A CYTOCHROME PEROXIDASE FROM PSEUDOMONAS FLUORESCENS*

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The nature of the physiological function of peroxidases has been the object of much research. The Altschul, Abrams, and Hogness (1) discovery of a cytochrome c peroxidase in brewers’ yeast was one of the first cases in which a peroxidase was found to have a physiologically important substrate. However, since its initial discovery, there have been relatively few reports concerning the presence and physiological function of cytochrome peroxidases in general. This paper is concerned with the study of some new features of a cytochrome peroxidase present in Pseudomonas fluorescens and of some physiological functions of the enzyme.

The cytochrome c peroxidase present in P. fluorescens differs from the enzyme described in yeast in that the Pseudomonas enzyme reacts specifically with the cytochrome c found in the Pseudomonas extracts. This cytochrome pigment is similar in spectral properties to animal cytochrome c; it differs from the animal pigment, however, in that it is not adsorbed on Amberlite IRC-50 (2) as is animal cytochrome c. On the other hand, reduced animal cytochrome c is not oxidized by the Pseudomonas enzyme.

The properties of the bacterial cytochrome and of cytochrome peroxidase will be described, as well as the relationship of the azide insensitivity of the peroxidase to “azide-insensitive” systems described previously.

Materials and Methods

Materials—DPNH was prepared as described by Pullman, Colowick, and Kaplan (3) from DPN¹ of approximately 90 per cent purity obtained from the Pabst Laboratories. Crystalline animal cytochrome c was obtained from the Sigma Chemical Company. Purified catalase was ob-

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¹ The following abbreviations are employed throughout this paper: DPN and DPNH for the oxidized and reduced diposphopyridine nucleotide, respectively; TPN for triphosphopyridine nucleotide; dye for 2,6-dichlorobenzenoneindo-3′-chlorophenol.

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tained from Armour and Company. Purified liver TPN-cytochrome c reductase (4) was kindly supplied by Dr. B. L. Horecker.

Preparation of Adsorbents—The Amberlite IRC-50 column was prepared by the method of Neilands (5). Acid-treated kaolin (fuller's earth) was prepared by adding 1000 ml. of concentrated HCl (specific gravity 1.18) to 300 gm. of kaolin. The mixture was boiled slowly for 12 hours over a period of 2 days. The HCl was poured off, and the kaolin was washed with distilled water by decantation. The acid treatment and washing were repeated. The kaolin was then washed with water until the washings had a nearly neutral pH, while the kaolin gave an acid reaction to litmus paper.

Growth of Bacteria—A strain of P. fluorescens, obtained from Dr. Carl Lamanna, was grown in a medium containing 5 gm. of sodium citrate, 5 gm. of NaNO2, 1 gm. of KH2PO4, 0.5 gm. of MgSO4·7H2O, and 4 gm. of powdered yeast extract (Difco) per liter adjusted to a pH of 6.9 to 7.1 with 4.5 ml. of 1 N NaOH per liter. The growth of the cells in 10 liters of media in a 20 liter carboy gave a high cytochrome peroxidase activity and a high cytochrome c concentration per cell. The cultures should not be aerated nor agitation during growth (7). 2 days growth at 30° usually gave 5 gm. of wet weight of cells per liter when harvested on a Sharples centrifuge. The harvested cells appear red in color.

Preparation of Extract—The cells were washed with 0.9 per cent NaCl, centrifuged, frozen, and ground in a cold mortar with an equal weight of Alumina powder (A-301). For each gm. of wet weight of cells, 5 ml. of cold 0.1 M phosphate buffer, pH 7.5, were slowly added to the cells and Alumina. The Alumina was removed by centrifugation at 2000 × g for 5 minutes. The remaining homogenate was centrifuged at 25,000 × g in the cold for 30 minutes, or longer periods, until the supernatant fluid was particle-free; the length of time varied with the individual homogenates.

Preparation of Reduced Dye—A 0.001 M solution of the reduced dye was prepared by dissolving 8.75 mg. of the oxidized dye, 2,6-dichlorobenzenoneindo-3'-chlorophenol, in 25 ml. of water, filtering, and reducing with 1 ml. of a 0.2 per cent suspension of 5 per cent palladium-asbestos and hydrogen gas, according to the method of Smith and Stotz (8). The reduced dye was filtered free from the palladium-asbestos catalyst through Whatman No. 42 filter paper. It has been observed that the reduction procedure will also form H2O2 by reducing the oxygen present in the solution. The amount of endogenous H2O2 formed is diminished by bubbling prepurified N2 gas through the solution of oxidized dye before reduction with H2; this procedure lowers the concentration of dissolved O2.

Assay for Cytochrome Peroxidase—The method of assay for cytochrome peroxidase is based upon the observation that the reduced dye, 2,6-di-
chlorobenzenoneindo-3'-chlorophenol will chemically reduce small amounts of cytochrome pigment; the cytochrome is oxidized enzymatically by hydrogen peroxide. The reaction is followed spectrophotometrically by measuring the rate of formation of the oxidized dye at 575 mλ. Since the reaction is rapid, the most accurate results are obtained with a Beckman model B spectrophotometer. The reaction mixture consists of 2.3 ml. of reduced dye, 0.1 ml. of $3 \times 10^{-3}$ M $\text{H}_2\text{O}_2$ (with a final molarity of $10^{-4}$), and 0.1 M orthophosphate buffer, pH 7.5, to bring the total contents of the cuvette to 3.0 ml. The reaction is started by the addition of the bacterial extract. In order to correct for a slight autoxidation of the dye, a 30 second reading is taken before the addition of the enzyme. If the $\text{H}_2\text{O}_2$ is omitted from the reaction mixture, an initial rapid oxidation of the dye will occur on the addition of the enzyme; this is due to endogenous $\text{H}_2\text{O}_2$ formed on the reduction of the dye. The specific activity is expressed as a 0.01 change in optical density of the dye at 575 mλ per mg. of protein in 30 seconds. Protein concentration was determined by the method of Lowry et al. (9). As presented in Fig. 1, the activity is proportional to the concentration of the enzyme.

**Assay for Pseudomonas Cytochrome Pigment**—By determining the protein content and the change in optical density at 550 mλ of the respective states of the *Pseudomonas* pigment and by assuming an approximate molecular weight of 14,000 for the pigment, the percentage of the soluble pigment.

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**Fig. 1. Proportionality of concentration of enzyme to dye oxidized.** The standard cytochrome peroxidase assay was used as stated in the text.
pigment per 100 mg. protein as the *Pseudomonas* cytochrome pigment may be calculated. One may obtain the percentage of soluble cytochrome pigment present in the bacterial extract by using the following formula:

\[
\text{Dilution factor} \times \Delta \text{ optical density } 550 \mu \text{m} \times \frac{14}{\text{mg. protein per ml.} \times 18} \times 100
\]

\[
= \text{ per cent soluble protein which is cytochrome pigment}
\]

The value 18 is the difference of the extinction coefficients of the reduced and oxidized animal cytochrome *c* at 550 \(\mu\)m. The above figures are corrected for a light path of 1.0 cm. An average extract of 3.0 mg. of protein per ml. of extract had a \(\Delta\ 550 \mu m\) of 0.047 and thus contained 3.65 per cent of the cytochrome *c*. If the cytochrome is in the reduced state, it may be oxidized by the addition of \(H_2O_2\) at a final concentration of \(10^{-4}\ M\) or by a few small crystals of \(K_3Fe(CN)_6\). The pigment is chemically reduced by the addition of a few crystals of \(Na_2S_2O_4\).

**Purification of Cytochrome Peroxidase**—The extract, prepared as described under "Methods," was fractionated with solid \((NH_4)SO_4\). The protein fraction, precipitating between 40 and 80 per cent saturation and containing most of the activity, was redissolved in 0.1 M orthophosphate at pH 7.5 to 20 per cent of the original volume.

Small aliquots of the 40 to 80 per cent \((NH_4)SO_4\) fraction were added to a chromatographic column, 1 cm. \(\times\) 15 cm., consisting of acid-treated kaolin. Since the cytochrome peroxidase activity is associated with the cytochrome pigment, which is darker in the reduced state, the enzyme was best followed by reducing the pigment with a few crystals of \(Na_2S_2O_4\) before the fraction was added to the column. The proteins moved very slowly on the column; the rate of flow, however, was increased by the application of air pressure by means of a rubber pressure bulb. After the pigmented band moved 5 cm., the column was washed with 0.1 M phosphate buffer, pH 7.5, in order to remove most of the contaminating proteins; the washing was stopped when the cytochrome band moved 10 cm. The column was then eluted with 20 ml. of saturated ammonium acetate. Five fractions of approximately 3 ml. volume were collected. However, the number and volume of the fractions may vary. Nearly 100 per cent of the cytochrome peroxidase activity was recovered with various degrees of purity, but the fourth fraction had the highest specific activity. A summary of this purification procedure is given in Table I.

**pH Optimum**—The enzyme has a pH optimum in the region of neutrality. Most of the enzyme studies were carried out at pH 7.5; the enzyme is stable at this pH, while the reaction rate is slower and, therefore, easier.
to follow. In the alkaline range, the reduced dye is autoxidizable, which makes accurate measurements difficult.

Effect of Azide—The cytochrome peroxidase is quite insensitive to azide, as concentrations of $10^{-3} \text{ M}$ produce only a small inhibition at pH 7.5. Further studies, however, demonstrated that at a more acid pH the azide inhibition is more marked (Table II). Since the concentration of hydrazoic

<table>
<thead>
<tr>
<th>Step</th>
<th>Units $\times 10^3$</th>
<th>Specific activity</th>
<th>Total protein mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>1180</td>
<td>520</td>
<td>2692</td>
</tr>
<tr>
<td>40–80 (NH$_4$)$_2$SO$_4$</td>
<td>1148</td>
<td>1045</td>
<td>110.1</td>
</tr>
<tr>
<td>4th ammonium acetate eluate from kaolin column</td>
<td>84</td>
<td>6480</td>
<td>13</td>
</tr>
</tbody>
</table>

1 unit is defined as a 0.01 change in optical density of the dye at 575 m$\mu$ in 30 seconds. Units per mg. of protein.

<table>
<thead>
<tr>
<th>pH</th>
<th>Sodium azide concentration $10^{-3} \text{ M}$</th>
<th>Per cent azide as hydrazoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>7.0</td>
<td>0.03</td>
</tr>
<tr>
<td>7.5</td>
<td>18.8</td>
<td>0.17</td>
</tr>
<tr>
<td>7.0</td>
<td>26.6</td>
<td>0.52</td>
</tr>
<tr>
<td>6.6</td>
<td>63.2</td>
<td>1.3</td>
</tr>
<tr>
<td>6.1</td>
<td>84.5</td>
<td>4.17</td>
</tr>
<tr>
<td>5.5</td>
<td>94.4</td>
<td>12.28</td>
</tr>
</tbody>
</table>

The conditions were as in the standard assay and utilized 5 $\gamma$ of enzyme purified thirteen times. The pH was varied by using appropriate 0.1 M phosphate buffer. The indicated amount of sodium azide was added.

acid increases with a decrease in pH, these results suggest that hydrazoic acid is a more potent inhibitor of the enzyme than is the azide ion.

Other Inhibitors—50 per cent inhibition was obtained with $3.2 \times 10^{-5} \text{ M}$ hydroxylamine and $10^{-4} \text{ M}$ cyanide. Potassium ethyl xanthate, carbon monoxide, and hydroxyquinoline produced no appreciable inhibition.

Spectra of Pseudomonas Cytochrome Pigment—Particle-free extracts of *P. fluorescens* were found to have a high content of a reddish pigment. The absorption spectra of both the oxidized and reduced states of the pigment are shown in Fig. 2. The spectra are almost identical to the spectra of the
respective states of animal cytochrome c. The reduced pigment has an 
α-band at 550 mμ, a smaller β-band at 520 mμ, and a large γ-band at 415 mμ 
in the Soret region. The oxidized pigment absorbs slightly at 530 mμ and 
has its Soret band at 408 mμ. The bacterial cytochrome could be re-
peatedly reduced by sodium dithionite and enzymatically oxidized by hy-
drogen peroxide, indicating that the reduced cytochrome is one of the sub-
strates of the peroxidatic reaction.

Differences between Pseudomonas Cytochrome Pigment and Animal Cyto-
chrome c—The Pseudomonas cytochrome pigment was found to differ from

![Spectra of the oxidized and reduced states of the bacterial cytochrome c.](http://www.jbc.org/)

animal cytochrome c in the number and kind of charged groups available 
to the resinous exchange column, IRC-50. Both Neilands (5) and Mar-
goliash (10) have shown that animal cytochrome c is readily adsorbed on 
this column. This bacterial pigment, however, was not adsorbed at all 
on the resin. In order to check the possibility that some component in the 
bacterial preparation might have been masking the potential adsorbing 
groups on the Pseudomonas cytochrome, a mixture of animal and bacterial 
cytochrome c was incubated before being placed on the column. When 
the mixture was placed on the column, only the animal cytochrome c formed 
a sharp red band at the top of the column, whereas all the bacterial cyto-
chrome, and nearly 100 per cent of the cytochrome peroxidase activity, 
passed through the column.

The two cytochromes also differed in their biochemical specificity to TPN
cytochrome c reductase of liver. Animal cytochrome c was reduced by this system; in contrast, the \textit{Pseudomonas} pigment was not reduced. Animal cytochrome c was found not to increase the activity of the \textit{Pseudomonas} cytochrome peroxidase system as measured by its failure to increase the rate of oxidation of the reduced dye. However, the lack of effect may be due to the fact that the system is saturated with the bacterial cytochrome. 

One other observation suggests another possible difference between the two cytochromes. All dialyzed fractions of the bacterial extracts that contained the cytochrome component gave a red fluorescence when the extracts exhibited the reduced spectrum of the pigment; on oxidation of the pigment by hydrogen peroxide, the red fluorescence disappeared.\textsuperscript{6} Dialyzed fractions of the extract obtained from cells grown with aeration and therefore having only a trace of cytochrome c (7), and from 

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
& Specific activity & Per cent soluble protein & Ratio, (A)/(B) \\
& cytochrome peroxidase & as cytochrome pigment & \\
(A) & (B) & \\
\hline
Crude extract & 500 & 3.65 & 137 \\
Ammonium acetate eluate & 7500 & 5.4 & 1390 \\
Grown in 10\% oxygen & 44 & 1.2 & 36 \\
\hline
\end{tabular}
\caption{Ratio of Cytochrome Peroxidase to Cytochrome Pigment}
\end{table}

Crude extracts of cells grown in a lower oxygen tension as described in the following paper (7). 

cells grown in an iron-deficient medium with and without aeration (7), do not exhibit this red fluorescence on reduction. It cannot be definitely said whether the fluorescence is due to the \textit{Pseudomonas} cytochrome or to another soluble non-dialyzable component which contaminates the cytochrome preparation. Animal cytochrome c is not fluorescent in this manner. 

The bacterial cytochrome could not be purified completely free of the very active cytochrome peroxidase. Therefore, it was impossible to obtain a substrate saturation curve of the enzyme with the cytochrome. However, as demonstrated in Table III, the ratios of the peroxidase to the pigment obtained from different fractions of the bacterial extracts vary significantly, indicating that the cytochrome pigment and the cytochrome peroxidase are different proteins. Thus far, it has not been possible to obtain two fractions during the purification procedures that together gave an increased cytochrome peroxidase activity. 

\textit{Requirement of Hydrogen Peroxide for Dye Oxidase Activity—Preliminary}

experiments demonstrated that either the crude or the purified *Pseudomonas*
extracts would cause the rapid oxidation of the dye 2,6-dichlorobenzene-
oneindo-3'-chlorophenol. This activity is similar to the "dye oxidase"
activity of plant tissue described by Smith and Stotz (8). However, the
rate of dye oxidation caused by the *Pseudomonas* extract usually reached
a plateau after 90 seconds, most of the dye being left in the reduced state
(Fig. 3, Curve A). Furthermore, hydrogen peroxide was found to be essen-

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

**Fig. 3.** The effect of catalase and hydrogen peroxide on dye oxidase activity.
Curve A represents the conditions of the standard assay without the addition of exogenous hydrogen peroxide. The reaction was started with 80 μg of the three times purified extract. The conditions represented in Curve B are the same as those of Curve A, except that 1.0 mg. of Armour catalase is incubated with the reaction mixture for 1 minute before the enzyme is added. Curve C represents the reaction mixture exactly under the conditions of the standard assay, i.e. with 0.1 ml. of 3 × 10⁻³ m H₂O₂ added.

tial for the dye oxidation, for the addition of exogenous hydrogen peroxide
promoted the complete oxidation of the dye (Curve C) and the incubation
of catalase with the reduced dye before the addition of the enzyme inhibited
the dye oxidation completely (Curve B). The initial burst of dye oxid-
ation was found to be due to the presence of endogenous hydrogen peroxide
in the dye solution; this hydrogen peroxide, as discussed above, was formed
by the reduction of dissolved oxygen when hydrogen gas was passed through
the dye solution in the presence of palladium-asbestos. The dye oxidation
was found to be due to the chemical reduction of the cytochrome pigment
of these extracts by the dye and the subsequent oxidation of the reduced
cytochrome by the hydrogen peroxide and peroxidase. A similar diffi-
culty was encountered by Altschul, Abrams, and Hogness (11) in their studies on yeast. They found that their apparent soluble cytochrome oxidase activity was caused by a cytochrome c peroxidase and that the hydrogen peroxide was generated during the reduction of the cytochrome c by H₂ and palladium-asbestos.

Enzymatic Oxidation of DPNH by H₂O₂—Crude particle-free extracts of *P. fluorescens* oxidized DPNH only slightly. The anaerobic addition of a trace of dye to the extract and DPNH resulted in only partial oxidation of the DPNH, while the dye and the cytochrome pigment (present in these extracts) were completely reduced. The dye accepted electrons from DPNH in the presence of diaphorase in the extract and passed them chemically to the cytochrome pigment. When the reaction mixture was carried out in the presence of oxygen, a rapid oxidation of the DPNH ensued (Fig. 4, Curve A). The aerobic addition of hydrogen peroxide to the extracts and DPNH resulted in an even faster oxidation of the DPNH (Fig. 4, Curve B). From these studies it was apparent that the *Pseudomonas* extracts produced hydrogen peroxide, which was subsequently used in the cytochrome peroxidase system. The addition of exogenous hydrogen peroxide provided more substrate for the peroxidase. The following sequence

![Fig. 4. The effect of H₂O₂ on DPNH oxidation by the extracts of *P. fluorescens*. The reaction mixture represented by Curve A consisted of 0.5 ml. (0.528 mg. of protein) of the crude bacterial extract, 0.04 ml. of DPNH (10 mg. per ml.), 0.4 ml. of phosphate buffer, pH 7.5, 0.1 ml. of oxidized dye, and water to bring the total content to 3.0 ml. The reaction mixture represented by Curve B was the same as Curve A, except for the addition of 0.1 ml. of 0.05M H₂O₂ before the enzyme was added.](http://www.jbc.org/)

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of reactions may be involved:

1. \[ \text{DPNH} + \text{H}^+ + \text{dye} \xrightarrow{\text{diaphorase}} \text{DPN}^+ + \text{dye} \cdot 2\text{H} \]

2. \[ \text{DPNH} + \text{H}^+ + \text{O}_2 \xrightarrow{\text{flavin}} \text{H}_2\text{O}_2 + \text{DPN}^+ \]

3. \[ \text{Dye} \cdot 2\text{H} + 2 \text{cytochromes Fe}^{+++} \xrightarrow{\text{cytochrome peroxidase}} 2 \text{cytochromes Fe}^{++} + \text{dye} + 2\text{H}^+ \]

4. \[ 2\text{H}^+ + 2 \text{cytochromes Fe}^{++} + \text{H}_2\text{O}_2 \xrightarrow{\text{cytochrome peroxidase}} 2 \text{cytochrome Fe}^{+++} + 2\text{H}_2\text{O} \]

Net: \[ 2\text{DPNH} + 2\text{H}^+ + \text{O}_2 \rightarrow 2\text{DPN}^+ + 2\text{H}_2\text{O} \]

That hydrogen peroxide can function as the electron acceptor in the absence of oxygen was demonstrated by the addition of the peroxide anaerobically, as seen in Fig. 5. Since a trace of the dye links the diaphorase to the cytochrome in the aerobic system and thereby allows the rapid oxidation of DPNH, it was necessary to eliminate the dye from the reaction cuvette until anaerobiosis was attained; the dye was then added with the peroxide. On anaerobic addition of the dye alone to the reaction mixture, shown in Curve A, Fig. 5, only the DPNH necessary for dye reduction was oxidized. Curve B demonstrates that the addition of both hydrogen peroxide and the dye gives a rapid and complete oxidation of the DPNH. The possibility exists that the catalase in these preparations decomposed some of the hydrogen peroxide and thereby supplied enough oxygen for an aerobic reaction. However, the production of oxygen was minimized by the addition of \(10^{-3}\) M azide to inhibit selectively most of the catalase activity and by the use of only minimal amounts of hydrogen peroxide.

The \textit{Pseudomonas} extracts required the dye in order to oxidize DPNH by \(\text{H}_2\text{O}_2\); therefore, these extracts did not exhibit any of the DPNH-peroxidase activity described by Dolin (12). The ferrocytochrome pigment, rather than the reduced dye, was demonstrated to be the actual substrate of the peroxidase. The cytochrome pigment, chemically reduced by a few crystals of sodium dithionite, remained in the reduced state indefinitely in the presence of oxygen. However, on the addition of \(\text{H}_2\text{O}_2\) the pigment was oxidized by means of the cytochrome peroxidase. This alternate reduction and oxidation was repeated a number of times without destroying the cytochrome pigment or the peroxidase activity.

\textit{Hydrogen Peroxide Saturation of Cytochrome Peroxidase}—In determining the Michaelis-Menten constant of cytochrome peroxidase towards hydrogen peroxide, two difficulties were encountered. The reduced dye contained endogenous hydrogen peroxide, making quantitative measurements impossible, and the cytochrome peroxidase preparation was not free from the bacterial catalase which competed with the peroxidase for \(\text{H}_2\text{O}_2\). In order to remove the endogenous \(\text{H}_2\text{O}_2\) present in the solution of reduced dye, the cytochrome peroxidase was added to the reaction mixture before the exog-
When the endogenous H$_2$O$_2$ was completely consumed, a known amount of hydrogen peroxide was added. The second difficulty was overcome by adding $10^{-3}$ M sodium azide to the reaction mixture at pH 7.5. As mentioned previously, this concentration of azide at the pH of the assay system inhibited the cytochrome peroxidase to only a slight extent, while under the same conditions most of the bacterial catalase was inhibited. As in Fig. 6, the substrate saturation curve with both peroxidase and catalase active was atypical and gave an apparent $K_m$ of $10^{-5}$ M. However, when the catalase was selectively inhibited by the azide, most of the hydrogen peroxide was made available to the cytochrome peroxidase, and the saturation curve appeared to be more typical with a corrected $K_m$ of $5 \times 10^{-6}$ M. It is likely that the $K_m$ is even lower, since the catalase was not completely inhibited by the azide.

**Distribution**—The cytochrome c peroxidase described by Altschul et al., which is assayed by the oxidation of reduced cytochrome c, was initially observed in brewers' yeast (2) and more recently in Neurospora tetra-opsorma (13) and the petite yeast mutant of Ephrussi et al. (14). The crude particle-free extracts of a number of organisms were tested for cytochrome peroxidase activity by the method discussed in this paper (Table...
Fig. 6. Hydrogen peroxide saturation of the enzyme. Conditions of the reactions represented by the saturation curve without the presence of sodium azide are the same as those of the standard assay with 6.3 γ of the (12.5 × purified) enzyme. In these cases the endogenous H₂O₂ of the reduced dye was removed by the addition of the purified enzyme to the reduced dye and buffer. After the endogenous H₂O₂ was utilized, the known amount of H₂O₂ was added. The conditions for the saturation curve in the presence of 1 × 10⁻³ M azide were exactly the same.

**Table IV**

_Distribution of Cytochrome Peroxidase Activity_

<table>
<thead>
<tr>
<th>Active</th>
<th>Specific activity</th>
<th>Specific activity when 10⁻² M azide added to reaction cuvette</th>
<th>Inactive</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. florescens</em></td>
<td>520 1000</td>
<td></td>
<td><em>Achromobacter fisheri</em></td>
<td>0</td>
</tr>
<tr>
<td><em>A. vinelandii</em></td>
<td>254</td>
<td></td>
<td><em>Pigeon liver</em></td>
<td>0</td>
</tr>
<tr>
<td><em>N. crassa, poky mutant</em></td>
<td>87</td>
<td></td>
<td><em>Paramecium caudatum</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Soy bean</em></td>
<td>2550</td>
<td></td>
<td><em>Lactobacillus arabinosus</em></td>
<td>0</td>
</tr>
<tr>
<td><em>B. Calmette-Guerin</em></td>
<td>1 14</td>
<td></td>
<td><em>Aerobacter aerogenes</em></td>
<td>0</td>
</tr>
<tr>
<td><em>N. crassa 146</em></td>
<td>4 37</td>
<td></td>
<td><em>Proteus morganii</em></td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1 10</td>
<td></td>
<td><em>Euglena</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. kluyveri</em></td>
<td>2 19</td>
<td></td>
<td><em>Drosophila melanogaster</em></td>
<td>0</td>
</tr>
<tr>
<td><em>A. agile</em></td>
<td>2 21</td>
<td></td>
<td><em>Acetobacter xallinum</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Bacillus brevis</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Leuconostoc mesenteroides</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Mycobacterium butyricum</em></td>
<td>0</td>
</tr>
</tbody>
</table>

IV). Appreciable activity was found in the poky mutant⁶ of _Neurospora_ and in _Azotobacter vinelandii_. Assays of extracts of _Neurospora crassa_, _Escherichia coli_, _Bacillus Calmette-Guerin_, and _Azotobacter agile_ demon-

⁶ A culture of the poky mutant was kindly supplied by Dr. H. K. Mitchell.
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strated the existence of small amounts of cytochrome peroxidase only in the presence of $10^{-3}$ M sodium azide. The azide was added in order to inhibit catalase; it had no significant effect on the peroxidase. Apparently the ratio of catalase to cytochrome peroxidase was high in these organisms, and the hydrogen peroxide was available to the peroxidase only when the catalase system was inhibited. Soy bean extracts exhibited a very high activity. However, it cannot be definitely stated whether this activity was due to cytochrome peroxidase, because it has not been possible to observe the cytochrome pigment in these extracts. The activity may be attributed to another peroxidase mediating the oxidation of the dye. In any case, the activity of the soy bean extract is solely due to a peroxidase, as it is completely inhibited by catalase.

DISCUSSION

The significance of the peroxidase complex described above in the normal respiration is not entirely clear at the present time. Recently there have been reports which cast doubt on the presence of a cytochrome oxidase in P. fluorescens (15, 16), thus enhancing the probability that the cytochrome peroxidase acts in oxidizing the highly abundant cytochrome pigment in this organism. On extraction of the bacterial cells, the cytochrome is usually in the reduced state, suggesting that it plays an active part in electron transfer. The cytochrome peroxidase unites two relatively inefficient DPNH oxidizing systems to form a very effective one. In the first system, the DPNH supplies electrons to oxygen forming hydrogen peroxide, while, in the second, the cytochrome is reduced. The cytochrome peroxidase catalyzes the reaction of the products of the two systems and yields ferri-cytochrome and water. This series of reactions is depicted in the accompanying scheme.

![Scheme of reactions](https://via.placeholder.com/150)

Other cytochrome c-containing organisms that have a cytochrome peroxidase and are void of cytochrome oxidase are the petite yeast (14) and poky Neurospora (17). Chantrenne has found a cytochrome c peroxidase in the petite yeast (18), and we have demonstrated the presence of some cytochrome peroxidase activity in the poky Neurospora.

Although an active cytochrome peroxidase activity was found in soy bean extracts, it is difficult at this time to conclude that this peroxidase activity is promoted by the peroxidation of a ferrocytochrome. No cytochrome
spectra could be observed in these extracts owing to the interfering chlorophyll pigments. The possibility exists that the activity observed in the soy bean extracts is the same as the "dye oxidase" activity observed by Smith and Stotz (8) in other plant tissues, since catalase was not added in their experiments.

Chance (19) has demonstrated that the yeast cytochrome c peroxidase acts with hydrogen donors other than cytochrome c. The cytochrome peroxidase of *P. fluorescens* reacts specifically with the *Pseudomonas* cytochrome. It will not promote the purpurogallin test as do most peroxidases, and it does not appear to react with animal cytochrome c.

Chance (20) also has presented evidence that the yeast cytochrome c peroxidase can oxidize ferrocytochrome c at a faster rate than cytochrome oxidase and that peroxide utilization is greater in the anaerobic yeast cell than in the aerobic yeast cell. It seems likely that the cytochrome c peroxidase would therefore be most active physiologically at an oxygen tension high enough to allow peroxide production, and yet low enough to permit the peroxidase to act at its maximal rate. The increased function of cytochrome peroxidase at low oxygen tensions is described more fully in the next paper (7).

In addition to our report of the *Pseudomonas* cytochrome, which is similar in spectra to animal cytochrome c (2), three other laboratories have independently reported a similar bacterial pigment. The cytochromes from an unidentified halotolerant organism (21), from *Rhodopseudomonas spheroides* (22), and from *A. vinelandii* (23) all have spectra similar to the *Pseudomonas* pigment, and likewise do not adsorb on an Amberlite IRC-50 column, in contrast to animal cytochrome c, which does adsorb. The possibility exists that the animal and bacterial cytochromes have similar prosthetic groups with nearly identical spectra. On the other hand, the protein components may differ greatly, accounting for the difference in adsorption on the Amberlite IRC-50 column. It is interesting to note that the extracts of *A. vinelandii*, which have a high content of bacterial cytochrome c, have also been found to have an active cytochrome peroxidase system (Table IV).

The *Pseudomonas* cytochrome peroxidase is unusual in that it is inhibited by sodium azide only at an acid pH, suggesting that the hydrazoic acid molecule inhibits the enzyme, since the dissociation constant for this acid is $1.9 \times 10^{-3}$. This inhibition differs from the similar phenomenon found by Keilin (24) with yeast cells when it appears that hydrazoic acid is more permeable to the yeast cell than the azide ion; no permeability problem is encountered with the *Pseudomonas* system. On the other hand, this work is similar to the Horecker and Stannard (25) observation that the inhibition of rat liver cytochrome oxidase by azide increased as the pH decreased, the only difference being that these workers used a cell-free particulate
preparation, while the studies presented in this paper were with an aqueous extract.

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SUMMARY

1. A cytochrome peroxidase obtained from *Pseudomonas fluorescens*, acting specifically with the *Pseudomonas* cytochrome pigment, has been purified 13-fold. The enzyme was assayed by its capacity to oxidize the reduced form of the dye, 2,6-dichlorobenzenoneindo-3'-chlorophenol.

2. Cytochrome peroxidase has a pH optimum at pH 7.0. Sodium azide was not inhibitory at this pH but was inhibitory at pH 5.5. Cyanide and hydroxylamine were inhibitors, while carbon monoxide was not.

3. The *Pseudomonas* cytochrome c is similar in spectral properties to animal cytochrome c, but differs in that it is not reduced by the TPN-cytochrome c reductase of liver and is not adsorbed on an Amberlite IRC-50 chromatographic column as is animal cytochrome c.

4. The $K_m$ for hydrogen peroxide was determined to be $5 \times 10^{-6}$ M. The peroxide stimulated the oxidation of DPNH in the presence of the crude bacterial extract and a trace of the dye, and it also promoted the oxidation of the reduced coenzyme under anaerobic conditions.

5. The enzyme was present in large amounts in *Pseudomonas fluorescens*, *Azotobacter vinelandii*, and in the poky mutant of *Neurospora crassa*. It was present in smaller amounts in *Neurospora crassa*, *Escherichia coli*, *Bacillus Calmette-Guerin*, *Clostridium kluyveri*, and *Azotobacter agile*.

6. The significance of cytochrome peroxidase in cellular respiration is discussed.

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