Plant Physiol. (1981) 67, 591–593
0032-0889/81/67/0591/03/$00.50/0

Short Communication

Vacuolar Deposition of Ascorbate-derived Oxalic Acid in Barley[1]

Received for publication August 5, 1980 and in revised form October 8, 1980

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ABSTRACT

L-[1-^{14}C]Ascorbic acid was supplied to detached barley seedlings to determine the subcellular location of oxalic acid, one of its metabolic products. Intact vacuoles isolated from protoplasts of labeled leaves contained [^{14}C]oxalic acid which accounted for about 70% of the intraprotoplast soluble oxalic acid. Tracer-labeled oxalate accounted for 36 and 72% of the ^{14}C associated with leaf vacuoles of seedlings labeled for 22 and 96 hours, respectively.

OA[2] is a widely occurring natural product of plants, yet little is known about its metabolism and function (3, 10, 15). The amount of OA formed and accumulated in different plant species can vary greatly (3, 9, 10, 14). Moderate to high accumulators, such as spinach and rhubarb, may contain 0.2 to 1% OA (on a fresh weight basis), whereas low accumulators, such as barley and tomato, may contain one-tenth this amount. In the very high accumulator *Halogeton glomeratus*, Ca-OA can account for 30% of the dry weight of the plant (10). Glycolic, glyoxylic, and isocitric acids (8) and oxaloacetic (1) and ascorbic (7, 13) acids are known to donate carbon to OA in plants. The relative significance of these metabolites as precursors of OA has not been established.

Ca-OA, in various crystalline forms, often is observed in plant vacuoles (2). It also has been reported to occur in cytoplasm, in cell walls, and in extracellular spaces. However, ultrastructural observations suggest that extracellular Ca-OA originates in vacuoles (see ref. 4 and references therein). Soluble OA salts are thought to be contained in vacuoles. In the study presented here, vacuolar deposition of OA formed from AA was tested in barley, a low level OA accumulator in which AA to OA conversion has been reported (9).

MATERIALS AND METHODS

Six 13-day-old seedlings of *Hordeum vulgare* cv. Moore were cut at soil level and recut under water in preparation for labeling. Cut stems were immersed in 100 μl water containing 7.3 μCi of [1-^{14}C]AA (8.44 mCi/mmol, New England Nuclear). Plants were incubated at room temperature under room fluorescent light (9 h/day) in a bell jar through which dry air was drawn by vacuum. No effort was made to recover $^{14}CO_2$ resulting from metabolism of [1-^{14}C]AA. Water was added as required. After 22 h, three plants were removed and their leaves were surface-sterilized with 70% ethanol. To prepare protoplasts, the leaves were sliced into 1-mm strips and the strips were incubated in a sterile solution containing 1% Cellulysin, 0.5% Macerase (both from Calbiochem), and 0.7 M mannitol. Leaves from 24 plants which had not been labeled were similarly treated to provide carrier protoplasts and vacuoles. Tissues were about 75% digested after incubation at 25 C and in room light for 4 h. After removal of undigested materials, labeled-tissue and carrier-tissue digests were combined and protoplasts were recovered by sedimentation at 500*g*. Protoplasts (0.4 ml) were washed three times with 40 ml 20 mM Mes-KOH (pH 5.5). One-fifth was removed to be used as a protoplast sample before protoplasts were sedimented from the third wash. The remaining four-fifths was used to prepare vacuoles, essentially as previously described for the preparation of tulip leaf vacuoles (12). Vacuoles were released in 0.17 M KH_2PO_4/K_2HPO_4 (pH 8.0); K-phosphate), and most residual protoplasts and much of the resulting particulate material were sedimented at 500*g*. The supernatant was made 17% (w/v) with sucrose by addition of 60% (w/v) sucrose and this solution was overlayered with a vacuole flotation zone consisting of 10 ml 18% (w/v) sucrose, 20 mM Hepes-NaOH (pH 8.0). The supernatant made 17% (w/v) with sucrose contained K-phosphate (0.12 M) and was more dense than buffered 18% (w/v) sucrose. Centrifugation at 5000*g* for 10 min at 20 C caused vacuoles to float to the top of the 18% sucrose zone. The top 2 ml of this zone was recovered as the vacuole fraction. The remainder was treated separately (see below) but considered as part of the cytosol in the calculation of results (see Table I, footnote c).

A fraction representing cytosol was recovered from the 17% sucrose zone. Soluble protoplast components were recovered from the sample, previously set aside, by lysis of protoplasts with 0.17 M K-phosphate.

The remaining three labeled plants were similarly treated 96 h after their initial exposure to the label. It was thought that this period would provide ample time for transfer of metabolically formed OA to the vacuole. The oldest leaves of cuttings began to show signs of senescence after 70 h, but cuttings were fresh and turgid at 96 h. Numbers and quality of protoplasts and vacuoles were like that obtained after 22 h.

Under these conditions of isolation, the vacuole fraction of barley contains only traces of Chl and Cyt *c* oxidase (<0.1 and 0.5% of protoplast levels, respectively) and less than 0.75% of the cytosol fraction-soluble material (determined to be less than 0.1%). A potential internal marker for barley protoplast cytosol, UDP-glucose:quercetin glucosyl transferase, was also monitored. UDP-glucose:anthocyanidin glucosyl transferase was observed to be largely located in cytosol and to be clearly extravacuolar in *Hippeastrum* and *Tulipa* protoplasts (5). After protoplast preparation and fractionation, the soluble materials representing 1.6×10^5 protoplasts, 0.19×10^5 protoplast equivalents of cytosol, and 0.15×10^5 vacuoles (yield for this experiment) were assayed as previously described (5) but using quercetin as substrate. Products of UDP-[^{14}C]glucose metabolism plus authentic carrier quercetin-3-

[1] This research was carried out at Brookhaven National Laboratory under the auspices of the United States Department of Energy.

[2] Abbreviations: OA, oxalic acid; Ca-OA, calcium oxalate; AA, ascorbic acid.

Table I. *Distribution of [1-^{14}C]AA-derived OA and Non-OA Label in Barley Protoplasts*

The cpm were standardized to 1.4 × 10^6-labeled protoplasts in experiments 1 and 2.

Experiment Number and Time	Fraction	[^{14}C]OA		Protoplast-soluble OA[a]	Non-OA ^{14}C[b]		Fraction ^{14}C in OA[a]
		Actual	Calculated[c]		Actual	Calculated[c]	
		cpm		%	*cpm*		%
1, 22 h	Protoplast	13,900			37,300		
	Cytosol[d]	13,150	4,950	32	34,700	19,450	20
	Vacuole	530	10,600	68	940	18,860	36
	Remainder of 18% sucrose zone	1,870			2,670		
2, 96 h	Protoplast	26,800			16,190		
	Cytosol[d]	24,520	7,280	27	16,670	10,290	41
	Vacuole	980	19,620	73	380	7,600	72
	Remainder of 18% sucrose zone	1,400			840		

[a] Determined from calculated values.

[b] Label remaining after removal of Ca-OA.

[c] Calculated vacuole = actual vacuole × 20 (to adjust for 5% yield in purified vacuole recovery; both experiments). Calculated cytosol = actual cytosol + ^{14}C in the remainder of the 18% sucrose zone minus label calculated to have been contributed to these fractions by lysed vacuoles.

[d] Fraction representing cytosol.

glucoside were separated by descending paper chromatography using the aqueous phase of the solvent mixture, butyl alcohol: acetic acid:H_2O (4:1:5, v/v/v). Quercetin-[3-^{14}C]glucoside formed was determined by quantitating radiochromatogram scans of the paper chromatograms. Protoplast, cytosol, and vacuole fractions contained 0.18 × 10^6 cpm, 0.39 × 10^6 cpm, and no detectable label, respectively, in this product after standardation of fractions to 47 × 10^6 protoplasts. Similar results were obtained for the distribution of this enzyme activity in fractions of wheat prepared in the same fashion (G. J. Wagner and G. Hrazdina, unpublished data). The occurrence of higher glucosyl transferase activity in the cytosol fraction than in the protoplast fraction was reported and discussed in an earlier publication (5).

Fractions from both experiments including the protoplast lysates, vacuole, the fraction representing cytosol, and the remaining 18% sucrose zones were centrifuged at 100,000*g* (1 h) to remove particulate materials. The supernatants were made 0.1% with oxalic acid to provide carrier OA and the total OA was recovered as insoluble Ca-OA after addition of a slight molar excess of calcium formate. The Ca-OA precipitates were washed twice with cold water, dried, and weighed, and aliquots were assayed for radioactivity in Aquasol (New England Nuclear)-water after solution in 6 N HCl. Attempts to purify OA from Ca-OA products by sublimation following the procedure described earlier (13) were unsuccessful. Passage of HCl solubilized salt through a Dowex 50 (Na^+) column and subsequently a Dowex 50(H^+) column yielded free acid which was purified by sublimation (yields averaged 80%). Portions of sublimed OA were dissolved in 1 ml water and counted after addition of 10 ml Aquasol.

Samples of sublimed OA prepared from the vacuole fractions of both experiments were converted to sodium-OA by passage through a Dowex 50(Na^+) column, diluted with carrier OA and converted to *S*-benzylisothiuronium oxalate following the procedure of Vogel (11). Specific radioactivity of sublimed OA was retained in the derivative (m.p., 198 C) and in derivative recrystallized from hot water.

RESULTS AND DISCUSSION

OA formation from [1-^{14}C]AA has been observed in barley seedlings which were pulsed with label and subsequently held in water for 22 and 96 h. In both experiments, vacuoles derived from protoplasts of labeled leaves were found to contain about 70% of the soluble [^{14}C]OA recovered from protoplass (Table I). Thus, approximately the same ratio of vacuole to cytosol OA was observed in the two experiments. However, more [^{14}C]OA and less non-OA^{14}C was found 96 h after labeling. Radiolabeled OA accounted for 36 and 72% of the total ^{14}C in vacuoles of the 22- and 96-h experiments, respectively. In comparison, the enriched cytosol fractions contained less OA and more label in uncharacterized, non-OA products. A portion of this activity may be in unmetabolized AA. No attempt was made here to assess the possible formation of 0.17 M K-phosphate (pH 8)-insoluble OA. The extent of labeling of particulate materials of protoplasts and the level of [1-^{14}C]AA conversion to CO_2 were also not determined. Regarding the latter, Nuss and Loewus (9) reported little conversion of this label to CO_2 in *H. vulgare* var. Laker. Labeled OA recovered as Ca-OA was purified by sublimation as described. The *S*-benzylisothiuronium oxalate formed from sublimed, vacuole-derived OA of both experiments retained specific radioactivity on recrystallization from hot water.

It is again noted that only ^{14}C which was soluble in the phosphate buffer used to prepare fractions was analyzed in these experiments. Insoluble OA salts may have been formed but not recovered. That this did occur is suggested by results obtained when particulate materials of protoplasts lysed in K-phosphate were treated with 0.2% OA. About as much [^{14}C]OA was recovered as was found in the phosphate lysate (data not shown).

Nuss and Loewus (9) found that 2% of the label supplied as [1-^{14}C]AA to detached seedlings of *H. vulgare* cv. Laker was recovered as OA upon acid extraction of tissues. Here, using *H. vulgare* cv. Moore, the yield was estimated to be about 0.7%. Essentially all the label was taken up by detached seedlings, but only leaves were used to prepare protoplasts. The extent of digestion of leaves was estimated to be 75%, survival of protoplasts through the washing procedures was 70% (determined experimentally, ref. 12), and acidic extractions (9) were not employed. The results obtained suggest that *H. vulgare* cv. Moore, like *H. vulgare* cv. Laker, is a low AA to OA converter.

Attempts to isolate vacuoles from several moderate and high OA accumulators including *Portulaca*, *Pelargonium*, *Begonia*, *Impatiens*, and *Halogeton* produced relatively impure vacuoles in low yield.

The study presented here indicates that the primary site of

deposition of K-phosphate-soluble OA formed from AA in barley is the vacuole, but it does not reveal the subcellular site of AA to OA conversion. The observed decrease in vacuolar non-OA ^{14}C from 22 to 96 h and the corresponding increase in [^{14}C]OA in the vacuole during that period (Table I) may represent conversion of AA to OA in the vacuole. Alternatively, this conversion may occur in the cytoplasm or in cytoplasmic organelles and the OA transported to the vacuole. Diurnal changes in the malic acid content of *Sedum* vacuoles has been reported (6) suggesting transport of that acid across the tonoplast. In future experiments, attempts will be made to monitor the fate of unmetabolized AA and of labeled products other than OA in an effort to determine the site of AA to OA conversion. It is noted that the subcellular site(s) of AA biosynthesis and storage in plants is not established.

Acknowledgment—I thank Dr. G. Hrazdina for the sample of quercetin-3-glucoside.

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