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Excretion of Glycolate, Mesotartrate and Isocitrate Lactone by Synchronized Cultures of *Ankistrodesmus braunii*[1]

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ABSTRACT

Fixation of $^{14}CO_2$ by synchronized cultures of *Ankistrodesmus braunii* was highest for young growing cells, low for mature cells, and lowest for dividing cells. The amount of ^{14}C excreted during photosynthesis followed the same trend. Cells at the end of the growing phase, after 10 hours of a 16-hour light phase, excreted nearly 35% of the total ^{14}C fixed as one product, glycolate. Dividing cells from the dark phase, when tested in the light, excreted only 4% as much glycolate-^{14}C as the young growing cells. Dividing cells also excreted as much mesotartrate as glycolate and also some isocitrate lactone and an unidentified acid. None of these excreted acids were found inside the cells in significant amounts. Methods for isolation and identification of the excreted acids are present. With ^{14}C-labeled algae, it was shown that the excretion of glycolate was light-dependent and inhibited by 1,1-dimethyl-3-(*p*-chlorophenyl) urea. The excretion of labeled mesotartrate, isocitrate lactone, and an unknown acid, but not glycolate, also occurred in the dark. The excreted mesotartrate was predominantly carboxyl-labeled even after long periods of $^{14}CO_2$ fixation. Since glycolate is known to be uniformly labeled, glycolate could not be the precursor of the carboxyl-labeled mesotartrate. The reason for the specific excretion of glycolate, mesotartrate, and isocitrate lactone is not known, but the metabolism of all three acids by the algae may be limited and each can form dilactides or lactones by dehydration. In this context isocitrate lactone was excreted rather than the free acid.

Algae excrete numerous organic compounds, and in 1956 (38) glycolate was identified as the major component which is now recognized as excreted by most algae (12, 14, 17, 20, 26) and perhaps chloroplasts (37). Currently glycolate excretion is attributed to the repression by CO_2 (21) of the enzyme glycolate dehydrogenase (22) that is involved in its further metabolism by the glycolate pathway (3, 13). A study on the excretion of glycolate during the life cycle of synchronized algal cultures was undertaken, because literature citations indicated that such a relationship might exist. Tolbert and Zill (38) observed that rapidly growing *Chlorella* cultures excreted glycolate; Allen (1) claimed that the amount of excretion by *Chlamydomonas* paralleled growth; but Forsberg *et al.* (7) stated that organic compounds were excreted during phases of declining relative growth rates and in the stationary phase. In a preliminary report of this work (4), it was shown that young growing cells were most active for glycolate excretion, and these results have been confirmed (8).

Except for glycolate, the excretion of other acids and compounds seems to be specific for certain algae. Excreted acids other than glycolate have not generally been identified. In the supernatant media from dividing cells of *Ankistrodesmus braunii* we have identified mesotartrate and isocitrate lactone as major components which are excreted. The excretion of these rather unusual acids, along with glycolate, raises further questions about the physiological significance of their excretion by algae. Mesotartrate and isocitrate lactone have not been associated with metabolic or physiological processes, and in fact their presence in algae has not been reported and their function in higher plants has not been elucidated.

Rhythmic changes of photosynthetic activity during the life cycle of algae, shifts in metabolic pathways, and changes in enzymic activity have been recognized by many investigators. In this report glycolate excretion during the life cycle has been found to follow the total photosynthetic activity. On the other hand, mesotartrate and isocitrate lactone were excreted only by dividing cells of *Ankistrodesmus braunii* when photosynthetic activity was at a minimum. Presently there is little information for correlating the significance of these results.

MATERIALS AND METHODS

Algae. Strains of algae were obtained from the culture collection of algae at Indiana University, Bloomington. *Ankistrodesmus braunii* (Naeg.) Collins (No. 245), *Scenedesmus obliquus* (Turp.) Krüger (No. 393) and *Scenedesmus quadricauda* (Turp.) Breb (No. 77) were cultured in 1-liter batches of medium V of Norris *et al.* (23) in 2.8-liter low form Fernbach flasks. *Chlamydomonas reinhardtii* Dangeard, (−) strain (N. 90) was cultured on a high phosphate medium (24). Cultures were aerated with about 0.2% CO_2 in air and shaken at 60 cpm. Illumination of 1200 ft-c was obtained from cool white fluorescent bulbs, and the temperature was maintained at 20 C for random cultures and at 30 C for synchronized cultures. An algal life cycle was synchronized at 16 hr light and 8 hr dark. At the end of each dark period the cell population was counted with a Levy and Levy-Hauser counting chamber and diluted to a population of 4×10^6 cells/ml, which represented about 1:4 dilution. Details concerning the growing of the algae as well as other methods employed are given elsewhere (4).

Chromatography. Two-dimensional paper chromatography and radioautography were carried out according to the procedures described by Benson *et al.* (2). In the first direction water-

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saturated phenol was used, and in the second direction a mixture of 1-butanol–propionic acid–water. In some cases the second solvent was modified by adding 0.1 ml of 6 N NaOH to each 100 ml of the solvent mixture (10). This modification gave preferential separation of the unknown organic acids and allowed much better visualization of the acid spots by the bromcresol green spray (0.04% in 95% ethanol). For one-dimensional separation of organic acids by paper chromatography, a solvent consisting of butanol–ethyl acetate–formic acid (1:1:1, v/v) was used (29). After drying paper chromatograms overnight in air, the approximate glycolic acid area was sprayed with 0.1 M Na_2CO_3 in 50% ethanol in order to prevent subsequent sublimation of the free acid during radioautography.

Organic acids were quantitatively separated on an anion exchange resin, AG1X8, acetate form, by modification of the method described by Palmer (25). Columns, 0.4 × 8.0 cm, were eluted by a pH gradient of acetic acid and then formic acid. Fractions of 4 ml were collected, and radioactivity in 0.1- or 0.2-ml aliquots was measured in 10 ml of Kinard's liquid scintillator. The radioactivity in each peak was combined, and the solution was evaporated to dryness at 35 to 38 C by a Buchler rotary evapomix.

Gas-liquid chromatography of organic acids as their trimethylsilyl derivatives was run on a 3% OV-1 column coated on Gas-Chrom Z, 80 to 100 mesh (36). Cochromatography was accomplished by mixing a radioactive sample with authentic material, evaporating to dryness, trimethylsilylating, and then chromatographing the trimethylsilyl derivatives. Chromatography was run either by linear temperature programming of 5 C/min starting at 100 C, or under isothermal conditions at 140 to 160 C. While a part of the trimethylsilyl derivatives was located by a recorder connected to a hydrogen flame ionization detector, the major portion of the radioactive derivatives was recovered at a collection port in small fractions, dissolved in a liquid scintillator, and counted, and coincidence of the peak for the authentic trimethylsilyl derivative with the radioactivity peak was established.

Photosynthetic Experiments. Algal cells at designated stages of their life cycle were centrifuged from the growth medium at 1000*g* for 5 min. The packed cells were washed once, centrifuged again, and then resuspended in 0.001 M phosphate at pH 6.0, so that final suspension of 1% (v/v) cells were obtained. $^{14}CO_2$ fixation experiments were run in lollipops of 20- or 100-ml volume maintained in a 20 C water bath and exposed to 3000 ft-c of light from each side by two 300-w reflector flood lamps. The algae were preilluminated and aerated by a stream of air for 5 min, and then 50 μl of $NaH^{14}CO_3$ solution per 10 ml of the algal suspension were injected at zero time. The $NaH^{14}CO_3$ solution contained 0.5 μc of ^{14}C per μl, and the percentage of ^{14}C varied between 25 and 50%. When experiments were run longer than 5 min, another aliquot of the $NaH^{14}CO_3$ was added. Aeration of *A. braunii* suspensions was not possible because severe foaming flooded the algae out of the lollipop. These suspensions were occasionally shaken by hand during the experiments.

At designated time intervals 2- to 4-ml aliquots were removed with a pipette and quickly filtered through a Millipore filter (AAWP-025) with suction and rinsed with 1 ml of water. The entire process of filtration and washing was completed within 15 sec. The combined filtrate and washing in a graduated test tube was acidified with glacial acetic acid, aerated for 10 min with $^{12}CO_2$, and made to a designated volume; an aliquot was counted; and the total ^{14}C excreted was calculated as cpm per ml algal suspension. The rest of the algal supernatant was evaporated to dryness at 35 to 38 C under reduced pressure, and the residue was analyzed chromatographically.

The cells on the Millipore filter were immediately transferred to 5 ml of boiling 80% (v/v) methanol in a beaker for 5 min, filtered through a Millipore filter, and washed twice with 2 to 3 ml of boiling 80% methanol. These combined fractions, called cell extract, were counted for ^{14}C and analyzed for labeled products of photosynthesis.

Degradation of Mesotartrate-^{14}C. Periodate degradation of tartrate to two molecules of glyoxylate has been described (33). This procedure is similar to Sakami's procedure (28) for the degradation of serine, which has been somewhat modified here (5). Mesotartrate was degraded by two similar procedures. The first method was used through most of the investigation when the identity of the acid was not known. To the degradation flask was added 1000 or more cpm of the ^{14}C-organic acid in less than 1 ml of water, 0.2 mmole of nonradioactive DL-serine in 1 ml of water, and 2 ml of 0.5 M phosphate at pH 5.8. To the closed system was added 0.75 mmole (160 mg) of $NaIO_4$ in 3 ml of water, and it was aerated at room temperature for 1 hr. Excess $NaIO_4$ was consumed with a second addition of 0.2 mmole of serine during another hour of aeration at room temperature. This step released the carboxyl groups of mesotartrate as CO_2 and carbons 2 and 3 as HCOOH. In the second step, the HCOOH was oxidized to CO_2 by a boiling solution of $HgCl_2$. The last step was the oxidation of HCHO, as well as all other carbon compounds, to CO_2 by boiling with $K_2S_2O_8$.

During each step of the degradation, CO_2 was carried by aeration through a water condenser into a U-tube partially filled with 5 ml of CO_2-trapping solution and divided by a sintered glass filter to produce very small bubbles (13, 42). The sum of radioactivity in cpm obtained through the three steps of degradation was expressed as % of ^{14}C in CO_2, HCOOH, and HCHO plus other carbon compounds.

A second procedure for periodate degradation of mesotartrate was similar to that for serine, except that 0.2 mmole (33.6 mg) of nonradioactive mesotartaric acid was used as carrier rather than serine. In this case the closed system was flushed with N_2 for 15 min before periodate was added, and aeration throughout the procedure was with N_2. The decomposition of excess $NaIO_4$ by the second addition of carrier serine was omitted. This procedure was used to determine specific radioactivity. The CO_2 released in each step was trapped in a known amount of $Ba(OH)_2$-$BaCl_2$ solution, and the amount was calculated by back-titrating the excess base with standard HCl. $Ba^{14}CO_3$ planchets were prepared, weighed, and counted by a gas flow counter for specific radioactivity (39).

RESULTS

CO_2 Fixation and Excretion by Synchronized Cells of *A. braunii*. In Table I is depicted the life cycle of the cells which had been synchronized by a 16-hr light and 8-hr dark regimen with about 1:4 dilution at the end of each dark period. During the life cycle, algal cells were measured for their ability to fix $^{14}CO_2$ and to excrete ^{14}C compounds during a 5-min period in the light (Fig. 1). This experimental design necessitated placing dividing cells, which could only be obtained from the dark period, into the light to measure their activity. The photosynthetic ability, expressed as cpm of ^{14}C fixed per ml of 1% (v/v) cell suspension, followed a distinct trend. Growing cells (2–10 hr light stage) were most active, while mature cells in the light (14–16 hr light stage) showed much less activity. Dividing cells in the dark stages, when placed in the light, had low CO_2 fixation capacity. Similar trends for photosynthetic ability as measured either by O_2 evolution, CO_2 fixation, or relative quantum efficiency have been reported for *Chlorella* species (30, 31, 32) and for *Chlamydomonas reinhardtii* (15). We, too, have verified these trends for *Chlorella* and *Chlamydomonas*. The decline in photosynthetic activity with aging of synchronized

Table I. *A Life Cycle of A. braunii Cells in Synchronized Culture, Relationship to Photosynthetic Activity, and ^{14}C Compounds Excreted during 5-min Photosynthesis in $NaH^{14}CO_3$*

Life Cycle	Cell Type	Photosynthetic Activity[1]	Excretion[2]					
			Total	Glycolate	Malate	Isocitric lactone (U_1)	Meso-tartrate (U_2)	U_3
			cpm × 10^3/ml 1% cell suspension					
Light, 0 hr	Daughter cells; not growing	Low	62	38.9 (62.7)	1.7 (2.7)	0.2 (0.3)	20.7 (33.5)	0.5 (0.8)
Light, 2 hr	Fast growing cells	Increasing	245	235.4 (96.1)	0.3 (0.1)	0	9.3 (3.8)	0
Light, 6 hr	Growing cells; cannot divide in dark	Highest	741	735.1 (99.2)	0	0	5.9 (0.8)	0
Light, 10 hr	Premature cells; can hardly divide in dark	Highest	1,044	1,044.0 (100.0)	0	0	0	0
Light, 14 hr	Mature cells; ready to divide	Decreasing	355	343.3 (96.7)	0.3 (0.1)	0	11.4 (3.2)	0
Light, 16 hr (Dark, 0 hr)	Fully mature cells; starting to divide	Decreasing	101	80.5 (79.7)	1.0 (1.0)	0	19.0 (18.8)	0.5 (0.5)
Dark, 2 hr	Dividing cells; daughter cells not yet released	Low	48	28.3 (59.0)	0.8 (1.6)	0.1 (0.2)	18.5 (38.6)	0.3 (0.6)
Dark, 4 hr	Dividing cells; starting to release daughter cells	Lowest	29	10.9 (37.8)	1.1 (3.8)	0.6 (2.0)	14.2 (48.9)	2.2 (7.5)
Dark, 6 hr	Dividing cells; releasing daughter cells	Low	48	23.6 (49.1)	1.5 (3.2)	0.5 (1.0)	20.9 (43.5)	1.5 (3.2)
Dark, 8 hr	Daughter cells; not growing	Low	72	42.8 (59.4)	2.1 (2.9)	0.5 (0.7)	25.3 (35.1)	1.3 (1.9)

[1] As measured both by $^{14}CO_2$ fixation and ^{14}C excretion (for details see Fig. 1).

[2] Values in parentheses are percentages of the total ^{14}C excreted.

cells is characteristic of normal development. In the present experiments, the daughter cells toward the end of the dark period had recovered some photosynthetic capability, if placed in the light.

Excretion of ^{14}C compounds approximately followed the same trend as $^{14}CO_2$ fixation during the developmental stages (Fig. 1) of the algal life cycle. However, excretion of ^{14}C compounds peaked a little later than the period of maximal photosynthesis. This trend for the period of maximal excretion was more clearly evident when the ^{14}C excreted was plotted as a percentage of the total ^{14}C fixed (Fig. 1). About 35% of the total ^{14}C fixed was excreted over a 5-min period by cells at the 10-hr light stage. This value dropped to less than 4% of the total ^{14}C which was excreted by dividing cells. Since the dividing cells could only fix about 25% as much CO_2 as growing cells, the amount of ^{14}C excreted by the dividing cells was low compared to growing cells. The results show that the amount of excretion by *A. braunii* is closely related to their photosynthetic ability.

Compounds Excreted by *A. braunii* at Different Stages of the Life Cycle. After 5 min of $^{14}CO_2$ fixation, the algal medium or supernatant was analyzed by paper chromatography and radioautography. A schematic chromatographic map of the compounds excreted is shown in Figure 2. The compounds excreted by the synchronized cells of *A. braunii* were different at various stages of the life cycle (Tables I and II).

Only glycolate was excreted in large or significant amounts by the growing young cells. Thus the growing cells possessed the greatest photosynthetic capacity and excreted about 10 to 35% of the total ^{14}C fixed as only glycolate. On the other hand, mature cells and particularly dividing cells, which fixed less total $^{14}CO_2$ and which excreted much less total ^{14}C, excreted as many as seven compounds. These were glycolate, mesotartrate, isocitrate lactone, glycerate, malate, citrate, and an unidentified acid, which was designated as U_3. Glycolate was always a major component excreted even by the dividing cells. However, at the time of cell division after 4 to 6 hr of darkness, the algae excreted as much ^{14}C in mesotartrate as in glycolate. In the supernatant from the dividing cells of *A. braunii* were also several per cent of the ^{14}C in isocitrate lactone, in malate, and in U_3. Glycerate and citrate were seen on the chromatograms of the supernatants only occasionally and in only small amounts relative to glycolate. Chromatograms of the cell extracts after 5 min of photosynthetic $^{14}CO_2$ fixation showed the usual large number of labeled sugar phosphates, free sugars, amino acids, and organic acids. Radioactive glycolate, mesotartrate, isocitrate lactone, and U_3 were found almost exclusively in the supernatant, and, therefore, these acids seemed to be specific for the excreted components. The cell fraction contained so much more ^{14}C-labeled glycerate, malate, and citrate than the amount of these excreted into the supernatant medium, that the excretion of the latter three acids did not seem significant.

Two patterns of excretion are visualized by plotting the percentage of the ^{14}C which was excreted for each compound during the life cycle (Fig. 3). Glycolate was excreted by the growing cells. Dividing cells excreted mesotartrate and other acids in a reciprocal relationship during the reduction of glycolate excretion.

Rates of $^{14}CO_2$ fixation and ^{14}C excretion with synchronized cultures of *Scenedesmus obliquus*, *Scenedesmus quadricauda*, and *Chlamydomonas reinhardtii* were examined only qualitatively. Analyses of the supernates revealed that glycolate excretion was maximal by growing cells and minimal by dividing cells, as was the case with *A. braunii*. Glycolate was the only major compound excreted by these algae throughout their life cycle, and formation in appreciable amounts of mesotartrate or isocitrate lactone by mature or dividing cells was not observed. Conditions for detecting the excretion of the other acids by different algae have not been exhaustively studied.

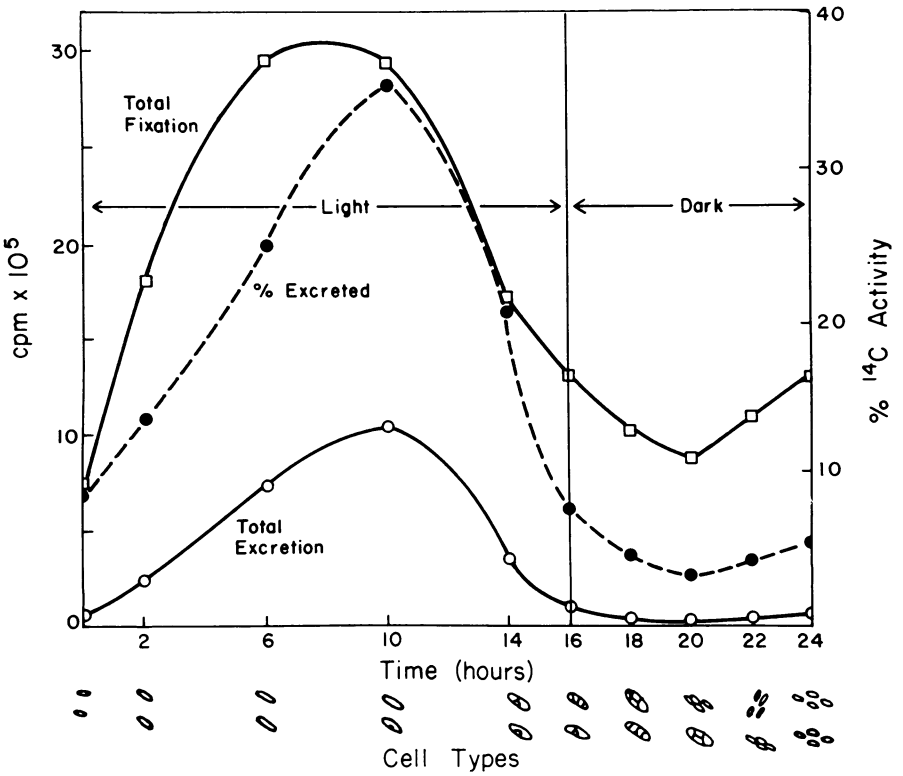


FIG. 1. Changes in the activity of photosynthetic $^{14}CO_2$ fixation and ^{14}C excretion during the life cycle of *A. braunii*. □: ^{14}C activity fixed in 5 min/ml 1% cell suspension; ○: ^{14}C activity excreted in 5 min/ml 1% cell suspension; ● percentage ^{14}C excreted of the total ^{14}C fixed.

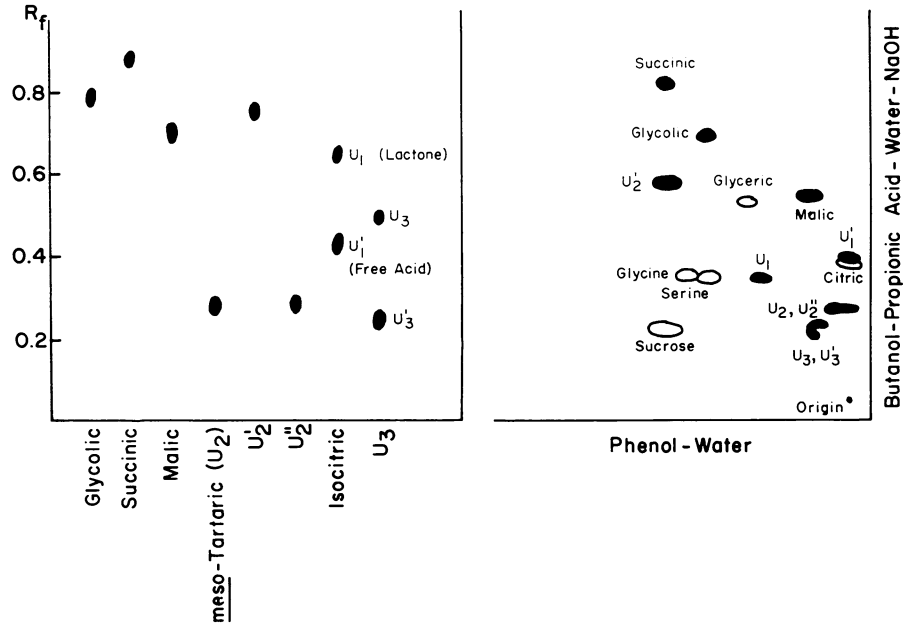


FIG. 2. Paper chromatographic maps of organic acids excreted by *A. braunii*. The solvent system for the one-dimensional chromatogram was butanol-ethyl acetate-formic acid. U_1 was identified as isocitrate lactone, U_2 as mesotartrate, but U_3 was not identified.

Since the synchronized algal cultures were not strictly sterile, the possibility existed that bacterial growth might have converted the excreted glycolate into the other acids. This possibility was discounted, because these same cultures of mature or dividing cells of *A. braunii* failed to metabolize added glycolate-^{14}C, P-glycolate-^{14}C, or mesotartrate-^{14}C in the light or dark. Further, the other algae which were grown at the same time in a similar manner and which excreted glycolate did not form the other excreted acids.

Kinetics of Photosynthetic Excretion. To *A. braunii* cells at different developmental stages was given $NaH^{14}CO_3$ in the light for 1- to 30-min periods by removing aliquots at the designated time. The supernatants were separated from the cells by a Millipore filter, and the ^{14}C compounds in the supernatant were

Table II. *Amount and Percentage Distribution of ^{14}C in Compounds Excreted by A. braunii at Different Stages of Their Life Cycle*

Min of $^{14}CO_2$ Fixation	Total cpm $\times 10^3$/ml 1% cell suspension	^{14}C in Excreted Compounds					
		Glycolate	Meso-tartrate	Iso-citrate lactone	U_3	Malate	Glycerate
		%	%	%	%	%	%
2-hr light stage							
1	3	98.9	1.1	0	0	0	0
2	17	98.2	1.7	0	0	0.1	0
5	176	98.8	1.0	0	0	0.2	0
10	308	99.0	0.8	0	0.1	0.1	0
30	1055	98.8	0.4	0	0.7	0.1	0
8-hr light stage							
1	6	91.4	4.5	0	0	4.1	0
2	230	99.8	0.1	0	0	0.1	0
5	978	99.8	0.2	0	0	0	0
10	1327	99.7	0.3	0	0	0	0
30	3498	99.0	0.6	0	0.1	0.1	0.2
0-hr dark or 16-hr light stage							
1	1	46.8	25.3	0	0	16.5	11.4
2	3	51.4	30.7	0	0	9.8	8.1
5	28	80.7	15.5	0.5	0.6	1.4	1.3
10	71	71.3	13.8	1.3	11.1	1.2	1.3
30	541	13.2	6.2	12.9	66.5	0.7	0.5
3-hr dark stage							
1	1	0	75.0	0	0	25.0	0
2	2	0	72.0	0	6.7	13.3	8.0
5	6	5.1	69.6	1.1	14.3	6.3	3.6
10	14	4.2	69.1	2.2	19.5	3.3	1.7
30	153	1.2	41.5	11.8	43.9	1.1	0.5
5-hr dark stage							
1	1	0	75.9	0	0	24.1	0
2	3	3.3	85.3	0	0	11.4	0
5	18	48.2	44.4	0	0	7.4	0
10	47	58.6	34.9	0.5	1.7	4.3	0
30	196	72.9	14.2	3.6	6.7	2.3	0.3
8-hr dark stage							
1	5	44.5	45.2	0	2.7	7.6	0
2	12	52.2	41.1	0	3.0	3.7	0
5	71	61.5	32.1	0.1	3.6	2.7	0
10	159	66.7	26.1	0.6	4.2	2.4	0
30	710	73.9	9.6	2.3	12.4	1.8	0

assayed by paper chromatography and radioautography (Table II). The amount of ^{14}C excreted increased with increasing periods of photosynthesis. However, the percentage distribution of ^{14}C among the excreted compounds varied with the stage of algal development and the length of the $^{14}CO_2$ fixation test. After 2 to 10 hr in the light, when the cells were rapidly growing and excreting a large amount of ^{14}C, glycolate represented over 98% of the total excretion throughout the 30-min test period. When the cells were starting to divide at the 0- and 3-hr dark periods, the maximal percentage of ^{14}C excreted as glycolate appeared only after 5 min of $^{14}CO_2$ fixation, and even then the total amount was much less. In most cases the highest percentage of ^{14}C excreted as mesotartrate, as well as malate and glycerate, occurred within 1 or 2 min of photosynthesis by dividing cells when the total amount of excretion was low. Isocitrate lactone and U_3, in contrast, contained a consistently increasing percentage of the ^{14}C excreted by dividing cells with increasing length of time for the test period. The significance of these

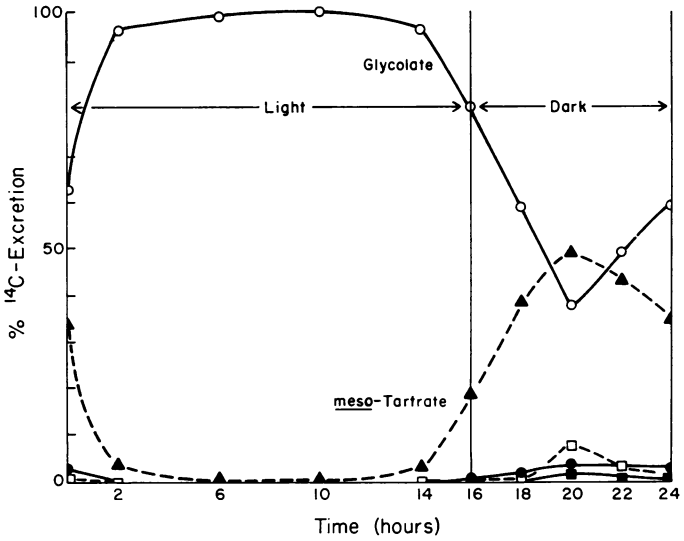


FIG. 3. Percentage distribution of ^{14}C among the compounds excreted during 5-min photosynthesis in $NaH^{14}CO_3$ by synchronized cells of *A. braunii*. ○: Percentage ^{14}C excretion in glycolate; △: percentage ^{14}C excretion in mesotartrate (U_2); □: percentage ^{14}C excretion in U_3; ●: percentage ^{14}C excretion in malate; ■: percentage ^{14}C excretion in isocitric lactone (U_1).

results and the metabolic relationships among the compounds are not clear.

From these results, the best condition for the excretion of the largest amounts of mesotartrate, isocitrate lactone, and U_3 by *A. braunii* was at the early stages (2 to 4 hr) of the dark period. For the highest yields of these acids, the fixation of $NaH^{14}CO_3$ should continue for at least 30 min. In many experiments with dividing cells, the percentage of the excreted ^{14}C which was found in mesotartrate, isocitrate lactone, and U_3 ranged from 30 to nearly 100%. Occasionally, with dividing cells from fresh cultures which had been synchronized for only 1 or 2 cycles, as described by Stange *et al.* (35), nearly 100% of the excreted ^{14}C was in acids other than glycolate. Thus the dividing cells lost nearly all of their ability to synthesize and excrete glycolate when they were placed back in the light. When this happened, they then excreted the other acids.

Excretion of Labeled Acids after $^{14}CO_2$ Photosynthesis. Algal cells were labeled with ^{14}C in a photosynthetic period of 15 min. They were washed free of supernate and excess $NaH^{14}CO_3$ by centrifugation and then filtration and washing on a Millipore filter. After resuspension to a 1% (v/v) mixture in 0.001 M phosphate, pH 6.0, the further excretion of labeled compounds in the light or dark in 15 min was measured in the absence of added $NaH^{14}CO_3$ (Table III). Growing cells mainly excreted glycolate in the light during $NaH^{14}CO_3$ fixation or from metabolites in the absence of added $NaHCO_3$. The continued excretion of glycolate in the light by *A. braunii* in the absence of further $NaH^{14}CO_3$ fixation is similar to that reported by Hess *et al.* for *Chlorella* (14). In the dark these algae excreted less ^{14}C, and little or none of it was glycolate. In the presence of CMU[3] the algal excretion was similar to that in darkness; *i.e.*, glycolate excretion was inhibited. In the dark the main excretion products were mesotartrate, isocitrate lactone, and U_3, which must have been synthesized from cellular ^{14}C-labeled photosynthetic compounds. Aeration with O_2 in the dark inhibited the excretion of isocitrate lactone (data not shown).

The results indicate that glycolate production and excretion are unique to photosynthesis and require light. The excretion of

[3] Abbreviation: CMU: 1,7-dimethyl-3-(*p*-chlorophenyl)urea.

Table III. *Amount of ^{14}C Excreted by ^{14}C-labeled Cells of A. braunii in Light or Darkness*

Cells fixed $NaH^{14}CO_3$ for 15 min in the light. They were then separated into supernatant and cells by centrifugation followed by filtration and washing on a Millipore filter. The cells were then resuspended in 1% (v/v) 1 mM phosphate at pH 6.0 and stirred for 15 min in light or darkness, but without addition of any $NaH^{14}CO_3$. Again the ^{14}C excreted was measured.

	Excreted ^{14}C		^{14}C Among Compounds Excreted					
			Glycolate	Malate	Isocitrate lactone	Meso-tartrate	U_3	Unidentified
	cpm/ml 1% cells	*% of total fixation*	%	%	%	%	%	%
Growing cells (8-hr light stage) synchronized for 2–4 days								
Initial supernate	34,780	43.7	98	0	0	0	0	2
Excretion afterwards								
In light	7,190	9.4	90	2	0	1	tr	7
In dark	1,366	1.7	5	9	0	7	2	80
Growing cells (7-hr light stage) synchronized for 2 days								
Initial supernate	10,031	19.3	94	1	0	2	tr	2
Excretion afterwards								
In light	3,917	7.6	79	0	2	8	4	7
In light and CMU	2,667	5.2	36	0	4	28	14	18
In dark	1,535	2.9	0	0	6	33	19	42
Dividing cells (4-hr dark stage) synchronized for 2–4 days								
Initial supernate	2,707	11.1	25	5	7	50	8	5
Excretion afterwards								
In light	1,275	5.2	11	7	17	12	18	36
In dark	1,110	4.5	0	6	30	16	21	28

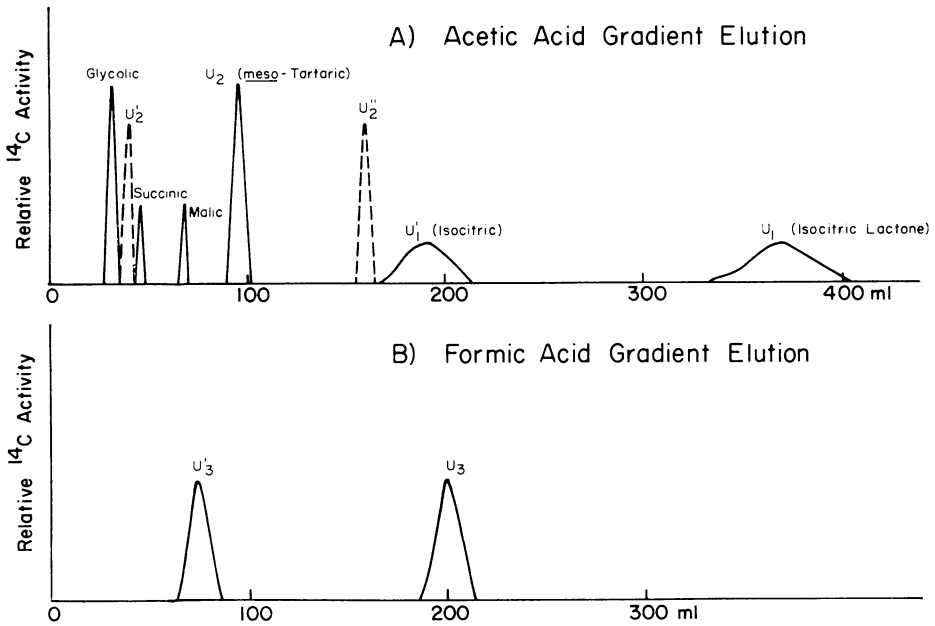


FIG. 4. pH gradient elution of organic acids excreted by *A. braunii* with an AG1 resin column. A: Acetic acid gradient elution; 3 N acetic acid was mixed into 200 ml of water. B: Formic acid gradient elution; after eluting 500 ml by gradient A, 3 N formic acid was mixed into 200 ml of the acetic acid remaining in the mixing flask.

the other acids, isocitrate lactone, mesotartrate, and U_3, occurs in the dark and is therefore probably related to some metabolic process not associated with photosynthesis.

Isolation of Acids by Resin Chromatography. Larger scale preparations of the excreted acids were made with 80- to 250-ml suspensions of 1% cells of *A. braunii* at an early dark stage (3–4 hr dark) of cell division by exposure to $NaH^{14}CO_3$ in the light for 30 min. The supernatant after concentration was passed through a cation exchange column of AG50W resin in the H-form. Paper chromatography before and after treatment with the AG50W resin showed that none of the ^{14}C-labeled acids were absorbed. Because filtration of larger volumes took a much longer time, small losses of other cellular constituents occurred which were in part removed by the AG50W resin. The eluates from the AG50W resin were put on columns of AG1 resin in the acetate form in order to absorb the organic

acids. The AG1 resin columns were then eluted by an increasing pH gradient, first with acetic acid and then with formic acid as idealized in Figure 4. The radioactive peaks were located by ^{14}C counting, then pooled and concentrated, and the identity of ^{14}C components was checked by paper chromatography and radioautography. The fast, one-dimensional separation with the butanol-ethyl acetate-formic acid system was generally employed for this purpose (Fig. 2). Pure samples of radioactive acids were obtained by repeated anion exchange chromatography. Mesotartrate was particularly difficult to purify, because it tended to give double spots on the two-dimensional paper chromatographic procedure, and because it was contaminated with malate and phosphate. During paper chromatography of isocitrate lactone, two radioactive spots were obtained and later identified as the lactone and the free acid.

Identification of Isocitrate Lactone. ^{14}C-Labeled spots at the R_F of isocitrate lactone were eluted from paper chromatograms or a corresponding peak from the AG1 resin columns was used. When a solution of the compound was reduced to dryness at 35 to 38 C under partial pressure of 5 to 10 mm, two labeled components, the lactone and the free acid, were obtained upon rechromatography. The ratio of ^{14}C in the two varied. Evaporation at 35 to 38 C under reduced pressure in the presence of acetic acid from the AG1 column promoted 10 to 30% hydrolysis of the lactone. At room temperature in water there was little conversion of the lactone in the time periods used. Lyophilization of the samples prevented hydrolysis of the lactone.

In three solvent systems, the ^{14}C-labeled spot cochromatographed, as detected by bromcresol green spray test for acids, with commerical isocitrate lactone or the lactone prepared by us from isocitrate. The hydrolysis product from the ^{14}C spot cochromatographed with isocitrate. The isocitrate lactone-^{14}C and isocitrate-^{14}C were eluted from the AG1 columns in approximate coincidence of the retention volume with those reported by Palmer (25). Isocitrate lactone was identified by converting it to α-ketoglutarate and then to glutamate by a slight modification of the procedure described by Grafflin and Ochoa (9). By alkaline hydrolysis with 0.1 N NaOH, 30,000 cpm of the lactone plus about 200 μg of unlabeled lactone carrier were converted to the free acid when heated in a boiling water bath for 10 min (6). To the hydrolysate, after neutralizing to pH 7.5, and diluting to 2 ml, were added 0.1 ml of 0.1 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.5), 0.2 ml of 0.02 M $MnCl_2$, 0.05 ml of 0.003 M NADP, and 0.4 ml of 0.05 M aspartate to serve as amino group donor. The reaction was initiated by addition of isocitrate dehydrogenase and aspartic aminotransferase, and the mixture was incubated for 1 hr at 37 C. The amino acids from the reaction mixture were absorbed on a small column of AG50W resin in the H-form, and the column was washed with water and then eluted with N NH_4OH. The radioactivity in the reaction mixture before addition of the enzymes cochromatographed with isocitric acid, and there was no glutamate spot on the chromatogram by either ninhydrin spray test or ^{14}C radioautography. The radioactivity after enzymic conversions cochromatographed with glutamic acid, as detected by the ninhdrin reaction.

Excretion of Isocitrate Lactone. The natural occurrence of isocitate lactone in biological material has not been well documented, and in the present work we were concerned that the presence of the lactone might be an artifact of the experimental procedure. The most likely step in the procedure which would cause lactonization was the acidification of the supernatant with acetic acid to remove excess $^{14}CO_2$ and then concentration of the supernatant at 35 to 38 C under partial vacuum. Experiments were repeated in which these steps were modified to avoid or minimize lactonization. Lyophilization was used as a substitution for heat and vacuum concentration, and the acidification with acetic acid was substituted by 30 min of aeration with $^{12}CO_2$. These changes did not alter to an appreciable extent the large amount of ^{14}C isocitrate lactone in the supernatant to the small amount of ^{14}C in isocitrate plus citrate. Also chromatography of the cell extracts which were treated similarly to the supernatants showed ^{14}C in isocitrate and citrate but no isocitrate lactone. It was thus unlikely that the chromatographic procedures were forming the lactone from free isocitrate.

In some experiments algal supernatants contained ^{14}C-labeled isocitrate lactone without any detectable label in isocitrate or citrate. Incomplete lactonization of isocitrate by chemical means has been reported by Pucher (27), Vickery (40), and Kato and Dickman (16), and in several conversions of isocitrate-^{14}C to the lactone we never exceeded a 70% lactonization. It is, therefore, very probable that the presence of only isocitrate lactone in the supernatant was due to its excretion by the algal cells. In fact, the cells may only be able to excrete the lactone, and the presence of a little free acid in some cases might be due to lactone hydrolysis after excretion. Such a conclusion suggests that in the excretion process there exists an enzyme for lactonization.

Identification of Mesotartrate. The unknown compound designated as U_2 in Figure 2 formed double spots on two-dimensional paper chromatograms, but a single spot in the butanol-ethyl-acetate-formic acid system. When either portion of the double spots was eluted and rechromatographed, the same results were obtained.

U_2 was partially purified by repeated anion exchange chromatography with an AG1-acetate column, and the fractions were concentrated with a shaking evaporator at 35 to 38 C several times to remove acetic acid. When this sample was again subjected to the same anion exchange chromatography, a tremendous shift of part of the radioactivity from U_2 (retention volume 72–124 ml) to peak U_2' (retention volume 28–52 ml) was observed (Fig. 4). The substance in this new peak, U_2', when concentrated by evaporation and rechromatographed on a AG1-acetate column, partially or completely reverted to U_2 or to another peak designated as U_2''. The compound, U_2', could also be distinguished from U_2 on paper chromatograms, but U_2'' and U_2 were indistinguishable by paper chromatography (Fig. 2). The conversion of U_2 to U_2' was observed when U_2 was heated to dryness in the presence of acetic acid. The reconversion of U_2' back to U_2 readily took place when heated in dilute acetic acid, water, or if neutralized. The best way to protect U_2' from reverting to U_2 during concentration was to lyophilyze. Other such experiments suggested that the conversion of U_2 to U_2' required acid and some heat, while the reversion of U_2' to U_2 was rapid in an aqueous solution. When U_2 was identified as mesotartrate, it seemed reasonable to assume that its conversion to U_2' was due to intermolecular esterification, but U_2' and U_2'' were not identified.

Samples of U_2 from the anion exchange chromatography were usually contaminated with some malate and larger amounts of phosphate. Further purification of U_2 was achieved by concentrating to dryness at 35 to 38 C a solution of it in acetic acid as eluted off the AG1-acetate column. The dried sample, which contained much U_2', was dissolved in a small amount of water and immediately introduced onto another AG1-acetate column for acetic acid gradient elution. The material in the U_2' peak was collected and evaporated to dryness to remove acetic acid. The material was dissolved in water, neutralized to pH 7 to 8 to revert it to U_2, and then rechromatographed again on an AG1-acetate column. Pure samples of U_2 were thus obtained free from malic acid, phosphate, and other contaminating components in the original U_2.

U_2 was not cationic, since it was not absorbed by AG50W-H^+. It was stable to boiling for 1 hr in N HCl or N NaOH, but it was destroyed by periodate oxidation at room temperature, as would

be the case for a tartaric acid. The purified U_2 cochromatographed in three different solvent systems with mesotartaric acid, but not with (+)-tartaric acid, which gave a slightly higher R_F. The meso- and (+)-isomers of tartaric acid were clearly separated by gas-liquid chromatography of their trimethylsilyl derivatives. When the purified U_2 was gas-liquid cochromatographed separately with two isomers as their trimethylsilyl derivative, the radioactivity coincided in location with the mass peak of the trimethylsilyl derivative for the carrier mesotartaric acid, but not with the (+)-form (Table IV).

U_2 has tentatively been identified as mesotartaric acid by three criteria. The ^{14}C-labeled unknown cochromatographed with mesotartaric acid in a number of solvents and on ion exchange columns. The unknown gas chromatographed with mesotartaric and not with other acids. The unknown and carrier mesotartaric acid were degraded by periodate in a similar manner.

Distribution of ^{14}C-Mesotartaric Acid. Periodate oxidation at room temperature of tartaric acid will give rise first to two molecules of glyoxylic acid, which are then further oxidized to CO_2 and HCOOH. Thus the carboxyl groups of mesotartaric acid are converted to CO_2 and carbons 2 and 3 to HCOOH, which can be subsequently oxidized to CO_2. Different samples of ^{14}C-labeled mesotartaric acid, which had been excreted during a 10-min period of photosynthesis in $^{14}CO_2$ by *A. braunii*, were degraded by periodate. Seventy to 80% of the radioactivity was located in the carboxyl groups, while the two middle carbons were relatively inactive (Table V). Degradation of U_2' and U_2'' gave similar results. Owing to the rapidity of photosynthesis, the distribution of ^{14}C among the carbon atoms of compounds synthesized by leaves or algae has been found to be nearly uniform after 1 min. Certainly after 10-min periods of photosynthesis a uniform labeling pattern would be expected in such acids, and such results were observed by Stafford and Loewus (34) for (+)-tartaric acid formed by grape leaves. Thus the retention of carboxyl labeling in mesotartaric acid which was excreted by the algae during the course of 10 min of photosynthesis seems unique and is probably related to the fact that it was excreted.

Table IV. *Gas-Liquid Chromatography of Trimethylsilyl Derivatives of Tartaric Acids and U_2*

A. By Linear Temperature Programming of 5 C/min Starting at 100 C

Sample (as Trimethylsilyl Derivative)	Mass Peak	^{14}C Activity
	C	*C*
Mesotartaric acid (250 μg)	165–172	None
L(+)-Tartaric acid (200 μg)	170–175	None
Mesotartaric acid (250 μg) plus ^{14}C-labeled U_2	162–170	162–170

B. Under Isothermal Conditions at 148 C

Sample (as Trimethylsilyl Derivative)	Mass Peak	^{14}C Activity
	min	*min*
Mesotartaric acid (2 μg)	12.0–13.7	None
L(+)-Tartaric acid (2 μg)	16.5–18.0	None
Mesotartaric acid (20 μg) plus ^{14}C-labeled U_2	11.5–13.2	11.5–13.2

Partial Purification and Properties of U_3. The chromatographic properties of this unknown compound which was excreted by *A. braunii* were those of a strong organic acid (Figs. 2 and 4). On paper chromatograms it had R_F values in the general location of oxalic or gluconic acid, or orthophosphate. Among the organic acids excreted by the algae, the elution of only U_3 from an AG1-acetate column required formic acid. Thus it was a very strong acid and could be prepared free of other ^{14}C contamination and phosphate. It existed in two interchangeable forms, U_3 and U_3', which had properties similar to an acid and a lactone form, as was the case with isocitric acid and isocitric lactone. U_3 was not absorbed by a cation exchange resin. It was not hydrolyzed by phosphatases. Periodate oxidation at room temperature released no CO_2, but about 60 to 70% of the ^{14}C was oxidized to HCOOH, and the rest to formaldehyde and other breakdown products. U_3 did not cochromatograph with oxalic or gluconic acid or many other acids which were tested. U_3 remains unidentified, but it is most likely to be an organic acid with secondary alcohol groups to account for the lactone formation and periodate attack (Table V).

DISCUSSION

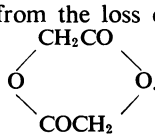
The physiological significance for the excretion of mesotartrate and isocitrate lactone by *A. braunii* is unknown, just as the significance of glycolate excretion by most algae remains in doubt. Hess and Tolbert (13) have postulated that algae excrete glycolate as an end product of photosynthesis, and Vickery and Palmer (41) proposed earlier a similar reason for the formation of (+)-tartrate in tobacco leaves.

The products of $^{14}CO_2$ fixation which were excreted by *A. braunii* had several common properties. (*a*) They were hydroxy carboxylic acids. (*b*) They were not accumulated inside the cell in significant amounts. (*c*) Metabolic pathways for the excreted products may be limited or are unknown in the algae. Glycolate was excreted, as glycolate dehydrogenase activity was low, from growing the cells on 0.2% CO_2 (22). The cells would not metabolize added glycolate-^{14}C or mesotartaric-^{14}C acid (data not shown). (*d*) The excreted acids could easily form lactones or dilactides by dehydration. Thus glycolate can exist in six other

Table V. *Periodate Degradation of Mesotartrate and U_3*

Values are averages of complete biological experiments and degradations. Experiments with carrier serine were repeated 13 times, with carrier mesotartrate 2 times, and for U_3 5 times.

Step of The Degradation	Carbons Released as CO_2	Mesotartrate (U_2)			U_3
		Oxidation with carrier serine	Oxidation with carrier mesotartrate		Oxidation with carrier serine
		%	*mμc/mg C*	%	%
Periodate oxidation at room temperature	Carboxyl groups of mesotartrate	75	3.60	78	2
$HgCl_2$ or boiling periodate oxidation of HCOOH	Carbons 2 and 3 of mesotartrate	20	1.02	22	63
Persulfate oxidation for all other carbons		5			35

forms, resulting from the loss of water, which are less acidic, such as glycolide  O. Mesotartrate existed in forms (U_2' and U_2'') which would revert to the free acid in acidic aqueous solutions and which we postulate to be dilactide structures. The excretion of lactate by algae under anaerobic conditions (38) may be similar in that lactate would be an end product of anaerobic metabolism and it, too, can exist in the lactide form. In the case of isocitrate, only the lactone form was excreted, and the rate of hydrolysis was sufficiently slow so that little free isocitrate was found in the algal suspension medium. Isocitrate lactone is probably metabolically inactive. The excretion of the lactone form of isocitrate suggests that the more active excretion of glycolate may also occur as the glycolide, but that the hydrolysis of glycolide is so rapid that it has not been detected, particularly in the chromatographic solvents used to separate the organic acids. The excreted organic acid, U_3, which was not identified, also was in reversible equilibrium with another form, U_3' (Figs. 2 and 4), which was probably a dehydrated structure. Excretion of the lactone forms would give specificity to the excretion process and would be advantageous to the cells in that accompanying losses of cations need not occur. Excretion of the lactone, followed by hydrolysis, might also be a chemical form of an acid pump which has been postulated as necessary for membrane transport. The observed loss of some malate, glycerate, and citrate or isocitrate by the dividing cells is not consistent with the above ideas. However, the latter three acids were only excreted in small amounts relative both to their pool sizes inside the cell and to the amount of total acid excretion.

The excretion of carboxyl-labeled mesotartrate over long periods of time by *A. braunii* seems unique. (+)-Tartrate is only very slowly labeled by plants and has been reported to be more labeled in the central carbons than in the carboxyl carbons (34). Maroc (18, 19) has recently proposed that *Pelargonium* leaves could form mesotartrate from two molecules of glycolate via glyoxylate and oxaloglycolate as intermediates. Since the glycolate formed from $^{14}CO_2$ is uniformly labeled, this pathway for mesotartrate biosynthesis by *A. braunii* does not seem feasible. Thus alternate pathways for mesotartrate synthesis must be considered. If mesotartrate were being formed from products of the path of carbon in photosynthesis, it should have been uniformly labeled after 5 or 10 min. That mesotartrate retained a 70 to 80% carboxyl labeling pattern suggests that it was formed by a carboxylation of a C_3 acid precursor and then immediately excreted. An analogous labeling pattern has been found in C_4 dicarboxylic acids (malate and aspartate) formed by certain tropical grasses (11). In the latter case, the ^{14}C from the $^{14}CO_2$ remains predominantly in the gamma carboxyl position over long periods of time, even though the compounds of the photosynthetic carbon cycle become uniformly labeled. By analogy for the excreted mesotartrate, the 70 to 80% ^{14}C in the carboxyl groups could have been mainly incorporated into one carboxyl position. The symmetrical nature of mesotartrate makes a test of this idea impossible by chemical degradation.

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