Plant Physiol. (1990) 94, 1496–1500
0032-0889/90/94/1496/05/$01.00/0

Received for publication July 10, 1990
Accepted August 20, 1990

Communication

D-Glucosone and L-Sorbosone, Putative Intermediates of L-Ascorbic Acid Biosynthesis in Detached Bean and Spinach Leaves[1]

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ABSTRACT

D-[6-^{14}C]Glucosone that had been prepared enzymically from D-[6-^{14}C]glucose was used to compare relative efficiencies of these two sugars for L-ascorbic acid (AA) biosynthesis in detached bean (*Phaseolus vulgaris* L., cv California small white) apices and 4-week-old spinach (*Spinacia oleracea* L., cv Giant Noble) leaves. At tracer concentration, ^{14}C from glucosone was utilized by spinach leaves for AA biosynthesis much more effectively than glucose. Carbon-14 from [6-^{14}C]glucose underwent considerable redistribution during AA formation, whereas ^{14}C from [6-^{14}C]glucosone remained almost totally in carbon 6 of AA. In other experiments with spinach leaves, L-[U-^{14}C]sorbosone was found to be equivalent to [6-^{14}C]glucose as a source of ^{14}C for AA. In the presence of 0.1% D-glucosone, conversion of [6-^{14}C] glucose into labeled AA was greatly repressed. In a comparable experiment with L-sorbosone replacing D-glucosone, the effect was much less. The experiments described here give substance to the proposal that D-glucosone and L-sorbosone are putative intermediates in the conversion of D-glucose to AA in higher plants.

Conversion of glucose[3] to AA in higher plants involves a pathway wherein the carbon chain and the hydroxymethyl group at carbon 6 of glucose are conserved. Oxidation of carbon 1 and carbons 2 or 3, and epimerization of carbon 5 represent minimal requirements for this conversion (10, 11, 14). None of the intermediates involved have been identified although a tentative scheme has been proposed (11):

D-glucose → D-glucosone → L-sorbosone → L-ascorbic acid

The first step, formation of glucosone, has been reported to occur in a red alga (5) and several species of macrofungi (2). Evidence of epimerization rests solely on isotopic studies involving overall conversion of glucose to AA (10, 13). The last step, oxidation of sorbosone to AA, has been described in bean and spinach (15).

An opportunity to test the role of glucosone as an intermediate in AA biosynthesis emerged when Liu *et al.* (9) developed a simple, one-step, enzymatic conversion of glucose to glucosone, readily adaptable to preparation of labeled substrate. Here, we present experiments that compare the relative efficiencies of [6-^{14}C]glucose and [6-^{14}C]glucosone as ^{14}C donors to AA under selected conditions in bean and spinach leaves. A limited study of sorbosone metabolism is also included.

MATERIALS AND METHODS

Preparation of Glucosone

Partially purified pyranose-2-oxidase, EC 1.1.3.10, from *Polyporus obtusus* (ATCC 26733) was immobilized on activated CH-Sepharose 4B (Pharmacia, Piscataway, NJ) (9). In a typical preparation, glucose (500 mg) and catalase (200 units, crystalline suspension from bovine liver, Sigma Chemical Co.) were added to sterile 15 mM potassium phosphate buffer (pH 6, 125 mL) in a 250-mL flask. To this was added immobilized pyranose-2-oxidase (5 gm, dry weight). The suspension was shaken (150 rpm, gyratory shaker, 25°C) and monitored for glucosone by HPLC (7). After complete conversion (usually 40 h), the suspension was filtered successively through Whatman No. 4 paper, and 0.8, 0.45, and 0.2 μm regenerated cellulose disks (Bioanalytical Systems, West Lafayette, IN). The filtrate was lyophilized or stored at −20°C. Glucosone was characterized as its hydrated 1,2:2,3:5,6-tri-*O*-isopropylidene derivative, melting point 125°C, theory 125°C (3).

To prepare [6-^{14}C]glucosone the procedure was downscaled as follows. [6-^{14}C]Glucose (0.6 mg, 12 μCi, NEN Corp.) was dried down in a 0.3-mL Reacti-vial (Aldrich Chem. Co.). To this was added catalase (1 mg, purified powder from bovine liver, Sigma Chemical Co.), 15 mM potassium phosphate buffer (pH 6.0, 20 μL), and immobilized pyranose-2-oxidase (150 μL containing 6 mg dry weight of immobilized enzyme). The suspension was stirred (30 h, 30°C, magnetic stirrer) and filter-centrifuged (0.2 μm regenerated cellulose membrane, Bioanalytical Systems, West Lafayette, IN), and the immobilized enzyme and filter were washed with buffer (60 μL). Combined filtrate and wash was purified by HPLC (7). Com-

[1] Supported by a grant from Hoffmann-LaRoche Inc. and Project 0266, College of Agriculture and Home Economics Research Center, Washington State University, Pullman, WA 99164.

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[3] Abbreviations: glucose, D-glucose; AA, L-ascorbic acid (L-*threo*-hexen-2-ono-1,4-lactone); glucosone, D-glucosone (D-*arabino*-hexos-2-ulose); sorbosone, L-sorbosone (L-*xylo*-hexos-2-ulose).

plete separation of glucosone from traces of glucose and gluconic acid was obtained in 1 mL fractions (2 mL/min solvent flow, 30°C, refractive index detection). Radiochemical purity was checked by TLC (9) or HPLC (7).

Treatment of Plant Tissues

Detached apices (growing tip plus two open leaves) from 2- to 3-week-old bean seedlings (*Phaseolus vulgaris* L., cv California small white) or individual leaves from 4-week-old spinach seedlings (*Spinacia oleracea* L., cv Giant Noble) were held in water or unlabeled solution for 1 to 4 h prior to labeling to check for signs of wilting. An individual bean apex or two spinach leaves were placed in each vial containing 100 μL of labeled solution. Uptake, completed in 1 to 2 h, was followed by water or unlabeled solution for the remainder of the metabolic period. Throughout the experiment, tissues were illuminated (10 Wm^{-2}, two 20-W fluorescent cool-white lamps and [bean only] one miniature high-intensity incandescent bulb). A continuous flow of air was drawn over the tissues (200 mL/min) and into a column of 2 M NaOH to trap respired CO_2. At the conclusion of the metabolic period, tissues from each vial in an individual experiment were pooled, sealed in aluminum foil and stored at −20°C until processed.

Extraction and Recovery of AA and Glycosyl Residues

Bean Apices

Frozen apices were pulverized, ground in 0.1 M formic acid containing 0.1% DTT (5 mL), and transferred to a centrifuge tube with additional medium (10 mL) containing AA (25 mg). Insoluble residue was removed (8000g, 10 min, 4°C), and the supernatant fractionated by ion exchange chromatography (17) with the slight modification that 0.01% DDT was included in all steps. Fractions under the AA peak were pooled, diluted with additional AA to assure sufficient material for degradative analysis, evaporated to a syrup, and crystallized from glacial acetic acid. Subsequent recrystallizations produced little or no change in specific radioactivity. Carbon-14 in carbon 1 was recovered by acid-catalyzed decarboxylation and that in carbon 6 by oxidative degradation followed by recovery of formaldehyde as its dimedon derivative corresponding to carbon 6 (12).

Insoluble residue from the 0.1 M formic acid extraction was resuspended in water (10 mL), filtered on Whatman No. 1 paper, dried, and pulverized. Separate aliquots were hydrolyzed in 2 M trifluoroacetic acid (2 h, 95°C) (1), filtered, concentrated to <0.5 mL, and subjected to descending chromatography on Whatman No. 1 paper in ethyl acetate-pyridine-water (8:2:1, v/v, 66 h). Regions corresponding to galactose and glucose were eluted with 50% ethanol, diluted with carrier sugar (200 mg), evaporated to syrups, and crystallized from ethanol. Oxidative degradation before and after sodium borohydride reduction yielded carbon 6 or carbons (1 + 6) as formaldehyde that was recovered as its dimedon derivative (6).

Spinach Leaves

Frozen leaves were pulverized and ground in 0.1% oxalic acid (2 × 12 mL) containing carrier AA (60 mg). After centrifugation to remove insoluble residues, oxalate was recovered as its insoluble calcium salt by adding an equivalent amount of calcium acetate (52.6 mg/mL). The neutral supernatant was fractionated by ion exchange chromatography to recover AA as described above.

Insoluble residues from the 0.1% oxalic acid extraction were assayed for ^{14}C by combustion in a biological oxidizer and hydrolyzed as described above. After centrifugation to remove insoluble material, the hydrolysates were lyophilized and extracted with 70% ethanol. These ethanol extracts were brought to dryness and taken up in a minimal volume of water (0.3 mL) for separation of sugar residues by HPLC on a 300 × 7.8 mm REZEX RQA-organic acid column (Phenomenex, Torrance, CA). The eluant was 1 mM H_2SO_4 at a flow rate of 0.6 mL/min and components were detected by refractive index. Glucose and galactose, the sugars of interest, gave baseline separation and were collected into separate fractions. After analysis of each fraction for ^{14}C, 150 mg of carrier sugar was added and each sample recovered by crystallization.

AA and residue-derived sugars, glucose and galactose, were degraded as described earlier.

Oxidation of AA with Ascorbate Oxidase

To characterize the labeled AA produced by [6-^{14}C]glucosone in spinach leaves, 6 mg of recrystallized product from experiment 4 (see Table II below) was dissolved in 0.2 M citrate-phosphate buffer (pH 5.6, 2 mL). To this solution was added 1 unit of ascorbate oxidase, EC 1.10.3.3 (Sigma Chemical Co.), 25°C. Ten-μL aliquots, diluted to 1 mL with water, were taken at intervals for spectrophotometric assay at 245 nm. Dehydroascorbic acid was separated from AA by placing the reaction mixture on a tandem set of ion exchange columns (Dowex 1 H^+ followed by Dowex 50 formate, 200 to 400 mesh, 8% cross-linked, 1 × 10 cm) and washing the columns with 100 mL of water. Eluate was collected in 10 mL fractions. Subsequently, a gradient of 250 mL water/500 mL 0.1 M

Table I. *Distribution of ^{14}C in Terminal Positions of Ascorbic Acid and Glycosyl Residues from [6-^{14}C]Glucose- and [6-^{14}C]Glucosone-fed Bean Apices*

Product Analyzed	^{14}C Recovered as % of Total ^{14}C		
	Carbon 1	Carbon 2–5	Carbon 6
		by difference	
D-[6-^{14}C]Glucose-fed apices			
Ascorbic acid	40	15	45
Glucosyl residues	15	47	38
Galactosyl residues	14	43	43
D-[6-^{14}C]Glucosone-fed apices			
Ascorbic acid	10	0	90
Glucosyl residues	15	57	28
Galactosyl residues	13	54	33

Table II. *Composition of Substrate Supplied to Detached Spinach Leaves*

Experiment No.	^{14}C-Labeled Substrate Compound	μCi	Unlabeled Substrate Compound	% (final)
1	D-[6-^{14}C]Glucose	10	None	
2	D-[6-^{14}C]Glucose	10	D-Glucosone	0.1
3	D-[6-^{14}C]Glucose	10	L-Sorbosone	0.4
4	D-[6-^{14}C]Glucosone	10	None	
5	D-[6-^{14}C]Glucosone	10	L-Sorbosone	0.2
6	L-[U-^{14}C]Sorbosone	4	None	

formic acid was applied to elute unreacted AA. Fractions were analyzed for ^{14}C.

Characterization of AA as its Isopropylidene Derivative

A portion of recrystallized AA from experiment 4 was converted to its 5,6-isopropylidene derivative (8). Another portion of the same AA was combined with 20-fold excess of D-erythorbic acid (D-*erythro*-hexen-2-ono-1,4-lactone), recrystallized, and converted to the 5,6-isopropylidene derivative.

RESULTS

Enzymatically prepared glucosone avoids problems caused by trace by-products commonly present in more classical approaches to glucosone synthesis (4). The pyranose-2-oxidase reaction mixture contains only unreacted glucose, glucosone, and traces of gluconic acid which are readily resolved by HPLC (7).

At concentrations greater than 0.2%, glucosone caused wilting and dessication in both bean and spinach seedlings within 24 h. When 0.1% glucosone was supplied to detached seedlings over a 24 h period, no toxic effects were seen. Spinach was unaffected by concentrations of sorbosone as high as 0.6%. In spinach experiments, where unlabeled glucosone accompanied labeled glucose, 0.1% glucosone was used; and where unlabeled sorbosone accompanied labeled glucosone, 0.2 or 0.4% sorbosone was used. In bean and spinach experiments involving only labeled glucose, glucosone or sorbosone, the sugar concentration was at tracer level.

Bean Apices

Detached 21-d-old seedlings (3 g) were supplied with [6-^{14}C]glucose (5 μCi, 0.2 mg) or [6-^{14}C]glucosone (4.77 μCi, 0.25 mg) under prescribed conditions for 25 h. In both experiments, 3% of the ^{14}C was recovered as $^{14}CO_2$, 22% as soluble constituents, 0.1% formic acid-extractable material, and 75% (by difference) remained as insoluble residues. Of the soluble portion, 0.4% was recovered as AA in each experiment. Distribution of ^{14}C between carbon 6 and carbon 1 in AA and glycosyl residues (glucose and galactose) is reported in Table I. [6-^{14}C]Glucose-labeled AA had almost as much ^{14}C in carbon 1 as in carbon 6 and 85% of the label was in these two positions. [6-^{14}C]Glucosone-labeled AA retained 90% of its ^{14}C in carbon 6 and all of the label was recovered in terminal carbons. By contrast, glycosyl units from insoluble residues contained only 43 to 57% of their ^{14}C in terminal positions and approximately 70% of this was in carbon 6. Considerable label entered internal positions in these sugar residues.

Spinach Leaves

Two sets of experiments were undertaken (Table II). In the first set, [6-^{14}C]glucose (10 μCi, 0.04 mg) was supplied to

Table III. *Metabolism of D-[6-^{14}C]Glucose, D-[6-^{14}C]Glucosone, and L-[U-^{14}C]Sorbosone in Detached Spinach Leaves*

Fraction Analyzed	Experiment No.[a]					
	1	2	3	4	5	6
	% of total ^{14}C					
Respired CO_2	10.7	17.8	11.6	8.0	6.5	2.6
Soluble fraction						
Neutrals	5.8	3.3	9.0	15.8	22.8	68.8
Cations	5.8	6.7	7.4	8.3	10.0	2.0
Anions	15.6	17.6	15.0	19.1	28.7	19.4
Oxalic acid	4.3	10.4	2.3	2.5	3.0	0.3
Ascorbic acid	0.6	0.05	0.2	4.1	5.7	0.5
Malic acid	2.3	0.6	3.6	1.6	1.8	<0.1
Citric acid	1.0	0.7	1.9	1.7	1.1	<0.1
Other	7.4	5.8	7.0	10.9	15.5	15.7
Insoluble residues	62.1	54.6	57.0	46.1	32.0	7.2

[a] Refer to Table II for details regarding each experiment.

detached spinach leaves alone, with 0.1% unlabeled glucosone, or with 0.4% unlabeled sorbosone. The leaves (0.7 ± 0.06 g/experiment) were held in water or in unlabeled substrate for 4 h before administrating [6-^{14}C]glucose. Label was taken up within 2 h and again water or unlabeled substrate (0.1% glucosone or 0.2% sorbosone) was supplied for 22 h.

In the second set of experiments with spinach, [6-^{14}C] glucosone (10 μCi, 0.04 mg) was supplied to leaves (0.76 ± 0.12 g/experiment) alone or with 0.2% unlabeled sorbosone. [U-^{14}C]Sorbosone (4 μCi, 0.02 mg) was supplied alone. Administration of labeled compounds followed the timing used in the first set of experiments.

Table III summarizes data on the distribution of ^{14}C for each experiment. When compared to spinach leaves supplied with [6-^{14}C]glucose alone (experiment 1), inclusion of 0.1% glucosone (experiment 2) depressed incorporation of ^{14}C into AA over 10-fold. Other glucosone effects on [6-^{14}C]glucose metabolism included increased incorporation of ^{14}C into oxalic acid and respired CO_2, and decreased incorporation of ^{14}C into malic acid. The presence of 0.4% sorbosone (experiment 3) reduced incorporation of ^{14}C into AA slightly but had little effect on the flow of ^{14}C into other constituents. Spinach leaves that were supplied with [6-^{14}C]glucosone either alone (experiment 4) or with 0.2% sorbosone (experiment 5) incorporated 4.1 and 5.7% of the ^{14}C in soluble constituents as AA, values 7- and 28-fold greater than those obtained in comparable [6-^{14}C]glucose experiments. Less ^{14}C from [6-^{14}C] glucosone was respired as CO_2 than observed when [6-^{14}C] glucose was used but much more ^{14}C appeared in soluble constituents. The composition of these labeled compounds was not determined. Almost as much ^{14}C reached cell wall polysaccharides (major component of insoluble residues) as when [6-^{14}C]glucose was used, an indication that much of the [6-^{14}C]glucosone was available for sugar phosphate interconversions. [U-^{14}C]Sorbosone (experiment 6) was converted to AA with roughly the same efficiency as [6-^{14}C]glucose but its entry into tricarboxylic acid cycle acids and sugar phosphate metabolism was much lower.

Distributions of ^{14}C within AA and glycosyl residues from labeled spinach leaves are summarized in Table IV. In contrast to the redistribution of ^{14}C found in AA from [6-^{14}C] glucose-labeled leaves (experiments 1–3), AA from [6-^{14}C] glucosone-labeled leaves was labeled almost exclusively at carbon 6 (experiments 4, 5) as noted earlier in bean apices. Glycosyl residues exhibited considerable redistribution of label whether the source of ^{14}C was [6-^{14}C]glucose or [6-^{14}C] glucosone (experiments 1–5). AA and glucosyl residues in experiment 6 reflect the distribution expected of [U-^{14}C] sorbosone. The greater accumulations of ^{14}C noted in terminal carbons of galactosyl residues require further study.

To establish that ^{14}C was present as AA, specifically the L isomer, a portion of the recrystallized labeled AA from [6-^{14}C]glucosone-labeled spinach leaves (experiment 4, Table II) was oxidized with ascorbate oxidase, an enzyme highly specific for compounds that contain the enediol lactone configuration as present in carbons 1 through 4 of AA. Under conditions in which 33% of the AA was oxidized in 30 min, the ^{14}C ratio of dehydroascorbic acid to AA was 0.62 (38% oxidized). After 4 h, all ^{14}C was recovered as dehydroascorbic acid. The slightly greater measure of oxidation observed at 30 min using the ^{14}C ratio undoubtedly reflected further enzymic oxidation of AA during the water-washing step on ion exchange columns. Since ascorbate oxidase will also accept D-erythorbic acid as substrate (16), a portion of [6-^{14}C]glucosone-derived AA from experiment 4 was mixed with 20-fold excess of D-erythorbic acid, recrystallized and converted to a 5,6-isopropylidene derivative. Virtually all ^{14}C present in the AA (>99.9%) was lost in this procedure in which >50% of the D-erythorbic acid was recovered as its 5,6-isopropylidene derivative (melting point 170–171°C). When recrystallized [6-^{14}C]glucosone-derived AA from experiment 4 was converted to its 5,6-isopropylidene derivative directly, all ^{14}C was retained in the product (melting point 220–221°C).

DISCUSSION

Although concentrations of glucosone greater than 0.2% are toxic to bean apex or young spinach leaf when supplied through the detached stem, these tissues will tolerate lower concentrations for as long as 48 h. When 0.1% glucosone was included in the solution of [6-^{14}C]glucose taken up by spinach leaf, it reduced the amount of ^{14}C converted to AA 12-fold. If only a tracer amount of [6-^{14}C]glucosone was given to a leaf, incorporation of ^{14}C into AA was 7-fold greater than the AA labeling obtained in a comparable [6-^{14}C]glucose-fed leaf.

Table IV. *Distribution of ^{14}C in Ascorbic Acid and Glycosyl Residues from D-[6-^{14}C]Glucose-, D-[6-^{14}C]Glucosone-, and L-[U-^{14}C]Sorbosone-labeled Spinach Leaves*

Experiment No.[a]	Product Analyzed	^{14}C Recovered as % of Total ^{14}C		
		Carbon 1	Carbons 2–5	Carbon 6
			by difference	
D-[6-^{14}C]Glucose-labeled leaves				
1				
	Ascorbic acid	7	32	61
	Glucosyl residues	25	13	62
	Galactosyl residues	27	6	67
2				
	Ascorbic acid	4	48	48
	Glucosyl residues	35	5	60
	Galactosyl residues	16	8	76
3				
	Ascorbic acid	13	27	60
	Glucosyl residues	21	13	66
	Galactosyl residues	36	11	53
D-[6-^{14}C]glucosone-labeled leaves				
4				
	Ascorbic acid	2	0	98
	Glucosyl residues	24	16	60
	Galactosyl residues	30	2	68
5				
	Ascorbic acid	3	1	96
	Glucosyl residues	35	12	53
	Galactosyl residues	23	8	69
L-[U-^{14}C]Sorbosone-labeled leaves				
6				
	Ascorbic acid	14	65	21
	Glucosyl residues	10	70	20
	Galactosyl residues	35	37	28

[a] Refer to Table II for details regarding each experiment.

The enantiomeric identity of AA was established by recrystallization with authentic AA, by oxidation with ascorbate oxidase, and by preparation of the 5,6-isopropylidene derivative which distinguished AA from D-erythorbic acid. Adding 0.2% sorbosone to the [6-^{14}C]glucosone had no significant effect on AA labeling. These observations suggest that glucosone has a putative intermediary role as the first oxidation step beyond glucose. Epimerization at carbon 5 converts glucosone to sorbosone. As the data indicate, sorbosone had little influence on the conversion of [6-^{14}C]glucosone to AA but was, itself, utilized for AA biosynthesis as efficiently as glucose. In another study now being prepared for publication, we report that bean and spinach contain a NADP$^+$-dependent L-sorbosone dehydrogenase that is able to catalyze the final step from sorbosone to AA. Taken together, the results suggest a pathway of conversion from glucose to AA resembling the one given in the Introduction. Obviously, much more work is needed but this is the first time that intermediates have been identified which will fulfill the requirements of a pathway from glucose to AA in plants as established by isotopic labeling studies.

ACKNOWLEDGMENT

The authors wish to thank Ronald Manning, Hoffmann-LaRoche Inc., for providing the labeled and unlabeled L-sorbosone used in this study and for his continued interest.

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Corrections

Vol. 93: 825–828, 1990

Marshall D. Hatch and James N. Burnell. Carbonic Anhydrase Activity in Leaves and Its Role in the First Step of C_4 Photosynthesis.

Page 826, left column, second line from the bottom, change "0.105 μmol H^+" to "1.05 μmol H^+."

Vol. 94, October, 1990: Table of Contents, Contents by Subject Group, and Author Index

On page iii and page v of the October 1990 issue of *Plant Physiology*, the article listed for page 796 is incorrect. (The article incorrectly listed is printed in this issue of the journal on pages 1365–1375). The correct citation should be:

796 Effects of Heat Shock on Amino Acid Metabolism of Cowpea Cells—Randall R. Mayer, Joe H. Cherry, and David Rhodes

On pages vii and viii, authors Randall R. Mayer, Joe H. Cherry, and David Rhodes should be added to the author index; authors Sandra M. Mayer and Samuel I. Beale should be deleted (they are included in this month's author index).