*Journal of Chromatography*, 512 (1990) 41–56
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1933

# High-performance liquid chromatography of amino acids, peptides and proteins

## XCVIII[a]. The influence of different displacer salts on the bandwidth properties of proteins separated by gradient elution anion-exchange chromatography

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

ABSTRACT

The influence of eight different displacer salts on the bandwidth properties of four globular proteins separated by a high-performance anion-exchange chromatography has been investigated. Proteins were eluted under gradient conditions with a range of alkali metal halide salts, in which the anion and cation were varied in the series $F^-$, $Cl^-$ and $Br^-$ and $Li^+$, $Na^+$ and $K^+$, respectively. The experimentally observed bandwidths ($\sigma_{v,exp}$) were found to deviate significantly from peak widths ($\sigma_{v,calc}$) predicted on the basis of plate theory for small molecules. For data accumulated under conditions of varied gradient time and constant flow-rate the solute bandwidth ratios ($\sigma_{v,exp}/\sigma_{v,calc}$) increased in the order $Br^- < Cl^- < F^-$ at low values of the gradient steepness parameter, $b$, or increasing column residence times. In addition, systematic changes in the cation influenced the bandwidth ratios ($\sigma_{v,exp}/\sigma_{v,calc}$) in the order $K^+ < Na^+ < Li^+$. Significant deviations between predicted and observed bandwidth values were also observed under elution conditions of constant gradient time and varied flow-rate. The results of the present study further demonstrate the complex nature of the interaction between protein solutes and coulombic chromatographic surfaces.

## INTRODUCTION

Our understanding of the physicochemical basis of the high-performance ion-exchange chromatography (HPIEC) of proteins has increased significantly over recent years due to the availability of retention models to assess protein chromato-

[a] For part XCVII, see ref. 23.

0021-9673/90/$03.50 

graphic behaviour. In particular, the application of stoichiometric displacement models[1,2] and the linear solvent strength (LSS) model[3], have considerably facilitated the quantitative evaluation of the influences on protein retention of the mobile phase pH, displacer salt concentration, the type of displacer salt and the elution mode[1,4–9].

Despite the widespread application of HPIEC in the purification of biomacromolecules, relatively little work has focused on mobile phase factors contributing to bandbroadening or methods to characterise the origin of zone spreading due to stationary phase induced phenomena. Recently, the LSS model has incorporated the general plate-height theory to provide a quantitative, descriptive model to estimate solute bandwidths associated with the elution conditions employed in HPIEC[9]. This approach has provided a useful basis for selecting criteria to optimise protein separations and enables investigations into the chromatographic behaviour of the HPIEC of proteins to assume quantitative, rather than qualitative details. This paper further investigates the application of these theoretical approaches for predicting protein bandwidth behaviour in gradient high-performance anion-exchange chromatographic systems. In particular, the effects of mobile phase composition and the gradient elution mode on peak widths associated with the mass transport of the globular proteins, lysozyme, myoglobin, carbonic anhydrase and ovalbumin as they migrate along a quaternary ammonium sorbent were investigated.

## MATERIALS AND METHODS

All chromatographic experiments were performed with a Pharmacia (Uppsala, Sweden) fast protein liquid chromatographic (FPLC) system, as previously described[6]. All chemicals and reagents used and experimental chromatographic procedures have also been given in detail in previous studies[5,7,8]. Gradient bandwidth parameters ($\sigma_{v,calc}$, $\sigma_{v,exp}/\sigma_{v,calc}$, $G$, $C$, $B$, $D_m$ and $N$) were calculated using the Sigma programme written in this laboratory in Basic language for IBM AT computers. The input values required for this programme were $T$, $\eta$, $t_o$, MW, $d_p$, $a'$, log $C_f/C_o$, $F$, $Z_c$, $b$, $t_G$ and $\sigma_{v,exp}$.

## RESULTS AND DISCUSSION

### *Theoretical gradient bandwidth relationships*

The general plate-height theory provides a basis for assessing the diffusional behaviour of small molecules in chromatographic systems. Snyder and co-workers[3,10] have extended the use of retention parameters derived from the LSS model[3] in conjunction with the plate-height theory to provide a method for predicting solute bandwidths so that overall chromatographic resolution can be evaluated and optimised. Using this elegant method of approach, reversed-phase high-performance liquid chromatographic (HPLC) studies (see for example ref. 4) with small peptides and some small proteins have shown good correlations between the experimentally and theoretically derived solute bandwidths. Recently, this approach has been adapted by Stout *et al.*[10] for the analysis of high-performance ion-exchange bandbroadening data. In these studies, good correlations were observed between the experimental and calculated bandwidths for lysozyme and ribonuclease A eluted using sodium acetate as the displacer salt with the DuPont Zorbax Bio-Series SCX, WCX, SAX and WAX strong and weak cation and anion exchangers.

While different salts have been known for many years to strongly influence protein retention in HPTEC, their influence on protein bandwidths has not generally been as fully assessed. The present paper specifically addresses the important issue of choice of monovalent salts. Associated studies to be reported from this laboratory have also examined the effect of di- and polyvalent ions on bandbroadening of proteins in HPIEC. Under ideal HPIEC conditions of gradient elution, the relationship between peak width, represented by $4\sigma_v$, and the median capacity factor, $\bar{k}$, for linear solvent systems can be expressed[3] as

$$\sigma_{v,calc} = [(k/2) + 1]\, G V_m N^{-1/2} \quad (1)$$

where $V_m$ is the column void volume ($V_m = t_o F$, $t_o$ is the retention time of an unretained compound, $F$ is the flow-rate), $N$ is the plate number and G is the band-compression factor[3,10] which arises from the increase in solvent strength across the solute zone as the gradient develops along the column. The parameter G is given by the expression

$$G^2 = [1 + 2.3b + 1/3(2.3b)^2]/(1 + 2.3b)^2 \quad (2)$$

where $b$ is the gradient steepness parameter. Under how-rate conditions typically used in gradient elution HPLC, $N$ can be approximated to

$$N = D_m t_o / C d_p^2 \quad (3)$$

where $d_p$ is the particle diameter. The diffusion coefficient, $D_m$, of the solute in the mobile phase can be expressed in terms of solute molecular weight (MW) as

$$D_m = 8.34 \cdot 10^{-10} T / \eta\, (MW)^{1/3} \quad (4)$$

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

$$c = \frac{[(1 - x + \bar{k}) / (1 + \bar{k})]^2}{15\rho^* a' + 15\rho^* b' \bar{k} - 19.2\rho^* x} \quad (5)$$

where x is the interstitial column fraction, found to be 0.62 for the Mono-Q anion exchange sorbent in well packed columns'*, a' is assumed to equal 1.1, and the term $b'$ is the surface diffusion parameter, calculated from the relationship

$$B = a' + b'k \quad (6)$$

The Knox equation constant, $B$, which arises from zonal dispersion due to longitudinal diffusion, was previously determined' in this laboratory from isocratic bandwidth data at different flow-rates and substituted into eqn. 6 to obtain a value of $b'$ equal to

0.72. The restricted diffusion parameter, $\rho^*$, a molecular-weight-dependent term, was calculated by using the Renkin relationship so that

$$\rho^* = 1 - 2.104\rho + 2.09\rho^3 - 0.95\rho^5 \quad (7)$$

where $\rho$ is equal to the ratio of the solute diameter to the sorbent pore diameter ($s_d/p_d$). The linear logarithmic relationship (correlation coefficient, $r^2 = 0.99$) found[9] experimentally between $\rho^*$ 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 daltons was

$$\log \rho^* = 0.19 - 0.06 \log \mathrm{MW} \quad (8)$$

The use of the general plate height theory to derive the above bandwidth relationships, and in particular eqns. 1–5, assumes that the solute migrates as a unique, conformationally rigid molecule. With a particular sorbent, fixed salt and defined set of elution conditions the influences of secondary multimodel retention processes due to the interplay of coulombic and hydrophobic effects on bandbroadening are anticipated to make essentially constant contributions to $\sigma_v$ over the linear operational range of $\bar{k}$ values. In addition, it is generally accepted that peptides and proteins can explore a variety of conformations in solution. These secondary equilibria processes can also be further enhanced or inhibited by the presence of either chaotropic or kosmotropic salts in the chromatographic eluent. If it is assumed that these conformational processes or any additional secondary phenomena, such as monomer–oligomer, or subunit association–dissociation, are extremely rapid compared to the chromatographic separation time, then the ratio between the experimentally observed bandwidth $\sigma_{v,exp}$ and the bandwidth $\sigma_{v,calc}$, determined by eqn. 1, should approach unity over the normal operational range or retention values commonly used in optimisation studies, *i.e.* $1 < \bar{k} < 10$. Similar considerations will also apply when these secondary processes are relatively slow with regard to chromatographic mass transfer. Under these conditions, the protein or peptide solute will effectively migrate as a single time averaged conformer. Such migratory behaviour can be considered "ideal", although divergence from the small molecule plate theory can still be anticipated due to differences in the hydrodynamic shape of various biosolutes, *e.g.* differences arising between globular and ellipsoidal proteins in terms of their intrinsic radii of gyration and molecular weights and their consequences for the calculation of effective $D_m$ values (*cf.* eqn. 5).

However, if time-dependent, mobile or stationary phase induced changes in the secondary, tertiary or quaternary structure of the protein or peptide solute occur with similar time scales as the chromatographic migration, the resulting changes in the diffusional and interactive properties of the solute will lead to a pattern of differential zone migration which is more complex than that anticipated by conventional plate height theory. Two cases of such complex bandbroadening behaviour can be especially identified. The first relates to significant alteration in the molecular dimensions of the peptide or protein solute in the bulk mobile phase due to specific ion interactions affecting the solvated structure, *e.g.* congruent and non-congruent mobile phase unfolding–refolding processes which also have similar half-lives to the separation trans-

port time. The second case relates to changes in the shape and surface topography of the solute as it binds, re-orientates or subsequently desorbs from the sorbent surface, *e.g.* stationary phase induced processes where the apparent time for interconversion is similar to the mass transfer time. These types of kinetic changes in the macroscopic properties of the biosolute will ultimately be revealed as experimental bandwidths which deviate from the values predicted by eqn. 1 with $\sigma_{v,exp}/\sigma_{v,calc}$ becoming significantly greater than unity.

*The influence of displacer salt type on solute bandwidth behaviour under conditions of varied gradient time and constant flow-rate*

It is well known that many characteristics of the mobile phase can be altered to influence protein retention behaviour in HPIEC[1–10,12–14]. However, there have been relatively few systematic investigations into the effects of the mobile phase composition on protein bandwidth behaviour. Such studies are of importance for the optimisation of sample resolution as well as the preservation of the native structure of the biosolute through minimisation of adverse mobile or stationary phase induced secondary equilibria.

Recently, the LSS theory has been utilised to analyse bandwidth data obtained for several proteins eluted with sodium chloride from a Mono-Q strong anion-exchange column under conditions of varied gradient time and constant flow-rate[9]. In this earlier investigation, variation in the rate of change of the displacer salt, associated with differences in gradient time, was found to dramatically affect the kinetic processes for several protein solutes, *e.g.* ovalbumin, bovine carbonic anhydrase and sperm whale myoglobin. In particular, increased column residence times resulted in significant deviations from the calculated bandwidths, obtained using eqns. 1–8. The divergencies from the predicted peak width were attributed to solute-specific physicochemical phenomena, (the so-called column "dwell" effect) associated with solute solvation or changes in the approach depth of the penetration of the protein at or near the Stern double layer.

In the present study, the effect of systematic changes in the displacer salt composition on protein bandwidth behaviour has been investigated under elution conditions of varied gradient time and constant flow-rate. The proteins listed in Table I were eluted from a Mono-Q strong anion-exchange column by salt gradients (0 to 300 m*M*) varying in time from 8.6 to 171.4 min at a constant flow-rate of 1 ml/min. Eluents A (20 m*M* piperazine) and B (20 m*M* piperazine and 300 m*M* displacer salt)

TABLE I

PROTEIN PHYSICAL PROPERTIES

| *Protein* | *MW* | $D_m$[a] *(m²/min)* |
|---|---|---|
| Ovalbumin | 43 000 | 4.24 |
| Carbonic anhydrase | 30 000 | 4.80 |
| Myoglobin | 17 500 | 5.74 |
| Lysozyme | 14 300 | 6.12 |

[a] Calculated from eqn. 5.

were adjusted to pH 9.6 by the addition of either HF, HCl or HBr as appropriate for the salt type. Theoretical bandwidth values were calculated, using eqn. 1, over the range of experimental conditions used to elute each protein solute. These values were then compared to the corresponding experimental bandwidths and plotted as a function of the reciprocal of the gradient steepness parameter, $b$.

Figs. 1–6 show plots of $\sigma_{v,exp}/\sigma_{v,calc}$ as a function of $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme eluted with LiCl, LiBr, NaF, NaCl, NaBr, KF, KCl and KBr. These figures reveal that each salt type influences protein mass transfer in a unique manner, with the largest deviations from unity for the $\sigma_{v,exp}/\sigma_{v,calc}$ ratio occuring with decreasing $b$ values or increased column residence times. At higher $b$ values, associated with relatively large rates of change in the displacer salt (*i.e.* 17.5 and 35 m$M$/min) and decreased residence times, the $\sigma_{v,exp}/\sigma_{v,calc}$ ratios were generally found to approach unity as the participation of secondary equilibria effects were reduced. In some instances, for example, carbonic anhydrase eluted with NaF or KCl (Figs. 2a and 3a) or ovalbumin eluted with NaF or KF (Figs. 2c and 3c) the $\sigma_{v,exp}/\sigma_{v,calc}$ values initially decrease with decreasing $b$ values then passed through a minimum at or near unity before increasing again with smaller $b$ values.

The bandwidth ratios for lysozyme shown in Figs. 1–6d were much larger than those observed for the other three proteins evaluated in this study. These values were in the order of magnitude of $5 \leqslant \sigma_{v,exp}/\sigma_{v,calc} \leqslant 60$. Currently, LSS theory has no

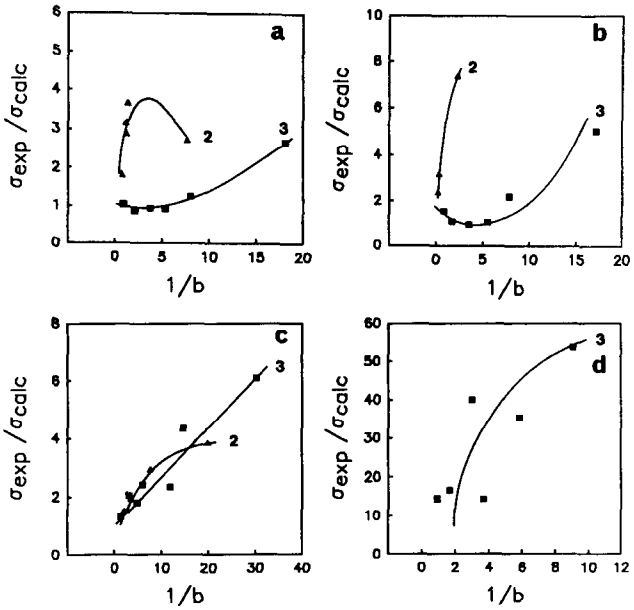


Fig. 1. The influence of anion type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the anions of lithium salts where ▲ or 2 = LiCl and ■ or 3 = LiBr. Data were acquired under conditions of varied gradient time at a flow-rate of 1 ml/min as described in the Materials and Methods section, $\sigma_{v,calc}$ was evaluated using eqn. 1.

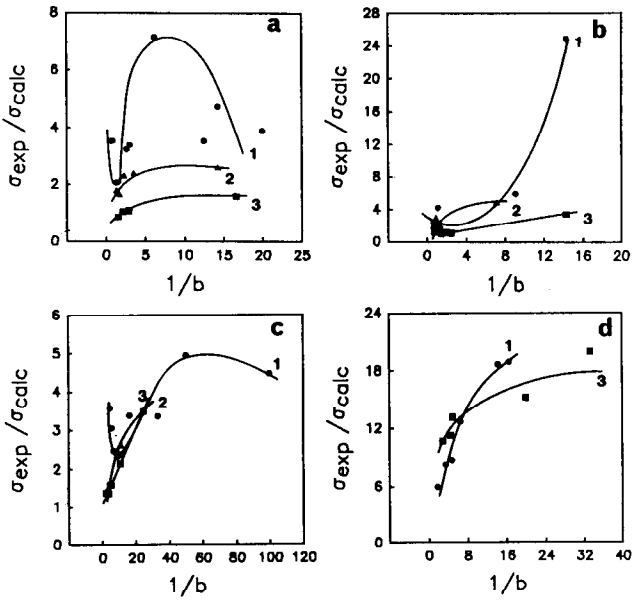


Fig. 2. The influence of anion type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the anions of sodium salts where ● or 1 = NaF, ▲ or 2 = NaCl and ■ or 3 = NaBr. See Fig. 1 for other details.

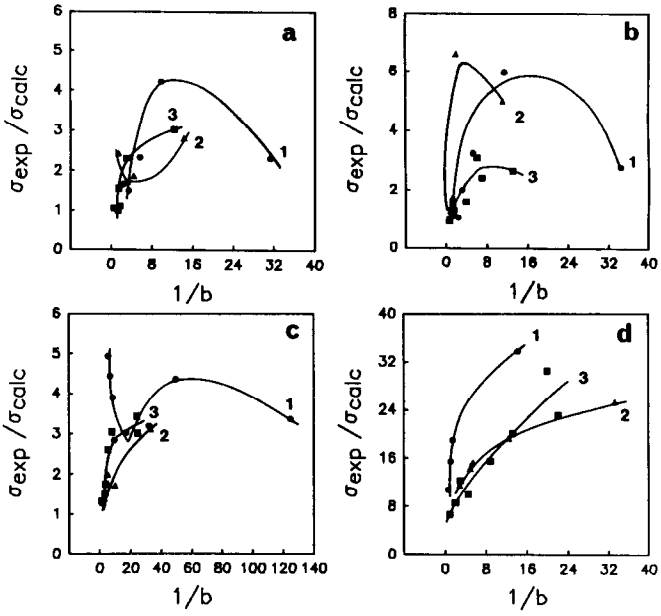


Fig. 3. The influence of anion type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the anions of potassium salts where ● or 1 = KF, ▲ or 2 = KCl and ■ or 3 = KBr. See Fig. 1 for other details.

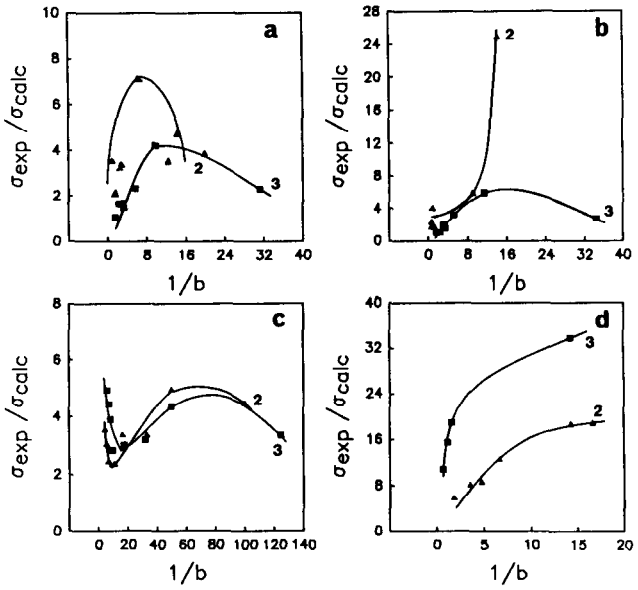


Fig. 4. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of fluoride salts where ▲ or 2 = NaF and ■ or 3 = KF. See Fig. 1 for other details.

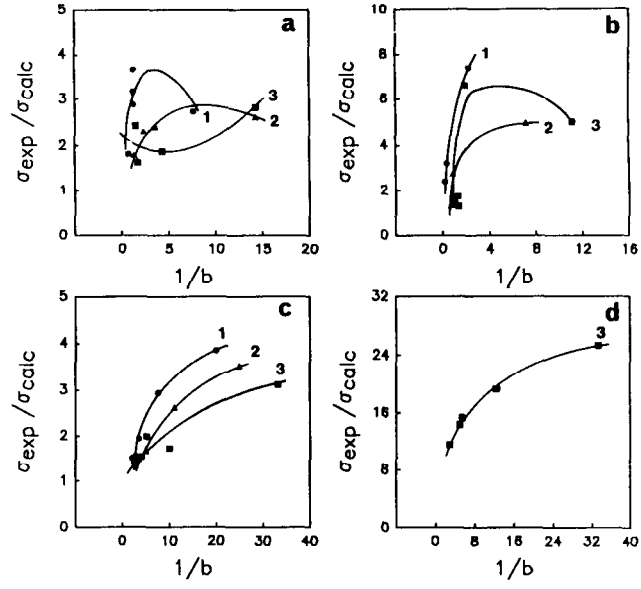


Fig. 5. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of bromide salts where ● or 1 = LiBr, ▲ or 2 = NaBr and ■ or 3 = KBr. See Fig. 1 for other details.

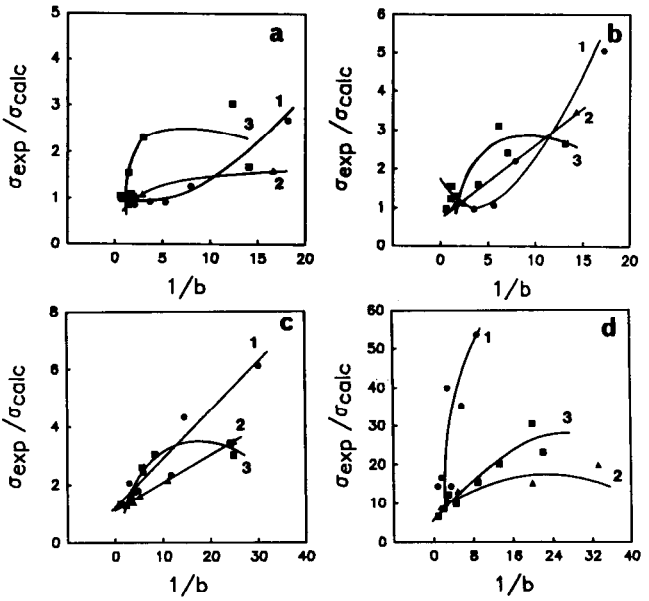


Fig. 6. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of bromide salts where ● or 1 = LiCl, ▲ or 2 = NaCl and ■ or 3 = KCl. See Fig. 1 for other details.

provision to account for secondary equilibrium processes such as the hinge motion of the lysozyme domains[15,16] or the dimerisation/polymerisation of lysozyme reported[17–19] to occur at high pH values. Participation of these effects would result in the predicted bandwidth values being much smaller than the experimental bandwidths due to the errors in the calculation of, for example, the diffusion coefficient, $D_m$ and the Knox parameter $C$.

The data in Figs. 1–6 demonstrate that changes in both the anion or cation species of the displacer salt can dramatically influence protein bandwidths under otherwise identical settings of chromatographic parameters. These data demonstrate that the selection of the most appropriate displacer salt to minimise secondary equilibria effects is clearly essential if optimisation of the resolution behaviour of proteins is to be achieved. For example, salt systems could be empirically selected which provide apparently equivalent behaviour under one set of chromatographic parameters, *e.g.* gradient time, gradient shape, etc., but which generate dramatically different resolution behaviour under another set. This type of behaviour is demonstrated in the band broadening effects of the displacer anion type, *i.e.* $F^-$ to $Cl^-$ to $Br^-$, with the selected proteins. Under the elution conditions used with high $b$ values, the plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for the various proteins were frequently coincidental with no apparent preference for a particular salt system evident. However, when small $b$ values or increased column residence times were used, salt specific trends in protein bandwidth behaviour emerged. For example, it was found for the anion series with the lithium, sodium and potassium salts that the bandwidth ratios for each protein

increased in the order $Br^- < Cl^- < F^-$. Systematic changes in the cation species as the fluoride, chloride and bromide salts also influenced protein bandwidth ratios, which increased in the order $K^+ < Na^+ < Li^+$. These results also indicate that the influence of the anion or cation series on the $\sigma_{v,exp}/\sigma_{v,calc}$ ratio was independent of the type of counter ion species present in the displacer salt.

These dependencies of $\sigma_v$ on salt type can be contrasted to the results observed for anion and cation effects on the value of the slope term $Z_c$ derived from plots of log $k'$ *versus* log $1/c$ (where $c$ = displacer salt concentration) for the same proteins reported previously[6,7]. The effects of various combinations of anions and cations on $Z_c$ values were found to be additive in terms of their position in the lyotropic series. Significant changes in $Z_c$ represent structural variations in the chemical composition or charge density of the solute binding site(s) [the ionotope(s)]. Thus, the two fundamental chromatographic parameters, namely average retention or $k'$, and peak variance $\sigma_v^2$, which reflect changes in the thermodynamic and kinetic properties of the ionotopic structure of the solute during column migration respond differently to the type of anion and cation species present in the displacing salt.

*The influence of displacer salt type on solute bandwidth behaviour under conditions of varied flow-rate and constant gradient time*

As evident from the preceding data, longer gradient times promoted experimental bandwidths which were larger than predicted by eqn. 1. Inspection of the same

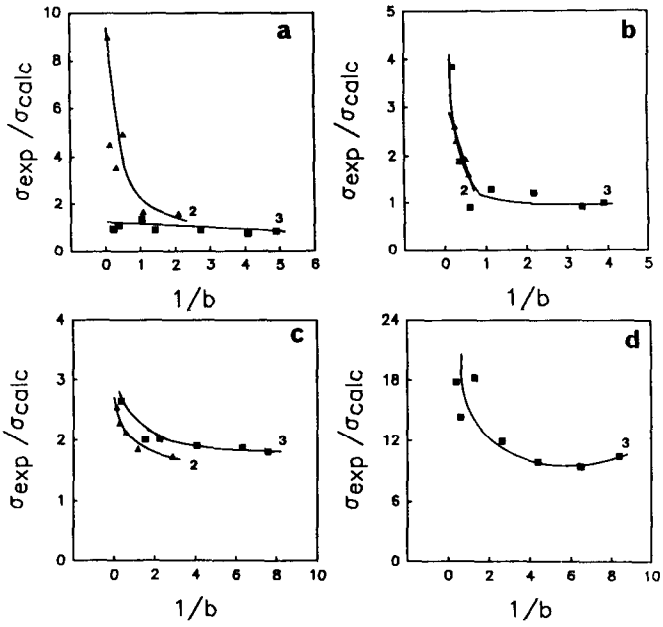


Fig. 7. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of lithium salts where ▲ or 2 = LiCl and ■ or 3 = LiBr. Data were acquired under conditions of varied flow-rate at a gradient time of 17.1 min as described in the Materials and Methods section. $\sigma_{v,calc}$ was evaluated using eqn. 1.

data also revealed that peak broadening, presumably due to secondary equilibria effects influencing protein diffusional properties, could be minimised by reducing the column residence time, such that the gradient time ($t_G$) < 20 min. To gain further insight into this phenomenon, the influence of displacer salt type on protein bandwidth behaviour was investigated using varied flow-rate and constant gradient time conditions with $t_G$ set at 17.1 min.

Figs. 7–12 show comparisons of the ratio of experimental solute bandwidth, $\sigma_{v,exp}$, to the predicted solute bandwidth, $\sigma_{v,calc}$, as a function of the reciprocal of the gradient steepness parameter, $b$. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ were found to decrease with increasing flow-rate or decreasing $b$ values. With myoglobin, carbonic anhydrase and ovalbumin these curves generally approached unity as the flow-rates became $\geqslant$ 0.5 ml/min. The bandwidth curves for lysozyme were an exception to this observation. Figs. 7–12d show bandwidth ratios in the order of $5 < \sigma_{v,exp}/\sigma_{v,calc} < 20$ for lysozyme eluted with various salts. The known[15–19] polymerisation or hinge motion of lysozyme at high pH values could account for this behaviour and significantly influence the diffusional properties of this protein resulting in larger than predicted experimental bandwidths. These observations are consistent with those in the previous section and further exemplify the inability of the plate theory of small molecules to accommodate for these types of secondary equilibria with proteins such as lysozyme.

Large $\sigma_{v,exp}/\sigma_{v,calc}$ ratios were also observed for ovalbumin eluted with either NaF or KF (Figs. 8c, 9c and 10c). The poor displacing ability of these fluoride salts results in very small $b$ values and the slow desorption kinetics. Consequently, oval-

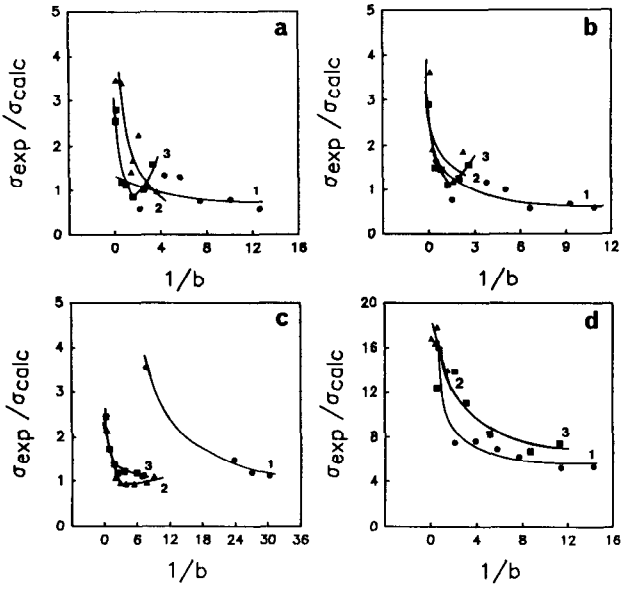


Fig. 8. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of sodium salts where ● or 1 = NaF, ▲ or 2 = NaCl and ■ or 3 = NaBr. See Fig. 7 for other details.

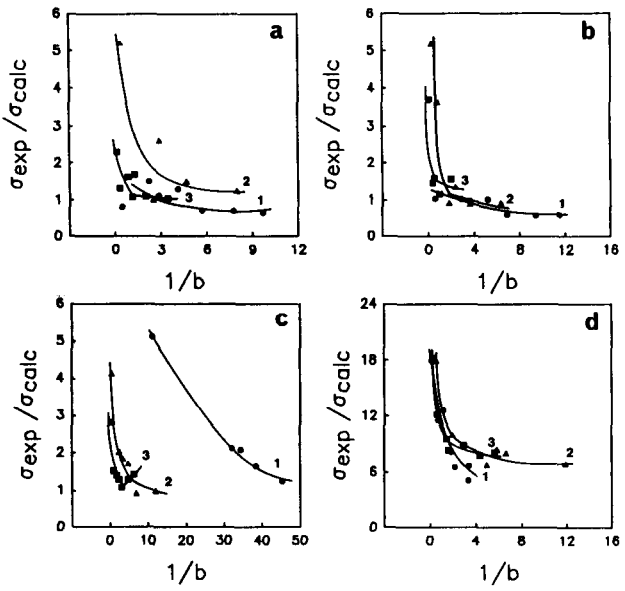


Fig. 9. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of potassium salts where ● or 1 = KF, ▲ or 2 = KCl and ■ or 3 = KBr. See Fig. 7 for other details.

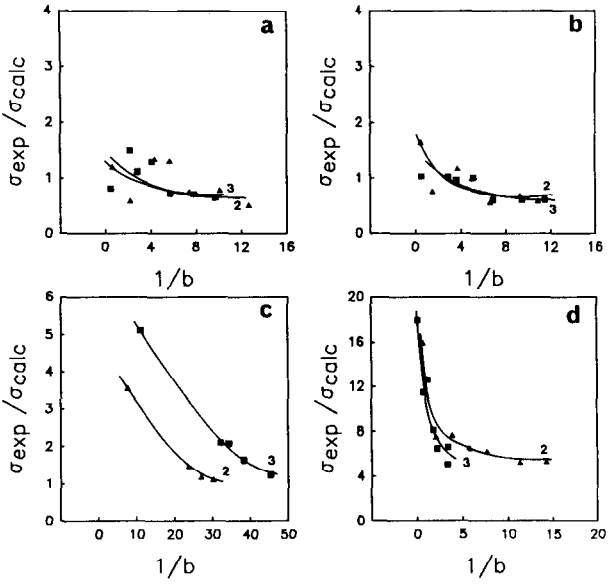


Fig. 10. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of fluoride salts where ▲ or 2 = NaF and ■ or 3 = KF. See Fig. 7 for other details.

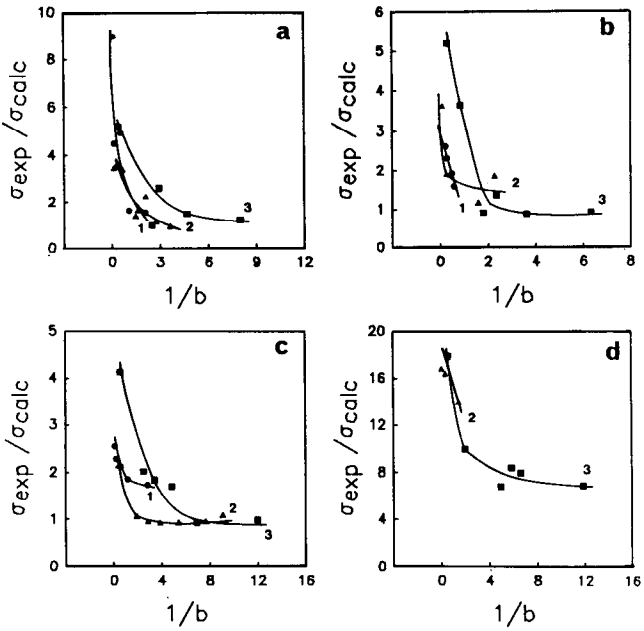


Fig. 11. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of chloride salts where ● or 1 = LiCl, ▲ or 2 = NaCl and ■ or 3 = KCl. See Fig. 7 for other details.

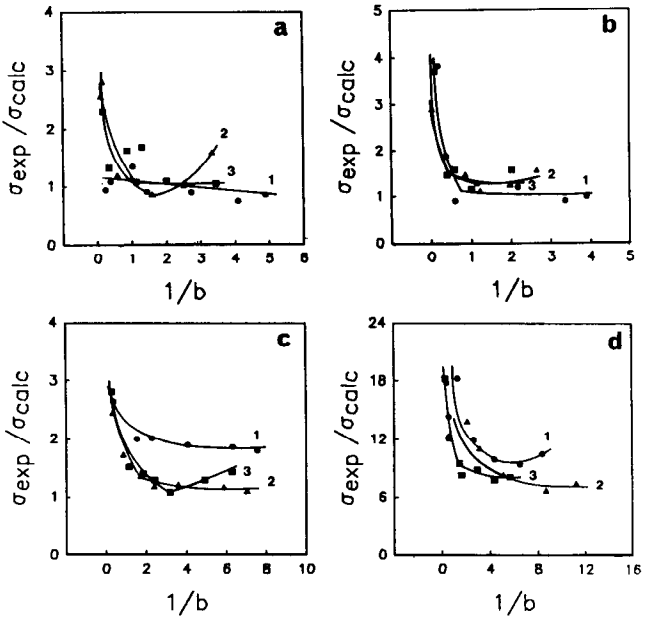


Fig. 12. The influence of cation type on the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for (a) carbonic anhydrase, (b) myoglobin, (c) ovalbumin and (d) lysozyme. Bandwidth data were derived as a result of systematic changes in the cations of bromide salts where ● or 1 = LiBr, ▲ or 2 = NaBr and ■ or 3 = KBr. See Fig. 7 for other details.

bumin bandwidth data for the NaF and KF salts are displaced to the right, causing increased $\sigma_{v,exp}/\sigma_{v,calc}$ values over the normal $1/b$ range, *i.e.*, $0 < 1/b < 10$.

*Influence of different salts on the J effect*

In RP-HPLC systems, the initial decrease in a solute's $\sigma_{v,exp}/\sigma_{v,calc}$ ratio at large $b$ values has been referred to as the "$J$ effect"[3,4]. Solutes eluted by steep gradients have reduced column residence times and their elution behaviour is therefore influenced predominantly by mobile phase parameters. Differential zone migration resulting from this occurrence is believed to generate this effect. To date, very little information has been reported[8–10] on the occurence or significance of this phenomenon in HPIEC systems. For many of the protein–salt systems examined in this study most bandwidth curves were observed to increase at high $b$ values. Eqn. 9 shows that the calculation of the $J$ value, as derived by Stadalius *et al.*[4], is dependent solely upon the gradient steepness parameter such that

$$J = 0.99 + 1.70b - 1.35b^2 + 0.48b^3 - 0.062b^4 \quad (9)$$

The origin of the phenomenon, numerically described by the $J$ value, has not been fully elucidated. The $J$ effect, however, is thought to arise from non-LSS gradient conditions associated with steep gradients and the increasingly important effect of the gradient elapse time, $t_e$, at low flow-rates resulting in almost stepwise elution[20–22]. The experimental bandwidth data were consequently used to further investigate anomalous bandbroadening at high $b$ values.

As illustrated in Fig. 9b, a change in displacer salt from KF to KCl or KBr generates different $J$ effects for myoglobin. Similarly in Fig. 7a there is a large difference in the $\sigma_{v,exp}/\sigma_{v,calc}$ ratio for carbonic anhydrase eluted with LiCl and LiBr. Examples such as these demonstrate that the $J$ effect is influenced by the mobile phase composition. With low flow-rates and small gradient times, protein solutes will spend relatively longer periods in the bulk mobile phase than orientated at the stationary phase surface. Therefore, the $J$ effect appears to reflect mobile phase compositional effects, as opposed to stationary phase–double layer effects on the solute's bandbroadening behaviour.

When zone broadening occurs with increasing $b$, the $J$ value can be used to numerically compensate for the deviation of $\sigma_{v,exp}/\sigma_{v,calc}$ from unity[22]. Initial studies by Stout *et al.*[10] with HPIEC using sodium acetate to elute several proteins from a strong cation exchanger have shown good correlation between experimental and predicted bandwidths calculated from eqn. 1 compensated with a $J$ value derived from eqn. 9. However, the utility of eqn. 9 has yet to be extensively verified over a range of IEC elution conditions. Theoretically, eqn. 9 permits bandwidth ratios which deviate from unity to be adjusted by a particular $J$ factor which is solely dependent on the $b$ value and independent of the type of displacer salt. For example, at $b = 0.5$ eqn. 9 predicts the $J$ value will have a magnitude of $J = 1.6$. When the $b$ value is $b = 2.0$ the $J$ value will increase to $J = 1.8$, *i.e.* over a typical range of slope conditions for gradient elution of $0.5 < b < 2$, the $J$ values will range between 1.6 and 1.8. Table II was derived from data in Figs. 7–11 and shows the effect of displacer salt type on protein bandwidth ratios at $b = 0.5$ and $b = 2.0$. Inspection of the data in Table II reveals that the $J$ factor required to normalise the experimental bandwidth ratios to a value equal

TABLE II

BANDWIDTH RATIOS ($\sigma_{v,exp}/\sigma_{v,calc}$) AT SELECTED $b$ VALUES

Using eqn. 9: $J_{b=0.5} = 1.6$ and $J_{b=2.0} = 1.8$.

| *Protein* | *b Value* | $\sigma_{v,exp}/\sigma_{v,calc}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Displacer salt* | | | | | | | |
| | | *LiCl* | *LiBr* | *NaF* | *NaCl* | *NaBr* | *KF* | *KCl* | *KBr* |
| Carbonic anhydrase | 2.0 | 3.5 | 1.1 | 1.2 | 3.6 | 1.4 | 1.4 | 5.1 | 1.7 |
| | 0.5 | 1.2 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 | 1.6 | 1.0 |
| Ovalbumin | 2.0 | 2.1 | 2.5 | >5 | 2.0 | 2.7 | >5 | 3.3 | 4.1 |
| | 0.5 | 1.7 | 1.8 | >5 | 1.0 | 1.2 | >5 | 1.7 | 1.1 |
| Myoglobin | 2.0 | 1.8 | 1.3 | 1.7 | 1.9 | 1.5 | 1.4 | 4.9 | 1.5 |
| | 0.5 | NE[a] | 1.0 | 1.0 | 1.5 | 1.2 | 1.0 | 1.0 | 1.5 |
| Lysozyme | 2.0 | ND[a] | 20.0 | >15 | 16.3 | >15 | 12.5 | 18.0 | 12.5 |
| | 0.5 | ND[a] | 9.9 | 7.7 | 12 | 9.6 | 5.6 | 8.4 | 8.1 |

[a] ND = Not determined; NE = solute not eluted at this $b$ value.

to one would vary significantly from those predicted by eqn. 9 for each value of $b$. The data for each protein in Table II indicates that salt-induced secondary equilibria can influence protein diffusional behaviour at higher $b$ values. Provisions for these secondary equilibria have yet to be incorporated into eqn. 9 and are clearly required in order to permit such empirical adjustment routines to find general applicability for characterising protein bandbroadening in high-performance anion-exchange systems.

## CONCLUSION

This study has shown that the bandbroadening behaviour of proteins in HPIEC can be regulated by the nature of the displacing salt in addition to other operating parameters such as the gradient time or flow-rate. The effect of displacer anions and cations on the magnitude of protein bandwidths was found to be dependent upon the relative position of the ions in the lyotropic series.

Increased column residence times associated with longer gradient times were found to lead to aberrant bandbroadening presumably as a consequence of secondary equilibria effects on protein structure. These results demonstrate that the associated decrease in mass transfer efficiency can be eliminated, by selecting experimental elution conditions which reduced the solute residence time at the stationary phase surface.

Anomalous bandbroadening was also observed at high $b$ values. The $J$ value derived from LSS theory, was unable to satisfactorily account for such HPIEC bandwidth behaviour. However, the $J$ effect was demonstrated to be influenced by the salt composition in a solute specific manner. This information clearly has a bearing on the selection of different salts in the optimisation of preparative HPIEC separations which often incorporate steep gradient or step-wise elution conditions.

Overall, this study demonstrates that mathematical models currently used to

describe bandbroadening behaviour require further development to incorporate the influence of mobile phase composition, stationary phase type and solute solvation effects on protein kinetic behaviour in HPIEC. Experimental parameters defining the origin of such complex phenomena need to be established before current models can be adapted to achieve general predictive applications. This will only occur as additional systematic studies exploring the mechanistic basis linking solute structure to chromatographic behaviour, find their way into the scientific literature.

ACKNOWLEDGEMENTS

The support research grants to M.T.W.H. from the National Health and Medical Research Council of Australia, Australian Research Grants Committee and Monash University Special Research fund are gratefully acknowledged.

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