ASCORBIC ACID OXIDASE IN BARLEY ROOTS[1,2]

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Two possible terminal metallo-oxidases have been found in barley, ascorbic oxidase by James and Cragg (14) and cytochrome oxidase by Merry and Goddard (23). Studies on the intracellular distribution of various ascorbic oxidases by Mandels (21) and Newcomb (25) indicated that the enzymes are localized at the surface. Newcomb (25) has shown that in many higher plants ascorbic oxidase is attached to or situated near the cell wall. According to the reviews of Goddard and Meeuse (7) and Goddard and Stafford (8), cytochrome oxidase is localized in the mitochondria. Thus, it may be possible to distinguish between ascorbic oxidase and cytochrome oxidase by their intracellular distribution. However, there are differing views on the intracellular distribution of cytochrome oxidase in higher plants. Lundegårdh (18) postulated a coating of cytochrome oxidase on the inner surface of the cell walls and in the tonoplast membrane of wheat roots, while Butler (3) considered that it is localized both in the tonoplast membrane and in the mitochondria. Lundegårdh (18) suggested that the peripheral localization facilitates salt respiration and accumulation which have been found associated with the cytochrome system (17, 31). An added complication raised by James (12) questions the existence of a functional cytochrome oxidase in root tips from older barley seedlings. Consequently, it was of interest to determine the identity of a barley root enzyme utilizing ascorbic acid and its localization in the cell.

METHODS AND MATERIALS

Barley grains, varieties Moore and Atlas 46, were germinated following the methods of Machlis (20). The entire procedure was carried out at 28° C and 90 % relative humidity in a dark room occasionally illuminated by red light. Roots from the barley seedlings were harvested 5 days after soaking the grains. Segments 5 mm long were cut from the roots, washed with distilled water, and stored for ca 1 hour at 7° C in distilled water.

Homogenates of the root segments were prepared essentially by the method of Newcomb (25). Cell-free homogenates (25 % w/w) were obtained after grinding the roots for 3 to 5 minutes in either ice-chilled water or media initially 0.8 M tonicity and adjusted to 0.4 M final exogenous tonicity.

Centrifugal fractionations of the homogenates were carried out in a refrigerated centrifuge at 0 to 2° C. The wall fragments were resuspended three times in the appropriate medium and the fragments resedimented, for 5 minutes at $225 \times g$ if not otherwise noted. The washed preparations were then resuspended to their original volumes. Recovery of activity was determined by assay of equivalent portions of the fractionated homogenate.

Ascorbic oxidase and cytochrome oxidase were assayed by Warburg manometric procedures. Temperature equilibration was carried out for 30 minutes before the assay. Substrates and other reagents were then tipped into the main vessel compartments which contained 0.5 ml enzyme preparation, unless otherwise specified. Concentrations of reagents before and after substrate addition were maintained constant. Exogenous inorganic ions were usually excluded. For example, ascorbate was added as the acid form and not as the sodium or potassium salt. This accounts for the low pH in experiments completed before tris-hydroxymethyl-amino-methane (Tris.), an organic base, was available. In later experiments sufficient organic base was added to maintain the pH. The final pH values of the assay solutions after the last manometer readings were determined with glass-calomel electrodes.

The activities quoted are corrected for non-enzymatic oxidation of substrates as computed from similar treatments either without the enzyme preparations or with heat inactivated preparations. No effects of tonicity on the activity were found. Consequently, precautions were not taken to assay activity under a given tonicity.

Preparations of various ages were employed since the homogenate was relatively stable with more than 70 % of the activity remaining after 10 days at 7° C.

The gases for experiments on carbon monoxide effects were commercial oxygen and nitrogen, and carbon monoxide generated from formic acid and washed through 6 M KOH. Gas mixtures were made up in 20-liter bottles with a gas displacing solution saturated with Na_2SO_4 and made acid to methyl red with sulphuric acid. The manometer vessels were gassed with 1 liter of the appropriate mixture while being shaked in the temperature bath.

The light source for the attempted reversal of carbon monoxide inhibition was a tungsten filament cylindrical bulb of intensity 1×10^2 fc at the manometer vessel position.

The nitrogen estimations were carried out according to the modified Nessler procedure of Umbreit et al (29).

EXPERIMENTAL RESULTS AND DISCUSSION

ASCORBIC OXIDASE IN ROOT HOMOGENATES: Stimulation by cytochrome c of an enzymatic oxidation of ascorbic acid may be considered as evidence for the presence of cytochrome oxidase. (In the assay for cytochrome oxidase, ascorbic acid is preferred by Slater (27) to hydroquinone or *p*-phenylenediamine

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because the latter produce toxic effects and non-enzymatic oxidations after their initial dehydrogenations. It has been reported by Friedkin and Lehninger (6) that oxidative phosphorylations do not occur with hydroquinone or *p*-phenylenediamine whereas a low rate of phosphorylation accompanies the oxidation of ascorbic acid by cytochrome.) An oxygen uptake by some root homogenates stimulated by cytochrome c suggested the presence of cytochrome oxidase (fig 1). The total oxygen uptake in this experiment was less than the oxidative equivalent of the added ascorbate, being 87 % and 91 % of the expected, with and without cytochrome c, respectively. In other experiments oxygen uptake equivalent to a complete oxidative reaction was obtained. Many other preparations showed only a high native activity with ascorbic acid and no extra activity with cytochrome c. Therefore, it was questionable whether the activity could be ascribed solely or even partially to cytochrome oxidase. A lack of stimulation owing to a sufficiency of a bound-cytochrome c system was unlikely. No stimulations were observed with these preparations which were homogenized in water, a treatment almost certain to elute cytochrome c from the cytochrome system. Preliminary experiments thus showed in all cases a marked native "ascorbic oxidase" and in some cases an additional "cytochrome oxidase" activity.

TABLE I

UTILIZATION OF SUBSTRATES BY ASCORBIC OXIDASE IN THE GROSS HOMOGENATE

TREATMENT *	μL O_2 UPTAKE/60 MIN
Ascorbate **	86
Ascorbate + cyt c	125
p-Phenylenediamine **	1
p-Phenylenediamine + cyt c	2
Hydroquinone **	2
Hydroquinone + cyt c	+7

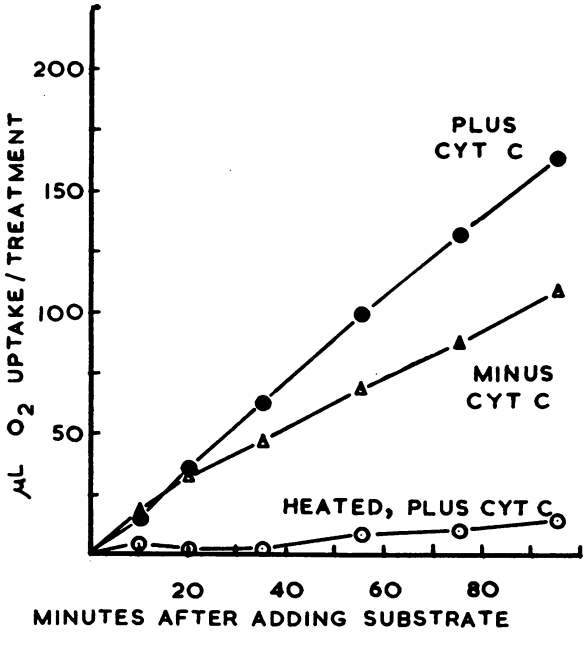
Moore roots were homogenized in 10 % gum ghatti + 0.1 M mannitol (38 % w/w, fresh wt). Activities of the fresh preparation (Expt. R-150 B) were assayed at final pH 6.7 to 6.9 and 25° C.

* Additional reagents were K-maleate, 4.6×10^{-2} M, adjusted to pH 7, and cyt c, 1.0×10^{-4} M.

** Concentration in 3.5 ml final volume was 6.4×10^{-3} M.

Characterization of the enzymatic activity was carried further by testing the substrate specificity, inhibition by copper enzyme and iron enzyme poisons, and the pH tolerance of the enzyme during homogenization.

Many preparations utilized only ascorbic acid as substrate (table I). These data suggest the absence of active cytochrome oxidase and constitute positive evidence for ascorbic oxidase. The stimulation by



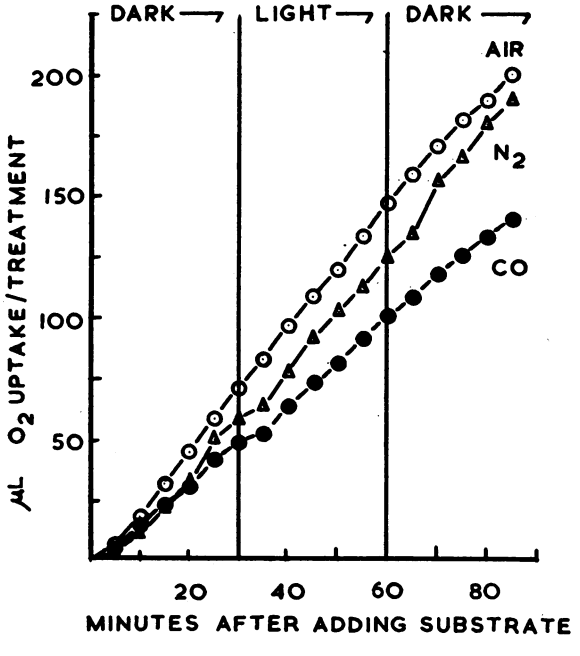


FIG. 1 (*left*). Apparent "cytochrome oxidase" in the gross homogenate. Moore roots were homogenized in water and yielded a 22.7 mg dry wt/ml gross homogenate. Activities of the 4-day-old homogenate (Expt. R-51) were tested at final pH 5.0 and 25° C. Substrate in 2.5 ml final volume was ascorbic acid, 7.5×10^{-3} M. Additional reagents were glucose, 2 %; cytochrome c, 1.7×10^{-5} M; and nicotinamide (NA), 1.0×10^{-2} M.

FIG. 2 (*right*). Inhibition of ascorbic oxidase in the gross homogenate by carbon monoxide. Moore roots were homogenized in water and yielded a 22.5 μM N/ml gross homogenate. Activities of the 8-day-old homogenate (Expt. R-174) were tested at final pH 5.8 to 5.9 and 20° C. Substrate in 2.0 ml final volume was ascorbic acid, 1.0×10^{-2} M. Additional reagents were maleic acid, 2.2×10^{-2} M; and TRIS, 2.4×10^{-2} M. Gas mixtures were air, N_2 90 % + O_2 10 %, and CO 90 % + O_2 10 %.

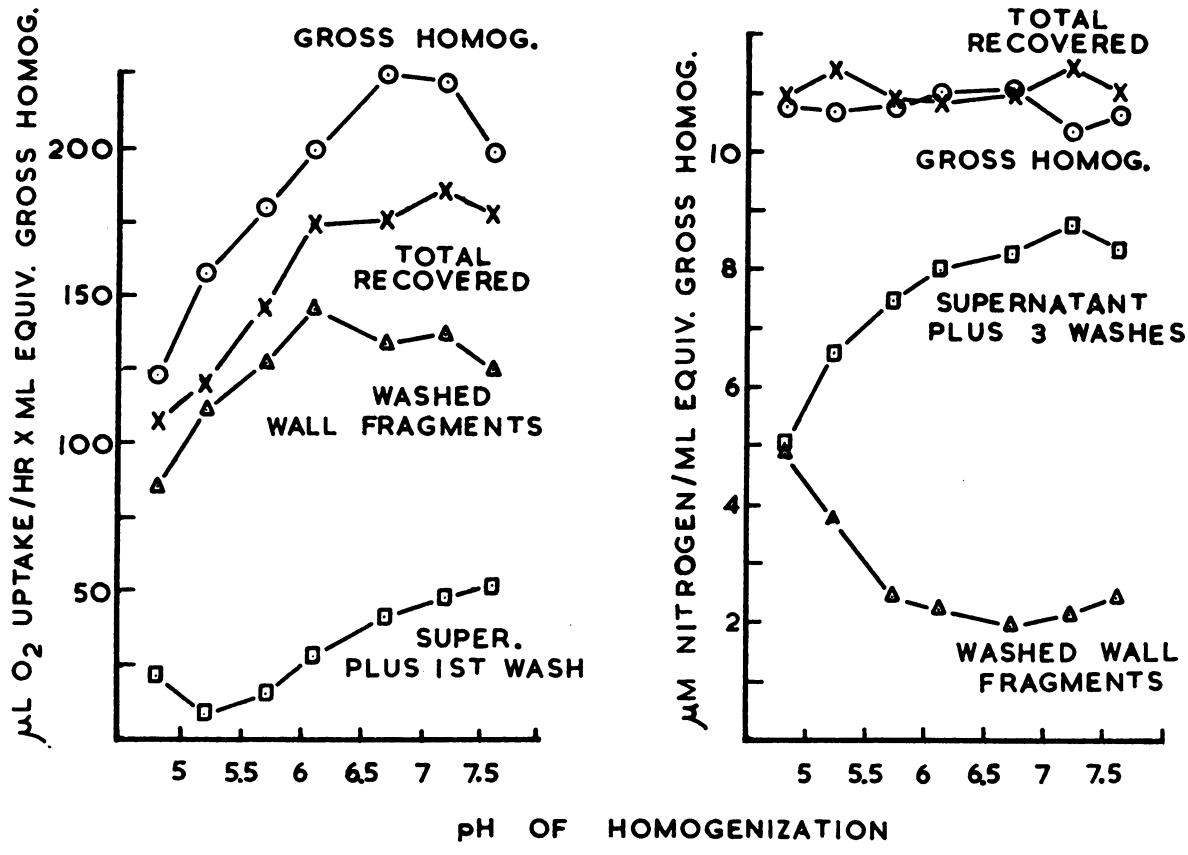


FIG. 3. Effect of pH of homogenization on the partitioning of ascorbic oxidase and nitrogen content. Atlas 46 roots, 390 segments of 10 mm per treatment, were homogenized in 0.2 M phosphate buffer and diluted to 0.1 M phosphate buffer. Fractionation was carried out in the usual manner. Activities of the fresh preparations (Expt. H-245) were tested at final pH 5.5 to 5.9 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 1.1×10^{-3} M. Additional reagents were maleic acid, 8×10^{-2} M; and TRIS, 1.2×10^{-1} M.

cytochrome c (table I) was apparently not specific since ferric tartrate also accomplished equal, if not greater results. The action of tartrate, and perhaps of cytochrome c, presumably was to remove inhibitory substances by complex formation (cf. the effects obtained with complexing agents by Altman and Crook (1)). Perhaps the cytochrome c stimulations were non-specific protein effects. Thus, the difference shown in figure 1 may have resulted from a stabilization of ascorbic oxidase rather than a stimulation of cytochrome oxidase.

Activity of the gross homogenates was decreased by carbon monoxide to 80 % of the nitrogen control. This inhibition was not reversed by light (fig 2). The lack of reversibility by light is evidence against this activity being mediated by cytochrome oxidase. The ascorbic oxidase studied by Matsukawa (22) similarly possessed a sensitivity to carbon monoxide which was not light reversed.

Copper complexing agents such as azide, sodium diethyldithiocarbamate (DDC), cyanide, and 8-hydroxyquinoline (8-OH-Q) inhibited the activity (see fig 6 for the type of results obtained). The amount of DDC present in the reaction mixture was not enough to compensate for oxygen uptake by its decomposition into carbon-oxy-sulfide under acidic conditions (15). Lack of inhibition by *p*-nitrophenol at 1.33×10^{-4} M apparently rules out tyrosinase as a possible enzyme present sensitive to 8-OH-Q or DDC.

The pH tolerance of the enzyme was determined while studying the effects of pH on the fractionation of homogenates. Above and below the pH range 6.7 to 7.2 the enzyme was inactivated to various degrees as shown by assay in the optimal pH range (see activities of the gross homogenates, fig 3). The optimal pH region for homogenization is distinct from that for activity which occurs between pH 5 and 6. Inactivation by low pH is one property of ascorbic oxidase (5). Consequently, the type of cytochrome c stimulation, or maintenance of activity, shown in figure 1 may have resulted from a protective action against the acidic conditions of the assay.

The above results, although individually inconclusive, all point to ascorbic oxidase as the source of activity towards ascorbic acid. The most convincing single point for this is the enzyme specificity for ascorbate.

CYTOCHROME OXIDASE IN ROOT HOMOGENATES:

The failure to detect cytochrome oxidase in the majority of preparations may have resulted from several factors.

Cytochrome oxidase may be present in such low amounts in roots that under conditions of the assay 0.5 ml of the gross homogenates, representing at most 380 mg of roots, was not sufficient to exhibit activity. A masking of cytochrome oxidase by a greater activity of ascorbic oxidase is possible.

The enzymes may have been inactivated by osmotic fluctuation, inhibitor action, or by acidic conditions. Osmotic fluctuations may have had little effect, although the mitochondrial system is noted for its tonicity requirements for activity (16, 19). No precautions against osmotic variations were taken in the majority of experiments since chemical reduction of cytochrome c obviates the need for better structural integrity of the mitochondrion in the cytochrome oxidase assay. On the other hand, both inhibitor action and especially the acidic conditions probably contributed to cytochrome oxidase inactivation. An inhibitor of cytochrome oxidase has been detected in preparations of particles from barley roots (10). The acid pH of homogenization and assay may have inactivated cytochrome oxidase to a greater extent than ascorbic acid. In this event the amount of tissue used would have been critical for demonstration of cytochrome oxidase. It should be reiterated that the system was studied as far as possible without exogenous salts. Consequently, in many preparations the absence of buffer determined the acidic conditions of homogenization and assay unfavourable for cytochrome oxidase.

A possible transient cytochrome oxidase in barley may have been a reason for erratic detection of the enzyme. James (12) did not find the cytochrome system in root tips of 10-day-old barley seedlings either by spectrophotometric means or enzyme inhibitor studies. On the other hand, cytochrome oxidate was detected in root tips from 3-day-old barley by James (13). The development of roots from the 5-day-old barley used in the experiments may have been on the borderline for the amount of enzyme detectable by the assay. The possible transcience is likely to be different from that of succinoxidase isolated by Goodwin and Waygood (9) from barley seedlings since cytochrome oxidase was always detected in their preparations. It is possible also that the bulk of their cytochrome oxidase is from the shoot and not from the barley root.

A situation similar to that for barley exists for older wheat roots from which Waygood (30) and Butler (4) were not able to extract an active preparation of cytochrome oxidase. The difficulty appears to be one of method since Lundegårdh (18) has shown spectrophotometrically the existence of a functioning cytochrome system in intact wheat roots.

The technique for extraction and assay of Millerd et al (24) applied to 5-day-old roots yielded a "mitochondrial" preparation which on an absolute oxygen uptake basis was weakly active. However, it possessed a $Q_{O_2}(N)$ of 130 with succinate and 280 with *p*-phenylenediamine plus cytochrome c. It is clear that under certain conditions of extraction and assay, cytochrome oxidase may be demonstrated in roots from 5 day old seedlings. A method for extraction and assay for cytochrome oxidase and succinoxidase from roots of older barley of another variety is reported separately (10).

Localization of Ascorbic Oxidase: As Newcomb (25) found for many higher plants, ascorbic oxidase of barley root cells was peripherally localized. Three lines of evidence were obtained from barley roots in corroboration of Newcomb's findings: (1) the partitioning of nitrogen content and ascorbic oxidase by homogenization and centrifugal fractionation; (2) the change in recovery of activity with the wall fraction induced by plasmolysis of the root cells; and (3) the effect of external solution pH upon ascorbate-induced oxygen uptake of root segments.

If homogenization does not change the distribution of enzymes in situ, then centrifugal fractionation should reveal the localization of ascorbic oxidase. The

Table II

Recovery of Ascorbic Oxidase in the Cell Wall Fraction

Expt.	Fraction	Activity recovery %	μM N/ml	$Q_{O_2}(N)$
R-160 Moore	Gross *	...	31.7	1070
"	Wall fragments, unwashed	90	16.5	1890
R-171 Moore	Gross *	...	18.7	965
"	Wall fragments, 3× washed	98	8.6	2070
R-167 Moore	Gross **	...	22.5	1320
"	Wall fragments, 3× washed	100	6.6	4700
HR-216 Moore	Gross **	...	30.2	1280
"	Wall fragments, 3× washed	91	3.6	9800
HR-220 Moore	Gross **	...	11.9	2040
"	Wall fragments, 5× washed	70	3.0	5670
HR-239 Atlas 46	Gross **	...	10.3	751
"	Wall fragments, 3× washed	74	0.9	6360
HR-242 Atlas 46	Gross **	...	16.0	772
"	Wall fragments, 7× washed	74	5.4	1690

* Homogenized in mannitol.
** Homogenized in water.

ascorbic oxidase activity of the gross homogenate was found to be easily sedimented under low centrifugal forces. The recovery of activity with the wall fragments ranged from 79 to 94 % under 9 to 560 × g, respectively. The high and relatively constant recovery of activity, although the nitrogen content of the wall fraction itself was low and variable (table II), is evidence for the peripheral localization. The specific activities varied presumably because different amounts of nitrogen other than that of ascorbic oxidase were removed from the wall. However, under the conditions of the homogenization ascorbic oxidase itself may be adsorbed from the cytoplasm onto the wall fragments through an isoelectric or precipitation effect. Wildman and Jagendorf (32) have pointed out the precipitating effect of low pH which may occur during the mixing of the vacuolar contents with the cytoplasm.

To determine a possible isoelectric effect, barley roots were homogenized in phosphate buffers of various pH values and the distribution of activities and nitrogen in the various fractions were determined. Enzyme assays were made in the optimal range of pH which was previously determined. To compare strictly the pH effect only root segments of similar development were used, i.e., segments from the same regions measured from the root apex. An isoelectric effect is characterized by a minimum protein solubility near the isoelectric point and increased solubility on both sides of it. The absence of a peak of recovery of nitrogen with the wall fragments probably is evidence against an isoelectric effect (fig 3). The data do show an acid-induced precipitation of nitrogen containing substances on the wall fragments but the trend is opposite to that for recovery of activity. However, the nitrogen content is a relatively gross means of detecting distribution changes affecting a single enzyme. Thus, the high recovery peak of activity and the low peak in the non-wall fractions may indicate a displacement of ascorbic oxidase. Nevertheless, if an isoelectric effect were present it was apparently negligible since these peaks do not coincide and especially as a relatively constant percentage of recovery was found in the wall fractions. There was a small but sharp increase in recovery between pH 6.7 and 6.1 from ca 60 to 70 % of the gross homogenate activity. Above and below this pH range, enzymatic recovery was constant at the percentage noted. The impracticability of determining the activity of the second and third wash solutions contributed to an incomplete recovery in the various fractions.

Waygood (30) found that dilute salt solutions eluted ascorbic oxidase from the residue of the centrifuged homogenate of wheat seedlings. The residue undoubtedly contained cell wall fragments. The effect noted by Waygood possibly was superimposed upon the effect of pH. The enzyme recovery in the barley root wall fractions was ca 90 % for homogenates prepared in water compared to the 70 to 60 % recovery for homogenates prepared in phosphate buffers at the corresponding pH (between pH 6 and 6.5).

TABLE III

EFFECT OF PLASMOLYSIS IN LOWERING THE RECOVERY OF ASCORBIC OXIDASE WITH THE CELL WALL FRAGMENTS OF ROOTS

FRACTION	MEASUREMENTS	SOAK TREATMENT	
		WATER	1.5 M GLUCOSE
Gross homogenate	μM N/ml	11.2	11.4
	$Q_{O_2}(N)$	1100	979
Wall fragments, 3 × washed	μM N/ml	4.2	4.0
	$Q_{O_2}(N)$	2650	2000
	Activity recovery	90 %	72 %

Moore roots were presoaked in 1.5 M glucose or in water for 1 hr, rinsed, and then homogenized in water. The wall fragments were sedimented under 125 × g for 5 min. Activities of the fresh preparation (Expt. HR-231) were assayed at final pH 5.7 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid 1.0×10^{-2} M. Additional reagents were maleic acid, 4×10^{-2} M, and TRIS, 6×10^{-2} M.

The high recovery with the cell wall fragments, in spite of the salt elution, and the absence of a significant isoelectric effect in the fractionation of homogenates suggest the peripheral localization of ascorbic oxidase.

If ascorbic oxidase were peripherally localized any treatment which separates the cytoplasm from the cell wall prior to homogenization should tend to decrease the recovery of activity with the wall fractions. However, if current concepts of the intermingling of peripheral cytoplasm with the cell wall are correct (26) plasmolysis should not greatly affect the distribution of ascorbic oxidase. The data in table III show that a hypertonic soak treatment of the root segments resulted in a decreased yield with the cell wall fragments. The small decrease in recovery is in accord with an intimate association of ascorbic oxidase with the cell wall. A coincidence to note is that the decrease in recovery is of the same order of magnitude as the salt elution effect, about 20 %.

The surface localization of an enzyme subjects it to a greater influence of the external environment. As Mandels (21) proposed, the pH of external solution would thus markedly affect a surface localized ascorbic oxidase. The evidence of Hope (11) and Butler (3) have affirmed the earlier conclusion of Brooks and Moldenhauer-Brooks (2) as to the freely permeable character of the cell cytoplasm to ions. However, the cytoplasmic proteins probably are capable of buffering externally added hydrogen ions. Consequently, an ascorbic oxidase activity of intact roots as a function of the external solution pH may be regarded as evidence for the surface localization of ascorbic oxidase. In the comparison of roots for the

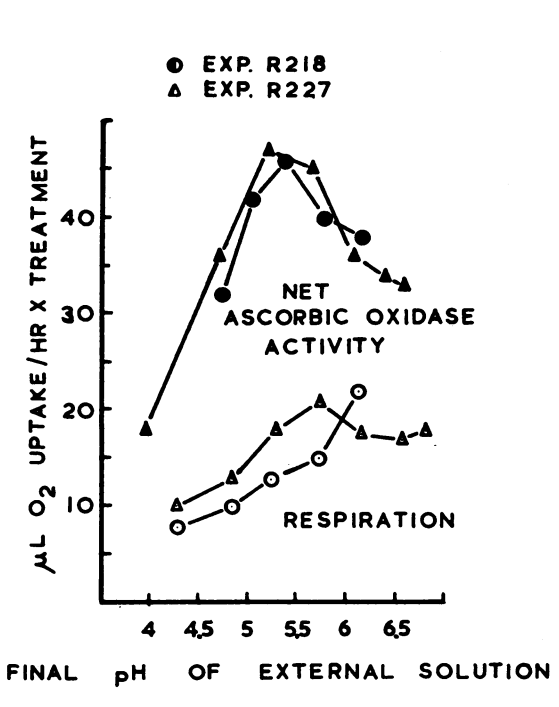
pH effect, only segments from similar regions were tested. As shown in figure 4 oxygen uptake by roots supplied with ascorbate was greatly affected by external solution pH. Moreover, the optimal pH range was within 5.0 to 6.6 which has been reported as optimal for various ascorbic oxidase preparations (5). The pH is not considered to act through its effect on the permeability of ascorbic acid. The mono-nodal activity-pH curve cannot be predicted from a consideration of pH, the pK of ascorbic acid, and the effect of pH on the weak electrolytes of the cytoplasm. Thus, the activity-pH relationship agrees with a peripheral localization of ascorbic oxidase. The assumption is made that the activity is due substantially to ascorbic oxidase and is not, for example, a consequence of ascorbate reaching cytochrome oxidase.

The peripheral localization of ascorbic oxidase found by Newcomb (25) for many higher plants is supported by the lines of evidence given above. A surface localized enzyme is not without precedent. The failure to localize ascorbic oxidase prior to the efforts of Newcomb can probably be attributed to the lack of interest in the enzymatic properties of the wall fragments and to the fortuitous release upon homogenization of ascorbic oxidase sufficient to provide an enzyme source.

It appears that substantially all the ascorbic oxidase is associated with the cell wall in barley roots which were soaked in water prior to homogenization. Salt solutions can elute ascorbic oxidase from the cell periphery. It was not established that the elution occurred after homogenization. It may be possible that elution in vivo occurs.

Identification of the Wall-bound Ascorbic Oxidase: It has been tacitly assumed in the previous section that the activity isolated with the wall fragments is attributable to only the ascorbic oxidase in the gross homogenate. However, active cytochrome oxidase on the wall would give similar results. The properties of the wall-bound enzyme were examined and found to agree with those of ascorbic oxidase. The pH optimum for activity conformed to the previously reported optimum for ascorbic oxidase (5) and the activity showed no cytochrome c stimulation even with preparations homogenized in water (fig 5). The system was inhibited by copper complexing agents (fig 6).

One property of the wall-bound enzyme differs



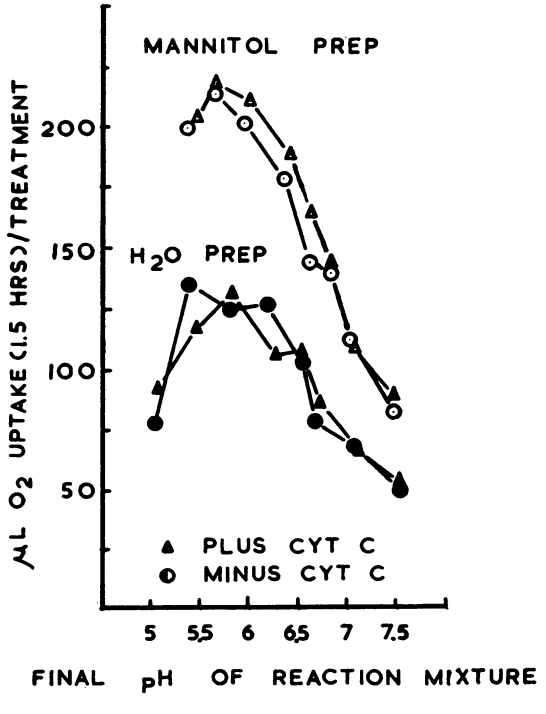


Fig. 4 (*left*). Effect of external solution pH on oxygen uptake by root segments supplied with ascorbic acid. Moore roots were stored 2 to 3 days in distilled water at 7° C. Activities of 175 segments of 5 mm (Expt. R-218) and 60 segments of 10 mm (Expt. R-227) per treatment were tested in 2.5 ml final volume at 25° C. The assay medium consisted of ascorbic acid, 6.9×10^{-3} M; TRIS, 6.6×10^{-3} M; and various amounts of a TRIS-maleate buffer ranging from 4.0 to 7.5×10^{-2} M TRIS and maleic acid 1.9 to 4.0×10^{-2} M.

Fig. 5 (*right*). pH activity curve of the wall-bound ascorbic oxidase. Moore roots were homogenized in water and 0.8 M mannitol. Nitrogen content of the water preparation was 2.2 μM N/ml wall fraction. Activities of 4- and 5-day-old preparations (Expt. R-176) were tested at 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 2.3×10^{-2} M. Additional reagents were cyt c, 1.0×10^{-5} M; and TRIS-maleate buffer ranging from 1.23 to 1.99×10^{-1} M TRIS and 3.6 to 10.0×10^{-2} M maleic acid.

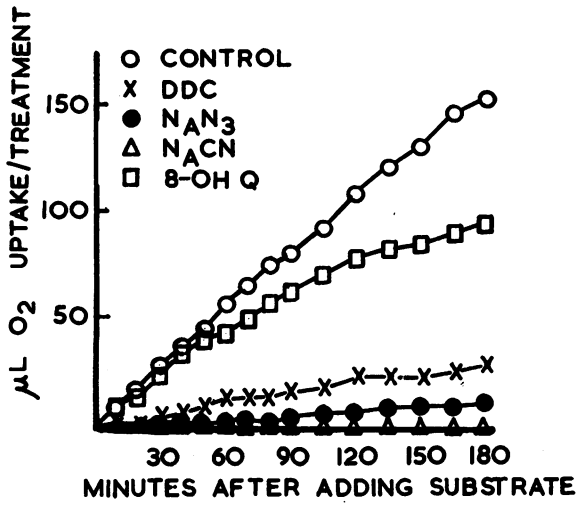


FIG. 6. Inhibition by copper complexing agents of the wall-bound ascorbic oxidase. Moore roots previously soaked in 1.5 M glucose for 1 hr were homogenized in water. The wall fraction was sedimented under $125 \times g$ for 5 min and contained 4.0 μM N/ml wall fraction. Activities of the 1-day-old preparation (Expt. HR-232) were tested at final pH 5.4 to 5.7 and 25° C. Substrate in 1.5 ml final volume was ascorbic acid, 1.1×10^{-2} M. Additional reagents were maleic acid, 4×10^{-2} M; TRIS, 6×10^{-2} M; sodium diethyldithiocarbamate (DDC), 1.0×10^{-3} M; 8-hydroxy-quinoline (8-OH-Q), 1.0×10^{-3} M; NaCN, 1×10^{-3} M; and NaN_3, 5×10^{-4} M.

from soluble ascorbic oxidase found by Steinman and Dawson (28) characteristically to be inactivated during the oxidation of ascorbic acid. The wall-bound enzyme was found to be only slightly less active after a second addition of ascorbate after a previous 1-hour reaction period. The $Q_{O_2}(N)$ decreased from 3750 to 3610 and from 2070 to 1710 when prepared in water and mannitol, respectively. Extreme sensitivity of soluble ascorbic oxidase possibly may be induced by change in structure which is caused by solubilization.

In addition to displaying the properties of ascorbic oxidase, the wall-bound enzyme was not able to catalyze the oxidation of *p*-phenylenediamine or succinate, both with cytochrome c added. No activity was found under conditions in which the cytochrome system would be expected to oxidize these compounds, e.g., hypertonic conditions of homogenization and assay, pH of assay near 7, addition of adenosine-triphosphate, magnesium chloride, and cytochrome c.

It is concluded that the wall-bound enzymatic activity cannot be ascribed to cytochrome oxidase but only to ascorbic oxidase. The lack of evidence for the peripheral localization of cytochrome oxidase contrasts with Lundegårdh's suggestion. In accordance with his views it would be unexpected to find no cytochrome oxidase attached to the wall fragments and instead to find it present on the cellular debris, as alleged to result after homogenization (18). Lundegårdh's evidence for a peripheral localization of cytochrome oxidase is indirect. Much weight is given to the observation of a rapid inhibition of respiration by cyanide and an oxidation of cytochrome c in solutions bathing wheat roots (18).

SUMMARY

Ascorbic oxidase has been identified by its various properties in the homogenates of barley roots.

The retention of activity with the cell wall fragments as the pH of extraction varies, the optimal pH range of external solution for oxygen uptake by root segments supplied with ascorbic acid, and the recovery of activity after plasmolysis of the barley root cells affirm the previously reported association of ascorbic oxidase with the cell walls of various plants.

No evidence was found to suggest that the wall-bound activity was due to cytochrome oxidase.

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