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Study of the retention mechanism of proteins in hydrophobic interaction chromatography

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SUMMARY

A stoichiometric displacement retention model for proteins based on hydrophobic interaction chromatography (HIC) is presented. Several methods were used to demonstrate that water is the displacing agent in this process. Salt not only affects the molar concentration of water, but also changes the conformation of proteins, hydrophobic interaction forces and the number of water molecules in a series of hydrated protein molecules. An equation that relates the capacity factor of proteins, k', to the water concentration and the stoichiometric displacement factor, Z (the number of water molecules required to displace a protein from ligands) was derived. The intercept of this equation, log I, contains a number of constants that relate to the affinity of protein to the ligands. There is a good linear relationship between log k' and log $[H_2O]$ under different chromatographic conditions. Although Z and log I varied with pH, salt temperature, the plot of log I *vs.* Z was always an excellent straight line, with a slope $j \approx 1.74$, the logarithm of the molar concentration of pure water. Hydrophobic interactions dominate the retention of proteins in HIC at high salt concentrations.

INTRODUCTION

Since Shaltiel and Er-el[1] first described hydrophobic interaction chromatography (HIC), in which agarose gels bonded to alkylamines were used as the stationary phase, this branch of chromatography has developed tremendously. The development of high-performance HIC (HPHIC), in which a rigid material, such as silica gel, is selected as the matrix has made the technique more convenient for the separation and purification of proteins with high biological activity. Chang and Geng[2] recently published a review.

The retention mechanism of proteins on an HIC column has been widely studied. Melander *et al.*[3] presented a thermodynamic model based on the cavity theory of Sinanoglu and Abdulnur[4] for the hydrophobic interaction between a protein and a hydrophobic matrix, and attributed the quantitative relationship between retention of the protein and the concentration of salt in the mobile phase to that between

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retention and the changes in surface tension. Fausnaugh *et al.*[5] and Jennissen and Botzet[6] explained the retention mechanism by an increase in entropy of the mobile phase when the hydrophobic groups are excluded from the polar environment and the interaction of the protein with matrix surface. Jennissen[7,8] suggested that the adsorption of proteins is due to multivalent interaction on the stationary phase. Arakawa[9] and Wyman[10] presented a preferential hydration model.

Although Karger and co-workers[11,12] obtained a linear log–log plot of the capacity factor of proteins, k', *versus* the concentration of a strong eluent (volume fraction) consisting of salt solution, which was very similar to the stoichiometric displacement model of retention (SDM-R) in reversed-phase liquid chromatography (RP-LC), they did not consider the retention mechanism of proteins in HIC.

Zhao *et al.*[13] investigated the influence of salts on the retention of small molecules in RP-LC and concluded that water is a displacing agent during the stoichiometric process of the displacement of solute to water molecules but the concentration unit of water was taken as the molar fraction. Therefore, the stoichiometric displacement factor Z is only a constant related to the real value.

Traditionally, one kind of interaction in one type of chromatography mainly dominates the chromatographic behaviour of solutes, and individual retention mechanisms have been considered on this basis. However, because each ligand bonded on silica or other matrices in HIC contains both polar and apolar groups, the polarities at different locations are unequal. Therefore, there are at least two different kinds of interaction forces between proteins and the stationary phase in HIC. Usually the hydrophobic interaction force is used to describe the interactions between proteins and bonded ligands on the HIC column. According to the definition by Ben-Naim[45], the hydrophobic interaction force is the indirect interaction between the same or different kinds of molecules in an aqueous solution, and it is very difficult to measure it exactly. In addition, except for the direct interaction forces between protein molecules and ligands, the influence of salt on the hydration of proteins and bonded ligands and the conformation of protein molecules make investigations of the retention mechanism of proteins on HIC columns very complicated. Fortunately, based on the recent result[14] that "No matter how different the interactions between adsorbent and solute or solvent molecules are, or how heterogeneous the distribution of these active sites is, the more rational mechanism for adsorption in a liquid–solid system should be stoichiometric displacement for solute adsorption", and the fact that adsorptions for both liquid–solid system in the physical chemistry of surfaces and liquid–solid chromatography were shown to have the same mechanism[15], the retention mechanism of proteins in HIC should also be a stoichiometric displacement process. This paper presents an SDM-R in HIC in which the contributions of salt, protein hydration, conformational changes, *etc.*, to the retention of proteins are considered, which was not the case in RP-LC.

THEORETICAL

When the separations of proteins on an HIC column are carried out with aqueous solutions of salts and the retention mechanism of proteins in HIC is assumed to be a stoichiometric displacement, the first question that needs to be answered is what the displacing agent is, salt, water or both. Strictly, both salt and water should

contribute to the retention of a protein. For convenience, let us assume that only one of them dominates the displacement process. If this is true, the second question that has to be answered is which one should be the displacing agent.

Compared with salt, the polarity of water is lower and stationary phase with moderate polarity in HIC prefers water. On the other hand, as is well known, salts retard the elution of proteins from an HIC column, and the higher the concentration of the salt the stronger is the adsorption of protein on the column. Therefore, it is reasonable to assume that water is the displacing agent. The functions of salt in HIC are very complicated, the most important being as a diluent for water. This will be discussed later.

Proteins in aqueous solutions of salts may hydrate with water[5,6,16,17] stepwise as follows:

$$\begin{array}{ccc} P_0 + H_2O & \rightarrow & P_1 \\ P_1 + H_2O & \rightarrow & P_2 \\ \vdots \quad\quad\quad & \vdots & \vdots \\ P_{n-1} + H_2O & \rightarrow & P_n \end{array} \tag{1}$$

where P denotes protein, the subscripts, 0–n represent the number of water molecules linking to a protein molecule and n is related to temperature[18], pH[19] and the kinds of proteins and salts[20–22]. The changes in the concentration of water in the usual separations of proteins by HIC are not very great. Therefore, it may be considered that the changes in n are not significant and it can be assumed that the hydrate–protein complexes in the mobile phase exist mainly as one species, P_i ($0 < i < n$). It also appears that a few hydrate–protein species occur in which the number of water molecules linked to the protein molecule are around i. For convenience, their mean value $\bar{m}$ is taken as the following, based on the definition of the mean value of water molecules for a series of hydrated ions[23]:

$$\bar{m} = \frac{[P_1] + 2[P_2] + \ldots + n[P_n]}{[P_0] + [P_1] + [P_2] + \ldots + [P_n]} \tag{2}$$

where the square brackets represent molar concentrations.

A simplified expression of eqn. 2 is

$$P_0 + \bar{m}H_2O \rightleftharpoons P_m \tag{3}$$

The ligands on the surface of the HIC column can also adsorb water and form a hydrated ligand complex:

$$L_0 + rH_2O = L_d \tag{4}$$

where L_0 is the bare ligand, L_d is the hydrated ligand and r is the number of water molecules bonding to a ligand in salt solution.

There are some hydrophobic amino acid residues on the surface of proteins and their distribution may be heterogeneous. As a result, some hydrophobic regions on the surface of a protein may interact with the stationary phase. The stronger the

hydrophobicities of these hydrophobic regions, the greater is the probability of contact with the stationary phase. Consequently, the hydrophilic side of protein molecules is far away from the stationary phase. This is the orientation action of protein molecules during its adsorption process. The orientation action of protein molecules depends mainly on the distributive heterogeneity of hydrophobic regions on the surface of the protein molecules and the chromatographic conditions. When experimental conditions are given, they relate only to the specific kinds of proteins.

Each ligand on the HIC column usually consists of two or three groups with different polarities and the end group of each ligand on the surface is the same. For convenience, assume that each ligand may be considered as a structural unit with a moderate polarity and the moderate hydrophobicity of the HIC column is the total result of these ligands, the distribution of which on the surface of the stationary phase may be considered to be homogeneous.

Compared with the size of the ligand noted above, a molecule of a protein is very large and has many hydrophobic regions, which may make contact with many ligands. Therefore, when protein molecules are adsorbed by an HIC column, the interactions between the stationary phase in HIC and protein molecules may be considered as if the latter had interacted with many of the same particles distributed homogeneously in a salt solution.

For convenience, the assumption in RP-LC is that the conformations of protein molecules in the mobile phase and on the stationary phase are identical[24,25]. However, this assumption is not valid for HIC. Pahlman *et al.*[26] proved that there is a difference by circular dichroism. This difference depends on the kinds of salts and ligands and also the characteristics of the protein[26–30]. This is because the conformation of the protein molecules in mobile phase is only affected by salt and water, whereas on the stationary phase it is influenced by ligands, salts and water molecules. Compared with the mobile phase, the surface of the HIC column is a relatively apolar region where the ligands can interact strongly with the hydrophobic regions on the surface of protein molecules with a three- or four-dimensional structure and draws these hydrophobic regions near the contact area towards the ligands and, as result, increases the contact surface area between the stationary phase and protein molecules. The conformation of the protein molecules in adsorbed state is therefore different from that in the mobile phase. The more apolar are the ligands, the stronger is the drawing force, and the greater are the changes in the conformation of the protein molecules[31–33]. The changes in the conformation of protein molecules also cause changes in the number of water molecules linked to the protein molecules[11,27,28]. From the foregoing discussion, it may be concluded that the conformation of protein molecules in the adsorbed state is different from that in the mobile phase under the elution conditions.

Suppose that both the conversion of the conformations of protein molecules between two states and the conversion between adsorption and desorption are rapid and reversible processes, the displacement of protein by water molecules in an aqueous solution may be given by

$$P_b + ZH_2O \rightleftharpoons P_m + nL_d \tag{5}$$

where P_m is the hydrated protein in the mobile phase, Z is the number of water molecules needed to displace a protein molecule adsorbed on the stationary phase and

n is the number of ligand interactions with a protein molecule. Here we mention again that the difference between P_b and P_m in eqn. 5 is not only with regard to the number of water molecules linked to the protein molecule, but their molecular conformations.

In order to elucidate clearly the physical meanings of each term in the foregoing equations and understand fully all of the changes in the conformations of proteins and the number of water molecules bonding to proteins as the environment around ligands and proteins changes, suppose the retention process of a protein on an HIC column involves the following steps.

(1) When a protein molecule interacts with ligand under vacuum, the conformation of the protein molecule under vacuum, P_0, is different from that in the adsorbed state, P'_a, then,

$$P_{0(\text{vacuum})} \rightleftharpoons P'_{a(\text{adsorbed state})} \tag{6}$$

If the number of ligands interacting with P'_a is assumed to be n_w, then we have

$$P'_a + n_w L_0 \rightleftharpoons P'_b \tag{7}$$

where P'_b represents protein–ligand complex in a vacuum environment.

(2) Suppose equilibrium 6 occurs in a system consisting of pure water, all the ligands, proteins and the conformations of protein molecules in both vacuum and pure water are identical and the proteins, ligand and their complex interact only with water molecules. The hydration process noted above and the reversible process of adsorption and desorption for proteins may be written as

$$P_0 + m_w H_2O \rightleftharpoons P_m^w \tag{8}$$

$$L_0 + r_w H_2O \rightleftharpoons L_d^w \tag{9}$$

$$P'_b + (m_w - b_w - q_w)H_2O \rightleftharpoons P_b^w \tag{10}$$

where P_m^w, P_b^w and L_d^w are hydrated protein, hydrated protein–ligand complex and hydrated ligand in pure water, respectively. When P'_b interacts with water, the number of water molecules bonding to it should be equal to the mean number of water molecules of hydrated protein molecule in pure water, m_w, minus the decreased number of water molecules due to the changes in the conformation of protein molecule b_w and the number of it released, q_w, from the contact surface area between hydrated protein molecule and ligands during the hydration process of protein in the adsorbed state.

When the hydrated ligands adsorb a hydrated protein molecule, r_w and q_w water molecules are released from the interface between the ligands and each protein molecule, respectively. Because the chromatographic process is a reversible adsorption–desorption process, the number of water molecules released from the interface between hydrated ligands and each hydrated protein as adsorption occurs should be equal to the water molecules adsorbed by both as desorption occurs. Suppose desorption of a protein molecule from the stationary phase occurs in two ways: (i) the conformation of the protein molecule remains the same for both the adsorbed state

P_b^w and the desorbed state P_a^w; (ii) the adsorbed and desorbed states at the moment of desorption of the protein vary in their conformations. For the former,

$$P_b^w + (r_w n_w + q_w)H_2O \rightleftharpoons P_a^w + n_w L_d^w \tag{11}$$

and for the latter,

$$P_a^w + b_w H_2O \rightleftharpoons P_m^w \tag{12}$$

Eqn. 12 shows that the desorbed state P_m^w has b_w water molecules more than the adsorbed state P_a^w as water is selected as the mobile phase.

(3) If the mobile phase is an aqueous salt solution, the presence of salt will change both the molar concentration of water in mobile phase and the conformation of the protein[13,26]. The changes in the conformation of protein molecule cause changes in its surface area, and also the contact area and the number of water molecules linked to it[19,27,28]. When a hydrated protein in water P_m^w is transferred into an aqueous salt solution or the usual mobile phase in HIC, a hydrated protein P_m^s in the latter instance will lose m_s water molecules:

$$P_m^w \rightleftharpoons P_m + m_s H_2O \tag{13}$$

In addition, the presence of a salt will influence the number of water molecules linked to each ligand which will be covered by protein molecules:

$$L_d^w \rightleftharpoons L_d + r_s H_2O \tag{14}$$

where L_d^w is the hydrated ligand complex in water, L_d is that in an aqueous salt solution and r_s is the change in the number of water molecules between the two states.

The change in the conformation of protein molecules due to the presence of salt causes a change in the contact surface area, and consequently the change in the number of ligands, n', linked to each protein molecule is

$$P_b^w + (q_s - b_s + rn')H_2O \rightleftharpoons P_b + n'L_d \tag{15}$$

where q_s is the decreased number of water molecules on the contact surface area as salt is added to water and b_s is that due to the conformation difference of each protein molecule in water P_b^w and the aqueous salt solution P_b. The term r denotes the difference between r_w and r_s, *i.e.*, $r = r_w - r_s$. When the mass action law can be used to describe the quantitative relationship for the foregoing eight thermodynamic equilibria (eqns. 6–10 and 13–15) in which the equilibrium constants are K_a, K_b, K_c, K_d, K_e, K_f, K_g and K_h, respectively, then

$$K = \frac{K_h}{K_f K_g^{n_w}} \cdot \frac{K_a K_b K_e}{K_c K_d^{n_w}} \tag{16}$$

or

$$K = \frac{[P_b]}{[P_m][L_d]^{n_w - n'}} [H_2O]^{(r_w n_w + b_w + q_w - q_s + b_s - n'r - r_s n_w - m_s)} \quad (17)$$

suppose

$$r_w - r_s = r \quad (18)$$
$$n_w - n' = n \quad (19)$$
$$r_w n_w + b_w + q_w = Z_{H_2O} \quad (20)$$
$$m_s + r_s n_w + q_s - b_s + rn' = Z_s \quad (21)$$
$$Z_{H_2O} - Z_s = Z \quad (22)$$

where Z denotes the number of water molecules released as a protein molecule is adsorbed by the HIC column, Z_{H_2O} represents the Z value as water is used as the mobile phase and Z_s is the change in the number of water molecules as protein molecules are transferred into an aqueous salt solution from water. For a given chromatographic system, when the salt, ligand and temperature are fixed, Z will be a characteristic constant of a protein.

Combining eqns. 17–22,

$$K = \frac{[P_b]}{[P_m][L_d]^n} [H_2O]^Z \quad (23)$$

Eqn. 23 is an exponential expression of eqn. 5.

In HPLC, the capacity factor of a solute, k', may be related to the partition coefficient, K_p, of the solute in both solid and liquid phases and the phase ratio, φ, of the column:

$$k' = K_p \varphi \quad (24)$$

From eqns. 23 and 24,

$$K_p = \frac{[P_b]}{[P_m]} = \frac{K [L_d]^n}{[H_2O]^z} \quad (25)$$

or

$$k' = K [L_d]^n \varphi / [H_2O]^z \quad (26)$$

When both the temperature and column are given, K and φ are constants. Suppose that the changes in the range of $[H_2O]$ is not too large, the term $[L_d]$, as that in the previous[24], may still be considered as approximately constant.

Assuming

$$I = K [L_d]^n \varphi \quad (27)$$

then combining eqns. 26 and 27,

$$\log k' = \log I - Z \log [H_2O] \quad (28)$$
$$\log I = \log K + n \log [L_d] + \log \varphi \quad (29)$$

Because both $\log I$ and Z in eqn. 28 are constants, eqn. 28 is linear and the plot of $\log k'$ *vs.* $\log [H_2O]$ should be a straight line. Although there are more equilibria in HIC than in RP-LC, eqn. 28 is the same expression as in RP-LC[24]. However, the Z values contain different terms in each instance.

EXPERIMENTAL

Equipment

An LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) was used, consisting of three pumps (LC-6A), a detector (SPD-6A V, variable-wavelength UV–visible), column oven (CTO-6A), controller system (SCL-6A), data system (CRT-3A) and recorder (R-112). Stainless-steel columns (5–10 cm × 4.1 mm I.D.) were used, packed with a slurry-packing apparatus (Chemico, Japan). A freezing system was set up *in situ*. A ZD-2 pH-meter was obtained from Second Analytical Instrument Co. (Shanghai, China).

Packings

Silica (7 μm, 500 Å) was removed with hydrochloric acid and then linked to two different ligands. Both ligands consist of a modified ether chain with the same ligand density, but the hydrophobicity of the first end-group is weaker than that of the second. The former is named HIC-I (alcohol group) and the latter HIC-II (keto group). The two kinds of columns were used continuously for 8 months without a significant decrease in the column efficiency.

Chemicals

Myoglobin (MYO, horse heart), cytochrome *c* (Cyt-*c*, horse heart, type III), α-chymotrypsinogen-A (α-CTY-A, bovine pancreas, type II) and α-amylase (α-AMY, *Bacillus anthracis*, type II A) were purchased from Sigma (St. Louis, MO, U.S.A.). Bovine serum albumin (BSA), ribonuclease (RNase), lysozyme (Lys), egg white and ovalbumin (OVA) were obtained from the Institute of Biochemicals (Shanghai, China). The other chemicals used were of analytical-reagent grade. Water was re-distilled (quartz).

Procedures

All retentions were determined with isocratic elution with a flow-rate of the mobile phase of 1.0 ml/min and the detection at 280 nm. The temperature of the column was adjusted by the column oven and a freezer. Each concentration was obtained by mixing a salt solution with a high concentration (A) and a low concentration of salt or water (B) by the SCL-6A controller. Before injecting a sample solution, the column was brought to equilibrium with 20 column volumes of new mobile phase. The dead volume of the column was determined as the minimum retention volume of the same protein by using a series of mobile phases with different

concentrations of water, including pure water. The capacity factor of a protein, k', is calculated as

$$k' = (t_R - t_0)/t_0 \tag{30}$$

where t_R is the retention time and t_0 is the dead time.

In order to prevent abnormal retentions of proteins owing to the very low salt concentration[34], the minimum concentration used here was not lower than 0.50 *M*.

The concentration of water [H_2O] is calculated as

$$[H_2O] = \frac{d_A\varphi_A + d_B\varphi_B - W_s}{0.018} \text{ (mol/l)} \tag{31}$$

where d_A and d_B are the densities of solutions A and B, respectively, φ_A and φ_B are the volume fractions (v/v) of solutions A and B, respectively, and W_s is the weight of salt in mixed solution. For example, when a mixture of 40% B (water) and 60% A [3 *M* $(NH_4)_2SO_4$] as used as the mobile phase at 20°C (d_A = 1.189 g/ml, d_B = 1.000 g/ml, $MW_{(NH_4)_2SO_4}$ = 132.1), the concentration of water is 48.64 *M*.

For different solutions of salts, when the concentration is 1.00 *M*, the corresponding concentration of water and the difference, ΔH_2O, between pure water and the solution are as listed in Table I.

Determination of adsorption isotherm

The method for the determination of the adsorption isotherm used in this paper is according to frontal elution as described by Horváth and co-workers[35,36]. When the equilibrium concentration of proteins in mobile phase is P_m, the concentration of proteins adsorbed on the stationary phase [P_b] is calculated as

$$P_b = P_{b-1} + \frac{([P_m] - [P_{m-1}])(V_F - V_D)}{V_{sp}} \text{ (mg/ml)} \tag{32}$$

where [P_m] and [P_{m-1}] are two different equilibrium concentrations of protein in mobile phase, P_{b-1} is the concentration of proteins adsorbed on the stationary phase when the equilibrium concentration of proteins in the mobile phase is P_{m-1}, V_F is the frontal retention volume, V_D is the dead volume of the system including column hold-up volume and V_{sp} is the volume of the packing in the column. The correlation coefficient, *C*, for linear regression analysis was taken to four digits. When *C* was greater than 0.99995, it was simply rounded to 1.000.

TABLE I

MOLAR CONCENTRATIONS OF WATER AND ITS CHANGES IN 1.00 *M* SALT SOLUTIONS

Parameter	*Na_2SO_4*	*$(NH_4)_2SO_4$*	*NaCl*	*KCl*	*NaBr*	*NH_4Cl*
M_{H_2O}	54.02	52.16	54.45	53.88	54.09	53.39
ΔH_2O	1.54	3.40	1.11	1.68	1.47	2.17

RESULTS AND DISCUSSION

Linear plot of log k' vs. log [H_2O]

In accordance with eqn. 28, the plot of log k' *vs.* log [H_2O] should be linear. So long as the displacement between a protein and water molecules is reversible and stoichiometric, this linear relationship should exist in all cases. Fig. 1a–d show these cases for (a) the different salts, (b) temperature changes, (c) two kinds of columns and (d) various pH values, the other experimental conditions remaining fixed in each instance. All the correlation coefficients, C, for linear regression analysis are larger than 0.99. Hence the experimental results fitted the expected values well.

Compared with a few popular models or plots in HIC, as shown in Fig. 2a–d, the linear relationships are straight lines in all instances: (a) SDM-R in this work; (b) ln k' *vs.* m (m = molar concentration of salt) by Horváth and co-workers[3,37]; (c) log k' *vs.* log $(1/X_{[H_2O]})$ by Zhao *et al.*[13] (X_{H_2O} = molar fraction of water); and (d) log k' *vs.* log φ_B (φ_B = volume fraction of strong eluent for gradient elution) by Karger and co-workers[11,12]. We selected eight kinds of proteins and measured their k' values

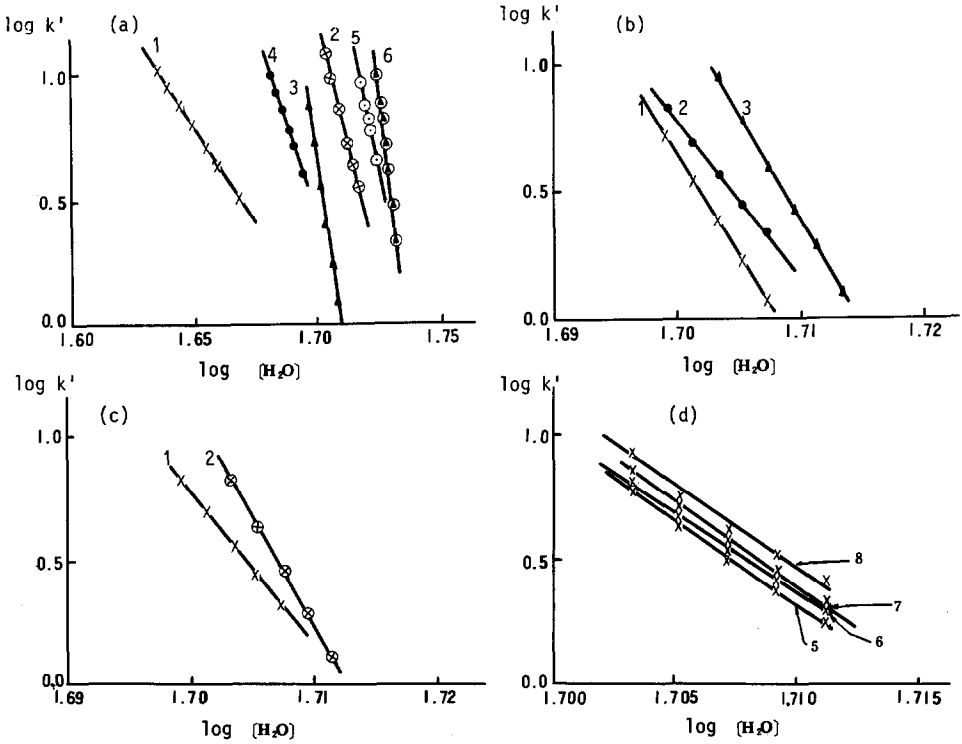

Fig. 1. Plot of log k' *vs.* log [H_2O] for lysozyme with different chromatographic conditions. (a) Lysozyme with various aqueous salt solutions. HIC-I column, 30°C, pH = 7. 1 = NH_4Cl; 2 = KCl; 3 = $(NH_4)_2SO_4$; 4 = NaBr; 5 = NaCl; 6 = Na_2SO_4. (b) Lysozyme at different temperatures. HIC-I column, $(NH_4)_2SO_4$–0.01 M KH_2PO_4, pH = 7. 1 = 10°C; 2 = 30°C; 3 = 50°C. (c) Lysozyme with different columns. $(NH_4)_2SO_4$–0.01 M KH_2PO_4, pH = 7, 30°C. 1 = HIC-I column; 2 = HIC-II column. (d) Lysozyme at pH values 5, 6, 7 and 8, as indicated. HIC-I column, 30°C, $(NH_4)_2SO_4$–0.01 M KH_2PO_4.

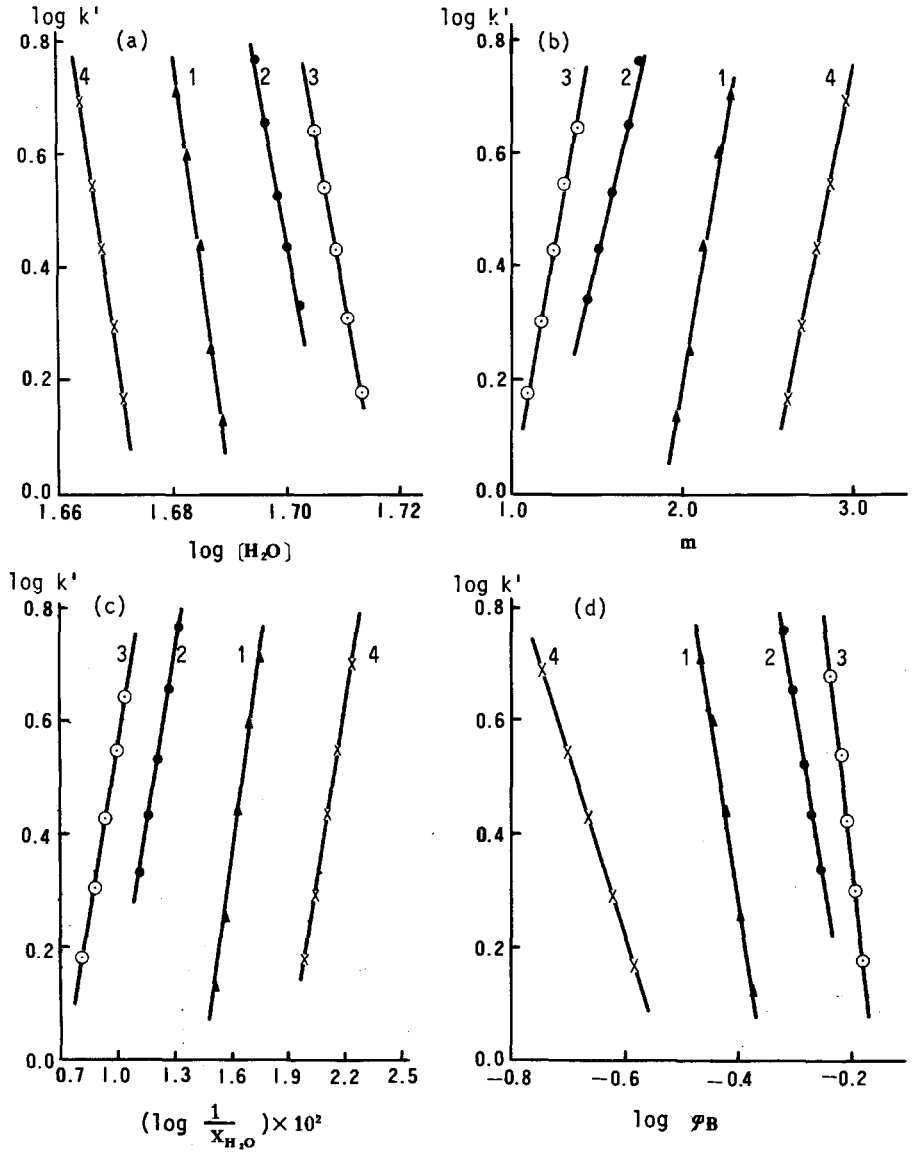

Fig. 2. Comparisons of four models or plots in HIC. (a) SDM-R in this work; (b) plots of log k' *vs.* m, from Melander *et al.*[3]; (c) plots of log k' *vs.* log $(1/X_{H_2O})$, from Zhao *et al.*[13]; (d) plots of log k' *vs.* log φ_B, from Karger and co-workers[11,12]. 1 = RNase; 2 = Lys; 3 = α-AMY; 4 = Cyt-*c*. HIC-I column, 0°C, $(NH_4)_2SO_4$–0.01 *M* KH_2PO_4, pH 7.

under the same experimental conditions. The same k' values were calculated with the foregoing four models (a–d). All the C values were larger than 0.99 and the agreement between the experimental and theoretical data may be considered to be satisfactory. Although such agreement is the most important criterion for evaluating a model, it is not the sole one, and the elucidation of some discrepancies with each of the above models needs detailed investigation.

Linear parameters log I and Z

When the intercepts are plotted against the slopes for each model or plot considered above, as shown in Fig. 3a–d, there is a good linear relationship only for the model presented in this paper (see Fig. 3a) with $C = 0.9998$. From previous studies[38–41] this linear relationship also exists in RP-LC for small molecules:

$$\log I = jZ + \log \varphi \tag{33}$$

where j is a constant related to the affinity of the solvent for the stationary phase in RP-LC and is independent of the kind of solute and temperatures changes[40]. The term

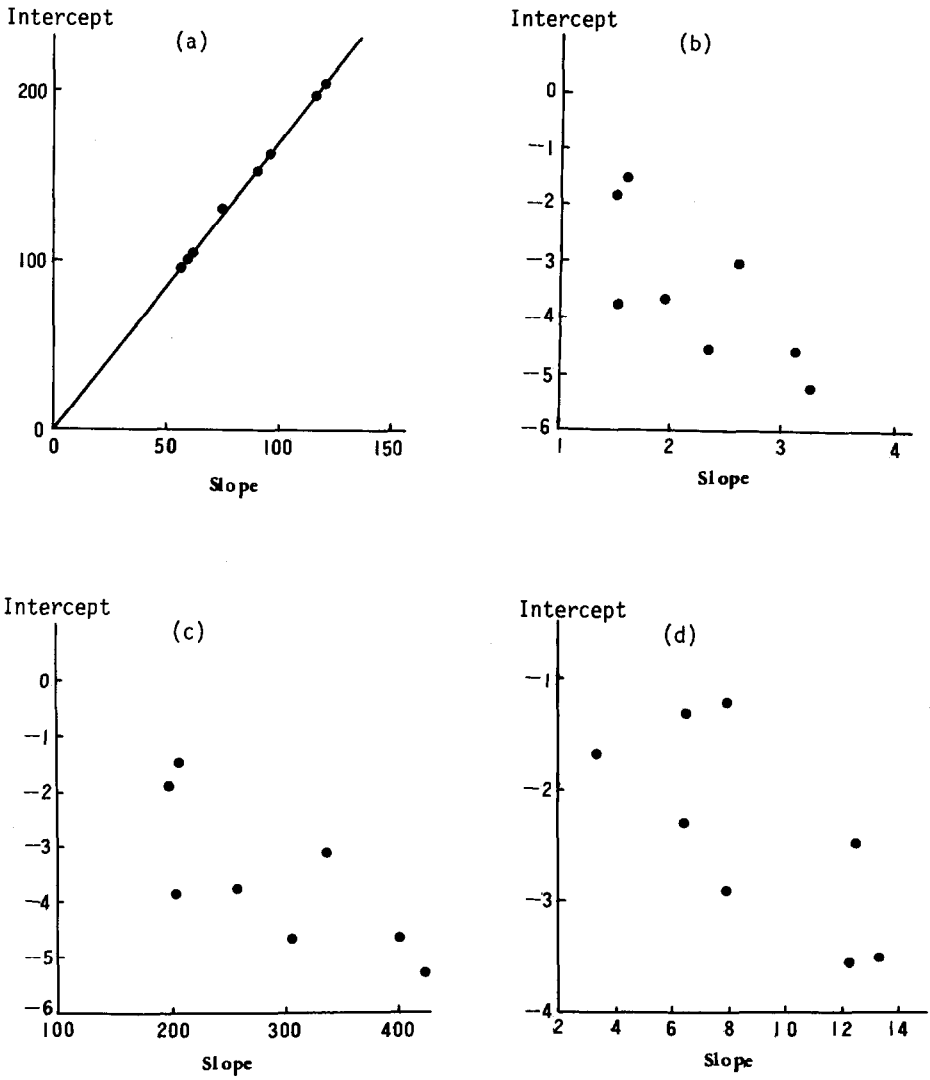

Fig. 3. Comparisons of plots of intercepts *vs.* slopes for each plot in Fig. 2. (a)–(d) as in Fig. 2.

φ is the phase ratio, which is defined from the point of view of thermodynamics[40], considering the value of the capacity factor of solute k' when the free energy of the solute in the displacement process equals to zero.

In order to confirm the applicability of this model, three different experimental conditions were selected to test eqn. 33 and these plots are shown in Fig. 4a–c. Although each log I or Z value for different cases is variable, each obeys the linear

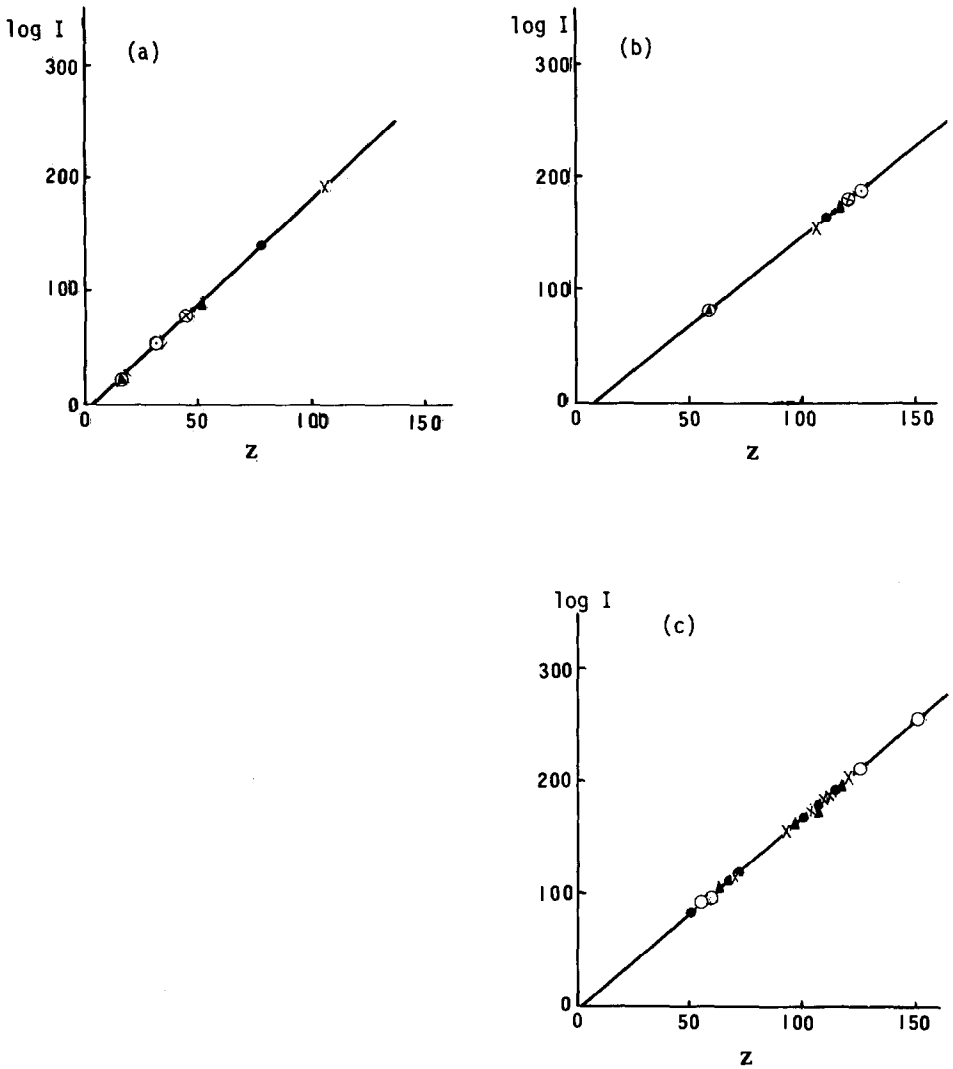

Fig. 4. Plots of log I *vs.* Z for the different cases. (a) Different mobile phases. Experimental conditions as in Fig. 1a. (b) Different temperatures. Experimental conditions as in Fig. 1b, but the temperatures are 0, 10, 20, 30, 40 and 50°C. (c) Different pH values. HIC-I column; $(NH_4)_2SO_4$–10 mM KH_2PO_4; seven kinds of proteins: Lys, Cyt-*c*, α-CTY-A, RNase, MYO, BSA and OVA, 30°C. pH: × = 5; ● = 6; ▲ = 7; ○ = 8.

relationship in eqn. 33. Further, we found that all 44 C values for these kinds of plots are larger than 0.9970 and, except for five C values which are less than 0.9990 and one equal to 0.9990, the other 38 C values are larger than 0.9995. Of the five cases where C was less than 0.9990, four involve temperature changes with the same protein.

Although the SDM-R was proved to be valid for normal-phase chromatography (NPC)[39], *i.e.*, eqn. 28 can be used to explain the quantitative relationship between k' and the concentration of strong solvent in the mobile phase, as shown in Fig. 5d, eqn. 33 is usually not valid as the selective force between silica and the solute predominates over the chromatographic behaviour. The linear plots in RP-LC have been tested and shown to be valid for small molecules (Fig. 5a) but not to be valid for

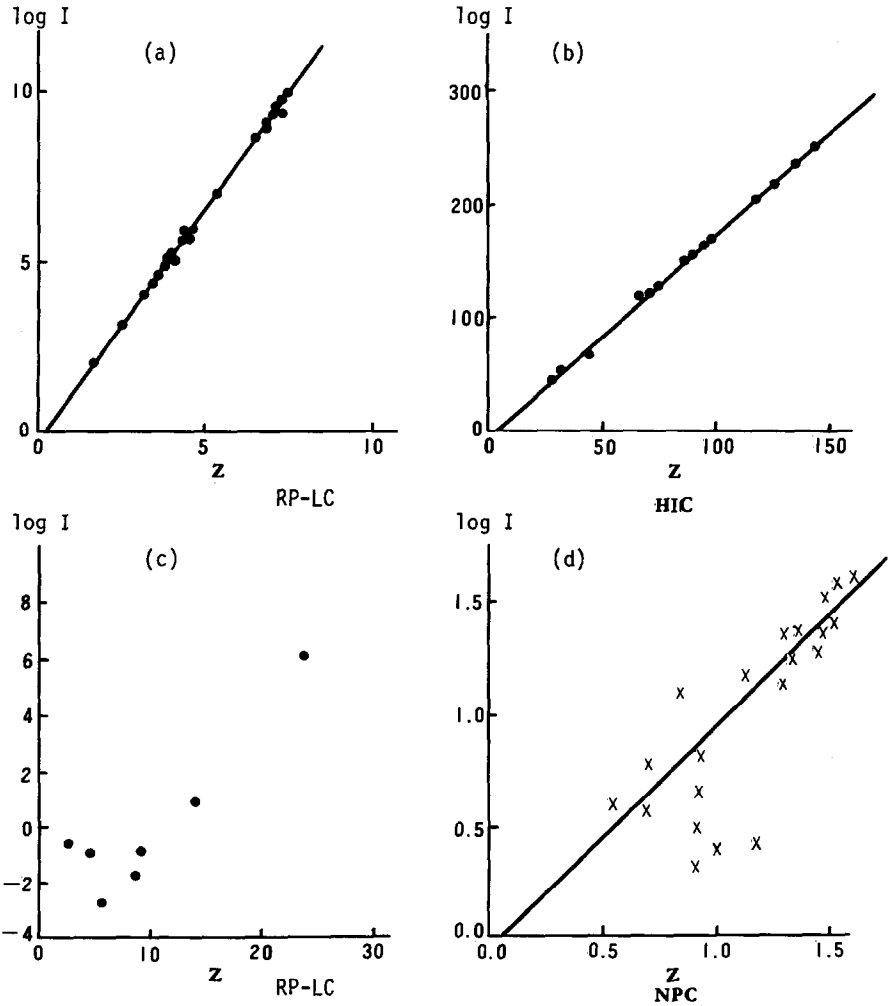

Fig. 5. Plot of log I *vs.* Z. (a) Small apolar solutes in RP-LC (original k' values were taken from ref. 38). (b) Proteins in HIC in this work. pH = 7, 30°C, four proteins (Lys, OVA, α-CTY-A and α-AMY) and four salt solutions [$(NH_4)_2SO_4$, NaCl, NH_4Cl and KCl]. (c) Proteins in RP-LC (data taken from ref. 24). (d) Small non-homologous solutes in NPC (ref. 39).

proteins (Fig. 5c), because the non-selective force controls the retention of small apolar or polar molecules. Fig. 5b shows this in HIC. Compared with both NPC and RP-LC, in accordance with Fig. 5a–d, it may be concluded that HIC is closer to RP-LC for solutes with small molecules and it seems that non-selective forces predominate over the interactions between the bonded phase and protein molecules in HIC or the hydrophobic interaction force has the characteristics of a non-selective force. Further, the stoichiometric displacement of protein molecules to water molecules is a very similar process to that of the displacement of small molecules including apolar and polar small molecules to organic solvent molecules in RP-LC. If this conjecture is reasonable, the method of investigating the retention mechanism of small apolar molecules on RP-LC columns may be applied to that of protein molecules on an HIC column.

The statistical result for j values from the experiments is a constant 1.74 whereas the j values on the HIC-I column (alcohol group) and the HIC-II column (keto group) are 1.77. These results are reasonable. The difference in j values between the two columns is only 0.03, but it is significant statistically. This result is consistent with the j value being a constant independent of the kinds of proteins and salts, pH and temperature but dependent on the type of column.

The most reasonable values of j should be the constant for the affinity of 1 mol of water to the HIC column without any influence of salts. When pure water is used as the mobile phase, the foregoing experimental conditions may be satisfactory. Inserting eqn. 33 for log I into eqn. 28, we obtain

$$\log k' = Z(j - \log [H_2O]) + \log \varphi \tag{34}$$

From eqn. 34 we know that, provided that

$$j = \log [H_2O] \tag{35}$$

then

$$\log k' = \log \varphi$$

In other words, the retention of protein in this case is due not to the protein itself but to pure water. If the retention mechanism of proteins in HIC as a stoichiometric displacement process between the protein and water molecules is true, then the logarithm of the molar concentration of pure water should be the most reliable value of j in an HIC system for the reasons discussed above. Table II gives the experimental and theoretical j values in HIC. In order to confirm the reliability of the foregoing conjectures, some j values from the experiments and expected values in RP-LC published in the literature are also given in Table II. The experimental and theoretical j values agree well.

Log k' and Ostwald absorption coefficient

It was pointed out above that we may use the method for the investigation of the chromatographic behaviour of small molecule in RP-LC to study that of protein molecules in HIC. Some workers have correlated the relationship between the k' values

TABLE II

COMPARISONS OF j AND THE LOGARITHM OF THE CONCENTRATIONS OF THE PURE DISPLACING AGENTS IN HIC AND RP-LC

Mobile phase	*Displacing agent*	*M (pure)*	*Log M*	*j*	*Ref.*
Methanol–water	Methanol	24.75	1.39	1.44	40
				1.42	40
				1.40	40
				1.38	38
				1.41	40
				1.35	39
2-Propanol–water	2-Propanol	13.06	1.17	1.14	40
THF[a]–water	THF[a]	10.23	1.01	1.03	40
ACN[b]–water	ACN[b]	19.07	1.28	1.29	40
				0.94	40
Salt–water	Water	55.56	1.74	1.74	

[a] Tetrahydrofuran.
[b] Acetonitrile.

of solutes in RP-LC and their partition coefficients in liquid–liquid extraction. The influence of salts on the k' values of proteins in HIC is of interest. The Ostwald absorption coefficients, γ_s, of methane or argon in water or solutions of salts in water and other solvents[42] have been considered as a criterion to describe the effects of salts on the free energy of transfer of a solute between two phases. In other words, γ_s can be used to explain quantitatively the influences of each salting-out effect on the partition coefficient of methane or argon. Some γ_s values are listed in Table III. The term k' in HIC has the same function of elucidating the energy of transfer of proteins between two phases. The γ_s value represents the interactions among molecules in an electrolyte solution. For the apolar solute methane, the interactions should be the hydrophobic interaction force. If the same salt solution used to investigate the γ_s value is used as the mobile phase in HIC, and the ligands on the HIC column are considered to be equivalent to a mechanically held moderately apolar liquid phase in liquid–liquid extraction, then the interactions among protein and ligand molecules can also be

TABLE III

OSTWALD ABSORPTION COEFFICIENT FOR METHANE IN VARIOUS SALT SOLUTIONS

Conditions: 25°C and 1 atm. From ref. 42.

Salt solution	$\gamma_s \times 10^3$
1 *M* NaCl	24.2
1 *M* KCl	24.9
1 *M* NH_4Cl	27.9
Pure H_2O	34.1

considered to be the same as the interactions between an organic liquid and argon. Provided that the kinds and concentrations of salts in both the aqueous solution for measuring γ_s and the mobile phase for measuring k' in HIC are the same, k' and γ_s should have a parallel relationship, because the same interaction force should show the same characteristics[37,43]. We found that there is a linear relationship between the log k' values of some proteins in HIC and log γ_s for methane in 1.00 M solutions of NaCl, NH_4Cl and KCl (results not shown). The linear relationship between log k' and log γ_s explains why the hydrophobic force mainly dominates the chromatographic behaviour of proteins in HIC.

Adsorption isotherm

The study of the adsorption isotherm of a solute on a stationary phase in RP-LC is an important tool for investigating the retention mechanism of a solute under the same chromatographic conditions and is receiving increasing attention. In a previous paper[14] it was concluded that the quantitative relationship between the concentration of a solute on the stationary phase and the equilibrium concentration in solute may be expressed as:

$$\log K_p = \beta_C + (q/Z) \log (1/[P_m]) \tag{36}$$

or

$$\log [P_b] = \beta_C + (nr/Z) \log [P_m] \tag{37}$$

where K_p is the partition coefficient of the solute in both phases, β_C contains a number of constants related to the affinity of the solute to the adsorbent, $[P_m]$ is the equilibrium concentration of solute in mobile phase, $[P_b]$ is the concentration of solute on the adsorbent and n, q, r and Z have the same meanings as before. The term q is the number of water molecules displaced from the contact surface area of the protein when it adsorbs to a HIC support.

Both eqns. 36 and 37 are expressions of the stoichiometric displacement model of adsorption (SDM-A) for solutes[14] and eqn. 37 is equivalent to the Freundlich empirical equation mathematically. In other words, the Freundlich empirical equation can be derived by SDM and gives the exact physical meaning of each term. Further, if an adsorption isotherm for a solute in a liquid–solid system belongs to the Freundlich type, its adsorption mechanism can be shown to be a stoichiometric displacement of solute to solvent molecules. Although Jennisson[8,44] reported that the adsorption isotherms of phosphorylase kinase on alkylamine–Sepharose derivatives obtained by a static method are of the Freundlich type, the results obtained by the static method are still far from valid for a complicated chromatographic system.

Horváth and co-workers[35,36] presented a dynamic method with frontal elution for measuring the adsorption isotherms of solutes. Their results should be much closer to real chromatographic conditions. We used their dynamic method for the HIC system, column–lysozyme–$(NH_4)_2SO_4$–KH_2PO_4 (pH 7.0), to obtain the Freundlich-type isotherm as shown in Fig. 6. The results demonstrate that the retention mechanism of proteins in HIC obeys the SDM-R.

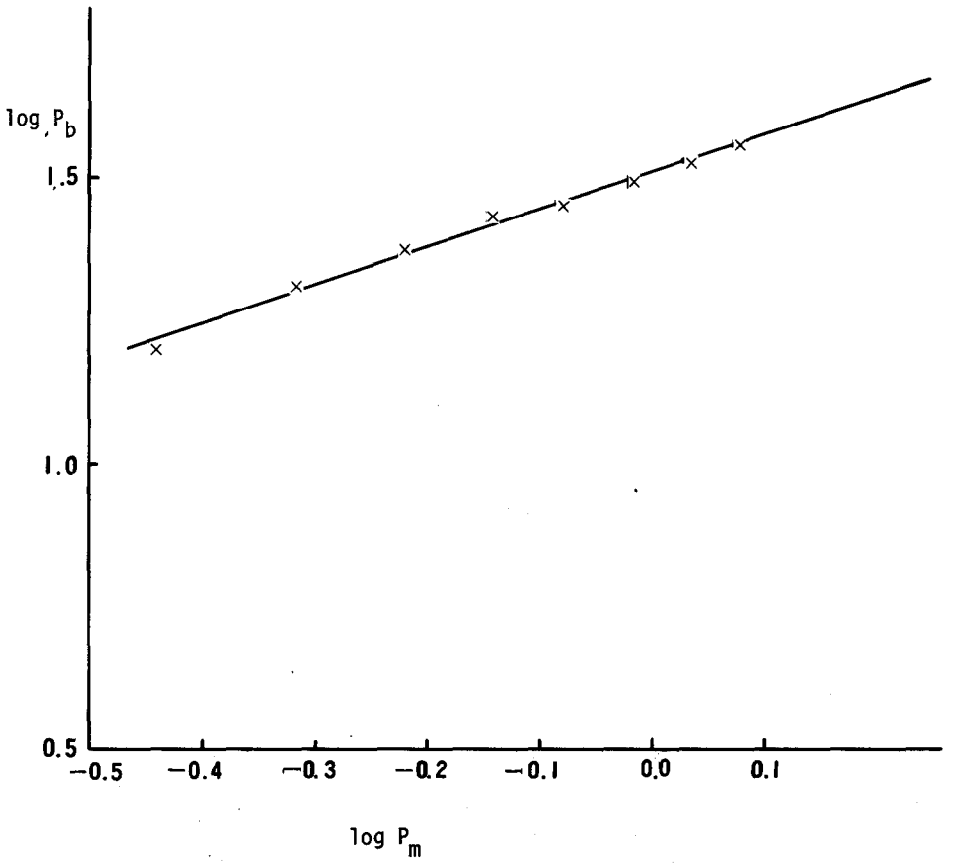

Fig. 6. Adsorption isotherm of lysozyme on HIC-I column (room temperature). $(NH_4)_2SO_4$–0.01 *M* KH_2PO_4, pH 7.

Functions of salts

As pointed out under Theoretical, the most important role of salt is as a diluent. According to Table I, the ability to act as a diluent for salts of concentration 1.00 *M* decreases in the order $(NH_4)_2SO_4 > NH_4Cl > KCl > Na_2SO_4 > NaBr > NaCl$. This order shows that the larger the changes in the concentration of water or ΔH_2O (also shown in Table I), the broader is the potential range of gradient elution in HIC, or the better is the mobile phase. From Table I it may be concluded that ammonium sulphate is the best diluent for making mobile phases in HIC. As discussed under Theoretical, the adsorption ability of proteins on an HIC column depends on the kind of salts and may be measured with log *I*. The adsorption of a particular protein decreased in the order $Na_2SO_4 > (NH_4)_2SO_4 > NaCl > KCl > NaBr > NH_4Cl$, as shown in Table IV.

The effect of salts on the adsorption ability may be attributed to the values of log *I*. As shown in eqns. 23 and 29, salts will affect the thermodynamic equilibrium constants *K* and the concentration of water $[L_d]$ on the surface of an HIC packing in addition to the number, *n*, of water molecules released from this surface. This involves

TABLE IV

VALUES OF Z AND LOG I FOR SDM-R ON AN HIC-I COLUMN WITH DIFFERENT SALT SOLUTIONS

Conditions: 30°C, pH = 7.00.

Salt	*Lys*		*OVA*		*α-CTY-A*		*α-AMY*	
	Z	*Log I*	*Z*	*Log I*	*Z*	*Log I*	*Z*	*Log I*
Na_2SO_4	110.1	192.1	–	–	263.9	459.1	369.0	641.4
$(NH_4)_2SO_4$	79.9	136.4	118.3	202.1	104.7	180.0	118.3	203.6
NaCl	50.1	87.0	50.1	86.0	89.4	154.6	64.8	111.6
KCl	45.1	78.0	45.1	77.8	66.6	114.6	67.4	115.6
NaBr	31.1	53.4	29.5	50.6	39.4	67.3	–	–
NH_4Cl	16.4	27.8	17.6	29.8	25.3	43.0	–	–

the kinds of HIC packings and proteins and also the composition of the mobile phase. When a chromatographic system is given, it depends only on the kind of protein.

The effect of salts on the conformation of protein molecules and various hydrations, such as ligands, proteins and some protein complexes, may cause a change in the total Z values, as shown in eqns. 18–22.

As shown in eqn. 21, Z includes many terms, and so far, we do not have the ability to measure all of them. However, from eqn. 28 the Z value may be measured accurately and some Z and log I values are listed in Table IV. The decrease in the order of Z values for a particular protein is Na_2SO_4 > $(NH_4)_2SO_4$ > NaCl > KCl > NaBr > NH_4Cl. This order is identical with that for the adsorption ability discussed above, and eqn. 32 already proves it theoretically.

In Summary, the effect of salts on the retention of proteins in HIC includes changes in the concentration of water, hydrophobicity (or adsorption affinity) and the conformation of protein molecules in addition to the hydration of ligands, protein molecules and ligand-protein complexes, *i.e.*, salts may affect each term on the right-hand side of eqn. 28, log I, Z and $[H_2O]$.

CONCLUSIONS

We believe that the most rational retention mechanism of proteins in hydrophobic interaction chromatography is a stoichiometric displacement process between protein and solvent molecules, with water as displacing agent. The adsorption of a protein molecule should be accompanied by the release of a stoichiometric number of water molecules to the solution from the interface between the protein molecule and the HIC ligands.

The mathematical model expressing the stoichiometric displacement process of retention may be described by saying that the logarithm of the capacity factor of the protein is proportional to the logarithm of the concentration of water in the mobile phase.

The stoichiometric number of water molecules Z displaced by one protein molecule is believed to involve in many factors, such as the characteristics of the

ligands, proteins, salts, many kinds of hydrations and the conformations of protein molecules.

The parameter for measuring the adsorption ability of proteins on HIC columns, log I, also measures the hydrophobic interactions between ligands and protein molecules.

The excellent linear relationship between Z and log I shows that HIC is closer to RP-LC or, more exactly, closer to the RP-LC of small molecules. The method of investigating the contribution of hydrophobic interaction forces to the retention of proteins in HIC by following the method used to study the relationship of the Ostwald absorbent coefficient in the same solutions of salts is valid and parallel relationships were found between log k' and log γ_s for 1.00 M solutions of various salts.

The functions of salts in HIC are believed to be as diluents and as reagents to change the affinity of proteins to ligands and the conformation of protein molecules, and also various hydrations such as of ligands, protein molecules and a series of protein complexes.

The adsorption isotherm of proteins on an HIC column was demonstrated to be of the Freundlich type by a dynamic chromatographic method, and showing again that the adsorption mechanism of proteins on an HIC column is a stoichiometric displacement process.

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SYMBOLS

b_w	changed number of water molecules linked to a protein molecule due to its different conformations between the adsorbed and desorbed states in pure water.
b_s	changed number of water molecules linked to a protein molecule due to its different conformations in the adsorbed state between pure water and salt solution.
C	correlation coefficient for linear regression analysis.
d_A, d_B	densities of eluates A and B for gradient elution, respectively.
I	contains a number of constants related to the affinity of a protein to the HIC column.
j	constant related to the affinity of water to the HIC column.
K	general equilibrium constant of the stoichiometric displacement process.
K_a, K_b, K_c, K_d, K_e, K_f, K_g	individual equilibrium constants during the displacement process.
K_p	partition coefficient of a protein between two phases.
k'	capacity factor.

L_0	bare ligands.
L_d, L_d^w	hydrated ligands in salt solution and pure water, respectively.
m	molar concentration of salt.
$\bar{m}$, m_w	mean numbers of water molecules linked to a protein molecule in salt solution and pure water, respectively.
m_s	changed number of water molecules linked to a protein molecule between pure water and salt solution, *i.e.*, $m_s = m_w - \bar{m}$.
n, n_w	numbers of ligands bonded to a protein molecule in salt solution and pure water, respectively.
n'	changed number of water molecules linked to a protein molecule between pure water and salt solution, *i.e.*, $n = n_w - n'$.
P_0	bare protein.
P'_a, P_a^w	protein at the moment it just desorbs from ligands and their molecular conformations are in an unstable state in vacuum and pure water, respectively.
P_b, P'_b, P_b^w	proteins in the adsorbed state in salt solution, vacuum and pure water, respectively.
P_m, P_m^w	hydrated proteins in salt solution and pure water, respectively.
P_{b-1}	concentration of proteins adsorbed on the stationary phase when the equilibrium concentration of protein in the mobile phase is P_{m-1}.
P_{m-1}	equilibrium concentration of protein in salt solution.
q	number of water molecules displaced from the contact surface area of the protein when it adsorbs to a HIC support.
q_w, q_s	changed numbers of water molecules on the surface of a protein molecule linked to ligands in the presence of pure water and salt solution, respectively.
r, r_w	numbers of water molecules bound to a ligand in the presence of salt solution and pure water, respectively.
r_s	changed number of water molecules bonded to a ligand when salt is added into pure water, *i.e.*, $r = r_w - r_s$.
t_0	dead time.
t_R	retention time.
V_D	dead volume of a system including the column hold-up volume.
V_F	retention volume of frontal elution.
V_{sp}	volume of adsorbent in a chromatographic column.
W_s	weight of salt in solution.
X_{H_2O}	molar fraction of water.
Z, Z_{H_2O}	total numbers of water molecules released from the interface between the protein molecule and the HIC column in the salt solution and pure water, respectively.

Z_s	change in the number of water molecules when protein molecules are transferred from water into an aqueous salt solution.
β_C	contains a number of constants related to the affinity of the solute to the absorbent (see eqns. 36 and 37).
φ	phase ratio of a column.
φ_A, φ_B	volume fractions of solutions A and B in a mixed solution, respectively.
γ_s	Ostwald absorption coefficient.

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