Effects of Anions on the Activation Thermodynamics and Fluorescence Emission Spectrum of Alkaline Phosphatase: Evidence for Enzyme Hydration Changes during Catalysis†

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ABSTRACT: The effects of anions on the thermodynamic activation functions for a model enzyme, calf intestinal alkaline phosphatase (EC 3.1.3.1), have been studied in order to examine the role of protein hydration changes in establishing the energetics of enzyme catalysis. The influences of these anions on the activation volume ($\Delta V^{\ddagger}$) and activation free energy ($\Delta G^{\ddagger}$) reflected clear Hofmeister (lyotropic) series effects, in the order $F^- > Cl^- > Br^- > I^-$ (order of increasing salting-out potential). A pronounced covariation was observed between the influences of these anions on V_{max}, which is proportional to $\Delta G^{\ddagger}$, and on the negative activation volume of the reaction. Fluoride was able to counteract the influences of Br^- and I^- on both V_{max} and $\Delta V^{\ddagger}$ when combinations of these anions were employed. The effects of Br^- and I^- on V_{max} and $\Delta V^{\ddagger}$ were more pronounced at lower temperatures. The control $\Delta V^{\ddagger}$ was increasingly negative at reduced temperatures. The effects of the neutral salts and propanol on $\Delta V^{\ddagger}$ and $\Delta G^{\ddagger}$, as well as the effects of salting-in anions on the activation enthalpy and the negative activation entropy of the reaction, are consistent with a model which proposes that peptide groups or polar side chains on the native enzyme exergonically increase their exposure to solvent during the catalytic activation event. These conclusions are in accord with the known free energy, enthalpy, entropy, and volume changes which occur when model peptide groups are transferred between water and concentrated salt solutions. Consistent with the kinetic results, the fluorescence emission wavelength maximum of alkaline phosphatase increased in the presence of anions in the order $F^- > Cl^- > Br^- > I^-$. The salting-out ion (F^-) and the salting-in ions (Br^- and I^-) shifted λ_{max} in different directions, and these λ_{max} shifts could be counterbalanced by using equimolar combinations of salting-in and salting-out anions. Control experiments with a model compound, *N*-acetyltryptophanamide, showed that the spectral shifts caused by the salts did not result solely from differential quenching by the anions of the solvent-exposed tryptophan(s) on the enzyme. Hofmeister additivity phenomena indicated that the solvent is at the basis of these salt-induced enzyme structural changes. It is concluded that changes in protein solvation during enzymic reactions contribute significantly to the thermodynamic activation parameters in both the native and the salt-perturbed enzyme.

One of the most challenging problems remaining for biochemists to solve in their attempt to understand the energetics of enzymic catalysis concerns the role of changes in hydration of the enzyme–substrate complex during catalysis. As Gutfreund (1972) states, "The overwhelming difficulty of interpreting thermodynamic parameters in terms of chemical change is due to the large effects of changes in solvation and solvent structure."

Attempts to resolve questions about the energetic significance of hydration changes during catalysis, especially the changes in water organization that result from altered exposure of protein groups (side chains and peptide linkages) during catalytic conformational changes, have had to rely on rather indirect evidence (Low & Somero, 1975a,b; Somero et al., 1977). The available data on the energy changes accompanying alteration of protein hydration derive largely from equilibrium studies of protein denaturation, on the one hand (Brandts, 1964a, 1969; Zipp & Kauzmann, 1973), and of protein model compound solubilities in organic and aqueous phases, on the other hand (Kauzmann, 1959; Nemethy & Scheraga, 1962; Brandts, 1964b; Cohn & Edsell, 1965; Edelhoch & Osborne, 1976). Whereas both types of studies have provided unequivocal evidence that even minor changes in protein conformation will be accompanied by energetically significant changes in water organization, to discover the energetic role played by the hydration changes which occur during rapid catalytic conformational changes, a method must be developed for detecting these short-lived changes in water organization.

One promising experimental approach for achieving the latter goal is suggested by the theories and results of high-pressure biochemistry. Large changes in system (protein complex plus water) volume result from alterations in water organization during catalysis (Laidler & Bunting, 1973). These hydration changes may be due to increases in polarity of the enzyme–substrate system arising from charge separation or altered exposure of protein groups. As shown by the results of model transfer studies, the movement of a protein group from a nonpolar phase (simulating the "interior" of the protein molecule) to water generally occurs with a decrease in system volume due to water organization around the exposed group (Kauzmann, 1959; Nemethy & Scheraga, 1962; Friedman & Scheraga, 1965; Bøje & Hvidt, 1972). Thus, to at least a first approximation, the study of volume changes during catalysis using hydrostatic pressure as an experimental perturbant (see Materials and Methods) may provide a means for detecting hydration changes during catalysis.

A second experimental approach to the analysis of enzyme hydration changes involves a method for modifying these hydration changes using neutral salts of the Hofmeister series (von Hippel & Schleich, 1969). Neutral salts which differ in their effects on protein group solubilities (Nandi & Robinson, 1972a,b) will be expected to differentially modify protein hydration. If hydration changes during catalysis contribute

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significantly to activation free energies, enthalpies and entropies, predictable solute effects on these thermodynamic parameters, as well as on activation volumes (see Materials and Methods), should be observed. Thus, the use of Hofmeister series salts and hydrostatic pressure as experimental variables in enzyme kinetic and structural studies may allow new insights into the roles played by hydration energies in catalysis and in salt activation and inhibition of catalysis.

Past studies of pressure and solute influences on enzymes generally have involved complex, multisubstrate enzymes and have not included detailed structural analyses of solute perturbation of enzyme structure (Low & Somero, 1975a,b; Somero et al., 1977). In the present studies, we thus have focused on a simple, single-substrate enzyme, calf intestinal alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], which seems ideally suited for studies of this type. The enzyme is markedly pressure sensitive, the rate-limiting step in the reaction is well understood, and the influences of salts on reaction velocity reflect clear Hofmeister series effects. The results of our kinetic and fluorescence studies support the hypothesis that hydration changes during catalysis involving both the native and the salt-perturbed enzyme contribute significantly to thermodynamic activation parameters.

Materials and Methods

Chemicals. Alkaline phosphatase (analytical grade I) purified to homogeneity from calf intestinal mucosa was obtained from Boehringer Mannheim Biochemicals. The enzyme had a specific activity of 400 units/mg of protein at 25 °C as received and was stored at 4 °C as a suspension in a 3.2 M $(NH_4)_2SO_4$ solution, pH 7.0, containing 0.1 mM $ZnCl_2$ and 1 mM $MgCl_2$. *p*-Nitrophenyl phosphate was from Boehringer Mannheim. Tris,[1] Ches, and NATA were reagent grade from Sigma Chemical Co. The neutral salts and propanol were reagent grade. Glass-distilled deionized water was used for the preparation of all solutions.

Enzyme Preparation and Assay Procedures. Ammonium sulfate suspensions of the enzyme (2.0 mg/mL) were diluted 200-fold and dialyzed against 0.1 M Tris, pH 9.20, containing 0.05 M KCl, 1.0 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ for 3 days at 4 °C. A hyperbolic increase in enzyme activity was observed during this period to ~4 times the original activity. Subsequent to this, the activity stabilized and remained constant for several days when the dialysate was stored at 4 °C. This phenomenon is perhaps due to the removal by dialysis of tightly bound endogenous inorganic phosphate, a product inhibitor of the alkaline phosphatase reaction (Fernley, 1971). For each day's assays an enzyme stock solution was prepared by dilution of the dialysate two- to threefold with the standard assay buffer (see below).

Tris-HCl buffer was used in all phases of this study because of its pressure insensitivity (Neuman et al., 1973). The standard assay medium contained, in a total volume of 5 mL, 0.1 M Tris-HCl, pH 9.20 at the assay temperature, and 0.05 M KCl. This buffer will henceforth be referred to as the control buffer. Varying concentrations of the substrate, *p*-nitrophenyl phosphate, and various other solutes were added as described in the figure legends. Addition of zinc or magnesium ions to the assay medium resulted in no observable changes in the activation volume of the reaction, and so they were omitted from routine assays. In the preparation of buffer solutions containing salts, care was taken to correct for minor pH changes caused by the addition of these solutes (~0.2 pH unit). Solutions of potassium iodide were prepared each day of use. All other salt solutions were stable indefinitely.

The reaction was initiated by the addition of 25 μL of enzyme stock solution and followed by monitoring the increase in absorbance at 400 nm on a Varian Model 634 spectrophotometer. The change in absorbance varied linearly with time for several minutes, provided that the total change in absorbance was less than ~0.35 OD unit. At the lower temperatures used in this study (10 and 15 °C), an initial nonlinearity was observed in the reaction rate in the presence of some neutral salts (KBr and KI). After an appropriate time (~1 min), however, the rate assumed linearity and remained so for several minutes. This linear portion was taken as the velocity of the reaction. Several lines of evidence support the assertion that the phenomenon of initial nonlinearity in the assay does not result from irreversible salt inactivation. (1) Initial velocities calculated from the linear portions of the tracings yielded classical Michaelis–Menton kinetics. (2) Increase to a higher pressure, followed by release back to the original pressure, reproduced the original velocity (see below). (3) The initial nonlinearity was observed whether or not the enzyme was preincubated in the presence of the salt. Moreover, the same velocity was calculated from the linear portion of the tracing regardless of whether the enzyme was preincubated with the salt or added immediately prior to assay. (4) The nonlinear effect was not observed at higher temperatures. However, velocities at all temperatures produced linear Arrhenius plots and linear plots of ln V vs. pressure (see Results). The mechanism responsible for the initial nonlinearity in the progress curve of the alkaline phosphatase reaction in the presence of these salts remains unknown. However, we feel that the phenomenon does not influence our results, provided that the reaction is allowed to reach linearity.

Specific activities were calculated from the absorbance slopes by using an extinction coefficient of $E_{400}^{mM} = 16.2$ for *p*-nitrophenol and $E_{278}^{1\%} = 7.8$ for alkaline phosphatase (Fernley, 1971). $NaDodSO_4$–polyacrylamide gel electrophoresis of the enzyme revealed a single band corresponding to a subunit molecular weight of 79 000. Since the enzyme is a dimer (Fernley, 1971), the molecular weight of the enzyme was taken to be 158 000.

The high-pressure optical cell used in these studies was similar to the cell described by Mustafa et al. (1971). High-grade mineral oil was used as the pressurizing fluid, and a minute amount of mineral oil entered the top of the cell during pressurization. No effect of the mineral oil on the enzyme was noted, and with the addition of at least 50 mM KCl to all buffers, mineral oil contamination in the cell was minimized. Initial pressurization was achieved in ~5–10 s, and changes in pressure during a reaction were achieved virtually instantaneously. The pressure range used was between 1 and 408 atm. The cell was kept at a constant temperature (±0.1 °C) with the aid of a circulating water bath continuous with the pressure cell.

Under no conditions of solute concentration, composition, and pressure did pressure-induced denaturation of the enzyme occur. Increases or decreases in pressure were followed by reproducible and instantaneous changes in rate, and the new rate remained stable during the period of the assay (generally 1–5 min at the low enzyme concentrations used). The instantaneous change to a new stable rate is strong evidence that pressure did not alter the enzyme since relaxation times for

[1] Abbreviations used: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ES, enzyme–substrate; K_d, dissociation constant; NATA, *N*-acetyltryptophanamide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

protein unfolding are between approximately 10 s and 1 min (Tsong et al., 1972; Brandts et al., 1975). Thus, pressure denaturation would have been observed as a continuous nonlinearity in reaction rate.

Fluorescence Studies. Ammonium sulfate suspensions of the enzyme (5.0 mg/mL) were diluted to a concentration of 1.0 mg/mL and dialyzed against 4 L of a 0.1 M Tris-HCl buffer, pH 9.20, containing 0.05 M KCl. Enzyme concentrations used for fluorescence studies were generally 0.125 mg/mL and were achieved by dilution with either control buffer (i.e., the dialysis buffer) or buffer containing the desired neutral salt concentrations. I^- reacts with oxygen in solution to form I_3^-, which can absorb emitted light. For minimization of production of I_3^-, freshly prepared solutions of KI were used which were then exposed to the exciting light only for the brief period required for a wavelength scan.

All solutions were passed through a Millipore filter (0.45 μm) prior to measurements to remove dust particles. Fluorescence spectra were recorded with a Perkin-Elmer Model MPF-44A fluorescence spectrophotometer equipped with a thermostated cell holder. Both the excitation and emission bandwidths were 10 nm. Spectra were not corrected for the spectral responses of the instrument.

Polarization of fluorescence was measured with the accessory supplied by the manufacturer. The corrected polarization, p, was determined from the equation (Chen & Bowman, 1965)

$$p = \frac{I_v^v - I_H^v(I_v^H/I_H^H)}{I_v^v + I_H^v(I_v^H/I_H^H)}$$

where the superscript of the fluorescence intensity, I, is the horizontally (H) or vertically (v) polarized excitation and the subscript indicates the corresponding emission values.

All fluorescence measurements were made at 15 °C (±0.2 °C).

Determination of the Activation Parameters. Mechanism. The single-substrate reaction catalyzed by alkaline phosphatase was analyzed according to the simple two-intermediate scheme (Fernley, 1971)

$$EH + ROP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH{\cdot}ROP \xrightarrow[ROH]{k_2} EP \xrightarrow[H_2O\ \ P_i]{k_3} EH$$

where ROP represents the substrate, EH and EP are the free and phosphoryl enzymes, respectively, and EH·ROP is the Michaelis complex. The Michaelis parameters in terms of the various rate constants are given in eq 1 and 2

$$K_m = \frac{(k_{-1} + k_2)k_3}{k_1(k_2 + k_3)} \quad (1)$$

$$V_{max} = \frac{E_0 k_2 k_3}{k_2 + k_3} \quad (2)$$

where E_0 is the concentration of active sites (equal to twice the enzyme concentration).

It has been rigorously established that at high pH the rate-limiting step in the reaction sequence is the relatively slow dissociation of noncovalently bound phosphate from the enzyme (i.e., $k_2 \gg k_3$) (Hull & Sykes, 1976a,b). Therefore, eq 2 reduces to

$$V_{max}/E_0 = k_3 \quad (3)$$

Thus, V_{max} is directly proportional to k_3.

According to transition-state theory, the velocity constant is related to the free energy of activation ($\Delta G^\ddagger$) as follows:

$$k_3 = \frac{\kappa KT}{h} e^{-\Delta G^\ddagger/RT} \quad (4)$$

where k_3 equals the turnover of substrate per active site (in seconds^{-1}), K is Boltzman's constant, h is Planck's constant, R is the universal gas constant (equal here to 1.987 cal mol^{-1} K^{-1}), and T is the absolute temperature. The transmission coefficient (κ) was assumed to be unity under all experimental conditions.

The enthalpy of activation ($\Delta H^\ddagger$) was calculated from eq 5

$$\Delta H^\ddagger = E_a - RT \quad (5)$$

where E_a is the activation energy as determined from the slope of an Arrhenius plot (see Results).

The entropy of activation ($\Delta S^\ddagger$) was calculated by using eq 6. Thus, by measuring the rate constant by which the enzyme–substrate complex breaks down to free enzyme and products, and the known enthalpy of activation, one can calculate the entropy of activation.

$$k_3 = \frac{\kappa KT}{h} e^{-[(\Delta H^\ddagger/RT) - (\Delta S^\ddagger/R)]} \quad (6)$$

A detailed treatment of the effects of pressure on enzymic reaction rates is given by Laidler (1951). Under maximum velocity conditions, where substrate binding volumes do not contribute to the total reaction volume change, the effect of pressure on reaction rate is due entirely to the activation volume, $\Delta V^\ddagger$, if no pressure-induced denaturation of the enzyme occurs. Activation volume is computed from the slope of a plot of ln V_{max} vs. pressure (in atmospheres) (Johnson et al., 1954), according to the equation

$$\Delta V^\ddagger = -RT\frac{d \ln k}{dP} \quad (7)$$

where k is the value of V_{max} at gauge pressure P (in atmospheres), R is the universal gas constant (equal here to 82 cm^3 atm K^{-1} mol^{-1}), and T is the absolute temperature.

Results

Measurement of Activation and Binding Volumes. The effects of increased pressure on the rates of enzymic reactions may derive from binding volumes (ΔV_b) and activation volumes ($\Delta V^\ddagger$) (see Materials and Methods). At nonsaturating substrate concentrations, substrate binding volumes may contribute significantly to the total volume change calculated from the effect of pressure on ln velocity. Thus, to accurately determine $\Delta V^\ddagger$, it is essential to use substrate concentrations which are high enough to eliminate ΔV_b effects (Laidler & Bunting, 1973). To this end, we measured the effects of pressure on reaction rate as a function of *p*-nitrophenyl phosphate concentration at two pressures (Figure 1A). Pressure is seen to have a marked effect on both the V_{max} and apparent K_m of the reaction. Table I gives the $\Delta V^\ddagger$ calculated from the theoretical V_{max} values at 68 and 340 atm as well as the apparent binding volume (assuming that K_m approximates K_d) calculated from the pressure dependence of the K_m according to the equation (Laidler, 1951)

$$\Delta V_b = -RT\frac{d \ln K_m^{-1}}{dP} \quad (8)$$

The effect of pressure on reaction velocity was also examined at a series of pressures between 1 and 408 atm by using a substrate concentration of 2.0 mM. The natural logarithm of velocity varied linearly with pressure over this entire pressure range (Figure 2), and the slope of the plot of ln velocity vs. pressure yielded an activation volume of −21.6 cm^3 mol^{-1}, a value which was in excellent agreement with the $\Delta V^\ddagger$ obtained

Table I: Pressure and Salt Dependence of Kinetic Parameters for the Alkaline Phosphatase Reaction at 20 °C, pH 9.2

	K_m ± 95% CL (μM)[a]		ΔV_b	k_3 ± 95% CL ($\times 10^{-2}$) (s^{-1})[a,b]		$\Delta V^{\ddagger}$ (theor)[c]	$\Delta V^{\ddagger}$ (app)[d]
	68 atm	340 atm	(cm^3 mol^{-1})	68 atm	340 atm	(cm^3 mol^{-1})	(cm^3 mol^{-1})
control	33.3 ± 1.5	54.4 ± 6.3	43.6	4.66 ± 0.06	5.89 ± 0.23	−20.8	−21.6 ± 0.7
1.0 M KI	58.5 ± 3.7	114.6 ± 12.5	60.9	3.69 ± 0.06	4.15 ± 0.14	−10.3	−8.6 ± 0.9

[a] K_m and theoretical V_{max} and 95% confidence limits (CL) were calculated by using a weighted linear regression method according to the technique of Wilkinson (1961). [b] k_3 = micromoles of *p*-nitrophenyl phosphate cleaved per second per micromole of enzyme active sites. [c] Activation volumes as calculated from the theoretical V_{max} at 68 and 340 atm. [d] Apparent activation volumes were determined from plots of ln V_{max} vs. pressure at 2.0 mM *p*-nitrophenyl phosphate. The slopes and 95% confidence limits were determined from a nonweighted least-squares linear regression analysis.

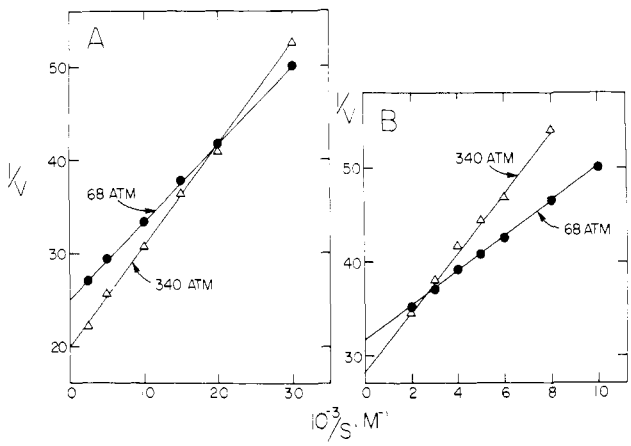

FIGURE 1: Double-reciprocal plots of reaction velocity vs. substrate concentration showing the pressure dependence of the alkaline phosphatase reaction. (A) Assays with standard assay medium (control) at 68 (●) and 340 (△) atm. (B) Assays in the presence of 1.0 M KI at 68 (●) and 340 (△) atm. All assays were performed at 20 °C, pH 9.20.

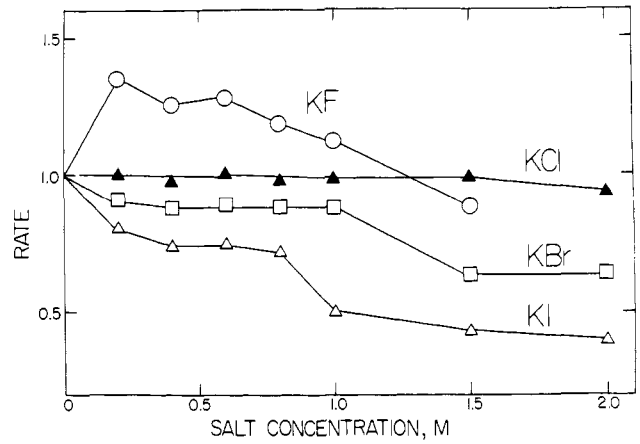

FIGURE 3: Effects of different Hofmeister anions on the maximal rate of the alkaline phosphatase reaction. KF (○); KCl (▲); KBr (□); KI (△). Velocities were determined at 1 atm, 15 °C, and pH 9.20 and were normalized to the velocity in the control buffer with no additional salts. The standard errors are less than the heights of the symbols.

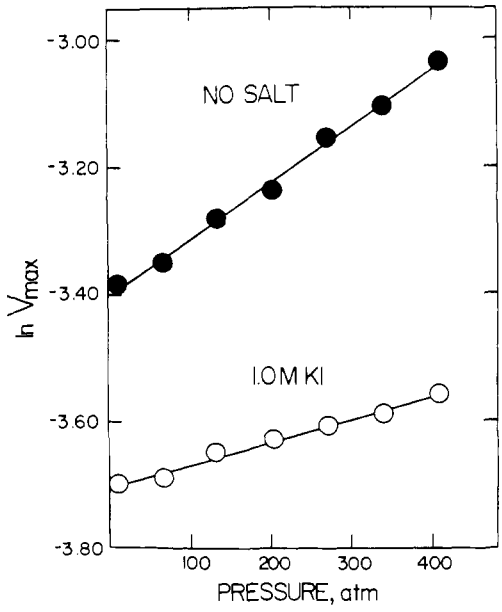

FIGURE 2: Plot of the variation of the natural logarithm of V_{max} vs. pressure for alkaline phosphatase. Closed symbols (●) are assays in control buffer, and open symbols (○) refer to assays in the presence of 1.0 M KI. V_{max} values (ΔOD/min) are optimum velocities obtained by using a *p*-nitrophenyl phosphate concentration of 2.0 mM. Temperature = 20 °C; pH 9.20.

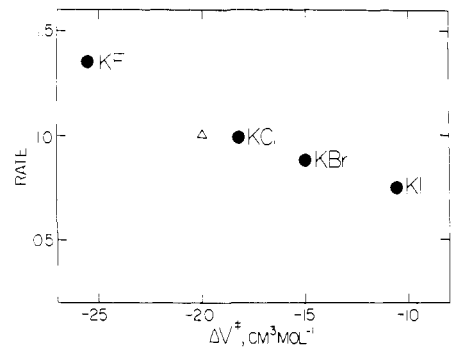

FIGURE 4: Relationship between V_{max} and $\Delta V^{\ddagger}$ for various Hofmeister anions at concentrations of 0.4 M (or 0.2 M in the case of KF) added to the control buffer. The open triangle represents the values obtained with 0.2 M KF + 0.2 M KI. Velocities were recorded at 1 atm, 15 °C, and pH 9.20.

by determining the effect of pressure on the theoretical V_{max} of the reaction (Table I). A parallel experiment was conducted by using 1.0 M KI, an inhibitory salt (see below) which increases $\Delta V^{\ddagger}$ (Figures 1B and 2). Again, plots of ln velocity vs. pressure were linear over the full pressure range tested, and the $\Delta V^{\ddagger}$ estimated using velocity values based on 2.0 mM concentrations of substrate agreed within experimental error (95% confidence limits) with the $\Delta V^{\ddagger}$ determined from theoretical V_{max} values (Figures 1 and 2; Table I).[2] We therefore routinely measured $\Delta V^{\ddagger}$ by using a *p*-nitrophenyl phosphate concentration of 2.0 mM. Both velocity and $\Delta V^{\ddagger}$ remained constant at higher substrate concentrations (up to 5.0 mM, where substrate inhibition could first be detected). Assay pressures were generally 68 and 340 atm (1000 and 5000 psi, respectively), pressures well within the range over which ln velocity varied linearly with pressure. Accordingly, from this point on we report activation volumes which were determined

[2] Activation volumes less than |10| cm^3 mol^{-1} have higher error associated with them due to the fact that a few percent change in V_{max} is associated with a $\Delta V^{\ddagger}$ of this magnitude.

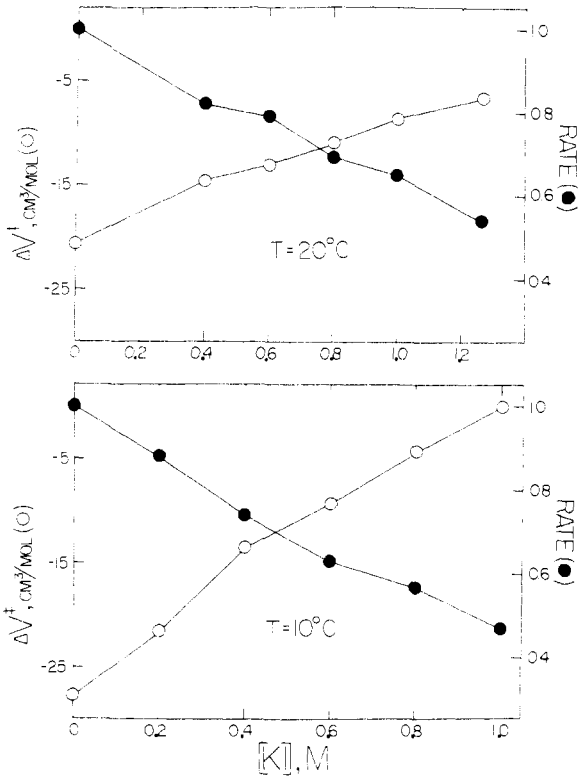

FIGURE 5: Effect of different concentrations of KI on the maximal rate (●) and activation volume (○) of the alkaline phosphatase reaction, indicating the reciprocal covariation of V_{max} and $\Delta V^{\ddagger}$. The upper curve is for data obtained at 20 °C, and the lower curve is for data collected at 10 °C. Maximal rates are for 340 atm.

on a single enzyme assay at pressures of 68 and 340 atm, using a substrate concentration of 2.0 mM. The $\Delta V^{\ddagger}$ values represent averages of at least two independent determinations which varied between 5 and 20%, depending upon the magnitude of $\Delta V^{\ddagger}$.[2]

Neutral Salt Effects on Velocity and $\Delta V^{\ddagger}$. The neutral salts tested displayed effects on both velocity and $\Delta V^{\ddagger}$ which reflected the salts' positions in the Hofmeister series of anions (von Hippel & Schleich, 1969): $F^- < Cl^- < Br^- < I^-$ (increasing salting-in behavior) (Figures 3 and 4). Figure 3 illustrates the effects of these four anions (potassium was the counterion in all cases) on reaction velocity. KF, a salting-out salt (decreases protein group solubility), activated the reaction by ~30% at a concentration of 0.2 M and reduced the value of the activation volume (Figure 4). Higher concentrations of KF were less activating. KCl, which has only weak effects on the solubilities of protein groups (von Hippel, 1975), had negligible effects on the velocity (Figure 3), even at concentrations as high as 2.0 M. This observation reveals that the salt effect on rate is not in significant measure due to simple ionic strength effects. However, 0.4 M KCl caused a small increase in $\Delta V^{\ddagger}$ from −22.5 to −18.2 cm^3 mol^{-1} (Figure 4), indicating the presence of a salt-sensitive component of $\Delta V^{\ddagger}$ which is not correlated with a corresponding rate effect.

Both of the salting-in salts tested, KBr and KI, were inhibitory of velocity and decreased the absolute value of the negative activation volume (Table I; Figures 4 and 5). Increases in the concentrations of KBr and KI led to quite regular decreases in velocity and increases in $\Delta V^{\ddagger}$ over the salt concentration ranges used (Figure 5).

Figure 4 illustrates that the salting-out salt KF was able to counteract the effect of a salting-in salt, KI, on both V_{max} and $\Delta V^{\ddagger}$. Equimolar concentrations of KF and KI returned both V_{max} and $\Delta V^{\ddagger}$ to values near those observed in the presence of KCl at an identical ionic strength (see Figure 4).

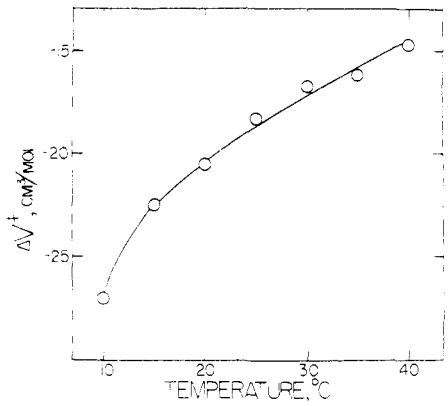

FIGURE 6: Plot of the variation of $\Delta V^{\ddagger}$ with temperature for alkaline phosphatase, pH 9.20.

Unlike the neutral salts, propanol was without effects on velocity and $\Delta V^{\ddagger}$ at alcohol concentrations as high as 0.6 M (not shown).

Tris buffer is known to activate alkaline phosphatase by virtue of its ability to replace water as a phosphate acceptor (Fernley, 1971). Therefore, as a control, the effect of buffer type and concentration on $\Delta V^{\ddagger}$ and on salt effects on $\Delta V^{\ddagger}$ was examined. No effect of Tris buffer concentration was observed. Changing to Ches buffer (0.1 M) resulted in a 30% more negative $\Delta V^{\ddagger}$, but the influences of neutral salts on $\Delta V^{\ddagger}$ were identical with those observed in Tris buffer.

Temperature Effects on $\Delta V^{\ddagger}$ *and Salt Sensitivity*. As shown by the data of Figure 5, temperature has significant effects on the salt sensitivity of reaction velocity and $\Delta V^{\ddagger}$. For example, at 10 °C 1 M KI inhibited the rate by 53% and increased $\Delta V^{\ddagger}$ to nearly zero, whereas at 20 °C the same concentration of KI reduced the rate by only 35% and increased $\Delta V^{\ddagger}$ to −8.5 cm^3 mol^{-1}.

Figure 6 shows the dramatic effect of temperature on the $\Delta V^{\ddagger}$ with no added salts. At reduced temperatures, $\Delta V^{\ddagger}$ was more negative. $\Delta V^{\ddagger}$ increased by ~50% (from −27.5 to −14.0 cm^3 mol^{-1}) as the experimental temperature increased from 10 to 40 °C.

Correlation of $\Delta V^{\ddagger}$ *with Other Thermodynamic Parameters*. The correlation between salt effects on the activation volume and the free energy of activation has already been demonstrated since rate is related to $\Delta G^{\ddagger}$ by way of eq 4.[3] This correlation between $\Delta G^{\ddagger}$ and $\Delta V^{\ddagger}$ (Figures 4 and 5) would be strengthened if a thermodynamically consistent relationship between $\Delta V^{\ddagger}$ and the other experimentally measurable activation parameter, the activation energy (E_a), was observed. Accordingly, activation energies were determined at various concentrations of KI and KBr from the slopes of plots of ln V_{max} vs. the reciprocal of absolute temperature, over the temperature range 5–30 °C. Such plots had correlation coefficients greater than 0.995 under all solute conditions. The determined activation energies were as follows (in kcal mol^{-1} ± 95% confidence limits): control, 10.68 ± 0.24; 0.4 M KI, 11.71 ± 0.60; 0.8 M KI, 12.44 ± 0.17; 0.8 M KBr, 12.03 ± 0.55.

Experimentally determined values for E_a allow one to calculate the enthalpy and entropy of activation for the reaction

[3] Low & Somero (1975b) have pointed out that this correlation cannot result simply from the $P\Delta V^{\ddagger}$ term in eq 6 ($\Delta H^{\ddagger} = \Delta E^{\ddagger} + P\Delta V^{\ddagger}$) which predicts only a 1% change in rate for a 20 cm^3 mol^{-1} change in $\Delta V^{\ddagger}$. Thus, the change in $\Delta V^{\ddagger}$ is a correlate of, but not the cause of, salt-induced changes in reaction velocity.

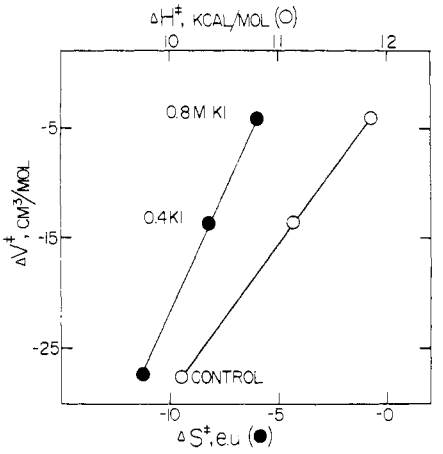

FIGURE 7: Relationship between the activation volume and activation enthalpy (○) and activation entropy (●) for alkaline phosphatase as a function of iodide concentration. The activation volumes and entropies are for 10 °C.

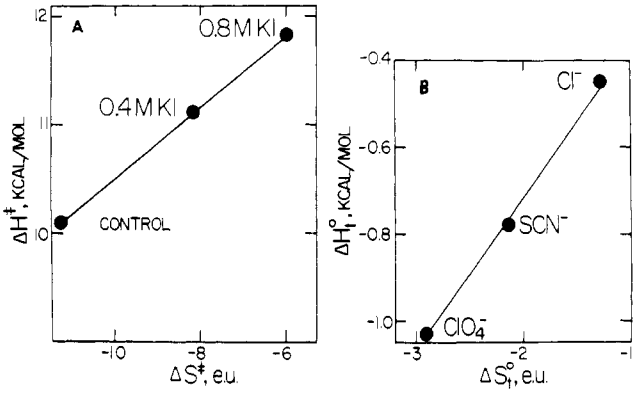

FIGURE 8: (A) Correlation between the effects of iodide concentration on the activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) for the alkaline phosphatase reaction. (B) Compensation between the entropy of transfer (ΔS_t°) and the enthalpy of transfer (ΔH_t°) of the peptide group from water to 2 M concentrations of some Hofmeister anions. The thermodynamic transfer parameters were calculated by Formisano et al. (1978) from the solubility data of Nandi & Robinson (1972a,b).

Table II: Thermodynamic Activation Functions for Alkaline Phosphatase in the Presence and Absence of KI and KBr at 15 °C and pH 9.2[a]

	$\Delta V^{\ddagger}$ ± SD (cm³ mol⁻¹)	$\Delta G^{\ddagger}$ ± SD (cal mol⁻¹)	$\Delta H^{\ddagger}$ ± 95% CL (cal mol⁻¹)	$\Delta S^{\ddagger}$ (eu)
control	−22.5 ± 0.5	13 361 ± 22	10 100 ± 227	−11.32
0.8 M KBr	−16.1 ± 1.7	13 495 ± 19	11 460 ± 528	−7.06
0.8 M KI	−10.3 ± 1.6	13 625 ± 20	11 870 ± 162	−6.09

[a] The rate data were obtained at 340 atm.

(see Materials and Methods). Figure 7 illustrates the linear correlation between the effects of varying concentrations of KI on $\Delta V^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$. Increased concentrations of salting-in salts increase all four activation thermodynamic functions ($\Delta V^{\ddagger}$, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$) in a manner reflecting the salts' positions in the Hofmeister series (Table II). Moreover, these effects are highly correlated with one another.

It is noteworthy that a pronounced entropy–enthalpy compensation effect is characteristic of the salt effect on the activation thermodynamics (Figure 8). The slope of such a plot, the compensation temperature (T_c), was 329 ± 1 K (90% confidence limits) for both the KI and KBr (not shown) effects. The compensation temperatures observed for the effect of KI and KBr are consistent with those values associated with chemical reactions involving the making or breaking of weak

Table III: Dependence of Fluorescence Emission Wavelength Maximum (λ_{max}) and Maximum Fluorescence Intensity of Alkaline Phosphatase on Various Hofmeister Anions

salt	λ_{max}[a] (nm)	rel max fluorescence[b]
0.4 M KF	324.1	1.00
0.4 M KCl	321.4	0.96
0.4 M KBr	320.0	0.93
0.4 M KI	317.9	0.84
0.4 M KF + 0.4 M KI	321.1	0.85
0.4 M KF + 0.4 M KBr	321.9	0.96
0.8 M KCl	321.0	0.96
control[c]	322.2	0.94

[a] Excitation wavelength was 280 nm. Values represent averages of three independent determinations (SEM = 0.3 nm). [b] Excitation wavelength was 290 nm to avoid absorption by iodide at 280 nm. [c] 0.1 M Tris-HCl and 0.05 M KCl, pH 9.2.

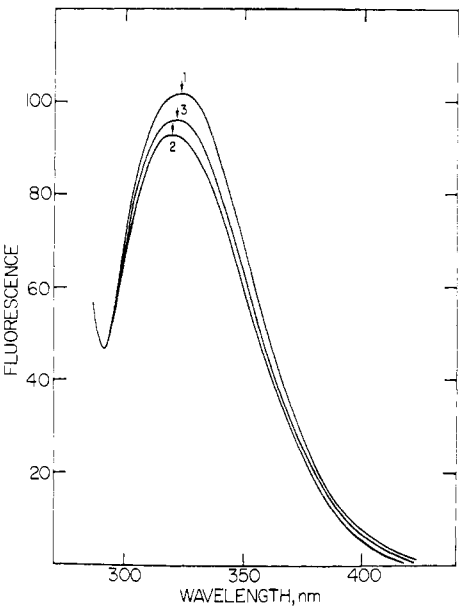

FIGURE 9: Fluorescence emission spectra of alkaline phosphatase in 0.1 M Tris-HCl and 0.05 M KCl, pH 9.20, with (1) 0.4 M KF, (2) 0.4 M KBr, and (3) 0.4 M KBr + 0.4 M KF. Excitation wavelength was 280 nm. Enzyme concentration was 0.12 mg/mL. Arrows indicate the location of fluorescence peaks.

bonds ($T_c \simeq 330$ K) (Stearn, 1949). More significantly, the transfer of peptide groups from water to solutions of Hofmeister anions is characterized by a T_c of 354 ± 13 K (90% confidence limits) (Figure 8).

Fluorescence Studies. Table III lists the wavelength maxima of the fluorescence emission spectrum (λ_{max}) in the presence of 0.4 M concentrations of different salts. Consistent with our kinetic data, we found that these salts affected λ_{max} in relation to their position in the Hofmeister series. Specifically, KF shifted the emission maximum to longer wavelengths (red shift), while a blue shift occurred in the presence of the other salts, in the increasing order of effectiveness $Cl^- < Br^- < I^-$. KF was able to counteract the blue shifts caused by KI and KBr (Table III and Figure 9), similar to the counteracting effects on V_{max} and $\Delta V^{\ddagger}$ described previously. Equimolar concentrations of KF with either KI or KBr returned λ_{max} to the initial value observed in the absence of salts. No effects of various ligands which can bind to the enzyme, Zn^{2+} (0.1 mM), Mg^{2+} (1.0 mM), and PO_4^{2-} (0.01 M), were observed on the salt-induced changes in protein fluorescence. Furthermore, removal of the salts by dialysis returned λ_{max} of the protein to the control value, indicating that salt-induced shifts in λ_{max} were reversible.

Table IV: Maximum Intensity of Fluorescence of NATA in the Presence of Various Hofmeister Anions[a]

salt	rel fluorescence[b]
0.4 M KF	1.00
0.4 M KCl	0.99
0.4 M KBr	0.78
0.4 M KI	0.18
0.4 M KF + 0.4 M KI	0.19
0.4 M KF + 0.4 M KBr	0.78
0.8 M KCl	0.99
20 mM Tris, 0.4 M KCl	1.04

[a] Excitation wavelength was 295 nm. Emission wavelength was at λ_{max} (348 nm). The concentration of NATA was 2.0 μM. [b] Standard deviations do not exceed ±0.03.

Lehrer (1971) has shown that tryptophyl fluorescence is quenched by iodide ion in model compounds and lysozyme by a mechanism involving selective collisional quenching of tryptophyl side chains which are exposed to solvent. Shaklai et al. (1978) have recently shown a similar quenching by I^- for apohemocyanin. Quenching by I^- is invariably accompanied by a blue shift in fluorescence emission since the selectively quenched solvated tryptophyls emit at longer wavelengths (Lehrer & Leavis, 1977). Table III shows that the maximum fluorescence intensity of alkaline phosphatase is also sensitive to Hofmeister anions in the order $I^- < Br^- < Cl^- < F^-$. Difference spectra between the salts displayed maxima near 350 nm, indicating perturbation of tryptophyl fluorescence. Therefore, it was critical to determine if the observed shifts in λ_{max} resulted from differential quenching by the salts of solvent-exposed tryptophyl residues on the enzyme. As a control, the influence of Hofmeister anions on the fluorescence of NATA was studied. The λ_{max} of this model compound was unaffected by all of the salts used in this study. The salt-induced λ_{max} shifts are therefore a property of the intrinsic protein fluorescence only. Table IV shows that no significant difference in fluorescence intensity of NATA was apparent for Cl^- and F^-. Furthermore, an increase in the concentration of Cl^- from 0.4 to 0.8 M resulted in no change in fluorescence of the model compound. Bromide ion decreased the fluorescence to 80% of the value observed in the presence of Cl^- [due in part perhaps to the absorption of excitation radiation (295 nm) by this ion]. However, the quenching by iodide was much stronger (20% of the Cl^- value) (Table IV). In addition, Tris buffer was without effect on fluorescence intensity since a reduction in the Tris concentration from 100 to 20 mM resulted in no dramatic change (Table IV).

Unlike the additive nature of the Hofmeister anions on the λ_{max} shift for alkaline phosphatase, the effects of F^-, Br^-, and I^- on the quenching of NATA were not additive. Fluoride ion was unable to counter the quenching of NATA fluorescence by either iodide or bromide ion (Table IV).

The studies with the indole derivative, NATA, do not support an interpretation for the salts' effects on protein λ_{max} which is based solely on the differential quenching by the salts of solvent-exposed tryptophyls. Instead, the Hofmeister-sensitive shifts in emission maximum may find at least a partial explanation in a salt-induced change in fluorophore environment.

Figure 10 illustrates the influence of increasing concentrations of I^- on the fluorescence emission maximum of alkaline phosphatase. The curve resembles a saturation isotherm with a total shift of 10 nm in λ_{max} being realized at ~1.5 M KI. It is very significant that quenching of alkaline phosphatase fluorescence by I^- is greatly diminished beyond I^- concentrations of 0.4 M (unpublished experiments). Therefore, the

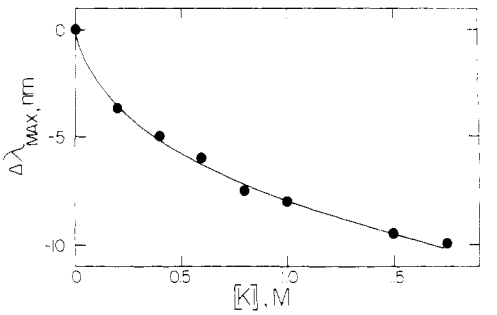

FIGURE 10: Effect of increasing concentrations of KI on the blue shift in the fluorescence emission wavelength maximum of alkaline phosphatase. Excitation wavelength was 280 nm. The blue shift is expressed as the difference between the emission maximum with control buffer and with iodide added.

Table V: Values of Fluorescence Polarization for Alkaline Phosphatase at Various Excitation Wavelengths and with Different Hofmeister Anions

	p (excitation at)[a]			
	280 nm	285 nm	290 nm	295 nm
control[b]	0.151	0.159	0.199	0.223
1.0 M KBr	0.152	0.158	0.193	0.220
0.4 M KF	0.148	0.153	0.182	0.224

[a] Emission monitored at 320 nm. [b] 0.1 M Tris-HCl and 0.05 M KCl, pH 9.2.

decrease in λ_{max} at the higher iodide concentrations is due to a salt-induced change in the environment of one or more accessible fluorophores since the quenching effect has been "titrated".

Selective quenching of solvent-exposed tryptophyls by iodide ion has been invoked to explain the increase in steady-state fluorescence polarization of apohemocyanin (Shaklai et al., 1978) since tryptophan heterogeneity dictates the weighting of the different emitters to the observed polarization. The increase in polarization should be apparent at all excitation wavelengths. Table V shows that at several excitation wavelengths there was no significant difference in the observed polarization in the presence of 1 M bromide ion as compared to a control with no added salts (Cl^- had no effect on the polarization). Fluoride slightly decreased the observed polarization at excitation wavelengths less than 295 nm. At 295-nm excitation, no significant difference in the observed polarization was apparent between the salts.

Discussion

Throughout all phases of this study we have carefully chosen experimental conditions designed to exclude the possibility that the salt perturbation of V_{max} and ΔV^* might result from salt-induced inhibition of ES complex formation or enzyme denaturation. Thus, the observed salt effects result from perturbations of the ES complex which directly affect the energy and volume changes that accompany the activation event in the alkaline phosphatase reaction.

Lazdunski et al. (1965) have found that the influence of dioxane and ethanol on the maximal rate of hydrolysis of *p*-nitrophenyl phosphate in the presence of calf alkaline phosphatase can be interpreted in terms of a dielectric constant effect. They proposed that this effect, as well as the influence of ionic strength (up to 0.1 M), could be accounted for by a change in the diameter of the ES complex in the transition state. Since the dissociation of noncovalently bound phosphate is the rate-limiting step in hydrolysis at high pH (Hull & Sykes, 1976a,b),[4] their interpretation is consistent with a sep-

aration of charge as the enzyme and negatively charged orthophosphate separate in the transition state. Such a charge separation will result in large electrostrictive volume changes due to reorganization of solvent around the transition-state dipole (Laidler & Bunting, 1973)[5] and is consistent with the large negative $\Delta V^{\ddagger}$ and $\Delta S^{\ddagger}$ which we observed for the alkaline phosphatase reaction. However, the solute effects on rate and $\Delta V^{\ddagger}$ reported in the present study were observed at higher concentrations of salts (0.2–1.0 M) where Debye–Hückel effects play only a minor role. To develop a model which is able to explain the Hofmeister effects on rate and $\Delta V^{\ddagger}$, it is first necessary to consider alternate sources of volume changes during the alkaline phosphatase reaction and the energetic significance of the events which are responsible for these volume changes.

The alternative water structure based mechanism for accounting for activation volumes, and the salt perturbation thereof, is based on well-established volume and energy characteristics of protein group (peptide backbone linkages and amino acid side chains) solvation processes. The hydration of protein groups, as might occur, for example, when groups are transferred from a nonpolar solvent to an aqueous solution, occurs with large changes in free energy, enthalpy, entropy, and volume, reflecting major changes in water organization around both polar and nonpolar groups. Furthermore, the energetics of these transfer processes are markedly influenced by the salt composition and concentration of the aqueous phase (von Hippel & Schleich, 1969). We argue that the salt effects observed in the present study can be explained largely, if not entirely, on the basis of these well-known salt effects on the solubilities of protein groups, particularly peptide groups.

The Hofmeister ranking of anions, in the order of increasing ability to salt-in polar protein groups such as peptide linkages, is

$$PO_4^{3-} < SO_4^{2-} < F^- < Cl^- < Br^- < I^- < CNS^-$$

Studies by von Hippel and co-workers (Hamabata & von Hippel, 1973; von Hippel & Hamabata, 1973; von Hippel et al., 1973) have demonstrated that the Hofmeister specificity in salt effects is due to the influences of nonpolar moieties vicinal to the peptide group. All salts increase the solubilities of peptide groups and decrease the solubilities of nonpolar groups. The net salting-in or salting-out effect of a salt or ion results from the relative magnitude of the polar vs. nonpolar effect. For ions to the "left" of Cl^- in the series as shown above, the solubilities of proteins are decreased; i.e., F^-, SO_4^{2-}, and PO_4^{3-} are salting-out ions. Ions to the "right" of Cl^- salt-in. Cl^- has only very small effects on protein solubility.

The enthalpy and entropy changes accompanying protein group solvation in different aqueous media have also been determined (von Hippel & Schleich, 1969; Nandi & Robinson, 1972a,b). For the transfer of peptide groups, the enthalpy and entropy, as well as the free energy, changes are all negative when transfer occurs from water to concentrated salt solutions. Each of these values decreases with the greater salting-in potential of the salt. Furthermore, for peptide group transfers, $|\Delta H_t^\circ| > |T\Delta S_t^\circ|$; i.e., the driving force for the transfer is the favorable enthalpy change. A different situation is found for the transfer of nonpolar groups between water and salt solutions. The free energy, enthalpy, and entropy of transfer are all positive, while again $|\Delta H_t^\circ| > |T\Delta S_t^\circ|$. Transfer studies with model protein groups have also shown a high degree of enthalpy–entropy compensation, such that large changes in transfer enthalpy are associated with relatively small changes in transfer free energy (see Figure 8).

The thermodynamic transfer functions are calculated from solubility studies and, therefore, depend upon the interaction of protein groups with water. In order to compare these studies to those of alkaline phosphatase, we must consider the exposure of groups which are relatively buried in the initial state. The basic tenet of our model is that the transfer of protein groups at the water–protein interface during catalytic conformational changes contributes significantly to the energy ($\Delta G^{\ddagger}$) and volume ($\Delta V^{\ddagger}$) changes of catalysis (Low & Somero, 1975b). Furthermore, anion-induced changes in ES complex hydration either increase (salting-in salts) or decrease (F^-) the activation free energy and activation volume of the reaction.

In the presence of Br^- or I^-, the ES complex will be more hydrated than in the absence of these salting-in ions. Thus, by salting-in near-surface groups that are normally well hydrated only in the transition state, Br^- and I^- will eliminate a negative contribution to $\Delta G^{\ddagger}$. A negative contribution to $\Delta V^{\ddagger}$ will similarly be removed by these salting-in ions. Thus, we propose that salting-in anions may exert their effects on the energy and volume changes accompanying catalysis by affecting the hydration changes that accompany alterations in enzyme conformation during the activation event.

The bases for concluding that polar or peptide groups are the likely class of protein groups to make the major energetic and volume contributions during the conformational change which occurs during the activation event of the alkaline phosphatase reaction are as follows. First, the transfer of predominantly nonpolar groups would be endergonic. An exergonic transfer could only be effected by the net burial of nonpolar groups, and this would lead to a volume increase, which is not observed. Second, the absence of any effect of propanol on rate and $\Delta V^{\ddagger}$ argues that nonpolar group transfers are not the major class of transfer which takes place during the conformational change of the activation event.[6] The increase in $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ in the presence of salting-in salts is consistent with a *net* decrease in free energy, enthalpy, and entropy of the ES complex relative to the unperturbed complex. This follows directly from the known negative free energies, enthalpies, and entropies of transfer of the peptide group and polar side chains from water to solutions of Hofmeister salts.

The closely coupled changes in activation enthalpy and entropy, with a compensation temperature of 330 K, are also consistent with altered exposure of a polar group (Figure 8).[7] Lumry & Rajender (1970) have suggested that the existence of entropy–enthalpy compensation is a direct result of the role of liquid water in many protein processes and have proposed that the linear enthalpy–entropy pattern be used as a "diagnostic test for the participation of water in protein

[4] This conclusion is based upon studies with the *Escherichia coli* enzyme. However, the active-site amino acid sequences are identical in both *E. coli* and mammalian alkaline phosphatases (Engström, 1964) and it is assumed that the mechanism deduced for the bacterial enzyme is similar to that for the mammalian homologue (Fernley, 1971).

[5] The volume changes resulting from hydration effects are generally more important than structural volume changes for reactions involving fairly strong transition-state dipoles (Laidler & Bunting, 1973).

[6] It should be emphasized that nonpolar groups do play a role in that nearby hydrophobic residues confer the specificity of the Hofmeister salts (von Hippel & Hamabata,1973). However, at the temperatures used in our study, the data indicate that the energetics are dominated by the salting-in or salting-out of polar side chains or peptide groups.

[7] Entropy–enthalpy compensation can be a consequence of experimental error, but we feel that the precision of the data is sufficient to demonstrate that the compensation is real. Moreover, the possibility of a statistical interpretation of the compensation pattern becomes much greater when the value of the compensation temperature approaches the experimental temperatures (Krug et al., 1976).

processes". In this context, the observation of a specific linear relationship between the influences of salting-in salts on $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ is additional thermodynamic evidence which supports a mechanism involving salt-induced changes in enzyme hydration.

In the case of a salting-out ion such as F^-, the rate and $\Delta V^{\ddagger}$ effects may be due to decreased hydration of near-surface groups which are more fully exposed to the solvent during the activation step; i.e., the salt activation due to F^- at concentrations less than ~1 M may derive from just the opposite effects that result from Br^- and I^-. Stated in another way, the free energy and volume of the ES complex are increased in the presence of F^-.

A final series of arguments in support of the hydration-based rate and volume effects of salts and, indeed, in support of the role of hydration changes during catalysis under non-salt-perturbed conditions can be based on the temperature effects noted on the size of $\Delta V^{\ddagger}$. As shown in Figure 6, the $\Delta V^{\ddagger}$ of the alkaline phosphatase reaction becomes less negative as temperature increases. This observation is consistent with a "melting" of water from around water-constricting polar groups that are exposed to solvent during the catalytic conformational change. At higher temperatures, water organization around these transferred polar groups is reduced, leading to net increase in $\Delta V^{\ddagger}$. The observed increases in KI effects on $\Delta V^{\ddagger}$ at lower temperatures (Figure 5) are also explainable in terms of hydration of polar side chains or peptide groups. Since ΔH_t° is negative for peptide group transfers between water and salt solutions, the salting-in abilities of Br^- and I^- increase as temperature is reduced and rate inhibition due to the model proposed above would be expected to increase as temperature is lowered.

At this juncture it is appropriate to emphasize that the salt-induced rate inhibition noted with Br^- and I^-, which is at most ~60% at the highest KI concentrations used, should not be taken as a quantitative index of the contributions which exergonic transfer reactions make to lowering of $\Delta G^{\ddagger}$ values. A reduction in rate of 50% by 1 M KI, for example, does not imply that hydration events are contributing only the caloric equivalent of this rate reduction (~0.2 kcal mol^{-1}) to $\Delta G^{\ddagger}$. Rather, these inhibition data only suggest that hydration energy contributions are involved in establishing $\Delta G^{\ddagger}$. The actual magnitude of hydration energy contributions to reductions in $\Delta G^{\ddagger}$ cannot be determined from salt perturbation experiments since complete titration of these hydration changes by inhibiting salts would probably not occur before these salts denatured the enzyme. Also, the free energy of transfer of a peptide group from a nonpolar solvent to an aqueous phase (ΔG_{tr}°) is large relative to the *change* in ΔG_{tr}° due to the addition of a salting-in salt to the aqueous phase. This follows from the fact that salts decrease both ΔS_{tr}° and ΔH_{tr}°, which results in a compensating effect on ΔG_{tr}°. For example, the ΔG_t° for the peptide group taken from water to 2 M KSCN is less than 0.2 kcal mol^{-1} at 0.5 °C (Nandi & Robinson, 1972a), whereas the transfer free energy for the peptide group taken from ethanol to water is −1.14 kcal mol^{-1} at 25 °C (Cohn & Edsall, 1965).[8] Thus, while salt inhibition studies argue for the importance of hydration energy effects in establishing $\Delta G^{\ddagger}$, these studies must not be regarded as providing quantitative estimates of these contributions to enzyme rate enhancement of reaction velocities.

It is also important to consider a possible alternative explanation for the salt effects on rate and $\Delta V^{\ddagger}$. von Hippel and co-workers (Hamabata & von Hippel, 1973; von Hippel et al., 1973) have shown that relative to water, F^- binds with a lower affinity to the amide dipole and the salting-in salts bind with a higher affinity in the order $Br^- < I^- < CNS^-$. The Hofmeister specificity was shown to be modulated by the nearby methyl groups. Therefore, the rate/volume effects may originate in pressure-enhanced rate inhibition which results from pressure favoring the equilibrium

$$\text{peptide} + \text{ion} \rightleftarrows \text{peptide–ion}$$

However, the electrostatic partial molal volume of all of the ions tested in this study is negative; i.e., in solution these ions tend to electrostrict solvent molecules (Noyes, 1964). Pressure increases would thus be expected to shift the above equilibrium to the left, an effect which would be expected to favor pressure-induced relief of enzyme inhibition. This is not noted in our studies.

The effects of anions on the fluorescence emission spectrum of alkaline phosphatase are in accord with a mechanism which proposes salt-induced changes in enzyme hydration. Consistent with our kinetic observations, we found that a blue shift in the wavelength maximum of fluorescence for alkaline phosphatase is sensitive to Hofmeister anions in the order $F^- < Cl^- < Br^- < I^-$ as well as to increasing concentrations of the same ion.

Shifts in the λ_{max} of fluorescence emission spectra are commonly interpreted as an indication of a change in the polarity of the environment of protein tryptophyl groups. Specifically, a red shift is interpreted as greater exposure to the solvent, while a blue shift is associated with the burial of protein fluorophores in the relatively nonpolar protein interior. These interpretations are based upon the fact that the fluorescence emission spectra of indole derivatives are red-shifted in aqueous solutions relative to more nonpolar solvents (Brand & Witholt, 1967).

The nature of the conformational changes responsible for the salt-induced changes in λ_{max} cannot, of course, be deduced from the spectral data presented in this paper. The key finding is that a salting-out ion (F^-) and salting-in ions (Br^- and I^-) shift λ_{max} in different directions and that these emission maxima shifts can be counterbalanced with salt combinations. One cannot be certain if the salts are exerting their influence upon the nonpolar tryptophyl side chains directly or whether they are modifying nearby groups with a resulting change in fluorophore environment or some combination of both processes. What is certain, however, is that the changes in λ_{max} observed are consistent with an interpretation of the protein group(s) experiencing different environmental polarities depending upon the nature of the Hofmeister anion in solution.

Indole derivatives have a somewhat greater quantum yield in dioxane than in water (Van Duuren, 1963), which is inconsistent with F^- both enhancing protein fluorescence and causing a red shift (relative to the chloride ion) in λ_{max}. However, quantum yields of tryptophyl fluorescence can potentially be influenced by neighboring α- and ϵ-amino groups, carboxyl groups, and tyrosine, imidazole, and mercapto groups as well as by solvent molecules (Brand & Witholt, 1967). For example, Kronman & Holmes (1971) have shown that tryptophan quantum yields in native proteins are both higher and lower than that of the free amino acid in aqueous solution. Their studies on native and denatured proteins indicated that tryptophan quantum yield is an unreliable index for the "degree of exposure" of the fluorophore to the solvent. More significant to the structural study is the fact that control experiments with the model compound NATA, as well as the polarization data, indicate that the salt-sensitive shifts in the fluorescence emission maximum could not be due entirely to

[8] For the transfer studies between water and salt solutions, chloroform and diethyl ether were used as the reference solvents.

Hofmeister-sensitive fluorescence quenching of solvated tryptophyl side chains.

The fluorescence data suggest that it is possible, at least in principle, to have a *net* salting-in or salting-out effect on protein groups on the enzyme. Warren & Cheatum (1966) observed somewhat similar results by using sulfhydryl group reactivity with DTNB as an index of the degree of protein group exposure. They interpreted their results in the context of a structure disruption by salt. The absence of a significant change in the fluorescence polarization spectrum in the presence of 1 M KBr, however, is evidence that no major denaturation or unfolding of alkaline phosphatase is occurring in the presence of these salts.[9]

Perhaps the most convincing evidence that the Hofmeister series anions are acting in their traditional roles as modulators of protein group solubilities in the native enzyme lies in the observation that the effects of salting-in and salting-out anions are additive, both in their effects on V_{max} and $\Delta V^{\ddagger}$ and on the wavelength shift of the fluorescence emission maximum. Studies with model protein peptide and nonpolar groups have clearly shown that the influences of Hofmeister anions on protein group solubilities are additive. We regard the latter finding as particularly strong evidence in support of our hypothesis that the changes in λ_{max} induced by the anions are the result of classical Hofmeister series effects on protein group solubility. We are unable to offer an alternative hypothesis to the hydration hypothesis to account for these counteracting effects.

The whole of the thermodynamic and structural evidence presented here is consistent with an interpretation of salt effects on enzymes which includes the aqueous environment in the overall energy picture of enzyme catalysis. While the concept of negative free-energy changes arising from changes in hydration during reactions of proteins is not a new one to biochemistry (Brandts, 1969; Lumry & Biltonen, 1969; Lewin, 1974; Lauffer, 1975; Edelhoch & Osborne, 1976), to date relatively little attention has been given to the potential role of this phenomenon during enzyme catalysis and binding. Observations of similar rate/volume salt effects on several other enzymes (Low & Somero, 1975a,b; Somero et al., 1977) support the hypothesis that enzyme hydration plays a significant role in the energetics of many types of enzyme reactions.

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[9] Intrinsic steady-state polarization of fluorescence can supply information on the extent of *local* freedom of rotation of tryptophyl residues since they do not emit light before the fluorescent amino acid side chains can rotate relative to the residues which connect them to the rest of the protein (Brewer & Weber, 1966). Thus, large depolarization effects usually accompany proteins in concentrated urea solutions and are due in part to increases in local rotational freedom of the tryptophyl residues (Weber & Bablouzian, 1966).

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p-(Bromoacetamido)phenyl Uridyl Pyrophosphate: An Active-Site-Directed Irreversible Inhibitor for Uridine Diphosphate Galactose 4-Epimerase†

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ABSTRACT: The synthesis of *p*-(bromoacetamido)phenyl uridyl pyrophosphate (BUP) is described. This compound is an active-site-directed irreversible inhibitor of *Escherichia coli* UDP-galactose 4-epimerase. The inactivation follows pseudo-first-order kinetics at pH 8.5 in nonnucleophilic buffers, and a saturation effect is seen in the pseudo-first-order rate constant as the concentration of BUP is increased. The half-saturation parameter for BUP in the inactivation is 0.21 ± 0.02 mM, which compares favorably with the inhibition constant of 0.3 ± 0.05 mM for BUP acting as a competitive reversible inhibitor of the enzyme. The inactivation rate is slow, however, with a minimum half-time of 12 h at pH 8.5 and 27 °C. Both specific alkylation and nonspecific alkylation by BUP occur, but nonspecific alkylation is faster than the inactivation and the rate of inactivation correlates well with the rate of covalent incorporation of one molecule of [^{14}C]BUP at the active site.

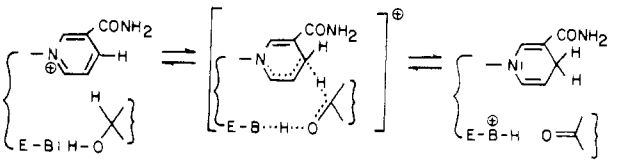
The mechanism of the interconversion of UDP-galactose and UDP-glucose catalyzed by UDP-galactose 4-epimerase is known to involve the reversible formation of an epimerase·DPNH·UDP-4-ketohexose intermediate complex, eq 1 and 2

$$E\cdot DPN^+ + \text{UDP-Gal} \rightleftharpoons E\cdot DPN^+\cdot\text{UDP-Gal} \rightleftharpoons E\cdot DPNH\cdot\text{UDP-4-ketohexose} \quad (1)$$

$$E\cdot DPNH\cdot\text{UDP-4-ketohexose} \rightleftharpoons E\cdot DPN^+\cdot\text{UDP-Glc} \rightleftharpoons E\cdot DPN^+ + \text{UDP-Glc} \quad (2)$$

(Nelsestuen & Kirkwood, 1971; Maitra & Ankel, 1971; Wee & Frey, 1973; Adair et al., 1973). The interconversions of this central complex with the epimerase·DPN$^+$·UDP-hexose complexes requires general acid–base catalysis, i.e., general base catalyzed removal of the proton from the glycosyl C-4 hydroxyl groups of UDP-hexose substrates concomitant with their conversion to the ketonic intermediate and general acid catalyzed protonation of the ketonic oxygen in concert with its reduction to the galactosyl or glucosyl groups. Since the reduction process is the microscopic reverse of the oxidation process, or nearly the microscopic reverse, the general acid and general base functions can be expected to be performed by a single functional group which acts as a general base in the oxidation of glycosyl groups and as a general acid in the reduction of the 4-ketohexopyranosyl group (Scheme I). While the identity of this group is not known, it is almost certainly present, because in the absence of such a group with a pK_a in the near-physiological range the reversible redox process would involve the compulsory formation of high-energy intermediates, either the protonated 4-ketohexopyranosyl group or the alkoxide ion.

Scheme I



A similar situation exists for all such reactions involving oxidation of an alcohol to a ketone, and general bases corresponding to that in Scheme I are known to be present in the active sites of pyridine nucleotide dependent dehydrogenases (Holbrook et al., 1975; Banaszak & Bradshaw, 1975). In these cases a histidyl residue appears to be appropriately situated to function as the prototropic catalyst.

In order to identify this functional group, we have undertaken to synthesize an active-site-directed irreversible inhibitor which may alkylate the functional group when it is in its unprotonated, nucleophilic form. In the design of the inhibitor we have taken into account the binding properties of the active site of this enzyme as well as the degradation of the alkylated enzyme to an identifiable product. These considerations led us to synthesize *p*-(bromoacetamido)phenyl uridyl pyrophosphate (BUP),[1] **1**.

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[1] Abbreviations used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; UMP, uridine 5′-monophosphate; Ans, 8-anilino-1-naphthalenesulfonate; NUP, *p*-nitrophenyl uridyl pyrophosphate; AUP, *p*-aminophenyl uridyl pyrophosphate; BUP, *p*-(bromoacetamido)phenyl uridyl pyrophosphate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

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