CHROMSYMP. 1426

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

LXXXVII*. COMPARISON OF RETENTION AND BANDWIDTH PROPERTIES OF PROTEINS ELUTED BY GRADIENT AND ISOCRATIC ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The high-performance ion-exchange gradient-elution behaviour of a range of globular proteins has been investigated, using a strong anion exchanger as the stationary phase and sodium chloride as the displacer salt. Deviations were observed between the Z_c values obtained from isocratic experiments and from gradient experiments with varied gradient time and varied flow-rate. These results indicate that theoretical treatments which relate gradient and isocratic elution processes do not adequately describe the retention behaviour of protein solutes separated by ion-exchange methods. Furthermore, the experimentally observed bandwidths deviated significantly from values predicted on the basis of plate theory for low-molecular-weight molecules. The significance of these results is discussed in terms of the influence of experimental parameters on the ability of particular electrostatically interactive areas on the surface of protein solutes to control the thermodynamic and kinetic properties of these polyelectrolyte molecules during ion-exchange chromatographic processes.

INTRODUCTION

High-performance ion-exchange chromatography (HPIEC) is now a widely used and important method for the separation of peptides, proteins and polynucle-otides. Despite the widespread application of the technique in the biological sciences, the mechanistic details of protein retention in the ion-exchange process have not yet been fully elucidated. This is mainly due to the complex three-dimensional structure and amphoteric nature of proteins, which leads to complex electrostatic interactions between the solute and stationary phase surface. Previous studies[1,2] on the isocratic anion-exchange elution of proteins have indicated that protein retention in HPIEC is dependent on both the number and distribution of charged sites on the protein surface.

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* For Part LXXXVI see ref. 3.

Thus, changes in mobile-phase pH and ionic strength, which will alter the surface interactive potential of the protein, can be used to manipulate the selectivity of proteins in HPIEC. The nature of the displacer ion and co-ion have also been shown to significantly influence the retention properties of proteins in HPIEC. In a previous paper[3], we reported that while the anions F^-, Cl^- and Br^- reduce protein retention as anticipated on the basis of their decreasing hydrated ionic radii and increased affinity for the stationary phase, cationic co-ions also significantly influence the retention behaviour of some proteins through preferential interactions with the solute. Furthermore, the extent of protein band-broadening under isocratic conditions depends on the choice of the anion/cation combination of the displacer salt, effects which are related to known Hofmeister effects on protein aggregation and solubility. While isocratic elution conditions offer an additional dimension for high-resolution protein purification, complex mixture are more commonly separated under gradient-elution conditions, where advantage can be taken of band-compression effects. The fundamental principles of gradient elution of low-molecular-weight solutes are now well established[4]. Mathematical expressions have been developed which relate isocratic and gradient-elution parameters under a wide range of experimental conditions and in some instances allow the accurate prediction of solute retention and bandwidth properties in ion-exchange[5,6], reversed-phase[7,8] and hydrophobic-interaction chromatography[9] of macromolecules. However, the application of models such as the linear solvent strength (LSS) approach to HPIEC of peptides and proteins has not been extensively verified, particularly with respect to the influence of solvent constituents on the ion-exchange properties of protein solutes. This paper describes the results of detailed investigations on the gradient-elution behaviour of several globular proteins in an anion-exchange chromatographic system.

EXPERIMENTAL

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 (Norwalk, CT, U.S.A.) LCI-100 integrator. Isocratic and gradient elution were 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 Research Products (Cambridge, MA, U.S.A.) SA 520 meter, equipped with a combination glass electrode. Column effluents for gradient elapse time, t_e, measurements were collected on a Pharmacia FRAC-100 fraction collector. The conductivity of the effluent was measured with a Radiometer (Copenhagen, Denmark) Model Cdm3 conductivity meter with a Model CDC 304 glass electrode. All chromatographic separations were carried out at ambient temperatures of *ca.* 20°C.

Chemicals and reagents

Bovine erythrocyte carbonic anhydrase, sperm whale skeletal muscle myoglobin (type iii), hen egg ovalbumin (grade V) and piperazine were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium chloride (AnalaR grade) was 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 with hydrochloric acid (sp. gr. 1.16, AnalaR grade), which was purchased from BDH.

Chromatographic procedures

Eluent A was a 0.02 M piperazine solution, adjusted to pH 9.60. Eluent B consisted of 0.02 M piperazine and 0.3 M sodium chloride 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.

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. Gradient elution was run 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. 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) and correlation coefficient (r^2). The various chromatographic parameters were calculated using the ChromoCalc programme series, developed in this laboratory and written in BASIC language for an IBM XT or AT computer.

RESULTS AND DISCUSSION

Retention relationships

Several studies have addressed the theoretical relationship between protein retention and mobile-phase composition in multivalent adsorption chromatography. Most work on retention optimisation and simulation has centred on reversed-phase HPLC, although more recent studies have also extended similar concepts to the hydrophobic-interaction and ion-exchange mode. For example, isocratic and gradient-elution theory, developed for simple organic acids and bases, has been adapted to evaluate the retention behaviour of polypeptides separated under regular reversed-phase conditions. It is well known that polypeptides frequently show pronounced retention dependencies on the volume fraction, φ, of the organic solvent modifier; in fact, only small changes in φ (*e.g.*, $\Delta\varphi < 0.1$) may be sufficient to encompass the solute's operational chromatographic range of $1 < k' < 100$ (refs. 10–12). This

dependency of the capacity factor, k', on the volume fraction, φ, for a polypeptide separated under regular reversed-phase isocratic conditions can be represented as

$$\log k' = \log k_0 - S\varphi \tag{1}$$

According to eqn. 1, linear dependencies of $\log k'$ on φ over relatively narrow ranges of φ, are anticipated with the intercept and slope equal to $\log k_0$ and S, respectively. However, experimental plots of $\log k'$ *versus* φ have been generally observed[10–12] to be curved rather than linear, over the operational range of mobile-phase compositions, *i.e.* over $0 < \varphi < 0.7$. This general observation is consistent with multisite binding between the protein solute and the stationary phase. The slope, S, can be used as a measure of the hydrophobic contact area established between the protein and stationary phase under specific chromatographic conditions. Evaluation of the S value, together with the $\log k_0$ value, represents critical experimental parameters in the development of structure–retention relationships and provides the basis for a quantitative approach to the optimisation of separation conditions.

Since the same reversed-phase chromatographic variables that control retention, resolution and bandwidth in isocratic elution are also relevant in gradient elution, recourse is usually to the latter elution mode, which provides a useful expedient for reducing separation times and decreasing peak volumes (σ). Several theoretical models have been developed for the prediction of the gradient-elution behaviour of small-molecular-weight solutes. For example, the retention time of a solute, t_g, of a conformationally rigid solute under linear solvent strength (LSS) gradient-elution conditions can be expressed as

$$t_g = \frac{t_0}{\beta} \log 2.3 \, k_0 b + t_0 + t_e \tag{2}$$

where t_0 is the column dead-time, k_0 is the capacity factor for the peptide solute under the initial gradient conditions, t_e is the time taken for eluent B to reach the column inlet and b is the gradient steepness parameter. Evaluation of b can be easily achieved by several experiments in which different gradient times or flow-rates are used. Thus, for elution conditions with fixed column, mobile-phase composition and flow-rate the gradient steepness parameter, b, can be derived from the relationship

$$b_1 = t_0 \log \beta \Bigg/ \left(t_{g1} - \frac{t_{g2}}{\beta} + \frac{t_{G1} - t_{G2}}{t_{G2}} \right) \tag{3}$$

where t_{g1} and t_{g2} are the solute gradient retention times of gradient times t_{G1} and t_{G2}, respectively, and β is the ratio of the gradient times (t_{G2}/t_{G1}). However, if chromatograms are obtained at flow-rates F_1 and F_2, whilst the gradient time is maintained constant, then b values may be obtained from

$$b_1 = \log (F_2/F_1) \Bigg/ \left(X_1 - X_2 \cdot \frac{F_1}{F_2} \right) \tag{4}$$

where

$$X_1 = \frac{t_{g1} - t_{0.1}}{t_{0.1}} \tag{5}$$

and

$$X_2 = \frac{t_{g2} - t_{0.2}}{t_{0.2}} \tag{6}$$

By definition, LSS gradients require the value of b for all components to be constant, and this can be achieved for low-molecular-weight solutes, such as amino acids, polyaromatic hydrocarbons or drug analogues. In most gradient systems as currently used, the value of the parameter b usually varies for different polypeptides. The gradient steepness parameter, b, can also be related to the variables which quantitatively define structure–retention dependencies on LSS gradients in reversed-phase separations through the expression

$$b = \frac{SV_m \, \Delta\varphi}{Ft_G} \tag{7}$$

It is evident from eqn. 7 that changes in gradient time or flow-rate will affect the value of b and consequently alter solute selectivity (α), as reflected by changes in the S value. Conversely, both the b value and selectivity will remain constant if t_G and F are varied proportionally, provided no change in the interactive contact area of the solute occurs due to slow secondary equilibrium phenomena. Optimisation of selectivity and peak capacity can therefore be carried out through rational manipulation of these experimental variables.

In reversed-phase chromatographic systems, application of the LSS concept has proved useful for the analysis of macromolecular retention data. Similarly, extension of the LSS model to HPIEC provides a useful basis for comparing isocratic and gradient data, although the full implications of multisite interaction and orientation effects in these systems have yet to be established. For small, charged molecules, separated under isocratic ion-exchange conditions, the capacity factor, k', can be related to the concentration of the displacer salt, c, by the following empirical relationship

$$\log k' = \log K + Z \log\frac{1}{c} \tag{8}$$

where K is an ion-exchange distribution constant and Z is the tangent to the curve obtained from a plot of $\log k'$ versus $\log 1/c$. Previous studies[5] indicate that linear salt gradients may result in a non-linear dependence of k' on gradient time, a finding which is not consistent with the assumptions of LSS-type separations. Eqn. 2 can still be used in such HPIEC systems, provided the gradient is assumed to approximate the LSS condition during the time that a solute molecule migrates through a column, i.e. over a limited k' range, such as $1 < k' < 10$. The resultant value for t_g can then be used to

obtain a solution for b from eqns. 3 or 4. The median capacity factor, $\bar{k}$, for a particular solute, eluted under gradient conditions can then be obtained from

$$\bar{k} = 1/1.15b \tag{9}$$

where the value of $\bar{k}$ corresponds to the capacity factor for a solute band at the column midpoint. Similarly, the concentration, $\bar{c}$, of the eluting salt when the sample band has reached the column midpoint is given by

$$\bar{c} = c_0 + \left(t_g - t_0 - t_e - 0.30\frac{t_0}{b}\right)\Delta c/t_G \tag{10}$$

where $\Delta c = c_f - c_0$ and c_0 and c_f are the initial and final salt concentrations for the gradient. Values of Z and $\log K$ can then be obtained from eqn. 8 by using iterative regression analyses of plots of $\log \bar{k}$ versus $\log 1/\bar{c}$.

If the physicochemical basis of isocratic and gradient elution in HPIEC is assumed to be the same, plots of $\log \bar{k}$ versus $\log 1/\bar{c}$, when approximated by linear dependencies derived from the data for both isocratic and gradient experiments for a particular solute, should be superimposable. However, if the interactive properties of the protein solute are sensitive to time-dependent changes, including changes mediated by the displacer salt concentration, conformational effects, ion-binding and salt-bridging effects or other secondary equilibria, the experimental values of Z and $\log K$, derived according to the LSS gradient model, will not coincide with the values determined isocratically. As part of further investigations on the mechanistic details of HPIEC of peptides and proteins, this paper describes the results of detailed studies on the comparative macromolecular retention behaviour in isocratic and gradient anion-exchange elution systems, using the LSS retention model summarised by eqns. 1–10 as the basis of data analysis.

The retention data for three proteins, listed in Table I, were obtained with a Mono-Q strong anion-exchange stationary phase, under gradient and isocratic elution conditions. Isocratic data were obtained at pH 9.60 with sodium chloride concentrations varying between 0 and 300 mM at a flow-rate of 1 ml/min. Gradient data were also collected at pH 9.60 with a 0 to 300 mM sodium chloride gradient. The procedure for the accumulation of gradient elution data involved either fixing the flow-rate at 1 ml/min, with t_G 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. Fig. 1 shows retention plots of $\log k$ versus $\log 1/c$, derived from the isocratic and two sets of gradient data for the proteins listed in Table I. The slopes (Z_{comb}) of these plots and the correlation coefficient, r^2, for the combined isocratic and gradient data are also listed in Table I. Values of r^2 approaching unity, as observed for ovalbumin ($r^2 = 0.95$) in Table I, indicate that both gradient and isocratic data are highly congruent and that the LSS theory provides an accurate model of gradient elution behaviour for this protein. However, lower r^2 values for Z_{comb} were seen for carbonic anhydrase ($r^2 = 0.77$) and myoglobin ($r^2 = 0.74$). For elution systems involving monovalent displacing ions, Z is generally defined as the number of electrostatic interactions between the protein solute and the charged stationary-phase surface. Furthermore, if the desorption process were solely dependent on the concentration of displacer salt in

TABLE I

PHYSICAL PARAMETERS AND Z VALUES FOR PROTEINS USED IN THIS STUDY

Protein	pI	MW	$D_m^\star \cdot 10^{-10}$ (cm^2/s)	$Z_{iso}^{\star\star}$	$Z_{VF}^{\star\star\star}$	$Z_{VTG}^{\S}$	$Z_{comb}^{\S\S}$
(1) Ovalbumin	4.70	43 500	4.24	7.7	7.4	9.4	7.3
(eggwhite)				(0.99)	(0.97)	(0.99)	(0.95)
(2) Carbonic	5.89	30 000	4.80	4.4	4.5	2.9	2.4
anhydrase				(0.99)	(0.87)	(0.98)	(0.77)
(bovine							
erythrocytes)							
(3) Myoglobin	7.68	17 500	5.74	2.8	5.1	4.0	1.7
(sperm whale	8.18			(0.98)	(0.98)	(0.94)	(0.74)
muscle)							

    $\star$ Calculated from the Stokes-Einstein equation[29].
   $\star\star$ Z value for isocratic data.
 $\star\star\star$ Z value for gradient varied F, constant t_G data.
    $\S$ Z value for gradient varied t_G, constant F data.
  $\S\S$ Z value for combined isocratic and gradient data.

the mobile phase, it would be anticipated that similar Z values should be obtained 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, significant differences were observed between the three sets of data, i.e. Z_{iso}, Z_{VF} and Z_{VTG} for myoglobin and carbonic anhydrase.

While gradient and isocratic elution processes can be mathematically related through such theoretical treatments as the LSS model, the experimental data indicate that the two modes of elution are not directly comparable for protein solutes separated by these two HPIEC methods. The main reason for these divergencies is that different sorption/desorption dynamics will occur in each process. For example, solutes eluted isocratically are exposed throughout column migration to a constant salt concentration. In contrast, under the condition of gradient elution, as used in the current study,
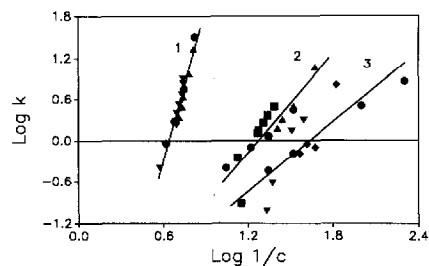

Fig. 1. Retention plots for isocratic (log k' versus log $1/c$) and gradient experiments (log $\bar{k}$ versus log $1/\bar{c}$) for the proteins listed in Table I. Isocratic data were derived from experiments with varied sodium chloride concentrations at pH 9.6 and a flow-rate of 1 ml/min. Gradient data 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. Other chromatographic conditions are given in Experimental. See Table I for the code to protein solutes and for the derived Z_c values. For protein 1: ● = isocratic, ▲ = varying gradient time, ▼ = varying flow-rate. For protein 2: ● = isocratic, ▲ = varying gradient time, ■ = varying flow-rate. For protein 3: ● = isocratic, ◆ = varying gradient time, ▼ = varying flow-rate.

solutes are initially immobilised on the anion exchanger at low ionic strength and then subjected to either varying rates of change in the salt concentration (varied t_G and constant F) and/or varying flow-rates including a constant rate of change in the salt concentration (varied F and constant t_G). In the latter case, at very low flow-rates, the system dead-volume, t_e, becomes a major portion of the total gradient volume of the system and consequently, the gradient condition approximates a stepwise elution, $i.e.$ b approaches infinity as t_G and F approach zero. Under these conditions, the sorbed solute is exposed to a smaller displacement volume, effectively at a higher rate of change of salt concentration per unit volume, rather than the more normal gradient development under elution conditions. Low flow-rates and short t_G values consequently promote increased solute presence in the mobile phase and lead to shorter elution times. Linear retention plots for log k' $versus$ log $1/c$ or log $\bar{k}$ $versus$ log $1/\bar{c}$ were observed for most of the varied flow-rate retention data obtained in this study. However, at very low flow-rates, such as 0.1 ml/min, data points for carbonic anhydrase and myoglobin deviate from this linear relationship, and the log k' or the log $\bar{k}$ $versus$ log $1/c$ $(1/\bar{c})$ plots exhibit a curvature, particularly when $F < 0.2$ ml/min. While the effect of t_e on elution conditions may account for deviations at low k or high b values, chromatographic residence times, particularly at low b values, will also significantly influence the retention behaviour of proteins.

The influence of salts on the stability, solubility and biological activity of proteins in solution has attracted some attention[13–18]. The implications of these effects for the chromatographic behaviour of proteins in HPIEC have not yet been incorporated into a general retention model. While sodium chloride is known not to greatly influence protein structure and stability, it is clearly apparent that changes in the concentration of this salt over the ranges of gradient times adopted in this study, modify the interactive properties of different solutes, an effect which cannot be predicted on the basis of the LSS model. Furthermore, conformational changes induced by the type or concentration of the salt or pH changes in the microenvironment of the protein would be expected to give rise to changes in both Z_c and K. Where such changes are discrete rather than monotonously continuous, breaks in the log k' $versus$ log $1/c$ plots will occur, leading to more than one apparent Z_c value. Such behaviour has been noted with subtilisin variants[19] as well as with several hormonal proteins[20]. The degree of solute exposure to mobile-phase constituents clearly represents a major factor contributing towards non-coincident retention behaviour for proteins eluted by gradient and isocratic HPIEC methods. The process of solute desorption is a complex interaction between the protein, the displacing salt species and the charged stationary-phase surface. If Z is also considered to be a measure of the electrostatic interaction area, A_{cont}, larger values of Z_{VTG} may be anticipated as the solute is initially sorbed in the absence of displacer salt, which should promote maximum interaction. However, this was only observed for ovalbumin and not myoglobin or carbonic anhydrase. The mechanism of protein desorption in HPIEC involves an ionic displacement hierarchy as the protein is desorbed from the charged stationary phase, in which certain charged centres on the solute surface dominate the interaction. The influence of solvent conditions on the interactive nature of these groups will thus not be solute-specific $per\ se$, but rather orientation- and electrostatic-contact-area-specific. As discussed elsewhere[21], these electrostatic contact areas or ionotopes define the structure–retention dependency for different proteins. It thus

follows that structurally different proteins with the same ionotopic contact area can have the same apparent retention under a particular HPIEC condition. If the protein surface structure is not influenced by the nature or the concentration of the displacer salt, similar Z values will be obtained for both isocratic and gradient experiments. However, if the interactive potential of the charged centres, which constitute the coulombic interaction area is diminished or enhanced in the presence of displacer salts, the desorption process will clearly differ between isocratic and gradient-elution conditions. This will give rise to subtle but important selectivity differences, which can be exploited in both analytical and preparative applications.

Salt-mediated changes in the surface electrostatic structure of protein solutes will also influence the kinetic properties of solute sorption and desorption, and these rate effects will be manifested as anomalous band-broadening behaviour. The LSS model was therefore further evaluated as a theoretical approach to aid in the prediction of solute bandwidth and to provide further insight into the kinetic behaviour of proteins at charged stationary phase surfaces.

Bandwidth relationships

The general plate height theory[22] provides a basis for assessing the diffusional behaviour of small molecules in chromatographic systems. Snyder and co-workers[4,5] have extended the use of retention parameters derived from their LSS model in conjunction with the plate-height theory to provide a method for predicting solute bandwidths so that overall chromatographic resolution can be evaluated and, if necessary, improved. Under chromatographic conditions of gradient elution, the resolution between two adjacent solute zones can be defined as

$$R_{s_{i/j}} = (\alpha_{i/j} - 1)\sqrt{N}\frac{k_i}{1 + k_i} \tag{11}$$

where $\alpha_{i/j}$ is the gradient separation factor (or selectivity) between two solutes, P_i and P_j, as they traverse the midpoint of the column, and N is the theoretical plate number. Furthermore, the peak capacity (PC) for a chromatographic separation of gradient time (t_G), flow-rate (F) and average resolution $(R_s = 1)$, for all adjacent peaks can be expressed by

$$PC = \frac{(t_G - t_0)F}{4\sigma_v} \tag{12}$$

where σ_v is the bandwidth measured in volume units (1 S.D.). When α is kept constant, maximising the peak capacity will, accordingly, optimise the resolution. The relationship between σ_v and $\bar{k}$ for linear solvent systems can be expressed as

$$\sigma_{v,calc} = \frac{(0.5\bar{k} + 1)GV_m}{\sqrt{N}} \tag{13}$$

where V_m is the column void volume $(V_m = t_0F)$ and G is the band-compression factor

which arises from the increase in solvent strength across the solute zone as the gradient develops along the column, given by the expression

$$G^2 = \left(1 + 2.3b + \frac{(2.3b)^2}{3}\right)\Big/\left(1 + 2.3b\right)^2 \tag{14}$$

Under normal flow-rate conditions in gradient elution, the plate number, N, can be approximated to

$$N = \frac{D_m t_0}{C d_p^2} \tag{15}$$

where d_p is the particle diameter and D_m is the diffusion coefficient (cm^2/s) of the solute in the mobile phase, which can be expressed in terms of solute molecular weight (MW) as

$$D_m = \frac{8.34 \cdot 10^{-10} T}{\eta MW^{1/3}} \tag{16}$$

where T is the absolute temperature and η is the eluent viscosity. The Knox equation parameter, C, which accounts for resistance to mass transfer at the stationary phase surface can be estimated by

$$C = \frac{[(1 - x + \bar{k})/(1 + \bar{k})]^2}{15\rho^* a' + 15\rho^* b' \bar{k} - 19.2\rho^* x} \tag{17}$$

where x is the interstitial column fraction found[23] to be 0.62 for a Mono-Q anion-exchange column, a' is assumed to equal 1.1 and b' is the surface diffusion parameter, calculated from the relationship

$$B = a' + b'k \tag{18}$$

The Knox equation constant, B, which arises from zonal dispersion due to longitudinal diffusion, was obtained experimentally from isocratic bandwidth data at different flow-rates and substituted into eqn. 18 to obtain a value of b' equal to 0.72. The restricted diffusion parameter, ρ^*, a molecular-weight-dependent term, was calculated by using the Renkin relationship[21] so that

$$\rho^* = 1 - 2.104\rho + 2.09\rho^3 - 0.95\rho^5 \tag{19}$$

where ρ is equal to the ratio of the solute diameter to the sorbent pore diameter (d_s/d_p). The linear logarithmic relationship ($r^2 = 0.992$) found experimentally between ρ^* and solute MW for a Mono-Q column, assuming an average pore size of 800 Å and a protein molecular-weight range between 12 000 and 69 000 was

$$\log \rho^* = 0.19 - 0.06 \log MW \tag{20}$$

Although the above bandwidth relationships provide reasonable correlations with experimental data of conformationally rigid low-molecular-weight solutes, it has been found in, *e.g.* reversed-phase HPLC that the chromatographic behaviour of proteins can be much more complex than described by these equations. In the present study, these bandwidth relationships have therefore been employed to further investigate protein kinetic behaviour during high-performance anion-exchange chromatography. Application of the bandwidth eqns. 11–20, derived from the general plate-height theory, assumes that the solute migrates as a single interactive species with an invariant surface charge distribution and shape. Divergencies between experimental and theoretical bandwidths will arise when changes in surface structure occur as a result of preferential salt interactions, which may or may not lead to more specific conformational changes. If it is assumed that these secondary equilibrium processes are either very rapid or very slow compared to the solute chromatographic separation time, then the ratio between the experimentally observed bandwidth, $\sigma_{v,exp}$, and the calculated bandwidth, $\sigma_{v,calc}$, should approach unity over an optimal range of retention values, *i.e.* $1 < \bar{k} < 10$. However, there is an increasing number of examples where the average relaxation times associated with solute-dependent secondary equilibrium process, such as conformational effects, are of a magnitude comparable to the mass transfer time[25–27]. In such cases where the shape and surface characteristics of the polypeptide molecule are changing in a relatively slow, time- or condition-dependent manner, the corresponding changes in the diffusional and interactive properties of the solute will lead to differential zone migration. These increases or decreases in the kinetics of solute mass transfer properties will ultimately be revealed as experimental bandwidths that deviate significantly from values predicted by eqn. 13.

Theoretical bandwidths for the proteins listed in Table I were therefore calculated over the range of experimental conditions employed by using eqns. 13–20 and compared to the experimental peak widths as a function of the gradient steepness parameter, *b*. Fig. 2 shows plots of $\sigma_{v,exp}/\sigma_{v,calc}$ as a function of $1/b$ for experiments carried out at a constant flow-rate of 1 ml/min. In these plots, the bandwidth ratio is seen to increase with decreasing *b*. Eventually, a maximum $\sigma_{v,exp}/\sigma_{v,calc}$ value is obtained for each protein where the bandwidth ratio approaches a plateau limit and remains essentially constant with decreasing *b* values. As is evident from Fig. 2, the variation in the rate of change of displacer salt concentration, associated with differences in gradient time, dramatically affects the kinetic processes for each solute.
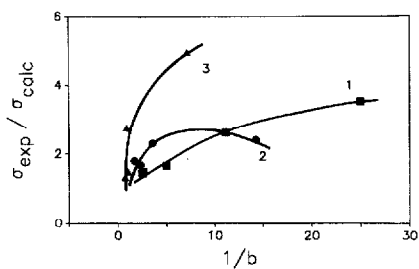

Fig. 2. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for proteins listed in Table I. The dependencies shown correspond to data acquired under conditions of different gradient times at a flow-rate of 1 ml/min. $\sigma_{v,calc}$ was evaluated by using the eqn. 13. See Table I for code to protein solutes.
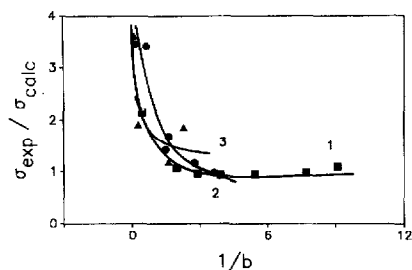
Fig. 3. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for data acquired under conditions of varying flow-rates and a constant gradient time of 17.1 min. See legend to Fig. 2 for other details.

The deviation of these plots from unity indicates that the assumptions inherent in the plate-height theory do not satisfactorily accommodate these secondary equilibrium effects. Detailed explanations of the underlying reasons for solute-dependent divergencies of $\sigma_{v,exp}$ and $\sigma_{v,calc}$ for very shallow gradients, *i.e.*, small b values, have yet to be offered. However, these divergencies may arise as a consequence of solute-specific physicochemical phenomena, associated with solute solvation or changes in the approach depth of penetration of the protein at or near the Stern double layer. Values for $\sigma_{v,exp}/\sigma_{v,calc}$ in reversed-phase systems with polypeptides/proteins[7,29-31] have been reported to deviate much less from unity over similar b-value ranges. The larger $\sigma_{v,exp}/\sigma_{v,calc}$ values found for proteins eluted in ion-exchange chromatography can be related to the physiochemical nature of the kinetics of interaction between solute and sorbent surface. The solute/stationary phase electrostatic interactions in HPIEC are much stronger in terms of free energy changes than the Van der Waals interactions that dominate reversed-phase separations, *i.e.* ΔG_{iec} *versus* ΔG_{vdw} of *ca.* 20 and 4kJ/mol, respectively[32]. As a result, the differences in the affinity of the interacting groups in HPIEC will be much larger than in reversed-phase systems. This will then cause differential zonal migration of the solute band and will contribute to anomalous bandbroadening behaviour, particularly at longer column residence times.

Fig. 3 shows a plot of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for the same proteins as shown in Fig. 2, where t_G was held constant at 17.1 min and the flow-rate was varied systematically between 0.1 and 2.0 ml/min. The curve shape and magnitude of the bandwidth ratios for the three non-related globular proteins were similar, and at lower
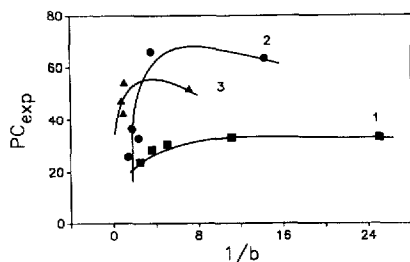

Fig. 4. Plots of peak capacity (PC) *versus* $1/b$ for data obtained under conditions of varying gradient time and a flow-rate of 1 ml/min. The PC value was determined according to eqn. 12. See legend to Figs. 1 and 2 for other details.

b-values or higher flow-rates these plots all approached unity. In general, at flow-rates of less than 0.5 ml/min, *i.e.* corresponding to large b values, there is a rapid increase in the bandwidth ratio. Such increases in $\sigma_{v,exp}/\sigma_{v,calc}$ have been attributed to the so-called J-effect[8], which is believed to be associated with rapid gradient generation, leading to stepwise elution. With decreasing flow-rate and a small t_G, the gradient elapse time, t_e, becomes a more significant proportion of the total gradient time, resulting in conditions where solute retention is governed predominately by stepwise rather than gradient elution.

This bandbroadening behaviour is also evident from analysis of the experimental peak capacity data. Fig. 4 shows plots of PC *versus* $1/b$ for the proteins listed in Table I at a constant flow-rate of 1 ml/min and varied t_G, where PC is calculated according to eqn. 12. The plots for all proteins were observed to increase to a maximum with decreasing b. The plots of PC *versus* $1/b$ for ovalbumin approached an apparent asymptotic limit, while the data for myoglobin and carbonic anhydrase indicated an apparent PC maximum at intermediate b values of 0.25–0.5, followed by decrease in PC. The relationship used to calculate $\sigma_{v,calc}$ assumes that the electrostatic surface structure of the protein solute is constant throughout its passage along the column. However, the observed differences between $\sigma_{v,exp}$ and $\sigma_{v,calc}$ (and hence the differences noted for PC_{exp}) with decreasing rates of change in sodium chloride concentration indicates that the interactive potential of the protein is, in fact, changing under the different elution conditions. This observation suggests that values calculated for various input parameters, such as D_m and the simple form of the dependency of the Knox C parameter on $\bar{k}$ (eqn. 17) may not adequately describe the diffusional and interactive properties of the protein solute as it passes along a charged stationary phase. Consequently, the incorporation of additional factors into bandwidth models is required to accommodate the influence of specific solute–solvent–stationary interactions and thus allow a mechanistic approach to optimisation of protein separation in HPIEC. These issues will be addressed in a subsequent manuscript.

Effect of buffer ion concentration

The magnitude of electrostatic interactions between solutes and the sorbent surface in HPIEC is clearly dependent upon several key structural and chromatographic factors. These include (i) the number and distribution of charged sites on the solute molecule that define the surface topography and contact area of the protein ionotopes that interact simultaneously with the packing, (ii) the charge density of the packing and (iii) the mobile phase composition. Of these factors, the mobile-phase composition is the easiest and most convenient to manipulate systematically. It is known that the mobile phase composition can be changed in a number of ways to influence solute retention, including variation in the type of displacer ion, ion concentration and solvent pH[1,3,33,34]. However, little has been reported on the effect of buffer ion concentration on solute retention for gradient HPIEC systems. In order to address this issue systematically, the following experimental data were obtained by simultaneously varying the concentration of piperazine in eluents A and B. Piperazine has pK_a values of 5.68 and 9.73 and at a solvent pH of 9.60 carries either a uni- or bi-valent positive charge, which may then influence chromatographic behaviour through preferential interactions with the protein solute.

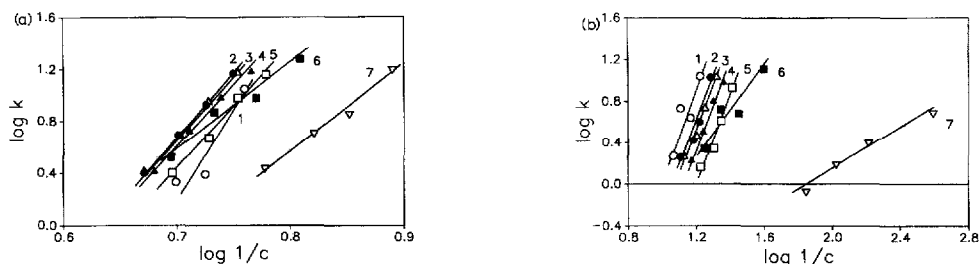Fig. 5 shows retention plots for carbonic anhydrase and ovalbumin, eluted under

Fig. 5. Retention plots of log k *versus* log $1/c$ for (a) ovalbumin and (b) carbonic anhydrase, eluted under gradient conditions with varying piperazine buffer concentrations of 0–80 mM in eluents A and B. At each buffer concentration, retention data were obtained from four gradient times (t_G = 17.1, 34.3, 60 and 100 min) at 1 ml/min. Piperazine concentrations were as follows: (1) 0 mM, (2) 5 mM, (3) 10 mM, (4) 20 mM, (5) 30 mM, (6) 40 mM and (7) 80 mM.

gradient conditions with varying piperazine buffer concentrations. The gradient data were collected at pH 9.60, using a Mono-Q strong anion-exchange column and sodium chloride concentrations varying between 0 and 300 mM. At each buffer concentration, solute retention data were obtained for four different gradient times (t_G = 17.1, 34.3, 60 and 100 min). In Fig. 5, the high degree of parallelism between different retention plots derived for the same solute becomes immediately evident. Table II shows the Z_{obs} values obtained from each series of gradient experiments at the different buffer concentrations. The retention plots for carbonic anhydrase remain essentially parallel until a critical piperazine concentration between 30 and 40 mM is used in eluents A and B. Parallel retention plots also exist for ovalbumin between 5 and 30 mM piperazine inclusive. The existence of these parallel retention curves indicates that the interaction properties between the solute and support surface are conserved over the corresponding piperazine concentration range. Analysis of the data shown in Table II reveals that significant changes in selectivity occur for both carbonic anhydrase and ovalbumin when the piperazine concentration exceeds 40 mM or is less than 5 mM, respectively. These variations in selectivity reflect changes in both the solute surface interactive properties and the affinity of the solute for the stationary phase surface. Furthermore, the log K term in eqn. 8 is related to the equilibrium constant for the ion-exchange process, K_b, so that changes in K_b reflected in log K represent a change in both the solute affinity toward the sorbent surface and the average distance of the buffer ion from the polyelectrolyte surface. It can thus be concluded that at higher piperazine concentrations (*i.e.* 40–80 mM) the number of charged interactions between

TABLE II

PROTEIN Z_{obs} VALUES AND STATISTICAL ANALYSIS FOR VARIED PIPERAZINE CONCENTRATIONS

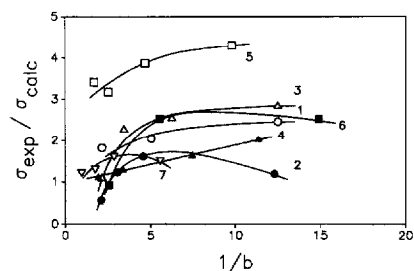| Protein	Piperazine concentration (mM)							$\bar{x} \pm 3$ S.D.
	0	5	10	20	30	40	80	
Carbonic anhydrase	4.0	4.4	4.1	4.1	4.2	2.0	1.0	4.2 ± 0.4
Ovalbumin	12.2	9.7	9.3	9.0	9.4	6.3	6.6	9.4 ± 0.9

Fig. 6. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for carbonic anhydrase eluted under gradient conditions and varied piperazine buffer concentrations. See legends to Figs. 2 and 5 for other details.

solute molecules and sorbent surface (Z_{obs}) is significantly reduced because of increased buffer ion competition and/or complexation of a new protein shape at the stationary phase surface. Conversely, in the case of ovalbumin at 0 mM piperazine, a significant increase in Z results when a decrease in the total ionic strength occurs, associated with a lack of buffer ions to complex competitively with the protein surface. Recent studies[19] on the HPIEC behaviour of subtilisin variants have documented a similar phenomena of elution-condition-dependent Z values. Further insight into the origin of this complex solute behaviour, mediated by the buffer concentration at the solute surface during gradient elution, can be obtained from bandbroadening studies. In Figs. 6 and 7 the effect of varying buffer concentration on bandwidth is shown for carbonic anhydrase and ovalbumin as plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$. The curves observed are similar in shape and magnitude to those found for each protein in Fig. 2, signifying the absence of any significant new kinetic effects induced by varying the buffer concentration. Close examination of the chromatographic profiles for ovalbumin and carbonic anhydrase at different piperazine concentrations, which are shown in Fig. 8, reveals the presence of minor isoform peaks. The identity of these isoform peaks was verified by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and isoelectrofocusing. Little change in the resolution between ovalbumin and its isoforms was observed. However, between 0 and 20 mM piperazine, the major peak 1 for carbonic anhydrase was well separated from those of the minor isoforms 2 and 3. However, at 30 mM piperazine, isoform peaks are observed to partially overlap with the major peak of carbonic anhydrase resulting in larger than predicted experimental bandwidths. As the buffer concentration is increased further the separation between carbonic anhydrase and its isoforms
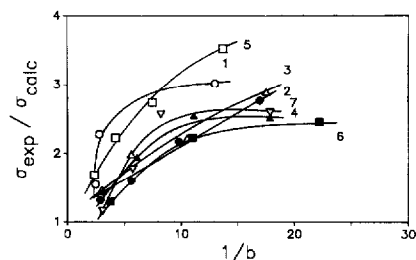

Fig. 7. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for ovalbumin, eluted under gradient conditions and varied piperazine buffer concentrations. See legend to Fig. 6 for other details.
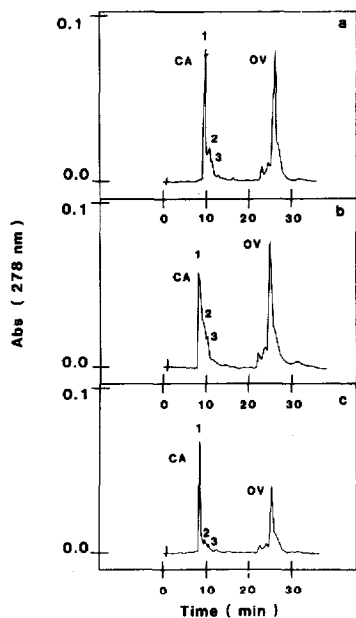
Fig. 8. Chromatograms indicating selectivity changes of carbonic anhydrase isoforms 1, 2 and 3 and of ovalbumin under gradient conditions with piperazine concentrations equal to (a) 20, (b) 30 and (c) 40 mM.

improves, while at 40 and 80 mM piperazine a decrease in the $\sigma_{v,exp}/\sigma_{v,calc}$ ratio is again observed. These trends are also evident in the peak capacity data shown in Figs. 9 and 10, where PC is plotted as a function of the inverse of the gradient steepness parameter, b, for carbonic anhydrase and ovalbumin, respectively.

CONCLUSION

The results of the current investigation clearly indicate that the interaction between protein solutes and the mobile-phase constituents plays a crucial role in the chromatographic behaviour of proteins in HPIEC. Furthermore, while the LSS theory
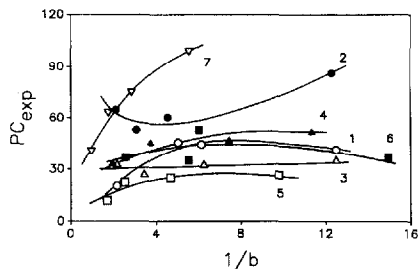

Fig. 9. Plots of peak capacity (PC) *versus* 1/b for carbonic anhydrase, eluted under gradient conditions and varied piperazine buffer concentrations. See legend to Figs. 4 and 5 for other details.
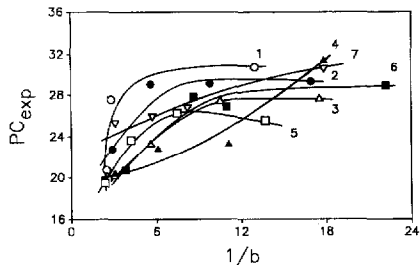
Fig. 10. Plots of peak capacity (PC) *versus* 1/b for ovalbumin eluted under gradient conditions and varied piperazine buffer concentrations. See legend to Figs. 4 and 5 for other details.

for gradient elution provides a useful basis for the analysis of the retention properties of macromolecules in HPIEC, the interactive properties of proteins are strongly influenced not only by the concentration of the displacer salt, but also by other eluent components, such as the choice and concentration of the mobile-phase buffer. The differences in the experimentally observed Z_c values in isocratic and gradient systems indicates that the orientation of the solute at the stationary phase surface can be controlled by experimental conditions. These results therefore have significant implications for the HPIEC of closely related or microheterogenous proteins. The choice of either isocratic or gradient conditions to enhance selectivity differences represents an additional parameter available for the optimisation of protein separations. It is now apparent that the concept of the coulombic interaction site or ionotype and the ability to identify their location within the surface structure of specific proteins is central to an understanding of the mechanistic basis of HPIEC of macromolecules. Studies currently in progress on the correlation of chromatographic data with the known tertiary structure of proteins, and hence, the electrostatic surface structure, will aid in the characterisation of the "molecular-docking" process between the solute and the stationary phase. The mechanistic details of this process, which is controlled by the charged groups within the ionotopic region, are required before further significant understanding of the thermodynamic and kinetic aspects of HPIEC of biological and non-biological macromolecules can be achieved. The next paper in the series will present the results of investigations into the influence of the type of displacer salt on the gradient elution of proteins in HPIEC by systematic changes in the anion or cation combination.

ACKNOWLEDGEMENTS

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