The Preparation and Some Properties of a Reduced Diphosphopyridine Nucleotide Dehydrogenase from the Snake Venom Digest of a Heart Muscle Preparation*

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Particulate preparations from mammalian heart which are able to transfer electrons in successive steps from reduced diphosphopyridine nucleotide (DPNH) to molecular oxygen or to intermediate components of the respiratory chain have been reported from time to time (1, 2). Likewise, lipid-free, soluble DPNH dehydrogenases have been obtained from sarcosomal particles. Among them, the diaphorase of Straub (3, 4) and the DPNH-cytochrome c reductase of Mahler (5, 6) have been extensively studied. Recently, it has been shown that diaphorase is actually a lipoyl dehydrogenase (7, 8). Indeed, the enzyme is a component of the α-ketoglutaric dehydrogenase complex (9, 10). On the other hand, the status and physiological action of DPNH-cytochrome c reductase are still obscure.

In the search for DPNH dehydrogenase in heart particles, another soluble enzyme has been isolated from the snake venom digest of the Keilin-Hartree preparation. This enzyme is different from the diaphorase of Straub. Although in certain aspects it resembles the reductase of Mahler, other properties have demonstrated the nonidentity of these two enzymes.

This paper reports on the enzyme isolated from the snake venom digest of the heart muscle preparation with respect to its solubilization, purification, and properties. Differences from and relations with other soluble DPNH dehydrogenases are discussed. The effect of venom digestion of the heart muscle preparation on its oxidative capacities toward DPNH is also described. Preliminary reports (11-13) have already appeared.

EXPERIMENTAL PROCEDURE

Materials—Cytochrome c (type III), DPNH (95% pure), and lipoic acid were obtained from the Sigma Chemical Company. Potassium ferricyanide, reagent grade, of the J. T. Baker Chemical Company was recrystallized three times from water. Both “desiccated” and “lyophilized” preparations of snake (Naja naja) venom were obtained from Ross Allen’s Reptile Institute, Silver Springs, Florida. Unless otherwise indicated, the desiccated preparation was used. Diethyldiaminethyl (DEAE)-cellulose was purchased from the Eastman Kodak Company and from Brown Company. Other chemicals in the purest grades available were obtained commercially. Liposomide was kindly supplied by Drs. L. J. Reed and Karl Folkers. Coenzyme Q10 (ubiquinone) was kindly supplied by Drs. D. E. Green, R. A. Morton, and F. R. Redfearn. The water used was first distilled and then glass-redistilled.

Enzyme Preparations—The Keilin-Hartree beef heart preparation was obtained by modifications (14) of the original method (15). Heart mince was washed once with phosphate buffer, and centrifugation was used instead of acid precipitation. DPNH dehydrogenase used for spectral study (Fig. 7, A and B) was isolated from heart muscle preparation prepared from heart mince washed three times with phosphate buffer.

Apo-d-aminoo acid oxidase was prepared from pig kidney according to the method of Burton (16) up to Step 5. TPNH-cytochrome c reductase was prepared according to the method described by Huennekens and Felton (17) and Haas, Horecker, and Hoggess (18). Several samples of yeast were tried. Among them, the yeast kindly supplied by the Rainier Brewery, Seattle, Washington, gave the best yield and the most complete removal of FMN from the partially purified enzyme.

Methods—The fat-free dry weight was used to measure total protein content of the heart muscle preparation (19). The method of Warburg and Christian (20) was used for the determination of the protein content of fractions obtained by chromatography, whereas the turbidimetric method with trichloroacetic acid was employed for other samples. Non-heme iron was determined by the Massey method (21). Systems for the determination of DPNH oxidase and dehydrogenase activities with oxygen and other electron acceptors are listed in Table I. The unit of activity was defined as a change of 1 optical density unit per minute, and specific activity, as units per mg of protein.

Exposing the sample to boiling water for 10 minutes, to 10% perchloric acid at 0° for 10 minutes, or to 10% trichloroacetic acid at 0° for 30 minutes was used to dissociate the flavin from the enzyme. The flavin sample from the dehydrogenase which were used for d-aminoo acid oxidase tests were always obtained by

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1 Advice from Dr. F. M. Huennekens on the flavin analysis is acknowledged.

2 The abbreviations used are: DCI, 2,6-dichlorophenolindophenol; FMN, riboflavin phosphate. The heart muscle preparation referred to in this paper is the Keilin-Hartree preparation.

3 The iron content of the enzyme was kindly determined by Dr. C. P. Lee.
heat treatment or trichloroacetic acid precipitation. The excess trichloroacetic acid was removed by extracting the supernatant liquid with peroxide-free ether six times at 0-4°C. The aqueous layer from the last extraction was neutralized and then lyophilized to dryness. The yellow powder thus obtained was dissolved in water. Perchloric acid was removed as its potassium salt by neutralization and concentration at 0°C.

FAD was determined, with apo-n-amino acid oxidase, manometrically (17) and spectrophotometrically (28). FMN was then lyophilized to dryness. The yellow powder thus obtained was redissolved in water and used by Mahler et al. (24), and Tris buffer or 2-amino-2-methyl-1,3-propanediol was also used by Mahler et al. (24). The enzyme solution was diluted with bicarbonate, 0.02 M, when necessary.

Varied amounts of ferricyanide were used in order to determine the maximal velocity by the Lineweaver-Burk plot.

### RESULTS

#### Behavior of Heart Muscle Preparation and Effect of Venom On Its Capacity to Oxidize DPNH

The heart muscle preparation prepared according to our adaptation contained a DPNH oxidase system (cf. 22) with activity of approximately 2.5 μmoles of the substrate oxidized per minute per mg of protein. The oxidation was almost completely inhibited by antiymin A (cf. also 29, 30). The preparation also catalyzed DPNH oxidation by lipoic acid at approximately 15 μmoles per minute per mg of protein when cyanide was used to block the cytochrome oxidase action. However, the preparation did not catalyze the oxidation of α-ketoglutarate by ferricyanide, tested manometrically according to the method of Sandel, Littlefield, and Bock (31), even in the presence of thio-ine pyrophosphate, lipoic acid, coenzyme A, glutathione, DPN, and magnesium chloride.

The DPNH oxidase activity of the preparation was rapidly inactivated in the presence of venom, even at a concentration as low as 0.5 μg per mg of protein. The rate of inactivation of DPNH oxidase varied with preparations of snake venom. A typical response with a lyophilized venom preparation is shown in Fig. 1B. When desiccated preparations were used, 50% of the DPNH oxidase activity was lost in approximately 20 minutes at 37°C and 240 minutes at 25°C. The oxidase activity was also labile to heat; in the absence of venom, the half-life of the enzyme under these conditions was approximately 90 minutes at 37°C and 8 hours at 25°C.

As shown in Fig. 1B, the cytochrome c activity was likewise impaired during the venom digestion. However, after approxi-
FIG. 1. Action of venom on DPNH oxidase, cytochrome c, and DC1 activities of heart muscle preparation. Heart muscle preparation (40 ml) was diluted to 100 ml with distilled water and adjusted to pH 7.5 with N acetic acid. Of this mixture, 8 ml were used as the zero control, and 92 ml were mixed with 0.23 ml of boiled venom (1 mg per ml) and 0.69 ml of 0.1 M CaCl₂. The resulting mixture was incubated with constant shaking at 37° for the intervals indicated. Final concentrations were: heart muscle preparation protein, 10.4 mg per ml; venom, 0.5 μg per mg of heart muscle preparation protein; CaCl₂, 7.5 X 10⁻⁷ M. After incubation, 8.0 ml were removed. A 0.5-ml aliquot was added to 4.5 ml of 0.02 M bicarbonate at 0°. Another aliquot of 6.5 ml was chilled and centrifuged at 40,000 r.p.m. (140,000 X g) for 25 minutes. The supernatant liquid was removed and diluted, as was the mixture. The residue from the centrifugation was resuspended in a final volume of 6.5 ml of 0.1 M borate-phosphate buffer and then diluted 10-fold with 0.1 M phosphate buffer, pH 7.4.

A, DPNH oxidase activity in the mixture; B, Cytochrome c activity in the supernatant fraction (Curve I), in the residue (Curve II), in the mixture as directly determined (Curve Z), and in the mixture by summation of the results from Curves Z and ZZ (Curve IV); C, DC1 activity in different fractions; the numbering system is the same as in B. All of the activities are represented as the percentage of original activity in the heart muscle preparation.

Marily 1 hour, the activity gradually returned and was found in the soluble fraction. The activity which thus emerged was no longer sensitive to antimycin A. The behavior toward 2,6-dichlorophenolindophenol followed the same pattern (see Fig. 1C). The response was, nonetheless, not so dramatic as that for cytochrome c. Evidently, this reflected different loci for cytochrome c and DC1 activities in Systems 1 and 2, respectively, except that in the system with residue, 0.058 M phosphate, pH 7.4, was used instead of glycylglycine, and 1 X 10⁻⁴ M KCN was also present. A, DPNH oxidase activity in the mixture; B, Cytochrome c activity in the supernatant fraction (Curve I), in the residue (Curve II), in the mixture as directly determined (Curve III), and in the mixture by summation of the results from Curves I and II (Curve IV); C, DC1 activity in different fractions; the numbering system is the same as in B. All of the activities are represented as the percentage of original activity in the heart muscle preparation.
FIG. 2. The effect of the ratio of venom to heart muscle preparation protein on the solubilization of DPNH dehydrogenase from the preparation under varying conditions. A, *Naja naja* venom solution in 0.01 M phosphate, pH 5.9, was heated in a boiling water bath for 5 minutes. An appropriate amount of the venom solution was added to a preparation of heart muscle preparation equivalent to 70 mg of protein; the final volume was then adjusted to 7.0 ml. The final concentration of CaCl$_2$ was $7.5 \times 10^{-4}$ M, except for Curve III (CaCl$_2$ absent). The pH of the systems represented in Curves I and II was 7.4, and that of Curve III was 6.5. The mixture was incubated at 37° with constant shaking for 90 minutes. At the end of incubation, the mixture was immediately cooled to 0°; the pH was adjusted back to 7.4, if necessary. The supernatant fraction, after centrifugation for 30 minutes at 140,000 X g, was assayed for cytochrome c and DCI activities in Systems 5 and 6 of Table I. Only the activity toward cytochrome c is given as an indication of the percentage of solubilization.

B, The systems and the operation were the same as in A, except that the venom used was not boiled; Curve IV, pH 7.4, without CaCl$_2$; Curve V, pH 6.5, without CaCl$_2$; and Curve VI, pH 6.5 in the presence of $7.5 \times 10^{-4}$ M CaCl$_2$. 
Some variations occurred in these experiments from time to time, but no correlation was found between the degree of inactivation and the age of the heart muscle preparation. It seemed that the “lyophilized preparation” of venom was somewhat more potent than the “desiccated preparation.” Edwards and Ball (32), using one sample of crude cobra venom, also observed considerable differences from experiment to experiment in their study of the effects of phospholipase on the succinic oxidase system and likewise observed that these effects were independent of age and activity of the muscle preparations.

Although the addition of Ca++ to the system did not enhance the inactivation of DPNH oxidase or the liberation of the dehydrogenase, the action of venom was not lost by boiling. The slightly lower activity of the boiled venom at suboptimal levels compared with that of the unboiled venom (Fig. 2) might be due to surface effects on phospholipase A rather than to actual heat denaturation. Albumin was used as a protective colloid by Edwards and Ball (32), but in our experiments, protective colloid was not added in order to avoid complications. In view of these facts, as well as other aspects of behavior, the inactivation of the DPNH oxidase system was probably due to the specific action of phospholipase.

“Solubilization” and Purification of DPNH Dehydrogenase

The soluble enzyme liberated from heart muscle preparation was purified by calcium phosphate gel adsorption, ammonium sulfate fractionation, and DEAE-cellulose chromatography.

All operations were performed at 0-4° unless otherwise indicated. Seven hundred milliliters of a mixture at pH 7.4, containing 2.5 X 10⁻² M phosphate, 1.5 X 10⁻² M borate, 7.4 X 10⁻⁴ M CaCl₂, 11 mg of heart muscle preparation protein per ml, and 32 µg of boiled venom per mg of protein, were incubated at 37° for 105 minutes with constant stirring. In early experiments,
the pH value used at this stage was 6.5 and was adjusted to 7.4 after incubation. At the end of the incubation, the mixture was cooled and then centrifuged at 59,000 × g for 75 minutes. The clear yellow supernatant liquid (690 ml) was separated.

Of the supernatant liquid, 630 ml were adjusted to pH 6.5 with 1 N acetic acid. Calcium phosphate gel (2.1 ml, 44 mg per ml; or enough to adsorb approximately 10% of the activity) was added. The mixture was stirred for 15 minutes and then centrifuged. The clear supernatant fraction was further treated with 39 ml of gel as before or until approximately 80% of the activity was adsorbed. The gel was separated by centrifugation and eluted twice with 90 ml of 0.2 m Na₂HPO₄ each time. The combined eluates (17.9 ml) were treated with 26 g of solid ammonium sulfate (0 to 0.30 saturation). The mixture was allowed to stand for 15 minutes with occasional stirring and was then centrifuged. The supernatant liquid, 25 g of ammonium sulfate, was dialyzed against 2 liters of buffer for approximately 12

**TABLE II**

**Summary of purification of DPNH dehydrogenase**

The assays were performed in Systems 5 and 6 of Table I for cytochrome c and DCI, respectively. Assay systems for heart muscle preparation were the same, except that 0.022 m phosphate, pH 7.4, was used instead of glycylglycine, and 1 × 10⁻⁴ M KCN was also present.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Activity rate (cytochrome c/DCI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart muscle preparation</td>
<td>0.607</td>
<td>1.50</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant of venom digest</td>
<td>0.934</td>
<td>2.98</td>
<td>69</td>
</tr>
<tr>
<td>Gel eluate</td>
<td>5.89</td>
<td>18.4</td>
<td>44</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.30 to 0.55 saturation)</td>
<td>11.8</td>
<td>35.1</td>
<td>24</td>
</tr>
<tr>
<td>First peak from chromatography</td>
<td>63.5</td>
<td>209</td>
<td>8</td>
</tr>
</tbody>
</table>

**TABLE III**

**Relative rates of acceptor reduction**

The assays were performed with appropriate amounts of DPNH dehydrogenase in Systems 5, cytochrome c, 11 (ferricyanide), 12 (menadione), 6 (DCI) and 2 (lipoic acid), Table I, with the exception that the acceptor concentrations were varied in order to calculate the maximal velocities in terms of the acceptor reduced. Lipoamide activity was determined in System 2 by substituting the amide for the acid. Coenzyme Q₁₀ was assayed in Systems 5, 6, (cytochrome c), 11 (ferricyanide), and 12 (menadione), Table I, with the exception that the acceptor concentrations were varied in order to calculate the maximal velocities in terms of the acceptor reduced.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>100</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>100</td>
</tr>
<tr>
<td>DCI</td>
<td>37</td>
</tr>
<tr>
<td>Menadione</td>
<td>36</td>
</tr>
<tr>
<td>Lipoic acid</td>
<td>0</td>
</tr>
<tr>
<td>Lipoamide</td>
<td>0</td>
</tr>
<tr>
<td>Coenzyme Q₁₀</td>
<td>0</td>
</tr>
</tbody>
</table>

sulfate (0.30 to 0.55 saturation) were added. After 15 minutes with occasional stirring, the precipitate was separated and suspended in 2.5 ml of 0.01 M phosphate, pH 7.4. The suspension was dialyzed against 2 liters of buffer for approximately 12

**TABLE IV**

**Some kinetic constants for DPNH dehydrogenase**

Assays were performed in Systems 5, 6, 11, and 12 of Table I for cytochrome c, DCI, ferricyanide, and menadione, respectively, except that the concentrations of DPNH or acceptor were varied in order to calculate maximal velocities and Michaelis constants by the conventional Lineweaver-Burk plot. $V_{max}$ and $K_{m}$ values are maximal velocities at infinite concentration of acceptor and DPNH, respectively, and are expressed in micromoles of acceptor and micromoles of DPNH reacting per minute per mg of enzyme (corrected to specific activity of 225 toward cytochrome c although the actual determinations were made with samples of lower activities) in the assay system of 3.0 ml at room temperature (approximately 25°C). $K_{m,acceptor}$ and $K_{m,DPNH}$ are the Michaelis constants of the enzyme for acceptor and DPNH, respectively. Turnover numbers were calculated from the values of maximal velocities and are expressed in moles of DPNH oxidized per minute per mole of flavin in the assay system of 3.0 ml at room temperature.

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>$V_{max}$</th>
<th>$V_{max}$</th>
<th>$K_{m,DCI}$</th>
<th>$K_{m,DPNH}$</th>
<th>$K_{m,acceptor}$</th>
<th>$K_{m,DPNH}$</th>
<th>Turnover No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>116</td>
<td>21</td>
<td>7.8 × 10⁻⁴</td>
<td>8.5 × 10⁻⁶</td>
<td>7.1</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>2,6-Dichlorophenolindophenol</td>
<td>43</td>
<td>14</td>
<td>13 × 10⁻⁴</td>
<td>1.7 × 10⁻⁴</td>
<td>5.3</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>114</td>
<td>20</td>
<td>5.2 × 10⁻⁴</td>
<td>7.3 × 10⁻⁶</td>
<td>7.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Menadione</td>
<td>41</td>
<td>95</td>
<td>3.8 × 10⁻⁴</td>
<td>4.6 × 10⁻⁴</td>
<td>5.0</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

* High concentrations of DPNH were inhibitory (see Fig. 5). The values listed were estimated from the extrapolation of the linear part of the Lineweaver-Burk plot.

![Fig. 5. The effect of DPNH concentration on DPNH dehydrogenase action toward various electron acceptors. The unit for the ordinate is the reciprocal of the number of micromoles of DPNH oxidized per minute per mg of enzyme. The upper abscissa is for Curves 1, 4, and 5, and the lower abscissa for Curve 3. Assays for cytochrome c (Curve 1), DCI (Curve 2), ferricyanide (Curve 3), and menadione (Curve 4) were performed in Systems 5, 6, 11, and 12, respectively, except that the concentration of DPNH is indicated in the figure.](http://www.jbc.org/)

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T. E. King and R. L. Howard
Effect of various reagents on DPNH dehydrogenase reactions

Assay systems 5 and 6 of Table I were used for the activity determination toward cytochrome c and DCI, respectively. The operation for testing the action of inhibitors for Nos. 1, 2, 3, 4, 7, 8, 9, 10, 20, and 21 was strictly the same as that used by Mahler and Elowe (34); i.e. the inhibitor was added to an enzyme solution to give final concentrations of $1.0 \times 10^{-5}$ M inhibitor; $0.1 \mu$g per ml of enzyme. The mixture was incubated for 5 minutes at 0°C, and 0.02 ml was then tested in Systems 5 and 6 of Table I.

Nos. 5, 6, 9A, 10A, 11, 12, 13, 14, 15, 16, 17, 18, and 19 were done by addition of the inhibitor indicated to the assay media containing 1.3 μg of enzyme. The reaction was started with DPNH. In all cases, the enzyme was used from chromatography and possessed a specific activity of 180 toward cytochrome c. Nos. 1 and 3 also contained $1 \times 10^{-6}$ M DPNH during the incubation. The mixture was incubated for 5 minutes at 0°C, and 0.02 ml was then tested in Systems 5 and 6 of Table I.

In incubation (-) were the final concentrations during the enzymatic assay.

The concentrations listed with prior incubation (+) were the final concentrations during the incubation; those without prior incubation (-) were the final concentrations during the enzymatic assay.

The data for Mahler's enzyme are from Mahler and Elowe (34) and Vernon, Mahler, and Sarkar (35).

a At $1 \times 10^{-4}$ M.

b At $1 \times 10^{-3}$ M.

c Incubated at 30 degrees in the presence of $10^{-4}$ M FAD or $10^{-3}$ M FMN.

Table V—Continued

| No. | Reagent | Prior incubation | Concentration | Percentage of stimulation (+) or inhibition (−) toward the activity of
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>with inhibitor</td>
<td>2,6-Dichlorophenolindophenol</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Present enzyme</td>
<td>Mahler's enzyme</td>
</tr>
<tr>
<td>19</td>
<td>Dicumarol</td>
<td>−</td>
<td>$1 \times 10^{-3}$</td>
<td>−28</td>
</tr>
<tr>
<td>20</td>
<td>FMN</td>
<td>+</td>
<td>$1 \times 10^{-2}$</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>FAD</td>
<td>+</td>
<td>$1 \times 10^{-3}$</td>
<td>$+5$</td>
</tr>
</tbody>
</table>

Fig. 6. The effect of pH on the DPNH dehydrogenase activity toward DCI and cytochrome c. For DCI, Assay System 6 of Table I was used with glycylglycine buffer (Curve 1), histidine buffer (Curve 2), or phosphate buffer (Curve 3) at different pH values. For cytochrome c (Curves 4), System 5 was used with glycylglycine.
FIG. 7. Absorption spectra of DPNH dehydrogenase. A, Absorption spectrum of a sample with specific activity of 225 for cytochrome c; protein, 3.08 mg per ml. B, An enlarged scale of A at the region indicated. C, Absorption spectra of a less pure sample with specific activity of 154 for cytochrome c; protein, 0.84 mg per ml. Curve 1, oxidized; Curve 2, reduced by $1.5 \times 10^{-4} \text{M} \text{DPNH}$; Curve 3, further reduced by dithionite. D, Absorption spectra of a preparation from rechromatography of a less pure sample on a DEAE-cellulose column, with specific activity of 250 for cytochrome c; protein, 1.15 mg per ml. Curve 1, oxidized; Curve 2, reduced by $1.5 \times 10^{-4} \text{M} \text{DPNH}$; Curve 3, further reduced by dithionite.

Measurements were made with a Cary recording spectrophotometer at room temperature. The enzyme was in phosphate buffer, pH 7.8, at approximately 0.04 M.

Table VI

Fluorescence of DPNH dehydrogenase

The fluorescent intensity was measured at 330 mp with activation at 280 mp. The enzyme solution contained 0.8 mg of protein per ml with specific activity of 110 toward cytochrome c.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Arbitrary unit</th>
<th>Percentage of decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>DPNH, $2.75 \times 10^{-4} \text{M}$</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>DPNH, $5.5 \times 10^{-4} \text{M}$</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>Dithionite</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table VII

$R_F$ values of flavin from DPNH dehydrogenase

Solvent systems 1, 2, and 3 were butanol-acetic acid-water (4:1:5), 8% Na$_2$HPO$_4$ \cdot 7H$_2$O in water, and water saturated tert butyl alcohol, respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>System 1</th>
<th>System 2</th>
<th>System 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>30</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td>FAD</td>
<td>7</td>
<td>37</td>
<td>18</td>
</tr>
<tr>
<td>FMN</td>
<td>11</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Dehydrogenase flavin</td>
<td>14</td>
<td>50</td>
<td>22</td>
</tr>
</tbody>
</table>

Table II is the summary of a representative, but not the best, preparation. The results were reproducible; more than 30 preparations were made. The "lyophilized preparation" of venom was also used and showed no observable difference through the isolation. The concentration of the heart muscle preparation in the incubation mixture was not critical. Specific activities toward cytochrome c ranging from 170 to 230 were obtained. Samples with lower specific activities were further purifiable by rechromatography. The maximal activity seemed to be limited to approximately 250.

Properties of Enzyme

Two peaks with DPNH dehydrogenase activity were obtained from DEAE-cellulose chromatography, as shown in Fig. 4; most of the activity was present in the first peak. The enzyme to be described was in this peak. However, no difference was observed between the fractions collected at these two peaks with respect to the specificity toward electron acceptors or the activity ratio. The reason for the appearance of two peaks is not clear and has not been further investigated. Rechromatography of the first peak gave only one active peak.

The plot of the enzymatic activity for all acceptors tested was linear with the amount of the heart muscle preparation, the crude digest, or the final preparation of the dehydrogenase in the assay system when the absorbancy change was lower than 0.2 unit.

Specificity Toward Electron Acceptors—The purified enzyme catalyzed the oxidation of DPNH by cytochrome c, DCI, ferricyanide, or menadione, but coenzyme Q$_0$ (ubiquinone), lipoic acid, lipoisamide, or oxygen showed no measurable activity. Table III shows the relative rates of the reactions at infinite concentration of acceptor with the rate of cytochrome c reaction arbitrarily chosen as 100. TPNH was not oxidized under these conditions in the presence of the enzyme from either the first or the second peak. In fact, even the preparations at less pure stages did not catalyze the oxidation of TPNH.
reported (36).

However, by choosing 520 nm as the base in these curves, extrapola-
tion of the linear part of a Lineweaver-Burk plot gave $V_{max}$ and $K_m$
values for DPNH as listed in Table IV. No inhibition was ob-
served when DCCl was used as acceptor. Ringler, Minakami,
and Singer have reported a similar inhibition by high concen-
trations of DPNH (33).

Inhibition—The effect of various inhibitors on the DPNH
dehydrogenase reaction is summarized in Table V. The results
reported by Mahler and Elowe (34) and Vernon, Mahler,
and Sarkar (35) are also listed for comparison. Neither Amytal,
$5 \times 10^{-4} M$, nor antimony $A$, 5 $\mu$g per mg of enzyme, produced
inhibition when cytochrome $c$ or DCCl was used as the acceptor.
Citrate or pyrophosphate, tested under the conditions of Mahler
and Elowe (34) with incubation, did not produce any inhibition.
When the concentration was $1 \times 10^{-4} M$ in the assay systems,
inhibitions of 60 and 75%, respectively, were observed toward
cytochrome $c$ and DCCl. The DPNH dehydrogenase action
toward DCCl was stimulated by CaCl$_2$, MgCl$_2$, cyanide, phos-
phate, and perchlorate, whereas toward cytochrome $c$ was
invariably inhibited.

pH Optima—The optimal pH for the reduction of cytochrome $c$
fund to be at 8.8 to 9.0, as shown in Fig. 6. Under the
same conditions, the activity toward DCCl showed two ill defined
optima at pH 7.5 and 8.5 (Fig. 6).

Stability—The purified enzyme was highly unstable, losing in
20 hours' storage in air at $-15^\circ$ approximately 25% of its original
activity toward either DCCl or cytochrome $c$ and approximately
50% at $0^\circ$. In the absence of oxygen, it was relatively stable.
No significant loss of the activity was observed when the enzyme
was stored in a vacuum for 36 hours at $0^\circ$.

Absorption Spectra—The purified enzyme exhibited absorption
spectra as shown in Fig. 7A. The absorption maxima of the
enzyme in the oxidized form were at 450, 335, and 275 nm,
with very small shoulders at 415 and 550 nm. These shoulders
were more distinct when the heart muscle preparation used was pre-
pared without preliminary washing with phosphate buffer.
Rechromatography of a sample with specific activity of 150,
which originally had shown a distinct peak at 415 nm (Fig. 7C),
yielded the enzyme without this maximum, and the specific
activity increased to approximately 250. Upon the addition
of DPNH, the 450 nm maximum decreased; the absorption
was further decreased by reaction with dithionite as shown in Fig.
7, C and D.$^6$ It should be pointed out that DPNII reduced the
absorbance at 450 nm only approximately two-thirds as much as
dithionite did. Moreover, even dithionite did not completely
abolish the absorption. The reason for this is not clear, but the
possibility that iron in a special coordination complex contributes
to the absorption cannot be neglected. The effect of ligands on
the absorption spectra of ferrous and ferric complexes has been
reported (38).

$^6$ Direct comparison of the absorbance as such at 450 nm among
the curves of Fig. 7 cannot be made, because different wave lengths
were used in these experiments to set the zero reference for scan-
inning in the spectrophotometer. Comparison can easily be made,
however, by choosing 320 nm as the base in these curves.

Fluorescence—The enzyme possessed a fluorescence peak at
330 nm when activation energy at 280 nm was used. When an
activation wavelength of 370 nm was used, a fluorescent peak
occurred at 520 nm. The latter possessed approximately 3% of
the fluorescent intensity of free FMN. Authentic free FMN
at concentrations comparable to the flavin content of the enzyme
sample, when activated at 280 nm, showed a fluorescent peak at
520 nm, but no peak was observed at 330 nm. The fluorescence
of the enzyme was reduced to 40% of its original amount upon
the addition of DPNH, and to zero by sodium dithionite, as
shown in Table VI.

Prosthetic Groups—The flavin of the enzyme could be disso-
ciated from the protein by treatment with 10% cold trichloro-
acetic acid, 10% perchloric acid, or heating at 100°. The flavin
thus liberated was completely inactive for apo-$d$-amino acid
oxidase. Quantitative recovery was assured by adding authen-
tic FAD to the sample for the entire course of the operation.
The nature of the flavin was established as FMN by three inde-
pendent methods. Chromatography in three different solvent
systems of the dehydrogenase flavin gave $R_f$ values very close
to authentic FMN, as shown in Table VII. The deviation from
the authentic FMN in water-saturated tert-butyl alcohol may
have been due to the fact that the $R_f$ values of these flavins were
rather sensitive to the impurities present in samples, and that
the spot from the dehydrogenase flavin was diffuse.

The flavin liberated from the enzyme was also analyzed fluoro-
metically. It exhibited the typical behavior of FMN. No
increase in fluorescence was obtained after acid hydrolysis.
Finally, the flavin was characterized with a FMN specific
TPNH-cytochrome $c$ reductase of yeast. By this method,
there were found 7.2 mmoles of FMN per mg of enzyme with
specific activity of 225 toward cytochrome $c$. Spectrophot-
ometric determinations made directly on the intact enzyme by
assuming the absorbancy index as $1.04 \times 10^4$ cm$^2$ mole$^{-1}$ for
the difference of absorbancy between the oxidized and the reduced
forms at 450 nm with the reference wave length of 520 nm gave
8.1 mmoles of FMN per mg of protein. Incubation of 10 mg
of trypsin with the washed residue from the trichloroacetic acid-
extracted dehydrogenase (5 mg) at pH 7.4 and 37° did not yield
any additional flavin measured spectrophotometrically. The
difference of approximately 15% in the results from these two
methods might be due to the accuracy of the absorbancy index
employed, to the contribution (see "Absorption Spectra") of
another group on the enzyme to the absorbancy, or to both.

Iron analysis$^3$ showed that the dehydrogenase contained
$3 \times 10^{-4}$ g atom of non-heme Fe per mg of protein, or 3.6 g atoms per
1.2 $\times 10^9$ g (see below) of the sample with specific activity of 225.

Sedimentation and Electrophoresis—In 0.1 M phosphate, pH 7.4,
a sample of the purified enzyme with specific activity of 250
toward cytochrome $c$ was studied for its behavior during ultra-
centrifugation at 50,740 r.p.m. for 120 minutes. Only one peak
was observed. The sedimentation constant was found to be
$s_{0.0} = 6.3 S$. A less pure sample with specific activity of more
than 160 showed two peaks under the same conditions. The
major peak was found to have $s_{0.0} = 6.24 S$, whereas the minor
peak had $s_{0.0} = 16$.

A sample of the enzyme with specific activity of 225 at 4 mg
per ml of glycylglycine-HCl buffer, pH 8.25, ionic strength 0.05,
was studied by free boundary electrophoresis. A single sym-
metrical peak was observed in 07 minutes at 7.5 ma and 100
volts. The limited supply of concentrated samples of the
enzym and the aforementioned instability in air prevented the
determination of the diffusion coefficient and other characteristics. However, samples with specific activities higher than 225 approached homogeneity.

Data available did not permit the precise calculation of the molecular weight of the enzyme. On the assumption of 1 mole of flavin per mole of protein, the empirical molecular weight is approximately 1.2 \( \times 10^4 \). If the diffusion coefficient and the partial specific volume of the enzyme were identical with those of the old yellow enzyme, the molecular weight would be approximately 1.1 \( \times 10^4 \). Thus, most probably, the dehydrogenase contains 1 mole of flavin per mole of enzyme.

**Differential Extractions of Heart Muscle Preparation**

In order to ascertain whether any other DPNH-dehydrogenating enzymes remained in the residue from venom-digested heart muscle preparation, experiments on differential extraction were performed. The residue from the venom digestion was extracted with 10% ethanol for cytochrome \( c \) reductase according to Mahler's method (24). It was found from several experiments that this fraction contained 10 to 35% of the cytochrome \( c \) activity based on the preparation originally used. The residue before the extraction was, however, practically inactive toward cytochrome \( c \). Thus it was evident that this additional activity was further released by the alcohol extraction. On the other hand, when the residue was extracted according to the Savage method (4) for diaphorase, the extract showed more than 50% of the original lipoypeptide activity of the heart muscle preparation but was inactive toward cytochrome \( c \).

The reproducibility in these experiments was not as good as might be desired. When the manipulations of suspensions in several steps and determination of low activities involved are considered, poor precision was perhaps to be expected. The trend was very clear, however; the Mahler type of enzyme was liberated largely in the soluble fraction in the snake venom digestion, whereas more than half of the lipoyl dehydrogenase or diaphorase remained in the residue. The total recovery of DCI and cytochrome \( c \) activities was always larger than the original activities in the heart muscle preparation and ranged between 120 to 170% (also cf. Fig. 2).

**Table VIII**

**Effect of alkali treatment of DPNH oxidation of heart muscle preparation**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Acceptor</th>
<th>Oxygen</th>
<th>Cytochrome ( c )</th>
<th>DCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>°</td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>22</td>
<td>10</td>
<td>16</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>28.5</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Incubation of the heart muscle preparation at pH 9.4 abolished its ability to oxidize DPNH by oxygen, cytochrome \( c \), or DCI, as shown in Table VIII. The action of alkali on the DPNH system was similar to that on succinic oxidase (37, 38). This alkali-treated preparation, together with a soluble succinic dehydrogenase preparation, has been used successfully in reconstitution of succinic oxidase (38).

DPNH oxidation by the alkali-treated preparation could be somewhat restored by the addition of pure diaphorase plus menadione or \( \alpha \)-tocopherol in a system whose DPNH was generated in the presence of sorbitol, rat liver sorbitol dehydrogenase (39), and DPN. The system with menadione might be considered as a model system, since reduced menadione could also be oxidized by oxygen nonenzymatically as well as through cytochrome \( c \) and cytochrome oxidase. In the reconstituted oxidase system with tocopherol when diaphorase was limiting, the rate of DPNH oxidation was less than 5% of the rate of the DCI reduction by diaphorase.

Numerous efforts were made to reconstitute the alkali-treated heart muscle preparation with the dehydrogenase described or with the Mahler enzyme. All, without exception, failed to show restoration of the DPNH oxidase activity to a significant degree, even in the presence of \( \alpha \)-tocopherol, coenzyme Q\(_1\) (ubiquinone), mitochondrial lipid, or all three. The reconstitution of DPN oxidase was equally unsuccessful when a soluble preparation of succinic dehydrogenase (38) was also present. It was, of course, realized that the failure of the reconstitution could be due to the alkali-treated heart muscle preparation. The latter is a suitable source for the components of the succinic oxidase system but may not be for the DPNH oxidase.

**DISCUSSION**

As early as 1933, Warburg and Christian (40) recognized the role of flavoproteins in the catalytic oxidation of DPNH during intracellular respiration. Since then, numerous attempts have been made to isolate the native DPNH dehydrogenase. Although a number of particulate preparations with varying complexity have been reported, only a few lipid-free, soluble preparations have been rigorously studied. Straub (3), Savage (4), and Massey (28) extracted diaphorase from heart muscle particles with dilute ammonium sulfate in the presence or absence of ethanol, whereas Edelhoch, Hayashi, and Teply (41) and Mahler et al. (24) succeeded in isolating cytochrome \( e \) reductase by heating heart particles with 10% ethanol at approximately 44°. Another enzyme, similar to the reductase, has been reported by de Bernard (42). Its properties resemble those of the Mahler enzyme. Indeed, the method of solubilization is practically the same as that used by Edelhoch, Hayashi, and Teply and by Mahler et al.

These treatments are relatively drastic. Modifications of unstable enzymes readily occur in alcoholic solution at elevated temperatures. In a search for milder methods, the work of Nygaard and Sumner (43) and Edwards and Ball (42) came to our attention. They found that phospholipase A inactivates succinic oxidase with concomitant release of fatty acids but exerts little adverse effect on succinic dehydrogenase tested with

Part of the experiments described in this section were done at the Molteno Institute, Cambridge University, Cambridge, England.

A very pure preparation of diaphorase made according to the Savage method was kindly supplied by Dr. Malcolm Dixon.
The importance of phospholipids has been gradually demonstrated in electron transport systems (see, for example, (44)). Phospholipase may act on DPNH oxidase as it does on succinic oxidase. With this premise in mind, we designed systematic experiments for the study of phospholipase activity on DPNH oxidase. At approximately the same time, Ambe and Crane (45), studying the extractibility of cytochrome c, reported the inactivation of DPNH oxidase in their electron transport particles by snake venom, but did not investigate the fate of the dehydrogenase after the treatment.

When the heart muscle preparation was incubated with boiled venom at low concentrations, only a small amount of DPNH dehydrogenase was found in the soluble fraction. The liberation of the soluble enzyme was, however, rapidly increased by increasing the venom concentrations (Fig. 2). By our method (12), Ringler, Minakami, and Singer (33) have confirmed our results on the ability of snake venom to liberate DPNH dehydrogenase from "DPNH oxidase" particles or a fragment of electron transport particles (46). However, their enzyme differs from our dehydrogenase in one important aspect, i.e., it shows very little activity toward cytochrome c. We reported in our preliminary communication (12) that the enzyme preparation up to the stage of gel elution showed activity ratios of 200:100:1 for ferricyanide, DCI, and methylene blue at finite concentrations of acceptor in Systems 10, 8, and 9 (Table I), respectively. The activity for cytochrome c assayed in System 7 and for lipoic acid in System 2 was less than 1% of that for ferricyanide. Later, it was found that these activities, especially for cytochrome c and methylene blue, vary greatly with concentration of acceptor. The most important factor, however, is the phosphate concentration; the phosphate in Assay System 7 originally used is sufficient to inhibit the activity toward cytochrome c by more than 95%. On the other hand, the activity toward DCI is somewhat stimulated by phosphate (Table V).

The present enzyme may be readily distinguished from diaphorase; the same may not be claimed for cytochrome c reductase. Both enzymes are sensitive to oxygen (cf. 47), contain approximately 4 iron atoms per flavin, are free from heme, and can catalyze the oxidation of DPNH by cytochrome c, DCI, and ferricyanide, and menadione. The de Bernard enzyme (42) contains 2 atoms of iron and approximately 0.05 mole of heme. The enzyme of Ringler, Minakami, and Singer (33) also shows heme absorption with a pronounced peak at 410 mp.

On the other hand, the DPNH dehydrogenase differs from the Mahler enzyme in many aspects. The flavin moiety of cytochrome c reductase exhibits three flavin spots on paper chromatograms, whereas the flavin from the dehydrogenase can be quantitatively accounted for as FMN. It must be noted that the aci-d-soluble flavins of the heart muscle preparation contain both FMN and FAD (48). Differences between these two enzymes toward certain inhibitors, as shown in Table V, are also distinct. Calcium chloride at \(10^{-4} \text{ M}\), for example, completely inhibits the activity of the Mahler enzyme toward DCI but stimulates the activity of the dehydrogenase nearly 2-fold. Magnesium chloride, 8-hydroxyquinoline, citrate, and pyrophosphate tested under Mahler's conditions (34) have very little effect upon our enzyme but inhibit cytochrome c reductase (34, 35) to varying degrees. The molecular weight of the dehydrogenase is approximately 50% higher than that of the reductase. The argument that the dehydrogenase may be an impure state of the reductase can be ruled out; the specific activity of the dehydrogenase is already higher than the highest value of the pure reductase either from experiments or from calculations given by Mahler et al. (24). Moreover, other physical tests already mentioned have not indicated any gross contamination in the final dehydrogenase preparations. All of these results would indicate that DPNH dehydrogenase and cytochrome c reductase per se are different entities. Other similarities and differences among various soluble DPNH-dehydrogenating enzymes have been reported elsewhere (48).

In view of the flavin distribution of heart muscle preparation, it is reasonable to assume that at least two DPNH-dehydrogenating enzymes exist in it. One is diaphorase, that is, a component of the \(\alpha\)-ketoglutaric dehydrogenase complex, although the heart muscle preparation does not contain the complete complex. Diaphorase may account for the FAD content of the acid-extractable flavins of the heart muscle preparation. In mitochondria, diaphorase may play the role of the reverse reaction, transferring the hydrogen atoms from \(\alpha\)-ketoglutarate to DPNH through lipico acid by way of the \(\alpha\)-ketoglutaric dehydrogenase complex. The DPNH and succinate thus formed are oxidized by molecular oxygen through the respiratory chain enzymes. Therefore, the failure to reconstitute DPNH oxidase with diaphorase is expected.

The fact that only a small amount of cytochrome c reductase can be further extracted from the residue of the venom-digested heart muscle preparation, and that the Mahler enzyme is similar to, but not identical with, our dehydrogenase, suggests that our enzyme and the reductase are derived from the same segment of the respiratory chain. Through extractions with alcohol, cytochrome c reductase emerges; with venom digestion, the DPNH dehydrogenase is liberated. This is reconcilable with the recent work of Huennekens et al. (49), who employed a modification of the Mahler method for solubilization and obtained from "DPNH oxidase" particules, an enzyme slightly different from the reductase. Its prosthetic group was characterized as FMN (49). The de Bernard enzyme (42) may be classified in the same family by the same reasoning and accounts for the FMN in the acid-extractable flavin fraction. The original segment containing FMN in the respiratory chain could yield different enzymes under different conditions of solubilization. It should be noted that none of these enzymes is antimycin A- or Amytal-sensitive. In fact, the antimycin-sensitive cytochrome c activity is rapidly destroyed by very low concentrations of venom. On the other hand, the antimycin-insensitive cytochrome c activity does not emerge until prolonged digestion or, more effectively, high concentrations of venom are employed. Thus, it is conceivable that the loci which react with cytochrome c in the intact heart muscle preparation may be different from those in the isolated dehydrogenase systems, or, in other words, the original sites lose access to the next member of the respiratory chain (the chain is ruptured); new sites are then exposed through the action of alcohol or venom. The new electron pathway is no longer antimycin A-sensitive.

B. Mackler and F. M. Huennekens, personal communication, 1961. After the present manuscript was submitted, two papers (50, 51) on their enzyme have appeared.

At this time, it is immaterial whether the inactivation is due to the action of venom per se or to the effect of products thus released. Nonetheless, the evidence presented would suggest the former as the mechanism. Edwards and Ball (32) have discussed this problem with respect to the succinic oxidase system. The new sites of the isolated dehydrogenase may or may not be the same sites as those in the intact respiratory chain.
and Amytal-sensitive. Actually, the identity of the component labile to the antibiotic has already been suggested by Chance and other investigators (cf. (30)) and is relatively far from the dehydrogenase on the chain. With regard to the narcotic, it has been proposed (52) that in phosphorylating systems such as ascites cells and intact mitochondria, Amytal acts on the respiratory chain somewhere near the flavoenzyme. But the evidence cited may not permit the conclusion that Amytal acts directly on the enzyme. Indeed, in nonphosphorylating systems (53), the rate of reduction of both cytochromes b and c has decreased to almost zero in the presence of Amytal, whereas the rate of reduction of flavoprotein appears to be as fast as in the uninhibited state. These observations would suggest that Amytal acts on a site between the flavoprotein and the next member of the chain, but not between DPNH and the flavin ((53), cf. also (54)).

Consequently, with respect to the behavior of cytochrome c in the isolated dehydrogenase systems, the pigment may be considered as an "artificial" electron acceptor, just like other dyes. Therefore, the action is not sensitive to antimycin or Amytal. This pattern is equally applicable to the DCI activity, although the initial activity in the heart muscle preparation is also antimycin and Amytal insensitive and the active site is evidently different from that of cytochrome c. The latter argument is further borne out by the demonstration of the nonidentity of $V_{max}$ for DCI and cytochrome c. Because of these facts, the recovery of either DCI or cytochrome c activity can be higher than 100% of the original. The term "recovery" as used does not hold very much meaning.

Superficially, the above view may confront serious obstacles from the work by Ziegler, Green, and Doeg on a lipoflavoprotein (55). This enzyme, with a molecular weight of at least $4 \times 490,000$, is antimycin A-insensitive and can be converted to the de Bernard, Mahler, or Straub enzymes depending upon the conditions used. Actually, it is possible that diaphorase lies proximate to the respiratory DPNH dehydrogenase. The method used by Ziegler, Green, and Doeg may cleave the respiratory chain in such a way that the resulting fragment contains both enzymes together with a large part of the lipid, but the Amytal- and the antimycin-sensitive sites are excluded. The real dilemma lies in the fact that the flavin from either lipoflavoprotein or electron transport particles is exclusively in the form of FAD (55), in contrast to the pre-existing FMN and FAD in the Keilin-Hartree preparation (48). However, a very recent communication (40) reports that electron transport particle, or a particulate "DPNH oxidase" derived therefrom, does contain FMN as well as FAD.

However strong may appear the evidence supporting the view that the same segment of the respiratory chain can yield various enzymes of the Mahler "family," the view will be definitely established only by the successful reconstitution, with isolated components, of DPNH oxidase under well defined conditions and the concomitant interconversions of the soluble enzymes. Searching for a suitable method of enabling the isolation of the primary dehydrogenase in a more "native" form, in addition to studying the lipoprotein particles involved, will prove useful.

**SUMMARY**

The Keilin-Hartree preparation from bovine heart contains reduced diposphopyridine nucleotide (DPNH) oxidase and diaphorase (lipoyl dehydrogenase). The oxidase activity is labile to digestion with snake venom at even as low a concentration as 0.5 $\mu$g per mg of protein of the heart muscle preparation. During the treatment, the activities of the heart muscle preparation toward cytochrome c and 2,6 dihydroxyphenolindophenol are also decreased. These activities reappear in the soluble fraction after prolonged digestion, or at higher concentrations of venom. The activities thus liberated are not antimycin A-sensitive.

A soluble DPNH dehydrogenase has been isolated from the venom digest of the heart muscle preparation. It catalyzes the oxidation of DPNH by ferriyanide, cytochrome c, 2,6-dichlorophenolindophenol, and menadione but not by coenzyme Q, lipoic acid, and lipoyl. The enzyme contains 1 mole of riboflavin phosphate and 4 atoms of iron per mole of protein, with an approximate molecular weight of $1.2 \times 10^{5}$. By comparison of its kinetic behavior and other properties with soluble DPNH-dehydrogenating enzymes from heart particles reported in the literature, it is concluded that the DPNH dehydrogenase described is different from the diaphorase of Straub, the DPNH-cytochrome c reductase of Mahler et al., and the de Bernard enzyme.

Evidence is presented to indicate that the enzymes of Mahler and de Bernard and the one described in this paper are derived from the same segment of the respiratory chain, whereas the diaphorase of Straub is from another part in proximity. The loss for the cytochrome c and 2,6-dichlorophenolindophenol action in the isolated enzymes are different from those on the intact respiratory chain.

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A DPNH Dehydrogenase from Heart Particles

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