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SALT-MEDIATED RETENTION OF PROTEINS IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

APPLICATION OF SOLVOPHOBIC THEORY

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SUMMARY

Retention behavior in hydrophobic-interaction chromatography was examined within the framework of the solvophobic theory. The principal parameters which determine the effect of salt on the retention are salt molality and the molal surface tension increment of the salt. According to the theory, in the absence of special binding effects, increase in salt molality in the mobile phase or change of salt to one of greater molal surface tension increment will result in increased retention of proteins in hydrophobic chromatography.

The theory is expanded to treat retention in gradient elution with linear decrease in salt concentration that is equivalent to linear increase in eluent strength. The results of the simple model lead to an expression with two parameters: the adjusted isocratic retention volume of the eluite with the gradient former and the slope of plot of logarithmic adjusted elution volume against salt molality, λ. The latter parameter is linearly dependent on molal surface tension increment if no specific interactions between the eluite and the stationary phase and/or salt are present. In practice, deviations are to be expected from the predicted behavior due to such effects.

The results of calculations are consistent with experimental results obtained with several proteins as the eluites and various salts in the eluent. Although unique values of the critical parameter λ could not be obtained from the data, the trends showed that λ is strongly correlated with the value of the molal surface tension increment. The prediction that increase in salt concentration in the initial eluent leads to increase in retention volume was found to be generally true, even when the isocratic retention volumes obtained with use of eluent having low salt concentration were small. Use of $NaClO_4$ in the starting eluent led in some cases to decrease in retention volume with increase in the salt concentration at the beginning of the gradient elution. This effect may be due to specific binding effects.

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INTRODUCTION

Reversed-phase chromatography with aqueous–organic eluents has been used to separate proteins[1,2]. However, in many cases, proteins are found to bind irreversibly to the stationary phase or to undergo denaturation due to their interactions with the mobile or stationary phase or both. Thus, the biological activity of the protein, or the protein itself is usually irretrievably lost in these cases. Hydrophobic-interaction chromatography with weakly hydrophobic stationary phases and mostly with the use of decreasing salt gradient is used to circumvent this difficulty[3–5]. Insofar as retention is expected to occur via interactions between the short hydrocarbonaceous chains of the otherwise inert stationary phase and the protein or other eluites of interest, this technique can be regarded as a branch of reversed-phase chromatography. Since the surface density of alkyl moiety is sparse with respect to those in most silica-bound hydrocarbonaceous stationary phases and the surface is non-interactive, the technique is formally similar to hydrophobic-affinity chromatography[6,7].

In both reversed-phase and hydrophobic-affinity chromatography, retention can be modulated by use of neutral salts in the mobile phase. In an earlier study we used the solvophobic theory to analyze the effects of neutral salts on retention in reversed-phase chromatography[8] as well as on salting-out and isocratic retention of proteins in hydrophobic-affinity chromatography[9]. Here we extend the scope of that analysis to hydrophobic-interaction chromatography including the use of linear gradient elution of several proteins and peptides.

THEORY

In a previous study, the effects of neutral salts on protein solubility and retention in hydrophobic-affinity chromatography were examined theoretically by use of solvophobic theory[9]. Since the physico-chemical basis of retention in hydrophobic-affinity chromatography and hydrophobic-interaction chromatography is essentially the same, we recapitulate the chief points of the previous theoretical treatment appropriate to isocratic elution in order to develop the present theory for elution with linear salt gradient. In hydrophobic-affinity chromatography the isocratic retention factor, k, was expressed as

$$\ln k = -\frac{1}{RT}(\Delta G^0_{cav} + \Delta G^0_{es} + \Delta G^0_{vdw} + \Delta G^0_{assoc} + \Delta G^0_{red}) + \ln\frac{RT}{PV} + \Phi \quad (1)$$

In the hermeneutics of the solvophobic theory[10,11] ΔG^0_{cav}, ΔG^0_{es}, and ΔG^0_{vdw} are overall differences between mobile and stationary phases in free energy, associated with cavity formation, electrostatic effects, and Van der Waals interactions, respectively. ΔG^0_{assoc} is the free energy change for ligate–eluite association in the absence of surrounding solvent, *i.e.*, in a gas phase, and ΔG^0_{red} expresses the reduction of that free energy due to solvent–ligate and solvent–eluite interactions not treated in the first

three terms. V and P are the mean molar volume of solvent and the operating pressure, respectively. The constant Φ is determined by the properties of the column and is related to the concentration of accessible ligates in the column.

In hydrophobic-interaction chromatography, the magnitude of ΔG^0_{es} varies with the salt concentration, m, and, according to our prior analysis, the dependence of protein activity coefficient in the mobile phase can be expressed by the extended Debye–Hückel equation as

$$\Delta G^0_{es} = A_{mp} - \frac{B_{mp}\,(m^{1/2})}{1 + C\,(m^{1/2})} - D_{mp}\,\mu m \tag{2}$$

where μ is the dipole moment of the protein, and the coefficients A_{mp} and B_{mp} are both proportional to the net charge on the protein. The coefficients C and D_{mp} are constants peculiar to each eluite because their magnitudes are functions of the dimensions of the macromolecule. The subscript mp refers to quantities pertinent to the mobile phase. The coefficient A_{mp} is inversely dependent on protein size as well. Further discussion of the physical significance of the coefficients can be found in the text by Edsall and Wyman[12]. As the relationship between salt concentration in the mobile phase and activity coefficient of protein bound to the stationary phase is unknown, we shall assume it to be similar in form to that given by eqn. 2. The electrostatic free energy change associated with the chromatographic retention process is given by

$$\Delta G^0_{es} = \Delta G^0_{es,sp} - \Delta G^0_{es,mp} \tag{3}$$

(where the subscript sp refers to the stationary phase), and can be expressed as a function of salt concentration by

$$\Delta G^0_{es} = A - \frac{B\,(m^{1/2})}{1 + C\,(m^{1/2})} - D\mu m \tag{4}$$

where A, B, and D are the net values of the corresponding coefficients in the expressions for electrostatic free energy changes in the mobile and stationary phases.

The energy of cavity formation in the mobile phase is related to the surface tension, γ, and surface area of the molecule, A_s, as

$$\Delta G^0_{cav} = [NA_s + 4.8\,N^{1/3}\,(\kappa^e - 1)\,V^{2/3}]\,\gamma \tag{5}$$

where κ^e is a constant which corrects for the curvature of the cavity and N is Avogadro's number. The surface tension of aqueous salt solutions is a function of the molal salt concentration, m, and is given by

$$\gamma = \gamma^0 + \sigma m \tag{6}$$

where γ^0 is the surface tension of neat water and σ is a constant characteristic of each salt, which we have called the molal surface tension increment[9]. Therefore, if the magnitude of the salt concentration has no effect on κ^e, A_s or V, the free energy change associated with cavity formation in the mobile phase $\Delta G^0_{cav,mp}$ is found to be

$$\Delta G^0_{cav,mp} = [NA_s + 4.8\, N^{1/3}\,(\kappa^e - 1)\, V^{2/3}]\,\gamma^0 + [NA_s + 4.8\, N^{1/3}\,(\kappa^e - 1)\, V^{2/3}]\,\sigma m \quad (7)$$

Thus the overall energy of cavity formation will be

$$\Delta G^0_{cav} = -[N\Delta A_s + 4.8\, N^{1/3}\,(\kappa^e - 1)V^{2/3}]\,\sigma m + \text{constant} \quad (8)$$

which can be expressed in a simplified form as

$$\Delta G^0_{cav} = -\Delta A_s \sigma m + \text{constant} \quad (9)$$

where ΔA_s is the difference in surface area of ligate and protein exposed to mobile phase between the bound and unbound states, *i.e.*, equivalent to the molecular contact area upon binding.

In the previous treatment[9] ΔG^0_{vdw} was assumed to be unaffected by exogeneous salts. Even if this assumption is incorrect, the form of the equations for analysis of salt effects does not change in a major way. The dominant term for Van der Waals interactions stems from pairwise interactions and, as a consequence, the first-order expression for the energy would be linear in salt number density. Therefore, the net free energy change due to Van der Waals interactions is expected to be nearly linear in salt concentration:

$$\Delta G^0_{vdw} = \Delta G^0_{vdw,sp} - \Delta G^0_{vdw,mp} = \Delta G^0_{vdw} + vm + \text{constant} \quad (10)$$

where v is a constant for a given salt–macromolecule pair and stationary phase.

Combination of eqns. 1–10 allows us to express the dependence of the logarithmic retention factor on the salt concentration for isocratic elution by

$$\ln (k/k_0) = -Bm^{1/2}/(1 + Cm^{1/2}) - D\mu m + \Delta A_s \sigma m + vm + \text{constant} \quad (11)$$

where k_0 is the retention factor at zero salt concentration.

At sufficiently high ionic strengths the leading term on the right-hand side approaches a constant value and then the logarithmic retention factor becomes linear in salt molality:

$$\log (k/k_0) = \lambda m \quad (12)$$

where λ is a parameter which measures the retentive strengths of the salt and is similar to the salting-out constant[9]. Eqn. 12 can be expressed in terms of adjusted retention volumes as

$$\log (V_R/V_0) = \lambda m \quad (13)$$

where V_R is the adjusted retention volume at sufficiently high salt molality, m, in the

eluent and V_0 is the adjusted retention volume in the absence of salt in the mobile phase.

Snyder[13] has shown that the relationship between isocratic retention factor and the elution in gradient elution is given by

$$1 = \int_0^{V_R} \mathrm{d}V/V_i \tag{14}$$

where $\mathrm{d}V$ is a differential volume of mobile phase that passes through the band center during its migration on the column, and V_i is the instantaneous value of the corrected retention volume at a given time. This relationship takes a simple analytical form when the change in solvent strength is linear.

In light of eqns. 13 and 14 we examine the effect of salt concentration under conditions of linear gradient elution, preceded by isocratic elution in the same chromatographic experiment. Such an elution strategy is frequently used in practice in order to separate sample components of widely different retention behavior in a single chromatographic experiment comprising both isocratic and gradient elution steps. Prior to gradient development during isocratic elution, the volume V_I passes through the column, and during gradient development, the volume V_G leaves the column. Elution of the eluite under consideration occurs at the retention volume V_R.

In the simplest case, the retention volume of the eluite is smaller than V_I, *i.e.*, it is eluted prior to the onset of the gradient so that the retention volume is obtained from eqn. 13 as

$$V_R = V_{R,0} \exp(\lambda m) \tag{15}$$

When elution of the eluite occurs during gradient development following the initial isocratic development, the retention volume is expressed from eqns. 13 and 14 as

$$V_R = V_G/\lambda m \cdot \log \{\lambda m[V_{R,0} \exp(\lambda m) - V_I]/V_G + \exp(\lambda m V_I/V_G)\} \tag{16}$$

If, upon completion of the gradient development, isocratic elution at the salt concentration of the gradient former is required to elute the eluite in consideration, combination of eqns. 13 and 14 yields the following expression for the retention volume

$$V_R = V_{R,0} + V_I + V_G - V_I \exp[-\lambda m] - V_G/\lambda m \cdot \exp[-\lambda m] \cdot \cdot \{\exp[\lambda m(V_I/V_G + 1)] - \exp[\lambda m V_I/V_G]\} \tag{17}$$

EXPERIMENTAL

Apparatus

A Kratos (Ramsey, NJ, U.S.A.) Spectroflow 430 gradient former with Kratos Spectroflow 400 solvent delivery system and a Perkin-Elmer (Norwalk, CT, U.S.A.) LC-85 variable-wavelength UV–VIS detector were used with a Rheodyne (Berkeley,

CA, U.S.A.) Model 7125 sample injection valve containing a 20-μl sample loop. The chromatograms were recorded on a Honeywell (Minneapolis, MN, U.S.A.) Electronic 194 recorder.

Column and chemicals

Experiments were carried out with 150 × 4.6 mm columns, packed with SynChropak-Propyl (6.4 μm), supplied by SynChrom (Linden, IN, U.S.A.).

All samples were from Sigma (St. Louis, MO, U.S.A.) except insulin which was obtained from Calbiochem (LaJolla, CA, U.S.A.). Reagent-grade chemicals (Fisher, Pittsburgh, PA, U.S.A.) were used exclusively. Distilled water was obtained with a Barnstead distilling unit in our laboratory.

Operating conditions

The flow-rate and the chart speed of the recorder were 1.5 ml/min and 0.1 or 0.5 in./min, respectively. Decreasing salt gradients linear in salt concentration were used in neat aqueous eluents. The chromatographic experiments consisted of three segments: a, isocratic elution with the starting eluent for 2 min; b, linear gradient from 0 to 100% of the gradient former in 25 min; c, isocratic elution with the gradient former proper. The solutions used in these experiments are described in the Results and discussion section.

The background electrolyte was in most cases 50 m*M* phosphate buffer, prepared by mixing 25 m*M* monobasic sodium phosphate with 25 m*M* dibasic sodium phosphate, and triethylamine was added to obtain a 6 m*M* solution. In some cases 50 m*M* boric acid solution with 6 m*M* triethylamine was used. Eluted pH was 6.80 throughout the experiments. All solutions were filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) before use as eluents.

Proteins were dissolved in 50 m*M* phosphate buffer (pH 6.80) at concentrations ranging between 1 and 3 μg/ml. Proteins were detected by monitoring the column effluent at 210 nm.

Column hold-up volume was determined from the retention volume of sodium nitrate by using 50 m*M* phosphate buffer as the mobile phase.

RESULTS AND DISCUSSION

Practical implications of the theory

Before evaluating the experimental results, it is useful to examine eqn. 11 cursorily for its implications in hydrophobic-interaction chromatography in view of the three distinct problems frequently observed in protein chromatography[14]: (i) protein is unretained, (ii) protein is not eluted and (iii) insufficient selectivity between two proteins is found.

As shown in eqn. 11 the magnitude of chromatographic retention is governed not only by the electrostatic effect of the salt in the eluent but also by salt-mediated changes in the surface tension, which depends both on the concentration and the nature, *i.e.*, the molal surface tension increment of the salt. At sufficiently high salt concentrations an increase in either quantity will lead to an increase in retention volume which depends on the magnitude of the molecular surface area change in the binding process. Consequently, changes in retention volume with salt concentration

are expected not to be identical for different proteins so that the chromatographic selectivity for various proteins will depend on the nature and concentration of the salt. The magnitude of the retention factor in the limit of zero salt concentration will depend on net charge on the protein, which in turn depends on the pH of the mobile phase, according to the previous treatment[9]. Thus, retention will be increased or decreased with changes of mobile phase pH toward or away from the isoelectric point of the protein.

Salts can manifest specific effects also. The presence of certain amines in the mobile phase may reduce or eliminate losses or tailing due to interaction with residual silanols at the stationary phase surface. Particular salts may be allosteric effectors of a given protein, and the resulting conformational changes may affect the retention significantly. The magnitude and direction of such changes are difficult to predict in advance of detailed knowledge of the properties of the proteins and of the nature of the protein–salt interaction.

Of course, the stationary phase itself affects the magnitude the retention in a major way. Besides various effects arising from the nature of the sorbent, both the number density on the surface and the length of the hydrocarbonaceous ligates affect retention in view of solvophobic theory. Thus an increase in the size of the hydrocarbonaceous functions and/or their concentration in the stationary phase will cause an increase in elution volume of all species retained by solvophobic interactions.

In view of this, there are many ways to modulate retention behavior in hydrophobic-interaction chromatography. It is noted that the surface of the stationary phase employed in this study is not considered to be electrostatically neutral, due to the presence of a polymer layer containing secondary and tertiary amino functions. Some of the predictions of practical significance are summarized in Table I.

Calculation of retention behavior

Retention volumes were calculated by use of eqns. 15–17 for various values of $V_{R,0}$ and λ. The values of the parameters were chosen to be appropriate to the ex-

TABLE I

ADJUSTMENT OF RETENTION BEHAVIOR IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY ACCORDING TO PREDICTIONS BY THE SOLVOPHOBIC THEORY

Observation	*Recommendation*
Protein poorly retained	Increase salt concentration
	Change salt to one with greater molal surface tension increment
	Change pH toward isoelectric point
	Change column to one with smaller hydrocarbonaceous moieties and/or lower ligate density, *i.e.*, reduce phase ratio
Insufficient selectivity	Change salt
	Use additives that selectively affect protein, *e.g.*, inhibitors, allosteric effectors
Protein is not eluted	Decrease salt concentration if "high", increase if "low"
	Change salt to one with lower molal surface tension increment
	Add amine or other silanophile to mobile phase
	Change column to one with longer ligate and/or greater chain density *i.e.*, increase phase ratio
	Change pH away from isoelectric point

perimental conditions reported below. Hence, the value of $V_{R,0}$ ranged from 10^{-3} to 100 ml and λ ranged in value from -4 to 4 mol^{-1}. The results of calculations for selected values of the parameters are shown in Fig. 1. The curves demonstrate that magnitudes of both $V_{R,0}$ and λ are significant in determining the dependence of the retention volume on salt molarity. With λ set equal to 0.1 mol^{-1}, the plots are practically linear, but the slope decreases to nearly zero as $V_{R,0}$ decreases from 4.1 to 0.1 ml.

Comparison of the three curves drawn with $V_{R,0}$ set equal to 0.1 ml indicates that an increasing value of λ causes a marked increase in the slope of the plot and departure from linearity. The results based on the solvophobic theory corroborate the practical observations[3,4] that the retention factor increases with the salt concentration in the eluent. Under conditions examined here, the logarithmic retention volume in gradient elution can increase linearly with salt concentration or even more rapidly according to the model calculations and as illustrated in Fig. 1.

However, qualitatively different results were obtained when larger values of $V_{R,0}$ and λ were used in the calculation. Fig. 2 shows plots of the logarithm of predicted retention volume against salt concentration at the beginning of the gradient. The retention volumes were calculated with $V_{R,0}$ values ranging from $5 \cdot 10^{-3}$ to 10 ml and λ fixed at 1.78 mol^{-1}. The curves obtained with a small value of $V_{R,0}$ show an increase in retention volume with the salt concentration that is qualitatively similar to that seen in Fig. 1. However, for higher $V_{R,0}$ values, the curves manifest a plateau in retention volume when the initial salt concentrations are high. The magnitude of the retention volume in the plateau region increases with $V_{R,0}$ in a non-linear fashion. The range of salt concentrations over which the retention volume increases to its limiting value is fairly small, *i.e.*, less than 1 *M*, and the concentration at which this occurs appears to decrease with increasing $V_{R,0}$. Fig. 3 shows the effect of λ on retention for values ranging from 4 to 20. These values bracket typical values of salting-out constants observed with proteins[9]. The curves observed with all values of λ, except those for small λ values are qualitatively similar to those depicted in Fig. 2 insofar as the retention volume increases rapidly with an increase in starting salt concentration to a limiting value. The rate of change with respect to salt concentration is much more sensitive to a change in the parameter λ than to that in the value of $V_{R,0}$. In this example, a change in λ from 4 to 16 affects retention behavior comparable to that observed upon a 3 order of magnitude change in the value of V_0.

The physical basis for the dependence of retention volume on salt concentration can be inferred from Figs. 4–6, which present time–distance diagrams for eluite movement through the column with the parameters used before in Figs. 2 and 3. Fig. 4 shows time–distance diagrams, calculated for different values of $V_{R,0}$, with λ and the initial salt molality fixed at 4 mol^{-1} and 2 molal, respectively. Fig. 5 shows the effect of salt concentration on movement of the band, with λ and $V_{R,0}$ fixed at 8 mol^{-1} and 0.5 ml, respectively. Fig. 6 shows the effect of change in λ value, with $V_{R,0}$ and the initial salt molality set equal to 0.05 ml and 2 molal, respectively. Inspection of Fig. 4 suggests that little movement of the eluite band occurs in the initial 70% of the duration of the chromatographic experiment. Rapid elution occurs, however, after the salt concentration falls to a level appropriate to reduce the binding of the eluite under consideration. Comparison of Figs. 4 and 5 suggests that the magnitude of the delay is more sensitive to a variation in initial salt concentration than it is to

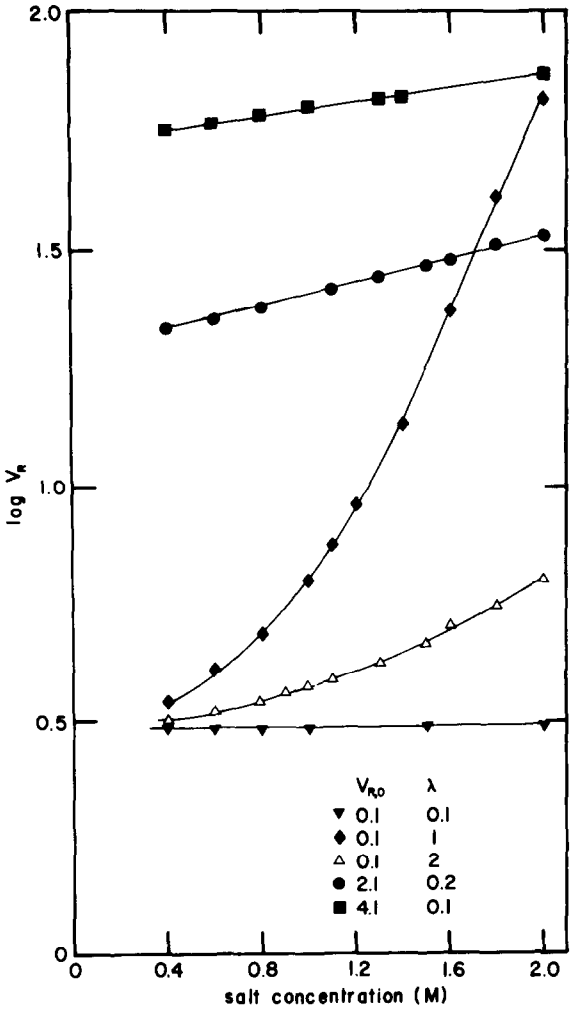
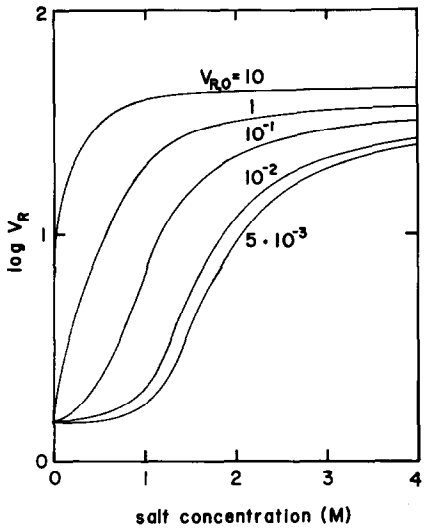


Fig. 1. Plots of logarithmic retention volume, calculated as a function of salt concentration in the starting eluent with gradient elution. The curves are plotted for various values of the parameter $V_{R,0}$ and λ.

$V_{R,0}$. The most interesting result is found in Fig. 6, which shows that the value of λ has a large effect on retention. As seen in Fig. 6, at sufficiently large λ values, the dependence of retention on the salt concentration is very nearly a step function, so that no movement occurs until a critical salt concentration is reached, but thereafter the eluite moves through the column almost unretained. This behavior is typical of interactive macromolecular chromatography and has been treated extensively in the literature[15]. The results suggest that if no elution occurs until a very low salt concentration is reached in the course of gradient elution, the retention volume in gradient elution would be largely insensitive to the initial conditions when the salt concentration is high. As seen in Figs. 3 and 6, however, when the value of λ is small, a rapid increase in retention volume with initial salt concentration would be expected.



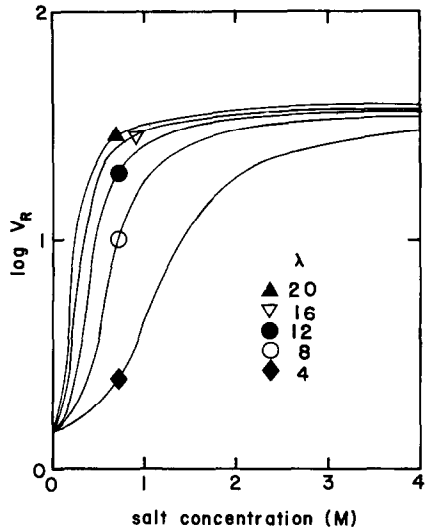


Fig. 2. Graph illustrating the dependence of the logarithmic retention volume on the initial salt concentration in the mobile phase with $V_{R,0}$ as the parameter, according to eqns. 15–17. The value of λ was fixed at 4 mol^{-1}. Conditions of linear gradient elution apply with a 4%/min decrease in salt concentration and a flow-rate of 1.5 ml/min.

Fig. 3. Graph illustrating the dependence of the logarithmic retention volume on the initial salt concentration in the mobile phase, according to eqns. 15–17. Conditions of linear gradient elution apply with a 4%/min decrease in salt concentration and a flow-rate of 1.5 ml/min. The plots represent different values of the critical parameter λ (mol^{-1}), and the value of $V_{R,0}$ was fixed at 0.05 ml.

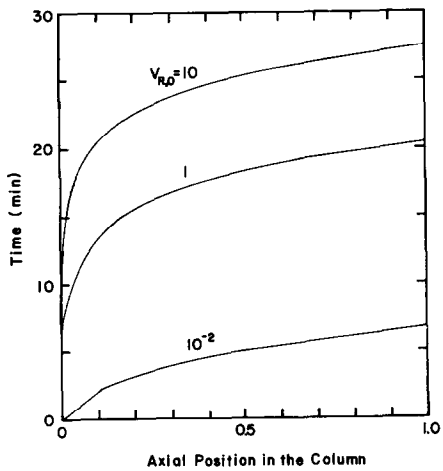


Fig. 4. Time–distance diagram illustrating the relationship between the elapsed time and the distance traversed by the eluite in gradient elution, according to eqns. 11 and 14. In this representation, λ and m were fixed at 4 mol^{-1} and 2.0 molal, respectively, and $V_{R,0}$ is the parameter.

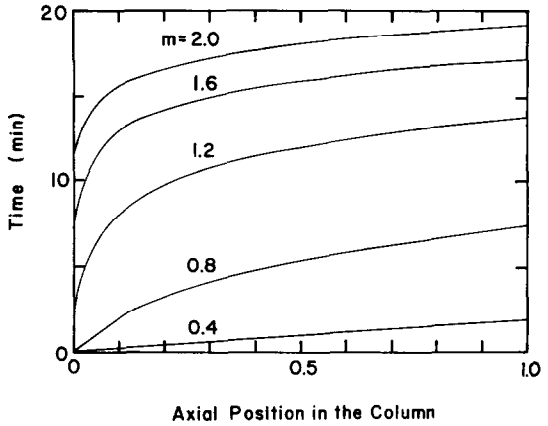


Fig. 5. Time–distance diagram illustrating the relationship between the elapsed time and the distance traversed by the eluite in gradient elution, according to eqns. 11 and 14. In this representation, the molal salt concentration is used as the parameter and the value of λ and $V_{R,0}$ were fixed at 8 mol^{-1} and 0.5 ml, respectively.

In this treatment, specific salt effects on the retention were neglected. Therefore, it should be noted that if binding of salt to protein causes a decrease in isocratic retention volume with use of the limiting buffer as the mobile phase, the plot of retention volume against salt concentration can have a maximum and minimum if the stability constant for salt–protein interaction is small or large, respectively.

Observed retention behavior of proteins

Retention volumes of myoglobin, ribonuclease A, insulin and its A and B chains, cytochrome *c*, trypsinogen and trypsin inhibitor were measured in hydrophobic-interaction chromatography with use of gradient elution, as described in Table II.

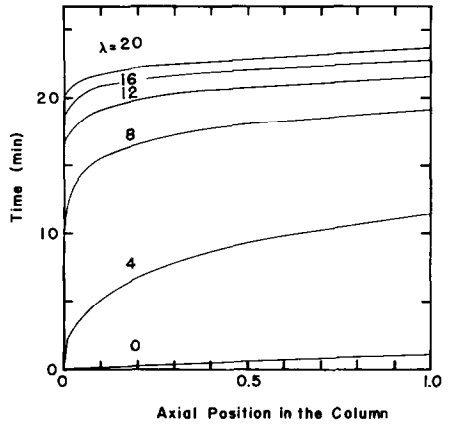


Fig. 6. Time–distance diagram, illustrating the relationship between the elapsed time and the distance traversed by the eluite in gradient elution, according to eqns. 11 and 14. In this representation the value of the critical parameter λ was varied, and the value of $V_{R,0}$ and m were fixed at 0.05 ml and 2 molal, respectively.

TABLE II

OPERATING CONDITIONS USED IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF PROTEINS

The starting eluent was the solution of the salt in the gradient former at one of the concentrations noted. Gradients were formed by a linear decrease in salt concentration at the rate of 4%/min.

Salt	*Initial salt molarity*	*Gradient former*
$(NH_4)_2SO_4$	1.4, 1.6, 1.8, 2.0	50 m*M* Phosphate buffer, 6 m*M* Triethylamine (pH 6.80)
Li_2SO_4	0.9, 1.1, 1.3, 1.5	50 m*M* Borate buffer, 6 m*M* Triethylamine (pH 6.80)
Na_2SO_4	0.4, 0.6, 0.8, 1.0	50 m*M* Phosphate buffer, 6 m*M* Triethylamine (pH 6.80)
$NaClO_4$	1.2, 1.6, 2.0, 2.4, 2.8, 3.2	50 m*M* Phosphate buffer, 6 m*M* Triethylamine (pH 6.80)

The retention volumes obtained in gradient elution with decreasing concentrations of $(NH_4)_2SO_4$, Li_2SO_4 and Na_2SO_4 are shown as functions of the initial salt concentration for the eight eluites in Figs. 7–9. In all cases, the data confirm the general predictions obtained from numerical solution of eqns. 15–17. An increase in salt concentration leads to an increase in protein retention on the column. In some cases, *e.g.* with myoglobin or ribonuclease A as the eluites and mobile phases containing $(NH_4)_2SO_4$ or Na_2SO_4, the plots appear to approach a limiting asymptote at high salt concentrations. Furthermore, comparison of the retention volumes observed with different salts in the weak eluent shows that the retention volume increases with the value of molal surface tension increment when the concentration of the various salts in the same.

The data were subjected to non-linear regression analysis according to eqns. 15–17 in order to obtain the parameters $V_{R,0}$ and λ. In most cases, the data obtained for a given protein in gradient elution with starting salt solutions of different compositions failed to converge to the theoretical equation. This may be a consequence of the small number of data points, 4, in each set. The data were compared with the theoretical values by calculation of the retention volumes at low and high salt concentration according to eqns. 15–17 with various values of the parameters. If both predicted retention volumes agreed with experimental values to within 5%, the values of the parameters and the predicted retention volumes were recorded. The results did not lead to unique values of the parameters. For example, calculations for the retention of myoglobin in Na_2SO_4 solutions gave values of V_0 ranging from 0.1 to 1.2 with a corresponding range in λ from 2.11 to 0.7. Therefore, determination of the coefficients by comparison of experimental and predicted results is not satisfactory. However, it is noteworthy that in most cases the range of V_0 values obtained with different salts overlap, as would be predicted from eqn. 13. Furthermore, cursory examination of λ values, obtained with different salts on a given protein at common values of V_0, suggests that they are strongly correlated with the value of σ.

The results obtained with $NaClO_4$ in the eluent are shown in Fig. 10. It is seen that they are qualitatively different from those obtained with sulfates. Retention of

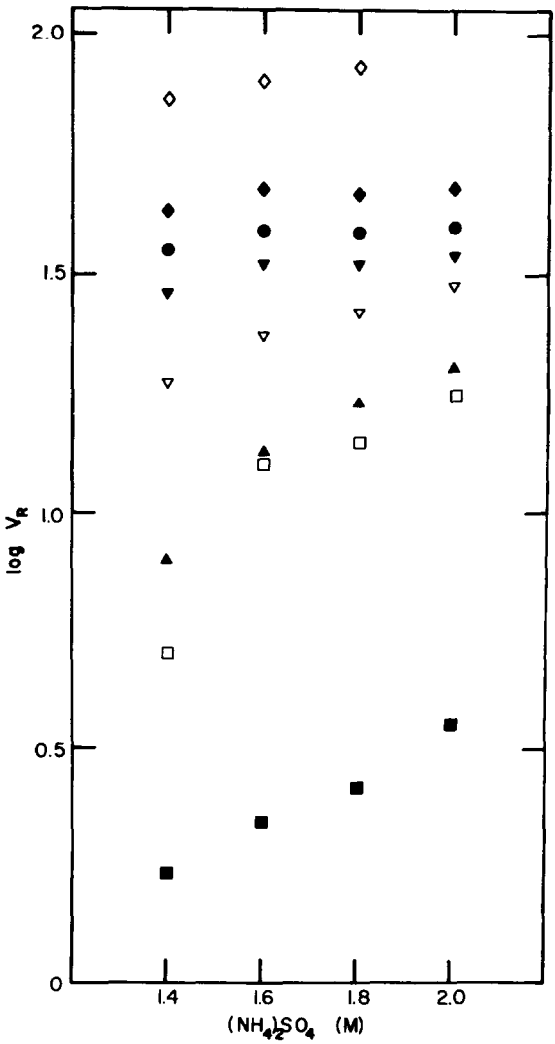


Fig. 7. Plots of logarithmic retention volume of eight polypeptides and proteins against $(NH_4)_2SO_4$ concentration in the starting eluent with gradient elution. The samples are insulin (◇), insuline B-chain (◆), trypsin inhibitor (●), trypsinogen (▼); insulin A-chain (▽), ribonuclease (▲), myoglobin (□) and cytochrome *c* (■).

insulin decreases before the usual increase with salt concentration, and the retention factor of insulin A-chain, trypsinogen, ribonuclease A, cytochrome *c* and myoglobin rapidly decreases with increasing salt concentration. Only trypsin inhibitor and insulin B-chain manifest the general pattern of retention found with use of sulfate. The reason for this difference is unclear and may be attributed to specific binding of perchlorate to the protein or stationary phase or to both. Any of these phenomena can cause deviation from the retention behavior expressed by eqn. 11. However, eqn. 11 also implies that an increase in salt concentration will lead to a decrease in retention due to electrostatic interactions if the value of λ and/or σ is small. The σ values of the salts used here as well as related ones are given in Table III. As the estimated σ

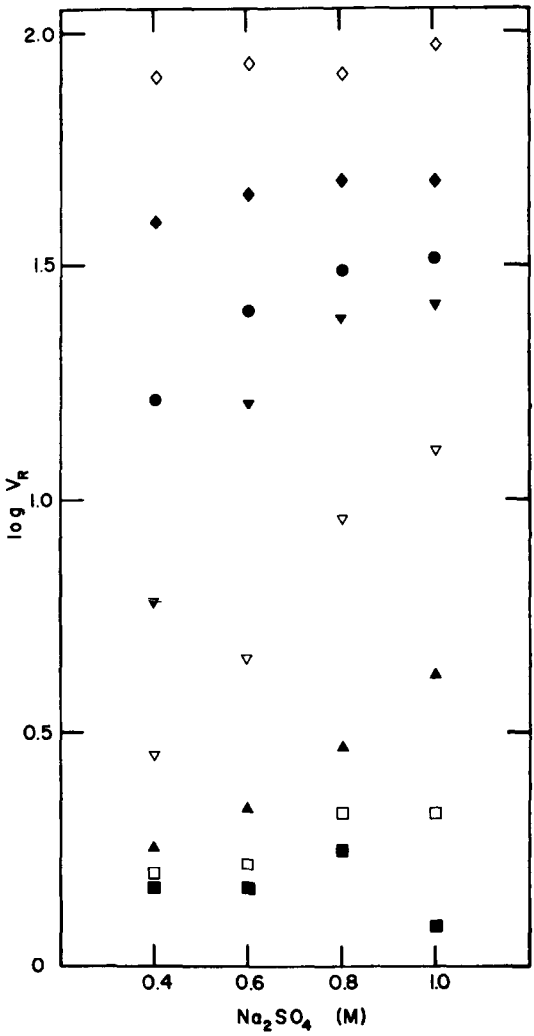


Fig. 8. Plots of logarithmic retention volume of eight polypeptides and proteins against Na_2SO_4 concentration in the starting eluent with gradient elution. For identification of symbols, see Fig. 7.

TABLE III

MOLAL SURFACE TENSION INCREMENTS OF SELECTED SALTS

An extended compilation of σ values is given in ref. 9.

Salt	$\sigma \times 10^3$ *(dyn-g/cm-mol)*
$(NH_4)_2SO_4$	2.16
Li_2SO_4	2.78
$MgSO_4$	2.10
K_2SO_4	2.58
Na_2SO_4	2.73
$KClO_4$	1.40
KSCN	0.45

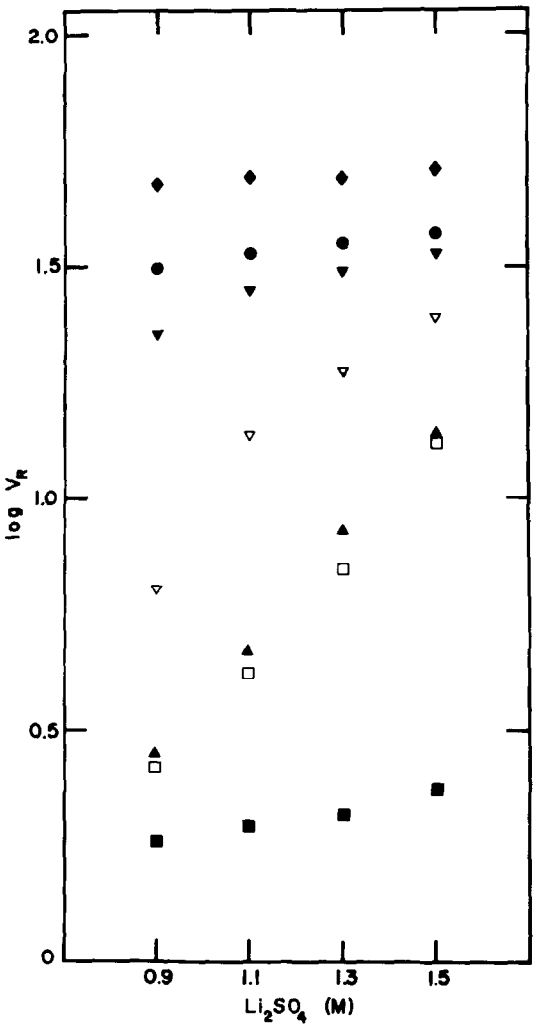


Fig. 9. Plots of logarithmic retention volume of eight polypeptides and proteins against Li_2SO_4 concentration in the starting eluent with gradient elution. For identification of symbols, see Fig. 7.

value of $NaClO_4$ lies between 500–700 dyn-g/cm-mol (*i.e.*, much smaller than that of the sulfates used, which are higher than 2000 dyn-g/cm-mol), this condition may be satisfied.

Examination of eqns. 15–17 suggests that plots of logarithmic adjusted retention volume against salt concentration would be linear in most cases, especially if elution occurred prior to completion of the gradient development. The parameters presented in Table IV were obtained from regression of the data by using the following relationship:

$$\log_{10} V'_R = V'_{R,0} + \lambda' m \tag{18}$$

where $V'_{R,0}$ and $\lambda' m$ are related to but not necessarily identical with the values of $V_{R,0}$ and λ, given in eqns. 11–17.

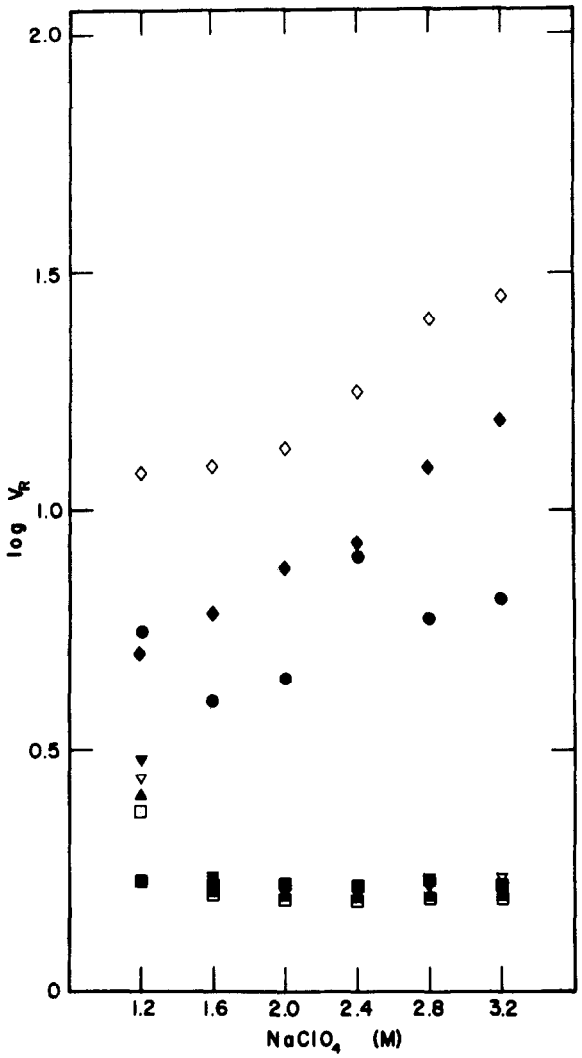


Fig. 10. Plots of logarithmic retention volume of eight polypeptides and proteins against $NaClO_4$ concentration in the starting eluent with gradient elution. For identification of symbols, see Fig. 7.

The slopes obtained from linear regression by using eqn. 18 are plotted against σ for some eluites in Fig. 11. Although in most cases the plots appear to be linear, data obtained with insulin A- and B-chains exhibit discrepancies between the values found for Na_2SO_4 and Li_2SO_4, which probably arise from experimental uncertainties in elution volume and void volume. Nonetheless, the data do tend to confirm the view that the dependence of retention in hydrophobic-interaction chromatography can be related in a simple way to salt concentration and the molal surface tension increment of the salt.

The results obtained with insulin and its constituent A- and B-chains as the eluites are especially interesting in this regard. The plots of data obtained with both

TABLE IV

SLOPES AND INTERCEPTS OBTAINED FROM PLOTS OF LOGARITHMIC ADJUSTED RETENTION VOLUMES AGAINST SALT MOLALITY

Eluite	*Slope*				*Intercept*			
	Salt				*Salt*			
	Na_2SO_4	$(NH_4)_2SO_4$	Li_2SO_4	$NaClO_4$	Na_2SO_4	$(NH_4)_2SO_4$	Li_2SO_4	$NaClO_4$
Ribonuclease A	3.44	1.71	3.83	−1.01	−2.78	−0.78	−3.56	0.56
Myglobin	3.20	2.32	3.98	−2.07	−3.80	−2.07	−3.90	0.42
Trypsinogen	2.77	0.30	0.63	−0.88	0.32	2.53	2.12	−0.19
Trypsin inhibitor	1.27	0.19	0.41	0.24	1.90	2.89	2.57	0.43
Insulin A-chain	3.65	0.77	2.46	−0.69	−1.46	1.42	−0.86	−0.72
Insulin B-chain	0.39	0.12	−0.06	0.69	3.10	3.22	3.42	−0.27
Insulin	0.24	0.49	0.12	0.51	3.86	3.18	3.29	1.19
Cytochrome *c*	–	3.46	1.65	−0.10	–	−6.49	−3.12	−2.27

insulin and the A-chain have a positive slope in Fig. 11, but the corresponding plot for the B-chain has a negative slope. The result can be understood in light of eqn. 11 if the B-chain has a large dipole moment and/or low hydrophobic surface area. Under the conditions of the experiment, the A and B chains both have a significant net charge and the insulin molecule itself is nearly neutral in view of its amino acid composition and isoelectric point of 5.54 (ref. 16). Insofar as the A-chain is acidic, it could also form ion pairs with triethylamine in the mobile phase; however, the effect is reduced with an increase in salt concentration. Since no decrease in retention of the A-chain with increasing salt concentration is observed, the effect of ion pairing under the conditions investigated here must be small. The amino acid sequence of the B-chain suggests a rather large dipole moment and, therefore, the unexpected negative slope shown in Fig. 11 can be rationalized. The combined A- and B-chains is believed to have a more compact structure than the individual chains; therefore, insulin proper behaves more like other large peptides or proteins.

It is noteworthy that the slope of the plots is not a simple function of molecular weight. This is agreement with the general argument of the solvophobic theory, which regards the area change upon binding as governing the magnitude of the slope. The area change, however, is a sensitive function of the number, nature, and distribution of groups on the exterior of such complex macromolecules which can be in contact with the stationary phase ligates. The combination of the insulin A- and B-chains is expected to result in a decrease both in the number of accessible hydrophobic groups and in the net charge on the insulin molecule. Therefore, it may explain why the slope of the plot of insulin data is smaller than that obtained for the A-chain.

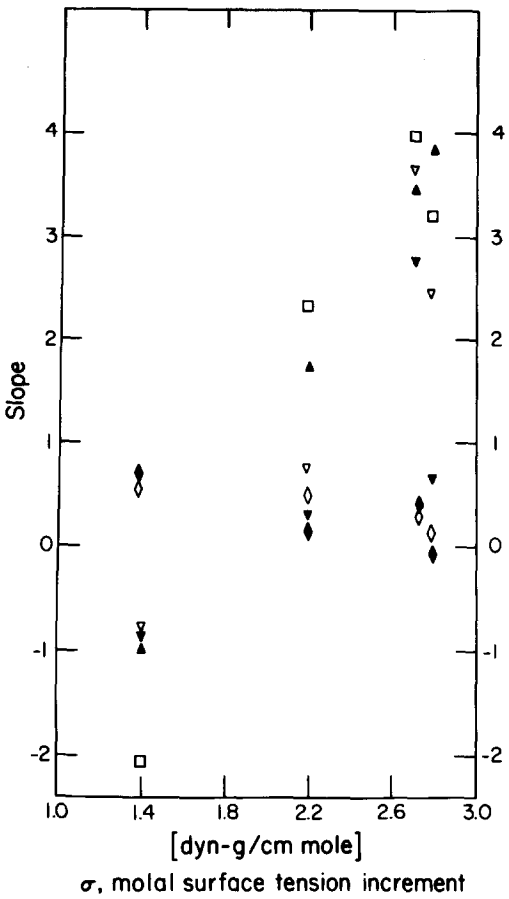


Fig. 11. Graph illustrating plots of the slopes obtained from plots of logarithmic adjusted retention factor against salt molarity versus the molal surface tension increment of the corresponding salt. For identification of symbols, see Fig. 7.

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