Kinetic Properties of Adenosine Deaminase in Mixed Aqueous Solvents*

D. W. Bolen† and J. R. Fisher

ABSTRACT: Hoagland and Fisher showed that chicken duodenal adenosine deaminase exhibits a rate equation over a very wide range of substrate and product concentrations in a neutral aqueous environment (see eq 1 of text). Since this reaction is irreversible and inosine and adenosine are the only substrate or product variables that can be used, it was difficult to get more information about the mechanism using standard steady-state kinetic techniques. Consequently a detailed study was made of this system in a variety of solvents at many concentrations, temperatures, and pH values. The same rate equation held under all of these conditions even though reaction rates were changed several orders of magnitude. Since in all probability such a detailed study would have allowed other terms in the rate equation to appear if they were present, we have concluded that there are no other terms and have proposed a mechanism based upon this assumption. Studies in a variety of solvents have allowed us to conclude that water is not a limiting substrate in this system and we have assumed that it is saturating. An ordered-sequence mechanism with a ternary complex is suggested which satisfies all the known properties of this system. The kinetic parameters (α, β, and γ) were drastically changed by the various solvents, *e.g.*, α decreased 1000-fold in the presence of 70% v/v ethylene glycol without irreversible inactivation of the enzyme. Specific properties of the solvent seem to induce these enormous changes without affecting the conformation of the enzyme. Log β is a linear function of the reciprocal of dielectric constant and log α appears to be a linear function of internal pressure. Log γ could not be correlated with any bulk solvent property examined.

Hoagland and Fisher (1967) made a detailed study of chicken duodenal adenosine deaminase in a neutral aqueous environment. They varied the concentration of adenosine over a 2000-fold range, used a wide variety of inosine concentrations for product inhibition, and studied the behavior of the enzyme in the presence of a number of sulfhydryl reagents. It was shown that some of these reagents activate the enzyme and apparently do so by forming a derivative with a single sulfhydryl group on the enzyme. A simple rate equation (see eq 1) was found to hold over these wide ranges of substrate and product concentrations and for sulfhydryl derivatives of the enzyme. Since it is always possible that a simple rate equation may represent a complex mechanism and that many other terms exist which are too small to have any significant effect under the conditions used, an attempt was made to demonstrate the presence of such terms by using a wide variety of environmental conditions including several mixed aqueous solvents.

Investigations of proteins in mixed aqueous binaries are potentially useful in the study of many aspects of enzyme chemistry. From a kinetic point of view such systems allow continuous variation in the reaction medium for the study of reaction mechanisms, an approach used in organic chemistry (Arnett, 1967). Aqueous organic solvents can also be useful in the study of forces involved in enzymatic reactions (Lumry, 1959; Barnard and Laidler, 1952; Castañeda-Agulló and Del Castillo, 1959a,b) and protein denaturation (Brandts and Hunt, 1967; Schrier *et al.*, 1965; Gerlsma, 1968). Solvent perturbation difference spectroscopy is currently achieving considerable success in investigations of conformational changes in proteins (Herskovits and Laskowski, 1962; Laskowski, 1966; Wetlaufer, 1962).

Our efforts in the investigation of the behavior of adenosine deaminase in mixed aqueous solvents have been directed to certain aspects of the mechanism of action and to attempt where possible to correlate physical properties of mixed aqueous solvent systems with changes in kinetic constants. The framework from which this study initiated was provided from the results of solution properties on organic reactions as discussed by Amis (1949), Laidler and Eyring (1940), Glasstone *et al.* (1941), Arnett (1967), and Scatchard (1932). The extensive work on enzyme reactions in aqueous binaries by Laidler and coworkers (Barnard and Laidler, 1952; Kaplan and Laidler, 1967; Laidler and Ethier, 1953) along with most helpful review articles on biophysical systems by Lumry (Lumry and Biltonen, 1968; Lumry, 1969) provides a reasonable basis for the interpretation of much of the work presented here.

Materials and Methods

Adenosine deaminase was prepared from acetone powder of fresh chicken duodena and its purity was established as described previously (Hoagland, 1967; Hoagland and Fisher, 1967). Specific activities ranged between 13 and 19 depending upon the particular batch of acetone powder used. However, all preparations were chromatographically and electrophoretically homogeneous.

Adenosine and inosine were obtained from Calbiochem,

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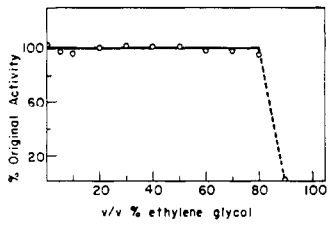

FIGURE 1: Effect of exposure of adenosine deaminase to aqueous ethylene glycol solutions.

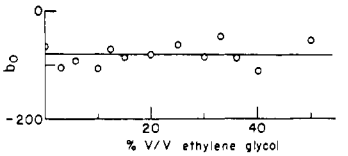

FIGURE 2: Variation of b_0 with ethylene glycol composition.

Matheson, Coleman & Bell supplied *t*-butyl alcohol, sorbitol was brought from Eastman Organic Chemicals, and ACS Spectroanalyzed acetone and Fisher Certified ethylene glycol were procured from Fisher Scientific Co. Urea, 1,4-dioxane, glycerol, and methanol were Baker Analyzed reagents. Sucrose was commercial grade. The solvents and other chemicals used for altering dielectric strength and surface tension of the medium were of the best grade commercially available and were used without further purification.

The decrease in optical density at 265 mμ was used to measure the activity of the enzyme preparation. A Gilford Model 2000 recording spectrophotometer equipped with thermospacers for constant-temperature control was used for all measurements of activity. Unless otherwise stated all measurements were made at 38 ± 0.2° using 1.0-cm pathlength cells containing 3 ml of adenosine solution of specified concentration in 0.1 M potassium phosphate buffer (pH 7.0). Between 2 and 100 μl of enzyme solution were used to initiate the reaction which was generally followed for 1–5 min. Protein concentration was determined by the spectrophotometric method of Warburg and Christian (1941). Ultraviolet spectra of substrates and products were taken on a Cary 15 spectrophotometer.

Solvent perturbation difference spectroscopy was carried out at room temperature on a Cary 15 spectrophotometer equipped with a 0.1 absorbance slide-wire. The optics of the instrument were flushed overnight with dry nitrogen and both sample and reference compartments were also flushed during the experiments. The compartments swept by nitrogen were not thermostated but their temperature was monitored by a thermistor after every third scan and ranged between 28 and 29°. Four concentrations of indole were used to demonstrate adherence to Beer's law and to show that stray light was not a problem over the spectral region of interest (Gratzer, 1967).

The experimental procedure outlined by Herskovits and Laskowski (1962) and Laskowski (1966) was followed for obtaining all difference spectra. Two matched pairs of Beckman standard silica cells with 1-cm path lengths were placed in tandem in the reference and sample compartments of a Cary 15. Aqueous and aqueous ethylene glycol solutions of adenosine deaminase (8.8 mg/ml) buffered at pH 7.0 by 0.025 M potassium phosphate were placed in reference and sample compartments, respectively. To compensate for the solvent contribution to the difference spectrum, water was placed in the front cell of the sample compartment (cell first receiving the incident beam); aqueous glycol without protein was placed in the front cell of the reference compartment to properly balance the solvent contribution of the sample compartment. The four cells were positioned in the instrument such that cells containing the blanks receive the incident beam first and cells containing protein are last to receive the sample and reference beams. A satisfactory base line for each set of experiments was established by adjustment of the instrument with all four cells in place and filled with respective solvents but without protein. The cells were then rinsed, dried, and filled with the proper experimental solutions. A base line was recorded after each scan.

Optical rotatory dispersion data were obtained at room temperature on a JASCO ORD–CD instrument purged overnight with nitrogen. Scans were made of adenosine deaminase in various compositions of ethylene glycol–water using a 1-cm cell. A base line consisting of the solvent without protein was determined for each new solvent system.

A FORTRAN IV computer program was written for routine determination of slope and intercept parameters of $1/v$ *vs.* $1/S$ plots. The program provides a least-squares regression analysis of these reciprocal plots using the method of normal equations with weighting (V^{-4}) of all points. Standard errors for all computed parameters were recorded along with plots of residuals for use in checking random behavior of the data.

Results

Characteristics of Adenosine Deaminase in Mixed Aqueous Solvents. The presence of an organic component in a solution containing an enzyme raises the possibility of irreversible denaturation of the protein. Consequently, a study of the stability of adenosine deaminase in various glycol–water mixtures was undertaken (a similar study was made with each organic additive). Solutions of enzyme were prepared in glycol–water mixtures having final compositions of from 0 to 90% v/v glycol, pH 7. Aliquots (0.1 ml) were taken after 1 hr at room temperature and placed in a standard aqueous assay system consisting of 3.0 ml of 1.0×10^{-4} M adenosine in 0.1 M potassium phosphate buffer at pH 7.0. The resulting velocities are shown in Figure 1 as per cent original activity *vs.* volume per cent composition of ethylene glycol. Beyond 80% v/v ethylene glycol the enzyme is inactivated and is incapable of regaining its catalytic ability upon dilution with water within a 5-min time span.

The above data indicate that the enzyme is not irreversibly denatured in the presence of mixed aqueous solvents (within the limits specified) but the possibility remained that major reversible changes in conformation could occur. Consequently, optical rotatory dispersion and solvent perturbation difference spectroscopy were used in an attempt to detect such conformational changes in the various solvent mixtures.

Measurements were taken on a JASCO ORD–CD recording instrument at room temperature with 1-cm cells and a spectral range of 250–400 mμ. Final concentrations of enzyme were 3.3 mg/ml. Refractive indices of aqueous ethylene glycol were kindly supplied by Dr. C. C. Bigelow

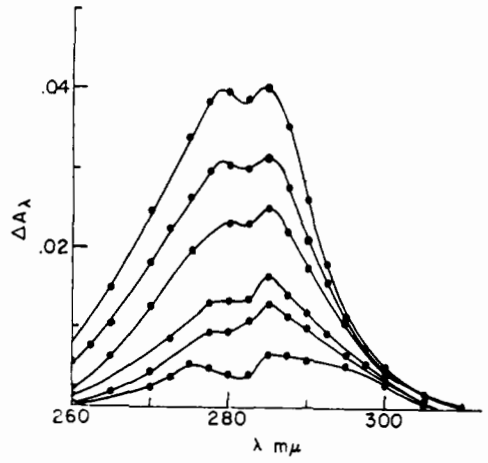

FIGURE 3: Solvent perturbation difference spectra of adenosine deaminase (4.4 mg/ml) in aqueous ethylene glycol. Solvent compositions beginning with the spectrum of lowest ΔA_λ include 10, 15, 20, 30, 40, and 50% v/v ethylene glycol.

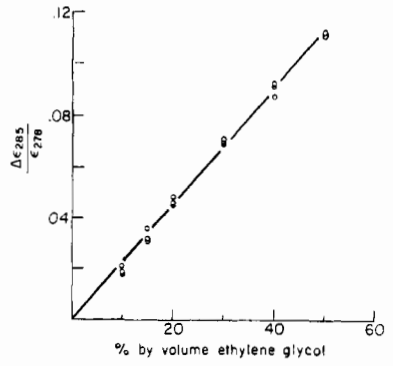

FIGURE 4: Plot of $\Delta E_{285}/E_{278}$ against volume per cent ethylene glycol.

and Dr. M. Kientz. These values of solvent refractive indices were used to calculate the optical constants from the Moffitt–Yang equation. The variation of b_0 with solvent composition is given in Figure 2. These data are somewhat scattered prohibiting detection of possible subtle conformational changes. Nonetheless, the scatter does lie within the range found by Tanford *et al.* (1962) in the study of β-lactoglobulin in glycol–water systems and it is permissible to conclude that the protein is not undergoing any extensive changes in structure, if indeed the conformation is changing at all.

Since optical rotatory dispersion is not particularly useful in detecting small conformational changes especially if they occur in amorphous regions of the protein, it was necessary to augment our structural studies with another, generally more sensitive, technique, *i.e.*, the method of solvent perturbation difference spectroscopy developed by Laskowski (1966), Herskovits and Laskowski (1962), and Bigelow and Geschwind (1960).

A representative plot of a single set of difference spectra is presented in Figure 3. Numerous scans were made at each concentration of ethylene glycol and the experiment was repeated on the same instrument using a different enzyme preparation giving the same results. The peaks obtained at 285 mμ and at 277–280 mμ identify tyrosine as the perturbed residue. The absence of tryptophan peaks indicates that no tryptophan is exposed to the solvent.

If solvent induces a conformational change in a protein effecting the exposure of a tyrosine residue to solvent, a discontinuity in the difference spectra as a function of solvent composition would result. Figure 4 gives a plot of the ratio of the difference in extinction at the maximum wavelength of the difference spectrum to the extinction of the protein itself at its maximum wavelength against volume per cent ethylene glycol. It can be seen that within experimental error the reduced quantity, $\Delta E_{285}/E_{278}$, is a linear function of solvent composition and that the line passes through the origin. This result indicates that with regard to the aromatic residues in the protein no significant change in exposure to solvent is apparent up through 50 vol % ethylene glycol.

Concentrations of ethylene glycol in excess of 80% v/v caused a strong blue shift in the protein difference spectrum indicating extensive structural changes. For example, 90% v/v ethylene glycol gave a $\Delta E_{285}/E_{278}$ ratio of -0.96. These results are consistent with the irreversible loss of catalytic activity above 80% v/v ethylene glycol shown in Figure 1.

Characteristics of the Adenosine Deaminase Catalyzed Reaction in Mixed Aqueous Solvents. The presence of ethylene glycol in reaction mixtures raises the possibility that an ether derivative of inosine could be formed rather than inosine itself. Consequently, ultraviolet spectral analysis of the products of the enzyme-catalyzed reaction in various glycol–water mixtures at pH 7.0 were performed for comparison with prepared inosine solutions in the same solvents. In all cases the spectra of products formed corresponded very well with equivalent concentrations of inosine dissolved in the appropriate glycol–water binary. Furthermore spectra of the products were taken after adjustment to pH 12.6 in order to establish the nature of the product more definitively. Inosine shows a characteristic change in its spectrum under alkaline conditions due to ionization of the hydroxyl group on the purine ring. Ether derivatives of inosine would not show this change. The spectra of the products were found to be essentially the same as inosine solutions. Therefore, we believe that the reaction occurring in the presence of ethylene glycol is the same as that which occurs in a strictly aqueous environment.

Hoagland and Fisher (1967) showed that eq 1 holds for chicken duodenal adenosine deaminase in a neutral aqueous environment. Care was taken to show that this equation

$$\frac{1}{\hat{V}} = \frac{1}{\beta} + \frac{1}{\alpha[\mathrm{Ad}]} + \frac{[\mathrm{In}]}{\gamma[\mathrm{Ad}]} \qquad (1)$$

holds over all conditions of solvent composition, pH, and temperature used in these studies. Velocities were usually determined for a 10-fold range of adenosine concentrations and plotted in accordance with the above equation.

Velocities for each adenosine concentration were measured in triplicate in each solvent mixture. Rate parameters (α and β) were then calculated from these data. A typical set of reciprocal plots for various solvent compositions can be seen in Figure 5. When inosine was added to reaction mixtures, $1/\hat{V}$ was plotted against [In]/[Ad] in order to establish linearity and evaluate the kinetic constant, γ. Under all conditions used linearity was observed and inosine was a strictly com-

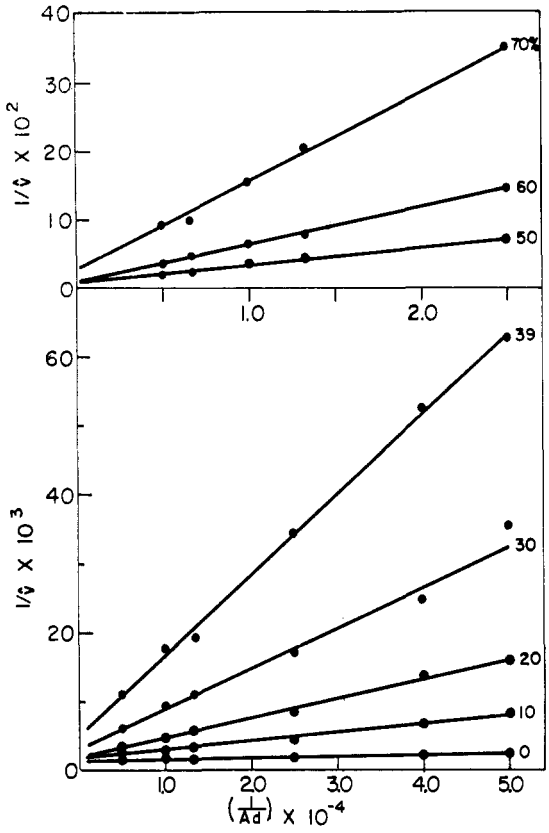

FIGURE 5: Reciprocal plots for adenosine concentrations ranging from 20 to 200 μM in the presence of 0–70% ethylene glycol. $\hat{V}$ is moles of adenosine deaminated per minute per mole of enzyme.

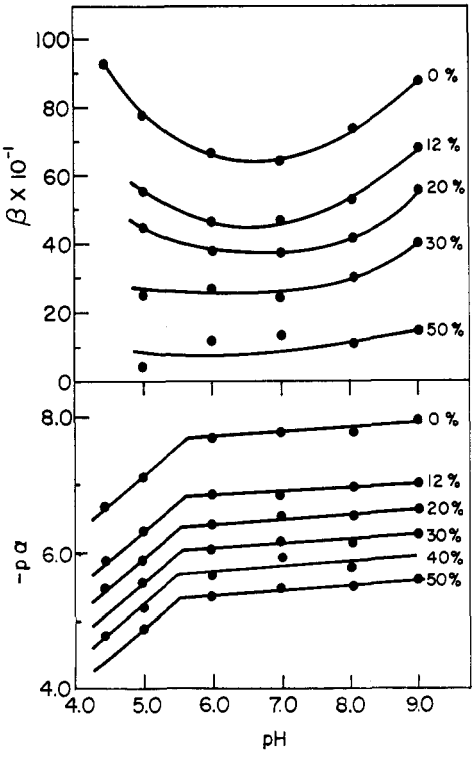

FIGURE 6: Effect of pH on the α and β rate parameters in 0–50% aqueous ethylene glycol mixtures.

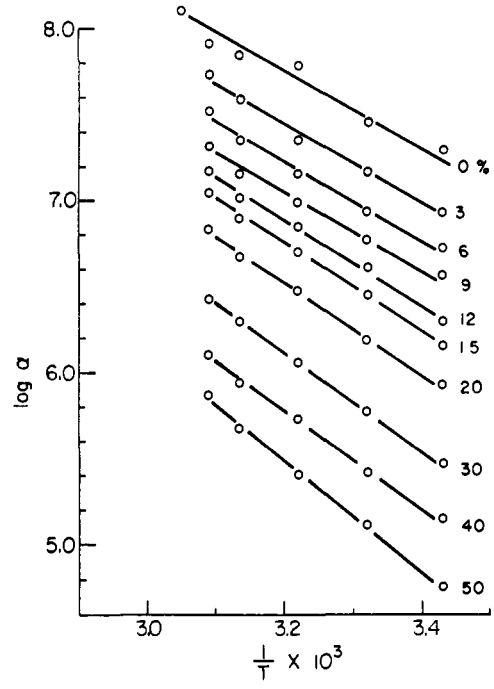

FIGURE 7: Arrhenius plot of the α rate parameter in the presence of 0–50% ethylene glycol.

petitive inhibitor. Therefore the equation presented by Hoagland and Fisher holds under all conditions we have studied.

To further ascertain if the mechanism operating in the fully aqueous systems is the same as that in mixed aqueous binaries, variation of the catalytic ability with pH was studied. The results obtained are described in Figure 6, and data for the fully aqueous system are identical in form with those reported by Chilson and Fisher (1963). The similarity of these curves indicates little change of pH dependence and it is concluded that as far as pH behavior is concerned the mechanism in the aqueous system is essentially the same as that in aqueous glycol mixtures. The temperature dependence of the rate parameters for the adenosine deamination reaction are shown in Figures 7 and 8 at different volume per cent ethylene glycol. Log α is a linear function of the reciprocal of temperature at all concentrations of ethylene glycol studied and the energy of activation increases with increasing glycol. Log β is a nonlinear function of the reciprocal of temperature and the degree of nonlinearity increases with increasing ethylene glycol. This curvature can be explained in terms of a shift in dominance of the part reactions composing the rate parameter (Lumry, 1959). The different behavior of the α and β parameters shows that they are primarily dependent upon different rate constants.

Solvent Effects on Rate Parameters. Results presented in the preceding sections show that there are no detectable conformational changes in the enzyme when it is exposed to glycol–water mixtures up to 70% v/v glycol. Also, these studies indicate that there is no change in the nature of the reaction occurring or in the mechanism by which the enzyme catalyzes the reaction, yet it has been shown that these changes in solvent concentration inhibit the rate of the reaction up to three orders of magnitude. There appear to be at least two ways this inhibition could occur. First, there is a

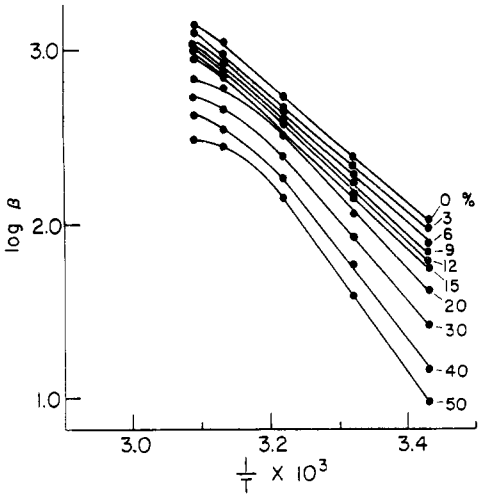

FIGURE 8: Arrhenius plot of the β rate parameter in the presence of 0–50% ethylene glycol.

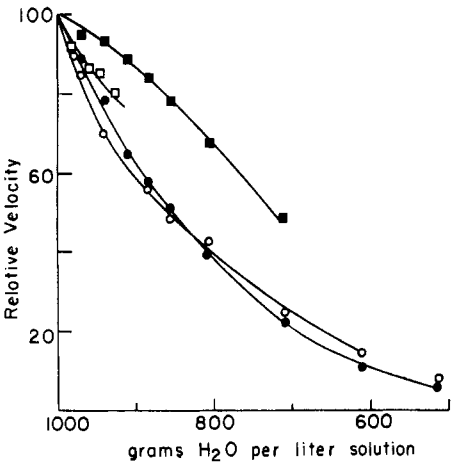

FIGURE 9: Effect of water concentration on reaction velocity. Solvent systems include aqueous methanol (■), aqueous glycerol (●), aqueous ethylene glycol (○), and aqueous sorbitol (□).

decrease in the concentration of the second substrate, water, as the glycol concentration is increased. Second, the presence of the glycol could cause large decreases in the kinetic parameters for the reaction.

Westhead and Malmström (1955) studied the effects of a variety of solvents on the catalytic activity with enolase. They found that the reaction velocity was highly dependent upon water concentration and that the velocities in various solvent systems formed a single line when velocities were plotted as functions of water concentration. Results of similar studies with adenosine deaminase are shown in Figure 9. Beyond 9% methanol and with other solvent systems the velocity decrease is far greater than could be expected on the basis of water concentration alone. When the chemical activity of water is plotted rather than concentration, the results are not substantially changed. Thus, it appears that water concentration is not the controlling factor for the adenosine deaminase catalyzed reaction. In addition, it is reasonable to assume that water, as a substrate, is saturating.

Since our spectroscopic and kinetic information indicates no structural change in the enzyme within certain limits of solvent composition and water concentration alone does not appear to be responsible for the large decreases in reaction velocity, research was begun to identify the properties of the bulk solvent which could be correlated with changes in the rate parameters.

A number of investigators have treated simple ion–ion, ion–dipole, and dipole–dipole interactions for those reactions in which electrostatic interactions are more important than nonelectrostatic ones (Laidler and Eyring, 1940; Glasstone *et al.*, 1941; Amis, 1949, Scatchard, 1932). Barnard and Laidler (1952) expanded the concepts of the dielectric constant effects derived from reactions of smaller molecules to enzyme kinetics. The various approaches differ in sophistication but predict essentially the same behavior of reaction rate with dielectric, *i.e.*, the log of velocity should be a linear function of the reciprocal of the dielectric constant for the bulk medium. Results presented in Figure 10 show the velocities of the adenosine deaminase reactions in the presence of high concentrations of adenosine (200 μM) in mixtures of five organic solvents with water. Since a given composition of these solvent systems give quite different dielectric constants, the close correspondence except for methanol and the linearity of the plots at very high composition of organic additive, suggests the over-all reaction velocity is indeed quite dependent upon electrostatic forces. The difference in slopes for the various binaries is presumably due to differences in nonelectrostatic forces. Since a homologous family of organic additives was used in the binary systems studied, the possibility exists that velocity behavior may be due to some other property of these binaries which also varies in a parallel manner with dielectric constant (Castañeda-Agulló and Del Castillo, 1959a,b). Accordingly, velocities of chemically nonrelated aqueous organic solvent systems were run,

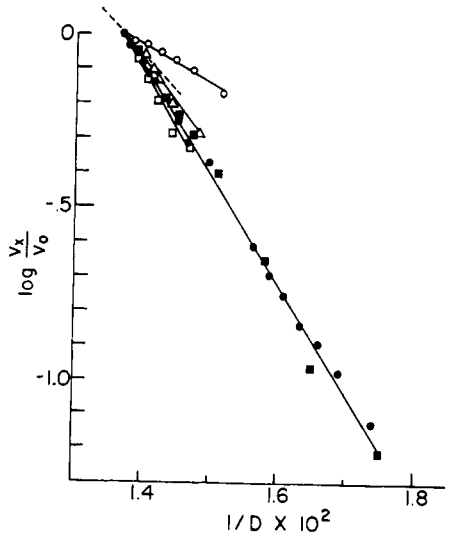

FIGURE 10: Dielectric behavior of the adenosine deamination reaction in aqueous polyhydroxy organic solvents. Solvent systems include aqueous methanol (○), ethylene glycol (●), sucrose (△), glycerol (■), and sorbitol (□). Dielectric data from Akerlof (see Table I). The dashed line represents the line in Figure 11. V_x represents the reaction rate in the mixed aqueous solvent and V_0 the reaction rate in the fully aquoeus system both at 200 μM adenosine.

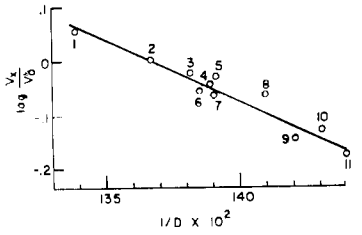

FIGURE 11: Effect of highly aqeuous organic solvents on the dielectric behavior of the adenosine deamination reaction. Sources for all points are listed in Table I. V_x represents the reaction rate in the mixed aqueous solvent and V_0 the reaction rate in the fully aqueous system both at 200 μM adenosine.

and to prevent possible adverse effects on the enzyme, less than 1 M binary solutions were used. Figure 11 shows the velocity–dielectric dependence obtained. For comparison, the line in Figure 11 is shown in Figure 10 as a dashed line. Mention should be made of the fact that urea increases the dielectric constant of water and therefore should increase the reaction velocity. This prediction is experimentally verified as seen in Figure 11.

Results involving the logarithms of extrapolated maximum velocities, β, have also been found to be linear functions of the reciprocal of dielectric for the aqueous polyhydroxy solvent systems. However, the different solutes tend to give results falling on straight lines which are more divergent than those seen in Figure 10. The corresponding log α and log γ parameters were found to be nonlinear functions of the reciprocal of dielectric. Consequently, an attempt was made to find other properties of bulk solvents which could be correlated with these parameters.

A number of organic reactions in mixed aqueous media demonstrate that nonelectrostatic forces influence nonenzymatic reaction kinetics (Amis, 1949; Hammond and Gutfreund, 1959; Laidler and Eyring, 1940). Nonelectrostatic forces which must be considered as possible factors in the adenosine deaminase catalyzed reaction include specific binding of the organic solvent to the enzyme and forces known to influence organic reactions such as internal pressure.

TABLE I: Source of Dielectric Data for Aqueous Organic Solvents.

Concn of Org Component (M)	Reference
Urea (0.5)	Wyman (1933)
Water	Akerlof (1932)
Methanol (0.776)	Akerlof (1932)
Glycerol (0.438)	Akerlof (1932)
Ethanol (0.5)	Akerlof (1932)
Ethylene glycol (0.549)	Akerlof (1932)
Sorbitol (0.432)	Akerlof (1932)
Dioxane (0.5)	Timmermans (1960)
Sucrose (0.343)	Akerlof (1932)
t-Butyl alcohol (0.5)	Akerlof (1932)
Sucrose (0.464)	Akerlof (1932)

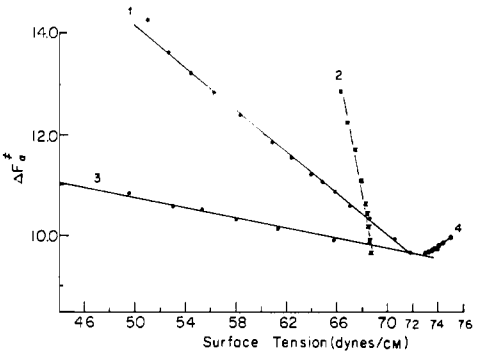

FIGURE 12: Variation of the activation free energy of the α parameter at 38° with surface tension at the various temperatures for which data are available. Line 1 gives the dependence in 0–75% v/v ethylene glycol, σ data at 25° from Curme and Johnston (1953); line 2 represents 0–50% glycerol, σ data at 38° from Miner and Dalton (1953); line 3 is aqueous methanol system, σ data at 25° from Butler and Wightman (1934), which holds up to the composition (30%) in which enzyme inactivation occurs; and line 4 gives the sucrose dependence, σ data at 20° from Timmermans (see Table I). $\alpha = (kT/h)e^{-\Delta F^{\mp}\alpha/RT}$ (Lumry, 1959).

A model for considering the combination of two molecules in solution has recently been advanced by Sinanoglu and Abdulnur (1964, 1965). The model provides a convenient way of looking at the interaction of combining molecules solely in terms of a bulk solvent property. This is accomplished by considering the solvent sheath around the molecules as the boundary of a cavity in solution, the solvent side being a macroscopic phase and the cavity being microscopic in character. This model is applicable only insofar as the molecules which make up the solvent are much smaller than those destined to react, so that the concept of macroscopic surface tension would have some validity. The gain in free energy accompanying the loss of surface area for the process of two molecules combining to form a complex is approximated by the equation $\Delta F = \Delta A\sigma$, where ΔA represents the loss in surface area and σ represents surface tension. Results presented in Figure 12 illustrate a rather marked linear dependence of the α rate parameter upon surface tension. The relationship holds up to the highest concentrations of methanol and ethylene glycol in which the enzyme remains undenatured. No upper limit was established for glycerol or sucrose which was determined through 50% v/v and 25% w/w, respectively. Sucrose, unlike other organic solutes, increases the surface tension when added to water. Thus, if the rate parameter, α, was indeed dependent upon the macroscopic surface tension,

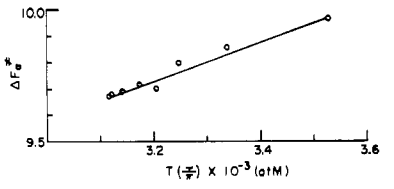

FIGURE 13: Variation of the activation free energy of the α rate parameter with internal pressure of the aqueous sucrose solvent.

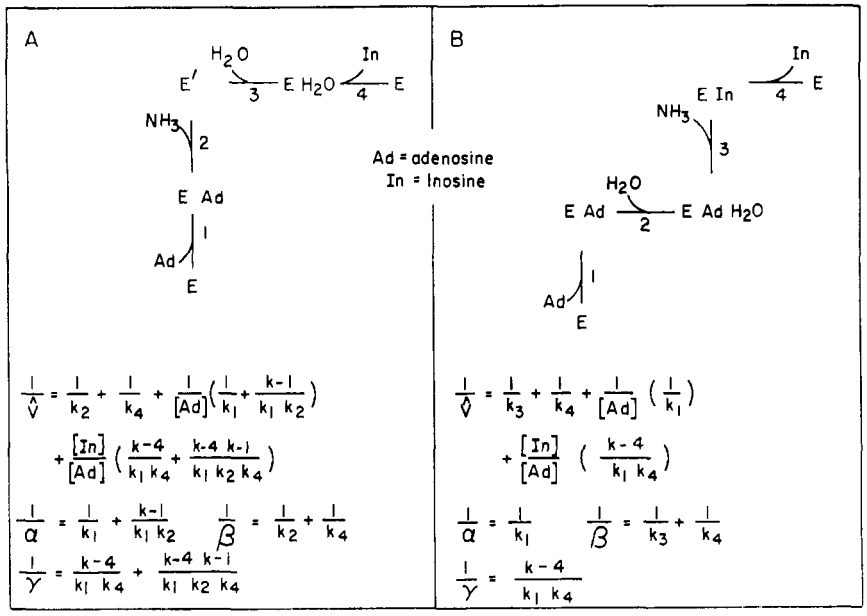

FIGURE 14: "A" represents a Ping-Pong-ordered sequence with the appropriate rate equation assuming that the ammonia release step is irreversible and water is saturating. "B" represents a ternary complex ordered sequence with the appropriate rate equation assuming that the ammonia release step is irreversible and water is saturating.

one would expect α to increase when sucrose was present. It is evident from Figure 12 that this prediction was not experimentally realized and that the surface tension model fails to explain all the kinetic data. However, it should be pointed out that the linearity observed is not trivial since α is changed by as much as 1000-fold accompanying the addition of up to 70% v/v ethylene glycol. This implies that some other property which varies in a parallel manner with surface tension for all solutes except sucrose could be responsible for the phenomenon.

The most likely candidate for consideration is the internal pressure of the liquid because surface tension and internal pressures are measures of the cohesive nature of the surface and bulk liquid, respectively. Both properties are direct functions of one another in nonpolar liquids and are somewhat more loosely related in associated liquids (Hildebrand and Scott, 1964). Indeed, the rates of certain organic reactions are reported to depend upon the internal pressure of the medium (Richardson and Soper, 1929) and such dependence has been treated theoretically (Glasstone *et al.*, 1941). Lumry (1969) and Lumry and Biltonen (1968) have considered the possible importance of internal pressure on enzyme catalysis in the context of a balance between internal pressure and free volume of solution and protein as the enzyme undergoes expansion and contraction during the course of catalysis. It is fully expected then that the rate parameters could respond to changes in internal pressure of the medium. Data for the coefficients of compressibility and expansion at temperatures near 38° are minimal and values could not be found for mixed aqueous glycol, glycerol, or sorbitol. Fortunately, however, compressibility and thermal expansion data were available for sucrose at 40°, close to the temperature at which the reactions were measured (38°) and internal pressures were calculated (Hildebrand, 1928). It was observed from calculated data (Timmermans, 1960) that over the concentration range used in the kinetic experiments the internal pressure of the solution increased with addition of sucrose. The same can be said for the other organic solutes for which compressibility and thermal expansion data exist. Therefore, when internal pressure rather than surface tension is considered, sucrose does behave like the other organic solutes.

A plot illustrating the correlation between $\Delta F_\alpha^{\ddagger}$ (hence log α) and the internal pressure of sucrose containing solutions can be seen in Figure 13. These observations lead us to believe that nonelectrostatic forces like internal pressure can exert a significant influence on certain rate constants of some enzyme-catalyzed reactions. Rigorous verification on this suggestion must await internal pressure data on several aqueous organic systems.

Log γ, log γ/α, and other ratios of the kinetic constants were not found to be linear functions of either the reciprocal of dielectric, $1/D$, or surface tension. Since the γ parameter could be more complex in terms of the rate constants of which it is composed, this lack of correlation is not too surprising.

Discussion

Results presented in the previous section have shown that chicken duodenal adenosine deaminase exhibits the same simple operating rate equation under a very wide range of experimental conditions. Eight different organic additives were used and wide concentration ranges were studied with five of these components. The pH was varied from 4.2 to 9.0 and temperatures from 20 to 48° were used. The system showed simple Michaelis–Menten kinetics under all conditions and the product inosine was always a strictly competitive inhibitor. On the basis of these results it seems reasonable to assume that there are no other terms in the rate equation. The adoption of this hypothesis leads to the conclusion that the adenosine deamination reaction is an ordered sequence

and that this sequence is of such a nature that inosine is a strictly competitive product inhibitor. There are two basic mechanisms which are consistent with these facts (Figure 14). It seems reasonable to assume that water is saturating and on the basis of the irreversibility of the reaction and the fact that ammonia is not an inhibitor (Hoagland and Fisher, 1967), it also seems reasonable to believe that the ammonia-releasing step is irreversible. The equations presented in Figure 14 were derived on the basis of the mechanism shown and these assumptions.

Extensive studies of the parameters α, β, and γ at a variety of temperatures and in a variety of solvents have led to several correlations. Log β was found to be a linear function of the dielectric of the medium but was observed to be a nonlinear function of Arrhenius plots in the presence of high concentrations of ethylene glycol. On the basis of these results it seems clear that β represents more than one rate constant. Log γ was found to be a nonlinear function of every property of the bulk solvent studied suggesting that it also is not a simple function. On the other hand, α behaved as a simple function in all experiments. Log α was a linear function of surface tension in a wide variety of solvents and linear Arrhenius plots were obtained in all solvent systems tested. In view of these observations we favor the ternary complex mechanism shown in Figure 14B. In this case α is k_1, whereas β and γ are more complex functions. On the basis of all these results, we favor a simple-ordered sequence mechanism involving a ternary complex for the chicken adenosine deamination reaction.

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