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

LXXXIX[a]. THE INFLUENCE OF DIFFERENT DISPLACER SALTS ON THE RETENTION PROPERTIES OF PROTEINS SEPARATED BY GRADIENT ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The influence of eight different displacer salts on the retention properties of four globular proteins, ranging in molecular weight from 14 000 to 43 000, was investigated by using the Mono-Q strong-anion-exchange resin as the stationary phase. Proteins were eluted under gradient conditions with a range of alkali metal halides to vary systematically the anion and cation species in the series F^-, Cl^-, and Br^- and Li^+, Na^+ and K^+. Protein Z_c values (*i.e.* slopes of the ion-exchange retention plots, as derived from the dependency of the logarithmic capacity factor log k' on the concentration of the ionic displacer) generally increased when both the anion and cation were either chaotropic, *e.g.* KBr, or kosmotropic, *e.g.* NaF, in nature. Conversely, Z_c values decreased when the displacer salt contained an anion–cation combination of a chaotropic and a kosmotropic ion, *e.g.* KF. These results indicate that the lyotropic properties of salts are additive in their effect on the interactive properties of proteins in anion-exchange chromatography. The Z_c values were also found to depend on the manner in which the ionic strength was manipulated to affect elution, *i.e.* isocratic or gradient change in concentration of the displacing salt. Thus, isocratic experiments and gradient experiments with varied gradient time or varied flow-rate were observed to result in log k' *versus* log $1/c$ dependencies with non-coincident Z_c values. The relationship between protein Z_c values, the electrostatic contact area or ionotope, A_c, and the electrostatic potential of the protein surface, ψ_s, is discussed.

INTRODUCTION

Several recent studies[1–6] on the theoretical and experimental aspects of protein retention behaviour in high-performance ion-exchange chromatography (HPIEC) have placed increasing emphasis on the mechanistic details of the electrostatic

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interaction between the solute and the sorbent surface. These investigations have demonstrated that composite contributions from the protein surface structure, protein–ion interactions and the properties of the double layer all have an important bearing on the retention mechanism. Thus, protein retention in HPIEC is dependent on both the number, *i.e.* the ionisation state, and distribution, *i.e.* the ionisation asymmetry, of charged sites on the protein surface. Protein selectivity in HPIEC can therefore be manipulated through changes in mobile phase pH and ionic strength, by altering the electrostatic surface potential (ψ_s) of the protein surface.

The nature of the displacer ion and co-ion have also been shown to influence significantly the ion-exchange retention properties of proteins, through their effects on protein solubility and aggregation. Furthermore, it is well known that elution methods also influence protein retention behaviour. For example, in a previous paper[3] we reported that deviations occurred between the value of the protein Z_c[4] as derived from isocratic and gradient experiments with varied gradient time or varied flow-rate. These results indicated that, while empirical treatments, such as the linear-solvent-strength (LSS) model of gradient elution may, in some selected situations, be equated with isocratic models to evaluate retention dependencies of proteins in HPIEC, these approaches do not yet adequately account for changes in protein surface interactive potential induced by specific solvent effects or elution mode effects. The present paper describes the results of our further investigations into the effect of various monovalent alkali halide salts on the gradient elution behaviour of several proteins, further tests the validity of the LSS model in the HPIEC of proteins, and examines the physicochemical relationship between Z_c, ψ_s, and the ionotopic contact area, A_c, of a protein.

MATERIALS AND METHODS

Apparatus

All chromatographic experiments were performed with a Pharmacia (Uppsala, Sweden) fast protein liquid chromatography (FPLC) system, as previously described[3].

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale skeletal muscle myoglobin (Type iii), hen egg ovalbumin (Grade V), hen egg white lysozyme (Grade I) and piperazine were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium fluoride (Univar grade), sodium bromide (Unilab grade), lithium fluoride (Unilab grade), lithium chloride (Unilab grade) and potassium bromide (Univar grade) were obtained from Ajax (Sydney, Australia). Lithium bromide (LR grade), sodium chloride (AnalaR grade), potassium fluoride (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 (specific gravity 1.16, AnalaR grade) or hydrobromic acid (specific gravity 1.46–1.49, AnalaR grade), all of which were purchased from BDH.

Chromatographic procedures

Eluent A was 0.02 *M* piperazine solution, adjusted to pH 9.60. Eluent B consisted of 0.02 *M* piperazine and 0.3 *M* displacer salt at pH 9.60. Eluents A and

B were filtered (0.45-μm cellulose acetate HAWP 04700, from Millipore) and degassed under vacuum. Protein solutions were prepared by dissolving purified protein in eluent A at a concentration of 5 mg/ml, unless otherwise specified. Before use, protein solutions were filtered through 0.22-μm ACRO LC13 filters (Gelman Sciences, Sydney, Australia). Protein sample sizes ranged from 5 to 1000 μg in injection volumes between 10 and 200 μl.

Gradient elution was performed from 0 to 100% eluent B under conditions of either constant flow-rate (F) and varied gradient time t_G or constant t_G and varied F. Varied-flow gradient experiments were conducted at flow-rates between 0.1 and 2.0 ml/min, while varied-gradient-time experiments were run with t_G varying between 17.1 and 171.1 min. 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). The gradient elapse time, t_e, required for eluent B to reach the column inlet, was determined from plots of conductivity *versus* time. Protein samples were eluted isocratically, starting at 100% eluent B, and elution was repeated at decreasing concentrations of B until the elution volume was greater than 30 column volumes. All data points represent the average of at least duplicate measurements.

In each type of elution experiment $\log k'$ or $\log \bar{k}$ and $\log 1/c$ or $\log 1/\bar{c}$ 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 (r^2). The iterative regression analyses were performed by statistical packages on a Monash University Computer Centre VAX11780 mini-computer system. The various chromatographic parameters were calculated by using the ChromoCalc programme series, developed in this laboratory and written in BASIC language for an IBM XT or AT computer.

RESULTS AND DISCUSSION

Influence of salt type on Z_c values

Protein retention on ion-exchange surfaces arises from electrostatic interactions between the protein surface and the charged stationary phase. Various theoretical models which describe protein retention behaviour have been developed from retention models for small organic molecules by the incorporation of an interactive charge term to account for multivalent attachment[7]. Thus, a non-mechanistic, stoichiometric equation, which is often written to describe the mass distribution in IEC of a polyelectrolyte solute molecule, $P^{\pm a}$, of net charge $\pm a$, 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 phase and stationary phase, respectively. Over a limited range of solvent composition, the relationship between the capacity factor, k', and the concentration, c, of the displacer ion is often approximated to a linear dependency of the form

$$\log k' = \log K + Z_c \log 1/c \quad (2)$$

where K is the distribution coefficient which incorporates several terms, including the binding constant, K_b, for the equilibrium process shown in eqn. 1, the phase ratio, φ, and the stationary phase ligand concentration D_b such that

$$K = \frac{K_b \varphi [D_b]^{Z_t}}{(z_a)(z_b)} \tag{3}$$

with the constants z_a and z_b adjusted for solute and salt valency. Under isocratic elution conditions, values for Z_c and log K can be determined over a narrow range of ionic strengths by linear regression analyses of plots of log k' *versus* log $1/c$. Alternatively, if gradient elution conditions are employed, application of the LSS theory[3,8] allows the determination of the median capacity factor, $\bar{k}$, and the corresponding salt concentration, $\bar{c}$, according to the relationships

$$\bar{k} = 1/1.15\, b \tag{4}$$

$$\bar{c} = c_0 + [t_g - t_0 - t_e - 0.30(t_0/b)]\Delta c/t_G \tag{5}$$

where b is the gradient steepness parameter, t_G is the gradient time and $\Delta c = c_f - c_0$, c_0 and c_f being the initial and final salt concentrations, respectively, for the gradient. Values of Z_c and log K can then be obtained from eqn. 2 by regression analysis of plots of log $\bar{k}$ *versus* log $1/\bar{c}$.

The central hypothesis of the stoichiometric relationship given by eqn. 1 assumes that the displacement of a protein solute from the charged surface is accompanied by the adsorption of a stoichiometric amount of displacing counter-ion. The value of Z_t is then defined as the ratio of the interactive charge on the solute to that on the counter-ion. For monovalent salt systems, the Z_t value is then equivalent to the average number of point charge interactions occurring between the protein solute and the charged sorbent support surface. The relative selectivity of protein solutes separated under anion-exchange conditions is thus anticipated to be directly related to the number of interacting charges and the displacing power of the counter-ion. According to the stoichiometric model (eqn. 1) values of Z_t, and as a consequence values of Z_c, should be independent of the chemical nature of the displacer ion and the type of elution conditions. However, previous studies[2] on the influence of different displacer salts on the retention and bandwidth properties of proteins separated by isocratic anion-exchange chromatography indicated that protein Z_c values vary with the type of anion used, and that the cationic co-ion also influences solute retention properties. In addition, deviations have been observed[3] between the Z_c values obtained from isocratic experiments and from gradient experiments with varied gradient time and varied flow-rate. These studies indicate that the chaotropic and kosmotropic properties of various salts, coupled with the degree of solute exposure to the mobile phase associated with varying elution conditions, could significantly alter the interactive properties of protein solutes in ion-exchange systems. Acquisition of experimental support for this concept formed the basis of the following investigations.

Based on these considerations, the present paper extends the results of our earlier investigations on both the influence of various monovalent alkali metal halides and the validation of the LSS model for gradient elution in HPIEC. The influence of the

TABLE I

PHYSICAL PARAMETERS OF PROTEINS

No.	*Protein (source)*	*pI*	*MW*
1	Ovalbumin (egg white)	4.70	43 500
2	Carbonic anhydrase (bovine erythrocytes)	5.89	30 000
3	Myoglobin (sperm whale muscle)	7.68 8.18	17 500
4	Lysozyme (hen egg white)	11.00	14 300

counter-ion and the co-ion on solute retention and bandwidth behaviour was studied by using LiCl, LiBr, NaF, NaCl, NaBr, KF, KCl and KBr. LiF was not used, due to limited solubility. Gradient retention data were obtained for four globular proteins, listed in Table I, by using a Mono-Q strong-anion-exchange stationary phase. All proteins were chromatographed by using a piperazine (20 m*M*) buffer at pH 9.60. Gradient data were obtained by either a constant flow-rate of 1 ml/min, with t_G

TABLE II

Z_c VALUES OBTAINED BY LINEAR REGRESSION FOR VARIED GRADIENT TIME

Correlation coefficient given in parentheses.

Salt	*Protein*			
	Carbonic anhydrase	*Ovalbumin*	*Myoglobin*	*Lysozyme*
LiF	Not done	Not done	Not done	Not done
LiCl	5.60 ± 2.12(0.78)	9.92 ± 2.90(0.80)	17.75 ± 3.23(0.97)	2.50 ± 0.26(0.98)
LiBr	3.14 ± 0.71(0.80)	7.30 ± 0.46(0.98)	1.96 ± 0.66(0.69)	4.21 ± 0.66(0.93)
NaF	3.55 ± 0.45(0.91)	6.56 ± 0.71(0.95)	5.49 ± 1.71(0.78)	2.99 ± 0.31(0.96)
NaCl	3.07 ± 0.21(0.99)	9.40 ± 0.50(0.99)	4.61 ± 1.57(0.90)	1.48 ± 0.17(0.97)
NaBr	2.92 ± 0.17(0.99)	7.83 ± 0.47(0.99)	1.43 ± 0.02(1.00)	2.41 ± 0.44(0.94)
KF	2.37 ± 0.25(0.95)	6.37 ± 0.57(0.97)	1.98 ± 0.27(0.93)	3.58 ± 0.14(1.00)
KCl	3.84 ± 0.05(1.00)	8.40 ± 0.76(0.98)	4.54 ± 0.51(0.99)	1.16 ± 0.15(0.95)
KBr	5.60 ± 0.75(0.92)	9.48 ± 0.69(0.97)	4.84 ± 0.75(0.89)	3.28 ± 0.24(0.98)[a] 0.98 ± 0.14(1.00)[b]
LiF	Not done	Not done	Not done	Not done
NaF	3.55 ± 0.45(0.91)	6.56 ± 0.71(0.95)	5.49 ± 1.71(0.78)	2.99 ± 0.31(0.96)
KF	2.37 ± 0.25(0.95)	6.37 ± 0.57(0.97)	1.98 ± 0.27(0.93)	3.58 ± 0.14(1.00)
LiCl	5.60 ± 2.12(0.78)	9.92 ± 2.90(0.80)	17.75 ± 3.23(0.96)	2.50 ± 0.26(0.98)
NaCl	3.07 ± 0.21 (0.99)	9.40 ± 0.50(0.99)	4.61 ± 1.57(0.90)	1.48 ± 0.17(0.97)
KCl	3.84 ± 0.05(1.00)	8.40 ± 0.76(0.98)	4.54 ± 0.51(0.99)	1.16 ± 0.15(0.95)
LiBr	3.14 ± 0.71(0.80)	7.30 ± 0.46(0.98)	1.96 ± 0.66(0.69)	4.21 ± 0.66(0.93)
NaBr	2.92 ± 0.17(0.99)	7.83 ± 0.48(0.99)	1.43 ± 0.02(1.00)	2.41 ± 0.44(0.94)
KBr	5.60 ± 0.75(0.92)	9.48 ± 0.69(0.97)	4.84 ± 0.75(0.89)	3.28 ± 0.24(0.98)[a] 0.98 + 0.14(1.00)[b]

[a] Z_c determined at low log $1/\bar{c}$ values.
[b] Z_c determined at high log $1/\bar{c}$ values.

ranging between 17.1 and 171 min, or setting the gradient time to 17.1 min and varying the flow-rate between 0.1 and 2.0 ml/min. Table II shows the Z_c values obtained for carbonic anhydrase, ovalbumin, myoglobin and lysozyme, eluted at gradient times ranging from 8.6 to 171.1 min at a constant flow-rate of 1 ml/min. Z_c values were determined by regression analysis of the linear portions of the log $\bar{k}$ *versus* log $1/\bar{c}$ curves. Values of Z_c are tabulated with an error range of one standard deviation; the correlation coefficient, r^2, is listed in parentheses. The ions used in this study were classified either as chaotropes (*e.g.*, Br^- and K^+), kosmotropes (*e.g.*, F^- and Li^+) or neutral (*e.g.*, Cl^- and Na^+). This relates to their relative position in the lyotropic series. Chaotropic ions exhibit properties which disrupt water structure and are known to destabilise protein structure while kosmotropic species stabilise water structure and have a stabilising effect on protein structure. When combinations of the above ions were used to elute proteins from the anion-exchange columns, several general trends concerning the change in the Z_c values were apparent:

(1) Z_c increased as the anion and cation of the displacer salt became more chaotropic in nature (*e.g.*, potassium or bromide salt series).

(2) Z_c increased as the anion and cation of the displacer salt became more kosmotropic in nature (*e.g.*, lithium or fluoride salt series).

(3) Z_c decreased when the displacer salt contained a combination of a chaotropic and kosmotropic ion (*e.g.*, LiBr in the bromide series and KF in the potassium series).

(4) Sodium and chloride ions generally resulted in intermediate values of Z_c in an anion or cation series (*e.g.* potassium and bromide salts).

More specifically, the anion effect on Z_c values for carbonic anhydrase, ovalbumin and myoglobin, was $Br^- < Cl^-$ for lithium, and $Br^- > Cl^- > F^-$ for potassium. For the sodium salt, the anion effect was $Br^- \approx Cl^- \approx F^-$ for carbonic anhydrase, $Br^- < Cl^- > F^-$ for ovalbumin, and $Br^- < Cl^- < F^-$ for myoglobin. Overall, there is a reversal in the effect that anion species exert on Z_c, as the cation is changed from potassium through to sodium and lithium. In anion-exchange chromatography, the elution process is often assumed to be independent of the cationic co-ion. However, the changes in Z_c observed with different cations in solution, as shown in Table II, indicate that cation selection can significantly influence solute retention behaviour. The effects of the cation on experimental Z_c values for all proteins, except lysozyme, were $K^+ > Na^+ \approx Li^+$ for bromide, $K^+ \approx Na^+ < Li^+$ for chloride, and $K^+ < Na^+$ for fluoride. These results indicate that the influence of the cationic species on Z_c can also be reversed as the anion is systematically changed from bromide to chloride to fluoride.

Recent studies[9] on the characterisation of ionic species that affect protein stability indicated that relative to bulk water chaotropic species, which destabilise protein structure, interact less strongly with the first layer of adjacent water molecules. In contrast, polar kosmotropes, which are known to stabilise protein tertiary structure, interact with the first layer of adjacent water molecules more strongly than bulk water itself. Furthermore, chaotropes increase the solubility and hydrodynamic radii of other solutes, while kosmotropes decrease their solubility and hydrodynamic radii. These findings suggested that the lyotropic properties of ions from neutral salts arise from their influence on water structure and are additive over all ions in solution. The observed changes in Z_c values suggest that the contact area or ionotopic region of the protein is altered in the presence of the different ions. Furthermore, the variation in Z_c

values with different combinations of chaotropic and kosmotropic ions result in both synergistic and antagonistic effects between the anions and cations selected as displacer salts. Thus, the influence of different salts on protein interactive behaviour, which is experimentally manifested as changes in Z_c values, is additive in the presence of salts comprised of only chaotropes (KBr) or kosmotropes (LiCl). However, a combination of chaotropic and kosmotropic ions (*i.e.* KF, LiBr) results in opposing effects on the magnitude of Z_c. Minor perturbations in protein tertiary structure or even small shifts in protein–ion interactions would result in changes in the ionotopic contact area, A_c, thereby altering the experimentally observed Z_c values. The question then immediately arises whether any fundamental physicochemical relationship exists between Z_c and A_c and the distribution coefficient, K.

The stoichiometric displacement model (SDM) also defines Z_t as arising solely from solute–sorbent surface electrostatic interactions. However, in a highly charged and polar environment, additional types of dipolar interactions may also contribute to the retention mechanism, as the solute molecule interacts with the support surface. Table III lists the ranges in which various types of dipolar interactions significantly occur and their relative magnitudes. One can see that most of these dipole interactions are significant as the protein approaches, interacts with, and departs from the stationary-phase surface. These dipolar forces are also known to be important in other forms of macromolecular association/dissociation in biologocal systems, *e.g.* antigen–antibody complexes. If all these dipolar interactions contribute to the retention process and are reflected in the experimentally derived Z_c value, then the definition of Z_c becomes far more complex than that delineated by the SDM.

TABLE III

MAGNITUDE AND EFFECTIVE RANGE OF VARIOUS DIPOLE INTERACTIONS

Interaction	*Magnitude*[10] *(kJ mol*$^{-1}$*)*	*Effective range*[11] *(Å)*
Ion–ion	40–400	100
Ion–dipole	4–40	
Dipole–dipole	0.4–4	
Dipole–induced-dipole	0.4–4	100[a]
Ion–induced-dipole	0.4–4	
Dispersion	4–40	
Hydrogen bond	4–40	1.5–5

[a] Some long-range Van der Waals forces may be operative at distances greater than 1000 Å.

Influence of elution conditions on Z_c values

If the desorption process in HPIEC were solely dependent on the concentration of displacer salt in the mobile phase, it would be anticipated that similar Z_c values should be observed for isocratic and gradient experiments, and that no difference should exist between gradient elution data obtained under conditions of varying gradient time and varying flow-rate. However, previous studies[3] on the comparison of retention properties of proteins, eluted by gradient and isocratic HPIEC with NaCl as the eluting salt, indicated that deviations existed between the Z_c values obtained under these different elution conditions. In light of the effects of various displacer salts on the

TABLE IV

Z_c VALUES OBTAINED BY LINEAR REGRESSION FOR VARIED FLOW-RATE

Correlation coefficient given in parentheses.

Salt	*Protein*			
	Carbonic anhydrase	*Ovalbumin*	*Myoglobin*	*Lysozyme*
LiF	Not done	Not done	Not done	Not done
LiCl	6.76 ± 0.80(0.95)	20.40 ± 3.57(0.92)	6.72 ± 3.11(0.82)	4.46 ± 0.25(0.99)
LiBr	4.16 ± 0.40(0.96)	7.45 ± 0.68(0.96)	4.11 ± 0.44(0.95)	3.96 ± 0.77(0.93)[a] 1.96 ± 0.09(1.00)[b]
NaF	1.57 ± 0.07(0.99)	4.09 ± 0.36(0.99)	1.46 ± 0.14(0.96)	2.91 ± 0.12(0.99)
NaCl	3.31 ± 0.16(0.99)	7.42 ± 0.79(0.95)	5.09 ± 0.80(0.95)	1.88 ± 0.23(0.96)
NaBr	4.53 ± 0.53(0.94)	8.05 ± 0.36(0.99)	3.48 ± 0.59(0.92)	3.30 ± 0.33(0.99)[a] 0.56 ± 0.00(1.00)[b]
KF	2.01 ± 0.22(0.95)	4.43 ± 0.21(1.00)	1.32 ± 0.12(0.97)	3.23 ± 0.50(0.89)
KCl	2.17 ± 0.24(0.97)	5.65 ± 0.63(0.95)	1.47 ± 0.15(0.97)	1.73 ± 0.11(0.99)
KBr	5.95 ± 1.19(0.83)	10.92 ± 0.88(0.97)	6.03 ± 2.26(0.70)	4.52 ± 0.75(0.95)[a] 2.53 ± 0.28(0.97)[b]
LiF	Not done	Not done	Not done	Not done
NaF	1.57 ± 0.07(0.99)	4.09 ± 0.36(0.99)	1.46 ± 0.14(0.96)	2.91 ± 0.12(0.99)
KF	2.01 ± 0.22(0.95)	4.43 ± 0.21(1.00)	1.32 ± 0.12(0.97)	3.23 ± 0.50(0.89)
LiCl	6.76 ± 0.80(0.95)	20.40 ± 3.57(0.92)	6.72 ± 3.11(0.82)	4.46 ± 0.25(0.99)
NaCl	3.31 ± 0.16(0.99)	7.42 ± 0.79(0.95)	5.09 ± 0.80(0.95)	1.88 ± 0.23(0.96)
KCl	2.17 ± 0.24(0.97)	5.65 ± 0.63(0.95)	1.47 ± 0.15(0.97)	1.73 ± 0.11(0.99)
LiBr	4.16 ± 0.40(0.96)	7.45 ± 0.68(0.96)	4.11 ± 0.44(0.95)	3.96 ± 0.77(0.93)[a] 1.96 ± 0.09(1.00)[b]
NaBr	4.53 ± 0.53(0.95)	8.05 ± 0.36(0.99)	3.48 ± 0.59(0.92)	3.30 ± 0.33(0.99)[a] 0.56 ± 0.00(1.00)[b]
KBr	5.95 ± 1.19(0.83)	10.92 ± 0.88(0.97)	6.03 ± 2.26(0.70)	4.52 ± 0.75(0.95)[a] 2.53 ± 0.28(0.97)[b]

[a] Z_c determined at low log $1/\bar{c}$ values.
[b] Z_c determined at high log $1/\bar{c}$ values.

retention properties of proteins in HPIEC, these investigations were extended to study the influence of flow-rate and gradient time conditions on the effects of anions and cations on protein Z_c values. Table IV lists the Z_c values for the four model proteins, eluted with a gradient time of 17.1 min and flow-rates ranging from 0.1 to 2 ml/min. Consider first the effect of varying the displacer anion on the value of Z_c. For these studies, fluoride, chloride, and bromide salts of sodium and potassium, and the chloride and bromide salts of lithium were investigated. The value of Z_c for the potassium salts was found to increase under both sets of gradient conditions in the order $F^- < Cl^- < Br^-$ for all proteins, except lysozyme, where other phenomena complicated the observed trend (see below). The varied gradient time and isocratically derived Z_c values obtained for the sodium salts generally decreased in the order $F^- > Cl^- > Br^-$ whereas the order was reversed for the varied flow experiments, such that Z_c increased as $F^- < Cl^- \approx Br^-$. The influence of the cationic co-ion was also found to be dependent on the elution conditions. The value of Z_c for the bromide salts was

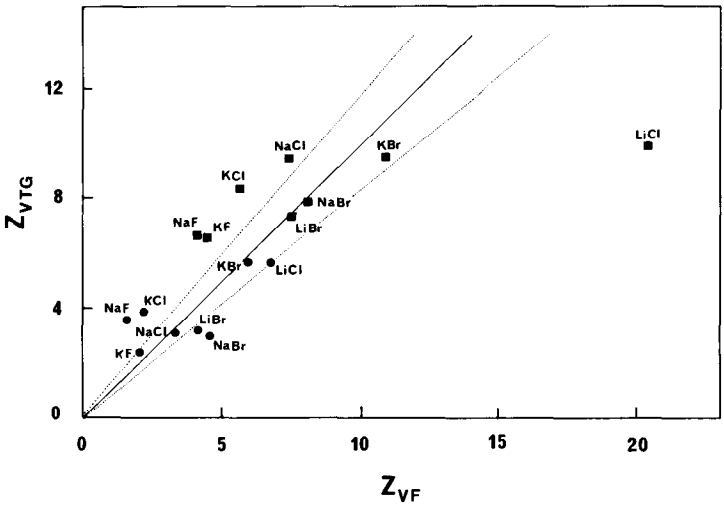

Fig. 1. Plot of Z_c values derived from varied gradient time experiments (Z_{VTG}) versus Z_c values for varied flow-rate experiments (Z_{VF}) for ovalbumin (■) and carbonic anhydrase (●) eluted with different salts as indicated. Gradient data were obtained with t_G varying between 17.1 and 171.1 min at a flow-rate of 1 ml/min or a constant t_G of 17.1 min and the flow-rate varying between 0.1 and 2.0 ml/min. The straight lines shown correspond to the theoretical relationship $Z_{VTG} = Z_{VF}$, with a ± 20% error margin.

found to decrease under both sets of gradient data in the order $K^+ > Na^+ \approx Li^+$ for all proteins, except lysozyme. Both sets of gradient data for the chloride salts have Z_c values increasing with the series $K^+ \approx Na^+ < Li^+$. For the fluoride salts, the order of cations leading to increased Z_c values was also dependent on the gradient elution mode. The Z_c value was found to increase with $K^+ < Na^+$ for the varied gradient time data while $K^+ \approx Na^+$ for the varied flow experiments. Thus for the fluoride salt series, a change in elution mode, from varied gradient time and constant flow-rate to constant gradient time and varied flow-rate conditions caused a decrease in Z_c values while for the sodium salt series there was a reversal of the effect of ions on Z_c. In Fig. 1 the Z_c values derived from varied gradient time (fixed flow-rate) experiments (Z_{VTG}) with ovalbumin and carbonic anhydrase are plotted against the Z_c values derived from varied flow-rate (fixed gradient time) experiments (Z_{VF}). According to the stoichiometric retention model and LSS theory, Z_{VTG} and Z_{VF} values should all fall on a common line (slope = 1). As is evident from the number of data points which fall outside the error margin of ± 20%, divergencies from this ideal behaviour predicted by these theoretical relationships occur. These results suggest that changes in the physicochemical basis of the adsorption/desorption process, (*e.g.* the results for the sodium and fluoride salts investigated) can be induced by experimental factors controlling the elution mode. In this instance, the rate of change of displacer salt concentration is the primary difference between the two gradient elution modes. The difference between a variable and a constant rate of change could influence the number and type of solute species interacting with the sorbent surface and ultimately the thermodynamics involved with the adsorption/desorption process.

Comparison of gradient and isocratic retention data

The linear solvent strength (LSS) model is a non-mechanistic theoretical treatment, designed to relate solute retention in gradient and isocratic elution. An assumption inherent in the development of this model is that identical physicochemical phenomena control the migration of a solute in both isocratic and gradient elution modes, giving rise (in principle) to superimposable retention plots. Hence, from a minimum of two different gradient experiments, sufficient data can be obtained to predict solute retention behaviour in a corresponding isocratic system and *vice versa*. The advantage of using models such as the LSS is immediately evident, since these approaches represent quick, cost-saving methods for optimising sample retention and provide alternative, more systematic procedures than the trial-and-error techniques usually employed. In particular, the LSS model has been used successfully in reversed-phase HPLC to optimise purifications of both small and large molecules[12–14] and has recently been extended to assess protein retention in hydrophobic interaction chromatography (HIC)[15] and ion-exchange HPLC systems[3,8].

However, recent studies[3] indicate that in the case of HPIEC, the physicochemical phenomena controlling gradient and isocratic elution may not be totally synonomous, and the differences could ultimately affect the accuracy and general utility of the LSS model in HPIEC systems. In order to investigate further the relevance of LSS methods to the physicochemical basis of the elution process in HPIEC, solute retention data from isocratic and gradient elution were compared for a variety of monovalent alkali metal halide salts.

Gradient retention data were obtained by varying the gradient time, t_G, between 8.6 and 171.1 min at a flow-rate of 1 ml/min or by varying the flow-rate from 0.1 to 2.0 ml/min with t_G = 17.1 min. Values of log $\bar{k}$ and log $1/\bar{c}$ for retention mapping were obtained by using eqns. 4 and 5 from the LSS model. Isocratic retention data were collected by systematically decreasing the concentration of the displacer salt in the eluent at a constant flow-rate of 1 ml/min. Values of log k' and log $1/c$ for the isocratic elutions were obtained directly from experimental data. Both gradient and isocratic experiments were conducted with a mobile phase pH equal to 9.6.

Table V lists the Z_c values obtained from linear regression of the combined isocratic and varied gradient time and varied flow conditions. The correlation coefficient was also used to assess the superimposability of the combined data, *i.e.* the

TABLE V

Z_c VALUES OBTAINED BY LINEAR REGRESSION DATA FOR COMBINED DATA

Salt	*Protein*			
	Carbonic anhydrase	*Ovalbumin*	*Myoglobin*	*Lysozyme*
NaF	2.24 ± 0.27(0.78)	4.52 ± 0.46(0.88)	1.84 ± 0.36(0.62)	−0.81 ± 0.63(0.09)
NaCl	2.41 ± 0.35(0.77)	7.37 ± 0.47(0.95)	1.70 ± 0.32(0.74)	0.37 ± 0.21(0.11)
NaBr	2.72 ± 0.25(0.90)	5.85 ± 0.44(0.92)	1.93 ± 0.23(0.85)	1.26 ± 0.31(0.55)
LiCl	3.63 ± 0.48(0.80)	9.97 ± 1.80(0.74)	0.92 ± 0.50(0.26)	0.66 ± 0.14(0.58)
NaCl	2.41 ± 0.35(0.77)	7.37 ± 0.47(0.95)	1.70 ± 0.32(0.74)	0.37 ± 0.21(0.11)
KCl	2.14 ± 0.19(0.90)	5.61 ± 0.38(0.94)	0.85 ± 0.25(0.39)	−0.14 ± 0.86(0.00)

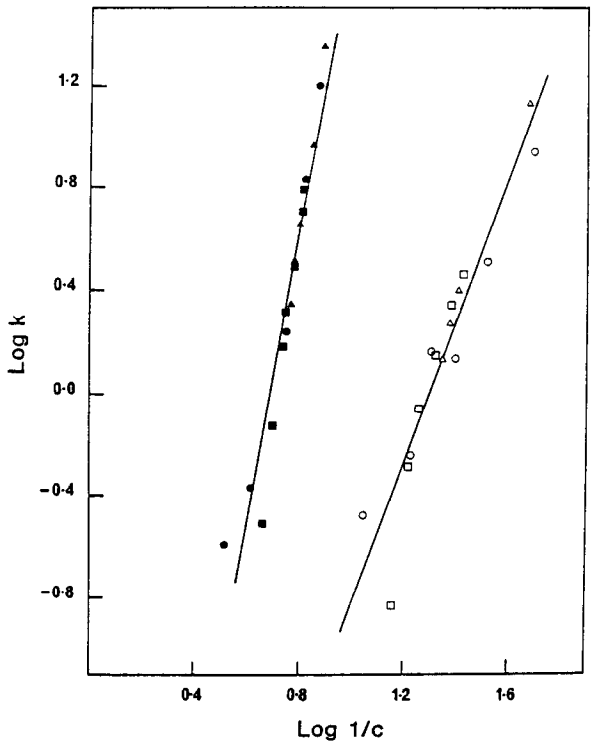

Fig. 2. Retention plots for isocratic (log k' *versus* log $1/c$) and gradient experiments (log $\bar{k}$ *versus* log $1/\bar{c}$) for ovalbumin and carbonic anhydrase, eluted with sodium bromide (isocratic data were derived from experiments with varied salt concentrations at pH 9.60 and a flow-rate of 1 ml/min). Other chromatographic conditions are given in the Materials and Methods section and legend to Fig. 1. See Tables II, IV, and V for the derived Z_c values. For ovalbumin: (●), Isocratic; (▲), VTG; (■), VF. For carbonic anhydrase: (○), Isocratic; (△), VTG; (□), VF.

level of correlation, as predicted by the LSS theory. Fig. 2 and 3 show plots of log k versus log $1/c$ for the four proteins eluted with NaBr. These data illustrate the variation in the degree of correlation between isocratic and gradient data for different experimental conditions. The extent of divergency in the correlation between theoretically and exprimentally derived dependencies of log $\bar{k}$ on log $1/\bar{c}$ generally followed the order ovalbumin < carbonic anhydrase < myoglobin < lysozyme for correlation of the superimpossibility of isocratic to gradient data. These results suggest that gradient and isocratic elution do not have an identical physicochemical basis for controlling solute retention. In particular, an assumption inherent in the derivation of equations that relate isocratic and gradient retention data is that each k' value is measured under equilibrium or near-equilibrium conditions. However, if the interactive properties of the protein solute are dependent on, for example, the mobile phase composition or the dwell-time of the system, then time-dependent changes in salt concentration associated with varied gradient time will result in protein–ligand interactions that are not at the identical point in the equilibrium trajectory. In particular, it was observed that r^2 for superimposability of retention data approached

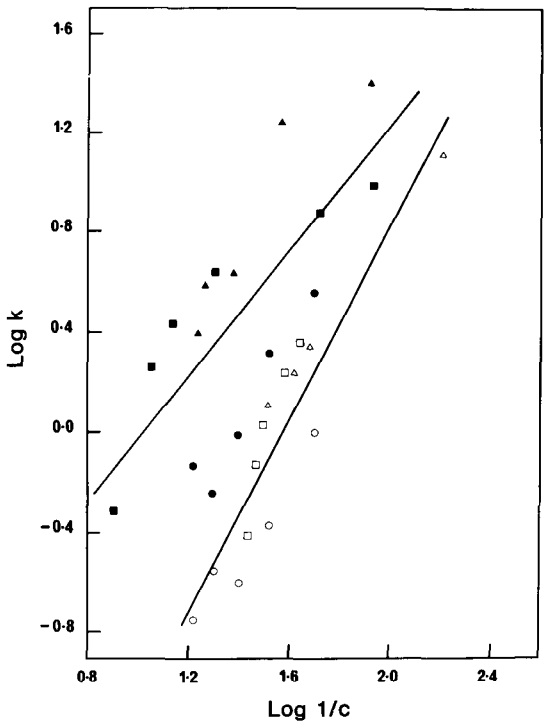

Fig. 3. Retention plots for isocratic and gradient experiments with myoglobin and lysozyme eluted with sodium bromide. See legend to Fig. 2 for experimental details. For myoglobin: (○), Isocratic; (△), VTG; (□), VF. For lysozyme: (●), Isocratic; (▲), VTG; (■), VF.

zero for myoglobin eluted with KCl or for lysozyme eluted with NaF. In such an event, isocratic, varied gradient time and varied flow-rate retention data were observed to form three distinct and separate plots, corresponding to each elution mode. In the example of lysozyme, eluted with NaF, an additional complication arises. Gradient-derived Z_c and log K values are used to obtain the isocratic k' and c values by substitution into eqn. 5. Lysozyme in this instance has similar Z_c values for both varied gradient time and varied flow-rate modes, but different log K values. Hence, the theoretical isocratic k' and c values obtained, which are derived from the gradient retention data used in the calculation, will not correspond to the true experimental isocratic values.

In evaluating the LSS model for use in HPIEC, Stout *et al.*[8] observed small, consistent differences between the theoretical and experimentally derived gradient Z_c values. They postulated that by using a simple correction factor, the experimentally derived Z_c value could be corrected for non-LSS behaviour. This factor was to ensure improved accuracy in calculating the corresponding isocratic k' and c values. This approach assumed that all displacer salts have a similar effect on this correction factor. In light of the data shown in Tables II and IV, where substantial differences were found to exist between solute Z_c values obtained with different displacer salts, this assumption requires modification. Furthermore, for a particular salt system, it is

evident from the data that significant differences in Z_c for a solute can arise from the use of different elution modes, *i.e.* varied gradient time, varied flow-rate or isocratic elution. These results therefore indicate that an accurate correction factor cannot yet be substantiated for use in all HPIEC systems.

Finally, for the displacer salts used in this study, it was found that r^2 values for the correlation of data from isocratic and gradient experiments generally improved as the hydrated-ion size decreased within a particular salt series. A reason for such an occurrence is not apparent at the present time, but the existence of such a trend may be a useful criterion for selecting alkali metal halide displacer salts that improve LSS behaviour in solute retention.

Influence of displacer salt and elution conditions on anion-exchange retention properties of proteins, where eluent pH < protein pI

According to the net charge concept[7], proteins chromatographed on a strong-anion-exchange column at mobile phase pH values below their known p*I* will not be retained due to the existence of a net positive surface charge. However, various examples of proteins have been reported where significant deviations occur[1,14] from the retention behaviour, expected on the basis of this assumption. For example, lysozyme, with a p*I* equal to 11.0, will exhibit a net positive charge under the mobile phase conditions of pH 9.6 used in the present study. However, in most cases, this protein exhibits a significant degree of retention on the anion-exchange column *e.g.* $\bar{k}$ values ≥ 10. Furthermore, in some solvent systems, the decreased solubility and the tendency of lysozyme to aggregate results in retention behaviour which does not conform to the simple dependency between k' and c, as expressed by eqn. 2. However such divergencies can give valuable insight into IEC retention mechanism(s) that are not always evident with the relatively well-behaved proteins. Examination of Tables II and IV and Figs. 4 and 5 reveals that the elution mode can not only modulate the effect of various ions on Z_c but can also influence the elution mechanism. For example, when lysozyme is isocratically eluted with KCl as the displacer salt, a downward curving retention plot (with a negative Z_c value) is obtained (Fig. 4) on adsorption which is indicative of a "salting out" phenomenon. However, under gradient elution conditions, again with KCl as the displacer salt, lysozyme exhibits "normal" ion-exchange retention, giving rise to positive Z_c values with increasing retention plots.

Plots of $\log \bar{k}$ versus $\log 1/\bar{c}$, which are used to map solute retention behaviour of proteins, are generally presented as linear dependencies with a slope corresponding to Z_c. In situations where the IEC retention process ceases to dominate the retention mechanism, *i.e.* when hydrophobic or salt-bridge interactions occur, then curvilinear dependencies of $\log \bar{k}$ on $\log 1/\bar{c}$ will be observed. Under these conditions, multiphasic retention plots will exhibit individual slope components, which are characteristic of the protein, the displacing salt system, the pH, and the chemical nature and concentration of the buffer salt. Recently, HPIEC retention maps for subtilisins[6] were also found to be curvilinear, consisting of two distinct lines with independent slopes and intercepts. These observations were believed to result from a change in the coulombic interactive area or ionotope, which was dependent upon mobile phase ionic strength. In the current study, the elution of lysozyme with a series of bromide salts resulted in similar biphasic retention plots. Fig. 5 shows retention plots for lysozyme, eluted by potassium salts under conditions of varied gradient time and constant flow-rate. Examination of

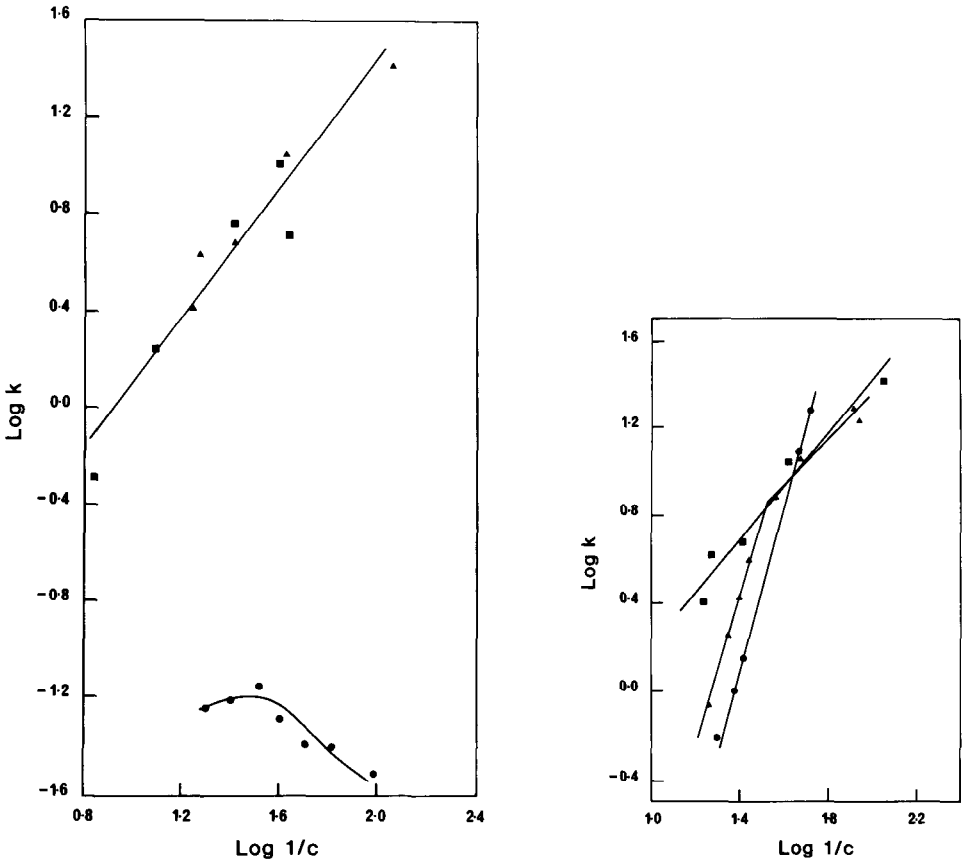

Fig. 4. Retention plots for isocratic and gradient experiments with lysozyme, eluted with potassium chloride. See legend to Fig. 2 for experimental details. (●), Isocratic; (▲), VTG; (■), VF.

Fig. 5. Retention plots for lysozyme eluted under gradient conditions, with t_G varying between 17.1 and 171.1 min at a flow-rate of 1 ml/min. The displacer salts used were: (●), KF; (■), KCl and (▲), KBr.

Table II reveals that the slope of the retention plot for lysozyme, eluted with KBr, at longer column residence times is similar to that for KCl and at shorter column residence times approximates the slope for KF. These observations clearly reflect the existence of several potentially interactive binding sites (*i.e.* different ionotopes) at the surface of the lysozyme molecule. The selection of these binding sites may thus not only depend upon column residence time but also the ionic species present in the eluent. For short column residence times, the ionotope for lysozyme, eluted with KBr, recognises the sorbent surface in a manner similar to that for KF, whilst at longer residence times the ionotope is similar to that for the KCl salt system. The sharp transition at intermediate ionic strength suggests a two-state surface-mediated phenomenon, in which the transition between the two ionotopic forms is very fast, because no unusual curve deformity is noted in the bandwidth data[17]. Similar results are also observed for lysozyme eluted with the potassium salt series under conditions of constant gradient time and varied flow-rate (Table IV). However, the biphasic phenomenon was not as

pronounced, and the ionic strength of KBr at the break point corresponded to a higher value, *i.e.* 30 m*M*, for varied gradient time and 65 m*M* for varied flow-rate conditions.

The retention plots for lysozyme, eluted with all bromide salts under conditions of constant gradient time and varied flow-rate were also found to be biphasic. At lower flow-rates (lower log $1/\bar{c}$ values) and at higher flow-rates (higher log $1/\bar{c}$ values) beyond the break points, significant differences between Z_c values for each salt system were observed with $K^+ > Li^+ > Na^+$. This indicates that the magnitude of the area of the lysozyme ionotope in each of the two-state systems was significantly different for each bromide salt. The break points in the retention plot for the bromide salts was found to vary, depending upon the cation, *i.e.* LiBr = 102 m*M*, NaBr = 69 m*M* and KBr = 65 m*M*. The data for the potassium salt indicated that the transition between each of the lysozyme ionotopic states is also fast, as there was no anomalous bandbroadening observed for this cation series[17].

The choice of gradient elution mode can also influence the expression of these bi-linear retention dependencies. For example, comparison of the retention plots of lysozyme eluted with LiBr (Fig. 6) under conditions of either varied flow-rate and constant gradient time or varied gradient time and constant flow-rate, indicates that the results of the varied gradient time experiments more closely approximate a first-order model of retention.

The nature of the displacer ion clearly has a significant influence on the ability of lysozyme, a protein with a net positive surface charge, to interact with a positive anion-exchange surface. In a previous study[2], it was found that under isocratic elution conditions lysozyme exhibited significant retention with eluents containing NaF, NaBr, and LiCl, yet no retention was observed with NaCl or KCl as the displacer salt. However, in the present study, involving the gradient elution mode, the elution of lysozyme with LiCl or NaCl consistently resulted in breakthrough elution profiles as shown in Fig. 7. In these instances, Z_c values were calculated from retention data

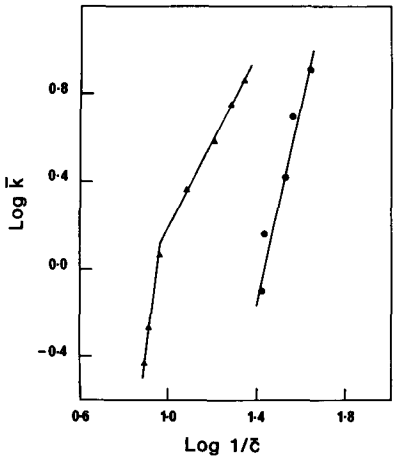

Fig. 6. Retention plots for lysozyme eluted under gradient conditions with LiBr as the displacer salt. Gradient data were derived with varied gradient time or varied flow-rate, as described in Fig. 1. (●), VTG; (▲), VF.

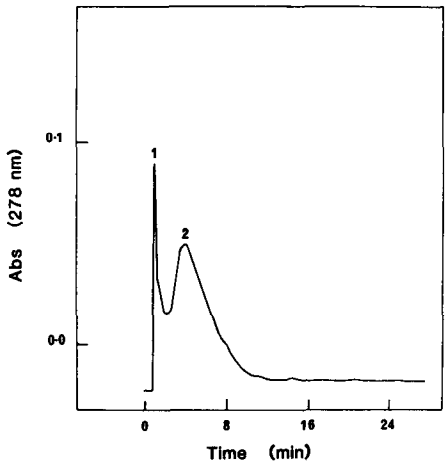

Fig. 7. Chromatogram illustrating pseudo-overload conditions with LiCl as displacer salt. t_G = 17.1 min; flow-rate = 0.7 ml/min; 100 μg of lysozyme.

associated with peak 2. It appears therefore that the time-dependent changes in the salt concentration have resulted in retention behaviour which is typical of column-overload conditions. However, only analytical loads (10–100 μg) were employed, and this indicates that the ionic capacity of the column has not been exceeded. In these cases, it appears that the delay in the exposure of lysozyme to the displacer salt, which occurs in gradient modes, results in a interactive process different from the mechanism of isocratic elution, in which the protein is loaded in the presence of varying salt concentrations. In particular, the elution of two different protein zones suggests that there is either a mobile-phase-dependent equilibrium between two alternate forms of lysozyme with different interactive properties or, alternatively, kinetic competition between diffusion and adsorption. This is evidenced by comparison of Z_c values, obtained, *e.g.*, for LiCl and LiBr. The value of Z_c in LiBr remains essentially constant ($\approx$ 4) in both gradient elution modes, and the absence of a breakthrough peak indicates the presence of a single interactive species. With LiCl, however, the Z_c values almost doubles from 2.5 with varied gradient time to 4.5 under varied flow-rate conditions. These results, therefore, graphically demonstrate the influence of both the mobile phase composition and the rate of change of displacer salt concentration on the retention properties of proteins in HPIEC.

The ion-exchange distribution constant

The K term, as defined in eqn. 2, is related to the overall distribution coefficient and incorporates several factors including the equilibrium binding constant, K_b, the phase ratio, φ, the stationary-phase ligand concentration, D_b, and the fractional occupancy of the ligands. According to eqn. 2, graphic extrapolation of the linear dependency between log k' and log $1/c$ yields log K as the intercept in the limit case of log $1/c \rightarrow 0$, *i.e.* when the displacer salt concentration is equal to 1 mol/l. However, this extrapolation assumes that the retention dependencies remain linear at high salt concentrations. Several examples have been reported[1,2] where curvilinear dependen-

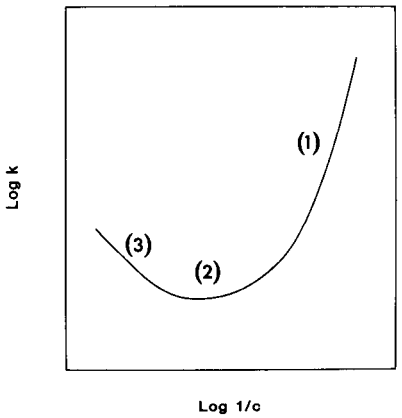

Fig. 8. General dependence of retention on salt concentration for proteins separated by HPIEC, illustrating (1) electrostatic, (2) size exclusion and (3) hydrophobic-interaction phenomena.

cies between log k' and log $1/c$ have been observed, such as that depicted by the hypothetical curve in Fig. 8. As is evident from this diagram, section 1 of the curve corresponds to retention behaviour dominated by ion-exchange interactions, while hydrophobic interactions govern the retention mechanism in section 3 leading to increasing k' values with increasing salt concentration. In section 2 of the curve size-exclusion phenomena dominate under conditions of displacing-ion concentrations where minimal interaction occurs between the solute and the sorbent surface. Thus, the K value obtained by linear extrapolation of section 1 to the y intercept will not represent the distribution coefficient at this particular salt concentration. Furthermore, numerical evaluation of this parameter bears no physicochemical relationship to an interactive distribution constant, which must be derived under conditions of infinite dilution of buffer constituents.

Analogous circumstances have been discussed for HIC and protein solubility[18,19]. In a subsequent paper the involvement of hydrophobic interaction effects and salting-out effects in the HPIEC of these proteins will be evaluated. These additional studies also have permitted the dependency of surface tension on log k' to be assessed. In the absence of displacer salt, the chromatographic capacity factor, K_0, will be related to the interactive affinity of the protein solute for the charged stationary-phase surface. This, in turn, is related to the area and charge density of the interactive region, *i.e.* the ionotope. According to the Debey-Hückel theory of electrostatics, the electrostatic free energy, W_e, of a spherical ion of radius r_p is given by

$$W_e = \int_0^{Z_t} \psi_s \, de \tag{6}$$

where Z_t is the full charge of the ion, and ψ_s is the potential at its surface. Because of the spherical symmetry, the electrical intensity, E, will be constant over this surface, and according to Gauss' law is given by

$$E = \frac{Z_t \varepsilon}{Dr^2} = \frac{4\pi Z_t \varepsilon}{DA_t} = \frac{-d\psi_s}{dr} \tag{7}$$

where ε is the protonic charge, D is the dielectric constant of the medium, and A_t is the total surface area of the charged sphere. For a protein solute with a single ionotopic region that dominates the coulombic interaction with an ion-exchange surface, the electrical intensity of the ionotopic surface may be given by

$$E_c = \frac{Z_c \varepsilon}{D \Sigma r_i r_j} = \frac{C_c Z_c \varepsilon}{DA_c} \tag{8}$$

where $\Sigma\ r_i r_j$ accounts for the ionotopic surface area, A_c, of three-dimensional coordinates, r_i, r_j, r_z, whilst C is a constant.

If it is assumed[20] that the distance, a, between the ionotope and the charged ligand corresponds to the distance of closest approach between the vector centres of the protein ion of radius r_p and the charged ligand, then integration of eqn. 6 with respect to r yields

$$\psi = \frac{CZ_c \varepsilon r_z}{D} \frac{1}{A_c} + F \tag{9}$$

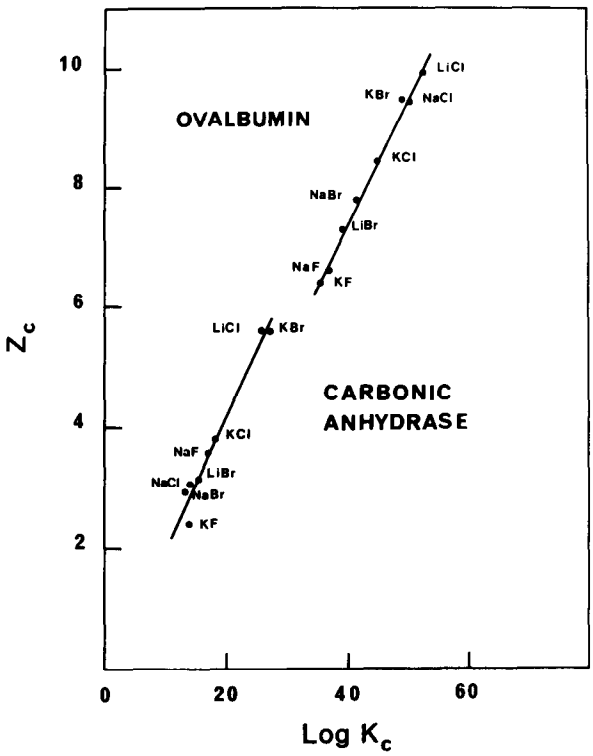

Fig. 9. Plots of Z_c *versus* log K_c for ovalbumin and carbonic anhydrase, separated under conditions of varied gradient time.

for $r_p \leqslant r \leqslant a$, and where F is given by

$$F = \frac{-Z_c\varepsilon}{D} \frac{\kappa}{1 + \kappa a} \tag{10}$$

and

$$\kappa^2 = \frac{4\pi\varepsilon^2}{DkT} \Sigma c_i Z_i^2 \tag{11}$$

The term κ depends only on the composition of the solution, and the $\Sigma\ cZ^2$ term represents the summation of all charged species of concentration c and charge Z. The term F of eqn. 9 corresponds to the potential due to the ion atmosphere surrounding the ionotope and is proportional to the charge of the ionotope. For a particular ligand type and mobile phase composition, F will be constant for a defined protein in the ionisation state Z_i. Furthermore, if solute elution is carried out under conditions that maximise electrostatic interactions, the electrostatic free energy term, ΔG_e, for ion-exchange solute retention is equivalent to W_e. Thus, integration of ψ with respect to an incremental charge increase, de, yields

$$W_e = \frac{Z_c Z_i \varepsilon^2}{2D} \frac{r_p}{A_c} - \frac{\kappa}{1 + \kappa a} = \frac{-RT}{2.303} \log K_0 \tag{12}$$

Hence, if the above assumptions apply, it follows that log K_0 is proportional to the ionotopic constant area, A, and, from eqn. 12, a linear dependency of log K_0 on Z_c is anticipated.

Fig. 9 shows plots of Z_c *versus* log K_c for ovalbumin and carbonic anhydrase. For these calculations, values of log K_c were determined by extrapolation of the log $\bar{k}$ *versus* log $1/\bar{c}$ plots to the limit case of $c \rightarrow 10^{-6}$ mol/l. While these values are not equivalent to the true log K_0 value, which by definition is a constant for a particular protein solute, log K_c values will provide some indication of the relative influence of different displacer salts on protein retention behaviour. The high correlation of the data shown in Fig. 9. ($r^2 = 0.99$ and 0.98, respectively) provide support for the conclusions summarised in the relationship shown in eqn. 12.

CONCLUSION

The current investigation provides a detailed experimental and theoretical basis for the quantitative characterisation of the electrostatic contact area and the electrostatic potential of the protein surface in HPIEC systems. In particular, the present data clearly demonstrate the influence on protein interactive behaviour of different cations and anions with known chaotropic or kosmotropic properties. Changes in the ionotopic surface of a protein under different environmental conditions can now be rapidly assessed by chromatographic analysis. If it is assumed that NaCl has little influence on protein structure, different displacer salts can be categorised on the basis of their effects on Z_c and log K_c. Correlation of these effects with

spectroscopic analysis of the eluted protein zone will then enable full optimisation of HPIEC separations to maximise chromatographic performance in both analytical and preparative HPIEC.

SYMBOLS

a	Closest approach distance between protein and charged ligand
A_c	Ionotopic surface area
A_t	Total surface area of a charged sphere
b	Gradient steepness parameter
$\bar{c}$	Median salt concentration during gradient elution
C	Constant of integration in eqn. 8
c_i	Concentration of a charged species in solution (eqn. 1)
c_0	Initial salt concentration for the gradient
c_f	Final salt concentration for the gradient
Δc	Change in concentration equivalent to $c_f - c_0$
D	Dielectric constant of medium
D_b	Stationary phase ligand concentration
E	Electrical intensity for a spherical ion
E_c	Electrical intensity for a spherical protein in an ion-exchange system
ε	Protonic charge
F	Potential due to the ion atmosphere surrounding the ionotope, *i.e.* proportional to the charge on the ionotope
k	Boltzmann constant
K	Distribution coefficient, as defined by eqn. 3
K_c	Capacity factor at $C = 10^{-6}$ mol/l, *i.e.* an estimate of K_0
K_b	Binding constant for the equilibrium shown in eqn. 1
K_0	Capacity factor in the absence of displacer salt
k'	Isocratic capacity factor
$\bar{k}$	Gradient capacity factor
κ	Debye length
LSS	Linear solvent strength model
R	Gas constant
r_i, r_j, r_z	Three-dimentional coordinates of protein ionotope
r_p	Radius of the protein
SDM	Stoichometric displacement model
T	Temperature
t_e	Gradient elapse time
t_G	Gradient time
t_g	Solute gradient retention time
t_0	Column dead-time
VF	Varied flow-rate conditions
VT_G	Varied gradient time conditions
W_e	Electrostatic free energy
z_a	Constant, adjusting for solute valency in eqn. 3
z_b	Constant, adjusting for salt valency in eqn. 3
Z_i	Valency of a charged species in solution (see eqn. 11)

Z_c Number of charges on a protein associated with adsorption, obtained from the slope of log k' *versus* log $1/c$ plots
Z_t Ratio of the interactive charge on the protein to that on the displacer ion
ψ_s Electrostatic potential of the protein
φ Phase ratio

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