drêche est repris par 2 ml de chloroforme.

les rayonnements de courte longueur d'onde. Les résultats de l'analyse des chromato grammes par cette technique sont rapportés dans le Tableau II. Les mesures n'ont été effectuées que sur la fumitoxine A, la plus abondante dans les extraits de cultures fongiques et l'une des plus toxiques sur l'embryon de poulet[4,5], mais les constantes présentées plus haut montrent que la méthode peut être également utilisée pour les trois autres.

La visualisation au moyen de la plaque de cellulose est possible pour des quantités comprises entre 0.02 et 0.04 µg mais cette évaluation approximative n'est pas applicable à un dosage.

Par photodensitométrie, la limite de détection est environ 0.03 µg; mais au delà de 0.1 µg, l'erreur est égale ou supérieure à 10%.

L'application de la méthode à l'analyse d'un produit végétal n'apparait possible que dans le cas de concentrations de fumitoxine supérieures à $0.5 \cdot 10^{-6}$ (Tableau III); pour des concentrations plus faibles, le volume d'échantillon à déposer est incompatible avec une résolution correcte sur couche mince. En revanche la méthode est utilisable dans l'étude de la production des fumitoxines par des cultures d'*A. fumigatus*, les concentrations dans les extraits étant de l'ordre de $1 \cdot 10^{-2}$ à $1 \cdot 10^{-4}$.

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Note

Separation of optical isomers of metal complexes by paper electrophoresis in mixtures of aluminium chloride and tartrate

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(Received May 10th, 1978)

The first paper dealing with the electrophoretic separation of optical isomers is that of Yoneda and Miura[1] who separated the two optical forms of $Co(en)_3^{3+}$ using a mixture of optically active disodium tartrate and aluminium chloride as electrolyte.

This work was then extended to tris–ethylenediamine complexes of rhodium and chromium[2]. These separations have one feature in common, they all have rather large differences in electrophoretic movement and the slower band is always much longer than the faster band.

The same mixture of tartrate and aluminium salts also produced separation of the optical forms of $Co(en)_3^{3+}$ in chromatography on silica gel. Again the slower spot is rather an elongated trail and the faster spot well-defined and close to the liquid front[3].

While Yoneda states that with his electrophoretic technique (low potential: 250 V/34 cm for 2–3 h) no separations of optical isomers were observed in tartrate solutions in the absence of aluminium, Ossicini and Celli[4] did obtain separations in tartrate alone with high-voltage electrophoresis. In tartrate alone both spots are round and of the same size, and the separation distance is always much lower than in the tartrate–aluminium mixtures.

The high-voltage separations were then extended by Cardaci *et al.*[5] to numerous metal complexes and to a number of optically active electrolytes. The separations in, for example, antimony tartrate or arsenic tartrate were all similar to those in tartrate *i.e.* with rather round spots and small differences in mobility. Thus so far the tartrate–aluminium system was found to be unique both for large separation factors and elongated spots.

We therefore wanted to extend this work with the hope of discovering something about the mechanism of the separations in tartrate–aluminium solutions and our results are reported here.

EXPERIMENTAL

Chromatographic experiments

We found that the separations described by Yoneda and Baba[3] were highly reproducible and even improved on Merck silica gel thin layers for high-performance

thin-layer chromatography. We then also noted that these separations are independent of the support and are obtained both on cellulose thin layers and paper strips. Typical results are shown in Fig. 1. Thus the mechanism is unlikely to involve specific adsorption on silica gel. We have also chromatographed some other Co(III) complexes on Whatman No. 3MM paper. $Co(NH_3)_6^{3+}$, which precipitates as a long trail, $Co(pn)_3^{3+}$, $Co(tn)_3^{3+}$, $Co(dip)_3^{3+}$ and $Co(o\text{-phen})_3^{3+}$ all move close to the liquid front when developed with 0.2 *M* ammonium *d*-tartrate + 0.1 *M* $AlCl_3$.

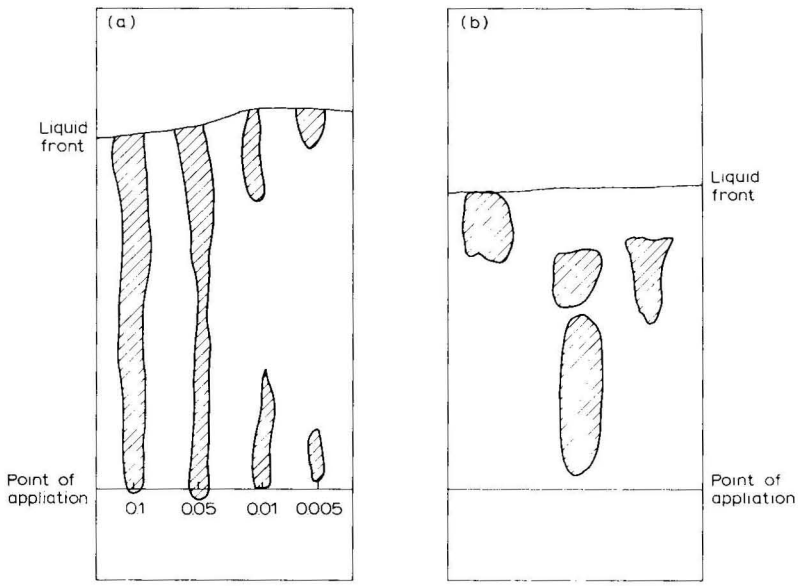


Fig. 1. Typical chromatograms on Whatman No. 3MM paper developed acendingly with an aqueous solution of 0.2 *M* ammonium-*d*-tartrate and 0.1 *M* $AlCl_3$. (a) Various concentrations of $Co(en)_3Cl_3$ solution chromatographed side by side. With 0.1 and 0.05 *M* solutions only long streaks are formed. With 0.01 and 0.005 *M* solutions good separations are obtained. (b) Several complexes are chromatographed side by side: (from left to right) $Co(o\text{-phen})_3^{3+}$, $Co(en)_3$ and $Co(pn)_3^{3+}$.

Paper electrophoretic experiments

In paper electrophoretic experiments if one carries out a separation and then reverses the current for the same length of time, all spots should return to the origin unless they were altered chemically during the electrophoretic run.

In tartrate–aluminium solutions we observed that the faster round spot did indeed return to the origin on reversing the current, while the slower elongated spot remained where it was. Thus the species did not migrate in the normal manner in solution.

Mixing the electrolyte and the solution of $Co(en)_3^{3+}$

When the two solutions were mixed a cloudy appearance was noticed immediately and a rather gelatinous precipitate formed within seconds. We have prepared this precipitate using various ratios of the two solutions. It is always gelatinous and does not show any crystalline structure under a low power microscope even on

aging and drying. The analyses for the Co–Al ratio varied with the ratio of the $Co(en)_3^{3+}$–electrolyte mixed to prepare it.

Typical results obtained are:

(1) The precipitate formed by mixing $Co(en)_3Cl_3$ and a solution of 0.4 *M* ammonium *d*-tartrate and 0.2 *M* $AlCl_3$ (12.1% Al; 8.1% Co).

(2) The precipitate formed by mixing $Co(en)_3Cl_3$ and a solution of 0.1 *M* ammonium tartrate and 0.05 *M* $AlCl_3$ (3.22% Al; 4.58% Co).

(3) The precipitate formed by mixing $Co(en)_3Cl_3$ and a solution containing 0.2 *M* ammonium *d*-tartrate and 0.1 *M* $AlCl_3$ (7.95% Al; 4.96% Co).

CONCLUSIONS

Our observations seem to indicate that the separations obtained in tartrate–aluminium solutions are best described as a form of precipitation chromatography specific for an optical configuration. The gelatinous nature of the precipitate would suggest that there is some form of hydrolytic process in which the addition of the cobalt complexes starts a small pH change and a gelatinous aluminium hydroxide–tartrate then preferentially adsorbs one of the optically active forms of the $Co(en)_3^{3+}$.

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Book Review

Affinity chromatography, by J. Turková, Elsevier, Amsterdam, Oxford, New York, 1978, X + 406 pp., price Dfl. 167.000, US$ 69.75, ISBN 0-444-41605-6.

This lucid book provides a readable introduction to, as well as a good text and cookbook on, affinity chromatography. The concept, the components and the art behind affinity chromatography are all explained. In addition, chemical techniques and considerations necessary for the preparation of affinity columns and their implementation are clearly described.

Following a short introduction in which the advantages of the use of affinity properties over physico-chemical properties are discussed, the author considers a working definition of affinity chromatography. Next, the relationship is examined between the fields of immobilized enzymes and affinity chromatography, which according to the author developed concomitantly. Subsequently, a collection of references is given from books and papers related to the subject.

In Chapter 2 the author describes the principles of affinity chromatography and the many possible applications. Chapters 3 and 4 are dedicated to the theory behind affinity chromatography. Chapter 3 will be difficult for the layman and probably only a theoretical chemist will appreciate this chapter. On the other hand Chapter 4 will be useful from both the theoretical and practical points of view, since every biochemist would like to determine whether his affinity procedure is authentically affinity based or whether other factors are also involved. Chapter 4 also includes procedures for the determination of dissociation constants for enzymatic reactions using partially purified enzymes.

Chapter 5 contains practical considerations involved in the purification of a protein: the requirement of spacers, concentration of ligands, effect of temperature, pH, ionic strength, etc. Chapter 6 provides examples illustrating the purification of different compounds and the rationale behind the various approaches. The isolation of experimental systems is given in increasing complexity from the purification of single proteins up to whole cells. In the last part of Chapter 6 the author provides a solution for lazy people by presenting a list of companies and the covalently bound ligands which they market. Chapter 7 includes all the other kinds of separation techniques which have evolved from the original concept of affinity chromatography, such as hydrophobic chromatography which developed from the spacers introduced for better purification. Other kinds of separation procedures included in this chapter are covalent chromatography, affinity elution and affinity electrophoresis.

Chapters 8, 9 and 10 describe experimental procedures for the coupling of ligands to solid supports, together with experimental details. Chapter 9 contains analytical methods for the determination of the amount of ligand bound. Chapter 10 describes factors involved in the elution of proteins from the column. One of the most important chapters of the book is Chapter 11, which contains a table of most

of the compounds purified up until 1976, making the book an excellent reference work. Finally Chapter 12 gives a short description of immobilized enzymes.

In conclusion, this book will be of great value to technicians, to undergraduate and graduate students (for whom their professors have no extra time) for chemists and biochemists, and even for specialists in the field as a valuable collection of references and an up-to-date record of progress in this exponentially growing area of affinity purification. Finally, as mentioned earlier, this is a text, a reference work and a cookbook combined. People interested in the subject will save a great deal of time by not being required to expend their efforts on original literature. The book not only fulfils its own goal in being a bibliographical review on affinity chromatography, but serves as an encyclopaedia on the subject.

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Book Review

Hochdruck-Flüssigkeits-Chromatographie, by H. Engelhardt, Springer, Berlin, Heidelberg, New York, 2nd ed., 1977, X + 257 pp, 62 figs., 18 tables, price DM 64.00, US$ 28.20, ISBN 3-540-08263-8

The present second edition of Engelhardt's book was edited only two years after the first one which may reflect the rapid development in the field of high-performance liquid chromatography (HPLC). Although the general outline of the book —12 chapters on the most relevant topics— remains unchanged, the revised edition contains significant improvements and up-to-date information.

In Chapter II, dealing with the principles of HPLC, a discussion on peak broadening and particle diameter of packing was newly included with consideration of the ultimate developments of small, totally-porous microparticle packings. Chapter III, which is concerned with the basic components of an LC system, was extended by a section on the characterisation and on test procedures of columns which enables the chromatographer to distinguish between well and badly packed columns. Chapter IV, which is especially reserved for detectors, covers the newest developments in this field, particularly in post-column chemical reactors. Chapter IV on adsorbents has been rewritten taking into account the major importance of reversed-phase silica packings over bare silica. The different techniques in column operation such as column coupling etc. are also discussed here. Chapter IX on size exclusion chromatography was extended to the separation of biopolymers exemplified by some results of the authors own work.

The book provides a helpful means for all who start to use HPLC as a separation technique and look for an appropriate phase system and conditions for their separation problems.

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Book Review

Radiochromatography —The chromatography and electrophoresis of radiolabelled compounds (Journal of Chromatography Library, Vol. 14), by T. R. Roberts, Elsevier, Amsterdam, Oxford, New York, 1978, X + 174 pp., price Dfl. 90.00, US$ 39.95, ISBN 0-444-41656-0.

Studies aimed at elucidating the mechanism of chemical reactions or at following the course of the biosynthesis and metabolism of a substance are greatly facilitated by the use of compounds labelled with radio-isotopes. Chromatographic techniques are indispensable for the analysis and purification of such labelled substances and for the separation of complex mixtures derived from them *in vitro* or *in vivo*. Radioactively labelled compounds that have been resolved by chromatography can be detected easily and measured very accurately. Thus, the use of radio-isotopes and the application of chromatographic methods complement each other in many ways.

In view of the significance and widespread use of radiochromatography in chemical and biomedical research, it is surprising that the book by Roberts is the first to cover this topic systematically and comprehensively.

First, various methods for the detection and quantitative determination of radioactive material are discussed in detail. The following chapters are devoted to paper chromatography, thin-layer chromatography and other chromatographic techniques as well as electrophoresis. Each of these chapters is divided into a theoretical section including a discussion of the choice of method, a description of practical aspects, a survey of applications and a list of pertinent references. An appendix contains the addresses of manufacturers of liquid scintillation counters, scanners for paper and thin-layer chromatograms, radio-gas chromatographs and other instruments.

The book is well organized and concisely written. It contains a wealth of information and, therefore, it should be of value to all working with labelled compounds. The reviewer is certainly happy to have it.

Like the preceding volumes of the *Journal of Chromatography Library*, the present book has been produced with great care and its price appears reasonable.

Münster (G.F.R.) H. K. MANGOLD

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Errata

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Page 458, legend to Fig. 7, last line should read "stationary phase, 3% OV-17 on Chromosorb Q (80–100 mesh). Other conditions as in Fig. 8".

Page 459, legend to Fig. 8, 2nd line, "3% OV-17" should read "5% OV-101".

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford. New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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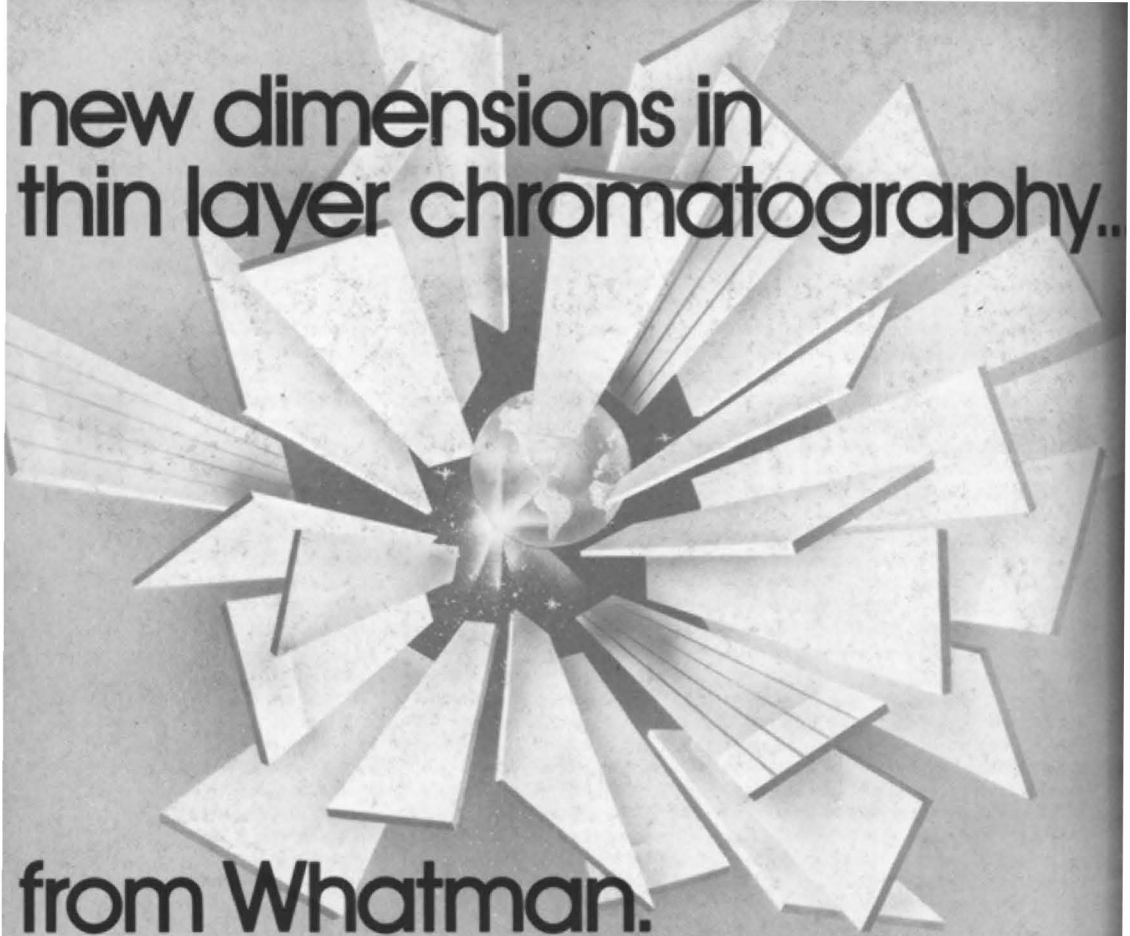
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