Nuclear Magnetic Resonance Studies of Alcohol Dehydrogenases. Binding of Inhibitors to the Yeast and Liver Enzymes†

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ABSTRACT: The alcohol dehydrogenases from yeast, or from equine liver, cause a broadening of the adenine proton nmr signal of reduced nicotinamide adenine dinucleotide and of some adenine nucleotides known to be inhibitors for the coenzyme. These line-broadening effects have been shown to be dependent on enzyme concentration and on coenzyme or inhibitor concentration. These findings indicate a specific molecular association of the coenzyme or inhibitor with the protein. A study of the effect of temperature on these line broadenings has allowed an estimate of the lifetime of these species in the bound state. The lifetime at NADH bound to yeast alcohol dehydrogenase is consistent with the activity of the enzyme. The lifetime of NADH bound to liver alcohol dehydrogenase has been found to be significantly shorter than that found in previous studies by direct kinetics. This result indicates a step in the unbinding process which has hitherto been undetected.

Nuclear magnetic resonance (nmr) spectroscopy has been used considerably in recent years as a means of studying the binding of small molecules to proteins (reviews include: Jardetzky, 1964; Sheard and Bradbury, 1971; Hollis, 1972). We wish to report here some results on the binding of NADH[1] and some of its fragments to the alcohol dehydrogenases of yeast and equine liver. An earlier report from this laboratory (Hollis, 1967) indicated that the presence of liver or yeast alcohol dehydrogenase had pronounced effects on the nmr spectrum of NADH but it was not always clear whether these were changes in line width, intensity, or both. In the present investigation we have used temperature and concentration studies to clarify the earlier results and have extended the study to include adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine diphosphoribose which are inhibitors of yeast and liver alcohol dehydrogenases. Since the usual techniques rely on the optical spectroscopic properties of the nicotinamide ring, they can only study the binding of these inhibitors through observation of their effects on the coenzyme. By contrast, nmr allows the direct study of the changes in molecular motion of these species as a result of their interaction with the protein.

The line broadening of the nmr spectrum of a small molecule due to a binding equilibrium with a macromolecule can be treated by the method of Swift and Connick (1962), which applies to exchange of nuclei between two sites, one being in large excess over the other. According to Swift and Connick

$$\frac{1}{T_{2p}} = \frac{f\left(\frac{1}{T_{2m}}\frac{1}{\tau_m} + \frac{1}{(T_{2m})^2}\right) + (\Delta\omega_m)^2}{\tau_m\left(\frac{1}{T_{2m}} + \frac{1}{\tau_m}\right)^2 + (\Delta\omega_m)^2} \quad (1)$$

where

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_{2,0}} \quad (2)$$

and $1/T_2$ is the nuclear magnetic transverse relaxation rate constant, measured for the species in the presence of the broadening agent, in this case, the enzyme. The quantity $1/T_{2,0}$, is the same rate constant measured in the absence of the enzyme. In eq 1, f is the fraction of coenzyme or inhibitor molecules which are bound to the protein, τ_m is the average lifetime of the bound species on the enzyme, $1/T_{2m}$ is the transverse relaxation rate constant for the species bound to the enzyme, and $\Delta\omega_m$ is the chemical-shift difference between the signals for free and for bound coenzyme or inhibitor. Under conditions where $\Delta\omega_m$ is small or zero, as in the present case, eq 1 reduces to

$$\frac{1}{T_{2p}} = \frac{f}{\tau_m + T_{2m}} \quad (3)$$

It is possible to show (Becker, 1969) that under conditions of a low intensity of the applied radiofrequency (rf) field, terms such as $1/T_2$ are adequately given by the line width of the resonance. This relationship can be combined with an expression for f, the fraction bound, in terms of the dissociation constant K for n equivalent sites and the total concentration of enzyme and substrate, $[E]_0$ and $[S]_0$, respectively. The final result, discussed in detail previously in the literature (Sykes *et al.*, 1970; Navon *et al.*, 1970; Lanir and Navon, 1971) is

$$\frac{1}{W_p} = \frac{\pi(\tau_m + T_{2m})}{n[E]_0}(K + [S]_0) \quad (4)$$

† From the Department of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore, Maryland 21205. *Received October 31, 1972.* This work was supported by National Institutes of Health Grant GM 17172. Presented in part at the 56th Annual Meeting of the Federation of American Societies of Experimental Biology, Atlantic City, N. J., April, 1972.

‡ Public Health Service Postdoctoral Fellow, National Institutes of Health Grant No. I-F02-GM 52879-01.

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[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide.

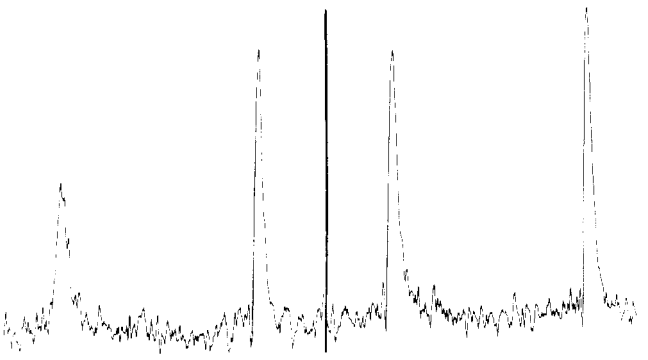

FIGURE 1: Effect of the addition of EDTA on the adenine region of the 100-MHz nmr spectrum of 10 mM NADH in 0.1 M phosphate buffer, pD 7.7, at 17.5°. The trace on the left is without EDTA. The trace on the right is with 10^{-4} M EDTA.

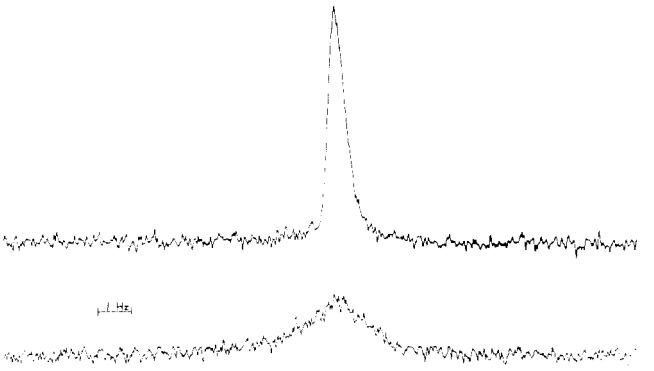

FIGURE 2: Effect of the addition of yeast alchol dehydrogenase on the nmr signal for the adenine H-2 proton of 10 mM ADP in 0.1 M phosphate buffer, pD 7.7, at 17.5°. The upper trace is without enzyme. The lower trace is with 15 mg/ml of the yeast enzyme.

where the line broadening W_p is the difference in line width between broadened and unbroadened signals. When the lifetime τ_m is long compared to T_{2m}, the exchange is termed slow. W_p increases as the temperature increases since τ_m decreases as the temperature is raised. By contrast, when τ_m is short, relative to T_{2m}, the line width decreases as the temperature is raised since T_{2m} increases. The temperature variation of W_p is described fully by Sykes *et al.* (1970).

Equation 4 makes clear that a plot of $1/W_p$ *vs.* $[S]_0$ will be linear with intercept on the abscissa at $-K$. The only requirement in the use of such a plot is that the concentration range extend to values of $[S]_0$ which are equal to, or comparable with, the value of K. Thus, if the lowest value of $[S]_0$ were much larger than K, the intercept to which the data extrapolated would be indistinguishably different from zero.

Other contributions to the line broadening which must be eliminated, if eq 4 is to be used, include magnetic field inhomogeneity, viscosity, and the presence of paramagnetic species. Since the broadening due to field inhomogeneity is additive, and the same for every signal, it will be eliminated in computing W_p. Changes in the viscosity depend essentially only on changes of the enzyme concentration. We can determine whether the effect is significant by establishing whether W_p depends on substrate concentration as well. If W_p does, in fact, vary with substrate concentration, then the viscosity effect is small compared with the broadening due to specific molecular association.

The molecules employed in this study all involved an adenosine moiety, known to bind metal ions. Therefore, the presence of paramagnetic ions, which could be bound to the coenzyme or inhibitors, could result in spurious line broadening effects. The effects of paramagnetic ions on the nmr spectra of nucleotides and related compounds have recently been reviewed by Izatt *et al.* (1971). The effects of such impurities can be eliminated by appropriate purification techniques or by the addition of a suitable scavenger such as ethylenediaminetetraacetic acid (EDTA). That this method of eliminating effects due to paramagnetic ions was effective in the present study is shown in the values of T_{2m} obtained for the various substrates. If paramagnetic ions were contributing to the observed line broadening then the values to T_{2m} would have been considerably smaller than those actually observed.

Experimental Section

All chemicals used in this study were reagent grade. Yeast and liver alcohol dehydrogenases were purchased from Worthington Biochemical Corporation (Freehold, N. J.). AMP was obtained as the free acid monohydrate from Schwarz/Mann Biochemicals (Orangeburg, N. Y.). ADP, adenosine diphosphoribose, and reduced coenzyme (NADH) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Nicotinamide was obtained from Eastman Organic Chemicals (Rochester, N. Y.). Chelex-100 ion exchange resin (50–100 mesh) was obtained from Bio-Rad Laboratories (Richmond, Calif.). EDTA (tetrasodium salt) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Deuterium oxide (99.8% minimum isotopic purity) was obtained from Diaprep, Inc. (Atlanta, Ga.).

All samples were dissolved in 0.1 M phosphate buffer in D_2O at pD 7.7. The samples contained approximately 1×10^{-4} M EDTA or were treated with Chelex-100 resin to remove paramagnetic ions.

Temperature studies were limited to temperatures lower than 30° due to slow denaturation of the enzymes at higher temperatures.

Spectra were obtained with the Varian HA-100 high resolution nmr spectrometer equipped with a 15-in. magnet. Nmr tubes were 12 mm o.d. (Wilmad Glass Co., Inc., Buena, N. J.) and were fitted with Teflon vortex plugs to allow use of small sample volumes (3–5 ml). Spectra were recorded with the Varian C-1024 computer of average transients for purposes of enhancing the signal-to-noise ratio. The internal lock was residual water (HOD) in the D_2O solvent.

Results and Discussion

Effect of Paramagnetic Impurities. Line broadening due to the presence of paramagnetic impurities is demonstrated in Figure 1. The narrowing of the adenine H-8 proton signal of NADH on the addition of EDTA is consistent with other studies (Izatt *et al.*, 1971) showing that the N-7 of the adenine ring is a chelating site.

Figures 2 and 3 show typical effects of the presence of yeast and liver alcohol dehydrogenases, respectively, on the H-2 and H-8 signals of ADP and AMP. The effects of both enzymes on the H-2 and H-8 signals always appear to be similar. In most cases, therefore, we have studied only the H-2 signal.

Concentration Dependence of Line Widths. The data presented in Table I show that the presence of yeast alcohol dehydrogenase causes a broadening of the adenine H-2 signal of AMP, ADP, adenosine diphosphoribose, and NADH. The line broadening, W_p, depends upon inhibitor or coenzyme

TABLE I: Effect of Concentration on Line Broadening in the Presence of Yeast Alcohol Dehydrogenase.[a]

Species	$[S]_0$ (mM)	W_p (Hz)[b]	$\tau_m + T_{2m}$ (msec)[c]
AMP	6	2.3	9 ± 0
	10	1.5	9 ± 1
	15	1.1	8 ± 1
ADP	10	1.4	9 ± 1
	15	1.0	9 ± 1
Adenosine diphosphoribose	10	0.8	16 ± 1
	15	0.5	17 ± 3
NADH	10	0.6	22 ± 3

[a] Enzyme concentration 15 mg/ml. [b] Line broadening measurements made at 17.5°. [c] Limits based on an estimated uncertainty in W_p of 0.1 Hz.

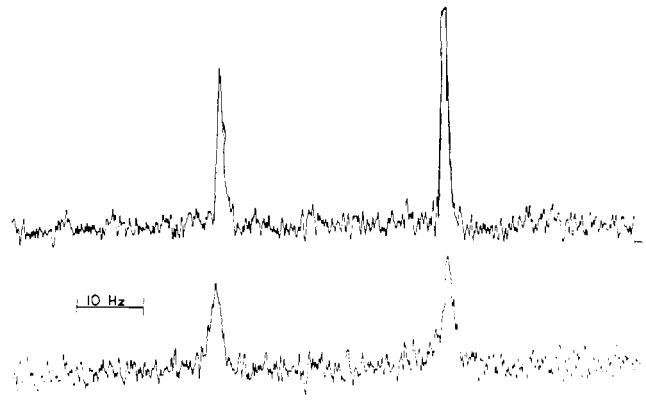

FIGURE 3: Effect of the addition of liver alcohol dehydrogenase on the nmr signals for the H-8 and H-2 protons (left and right, respectively) of 10 mM AMP in 0.1 M phosphate buffer, pD 7.7, at 17.5°. The upper trace is without enzyme. The lower trace is with 16 mg/ml of the liver enzyme.

concentration as well as enzyme concentration. Thus, viscosity effects are small. Moreover, additions of either bovine serum albumin or lysozyme cause only slight broadening of the nmr signals for the various protons of NADH (Hollis, 1967), again indicating negligible viscosity effects for such molecules.

Table II shows similar data for the liver enzyme. Again viscosity effects are small, since W_p depends on both substrate and enzyme concentrations. Presumably, for each enzyme, the adenine ring systems of the various species all bind at the same site on the protein. Hence H-2 is likely to be in similar environments regardless of the nature of the rest of the molecule. As a consequence the value T_{2m}, for each of those species bound to a given enzyme, should be similar. The relative values of τ_m and T_{2m} can be estimated, in each case, by measurements of W_p as the temperature is varied.

Values of $\tau_m + T_{2m}$ appear in Tables I and II. They have been calculated using eq 4. The value of $n = 4$ for yeast alcohol dehydrogenase was reported by Hayes and Velick (1954) and by Kägi and Vallee (1960). A value of $n = 2$ for liver alcohol dehydrogenase was used consistent with the work of Theorell and Bonnichsen (1951), Ehrenberg and Dalziel (1958), and Theorell and Yonetani (1963).

TABLE II: Effect of Concentration on Line Broadening in the Presence of Liver Alcohol Dehydrogenase.

Species	$[S]_0$ (mM)	$[E]_0$ (mg/ml)	W_p (Hz)[a]	$\tau_m + T_{2m}$ (msec)[b]
AMP	10	8	0.5	13 ± 3
	6	16	2.0	11 ± 1
	10	16	1.3	10 ± 1
	15	16	0.9	10 ± 1
ADP	6	8	1.0	11 ± 1
	10	8	0.5	13 ± 3
Adenosine diphosphoribose	6	8	0.6	17 ± 1
	10	8	0.2	30 ± 15
NADH	6	8	0.4	28 ± 7
	10	8	0.0	
	6	16	1.1	20 ± 2

[a] Line broadening measurements made at 17.5°. [b] Limits based on an estimated uncertainty in W_p of 0.1 Hz.

The data in Tables I and II clearly indicate a specific binding interaction. Whether the observed interaction involves the enzyme active site, however, remains to be shown. This might be done by measurement of the dissociation constant of the various molecules, using eq 4, and comparison of these values with the dissociation constants obtained from kinetic measurements. AMP, ADP, and adenosine diphosphoribose are known to be competive inhibitors of NAD^+ for the yeast enzyme (Hoch *et al.*, 1960; Wratten and Cleland, 1963; Anderson and Reynolds, 1965; Anderson *et al.*, 1965) and for the liver enzyme (Wratten and Cleland, 1963; Sigman, 1967; Plapp, 1969) as well. The dissociation constant for NADH bound to yeast alcohol dehydrogenase is known to be of the order of 10^{-5} M (Hayes and Velick, 1954; Nygaard and Theorell, 1955). This value is much too low to allow an accurate determination of the binding constant by the nmr method for reasons already discussed. We can, however, determine the dissociation constant of AMP binding to these enzymes and compare the results with values of K_i, the inhibition constant, for AMP, from kinetic studies (Anderson and Reynolds, 1965). The plot of line broadening data, according to eq 4, for AMP in the presence of yeast alcohol

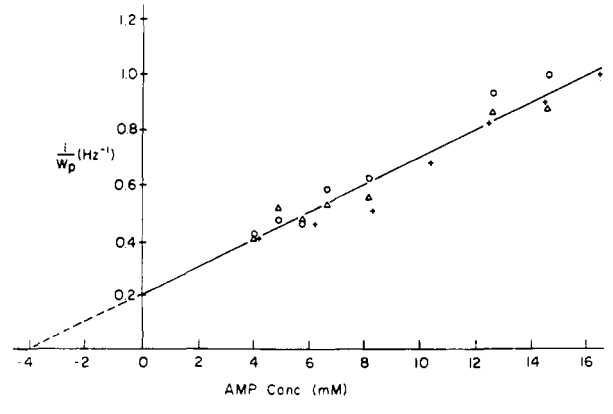

FIGURE 4: Concentration dependence of the reciprocal of the line broadening, W_p, of the adenine signals for AMP in 0.1 M phosphate buffer (pD 7.7) in the presence of yeast alcohol dehydrogenase: (+) H-2 at 17.7°; (○) H-2 and (△) H-8 at 18.3°.

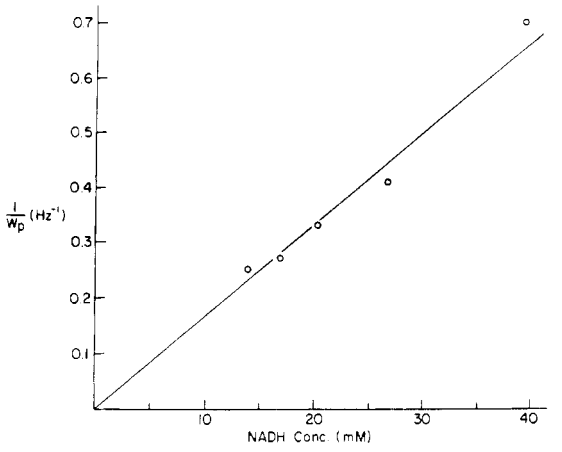

FIGURE 5: Concentration dependence of the reciprocal of the line broadening, W_p, of the adenine H-2 signal for NADH in 0.1 M phosphate buffer (pD 7.7, 28°) in the presence of yeast alcohol dehydrogenase. The enzyme concentration is 63 mg/ml.

TABLE III: Effect of Temperature on Line Broadening in the Presence of Yeast Alcohol Dehydrogenase.

Species	$[S]_0$ (mM)	Temp (°C)	W_p (Hz)
AMP[a]	10	7.3	2.4
		17.7	1.5
		27.8	1.1
ADP[a]	10	17.5	1.4
		27.3	1.3
Adenosine diphosphoribose	15	17.5	0.5
		27.3	0.5
NADH[b]	14	8.0	3.7
		17.4	4.1
		28.0	4.0

[a] Enzyme concentration 15 mg/ml. [b] Enzyme concentration 63 mg/ml.

dehydrogenase is shown in Figure 3. The data for the H-2 and H-8 protons give approximately the same results. Extrapolation of these data yields a value for the intercept of −4 mM. Thus the dissociation constant is 4×10^{-3} M at 18° which compares well with the value of 8.9×10^{-3} M at 25° obtained from kinetics (Anderson and Reynolds, 1965).

Figure 4 shows a plot of line broadening data for NADH at high enzyme concentration. In this case an extrapolation of the data to the abscissa gives an intercept not distinguishably different from zero. Thus, the dissociation constant is significantly smaller than approximately 5×10^{-3} M, a result consistent with the value of 10^{-5} M reported by Nygaard and Theorell (1955). Figure 5 is a plot of the line broadening, according to eq 4, for AMP in the presence of liver alcohol dehydrogenase. The extrapolation of the data intercepts the abscissa at a value indistinguishably different from zero. Thus the dissociation constant is smaller than about 2×10^{-3} M. The value for the dissociation constant for AMP bound to liver alcohol dehydrogenase is 1.4×10^{-4} M at 25° (Sigman, 1967). Our line broadening studies cannot reasonably be extended to concentrations of the order of $2\text{–}3 \times 10^{-4}$ M. Thus, despite the fact that this experiment produces no specific value for the dissociation constant, the results are consistent with the value obtained from kinetics, in the sense that K must be considerably less than 2×10^{-3} M.

Although there is no certainty that our equilibrium spectroscopic results measure binding to the same site as the enzyme kinetics studies, the consistency between the equilibrium constants obtained by the two methods lends credence to the tentative conclusion that we are detecting a dynamic process associated with the enzymatic catalysis.

Temperature Dependence of Line Widths. The data in Table III show that the line broadening decreases significantly for AMP in the presence of yeast alcohol dehydrogenase as the temperature is increased. According to our earlier discussion this is evidence of fast exchange. There appears to be some decrease of the line broadening with increasing temperature for ADP as well. This effect, however, is significantly less pronounced than for AMP. The data for adenosine diphosphoribose indicate that changes in the line broadening with temperature are insignificant. The data in Table III for NADH were carried out at high enzyme concentration in order to distinguish between a slight temperature dependence of W_p and temperature independence of that quantity. Even at large values of W_p an increase in temperature has very little effect, or results in a very slight line broadening. Thus, in the progression from AMP to NADH, τ_m is increasing relative to T_{2m}. The temperature dependences of W_p shown in Table III indicate that, for AMP, $\tau_m \ll T_{2m}$, while for NADH $\tau_m \geq T_{2m}$. Using the entry in Table I for NADH we have a value of $\tau_m + T_{2m}$ for this species. We can then apply the results of the temperature dependence as discussed above, concerning the approximate equality of τ_m and T_{2m}, to arrive at a value of τ_m of 11 msec for NADH. This result (17.3°) can be compared with the lifetime calculated from the turnover rate based on the activity of the enzyme (350 μmol per min per mg). This activity and an assumption of four sites per molecule (Hayes and Velick, 1954; Kägi and Vallee, 1960) lead to a value for the lifetime of approximately 4 msec at 25°. The somewhat longer lifetime measured at 17.3° is consistent with the activation energies associated with the catalysis reported by Müller-Hill and Wallenfels (1964). A study of the effect of deuterium substitution on the rate of catalysis has led Mahler and Douglas (1957) to suggest that the rate limiting step is the transformation of the ternary enzyme–coenzyme–substrate complex, and that the mechanism includes random binding–unbinding. However, Wratten and Cleland (1963) investigated the catalytic mechanism of this enzyme by product inhibition studies and concluded that the mechanism was ordered. These workers also suggested that the rate limiting step for yeast alcohol dehydrogenase is the ternary complex interconversion and/or the coenzyme dissociation. Silverstein and Boyer (1964) studied the catalysis by the yeast enzyme using equilibrium reaction rate measurements. They concluded that coenzyme dissociation is slow compared to ternary complex interconversion and that this dissociation is therefore rate limiting. They also suggested that the coenzyme dissociation becomes less important as the rate determining step, as substrate (acetaldehyde or ethanol) concentration is increased. This implies that in the limit of zero substrate concentration coenzyme dissociation is completely rate limiting. Therefore, the agreement of the bound lifetime for NADH in the absence of substrate, as measured by nmr, with the lifetime based on the turnover number, appears to be in accord with the analysis of Silverstein and Boyer. Note also that for AMP, since $\tau_m \ll$

T_{2m}, we can state that $T_{2m} \approx 9$ msec, whereas for NADH $T_{2m} \approx \tau_m \approx 11$ msec. This is consistent with our earlier remarks concerning the likelihood of similar environments for the adenine ring regardless of the nature of the remainder of the molecule. The data in Table IV show the temperature dependence of the line broadening for the various species in the presence of liver alcohol dehydrogenase. It appears that, in this case, each of the substrates shows some slight decrease in line broadening effects as the temperature is increased. No increases in line broadening with increasing temperature are observed, thus ruling out slow exchange. Since W_p does not show pronounced decreases with increases in temperature, the exchange rate for these molecules must be considered intermediate to fast, hence $\tau_m \leq T_{2m}$. For AMP $\tau_m \leq 5$–7 msec and T_{2m} is also approximately in this range. For NADH we have $\tau_m \leq 10$–14 msec and T_{2m} is in this range as well. Thus the difference in the lifetimes of AMP and NADH is much less pronounced in the case of liver than that found for yeast alcohol dehydrogenase. This could be interpreted to indicate that the second phosphate and ribose and the reduced nicotinamide ring contribute to holding the coenzyme to the enzyme in the case of yeast alcohol dehydrogenase. However, in the case of the liver enzyme the adenine nucleotide portion of the molecule is the main factor in determining the lifetime. Moreover, the activity of the enzyme is approximately 2 μmol mg^{-1} min^{-1} from which we calculate a lifetime for NADH of 360 msec at 25°. Unlike the results for NADH and yeast alcohol dehydrogenase, the nmr and kinetic measurements for NADH and liver alcohol dehydrogenase clearly do not yield congruent values of the bound lifetime. The nmr data reflect an unbinding process much faster than the rate-limiting step in the overall mechanism of reduction of NAD$^+$ to NADH by liver alcohol dehydrogenase. Theorell and Chance (1951) concluded from their fast kinetic study of the liver enzyme–NADH interaction that the dissociation of the liver alcohol dehydrogenase–NADH complex is the slow step in the reaction mechanism. More recent values of the rate constant for the dissociation of NADH from liver alcohol dehydrogenase have been reported. Shore (1969) found that, in the absence of substrate, $k_{off} = 1.7$ sec^{-1} (pH 7.0, 0.1 μ of phosphate buffer, 8°) by stopped flow measurements. Since $\tau_m = k_{off}^{-1}$, this corresponds to a lifetime for NADH bound to the protein of 590 msec. Shore and Gutfreund (1970) reported a value of 2.8–3.2 sec^{-1} for k_{off} (pH 7.0, 0.1 μ of phosphate buffer, 23°) corresponding to a lifetime of approximately 330 msec. Thus, even when measured directly, the average lifetime is significantly longer than that measured by nmr line broadening. The difference in pH (or pD) and phosphate ionic strength between the present studies and those of Shore (1969) and Shore and Gutfreund (1970) cannot account for the differing results. Theorell *et al.* (1955) have shown that the rate of NADH unbinding is nearly independent of pH above pH 7. Moreover, Theorell and Winer (1959) have shown that a fourfold increase in phosphate ionic strength results in an increase of the rate by a factor of approximately 1.5. Thus, under the conditions used in the present study the ionic strength of approximately 0.2 μ would result in an increase in the rate by a factor of 1.25 above that measured in the studies by the stopped-flow technique. Such an effect cannot be the cause of the much faster unbinding rate measured in this study.

There is an additional mechanism by which magnetically relaxed substrate could be carried into the bulk phase and thus contribute to line broadening. The mechanism is a nonproductive bimolecular displacement, in addition to the monomolecular dissociation, *i.e.*

TABLE IV: Effect of Temperature on Line Broadening in the Presence of Liver Alcohol Dehydrogenase.

Species	$[S]_0$ (mM)	$[E]_0$ (mg/ml)	Temp (°C)	W_p (Hz)
AMP	10	16	17.5	1.3
			25.5	1.2
ADP	10	8	7.5	0.9
			17.5	0.6
			27.8	0.5
Adenosine diphosphoribose	10	8	8.8	0.3
			17.5	0.2
			24.7	0.0
NADH	6	8	17.5	0.4
			26.7	0.3
	6	16	17.5	1.1
			24.6	0.8

$$E\cdot S \xrightarrow{k_{-1}} E + S$$
$$+$$
$$S^* \xrightarrow{k_b} E\cdot S^* + S$$

If k_{-1} represents a slow step the line broadening would occur only if the bimolecular displacement were sufficiently fast. The rate expression for the fast step in this mechanism would be rate(off) = $k_b[E\cdot S][S]_0$. The term τ_m would then be the reciprocal of a pseudo-first-order rate constant since our usage assumes the off rate to be unimolecular, *i.e.*, rate(off) = $k_{-1}[E\cdot S]$. Hence, $k_{-1} = k_b[S]_0$ or $\tau_m = (k_b[S]_0)^{-1}$. The value of τ_m for AMP binding to liver alcohol dehydrogenase (Table II) is approximately 3–5 $\times 10^{-3}$ sec, when [AMP] = 10^{-2} M and thus $k_b \approx 2$–3×10^4 sec^{-1} mol^{-1}. In the event that the bimolecular process is important, eq 4 becomes

$$\frac{1}{W_p} = \frac{\pi T_{2m}}{n[E]_0}\left(\frac{1}{k_b T_{2m}} + [S]_0\right)$$

$$[S]_0 \gg K$$

Thus, a plot of $1/W_p$ *vs.* $[S]_0$ would give an intercept on the abscissa of $-1/k_bT_{2m}$. Since our values of T_{2m} are of the order of 10^{-2} sec the intercept should be at 3–5 mM, a value readily detectable as different from zero. Yet the data shown in Figure 5 for AMP and liver alcohol dehydrogenase give a value indistinguishably different than zero. Thus, for AMP, the data appear to rule out the bimolecular process. This certainly implies that such a process is also not operable in the case of NADH since, for the coenzyme, there would be a further increase in an already unfavorable entropy of activation, due to the more complex structure of the coenzyme. That this second method of unbinding does not occur is shown by the work of Shore and Gutfreund (1970) which included a study of the rate of unbinding of NADH from liver alcohol dehydrogenase in the presence of NAD$^+$. The latter species presumably rapidly occupied the sites vacated by the reduced coenzyme. If a biomolecular process were operative in producing a high rate of unbinding of NADH it should have been observed in such a study. Since NAD$^+$ does not rapidly and bimolecularly replace NADH we believe this

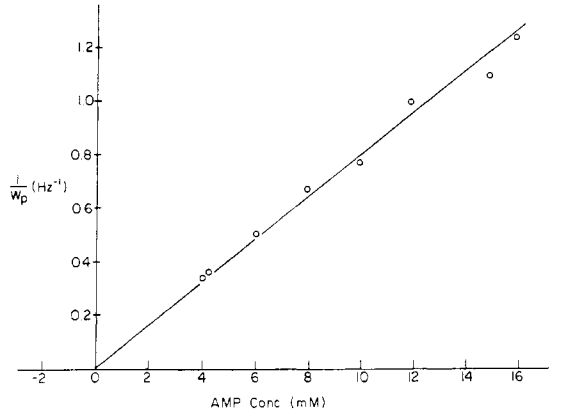

FIGURE 6: Concentration dependence of the reciprocal of the line broadening, W_p, of the adenine H-2 signal for AMP in 0.1 M phosphate buffer (pD 7.7, 17.5°) in the presence of liver alcohol dehydrogenase. Enzyme concentration is 16 mg/ml.

constitutes strong evidence that free NADH itself cannot rapidly replace bound NADH in a bimolecular process. We therefore need only consider the monomolecular unbinding process. A fuller discussion of the case of bimolecular displacements has been given by Navon *et al.* (1970). Shore and Theorell (1967), Shore (1969), and Shore and Brooks (1971) have studied the association and dissociation rates of the 3-acetylpyridine analog of NAD^+–NADH, with liver alcohol dehydrogenase. Shore and Brooks (1971) found that the reduced form of the analog dissociated, in the presence of excess adenosine phosphoribose, with a first-order rate constant of 100 sec^{-1} (τ_m = 10 msec), while Shore and Gutfreund (1970) and Shore (1969) found the dissociation rate constant for NADH to be 3 sec^{-1}. These workers also pointed out that the dissociation rate for the reduced 3-acetylpyridine analog agrees with that obtained by Dalziel (1963) for NAD^+. This could imply that the observed rate constant is for the dissociation of the adenine ring. The nmr data reported in the present work give direct evidence for a dissociation rate constant of 100 sec^{-1}. However, in this connection Shore (1969) has reported that the binding of the adenine group to liver alcohol dehydrogenase takes place prior to the binding of the nicotinamide ring, and has suggested different bound lifetimes for the two ends of the coenzyme. In the present study the nmr results are those for the adenine ring.

The signal for the C-2 proton of the reduced nicotinamide ring of NADH is visible in the nmr spectrum; however, in the absence of enzyme, its width is too large for a single proton with no very near neighbors. This can only be due to long-range spin–spin coupling with the two protons at C-4 and the single proton at C-6. Consequently a reliable line width cannot be determined and quantitative studies have therefore not been possible. A previous report of one of the present authors (Hollis, 1967) showed a distinct effect on the nmr signal for the C-2 proton resulting from an addition of liver alcohol dehydrogenase to the solution of reduced coenzyme. A more recent study (Sarma and Woronick, 1972) has reported similar spectra and shown the effect to clearly be line broadening. This result means that the upper limit on $\tau_m + T_{2m}$, and hence on τ_m, for the nicotinamide moiety of NADH is similar to that reported here for the adenine ring system. These observations indicate that both nicotinamide and adenine rings are exchanging at a rate greater than that previously measured by optical means. While such a comparison does not give information regarding the order of binding, it is significant to note that the data in Table IV show that the adenine lifetime is not independent of the rest of the molecule. Such an observation is consistent with binding which is simultaneous, or very nearly so. The more significant statement is that the unbinding of NADH as measured by nmr is at a very much higher rate than that previously measured. Apparently, the nmr measurement is sensing a particular step in the monomolecular unbinding process. This implies that the binding of NADH to liver alcohol dehydrogenase is a process of at least two steps as shown in the following scheme

$$\text{liver alcohol dehydrogenase} + \text{NADH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$\text{liver alcohol dehydrogenase}\cdot\text{NADH} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$$

$$(\text{liver alcohol dehydrogenase}\cdot\text{NADH})^*$$

In this scheme the step corresponding to k_{-2} is the slow step which produces the shift in the 340-mμ absorption of NADH, used by Theorell and Chance (1955). Presumably this step also produces the fluorescence changes used by various workers to study the NADH–liver alcohol dehydrogenase binding directly. The dissociation involving k_{-1} is a hidden step with respect to optical spectroscopic studies reported so far, but is visible to the nmr technique. Since the line broadening measurements are sensitive to the rate at which the bound inhibitor is carried into the bulk phase we suggest that our measured lifetime gives a value for k_{-1} of 100 sec^{-1}. Thus this rate is much too large to be the rate limiting step measured by earlier workers (Theorell and Chance, 1951; Shore, 1969; Shore and Gutfreund, 1970). It is not possible to say with any certainty to what process the second step corresponds. However, since the second step is slow it may well correspond to a sluggish change in the conformation of the protein (Theorell, 1968; Luisi and Favilla, 1970, 1972; Bernhard *et al.*, 1970; Brändén *et al.*, 1970).

Dalziel (1963) has reported the activation parameters for the kinetics by fluorescence measurements and gives a value of 4.8 kcal/mol^{-1} for the enthalpy of activation of the unbinding process. This would lead to the expectation that the line broadening for the NADH–liver alcohol dehydrogenase system would be more sensitive to temperature than is observed. This temperature insensitivity suggests that the process which contributes to the nmr line broadening is not the same process to which Dalziel (1963) referred.

The lack of an optical spectroscopic change to accompany the mechanistic step reported here may be due to the incomplete immobilization of the nicotinamide ring of NADH on the enzyme. Since the molecular correlation time which is most relevant to nmr relaxation is the rotational correlation time, it would be possible for the nicotinamide ring to become detached from the enzyme, but held in place by the adjoining group, the phosphoribose moiety. It could, in such a situation, gain vibrational freedom, while retaining the same rotational time as the protein itself. Its continued proximity to the enzyme would provide for relaxation of the magnetic nuclei and would be sensed by the nmr but not by observation of its optical chromophore.

In summary, the results reported in this work, in terms of the broadening of nmr lines of coenzyme and inhibitors binding to proteins, have yielded estimates of the lifetimes of those species in the bound state. In the case of yeast alcohol dehydrogenase the nmr data lead to a lifetime for the reduced

coenzyme which is in satisfactory agreement with the value calculated from the known activity of the enzyme. This observation is consistent with the view that the mechanism for yeast enzyme is ordered and that, in the limit of vanishing substrate concentration, the dissociation of reduced coenzyme is rate limiting. By contrast the rate constant for the unbinding of the coenzyme from its complex with liver alcohol dehydrogenase was found, by nmr, to be considerably greater than in previous investigations by techniques using optical spectroscopy. The nmr data also indicate that the adenine and nicotinamide moieties of NADH undergo this process with similar rate constants. Since the nmr technique detects the carrying of relaxation information from the bound state into the free solution phase we have proposed that the nmr detects the true unbinding process. It follows that previous investigators have studied a different step in the mechanism of action of this enzyme, possibly a conformational change of the enzyme–coenzyme complex, which involves a measurable change in the chromophore of the coenzyme. No such chromophoric variation appears to accompany the process which is sensed by the magnetic resonance method, since this step has failed to be observed in previous studies. It seems likely that the chromophorically measured slow step directly precedes the actual unbinding. It is interesting to note that Lanir and Navon (1971) have reported a study using nmr line broadening to investigate the binding of various sulfonamide inhibitors to bovine carbonic anhydrase. They found the bound lifetimes of such inhibitors to be much shorter than those calculated from studies done by other methods.

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