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A CRITICAL STUDY OF THE INTRACELLULAR DISTRIBUTION OF ASCORBATE OXIDASE AND A COMPARISON OF THE KINETICS OF THE SOLUBLE AND CELL-WALL ENZYME

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Abstract—Ascorbate oxidase is present in both the soluble and cell-wall preparations from many plant tissues, and can be distinguished by its kinetic characteristics from other catalysts of ascorbate oxidation. The soluble and cell-wall ascorbate oxidases from cabbage leaves have the same Michaelis constant (10^{-2} M) and optimal pH (6·0) but the cell-wall enzyme has a higher energy of activation and is less sensitive to inhibition by 8-hydroxyquinoline, azide, and diethyldithiocarbamate than the soluble enzyme. The cell-wall enzyme cannot be efficiently released by incubation with 0·15 M NaCl, 0·15 M $CaCl_2$, 1·5 M sucrose or 5% deoxycholate. Incubation of the walls with cellulase releases some activity. Up to 50% of the ascorbate oxidase of barley root-tips is freely accessible to ascorbate in medium bathing the roots and is postulated to be located *in vivo* in the cell walls. It is concluded that the ascorbate oxidase activity of cell-wall and soluble fraction from cabbage leaves is attributable to the same enzyme, and that *in vivo* the majority of the ascorbate oxidase is located in the cell walls. The data do not exclude the possibility that the remainder, recovered *in vitro* in the supernatant, may *in vivo* be very loosely associated with some subcellular particle.

INTRODUCTION

ASCORBATE oxidase (E.C. 1.10.3.3) activity has been detected frequently in the soluble fraction from plant tissues,[1] less often in the cell debris.[2,3] It has not been identified in any other fraction. The distribution of activity between the two fractions varies with the type of tissue; in general more is associated with the cell wall in preparations from mature tissues than from young tissues.[4] The presence of an activity in two fractions from a cell-free preparation does not exclude the possibility that *in vivo* the enzyme is wholely associated with one site, since during processing some activity could be lost from that site and appear in a second subcellular fraction.

If the activity is located in two sites *in vivo* both the activities may be due to one enzyme or kinetically distinct enzymes may be responsible for the activity in the different sites. There have been few detailed studies of either the localization of ascorbate oxidase *in vivo*[2,3,5] or of the comparative kinetics of an enzyme associated with both the cell wall and soluble fractions.[6] We have therefore determined the kinetics of the ascorbate oxidase of the soluble and cell-wall fractions prepared from leaves of cabbage (*Brassica oleracea*) and carried out an investigation into the intracellular location of the enzyme in both leaf and root tissue.

[1] W. D. BONNER, *Ann. Rev. Plant Physiol.* **8**, 427 (1957).
[2] S. I. HONDA, *Plant Physiol.* **30**, 174 (1955).
[3] H. M. VINES and M. F. OBERBACHER, *Plant Physiol.* **38**, 333 (1963).
[4] V. S. BUTT and M. HALLAWAY, Proc. Intern. Cong. Biochem. 4th 1958, Vienna, Vol. **15**, 144 (1960).
[5] D. MERTZ, *Am. J. Bot.* **48**, 405 (1961).
[6] J. L. HALL and V. S. BUTT, *J. Exp. Bot.* **19**, 276 (1968).

The results of the comparison of the kinetics of soluble and insoluble (cell-wall) ascorbate oxidase are relevant, also, in assessing the validity of using enzymes synthetically attached to an insoluble matrix as models of naturally occurring particle-bound enzymes.[7–9]

RESULTS AND DISCUSSION

Presence of Ascorbate Oxidase in Subcellular Fractions From Cabbage Leaves and Barley Roots

About 70% of the ascorbate oxidizing activity of a homogenate of cabbage leaves, and 80–100% of the activity of a homogenate of barley root-tips is recovered in the cell-wall fraction (Table 1), and less than 10% of the activity of the cell-wall supernatant is sedimented

TABLE 1. DISTRIBUTION OF ASCORBATE OXIDASE ACTIVITY IN PREPARATIONS FROM CABBAGE LEAVES AND BARLEY ROOT-TIPS

Experiment No.	Ascorbate oxidase activity (μl O_2/hr/100 mg tissue or 50 root tips)			
	Homogenate	Cell walls	Supernatant	Recovery (%)
(*a*) Cabbage leaves				
1	270	183 (68%)	92 (34%)	102
2	135	94 (69%)	45 (33%)	103
(*b*) Barley root tips				
3	384	262 (68%)	78 (20%)	89
4	4-day old 352	288 (81%)	—	—
5	14-day-old 293	304 (103%)	—	—

The leaf or roots were homogenized in 0·067 M, pH 6·0, phosphate buffer (1 ml/0·1 g tissue). The homogenates were centrifuged at 500 *g* for 5 min. The sediment was resuspended in phosphate buffer. Ascorbate oxidase activity determined under standard conditions. Figures in brackets are % activity of homogenate.

by centrifugation at 100,000 g for 30 min. Microscopic examination of the cell-wall fraction shows it is composed of fragments of wall and xylem strands, it contains no intact cells, chloroplasts or starch grains. The nitrogen content of the preparations ranged from 0·3% dry wt. to 0·9% dry wt., the higher level being in the walls from younger tissues. This content is at the lower end of the range reported in the literature (0·4–2%)[10] and indicates that cytoplasmic contamination of the preparations can only be slight.

Ascorbic acid is, however, readily oxidized at pH values below 6·5 in the presence of copper ions, free or loosely bound to protein, or of phenolase in the presence of a trace of dihydroxy phenol, as well as by a specific enzyme.[11–13] Above pH 6·8 the uncatalysed aerobic oxidation is rapid. The systems active below pH 6·5 can be distinguished from each other by

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[9] J. H. SILMAN and E. KATCHALSKI, *Ann. Rev. Biochem.* **35**, 873 (1966).
[10] D. T. A. LAMPORT, *Advan. Botan. Res.* **2**, 151 (1965).
[11] C. R. DAWSON, in *Copper Metabolism* (edited by W. E. MCELROY and B. GLASS), p. 18, Johns Hopkins Press, Baltimore (1950).
[12] V. S. BUTT and M. HALLAWAY, *Biochem. J.* **69**, 20 P (1958).
[13] C. R. DAWSON, in *The Biochemistry of Copper* (edited by J. PEISACH, P. AISEN and W. E. BLUMBERG), p. 305, Academic Press, New York (1966).

their response *a* to changes in ascorbate concentration and *b* to the presence of ethylene-diaminetetra-acetic acid (EDTA) or catechol[12] (Table 2). Ascorbate oxidase, prepared from

TABLE 2. CHARACTERISTICS OF ASCORBATE OXIDIZING ACTIVITY OF VARIOUS PREPARATIONS

Preparation	μl O_2 taken up/hr				
	Additions				
	4 mM HA^-	4 mM HA^- +2 mM EDTA	40 mM HA^-	40 mM HA^- +2 mM EDTA	4 mM catechol
Ascorbate oxidase, 0·5 ml (from *C. pepo*)	214	220	218	212	0
12×10^{-9} moles $CuSO_4$ + 20 mg gelatin	28	0	81	0	0
Lettuce seedling homogenate (200 mg)	271	625	380	630	880
Cabbage leaf cell wall	187	180	182	186	0
Cabbage leaf soluble fraction	80	74	82	78	0
Barley root cell wall	268	262	272	284	0
Barley root soluble fraction	71	73	74	78	0
Vegetable marrow cell wall	102	101	102	114	0
Vegetable marrow soluble fraction	324	334	336	342	0

O_2 uptake measured under standard conditions; values for cabbage, barley and vegetable marrow are for preparation from 100 mg tissue. (HA^- = ascorbate.)

vegetable marrow (*Cucurbita pepo*) shows zero order kinetics with respect to ascorbate over the range 4–40 mM ascorbate, is not inhibited by 2 mM EDTA and will not oxidize catechol. In contrast, the rate of oxidation catalysed by copper ions increases with ascorbate concentration and is totally inhibited by EDTA; the system will not oxidize catechol. The rate of oxidation catalysed by homogenates rich in phenolase, e.g. from lettuce (*Lactuca sativa*) seedlings, increases with ascorbate concentration, is not inhibited (and may be stimulated) by EDTA and the preparations oxidize catechol rapidly. By these criteria, the cell-wall and supernatant fractions from barley roots, cabbage leaves and vegetable marrow fruits (a typical source of the soluble enzyme[11,13]), all contain an ascorbic oxidase. The activity in all is independent of ascorbate concentration over range 4–40 mM, is unaffected by 2 mM EDTA and none oxidizes catechol.

Comparison of the Reaction Characteristics of the Cell-Wall and Soluble Ascorbate Oxidases of Cabbage Leaves

Table 3 lists the characteristics of the ascorbate oxidation catalysed by soluble and cell-wall preparations from cabbage leaves, and for comparison are included the corresponding figures for the reaction catalysed by a partially purified ascorbate oxidase from vegetable marrow.

The leaf soluble and cell-wall preparations have the same optimal pH (6·0) and the same Michaelis constant (10^{-2} M) as determined by the Lineweaver–Burk method. The soluble enzyme is more sensitive than the cell-wall enzyme to the chelating agents 8-hydroxyquinoline (oxine), sodium diethyldithiocarbamate (dieca) and to sodium azide. Neither preparation is

TABLE 3. PROPERTIES OF THE ASCORBATE OXIDASE OF THE CELL WALL AND SOLUBLE FRACTION FROM CABBAGE LEAVES AND OF A PURIFIED ASCORBATE OXIDASE FROM VEGETABLE MARROW

Property	Enzyme		
	Cell-wall preparation	Soluble fraction	Marrow enzyme
K_m	10 mM	10 mM	0·01 mM
Optimal pH	6·0	6·0	5·8
Energy of activation (15–40°) (kcal/mole)	12	4·4	3·5–4
% Inhibition by			
8 hydroxyquinoline (1 mM)	0	42	100
8 hydroxyquinoline (0·5 mM)	0	27	90
sodium diethyl dithiocarbamate (1 mM)	60	75	100
(0·5 mM)	0	45	100
(0·1 mM)	0	0	100
sodium azide (1 mM)	0	42	50
p-chlormercuribenzoate (1 mM)	0	0	0

inhibited by *p*-chloromercuribenzoate which reacts with sulphydryl groups. Apart from the difference in sensitivity to inhibitors, the major difference between the two is that the energy of activation of the cell-wall enzyme is more than twice that of the soluble enzyme. Similarly the energy of activation of the reaction catalysed by cell walls from vegetable marrow is 6·7 kcal/mole while the value for the corresponding soluble enzyme is 3·5–4 kcal/mole.

Despite these differences, consideration of the effects on its kinetics of binding a soluble enzyme into an insoluble matrix suggest that it is likely the same enzyme is responsible for the activity in the cell-wall and supernatant fractions. Bernfeld and Bieber[7] showed that the energy of activation of enolase embedded in an insoluble polymer was 15·3 kcal/mole, whereas that of the free enzyme was 11·8; but Weliky, Brown and Dale[8] found that binding peroxidase onto carboxymethyl cellulose raised the energy of activation only from 6·5 for the free enzyme to 7·2 kcal/mole for the bound form. They also observed that 1 mM azide inhibited the free peroxidase by 82 per cent and the bound by 55 per cent;[8] similarly, 0·27 mM $ZnCl_2$ inhibited soluble enolase by 60 per cent and the insoluble form by 28 per cent.[7] Finally, binding an enzyme into a neutral matrix does not in general alter the K_m or the optimal pH significantly.[7–9] We therefore conclude that the ascorbate oxidase of cell walls and soluble fraction of cabbage leaves, and probably also of other tissues, is attributable to the presence of the same enzyme in the two fractions.

Intracellular Location of Ascorbate Oxidase in vivo

There are three possible explanations for the occurrence of the same enzyme in both the cell walls and supernatant *in vitro*: i. the activity detected in the cell walls could have been adsorbed onto them, during their isolation, from the soluble phase to which it is restricted *in vivo*; ii. the enzyme present in the supernatant might *in vivo* be associated solely with the wall but be released from it during fractionation; iii. the enzyme might be present in both sites *in vivo*. Experiments were carried out to investigate these possibilities.

a. If the cell-wall enzyme is derived by adsorption of a soluble enzyme, then physical or chemical treatment of the walls should release it. The results given in Table 4 show the enzyme is held tenaciously. 0·15 M NaCl, which readily removed adsorbed pectin methyl esterase

TABLE 4. EFFECT OF VARIOUS CHEMICAL AND PHYSICAL TREATMENTS ON THE ASCORBATE OXIDASE ACTIVITY OF CABBAGE CELL-WALL PREPARATIONS

Treatment	Ascorbate oxidase activity μl O_2/hr/100mg Cell walls			
	Before treatment	After treatment		Recovery (%)
	Cell walls	Supernatant	Cell walls	
0·15 M NaCl, 1 hr 25°	275	15	282 (102)	109
0·15 M $CaCl_2$, 1 hr 25°	300	20	310 (103)	110
0·5% deoxycholate wash	265	8	250 (95)	98
2·5 M glucose, 1 hr 25°	310	17	296 (96)	102
Additional homogenization in Potter homogenizer	262	68	217 (83)	109
Additional homogenization in Ultra-turrax	312	34	281 (90)	101
Hughes press	306	6	250 (82)	84
Ultrasonication at 0°	229	10	162 (71)	75

Cell walls were incubated, washed, or homogenized in 0·067 M phosphate buffer, pH 6·0. The samples were then centrifuged at 1000 *g* for 5 min. The sediment (cell walls) was resuspended in buffer. Assays of ascorbate oxidase described in Experimental section. Figures in bracket % activity of original cell-wall preparation.

from oat coleoptile walls[14] and 50 per cent of the glycerophosphatase activity from barley root walls,[6] and 0·15 M $CaCl_2$ which releases amylase from cultured cells[15] do not reduce the activity of the walls significantly. Deoxycholate, which would disrupt endoplasmic reticulum, also has little effect, indicating that the enzyme is not associated with strands of endoplasmic reticulum (attached to the wall fragments) which had survived homogenization. Further grinding of the walls reduced the activity by up to 20 per cent, but recovery of the wall material in these experiments was incomplete owing to losses during transfers. 0·15 M NaCl will remove adsorbed ascorbate oxidase, since washing cell walls of vegetable marrow, which had been incubated with a concentrated preparation of ascorbate oxidase, with 0·15 M NaCl removed 70 per cent of the adsorbed activity (Table 5). (Incubating either marrow or barley-root walls with the unconcentrated corresponding soluble fraction resulted in no adsorption of activity; only concentrated preparations were adsorbed.) It therefore seems unlikely that the activity found in cell-wall fractions has been adsorbed from the soluble phase during processing. Mertz[5] observed that the ascorbic oxidase of cells walls from maize roots was not eluted by 0·25 M sucrose, nor by deoxycholate, and he concluded it was structurally associated with the cell wall.

b. Incubating cell walls with cellulase partially degrades the wall and releases some ascorbate oxidase, thus confirming the structural connexion of the enzyme with the wall. Figure 1 shows that during 60 min incubation with cellulase the activity in the cell wall fell, that in the supernatant rose sharply and the total activity (cell wall + soluble) increased by about 20 per cent.

Cellulase itself has no ascorbate oxidase activity and incubating the cell walls without cellulase does not release or activate ascorbate oxidase. It is of interest that the total activity increases on release of the wall-bound enzyme, for treating the walls with ionic reagents which did not greatly lower the activity of the walls releases a trace of oxidase so that the recovery of activity is greater than 100 per cent. Adsorption or binding of enzymes has been shown to

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[15] J. STRAUS and W. A. CAMPBELL, *Life Sci.* 1, 50 (1963).

TABLE 5. ADSORPTION OF ASCORBATE OXIDASE ONTO CELL WALLS AND ELUTION BY IONIC REAGENTS

Preparation	Ascorbate oxidase activity (μl O_2/hr/100 mg walls or hr/ml enzyme)	Activity as % of initial cell-wall activity	Activity as % of adsorbed enzyme
1. Vegetable marrow cell walls	150	100	—
2. Purified marrow ascorbate oxidase	1478	—	—
3. Cell walls + adsorbed enzyme	342	230	100
4. Walls from 3 washed			
a. with 3 × 3 ml of 0·1 M phosphate buffer, pH 6·0	227	151	40
b. then with 3 × 3 ml 0·15 M NaCl	200	133	26
c. then with 3 × 3 ml 0·15 M $CaCl_2$	208	139	30

Ascorbate oxidase activity determined under standard conditions. 100 mg cell-wall preparation was incubated with 1 ml of purified ascorbate oxidase for 1 hr at 20°. The cell walls were centrifuged at 100 g for 5 min, and assayed in 0·1 M phosphate buffer. They were washed by suspension and re-centrifugation.

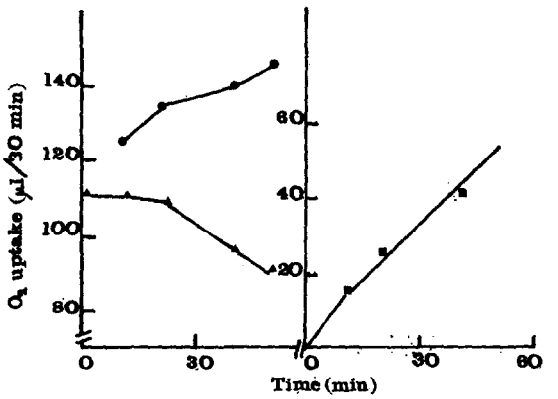

FIG. 1. REDISTRIBUTION OF ASCORBATE OXIDASE ACTIVITY OF CABBAGE CELL-WALLS DURING INCUBATION WITH CELLULASE.

Aliquots of cell walls were incubated at pH 5·0 in 0·05 M acetate buffer, 2 mM EDTA at 30° with purified cellulase. At intervals the ascorbate oxidase activity of the incubation mixture (● —— ●), the 1000 *g* sediment (▲ —— ▲) and the supernatant (■ —— ■) were measured at pH 5·0 and otherwise standard conditions. Results expressed in μl O_2 consumed/30 min calculated from first 15 min of oxidation, 100 mg of cell-wall preparation.

reduce their activity,[9] and release of an enzyme from a particle is sometimes accompanied by an increase in activity.[16,17]

c. If the ascorbate oxidase is present in the cell wall, then *in vivo* it should be freely accessible to ascorbate provided in the medium. If ascorbate is taken into the cell only slowly then the kinetics of the oxidation by the tissue should closely resemble those of the cell-wall enzyme, the oxidation should proceed without a lag and without modifying CO_2 production of the tissue. Although accumulation of ascorbate or dehydroascorbate within the tissue is not evidence against the functioning of a surface enzyme, if during the oxidation all the ascorbate and dehydroascorbate can be recovered outside the tissue, this clearly supports the view that

[16] M. HALLAWAY, in *Plant Cell Organelles* (edited by J. PRIDHAM), p. 1, Academic Press, London (1968).
[17] J. GAHAN, in *Plant Cell Organelles* (edited by J. PRIDHAM), p. 228, Academic Press, London (1968).

the catalyst responsible is located on the surface of the tissue rather than within the cytoplasm. These considerations were investigated using 1 cm barley root tips as the experimental material and all the features of the oxidation of ascorbate indicate a peripheral site for the oxidase.

Homogenates of barley root tips contain an active ascorbic oxidase, the wall-bound enzymes accounts for about 80 per cent of the activity of homogenates of 4-day-old root tips rising to 100 per cent for 8-day-old root tips (Table 1). Root tips oxidize added ascorbate solutions, without any time lag and, since the rate of oxidation by the roots is 25–50 per cent of that of the homogenate, it indicates that the same percentage of the ascorbic oxidase of the

TABLE 6. RATE OF ENDOGENOUS O_2 UPTAKE BY BARLEY ROOT-TIPS AND OF ASCORBATE OXIDATION BY INTACT ROOTS, HOMOGENATES AND CELL-WALL PREPARATIONS

Age of seedlings (days)	O_2 uptake/hr/50 1-cm root tips (or equivalent amount of cell-free preparation)			
	Endogenous O_2 uptake	Rate HA^- oxidn. by homogenate	Rate HA^- oxidn. by cell walls	Rate HA^- oxidn. by roots
5	90	450	355	—
6	78	555	493	—
8	84	420	426	108
9	86	382	—	130
11	114	330	—	114
14	84	293	304	126

15 μmoles ascorbate added per flask; O_2 uptake measured under standard conditions. Homogenate and cell walls prepared as described in Experimental section. Rate ascorbate oxidation by roots corrected for endogenous O_2 uptake.

tissue is freely accessible to the external ascorbate (Table 6). The rate of oxidation is independent of the ascorbate concentration from 1·5 to 4 mM so diffusion to the site does not limit the rate of oxidation; and the oxygen uptake by the roots after the oxidation is complete is not altered appreciably from the initial rate (Table 7). The amount of additional oxygen uptake is almost exactly equivalent to the amount of ascorbate added (Table 7). The CO_2 production by the roots is unaffected by the addition of ascorbate (Table 8). The respiration rate of

TABLE 7. EFFECT OF ASCORBATE CONCENTRATION AND NUMBER OF ROOTS ON RATE OF O_2 CONSUMPTION BY ROOT TIPS

No. roots	Endogenous O_2 uptake (μl/hr)	μmoles HA^- added	Rate O_2 uptake + HA^- (μl/hr)	μg atoms O_2 taken up	Final rate O_2 uptake (μl/hr)
40	47	4·5	140	4·9	46
40	50	9·0	140	9·7	50
40	47	10·5	140	10·9	50
40	48	15·0	140	14·0	50
60	68	8·0	200	7·8	73

O_2 uptake measured under standard conditions. Ascorbate added after measuring endogenous O_2 uptake for 45 min. Rate O_2 uptake + ascorbate corrected for endogenous O_2 consumption.

TABLE 8. CO_2 PRODUCTION BY ROOT TIPS IN THE PRESENCE AND ABSENCE OF ASCORBATE

Age of seedlings (days)	Rate CO_2 production (μl CO_2/hr/50 root tips)	
	Endogenous	+12 μmoles ascorbate
5	99, 98	99, 100
8	84, 87	87, 90
9	69	70

CO_2 production measured under standard conditions.

segments from different parts of barley root varies with the position of the segment.[18] However, the rate of ascorbate oxidation is almost constant for segments of the same length (Table 9). This fact also indicates that uptake of ascorbate through the cut end is not a controlling factor since the tip (with only one cut end) does not oxidize the ascorbate more slowly

TABLE 9. RATES OF OXYGEN UPTAKE IN PRESENCE AND ABSENCE OF ASCORBATE BY BARLEY-ROOT SEGMENTS

Segment (cm)	O_2 uptake (μl/hr/50 segments)	
	Endogenous	+9 μmoles ascorbate
0–1	144	54
1–2	114	50
2–3	96	48
3–4	88	48
4–5	76	44
5–6	72	48
7–8	52	48

O_2 uptake measured under standard conditions, and rate + ascorbate corrected for endogenous oxygen uptake. 7-day-old seedlings employed.

than the subsequent segments (with two cut ends). When the amount of ascorbate remaining at intervals during the oxidation was determined and also the total (ascorbate + dehydroascorbate) it was found that at each time the loss of ascorbate was equivalent to the additional oxygen consumption, and the total ascorbate + dehydroascorbate remained effectively constant (Fig. 2). This experiment demonstrates the stoichiometry of the oxidation and also shows that any retention of substrate of product within the roots was less than 4 per cent. From these experiments it can be concluded that *in vivo* some of the ascorbate oxidase of barley roots is located at a site freely accessible to the external medium, which we suggest is in the cell walls.

Thus, the balance of evidence indicates that *in vivo* some of the ascorbate oxidase is associated with the cell wall; it does not exclude the possibility that *in vivo* all the ascorbate oxidase is associated with the wall and some is released during cell fractionation. If this is the case there must be at least two classes of wall-bound enzyme, enzyme held strongly within the wall and released only by hydrolysis of the wall, and enzyme held very loosely and lost into

[18] L. MACHLIS, *Am. J. Bot.* **31**, 281 (1944).

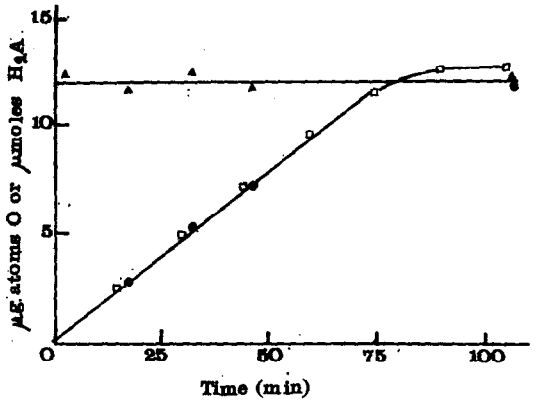

FIG. 2. RECOVERY OF ASCORBATE AND DEHYDROASCORBATE AND RATES OF OXYGEN UPTAKE AND ASCORBATE LOSS DURING ASCORBATE OXIDATION.
Forty 8-day-old root tips incubated under standard conditions, 12·5 μmoles ascorbate added at zero time. ▲ —— ▲ = total ascorbate + dehydroascorbate; ● —— ● = ascorbate lost; □ —— □ = oxygen uptake corrected for endogenous O_2 uptake.

the cytoplasm during fractionation. Honda[2] has shown that in barley roots the amount of ascorbate oxidase recovered in the soluble fraction is not altered by grinding the tissue in media of differing pH and tonicity which indicates the enzyme is unlikely to be attached to subcellular particles *in vivo*.

The pattern of similarities and differences between the kinetics of the soluble and cell-wall ascorbate oxidase is qualitatively the same as that established for enzymes free and synthetically bound into an inert matrix. This identity of pattern indicates that the same enzyme is probably responsible for the activity in the wall and the soluble fraction; it also provides further support for the validity of using enzymes chemically linked to an insoluble matrix as model systems for studying the behaviour of particle-bound enzymes.

EXPERIMENTAL

Preparation of Enzymes

Barley (*Hordeum vulgare* var. "Proctor") seeds were soaked for 12 hr in running water and spread on muslin on stainless-steel grid over water and grown at 20–22° in the dark. Roots, 4–6 days old, were used unless otherwise stated. Cabbages (*Brassica oleracea*) and vegetable marrows (*Cucurbita pepo*) were purchased locally and used without storage. Roots were homogenized in a power-driven Potter homogenizer, marrows and cabbage leaves in either a Kenwood Liquidizer or an Ultra-turrax; unless otherwise stated the medium was 0·067 M phosphate buffer, pH 6·0. The cell walls from roots and vegetable marrow homogenates were isolated by centrifugation at 500 *g* for 5 min, and were washed three times by suspending in buffer and re-centrifuging. Cell walls from cabbage were prepared in this way and also by filtration on a sintered glass funnel (No. 1 porosity); the residue was washed on the funnel. Cell walls separated by the two methods had identical kinetic properties. The first supernatant (or filtrate) was called the soluble fraction. All preparations were used immediately except for root tips, which were floated on water for 2–3 hr before use. Purified ascorbate oxidase was prepared according to the method of Dawson and Magee[19] (stopping at the end of their third stage).

Cellulase (practical grade, Type II, Sigma Chemical Co.) dissolved in 0·01 M acetate buffer, pH 5·0, was dialysed against buffer, and partially purified by chromatography on Sephadex G-75. Cellulolytic activity occurred in four peaks, the main peak had no proteolytic activity. Its molecule weight, by the method of Andrews,[20] using ribonuclease, ficin, pepsin and bovine serum albumin as markers, was 40,000.

[19] C. R. DAWSON and R. J. MARGEE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. II, p. 831, Academic Press, New York (1955).
[20] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).

Assay Methods

Ascorbate oxidase activity was measured manometrically under the standard conditions: at 30° in 0·067 M phosphate buffer, pH 6·0, 2 mM EDTA and 5 mM ascorbate in a final reaction volume of 3 ml. In all experiments, enzyme-free and substrate-free controls showed negligible oxygen uptake. The activity of the soluble fraction was occasionally measured spectrophotometrically.[21]

The cellulolytic activity of the purified cellulase was assayed by measuring the release of glucose from cellulose powder at pH 5 in 0·01 M acetate buffer, by Nelson's method. The proteolytic activity was assayed by measuring increase in absorbance at 280 nm on incubating cellulase fractions with bovine serum albumin, at pH 5·0, then precipitating the proteins with 10% trichloracetic acid.

Nitrogen was assayed by the Micro Kjeldahl procedure. Ascorbic acid was measured by the method of Roe,[22] and dehydroascorbate by reducing it to ascorbic acid with H_2S and then assaying the ascorbic acid. CO_2 evolution was followed by the direct method of Warburg.

Acknowledgements—We wish to thank the Agricultural Research Council for a research studentship to P. D. P. and the University of Liverpool for a Scholarship to J. T.

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