Ascorbic Acid Oxidase of Myrothecium verrucaria

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Ascorbic acid oxidase is widely distributed in higher plants. The enzyme from certain tissues has been highly purified, and some of its properties are well established, especially its copper protein character (6, 19, 23, 24). The function of the enzyme and the role and metabolism of ascorbic acid in plant tissues has been studied extensively (19). Although enzymatic pathways of electron transfer between dehydrogenase systems and ascorbic acid are known, the significance of these and of ascorbic acid oxidase, itself, in respiration or other metabolic phenomena remains obscure.

Two atypical ascorbic acid oxidases in lower plants have been investigated, one by Mandels (17, 18) in spores of the fungus Myrothecium verrucaria, the other by Ward (29) in the slime mold Physarum polycephalum. These enzymes were termed "atypical" because they differed from the copper enzyme in response to inhibitors of metallo-enzymes, in reaction products, or in substrate specificity. Furthermore, the slime mold enzyme appeared to require an intermediate thiol compound for activity.

The mycelium of M. verrucaria also contains an atypical ascorbic acid oxidase (18, 30) with certain properties distinctly different from those of the spore enzyme as well as the slime mold and higher plant oxidases. The present study deals mainly with the general properties and cellular location of the mycelial oxidase and its role as an electron transfer agent in the respiration of mycelium.

Materials & Methods

Myrothecium verrucaria (Alb. & Schw.) Dit. ex. Fr., strain QM 460, was maintained by point inoculation on filter paper supported by a solid agar-salts medium (13). Spore suspensions from cultures free of visible variants were used to inoculate the solid medium for production of uniform-aged spores. Spore formation was initiated at about two days and completed after 4 or 5 days. Spores from cultures older than 3 weeks were not used as inoculum since mycelial growth from these tended to be irregular.

Mycelial pellets were grown in a liquid medium recommended by Darby and Goddard (4). The pellets were cultured under the conditions described by Hilton and Smith (13). After 24 to 30 hours, the liquid medium was filtered off with suction and the mycelium washed twice with about 100 ml of distilled water and then resuspended and further washed with 200 ml of cold, 0.01 M KH₂PO₄-K₂HPO₄ plus 0.005 M citrate buffer at pH 6.3. Following filtration, the wet mat of about 12 g was sectioned, mixed with an equal weight of 100-mesh pyrex glass and 15 ml of cold buffer and ground in a Potter-Elvehjem homogenizer. The homogenate plus washings was centrifuged at 1,000 × g at 0 C for 10 minutes and the supernatant extract decanted. The sediment was again suspended in buffer, ground, and centrifuged. The pooled, cell-free extracts then were centrifuged at 0 C for 30 to 40 minutes at 20,000 × g. The resulting supernatant was separated from the particulate residue and diluted to 60 ml with the phosphate-citrate buffer. The enzyme solution could be stored at −20 C for several months without serious loss of activity. One milliliter of such preparations gave initial rates of ascorbic acid oxidation equivalent to 200 to 500 μl of oxygen per hour. Prior to measuring activity, the extract was acidified with 1 N HCl to pH 4.5, which was optimum for the mycelial enzyme.

Extracts of M. verrucaria spores were prepared by grinding approximately 4 ml of washed, centrifuged spores in a Potter-Elvehjem homogenizer with cold, pH 6.3 phosphate-citrate buffer and 12 g of 100-mesh glass. The debris was spun down at 0 C for 10 minutes at 500 × g and the spore residue again ground in cold buffer. The combined extracts were centrifuged in the cold for 30 minutes at 20,000 × g and the opaque supernatant diluted to 20 ml with buffer.

All reaction measurements were made at 30 C in air by standard Warburg technique unless otherwise specified. The standard reaction mixture contained 0.0112 M ascorbate and 0.034 M phosphate-0.017 M citrate buffer at pH 4.5, in a fluid volume of 2.0 ml. Since carbon dioxide evolution was not observed in the oxidase reaction, alkali was added to the flasks only for study of mycelial respiration. Commercial
Results & Discussion

General Properties: Absence of phenol oxidase or cytochrome oxidase in high speed supernatants of mycelial extracts (5) was confirmed by failure of the latter to oxidize p-cresol, catechol, or tyrosine and ferrocytochrome c. High catalase, but little or no peroxidase activity was present. This evidence and inhibitor results to follow, indicated that ascorbic acid oxidase was solely responsible for substrate oxidation.

Acidification (pH 5.0 or lower) of the enzyme extract precipitated an insoluble fraction carrying from 20 to 70% of the total activity. The insoluble fraction was separated by low-speed centrifugation in the cold and was resuspended in pH 4.5, 0.01 M phosphate-0.005 M citrate buffer for activity tests. About 15% of the total activity in the insoluble fraction was removed by a single washing, but further washing had no effect. No constant proportion of soluble to insoluble fraction was observed. It would appear that the insoluble fraction represents a partially denatured form of the enzyme.

The variation in activity with pH was studied at a buffer strength of 0.034 M phosphate-0.017 M citrate, the highest permissible without reduction in activity. Figure 1 shows the pH-activity curves of the whole extract and of both fractions. The curves for the whole extract and insoluble fraction were the same and were fairly symmetrical while those for the soluble fraction were consistently skewed to the alkaline side. Phosphate alone gave the same type of pH response. The observed pH optimum at about 4.5 was independent of substrate concentration over a range of 0.0028 to 0.028 M ascorbate. In a limited study of pH stability, the enzyme proved to be rapidly and irreversibly inactivated at pH 2.5 but was somewhat more stable at alkaline pH's.

The effect of pH on activity of the mycelial enzyme is different from that reported for the spore enzyme (17) and the purified copper oxidase of higher plants (24). Both of the latter enzymes in phosphate-citrate buffer had broader optima above pH 5. LuValle and Goddard (16) suggested that catalysis by the copper enzyme involves the monovalent ascorbate ion. This also could be true for the mycelial oxidase, but the rapid decrease in activity above pH 5 would indicate that ionization of the enzyme itself is involved in the pH-activity relationship.

In a study of the effect of substrate concentration on the enzyme, the rate was found to increase rapidly to about 0.004 M ascorbate and then more slowly, reaching a maximum at about 0.015 M. Furthermore, the rate of oxidation and the amount of substrate required for maximal velocity increased with the oxygen tension. The reciprocal plot gave a $K_m$ in air of 0.0012 M and in oxygen of 0.0048 M. In five experiments, the calculated $K_m$ in air ranged from 0.00115 M to 0.00135 M. In a single experiment, the separate enzyme fractions had the same $K_m$'s as the whole extract.

There is insufficient data in Mandels' paper (18) to compute a $K_m$ value, but it would appear that the spore and mycelial enzymes have similar affinities for ascorbate. In the case of the purified higher plant enzyme, however, Frieden and Maggiolo (11) recently reported a $K_m$ of about 5 $\times$ 10^{-3} M using the Warburg and a much lower value of 3.9 $\times$ 10^{-5} M by a spectrophotometric method. Dependence of the $K_m$ on oxygen concentration also has been indicated for the spore (18) and the higher plant ascorbic acid oxidases (9, 11, 18). This is to be expected in an oxidative reaction where both donor and acceptor substrates are involved and the former is present in sufficient concentration (2).

Under the assay conditions used, reaction rates were linear for only 10 to 15 minutes, then fell off gradually as shown in figure 3. The decrease in rate with time was not due to substrate depletion since it occurred even at high levels of substrate (0.028 M). Whether expected stoichiometry eventually could be reached depended on the relative concentrations of substrate and enzyme and the rate of enzyme inactivation, which will be discussed further in the kinetics section. Using the linear portions of the curves, reaction rates were proportional to enzyme concentration as illustrated in figure 4. The same relationship held for the soluble and insoluble fractions. This proportionality extended to rates of 300 to 350 $\mu$l of oxygen uptake per hour. The departure from proportionality at higher rates could not be attributed to substrate depletion, product inhibition, or limitation in oxygen diffusion. As with the typical copper enzyme (27) and the spore oxidase (17), the stoichiometry found was $\frac{1}{2}$ mole of oxygen reduced per mole of ascorbate oxidized (table 1). There was no manometric evidence of dehydroascorbic acid reaction since no excess oxygen consumption nor any carbon dioxide evolution was observed. The addition of dialysed crystalline liver catalase (Worthington Biochemicals) or the inhibition of endogenous catalase with azide had no effect on the total amount.
Fig. 1. Effect of pH on ascorbate oxidation by the mycelial extract and the soluble and insoluble fractions. Standard reaction conditions.

Fig. 2. Lineweaver-Burk plot of initial reaction rates as a function of ascorbate concentration with the mycelial enzyme in air and pure oxygen. Standard reaction conditions.

Fig. 3. Typical reaction curve for ascorbate oxidation by the mycelial enzyme. Standard reaction conditions except 0.014 M ascorbate.

Fig. 4. Relation of initial rate of reaction to concentration of mycelial enzyme. Standard reaction conditions except 0.014 M ascorbate.

Fig. 5. First order reaction plot of ascorbate oxidation by the mycelial enzyme in air. Standard reaction conditions with ascorbate concentrations from 0.003 to 0.014 M. $a$: initial amount of ascorbate; $x$: amount of ascorbate used in time $t$. 
of oxygen used in contrast to Ward's slime mold system (29). This provided evidence against peroxide formation but not necessarily against an intermediate enzyme-peroxide complex. Mandels (17) also found no peroxide formation by the spore enzyme. However, Dawson and Tokuyama (7) recently reported evidence of peroxide formation by the highly purified squash enzyme.

A number of compounds known to inhibit metal-containing and sulphydryl enzymes were tested on the mycelial oxidase to obtain information about its prosthetic group. Some of these results are given in Table II. A range of inhibition values is shown where a significant variation in response occurred. The enzyme was relatively insensitive to high concentrations (0.01 M) of metallo-oxidase inhibitors. Cyanide and azide inhibited only 30 to 35% at 0.01 M and were about as effective at 0.001 M. Carbon monoxide to oxygen in the ratio of 19 to 1 had no effect in light or dark. The lack of strong inhibition by these compounds would appear to rule out presence of cytochrome oxidase or phenol oxidase. Diethylthiocarbamate, 8-hydroxyquinoline, and phenylthiourea, which are powerful inhibitors of the copper enzyme at 0.01 M (8, 20), had no comparable effect on the atypical enzyme. In contrast to Ward's system (29), catalytic amounts of diethylthiocarbamate (1 x 10^{-3} M) failed to stimulate oxygen uptake. Ethylenediaminetetraacetate (disodium salt of EDTA) inhibited 10 to 20% at concentrations from 0.001 M to 10^{-6} M. The effects of the metallo-enzyme inhibitors clearly differentiate the atypical enzyme from the copper oxidase. The possibility of iron participation is considered unlikely in view of the results with cyanide, azide, and CO. However, an answer to the question of metal ion function in electron transfer must await purification of the enzyme. Mandels (17) also found that the spore enzyme was insensitive to various metal complexing agents.

Unlike the copper enzyme (11), there was no evidence for essential sulphydryl groups in the mycelial enzyme. Iodoacetate and p-chloromercuribenzoate were stimulatory rather than inhibitory, and iodoacetamide, p-iodosobenzoate, and phenylarsenoxide had little or no effect on activity. Treatment with inhibitors of flavin enzymes (25) such as riboflavin, atabrine, and isoriboflavin or with the flavin nucleotides, FAD and FMN, had no effect on this impure enzyme.

### Table I

<table>
<thead>
<tr>
<th>Ascorbate (mg)</th>
<th>Maximum O₂ uptake (µl)</th>
<th>Obs.*</th>
<th>Calc.**</th>
<th>% of Theory</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>51</td>
<td>neg.</td>
<td>...</td>
<td>100</td>
</tr>
<tr>
<td>0.8</td>
<td>68</td>
<td>51</td>
<td>64</td>
<td>106</td>
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<td>136</td>
<td>128</td>
<td>106</td>
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<tr>
<td>2.0</td>
<td>194</td>
<td>192</td>
<td>101</td>
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</tbody>
</table>

* Flasks contained 0.034 M phosphate-0.0017 M citrate.
** Calculated on the basis of 1/2 mole of O₂ per mole of ascorbate.

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**Fig. 6.** Plots of the mixed order, integrated form of the Michaelis-Menten equation. Curve 1: mycelial enzyme in pure oxygen, 0.014 M ascorbate. Curve 2: mycelial enzyme in air, 0.014 M ascorbate (cf. fig 5). Curve 3: cell surface-localized enzyme in air, 0.0112 M ascorbate. Otherwise standard reaction conditions.

**Fig. 7.** Lineweaver-Burk plot of initial reaction rates as a function of oxygen tension with the mycelial enzyme. Standard reaction conditions.

**Fig. 8.** Plot of the mixed order, integrated form of the Michaelis-Menten equation for the spore enzyme. Reaction conditions: 0.0168 M ascorbate, pH 6.2, 0.034 M phosphate-0.017 M citrate, 50 µg glutathione, in air. a: initial amount of ascorbate; x: amount of ascorbate used in time t.

**Fig. 9.** Semilog plot of k_e values calculated from data of figure 6. Curve 1: mycelial enzyme in pure oxygen. Curve 2: mycelial enzyme in air. Curve 3: surface-localized mycelial enzyme in air.

**Fig. 10.** Effect of pH on mycelial respiration and on activity of the surface-localized enzyme. Net oxidase activity curve (dashes) is from differences between QO₂ values for respiration plus oxidase activity and for respiration. Vessel contents: starved mycelial suspension, 0.014 M ascorbate, 0.034 M phosphate-0.017 M citrate, KOH in center well. Mycelium was starved to reduce endogenous respiration by shaking washed mycelium in 0.01 M phosphate (pH 6.3) and 0.001 M Mg for 28 to 30 hours at 30 °C. QO₂ on a dry basis.
The inhibitor response of the soluble and insoluble enzyme fractions was similar in general. However, some differences were observed, and table III gives some results for individual experiments on fractions separated from the same enzyme preparations. The soluble fraction was less sensitive to cyanide and azide but was much more sensitive to 8-hydroxyquinoline and phenylthiourea. Diethylthiocarbamate may have had a greater stimulatory effect on the insoluble fraction. What these differences mean is not yet clear.

The copper prosthetic group of the typical ascorbic acid oxidase can be removed with cyanide and the resulting inactivation reversed by adding copper (21). With this dialysis procedure the mycelial enzyme lost only about 30% of its activity. There was no restoration by addition of ionic copper or iron. This constitutes further evidence against a copper prosthetic group in the atypical ascorbic acid oxidase and also indicates the lack of a soluble cofactor.

**Kinetic Analysis:** The nature of enzyme-substrate interaction was explored by a study of the apparent reaction order. Kinetic analysis of the mycelial enzyme data in air by the conventional first order equation gave a family of curves changing in slope and in shape with initial ascorbate concentration. Figure 5 shows a typical plot of such data for concentrations from 0.0031 M to 0.014 M. It appeared from the change in initial slope with substrate concentration that zero order effects were significant and from the decreasing slopes at higher substrate concentrations that higher order effects developed. Similar curves were obtained when enzyme activity was varied at a constant substrate level.

Further kinetic analysis was carried out according to Elkins-Kaufman and Neurath (10) using an integrated form of the Michaelis-Menten equation:

\[ k' et = 2.3K_m \log \frac{a}{a-x} + x \]

where \( a \) is initial ascorbate concentration, \( x \) is amount of ascorbate reacting in time \( t \), \( e \) is enzyme concentration, \( K_m \) is steady state constant governing enzyme-substrate complex concentration, and \( k' \) is the reaction velocity constant. This is a composite first and zero order expression in which the contributions of the two terms depend on the magnitude of \( K_m \) and the initial substrate concentration. The term \( k'e \) is a constant, providing higher order effects such as enzyme inactivation are not present. The first order contribution predominates when substrate concentration is small with respect to \( K_m \) and the zero order term increases in magnitude with initial substrate concentration.

Plots of data for reactions in air by the mixed order treatment gave curves which were linear for 10 to 20 minutes then decreased in slope. An example is shown in figure 6 (curve 2) for the 0.014 M data of figure 5. Values of \( k'e \) for the linear portion of the curves were essentially constant over a tenfold range of ascorbate concentration. Data for a typical experiment are given in table IV. Examination of the separate terms of the equation showed the zero order effect contributed about 90% to the initial \( k'e \) at 0.014 M ascorbate and was predominant even at concentrations as low as 0.0031 M. As expected, the first order effect increased with reaction time. The decreasing slope (\( k'e \)) as the reaction proceeded indicated higher order effects due to enzyme inactivation. At intermediate substrate concentrations, such as the 0.0084 M in figure 5, the combination of zero, first, and higher order results resulted in a pseudo-first order reaction extending to about 90% substrate utilization.

A similar analysis was made by the mixed order equation of the effect of ascorbate concentration on the reaction kinetics in an oxygen atmosphere.

### Table III

Comparison of Inhibitor Response of Soluble & Insoluble Fractions of Mycelial Enzyme*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(M)</th>
<th>Inhibition (%)**</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Insol.</td>
</tr>
<tr>
<td>Cyanide</td>
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<td>30</td>
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<tr>
<td>Azide</td>
<td>0.001</td>
<td>38</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>0.01</td>
<td>6</td>
</tr>
<tr>
<td>Phenylthiourea</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Diethylthiocarbamate</td>
<td>0.001</td>
<td>-30</td>
</tr>
</tbody>
</table>

* Standard reaction conditions. Ascorbate added at start of reaction.
** Negative values indicate stimulation.

### Table IV

Comparison of Initial \( k'e \) Values at Varying Substrate Concentrations for Mycelial Enzyme in Air & Oxygen Spore Enzyme in Air*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Ascorbate (M)</th>
<th>( k'e )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Moles/min × 10^4)</td>
<td></td>
</tr>
<tr>
<td><strong>Mycelial enzyme</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air</td>
<td>0.0014</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>0.0017</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>0.0031</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>0.0059</td>
<td>2.05</td>
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<tr>
<td></td>
<td>0.0112</td>
<td>2.10</td>
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<tr>
<td></td>
<td>0.0140</td>
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<td>0.0056</td>
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<td>0.0084</td>
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<td></td>
<td>0.0112</td>
<td>4.40</td>
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<td>0.0140</td>
<td>2.05</td>
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<tr>
<td><strong>Spore enzyme</strong></td>
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<tr>
<td>Air</td>
<td>0.0031</td>
<td>2.00</td>
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<tr>
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<td>1.95</td>
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<tr>
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<td>0.0226</td>
<td>2.05</td>
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</table>

* Standard reaction conditions except pH 6.25 with spore enzyme.
** Mixed order rate constant (see text).
about 0.01 μ ascorbate, rates were increased 2 to 2.5 times in pure oxygen compared to air. The representative plot in figure 6 (curve 1) shows the higher k'e value and its more rapid decline during the reaction in oxygen than in air. In oxygen, the initial k'e values decreased with increasing ascorbate concentration (table IV). As expected from the K_m's (fig 2), the calculated first order contribution was greater in oxygen. It was evident that first and higher order effects were more prominent at the higher oxygen concentration.

The kinetic situation is similar to that described by Chance (2) for peroxidase where both donor (D) and acceptor (A) substrates are involved:

\[
\begin{align*}
E (e-p) + D (d) \xrightarrow{k_1} ED, \\
ED (p) + A (a) \xrightarrow{k_2} E + D_{ox} + A_{rd},
\end{align*}
\]

\[K_m = \frac{k_1(a) + k_2}{k_1}\]

In the present reaction, (d) is ascorbate concentration (a) is oxygen concentration, and (p) is the concentration of the enzyme-substrate complex. If k_1(a) is large compared with k_2, K_m is proportional to (a). This was approximately true for the mycelial oxidase between 0.2 and 1 atm of oxygen (fig 2). By this formulation, there is no optimum condition, since enzyme activity would increase linearly with (a) as long as (d) is large compared with K_m. This does not apply strictly to the present study, probably because the ascorbate concentrations were not high enough. Rates in excess of 90% of V_max were obtained at 1 atm of oxygen with ascorbate concentrations of about 0.01 μ. The dependence of rate on oxygen tension is shown in a reciprocal plot of typical data in figure 7. The linearity indicates the effect of oxygen tension on the mycelial enzyme reaction was consistent with Michaelis-Menten kinetics.

Since the mycelial enzyme appears to be on the cell surface (next section), it was possible to compare its kinetics in vivo and in vitro, so to speak. No significant differences have been observed so far. The similarity in mixed order plot is shown in figure 6 (curves 2 & 3). In particular, the enzyme in its native and extracted state seemed to be about equally susceptible to inactivation. This is of interest since Honda (12) found no reaction inactivation of the wall-localized oxidase in barley roots. The barley enzyme was inactivated in vitro.

Analysis of the kinetics of the extracted spore enzyme in air by the mixed order treatment gave results like those with the mycelial enzyme. Representative data are given in figure 8 and table IV. Zero, first, and higher order effects all contributed, their magnitude depending on relative enzyme and substrate concentrations. The apparent first order kinetics observed by Mandels (17) probably resulted from compensating effects under his reaction conditions.

The higher order effects observed with the fungal ascorbic acid oxidase could be attributed to reaction inactivation. No loss in activity occurred under the reaction conditions when enzyme and ascorbate were incubated under nitrogen or with the enzyme alone under oxygen. Furthermore, the reaction product, dehydroascorbate, at 0.01 μ had no effect on enzyme activity. Semilog plots of k'e values from the mixed order curves were linear for most of the reaction course. Figure 9 gives examples for the mycelial enzyme using the data in figure 6. The spore enzyme gave similar results. These observations indicated inactivation was first order with respect to enzyme concentration. Since this was true in both air and pure oxygen, it is unlikely that oxygen, as such, inhibited the enzyme during catalysis. The greater rate of inactivation in oxygen may be ascribed to the higher k'e or more rapid turnover. The rate of reaction inactivation varied among enzyme preparations for the same reaction conditions. In terms of negative slopes of log k'e curves, the following ranges were observed for the mycelial enzyme: in vitro-air, 0.0023 to 0.0040; in vitro-oxygen, 0.0059 to 0.0077; in vivo-air, 0.0022 to 0.0025.

The purified ascorbic acid oxidase of higher plants also is susceptible to reaction inactivation and several studies of the mechanism have been reported (7,11,23). The mechanism with the atypical oxidase is probably different, however, since there is no evidence that sulphydryl groups or copper are involved in catalysis. Furthermore, unlike the copper enzyme (23), solutions of protein such as gelatin and albumin or complexers like EDTA or cysteine did not protect or activate the fungal enzyme. The present kinetic results suggest that the rate of enzyme inactivation may depend on the concentration of some
transient intermediate, such as the hydro-peroxide ion (16), which alters the reaction site in a certain fraction of the turnovers.

- Location of Mycelial Enzyme: Mandels (17) took advantage of the acid resistance of Myrothecium spores to demonstrate that the ascorbic acid oxidase was at the cell surface. A similar method was used in this study with the mycelial oxidase. Table V shows that short term exposure of mycelium to dilute HCl completely inactivated the oxidase with only slight effect on the endogenous respiration of either starved or unstarved mycelium. The inactivation was pH-irreversible as with the extracted enzyme. These results indicate a surface location, since it is unlikely that acid treatment could inactivate the oxidase inside the cell without inhibiting respiratory enzymes. In addition, no ascorbic acid oxidase activity could be extracted by the standard procedure from mycelium exposed for 5 minutes to 0.01 N HCl. Apparently, virtually all the oxidase is at the cell surface.

Further evidence of surface location is provided by pH-activity curves. Since respiratory activity was little influenced by pH, the pH-response curve of net oxidase activity could be established by difference. The similarity in pH-response curves in figures 1 and 10 for the extracted and native enzyme indicated the latter is in contact with the external medium. In addition to the low pH optimum, two other observations seemed to rule out participation of surface-localized cytochrome oxidase in ascorbate oxidation. First, addition of cytochrome c did not affect the oxygen uptake of mycelium with added ascorbate. Second, 0.001 M cyanide did not inhibit the native ascorbic acid oxidase on the basis of differences in oxygen uptake of mycelium with and without added ascorbate.

Role of Oxidase in Mycelial Respiration: The nature of the terminal oxidation system in M. verrucaria mycelium is not altogether clear. Darby and Goddard (5) reported an active cytochrome oxidase in extracts but found that mycelial respiration was not inhibited by 95% CO or appreciably by 0.001 M cyanide. They suggested that another terminal oxidase might be present. On the other hand, Hilton and Smith (13), using younger mycelium (24 instead of 48 hr cultures), observed much greater cyanide inhibition, averaging about 65% at 4.6 x 10^{-4} M. Furthermore, they found mycelial respiration was inhibited up to 95% by the alkynaphthoquinone, S949, which blocks cytochrome c reduction (1). Thus, while there is substantial evidence for a cytochrome pathway, the possibility of another terminal oxidase must be considered, presumably one insensitive to CO and less sensitive to cyanide. The atypical ascorbic acid oxidase was such a possibility.

The first approach to the problem was to compare the oxygen affinity of the extracted oxidase with that of mycelial respiration. Other workers (9, 28, 31) have employed this technique to determine the primitive terminal oxidase in plants on the assumption that tissue possessing a high affinity, compared with ascorbic acid oxidase, depends on cytochrome oxidase or a flavin terminal system. The oxygen affinity of the mycelial oxidase was calculated from data like that plotted in figure 7. Expressed as pO_{2,50, the partial pressure of oxygen in atmospheres for half-maximum velocity, the affinity was 0.25 atm. The corresponding value for the spore oxidase estimated from a curve given by Mandels (18) is 0.37 atm. These affinity values for the atypical ascorbic acid oxidase are in the same range as those for ascorbic acid oxidases from higher plants, for example, 0.13 atm and 0.32 atm for the pea internode (9) and squash enzymes (18), respectively. The ascorbic acid oxidase values are all higher than those for phenol oxidase, various flavin oxidases, and especially cytochrome oxidase (3, 26, 31).

Measurement of an oxygen affinity value for the respiration of fungus mycelial pellets presents special problems. In Darby and Goddard's work with submerged pellets (4), maximum rate was reached only at 40% oxygen by volume, and they concluded that oxygen diffusion within the pellet was a limiting factor. From their data, one can estimate by reciprocal plots a pO_{2,50} value for respiration of 0.1 atm, much higher than observed for typical cytochrome-mediated respiration.

To minimize the diffusion problem, a different technique was devised for the present work, similar in principle to that used with slices of higher plant tissues (9, 28, 31). The extra-cellular liquid diffusion path was reduced by using thin mats of mycelium exposed directly to the gas phase. Appropriate amounts of mycelium were washed, suspended in cold, pH 6.0, 0.01 M phosphate, and filtered under suction on filter paper over a thick layer of paper pulp to give a uniform mat of about 0.5 mm thickness. The mat was transferred to dry filter paper, and discs of 14 mm diameter were cut with a cork borer. These discs were allowed to dry in air until they assumed a light color which by microscopic observation indicated surface water was removed. This treatment did not appear to affect endogenous respiration, but for reproducible results it was important to regulate disc thickness and dryness carefully. Oxygen uptake was measured at 30°C using three to five discs per flask in a water-saturated atmosphere at oxygen tensions down to 0.01 atm. Analysis of results from four cultures of mycelium showed no significant difference in rates at 0.2 and 0.01 atm of oxygen. In several experiments at low oxygen tension where all oxygen was used up, it was estimated from slopes of the curves that respiratory rates at 0.005 atm were still greater than half-maximal. Therefore, the pO_{2,50} value for mycelial respiration under these conditions was somewhat less than 0.005 atm. In other words, if one assumes the terminal oxidase was rate limiting, its oxygen affinity was over 50 times as great as that of ascorbic acid oxidase. This affinity value is in the range calculated from published data for cyto-

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Table VI
Effect on SN 5949 & Antimycin A on Respiration of Intact Mycelium & on Ascorbic Acid Oxidase Activity

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Inhibitor*</th>
<th>Amount (µg)</th>
<th>Oxidative activity (µl O₂/hr)</th>
<th>Respiration</th>
<th>Inhib. (%)</th>
<th>Oxidase</th>
<th>Inhib. (%)</th>
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* Dissolved in 95% ethanol. Control contained same volume of ethanol.
** For respiration measurement, flasks contained mycelial suspension, pH 5, 0.034 M phosphate, 0.0004 M Mg, and KOH in center well. For oxidase assay, standard extraction technique and reaction conditions were used.
*** For respiration measurement and oxidase assay, flasks contained mycelial suspension, pH 4.5, 0.032 M phosphate-0.016 M citrate, 0.0004 M Mg, and KOH in center well. Oxidase activity was determined by difference in oxygen uptake with and without 0.01 M ascorbate.

The second approach to the role of the atypical ascorbic acid oxidase in terminal respiration was through the use of selective inhibitors of the cytochrome system. The most useful were antimycin A and SN 5949 which block electron transfer between cytochromes b and c (1,22). In table VI are presented typical results. Inhibition of respiration was essentially complete with 10 µg per ml of either inhibitor. Activity of extracted ascorbic acid oxidase (in vitro), on the other hand, was unaffected by even higher concentrations. A single experiment (No. 3) with the native oxidase (in vivo) also indicated no inhibition by SN 5949. On the basis of these results, it is doubtful that ascorbic acid oxidase participates in respiration to any extent. The work in the previous section on short term acid exposure also supports this conclusion. It would appear that the mycelial oxidase must have a role other than in respiratory metabolism. One must recognize, however, that the situation may be different at other stages of the life cycle or under other nutritional or environmental conditions.

Summary
Mycelium of the fungus Myrothecium verrucaria (Alb. & Schw.) contains an atypical ascorbic acid oxidase having properties different from those of the classical copper enzyme of higher plants and the enzyme in spores of the same organism. The enzyme extracted from mycelium had maximum activity at pH 4.2 to 4.8. Acidification of the extract to pH 5 or lower separated soluble and insoluble fractions with somewhat different properties. Reaction kinetics of both the mycelial and spore enzymes can be described, in part, by a mixed order form of the Michaelis-Menten equation. Decrease in the reaction constant with time was attributed to reaction inactivation of the enzyme. The reaction stoichiometry appeared to be ½ mole of oxygen per mole of ascorbate with no evidence of peroxide formation. The mycelial enzyme was relatively insensitive to inhibitors of metallo-enzymes such as cyanide, azide, and diethyldithiocarbamate and was stimulated by various sulphydryl group reagents. The oxygen tension required for half-maximal velocity was about 0.25 atm, and the apparent Kₘ increased from about 0.0012 m ascorbate in air to 0.0048 m ascorbate in pure oxygen. Acid inactivation and pH-response data indicated the mycelial enzyme was localized at the cell surface. The effect of oxygen tension and selective inhibitors showed that the respiration of mycelium was dependent on the cytochrome system and not the ascorbic acid oxidase.

Acknowledgment
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Literature Cited
1. Ball, E. G., C. B. Anfinsen, & O. Cooper. 1947. The inhibitory action of naphthoquinones on res-


