THE PURIFICATION AND PROPERTIES OF MICROSMAL CYTOCHROME REDUCTASE*  

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In earlier work (1) a DPNH-specific microsomal cytochrome reductase, liberated by alcohol extraction and partially purified, was identified in liver microsome fractions from rats and rabbits. Microsomal cytochrome, but not cytochrome c, was found to act as electron acceptor in the oxidation of DPNH catalyzed by the enzyme. Through a rapid cytochrome to cytochrome reaction, cytochrome c was reduced in this system when the microsomal cytochrome was added as an intermediate electron carrier. Although flavin was found in the preparations, the large amount of contaminating protein made it impossible to determine conclusively whether the flavin was a prosthetic group on the reductase. The present report describes the isolation of this enzyme as an essentially homogeneous flavoprotein from calf liver microsomes by a new method. Measurements of the molecular weight and physical properties, the characterization of the coenzyme and other active groups, analysis for possible metal cofactors, and studies of the general enzymatic properties of the reductase system are presented. The yield of microsomal cytochrome reductase establishes this enzyme as a major part of the so called DPNH cytochrome c reductase in microsomes.

Methods

Most enzymatic assay systems were similar to those described previously (1–3), except that microcells* containing 0.2 ml. were used for aerobic and anaerobic measurements. All systems contained DPNH as electron donor.

* This investigation was supported in part by research grants No. H-2768 and H-2732 from the National Heart Institute, Public Health Service. A preliminary account of this work has been submitted for publication.

1 The following abbreviations are used: DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PCMBS, p-chloromercuribenzenesulfonate.

2 Aerobic microcells (0.50 ml.) were obtained from the Pyrocell Manufacturing Company. The anaerobic microcell is similar to the larger cell described previously (3) with a microcell at the optical end.
When microsomal cytochrome was used as acceptor, cytochrome c was added to reoxidize the microsomal cytochrome. DPNH oxidation was followed at 340 m\(\mu\) with other electron acceptors. For anaerobic systems Linde nitrogen was freed from residual oxygen by being passed through a vanadyl sulfate tower. Measurements were made with a calibrated Beckman model DU spectrophotometer with use of the photomultiplier attachment to maintain small slit widths. For measuring activities during purification and as a general standard of enzyme activity, the optical density change at 550 m\(\mu\) was followed at 25\(\degree\) in an aerobic silica microcell containing 0.01 \(\mu\)mole of DPNH, 0.001 \(\mu\)mole of microsomal cytochrome, and 0.01 \(\mu\)mole of cytochrome c in 0.20 ml. of 0.1 M Tris-acetate and 0.001 M EDTA at pH 8.10. The concentration of microsomal cytochrome, as shown below, is not optimal in this assay system, but the specific activity of any preparation was arbitrarily calculated from the micromoles of cytochrome c reduced per minute per mg. of protein in this standard system.

Protein analyses were usually made by the colorimetric method of Lowry et al. (4), with crystalline bovine serum albumin as standard. \(\alpha\)-Amino acid oxidase was carried to Step 4 of the method of Negelein and Brömel (5), and the Cy gel\(^3\) used was prepared by the method of Willstätter, Kraut, and Erbacher (6). Microsomal cytochrome was isolated from calf liver essentially as described previously for preparation from rabbit liver (2). The initial suspensions of calf liver microsomes, however, were made as described below in Steps 1 and 2 of the preparation of microsomal cytochrome reductase.

The various substituted indigo dyes were synthesized by Dr. P. W. Preisler; other dyes used were commercial preparations. The beef heart preparation of cytochrome c, PCMBS, FAD, FMN, riboflavin, DPNH, and TPNH were products of the Sigma Chemical Company. Cobra venom (\(Naja naja\)) was obtained from Ross Allen’s Reptile Institute.

**EXPERIMENTAL**

*Preliminary Experiments*

Microsomes catalyze the reduction of added microsomal cytochrome by DPNH at 40 to 60 per cent of the rate at which cytochrome c is reduced. The specific microsomal cytochrome reductase (1) together with the large amount of microsomal cytochrome in the particles can therefore account for at least 40 to 60 per cent of the cytochrome c reductase activity of microsomes. A major problem in attempting to purify the microsomal cytochrome reductase was to find a procedure which would free the enzyme in a soluble form from the particles in good yield. This was achieved by in-

\(^3\) The Cy gel was a generous gift of Dr. R. K. Crane.
cubating calf liver microsomes with cobra venom at 37° and pH 6.55. Reductase was released continuously during this treatment in amounts directly proportional to the time of incubation. At the end of 5 hours, over 80 per cent of the enzyme had been freed in a soluble form. During the incubation the pH dropped to 5.9 to 6.1, indicating the release of acid groups. When the initial pH was raised to 7.3, the yield of reductase was only 20 to 40 per cent. Although a pH of 6.55 is within the pH range for maximal lecithinase A activity, it remains to be decided whether or not release of reductase from microsomes is due in part or entirely to the action of this hydrolytic enzyme of crude snake venom.

Preparation of Microsomal Cytochrome Reductase

Materials—Approximately 18 liters of sucrose media, containing 0.25 m sucrose and 0.001 m EDTA at pH 7.6, are prechilled in a cold room at 5°. The calf liver, obtained fresh from the slaughterhouse, is chilled in ice within 20 minutes after death of the animal and is used within 1 hour. All operations, unless otherwise indicated, are carried out at 2–5°.

Step 1—2000 gm. of calf liver are passed through a cold meat grinder and then homogenized in approximately 150 gm. batches for 30 seconds in Waring blenders with a total of 6 liters of sucrose media. This suspension is then diluted with an additional 12 liters of sucrose media, filtered quickly through two layers of gauze, and passed through a Sharples centrifuge (12,000 X g) at the rate of 1 liter per minute. This removes red blood cells, nuclei, and unbroken cells, and yields 18 to 19 liters of supernatant fluid. The elapsed time for this procedure is about 1 hour.

Step 2—The supernatant fluid from Step 1 is next passed through the centrifuge (12,000 X g) at a rate of 1 liter per 6 to 10 minutes to remove the largest portion of the mitochondrial fraction. The supernatant fluid (17 to 18 liters) is saved.

Step 3—The supernatant fluid from Step 2 is brought to pH 5.35 with 1 N acetic acid and centrifuged for 7 minutes at 8500 X g. The precipitate, obtained by centrifuging this suspension in the Sharples centrifuge (12,000 X g) at a flow rate of 1 liter per 6 to 10 minutes, is suspended in 1500 ml. of 0.05 m Tris-acetate plus 0.001 m EDTA, pH 8.15, and stored overnight.

Step 4—The suspension from Step 3 is brought to pH 5.35 with 1 N acetic acid and centrifuged for 7 minutes at 8500 X g. The precipitate is resuspended with 925 ml. of 0.05 m Tris-acetate plus 0.001 m EDTA, pH 8.1. Slowly, 160 mg. of cobra venom in 50 ml. of 0.1 m Tris-acetate plus 0.001 m EDTA, pH 8.1, are added to the suspension, and the pH is adjusted to 6.55 at 5° with 1 N acetic acid. (This pH is critical.) The preparation is then stirred gently at 37° for 5 hours, after which the suspension
is cooled to 5° in an ice bath and centrifuged at 8500 X g for 20 minutes to yield approximately 1000 ml. of supernatant fluid containing the reductase. The pH of this solution is immediately brought to 8.4 with 1 N NaOH, to avoid loss of activity, and stored overnight.

**Step 5**—The supernatant fluid from Step 4 is fractionated with solid ammonium sulfate. The pH is maintained at 8.4, measured at 5°, with 1 N NaOH. The protein precipitated between 45 and 85 per cent saturation with ammonium sulfate is centrifuged at 8500 X g for 10 minutes, dissolved in approximately 100 ml. of 0.1 M Tris-acetate plus 0.001 M EDTA, pH 8.1, and dialyzed against three 2 liter volumes of 0.1 M Tris-acetate plus 0.001 M EDTA, pH 9.1. The volume of dialyzed preparation is 160 ml.

**Step 6**—Two columns, 2 by 40 cm., which contain a mixture of 10 gm. of Celite and 40 ml. of Cγ gel (15.56 mg. of dry weight per ml.), are washed with water and then with 0.1 M Tris-acetate plus 0.001 M EDTA, pH 9.1. The dialyzed enzyme from Step 5 is then treated successively with 5 ml. portions of Cγ gel, the mixture being stirred each time for 20 minutes and then centrifuged at 1000 X g. Impurities, largely tan at first and then pink in color, are removed in this manner with about 25 ml. of gel and discarded. The reductase is then adsorbed on two 30 ml. aliquots of gel, each of which is stirred in the enzyme solution for 2 hours. The gel, containing the enzyme, is suspended in about 100 ml. of 0.1 M Tris-acetate plus 0.001 M EDTA, pH 9.1, 16 gm. Celite is added and one-half of the suspension is layered on each of the Cγ gel-Celite columns.

The columns are washed first with 70 to 80 ml. of 1 M Tris-acetate, pH 9.1 (24 hours under 4 feet of hydrostatic pressure), and then with 100 ml. of 1 M Tris-acetate, pH 9.1, containing 10 ml. of saturated ammonium sulfate per liter (48 hours under 4 feet of hydrostatic pressure). By this time the reductase has moved to within 5 cm. of the bottom of the column, forming a bright yellow band and leaving over 90 per cent of the protein in the upper part of the column. All except the lower 8 cm. of gel Celite mixture is then removed from each column and the enzyme is eluted with 1 M Tris-acetate, pH 9.1, containing 20 ml. of saturated ammonium sulfate per liter. Two 10 to 14 ml. fractions of lemon-yellow reductase are obtained from each column and combined.

**Step 7**—The reductase from Step 6 is fractionated with ammonium sulfate. Increasing amounts of a saturated solution of ammonium sulfate (5°) are added to the reductase solution and the precipitate between 60 and 85 per cent saturation is centrifuged and dissolved in 10 ml. of 0.1 M Tris-acetate plus 0.001 M EDTA, pH 8.1. This last stage of purification was also accomplished by preparative electrophoresis at pH 8.1.

The reductase from Step 7 is between 90 and 100 per cent pure as judged
by several criteria (see below). A summary of the steps of a typical preparation is shown in Table I. The enzyme can be stored either in 0.1 M Tris-acetate plus 0.001 M EDTA, pH 8.1, for 1 week or in a 50 per cent saturated solution of ammonium sulfate, pH 8.1, for at least several weeks at 5°C with negligible loss of activity. When frozen at -20°C in varying concentrations of Tris-acetate from 10^-4 to 10^-1 M at pH 8.1, the reductase is stable for months.

Properties of Microsomal Cytochrome Reductase

Absorption Spectra—Fig. 1 shows the absorption spectra of the microsomal cytochrome reductase in the oxidized and reduced forms, with either excess DPNH or sodium hydrosulfite as the reducing agent. The milli-

<table>
<thead>
<tr>
<th>Step No.</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (cobra venom)</td>
<td>1050</td>
<td>8700</td>
<td>12,800</td>
<td>1.5</td>
</tr>
<tr>
<td>5 (ammonium sulfate)</td>
<td>162</td>
<td>2300</td>
<td>9,700</td>
<td>4.2</td>
</tr>
<tr>
<td>6 (gel)</td>
<td>27</td>
<td>35</td>
<td>4,400</td>
<td>125</td>
</tr>
<tr>
<td>7 (ammonium sulfate)</td>
<td>10</td>
<td>24</td>
<td>3,250</td>
<td>136</td>
</tr>
</tbody>
</table>

* Standard assay system as described under “Methods.” Extraction with cobra venom leads roughly to a 15-fold purification of the enzyme over the level in whole microsomes. The over-all purification is approximately 1300-fold.

molar absorption coefficients ($E_{\text{mm}}$) are based on flavin content as determined by Dr. Helen B. Burch, with use of the method of Burch, Bessey, and Lowry (7). Since both reducing agents absorb in the 300 to 400 mm region, the spectrum of the reduced enzyme is plotted only above 400 mm. The oxidized form of the reductase has absorption maxima at 273, 390, 461, and 485 mm. While the first three are typical of flavoproteins, an absorption at 485 mm as marked as that of microsomal cytochrome reductase is not found in other cytochrome reductase spectra (8, 9). Theorell and Åkeson (10), however, have recently reported a similar absorption maximum for old yellow enzyme. Both the 461 and 485 mm absorption maxima disappear on DPNH or sodium hydrosulfite reduction.

Identification of Prosthetic Group—The flavin prosthetic group could be split from the protein in three ways: (a) by heating at 90°C for 5 minutes; (b) by precipitating the protein with ammonium sulfate at pH 1; or (c) by treatment with 7 per cent trichloroacetic acid. Each of these methods yielded, after centrifugation, a protein-free solution containing the flavin.
The spectrum of this flavin coincided quantitatively with that of FAD. On paper chromatograms, in a butanol-phosphate solvent system (11), the reductase flavin and FAD exhibited exactly the same relative mobility ($R_F$). The fluorescence of the reductase flavin at a neutral pH is 14 per cent of the fluorescence after trichloroacetic acid hydrolysis at $37^\circ$ for 24 hours. This is what would be expected for FAD (12). Finally, the reductase flavin fully reactivated d-amino acid oxidase apoenzyme with the same Michaelis constant ($K_m$) as FAD. In view of these data, the reductase flavin is assumed to be FAD.

Reversible Removal of FAD—A number of procedures were examined for splitting FAD from the reductase to yield an apoenzyme which could be reactivated by added FAD. Some reversible splitting was achieved with acid ammonium sulfate precipitation (13). Table II shows that, when the reductase is brought to pH 2.8 in 66 per cent saturated ammonium sulfate at 0–5°, the protein which precipitated had only 8 per cent of its original activity. This activity was increased 2.5-fold to 20 per cent of

![Absorption spectra of oxidized and reduced microsomal cytochrome reductase.](http://www.jbc.org/)

**Fig. 1.** Absorption spectra of oxidized and reduced microsomal cytochrome reductase. Optical density readings were taken at 5 $\mu$m intervals over the entire wave length range and at 1 $\mu$m intervals at each absorption maximum or minimum. The protein spectra were read at 5° in 0.1 M Tris-acetate and 0.001 M EDTA, pH 8.10. For reduction excess sodium hydrosulfite or 0.2 $\mu$ mole of DPNH per ml. was added. The control tubes contained the same buffer and the same amount of reducing agent. $E_{\text{mm}}$ values were based on flavin analyses by the method of Burch, Bessey, and Lowry (7). For oxidized reductase, the $E_{\text{mm}}$ values at 273, 390, 461, and 485 $\mu$m are 71.3, 11.7, 10.2, and 8.3, respectively; for reduced enzyme, at 461 and 485 $\mu$m, 2.6 and 2.1, respectively.
the original activity on the addition of 0.001 μmole of FAD to the assay system. The addition of magnesium chloride with the FAD caused no further reactivation (see "Metal analysis" and "Discussion").

**Protein Analysis and Minimal Molecular Weight—**Table III shows the minimal molecular weight of the reductase calculated from the $E_{\text{mM}}$ values (oxidized) and the determination of the protein concentration by the method of Lowry *et al.* (4) and by dry weight. The protein samples were first dialyzed against glass-distilled water for 2 days at 5°. In the case of dry weight determinations, the samples were then dried *in vacuo* over phosphorus pentoxide at room temperature and then at 110° in air to constant weight. Although these calculations are subject to the cumulative errors in $E_{\text{mM}}$, in drying and weighing small samples, in all possible contamination by salts and residual water, and in variations in color production by

<table>
<thead>
<tr>
<th>Table II</th>
<th>Reactivation of Acid Ammonium Sulfate-Precipitated Reductase by FAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reductase preparation</td>
<td>Addition to assay system</td>
</tr>
<tr>
<td></td>
<td>μmole per 0.20 ml.</td>
</tr>
<tr>
<td>A†</td>
<td>None</td>
</tr>
<tr>
<td>B‡</td>
<td>“”</td>
</tr>
<tr>
<td>B‡</td>
<td>0.001 FAD</td>
</tr>
<tr>
<td>B‡</td>
<td>0.001 MgCl₂</td>
</tr>
</tbody>
</table>

* The standard assay system indicated under "Methods" was used.
† Preparation A was 82% of microsomal cytochrome reductase.
‡ Preparation B was the precipitate obtained at 0–5° by diluting Preparation A to 2.0 ml. with distilled water, adding 2.0 ml. of saturated ammonium sulfate, lowering the pH to 2.8 with 1 N HCl, adding 1.0 ml. of saturated ammonium sulfate, and centrifuging at 8000 × g for 10 minutes.

<table>
<thead>
<tr>
<th>Table III</th>
<th>Minimal Molecular Weight Based on $E_{\text{mM}}$ Values and Protein Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>Reductase concentration*</td>
</tr>
<tr>
<td></td>
<td>μmole per ml.</td>
</tr>
<tr>
<td>1</td>
<td>0.0408</td>
</tr>
<tr>
<td>2</td>
<td>0.0408</td>
</tr>
<tr>
<td>3</td>
<td>0.0408</td>
</tr>
<tr>
<td>4</td>
<td>0.0408</td>
</tr>
</tbody>
</table>

* Based on an $E_{\text{mM}}$ of 10.2 at 461 mμ for the oxidized form.
† By the method of Lowry *et al.* (4).
the Lowry protein method, the results are in good agreement with those obtained from sedimentation and diffusion analysis, as described in the section on molecular weights.

**Sedimentation Coefficient**—Two solutions of the reductase, 0.16 per cent protein in 0.2 M Tris-acetate, pH 8.27, at 5°, and 0.24 per cent protein in 0.2 M Tris-acetate, pH 8.75, at 5°, sedimented as a single boundary in sedimentation runs at 5° in the analytical ultracentrifuge. The results at pH 8.27, corrected to an average rotor temperature at 20° in water, give a sedimentation coefficient, $s_{20,w}$, of $2.8 \times 10^{-13}$ sec.$^{-1}$.

**Diffusion Coefficient**—The diffusion coefficient was determined at 3.9° with a 0.16 per cent protein solution in 0.20 M Tris-acetate, pH 8.27, at 5°, in duplicate. Boundaries were formed in both limbs of a standard Tiselius electrophoresis cell (Klett) and, after compensation, were followed at intervals with Longsworth schlieren scanning photographs. Normalized curves at three time intervals fell within close limits on the Gaussian distribution curve. The diffusion coefficients, $D_{3.9,w}$, calculated by the maximal ordinate area method and by the method of moments (14), by using twelve time points, are $4.20 \times 10^{-7}$ and $4.27 \times 10^{-7}$ cm.$^{2}$ sec.$^{-1}$, respectively. For molecular weight determination these measurements, made at 3.9°, were extrapolated to the conditions prevailing in water at 20°, giving a $D_{20,w}$ of $6.8 \times 10^{-7}$ cm.$^{2}$ sec.$^{-1}$.

**Partial Specific Volume**—By the density gradient method (15) this quantity ($\bar{V}$) was found to be 0.737 at 20°.

**Molecular Weight**—The molecular weight of the reductase was calculated to be 38,400 by substituting the above quantities in the standard equation for molecular weight (14).

**Electrophoretic Analysis**—A 0.24 per cent reductase solution showed a single sharp and symmetrical boundary for as long as 7 hours in experiments carried out in 0.20 M Tris-acetate at either pH 8.87 or 8.27 at 5°. At these two pH values the mobilities are $-6.6 \times 10^{-5}$ and $-1.5 \times 10^{-5}$ cm.$^{2}$ volt.$^{-1}$ sec.$^{-1}$, respectively. The reductase, therefore, behaves as a homogeneous protein with a net negative charge in the pH range examined.

**Metal Analysis**—Of a group of eleven metals tested, only magnesium was found in the reductase in more than trace amounts, approximately 2 moles per mole of flavin (Table IV). Iron, molybdenum, copper, manganese, silver, mercury, zinc, lead, cobalt, and nickel were present in concentrations of less than 0.1 of a mole per mole of flavin. The analytical methods used were microadaptations of colorimetric procedures (16, 17) and enzymatic assay (18). For every metal, standards were run through the full procedure at concentrations from 0.1 to 10 times the amount that would be present in the reductase if it contained 1 equivalent of the metal. Internal standards were also used for iron and molybdenum. Since mag-
nesium was found to be leached from glass in wet ashing procedures, the magnesium determinations were carried out after dry ashing.

_Sulfhydryl Group Analysis_—The visible spectrum of the oxidized reductase is unaffected by PCMBS. However, in the presence of this reagent the flavin is no longer reduced by DPNH. The quantitative nature of the effect is shown in Fig. 2. Here the reductase was assayed after the addition of varying amounts of PCMBS. As can be seen, the addition of PCMBS leads to a proportionate inactivation of reductase and 1 equivalent almost completely inhibits 1 equivalent of reductase. Reoxidation of the reduced flavin is unaffected by PCMBS since the reductase, first reduced

### Table IV

<table>
<thead>
<tr>
<th>Reductase</th>
<th>Flavin</th>
<th>Sample treatment*</th>
<th>Method</th>
<th>Mg**</th>
<th>μmole Mg**</th>
<th>μmole flavin</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>μmole</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.106</td>
<td>0.00265</td>
<td>Dry ash</td>
<td>Eriochrome (16)</td>
<td>0.0037</td>
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<tr>
<td>0.212</td>
<td>0.0053</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.0100</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>0.212</td>
<td>0.0053</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.0096</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>0.212</td>
<td>0.0053</td>
<td>&quot;</td>
<td>Pyrophosphatase activated†</td>
<td>Qualitatively</td>
<td>present</td>
<td></td>
</tr>
</tbody>
</table>

* Dry ashing was carried out in a platinum crucible in an oxygen stream at 500-700°.

† Magnesium was assayed in a microsystem with the pyrophosphatase assay procedure described by Kunitz (18) with magnesium as the limiting factor. Blanks and a sample of protein before incubation gave no activity in this system. The crystalline pyrophosphatase used was prepared in the laboratory of Dr. Kunitz.

with excess DPNH and then treated with 1 to 10 equivalents of PCMBS, is rapidly reoxidized by potassium ferricyanide.

_Anaerobic Reductive and Oxidative Titrations_—Microsomal cytochrome reductase was titrated with DPNH and then reoxidized with aliquots of potassium ferricyanide in anaerobic microcells (see “Methods” and Velick and Strittmatter (3)). As shown in Fig. 3, each addition of DPNH or ferricyanide results in the same relative changes in both the 461 and 485 μm absorption peaks, and the curves also begin and end abruptly. The $E_{\text{mM}}$ calculated from the slopes of these titration curves during reduction by DPNH or oxidation by ferricyanide give values of 6.9 and 7.4, respectively, at 461 μm, assuming a 2 electron change for the flavoprotein. The relatively small variation between the two values can be attributed to the difficulty in preparing and standardizing the dilute DPNH and ferricyanide and in excluding oxygen completely during the titration procedure. The observations above indicate that the absorption spectra for oxidized re-
ductase represent a single FAD protein spectrum and reveal no other oxidizable or reducible group.

Oxidation-Reduction Potential—Excess DPNH \((E'_0, -0.35 \text{ at } 30^\circ \text{ and pH } 8.1)\) (19) must be used to achieve more than 80 to 90 per cent reduction of reductase flavin at pH 8.1. Titration of a mixture of indigomonsulfonate \((E'_0, -0.21 \text{ at } 25^\circ \text{ and pH } 8.1)\) (20) and reductase with DPNH results in only 5 to 10 per cent reduction of the reductase when over 90 per cent of the dye is reduced at pH 8.1. The oxidation-reduction potential \((E'_0)\) of the reductase, therefore, probably lies between \(-0.33\) and \(-0.25\) mv. at pH 8.1 and 25°.

pH Optima—The pH-activity curve for the reductase system under standard assay conditions, with either microsomal cytochrome or ferricyanide as acceptor, is optimal between pH 5.5 and 8.2 as indicated previously for crude reductase preparations (1).

Electron Donors—The enzyme is specific for DPNH; TPNH is inactive when substituted for DPNH as electron donor.
Maximal Rates and Apparent Michaelis Constants for DPNH and Electron Acceptors—Table V shows the maximal velocities \( (V_{\text{max}}) \) and the apparent Michaelis constants \( (K_m) \) for several electron acceptors in the reductase system in 0.1 M Tris-acetate and 0.001 M EDTA at pH 8.1. These values, \( K_m \) and \( V_{\text{max}} \), were calculated from Lineweaver-Burk plots of \( 1/V \) and \( 1/S \) in the usual way (21). The maximal turnover numbers were then calculated from the \( V_{\text{max}} \) values and the moles of reductase present. The \( K_m \) for DPNH with microsomal cytochrome as acceptor is also given. Reaction of the flavin with oxygen is 0.00025 times the rate observed with ferricyanide.

### Table V

<table>
<thead>
<tr>
<th>Substance tested</th>
<th>( V_{\text{max}} ) *</th>
<th>( K_m )</th>
<th>Reductase</th>
<th>Turnover No.</th>
<th>DPNH</th>
<th>Acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{mole per min.} )</td>
<td>( \mu \text{mole} )</td>
<td>( \text{moles electrons per min. per mole enzyme} )</td>
<td>( \mu \text{mole per min.} )</td>
<td>( \mu \text{mole per min.} )</td>
<td></td>
</tr>
<tr>
<td>DPNH.............</td>
<td>2.7 ( \times 10^{-6} )</td>
<td>3.4 ( \times 10^{-7} )</td>
<td>0-2 ( \times 10^{-5} )</td>
<td>5.5 ( \times 10^{-6} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal cytochrome†</td>
<td>2.0 ( \times 10^{-5} )</td>
<td>3.4 ( \times 10^{-7} )</td>
<td>15,600</td>
<td>6 ( \times 10^{-5} )</td>
<td>0-5 ( \times 10^{-5} )</td>
<td></td>
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<tr>
<td>Potassium ferricyanide.............</td>
<td>2.2 ( \times 10^{-6} )</td>
<td>1.6 ( \times 10^{-7} )</td>
<td>31,800</td>
<td>12 ( \times 10^{-5} )</td>
<td>0-10^{-4}</td>
<td></td>
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<tr>
<td>Indigotetrasulfonate.............</td>
<td>7 ( \times 10^{-5} )</td>
<td>1.6 ( \times 10^{-6} )</td>
<td>4,800</td>
<td>12 ( \times 10^{-5} )</td>
<td>0-10^{-4}</td>
<td></td>
</tr>
</tbody>
</table>

System: aerobic, 25°, 0.20 ml. containing 0.1 M Tris-acetate plus 0.001 M EDTA, pH 8.1, and the concentrations of reactants indicated.

* Either disappearance of DPNH at 340 \( \mu \text{m} \) or cytochrome c reduction at 550 \( \mu \text{m} \) was used to determine the rate of reaction. For microsomal cytochrome, when the concentrations of the oxidized form remained constant, rates were read each 15 seconds and were linear for at least 2 minutes. With indigotetrasulfonate, initial velocities were used the first 15 seconds of reaction. In the experiments with low and changing amounts of DPNH and ferricyanide, the reaction was followed to completion. In the concentration range in which either reactant became limiting, the average velocity during each 15 second interval was plotted against the average concentration during this time interval. Excess DPN, varied over a 100-fold concentration range, had no measurable effect on the velocities.

† In systems in which microsomal cytochrome is acceptor, 0.01 \( \mu \text{mole of cytochrome c} \) was present to maintain the microsomal cytochrome in an essentially oxidized form.

Inhibition of Reduction by Pyrophosphate, Citrate, and EDTA—Table VI shows that reduction of microsomal cytochrome is inhibited by these three reagents. Pyrophosphate inhibition is also observed with ferricyanide or indigotetrasulfonate as electron acceptor, but the concentration of inhibi-
TABLE VI
Inhibition by Pyrophosphate, Citrate, and EDTA

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Concentration of acceptor $M$</th>
<th>Inhibitor</th>
<th>Concentration of inhibitor $M$</th>
<th>Per cent inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal cytochrome</td>
<td>$6.25 \times 10^{-6}$</td>
<td>Pyrophosphate</td>
<td>$0.01$</td>
<td>47</td>
</tr>
<tr>
<td>&quot;</td>
<td>$6.25 \times 10^{-6}$</td>
<td>&quot;</td>
<td>$0.02$</td>
<td>67</td>
</tr>
<tr>
<td>&quot;</td>
<td>$6.25 \times 10^{-6}$</td>
<td>Citrate</td>
<td>$0.01$</td>
<td>30</td>
</tr>
<tr>
<td>&quot;</td>
<td>$6.25 \times 10^{-6}$</td>
<td>&quot;</td>
<td>$0.02$</td>
<td>43</td>
</tr>
<tr>
<td>&quot;</td>
<td>$6.25 \times 10^{-6}$</td>
<td>EDTA</td>
<td>$0.05$</td>
<td>64</td>
</tr>
<tr>
<td>&quot;</td>
<td>$6.25 \times 10^{-6}$</td>
<td>&quot;</td>
<td>$0.10$</td>
<td>80</td>
</tr>
<tr>
<td>Ferriyanide</td>
<td>$1 \times 10^{-4}$</td>
<td>Pyrophosphate</td>
<td>$0.20$</td>
<td>30</td>
</tr>
<tr>
<td>Indigotetrasulfonate</td>
<td>$5 \times 10^{-5}$</td>
<td>&quot;</td>
<td>$0.02$</td>
<td>57</td>
</tr>
<tr>
<td>&quot;</td>
<td>$5 \times 10^{-5}$</td>
<td>&quot;</td>
<td>$0.20$</td>
<td>75</td>
</tr>
</tbody>
</table>

* Assay system was aerobic, at $25^\circ$, and in 0.20 ml. containing 0.1 M Tris-acetate, pH 8.1, the acceptor and inhibitors indicated, 0.01 $\mu$ mole of DPNH, and reductase to give a conveniently measurable rate. When microsomal cytochrome was the acceptor, 0.02 $\mu$ mole of cytochrome c was also present.

Fig. 4. Competitive inhibition of electron transfer to various acceptors by pyrophosphate. Systems were aerobic, at $25^\circ$, and contained 0.20 ml. of 0.1 M Tris-acetate, pH 8.1, with the concentrations of the reactants indicated. All the rates are expressed as micromoles of electrons transferred per minute. A, cytochrome c reduction was measured at $550 \text{ m}\mu$ every 15 seconds in the system shown plus 0.02 $\mu$ mole of cytochrome c, $7.2 \times 10^{-7}$ $\mu$ mole of reductase, and 0.02 $\mu$ mole of DPNH. B, DPNH oxidation was measured at $340 \text{ m}\mu$ every 15 seconds in the system shown plus $1.6 \times 10^{-7}$ $\mu$ mole of reductase and 0.02 $\mu$ mole of DPNH. C, DPNH oxidation was measured at $340 \text{ m}\mu$ every 15 seconds in the system shown plus $4 \times 10^{-6} \mu$ mole of reductase and 0.02 $\mu$ mole of DPNH.
tor needed to obtain an appreciable effect is much higher. This can be attributed to the fact that these acceptors are present in large and optimal amounts. The inhibition with pyrophosphate is competitive with respect to each of the three electron acceptors (Fig. 4).

**DISCUSSION**

With the isolation of microsomal cytochrome (1) it was possible to show that the microsomal fraction of liver contains a specific DPNH microsomal cytochrome reductase system in which this heme protein, but not cytochrome c, can function as electron acceptor (1). That this reductase system is a major pathway of electron flow from DPNH to microsomal cytochrome in microsomes has been demonstrated in the present report. This pathway also accounts for a large part of the so-called cytochrome c reductase activity in microsomes. The incubation of microsomes with cobra venom has provided the basis for obtaining the reductase in a pure form. With the purification procedure indicated, reproducible preparations are obtained that meet several criteria of homogeneity.

The reductase could be isolated in sufficient quantity to permit the study of certain features of the reaction sequence by use of substrate concentrations of enzyme. The first part of the reaction (Reaction 1) is very rapid and can be followed best

\[
\text{DPNH} + \text{H}^+ + \text{FAD protein} \rightarrow \text{DPN}^+ + \text{FADH}_2 \text{protein}
\]

anaerobically. The equilibrium favors flavin reduction but requires an excess of DPNH to go to completion. In the second part of the reaction, transfer of electrons from the FAD to either one or two electron acceptors occurs (Reaction 2 or 3).

\[
\text{(2) } \text{FADH}_2 \text{protein} + 2\text{A}_{\text{oxidized}} \rightarrow \text{FAD protein} + 2\text{A}_{\text{reduced}} + 2\text{H}^+
\]

\[
\text{(3) } \text{FADH}_2 \text{protein} + \text{indigotetrasulfonate (oxidized)} \rightarrow \text{FAD protein} + \text{indigotetrasulfonate (reduced)}
\]

It is Reaction 1 which is inhibited by PCMBS and requires one essential free sulfhydryl group on the enzyme. Since the reoxidation of the previously reduced flavoprotein occurs rapidly in the presence of PCMBS, it is likely that the sulfhydryl group is involved in the interaction of the DPNH with the protein. A characteristic of the reoxidation of the bound FAD is the extreme slowness with which it occurs when oxygen is the acceptor. Since inhibition by pyrophosphate is competitive with the acceptors, it is suggested that the chelating agents affect interaction of the cytochrome with the enzyme. Magnesium would provide a natural site of attack for these inhibitors and one might suppose that magnesium is involved in the interaction of acceptors with the flavoprotein. It must be pointed
out, however, that inhibition by chelating agents does not constitute proof of metal involvement in this reaction. At the high concentrations of reagent used, other mechanisms of inhibition might be possible. No direct evidence has been obtained as yet that magnesium is a functional part of the reaction sequence on the reductase rather than a passive contaminant. The data, so far, make it unlikely that another metal, capable of undergoing oxidation and reduction, is involved in the transfer of electrons from the FAD to acceptors (Reaction 2 or 3). A large number of likely metals in the group of transition elements has been ruled out by analysis. Reductive and oxidative titrations of the reductase also do not reveal any group, other than FAD, undergoing alternate reduction and oxidation.

SUMMARY

1. The isolation of microsomal cytochrome reductase as an essentially homogeneous protein in good yield has been described.

2. The spectrum of the oxidized reductase shows absorption maxima at 273, 390, 461, and 485 μ. The 461 and 485 μ absorption peaks disappear on reduction with reduced diphosphopyridine nucleotide (DPNH) or sodium hydrosulfite.

3. Flavin adenine dinucleotide (FAD) was identified as the prosthetic group on the reductase. Flavin analysis and protein determinations give a minimal molecular weight of 42,000 ± 2000.

4. By sedimentation, diffusion, and electrophoretic analysis the reductase appeared homogeneous. The molecular weight, by physical measurements, was calculated to be 38,400.

5. Approximately 2 moles of magnesium are present per mole of enzyme. Ten other metals were shown to be absent or present in only trace amounts.

6. One labile sulfhydryl group was shown to be involved in the DPNH-flavoprotein interaction by titration of the reductase with p-chloromercuribenzenesulfonate.

7. Reductive and oxidative titrations of the reductase in an anaerobic system with DPNH and ferricyanide, respectively, indicate that the absorption spectra for oxidized reductase represent a single FAD protein spectrum and reveal no other oxidizable or reducible group.

8. The calculated maximal turnover rates for the reductase are 15,600, 31,800, and 4800 with microsomal cytochrome, ferricyanide, and indigotetrasulfonate, respectively, as acceptors. Oxygen reacts very slowly with the reductase, only 0.00025 times as fast as ferricyanide.

9. The apparent Michaelis constants for DPNH and the various acceptors were determined.

10. Reaction of the acceptors with the reductase was competitively inhibited by pyrophosphate. Citrate and ethylenediaminetetraacetic acid also inhibited this reaction.
The technical assistance of Mary Strittmatter and Carmelita Lowry is gratefully acknowledged.

BIBLIOGRAPHY

The Purification And Properties Of Microsomal Cytochrome Reductase
Philipp Strittmatter and Sidney F. Velick


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