THE REDUCTION OF CYTOCHROME c BY XANTHINE OXIDASE

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A number of substances such as oxygen, dyes, and nitrate can act as hydrogen acceptors for xanthine oxidase (1). It has now been found that cytochrome c is also reduced by this system. Since only one other enzyme has been isolated which reduces cytochrome c (2), it was of interest to study the reaction, particularly since Ball (3) has indicated that xanthine oxidase is a flavoprotein. The activity toward cytochrome c was found to parallel the oxygen activity over a 200-fold range of purification, with the same substances active in the reduction of cytochrome c as in the reaction with oxygen. Improvement of the purification procedure has resulted in a preparation of xanthine oxidase which has a Qo2 3 times higher than Ball's best preparation, and a definite relation has been established between enzyme activity and flavin-adenine dinucleotide (FAD) content.

EXPERIMENTAL

Cytochrome c was prepared from beef heart according to Keilin and Hartree (4). Catalase was recrystallized three times from beef liver by the method of Sumner and Dounce (5). Hypoxanthine and xanthine solutions were 0.05 and 0.025 M, respectively, in 0.05 M NaOH, and acetaldehyde was 0.04 M in H2O. Albumin was crystalline bovine serum albumin (Armour Laboratories).

Spectrophotometric and Manometric Tests—The reduction of cytochrome c was measured at 550 m\textmu in 1.00 cm. absorption cells in a Beckman model DU quartz spectrophotometer with a slit width of 4.6 m\textmu. Catalase was required to prevent the reoxidation of reduced cytochrome c, probably by the action of milk peroxidase which was present even in purified xanthine oxidase preparations. The test solution contained 2.48 \times 10^{-8} mole of cytochrome c, 0.5 unit of catalase, 0.6 mg. of albumin, 0.04 cc. of diluted enzyme solution, and 5.00 \times 10^{-7} mole of hypoxanthine, in 0.06 M phosphate buffer, pH 7.4, in a total volume of 1.55 cc. The gas space contained air unless otherwise indicated. The hypoxanthine solution was added last, readings were taken every minute for 7 minutes, and then about 1 mg. of solid Na2S2O4 was added to reduce the cytochrome completely.
XANTHINE OXIDASE AND CYTOCHROME c

The concentration of oxidized cytochrome (ferricytochrome) was calculated from the equation

$$\text{Ferricytochrome} = \frac{d_R - d_t}{1.96 \times 10^4} \text{ moles per liter}$$

where $d_R$ and $d_t$ are the density readings after addition of Na$_2$S$_2$O$_4$ and at any time $t$ during the rate determination, respectively. 1.96 $\times$ 10$^4$ is the difference between the molecular extinction coefficients for reduced and oxidized cytochrome c at 550 m$\mu$. The reaction followed a first order course until at least 80 per cent reduction, beyond which measurements were not made. A unit of enzyme was defined as the quantity which gave a value of 1.0 for $(\Delta \log \text{ferricytochrome})/\Delta t$, the first order rate, where $t$ was expressed in minutes. The activity, $Q_{cytochrome}$, is the number of units per mg. of protein in the test. As shown in Table I, the rate of reaction is proportional to enzyme concentration.

The rate of oxygen consumption was measured manometrically at 25° essentially as described by Ball (3) except that 2.4 mg. of albumin were added to each test. Purified preparations gave little or no oxygen uptake without added albumin. Philpot (6) has reported a similar observation with the methylene blue test. The albumin can be replaced by other proteins, such as hemoglobin or cytochrome, but not by low concentrations of catalase, indicating that the protective action is not due to a removal of hydrogen peroxide.

Protein was determined by the turbidimetric procedure of Bücher (7), calibrated with a standardized rabbit serum solution and measured at 340 m$\mu$ with the Beckman spectrophotometer.

Purification of Enzyme—Fresh raw cream containing 40 to 42 per cent butter fat was churned in a mechanical mixer until the butter separated as fine hard particles, which were removed by straining through several

<table>
<thead>
<tr>
<th>Table I</th>
<th>Proportionality of Cytochrome Reduction and Oxygen Uptake to Xanthine Oxidase Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \log \text{ferricytochrome}/\Delta t$</td>
<td>0.0116 0.0236 0.0491 0.0960</td>
</tr>
<tr>
<td>Xanthine oxidase preparation, mg.</td>
<td>0.0028 0.0036 0.011 0.022</td>
</tr>
<tr>
<td>$Q_{cytochrome}^*$</td>
<td>4.15 4.22 4.46 4.36</td>
</tr>
<tr>
<td>$O_2$ per hr., c.m.m.</td>
<td>67 143 196 250 358</td>
</tr>
<tr>
<td>Xanthine oxidase preparation, mg.</td>
<td>0.077 0.128 0.193 0.256 0.384</td>
</tr>
<tr>
<td>$Q_{O_2}$</td>
<td>870 973 1015 977 935</td>
</tr>
</tbody>
</table>

* Different preparations of enzyme were used for the manometric and spectrophotometric measurements.
layers of cheese-cloth. After addition of 0.6 volume of 0.2 M Na$_2$HPO$_4$ to the buttermilk, it was digested with crude trypsin and clarified with calcium chloride as described by Ball (3). The calcium chloride supernatant was warmed to 60° over a period of 5 minutes and kept there for 5 minutes, after which it was cooled in an ice bath. At this point the enzyme loses little activity in several weeks. From 2 quarts of cream about 1100 cc. of calcium chloride supernatant were usually obtained.

200 cc. of the solution were treated with 45.2 gm. of ammonium sulfate and filtered. The filtrate was treated with 12.0 gm. of ammonium sulfate per 100 cc., and the precipitate was collected by centrifugation and dissolved in 40 cc. of water. 42 cc. of this solution were treated with 25.5 mg. of aluminum hydroxide gel C$_4$ (8). The centrifuged gel was eluted with three 2.0 cc. portions of 0.5 M phosphate buffer, pH 7.5. The combined eluates were treated with 9.0 cc. of saturated ammonium sulfate solution, and the precipitate was collected by centrifugation and dissolved in 10 cc. of water. The solution obtained was treated with 16.5 mg. of calcium phosphate gel (9) (aged about 3 months) and the centrifuged gel washed twice with 2 cc. portions of 0.1 M phosphate buffer, pH 6.2. The enzyme was then eluted with two 2.0 cc. portions of 0.5 M phosphate buffer, pH 6.2. 6.0 cc. of saturated ammonium sulfate solution were added to the combined eluates, and the precipitate was collected by centrifugation and dissolved in 5 cc. of water. All operations except the two adsorption steps were carried out at 0-2°. The final solution is stable for several days, and the enzyme may be kept in saturated ammonium sulfate for several weeks.

In Table II are shown the over-all yield and the activity with respect

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**Table II**

Comparison of Rate of Oxygen Consumption and Cytochrome c Reduction during Purification of Xanthine Oxidase

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Total units of cytochrome activity</th>
<th>Over-all yield</th>
<th>(\frac{Q_{cytochrome}}{Q_{O_2}})</th>
<th>(Q_{O_2})</th>
<th>(Q_{cytochrome})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buttermilk</td>
<td>1300</td>
<td>96</td>
<td>0.031</td>
<td>4.9</td>
<td>158</td>
</tr>
<tr>
<td>After trypsin and heating</td>
<td>800</td>
<td></td>
<td>0.35</td>
<td>62</td>
<td>177</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>624</td>
<td></td>
<td>0.81</td>
<td>145</td>
<td>179</td>
</tr>
<tr>
<td>Alumina C$_4$ eluate</td>
<td>400</td>
<td></td>
<td>3.50</td>
<td>545</td>
<td>150</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$</td>
<td>223</td>
<td></td>
<td>4.96</td>
<td>742</td>
<td>150</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Purified from a more active buttermilk preparation.*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* Purified from a more active buttermilk preparation.*
to oxygen consumption and cytochrome reduction at each step. The ratio of the two activities was essentially unchanged during a 220-fold purification. The reaction with xanthine was 0.6 times that with hypoxanthine in either test system. Acetaldehyde was 0.75 times as active as hypoxanthine in the spectrophotometric test; the manometric test was complicated by inactivation of the enzyme (3).

The oxygen uptake at 20° was 0.75 and 0.4 of that observed at 25° and 37°, respectively. Calculation of the $Q_{O_2}^{20}$ of the last preparation in Table II gives a value of 800, compared with a $Q_{O_2}^{20}$ of 270 reported by Ball.

Effect of Oxygen on Rate of Cytochrome c Reduction—Oxygen was essential for the reduction of cytochrome c, which was very slow under anaerobic conditions. As shown in Fig. 1, this was true with acetaldehyde as well as with hypoxanthine, indicating that cytochrome c reduction was not due to an oxidation product of the substrate. Further, prior incuba-
tion in air with substrate before addition of cytochrome c did not increase the rate of reaction in the absence of air. In Fig. 2 are compared the effects of oxygen tension on the rates of reaction with oxygen and cytochrome. The reduction of cytochrome c continued to be accelerated up to 100 per cent oxygen. In confirmation of earlier work (10), nitrate was found to act as a hydrogen acceptor but here the reduction was inhibited by oxygen. Nitrate was reduced very much more slowly than cytochrome c.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{A comparison of the oxygen effect in the manometric and spectrophotometric tests. Test conditions were as described in the text.}
\end{figure}

Absorption Spectrum—While the shape of the absorption spectrum of the purified enzyme was not significantly different from that reported by Ball, the absorption coefficient, \( \beta_{450} \), was 2.5 times as high (\( 3.7 \times 10^3 \) sq. cm. per gm. of N \textit{versus} \( 1.5 \times 10^3 \)). Reduction with excess hypoxanthine in the absence of \( O_2 \) resulted in partial discharge of the color; with hydroxysulfite it was almost completely bleached.

From the decrease in density on reduction with hypoxanthine, the flavoprotein content of the enzyme could be calculated, assuming that the
extinction coefficient $\epsilon$ was $1.04 \times 10^3$ liters $\times$ cm.$^{-1}$ $\times$ mole$^{-1}$ (11, 12) and that the reduced form did not absorb at this wave-length. The flavoprotein content for the preparation in Fig. 3 was $0.079 \times 10^{-4}$ M. With the further assumption that the molecular weight is 70,000 (13), the enzyme concentration would be 0.55 mg. per cc. The purity would therefore be $(0.55/0.89) = 0.62$ (see Fig. 3).

![Graph showing the spectrum of xanthine oxidase](image)

**FAD Content**—With a fluorometric method developed in this laboratory$^1$ a direct relation was established between the enzyme activity and the FAD content. FAD was liberated from the protein by heating in a boiling water bath for 5 minutes and the precipitated protein removed.

$^1$ FAD was determined by an unpublished method of A. Kornberg, based on the rate of increase in fluorescence when the dinucleotide was converted to alloxazine mononucleotide by the action of potato nucleotide pyrophosphatase (14).
by centrifugation. In Table III are given the results obtained with the preparations described in Table II. It is evident that the enzymatic activity and FAD content are proportional over a purification of 18-fold, in confirmation of the previous findings of Corran et al. (15). The last solution listed in Table III had a flavoprotein content calculated from the change in density with excess hypoxanthine of $12.1 \times 10^{-3}$ micromole per cc., which is in agreement with the FAD content of $10.2 \times 10^{-3}$ micromole per cc. from the fluorometric method. The residual absorption after reduction by hypoxanthine is therefore not due to a FAD-containing enzyme, since all of the FAD is present as xanthine oxidase flavoprotein.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Flavin-Adenine Dinucleotide Content of Xanthine Oxidase Preparations</th>
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<tbody>
<tr>
<td></td>
<td>$Q_{cytochrome}$</td>
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<td>Ammonium sulfate fraction</td>
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</tr>
<tr>
<td>Alumina $C\gamma$ eluate</td>
<td>3.50</td>
</tr>
<tr>
<td>$Ca_2(PO_4)_2$</td>
<td>4.96</td>
</tr>
<tr>
<td>&quot;*&quot;</td>
<td>6.45</td>
</tr>
</tbody>
</table>

*From a different lot of buttermilk.

**DISCUSSION**

For the reduction of cytochrome $c$ the turnover number in 20 per cent oxygen is 37 moles per mole of flavoprotein per minute, compared to a turnover number of 85 moles per mole of flavoprotein per minute in the reaction with oxygen. Xanthine oxidase is thus far less active than other flavoproteins in its reaction with oxygen (16) or cytochrome $c$ (2). The effect of oxygen on the reduction of cytochrome requires elucidation before the role of cytochrome in the physiological mechanism can be understood. It would appear that the reaction with cytochrome proceeds rapidly only in the second step of the leucoflavoprotein oxidation and that oxygen is required to generate the intermediate oxidation product. Some evidence is available in support of this hypothesis, since the initial rate of reduction of cytochrome with equivalent amounts of leucoflavoprotein is very slow in the absence of oxygen, but increases as the reaction progresses.

Ball identified FAD as a constituent of his xanthine oxidase preparations, although FAD did not replace the heat-stable factor which reactivated the enzyme after prolonged dialysis. It has now been shown that
FAD quantitatively accounts for the decrease in absorption at 450 m\(\mu\) when the enzyme is reduced with hypoxanthine. On the basis of this decrease in absorption the purity of the xanthine oxidase calculated as flavoprotein is 62 per cent. It should be pointed out that with the best preparations obtained in the course of the present work the absorption at 450 m\(\mu\) was decreased by hypoxanthine only to the extent of 36 per cent, whereas Ball obtained a decrease of 62 per cent with a preparation with values of \(Q_{O_2}\) and \(\beta_{450}\) only one-fourth to one-third as high. Corran et al. (15), with a highly purified milk xanthine oxidase preparation, also found the flavin to account for only 35 per cent of the absorption at 450 m\(\mu\).

**SUMMARY**

1. Xanthine oxidase purified from milk reduces cytochrome c in the presence of hypoxanthine, xanthine, or acetaldehyde. The activity toward cytochrome c parallels that toward oxygen over a 220-fold purification.

2. The rate of reduction of cytochrome c is accelerated by oxygen. In 20 per cent oxygen the reaction with cytochrome is about one-half as fast as the reaction with oxygen.

3. An improved method of purification results in a xanthine oxidase preparation which is 3 times as active as any previously reported.

4. The flavin-adenine dinucleotide (FAD) content is proportional to the enzymatic activity over a wide range in purification. The purity calculated from the FAD content is 62 per cent. FAD accounts quantitatively for the decrease in absorption at 450 m\(\mu\) when the enzyme is reduced with hypoxanthine.

**BIBLIOGRAPHY**

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