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EVALUATION OF A RETENTION MODEL FOR HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY USING TWO DIFFERENT DISPLACING SALTS*

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SUMMARY

A model proposed for the retention of proteins on high-performance ion-exchange chromatographic supports was further investigated via the use of NaCl *vs.* $MgCl_2$ as the chromatographic displacing salt. Evaluations of the Z term generated by this model were carried out using selected proteins on a strong anion-exchange support in an effort to define its meaning more clearly.

A positive correlation between Z and protein retention was found. Higher values for Z_{MgCl_2} were attributed to enhanced ionization of protein functional groups by Mg^{2+}. Findings suggest that Z could be more precisely defined as the number of electrostatic interactions between the protein and the ion-exchange support.

INTRODUCTION

High-performance ion-exchange chromatography (HPIEC) is becoming increasingly important in the separation of proteins and other biopolymers, yet the ion-exchange process itself is not clearly understood. Although governed by electrostatic forces, the amphoteric character and three-dimensional structure of proteins make their interaction with HPIEC columns complex. Recent work[1] suggests that protein retention depends on both the distribution of charge within a molecule and the number of charged sites interacting with the support surface. Although the latter tends to decrease with decreasing net charge, it may be greater than the net charge at the isoelectric point. (pI) of a protein, and less than the net charge several pH units away from the pI.

The model proposed[1] for the retention of proteins on HPIEC supports was based on the equilibrium:

$$P \cdot C_i + ZD_b \rightleftharpoons P_b + Z \cdot aD_0 + Z \cdot bC_i \quad (1)$$

where $P \cdot C_i$ refers to protein in solution with accompanying counter-ion, C_i; P_b is protein bound on the ion-exchange column; and D_b and D_0 refer to displacing ions

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associated with the ion-exchange surface and in the mobile phase, respectively. From this, an expression was derived which relates the retention of a solute (expressed as the capacity factor, k') to the concentration of displacing agent in the mobile phase (D_0) and the number of charged groups involved in the adsorption/desorption process (Z):

$$k' = K_Z/[(D_0)(C_i)]^Z \tag{2}$$

For a monovalent displacing salt, such as NaCl, eqn. 2 is further reduced to

$$k' = K_Z/[\mathrm{NaCl}]^{2Z} \tag{3}$$

and Z may be evaluated graphically as (one-half) the slope of the line

$$\log k' = 2Z \log (1/[\mathrm{NaCl}]) + \log K_Z \tag{4}$$

For a divalent cation/monovalent anion-containing salt, *e.g.* $MgCl_2$, eqn. 2 is reduced to

$$k' = K_Z/[\mathrm{MgCl_2}]^{3Z/2} \tag{5}$$

and Z is obtained from

$$\log k' = 3/2\ Z \log (1/[\mathrm{MgCl_2}]) + \log K_Z \tag{6}$$

Evaluations of Z in the above manner using NaCl as the displacing salt* and a silica-based strong anion-exchange (SAX) support were made for β-lactoglobulin[1] and conalbumin (unpublished data). Results indicated a positive correlation between Z and protein retention, and suggested that Z is simply the number of charged sites on a protein which are interacting with the surface of the chromatographic support. It was assumed that, if the above is true, Z values for a given protein would be independent of the displacing salt used in the chromatographic eluent. Conversely, if Z was entirely (or partially) dependent on physiochemical properties of the displacing ion (*i.e.* valence, activity coefficient, etc.) one would expect to see differences in Z values when different displacing salts were used. In this study, an attempt was made to more clearly define the meaning of this Z term with regard to the ion-exchange process by investigating the influence of displacing salt composition (via a mono- *vs.* divalent cation-containing salt) on Z for selected proteins.

MATERIALS AND METHODS

Proteins and reagents

Proteins analyzed were β-lactoglobulin A (β-LAC), conalbumin (CON), ovalbumin (OVA) and soybean trypsin inhibitor (STI). These proteins were selected be-

* The value "2" was inadvertently omitted from eqns. 18 and 19 in ref. 1. The Z values previously reported for β-lactoglobulin[1] should have been divided by two.

cause their retention behavior had already been studied in detail[1,2]. All were purchased from Sigma (St. Louis, MO, U.S.A.) except OVA which was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Inorganic reagents were AR grade or of comparable quality.

All samples were prepared by dissolving the protein (0.25 mg/ml) in a solution consisting of one part 1 *M* NaCl to 19 parts 0.01 *M* buffer of either pH 5.0 or 7.0 (so as to be within one unit of the eluent pH).

Chromatography

Data were obtained using an organic-based SAX column (Pharmacia "Mono Q"; functional group, $-CH_2{}^+N(CH_3)_3$; ionic capacity, 0.28–0.36 mmol/ml) on a Pharmacia FPLC instrument system. A Model 153 Altex UV detector (Anspec, Ann Arbor, MI, U.S.A.) was used to monitor absorbance at 254 nm, while conductance was monitored with an Anspec AN 400 ion chromatograph.

The pH range of 4.0–8.0 was covered at one-half unit intervals. (Determinations were not carried out at higher pH values owing to the precipitation of $Mg(OH)_2$ from the $MgCl_2$-containing eluent.) At each pH, samples were eluted isocratically, starting at 100% eluent B and repeating at decreasing concentrations of B until the protein was completely retained on the column. This generated a series of retention times for each protein.

Eluent A was 0.01 *M* solution of a suitable buffering ion. (Buffers used were sodium acetate for pH 4.0–5.5; hydroxylamine·HCl, pH 6.0–7.0; and tris(hydroxymethyl)aminomethane, pH 7.5 and 8.0). Eluent B contained either NaCl or $MgCl_2$ as the displacing salt, at an ionic strength (I) of 0.5, in the same buffer. NaCl or $MgCl_2$ were used because they had been evaluated in a recent study on mobile phase selection for HPIEC[2], and because they contain the same anion; thus, comparisons could be limited to the effect of a monovalent *vs.* a divalent cation. Two independent series of determinations were carried out with NaCl in order to estimate experimental error.

Calculations

Retention times were converted into k' values

$$k' = (t_R - t_0)/t_0$$

where k' = column capacity factors, t_R = retention time of protein of interest and t_0 = retention time of all unretained proteins.

In order to include the contribution from A to the salt concentration of the final eluent mixture (at less than 100% B), the evaluation of both salts was carried out using log (1/I), where I is the ionic strength of the mobile phase as monitored by conductance.

For a given protein, at each pH, values of log k' and log (1/I) at every %B at which the protein was unequivocally retained were fitted to a straight line via a least-squares calculator program. This generated by the y intercept (K_Z), slope (subsequent division by the appropriate factor gave Z) and a correlation coefficient.

RESULTS AND DISCUSSION

For both displacing salts, isocratic elution at decreasing ionic strength (over the

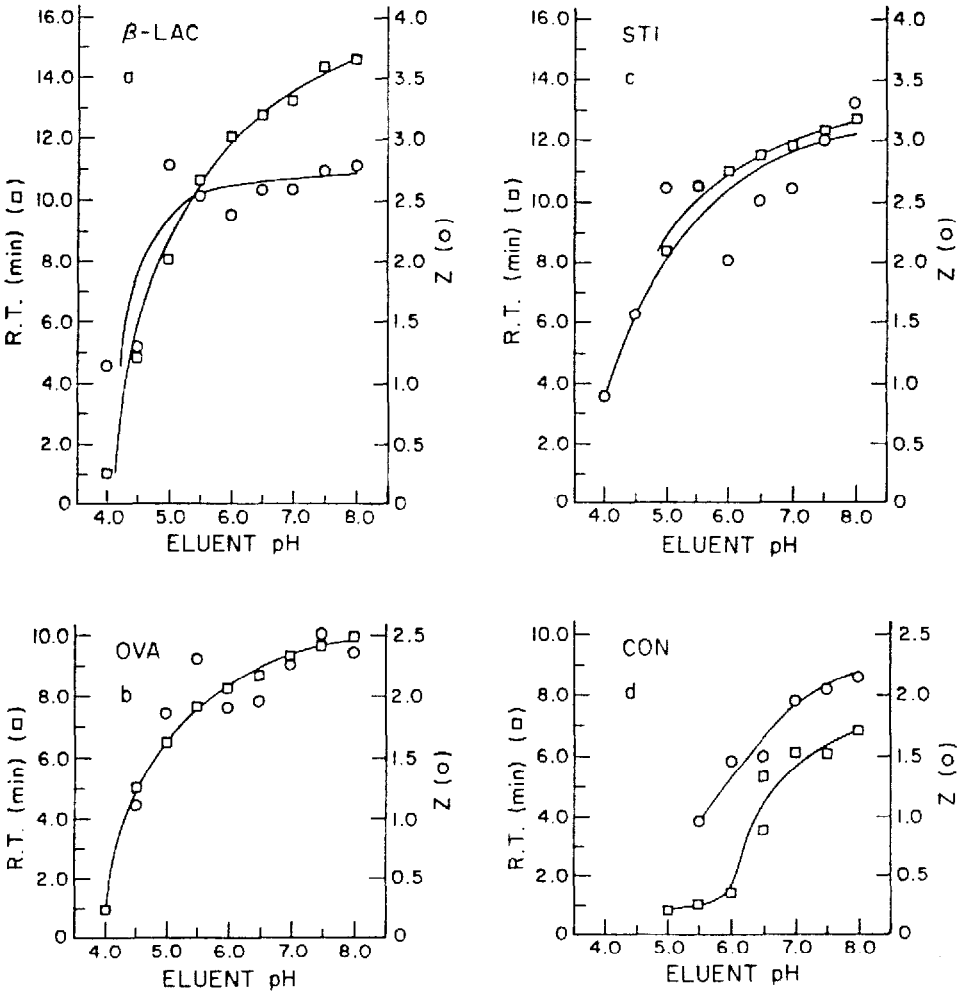

Fig. 1. Change in protein retention C.R.T. (□) and Z (○) with eluent pH, using NaCl as the displacing salt. Retention data were replotted from ref. 1; pI values: β-LAC, 5.1; OVA, 4.7; STI, 4.5 and CON, *ca.* 6.3 (see ref. 1).

pH range 4.0–8.0) generated a series of retention times for each of the four proteins evaluated. Analysis of the data was conducted by using the slope of a log–log plot (as described above) to obtain Z.

Validity of method and experimental error

Ninety-six percent of the correlation coefficients for slopes obtained were 0.97 or higher (80% were above 0.98), indicating a good fit of the data to a straight line (based on 73 calculations). Between pH 5.5 and 7.0, the variation between two NaCl runs, using β-LAC as the probe, was less than 10%. A greater variation was observed at the pH extremes with the maximum difference at eluent pH 5.0. It is possible that this variation was due to changes in the hydrodynamic volume and/or conformation of

TABLE I

Z VALUES FOR SELECTED PROTEINS ON A STRONG ANION-EXCHANGE COLUMN

Displacing salt, NaCl *vs.* $MgCl_2$

Chromatography was performed using a Pharmacia "Mono Q" column and isocratic elutions at decreasing salt concentrations, starting with 100% B eluent of ionic strength 0.5. All Z values are based on 3–7 retention times (each converted to k' for plotting as log k' *vs.* log $1/I$) except those in brackets, where the protein was retained at only two salt concentrations.

Eluent pH	*β-LAC**			*STI*		*OVA*		*CON*	
	Z_{NaCl}		Z_{MgCl_2}	Z_{NaCl}	Z_{MgCl_2}	Z_{NaCl}	Z_{MgCl_2}	Z_{NaCl}	Z_{MgCl_2}
	1	*2*							
4.0	—	[1.2]	1.4	0.9	1.1	—	[1.2]	—	—
4.5	1.0	1.3	1.9	1.5	2.2	1.1	2.1	—	—
5.0	2.0	2.8	3.0	2.6	2.9	1.8	2.9	—	—
5.5	2.5	2.5	4.3	2.6	3.3	2.3	3.5	1.0	[2.2]
6.0	2.2	2.4	3.6	2.0	3.5	1.9	3.7	1.5	2.9
6.5	2.6	2.6	3.8	2.5	3.7	1.9	4.2	1.5	3.5
7.0	2.7	2.6	5.0	2.6	4.4	2.3	4.7	2.0	3.2
7.5	—	2.8	5.0	3.0	4.3	2.5	4.0	2.1	3.1
8.0	2.5	2.8	4.7	3.3	4.2	2.4	4.1	2.2	2.8

* Two completely independent sets of determinations were carried out using NaCl as the displacing salt and β-LAC as a probe. (Z_{NaCl} values for the other three proteins were obtained during the second series.)

the protein itself, since investigators have reported[3–7] such changes for β-LAC at both pH 5 and near pH 7.5.

Relationship between Z and protein retention

Earlier data for β-LAC showed that Z and protein retention increased concomitantly with an increase in eluent pH from 5 to 8 (with NaCl as the displacing salt)[1]. In this study, Z values for β-LAC, OVA, STI and CON chromatographed on the

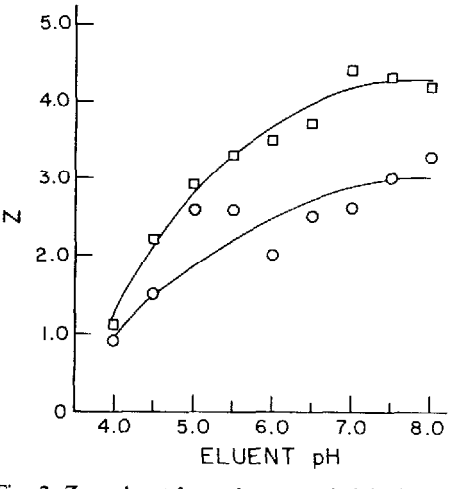

Fig. 2. Z *vs.* eluent for soybean trypsin inhibitor (pI=4.5), using NaCl (○) or $MgCl_2$ (□) as the displacing salt (on Pharmacia Mono Q SAX column).

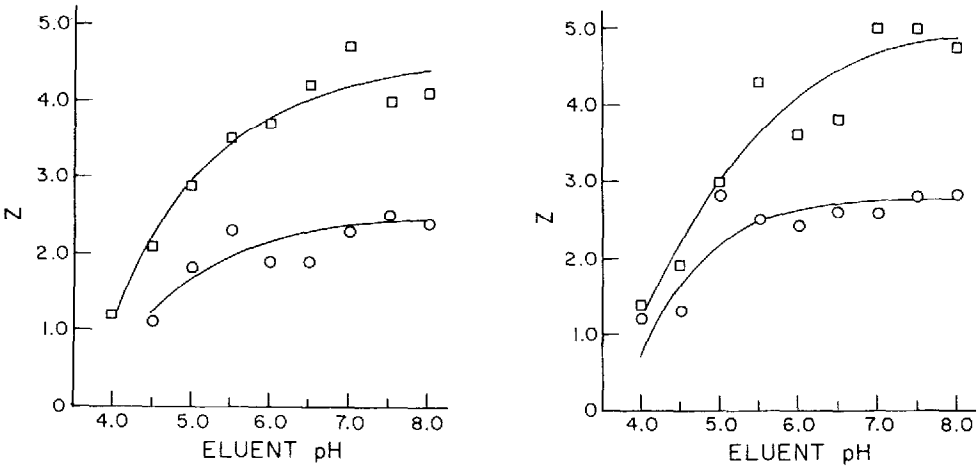

Fig. 3. Z *vs.* eluent pH for ovalbumin (pI=4.7), using NaCl (○) or $MgCl_2$ (□) as the displacing salt (on Pharmacia Mono Q SAX column).

Fig. 4. Z *vs.* eluent pH for β-lactoglobulin A (pI=5.1), using NaCl (○) or $MgCl_2$ (□) as the displacing salt (on Pharmacia Mono Q SAX column; Z_{NaCl} values plotted are from column 2 in Table I).

Mono Q SAX support all showed a positive correlation with retention (Fig. 1). In all four cases both retention and Z increased as eluent pH increased and, for β-LAC, OVA and STI, both began to level off about one pH unit above the protein's isoelectric point. (Curves for CON do not extend far enough the pI of this protein to be certain of the trend.) In the case of OVA, both sets of data points first the same curve (Fig. 1b).

NaCl vs. $MgCl_2$ as the displacing salt

The data in Table I show that Z values obtained with both displacing salts were relatively small, ranging from 0.9 to 5.0. In all cases except CON with $MgCl_2$ as the displacing salt, Z (both salts) increased with increasing eluent pH and leveled off as basic pH was approached (Figs. 2–5). For the three proteins of low pI, (STI, OVA and β-LAC) Z_{MgCl_2} began to increase significantly over Z_{NaCl} at the protein's pI (Figs. 2–4).

Soybean trypsin inhibitor is a relatively small (M.W. = 20,100[8]) protein and is stable in the pH range 1–12[9]. For these reasons, and the fact that it is less likely than the other three proteins to be affected by metal ions[10], specific findings for OVA, β-LAC and CON were considered relative to the data for STI.

Despite the fact that its molecular weight (45,000[11]) is more than twice that of STI, Z values for OVA were lower than those for STI when NaCl was used as the displacing salt. (Z_{MgCl2} values were essentially the same for both proteins.) A comparison of the "retention maps"[1] for these proteins (see also Fig. 1) showed that at any given pH retention times were shorter for OVA indicating that it was less tightly bound to the Mono Q SAX column than was STI. This correlation would support a definition of Z as the number of charged sites on the protein which are interacting with the support surface.

As illustrated in Fig. 1, the Z_{NaCl} *vs.* pH curve for β-LAC leveled off more sharply than did those for the other three proteins, with little change in Z_{NaCl} above pH 5.5.

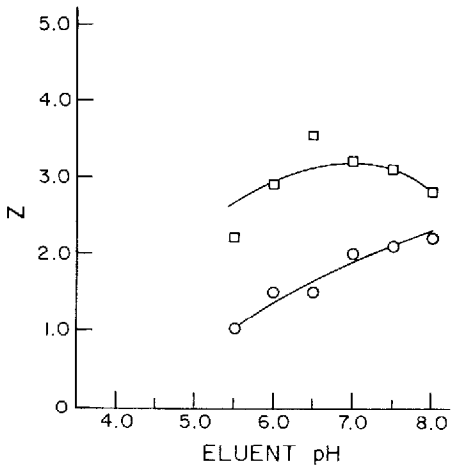

Fig. 5. Z *vs.* eluent pH for conalbumin (p*I ca.* 6.3), using NaCl (○) or $MgCl_2$ (□) as the displacing salt (on Pharmacia Mono Q SAX column).

β-Lactoglobulin (dimer M.W. = 36,000[4,5]) is one of only a few proteins known to bind Na^+ (refs. 12–14). (Saroff[14] has proposed a structure for the binding site, with an association constant of 10^2—10^3 for Na^+.) A study of the pH dependence of Na^+ binding to β-LAC indicated no binding near pH 5[12]. As pH increased, Na^+ binding gradually increased up to *ca.* four sodium ions per mole of β-LAC near pH 9 (refs. 12 and 13). In view of the concentrations of Na^+ used in this study (100% B = 0.5 *M* NaCl), it seems possible that binding of Na^+ to β-LAC could occur before the protein was bound to the positively charged anion-exchange column. (These Na^+ ions are not to be considered equivalent to the counter-ions, C_i, in eqn. 1.) A decrease in the number of negatively charged sites on the protein as a result of such binding would be consistent with the fact that Z_{NaCl} leveled off more rapidly with increasing eluent pH for β-LAC than for the other proteins evaluated.

As noted earlier, the pH dependency of Z for CON, when $MgCl_2$ was used as the displacing salt (Fig. 5), differed from that exhibited by STI, OVA and β-LAC. This apparent anomaly can be reconciled in terms of the properties of this particular protein. Conalbumin, or ovotransferrin (M.W. = 76,000), is capable of binding Fe^{3+} and a variety of other di- and trivalent metal cations[11,15,16]. It has recently been determined that the two metal binding sites in this protein each contain three histidine residues, one of which binds to a divalent anion (usually carbonate; simple monovalent inorganic ions such as Cl^- are not effective[15]). Upon binding iron, CON apparently becomes more compact, supporting an earlier postulate that a marked conformational change occurs[16].

In view of the very low protein concentrations used in this study (0.25 mg/ml ≈ $3.3 \cdot 10^{-6}$ *M* CON), it is conceivable that traces of CO_3^{2-} in the chromatographic eluent could bring about binding of Mg^{2+} to CON at alkaline pH. If this binding resulted in a more compact protein configuration, a decrease in the number of charged sites available for interaction with the ion-exchange support might be anticipated. This could explain the decrease in Z_{MgCl_2} above pH 7.0 (if Z does refer to the number of available charged sites as just described).

The magnitude of the values obtained and the general nature of the pH depen-

dency of Z strongly suggest that Z is closely related to the number of charged sites on the protein which are interacting with the surface of the HPIEC support. However, Z was obviously affected by the displacing salt used, since values of Z_{MgCl_2} were higher than Z_{NaCl}. Because the anion was constant, these differences must be due to the cation, *i.e.* sodium *vs.* magnesium*.

Mg^{2+} binds more thightly to carboxylic acids than does Na^+ (ref. 19). However, a protein · Mg^{2+} complex of this nature would be expected to result in $Z_{MgCl_2} > Z_{NaCl}$. Since this was not the case, such an association is probably of minor importance, and other factors are assumed to be more important to the relative Z values obtained with NaCl and $MgCl_2$. For example, Mg^{2+} is a Lewis acid and could enhance the ionization of dissociable functional groups on a protein molecule. (In the pH range 4.0–8.0, ionizable groups are carboxyl and imidazole.) Such an increase in the number of negatively charged groups at a given pH (*i.e.* a shift of the titration curve toward lower pH values) would be consistent with both $Z_{MgCl_2} > Z_{NaCl}$ and with a definition of Z as the number of charged sites on the protein which are interacting with the ion-exchange support**. In a classic study of the titration curve of egg albunim, *i.e.* OVA, in which several different salts were use, Cannan *et al.*[20] found that substituting MCl_2 (M = metal) for KCL (at constant ionic strength) gave a parallel displacement of the whole dissociation curve toward lower pH. The displacements due to M = Mg^{2+} or Ca^{2+} were nearly identical and were slightly greater than when M = Sr^{2+} or Ba^{2+}.

CONCLUSIONS

Additional study of a proposed retention model for HPIEC[1] was conducted using selected proteins and both a mono- and divalent cation-containing displacing salt in an effort to more clearly define the meaning of the Z term in this model. Correlation coefficients for the slopes obtained indicated that the method described herein is valid. A comparison of data from two independent series of determinations suggested that experimental error was less than 10% at moderate pH values.

Z values for four proteins chromatographed on an organic-based SAX support, with NaCl as the displacing salt, all showed a positive correlation with retention (from pH 4.0 to 8.0). This is in agreement with earlier findings on a silica-based anion-exchange column[11].

The magnitude of the Z values obtained, as well as the general nature of the pH dependency of Z, suggests that Z is closely related to the number of charged sites on the protein which are interacting with the surface of the ion-exchange support. (In retrospect, β-LAC and CON were less-than-ideal choices for probes in this study because of pH-induced structural changes and metal binding tendencies. Minor discrepancies in the data were attributed to these factors.)

Higher values for Z when $MgCl_2$ was used as the chromatographic displacing salt were attributed to a shift in the protein titration curves brought about by Mg^{2+}.

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* In aqueous solutions, some change in the nature of the magnesium ion itself occurs as Mg^{2+} is hydrolyzed to $MgOH^+$ ($pK_h = 11.42$)[17]. However, the contribution of this species is negligible, even at pH 8.0 ($[MgOH^+]/[Mg^{2+}] = 3.8 \cdot 10^{-4}$). Likewise, the formation of $MgCl^+$ is negligible[18].

** Alternatively, larger Z_{MgCl_2} values may simply reflect tighter binding of the same number of sites to the support surface.

The findings discussed in this paper suggest that, with regard to HPIEC, Z could be more precisely defined as the number of electrostatic interactions between the protein and the ion-exchange support. It must be remembered, however, that physiochemical properties of the displacing salt may contribute to apparent Z values, and the relationship of variables in this type of study is quite complex.

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