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Interaction of a Spin-Labeled Analog of Nicotinamide–Adenine Dinucleotide with Alcohol Dehydrogenase. I. Synthesis, Kinetics, and Electron Paramagnetic Resonance Studies*

Henry Weiner

ABSTRACT: An analog of nicotinamide–adenine dinucleotide containing an unpaired electron has been prepared and shown to inhibit liver alcohol dehydrogenase competitively with respect to nicotinamide–adenine dinucleotide (K_I = 5 ± 2 μM). The compound, adenosine 5′-diphosphate-4(2,2,6,6,-tetramethylpiperidine-1-oxyl), was formed by coupling adenosine monophosphate to 2,2,6,6-tetramethyl-4-phosphopiperidine-1-oxyl. The electron paramagnetic resonance of the spin-labeled compound broadened when bound to liver alcohol dehydrogenase.

Titrating the enzyme with the spin-labeled analog and measuring the decrease in amplitude of the electron paramagnetic resonance spectrum revealed that the enzyme possessed two classes of binding sites: two sites which bind the analog with a K_D = 17 ± 8 μM, in agreement with its K_I, and five to six sites which bind with a K_D = 75 ± 9 μM. Reduced nicotinamide–adenine dinucleotide can only displace the bound radical from the strong binding sites, not from the five or six weak binding sites. Zinc-free liver alcohol dehydrogenase has been prepared and was shown to possess the two strong binding sites for the spin-labeled analog with a K_D = 27 ± 6 μM. The apoenzyme does not possess the weak binding sites. The analog can be displaced by reduced nicotinamide–adenine dinucleotide, showing that the enzymatically inactive apoenzyme can still bind coenzyme.

Fluorescence, absorption, and paramagnetic resonance spectroscopy have been used to study thermodynamic, kinetic, and structural properties of enzymes. The interactions of cofactors and enzymes have also been studied by these methods. For example, Boyer and Theorell (1956) have used fluorescence to study the binding of NADH to alcohol dehydrogenase (EC 1.1.1.1), and Beinert (Beinert *et al.*, 1962) used electron paramagnetic resonance to study flavoenzymes.

If the enzyme did not contain a group possessing the desired spectral properties, small molecules have been either chemically conjugated or bound to the enzyme in order to obtain the desired property. Enzymes conjugated to fluoroscein have been used for fluorescence studies (Steiner and Edelhoch, 1962), while "reporter" groups containing nitrophenol have been used for their absorp-

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tion property (Koshland *et al.*, 1964). Recently McConnell and coworkers (Hamilton and McConnell, 1968) have covalently attached spin-labeled compounds to proteins in order to study the perturbation of the electron paramagnetic resonance signal of the conjugated group. Metal ions, especially manganese, have also been bound to enzymes in order to study the electron paramagnetic resonance and nuclear magnetic resonance changes in the system (Cohn, 1963; Mildvan and Scrutton, 1967).

In most cases the location of the conjugated group relative to the active site of the enzyme is not known. It is the aim of this report to describe both the synthesis of a coenzyme analog containing an unpaired electron and the interactions of the compound with a dehydrogenase. This compound is bound at the active site of the enzyme so any perturbation of the electron paramagnetic resonance spectrum reflects changes that are taking place near the region of the active site of the enzyme.

Since ADP ribose is a known competitive inhibitor for alcohol dehydrogehase (Dalziel, 1963), the desired derivative was synthesized by replacing the ribose in ADP ribose with a nitroxide containing organic spin-labeled molecule.

Experimental Section

Synthesis. The synthesis of ADP-R·, V,[1] is schematically represented in Figure 1.

Triacetone Amine (I). The compound was synthesized by a method similar to that of Harris (1927). Ammonia gas was passed into a flask containing 1.5 l. of acetone and 30 g of $CaCl_2$. After *ca.* 2 hr the solid $CaCl_2$ became gelatinous and the resulting mixture was allowed to react for 2 days at room temperature in a flask equipped with a Dry-Ice trap. The contents were allowed to reflux for 10 hr with the trap attached, then for 10 hr without the trap to distill the excess ammonia. Unreacted acetone was removed by vacuum distillation. The resultant oil was added to 1 l. of water and extracted with *ca.* 1 l. of ether. The product was isolated by saturating the aqueous solution with NaCl and collecting the yellow precipitated product. Additional products could be isolated by extracting the saturated salt solution with acetone. The product was purified by dissolving in ether, drying over calcium sulfate, and recrystallizing from hot ether: yield 85 g, mp 35–38°, lit. (Harris, 1927) mp 40°.

2,2,6,6-Tetramethyl-4-hydroxypiperidine (II). To 5 g of I in 15 ml of methyl alcohol was added 1 g of $NaBH_4$ in small increments. After 2.5 hr the solvent was removed by evaporation and 4 ml of water was added and enough HCl to destroy the unreacted $NaBH_4$. Cold NaOH was added to precipitate the product, which was crystallized from ether: yield 2.7 g, mp 128–129°, lit. (Rozantzev, 1965) mp 128–129°.

[1] Abbreviations used: ADP-R·, adenosine 5′-diphosphate-4-(2,2,6,6-tetramethylpiperidine-1-oxyl); subscripts, t, b, f (*i.e.*, [ADP-R·]$_t$), total, bound, and free; apoenzyme, zinc-free alcohol dehydrogenase.

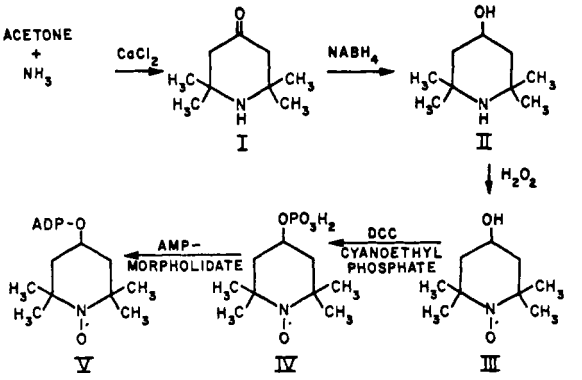

FIGURE 1: Schematic representation of the synthesis of ADP-R· (V) from acetone and ammonia.

2,2,6,6-Tetramethyl-4-hydroxypiperidine-1-oxyl (III). The paramagnetic compound was prepared by the general procedure of Rozantzev and Krinitskaya (1965). To 1.8 g of II in 10 ml of water was added 0.2 g of EDTA, 0.2 g of $NaWO_4$, and 3 ml of 30% H_2O_2. The solution was left at room temperature for 6 days. Upon extraction with chloroform the product was isolated and recrystallized from 1:3 ether–pentane: yield 700 mg, mp 70–71°, lit. (Rozantzev, 1965) mp 71.5°.

2,2,6,6-Tetramethyl-4-phosphopiperidine-1-oxyl (IV). The phosphorylation of III was based on the procedure outlined by Tener (1961). To 1 mmole of III (172 mg) was added 4 ml of 1 M cyanoethyl phosphate in aqueous pyridine. The solution was evaporated to dryness and reconstituted with 5 ml of pyridine. This was repeated four times to remove the water. To the final mixture, in 5 ml of pyridine, was added 1.5 g of *N,N′*-dicyclohexylcarbodimide. After 3 days at room temperature the reaction was terminated by adding 2 ml of water. The urea formed was removed after 1 hr by filtration and washed with 25 ml of water. The wash was combined with the yellow solution and the resulting solution was made 0.4 M in LiOH. The basic solution was refluxed for 1 hr to hydrolyze the cyanoethyl group, then filtered. To the yellow supernatant was added 6 ml of water and additional precipitate was removed. The yellow solution was passed through a 2 × 28 cm Dowex 50X-8 ion-exchange column to remove the lithium. The phosphorylated product was made basic with pyridine, and used without further purification.

Adenosine 5′-Diphosphate-4(2,2,6,6-tetramethylpiperidine-1-oxyl) (V). The solution of IV in pyridine was evaporated to dryness many times. To the flask was added 5 ml of dry pyridine and 600 mg of AMP morpholidate (Calbiochemical Co.); the resulting solution was evaporated to dryness and 5 ml of dry pyridine was added and the evaporation was repeated five more times. The reaction proceeded at room temperature in 5 ml of pyridine for 5 days and was terminated by the addition of 15 ml of water. The solvents were removed under vacuum on a rotary vacuum evaporator keeping the temperature below 40°. More water was added and the evaporation was continued until the odor of pyridine was absent. Finally the precipitate was dissolved in 20 ml of water and the insoluble material was removed by filtra-

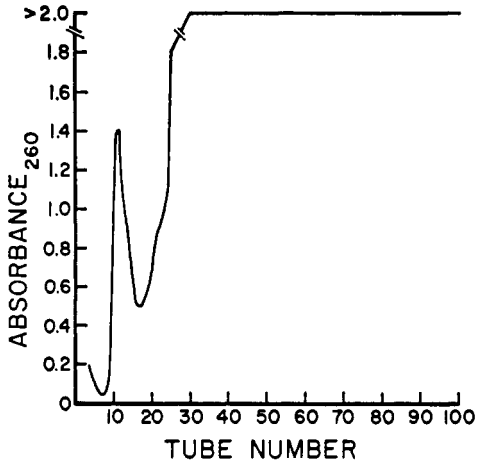

FIGURE 2: Chromatographic separation of 10 mg of ADP-R· from excess AMP on a 25 × 1 cm DEAE-cellulose column, using a phosphate gradient between μ = 0.006 and 0.2 at pH 6.0.

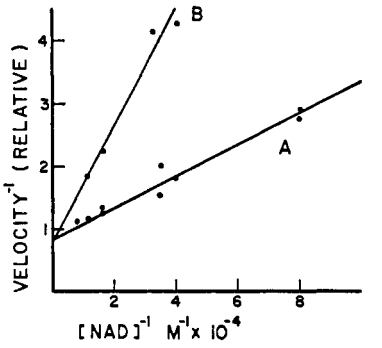

FIGURE 3: Double-reciprocal plot of the initial velocity (expressed in relative fluorescent changes per minute) of the oxidation of NAD by liver alcohol dehydrogenase. Line A is in the absence of inhibitor and line B is in the presence of 14.1 μM ADP-R·. The reaction was initiated by adding 0.1 μM liver alcohol dehydrogenase to the coenzyme and 8.7 mM ethanol in 2 ml of phosphate buffer at pH 6.0, μ = 0.05, at 25°.

tion. To the aqueous solution was added 350 mg of barium acetate and 100 ml of ethyl alcohol and the solution was left at −15° for 12 hr. The white precipitate was collected and dissolved in pH 6, μ = 0.006, phosphate and the barium salts were removed by centrifugation. The product was separated from AMP by chromatography on a DEAE-cellulose column using a linear gradient between μ = 0.006 and 0.2 phosphate. The results of the separation are presented in Figure 2.

The product was homogeneous on paper chromatography using ethanol–1.0 M ammonium acetate (pH 7.5, 7:3) or *n*-butyl alcohol–acetone–acetic acid–5% NH_4-OH–water (4.5:1.5:1:1:2) (Mangold, 1965). The migration of ADP-R·, V, was identical with that of commercial ADP ribose (Sigma Chemical Co.). The molar extinction coefficient for ADP-R· is ϵ_{260} 17,000.

Reagents. Sodium phosphate buffers were used. Metal-free buffers were prepared by extracting a concentrated buffer solution with dithizone (100 mg/l. of CCl_4) (Vallee and Gibson, 1948). Pyridine was dried by storing over calcium hydride.

Enzyme. Horse liver alcohol dehydrogenase was prepared by a method similar to that of Taniguchi *et al.* (1967). The preparation was homogeneous on starch gel, acrylamide gel, and cellulose acetate electrophoresis. Concentration of enzyme was determined by using A_{280} × 2.2 = mg/ml (Taniguchi *et al.*, 1967). The specific activity was 4000 μmoles/min per mg of protein and the purity was estimated to greater than 95%.

Yeast alcohol dehydrogenase was purchased from Sigma Chemical Co. and used without further purification.

Apoenzyme. The four zincs atoms of liver alcohol dehydrogenase (Åkeson, 1964) were removed by dialyzing the enzyme against 10^{-4} M EDTA in pH 6, μ = 0.05, phosphate for 36 hr at 4°. The apoenzyme had less than 5% catalytic activity and less than 0.2 g-atom of zinc/mole. The zinc content was determined by atomic absorption spectroscopy. A detailed discussion of the properties of apoenzyme liver alcohol dehydrogenase prepared in this manner will be presented elsewhere.[2]

Kinetics. Assays under V_{max} conditions were carried out as outlined by Dalziel (1957) in a 0.1 M glycine buffer. To test for competitive inhibition by ADP-R·, ADP ribose, or ADP glucose fluorescence assays were performed using an Aminco Bowman spectrofluorimeter. The samples were excited at 340 mμ and emission at 460 mμ was observed. The initial velocity as a function of substrate concentration was determined and the dissociation constants were calculated from Lineweaver–Burk plots. A representative example is presented in Figure 3.

Electron Paramagnetic Resonance Measurements. A Varian Model E-3 electron paramagnetic resonance spectrometer and quartz capillaries containing 0.05–0.1-ml samples were used as described by Mildvan and Cohn (1963). Spectra were recorded at a modulation amplitude of 1 gauss with a time constant of 0.3 sec and a scan time of 4 min at 22 ± 1°.

Binding Studies. All electron paramagnetic resonance binding studies were performed in metal-free phosphate buffer, pH 6.0, μ = 0.05, 22 ± 1°. As the ratio of enzyme to ADP-R· increased the electron paramagnetic resonance signal decreased as can be seen in Figure 4. To determine if NADH or 1,10-phenanthroline was competitive with ADP-R·, binding studies were conducted in their presence. A Varian C 1024 time-averaging computer was used with selected samples to improve the signal to noise ratios.

Results

Kinetic Inhibitory Properties of ADP-R·. The spin-labeled derivative was a coenzyme competitive inhibitor for liver alcohol dehydrogenase. Its inhibitory constant, K_I, was lower than that of ADP ribose or ADP glucose.

[2] H. Weiner and C. W. Hoagstrom, manuscript in preparation.

TABLE I: Dissociation Constants for Competitive Inhibitors of Liver Alcohol Dehydrogenase.

Inhibitor	K_I (μM)	K_I (μM) Lit.
ADP-R·[a]	9 ± 2	
ADP-R·[b]	5 ± 2	
ADP ribose		9.3[c]
ADP ribose[a]	88 ± 10	31[d]
ADP glucose[a]	480 ± 50	
ADP		380[e]

[a] pH 8, 0.02 M Tris, 25°. [b] pH 6, μ = 0.05 phosphate, 25°. [c] pH 7, μ = 0.1 phosphate, 23°; Yonetani (1963a). [d] pH 7, μ = 0.1 phosphate, 23°; Yonetani (1963a). [e] pH 7, μ = 0.1 phosphate, 23°; Yonetani (1964).

The results are summarized in Table I. As was to be expected, ADP-R· was also a competitive inhibitor toward yeast alcohol dehydrogenase; K_I = 20 μM.

Electron Paramagnetic Resonance Properties of ADP-R·. The electron paramagnetic resonance spectrum of ADP-R· is presented in Figure 4A. The spectrum is identical with those of the spin-labeled derivative prepared by McConnell and coworkers (Stone *et al.*, 1965; Griffith and McConnell, 1966; Berliner and McConnell, 1966).

The presence of liver alcohol dehydrogenase produced a marked decrease in the height of the electron paramagnetic resonance signal of the radical as can be seen in Figure 4B. When the ratio of radical to enzyme was high, as in the example presented in Figure 4, no broadening or alteration of the electron paramagnetic resonance signal could be observed. However, where the ratio was less than unity a marked change in the shape of the electron paramagnetic resonance signal could be observed.

The change in the shape of the bound radical signal could be best observed when repeated electron paramagnetic resonance scans were compiled in a time-averaging computer. Figure 5 presents the spectrum for 10 scans of a sample with a low radical to enzyme ratio. These spectra may be somewhat distorted due to the time-averaging techniques and possibly by over modulation. However, the qualitative differences between the bound and unbound radical can be observed. Figures 4A and 6A present typical spectra of the unbound radical which appear to be nearly identical with that of the unbound radical in Figure 5B.

The broadened center peak in Figure 5 and the new small peaks with a separation of 53 gauss are qualitatively similar to the findings of Griffith and McConnell (1966). Their work was with bovine serum albumin and covalently attached radical.

Titration of Liver Alcohol Dehydrogenase with ADP-R·. To measure the dissociation constant of the bound radical a titration of liver alcohol dehydrogenase with increasing radical concentration was performed. It was assumed that the bound radical made no contribution to the height of the electron paramagnetic resonance signal so that the ratio of the height of the signal in the

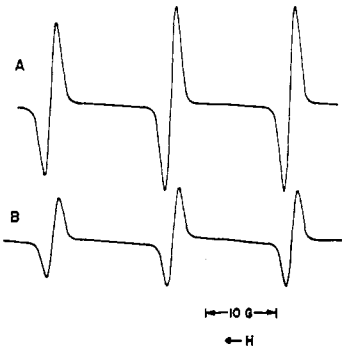

FIGURE 4: Electron paramagnetic resonance spectra of ADP-R· in the presence and absence of alcohol dehydrogenase. The upper curve (A) shows the spectrum for 0.57 mM ADP-R· and the lower curve (B) was the same concentration of ADP-R· in the presence of 50 μM liver alcohol dehydrogenase. Both curves obtained at pH 6.0 phosphate, μ = 0.05, 22°.

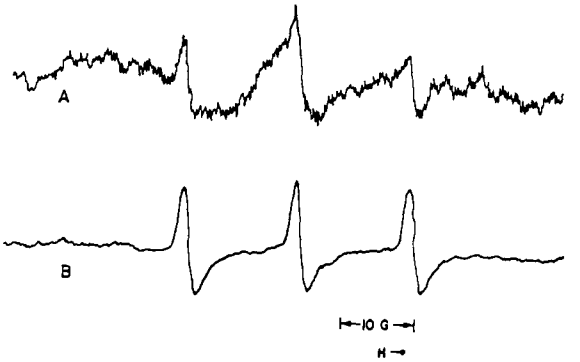

FIGURE 5: Electron paramagnetic resonance spectra for ADP-R· in the presence of an excess of alcohol dehydrogenase. Spectra obtained are the average of 10 scans programmed into a time-averaging computer. In the upper curve (A) 17.9 μM ADP-R· and 35.5 μM liver ADH are present. The lower curve (B) contains the radical in the absence of enzyme. The height of the two curves are not comparable for each was obtained using a different instrument gain. See Figure 6 for same samples run under identical conditions with no time averaging. Buffer was the same as described in Figure 4.

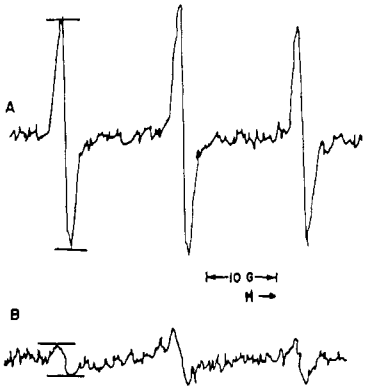

FIGURE 6: Electron paramagnetic resonance spectra for 17.9 μM ADP-R· in the presence and absence of liver alcohol dehydrogenase. The upper curve (A) shows the radical in absence of enzyme while the lower curve (B) was obtained in the presence of 35.5 μM liver alcohol dehydrogenase. The electron paramagnetic resonance signal was decreased in the presence of liver alcohol dehydrogenase. The height of the signal, indicated by the lines could then be used to determine the concentration of $[ADP\text{-}R\cdot)_f$. Buffer was the same as described in Figure 4.

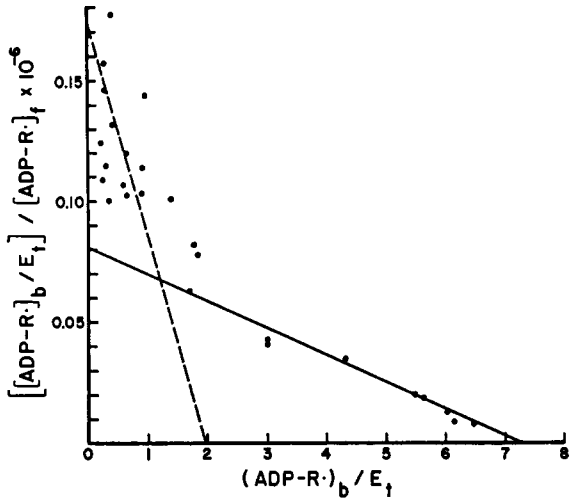

FIGURE 7: Scatchard plot of the electron paramagnetic resonance data for ADP-R· binding to liver alcohol dehydrogenase. The biphasic nature of the data reveals that two different types of binding sites are available for ADP-R·. The line intercepting at $[ADP\text{-}R\cdot]_b/E_t = 2$ was determined by a least-square computer fit of the data obtained at low values $[ADP\text{-}R\cdot]_b/E_t$ assuming that the x intercept was 2.

presence and absence of enzyme would be proportional to the concentration of unbound radical. Figure 6 presents a spectrum of the same solutions used to obtain the curves in Figure 5 except that in Figure 6 the curves were obtained under identical instrument conditions. It can be seen that the sample containing enzyme had very little signal when compared with the sample without enzyme. The assumption that the bound radical made a zero contribution to the net height of the electron paramagnetic resonance signal is not completely valid at low ratios of $[ADP\text{-}R\cdot]_f/[ADP\text{-}R\cdot]_b$; it does, however, permit one to evaluate dissociation constants by extrapolation.

Dissociation of ADP-R· from Liver Alcohol Dehydrogenase. The data obtained from the electron paramagnetic resonance measurements was treated by the method of Scatchard (1949) to determine both the dissociation constant and the moles of radical bound per mole of enzyme. The results of such a titration is presented in Figure 7. The biphasic nature of the curve suggests that two different types of binding sites are available for the radical. The data show that a total of 7–8 moles of radical bind per mole of enzyme. The experimental error at low radical concentrations was large. This was due partly to the low signal to noise ratio and partly to the assumption that the bound radical did not significantly contribute to the height of the electron paramagnetic resonance signal. An extrapolation to $[ADP\text{-}R\cdot]_b/E_t = 2$, which fits the data, leads to $K_D = 17$ μM. However, the scatter of the data leads to an experimental error in these constants of ±25%. Previous work had revealed that there were two coenzymes binding sites per mole of liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951).

The number of radical molecules bound to the second type of site was more easily obtained. An extrapolation to $(ADP\text{-}R\cdot)_b/E_t = 7\text{–}8$ could be made from the data in Figure 7. If there are two "active sites" and the five to six remaining sites all are identical, then the dissociation from the second type of "nonactive site" is 75 μM. In neither case is there any evidence for cooperative binding between the different sites or between members in the same type of site.

Effect of NADH on the Electron Paramagnetic Resonance Spectrum of ADP-R· with Liver Alcohol Dehydrogenase. NADH was added to a sample of ADP-R· and liver alcohol dehydrogenase and the results are presented in Table II. In the presence of 7.2 mM NADH all of the ADP-R· should have been displaced from the enzyme ($K_D = 0.2$ μM; Yonetani, 1963a) and the new spectrum should have been the same as that of 100% unbound ADP-R·. Only 69% of the radical was displaced from the enzyme in the presence of NADH; this implies that the radical binding to the nonspecific sites was not displaced by NADH.

In the absence of NADH, 81% of the radical was bound under the conditions of the experiment. After NADH addition, 31% of the radical remains bound (33.5 μM). Therefore the amount of radical bound to the "active site" was

$$[ADP\text{-}R\cdot]_b = [ADP\text{-}R\cdot]_t - [ADP\text{-}R\cdot]_f - [ADP\text{-}R\cdot]'_b$$
$$[ADP\text{-}R\cdot]_b = 108 - 20.5 - 33.5 = 54\ \mu M \quad (1)$$

where $[ADP\text{-}R\cdot]'_b$ is ADP-R· bound to the nonactive sites.

Thus a dissociation constant for the active sites radical can be calculated from eq 2.

$$K_D = \frac{(E_t - [ADP\text{-}R\cdot]_b)[ADP\text{-}R\cdot]_f}{[ADP\text{-}R\cdot]_b} \quad (2)$$

If two independent binding sites are assumed then with $E_t = 95.2$ μM, $K_D = 15.7$ μM.

The calculated dissociation constant represents an upper limit for K_D. In the above example 31% of the ADP-R· was bound to the weak sites when NADH was bound to the active sites. In the absence of NADH some of the $[ADP\text{-}R\cdot]_f$ would bind to the active site lowering

TABLE II: Effect of NADH on the Electron Paramagnetic Resonance Spectrum of a Sample Containing 108 μM ADP-R· and 47.6 μM Liver Alcohol Dehydrogenase.

[NADH] (mM)	Peak Height[a]	Unbound Radical (%)
	53[b]	100
	10	19
7.2	36	68
1.4	37	70
0.14	17	32
0.028	11	21

[a] Arbitrary units. [b] No liver alcohol dehydrogenase present.

TABLE III: Dissociation Constants for ADP-R· from Liver Alcohol Dehydrogenase Determined by Different Methods.

Method	K_D (μM)	N^a
Active Site		
Kinetic inhibition	5 ± 2	
Electron paramagnetic resonance binding	17 ± 8	2 ± 0.5
NADH displacement[b]	15	2 ± 0.5
Additional Site		
Electron paramagnetic resonance binding	75 ± 9	5.2 ± 0.3
NADH displacement[b]	146	5.2 ± 0.3

[a] N is the assumed number of binding sites. [b] See text for details.

both [ADP-R·]$_f$ and [ADP-R·]'$_b$. This would lead to a larger value of [ADP-R·]$_b$ and hence a lower K_D. The results for the various methods of calculating the dissociation constant summarized in Table III are in reasonable agreement.

Dissociation Constant for NADH from Liver Alcohol Dehydrogenase. From the data presented in Table II it was possible to calculate a dissociation constant for NADH from liver alcohol dehydrogenase. If one assumes that NADH does not bind to the "nonactive" sites, then its K_D = 50 ± 10 μM can be calculated. This value is much larger than the literature value of 0.2 μM obtained by fluorescence titrations (Yonetani, 1963a). It is not apparent why the different methods give such different answers.

Effect of o-Phenanthroline on the Electron Paramagnetic Resonance Spectrum of ADP-R· Bound to Liver Alcohol Dehydrogenase. *o*-Phenanthroline was added to a sample containing ADP-R· and liver alcohol dehydrogenase and the change in the height of the electron paramagnetic resonance signal was observed. Results are tabulated in Table IV. Even though *o*-phenanthroline's concentration was 100 times its K_I (K_I = 8 μM) (Yonetani, 1963a) and ADP-R· was only at 10 times its K_I, virtually no radical was displaced by the compound.

TABLE IV: Effect of *o*-Phenanthroline on a Sample Containing 123 μM ADP-R· and 46 μmoles of Liver Alcohol Dehydrogenase.

o-Phenanthroline (μM)	Unbound Radical (%)
	20.6
191	23.6
382	21.6
573	25.5
764	27.5

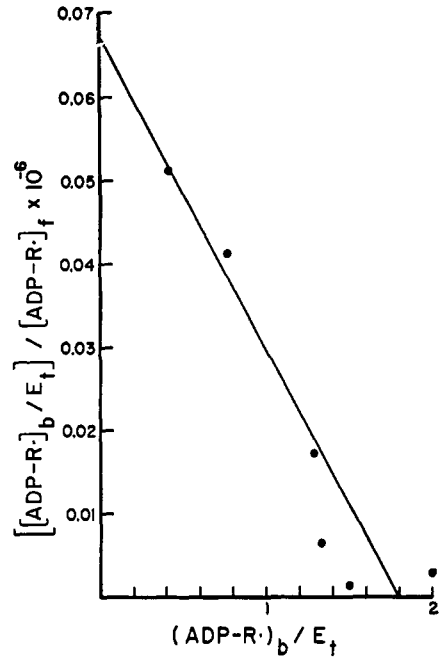

FIGURE 8: Scatchard plot of the electron paramagnetic resonance data for ADP-R· binding to apo liver alcohol dehydrogenase.

Yonetani (1963b) reported that alcohol dehydrogenase forms a ternary complex with ADP ribose and *o*-phenanthroline. The finding that *o*-phenanthroline did not displace the radical from liver alcohol dehydrogenase indicates that the radical binds in a manner which is similar to that of ADP ribose.

Effects of Ethanol, Acetaldehyde, and Isobutyramide on the Electron Paramagnetic Resonance Spectrum of ADP-R· to Liver Alcohol Dehydrogenase. Neither the substrates, ethanol and acetaldehyde, nor an inhibitor, isobutyramide, had any effect on the electron paramagnetic resonance spectra of the bound radical.

Dissociation Constant of ADP-R· from Apoenzyme. A titration of apoenzyme by ADP-R· was performed in a manner similar to the titration of liver alcohol dehydrogenase by radical. The electron paramagnetic resonance data obtained was treated by the method of Scatchard (1949) and the results are presented in Figure 8. The radical binds to the extent of *ca.* 1.8 ± 0.3 moles/mole of enzyme compared with *ca.* 2 ± 0.5 moles/mole with native enzyme. The dissociation constant is 27 ± 6 μM.

Most significant is that the ancillary sites which are present in native enzyme are absent in the apoenzyme. The dissociation constants are similar for the major site: 17 and 27 μM for native and apoenzyme.

Displacement of ADP-R· from Apoenzyme by NADH. When 239 μM NADH was added to a solution of 45 μM apoenzyme and 27.5 μM ADP-R· the amount of [ADP-R·]$_b$ decreased from 18.6 to 6.6 μM. From these data and using a K_D of 27 μM it was possible to calculate a dissociation constant of 22 μM for NADH if 1.8 sites/mole of enzyme was present. If there are two sites per mole then $K_{NADH\ apo}$ = 19 μM. The data for the binding of coenzymes to apoenzyme are summarized in Table V.

TABLE V: Dissociation Constants from Apo Liver Alcohol Dehydrogenase.

Compound	Sites/ Mole of Enzyme	K_D (μM)
ADP-R·	1.8 ± 0.3	27 ± 6
NADH[a]	1.8[b]	22
NADH[a]	2.0[b]	19

[a] NADH binding measured by its displacement of ADP-R· from enzyme. [b] Assumed number of sites per mole per enzyme.

Discussion

ADP-R· is found to bind as tightly to liver alcohol dehydrogenase as does ADP ribose. The nature of the "R" group in ADP-R· is important to some extent in determining the affinity because ADP glucose is as poor an inhibitor as is ADP (Table I). However one cannot predict the K_I from the size or hydrophilic character of the R group. The six-membered ring of the spin-labeled derivative and glucose are closer in size than are the radical and ribose, but the latter pair have nearly the same inhibitory ability. Glucose and ribose are more similar in hydrophilic character than are ribose and the radical, but differ by a factor of 10 in binding.

The electron paramagnetic resonance spectrum of ADP-R· changed dramatically when the radical was bound to the enzyme. Not only did the intensity of the signal decrease, but the basic shape of the curve also changed.

It has long been known that the electron relaxation times of organic radicals and of paramagnetic ions are altered by changing the environment resulting in a change in line width of the electron paramagnetic resonance signal. Cohn and Townsend (1954) used the broadening of the electron paramagnetic resonance spectrum of manganese to study its binding to proteins. McConnell (Stone *et al.*, 1965) has shown that the line width of the nitroxide radical is increased when the radical is immobilized on a macromolecule.

A broadening of the electron paramagnetic resonance signal can be expected when ADP-R· is bound to the enzyme if the radical is held firmly to the enzyme's surface. It has been shown that the nicotinamide ring of NADH has no freedom of motion independent of that of liver alcohol dehydrogenase (Weiner, 1968). It is conceivable that the ribose, or in this case the organic radical, will be held rigidly to the enzyme's surface since these groups are located between the fixed ADP and fixed nicotinamide portions of the coenzyme. The alteration and diminuation of the electron paramagnetic resonance signal are consistent with the hypothesis that all portions of the coenzyme are held rigidly to the enzyme surface.

Because of the multiple binding sites for the radical, an additional mechanism of broadening may exist. It is possible that one radical will broaden the electron paramagnetic resonance signal of another radical molecule if the two are close to each other. A plot of electron paramagnetic resonance signal intensity with ADP-R· alone did not deviate from linearity over a concentration range of 10^{-6}–10^{-3} M. However, it is possible that on the enzyme surface the two radicals are held very closely together and hence produce a diminution of the amplitude of the radical's electron paramagentic resonance signal.

There are two coenzyme binding sites in liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951). Kinetic experiments would not be expected to reveal that ADP-R· was binding to weak binding sites, but the electron paramagnetic resonance titration data reveal that two distinct types of binding sites are available to the radical. The strong binding site for ADP-R· is presumably the coenzyme binding site.

The five or six weak sites could simply be due to a nonspecific hydrophobic interaction between ADP-R· and the enzyme.[3] If this were the case one would not expect the radical portion of ADP-R· to be in the same environment in the two different types of binding sites on liver alcohol dehydrogenase. However, from the electron paramagnetic resonance spectra of the bound form it was not possible to differentiate between the two types of binding sites. It is difficult to assess the possible biological significance of the additional binding sites. The electron paramagnetic resonance data obtained in the presence of NADH suggests that NADH does not bind to the weak site. It is possible, however, that the sites have a very low affinity for NADH. The nature of the five or six weak sites is currently being investigated.

A catalytically inactive apoenzyme has been described (Åkeson, 1964; Drum *et al.*, 1967; Oppenheimer *et al.*, 1967). The apoenzyme prepared by EDTA treatment is still capable of binding either the coenzyme or the radical analog. The coenzyme is bound tightly to both native and apoenzyme thus suggesting that the two forms of the enzyme are similar. However some alteration of the apoenzyme had occurred since the five or six ancillary binding sites were not detected. Hence, zinc is not essential for binding coenzyme to the active site of liver alcohol dehydrogenase but is necessary for some other aspect of the over-all enzyme activity as suggested by Theorell and Yonetani (1963).

o-Phenanthroline is a competitive inhibitor for the coenzyme with liver alcohol dehydrogenase, but not against ADP ribose (Yonetani, 1963b). Theorell has shown that a ternary complex of ADP-R· and *o*-phenanthroline crystallizes identically with that of liver alcohol dehydrogenase (Zeppezauer *et al.*, 1967). The results of the data presented in Table IV indicate the existence of an analogous ternary complex between ADP-R·–*o*-phenanthroline–alcohol dehydrogenase.

The use of spin-labeled conjugates has led to a better

[3] ADP-R· was found to also bind to pyruvate kinase, but was not displaced by the substrate, ADP. This suggests that the radical binds to "nonactive" sites. But ADP-R· did not bind to creatine kinase, an enzyme which also possess an adenine binding site (A. S. Mildvan and H. Weiner, unpublished observations). These observations suggest that the radical will not bind to any protein but could bind preferentially to "nonactive" sites in selected systems. It is important, then, to differentiate between the active and nonactive site interactions.

understanding of the subunit interaction in hemoglobin (Boeyens and McConnell, 1966; Ohnishi *et al.*, 1966; Ogawa and McConnell, 1967), and to the active site region of chymotrypsin (Berliner and McConnell, 1966). The extension of the original concept of spin-labeled compound to coenzymes will allow an investigation of the region of the active site of the dehydrogenases. In addition to electron paramagnetic resonance spectroscopy, the introduction of an unpaired electron into a nonparamagnetic enzyme permits one to apply other paramagnetic resonance techniques to study the interactions of substrates with the enzymes. Such experiments will be described in future publications.

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