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Stereoisomeric Characterization of Tartaric Acid Produced during L-Ascorbic Acid Metabolism in Plants[1]

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ABSTRACT

Labeled tartaric acids from *Pelargonium crispum* apices which had been fed L-ascorbic acid-6-^{14}C and *Vitis labrusca* and *Parthenocissus inserta* tissues which had been fed L-ascorbic acid-1-^{14}C were examined by chemical means to determine chiral configuration. In each instance, label was associated with (+)-tartaric acid.

Similar experiments with labeled tartaric acid from *P. crispum* which had been labeled with D-glucose-1-^{14}C or -6-^{14}C led to the same result. No evidence was obtained for formation of labeled meso-tartaric acid in experiments described above. The recent suggestion of H. Ruffner and D. Rast (Z. Pflanzenphysiol. 73: 45–55, 1974) that conversion of L-ascorbic acid to tartaric acid in plants is a nonenzymatic process is re-examined in the light of present findings.

All three stereoisomeric forms of tartaric acid have been found in higher plants; (+) TA[4] in numerous plant families (4) notably the Vitaceae and Geraniaceae (23, 24); (−) TA in *Bauhinia reticulata* (18) and as esters of caffeic acid in *Cichorium intybus* L., *C. endivia* L. and *Lactuca sativa* L. (6, 21, 22, 30); and meso-TA as an ester of *p*-coumaric acid in spinach leaves and isolated spinach chloroplasts (16, 25) or as an excreted product (5). Only the (+) form has received much attention as regards its metabolic origin. Literature pertinent to biosynthesis of (+) TA is summarized in recent publications (19, 27–29). These biosynthetic studies employed labeled precursors and, in most instances, recovered TA from solution as its poorly soluble KH salt. In one study (13, 14), formation of (+) TA from glycolate-1-^{14}C in *Pelargonium zonale* L. was accompanied by (−) TA and/or meso TA.

This latter observation prompted a careful study of the isomeric form of labeled TA recovered from *Pelargonium crispum* L. after feeding L-ascorbic acid-6-^{14}C (28) and from *Vitis labrusca* L. and *Parthenocissus inserta* after feeding L-AA-1-^{14}C (29). Data on labeled TA from *P. crispum* after feeding D-glucose-1-^{14}C or -6-^{14}C were also gathered (12). Methods used in this study separate all three stereoisomers of TA and show that the (+) form is a metabolic product of L-AA and D-glucose.

MATERIALS AND METHODS

Sources of Labeled TA. Samples of radioactive tartaric acid from *Pelargonium crispum* apices which were labeled with L-AA-6-^{14}C (28) and from *Vitis labrusca* berries and *Parthenocissus inserta* leaves which were labeled with L-AA-1-^{14}C (29) were used in this study. Samples from *P. crispum* apices which were labeled for 72 hr with D-glucose-1-^{14}C or -6-^{14}C by the same procedure used in AA experiments (12) were also studied.

Separation of Diastereomeric Forms of TA. Ion exchange chromatography with Dowex 1 (formate) resin and a 0 to 4 M formic acid gradient readily separated meso-TA from the chiral forms. With the two step gradient procedure described earlier (28), meso-TA appeared in the second step of the gradient in the volume eluted between 60 to 110 ml and chiral TA between 100 to 160 ml. Further purification by paper chromatography (28) and GLC (27) was used to identify TA.

Resolution of Labeled TA. Samples used for this study had been recovered from plant extracts by ion exchange chromatography using (+) TA as carrier. Chiral TA fractions were combined and adjusted to pH 3.5 with KOH to recover the KH salt of TA. A weighed aliquot containing 0.05 to 0.1 μCi was converted to free TA and combined with an equimolar amount of (−) TA to make a racemic mixture. Following further dilution with unlabeled racemic TA, the mixture was resolved by the method of Haskins and Hudson (8) as described by Richtmyer (17) using 2-(D-glycero-D-gulo-hexahydroxyhexyl)-benzimidazole. The benzimidazole salt of (−) TA readily crystallized from the reaction mixture and was recrystallized three times in 30% ethyl alcohol. After conversion of the (−) TA salt to the free acid by passage over a column of Dowex 50 H^+ resin,[5] it was converted to the KH salt and recrystallized from H_2O. Weighed aliquots of the KH salt were reconverted to the free acid and adjusted to a predetermined volume in aqueous solution for polarimetric analysis.

After reducing the volume of the mother liquor of the benzimidazole salt of (−) TA, additional crops of (−) TA salt were obtained by further crystallization. In this way, four crops

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[4] Abbreviations: TA: tartaric acid; AA: ascorbic acid.

[5] The benzimidazole base was readily recovered from Dowex 50 resin by elution with a 0 to 1 M ammonium hydroxide gradient. After removal of the ammonium hydroxide and several water washes, the residue was crystallized from 50% ethyl alcohol to give essentially 100% recovery. The melting point, decomposition 218 C, was unchanged (14).

Table I. *Specific Rotations and Specific Radioactivities of Tartaric Acid*

Labeled Precursor	Tissue	Configuration of Tartaric Acid	Specific Rotation[1]	Specific radioactivity: Value if all ^{14}C was located in (+) tartaric acid	Specific radioactivity: Experimental value
			$[\alpha]_D^{21}$ (c 4, H_2O)	cpm/μmole	
AA-6-^{14}C	*P. crispum*	+	+11.1	1.23	1.17
		−	−15.2	0	0
AA-1-^{14}C	*V. labrusca*[2]	+	+13.3, +12.8	1.56, 1.35	1.64, 1.37
		−	−14.8, −14.9	0, 0	0, <0.01
AA-1-^{14}C	*P. inserta*	+	+14.0	2.25	2.11
		−	−15.1	0	<0.01
Glc-1-^{14}C	*P. crispum*	+	+15.6[3]	13.5	13.0
		−	−14.1	0	<0.1
Glc-6-^{14}C	*P. crispum*	+	+15.5[3]	8.22	7.5
		−	−14.0	0	<0.1

[1] Specific rotations of reagent TA used as carrier or for dilution: $[\alpha]_D^{21}$ (c 4, H_2O) (+) TA, +14.3; (−) TA, −14.2; racemic TA, 0.

[2] Values from two separate resolution experiments on the same sample.

[3] Rotations taken at 0.8 g/100 ml and corrected to 4 g/100 ml using a standard curve. All other samples were read at 4 g/100 ml so no correction was necessary.

were removed, although only the first was used for recovery of (−) TA. Mother liquor remaining from the fourth crop contained the soluble (+) TA salt as well as residual (−) TA salt. This solution was passed through Dowex 50 H^+ resin and adjusted pH 3.5 with KOH to obtain the KH salt of (+) TA. The latter was crystallized twice from water. Weighed samples were used for polarimetric analysis as described above.

Rotations were measured in a 1-dm tube (1 ml) at 586 nm using a Perkin-Elmer Model 141 Polarimeter. The radioactivity of samples was determined immediately thereafter (28).

RESULTS AND DISCUSSION

In the present study, labeled TA which had been trapped with carrier (+) TA and isolated by ion exchange chromatography as the chiral TA peak was converted to a racemic mixture with addition of an equimolar amount of (−) TA and then chemically resolved in order to trace the fate of label distribution between (+) and (−) chiral forms. Specific rotations of resolved (+) TA and (−) TA and the specific radioactivity of these samples are listed in Table I. For purposes of comparison, the specific radioactivity to be expected if all label present in the racemic mixture appeared in (+) TA is also given. Specific rotations of the (+) TA, (−) TA, and racemic TA used in this study as carrier or diluent are also listed.

In each experiment, whether TA was produced from labeled L-AA or D-glucose, (+) TA retained virtually all radioactivity after resolution of the artificially mixed racemic TA. Considerable significance is attached to the observation that (−) TA lacked label. Its diastereomeric benzimidazole salt was poorly soluble in ethyl alcohol and crystallized from a radioactive solution yet was devoid of label.

In *P. crispum* apices labeled with L-AA-6-^{14}C and *V. labrusca* and *P. inserta* tissues labeled with L-AA-1-^{14}C, virtually all label appearing in the chiral TA region of the ion exchange was recovered as the KH salt of TA, and the specific radioactivity remained unchanged during subsequent recrystallizations. Since resolution of an artificially prepared racemic mixture revealed all label to be associated with (+) TA, it is certain that (+) TA is the only chiral form produced during L-AA metabolism in these tissues regardless of the path of conversion (28, 29).

In *P. crispum* apices labeled with D-glucose-1-^{14}C or -6-^{14}C, recovery of virtually all label from the chiral TA region of the ion exchange profile as the KH salt and appearance of all of this label in (+) TA showed that label from the hexose pool contributed to (+) TA only. It was reported elsewhere (12) that TA obtained from *P. crispum* labeled with D-glucose-1-^{14}C contained more ^{14}C in internal carbons than tissues labeled with D-glucose-6-^{14}C, presumably an indication that label from D-glucose-1-^{14}C followed a less direct path of conversion to TA (11).

Although present results establish the biosynthesis of only one chiral form of TA during D-glucose and L-AA metabolism, possible formation of meso-TA in certain experiments must also be considered. In AA-labeled *P. crispum* experiments, only traces of radioactivity were found in the meso-TA region of the ion exchange profile and only when L-AA-6-^{14}C was provided, never when the source of label was L-AA-1-^{14}C. With AA-labeled *V. labrusca* and *P. inserta*, however, a substantial peak of radioactivity appeared in the meso-TA region when L-AA-6-^{14}C was supplied, much less when L-AA-1-^{14}C was given in the light, and none when L-AA-1-^{14}C was given in the dark (27). In one experiment, *P. inserta* labeled with L-AA-6-^{14}C for 14 hr (26, see Table IV), 50 mg each of glycolate, glyoxylate, L-AA, meso-TA, and (+) TA were added as carriers to the plant extract prior to removal of oxalic acid used in the grinding medium. Acidic peaks corresponding to each of these additions were obtained in appropriate eluted fractions after ion exchange chromatography using the two-step gradient, an indication that none of these added acids were lost during precipitation of calcium oxalate.

Radioactive components in the meso-TA region of all experiments mentioned earlier were further examined by paper chromatography in systems which separate malic acid, citric acid and meso-TA. In none, including the experiment involving added meso-TA, did any of the radioactive components correspond to meso-TA. Label from C1 or C6 of L-AA is not converted to meso-TA by *P. crispum*, *V. labrusca*, and *P. inserta*.

This same conclusion probably applies to conversion of D-glucose-1-^{14}C or -6-^{14}C to TA in *P. crispum*. Despite the large amount of radioactivity found in fractions associated with the meso-TA region after ion exchange chromatography, virtually all of this label separated from meso-TA after paper chromatography.

In the course of a recent study of TA biosynthesis in grape tissue using specifically labeled D-glucose and glyoxylic acid, Ruffner and Rast (19) gave brief consideration to the role of L-AA as a TA precursor. They dismissed Saito and Kasai's findings (20) as artifacts, the consequence of nonenzymatic decomposition of L-AA-1-^{14}C during extraction and recovery procedures, citing as evidence an experiment of their own in which addition of L-AA-1-^{14}C to an acidic homogenate of grape leaves resulted in conversion of 25% of the label to organic acid. Gas-liquid chromatography of this fraction after trimethylsilylation revealed that a considerable portion of the label was associated with the TA peak. Another portion of the radioactivity was recovered in an unidentified peak, presumably a 2-carbon fragment. They attribute Saito and Kasai's "extraordinarily high incorporation rate" of label into TA from L-AA-1-^{14}C to a combination of circumstances involving the presence of halide in the extracting medium, use of strongly acidic ion exchange resin, and elevated temperatures.

Experiments with labeled AA in *P. crispum* (28) and Vita-

ceae species (29) argue against Ruffner and Rast's conclusions. When L-AA-1-^{14}C was supplied to *P. crispum* apices, virtually no label appeared in (+) TA even after 72 hr of metabolism, but when L-AA-6-^{14}C was supplied there was a gradual increase in labeled TA from 2% of the soluble label in 4 hr to 32% in 72 hr (12, 28). Moreover, Saito and Kasai's results were reproducible using a mild extraction procedure that excluded halide ions and high temperatures (29). Further, when L-AA-1-^{14}C was supplied to oxalate accumulating plants such as spinach or *Oxalis,* label was recovered in large measure as oxalic acid, not TA (12). Barley seedlings accumulate neither oxalic acid nor TA. When fed L-AA-1-^{14}C, barley seedlings formed neither labeled oxalic acid nor labeled TA (J. C. Yang, unpublished observations). Finally, one might expect to find both chiral forms of TA labeled after supplying L-AA-1-^{14}C if a nonenzymatic degradation was involved. Results obtained in this study show that only the (+) form was produced.

TA Nomenclature and Stereochemistry. The absolute configuration of (+) TA, established by Bijovet *et al.* in 1951 (3) can be assigned either D or L prefix (1, 9, 10, 15, 26). The Cahn-Ingold-Prelog system of nomenclature, which assigns a priority to functional groups of each asymmetric carbon in TA, designates (+) TA as 2R, 3R-TA and (−) TA as 2S,3S-TA (2, 7, 9, 10).[6] Although unambiguous, this convention has net yet been widely adopted for chiral forms of TA. It would be useful to find a biochemical viewpoint, based on biosynthesis, that would provide a stereochemical relationship between a known structure and (+) TA. The relationship between L-AA and (+) TA in *P. crispum* promises to provide such information.

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[6] Alworth (2) designates (+) TA as 1R,2R-TA and (−) TA as 1S,2S-TA.