Terminal Oxidases of the Mature Sugar Beet Root[1]

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Few reports concerning the identity or relative activity of the terminal oxidase enzymes of the sugar beet root have been published. Phenol oxidase has been shown to be active in the leaves of the sugar beet by Arnon (1)[3], and Dawson and Tarpley (4) also detected laccase activity. Bhagvat and Hill (2) included the sugar beet root in an extensive list of plants tested spectrophotometrically for cytochromes and cytochrome oxidase. The bands of cytochromes a, b, and c were observed but no cytochrome oxidase activity was detected.

Stout and Spikes (10) described the effects of various chemical inhibitors on the oxygen consumption of sugar beet root tissue discs. Lack of inhibition by 8-hydroxyquinoline, coupled with inhibition by cyanide and azide led the authors to the presumption that respiration in the sugar beet root is mediated by way of a cytochrome oxidase or polyphenolase rather than by ascorbic acid oxidase. No direct evidence of cytochrome oxidase participation was present, however.

The experiments described below were designed to investigate the active terminal oxidases of the sugar beet root through Q_{O_2} effects of particular inhibitors and substrates added to a standard respiratory medium, the use of carbon monoxide inhibition, and spectrophotometric techniques for the detection of cytochrome oxidase activity.

Materials and Methods

Sugar beet seed, SKE-R-11 (supplied by the British Columbia Sugar Refining Company, Vancouver, B. C.) was sown in flats in the greenhouse on February 23, 1957 and uniform seedlings planted in the field May 11, 1957. Beginning July 15, beets whose diameters were five inches or over, were taken at random for the oxidase studies.

After the roots had been cleaned and trimmed, a trans-section 3.75 cm. in height was cut from each root just below the region of greatest diameter. Trans-slices one mm. in thickness were cut from these cylinders by a Spencer hand microtome and from

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[3] Numbers in parentheses refer to literature cited.

these slices discs one cm. in diameter were cut by a steel cork borer, avoiding the periphery and the core of the beet. The discs (one cm. in diameter, one mm. thick) were thoroughly mixed in distilled water and after 30 minutes removed and rinsed with 50 ml. of the medium described below.

Twenty discs (average fresh weight ca 1.62 g., dry weight ca 0.29 g.) were blotted dry, quickly weighed, and placed in a Warburg vessel containing a total of 4.0 ml. of solution whose composition was 0.4 M sucrose, 0.05 M phosphate buffer at pH 6.8, and 0.04 M KCl. Inhibitors, selected from those suggested by James (8), when used, were included in the medium. Substrates, when used, were placed in the sidearm and added at zero time. Carbon dioxide was absorbed by 0.2 ml. of 20 percent KOH (or of 2.0 M. KCN in the cyanide inhibition trials) on fluted filter paper in the center well.

After 15 minutes equilibration, the oxygen consumption at 25° C. was determined at 20 minute intervals for periods up to two hours. The rate of shaking was 120 strokes per minute. Respiration rates were computed as microliters of oxygen consumed per hour per gram fresh and per gram dry weight of the discs. Because of their parallelism, the rates are reported on the fresh weight basis only.

In the determinations of carbon monoxide inhibition the gas atmosphere of the reaction flasks was changed using the evacuation method of Burris (3). An atmosphere of 95% CO and 5% O_2 was used and the rate of respiration in the carbon monoxide was compared with that obtained in a mixture of 95% N_2 and 5% O_2. Since CO is absorbed in small amounts by KOH, a control containing no tissue was used.

The desirability of the spectroscopic method of determining cytochrome oxidase activity has been re-emphasized by Fritz and Beevers (5). Cytochrome c was dissolved in 0.03 phosphate buffer (pH 7.3) at the rate of 0.5 mg./ml., and reduction was accomplished by adding sodium hyposulfite. The excess hyposulfite was oxidized by intermittent shaking of the solution for 20 minutes. Sugar beet root tissue was homogenized in a Potter-type homogenizer with an equal weight/volume of cold 0.03 M phosphate buffer (pH 7.3). The homogenate was centrifuged at 1000 g for five minutes and the supernatant used as the enzyme source. To 2.5 ml. of the reduced cytochrome c solution 0.5 or 0.05 ml. of the enzyme preparation was added. Measurement of absorbance at 550 mμ was made at room temperature with a Beckman Model B spectrophotometer.

The effect of pH upon ascorbic acid oxidation was followed in an attempt to determine whether an increase in oxygen consumption obtained by the addition of ascorbic acid as substrate to sugar beet root tissue discs were the result of increased activity of laccase or of ascorbic acid oxidase. Root tissue was homogenized in an equal weight/volume of cold 0.4 M sucrose and centrifuged at 1000 g for five minutes. The supernatant was used as a source of enzyme. To 2.9 mls. of a 5.0 mM solution of ascorbic acid in 0.03 M phosphate buffer over a range of pH values from 4 to 8, 0.1 ml. of the enzyme preparation was added. The changes in optical density were followed at 265 mμ with a Beckman Model DU spectrophotometer at room temperature.

Results

Manometric determinations were replicated up to four times. At least four respirometers were used for each treatment at each replication. The average Qo_2 and percentage inhibition or activation in each replication is given in the tables, but analysis of variance involved all the individual readings of the respirometers.

Inhibitors

The results obtained with the inhibitors at a concentration of 0.01 M are given in Table 1.

Cyanide and azide: In the respiratory medium containing cyanide, the oxygen uptake by the sugar beet tissue slices was reduced 92.2 percent. The inhibitions, as determined by four replications involving different beets, were 90.4, 89.9, 91.1, and 87.4 percent. The average inhibition obtained with sodium azide was 85.0 percent. Cyanide and azide strongly affect metal-containing enzymes, particularly the iron-containing cytochromes and the copper-containing oxidases. Ascorbic acid oxidase, however, appears insensitive to azide. The high sensitivity to cyanide and azide suggests that metallo-oxidases are the ones primarily active in the respiration of sugar beet root.

Diethyldithiocarbamate (Dieca): Dieca is a powerful inhibitor of the copper enzymes. The average inhibition produced by the presence of dieca in the respiratory substrate was 37.7 percent. This is positive evidence that copper enzymes are active.

Resorcinol: Resorcinol, which is a competitive inhibitor of phenolase, (polyphenoloxidase) produced a reduction of 22 percent in oxygen consumption, compared with the respiration of discs in the control medium. The value is significant at the 0.01 level.

Thiourea: In two replications, involving five respirometers each, the presence of thiourea resulted in a lowering of the

Table 1.—Effect of Inhibitors on the Respiration of Sugar Beet Root Tissue Discs. Respiration (Microliters 0_2/Hour/Gram Fresh Weight)

Inhibitor	Replicates 1 Control	1 With Inhibitor	1 % Inhibition	2 Control	2 With Inhibitor	2 % Inhibition	3 Control	3 With Inhibitor	3 % Inhibition	4 Control	4 With Inhibitor	4 % Inhibition	Average % Inhibition	Level of Significance
Cyanide	77.4	7.4	90.4	85.0	8.6	89.9	84.3	7.5	91.1	69.5	8.8	87.4	92.2	.001
Azide	67.7	11.2	83.4	72.8	10.7	85.5	77.9	13.7	86.2				85.0	.001
Dieca	54.5	32.8	40 0	62.3	39.0	37.4	60.4	38.6	36.1				37.8	.001
Resorcinol	61.4	46.6	24.1	60.6	48.6	20.0							22.0	.01
Thiourea	68.1	65.1	4.4	60.6	56.4	7.0							5.7	N.S.[1]
8-hydroxyquinoline	66.0	65.8	0.2	64.0	63.0	1.6							0.9	N.S.
CO (light)	67 0	57.5	14.0	36.4	28.4	22.0	81.1	71.9	11.0	66.9	57.8	13.0	15.0	.01
CO (dark)	67.0	60.9	9.1	36.4	27.0	26.0	81.1	68.1	16.0	66.9	57.6	14.0	13.8	.01
CO (light vs. dark)														N.S.

[1] Almost significant at the .05 level.

respiratory rate. The two average inhibition values were 4.4 and 7.0 percent, giving an over-all average of 5.7 percent. This is almost, but not quite, significant at the 0.05 level. Thiourea is an inhibitor of such copper enzymes as ascorbic acid oxidase, phenolase, and laccase.

8-hydroxyquinoline: In the two replications 8-hydroxyquinoline did not reduce the rate of oxygen consumption. The average increase of 0.9 percent lacked significance. This inhibitor is active against ascorbic acid oxidase. The result obtained, which is similar to that reported by Stout and Spikes (10), casts doubt on the presence or activity of ascorbic acid oxidase.

Carbon monoxide: Respiration rates in an atmosphere of 95% N_2 and 5% O_2 did not differ significantly from the rates in air. Compared with rates in the 95% N_2/5% O_2 atmosphere the mixture 95% CO/5% O_2 produced an average decrease in respiratory rate of 15 percent in light, and 13.8 percent in the dark. Both values are significant at the 0.01 level. No significance exists between the inhibition in light and in darkness. The results tend to question the participation of cytochrome oxidase in the respiratory sequence.

Taken together, the results obtained with the inhibitors suggest 1.) metallo-oxidases are involved in sugar beet root respiration, 2.) ascorbic acid oxidase is non-active or absent, 3.) there is no evidence of cytochrome oxidase activity and 4.) the active oxidases are probably copper-containing enzymes.

Substrates

The substrates were added to the respiratory medium at the rate of 0.5 or 1.0 mg./ml. The effects on oxygen uptake by sugar beet root discs are given in Table 2.

The laccase substrates, hydroquinone and p-phenylenediamine, increased the rate of oxygen utilization by an average of 11.5 and 30.5 percent, respectively. The values are significant at the 0.05 and 0.01 levels.

The addition of protocatechuic acid and tyrosine, substrates for phenolase, did not result in as great increments in oxygen consumption. Although the average increase occasioned by protocatechuic acid was 11.5 percent, this value just lacked significance at the 0.05 level. The average increase, 9.0 percent, produced by the addition of tyrosine was significant at the 0.05 level. Catechol, which serves as a substrate for both laccase and phenolase, produced an average increase of 32.7 percent in respiration. This was significant at the 0.01 level.

Table 2.—Effect of Substrates on the Respiration of Sugar Beet Root Tissue Discs. Respiration (Microliters O_2/Hour/Gram Fresh Weight)

Substrate	Mg./ml.	Replicates 1 Control	1 With Substrate	1 % Activation	2 Control	2 With Substrate	2 % Activation	% Average Activation	Level of Significance
Hydroquinone	0.5	65.5	70.0	7.0	63.8	73.9	16.0	11.5	.05
p-phenylenediamine	0.5	65.5	87.8	34.0	63.8	81.2	27.0	30.5	.01
Protocatechuic acid	1.0	87.6	99.6	14.0	70.5	76.8	9.0	11.5	N.S.[1]
Tyrosine	0.5	67.0	72.9	9.0				9.0	.05
Catechol	1.0	87.6	95.1	8.0	70.5	110.8	57.0	32.7	.01
Glycolic acid	1.0	73.5	71.1	−3.2	58.1	49.1	−15.1	−9.2	N.S.
Lactic acid	1.0	74.5	70.7	−5.1	82.4	81.4	−1.2	−3.7	N.S.
Ascorbic acid	1.7	45.0	98.5	118.0	87.6	166.4	90.0	104.0	.01

[1] Almost significant at the .05 level.

The above results, coupled with those obtained when inhibitors were used, strongly suggest that laccase and phenolase are active in the root of the sugar beet.

The changes in respiratory rate, a decrease of 9.2 percent with glycolic acid and a decrease of 3.7 percent with lactic acid, lacked significance. The probable absence of glycolic acid oxidase from the root tissue was thus indicated.

The increase in oxygen consumption, 104.0 percent, consequent to the addition of ascorbic acid to the respiratory medium of the root dics could signify either ascorbic acid oxidase or laccase activity, or both (11). The lack of inhibition of respiration by 8-hydroxyquinoline threw doubt on the participation of ascorbic acid oxidase. To clarify this situation the effect of pH on the oxidation of ascorbic acid by root homogenate was determined. The results are given in Figure 1.

The optimum pH for the beet root enzyme system capable of oxidizing ascorbic acid was found to be 6.6. This strongly suggests laccase was responsible, since this enzyme has an optimum pH range of 6.2 to 6.8, depending on source and purity (11). Ascorbic acid oxidase has a pH optimum of approximately 5.6. No evidence of a secondary peak in the curve at this PH was obtained.

The spectrophotometric determination gave no evidence of cytochrome oxidase activity. The change in optical density at 550 Mμ was the same in the presence and absence of enzyme preparation, *viz.* 0.05 optical density units per 10 minutes.

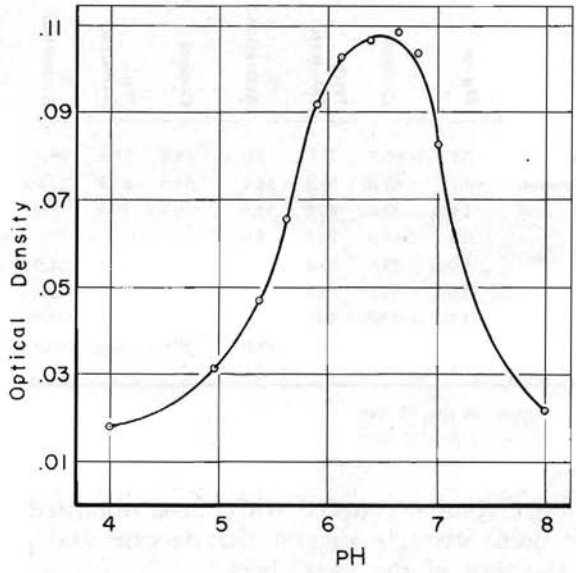

Figure 1.—Oxidation of ascorbic acid as affected by pH of sugar beet tissue homogenate. Optical density changes were followed at 265 mμ.

Discussion

The severe inhibition of oxygen uptake by sugar beet root discs which resulted from the inclusion of cyanide and azide in the respiratory medium strongly suggests that metallic oxidases are involved in the respiration of the tissue. In spite of the low level of inhibition obtained with thiourea, which may have resulted from lack of penetration of the tissue by this inhibitor, the marked inhibition produced by dieca indicates the activity of copper enzymes. While Honda (7) has shown that confusing results can be obtained with dieca, James (8) considers this chelating agent to be the most useful for distinguishing the copper enzymes from cytochrome oxidase in situ.

The inhibition obtained by the use of resorcinol, which acts primarily as a competitive inhibitor for phenolase (8), suggests that this enzyme may play a part in the respiratory process in root discs. The addition of catechol, a substrate of both laccase

and phenolase, resulted in a very considerable increase in respiration. Further evidence of the activity of these enzymes was furnished by the activation produced by p-phenylenediamine and hydroquinone, both of which are laccase substrates, and by tyrosine and protocatechuic acid, substrates of phenolase. The light stable inhibition of respiration by CO admits the possibility that phenolase may be present in the beet root tissue.

Ascorbic acid oxidase activity is questioned by the lack of response to the inhibitor 8-hydroxyquinoline, as Stout and Spikes (10) found, and to the absence of the characteristic ascorbic acid oxidase pH optimum in the oxidation of ascorbic acid by root homogenate. In this instance the optimum pH observed, pH 6.6, strongly suggests that laccase is the active oxidase.

In spite of the ubiquitous occurrence of cytochromes in plant tissue, as suggested by Smith and Chance (9), and others, the techniques used in this investigation failed to identify cytochrome oxidase as an active participant in the respiration of sugar beet root tissue. It has been emphasized by numerous writers that while light reversibility of CO inhibition of respiration shows the presence of cytochrome oxidase, the absence of light sensitivity in CO inhibited respiration is not conclusive evidence that an enzyme other than cytochrome oxidase is opertive (6, 9). The absence of cytochrome oxidase activity in beet root homogenate, as shown by the spectrophotometric technique, is difficult to explain away, however.

Summary

The results obtained by the use of numerous inhibitors and substrates of cytochrome oxidase, ascorbic acid oxidase, phenolase, laccase and glycolic acid oxidase; the absence of light-sensitive carbon monoxide inhibition; and the failure to detect cytochrome oxidase activity spectrophotometrically strongly suggest that, under the various conditions imposed by this investigation, the oxidases active in the respiration of mature sugar beet root tissue are laccase and phenolase.

Literature Cited

(1) ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24:1-25.

(2) BHAGVAT, K., and HILL, R. 1951. Cytochrome oxidase in higher plants. New Phytol. 50:112-120.

(3) BURRIS, R. H. 1957. In Manometric Techniques. eds. Umbreit, H. W., Burris, R. H., and Stauffer, J. F. Burgess Publishing Co., Minneapolis.

(4) DAWSON, C. R., and TARPLEY, W. B. 1951. In The Enzymes eds. Sumner, J. B., and Myrback, K. Academic Press. New York.

(5) FRITZ, G., and BEEVERS, H. 1955. Cytochrome oxidase content and respiratory rates of etiolated wheat and barley seedlings. Plant Physiol. 30:309-317.

(6) HILL, R., and HARTREE. 1953. Hematin compounds in plants. Ann. Rev. Plant Physiol. 4:115-150.

(7) HONDA, S. I. 1955. Succinoxidase and cytochrome oxidase in barley roots. Plant Physiol. 5:402-410.

(8) JAMES, W. O. 1953. The use of respiratory inhibitors. Ann. Rev. Plant Physiol. 4:59-90.

(9) SMITH, L., and CHANCE, B. 1958. Cytochromes in plants. Ann. Rev. Plant Physiol. 9:449-482.

(10) STOUT, M., and SPIKES, J. D. 1957. Respiratory metabolism of the sugar beet. Jour. Amer. Soc. Sugar Beet Tech. 9 (6) :469-475.

(11) SUMNER, J. B., and SOMERS, G. F. 1953. Chemistry and methods of enzymes. Academic Press. New York.