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## Uptake and Conversion of Radioactive Carbon Dioxide and Glucose in the Acerola and their Relationship to Ascorbic Acid Biosynthesis<sup>1</sup>

A. Mavetzi, H. J. Teas, and C. F. Asenjo<sup>2</sup>

### INTRODUCTION

The West Indian Cherry, *Malpighia punicifolia* L., called "acerola" in Puerto Rico, is well known for the extraordinarily high content of ascorbic acid in the fruit (1).<sup>3</sup> The phenomenon of excessively large amounts of a substance accumulating in a plant system is not rare. High concentrations of such compounds as alkaloids, lipids, and certain proteins or carbohydrates are well known in the tissues of a variety of plants. We are attempting to answer the question: Why does ascorbic acid accumulate in such large amounts in the fruit of the West Indian Cherry? A number of strains of this plant occur in Puerto Rico. Variations of up to 25 percent in the ascorbic acid levels of their mature fruit have been reported (2). It was thought possible that with this spectrum of types available, we might obtain a generally applicable understanding about the interplay of genetic and metabolic systems involved in the determination of ascorbic acid levels in plants.

Radioactive precursors were chosen which would encompass as wide an area of metabolism as possible, while yielding maximum information about the substances produced. The present study used developing acerola fruit and leaves to explore the fate of radiocarbon-labeled carbon dioxide and glucose.

<sup>1</sup> The authors are grateful to Mr. H. Ayguabibus for his care of the acerola trees at the Puerto Rico Nuclear Center, Mayagüez, P.R.

<sup>2</sup> Associate Scientist, Puerto Rico Nuclear Center, Caparra Heights, P.R., and Associate, Department of Biochemistry and Nutrition, School of Medicine, University of Puerto Rico, San Juan, P.R.; Chairman, Division of Biological Sciences, University of Georgia, Athens, Ga.; and Professor and Head, Department of Biochemistry and Nutrition, School of Medicine, University of Puerto Rico, San Juan, P.R., respectively.

<sup>3</sup> Italic numbers in parentheses refer to Literature Cited, pp. 8-9.

## MATERIALS AND METHODS

Fruit and leaves of acerola for the rate studies were obtained from B-17 and B-19 strains grown on plots at the Puerto Rico Nuclear Center and the College of Agriculture located at Mayagüez. For other experiments a tree at the Acerola Corp., Sabana Seca, of unknown strain but similar ascorbic acid content and morphology to B-17 and B-19, was selected. The fruits used were between 1 and 6 mm. in diameter (at maturity these strains reach a size of approximately 18 mm. in diameter) and, at this stage of development, contained approximately 800 mg./percent fresh weight of ascorbic acid.

The radiocarbon-labeled materials employed had the following specific activities: Uniformly  $C^{14}$ -labeled glucose, 4.66 mc/millimoles; 1- $C^{14}$  glucose, 3.49 mc/millimoles;  $C^{14} O_2$ , as produced by a generator system from barium carbonate, 0.20 mc/mg.; and  $C^{14} O_2$  produced endogenously from bicarbonate by the acidity of acerola tissues, 5.44 mc/millimoles.

Fruits were cut into slices of 0.1 to 0.5 mm. in thickness; leaf disks were punched out with a No. 6 cork borer (10-mm. diameter). Slices and disks were incubated with the radioisotope solution in closed, small, widemouth weighing bottles. Incubations were terminated by the addition of hot 70-percent ethanol and submersion of the bottle in a boiling water bath. Subsequently the plant tissues and solution were decanted into a centrifuge tube and ground to a pulp with a glass rod while heated in a water bath. The suspensions were centrifuged at  $1,500 \times gm.$  for 10 minutes which gave a clear supernatant liquid. The residual pulp was dried and weighed. The pulp weight was consistently found to be between 9 and 10 percent of the fresh weight. In rate studies an aliquot of the supernatant liquid was used directly for two-dimensional chromatography.

Small branches bearing leaves or fruit were cut under water and placed in a beaker containing water inside a Virtis freeze-drying flask. The flask was fitted with a separate device for gas generation and a sodium hydroxide trap. The apparatus was connected to a vacuum pump to aid in the infiltration of tissues. Radiocarbon dioxide was released from barium carbonate C 14 by the addition of 10-percent lactic acid.

Two-dimensional paper chromatography of the hot-alcohol supernatant solution was carried out using redistilled phenol saturated with water in the first dimension, followed by freshly prepared *n*-butyl alcohol, acetic acid, water (4:1:1, vol./vol./vol.) in the second (short) direction. Whatman No. 1 filter paper, 18  $\times$  22-inch sheets, were ordinarily used. For radioautography, chromatograms were exposed against 14  $\times$  17-inch. No-screen X-ray film (Kodak) and the film was developed after 16 to 20 days.

Carbohydrates were detected on paper with ammonium molybdate

reagent (3); organic acids by the method of Paskova and Munk (4); keto acids with 2-percent dinitrophenylhydrazine in 9 *N* sulfuric acid; and amino acids with 0.15-percent ninhydrin plus 2 ml. of pyridine per 500 ml. of 95-percent ethyl alcohol.

For other than rate studies, the ethanol extracts were evaporated, dissolved in water and neutralized before passage through a column of AG-1  $\times$  8 resin in the formate form. The neutral and basic substances were eluted by irrigation of the column with water. The acids were separated in 2- to 4-ml. fractions collected from a water to 1 *N* formic acid gradient, followed by further elution of the column successively with 3 *N* and 6 *N* formic acid. Fractions were combined according to acid peaks after evaporation of the formic acid. The two-dimensional solvent systems of Ladd and Norsal (5) were used for further confirmation of the identity of individual acids by paper chromatography.

For quantitative determinations, all the solvent systems already described were used one-dimensionally on Whatman 3MM paper further to separate neutral, basic or acidic components from one another, if necessary. Bands of the substances of interest were eluted from the paper with water. Only in the case of glutamine was it found necessary to chromatograph in a second dimension to obtain separation from alanine.

Sucrose and fructose were determined quantitatively by the anthrone method (6); glucose by the use of 2,4-dinitrosalicylic acid (7); and glutamine, alanine, aspartic acid, and glutamic acid by the ninhydrin method of Cocking and Yemm (8). Acids were determined by titration with sodium hydroxide, using phenolphthalein or methyl red as an indicator.

Chromatographic spots were counted directly. Radioactivity was measured as counts per minute using a thin mica window TGC-2 (Tracerlab) Geiger-tube. This method had an efficiency of approximately 10 percent. Counting time was routinely 3 minutes per chromatogram area.

## RESULTS AND DISCUSSION

Small excised branches carrying fruit and blossom as well as leaves were found to be the most satisfactory for introduction of the labeled precursors under controlled conditions. It can be seen in table 1 that  $C^{14}$  glucose was readily absorbed into the tissues by this procedure. Within 2 to 3 hours the total volume of radioisotope solution (usually 2 ml.) was taken up through the cut stem; for longer incubation periods the isotope solution was then replaced with water. Respiration losses were very high.

Over longer periods of metabolism changes in levels of radioactivity were noted in the tissues. Total specific radioactivity was about 25 percent higher in leaves than in fruit after 3 hours of glucose uptake under normal conditions of light. Radioactivity in fruit increased almost 100 percent over

that in leaves when this period was followed by darkness; blossoms accumulated four times the level of  $C^{14}$  found in leaves. The relative surface areas of the leaves, fruit, and blossom may account for respiratory differences, and translocation would also play a part in these changes.

The levels of ascorbic acid in fruit of 3 to 6 mm. and 6 to 9 mm. diameters are shown in table 2. These sizes were chosen because it had been

TABLE 1.—Uptake of glucose  $C^{14}$  through severed stems of acerola twigs:  
(Counts per minute per milligram of tissue, fresh weight)

Part of plant	Experiment 1— uniform $C^{14}$ , 3.5 hr. light, <sup>1</sup> 10 $\mu$ c <sup>2</sup>	Experiment 2— uniform $C^{14}$ , 5 hr. light, 12 hr. dark, 12 $\mu$ c <sup>2</sup>	Experiment 3— uniform $C^{14}$ , 9 hr. light, 12 hr. dark, 10 $\mu$ c <sup>2</sup>	Experiment 4— uniform $C^{14}$ , 9 hr. light, 12 hr. dark, 10 $\mu$ c <sup>2</sup>	Experiment 5— i- $C^{14}$ 5 hr. light, 12 hr. dark, 24 $\mu$ c <sup>2</sup>
Leaves	74	51	61	25	290
Fruit (2-5 mm. diam.)	55	101	117	—	666
Blossoms	59	—	—	84	1,296

<sup>1</sup> Specific activity 4.66 mc/millimoles.

<sup>2</sup> Specific activity 3.49 mc/millimoles.

<sup>3</sup> Total radioactivity taken up.

TABLE 2.—Concentrations of major primary acceptors of  $C^{14}$  in acerola fruit<sup>1</sup>

Fruit size	Glucose	Fruc- tose	Sucrose	Glu- tam- ic acid	Aspar- tic acid	Succinic acid	Ascor- bic acid <sup>1</sup>	Malic acid	Alanine	Glu- tamine
3-6 mm. diam., millimole/gm. }	4.5	5.5	3.0	36.0	6.6	1.3	66.4	2.1	20.3	17.5
	5.0	6.0	3.4	35.4	6.9	.7	67.0	1.4	—	—
9-12 mm. diam., millimole/gm. }	17.5	17.0	4.1	39.0	9.9	.8	105.0	1.3	20.3	17.6
	17.5	16.0	3.8	40.2	10.7	.7	106.0	1.0	—	—

The acids are listed in the order of their emergence from the AG 1 x 8 (formate) column, water to 1 N formic acid gradient.

<sup>1</sup> Duplicate readings on each size.

reported (9) that ascorbic acid increases most actively in the very early stages of fruit formation, reaching a maximum level when fruit has attained mature size but before the ripening process begins. We found that the maximum rate of ascorbic acid formation was attained before fruit reached 3 mm. in size, or ca. 70 mg. in weight. Even during the advanced stages of fruit formation from which the data in table 2 are derived, endogenous ascorbic acid levels almost doubled with a similar increment in size of the fruit, whereas relatively little change occurred in the levels of the other major acceptors of the  $C^{14}$  label, except for glucose and fructose.

The results shown in table 3 are in terms of percentage distribution of radioactivity and do not reflect the degree of efficiency of ascorbic acid formation compared to that reported for some other fruits, for instance, for strawberries (10). It is evident that the blossom is already actively engaged in ascorbic acid formation.

This is not surprising, because cell division in the flesh of fruit is often completed at an early stage, that is, in the flowerbud (11). Whether ascorbic acid biosynthesis takes place also in the leaves of acerola and is translo-

TABLE 3.—Distribution of radioactivity in 70-percent ethanol extracts of acerola<sup>1</sup>

Extract	Percentage of total radioactivity for --						
	CO <sub>2</sub> , 9 hr. light, 12 hr. dark, excised branch		Bicarbonate C <sup>14</sup>		1-C <sup>14</sup> glucose, 5 hr. light, 12 hr. dark, stem fed through excised branch		
	Leaves	Fruit	3 hr. light Leaf disks	2 hr. light Fruit slices	Leaves	Fruit	Blossom
Aspartic acid	1.8	6.7	1.8	8.2	2.0	3.2	2.4
Malic acid	2.6	9.3	8.3	33.1	6.8	6.2	10.1
Glutamic acid	3.0	.1	2.5	6.3	8.0	3.2	2.5
Succinic acid	.7	1.6	.4	5.2	3.2	.6	.1
Ascorbic acid	.1	1.1	0	3.4	.1	3.8	4.2
Citric acid	1.1	3.0	1.1	2.4	9.5	3.4	2.6
Glutamine	1.0	1.0	.3	2.9	.1	1.2	.8
Alanine	1.6	4.7	.8	9.9	.1	.5	.7
Sucrose	41.0	12.2	81.0	7.8	26.2	21.0	20.6
Fructose	17.5	13.1	.6	.9	20.0	12.3	20.6
Glucose	11.8	18.7	.1	2.6	14.4	12.0	20.6
Chromatogram origin	14.0	22.8	1.7	12.5	8.1	29.4	16.6
Other	2.9	6.3	1.5	4.7	0	0	6.4

<sup>1</sup> Total radioactivity does not take into account substances too weakly radioactive to be counted.

cated into the fruit, as suggested for acids in general (12), cannot be determined from our results. In preliminary experiments we found that the level of ascorbic acid in acerola leaves fluctuated between ca. 22 millimoles/gm. at periods of active fruit development, and 10 millimoles/gm. during relatively dormant periods. This finding can be interpreted as indirect evidence that leaves are able to carry out ascorbic acid synthesis independently; however, the radioactivity in ascorbic acid from leaf extracts was too low to provide further evidence.

Fruit slices and leaf disks could be better manipulated and controlled than intact branches. Measurable quantities of the label reached the pri-

mary acceptors when incubations were carried out with  $C^{14}$  bicarbonate. The acid pH of acerola fruit slices (between pH 4 and 5) rapidly converted the added bicarbonate to  $C^{14}O_2$ . Chromatographed extracts of fruit slices and leaf disks treated in this way showed a pattern of  $C^{14}$  distribution strikingly similar to those observed in other plants (13). Carbon dioxide fixation into malic, glutamic, and aspartic acids, alanine, glutamine,

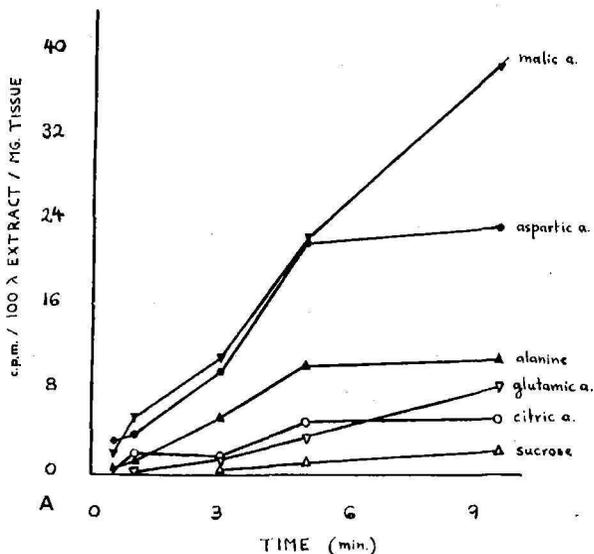


FIG. 1—A,  $C^{14}O_2$  (generated from  $C^{14}$  bicarbonate) uptake into sliced acerola fruit as a function of time (from 30 seconds to 10 minutes). Plotted from 2-dimensional chromatographic separation of extracts. B,  $C^{14}O_2$  (generated from  $C^{14}$  bicarbonate) uptake into sliced acerola fruit as a function of time (from 5 to 60 minutes). Plotted from 2-dimensional chromatographic separation of extracts. (See B on p. 7).

and sucrose occurred in a pattern similar to that reported by Bassham and Calvin (14). It has been assumed that the leaf disks and fruit slices were undergoing substantially normal metabolism, because, when incubation was carried out in darkness, there was almost complete shutoff of the photosynthetic pathways leading to sucrose labeling. A relatively high percentage of radioactivity remained at, or very close to, the chromatogram origin, particularly in fruit.

In the chromatographic solvent systems employed in these investiga-

tions one would expect to find sugar phosphates, polysaccharides, and proteins in this location on the paper. Presumably these substances are all synthesized by the system.

In Fig. 1,A and 1,B the time course of  $C^{14}$  uptake into primary detectable intermediates from  $C^{14}O_2$  may be seen for fruit slices. The rates of  $C^{14}$  utilization by some of the early receptors reached a peak value prior

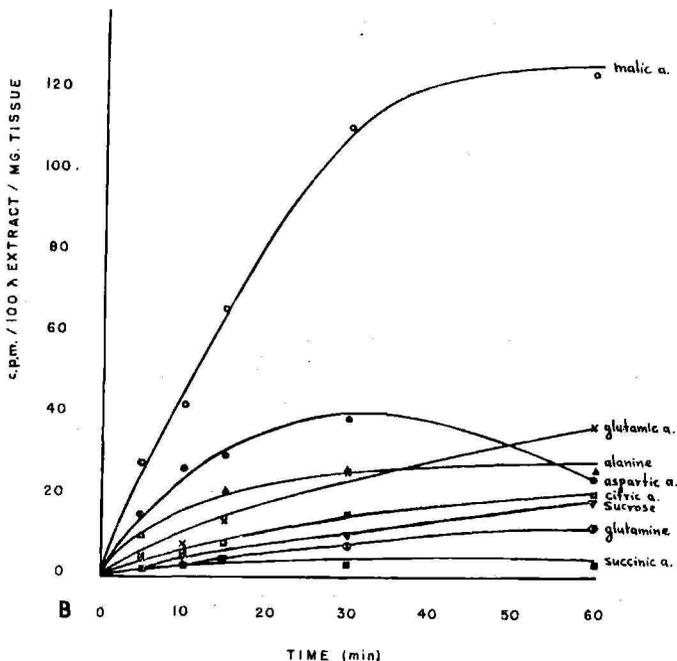


FIG. 1B

to the shortest time period (30 seconds) in which reliable rate values could be obtained (fig. 1A). The predominant early labeling of malic acid in *Chlorella* was reported by Varner and Burrell (15). The early labeling we found in the acerola system is of interest because of the low endogenous ratio of malic to ascorbic acid in the early stage of fruit development. According to determinations reported by Santini (16), malic acid may reach levels higher than those of ascorbic acid; however, in our experiments the malic acid levels were about  $\frac{1}{10}$  as high, and there was no increase

in concentration as the acerola fruit matured. Ascorbic acid could not be detected during the first 60 minutes of incubation with  $C^{14}$  bicarbonate. A possible interpretation of the rapid malate labeling we found in acerola is that it may derive from ribulose phosphate conversion to phosphoenolpyruvate which is converted to four carbon acids (*i.e.* oxalate and malate) through  $C^{14}O_2$  fixation, as suggested by Bradbeer *et al.* (17).

These investigations have served as a base for further study of the problem of ascorbic acid biosynthesis in the acerola. For a more comprehensive picture it will be necessary to examine the specific activities of the intermediate substances herein reported. Because of the high content of ascorbic acid in the fruit, relative to that of other components, and the low percentage of total radioactivity incorporated into this substance, we cannot conclude that we are dealing with an exceptionally efficient pathway of ascorbic acid biosynthesis. Rather, these results suggest that the degradative fate of ascorbic acid may yield important information for a satisfactory explanation of the high ascorbic acid levels in acerola; however, metabolic regulation of ascorbic acid synthesis and degradation remain to be explored.

#### SUMMARY

A study of the fate of radiocarbon-labeled glucose and carbon dioxide in the acerola (West Indian Cherry) has shown that these substances follow patterns of conversion comparable to those observed in other plants. These substances were not preferentially incorporated into ascorbic acid by acerola fruit. Sucrose, several amino acids, and malic acid appear to compete successfully with ascorbic acid for the label from these precursors.

#### RESUMEN

El destino de la glucosa y del anhídrido carbónico marcados con carbón radioactivo, respectivamente, en la acerola, indica que estas sustancias se ajustan a patrones de conversión similares a las ya observados en otras plantas. Por lo tanto, la acerola no incorpora estos precursores preferentemente en la formación de ácido ascórbico. Aparentemente la sacarosa, varios amino ácidos y el ácido málico compiten favorablemente con el ácido ascórbico por la marca radiactiva presente en estos precursores.

#### LITERATURE CITED

1. Asenjo, C. F., and Freire de Guzmán, A. R., The high ascorbic acid content of the West Indian Cherry, *Science* 103: 219, 1946.
2. Jackson, G. C. and Pennoek, W., Fruit and vitamin C production of 5- and 6-year-old acerola tress, *J. Agr. Univ. P.R.* 42: 196-205, 1958.
3. Aronoff, S. I. and Vernon, L., Metabolism of soybean leaves: I, The sequence of formation of the soluble carbohydrates during photosynthesis, *Arch. Biochem.* 28: 424-39, 1950.

4. Paskova, J. and Munk, V., A combined detecting reagent for the identification of organic acids on paper chromatograms, *J. Chromatogr.* 4: 241-3, 1960.
5. Ladd, J. N. and Nossal, P. M., The chromatographic separation and identification of organic acids and their application to yeast, *Austral. J. Exp. Biol. & Med. Sci.* 32: 523-31, 1954.
6. Trevelyan, W. E. and Harrison, J. S., Studies on yeast metabolism: I, Fractionation and microdetermination of cell carbohydrates, *Biochem. J.* 50: 298-304, 1952.
7. Alexander, A. G., Sucrose-enzyme relationships in immature sugarcane supplied with variable nitrate and potassium in sand culture, *J. Agr. Univ. P. R.* 48: 165-231, 1964.
8. Cocking, E. C. and Yemm, E. W., Estimation of amino acids by ninhydrin, *Biochem. J.* 56 (XII), 1954.
9. Del Campillo, A. and Asenjo, C. F., The distribution of ascorbic acid, dehydroascorbic acid, and diketogulonic acid in the acerola fruit at different stages of development, *J. Agr. Univ. P. R.* 41: 161-66, 1957.
10. Loewus, F. A., Tracer studies on ascorbic acid formation in plants, *Phytochem.* 2: 109-28, 1963.
11. Jackson, G. A. D. and Barakat, S. E. Y., The organic acids in rose hips, *Nature* 184 (Sup. 25), 1953-5, 1959.
12. Nitsch, J. P., The physiology of fruit growth, *Ann. Rev. Plant Physiol.* 4: 199-227, 1953.
13. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W., The path of carbon in photosynthesis. V. Paper chromatography of the products, *J. Amer. Chem. Soc.* 72: 1710-18, 1950.
14. Bassham, J. A. and Calvin, M., The way of CO<sub>2</sub> in plant photosynthesis, *Comp. Biochem. and Physiol.* 4: 187-204, 1962.
15. Varner, J. E. and Burrell, R. C., Use of C 14 in the study of the acid metabolism of *Bryophyllum calycinum*, *Arch. Biochem.* 25: 280-87, 1950.
16. Santini, R., Identification and determination of polybasic organic acids present in West Indian Cherries (*Malpighia punicifolia*) and in three varieties of guava (*Psidium guajava*) *J. Agr. Univ. P. R.* 37: 195-8, 1953.
17. Bradbeer, J. W., Ranson, S. L., and Stiller, M., Malate synthesis in Crassulacean leaves, I, The distribution of C<sup>14</sup> in malate of leaves exposed to C<sup>14</sup>O<sub>2</sub> in the dark, *Plant Physiol.* 33: 66-70, 1958.