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L-Ascorbic Acid Biosynthesis in Higher Plants from L-Gulono-1,4-lactone and L-Galactono-1,4-lactone[1]

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ABSTRACT

Detached bean (*Phaseolus vulgaris*) and strawberry (*Fragaria*) fruits fed L-gulono-1,4-lactone or L-galactono-1,4-lactone convert this compound, in part, to L-ascorbic acid. When L-galactono-1,4-lactone is given as a 0.25% solution to detached bean shoots, the ascorbic acid content is tripled in less than 10 hours. L-Gulono-1,4-lactone is only 5 to 10% as effective as its epimer. Experiments with specifically labeled L-gulono-1,4-lactone and L-galactono-1,4-lactone prove that conversion is direct. Ascorbic acid is labeled at the same carbon as its precursor.

A method is described for preparation of L-galactono-1,4-lactone-2-^{14}C from *myo*-inositol-2-^{14}C. This method can be extended to the preparation of L-ascorbic acid-2-^{14}C on the basis of results obtained in the present study.

During the conversion of an uronic acid ester or lactone to ascorbic acid in higher plants, reduction of carbon-1 precedes the oxidative step (6, 15). Thus, D-galacturonate methyl ester is converted to L-galactono-1,4-lactone, and D-glucurono-1,4-lactone is converted to L-gulono-1,4-lactone. These L-aldono-1,4-lactones, but not the corresponding acids, are then oxidized to L-ascorbic acid. Experiments with labeled D-galacturonate methyl ester and D-glucurono-1,4-lactone have confirmed these observations. Cell-free preparations indicate that the specificity of the lactone that is finally oxidized may be limited to L-galactono-1,4-lactone or a compound configurationally similar to the lactone (16).

Further information concerning this portion of the pathway of L-ascorbic acid biosynthesis from uronic acids is now obtained by examining conversion of labeled L-gulono-1,4-lactone and L-galactono-1,4-lactone in detached plant tissues. Preliminary reports of this work have been presented.[4]

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[4] See S. Kelly and F. Loewus, Abstract No. 32, Annual Meeting of the Western Section, American Society of Plant Physiologists, Palo Alto, Calif., June, 1963; and M. M. Baig and F. Loewus (1), National Meeting, American Society of Plant Physiologists, Amherst, Mass., August, 1968.

MATERIALS AND METHODS

Preparation of L-Galactono-1,4-lactone-2-^{14}C. *myo*-Inositol-2-^{14}C is readily converted to D-galacturonic acid-5-^{14}C in detached ripening strawberries (13). In this study, three ripening strawberries (5 g) were administered *myo*-inositol-2-^{14}c (2 μc, 0.5 μmole in 0.1 ml of water) through freshly severed stems. Label was taken up by the berries in about 2 hr. After 24 hr, berries were ground in 70% ethanol (v/v), and the insoluble residue was washed with fresh portions of solvent until all of the soluble ^{14}C was removed. The residue was hydrolyzed with Pectinol R-10 (Rohm and Haas' commercial pectinase) and D-galacturonic acid (14 mg, containing 21% of the ^{14}C supplied) was recovered by procedures already described (13). The labeled D-galacturonic acid was converted to its sodium salt and was reduced to the corresponding L-galactonate with sodium borohydride. Recovery of the free acid was achieved by placing the borohydride reaction mixture (after destroying excess borohydride with formic acid) on a column of Dowex 1 exchange resin (formate form) and then by eluting with a formic acid gradient (13). The free acid was converted to its 1,4-lactone by flash evaporation from glacial acetic acid and finally, by heating the residue to 60 C under reduced pressure. A portion of the lactone was converted to its trimethylsilyl ether, and was found to have the same retention time on SE-30 and OV-1 gas chromatographic columns as an authentic sample of tetrakis-*O*-trimethylsilyl-L-galactono-1,4-lactone (9). The final product had a specific radioactivity of 6 μc per mmole.

Preparation of L-Gulono-1,4-lactone-1-^{14}C and -6-^{14}C. D-Glucuronic acid-6-^{14}C and-1-^{14}C, respectively, were converted to sodium salts, were reduced to L-gulonic acids, were purified and lactonized as described above. The labeled D-glucuronic acids used in this preparation had been purified previously (20). Final specific radioactivity of L-gulono-1,4-lactone-1-^{14}C was 78 μc per mmole, and that of L-gulono-1,4-lactone-6-^{14}C was 158 μc per mmole.

Plant tissue. Strawberries (*Fragaria*, cv. 'Redglow') were harvested from greenhouse-grown plants during summer months. Only berries at the green-white stage of ripening were used.

Young bean shoots (*Phaseolus vulgaris*, var. 'Pinto') were harvested from 7- to 8-day-old greenhouse-grown seedlings. Shoots were detached just above the cotyledons. Each shoot bore a single pair of leaves, 3 to 4 cm in width.

Labeling Procedure and Recovery of Labeled Products. A freshly severed stem of a detached berry or shoot was placed in a vial containing an aqueous solution of the labeled lactone. When nearly all of the radioactive solution had been taken up, small increments of water were added to keep the cut surface of the stem moist and to ensure uptake of remaining activity.

Tissues were ground in a Sorvall Omnimixer in 0.1% oxalic acid (10 ml/g fresh weight) for 1½ min at full speed. Insoluble residue was removed by centrifuging the suspension for 1 hr at 10,000*g*. Ascorbic acid was determined by titrating an aliquot of the supernatant solution with 2,6-dichlorophenolindophenol

Table I. *The Ascorbic Acid Content of Detached Bean Shoots after Feeding Aqueous Solutions of L-Gulono-1,4-lactone and L-Galactono-1,4-lactone*

Lactone Concn	No. Hr in Lactone Solution		
	6	24	48
%	*mg/100 g fresh tissue*[1]		
L-Gulono-1,4-lactone			
0	70	70	50
0.1	70	90	35
0.25	70	85	30
0.5	85	105	30
1.0	90	95	30
1.5	90	95	30
2.0	110	100	30
L-Galactono-1,4-lactone			
0	70	70	50
0.1	170	[2]	[2]
0.25	245	[2]	[2]
0.5	255	[2]	[2]

[1] Values given are the average (±10 mg/100 g fresh tissue) of two separate experiments.

[2] Shoots wilted.

Table II. *Ascorbic Acid Content and Incipient Wilting Time of Detached Bean Shoots after Feeding Aqueous Solutions of L-Galactono-1,4-lactone in the Absence and Presence of 0.5% myo-Inositol*

L-Galactono-1,4-lactone Concn	No. Hr in Lactone Solution			
	7	9	39	57
%	*mg/100 g fresh tissue*			
No *myo*-inositol added				
0	70	70	70	50
0.05	—	—	—	170[1]
0.1	—	—	200[1]	—
0.25	—	260[2]	—	—
0.5	270[2]	—	—	—
myo-Inositol added				
0	70	70	70	50
0.05	—	—	—	145[1]
0.1	—	—	225[1]	—
0.25	—	265[2]	—	—
0.5	260[2]	—	—	—

[1] No evidence of wilt at the time shoots were assayed for ascorbic acid.

[2] Shoots had just begun to wilt.

(19). As soon as possible after determining the ascorbic acid content, a preweighed quantity of L-ascorbic acid (20–40 mg) was added to minimize loss of labeled ascorbic acid due to oxidation. Oxalic acid was removed from solution as its insoluble calcium salt by adding an amount of 2 M calcium formate equivalent to oxalic acid initially added for grinding.

Ascorbic acid was recovered by ion exchange chromatography (3) and was further purified by crystallization from glacial acetic acid. A portion of the crystallized ascorbic acid was converted to 5,6-*O*-monoisopropylidene derivative and was recrystallized. Comparison of the specific radioactivity of the unsubstituted ascorbic acid with its isopropylidene derivative established the specific radioactivity of the product in each experiment.

Other labeled components from neutral effluent and acidic fractions that were recovered during ion exchange chromatography were set aside for separate investigation.

Degradation of L-Ascorbic Acid. A detailed description of methods used to determine the distribution of ^{14}C in specific carbons of ascorbic acid is presented elsewhere (10). In this study, decarboxylation of ascorbic acid in 8 N sulfuric acid yielded carbon-1 as carbon dioxide. Carbons-1 + 2 were recovered as calcium oxalate after sodium hypoiodide oxidation. Calcium oxalate was converted to its free acid and was further purified by sublimation. Carbon-6 was recovered as its formaldehyde dimedon derivative after ascorbic acid had been oxidized with sodium periodate.

Gas-liquid chromatography and radioisotope-counting procedures were the same as those used in previous studies (9, 20).

RESULTS

Prior to experiments with labeled material, ascorbic acid biosynthesis was examined in detached bean shoots that had been given unlabeled L-gulono-1,4-lactono 1,4-lactone (Table I). When L-gulono-1,4-lactone was fed to bean shoots, there was a 25 to 30% increase in ascorbic acid content within 6 hr, if the lactone concentration was above 0.5%. At 24 hr, even the lowest lactone concentration used, 0.1%, caused this increase. Beyond 24 hr, the content of ascorbic acid declined in all samples. Wilting was absent, even at 2% L-gulono-1,4-lactone, up to 48 hr.

Compared to results obtained with L-gulono-1,4-lactone, detached bean shoots fed L-galactono-1,4-lactone produced substantial amounts of ascorbic acid over that present in controls. This increase was accompanied by a pronounced tendency to wilt. Shoots held in 0.25% L-galactono-1,4-lactone for 6 to 10 hr tripled their ascorbic acid content; when they were held for longer periods, wilting occurred. Incipient wilting corresponded to a level of approximately 250 to 260 mg of ascorbic acid per 100 g of fresh tissue. This level was observed over a range of concentrations of lactone fed to shoots (Table II). At 0.1% lactone, shoots seldom reached the ascorbic acid level associated with wilting. In these samples, wilt was not observed even after 57 hr.

The possible relationship between wilt and L-ascorbic acid was further examined. Experiments were run in which 0.25, 0.5, and 1% solutions of L-ascorbic acid, unbuffered or buffered to pH 4, 4.5, or 5 with sodium bicarbonate, were fed to detached bean shoots. Similar results were obtained regardless of the pH of the solution fed. At 0.25%, shoots seldom accumulated more than 150 mg of ascorbic acid per 100 g fresh weight of tissue during the first 30 hr of uptake, and wilting was not observed. At 0.5 to 1%, the ascorbic acid content of the shoots rose rapidly above 250 mg/100 g fresh weight of tissue, and wilting occurred invariably when the threshold value was reached (29 hr for shoots held in 0.5% solution of L-ascorbic acid, and 9 hr for those held in 1%). These results indicated that 7- to 8-day-old bean shoots could tolerate L-ascorbic acid at tissue concentrations approximately twice as high as normal, but that higher levels caused wilt.

The effect of *myo*-inositol on L-ascorbic acid biosynthesis from L-galactono-1,4-lactone was tested inasmuch as *myo*-inositol, exogenously supplied to detached plants, is converted in part to L-gulonic acid (14). The presence of 0.5% *myo*-inositol, either in water controls or solutions of L-galactono-1,4-lactone ranging from 0.05 to 0.5%, had no effect on L-ascorbic acid biosynthesis, and the onset of wilting was similar to that observed in shoots given only L-galactono-1,4-lactone (Table II).

The effect of *myo*-inosose-2 on L-ascorbic acid biosynthesis from L-galactono-1,4-lactone was examined briefly. At 0.1 and 0.5% *myo*-inosose-2, ascorbic acid levels remained similar to water controls up to 48 hr (Table III). However, the rate of L-ascorbic acid biosynthesis from 0.1% L-galactono-1,4-lactone was reduced in the presence of *myo*-inosose-2 although the level

Table III. *Ascorbic Acid Content of Detached Bean Shoots after Feeding Aqueous Solutions of myo-Inosose-2 in the Absence and Presence of 0.1% L-Galactono-1,4-lactone*

myo-Inosose-2 %	No. Hr in Inosose Solution			
	6	12	24	48
	mg/100 g fresh tissue			
No L-galactono-1,4-lactone added				
0	—	—	70	50
0.1	—	—	68	50
0.5	—	—	70	48
L-Galactono-1,4-lactone added				
0	70	70	70	—
0.1	150	175	180	—
0.25	115	150	220	—
0.5	90	100	180	—

finally reached at 24 hr (approximately 200 mg of L-ascorbic acid/100 g fresh weight) remained unchanged. In the absence of detailed information on the metabolism of *myo*-inosose-2 in bean shoots, further study of this effect was deferred.

Experiments with Labeled Lactones. Table IV summarizes results on the metabolism of L-galactono-1,4-lactone-2-^{14}C, L-gulono-1,4-lactone-1-^{14}C, and L-gulono-1,4-lactone-6-^{14}C by detached ripening strawberries and bean shoots in six experiments. Of the radioactivity supplied, 80% or more was taken up by the tissues in the metabolic period. Most of this was ultimately recovered as water-soluble components with a distribution of approximately two to one between acidic and neutral compounds. Very little ^{14}C was released as carbon dioxide, even in tissues fed L-gulono-1,4-lactone-1-^{14}C. About 10 to 15% of the ^{14}C in the tissues was trapped in discarded residue. Vigorous extraction would have released most of this radioactivity into solution; however, this was not done because it also decreased the amount of ascorbic acid that could be recovered in reduced form.

Neutral components in the soluble fraction accounted for 15 to 29% of the label fed. Paper chromatography showed that none of this neutral radioactivity was associated with soluble sugars. If the neutral fraction was first treated with dilute ammonium hydroxide, and then separated on paper in a solvent system that resolved simple sugars, all of the ^{14}C remained on the origin, and none migrated with the sugar components. A considerable portion of this labeled material appeared to be either starting material or closely related products. Identification of labeled components in this fraction is under investigation.

From 40 to 62% of the soluble radioactivity was present as acidic substances. It was eluted from the ion exchange column as two distinct peaks, one in the eluate volume occupied by gulonic and galactonic acids, and the other in the volume corresponding to ascorbic acid. Tissues fed L-galactono-1,4-lactone-2-^{14}C produced a very small radioactive peak in the aldonic acid region and a very substantial peak in the ascorbic acid region, whereas those fed L-gulono-1,4-lactone-1-^{14}C or -6-^{14}C had their labeled acidic components primarily in the aldonic acid peak. In a preliminary attempt to identify the labeled acids recovered as "aldonic" acids, portions of each sample were lactonized and were separated by paper chromatography (22). Both L-gulono- and L-galactono-1,4-lactone fed tissues contained a labeled "aldonic" acid component that lactonized and migrated with about the same speed is L-galactono-1,4-lactone; however, further attempts to recover and identify the radioactivity as L-galactono-1,4-lactone by carrier dilution were unsuccessful (G. Wagner, unpublished observation) and, therefore, further study is indicated.

Table IV. *Distribution of Radioactivity in Soluble Constituents from Detached Strawberry Fruits and Bean Shoots after Feeding Specifically Labeled L-Galactono-1,4-lactone and L-Gulono-1,4-lactone*

	Galactonolactone ^{14}C-2		Gulonolactone ^{14}C-1		Gulonolactone ^{14}C-6	
	Bean	Strawberry	Bean	Strawberry	Bean	Strawberry
Tissue (g fresh wt.)	2.0	5.9	1.9	6.1	1.9	6.6
Lactone fed						
(mg)	4.5	4.5	1	1	1	1
(μc)	0.11	0.11	0.5	0.5	0.9	0.9
Metabolism (hr)	24	41	24	41	24	41
Uptake (% of ^{14}C)	84	80	100	86	84	90
Recovery (% of ^{14}C)[1]						
Neutral components	29	22	25	26	15	24
Acidic components[2]	55	42	62	40	55	52
Aldonic peak	(7)	(13)	(49)	(31)	(49)	(47)
Ascorbic peak	(48)	(29)	(13)	(9)	(6)	(5)

[1] Uptake = neutral components + acidic components + unrecovered radioactivity in the sedimented fraction.

[2] All of the radioactivity in the acidic components was recovered in the aldonic and ascorbic peaks and represents the sum of values given in parentheses.

Table V. *Distribution of Radioactivity in Ascorbic Acid from Detached Strawberry Fruits and Bean Shoots after Ion Exchange Chromatography*

The values given in this table refer to undiluted ascorbic acid.

	Galactonolactone ^{14}C-2		Gulonolactone ^{14}C-1		Gulonolactone ^{14}C-6	
	Bean	Strawberry	Bean	Strawberry	Bean	Strawberry
Ascorbic acid recovered						
(mg)	3.1	6.7	0.7	4.4	1.2	3.2
mg/g fresh wt.	1.5	1.1	0.4	0.7	0.7	0.5
Specific radioactivity						
dpm/mmole	240	45	1000	130	590	170
dpm/mmole[1]	238	47	990	134	630	176
Internal distribution[2]						
Carbon-1	<1	<1	99	99	<1	<1
Carbons-1 and 2	99	99	99	99	<1	<1
Carbon-6	<1	<1	<1	<1	99	99

[1] 5,6-*O*-Isopropylidene derivative of L-ascorbic acid.

[2] Reported as percentage of total ^{14}C in ascorbic acid.

Labeled ascorbic acid was recovered in each of the six experiments (Table V). In tissues given labeled L-gulono-1,4-lactone, the ascorbic acid yield approximated that in water controls (see Table I). In tissues given labeled L-galactono-1,4-lactone, the yield was 2 to 3 times greater than the water control (see Table II), evidence that a net synthesis of ascorbic acid had occurred. After adding unlabeled L-ascorbic acid as carrier, each product was crystallized and its specific radioactivity was measured. A portion was further converted to its 5,6-*O*-mono-isopropylidene derivative and was recrystallized. Comparison of specific radioactivities of ascorbic acid and its mono-isopropylidene derivative for each sample showed that there was very little change, thus confirming the radiochemical identity of the product.

Part of each ascorbic acid sample was degraded to determine the location of ^{14}C in the carbon chain. In each sample (Table V), the ^{14}C was recovered from ascorbic acid in the carbon corresponding to the site labeled in the precursor lactone. None of the ^{14}C redistributed into other carbons of the chain. Results indicated that the carbon chains of L-gulono-1,4-lactone and L-galactono-1,4-lactone were conserved in the oxidative step.

DISCUSSION

D-Glucurono-1,4-lactone is converted by detached strawberry fruits, in part, to L-gulonic acid (12) and L-ascorbic acid (3). Although direct experimental evidence must still be obtained, it is likely that L-gulono-1,4-lactone is an intermediate. Pea extracts reduce D-glucurono-1,4-lactone at about one-third of the rate shown by D-galacturonate methyl ester (17). Hydrolysis of L-gulono-1,4-lactone to L-gulonic acid may be nonenzymatic, because Mapson is unable to detect lactonase activity in plant tissues (15, see p. 378). Peas also contain a particulate fraction that oxidizes L-gulono-1,4-lactone to L-ascorbic acid, albeit at a fraction of the rate obtained with L-galactono-1,4-lactone (18). A particulate preparation from cauliflower florets, however, oxidizes only L-galactono-1,4-lactone (16).

In the present study, conversion of L-gulono-1,4-lactone and L-galactono-1,4-lactone to L-ascorbic acid is examined in two tissues, detached bean shoots and detached strawberry fruits. Bean shoots held in 0.5% L-gulono-1,4-lactone show a small increase in L-ascorbic acid; nevertheless, this increase is only about one-tenth of that obtained in 0.5% L-galactono-1,4-lactone. Experiments with specifically labeled L-gulono-1,4-lactone and L-galactono-1,4-lactone show that conversion to L-ascorbic acid is real, and is not an indirect stimulation of L-ascorbic acid formation from other endogenous compounds. Further, this conversion takes place without altering the carbon skeleton.

In mammalian systems, conversion of L-gulono-1,4-lactone to L-ascorbic acid is probably a single enzymatic step (15). Whether this is so in plants is not known. The present findings demonstrate that such a conversion is effected, but it does not reveal the enzymatic path. At least three possibilities can be considered: direct conversion over the same enzyme that oxidizes L-galactono-1,4-lactone; direct conversion over a separate enzyme with a specificity for the L-gulono rather than the L-galactono configuration; or a two-step process in which epimerization of carbon-3 precedes oxidation. Should the third possibility pertain, the rate-limiting step must reside at epimerization, because appreciable quantity of L-gulono-1,4-lactone is recovered from our labeled tissue as L-gulonic acid, and yet there is no trace of the corresponding L-L-galactonic acid, even at the limits of detection of the gas chromatograph (G. Wagner, unpublished observations).

Two pathways of ascorbic acid biosynthesis are present in higher plants (8). Both require a six-carbon product of hexose metabolism, and this carbon chain is conserved during conversion. The pathway from D-glucose involves an oxidation of carbon-1 of the sugar, an epimerization of carbon-5, and retention of the primary alcohol function at carbon-6. New information pertinent to this pathway is found in a report that describes a naturally occurring derivative of 5-keto gluconate (2) and also in the observation that carbons 1 through 4 of L-ascorbic acid are converted to (+)-tartaric acid in grape berries (21).

The other pathway, from uronic acid esters or lactones, involves reduction of the aldehydic function which eventually becomes carbon-6 of L-ascorbic acid. The carboxyl function is conserved as carbon-1 of L-ascorbic acid. A theoretical scheme of L-ascorbic acid biosynthesis in plants, first proposed by Isherwood *et al.* (5) in analogy to that found in animals (4, 5), stresses the role of the galacto configuration and names D-galactose as the ultimate precursor (15). There is no evidence to indicate that the conversion of D-galactose to L-ascorbic acid differs significantly from that of D-glucose (8). As regards subsequent steps from a uronic acid derivative and its reduction product, L-aldono-1,4-lactone, plants utilize both D-glucurono-1,4-lactone (L-gulono-1,4-lactone) and D-galacturonate (L-galactono-1,4-lactone). Whether these compounds share a common intermediate at a point short of L-ascorbic acid formation with the pathway from D-glucose is not known (7, 11).

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