Journal of Chromatography, 266 (1983) 3-21
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 052

RETENTION MODEL FOR HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY*

W. KOPACIEWICZ, M. A. ROUNDS, J. FAUSNAUGH and F. E. REGNIER*
Department of Biochemistry, Purdue University, West Lafayette, IN 47907 (U.S.A.)

SUMMARY

Protein retention on an ionic surface is the result of protein charge, surface charge and the charge characteristics of the surrounding medium. To date, a "net charge" model has been used to explain this phenomenon; however, retention mapping studies on strong ion-exchange columns showed it to be inadequate. Deviations may result from charge asymmetry, since it appears that only a fraction of the protein surface interacts with the stationary phase. Retention is also altered by the type of displacing salt. A non-mechanistic model has been developed which shows a positive correlation between protein retention and the number of charges associated with the adsorption-desorption process. lntegration of these observations and concepts provides a deeper understanding of protein retention on ion-exchange surfaces.

INTRODUCTION

Advances in the preparation of pressure-stable, microparticulate ion-exchange packing materials with pore diameters greater than 200 Å have led to high-performance ion-exchange chromatography (HPIEC) of biopolymers. In the case of proteins, high-performance separations may be achieved 10–100 times faster than on conventional gel-type columns with equal or superior resolution[1]. These dramatic achievements have made HPTEC a major fractionation technique for proteins. Although many biopolymers have been separated by this method, a useful understanding of the ion-exchange process has not been developed.

The ion-exchange process is largely governed by electrostatic interactions. Retention is thought to be the result of the strength of these interactions. In the most simplistic terms, the attractive force between two oppositely charged bodies can be represented by Coulomb's law:

$$F = q_1 q_2 / \mathrm{d} r^2 \qquad (1)$$

where q_1 and q_2 are point charges of opposite sign, r is their separation distance and d

* This is Journal Paper No. 9395 from the Purdue University Agricultural Experiment Station.

0021-9673/83/$03.00

is the dielectric constant of the medium. The ion-exchange process with proteins is considerably more complex since the protein and support are not point charges. However, the proportionality of the relationship remains the same.

Since there are both acidic and basic residues within the same protein molecule, its net charge is pH-dependent. Under acidic conditions, basic amino acids are ionized, while carboxyl ionization is suppressed, and the protein obtains a net positive charge. Conversely, under basic conditions carboxyl groups are ionized, while the amino groups are neutral, and the protein accumulates a negative charge. Depending on the ratio of acidic to basic amino acids, at some intermediate pH, the net charge will be zero and a protein is said to be at its isoelectric point (p*I*). The exact pH of the isoelectric point is determined by both the type of amino acids and molecular structure. The amphoteric nature of a protein is best examined by a pH titration curve. Since a protein can exhibit net positive, neutral or net negative charge, depending on its p*I* and solution pH, the "net charge" concept has been used to predict retention behavior on ion-exchange columns. The general features of this concept are that proteins: (1) will not be retained on ion-exchange columns at their p*I* because they have no net charge; (2) will be retained above their p*I* on anion-exchange columns because they have a net negative charge; (3) will be retained below their p*I* on cation-exchange columns because they have a net positive charge; and (4) will show a correlation between net charge, as demonstrated by the titration curve and retention on ion-exchange columns. This hypothetical relationship between net charge and chromatographic retention is illustrated in Fig. 1. A titration curve depicts net charge (*Z*), and a retention map (retention time *versus* pH) represents pH-dependent chromatographic behavior. This paper examines the suitability of the "net charge" concept in explaining chromatographic retention on high-performance ion-exchange columns.

MATERIALS AND METHODS

Proteins and reagents

All proteins were purchased from Sigma (St. Louis, MO, U.S.A.). Those used

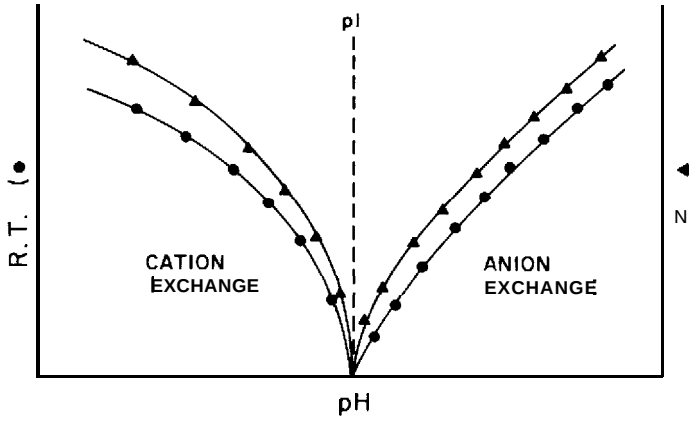

Fig. 1. The hypothetical relationship between protein net charge and chromatographic retention. Net charge, Z, is depicted by a titration curve, and pH-dependent chromatographic behavior is represented by a retention map (retention time *vs.* pH).

in the retention mapping studies were selected so as to cover a wide range of isoelectric points and molecular weights (see Table I). Other reagents were of AR grade or comparable quality.

Chymotrypsinogen was dissolved in 0.001 *M* HCl to prevent autolysis. All other proteins were dissolved in the appropriate chromatographic eluents so that sample pH was within one unit of eluent pH, although previous work indicated that this was probably not necessary'.

Retention mapping

Retention map data were obtained with Pharmacia strong cation- ("Mono S") and strong anion- ("Mono Q") exchange columns on a Pharmacia FPLC instrument system (Pharmacia, Piscataway, NJ, U.S.A.). A Model 150 Altex UV detector (Anspec. Ann Arbor, MI, U.S.A.) was used to monitor absorbance at 254 nm, and an Anspec AN 400 ion chromatograph to monitor conductance. Samples were eluted at 1 mljmin with a 20-min linear gradient from 0.01 *M* buffer to 0.5 *M* NaCl in 0.01 *M* buffer. unless otherwise noted. Buffers were selected so that buffer pK_a was within one pH unit of the desired eluent pH. An ElectroMark Analyzer (Markson Science, Del Mar. CA, U.S.A.) was used for all pH measurements.

Double layer dimensions

Ion-exclusion effects were examined by using a SynChropak CM 300 (SynChrom, Linden. IN. U.S.A.) column on a Micromeritics Model 7000 liquid chromatograph (Micromeritics, Norcross. GA, U.S.A.) with an LDC Model 1107L Refracto-Monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) as the detector. Glucose. sodium citrate and potassium oxalate samples. prepared in the appropriate

TABLE I

PHYSICAL PARAMETERS OF PROTEINS USED IN RETENTION MAPPING STUDY

Protein (origin)	pI*	M.W.
Ovalbumm (egg white)	4.7	43,500
Soybean trypsin inhibitor	4.5	20,100
Bovine serum albumin	4.98. 5.07, 5.18	69.000
β-Lactoglobulin A (bovine milk)	5.13	35,000
α-Amylase (bacterial)	*ca.* 6 (human)	55.000 (human)
Conalbumin (chicken egg white)	6.0, 6.3. 6.6	77,000
β-Glucosidase (almonds)	7.3	two subunits a 65,150
Carbonic anhydrase (bovine erythrocytes)	7.3 (pig erythrocytes)	30,375 (pig)
Myoglobin (horse heart)	6.9. 7.3	17.500
Immunoglobulin G (equine)	*ca.* 7.7	*ca.* 150,000
Ribonuclease-A (bovine pancreas)	8.7. 8.8 (guinea pig pancreas)	13,500 (guinea pig)
α-Chymotrypsinogen-A (bovine pancreas)	8.8. 9.2. 9.6	25.000
Cytochrome *c* (horse heart)	9.0. 9.4	12,200 (beef heart)
Lysozyme (egg white)	11	13.930

* Data from refs. 2-4.

buffer, were eluted isocratically (0.5 ml/min) with a potassium phosphate buffer (pH 6.0) at ionic strengths (μ) of 0.002-0.28.

Stationary and mobile phase contributors

The pH-dependence of retention on a weak and strong anion-exchange support was measured on a SynChropak AX 300 (SynChrom) column and on a quaternized version of this support. (AX 300 (1 g) was exhaustively methylated overnight with a 20 % solution of iodomethane in absolute ethanol.) A 20-μl volume of a mixture of carbonic anhydrase (CA) (3 mg/ml), ovalbumin (OVA) (7 mg/ml) and soybean trypsin inhibitor (STI) (15 mg/ml) prepared in Buffer A, was chromatographed on each column at various pH values. Buffer A was a 0.01 μ solution of buffering ion, and Buffer B was a combination of buffering ion (0.01 μ) and displacing salt (0.5 μ). In all cases, proteins were eluted with a 20-min linear gradient from A to B at 1 ml/min.

Salt effects were then examined for the same protein mixture on strong cation- and anion-exchange columns (Pharmacia Mono S and quaternized AX 300 columns, respectively). A 20-min linear gradient, as described above, was used, with the ionic strength of Buffer B kept constant at 0.5 μ. An LDC Constametric I and II G pumping system with a Gradient Master controller and a Model 153 Altex UV detector was used throughout these studies.

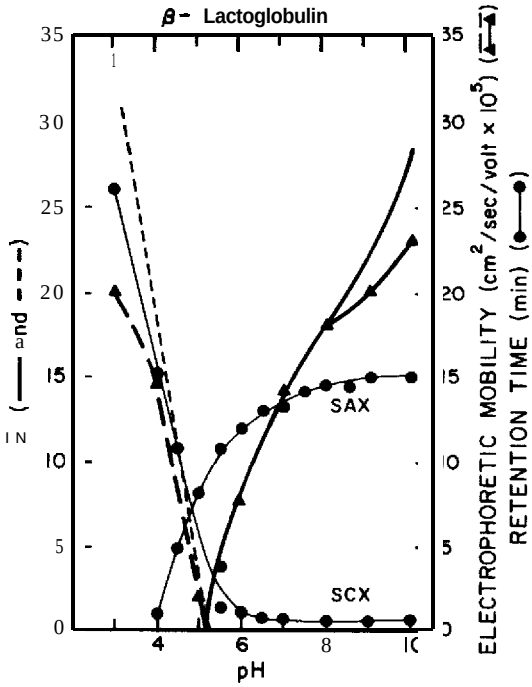

Fig. 2. Chromatographic retention as compared to titration (data from ref. 5) and electrophoretic mobility (data from ref. 6) curves for β-lactoglobulin, p*I* = 5.1. SAX refers to the Pharmacia Mono Q column, and SCX to the Pharmacia Mono S column. (A 40-min linear gradient to 1.0 *M* NaCl was used at eluent pH 3.0 on the SCX column.)

RESULTS AND DISCUSSION

Retention maps

A retention map, titration curve[5] and electrophoretic mobility[6] plot for β-lactoglobulin (β-LAC) is illustrated in Fig. 2. Examination of the retention plot shows that β-LAC was retained *ra.* one pH unit below its isoelectric point (pH 5.1) on the Mono Q anion-exchange column and *ca.* half a pH unit above the p*I* on the Mono S cation-exchange column. Although the retention curves cross at pH 4.8, which is near the p*I*, these results clearly do not fit the "net charge" retention concept, as illustrated in Fig. 1.

Another feature of the "net charge" concept is that protein retention should parallel net charge. In the case of β-LAC on the cation-exchange column, there is good correlation between the titration curve and chromatographic retention. In fact. chromatographic retention corresponds more closely to the titration curve than electrophoretic mobility below the p*I*. In contrast, retention on the anion-exchange column deviates widely from both the titration and electrophoretic mobility curves. Chromatographic retention is greater than the other variables near the isoelectric point but gradually shifts to a negative deviation two pH units above the p*I*. (Plotting chromatographic retention as a function of eluent ionic strength instead of retention time in minutes made little difference in the general shape of the plot). Thus, the "net charge" concept of retention is defective for β-LAC in two respects: (1) significant retention occurs at the p*I* and (2) there is not a good correlation between the net charge of the protein and its retention. Is β-LAC atypical or do other proteins behave

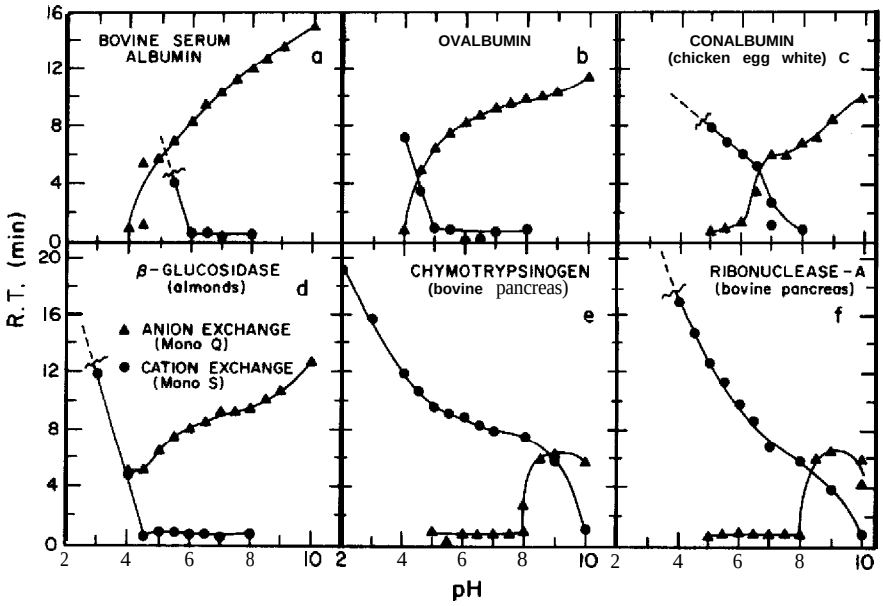

Fig. 3. Retention maps for six proteins which were retained on at least one of the strong ion-exchange columns at their respective p*I* values (see Table I for approximate p*I* values). Two points on a retention curve, e.g. BSA at pH 4.5, indicate that two major peaks were observed at this eluent pH.

similarly? This question was addressed by examining the retention maps of thirteen other commercially available proteins.

In six cases, shown in Fig. 3. the protein was retained at its isoelectric point on at least one of the ion-exchange columns, and the retention curves of five of these proteins (all except β-glucosidase, β-GLU) cross near the p*I*. Bovine serum albumin (BSA) (Fig. 3a) was retained one pH unit beyond its p*I* (cu. 5) on both ion-exchange columns. (Because recovery of BSA from the cation-exchange column was poor below pH 5.5, retention data are not reported.) The retention of OVA (Fig. 3b) decreased to zero at its pZ(4.7) on the cation-exchange column as the "net charge" concept would predict. but it was retained one pH unit below the p*I* on the anion-exchange column. Conversely, conalbumin (CON) retention declined to zero near its p*I* (ca. 6.3) on the anion-exchange column, but it was retained up to nearly two pH units above the p*I* on the cation-exchange column. Clearly, deviations from retention as predicted by the "net charge" concept do not seem to occur in any systematic way. Indeed, a completely different type of deviation was shown by a-amylase (α-AMY) as seen in Fig. 4. The retention of this protein dropped to zero almost one pH unit before the p*I* (*ca.* 6.0) was reached on both the anion- and cation-exchange columns.

"Zero" retention at (or very near) the p*I* on both ion-exchange columns in agreement with the "net charge" concept was observed for only three of the fourteen proteins studied: carbonic anhydrase (CA), myoglobin (MYO) and immunoglobulin G (IGG) (Fig. 5). The retention maps for lysozyme (LYS) and soybean trypsin inhibitor (STI) (Fig. 6) are incomplete because the p*I* of LYS is above the maximum pH used in this study and STI is insoluble below pH 5.

From the data shown in Figs. 3-5, it is obvious that the retention of β-LAC on strong ion-exchange columns at its p*I* is not an exception. The majority of the proteins examined in this study (75 %) did not behave in agreement with the "net charge" concept.

It should be noted that five proteins, CA, MYO, cytochrome *c* (CYT c), ribonuclease (RNase) and chymotrypsinogen (CHY), decreased in retention above pH 9 on the Mono Q column while the net charge on the proteins was either constant or increasing. 'Possible explanations for this are that the support was not fully quater-

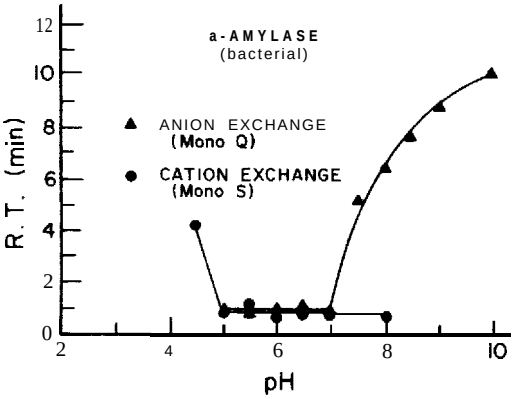

Fig. 4. Retention map of α-amylase, the only protein in this study for which retention on both columns was zero nearly one pH unit before its p*I* was reached.

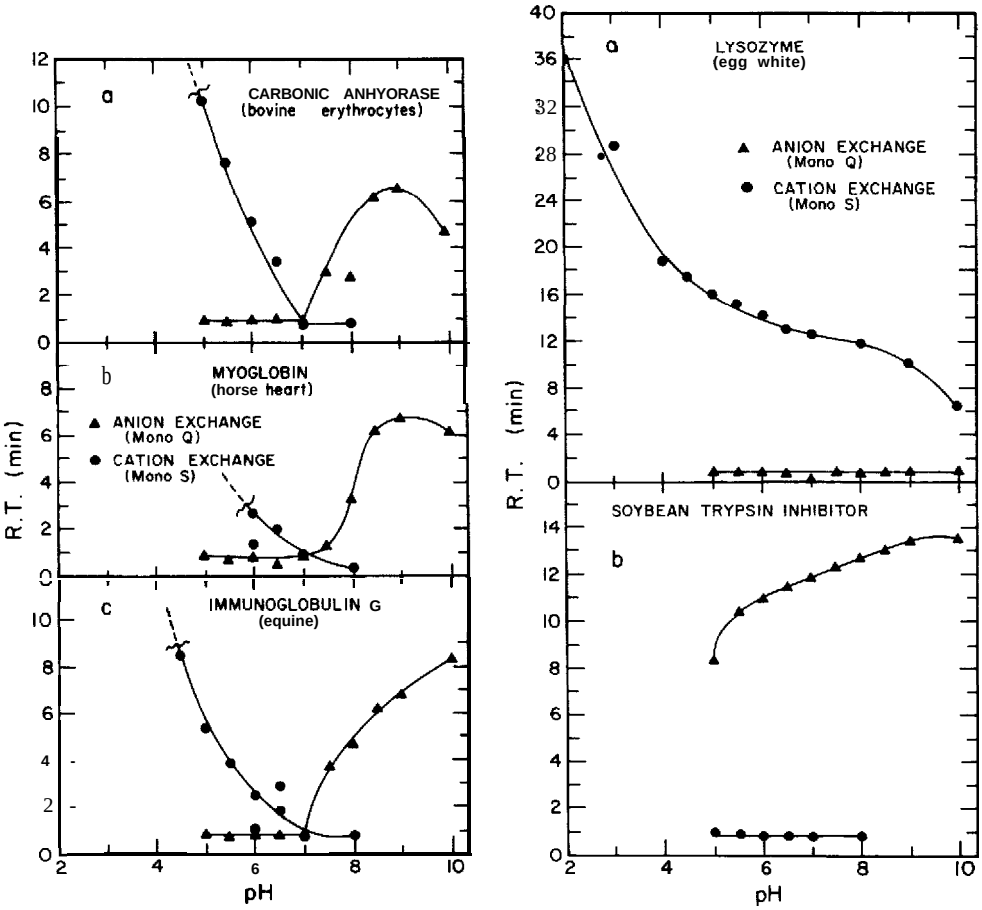

Fig. 5. Retention maps of three proteins which behaved in accordance with the "net charge" concept; *i.e.* were not retained on either ion-exchange column at. or very near. their p*I* values.

Fig. 6. Partial retention maps for lysozyme and soybean trypsin inhibitor; the p*I* of LYS is above pH 10 and STI is insoluble below pH 5. (Retention times for LYS at pH 3 and 2 on the Mono S column were obtained by using a 40-min linear gradient to 1 .0 *M* NaCl.)

nized and was losing charge, or that the structure of these proteins was altered above pH 9.0. The fact that chromatographic peaks for these five proteins became very broad as mobile phase pH was increased from 9 to 10 supports the latter explanation.

Other sorptive effects

Multiple-mode separations are common in liquid chromatography. In the event that a protein is adsorbed to a column by multiple modes, the total attractive force (F_t) of a protein for the surface will be the sum of attractive forces due to the individual modes as expressed by the equation

$$F_t = F_e + F_s + F_h + \ldots + F_n \qquad (2)$$

where F_e represents electrostatic force, F_s is due to solvophobic effects, F_h is due to hydrogen bonding and F_n represents any of a series of other forces that may be working in the system. The possibility that solvophobic forces were involved in sorptive behavior at the p*I* was examined by adding 1 % of 2-propanol to the mobile phase. This technique has been shown' to reveal and partially overcome hydrophobic effects in high-performance ion-exchange chromatography of proteins. Retention near the pi and one pH unit on either side of the p*I* was examined using β-LAC on both Pharmacia ion-exchange columns. (Retention on each column was measured at pH 4.5, 5.5 and 6.5 both in the presence and absence of 2-propanol.) In addition, LYS and CYT *c* were examined on the cation-exchange column and BSA and OVA on the anion-exchange column. These proteins were chosen because they were strongly retained and have been previously used to determine gross column hydrophobicity[8]. In most cases. retention time was decreased by less than 5 % with the addition of 2-propanol. The only significant exception was elution of BSA at pH 4.5 from the anion-exchange column, where addition of propanol decreased retention time by 17 %. These findings indicate that the Pharmacia columns have little, if any, hydrophobicity and that retention of proteins at the p*I* is not the result of solvophobic effects. The retention curves one pH unit away from the p*I* of a protein were observed to be equal to or less than what the "net charge" concept would predict, based on purely electrostatic forces. This further discounts additional sorptive forces playing a significant role in protein retention.

Influence of charge asymmetry

The fact that the net charge of a protein is zero at its p*I* does not mean that it is devoid of charge. Depending on its amino acid composition, a protein can have a dozen or more charged groups at the p*I*, the spatial arrangement of which is a function of primary, secondary, tertiary and quaternary structure. In large, complex molecules, the distribution of charged species throughout the structure may not be uniform. The question of whether the arrangement of these charged groups in space can have an influence on ion-exchange retention may be dealt with, theoretically, by examining the electrostatic potential. The electrostatic potential (W) is defined[9] as the amount of work required to bring a unit charge from inifinity up to a certain point relative to a charge (q_1). The electrostatic potential for a single charge (q_1) is given by the equation

$$W = q_1/DR \tag{3}$$

where D is the dielectric constant of the medium and R is the distance between charges. A charge and its resulting electrostatic potential has direction and can be treated as a vector. When dealing with a multiply charged system such as a protein, the electrostatic potential (W_p) at any point on the protein surface is the sum of contributions of individual amino acid residues and will have magnitude and direction :

$$W_p = \sum_{i=1}^{n} q_i/DR_i \tag{4}$$

In this case, q_i is the charge on the i^{th} amino acid residue and R_i is its distance from a

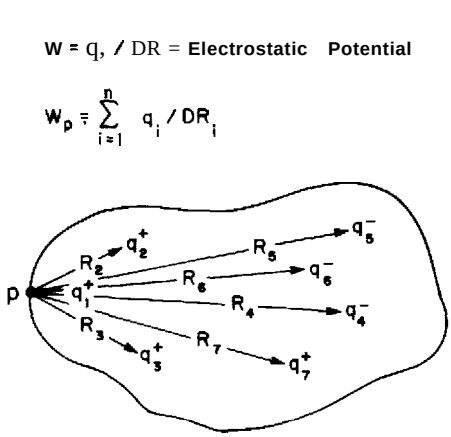

Fig. 7. Illustration of the effect of asymmetry on the electrostatic potential at a point (p). The symbols q_{1-7} represent charges which have **an effect on the** electrostatic potential (W) at point p (W_p). Since W_p has magnitude and direction. its value is the vectorial sum of all interacting charge potentials relative to p.

point (p) on the protein's surface. A graphic representation of such a system is seen in Fig. 7. Non-uniform distribution of charge within a protein would be expected to give unequal distribution of charge at various points on the surface.

Obviously. the interaction of a protein with an ion-exchange column does not occur at point sources and eqn. 4 would not strictly apply. A more rigorous treatment would establish that areas of electrostatic potential on the surface of a protein adjacent to regions rich in anionic amino acids would have a potential substantially different from those in regions rich in cationic amino acids. Eqn. 4 predicts that even within spherical proteins, charge asymmetry at the pI would produce areas of different electrostatic potential at their surface.

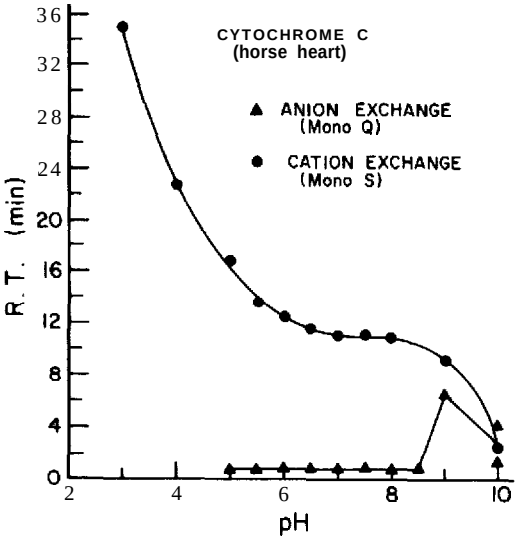

Fig. 8 Retention map of CYT c (retention times at pH 4 and 3 on the Mono S column were obtained by using a 40-min linear gradient to 1.0 M NaCl). The downturn in the Mono Q column curve at pH 10 may have been due to structural alteration of the protein. (Two major peaks were observed at eluent pH 10.)

The globular protein CYT *c* provides a model for studying the importance of charge asymmetry in ion-exchange chromatography. Horse heart CYT c (M.W. cu. 12,200) has a p*I* of *ca.* 9, is retained on a strong cation-exchange column both at and below its p*I* (Fig. 8) and contains nineteen lysine residues". Brautigan *et al.*[10] have shown that the c-amino groups of lysine in CYT *c* can be derivatized with 4-chloro-3,5-dinitrobenzoic acid (CDNB). From each amino group which reacts with this reagent. a potential negative charge (from the aromatic carboxyl group) is acquired. Thus, the "net charge" of the CYT *c* molecule can be systematically varied by controlling the extent of this reaction. These stable derivatives have been separated by conventional CM-cellulose cation-exchange chromatography. In addition to separating native. mono-, di- and multi-substituted cytochromes *c*, Brautigan et *al.*[10] have shown that the monosubstituted products (those with only one CDNP-lysyl residue per molecule) can be further resolved into four major fractions. Since all of these monosubstituted products have the same net charge and. consequently, the same p*I*, separation of these four fractions must be due to the fact that they are structural isomers: i.e.. they differ in the location of the single CDNP-lysyl group within the molecule".

This finding directly contradicts the "net charge" concept which implies that molecules with the same p*I* cannot be resolved by ion-exchange chromatography. These data also suggest that not only the net charge. but also the distribution of charge within a molecule is important. Apparently. protein binding during the ion-exchange process is not totally random. Sites of high electrostatic potential on the surface of the protein may orient its approach to the charged support. As the charge asymmetry in a protein increases, this "steering effect" should increase accordingly.

Ion-exclusion layer thickness

Charged surfaces have been shown to have charged layers of ions extending into the external medium. Helmholtz[11], Gouy[12] and Stern'" have investigated these charged layers which are often referred to as "double layers". The concentration and charge of ions in the double layer are quite different from that of the surrounding medium. The double layer forms a gradient of both charge and ionic concentration between the solid phase and the bulk solution which is dependent upon the pH and nature of the ionic species in the medium.

In HPIEC of proteins. the determination of double-layer dimensions relative to protein dimensions is important. A relatively thick double layer may affect the retention of a protein by engulfing the entire charged surface of the protein. Charge localization within the protein would then be less important. In contrast, a thin double layer would limit the area of contact with the protein, and the protein might be envisioned as rolling or orienting itself through electrostatic steering to charges on the support surface.

Double-layer thickness of an ion-exchange support was measured indirectly by examining ion-exclusion effects. Ion-exclusion studies were carried out with a 300-A pore diameter CM cation-exchange column operated at low ionic strength. Sodium citrate and potassium oxalate were used as ion-exclusion probes while glucose was used to determine the total permeation volume. As mobile-phase ionic strength was decreased, anionic species were excluded from increasingly greater volumes of liquid at the support surface. The result of this exclusion was a decrease in the elution

volume, V_{ie}, of the ion-excluded species. The difference between the elution volume of the totally permeating species, V_e, and V_{ie} is the ion-exclusion volume (ΔV_x) as shown in eqn. 5.

$$\Delta V_x = V_e - V_{ie} \tag{5}$$

Although the 300-Å support is a porous medium. there is no size-exclusion contribution when small species such as these are used as probes. The surface can be treated as if it were planar and the thickness (d) of the ion-exclusion layer may be related to V_x as shown in eqn. 6

$$\Delta V_x = d A_s \tag{6}$$

where A_s is the total surface area of the packing material in the column. The thickness (d) of the ion-exclusion layer for sodium citrate and potassium oxalate as a function of ionic strength is illustrated as a semi-log plot in Fig. 9.

The dimensions of the surface double layer are assumed to be of the same magnitude as the ion-exclusion layer. Since ion exchangers are seldom operated at an ionic strength of less than 0.01 and most proteins have diameters greater than 20 Å, double-layer dimensions. as seen in Fig. 9. are substantially smaller than the molecular dimensions of proteins. It may be concluded that only a small portion of the total surface area of a protein may encounter the double layer. This would allow areas of greater electrostatic potential (due to charge asymmetry) on the surface of a protein to cause a molecular orientation at the surface of the support.

Stationary phase contributions

Before undertaking ion-exchange chromatography of proteins, a decision must be made whether to use a so-called "strong" or "weak" ion-exchange column. The

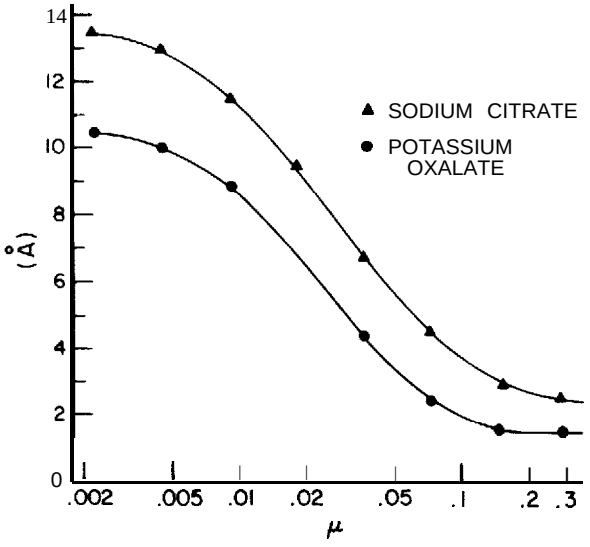

Fig 9. The double-layer thickness (in Å) of a SynChropak CM 300 cation-exchange column as a function of the ionic strength, μ. of the phosphate buffer.

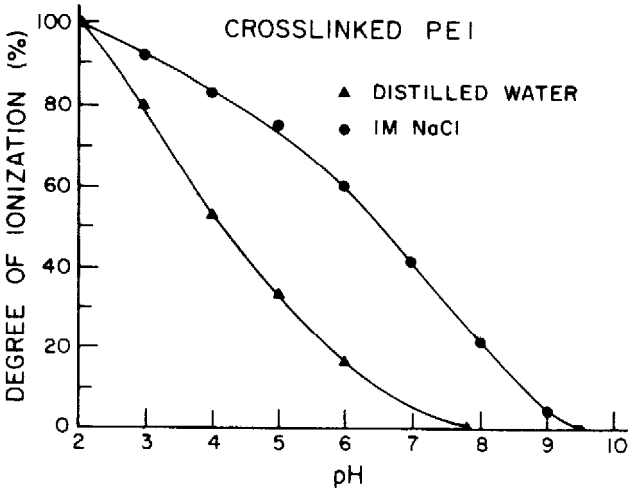

Fig 10 The effect of pH on the ionization of a weak anion exchanger. A pellicular polyethyleneimine (PEI) coating was titrated with 0 1 M HCI in both distilled water and 1.0 M NaCl. (Redrawn from ref. 14. Fig 3)

functional groups in strong anion and cation exchangers are quaternary amines and sulfonic acids. respectively. These groups are essentially always charged and retain the same charge density regardless of operating pH. Weak ion-exchange functionalities, such as carboxymethyl, or primary, secondary and tertiary amines, are seldom completely ionized and have a ligand density dependent on mobile phase pH. In fact, the ionization curve of a cross-linked PEI anion-exchange support extends over the pH range from 2.0 to 9.5 (Fig. 10). This causes a variation of ligand density up to five-fold throughout the normal operating range of the ion exchanger. Thus. retention properties are more difficult to predict. because the net charge on both the support and the protein vary simultaneously. Because strong ion-exchange columns are much simpler in this respect, they were chosen for the retention mapping studies.

Although weak ion-exchange supports are less predictable than strong ion-exchange materials. it does not mean that they are less useful. In our studies, SAX columns were generally found to be superior, but the resolving power of weak anion-exchange (WAX) columns was comparable. Under limited circumstances, WAX ionization could be exploited, thereby enhancing resolution (data not shown).

Displacing ions

The influence of displacing ion on protein retention was examined with a variety of salts. Four proteins (OVA. STI. CYT c and LYS) were chromatographed on either a SAX or SCX column. with only the type of displacing ions being varied. Through the examination of relative retention values and retention time ratios (Tables II and III), two types of ion were identified: those which changed retention wrthout changing selectivity (as indicated by retention ratios), and those which altered both retention and selectivity.

On the SAX column, relative retention times of OVA and STI could be altered by varying either the anion or the cation (Table II). When the anion (chloride) was

TABLE II

INFLUFNCE OF VARIOUS IONS ON THE RETENTION OF OVALBUMIN (OVA) AND SOYBEAN TRYPSIN INHIBITOR (STI) ON A STRONG ANION-EXCHANGE (SAX) COLUMN

	*Relative retention** on SAX*** column*		*Retention ratio§ STI/OVA*
	OVA	*STI*	
Anion* *(sodium salt)*			
Chloride	*0.83*	*0.64*	**1.50**
Bromide	*0.72*	*0.56*	*1.46*
Perchlorate	*0.62*	*0.64*	*1.93*
Bicarbonate	*0.82*	*0.71*	1.63
Formate	1.00	*0.72*	1.73
Acetate	1.00	*0.95*	1.91
Propionate	1.00	1.00	1.89
Sulfate	0.68	0.79	2.20
Tartrate	0.72	0.80	2.09
Citrate	0.50	0.69	2.61
Cation* *(chloride salt)*			
Lithium	**1.00**	*0.82*	*1.52*
Sodium	*0.83*	*0.65*	1.41
Potassium	*0.85*	*0.68*	1.49
Ammonium	*0.81*	*0.64*	1.46
Magnesium	*0.68*	*0.50*	1.38
Calcium	*0.68*	*0.47*	1.28
Sodium §§	*0.67*	*1.00*	1.92
Magnesium §§	*0.83*	*0.82*	1.91

* Chromatography was performed at pH 8. The ionic strength of Buffer B was 0.5.
** Unity refers to the longest retention time obtained with a 20-min gradient.
*** SAX column refers to a silica-based strong anion-exchange column.
§ Ratios were calculated from the actual retention times.
§§ Acetate salts

held constant and the cation was changed, relative retention times for OVA and STI varied. but the retention ratio (STI/OVA) remained fairly constant, suggesting only slight differences in selectivity. However, when the cation (sodium) was held constant and the anion was varied, both retention and column selectivity for these two proteins changed. For STI. bromide decreased retention the most and propionate the least. In contrast, citrate was the most effective in displacing OVA, and acetate and propionate were the least effective. The most significant finding was that the selectivity indicator (STI/OVA retention ratio) varied from, 1.46 to 2.61 depending on the displacing anion. This implies that SAX column selectivity can be altered by changing the mobile phase anion a technique which could be very useful in the separation of complex mixtures.

Kunin and McGarvey[15] claim that the displacing power of anions for small molecules on strong anion-exchange columns is in the order: citrate > sulfate > oxalate > bromide > chloride > formate > fluoride > acetate. A rough correlation

T4BI.F III

INFLUENCE OF VARIOUS IONS ON THE RETENTION OF CYTOCHROME *c* AND LYSOZYME ON 4 STRONG CATION-EXCHANGE(SCX) COLUMN

	*Relative retention** on SCX*** column*		*Retention ratio§ LYS/CYT c*
	CYT c	*LYS*	
*4nion**			
sodium salt			
Fluoride	*0.79*	**0.88**	1.38
Chloride	*0.68*	*0.64*	1.16
Bromide	*0.58*	*0.51*	1.10
Phosphate	*0.54*	*0.57*	1.29
Sulfate	*0.90*	*0.85*	1 16
Acetate	*0.74*	*0.74*	1 22
Tartrate	*0.98*	*0.99*	1 25
Citrate	1.00	*1.00*	1 23
*Cation**			
chloride salt			
Lithium	1.00	1.00	1.22
Sodium	0.58	0.55	1.16
Potassium	0.57	0.55	1.19
4mmomum	0.59	0.58	1.19
Magnesium	0.56	0.54	1.18

* Chromatography was performed at pH 6. The ionic strength of Buffer B was 0.5
• * Unity refers to the longest retention time obtained with a 20-min gradient.
*** SCX refers to the Pharmacia Mono S column.
§ Ratios were calculated from the actual retention times.

to this relationship was obtained in the retention values for OVA, but not for STI. Further studtes are needed to determine whether this anomalous behavior is unique for STI. The affinity of inorganic ions for an ion-exchange column depends on the structure. size and valence of the ion: could the same be true of protein?

The retention of CYT *c* and LYS on a SCX column was also investigated with regard to displacing ions (Table 111). With the SCX column, varying the mobile phase cation did not significantly change the retention ratio (LYS/CYT *c*). Although the use of different anions did alter both retention and retention ratio, the changes were not as great as those found for the SAX column. It was concluded that the use of different anions produces only small changes in selectivity for SCX columns.

The effect of salts on protein retention appears to be similar for SAX and SCX columns. For example, sodium bromide significantly decreased retention on both columns. However, the salts which provided optimal selectivity were quite different for the two types of column. Trisodium citrate enhanced selectivity the most for the SAX column while sodium fluoride was superior for the SCX column. At this time, it is difficult to provide a rationale for these complex and diverse effects. They are undoubtedly linked to ionic size, density. and thermodynamic activity. In any event, the constituents of the mobile phase play an important role in the retention process, along with the ionic character of both the protein and the support.

Ion-exchange process

As noted above, the ion-exchange support is covered by layers of ions in order to maintain electroneutrality with respect to ionic character. The double-layer thickness is in direct proportion to support ligand density. Macromolecules, which also have charged surfaces. exhibit the same phenomenon. When a macromolecule encounters the double layer on the support surface during the ion-exchange process, a redistribution of ions in the double layers of both support and protein occurs. It has been assumed that ions are expelled during this process because an increasing concentration of ions must be used to desorb proteins from an ion-exchange support. The exact mechanism by which this absorption and desorption process occurs is still unknown.

Boardman and Partridge[16] have given a theoretical treatment for ion exchange of polyelectrolytes on weak cation exchangers. Polyelectrolytes in equilibrium at the ion-exchange surface with smaller displacing ions are assumed to be bound to the surface at multiple (Z) sites. Their mathematical analysis shows that as the number of binding sites (Z) increases, desorption curves should become increasingly convex. By simple graphic comparisons. they estimated that CYT *c* was bound to a weak cation exchanger at six sites.

A non-mechanistic model for this process similar to that of Boardman and Partridge[16] is given by the equilibrium expression

$$P \quad C_{i\,t}\, Z\, D_b \rightleftharpoons P_b + \quad Z\, aD_0 \quad t \quad z \cdot bC_i \tag{7}$$

The symbol D_b represents the concentration of displacing ions associated with the surface and is in direct proportion to the ion-exchange stationary phase density (i.e. ligand density) in moles m^2. $P \cdot C_i$ is the concentration (moles/l) of protein in solution above the surface with accompanying counter-ion concentration (C_i). P_b signifies the protein concentration on the ion-exchange column in moles/m^2, while D_0 is the displacing ion concentration of the mobile phase in moles/l. It is known, however, that the displacing power of an ion is proportional to its ionic strength, and the constants *a* and *b* are needed to adjust for valence, activity coefficient and relative displacing-power differences between ions. The Z term in the formula is the number of charges that are associated with the adsorption desorption process.

The equilibrium constant for the ion-exchange process may be expressed as

$$K_b = \frac{(P_b)\,(aD_0)^z\,(bC_i)^z}{(P \cdot C_i)\,(D_b)^z} \tag{8}$$

where K_b is a binding constant. As the equation is written, it would appear that there is no limit to how much protein a support will bind. Obviously. this is not true. All supports have a maximum load capacity (P,). and bound protein (P_b) can be expressed as

$$f = (P_b)/(P_m) \tag{9}$$

where f is the fraction of the surface loaded with protein.

The same logic may be used to express ligand concentration (D_b) on a surface.

As protein is bound to the surface, D_b decreases

$$D_b = D_{bi}(1 - f) \tag{10}$$

where D_{bi} is the initial ligand concentration and $(1 - f)$ is the fraction of the surface still available for binding. Substituting eqns. 9 and 10, eqn. 8 may be rewritten as

$$K_b = \frac{(P_m)(aD_0)^z (bC_i)^z}{(P \cdot C_i)(D_{bi})} \cdot \frac{f}{(1 - f)} \tag{11}$$

Expression of eqn. 11 in chromatographically meaningful terms requires that it be related to the chromatographic distribution coefficient (K_i) as in eqn. 12

$$K_i = \frac{C_s}{C_m} = \frac{(P_m)P_b)}{(P \cdot C_i)} \quad (P \cdot C_i) \cdot f \tag{12}$$

C_s is the concentration of solute on the stationary phase in moles/m^2 while C_m is the mobile phase concentration in moles/l. If eqn. 11 is rearranged to give an expression for f

$$f = [K_b(1 - f)] \cdot \frac{(P \cdot C_i)(D_{bi})^z}{(P_m)(D_0)^z (C_i)^z (a)^z (b)^z} \tag{13}$$

which is substituted into eqn. 12, an expression that relates K_i to the ion-exchange process is obtained

$$K_i = \frac{K_b}{(a)^z (b)^z} \frac{(D_{bi})^z}{(D_0)^z (C_i)^z} \cdot (1 - f) \tag{14}$$

A 25 x 0.41 cm l.D. ion-exchange column may be loaded with at least 10 mg of protein without showing any signs of overloading. When such a column is operated in the analytical mode (100-μg samples or less), the quantity $(1 - f)$ approaches one, and the constants K_b, $(D_{bi})^z$, $(a)^z$, and $(b)^z$ may be incorporated into a single constant, K_y. This allows the reduction of eqn. 14 to a simpler expression

$$K_i = K_y/[(D_0)(C_i)]^z \tag{15}$$

Although the partitioning process in liquid chromatography is defined in terms of K_i, solute retention on an ion-exchange column is usually expressed in terms of the capacity factor, k',

$$k' = K_i A_s / V_m \tag{16}$$

where A, and V_m designate available surface area in m^2/g and mobile phase volume, respectively, and are constants for a given solute/support combination. Combining eqns. 15 and 16, and incorporating K_v, A, and V_m into a new constant, K_z, leads to the expression

$$k' = K_z/[(D_0)(C_i)]^z \tag{17}$$

which relates retention of a solute to the displacing agent concentration of the mobile phase, and the number of charged groups involved in the adsorption-desorption process.

When sodium chloride is used as a displacing agent, it is assumed that (D_0) equals (C_i) and that eqn. 17 is further reduced to

$$k' = K_z/[\mathrm{NaCl}]^{2z} \tag{18}$$

Graphical evaluation of Z is simplified by expressing eqn. 18 in the log form

$$\log k' = 2\ Z\ \log\ (1/[\mathrm{NaCl}]) + \log K_z \tag{19}$$

where Z is the slope and $\log K_z$ the intercept of a plot of $\log k'$ *versus* $\log (1/[\mathrm{NaCl}])$. Fig. 11 is such a plot for β-LAC chromatographed on a silica-based SAX column. The value of Z increased as mobile-phase pH was increased from 5 to 8. At pH 5, near

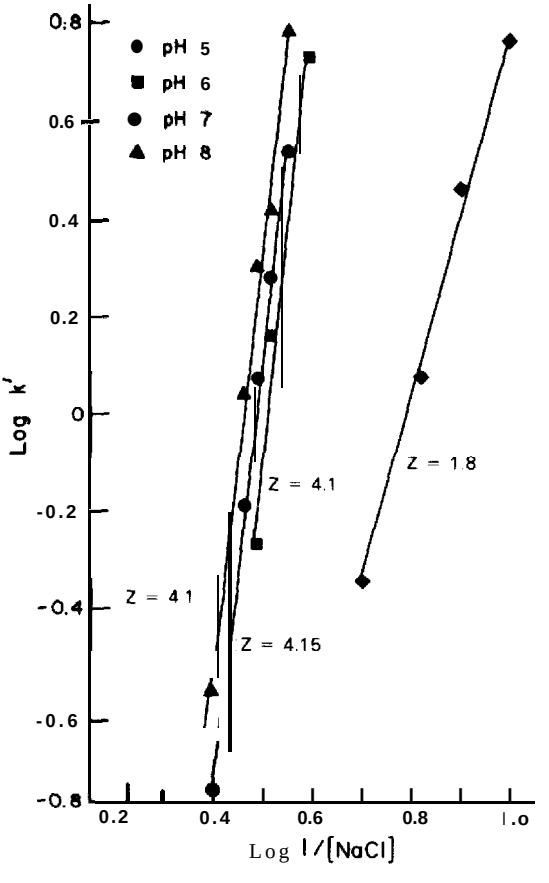

Fig. 11 An estimation of the number of charge interactions (Z) occurring between β-lactoglobulin and the surface of a strong anion-exchange column (see eqn. 19). β-LAC was chromatographed isocratically at several pH values on a 25 x 0.41 cm I.D. silica-based SAX column.

the p*I* of β-LAC (5.1), Z was 3.6. whereas from pH 6 to 8, Z remained nearly constant (x.2-8.3). The net charge of β-LAC is zero at pH 5 (by definition of p*I*) and - 18 at pH 8.0[5]. These data suggest that the number of charges interacting with a surface can either be greater or less than the net charge of the protein. At the p*I* it is likely that *Z* will be greater than net charge, while *Z* may be less than the net charge several pH units away from the p*I*. The retention map of β-LAC (Fig. 2) shows that retention on a SAX column increased concomitantly with an increase in pH from 5 to 8. Thus, increased protein retention on ion-exchange supports appears to be due to an increase in the number of sites in a molecule interacting with the surface. Data analogous to that presented for β-LAC were also obtained for CON but are not shown.

CONCLUSIONS

The retention of proteins on high-performance ion-exchange columns cannot be satisfactorily explained by the "net charge" concept alone. Approximately three-fourths of the proteins studied deviated from this concept in that they were retained at their respective p*I*. Thus, the importance of protein p*I* has apparently been overemphasized. Non-systematic deviations from this concept are not the result of non-ionic interactions but, rather. are due to the nature of the protein molecule itself. Intramolecular charge asymmetry promotes differences in electrical potential on the surface of the protein. These regions of localized charge are present even when the net charge of the molecule is zero (at the p*I*) and are postulated to orient or "steer" the protein with respect to oppositely charged surfaces, such as-ion-exchange support. An estimation of stationary-phase surface double-layer thickness indicates that it is small relative to protein dimensions: thus, this "steering effect" may be important during the adsorption desorption process.

In addition to the charge characteristics of the protein itself. the nature of the support surface and the mobile-phase displacing ion are also important. Retention on both strong anion- and cation-exchange columns was shown to be affected by the choice of displacing salt. The extent of this effect varied among columns. In some cases. protein retention was altered as much as 100%. Individual proteins may respond differently to specific salts. This phenomenon may be linked to the thermodynamic activity of the displacing ion itself.

A non-mechanistic model for the retention of proteins on HPIEC columns was developed. Calculations of Z (the number of charges associated with the adsorption/desorption process) for β-lactoglobulin indicates that the number of charged sites involved in binding may be greater or less than the net charge of the protein. This would explain the lack of correlation between retention and titration curves for β-LAC. There appears to be a positive correlation between Z and protein retention.

ACKNOWLEDGEMENTS

The authors thank Mr. Edward A. Pfannkoch and Mr. Randy R. Drager for helpful discussions regarding this research. This work was supported by grants from the National Institute of Health (NIH Grant No. GM 25431) and Hercules, Inc., Wilmington, DE.

RFFFRENCES

1 F. E. Regnier and K. M. Gooding, Anal. *Biochem.*. 103 (1980) 1.
2 W Kopaciewicr and F. E. Regnier. Anal. *Biochem.*, 126 (1982) 8.
3 P G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
4 P G. Righetti. G Tudor and K. Ek, *J. Chromarogr.*. 220 (1981) 115.
5 R. K Cannan. A H Palmer and A. C. Kibrick. *J. Biol Chem.*. 142 (1942) 803.
6 K. 0. Pedersen. *Biochem. J.*. 30 (1936) 961.
7 *Fed. Proc.*, 41 (1982) 875 (abstract 3541).
8 E. Pfannkoch. K. C. Lu, F. E. Regnier and H. G. Barth. *J. Chromatogr. Sci.*, 18 (1980) 430.
9 J T Edsall and J Wyman, *Biophysical Chemistry.* Academic Press, New York, NY, 1958, Ch. 5.
10 D. L. Brautigan, S. Ferguson-Miller and E. Margoliash, *J. Biol.* Chem., 253 (1978) 130.
11 H. Helmholtz. *Wied. Annal.*, 1 (1879) 337.
12 A. Gouy, *J. de Phys.*, 9 (1910) 457.
13 0. Stern. *Z. Electrochem.*, 30 (1924) 508.
14 A. J. Alpert and F. E. Regnier, *J. Chromatogr., 185 (1979) 375.*
15 R. Kunin and F. X. McGarvey, *Ind. Eng. Chem.*, 41 (1949) 1265.
16 N. K. Boardman and S. M. Partridge, *Biochem. J., 59 (1955) 543.*