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The Biosynthesis of (+)-Tartaric Acid in *Pelargonium crispum*[1]

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GEORGE WAGNER AND F. LOEWUS
Department of Biology, State University of New York at Buffalo, Buffalo, New York 14214

ABSTRACT

Metabolic conversion of L-galactono-1,4-lactone and L-ascorbic acid to (+)-tartaric acid and oxalic acid has been studied in *Pelargonium crispum*, cv. Prince Rupert. Experiments with specifically labeled substrates suggest a path of conversion involving cleavage of L-ascorbic acid, or a metabolic product of L-ascorbic acid, between C_2 and C_3, such that oxalic acid arises from the two carbon fragment and (+)-tartaric acid from the four carbon fragment.

Conversion of L-ascorbic acid-1-^{14}C to carboxyl-labeled tartaric acid in ripening grape berries has been reported by Saito and Kasai (31). They also showed that D-glucurono-6,3-lactone-6-^{14}C, a precursor of L-ascorbic acid-1-^{14}C (12), gave the same labeled product. From a number of other studies of the grape involving the use of $^{14}CO_2$ (13, 17, 27–30, 37), L-ascorbic acid-6-^{14}C (23), D-glucose-U-^{14}C, -1-^{14}C or -6-^{14}C (26, 29) uniformly labeled sucrose, glucose, fructose, and L-malic acid (14), and fumaric acid-2,3-^{14}C (18), as well as studies on the conversion of specifically labeled D-glucose to L-ascorbic acid (19), it was concluded that carbon 1 of glucose (or hexose from the hexose phosphate pool) furnished a carboxyl carbon to tartaric acid. By inference, carbons 1 through 4 of glucose provided the carbon chain of tartaric acid.

Tartaric acid occurs in a number of plant families (35), and its quantitative occurrence has been examined by Stafford (36) in 52 species of Geraniaceae. She found several species of *Pelargonium* which were high accumulators of tartaric acid. Earlier, Maroc-Gyr (25) had reported that in growing leaves of *Pelargonium zonale* L., tartaric acid is more heavily labeled from D-glucose-6-^{14}C than from D-glucose-1-^{14}C. Maroc-Gyr's (25) observation regarding tartaric acid labeling by glucose in *Pelargonium* was contrary to a similar study in the grape (*Vitis vinifera* L.) by Ribereau-Gayon (27, 29), who found D-glucose-1-^{14}C to be a better source of label for tartaric acid than D-glucose-6-^{14}C.

The present study examines both L-ascorbic acid and (+)-tartaric acid biosynthesis in *Pelargonium crispum* L. cv. Prince Rupert using the observations that L-galactono-1,4-lactone is readily converted to L-ascorbic acid by plants (12, 16) and that L-ascorbic acid has, in one case (31), clearly been shown to be a precursor of tartaric acid.

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MATERIALS AND METHODS

Preparation of L-Galactono-1,4-lactone-U-^{14}C and -6-^{14}C. L-Galactono-1,4-lactone-U-^{14}C and -6-^{14}C were prepared from D-glucose-U-^{14}C and D-glucuronic acid-1-^{14}C, respectively. D-Glucose is converted into both the galacturonic acid and arabinose moieties of boysenberry and strawberry pectin (32, 33). D-Glucuronate is converted into D-galacturonic acid of strawberry pectin (22). The labeled lactones used in this study were prepared by supplying labeled precursors to germinating *Lilium longiflorum* pollen cv. Ace, which had been pregerminated at 27 C in Dickinson's (11) pentaerythritol medium prior to addition of label. After 6 hr of metabolism with the label, the pollen was washed free of media, and the D-galacturonic acid residues of the pollen tube wall pectin were isolated and converted to L-galactono-1,4-lactone, as described for the preparation of L-galactono-1,4-lactone-2-^{14}C prepared from strawberry (20). To 80 mg of pollen, pregerminated for 4 hr in 8 ml of medium was added D-glucose-U-^{14}C (180 c/mole). During the following 6 hr of incubation, 93% of the label was taken up by the growing pollen tubes. In the case of D-glucuronic acid-1-^{14}C (1.54 c/mole), in which 500 mg of pollen (85–90%) germination was pregerminated for 5 hr in 30 ml medium, 64% of the label was taken up in 6 hr. The final specific radioactivity of L-galactono-1,4-lactone-U-^{14}C was 600 μc/mmole, and that of L-galactono-1,4-lactone-6-^{14}C was 13 μc/mmole.

L-Ascorbic acid-6-^{14}C (276 μc/mmole) and L-ascorbic acid-1-^{14}C (735 μc/mmole) were purified by ion exchange chromatography and crystallized (20) prior to use.

L-Galactono-1,4-lactone-6-^{14}C, L-ascorbic acid-6-^{14}C and L-ascorbic acid-1-^{14}C were degraded (20), and each was shown to contain essentially all of its radioactivity in the carbon atom indicated.

Labeling Procedure and Recovery of Labeled Products. *Pelargonium crispum* L. cv. Prince Rupert was grown from cuttings purchased from Logee's Greenhouse, Danielson, Connecticut. Plants, 3 to 18 months old, were routinely pruned to promote quantities of new tip growth. For the experiments reported here, foliated tips containing the first three unfolded leaves were detached and placed in individual glass vials containing the labeled precursor. As the label, in 50 to 200 μl of distilled water, was taken up during the first 1 to 7 hr, additional water was added. When all the label had been taken up, the cuttings were transfered to distilled water for the remaining period of metabolism.

Labeled tissues were ground (Sorvall Omnimixer, 50-ml stainless steel container) at top speed for 90 sec in 30 ml of 0.1% (w/v) oxalic acid at 0 C. The resulting suspension was centrifuged at 13,000*g* for 10 min at 0 C. Aliquots of the supernatant were removed for analysis of radioactivity, ascorbic acid (21), and tartaric acid (38). The latter aliquot (1 ml) was first treated by passage through a column containing 1 ml of Polyclar-AT (GAF Corp.) to remove compounds that inter-

ferred with the metavanadate assay. It has been determined that the extraction procedure used releases 87% of the tartaric acid contained in the tissues. Similar data for L-ascorbic acid and oxalic acid are not available.

Carrier L-ascorbic acid (50 mg) and (+)-tartaric acid (50 mg) were added to the supernatant fraction. Oxalic acid was recovered by addition of an equimolar amount of calcium formate. After centrifugation to recover the calcium oxalate, the supernatant fraction was applied first to a column of Dowex 50 (H^+) (0.9 × 15 cm) then to a column of Dowex 1 (formate) (0.9 × 15 cm). After thoroughly flushing the Dowex 1 column with water to remove all traces of radioactive neutral components, acidic components were eluted with a formic acid gradient in two steps, 0 to 0.1 N and 0.1 to 4 N formic acid. In the first step, 500 ml of 0.1 N formic acid was introduced into a mixing chamber containing 250 ml of water. In the second step, 100 ml of 4 N formic acid was introduced into a mixing chamber containing 100 ml of 0.1 N formic acid. Ascorbic acid was eluted by the first step in fractions from 260 to 330 ml. Tartaric acid was eluted by the second gradient in fractions from 135 to 170 ml.

Table I. *Metabolism of Labeled Precursors to L-Ascorbic Acid, (+)-Tartaric Acid, and Oxalic Acid by P. crispum*

Labeled Precursor	Period of Metabolism	Labeled Products		
		L-Ascorbic acid	(+)-Tartaric acid	Oxalic acid
	hr	*% of 0.1% oxalic acid-soluble radioactivity*		
L-Gal-1,4-lact-U-^{14}C[1]	53	10.0	8.5	2.0
L-Gal-1,4-lact-6-^{14}C[2]	73	2.6	15.4	0.4
L-Ascorbic acid-6-^{14}C[3]	72	22.3	32.3	0.03
L-Ascorbic acid-1-^{14}C[4]	74	18.2	0.4	12.1

[1] 1.3 × 10^6 cpm supplied to 1.92 g fresh wt of cuttings.
[2] 0.87 × 10^6 cpm supplied to 2.42 g fresh wt of cuttings.
[3] 2.07 × 10^6 cpm supplied to 2.86 g fresh wt of cuttings.
[4] 3.11 × 10^6 cpm supplied to 2.24 g fresh wt of cuttings.

Table II. *Identity of L-Ascorbic Acid and Tartaric Acid Recovered after Feeding P. crispum Labeled Precursors and the Distribution of Internal Radioactivity*

	L-Gal-1,4-lact-U-^{14}C	L-Gal-1,4-lact-6-^{14}C	L-Ascorbic Acid-6-^{14}C
L-Ascorbic acid			
Specific radioactivity			
cpm/mmole	102,300	26,550	326,300
cpm/mmole[1]	123,000		335,000
Internal distribution[2]			
C1 thru C5		9.8%	5.7%
C6		90.2%	94.3%
(+)-Tartaric acid			
Specific radioactivity			
cpm/mmole	100,500	106,800	2,300,000
cpm/mmole[3]	115,000	107,000	1,950,000
Internal distribution[4]			
C1 + C4		96.5%	99.0%
C2 + C3		3.5%	1.0%

[1] 5,6-*O*-Isopropylidene derivative of L-ascorbic acid, second crystallization.
[2] Percentage of total ^{14}C in L-ascorbic acid, C1 through C5 by difference.
[3] Diphenacyl derivative of tartaric acid, second crystallization.
[4] Percentage of total ^{14}C in tartaric acid.

Calcium oxalate was dissolved in 6 N HCl, evaporated to dryness at reduced pressure and sublimed as its free acid at 40 C under reduced pressure (Kontes Bantamware sublimation apparatus, No. K-306500).

Ascorbic acid was recovered by procedures already reported (20). A portion of the crystalline sample was converted to its isopropylidene derivative.[2] Ascorbic acid was degraded to determine the amount of radioactivity in carbon 1 and carbon 6 (20). L-Galactono-1,4-lactone-6-^{14}C was degraded by the same procedure to verify the location of ^{14}C.

Tartaric acid was recovered as its potassium acid salt (37). A portion was converted to diphenacyl tartrate using the procedure previously used to prepare diphenacyl malate (24). To determine the amount of ^{14}C in the carboxyl carbons, a portion of the tartaric acid was degraded (34). When ^{14}C formic acid was treated with $NaIO_4$ under the same conditions used for tartaric acid oxidation, no $^{14}CO_2$ was formed.

Determination of the Optical Rotation of *P. crispum* Tartaric Acid. To determine the optical rotation of tartaric acid from *P. crispum*, a large sample was extracted from unlabeled cuttings, purified by ion exchange chromatography, recovered as its potassium acid salt after two recrystallizations from water, and then reconverted to the free acid. Rotations were made in 1-dm tubes on a Perkin-Elmer Model 141 photoelectric polarimeter.

All radioactive measurements were made on a Packard Model 3320 liquid scintillation spectrometer. Samples were dissolved in 1 ml of water to which was added 10 ml of toluene-Triton X-100 liquid scintillation mixture. The efficiency of counting averaged 70%. When samples of potassium acid tartrate were counted, it was necessary to convert these samples to the free acid prior to addition of the liquid scintillation fluid.

RESULTS

Results from four experiments are given in Table I. When L-galactono-1,4-lactone-U-^{14}C was supplied as labeled precursor, 20.5% of the soluble radioactivity, corresponding to 18% of the lactone supplied, appeared as labeled ascorbic acid, tartaric acid, and oxalic acid. In this experiment, 41% of the galactonolactone was recovered unchanged or as its free acid.

When L-galactono-1,4-lactone-6-^{14}C was given, a substantial portion of the ^{14}C was found in tartaric acid while negligible label appeared in oxalic acid. Degradation of labeled L-ascorbic acid showed 90.2% of the ^{14}C to be incorporated in carbon 6. In the tartaric acid over 96% of the ^{14}C was located in the carboxyl carbons (Table II).

An experiment with L-ascorbic acid-6-^{14}C gave results similar to those obtained with L-galactone-1,4-lactone-6-^{14}C. Nearly one-third of the ^{14}C in the soluble fraction was recovered as tartaric acid, while oxalic acid was virtually devoid of label. Degradation of labeled tartaric acid from this experiment also gave similar results (Table II). Data identifying L-ascorbic acid and tartaric acid obtained from these experiments are shown in Table II. When L-ascorbic acid-1-^{14}C was used in the place of L-ascorbic acid-6-^{14}C, only 0.4% of the label in the soluble fraction appeared in tartaric acid and 12.1% appeared in oxalic acid.

[2] The melting point given in reference 20 is in error. The correct melting point is 219 to 220 C. The isopropylidene ascorbic acid is crystallized from acetone-*n*-heptane, 1:1.

Table III. *Time Course of Labeling P. crispum with L-Ascorbic Acid-6-^{14}C*

Fraction	Hr of Incubation				
	4[1]	10[1]	24[1]	72[1]	72[2]
	% of 0.1% oxalic acid-soluble radioactivity				
Neutral	20.2	15.0	13.9	12.6	12.9
Cationic	4.8	5.5	13.6	15.0	18.0
Anionic					
L-Ascorbic acid	54.7	52.1	43.1	36.3	22.3
(+)-Tartaric acid	2.2	6.9	9.1	17.8	32.3
Oxalic acid	0.01	0.01	0.01	0.01	0.03
Other	16.2	18.3	17.3	16.7	15.9

[1] 1.24×10^6 cpm supplied to each experiment. Average fresh wt of cuttings was 1.7 g/experiment.

[2] 2.07×10^6 cpm supplied to 2.24 g fresh wt of cuttings.

To follow the metabolic appearance of ^{14}C from L-ascorbic acid-6-^{14}C, several experiments ranging from 4 to 72 hr were run (Table III). For purposes of comparison, data from the corresponding experiment in Table I are included. Over the period examined, recoverable labeled ascorbic acid decreased to about 30% of its initial value. In the same period, there was a rise in the appearance of label in tartaric acid. Virtually no label appeared in oxalic acid over the time course of this study. Two other changes were noted. There was a progressive decrease in the amount of ^{14}C recovered as neutral compounds accompanied by a rise in the amount of ^{14}C bound to Dowex 50 (H^+) resin. The nature of these changes was not examined.

In another experiment, not reported here, a time course study of the metabolism of L-ascorbic acid-1-^{14}C, similar to that reported here for L-ascorbic acid-6-^{14}C, resulted in an increase in labeled oxalic acid which qualitatively resembled the increase in tartaric acid observed with time from L-ascorbic acid-6-^{14}C labeling.

In Table IV, the optical rotation of tartaric acid from *P. crispum* is compared with those values obtained on a commercial sample and on two samples of tartaric acid prepared by Stafford (35) from *Pelargonium hortorum* and *Parthenocissus*. All four samples were analyzed at the same time, at a single concentration and at the same temperature. Specific rotations of all four samples were similar and very close to values reported for (+)-tartaric acid.

DISCUSSION

It is clear from the results reported in this study that the biosynthesis of tartaric acid in *Pelargonium crispum* is related to L-ascorbic acid metabolism, but the path of conversion differs from that found for the grape berry by Saito and Kasai (31). In the latter, carbon 1 of L-ascorbic acid becomes one of the carboxyl carbons of tartaric acid. The observations of Loewus and Stafford (23) indicate that carbon 6 does not get converted to tartaric acid, although a substantial portion of this carbon is reutilized by the grape leaf, possibly as a two carbon fragment corresponding to C5+C6 of L-ascorbic acid which can enter normal metabolic processes since L-ascorbic acid-6-^{14}C, in the grape leaf, gives rise to hexose products predominantly labeled in carbons 1, 3, 4, and 6.

In *P. crispum*, it is carbon 6 of L-ascorbic acid, or its precursor L-galactono-1,4-lactone, that provides a carboxyl carbon to (+)-tartaric acid. Unlike the grape, where L-ascorbic acid-1-^{14}C acts as a source of carboxyl label, in *P. crispum* only a trace of ^{14}C appears in tartaric acid when this label is supplied. Further, it has been found that carbon 1 of L-ascorbic acid is converted to oxalic acid in *P. crispum*. This appears to be the first such observation in plant tissues although the conversion of carbons 1 and 2 of L-ascorbic acid to oxalic acid in animals has been shown (1, 3–10, 15).

Table IV. *Specific Rotations of Tartaric Acid*

Source	$[\alpha]_{24}^{D}$ (concn 4.8, H_2O)
(+)-Tartaric acid (Fisher Sci.)	+14.65
Pelargonium leaf (Stafford, 35)	+14.88[1]
Parthenocissus. leaf (Stafford, 35)	+14.70
Pelargonium apex (Wagner and Loewus)	+15.00

[1] Value reported for same sample in Stafford's (35) paper was +15.9.

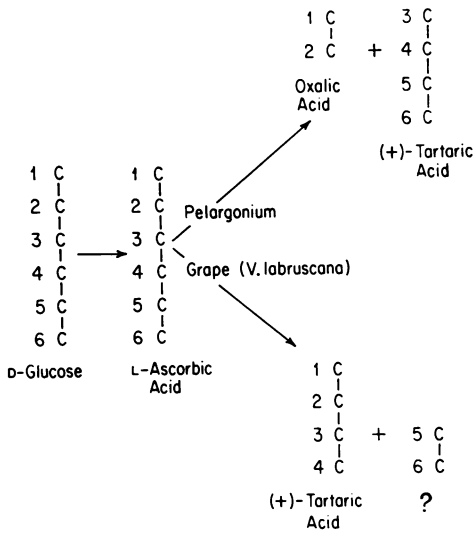

FIG. 1. A scheme for the biosynthesis of (+)-tartaric acid and oxalic acid from D-glucose and L-ascorbic acid in *P. crispum* and (+)-tartaric acid in the grape berry, Saito and Kasai (31).

The observations reported here lend support to Maroc-Gyr's (25) finding that D-glucose-6-^{14}C is a better source of label to tartaric acid in *Pelargonium* than D-glucose-1-^{14}C. If it is assumed that the conversion of D-glucose to L-ascorbic acid proceeds without inversion as has been found in several other plant species (19), then it follows from results obtained in the present study, that D-glucose-6-^{14}C would be a better source of label to tartaric acid than D-glucose-1-^{14}C in *Pelargonium*. Some redistribution of label does occur between carbon 1 and carbon 6 during passage of labeled hexose through the hexose phosphate pool, but this effect has seldom led to complete equilibration of label between carbon 1 and carbon 6 in previous studies of the type reported here.

Figure 1 summarizes the observations reported here for *P. crispum* and compares these findings with those reported earlier by Saito and Kasai (30, 31) for the grape berry.

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