Pyridine Nucleotide Transhydrogenase

VII. DETERMINATION OF THE REACTIONS WITH COENZYME ANALOGUES IN MAMMALIAN TISSUES*

ABRAHAM M. STEIN,† NATHAN O. KAPLAN, AND MARGARET M. CIOTTI

From the The Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

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Since our initial report (1) on the occurrence of pyridine nucleotide transhydrogenase activity in animal tissue mitochondria and the purification of such an enzyme from beef heart mitochondria, there have been a number of papers which have confirmed and extended our findings (2–5). In our first publication we reported that the animal tissue mitochondrial preparations catalyzed the following reversible reaction:

\[ \text{TPNH} + \text{DPN} \rightleftharpoons \text{TPN} + \text{DPNH} \]  

In addition, a second transfer reaction between the oxidized and reduced forms of DPN was described. This reaction was followed by means of DPN labeled with \(^{14}N\) in the nicotinamide moiety (6), or by a reaction involving deamino-DPN\(^{1}\) as illustrated in the following equation:

\[ \text{DPNH} + \text{deamino-DPN} \rightarrow \text{DPN} + \text{deamino-DPNH} \]  

During the course of studies with flavoproteins, it was noted that certain of these enzyme preparations catalyzed the oxidation of DPNH by the acetylpyridine analogue of DPN (7–9) according to Equation 3:

\[ \text{DPNH} + \text{acetylpyridine (DPN)} \rightarrow \text{DPN} + \text{acetylpyridine (DPNH)} \]  

This reaction could be followed directly in the spectrophotometer utilizing the difference in absorption maxima of DPNH and its reduced acetylpyridine analogue (8). DPNH and reduced deamino-DPN have identical spectra. Furthermore, Reaction 3 goes to completion because of the more positive potential of the acetylpyridine analogue of DPN (9).

Studies measuring transhydrogenase activity by cytochrome \(c\) reduction and oxygen uptake (10) indicated that the enzyme was present in rat liver mitochondria in higher concentrations than those obtained by our original assay system, which involved the generation of DPNH mediated by the transhydrogenase in the presence of the isocitric dehydrogenase system and catalytic amounts of TPN; the reaction was followed at 340 nm. It appeared to us that the oxidation of TPNH by the acetylpyridine analogue of DPNH, as given in the following equation

\[ \text{TPNH} + \text{acetylpyridine (DPN)} \rightarrow \text{TPN} + \text{acetylpyridine (DPNH)} \]  

could be a more sensitive and perhaps more meaningful method for measuring transhydrogenase activity.

It is the purpose of this paper to describe the application of the above reaction to the determination of transhydrogenase activity in animal tissues and also to give further information on reaction 3. Data will also be given on the following two reactions:

\[ \text{DPNH} + \text{acetylpyridine (TPN)} \rightarrow \text{DPN} + \text{acetylpyridine (TPNH)} \]  

\[ \text{TPNH} + \text{acetylpyridine (TPN)} \rightarrow \text{TPN} + \text{acetylpyridine (TPNH)} \]  

Information as to whether one or more enzymes are involved in the various transfer reactions of the pyridine nucleotides will also be presented.

EXPERIMENTAL

DPN, DPNH, and TPN were obtained from the Pabst Laboratories. TPNH was obtained from the Sigma Chemical Company. The acetylpyridine and pyridine-3-aldehyde analogues of DPN were obtained from the Pabst Laboratories. The acetylpyridine analogue of TPN was prepared by the transglycosidase reaction as described previously (8). The thionicotinamide analogue of DPN was prepared by Dr. B. M. Anderson (11). The digitonin used was a preparation of the Merck Chemical Company. L-Thyroxine and triiodothyronine were obtained from Smith, Kline and French Laboratories.

Preparation of Tissues—Mitochondria, microsomes, and soluble fractions were obtained from rat and rabbit tissues as described by Kielley and Kielley (12). The tissues were forced through a stainless steel press with holes about 1 mm. in diameter, and the weight of the tissue was estimated by displacement of 0.25 m sucrose in a graduated cylinder. Homogenization was carried out in a TenBroeck all-glass, hand homogenizer in a 10-fold dilution of 0.25 m sucrose. The particles sedimenting between 600 × g and 6,000 × g, and 25,000 × g and 105,000 × g were considered to be the mitochondrial and microsomal...
fractions, respectively. Beef heart mitochondria were prepared as described previously (1), or by a modification of the method of Singer (13).

Concentration of Analogues—The concentration of the acetylpyridine analogue of DPN was measured by reduction with yeast alcohol dehydrogenase and ethanol with semicarbazide as a trapping reagent. The extinction coefficient of the reduced analogue at 365 μm was taken as 7.8 (8). The concentration of the acetylpyridine analogue of TPN was determined by forming the cyanide addition product (8).

Estimation of Mitochondrial Protein—Of the mitochondrial suspensions, 0.5 to 1 ml. is precipitated with 5 ml. of 10 per cent trichloroacetic acid, extracted with hot 95 per cent ethanol and ether, and air dried. The residues are dispersed in 0.5 to 1 ml. of 0.5 N NaOH overnight. Aliquots of 0.1 ml. of the resulting solutions, diluted with 1.5 volumes of water, are assayed by the procedure of Lowry (14), with bovine serum albumin as a standard. By this method, approximately 20 mg. of mitochondrial protein are equivalent to a gm. of fresh rat liver.

RESULTS

Reaction Measurement—The reaction of TPNH and DPNH with pyridine nucleotide analogues was followed at room temperature in a 3-ml. cuvette with a 1-cm. light path in a Beckman model DU spectrophotometer. The contents of the cuvette were 0.3 mmole of potassium phosphate buffer, pH 6.5 or 7.5; 3 μmoles of KCN; 0.4 μmole of reduced pyridine nucleotide; and 0.6 μmole of acetylpyridine analogue of DPN in a final volume of 3 ml. The rate of reaction was estimated with the spectral difference of DPNH and its acetylpyridine analogue shown in Fig. 1. Generally the reaction is followed at 375 μm, where the spectral difference is 0.0051 optical density unit per μmole of analogue reduced. Small corrections for endogenous oxidation of reduced pyridine nucleotides were applied, when necessary, by subtracting the values obtained from a control cuvette without analogue acceptor. The rates of endogenous oxidation of reduced pyridine nucleotides in whole mitochondrial preparations are generally quite small, amounting to no more than 5 to 10 per cent of the optical density change at 375 μm. The reduced analogues under the conditions of the experiments are oxidized at a slow rate; no corrections were made for this oxidation during the course of the transhydrogenase reactions, and, therefore, the rates reported represent minimal values. A study of the rate of reaction followed at several wave lengths indicated no significant effect of the pyridine nucleotide analogues on the endogenous oxidation of TPNH or DPNH in mitochondrial preparations.

Efficiency of Acetylpyridine Analogues—The rates of reaction of the TPNH-DPN transhydrogenase reaction with the use of DPN and its acetylpyridine analogue as acceptors are compared in Fig. 2. Under the conditions studied, the reaction is 5 to 6 times faster with the analogue as acceptor than with DPN.

Effect of Digitonin on Rate of Reaction—Digitonin was used originally (1) to extract transhydrogenase activity in a non-sedimentable form from beef heart particles. Treatment of mitochondria with digitonin before assay has been adopted for routine estimation of rates of reactions in mitochondria. Table I shows the effect of digitonin concentration on the activities of Reactions 4 and 3. In a typical preparation, mitochondria are prepared in sucrose to a final concentration representing 0.2 to 0.25 ml. of tissue press per ml. of suspension. Before assay, 1 ml. of mitochondrial suspension is mixed with 1 ml. of 1 per cent digitonin adjusted to about pH 7.5 (0.02 m.eq. of NaOH per 10 ml. of solution). Routinely, 0.05 ml. of mixture is assayed 10

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1 Recent data from the Pabst Laboratories with the use of a purer preparation of the reduced acetylpyridine analogue of DPN indicate the μm extinction coefficient to be 9.1 rather than the 7.8 value originally reported.
TABLE I

Activation by digitonin of rat liver mitochondrial transhydrogenases

<table>
<thead>
<tr>
<th>Digitonin concentration</th>
<th>TPNH + acetylpyridine (DPN)</th>
<th>DPNH + acetylpyridine (DPN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Analogue reduced per min. per ml. of reaction mixture*</td>
<td>Analogue reduced per min. per ml. of reaction mixture*</td>
</tr>
<tr>
<td>%</td>
<td>mmoles</td>
<td>mmoles</td>
</tr>
<tr>
<td>0.125</td>
<td>4.65</td>
<td>8.24</td>
</tr>
<tr>
<td>0.25</td>
<td>7.57</td>
<td>11.7</td>
</tr>
<tr>
<td>0.50</td>
<td>7.78</td>
<td>14.0</td>
</tr>
<tr>
<td>1.0</td>
<td>8.47</td>
<td>13.9</td>
</tr>
</tbody>
</table>

* Mitochondria equivalent to 10 mg. of fresh liver were used. A mitochondrial suspension of 20 mg. per ml., fresh weight equivalent, was diluted with equal volumes of digitonin to yield the indicated concentrations.

Fig. 3. The effect of concentration of digitonized rat liver mitochondria on the TPNH-acetylpyridine (DPN) transhydrogenase. One volume of mitochondrial suspension is added to one volume of 1 per cent digitonin to yield the equivalent of 10 mg. of fresh tissue per 0.10 ml. mixture. The rates are expressed in mmoles per ml. of reaction mixture. The reaction is followed at 365 mμ. Conditions are described in the text. The reaction is carried out at pH 6.5 at 25°.

Fig. 4. Effect of digitonin on the reactions of TPNH and DPNH with the acetylpyridine analogue of DPN in pigeon liver mitochondria. Mitochondria from 18 mg. of liver, fresh weight. O—O, mitochondria preincubated 10 minutes in the reaction mixture; ●—●, mitochondria lysed in 0.5 per cent digitonin. Conditions are described in the text. The reaction is carried out at pH 6.5 at 25°.

Fig. 5. Rates of various transhydrogenases in digitonin extracts (1) of beef heart mitochondria. APDPN represents the acetylpyridine analogue of DPN; APTPN, the acetylpyridine analogue of TPN. The conditions are described in the text.

Occurrence of Reactions—Relative rates of reduction of the acetylpyridine analogues of DPN and TPN by reduced pyridine nucleotides are shown in Fig. 5 for a digitonin extract (1) of beef liver mitochondria. Pigeon liver mitochondria show little transhydrogenase ac-

Activity when assayed directly by the reduction of the acetylpyridine analogue of DPN when either TPNH or DPNH are donors. On treatment with digitonin there is a remarkable increase in both activities (see Fig. 4) and the rates are comparable to those found in rat liver. It is evident from these observations that precautions are essential in interpreting the relative concentrations of transhydrogenase activities in mitochondria.
Preciable amounts in the microsomal fraction of rat liver. It has closely that of the corresponding analogue reaction. The heart enzymes (1), the activity of the TPNH-DPN reaction follows closely that of the corresponding analogue reaction. The reaction of TPNH with the acetylpyridine analogue of TPN (Reaction 6) is virtually absent from all mitochondrial preparations tested. However, this reaction has been detected in appreciable amounts in the microsomal fraction of rat liver. It has also been found to be catalyzed by a diaphorase from chloroplasts and by a beef heart TPNH-cytochrome c reductase preparation by Weber and Kaplan (15), and lately by us in the soluble fraction of Chromatium sонicate. The low rate of reaction of DPNH with the acetylpyridine analogue of TPN is in accord with the relatively slow rate of the reverse transhydrogenase reaction (Reaction 1), previously observed in animal tissue mitochondria (1). The distribution of the major transhydrogenase reactions in rat and rabbit tissue fractions is shown in Table II. These results are in general agreement with previous data (1). The greater sensitivity of the present method allows more reliable estimates of rates in tissues with low activities. It appears, at least in the case of liver, that some of the older data may have been complicated by the impermeability of the mitochondria. Rat liver microsomes display much less transhydrogenase activity than do mitochondria. Fig. 6 shows the effect of anaerobic conditions on Reactions 4 and 3 in microsomes. The failure to observe the reaction of the acetylpyridine analogue of DPN with TPNH under aerobic conditions may be due to the fact that at the concentration used (0.001 M), the cyanide added fails to inhibit the oxidation of TPNH completely. It should be noted that the anaerobic activity of Reaction 4 in microsomes is only about 25 per cent of that obtained aerobically with digitonin. As previously noted (1), all pyridine nucleotide transfer reactions are either absent or present only in trace amounts in the 105,000 x g supernatants of mammalian tissue homogenates. Further, the amount of such activities recovered appears to be influenced by the length of time the samples are exposed to the high centrifugal field. We have detected a low rate of reduction of the acetylpyridine analogue of DPN by TPNH in the soluble fraction of human placenta in agreement with some of the observations reported by Talalay and Williams-Ashman (16). The results were complicated by an endogenous reduction of the analogue several times greater than found with added TPNH.

Reduction of Other Pyridine Nucleotide Analogues—We have found that the pyridine-3-aldehyde analogue of DPN (8, 9) can act as an acceptor in Reactions 3 and 4 in rat liver and beef heart mitochondria. We have studied in some detail the role of the thionicotinamide analogue of DPN4 as acceptor in these reactions in rat and rabbit tissues. This analogue (17) is only about 50 per cent as effective as the acetylpyridine analogue as an acceptor in Reaction 4. On the other hand, it is as active or more active than the acetylpyridine analogue in Reaction 3, in spite of its lower oxidation-reduction potential (17). This was particularly marked with the partially purified enzyme from rat liver mitochondria (18), here the activity with the thionicotinamide analogue was found to be 4 to 5 times greater than with the acetylpyridine analogue. These studies will be reported in detail elsewhere.

Affinity Constants—The Michaelis constants of the various pyridine nucleotides in Reactions 4 and 5 have been determined. K_m values for Reaction 4 are 7.5 x 10^{-4} M for TPNH and 1.5 x 10^{-6} M for the acetylpyridine analogue of DPN as determined by Lineweaver-Burk plots. Similarly, in Reaction 5, the values are 9.8 x 10^{-4} M and 7.7 x 10^{-6} M for DPNH and the acetylpyridine analogue of TPN, respectively. The latter determinations

\( \text{Table II} \)

Distribution of transhydrogenase reactions in digitonized mitochondria of rat and rabbit tissues as measured by reduction of acetylpyridine analogue of DPN

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>Analogue reduced/min./mg. protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TPNH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Liver</td>
<td>Mitochondria</td>
<td>181</td>
</tr>
<tr>
<td>Liver</td>
<td>Microsomes</td>
<td>14</td>
</tr>
<tr>
<td>Heart</td>
<td>Mitochondria</td>
<td>367</td>
</tr>
<tr>
<td>Kidney</td>
<td>Mitochondria</td>
<td>108</td>
</tr>
<tr>
<td>Brain</td>
<td>Mitochondria</td>
<td>34</td>
</tr>
<tr>
<td>Lung</td>
<td>Mitochondria</td>
<td>143</td>
</tr>
<tr>
<td>Testis</td>
<td>Mitochondria</td>
<td>112</td>
</tr>
<tr>
<td>Spleen</td>
<td>Mitochondria</td>
<td>137</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Novikoff Hepatoma</td>
<td>Mitochondria</td>
<td>27</td>
</tr>
<tr>
<td>Placenta†</td>
<td>Mitochondria</td>
<td>32</td>
</tr>
<tr>
<td>Placenta‡</td>
<td>Supernatant</td>
<td>0.8*</td>
</tr>
</tbody>
</table>

* Negligible activity.
† Prepartum placenta, average about 10 mm. in diameter.
‡ Supernatant of 25,000 X g centrifugation.

Fig. 6. Effect of anaerobiosis on Reactions 4 and 3 in rat liver microsomes. The reaction mixtures contained 0.1 M potassium phosphate, pH 6.8; 0.001 M KCN; 0.003 M MgCl_2; 600 μg. of acetylpyridine analogue of DPN (APDPN); 400 μg. of TPNH or DPNH; and 0.1 cc. of the microsomal preparation in a final volume of 3 cc. The reaction is started by the addition of the microsomes. The anaerobic experiments were carried out in a Thunberg-type cuvette.

Heart mitochondria. Digitonin extracts of rat liver mitochondria show a similar spectrum of activities. As in the case of the beef heart mitochondria, the rat liver mitochondrial extracts fail to catalyze Reaction 6 and catalyze Reaction 5 at a considerably slower rate than Reaction 4. In the purification of the beef heart enzymes (1), the activity of the TPNH-DPN reaction follows closely that of the corresponding analogue reaction. The reaction of TPNH with the acetylpyridine analogue of TPN (Reaction 6) is virtually absent from all mitochondrial preparations tested. However, this reaction has been detected in appreciable amounts in the microsomal fraction of rat liver. It has...
TABLE III

<table>
<thead>
<tr>
<th>Days stored at $^\circ$C</th>
<th>$\Delta$A$_{365}$ from 2 to 7 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPNH + acetylpyridine (DPN)</td>
</tr>
<tr>
<td>0</td>
<td>0.243</td>
</tr>
<tr>
<td>1</td>
<td>0.230</td>
</tr>
<tr>
<td>4</td>
<td>0.213</td>
</tr>
<tr>
<td>5</td>
<td>0.205</td>
</tr>
<tr>
<td>6</td>
<td>0.215</td>
</tr>
<tr>
<td>11</td>
<td>0.227</td>
</tr>
</tbody>
</table>

* The preparation used was a calcium phosphate gel supernatant of a digitonin extract of rat liver mitochondria.

TABLE IV

**Effect of thyroxine and triiodothyronine on beef heart transhydrogenases**

Suspension of beef heart mitochondria added to cuvette at zero time; acetylpyridine analogue of DPN used in all reactions.

<table>
<thead>
<tr>
<th>Reduced pyridine nucleotide</th>
<th>Inhibitor</th>
<th>Concentration</th>
<th>$\mu$moles min.$^{-1}$ ml.$^{-1}$ of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPNH</td>
<td>None</td>
<td></td>
<td>8.09</td>
</tr>
<tr>
<td>TPNH</td>
<td>Thyroxine</td>
<td>$3.7 \times 10^{-5}$</td>
<td>5.37</td>
</tr>
<tr>
<td>TPNH</td>
<td>Triiodothyronine</td>
<td>$3.7 \times 10^{-5}$</td>
<td>2.84</td>
</tr>
<tr>
<td>DPNH</td>
<td>None</td>
<td></td>
<td>19.9</td>
</tr>
<tr>
<td>DPNH</td>
<td>Triiodothyronine</td>
<td>$3.7 \times 10^{-5}$</td>
<td>19.9</td>
</tr>
</tbody>
</table>

The transhydrogenase reaction between TPNH and DPNH does not appear to be associated with the diaphorase activities of mitochondria (19, 18), since the latter are readily extractable by sonic oscillation in contrast to the former. Further, it appears that TPNH diaphorase is quite resistant to heating, whereas the transhydrogenase which catalyzes the interaction between the two forms of the pyridine coenzymes is readily destroyed (Table V). We have failed to obtain any evidence for Reaction 4 either in reaction mixtures containing DPNH or TPNH specific diaphorases, or the nonspecific diaphorase A of rat liver mitochondria (18). This latter enzyme reacts with both TPNH and DPNII.

DISCUSSION

The data presented in this paper illustrate the advantage of the use of pyridine coenzyme analogues in measuring the pyridine nucleotide transhydrogenase reactions. The higher values obtained with the analogues may be related, in part, to the high oxidation-reduction potential of the acetylpyridine coenzymes (19). Furthermore, the difference in absorption maxima and the higher extinction coefficients of the reduced analogues makes possible a simple and direct method for following the transhy-
indicated considerably higher activity for rat liver mitochondria. The reduction potential of the acetylpyridine nucleotides, the reactions proceed to completion.

Measurement of TPNH-DPN transhydrogenase activity, either by cytochrome c reduction or by oxygen consumption, indicated considerably higher activity for rat liver mitochondria than found with the original assay procedure (19). The value for the reaction of TPNH with the acetylpyridine analogue of DPN is more in line with the results obtained with the former methods. It might be expected that the cytochrome c reduction reaction is more favorable for the assay of transhydrogenase, because of the removal of DPNH, as indicated by the following equations:

\[
\text{TPNH} + \text{DPN} \rightleftharpoons \text{TPN} + \text{DPNH}
\]

\[
\text{DPNH} + \text{cytochrome c Fe}^{++} \rightarrow \text{DPN} + \text{cytochrome c Fe}^{+}
\]

Similar considerations would hold for the removal of DPNH by oxygen uptake. With the use of the acetylpyridine analogue of DPN as acceptor, the equilibrium problems are avoided and the reaction proceeds much more rapidly.

In discussing the equilibrium of the transhydrogenase reaction, it should be pointed out that under normal conditions in mitochondria the DPN is probably bound to protein. As indicated by Theorell and Bonnichsen (20) the binding of DPN increases the oxidation-reduction potential of the DPNH-DPN couple some 0.06 volt. This, therefore, would favor energetically the reduction of DPN in the transhydrogenase reaction as occurring under natural conditions.

It is difficult to attempt to account for the respiratory rates in terms of measurement of individual enzymatic steps. This is pointed out by Ernst and Navazio (21) in reference to a role played in respiration by a possible DPN-linked isocitrate dehydrogenase of rat liver mitochondria. These authors, on the basis of low values obtained with their transhydrogenase assays on rat liver mitochondria, have ruled out transhydrogenase (Reaction 1) as a factor in isocitrate oxidation by mitochondria in favor of the aforementioned DPN-linked isocitrate dehydrogenase. With our present method of assay, we have obtained values for the rate of TPNH-DPN transhydrogenase 40 to 50 times greater than those reported by Ernst and Navazio (21), sufficient to accommodate the rate of reduction of TPN by isocitrate and about 5 times greater than the isocitrate-coupled oxygen uptake observed by these authors. We have observed the rate of the transhydrogenase reaction to be in the same order of magnitude as TPN isocitrate dehydrogenase and DPNH-cytochrome c reductase in rat liver mitochondria.

Although the acetylpyridine analogues of DPN and TPN are active with the animal tissue dehydrogenases, they will not act as acceptors in the *Pseudomonas fluorescens* transhydrogenase system (15). Hence, caution must be used when applying the analogue method for the assay of transhydrogenase activities of different sources. As noted above, DPNH will reset with the thionicotinamide analogue of DPN. The relative rates of reaction with the two analogues appear to be different in different tissues. This suggests some structural differences between the enzymes of different tissues catalyzing Reaction 3.

The *Pseudomonas* enzyme (22) catalyzes the exchange between TPNH and DPN as well as the DPNH-DPN reaction. These two activities have not been separated, and it appears possible that they are due to one protein. The experiments reported in this paper indicate that these reactions are catalyzed by two different proteins in the animal tissue mitochondria. The reaction of DPNH with the acetylpyridine analogue of DPN has been solubilized by sonic oscillation and under these conditions the reaction of TPNH with the acetylpyridine analogue of DPN remains in the insoluble fraction. Although the reaction of DPNH with the acetylpyridine analogue of DPN appears to be associated with diaphorase activity, the enzyme appears to be distinct from the nonspecific diaphorase which is also present in mitochondria (18).

It is of interest that the reaction of DPNH with the acetylpyridine analogue of TPN proceeds at a much slower rate than the converse reaction of TPNH with the acetylpyridine analogue of DPN. This difference in rate has also been observed with the natural coenzymes (1). It thus appears that the animal tissue transhydrogenase has some properties in common with the bacterial system, since with the latter enzyme the DPNH-TPN reaction is very sluggish and will not proceed unless 2'-adenylic acid is added (23). In the rat liver TPNH-DPN transhydrogenase, the affinities of the reduced pyridine nucleotides and pyridine nucleotide analogue acceptors are such as to possibly afford an explanation for the difference in rates for the forward and reverse reactions. It will be noted that the preferred direction for Reaction 1 is consistent with Reaction 4 being more active than Reaction 5 and is in the direction of the nucleotides with the lowest K_m value. The difference in rate of the forward and reverse reaction may be of significance as a physiological regulating mechanism of electron transfer in mitochondria.

Talalay and Williams-Ashman (16) have reported that the estradiol-β-17 dehydrogenase from human placenta can act as a transhydrogenase. This dehydrogenase is both TPN and DPN linked and Talalay and Williams-Ashman have suggested that the net transfer of hydrogen or electrons from TPNH to DPN mediated by placental extracts is effected by alternate oxidation and reduction of catalytic amounts of estradiol-β-17. It is suggested by these authors that transhydrogenase may be such an enzyme. It should be emphasized that the steroid dehydrogenase activity is located in the soluble fraction of the cytoplasm whereas essentially all of the transhydrogenase activity which we have detected is present in particulate fractions of the cell. It is of interest that the properties of rat liver mitochondrial transhydrogenase are different from those reported by Talalay et al. (24) for his placental enzyme, both with respect to affinity of pyridine nucleotides for the enzyme and the relative rates with pyridine nucleotide analogues. The possibility exists that the TPNH-DPN transhydrogenase of the mitochondria may be due to a TPN-DPN-linked dehydrogenase which conceivably may involve steroids. However, this has yet to be demonstrated, and until this point has been clarified, confusion might arise if the activity described by Talalay and Williams-Ashman is linked with the transhydrogenase system which we have studied in mitochondria. Further work on the purification of mitochondrial transhydrogenases is now under way.

Navazio et al. (25) have attempted to account for the oxidation of TPNH in rat liver by the lactate dehydrogenase activity of the soluble fraction of rat liver homogenates. This attempt becomes necessary if one ignores the potent TPN-DPN transhydrogenase we have demonstrated in mitochondria of rat liver and other tissues, and assumes a DPN-linked isocitrate dehydrogenase in mitochondria to account for isocitrate oxidation. In spite of many attempts, we have failed to demonstrate any
formed stoichiometrically with pyruvate during glycolysis would be preferentially oxidized by the pyruvate. Navazio et al. (25) process could account for a net TPNH oxidation since DPNH isocitrate dehydrogenase in rat liver. It is doubtful that this absence of convincing evidence for a mitochondrial DPN-linked oxidations, which must be assumed to be associated with TPN in the reactions, where the pre-existing pyridine nucleotides have been removed by proper depletion (26). The results of such an experiment are shown in Fig. 8. The concept that the soluble lactic dehydrogenase of rat liver will account for TPNH oxidation of the rat liver cell can be criticized on several counts. It obviously cannot account for isocitrate oxidation in mitochondrial preparations, which must be assumed to be associated with TPN in the absence of convincing evidence for a mitochondrial DPN linked isocitrate dehydrogenase in rat liver. It is doubtful that this process could account for a net TPNH oxidation since DPNH formed stoichiometrically with pyruvate during glycolysis would be preferentially oxidized by the pyruvate. Navazio et al. (25) have reported that the oxidation of TPNH by pyruvate with lactic dehydrogenase is about 2.5 per cent that of DPNH. Furthermore, the presence of high concentrations of DPN in the rat liver soluble fraction (27) would materially decrease this reaction, according to the data of Navazio et al. (25) on the inhibition of TPNH oxidation by pyruvate in the presence of DPN. As indicated previously (28), lactic dehydrogenase does not carry out a transfer of reduction state from TPNH to DPN. We have confirmed this with the use of the acetylpyridine analogue of DPN in the presence of catalytic or substrate concentrations of pyruvate. It should be noted that a net transfer of hydrogen or electrons from TPNH to DPN is implicit in the suggestion that lactic dehydrogenase can account for TPNH oxidation, unless it is further suggested that an alternate pathway exists for lactate metabolism, not involving DPN.

In confirmation of Ball and Cooper’s work (3), we have found that thyroxine and triiodothyronine, as well, will inhibit the transhydrogenase reaction as measured by Reactions 4 and 5. It is of interest that these compounds do not affect the reaction of DPNII with the acetylpyridine analogue of DPN. The significance of this inhibition is as yet not clear since we have found in preliminary experiments that hyper- and hypothyroid states do not produce notable changes in transhydrogenase levels of rat liver mitochondria.

**SUMMARY**

1. A method is described for the assay of transhydrogenase activity through the use of the acetylpyridine analogues of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). This method, which involves the transfer of hydrogens or electrons from reduced triphosphopyridine nucleotide (TPNH) to the acetylpyridine analogue of DPN, gives values considerably higher than those obtained by other methods. It has also been found, in confirmation of earlier work, that the highest levels of transhydrogenase are located in mitochondria of heart, kidney, and liver. Relatively little activity is found in brain. Nearly all of the transhydrogenase activity is located in the mitochondria, although some evidence has been obtained for the presence of low levels of transhydrogenase activity in rat liver microsomes.

2. It has been found that the rat liver and beef heart transhydrogenases promote the reaction between TPNH and the acetylpyridine analogue of DPN at a much faster rate than the analogous reverse reaction, the reaction of reduced diphosphopyridine nucleotide (DPNH) with the acetylpyridine analogue of TPN.

3. Mitochondrial preparations will also promote a reaction between DPNH and the acetylpyridine analogue of DPN. Evidence is presented which indicates that this reaction is catalyzed by a different enzyme than that which is responsible for the reaction of TPNH with the acetylpyridine analogue of DPN.

4. Thyroxine and triiodothyronine have been found to inhibit the reaction of TPNH and the acetylpyridine analogue of DPN, whereas these compounds do not influence the reaction of DPNH with the acetylpyridine analogue of DPN.

5. The significance of the results of the acetylpyridine analogue assay for transhydrogenase reactions in whole mitochondrial preparations is discussed. A discussion of the various transhydrogenases and their possible nature is presented.

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