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CONVERSION OF TARTRATE TO MALATE AND MONOETHYL TARTRATE IN GRAPE LEAVES

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Key Word Index—*Vitis labruscana*; Vitaceae; grape; leaf; tartrate; malate; monoethyl tartrate; metabolic conversion.

Abstract—$^{14}CO_2$ evolution from L(+)-tartrate-[U-^{14}C] in grape leaves was observed confirming the dissimilation of tartrate. 30 sec after the administration of L(+)-tartrate-[U-^{14}C], 3 compounds were found to contain ^{14}C. Two of them were identified as monethyl tartrate and malate by chromatographic and MS studies. It is suggested that the $^{14}CO_2$ evolved is derived from malate-[U-^{14}C] which is metabolically formed from L(+)-tartrate-[U-^{14}C], and that monoethyl tartrate is not an intermediate for the conversion to malate.

INTRODUCTION

Tartrate has been found in certain species of plants, such as grape, geranium and pelargonium [1–4]. Saito and Kasai have reported the conversion of L-ascorbate-[1-^{14}C] to carboxyl-labelled tartrate in grape berries [5]. In pelargonium conversion of L-ascorbate-[6-^{14}C] to carboxyl-labelled tartrate has been reported by Wagner and Loewus [6]. However, in both cases the metabolic intermediates between L-ascorbate and tartrate have not been determined. Kotera *et al.* proposed a biosynthetic pathway of L(+)-tartrate via 1,2-dihydroxyethyl hydrogen L(+)-tartrate (named pretaric acid) from 5-keto-gluconic acid in *Gluconobacter suboxydans* [7–9].

Only a few studies have been made on the dissimilation of tartrate in higher plants. Vickery and Palmer reported that L(+)-tartrate is not metabolized in tobacco leaves [10, 11]. On the contrary, Stafford proposed that D(−)- or *meso*-tartrate is first metabolized to either the enol or keto form of dihydroxyfumaric acid by crude enzyme preparations from peas, beans and wheat germ [12]. Evidence for the dissimilation of tartrate in higher plants has been shown by the experiments with ^{14}C-labelled tartrate. Hardy observed $^{14}CO_2$ evolution from D,L-tartrate-[1,4-^{14}C] in grape berries [13], which are the best known accumulator of tartrate. Saito and Kasai also demonstrated $^{14}CO_2$ evolution from grape berries administered L(+)-tartrate-[1,4-^{14}C] [14]. In grapes tartrate occurs in the L(+) form [15]. Recently, we investigated dissimilation of L(+)-tartrate in grape berries attached to the vines throughout their ripening process, using L(+)-tartrate-[U-^{14}C] [16]. $^{14}CO_2$ evolution was observed at any ripening stages of grape berries and a diurnal variation was observed.

Tartrate is also dissimilated in micro-organisms and animal tissues. In *Pseudomonas, Aspergillus, Rhodotorula* and *Aerobacter* utilization of tartrate has been demonstrated [17–24]. Two main pathways have been described for microbial utilization of tartrate. One is dehydration to oxaloacetic acid in *Pseudomonas* [18, 25, 26] and the other is conversion to glycerate in *Pseudomonas* and *Rhodotorula* [17, 23]. Tartrate is oxidized in pigeon liver [27]. Kun *et al.* reported that mitochondrial enzymes from rat and beef tissues dehydrate tartrate to dihydroxyfumaric acid or oxaloglycolate [28, 29].

In grape leaves as well as berries, tartrate is a major organic acid [30], but its dissimilation has not been investigated except for the work by Stafford and Loewus [30]. The present work was designed to investigate the dissimilation of L(+)-tartrate and to determine the metabolic products in order to elucidate metabolic conversion of tartrate in grape leaves. Here we report the dissimilation of tartrate in grape leaves and identification of malate and monoethyl tartrate as the metabolic products. In the following, tartrate refers to the L(+)-form, except where otherwise indicated.

RESULTS

$^{14}CO_2$ evolution from tartrate-[U-^{14}C] in grape leaves

Tartrate-[U-^{14}C] was fed into a grape leaf attached to the cutting and $^{14}CO_2$ evolution was measured; $^{14}CO_2$ was evolved at a constant rate (Fig. 1). The ratio of the total $^{14}CO_2$ evolved for 21 hr to the ^{14}C remaining in leaf tissues was *ca* 10%. $^{14}CO_2$ evolution was also observed when tartrate-[U-^{14}C] was administered from the surface of leaf segments under reduced pressure. The rate of $^{14}CO_2$ evolution was nearly constant until 10 hr after the administration (Fig. 2). In the following experiments ^{14}C-labelled compounds were administered by the vacuum infiltration method.

Metabolic products of tartrate in grape leaves

After incubation at 30° for 30 min, leaf segments administered tartrate-[U-^{14}C] were extracted with water,

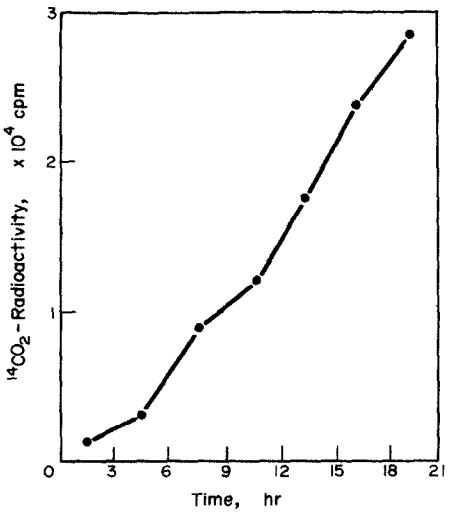

Fig. 1. $^{14}CO_2$ evolution from tartrate-[U-^{14}C] in a grape leaf cutting; 2 μCi of tartrate-[U-^{14}C] was fed. The experiment was carried out at 30° under illumination (10^4 lx).

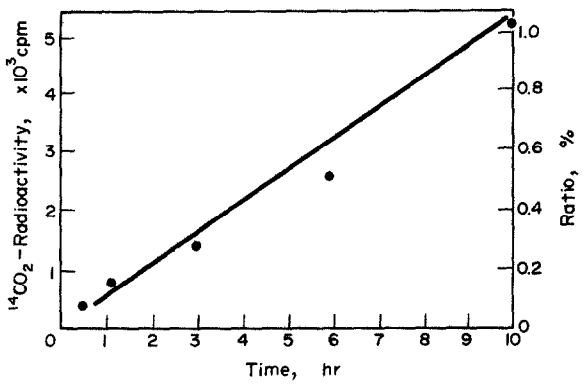

Fig. 2. $^{14}CO_2$ evolution from tartrate-[U-^{14}C] in grape leaf. About 0.25 μCi of tartrate-[U-^{14}C] was absorbed into tissues of leaf segments under reduced pressure. Per cent ratio shows the ratio of the amount of $^{14}CO_2$ evolution to tartrate-[U-^{14}C] absorbed in leaf tissues. No $^{14}CO_2$ was detected from a boiled leaf.

and the extracts were centrifuged. No radioactivity was detected in the sediments. The water-extracts were separated into anionic, cationic and neutral fractions with ion exchange resins. As shown in Table 1, almost all radioactivity was found in the anionic fraction. This fraction was subjected to Si gel column chromatography giving several ^{14}C-radioactive fractions in additions to the ^{14}C-tartrate fraction. As shown in Fig. 3, 8 fractions observed at 30 min incubation decreased to 3 fractions: I, II and III, at 30 sec incubation. It is thus supposed that these 3 compounds are early products in the metabolic conversion of tartrate in grape leaves.

The TMSi derivative of the KOH-treated compound I gave the same GLC R_t as that of tartrate. The MS of the TMSi derivative of the alkali-treated compound I was also identical with that of tartrate. MS studies using a chemical ionization source were carried out to determine the MW of the TMSi derivative of compound I, giving a value of 394; high reslution MS gave an *m/e* value of 394.17157 (PFK). This *m/e* value was analyzed by a computer system to determine the number of constituents of the TMSi derivative of compound I assuming that the derivative was composed of only C, H, O and Si. The resulting molecular formula for compound I was $C_6H_{10}O_6$. One possible compound having this formula is monoEt tartrate and I is essentially identical with the synthetic monoEt tartrate by GLC,

Table 1. Fractionation of the extracts of leaf segments administered tartrate-[U-^{14}C]

Fractions	cpm	Ratio (%)
Anionic	341000	99.3
Cationic	2500	0.7
Neutral	0	0
Total	343500	100.0

Leaf segments adminstered tartrate-[U-^{14}C] under reduced pressure were incubated at 30° for 30 min. Water extracts were separated with Amberlite IR-120B(H^+) and then with IR-45(OH^-).

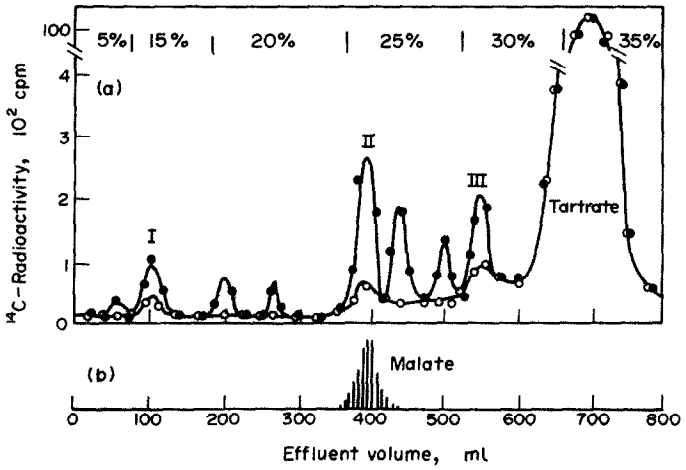

Fig. 3. Si gel column chromatogram of an anionic fraction of leaf extracts (a) and authentic malate (b). Leaf segments administered tartrate-[U-^{14}C] were incubated at 30° for 30 sec (○) or 30 min (●). Samples were eluted with chloroform–*tert*-butanol; the per cent ratios shows the concentration of *tert*-butanol.

Table 2. Intramolecular distribution of ^{14}C in malate-[^{14}C]

Malate	Carboxyl (%)	$C_2 + C_3$ (%)
a	66.2	33.8
b	99.3	0.7
c	51.9	48.1

a: Malate-[^{14}C] from leaf administered tartrate-[U^{14}C] and incubated at 30° for 15 min.
b: Malate-[^{14}C] from leaf exposed to $^{14}CO_2$ for 15 min.
c: Uniformly labelled malate-[^{14}C].

MS and PC. Compound I was therefore identified as monoEt tartrate. The monoEt tartrate content in grape leaves was determined by GLC, giving 0.025% fr. wt basis (wt %), and 2.7% of malate and 3% of tartrate (molar ratio).

A Si gel column chromatogram of compound II corresponds to that of malate (Fig. 3). Subsequent PC, GLC and MS studies confirmed its identity. R_f values of the radioactive peak and coloration spot of compound II are identical with those of malate. The GLC R_t and MS of the TMSi derivative of the compound II are also identical with those of malate.

It is known that CO_2-fixation to phosphoenolpyruvate is one of the pathways for the formation of malate in plants. It was therefore necessary to determine whether the malate-[^{14}C] formed was due to metabolic conversion of tartrate-[U-^{14}C], or due to re-fixation of $^{14}CO_2$ evolved by dissimilation of tartrate-[U-^{14}C]. In the former case ^{14}C should be uniformly distributed in malate, but in the latter case ^{14}C should be localized in its carboxyl-carbon. Malate-[^{14}C], which was obtained from the leaf segments administered tartrate-[U-^{14}C] and incubated for 15 min, was degraded in order to determine the ratios of ^{14}C in carboxyl-carbon and in ($C_2 + C_3$) in this molecule. As shown in Table 2, 66.2% of radioactivity of malate-[^{14}C] was found in the carboxyl-carbon and 33.8% in ($C_2 + C_3$), indicating that the malate-[^{14}C] was a mixture of uniformly labelled malate and some carboxyl-labelled ones. However, in case of malate-[^{14}C] formed in leaf segments exposed to $^{14}CO_2$ (0.3 mCi) for 15 min, the ^{14}C incorporation ratios in carboxyl-carbon and in ($C_2 + C_3$) were 99.3% and 0.7%, respectively, indicating carboxyl-labelled malate. It was similar to the result reported by Stafford and Loewus [30]. It is therefore suggested that malate-[^{14}C] formed in leaf segments administered tartrate-[U-^{14}C] in 15 min is a direct conversion product of tartrate-[U-^{14}C] and some refixation product. Compound III remains unidentified.

$^{14}CO_2$ evolution from monoethyl tartrate-[^{14}C] and malate-[U-^{14}C], and their metabolic conversion

$^{14}CO_2$ evolution from tartrate-[U-^{14}C] in leaf segments was compared with that from monoEt tartrate-[^{14}C] and malate-[U-^{14}C]. The ratios of $^{14}CO_2$ evolved for 1 hr to the administered tartrate-[U-^{14}C], monoEt tartrate-[^{14}C] and malate-[U-^{14}C] were 0·1%, 0.016% and 18.2% respectively (Table 3). In the leaf administered monoEt tartrate-[^{14}C], incorporation of ^{14}C into tartrate and malate was observed. Incorporation of ^{14}C into malate from monoEt tartrate-[^{14}C] was lower than that from tartrate-[U-^{14}C]. On the other hand, the conversion of malate to tartrate and monoEt tartrate was not detected (Table 3).

DISCUSSION

We observed $^{14}CO_2$ evolution from tartrate-[U-^{14}C] and confirmed dissimilation of tartrate in grape leaves (Figs 1 and 2). Hardy [13], and Saito and Kasai [14] have reported the dissimilation of tartrate in detached grape berries, and lately in intact grape berries the dissimilation of tartrate was demonstrated [16]. In the present work we have proved the dissimilation of tartrate in grape leaves. A nearly constant rate of $^{14}CO_2$ evolution (Fig. 2) suggests that tartrate-[U-^{14}C] is rapidly mixed with endogenous tartrate.

It has been reported that tartrate occurs as several kinds of phenolic esters in higher plants. Scarpati and Oriente isolated dicaffeyl ester of D(−)-tartrate from chicory leaves [31]. The presence of monocaffeyl, mono-*p*-coumaryl and monoferulyl esters of *d*-tartrate in grapes has been reported [32]. Suzuki *et al.* isolated and determined mono-*p*-coumaryl ester of *meso*-tartrate from spinach leaves [33, 34]. We have found for the first time monoEt tartrate in grape leaves. These findings suggest that tartrate occurs not only in the free- or salt form [35, 36], but also in the form of esters in plants. MonoEt tartrate is a minor organic acid in grape leaves, and the observed molar ratios of monoEt tartrate to malate and tartrate were 2.7% and 3‰ respectively.

Conversion of monoEt tartrate-[^{14}C] to both malate and tartrate was 5.5 times that of tartrate-[U-^{14}C] to monoEt tartrate (Table 3), but the pool size of tartrate was *ca* 33 times that of monoEt tartrate. It is therefore predicted that the turnover rate from tartrate to monoEt tartrate is higher than that from monoEt tartrate to

Table 3. $^{14}CO_2$ evolution and incorporation of ^{14}C into tartrate, monoethyl tartrate and malate from ^{14}C-substrates

	Substrates		
^{14}C Incorporated substances	Tartrate -[U-^{14}C] (%)	Monoethyl tartrate-[^{14}C] (%)	Malate -[U-^{14}C] (%)
CO_2 (1 hr)	0.10	0.016	18.2
Tartrate (1 min)	—	0.510	0
Monoethyl tartrate (1 min)	0.13	—	0
Malate (1 min)	0.60	0.200	—

Figures show the ratio of $^{14}CO_2$ evolved or ^{14}C incorporated into each substance to the total ^{14}C radioactivity fed into leaf tissues.

both malate and tartrate, and monoEt tartrate may be converted to other substances besides malate and tartrate.

The malate-[^{14}C] produced in leaf segments administered tartrate-[U-^{14}C] was a mixture of uniformly labelled and carboxyl-labelled malates (Table 2). On the other hand, the malate-[^{14}C] produced in leaf segments exposed to $^{14}CO_2$ was carboxyl-labelled only. This indicated that tartrate is converted to malate in grape leaves. Malate is a ubiquitous organic acid in plants and metabolically active. As shown in Table 3, high $^{14}CO_2$ evolution from malate-[U-^{14}C] in grape leaf was demonstrated. From monoEt tartrate-[^{14}C], $^{14}CO_2$ evolution and ^{14}C incorporation into malate in grape leaf were both lower than these from tartrate-[U-^{14}C], suggesting that tartrate is converted to malate and that monoEt tartrate is not an intermediate to malate.

We, therefore, propose that there are at last two pathways of metabolic conversion of tartrate in grape leaves; one is the conversion to malate and the other is to monoEt tartrate. Incorporation of ^{14}C into tartrate from malate-[U-^{14}C] was not observed, so that the reaction from tartrate to malate is irreversible. Compound III remains unidentified. In the leaf segments fed malate-[U-^{14}C] or monoEt tartrate-[^{14}C], incorporation of ^{14}C into compound III was not detected. It is therefore supposed that compound III is an intermediate product in another pathway of the dissimilation of tartrate in grape leaves.

EXPERIMENTAL

Berries and leaves of *Vitis labruscana* B 'Delaware' from the vineyard at Kyoto University were used. Leaves were grown from cuttings.

Preparation of ^{14}C-labelled compounds and determination of their intramolecular distribution of ^{14}C. Tartrate-[U-^{14}C] and malate-[U-^{14}C] were prepared according to ref. [16]; the berries, 10 days after flowering, were exposed to 5 mCi of $^{14}CO_2$ for 48 hr under continuous illumination (10^4 lx) and then detached. Berries were extracted with 80% EtOH and subsequently with 50 mM HCl. Extracts were separated with Amberlite IR-120B(H^+) and IR-45(OH^-), and then the anionic fraction was subjected to Si gel column chromatography. About 0.13 mCi (0.12 mCi/mmol) of tartrate-[^{14}C] and 0.17 mCi (1.41 mCi/mmol) of malate-[^{14}C] were obtained. The tartrate-[^{14}C] thus isolated was degraded according to ref. [5]. The ratios of ^{14}C in carboxyl-carbon and in (C_2 + C_3) were 53.5% and 46.5%, rdspectively, indicating uniform labelling. The ratios of ^{14}C in carboxyl-carbon and in (C_2 + C_3) of malate-[^{14}C] were 51.9% and 48.1%, respectively. It was therefore proved that malate-[^{14}C] isolated was uniformly labelled. Malate-[^{14}C] was oxidized with $KMnO_4$ into $^{14}CO_2$ (derived from carboxyl group) and acetaldehyde-[^{14}C] (derived from C_2 + C_3) by the method of ref. [37].

Administration of ^{14}C-labelled compounds and collection of $^{14}CO_2$. ^{14}C-labelled compounds were fed into grape leaf by the following procedures. Feeding of tartrate-[U-^{14}C] into leaf cuttings was carried out with cotton thread passed through the petiole [16], and the leaf placed in a glass vessel. $^{14}CO_2$ evolved in a glass vessel was collected into CO_2-absorber (ethanolamine–ethylcellosolve, 1:9) by suction [16, 38]. Detached leaves were soaked in 5% Triton X-100 for *ca* 30 min and then washed thoroughly with H_2O. Leaves were then cut into segments of *ca* 1 × 1 cm. A small aliquot of ^{14}C-labelled compound, (0.05 to 0.1 ml) was placed on the segments which were then subjected to red. pres. to infiltrate the labelled compound. Any residual ^{14}C-compound on the segments was washed off with H_2O. The segments were placed in a glass vial and $^{14}CO_2$ evolved was collected into a CO_2-absorber by suction.

Analytical procedure. Leaf segments administered ^{14}C-compound were incubated at 30° and the reaction was terminated by dipping in dry ice–EtOH slush. Frozen segments were homogenized with H_2O, followed by centrifugation. The soluble fraction was passed through columns of Amberlite IR-120B(H^+) and then of IR-45(OH^-). The anionic fraction adsorbed on IR-45 was eluted with 2M $(NH_4)_2CO_3$ and the cationic fraction absorbed on IR-120B was eluted with 4M NH_4OH.

GLC. Instruments equipped with TC and FID detectors were used. The columns were: (a) 2.25 m × 3 mm i.d. glass packed with 3% OV-17 (G-80), and (b) 2 m × 3 mm i.d. glass packed with 1.5% SE-52 (4BM). Column temps were 160° isothermal for column (a), and temp. programmed from 100° to 200° at 6°/min for column (b). Injector temp. was 180° and He (G-80) and N_2 (4BM) flow rates were 30 ml/min. Lyophilized samples were silylated with trimethylchlorosilane and hexamethyldisilazane. The reaction mixture was directly injected into the chromatograph.

MS. Spectra were recorded at an accelerating voltage of 3.5 kV, an ionizing electron energy of 70 eV (500 eV for chemical ionization) and an ion source temp. of 270° (210° for chemical ionization). A glass column 2 m × 3 mm i.d. packed with 3% SE-52 was used for the chromatographic separation.

Synthesis of monoEt tartrate [39]. Tartrate (4g) were refluxed at 70° in 6 g of dry EtOH for 7 hr. H_2O (6 g) and $BaCO_3$ (8g) were then added to the mixture with stirring, followed by centrifugation. The supernatant was passed through a column of Amberlite IR-120B(H^+) and then purified by Si gel column chromatography. MonoEt tartrate-[^{14}C], in which only the tartrate moiety was uniformly labelled, was synthesized from tartrate-[U-^{14}C] by the same procedure.

Measurement of radioactivity. ^{14}C-Radioactivity was counted by liquid scintillation. The counting mixture was composed of toluene, PPO and POPOP for $^{14}CO_2$ collected in CO_2-absorber, or dioxane, PPO, POPOP and naphthalene for aq. samples.

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