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Prediction of neutral salt elution profiles for affinity chromatography

(blue dextran-Sepharose/viscosity *B* coefficient/N^6-ATP-agarose)

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ABSTRACT **Neutral salts exhibit very marked differences as eluants of proteins from affinity columns. We observe: (*i*) that the relative potencies of neutral salts as eluants are independent of the protein or the affinity ligand in the systems studied, (*ii*) that the absolute salt concentration necessary to elute any given protein bound to the affinity matrix is proportional to the algebraic sum of a set of elution coefficients defined herein for the separate ions present in the solution, and (*iii*) that the proportionality between elution potency and elution coefficient is a function of the affinity of the protein for the immobilized ligand. Given the concentration of one neutral salt required for elution of a protein of interest from an affinity column, the elution capability of any neutral salt at any temperature can be quantitatively predicted for that protein. Accordingly, application and elution protocols for affinity chromatography can be designed to optimize the yield and fold purification of proteins.**

While affinity chromatography has expedited the purification of a wide range of proteins, use of a substrate or substrate analog as a selective eluant is often very expensive, particularly in the large-scale purification procedures required for acquisition of useful quantities of proteins of low natural abundance. For such situations various neutral salt solutions have been utilized as an eluant (1, 2), often resulting in a fold purification inferior to that with a specific ligand as the eluant. We find that various properties of the solutions under study correlate closely with the concentration of salt sufficient to remove enzyme bound to an affinity system, with the most useful correlation being found with the *B* coefficient in the equation postulated by Jones and Dole (3). This correlation can be exploited to optimize the fold purification and yield achieved by affinity chromatography by neutral salt elution.

MATERIALS AND METHODS

Rabbit muscle lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) were purchased from Sigma and bovine serum albumin, from Miles Laboratories. Particulate nucleosidediphosphate kinase (EC 2.7.4.6) was purified from bovine brain by using blue dextran-Sepharose. Horse liver alcohol dehydrogenase (EC 1.1.1.1) was a gift from B. V. Plapp. Blue dextran-Sepharose was prepared as described by Ryan and Vestling (4) and found to contain 1 mmol of blue dye per ml of gel after mild acid hydrolysis (5). Agarose-hexane-adenosine 5′-triphosphate, type 2 (N^6-ATP-agarose), was purchased from P-L Biochemicals. Reactive blue 2 (C.I. 61211) was purchased from Polysciences (Warrington, PA).

Blue dextran-Sepharose chromatography of purified proteins was done routinely at 23°C, using a 0.6 × 20 cm column equilibrated with 50 mM Tris·HCl buffer, pH 7.5 (5 mM $MgSO_4$ was also present when kinases were chromatographed). Linear gradients of neutral salts were obtained by mixing 75 ml of the equilibration solvent with 75 ml of the same solvent containing 1 M desired neutral salt. Fractions (110 drops) were collected at a flow rate of one drop per 3 s. The concentration of protein in each fraction was measured by the procedure of Bradford (6) or spectrophotometrically at either 222 or 280 nm. The concentration of enzyme in each fraction was measured by using the standard catalytic assay procedure. The concentration of neutral salt in the fraction containing the maximal protein concentration, C_p, was determined by the relationship:

$$C_p = \frac{C_f(F_p - F_i)}{F_t - F_i},$$

in which C_f is the final concentration of elution salt in the gradient device, F_i is the fraction containing the minimal concentration of the elution salt, F_p is the fraction containing the maximal concentration of protein, and F_t is the total number of fractions collected. Conductivity measurements obtained by using a Radiometer CDMS conductivity meter ensured the linearity of the initial portion of each gradient. The indirect method of salt concentration determination was adopted due to the loss of the proportionality between conductivity and concentrations in high salt concentrations.

For the chromatographic study of rat liver cytoplasmic enzymes, 20 g of rat liver was homogenized in 200 ml of 250 mM sucrose containing 100 mM Tris·HCl buffer, pH 7.5, using a Waring Blendor. The homogenate was centrifuged for 1 hr at 48,000 × *g* and 4°C and the supernatant was separated into 15-ml portions, which were stored at −20°C until needed. Each thawed portion was applied to a 2 × 10 cm blue dextran-Sepharose column equilibrated with 50 mM Tris·HCl buffer, pH 7.5 (23°C), maintained at 4°C. The column was washed with equilibration buffer until the A_{280} was less than 0.1. A 0–2 M gradient (except for KI, for which a 0–0.5 M gradient was used due to the density of 2 M KI) was applied, and 220-drop fractions were collected at a flow rate of one drop per s.

For the solubility study, 1 g of reactive blue 2 was placed in each of a series of small polycarbonate centrifuge tubes. Five milliliters of 50 mM Tris·HCl buffer, pH 7.5, containing either no added neutral salt or a given neutral salt at 200 mM was added to each tube. The contents of each tube were vigorously stirred by using a Vortex mixer and allowed to equilibrate for 96 hr at 23°C. These samples were then centrifuged at 48,000 × *g* and 23°C and appropriate samples of supernatant were removed for spectrophotometric measurement of dye concentration at 610 nm, using an extinction coefficient of 13.6 mM^{-1} cm^{-1} (7).

The dissociation constants for protein–dye complexes were obtained from equilibrium dialysis, zone electrophoresis, dif-

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ference spectral titration, and catalytic inhibition measurements as described (7, 8). Polyacrylamide slab gels were made from 7% acrylamide solutions and electrophoresis was done in 0.1% sodium dodecyl sulfate.

RESULTS

We initially compared the abilities of simple (i.e., biatomic) neutral salts for elution of rabbit muscle lactate dehydrogenase from blue dextran-Sepharose under the routine chromatographic conditions defined in *Materials and Methods*. This was done by elution of a series of identical immobilized columns containing the enzyme with a gradient of each neutral salt. In all cases the enzyme was quantitatively eluted as a single symmetrical component. The concentration of salt in the elution fraction containing the highest concentration of enzyme was defined as the elution concentration. As shown in Table 1, the elution concentrations for the simple neutral salts ranged from 105 to 595 mM, indicating that ionic strength is not the significant variable. Perusal of these results also indicates that the relative potencies of the simple neutral salts do not adhere to the ordering frequently observed in the lyotropic or Hofmeister series. However, the relative potencies correlate both qualitatively and quantitatively with the Jones–Dole viscosity *B* coefficients as shown in Fig. 1*A*. This coefficient originates in the expression

$$\eta/\eta_0 = 1 + Ac^{1/2} + Bc,$$

in which η is the viscosity of an aqueous salt solution, η_0 is the viscosity of the solvent, c is the concentration of salt, A is an electrostatic term not operative for the salts of interest at concentrations in excess of 20 mM, and B is the Jones–Dole coefficient (9–12).

The correlation between *B* coefficient and elution concentration was probed further in several ways. The published dB/dT (T being absolute temperature) predicts that the *B* coefficient for KCl will be decreased from −0.014 at 25°C to −0.064 at 4°C. The relationship for lactate dehydrogenase shown in Fig. 1 predicts that the elution concentration with KCl at 4°C should be 220 mM, the exact value that is observed experimentally.

Table 1. Elution of lactate dehydrogenase from blue dextran-Sepharose

Salt	Identification number	Elution concentration, mM	*B* coefficient (9–12)	Elution coefficient
$BaCl_2$	1	105	0.206	−0.124
KSCN	2	120	−0.110	−0.110
KI	3	180	−0.075	−0.075
KBr	4	250	−0.039	−0.039
CsCl	5	260	−0.052	−0.052
$CaCl_2$	6	260	0.271	−0.045
KNO_3	7	275	−0.053	−0.037
RbCl	8	300	−0.037	−0.037
$MgCl_2$	9	300	0.371	−0.025
$(CH_3CH_2)_4NCl$	10	300	0.374	−0.025
KCl	11	330	−0.014	−0.014
K phosphate buffer, pH 7.5	12	330		−0.009
NH_4Cl	13	430	−0.014	0.041
NaCl	14	500	0.079	0.079
Tris·HCl	15	510	0.343	0.081
CH_3CO_2K	16	525	0.243	0.089
$(NH_4)_2SO_4$	17	540	0.194	0.096
KF	18	570	0.093	0.093
LiCl	19	595	0.142	0.123

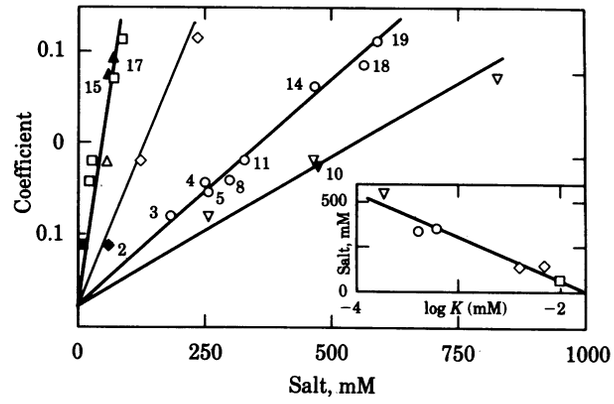

FIG. 1. Elution of proteins from blue dextran-Sepharose by neutral salts. ○, Lactate dehydrogenase; □ and ■, alcohol dehydrogenase; △ and ▲, serum albumin; ◇ and ◆, pyruvate kinase; ▽ and ▼, nucleoside diphosphate kinase. Open symbols represent measurements with simple salts plotted as *B* coefficients. Filled symbols represent measurements with complex salts plotted as elution coefficients. The numbers identify the salt employed as indexed in Table 1. (*Inset*) Dependence of the dissociation constants, *K*, for the dye–protein complexes of these enzymes on the elution concentration for a hypothetical salt having an elution coefficient of zero. The latter value was obtained by interpolation of the main portion of this figure. The dissociation constant for the nucleosidediphosphate kinase–dye complex was obtained from equilibrium dialysis measurements, the constants for the lactate dehydrogenase complex from equilibrium dialysis and electrophoresis measurements, those for the pyruvate kinase complex from difference spectral titration and electrophoresis measurements, and the constant for the alcohol dehydrogenase complex from competitive inhibition catalytic measurements.

Second, it can be anticipated that if neutral salts act independently, the elution volume for a combination of salts should be the average of their individual elution concentrations. Accordingly, lactate dehydrogenase retained by blue dextran-Sepharose was eluted by a linear gradient of 0–250 mM KCl and 0–250 mM LiCl simultaneously. Reference to Table 1 indicates that 250 mM of either salt individually should not result in the elution of the enzyme. However, the enzyme was eluted by the simultaneous gradient at a position corresponding to 440 mM of total salt. This concentration is within experimental error (23 mM, the change in salt concentration within the volume of each fraction) of the average elution concentration of the two salts employed, 460 mM. Third, we have used a nonionic compound, urea, to examine whether solvent viscosity *per se* is the operative principle. The *B* coefficient for urea, 0.038, would predict that a 430-mM solution of urea would elute lactate dehydrogenase from a blue dextran-Sepharose column according to Fig. 1. However, no elution of the enzyme was observed in a 0–1 M gradient of urea.

Correlation of the data with the *B* coefficient of viscosity also allows other correlations to be established. That the partial molal entropy of hydration and the temperature coefficient of ionic mobility are proportional to the *B* coefficient is well documented (10–12), and these correlate well with elution concentration. However, the precision and extent of data available for the *B* coefficient predisposed us towards its correlation, and further analysis was limited to this quantity.

The linear correlation between *B* coefficient and salt elution concentration is not restricted to the elution of lactate dehydrogenase from blue dextran-Sepharose. As shown in Fig. 1, such a correlation also pertains to the elution of alcohol dehydrogenase, serum albumin, pyruvate kinase, and nucleoside-diphosphate kinase from blue dextran-Sepharose. These indi-

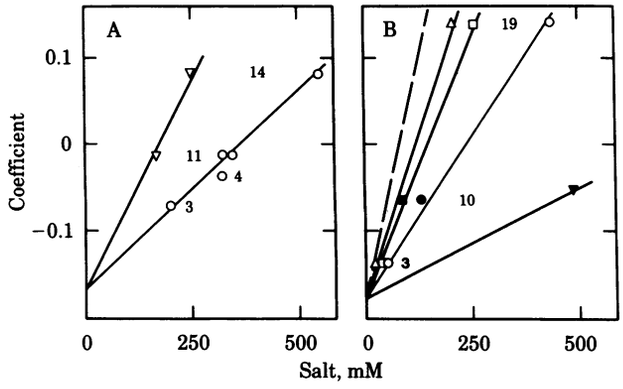

FIG. 2. Elution of purified enzymes from ATP-Sepharose (*A*) and rat liver enzymes from blue dextran-Sepharose (*B*). ○ and ●, Lactate dehydrogenase; ▽ and ▼, nucleosidediphosphate kinase; □ and ■, malate dehydrogenase; ▽, citrate synthase. Open symbols represent measurements with simple salts plotted as *B* coefficient and filled symbols represent complex salts plotted as elution coefficients. The numbers identify the salt employed as indexed in Table 1. The broken line in panel *B* represents the elution concentration for the bulk protein (as defined in the legend to Fig. 3) retained by the affinity column.

vidual correlations exhibit a common intercept and various slopes. The concentration of a salt of a given *B* coefficient—e.g., zero—required to elute these proteins is a linear function of the logarithm of the affinity of the enzyme for the immobilized ligand, as shown in the *Inset* to Fig. 1. The elution concentration for a salt of known elution coefficient (*EC*, as defined below) required to elute a protein having a reciprocal affinity constant for this affinity system (K_d) is given by the empirical relationship

$$\text{Salt concentration} = \frac{EC + 0.169}{0.000661(\log K_d) + 0.00272},$$

in which all concentration terms have the units mM.

The correlation between *B* coefficient and salt elution concentration also applies to the elution of nucleosidediphosphate kinase and lactate dehydrogenase from an ATP-Sepharose column as shown in Fig. 2*A*. Again, two divergent linear relationships with a common intercept are observed. While the intercept values observed for blue dextran-Sepharose and ATP-Sepharose are the same, the relative affinities of the immobilized ligands for the kinase and dehydrogenase are reversed. Finally, rat liver citrate synthase, malate dehydrogenase, lactate dehydrogenase, and nucleosidediphosphate kinase applied to blue dextran-Sepharose in a crude homogenate are eluted by the concentrations of simple salts LiCl and KI proportional to their *B* coefficients as shown in Fig. 2*B*.

As shown in Fig. 3, rat liver lactate dehydrogenase is relatively well resolved from the bulk rat liver protein in the LiCl elution gradient, resulting in a 60-fold purification of this enzyme relative to the crude liver homogenate. The polyacrylamide gel electrophoresis patterns indicate that although the absolute A_{280} values toward the end of the 0–1 M LiCl gradient are small, a variety of polypeptides are eluted at 0.6 and 0.9 M LiCl and that different proteins would be predicted to be eluted up to a LiCl concentration of 3.5 M. Such retention indicates that proteins of very high affinity for immobilized blue dye exist, having K_d values equal to or smaller than the 20–50 nM values reported for prostaglandin reductase (13) and choline acetyltransferase (14), respectively.

In contrast to the biatomic neutral salts, the concentration of polyatomic neutral salts required for elution of lactate dehydrogenase from blue dextran-Sepharose are generally unrelated to the *B* coefficients of the polyatomic salts. These elution concentrations can be assigned to an effective *B* coefficient, termed the elution coefficient, using the relationship for lactate dehydrogenase shown in Fig. 1. The elution coefficients for the polyatomic salts and ions obtained by this procedure are listed in Tables 1 and 2. As shown in Figs. 1 and 2, the elution coefficient values for polyatomic neutral salts are not restricted to lactate dehydrogenase chromatography on blue dextran-Sepharose but also pertain to elution of serum albumin and nucleosidediphosphate kinase from blue dextran-Sepharose and rat liver cytoplasmic malate and lactate dehydrogenases from blue dextran-Sepharose, with a crude homogenate as the enzyme source. It should be noted that these comparisons were made while using complex salts whose elution and *B* coefficients are markedly different.

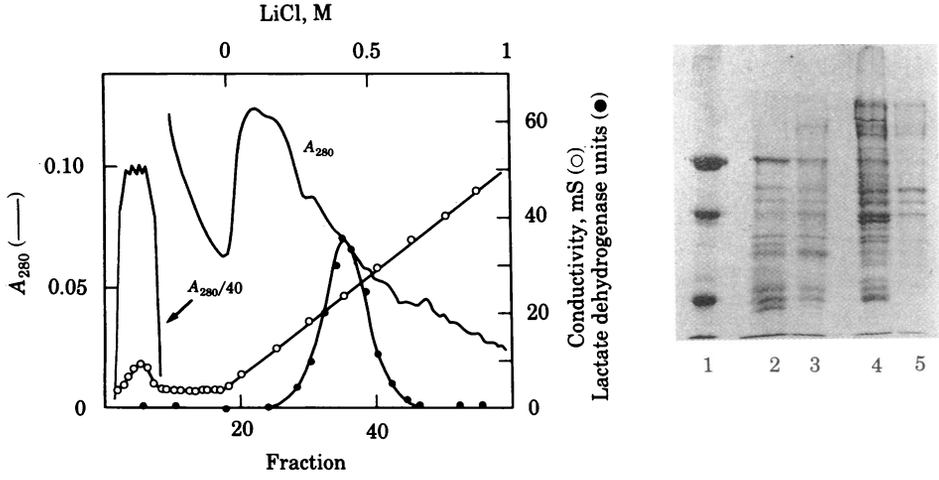

FIG. 3. Fractionation and analysis of a rat liver extract. (*Left*) Affinity chromatography on blue dextran-Sepharose at 4°C with a 0–1 M gradient of LiCl as the eluant. The bulk protein is defined as that in the fraction containing the highest protein concentration in the gradient elution profile. (*Right*) Polyacrylamide gel electrophoresis in detergent. Protein was stained with 0.1% Coomassie blue R in 50% trichloroacetic acid. Lane 1 contains the standard proteins serum albumin, ovalbumin, and trypsinogen (from top to bottom); lanes 2 and 3 illustrate the polypeptides in fractions containing 0.6 and 0.9 M LiCl, respectively; lanes 4 and 5 illustrate the polypeptides expected in fractions containing 2.2 and 3.5 M LiCl, respectively. The latter two samples were actually obtained from a KI gradient elution of the rat liver extract. The LiCl concentrations corresponding to the actual KI concentration in the fractions selected were extrapolated by using Fig. 2*B*.

Table 2. Ion coefficients

Ion	Coefficient at 25°C		
	B (9–12)	Elution	*dB/dT* (12)
Ba^{2+}	0.22	−0.11	
SCN^-	−0.103	−0.103	
I^-	−0.068	−0.068	0.0017
Cs^+	−0.045	−0.038	
Br^-	−0.032	−0.032	0.0011
Ca^{2+}	0.285	−0.031	
Rb^+	−0.030	−0.030	
NO_3^-	−0.046	−0.030	
$(CH_3CH_2)_4N^+$	0.381	−0.018	−0.0011
Mg^{2+}	0.385	−0.011	0.0024
K^+	−0.007	−0.007	0.0012
Cl^-	−0.007	−0.007	0.0012
SO_4^{2-}	0.208	0.001	−0.0019
NH_4^+	−0.007	0.048	0.0005
Na^+	0.086	0.086	0.0000
$CH_3CO_2^-$	0.250	0.096	
F^-	0.10	0.10	
Li^+	0.150	0.132	−0.0011

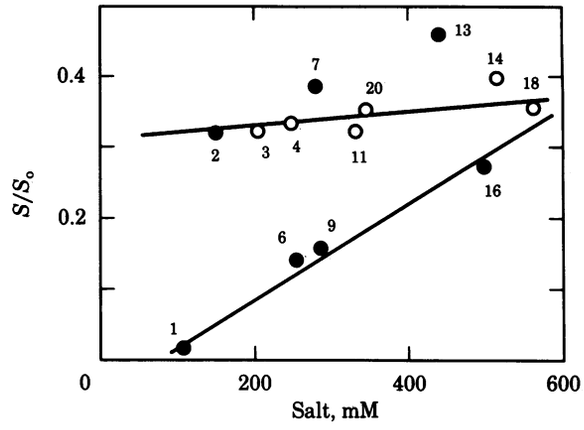

FIG. 4. Solubility of reactive blue 2 in neutral salt solutions. The term S/S_o represents the solubility of the dye in 0.2 M salt relative to its solubility in water. This term is plotted against the concentration of the salt that elutes lactate dehydrogenase from blue dextran-Sepharose (Table 1). The number beside each experimental value indicates the identity of the salt as defined in Table 1. Simple salts are indicated by ○s and complex salts by ●s.

The effect of neutral salts on lactate dehydrogenase and on the blue dye were examined individually. As shown in Table 3, individual simple salts at the elution concentrations observed for lactate dehydrogenase on blue dextran-Sepharose caused similar perturbations in the catalytic parameters of lactate dehydrogenase. The solubility of the blue dye of blue dextran, reactive blue 2, is decreased in the presence of neutral salt. As shown in Fig. 4, the solubility of dye is essentially the same in all the biatomic neutral salts and in KSCN and KNO_3—i.e., those salts whose *B* coefficient and elution coefficient are nearly identical. However, the dye was less soluble in the presence of those salts whose *B* coefficient is greater than elution coefficient—$BaCl_2$, $CaCl_2$, $MgCl_2$, and potassium acetate—and more soluble in the presence of the single polyatomic salt, NH_4Cl, whose *B* coefficient is less than its elution coefficient.

DISCUSSION

The major observations of this work are illustrated in Figs. 1 and 2; (*i*) neutral salts are not equipotent as eluants of proteins from affinity columns; (*ii*) the relative potencies of neutral salts as eluants are independent of the protein or the immobilized ligand; and (*iii*) the absolute concentration of neutral salt(s) required to elute a given protein from an immobilized ligand column is quantitatively related to the algebraic sum of the elution coefficient(s) of the constituent salt(s) and to the affinity of the protein for the immobilized ligand. These observations have several important practical aspects for affinity chromatography in general and provide several insights into the nature of dye–protein complexation.

Table 3. Effect of salts on lactate dehydrogenase catalysis

Additive		K_m	V_{max},
Salt	Conc., mM	for NADH, μM	μmol/min
None		10 ± 1	17 ± 0.5
KSCN	120	30	10
KI	180	26	11
KBr	250	27	13
KCl	330	24	13
NaCl	500	28	11
LiCl	600	40	12

± indicates SD.

As noted in the Introduction, affinity chromatography, particularly that employing nonbiodegradable textile dyes, is frequently very productive when employed early in a protein purification protocol. Rational use of inexpensive neutral salts in the application of a crude protein mixture, washing, and elution should in principle greatly expand the utility, yield, and fold purification of this fractionation procedure. Because the lines in Fig. 1 have a common intercept, it should be possible to predict the elution concentration of any neutral salt of known elution coefficient from the elution concentration of a single neutral salt in the affinity systems we have studied. For other affinity columns, determination of the elution concentrations for two neutral salts having known elution coefficients would adequately define the slope and intercept for a plot analogous to Fig. 1 for the immobilized ligand–protein complex of interest. We suggest that a minimal sample of a tissue extract be applied to a small affinity column, washed, and then eluted with a gradient of a common relatively innocuous salt having an elution coefficient near zero—e.g., KCl. Fractions of the flow-through, wash, and gradient elution should be analyzed for total protein—e.g., by A_{280}—and the desired biological activity as shown in Fig. 3 *Left*. Assuming the protein of interest both binds to the affinity column and is eluted in the test salt gradient chosen, a plot relating elution coefficient and salt concentration analogous to Fig. 2*B* can be constructed from the intercept and salt elution concentration for the bulk protein and for the protein of interest. At this point several decisions can be made: (*i*) The ionic strength range and temperature necessary to retain maximal stability/function of the desired protein will limit the concentration range of a given neutral salt. In addition, salts deleterious to function via selective interaction should be avoided. (*ii*) Within these constraints, if the protein of interest emerges after the bulk protein, a salt and concentration should be selected for addition to the tissue extract that will result in maximal retention of the protein of interest with minimal retention of the bulk protein. This should be followed by batch or gradient elution of the protein of interest, using the same or a different salt of advantageous concentration(s). Using Fig. 2*B* as an example, one can see that malate dehydrogenase could be purified by application of the crude extract in 175 mM NaCl followed by elution in a 175–300 mM gradient in NaCl; nucleosidedi-

phosphate kinase could be applied in crude extracts containing 300 mM KI and batch eluted by using 300 mM KSCN. (*iii*) If the elution of the protein of interest precedes that of the bulk protein, a salt and salt concentration should be selected to elute the maximal amount of protein of interest and the minimal amount of bulk protein. (*iv*) If the protein of interest and the bulk protein virtually coelute, a different immobilized ligand might be explored. Fig. 2 illustrates the inversion of the relative affinities of lactate dehydrogenase and nucleosidediphosphate kinase for immobilized ATP and reactive blue 2.

The results obtained by using the mixed LiCl/KCl gradient indicate that the elution coefficients of all ionic components present must be considered in the prediction and utilization of the relationships described here. The 42 mM Tris·HCl buffer component makes a trivial addition to the elution of lactate dehydrogenase by neutral salts in Fig. 1, due to its low concentration and high elution coefficient and to the high affinity of the enzyme for the immobilized ligand. However, for enzymes of relatively low affinity and for solvent components of higher concentration or lower elution coefficient, the presence of such components may contribute significantly to enzyme retention and elution.

It is not uncommon that a protein cannot be eluted from some affinity columns, particularly those containing biospecific ligand immobilized by using apolar spacers, without using a mild denaturant. Salts having very negative elution coefficients or combinations thereof would be useful in this context, because they should reduce the inevitable loss of enzyme resulting from irreversible denaturation. It should also be noted that many proteins in relatively small abundance strongly adhere to blue dextran-Sepharose (Fig. 3) and should be elutable in such neutral salt-containing solvents. Finally, use of a salt having a low elution coefficient should clear the affinity column of all bound protein at high salt concentration. This is not a trivial observation, because the capacity of preparative affinity columns is markedly reduced upon repeated use due to accumulation of strongly bound protein not removed during most regeneration protocols.

As to the nature of the immobilized dye–protein complexation, Table 1 clearly indicates that it is not principally electrostatic as is commonly assumed. Because in the classical analysis of the solution parameters affecting *B* coefficient (10), the only parameter leading to a decrease in this value is disruption of solvent–solvent interactions, and because elution ability of a given salt is inversely proportional to its *B* coefficient, it is reasonable to surmise that the salts are weakening a dye–protein hydrophobic interaction by altering the structure of the aqueous solvent. This can also be seen in the effect of salt on the catalytic parameters of lactate dehydrogenase as listed in Table 3. Such a mechanism, by which the ability of a salt to lower the solvent's repulsion for the organic ring systems under consideration (whether nucleotide or dye), would affect the complexation of all immobilized ligand–protein interactions to the extent of their hydrophobic nature. Because the strength of ligand complexation can be related to the surface area buried (15), the stronger the affinity the greater the water structure must be perturbed to effect elution as seen in the *Inset* to Fig. 1. It is useful to note as an aside that the limiting value of the line in the *Inset* to Fig. 1 implies that a blue dextran-Sepharose–protein complex must have a $K_d < 20\ \mu M$ in order for the protein to be retained by the column due to the dynamics of chromatography. This limiting affinity agrees with empirical observations.

Finally, the source(s) of the difference in *B* coefficient and elution coefficient listed in Tables 1 and 2 merits comment. As shown in Fig. 4, those salts having such differences also effect dye solubility in a manner appropriate to the sign of the difference—i.e., NH_4Cl enhances solubility and has a positive difference, while the remaining salts decrease solubility and have negative differences. In the case of $BaCl_2$, $MgCl_2$, and $CaCl_2$, the cationic component is the one whose coefficient deviates from predicted values (Table 2). Molecular models indicate that the three sulfonates on the dye can simultaneously complex with a divalent cation. Thus these cations could compete with protein for the dye, providing a specific interaction to supplement the solvent effect proposed for monovalent cations. Unfortunately, no insight into the deviation exhibited by acetate, ammonium, or tetraethylammonium is evident to us, other than the obvious conclusion that the polyatomic nature of these compounds sets them apart from the monatomic ions that follow *B* coefficient in elution.

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