Interactions of Substrates, Inhibitors, and Coenzymes at the Active Site of Horse Liver Alcohol Dehydrogenase*

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DAVID S. SIGMAN

From the James Conant Laboratory of the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

SUMMARY

2,2-Bipyridine chelates 2 zinc ions of horse liver alcohol dehydrogenase with a dissociation constant of $4.0 \times 10^{-4}$ M. The complex shows an absorption maximum at 308 nm with a difference extinction coefficient of $1.1 \times 10^{4} M^{-1} cm^{-1}$ per zinc ion. Because bipyridine binds the enzyme less tightly than o-phenanthroline, and because the principal absorption maximum of the difference spectrum appears at longer wavelengths, this reagent is preferred as a tool in the determination of the binding constants of inhibitors, substrates, and coenzymes.

The dissociation constants for binary complexes of enzyme with alcohols, aldehydes, mercaptans, carboxylic acids, and amides, as well as with imidazole and pyrazole, have been determined by measuring the diminution that they cause in the intensity of the enzyme-bipyridine spectrum.

The competitive inhibitors ADP-ribose, AMP, ADP, and 4-biphenylcarboxylic acid do not interfere with the enzyme-bipyridine spectrum, whereas the coenzymes NAD$^{+}$ and NADH diminish this spectrum. These results confirm the conclusion, previously advanced by Yonetani, that the nicotinamide moiety of the coenzyme lies near the zinc ion of the active site, and that this zinc ion is part of the substrate-binding site.

Ternary complexes of enzyme, coenzyme, and inhibitor have been detected from the changes in enzyme-bipyridine spectrum; these complexes therefore require the enzymic zinc ion at the active site, or form near it, or both.

Horse liver alcohol dehydrogenase in the presence of NAD$^{+}$ catalyzes the reversible oxidation of a variety of primary and secondary alcohols to their corresponding aldehydes (1). The enzyme is a stable protein of molecular weight 84,000 (2, 3), contains 28 free sulfhydryl groups (4), and has two independent active sites per molecule (5, 6). Following the discovery of zinc ion in yeast alcohol dehydrogenase (7), nondialyzable zinc ion was also found in the horse liver enzyme (8, 9). Although 2 zinc ions per molecule of enzyme were reported initially (8, 9), the most recent analytical data suggest that the enzyme may contain as many as 4 zinc ions per molecule (10). Two of the enzymic zinc ions can be exchanged for $^{65}$Zn by equilibrium dialysis in 0.1 M succinate buffer, pH 6.0 (11, 12). 1,10-Phenanthroline competitively inhibits the enzyme with respect to the coenzymes (13). The similarity between the characteristic ultraviolet absorption spectrum of phenanthroline when it chelates ionic zinc and that observed when the chelating agent is mixed with the enzyme indicates that phenanthroline binds the zinc ions of horse liver alcohol dehydrogenase (14, 15). These spectrophotometric and kinetic results have suggested that the zinc ions are at or near the binding site for the coenzymes (16).

The characteristic enzyme-phenanthroline spectrum has been used as a probe of the interactions taking place at the enzymic zinc ion site. For example, Yonetani (17, 18) showed that both NAD$^{+}$ and NADH diminish the intensity of the enzyme-phenanthroline spectrum, whereas adenosine diphosphoribose, which is a potent kinetic inhibitor, does not. The zinc ion of this enzyme is therefore near the nicotinamide moiety of the coenzyme, and remote from the ADP-ribose-binding site. Ulmer, Li, and Vallee (19-23) have utilized the distinctive optical rotatory dispersion curve of the enzyme phenanthroline complex to investigate interactions near the enzymic zinc ion.

Although the coenzyme has been shown to interfere with the ability of phenanthroline to chelate the enzymic zinc ion, no evidence has previously been presented which shows that substrates or substrate inhibitors interact at or near the enzymic zinc ion. It has now been found that 2,2-bipyridine is a useful reagent with which to study the interaction of substrates, inhibitors, and coenzymes at the active site of horse liver alcohol dehydrogenase and that zinc ion is a part of the binding site for the substrates at the active site.

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† National Institutes of Health Predoctoral Fellow, 1963 to 1965. Present address, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115.

† In this paper phenanthroline represents 1,10-phenanthroline, and bipyridine, 2,2-bipyridine.
EXPERIMENTAL PROCEDURE

Enzyme—Horse liver alcohol dehydrogenase was purchased from either Worthington or Boehringer. Both commercial sources prepared the enzyme according to the method of Bonnichsen and Brink (24).

Each commercial preparation of enzyme was chromatographed by the method of Dalziel (25) and revealed two chromatographically distinct species. However, the relative amounts of the two proteins are reversed from those reported (25) for a newer method of preparation. The material from the larger peak in the commercial preparation was used in these studies. Experiments performed with enzyme prepared by both the old and new methods have yielded similar kinetic results (26).

After the enzyme had been chromatographed, it was crystallized twice and stored as a crystalline slurry in 0.02 M phosphate containing 10% ethanol. Some experiments were performed with unchromatographed enzyme with essentially the same results as those from a single chromatographic peak. A slurry of enzyme prepared by both the old and new methods was assayed spectrophotometrically as 89% pure on the basis of a molecular weight of 763 (NADH NA2.3H2O) and an extinction coefficient of 8.8 x 10^6 

Enzyme Assay—Enzyme activity was followed by the appearance or disappearance of the fluorescence of NADH. With a Photovolt multiplier fluorescence meter, model 540, coupled to a Sargent recorder, model S-1R. Incident light from a mercury lamp was filtered to isolate the 366 m wavelength, and emission was observed in the 410 to 580 m wavelength region. All kinetic studies were carried out either at 25° ± 0.1° or 30° ± 0.1°.

The difference spectra were determined with a Cary model 14 recording spectrophotometer, with a 0 to 0.1 slide wire attachment. The reference cuvette contained the same concentrations of reagents as the sample cell, except for ione and the enzyme. Since the enzyme absorbs at some of the wavelengths studied, its absorption was subtracted from the difference absorbance prior to calculating the difference extinction coefficient for complexes involving the enzyme.

RESULTS

Interaction of Chelating Agents with Dehydrogenase—Chelating agents can interact with metalloproteins in one of three ways. They can chelate the metal while it is simultaneously bound to the protein, as in Equation 1,

\[ E-Me + C \rightleftharpoons E-Me-C \]  
(1)

(where E represents the chelation site of the enzyme, Me the metal, and C the chelating agent), or they can compete with the protein for the metal according to Equation 2.

\[ E-Me + xC \rightleftharpoons E + Me(C)_{x} \]  
(2)

If the protein is denatured in the absence of the metal, the second equilibrium will be essentially irreversible and the chelating agent can remove all the metal from the protein. Finally, chelating agents can interact by a combination of the above processes as indicated in Equations 1 and 3.

\[ E-Me + C \rightleftharpoons E-Me-C \]  
(1)

\[ E-Me-C + xC \rightleftharpoons E + Me(C)_{x} \]  
(3)

In this case, the E-Me-C complex is stable, but if it is exposed to high concentrations of chelating agents for long periods of time, irreversible inactivation will occur. If low concentrations of chelating agents are used and measurements are made soon after the chelating agent is mixed with the enzyme, the reaction represented by Equation 3 can be minimized and the E-Me-C complex will be stable.

1,10-Phenanthroline—This compound interacts with horse liver alcohol dehydrogenase according to Equation 1 at low phenanthroline concentrations. The dissociation constant of the enzyme-phenanthroline complex has been found by Yonetani (17) to be 8 x 10^{-10} at pH 7.0 and by Vallee and Coombs (15) to be 3.3 x 10^{-14} at pH 7.0. The present author found the value to be 8.8 x 10^{-8} at pH 7.0. In order to calculate the dissociation constants, 2 noninteracting zinc ions were considered to be chelated per molecule of enzyme (15, 17, 18).

2,2'-Bipyridine—This compound also interacts with the enzyme according to Equation 1 at concentrations less than 5 x 10^{-7} M. The distinctive enzyme-bipyridine (E-BP) spectrum (Fig. 1) appears immediately upon mixing the chelating agent and the enzyme. A solution of enzyme and bipyridine has the absorption at 308 m which in the presence of either 2 x 10^{-3} M EDTA or 2 x 10^{-3} M EDTA at pH 7.0. The dissociation constant for E-BP was calculated by a graphical procedure by using the absorbance at 308 m as the measure of complex formation. If
\[
K = \frac{(E)(BP)}{(E-BP)}
\]

then
\[
\frac{1}{A} = \left(\frac{K}{\epsilon E_t}\right) \left(\frac{1}{BP}\right) + \frac{1}{\epsilon E_t}
\]

where

- \(E_t\) = total enzyme concentration in active sites
- \((E)\) = free enzyme concentration in active sites
- \((BP)\) = free bipyridine concentration
- \((E-BP)\) = E-BP concentration
- \(A\) = absorbance at 308 mp = \(\epsilon(BP)\)
- \(\epsilon\) = difference extinction coefficient of E-BP at 308 mp

Values for \(K\) and \(\epsilon\) were then obtained in the following way. As a first approximation, \((BP)\), the free bipyridine concentration, was assumed to be equal to \((BP_t)\), the total bipyridine concentration. This assumption is justified because bipyridine was generally present in a large molar excess (usually greater than 10-fold) over the enzyme. \(1/A\) was then plotted with respect to \(1/(BP)\).

The value of \(\epsilon\) that was obtained from this plot was then used to determine the concentration of the enzyme-bipyridine complex at a given total bipyridine concentration. Since

\[
(BP_t) = (E-BP) + (BP)
\]

\((BP)\), the free bipyridine concentration at each point, could be obtained. \(1/A\) was then plotted as a function of \(1/(BP)\), and a new value of \(\epsilon\) was obtained. This procedure was repeated until constant values of \(\epsilon\) and \(K\) were obtained. In practice, only a single iteration was necessary.

A typical determination of \(K\) and \(\epsilon\) is illustrated by Fig. 2, in which \(1/A\) is plotted with respect to \(1/(BP)\). The values of \(K\) and \(\epsilon\) obtained at \(24^\circ\) in 0.045 M phosphate buffer at pH 7.0, are

\[
K = 4.0 \pm 0.4 \times 10^{-4} \text{M}^{-1}\text{cm}^{-1}, \quad \epsilon = 1.1 \pm 0.1 \times 10^4 \text{M}^{-1}\text{cm}^{-1}
\]

chiometry of the complex of bipyridine and enzyme is 1:1 per active site and that the active sites are independent. The constancy of the calculated values of \(K\) and \(\epsilon\) over a wide range of concentrations of bipyridine supports the 1:1 stoichiometry. The formation of a complex with a stoichiometry of 2:1 can be ruled out, since such a complex would require that a plot of \(1/A\) with respect to \(1/(BP)\) be linear, and such is not the case.

There are two findings which are consistent with (but not proof of) the binding of 1 bipyridine molecule per active site of enzyme. First, the value of the difference extinction coefficient for the E-BP complex is \(1.1 \times 10^4 \text{M}^{-1}\text{cm}^{-1}\). This is roughly one-third of the difference extinction coefficient per zinc for the 3:1 complex (Fig. 1) of bipyridine and ionic zinc, but significantly less than \(1.68 \times 10^4 \text{M}^{-1}\text{cm}^{-1}\), which is the value for the 1:1 complex. If 4 zinc ions of the enzyme were all bound by bipyridine, the difference extinction coefficient per zinc site would be \(5.5 \times 10^3 \text{M}^{-1}\text{cm}^{-1}\), which is low when compared to either possible value of the model system. Second, Vallee and Coombs (15) and Yonetani (17) have demonstrated that phenanthroline binds 1 enzymic zinc ion per active site. Since phenanthroline and bipyridine are very similar chelating agents, the latter probably also binds 1 zinc ion per active site.

A comparison of the various constants of the enzyme-bipyridine and enzyme-phenanthroline complexes is made in Table I.
### Table I

**Comparison of relevant constants of enzyme-bipyridine and -phenanthroline complexes**

<table>
<thead>
<tr>
<th>Constant</th>
<th>Bipyridine</th>
<th>Phenanthroline</th>
</tr>
</thead>
<tbody>
<tr>
<td>E of C &lt;i&gt;&lt;sub&gt;Xmax&lt;/sub&gt;&lt;/i&gt;</td>
<td>8 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>8 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
</tr>
<tr>
<td>Difference extinction coefficient at &lt;i&gt;&lt;sub&gt;Xmax&lt;/sub&gt;&lt;/i&gt;</td>
<td>308 nu</td>
<td>297 nu</td>
</tr>
<tr>
<td>&lt;i&gt;&lt;sub&gt;C&lt;/sub&gt;&lt;/i&gt; at &lt;i&gt;&lt;sub&gt;Xmax&lt;/sub&gt;&lt;/i&gt;</td>
<td>4 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>4 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
</tr>
<tr>
<td>&lt;i&gt;&lt;sub&gt;C&lt;/sub&gt;&lt;/i&gt; of enzyme at &lt;i&gt;&lt;sub&gt;Xmax&lt;/sub&gt;&lt;/i&gt;</td>
<td>4 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
<td>4 x 10&lt;sup&gt;-4&lt;/sup&gt; M</td>
</tr>
<tr>
<td>Dissociation constant of E-C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>308 nm</td>
<td>297 nm</td>
</tr>
</tbody>
</table>

<sup>a</sup> C represents either bipyridine or phenanthroline.

### Table II

**Dissociation constants for complexes of enzyme with substrates and substrate inhibitors at pH 7.0**

The concentrations of bipyridine, substrates, and substrate inhibitors at which diminution of the enzyme-bipyridine spectrum can be observed are included in the table. The enzyme concentrations generally used ranged from 1 to 3 x 10<sup>-5</sup> M (in active sites).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>(BP&lt;sub&gt;&lt;i&gt;&lt;i&gt;B&lt;/i&gt;&lt;/i&gt;)&lt;sub&gt;1&lt;/sub&gt;)</th>
<th>K&lt;sub&gt;&lt;i&gt;&lt;i&gt;B&lt;/i&gt;&lt;/i&gt;&lt;/sub&gt;&lt;sub&gt;&lt;i&gt;&lt;i&gt;P&lt;/i&gt;&lt;/i&gt;&lt;/sub&gt;</th>
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<tr>
<td><strong>Carboxylic acids</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Acetic</td>
<td>0.442</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.9</td>
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<tr>
<td>Butanoic</td>
<td>6.5 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.8 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexanoic</td>
<td>9.69 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>7.7 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octanoic</td>
<td>6.07 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>1.31 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>2.1 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>Decanoic</td>
<td>3.00 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.30 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>6.8 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<td><strong>Alcohols</strong></td>
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<td>Ethanol</td>
<td>0.355</td>
<td>4.11 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.27</td>
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<td>1-Propanol</td>
<td>0.165</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>5.2 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>1-Butanol</td>
<td>6.50 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.1 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>1-Pentanol</td>
<td>0.131</td>
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<td>1.7 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>1.60 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1.31 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>7.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>Trifluoroethanol</td>
<td>0.373</td>
<td>1.27 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.502</td>
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<td><strong>Mercaptans</strong></td>
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<tr>
<td>Ethanethiol</td>
<td>5.66 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.8 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>Butanethiol</td>
<td>6.44 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.31 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<td><strong>Amides</strong></td>
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<tr>
<td>Formamide</td>
<td>0.500</td>
<td>1.30 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
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<tr>
<td>Acetamide</td>
<td>0.477</td>
<td>1.30 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.61</td>
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<td>n-Butyramide</td>
<td>0.111</td>
<td>4.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>6.8 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>n-Hexamid e</td>
<td>2.09 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>2.10 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.0 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>Isobutamide</td>
<td>7.60 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.96 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.12</td>
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<tr>
<td>Trimethylacetamide</td>
<td>5.32 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.11</td>
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<tr>
<td>Benzamide</td>
<td>4.28 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.15</td>
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<tr>
<td>N,N-Dimethylamides</td>
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<tr>
<td>N,N-Dimethylformamide</td>
<td>0.469</td>
<td>1.30 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>9.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<td>N,N-Dimethylacetamide</td>
<td>0.312</td>
<td>3.96 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.15</td>
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<tr>
<td>N,N-Dimethyl-n-butyramide</td>
<td>7.28 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3.96 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2.8 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<td><strong>Aldehydes</strong></td>
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<tr>
<td>Acetaldehyde</td>
<td>0.379</td>
<td>4.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.37</td>
</tr>
<tr>
<td>n-Butyraldehyde</td>
<td>4.72 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2.8 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td><strong>Nitrogen bases</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Imidazole&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.82 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.20 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyrazole</td>
<td>3.09 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.4 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>Nicotinamide</td>
<td>9.90 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>1.9 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<tr>
<td>Nicotinic acid</td>
<td>7.13 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>8.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Imidazole constant was calculated for the free base with a pK<sub>a</sub> of 7.10.
enzymic zinc ions. All the compounds in Table II reduced the intensity of the enzyme-bipyridine spectrum if added to a solution of bipyridine and the enzyme in the concentration ranges indicated.

With all the compounds studied, the shape and wave length maximum of the enzyme-bipyridine spectrum in the region of 308 m\(\mu\) were unchanged. Difference spectra were always used; the various substrates and substrate inhibitors were present in the reference cell as well as the sample cell. The only compounds studied which had significant absorption at 308 m\(\mu\) were acetaldheyde and butyraldehyde; even they, however, presented no great experimental problem. Routinely, EDTA, at a concentration of about 0.01 M, was added to chelate any nonenzymic metal impurity that might be present.

The simplest possible mechanism to account for the reduction of the characteristic enzyme bipyridine spectrum by substrates and substrate inhibitors is competition by these compounds with bipyridine for the binding site for bipyridine. It is assumed that the enzyme-bipyridine complex is replaced by the enzyme-substrate (or enzyme-inhibitor) complex; the extent of this displacement is measured by the absorbance change at 308 m\(\mu\).

If one uses a dissociation constant of 4 \(\times\) 10^{-4} M and a difference extinction coefficient of 1.1 \(\times\) 10^{4} M^{-1} cm^{-1} at 308 m\(\mu\) for the enzyme-bipyridine complex, binary dissociation constants for the substrates and substrate inhibitors can be calculated. These dissociation constants are defined by the equation

\[
K_{E,S} = \frac{(E)(S)}{(E-S)},
\]

where \((S)\) represents the concentration of free substrate or inhibitor. \(K_{E,S}\) can be computed by making use of the relation

\[
K = \frac{(E-S)(BP)}{(E-BP)(S)} = \frac{K_{E,BP}}{K_{E,S}}
\]

where \((BP)\) is total concentration of bipyridine present. \((E-S)\) can be computed from the relation

\[
(E-S) = \frac{1}{1} \frac{K_{E,BP}}{K_{E,S}} [E - (E-BP)]
\]

where

\[
K_{E,BP} = \frac{[E] - (E-BP)(BP)}{(E-BP)(S)},
\]

\[
K_{E,S} = \frac{[E] - (E-BP)(S)}{(E-BP)(BP)}
\]

The failure of the coenzyme inhibitors to diminish the intensity of the enzyme-bipyridine spectrum is explained by the fact that these compounds do not compete with bipyridine for any part of the binding site of the chelating agent. The failure of the coenzyme inhibitors to diminish the intensity of the benzyl carboxamide group in the bipyridine spectrum. Table III indicates the results obtained.

The failure of the coenzyme inhibitors to diminish the intensity of the inhibition or dissociation constants suggests that these compounds do not compete with bipyridine for any part of the binding site of the chelating agent. The effect of coenzyme and coenzyme analogues on enzyme-bipyridine spectrum—The coenzymes NAD+ and NADH diminish the intensity of the enzyme-bipyridine spectrum. However, kinetic studies revealed that bipyridine, unlike phenanthroline, does not inhibit the enzyme competitively. Fig. 3 shows that, depending on the concentration of coenzyme, bipyridine can either accelerate or inhibit the enzyme reaction. Therefore, attempts to use changes in the enzyme-bipyridine spectrum to evaluate the thermodynamic dissociation constants of the enzyme coenzyme complexes necessarily failed.

McKinley-McKee (31) previously found that imidazole exhibits similar complex kinetics. These authors explained the rate acceleration in terms of a ternary complex composed of enzyme, coenzyme, and imidazole, in which the rate of dissociation of the ternary complex is greater than the rate of dissociation of the coenzyme from a binary complex composed of the enzyme and coenzyme alone. The analysis of these spectrophotometric data according to the kinetic scheme of Theorell and McKinley-McKee (29) has been reported elsewhere (32).

In order to assess the importance of the carbamoyl group in preventing bipyridine from binding to the enzymic zinc ions, NAD+ and the coenzyme analogues PyAlAD+ were studied to see which was more effective in diminishing the characteristic spec-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>(K_d) (BP)</th>
<th>(j)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M)</td>
<td>(M)</td>
<td>(M)</td>
</tr>
<tr>
<td>4-Biphenylycarboxylic acid</td>
<td>1.11 (\times) 10^{-3}</td>
<td>6 (\times) 10^{-4}</td>
<td>1.60 (\times) 10^{-3}</td>
</tr>
<tr>
<td>4-Biphenylcarboxylic acid</td>
<td>1.09 (\times) 10^{-4}</td>
<td>6 (\times) 10^{-4}</td>
<td>1.33 (\times) 10^{-3}</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>1.23 (\times) 10^{-2}</td>
<td>2.6 (\times) 10^{-2}</td>
<td>1.27 (\times) 10^{-3}</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>2.33 (\times) 10^{-2}</td>
<td>2.6 (\times) 10^{-2}</td>
<td>1.33 (\times) 10^{-3}</td>
</tr>
<tr>
<td>AMP</td>
<td>1.80 (\times) 10^{-4}</td>
<td>1.4 (\times) 10^{-4}</td>
<td>2.53 (\times) 10^{-4}</td>
</tr>
<tr>
<td>ADP</td>
<td>7.1 (\times) 10^{-4}</td>
<td>1.4 (\times) 10^{-4}</td>
<td>3.1 (\times) 10^{-4}</td>
</tr>
<tr>
<td>NMN</td>
<td>4.78 (\times) 10^{-4}</td>
<td>3.9 (\times) 10^{-4}</td>
<td>2.53 (\times) 10^{-4}</td>
</tr>
<tr>
<td>N-Benzylcytosine</td>
<td>1.30 (\times) 10^{-4}</td>
<td>9.7 (\times) 10^{-5}</td>
<td>5.96 (\times) 10^{-4}</td>
</tr>
<tr>
<td>N-Benzylnicotinamide chloride</td>
<td>5.16 (\times) 10^{-2}</td>
<td>3.1 (\times) 10^{-2}</td>
<td>2.52 (\times) 10^{-2}</td>
</tr>
<tr>
<td>N-Butylnicotinamide chloride</td>
<td>9.56 (\times) 10^{-2}</td>
<td>1.33 (\times) 10^{-2}</td>
<td>1.07</td>
</tr>
<tr>
<td>N-Pentynicotinamide chloride</td>
<td>6.87 (\times) 10^{-2}</td>
<td>5 (\times) 10^{-2}</td>
<td>3.96 (\times) 10^{-2}</td>
</tr>
<tr>
<td>N-Methylcytosine</td>
<td>8.13 (\times) 10^{-4}</td>
<td>7 (\times) 10^{-4}</td>
<td>2.96 (\times) 10^{-4}</td>
</tr>
</tbody>
</table>

\(j\) is the ratio of the absorbance increase at 308 m\(\mu\) in the presence of the indicated compound to the absorbance increase at 308 m\(\mu\) in the absence of the compound, but at the same concentration of bipyridine and enzyme.

\(K_d\) values obtained from Reference 30.

\(c\) Done at pH 9.0.
trum of the enzyme-bipyridine complex. Both were effective, although NAD$^+$ inhibited the binding of bipyridine to the enzymic zinc ion more than PyAlAD$^+$.

**Method for Demonstrating Competitive Inhibition with Respect to Coenzymes—**Since inhibitors such as ADP-ribose, AMP, and ADP do not diminish the intensity of the enzyme-bipyridine spectrum whereas the coenzymes do, the enzymic zinc ions must be near the nicotinamide moiety of the coenzyme. This conclusion has already been advanced by Yonetani (17, 18) on the basis of his work with phenanthroline. The enzyme-bipyridine spectrum can be conveniently used to demonstrate that an inhibitor competes with the coenzyme for the ADP-ribose-binding site. For example, NADH diminishes the intensity of the characteristic enzyme-bipyridine spectrum if added to a solution of a given enzyme and bipyridine concentration. If ADP-ribose is then mixed with the solution of NADH, enzyme, and bipyridine, a molecule which interferes with the binding of bipyridine at the zinc site is replaced by one that does not. As a result, the characteristic enzyme-bipyridine spectrum is restored. Fig. 4 illustrates the changes involved. Similar results have been obtained with ADP-ribose and NAD$^+$, or with AMP and 4-biphenylcarboxylic acid and either NAD$^+$ and NADH.

**Ternary Complexes—**Horse liver alcohol dehydrogenase and NAD$^+$ form several ternary complexes with a variety of substrate inhibitors, such as pyrazole (6), hydroxylamine (33), and aliphatic mercaptans (34). The enzyme presumably catalyzes the addition of these nucleophiles to position 4 of the nicotinamide ring. The complexes all have characteristic absorption spectra that are similar to that of NAD$^+$.

Yonetani (17, 18) has demonstrated that when ternary complexes composed of enzyme, NAD$^+$, and pyrazole are formed, phenanthroline is unable to bind at the enzymic zinc ion. Similarly, bipyridine is not able to chelate the enzymic zinc ion of this ternary complex, or of that composed of the coenzyme analogue AcPyAD$^+$, pyrazole, and the enzyme. Similar results have been obtained with PyAlAD$^+$, pyrazole has only a small effect on the enzyme-bipyridine spectrum at the concentrations used in this experiment.

The following experiment indicates that the ternary complex composed of enzyme, NAD$^+$, and pyrazole is stable. A solution composed of 1.88 X 10$^{-3}$ m NAD$^+$, 2.53 X 10$^{-3}$ m ADP-ribose, 1.32 X 10$^{-4}$ m bipyridine, and 2.01 X 10$^{-6}$ m enzyme (in active sites) had the same difference absorbance at 308 mp as a solution containing only bipyridine and enzyme at the same concentrations. If 2.28 X 10$^{-3}$ m pyrazole then was added, the spectrum of the enzyme-bipyridine complex completely disappeared. NAD$^+$ alone cannot compete effectively with ADP-ribose for the coenzyme-binding site, but since the ternary complex prevents the chelation of zinc by bipyridine, the absorbance at 308 mp is abolished. If pyrazole is added to a solution containing only enzyme, bipyridine, and ADP-ribose, no diminution of the enzyme-bipyridine spectrum is observed.

The ternary complexes composed of the enzyme, NAD$^+$, and either ethanethiol or butanethiol also prevent the binding of bipyridine to the enzymic zinc ion. Similar results are obtained with PyAlAD$^+$ or AcPyAD$^+$. Ternary complexes composed of NAD$^+$ and hydroxylamine, O-methylhydroxylamine, and N-methylhydroxylamine also have characteristic absorption spectra and are able to abolish the enzyme-bipyridine spectrum.

FIG. 4. Effect of NADH on enzyme-bipyridine spectrum at pH 7.0 in 0.045 m phosphate buffer, at 24°C in a 1-cm path length cell. The contents of the sample cell are indicated. The reference cell was identical except it lacked enzyme and NADH. Curve a, 1.53 X 10$^{-4}$ m NADH and 3.72 X 10$^{-3}$ m EDTA, or 1.53 X 10$^{-4}$ m NADH, 5.72 X 10$^{-3}$ m EDTA, and 2.94 X 10$^{-4}$ m ADP-ribose; Curve b, 3.44 X 10$^{-2}$ m EDTA, 2.72 X 10$^{-3}$ m ADP-ribose, 1.42 X 10$^{-3}$ m NADH, and 2.16 X 10$^{-3}$ m enzyme (active sites); Curve c, 3.20 X 10$^{-2}$ m EDTA, 1.32 X 10$^{-3}$ m bipyridine, 2.53 X 10$^{-4}$ m ADP-ribose, 1.52 X 10$^{-4}$ m NADH, and 2.01 X 10$^{-4}$ m enzyme; Curve d, 1.42 X 10$^{-4}$ m NADH, 3.44 X 10$^{-3}$ m EDTA, and 2.16 X 10$^{-4}$ m enzyme (active sites); Curve e, 1.52 X 10$^{-4}$ m NADH, 3.20 X 10$^{-3}$ m EDTA, 1.32 X 10$^{-4}$ m bipyridine, and 2.01 X 10$^{-6}$ m enzyme; Curve g, 3.44 X 10$^{-2}$ m EDTA and 2.16 X 10$^{-4}$ m enzyme.
Table IV

Effect of aliphatic carboxylic acids and NAD⁺ on enzyme-bipyridine spectrum

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Concentration</th>
<th>Acid</th>
<th>Concentration</th>
<th>(BPd)</th>
<th>f</th>
<th>f₁ × f₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>2.77 X 10⁻⁴</td>
<td>None</td>
<td>4.80 X 10⁻⁴</td>
<td>0.715⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77 X 10⁻⁴</td>
<td>Acetic</td>
<td>4.80 X 10⁻⁴</td>
<td>0.805⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77 X 10⁻⁴</td>
<td>Acetic</td>
<td>4.80 X 10⁻⁴</td>
<td>0.128⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77 X 10⁻⁴</td>
<td>Hexanoic</td>
<td>4.80 X 10⁻⁴</td>
<td>0.578⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.77 X 10⁻⁴</td>
<td>Decanoic</td>
<td>4.80 X 10⁻⁴</td>
<td>0.405⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PyAlAD⁺</td>
<td>1.57 X 10⁻⁴</td>
<td>None</td>
<td>4.20 X 10⁻⁴</td>
<td>0.940⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.57 X 10⁻⁴</td>
<td>Acetic</td>
<td>4.20 X 10⁻⁴</td>
<td>0.354⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcPyAD⁺</td>
<td>1.31 X 10⁻⁴</td>
<td>None</td>
<td>1.29 X 10⁻³</td>
<td>0.855⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.31 X 10⁻⁴</td>
<td>Acetic</td>
<td>1.29 X 10⁻³</td>
<td>0.185⁺</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Experimentally observed f.
* Calculated f value assuming no ternary complex formation between the coenzyme and carboxylic acid.

All the ternary complexes previously mentioned have distinctive spectra. In addition, the enzyme-bipyridine spectrum can be used to detect the existence of ternary complexes which do not have readily accessible absorption spectra. Suppose that a compound diminishes the absorbance at 308 µ, under given conditions, to an f value (defined in Table III) of f₁, whereas NAD⁺ or an NAD⁺ analogue, under the same conditions, reduces the absorbance to an f value of f₂. If both the compound and NAD⁺ were present together, under otherwise identical conditions, the absorbance at 308 µ should be reduced to an f value not less than f₁ × f₂ unless a ternary complex were present. If the absorbance is diminished to an f value significantly less than f₁ × f₂, the existence of a ternary complex can be inferred.

Ternary complexes consisting of enzyme, aliphatic carboxylic acids, and NAD⁺ have previously been postulated and their dissociation constants have been measured (26). Table IV shows that aliphatic carboxylic acids and NAD⁺ have a concerted effect in diminishing the enzyme-bipyridine spectrum and hence form a ternary complex with the enzyme at or near the enzymic zinc ion. Druyan and Vallee (12) have reached the same conclusion by showing that coenzymes and certain coenzyme inhibitors, in combination with substrates or substrate inhibitors, greatly retard the rate of exchange of the enzymic zinc ions.

Inhibition by Trifluoroethanol—Trifluoroethanol is a competitive inhibitor with respect to ethanol (Fig. 5) and an uncompetitive inhibitor with respect to NAD⁺. The inhibition constant calculated from the Lineweaver-Burk plot, in which the ethanol concentration is varied, is 7.2 X 10⁻⁴ M. Table II shows that trifluoroethanol, in the absence of NAD⁺, has a low affinity for the enzyme. However, NAD⁺ and trifluoroethanol when present together are effective in diminishing the characteristic enzyme-bipyridine spectrum (Table V). Thus, trifluoroethanol is a potent inhibitor of the enzyme because it forms a stable ternary complex with the enzyme and NAD⁺.

Inhibition by Aromatic Carboxylic Acids—4-Biphenylcarboxylic acid and 9-phenanthroic acid are effective inhibitors of horse liver

![Fig. 5. Kinetics in the presence of trifluoroethanol at a constant NAD⁺ concentration of 6.04 X 10⁻⁴ M and varied ethanol concentration; (Et) = 2.72 X 10⁻⁴ M, 25°, pH 7.0, in 0.045 M phosphate buffer. O---O, control; O---O, total trifluoroethanol = 4.68 X 10⁻⁴ M.]
alcohol dehydrogenase; their inhibition constants are $6 \times 10^{-5}$ M and $2 \times 10^{-4}$ M, respectively, in phosphate buffer, pH 7.0, at 30°C.

While aliphatic carboxylic acids are competitive inhibitors with respect to ethanol, these aromatic carboxylic acids are competitive with respect to the coenzyme. 4-Biphenylcarboxylic acid, like ADP-ribose, fails to reduce the intensity of the enzyme-bipyridine spectrum (Table III) and interferes with the normal action of NAD$^+$ in diminishing this spectrum.

### DISCUSSION

For two reasons, 2,2-bipyridine is frequently a more useful reagent than o-phenanthroline for studying the interactions of substrates and inhibitors with horse liver alcohol dehydrogenase. First, the enzyme-bipyridine complex shows a maximum in its difference spectrum from enzyme and reagent at 308 m$\mu$. At this wavelength both enzyme and bipyridine have low extinction coefficients, while that for the complex is $1.1 \times 10^4$ M$^{-1}$cm$^{-1}$. Second, the dissociation constant for the bipyridine complex is $4 \times 10^{-4}$ M, whereas that for o-phenanthroline is $8 \times 10^{-4}$ M; thus, those substrates and inhibitors with low affinity for the enzyme will cause a greater change in absorbance when competing with bipyridine.

The diminution of the enzyme-bipyridine spectrum by substrates and substrate inhibitors indicates that these compounds bind at or near the enzymic zinc ion. This result is consistent with the conclusion that the zinc ion is also near the binding site of the nicotinamide ring of the coenzyme. Since the enzymic reaction proceeds by direct hydrogen transfer (35), the substrate and the nicotinamide ring would most likely occupy contiguous positions on the enzyme surface.

Two factors are apparently important in determining the affinity of a substrate or a substrate inhibitor for the enzyme. Table II shows that the dissociation constants of aliphatic acids, alcohols, amides, aldehydes, dimethylamides, and mercaptans decrease with increasing chain length. Winer and Theorell (26) have previously postulated the existence of a hydrophobic region at the substrate-binding site, and the present results support their conclusion. Apparently, this hydrophobic region is specific for aliphatic compounds; 4-biphenylcarboxylic acid, although it is a potent inhibitor, is not bound at the hydrophobic site near the zinc, while hexanoic, octanoic, and decanoic acids are.

The second factor that appears important in determining the magnitude of the dissociation constants is the nature of the functional group. Ethyl mercaptan and butyl mercaptan have dissociation constants which are only $10^{-2}$ those of ethanol and butanol, respectively. The binding of both mercaptans and alcohols to the enzyme is probably stabilized by the same hydrophobic binding site of the enzyme, since an increase in length of the aliphatic chain from ethyl to butyl decreases the dissociation constants of both these types of compounds by a factor of 10. The greater affinity of the mercaptans for the enzyme compared to the alcohols can be most simply ascribed to the greater ability of the —SH group to form complexes as compared to the hydroxyl group at pH 7.0. The lower dissociation constants of the mercaptan complexes compared to those of the alcohol complexes are consistent with the postulate that the functional group of the aliphatic compounds actually undergoes complex formation with the enzymic zinc ion, and probably competes for one of the coordinate positions of the zinc ion that bipyridine occupies.

The dissociation constants at pH 7.0 for enzyme-substrate and enzyme-substrate-inhibitor complexes that have been obtained by using the enzyme-bipyridine spectrum are compared with those obtained by other methods in Table II. The values of the constants for the aliphatic carboxylic acids, ethanol, and imidazole are generally in good agreement. However, the constants for complexes with aliphatic amides and pyrazole determined in the present work are in serious disagreement with the values presently in the literature. Possible sources for the discrepancy have been discussed elsewhere (32). Dissociation constants for alcohols other than ethanol have not been determined previously, nor have constants for mercaptans, dimethylamides, and aldehydes.

Table VI indicates that complexes of alcohols, acids, dimethylamides, amides, and aldehydes of the same chain length have very similar dissociation constants. This similarity suggests that (a) the complex-forming capability of the various types of functional groups (with the exception of the —SH group) are roughly equal.
the same, and (b) the hydrophobic interaction between the aliphatic chains and the enzyme is a major force binding these compounds to the enzyme. Although n-butanethiol binds at the same hydrophobic sites as the other n-butyl derivatives, its affinity for the enzyme is greater than the other compounds as a result of the strong ability for complex formation of the -SH group.

4-Biphenylcarboxylic acid behaves in a markedly different manner from aliphatic carboxylic acids (e.g. decanoic acid); the affinity for the enzyme is greater than the other compounds as a result of the hydrophobic interactions between the aromatic chains and the enzyme. This latter site is remote from the zinc ion.

Forms ternary complexes with the coenzyme and enzyme that eliminate the enzyme-bipyridine spectrum, whereas 4-biphenylcarboxylic acid interferes with the normal action of NAD+ in diminishing the band at 308 nm. Finally, decanoic acid diminishes this spectrum. The contrasting behavior of the two inhibitors suggests that they are bound at different sites on the enzyme. The aliphatic carboxylic acids are bound at a substrate site near the zinc ion; the aromatic acids are bound at the site that holds the ADP-ribose moiety of the coenzyme. This latter site is remote from the zinc ion.

The ability of substrates to diminish the characteristic enzyme-bipyridine spectrum suggests that the presence of the coenzyme is not essential for the binding of the substrates to the enzyme. The Theorell-Chance mechanism, which is formulated as

\[ E + \text{NADH} \Rightarrow E - \text{NADH} \]

\[ \text{H}^+ + E - \text{NADH} + \text{aldehyde} \Rightarrow E - \text{NAD} + \text{alcohol} \]

\[ E - \text{NAD} \Rightarrow E + \text{NAD} \]

has been supported by extensive kinetic and equilibrium investigations (1). The absence of a term for binary complexes consisting of enzyme and substrate has led to the description of the Theorell-Chance mechanism as a compulsory order mechanism (36); according to this interpretation, substrates are unable to bind to the enzyme without prior binding of the coenzyme. The direct observation of binary complexes of aldehydes and alcohols with the enzyme, by means of the enzyme-bipyridine complex, prohibits this interpretation of the Theorell-Chance mechanism. Studies of the equilibrium reaction rates of horse liver alcohol dehydrogenase have also shown that the enzymic reaction does not proceed with a strictly compulsory order of substrate binding (30, 37).

Substrate inhibitors and coenzymes have a concerted effect in diminishing the enzyme-bipyridine spectrum because of the formation of ternary complexes. The ability of PyAlAD+ and AcPyAD+ to form ternary complexes which abolish the enzyme-bipyridine spectrum suggest that the carboxamide group of NAD+ is not essential in binding the coenzyme to the enzymic zinc ion. But, since the potent inhibitor ADP-ribose does not diminish the enzyme-bipyridine spectrum, the nicotinamide moiety of the coenzyme must lie near the zinc ion, as previously postulated by Yonetani (17).

Two types of ternary complexes have been found to abolish the enzyme-bipyridine spectrum. In one type, a nucleophile such as pyrazole or butanethiol adds to position 4 of the nicotinamide ring of NAD+. In this case, the abolition of the enzyme-bipyridine spectrum may arise from a steric effect. Ternary complexes in which no addition to the nicotinamide ring takes place, such as those observed with NAD+ and carboxylic acids, NAD+ and trifluoroethanol, NADH and amides, or NADH and ethanol, probably diminish the enzyme-bipyridine spectrum by a different mechanism. All these substrate inhibitors have already been shown to bind at the zinc ion that is chelated by bipyridine. Since the substrates or substrate inhibitors bind more tightly in ternary complex than in binary complexes, they will compete more effectively with bipyridine for the zinc site in the presence of the coenzyme than in its absence.

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REFERENCES


Interactions of Substrates, Inhibitors, and Coenzymes at the Active Site of Horse Liver Alcohol Dehydrogenase
David S. Sigman


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