# Interaction of a Spin-Labeled Analog of Nicotinamide–Adenine Dinucleotide with Alcohol Dehydrogenase. II. Proton Relaxation Rate and Electron Paramagnetic Resonance Studies of Binary and Ternary Complexes*

A. S. Mildvan and H. Weiner

ABSTRACT: Adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl), a paramagnetic analog of nicotinamide–adenine dinucleotide, increases the longitudinal proton relaxation rate of water. The binding of adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl) to liver alcohol dehydrogenase enhances the effect of the unpaired electron on the proton relaxation rate of water by a factor ($\epsilon$) as great as 81. Hence, the relaxation enhancement phenomenon, previously discovered for complexes of $Mn^{2+}$ and $Cu^{2+}$ with proteins and nucleic acids, is extended to complexes of organic radicals with macromolecules. From temperature studies, $\epsilon$ is ascribed to a hindrance, by the protein, of the relative rotational motion of the bound radical and its hydration shell. Titration of alcohol dehydrogenase with adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl) measuring $\epsilon$ reveals two tight binding sites ($K_D$ = 15 $\mu$M) and five or six weak binding sites ($K_D$ = 83 $\mu$M) on the enzyme, in agreement with previous results obtained by electron paramagnetic resonance line broadening. The tight binding sites for adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl) correspond to the reduced nicotinamide–adenine dinucleotide binding sites as determined by competition studies. The electron paramagnetic resonance spectrum of adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl) bound at these sites is broadened, suggesting immobilization of the radical. The enhancement factor of the bound radical, $\epsilon_b$, decreases from 81 to 13 as the occupancy of the tight binding sites increases from 0 to 2, indicating site–site interaction which was not reflected in the dissociation constant. This interaction results in an "opening" of the site facilitating water rotation in the environment of the unpaired electron, and may be necessary to permit substrate entry. Similar site–site interaction is detected by changes in $\epsilon_b$ from 32 to 4.5 in the Zn-free apoenzyme, where only the two tight binding sites exist. The lower $\epsilon_b$ values in the apoenzyme–(adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl))$_2$ complex suggest that Zn immobilizes water molecules in the environment of the bound radical (and coenzyme). In addition to displacing adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl), the coenzyme, reduced nicotinamide–adenine dinucleotide, decreases $\epsilon_b$ of the residual bound radical probably by "opening" the site. The inhibitor *o*-phenanthroline, which does not displace adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl), decreases $\epsilon_b$ at high concentrations ($K_3$(*o*-phenanthroline) = 0.4 mM) indicating the formation of a ternary complex ($\epsilon_T$ = 3). The substrate, ethanol, also decreases $\epsilon_b$ without displacing adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl) forming a ternary complex ($\epsilon_T$ = 5.8) with a dissociation constant ($K_D$ = 1.1 mM) in agreement with its $K_M$ and with the $K_D$ of the E-NAD-ethanol complex from the literature. Ethanol does not alter the electron paramagnetic resonance spectrum of bound adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl). The activation energy for the paramagnetic contribution to the proton relaxation rate in the ternary enzyme–(adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl))$_2$(ethanol)$_2$ complex (5.2 kcal/mole) is indistinguishable from that of the binary enzyme–(adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl))$_2$ complex (5.0 kcal/mole) suggesting that ethanol reduces $\epsilon$ by displacing water molecules which are hydrogen bonded to the nitroxide group of adenosine diphosphate 4-(2,2,6,6-tetramethylpiperidine-1-oxyl). Since the unpaired electron of the analog is localized in a region which corresponds to the pyridine–*N*-ribose $C_1$ bond of nicotinamide–adenine dinucleotide, it is suggested that ethanol binds to the solvent side of the bound coenzyme and overlies the ribosidic bond to pyridine. This structure is consistent with the preferred order kinetic scheme of liver alcohol dehydrogenase proposed by Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127. It is concluded that proton relaxation rate studies may be used to determine stoichiometry, binding constants, and structural properties of binary and ternary complexes of organic radicals with macromolecules.

When an enzyme contains atoms or groups with unpaired electrons, the methods of electron spin resonance (Beinert, 1966) or nuclear magnetic relaxation (Mildvan *et al.*, 1966; Mildvan and Scrutton, 1967) can give detailed insight into the mechanism of the reaction. The use of paramagnetic probes for diamagnetic systems in the study of enzyme mechanisms has been pioneered by Mildred Cohn who replaced magnesium with manga-

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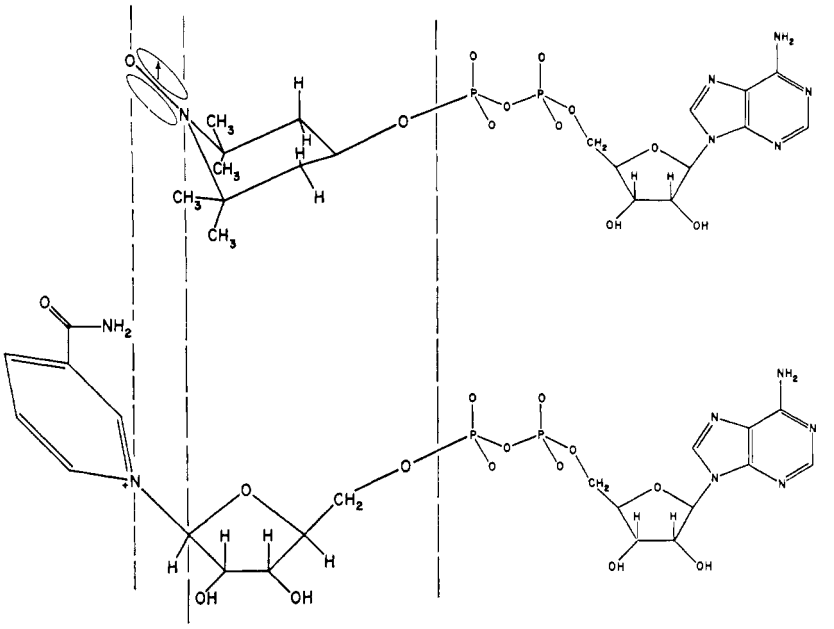


FIGURE 1: Comparison of the structures of ADP-R· and NAD, showing the location of the unpaired electron.

nese as the activator of various kinases (Cohn and Leigh, 1962; Cohn, 1963). Using the paramagnetic manganous ion, one can study thermodynamic, kinetic, and structural properties of enzyme–metal–substrate complexes by electron spin resonance and nuclear magnetic relaxation of the solvent (Mildvan and Cohn, 1965, 1966) or of the substrates (Mildvan *et al.*, 1967).

McConnell (1967) has extended this approach by covalently attaching paramagnetic organic compounds to enzymes and studying them by electron spin resonance. Using this method, McConnell and coworkers (Hamilton and McConnell, 1968) have shown that one can determine whether the bound radical or "spin label" is able to rotate freely or is restricted in its motion, and how the motion of the radical changes as the environmental conditions are altered.

The spin-labeling technique has recently been extended further to electron paramagnetic resonance studies of noncovalently bound compounds by the preparation of a paramagnetic hapten (Stryer and Griffith, 1965) and a paramagnetic analog of NAD (Weiner, 1969). The latter compound (Figure 1), ADP-R·,[1] is a competitive inhibitor of liver and yeast alcohol dehydrogenases with respect to the coenzymes and has been shown by electron paramagnetic resonance line broadening to bind to liver alcohol dehydrogenase in a manner which is indistinguishable from that of the competitive inhibitor ADP-ribose (Weiner, 1969).

The present work describes the effects of this spin-labeled substrate analog and its binary and ternary complexes with liver alcohol dehydrogenase and ethanol, on the nuclear magnetic relaxation rate of the solvent protons. From studies of the enhancement of the proton relaxation rate one can gain detailed information about the structural and thermodynamic properties of the binary and ternary complexes. Paper III of this series will further examine the properties of ternary complexes with substrates and inhibitors by proton relaxation studies of the solvent, substrates, and the inhibitors (Mildvan and Weiner, 1969).

## Experimental Section

*Materials.* Alcohol dehydrogenase from horse liver was prepared by a method similar to that of Taniguchi *et al.* (1967). From its specific activity (4000 $\mu$m/(min mg)) and its behavior on starch gel, acrylamide gel, and cellulose acetate electrophoresis, the purity of the enzyme was estimated to be greater than 95% (Weiner, 1969).

The paramagnetic analog of NAD was prepared as reported (Weiner, 1969). The structure of this analog is compared with that of NAD in Figure 1. By superposition of molecular models of the analog and of NAD we conclude that the unpaired electron (which is delocalized between the nitrogen and oxygen of the nitroxide; Luz *et al.*, 1966) is located in a position that would lie between the ring nitrogen of the pyridine and the C-1 of the ribose of NAD. Metal-free buffers were used as previously described (Weiner, 1969) and all compounds used were of the highest purity commercially available.

*Magnetic Resonance Techniques.* Electron spin resonance spectra of 0.1-ml samples of dilute solutions of the analog (0.01–1.0 mM) and of the enzyme (0.05–0.20 mM) were obtained with a Varian E-3 electron paramagnetic resonance spectrometer with temperatures controlled to $\pm 1°$ as previously described (Weiner, 1969). The signal to noise ratio was improved by use of the Varian C1024 time-averaging computer.

[1] Abbreviations used: ADP-R· refers to ADP-4-(2,2,6,6-tetramethylpiperidine-1-oxyl), the structure of which is given in Figure 1; ADP ribose, adenosine diphosphoribose. All symbols related to enhancement are defined in the text and in previous references (Mildvan and Cohn, 1963, 1965, 1966).

The longitudinal relaxation rate, $1/T_1$, of the protons of water in the same solutions was measured by the pulsed method at 24.3 MHz as previously described (Mildvan and Cohn, 1963). The theory of relaxation enhancement has been discussed elsewhere in connection with coordination complexes of paramagnetic ions (Cohn and Leigh, 1961; Cohn, 1963; Eisinger *et al.*, 1962). The paramagnetic contribution to the observed longitudinal relaxation rate of water protons, $1/T_{1p}$, was determined by subtracting the corresponding relaxation rate in the absence of ADP-R· from the relaxation rate observed in its presence.

The enhancement factor, $\epsilon^*$, is defined as previously described for metal complexes (Cohn and Leigh, 1962; Eisinger *et al.*, 1962) as the ratio of $1/T_{1p}$ in the presence of protein (as indicated by the asterisk) to $1/T_{1p}$ in the absence of protein but in the presence of the same concentration of ADP-R·

$$\epsilon^* = \frac{(1/T_{1p})^*}{(1/T_{1p})} \tag{1}$$

Luz and Meiboom (1964) have shown that $1/T_{1p}$ of solvent protons is a function of the residence time, $\tau_M$, and the relaxation time, $T_{1M}$, of a solvent molecule in the "inner coordination sphere" of the paramagnetic atom

$$1/T_{1p} = \frac{pq}{T_{1M} + \tau_M} \tag{2}$$

In eq 2, $p$ = [ADP-R·]/2[$H_2O$] and $q$ is the "coordination number," the number of solvent protons which can simultaneously interact with the nitroxide. Since chemical bonding between these protons and ADP-R· is very weak (hydrogen bonds), the hyperfine coupling, which operates through chemical bonds, may be assumed to be vanishingly small compared with the dipolar interaction between the unpaired electron and the relaxing protons. Therefore $T_{1M}$ is inversely proportional to the correlation time, $\tau_c$, for dipolar interaction between the unpaired electron of ADP-R· and the adjacent water protons (Solomon, 1955; Bloembergen, 1957). Hence eq 2 may be rewritten as

$$1/T_{1p} = \frac{pq}{c/\tau_c + \tau_M} \tag{3}$$

where $c$ is a constant. Because the electron spin relaxation time of nitroxide radicals is long ($10^{-9}$ sec $\leq \tau_s \leq 10^{-8}$ sec) compared with rotation times ($10^{-13} \leq \tau_r \leq 10^{-11}$), $\tau_c$ probably is a rotation time, from 4.

$$1/\tau_c = 1/\tau_r + 1/\tau_s \tag{4}$$

Hence $\tau_c$ in eq 3 may be replaced by $\tau_r$.

It will be shown that $1/T_{1p}$ decreases with increasing temperature in the presence and absence of protein suggesting that $\tau_M \ll T_{1M}$ (Luz and Meiboom, 1964). Therefore eq 3 reduces to

$$1/T_{1p} \doteq \frac{pq\tau_r}{c} \tag{5}$$

and eq 1 can be rewritten as

$$\epsilon^* = \frac{(1/T_{1p})^*}{(1/T_{1p})} \doteq \frac{(q\tau_r)^*}{(q\tau_r)} \tag{6}$$

Relaxation enhancements (*i.e.*, $\epsilon^* > 1$) can therefore occur by the formation of complexes of ADP-R· with macromolecules in which the relative rotational motion of the radical and the relaxing solvent proton is hindered, *i.e.*, in which $\tau_r$ is increased. Similar effects are well known in complexes of the manganous ion (Cohn and Leigh, 1962; Eisinger *et al.*, 1962; Mildvan and Cohn, 1963, 1965; Scrutton *et al.*, 1966).

## Results

*Binding of ADP-R· to Liver Alcohol Dehydrogenase as Studied by Electron Paramagnetic Resonance.* It has previously been shown that the binding of ADP-R· to liver alcohol dehydrogenase results in a decrease in the amplitude of the electron paramagnetic resonance spectrum which may be used to determine dissociation constants (Weiner, 1969). In addition, the broadened spectrum of the bound radical could also be discerned.

Three techniques are used to improve the resolution of the electron paramagnetic resonance spectrum of the bound radical. As before, time averaging and a ratio of ADP-R· to liver alcohol dehydrogenase was used (Weiner, 1969) where most of the radical is bound (Figure 2, spectra 1 and 2). In addition a selective amplification of the broadened resonance of the bound radical is obtained by increasing the modulation amplitude of the electron paramagnetic resonance spectrometer to 10 gauss (Figure 2, spectrum 4) but this distorts the spectrum of the unbound radical (spectrum 3). Spectrum 5 gives the difference electron paramagnetic resonance spectrum of the bound radical. The appearance of this spectrum is similar to that reported for the spin label which was covalently attached to bovine serum albumin (Griffith and McConnell, 1966) and indicates that the bound radical is strongly immobilized, *i.e.*, it tumbles at a rate $< 10^7$/sec (Hamilton and McConnell, 1968). Ogawa and McConnell (reported in Hamilton and McConnell, 1968) have found that a spin label covalently attached to a sulhydryl group of yeast alcohol dehydrogenase is highly immobilized, as determined by electron paramagnetic resonance.

In another experiment at a modulation amplitude of 10 gauss (Figure 3), the addition of 96 $\mu$M alcohol dehydrogenase to 118 $\mu$M ADP-R· results in the binding of 83% of the ADP-R· (spectra 1 and 2). Spectrum 2 reveals the separate resonances of both the free and the bound radical indicating that chemical exchange between these two forms occurs at a rate $< 5 \times 10^7$ sec$^{-1}$. The addition of 4.5 mM ethanol (spectrum 3) produces no visible change in the electron paramagnetic resonance spectrum, but the addition of 1.07 mM NADH (spectrum 4) causes a fivefold reduction in the intensity of the broadened resonance of the bound radical and a two- to fivefold increase in the intensity of the signal due to free ADP-R·. When the amplitude of the narrow resonances

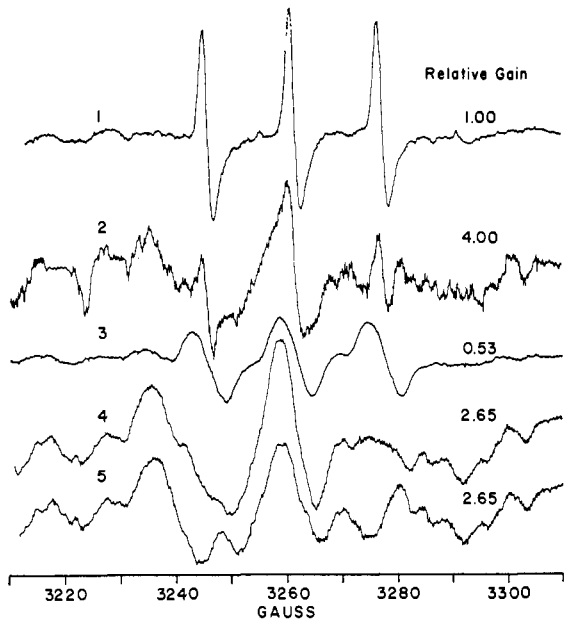
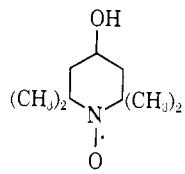



FIGURE 2: The electron paramagnetic resonance spectra of free and bound ADP-R·. The concentrations of components and sweep conditions are: curve 1: [ADP-R·] = 12.8 μM, 25 sweeps at 1-gauss modulation amplitude; curve 2: [ADP-R·] = 12.8 μM, [liver alcohol dehydrogenase] = 35.5 μM, 25 sweeps at 1-gauss modulation amplitude; curve 3: [ADP-R·] = 12.8 μM, 2 sweeps at 10-gauss modulation amplitude; curve 4: [ADP-R·] = 12.8 μM, [liver alcohol dehydrogenase] = 35.5 μM, 10 sweeps at 10-gauss modulation amplitude; and curve 5: difference electron paramagnetic resonance spectrum of curve 4 minus curve 3. Solutions contained 0.04 M $NaH_2PO_4$–$NaHPO_4$ buffer, pH 6.1, $T$ = 23°.

in spectrum 4 is corrected for the 1.12-fold dilution of ADP-R·, it is calculated that 15% of the radical has remained bound. These observations are consistent with the competition between NADH and ADP-R· previously reported (Weiner, 1969).

*Binding of ADP-R· to Liver Alcohol Dehydrogenase as Studied by Proton Relaxation Rate.* The data of Table IA indicates that the analog of NAD increases the longitudinal relaxation rate, $1/T_{1p}$, of the protons of water in proportion to its concentration. The molar relaxivity of solutions of the radical ($1/T_p$[ADP-R·] = 44.1 ± 6.4 $M^{-1}$ $sec^{-1}$) is small compared to that of the simpler compound, 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (282 ± 3 $M^{-1}$ $sec^{-1}$, in which a hydroxyl group is substituted for ADP




or of $Cu^{2+}$ (875 ± 10 $M^{-1}$ $sec^{-1}$)[2] which also has one unpaired electron, but is of the same order of magnitude as peroxylaminedisulfonate (72 ± 15 $M^{-1}$ $sec^{-1}$).[3]

These differences in relaxivity result, in part from differences in accessability of solvent protons to the unpaired electron as well as differences in correlation times for the interactions. The low value for ADP-R· may reflect, in part, diminished access of water protons to the unpaired electron due to a folded structure of the type proposed by Velick (1961) for NADH.

The addition of 50.5 μM liver alcohol dehydrogenase to 570 μM ADP-R· results in the binding of 47% of the radical, as detected by electron paramagnetic resonance and an enhancement of the effect of the radical on the water by a factor of 6.7. At a lower concentration of radical (Table IB), where a larger fraction is bound, the observed enhancement $\epsilon^*$ = 48. Thus the enhancement phenomenon originally discovered for Mn complexes of proteins (Cohn and Leigh, 1962) and nucleic acids (Eisinger *et al.*, 1962) is here demonstrated for complexes of organic radicals with macromolecules. It has previously been shown for Mn–protein complexes that the enhancement phenomenon may be used to determine thermodynamic constants and stoichiometry (Mildvan

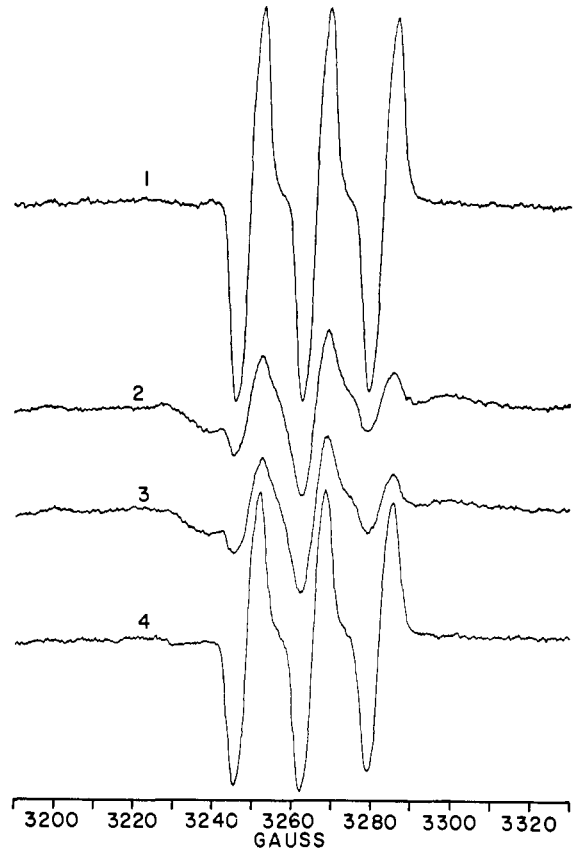



FIGURE 3: The effect of ethanol and NADH on the electron paramagnetic resonance spectra of free and bound ADP-R·. The concentrations of components are: curve 1: [ADP-R·] = 118 μM; curve 2: [ADP-R·] = 118 μM, [liver alcohol dehydrogenase] = 96 μM; curve 3: [ADP-R·] = 112 μM, [liver alcohol dehydrogenase] = 92 μM, [ethanol] = 4.6 mM; curve 4: [ADP-R·] = 103 μM, [liver alcohol dehydrogenase] = 84 μM, [ethanol] = 4.2 mM, [NADH] = 1.07 mM. Other components are as described in Figure 2. Each spectrum is a single sweep at a modulation amplitude of 10 gauss and all have the same gain; $T$ = 19°.

[2] B. Joyce and M. Cohn, unpublished observations.

[3] A. S. Mildvan and J. S. Leigh, Jr., unpublished observation.

TABLE I: Effect of ADP-R· and Its Complexes with Liver Alcohol Dehydrogenase on the Proton Relaxation Rate of Water.

| Expt | Addn to 0.04 M $NaH_2PO_4$–$Na_2HPO_4$ Buffer | $1/T_1$ or Proton Relaxation Rate ($sec^{-1}$) | $1/T_{1p}$ ($sec^{-1}$) | $\epsilon^*$ [a] | [ADP-R·]$_f$/[ADP-R·]$_t$ [b] | $\epsilon_b$ [c] |
|---|---|---|---|---|---|---|
| A | None | 0.380 | | | | |
| | ADP-R· (0.57 mM) | 0.400 | 0.020 | | 1.00 | |
| | ADP-R· (1.14 mM) | 0.420 | 0.040 | 1.00 | 1.00 | |
| | Liver alcohol dehydrogenase (50.5 μM) | 0.386 | | | | |
| | ADP-R· (0.57 mM) + liver alcohol dehydrogenase (50.5 μM) | 0.520 | 0.134 | 6.7 | 0.525 | 13.0 |
| | ADP-R· (1.14 mM) + liver alcohol dehydrogenase (50.5 μM) | 0.574 | 0.188 | 4.7 | 0.706 | 13.6 |
| B | ADP-R· (11.4 μM) | 0.381 | 0.00040 [d] | 1.00 | 1.00 | |
| | ADP-R· (11.4 μM) + liver alcohol dehydrogenase (50.5 μM) | 0.405 | 0.019 | 48 | 0.150 | 56 |
| C | None | 0.390 | | | | |
| | ADP-R· (114 μM) | 0.396 | 0.006 | 1.00 | | |
| | Liver alcohol dehydrogenase (50.5 μM) | 0.400 | | | | |
| | ADP-R· (114 μM) + liver alcohol dehydrogenase (50.5 μM) | 0.471 | 0.071 | 11.8 | 0.265 | 15.7 |
| | Ethanol (2.24 mM) | 0.440 | 0.040 | 6.7 | | |
| | Ethanol (6.4 mM) | 0.422 | 0.022 | 3.7 | | |
| | Ethanol (10.1 mM) | 0.423 | 0.023 | 3.8 | 0.264 | 4.8 |

[a] Calculated from the definition of $\epsilon^*$ given in eq 1. [b] Determined by electron paramagnetic resonance (Weiner, 1969). [c] Calculated from *a* and *b* using eq 7. [d] Calculated from experiment A based on linearity of $1/T_{1p}$ with [ADP-R·]. $T = 23°$.

and Cohn, 1963, 1965, 1966) because the observed enhancement, $\epsilon^*$, is a weighted average of the enhancements of the free, $\epsilon_f$, and bound, $\epsilon_b$, Mn. Making a similar assumption here, we argue that

$$\epsilon^* = \frac{[\text{ADP-R}\cdot]_f}{[\text{ADP-R}\cdot]_t}\epsilon_f + \frac{[\text{ADP-R}\cdot]_b}{[\text{ADP-R}\cdot]_t}\epsilon_b \qquad (7)$$

where the subscripts f, b, and t refer to the concentration of free, bound, and total radical, respectively. By definition, $\epsilon_f = 1$ (eq 1).

If $\epsilon_b$ is known, then [ADP-R·]$_f$ and [ADP-R·]$_b$ (= [ADP-R·]$_t$ − [ADP-R·]$_f$) may be found for each value of $\epsilon^*$. As has previously been shown (Mildvan and Cohn, 1963), $\epsilon_b$ may be determined by titration of a constant concentration of protein with variable concentrations of the paramagnetic substance plotting $1/\epsilon^*$ against [ADP-R·]$_t$ and extrapolating to [ADP-R·]$_t$ = 0. If the dissociation constant of the enzyme radical complex is small compared with the concentration of binding sites in solution then $1/\epsilon^*$ approaches $1/\epsilon_b$ as [ADP-R·]$_t$ approaches 0 (Mildvan and Cohn, 1963). Such a plot, which is shown in Figure 4, reveals a break in the curve giving two values for $\epsilon_b$. For those concentrations of [ADP-R·]$_t$ below the concentration of the enzyme $\epsilon_b = 100 \pm 48$ and for concentrations of [ADP-R·]$_t$ greater than the enzyme concentration $\epsilon_b = 13.2 \pm 1.7$. The concentrations of free and bound radical calculated from eq 7 using the latter value of $\epsilon_b$ and the $\epsilon^*$ values for [ADP-R·]$_t \geq$ [LADH] are plotted as a Scatchard plot in Figure 5A. The results, which are summarized in Table II, are in good agreement with those obtained previously by electron paramagnetic resonance (Weiner, 1969).

A more accurate nethod of determining $\epsilon_b$ is by measuring the value of [ADP-R·]$_f$/[ADP-R·]$_t$ by electron paramagnetic resonance (Weiner, 1969) and $\epsilon^*$ by proton relaxation rate in the same solutions and directly substituting these values in eq 7 (Mildvan and Cohn, 1963). Representative values so obtained are indicated in Table IA and B and are plotted as a function of [ADP-R·]$_b$/[liver alcohol dehydrogenase] in Figure 5B.

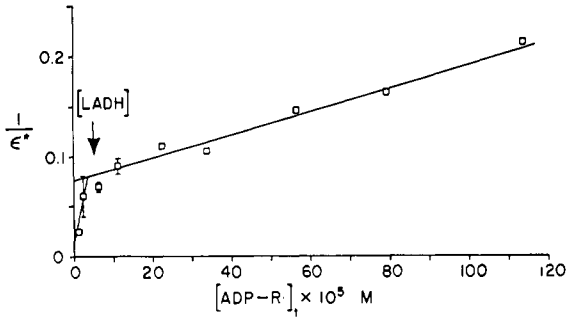



FIGURE 4: Titration of alcohol dehydrogenase (50.5 μM) with ADP-R· measuring the enhancement of the proton relaxation rate. The observed enhancement factors, $\epsilon^*$, are plotted in reciprocal form for determination of $\epsilon_b$, the enhancement of bound ADP-R· (Mildvan and Cohn, 1963). Other components present are indicated in Figure 2, $T$ = 23°.

In agreement with the previous experiment, the values of $\epsilon_b$ decrease from 62 ± 19 to 13.0 ± 1.3 as the first molecule of [ADP-R·] is bound and remain constant at 13 thereafter as a total of 8 molecules of ADP-R· is bound. The agreement of the two methods of calculating $\epsilon_b$ indicates that eq 7 fits the data.

Hence the $\epsilon_b$ value of the two tight binding sites varies from ~80 to ~13 as the occupancy increases from 0 to 2 (Figure 5B) presumably due to site–site interaction which is not detected thermodynamically (Figure 5A). The decrease in $\epsilon_b$ probably results from a decrease of $\tau_r$ (eq 6), *i.e.*, there is less hindered rotation of water molecules in the environment of the bound radical due to a more open site (*vide infra*). The five to six weak binding sites have an $\epsilon_b$ value which is ~13 (Table II).

*The Effect of NADH and o-Phenanthroline on* $\epsilon_b$. It has previously been shown that liver alcohol dehydrogenase binds two molecules of NADH (Theorell and Bonnichsen, 1951) and two molecules of NAD (Theorell and Yonetani, 1963) per molecule of enzyme and that ADP-R· is a competitive inhibitor of the enzyme with

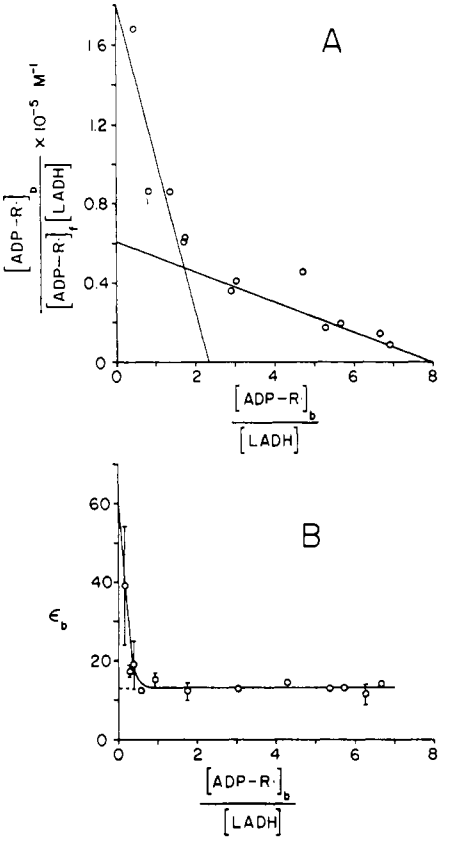



FIGURE 5: Stoichiometry and properties of ADP-R· binding sites on alcohol dehydrogenase. (A) Scatchard plot of the free and bound ADP-R· calculated from the data of Figure 4 assuming $\epsilon_b$ = 13.2 as described in the text. (B) The enhancement of bound ADP-R· $\epsilon_b$, as a function of the number of enzyme sites occupied. $\epsilon_b$ was calculated from the observed enhancement, $\epsilon^*$, using eq 7. [ADP-R·]$_f$/[ADP-R·]$_t$ was determined for each point by electron paramagnetic resonance (Weiner, 1969). Conditions are as described in Figures 2 and 4.

TABLE II: Stoichiometry, Dissociation Constants, and Enhancements of Binary Complexes of Liver Alcohol Dehydrogenase and ADP-R·.

| Method | Tight Binding | | | Weak Binding | | |
|---|---|---|---|---|---|---|
| | $n$ | $K_D$ (μM) | $\epsilon_b$ | $n$ | $K_D$ (μM) | $\epsilon_b$ |
| Electron paramagnetic resonance[a] | 2.0 ± 0.5 | 17 ± 8 | | 5.2 ± 0.3 | 73.3 ± 9.4 | |
| Proton relaxation rate | 2.3 ± 0.4 | 15 ± 6 | 100 ± 48<br>13.2 ± 1.7 | 5.7 ± 0.7 | 93.2 ± 19.6 | 13.2 ± 1.7 |
| Both electron paramagnetic resonance and proton relaxation rate | | | 62 ± 19<br>13.0 ± 1.3 | | | 13.0 ± 1.3 |
| Av | 2.2 ± 0.5 | 16 ± 7 | 81 ± 33<br>13.1 ± 1.5 | 5.4 ± 0.5 | 83.3 ± 14.5 | 13.1 ± 1.5 |

[a] From Weiner (1969). Conditions are given in Table I and in Figure 2.

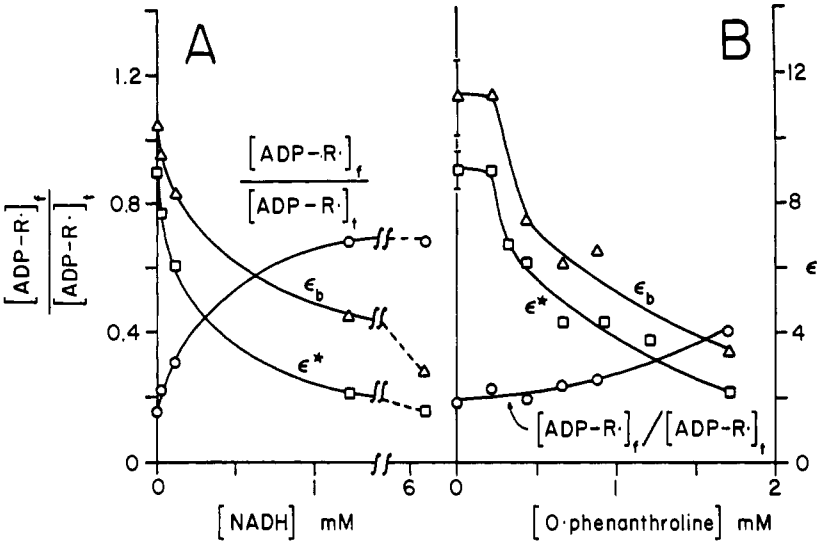



FIGURE 6: Effect of NADH and *o*-phenanthroline on the enhancement of ADP-R· bound to liver alcohol dehydrogenase. (A) NADH titration of solutions containing 108 $\mu$M ADP-R· and 48.1 $\mu$M liver alcohol dehydrogenase, measuring the enhancement $\epsilon^*$ by proton relaxation rate, and the fraction of ADP-R· which is free by electron paramagnetic resonance. The enhancement of bound ADP-R· was calculated from these values using eq 7. (B) *o*-Phenanthroline titration of solutions containing 121 $\mu$M ADP-R· and 46 $\mu$M liver alcohol dehydrogenase. Parameters were measured and calculated as in A. Other conditions are as given in Figure 2.

respect to the coenzymes as determined by kinetics and by direct binding studies using electron paramagnetic resonance (Weiner, 1969) (Figure 3). The effect of NADH on the binding of the analog by liver alcohol dehydrogenase was studied by proton relaxation rate and electron paramagnetic resonance to determine the enhancement factor $\epsilon_b$ of the residual bound radical, at concentrations of ADP-R· and liver alcohol dehydrogenase at which binding occurs predominantly at the tight binding sites. The results, summarized in Figure 6A, indicate that as NADH displaces ADP-R·, $\epsilon_b$ decreases from 10.8 to a value approaching 2, when only the weak binding sites remain occupied by the radical. The reduction in $\epsilon_b$ suggests an "opening" of residual ADP-R· binding sites as NADH binds, resulting in less hindered rotation of water molecules near the bound radical (*i.e.*, a decrease in $\tau_r$).

*o*-Phenanthroline has been shown to compete against NADH and NAD ($K_I \leq 11$ $\mu$M; Yonetani, 1963) but not against ADP ribose (Yonetani and Theorell, 1964) or ADP-R· (Weiner, 1969). The experiment summarized in Figure 6B indicates that below 0.7 mM *o*-phenanthroline has no effect on the binding of ADP-R·, and below 0.2 mM has no effect on the enhancement, $\epsilon_b$, of the bound radical. Above 0.2 mM, *o*-phenanthroline causes a small increase in $[ADP\text{-}R\cdot]_f$ but a large progressive decrease in $\epsilon_b$ indicating the formation of higher complexes of the form liver alcohol dehydrogenase $(ADP\text{-}R\cdot)_2(o\text{-phenanthroline})_n$ with an apparent dissociation constant

$$K_s = \frac{[E(ADP\text{-}R\cdot)][o\text{-phenanthroline}]}{[E(ADP\text{-}R\cdot)(o\text{-phenanthroline})]} = 0.4 \pm 0.1 \text{ mM}$$

Hence *o*-phenanthroline can bind to liver alcohol dehydrogenase in several ways, which could account, in part, for the variability in the published $K_I$ values of this inhibitor (Sund and Theorell, 1963). The decreased enhancement of this ternary complex, $\epsilon_t \sim 3$, suggests either an "opening" of the tight binding site for ADP-R· (a decrease in $\tau_r$) or an inaccessability of the radical to water protons in the ternary complex (a decrease in $q$), as may be seen from eq 6.

*Binding of [ADP-R·] to the Zn-Free Apoenzyme.* Liver alcohol dehydrogenase has been shown to contain Zn (Theorell *et al.*, 1955; Vallee and Hoch, 1957) in a stoichiometry of 4 per mole of enzyme (Åkeson, 1964) which may participate in the binding of *o*-phenanthroline (Vallee *et al.*, 1958; Yonetani, 1963; Piette and Rabold, 1967) and of NADH (Sund and Theorell, 1963). It was of interest, therefore, to determine the role of Zn in the binding of [ADP-R·]. The apoenzyme was prepared as previously described (Weiner, 1969) by dialysis of liver alcohol dehydrogenase for 36 hr at 4° against $NaH_2PO_4$ buffer, $\mu = 0.05$, pH 6.0, containing 0.1 mM EDTA followed by extensive dialysis against Zn-free buffer. Such treatment removed 94% of the Zn as judged by residual specific activity. The half-life of Zn under these conditions is 7 hr as determined by atomic absorption spectroscopy and specific activity.[4]

It has previously been shown that the apoenzyme prepared in this way binds approximately two molecules of ADP-R·. tightly but has no detectable weak binding sites (Weiner, 1969). The values of $\epsilon^*$ and $[ADP\text{-}R\cdot]_f$ were measured in the same set of solutions by proton relaxation rate and electron paramagnetic resonance, respectively, and the values of $\epsilon_b$ calculated from eq 7 are plotted in Figure 7. As the two binding sites for ADP-R· are occupied, $\epsilon_b$ decreases progressively from 31.6 to 4.5. Hence the apoenzyme manifests the same kind of site–site interaction as does the native enzyme. However, the lower values of $\epsilon_b$ in the apoenzyme suggest that

[4] C. Hoagstrom and H. Weiner, unpublished observations.

the solvent, in the environment of the bound radical, is more free to rotate. A role of the Zn therefore is to immobilize water molecules near the binding sites of ADP-R· either by direct coordination of water and/or by an effect on the protein conformation. The Zn also stabilizes a protein conformation which permits the binding of five or six additional molecules of ADP-R· (Weiner, 1969).

*Detection of a Ternary Complex of Liver Alcohol Dehydrogenase, ADP-R·, and Ethanol by Proton Relaxation Rate.* The addition of ethanol (10.1 mM) to a solution of liver alcohol dehydrogenase–(ADP-R·)$_2$ causes a decrease in $\epsilon^*$ from 11.8 to 3.8 (Table IC) but no change in [ADP-R·]$_f$ as detected by electron paramagnetic resonance, indicating that the decrease in $\epsilon^*$ represents a change in the enhancement by the bound radical, $\epsilon_b$. No significant change in the electron paramagnetic resonance spectrum of the bound radical was detected upon addition of ethanol (Figure 3). Hence a ternary complex of the form E(ADP-R·)$_2$(ethanol)$_n$ has formed which is detectable by proton relaxation but not by electron paramagnetic resonance.

A titration of liver alcohol dehydrogenase(ADP-R·)$_2$ with ethanol, measuring $\epsilon^*$ (Figure 8A), reveals a dissociation constant $K_3$ of ethanol from the ternary complex of 1.3 ± 0.2 mM, and an enhancement factor $\epsilon_T$ = 5.6 ± 0.6 when the enhancement at the end point (4.4 ± 0.5) is corrected for the bound ADP-R·.

A more precise analysis of this titration is given in the reciprocal plot of Figure 8B (Mildvan and Cohn, 1966). The linearity of this plot is consistent with simple binding of ethanol in a 1:1 stoichiometry with the bound analog, forming a ternary complex liver alcohol dehydrogenase(ADP-R·)$_2$(ethanol)$_2$ with no site–site interaction. From this analysis $K_3$ = 1.1 ± 0.2 mM and $\epsilon_T$ = 5.8 ± 0.3.

The dissociation constant of ethanol from the ternary complex with ADP-R· (1.2 mM) is in good agreement with its dissociation constant from liver alcohol dehydrodenase(NAD)$_2$(ethanol)$_2$ reported by Theorell and Yonetani (1962) as 1–2 mM, and is of the same order of magnitude as the $K_M$ of ethanol obtained kinetically (2.5 mM) (Theorell *et al.*, 1955).

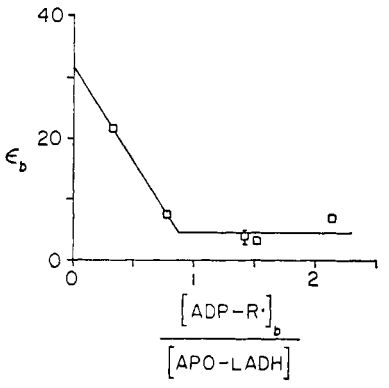



FIGURE 7: Enhancement of ADP-R· bound to Zn-free apoalcohol dehydrogenase (42.9 μM). The values of $\epsilon_b$ were obtained as described in Figure 6 and in the text. Other conditions are given in Figure 2.

The decrease in enhancement from a value of $\epsilon_b$ = 17.4 (in this experiment) to $\epsilon_T$ = 5.7 is probably due to a decrease in $q$ (eq 6), *i.e.*, the displacement of water molecules in the environment of the bound ADP-R· by ethanol. Evidence for this will be given in the next section.

*Temperature Dependence of the Relaxation Rate of Water Protons in the Presence of [ADP-R·] and Its Complexes.* The Arrhenius plot of Figure 9 illustrates the temperature dependence of the paramagnetic contribution to the longitudinal relaxation rate of water protons

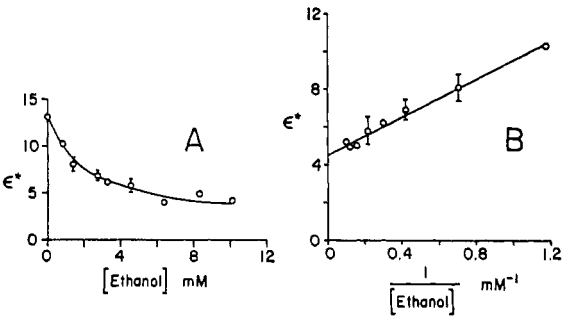



FIGURE 8: The binding of ethanol to the alcohol dehydrogenase(ADP-R·)$_2$ complex. (A) Titation of 50.5 μM liver alcohol dehydrogenase and 114 μM ADP-R· with ethanol, measuring $\epsilon^*$. As determined by electron paramagnetic resonance 26.4% of the ADP-R· was free at the beginning and 26.5% was free at the end of the titration. Conditions are as described in Figure 2. (B) Reciprocal plot of the data in A for determination of $\epsilon_b$ and $K_3$ (Mildvan and Cohn, 1966).

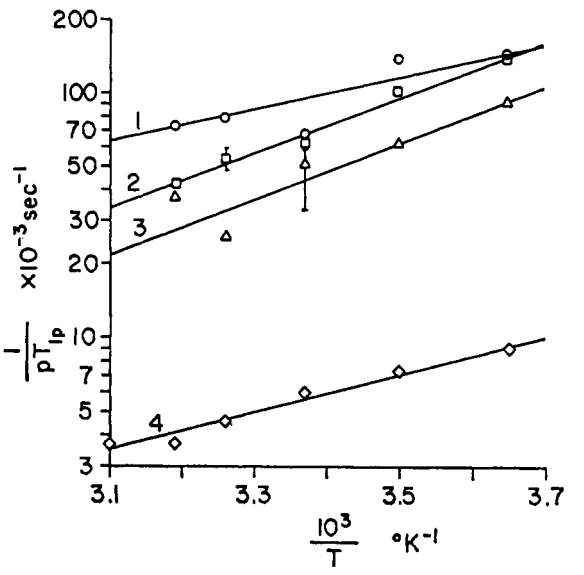



FIGURE 9: Arrhenius plot showing the effect of temperature on the proton relaxation rate of solutions of ADP-R· and its complexes. The relaxation rates were corrected to those of the bound form of ADP-R· and normalized as described in the text. The concentrations of components used and the energies of activation, $E_a$, in kilocalories per mole are: curve 1: [ADP-R·] = 49.6 μM, [liver alcohol dehydrogenase] = 50.5 μM, $E_a$ = 2.9 ± 0.7; curve 2: [ADP-R·] = 99.2 and 248 μM, [liver alcohol dehydrogenase] = 50.5 μM, $E_a$ = 5.0 ± 0.2; curve 3: [ADP-R·] = 99.2 μM, [liver alcohol dehydrogenase] = 50.5 μM, [ethanol] = 4.0 mM, $E_a$ = 5.2 ± 0.8; curve 4: [ADP-R·] = 1.24 mM, $E_a$ = 3.4 ± 0.8. Other components are as described in Figure 2.

in the presence of ADP-R·, $1/T_{1p}$, normalized by dividing by $p$ = [ADP-R·]/2[$H_2O$]. In solutions of liver alcohol dehydrogenase and ADP-R·, $1/T_{1p}$ was determined by pulsed nuclear magnetic resonance and [ADP-R·]$_f$ by electron paramagnetic resonance, over the same temperature range. The latter measurements permitted correction of the $1/pT_{1p}$ values to those values characteristic of the bound forms. Curve 1 was obtained at a ratio of [ADP-R·]/[liver alcohol dehydrogenase] < 1 and reflects in part the initial high enhancement. Curve 2 which was obtained at ratios of [ADP-R·]/[liver alcohol dehydrogenase] = 1.96 and 4.91 reflects the tightly bound but less enhanced radical.

Curve 3 is the corrected value observed in the presence of [ADP-R·]:[liver alcohol dehydrogenase]:[ethanol] at a ratio of 1.96:1:79.2 and represents the $1/pT_{1p}$ value of the ternary enzyme–radical–ethanol complex. Further studies of this and other ternary complexes will be described in the next paper in this series.

All of the complexes of [ADP-R·] show a decrease in the relaxation rate with increasing temperature suggesting rapid chemical exchange of water protons into the environment of the spin in all of the complexes (Luz and Meiboom, 1964), *i.e.*, $\tau_M \ll T_{1M}$ in eq 2. Hence the relaxation rate ($1/pT_{1p}$) is a measure of the relaxation rate of water molecules "coordinated" or hydrogen bonded to the unpaired electron ($q/T_{1M}$), and changes in $1/pT_{1p}$ or $\epsilon$ can be interpreted as being due to changes in either the number, $q$, of interacting water molecules or $\tau_r$, the correlation time for the interaction (eq 5).

The energy of activation of $1/pT_{1p}$ represents the energy of activation of $\tau_r$ (eq 5). For the binary liver alcohol dehydrogenase(ADP-R·)$_2$ complex, the energy of activation (5.0 ± 0.2 kcal/mole) is experimentally indistinguishable from that of the ternary liver alcohol dehydrogenase(ADP-R·)$_2$(ethanol)$_2$ complex (5.2 ± 0.8 kcal/mole) yet $1/pT_{1p}$ is decreased by ethanol suggesting that ethanol displaces a water molecule from the environment of the bound radical. Further evidence for this view will be presented in the next paper in this series. No significant alteration of the electron paramagnetic resonance spectrum of the bound radical was detected upon addition of ethanol (Figure 3).

## Discussion

The present results indicate that large enhancements of the proton relaxation rate may be observed in complexes of organic radicals with macromolecules and are, therefore, not limited to complexes of paramagnetic metal ions with proteins (Cohn and Leigh, 1962) or nucleic acids (Eisinger *et al.*, 1962). The enhancements may be used to give thermodynamic and structural parameters of binary complexes of radicals and macromolecules. Moreover, as suggested by the experiments with *o*-phenanthroline and ethanol, the proton relaxation rate method can also be used to detect and characterize ternary complexes, under conditions where the electron paramagnetic resonance spectrum failed to detect such complexes.

The number of tight binding sites for ADP-R· on liver alcohol dehydrogenase corresponds to the number of NADH binding sites and competition has been observed between ADP-R· and NADH for these sites by kinetics and by binding studies (Weiner, 1969). Moreover, the dissociation constant of ADP-R· obtained in binding studies by two independent methods, electron paramagnetic resonance and proton relaxation rate (16 ± 7 μM) (Table II), and the inhibitor constant obtained kinetically (5 ± 2 μM) are in reasonable agreement. Hence the tight binding sites for ADP-R· may be equated with the NADH binding sites.

The broadened electron paramagnetic resonance spectrum of ADP-R· when it is bound to these sites (Figure 2) may be interpreted to indicate that the analog is immobilized by the protein, *i.e.*, it tumbles at a rate $\leq 10^7$ $sec^{-1}$, which is characteristic of the entire protein molecule (Hamilton and McConnell, 1968).

The decrease in $\epsilon_b$ from 81 to 13 as the two tight binding sites for ADP-R· are occupied might be due, in part, to aggregation of the protein at the high concentrations used and disaggregation by ADP-R·. However, sedimentation studies at high concentrations of liver alcohol dehydrogenase have failed to detect aggregation (Ehrenberg and Dalziel, 1958). Moreover, a similar reduction in $\epsilon_b$ was observed when the same amounts of ADP-R· were bound to the Zn-free apoenzyme, a protein with altered properties (Oppenheimer *et al.*, 1967). A more likely explanation for the reduction in $\epsilon_b$ as ADP-R· is bound is that it reflects a "substrate-induced" conformational change (Koshland, 1958) which may be relevant to catalysis. Coenzyme binding has been found to cause space group changes in crystalline liver alcohol dehydrogenase (Zeppezauer *et al.*, 1967) and dogfish muscle lactic dehydrogenase (Wonacott *et al.*, 1968).

The relaxation data permits a more detailed insight into the nature of the conformation change at the active sites. From the temperature dependence of the relaxation rate (Figure 9), a decrease in $\epsilon_b$ may be interpreted as being due to either a decrease in $q$, the number of water molecules in the immediate environment of the unpaired electron of ADP-R·, or a decrease in $\tau_c$ which is dominated by the relative rotation time, $\tau_r$, of water molecules and the bound ADP-R· (eq 6). The change in the $E_{act}$ for $\tau_r$ as the second molecule of ADP-R· is bound (Figure 9) indicates the latter alternative, namely, that $\tau_r$ must have changed. Hence the water molecules in contact with the radical must have greater freedom of rotation due to an "opening up" of the site. It is of interest that the coenzyme NADH also decreases $\epsilon_b$ of the residual bound ADP-R· in the competition experiment (Figure 6A). Opening of the site in the region of the coenzyme may be necessary to permit entry of the alcohol or aldehyde substrates, and would afford a structural explanation for the preferred binding of the coenzyme prior to the substrates in the kinetic scheme of liver alcohol dehydrogenase (Theorell and Chance, 1951). Such structural alterations need not affect the affinity for coenzymes or substrates and may, therefore, be missed by simple binding studies. Only selected structural parameters, such as $\epsilon_b$, reflect these alterations (O'Sullivan and Cohn, 1966; Mildvan and Cohn, 1964). Hollis (1967) has recently shown that very high concentrations of yeast alcohol dehydrogenase (1.1 mM) di-

minished the amplitude and slightly broadened the methyl resonance in the nuclear magnetic resonance spectrum of ethanol (1 mM) only in the presence of excess NAD.

Little is known about the nature of the five or six weak binding sites for ADP-R·, but it is of great interest that they disappear upon removal of the Zn. Hence, one role of the metal is to stabilize a protein conformation which permits the weak and nonspecific binding of five or six molecules of ADP-R·. Conformational differences between the native and apoenzyme have been described (Oppenheimer *et al.*, 1967). A more specific role of Zn at the active site may be inferred from the observation that the $\epsilon_b$ values of the two tight binding sites for ADP-R· are greater by a factor of three in the native enzyme than in the Zn-free apoenzyme. This suggests that the presence of Zn increases either $q$ or $\tau_r$, *i.e.*, Zn immobilizes water molecules near the binding site of ADP-R· (and of NADH) either by direct coordination of water or by an indirect effect on protein conformation, or both.

The reduction in the enhancement of liver alcohol dehydrogenase $(ADP\text{-}R\cdot)_2$ brought about by the addition of ethanol is due to the formation of a ternary complex liver alcohol dehydrogenase$(ADP\text{-}R\cdot)_2(ethanol)_2$, the dissociation constant of which agrees with that of the kinetically active species liver alcohol dehydrogenase-$(NAD)_2(ethanol)_2$. In the liver alcohol dehydrogenase-$(ADP\text{-}R\cdot)_2(ethanol)_2$ complex, either the correlation time, $\tau_r$, or the number of water molecules near the unpaired electron are diminished, since $\epsilon_T < \epsilon_b$. The agreement of the activation energies of $\tau_r$ in the binary and ternary complexes (Figure 9), and the evidence to be presented in the next paper which indicates the ethanol molecule to be very near the unpaired electron in this ternary complex ($\leq 4.5$ Å), suggest that ethanol decreases $\epsilon_b$ by decreasing $q$, *i.e.*, by displacing water molecules which can hydrogen bond to the enzyme-bound ADP-R·. Thus ethanol appears to bind on the solvent side of ADP-R·. Since the unpaired electron of ADP-R· is localized in a region which corresponds to the pyridine–*N*-ribose–$C_1$ bond of NAD (Figure 1), ethanol may bind on the solvent side of the bound coenzyme and overlie the ribosidic bond to pyridine. Such a structure for liver alcohol dehydrogenase–NAD–ethanol would be consistent with the preferred-order kinetic scheme in which the coenzyme binds first (Theorell and Chance, 1951). Detailed structural, and kinetic properties of this and other ternary complexes of liver alcohol dehydrogenase will be presented in the next paper in this series.

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# Succinic Thiokinase of *Escherichia coli*. Purification, Phosphorylation of the Enzyme, and Exchange Reactions Catalyzed by the Enzyme*

Frederick Lawrence Grinnell and Jonathan S. Nishimura

ABSTRACT: Succinic thiokinase (succinate:coenzyme A ligase (adenosine diphosphate, EC 6.2.1.5) has been isolated in highly purified form from *Escherichia coli* (ATCC 4157) by a procedure which includes DEAE-cellulose chromatography and gel filtration on Sephadex G-150. Assuming a molecular weight of 141,000 (Ramaley, R. F., Bridger, W. A., Moyer, R. W., and Boyer, P. D. (1967), *J. Biol. Chem. 242* (4287), close to two phosphoryl groups are incorporated per mole of enzyme, whether the phosphorylating agent is adenosine triphosphate or inorganic phosphate (in the presence of succinyl-coenzyme A). Capability for phosphorylation appears to be related to enzyme activity and extent of phosphorylation by adenosine triphosphate is not significantly affected by coenzyme A at $2.5 \times 10^{-5}$ M. At this concentration coenzyme A strongly stimulates the adenosine triphosphate $\rightleftarrows$ adenosine diphosphate exchange reaction catalyzed by the enzyme. It has been concluded that under these conditions coenzyme A is not bound covalently or involved in a high-energy nonphosphorylated form of the enzyme. It has also been found that inorganic phosphate is an almost complete requirement for the succinate $\rightleftarrows$ succinyl coenzyme A exchange reaction catalyzed by the enzyme, adding support to the hypothesis that enzyme-bound succinyl phosphate is an intermediate in the over-all reaction.

Previous publications from this laboratory (Nishimura and Meister, 1965; Nishimura, 1967) described experiments with partially purified preparations of succinic thiokinase from *Escherichia coli*, which provided evidence for enzyme-bound succinyl phosphate as an intermediate in the reaction catalyzed by this enzyme. Inferred from these experiments was a mechanism which can be summarized as follows:

$$\text{enzyme} + \text{ATP} \rightleftarrows \text{enzyme-P} + \text{ADP} \quad (1)$$

$$\text{enzyme-P} + \text{succinate} \rightleftarrows \text{enzyme–succinyl-P} \quad (2)$$

$$\text{enzyme–succinyl-P} + \text{CoA} \rightleftarrows \text{enzyme} + \text{succinyl-CoA} + P_i \quad (3)$$

$$\text{ATP} + \text{succinate} + \text{CoA} \rightleftarrows \text{ADP} + \text{succinyl-CoA} + P_i \quad (4)$$

Reaction 1 has been demonstrated and studied in detail by several laboratories (Upper, 1964; Cha *et al.*, 1965; Nishimura, 1967; Ramaley *et al.*, 1967; Moyer *et al.*, 1967) and the site of phosphorylation has been shown to be the N-3 position of a histidine residue of the enzyme protein (Hultquist *et al.*, 1966). Evidence for reactions 2 and 3 was based on the observation that chemically synthesized succinyl phosphate gave rise to succinyl-CoA when incubated with the enzyme, $Mg^{2+}$, and CoA, and to enzyme-P when incubated with enzyme and $Mg^{2+}$ (Nishimura and Meister, 1965). Reaction 2 was subsequently demonstrated directly (Nishimura, 1967). However, the release of succinyl phosphate from the enzyme was slow compared with the release of $P_i$ from enzyme-P in the presence of succinate and CoA. For this reason it was suggested that CoA might be required to facilitate formation of the enzyme–succinyl phosphate complex. It was, therefore, desirable to obtain more information concerning the possibility of such a role of CoA and evidence that the succinyl phosphate pathway represents the major catalytic route of the succinic thiokinase reaction.

In the present investigation succinic thiokinase has

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