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# INTERPLAY OF HYDROPHOBIC AND ELECTROSTATIC INTERACTIONS IN BIOPOLYMER CHROMATOGRAPHY

## EFFECT OF SALTS ON THE RETENTION OF PROTEINS[a]

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SUMMARY

The effect of salt on the retention behavior of proteins in electrostatic and hydrophobic interaction chromatography is described by a three-parameter equation, $\log k' = A - B \log m_s + Cm_s$, where $k'$ is the retention factor and $m_s$ is the molality of the salt in the eluent. Parameter $B$, termed the electrostatic interaction parameter, depends on the characteristic charge of the protein and the salt counterion and governs the change of retention with the salt concentration in ion-exchange chromatography. According to the model the magnitude of the hydrophobic interaction parameter $C$ is determined by the hydrophobic contact area upon protein binding at the stationary phase surface and the properties of the salt as measured by its molal surface tension increment. Retention data measured at different salt concentrations in the eluent on a variety of ion exchangers can be fitted to the above equation which yields U-shaped plots of $\log k'$ against $\log m_s$. The limiting slopes of the appropriate plots at sufficiently low and high salt concentrations can be used to evaluate the electrostatic and hydrophobic interaction parameters, respectively. The approach, which is based on a combination of established treatments of electrostatic and hydrophobic interactions offers a convenient framework for analyzing retention data in biopolymer high-performance liquid chromatography and for the characterization of stationary phases. Furthermore, it may facilitate some characterization of protein molecules on the basis of their retention behavior as a function of the concentration and nature of the salt in the eluent.

In the treatment of electrostatic interactions use is made of the counterion condensation theory that is believed to make possible a more comprehensive analysis

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than the traditional stoichiometric ion-exchange model which assumes binding of the proteins by coulombic interactions at discrete sites. The treatment of hydrophobic interactions is based on an adaptation of the solvophobic theory which predicts that the hydrophobic portion of the free energy of binding is proportional to the hydrophobic contact area and the microthermodynamic surface tension of the aqueous salt solution. Despite its simplicity the theory was successful in explaining the observed effect of the nature and concentration of salt in the eluent, the pH and the effect of the density of fixed charges at the surface of the stationary phase in the absence of specific salt effects.

## INTRODUCTION

Electrostatic interaction (ion-exchange) and hydrophobic interaction chromatography (HIC) with high-performance columns and equipment are emerging as major branches of biopolymer chromatography both on an analytical and preparative scale. As the nature and concentration of the salt in the eluent is the basic retention modulator in both types of chromatography, the effect of salt is of fundamental significance. Since in many instances both types of interactions are involved in the retention process it is of interest to develop a comprehensive treatment of the underlying physico-chemical phenomena. In this work we attempt to account for the salt effect on electrostatic and hydrophobic interactions responsible for the retention of biopolymeric eluites.

The classical stoichiometric theory of salt elution in ion-exchange chromatography of biopolymers was developed by Boardman and Partridge[1] and a more detailed treatment can be found in the monograph of Morris and Morris[2]. Recently the theory was expanded by Regnier and co-workers[3,4] who presented retention data obtained from measurements by using high-performance liquid chromatography (HPLC). A major shortcoming of the stoichiometric theories, however, is their failure to consider explicitly stationary phase properties and the implications of hydrophobic interactions which may occur concomitantly with electrostatic interactions. The "ion-exchange" model assumes that the polyion is site bound, and its popularity stems from the simplicity of the appurtenant stoichiometric treatment. Moreover, no other models are currently available because of the lacuna in applying modern polyelectrolyte theories to such complex systems.

Another way of looking at the problem is to assume that both the salt and polyionic eluite are territorially or atmospherically bound, *i.e.*, they are retained by the electrostatic field at the stationary phase surface, but remain free to move within a certain layer above it. In such a case, the biopolymeric eluite is expected to experience little change in hydration upon transfer from the bulk mobile phase to the stationary phase domain. A modified Poisson–Boltzmann theory or its descendant, the Gouy double-layer theory, could be of interest here for treatment of electrostatic interactions. We have chosen instead to adopt Manning's counterion condensation theory because of the analytical solutions it has provided[5–10] and its experimental verification[11,12]. For the treatment of hydrophobic interactions we have employed our earlier adaptation of Sinanoglu's[13] solvophobic theory to the salting-out of proteins and their retention in hydrophobic interaction chromatography[14,15]. The three-parameter equation that we obtain by combining the electrostatic and hydrophobic theory offers

a means to interpret the physico-chemical phenomena underlying the retention process and a method to characterize the properties of the stationary phase. In this paper, this approach is used to analyze isocratic retention data obtained with siliceous ion exchangers having a "soft" surface composed of a highly hydrated polymeric layer in a broad range of salt concentration in the eluent.

## THEORY

### *Simplified model of retention*

A simplified model is proposed to describe the effect of salt concentration on retention in biopolymer chromatography with stationary phases which have a "soft", highly hydrated surface with fixed charges or weakly hydrophobic binding sites or both. It is based on the following assumptions. (i) The dimensions of the smooth walled pores of the rigid support are large with respect to the size of the eluite molecules so that pores can be approximated by cylinders of infinite radius and size-exclusion effects are absent. (ii) The fixed charges and hydrophobic binding sites are uniformly spaced and equi-accessible in the hydrated stationary phase layer at the pore wall. (iii) The biopolymer eluites are spherical molecules with uniformly distributed and equi-accessible fixed charges and hydrophobic patches at the surface. (iv) The conformation of the biopolymer molecule upon transfer between the two phases and interaction with the functional groups in the stationary phase domain is conserved. (v) A single valued phase ratio expresses the ratio of the volume occupied by the fixed charges and hydrophobic binding sites, and the volume of the mobile phase in the column. (vi) Conditions of linear elution chromatography prevail, *i.e.*, only a small fraction of the binding domains are occupied by the eluite. (vii) No specific interactions of the eluting salt with the biopolymer have to be considered.

The magnitude of the retention when both electrostatic and hydrophobic interactions are involved is determined by the sum of the free energies for the equilibrium distribution of the biomacromolecular eluite between the bulk mobile phase and the stationary phase domains. The corresponding equilibrium constant, $K$, can be written formally as

$$\log K = (-\Delta G^{\circ}_{es}/2.3RT) - (\Delta G^{\circ}_{h\phi}/2.3RT) \tag{1}$$

where $\Delta G^{\circ}_{es}$ and $\Delta G^{0}_{h\phi}$ are the Gibbs free energies for retention by electrostatic and hydrophobic interactions, respectively, $T$ is the absolute temperature and $R$ is the universal gas constant.

We assume that the retention factor, $k'$, which can be directly measured from the chromatogram, is related to $K$ as

$$k' = \phi K \tag{2}$$

where $\phi$ is the phase ratio, *i.e.*, the ratio of the two appurtenant domains of the stationary and mobile phases.

### *Electrostatic interactions between biopolymers and the stationary phase*

Recent developments in polyelectrolyte theory, particularly the counterion

condensation theory of Manning[5–10] as well as Record and co-workers[11,12] provides the basis for our analysis of the pertinent retention thermodynamics in electrostatic interaction chromatography. Although the counterion condensation theory is particularly appropriate to linear polyelectrolytes with discrete charges, we assume that it is applicable to electrostatic interaction chromatography with stationary phases which contain wide cylindrical pores and an array of fixed charge bound via the organic moiety to the pore walls[16–18]. The validity of this assumption is discussed later.

A detailed description of the counterion condensation theory is found in Manning's review[5]. In his treatment the charged surface is characterized by a dimensionless structural parameter $\xi$, that is given by

$$\xi = q^2/\varepsilon b k_B T \tag{3}$$

where $q$ is the protonic charge, $\varepsilon$ is the dielectric constant of the bulk mobile phase, $b$ is is the average spacing of fixed charges on the surface and $k_B$ is Boltzmann's constant. It is assumed that the polyionic protein having a characteristic charge of $Z_p$ is

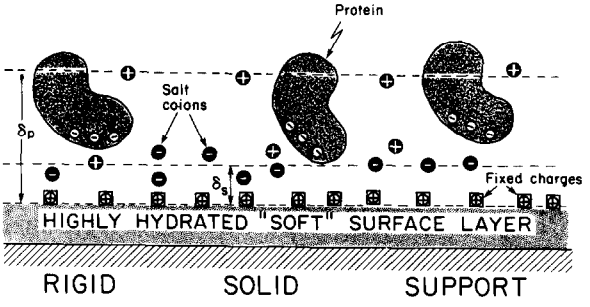


Fig. 1. Schematic illustration of atmospheric binding of proteins in view of the counterion condensation theory. The thickness of the layers containing the condensed salt counterions and the bound protein molecules is given by $\delta_s$ and $\delta_p$, respectively. The protein, as illustrated, carries a characteristic charge of $-3$.

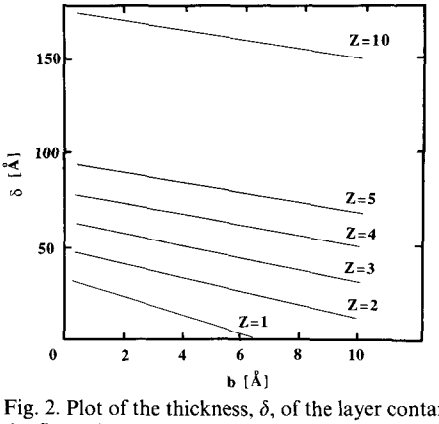


Fig. 2. Plot of the thickness, $\delta$, of the layer containing condensed counterions against the distance between the fixed charges at the surface, $b$, with the charge on the counterion, $Z$, as the parameter. The following values were used in the calculation: $q = 4.8 \cdot 10^{-10}$ e.s.u.; $\varepsilon = 80$; $k_B T = 4.11 \cdot 10^{-14}$ ergs.

atmospherically bound and found in a "condensation" layer having a thickness of $\delta_p$ over the surface of the stationary phase where each fixed charge occupies an area of $b^2$. For our case, the treatment by Manning[5] has been adapted to evaluate the relationship:

$$\delta_p = be(1 + Z_p)(\xi - Z_p^{-1}) \tag{4a}$$

where e is the base of the natural logarithm.

The corresponding layer thickness for salt counter-ions having a valence of $Z_s$ is given by

$$\delta_s = be(1 + Z_s)(\xi - Z_s^{-1}) \tag{4b}$$

The free energy of binding of the polyionic eluite to the oppositely charged surface of the stationary phase in the presence of the salt counterion that is expelled in the process, is found to be

$$-\Delta G^\circ_{es}/2.3RT = \log(N_{AV}b^2\delta_p/1000e) + (Z_p/Z_s)\log[1000e/(N_{AV}b^2\delta_s m_s)(1 - Z_s\xi)] \tag{5}$$

where $m_s$ is the molal salt concentration and $N_{AV}$ is Avogadro's number.

The atmospheric binding of the protein and the salt counterions is schematically illustrated in Fig. 1. It is seen that the protein molecules are not bound to any of the charged sites at the surface but are kept in the stationary phase domain by the electrostatic field generated by the plurality of fixed charges in close proximity at the surface. A feature of such territorial binding is believed to be the freedom for lateral movement by the bound species. Fig. 2 represents a graphical illustration of eqn. 4 and shows the dependence of the thickness of the counterion condensation layer on the distance between the charges for counterions having different electronic charges.

*Hydrophobic interactions between biopolymer and stationary phase*

In the present treatment of the energetics of retention in hydrophobic interaction chromatography, retention is assumed to occur due to contact between the hydrophobic patches at the biopolymer surface and the hydrophobic binding sites on the stationary phase as illustrated in Fig. 3. In the hermeneutics of the solvophobic theory[13], the free energy change for hydrophobic interactions, $\Delta G^\circ_{h\phi}$ has been expressed as

$$\Delta G^\circ_{h\phi} = \Delta G^\circ_{es} + \Delta G^\circ_{vdw} + \Delta G^\circ_{assoc} + \Delta G^\circ_{red} + N_{AV}(\gamma'_S A_S - \gamma'_M A_M) + RT\ln(RT/P^\circ V) \tag{6}$$

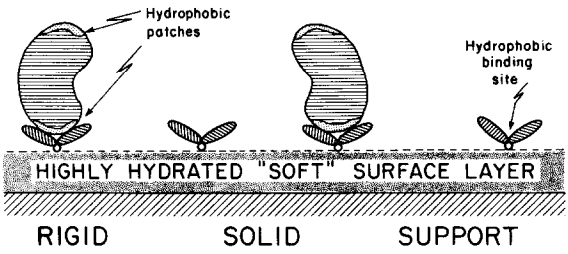


Fig. 3. Illustration of hydrophobic interactions between the hydrophobic ligates of the stationary phase and the hydrophobic patches on the surface of the protein.

where, according to the previous treatment[14], $\Delta G^{\circ}_{es}$ is the free energy change associated with electrostatic effects upon binding, $\Delta G^{\circ}_{assoc}$ is the free energy change for eluite–ligate association in the absence of surrounding solvent, *i.e.*, in the gas phase, and $\Delta G^{\circ}_{vdw}$ is the free energy change due to Van der Waals interactions. $\Delta G^{\circ}_{red}$ expresses the reduction of free energy due to solvent–ligate and solvent–eluite interactions not treated in the preceding terms. In the last term which takes care of the free volume, $V$ and $P^{\circ}$ are the mean molar volume of solvent and the standard pressure, respectively. The respective microthermodynamic surface tensions pertinent to the mobile and stationary phase domains are $\gamma'_M$ and $\gamma'_S$, whereas the molecular surface areas of the free and bound eluite exposed to the aqueous mobile phase are $A_M$ and $A_S$, respectively.

Electrostatic effects indicated by the term $\Delta G^{\circ}_{es}$ in eqn. 6 had been evaluated by combining the Debye–Hückel treatment with the solvophobic theory[15,19]. In the absence of fixed charges at the surface the electrostatic contribution would be solely determined by this term, which vanishes at sufficiently high salt concentrations. When there are fixed charges at the stationary surface eqn. 5 can be used to determine the value of $\Delta G^{\circ}_{es}$.

Examination of eqn. 6 suggests that biopolymer size does not enter explicitly into the calculation of retention energetics. The important quantity is the change in the molecular surface area $\Delta A'$, which is given by the change in the total surface area upon hydrophobic binding ($A'_M - A'_S$) and is thus proportional to the "hydrophobic contact surface area". In general, the total hydrophobic surface area is expected to increase with the molecular weight of the biopolymer eluite. Of course, the hydrophobic surface area of each protein will be determined by its primary, secondary, and tertiary structure under the prevailing experimental conditions.

In the present treatment it is assumed that the sole effect of increasing salt concentration at sufficiently high salt concentrations is to increase the microthermodynamic surface tension and to augment hydrophobic interactions[15,20,21]. Thus, specific interactions between the biopolymeric eluite and the salt are assumed to be absent. As the surface tension of salt solutions is often a linear function of the salt concentration in such cases, the logarithmic equilibrium constant is expected to increase linearly with the salt concentration in the mobile phase when hydrophobic interactions dominate retention. We also may assume as a first approximation, that with stationary phases having "soft", *i.e.*, very hydrophilic surface in contact with neat water the microthermodynamic surface tension is the same in both the mobile and stationary phase domains and given by $\gamma$ so that $\gamma \equiv \gamma'_M \equiv \gamma'_S$. On the other hand, for salt solutions the appropriate surface tension can be expressed by adapting earlier treatment as $\gamma = \gamma_o + \sigma_s m_s$ where $\gamma_o$ is the surface tension of the neat water, $\sigma_s$ is the molal surface tension increment of the neutral salt and $m_s$ is the molal salt concentration[15].

The simple approach outlined above allows us to express the dependence of the free energy of hydrophobic interactions on the molal surface tension increment of the neutral salt, $\sigma_s$, in the absence of specific salt effects[15] as

$$\Delta G^{\circ}_{h\phi} = \Delta G^{\circ}_{aq} - \Delta A' \sigma_s m_s \tag{7a}$$

where $\Delta G^{\circ}_{aq}$ includes all contributions to the retention free energy in the system under

investigation according to eqn. 6, except that due to salt mediated hydrophobic interactions and is given by

$$\Delta G^{\circ}_{aq} = \Delta G^{\circ}_{es} + \Delta G^{\circ}_{assoc} + \Delta G^{\circ}_{red} - \Delta A'\gamma_o + RT \ln(RT/P^oV) \quad (7b)$$

Thus the product $\Delta A'\gamma_o$ accounts for free energy differences of cavity formation in neat solvent in the absence of any electrostatic effects.

*Retention by combined coulombic and hydrophobic interactions*

In order to express the dependence of the retention factor on the salt concentration in the eluent when both electrostatic and hydrophobic interactions are involved, eqns. 1–7 are combined to yield

$$\log k' = \log(N_{AV}b^2\delta_P/1000e) + (Z_p/Z_s)\log[1000e/(N_{AV}b^2\delta_s m_s)(1 - Z_s\xi)] - + (\Delta G^{\circ}_{aq}/2.3RT) + (\Delta A'\sigma_s m_s/2.3RT) + \log\phi \quad (8)$$

where $\phi$ is the phase ratio.

In view of eqn. 8 the dependence of the logarithmic retention factor on the salt concentration for the combined effect of hydrophobic and electrostatic interactions can be expressed in a simplified form as

$$\log k' = A - B \log m_s + Cm_s \quad (9)$$

where $B$ and $C$ are the appropriate electrostatic and hydrophobic interaction parameters, respectively, and $A$ is a constant encompassing all characteristic system parameters. The value of the parameters in eqn. 9 depends, among other factors, on the number, size, and distribution of charges and hydrophobic sites of the biopolymer, as well as on the nature and density of the binding sites on the stationary phase.

Comparison of eqns. 8 and 9 yields for parameter $A$ the following expression

$$A = \log(N_{AV}b^2\delta_P/1000e) + (Z_p/Z_s)\log[1000e/(N_{AV}b^2\delta_s)(1 - Z_s\xi)] - + \Delta G^{\circ}_{aq}/2.3RT + \log\phi \quad (10)$$

The electrostatic interaction parameter $B$ is evaluated in a similar fashion as

$$B = Z_p/Z_s \quad (11)$$

The expression for the hydrophobic interaction parameter $C$ is obtained as

$$C = \Delta A'\sigma_s/2.3RT \quad (12)$$

The analysis is further complicated if $n$ co-ions accompanying the biopolymer are expelled when the biopolymer is bound to the stationary phase. In such a case with monovalent salt, the electrostatic interaction parameter may be modified[12] as

$$B = (Z_p/Z_s) + n \quad (13)$$

Eqn. 9 expresses the relationship between the retention factor and the molality of salt in the mobile phase, when chromatography proceeds within the constraints of the simplifying assumptions stated above, via electrostatic interactions, hydrophobic interactions or both. The equation implies that under conditions of purely hydrophobic interaction chromatography, the plots of log $k'$ against $m_s$ will be linear, whereas plots of log $k'$ against log $m_s$ yield straight lines under conditions of electrostatic interaction chromatography over a certain range of salt concentrations.

The salt concentration $m_{s,o}$ at the minimum of log $k'$ in such plots is determined by differentiating eqn. 9 with respect to concentration and is given by

$$m_{s,o} = B/2.3C \tag{14}$$

In view of the previous treatment, $m_{s,o}$ is a complex function of the properties of the salt, the stationary phase and the macromolecule. The minimum retention factor, $k'_{min}$ at salt concentration $m_{s,o}$ is given by

$$\log k'_{min} = A - B \log(B/2.3C) + B/2.3 \tag{15}$$

so that the minimum value of the retention factor is a single function of the three parameters in eqn. 9. Although at log $k'_{min}$ there may be no interaction between the biopolymeric eluite and the stationary phase surface, significant size exclusion effects still may yield a measurable value for $k'_{min}$ (ref. 22).

We would expect the eluent pH to have no effect on the value of $\xi$ which expresses the charge density on stationary phases having the properties of strong anion or strong cation exchangers. On the other hand, weak anion and weak cation exchangers have ionogenic functions which may dissociate to different degrees in the operational pH range of mobile phase. Hence, their values may be dependent on the eluent pH used in the experiment. Although Manning[5] does not consider such titratable sites independent and equivalent, we may make this simplifying approximation to express the pH dependence of $\xi$ for anion exchangers as

$$\xi = \xi_{max}[1/(1 + 10^{pH - pK_a})] \tag{16}$$

and for cation exchangers as

$$\xi = \xi_{max}[10^{pH - pK_a}/(1 + 10^{pH - pK_a})] \tag{17}$$

where $\xi_{max}$ is the value of the $\xi$ parameter when all fixed ionogenic groups at the surface are dissociated and $pK_a$ is the negative logarithm of their acid dissociation constant.

Another effect of pH is to modify the value of the protonic charge on the biomacromolecule. Thus, the overall effect of pH on the retention is the result of changing the electrostatic potential of both the stationary phase and the biopolymeric eluite. As a result both electrostatic and hydrophobic interactions are affected by the change in pH.

*Apparent values of the interaction parameters*

Evaluation of the parameters $A$, $B$ and $C$ requires retention data measured over

a sufficiently wide range of salt concentrations in the eluent. In the domain of electrostatic interaction chromatography, *i.e.*, at relatively low salt concentrations, it has been customary to make log $k'$ *vs.* log $m_s$ plots which yield apparent straight lines with slope denoted by the symbol $B'$ here. In the literature the symbol $Z$ has frequently been used for the slope thus obtained[1–3].

Neglect of solvophobic effects by omitting the linear dependence of the logarithmic retention factor on the salt concentration in eqn. 9 modifies the interpretation of the coefficients $A$ and $B$. Eqn. 9 can be differentiated with respect to logarithmic salt concentration to obtain $A'$ and $B'$, the apparent values of $A$ and $B$ respectively as given by

$$A' = A + Cm_s(1 - 2.3 \log m_s) \tag{18}$$

and

$$B' = B - 2.3Cm_s \tag{19}$$

At low salt concentrations the magnitude of the product of the hydrophobic interaction parameter and salt molality, $Cm_s$, is usually negligible with respect to $B$ and only minor deviations from linearity are expected in plots of log $k'$ *vs.* log $m_s$ at low salt concentrations. According to eqn. 11, the coefficient $B$ is equal to $Z_p/Z_s$, *i.e.*, the ratio of the effective charges on the biopolymeric and the salt counterions, but as seen from eqn. 19, hydrophobic interactions may also affect the value of the slope of such a plot of experimental data. Nevertheless, even at salt concentrations of the order of 0.1 $m$ the value of the term 2.3 $Cm_s$ in eqn. 19 is only slightly greater than 20% of $B$ when $B$ and $C$ are of similar magnitude. This condition is satisfied for the proteins examined here. However, the similarity in magnitude of $B$ and $C$ parameters needs to be determined for each protein and set of experimental conditions, because, if $C$ is significantly greater than $B$ at the error in evaluating $B$ at $m_s > 0.1$ from a quasi-linear plot can be more substantial. In these cases, the measured value of $Z_p$ would be significantly affected by the magnitude of hydrophobic interactions as well, as illustrated in Fig. 4.

The implications of eqns. 18 and 19 are schematically illustrated in Fig. 4. Fig. 4a

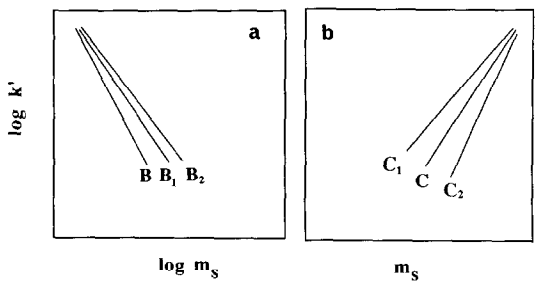


Fig. 4. Schematic illustration of the true and apparent interaction parameters. (a) When hydrophobic interactions are significant at low salt concentrations experiments may yield instead of the intrinsic electrostatic interaction parameter $B$ the apparent values of $B_1$ or $B_2$. (b) Attractive or repulsive electrostatic interactions can affect the apparent value of the hydrophobic interaction parameter, and experiments may yield the respective slopes $C_1$ or $C_2$ instead of the intrinsic hydrophobic interaction parameter $C$.

shows the log $k'$ *vs.* log $m_s$ plots obtained in the low salt concentration regime at three different values of $C$. As the value of $C$ increases, the slope of the curve decreases and so the apparent value of $B$ decreases as suggested on the graph by $B_1$ and $B_2$. Fig. 4b shows that neglect of electrostatic effects in the high salt concentration regime may produce analogous ambiguities in the estimated value of $C$. The line marked $C$ corresponds to the true value of the hydrophobic interaction parameter obtained if electrostatic effects are absent or correctly treated, whereas $C_1$ and $C_2$ show the effect when electrostatic effects are present but neglected. Similar results could be expected for instance in hydrophobic interaction chromatography on ion exchangers or other stationary phases with fixed charges at high salt concentrations.

## EXPERIMENTAL

### *Materials*

Cytochrome *c* from horse heart, ovalbumin, myoglobin from equine skeletal muscle, ribonuclease A, and α-chymotrypsinogen A, both from bovine pancreas, bovine serum albumin, lysozyme from chicken egg white and Tris were purchased from Sigma (St. Louis, MO, U.S.A.). $H_3PO_4$, $NaH_2PO_4$, $Na_2HPO_4$, $(NH_4)SO_4$, HCl, NaOH, NaCl, $MgSO_4$, acetic and citric acids were supplied by Fisher Scientific (Pittsburgh, PA, U.S.A.). Distilled water was prepared with a Barnstead Nano pure unit.

### *Instruments*

The liquid chromatograph was assembled from a Model 750 solvent delivery pump with a Model 753 ternary solvent mixer and a Model 740 control module supplied by Micromeritics (Norcross, GA, U.S.A.) or of two Model 100A pumps, a Model 420 gradient controller, an Altex (Berkeley, CA, U.S.A.) magnetic mixer, a Rheodyne (Berkeley, CA, U.S.A.) Model 7010 sampling valve with 20- or 100-μl sample loop and a Kratos (Ramsey, NJ, U.S.A.) Model 770R variable-wavelength UV detector. The column effluent was monitored at 280 nm, and chromatograms were obtained with a Schlumberger (Benton Harbor, MI, U.S.A.) Model SR-204 strip chart recorder or with a Shimadzu (Columbia, MD, U.S.A.) Model Chromatopac C-R3A recording data processor.

### *Columns*

The Zorbax BioSeries 80 × 6.2 mm I.D. columns WCX-300, WAX-300, SCX-300 and SAX-300 were gifts from DuPont (Wilmington, DE, U.S.A.). The acronyms used for column designation are as follows: WCX, weak cation exchanger; WAX, weak anion exchanger; SCX, strong cation exchanger; SAX, strong anion exchanger.

### *Data analysis*

Retention factors determined under isocratic conditions were fitted to eqn. 9 by non-linear regression analysis and thus the coefficients, $A$, $B$ and $C$ were evaluated. The degree of fit was checked by comparison of experimental values to those calculated by use of the fitted parameters.

## RESULTS AND DISCUSSION

The aim of this paper is to analyze biopolymer retention over a wide range of salt concentrations in a more comprehensive way than is possible by previous treatments. By considering both hydrophobic and electrostatic effects, the two chief factors which determine the magnitude of retention are treated conjointly. Although greatly oversimplified and restricted to "well behaved" systems, the analysis can provide a means to extract from experimental data parameters which are related to the properties of the biopolymer eluite and the salt in a given chromatographic system. Furthermore, these parameters could be useful in characterizing the stationary phases used in this kind of chromatography.

The electrostatic interaction parameter $B$, as defined in eqn. 11, is independent of the charge density $\xi$ at the surface. This may correspond to a situation of low counterion condensation according to Manning[5] and is of interest in linear elution chromatography for which eqn. 5 is believed to apply. When the electrostatic interaction between the polyionic eluite and the stationary phase is very strong, a high level of condensation occurs[5]. Binding of proteins by ion exchangers at very low salt concentrations, such as observed in preparative chromatography under certain conditions, may exemplify this condition. The value of the electrostatic interaction parameter for high levels of condensation, $B^*$, is obtained from Manning[5] as

$$B^* = Z_p\xi(1 - Z_p\theta_p)/Z_s \tag{20}$$

where $\theta_p$ is the number of protein counterions associated with a fixed charge at the surface. A significant conclusion from eqn. 20 is that at high level of condensation the charge density of the ion exchanger has a strong effect on the electrostatic interaction parameter and a comparison of eqns. 11 and 20 shows that the electrostatic interaction parameters at low and high levels of condensation differ by a factor of $\xi(1 - Z_p\theta_p)$. The following discussion will be restricted to low levels of condensation believed to occur in ion-exchange chromatography with salt elution.

Record has given an alternative formulation of the electrostatic interaction parameter, $B^{**}$ (refs. 11 and 12) that applies to situations of low levels of counterion condensation. The dependence on the charge density is as follows

$$B^{**} = (Z_p/Z_s)[1 - (2\xi)^{-1}] \tag{21}$$

According to this relationship, the absolute value of $B$ is expected to increase with the charge density, *i.e.*, with decreasing spacing between the fixed charges at the surface of the stationary phase. From this it follows that the shape of the $\log k'$ *vs.* logarithmic salt concentration plots will also depend on the charge density of the ion exchanger even at low levels of condensation.

Thus, the electrostatic interaction parameter $B$ provides a measure of electrostatic effects and is a function of $Z_p$, the effective charge on the biopolymer eluite. It is an indirect function of the charge density on the surface of the stationary phase. The counterion condensation theory begins with the insight that in order to interact charges must be within the Bjerrum length. This length is defined as the distance at which two unit charges interact with an energy of $k_BT$ in the relevant dielectric medium

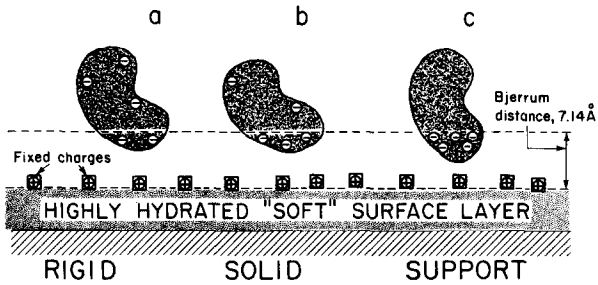


Fig. 5. Schematic illustration of the atmospheric binding of proteins having the same net charge (−5) but (a) uniform, (b) moderately asymmetric and (c) highly asymmetric charge distribution. It is seen that the effective (characteristic) charge on the protein, which is represented by these charges present within the layer of a thickness equal to the Bjerrum distance, is determined by the charge distribution.

and its value is 7.14 Å in water at 25°C. Many proteins can be regarded as spherical molecules having diameters in excess of this value with charges randomly dispersed on the protein surface. Therefore, only a fraction of the total charges on the protein molecule can interact with the charged surface of the stationary phase unless the charge distribution on the protein is highly asymmetrical as suggested by the schematic illustrations in Fig. 5. Thus, in the usual case, the magnitude of $Z_p$ does not reflect the macromolecular net charge but an "effective" or "characteristic" charge of the protein. This "effective" charge can probably be regarded as the number of charges involved in the most energetically favorable interactions between protein and stationary phases.

The hydrophobic interaction parameter $C$ is expected to depend on that hydrophobic surface area of the eluite that contacts the hydrophobic ligates at the stationary phase surface[15,19,20]. Thus it will increase with the density and size of hydrophobic patches on the surface of the protein. Generally, $C$ is likely to increase with the molecular size of the protein, and decrease with the number of ionized groups on the protein. Concomitantly, $C$ is expected to increase with the density and size of the hydrophobic binding sites at the stationary phase surface, particularly at low ligate concentrations. The nature of the salt used in the eluent also affects the value of $C$, which increases in the absence of specific salt effects with the molal surface tension increment[15]. In many cases, salts may enter into dipole–dipole interactions with the large dipolar protein molecules (see refs. in ref. 15), and other types of specific ion binding may also occur[23]. In such cases the above simple interpretation of the hydrophobic interaction parameter is not expected to be applicable.

The theoretical treatment presented here facilitates the interpretation of retention data obtained in protein chromatography with stationary phases having fixed charges at the surface and with increasing and decreasing salt gradients in the electrostatic and in hydrophobic interaction modes of chromatography, respectively. Since different physico-chemical properties of the proteins are responsible for their retention in the two techniques, widely different separation selectivities can be achieved by using the same column in the dual operation mode. The theory allows us to evaluate the interaction parameters that determine the effect of salt on the retention over a wide range of conditions, and at the same time, are characteristic of both the protein and the chromatographic system. Fig. 6 illustrates the effect of these

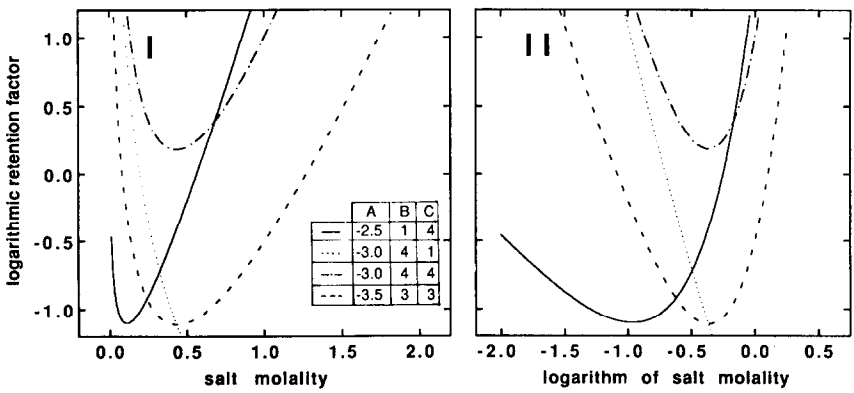


Fig. 6. Graphs illustrating the dependence of the logarithmic retention factor against the salt molality (I) and against the logarithmic salt molality (II) for the parameter values *A*, *B* and *C*.

parameters on protein retention. Plots similar to those shown in Fig. 6I and II can be used to evaluate from the respective limiting slopes the electrostatic and hydrophobic interaction parameters.

The effect of salt concentration on protein retention has been investigated under various experimental conditions; representative plots of the measured logarithmic retention factors against the salt concentration in the eluent are shown in Figs. 7–9, where the solid lines were drawn by fitting the data points according to eqn. 9. The corresponding parameter values calculated are listed in Table I.

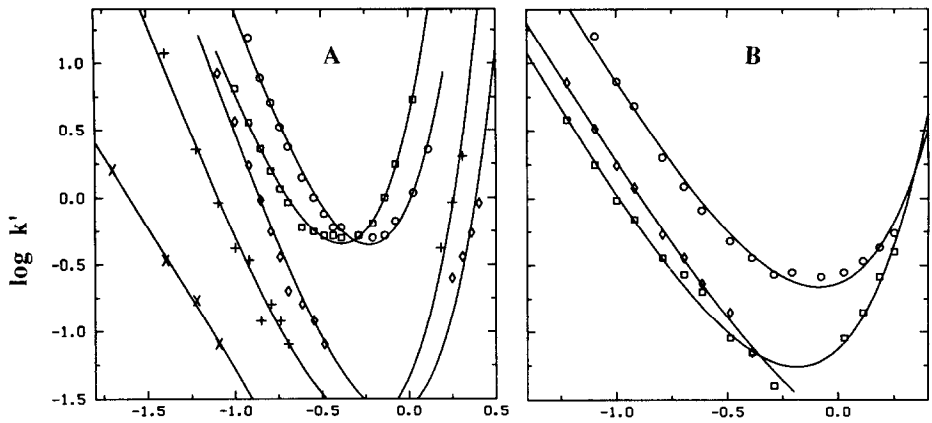


Fig. 7. Plot of the logarithmic retention factor of proteins obtained on weak cation-exchange (A) and strong cation-exchange (B) columns against the logarithmic molality of ammonium sulfate in the mobile phase. The solid curves were obtained by use of eqn. 9 and the parameters listed in Table I. Column, 80 × 6.2 mm I.D.; mobile phase, 20 m*M* phosphate buffer containing ammonium sulfate, pH 6.0; flow-rate, 1.5 ml/min; temperature, 25°C; UV detection at 280 nm. Sample components, (×) myoglobin; (+) ribonuclease; (◇) cytochrome *c*; (□) α-chymotrypsinogen A; (○) lysozyme.

Fig. 7 shows results obtained with the use of a weak and a strong cation exchanger and ammonium sulfate in the eluent at pH 6.0. It is seen that on both columns the isocratic retention factor first decreases with increasing salt concentration in the mobile phase until a minimum is reached after which further increase in salt concentration results in increasing retention factors in accordance with the predictions of eqn. 9. Fig. 8 shows results obtained by using weak and strong anion exchanger columns and salt at pH 7.8 with another set of proteins. As seen in Fig. 8A, the value of $B$ for α-chymotrypsinogen on both the weak and strong anion-exchange columns is rather small. This is not surprising because this protein has a net positive charge at the pH of the eluent. On the other hand ovalbumin and bovine serum albumin were not retained on weak anion exchangers by eluents having ammonium sulfate concentrations in the range 0.25–2.0 *m*.

Fig. 8B shows results obtained with the same proteins on a strong anion exchanger. Both bovine serum albumin and α-chymotrypsinogen A are retained by electrostatic and hydrophobic interactions at low and high salt concentrations, respectively, whereas ovalbumin is retained only by electrostatic interactions in the experimental salt concentration range.

Fig. 9 illustrates results obtained with α-chymotrypsinogen A on a weak cation exchanger column by using isocratic elution with 25 m*M* phosphate buffer, pH 6.0, that contained sodium acetate, sodium chloride or ammonium sulfate over a wide range of concentration. As expected the $B$ values are of the same order of magnitude within experimental error given the fact that the valence of the salt counterion, $Z_p$, is the same in all cases and equal to unity. In contradistinction, the value of the hydrophobic interaction parameter, $C$, is a function of the molal surface tension increment of salts; therefore, the decreasing order of $C$ values for ammonium sulfate, sodium acetate and sodium chloride reflects the relative magnitude of the $\sigma_s$ values.

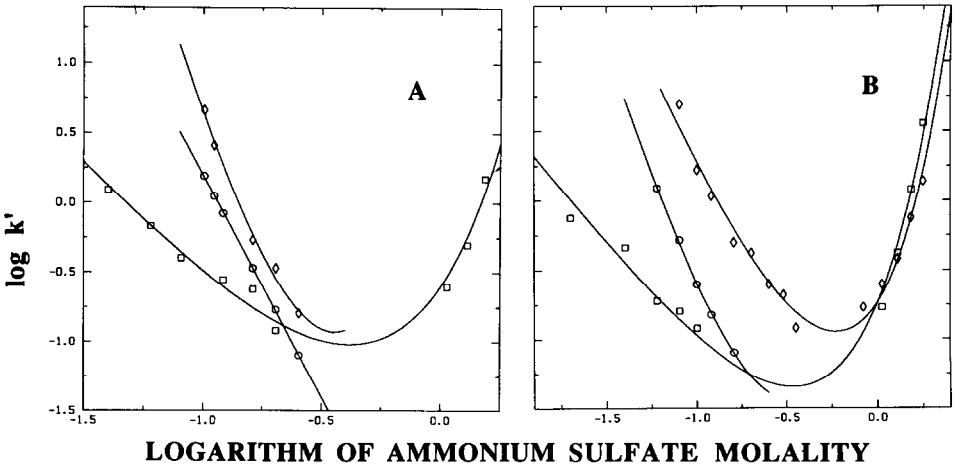


Fig. 8. Plot of the logarithmic retention factor of proteins obtained on weak anion-exchange (A) and strong anion-exchange (B) columns against the logarithmic molality of ammonium sulfate in the mobile phase. The solid curves were obtained by use of eqn. 9 with the parameters listed in Table I. Mobile phase, 20 m*M* Tris-HCl containing ammonium sulfate, pH 7.8. Sample components, (□) α-chymotrypsinogen, (○) ovalbumin, (◇) bovine serum albumin. Other conditions as in Fig. 7.

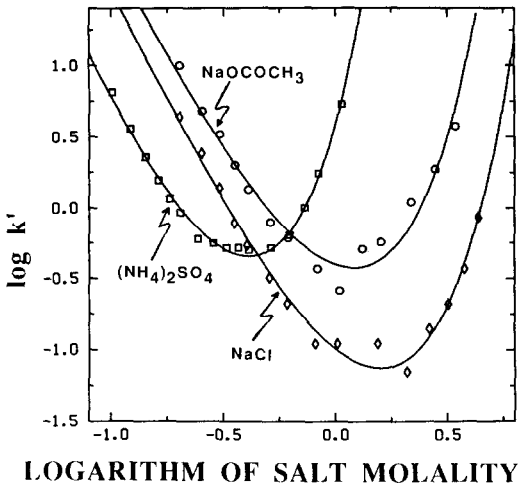


Fig. 9. Graph illustrating plots of the logarithmic retention factor of α-chymotrypsinogen A against the concentration of various salts in the eluent. The solid curves were obtained by use of eqn. 9 with the parameters listed in Table IV. Column: WCX, 80 × 6.2 mm I.D.; mobile phase, 20 m*M* phosphate buffer, pH 6.0 containing various salts; flow-rate, 1.5 ml/min; temperature, 25°C.

Figs. 7–9 suggest that the present analysis of salt effects probably is of broad applicability. Indeed, data obtained with a limited number of salts but with a variety of proteins and other polyionic biological substances on stationary phases of various provenance conforms at least qualitatively to the predicted shape of the logarithmic retention factor *versus* salt concentration plots. The pore morphology of the silica

TABLE I

PARAMETERS OF EQN. 9 FOR VARIOUS PROTEINS AND ION EXCHANGERS

Retention data were measured with buffered eluents containing different concentrations of $(NH_4)_2SO_4$.

| *Protein* | *Stationary phase* | *Parameter* | | |
|---|---|---|---|---|
| | | *A* | *B* | *C* |
| Lysozyme | WCX[a] | −3.17 | 4.25 | 3.12 |
| | SCX[a] | −2.15 | 2.87 | 1.51 |
| α-Chymotrypsinogen A | WCX[a] | −3.58 | 3.94 | 4.16 |
| | SCX[a] | −2.93 | 2.78 | 1.84 |
| | WAX[b] | −2.51 | 1.83 | 1.91 |
| | SAX[b] | −2.82 | 1.64 | 2.07 |
| Cytochrome *c* | WCX[a] | −3.83 | 4.13 | 2.20 |
| | SCX[a] | −2.06 | 2.35 | – |
| Ribonuclease A | WCX[a] | −3.99 | 3.46 | 2.69 |
| Bovine serum albumin | WAX[b] | −2.96 | 3.55 | – |
| | SAX[b] | −2.92 | 2.99 | 2.18 |
| Ovalbumin | WAX[b] | −2.98 | 3.18 | – |
| | SAX[b] | −3.37 | 2.81 | – |

[a] 20 m*M* phosphate buffer, pH 6.0.
[b] 20 m*M* Tris buffer, pH 7.8.

support often has a great influence on the retention behavior of proteins, but little is known about either the detailed pore topology of the various stationary phase supports or the nature of its effect on retention. In order to facilitate the comparison of the results presented here, therefore, Zorbax BioSeries ion exchangers, which are based on the same silica gel support, were used throughout the study.

*Effect of ligate density*

The density of the ligates, *i.e.*, the covalently bound functions serving as the binding sites at the stationary phase surface cannot usually be measured directly with confidence. However, as discussed above, the value of the electrostatic parameter $B$ for a given protein is likely to be directly proportional to the surface density of the ionized ligates on the stationary phase. Thus, by comparing the values of $B$, obtained from retention data measured on different stationary phases, we can estimate the relative concentrations of fixed charges at their surface.

In general, the ratio of ligate densities can be estimated from the results of appropriate elemental analysis of the stationary phases provided they were made from the same support by using similar chemistries. In our case, the ratio of ligate densities, $\delta_1/\delta_2$, was calculated by using data from the supplier[24] as 1.07 and 1.78 for the stationary pairs WAX/SAX and WCX/SCX, respectively. As shown in Table II, the electrostatic interaction parameters measured with different proteins on these ion exchangers yield ratios that are fairly constant and commensurate to the above values. Moreover, the ratios of the pertinent $B$ parameters are nearly invariant with protein, and this observation also supports the usefulness of this approach.

TABLE II

VALUES OF THE ELECTROSTATIC INTERACTION PARAMETER, $B$, OBTAINED UNDER VARIOUS EXPERIMENTAL CONDITIONS AND THE EFFECT OF THE RELATIVE LIGATE DENSITY AT THE STATIONARY PHASE SURFACE, $\delta_1/\delta_2$

Retention data were measured on Zorbax BioSeries columns and with $(NH_4)_2SO_4$ in the eluent. Subscripts denote the column number.

| *Protein* | *pH* | *Column* | | $B_2$ | $B_1/B_2$ | $\delta_1/\delta_2$ |
|---|---|---|---|---|---|---|
| | | *1* | *2* | | | |
| α-Chymotrypsinogen A | 6.0 | WAX | SAX | 1.43 | 1.29 | 1.07 |
| | 7.0 | WAX | SAX | 1.66 | 1.10 | 1.07 |
| | 7.8 | WAX | SAX | 1.64 | 1.12 | 1.07 |
| | 6.0 | WCX | SCX | 2.78 | 1.42 | 1.78 |
| Ovalbumin | 6.0 | WAX | SAX | 3.08 | 0.98 | 1.07 |
| | 7.0 | WAX | SAX | 2.47 | 1.47 | 1.07 |
| | 7.8 | WAX | SAX | 2.82 | 1.13 | 1.07 |
| α-Chymotrypsinogen A | 6.0 | WCX[a] | WCX[b] | 3.94 | 0.78 | – |
| | 7.0 | WCX[a] | WCX[b] | 4.11 | 0.87 | – |
| Lysozyme | 6.0 | WCX[a] | WCX[b] | 4.25 | 0.79 | – |
| | 7.0 | WCX[a] | WCX[b] | 4.17 | 0.90 | – |
| | 6.0 | WCX | SCX | 4.25 | 1.48 | 1.78 |
| Cytochrome *c* | 6.0 | WCX | SCX | 4.13 | 1.54 | 1.78 |

[a] Column after losing a part of its retentive capacity.
[b] Column before losing a part of its retentive capacity.

In the course of the experiments, the weak cation-exchange column was operated at elevated temperatures with concomitant reduction of its retentive properties due to loss of organic ligates. The $B$ values calculated from the data obtained on this cation-exchange column before and after the drop in its retention capacity were compared to gain information on the effect of reduced ligate density. Although the actual reduction can only be inferred from the significantly lower retention factors measured upon the heat treatment of the column, the $B_1$ to $B_2$ ratios in Table II clearly indicate a loss of surface charges. It should also be noted that this analysis confirms the general observation that the surface density of the negatively charged groups is significantly greater for the weak than the strong cation exchanger. Repulsion between the negatively charged sulfonic acid groups may hinder the attainment of such a high charge density at the surface that is possible with neutral carboxyl groups in the preparation of the stationary phase at sufficiently low pH. Therefore, the "weak" ion exchangers may have greater capacity than do "strong" ion exchangers.

The ratio of the two parameters $B$ and $C$ may also be of significance in the characterization of stationary phases. The value of $B/C$ expresses the relative magnitude of the two major salt-mediated interactions that determine retention. The values of $B/C$ have been calculated for several proteins on various columns and are presented in Table III. In order to assure that parameter $C$ employed in the calculations truly represents hydrophobic interactions, only data obtained with

TABLE III

RATIOS OF THE ELECTROSTATIC, $B$, AND HYDROPHOBIC, $C$, INTERACTION PARAMETERS OBTAINED FROM THE RETENTION DATA OF VARIOUS PROTEINS ON DIFFERENT ION-EXCHANGE COLUMNS WITH AMMONIUM SULFATE IN THE ELUENT

| *Protein* | *pH* | *Column* | *B/C* |
|---|---|---|---|
| α-Chymotrypsinogen A | 6.0 | SCX | 1.51 |
| Lysozyme | 6.0 | SCX | 1.90 |
| Bovine serum albumin | 6.0 | SAX | 1.19 |
| | 7.0 | SAX | 1.31 |
| | 7.8 | SAX | 1.37 |
| α-Chymotrypsinogen A | 6.0 | SAX | 0.71 |
| | 7.0 | SAX | 0.77 |
| | 7.8 | SAX | 0.79 |
| | 6.0 | WAX | 0.83 |
| | 7.0 | WAX | 0.91 |
| | 7.8 | WAX | 0.96 |
| | 6.0 | WCX[a] | 0.95 |
| | 6.0 | WCX[b] | 0.99 |
| | 7.0 | WCX[a] | 1.10 |
| | 7.0 | WCX[b] | 1.15 |
| Lysozyme | 6.0 | WCX[a] | 1.36 |
| | 6.0 | WCX[b] | 1.47 |
| | 7.0 | WCX[a] | 1.60 |
| | 7.0 | WCX[b] | 1.62 |

[a] Column before losing a part of its retentive capacity.
[b] Column after losing a part of its retentive capacity.

proteins which exhibited a major increase in retention with the salt concentration are included in Table III. As seen the $B/C$ values for a given protein depend only weakly on the eluent pH. This is expected because the extent of ionization of the major acidic or basic groups and consequently the density of the cationic and anionic charges on the proteins may not change significantly in the pH range studied here. The $B$ to $C$ ratios obtained from data on the weak cation exchanger before and after the reduction in its retention capacity are very similiar. This finding suggests that loss of both hydrophobic and charged functions occurred to the same extent. This is not unexpected because each ligate embodies both kinds of functions and therefore their densities are likely to be proportional. The $B$ to $C$ ratio obtained on a given ion exchanger with different proteins can also be interpreted as some kind of a weighted number of charges per unit hydrophobic area of protein and such values measured for various proteins on a given stationary phase can be used to rank them according to their charge density thus defined. Accordingly the data in Table III suggests that both the anionic charge density on bovine serum albumin and the cationic charge density on lysozyme are greater than the respective charge densities on α-chymotrypsinogen A. In turn, $B/C$ data obtained with selected proteins on various columns may be useful to gain information on the relative densities of the electrostatic and hydrophobic functions on the surface of different stationary phases. For instance, such information can be extracted from the $B$ to $C$ ratio for α-chymotrypsinogen at pH 6.0 that is greater on the strong than on the weak cation exchanger and greater on the weak than on the strong anion exchanger.

*Effect of salt on the electrostatic interaction parameter*

The stoichiometric model of Boardman and Partridge[1] for electrostatic interaction chromatography, in agreement with eqn. 11, predicts that the electrostatic interaction parameter $B$, is directly proportional to the characteristic charge on the protein, and inversely proportional to the charge on the salt co-ion. On the other hand, the model of Regnier and co-workers[3,4] postulates a more complex relationship for the dependence of B on the valence of the salt co-ion.

Values of the parameter $B$ obtained on a weak cation exchanger for several proteins are shown in Table IV. The data, which were obtained at pH 6.0 and 7.0 with univalent and divalent salt in the eluent, suggests an inverse dependence of the parameter $B$ on the valence of the salt co-ion. In view of eqn. 11 the parameter $B$ measured on the cation exchanger with a sodium salt should be twice as large as that measured with magnesium salt for a given protein in the event of simple ion exchange. It is seen from Table IV, however, that it is not the case and this discrepancy can be explained by the well known specific binding of $Mg^{2+}$ in proteins that has been found to result in a deviation from the usual salt effect on hydrophobic interactions[23].

In order to investigate this phenomenon we used the $B$ values in Table IV and estimated the magnitude of magnesium binding assuming that eqn. 13 is applicable. The corresponding numbers of $Mg^{2+}$ ions bound, $n$, per molecule of cytochrome $c$, α-chymotrypsinogen A and lysozyme were 1.24, 0.86, and 0.79, respectively. This finding suggests that in the case of specific salt binding the physico-chemical phenomena determining the value of $B$ are more complex than those considered in the present treatment.

According to the theory, the interaction strength should be proportional to the

TABLE IV

EFFECT OF SALT ON THE ELECTROSTATIC INTERACTION PARAMETER *B*

The retention data were obtained on the weak cation-exchange column of reduced retentive capacity

| *Protein* | *pH* | *Salt* | *B* |
|---|---|---|---|
| Cytochrome *c* | 6.0 | Sodium acetate | 3.26[a] |
| | 6.0 | NaCl | 3.07 |
| | 6.0 | $(NH_4)_2SO_4$ | 2.31[a] |
| | 6.0 | Sodium citrate | 2.03 |
| | 7.0 | $MgSO_4$ | 2.29[a] |
| | 7.0 | $(NH_4)_2SO_4$ | 2.99[a] |
| α-Chymotrypsinogen A | 6.0 | Sodium acetate | 3.11 |
| | 6.0 | NaCl | 3.48 |
| | 6.0 | $(NH_4)_2SO_4$ | 3.08 |
| | 6.0 | Sodium citrate | 3.33 |
| | 7.0 | $MgSO_4$ | 3.02 |
| | 7.0 | $(NH_4)_2SO_4$ | 3.56 |
| Lysozyme | 6.0 | Sodium acetate | 3.43 |
| | 6.0 | NaCl | 3.48 |
| | 6.0 | $(NH_4)_2SO_4$ | 3.38 |
| | 6.0 | Sodium citrate | 3.16 |
| | 7.0 | $MgSO_4$ | 2.73 |
| | 7.0 | $(NH_4)_2SO_4$ | 3.75 |

[a] Insufficient data in the hydrophobic interaction domain at high salt concentrations.

effective charge on the macromolecule and the electrostatic potential at the stationary phase surface. The effective or characteristic charge at present has to be obtained from experimental data since it is not necessarily the same as the net charge on the protein, *e.g.*, due to asymmetric charge distribution. This can dramatically affect retention behavior; for instance, Haff *et al.*[25] and Fausnaugh *et al.*[26] have found that β-lactoglobulin A was retained on anion exchangers at eluent pH values equal to or even below the isoelectric point of the protein.

*Effect of eluent pH*

According to the model, parameters *B* and *C* are directly proportional to the characteristic charge on the biopolymer eluite and to the net decrease in exposed surface area upon its binding to the stationary phase ligates, respectively. As the number of anionic and cationic sites on the macromolecule changes with the pH at least parameter *B* is strongly pH dependent even with strong ion exchangers. The degree of ionization of weak cation and anion exchangers may also change with the pH so that more complex pH dependence of the parameter *B* is expected with such stationary phases. The hydrophobic interaction parameter *C* may be affected by pH dependent changes in certain binding sites on the stationary phase and a marked pH dependence is expected when the hydrophobic binding sites contain ionogenic groups. The situation might further be complicated by pH induced conformation changes in the eluite molecule or in the ligates at the surface of the stationary phase.

Fig. 10 illustrates the pH dependence of each parameter. For the proteins under investigation, data were obtained over a sufficiently broad range of salt concentration to assure reliable values of both parameters. The observed dependence of *B* on the

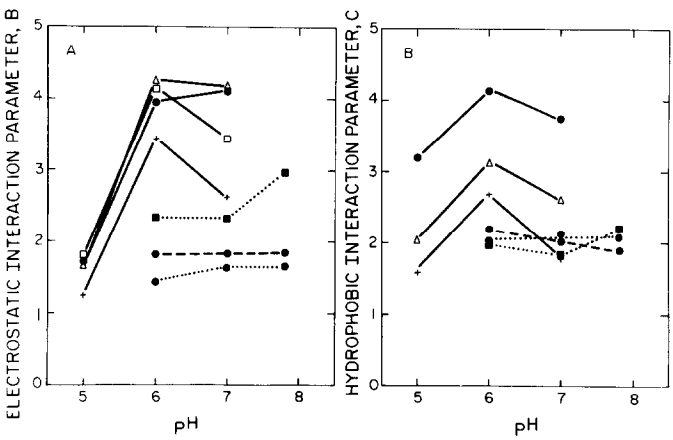


Fig. 10. Graphs illustrating the dependence of the electrostatic interaction (A) and the hydrophobic interaction (B) parameters on the pH of the eluent containing ammonium sulfate. Columns: WCX (——); WAX (– – –); SAX (· · ·). Mobile phase, 20 m*M* phosphate at pH 5.0 and 6.0, or 20 m*M* Tris–HCl at pH 7.0 and 7.8. Sample components: (●) α-chymotrypsinogen A; (△) lysozyme; (+) ribonuclease A; (■) bovine serum albumin; (□) cytochrome *c*.

eluent pH, depicted in Fig. 10A, is generally consistent with the behavior inferred above. The *B* values obtained for α-chymotrypsinogen A on both strong and weak anion exchangers were nearly constant or increased slightly with pH because this protein has a relatively high p*I* value. However, the value of *B* for bovine serum albumin increased with the eluent pH, indicating an increase in the dissociation of the side chain carboxylic acids of this acidic protein (p*I* = 4.5).

As mentioned above, the interpretation of the results obtained with the weak cation exchanger is less straightforward. The electrostatic interaction parameters for lysozyme, ribonuclease A, cytochrome *c* and α-chymotrypsinogen A all increase upon a change in pH from 5 to 6, but only for α-chymotrypsinogen A is seen an increase in the value of *B* between pH 6 and 7. The general increase in the pH range from 5 to 6 is probably due to the increasing degree of ionization of the carboxylic groups on the stationary phase, since changes in the degree of ionization of cationic groups on the protein, *i.e.*, the histidine, lysine and arginine side chains, are very small in this pH range. In contrast, the ionization of the carboxylic ligates would not be expected to increase significantly in the pH range from 6 to 7. Where protonation of the histidine side chains occurs in the pH domain, the retention can either decrease or increase according to the relative significance of the histidine ionization.

The pH dependence of the hydrophobic interaction parameter *C* is shown in Fig. 10B. On both types of anion exchangers the valence of the parameter is practically independent of pH over the range from pH 6.0 to 7.8. However, the results on the weak cation exchanger are more complex. The values of *C* are lower at pH 7 than at pH 6, suggesting that the number of hydrophobic binding sites on the stationary phase decreases due to increasing ionization of the carboxylic functions. The observed increase in *C* between pH 5 and 6, which should reflect an increase in the hydrophobicity characteristic of the protein over that pH range, is more difficult to explain. The two major groups which undergo ionization in that pH range are the

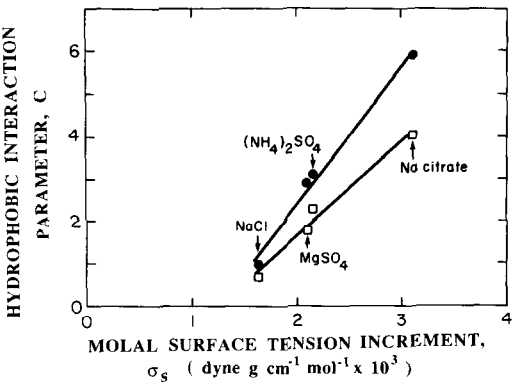


Fig. 11. Graph illustrating the dependence of the hydrophobic interaction parameter on the molal surface tension increment of the salt in the eluent. Weak cation-exchange column was used with NaCl, $MgSO_4$, $(NH_4)_2SO_4$ and sodium citrate in the eluent and the proteins were (●) α-chymotrypsinogen A and (□) lysozyme. The values of the surface tension increment were taken from ref. 15.

carboxyls of the glutamic and aspartic acid side chains and the basic group of histidine. Only the latter would be expected to become more hydrophobic with increasing pH so that the ionization of these groups and/or pH induced configuration changes may be responsible for the observed effect.

*Effect of salt on the hydrophobic interaction parameter*

The data of Fausnaugh *et al.*[26] support the theoretical prediction[15] that the retention augmenting effect of neutral salts parallels their molal surface tension increment in hydrophobic interaction chromatography. Gooding *et al.*[27] investigated the effect of sodium chloride and ammonium sulfate on the retention of seven proteins by using two kinds of stationary phases in hydrophobic interaction chromatography and their results are also in qualitative agreement with the expected behavior.

According to eqn. 12, which stems from a rather simple approach to the effect of neutral salts on protein interactions, the hydrophobic interaction parameters should be a linear function of the molal surface tension increment[15].

In this study, values of $C$ were obtained for α-chymotrypsinogen and lysozyme from retention data on a weak cation exchanger with sodium chloride, ammonium sulfate, sodium citrate and magnesium sulfate at pH 6 and 7, respectively. Fig. 11 shows plots of parameter $C$ against the molal surface tension increment that have the dimensions of (dyne g $cm^{-1}$ $mol^{-1}$) · $10^3$. The values used were 1.64 for NaCl, 2.10 for $MgSO_4$ 2.16 for $(NH_4)_2SO_4$ and 3.12 for sodium citrate[15]. The hydrophobic interaction parameters obtained with ammonium sulfate at pH 6 and 7 were 3.11 and 3.10 for α-chymotrypsinogen A and 2.29 and 2.32 for lysozyme, respectively. The plots of the $C$ values obtained with NaCl, $MgSO_4$, $(NH_4)_2SO_4$ and citrate yield straight lines for both proteins. Thus, the results are in agreement with the predictions of the model presented here. Nevertheless, we recognize that the surface tension argument is an oversimplification when dealing with such a complex system as protein binding and cannot be generally applicable in view of specific salt binding effects. Nevertheless, in many cases it offers a very convenient approach to explain the effects of salt on

hydrophobic interactions. Only values for preferential hydration[23], which were found to parallel the $C$ parameters measured with different salts in the eluent for lysozyme[28], might offer an alternative.

*Effect of salt concentration on column efficiency*

The concentration of the eluting salt affects not only the magnitude of retention but also the efficiency of the column with isocratic elution as shown in Fig. 12. On a given ion exchanger many proteins can be retained to a similar extent by electrostatic and hydrophobic interactions at low and high salt concentrations, respectively. We have found that for a given protein and retention factor the efficiency of the column under conditions of isocratic elution as measured by the plate height is significantly lower at high than at low salt concentrations in the eluent, *i.e.*, the same column exhibits a lower efficiency in the hydrophobic than in the electrostatic interaction mode with the columns under investigation. Two factors may be responsible for this behavior. First, the viscosity of the mobile phase increases with the salt concentration and concomitantly the diffusivity of the eluite decreases. Second, the binding kinetics for hydrophobic interactions, which are believed to involve contact between the hydrophobic moieties of the protein and stationary phase ligates, are expected to be less favorable than those for electrostatic interactions, which involve atmospheric binding, according to the treatment presented here. However, since most commonly gradient elution is used in both electrostatic or hydrophobic interaction chromatography, in practice, the apparent column efficiencies may not differ appreciably. It should also be noted that in bona fide hydrophobic interaction chromatography with mildly hydrophobic stationary phases that do not have fixed charges, lower salt concentration in the eluent suffices to retain the proteins than in the cases discussed here.

*Potential use of the interaction parameters*

In light of the above analysis parameters $B$ and $C$ may provide some physico-chemical information on the eluite molecule upon comparing data obtained

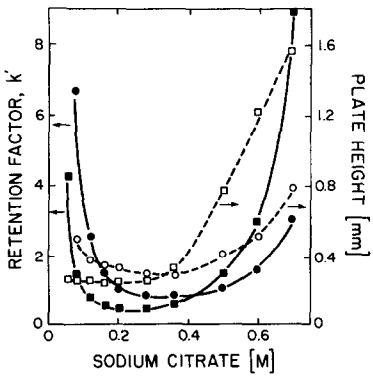


Fig. 12. Effect of salt concentration in the eluent on the magnitude of retention factor and column efficiency. Column: Zorbax BioSeries WCX 300, 80 × 6.2 mm I.D. Eluent: 20 m$M$ phosphate buffer, pH 6.0, containing sodium citrate; flow-rate, 1.5 ml/min; temperature 25°C; UV detection at 280 nm. Sample components: (□ and ■) α-chymotrypsinogen A; (○ and ●) lysozyme.

on reference columns under different elution conditions. The dependence of $B$ on salt type is likely to give insight into salt binding by the proteins in solution and the corresponding stoichiometric coefficients. The equilibrium constants for these interactions are implicit in the parameter $B$ and it may be possible to extract them mathematically. On the other hand, the hydrophobic surface of the eluite is characterized by the parameter $C$. Therefore, ranking of eluites in ascending order of the parameter obtained from data measured on a suitable HIC column $C$ can provide a semi-quantitative scale of the hydrophobic character of the surface for biopolymer eluites. Since $C$ is a measure of eluite interaction with a hydrophobic surface, the information thus obtained may afford further insights into the interactions of proteins with membranes or other surfaces of biological interest.

Alternatively, the interaction parameters may be used to characterize columns by comparing data obtained on different columns with an appropriately chosen set of eluites used as probes. The charge density of a given stationary phase relative to that of a reference stationary phase having similar properties can be inferred from the comparison of the corresponding values of parameter $B$ obtained with such standard eluites. That should provide a valid basis for intercolumn comparison. In similar fashion, the relative magnitude of parameter $C$ obtained with retention data of the standard eluites on different stationary phases should give a comparison of the effective size and/or density of the hydrophobic functions at the surface. We note that in general no such information can be obtained from a comparison of retention factors measured with different columns at the same salt concentration in the eluent, as seen from the illustration of simulated data in Fig. 6.

*Interrelationship of the interaction parameters*

Parameter $A$ contains information on both electrostatic and hydrophobic interactions as seen from eqns. 10–12 that reveal a mathematically simple relationship between the three parameters. Eqn. 10 contains the ratio $Z_p/Z_s$ which is identical to $B$ in the simple case represented by eqn. 11, and suggests a linear relationship between

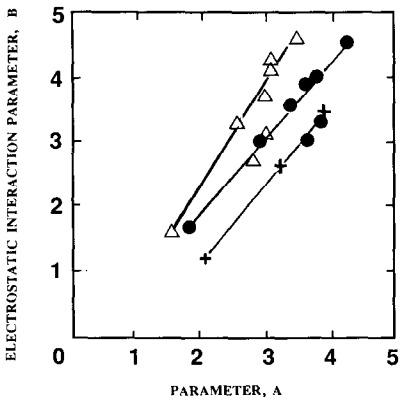


Fig. 13. Illustration of the relationship between parameters $A$ and $B$. The data were obtained from measurements on weak ion exchange columns in a wide range of eluent pH and salts. Each data point represents a pair of parameters evaluated from measurements with the same salt at the same pH. Sample components: (△) lysozyme; (●) α-chymotrypsinogen A; (+) ribonuclease A.

$A$ and $B$. Indeed, the plots of the parameters obtained from experimental data shown in Fig. 13 confirm the prediction. On the other hand the term $-\Delta G^{\circ}_{aq}/2.3RT$ in eqn. 10 is directly proportional to the molecular surface area change, $-\Delta A'$, the term $C$. This implies that electrostatic effects or hydrophobic interactions contribute to retention even outside the salt concentration range where they are predominant. For example, the intercepts of log $k'$ *vs.* $m_s$ plots in the domain of hydrophobic interaction chromatography should have increasingly greater negative intercepts when the characteristic charge of the eluite, $Z_p$ increases or its hydrophobic character as measured by the $\Delta G^{\circ}_{h\phi}$ term decreases. Usually, the apparent intercept of log $k'$ *vs.* log $m_s$ plots increases with $Z_p$ and decreases with the appropriate hydrophobic surface area. The determination of pertinent equilibrium constants from the values of $A$, $B$ and $C$ would be theoretically possible but difficult in practice due to experimental uncertainties and the simplifying assumptions underlying the model.

## CONCLUSION

Elucidation of the physico-chemical phenomena underlying the retention process of complex molecules in interactive chromatography is one of the greatest challenges for chromatographic science today. The intricate properties of the dynamic surface of the biopolymeric eluites are still largely unknown and therefore a detailed mechanistic study of the interactions is not yet possible. This paper represents an attempt to treat together both electrostatic and hydrophobic interactions which are implicated in salt mediated elution chromatography of proteins. Although it inevitably entails gross oversimplifications, it provides a framework for the analysis of chromatographic data and facilitates the study of the effect on the retention of the surface properties of both the biopolymer and the stationary phase as well as of mobile phase components. The concepts presented here are not restricted to linear elution chromatography and can be extended to non-linear chromatography of proteins, which is of growing interest in preparative scale separations.

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