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FORMATION OF L-ASCORBIC ACID AND OXALIC ACID FROM D-GLUCOSONE IN *LEMNA MINOR*

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Key Word Index—*Lemna minor* L.; L-ascorbic acid biosynthesis; chlorpromazine; duckweed; D-glucosone; oxalic acid biosynthesis.

Abstract—The conversion of D-[1-^{14}C]glucosone to L-ascorbate and oxalate occurs in *Lemna minor* L. to the same extent as reported earlier in spinach and *Pelargonium*. A significant amount of L-[1-^{14}C]ascorbate is converted to oxalate, but virtually none to either glycine or serine. On the other hand, little [2-^{14}C]glycolate is converted to oxalate but a significant amount to glycine and serine. A time course study on *Lemna* pulse-labelled with D-[1-^{14}C]glucosone revealed that the decrease in labelled L-ascorbate is nearly equivalent to the increase in oxalate. These results strongly suggest that L-ascorbate, which has been formed from D-glucosone in *Lemna*, is converted to oxalate without passing through glycolate and/or glyoxylate as intermediates. Chlorpromazine, a calmodulin antagonist which is known to be an inhibitor of electron transport in photosystem II of spinach chloroplast, markedly lowered the content of L-ascorbate in *Lemna* possibly due to stimulation of L-ascorbate breakdown.

INTRODUCTION

Oxalic acid (OxA) is common in higher plants [1]; free OxA and sometimes crystalline deposits of calcium oxalate (CaOx) are found in tissues of most higher plants. Processes leading to OxA formation include oxidation of glycolic acid produced by photorespiration [2], catabolism of L-ascorbic acid (AA) [3], cleavage of isocitric acid by isocitrate lyase [4], and cleavage of oxalacetic acid by oxalacetase [5]. Conclusive experimental evidence indicating the major precursor of OxA in higher plants is still lacking.

Franceschi [6] demonstrated formation of labelled crystalline CaOx in idioblasts from *Lemna minor* when [^{14}C]glycolate, [^{14}C]glyoxylate, or [1-^{14}C]AA was administered. The formation of Ca-[^{14}C]Ox from these precursors was inhibited by the glycolate oxidase inhibitors (2-pyridylhydroxymethanesulphonic acid or methyl-2-hydroxy-3-butynoic acid), suggesting that glycolate and/or glyoxylate are possible intermediates between AA and OxA [6]. He also showed that formation of crystals of CaOx in root idioblasts could be reversibly controlled either by changing the calcium concentration of the medium or by adding or eliminating chlorpromazine, a calmodulin antagonist [7].

Labelled OxA is a major metabolite in several species of OxA-accumulating plants which have been supplied with [1-^{14}C]AA [8, 9]. Apart from one suggestive report which deals with the hydrolysis of diethyl monothioloxalate, a putative intermediate in AA catabolism [10], biochemical reaction mechanisms for the cleavage of AA to produce OxA must still be sought.

In higher plants, biosynthesis of AA is considered to be a hypothetical sequence of reactions which include oxidation of C-1 of D-glucose, oxidation of C-2 or C-3 to form an enediol group, and epimerization of C-5 [3]. None of these intermediates between D-glucose and AA have been identified, although a tentative scheme involving D-glucosone, an oxidized product of D-glucose at C-2, has been proposed [11]. Recently, the author has demonstrated significant formation of AA and OxA from labelled D-glucosone in young leaves from bean [12], spinach [12], and *Pelargonium* [13].

This paper traces the fate of several labelled precursors, which are putatively linked to OxA biosynthesis, in *Lemna minor*. The considerable conversion of D-[1-^{14}C]-glucosone to labelled OxA in *Lemna* also prompted a time course study to analyse the kinetics of the precursor–product relationship between AA and OxA in *Lemna*. Results indicate that AA, but not glycolate, is a precursor of OxA in *Lemna*, and that the decrease of labelled AA corresponds to the increase of labelled OxA, both of which are formed in *Lemna* after pulse-labelling with D-[1-^{14}C]glucosone. It is also noted that chlorpromazine, calmodulin antagonist markedly decreases the AA content in *Lemna*.

RESULTS AND DISCUSSION

Metabolism of putative labelled precursors of AA and OA

D-[1-^{14}C]glucosone or [6-^{14}C]glucosone, putative precursors of AA in higher plants [11–13], and [1-^{14}C]AA

or [2-^{14}C]glycolate, putative precursors of OxA in *Lemna* [6], were administered to *Lemna* for 24 hr (Experiment 1). [1,5-^{14}C]Citrate was also used because it can be metabolized to OA via isocitrate or oxalacetate, constituents of the TCA cycle. [6-^{14}C]AA was also used as a positive control for the D-[6-^{14}C]glucosone experiment (Table 1).

In D-[1-^{14}C]glucosone or [6-^{14}C]glucosone experiments, a considerable amount of label was incorporated into insoluble residue and into CO_2, as previously noted [12, 13]. In these experiments, *ca* 4% of the label was incorporated into AA, as was observed in spinach [12] and *Pelargonium* [13]. This confirms that D-glucosone is a precursor of AA in *Lemna* also. The incorporation of D-[1-^{14}C]glucosone into AA was depressed to *ca* 40% of the control when 13 mM ascorbate was added to the medium; this is similar to the result observed in *Pelargonium* [13]. The effectiveness of D-[6-^{14}C]glucosone as an OxA precursor in *Lemna*, about 30% of the effectiveness of D-[1-^{14}C]glucosone, probably was due to metabolic redistribution of label from C-6 to C-1 as previously observed in *Pelargonium* [13].

In *Lemna* supplied with [1-^{14}C]AA, about half of the label was recovered as unmetabolized labelled AA, even after 24 hr. About 33% of the label, corresponding to 68% of the [1-^{14}C]AA metabolized, was converted to soluble and insoluble OxA, with the latter primarily CaOx. Conversion of [6-^{14}C]AA into labelled OxA was negligible. On the other hand, only 0.7% of the [2-^{14}C]glycolate was metabolized to OxA despite the presence of significant levels of glycolate oxidase in *Lemna* [8]. Taken collectively, these results indicate that AA is a major precursor of OxA in *Lemna*. Only a small amount (0.3%) of [1,5-^{14}C]citrate was metabolized to OxA, suggesting that oxalacetate and isocitrate are not effective precursors for OxA in *Lemna*.

Glycine and serine are major metabolites of glycolate and/or glyoxylate in higher plants [14]. A considerable amount of labelled glycine and serine was formed from [2-^{14}C]glycolate (2.41% of the total label), but not from L-[1-^{14}C]AA (0.07% of the total label). The metabolism of [1-^{14}C]AA and [2-^{14}C]glycolate to OxA, glycine and serine strongly suggests that glycolate and/or glyoxylate are not involved in the conversion of AA and OxA as intermediates.

Franceschi [7] observed that the formation of CaOx crystals in the roots of *Lemna* was blocked by 0.3 mM chlorpromazine, a calmodulin antagonist. To examine the effect of chlorpromazine on OxA metabolism in *Lemna*, [1-^{14}C]AA was given to *Lemna* pre-treated with 0.3 mM chlorpromazine. After 24 hr, most of the unmetabolized labelled AA (compare control to 0.3 mM chlorpromazine-treated sample, Table 1) had disappeared. At the same time, the formation of labelled OxA (especially insoluble Ox) was considerably lower than the control experiment. In this case, an amount of label roughly corresponding to the decreases observed in AA and OxA was recovered as CO_2, an indication that the decarboxylation reaction of AA was stimulated by chlorpromazine.

Table 1. Metabolism of respective labelled compounds in *Lemna minor* for 24 hr (recovered % of ^{14}C supplied to plants)

	D-[1-^{14}C]Glucosone		D-[6-^{14}C]-Glucosone	[1-^{14}C]AA		[6-^{14}C]AA	[2-^{14}C]-Glycolate	[1,5-^{14}C]-Citrate
	Control	12.6 mM Na-AA	Control	Control	0.3 mM Chlorpromazine	Control	Control	Control
$^{14}CO_2$	14.9	11.3	6.1	3.0	76.9	1.3	10.3	6.8
Neutral fraction	8.7	10.2	8.9	4.0	2.0	14.4	7.3	2.3
Cationic fraction		2.3	4.8		0.4	1.0		
Gly + Ser	0.23			0.07			2.41	0.59
Ala + Asp + Glu	1.99			0.25			2.03	5.34
Other compounds	3.88			0.68			2.16	5.57
Anionic fraction								
AA	4.0	0.4	4.4	51.0	2.2	50.2	0.8	0.2
OxA (soluble)	0.6	0.3	0.2	16.2	3.0	0.1	0.3	0.2
OxA (insoluble)	0.7	0.2	0.2	17.1	0.9	0	0.4	0.1
Other acids	12.2	17.4	11.4	5.0	10.8	28.2	14.4	63.4*
Adsorbed compounds	2.2	5.8	3.2	0	0	0	0.7	0
Insoluble residue	50.6	52.1	60.8	2.8	3.8	4.8	59.2	11.5

*Citric acid (39.3%) and malic acid (4.4%) were the major components.

Decrease of AA content in Lemna *plants which were treated with chlorpromazine*

AA has an important role in preventing photoinhibition of green plant tissues by scavenging active oxygen species [15]. Consequently, a decrease of AA content may cause a photoinhibition of *Lemna*. On the other hand, it was already reported that calmodulin antagonists like chlorpromazine inhibit electron transport in photosystem II of spinach chloroplasts [16].

In this experiment, *Lemna* gradually became yellow when 0.3 mM chlorpromazine was added under the specified light conditions. This, combined with the metabolic behaviour of [1-^{14}C]AA in *Lemna* reported above, prompted a determination of the AA content of chlorpromazine-treated *Lemna*.

The AA content of *Lemna* grown in medium without sucrose ((−) sucrose medium) was 1.4 μmol g^{-1} fr. wt (Table 2). The AA content of *Lemna* grown in medium containing sucrose ((+) sucrose medium) was greater, but decreased gradually over 2 days when transferred to (−) sucrose medium to reach the same AA content as *Lemna* originally grown in (−) sucrose medium.

The AA content of *Lemna* grown in (−) sucrose medium rapidly decreased after treatment with 0.3 mM chlorpromazine, to about 60% after 1 hr, and to virtually 0% after 24 hr. *Lemna* treated with 75 μM chlorpromazine for 24 hr had 46% of the AA present in the control. When less than 30 μM chlorpromazine was present, no decrease in AA content was observed up to 24 hr. The effect of 0.3 mM chlorpromazine on AA content of *Lemna* grown in (+) sucrose medium resembled that observed on *Lemna* grown in (−) sucrose medium.

As determined by fluorescent emission of chlorophyll in intact *Lemna*, there was a significant decrease in photosystem II activity 1 hr after adding 0.3 mM chlorpromazine. This may have some bearing on the observation that the AA content of *Lemna* was significantly lower under those conditions. By contrast, the AA content of intact chloroplasts isolated from spinach was not effected after treatment with 0.3 mM chlorpromazine (Asada, K., pers. commun.). The reaction mechanism by which chlorpromazine produces a decrease in the AA content of intact *Lemna* is not known. Soluble and insoluble OA in *Lemna* were scarcely affected by the presence of sucrose or chlorpromazine in the inorganic medium (Table 2). These observations suggest that catabolism of AA, a precursor of OxA, but not catabolism of OxA itself was the reason only small amounts of label from [1-^{14}C]AA were incorporated into OxA in *Lemna* which had been treated with 0.3 mM chlorpromazine (Table 1).

Kinetic analysis of OxA formation in Lemna *labelled with* D-[1-^{14}C]*glucosone*

The preceding experiments with D-[1-^{14}C]glucosone and [1-^{14}C]AA in *Lemna* suggested that OxA was formed in *Lemna* from D-glucosone via AA as already shown in *Pelargonium* [13]. To confirm this, a time course study (Experiment 2) of *Lemna* labelled with D-[1-^{14}C]glucosone was carried out. After administering D-[1-^{14}C]glucosone to *Lemna* for 24 hr, the labelling medium was replaced with fresh inorganic medium. Thirty plants were removed on each of the following six days to determine the specific radioactivity (s.r.) of AA, free OxA and insoluble Ox. On the first day, s.r. values were 2 000 000, 25 000, and 2300 dpm μmol^{-1}, respectively. The s.r. of AA gradually decreased over the following five days to 600 000 dpm μmol^{-1}, evidence that the supply of labeled precursor had been effectively eliminated when the labelling medium was replaced by fresh inorganic medium. The s.r. of soluble OxA rose to 6300 dpm μmol^{-1} during the four days following labelling and then decreased. Insoluble Ox increased, rapidly so in the final three days of the experiment, reaching a final value of 21 000 dpm μmol^{-1}.

To calculate the amount of ^{14}C in AA, free OxA and insoluble Ox for each 24 hr period, the μmol g^{-1} fr. wt of each component (Table 2) was multiplied by its s.r. Data are given in Fig. 1.

Figure 1 shows that the amount of labelled AA lost during each metabolic period was almost equal to the corresponding total amount of soluble and insoluble labelled OxA formed during the same period of time, especially between 2 and 6 days after administering D-[1-^{14}C]glucosone into the *Lemna*. These results clearly indicate that a precursor–product relationship exists

Table 2. Effect of chlorpromazine on AA, soluble OxA, and insoluble OxA contents in *Lemna minor* cultured under various conditions (μmol g^{-1} fr. wt)*

	(−) Sucrose medium†			(+) Sucrose medium		
	Control	75 μM Chlorpromazine	0.3 mM Chlorpromazine‡	Control	75 μM Chlorpromazine	0.3 mM Chlorpromazine‡
AA	1.4	0.65	0	2.0	1.3	0
Soluble OxA	16	—	12	17	—	13
Insoluble OxA	34	—	33	32	—	33

*Data represent mean values from two independent experiments.

†2 Days after transferring *Lemna* to (−) sucrose medium, chlorpromazine with respective concentration was added to the culture medium for 24 hr.

‡Treatment with 0.3 mM chlorpromazine for 24 hr caused 100% root abscission.

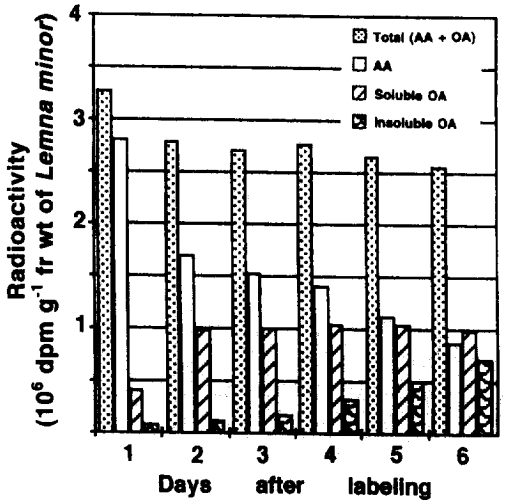

Fig. 1. Time course of ^{14}C-radioactivity distributed into AA, soluble OxA, and insoluble OxA in *Lemna minor* pulse-labelled for 24 hr with D-[1-^{14}C]glucosone.

between AA and OxA. The conversion of soluble OxA to insoluble Ox was also evident from Fig. 1. These kinetic data are therefore indicative of the metabolic relationship between AA, soluble OxA, and insoluble Ox in intact *Lemna* plants.

EXPERIMENTAL

Plant material. Lemna minor L. (clone 601) was grown as an axenic culture in a growth chamber under fluorescent lamps (150 $\mu E\,m^{-2}\,s^{-1}$) at 25°. Cultures were grown in 500 ml conical flasks containing 150 ml of the medium previously described [6]. *Lemna minor* culture was started with 5–6 plants, which were allowed to grow until the surface was *ca* 30% covered by plants (time taken: 10 days in medium with 29 mM sucrose, or 15 days in medium without sucrose). Two days prior to a labelling experiment, cultures were transferred into fresh, inorganic medium without sucrose and held in this medium throughout the experimental period unless otherwise indicated.

Labelling experiments. D-[1-^{14}C]glucosone and 6-^{14}C]glucosone (both 2.22 TBq mol^{-1}) were synthesized from labelled D-glucose as described previously [17]. [1-^{14}C]AA (25.9 GBq mol^{-1}) was purchased from NEN-DuPont (Boston, MA, USA), and [2-^{14}C]glycolate (monocalcium salt, 1.11 TBq mol^{-1}) and [1,5-^{14}C]citrate (3.7 GBq $mmol^{-1}$) from ICN Pharmaceuticals (Irving, CA, USA). [6-^{14}C]AA (19.5 GBq mol^{-1}) was a gift from Dr Frank A. Loewus.

About 185 kBq of each labelled precursor was supplied to *ca* 30 *Lemna* plants (0.25–0.3 g fr. wt) which were floating on 3 ml of inorganic medium in a 21 mm Petri dish (Experiment 1). Incubation proceeded in a glass bell-jar in order to collect respired $^{14}CO_2$ [17]. In dilution or inhibition (chlorpromazine) experiments, Na ascorbate or chlorpromazine at designated concns were present in the inorganic medium 24 hr prior to precursor labelling. In the time course study (Experiment 2), 1 ml of 50% MeOH containing 1.79 MBq of D-[1-^{14}C]glucosone was administered to *ca* 180 *Lemna* plants in 28 ml of the inorganic medium for 24 hr. Then, the labelled medium was replaced with sterile, unlabelled medium, and 30 *Lemna* plants were harvested. Thereafter, *ca* 30 plants were harvested every 24 hr up to 6 days following pulse-labelling.

Separation and identification of metabolic products. Labelled plants were extracted in 25 ml of 0.1% OxA which contained 60 mg AA as a carrier. The extract was passed directly through a tandem pair of columns packed with Dowex 50W × 8 (H^+) and Dowex 1 × 8 (formate) ion exchange resins (200–400 mesh). Anionic metabolites were recovered from the Dowex 1 column by a two-step linear formic acid gradient [18]. OxA remained on the column. After flushing the Dowex 1 column with 100 ml of water, OxA was recovered by running a linear gradient of 0–2 M NH_4 carbonate. The fractions containing AA and OxA were pooled and concd at red. pres. (<40°). AA was recrystallized three times from 15 M HOAc [19]. Residual NH_4 carbonate in the NH_4 oxalate was removed by decomposition on a steam bath. NH_4Ox was converted to cyclohexylammonium Ox and recrystallized three times from H_2O–MeOH–EtOAc [20].

Insoluble OxA was extracted from the insoluble residues of plant tissues using 4.5 ml (1.5 ml × 3) of 0.5 M HCl containing 25 mg of OxA as carrier. To optimize extractions, samples were sonicated (15 min × 3). These OxA samples were purified by conversion to their cyclohexyl NH_4 salts using the same procedures as mentioned above.

Cationic metabolites were eluted from the Dowex 50W (H^+) column with a 3% NH_4OH in 80% EtOH. After concn into a small vol., the cationic compounds were separated by 2D TLC [21]. Incorporation of the label, especially into glycine and serine, was qualitatively determined using a Bio Imaging Analyzer (BAS 2000, Fuji Film Co. Ltd, Japan).

In the time course experiment, labelled plants were extracted successively with 25 ml of 0.1% DTT and then with 4.5 ml (1.5 ml × 3) of 0.5 M HCl without adding any carriers. Separation procedures for recovering AA and OxA by ion-exchange columns were the same as mentioned above except for adding 0.1% DTT (final concn) to the linear gradient with which AA was eluted from the column. Pooled AA or OxA peak fractions were concd and purified by HPLC (eluent: 0.5 ml of 0.001M H_2SO_4 min^-, column: Bio-Rad organic acid column, Aminex HPX-87H, 300 × 7.8 mm, detector: Jasco 840-EC electro chemical detector at 0.6 V for AA, or Jasco 875-UV detector at 213 nm for OxA). AA and OxA peaks were collected at 0.1 min intervals, and recovered fractions were radio-assayed to determine the s.r. values of the eluted AA and OA.

To determine the AA content, *Lemna* (*ca* 0.3 g fr. wt) were extracted with 0.1% DTT in 4% OA (8 ml × 3). The extract was adjusted to 25 ml with additional extracting soln and a 2 ml aliquot was passed through a small

(4 × 40 mm) column of Dowex 50W (H^+) followed by extract soln to a total vol. of 5 ml. 10 μl of the decationized extract was used for HPLC analysis.

Soluble OxA was extracted from *Lemna* with 0.1% DTT (8 ml × 3), and then insoluble OxA with 4.5 ml (1.5 ml × 3) of 0.5 M HCl. Each extract was loaded onto a tandem pair of the Dowex columns from which the OxA was isolated as mentioned above. Treatment through a short column of Amberlite IR-120 (H^+) produced free OxA for HPLC analysis. The total amount of label incorporated into AA and OxA was calculated by multiplying the specific radioactivities (dpm μmol^{-1}) of recrystallized AA or OxA by the content ($\mu mol\, g^{-1}$ fr. wt) of AA or OxA in *Lemna*. Radioactivity incorporated into insoluble residues was determined after combustion with a Packard Model 306 biological oxidizer.

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