Journal of Chromatography, 185 (1979) 429–444

CHROM. 12,037

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XX*. INVESTIGATION OF THE EFFECT OF pH AND ION-PAIR FORMATION ON THE RETENTION OF PEPTIDES ON CHEMICALLY-BONDED HYDROCARBONACEOUS STATIONARY PHASES

MILTON T. W. HEARN** and BORIS GREGO

Immunopathology Research Unit, Medical Research Council of New Zealand, University of Otago Medical School, P.O. Box 913, Dunedin (New Zealand)

and

WILLIAM S. HANCOCK

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North (New Zealand)

SUMMARY

The effects of different anionic and cationic reagents on the retention of a series of dipeptides chromatographed on μBondapak C_{18} reversed-phase supports are described. At low pH, hydrophobic anionic reagents result in increased retention of the peptide samples whereas hydrophobic cationic reagents cause decreased retention. These trends can be explained on the basis of either ion-pairing or ion-exchange interactions of the reagent with the protonated peptide. Equations based on the ligand adsorption model for ion-pair reversed-phase chromatography accurately describe the dependency of the capacity factor on the counter-ion concentration as well as on the pH. Hyperbolic and parabolic dependencies of the capacity factor on the counter-ion concentration were observed depending on the hydrophobic nature of the counter-ionic reagent. For short alkyl chain reagents, ion-pairing formation appears to govern retention whereas for long chain reagents ion-exchange mechanisms dominate. A local minimum in capacity factor occurs when the pH of the mobile phase corresponds to the pI of the peptide. Although it is possible to predict elution conditions based on pH from a minimum set of capacity factor and pH measurements for closely related peptides, such as those present in a crude synthetic preparation or in a fraction isolated from natural sources, there is little practical advantage in using pH variation as the sole method to influence selectivity. However, large selectivity differences can be easily achieved by the addition at suitable pH of low concentrations, *e.g.* 5 m*M*, of suitable reagents that can either undergo ion-pair formation with the peptides or modify the stationary phase to a dynamic ion-exchanger. These methods can be

* For Part XIX, see ref. 17.

** To whom correspondence should be addressed.

rigorously used to establish criteria for the homogeneity of peptides analogous to those based on thin-layer chromatography in several different solvent systems. Their value in the analysis of proteolytic digests of proteins is discussed.

INTRODUCTION

When high-performance liquid chromatography (HPLC) was introduced as an analytical tool, it was soon recognised that this technique had the potential to allow the selective and rapid separation of natural amino acids, as well as peptides of various molecular weights. Early application of HPLC to the analysis and isolation of underivatised peptides and proteins using polar adsorption supports, liquid–solid or liquid–liquid reversed-phase partition systems, although encouraging, were frequently associated with poor resolution, lack of reproducibility and low recoveries. Over the past several years, reversed-phase liquid chromatographic methods have attracted the most attention. The microparticulate bonded phases, in particular, have gained rapid acceptance due, in part, to their stability and consequential advantages of high column efficiency and good reproducibility when associated with water–alcohol or water–acetonitrile elution mixtures. To be of general application in peptide and protein chemistry, HPLC techniques must be able to take into account several practical considerations. First, wide structural and functional variation exists with amino acid derivatives, and the separation methods should have the potential to distinguish not only major differences in composition and sequence but also minor changes, *e.g.* an amino acid replacement or deletion. Secondly, the elution conditions must be compatible with high sensitivity detection systems since many natural peptidic molecules are available only in trace amounts. Obviously, chromatographic conditions which optimise the electrostatic, hydrogen bonding or hydrophobic interactions of the solute molecules with components in the mobile or stationary phase will determine the ease of a separation. In recent publications[1–4], we have explored several strategies for the analysis and isolation of underivatised amino acids, peptides and proteins from natural and synthetic sources. These studies, as well as reports from other laboratories[5–12], clearly demonstrated that the separation of peptides and proteins using ion-pair reversed-phase HPLC systems is a practical and very rapid alternative to conventional techniques of analysis or purification, *e.g.* thin-layer or ion-exchange chromatographic techniques.

Selectivity in chromatographic separations is mediated by differences in the distribution equilibrium of the analyte molecules between the mobile and stationary phases. However, secondary equilibria between the analyte molecules and components present in the mobile phase can dramatically influence the retention characteristics. For example, in a recent paper[13] we compared the effect of moderately hydrophobic, *e.g.* hexanesulphonate, and extremely hydrophobic, *e.g.* dodecylsulphate, and hydrophilic ion-pairing reagents on the retention time of a group of argininyl peptides. Major selectivity differences were noted with these compounds consistent with the involvement of different retention mechanisms depending on the nature of the ion-pairing reagent. Besides the participation, under suitable conditions, of ion-pairing equilibria, protonic equilibria in the mobile phase also have a pronounced effect on the retention of underivatised peptides with non-polar stationary phases. In order

to clarify the effect of pH and ion-pair formation, we have examined the chromatographic retention of a series of dipeptides on octadecylsilica columns under elution conditions where these two parameters are varied. The experimental data presented in this report allow these influences to be quantitatively assessed in terms of the secondary equilibria involved.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which included two M6000A solvent delivery systems, a M660 solvent programmer and an U6K universal liquid chromatograph injector, coupled to a M440 fixed wavelength or a M450 variable wavelength UV monitor and a Rikadenki dual-channel recorder. The μBondapak columns (10 μm, 30 cm × 4 mm I.D.) were obtained from Waters Assoc. Sample injections were made with Pressure-Lok liquid syringes (0–25 μl, 0–100 μl) Series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). Filtration of solvents was carried out using a Pyrex filter holder (Millipore Corp., Bedford, Mass., U.S.A.), and peptide samples were filtered using a Swinney Filter assembly (Millipore) with AP2500 filters.

Reagents

All solvents were AnalaR grade and were purified as described previously[1]. Orthophosphoric acid and potassium dihydrogen phosphate were from May and Baker (Dagenham, Great Britain). The ion-pairing reagents were obtained from the following suppliers: tetraethylammonium chloride, dodecylamine, sodium dodecylsulphate (specially pure grade), D- and L-camphor-10-sulphonic acid were obtained from BDH (Poole, Great Britain). Sodium hexane sulphonate and sodium heptane sulphonate were prepared from the corresponding alkyl bromides[14]. The peptides were prepared in this laboratory by solid-phase methods using standard procedures or purchased from Research Plus Labs. (Denville, N.J., U.S.A.) or Sigma (St. Louis, Mo., U.S.A.). All amino acids, except glycine, were of the L-configuration. Ovine 19-S thyroglobulin was isolated by established methods[15], and bovine thyrotrophin was provided by the National Pituitary Agency (NIH-bTSH-B8). Tryptic digests were carried out essentially as described by Chernoff and Liu[16].

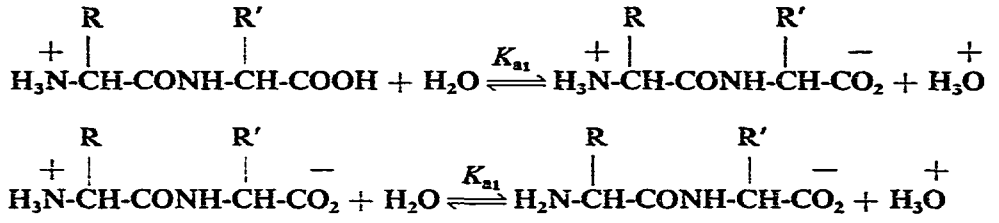
Methods

All chromatograms were carried out at room temperature (*ca.* 20°). All peptides were made up in the eluting solvent. Bulk solvents were degassed separately and the appropriate mobile phases prepared and equilibrated to operating conditions as reported previously[1]. All columns were equilibrated to new mobile phase systems for at least 30 min, and after gradient elution experiments, for 45–60 min with the initial mobile phase. Sample sizes varied between 10 and 100 μg of peptide material injected in volumes of 10–100 μl. The capacity factors were calculated in the usual way using dimethylformamide to calibrate the void time. The data were analysed using a non-linear least-squares fit programme with methods similar to those reported previously[7,17,18].

RESULTS AND DISCUSSIONS

Effect of pH on k'

Simple dipeptides with no ionogenic side-chains will ionise according to



and the change in the capacity factor for such compounds as a function of pH is given by[7,20]

$$k' = \frac{k_0 + k_1\left(\frac{[H^+]}{K_{a1}}\right) + k_2\left(\frac{K_{a2}}{[H^+]}\right)}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}} \qquad (1)$$

where k_0, k_1 and k_2 are the capacity factors for the isoelectric (zwitterionic), cationic and anionic forms, respectively, and K_{a1} and K_{a2} are the respective ionisation constants for the two steps in the ionisation processes. The amino acid side-chains, R and R', have only a small effect on the ionisation equilibria when they contain no ionisable groups. However, these side-chains will contribute to the relative hydrophobicities of the peptides, and their effect can be directly described in terms of hydrophobicity fragmental constants or other topological indices[19]. For peptides that contain additional ionisable centres, the fundamental equation relating the capacity factor to pH must be expanded. Thus for the series of dipeptides of the type X-Tyr used in the present study the relationship between k' and pH can be expressed as

$$k' = \frac{k_0 + k_1\left(\frac{[H^+]}{K_{a1}}\right) + k_2\left(\frac{K_{a2}}{[H^+]}\right) + k_3\left(\frac{K_{a2}K_{a3}}{[H^+]^2}\right)}{1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]} + \frac{K_{a2}K_{a3}}{[H^+]^2}} \qquad (2)$$

where k_0, k_1, k_2 and k_3 are the capacity factors for the isoelectric, cationic, anionic and doubly charged anionic species, respectively, and K_{a1}, K_{a2} and K_{a3} are the three ionisation constants. This equation predicts a progressive increase in capacity factor as the pH is lowered below the p*I* value of the peptide. When the pH and p*I* become coincident a local minimum occurs. Only at pH values greater than 8 will the ionisation of the phenolic group significantly influence the retention. For peptides containing amino acids with additional ionogenic side-chains, an additional term is required in eqn. 2 for each ionisable centre. The dependency of the capacity factor of a polypeptide on pH can thus be written in terms of a polynomial expression in terms of pH and pK_{a1} ... pK_{an}. Any change in the ionisation state of free carboxyl or amino groups will result in a variation in k'.

The general form of eqn. 1 has been shown to be valid for the adsorption of

weak organic acids, weak organic amines, amino acid and monoprotic phenol derivatives[7,10,20]. Fig. 1 shows the plots of the experimentally determined k' values on μBondapak C_{18} columns of the X-Tyr peptides as a function of pH at constant ionic strength and mobile phase composition. Because of the chemical instability of μBondapak C_{18} columns at pH values greater than 7.5, a pH range of 3.0–7.2 was used. These results are very similar to those observed for amino acids on μBondapak C_{18} supports, as well as for the retention characteristics of amino acids and peptides on Amberlite XAD-4 and XAD-7 reversed-phase adsorbants[7]. The observed plots for the X-Tyr peptides, as well as those reported for tetra-alanine and triglycine[7] over the pH range studied, closely follow that predicted by eqns. 1 and 2 with local minima in k' occurring near the isoelectric point. As the hydrophobic character of the side-chain increases, there is also a progressive increase in retention with k' for the X-Tyr peptides changing in the order X = Leu > Pro > Ala. It is noteworthy that at pH 3.0, Gly-Tyr was unresolved on a μBondapak C_{18} column from Ala-Tyr. With a series of related peptides, large selectivity differences generally arise as a consequence of major changes in the hydrophobicity of the side-chains, with pH-dependent effects involving the α-amino groups having only a secondary influence. For example, the presence of a lysinyl residue in a peptide will result, at low pH, in an additional cationic charged centre due to protonation of the ε-amino group. This will have the effect of decreasing the hydrophobicity of the lysinyl peptide relative to, say, a glycine analogue, and result in a shorter retention time. The converse will apply to peptides with acidic amino acid residues, *e.g.* aspartic acid, where protonation at low pH will cause ionic suppression of the carboxyl groups leading to enhanced retention. As can be seen from Fig. 1, there is a decreased column selectivity for the peptides X-Tyr as the pH is increased from 3.0 to 7.2. This observa-

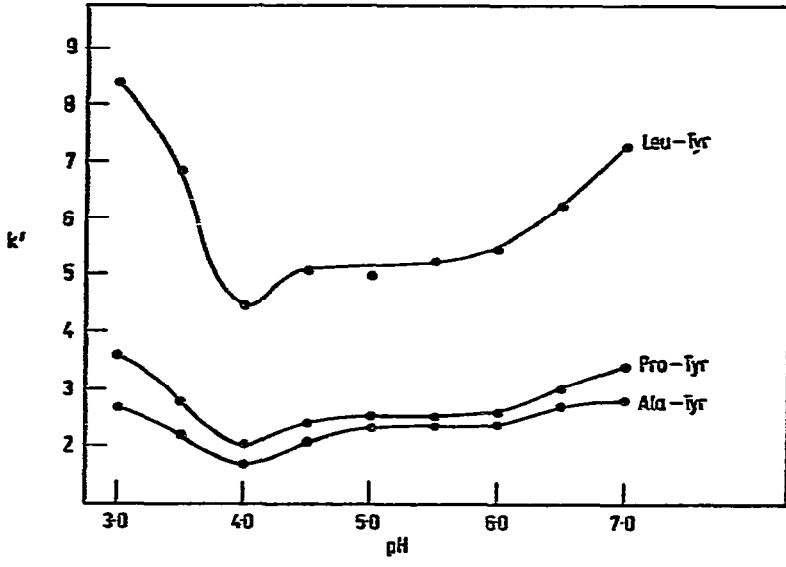


Fig. 1. Capacity factors for the X-Tyr peptides on a μBondapak C_{18} reversed-phase column as a function of pH. The eluent was 5% methanol–95% water–5 m*M* H_3PO_4 tritrated to the appropriate pH with NaOH, at a flow-rate of 2 ml/min.

tion is consistent with our early studies[1,21] with peptides related to angiotensin and other hormonal peptides, and with complementary data obtained by Kroeff and Pietrzyk[7] with X-Phe dipeptides separated on Amberlite XAD-2. Krummen and Frei[6] have also noted a similar trend with nonapeptides related to oxytocin. These results suggest that, although it is possible to predict the elution conditions based on pH from a minimum set of k' and pH measurements, there is little practical advantage in using pH variation for the separation of closely related peptides. One method by which large selectivity differences can however be achieved, is the addition to the mobile phase of low concentrations, *c.a.* 5 m*M*, of reagents that can either undergo ion-pair formation with the peptides or modify the characteristics of the stationary phase.

Effect of ion-pair formation on k'

Several fundamental mechanistic models for ion-pair chromatography on normal and reversed phases have recently been proposed in order to describe quantitatively the influence of the nature and concentration of the counter-ion, the phase volume ratio and the physical properties of the stationary and mobile phases. Conceptually, these models are based either on partition or on adsorption processes[18,22]. In the first case, solute retention is assumed to be related to the stationary phase volume through the partition coefficient, whereas for the second case, solute retention is related to the total interfacial surface area through the adsorption coefficient. The current surge of interest in reversed phase ion-pairing techniques with chemically bonded non-polar phases has highlighted several important considerations as far as the relevance of ion-pair extraction theory to ion-pair chromatography is concerned. One important requirement of the liquid–liquid partition model is that the stationary phase behaves as a bulk liquid. Recent theoretical and experimental studies, particularly by Horváth and his co-workers[18,20], have indicated that the partition model is inappropriate for chemically bonded reversed-phase systems, because the attached hydrocarbon will have restricted translational and rotational degrees of freedom compared with an unbound non-polar phase and, in addition, will have an effective film thickness similar to a monolayer. Solvophobic theory, as originally developed by Sinanoglu[23], can be adapted to describe quantitatively in thermodynamic terms the interaction of solute molecules with hydrocarbonaceous moieties bonded to porous silica particles[12]. In this treatment, the capacity factors can be directly related to the equilibrium association constants of a family of binding interactions between the charged solute molecules, the counter-ion and the immobilised non-polar ligand. These interactions are assumed to occur independently and reversibly and to be due to hydrophobic and not electrostatic or hydrogen-bonding effects. Under these conditions, the interaction of a cationic solute with an anionic counter-ion to form a neutral complex that can be adsorbed to a non-polar ligand, chemically bound to an inert support, will result in a modification in the capacity factor as given by

$$k' = \frac{(k_0 + \beta[\mathrm{B}])}{(1 + K_2[\mathrm{B}])\,(1 + K_3[\mathrm{B}])} \qquad (3)$$

where k_0 is the capacity factor in the absence of the counter-ionic species B, and K_2, K_3 and β are association constant terms describing the phenomena. (For the derivation of this equation and a discussion of the significance of K_2, K_3 and β see refs.

18 and 22.) If the direct binding of the counter-ion to the non-polar ligand (thus generating a dynamic liquid ion-exchanger) is not significant, *i.e.* $K_3[B] \ll 1$, then eqn. 3 simplifies to

$$k' = \frac{(k_0 + \beta[B])}{(1 + \gamma[B])} \quad (4)$$

The foregoing considerations assume that the counter-ion interacts stoichiometrically with a strong cationic electrolyte. In general, peptides will only exist as strongly cationic species when the pK_{a2} — pH difference is greater than 2. The pH 3.0 condition, used for the present study on the effect of counter-ion concentration on the capacity factors, was chosen to be well below the isoelectric points of the X-Tyr peptides. Under these conditions pK_{a2} — pH values for the X-Tyr peptides will all be greater than 7, *i.e.* all available amino groups will be fully protonated, whilst the degree of dissociation of the tyrosinyl carboxyl group will be essentially constant throughout the peptide series. The interaction of a charged peptide with a suitable counter-ion can be visualised as involving the formation of solvent cavities surrounding neutral caged species that can subsequently bind to the non-polar ligand. The retention of a series of peptides in the presence of ion-pairing reagents at low pH should thus follow the order of relative hydrophobicities determined by the nature of the amino acid side-chains and the polarity of the counterion. Thus, a peptide containing an *N*-terminal basic amino acid, *e.g.* arginine, will have a shorter retention time on non-polar phases at low pH with hydrophilic anionic ion-pairing reagents present in the mobile phase than a corresponding peptide with, say, an *N*-terminal alanine. However, major selectivity changes will occur when anionic hydrophobic ion-pairing reagents are present in the mobile phase at low pH owing to formation of ion pairs with the doubly charged argininyl residue (Table I). Very hydrophobic reagents, *e.g.* dodecylsulphate, may significantly bind to the non-polar stationary phase, thus modifying its characteristics. Under these circumstances, dynamic ion exchange may be the underlying mechanism. Selectivity changes with peptides have been observed[3] under conditions compatible with ion-pair formation, and parallel similar observations with amino acids. The data presented below verify the suitability

TABLE I

EFFECT OF ION-PAIRING REAGENTS ON THE RETENTION TIME OF ARGININYL PEPTIDES

Column: μBondapak alkylphenyl using methanol–water (50:50). (a) 5 m*M*, pH 2.5; (b) 5 m*M*, pH 6.5; (c) 5 m*M*, pH 7.15; (d) 2 m*M*, pH 4.0. The data are from references 4 and 13. The one-letter code for the amino acids is used by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Md, U.S.A., A = alanine; F = phenylalanine; L = leucine; M = methionine; R = arginine; W = tryptophan.

Peptide	*Retention time (min)*					
		(a), $H_2PO_4^-$	*(b)*, $C_6H_{13}SO_3^-$	*(c)*, $C_{12}H_{25}SO_4^-$	*(d)*, $C_{12}H_{25}NH_3^+$	*(d)*, $(C_3H_7)_4\overset{+}{N}$
M-R-F	32.5	2.4	3.6	>60	1.5	3.0
R-F-A	> 50	2.1	3.0	33.2	1.3	2.1, 2.7
L-W-M-R	112	2.3	4.0	16.2	1.6	3.5
L-W-M-R-F	>120	5.1	10.2	40.5	2.1	8.7

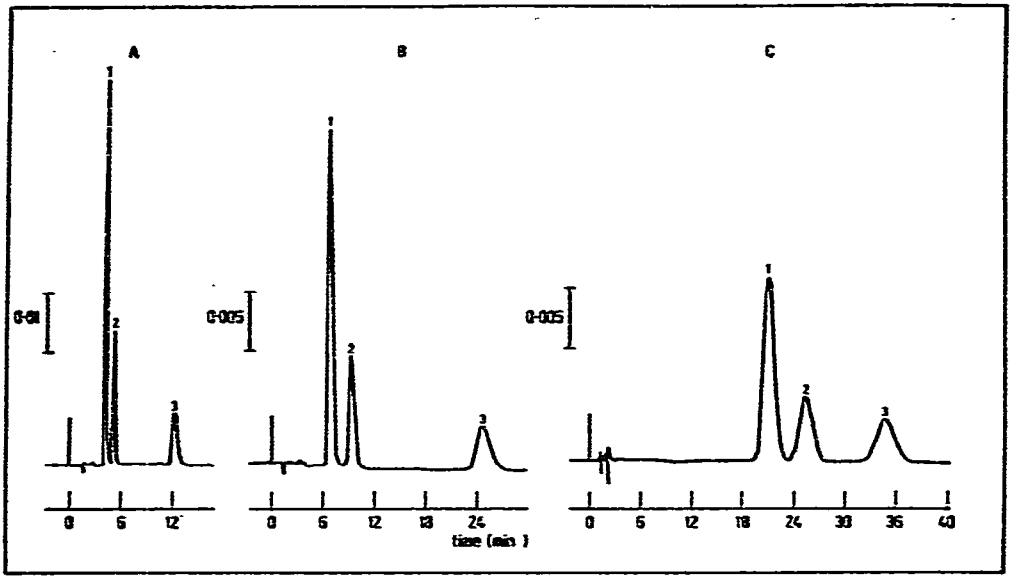


Fig. 2. Chromatograms illustrating the effect of ion-pair formation on the separation of the dipeptides Ala-Tyr (1), Pro-Tyr (2) and Leu-Tyr (3) by reversed-phase chromatography. Column, μBondapak C_{18}; flow-rate 2 ml/min; temperature 20°; eluents: A, 5% methanol–95% water–50 m*M* KH_2PO_4–5 m*M* H_3PO_4 pH 3.0; B, same as A and 5 m*M* hexylsulphonate; C, 50% methanol–50% water–50 m*M* KH_2PO_4–5 m*M* H_3PO_4–5 m*M* dodecylsulphate, pH 3.0.

of the ligand adsorption model for predicting the dependence of the capacity factors of peptides on the nature and concentration of ion-pairing reagents.

A typical chromatogram illustrating the separation of the X-Tyr dipeptides on a μBondapak C_{18} column in the absence and presence of 5 m*M* *n*-hexyl sulphonate in a 5% methanol–water–50 m*M* KH_2PO_4, pH 3.0, buffer is shown in Fig. 2 (a and b), and Fig. 2 (c) shows the separation of the dipeptides using a 50% methanol–water–50 m*M* KH_2PO_4, pH 3.0, eluent containing 5 m*M* dodecylsulphate. The higher methanol concentration was required in this case to obtain comparable retention times. Experiments were carried out with different concentrations of four anionic hydrophobic ion-pairing reagents, namely, *n*-hexyl-, *n*-heptyl-, D-camphor-10- and L-camphor-10-sulphonic acid. The two camphor derivatives, being chiral, were used to examine whether retention could be influenced by an optically active centre. If the binding of the hydrophobic counter-ion to the non-polar ligand is negligible, and the concentration of the neutral ion-paired species in the mobile phase is small, then the predicted plot of capacity factor against counter-ion concentration should initially increase but eventually become independent of increasing counter-ion concentrations, *i.e.* follows a rectangular hyperbola. As the binding of the counter-ion to the stationary phase, or the concentration of the neutral ion-paired species in the mobile phase, becomes more significant then the predicted shape of the plot of capacity factor against counter-ion concentration should follow more closely a parabolic dependency. With small polar molecules, *e.g.* catecholamines, both hyperbolic and

parabolic dependencies of the capacity factor on counter-ion concentration have been reported[18,24]. As is apparent from Fig. 3–6, dipeptides of the type X-Tyr behave in a similar fashion, and their retention characteristics on μBondapak C_{18} columns in the presence of varying amounts of anionic ion-pairing reagents can be discussed in terms of eqn. 3 or 4. Inspection of the data shown in Figs. 3–6 suggests, in addition, that the retention of the peptides under ion-pairing conditions is dependent on the hydrophobic area of the counter-ion and follows the order camphor- > heptyl- > hexyl-sulphonate. The finding that L-camphor-10-sulphonic acid had a uniformly smaller influence on the capacity factor of the various peptides than the corresponding D-isomer is interesting and indicates that a more detailed study of the effect of enantiomeric ion-pairing reagents would be justified. The above analysis of the influence of ion-pair formation on the capacity factor of peptides also permits an insight into the adsorption mechanism of very hydrophobic anionic reagents such as dodecylsulphate, as well as comparable cationic reagents. Probably the most important property of the counter-ionic species in influencing the retention is its charge density. Small, polar counter-ions, *e.g.* $H_2PO_4^-$, ClO_4^-, $(CH_3)_4N^+$, will tend to have minimal interactions with the stationary phase and operate with peptides *via* hydrophilic ion-pairing mechanisms, *i.e.* retention times are reduced by the addition of the counter-ion. Reagents of intermediate polarity, *e.g.* heptanesulphonate, $(C_4H_9)_4N^+$, will act *via* a hydrophobic ion-pairing mechanism with the retention times enhanced

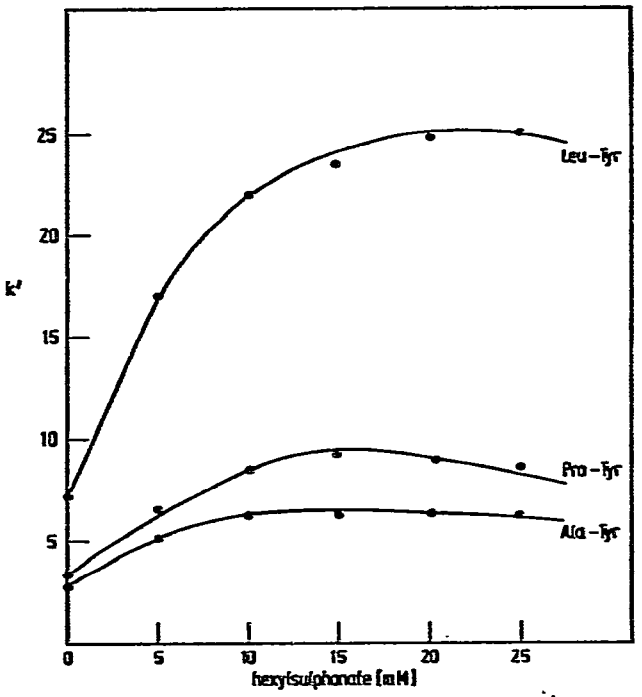


Fig. 3. Dependence of the capacity factor of the protonated X-Tyr peptides on the concentration of *n*-hexylsulphonate in the mobile phase. Column: μBondapak C_{18}; flow-rate 2 ml/min; temperature 20°; mobile phase 5% methanol–95% water–50 m*M* KH_2PO_4–5 m*M* H_3PO_4, pH 3.0, containing various concentrations of the ion-pairing reagent.

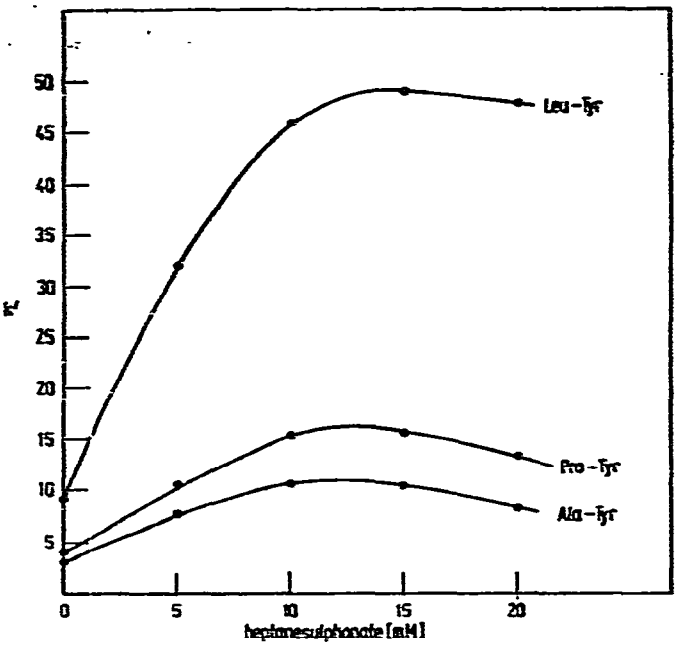


Fig. 4. Dependence of the capacity factor of the protonated X-Tyr peptides on the concentration of *n*-heptylsulphonate in the mobile phase. Other conditions as in Fig. 3.

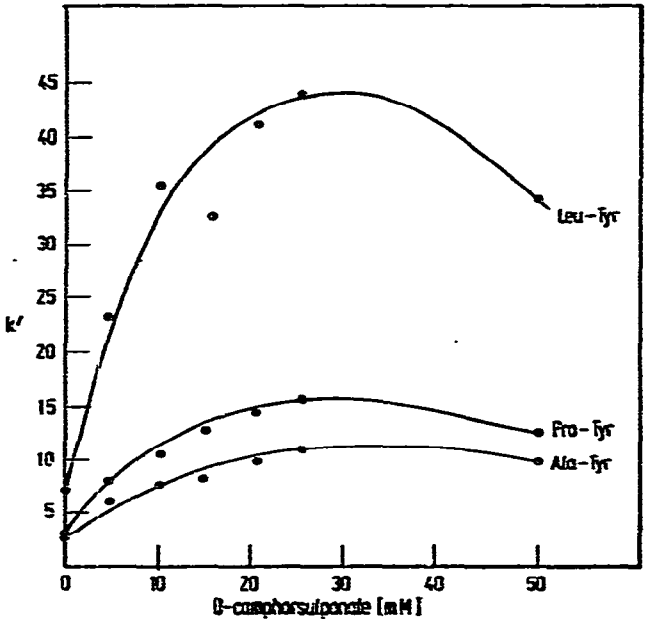


Fig. 5. Dependence of the capacity factor of the protonated X-Tyr peptides on the concentration of D-camphor-10-sulphonate in the mobile phase. Other conditions as in Fig. 3.

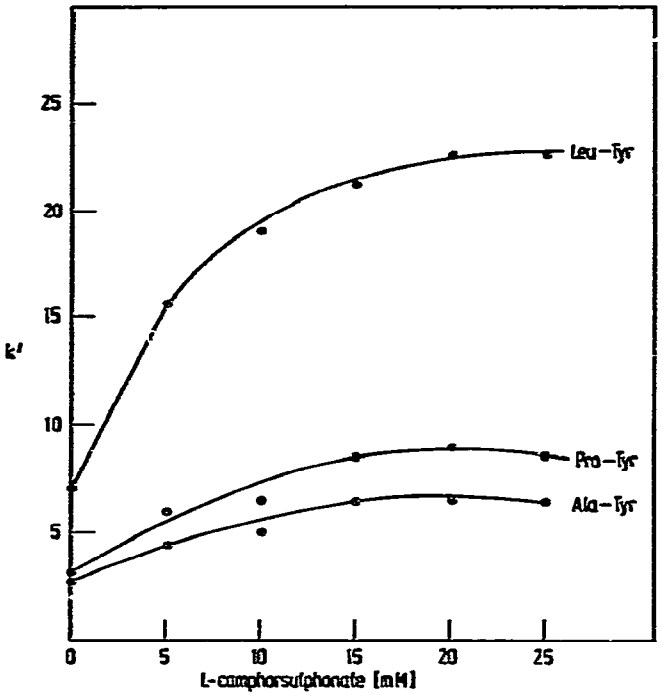


Fig. 6. Dependence of the capacity factor of the protonated X-Tyr peptides on the concentration of L-camphor-10-sulphonate in the mobile phase. Other conditions as in Fig. 3.

by the addition of the counter-ion. Amphiphatic counter-ions like dodecylsulphate or dodecylammonium, on the other hand, could preferentially bind to the stationary phase, which takes on the characteristics of a dynamic ion-exchanger. These solvent-generated stationary phase modifications can permit ionic interactions between the peptide and the stationary phase, and this effect may be a dominant mechanism for both anionic and cationic detergents. For example, at pH 3.0, the tetrapeptide Cys-Ala-Gly-Tyr will be predominantly protonated and should show minimum interaction with a dodecylammonium-modified cationic stationary phase. This will be reflected in reduced retention times when dodecylammonium acetate is present in the mobile phase (Fig. 7). A further consequence of stationary phase modification by amphipathic reagents is that selectivity differences for a series of similarly charged peptide molecules will be reduced. This effect can be seen by comparing the influence of 5 m*M* heptanesulphonate and 5 m*M* dodecylsulphate on the column selectivity for the X-Tyr peptide series shown in Fig. 2b and c. Similar selectivity changes have been observed under conditions of dynamic ion-exchange formation with amino acids and hormonal peptides[3,6,9].

Ion-pairing and stationary phase modification constitute a powerful method for varying the retention of peptides on reversed-phase systems. One area of application is in the analysis of a peptide sample using two different types of mobile phase, *e.g.* with hydrophilic and hydrophobic ion-pairing reagents, which can be used as strong evidence for the homogeneity of the sample similar to thin-layer

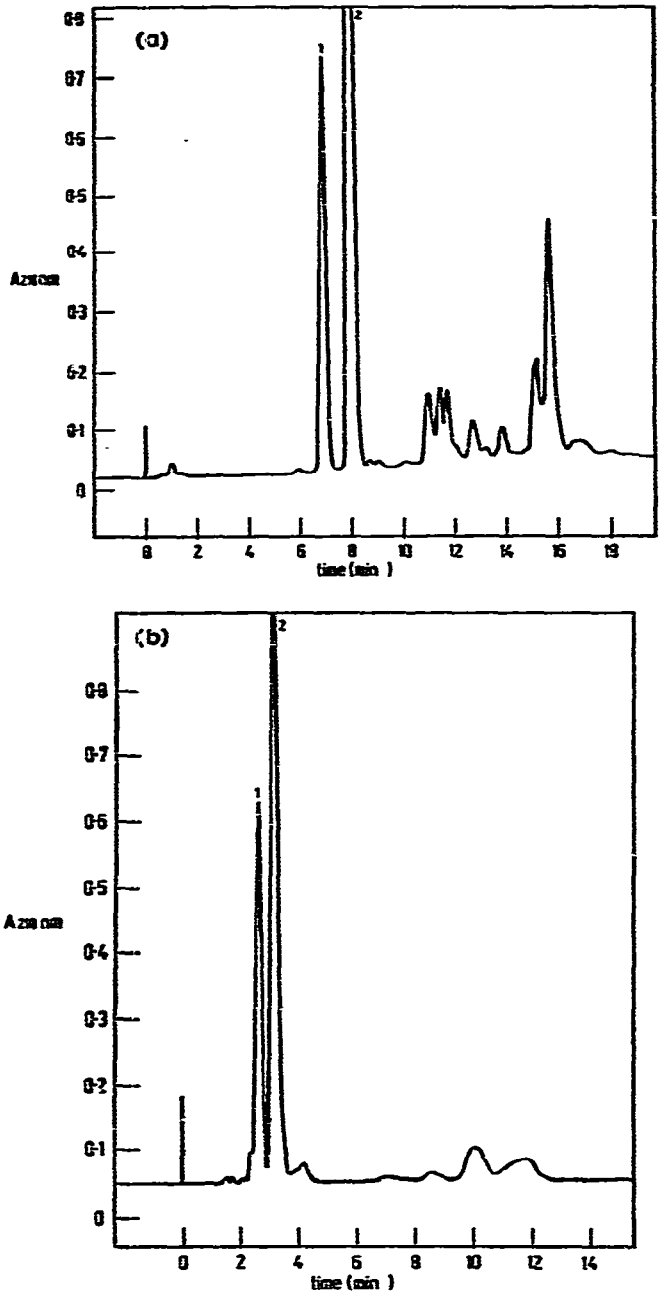


Fig. 7. Analytical chromatograms of a crude peptide mixture containing the tetrapeptide, Cys-Ala-Gly-Tyr, a fragment common to the β-chain of the pituitary glycoproteins, cholera toxin B protein and trypsin, obtained by solid-phase synthesis. Peak 2 corresponds to the tetrapeptide Cys-Ala-Gly-Tyr, peak 1 is a synthetic deletion contaminant. Column: μBondapak C_{18}; flow-rate 2 ml/min; elution conditions, a 10-min linear gradient, started 1 min after the injection of a 25 μg sample, was generated for (a) from water–2 m*M* H_3PO_4 to 40% methanol–60% water–2 m*M* H_3PO_4 and for (b) from water–0.2 m*M* dodecylamine–2 m*H* H_3PO_4 to 40% methanol–60% water–0.2 m*M* dodecylamine–2 m*M* H_3PO_4.

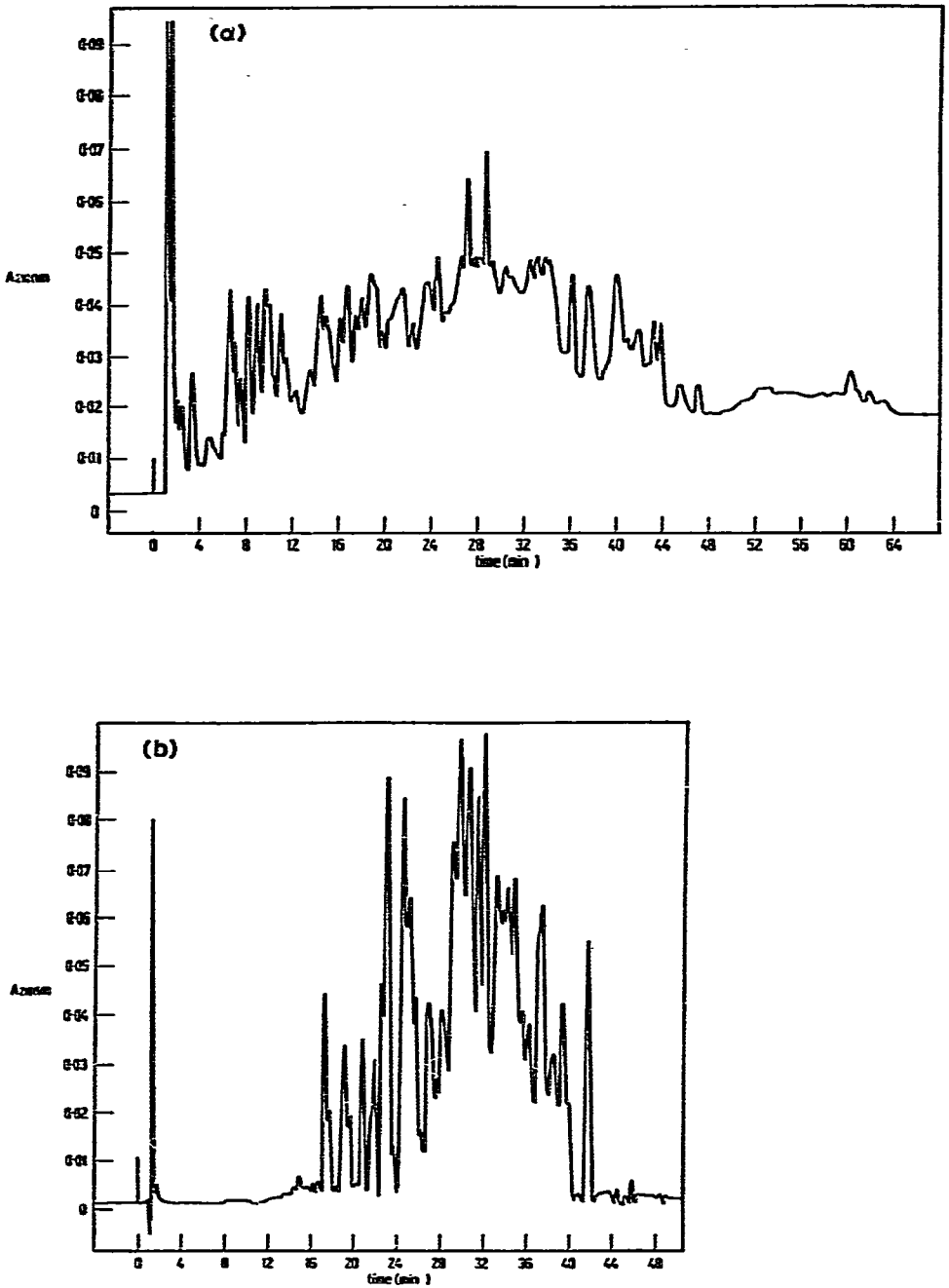


Fig. 8. Gradient elution profiles for the 12-h tryptic digest of sheep 19-S thyroglobulin (500 μg). Column: μBondapak C_{18}; flow-rate 2.5 ml/min; temperature 20°; elution conditions, a 60-min linear gradient was generated for (a) from water–0.1% H_3PO_4 to 50% acetonitrile–50% water–0.1% H_3PO_4 and for (b) water–0.1% H_3PO_4–5 m*M* hexanesulphonate to 50% acetonitrile–50% water–0.1% H_3PO_4–5 m*M* hexanesulphonate.

chromatography in several different solvent systems (*cf.* Fig. 7). An obvious extension of this approach is the assessment of homogeneity and structure of proteins *via* proteolytic digests. In a recent study[25], we described a reversed-phase HPLC method which allows the rapid analysis of the thermolysin digest of acyl carrier protein, as

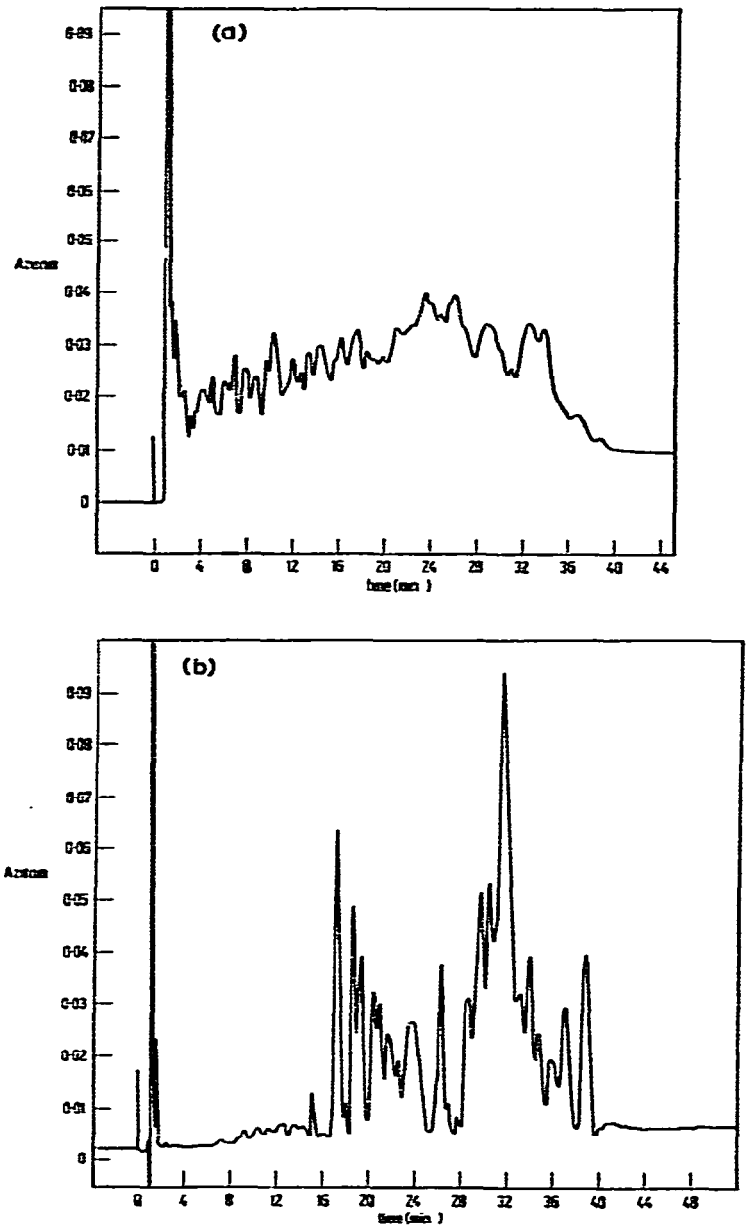


Fig. 9. Gradient elution profiles for the 12-h tryptic digest of bovine thyrotrophin (400 μg). Column: μBondapak C_{18}; flow-rate 3.0 ml/min; the elution conditions for (a) and (b) were the same as shown for Figs. 8a and 8b, respectively.

well as several other proteolytic digests. Although a considerable amount of information regarding the homogeneity and structure of the examined proteins was obtained, a single set of mobile phase conditions did not allow a complete "protein fingerprint" to be made, owing in part to the possibility of overlapping peaks. This is not surprising in view of the wide structural diversity of the proteolytic digestion products, and the fact that resolution under a single set of conditions may not necessarily be optimal. However, the use of two mobile phase conditions of similar composition, differing only in the presence or absence of a low concentration of ion-pairing reagent, provides a much more informative approach. Illustrative of this technique are the pairs of chromatograms, obtained for the tryptic digests of ovine 19-S thyroglobulin and bovine thyrotrophin in the presence and absence of 5 m*M* hexanesulphonate, shown in Figs. 8 and 9. Even with digests of such complexity as produced by these two microheterogeneous proteins, the simplicity of the method, the high sensitivity (only 500 μg of each protein was used) and the excellent resolution permit digestion profiles to be obtained in less than 60 min, with each pair of chromatograms representing a unique protein map analogous to that obtained with two-dimensional electrophoretic techniques. Preliminary preparative studies with these and other protein digests indicate that this approach considerably simplifies unequivocal structural assignments made during the sequencing of proteins. Their use in the determination of the primary structure of mutant haemoglobins and pituitary and thyroid glycoproteins will be reported elsewhere.

ACKNOWLEDGEMENTS

This investigation was supported by the Medical Research Council of New Zealand. Mr. Boris Grego is a recipient of a University Grants Committee Postgraduate Scholarship.

REFERENCES

1 M. T. W. Hearn, C. A. Bishop, W. S. Hancock, D. R. K. Harding and G. D. Reynolds, *J. Liquid Chromatogr.*, 2 (1979) 1.
2 M. T. W. Hearn and W. S. Hancock, *Trends in Biochem. Sci.*, 4 (1979) 58.
3 M. T. W. Hearn and W. S. Hancock, in J. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography*, Marcel Dekker, New York, 1979, pp. 243–271.
4 W. S. Hancock, C. A. Bishop, J. E. Battersby, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
5 J. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 347.
6 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 27.
7 E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, 50 (1978) 502.
8 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
9 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671.
10 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241.
11 J. J. Hansen, T. Greibrokk, B. L. Currie, K. N.-G. Johansson and K. Folkers, *J. Chromatogr.*, 135 (1977) 155.
12 I. M. Johansson and K. G. Wahlund, personal communication.
13 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
14 W. E. Truce and J. P. Norrell, *J. Amer. Chem. Soc.*, 85 (1963) 3231.
15 A. J. Paterson and M. T. W. Hearn, *Aust. J. Exp. Biol. Med. Sci.*, in press.
16 A. I. Chernoff and J. C. Liu, *Blood*, 17 (1961) 54.

17 M. T. W. Hearn and W. S. Hancock, *J. Chromatogr. Sci.*, submitted for publication.
18 Cs. Horváth, W. Melander, I. Molnár and P. Molnár, *Anal. Chem.*, 49 (1977) 2295.
19 R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977, p. 301.
20 Cs. Horváth, W. Melander and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
21 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *Science*, 200 (1978) 1168.
22 M. T. W. Hearn, *Advan. Chromatogr.*, Vol. 18, Ch. 12, in press.
23 O. Sinanoglu, in B. Pullmann (Editor), *Molecular Associations in Biology*, Academic Press, New York, 1968, p. 427.
24 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
25 W. S. Hancock, C. A. Bishop, R. L. Prestidge and M. T. W. Hearn, *Anal. Biochem.*, 88 (1978) 203.