DIPHOSPHOPYRIDINE NUCLEOTIDE ISOCITRIC DEHYDROGENASE FROM ANIMAL TISSUES*

BY G. W. E. PLAUT† AND SHAN-CHING SUNG‡
(From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin)

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In a study of the metabolism of heart mitochondria it was found that acetate and all substrates of the Krebs cycle except citrate, isocitrate, and cis-aconitate were oxidized (1). It was demonstrated that under certain conditions the oxidation of citrate could be promoted by the addition of diphosphopyridine nucleotide. Stimulation of citrate oxidation by DPN was also observed with certain tumor homogenates (2). It seemed possible that this effect could be attributed to the presence of a DPN-linked isocitric dehydrogenase. Such an enzyme, in addition to TPN-linked isocitric dehydrogenase, has been purified from yeast juice by Kornberg and Pricer (3); its occurrence and partial purification from animal tissues will be presented here.

EXPERIMENTAL

Evidence for DPN-Linked Isocitric Dehydrogenase—Mitochondria were isolated from guinea pig hearts as previously described (1). Acetone powders were prepared from these particles by the method of Drysdale and Lardy (4), except that the ether wash was omitted and the final drying was accomplished in vacuo at room temperature. A yield of about 1 gm. of dry powder was obtained from 20 to 25 gm. of heart. When this powder was stored for 1 month at -10°C, about 25 per cent of the DPN isocitric dehydrogenase was lost but none of the TPN enzyme activity. The soluble enzyme solution was prepared by mixing about 0.25 gm. of acetone powder with 10 ml. of 0.01 M phosphate buffer, pH 6.5, with a Potter-Elvehjem homogenizer (5). The suspension was then centrifuged for 30 min-

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† Established Investigator of the American Heart Association.
‡ Fellow of the National Heart Institute, 1952–53. Present address, Department of Biochemistry, College of Medicine, National Taiwan University, Taipei, Formosa.

The following abbreviations will be used: triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), adenosinetriphosphate (ATP), adenosine-5-phosphate (AMP), nicotinamide mononucleotide (NMN), crystalline yeast alcohol dehydrogenase (ADH).

All enzyme extractions and fractionations were done at 0–2°C unless otherwise specified.
utes at 18,000 × g. The resulting supernatant solution constituted the crude enzyme preparation. When DPN was incubated with such a mitochondrial extract under the assay conditions mentioned below, the addition of isocitrate resulted in an increase in optical density at 340 μ. The increase in absorption was due to the reduction of DPN. This was confirmed by stopping the reaction after 30 minutes by immersion of the reaction vessels in a boiling water bath for 5 minutes; the subsequent addition of excess ADH and acetaldehyde (CH₃CHO) at pH 6.5 led to the immediate reoxidation of the reduced DPN (Fig. 1). As expected, neither acetaldehyde nor alcohol dehydrogenase alone resulted in the reoxidation of reduced DPN.

This extract contains about 30 times more TPN isocitric dehydrogenase activity than the corresponding DPN enzyme; however, a difference between these enzymes is apparent from the pH optima. When TPN is used as the coenzyme, a broad range of reaction-pH dependency is obtained with a peak at about pH 7.8. The DPN-linked reaction appears to be much more sensitive to the pH of the reaction medium with an optimal pH at 6.5. The rates obtained with cacodylate and tris(hydroxymethyl)aminomethane buffer at given pH values were in agreement (Fig. 2).

Further support for the presence of two separate enzyme entities can be obtained by fractionation of the crude guinea pig heart mitochondrial extract with ammonium sulfate. In the example used in Table I, 9 ml. of
the enzyme extract were brought to 0.4 saturation\(^3\) with saturated ammonium sulfate solution. The centrifuged precipitate contained little activity and was discarded. The supernatant solution was adjusted to 0.6 saturation with ammonium sulfate. The resulting residue was taken up in 4.5 ml. of 0.01 M phosphate, pH 6.5, and ammonium sulfate was added to a saturation level of 0.5; after centrifugation the supernatant solution was brought to 0.6 saturation with ammonium sulfate. The protein sediments obtained in this fractionation were dissolved in 0.01 M phosphate and were termed R\(_{40-50}\) and R\(_{40-60}\), respectively. The 0.6 saturated ammonium sulfate solution obtained after the earlier removal of the R\(_{40-50}\) fraction was brought to 0.8 saturation with solid ammonium sulfate and the residue obtained was dissolved in buffer (R\(_{50}\)). As can be seen in

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td><strong>Fractionation of Isocitric Dehydrogenase from Guinea Pig Heart with Ammonium Sulfate</strong></td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Crude mitochondrial extract</td>
</tr>
<tr>
<td>R(_{40-50})</td>
</tr>
<tr>
<td>R(_{40-60})</td>
</tr>
<tr>
<td>R(_{50})</td>
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</tbody>
</table>

Table I, R\(_{40-50} + R_{50-60}\) contained 83 per cent of the original DPN activity but only 0.3 per cent of the TPN isocitric dehydrogenase, while in Fraction R\(_{50}\) 17 per cent of DPN and 70 per cent of the TPN enzyme activities were recovered.

**Occurrence**—The previous evidence established the presence of DPN isocitric dehydrogenase in guinea pig heart. It was of interest to determine the occurrence of this enzyme in other animal tissues. The results in Table II demonstrate that DPN isocitric dehydrogenase is widely distributed. It should be pointed out, however, that no attempt was made to separate the DPN and TPN enzymes except with guinea pig and beef heart and pigeon breast muscle. It is conceivable that the reduction of DPN by isocitrate in other non-fractionated crude preparations could, for example, be attributed to a mechanism involving TPN enzyme and transdehydrogenase (6).

\(^3\)The saturation levels referred to here are in terms of saturation of (NH\(_4\))\(_2\)SO\(_4\) at 25\(^\circ\), although fractionations were done at 0-2\(^\circ\).
Purification of Enzyme—Since only limited quantities of material could be obtained in a practical manner from guinea pig heart, DPN isocitric dehydrogenase was purified from beef heart.

Fresh beef hearts\(^4\) were packed in ice at the packing plant and transported to the laboratory within \(\frac{1}{2}\) hour. The hearts were trimmed to remove fat, ligament, and the auricles. The ventricles were cut into about 1 cm. cubes. 2 kilos of this tissue were ground with 5.5 liters of a medium containing 0.25 \(M\) sucrose and 0.03 \(M\) \(K_2HPO_4\) in a Waring blendor for 1 minute at "fast" and 1 minute at "slow" speed (about 150 gm. of wet tissue and 400 ml. of suspending medium were used per Waring blendor bowl). The resulting suspension was centrifuged at 600 \(\times g\) for 10 minutes to remove large particles. The resulting supernatant solution was passed through a double layer of cheese-cloth to remove floating pieces of fat. The filtrate was adjusted to pH 5.8 to 5.9 with dilute acetic acid (7) and centrifuged at 1800 \(\times g\) for 20 minutes. The residue was taken up in a minimal quantity of 0.25 \(M\) sucrose and centrifuged at 5000 \(\times g\) for 30 minutes. The well packed washed residue preparation was then converted to an acetone powder as previously described in the case of guinea pig heart mitochondria. About 25 gm. of dried preparation were obtained.

Because of the lability of the enzyme all of the operations described below and the assays must be done on the same day.

5 gm. of acetone powder were extracted with 100 ml. of 0.01 \(M\) phosphate, pH 6.5. The mixture was centrifuged at 18,000 \(\times g\) for 20 minutes. 80 ml. of supernatant solution were treated with 1 ml. of calcium phosphate gel (2.2 mg. of gel per mg. of protein), the latter prepared according to the

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\(\text{TABLE II}
\)

Distribution of DPN Isocitric Dehydrogenase

<table>
<thead>
<tr>
<th>Source*</th>
<th>Specific activity of extract</th>
<th>Activity per gm. dried powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole guinea pig heart</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>Guinea pig heart, mitochondria</td>
<td>50-70</td>
<td>2000-4000</td>
</tr>
<tr>
<td>Beef heart, washed residue</td>
<td>25-50</td>
<td>1300-2400</td>
</tr>
<tr>
<td>Whole pig heart</td>
<td>0.5 (Ca.)</td>
<td>100</td>
</tr>
<tr>
<td>Pigeon heart, mitochondria</td>
<td>70</td>
<td>3000</td>
</tr>
<tr>
<td>&quot; breast muscle, washed residue</td>
<td>20-40</td>
<td>1500</td>
</tr>
<tr>
<td>Rat kidney, mitochondria</td>
<td>50</td>
<td>5000</td>
</tr>
<tr>
<td>&quot; liver, &quot;</td>
<td>2</td>
<td>600</td>
</tr>
</tbody>
</table>

* All tissue preparations were acetone-dried powders. The extracts were made according to the directions given in the text.
† Total activity in the extract from 1 gm. of dried acetone powder.

\(\text{Kindly furnished by Oscar Mayer and Company, Inc.}\)
directions of Swingle and Tiselius (8). After a contact period of 10 minutes, the suspension was centrifuged for 10 minutes at 18,000 x g. The supernatant solution usually contained less than 5 per cent of the activity and was discarded. The residue was suspended in 80 ml. of 0.1 M phosphate, pH 6.5, and centrifuged. The supernatant solution contained 40 per cent of the protein but little activity. The residue was subsequently washed in a similar manner with 60 ml. of 0.6 saturated ammonium sulfate which removed a considerable amount of TPN enzyme. The DPN isocitric dehydrogenase was eluted from the gel with 30 ml. of 0.3 saturated ammonium sulfate. The ammonium sulfate concentration of the centrifuged eluate was adjusted to 0.6 with saturated ammonium sulfate and centrifuged, and the residue was taken up in 9 ml. of 0.01 M phosphate, pH 6.5. 6 ml. of saturated (NH₄)₂SO₄ were added to this solution. Upon centrifugation the supernatant layer of the 0.4 saturated solution was placed on a starch-Celite column (diameter 2.2 cm., height 2.6 cm.) prepared according to the directions of Fischer and Hilport (9). The column was washed with 25 ml. of 0.4 saturated (NH₄)₂SO₄ which removed none of the activity but considerable protein. The eluent concentration was then changed to 0.3 saturation and three 8 ml. fractions were collected. The second fraction contained most of the activity; its ammonium sulfate concentration was adjusted to 0.5, and the resulting precipitate was collected by centrifugation and dissolved in 5 ml. of 0.01 M phosphate, pH 6.5, for assay. When the initial extract was compared with the purified material, it was found that the DPN isocitric dehydrogenase increased in specific activity from 31 to 1410 units per mg., while the TPN activity decreased from 700 to 50 units per mg. Information pertinent to this fractionation is summarized in Table III.

Coenzyme Specificity Even though the purified DPN isocitric dehydrogenase still contained a small amount of TPN enzyme, no significant inhibitory or activating effect by TPN was observed (Fig. 3). Pyridine nucleotide transdehydrogenase, which was found by Colowick et al. (6) in extracts of Pseudomonas fluorescens, did not seem to be present in this preparation. Nicotinamide mononucleotide, which activates citrate oxidation in heart mitochondria in the presence of small amounts of ATP (1), was not active in the crude or purified preparation either in the absence or presence of ATP. As a matter of fact, the DPN enzyme was almost completely inhibited by 6 μM of ATP.

Properties of Enzyme—DPN isocitric dehydrogenase was completely inactive in the absence of certain metal ions. Manganous ion was found to be a more effective activator than magnesium (Fig. 4).

In contrast to the TPN enzyme which has a very high affinity for isocit-
rate \( (K_s < 1.2 \times 10^{-5} \text{ M}) \) the DPN enzyme has a much higher \( K_s \) (approximately \( 4.5 \times 10^{-4} \text{ M} \)) (Fig. 5). \( K_{DPN} \) was approximately \( 6 \times 10^{-5} \text{ M} \).

Evidence has been presented here that the addition of \( d \)-isocitrate\(^6\) leads to the reduction of DPN in the presence of the appropriate enzyme. In

<table>
<thead>
<tr>
<th>TABLE III</th>
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<tr>
<td>Purification of DPN-Linked Isocitric Dehydrogenase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity*</th>
<th>Recovery of original</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cs(_4)(PO(_4))(_2) gel</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Raffinate</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.1 M phosphate, pH 6.5, wash</td>
<td>5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>0.3 saturated (NH(_4))(_2)SO(_4) eluate</td>
<td>138</td>
<td>70</td>
<td>4.5</td>
</tr>
<tr>
<td>0.4 saturated (NH(_4))(_2)SO(_4) supernatant</td>
<td>261</td>
<td>60</td>
<td>1.9</td>
</tr>
<tr>
<td>Starch column effluent</td>
<td>1410</td>
<td>34</td>
<td>5.4</td>
</tr>
<tr>
<td>Over-all purification</td>
<td>1410</td>
<td>34</td>
<td>46</td>
</tr>
</tbody>
</table>

* Units per mg. of protein.

Fig. 3. Effect of TPN on purified DPN isocitric dehydrogenase. Enzyme specific activity 1410 units per mg. Curve a, 1 \( \mu \text{M} \) of DPN added initially; Curve b, 0.15 \( \mu \text{M} \) of TPN added first, followed after 225 seconds by 1 \( \mu \text{M} \) of DPN.

view of the studies with TPN enzyme from pig heart (10, 11) and DPN isocitric dehydrogenase from yeast (3) it was to be expected that the product of oxidation of the substrate was \( \alpha \)-ketoglutarate. When the reduction of DPN was compared with the appearance of \( \alpha \)-ketoglutarate, a stoichiometric relationship was observed (Table IV). When the product of this reac-

\(^6\) On a molar basis \( dl \)-isocitrate is one-half as effective as the \( d \) form (Fig. 5).
tion was converted to the 2,4-dinitrophenylhydrazone, it was found to migrate at the same rate as an authentic sample of the 2,4-dinitrophenylhydrazone of \( \alpha \)-ketoglutarate on silica gel columns with different solvent systems (12).

When crude dialyzed guinea pig or beef heart extracts were used to reduce DPN in the presence of isocitrate, the reduced DPN could be reoxidized by the addition of \( \alpha \)-ketoglutarate and bicarbonate or by oxalosuccinate. Similar results were obtained with purified fractions from guinea pig heart which contained much DPN enzyme but little TPN isocitric dehydrogenase.

We wish to thank Dr. D. O. Brummond for giving us the directions for this procedure prior to publication.
dehydrogenase. When DPNH was added directly to such preparations, reoxidation was observed with α-ketoglutarate and bicarbonate (Fig. 6). These observations suggested that the reaction catalyzed by DPN isocitric dehydrogenase was reversible (13). However, when more highly purified DPN isocitric dehydrogenase from beef heart was employed, practically no reoxidation of DPNH by either α-ketoglutarate or oxalosuccinate could be obtained (Fig. 6). Furthermore, it was found that, in crude preparations, DPNH could be reoxidized by α-ketoglutarate in the absence of added bicarbonate and that Mn++, which was absolutely required for the isocitric dehydrogenase reaction, was not needed for the oxidation of DPNH by α-ketoglutarate. Attempts to identify isocitrate as the product of the reaction between α-ketoglutarate and DPNH both in the presence and absence of bicarbonate were unsuccessful, and it would appear that some other product is formed. Further studies on this point are in progress.

**DISCUSSION**

DPN isocitric dehydrogenase from guinea pig heart mitochondria or beef heart washed residue preparations resembles in many ways the analogous enzyme prepared from yeast by Kornberg and Pricer (3). For example, it is more labile than the corresponding TPN enzyme, the metal requirement is similar, and finally with purified enzyme no reoxidation of DPNH can be demonstrated with either α-ketoglutarate or oxalosuccinate. How-
ever, in contrast to the enzyme from yeast, the addition of AMP is not absolutely required for activity, although it has been found to stimulate the reaction at times and was therefore routinely added to the reaction mixture.

The presence of DPN isocitric dehydrogenase in whole guinea pig heart mitochondria could not be demonstrated. However, the failure may have been due to the use of cyanide in this assay (14) to inhibit cytochrome oxidase which was subsequently found to be inhibitory to the soluble DPN enzyme.

**Methods and Materials**

Protein was determined by the method of Warburg and Christian (15) and a-ketoglutarate by a modification of the colorimetric procedure of Friedemann and Haugen (16). d-Isocitric acid (17) was kindly supplied by Dr. V. R. Potter from a sample prepared by Dr. Alton Meister. dI-Isocitric acid and oxalosuccinic acid were purchased from the California Foundation for Biochemical Research, 89 per cent DPN and 59 per cent TPN from the Sigma Chemical Company, and ATP from the Pabst Laboratories. 80 per cent NMN was prepared by a modification of the method of Kornberg and Pricer (18, 19). DPNH was prepared by reduction of DPN with alcohol in the presence of crystalline alcohol dehydrogenase (20), followed by heating and removal of denatured protein by centrifugation.

The following reaction mixtures were used for the assay of DPN and TPN isocitric dehydrogenase activity, respectively. The DPN system contained 1.0 ml of 0.1 M cacodylate buffer, pH 6.5, enzyme solution, 0.1 ml of 0.01 M DPN, 0.1 ml of 0.01 M AMP, 0.1 ml of 0.02 M MnSO₄, and 0.1 ml of 0.08 M dI-isocitrate; volume made up to 3 ml with water. The TPN system contained 1.0 ml of 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, enzyme solution, 0.2 ml of 0.0015 M TPN, 0.1 ml of 0.02 M MnSO₄, and 0.05 ml of 0.08 M dI-isocitrate; volume made up to 3 ml with water. In all cases a control containing all reaction components except isocitrate accompanied the particular sample tested. All assays were performed at room temperature in a Beckman model DU spectrophotometer in cuvettes of 1 cm light path.

A unit of enzyme activity was defined as the amount causing an increase in optical density of 0.01 per minute under conditions for which the rate of density increase remained linear for at least 5 minutes.

**SUMMARY**

DPN isocitric dehydrogenase activity was found in aqueous extracts of acetone powders of mitochondria (or washed residues) from various tissues. TPN and DPN isocitric dehydrogenase activity in such prepara-
tions was separated in the case of pigeon breast muscle, guinea pig heart, and beef heart. The DPN enzyme was purified 40- to 50-fold from beef heart extract. DPNH and \( \alpha \)-ketoglutarate were formed in equivalent amounts in this reaction between DPN and isocitrate. The DPN enzyme was activated by Mn\(^{++}\) and Mg\(^{++}\) and had a \( K_i \) of approximately \( 4.5 \times 10^{-4} \) M; the pH optimum was at pH 6.5 (TPN system, pH 7.8). In contrast to DPN isocitric dehydrogenase from yeast the addition of adenosine-5-phosphate was not required for activity. When purified DPN isocitric dehydrogenase was used, DPNH could not be reoxidized with \( \alpha \)-ketoglutarate and bicarbonate or oxalosuccinate.

### BIBLIOGRAPHY

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