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Oxidizing Ascorbic Acid in Fungus Spores

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### INTRODUCTION

The localization of enzymes at the cell surface has received increasing attention in recent years. Certain carbohydrases and phosphatases have been reported to be at the surface of yeast cells, and choline esterase at the surface of certain cells in the squid. [See review by Dounce (1).] In addition, several carbohydrases have been localized at the surface of spores of the fungi *Myrothecium verrucaria* and *Aspergillus luchuensis* (2, 3), and dehydrogenases at the surface of bacteria (4). Data presented in this communication show that an enzyme is present at the surface of *M. verrucaria* spores which actively oxidizes ascorbic acid.

Ascorbic acid can be oxidized directly by ascorbic acid oxidase, and indirectly by cytochrome oxidase, peroxidase, polyphenol oxidase, and laccase [see reviews by Michaelis (5), James (6), Ponting and Joslyn (7) and Dawson and Tarpley (8)]. Metals, particularly copper, catalyze its autoxidation. The characteristics of the enzyme in *M. verrucaria* spores, as shown in the present study, indicate that it is not identical with any of the above mentioned systems since it is resistant to inhibitors of heavy metal catalysts. Furthermore, substrates such as hydroquinone, tyrosine and catechol are oxidized very slowly or not at all.

### METHODS

Spores of the cellulolytic fungus *Myrothecium verrucaria* were obtained from cultures grown on filter paper and were suspended in water or buffer as described previously (2). Spore extracts were prepared by centrifuging spores into the mortar of a Potter mill, decanting off the supernatant, and grinding for about 30 min., keeping the mill chilled in an ice bath. Distilled water was then added to the ground preparation. After centrifuging, the supernatant was decanted and stored in a Deepfreeze until used.

Conventional Warburg techniques were employed to follow oxidation of the substrates. Unless specified to the contrary, 0.05 M  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  buffer at pH 6.25 in ordinary distilled water was used. Each vessel usually contained 4-5 mg. of spores or the equivalent of an extract of ca. 10 mg. of spores. One-half milliliter of substrate (containing 3-4 mg. ascorbic acid adjusted to the proper pH) was placed in the side arm. Alkali was usually omitted from the center wells to minimize any endogenous gas exchange.

Results for oxidation of ascorbate by spores are in terms of  $\mu\text{l. O}_2/\text{mg. dry wt. spores/hr.}$  calculated from the rate for the first 10 min. of the reaction. In experiments using spore extracts, rates are given as  $\mu\text{l. O}_2/\text{vessel/hr.}$  based on the rate for the first 10 min. of the reaction.

Since glutathione protects ascorbic acid against autoxidation (9), the ascorbic acid (Merck) was dissolved in buffer or water containing 60  $\mu\text{g./ml.}$  of glutathione (GSH) in most of the experiments reported here. This quantity of glutathione prevented autoxidation of the ascorbate, yet had no measurable effect on the course or rate of the reaction. If glutathione was omitted, controls for autoxidation were always included.

### RESULTS

#### *Oxidation of Ascorbic Acid by Spores and Spore Extracts*

Addition of ascorbic acid to suspensions of *M. verrucaria* spores results in an absorption of oxygen at a rate of about 90  $\mu\text{l./mg. dry wt. of spores/hr.}$  Within the limits of experimentation, no lag is observed between the addition of ascorbate and its oxidation by the spores. This rate of oxidation is much more rapid than respiratory rates with sugars such as glucose or sucrose, in which cases  $Q_{\text{O}_2}$  values of about 10 and 20, respectively, are found. The oxygen uptake when ascorbic acid is added is not a respiratory phenomenon in the usual sense of the term, since very little  $\text{CO}_2$  evolution occurs beyond that which can be ascribed to endogenous respiration. There are some data, however, obtained with high ascorbate concentration (ca. 1%), which indicate that either ascorbic acid or its oxidation product, dehydroascorbic acid, can be respired slowly—at a  $Q_{\text{O}_2}$  value of about 5.

Attempts to establish a linear relation between spore concentration and rate of oxidation of ascorbate were unsuccessful because of the rapid decrease in activity after addition of ascorbate at low spore concentrations (see Fig. 1). At high spore concentrations the rate of  $\text{O}_2$  uptake would be limited by diffusion of oxygen into the solution. This phenomenon at low concentrations is presumably the "reaction" inactivation described by Powers and Dawson (10) in their studies of purified ascorbic acid oxidase. While these investigators found that gelatin or

other proteins could partially protect the purified enzyme from inactivation, no such protection was found in the present study using gelatin and crude enzyme preparations.

The enzyme catalyzing the oxidation of ascorbate by the spores is readily inactivated by heating to boiling. The enzyme is not released from intact spores suspended in buffer overnight, nor does addition of toluene or merthiolate result in its release or inactivation. The enzyme

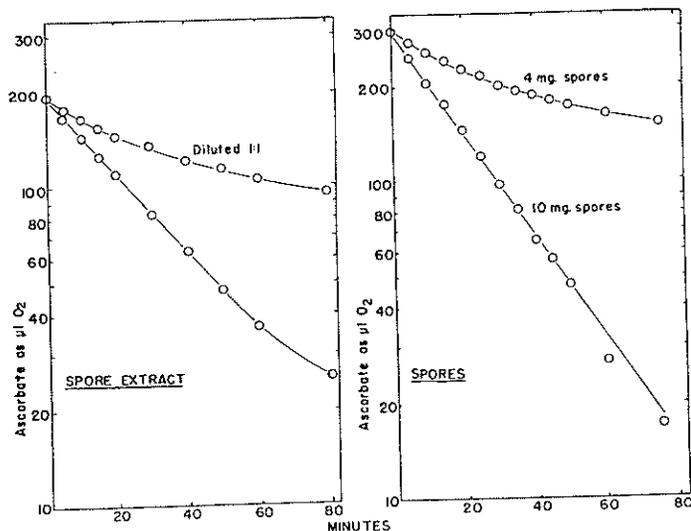


FIG. 1. Oxidation of ascorbic acid by spores and spore extracts. (Ascorbate remaining calculated on basis of potential  $O_2$  uptake and plotted on log scale against time for different dilutions of spore extract and spores.)

is easily obtained in solution by grinding the spores in a Potter mill. In such spore extracts the activity is not lost by storing in the frozen condition for periods of about a week. The activity of these extracts is not associated with particulate matter since centrifugation at  $70,000 \times g$  for 15 min. causes no reduction in activity. Dialysis of extracts against distilled water results in no loss in activity. The phenomenon of reaction inactivation is found in the spore extracts as well as in intact spores. Gelatin, again, gives no protection here.

### Stoichiometry and Kinetics of the Reaction

As in the oxidation of ascorbate by other enzyme systems, the theoretical  $\frac{1}{2}$  mole of  $O_2$  is absorbed per mole of ascorbate in the oxidation catalyzed by the spores (Table I). The reaction catalyzed by either spores or spore extracts follows a monomolecular course as shown by the linear relation between the logarithm of the concentration of substrate remaining and time (Fig. 1).

### Specificity of the Reaction

To determine the possible role of enzymes other than ascorbic acid oxidase in the oxidation of ascorbate by the spores, several other substrates were tested. The possible mediation of cytochrome oxidase is

TABLE I  
Calculated Versus Experimental  $O_2$  Uptake in Oxidation of Ascorbic Acid by  
*Myrothecium verrucaria* Spores  
(10.4 mg. spores/vessel;  $PO_4^{--}$  buffer, pH 6.25; 30  $\mu g$ . GSH/vessel)

Ascorbic acid mg.	Microliters $O_2$		$O_2$ corrected for endog.		Calcd. $O_2^a$ uptake
	60 min.	160 min.	60 min.	160 min.	
0	41	76	0	0	0
1	104	143	63	67	64
2	172	214	131	138	128
4	288	343	247	267	256

<sup>a</sup> On basis  $\frac{1}{2}$   $O_2$ /mole ascorbic acid.

negated by the failure to oxidize hydroquinone even when cytochrome c was added. The behavior toward inhibitors substantiates this conclusion. Since neither tyrosine nor catechol is oxidized rapidly, tyrosinase, laccase, and peroxidase are not responsible for the activity, as is also substantiated by the action of inhibitors. Qualitative tests with gum guaiac gave negative results for polyphenol oxidase and peroxidase using spores. With spore extract a slight positive test for peroxidase was found. Considerable catalase activity is found in the spore extract.

### Effect of pH and Buffer

In phosphate buffer, pH has no significant effect on the initial rate of oxidation by spores from pH 4.5 to above pH 7 or 8 (Fig. 2). Below

about pH 4.5 the rate falls off quickly, with no activity at pH 2. In citrate-phosphate buffer, the activity decreases more gradually, from pH 5.5. The pH-activity curve for spore extracts in phosphate buffer is essentially the same as for intact spores (Fig. 2). Some fragmentary data obtained with several other buffer systems using spore suspensions show that acetate is comparable to citrate. Borate is similar to phosphate. The activity in phthalate buffer is considerably below that in citrate, at least up to pH 5.

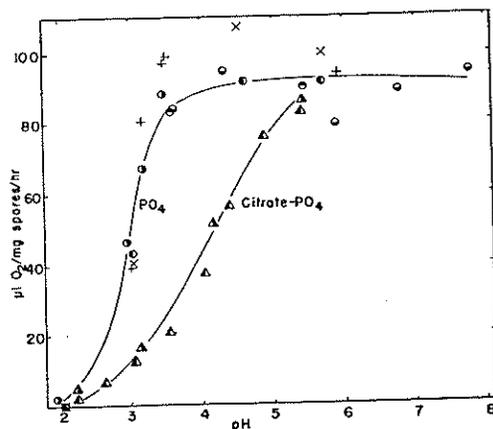


FIG. 2. Effect of pH and buffer on oxidation of ascorbic acid by spores and spore extract. (Data are from several experiments as indicated by different symbols: triangles are spores + citrate- $\text{PO}_4$  buffer; circles are spores +  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  buffer; crosses are spore extract +  $\text{KH}_2\text{PO}_4$ - $\text{K}_2\text{HPO}_4$  buffer. Rates calculated from  $\mu\text{l. O}_2$  absorbed in first 10 min. Data for spore extract are relative values.)

#### Action of Inhibitors

The enzyme responsible for the oxidation of ascorbic acid by spores and spore extracts is resistant to cyanide, azide, sulfide, diethyl dithiocarbamate, phenylthiourea, and 8-hydroxyquinoline (Fig. 3 and Table II). These inhibitors poison either the copper or iron-containing enzymes. Actually the system is stimulated by many of these inhibitors, particularly by  $\text{Na}_2\text{S}$ . In the presence of 0.01  $M$   $\text{Na}_2\text{S}$ , more than the theoretical quantity of oxygen is absorbed. Presumably this is due to reduction of dehydroascorbic acid to ascorbic acid by  $\text{H}_2\text{S}$ , the net effect being

an oxidation of both sulfide and ascorbate. At lower concentrations of sulfide (0.001  $M$ ) no effect was found. Certain poisons which characteristically inhibit enzymes requiring  $-\text{SH}$  groups for their activity are

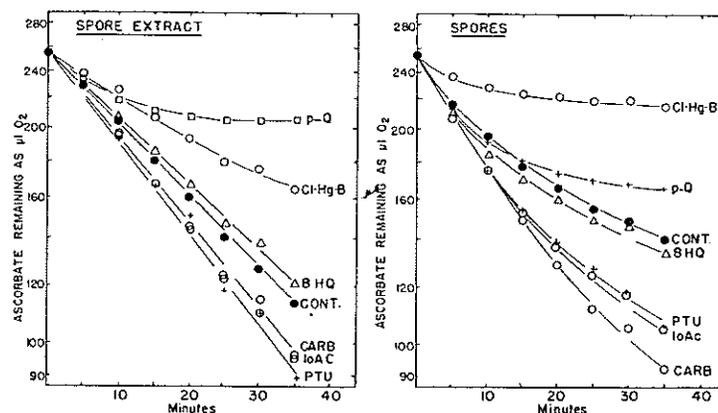


FIG. 3. Effect of inhibitors on oxidation of ascorbic acid. ( $p$ - $Q$  =  $p$ -quinone; 8 HQ = 8-hydroxyquinoline; PTU = phenylthiourea; Carb. = diethyl dithiocarbamate; Cl-Hg-B = chloromercuribenzoate; IoAc = iodoacetate; Cont. = control. All concentrations are  $10^{-2} M$  except Cl-Hg-B, PTU, and 8 HQ where the concentration was less because of limited solubility.  $\text{PO}_4$  buffer. Final pH, 5.5-6.5. For spore data, 3 mg. spores/vessel.)

TABLE II

#### Effect of Inhibitors on Oxidation of Ascorbic Acid

Inhibitor, $10^{-2}M$	Per cent inhibition <sup>a</sup>	
	Spores	Spore extract
KCN	5	-30
Azide	-9	-51
2,4-Dinitrophenol	5	-
$\text{Na}_2\text{S}$	-65	-87

<sup>a</sup> Calculated from rate of  $\text{O}_2$  uptake for first 10 min. Negative values indicate stimulation.

inhibitory—i.e., chloromercuribenzoate and  $p$ -quinone. Iodoacetate, however, is slightly stimulatory.

#### Location of the Enzyme

In another study (3), advantage was taken of the acid resistance of fungus spores to determine the location of carbohydrases at the surface

of the spores. Exposure of these spores to 0.1 *N* HCl inactivates certain enzymes of the spores, yet produces no concomitant decrease in viability, respiratory rate, or oxidation of glucose. Such data indicate surface location of the inactivated enzymes. With respect to the oxidation of ascorbic acid it was found that very brief exposure to 0.1 *N* HCl—as short as 30 sec., almost completely inactivated the oxidative system; exposures as long as 40 min. had only a moderate effect on the oxidation of glucose or on endogenous respiration (Table III). The results obtained by washing the acid from the spores were similar to those obtained by

TABLE III  
Effect of Acid Treatment on Oxidation of Ascorbic Acid by  
*Myrothecium verrucaria* Spores

Expt. No. <sup>a</sup>	Exposure time <sup>b</sup> min.	O <sub>2</sub>		
		Ascorbate	Glucose	Endogenous
1	0	61	14.4	3.7
	20	5.8	12.4	2.5
	40	4.5	8.5	3.2
2	0	50	11	
	5	3	10	
	10	3	9	
3	0	58		
	0.5	5		
	1.0	5		
	2.0	5		

<sup>a</sup> In Expts. 1 and 2, spores were washed by centrifugation after acid treatment. In Expt. 3, acid was neutralized with K<sub>2</sub>PO<sub>4</sub>. Spores were not washed after neutralization.

<sup>b</sup> To 0.1 *N* HCl (final concentration).

neutralizing the acid. The slow oxygen uptake by the acid-treated spores in the presence of ascorbate in excess of the endogenous respiration is ascribed to respiration of ascorbate rather than to simple enzymatic oxidation to dehydroascorbic acid.

#### DISCUSSION

The enzymatic nature of the oxidation of ascorbic acid by *M. verrucaria* spores and spore extracts is demonstrated by heat lability and by failure of the active principle to pass through a dialyzing membrane.

Furthermore, the stoichiometry of the reaction is identical with that of other enzyme-catalyzed oxidations of ascorbate (i.e., ½ mole O<sub>2</sub>/ascorbate), whereas in the heavy-metal catalysis more than this amount of O<sub>2</sub> is absorbed (11). The exact nature of the enzyme has not been established. Its properties, however, do not coincide with those of systems found in other organisms which oxidize ascorbate—i.e., ascorbic acid oxidase, cytochrome oxidase, tyrosinase, peroxidase, or laccase. Of all these systems, only ascorbic acid oxidase acts directly on ascorbate, the others acting indirectly thru the mediation of cytochrome or quinones. The enzymes ascorbic acid oxidase, tyrosinase, and laccase, as described from various sources, are all copper proteins and are inhibited by such "copper poisons" as diethyl dithiocarbamate, phenylthiourea, and 8-hydroxyquinoline. Peroxidase and cytochrome oxidase, being iron-proteins, are inhibited by poisons such as cyanide, azide, and sulfide. The enzyme studied here is inhibited by none of these reagents. The enzyme can be further differentiated from cytochrome oxidase in that it will not oxidize hydroquinone with added cytochrome c. Since neither catechol nor tyrosine is oxidized at rates comparable to that for ascorbic acid by the spores, this confirms the dissimilarity between the enzyme in the spores and tyrosinase, peroxidase, and laccase. With the data available it appears that the enzyme is most closely related to ascorbic acid oxidase in that it apparently oxidized ascorbate directly. While no evidence is available indicating that compounds other than oxygen can act as hydrogen acceptors (except that methylene blue cannot), the ability of dialyzed spore extracts to oxidize ascorbate as rapidly as the original preparations strongly suggests direct oxidation of ascorbate as is effected by ascorbic acid oxidase. This similarity is substantiated by the first-order kinetics of the reactions which obtain for ascorbic acid oxidase. The kinetics of the indirect peroxidase system is stated by Szent-Györgi (12) to be very complicated. The pH-activity curve of the enzyme is distinct from the published data for ascorbic acid oxidase from the drumstick plant (13), squash (14), apple (7), and the purified enzyme from squash (15), in that there is a more or less well-defined optimum at about pH 5-6 in these systems, whereas the enzyme from *M. verrucaria* spores has a broad plateau of optimum activity from pH 4 or 5, depending on the buffer, to pH 8 or higher. Further similarity to ascorbic acid oxidase is noted in the inactivation of the enzyme from spores by ascorbate. This is stated to be characteristic of ascorbic acid oxidase (1, 16). While gelatin and certain other proteins protect this reaction

inactivation in the latter case, gelatin has no such effect on the enzyme from *M. verrucaria* spores.

The data available indicate strongly that the enzyme is at the surface of the spores. The evidence is of two types. In phosphate buffer, pH has the same effect on the enzymic activity of the intact, viable spores as it does upon the cell-free enzyme extracted from the spores. Since the pH of the interior of the viable cell cannot be greatly affected by fluctuations in pH of the environment, such coincidence of *in vivo* and *in vitro* activity curves is strong indication of a surface location of the enzyme. Similar evidence has been used to locate certain carbohydrases at the cell surface. In these cases where no significant dissociation of substrate occurs, such evidence is fairly convincing, since it is unlikely that permeability of the cell to the substrate would be affected in the same manner as the activity of the enzyme. In the case of ascorbate, however, penetration of the cell could be very greatly affected by pH, depending on the concentration of the penetrating species—whether undissociated ascorbic acid or dissociated ascorbate ion. Since we cannot analyze this further with the data available, we can only conclude that it is rather unlikely that penetration into the cell should be affected to the same extent by pH as activity of the cell extract. The similarity between the pH-dissociation curve for ascorbic acid (17) and the enzyme-activity curve is very striking. Possibly combination of substrate with enzyme is with the univalent ion only, as has been claimed for the copper-catalyzed reaction (11).

Further evidence relating to the location of the enzyme has been obtained by an entirely different method, i.e., by acid inactivation studies. Previous work has shown (3) that spores of *M. verrucaria* can be immersed in 0.1 *N* HCl up to 20 min. without killing a significant percentage of the spores. Such treatment does not appreciably impair the respiratory activity of the spores on glucose, the endogenous respiration, nor glucose assimilation. Data presented here show that acid treatment for as short as 30 sec. completely inactivates the ascorbic acid oxidase of the spores, as well as that of spore extracts. This is readily interpreted as being due to the location of the enzyme at the external surface of the plasma membrane. It is rather inconceivable that the acid inactivation could occur within the cell without seriously disrupting the respiratory and assimilatory enzymes or killing the cell.

The presence of a very active oxidative system such as the one described here in spores is pertinent to interpretation of substrate utiliza-

tion studies based on respiratory measurements. Obviously consideration of oxygen uptake alone could be very misleading without parallel data showing comparable CO<sub>2</sub> uptake or increments in dry weight.

#### ACKNOWLEDGMENT

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#### SUMMARY

1. Spores of the fungus *Myrothecium verrucaria* actively oxidize ascorbic acid. The enzyme is located at the surface of the spores and is easily extracted by grinding the spores, but is not released from intact spores in the presence of toluene or merthiolate.

2. The enzyme differs from described ascorbic acid oxidizing systems in its resistance to inhibitors and in its specificity.

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