PHOTOSYNTHESIS AND METABOLISM OF ORGANIC ACIDS IN HIGHER PLANTS[1]

R. E. STUTZ[2] AND R. H. BURRIS

(WITH TWO FIGURES)

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The importance of organic acids as photosynthetic intermediates, first suggested by Liebig, has been subject to speculation for over 100 years. The free organic acids or their phosphate esters still are included in the most recent schemes for photosynthetic assimilation of carbon dioxide (**8, 12**). Recent advances in our knowledge of the path of carbon in photosynthesis have been described so adequately (**3, 8, 9, 12**), that any extensive review of the subject here is unwarranted. BENNET-CLARK (**2**) recently has reviewed the literature on the organic acids of plants.

Our early attempts to use higher plants for the biosynthesis of C^{14}-labeled organic acids (**7**) revealed that equilibrium in C^{14} distribution among the organic acids was not established even many hours after exposure to $C^{14}O_2$. The observed concentration of C^{14} in malic acid suggested the possible importance of this acid as an early photosynthetic product. Accordingly, the distribution of C^{14} among several organic acids from plants supplied $C^{14}O_2$ was investigated.

Materials and methods

GROWTH OF PLANTS AND EXPOSURE TO $C^{14}O_2$

Plants were grown in the greenhouse on sand or vermiculite and were supplied with nutrient solution 1 of HOAGLAND and ARNON (**13**). In some instances intact, mature plants were enclosed for treatment with $C^{14}O_2$; in other experiments small, intact plants were treated. Rhubarb leaves were removed from the plant and their petioles were immersed in water. Exposures of large plants to $C^{14}C_2$ were made in Pyrex glass cylinders (**7**, fig. 7); the CO_2 was generated internally by injecting 20% perchloric acid into a vial containing $BaC^{14}O_3$. Tobacco seedlings 1.5 to 2 inches high were grown in alundum extraction thimbles and exposed to $C^{14}O_2$ in stoppered 38×200 mm. Pyrex test tubes. Alternatively, these small plants were treated with $C^{14}O_2$ under a small bell jar type filtration chamber through which gas was circulated with a diaphragm pump.

Normally, after the plants were sealed in the chambers, they were illuminated to deplete CO_2 (and to generate any reducing agents active in photo-

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[2] Present address: Argonne National Laboratory, Chicago 80, Illinois.

synthesis). Dark treated plants were darkened prior to supplying $C^{14}O_2$ to deplete any reducing agents. Other plants were preilluminated and then darkened and immediately exposed to $C^{14}O_2$. In tests on the interconversion of photosynthetic products, the plants were kept in the dark after a period of photosynthesis of $C^{14}O_2$.

Preparation of extracts

After exposure to $C^{14}O_2$, the leaves, or entire plant, were dropped quickly into liquid air. Petioles and midribs were removed from the leaves, and the frozen tissue was ground to a fine powder in an iron mortar; liquid air was added occasionally during the grinding. Five parts of powdered leaf tissue then were combined with two parts by weight of 0.5 *N* H_2SO_4, adjusted to pH 1–2 with a few drops of 7 *N* H_2SO_4, filtered if there were over 10 gm. of tissue and stored frozen in the refrigerator.

Celite 545 and plant extract were combined, in the proportion 14 gm. celite per 10 ml. extract, and were mixed thoroughly with solvent (1 volume : 1 volume, butanol-chloroform equilibrated with 0.5 *N* H_2SO_4) in a glass mortar. Some losses of organic acids were incurred in early experiments before the necessity for removing metal from the celite by thorough washing with HCl and water was realized. The slurry of celite and extract was packed in a column and extracted with 400 ml. acid equilibrated 1 : 1 butanol-chloroform mixture in 25- to 50-ml. portions. When tissue was limited, celite was combined directly with the ground, acidified tissue and the whole mixture was extracted with a correspondingly smaller volume of solvent.

Twenty-five ml. of water was added to the flask containing the non-aqueous solvent, and the dissolved acids were titrated with 0.1 *N* NaOH to the endpoint of phenol red. The non-aqueous phase then was removed with a separatory funnel and washed with two 20-ml. portions of water. The combined aqueous phases were concentrated to a fraction of a ml. by vacuum distillation and acidified with an amount of 7 *N* H_2SO_4 calculated to liberate the organic acids present as the sodium salts. The volume was made to 1 ml. with water. To the extract in the flask was added 1 gm. of silica gel (prepared according to Isherwood (**14**)), and extract and gel were mixed by passing them through an 80-mesh sieve. The gel was made into a slurry with about 2 ml. of a mixture of equal volumes tertiary-amyl alcohol and chloroform, which had been equilibrated with 1.0 M Na_2SO_4, and was placed on a small column. The column was washed with successive 0.5-ml. portions of the tertiary-amyl alcohol–chloroform mixture until the volume of extract totalled 5 to 10 ml. This extract was stored in the cold room if analytical separation was delayed.

Preparation of columns

Analytical separations were made on 18-mm.-inside-diameter columns with 3 gm. of silica gel or on 8-mm. columns with 1 gm. of silica gel. The

silica gel was mixed with 1 ml. 0.5 N H_2SO_4 per gram of gel (as the gel dried out over P_2O_5 the amount of 0.5 N H_2SO_4 had to be increased at intervals until 1.16 ml. were used per gm. of gel), and the powder was passed through an 80-mesh-per-inch bronze wire sieve to assure complete mixing. The powder was made into a slurry with some of the solvent to be used in developing the column. The slurry was poured carefully into a column without forming bubbles and residual gel was washed in with additional solvent. The gel was allowed to settle and the column to drain; the column shrinks about 2 cm. after the solvent drains. Gel adhering to the glass above the packed column was removed with cotton wads. A tight-fitting disk of filter paper was placed on top of the column and the column was compressed until shortened 1.0 to 1.5 cm. The column was filled with solvent and connected to the air line; the pressure was increased gradually to the operating point. After about 60% of the solvent had passed through, the pressure was lowered gradually to avoid cracking. Unless cracked or dehydrated, the columns may be stored (filled with solvent), and reused for several separations.

Analytical separations

For separation, the organic acids in tertiary-amyl alcohol–chloroform mixture were pipetted onto the column; 1 ml. was used per gm. of silica gel in the column. Isherwood (**14**) rates the capacity of a 3-gm. column at 10 mg. of mixed acids; we have separated effectively as much as 16 mg. of acids on a 1-gm. column. The solution of acids was forced slowly into the column with a pressure of 5 lbs. per square inch; just as the top of the column became dry, the pressure was released and a small portion of the solvent (usually 30–35 volume per cent. butanol in chloroform equilibrated with 0.5 N H_2SO_4) was added. Two or three more small portions of solvent were used to wash down the sides of the tube and to force the sample into the column. The column tube was half filled with solvent, connected to a solvent reservoir, and the operating pressure was applied above the solvent in the reservoir. Fractions of effluent (usually 0.7–2.0 ml.) were collected with an automatic fraction collector.

To each tube from the fraction collector 2 to 3 ml. of CO_2-free water and one drop of 0.01% phenol red were added. The acid was titrated with 0.1 N $Ba(OH)_2$ delivered from a 1-ml. burette. The tip of the burette was submerged and the contents of the tube were mixed with CO_2-free air. The titration of each tube was plotted as a step on a bar graph; figure 1 represents a typical separation and indicates the resolution of organic acids which may be expected on a silica gel column. Malic acid always was present in the plant materials used and served as a convenient reference point in identifying other acids. The positions of known acids were mapped. Acids separated included succinic, oxalic, glycolic, malic, citric, and isocitric acids. The "succinic acid fraction" may include fumaric, pyruvic and acetic acids and esters of other acids, when 30 or 35% butanol in chloroform is used for the developing solvent. Under these conditions aconitic acid is

not separated well from oxalic acid. (Authentic oxalic acid appears at the position designated in figure 1, and material recovered from natural products at this position precipitates as its calcium salt from aqueous solution. Crystalline calcium oxalate has been recovered from preparative columns by this method; it reacted stoichiometrically with potassium permanganate.) Glyceric and isocitric acids appear together in the effluent from the column. (The lactone of isocitric acid comes off the column at almost the same position as succinic acid, but the free acid derived from plant extracts or from hydrolysis of the lactone appears at the position shown in figure 1.) The other acids are separated effectively. A solvent such as 10% butanol in chloroform should resolve the possible mixtures in the "succinic acid fraction." The peak including isocitric acid is labeled "isocitric fraction" because of possible contamination with glyceric acid. We have not found

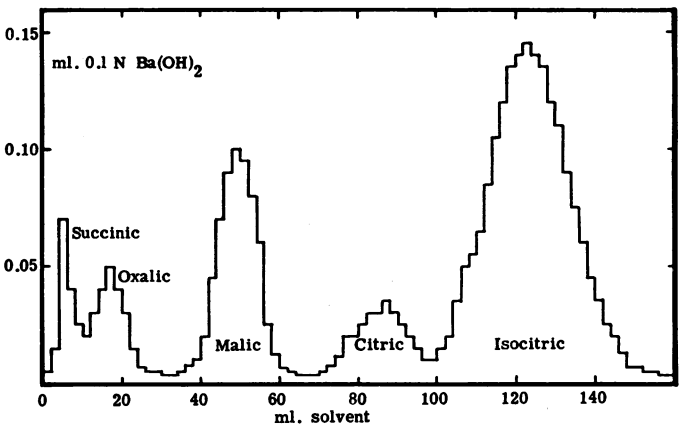

FIG. 1. Typical separation of organic acids from *Bryophyllum calycinum* on a silica gel column with 35 volume per cent. butanol in chloroform equilibrated with 0.5 N H_2SO_4 as solvent.

aconitic acid, so the oxalic peak is designated oxalic acid; the possibility of occasional contamination of oxalic by aconitic acid remains.

All tables list the mg. of each acid recovered, and these values are used in calculating the C^{14} as per cent. of the total in the acid fraction. The recovery of organic acids by the method employed is not complete, so the data should not be interpreted as strictly quantitative. The loss of organic acids occurs in their preliminary purification. In certain of the early experiments, the celite used was not thoroughly acid-washed; unwashed it contains heavy metal impurities, and added organic acids are not completely removed by the 1:1 butanol-chloroform mixture. In the second purification, the organic acids are placed on silica gel and eluted with a small volume of tertiary-amyl alcohol–chloroform mixture. This small volume of solvent does not remove the organic acids completely from the silica gel. The succinic and oxalic acid fractions are removed more completely

than malic and citric acids; in a second wash the ratio of malic and citric acids to succinic and oxalic acids will be higher than in the initial wash. ISHERWOOD (**14**) employed a more thorough extraction with the tertiary-amyl alcohol–chloroform mixture and reported almost complete recovery of organic acids. The small volume of solvent was used in our experiments to yield a more concentrated solution for addition to the analytical column and to insure that sufficient organic acids for a C^{14} analysis would be recovered. Although the data for amounts of organic acids present are not strictly quantitative, they indicate the approximate distribution of acids. As the specific activities of individual acids vary widely, there are probably few instances in which an entirely quantitative accounting would alter the order of the acids arranged according to their C^{14} content as per cent. of total in the acid fraction. The data of greatest importance in interpreting the sequence of formation and conversion of the compounds are their specific activities (counts/sec./mg. C), rather than their total amounts.

PREPARATION OF SAMPLES AND ANALYSIS FOR C^{14}

The contents of the tubes from an entire acid fraction, or the pure segment of a fraction, were pooled after titration; the non-aqueous phase was removed and washed, and the aqueous fraction was concentrated to a few ml. by vacuum distillation. This small volume of solution of the barium salts of the acids was stored in the refrigerator until it was sampled for measurement of C^{14}.

A sample of about 4 mg. of the barium salt of an organic acid was dried in a carbon oxidation flask (fig. 2) over P_2O_5. Oxidation then was effected with the Van Slyke and Folch oxidation mixture (**22**) in the apparatus shown in figure 2. Carbon dioxide was captured in freshly filtered, saturated $Ba(OH)_2$ to which NH_4Cl was added to suppress formation of bicarbonate (**11**). The centrifuge tube at the base of the Vigreaux column was loosened, and the column was rinsed with CO_2-free water. The centrifuge tube was capped and centrifuged. The precipitate was washed with CO_2-free water and centrifuged. The precipitate was suspended in methanol and centrifuged. A few drops of methanol were added to the $BaCO_3$ and a thick slurry was made. The slurry was spread evenly over a 2 sq. cm. circular area, inscribed with dividers on an aluminum dish, and was dried. The weight of the $BaCO_3$ was determined. Samples from a given experiment were all counted (thin window counter) at one time, and specific activities were calculated after appropriate correction for background, self-absorption and coincidence loss in counting. The error in counting the samples of low activity was less than 10%.

Results

PRELIMINARY EXPERIMENTS

In the initial experiment, *Bryophyllum calycinum* was exposed to $C^{14}O_2$ for 12 hours in the light and 12 hours in the dark. If a highly active tri-

carboxylic acid cycle were operative, one might anticipate that the C^{14} specific activities of the organic acids would approach equilibrium in 24 hours. As shown in table I a the specific activities differed widely. Malic acid had accumulated most of the total C^{14} and had the highest specific activity among the acids. Citric acid had a much higher specific activity than isocitric acid. In other early experiments with *B. calycinum,* an exposure to $C^{14}O_2$ for four hours in the light gave a distribution of C^{14} very similar to

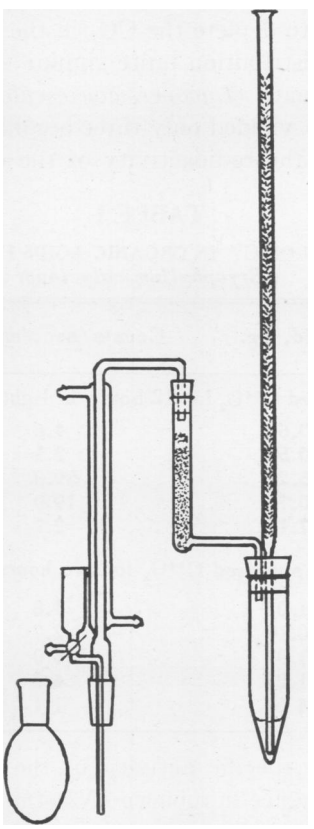

FIG. 2. Apparatus used in the wet oxidation of compounds to carbon dioxide. The oxidation flask has a 19/38 standard taper joint and has a capacity of about 45 ml. Air and oxidizing reagent are admitted through a 3-way stopcock. The tube preceding the 40-ml. centrifuge tube in the train contains 20-mesh granular zinc.

the 24-hour exposure; in a 2-hour exposure (table I b) the high percentage of the total activity in malic acid was accentuated.

At the time these first experiments were performed, there was much speculation on the possible importance of malic and succinic acids as early intermediates in photosynthesis (**5**). The abundance of C^{14} accumulated by malic acid in Bryophyllum was compatible with the idea that malic acid was a key compound in photosynthesis. Consequently, observations were

extended to other higher plants, and lengths of exposure to $C^{14}O_2$ were shortened to emphasize the differential distribution of the isotope. The description of subsequent experiments will be arranged according to the type of exposure to $C^{14}O_2$.

Exposure to $C^{14}O_2$ in light for 30 minutes

Several plant species were enclosed in glass chambers and exposed to $C^{14}O_2$ for 30 minutes in the light; prior to generating $C^{14}O_2$ the enclosed plants were illuminated to deplete the CO_2 in the chambers. *B. calycinum* (table II a) showed a distribution quite similar to that noted upon longer exposure to $C^{14}O_2$. Tomato (*Lycopersicon esculentum* Mill., variety John Baer) leaves (table II b) yielded only three organic acids in this separation. Malic acid had 92% of the radioactivity of the organic acid fraction and

TABLE I

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM LEAVES OF *Bryophyllum calycinum*

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plants supplied $C^{14}O_2$ for 12 hours in light, 12 hours in dark			
Succinic fraction	0.62	4.6	0.9
Oxalic	0.50	2.1	0.2
Malic	5.27	59.0	89.0
Citric	0.71	19.0	4.1
Isocitric fraction	7.16	2.7	5.8
b. Plants supplied $C^{14}O_2$ for two hours in light			
Succinic fraction	1.03	3.8	1.8
Oxalic	0.10	2.7	0.8
Malic	1.97	114.0	93.2
Citric	1.21	4.1	2.2
Isocitric fraction	4.07	1.1	2.0

had about 10 times the specific activity of the other acids. Malic acid again exhibited its dominance in tobacco (*Nicotiana tabacum*, variety Comstock Spanish) (table II c). In barley (*Hordeum vulgare*, variety Wisconsin no. 38) (table II d) the differential in specific activities was less, but the malic acid still had the highest total and specific activity. The isocitric acid fraction had greater specific activity than succinic or citric acids; this differed from the isocitric acid fraction of Bryophyllum which contains an enormous reservoir of this acid. Rhubarb (*Rheum hybridum*, variety Victoria) exhibited even less differential than barley in specific activities of its organic acids (table II e). Oxalic acid was relatively inert, but succinic and citric acids were active metabolites.

Exposure to $C^{14}O_2$ in dark for 15 minutes

To determine the capacity of higher plants to fix $C^{14}O_2$ in the dark and to determine the distribution of C^{14} among the organic acids formed in the

dark, plants enclosed in glass jars were pre-illuminated to deplete CO_2 and then were exposed in the dark to $C^{14}O_2$ generated within the chambers. After 15 minutes exposure to $C^{14}O_2$, the plants were harvested rapidly in a hood in subdued light.

The data in table III a indicate that in Bryophyllum, malic acid again carried over 91% of the C^{14} in the organic acid fraction. In specific activity it was even more dominant than it was when the plants fixed $C^{14}O_2$ in the light. The distribution of C^{14} among the other acids was not greatly differ-

TABLE II

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM LEAVES

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. *Bryophyllum calycinum* supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	0.98	3.0	1.8
Oxalic	0.29	....	....
Malic	4.07	40.6	89.2
Citric	2.31	3.8	4.9
Isocitric fraction	10.00	0.7	4.1
b. Tomato supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.15	8.8	1.8
Malic	6.66	90.6	92.3
Citric	3.80	9.6	5.9
c. Tobacco supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.18	8.1	2.4
Oxalic	0.26	6.1	0.3
Malic	10.10	43.0	95.3
Citric	1.23	7.3	2.0
d. Barley supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.97	55.0	10.3
Oxalic	0.45	16.2	0.5
Malic	4.26	233.0	83.5
Citric	1.09	41.7	4.0
Isocitric fraction	0.22	90.7	1.7
e. Rhubarb supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	0.39	325.0	15.9
Oxalic	0.53	136.0	6.0
Malic	0.59	820.0	53.7
Citric	0.82	254.0	24.4

ent from that in the light; isocitric acid was more favored relative to citric in the dark than in the light. The leaves from tomato (table III b) contained glycolic acid, and it ranked third in total and specific activity. Although malic acid had the highest specific activity, it was only about 2.5 times that of citric acid, whereas in the light the difference was almost tenfold. Mechanisms for formation of citric acid in tomato leaves apparently are favored in the dark relative to the light. This same response was apparent with tobacco, barley and rhubarb. Tobacco (table III c) was notable

for the higher specific activity of citric than malic acid. The total C^{14} in malic acid was much higher than in citric acid, however. Barley, representative of a plant which has a very small reservoir of organic acids, exhibited a distribution of C^{14} fixed in the dark which was quite different from that of the other plants examined (table III d). Malic acid contained less than

TABLE III

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM THE LEAVES OF PLANTS PREILLUMINATED THEN SUPPLIED $C^{14}O_2$ FOR 15 MINUTES IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
	a. *Bryophyllum calycinum*		
Succinic fraction	0.77	0.9	0.8
Oxalic	1.24	1.7	1.6
Malic	2.12	42.6	91.3
Citric	1.53	1.1	1.7
Isocitric fraction	6.23	0.7	4.6
	b. Tomato		
Succinic fraction	1.36	1.1	1.7
Oxalic	0.41	0.2	0.1
Glycolic	1.63	1.4	2.0
Malic	8.40	10.1	84.8
Citric	2.62	4.0	11.2
Isocitric fraction	0.29	0.8	0.2
	c. Tobacco		
Succinic fraction	0.98	2.0	2.5
Oxalic	0.16	1.5	0.2
Malic	13.70	5.7	87.5
Citric	0.98	8.5	9.8
	d. Barley		
Succinic fraction	2.18	58.2	42.7
Oxalic	0.19	6.1	0.3
Malic	3.74	42.6	47.5
Citric	1.20	23.4	8.7
Isocitric fraction	0.09	27.2	0.8
	e. Rhubarb		
Succinic fraction	0.97	3.0	4.7
Oxalic	0.50	0.3	0.2
Malic	6.70	6.1	57.0
Citric	5.05	5.1	38.1

half the total C^{14} of the organic acid fraction and its specific activity was exceeded by that of the "succinic acid fraction." The specific activities of citric and isocitric acids were relatively high. The fixation of $C^{14}O_2$ in the dark by rhubarb (table III e) yielded citric acid approaching malic acid both in total and specific activity. The shift in distribution between light and dark treatments was less pronounced than with most plants tested. Oxalic acid formed in the dark had very low C^{14} specific activity.

Exposure of depleted plants to $C^{14}O_2$ in dark for four hours

In the dark experiments described, the plants were illuminated until the time of addition of $C^{14}O_2$; one would expect that stored reducing compounds would be primarily responsible for the reduction of $C^{14}O_2$ assimilated in the dark. To find the distribution of C^{14} following "respiratory fixation" of $C^{14}O_2$, reducing compounds were depleted by darkening the plant for an hour before adding $C^{14}O_2$; the plants were kept in the dark for four hours after addition of the isotope before they were harvested. It is apparent from table IV that most of the C^{14} fixed in the organic acid fraction of tobacco and barley was incorporated into malic acid. Succinic acid had the next greatest amount of C^{14} in each instance. Oxalic acid from tobacco was inactive but from barley had a rather high specific activity. The specific

TABLE IV

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM THE LEAVES OF PLANTS DEPLETED IN THE DARK AND THEN SUPPLIED $C^{14}O_2$ FOR FOUR HOURS IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
		a. Tobacco	
Succinic fraction	1.28	0.6	3.7
Oxalic	0.42	0.0	0.0
Malic	15.10	1.4	93.9
Citric	0.48	1.1	2.4
		b. Barley	
Succinic fraction	0.94	2.3	8.3
Oxalic	0.12	4.5	1.4
Malic	3.60	7.0	85.6
Citric	0.34	3.8	4.6
Isocitric fraction	0.06	0.6	0.1

activity of citric acid was greater than half that of malic acid in both tobacco and barley.

Interconversion of acids

If a plant is allowed to photosynthesize $C^{14}O_2$ and then is placed in the dark for a period in the absence of $C^{14}O_2$, there should be an opportunity for the compounds containing C^{14} to interchange their carbon with other compounds by normal processes of degradation and synthesis occurring in the darkened plant. Under these conditions, the specific activity of malic acid would be expected to decrease if it were one of the early photoproducts, whereas citric acid and other acids might be expected to increase in specific activity at the expense of malic acid. This experimental technique appeared to be a simple and independent means for testing the observation of Vickery *et al.* (**25**) that malic acid in the darkened tobacco leaf is converted to citric acid.

Potted plants used to test the interconversion of compounds were sealed in glass jars and illuminated to deplete CO_2. Then $C^{14}O_2$ was generated within the chamber, and the plants were illuminated for 15 minutes. The plants were taken from the chamber, and alternate leaves were removed in dim light and immersed in liquid air. The remaining plant was covered with a cardboard drum and was not given additional $C^{14}O_2$; thus exposure to $C^{14}O_2$ was limited to the 15-minute period of illumination. After four hours in the dark, the remaining leaves on the plant were removed for analysis.

In table V are listed the analyses for Bryophyllum leaves exposed to $C^{14}O_2$ in the light and after four additional hours in the dark. Contrary to expectations, none of the organic acids decreased in specific activity during the 4-hour dark period. In fact, every organic acid isolated at least doubled

TABLE V

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF *Bryophyllum calycinum* IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plant supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.28	2.8	2.9
Oxalic	0.13	0.6	0.2
Malic	0.66	40.8	88.7
Citric	0.49	1.5	2.6
Isocitric fraction	2.50	0.7	5.6
b. Same plant after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	0.47	7.9	1.2
Malic	3.19	97.0	90.8
Citric	1.57	9.8	4.7
Isocitric fraction	6.95	1.6	3.3

in specific activity. The most notable increase in specific activity was in citric acid; the increase was over sixfold. Very likely some of this citric acid originated from the highly active malic acid. The failure to recover oxalic acid after darkening is unexplained. Malic acid contained about 90% of the total C^{14} of the organic acid fraction.

Data from an experiment to test interconversion of organic acids in tobacco are given in table VI. The experiment was performed on a potted tobacco plant about 12 inches high. After exposure to $C^{14}O_2$ in the light for 15 minutes, 88% of the C^{14} of the organic acid fraction was in malic acid but the "isocitric fraction" had a higher specific activity. During the 4-hour dark period the specific activity of all the acids except the "isocitric fraction" increased; the most spectacular rises in activity occurred in oxalic and citric acids. As the amount of acid in the "isocitric fraction" was small, it could not account for the increase in specific activity of the other acids. Glycolic acid disappeared during the dark period.

TABLE VI

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF TOBACCO IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plant supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.83	11.6	3.4
Oxalic	0.06	2.4	0.1
Glycolic	0.11	7.2	0.2
Malic	14.80	18.9	88.2
Citric	0.88	11.8	3.4
Isocitric fraction	0.42	33.9	4.7
b. Same plant after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	1.22	21.9	4.5
Oxalic	0.12	15.8	0.2
Malic	15.30	37.9	85.5
Citric	1.44	41.5	9.2
Isocitric fraction	0.17	22.8	0.6

The experiment with tobacco was repeated with the top 12 inches of two plants about 36 inches high; the cut ends were immersed in water. All acids (including the "isocitric fraction") except the "succinic fraction" increased in specific activity during the dark period. Again the glycolic acid disappeared in the darkened plant.

Barley, which has a lower content of organic acids and a more active respiratory metabolism than Bryophyllum and tobacco, differed from them also in its behavior in an interconversion experiment. Succinic, glycolic, malic, and isocitric acids all decreased in C^{14} specific activity during a dark period following photosynthesis of $C^{14}O_2$ (table VII). Only oxalic and citric acids increased in specific activity.

TABLE VII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF BARLEY IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plants supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.80	13.6	5.3
Oxalic	0.14	14.9	0.7
Glycolic	0.85	6.6	2.1
Malic	3.13	66.3	88.8
Citric	0.62	9.7	2.7
Isocitric fraction	0.11	8.3	0.4
b. Plants from same pot after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	0.96	10.6	6.0
Oxalic	0.25	45.8	4.4
Glycolic	0.25	3.1	0.4
Malic	4.16	36.1	78.3
Citric	0.50	36.2	9.9
Isocitric fraction	0.32	6.2	1.0

Discussion

The initial studies on biosynthesis of C^{14}-labeled compounds by higher plants revealed that even after several hours of photosynthesis of $C^{14}O_2$, the C^{14} was very unequally distributed among the organic acids. The early proposal by BENSON *et al.* (**5**) of a 4-carbon acid cycle in photosynthesis lent interest to the observation that malic acid regularly had the highest total and specific activity among the organic acids recovered. Subsequent work by BASSHAM *et al.* (**1**) has indicated that the 4-carbon acids may not function in the direct pathway of photosynthesis. The data presented here perhaps are of greater interest in respect to organic acid metabolism of plants than in respect to photosynthesis.

The distribution of C^{14} among the organic acids from leaves of Bryophyllum was very similar whether the plants were harvested two or 24 hours after exposure to $C^{14}O_2$ (table I). The $C^{14}O_2$ generated in the chamber was undoubtedly exhausted within an hour. The increase in activity of individual acids with time was largely at the expense of the malic acid or other compounds (*e.g.*, phosphorylated intermediates) not measured in our experiments. The lack of equilibration of C^{14} between malic and isocitric acid could be attributed to the great quantity of isocitric acid present. This explanation does not suffice for succinic acid, for the succinic acid is present in small quantities but has a far lower specific activity than malic acid even after 24 hours. If a tricarboxylic acid cycle functions in Bryophyllum, it is sluggish. A tricarboxylic acid cycle of greater activity may operate in plants other than Bryophyllum; for example, our tests with barley and rhubarb showed a more rapid approach to equal distribution of C^{14} among their organic acids. Even in these plants it is doubtful that the tricarboxylic acid cycle is of importance in photosynthesis, *per se*.

Several species of plants exposed to $C^{14}O_2$ in the light for a period of 30 minutes (table II) exhibited a remarkable similarity in the distribution of C^{14} among their organic acids. Apparently the organic acids are metabolized in a common manner in the various plants. In every instance, malic acid had the highest specific activity and the highest total activity. This consistently high activity of malic acid confirms it as the key organic acid in the metabolism of many plants. The extensive studies of VICKERY, PUCHER, and coworkers (**17, 25, 26**) also have led to the conclusion that malic acid is metabolically most active of the organic acids normally analyzed. Oxalic acid occupies the position of the least active acid.

As in the earlier experiments, succinic acid was much less active than malic acid but comparable to citric acid. This is compatible with, but not proof of, its formation from citric acid via a tricarboxylic acid cycle rather than directly from reduction of malate as early postulated by BENSON *et al.* (**5**). The relatively low activity of succinic acid indicates that the later view of CALVIN (**8**) is likely correct, that succinic acid is not directly functional in the cycle of photosynthesis but arises through side reactions.

The data obtained with Bryophyllum are interesting in relation to the plant's diurnal cycle of acidity. WOLF (**27**) reported that a high pCO_2 inhibits the loss of acidity from the Crassulaceae in the light. BONNER and BONNER (**6**) extended this observation and attributed the increased formation of acid at night to a lowered temperature and an enhanced pCO_2 within the tissues. They suggested the Wood and Werkman reaction as the most logical pathway for CO_2 fixation. THURLOW and BONNER (**21**), with the aid of C^{14}, established that leaves of Bryophyllum assimilated CO_2 in the dark. Analyses indicate that changes in the concentration of malic acid are chiefly responsible for the diurnal variations in the acidity of Bryophyllum (**18**). VENNESLAND (**24**) has found oxalacetic acid carboxylase to be widespread in the plant kingdom, but has reported no tests for its presence in the Crassulaceae. All the oxalacetic acid carboxylase preparations also were active in the reversible oxidative decarboxylation of malic acid (**10**). Our results on CO_2 fixation in the dark certainly are compatible with the primary formation of malic acid, most likely via oxalacetic acid. Specific activities of the organic acids isolated after a short exposure to $C^{14}O_2$ indicate that the participation of CO_2 in the formation of citric and isocitric acids in Bryophyllum is a much slower process than in the formation of malic acid.

Accumulated information suggests that diurnal, reversible shifts from starch to malic acid account for changes in acidity of Bryophyllum. One might postulate that the following sequence of reactions occurs: starch undergoes phosphorolysis to glucose-1-phosphate which is converted to pyruvic acid by the usual glycolytic mechanism; the pyruvic acid is carboxylated to oxalacetic acid (reaction enhanced by high pCO_2), which is reduced to malic acid. The reverse could occur under the influence of light. Such a scheme, though obviously an oversimplification of the many enzymatic reactions occurring simultaneously in the leaf, represents a feasible mechanism for acidification and deacidification of succulents.

PUCHER *et al.* (**19**) have demonstrated that starch is synthesized in some quantity in Bryophyllum leaves harvested in the morning and placed in the dark. The failure of VARNER and BURRELL (**23**) to verify such extensive starch formation in the dark might arise from use by the two groups of experimenters of Bryophyllum leaves in different physiological states. Varner and Burrell have followed the assimilation of $C^{14}O_2$ by *B. calycinum* under a variety of conditions and have found that among the organic acids malic acid accumulates the greatest concentration of C^{14}.

The role of isocitric acid may be compared in Bryophyllum and barley (table II, a and d). Bryophyllum contained large quantities of isocitric acid and its specific activity was low. Barley contained little isocitric acid, but its specific activity was high. Assuming a tricarboxylic acid cycle is operative in these plants, the data for Bryophyllum would support the subordinant role of isocitric to citric acid in the tricarboxylic acid cycle (**15**,

16), whereas the higher specific activity of isocitric than of citric acid in barley would support its direct function in the cycle. However, there is a good possibility that glyceric acid contaminated the isocitric fraction from barley. Peaks for glyceric and isocitric acids are coincident in the separation procedure employed, so contamination with a small amount of highly active glyceric acid could enhance greatly the activity of the small isocitric acid fraction found in barley. On the other hand, there is little doubt that isocitric acid comprises virtually all the isocitric fraction in Bryophyllum.

Among the plants tested, equilibrium of C^{14} was most closely approached in the leaf blade of rhubarb. From this evidence, one could postulate the more active operation of a tricarboxylic acid cycle in rhubarb than in the other plants tested.

The dark fixation of $C^{14}O_2$ by plants which have been illuminated until supplied the isotope gives a distribution of C^{14} as shown in table III. The similarity in C^{14} distribution when $C^{14}O_2$ is fixed in the light or in the dark by preilluminated plants seems sufficient to warrant the conclusion that the illuminated plant stores energy that can be used in the dark to effect a reduction of CO_2 very much like that accomplished in the light. BENSON and CALVIN (4) have found that the distributions of C^{14} fixed in the dark by preilluminated algae and barley is similar to that when $C^{14}O_2$ is fixed in the light.

When plants have been depleted of reducing power by storage in the dark before exposure to $C^{14}O_2$, their uptake of C^{14} is much less active than is that of illuminated or preilluminated plants (table IV). The differences in specific activities among the acids formed by what is often termed respiratory fixation are less striking than the differences among acids formed during photosynthesis. BENSON and CALVIN (4) have observed that incorporation of C^{14} into glutamic, succinic and isocitric acids is suppressed in the light but that radioactivity appears in these acids in darkened plants.

VICKERY *et al.* (25) have presented good evidence that malic acid in darkened tobacco leaves is converted to citric acid. It seemed that one might check this point by allowing a plant to photosynthesize $C^{14}O_2$ for a short time and then by allowing it to interconvert its labeled and unlabeled acids in the dark. Definitive experimental results on the interconversion of malic and citric acids were not obtained, for all of the organic acids in Bryophyllum and all but isocitric acid in tobacco increased in specific activity during the dark period (tables V, VI). The small amount of isocitric acid in tobacco could account for but little of the increased activity of the other organic acids. PUCHER and VICKERY (20) have been unable to demonstrate the presence of significant quantities of isocitric acid in tobacco leaves. This fact, in combination with the unusually high specific activity of the isolated fraction, suggests that the "isocitric fraction" in tobacco may have been predominantly glyceric acid. From the observed increases in C^{14} specific activities of the organic acids while the plants were darkened, one must conclude that during photosynthesis some compound(s) other than the

organic acids isolated was synthesized with high specific activity and that this compound(s) was converted to organic acids during the dark period. The results of the interconversion experiments imply that none of the organic acids accounted for in the separations are particularly early products of photosynthesis, but are preceded by another compound(s) of much higher specific activity.

Among the organic acids of barley, only citric and oxalic acids showed an increase in specific activity during the dark period (table VII). The oxalic acid was particularly active, but the quantity present was so small that considerable error was attached to its analysis. It is interesting that the citric acid increased to the same specific activity as malic acid in the dark. It is not clear why barley behaves so differently from tobacco and Bryophyllum, but it may be associated with the fact that barley stores comparatively little organic acid. Precursors may be channeled more extensively into carbohydrate rather than into organic acid synthesis in barley.

During the dark period, glycolic acid disappeared from tobacco and was reduced over threefold in barley. This decrease of glycolic acid in the dark has been noted by BENSON and CALVIN (**4**) in Chlorella and barley. The glycolic acid in our experiments was never particularly high in specific activity, although it has been shown to be an early product of photosynthesis (**4**).

Summary

Plants were exposed to $C^{14}O_2$, their leaves were removed, and the distribution of C^{14} among the organic acids of the leaves was determined. Even after 24 hours, the C^{14} was far from equally distributed among the organic acids of *Bryophyllum calycinum*. If a tricarboxylic acid cycle is operative in this plant, it is sluggish.

In leaves of Bryophyllum, tomato, tobacco, barley, and rhubarb exposed to $C^{14}O_2$ in the light for 30 minutes, malic acid had the highest C^{14} specific activity and the highest total activity among the organic acids isolated. Malic acid appears to be a key compound in the organic acid metabolism of the plants examined.

Plants allowed to fix $C^{14}O_2$ in the dark following a period of illumination assimilated particularly high concentrations and total amounts of C^{14} into malic acid. Fixation in the dark, following depletion of the plants in the dark, also yielded malic acid of high activity.

Plants were exposed to $C^{14}O_2$ in the light for 15 minutes, part of the leaves were removed for analysis, and the rest of the plant was kept for four hours in the dark without $C^{14}O_2$. In the dark the specific activity of citric and oxalic acids increased in barley and the specific activity of the other organic acids decreased. During the dark period, the C^{14} specific activity of all organic acids in Bryophyllum and all but the isocitric acid fraction in tobacco increased. Evidently a precursor of high specific activity was formed in the light by Bryophyllum and tobacco and was converted

to organic acids in the dark. Thus the organic acids isolated are not first products of photosynthesis but are formed from precursors synthesized in earlier steps of the photosynthetic process.

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF WISCONSIN
MADISON 6, WISCONSIN

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