Plant Physiol. (1982) 69, 465–468
0032-0889/82/69/0465/04/$00.50/0

L-Ascorbic Acid Biosynthesis in *Ochromonas danica*[1]

Received for publication June 2, 1981 and in revised form October 12, 1981

JOHANNES P. HELSPER, LEA KAGAN, CORAL L. HILBY, TRACY M. MAYNARD AND FRANK A. LOEWUS[2]
Institute of Biological Chemistry and Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164

ABSTRACT

Ochromonas danica **Pringsheim, a freshwater chrysomonad, converts D-glucose into L-ascorbic acid over a metabolic pathway that 'inverts' the carbon chain of the sugar. In this respect, L-ascorbic acid formation resembles that found in ascorbic acid-synthesizing animals. It differs from this process in that D-galacturonate and L-galactono-1,4-lactone, rather than D-glucuronate and L-gulono-1,4-lactone, enhance production of ascorbic acid and repress the incorporation of ^{14}C from D-[1-^{14}C]glucose into ascorbic acid.**

Two mutually exclusive, biosynthetic pathways of AA[3] formation are found in eukaryotic organisms. In both, D-glucose is implicated as the six-carbon precursor on the basis of studies with radioactive specifically labeled sugars. Both pathways conserve the six-carbon chain. One 'inverts' the sequence of carbons, as referred to the parent sugar by reduction of C-1 and oxidation of C-5 (or C-4) and C-6 with conservation of the chirality of C-2 and C-3. AA-Synthesizing animals such as the rat utilize this process according to the following scheme:

D-Glucose ---→ D-Glucuronate → L-Gulonate → L-Gulono-1,4-lactone → L-Ascorbate.

The other pathway requires that C-1 and C-2 (or C-3) be oxidized while an epimerization reverses the configuration of C-5. Higher plants utilize this latter process, which conserves the hydroxymethyl function of C-6 of hexose (15). Intermediate steps in the conversion of D-glucose to AA by higher plants have yet to be determined, but evidence of overall conversion is thoroughly established (18). Participation of D-glucuronate or products of D-glucuronate metabolism in this conversion is excluded (16).

Higher plants also synthesize AA when supplied with D-glucurono-3,6-lactone, D-galacturonate, L-gulono-1,4-lactone, or L-galactono-1,4-lactone (4, 6, 9, 19). Inasmuch as radiolabeling studies exclude these compounds as intermediates in the overall pathway from D-glucose to AA, these synthetic events appear to be dependent upon the exogenous source or, at best, salvage mechanisms (7). It is important to point out that higher plants, unlike AA-synthesizing animals, fail to utilize D-glucuronate or L-gulonate for AA formation.

Recently, Shigeoka *et al.* (23) tested the effect of a number of simple sugars and potential AA precursors on AA formation in *Euglena gracilis* Z, a unicellular alga with animal-like properties. Their results led them to propose a biosynthetic scheme from D-glucose to AA, in which D-galacturonic acid, L-galactonic acid, and L-galactono-1,4-lactone were intermediates. D-Glucurono-3,6-lactone and L-gulono-1,4-lactone also effected AA synthesis, and these compounds were included in their scheme as a minor alternate route that differed from the animal pathway in one respect, reduction of D-glucurono-3,6-lactone to L-gulono-1,4-lactone rather than reduction of D-glucuronate to L-gulonate (23).

Ochromonas danica Pringsheim, a unicellular chrysophycean alga with phagotrophic, heterotrophic, photoheterotrophic, and photoautotrophic properties, provides an exceptional opportunity to explore the phylogenetic connection between animal and plant as it pertains to AA biosynthesis. *O. danica* synthesizes and secretes into the medium a number of vitamins, including AA, when grown in an artificial medium (2). Advantage has been taken of this observation to explore the conversion of specifically labeled D-glucose into AA and to test the effect of possible intermediates in this process.

MATERIALS AND METHODS

Chemicals. L-[1-^{14}C]Ascorbic acid and specifically labeled forms of D-glucose were purchased from New England Nuclear Corp. Chemicals, including those used to prepare culture media, were reagent grade.

Algal Culture. *O. danica* Pringsheim was obtained from the Culture Collection of Algae at the University of Texas at Austin. Cells were dark-grown at 25°C in chemically defined medium containing 1% D-glucose (1). Inoculum (10 ml, approx. 10^8 cells) was added to 100 ml of medium. After 14 d, the entire 100 ml was added to 1 L of medium in a 2.5-L, low-form, culture flask in which growth was continued for an additional 10 d. Light-adapted cells were prepared by exposure of the final growth suspension for 24 h at 25°C to fluorescent white light (320 ft-c) plus a minor incandescent source. Batches of cells (1 L) were harvested by centrifugation (2,000 *g*, 6 min), washed (2×) with glucose-free medium, and finally resuspended in the same medium (100 ml). Aliquots (10 ml, 10^{10} cells) were transferred to the outer well of 50-ml center-well flasks (MRA Corp., Clearwater, FL) as described below.

Labeling Procedure. Labeled D-glucose or AA (2–10 μCi) and unlabeled carbohydrates as indicated were placed in the outer well of each 50-ml flask prior to addition of the cell suspension. Respired CO_2 was trapped with 1 N KOH (0.5 ml, center well). Flasks were sealed with rubber serum-bottle stoppers and incubated at 25°C in dark or light (320 ft-c) for 6 h. Experiments were terminated by adding 2% oxalic acid (0.5 ml) with 50 mg of AA as carrier. Before the flasks were opened, they were shaken (30 min, 20°C) to facilitate complete transfer of CO_2 to the center well. Aliquots from center wells of flasks used in ^{14}C-labeled studies were counted to determine respired $^{14}CO_2$. In experiments involving the use of ^3H-labeled substrates, aliquots from both the

[1] Supported by grants from National Institutes of Health (GM-22427), National Science Foundation (PCM-7813254), and the Netherlands Organization for the Advancement of Pure Research (Z. W. O.). Scientific Paper No. 5926, Project 0266, College of Agriculture Research Center, Washington State University, Pullman, WA 99164.

[2] To whom inquiries and requests for reprints should be addressed.

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

outer and the inner well were transferred to one arm of a 2-vessel sublimation apparatus, frozen, and sublimed to recover tritiated H_2O. Cells were recovered by centrifugation (12,000*g*, 20 min, 4°C) and washed (2×) with 2.5 ml 0.1% oxalic acid. When secreted and intracellular AA were examined separately, centrifugation and washing of cells with glucose-free medium preceded addition of oxalic acid and carrier AA to the separated cells and medium. Supernatant and washes were combined. When cells and medium were not separated, the acidified cell suspension was frozen and stored at −20°C until analyzed. Prior to isolation of AA, the thawed suspension was homogenized and separated by centrifugation (12,000*g*, 20 min, 4°C) into supernatant and residual pellet.

Separation of Labeled Products. Oxalic acid was removed from the combined supernatant and washes by precipitation as calcium oxalate. The oxalate-free solution was separated into cationic, anionic, and neutral fractions, as described previously (24). AA was separated from other acidic constituents in the anionic fraction and recrystallized from glacial acetic acid until constant specific radioactivity was attained (usually 2×). AA was further characterized by its conversion into 5,6-*O*-monoisopropylidene AA, as described by Lee *et al.* (13). The reaction was scaled down to 100 mg of finely powdered AA and run at 30°C in dry acetone (2 ml) with acetyl chloride (50 μl) as catalyst in a stirred 5-ml Reacti-Vial (Pierce Chem. Co., Rockford, IL). The AA dissolved within 10 min, and the product slowly crystallized from solution over the next 2 h. It was collected on a Hirsh filter and washed with cold hexane:acetone (7:4). The product melted at 219 to 221°C. After one recrystallization, the melting point was 220 to 221°C. Distribution of radioisotope in AA was determined by chemical degradation (17).

Aliquots of the cell pellet were combusted in a biological oxidizer (Packard Instrument Co., Model 306B) to determine the ^3H or ^{14}C content. Other aliquots were extracted once with 98% ethyl alcohol and then twice with 20% ethyl alcohol (70°C, 1 h) to solubilize the β-glucan storage product (11). Combined extracts were subjected to ultrafiltration (47-mm diameter UM-2 Diaflo membrane, 20 p.s.i., N_2) to remove low mol wt components. Less than 0.1% of the extracted radioactivity passed through the membrane. The polymeric material was hydrolyzed in concentrated HCl (25°C, 24 h) and evaporated to dryness to remove HCl. Glucose was recovered by descending paper chromatography on Whatman 3MM (72 h; ethyl acetate:pyridine:water, 8:2:1, v/v/v). Radioactive glucose was recovered, diluted with 150 mg carrier glucose, and recrystallized from ethyl alcohol. Distribution of radioisotope in D-glucose was determined according to Bloom (5). Quantitative analysis of AA was performed by titration with 2,6-dichloroindophenol (17).

RESULTS AND DISCUSSION

Secretion and Identification of AA. When dark-grown *O. danica* suspensions in 0.1% D-glucose were transferred into the light for 24 h preceding addition of label, secretion of [^{14}C]AA was measurable within 0.5 h after supplying D-[1-^{14}C]glucose to the medium (Fig. 1). Cells continued to release [^{14}C]AA into the medium at an almost linear rate up to 6 h. Very little [^{14}C]AA was retained by the cells. At 6 h, the ratio of [^{14}C]AA in the medium to that in the cells was 6:1. Breakdown and oxidation of secreted AA during the metabolic period was negligible. This was demonstrated experimentally by supplying 0.1% L-[1-^{14}C]AA to a suspension of cells that was grown on 0.1% D-glucose (Table I). At the end of 6 h, 87% of the labeled AA was recovered unchanged. Another 7% of the ^{14}C present in the neutral fraction probably represents labeled dehydro-AA.

To characterize the AA, an experiment was performed in which cells were grown on 0.1% D-[1-^{14}C]glucose for 6 h. At the end of this metabolic period, 500 mg of authentic AA was added to the medium. The labeled AA was recovered by ion exchange chromatography and carried through three successive crystallizations in glacial acetic acid with no loss in specific radioactivity (2 × 10^4 dpm/mmol). Conversion of the thrice-crystallized AA to its 5,6-*O*-monoisopropylidene derivative produced no change in specific radioactivity (20).

AA is found in species of algae from several classes, including the Cyanophyceae (25), Rhodophyceae (14), Chrysophyceae (2), Phaeophyceae (10), Bacillariophyceae (22), Chlorophyceae (3), and Euglenophyceae (23). Only the chrysophycean alga *O. danica* is known to secrete AA (2). Conversion of D-glucose to AA is

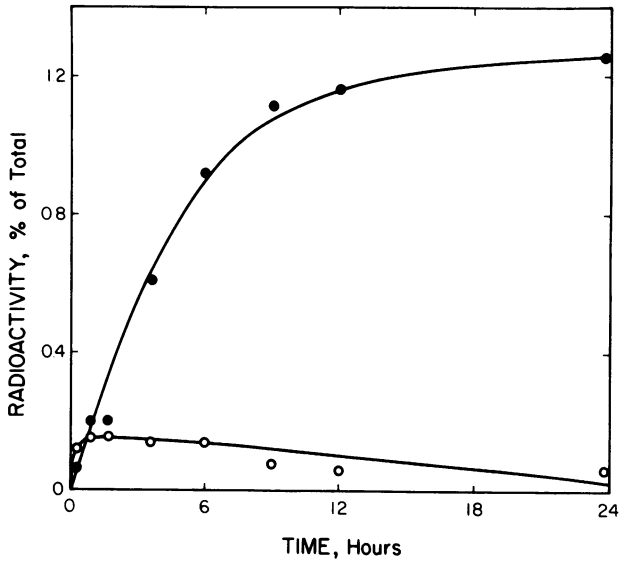

FIG. 1. Appearance of labeled AA in cells (○) and in medium (●) after the addition of 0.1% D-[1-^{14}C]glucose to a dark-grown suspension of *O. danica* that had been transferred to the light 24 h preceding addition of label.

Table I. *Distribution of Radioactivity after Metabolism of 0.1% Labeled D-Glucose or AA by O. danica in the Presence of Light*

Site of Radiolabel		Labeled Fraction					
		CO_2	H_2O	Insoluble residue	Neutral compounds	AA	Other compounds
		% total radioactivity					
D-Glucose	[1-^{14}C]	13		28	52	0.9	6
	[6-^{14}C]	12		29	51	0.9	7
	[1-^3H]		47	6	41	0.7	5
	[6-^3H]		54	7	36	0.2	3
AA	[1-^{14}C]	0.1		0.4	7	87	5

Table II. *Percentage of ^{14}C in Terminal Positions of AA and β-Glucan-Derived Glucose from Cells of O. danica after Incubation in Media Containing D-[1-^{14}C]- or D-[6-^{14}C]Glucose in the Presence of Light*

Form of Labeled Glucose	Terminal Carbon Analyzed	0.01% Glucose, AA	0.1% Glucose	
			AA	Glucose (β-glucan)
		% of ^{14}C in molecule		
[1-^{14}C]	C1	16	21	59
	C6	37	53	14
[6-^{14}C]	C1	42	77	8
	C6	18	10	66

Table III. *Effect of Putative Intermediates (Concentration 0.25%) on AA Biosynthesis from* D-*[1-*^{14}*C]Glucose in O. danica*

Compound Added to Medium	AA Synthesized	
	DCIP[a]-Titratable material	Radioactivity
	relative to control (=100)	
D-Glucose only (0.1%)	100	100
Plus D-glucuronic acid	106	97
Plus D-galacturonic acid	333	61
Plus L-gulonolactone	108	107
Plus L-galactonolactone	657	72

[a] 2,6-Dichloroindophenol.

reported in two studies (22, 23) as well as in this paper.

Conversion of D-Glucose to AA. Preliminary studies established the condition of illumination and the range of D-glucose concentrations used here. Dark-grown cells were preilluminated for 24 h before addition of labeled glucose. Illumination was maintained during the labeling period. In the absence of this light period, conversion of labeled glucose to AA was only 67% of that obtained in the light (data not shown). Addition of AA (5 mg/ml) as carrier at the beginning of the labeling period led to decreased production of labeled AA. To facilitate maximal secretion of AA, addition of carrier was delayed until completion of the metabolic period.

Cell suspensions supplied with D-[1-^{14}C]- or D-[6-^{14}C]glucose converted 0.9% of the ^{14}C to AA (Table I). The major labeled products were CO_2, isofloridoside, and β-glucan (12). When the neutral fraction was separated by descending paper chromatography (ethyl acetate:pyridine:water, 8:2:1, v/v/v; 24 h), virtually all of the radioactivity remained on or near the origin. The region corresponding to glucose contained a negligible amount of ^{14}C.

Cell suspensions supplied with D-[1-^{3}H]glucose produced labeled AA in an amount similar to that of those given D-[1-^{14}C]- or D-[6-^{14}C]glucose, if one takes into account the loss of ^{3}H due to exchange with the medium (Table I). AA from cell suspensions supplied with D-[6-^{3}H]glucose contained less ^{3}H. In these tritiated glucose experiments, much less ^{3}H appeared in the cellular residue, again a consequence of exchange processes. The displaced label was recovered as tritiated water.

Distribution of ^{14}C in AA and β-Glucan. At 0.01% glucose, there was rapid redistribution of label in the hexose carbon chain preceding conversion to AA (Table II). This could be suppressed somewhat by raising the concentration of glucose to 0.1%. Since the cells were labeled in the light, some of the respired $^{14}CO_2$ was photosynthetically recycled into hexose and eventually into AA. This ^{14}C would appear primarily in internal carbons of hexose products such as AA and the β-glucan, accounting in part for the lower percentages of ^{14}C in the terminal positions, C1 and C6. Even so, the relative percentages of ^{14}C found in terminal positions of AA indicate that the pathway of conversion from D-glucose to AA, involves inversion of the carbon chain. Confirmation was obtained by examining the distribution of ^{14}C between terminal carbons of glucose residues in the β-glucan (Table II) where the percentages of ^{14}C in C1 and C6 correspond inversely to those in AA.

Tritium linked to C1 of glucose was conserved during the conversion of glucose to AA (Table I), additional evidence that C1 of glucose was reduced. By contrast, AA from cell suspensions labeled with D-[6-^{3}H]glucose contained less label. The small amount observed here corresponds closely to the ^{3}H anticipated to be present as the result of redistribution of label from C6 into C1 during hexose phosphate metabolism. Only the ^{3}H at C1 of hexose is retained during the conversion to AA.

Effect of Putative Precursors of AA on AA Formation. Cells that were grown in media containing 0.25% L-galactono-1,4-lactone in addition to 0.1% D-glucose produced 6.6 times as much AA in a 6-h period as did cells lacking the lactone (Table III). Addition of 0.25% D-galacturonate instead of L-galactonolactone resulted in a 3.3-fold increase in AA. Under the same conditions, neither D-glucuronate nor L-gulono-1,4-lactone had an appreciable effect. D-Galacturonate and L-galactono-1,4-lactone also reduced the incorporation of ^{14}C from D-[1-^{14}C]glucose into AA (Table III). Again, D-glucuronate and L-gulono-1,4-lactone were relatively ineffective.

The enhanced production of AA by cells grown in the presence of D-galacturonate or L-galactono-1,4-lactone, as well as the diluting effect of these putative intermediates on the conversion of D-[1-^{14}C]glucose into AA, suggests a pathway to AA which involves both D-galacturonate and L-galactono-1,4-lactone as intermediates. Such a scheme has already been proposed by Shigeoka *et al.* (23) to explain their observations on AA biosynthesis in *E. gracilis*. An L-galactono-1,4-lactone oxidase, the final step in this scheme, has been isolated from yeast (21). Occurrence of AA in yeasts has been reported (8), but a more specific method for this identification of AA is needed to confirm this observation.

The presence of two mutually exclusive processes of AA formation in photosynthetic organisms poses an interesting question concerning the evolutionary development of alternative pathways. Higher plants convert glucose to AA by oxidizing C1 of the sugar and conserving the hydroxymethyl function of C6 (15, 18). The two algal systems that have been studied utilize a variant of the pathway found in animals in which glucose is oxidized at C6 and reduced at C1 during its conversion to AA (9). It will be of interest to examine representative species from other algal classes in this regard.

LITERATURE CITED

1. AARONSON S, H BAKER 1959 A comparative biochemical study of two species of *Ochromonas*. J Protozool 6: 282–284
2. AARONSON S, B DEANGELIS, O FRANK, H BAKER 1971 Secretion of vitamins and amino acids into the environment by *Ochromonas danica*. J Phycol 7: 215–218
3. AARONSON S, SW DHAWALE, NJ PATUI, B DEANGELIS, O FRANK, H BAKER 1977 The cell content and secretion of water-soluble vitamins by several fresh water algae. Arch Microbiol 112: 57–59
4. BAIG MM, S KELLY, FA LOEWUS 1970 L-Ascorbic acid biosynthesis in higher plants from L-gulono-1,4-lactone and L-galactono-1,4-lactone. Plant Physiol 46: 277–280
5. BLOOM B 1962 The simultaneous determination of ^{14}C and ^{3}H in the terminal groups of glucose. Anal Biochem 3: 85–87
6. FINKLE BJ, S KELLY, FA LOEWUS 1960 Metabolism of D-[1-^{14}C]- and D-[6-^{14}C] glucuronolactone by the ripening strawberry. Biochim Biophys Acta 38: 332–339
7. HAJRATWALA BR, L STEELE 1976 Vitamin C increase in aerated potato slices. Qualitas Plant 26: 365–388
8. HEICK HMC, GLA GRAFF, JEC HUMPERS 1972 The occurrence of ascorbic acid among the yeasts. Can J Microbiol 18: 597–600
9. ISHERWOOD FA, YT CHEN, LW MAPSON 1954 Synthesis of L-ascorbic acid in plants and animals. Biochem J 56: 1–15
10. JENSEN A 1961 Ascorbic acid in *Ascophyllum nodosum*, *Fucus serratus* and *Fucus vesiculosus*. Proc Int Seaweed Symp 9: 319–325
11. KAUSS H 1965 Isolierung und Identifizierung von Allose aus der chrysomonadale Alge *Ochromonas*. Z Pflanzenphysiol 53: 58–63
12. KAUSS H 1979 Osmotic regulation in algae. *In* L Reinhold, HB Harborne, T Swain, eds, Progress in Phytochemistry, Vol 9. Pergamon Press, New York, pp 1–27
13. LEE CH, PA SEIB, YT LIANG, RC HOSENEY, CW DEYOE 1978 Chemical synthesis of several phosphoric esters of L-ascorbic acid. Carbohydr Res 67: 127–138
14. LISO R, CALABRESE, M CHIEPPA 1978 Research on ascorbic acid physiology in red algae. IV. On the seasonal variation of AA/DHA ratio in *Pterocladia capillacea* (Gmel.) Born. et Thur. (Rhodophyta, Gelidiales). Phycologia 17: 143–147
15. LOEWUS FA 1963 Tracer studies on ascorbic acid formation in plants. Phytochemistry 2: 109–128
16. LOEWUS FA 1971 Carbohydrate interconversions. Annu Rev Plant Physiol 22: 337–364
17. LOEWUS FA, MM BAIG 1970 Biosynthesis and degradation of isotopically labeled ascorbic acid (plants). Methods Enzymol 18: 22–28
18. LOEWUS FA, JPFG HELSPER 1981 Metabolism of L-ascorbic acid in plants. *In* PA Seib, BM Tolbert, eds, Ascorbic Acid: Chemistry, Metabolism and Uses. American Chemical Society Advances in Chemistry, Washington DC. In press
19. LOEWUS FA, S KELLY 1961 The metabolism of D-galacturonic acid and its methyl

ester in the detached ripening strawberry. Arch Biochem Biophys 95: 483–493
20. LOEWUS FA, S KELLY 1961 Identity of L-ascorbic acid formed from D-glucose by the strawberry (Fragaria). Nature (Lond) 191: 1059–1061
21. NISHIKIMI M, E NOGUCHI, K YAGI 1980 Redox properties of L-galactono-lactone oxdiase purified from baker's yeast. Biochem Int 1: 155–161
22. SAUNDERS JA, RRL GUILLARD, FA LOEWUS 1974 Conversion of D-glucose to L-ascorbic acid in *Cyclotella cryptica* Reimann, Lewin and Guillard. Biol Bull 147: 497–498
23. SHIGEOKA S, Y NAKANO, S KITAOKA 1979 The biosynthetic pathway of L-ascorbic acid in *Euglena gracilis* Z. J Nutr Sci Vitaminol 25: 299–307
24. WAGNER G, FA LOEWUS 1973 The biosynthesis of (+)–tartaric acid in *Pelargonium crispum*. Plant Physiol 52: 651–654
25. WAHAL CK, NC BHATTACHARYA, ERS TALPASAYI 1973 Ascorbic acid and heterocyst development in the blue-green algae *Anabaena ambigua*. Physiol Plant 28: 424–429