Journal of Chromatography, 185 (1979) 241–257

CHROM. 12,000

RETENSION MECHANISM IN REVERSED-PHASE ION-PAIR CHROMATOGRAPHY OF AMINES AND AMINO ACIDS ON BONDED PHASES

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SUMMARY

The retention behaviour of a series of amines and amino acids was studied in systems consisting of a hydrophobic column packing and water or water–propanol mixtures containing low concentrations of alkylsulphonates of increasing chain length as the mobile phase. In these systems an ion-exchange mechanism governs retention. The amphiphilic sulphonate ions are adsorbed on to the hydrophobic surface and counter ions from the aqueous buffer solution are concentrated near the surface. Protonated amines and amino acids exchange with these counter ions.

INTRODUCTION

The development of *n*-alkyl chemically bonded phases as a suitable column packing material for reversed-phase high-performance liquid chromatography (HPLC) marks an important breakthrough. There has been a spectacular increase in the number of practical applications of LC following the introduction of the alkyl-modified silicas, and since 1976 an estimated 60–70% of the analytical work by LC has been carried out on these packings[1–3].

For separating ionic or ionizable compounds, two distinct modes of reversed-phase chromatography are commonly used. In the more conventional form an aqueous buffer, which may contain a water-miscible organic solvent, is used as the mobile phase. The retention mechanism in this so-called solvophobic chromatography has been investigated extensively by Horváth and co-workers[4,5]. In the other mode, which is commonly referred to as reversed-phase ion-pair chromatography, the retention of ionized compounds is strongly enhanced by the addition of a suitable lipophilic ion to the aqueous mobile phase.

Ion-pair chromatography in reversed-phase liquid–liquid partition systems was first investigated by Wahlund and Gröningsson[6]. It has been shown that the column performance could be improved considerably compared with the original work by using alkyl-modified silica as a support material and applying appropriate *in situ* coating techniques[7,8]. In this mode of chromatography, the charged solute in

the aqueous mobile phase combines with the lipophilic counter ion and the combination is extracted as an ion pair into an organic apolar liquid phase. Increased retention due to the presence of a suitable amphilic ion such as a long-chain quaternary ammonium compound, alkylsulphonates or alkylsulphates, is also observed on alkyl-bonded silica without any liquid coating. Haney and co-workers[9–11] and Knox and co-workers[12,13] have pioneered this technique, which has proved very useful for separating ionized organic substances. This technique is also referred to as paired-ion chromatography (PIC)[11], soap chromatography[12], dynamic solvent generated ion-exchange chromatography[14] or hetaeric chromatography[15]. The retention mechanism in this type of chromatography is still a matter of controversy[16]. The original concept supposes that ion-pair formation takes place in the aqueous mobile phase followed by strong retention of the neutral complex on the apolar surface. This concept is supported by a recent study that presents extensive data on the retention behaviour of catecholamines in a pure aqueous eluent containing alkylsulphates as lipophilic ions[15]. However, the adsorption of the lipophilic ion on to the hydrophobic surface of the column packing material seems to be underestimated in this work.

A different mechanism has been proposed by Kissinger[16] and Kraak *et al.*[14]. Taking into account the adsorption of the lipophilic ion, they suggest that the charged surface acts as an ion exchanger. This concept is also supported by experimental data[14,17,18]. Recently, Knox and Jurand[19] stressed the importance of a knowledge of the adsorption of the lipophilic ion in deciding which of the proposed retention mechanisms is really operative.

The main objective of this study was the further elucidation of the retention mechanism of ionized solutes in reversed-phase ion-pair chromatography on *n*-alkyl-modified silica. We restricted the study to eluents consisting of low-concentration solutions of alkylsulphonates in neat aqueous buffers or mixtures of these buffers with 1-propanol and 2-propanol. As solutes catecholamines and amino acids were used.

THEORETICAL

Amphiphilic substances such as long-chain quaternary ammonium compounds and alkylsulphonates are readily adsorbed at the interface between two immiscible liquids of very different polarity, *e.g.*, octane–water. The charges of the amphiphilic ions at the interface are neutralized by the adsorption of counter ions from the aqueous phase. Further, the ions at the interface show a selectivity towards the various counter ion species in the aqueous phase and behave like two-dimensional ion exchangers[20,21].

These ionized layers may serve as a model for the surface of the column packing material in reversed-phase ion-pair chromatography on bonded phases. It is assumed that the amphiphilic ion, *i.e.*, the alkylsulphonate X^-, is adsorbed on to the hydrophobic surface. Counter ions B^+ from the aqueous buffer solution are concentrated near the surface. Different types of interactions between the charged surface ion and the counter ions may be distinguished depending on the specific location of the counter ions in the surface layers[22,23].

Amines that are present in the eluent as tracer cations HA^+ are assumed to exchange with the counter ions:

$$\overline{B^+X^-} + HA^+ \leftrightharpoons \overline{HA^+X^-} + B^+$$

The bar denotes the complex at the surface. The equilibrium can be described by means of the molar selectivity coefficient for ion exchange[24]:

$$K_e = \frac{\overline{[HA^+X^-]}\,[B^+]}{\overline{[B^+X^-]}\,[HA^+]} \tag{1}$$

where $\overline{[HA^+X^-]}$ and $\overline{[B^+X^-]}$ denote the concentrations of the respective complexes at the surface.

If the pH of the eluent is sufficiently low, the amine will be present only in its protonated form, HA^+. For a partition process based on ion exchange the partition coefficient, K, of the amine can be written as

$$K = \frac{\overline{[HA^+X^-]}}{[HA^+]} \tag{2a}$$

$$= K_e \cdot \frac{\overline{[B^+X^-]}}{[B^+]} \tag{2b}$$

The capacity factor, k', is defined in the usual way as

$$k' = qK_e \cdot \frac{\overline{[B^+X^-]}}{[B^+]} \tag{3}$$

were q is the phase ratio.

An amino acid in aqueous buffers at low pH will exist only as a zwitterion, HG, and as a positive ion, H_2G^+. The retention behaviour is governed by two exchange equilibria:

$$K_{e1} = \frac{\overline{[H_2G^+X^-]}\,[B^+]}{\overline{[B^+X^-]}[H_2G^+]} \tag{4}$$

and

$$K_{e2} = \frac{\overline{[HGX^-]}\,[B^+]}{\overline{[B^+X^-]}\,[HG]} \tag{5}$$

The following expression for the capacity factor, k', can be derived:

$$k' = q\left(K_{e1} + \frac{K_{e2}K_{a1}}{[H^+]}\right) \cdot \frac{[H^+]}{[H^+] + K_{a1}} \cdot \frac{\overline{[B^+X^-]}}{[B^+]} \tag{6}$$

were K_{a1} is the dissociation constant for the ionization stage concerned.

EXPERIMENTAL

Apparatus

Various liquid chromatographs were used. Catecholamine separations were carried out on a Spectra-Physics Type 3500 liquid chromatograph equipped with a

Model 770 variable-wavelength UV photometer or a Kipp LC 771 liquid chromatograph equipped with a Zeiss Type PM-DLC spectrophotometer.

The instrument used for the separation of amino-acids has been described elsewhere[25]; the on-line reaction with *o*-phthalaldehyde was used for detection. All columns were 15 cm long and constructed from 4.6 mm I.D. and 6.35 mm O.D. precision-bore stainless-steel tubing. The columns were thermostatted by means of a circulating water-bath to within 0.1°. High-pressure sampling valves (Rheodyne Type 70-10) equipped with 20-μl sample loops were used in all instruments.

An LDC Type 1107 refractometer was used for monitoring breakthrough curves. The apparatus used for batch experiments has been described elsewhere[26].

Chemicals and materials

Quartz-distilled water and analytical-grade organic solvents were used in all experiments. Amino acids and catecholamines were obtained from Aldrich Milwaukee, Wisc., U.S.A.) and Serva (Heidelberg, G.F.R.). Buffers were prepared from Titrisol solutions of orthophosphoric acid and sodium hydroxide (Merck, Darmstadt, G.F.R.). Sodium salts of hexyl-, octyl- and dodecylsulphonic acids (zur Tensidanalyse) were purchased from Merck.

The bonded phases examined were LiChrosorb RP-18 (Merck) and Partisil 10-ODS (Whatman, Maidstone, Great Britain), both with an average particle size of 10 μm. Oxtadecyl-modified silicas were also prepared from *n*-octadecyltrichlorosilane and 10-μ m SI-100 (Merck)[27,28]; the carbon content of the resulting material was 16%. This packing material is coded as C-18.

The symbols used for denoting the sample components are as follows: nor ADR, noradrenaline; ADR, adrenaline; DOP, dopamine; TYR, tyramine; GLY, glycine; THR, threonine; SER, serine and ALA, alanine.

Procedures

The columns were packed by a balanced-density slurry technique with 1,1,2,2-tetrabromoethane–tetrachloromethane–dioxan (36:32:32) as the suspension medium and at pressure of about 50 MPa. The columns were washed successively with 100 ml each of 2,2,4-trimethylpentane, dichloromethane, 2-propanol and 100 ml of water before the mobile phase was applied.

The capacity factor, k'_i, for a component i was determined from its retention volume, V_{Ri}, and the liquid hold-up of the column V_{Ro}. The usual method for determining k'_i from the corresponding retention time, t_{Ri}, and the retention, t_{Ro}, of an unretarded component was not applied, as unretarded compounds are difficult to find for the phase systems concerned. Inorganic UV-absorbing anions, *e.g.*, nitrate, should particularly be avoided as they are excluded from the internal volume of the reversed-phase packing material "loaded" with sulphonates[16,25].

The liquid hold-up of the column, V_{Ro}, was determined as follows. The column was first filled with water, then rinsed with about 90 ml of 2-propanol. The eluate was collected in a 100-ml calibrated flask and diluted to 100 ml with 2-propanol. The water content was measured by Karl Fisher titration, and from the resulting value V_{Ro} was calculated. Sometimes the values found were verified by applying the reverse procedure, *i.e.*, filling the column with 2-propanol and rinsing it with water. The 2-propanol

in the eluate was determined by gas chromatography and the values were found to agree to within 5% or better.

Adsorption isotherms ware calculated from breakthrough curves as described by Huber and Gerritse[29]. Before measuring the breakthrough for the lowest concentration for a given sulphonate, the columns were equilibrated with the eluent without the detergent. Then the eluent containing a fixed amount of the sulphonate concerned was fed to the column and the elution curve was measured by the refractive index monitor. The eluate was collected in 1-ml fractions and the breakthrough of the sulphonate was verified by simply shaking, which caused foaming in the presence of the sulphonate, or by a colour reaction with methylene blue[30].

By using a series of eluents of increasing sulphonate concentration the adsorption isotherms could be readily constructed[29].

RESULTS AND DISCUSSION

Before starting the chromatographic experiments, the stability of the phase systems was verified. A typical result is shown in Fig. 1, illustrating the excellent reproducibility of the retention behaviour for a single column. The reproducibility for k' values for a given eluent composition between columns was better than 5%.

Two different types of reversed-phase packing material were used[31]. LiChrosorb RP-18 and the C-18 material are "brush"-type packings[32] in which the silica surface is covered almost completely by single carbon moieties bonded to single

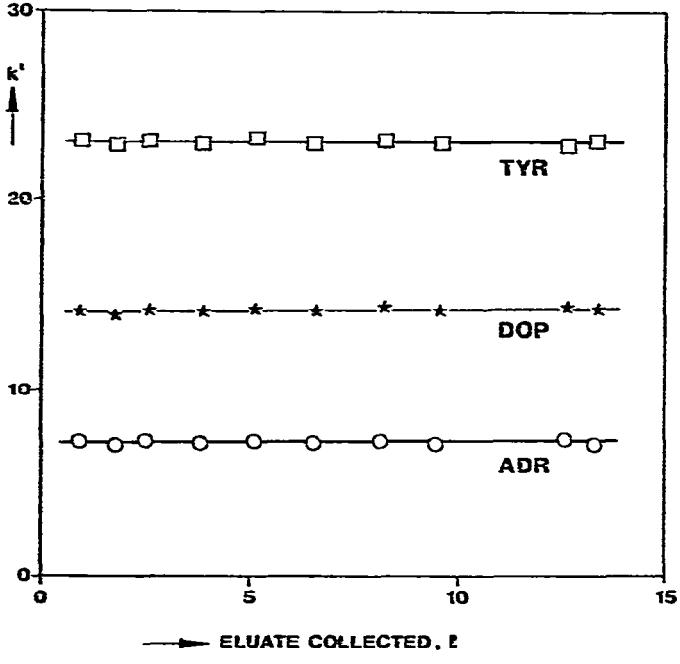

Fig. 1. Stability of the phase systems. Column (15 × 0.46 cm I.D.) packed with 10-μm LiChrosorb RP-18. Eluent: phosphate buffer, (0.01 M Na^+, pH 3.00) + 2% (v/v) 1-propanol and 0.005 M sodium octylsulphonate. Temperature: 40°.

surface sites and the resulting material is strongly hydrophobic. In Partisil 10-ODS, on the other hand, the surface is only partially covered and the material is readily wetted by water[31].

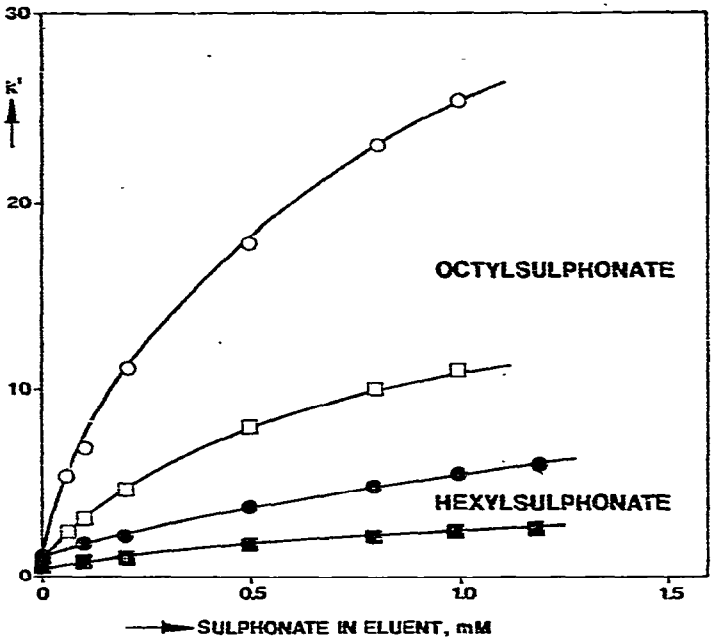

Fig. 2. Capacity factors of catecholamines as a function of sodium sulphonate concentration. Column packing: LiChrosorb RP-18. Eluent: phosphate buffer (0.05 *M* Na^+, pH 3.00). Temperature: 20°. ○, ●, ADR; □, ■, nor ADR.

Fig. 2 shows the retention behaviour of some catecholamines on the former material as a function of the sulphonate concentration in a neat aqueous eluent. The corresponding adsorption isotherms for hexyl- and octylsulphonate are given in Fig. 3. These isotherms are of L- or H-type[33] and obey the Freundlich equation:

$$\overline{[B^+X^-]} = a[X^-]^b \qquad (7)$$

As shown in Fig. 4, the dependence of the capacity factors on $\overline{[Na^+X^-]}/[Na^+]$ is in good agreement with eqn. 3. The slope of the straight lines through the data points is equal to qK_e. The value of the phase ratio, q, was estimated from the weight of the packing material in the column and the liquid hold up. Then K_e was calculated (see Table I). It appears that K_e increases markedly with increasing chain length of the amphiphilic ion. A similar effect is observed for ion-exchange phenomena in detergent films at the air-water interface[20]. As the sodium concentration in the buffer used in the chromatographic experiment is 0.05 *M* and that of the sulphonates is about 0.001 *M* at most, the sodium ion concentration remains virtually constant. Figs. 5 and 6 show values of k' plotted against the concentration of octyl- and dodecylsulphonate,

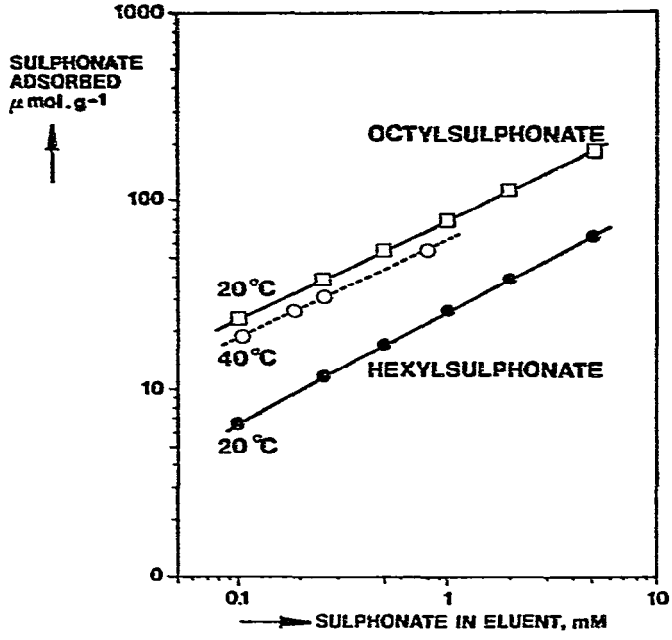

Fig. 3. Adsorption isotherms (Freundlich plots) of sodium sulphonate on LiChrosorb RP-18 from phosphate buffer (0.01 *M* Na^+, pH 3.00).

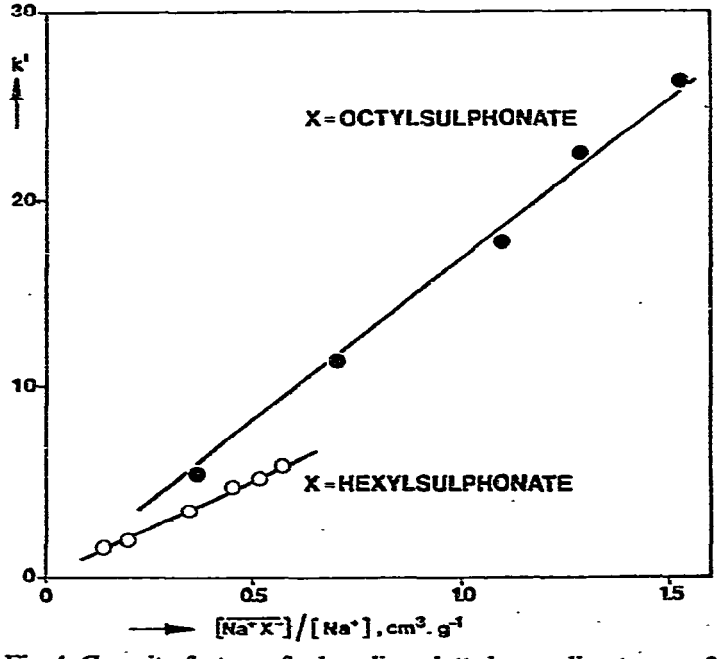

Fig. 4. Capacity factors of adrenaline plotted according to eqn. 3 for hexyl- and octylsulphonate. Phase system: see Fig. 2.

TABLE I

MOLAR SELECTIVITY COEFFICIENTS FOR ION EXCHANGE OF ADRENALINE *VERSUS* SODIUM

Phase system: Lichrosorb RP-18 and aqueous sodium phosphate buffers (pH 3.00).

Amphiphilic ion	*Temperature (°C)*	K_e
Hexylsulphonate	20	12.5
Octylsulphonate	20	21.3
Octylsulphonate	40	11.3

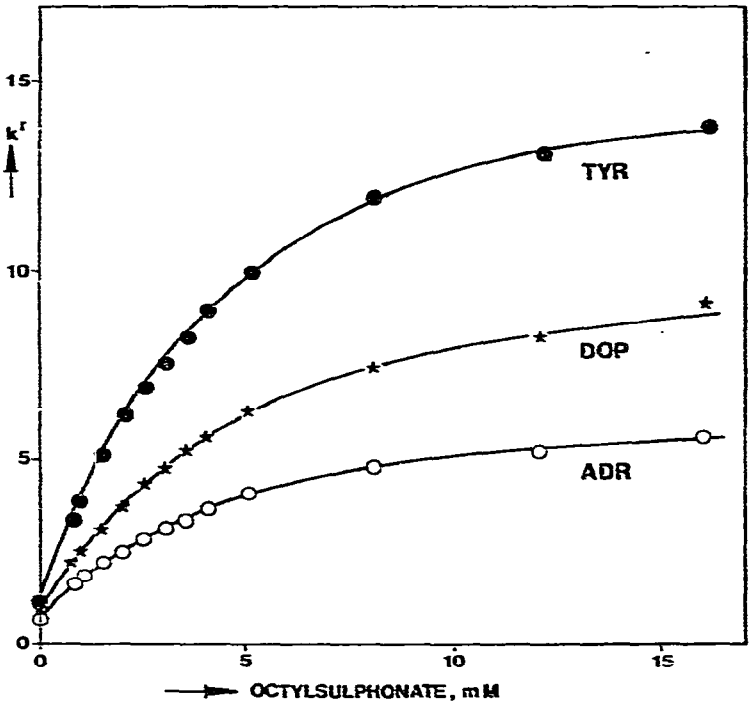

Fig. 5. Capacity factors of catecholamines as a function of sodium octylsulphonate concentration. Column packing: Partisil 10-ODS. Eluent: phosphate buffer (0.01 *M* Na^+, pH 3.00) + 0.5% (v/v) 2-propanol and sodium octylsulphonate. Temperature: 40°.

respectively, for Partisil-ODS. In these systems the addition of the sulphonates brings about a significant increase in the sodium concentration in the eluent.

The corresponding adsorption isotherms are shown in Fig. 7. From Figs. 8 and 9 it can be seen that eqn. 3 holds also for these systems. The K_e values are given in Table II. It appears, once again, that K_e increases with increasing alkyl chain length of the amphiphilic ion.

The influence of temperature on k' is shown in Fig. 10. The increase in k' at lower temperatures is due to the combined influence of enhanced adsorption of the sulphonate (see Fig. 7) and an increase in K_e (see Table II). In this instance, however, the influence of K_e predominates.

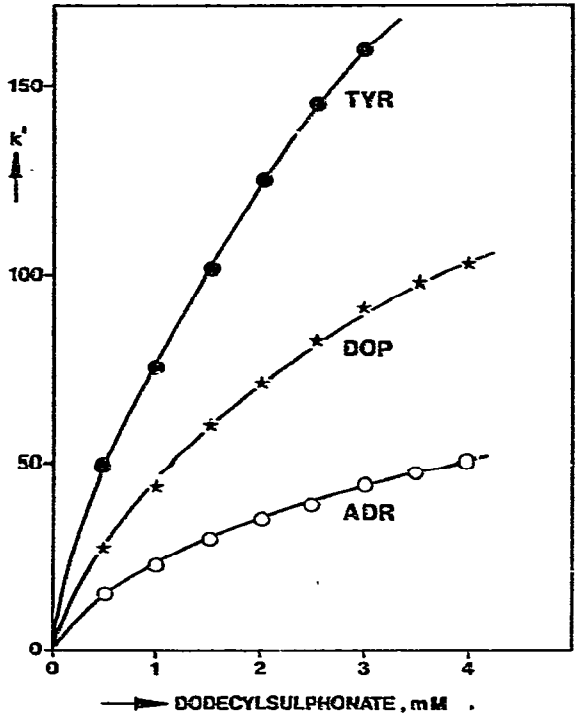

Fig. 6. Capacity factors of catecholamines as a function of sodium dodecylsulphonate concentration. Phase system: see Fig. 5.

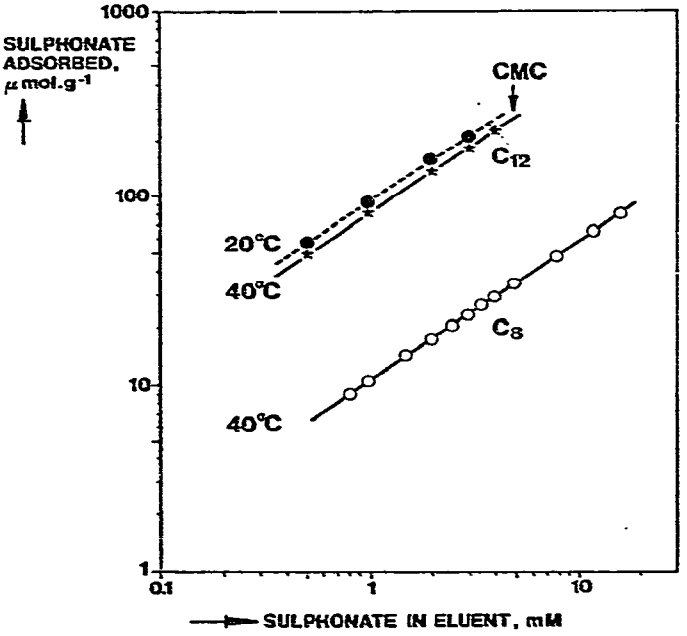

Fig. 7. Adsorption isotherms (Freundlich plots) of sodium sulphonate on Partisil 10-ODS from phosphate buffer (0.01 *M* Na^+, pH 3.00) + 0.5% (v/v) 2-propanol.

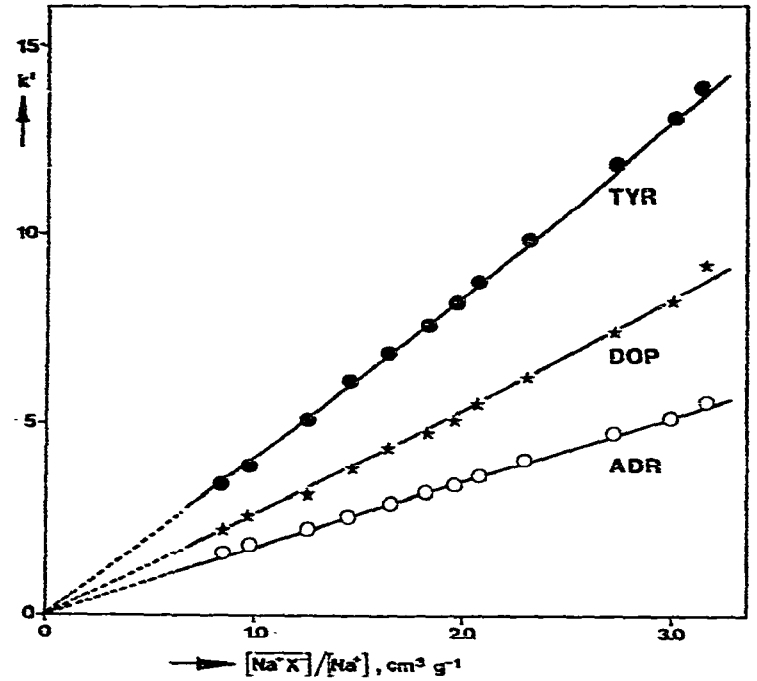

Fig. 8. Capacity factors of catecholamines plotted according to eqn. 3 for the phase system from Fig. 5 (sodium octylsulphonate).

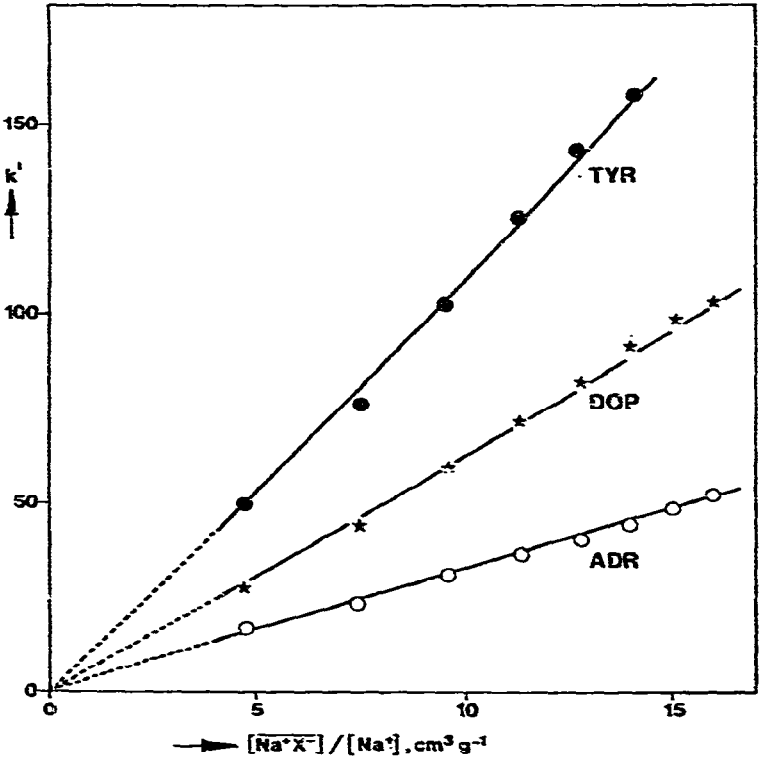

Fig. 9. Capacity factors of catecholamines plotted according to eqn. 3 for the phase system from Fig. 6 (sodium dodecylsulphonate).

TABLE II

MOLAR SELECTIVITY COEFFICIENTS FOR ION EXCHANGE OF CATECHOLAMINES *VERSUS* SODIUM

Phase system: Partisil 10-ODS and sodium phosphate buffers (pH 3.00) + 0.5% (v/v) 2-propanol.

Amphiphilic ion	*Temperature (°C)*	K_e		
		ADR	*DOP*	*TYR*
Octylsulphonate	40	2.2	3.5	5.5
Dodecylsulphonate	40	4.1	8.0	13.8
Dodecylsulphonate	20	6.3	14.9	28.0

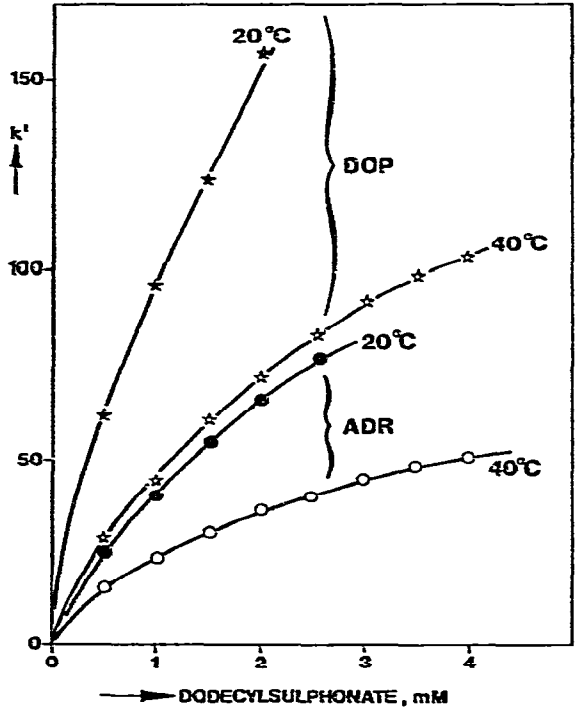

Fig. 10. Influence of temperature on the capacity factors of catecholamines. Phase system: see Fig. 6.

Fig. 11 shows the dependence of k' for tyramine on the octylsulphonate concentration in phase systems containing various amounts of added 1-propanol and sodium ions. The sodium ion concentration increases on increasing the strength of the phosphate buffer. The corresponding adsorption isotherms are given in Fig. 12. In this instance, however, no linear relationship between k' and $\overline{[Na^+X^-]}/[Na^+]$ was found. It turns out that K_e, as calculated from eqn. 3, increases with increasing coverage of the surface by the amphiphilic ion (see Fig. 13). At a loading of 200 μmol·g^{-1} a constant value of K_e appears to be attained. By way of comparison, it should be noted that the coverage by hydrocarbon ligands for the particular packing material cor-

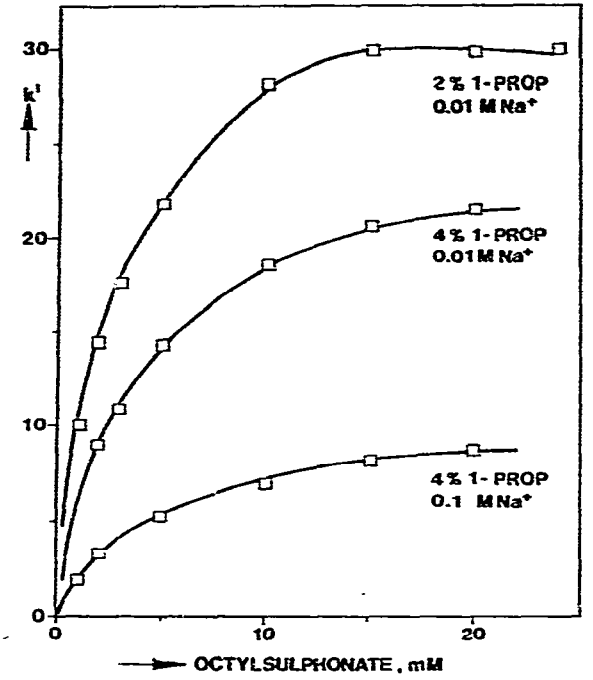

Fig. 11. Capacity factors of tyramine in sodium phosphate buffer (pH 3.00) containing various amounts of added 1-propanol as a function of sodium sulphonate concentration. Column packing: C-18 silica. Temperature: 40°.

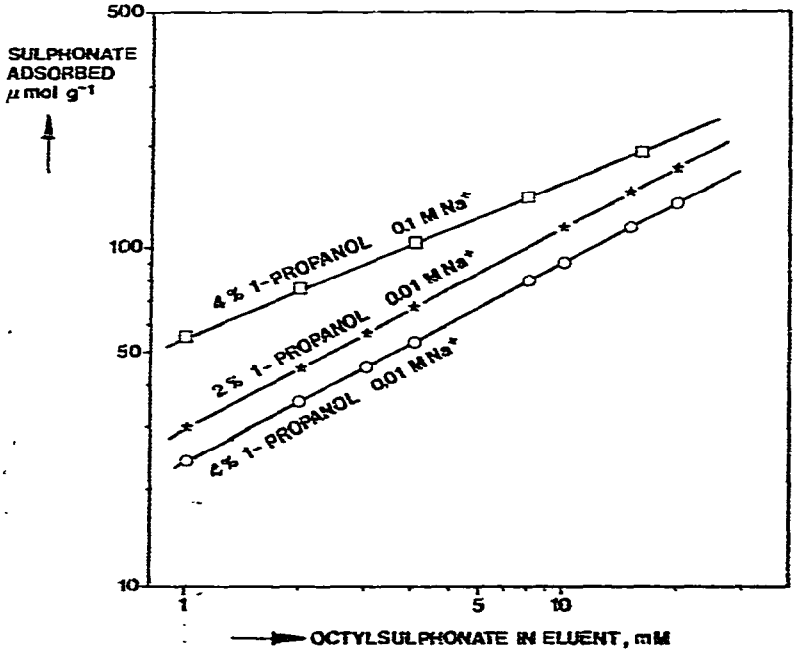

Fig. 12. Adsorption isotherm (Freundlich plots) of sodium octylsulphonate on C-18 silica for the phase systems from Fig. 11.

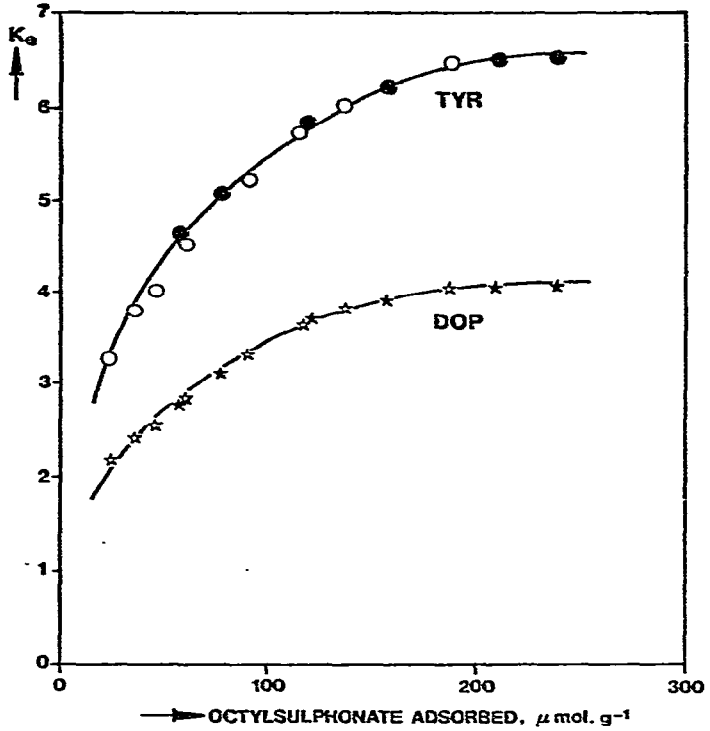

Fig. 13. Molar selectivity coefficient for ion exchange, K_e, for catecholamines *versus* sodium as a function of adsorbed octylsulphonate. Column packing: C-18 silica. Eluent: sodium phosphate buffer (pH 3.00) + 4% (v/v) 1-propanol. Temperature: 40°. ○, ★, 0.01 *M* Na^+; ●, ★, 0.1 *M* Na^+.

responds to about 800 $\mu mol \cdot g^{-1}$. A dependence of selectivity coefficients on surface density has also been observed in a study on ion-exchange phenomena on soap films[20].

Fig. 14 shows the capacity factors of three amino acids as a function of the concentration of dodecylsulphonate in an eluent containing 0.5% of 2-propanol at pH 3.00. The corresponding adsorption isotherm is given in Fig. 15. It should be noted that the flattening of the curve for sulphonate concentrations around 4 m*M* is certainly not due to micelle formation, because in the eluent the critical micellar concentration, as determined by conductivity measurements, is 4.9 m*M*. In fact, a maximum in the k' plot corresponds to a maximum in the fraction $\overline{[Na^+X^-]}/[Na^+]$ in eqns. 3 and 6. Now, $\overline{[Na^+X^-]}$ can be calculated according to eqn. 6, and $[Na^+]$ is found as the sum of the sodium concentration in the original buffer, $[Na^+]_o$, and the sodium associated with the added sulphonate $[X^-]$. Substituting $[Na^+] = [Na^+]_o + [X^-]$ and differentiating we find from eqn. 7 that a maximum should occur for $[X^-] = b[Na^+]_o/(1 - b)$. From the Freundlich plot of the adsorption isotherm we find $b = 0.31$. Then, for $[Na^+] = 0.01$ *M*, we find that k' should reach a maximum at $[X^-] = 4.5$ m*M*.

The influence of the concentration of the counter ion, $[Na^+]$, was measured at a constant concentration of the sulphonate in the eluent, $[X^-]$. The concentration of the counter ion was changed by adding sodium perchlorate (0–0.02 *M*). Fig. 16 shows

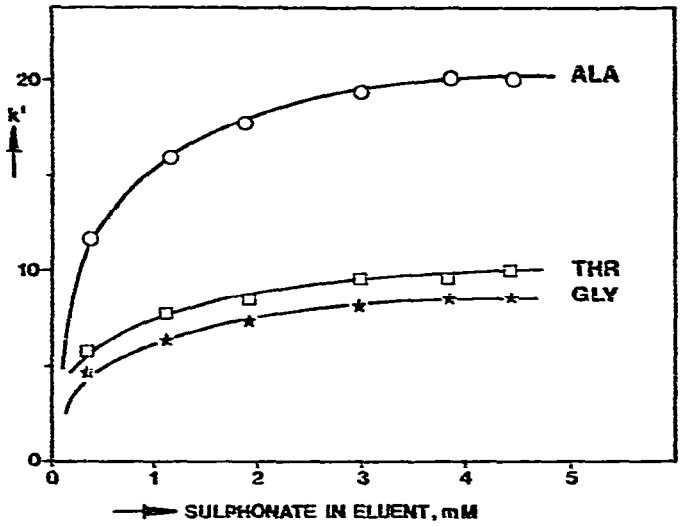

Fig. 14. Capacity factors of amino acids as a function of sodium dodecylsulphonate concentration. Column packing: C-18 silica. Eluent: phosphate buffer (0.01 *M* Na^+, pH 3.00) + 0.5% (v/v) 2-propanol. Temperature: 20°.

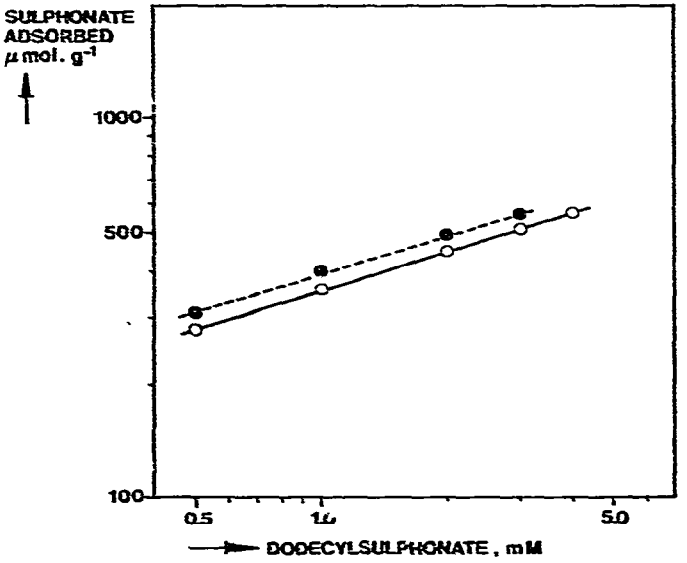

Fig. 15. Adsorption isotherms (Freundlich plots) of sodium dodecylsulphonate on C-18 silica for the phase system from Fig. 14. ○, Buffer; ●, buffer + 0.01 *M* $NaClO_4$.

k' values plotted against $\overline{[Na^+X^-]}/[Na^+]$. The data were taken both from Fig. 14 and from the experiments with increasing $[Na^+]$ at constant $[X^-]$. In the latter instance, the increase in $\overline{[Na^+X^-]}$ resulting from the addition of sodium perchlorate was taken into account. The dependence of k' on $\overline{[Na^+X^-]}/[Na^+]$ is in agreement with eqn. 6. Probably as a result of the high surface loading, no variation in K_e was observed.

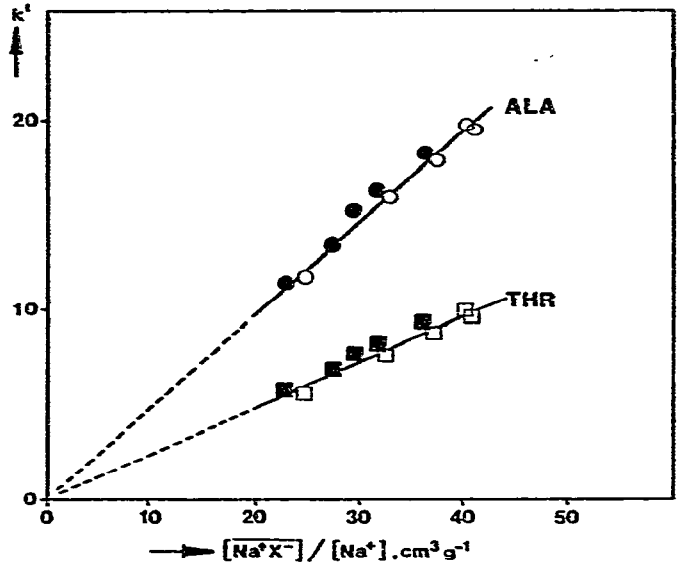

Fig. 16. Capacity factors of threonine and alanine plotted according to eqn. 3. Data from Fig. 13 (□) and from experiments with increasing [Na^+] produced by addition of sodium perchlorate (■).

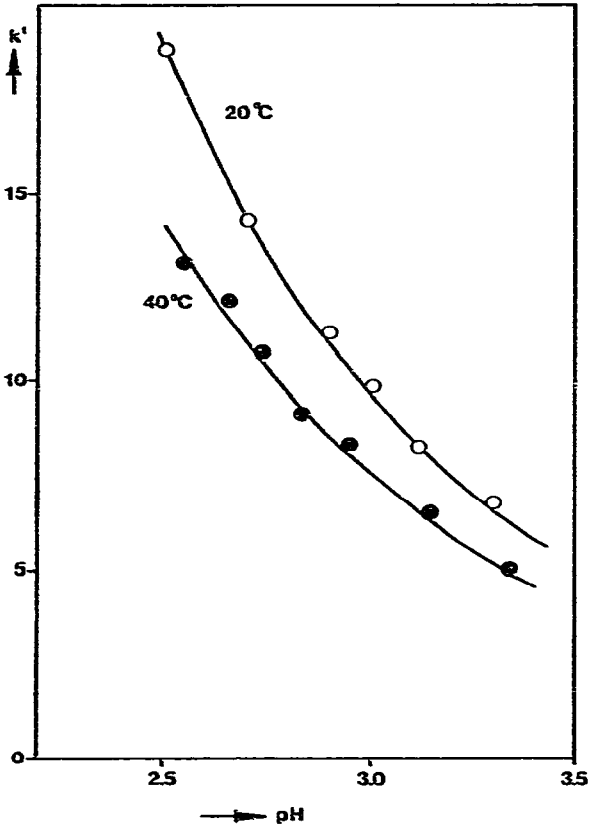

Fig. 17. Capacity factors of alanine as a function of pH. Phase system: see Fig. 14.

At otherwise constant eluent composition, the pH strongly influences the retention of amino acids (see Fig. 17). In these experiments the concentrations of organic modifier, counter ion and dodecylsulphonate were kept constant and only the pH of the buffer was changed by varying the amount of orthophosphoric acid. The K_e values for alanine were estimated from eqn. 6 by regression; the values are given in Table III. The full lines in Fig. 17 were calculated by using eqn. 6 and the estimated K_e values.

TABLE III

MOLAR SELECTIVITY COEFFICIENTS FOR ION EXCHANGE OF ALANINE *VERSUS* SODIUM

Phase system: C-18 silica and sodium phosphate buffers (for pH see Fig. 17) + 2% (v/v) 1-propanol and 0.002 *M* sodium dodecylsulphonate

Temperature (°C)	K_{e1}	K_{e2}
20	1.73	0.11
40	1.49	0.09

To verify the basic assumption in this work, *i.e.*, that the protonated amines are retained through the formation of a complex with an amphiphilic ion previously adsorbed on to the surface of the hydrophobic packing material rather than through adsorption of an amine–sulphonate ion pair previously formed in the eluent, the following experiment was carried out. A column packed with LiChrosorb RP-18 was equilibrated with a neat aqueous eluent at pH 3.00 and $[Na^+] = 0.01$ *M*, containing 0.01 *M* octylsulphonate. This column was placed in a recycle system. The solvent container and pumping system were filled with the same eluent; the total amount on liquid in the system was about 5 ml. Then, a known amount of adrenaline was added to the eluent in the reservoir and the recycling procedure was started. After equilibration, which was considered to be complete after 500 ml had passed through the column, the concentrations of the relevant ionic species in the eluent were determined by isotachophoresis. The compositions of the eluent before and after recycling are given in Table IV. Although a considerable amount of the amine is retained on the column packing material, no significant decrease in the sulphonate concentration in the eluent could be observed. Further, the decrease in the adrenaline concentration in the mobile phase is compensated for by an almost equivalent increase in $[Na^+]$. The final pH shift due to the addition of adrenaline was about 0.2.

TABLE IV

CHANGES IN ELUENT COMPOSITION IN A RECYCLE SYSTEM RESULTING FROM THE ADDITION OF ADRENALINE

Io	*In eluent*		*Difference (μmol)*
	Before recycling (μmol)	*After recycling (μmol)*	
Na^+	52	58	+6 (±2)
$(octylsulphonate)^-$	7	7	—
$(adrenaline)^+$	11	3	−8 (±2)

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