Proceedings of the Iowa Academy of Science

Volume 69 | Annual Issue Article 32

1962

Anomalous Gas Exchange in Ascorbate Oxidation by the Myrothecium Enzyme

E. B. Lillehoj
Iowa State University

F. G. Smith
Iowa State University

7. Brownley, F. I., and Howle, C. W., Anal. Chem., *24*, 1330 (1960)
8. Bumbstead, H. E., and Wells. J. C., Anal. Chem., *24*, 1595 (1962).
9. Curry, R. P., and Mellon, M. G., ibid., 24, 1567 (1956).
10. Fahey, J. J., Ind. Eng. Chem., Anal. Ed., *11*, 362 (1939)
11. Fennell, T.R.F.W., Chem. and Ind., 1404 (1955).
12. Fine, L. and Wynne, E. A., Microchem. J., *3*, 515 (1959).
13. Hoffman, J. I., and Lundell, G.E.F., Bur. of Standards J. Research, *3*, 581 (1929)
14. Kaufman, S., Anal. Chem. *21*, 582 (1949).
15. Megregian, S. and Maier, F. S., ibid., *26*, 1161 (1954).
16. Miller, J. F., Hunt, H. and McBee, E .T., ibid., *19*, 148 (1947).
17. Nielson, H. M., ibid., *30*, 1009 (1958).
18. Silverman, L. and Shideler, M. E., ibid., *31*, 152 (1959)
19. Stevens, R. E., Ind. Eng. Chem., ibid., *8*, 248 (1936).
20. Willard, H. H., and Horton, C. A., ibid., *22*, 1190 (1950).
21. Willard, H. H., and Winter, O. B., Ind. Eng. Chem., Anal. Ed., *5*, 7 (1933).

Anomalous Gas Exchange in Ascorbate Oxidation by the Myrothecium Enzyme

E. B. Lillehoj[1] and F. G. Smith[1]

Abstract. Manometric studies were employed to assay for ascorbic acid oxidase activity in cell free homogenates from the mycelium of the mold *Myrothecium verrucaria.* It was found that gas exchange measurements deviated from expected stoichiometry based on the utilization of one-half of a mole of oxygen for every mole of ascorbate oxidized. Oxygen was consumed in excess of the expected amount and carbon dioxide was produced during the course of the reaction. Apparently, the ascorbic acid oxidase reaction which produces dehydroascorbic acid is accompanied by or followed by other reactions involving oxygen consumption and carbon dioxide evolution.

Introduction

Two enzymatic oxidations have been implicated in the transfer of electrons from ascorbic acid directly to oxygen. The first of these is by the well-characterized ascorbic acid oxidase from higher plants. This enzyme has been highly purified and the active site is known to be a copper prosthetic group. A second oxidase has been observed in lower plants. One of the characteristics of the latter is that heavy metal inhibitors do not block the oxidation of ascorbic acid. Primarily for this reason the lower plant oxidase has been termed "atypical."

Dunn and Dawson (4) showed that the stoichiometry of the purified ascorbic acid oxidase from higher plant sources involves the utilization of one-half of a mole of oxygen per mole

[1] Deparment of Botany and Plant Pathology, Iowa State University, Ames, Iowa.
[2] This work was aided by a Public Health Service research grant, RG-7354, from the Division of General Medical Sciences, U. S. Public Health Service.

of ascorbate. They concluded that the initial product of the oxidase reaction is dehydroascorbic acid which is non-enzymatically converted to 2, 3-diketogulonic acid with no further breakdown involved.

Mandels (7, 8) found an atypical ascorbic acid oxidase in the spores and the mycelium of *Myrothecium verrucaria*. White (14) in this laboratory has extended Mandels' initial observations and has made a thorough analysis of some of the properties of the extracted mycelial enzyme. These workers concluded that a catalyst exists in the mold which transfers the electrons of ascorbic acid directly to oxygen. Furthermore, Mandels (8) reported that the spore oxidase yielded the same stoichiometry as the higher plant enzyme, one-half mole of oxygen per mole of ascorbate. Preliminary studies by White (14) indicated that this relationship also existed for the mycelial extracts.

Materials and Methods

The spore cultures of *Myrothecium verrucaria* (Alb. and Schw.) Dit. ex. Fr. were maintained by culturing on a modified Fries medium containing micronutrients (11). Point inoculations were used to detect variant strains. At 30° C. sporulation occurred about five days after inoculation on a filter paper which had been placed on the surface of the agar-salts medium. Spore production cultures were inoculated by flooding the filter paper with a spore suspension, other conditions being the same as for the point inoculation.

Suspensions of mycelial pellets were produced in a liquid medium described by Darby and Goddard (1). Twenty-five ml. of the culture medium was inoculated with approximately $2x10^8$ spores from the spore production cultures. Incubation was carried out on a rotary shaker for 24 to 30 hours at 30°C.

The culture medium was removed by filtration, and the mycelial mat was washed with water. Three g. of the mycelial material was placed in the grinding cup of the Servall Omni-Mixer along with about 5 g. of 100 mesh pyrex glass and 3 ml. of extractant per gram of mycelium. The grinding was carried out in an ice bath with the blendor operating at 15,000 r.p.m. for 10 minutes. After grinding, the mycelial debris was frozen. When the material was to be assayed, it was thawed and resuspended with the Servall. The debris and supernatant were transferred to pre-chilled centrifuge tubes which were centrifuged for 10 minutes at 1000 x g. at 0°C. The supernatant fraction, which was free of intact cells by microscopic examination, was centrifuged at 20,000 x g. for 30 to 40 minutes in the cold. This supernatant was the enzyme extract which was studied.

Standard manometric techniques were employed for assay of

the enzymatic activity. Carbon dioxide was measured by the direct method described by Umbreit, Burris and Stauffer (13). Gas exchange measurements were made at 30°C. with reaction mixtures consisting of enzyme extract, substrate and acetate buffer at pH 4.5.

Ascorbic acid analysis of the reaction mixture was carried out with 2, 6-dichlorophenolindophenol using the xylene method described by Gyorgy and Rubin (5). Absorbancy measurements were made with a Lumetron colorimeter.

Results and Discussion

Previous work on ascorbic acid oxidase from higher plants (4,9) and on Myrothecium extracts (7,8,14) had indicated that one-half mole of oxygen was consumed for every mole of ascorbate oxidized. There was no reason to expect products of the oxidase reaction other than dehydroascorbic or 2,3-diketogulonic acid (2,9). However, in studying extraction methods and reaction conditions for the mycelial enzyme, it was observed that reactions followed for an hour or more frequently showed unexpectedly high oxygen uptake. An examination of manometric

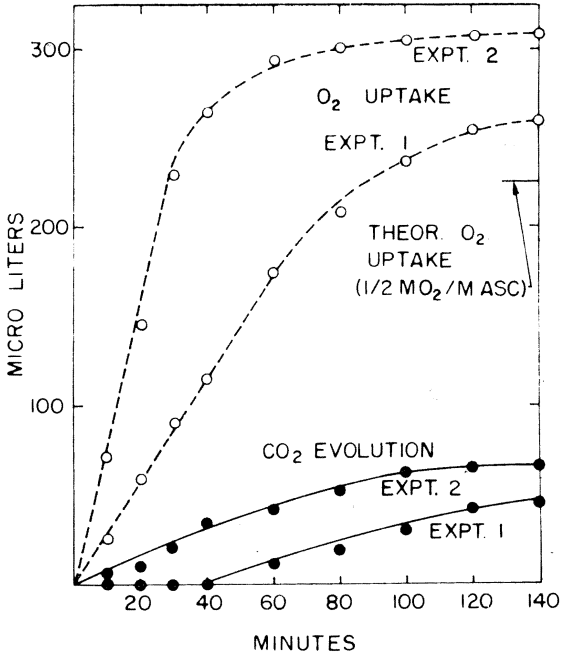

Figure 1. Oxygen uptake and carbon dioxide production of two different enzyme extracts. Expt. 1 shows a lag in carbon dioxide production which is not shown in Expt. 2. Both experiments exceeded theoretical oxygen uptake. Reaction conditions; 0.6 ml. 0.02 M acetate buffer, 0.8 ml. enzyme extract, 0.4 ml. of 0.05 M ascorbate and 0.2 ml. of 20% KOH or H_2O with a paper accordian in the center well. All flask components adjusted to pH 4.5 before assay.

Table 1. Excess oxygen uptake, carbon dioxide evolution and rates of reaction using standard assay conditions.[1]

O_2 Uptake (μl./hr.)		CO_2 Evol. (μl./hr.)		CO_2 Evolved (μl.)	Excess O_2 (μl.)
Initial	Final	Max.	Final		
268	0	62	0	82	56
270	20	70	16	112	68
300	12	30	15	73	83
320	12	30	18	37	41
340	10	90	30	89	97
348	6	46	5	76	99

[1] Reaction conditions; 0.6 ml. 0.02M acetate buffer, 0.8 ml. enzyme extract, 0.4 ml. of 0.05 M ascorbate and 0.2 ml. of 20% KOH or H_2O with a paper accordian in the center well. All flask components adjusted to pH 4.5 before assay.
[2] Excess oxygen is the amount of oxygen uptake in excess of the calculated stoichiometry which is 224 μl based on 1 mole of ascorbate per ½ mole oxygen.

results with extracts prepared in various ways and with differing reaction conditions revealed that 40 out of 45 experiments exhibited oxygen uptake in excess of calculated stoichiometry. The average excess was 86 microliters. Furthermore, carbon dioxide was produced in all experiments with an average yield of 70 microliters. Table 1 presents some representative data from these experiments. There was considerable variation among the gas exchange curves ranging between the two types illustrated in Figure 1. In some cases, such as Expt. 1, no carbon dioxide evolution occurred in the first 10 to 20 minutes; in others, such as Expt. 2, little or no lag was observed. In experiments where gas exchange was essentially complete, the mean ratio of oxygen excess over carbon dioxide production was 1.13. Again, however, a great deal of variability was present, and the significance of the ratio is in doubt.

One question of interest was whether the secondary oxidation and carbon dioxide evolution reactions accompanied or followed the primary oxidation. This question was explored in preliminary experiments by including periodic analyses of ascorbate with the gas exchange measurements. Figure 2 shows typical results. It is evident that most of the excess oxygen consumption and carbon dioxide evolution ocurred before the ascorbate was used up. However, slow gas exchange reactions continued after the primary oxidation was complete. Apparently there was no sharp separation in time between the primary oxidation attributable to the ascorbic acid oxidase and the secondary reactions.

Other factors which might affect the interpretation of the gas exchange data were considered. First, ionic copper can catalyze ascorbate oxidation with the production of hydrogen peroxide (2,3). This would change the stoichiometry if hydrogen peroxide accumulated or if it oxidized some substrate other than ascorbate. Non-enzymatic catalysis in our experiments was unlikely, however, since routine blanks with boiled extracts gave no significant oxygen uptake. Furthermore, removal of dialyzable endogenous substrates did not alter the gas exchange pattern (Table 2).

Table 2. The effect of dialysis and high speed centrifugation on the stoichiometry of the ascorbic acid oxidase reaction.[1]

Exp.		Initial Rate Oxygen Uptake (μl./hr.)	Observed Stoichiometry[2] (μl.) (theor. = 224 μl.)
Dialysis			
1.	Before	200	250
	After	180	243
2.	Before	320	325
	After	280	300
Centrifugation			
1.	20,000 x g.	480	272
	100,000 x g.	460	273
2.	20,000 x g.	368	358
	100,000 x g.	352	354
3.	20,000 x g.	330	326
	100,000 x g.	330	294
4.	20,000 x g.	362	288
	100,000 x g.	368	288

[1] Reaction conditions; 0.8 ml. 0.02 M acetate buffer, 0.8 ml. enzyme extract, 0.4 ml. 0.05 M ascorbate. All flask components adjusted to pH 4.5 before assay.
[2] Ascorbate added equivalent to 224 μl oxygen uptake on basis of 1 mole ascorbate per ½ mole oxygen.

Finally, addition of crystalline catalase to the reaction mixture had no apparent effect on the stoichiometry.

Other oxidative enzyme systems would be expected to affect the gas exchange data only if substrates other than ascorbate were present in significant amounts. Results of the dialysis experiments Table 2) appeared to rule out such interference.

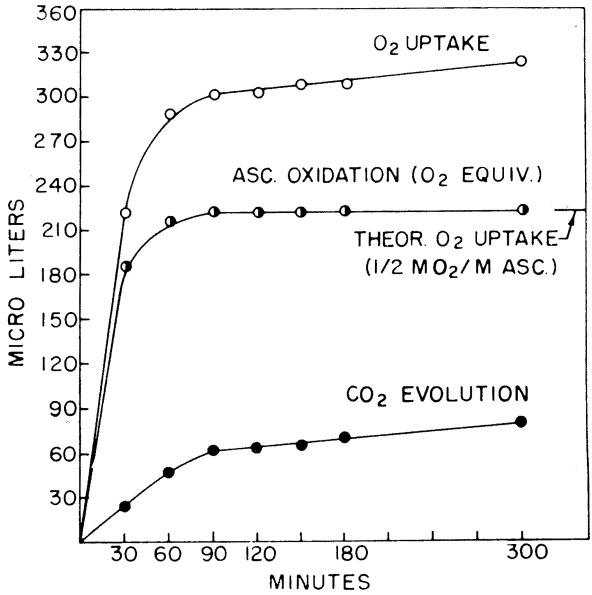

Figure 2. Manometric measurements of oxygen uptake and carbon dioxide production and assay of the disappearance of reduced ascorbate with the indophenol-xylene method expressed in oxygen equivalents.
Reaction conditions; 0.6 ml. 0.02 M acetate buffer, 0.8 ml. enzyme extract, 0.4 ml. of 0.05 M ascorbate and 0.2 ml. of 20% KOH or H_2O with a paper accordian in the center well. All flask components adjusted to pH 4.5 before assay.

Furthermore, White (14) in this laboratory found no cytochrome oxidase or phenol oxidase activity in 20,000 x g. supernatants of Myrothecium extracts. However, because of reports (12) that b-type cytochromes in submitochondrial particles can catalyze ascorbate-linked oxidation of other substrates, tests were made on supernatents certrifuged at 100,000 x g. for 40 minutes. The results presented in Table 2 show that the Myrothecium ascorbic acid oxidase is not particulate.

On the basis of evidence so far, is appears that the excess oxygen uptake and the carbon dioxide evolution observed during ascorbate oxidation by Myrothecium extracts is probably the result of further reactions of ascorbate oxidation products. The best possibility would seem to be the catabolic systems recently studied at the National Institutes of Health and the University of Tokyo. These groups have reported (6,10) enzymes which decarboxylate diketogulonic acid and oxidize one of the resulting pentonic acids. They also have evidence that this pathway may ultimately lead to glucose. In future work, we plan to examine Myrothecium extracts for the presence of these and related enzymes in an effort to elucidate the ascorbate metabolism of this organism.

Literature Cited

1. Darby, R. and D. Goddard. Studies on the respiration of the mycelium of the fungus Myrothecium verrucaria. Amer. Jour. Bot. 37: 379-387. 1950.
2. Dawson, R. The copper protein, ascorbic acid oxidase. In McElroy, W. D. and B. Glass, eds. Copper metabolism. pp. 18-47. Baltimore, Md., The John Hopkins Press, Inc. 1951.
3. Dekker, A. O. and R. G. Dickinson. Oxidation of ascorbic acid by oxygen with cupric ions as catalyst. Jour. Amer. Chem. Soc. 62: 2165-2171. 1940.
4. Dunn, F. J. and C. R. Dawson. On the nature of ascorbic acid oxidase. Jour. Biol. Chem. 189: 485-497. 1951.
5. Gyorgi, P. Vitamin methods. New York, Academic Press, Inc. 1950.
6. G. Ashwell, J. Kanfer, J. D. Smiley and J. J. Burns. Metabolism of ascorbic acid and related uronic acids, aldonic acids, and pentoses. Annals N. Y. Acad. Sci. Vol. 92, Art. 1, pp. 105-114. 1961.
7. Mandels, G. R. The properties and surface location of an enzyme oxidizing ascorbic acid in fungus spores. Arch. Biochem. 42: 164-173. 1953.
8. ———. The atypical ascorbic acid oxidase in fungus spores. Its activation by isoascorbate and its specificity. Arch. Biochem. 44: 362-377. 1953.
9. Mapson, L. W. Metabolism of ascorbic acid in plants: Part I. Function. Ann. Rev. Plant Physiol. 9: 119-150. 1958.
10. Shimazono, N. and Y. Mano. Enzymatic studies on the metabolism of uronic and aldonic acids related to L-ascorbic acid in animal tissues. Annals N. Y. Acad. Sci. Vol. 92, Art. 1, pp. 91-104. 1961.
11. Sinden, J. W., A. J. Mix and R. G. H. Siu. Effect of environment and mineral nutrition on cellulytic activity of fungi. Research report from Quartermaster General Laboratories. Microbiology series No. 8. 1948.
12. Staudinger, H., K. Kirsch and S. Leonhauser. Role of ascorbic acid in microsomal electron transport and the possible relationship to hydroxylation reactions. Annals N. Y. Acad. Sci. Vol. 92, Art. 1, pp. 212-222. 1961.

13. Umbreit, W. W., R. H. Burris and J. F. Stauffer. Manometric techniques. Minneapolis, Minn., Burgess Pub. Co. 1957.
14. White, G. A. Atypical ascorbic acid oxidase of *Myrothecium verrucaria*. Unpublished Ph.D. Thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1959.

Antibiotics Stimulating or Inhibiting Germination and Growth of Rice

ROBERT C. GOSS[1]

Abstract. This report is concerned with the effect of antibiotics on germination and translocation of native chemicals in the laboratory and on the plant's reaction to certain antibiotics used as soil drenches under greenhouse conditions.

The seeds treated with antibiotics active against Gram positive organisms were deficient in both carbohydrates and proteins. The seeds treated with antibiotics active against Gram negative organisms had a delayed germination but a normal physiological development. Streptomycin had the most adverse effect on translocation but stimulated germination. Analysis of variance of germination indicated that seed germination was dependent upon the antibiotic used. The analysis for the translocation resulted in the means being significantly different.

No adverse effects, caused by the soil treatment, were noted for germination. Neomycin, penicillin, and streptomycin appear to stimulate germination; tyrothricin and polymyxin inhibit germination the first week but by the second week have a higher germination percentage than the control.

Streptomycin, polymyxin and neomycin drenches have the most beneficial effect on the growth of Nato, Magnolia, and Upland varieties. A trigger agent is apparently released which stimulates growth from the first week until termination of the experiment. The three antibiotics inhibit the growth of Blue bonnet.

INTRODUCTION

The word antibotic has been devised to indicate a substance formed by one organism which is injurious to another organism. This phenomenon is not confined to microorganisms but is found, at least to some extent, in the higher plants and also in animals. As experience with the practical utilization of antibiotics has increased, and as more research has been made for new ones, the meaning has shifted to the point where antibiotic now means almost any substance isolated from a culture of an organism, and the activity is not only inhibitory but in some cases the substance can be growth-stimulating or growth-changing in nature.

[1] State College of Iowa, Science Department, Cedar Falls, Iowa.