THE REDUCTION OF INORGANIC IRON AND CYTOCHROME c
BY FLAVIN ENZYMES*

BY MORTON M. WEBER,† HOWARD M. LENHOFF,‡ AND
NATHAN O. KAPLAN

(From the McCollum-Pratt Institute, The Johns Hopkins University,
Baltimore, Maryland)

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During the course of an investigation of the components involved in
the electron transport pathway in Pseudomonas fluorescens (1), the problem
arose as to the nature of the carriers mediating the transfer of electrons
between the diaphorase and bacterial cytochrome c.

Mahler and Elowe (2) have reported the involvement of iron as a com-
ponent of the DPNH\textsuperscript{1} cytochrome c reductase from pig heart. Removal
of iron from the enzyme transformed the reductase to a diaphorase; addi-
tion of iron restored reductase activity. In view of this, it was deemed
advisable to investigate the possibility of reconstituting the cytochrome c
reductase of Pseudomonas by adding iron to the diaphorase rather than
the usual bacterial particles or dye. The addition of ferric iron to the
diaphorase did not cause a reduction of cytochrome c; however, the enzyme
could reduce the ferric iron, as indicated by the orange-red color which
developed when orthophenanthroline was used as a complex-forming agent.

In view of subsequent work from this laboratory demonstrating that
inorganic ferrous iron under appropriate conditions could reduce cyto-
chrome c non-enzymatically (3), it became clear that reduction of cyto-
chrome c does not necessarily indicate the reconstitution of a cytochrome
c reductase from inorganic iron and a diaphorase. Any enzyme possessing
the capacity to reduce ferric iron would appear to have some cytochrome
c reductase activity.

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† Postdoctoral Fellow in Cancer Research of the American Cancer Society.
‡ Lalor Predoctoral Fellow. Present address, Loomis Laboratories, Greenwich,
Connecticut.

\textsuperscript{1} The following abbreviations are used: DPN and DPNH, unreduced and reduced
diphosphopyridine nucleotide, respectively; TPN and TPNH, unreduced and re-
duced triphosphopyridine nucleotide, respectively; FMN, flavin mononucleotide;
FAD and FADH\textsubscript{2}, unreduced and reduced flavin adenine dinucleotide; GSSG and
GSH, unreduced and reduced glutathione, respectively; Tris, tris(hydroxymethyl)-
aminomethane; ADH, yeast alcohol dehydrogenase; TPD, rabbit muscle triosephos-
phate dehydrogenase.
Although the Pseudomonas cytochrome c was not reduced by the diaphorase plus ferric iron under aerobic conditions, anaerobic incubation of Pseudomonas diaphorase and ferric iron prior to the addition of cytochrome c caused an immediate reduction of the cytochrome. This is in confirmation of our earlier finding with the animal diaphorase and cytochrome c (3).

The present report is concerned with an investigation of iron-reducing systems, and it will be shown that iron reduction appears to be a property of flavin enzymes and is not limited to enzymes which normally reduce cytochrome c.

**Materials and Methods**

**Coenzymes and Other Materials**—DPN and TPN of 90 per cent purity were obtained from the Pabst Laboratories. DPNH was prepared enzymatically by the method of Pullman et al. (4) and TPNH enzymatically by the use of the pig heart isocitric dehydrogenase, as described by Nason and Evans (5). FAD of 40 per cent purity and FMN were obtained from the Sigma Chemical Company, 2,6-dichlorophenolindophenol from the Distillation Products Industries, and 1,10-orthophenanthroline monohydrate from the G. Frederick Smith Chemical Company. The concentrations of FAD and FMN were determined from their extinction coefficients at 450 mp of $1.13 \times 10^7$ sq. cm. × mole$^{-1}$ and $1.22 \times 10^7$ sq. cm. × mole$^{-1}$, respectively (6). Animal cytochrome c was obtained from the Mann Biochemical Company.

**Measurements**—All measurements were performed with a Beckman model DU spectrophotometer with 3.0 ml. cuvettes with a 1.0 cm. light path. All the reactions were run at room temperature. Cytochrome c reduction was determined by an increase in optical density at 550 mp, and 2,6-dichlorophenolindophenol reduction by a decrease in extinction at 610 mp. Ferrous-orthophenanthroline complex formation, in which 3 moles of orthophenanthroline bind 1 atom of Fe$^{++}$, was measured by an increase in optical density at 510 mp. The extinction coefficient was found to be $1.13 \times 10^7$ sq. cm. × mole$^{-1}$. Xanthine oxidase activity was determined by the increase in absorption at 290 mp resulting from uric acid formation from hypoxanthine, as described by Kalckar (7). Protein was estimated by the method of Warburg and Christian (8).

**Enzymes**—*P. fluorescens* extracts were prepared from cells grown in un aerated cultures by the method of Lenhoff and Kaplan (9). Diaphorase was prepared from pig heart by the method of Straub (10). TPNH cytochrome c reductase from pig liver, aldehyde oxidase from rabbit liver, and glutathione reductase from yeast were prepared by the methods of Horecker (11), Hurwitz (12), and Racker (13), respectively. Rabbit muscle crys-
talline TPD was kindly supplied by Dr. Gale Rafter. Xanthine oxidase, crystalline catalase, and crystalline ADH were obtained from the Worthington Biochemical Corporation. In some assays, as will be noted in the results, xanthine oxidase was dialyzed against 0.001 M phosphate buffer, pH 7.5, to remove (NH₄)₂SO₄.

![Graph](http://www.jbc.org/)

**Fig. 1.** Reduction of various concentrations of iron by diaphorase and *P. fluorescens*. Curves I, II, III, and IV designate 9, 4.5, 2.25, and 0.9 μmoles of FeCl₃·6H₂O, respectively. Curve Ia represents the same concentration of iron as Curve I, except that 3.6 μmoles of orthophenanthroline were used rather than the amount in the reaction mixture described below. The reactions were started with enzyme, as designated on the curve. A, pig heart diaphorase. The reaction mixture consisted of the concentrations of iron listed above, and 50 μmoles of Tris, pH 7.5, 25 μmoles of trisodium citrate·2H₂O, 0.6 μmole of DPNH, 12 μmoles of orthophenanthroline (except as indicated above in Curve Ia), and 0.035 mg. of enzyme protein in a total volume of 3.0 ml. B, *P. fluorescens* extract. The same reaction mixture as in diaphorase, except 0.21 mg. of enzyme protein.

**Results**

It was noted that high concentrations of DPNH and orthophenanthroline caused a slight non-enzymatic reduction of ferric iron. In order to minimize this effect, in determining the enzymatic reaction, low levels of DPNH and orthophenanthroline were used. Therefore, when high concentrations of ferric iron were employed, DPNH and orthophenanthroline were limiting in the reaction. Furthermore, as can be seen in Fig. 1, A by comparing Curves I and Ia, increasing the concentration of orthophenanthroline three times causes a 2-fold increase in the enzymatic rate.
The increased rate observed may be indicative of the greater rapidity with which ferrous iron complex is formed by the higher concentration of ortho-
phenanthroline, or the result of a displacement of equilibrium by removal
of ionized ferrous iron, or a combination of both phenomena. In any event,
however, we have previously demonstrated (3) that ferrous iron is formed
enzymatically in the absence of orthophenanthroline. In consequence of
these results, comparable conditions were adhered to in determining the
rate of iron reduction in all the enzymes tested.

Diaphorase—According to Fig. 1, A, diaphorase, the DPNH flavin en-
zyme from pig heart which catalyzes the reduction of dyes, but not cyto-
chrome c, can catalyze the reduction of ferric iron by DPNH. There is
a slow non-enzymatic rate of iron reduction; however, a marked increase
is noted after the addition of the enzyme. There is no apparent reduction
of iron when DPNH is omitted from the reaction mixture. Weber, Lenhoff,
and Kaplan (3) reported that, although this enzyme does not normally
reduce cytochrome c, it can be made into an apparent cytochrome c reduc-
tase by virtue of its ability to catalyze the reduction of ferric iron. The
ferrous iron so formed, in the presence of citrate (or any other complex-
forming anion such as pyrophosphate, which can decrease the oxidation-
reduction potential below that of cytochrome c) can now non-enzymatically
reduce cytochrome c.

P. fluorescens—As seen in Fig. 2, A and B, and Fig. 1, B, crude extracts
from P. fluorescens in the presence of DPNH catalyze the reduction of the
dye 2,6-dichlorophenolindophenol and ferric iron. Dialysis of these ex-
tracts against 0.001 M KCN (final concentration inside and outside of
dialysis bag) decreased the rate of both dye and iron reduction. It can
be observed that the addition of flavin to these extracts restored the ac-
tivity. FMN stimulated the rate of dye reduction more than either FAD
or riboflavin, while it appears that FAD stimulated the rate of iron reduc-
tion more readily than either FMN or riboflavin. Omission of either
DPNH or enzyme resulted in no appreciable iron reduction.

TPN Cytochrome c Reductase—This enzyme, from pig liver, was found
to catalyze the reduction of dye and ferric iron in addition to cytochrome
c. The rate of the reduction of ferric iron can be observed in Fig. 3, A.
Although not indicated in this paper, different concentrations of citrate
were used in all three assays. This was necessary, as it was found in pre-
liminary experiments that citrate inhibited the rate of reduction of cyto-
chrome c and increased the rate of dye reduction, and that an optimal
concentration was necessary for the maximal rate of iron reduction. The
interpretation of the results will be the subject of a subsequent publication.

Horecker (11) has shown that, although FAD appears to be the prosthetic
group of the enzyme, FMN will substitute in the reduction of cytochrome
c. The demonstration of the involvement of flavin in the enzymatic reduction of iron was tested by treating the enzyme with acid (NH₄)₂SO₄ to remove flavin from the enzyme according to the method of Warburg and Christian (14). The result on iron reduction is noted in Fig. 3, B. As can be seen, the addition of either FAD or FMN to the treated enzyme markedly stimulated the rate of reduction of iron. Similar stimulatory effects by flavin were obtained in the catalysis of reduction of both dye and cytochrome c by the acid (NH₄)₂SO₄-treated enzyme.

**Fig. 2.** Effect of the removal of flavin from *P. fluorescens* extracts on the reduction of dye (2,6-dichlorophenolindophenol) and iron. Curve I designates untreated enzyme; Curves II, III, IV, and V cyanide-dialyzed enzyme containing a final concentration of 8.0 × 10⁻⁷ M FMN, FAD, riboflavin, or no added flavin, respectively; and Curve VI, no enzyme. Cyanide-treated enzyme was incubated at room temperature for 5 minutes with the respective flavins before starting the reaction with DPNH. A, dye reduction. The reaction mixture contained, in addition to the designations on the curves, 50 μmoles of Tris, pH 7.5, 25 μmoles of trisodium citrate·2H₂O, 0.27 μmole of dye, 0.6 μmole of DPNH, and 0.21 mg. of untreated enzyme protein, or 0.19 mg. of cyanide-treated enzyme protein in a total volume of 3.0 ml. B, iron reduction. The reaction mixture contained the same concentrations of Tris, citrate, DPNH, and enzyme as in Fig. 2, A, but 9 μmoles of FeCl₃·6H₂O and 12 μmoles of orthophenanthroline instead of dye in a total volume of 3.0 ml.
being reduced by a mechanism different from that of the other flavin enzymes tested. As this enzyme reacts with \( O_2 \) in the presence of hypoxanthine to generate \( H_2O_2 \), it was thought that perhaps \( H_2O_2 \) was acting as the reducing agent. Kuhn and Wassermann (15) demonstrated that \( H_2O_2 \) could reduce ferric iron in the presence of orthophenanthroline. In view of this and the results of Horecker and Heppel (16), who dem-

![Fig. 3. Iron reduction by untreated and acid (NH_4)_2SO_4-treated pig liver TPNH cytochrome c reductase. A, untreated enzyme. The reaction mixture consisted of 50 \( \mu \)moles of Tris, pH 7.5, 0.15 \( \mu \)mole of trisodium citrate \( \cdot 2H_2O \), 12 \( \mu \)moles of orthophenanthroline, 0.6 \( \mu \)mole of TPNH, 0.08 mg. of enzyme protein, and 9.0, 4.5, 2.25, and 0.9 \( \mu \)moles of \( FeCl_3 \cdot 6H_2O \), as designated in Curves I, II, III, and IV, respectively, in a total volume of 3.0 ml. The reaction was started with enzyme. B, acid (NH_4)_2SO_4-treated enzyme. The reaction mixture contained the same concentrations of Tris, citrate, orthophenanthroline, and TPNH as in Fig. 3, A. It also contained 9.0 \( \mu \)moles of \( FeCl_3 \cdot 6H_2O \) and, as indicated on the graph, no added flavin, FAD, or FMN in a final concentration of \( 8.0 \times 10^{-6} \) M, and 0.18 mg. of enzyme protein in a total volume of 3.0 ml. The enzyme was incubated for 10 minutes at 0° with the flavin and the reaction started with TPNH.]

strated that \( O_2 \) was necessary for the reduction of cytochrome c by this enzyme, we attempted to reduce ferric iron anaerobically. The results (Fig. 4) indicate that very little iron was reduced under anaerobic conditions. However, when \( O_2 \) was bubbled into the reaction mixture, there was immediate reduction. Furthermore, the addition of increasing concentrations of catalase correspondingly decreased the amount of iron reduced (Fig. 4). Although not indicated on the graph, the addition of 1.0 mg. of catalase completely inhibited iron reduction.
In Fig. 5, Curve V, cytochrome c was reduced only when O₂ was introduced to the reaction mixture, thereby confirming the results of Horecker and Heppel (16). In the absence of small amounts of catalase, cytochrome c was reoxidized by a peroxidase present in the xanthine oxidase preparation. This peroxidatic effect was not evident in the iron reduction assay, as the Fe⁺⁺-orthophenanthroline complex is stable. Furthermore, as can be observed in Fig. 5, Curves I, II, and III, increasing concentrations of catalase, over the stimulatory level, decreased the amount of cytochrome c reduced. Here again (not indicated in Fig. 5), as with iron reduction, the addition of 1.0 mg. of catalase completely inhibited cytochrome c reduction under aerobic conditions.

Other Flavin Enzymes—As previously reported (17), crude extracts of Clostridium kluyveri reduce ferric iron in the presence of DPNH. Aldehyde oxidase, a flavoprotein from rabbit liver, catalyzes the reduction of ferric iron at a slow rate when N-methylnicotinamide serves as electron donor. D-Amino acid oxidase also catalyzed the reduction of iron at a slow rate. At present we are not certain as to the significance of this slow reduction.

Non-Flavin Enzymes—Yeast TPNH, GSSG reductase, yeast ADH, and
rabbit muscle TPD did not catalyze the reduction of dye or ferric iron at pH 7.5 with TPNH, ethanol, and 3-phosphoglyceraldehyde as electron donors, respectively. In the presence of TPNH and GSSG, the GSH formed by the action of GSSG reductase reduced both dye and ferric iron non-enzymatically.

**DISCUSSION**

It has been shown that, in addition to reacting with dyes, cytochrome c, and O₂, flavin enzymes can catalyze the reduction of inorganic iron.
This appears to be a general property of flavin enzymes, as non-flavin dehydrogenases do not seem to catalyze this reduction. Furthermore, flavin enzymes such as the animal and *Pseudomonas* diaphorases that normally do not reduce cytochrome c can reduce ferric iron.

Although it was highly suggestive that reduced flavin was the electron donor to ferric iron, it was important to demonstrate this relationship. Two approaches were used. First a demonstration of the non-enzymatic reduction of ferric iron by FADH₂, and, secondly, as noted in the text, direct indication by removal of the flavin from the enzyme by acid (NH₄)₂SO₄ treatment and restoring activity by the addition of flavin. The former demonstration was carried out by reducing free FAD anaerobically in a Thunberg tube with DPNH and a crude extract from *C. kluyveri*, as described by Weber and Kaplan (17). The reaction mixture was boiled to inactivate the enzyme and then cooled. The addition of ferric iron and orthophenanthroline from the side arm caused an immediate reduction to ferrous iron by the free FADH₂.

The apparent enzymatic reaction, in which *E* represents the enzyme, can be expressed as follows:

\[
\begin{align*}
\text{DPNH} + \text{H}^+ + \text{E-FAD} &\rightarrow \text{DPN}^+ + \text{E-FADH}_2 \\
\text{E-FADH}_2 + 2\text{Fe}^{3+} &\rightarrow \text{E-FAD} + 2\text{Fe}^{2+} + 2\text{H}^+ \\
\text{Net: DPNH} + 2\text{Fe}^{3+} &\rightarrow \text{DPN}^+ + 2\text{Fe}^{2+} + \text{H}^+
\end{align*}
\]

Xanthine oxidase appears to reduce both iron and cytochrome c by a different mechanism from that of the other flavin enzymes. As mentioned in the results, we have been able to confirm the results of Kuhn and Wassermann (15), which demonstrate that H₂O₂ in the presence of orthophenanthroline can reduce inorganic iron. It may be that iron reduction occurs when H₂O₂ is formed by the oxidation of hypoxanthine. Although it has not been possible to reduce cytochrome c with free H₂O₂, the possibility exists that enzyme-bound H₂O₂ can act as the reductant. This can account for the necessity of O₂ for the enzymatic reduction of cytochrome c and iron, and the inhibition by relatively high concentrations of catalase.

The stimulatory effect of low concentrations of catalase in cytochrome c reduction can be explained by assuming that free H₂O₂ is broken down by the relatively low concentrations of catalase, thereby not permitting the peroxidase, contaminating the enzyme preparation, to oxidize the reduced cytochrome c. High concentrations of catalase, however, may attack the H₂O₂ bound to the enzyme and prevent the bound H₂O₂ from acting as the reducing agent. The reduction of ferric iron can then be accounted for by both the free and bound H₂O₂, whereas cytochrome c reduction can be accounted for only by presumably enzyme-bound H₂O₂.
In this aspect it is significant that Horecker and Heppel (16) suggested that the requirement for O\textsubscript{2} for the reduction of cytochrome c by this enzyme was due to leucoflavoprotein oxidation which generated an intermediate oxidation product capable of reducing cytochrome c.

The reaction for the reduction of iron by this enzyme, where AH\textsubscript{2} and A refer to hypoxanthine and uric acid, respectively, can be expressed as follows:

1. \( \text{AH}_2 + \text{E-FAD} \rightarrow \text{E-FADH}_2 + \text{A} \)
2. \( \text{E-FADH}_2 + \text{O}_2 \rightarrow \text{E-FAD} + \text{H}_2\text{O}_2 \)
3. \( \text{H}_2\text{O}_2 + 2\text{Fe}^{+++}\text{-orthophenanthroline} \rightarrow 2\text{Fe}^{++}\text{-orthophenanthroline} + 2\text{H}^{+} + \text{O}_2 \)

Net: \( \text{AH}_2 + 2\text{Fe}^{+++}\text{-orthophenanthroline} \rightarrow \text{A} + 2\text{Fe}^{++}\text{-orthophenanthroline} + 2\text{H}^{+} \)

\textbf{Table I}

\textit{Comparison of Amount of Dye and Iron Reduced by Flavin Enzymes}

The reaction mixture contained either 0.27 \( \mu \text{mole} \) of dye (2,6-dichlorophenolindophenol) or 4.5 \( \mu \text{moles} \) of FeC\textsubscript{13}\cdot6H\textsubscript{2}O\textsubscript{5}, and the respective electron donors, buffers, and orthophenanthroline (in iron reduction), as noted in the figures representing the various enzymes, in a total volume of 3.0 ml.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Dye reduced in 1 min.</th>
<th>Fe\textsuperscript{+++} reduced in 1 min.</th>
<th>( \mu \text{mole} ) dye reduced</th>
<th>( \mu \text{mole} ) Fe\textsuperscript{+++} reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphorase</td>
<td>0.078</td>
<td>0.021</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>\textit{P. fluorescens}</td>
<td>0.213</td>
<td>0.045</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>TPNH cytochrome c reductase</td>
<td>0.057</td>
<td>0.028</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>0.105</td>
<td>0.015</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

In the above reaction, if orthophenanthroline were not present to trap the ferrous iron as it was formed, then Reaction 4 would result.

4. \( 2\text{Fe}^{++} + \text{O}_2 + 2\text{H}^{+} \rightarrow 2\text{Fe}^{+++} + \text{H}_2\text{O} \)

The reaction for the reduction of cytochrome c can be expressed as follows:

\( \text{AH}_2 + \text{E-FAD} \rightarrow \text{E-FADH}_2 + \text{A} \)

\( \text{E-FADH}_2 + \text{O}_2 \rightarrow [\text{E-FAD-H}_2\text{O}_2] \)

\([\text{E-FAD-H}_2\text{O}_2] + 2\text{cytochrome c Fe}^{+++} \rightarrow \text{E-FAD} + 2\text{cytochrome c Fe}^{++} + 2\text{H}^{+} + \text{O}_2 \)

Net: \( \text{AH}_2 + 2\text{cytochrome c Fe}^{+++} \rightarrow \text{A} + 2\text{cytochrome c Fe}^{++} + 2\text{H}^{+} \)

No attempt was made in the present study to resolve the rôle of molybdenum in the reduction of cytochrome c by this enzyme.

It is apparent from the data in Table I that all of the flavin enzymes tested seem to be different in their ability to catalyze the reduction of iron.
and dye. The most outstanding difference in the ratio of dye to iron reduced is that of the xanthine oxidase. The difference in iron reduction by this enzyme as compared to that of the other systems has already been discussed. It is of interest, however, that catalase does not effect the reduction of dye by this enzyme. This suggests that the reduced flavin on the enzyme may be directly responsible for the reduction of dye, in contrast to iron and cytochrome c reduction where other factors may be involved.

Animal diaphorase and P. fluorescens extracts do not normally catalyze the reduction of cytochrome c; yet they both can catalyze the reduction of inorganic iron. The possibility exists, of course, that the reduction of dye and iron is catalyzed by two different enzymes. This may be the case in P. fluorescens extracts, but is probably not so with the purified animal diaphorase.

The authors are grateful to Mr. John Choate for preparation of the enzymes used in this study.

SUMMARY

1. Flavin enzymes in general promote reduction of inorganic ferric iron, even though some of these enzymes do not catalyze the reduction of cytochrome c.

2. Flavin appears to be essential as an electron donor in the enzymatic reduction of iron. It has also been found that free reduced flavin adenine dinucleotide can reduce iron non-enzymatically.

3. The reduction of inorganic iron and cytochrome c by xanthine oxidase appears to require free or enzyme-bound H$_2$O$_2$, as lack of O$_2$ and relatively high concentrations of catalase inhibit this reduction. The reduction of iron and cytochrome c by xanthine oxidase appears to proceed through mechanisms different from those of other flavoproteins.

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