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INCORPORATION OF ^{18}O INTO OXALIC, L-THREONIC AND L-TARTARIC ACIDS DURING CLEAVAGE OF L-ASCORBIC AND 5-KETO-D-GLUCONIC ACIDS IN PLANTS

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Key Word Index—*Pelargonium crispum*; *Petroselium sativum*; *Phaseolus vulgaris*; *Vitis labrusca*; ascorbic acid cleavage; 5-keto-D-gluconic acid cleavage; ^{18}O-incorporation; oxalic acid; L-tartaric acid; L-threonic acid.

Abstract—L-[1,6-^{14}C]Ascorbic acid was administered to young leaves of *Pelargonium*. 5-Keto-D-[1-^{14}C]gluconic acid, a metabolic product of L-[1-^{14}C]ascorbic acid in grape, was supplied to young leaves of *Pelargonium*, parsley, bean and grape in the presence of $^{18}O_2$ or $H_2{}^{18}O$. From the ^{18}O incorporated into oxalic and L-threonic acids, which were C2/C3 cleavage products of L-ascorbic acid, and into L-tartaric acid, which was a C4/C5 cleavage product of 5-keto-D-gluconic acid, the number of oxygen atoms incorporated into the respective organic acids was calculated. The data strongly suggest that a cleavage mechanism of C2/C3 of L-ascorbic acid involves both oxygenase and hydrolase reactions but that cleavage of C4/C5 of 5-keto-D-gluconic acid is due only to hydrolase. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

L-Tartaric acid (TA) is a product of L-ascorbic acid (AsA) breakdown in higher plants [1]. Oxalic acid (OxA)-accumulating species cleave AsA at the C2/C3 bond to produce OxA and L-threonic acid (ThA). The latter is either decarboxylated [2] or oxidized to TA [3]. However, 5-keto-D-gluconic acid (5KGA), a major metabolite of AsA in grape species, is cleaved at C4/C5 to form TA and a C_2 product [4]. Formation of one or two carboxyl functions accompanies these cleavage processes but the mechanism is obscure. A fungal enzyme which cleaves AsA to OxA and ThA was reported as an oxygenase since one mole of O_2 was absorbed during the reaction [5] but spinach, an OxA-accumulator failed to incorporate $^{18}O_2$ into OxA when supplied with $^{18}O_2$ [6]. More recently, a theoretical mechanism for oxidative cleavage of AsA to OxA and ThA has been proposed [7].

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RESULTS AND DISCUSSION

Metabolism of labelled substrates

[1,6-^{14}C]AsA in *Pelargonium* and 5K[1-^{14}C]GA in *Pelargonium*, bean and grape were metabolized as already reported [1, 3, 4, 8]. 5K[1-^{14}C]GA, here administered to parsley for the first time, was metabolized similarly to other species, but with less activity. For example, the fractions of ^{14}C incorporated into TA from 5K[1-^{14}C]GA during 24 hr metabolism were 16% in *Pelargonium*, 9% in bean, 92% in grape and 5% in parsley.

Specific radioactivity of labelled organic acids recovered

The administered [^{14}C]AsA and 5K[^{14}C]GA were diluted by endogenous AsA or 5KGA in the leaves. We assumed that the specific radioactivities of labelled substrates, as recovered from leaves after 24 hr metabolism, represented the specific radioactivities available as substrate for the cleavage reaction throughout the metabolic period. The decrease in specific radioactivity of synthesized [^{14}C,^{18}O]organic acid compared with the [^{14}C]substrate (Tables 1 and 2) was due to dilution with the endogenous organic acid. These data

Table 1. Specific radioactivities (kBq μmol^{-1}) of organic acids recovered from *Pelargonium* after 24 hr metabolism

	$^{18}O_2$-Experiment	$H_2{}^{18}O$-Experiment
[1,6-^{14}C]AsA	7.3	7.6
[^{14}C]OxA	0.21	0.36
[^{14}C]ThA	0.49	0.64

[1,6-^{14}C]AsA was administered to young leaves of *Pelargonium* and labelled organic acids were recovered from leaves after 12 hr metabolism.

Table 2. Specific radioactivities (kBq μmol^{-1}) of 5K[^{14}C]GA and [^{14}C]TA recovered from leaves after 12 hr metabolism

	Pelargonium	Bean	Parsley	Grape
$^{18}O_2$-Experiment				
5K[^{14}C]GA	6.9	2.4	2.9	2.0
[^{14}C]TA	0.51	2.4	3.2	0.87
$H_2{}^{18}O$-Experiment				
5K[^{14}C]GA	7.3	1.6	3.0	1.9
[^{14}C]TA	0.36	1.6	3.1	0.26

5K[1-^{14}C]GA was administrated to young leaves of each plant and labelled organic acids recovered after 24 hr metabolism.

suggest that considerable amounts of OxA, ThA and TA were present in *Pelargonium* and grape, but there was no TA in bean [9] and parsley.

Average concentration of ^{18}O in $^{18}O_2$ and $H_2{}^{18}O$ administered

The $^{18}O_2$-concentration in total O_2 which existed in the chamber decreased from 95 to *ca* 35% based on GC-mass spectrometric analysis at 6 hr intervals throughout the metabolic period. The average concentrations of ^{18}O in O_2 are shown in Table 3, together with the $H_2{}^{18}O$ concentration in the labelled solution administered. We assumed that the $^{18}O_2$ and $H_2{}^{18}O$ with the same concentration of ^{18}O (Table 3) was supplied for the cleavage reaction throughout the metabolic period.

Concentration of ^{18}O in trimethylsilylated organic acids

The TMSi derivatives of the produced organic acids were analyzed by GC-SIM (selected ion monitoring) and the midpoint of the standard peak (M) was calculated (Table 4). Table 4 also shows similar data for authentic organic acids.

Number of oxygen atoms incorporated into organic acids produced by cleavage of ascorbic and ketogluconic acids

The midpoint of the GC peak of the TMSi organic acid gives the average mass number of the organic acid. When the difference between the mass number of the TMSi [^{18}O]organic acid and the corresponding authentic TMSi organic acid is divided by two, the quotient provided the number of ^{18}O atoms present in the [^{18}O]organic acid. The total number of oxygen atoms incorporated into respective organic acids was estimated by correcting the quotient above with the two dilution coefficients, regarding dilution of [^{18}O]organic acid with endogenous organic acid (which can be calculated from Tables 1 and 2) and also the dilution of $^{18}O_2$, or $H_2{}^{18}O$ with $^{16}O_2$ or $H_2{}^{16}O$ (Table 3). For example, the number of oxygen atoms incorporated from H_2O into OxA and its standard deviation can be calculated as follows:

$$N = \frac{(0.321-0.289)\pm\sqrt{(0.009)^2+(0.002)^2}}{2} \div \frac{0.36}{7.6/2} \div 0.19 = 0.89\pm0.26$$

The numbers of oxygen atoms incorporated into OxA, ThA and TA from O_2 and H_2O in respective leaves were calculated in the same way and are listed in Tables 5 and 6.

By administering radiolabelled substrates accompanied by a source of ^{18}O, the amount of O-atom incorporated into the metabolites could be calculated quantitatively. The data obtained here clearly indicate that cleavage of AsA between C2 and C3 is due to oxygenase and also hydrolase activities. A large standard deviation, however, was observed in cases where oxygen incorporated into OxA and TA from $H_2{}^{18}O$ in *Pelargonium* was evaluated. This is accounted for by the low concentration of ^{18}O in TMSi-[^{14}C,^{18}O]organic acid. Therefore, administration of greater amounts of $H_2{}^{18}O$ would be useful to overcome a part of the problems encountered here.

A proposed scheme for the cleavage of AsA is shown in Scheme 1. In this scheme, it is necessary for

Table 3. ^{18}O concentration in O_2 or H_2O administered to leaves during metabolism of each labelled compound

	Pelargonium [^{14}C]AsA	*Pelargonium* 5K[^{14}C]GA	Bean 5K[^{14}C]GA	Parsley 5K[^{14}C]GA	Grape 5K[^{14}C]GA
^{18}O in O_2	0.59	0.59	0.58	0.52	0.52
^{18}O in H_2O	0.19	0.15	0.074	0.12	0.12

(^{18}O in O_2) indicates average value of the $^{18}O_2$ concentration in total O_2 in the chamber.
(^{18}O in H_2O) indicates the $H_2{}^{18}O$ concentration in total H_2O which exists in the labelled solution.

Table 4. Mid-point of SIM-spectrum obtained from peaks of TMSi OxA, -ThA, and -TA formed from leaves in $^{18}O_2$ or $H_2{}^{18}O$

		$^{18}O_2$-Experiment	$H_2{}^{18}O$-Experiment
	Authentic OxA	M + (0.289 ± 0.009)	M + (0.289 ± 0.009)
(*Pelargonium*)	[^{14}C, ^{18}O]OxA	M + (0.299 ± 0.004)	M + (0.321 ± 0.002)
	Authentic ThA	M + (0.596 ± 0.004)	M + (0.596 ± 0.004)
(*Pelargonium*)	[^{14}C, ^{18}O]ThA	M + (0.753 ± 0.007)	M + (0.650 ± 0.0007)
	Authentic TA	M + (0.646 ± 0.003)	M + (0.646 ± 0.003)
(*Pelargonium*)	[^{14}C, ^{18}O]TA	M + (0.646 ± 0.003)	M + (0.661 ± 0.006)
(Bean)	[^{14}C, ^{18}O]TA	M + (0.681 ± 0.004)	M + (0.831)
(Parsley)	[^{14}C, ^{18}O]TA	M + (0.674 ± 0.003)	M + (0.919 ± 0.009)
(Grape)	[^{14}C, ^{18}O]TA	M + (0.643 ± 0.006)	M + (0.672 ± 0.003)

M: mass number of the standard peak which is 15 less than the mass number of the respective parent peak.

Table 5. Number of oxygen atoms incorporated into OxA and ThA produced by C–C cleavage of AsA in *Pelargonium* leaves

	OxA	ThA
From O_2	0.15 ± 0.15	0.99 ± 0.05
From H_2O	0.89 ± 0.26	0.85 ± 0.06

H_2*O generated from *O_2 in leaves to be diluted with a substantial amount of $H_2{}^{\#}O$ administered. This is possible since the *O_2 gas which can be dissolved in 40 ml of $H_2{}^{\#}O$ gives only 1 μl of H_2*O in this reaction. This scheme is consistent with the mechanism of another well-studied oxygenase reaction [10] and also the hypothesis proposed by Al-Arab *et al.* [7] for the synthesis of OxA and ThA by cleavage of AsA. In contrast, based on data in Table 6, cleavage of 5KGA between C4/C5 seems to involves only a hydrolase. On the other hand, a preliminary experiment showed that $H_2{}^{18}O$ but not $^{18}O_2$ was incorporated into TA when it was oxidatively formed from ThA in *Pelargonium* (data not shown).

EXPERIMENTAL

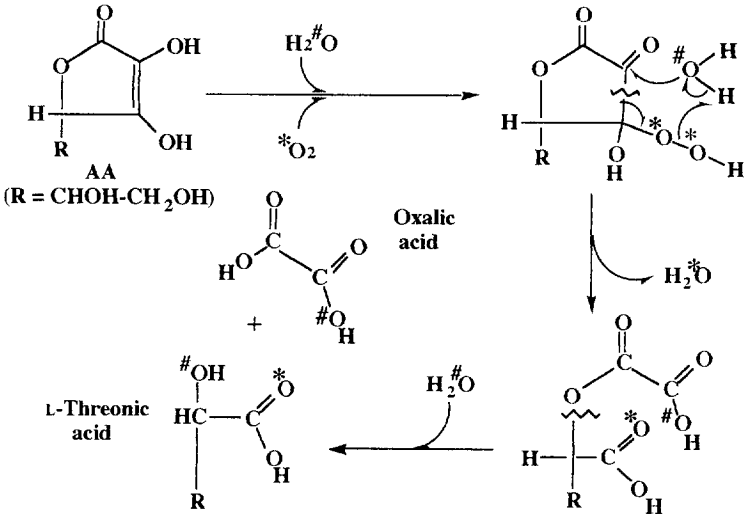
Plant materials. Developing leaves of *Pelargonium* (*Pelargonium crispum* L., 0.12 g fr. wt per leaf) were used to study AsA cleavage. Developing leaves of *Pelargonium*, parsley (*Petroselium sativum*, *ca* 3 cm, 0.25 g fr. wt per shoot) and young leaves of bean (*Phaseolus vulgaris* L. cv. Honkintoki, 0.5 g fr. wt per 10-day-old seedling with both primary leaves) and grape (*Vitis labrusca*, cv. Delaware, 0.2 g fr. wt per leaf) were used to study 5KGA cleavage.

Labelled substrates. [1-^{14}C]AsA (DuPont/NEN, USA) and [6-^{14}C]AsA (provided by F. A. Loewus) were repurified by chromatography on Dowex columns as described elsewhere [11]. [1,6-^{14}C]AsA was prepd by mixing equal amounts of [1-^{14}C]- and [6-^{14}C]AsA. 5K[1-^{14}C]GA was prepared from D-[1-^{14}C]glucose as described elsewhere [4]. Specific radioactivities of [1,6-^{14}C]AsA ranged from 12.9 to 13.7 kBq μmol^{-1} and 5K[1-^{14}C]GA from 1.7 to 6.8 kBq μmol^{-1} before administration. $^{18}O_2$ (99 atom % excess) and $H_2{}^{18}O$ (97.1 atom % excess) were purchased from Isotec, U.S.A.

Administration of labelled compounds. A diagram of the experimental setup is shown in Fig. 1. A soln of labelled substrate (plus $H_2{}^{18}O$ in the $H_2{}^{18}O$-expt) was added to a small glass vial (A, B). The stem portion of leaves was immersed in the soln to be covered at its fresh-cut surface. Then, the glass vial was placed in a transparent acrylic chamber (C, 300 ml) and plugged with a silicone-rubber stopper (K). Granular silica gel (L) was placed in the bottom of the chamber (5 mm layer). A circulating bypass was assembled with silicone-rubber tubing (8 mm i.d.), a plastic tube (E, 100 ml) containing silica gel and two, three-way valves (G, H). A manometer (D) on the bypass kept the inner pressure of the chamber nearly constant. In $^{18}O_2$-expts, the gas phase of the system was exchanged with N_2 completely, then *ca* 84 ml of ^{18}O (final concn of $^{18}O_2$ 21%) and 0.12 ml of CO_2 (final conc of CO_2 0.03%)

Table 6. Number of oxygen atoms incorporated into TA produced by C–C cleavage of 5KGA in leaves

	Pelargonium	Bean	Parsley	Grape
From O_2	0 ± 0.05	0.03 ± 0.004	0.03 ± 0.01	0 ± 0.01
From H_2O	1.01 ± 0.45	1.25 ± 0.02	1.14 ± 0.04	0.79 ± 0.13



Scheme 1. Proposed mechanism for cleavage reaction of ascorbic acid in *Pelargonium*.

was injected successively into the chamber through a silicone-rubber plug (J) using a glass syringe (M). During the period in which label was incorporated and metabolized (24 hr), the chamber was illuminated by fluorescent lamps supplemented with an incandescent source (300 μE m^{-1} s^{-1}) and the gas phase was circulated with a peristaltic pump.

Recovery of organic acids from leaves. Labelled leaves (less stems) were ground in a mortar and extracted with H_2O (8 ml × 3). The extract was separated on ion-exchange resin columns according to a modified version of the method of ref. [1] to obtain anionic, cationic and neutral frs. AsA, ThA, 5KGA and TA were eluted from a Dowex 1 × 8 (HCO_2H) column (1 cm i.d. × 10 cm). OxA which remained on the column was eluted with a linear gradient of 0 to 2 N $(NH_4)_2CO_3$. Frs containing [^{14}C]organic acids were collected. AsA and 5KGA were purified by HPLC to calculate their sp. radioactivity under the following conditions: detector, differential refractometer; column. Aminex HPX-87H (250 × 4.4 mm, Bio-Rad Labs, U.S.A.); eluent, 0.05 N HCO_2H at 0.5 ml min^{-1}. The eluate from the column was collected in small frs at regular intervals (12 s) and radioactivity was determined by liquid scintillation counting.

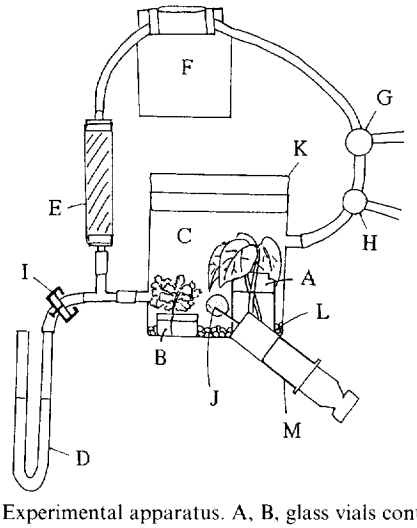


Fig. 1. Experimental apparatus. A, B, glass vials containing labelled solution; C, acrylic sealed chamber (300 ml); D, manometer; E, plastic tube (100 ml) containing granular silica gel; F, peristaltic pump; G, H, three-way valves; I, pinch cock; J, K, silicone rubber plugs; L, granular silica gel; M, glass syringe.

GC-SIM analysis of TMSi organic acids. Organic acid metabolites were converted to TMSi derivatives and analysed by GC-SIM by a modified version of the method described elsewhere [12]. A small amount (1 μg) of TMSi derivatives of [^{18}O]organic acid was injected into the GC-MS analyser (column: Ultra-1, 12 m × 0.2 mm × 0.33 μm film thickness of cross-linked Me silicone gum, Hewlett Packard; carrier gas: He at 20 ml min^{-1}). GC-SIM analyses were repeated at least 3 times with selection of $[M]^+$, $[M+1]^+$, $[M+2]^+$, $[M+3]^+$, $[M+4]^+$ and $[M+5]^+$ as the mass number to be monitored, where M, the mass number of the standard peak, is 15 less than the mass number of the parent peak. GC-SIM analysis of TMSi derivative of authentic organic acids was also made under the same conditions. The mid-point of each SIM-spectrum was calculated as follows. Suppose N_M, N_{M+1}, N_{M+2}, N_{M+3}, N_{M+4}, and N_{M+5} shows the abundance of ions located in each M number. Then the frs (F_M, F_{M+1}, F_{M+2}, F_{M+3}, F_{M+4} and F_{M+5}) of the ion abundance located in respective M number against the total ion abundance ($N_M + N_{M-1} + N_{M+2} + N_{M+3} + N_{M+4} + N_{M-5}$) of the SIM-spectrum were calculated. The midpoint of the SIM-spectrum was calculated from $[(M \times F_M) + \{(M+1) \times F_{M+1}\} + \{(M+2) \times F_{M+2}\} + \{(M+3) \times F_{M+3}\} + \{(M+4) \times F_{M+4}\} + \{(M+5) \times F_{M+5}\}]$.

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REFERENCES

1. Wagner, G. and Loewus, F. A., *Plant Physiology*, 1973, **52**, 651.
2. Helsper, J. P. and Lewus, F. A., *Plant Physiology*, 1982, **69**, 1365.
3. Saito, K., Morita, S. and Kasai, Z., *Plant Cell Physiology*, 1984, **25**, 1223.
4. Saito, K. and Kasai, Z., *Plant Physiology*, 1984, **76**, 170.
5. White, G. A. and Krupka, R. M., *Archives of Biochemistry and Biophysics*, 1965, **110**, 448.
6. Raven, J. A., Griffiths, H., Glidewell, S. M. and Preston, T., *Proceedings of the Royal Society Series B*, 1982, **216**, 87.
7. Al-Arab, M. M. and Hamilton, G. A., *Biorganic Chemistry*, 1987, **15**, 81.
8. Saito, K. and Loewus, F. A., *Plant Cell Physiology*, 1989, **30**, 629.
9. Saito, K. and Loewus, F. A., *Plant Science*, 1989, **62**, 175.
10. Fraser, M. S. and Hamilton, G. A., *Journal of the American Chemical Society*, 1982, **104**, 4203.
11. Williams, M. and Loewus, F. A., *Carbohydrate Research*, 1979, **63**, 149.
12. Saito, K. and Kasai, Z., *Plant Cell Physiology*, 1982, **23**, 499.