Interaction of Pyridine-Nucleotide linked Enzymes

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The oxidations of lactate by lactic dehydrogenase (LDH), glutamate by glutamic dehydrogenase, and alcohol by liver and yeast alcohol dehydrogenase (ADH) have been found to be somewhat slower with diphasopyridine nucleotide (DPN) bound to glyceraldehyde-3-phosphate dehydrogenase (TDH) than with free DPN. In contrast, the reduction of pyruvate by LDH and acetaldehyde by liver ADH was faster with reduced DPN (DPNH) bound to TDH than with free DPNH. The equilibrium constants of the systems were in all cases displaced towards the reduced substrate with bound DPN.

The overall kinetics of the liver ADH system using DPN(H) bound to TDH (TDH . DPN and TDH . DPNH) has been studied in some detail. The reaction mechanism appeared to be the same as with free DPN(H).

When liver ADH was added to TDH . DPNH which had been reduced by glyceraldehyde-3-phosphate, the absorption peak of DPNH shifted from 340 mμ to 330 mμ. If the bound DPN was reduced by liver ADH and alcohol, the absorption peak shifted down to 322 mμ, which is in the same region as the absorption peak of ADH-DPNH itself. No complex between liver ADH and TDH . DPNH could be observed in the ultracentrifuge.

Implications of these findings have been discussed.

Most oxidation-reduction reactions at the "substrate" level occur by virtue of pyridine nucleotide-linked (PN) enzymes. In the past it has often been explicitly or implicitly implied that the PN coenzymes themselves act as mobile oxidation-reduction (O/R) carriers between the various dehydrogenase systems. Several lines of evidence, however, allow alternative interpretations. Although these coenzymes are often readily dissociable from their respective apoenzymes, this is by no means always the case. Taylor et al.1 have shown that crystalline triosephosphate dehydrogenase (TDH) contains firmly bound diphasopyridine nucleotide (DPN) which cannot be removed by dilution or dialysis, but which rapidly exchanges with free DPN. Theorell and Bonnichsen2 observed that the liver alcohol dehydrogenase, on the other hand, has a very

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high affinity for reduced DPN (DPNH). As a matter of fact, the diversified manners by which pyridine nucleotides are bound may also be inferred from the different spectral manifestations of the coupling reactions of PN and PNH with their various apodehydrogenases.

These different types of binding of PN coenzymes could be the reflection of a complicated pattern of interaction between the PN enzymes themselves. In biological systems where the number of binding sites is of the same order of magnitude as the available PN molecules, interactions of apoproteins with bound PN could play a prominent role in physiological oxidation-reduction. It is therefore interesting that DPN-linked enzymes have been found to react with DPN of TDH (TDH - DPN). Cori, Velick and Cori observed that when TDH - DPN was reduced by glyceraldehyde-3-phosphate (G-3-P), it could be reoxidized by addition of pyruvate and lactic dehydrogenase (LDH). The rate of the reaction made the intermediation of dissociated DPNH unlikely. Similar reactions of TDH - DPNH with DPNH cytochrome-c reductase have been reported by Mahler, and with various DPN degrading enzymes as well as other dehydrogenases by Astrachan. Under the conditions studied, the rates of reactions were generally somewhat slower than with free DPN(H).

The implications of these data are manifest. The kinetic relations which have been the result of the study of isolated enzymes under the usual assay conditions involving high concentrations of free coenzyme and substrates may well not apply to situations in which apoprotein-holoenzyme interactions pre-dominate. For example, Theorell and Chance, and Theorell, Nygaard and Bonnichsen have been able to develop an apparently satisfactory kinetic treatment of the reactions of liver ADH without formal consideration of the formation of complex with the substrate. Under some experimental conditions, association-dissociation reactions of DPN(H) with the apoenzyme seemed to be rate-limiting steps. If, by virtue of interaction with other enzymes, the overall reaction occurred without association and dissociation of coenzyme, or if these reactions occurred at modified rates, then ADH might exhibit quite different reaction properties.

In this paper we wish to present some further information on the nature of the bound DPN(H) of TDH, especially its reactivity with various other dehydrogenases. Using the sensitive recording fluorimetric technique developed in this laboratory, it has been possible to study the kinetics of the interactions with liver ADH rather thoroughly. Some experiments on the mechanism of dehydrogenase interaction are also presented, and the implications of these data are discussed.

MATERIALS AND METHODS

Enzymes. TDH was prepared from rabbit muscle according to Cori et al. The preparation used was recrystallized 4 times, and contained 2.0 moles DPN/mole TDH as determined by reduction with G-3-P as well as with ADH and ethanol. The ratio of light absorption at 280 μμ vs 260 μμ was 1.12. This is in agreement with data previously reported by Velick and Hayes.

LDH was prepared from bovine heart muscle by the procedure of Straub. It was recrystallized three times and contained both A and B components.

Crystalline preparations of liver ADH and glutamic dehydrogenase were generously supplied by Dr. Roger Bonnichsen. Crystalline yeast ADH was procured from C. F.
Boehringer & Soehne. Crude Zwischenferment was prepared from bottom yeast according to the method of Warburg and Christian. DPN was prepared in this laboratory according to Akeson and Neilders. The sample used was 88% pure, as judged by the ADH test. A sample of approximately 90% pure TPN was obtained from Pabst laboratories. DPNH was prepared enzymatically according to Nygaard and Theorell. Substrates. Glyceraldehyde-3-phosphate (G-3-P) was prepared essentially according to Meyerhof. The other substrates were obtained commercially.

Kinetic measurements. The highly sensitive fluorimetric technique allowed kinetic measurements to be made without using inordinately large amounts of TDH. The experimental procedure is the same as used in the recent study of the ADH system. The calibration of the total amounts of free DPN or bound DPN added in a given experiment was made with yeast ADH and ethanol.

The experiments involving bound DPNH (TDH · DPNH) were carried out in the following manner: The amount of G-3-P necessary to reduce a known amount of TDH · DPN nearly to completion was determined, and that amount was used to form TDH · DPNH for oxidation by the different pyridine-nucleotide systems. To get more than 90% reduction of TDH · DPN it was not possible to avoid an excess of G-3-P. The initial velocities for oxidation of TDH · DPNH by the various dehydrogenase systems could accordingly never be maximum, because of the counter reaction.

RESULTS

The TDH reaction studied with the fluorescence technique

Before the proposed kinetic studies could be carried out, it was necessary to investigate with the fluorimetric technique certain aspects of the reaction in the presence of TDH. The basis of the technique as applied to the PN enzymes is that DPNH fluoresces while DPN does not. At the outset it was by no means certain that TDH · DPNH would exhibit the same fluorescence properties as free DPNH. It is known that FMN, for example, loses its fluorescence when it is coupled with the old yellow enzyme. Appropriate experiments demonstrated that TDH · DPNH does in fact fluoresce, and that the intensity of fluorescence of TDH · DPNH is the same as for free DPNH within experimental error (—5%). A similar situation has been found for ADH · DPNH.

It was originally noted by Velick et al. that DPNH undergoes a transformation with consequent loss of absorption in the 340 μ region in the presence of TDH. This interesting fact has been further studied by Krebs et al. Such a "destruction" of DPNH bound to TDH was observed in the fluorescence apparatus. The product(s) of the reaction are therefore non-fluorescent. The reaction cannot represent a simple reoxidation to DPN since further addition of substrate did not elicit additional fluorescence, whereas addition of free DPN resulted in a rapid reaction reflected by an immediate increase in fluorescence. Since no free DPNH was added in these experiments, the reaction probably occurred at the binding site of DPNH on the TDH molecule. There was no observable destruction of DPNH by any of the other dehydrogenases tested.

It was consistently observed that 2 molecules DPN/molecule TDH could be reduced by ADH and ethanol at pH 10. Similar results could be obtained with G-3-P only in the presence of low arsenate concentrations (—10^-4 M). At higher

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concentrations, less than 2 molecules DPN/TDH were reduced (in 0.1 M arsenate the reduction of approximately one of the two DPN molecules on TDH was observed). This effect was not due to a rapid destruction of DPNH \(^\text{16,17}\) by a reaction similar to that described above, since such destruction occurred very slowly, if at all, under the conditions of the experiments. High concentrations of arsenate did not inhibit the reduction of 2 DPN per TDH molecule by the ADH. The significance of these observations has not been investigated further, but a relationship to the inhibition of the reaction rate of TDH by arsenate as studied by Bücher \(^\text{18}\) is suggested.

Because of the very large effects of pH as well as salts on the binding of FMN to the old yellow enzyme \(^\text{19}\), several attempts to dissociate TDH · DPN by dialysis at different pH’s in the presence of various salts were made. Whenever dissociation was observed, so also was loss of enzyme activity.

**Interaction of LDH with TDH · DPN and TDH · DPNH**

The interaction of the LDH system with TDH · DPN and TDH · DPNH first studied by Cori, Velick and Cori \(^\text{4}\), has been re-investigated in an attempt to get more quantitative estimate of the relative reaction rates of LDH with bound and free DPN(H). The 4 times recrystallized TDH preparation used in these experiments exhibited small but definite lactic dehydrogenase activity. Appropriate experiments indicated that "endogenous" LDH had properties different from the crystalline LDH. Figs. 1 and 2 show that the Michaelis constant \((K_m)\) for pyruvate in the two systems is considerably different. Similar results were obtained using TDH · DPNH instead of free DPNH. In addition, the inhibition of activity of crystalline LDH in the presence of high pyruvate concentrations was not observed with "endogenous" LDH at pH 9.0, and was observed only at higher concentrations than with added LDH at pH 7.15. When crystalline LDH was added to the TDH preparation, an intermediate \(K_m\) for pyruvate was observed, the Lineweaver-Burk plot being almost the algebraic sum of the plots for the separate enzymes. "Endogenous" LDH activity was not immediately inhibited by 10⁻⁶ M \(p\)-chloromercuribenzoate. In this respect it resembles LDH, and contrasts with the known properties of TDH which is sensitive to SH reagents. It is possible that the LDH activity is due to an impurity of muscle LDH, which might have properties different from those of bovine heart muscle LDH.

At high pyruvate concentrations (— 1 mM), the endogenous LDH activity of TDH was great enough to make quantitative evaluation of the effects of added LDH difficult. However, at lower concentrations of the substrate the rate of the reaction due to "endogenous" LDH was much less and could be corrected for. The results with pyruvate concentrations around 20 \(\mu\)M (pH 8.8) showed that LDH oxidized TDH · DPN some 10 % faster than free DPNH. Since the rate of reaction with bound DPNH is submaximum (see materials and methods), this difference is all the more emphasized. An increased reaction rate of LDH with TDH · DPNH observed by Cori, Velick and Cori \(^\text{4}\) was attributed to stabilization of LDH by TDH. It is believed that the present results cannot be attributed to such an effect since they are based

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Fig. 1. Pyruvate reduction by 4 times crystallized triosephosphate dehydrogenase (TDH) (xx) and by LDH (ooo), pH 9.0.

Conditions of the experiment:
Conc. DPNH: 9.0 µM
Conc. TDH: 0.78 µM
Conc. LDH: 0.0003 µM
Glycine buffer 0.1 M 23°C.

Results:
K<sub>(pyruvate, TDH)</sub>: 6 000 µM
K<sub>(pyruvate, LDH)</sub>: 700 µM

Fig. 2. Pyruvate reduction by 4 times crystallized triosephosphate dehydrogenase (TDH) (xxx) and by LDH (ooo), pH 7.16.

Conditions of the experiment:
Conc. DPNH = 13 µM
Conc. TDH = 0.78 µM
Conc. LDH = 0.00054 µM

Buffer: phosphate, µ = 0.1 + M1.000 versene

K<sub>M (pyruvate, TDH)</sub> = 150 µM
K<sub>M (pyruvate, LDH)</sub> = 18 µM

on the initial rate of the reaction, and the enzyme appeared to be stable at least for 30 seconds after the onset of the reaction.

In the reverse reaction, the oxidation of lactate, LDH definitely reacts with TDH: DPN at a slower rate than with free DPN, and the equilibrium constant is displaced towards lactate. These results are shown in Table 1. In this connection it was of interest to examine the influence of p-chloromercuribenzoate (PCMB) on the reaction of LDH and lactate with TDH: DPN, since it has been shown by Racker and Krinsky that —SH— inhibitors affect the binding of DPN to TDP. As shown in Table 1, the reaction of LDH with TDH: DPN treated with PCMB proceeds at the same rate, and gives the same equilibrium constant as the reaction with free DPN. In contrast, incubation with iodoacetate (IAA) produced no effect, although TDH was completely inactivated by treatment with this inhibitor. Since Racker and Krinsky have shown that IAA partially reverses the spectral shift obtained on combination of TDH with DPN, these observations suggest that the DPN nevertheless still remains attached to TDH.

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**Table 1. Oxidation of lactate by lactic dehydrogenase with free and TDH-bound DPN. The effect of iodoacetate and p-chloromercuribenzoate.**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Inhibitor *</th>
<th>Rate ( \mu \text{M/sec} )</th>
<th>DPNH formed ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>None</td>
<td>0.061</td>
<td>2.6</td>
</tr>
<tr>
<td>Free</td>
<td>Iodoacetate</td>
<td>0.065</td>
<td>2.5</td>
</tr>
<tr>
<td>Free</td>
<td>p-Chloromercuribenzoate</td>
<td>0.065</td>
<td>2.5</td>
</tr>
<tr>
<td>Bound</td>
<td>None</td>
<td>0.048</td>
<td>1.9</td>
</tr>
<tr>
<td>Bound</td>
<td>Iodoacetate **</td>
<td>0.043</td>
<td>1.8</td>
</tr>
<tr>
<td>Bound</td>
<td>p-Chloromercuribenzoate</td>
<td>incubated 1 min.</td>
<td>0.064</td>
</tr>
<tr>
<td>Bound</td>
<td>p-Chloromercuribenzoate</td>
<td>incubated 5 min.</td>
<td>0.061</td>
</tr>
</tbody>
</table>

Test conditions: Buffer 0.1 M glycine
d- lactate: 8.3 mM
DPN, free or bound to TDH 8.2 \( \mu \text{M} \)
LDH: \( 6.8 \times 10^{-2} \) M
Final pH = 9.0

* To TDH - DPN (25 \( \mu \text{l} \), in phosphate \( \mu = 0.1 \), pH = 7.2) iodoacetate or p-chloromercuribenzoate to \( 5 \times 10^{-3} \) M and \( 4.5 \times 10^{-3} \) M, respectively, was added. Incubation at 0°. To the solutions with free DPN the same amounts of inhibitors were added.
** TDH was completely unable to oxidize G-3-P after incubation with iodoacetate.

Astrachan 6 has observed that DPN attached to reduced TDH reacts more slowly with a number of dehydrogenases than when attached to TDH in which some of the thiol groups had been oxidized. This is in contrast to our experiments with LDH as well as with those carried out with ADH from yeast and liver. Under our conditions it made no difference whether the solution of TDH was newly prepared, aged, or incubated with cysteine.

**Table 2. Rate of oxidation of ethyl alcohol and glutamate by yeast ADH and glutamic dehydrogenase, respectively, using free DPN and DPN bound to TDH.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration of Substrate mM</th>
<th>Concentration of DPN, free and bound ( \mu \text{M} )</th>
<th>Rate of reduction of DPN; ( \mu \text{M/sec.} )</th>
<th>DPNH formed ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>Bound</td>
<td>Free</td>
</tr>
<tr>
<td>Yeast ADH</td>
<td>5</td>
<td>8.1</td>
<td>0.058</td>
<td>0.018</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>50</td>
<td>8.1</td>
<td>0.035</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Buffer: phosphate \( \mu = 0.1 + M/1000 \) versene; pH = 7.11; temperature 23-25° C.

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Interaction of yeast ADH and glutamic dehydrogenase with TDH·DPN

Some results obtained with these two enzymes are summarized in Table 2. The rate of reduction for TDH·DPN is much less than for free DPN and the equilibrium is shifted towards oxidized DPN. The reduction of acetaldehyde with bound DPNH was at least as fast as with free DPNH.

Interaction of liver ADH with TDH·DPN and TDH·DPNH

We have investigated the initial rates of the reactions of liver ADH with free and bound DPN(H), with varying concentrations of DPN(H), acetaldehyde, and alcohol. The results are shown in Fig. 3. It can be seen that the reaction with bound DPN(H) can be slower or faster than with free DPN(H), depending upon the concentrations of substrates and coenzyme. TDH·DPN did not oxidize acetaldehyde appreciably under the conditions used in these experiments.

The kinetics of the liver ADH system reacting with free DPN or DPNH have been satisfactorily interpreted according to the following equations:

\[ \text{ADH} + \text{DPNH} \rightleftharpoons k_1 \text{ADH} \cdot \text{DPNH} \]  \hspace{1cm} (1)

\[ \text{ADH} \cdot \text{DPNH} + \text{ald} \rightleftharpoons k_4 \text{ADH} \cdot \text{DPN} + \text{alc.} \]  \hspace{1cm} (2)

\[ \text{ADH} \cdot \text{DPN} \rightleftharpoons k_3 \text{ADH} + \text{DP} \]  \hspace{1cm} (3)

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Table 3. Reaction velocity constants — in the liver ADH system, using free DPN(H) and DPN(H) bound to TDH.

<table>
<thead>
<tr>
<th>Velocity constants</th>
<th>Bound DPN</th>
<th>Free DPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^{-4} \times k_1)</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>(10^{-4} \times k_4)</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>(k_1)</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>(k_3)</td>
<td>7.0</td>
<td>4.9</td>
</tr>
<tr>
<td>(10^{-4} \times [H^+] k_4)</td>
<td>0.019</td>
<td>0.022</td>
</tr>
<tr>
<td>(10^{-4} \times k_4)</td>
<td>0.38</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Buffer: 0.1 M glycine + M/60 arsenate; final pH = 8.8; temperature 23.5°C.

In this scheme, no compound between substrate and enzyme is included. Such a complex is undoubtedly formed, but the kinetic data suggested that it must dissociate more rapidly (into product and ADH · DPN(H)) than does the protein · coenzyme complex, and that it therefore does not effect the kinetic constants indicated above. By means of the experimental data in Fig. 1 and the equilibrium constant we can test whether the above sequence of reactions is possible when the coenzyme is bound to TDH. The method of calculation of the constants \(k_1\) ....... \(k_3\) is the same as outlined previously \(8\), and the result is tabulated in Table 3. In Table 4 is shown the experimentally determined

Table 4. The equilibrium constant (\(K_{eq}\)) as related to reaction velocity constants for different reaction mechanisms \(7,8,11\).

| Slow intramolecular transformation of ternary complex*, affinity of ADH for DPN(H) not influenced by substrate, or vice versa \(11\). | Relative stable ADH-DPN(H) complexes, shortlived ternary complex formed \(7,11\). | A group in the enzyme is oxidized/reduced by substrate(or coenzyme) with the formation of enzyme-substrate intermediates \(11\). | \(K_{eq}\) (Exptl.) |
|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| \(K_{eq} = \frac{k_2k_3k_4}{k_1k_3k_4}\) | \(K_{eq} = \frac{k_3k_2k_4}{k_1k_2k_4}\) | \(K_{eq} = \frac{k_3k_2}{k_1}\) |

<table>
<thead>
<tr>
<th>Free DPN</th>
<th>Bound DPN</th>
<th>Free DPN</th>
<th>Bound DPN</th>
<th>Free DPN</th>
<th>Bound DPN</th>
<th>Free DPN</th>
<th>Bound DPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K \times 10^{11})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>2.0</td>
<td>0.76</td>
<td>4.9</td>
<td>5.8</td>
<td>0.9</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* ADH · DPN · alcohol \(\neq\) ADH · DPNH · aldehyde.

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equilibrium constants, and the equilibrium constants calculated from $k_1 \ldots \ldots k_8$ on the assumption of different reaction schemes\textsuperscript{12}.

It is problematic whether in the present system the constants mean what they say, but it is interesting that the association constants $k_1$ and $k_8$ of DPNH and DPN, respectively, are not significantly different, while $k_8$ is increased from 4.9 to 7.6 sec\textsuperscript{-1}, and $k_3$ is decreased from 2.0 to 1.2 sec\textsuperscript{-1}. The decrease in $k_3$ may also be significant, since the ratio $k_8/k_4$ which determines the equilibrium constant was decreased.

**Interaction of Zwischenferment with TDH·DPN**

If one dehydrogenase could react with PN bound to another PN-linked enzyme without the PN actually changing proteins during the course of the reaction, then it appears possible that a TPN linked enzyme might utilize bound DPN. The possibility whether a TPN specific enzyme might react with the bound DPN of TDH was therefore experimentally tested. In the presence of glucose-6-phosphate Zwischenferment reduced TPN at a rapid rate. When DPN was substituted for TPN, no reaction was detected. When TDH·DPN was substituted for TPN, still no reaction was detected.

**The interaction of liver ADH with TDH·DPNH studied spectrophotometrically**

Since liver ADH·DPNH has a markedly different spectrum in the 340 m\m region\textsuperscript{2} than TDH·DPN, it was possible to test whether a significant number of DPNH molecules reside on the ADH protein during the reactions.

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of ethanol and liver ADH with TDH·DPNH. The results are shown in Fig. 4. When TDH·DPN is reduced by G-3-P, it gives a spectrum with a maximum at 340 mμ. When TDH·DPN is reduced by G-3-P in the presence of ADH, however, the maximum is shifted downward to 330 mμ. If ethanol was used as substrate, then the maximum was shifted down to 322 mμ, in the same region as the ADH·DPNH compound itself. Under these latter conditions, therefore, most of the DPNH molecules must have been bound to ADH.

These results indicate ADH can compete successfully with TDH for DPNH and that the substrate may have an effect on the affinity of the coenzyme. The dissociation constant for liver ADH·DPNH has been found to be $4 \times 10^{-7}$, the dissociation constant for TDH·DPNH has not been accurately determined, but it is known that it must be larger than the dissociation constant for TDH·DPN whose upper limit according to Velick and Hayes\textsuperscript{10} is $2 \times 10^{-7}$. Our results suggest that the two dissociation constants (ADH·DPNH and TDH·DPNH) must be of the same order of magnitude.

**The interaction of liver ADH with TDH·DPNH studied ultracentrifugally**

When small amounts of another dehydrogenase are allowed to react with substrate quantities of TDH·DPN or TDH·DPNH, and the reaction proceeds rapidly, the two proteins must either form a complex which dissociates very readily or it is possible that a more stable complex is formed and a rapid exchange reaction occurs. The possibility that a more stable complex is formed in the ADH—TDH·DPNH reaction was tested ultracentrifugally in cooperation with A. Ehrenberg. Approximately equal molar concentrations of ADH and TDH·DPNH, both in 0.1 M phosphate, pH 7.4, were centrifuged at 59 000 RPM in the Spinco Model E ultracentrifuge. The sedimentation patterns were compared with those obtained by running the enzymes separately. Because of the sizeable difference in sedimentation constants of the two proteins, it was estimated that complexes involving 10% of the molecules would be readily detectable. In actuality the peaks in the combined sample were of the same height and symmetry as in the uncombined single runs, thus indicating no detectable formation of stable complexes between the two proteins.

**DISCUSSION**

The results obtained in these studies virtually eliminate the obligatory intermediation of free DPN in the reaction of various dehydrogenases with TDH·DPN and TDH·DPNH. This is obviously so in reactions where the rate of oxidation of TDH·DPNH was faster than with free DPNH, but it is also evident for reactions with TDH·DPN where observed rates were much too fast to be compatible with Velick and Haye's\textsuperscript{10} estimate of upper limit of the dissociation constant ($2 \times 10^{-7}$ M). In agreement with interpretations placed by previous investigators in their work, these data are most easily interpretable on the basis of direct interactions of the two dehydrogenases themselves. This is not to say that the PN coenzyme remains immobile during the

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reaction. Although our experiments permit no unequivocal conclusions to be drawn on this point, they suggest that the PN switches proteins during the reaction. The spectral shift observed during the reaction of ADH and ethanol with TDH·DPN indicates the formation of ADH·DPNH. The facts that specificity for a particular PN coenzyme is maintained, and also the reaction mechanism of the liver ADH, are most easily interpretable on the basis of a direct exchange of PN from TDH to apodehydrogenase during interaction of the proteins. The ultracentrifugal experiments suggest the complex of the two proteins to be readily dissociated at least for the case of TDH·DPN·ADH.

The kinetics of the reaction of liver ADH with TDH·DPN and TDH·DPNH do not appear to be markedly altered from the kinetics of its reaction with free DPN. The fact that under certain conditions different rate of reaction can be obtained with bound DPN compared with free emphasizes the fact that the turnover rate of the enzyme under usual assay conditions may not correspond to its turnover rate in the living cell.

It must be emphasized that in these experiments, enzymes from widely different sources were employed. In a given physiological system, subtle differences in individual enzymes might well elicit more specific and quantitatively greater effects than those observed. The fact remains, however, that all of the dehydrogenases studied behaved in nearly the same manner, they reacted slower with TDH·DPN and faster with TDH·DPNH compared to the free coenzyme. This indicates some change in the O/R potential of the coupled systems, and together with the observations that the substrate may affect the affinity of dehydrogenase for PN, provides the basis for possible selective interactions under in vivo conditions.

But it is perhaps most intriguing to consider the possibility in which certain enzymes along a given metabolic pathway were unable to react with bound PN, either because of structural organization, or chemical disinclination, and were dependent upon free PN for activity. This would provide a reasonable means for selecting important pathways, synthetic, or energy yielding, according to imposed conditions simply by regulating coenzyme levels. This argument needs not be restricted to PN coenzymes but can be extended to all of the so called mobile coenzyme systems.

We are indebted to Professor Hugo Theorell for his interest in this work. Thanks are also due to Miss Ann Louise Swanson for skilful technical assistance.

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