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

LXXXVI*. THE INFLUENCE OF DIFFERENT DISPLACER SALTS ON THE RETENTION AND BANDWIDTH PROPERTIES OF PROTEINS SEPARATED BY ISOCRATIC ANION-EXCHANGE CHROMATOGRAPHY

M. T. W. HEARN*, A. N. HODDER and M. I. AGUILAR
Department of Biochemistry, Monash University, Clayton, Victoria 3168 (Australia)

SUMMARY

The influence of different displacer salts on the retention behaviour of seven globular proteins ranging in molecular weight from 12 000 to 69 000 was investigated using the Mono Q anion-exchange resin as the stationary phase. Isocratic retention data were collected using several different alkali metal halides as the displacing salt, thereby systematically varying the anion and cation species in the series F^-, Cl^- and Br^- and Li^+, Na^+ and K^+. The different anions were found to reduce protein retention in order of their decreasing hydrated ionic radii. Protein Z_c values were found to be lower for fluoride and bromide than for chloride. It was demonstrated that the cationic co-ions also influence solute retention properties with this anion-exchange resin through, *inter alia*, preferential interactions with the protein solute.

Protein band-broadening was found to systematically vary with the choice of displacer salt. These changes were related to known Hofmeister effects on protein aggregation kinetics and solubility and the degree of ion penetration at the double layer of the stationary phase–mobile phase interface. These studies now provide a rapid comparative basis for evaluating the mechanism of co- and counter-ion interactions with proteins in high-performance ion-exchange chromatographic systems.

INTRODUCTION

Modern high-performance ion-exchange chromatographic (IEC) supports have now found extensive application in the high-resolution analysis and purification of many proteins and other biological macromolecules. However, many of the theoretical aspects pertaining to the structure–retention behaviour of these complex polyelectrolyte molecules during ion-exchange elution have not been fully elucidated. The "net-charge" concept has been widely used as a predictive basis to anticipate the

* For Part LXXXV see ref. 40.

retention characeristics of proteins eluted from both anion- and cation-exchange resins[1,2]. Various studies (see for example refs. 3–5) have, however, indicated that protein retention in IEC depends on a number of factors including the number of charged sites on the protein surface which interact with the support surface and the distribution of these surface charges. The quantitative consequences of these factors are pre-determined by the amino acid sequence and the secondary and tertiary structure of each protein solute. Furthermore, the retention of a specific protein is strongly influenced by the mobile phase composition so that the solute retention–eluent composition relationships rarely show direct proportionality for unrelated proteins.

Extensive studies have shown that the selectivity of proteins eluted in IEC can be manipulated by variation in solution pH and ionic strength, thus capitalising on changes in the electrostatic surface potentials of the proteins. An alternative method of optimising selectivity in IEC involves changes in the nature of the displacer ion. However, the roles that different salt species can play in the retention behaviour of proteins in IEC has not been fully rationalised to permit comprehensive predictability. For example, it would be anticipated that the well documented effect of different salts on protein solubility and stability, known as the Hofmeister effect, would have a significant influence on the chromatographic behaviour of proteins in IEC yet formal relationships between the Hofmeister or Setchenov coefficients and the electrostatic displacing strength of different ions in ion exchange systems have not been firmly documented. The Hofmeister effects become important at moderate concentrations in the range 0.01–1.0 *M* in bulk solution and tend to be dominated by anion effects which are approximately additive for many polyionic or amphoteric species in solution. In more complex systems such as in anion-exchange chromatography, the effects of anions on protein stability will not simply be manifested through their presence in the bulk mobile phase but will also arise due to time dependent protein interactions with anions complexed at the stationary phase–mobile phase interface. These charge and concentration gradients, which form to neutralise in the case of anion-exchange resins the positively charged stationary phase surface, are often referred to as double layers and are believed to exist predominantly at low ionic strengths[3]. Similarly the role of co-ions in the mechanism of protein retention in IEC has not been clearly defined. It is generally considered that the co-ions complex with the accessible protein surface as proposed in the ion-condensation theory[6]. As these thermodynamically favourable interactions may exert significant effects on protein structure and stability, the appropriate choice of both the anion and cation species in anion-exchange systems therefore represents an important consideration for the optimisation of protein separations in terms of both resolution and biorecovery. As part of a continuing study on the mechanistic details of protein retention in IEC, the present paper describes the results of detailed investigations addressing these fundamental questions related to the influence of various displacer salts, selected from the lyotrophic series, on the isocratic retention and bandwidth properties of several globular proteins.

MATERIALS AND METHODS

Apparatus

All chromatographic experiments were performed with a Pharmacia (Uppsala,

Sweden) fast protein liquid chromatography (FPLC) system consisting of two P-500 syringe pumps, a V-7 injector and a 278-nm fixed-wavelength single-path UV monitor, coupled to a two-channel REC 482 pen recorder and a Perkin-Elmer (Newalk, CO, U.S.A.) LCI-100 integrator. Isocratic elution was controlled with a Pharmacia GP-250 solvent programmer. Mono Q prepacked strong-anion-exchange columns (HR 5/5, 50 × 5 mm I.D.) were used throughout. The pH measurements were performed with an Orion (Cambridge, MA, U.S.A.) SA 520 meter, equipped with a combination glass electrode. All chromatographic separations were carried out at 20°C.

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale skeletal muscle myoglobin (type iii), hen egg white lysozyme (grade I), hen egg ovalbumin (grade V), horse heart cytochrome *c* (type iv), human serum albumin, bovine haemoglobin (type 1) and piperazine were all purchased from Sigma (St. Louis, MO, U.S.A.). Sodium fluoride (Univar grade), sodium bromide (Unilab grade) and lithium chloride (Unilab grade) were obtained from Ajax Chemicals (Sydney, Australia). Sodium chloride (AnalaR grade) and potassium chloride (AnalaR grade) were obtained from BDH (Port Fairy, Australia). Quartz-distilled water was further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Buffers were adjusted to pH 9.60 by either hydrofluoric acid (48% AnalaR grade), hydrochloric acid (spec. gravity 1.16, AnalaR grade) or hydrobromic acid (spec. gravity 1.46–1.49, AnalaR) all of which were purchased from BDH.

Chromatographic procedures

Eluent A was a 0.02 *M* piperazine solution adjusted to pH 9.60. Eluent B comprised 0.02 *M* piperazine and 0.3 *M* displacer salt at pH 9.60. The displacer salts used in this study were potassium chloride, sodium chloride, lithium chloride, sodium fluoride and sodium bromide. Eluents A and B were filtered (0.45-μm cellulose acetate, HAWP 04700, Millipore) and degassed under vacuum. Protein solutions were prepared by dissolving the repurified protein in eluent A at a concentration of 5 mg/ml unless otherwise specified. Protein solutions were filtered before use through 0.22-μm ACROLC 13 filters (Gelman Sciences, Sydney, Australia). Protein sample sizes ranged from 5 to 1000 μg with injection volumes between 10 and 200 μl. For each displacer salt, samples were eluted isocratically, starting at 100% eluent B and repeated, at decreasing concentrations of B, until the protein elution volume was greater than 30 column volumes. With each displacer salt used log k' and log $(1/c)$ data were collected for each protein and subjected to an iterative regression analysis to determine the y-intercept (log K), slope (Z_c) and correlation coefficient. Column efficiency, h_{eff}, was determined from peak width at half height measurements and second moments. The column dead time, t_0, was obtained from the retention time of a salt breakthrough peak following a 50-μl injection of 1 *M* sodium chloride in 100% eluent B (0.3 *M* sodium chloride).

RESULTS AND DISCUSSION

Retention relationships

Solute retention in IEC is conventionally formalised as arising from comple-

mentary electrostatic interactions between the solute molecules and the stationary phase surface. For large polyelectrolyte solutes such as proteins, the magnitude of these electrostatic interactions depends on several factors. These include the number of charged sites on the solute molecule that interact with the stationary phase, the charge density of the packing and the mobile phase composition. A non-mechanistic, stoichiometric equation which is often written to describe the bulk mass distribution of a polyelectrolyte solute molecule, $P^{\pm a}$, in IEC is as follows

$$(P^{\pm a})_m + (a/b)(D^{\pm b})_s \rightleftarrows (P^{\pm a})_s + (a/b)(D^{\pm b})_m \quad (1)$$

where $D^{\pm b}$ is the displacer counter ion and the subscripts m and s represent the mobile and stationary phase respectively. From the above equation the equilibrium constant for the interaction of a protein with an ion-exchange surface under static conditions can be represented as

$$K_b = \frac{[P^{\pm a}]_s [D^{\pm b}]_m^{Z_t}}{[P^{\pm a}]_m [D^{\pm b}]_s^{Z_t}} \quad (2)$$

where Z_t for large molecules, such as proteins, represents the stoichiometric charge ratio (a/b)[4,7]. If near equilibrium conditions can be assumed for the isocratic chromatographic process associated with the transport of a protein along an anion-exchange support then the following form of the Taylor series expression can also be employed to represent the relationship between retention of the polyelectrolyte solute and the concentration of the displacer salt, c

$$\log k' = \alpha + \beta [\log(1/c)] + \gamma[\log(1/c)]^2 + \delta[\log(1/c)]^3 + \ldots \quad (3)$$

where k' is the capacity factor and $\alpha, \beta, \gamma, \delta$ are coefficients dependent on the solubility parameter of the solute, the stationary phase ζ potential and mobile phase composition, polarisability and dielectric properties. Over a limited range of solvent composition the relationship between $\log k'$ and $\log 1/c$ is often approximated to a linear dependency given in the familiar form

$$\log k' = \log K + Z_c \log(1/c) \quad (4)$$

where K is the distribution coefficient which incorporates several terms in eqn. 2, including the binding constant K_b, the phase ratio φ and the stationary phase ligand concentration, D_b, in the following manner:

$$K = \frac{K_b \varphi [D_b]^{Z0}}{ab} \quad (5)$$

where the constants a and b adjust for solute and salt valency. Under linear elution conditions, values for Z_c and $\log K$ can therefore be determined for a narrow range of ionic strengths by linear regression analyses of plots of $\log k'$ *versus* $\log (1/c)$. It must be noted however, that the Z_c coefficient as defined in eqn. 4 is not formally equivalent in mathematical or physicochemical terms to the Z_t term of eqn. 2 or

indeed the Z_0 term as defined in eqn. 5 but reflects the apparent number of ionic charges associated with the adsorption–desorption process at the chromatographic surface.

According to eqn. 4 for eluents of fixed mobile phase pH, the logarithmic capacity factor of a protein separated in IEC is linearly related to the inverse of the logarithm of the concentration of displacer ion. In addition, solute desorption from IEC supports is anticipated to be closely associated with the affinity which the displacer ions exhibit with the support surface. At fixed ionic strengths, anions can be ranked[8] in their affinity for the quarternary ammonium anion-exchange resin, Dowex-2, as follows,

$$ClO_4^- > SCN^- > (CH_3)_3CH_2COO^- > p\text{-Tosyl}^- > I^- > Br^- > NO_3^- > Cl^- > H_2PO_4^- > CH_3COO^- > F^-$$

Experimentally, under normal IEC operating conditions, smaller elution volumes are observed for low-molecular-weight samples when elution is carried out with displacer ions with higher affinities for the stationary phase. For example, simple organic acid molecules are typically eluted from anionic resins with much smaller retention times using thiocyanate than fluoride ions. Similar trends are observed when small organic bases and other positively charged low-molecular-weight molecules are separated using cation-exchange chromatography. Under these conditions, retention times are found to increase with the choice of the displacing cation in the following manner[9]

$$Ba^{2+} < Ca^{2+} < Mg^{2+} < K^+ < NH_4^+ < Na^+ < Li^+$$

However, there is generally less variation in retention times of solutes eluted in cation-exchange chromatography as the range in the magnitude of the distribution coefficients and cation affinities for the support surface are much narrower than observed for anions due to smaller variations in the size and charge characteristics of the solvated cation, and the solvated characteristics of the complementary immobilised ionic ligand in terms of the double layer structure.

While a large amount of work has been reported on the characterisation of conventional soft IEC supports[1,2,10–12], there have been few detailed studies on the retention phenomena of proteins separated with modern high-performance IEC supports, particularly with respect to protein retention mechanisms. In the current study, the influence of anion and cation selection on the retention and bandwidth properties of several protein solutes has been investigated using the strong anion exchanger Mono Q. The experimental design used in this study included the selection of various monovalent alkali metal halides as displacer salts. The influence of the counter ion was studied using the series NaF, NaCl and NaBr whilst LiCl, NaCl and KCl were used to assess the effect of the co-ion on solute retention and bandwidth behaviour. Table I lists some physical properties of these metal halide salts. It is apparent that while the crystal radii (r_c) of both anions and cations become larger with increasing atomic weight, *i.e.* $r_{c,F} < r_{c,Cl} < r_{c,Br}$ and $r_{c,Li} < r_{c,Na} < r_{c,K}$, in aqueous solutions the relative size of the hydrated ionic radii (r_i) of these ions are such that these sequences are reversed. The stability of the hydrated species is also reflected in the respective enthalpy (ΔH_f^0), entropy (ΔS^0) and free energy (ΔG_f^0) changes associated

TABLE I

PHYSICAL PROPERTIES OF THE ALKALI METAL HALIDES USED IN THIS STUDY

	F^-	Cl^-	Br^-	Li^+	Na^+	K^+
Crystal radius (Å) (ref. 13)	1.36	1.81	1.95	0.60	0.95	1.33
Hydrated radius (Å) (ref. 13)	3.52	3.32	3.30	3.82	3.58	3.31
ΔH_f^0 (kJ mol^{-1}) (ref. 14)	−329.1	−167.4	−120.9	−278.4	−239.7	−251.2
ΔS^0 (kJ^{-1} mol^{-1}) (ref. 14)	−9.6	55	80.7	14.2	60.3	102.5
ΔG_f^0 (kJ mol^{-1}) (ref. 14)	−276.5	−131.2	−102.8	−293.8	−261.9	−282.3
$K_{(Dowex-2)}$ (ref. 15)	0.1	1.0	3.4	–	–	–
$\tau_r^{\pm}/\tau_r^0$ (ref. 16)	2.3	0.9	0.6	2.3	1.6	0.9

with aqueous ion formation. This sequence reversal has been attributed to the small mass/charge ratio found with the lower-atomic-weight ions leading to increased electrostriction of adjacent water molecules.

According to the stroichiometric model for IEC of proteins, and on the basis of ion affinities for the stationary phase, the relative selectivity of protein solutes separated under anion-exchange conditions is anticipated to be directly related to the number of interacting point charges and the displacing power of the counter ion. However, it is now well established that different salts can, in bulk solution, influence the stability, solubility and biological activity of macromolecules, effects which clearly have profound implications for the chromatographic behaviour of proteins in IEC. The effects of various salts on protein solubility was originally documented by Hofmeister[17] and has since been reviewed extensively[18–22]. Generally, anionic species having stronger affinities than Cl^- for the ion-exchange medium are classified as chaotropes or water-structure breakers and are known to destabilise protein structure while those anions which have a weaker affinity than Cl^- are referred to as polar kosmotropes[23] or water-structure makers and have a stabilising effect on protein structure. The chloride ion has little effect on water structure surrounding the protein molecule and in the range of molarities commonly used in IEC (*e.g.* 0.1–0.7 *M*) has little influence on protein three dimensional hierarchy or conformational stability. The influence of various salts on protein solubility has also been found to serendipidously coincide with anion affinities for anion-exchange surfaces. The effectiveness of chaotropic ions as displacing species in IEC and their destabilising effect on protein three dimensional structure is believed to be associated with the energetically favourable loss of loosely held water molecules from the ions' first hydrated shell as they preferentially complex with either the stationary phase medium or protein molecules[24]. The loss of water molecules from the hydration layer(s) of polar kosmotropes does not readily occur and consequently these ions tend to stabilise protein structure and complex less readily with IEC media. Clearly, experimental practice and observation qualitatively teach us that the IEC properties of proteins are strongly influenced by the presence of different salts. In the current study, our investigations on the systematic variation of the mobile phase composition are continued, whereby salts composed of chaotropic and kosmotropic ions were selected as mobile phase displacer additives in the isocratic elution of proteins from Mono Q strong anion-

exchange media. Isocratic retention and bandwidth data were obtained for seven globular proteins listed in Table II. The molecular weights of these proteins varied between 12 000 and 69 000 with p*I* values ranging between 4.7 and 11.0. All proteins were chromatographed using piperazine (20 m*M*) buffer at pH 9.60 and the displacer salt concentration varying between 0 and 300 m*M* at a flow-rate of 1 ml/min.

Fig. 1 represents plots of isocratic retention data as a function of the displacer salt concentration for each protein listed in Table II. In these investigations one ion, namely either the sodium or the chloride ion has been held constant to allow the effect of the selected complimentary anion or cation on solute retention to be characterised. Curvi-linear retention plots were obtained in all cases, a finding that is consistent with multi-site binding processes associated with biomolecule interactions with heterogeneous coulombic surfaces. Under the pH 9.6 elution conditions, the isoelectric points (p*I*) of lysozyme and cytochrome *c* are very close to the buffer pH (pH_b) values resulting in only a small net positive or near zero average charge respectively on these biomolecules. Several consequences arise when $pH_b \approx pI$ for proteins separated by IEC. Firstly, the solubility of proteins in solution will be greatly reduced. Secondly, the interplay between hydrophobic and coulombic effects will be accentuated such that mixed-mode retention mechanisms become more dominant as the eluent pH approaches the protein p*I* value. Although in the limit situation where $pH_b \rightarrow pI$, the net charge Z_n approaches zero, it must be born in mind that the protein solute will still exhibit an asymmetric surface charge distribution.

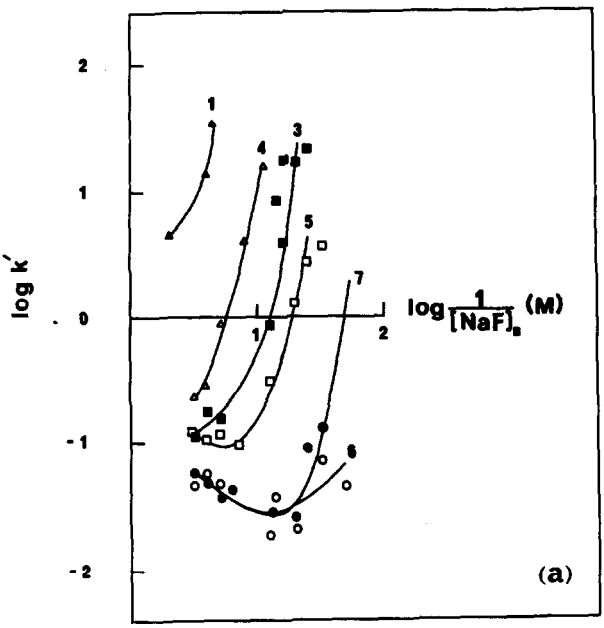
In Fig. 1, the relative retention effects induced by each salt type with the selected proteins can be observed from the shape of their retention plots. For most salt systems investigated, the plots for both lysozyme and cytochrome *c* exhibited a pseudo-parabolic dependence of log k' on log $(1/c)$. For example, at high ionic strength a downward curvature of the plots was observed, a finding which is contrary to that normally anticipated for IEC behaviour of proteins but which is consistent with hydrophobic partitioning behaviour of these solutes at the stationary phase boundary. This "salting out" process is characterised by decreasing solute retention as the salt concentration also decreases from relatively high values (*i.e.* from 0.3 *M*). With some protein/ion cases, such as the elution of cytochrome *c* with sodium fluoride or sodium bromide, there was a subsequently slight upward curvature of these plots as

TABLE II

PROTEIN PHYSICAL PARAMETERS

No.	*Protein (source)*	*pI*	*MW*	*Dm* × 10^{-10} (m^2/min)*
1	Ovalbumin (egg white)	4.70	43 500	4.24
2	Albumin (human serum)	5.85	69 000	3.64
3	Carbonic anhydrase (bovine erythrocytes)	5.89	30 000	4.80
4	Haemoglobin (bovine)	6.80	64 500	3.72
5	Myglobin (sperm whale muscle)	7.68 8.18	17 500	5.74
6	Cytochrome *c* (horse heart)	9.4	12 400	6.42
7	Lysozyme (hen egg white)	11.0	14 300	6.12

* Calculated from Stokes–Einstein equation[25].


log k'
2
1
0
-1
-2
1
2
3
4
5
6
7
log 1/[NaF]B (M)
(a)

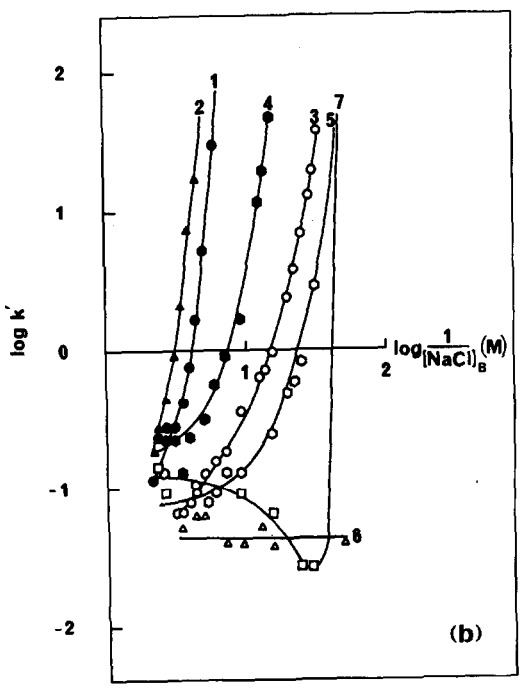
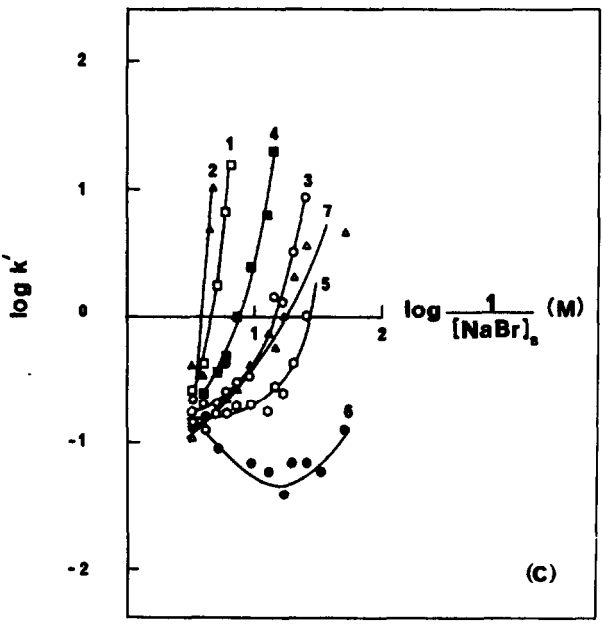

log k'
2
1
0
-1
-2
1
2
3
4
5
6
7
log 1/[NaCl]B (M)
(b)

Fig. 1.



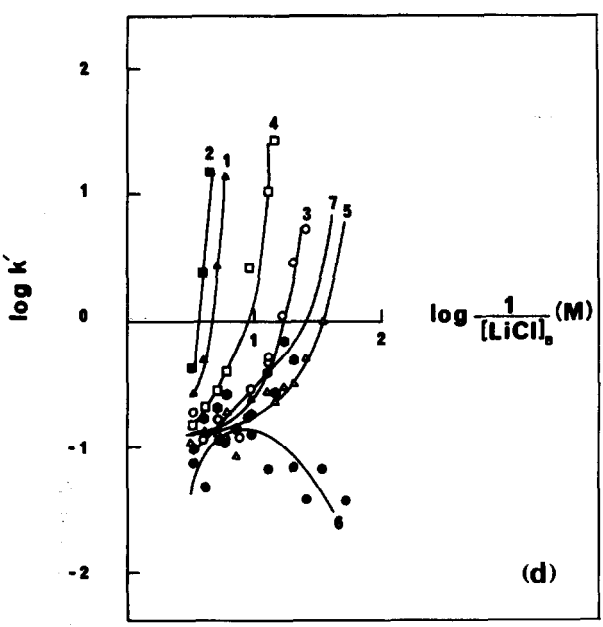


Fig. 1

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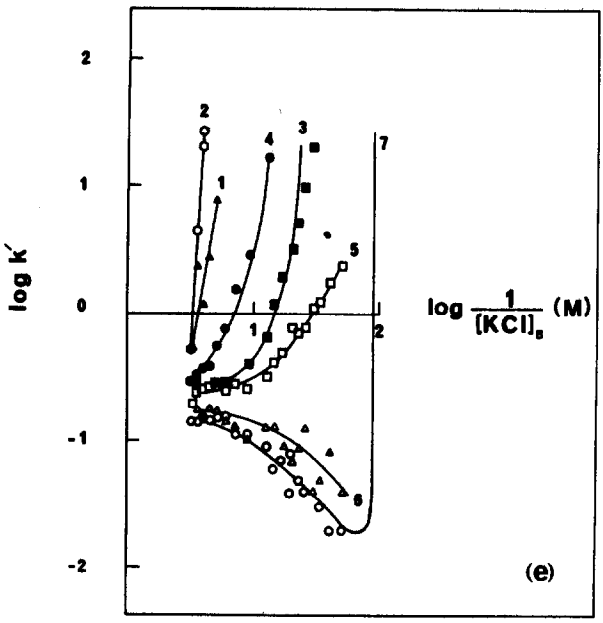


Fig. 1. Plots of log k' *versus* log (1/[salt]) for the seven proteins listed in Table II. The plots were derived from isocratic elution data of pH 9.6 and flow-rate of 1 ml/min for (a) sodium fluoride, (b) sodium chloride, (c) sodium bromide, (d) lithium chloride and (e) potassium chloride. Other chromatographic conditions are given in Materials and methods. See Table II for the code to protein solutes and Table III for the derived Z_c values.

the salt concentration was systematically lowered from 0.05 to 0.02 *M*. This behaviour is also associated with increasing solubility of the solutes in the presence of these ions such that these proteins begin to elute with selectivities consistent with "normal" ion-exchange processes. These observations again are indicative of the interplay of alternative mixed-mode retention mechanisms which may be operating. It should be noted that log–log plots such as those used in Fig. 1 greatly magnify the "salting-out" effect simply because over the retention range $-1.5 < \log k' < 0$ the relatively small changes in the experimental retention times fall outside the usual operational conditions for coulombic chromatography, *i.e.* $0.3 < \log k' < 2$.

Table III lists Z values (Z_c) obtained for each solute by regression analysis of the linear regions of the retention plots shown in Fig. 1 for the different displacer salt systems. Values of Z_c were not estimated for the salt systems where lysozyme and cytochrome *c* were eluted by mixed modes of retention. It was generally observed for the majority of proteins that small but significant differences in the Z_c values were obtained when the protein was eluted with different anions. In particular, Z_c values of proteins determined in the presence of the fluoride or bromide ion were in all cases slightly less than the Z_c values obtained with the chloride ion (sodium was the common cation throughout). Significant variation in the Z_c values were also observed with different cationic species. For example, myoglobin, ovalbumin and carbonic anhydrase showed differences in Z_c for all cations while haemoglobin and human

TABLE III

ISOCRATIC Z_c VALUES AND CORRELATION COEFFICIENT (r^2) OBTAINED FROM REGRESSION ANALYSIS OF PROTEIN RETENTION DATA

ND = Not determined due to mixed-mode retention mechanisms.

*Protein No.**	*NaF*	*NaCl*	*NaBr*	*KCl*	*NaCl*	*LiCl*
1	6.69 (0.98)	7.73 (0.98)	7.09 (1.00)	4.88 (0.77)	7.73 (0.98)	6.34 (0.96)
2	**	11.14 (0.98)	8.53 (0.72)	11.90 (1.00)	11.14 (0.98)	10.68 (0.96)
3	3.94 (0.85)	4.42 (0.98)	3.21 (0.96)	3.97 (1.00)	4.42 (0.98)	3.09 (0.92)
4	3.57 (0.98)	5.04 (0.96)	3.92 (0.98)	3.68 (0.98)	5.04 (0.96)	4.40 (0.98)
5	2.36 (0.72)	2.76 (0.96)	2.36 (0.85)	1.43 (1.00)	2.76 (0.96)	0.82 (0.79)
6	ND	ND	ND	ND	ND	ND
7	0.74 (0.53)	ND	1.59 (0.90)	ND	ND	0.80 (0.81)

* See Table II.

** Human serum albumin was not eluted with this salt.

serum albumin exhibited no significant variation in Z_c over the range of cations used. If the Z_c value represents the number of charged interactive sites involved in the binding between the protein solute and the stationary phase surface, and if the ionic species in solution have no influence on the spatial distribution of these interactive charges, it would be anticipated for a particular protein solute that experimentally observed Z_c values will be independent of the displacer ions used in solute elution. Although examples of this behaviour are known from the present and other studies[26], in general it can not be assumed that the mechanism of interaction between a displacer ion, a particular protein solute and the stationary phase surface will be identical for all salt species. Furthermore the results of the present study confirm that the derivation of Z_t as a stoichiometric charge ratio from chromatographic data requires caution, *i.e.* eqn. 2 represents an over-simplified description of the actual IEC process. In fact, the magnitude of Z_c values, which can be obtained through linear regression analysis of experimental retention data, was in each case not identical to the net charge of the protein as predicted on the basis of knowledge of the eluent pH and the p*I* of the protein. Since the physico-chemical properties of all constituents in the interactive double layer and diffuse layer zones, the average dynamic orientation of the protein solute to the stationary phase and the influence of the displacing anions and the cationic co-ions on the interactive properties will ultimately determine the slope of the log k' *versus* log $(1/c)$ plots, *i.e.* Z_c, such divergences are not unexpected. In agreement with a dominant IEC elution mode, generally high correlation coefficients were obtained ($r^2 \geqslant 0.95$) for the linear regression analysis of the experimental data shown in Fig. 1, over the elution range encompassing the retention region $0.3 < \log k < 1.5$. However, over the total range of ionic strengths examined in these investigations (0–300 m*M*), poorer correlations of log k' *versus* log $(1/c)$ in terms of eqn. 2 were observed. This observation was particularly evident as experimental divergences from "ideal" ion-exchange retention behaviour at high and low ionic strengths. These results, in accord with the interplay of multi-modal retention processes, are reflected as non-linear log k' *versus* log $(1/c)$ retention dependencies and

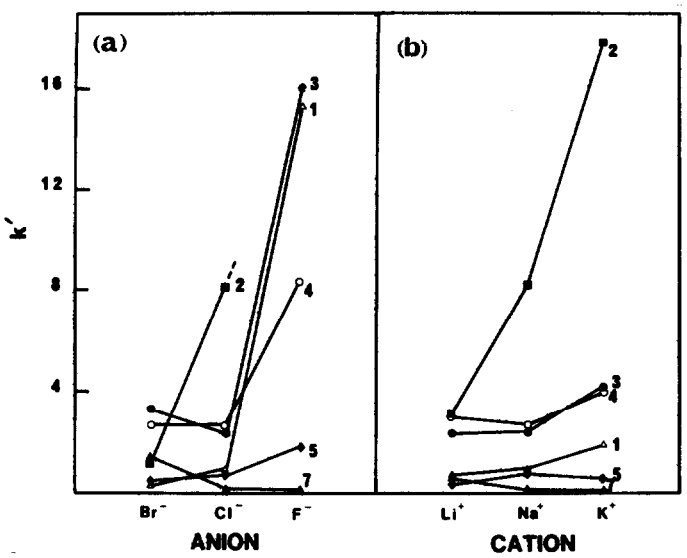


Fig. 2. Dependence of k' on ion type for displacer anions (a) and cationic co-ions (b). Capacity factor values for each protein were obtained from regression analysis data for each anion and cation type. The capacity factor was calculated at a salt concentration of 30 mM for carbonic anhydrase, myoglobin and lysozyme, 90 mM for haemoglobin and 240 mM for ovalbumin and human serum albumin.

demonstrate the requirement for theoretical descriptions of IEC retention processes to involve more complex expressions than either eqn. 1 or 4, if they are to provide any quantitative utility or insight into ion selective effects. Several other lines of evidence of ion selective influences on protein retention in IEC[27,28] and also HIC[29] have been documented which irrevocably lead to the same conclusion.

The relative displacing strength of each salt is illustrated in Fig. 2, where the capacity factor, k', at a particular salt concentration is plotted against the particular anion or cation. Values of k' were obtained by regression analysis of the linear regions of the log k' *versus* log $(1/c)$ plots shown in Fig. 1. It must be noted that the data in Fig. 2 do not formally represent selectivity curves as typically derived for different solutes [*i.e.* from $\alpha = k'$ (solute 1)/k' (solute 2)] but rather the intrasolute selectivity curve for different ions [*i.e.* $\alpha = k'$ (solute, 1, ion 1)/k' (solute 1, ion 2)]. As is evident from Fig. 2a and Table I, there is a general increase in solute retention time as the hydrated radius of the anion increases through the series bromide, chloride, fluoride. This result is also consistent with the different affinities of these ions for the anion-exchange support. Since the Z_c values for each protein were of similar magnitude for the range of anions used, large changes in log k' such as those observed for ovalbumin (1) and carbonic anhydrase (3) would, according to eqn. 4, be a consequence of changes in log K. If salts of the same valency are used with a particular column packing, eqn. 5 predicts that K will be directly related to K_b, the equilibrium constant for the ion-exchange process. If the interactive properties of a particular solute in the presence of different ions remain constant as evidenced by similar Z_c values, then changes in K_b will be proportional to the affinity of the anion for the stationary phase surface and the average distance (r_0) of the ion from the polyelectrolyte surface. In

an associated paper, determination of the r_0 values for the various proteins using the above chromatographic approach will be presented. These determinations based on derivative mathematical treatments of the Stern double layer theory and the diffuse boundary theory provide quantitative assessment of the number of ions, and the average distance of those ions from the solvated surface, which are involved in the desorption process.

Fig. 2b illustrates the effect of cation selection on protein retention. With the exception of human serum albumin, the retention times of all solutes were much less sensitive to changes in the cation species. While the co-ions have little effect on the affinities of the solutes for the stationary phase surface, overall they can exert a significant influence on the nature of the ionic binding domain involving the protein surface. This conclusion is in agreement with Manning's ion condensation theory[6] for polyelectrolytes which postulates[30,31] that cations are actively involved in stabilising polyelectrolyte structures by neutralising the repulsive effects of various anionic groups at the surface of the solute molecule. Furthermore, previous studies[30,31] on the binding of metal ions to DNA in aqueous solutions have suggested that all small cations, except those that actually penetrate the native DNA structure and enter into strong intercalations with the nucleoside bases, are associated to various extents with DNA as delocalised interactions.

The ratio of the reorientation time ($\tau_r^{\pm}$) of a water molecule in the first hydration shell of the diamagnetic ions used in this study, to the corresponding time in pure water (τ_r^0) at 25°C as determined by proton nuclear magnetic longitudinal (T_1) relaxation rates are listed in Table I. The higher $\tau_r^{\pm}/\tau_r^0$ ratio for the lithium cation corresponds to lower ionic mobility and higher contact viscosity. In order to accommodate these observations it is proposed that the positive charge on the lithium ion is delocalised throughout the water molecules of the inner solvation sheath. One consequence of this behaviour would be a preference for multi-site attachments between lithium ions and the protein solute. The data documented in this paper on the chromatographic behaviour of various proteins in the presence of different cations is in accord with this hypothesis which predicts that alteration of the interactive properties of the solute will effectively arise due to selective cation masking of the surface negative charges on the biosolute. This process will diminish with decreasing hydrated ionic radii across the series lithium > sodium > potassium. While this phenomena would clearly be solute dependent, the large change in k' observed on varying the cation, *e.g.* human serum albumin in the presence of potassium chloride compared to sodium chloride, demonstrates that the cationic species can and do significantly influence the anion-exchange chromatographic behaviour of protein solutes.

Changes in the experimentally derived Z_c values reflect changes in the effective surface area of the solute's coulombic binding site. Provided that the average coulombic contact area remains constant over a range of experimental conditions, it is feasible for two different proteins to exhibit identical Z_c values. Similarly, a particular protein could exhibit a constant Z_c value with a selection of ionic displacers, despite the fact that mechanistically different retention processes may prevail. The data with human serum albumin may represent one such example. In this case similar Z_c values were observed for human serum albumin with various cations although the relative retention factors were significantly different. This finding suggests that double-lay-

er-mediated orientational or conformational changes give rise to differences in the affinity of the protein for the stationary phase surface. In order to distinguish the various contributions of the displacer salts to the mechanism of protein retention in IEC, including the influence of different ions on the protein diffusional and interactive properties and binding kinetics, the relevant information must be retrieved from the analysis of the experimental bandwidth behaviour.

Bandwidth relationships

The efficiency of a well-packed column can be assessed, according to Kennedy and Knox[32], in terms of the following relationship

$$h_{\text{eff}} = Av^{1/3} + B\,v^{-1} + Cv \tag{6}$$

where v is the reduced velocity, h_{eff} is the effective reduced plate height obtained from the peak zone second moment data, the A term is related to eddy diffusion and interparticle mass transfer, whilst the B term accomodates longitudinal diffusion effects. When operating at high flow-rate conditions (where v is typically > 100) with a well packed ion-exchange column, the dependency of the reduced plate height on the reduced velocity can usually be approximated to

$$h_{\text{eff}} = Cv \tag{7}$$

where C is the intraparticulate mass transfer coefficient and includes the resistance to mass transfer at the stationary phase surface inside the pores of the column packing material. Giddings[33] has represented the dependency of C on the separation conditions as follows

$$C = \frac{1}{30}\,\frac{k''}{(1+k'')^2}\,\frac{D_{\text{m}}}{D_{\text{sz}}} \tag{8}$$

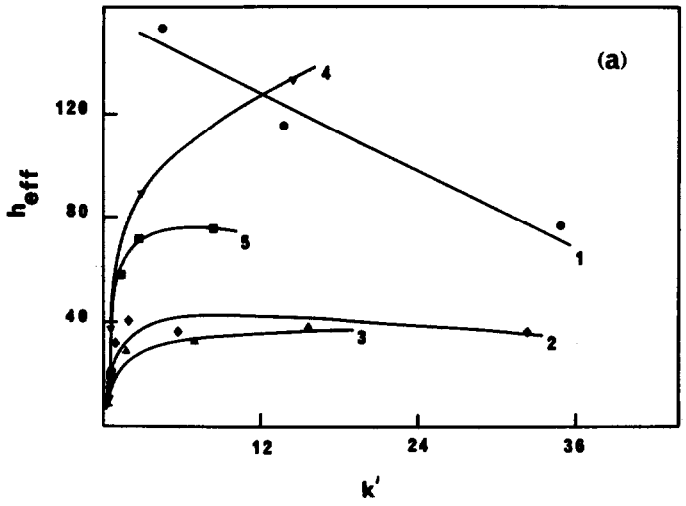
where D_{m} and D_{sz} are the solute diffusion coefficients in the mobile phase and stationary phase zone respectively, and k'' is the zone capacity factor equivalent to

$$k'' = \frac{\text{mass of solute in stationary phase}}{\text{mass of solute in mobile phase}} \tag{9}$$

In addition, k' can be related to k'' through the parameter β, which is the fraction of the mobile phase present in stagnant pools within the pores as follows

$$k' = k''\,(1 - \beta) - \beta \tag{10}$$

Previous studies[4] have shown that manipulation of mobile phase pH and ionic strength influences the diffusional properties of protein solutes. If it is assumed that ratios of D_{m} and D_{sz} of a particular protein in the presence of different salt systems remain constant then the contribution of the diffusion processes associated with mass transfer of the solute to the overall kinetics of the separation would, according to eqn. 8, be reduced to the dependence of the C term on the zonal capacity factor. If



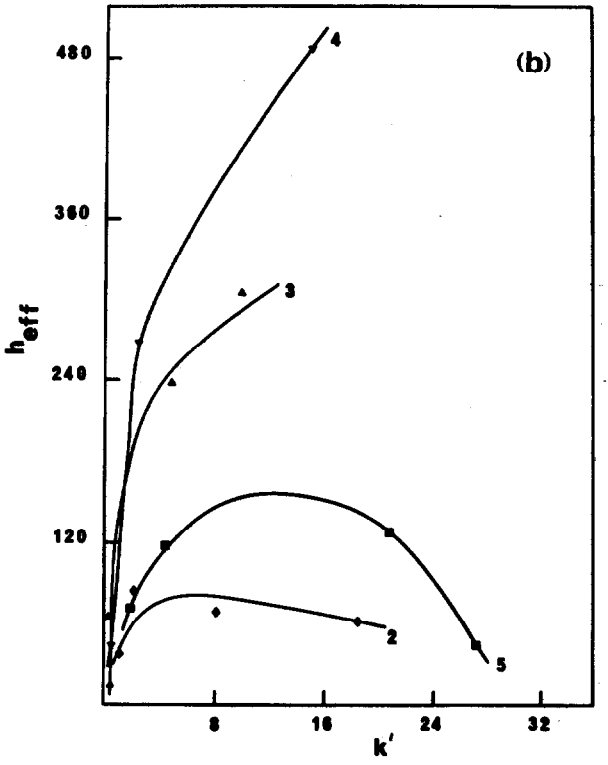
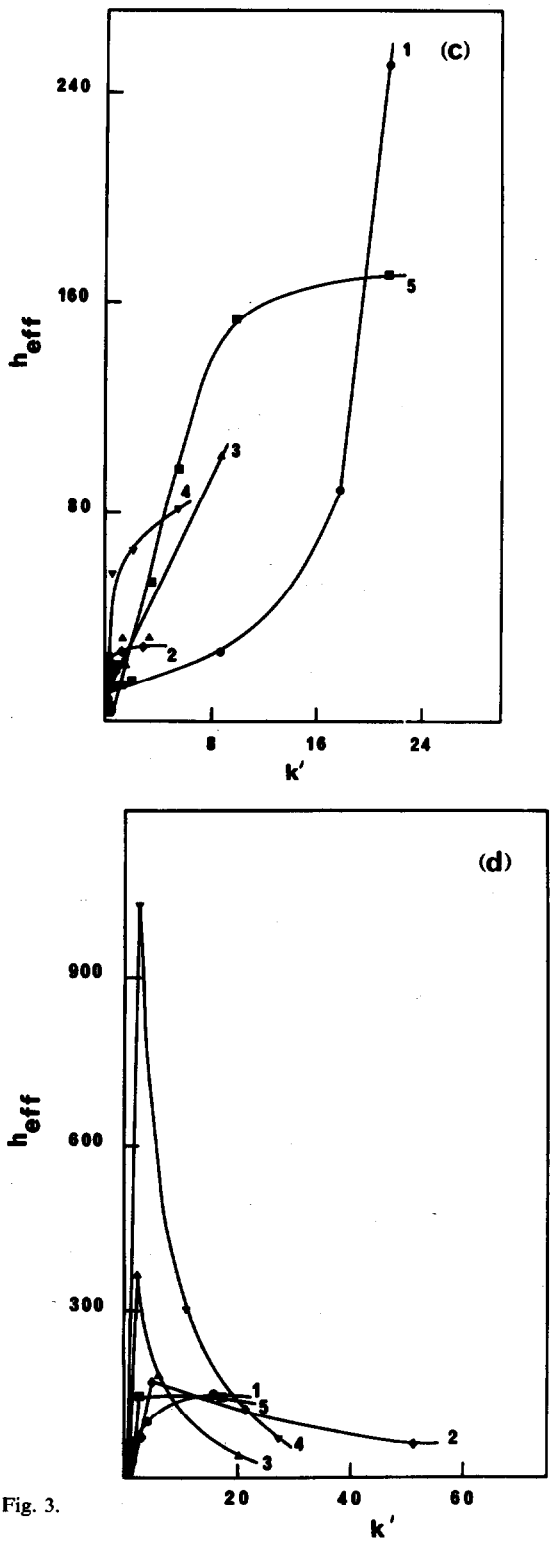


Fig. 3.

(*Continued on p. 112*)


(c)
240
160
80
h_{eff}
1
2
3
4
5
8
16
24
k'
(d)
900
600
300
h_{eff}
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2
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4
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20
40
60
k'

Fig. 3.

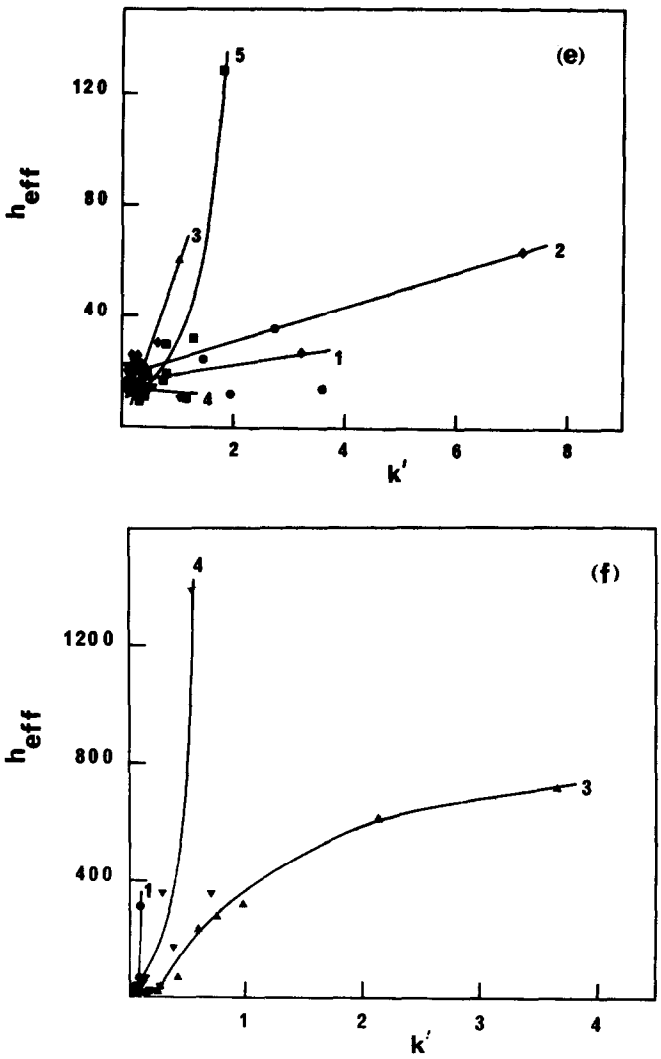

Fig. 3. The influence of anion and cation type on the dependence of h_{eff} *versus* k' for (a) ovalbumin, (b) human serum albumin, (c) carbonic anhydrase, (d) haemoglobin, (e) myoglobin and (f) lysozyme. Values of h_{eff} were determined from measurements of peak width at half height. The salts used were (1) sodium fluoride, (2) sodium chloride, (3) sodium bromide, (4) lithium chloride and (5) potassium chloride.

the experimental capacity factors are dependent on the concentration of the displacer ion exclusively, and the kinetics of solute retention are independent of the mobile phase composition in terms of the ions present, then, superimpossible plots of h_{eff} *versus* k' for a specific protein eluted at constant pH with different displacer salts would be anticipated on the basis of eqns. 7 and 8. Fig. 3 illustrates the dependence of h_{eff} on k' for the six proteins of the current study. As is evident from these data, h_{eff} values were found to vary considerably with the choice of anion or cation, a finding which immediately leads to the conclusion that adsorption–desorption ki-

netics are dependent on the nature of the displacing ion. For the anion series which act as the principal desorption agents, the h_{eff} values were closely dependent on the protein solute, and in some cases were seen to increase in the order $F^- < Cl^- < Br^-$. Divergencies in the plots become more evident with higher k' values or longer column residence times. The h_{eff} values under these conditions are not superimposible but for some proteins are still ranked in accordance with the anion positions in the Hofmeister series. Charged surfaces are known to generate charged layers of ions extending into the external medium[34–36]. In IEC the formation of these so called double layers and diffuse boundary layers results in a gradient of both charge potential and ionic concentration between the solid phase and the bulk solution. The magnitude of this electrostatic potential gradient is dependent upon the pH and the nature of the ionic and non-ionic species in the medium. Ion exclusion studies[3] using IEC media have shown that double layer formation is greatest at lower ionic strength. Since it can be also argued on stochastic grounds that only a small portion of the total surface area of a protein will encounter the double layer, areas of greatest electrostatic potential on the protein surface will dominate the equilibrium interaction but other areas of lower potential could kinetically participate in molecular orientation of the protein solute at the support surface. Prolonged exposure of the solute to the anion gradient at the double layer–diffuse boundary layer interface may then lead to increased band broadening with longer column residence time as shown in Fig. 3. This phenomenon may account for the stabilisation–destabilisation of the protein hierarchial structure and the loss of bioactivity observed with some proteins when separated under IEC conditions. The effect of having double layers formed with different types of anions which influence protein stability would also be expected to be reflected in the changes in the D_m/D_{sz} ratio and thus, a change according to eqns. 2 and 8, in C and h_{eff}.

In contrast to the results with several proteins, *e.g.* carbonic anhydrase, ovalbumin shows an order reversal for the effect of anion type on column efficiency. Application of known Hofmeister anion effects observed with proteins in solution studies can still accommodate these observations. Instead of the increase in h_{eff} values, which reflect the relative degree of destabilisation of protein structure due to conformational effects mediated by various anions as for example described above for carbonic anhydrase, the results with ovalbumin show an increasing h_{eff} effect across the anion series. In this case, the band broadening appears to arise as a consequence of the well known propensity of this protein to aggregate, with increases in h_{eff} induced by prolonged interaction with double layer anions and promoted in the following order: $F^- > Cl^- > Br^-$. This aggregation behaviour is in compliance with the "salting out" ability of these anions in the Hofmeister series. The increase in the C value of the Knox equation which arises due to aggregative events can be interpreted as a consequence of changes in the D_m/D_{sz} ratio in eqn. 8. The effect of anion type on the h_{eff} values for lysozyme, shown in Fig. 3f, follows the same ranking as seen for ovalbumin. Very broad elution profiles corresponding to extremely large h_{eff} values were obtained. Previous work[37–39] has shown that lysozyme not only dimerises but can also assume higher polymeric forms in buffers when the pH > 9. Such phenomena may account for the very broad elution profiles and low column efficiencies seen for lysozyme. In common with the results seen for ovalbumin, the elution of lysozyme with the bromide ion gives rise to decreased h_{eff} values relative to

the fluoride ion due to inhibition of polymerisation and disruption of protein–protein association.

Cationic co-ions in an anion-exchange system are believed to be principally involved in offsetting the repulsive electrostatic free energy of interaction between anionic groups at the accessible protein surface. As a result, the stabilising effect of

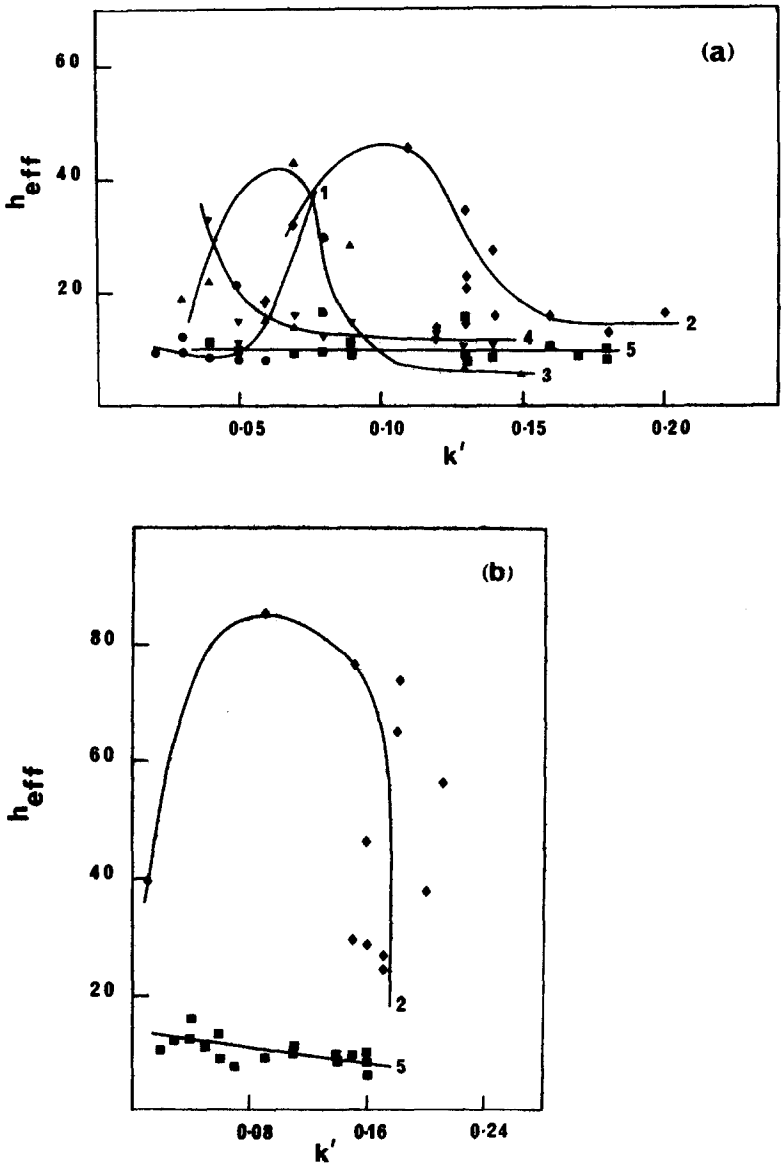

Fig. 4. The influence of anion and cation type on protein solubility as reflected in the dependence of h_{eff} *versus* k' for cytochrome *c* (a) and lysozyme (b) at high ionic strengths. Codes for displacer salts and values of h_{eff} determined as for Fig. 3.

various cations will be dependent on the protein structure and the cation effect on h_{eff} will therefore be solute specific. In Figs. 3 and 4 variation in the type of cation used in the displacer salt gave non-superimposable curves of h_{eff} *versus* k' plots for each solute. The effect of cations on h_{eff} values generally did not follow their ranking in the Hofmeister series. As shown in Fig. 2b, choice of any one of the cations used generally only leads to small changes in solute retention. Therefore, the major contributing factor to the choice of different cations in anion exchange relates to the potential for cation-induced changes in h_{eff} which may be a result of significant changes in the D_m/D_{sz} ratio arising from cation-induced changes in protein hierarchial stability.

Fig. 4 shows the effect of changes in salt type on the dependences between h_{eff} and k' for cytochrome *c* and lysozyme. These results have been considered separately to those in Fig. 3 since the retention behaviour of these two proteins as shown in Fig. 1 suggests that they are examples of proteins whose elution mode at pH 9.6 with Mono Q supports predominantly involves hydrophobic interaction rather than ion exchange even at relatively low ionic strengths. As is evident from these plots, different kinetic processes must apply since in both these cases increased k' values corresponding to increased "salting out" effects were observed as the ion concentration was increased. The influence of anions on the solubility of cytochrome *c* clearly follows their ranking in the Hofmeister series, whereas the effect that the various cations have on h_{eff} do not exhibit a similar predictability. The h_{eff} values for both lysozyme and cytochrome *c* are generally smaller than those seen for the other proteins (*cf.* Fig. 4 with data shown in Fig. 3). This difference may be due to the change in elution mode associated with the reduced contribution of coulombic interactions between the protein and the stationary phase. This results in narrow, sharp peaks with small retention times. Also supportive of the concept of multi-modal elution operating over a very narrow range of salt concentrations were the very large h_{eff} values seen for lysozyme when this protein was eluted isocratically at low ionic strengths.

CONCLUSION

The static equilibrium model for the interaction between a polyelectrolyte solute molecule and a charged surface as outlined in eqn. 1 is clearly insufficient to fully describe the various processes which contribute to the overall mechanism of separation of proteins chromatographed on porous microparticulate anion exchange supports. The retention and bandwidth data presented in the current study further demonstrates that the mechanism of protein retention in IEC involves complex multimodel interactions between the protein solute, the mobile phase constituents and the charged stationary phase surface. In this paper, an analysis of the influence of both the displacer ion and co-ion on the retention and bandwidth behaviour of proteins separated under isocratic high-performance IEC conditions, has been presented. Furthermore, the ability to assess the effect of ionic species in solution on protein structure and surface interactive properties provides further insight into the processes which control both mass recovery and biorecovery of protein solutes in IEC. The results of the present study also indicate that the potential now exists to rapidly monitor protein chromatographic performance under a wide range of eluent condi-

tions as part of the optimisation of experimental conditions for protein purification in IEC–high-performance liquid chromatography (HPLC). Furthermore, a major advantage of the use of isocratic elution for the optimisation of IEC of proteins is the ease of sample scale-up to a preparative mode using the principles of displacement chromatography. While isocratic conditions can in many instances be employed in the separation of peptides and proteins under well-defined conditions, complex mixtures are now typically separated under gradient elution conditions. The following paper in the series will therefore examine the results of detailed investigations on the influence of different displacer salts on the retention and bandwidth properties of proteins separated by gradient elution IEC–HPLC.

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