Journal of Chromatography, 204 (1981) 3–21
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,277

MECHANISM OF ION-PAIR LIQUID CHROMATOGRAPHY OF AMINES, NEUTRALS, ZWITTERIONS AND ACIDS USING ANIONIC HETAERONS

JOHN H. KNOX* and RICHARD A. HARTWICK*

Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ (Great Britain)

SUMMARY

The basis of retention in reversed-phase ion-pair chromatography is examined using C_{18}, C_{10} and C_{12} alkyl sulphates as hetaerons (pairing species) with a range of cations, neutrals, zwitterions and anions as solutes. The basic eluent comprises water–methanol (80:20) containing 20 m*M* phosphate buffer pH 6.00 and 50 m*M* sodium ion. Measurements were made of k' (column capacity ratio) as a function of hetaeron concentration in the mobile (C_m) and stationary phases (C_s) the latter being determined by the break-through method.

While the adsorption isotherms were non-linear, plots of k' *versus* surface concentration of hetaerons were essentially linear for cations up to a level at which the hetaeron formed micelles in the eluent whereupon k' declined. k' for neutrals fell slightly as C_s increased as did k' for small zwitterions; k' for anions fell drastically as C_s increased whereas small peptides behaved like cations. k' values for all types at any given surface concentration (molar) were more or less independent of the chain length of the hetaeron.

It is concluded that the degree of retention is directly related to the surface charge arising from the adsorbed hetaeron and that under most practical conditions ion-pair formation in the eluent is unimportant.

INTRODUCTION

Recently several papers have appeared on the so-called "mechanism" of ion-pair chromatography[1–6]. Two "mechanisms" were clearly distinguished by Horváth *et al.*[1] who wrote: "Retention of eluite in ion-pair chromatography on non-polar bonded phases can occur by dynamic ion-exchange, *i.e.* ion-pair formation takes place between the eluite and hetaeron (the pairing ion) bonded to the stationary phase, or by ion-pair formation in the mobile phase and binding the complex to the non-polar stationary phase". Deelder *et al.*[2] repeated this distinction in very similar words. Bidlingmeyer *et al.*[3] were more specific: "Two hypotheses are presently embraced. The first view stipulates the formation of an ion pair in the mobile phase

* Present address: School of Chemistry, Rutgers University, Piscataway, NJ 08854, U.S.A.

0021-9673/81/0000–0000/$02.50

prior to its adsorption onto the bonded hydrophobic stationary phase. The second view stipulates an ion-exchange mechanism ... (in which) ... the unpaired lipophilic alkyl ions adsorb onto the bonded surface and cause the column to behave as a dynamic ion exchanger". Terweij-Groen *et al.*[5] also accept the existence of two "mechanisms" arguing in favour of the first.

These statements and others similar to them[6–8] seem to imply that there are two alternative kinetic processes whereby partition of solute ions can occur in ion-pair chromatography. Thus in the "ion-exchange" mechanism the solute ions are supposed to approach the bonded surface and there to unite with adsorbed hetaerons to form ion pairs, whereas in the "ion-pair-formation-in-the-mobile-phase" mechanism the ion-pairs are supposed first to be formed in the eluent and subsequently to migrate to the bonded surface where they become adsorbed. *It can be stated categorically that, whether these are the processes occurring or not, they have no relevance to the basis or "mechanism" of retention.* The kinetics of processes occurring in chromatography affect only the peak width not peak retention[9]: peak retention is determined exclusively by thermodynamic equilibria[4,9,10]. How the equilibria come to be established is irrelevant provided that the processes are fast enough not to cause additional peak spreading. Thus it is a fundamental tenet of chromatographic theory that no deductions about the kinetics of chromatography can be made from measurements of solute retention.

The ion-exchange analogue as proposed by Knox and co-workers[11–13] in connection with the use of strongly hydrophobic hetaerons, referred to the idea that, at thermodynamic equilibrium, the hydrophobic surface of the support was nearly saturated with respect to adsorption of pairing agent so that the effect of counter ions added to the eluent was analogous to the effect in ion-exchange chromatography, namely a strong reduction in retention. It was also recognised[13] that under many conditions, especially with short-chain hetaerons, the surface would be far from saturated. While retention still arises from the partition of solute–hetaeron ion pairs into the bonded layer, the effect of added counter ions in the eluent is now relatively minor. Through a confusion of roles of thermodynamic equilibria and rate processes in chromatography these two thermodynamic situations appear to have become associated (wrongly) with two possible kinetic mechanisms for equilibration of solute ions between the eluent and stationary phase.

The main equilibria which are generally recognised to be important in ion-pair chromatography of an ionized species[1,2,4–7,11–13] are the following: (P^- is a hetaeron which can equally well be a cation with appropriate reversal of the charges on other ions, S^+ is a solute ion, C^+ and B^- are counter ions normally also present in the eluent, L represents the hydrocarbon or ligand groups bonded to the internal surface of the support, and the subscripts m and s refer to the mobile and stationary phases, respectively).

Adsorption of solute ion onto L	$S_m^+ + B_m^- + L_s \overset{1}{\rightleftharpoons} SBL_s$
Ion-pair formation in eluent	$S_m^+ + P_m^- \overset{2}{\rightleftharpoons} SP_m$
Adsorption of haeteron onto L	$P_m^- + C_m^+ + L_s \overset{3}{\rightleftharpoons} PCL_s$
Adsorption of solute and hetaeron giving ion pairs in stationary phase	$S_m^+ + P_m^- + L_s \overset{4}{\rightleftharpoons} SPL_s$

If it is assumed that S^+ is present in trace concentrations and that the total available ligand concentration [L] is constant, that is

$$[L] = [L_s] + [PCL_s] = \text{constant} \tag{1}$$

one can readily derive

$$k' = \varphi[L] \frac{K_1[B_m^-] + K_4[P_m^-]}{\{1 + K_2[P_m^-]\}\{1 + K_3[P_m^-][C_m^+]\}} \tag{2}$$

where k' is the column capacity ratio and φ is the ratio of stationary to mobile phase. This equation is essentially the same as that derived by Horváth *et al.*[1] except that their equilibria 1 and 3 did not contain the counter ions B^- and C^+ necessary to ensure the balance of electrical neutrality. Melin *et al.*[4] give a similar equation but do not include equilibrium 2.

In some circumstances it may be useful to consider other equilibria involving the hetaeron

Ion-pair exchange $\quad PCL_s + S_m^+ \overset{5}{\rightleftharpoons} PSL_s + C_m^+$

Ion-pair adsorption $\quad PS_m + L_s \overset{6}{\rightleftharpoons} PSL_s$

These equilibria are not, however, independent of 2, 3 and 4 since

$$K_4 = K_3K_5 = K_2K_6 \tag{3}$$

From the five equilibria 2, 3, 4, 5, 6 there are, in fact, eight possible combinations of three which are strictly equivalent. These may be represented as

$$\begin{aligned} 2 + 3 + 4 \equiv 2 + 3 + 5 \equiv 2 + 3 + 6 \equiv 2 + 4 + 5 \equiv 3 + 4 + 6 \\ \equiv 2 + 5 + 6 \equiv 3 + 5 + 6 \equiv 4 + 5 + 6 \end{aligned} \tag{4}$$

where the numbers represent the equilibria. Eqn. 2 is unalterable except for substitutions of K_4 derivable from eqn. 3. It is thus incorrect to maintain as do Terweij-Groen *et al.*[5] that equilibrium 5 is important while equilibrium 4 is unimportant, since 4 is the sum of 3 and 5.

In eqn. 2 the cause of retention on addition of the hetaeron P^- is seen unequivocably to be the operation of equilibrium 4, that is the partition of S^+ into the stationary phase by formation of solute–hetaeron ion pairs. The term $K_1[B_m^-]$ arises from the retention of S^+ in the absence of added hetaeron, and in many situations is negligible. Retention is counteracted by advancement of the equilibria 2 and 3 which correspond to formation of ion pairs in the eluent and to saturation of the ligand L by adsorption of counter ion-hetaeron ion pairs. In no sense can "formation of ion pairs in the mobile phase" be said to contribute to increasing retention: indeed it can only act to reduce retention, as was demonstrated by Terweij-Groen *et al.*[5].

When one, but not the other, of K_2 and K_3 is small so that the appropriate

factor is the denominator is always near unity, then k' will initially rise in proportion to $[P_m^-]$ but will flatten off to a constant value when $[P_m^-]$ is high. This behaviour is widely observed in practice[1-7,11-13].

Under these conditions the limiting value of k' is given by either

$$k'_{max.} = \varphi[L]\, K_5 \qquad (\text{when } K_2 = 0) \tag{5}$$

or

$$k'_{max.} = \varphi[L]\, K_6 \qquad (\text{when } K_3 = 0) \tag{6}$$

It is these two limiting conditions which may have led to Horváth *et al.*[1] to define their two "mechanisms". From circumstantial evidence on the effect (or rather lack of effect) of the chain length of the hetaeron on the concentration of P^- at which k' became constant, they deduced that K_3 was negligible in their system, that equilibrium 2 caused the flattening off, and therefore that eqn. 6 held. Since reaction 6 represents the equilibration of solute–hetaeron ion pairs between the eluent and stationary phase they deduced that ion-pair chromatography "proceeds through the formation of ion pairs in the mobile phase followed by adsorption onto the non-polar stationary phase". While the first deduction (*i.e.* that K_3 is negligible) may well be correct, the second is incorrect if it is meant to refer to the kinetics rather than the thermodynamics of the process. Taking K_3 as zero, Horváth *et al.* could then deduce that K_6 increased by a factor of about 1.7 for each CH_2 group added to the alkyl chain of the hetaeron.

It was unfortunate that in this work[1] the degree of adsorption of P^- by the surface was not directly measured since this would have at once established the shape of the isotherm and given a value for K_3. Terweij-Groen *et al.*[5] and subsequently Deelder *et al.*[2] measured this directly. Using the equilibria already given it is readily shown that k' can equally well be expressed by eqn. 7, which involves the concentration of adsorbed hetaeron, $[PCL_s]$, rather than its concentration in eluent $[P_m^-]$.

$$k' = \varphi \frac{K_5 + (K_1/K_3)\,[B_m^-]/[P_m^-]}{1 + K_2[P_m^-]} \cdot \frac{[PCL_s]}{[C_m^+]} \tag{7}$$

Terweij-Groen *et al.*[5] demonstrated that eqn. 7 held for the chromatography of acids using cetyltrimethylammonium as hetaeron. Deelder *et al.*[2] demonstrated that with aqueous eluents containing hexyl, octyl and dodecyl sulphonates as hetaerons, plots of k' against $[PCL_s]/[C_m^+]$ were linear, particularly when PCL_s was high ($\approx 200\ \mu mol\ g^{-1}$ or $\approx 1\ \mu mol\ m^{-2}$), having chosen conditions so that retention of S^+ was negligible in the absence of hetaeron. From both studies it may be concluded that $K_2[P_m^-] \ll 1$ in these systems. Deelder *et al.*[2] found that K_5 (their K_e) increased about twice going from the C_6 to C_8 sulphonate and by another twice from C_8 to C_{12} sulphonate.

In the present work we were concerned to provide further evidence on the basis of ion-pair chromatography and to test a very simple hypothesis, namely that the predominant factor causing enhanced retention in ion-pair chromatography is the interaction of the charge of adsorbed hetaeron with the opposite charges of the solute ions. If this is so then, to a first approximation, retention should be a linear function of the charge density on the surface of the packing material, which arises from adsorption of the hetaeron, and it should be more or less independent of the chain

length of the hetaeron at any surface concentration. As a further consequence of this hypothesis retention of ions of the same charge as the hetaerons (co-ions) should be reduced while that of neutrals should be almost unaffected. The retention of zwitterionic species would be particularly interesting since they might behave as either counter ions, or co-ions or neutrals showing increased, decreased or constant retention as the hetaeron concentration was charged.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment comprised the following components: a Waters Assoc. (Croydon, Great Britain) Model M6000 pump, a Waters Assoc. U6K valve, or a Shandon syringe/septum injector and a 100 or 125 × 5 mm I.D. internally polished stainless-steel column (Shandon Southern Products, Runcorn, Great Britain). For UV absorbing compounds, a Cecil Instruments (Cambridge, Great Britain) Model 212 UV photometer with an 8-μl flow cell was used; for fluorescent compounds, a Schoeffel Model FS 970 fluorometer (Kratos, Manchester, Great Britain) was used; for breakthrough curves, an Optilab Multiref 902 refractive index monitor (Techmation, London, Great Britain) was used.

Columns were slurry packed in the laboratory with 5-μm ODS-Hypersil (Shandon Southern Products) at 6000 p.s.i. using propanol followed by hexane, methanol and finally water–methanol (80:20, v/v). Columns were thermostatted by water jackets connected to a circulating bath at 25 ± 1°C. In addition the eluent was thermostatted before entry to the column by passage through a heat exchanger consisting of 1 m of 1/16 in. O.D., 0.020 in I.D. stainless-steel tubing immersed in the circulating bath.

For microelectrophoresis a Mark II electrophoresis equipment (Rank Bros, Bottisham, Great Britain) was used employing a rectangular quartz cell 0.80 mm deep, 9.7 mm wide and ≈50 mm long. Platinum electrodes were used in the end compartments with a constant potential difference of 50 V. Microscopic observations of the electrophoretic mobilities, U_e, of ODS-Hypersil particles were made at the so-called stationary levels in the cell to eliminate electroendosmotic effects, at least ten measurements being made in each direction. From the electrophoretic mobility the zeta potential, ζ, was obtained from

$$\zeta = \frac{U_e \eta}{\varepsilon_0 \varepsilon_r E} \tag{8}$$

where η = viscosity, ε_r = dielectric constant, ε_0 = permittivity of a vacuum, E = field strength and U_e = true electrophonetic velocity.

Reagents and standards

The alkyl sulfates used as pairing ions (sodium salts), were purchased from Sigma (Poole, Great Britain). HPLC-grade methanol (Rathburn Chemicals, Walkerburn, Great Britain) and AnalaR-grade phosphates (BDH, Poole, Great Britain) were used in the preparation of buffers.

The various amino acids, peptides and amine compounds were purchased from Sigma.

Preparation of eluents

Eluents were prepared in single large batches of sufficient quantity for experiments on a single pairing ion so that intra-study variations were eliminated. The standard eluents consisted of water–methanol (80:20) mixtures containing 0.018 *M* KH_2PO_4 and 0.002 *M* $NaHPO_4$ calculated to provide a pH of 6.0. A pH of exactly 6.00 was finally produced by small additions of either NaOH or HCl to this solution. After addition of the desired concentration of pairing agent as the sodium alkyl sulphate, the total sodium ion concentration was adjusted to exactly 0.050 *M* by the addition of NaCl. In this way, when the pairing salt concentration was changed, only the relative concentrations of the pairing ion and chloride were altered. In a few experiments other sodium chloride concentrations were used as described in the text.

Adsorption isotherms

The amounts of sodium alkyl sulphate adsorbed by the stationary phase from the standard eluents were determined by the break-through method using the Optilab Multiref 902 refractometer as detector. A "T-piece" leading to an on/off valve was fitted to the chromatograph immediately upstream of the injector so that when changing the pairing-ion concentration, the entire system could be flushed up to the point of injection with new eluent. The new eluent was then directed onto the column by closing the valve leading from the "T-piece". The breakthrough volume, V_b, was measured from the moment of closing the valve to the mid point of the eluted front. After subtraction of the void volume, V_m, the amount of pairing ion adsorbed, q_{ads}, is readily obtained[14] from

$$q_{ads} = (V_b - V_m)\, C_m = V_b' C_m = AC_s \tag{9}$$

where C_m is the concentration of pairing ion in the eluent, and V_b' is the net retention volume corresponding to the front, A is the surface area of the packing within the column and C_s the surface concentration of adsorbed hetaeron.

For the longer chain alkyl sulphates it was impossible to wash the pairing ions off the column between each concentration increase and a step-wise increase in concentrations was used with each previous concentration being used as the baseline. The total amount adsorbed onto the stationary phase was the sum of the individual increases obtained by successive applications of eqn. 9.

RESULTS AND DISCUSSION

Adsorption isotherms

Isotherms for the adsorption of the three alkyl sulphates, octyl, decyl and lauryl (dodecyl) from the standard eluent [that is water–methanol (80:20) containing 0.02 *M* phosphate buffer at pH 6.00 and sodium ion at 0.05 *M*] are shown in Fig. 1. To calculate the surface concentration, C_s, the surface area of ODS-Hypersil was required. This was measured by the BET method[16] and found to be 105 $m^2\ g^{-1}$. Taken with a measured weight of ODS-Hypersil in the column of 1.13 g the total surface area within the 4.7 × 125 mm column was 120 m^2. Fig. 2 shows that the adsorption isotherms of Fig. 1 are well fitted by the Freundlich equation as found by Deelder *et al.*[2]:

$$C_s = \alpha C_m^{\beta} \tag{10}$$

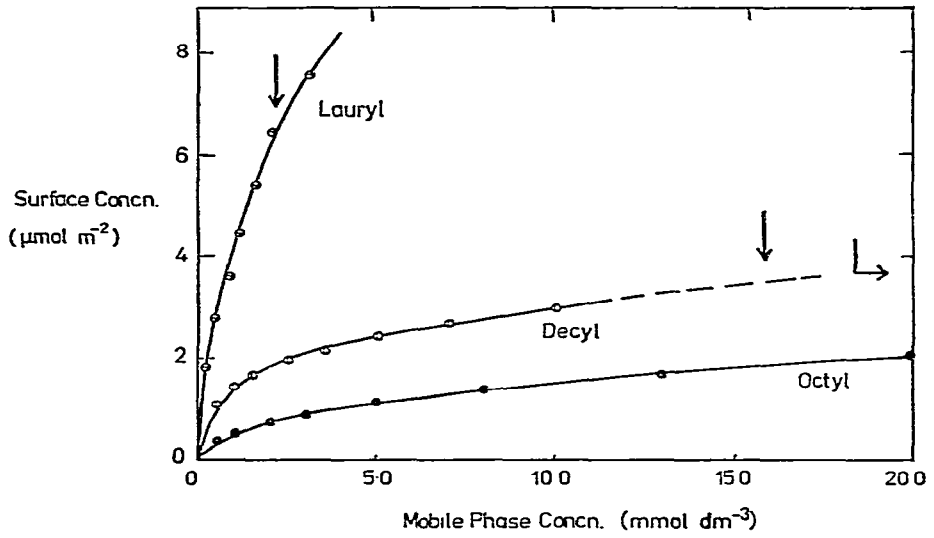

Fig. 1. Isotherms for adsorption of alkyl sulphates by ODS-Hypersil from standard eluent (water–methanol, 80:20 made 0.02 *M* in phosphate buffer pH 6.0 and 0.05 *M* in Na^+). Surface area of ODS-Hypersil 105 $m^2 g^{-1}$. Temperature, 298°K. Estimated critical micelle concentrations[15] are indicated by arrows.

From Fig. 1 it may be noted that the maximum concentrations of the adsorbed alkyl sulphates were approximately 2, 3 and 8 μmol m^{-2} for the C_8, C_{10} and C_{12} pairing agents, respectively even though the isotherms show no sign of reaching saturation (as seen by Fig. 2). These surface concentrations are relatively high and compare with the maximum concentration of trialkyl silyl groups bonded to silica surfaces of around 2 to 5 μmol m^{-2} (ref. 17) depending upon chain length of the alkyl groups. It

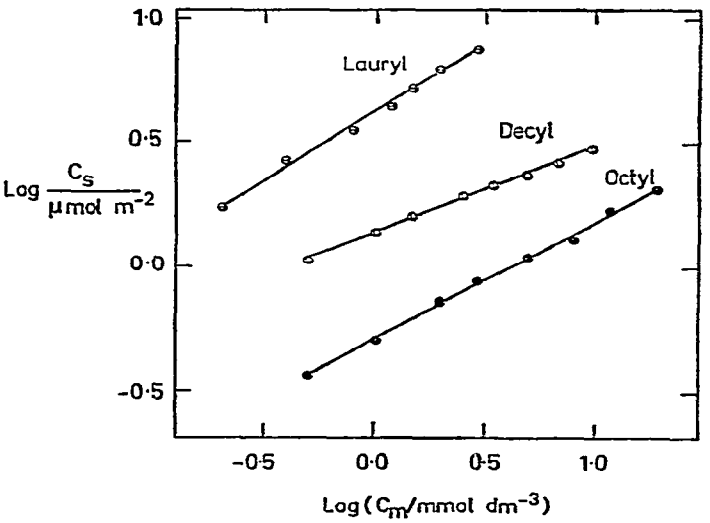

Fig. 2. Logarithmic plot of data of Fig. 1 according to eqn. 10. Values of β are: octyl sulphate, 0.49; decyl sulphate, 0.35; lauryl sulphate, 0.57.

may be noted that with lauryl sulphate the estimated critical micelle concentration (CMC) of sodium lauryl sulphate[15] is around the highest concentration used and therefore that catastrophic adsorption may be occurring. For octyl and decyl sulphates the CMC values are significantly above the highest concentration employed. Here the maximum surface concentrations are comparable to those of bonded groups indicating a near monomolecular layer of adsorbed hetaeron. For comparison it may be noted that the maximum surface concentrations of cetyltrimethylammonium observed by Knox and Laird[11] at an eluent concentration of 2% (w/v) were $\approx$0.25 μmol m^{-2} on SAS silica and by Terweij-Groen *et al.*[5] up to 1.5 μmol m^2 (300 μmol g^{-1}). Deelder *et al.*[2] measured maximum surface concentrations of 50 and 250 μmol g^{-1} for C_8 and C_{12} sulphonates adsorbed by Partisil 10 ODS corresponding to between 0.4 and 1.25 μmol m^{-2} of surface area.

The surface coverages found in this and other comparable studies thus show that surface active agents are strongly adsorbed by bonded phases to give coverages approaching those of the chemically bonded groups themselves.

Dependence of retention upon concentration of the pairing ions in mobile and stationary phases

Fig. 3 shows the effect of the mobile phase concentration of lauryl, decyl and octyl sulfates on the k' values of tyrosine amide. A maximum in retention occurs for

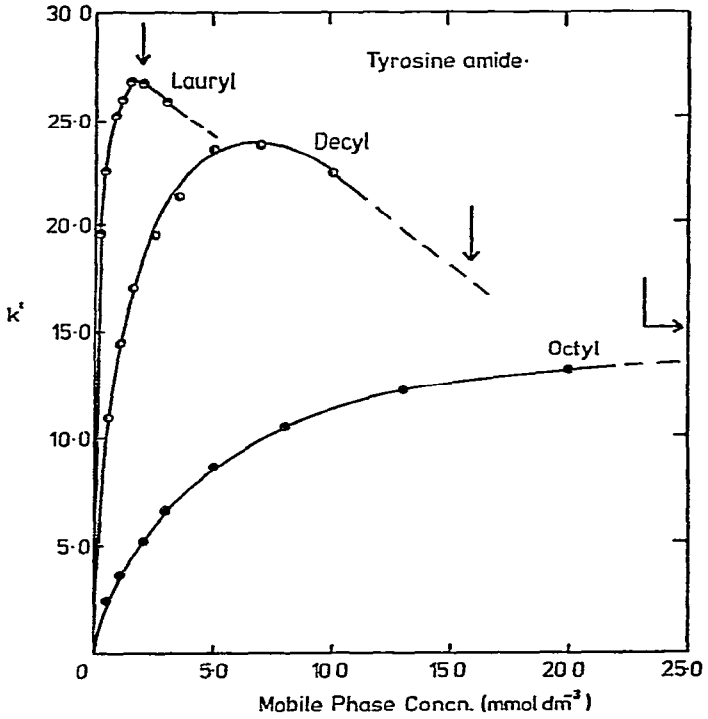

Fig. 3. Dependence of k' for tyrosine amide upon concentrations of alkyl sulphates added to standard eluent. Packing ODS-Hypersil. Estimated critical micelle concentrations[15] indicated by arrows.

the decyl and lauryl sulfates which is similar to that observed by Horváth *et al.*[1] with decyl sulphate as hetaeron. It is interesting to note that these maxima occur at lower mobile phase concentrations the longer the chain length of the pairing ion. These results contrast with those of Horváth *et al.* who found that the concentration of hetaeron at which k' became constant was independent of chain length for C_4 to C_8 alkyl sulphates and about 40 mM. However, for C_{10} sulphate a clear maximum was observed at a significantly lower hetaeron concentration of about 15 mM. In the present work the maxima occurred at ≈20 mM for octyl sulphate, ≈7 mM for decyl sulphate and ≈2 mM for lauryl sulphate. Estimated values of the CMC[15] in the eluent used by Horváth *et al.* and in our standard eluent are 100 mM for octyl, 20 mM for decyl and 2.5 mM for lauryl sulphate. It seems most likely that the eventual fall in k' as hetaeron concentration is increased in both this work and that of Horváth *et al.*, arises from the incipient micelle formation coupled with ion-pair formation in the eluent as proposed earlier to explain similar effects by Knox and Laird[11] and by Terweij-Groen *et al.*[5]. It is, however, notable that the adsorption of the alkyl sulphates is still increasing when k' is past its maximum value and indeed even when C_m exceeds the estimated CMC.

Fig. 4 shows how the k' values of positive, neutral and negatively charged solutes depend upon the concentration of the hetaeron decyl sulphate in both eluent and stationary phase.

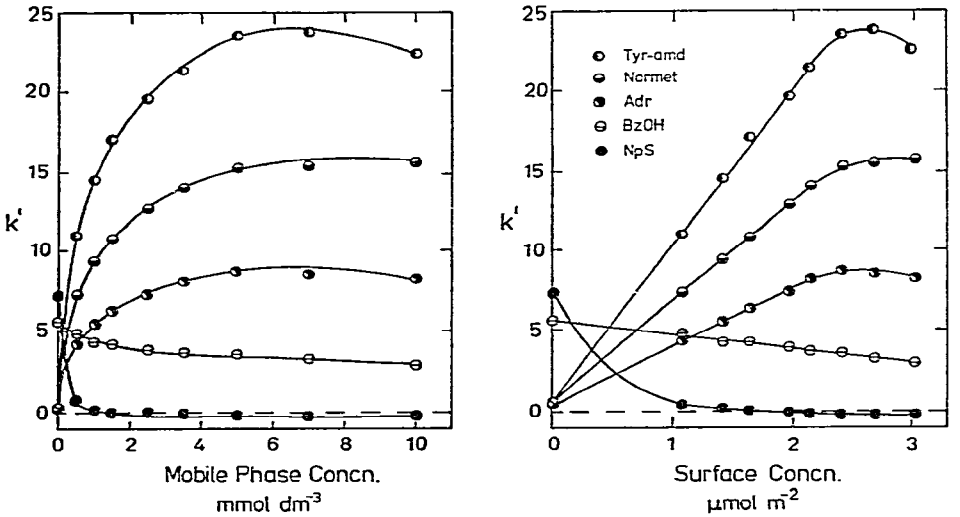

Fig. 4. Dependence of k' for various solutes upon concentrations of octyl sulphate in standard eluent. Left: k' *versus* C_m; right: k' *versus* C_s. Tyr-amd = tyrosine amide, Normet = normetadrenaline, Adr = adrenaline, BzOH = benzyl alcohol, NpS = naphthalene-2-sulphonate. Pairing ion: decyl sulphate.

For positively charged species k' increases with C_m in a concave manner to a maximum value when C_m is around 8 mmol dm^{-3}. When k' is plotted against C_s, however, an excellent linear relationship is observed almost up to the concentration corresponding to the maximum in k'. This result is in close agreement with those of previous workers[2,5] and strongly suggests that the major factor contributing to reten-

tion in this form of ion-pair chromatography is a direct interaction between the adsorbed pairing species and the solute ion of opposite charge, with interactions in the mobile phase being negligible until C_m reaches some critical concentration which is close to the CMC.

For the neutral species, benzyl alcohol, k' decreases slightly with increase in C_s as found by Melin *et al.*[4] indicating a slight lessening of the interaction with the stationary phase as this becomes progressively more polar through adsorption of the hetaeron. For the negatively charged solute naphthalene-2-sulfonate (NpS^-), on the other hand, there is a dramatic loss of retention when decyl sulfate is adsorbed onto the stationary phase, until finally it is partially excluded from the packing material (that is its k' value becomes negative). This occurs at about 2.7 μmol m^{-2} of decyl sulfate. This result is similar to those previously found by Knox and Jurand[13], Bidlingmeyer *et al.*[3] and by Melin *et al.*[4].

The dramatic decrease in k' of negatively charged solutes, the slight loss in k' of neutral species, and the linear increases in k' of the positively charged species with C_s, all point strongly to surface charge as the dominant factor governing retention, the surface becoming increasingly negatively charged with increasing adsorption of the hydrophobic negatively charged hetaeron ions.

The profound effect observed in practice from the addition of pairing ions on the retention of the solutes of various charges is well illustrated by the chromatograms shown in Fig. 5. When no octyl sulfate is present in the mobile phase, the positively charged species adrenaline (Adr^+), normetadrenaline ($Normet^+$) and tyrosine amide

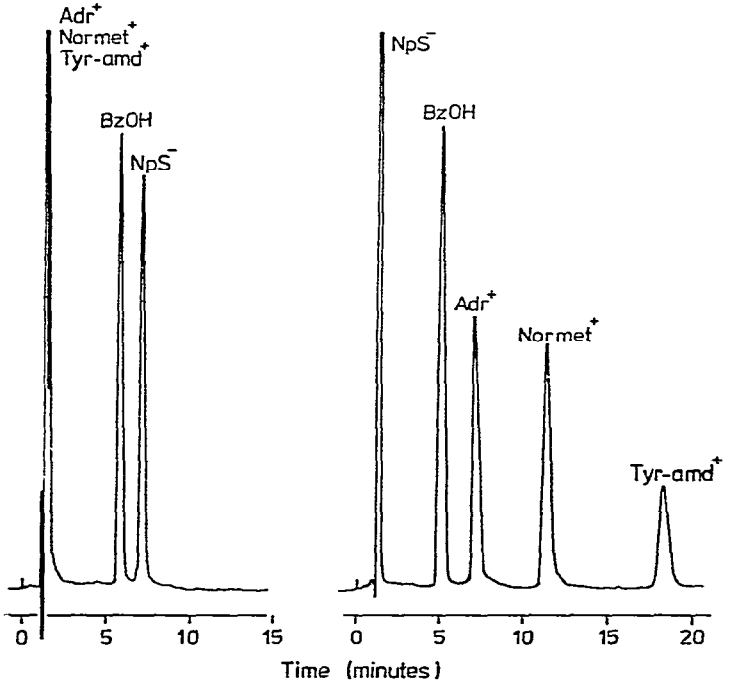

Fig. 5. Effect on k' of various species (for identification see Fig. 4) of the addition of octyl sulphate to standard eluent. Left: no octyl sulphate; right: 14 mmol dm^{-3} in octyl sulphate.

(Tyr-amd$^+$) are almost unretained, whereas the neutral solute benzyl alcohol (BzOH) and the negatively charged napthalene-2-sulfonic acid (NpS$^-$) have k' values of about 6 and 7, respectively. When the mobile phase contains 0.014 mol dm^{-3} octyl sulfate, NpS$^-$ loses almost all retention, while Adr$^+$, Normet$^+$ and Tyr-amd$^+$ become well retained. The retention of BzOH decrease slightly.

The type of effect shown in Fig. 5 is not, of course, limited to negatively charged pairing ions. Fig. 6 shows the effect of the addition of tetrabutyl ammonium iodide (TBAI) upon the retention of NpS$^-$, phenol (PhOH) and the O-methyl ester of tryptophan (Trp-OMe$^+$). Again, reversal of the elution order occurs, this time with the positively charged Trp-OMe$^+$ losing retention, while that of NpS$^-$ increases, and that of the neutral PhOH slightly decreased.

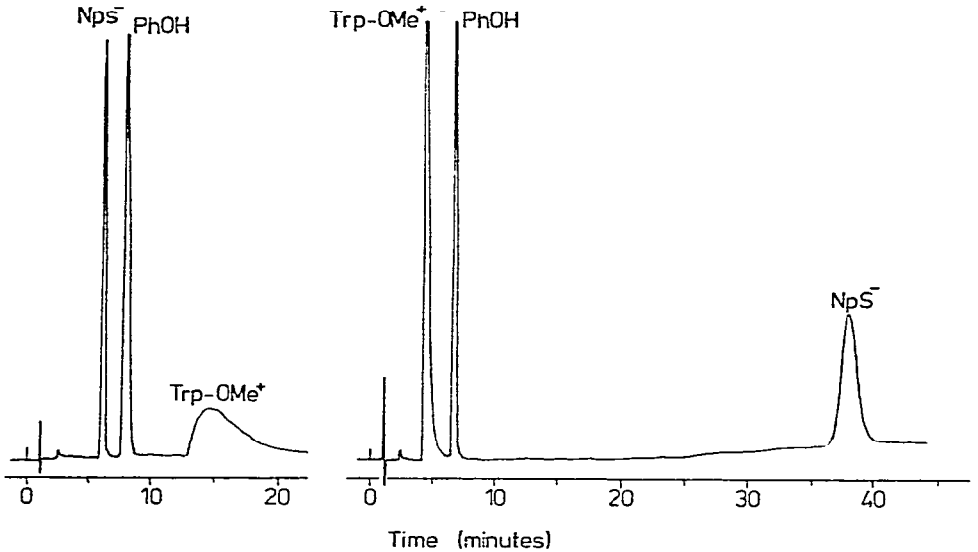

Fig. 6. Effect on k' of various species of the addition of tetrabutyl ammonium (4 mM). Eluent, water–methanol (75: 25, v/v) containing 0.10 M phosphate buffer pH = 6.0. NpS$^-$ = naphthalene-2-sulphonate, PhOH = phenol, Trp-OMe$^+$ = methoxy tryptophan. Left: No TBAI; right: 4 mmol dm^{-3} in TBAI.

Effect of pairing ions on zwitterionic solutes

Fig. 7 shows the retention behaviour of zwitterions of various chain lengths, in the presence of decyl sulfate at various concentrations. The simple amino acids tryptophan (Trp) and phenylalanine (Phe) show ambiguous behaviour like that of neutral species with no significant change in retention when the pairing-ion concentration is increased. However, with peptides, as the chain length is increased, the effect of addition of pairing ion becomes progressively greater so that with PheGlyGly and TrpGly the retention behaviour resembles that of the positively charged species of Fig. 4; that is, there exists a linear relationship between k' and the surface concentration of pairing ion. Apparently with the longer peptides, the positive charge on the amino group is able to function more or less independently of the negatively charged carboxyl group. This suggests that a singly charged pairing ion of either charge could effect the separation of the short chain peptides on reversed-phase materials, and that

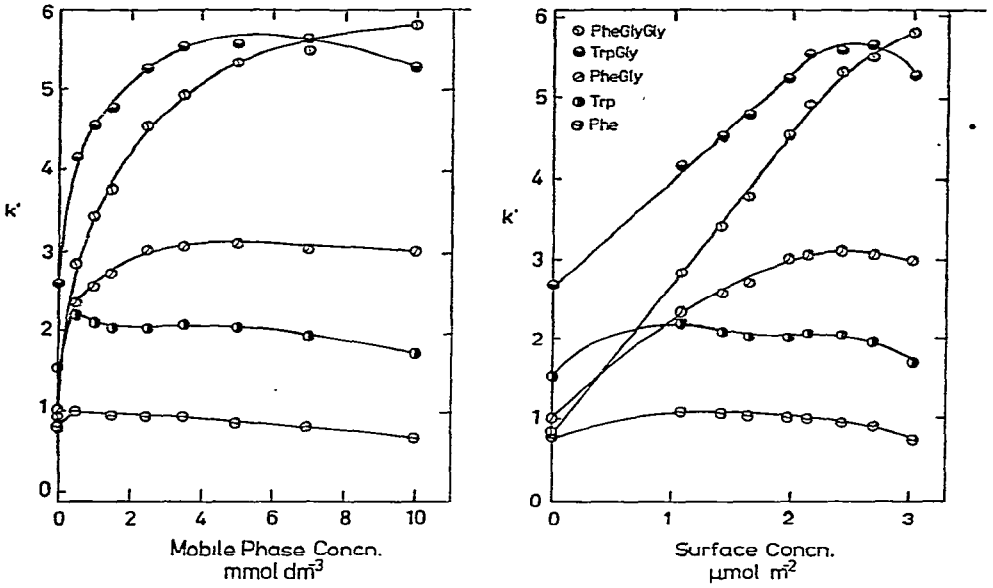

Fig. 7. Dependence of k' for various zwitterionic solutes upon concentration of decyl sulphate in standard eluent. Left: k' *versus* C_m; right k' *versus* C_s. Pairing ion: decyl sulphate.

it is unnecessary to employ extreme pH as previously though necessary[5] in order to suppress the ionization of either the amino or the carboxyl groups.

Dependence of retention upon chain length of pairing ion

If retention in reversed-phase ion-pair chromatography is predominantly dependent upon the interaction of a charged surface produced by adsorption of pairing ions, with solute ions of opposite charge, retention of a given solute ought to be independent of the chain length of the pairing ion *per se*. Figs. 8 and 9 illustrate the degree to which this was found to be true. Fig. 8 shows that when normetadrenaline (Normet$^+$) is chromatographed at various concentrations of lauryl, octyl and decyl sulfates in the presence of constant sodium ion concentration, k' shows the usual concave dependence upon C_m, the curves being steeper and showing maxima occurring at lower values of C_m the greater the length of the alkyl chain. However, when the k' values are plotted against surface concentration, C_s, fairly good linear relationships are observed up to the maximum k', but more important, the curves for the three hetaerons are nearly coincidental. Fig. 9 shows that this behaviour also occurs with neutral, negatively charged and other positively charged species.

Evidently the major factor governing retention is the interaction of charges and not of the non-polar alkyl groups with the solute ions. A secondary influence of chain length can nevertheless be detected in that for a given C_s, the retention is slightly greater with lauryl sulphate (C_{12}) than with octyl or decyl sulphates. This study thus indicates that K_S is little dependent upon chain length of the hetaeron, when the eluent is water–methanol (80:20) made 0.05 *M* in NaCl and 0.02 *M* in phosphate buffer. The results contrast with those of Deelder *et al.*[2] who found a two-fold increase in K_S in going from C_8 to C_{12} sulphonates.

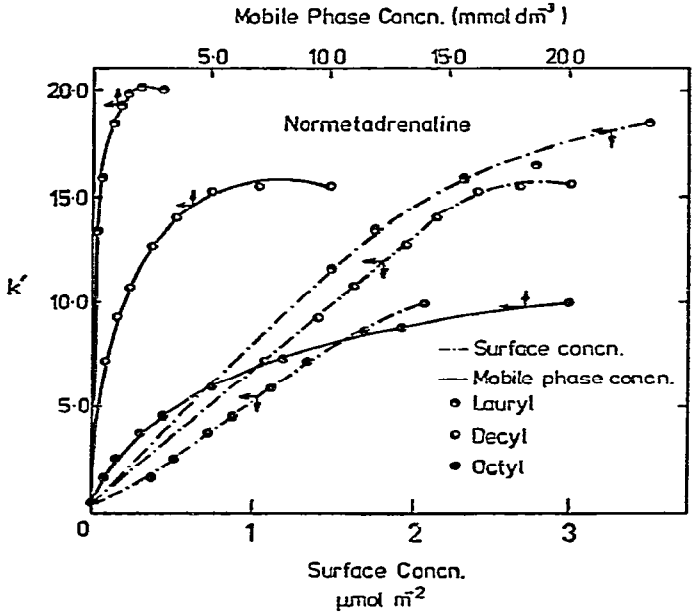

Fig. 8. Dependence of k' for normetadrenaline on concentration of alkyl sulphate for alkyl sulphates of different chain length. Full lines, plots of k' *versus* C_m; broken lines, plots of k' *versus* C_s.

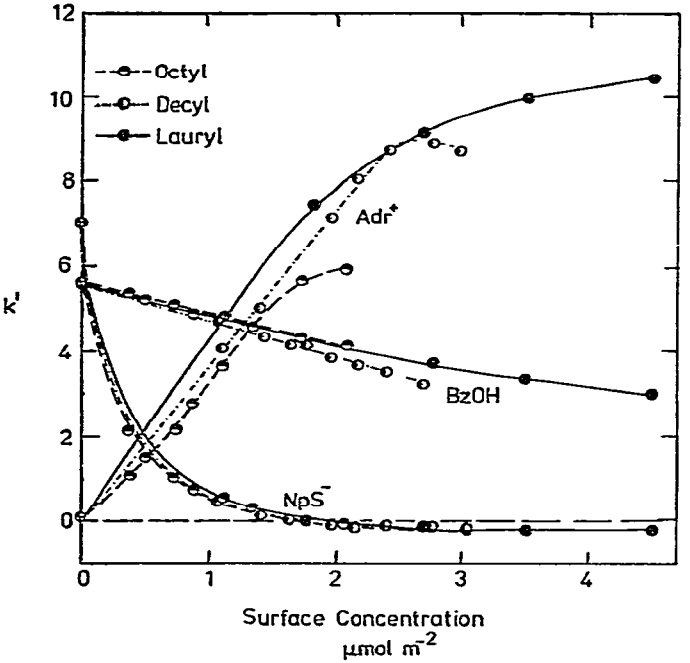

Fig. 9. Dependence of k' for positively charged, neutral and negatively charged solutes (for identification see Fig. 4) upon concentration of alkyl sulphate for alkyl sulphates of different chain length.

Effect of counter-ion concentration

According to eqn. 7, if K_1 and K_2 are taken as effectively zero, plots of k' for an ionized solute S^+ against $C_s/[Na_m^+]$ should be linear. This is demonstrated by Fig. 10 in which $[Na_m^+]$ was varied while keeping the mobile phase concentration of octyl sulphate constant at 5 mmol dm^{-3}. For cationic species excellent linear relationships are observed with small positive intercepts at $C_m = 0$. The results are similar to those found previously[2,5] except that in some cases[2] some curvature was found indicating a dependence of K_5 upon surface coverage by the hetaeron. Neutral benzyl alcohol and simple amino acids show indecisive behaviour as was observed when C_m was varied at constant $[Na_m^+]$. The behaviour of the dipeptide TryGly is, however, interesting in that increase of $C_s/[Na_m^+]$ brought about by changing $[Na_m^+]$ at constant C_m causes a slight decrease in k' whereas increase in C_s brought about by changing C_m at constant $[Na_m^+]$ increases k' (Fig. 7). These two apparently contradictory results are rationalised by observing that in the former case, because of the non-linearity of the adsorption isotherm, C_s increases as $C_s/[Na_m^+]$ decreases. Thus for both cases k' increases with C_s and $[Na_m^+]$ has little influence on k'. This is not unexpected for with a zwitterionic solute increase of ionic strength will have compensating effects on the two oppositely charged parts of the molecule so that charge interaction will appear more important than ion-exchange effects.

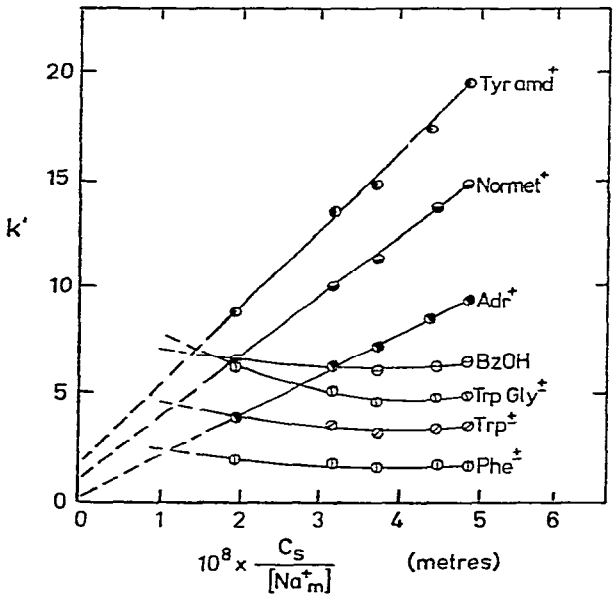

Fig. 10. Effect of counter-ion concentration (10–70 mM Na^+) on retention of various solutes in presence of octyl sulphate (5 mM). C_s determined by breakthrough technique. Plot according to eqn. 7 (simplified).

Buffer capacity

In choosing the optimum eluent for ion-pair chromatography several types of buffering must be considered. Throughout the chromatographic band the concentration of solute will very from zero to some maximum which will normally be in the

range of 10^{-3} to 10^{-6} M depending upon the concentration in the injected sample, the distance migrated along the column, the column volume and its plate efficiency. If the solute is wholly or partially ionized its retention or k' values may vary over this concentration range for some or all of the following reasons: (a) inadequate pH buffer capacity which allows the ratio of ionized to unionized form to vary, (b) too small an ionic strength of eluent which allows the ionic strength to vary within the peak, (c) inadequate pairing-ion capacity which causes C_s to vary throughout the peak as a result of additional ion pairs brought into the stationary phase associated with the adsorbed solute ions.

If any of (a) to (c) occur peak tailing or fronting will be observed. The combined importance of ionic strength and pH buffering is demonstrated by the two chromatograms of Fig. 11. Severe tailing of the ionized solutes but not of the neutral benzyl alcohol is observed when the ionic strength of the phosphate buffer is 0.005 M. This is largely eliminated when the ionic strength is increased to 0.10 M, the concentration of the pairing agent being 0.01 M in both cases. The initial sample size was around 10^{-8} mol injected in 10 μl of eluent (*i.e.* initial concentration was about 10^{-3} M). This emerged from the column with a peak maximum concentration of about 10^{-5} M.

Loading of the pairing ions onto the column

A direct consequence of adsorption of the pairing ions onto the stationary phase is that finite volumes of eluent must be passed before the column surface is

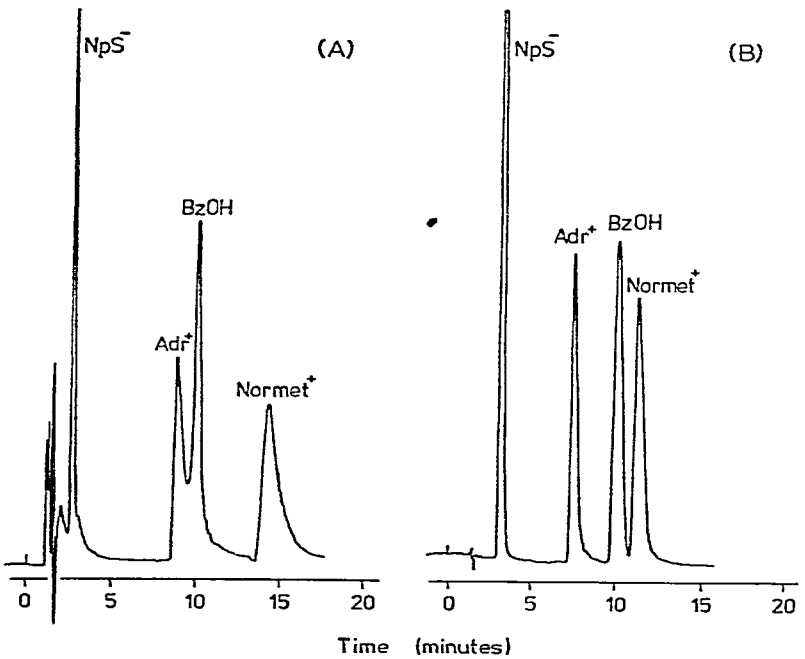

Fig. 11. Effect of inadequate buffer capacity in ion-pair chromatography. Eluents (A) 5 mM phosphate buffer, (B) 100 mM phosphate buffer in water–methanol (85/15, v/v) containing 10 mM octyl sulphate.

fully coated. Obviously, the more packing surface area there is to be covered, the more strongly adsorbed the pairing ion is, and the lower is its concentration in the mobile phase, the more volume will be required to coat the packing surface, as shown by eqn. 9. Since the adsorption isotherms follow Freundlich behaviour, eqns. 9 and 10 give

$$V_b' = A\alpha C_m^{(\beta-1)} \tag{11}$$

Fig. 12 shows the approximate V_b' values calculated from eqn. 11 for a 100-mm column packed with 5-μm particles of ODS-Hypersil containing a total surface area of 100 m^2. As the concentration of pairing ion in the mobile phase is decreased, greater volumes are required for equilibration and, for example, with lauryl sulfate at 0.1 mmol dm^{-3} $\approx$1.5 dm^3 of eluent are required. At a flow-rate of 1.0 cm^3 min^{-1}, 25 h would be needed to reach full equilibration! For the less strongly adsorbed octyl sulfate, equilibration is not such a problem with only about 100 cm^3 being required, even at this low concentration.

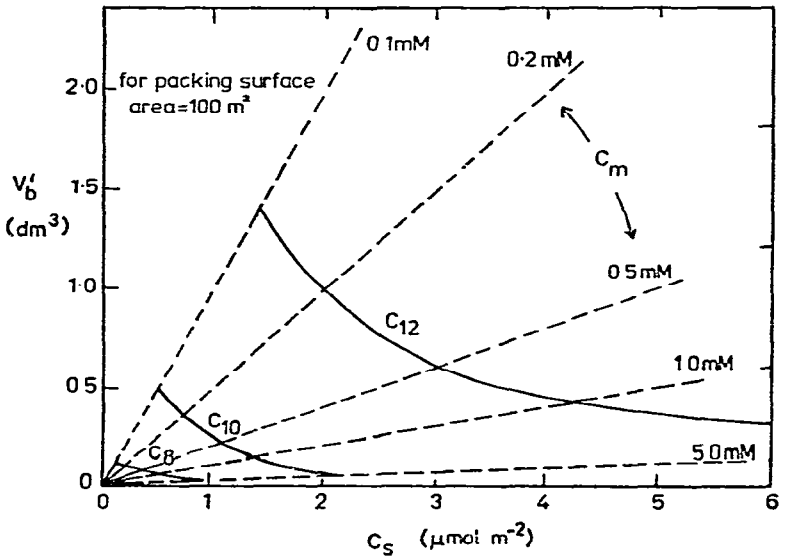

Fig. 12. Breakthrough volumes for alkyl sulphates at various mobile phase concentrations for a references surface area of 100 m^2 using standard eluent.

From Fig. 12 it may also be deduced that, for the longer chain pairing ions, it is preferable to load the column at high concentrations and then decrease the mobile phase concentration to the desired level, again waiting for re-equilibration to occur (see Knox and Jurand[13]).

The different rates of desorption of the three pairing agents are clearly shown in Fig. 13 which establishes that strongly adsorbed hetaerons, such as lauryl sulfate, require almost infinitely large volumes for complete desorption as a consequence of the high curvature and extreme slope of the adsorption isotherm at low concentrations of hetaeron.

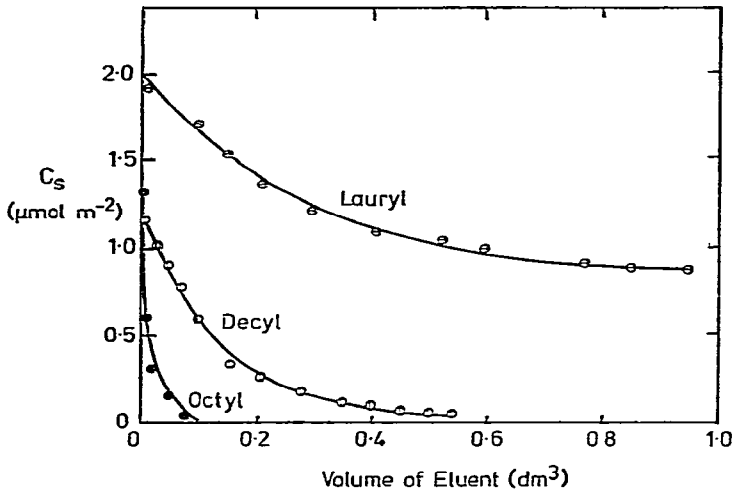

Fig. 13. Desorption of various alkyl sulphates from ODS-Hypersil by standard eluent containing no alkyl sulphate.

The practical consequence of this is that a column is often irreversibly altered if strongly adsorbed pairing ions are used: in this work, for example, we were unable to wash the lauryl ion off the column packing with even pure methanol and isopropanol.

Microelectrophoresis

Microelectrophoresis of ODS-Hypersil was carried out using standard eluents containing various concentrations of octyl sulphate in the range 10 to 20 mM. The results are shown in Fig. 14 from which it may be seen that zeta potential at first rises gradually from about 15 mV and then fairly rapidly when C_s is in the range 1.5 to 2 μmol m^{-2} corresponding to a surface charge of 0.15 to 0.2 C m^{-2}. The zeta potential for ODS-Hypersil in standard eluent containing no octyl sulphate is in agreement with that obtained in a wider ranging study by Knox and Kaliszan[18].

In a recent paper Cantwell and Puon[19] were able to explain the adsorption isotherm of diphenylguanidinium ($DPGH^+$) on Amberlite XAD-2 resin on the basis of the Stern-Gouy-Chapman (SGC) theory of the electrial doublelayer (see for example ref. 20). They showed that the charge density in the inner part of the double layer (the "molecular condenser") was that obtained by assuming that virtually all the adsorbed $DPGH^+$ contributed to the charge. From their data they were able to show that the Outer Helmholtz Plane (OHP) was about 0.4 nm from the surface. Their surface potentials, ψ, calculated from SGC theory and the observed isotherms, ranged from 30 to 120 mV, for activities of $DPGH^+$ from $3 \cdot 10^{-4}$ to $3 \cdot 10^{-2}$ M, and more or less fitted eqn. 12, where a_{DPGH^+} is the activity of $DPGH^+$. However, values

$$\psi = \psi^{\ominus} + (RT/F) \ln a_{DPGH^+} \qquad (12)$$

obtained from measurements of zeta potentials were much smaller and showed less variation being in the range 25–40 mV. For this latter calculation it was assumed

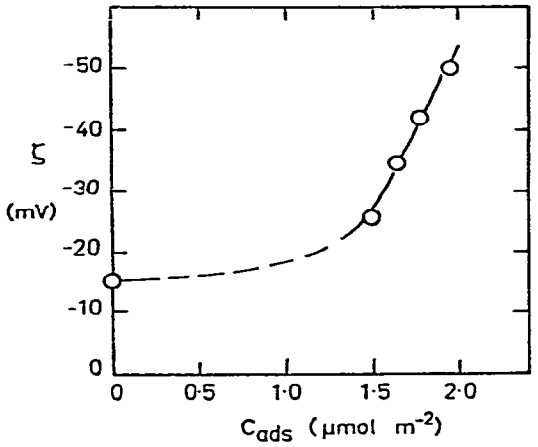

Fig. 14. Zeta potential of ODS-Hypersil as a function of surface concentrations of octyl sulphate for C_m between 10 and 30 m*M* and $[Na^+]$ = 50 m*M*. All values of ζ are negative.

by the authors that the zeta potential was the same as the potential at the OHP. The discrepancy between these values suggests that this assumption may be fairly seriously in error. This may arise from the porous nature of the particles and the fact that the zeta potential is determined by a surface dividing those ions which on average move with the particle during electrophoresis from those that remain with the surrounding fluid. This particular surface must reside entirely outside the porous particles whereas the OHP will penetrate and suffuse the pores within the particles. The plane defining the zeta potential will therefore separate the inside of the particle from the surrounding fluid by a potential barrier (whose height is given roughly by the zeta potential). The situation is then very similar to that associated with the Donnan membrane equilibrium when specific ions (such as $DPGH^+$) are restricted to one side of a membrane causing a chemical and electrical potential difference for any ions which are not so restricted (*e.g.* Na^+ and Cl^-). To a first approximation it may be appropriate to regard the entry ports to the inside of a porous particle as being protected by a potential barrier which will exclude ions of the same charge as those producing the barrier (negative in the case of alkyl sulphates) and which will welcome ions of opposite charge.

CONCLUSIONS

Eqns. 2 and 7 appear to give a good explanation of the enhancement of retention of ionized solutes arising from addition of hydrophobic ions (hetaerons) to the eluent. It appears that the enhanced retention can be attributed to the adsorbed charge and is to a first approximation independent of the hydrophobic part of the hetaeron. For ions of the same charge as the hetaeron eqns. 2 and 7 clearly do not apply and some form of electrostatic repulsion must be invoked.

At the same time it is clear that the electrical properties of the packing material change considerably as the concentration of adsorbed ions increases. Thus the absolute values of both the surface potential and the zeta potential increase with surface charge.

Since the zeta potential is determined by a surface external to the porous particles its existence should affect the chemical potential of solute ions distributed between the interior and exterior. The degree of retention or exclusion must be related exponentially to the potential rather than linearly. In the case of retention the operation of eqn. 12 produces a linear relationship between retentivity and surface charge and justifies eqns. 2 and 7. However, ions of like charge will become increasingly excluded from the particles so that superimposed on any changes in the partition ratio for similar but uncharged species will be a factor $\exp(-z\zeta F/RT)$, where z is the integral ionic charge and F is Faraday's constant. Typically ζ is of the order of 50 mV which will produce roughly a tenfold reduction in k'. This can, of course, be only a rough approximation (a) because the zeta potential may represent only a proportion of the potential difference between the pore volume and external volume, and (b) because there will be variations of potential within the pores, particularly close to the internal surface arising from the tightly held adsorbed hetaeron ions. Clearly further investigation of ionic exclusion will be necessary before this phenomenon is fully explained.

ACKNOWLEDGEMENTS

The authors warmly thank Kratos UK Ltd. and Waters Associates for the loan of equipment.

We also gratefully acknowledge the award of a NATO postdoctoral fellowship to one of us (R.A.H.) without which support the project could not have been carried out.

REFERENCES

1 Cs. Horváth, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
2 R. S. Deelder, H. A. J. Linssen, A. P. Konijnendijk and J. L. M. van de Venne, *J. Chromatogr.*, 185 (1979) 241.
3 R. A. Bidlingmeyer, S. N. Deming, W. P. Price, Jr., B. Sachok and M. Petrusek, *J. Chromatogr.*, 186 (1979) 419.
4 A. T. Melin, M. Ljungerantz and G. Schill, *J. Chromatogr.*, 185 (1979) 225.
5 C. P. Terweij-Groen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 161 (1978) 69.
6 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 883.
7 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
8 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 175 (1979) 51.
9 J. C. Giddings, *Dynamics of Chromatography*, Part I, Marcel Dekker, New York, 1965.
10 J. H. Knox (Editor), *High Performance Liquid Chromatography*, Edinburgh University Press, Edinburgh, 1978.
11 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1976) 17.
12 J. H. Knox and J. Jurand, *J. Chromatogr.*, 125 (1976) 89.
13 J. H. Knox and J. Jurand, *J. Chromatogr.*, 149 (1978) 297.
14 J. F. K. Huber and R. G. Gerritse, *J. Chromatogr.*, 58 (1971) 137.
15 *Critical Micelle Concentrations of Aqueous Surfactant Systems*, NSRDS-NBS-36, Natural Bureau of Standards, Washington, DC, 1971.
16 S. Brunnauer, P. H. Emmett and E. Teller, *J. Amer. Chem. Soc.*, 60 (1938) 309.
17 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, Oxford, New York, 1979.
18 J. H. Knox and R. Kaliszan, *J. Chem. Soc., Faraday Trans.*, in press.
19 F. F. Cantwell and S. Puon, *Anal. Chem.*, 51 (1979) 623.
20 J. T. G. Overbeek, in H. R. Kruyt (Editor), *Colloid Science*, Vol. 1, Elsevier, Amsterdam, 1952, p. 115.