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TARTARIC ACID SYNTHESIS FROM L-ASCORBIC ACID-1-^{14}C IN GRAPE BERRIES

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Abstract—Young grape berries were exposed to $^{14}CO_2$ for 10 min under light and dark conditions, and changes of ^{14}C-assimilation products was surveyed during a short period. In the light, radioactivity was found in tartaric acid immediately after $^{14}CO_2$-fixation, while in the dark, radioactivity was not detected in tartaric acid until 480 min after $^{14}CO_2$-fixation. D-Glucurono-γ-lactone-6-^{14}C, L-ascorbate-1-^{14}C, D-glucuronate-6-^{14}C and sucrose-U-^{14}C were fed to young grape berries, and the incorporation into tartaric acid was investigated. A considerable amount of radioactivity was incorporated into tartaric acid from D-glucurono-γ-lactone-6-^{14}C and L-ascorbic acid-1-^{14}C, and, in the case of L-ascorbic acid-1-^{14}C, 72 per cent of the total radioactivity was found in tartaric acid. The tartaric acid synthesized was partly degraded; when sucrose-U-^{14}C was fed, uniformly labeled tartaric acid was obtained, but when D-glucurono-γ-lactone-6-^{14}C or L-ascorbic acid-1-^{14}C was used, most of radioactivity was localized in the carboxyl groups.

INTRODUCTION

ALTHOUGH considerable effort has been devoted to the elucidation of the biosynthesis of tartaric acid* in plants,[1–4] the precursor of this acid is not known. After feeding labeled glucose, Gyr[3,4] supposed that D-glucose was a precursor. Recently, Ribéreau-Gayon[5] reported that more radioactivity was incorporated in the carboxyl groups of tartaric acid by feeding D-glucose-1-^{14}C than D-glucose-6-^{14}C to young grape berries and he suggested that tartaric acid was synthesized from the C_1–C_4 moiety of glucose.

Previous papers of this laboratory[6,7] showed that grape berries are highly active in synthesizing tartaric acid in their early stage of ripening. In addition, it was found that young grape berries have a capacity to fix CO_2 in the light and about 30 per cent of ^{14}C derived from $^{14}CO_2$-fixation was detected in tartaric acid. These studies raised questions which required further investigation of $^{14}CO_2$-fixation.

This communication describes a detailed study on the incorporation of $^{14}CO_2$ into tartaric acid in the light and the dark by young grape berries. The results indicate that tartaric acid is not formed through a carboxylation of C_2 or C_3 compound, but through sugars derived from $^{14}CO_2$-fixation products. Further investigations on feeding of labeled sugars establish that L-ascorbic acid is an effective precursor of tartaric acid and the C_1–C_4 moiety of L-ascorbic acid is a source of tartaric acid in grape berries.

* In this paper, tartaric acid refers to L(+)-tartaric acid.

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RESULTS AND DISCUSSION

Changes in $^{14}CO_2$-Assimilation Products After Fixation by Young Grape Berries

As described previously,[7] almost 30 per cent of the total fixed radioactivity was found in tartaric acid 2 days after $^{14}CO_2$-fixation of the young grape berries (10 days after flowering), suggesting that tartaric acid synthesis from CO_2 is very active in an early stage of ripening and that tartaric acid is an early assimilation product of CO_2. Using young grape berries, $^{14}CO_2$-fixation was performed in the light and dark and changes of $^{14}CO_2$-assimilation products was surveyed during a shorter time interval than in the previous experiment.

Tables 1 and 2 show the results of distribution of ^{14}C among the $^{14}CO_2$-assimilation products in the light and dark. The amount of radioactivity of "total extract" in the dark was about one-fourth of that found in the light, indicating that dark fixation of CO_2 plays an

TABLE 1. RADIOACTIVITY TRANSITION OF $^{14}CO_2$-ASSIMILATION PRODUCTS IN GRAPE BERRIES IN THE LIGHT

($\times 10^3$ cpm PER 20 BERRIES)

	Time after the beginning of $^{14}CO_2$-fixation			
	20 min	30 min	120 min	480 min
Total extract	237·8	242·0	160·0	153·8
Anionic fraction	87·1	87·0	80·8	78·6
Malic acid	43·4	54·0	21·4	19·2
Tartaric acid	1·8	1·9	6·7	16·8
Other free acids	20·4	16·2	21·5	11·1
Formic acid eluate	21·5	14·9	31·2	31·5
Cationic fraction	68·3	76·5	32·6	21·3
Neutral fraction	82·4	79·0	46·6	53·8

Grape berries: 8 days after flowering.
$^{14}CO_2$-fixation: in 200-ml bag containing 89·3 μmoles CO_2 (conc. 1·0%), labeled with 200 μc ^{14}C, for 10 min.

TABLE 2. RADIOACTIVITY TRANSITION OF $^{14}CO_2$-ASSIMILATION PRODUCTS IN GRAPE BERRIES IN THE DARK

($\times 10^3$ cpm PER 20 BERRIES)

	Time after the beginning of $^{14}CO_2$-fixation			
	20 min	30 min	120 min	480 min
Total extract	43·1	58·5	37·8	36·8
Anionic fraction	24·6	38·5	25·9	15·4
Malic acid	17·1	27·8	14·7	3·7
Tartaric acid	0	0	0	2·3
Other free acids	7·5	10·7	11·2	1·0
Formic acid eluate	0	0	0	8·4
Cationic fraction	18·5	20·0	5·7	10·0
Neutral fraction	0	0	6·2	11·4

Grape berries and the conditions of $^{14}CO_2$-fixation were the same as in Table 1, except the covering of the bag with aluminium foil.

important role in young berries. Recently, it was reported that the activity of phosphopyruvate carboxylase is especially high in young berries.[8]

On comparing Tables 1 and 2, it is apparent that in the dark radioactivity is not found in the neutral fraction until 120 min after fixation. On the other hand, the incorporation into malic acid is observed after a short period. It has been known that the first product of dark fixation of CO_2 by plants is oxaloacetic or malic acid, and that they are metabolized to other organic and amino acids. Furthermore the secondary products can ordinarily be converted to carbohydrates in the light.[9–11] The results of Table 2 seem to indicate that dark fixation of grape berries is the same as in other plants. In the light (Table 1), a considerable amount of radioactivity was found in the neutral fraction immediately after the $^{14}CO_2$-fixation and the radioactivity was already found in tartaric acid only 20 min from the beginning of the experiment. In the dark, the incorporation of ^{14}C into tartaric acid was found only at 480 min after $^{14}CO_2$-fixation and it is very interesting that the incorporation into tartaric acid is followed to the incorporation into sugars. These results suggest that tartaric acid is not formed through a carboxylation of C_2 or C_3 compounds but is synthesized from sugars, in agreement with results of earlier workers.[3–5]

Tartaric Acid Synthesis from D-*Glucuronate*-6-^{14}C, D-*Glucurono-γ-lactone*-6-^{14}C, L-*Ascorbic Acid*-1-^{14}C *and Sucrose-U-*^{14}C *in Grape Berries*

To verify the assumption that tartaric acid is formed from sugars, labeled sugars were fed to young grape leaf buds or seedlings. It was found that glucose-U-^{14}C, fructose-U-^{14}C and sucrose-U-^{14}C were equivalent to $^{14}CO_2$ as precursors judging from the ratio of the incorporation into tartaric acid. But they were not effective precursors because of their low ratio of the incorporation; indeed, the incorporation into malic acid was higher than into tartaric acid. A study of the incorporation of other labeled sugars into organic acids showed that the incorporation from *myo*-inositol-U-^{3}H was higher into tartaric acid than malic acid. In higher plants, the conversion of *myo*-inositol into D-glucuronic acid has already been established by Loewus.[12] Therefore, labeled D-glucurono-γ-lactone, a member of uronic acid cycle, was fed and it was found that 27 per cent of the radioactivity was found in tartaric acid 48 hr after administration of D-glucurono-γ-lactone-6-^{14}C into a grape leaf buds through the petiole. A high incorporation into tartaric acid from D-glucurono-γ-lactone suggests that a member of uronic acid cycle is a precursor of tartaric acid.

D-Glucuronate-6-^{14}C and L-ascorbate-1-^{14}C were then fed in addition to D-glucurono-γ-lactone-6-^{14}C, because they are closely related metabolically to D-glucurono-γ-lactone; sucrose-U-^{14}C was used as a control. Table 3 shows the results of distribution of ^{14}C derived from ^{14}C-labeled compounds. As can be seen,^{14}C-incorporation into tartaric acid was high from D-glucurono-γ-lactone-6-^{14}C and L-ascorbic acid-1-^{14}C 24 hr after feeding. In the case of L-ascorbic acid-1-^{14}C, it is remarkable that 72 per cent of the radioactivity was incorporated into tartaric acid. With D-glucuronate-6-^{14}C, tartaric acid-^{14}C was synthesized more than it was with sucrose-U-^{14}C. From D-glucuronate-6-^{14}C, a considerable amount of radioactivity was found in malic acid and other free acids. These results suggest that a portion of D-glucuronate-6-^{14}C is metabolized via the hexose monophosphate shunt and this may be one of the reasons why only a small quantity of tartaric acid is synthesized.

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TABLE 3. TARTARIC ACID SYNTHESIS FROM FOUR RADIOACTIVE SUBSTRATES IN GRAPE BERRIES (PERCENTAGE OF ^{14}C IN THE HCl-EXTRACT)

Sugars administered	Glucurono-γ-lactone-6-^{14}C		Glucuronate-6-^{14}C		Ascorbate-1-^{14}C		Sucrose-U-^{14}C	
Feeding time (hr)	24	48	24	48	24	48	24	48
Total extract	100%	100%	100%	100%	100%	100%	100%	100%
Anionic fraction	56·6	38·2	79·4	39·2	81·3	57·3	19·8	15·2
Oxalic acid	1·4	1·0	0	2·3	3·7	3·0	1·2	0
Malic acid	3·2	2·5	13·0	5·2	1·7	0·9	2·5	10·3
Tartaric acid	24·8	20·6	4·5	8·2	71·8	48·2	2·0	3·2
Other free acids	3·7	5·3	7·3	15·6	0·9	3·7	3·3	1·7
Formic acid eluate	23·5	8·8	55·0	7·9	3·2	1·5	10·8	0
Cationic fraction	2·0	2·6	2·9	3·8	0·9	0·1	9·3	12·7
Neutral fraction	19·4	21·7	15·8	22·4	3·1	3·8	62·7	72·1
Adsorbed fraction	22·0	37·5	1·9	34·6	14·7	38·8	8·2	0

Grape berries: 8 days after flowering.
Substrate: each substrate labeled with 10 μc was used.

In our studies, D-glucuronate, D-glucurono-γ-lactone and L-ascorbate were fractionated into the "formic acid eluate". It is assumed that the radioactivity in this fraction, especially in the case of D-glucuronate-6-^{14}C and D-glucurono-γ-lactone-6-^{14}C, is due to the unmetabolized labeled compounds, for the radioactivity in this fraction decreased with time. In Table 3, "adsorbed fraction" indicates the portion that was not recovered after fractionation by ion-exchange resins. This fraction probably contains polysaccharide, because glucuronate, its lactone and ascorbate are effective precursors for glucuronides, and furthermore the percentage of radioactivity in this fraction increased with time. From these results (Table 3) it can be concluded that L-ascorbic acid is an effective precursor of tartaric acid.

Degradation of Tartaric Acid

Degradation experiments were carried out to discover how tartaric acid is synthesized from each precursor. Because of the nature of this experiment, tartaric acid that was synthesized 24 hr after feeding was used as a sample for the degradation. Table 4 shows the results of the distribution of ^{14}C in tartaric acid derived from D-glucurono-γ-lactone-6-^{14}C, L-ascorbic acid-1-^{14}C and sucrose-U-^{14}C. In the case of sucrose-U-^{14}C, uniformly labeled tartaric acid was synthesized, but with D-glucurono-γ-lactone-6-^{14}C and L-ascorbic acid-1-^{14}C, it was found that most of the radioactivity was localized in carboxyl groups of tartaric acid. These

TABLE 4. DISTRIBUTION OF ^{14}C IN TARTARIC ACID SYNTHESIZED FROM EACH RADIOACTIVE SUBSTRATE FED TO GRAPE BERRIES

Labeled compounds administered	Glucurono-γ-lactone-6-^{14}C (%)	Ascorbate-1-^{14}C (%)	Sucrose-U-^{14}C (%)
COOH COOH HCOH—HOCH	94 6	95 5	47 53

Tartaric acid obtained in Table 3 (24-hr feeding) was subjected to the degradation.

results suggest that the C_1–C_4 moiety of L-ascorbic acid are converted directly into tartaric acid.

Loewus and Stafford have already fed L-ascorbic acid-6-^{14}C to excised grape leaves;[2] the radioactivity was not incorporated into tartaric acid, and it was concluded that L-ascorbic acid was not a direct precursor. Their results can be taken as indirect proof supporting the results of the present experiments. Recently, Ribéreau-Gayon[5] showed that in grape berries the incorporation into the carboxyl groups of tartaric acid from D-glucose-1-^{14}C was higher than from D-glucose-6-^{14}C. On the other hand, Loewus[13] presented evidence that in strawberries C_1 of L-ascorbic acid is derived from C_1 of D-glucose. If the biosynthesis of L-ascorbic acid from D-glucose in grape berries is the same as in strawberries, then the observation of Ribéreau-Gayon also supports the present results that tartaric acid is formed from the C_1–C_4 moiety of L-ascorbic acid.

EXPERIMENTAL

Plant Material

Grape berries were obtained from a 15-yr-old vine of *Vitis labruscana* B. "Delaware", growing in a vineyard at Kyoto University.

Labeled Compounds

Sucrose-U-^{14}C, D-glucuronate-6-^{14}C, D-glucurono-γ-lactone-6-^{14}C and DL-tartarate-1,4-^{14}C were purchased from the Radiochemical Centre, Amersham, England. L-Ascorbate-1-^{14}C was purchased from New England Nuclear Corp., Boston, U.S.A. DL-Tartarate-2,3-^{14}C was prepared by the oxidation of fumarate-2,3-^{14}C (product of the Radiochemical Centre) with OsO_4 according to Milas and Terry,[14] and identified by paper chromatography. *myo*-Inositol-U-^{3}H was kindly supplied by Dr. K. Asada, which was prepared by a Wilzbach method.[15] ^{3}H exchanged with a hydrogen atom of the hydroxyl groups of *myo*-inositol was removed by acetylation with acetic anhydride and subsequent hydrolysis with HCl.

$^{14}CO_2$-*Fixation and Administration of Labeled Compounds*

The grape cluster was exposed to $^{14}CO_2$ under illumination from fluorescent lamps (6000 lux) as described in a previous paper.[7] In the case of dark-fixation, the assimilation bag was wrapped with aluminium foil. Before $^{14}CO_2$-fixation, the cluster was pre-treated in the light or dark for 1 hr. After the pre-treatments, $^{14}CO_2$-fixation was carried out for 10 min. Subsequently, $^{14}CO_2$ was removed from the assimilation bag during the next 10 min, and the aluminium foil and the assimilation bag were removed and several berries were immediately picked from the cluster as the first sample. The berries were sampled at 30, 120 and 480 min after the beginning of $^{14}CO_2$-exposure to the cluster.

^{14}C-Labeled compounds were fed through a peduncle. The ends of two cotton threads were dipped into the solution of labeled compound in a small glass tube (5 × 40 mm). The other ends of threads were pierced through the peduncle. Whenever labeled compound in the tube was completely absorbed by the cluster, water was supplied. The feeding experiment was conducted alternately under illumination from fluorescent lamps (6000 lux) and under the dark condition with 12-hr intervals. A temperature of 25° was maintained during the entire cycle.

Analysis of Tartaric Acid

The berries were homogenized with 0·03 N HCl in a bowl-mill and the pH was re-adjusted to about 1 by adding 1 N HCl. The soluble fraction was then separated by centrifugation. The residue was re-extracted (× 3) with 0·03 N HCl. The combined extract was then fractionated by ion-exchange resins and the anionic fraction was separated by silica gel column chromatography, as described in the previous paper.[7]

Determination of ^{14}C

The radioactivity of each sample was determined with a liquid scintillation counter (Nuclear Chicago, type 6801). In the case of column chromatography of organic acids, the radioactivity of the effluents was determined after evaporating organic solvents.

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Degradation of Tartaric Acid

Tartaric acid was oxidized with HIO_4, according to the modified method of Sprinson and Shargaff.[16] The first test-tube contained tartaric acid-^{14}C (the final concentration was 0·16 M) isolated from the berries feeding labeled sugar, and the second test-tube contained 0·2 N NaOH. Both test-tubes were connected and degradation was started by adding HIO_4 (the final concentration was 1 M) to the first test-tube. N_2 gas was bubbled through the reaction mixture, and evolved CO_2 was trapped in the second tube. A sample from the second test-tube was used for counting radioactivity of $^{14}CO_2$, and the residue was used for determination of CO_2 evolved by titration. The specific activity of CO_2, which is derived from the both carboxyl groups of tartaric acid, was then calculated. Because the reaction did not proceed completely, the ratio of ^{14}C in the carboxyl groups of tartaric acid was calculated from the ratio of specific activities of CO_2 and tartaric acid. The validity of this procedure was confirmed by degradation of DL-tartaric acid-1,4-^{14}C and DL-tartaric acid-2,3-^{14}C as authentic samples.

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