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Hydrogen Exchange in Nucleosides and Nucleotides. Measurement of Hydrogen Exchange by Stopped-Flow and Ultraviolet Difference Spectroscopy†

Dallas G. Cross

ABSTRACT: Time-dependent changes in the ultraviolet absorbance of the adenine chromophore are observed in the stopped-flow spectrophotometer when adenosine and its analogs are rapidly transferred from protium oxide to deuterium oxide. These absorbance changes are shown to result from hydrogen exchange in the exocyclic amino groups of the purine ribonucleosides by using derivatives of adenosine in which methyl groups replace exchangeable hydrogens and by showing that the general characteristics of hydrogen exchange in adenosine analogs agree with those found here. A study of the dependence of hydrogen-exchange rate constants on adenosine, AMP, and phosphate concentration showed there is a second-order dependence on AMP concentration which is primarily due to intermolecular catalysis by the phosphate group of the nucleotide. The deuterium oxide perturbation difference spectrum, obtained at equilibrium, was found to contain two components that result from blue shifts of the adenine chromophore absorbance: (1) a shift cause by the substitution of deuterium for protium in the ring (N^1) nitrogen and exocyclic nitrogens, and (2) a shift associated with a change in the polarizability of the medium. Since the theory of solvent perturbation, which is used to measure the relative "exposure" of chromophores in macromolecules, assumes that the spectral shifts observed are solely due to (2) above, the use of deuterium oxide as a measure of chromophore exposure to perturbants the size of water must be reexamined.

The use of hydrogen exchange methodology to study both the chemistry of the constituent groups of proteins and polynucleotides and the structural properties of macromolecules has been reviewed by Englander *et al.* (1972). Hydrogen exchange data have been obtained using a number of instrumental methods that measure total and specific exchange over a variety of time ranges. Near-infrared absorption properties of amides have been used by Miller and Klotz (1973) to measure hydrogen exchange rate constants which are on the order of 0.02 sec^{-1}. Overall hydrogen exchange in nucleic acids has been measured with Sephadex column chromotography using tritium (Printz and von Hippel, 1968; McConnell and von Hippel, 1970; Hanson, 1971; Englander, 1972). In these experiments the earliest data were obtained at approximately 6 sec after initiation of exchange. Marshall and Grunwald (1969) and McConnell and Seawell (1972) using nuclear magnetic resonance (nmr) and Lang *et al.* (1974) using ultrasonic absorption techniques have measured much faster rates of hydrogen exchange to specific chemical groups in analogs of adenosine.

I show that there are changes in the ultraviolet absorption spectra of adenosine and other purine and pyrimidine analogs which reflect the number of hydrogens that exchange with deuterium at the ring nitrogen and amino nitrogens. The ultraviolet difference spectra associated with these exchanges can be used to identify the exchanging chromophoric moiety. I use these ultraviolet absorption changes to study the general properties of hydrogen exchange including the effect of adenosine and AMP concentration on the apparent first-order rate constant of exchange. Furthermore, I determine the spectral contributions from deuterium exchange to the total deuterium oxide perturbation difference spectrum and reexamine the use of deuterium oxide to measure the accessibility of water to chromophores in macromolecules.

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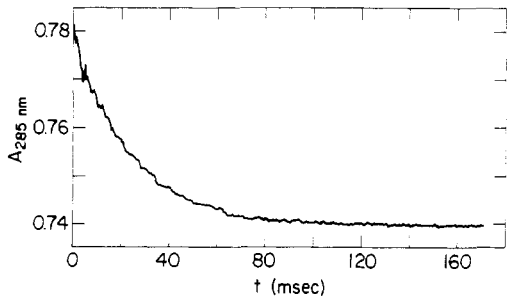

FIGURE 1: Progress curve resulting from mixing adenosine in protium oxide with deuterium oxide. In the stopped-flow experiment 1:1 ratio drive syringes were used to give a final concentration of 2.42 mM adenosine and 49.5% deuterium oxide at pH 7. The experimental curve represents an average of 10 individual rate measurements. The cell had a light path length of 0.54 cm and was thermostated to 20 ± 0.2°.

Experimental Procedure

Adenosine, AMP, N^1-methyladenosine, 2′-deoxyadenosine monophosphate, 2′-deoxy-3′:5′-cyclic adenosine monophosphate, and deuterium oxide (approximately 99.8%) were purchased at the highest purities available from Sigma Chemical Company. N^6-Methyladenosine and N^6-dimethyladenosine were products of the Aldrich Chemical Company, Inc. NaCl, NaH_2PO_4, NaOH, and HCl, all analytical reagent grade, were products of Mallinckrodt Chemical Works. A Radiometer PHM 26 pH meter was used to measure pH's; pD's were determined by the approximation of Glasoe and Long (1960), $pD = pH_{meter} + 0.4$. All experiments were conducted at pH or pD values of 7.0 unless otherwise designated. Concentrated HCl and NaOH were used to make pH and pD adjustments to minimize dilution of deuterium oxide solutions to less than 0.3%. The temperature was maintained at 20 ± 0.2° for all experiments. In the experiments in which the purine analogs were dissolved in deuterium oxide prior to use the solutions were prepared and used within a 6-hr time period.

Equilibrium Difference Spectra. Deuterium oxide perturbation difference spectra were measured in a Cary Model 14 spectrophotometer interfaced to a Varian 620i digital computer using a tandem cell arrangement in the sample and reference compartments as previously described (Cross and Fisher, 1969). Experimental base lines were recorded with solvent in the sample and reference compartments and difference spectra were obtained after addition of samples to the sample and reference cells with Lang-Levy pipets. Final concentrations of adenosine analogs were 70–80 μM in a 1.000-cm cell. The data points in a difference spectrum were collected at 0.5-nm intervals and each point represents an average of absorbances over a 0.2-nm range. The values of from 3 to 4 difference spectra were then averaged to obtain the data for the final difference spectrum.

Model Difference Spectra. Model difference spectra were derived by subtracting from an actual spectrum of an adenine analog in deuterium oxide the same spectrum which was mechanically shifted either 0.5 or 1.0 nm to longer wavelengths. These shifts are small enough in proportion to the absorption bandwidth to produce difference spectra which possess the same shape and which have amplitudes which are proportional to the wavelength shift (Chervenka, 1959; Fisher *et al.*, 1969).

The amplitudes of difference spectra obtained by the equilibrium method, from the stopped-flow results, or from mechanical wavelength shifts are expressed as peak-to-trough measurements. The difference spectra obtained with these three methods and using all of the adenosine analogs had, as common features, a peak and a trough.

Stopped-Flow Kinetics. Stopped-flow experiments were performed on a Durrum–Gibson stopped-flow spectrophotometer interfaced to a Varian 620i computer as described previously (Colen *et al.*, 1972). The light source was a 75-W xenon arc lamp and the slit width was 0.4 mm. Wavelength calibration against known standards resulted in an uncertainty of ±1 nm in the wavelength observed. All experiments were performed under conditions where Beer's law was obeyed by using combinations of observed wavelength, cell path length (0.54 and 1.74 cm), and chromophore concentrations. The data reported herein represent an average of from 7 to 10 reaction progress curves which were collected as 100-μsec samples taken at intervals of from 1 to 5 msec. The dead-time of the instrument was determined using the reaction of ferric nitrate with potassium thiocyanate in 0.1 N H_2SO_4 and was found to be 3 msec.

All the progress curves showed time-dependent changes in absorbance which were first order. Thus, a plot of log $(A_t - A_\infty)$ *vs.* t, where A_t is the absorbance at time t and A_∞ is the final absorbance, yields two measurable parameters: (1) the first-order rate constant (k_{app}) which is obtained from the slope of a least-squares linear fit of the data, and (2) the total signal amplitude (ΔA) resulting from the rate process which was determined by subtracting A_∞ from the extrapolated value of A_0. Most experiments were performed using 1:1 ratio drive syringes but in those experiments where high final concentrations of protium oxide or deuterium oxide were required drive syringes with ratios of 1:7 or 1:14 were used. Studies in which the effect of phosphate or NaCl concentration was measured were accomplished by including the salt in both drive syringes.

Results

When adenosine in protium oxide is rapidly mixed with deuterium oxide in the stopped-flow spectrophotometer an absorbance change is observed in the vicinity of the adenine absorbance maximum at 259 nm (Figure 1). Apparent first-order rate constants for this process were determined at several wavelengths and were found to be identical within experimental error. The total extinction coefficient change ($\Delta\epsilon$) was determined kinetically using the value of ΔA as determined at several wavelengths and as described under Experimental Procedures. The extinction changes are shown as a difference spectrum in Figure 2A. Figure 2B is a difference spectrum resulting from the effect of deuterium oxide on the spectrum of adenosine which was obtained in a static experiment. This latter type of difference spectrum is an equilibrium solvent perturbation difference spectrum (Herskovits and Laskowski, 1962) and has been used with deuterium oxide as a perturbant to determine the relative aqueous exposure of nucleic acid and aromatic amino acid chromophores in proteins (Cross and Fisher, 1966; Herskovits and Fuchs, 1972; Kronman *et al.*, 1972) and dinucleotides (Cross and Fisher, 1969). Figure 2C shows that a model difference spectrum of adenosine absorbance, produced using wavelength shifts as described under Experimental Procedures, is identical in shape to the experimental difference spectra, Figure 2A and B. This identity permits characterization of the experimental difference spectra in Figure 2 as arising from a blue shift of adenine absorbance in the presence of deuterium oxide. All of the experimental difference spectra encountered in this study could be exact-

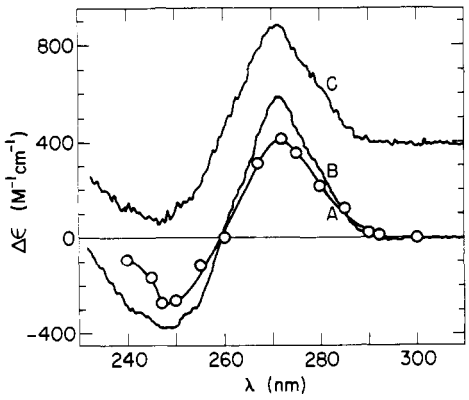

FIGURE 2: Experimental and derivative difference spectra of adenosine at pH 7 and 20°. (A) Difference spectrum derived from measurements of the first-order rate process as described in the text. Final concentrations of adenosine which were used to obtain the amplitudes of the optical density change associated with the rate process were varied from 0.029 to 2.5 mM. (B) Equilibrium deuterium oxide perturbation difference spectrum of adenosine. (C) Derivative difference spectrum produced mechanically using a 0.50-nm shift of the adenosine absorbance spectrum as described in the text. This curve is offset by 400 units for clarity. The two experimental difference spectra represent comparison of the spectrum of adenosine in 100% protium oxide *vs.* that in 100% deuterium oxide.

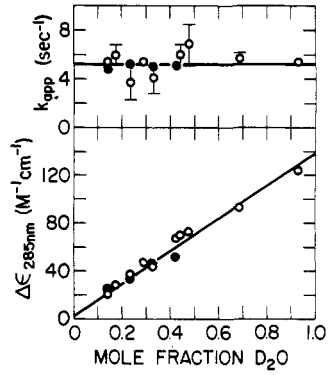

FIGURE 3: Dependence of the apparent first-order rate constant (k_{app}) and the molar difference extinction coefficient ($\Delta\epsilon$) on the final mole fraction of deuterium oxide at pH 7 and 20 ± 0.2°. (○) Data acquired from stopped-flow experiments in which adenosine, equilibrated in protium oxide, was rapidly mixed with deuterium oxide; (●) data from experiments in which adenosine was equilibrated in deuterium oxide before mixing with protium oxide. The rates were observed at 285 nm using a 1.73-cm path-length cell thermostated to 20°. Final concentrations of adenosine varied between 0.51 and 0.63 mM.

ly reproduced by shifting the appropriate analog spectrum less than 1 nm. Thus, the wavelength shift is used to provide a comparative measure of the effect of deuterium oxide rather than the amplitudes of the difference spectra which are dependent on absorption band shape as well as wavelength shift (Fisher *et al.*, 1969).

The equilibrium difference spectrum for the deuterium oxide perturbation of the adenosine spectrum is larger than that determined kinetically. Since no further absorbance changes are observed during periods to 30 min, the additional absorbance, representing the difference of the two spectra (Figure 2A and B), must have occurred within the dead-time of the stopped-flow apparatus (3 msec). The rate of the spectral shifts associated with refractive index change or medium polarizability change is most likely to be diffusion limited and thus, is much too rapid to observe in these stopped-flow experiments. The dependence of the first-order rate constants and the amplitudes of the difference spectra on the final mole fraction of deuterium oxide are shown in Figure 3. At pH 7 the apparent rate constant does not vary significantly from the average (5.3 ± 0.5 sec^{-1}) when adenosine is transferred from either protium oxide or deuterium oxide to any final mole fraction of hydrogen isotope. These data also show that the amplitudes of the time-dependent difference spectra are directly proportional to the final mole fraction of isotope. Thus, under these conditions (1) rate constants are comparable when obtained using any final mole fraction, and (2) the amplitudes of difference spectra or wavelength shifts associated with the production of these different spectra may be normalized by linear extrapolation to a constant isotopic fraction. Therefore, the data herein have all been normalized to a deuterium oxide concentration change of one mole fraction unless otherwise designated.

Analogs of Adenosine. Several analogs of adenosine were examined to determine if the observable time-dependent spectral shifts are related to the hydrogen exchange on the exocyclic amino group. Purine ribonucleoside and N^6,N^6-dimethyladenosine were found to show no time-dependent absorbance changes in the stopped-flow spectrophotometer when examined at wavelengths for which deuterium oxide perturbation difference spectra at equilibrium showed significant absorbance changes. On the other hand, time-dependent shifts in the adenine absorbance of AMP, 2′-deoxyadenosine monophosphate, and 2′-deoxy-3′:5′-cyclic adenosine monophosphate were observed when these analogs were transferred from deuterium oxide to protium oxide. In addition, time-dependent absorbance changes were observed for cytidine and guanosine indicating that these nucleosides can also be studied using these methods.

At pH or pD 7, adenosine, N^6-methyladenosine, and N^6,N^6-dimethyladenosine have 2, 1, and 0 dissociable hydrogens on their respective exocyclic amino groups. Transfer of these compounds from protium oxide to deuterium oxide produced a time-dependent shift in the spectrum of adenosine, a slower but smaller shift in the spectrum of N^6-methyladenosine, and no time-dependent shift in the spectrum of N^6,N^6-dimethyladenosine. Difference spectra, derived kinetically from the time-dependent absorbance changes observed above, were found to be identical in shape with, but smaller in magnitude than, the deuterium oxide difference spectra produced at equilibrium using these same analogs. Mechanically produced model difference spectra of N^6-methyladenosine and N^6,N^6-dimethyladenosine were also found to be identical in shape with the time-dependent and/or equilibrium difference spectra experimentally determined for these analogs. The spectral shifts required to reproduce exactly the equilibrium and time-dependent difference spectra of the two methyl analogs were determined and are shown together with the same data obtained for adenosine in Figure 4. The linear dependence of the data obtained at pH 7 in this figure shows an apparent correlation of the time-dependent portion ($-\Delta\lambda_t$) of the total wavelength shift obtained at equilibrium with the number of exchangeable hydrogens on the exocyclic amino moieties of the analogs. This apparent correlation prompted examination of the spectral properties of the same analogs at pH 1 where the ring nitrogen (N^1) is also protonated (Cochran, 1951; Bock *et al.*, 1956; Zubay, 1958; Miles, 1961; Jardetzky *et al.*, 1963) which would provide the nucleosides with another dissociable hydrogen. Hydrogen exchange at pH 1 is too rapid to be measured accurately in the stopped-flow apparatus, but the results from deuterium oxide perturbation difference spectra obtained at equilibrium using the an-

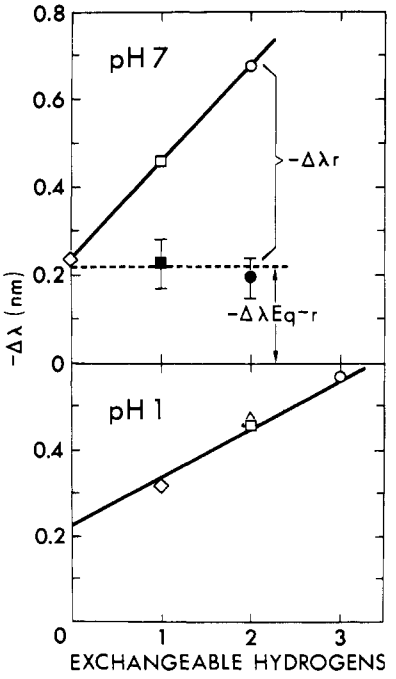

FIGURE 4: Correlation of the wavelength shifts of the spectra of adenosine and adenosine analogs with the number of dissociable hydrogens on N^6 and N^1 of the adenine moiety. The open symbols represent the wavelength shifts ($-\Delta\lambda$) required to reproduce the equilibrium difference spectra resulting from deuterium oxide perturbation of adenosine (○), N^6-methyladenosine (□), N^6,N^6-dimethyladenosine (◇), and N^1-methyladenosine (△). For the data obtained at pH 7 the number of exchangeable hydrogens refers to those at the N^6 position and at pH 1 this number refers to the sum of the N^6 and N^1 dissociable hydrogens. Since at either pH N^1-methyladenosine is an analog of the protonated form of adenosine, it is only included in the data for pH 1. At pH 7 the portion of the graph indicated by the brace labeled $-\Delta\lambda_r$ indicates the wavelength shifts resulting from the rate process as determined from the values of $A_\infty - A_0$ described in the text. The solid symbols and the arrows labeled $-\Delta\lambda_{Eq-r}$ indicate the apparently constant wavelength shift which represents the difference between the equilibrium and rate process values.

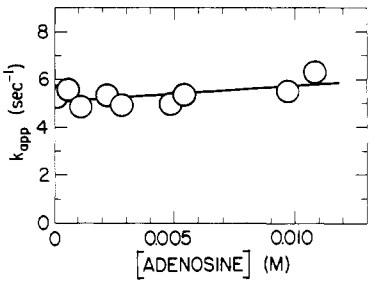

FIGURE 5: Variation of the apparent first-order rate constant on adenosine concentration at pH 7 and 20°.

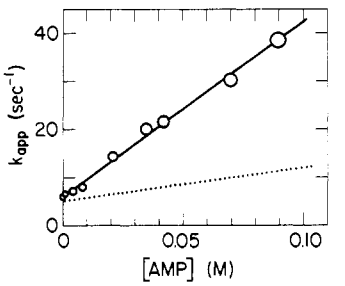

FIGURE 6: Variation of the apparent first-order rate constant on AMP concentration. The dotted line shows the dependence of the first-order rate constant on adenosine concentration as determined from Figure 5 and is included for comparison.

alogs (Figure 4) show a linear dependence of the total wavelength shift on the sum of the number of exchangeable hydrogens on the ring and exocyclic amino group. A line through these data intersects the abscissa quite near the intercept of the pH 7 plot. This common intersection indicates that the medium polarizability effect, represented by a wavelength shift of 0.23 nm, is, as expected, essentially the same at both pH values. This polarizability effect is also indicated by the apparently constant $-\Delta\lambda_{Eq-r}$ values in Figure 4 for the three analogs of unprotonated adenosine. The values of the slopes in Figure 4, 0.22 and 0.1 nm per exchangeable hydrogen atom, represent the blue shift associated with the total protium–deuterium exchange of the unprotonated and N^1-protonated forms of the adenine moiety, respectively. The different values for the two species are expected since the spectra of the protonated purine nucleosides are quite different from the unprotonated ones reflecting, of course, differences in electronic structure.

Second-Order Rate Constants. The rate of hydrogen exchange at the amino group of AMP has been calculated from nmr data (McConnell and Seawell, 1972) and has a second-order dependence on the concentration of the mononucleotide. This exchange can be studied by nmr only in concentration ranges where this nucleotide is known to undergo self-association. Since solubility of adenosine did not permit examination of concentration dependence of hydrogen exchange using this technique, the concentration dependence of the rates of exchange in these compounds was examined by measuring ultraviolet absorbance changes by the stopped-flow technique. The data in Figure 5 show that there is a very small concentration dependence of the apparent first-order rate constant on adenosine concentration. The second-order constant calculated from a least-squares fit to the data is 73 ± 15 M^{-1} sec^{-1}. In contrast to the adenosine data, the apparent rate constant obtained using AMP does show a significant second-order dependence (Figure 6) which extrapolates linearly and from which a second-order rate constant of 366 ± 100 M^{-1} sec^{-1} was calculated. The rate constants at infinite dilution were determined from the intercepts of the extrapolated data with the abscissas in Figures 5 and 6 and were found to be 5.0 ± 0.3 sec^{-1} and 5.7 ± 0.8 sec^{-1} for adenosine and AMP, respectively.

Phosphate catalysis of hydrogen exchange to the amino group has been indicated by line-width broadening in nmr studies (McConnell and Seawell, 1972). The dependence of the apparent rate constant for hydrogen exchange in adenosine was measured and found to be linear with sodium phosphate concentration between zero and 0.1 M. The second-order rate constant for phosphate catalysis at pH 7, calculated from the slope of the least-squares fit of these data, is 246 ± 80 M^{-1} sec^{-1}. A study of the effect of ionic strength on the apparent rate constant for hydrogen exchange in adenosine was performed and showed that there was less that 10% variation in this rate constant at pH 7 and 20° when sodium chloride concentration was varied from 0 to 2 M.

Discussion

Since time-dependent ultraviolet absorbance changes are observed only with those adenosine analogs which have exchangeable hydrogens on the exocyclic amino group and are not observed with analogs in which the possibility of this exchange is blocked or absent, it appears that the blue shifts associated with the deuterium oxide effect can be used to

measure the rate of hydrogen exchange to the exocyclic nitrogen of the adenine moiety. A blue shift from deuterium substitution of a carbon-bound hydrogen of benzene is observed in the ultraviolet region.[1] This result suggests that a blue shift in the spectrum of adenosine would result from a similar substitution at the more dissociable N^6 hydrogens. It is probable that deuterium exchange of the carbon-bond hydrogens of adenine would also produce spectral shifts but since these exchanges are at least 10^4 times slower than the rates measured here (Tomaz *et al.*, 1972), they will not contribute to the absorbance changes observed in this study. Nuclear magnetic resonance studies of hydrogen exchange of the amino group of adenosine analogs show acid–base catalysis, phosphate catalysis, and a second-order rate constant dependence on AMP concentration, the same general characteristics as observed in the present study. In addition the rates calculated from the nmr studies agree, within experimental limitations, with the rates observed directly in this study.

The use of ultraviolet absorption changes in the stopped-flow spectrophotometer to measure hydrogen exchange has several advantages. The capability to measure the actual rate of hydrogen exchange is certainly more desirable than derivation of rates from relaxation data such as is done in nmr and ultrasonic absorption methods. The concentration of the exchanging moiety required to measure hydrogen exchange by the stopped-flow technique is at least two orders of magnitude smaller than that required for nmr or for ultrasonic absorption permitting studies over larger concentration ranges and use of smaller quantities of sample materials. Perhaps the large time-range amenable to the study of hydrogen exchange by ultraviolet spectroscopy is of the greatest utility since this range includes that feasible for nmr and rapid column chromotography and is exceeded only by ultrasonic absorption methods. The only limitation is the time-resolving ability of the ultraviolet spectrophotometer being used.

Spectral identification of the chromophore with groups undergoing hydrogen exchange provides a specificity which is useful for the study of exchange in systems which exchange hydrogens at many groups. Insofar as the absorption changes in adenosine are due only to hydrogen exchange of the nitrogen-bound hydrogens involved in Watson–Crick hydrogen bond pairs, the method may be used to study the effect of structure on the rate of exchange in polynucleotides.

Rate Studies. Rhodes and Schimmel (1974), using ultrasonic absorption, have demonstrated an intermolecular catalysis between AMP molecules involving the protonated ring nitrogen of one molecule and the anionic phosphate group of another. McConnell and Seawell (1972) showed hydrogen exchange of the amino nitrogen in AMP to exhibit a second-order dependence of the apparent rate constant on AMP concentration. They attributed this dependence to intermolecular catalysis effected by the ribose–phosphate moiety of the mononucleotide. The results in this paper show that the sum of the rate constants for hydrogen exchange in adenosine and that of phosphate catalysis approximates that observed for hydrogen exchange in AMP confirming their suggestion that the phosphate, *per se,* is responsible for the second-order effect. We have also shown that adenosine and AMP have equal intrinsic rates for hydrogen exchange to the amino group which indicates that there is no intramolecular catalytic contribution from the phosphate moiety on AMP.

Broom *et al.* (1967) and Marshall and Grunwald (1969), using purines and derivative analogs of adenosine, have shown that the interactions involved in the intermolecular stacking of adenosine and AMP do not involve the formation of intermolecular hydrogen bonds by the amino moiety of the adenosine analogs. Hydrogen bonding has been shown to be responsible for a lowering of the amino proton exchange rate in polynucleotides (Hanson, 1971; Englander *et al.*, 1972; Englander and von Hippel, 1972; Hilbers *et al.*, 1973). Since we show here that the apparent rate constants for hydrogen exchange in adenosine and AMP show linear increases throughout the concentration ranges where both are known to undergo stacking, hydrogen bond formation involving the amino moiety is not indicated. This conclusion agrees with the description of the intermolecular AMP complex by Son and Chachaty (1973) who describe the complex as possessing amino groups which are in the same chemical environment as found in the monomeric nucleotide.

Solvent Perturbation Difference Spectroscopy. The total deuterium oxide perturbation difference spectrum obtained at equilibrium contains wavelength shift components derived from two processes: a change in the polarizability of the medium surrounding the chromophore which is the effect used to determine the relative "exposure" of chromophores in solvent perturbation studies (Herskovits and Laskowski, 1962; Cross and Fisher, 1969), and a specific effect resulting from deuterium substitution which I have shown to indicate the number of hydrogens exchanged on the N^1 and amino groups. The contributions from these two processes to the equilibrium difference spectrum requires a reexamination of the use of deuterium oxide as a perturbant to determine the relative exposure of chromophores in polymers.

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[1] The ultraviolet spectra of hexadeuteriobenzene and benzene, in the gas phase at 20°, were measured in this laboratory and a comparison of the two spectra showed an average spectral shift in the 260-nm region of 0.3 nm to shorter wavelengths for the deuterium-substituted compound.

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Relationship between Fluorescence and Conformation of ϵNAD$^+$ Bound to Dehydrogenases†

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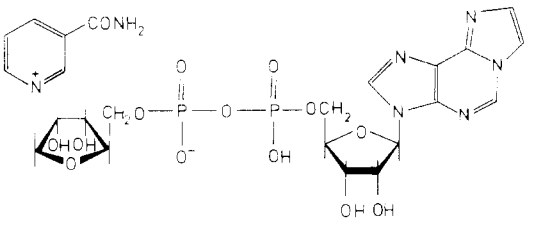
ABSTRACT: This work reports on the interaction of the fluorescent nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD$^+$) with horse liver alcohol dehydrogenase, octopine dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase from different sources (yeast, lobster muscle, and rabbit muscle). The coenzyme fluorescence is enhanced by a factor of 10–13 in all systems investigated. It is shown that this enhancement cannot be due to changes in the polarity of the environment upon binding, and that it must be rather ascribed to structural properties of the bound coenzyme. Although dynamic factors could also be important for inducing changes in the quantum yield of ϵNAD$^+$ fluorescence, the close similarity of the fluorescence enhancement factor in all cases investigated indicates that the conformation of bound coenzyme is rather invariant in the different enzyme systems and overwhelmingly shifted toward an open form. Dissociation constants for ϵNAD$^+$–dehydrogenases complexes can be determined by monitoring the coenzyme fluorescence enhancement or the protein fluorescence quenching. In the case of yeast glyceraldehyde-3-phosphate dehydrogenase at pH 7.0 and t = 20° the binding plots obtained by the two methods are coincident, and show no cooperativity. The affinity of ϵNAD$^+$ is generally lower than that of NAD$^+$, although ϵNAD$^+$ maintains most of the binding characteristics of NAD$^+$. For example, it forms a tight complex with horse liver alcohol dehydrogenase and pyrazole, and with octopine dehydrogenase saturated by L-arginine and pyruvate. One major difference in the binding behavior of NAD$^+$ and ϵNAD$^+$ seems to be present in the muscle glyceraldehyde-3-phosphate dehydrogenase. In fact, no difference was found for ϵNAD$^+$ between the affinities of the third and fourth binding sites. The results and implications of this work are compared with those obtained recently by other authors.

Nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD$^+$),[1] having the formula shown below, has a fluorescence maximum at around 410 nm when excited in the 305-nm region (Barrio *et al.,* 1972). It therefore offers the possibility of exploiting fluorescence techniques for studying the interaction between dehydrogenases and the oxidized form of the coenzyme. Moreover, since the fluorescence signal is localized on the adenine ring, information on the involvement of the adenosine moiety in the coenzyme binding can possibly be obtained. Our laboratory has al-



ϵNAD

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[1] Abbreviations used are: ϵNAD$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.