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Loss of Hydrogen from Carbon 5 of D-Glucose during Conversion of D-[5-^{3}H,6-^{14}C]Glucose to L-Ascorbic Acid in *Pelargonium crispum* (L.) L'Hér[1]

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ABSTRACT

Conversion of D-[5-^{3}H,6-^{14}C]glucose to L-ascorbic acid in detached apices of *Pelargonium crispum* (L.) L'Hér cv Prince Rupert (lemon geranium) was accompanied by complete loss of tritium in the product. Chemical degradation of D-glucose which was recovered from the labeled apices yielded D-glyceric acid (corresponding to carbons 4, 5, and 6 of glucose) with a ^{3}H:^{14}C ratio of 4 to be compared with 9, the ratio in D-[5-^{3}H,6-^{14}C] glucose initially. Conversion of D-[6-^{3}H,6-^{14}C]glucose in the same tissue was accompanied by retention of tritium in L-ascorbic acid with a ^{3}H:^{14}C ratio comparable to that of compounds from the hexose pool. Results indicate that during L-ascorbic acid biosynthesis from glucose in *Pelargonium crispum* hydrogen at carbon 5 undergoes exchange with the medium, suggesting an epimerization at this carbon atom.

L-Ascorbic acid is a product of D-glucose metabolism in plants as well as in those animals which have retained the capacity for AA[3] biosynthesis. In contrast to AA formation in animals, the process of conversion in higher plants proceeds without inversion of the carbon skeleton of D-glucose (8). The L-configuration of AA is inherent at carbon 2 of D-glucose for the 'inversion' pathway of AA-synthesizing animals. In plants, however, the conversion of D-glucose to AA must involve a configurational change at carbon 5 in addition to the oxidation steps at carbon 1 and carbon 2 or 3. Inasmuch as the intermediate steps between D-glucose and AA, in the case of plants, are unknown, one can only speculate on the enzymic mechanism of the epimerizing step. Possible mechanisms include a pyridine nucleotide-linked oxidation-reduction, an oxidation-reduction linked to hydrogen carriers other than pyridine nucleotide, or a keto-enol rearrangement (1, 4).

The first type, best characterized by UDP-D-glucose 4'-epimerase, EC 5.1.3.2, involves the removal of hydrogen from one side of the carbon atom that undergoes epimerization followed by the transfer of this same hydrogen back to the opposite side. There is no exchange of this hydrogen with the medium. In the second type, an exchange of hydrogen with the medium may or may not occur. One example in which an exchange does not occur is L-ribulose-5-phosphate 4-epimerase, EC 5.1.3.4, which catalyzes the conversion of L-ribulose 5-phosphate to D-xylulose 5-phosphate (3, 12). An example of the third type is D-ribulose-5-phosphate 3-epimerase, EC 5.1.3.1, which interconverts D-ribulose 5-phosphate and D-xylulose 5-phosphate accompanied by an exchange of carbon 3 hydrogen with the medium (1, 3).

Here we describe experiments in which D-[5-^{3}H,6-^{14}C]glucose is supplied to detached apices of *Pelargonium crispum* to test the fate of carbon 5-bound hydrogen during the epimerization that accompanies AA biosynthesis. *P. crispum*, previously examined with regard to AA biosynthesis (10) and metabolism (5, 16, 17), converts D-glucose to AA by the 'direct' pathway in which the carbon chain sequence of the sugar precursor is conserved. This study also examines the changes in the ^{3}H:^{14}C ratio of the characteristic metabolic products oxalic acid, L-tartaric acid, and L-threonic acid which in *P. crispum* are due to cleavage of AA between carbons 2 and 3.

MATERIALS AND METHODS

Chemicals. D-[5-^{3}H]Glucose (15.6 Ci/mmol), D-[6-^{3}H]glucose (30.0 Ci/mmol), and D-[6-^{14}C]glucose (56.1 mCi/mmol) were purchased from New England Nuclear Corp. Calcium L-threonate was prepared from AA (6). Other chemicals were of analytical or reagent grade.

Labeling Procedure. *Pelargonium crispum* (L.) L'Hér cv Prince Rupert (lemon geranium) was grown under greenhouse conditions and regularly pruned to induce new apical growth. Apices consisting of three to four unfolded leaves were detached from the plant while the stem section to be cut was submerged in water. Two detached apices (fresh weight, 1.62 g) were transferred to a glass vial containing 0.2 ml D-[5-^{3}H,6-^{14}C]glucose solution (50.1 μCi; initial ^{3}H:^{14}C ratio, 9.16). Uptake of labeled solution (1.5 h) was followed by 0.1- to 0.2-ml increments of 0.1% (w/v) D-glucose (total 0.6 ml). About 6 h after labeling had been initiated, the apices were transferred to distilled H_2O for the remainder of the metabolic period (18 h). Throughout the experiment, light (8,000 lux) was supplied from two 40-w cool-white fluorescent lamps and one high intensity incandescent bulb. Apices were enclosed in a bell jar through which a constant stream of air was passed. Respired $^{14}CO_2$ and transpired ^3H_2O were trapped by two series-connected 250-ml gas dispersion bottles, each holding 150 ml 0.5 N KOH (17).

Extraction of Apices. Labeled apices were frozen in liquid N_2, ground in 10 ml 0.1% (w/v) DTT in a 100-ml Omnimixer chamber (DuPont/Sorvall) for 1 min at full power, and centrifuged (27,000*g*, 20 min, 5°C). The residue was re-extracted in 10 ml 0.1%

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[3] Abbreviation: AA, L-ascorbic acid.

DTT and lyophilized. To the combined extracts were added 50 mg each of oxalic acid, AA, L-tartaric acid, and calcium L-threonate.

Oxalic Acid. Calcium oxalate was precipitated by addition of 105 mg calcium acetate. Free oxalic acid was obtained by stirring the suspended calcium oxalate in 2 ml H_2O with the appropriate amount of Dowex 50W (X8-400 mesh, H^+ form) exchange resin. The ion exchanger was filtered off and the solution was evaporated. The residue was further purified by sublimation at 40°C and 0.01 mm Hg (15). The crude oxalate-free extract (30 ml) was fractionated by ion-exchange resins into cationic, neutral, and anionic compounds with slight modification to previously described methods (16). The eluent of the anionic fraction (Dowex-1) was passed through an UV detector (model 200, Pharmacia Fine Chemicals, Inc.; 254 nm) at a flow of 2.3 ml/min and collected in 8- to 10-ml fractions.

Ascorbic Acid. This acid was detected as the main UV-positive peak. Aliquots of the fractions were analyzed by HPLC (pump 6000A, injector U6K, Waters Associates, Inc.) on a 300 × 7.8 mm Aminex HPX-87 column (Bio-Rad Laboratories) with guard column (Waters Associates) filled with Aminex Q-15S (Bio-Rad). The eluent was 0.1 M formic acid (40°C) at a flow rate of 0.7 ml/min. Ascorbic acid was detected electrochemically at +0.7 v (amperometric detector LC-4 with glassy carbon flow cell LC-16, Bioanalytical Systems, Inc., West Lafayette, IN) with a retention time of 8.5 min (11). The fractions containing AA were combined, and 70 mg carrier AA was added, followed by recrystallization from glacial acetic acid until constant specific radioactivity was reached (7).

L-Tartaric Acid. Aliquots of the Dowex-1 fractions were tested for tartaric acid by HPLC with refractive index monitoring (RI detector model R-400, Waters Associates). The HPLC conditions were the same as described under AA. The retention time of tartaric acid was 7.2 min. The tartaric acid-containing fractions were combined. After addition of 70 mg tartaric acid, it was converted to its monopotassium salt and recrystallized (3×) from hot water (15). Final purification of L-tartaric acid was accomplished by HPLC using repeated 25-μl injections of 2 M formic acid (0.2 ml) containing 5 to 7 mg of the monopotassium salt.

L-Threonic Acid. This acid was analyzed by GLC as its trimethylsilyl derivative. Aliquots of 50 μl were dried down, mixed with 0.1 ml pyridine and 0.1 ml *N,O*-bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane), and heated 40 min at 40°C. The product was separated on a 1.8 m × 0.64 cm column of 3% OV-17 on GasChrom Q (Applied Science Laboratories, Division of Milton Roy Co., State College, PA) at 130°C (GC model 7821, Packard Instrument Company). L-Threono-1,4-lactone had a retention time of 3.54 min and L-threonic acid, 6.15 min. Threonic acid-containing fractions were diluted with 70 mg calcium L-threonate plus 27 mg calcium acetate and recrystallized three times from water and methanol (5). Calcium threonate was converted to the free acid and further purified by descending paper chromatography (15) on Whatman 3 (ethyl acetate-pyridine-acetic acid-H_2O-methanol, 7:5:1:1.5:1, 7 h). The threonic acid zone (R_F = 0.18) was cut out and eluted with H_2O.

Sugars. To aliquots of the neutral fraction (30 to 50 ml) were added sucrose, D-glucose, and D-fructose (1 mg each). Sugars were separated by descending chromatography in ethyl acetate-pyridine-water, 8:2:1 (v/v), on Whatman 3 paper (25 h). Regions corresponding to each of the added sugars, detected according to Trevelyan *et al.* (14) were eluted with H_2O. To each eluate was added 70 mg of the appropriate sugar followed by crystallization from water with ethyl alcohol.

Distribution of Radioisotopes in AA and Glucose. AA was degraded to obtain carbon 6 as formaldehyde which was recovered as the dimedon derivative (7). Glucose which was isolated from the neutral fraction by paper chromatography was degraded by a chemical procedure that released carbons 4, 5, and 6 as D-glyceric acid (2).

Analysis of Radioactivity. Aliquots from water-soluble samples in 0.5 ml H_2O were added to 5 ml Aquassure (New England Nuclear Corp.) in Pica-vials and counted in a Packard model 460-CD scintillation spectrometer. Samples containing standard [^{3}H] toluene and [^{14}C]hexadecane were used to obtain a quench curve for conversion of cpm to dpm values. Insoluble samples were burned in a biological oxidizer (Packard model 306B). Products of combustion were trapped in 15 ml Monophase-40 (^3H_2O) and 5 ml Carbosorb II, 9 ml Permafluor V ($^{14}CO_2$).

RESULTS

Table I summarizes the distribution of radioactivity in respired CO_2, water, soluble constituents, and insoluble residue after feeding D-[5-^{3}H,6-^{14}C]glucose for 18 h. As in other experiments involving the use of D-[5-^{3}H]glucose, there was a substantial loss of ^{3}H during metabolism due to exchange processes (9). This tritiated H_2O, when corrected for the actual recovery, amounted to a loss of 76%. The remaining ^{3}H was recovered primarily in sugars and insoluble residue. Very little ^{3}H was found in cationic or anionic constituents. Carbon-14 accumulated in insoluble residue, organic acids, and sugars. Only 4% was lost as respired $^{14}CO_2$.

Table II lists ^{3}H:^{14}C ratios for insoluble residue and for sugars that were recovered from the neutral fraction. To obtain a better perspective on distribution of radiolabel within the glucose pool after metabolism, free glucose from the neutral fraction was degraded chemically (2). The intermediates in this degradation gave values comparable to that of glucose. The final degradation product D-glyceric acid, corresponding to carbons 4, 5, and 6 of the starting glucose, had a ^{3}H:^{14}C ratio significantly greater than that of the glucose which had been degraded (Table II).

The ^{3}H and ^{14}C analyses of oxalic acid, AA, L-threonic acid, and L-tartaric acid are given in Table III. Within the limits of detection, AA contained no ^{3}H but small amounts of ^{3}H were present in L-threonic acid and L-tartaric acid even after repeated recrystallization and further purification by chromatography. In the case of L-tartaric acid, a ratio of 0.16 was obtained.

Degradation of labeled AA showed that a considerable redistribution of label from administered D-[6-^{14}C]glucose occurred. Carbon 6 of AA contained 55% of the total ^{14}C present in the

Table I. *Distribution of Radioactivity as Percentage of Recovered Label*[a] *in Fractions from D-[5-^{3}H,6-^{14}C]Glucose-Labeled Geranium Apices*

Fraction	^{3}H	^{14}C
CO_2		3.8
H_2O[b]	52.6	
Soluble fraction		
Neutrals	5.4	11.1
Cationic	0.2	1.2
Anionic	0.4	9.8
Insoluble residue	41.4	74.2

[a] Recovery of administered label: ^{3}H, 69%; ^{14}C, 99%.

[b] Sum of ^3H_2O recovered from KOH traps, feeding vial and neutral fraction.

Table II. *^{3}H:^{14}C Ratios in Sugars and Insoluble Residue from D-[5-^{3}H,6-^{14}C]Glucose-Labeled Geranium Apices*

Fraction or Compound	
Insoluble residue (lyophilized)	3.55
Sucrose	1.45
Fructose	2.91
Glucose	3.03
Carbons 4, 5, and 6 of glucose (as D-glyceric acid)	4.00

Table III. *Distribution of Radioactivity, Specific Radioactivity, and ^3H:^{14}C Ratio in Organic Acids from D-[5-^3H,6-^{14}C]Glucose-Labeled Geranium Apices*

Organic Acid	Total Radioactivity		Specific Radioactivity		^3H:^{14}C
	^3H	^{14}C	^3H	^{14}C	
	%		*dpm μmol*$^{-1}$		*ratio*
Oxalic acid	0.0	1.3	0	24	0.00
L-Ascorbic acid[a]	0.0	5.4	0	84	0.00
L-Threonic acid	0.6	2.9	3	43	0.06
L-Tartaric acid	3.5	6.0	13	80	0.16
Other[b]	95.8	84.4			0.32

[a] Analysis of AA after periodate oxidation and recovery of carbon 6 as its dimedon derivative revealed that it contained 55% of the ^{14}C present in AA.

[b] Pooled fractions from ion-exchange chromatography, exclusive of oxalic acid, AA, threonic acid, and tartaric acid.

molecule. The redistribution of ^{14}C in AA seen here was reflected in the presence of a substantial amount of ^{14}C in oxalic acid, a metabolic product of AA corresponding to C-1 plus C-2 (16). This experiment was repeated with a 24-h labeling period and produced data virtually identical with that presented here for the 18-h period.

An analogous experiment involving the use of D-[6-^3H,6-^{14}C] glucose-labeled apices (initial ratio, ^3H:^{14}C = 11.02) was carried out for 18 h. It was determined that newly synthesized AA had a ratio of 5.27. Glucose and fructose had ratios of 8.31 and 4.21, respectively.

DISCUSSION

Apices labeled with D-[6-^3H,6-^{14}C]glucose produced an AA whose ^3H:^{14}C ratio was comparable to those of free glucose and fructose from the same tissue. This is consistent with the direct pathway from glucose to AA which requires the intact incorporation of the hydroxymethyl group (carbon 6) of glucose (10). In contrast to that, complete loss of ^3H from carbon 5 occurred during conversion of D-[5-^3H,6-^{14}C]glucose to AA in detached geranium apices. This total loss of ^3H was not found in other products of D-glucose metabolism such as D-fructose and sucrose, or in D-glucose that was recovered from apices at the end of the labeling period. Chemical degradation of the latter produced D-glyceric acid (corresponding to carbons 4, 5, and 6 of glucose) with a ^3H:^{14}C ratio of 4 as compared to the ratio of 9 in D-[5-^3H,6-^{14}C]glucose which was fed to the apices. In other words, the glucose pool from which AA was produced retained a significant portion of the ^3H on carbon 5 throughout the metabolic period despite substantial redistributive processes that occurred. These processes include the recombination of triose moieties derived from hexose carbon chains leading to the appearance of ^{14}C in carbon 1 of glucose and AA, respectively. Aldolase and triose-P isomerase activities are responsible for that equilibration whereas the loss of ^3H is caused by isomerase activity at carbon 2 during the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (9, 13). Additional evidence of triose equilibration is seen in the appearance of ^{14}C in oxalic acid, a metabolic product derived from carbons 1 and 2 of AA (10, 16).

L-Threonic acid and L-tartaric acid, C_4 products of AA cleavage at the carbon 2/carbon 3 bond, are natural constituents in geranium (5, 16). The presence of low, yet significant, amounts of ^3H in these acids was unexpected in view of the fact that AA was devoid of ^3H. This ^3H may have accompanied a minor pathway of L-threonic acid biosynthesis exclusive of AA. Further studies are needed to clarify these results.

The present study demonstrates the presence of an exchange of hydrogen with the medium during the change in configuration about carbon 5 that accompanies conversion of D-glucose to AA in geranium. Of the three general mechanisms for epimerization noted earlier, direct transfer is unlikely unless an exchange of hydrogen on carbon 5 takes place in a step preceding epimerization. Further work will require characterization of intermediates that are involved in AA biosynthesis.

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