A Nuclear Magnetic Resonance Study of Substrate Binding by Alcohol Dehydrogenases*

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ABSTRACT: The effect of the enzymes yeast alcohol dehydrogenase (YADH) and equine liver alcohol dehydrogenase (LADH) on the nuclear magnetic resonance (nmr) spectra of oxidized nicotinamide–adenine dinucleotide (NAD^+), reduced nicotinamide–adenine dinucleotide (NADH), ethanol, and acetaldehyde are reported. The resonances of the adenine C-2 and C-8 protons of NAD^+ and NADH are broadened in the presence of YADH. For NAD^+ the nicotinamide proton resonances are not observably broadened. The C-2 proton peak of NADH is not broadened in the presence of YADH but the C-4 proton peak is strongly affected by either broadening or by an intensity decrease or both. LADH produces an apparent intensity decrease in the nmr spectra of both the nicotinamide and adenine moieties of NAD^+ and NADH. These results suggest a preferential interaction of YADH with the adenine moiety of NAD^+ and NADH in the absence of substrate and are consistent with relatively rapid turnover of the coenzymes. The effect of LADH suggests binding of both the nicotinamide and adenine moieties to the enzyme with relatively slow turnover. The sharp methyl group spectra of neither ethanol nor acetaldehyde are noticeably affected in the absence of coenzyme by either enzyme at relative concentrations sufficient to bind a few per cent of the substrate present. These results appear to eliminate the possibility of rigid binding of substrates with rapid turnover and suggest that the presence of coenzyme is required for binding of the substrate in the rigid mode required to account for the stereospecificity of the enzymes. Other possible interpretations of the results are discussed.

The potential utility of high-resolution nuclear magnetic resonance (nmr) as a method for the study of the binding of small molecules by macromolecules is well recognized, and a review of the subject has appeared (Jardetzky, 1964). Briefly, the basis of the method is that line widths in nmr spectra are sensitive to the degree and kinds of molecular motion present in the sample. Increased line widths are generally associated with diminished rates of molecular motion, particularly rotational motion. When a small molecule is bound to a larger one increased line widths may be observed in its nmr spectrum since the motion of the bound molecule may be restricted in the complex.

Zimmerman and Brittin (1957) have developed a theory dealing with the case in which a small molecule exists in equilibrium between two or more phases (*i.e.*, bound and free) having different characteristic relaxation times and hence different line widths. It is predicted that the observed line widths depend on the rate of exchange of molecules between the bound and free phases as well as on the equilibrium binding constant. Particularly simple behavior is predicted for two limiting cases in which the average lifetimes of a molecule in the two states are either very long or very short compared to the corresponding relaxation times. For the slow-exchange case

$$\left(\frac{1}{T}\right)_{\text{exchange}} < \left(\frac{1}{T_2}\right)_{\text{free}} < \left(\frac{1}{T_2}\right)_{\text{bound}} \qquad \text{(case I)}$$

where T is the average lifetime of a molecule in either the free or bound states and $(1/T_2)_{\text{free}}$ and $(1/T_2)_{\text{bound}}$ are the relaxation rates of protons in the free and bound phases, respectively, molecules in the two phases relax independently and two separate relaxation times should be experimentally observable. Thus, the nmr spectrum should show a broad line and a narrow line corresponding to the bound and free phases, respectively. These lines need not be superimposed since a chemical shift difference may exist between the bound and free molecules. The integrated intensity of each line will be proportional to the number of molecules in the corresponding phase.

For the fast-exchange case (which we label case III to follow Fischer and Jardetzky, 1965)

$$\left(\frac{1}{T_2}\right)_{\text{free}} < \left(\frac{1}{T_2}\right)_{\text{bound}} < \left(\frac{1}{T}\right)_{\text{exchange}} \qquad \text{(case III)}$$

only a single, average relaxation time can be observed

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which is given by

$$\frac{1}{T_{av}} = \frac{P_F}{T_{2F}} + \frac{P_B}{T_{2B}} \quad (1)$$

where P_F and P_B are the fractions of the molecules in the free and bound phases and T_{2F} and T_{2B} are the corresponding relaxation times. The nmr spectrum will then show only one line whose width will be intermediate between those corresponding to the free and bound species and whose integrated intensity will represent the total concentration of free and bound molecules.

For the case in which the exchange rate is intermediate between the relaxation rates of the two phases

$$\left(\frac{1}{T_2}\right)_{free} < \left(\frac{1}{T}\right)_{exchange} < \left(\frac{1}{T}\right)_{bound} \quad \text{(case II)}$$

the nuclei in the bound phase will relax normally but the relaxation rate of the free molecules will be influenced by the exchange process. If most of the molecules are free the relaxation rate of the free phase will be determined by the exchange rate (Fisher and Jardetzky, 1965).

It is possible that the nuclei located in different portions of a complex molecule may experience differential changes in relaxation rate upon binding depending on the extent of involvement of the various portions of the molecule in the binding mechanism. Several examples of such "selective broadening" have been reported. Fischer and Jardetzky (1965) have thoroughly studied the interaction of penicillin and bovine serum albumin by this technique and several other examples are given in Jardetzky's review article. In all cases so far reported case III behavior (*i.e.*, rapid exchange) has best accounted for the experimental results.

It seems reasonable to expect that useful information might be obtained concerning the binding of substrates to enzymes by studying the effects of binding on the nmr spectra of the substrates. To our knowledge only a single brief report of such a study has appeared in the literature, this being a study of the effect of YADH[1] on the nmr spectrum of NAD^+ (Jardetzky *et al.*, 1963). In this study a time-averaging technique was used to partially overcome the inherent lack of sensitivity which has been a major factor in preventing the use of nmr in such studies. The availability in this laboratory of more advanced nmr and time-averaging equipment than that used by Jardetzky *et al.*, as well as an interest in the comparative properties of the alcohol dehydrogenases of yeast and liver, have prompted the initial study, to be reported here, of the effect of these two enzymes on the nmr spectra of their coenzymes and substrates.

[1] Abbreviations used: YADH, yeast alcohol dehydrogenase; LADH, liver alcohol dehydrogenase; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotides; TMS, tetramethylsilane; BSA, bovine serum albumin.

Materials and Methods

LADH and YADH were obtained as crystalline suspensions from Sigma Chemical Co. or Boeringer & Sohne and were dialyzed for 48 hr at 1° *vs.* 0.1 M phosphate buffer (pH 7.5). The resulting solutions were centrifuged to remove a small amount of insoluble material and then lyophylized.

NAD^+ and NADH (both grade III) were obtained from Sigma Chemical Co. (the former as the free acid and the latter as the disodium salt) and were used without further purification. The concentration of NAD^+ and NADH in approximately 0.05 M stock solutions in D_2O containing 0.1 M phosphate buffer at the desired pH was assayed by comparing the intensity of their nmr spectra to that of a standard 0.05 M sodium benzoate solution in the same solvent using identical instrumental conditions. The concentration of the ethanol and acetaldehyde solutions employed was similarly determined. The stock solutions were diluted by standard volumetric techniques to give solutions of the desired concentrations.

Methods

All nmr spectra were obtained using the Varian HA100 nmr spectrometer coupled to a Varian C-1024 time-averaging computer. The spectrometer was equipped with a Varian Auto Shim homogeneity controller which ensured maintenance of high-resolution conditions over the time required to accumulate the repeated spectra. The probe temperature remained constant at 30 + 2° throughout all experiments. The resulting solutions were analyzed for protein and specific activity according to the methods presented by Worthington Biochemical Corp., Freehold, N. J., in their 1966 catalog. The YADH preparations had specific activities of 170–180 μmoles/min mg, the LADH preparations 1.6–1.8 μmoles/min mg. The YADH activity decreased by as much as 20% during the course of some experiments but that of LADH was unaffected.

In a typical experiment 0.5 ml of a coenzyme or substrate solution of the desired concentration in buffered D_2O (0.1 M phosphate) was added to the required weight of the enzyme preparation and the resulting solution was placed in a standard nmr tube. A fine capillary tube containing TMS was placed in the tube to serve as a reference signal to which the spectrometer was "locked." After accumulating the required number of repeated spectra, the enzyme solution was replaced by the coenzyme or substrate solution containing no enzyme and the experiment was repeated exactly using identical instrumental settings. The apparent width of the peak arising from residual HDO in the sample was used as an index of resolution. The width of this peak was about 1–2 cycles/sec and was unaffected by the presence of the enzyme. This is an important fact since it eliminates any complications

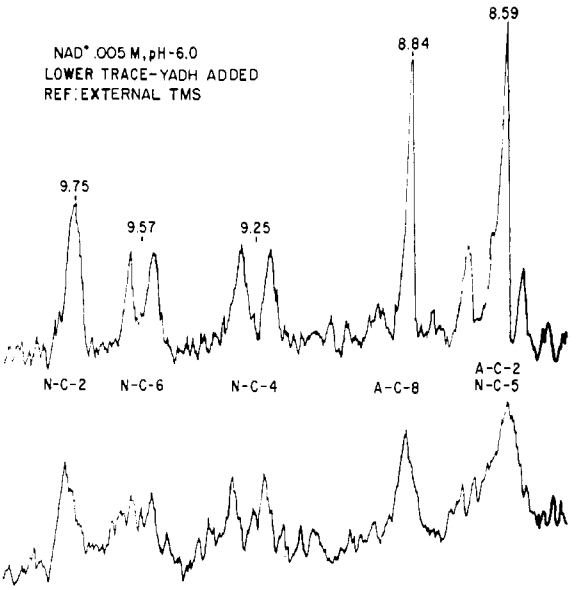

FIGURE 1: Nmr spectrum (100 Mcycles/sec) of 1.3 mM NAD^+ in D_2O (pH 6.0). Upper trace recorded in absence of enzyme; lower trace recorded in the presence of 0.6 mM YADH. The two spectra were obtained under identical instrumental conditions.

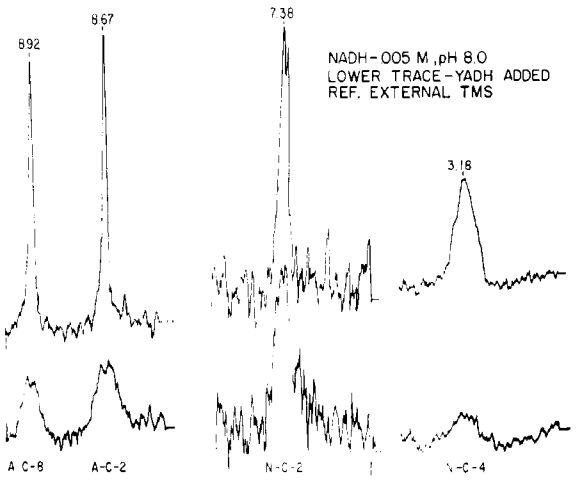

FIGURE 2: Nmr spectrum (100 Mcycles/sec) of 5 mM NADH in D_2O (pH 8.0). Upper trace recorded in absence of enzyme; lower trace recorded in the presence of 0.67 mM YADH. The two spectra were obtained under identical instrumental conditions.

due to the possible introduction of paramagnetic ions with the enzymes since these would strongly affect the width of the HDO peak (Jardetzky, 1964).

A sweep rate of two cycles per second per second was used for all spectra reported here. About 50 repetitive scans were found to give a useable spectrum on the 5 mM coenzyme solutions used. Since a single scan including flyback required 35 sec for a fifty cycle-per-second width such a segment of the spectrum could be obtained in about 30 min.

The NAD^+ and NADH samples were assayed by measurement of the ultraviolet absorption at 340 mμ, the former after reduction by excess ethanol in the presence of YADH. Both samples were nearly 98% pure without correcting for water contained in the crystalline samples. Examination of the nmr spectra of concentrated solutions in the region of 8–10 ppm below external TMS revealed no suggestion of impurities such as, for example, adenosine monophosphate. It is estimated that less than 0.5% of such impurities was present.

Results

Effect of YADH or the Nmr of NAD^+ and NADH. Figure 1 shows a portion of the 100-Mcycles/sec proton nmr spectrum of 5 mM solution of NAD^+ in D_2O at pH 6.0. The lower trace resulted when sufficient YADH was added to give a 0.67 mM concentration of binding sites based on a molecular weight of 150,000 and four sites per YADH molecule. Since the dissociation constant of the YADH–NAD^+ complex is about 10^{-4} M at pH 6.0 (Nygaard and Theorell, 1955; Hayes and Velick, 1954) about 13% of the NAD^+ should be bound at equilibrium.

The assignment of the NAD^+ spectrum as given in the figure has been made previously (Jardetzky *et al.*, 1963). The peaks are labeled A or N for protons of the adenine and nicotinamide moieties, respectively. The chemical shifts are in parts per million relative to external TMS.

First of all it should be noted that all of the peaks corresponding to the protons of the adenine and nicotinamide moieties are still detectable after addition of the enzyme, except that of N-C-5 which is probably partly obscured by the broadening of A-C-2. Furthermore, no drastic broadening of the nicotinamide portion of the spectrum relative to that of the adenine portion is evident, contrary to the findings of Jarketzky *et al.* (1963). In fact, the adenine resonances have apparently been broadened relatively more than those of the nicotinamide which, within experimental error, are not affected. The widths of all the observed peaks are similar and may be identical within experimental error. This same experiment was also carried out at pH 7.2 in order to more closely approximate the conditions used by Jardetzky *et al.* At this pH, however, a fairly rapid decomposition of the NAD^+ by the enzyme was observed. The peaks corresponding to the nicotinamide moiety moved upfield to a position corresponding to free nicotinamide while the peaks of the adenine portion were no longer prominent. Also, a four-line pattern corresponding to the C-5 proton of nicotinamide appeared at 7.95 ppm. By sweeping over a narrower region (50 cycles/sec) it was possible to observe the various NAD^+ resonances prior to decomposition. The result was similar to that at pH 6.0 in that the nicotinamide protons are only slightly broadened while

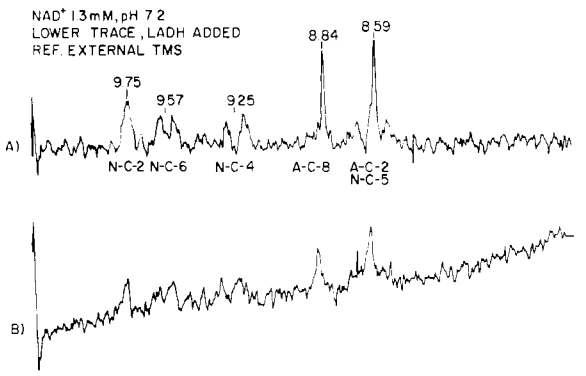

FIGURE 3: Proton nmr spectrum (100 Mcycles/sec) of 1.3 mM NAD^+ in D_2O (pH 7.2). Trace A recorded in absence of enzyme; trace B recorded in the presence of 0.6 mM LADH. Traces A and B were recorded under identical instrumental conditions.

the adenine peaks are strongly affected. Decomposition of NAD^+ also occurs at pH 6.0 but is considerably slower then at pH 7.2. This activity was found in all YADH preparation studied including several lots from Sigma Chemical Co. and Boeringer & Sons. Decomposition of NAD^+ to unknown products in the presence of high YADH concentrations has been observed previously by Burton and Wilson (1953) but not by Hayes and Velick (1954).

The effect of YADH on the nmr of NADH could be studied with less difficulty since the NADH was stable for at least 1 or 2 days in the presence of YADH concentrations similar to those causing decomposition of NAD^+. Figure 2 shows the result of an experiment identical with the one described above for NAD^+ except that 5 mM NADH was substituted for NAD^+ and the solution was buffered at pH 8.0. The lower trace again shows the effect of adding YADH to a concentration of 0.67 mM in binding sites. Thus about 13% of the NADH should be bound since the dissociation constant is about 10^{-5} M (Hayes and Velick, 1954). The peak assignments in the NADH spectrum are evident on inspection and have been given previously by Meyer *et al.* (1962). As in the case of NAD^+ the adenine protons are noticeably broadened while the N-C-2 peak may be slightly broadened. The N-C-4 peak seems to be affected most strongly but it is not possible to tell from this spectrum whether line broadening or an intensity decrease or both are responsible for the observed change. The initial width of the N-C-4 resonance is about 8 cycles/sec compared to 3 and 1.5 cycles/sec for N-C-2 and the adenine protons, respectively.

To determine whether the observed effects of YADH on the coenzyme nmr spectra might be a general effect common to all proteins, the above experiments were repeated using the same mass of BSA and lysozyme in place of the alcohol dehydrogenase. Only slight changes in the coenzyme spectra were observed with either protein.

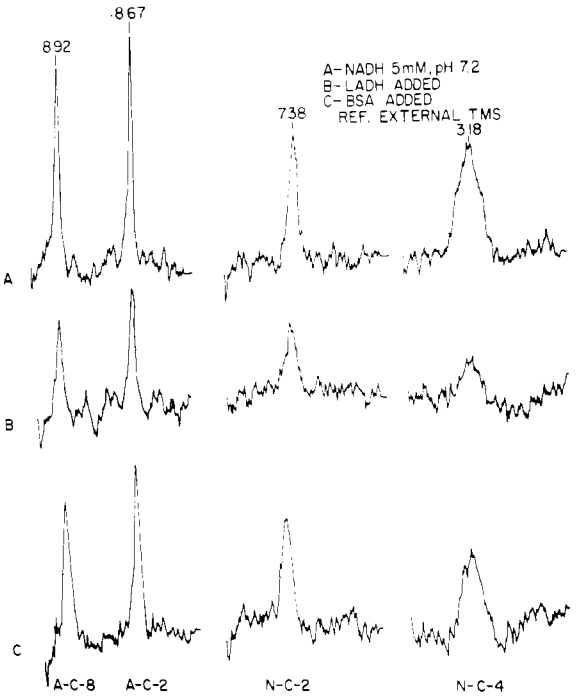

FIGURE 4: Proton nmr spectrum (100 Mcycles/sec) of 5 mM NADH in D_2O (pH 7.2). Trace A was recorded in the absence of enzyme. Trace B was recorded in the presence of 0.52 mM LADH. Trace C was recorded in the presence of the same mass of BSA in place of the LADH.

Effect of LADH on the Nmr of NAD^+ and NADH. Figure 3 shows the effect of LADH on the proton nmr spectrum of NAD^+. The upper trace shows the spectrum of a 1.3 mM solution of NAD^+ in 0.1 M phosphate buffer (pH 7.2). The lower trace is the spectrum of a 1.3 mM NAD^+, also containing 0.6 mM LADH binding sites assuming a molecular weight of 84,000 and two binding sites per molecule. Since the dissociation constant is about 0.2 mM (Sund and Theorell, 1963) about 40% of the NAD^+ should be bound. Although the two spectra were obtained under identical instrumental conditions, the signal-to-noise ratio is markedly reduced after addition of the enzyme. This reduced signal-to-noise ratio could arise from a broadening of the spectrum or from an intensity decrease or a combination of the two. The N-C-2 peak is the only nicotinamide peak which is sufficiently intense to allow a rough estimate of the line width. In the spectrum shown, the N-C-4 peak appears slightly sharper after addition of the enzyme. This is undoubtedly the result of experimental error in the line-width measurement. Examination of several similar spectra suggests that within an error of about $\pm 20\%$ the line width of the N-C-4 peak does not change on addition of LADH. Since the peak intensity decreases by 40–50% in these experiments the observed signal-to-noise decrease is probably due to a decrease in integrated intensity of the peak. Similarly, planimeter integration of the adenine reso-

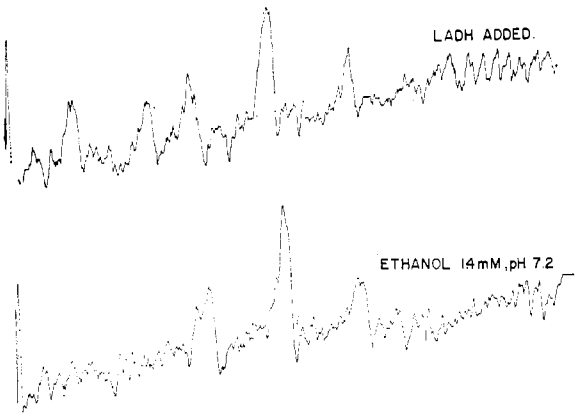

FIGURE 5: Effect of LADH on the nmr of ethanol. Lower trace shows the methyl resonance of a 1.4 mM ethanol solution in D_2O (pH 7.2). The upper trace illustrates the effect of 0.6 mM LADH. The origin of the two peaks to the left of the ethanol triplet is not known.

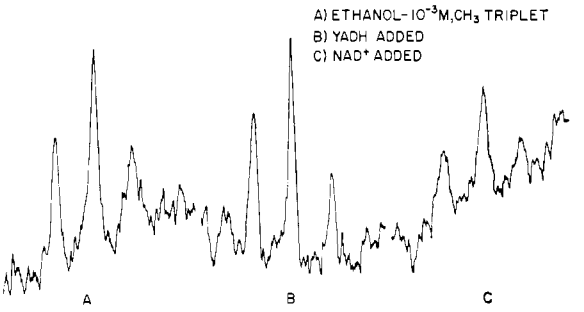

FIGURE 6: Effect of YADH on the nmr of ethanol. Trace A: Methyl resonance of 1 mM ethanol in D_2O (pH 7.2.). Trace B: Same as trace A except that solution contains 1.1 mM YADH. Trace C: Same as trace B except that 2/μmoles of NAD^+ was added.

nances in several spectra suggests an intensity decrease for these peaks. From Figure 3, however, it can be seen that the adenine peaks do appear somewhat broader in the presence of the enzyme.

Figure 4 shows the effect of LADH on the nmr spectrum of NADH. A 5 mM solution of NADH gave trace A while trace B resulted when sufficient LADH was added to produce a 0.52 mM concentration of binding sites. About 10% of the NADH should be bound since the dissociation constant is about 3×10^{-7} M (Sund and Theorell, 1963). As in the case of NAD^+, an intensity decrease has occurred in the presence of the enzyme. Trace C of Figure 5 shows the effect of adding an equal mass of BSA in place of the YADH. A small but definite increase in the line width of the adenine peaks has occurred on addition of LADH. A similar broadening occurred on addition of BSA but the latter did not produce a noticeable intensity decrease.

Effect of LADH and YADH on the Nmr of Ethanol and Acetaldehyde. The methyl resonance of ethanol has been examined in the presence of both LADH and YADH. The CH_2 group could not be observed because of interference by residual H_2O in the sample. No effect of enzyme on the spectrum has been observed in either case.

Figure 5 shows the methyl triplet from 1.4 mM ethanol. The upper trace was obtained under identical conditions except that the solution contained 0.6 mM LADH. Within experimental error the enzyme has no effect on either the line width or the intensity of the spectrum.

The results of a similar experiment using YADH rather than LADH is shown in Figure 6. Here trace A is the methyl resonance of a 1.4 mM ethanol solution, while trace B shows the same solution after YADH was added to give a 1.1 mM solution of binding sites assuming a molecular weight of 150,000 and four binding sites per YADH molecule. Here again the presence of the enzyme has no appreciable influence on the spectrum.

Figure 6C resulted when 2 μmoles of crystalline NAD^+ was added to the solution giving trace 7B, giving an NAD^+ concentration of 4 mM. A slight broadening of the spectrum as well as an approximately 40% intensity decrease has occurred on addition of the NAD^+. When only one equivalent of NAD^+ was added the intensity of the methyl resonance showed a considerably smaller decrease. When the same mass of BSA or lysozyme was substituted for YADH, no intensity decrease was observed in either case.

In the case of acetaldehyde, it was not possible to determine the effect of LADH because rapid dismutation of the aldehyde to acetic acid and ethanol occurred even in the absence of added NAD^+ or NADH. The formation of 1 mole of acetic acid and 1 mole of ethanol per mole of acetaldehyde was readily observed from the nmr spectrum. Presumably, this reaction results from the presence of a catalytic amount of NAD^+ or NADH in the solution although neither was deliberately added. On the other hand, this result may indicate a direct dismutation of acetaldehyde without the participation of the coenzyme. This question requires further study.

YADH caused no noticeable broadening of the methyl resonances of either the aldehyde or hydrated forms of acetaldehyde at concentrations of about 1 mM acetaldehyde and 1 mM YADH. However, the intensity of the acetaldehyde spectrum decreased with time in the presence of the enzyme. When an excess of enzyme was added considerable broadening was observed, but it did not seem to be due to a specific interaction since BSA produced about the same result.

Discussion

In the following discussion an attempt will be made to interpret the effects of LADH and YADH on the nmr spectra of the coenzymes in terms of the three

cases discussed above. The coenzyme is assumed to exist in only two states (*i.e.*, bound and free) characterized by two corresponding relaxation times. This is not meant to imply that only one mode of binding exists but only that the relaxation behavior for all bound species is approximately the same.

NAD^+–YADH. The present results on the effect of YADH on the nmr spectrum of NAD^+ are not in agreement with those obtained by Jardetzky *et al.* (1963). At both pH 6.0 and 7.2 we have found that all of the nmr peaks corresponding to the protons of the nicotinamide and adenine rings are still observable in a solution containing an approximately sevenfold stoichiometric excess of NAD^+ over enzyme binding sites. For a 2.5-fold excess of NAD^+ the previous workers observed the complete disappearance of the spectrum corresponding to the nicotinamide moiety while the adenine proton peaks were little affected. In our experiment the adenine peaks were doubled in width while the nicotinamide peaks appeared to be only slightly affected. The widths of all peaks were approximately the same in the presence of the enzyme. The reasons for these discrepancies are not apparent but may be connected with the above-mentioned decomposition of NAD^+ by the enzyme.

In any case, it seems clear that we are observing an interaction of the adenine protons with YADH. Since no sharp component due to adenine protons of free NAD^+ is observed the results cannot be explained on the basis of the slow-exchange case I, discussed above. The result may be in accord, however, with either the fast-exchange case III or intermediate exchange case II. Present data do not allow a clear distinction between the two possibilities. In principle, an intensity measurement should distinguish case II from case III since in the former case the protons of the bound molecules would not contribute to the observed resonance while in the latter case both bound and free protons would be observed as a single peak. Meaningful intensity measurements on the NAD^+–YADH system have not been possible, however, since a gradual decomposition of the NAD^+ is known to occur during the course of the experiment. Assuming fast exchange, the line width of the adenine protons of the bound NAD^+ can be calculated from eq 1 giving a result of about 20 cycles/sec. Since the activity of the YADH used is somewhat lower than that of the most active preparations and since lowered activity may reflect lowered capacity of the YADH for NAD^+ binding, this value may be somewhat higher perhaps 30–40 cycles/sec. These values are much lower than would be expected from the results of Fisher and Jardetzky (1965) on the binding of penicillin by BSA in which line widths of 800–2200 cycles/sec were estimated for various types of proton in the bound penicillin. This consideration is the most serious argument against case III behavior. For case II behavior, the line width is determined by the average lifetime of a free NAD^+ molecule. Thus, for this case, all protons of NAD^+ would exhibit the same width assuming that for all protons, the line width in the bound state is much greater than that in the free state. In the observed spectrum the widths of all peaks are the same within the rather large experimental error mentioned above. However, the widths of the nicotinamide protons are apparently unchanged on addition of the enzyme. If the intermediate exchange case applies then the lifetime of the free NAD^+ molecules must fortuitously be close to the reciprocal of the line width of the nicotinamide resonances in the free state or the line width for the nicotinamide protons in the bound state must be essentially unaffected by binding while the adenine protons are affected.

NADH–YADH. In the NADH–YADH system the situation is similar in that the adenine proton resonances are clearly broader than the N-C-2 proton resonance which is not affected by the enzyme. As discussed above, the situation with regard to the N-C-4 peak is less clear but it appears to have lost intensity corresponding to approximately one proton.

Although a detailed interpretation of the observed effects cannot be made from present data, the author tentatively favors case II behavior with selective broadening of the adenine protons as the most likely explanation. This implies that the adenine moiety is more intimately involved in the binding of the coenzyme to YADH than is the nicotinamide moiety. Even if fast-exchange behavior were the correct explanation selective broadening of the adenine peaks would still have to be invoked to account for the results. A possible alternative interpretation of this effect is that the adenine protons of the bound NAD^+ are chemically shifted from those of the free form and that the exchange rate is sufficiently great to produce line broadening in the spectrum of the free NAD^+ (Pople *et al.*, 1960). Regardless of which of these alternatives is correct the nmr spectrum indicates an interaction between the adenine moiety and the enzyme which does not appear to affect the nicotinamide moiety.

For LADH the results can be interpreted quite easily if it is assumed that the observed intensity decreases are the result of the enzymatically significant coenzyme binding and that the observed small linewidth changes are due to some additional interaction with the protein. In this connection, we recall that BSA produced a small line-broadening effect on NADH. If only an intensity decrease has resulted from addition of the enzyme to the NAD^+ or NADH solutions the slow-exchange case I appears to be the only plausible explanation. This implies that both the adenine and nicotinamide rings are bound to the enzyme in such a way that their mobility is severely restricted relative to that of the free NAD^+. It is of interest to note here that the 340-mμ band of NADH is shifted to 325 mμ on binding to LADH but is unaffected by YADH (Theorell and Bonnichsen, 1951; van Eys *et al.*, 1958). This is consistent with the present interpretation of the nmr results in that a strong interaction of enzyme with the nicotinamide moiety is indicated in the case of LADH but not in the case of YADH. Furthermore, the slow exchange of coenzymes in the case of LADH and their intermediate or fast exchange in the case of

YADH are in accord with the fact that coenzyme dissociation is rate determining for the interconversion of acetaldehyde and ethanol by LADH but not by YADH which catalyzes the reaction at about 100 times the LADH rate under comparable conditions. Again, however, the possibility that a chemical shift of the NADH spectrum with slow exchange produces the effect observed cannot be eliminated by the present results. However, a search of the region 100 cycles/sec above and below the adenine peak position of free NADH has not revealed a signal due to the bound form. Since a chemical shift of greater magnitude than this on binding does not seem likely line broadening is probably involved, perhaps in addition to a chemical shift.

It is not possible to determine from present data whether the marked change in the N-C-4 resonance brought about by both YADH and LADH is due to a line-width increase or an intensity decrease or both, due to the difficulty in determining the line width or intensity of a very weak or very broad peak. If the effect is due to broadening then the N-C-4 peak must be preferentially broadened relative to the N-C-2 proton and the adenine protons. A possible mechanism for intensity loss is the exchange of one of the N-C-4 protons for solvent deuterium as would occur according to the scheme discussed by Fernandez *et al.* (1962) in which NADH reduces a disulfide group of the enzyme to sulfhydryl group which subsequently exchanges with the solvent. Regardless of the ultimate interpretation of this effect, it appears that one or both of the N-C-4 protons are involved in a special interaction with the enzymes. This point is being investigated further.

Ethanol and Acetaldehyde. Although clear evidence exists that the binary complexes of LADH and YADH with NAD^+ and NADH exist, there is not clear agreement as to whether the binary complexes of the enzymes with ethanol and acetaldehyde exist in significant concentrations. Sund and Theorell (1963) concluded that all four binary complexes may form with LADH but Dalziel (1963) working with inhibitor-free coenzyme preparations found no definite evidence for kinetically significant binary complexes with ethanol or acetaldehyde. McKinley-McKee (1963) concluded from fluorimetric studies that binding sites for substrates do not exist unless the coenzymes are bound. For YADH most studies are consistent with random dissociation of coenzymes and substrates with interconversion of tertiary complexes the rate-limiting step. However, product inhibition studies of Wratten and Cleland (1963) are not in accord with this mechanism. Silverstein and Boyer (1964) concluded from equilibrium rate studies that for both enzymes all binary complexes exist.

Equilibrium constants for the dissociation of the binary enzyme–substrate complexes have been obtained for both LADH and YADH from kinetic studies. For LADH they are K_{EALC} = 4.6 mM and K_{EALD} = 10.2 μM according to Theorell and McKinley-McKee (1961). For YADH the values are K_{EALC} = 19 mM and K_{EALD} = 0.3 mM at pH 7.6, T = 22° (Mahler and Douglas, 1957). One might, therefore, hope to obtain direct physical evidence for substrate binding by observing the effect of the enzymes on the nmr spectra of the substrates in the absence of the coenzymes.

Considering first the case of YADH, the results shown in Figure 6 reveal no effect of the enzyme on the ethanol spectrum although about 6% of the ethanol present should be bound assuming the dissociation constant of 19 mM mentioned above applies. This result would seem to eliminate the possibility that in the absence of coenzyme the ethanol is bound to the enzyme in the rigid manner required to account for the stereospecificity of hydrogen transfer from ethanol to NAD^+, provided that either the rapid or intermediate exchange cases II or III apply. If this were the case, observable line broadening would be expected. Fischer and Jardetzky (1965), for example, observed marked broadening in the spectrum of penicillin under conditions where they estimated that only 1% of the penicillin was bound and the system obeyed case II. The possibility of the slow-exchange case I being operative cannot be definitely eliminated by the nmr results since in that case only an approximately 6% intensity decrease in the spectrum would be observed and this could not be reliably detected in the present spectra. However, this possibility is believed unlikely because the rate of dissociation of ethanol from the tertiary enzyme–coenzyme–substrate complex has been shown to be greater than that of the coenzyme (Silverstein and Boyer, 1964) and there is little reason to expect it to be slower in the absence of coenzyme. Since our nmr results on the NAD^+–YADH system indicate either intermediate or fast exchange, ethanol also should be expected to conform to one of these cases. It is hoped that this question may be settled by experiments now in progress which are designed to detect an intensity decrease in the ethanol spectrum.

As we reported previously (Hollis *et al.*, 1966) and as shown in Figure 6, addition of a fivefold excess of NAD^+ to the YADH–ethanol system produced a marked decrease (~40%) in the intensity of the ethanol spectrum while producing only slight line broadening. We tentatively interpreted this result as indicating binding of the ethanol with slow exchange in the presence of NAD^+. On further consideration, this interpretation does not appear warranted since the intensity decrease may be due in part to the combined effect of preferential binding of NADH and acetaldehyde over NAD^+ and ethanol, respectively, causing an equilibrium shift toward NADH and acetaldehyde in the presence of the high YADH concentrations employed. This point is being further investigated.

As shown in Figure 5, the ethanol spectrum is also apparently unaffected by LADH sufficient to bind about 6% of the ethanol present assuming a dissociation constant of 4.6 mM. In the interpretation of this result the same considerations apply as in the discussion above of the YADH–ethanol system. Thus rigid binding combined with rapid exchanges can be eliminated.

However, in this case there seems to be no objective basis for elimination of the possibility of rigid binding and slow exchange although it is hoped that experiments now in progress will be able to detect the intensity decrease expected for the slow-exchange case and thus allow this possibility to be confirmed or eliminated.

In view of the preceding discussion, it appears that in the absence of coenzyme, ethanol must be bound to YADH in a manner such that its rotational motion is not appreciably restricted. The rigidly bound ethanol which must be present in the tertiary transition complex of enzyme–ethanol–NAD^+ may exist in very small concentrations in the absence of coenzyme or it may exist only in the presence of coenzyme.

Concluding Remarks

Aside from the above-discussed tentative interpretations of the effects observed in the nmr spectra of the alcohol dehydrogenase systems an important aspect of the results presented here in the demonstration that the most advanced nmr equipment now available is sufficiently sensitive to allow convenient study of substrate nmr spectra at the low concentrations required. Future studies of the effects of inhibitors and activators on the substrate spectra are obviously suggested. An intriguing possibility for example is the study of the various interactions between enzyme, substrate, activator, and inhibitor in allosteric systems where in favorable cases nmr can be imagined as an important supplementary tool. Full exploitation of these possibilities will require still greater improvement in the sensitivity of the nmr technique but progress in this direction is being made as evidenced particularly by the recent development of a high-frequency spectrometer using a superconducting magnet whose theoretical sensitivity should be about three times that of the spectrometer employed in these studies.

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