THE PREPARATION AND PROPERTIES OF A SOLUBLE
DIPHOSPHOPYRIDINE NUCLEOTIDE
CYTOCHROME c REDUCTASE

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(Received for publication, December 26, 1951)

The transfer of electrons from the pyridine nucleotide-linked dehydro-
genases to cytochrome c is mediated by a class of enzymes which has been
termed the cytochrome c reductases (1). Two TPNH cytochrome c re-
ductases, both flavoproteins, have been purified, one by Haas et al. (1)
from yeast, and another by Horecker (2) from liver. Analogous enzymes
in the case of DPNH have been implicated in particulate preparations (3)
and crude extracts (4, 5), but our understanding of their nature and func-
tion is still fragmentary.

The relationship between the pyridine nucleotide-cytochrome c reduc-
tases and the diaphorase discovered by von Euler et al. (6,7) and Green and
Dewan (8), and isolated in purified form by Straub (9), remains obscure.
This enzyme catalyzes the reoxidation of DPNH by dyes, such as methyl-
ene blue, but is incapable of interaction with cytochrome c, the physi-
ological oxidant. This inability may be due to a structural modification of
a native cytochrome c reductase (3, 10).

On the basis of inhibition studies, Slater (11) has concluded that a factor
sensitive to BAL (2,3-dimercaptopropanol) is necessary for full reductase
activity. More recently Potter and Reif (12) have implicated a similar
factor, as a result of their studies on antimycin A inhibition.

From this laboratory Hayaishi (13) has already reported on the prepara-
tion and assay of a soluble DPN cytochrome c reductase from pigeon
breast muscle. This paper deals with an improved method for the prepa-
ration of this reductase.

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1 DPN+ = diprophosphopyridine nucleotide, DPNH = reduced DPN, TPN+ =
triphosphopyridine nucleotide, TPNH = reduced TPN.
Materials and Methods

Pyridine Nucleotides—DPN+ (Sigma) was found to be approximately 60 per cent pure by the hydrosulfite method of Gutcho and Stewart (14), or by enzymatic reduction with alcohol dehydrogenase. DPNH was prepared by the latter method. TPN+ (76 per cent purity) was kindly supplied by Dr. H. Beinert and was converted to the reduced form in the Zwischenferment system (15).

Cytochrome c—This material (Sigma) was found to be about 90 per cent cytochrome c, as determined from its extinction coefficient at 550 m\(\mu\) (16).

Antimycin—An alcoholic solution containing 100 \(\gamma\) of crystalline antimycin A per ml. was kindly provided by Professor F. M. Strong.

Other Materials—Zwischenferment was a gift of Dr. R. S. Schweet. Diol (2-amino-1,3-propanediol), glycylglycine, and Tris (tris(hydroxymethyl)-aminomethane) buffers were prepared from commercial preparations. Crystalline bovine serum albumin was obtained from Armour and Company.

Test Systems—Enzyme activities were determined spectrophotometrically with a Beckman DU spectrophotometer. For DPNH cytochrome c reductase assays, the experimental cuvette contained \(6 \times 10^{-6}\) mole of glycylglycine, pH 8.7, \(1.6 \times 10^{-7}\) mole of cytochrome c, \(2.3 \times 10^{-7}\) mole of DPNH, 0.1 ml. of enzyme solution, and water to 3.0 ml. The increase in optical density at 550 m\(\mu\) was measured at intervals of 1 minute.

In assays for diaphorase activity, \(1.2 \times 10^{-7}\) mole of 2,6-dichlorophenol indophenol was substituted for the cytochrome c, and the decrease in optical density at 600 m\(\mu\) was followed.

If enzyme preparations were diluted to give optical density changes of 0.015 or less per minute, rates were found to be linear for several minutes. In those instances in which linearity was not observed, the extrapolated value from zero to 1 minute was used for the calculation of enzyme activity. Blanks in the absence of DPNH or in the absence of enzyme produced optical density changes of 0.002 or less per minute. Under the conditions of the test, the measured activities were found to be linear with enzyme concentration over a wide range.

The unit of enzyme activity has been defined as the amount of enzyme producing a change of 1.00 in optical density per minute. The specific activity then is the number of units per mg. of protein.

Protein Concentrations—The weights of enzyme reported are based on absorption at 280 m\(\mu\), which was calibrated by using the average of carbon (17), nitrogen ((16) p. 161), and biuret (18) values on the preparations of highest purity. The carbon and biuret methods were calibrated against crystalline bovine serum albumin. Biuret and nitrogen determinations
agreed closely, while carbon analyses indicated slightly higher contents of organic matter.

Preparations from Pigeon Breast Muscle

Two methods have been developed for the extraction of a soluble DPN cytochrome c reductase from pigeon breast muscle; both utilize preliminary steps similar to those employed in the preparation of diacetyl mutase (19), pyruvic oxidase (20), and α-ketoglutaric oxidase (21).

![Fig. 1. Effect of pH on the solubilization of reductase. All samples adjusted to pH 7.0 before centrifugation.](image)

Method A (Acid Extraction)—Fresh pigeon breast muscle was diced and homogenized in 3 volumes of 0.01 M Na₂HPO₄ in a Waring blender for 90 seconds at 0°. The homogenate was centrifuged (at 0°) for 7 minutes at 5000 r.p.m. (in the angle head of the International centrifuge) and the slightly turbid supernatant was decanted and brought to pH 5.35 with dilute acetic acid. The precipitate which formed was collected by centrifugation at 5000 r.p.m. for 15 minutes. It was then washed with 10 volumes of cold water and diluted 3- to 4-fold with water. The pH of the suspension was carefully adjusted to 4.2 with dilute HCl. The solution was stored in the frozen state overnight. After thawing, the pH was raised to values ranging from 4.7 to 7.0, and the preparation was centrifuged at
16,000 r.p.m. (16,000 X g) in the high speed head of the International centrifuge for 20 minutes, the reductase appearing in the supernatant.

**Table I**

*PH Fractionation of Acid-Extracted Reductase*

<table>
<thead>
<tr>
<th>pH</th>
<th>Reductase activity</th>
<th>Diaphorase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7</td>
<td>5.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Mg. protein per ml. extract</td>
<td>7.1</td>
<td>2.5</td>
</tr>
<tr>
<td>AD per min. per mg.</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>Activity ratio, reductase to diaphorase</td>
<td>0.63</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Table II**

*Effect of Conditions on Efficiency of Extraction of Reductase*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Extraction medium</th>
<th>Temperature °C</th>
<th>Time min.</th>
<th>Mg. protein per ml. extract</th>
<th>Specific activity Reductase</th>
<th>Specific activity Diaphorase</th>
<th>Ratio, reductase/diaphorase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H₂O</td>
<td>43</td>
<td>15</td>
<td>0.8</td>
<td>0.02</td>
<td>0.02</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>3% alcohol, 2% (NH₄)₂SO₄</td>
<td>43</td>
<td>15</td>
<td>1.6</td>
<td>0.03</td>
<td>0.56</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>6% “ 4% “</td>
<td>43</td>
<td>15</td>
<td>2.2</td>
<td>0.06</td>
<td>0.30</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>H₂O, 2% (NH₄)₂SO₄</td>
<td>43</td>
<td>15</td>
<td>2.8</td>
<td>0.11</td>
<td>0.29</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>3% alcohol</td>
<td>43</td>
<td>15</td>
<td>0.40</td>
<td>3.25</td>
<td>2.39</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>H₂O</td>
<td>43</td>
<td>15</td>
<td>0.15</td>
<td>0.43</td>
<td>0.24</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>3% alcohol</td>
<td>43</td>
<td>15</td>
<td>0.16</td>
<td>0.63</td>
<td>0.44</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>6% “</td>
<td>43</td>
<td>15</td>
<td>0.18</td>
<td>1.37</td>
<td>0.79</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>9% “</td>
<td>43</td>
<td>15</td>
<td>0.18</td>
<td>3.50</td>
<td>1.90</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>5.6% alcohol</td>
<td>45</td>
<td>15</td>
<td>0.66</td>
<td>12.9</td>
<td>8.0</td>
<td>1.6</td>
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<tr>
<td></td>
<td>11.2% “</td>
<td>45</td>
<td>15</td>
<td>0.85</td>
<td>10.0</td>
<td>5.8</td>
<td>1.7</td>
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<tr>
<td></td>
<td>16.8% “</td>
<td>45</td>
<td>15</td>
<td>0.50</td>
<td>9.2</td>
<td>5.0</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>5.6% “</td>
<td>55</td>
<td>7.5</td>
<td>1.00</td>
<td>3.3</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>16.8% “</td>
<td>55</td>
<td>7.5</td>
<td>0.80</td>
<td>1.4</td>
<td>0.4</td>
<td>3.6</td>
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<tr>
<td>3</td>
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<td>0</td>
<td>15</td>
<td>0.10</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
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<td>9% “</td>
<td>25</td>
<td>15</td>
<td>0.15</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>9% “</td>
<td>43</td>
<td>15</td>
<td>0.42</td>
<td>16.7</td>
<td>2.9</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>9% “</td>
<td>43</td>
<td>5</td>
<td>0.36</td>
<td>12.2</td>
<td>4.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>9% “</td>
<td>43</td>
<td>18</td>
<td>0.36</td>
<td>12.0</td>
<td>4.7</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>9% “</td>
<td>43</td>
<td>32</td>
<td>0.44</td>
<td>10.0</td>
<td>3.4</td>
<td>2.9</td>
</tr>
</tbody>
</table>

As can be seen from Fig. 1, solubility of the reductase depends strongly on the pH, with best results at pH 4.2. Table I seems to indicate that considerable variability in specific activity and yield attended the subsequent pH adjustment of the suspension at pH 4.2; maximum specific activity...
appeared at pH 6.1, while maximum yields could be observed at pH 4.7. It is interesting to note that, in spite of these variations, the reductase-diaphorase ratios seemed to remain essentially constant throughout the range tested.

Method B (Alcohol Extraction)—This method was developed from Straub’s procedure (9) for the extraction of diaphorase from pig heart preparations. It accomplished in a single step a purification about 5 times greater than that obtained in Method A, even after subsequent purification. The initial homogenization was carried out in the manner already described. The preparation was similarly brought to pH 5.35 by means of acetic acid, and the sediment collected after centrifugation. After washing, freezing, and thawing2 at pH 5.35, the residue was collected by centrifugation at 5000 r.p.m. for 5 minutes. Extraction was effected by suspending the residue in 3 to 4 volumes of 9 per cent ethanol at 42–45° for 15 minutes and removing the insoluble protein by centrifugation at 5000 r.p.m. (2300 × g). Experiments leading to this procedure are summarized in Table II.

Experiment 1 demonstrated that (NH₄)₂SO₄ was extracting inert protein and not serving any useful purpose in this procedure. Experiments 1, 2, and 3 indicated that, upon increasing the alcohol concentration, maximum efficiency was achieved at 9 per cent. The effect of temperature was evaluated in Experiments 3 and 4. Below 25° no reductase was found in solution, while at 55° a substantial decline of activity was observed.

When 0.45 gm. of (NH₄)₂SO₄ was added to each ml. of a 1 per cent solution of lyophilized powder at 0°, the precipitate showed a 3-fold increase in specific reductase activity.

Properties of Enzyme Preparation

Diaphorase Activity—The last column in Table II lists the reductase-diaphorase activity ratio for various extracts. As can be seen, this ratio varies over a wide range and is dependent on the extraction conditions.

Reductase Activity—The alcohol extraction method usually resulted in a very dilute protein solution, 0.02 to 0.05 per cent, with a specific activity near 10 units. (NH₄)₂SO₄ fractionation further purified the preparation, as already indicated.

Stability—The alcoholic extract lost almost all of its activity upon storage at 0° for several days; if frozen, it retained its activity for several weeks, while a dry powder, obtained by lyophilization, was stable for several months if stored at 0°.

2 The freezing and thawing step in this procedure induces aggregation of the colloidal particles, thereby facilitating a sharp separation of the supernatant after the alcohol extraction step.
**Specificity**—When equivalent amounts of TPNH were substituted for DPNH, no activity was observed.

**pH Optimum**—The pH activity curve of the reductase is plotted in Fig. 2. Identical results were observed, on a relative scale, with enzyme preparations obtained with either extraction procedure, although the specific activities varied about 50-fold. Diol and Tris buffers could be substituted for glycylglycine in their respective buffering zones without any effect on the activity of the preparations.

**Salt Effects**—The effects of phosphate, pyrophosphate, chloride, and cyanide ions on the two activities have been studied. Pyrophosphate (100 μM) inhibited reductase activity about 80 per cent but stimulated diaphorase activity. Phosphate and chloride acted in the same manner, only the corresponding inhibitions and stimulations were less pronounced (60 and 30 per cent inhibition, respectively). Cyanide inhibition of reductase activity is probably due, at least in part, to its reaction with cytochrome c (22). Glycylglycine, the ion used as buffer in the routine assays, appeared to have a smaller inhibitory effect.

Crystalline bovine serum albumin (1 mg.), adjusted to pH 8.7, produced only a slight inhibitory effect on both enzyme activities, and no effect on the marked response of either test system to pyrophosphate.

**Pig Heart Preparations**

Straub's purified diaphorase preparations (9) were found to be devoid of reductase activity. When pig heart was extracted under conditions identical to those developed for pigeon breast muscle, i.e. at pH 5.35 and in 9 per cent alcohol at 45°, an extract similar to that of pigeon breast
Inhibition by Antimycin

Since Potter and Reif have recently shown that antimycin is an inhibitor of both the succinoxidase and DPNH cytochrome c reductase systems (12), it seemed appropriate to investigate this inhibition with the soluble reductase from pigeon breast muscle. With the standard test system, no inhibition of either reductase or diaphorase activity could be observed at pH 8.7 or 7.7, even at antimycin levels several-fold greater than those reported by the original authors.

When the neutralized suspension of the precipitate at pH 5.35, however, was tested spectrophotometrically in the presence of cyanide, inhibition of reductase activity was observed at antimycin levels approximately similar to those at which Potter and Reif found inhibition. Their findings were also confirmed as to the extent of the inhibition, with a maximum inhibition of 62 per cent. No inhibition was found in the test for diaphorase activity with the precipitate at pH 5.35 as the source of enzyme.

DISCUSSION

A 9 per cent alcohol solution has been employed for the extraction of a soluble DPNH cytochrome c reductase from a particulate preparation. In principle, this method resembles that claimed by Morton to yield soluble preparations of other hitherto refractory enzymatic activities (23).

The fact that, with the same procedure, pig heart produced a similar activity pattern to that of pigeon breast muscle indicates that Straub's initial extract probably possessed reductase activity, which was subsequently lost or destroyed upon further purification.

The results with antimycin suggest that the action of this agent may be on some site of the insoluble particle affecting reductase activity, but not on the reductase protein proper, or, alternatively, that the properties of the reductase may be critically dependent on environmental factors, such as attachment to larger particles.

Slater's BAL treatment was found to inactivate the soluble reductase and, therefore, could not be tested. Slater has similarly found that he could not use his procedure with soluble yeast TPNH cytochrome c reductase.

Preparations at this stage of purity have been examined in the analytical ultracentrifuge. The sedimentation behavior confirmed the soluble nature of the enzyme.

Personal communication from Dr. E. R. Slater.
SUMMARY

1. The partial purification of a soluble, DPNH-specific, cytochrome c reductase from pigeon breast muscle has been reported.
2. Pig heart preparations were shown to possess similar activity.
3. Antimycin was found ineffective as an inhibitor of the soluble reductase in marked contrast to its effect on the same activity in association with particles.

This investigation was aided by a grant from the National Heart Institute of the National Institutes of Health, and by a grant from the Commonwealth Fund.

The authors wish to thank Professor D. E. Green for his advice and encouragement throughout the course of this investigation.

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