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ASCORBIC ACID METABOLISM IN GERANIUM AND GRAPE*

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Key Word Index—*Pelargonium crispum*; Geraniaceae; geranium; *Vitis labrusca*; Vitaceae; grape; metabolism; L-ascorbic acid; D-ascorbic acid; biosynthesis; L-(+)-tartaric acid; oxalic acid.

Abstract—L-Ascorbic acid-[UL-^{14}C] has been used to follow the appearance of ^{14}C-labeled oxalic acid and tartaric acid as metabolic products of oxidative cleavage of ascorbic acid in geranium apices (*Pelargonium crispum*). The enantiomeric specificity of ascorbic acid metabolism was established in geranium by comparing the incorporation of D- and L-ascorbic acid-[6-^{14}C] in the presence of L-ascorbic acid–[4-^{3}H]. L-Ascorbic acid-[4-^{3}H] has been used to demonstrate the retention of ^{3}H during biosynthesis of L-(+)-tartaric acid in the geranium and its exchange with water during biosynthesis of L-(+)-tartaric acid in the grape.

INTRODUCTION

L-Ascorbic acid is an effective precursor of L-(+)-tartaric acid in grape and geranium plants [1–6]. In grape the C_4 fragment corresponding to C1-C4 of L-ascorbic acid is converted to tartaric acid whereas in geranium this C_4 fragment comes from C3-C6 of L-ascorbic acid [5]. Further, the C_2 fragment generated from C1 + C2 in L-ascorbic acid in geranium as well as other oxalic acid accumulating plants is converted to oxalic acid [2, 7, 8]. There is additional evidence to suggest that C6, and possibly C5 as well, of L-ascorbic acid are recycled by the grape for utilization in hexose biosynthesis [3, 9]. A summary of these conversions is given in Fig. 1.

The present study provides new evidence for simultaneous production of oxalic acid and tartaric acid during L-ascorbic acid metabolism by geranium and traces the fate of hydrogen attached to C4 of L-ascorbic acid in grape and geranium. The enantiomeric specificity of ascorbic acid metabolism in geranium is also examined.

RESULTS AND DISCUSSION

Ascorbic acid metabolism in geranium

A series of geranium apices (0.5–0.6 g fr. wt apex^{-1}) supplied with L-ascorbic acid-[UL-^{14}C] (1.08 μCi apex^{-1}) were sampled at intervals to determine distribution of ^{14}C among metabolic products, particularly

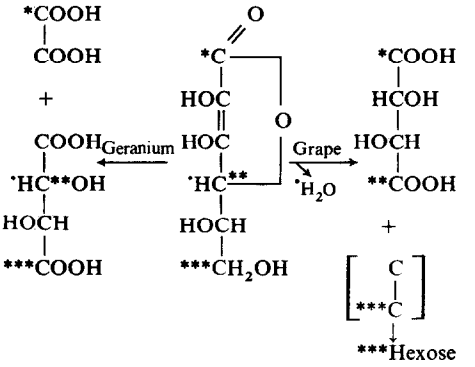

Fig. 1. Conversion of L-ascorbic acid to L-(+)-tartaric acid and other products in grape and geranium plants. Carbons marked with ^{14}C are identified by one or more asterisks (*C corresponds to C1 in ascorbic acid, **C to C4 and ***C to C6). Hydrogen marked with ^{3}H is identified by a solid circle.

oxalic acid, tartaric acid and respired CO_2. Results are given in Table 1.

Following a brief lag period, CO_2-[^{14}C] rose continuously during the metabolic period. Carbon-14 in L-(+)-tartaric acid increased at a comparable rate. Conversion of L-ascorbic acid-[UL-^{14}C] to oxalic

Table 1. Distribution of carbon-14 in L-ascorbic acid-[UL-^{14}C]-fed geranium apices as percent of administered label

Fraction	Hours of metabolism 6	12	18	24	48	72
CO_2	1	3	8	12	19	26
Ascorbic acid	50	49	31	16	8	3
Oxalic acid	2	2	4	8	10	10
Tartaric acid	4	5	8	15	19	26
Other acids*	15	16	19	19	20	17
Neutral compounds	26	21	22	19	12	4
Cationic compounds	<1	<1	1	2	1	<1
Insoluble residues	2	3	7	9	11	13

* Includes 0.1 M HCl-extract (see text).

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acid-[^{14}C] proceeded at a rate similar to that of tartaric acid-[^{14}C] formation for 24 hr, then slowly reached a plateau between 48 and 72 hr, possibly due to depletion of L-ascorbic acid-[UL-^{14}C] and continued conversion of oxalic acid-[^{14}C] to CO_2-[^{14}C] [8]. A similar observation was made after normalizing data obtained from time-course studies of L-ascorbic acid-[1-^{14}C] and -[6-^{14}C] metabolism in geranium [10]. During the first 48 hr, about twice as much ^{14}C reached tartaric acid as oxalic acid, an observation consistent with C_2/C_4 cleavage of a uniformly-labeled C_6 precursor. At 24 hr, about 80% of the L-ascorbic acid-[UL-^{14}C] was utilized. Less than 5% of this metabolized ^{14}C was recovered in the region corresponding to dehydroascorbic acid as measured by chromatography of the neutral fractions on paper in ethyl acetate–water–formic acid–acetic acid (18:4:1:3). Dehydroascorbic acid was probably a by-product of a non-enzymic process since, as will be seen shortly, D-ascorbic acid which was not a significant precursor of tartaric acid in the geranium also formed dehydroascorbic acid.

Treatment of 0.1% oxalic acid-extracted residues with dilute HCl (Table 1) removed additional ^{14}C, 1% at 6 hr, increasing to 5 or 6% at 24 hr with no further increase up to 72 hr. Neither oxalate nor tartrate was present in this acid extract. About one-half of the ^{14}C listed in Table 1 as 'other acids' appeared in the dilute formic acid elution from Dowex-1 formate resin as a single unidentified peak preceding ascorbic acid. Incorporation of ^{14}C into insoluble residue increased slowly during the metabolic period, reaching 13% at 72 hr. The ^{14}C-labeled constituents of this fraction were not identified.

To trace the fate of hydrogen attached to C4 of L-ascorbic acid during tartaric acid biosynthesis in geranium apices and to compare the utilization of epimeric ascorbic acids during this conversion, a set of experiments were undertaken in which D- or L-ascorbic acid-[6-^{14}C] was given to geranium apices together with L-ascorbic acid-[4-^{3}H]. The metabolic period was 72 hr. Results are given in Table 2. In experiment 1, 39% of the ^{3}H and 37% of the ^{14}C supplied as L-ascorbic acid appeared in tartaric acid, giving a ^{3}H/^{14}C ratio almost identical to that present in the labeled ascorbic acid. Unidentified acidic compounds, including some tightly bound label that resisted elution from the anionic exchange resin, accounted for 16% of each radioisotope. Very little label appeared in the neutral fraction which contained dehydroascorbic acid as well as other products. On the basis of non-volatile label recovered from the reservoir at the end of the experiment, it was estimated that about 80% of the L-ascorbic acid-[4-^{3}H,6-^{14}C] entered the apices. About 65% of the ^{3}H recovered as H_2O-[^{3}H] was found in the reservoir at the end of the experiment, presumably the result of passive leakage of products arising from tartaric acid metabolism. Some support for this conclusion was found in the difference between incorporation of ^{3}H and ^{14}C into insoluble products which contained 13% of the ^{14}C from C6 of ascorbic acid but virtually none of the ^{3}H from C4.

Table 2. Distribution of radioactivity in geranium apices after feeding D- or L-ascorbic acid-[6-^{14}C] together with L-ascorbic acid-[4-^{3}H] for 72 hr

Fraction	Distribution of radioactivity, % Experiment 1* L-Ascorbic acid-[4-^{3}H] + L-Ascorbic acid-[6-^{14}C]		Experiment 2† L-Ascorbic acid-[4-^{3}H] + D-Ascorbic acid-[6-^{14}C]	
	^{3}H	^{14}C	^{3}H	^{14}C
CO_2	—	3	—	7
H_2O‡	23	—	22	—
Ascorbic acid	2	2	6	16
Oxalic acid	0	2	0	1
Tartaric acid	39	37	46	2
Other acids	15	17	13	21
Neutral compounds	3	4	4	9
Cationic compounds	< 1	≪1	< 1	0
Insoluble residue	1	13	2	4
Non-volatile compounds recovered from reservoir	17	22	7	40

* ^{3}H/^{14}C ratio = 1.1 at start of experiment 1.

† ^{3}H/^{14}C ratio = 1.6 at start of experiment 2.

‡ Sum of H_2O-[^{3}H] present in sublimates from gas-dispersion bottles, final fluid in reservoir and neutral fraction after ion exchange chromatography.

Replacing L- with D-ascorbic acid-[6-^{14}C] (experiment 2) did not disturb the pattern of distribution among ^{3}H-labeled products derived from L-ascorbic acid-[4-^{3}H] but did produce a profound change in utilization of ^{14}C (Table 2). Only 2% of the ^{14}C appeared in tartaric acid. Repeated recrystallization of the tartaric acid as its racemic mono-potassium salt failed to free it of this ^{14}C and it is considered highly likely that this ^{14}C entered the tartaric acid as a result of secondary metabolism stemming from non-enzymic breakdown products of D-ascorbic acid rather than direct conversion due to a C2-C3 cleavage of ascorbic acid. The slightly greater amounts of ^{14}C recovered in CO_2, 'other acids' and neutral compounds when D- rather than L-ascorbic acid-[6-^{14}C] was provided probably arose from that D-ascorbic acid lingering in the apices unmetabolized and undergoing slow non-enzymic decomposition. There was indication of preferential uptake of L- as compared to D-ascorbic acid based on recovery of non-volatile ^{14}C-labeled compounds from the reservoir at the end of experiments 1 and 2. Additional experiments are needed to confirm this observation.

The results obtained here indicate that hydrogen attached to C4 of L-ascorbic acid was conserved during tartaric acid biosynthesis in the geranium (Fig. 1). Some decomposition of L-ascorbic acid accompanied this conversion under the experimental conditions used in these studies. Decomposition also occurred when D-ascorbic acid-[6-^{14}C] replaced the L-isomer. Since the D-isomer was not converted to tartartic acid in significant amount, non-enzymic breakdown of the ascorbic acid is considered to be the most probable source of ^{14}C in 'other acids', neutral compounds and, possibly, CO_2.

Oxidative cleavage of L-ascorbic acid at C2-C3 occurs in spore and mycelial extracts of *Myrothecium verrucaria* [11]. The products, oxalate and threonate, distinguish this oxygenase activity from ascorbate oxidase activity in the same preparation. A mechanism involving addition of dioxygen across the enolic double bond in ascorbic acid to produce oxalate and threonate directly is suggested. This type of activity has not been described

Table 3. Distribution of radioactivity in grape apice and leaf after feeding L-ascorbic acid-[1-^{14}C] or -[UL-^{14}C] together with L-ascorbic acid-[4-^{3}H] for 24 hr

Fraction	Distribution of radioactivity, % Experiment 3* L-Ascorbic acid-[4-^{3}H] + L-Ascorbic acid-[1-^{14}C]		Experiment 4† L-Ascorbic acid-[4-^{3}H] + L-Ascorbic acid-[UL-^{14}C]	
	^{3}H	^{14}C	^{3}H	^{14}C
CO_2	—	8	—	3
H_2O‡	33	—	69	—
Ascorbic acid	3	3	1	1
Oxalic acid	0	1	0	1
Tartaric acid	<1	15	<1	45
Other acids	23	18	14	14
Neutral compounds	24	29	9	12
Cationic compounds	2	7	<1	1
Insoluble residue	2	6	5	21
Non-volatile compounds recovered from reservoir	13	13	1	1

*^{3}H/^{14}C ratio = 1.2 at start of experiment 3.
† ^{3}H/^{14}C ratio = 0.6 at start of experiment 4.
‡ See footnote ‡ in Table 2.

in cell-free preparations from higher plants. *In vivo* cleavage of L-ascorbic acid at C2-C3 to produce oxalate and tartrate in the geranium and conservation of hydrogen attached to C4 during this conversion closely resembles the process catalysed by the oxygenase from *M. verrucaria.*

Ascorbic acid metabolism in the grape

In the grape, tartaric acid is a metabolic product of C1-C4 of L-ascorbic acid [3, 5, 6] and loss of hydrogen from C4 would be anticipated. To examine this, two experiments were run, one (experiment 3) with L-ascorbic acid-[4-^{3}H,1-^{14}C] as marker and the other (experiment 4) with L-ascorbic acid-[4-^{3}H,UL-^{14}C]. In both, the metabolic period was 24 hr. Experiment 3 involved the use of newly unfolded leaves from a detached vine segment that had been forced into new growth following five months of dormancy. Uptake of labeled L-ascorbic acid was slow and only 15% of the ^{14}C supplied to the leaves was converted to tartaric acid (Table 3). Less than 1% of the ^{3}H accompanied this ^{14}C into tartaric acid. A very substantial portion of the ^{3}H was recovered as H_2O-[^{3}H].

In experiment 4, young leaves were taken from actively growing vines. L-Ascorbic acid-[UL-^{14}C] rather than -[1-^{14}C] was used as the ^{14}C-label accompanying L-ascorbic acid-[4-^{3}H] in order to trace the fate of both C_4 and C_2 fragments, rather than just the C_4 fragment as in experiment 3. Tartaric acid from experiment 4 contained 45% of ^{14}C but less than 1% of the ^{3}H supplied as L-ascorbic acid. Since the ascorbic acid was uniformly labeled with ^{14}C, appearance of 45% in tartaric acid corresponded to 68% of the L-ascorbic acid-[4-^{3}H,UL-4C] supplied. That value compared favourably with the amount of ^{3}H recovered as H_2O-[^{3}H] due to exchange.

In previous studies involving feeding of L-ascorbic acid-[6-^{14}C] to detached leaves of *Parthenocissus inserta* and immature grape berries [3], about 40% of the ^{14}C was recovered in neutral compounds, mainly hexose. In the present study, 21% of the ^{14}C in experiment 6 was found in the insoluble residue, an amount comparable to that present in tartaric acid if one assumes a C_4/C_2 cleavage of 68% of the available L-ascorbic acid-[UL-^{14}C]. Other observations [9] indicate that the C_2 fragment from C5-C6 of L-ascorbic acid is rapidly recycled by the grape into hexose which is utilized by the young expanding leaf for polysaccharide biosynthesis. This would account for the substantial amount of ^{14}C found in the residual fraction after extraction with dilute acid.

Conversion of L-ascorbic acid to L-(+)-tartaric acid in the grape involves cleavage of the C_6 chain at C4-C5. The enzymic steps involved in this process have not been described. One possibility is a Baeyer–Villiger type oxidation similar to that postulated by Kotera *et al.* [12] for the conversion of D-*xylo*-hex-5-ulosonic acid to L-(+)-tartaric acid and glycolic acid in *Gluconobacter suboxydans.* Pretaric acid [2,3-dihydroxybutanedioic acid mono(dihydroxyethyl)ester, 2R-[1(S*, 2R*, 3S*)]], produced as an intermediate during the oxidation, breaks down to yield L-(+)-tartaric acid and glycoaldehyde. The latter is further oxidized to glycolic acid.

In a photosynthetic tissue such as grape leaf, glycolic acid is converted to carbohydrate through the glycolic acid pathway [13]. The expected C_2 fragment from L-ascorbic acid-[6-^{14}C] metabolism, glycolic acid-[2-^{14}C], would be converted to hexose in which label would appear in C1, C2, C5 and C6 [13]. An earlier study of the distribution of ^{14}C in glucose that was recovered from a grape leaf labeled with L-ascorbic acid-[6-^{14}C] showed nearly equal labeling in C1, C2, C4 and C6 [9]. This matter was re-examined [K. Saito and F. A. Loewus, manuscript in preparation] by feeding detached young grape leaves with L-ascorbic acid-[6-^{14}C] under the same conditions used in the present study in the presence and absence of inhibitors specific for the glycolic acid pathway [13]. Sucrose-derived glucose from labeled leaves used as controls was degraded to determine the pattern of ^{14}C distribution. Results similar to the ^{14}C distribution predicted by the glycolic acid pathway were obtained.

EXPERIMENTAL

Labeled ascorbic acids. L-Ascorbic acid-[1-^{14}C] and -[UL-^{14}C] were purchased from New England Nuclear Corp. (Boston, MA). These commercial preparations were diluted with L-ascorbic acid to 1 μCi mg^{-1}, recrystallized in glacial HOAc and stored as dry crystalline powders at −10° under N_2. L-Ascorbic acid-[4-^{3}H] (2.45 μCi mg^{-1}), L-ascorbic acid-[6-^{14}C] (1.15 μCi mg^{-1}) and D-ascorbic acid-[6-^{14}C] (1.16 μCi mg^{-1}) were prepared from D-glucopyranose-[3-^{3}H], -[1-^{14}C], and L-glucopyranose-[1-^{14}C], respectively, by a procedure recently published [14]. Labeled glucoses were purchased from New England Nuclear Corp. (Boston, MA). An unknown radioactive component present in L-glucopyranose-[1-^{14}C] accompanied the synthesis of D-ascorbic acid-[6-^{14}C] through its final step of purification on Dowex-1 (formate) during ion exchange chromatography. This impurity was separated from ascorbic acid by TLC on HPO_3–Si gel (1:33) using 3 × development in MeCN–PrCN–H_2O (66:33:2); the ascorbic acid (R_f 0.32) separated cleanly from the unknown component (R_f 0.16).

The position of ^{14}C in D- and L-ascorbic acid-[6-^{14}C] was established by periodate oxidation and isolation of the formaldehyde fragment corresponding to C6 as its dimedon derivative [15]. Carbon 6 contained >96% of the ^{14}C. The location of ^{3}H in L-ascorbic acid-[4-^{3}H] was inferred from the established location of ^{3}H in the starting product, D-glucopyranose-[3-^{3}H], which was prepared by catalytic reduction of 1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hex-3-ulofuranose using tritium gas, and from the absence (<0.02%) of ^{3}H in dimedon–formaldehyde after periodate oxidation of the labeled ascorbic acid.

Plant tissue. Lemon geranium apices (*Pelargonium crispum* (L.) L'Hér. cv Prince Rupert) were obtained from greenhouse-grown 2-yr-old plants which had been heavily pruned to induce new apical growth. Grape apices (*Vitis labrusca* L.) bearing two unfolded leaves and a floral bud were obtained from 1-yr-old vine segments (20 cm) which had been cut from dormant garden-grown vines (Feb. 1978). The lower end of each segment was dipped in 0.1% indolebutyric acid (Hormodin 1, Merck, Inc.) and placed in moist vermiculite until buds began to open. Grape leaves were removed from the same garden-grown vines used to obtain vine segments during May 1978. Leaves in positions opposite to flower clusters were chosen for use.

Detached tissues were labeled by placing a measured drop (50–200 μl) of the labeled solution (0.5% ascorbic acid containing 0.5–1.0 μCi of ^{3}H or ^{14}C or both) on Parafilm directly under the freshly-cut surface of stem or petiole. Uptake of label, which usually took less than 1 hr, was followed by several small increments of H_2O to flush all label through the vascular system into the leafy tissues. Leaves and apices were then transferred to plastic containers so cut surfaces were submerged in H_2O for the remainder of the metabolic period. Light was continuously supplied from two 15 W cool-white fluorescent lamps (4000 lx) throughout the experiment. Respired CO_2 and transpired H_2O were trapped by enclosing the experiment in a bell-jar and aspirating CO_2-free air through the jar into two series-connected 250 ml gas dispersion bottles, each containing 100 ml M KOH. Over 95% of the CO_2-[^{14}C] and H_2O-[^{3}H] from the experiment was trapped in the first bottle.

Recovery of labeled fractions. Procedures for fractionation generally followed those used in previous studies [2, 3, 5]. This involved extraction of ground tissues in 0.1% oxalic acid, further extraction of the residue with 0.1 M HCl and combustion of the final residue in a biological oxidizer to obtain ^{3}H and ^{14}C content. The 0.1% oxalic acid extract was treated with $Ca(OAc)_2$ to precipitate Ca oxalate and then fractionated into cationic, anionic, and neutral components by ion exchange chromatography. Aliquots from the gas-dispersion bottles, the final fluid in the reservoir and the neutral effluent after ion exchange chromatography were sublimed to recover the H_2O component for determination of ^{3}H exchange. Ascorbic acid and tartaric acid were recovered separately from other acids by gradient elution of the anionic exchange column (Dowex-1 formate) with formic acid and analysed quantitatively [2].

Radioactive determinations. Soluble fractions were counted with 0.5 ml of H_2O in 5 ml of counting fluid [16] using 7 ml glass liquid scintillation vials (PICO-VIAL, Packard Instr. Co.). Insoluble fractions including radioactivity bound to ion exchange resins were oxidized in a Model 306 biological oxidizer (Packard Instr. Co.) to obtain H_2O and CO_2 combustion products for determination of ^{3}H and ^{14}C by liquid scintillation counting. All determinations of radioactivity, after correction for background and quench, were converted to dpm by comparison with appropriate standards.

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