Journal of Chromatography, 331 (1985) 1–10
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 847

AFFINITY CHROMATOGRAPHIC EXAMINATION OF A RETENTION MODEL FOR MACROMOLECULES

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(First received December 19th, 1984; revised manuscript received February 26th, 1985)

SUMMARY

Plots of log k' *vs.* log (1/[mobile phase modifier]) were made for a monovalent and a divalent solute using affinity chromatography. Some of the plots were curved and all exhibited slopes (Z values) of less than the theoretical integer values. It was shown that this was an expected result when lower forms of the solute were present, *e.g.*, a divalent solute adsorbed monovalently.

INTRODUCTION

The retention of macromolecules on various chromatographic stationary phases is a subject of considerable fundamental and practical interest. Knowledge of the mechanism of retention aids in the design of stationary phases with improved selectivity and in the choice of appropriate elution conditions. Several authors have described models for the retention of macromolecules in ion-exchange[1–4], reversed-phase[5–8] and hydrophobic interaction[9–11] chromatography. A parameter in many of these models is the number of sites on the surface of the macromolecule which adsorb to the stationary phase. Unfortunately, this number is seldom known from independent measurements so the models are difficult to verify.

Affinity chromatography provides a means to examine some aspects of these retention models since the stoichiometry and the binding constants between stationary phase ligand, analyte, and mobile phase modifier are sometimes known when competitive elution is used[12–15].

The model of interest here has been widely utilized for small solutes and more recently by Regnier and co-workers for ion-exchange[3,4], and reversed-phase[8] chromatography of proteins. In analogy with Regnier's work, we write the adsorption process as

$$EI_Z + Z \cdot L \rightleftharpoons EL_Z + Z \cdot I \quad (1)$$

where E is the macromolecule, I is the mobile phase modifier (inhibitor), and L is the immobilized ligand. One can then derive an equation for the capacity factor, k':

$$\log k' = \log c + Z \log (1/[I]) \quad (2)$$

0021-9673/85/$03.30

where c is a constant involving the equilibrium constant for reaction 1, the concentration of immobilized ligand, and the phase ratio. A plot of log k' *vs.* log (1/[I]) should have a slope equal to Z, the number of sites of adsorption[3]. This will be referred to as a log k' plot in this paper.

In affinity chromatography, E usually contains 1–4 binding sites. Steric considerations generally limit Z to no more than 2. In this study the validity of eqn. 2 was examined using concanavalin A (Con A) and various sugars.

EXPERIMENTAL

Reagents

Con A (type IV), bovine serum albumin (BSA), D(+)-glucosamine hydrochloride, *p*-aminophenyl α-D-mannopyranoside (PAPM), *p*-nitrophenyl α-D-mannopyranoside (PNPM), 4-methylumbelliferyl α-D-mannopyranoside (MUM), methyl α-D-mannopyranoside (MDM, grade III) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma (St. Louis, MO, U.S.A.). Orcinol monohydrate, succinic anhydride and 1,1'-carbonyldiimidazole (CDI) were obtained from Aldrich (Milwaukee, WI, U.S.A.). The 10-μm LiChrospher SI 500 was obtained from Rainin (Woburn, MA, U.S.A.). All other chemicals were reagent grade. Dioxane and acetonitrile were stored over molecular sieves.

Con A was further purified according to the procedure of Cunningham *et al.*[16] with two exceptions: dialysis of the Con A supernatant was against the mobile phase sodium acetate buffer instead of water and no lyophilization was done. Orcinol was purified according to the following procedure: 50 g of orcinol was dissolved in 100 ml of boiling water. The solution was cooled to room temperature and the white crystals were filtered and then washed with ice-cold water on a medium porosity glass filter. The filtrate was concentrated to one-third volume and then the recrystallization procedure repeated. The crystals were vacuum-dried at room temperature.

Apparatus

A Model 344 gradient liquid chromatograph (Beckman, Berkeley, CA, U.S.A.) and a V[4] variable-wavelength absorbance detector (ISCO, Lincoln, NE, U.S.A.) were used. Data was collected and processed on an Apple IIe computer via an ADALAB interface board (Interactive Microware, State College, PA, U.S.A.). A magnetic switch (Radio Shack) was attached to the injector to automatically initiate data collection. Columns were of a published design[17] with the outer connector modified as a water jacket. Column temperature was controlled by a Lauda K-2/RD refrigerated circulator (Brinkmann, Westbury, NY, U.S.A.).

A 100-W ultrasonic cleaner (Fisher, St. Louis, MO, U.S.A.) and a wrist-action shaker (Burrell, Pittsburgh, PA, U.S.A.) were used for the stationary phase preparation. A Haskel air-driven pump (Alltech, Deerfield, IL, U.S.A.) and a Model 705 stirred-slurry column packer (Micromeritics, Norcross, GA, U.S.A.) were used for column packing.

Stationary phase preparation

A CDI activation method[18] was used for the immobilization of PAPM. Diol-bonded LiChrospher SI 500 was prepared as described earlier[19]. An amount of 2.0

g of diol-bonded silica was activated by addition of 0.96 g CDI in 16 ml anhydrous acetonitrile, sonicated under vacuum for 10 min, and shaken for an additional 30 min at room temperature. The activated silica was washed with anhydrous acetonitrile and suction-dried over a medium porosity glass filter. To each of two test tubes was added 1 g activated silica, 4 ml of 0.1 *M* sodium phosphate buffer, pH 7, and 81 mg or 10 mg of PAPM. This reaction mixture was sonicated under vacuum for 10 min, flushed with nitrogen, stoppered and shaken at room temperature for 48 h. The silica was washed with 2 *M* sodium chloride and water.

A CDI activation procedure was also used for the immobilization of Con A with the following changes in the above procedure: to 2.0 g LiChrospher SI 500 diol was added 0.64 g CDI and 25 ml acetonitrile. Sonication time for the activation step was 20 min. For immobilization of Con A, 10 ml of 3.4 mg/ml purified Con A in sodium acetate buffer (buffer composition was the same as the mobile phase described below with no MDM) was added to 2 g of activated silica. Sonication was performed for 15 min under conditions in which a vacuum was repeatedly applied and released so that the solution did not foam excessively. Flushing the sample with nitrogen was not necessary. The solution was shaken for 5 days at 4°C.

An ester-amide (EA) activation procedure[20] was used for the immobilization of glucosamine. To 1.5 g of LiChrospher SI 500 diol was added 0.38 g of succinic anhydride in 75 ml of anhydrous dioxane. The reaction was allowed to proceed for 24 h at room temperature with shaking. The carboxylated silica was then collected on a medium porosity glass filter and washed with several warm and room temperature portions of anhydrous dioxane, and dried under vacuum. An 8 ml volume of 0.1 *M* glucosamine in 0.1 *M* sodium phosphate (pH 7) solution and 0.154 g EDC were added to 0.61 g of carboxylated silica. This reaction mixture was sonicated under vacuum for 5 min, flushed with nitrogen, stoppered, and shaken for 24 h. The glucosamine silica was then filtered, washed with 0.1 *M* sodium phosphate buffer (pH 7), water and methanol.

Assay of immobilized ligands

All silica samples used in the assays were vacuum-dried at room temperature. PAPM silica samples were assayed by an orcinol method[21] which was adapted to silica samples as described below. The orcinol reagent consisted of 0.5 g of recrystallized orcinol dissolved in 1 l of 70% v/v sulfuric acid and stored in a brown bottle. Orcinol reagent (5.0 ml), aliquots of standard PAPM solutions or silica samples, and additional water to bring total volume to 5.5 ml were added to test tubes. Sample and standards were sonicated for 10 min and allowed to sit for an additional 5 min. The test tubes were heated in boiling water for 10 min, cooled and the absorbance of the solution measured at 420 nm. Silica-containing samples were centrifuged and decanted prior to absorbance measurements. Analyses of the glucosamine silica and Con A-silica were by the alkaline ferricyanide[22] and Lowry[23] methods, respectively. The results of the analyses are summarized in Table I.

Chromatography

Pertinent chromatographic conditions for each column are summarized in Table I. All columns were packed at 3000 p.s.i. using the acetate buffer described below and stored at 4°C when not in use.

Chromatography was performed with the column thermostated at 25.0°C. The mobile phase consisted of a 0.5 *M* sodium acetate buffer, pH 5.0, containing 1.00 m*M* calcium chloride and manganese chloride, and MDM of various concentrations. The pH was adjusted with hydrochloric acid. The flow-rate was 1.0 ml/min. Samples were pre-equilibrated with the mobile phase. The detector wavelength was 280 nm for Con A, uracil, sodium nitrate and BSA; 316 nm for MUM; and 305 nm for PNPM.

Statistical moments of the peaks were determined by the modified $B/A_{0.1}$ and $B/A_{0.5}$ methods[24]. Samples of either 4.6 mg/ml sodium nitrate, 16 μg/ml uracil, or 90 μg/ml BSA were injected and the first moment was taken to be the void time. The capacity factor (k') was calculated from the first moments of the peaks.

RESULTS AND DISCUSSION

The conditions of pH and ionic strength were chosen such that Con A would be present primarily in the form of a dimer[25] with two identical binding sites[26]. With Con A as analyte, one would expect Z values of 1–2, depending on the surface density of sugar ligands and other steric effects. With monovalent sugars used as analytes and immobilized Con A, one would expect $Z = 1$.

The Z values were determined for the five systems given in Table I. Plots of $\log k'$ *vs.* $\log(1/[I])$ are shown in Fig. 1. The plots for the immobilized PAPM columns were linear. The Z value for the high-coverage PAPM column was 1.8, indicating primarily divalent binding. The Z value decreased to 1.5 on the low-coverage column, indicating that divalent binding occurred less frequently as the surface concentration of ligand decreased.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Column number	*Immobilized ligand*	*Conc. immobilized ligand sites (μmol/m²)**	*Column parameters*		*Analyte*	*Analyte conc.*	*Amount injected (μl)*
			I.D. (mm)	*Length (mm)*			
1	PAPM	0.98	4.6	45.0	Con A	4 mg/ml	10
2	PAPM	0.28	4.6	50.0	Con A	4 mg/ml	10
3	Glucosamine	0.73	4.1	50.3	Con A	0.06 mg/ml	10
4	Con A	0.012	4.1	100.0	MUM	6 μ*M*	20
					PNPM	5 μ*M*	20

* Based on ligand assays and manufacturer's (Merck, Darmstadt, F.R.G.) estimates of surface area.

The remaining three studies yielded non-linear plots. Not shown in Fig. 1 are three points measured at [I] = 0 which clearly indicated that all three curves flattened out at large log (1/[I]). The high-coverage glucosamine column had a slope of only 0.7 in the linear portion of the curve even though the surface concentration of ligand was comparable to the PAPM columns. The Z values for two analyte sugars on the

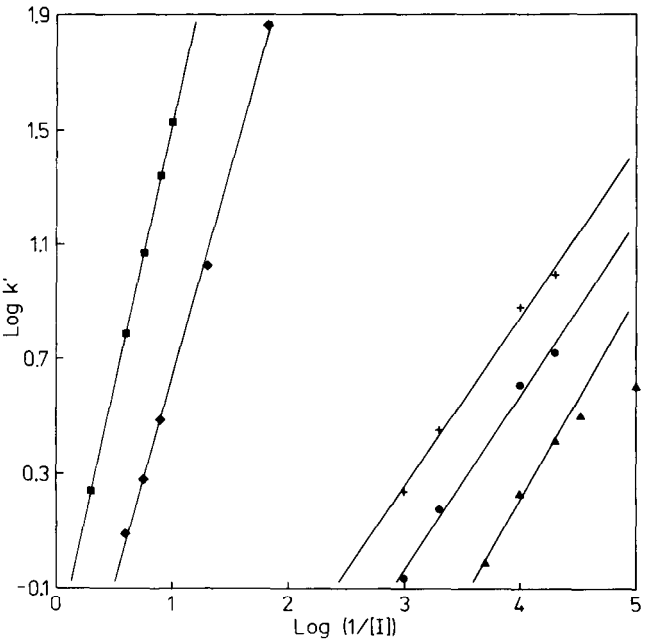

Fig. 1. Affinity chromatographic retention data. The slope of the fitted line is given in parenthesis after the column number (see Table I): ■, 1 (1.8); ◆ 2 (1.5); ▲, 3 (0.7); +, 4-MUM (0.6); ●, 4-PNPM (0.6).

Con A column were also significantly less than the expected value of one. These discrepancies and the curvature of the plots were, as will be shown below, due to limitations of the model used to derive eqn. 2.

The data were also plotted according to the empirical relationship used by Stadalius *et al.*[7] for reversed-phase chromatography (log k' *vs.* [I]). All of these plots exhibited considerable curvature. Thus, such plots appear to have little practical or fundamental use in affinity chromatography.

Modification of the model

Examination of reaction 1 indicates that a limitation of the model is likely to be the presence of other forms of the analyte such as E, EI and LEI. For the biochemical system used here, it is possible to experimentally determine how these lower forms affect the log k' plots.

Fig. 2 shows the many different equilibria that can occur in a divalent system.

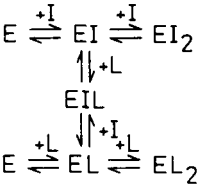

Fig. 2. Equilibria in a competitive-binding affinity chromatographic system in which the solute (E) is divalent and the ligand (L) and mobile phase modifier (I) are monovalent.

Listed below are the equilibrium constants that govern the equilibria:

$$K_2 = \frac{[EI]}{[E]\,[I]} = \frac{[EI_2]}{[EI]\,[I]} = \frac{\{LEI\}}{\{EL\}\,[I]} \tag{3}$$

$$K_3 = \frac{\{EL\}}{[E]\,\{L\}} = \frac{\{LEI\}}{[EI]\,\{L\}} \tag{4}$$

$$K_4 = \frac{\{EL_2\}}{\{EL\}\,\{L\}} \tag{5}$$

The { } represents a surface concentration (mol/dm^2). An assumption made in eqn. 3 is that the two binding sites are identical even when E is adsorbed, hence K_2 is the same for the binding of I to either E, EI or EL. Note that K_2 and K_3 have units of 1/mol, while K_4 has units of dm^2/mol. K_4 is expected to be highly sensitive to steric effects. Also, since the immobilized ligand sites are not likely to be perfectly uniformly distributed, K_4 is an "average" divalent binding constant.

From the definition of k' one can write

$$k' = \frac{A}{V_m}\,\frac{2\{EL\} + 2\{LEI\} + \{EL_2\}}{[E] + 2[EI] + [EI_2]} \tag{6}$$

where A is the column surface area (dm^2) and V_m is the void volume (l). The coefficients of 2 are due to the multiple microscopic forms of some of the species, *e.g.* LEI and IEL.

Substitution of eqns. 3–5 into eqn. 6 yields:

$$k' = \frac{K_3\{L\}A}{V_m}\,\frac{2(1 + K_2[I]) + K_4\{L\}}{(1 + K_2[I])^2} \tag{7}$$

The logarithmic form of this equation is:

$$\log k' = \log\left(\frac{K_3\{L\}A}{V_m}\right) + \log(2 + 2K_2[I] + K_4\{L\}) + \\ + 2\log\left(\frac{1}{1 + K_2[I]}\right) \tag{8}$$

In general, a plot of log k' *vs.* log (1/[I]) will not be linear. A Z value of two will be observed only under a limited range of conditions.

For the monovalent case, a similar derivation yields:

$$k' = \frac{K_3\{L\}A}{V_m\,(1 + K_2[I])} \tag{9}$$

$$\log k' = \log\left(\frac{K_3\{L\}A}{V_m}\right) + \log\left(\frac{1}{1 + K_2[I]}\right) \tag{10}$$

where $\{L\}$ is the surface concentration of immobilized ligand sites. The Z value will be one only if $K_2[I] \gg 1$. Expressions similar to eqns. 7 and 9 can be obtained from the work of Dunn and Chaiken[12], Eilat and Chaiken[13] and Hethcote and DeLisi[15].

Experimental values for K_2, K_3 and K_4 were determined for the immobilized sugar columns using eqn. 7 and a non-linear least squares program. Experimental values for K_2 and K_3 were determined for the immobilized Con A column using eqn. 9 and a linear least squares program. In every case the fits to the data were excellent and indicated mixed divalent–monovalent interactions on the immobilized sugar columns and only monovalent interactions on the immobilized Con A columns. These results will be discussed in more detail elsewhere. An important conclusion from the data was that divalent adsorption was a highly cooperative[27] process. A comparison of the dimensionless quantities $K_3 m_L/V_p$ and $K_4\{L\}$ (m_L = moles of ligand in column, V_p = pore volume) indicated that divalent binding was approximately ten times stronger than monovalent binding on the high-coverage PAPM column. This occurred in spite of the fact that the binding sites of Con A were identical. Cooperative binding of alkyl-agaroses to proteins has been extensively studied by Jennissen[9–11].

The fitted parameters were then used to prepare log k' *vs.* log (1/[I]) plots according to eqns. 8 and 10. Fig. 3 shows that these plots contained straight regions and curved regions. For example, the glucosamine column data clearly lay on a curved region.

The divalent (upper curves) and monovalent (lower curve) plots of eqns. 8 and 10, respectively, are expanded in Fig. 4 to clearly show all of the regions. At large [I],

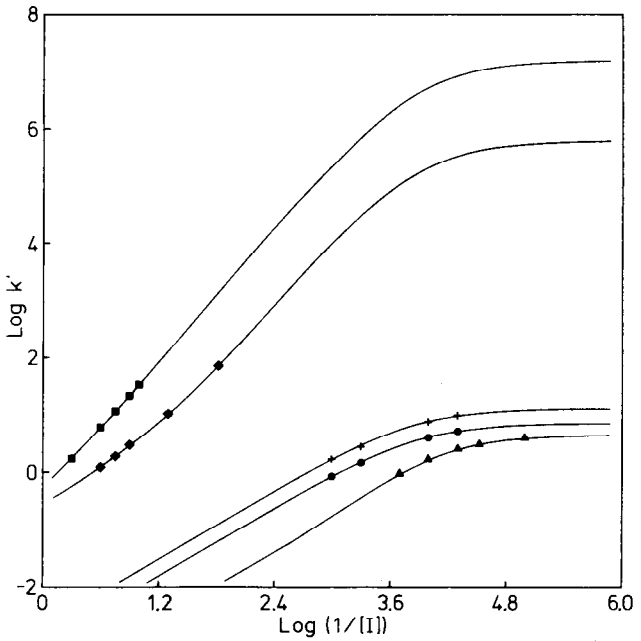

Fig. 3. Expanded plots of the data of Fig. 1 fitted with eqns. 7 and 9. The fitted parameters are given in parenthesis after the column number (see Table I): ■, 1 (K_2 = 7000, $K_3\{L\}A/V_m$ = 880, $K_4\{L\}$ = 17 400); ◆, 2 (K_2 = 7000, $K_3\{L\}A/V_m$ = 920, $K_4\{L\}$ = 680); ▲, 3 (K_2 = 8600, $K_3\{L\}A/V_m$ = 0.69, $K_4\{L\}$ = 4.6); +, 4-MUM (K_2 = 6700, $K_3\{L\}A/V_m$ = 13.1); ●, 4-PNPM (K_2 = 7400, $K_3\{L\}A/V_m$ = 7.2.).

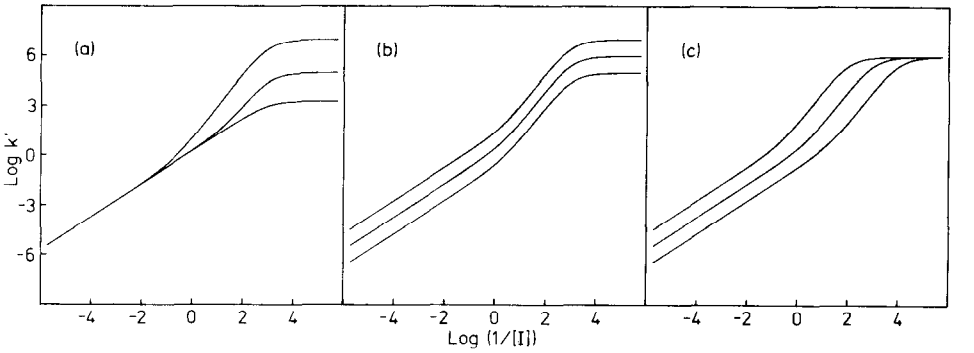

Fig. 4. General plots of eqn. 7 for a divalent system. $K_2 = K_3\{L\}A/V_m = K_4\{L\} = 1000$ except as follows: (a) from top to bottom, $K_4\{L\} = 10\,000$, 100, 0; (b) from top to bottom, $K_3\{L\}A/V_m = 10\,000$, 1000, 100; (c) from left to right, $K_2 = 100$, 1000, 10 000.

the slope is 1. At intermediate [I], the slope is 2. At small [I], the slope is 0. We will call these regions 1, 2 and 0, respectively. The monovalent plot has no region 2. At the transition between each region there is a curved region. Experimentally, only a small part of the plot ($1 < k' < 10$) is accessible. It is apparent that a plot of experimental data may be either linear or curved, and that the slope may be less than the maximum number of binding sites. The boundary between regions 1 and 2 occurs at $[I] \approx K_4\{L\}/K_2$, while the boundary between regions 2 and 0 (or 1 and 0 in the case of monovalent binding) occurs at $[I] \approx 1/K_2$. Computer calculations show that in the mobile phase the dominant form of E in region 0 is free E, while in regions 1 and 2 the dominant form is EI_2. EI dominates at the transition between regions 0 and 2. On the surface, EL_2 dominates in regions 0 and 2 and LEI dominates in region 1. EL is not present in significant amounts at any inhibitor concentration.

There are three factors which determine the position and size of the various regions. These are: (1) the strength of monovalent binding of the analyte to the ligand (determined by K_3, $\{L\}$, and A/V_m); (2) the strength of the mobile phase modifier (K_2); and (3) the strength of divalent interaction (K_4). The effect of each factor was determined separately by generating plots of eqn. 8 on a computer using arbitrarily chosen values of the parameters.

The amount of divalent interaction is reflected in the $K_4\{L\}$ term of eqn. 8 relative to the terms $2K_2[I]$ and 2 (which represent the amounts of LEI and EL, respectively). Fig. 4a shows that when K_4 is 0, there is no region 2 and the plot is the same as for a monovalent interaction. As K_4 increases, the width of the region with a slope of two increases, and the height of region 0 increases. The latter region is the region where I does not significantly affect k', and so the plot plateaus. As K_4 increases, this plateau k' value also increases. These effects can be observed in comparing the immobilized sugar columns (Fig. 3). The high-coverage PAPM column had a wide region 2, while the low-coverage PAPM column had a narrower region 2. The glucosamine column K_4 was so small that region 2 was nearly absent (slope just slightly greater than one).

Fig. 4b shows the effect of changing the monovalent binding strength, $K_3\{L\}A/V_m$. The shape of the plot is unaffected, but increasing binding strength shifts

the curve vertically to higher k'. This effect can be observed in comparing the high-coverage immobilized PAPM and glucosamine columns (Fig. 3) which differed primarily in that K_3 was larger for the PAPM column (solution K_3 values for PNPM and N-acetyl-D-glucosamine are 71 400 and 140 M^{-1}, respectively, at 5°C)[28]. The larger K_3 caused the observed data to shift from the curved transition area between regions 0 and 2 to region 2.

Fig. 4c shows that the value of K_2 influences the horizontal position of the plot but not the shape. No new information is obtained by making measurements with different inhibitors. The same curve is obtained, but at different concentrations of I.

It is clear that the log k' plotting method generally yields a Z value which is less than the true valency of the analyte. In general, there is no way to determine the true value of Z, although in affinity chromatography it will usually be the next highest integer. The exception is for monovalent interactions, where a plot of log k' *vs.* log $(1/(1 + K_2[I]))$ should have a slope of exactly 1. Of course, this requires a preliminary determination of K_2, for which Z needs to be known in advance.

The log k' plotting method does have value in affinity chromatography for the semi-quantitative estimation of the degree of monovalent or divalent binding. A Z value of 1 or less indicates primarily monovalent interactions, while values approaching two indicate increasing strength of divalent interactions. A curved plot indicates a transition between two regions.

Another potential use of the log k' plotting method in affinity chromatography may be in the more common chromatographic cases where elution of analyte is caused by pH, ionic strength or other mobile phase modifiers rather than by competitive elution with inhibitors. For example, a plot of log k' *vs.* log $(1/[H^+])$ might help to indicate the mechanism of elution during pH changes. The slope of the plot might indicate how many critical sites in the protein are being protonated or deprotonated during elution.

Extension to other chromatographic methods

The more general affinity chromatographic model can be extended to other types of chromatography by making reasonable assumptions about the forms of solute present in each phase. Equilibrium constants can then be calculated for individual adsorption sites using retention data. The model can be used to explain the frequently-observed curvature in log k' plots of solutes with $Z > 1$, and to explain why the measured slopes may not be integer values.

A particularly interesting conclusion one can draw from such studies is that the individual site equilibrium constants must decrease as Z increases. This is particularly apparent from the reversed-phase studies of Geng and Regnier[8] which yielded Z values of 2–24 for a series of proteins. The straight log k' plots and large slopes indicated a high degree of cooperativity ($K_Z > K_{Z-1} > K_{Z-2}$, etc.). One would have expected that the mobile phase modifier concentration needed to elute the protein with $Z = 24$ to have been many orders of magnitude greater than that of the proteins with smaller Z values (*e.g.*, note the large range of [I] in Fig. 1 where Z only changed by 1). However, only a fifteen-fold difference was observed. This indicated that the individual equilibrium constants must have decreased as Z increased. The use of the general model thus provides some additional insight into the mechanism of retention.

ACKNOWLEDGEMENTS

The authors thank Mary Landgrebe for synthesizing the glucosamine packing. This work was supported by the National Science Foundation under Grant CHE-8305057.

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