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This is to certify that the

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Excretion of Organic Acids During
Photosynthesis by Synchronized Algae

presented by

Wei-Hsien Chang

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PhD

N. E. Tolbert
Major professor
Dept Biochemistry

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ABSTRACT

EXCRETION OF ORGANIC ACIDS DURING PHOTOSYNTHESIS BY SYNCHRONIZED ALGAE

by Wei-Hsien Chang

Synchronized cultures of Ankistrodesmus braunii were grown during a 16-hr light and 8-hr dark regimen at 30° with a 1 to 4 dilution at the end of each dark period. The photosynthetic ability, as measured by $^{14}CO_2$ fixation, was the 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.

The ^{14}C-compounds excreted during photosynthesis changed during the algal life cycle. Young growing cells excreted glycolate in large amounts, but none of the other acids. Dividing cells excreted only about 4% as much glycolate-^{14}C as young growing cells. Dividing cells also excreted meso-tartrate, isocitric lactone, malate, and an unidentified acid, U_3, and occasionally some citrate and glycerate. Of the acids excreted by dividing cells, glycolate and meso-tartrate were the major ones and they were excreted in comparable amounts. Excretion of meso-tartrate when glycolate excretion decreased implied that the two acids might be metabolically and physiologically related.

Kinetic studies on the excretion of these acids by the synchronized cells were done during 30 minutes of photosynthesis. Growing cells excreted glycolate. With dividing cells, meso-tartrate, malate and glycerate were excreted in relative largest amounts within 1 or 2 minutes of photosynthesis, glycolate in 5 minutes, and isocitric lactone and U_3 in 30 minutes.

Large-scale separation and purification of the excreted acids were carried out by repeated anion exchange chromatography on AG1-acetate columns with acetic acid gradient elution followed by formic acid gradient elution. The acids were eluted from the column with the acetic acid gradient in the order of glycolic, malic, meso-tartaric, isocitric and citric acids, and isocitric lactone; U_3 was eluted with a subsequent formic acid gradient.

The meso-tartaric acid was identified by cochromatography on paper chromatograms and by gas-liquid cochromatography as the trimethylsilyl derivative. Isocitric lactone was identified by cochromatography on paper, and by hydrolyzing it to isocitrate. The isocitrate was then identified by cochromatography and by converting it to glutamate by a reaction catalyzed by isocitric dehydrogenase and glutamic-aspartic transaminase. Isocitric lactone was experimentally shown to be excreted as such by the dividing cells.

After a period of photosynthesis in $NaH^{14}CO_3$, subsequent excretion of ^{14}C-labeled acids in the light and in the dark was analyzed both for the amount and for the components. In the light, excretion of glycolate and the other acids continued. In the dark, glycolate excretion was completely stopped, while the excretion of the other acids continued in even larger amounts. Upon addition of CMU during the light, the excretion pattern was similar to that in the dark. Aeration with oxygen during the dark prevented the excretion of isocitric lactone.

The distribution of ^{14}C in meso-tartaric acid was determined. The carboxyl carbons were about 4 times as radioactive as the middle carbons. Since glycolate is known to be uniformly labeled, glycolate

could not be the precursor of carboxyl labeled meso-tartrate, nor meso-tartrate a direct precursor of glycolate. The biosynthesis of meso-tartrate by these algae is unknown, but the carboxyl labeling pattern suggests that a carboxylation to form a C_4-precursor of the tartrate might exist. From the data, it was speculated that glycolate might be formed from a precursor common to both meso-tartrate and glycolate. The reason for the specific excretion of glycolate, meso-tartrate and isocitric lactone is not known, except that all three acids may not be further metabolized by the algae.

EXCRETION OF ORGANIC ACIDS DURING PHOTOSYNTHESIS BY SYNCHRONIZED ALGAE

By

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INTROD

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INTRODUCTION AND LITERATURE REVIEW

Excretion of organic compounds by algal cells has often been reported. Both the quantity and components excreted may vary for different species, and for different environmental conditions. Allen (1) and Antia, et al. (3) reported that from 10% to as high as 45% of the organic matter formed by algal cells was excreted. Recent reports (25, 30, 56), however, agree that the excretion of organic compounds by algal cells amounts to 2 to 6% of the total carbon fixed. According to Allen (1), the amount of excretion by Chlamydomonas species was parallel to the growth. However, working with other green algae, Forsberg, et al. (25) claimed that organic compounds were excreted during the phase of declining relative growth rate and in the stationary phase of growth. In fact they reported some uptake of external carbon compounds before excretion started. High light intensity (1, 23, 30) and nitrate as nitrogen-source (1) have been found to favor excretion.

Various compounds have been found to be excreted by algae. Beside organic acids, which are the major excretion compounds, amino acids, other nitrogenous compounds, and various carbohydrates have been reported. Glycolic acid is the common compound which is excreted in relatively large amounts by all the algae. Except for glycolic acid, the types of other compounds excreted seem to be specific to the different algal species. Chlamydomonas (1) excreted some polysaccharides, in addition to glycolic, oxalic and pyruvic acids, but

no nitrogenous compounds. Hellebust (30), who surveyed a large number of green algae found 9 to 38% of the total carbon excreted as glycolate, 0.2 to 5.9% as protein, and 2.8 to 10.3% as lipid, together with some amino acids and peptides.

Excretion of nitrogenous compounds has also been analyzed. From 25 to 40% of total N-fixation was excreted according to Henriksson (32, 31), but 5 to 8% was reported by Magee and Burris (51) and Allen and Arnon (2). Henriksson (32) found that shaking of the culture and high nitrate could decrease the excretion of nitrogenous compounds. According to Watanabe (95), nitrogen-fixing blue-green algae excreted only 3 amino acids, i.e., aspartic and glutamic acids and alanine. Stewart (81) found many kinds of amino acids were excreted, among which alanine, threonine, leucine, glutamic acid and basic amino acids were the main ones. Other minor compounds reported in algal excretion have been glycerol (30), mannitol (30), some analogue of acetylcholine (93), dimethylsulfide or related sulfur compound (4), and a yellow, nitrogeneous compound, probably a protein-carotenoid complex (22).

The studies mentioned above were, in most cases, done from an ecological viewpoint. Therefore, they were either run in the field or in the ocean under natural conditions, or carried out in a large scale for a long period of time. Few experiments were performed in a laboratory with biochemical equipment and techniques. Glycolate excretion is the only subject that has been extensively studied from a biochemical point of view, and with biochemical equipment and techniques.

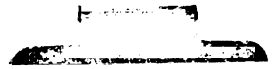
Rapid formation of glycolate by algae during photosynthesis was first reported by Benson and Calvin (8) in 1950, but it was not until 1956 that Tolbert and Zill (86) first reported that the glycolate

formed by Chlorella was excreted into the medium. The glycolate excretion by algae has since been confirmed by many investigators, including Lewin (50) for Chlamydomonas, Pritchard, et al. (62) for Chlorella, Miller, et al. (55) for Chlorella, and Hess, et al. (35) for Scenedesmus. One group with Fogg in England is investigating the ecological significance of glycolate excretion. The amount of glycolate excretion during photosynthesis under normal conditions was reported by Tolbert and Zill (86) to be 3 to 10% of the total carbon fixed, or 3 to 8 mg glycolic acid per liter of algal suspension. However, it can be enhanced by low CO_2 concentration (55, 62), high O_2 partial pressure (55, 86, 99), high pH (21, 57, 60), and high light intensity (62, 86). Light and manganese have been shown to be indispensable for glycolate excretion (35, 55, 62, 86). Although there have been reports that glycolate can be taken up by algal cells (21, 55, 99), especially when illuminated, Hess and Tolbert (34) have recently claimed that no significant uptake by algae of either glycolate or phosphoglycolate was observed. In spite of intensive studies and numerous speculations, the physiological significance of glycolate excretion by algae still remains unexplained. Hess and Tolbert (34) speculated that glycolate might be an end product of carbon metabolism in algal cells because it was excreted but not taken up, and because of the absence in algae of glycolate oxidase which is necessary for its metabolism.

The pathway of glycolate biosynthesis is unknown, although there are two hypothesis in the current literature. The most likely pathway was based on kinetic studies of ^{14}C-incorporation into products of photosynthesis (98). This hypothesis postulates that glycolate may be derived from a photosynthetic product of the carbon reduction cycle.

Since Schou, et al. (68) found that the glycolate molecule is uniformly labeled, the possible source of such a glycolate molecule would be the top two carbon atoms of a sugar phosphate which are also uniformly labeled. Thus, Bassham (5) and others have proposed that glycolate arose from a sugar phosphate with the participation of thiamine pyrophosphate. Holzer's group isolated a glycolaldehyde-TPP intermediate, which was oxidized nonenzymatically to glycolyl-TPP, and then hydrolyzed to free glycolate (24, 36). Racker's group were also able to demonstrate glycolate formation from fructose-6-phosphate by chloroplasts with ferricyanide, a strong oxidant (9). They also isolated a glycolaldehyde-phosphoketolase intermediate (26). In analogy, Tolbert (85) has suggested that phosphoglycolate might be formed by the cleavage of a ketose diphosphate molecule, probably through a TPP-C_2-phosphate intermediate. However, none of these reactions have been shown to occur enzymatically or *in vivo*. Another hypothesis for glycolate biosynthesis is a *de novo* formation by direct condensation of two molecules of CO_2 (82). From data of specific activity measurements, Zelitch (101) favored the *de novo* synthesis. However, Hess and Tolbert (33) did not confirm Zelitch's results.

A biochemical study of acid excretion by algal cells during photosynthesis could best be done with the use of a synchronized culture. Shifts in metabolic pathways and change in enzyme activity of algal cells during their life cycle have been recognized (17, 39, 41). The most significant correlation between cellular activity of algae and the life cycle was observed for the photosynthetic activity. Thus, Sorokin (71, 72, 74) and Kates and Jones (40) were able to show a rhythmic change of photosynthetic activity during the life cycle of



Chlorella or Chlamydomonas species. Sorokin (73) further separated the small younger cells and the larger older cells by fractional centrifugation. He clearly showed that the small younger cells were invariably more active than the large older cells. He, therefore, concluded that the change in photosynthetic activity during the life cycle is characteristic of normal cell development. However, there are some reports (15, 75) in which no rhythmic change of photosynthetic activity was observed during the life cycle.

If a minor phenomenon is taking place, or a minor component is being formed, simultaneously with a major one, the minor one may be obscured by the major one and tends to be overlooked. This is usually the case when a random algal culture is used in a biochemical study of some cellular activity. The activity of the rapidly growing young cells dominates. In this study, therefore, excretion of organic acids was measured, both quantitatively and qualitatively, during the life cycle of a synchronized algal culture. Ankistrodesmus braunii was used in this study because it was easily synchronized, its morphological change during the life cycle could be clearly distinguished microscopically, and the cells were easily filtered through a Millipore filter. As reported in this thesis, the amount of photosynthetic excretion changed during the life cycle as did the photosynthetic activity, and the compounds excreted also changed. In part the results are a study of changes in glycolate excretion during the algal life cycle. Most of my effort was devoted to the identification of meso-tartaric acid, which was the second most important acid excreted by the dividing cells of A. braunii, and of isocitric lactone which was also being excreted.

MATERIALS AND METHODS

Algae:

The strains of algae used in these experiments were obtained from the "Culture Collection of Algae" at Indiana University, Bloomington, Ind. (80). Ankistrodesmus braunii (Naeg.) Collins (No. 245), Scenedesmus obliquus (Turp.) Krüger (No. 393), and Scenedesmus quadricauda (Turp.) Bréb. (No. 77) were obtained on proteose-agar slants and cultured on an inorganic salt medium with Hoagland's micronutrients (58); Chlamydomonas reinhardtii Dangeard, (-) strain (No. 90) was obtained on a soil-extract-agar slant and cultured on the high phosphate medium described by Orth, et al. (60).

Inocula were first taken from stock cultures on agar slants into 100 ml of the respective culture medium in a 250 ml Erlenmeyer flask for growth and multiplication over a period of a week to 10 days. This culture was then transferred to 1.5 l of fresh sterile culture medium in a 2.5 l "low form" Fernbach flask for further growth and multiplication. The continuous cultures were diluted 1 to 10 with fresh medium about every 3 days before becoming dark green or brownish, and each culture was renewed from the respective stock culture every 2 to 3 months.

The culture flasks were fitted with air inlet and outlet, both provided with a cotton filter. Aeration with 0.2% (v/v) CO_2 in air was maintained by passing air from an oil-free compressor through a cotton filter and distilled water, and then mixing the air with

appropriate volumes of CO_2 which was monitored by bubbling through distilled water. The flasks were placed on a reciprocating shaker (Eberbach Corp., Ann Arbor, Mich.) of about 60 excursions per min., thus providing a gentle, but thorough agitation of the 1.5 l medium in the large flasks. The shaker was held in a controlled environment chamber ("Sherer Controlled Environment Lab," Model CEL 37-14; Sherer-Gillett Co., Marshall, Mich.) kept at 15°. Continuous light from cool white super high fluorescent bulbs (General Electric, F72T12-CW-1500) on the top of the chamber provided a light intensity at the level of the flasks of 1,200 ft-c. These conditions maintained a temperature in the culture medium of 20° as monitored by a thermometer.

Synchronous culture of algae:

For synchronization of the algal cultures, Tamiya's method (84) of "programmed" light-dark regimen with periodic dilution was slightly modified according to Wanka's light-dark time schedule (94). Initially a dense, random culture of algae was diluted one to one with fresh medium and kept growing under continuous light for another day or two. By this method, which is similar to that reported by Stange, et al. (79), a partially synchronized culture was obtained, in which most of the algal cells were resting at a certain stage of growth and only a few dividing cells could be observed microscopically. The algal cell population was then counted with a "Levy and Levy-Hausser counting chamber" in which each of the smallest square units was equivalent to 2.5×10^{-7} ml. The algal cells were diluted with fresh medium to a population of 4×10^6 cells/ml, or 1 cell/smallest square unit.

One liter of the diluted algal culture in a low form Fernbach flask was placed on a reciprocating shaker held in another controlled environment chamber. Illumination in this chamber was set for cycles of 14-hr light of the same intensity and of 8-hr dark. The temperature during the light period of the chamber was kept at 25° in the chamber, which was sufficient to maintain a temperature in the culture medium of 30°. During the dark period the temperature was kept at 30° by means of additional heat from darkened tungsten lamps which were connected to a thermostat. Since the diluted algal cells were already partially synchronized in a certain growth stage, they were put into the growth chamber at about the middle of the light period, so that the remaining period of illumination would bring them up to mature cells which would divide during the following dark period.

At the end of the dark period the population of daughter cells was counted again and the "division number" was calculated. In most cases, the division number for Ankistrodesmus braunii was between 3 and 4 and that for Scenedesmus obliquus, Scenedesmus quadricauda, or Chlamydomonas reinhardtii was between 6 and 8. The daughter cells were diluted at the end of the dark period again to 4×10^6 cells/ml, or 1 cell/smallest square unit, and the next cycle of light and dark was started. Two generations of such a light-dark-dilution cycle were in most cases enough to bring a partially synchronized culture to complete synchronization. The algal cells were examined microscopically and classified, as described in Results and Discussion.

Chemicals:

All of the chemicals used in this work were standard reagent grade. Authentic compounds for chromatography were calcium, gluconate,

fumaric, maleic, and malonic acids as obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio); dihydroxymaleic and dihydroxytartaric acids from Aldrich Chemical Co. (Milwaukee, Wisc.); DL-isocitric lactone, (+) tartaric, and meso-tartaric acids from Calbiochem (Los Angeles, Calif.); oxalic acid from J. T. Baker Chemical Co. (Phillipsburg, N. J.); tartronic acid from General Biochemicals (Chagrin Falls, Ohio); and mesoxalic acid prepared by N. E. Tolbert.

Organic solvents used for paper chromatography were n-butyl alcohol, ethyl acetate, and 88% phenol (without preservative for chromatographic purposes) from Mallinckrodt Chemical Works (New York, N. Y.); formic acid from J. T. Baker Chemical Co.; and propionic acid from Eastman Organic Chemicals. Enzymes used in this work were acid phosphatase, glutamic oxalacetic transaminase, and isocitric dehydrogenase from Sigma Chemical Co. (St. Louis, Missouri); alkaline phosphatase from Nutritional Biochemicals Corp.; and phosphoglycolate phosphatase from Donald Anderson. Bio-Rad Laboratories (Richmond, Calif.) supplied anion exchange resin AG1 x 8 (100-200 mesh, Cl-form) and cation exchange resin AG50W x 8 (100-200 mesh, H-form).

Chemicals used for gas-liquid chromatography were pyridine from Eastman Organic Chemicals; hexamethyldisilazane from Peninsular Chemresearch (Gainesville, Florida); trimethylchlorosilane from General Electric Co., Silicone Products Dept. (Waterford, N. Y.); and OV-1 and Gas-Chrom Z from Applied Science Laboratories (State College, Pa.). Those used in degradation experiments were mercuric chloride and sodium meta periodate from Fisher Scientific Co. (Fair Lawn, N. J.); potassium persulfate from Allied Chemical (Morristown,

N. J.); DL-serine from Nutritional Biochemicals Corp., silver nitrate from D. F. Goldsmith Chemical & Metal Corp. (Chicago, Ill.), barium hydroxide and barium chloride from J. T. Baker Chemical Co. Chemicals used for liquid scintillation counting were p-dioxane and phenethylamine from Eastman Organic Chemicals, naphthalene and xylene from J. T. Baker Chemical Co., toluene from Mallinckrodt Chemical Works, absolute ethyl alcohol from Commercial Solvents Corp. (Terre Haute, Ind.); and α-NPO (α-naphthylphenyloxazole), POPOP (1,4-bix-2-(5-phenyloxazolyl)benzene) and PPO (2,5-diphenyloxazole) from Packard Instrument Co. (Downers Grove, Ill.). Other chemicals used were: TES (N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid) from Calibiochem, manganous chloride from J. T. Baker Chemical Co., NADP from Sigma Chemical Co., DL-aspartic acid from Nutritional Biochemicals Corp., and CMU (3-(p-chlorophenyl)-1,1-dimethylurea) from E. I. DuPont DeNemours & Co., Wilmington, Del.

Radioactive compounds used in this work were $Ba^{14}CO_3$ received from either Oak Ridge National Laboratories (Oak Ridge, Tenn.) or New England Nuclear Corp. (Boston, Mass.), orthophosphoric-^{32}P acid from Tracerlab (Waltham, Mass.), benzoic-^{14}C acid from Packard Instrument Co., oxalic-U-^{14}C acid from Volk Radio Chemical Co. (Chicago, Ill.); and calcium salts of glycolic-1-^{14}C, glycolic-2-^{14}C, and phosphoglycolic-^{14}C acids from Orlando Research Inc. (Orlando, Florida). Solutions of $NaH^{14}CO_3$ for photosynthetic experiments were prepared by generating $^{14}CO_2$ gas from $Ba^{14}CO_3$ by the addition of lactic acid in one arm of an evacuated, Y-shaped apparatus, and capturing the $^{14}CO_2$ gas in a calculated amount of an NaOH solution in the other arm of the apparatus. The specific activity of the

$NaH^{14}CO_3$ was generally 50%, and the final solution always contained 1 μc ^{14}C/2 μl.

Photosynthetic experiments:

The normal photosynthetic experiments were similar to those of Bassham and Calvin (6). The algal cells at a designed stage of their life cycle were centrifuged from the medium at 1,000 x g for 5 min. The packed cells were washed once by resuspending in a small volume (usually less than 10 ml) of distilled water in a graduated centrifuge tube and centrifuged again at 1,000 x g for 5 min. The packed volume was recorded and the cells were then resuspended in a volume of 0.001 M phosphate buffer at pH 6.0, so that a final suspension of 1% (v/v) algal cells was obtained.

Small scale (5 to 20 ml) and medium scale (20 to 80 ml) photosynthetic experiments were performed in a "lollipop" (13) of the corresponding volume. White light of 3,000 ft-c was obtained from two 300-watt KEN-RAD reflector flood lamps, positioned perpendicular to the plane of the lollipop, one on each side. The lollipop was placed in a water bath maintained at 20°. Five-liter diphtheria toxin culture bottles, filled with distilled water, were placed between the lamps and the water bath in order to absorb excess heat from the lamps.

After 5 min. preillumination and aeration by a stream of air, 50 μl of $NaH^{14}CO_3$ solution per 10 ml of algal suspension was injected at zero time and the lollipop was closed and briskly shaken in the light path. The $NaH^{14}CO_3$ solution contained 1 μc ^{14}C per 2 μl, and the percent ^{14}C varied between 25 and 50%. Aeration of an Ankistrodesmus

suspension was not possible because severe foaming forced the cells to flood out of the lollipop, but instead, the lollipop was occasionally shaken in the light path in order to keep the cells from settling. When the photosynthetic experiments were to run longer than 5 min., another aliquot of the $NaH^{14}CO_3$ solution was added either at zero time or after 5 min. of photosynthesis.

At various time intervals as designed, 2 to 4-ml aliquots of the suspension after shaking were taken with a pipette and quickly filtered through a Millipore filter (AAWP-025) with suction. The cells on the filter were immediately rinsed under continuous suction with 1 ml of distilled water. The entire process of filtration and washing was usually completed within 15 sec. The filtrate and the washing were combined in a graduated test tube, acidified with 1 or 2 drops of glacial acetic acid, aerated for 10 min. with $^{12}CO_2$ gas, made up to a certain volume by adding water, and then thoroughly mixed. An aliquot of less than 0.5 ml of this solution, called "supernate," was counted in 10 ml of Kinard's liquid scintillator (44) with a Packard Tri-carb Liquid Scintillation Spectrometer, Model 3310. This liquid scintillator, suitable for counting aqueous solutions, was made by dissolving 0.1 g α-NPO, 10 g PPO, and 160 g naphthalene in a mixture of 770 ml xylene, 770 ml p-dioxane, and 462 ml absolute ethyl alcohol. The total ^{14}C-activity excreted was calculated as cpm/ml algal suspension. The remaining portion of the supernate was evaporated to dryness with a shaking evaporator (Buchler's Rotary Evapo-Mix) at 35-38° under reduced pressure, and the residue was analyzed by paper chromatography and radioautography.

The washed cells on the filter were immediately transferred

into a beaker containing about 5 ml of boiling 80% methanol, the extract filtered through a Millipore filter, and the broken cells washed twice with 2 to 3 ml of boiling 80% methanol. The filtrate and the washing were combined in a graduated test tube, acidified with glacial acetic acid, aerated with $^{12}CO_2$, made up to a certain volume (usually 10 ml) and thoroughly mixed. A 0.1 or 0.2-ml aliquot of the solution, called "cell-extract," was counted in 10 ml of Kinard's liquid scintillator. The ^{14}C-activity in the cell-extract was also calculated as cpm/ml algal suspension, and the total ^{14}C-fixation was then calculated by summation of the ^{14}C-activity excreted and in the cell-extract. It is obvious, therefore, that this figure of total ^{14}C-fixation does not include the fixation into compounds insoluble in 80% methanol, such as some proteins or polysaccharides. An adequate aliquot of the cell-extract was evaporated to dryness and analyzed by paper radioautography, as described for the supernate.

Paper chromatography and radioautography:

Two-dimensional paper chromatography was carried out according to the procedures described by Benson, et al. (7). The solvent systems were water-saturated phenol (made by mixing 4 vol. of 88% phenol with 1 vol. of water) for the first direction and butanol-propionic acid-water (freshly made by mixing equal volumes of butanol-water (1,246 ml:84 ml) and propionic acid-water (620 ml:790 ml) for the second direction. In many cases the second solvent system was modified, according to Hartley and Lawson (28), by adding 0.1 ml of 6 N NaOH to 100 ml of the solvent mixture. This modification gave preferential separation of particular compounds and better visualiza-

tion on paper of acid spots of lower R_f values by bromcresol green spray (0.04% solution in 95% ethanol).

Another solvent system, which was used for one-dimensional separation of various organic acids, consisted of butanol-ethyl acetate-formic acid (1:1:1, by vol.) as reported by Schramm (69).

Whatman No. 1 chromatographic paper was used throughout these experiments. After drying overnight in the air, the approximate glycolic acid area on the chromatograms was sprayed with 0.1 M Na_2CO_3 solution in 50% ethanol in order to prevent sublimation of the free acid. Cochromatography on paper was performed by mixing the radioactive sample with a nonradioactive, authentic acid in a test tube, evaporating the mixture to dryness, and spotting the residue on the origin as usual.

Radioautograms were made by exposing the dried paper chromatograms to "Kodak Medical X-ray Film, Blue Brand" from Eastman Co., Rochester, N. Y. After an appropriate period (3 days to 2 weeks) of exposure, the films were developed to locate radioactive spots on the chromatograms. The radioactive areas were then counted with a thin window (DuPont Mylar film) gas flow counter, using a Nuclear Chicago Scaler, Model 161A. Helium, passing through ethanol which was cooled with an ice bath, provided the gas for the counting chamber.

Anion exchange chromatography:

Analyses of organic acids by anion exchange chromatography were performed by a modification of the method described by Palmer (61). The anion exchange resin, AG1 x 8, Cl-form, was converted to the acetate form and packed to about 8 cm high in a cylinder of 0.4 cm inner diameter and 12 cm in length. The total volume of the resin

bed was about 1 ml, which was approximately equivalent to an exchange capacity of 1.4 milliequivalent.

After thorough washing with deionized water a radioactive sample solution was introduced onto the column, which was again washed with deionized water until no radioactivity could be detected by liquid scintillation counting in the effluent. Fractional elution of the organic acids from the resin column was effected by the use of a pH-gradient provided by a simple apparatus described by Palmer (61). The gradient elution was initiated by introducing 3 N acetic acid from a reservoir flask into a mixing flask containing 200 ml of deionized water. A constant pressure of 3 pounds per square inch was applied to the reservoir flask in order to maintain a flow rate of 1-2 ml/min., and the effluent was collected in 4-ml fractions with a Gilson fraction collector. After 500 ml of 3 N acetic acid had been used, the reservoir was refilled with 3 N formic acid, leaving the acetic acid solution in the mixing flask, and the gradient elution was continued.

The peaks of radioactive acids in the effluent were first approximately located by counting 0.1 or 0.2-ml aliquots of every other tube in 10 ml of Kinard's liquid scintillator, and then precisely located by counting every tube around each peak. The radioactive compounds contained in each peak were collected by evaporating the combined solution to dryness with a shaking evaporator, and were then analyzed by paper radioautography.

Gas-liquid chromatography:

Gas-liquid chromatography of organic acids as their trimethylsilyl (TMS) derivatives was performed by the method of Sweeley, et al. (83). An F & M Scientific 402 high efficiency gas chromatograph

(Hewlett-Packard, F & M Scientific Division, Avondale, Pennsylvania) equipped with a 3% OV-1 column (6 ft x 0.25 inches) coated on Gas-Chrom Z, 80-100 mesh, was used. The carrier gas, Argon, was passed through the column at an optimal flow rate of approximately 50-60 ml/min., and the effluent gas was split with a device into two streams, one leading to a hydrogen flame ionizing detector and the other to a collection port (18), which enabled the collection of fractions of the TMS derivatives by inserting a long-tip, disposable pipette.

A trimethylsilylating reagent (96), made by mixing in order 1.7 ml anhydrous pyridine, 0.2 ml hexamethyldisilazane, and 0.1 ml trimethylchlorosilane, was used for trimethylsilylation of organic acids. One-tenth ml of the fresh reagent was added to 0.1-1.0 mg of authentic organic acids, or 1,000-100,000 cpm of unknown organic acids which had been collected from an anion exchange column and dried overnight in a centrifuge tube over KOH pellets in a vacuum desicator. The mixture was shaken vigorously for about 30 sec. and allowed to stand for 5 min. or longer at room temperature, and then a 1 to 30-µl aliquot was injected with a microsyringe into the column for chromatography. The chromatography was ran either by linear temperature-programming of 5°/min. starting at 100°, or under isothermal conditions at a desired temperature for better resolution (83). While peaks of TMS derivatives in the effluent were located by an automatic recorder connected to the hydrogen flame ionization detector, the major portion of the radioactive derivatives was collected at the collection port in fractions. The radioactivity from each collection was dissolved in 10 ml of Kinard's liquid scintillator and counted.

Cochromatography was accomplished by mixing a radioactive acid sample with a nonradioactive, authentic acid in a centrifuge tube, drying, trimethylsilylating, and then chromatographing the TMS derivatives. Fractional collection of the radioactive TMS derivative was programmed in accordance with the appearance of the peaks due to the nonradioactive derivatives recorded on a recording chart.

Degradation of ^{14}C-organic acids:

The distribution of ^{14}C in the molecules of ^{14}C-organic acids, purified either by paper chromatography or by anion exchange chromatography, was determined by a modified technique (14) of Sakami's procedures (66) for the degradation of serine, which gave 1 mole each of CO_2, HCOOH, and HCHO on periodate oxidation at room temperature. The degradation vessel was a 50 ml, 3-necked, pear-shaped flask fitted with a separatory funnel for adding reagents, a condenser with an attached CO_2 trap, and an inlet tube for aeration. The degradation followed three separate steps as outlined below.

The first step was cleavage of serine into 1 mole each of CO_2, HCOOH, and HCHO by periodate oxidation at room temperature. To the flask were added 1,000-10,000 cpm of the ^{14}C-organic acid in less than 1 ml water, 0.2 mmole (21 mg) of nonradioactive DL-serine in 1 ml water as a carrier, and 2 ml of 0.5 M phosphate buffer at pH 5.8. Then, to the closed system, 0.75 mmole (160 mg) of $NaIO_4$ in 3 ml water was introduced through the funnel and the system was aerated at room temperature for an hour. Most of the excess $NaIO_4$ was consumed by reaction with additional 0.2 mmole of carrier serine during another hour of aeration at room temperature.

The second step was oxidation of HCOOH to CO_2 by boiling with

$HgCl_2$. After adding 3 ml of 1.5 M phosphate buffer at pH 2.5 to the flask, the system was closed and 1 g of $HgCl_2$, dissolved in 5 ml of hot water, was introduced through the funnel. The system was then aerated for an hour while boiling gently. The last step was the oxidation of HCHO, as well as all other carbon compounds, to CO_2 by boiling with $K_2S_2O_8$. After adding 1 ml of 5% $AgNO_3$ solution to the flask, the system was closed and 1.5 g of $K_2S_2O_8$, dissolved in 5 ml of hot water, was introduced through the funnel. The system was first aerated for 45 min. with gentle boiling, and then aeration continued 30 min. longer with vigorous boiling.

In some cases, for the determination of the ^{14}C-activity in HCHO formed by periodate oxidation, the second step was modified by eliminating the second addition of 0.2 mmole carrier serine to decompose the excess $NaIO_4$. Instead, the closed system was heated in the presence of the excess $NaIO_4$ which oxidized both HCOOH and HCHO to CO_2. The increase in $^{14}CO_2$-yield in this step over that in the normal $HgCl_2$-oxidation step was considered as the ^{14}C-activity due to $H^{14}CHO$. ^{14}C-Activity not recovered after this step was attributed to carbon atoms in molecules other than CO_2, HCOOH, and HCHO.

During each step of the degradation, CO_2 evolved was carried by aeration through the condenser and introduced into a U-tube with a sintered glass filter fused at the middle. The U-tube was filled with 5 ml of CO_2-trapping solution (100) which was prepared as follows. Five grams of PPO and 100 mg of POPOP were dissolved in a solution of 270 ml redistilled phenethylamine (b.p. 65-67° at 6 mm Hg pressure) and 270 ml absolute methanol, and then diluted to 1 liter with toluene and stored in the dark. After each step of the

degradation procedure the trapping solution was carefully transferred to a counting vial, and the trap was rinsed twice with 5 ml of scintillation fluid (100), made by dissolving 5 g PPO and 100 mg POPOP in 1 liter of toluene. The final volume of the counting solution in the vial was approximately 15 ml. The sum of radioactivity in cpm obtained through the three steps of degradation was calculated as 100%, and the % distribution of ^{14}C in CO_2, HCOOH, HCHO, and other carbon compounds was calculated on this basis.

Determination of specific activities of carbon atoms:

Determination of the specific activity of carboxyl (i.e., 1 and 4) and the middle (i.e., 2 and 3) carbon atoms of meso-tartaric acid molecules was performed essentially according to the method of Van Slyke, et al. (89). The first step of the degradation by periodate oxidation was the same as that described for serine degradation, except that 0.2 mmole (33.6 mg) of nonradioactive meso-tartaric acid was used in place of serine as the carrier. An important difference was that the closed system had been flushed with N_2 gas for 15 min. before periodate was introduced into the flask, and instead of aeration, the N_2 gas was continuously passed through during the entire process. The decomposition of excess $NaIO_4$ by the second addition of nonradioactive carrier was omitted. The second step of the degradation by $HgCl_2$-oxidation was the same except that N_2 gas was continuously passed through the closed system. The CO_2 evolved in each step was trapped in a known volume and concentration of a $Ba(OH)_2$-$BaCl_2$ solution (88). The recovery of CO_2 in each step of the degradation was calculated by back-titrating the $Ba(OH)_2$-$BaCl_2$ trap with a standardized HCl solution. Planchets were then prepared from the

$BaCO_3$ suspensions and, after drying to a constant weight, the radioactivities were determined with a Nuclear Chicago low background gas flow counter, Model C 115. The specific activity of each carbon was calculated by the following equation:

$$A = \frac{N_s}{FN_k} \times \frac{\text{mg C in sample + mg C in blank}}{\text{mg C in sample}}$$

where A gives specific activity in mμc./mg C; N_s is observed sample count; F, "infinite thickness" factor, determined from a self-absorption curve (65); and N_k, the counter constant, determined from a $BaCO_3$ sample produced by a total combustion of standard benzoic-^{14}C acid.

Large-scale preparation of unknown acids excreted by A. braunii:

Fully mature or dividing cells of A. braunii were collected by centrifugation from a synchronized culture between the 3rd and the 4th hour of the dark period. They were washed and resuspended in an enough volume of 0.001 M phosphate buffer to give a final concentration of 0.5-0.8% algal cells. A photosynthetic experiment in $NaH^{14}CO_3$ was run with 80 ml of the suspension in a medium-size lollipop, and a large-scale experiment using air but no $^{14}CO_2$ was carried out in parallel in a "high form" Fernbach flask containing 200-400 ml of the suspension. The flask was placed between two diphtheria toxin culture bottles filled with water for absorption of heat, and illumination by two 300-watt KEN-RAD lamps, one from each side, was continued for 30 min. while the suspension was slowly aerated with air and occasionally shaken.

After 30 min. the supernates were collected separately by centrifugation, then filtered through a Millipore filter and stored at -18°. A 2 to 4-ml portion of the radioactive supernate was evaporated to dryness and analyzed by paper radioautography. In case the ^{14}C-activity in the unknown acids matched or exceeded that in glycolic acid, the remaining portion of the radioactive supernate was combined with the larger volume of the nonradioactive supernate, and the mixture was evaporated to a small volume with a rotary evaporator (Buchler's Flash Evaporator). The concentrated supernate was then passed through a column of cation exchange resin, AG50 in H-form, and the column was thoroughly washed with deionized water. The eluate and the washing were combined in a test tube and again evaporated to a small volume with a shaking evaporator. The concentrate was neutralized and introduced onto a column of anion exchange resin, AG1 in acetate form, and then chromatographic separation of the acids was carried out by pH-gradient elution as described before. Radioactive acids in each peak were analyzed by paper radioautography, and peaks containing the unknown acids were saved for further purification by repeated anion exchange chromatography.

RESULTS AND DISCUSSION

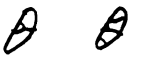
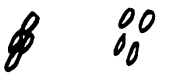
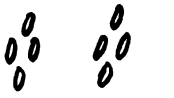
Photosynthetic CO_2-fixation and excretion by synchronized algal cells during their life cycle:

The life cycle of A. braunii cells, kept synchronous by a 16-hr light and 8-hr dark regimen with 1 to 4 dilution at the end of each dark period, is depicted in Table 1. Along the life cycle, the algal cells were collected at different stages of development, and 5-min. photosynthesis and excretion experiments were carried out as described in Methods. The changes in photosynthetic activity, as measured by $^{14}CO_2$-fixation and ^{14}C-excretion for 5 min., are shown in Figure 1 and also summarized in Table 1.

The photosynthetic ability, measured by $^{14}CO_2$-fixation and expressed as ^{14}C-activity fixed per unit volume of cell suspension, followed a distinct trend: growing cells were more active than mature cells, and mature cells more active than dividing cells. Since the amount of $^{14}CO_2$-fixation at the peak of the activity, i.e., at 6 and 10-hr stages in the light period, exceeded 90% of the $NaH^{14}CO_3$ added, the real photosynthetic ability at these stages was probably even higher than the values in Figure 1. A similar trend for change in photosynthetic ability as measured either by O_2-evolution, CO_2-fixation, or relative quantum efficiency during the life cycle of synchronized algal cells has been reported by Sorokin and his coworkers for Chlorella species (71, 72, 74), and by Kates and Jones for Chlamydomonas reinhardtii (40). Sorokin (73), with

TABLE 1

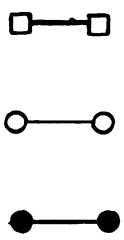
A Life Cycle of A. braunii Cells in A Synchronous Culture and Their Relationship to Photosynthetic Activity

Stage of Life Cycle	Morphological Observation		Photosynthetic Activity*
	Pictorial Drawing	Description	
Light, 0 hr		Daughter cells; not growing	Low, increasing
Light, 2 hr		Fast growing cells	Higher, increasing
Light, 6 hr		Growing cells; cannot divide if put in dark	Highest, still increasing
Light, 10 hr		Premature cells; can hardly divide if put in dark	Highest, started decreasing
Light, 14 hr		Mature cells; ready to divide	Low, decreasing
Light, 16 hr (Dark, 0 hr)		Fully mature cells; starting to divide	Lower, decreasing
Dark, 2 hr		Dividing cells; daughter cells not yet released	Lower, still decreasing
Dark, 4 hr		Dividing cells; starting to release daughter cells	Lowest
Dark, 6 hr		Dividing cells; still releasing daughter cells	Low, increasing
Dark, 8 hr (Light, 0 hr)		Daughter cells; not growing	Low, increasing

*As measured both by $^{14}CO_2$-fixation and ^{14}C-excretion. (For details see Figure 1.)

FIGURE 1

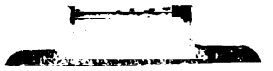
Changes in the Activity of Photosynthetic $^{14}CO_2$-Fixation and ^{14}C-Excretion During the Life Cycle of A. braunii

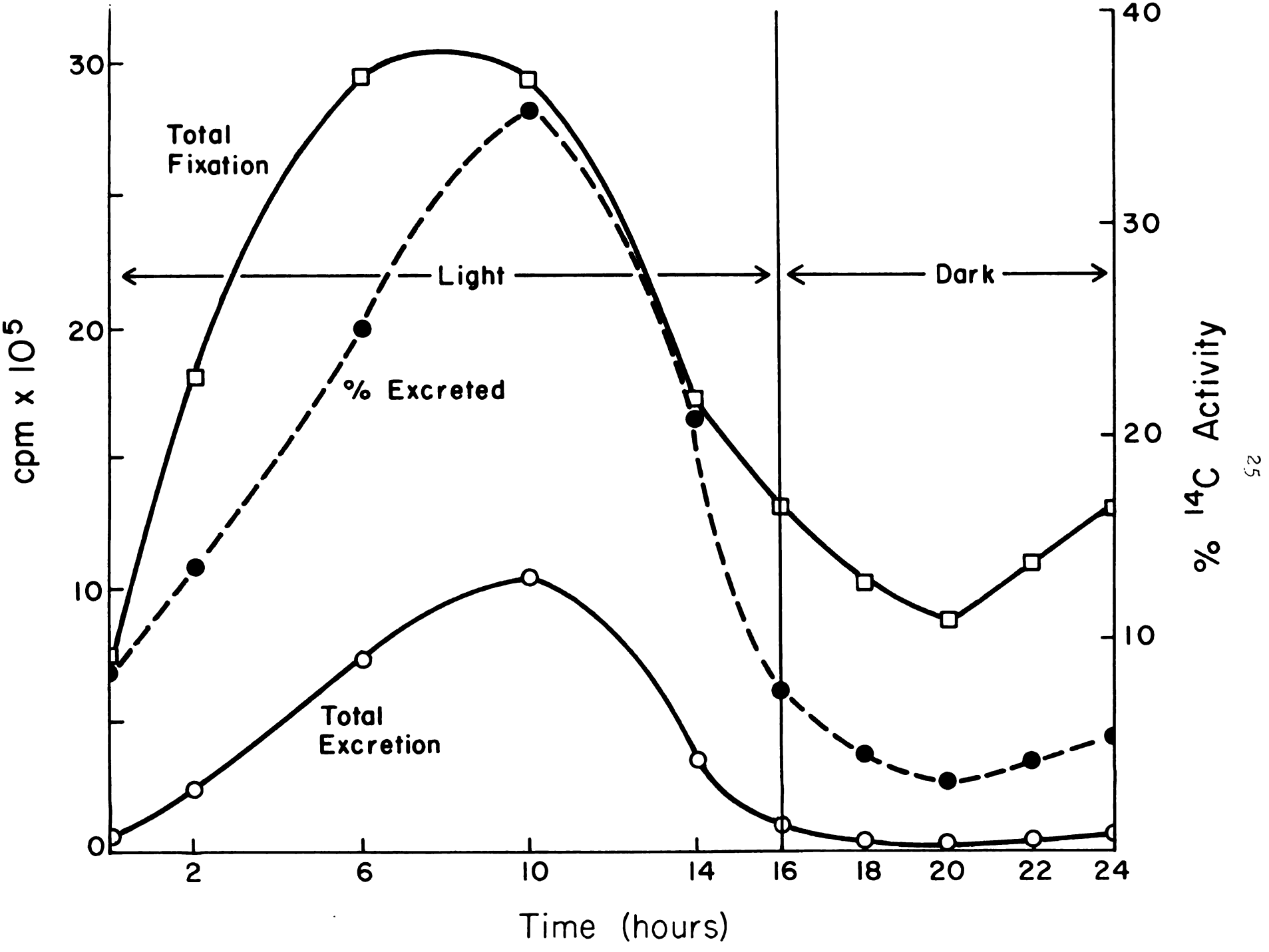


^{14}C-Activity fixed in 5 min./ml 1% cell suspension.

^{14}C-Activity excreted in 5 min./ml 1% cell suspension.

% ^{14}C excreted of the total ^{14}C fixed.




Total Fixation
% Excreted
Total Excretion
Light
Dark
cpm x 10⁵
% ¹⁴C Activity
Time (hours)
0
10
20
30
40
2
6
10
14
16
18
20
22
24

the use of younger (small) and older (large) cells of Chlorella 7-11-05 which had been separated from a nonsynchronized suspension by fractional centrifugation, also showed that younger cells invariably possessed higher photosynthetic activity than older cells. It has been concluded that the decline in photosynthetic activity with the age of synchronized cells must be assumed to be characteristic of normal cell development.

However, there have been different reports by Spektorov, et al. (75) for Chlorella pyrenoidosa Pringsh. 82 and by Cook (15) for Euglena gracilis, in which no decrease in photosynthetic activity of the mature or dividing cells was observed. This difference has been attributed by Spektorov, et al. (75) to the fact that different species of algae exhibited different degrees of light-inhibition particularly of the photosynthetic process, not only during the division processes but also during the entire life cycle of the cells. It is noteworthy that, in the present experiments, the daughter cells had recovered some photosynthetic capability at the end of the dark period, although they were not yet growing (Figure 1).

No correlation of the ^{14}C-activity excreted during photosynthesis by algal cells at different stages of development has been previously reported. As shown in Figure 1, photosynthetic excretion of ^{14}C-activity approximately followed the same trend as the change in photosynthetic $^{14}CO_2$-fixation during the developmental stages. The two curves were not quite parallel, since the peak of the curve for photosynthetic excretion appeared a little later than that of photosynthetic activity. This trend for photosynthetic excretion was more clearly shown when the ^{14}C excreted was plotted as a % of the

total ^{14}C fixed. The results imply that the process of photosynthetic excretion, or the compounds excreted, must be closely related to the photosynthetic processes.

Compounds excreted during photosynthesis:

The supernates from 5-min. photosynthetic experiments were analyzed by paper chromatography and radioautography. Representative radioautograms were presented in Figure 2. The compounds excreted by synchronized cells of A. braunii were different at various stages of the life cycle. Primarily, only glycolate was excreted in a large amount by the growing young cells. Mature and dividing cells excreted as many as seven compounds, i.e., glycolate, glycerate, malate, citrate, and three unknown compounds designated by U_1, U_2 and U_3, as shown in Figure 2-B. Glycolate was always the major acid excreted, but dividing cells excreted a large amount of U_2 (Table 2). U_2 tended to form double spots as seen in Figure 2, B and C. The separation of U_3 from U_2 was much improved by the addition of a small amount of NaOH to the second solvent system, butanol-propionic acid-water, as recommended by Hartley and Lawson (28). Glycerate and citrate were seen only occasionally and rather irregularly, and only small amounts of radioactivity as glycerate, malate and citrate were excreted in comparison to glycolate. By comparing the chromatograms of supernates (Figure 2, A and B) with those of cell-extracts (Figure 2, D and E) of the same experiment, it is noted that among the seven compounds excreted, only glycerate, malate and citrate existed in considerable amounts in cell-extracts. The other four compounds (glycolate, U_1, U_2, and U_3) were present almost exclusively in the supernates and therefore seemed to be specifically excreted. The identification of these

FIGURE 2

Radioautograms of Supernates and Cell-Extracts After 5-min. Photosynthesis in $NaH^{14}CO_3$ by Synchronized Cells of *A. braunii*

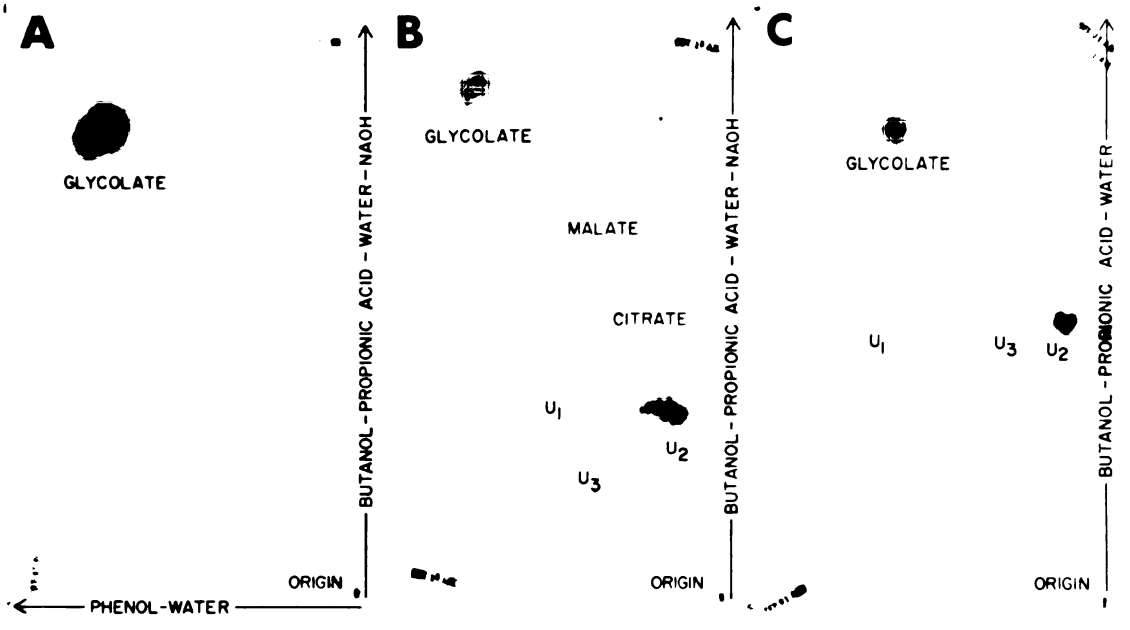
A: Supernate from growing cells (light, 10 hr).

B: Supernate from dividing cells (dark, 4 hr).

C: The same as B, except that NaOH was omitted from the second solvent.

D: Cell-extract of growing cells (light, 10 hr).

E: Cell-extract of dividing cells (dark, 4 hr).


A
GLYCOLATE
BUTANOL - PROPIONIC ACID - WATER - NAOH
ORIGIN
PHENOL - WATER
B
GLYCOLATE
MALATE
CITRATE
U1
U2
U3
BUTANOL - PROPIONIC ACID - WATER - NAOH
ORIGIN
C
GLYCOLATE
U1
U3
U2
BUTANOL - PROPIONIC ACID - WATER
ORIGIN

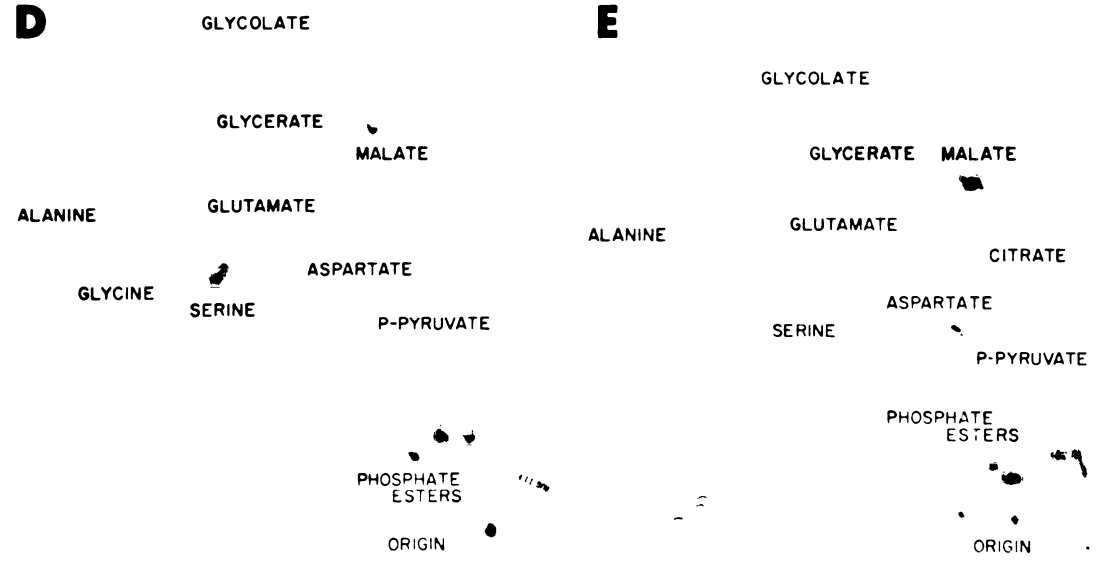

D
GLYCOLATE
GLYCERATE
MALATE
ALANINE
GLUTAMATE
ASPARTATE
GLYCINE
SERINE
P-PYRUVATE
PHOSPHATE ESTERS
ORIGIN
E
GLYCOLATE
GLYCERATE
MALATE
ALANINE
GLUTAMATE
CITRATE
ASPARTATE
SERINE
P-PYRUVATE
PHOSPHATE ESTERS
ORIGIN

TABLE 2

Amount and % Distribution of ^{14}C in the Compounds Excreted During 5-min. Photosynthesis in $NaH^{14}CO_3$ by Synchronized Cells of A. braunii. Values are cpm x 10^3/ml 1% cell suspension; in parentheses are the % of the total ^{14}C excreted.

Stage of Life Cycle	Compound Excreted					
	Glycolate	Malate	Isocitric Lactone (U_1)	meso-Tartrate (U_2)	U_3	Total
Light, 0 hr	38.9 (62.7)	1.7 (2.7)	0.2 (0.3)	20.7 (33.5)	0.5 (0.8)	62.0
Light, 2 hr	235.4 (96.1)	0.3 (0.1)	0	9.3 (3.8)	0	245.0
Light, 6 hr	735.1 (99.2)	0	0	5.9 (0.8)	0	741.0
Light, 10 hr	1,044.0 (100.0)	0	0	0	0	1,044.0
Light, 14 hr	343.3 (96.7)	0.3 (0.1)	0	11.4 (3.2)	0	355.0
Light, 16 hr (Dark, 0 hr)	80.5 (79.7)	1.0 (1.0)	0	19.0 (18.8)	0.5 (0.5)	101.0
Dark, 2 hr	28.3 (59.0)	0.8 (1.6)	0.1 (0.2)	18.5 (38.6)	0.3 (0.6)	48.0
Dark, 4 hr	10.9 (37.8)	1.1 (3.8)	0.6 (2.0)	14.2 (48.9)	2.2 (7.5)	29.0
Dark, 6 hr	23.6 (49.1)	1.5 (3.2)	0.5 (1.0)	20.9 (43.5)	1.5 (3.2)	48.0
Dark, 8 hr (Light, 0 hr)	42.8 (59.4)	2.1 (2.9)	0.5 (0.7)	25.3 (35.1)	1.3 (1.9)	72.0

excreted compounds and elucidation of the physiological conditions responsible for their excretion have been a major effort to be reported in this thesis. In subsequent sections will be given results for the identification of U_1 as isocitric lactone and of U_2 as meso-tartrate. U_3 remains unidentified.

The radioactivity of all the spots on a radioautogram of a supernate was added to make 100%, and the % distribution of radioactivity among the compounds excreted was computed on this basis (Table 2). The actual radioactivity in cpm of each compound excreted by 1 ml cell suspension was then calculated from the % distribution and the respective amount of total ^{14}C-excretion as shown in Figure 1. It is obvious from the data shown in Table 2 that maximum amount of glycolate was excreted by the rapidly growing cells and that mature or dividing cells excreted much less glycolate. Maximum amounts of the other five compounds were excreted by mature or dividing cells, or in other words, the excretion of the other five compounds was approximately in a reciprocal relationship to glycolate excretion. This relationship is also shown in Figure 3 by plotting along the life cycle the change in the ^{14}C-activity in glycolate excreted, together with that of total excretion. Thus total ^{14}C-activity in the other five compounds is represented by the difference of the two curves, i.e., the shaded area in the Figure. The two different patterns of photosynthetic excretion are further clarified in Figure 4 by plotting along the life cycle the change in % distribution of ^{14}C among the compounds excreted. Figure 4 emphasizes that the curve for glycolate excretion is complementary to that for meso-tartrate excretion. Excretion of U_3 followed the same trend as that for meso-tartrate, but the results were less clear,

FIGURE 3

^{14}C-Activity in the Compounds Excreted During 5-min. Photosynthesis in $NaH^{14}CO_3$ by Synchronized Cells of A. braunii

Total excretion in cpm/ml 1% cell suspension.

Glycolate excretion in cpm/ml 1% cell suspension.

Shaded area: Excretion of glycerate, malate, U_1, U_2 and U_3.

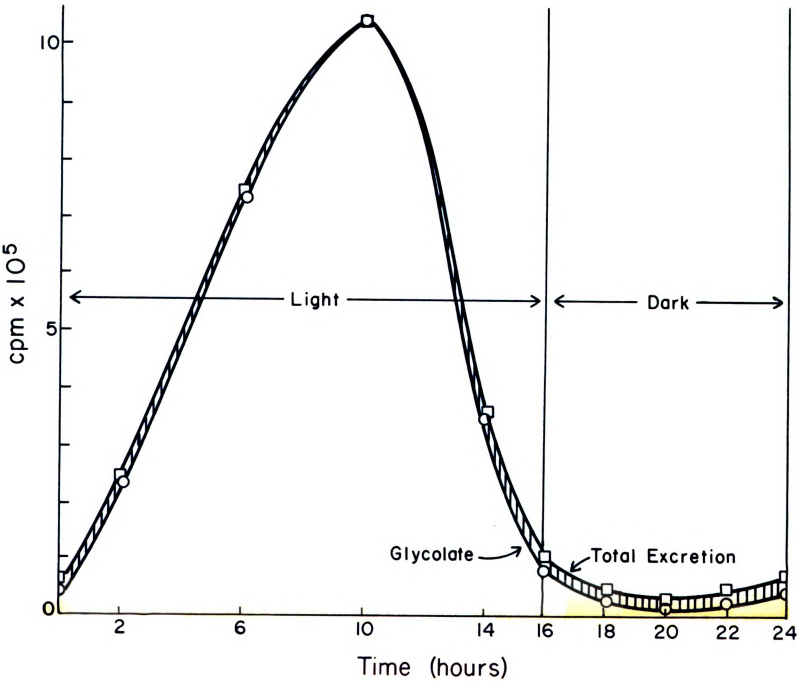
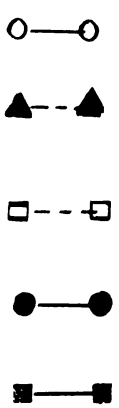

10
5
0
cpm x 10^5
Light
Dark
Glycolate
Total Excretion
2
6
10
14
16
18
20
22
24
Time (hours)

FIGURE 4

% Distribution of ^{14}C Among the Compounds Excreted During 5-min. Photosynthesis in $NaH^{14}CO_3$ by Synchronized Cells of A. braunii



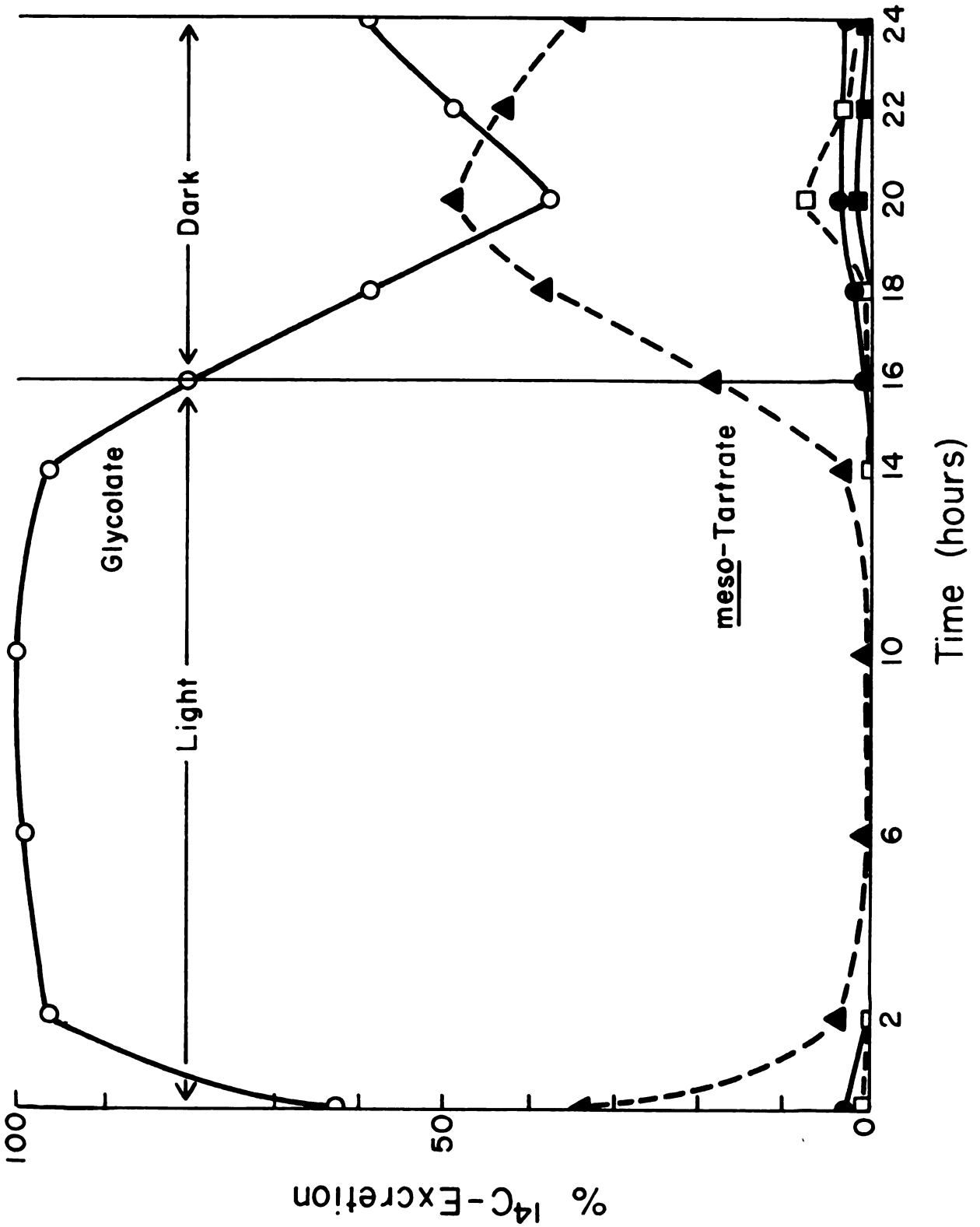
% ^{14}C-excretion in glycolate.

% ^{14}C-excretion in meso-tartrate (U_2).

% ^{14}C-excretion in U_3.

% ^{14}C-excretion in malate.

% ^{14}C-excretion in isocitric lactone (U_1).


% ^{14}C-Excretion
100
50
0
2
6
10
14
16
18
20
22
24
Time (hours)
Light
Dark
Glycolate
meso-Tartrate

perhaps because of less formation during 5-min. photosynthesis.

Rates of photosynthetic $^{14}CO_2$-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 maximum by growing cells and minimum by dividing cells. However, glycolate was the only major compound excreted by these algae throughout their life cycle, and no formation in appreciable amounts of the unknown compounds by mature or dividing cells was observed.

Since the synchronous algal cultures were not strictly sterile, and some bacterial cells were sometime observed microscopically, there existed the possibility that the bacterial growth might have been favored during the dark period, and that the bacteria might have changed the amount of glycolate excreted by mature or dividing algal cells in relation to the unknown compounds. This possibility, however, was eliminated by two facts. First, mature or dividing cells of A. braunii fed either glycolate-^{14}C or phosphoglycolate-^{14}C in the light or dark failed to metabolize the radioactive compounds. Secondly, mature or dividing algal cells of other species grown under the same circumstances and conditions did not excrete significant amounts of the unknown compounds during 5-min. photosynthesis in $NaH^{14}CO_3$. It was therefore concluded that these compounds in the supernates were formed and excreted by the algal cells, that glycolate was the major excretion product of all the algae, and that excretion and accumulation of large amounts of the other compounds were characteristic of mature or dividing cells of A. braunii. However, conditions for detecting the excretion of the unknown compounds by the other algae

have not been carefully studied.

Kinetic studies of the photosynthetic excretion:

Kinetic studies were performed to examine further the mode of formation and excretion of those compounds during photosynthesis, and to establish the best conditions for isolation of enough of the unknown compounds for identification. During the course of the standard photosynthetic experiments in $NaH^{14}CO_3$ with A. braunii cells at different developmental stages (as described in Table 3), 2-ml portions of the cell suspension were removed after 1, 2, 5, 10 and 30 min. The supernates were separated with Millipore filters and analyzed both for total ^{14}C-activity by scintillation counting and for radioactive components by paper radioautography. The % distribution of ^{14}C among those compounds excreted at each stage of development was computed from the respective radioautogram, as described before, and then the actual ^{14}C-activity in cpm of each compound per ml cell suspension was calculated (Table 3).

In almost all cases, the amount of an excreted compound increased with the increasing period of photosynthesis. The rate of increase in amount, however, varied from one compound to the other, and thus there occurred different values for the % distribution of the excreted compounds during the course of photosynthesis. In most cases the percent of ^{14}C excreted as malate, glycerate, and meso-tartrate was the highest (as underlined in Table 3) within 1 or 2 min. of photosynthesis, while the total amount of excretion was still low. Isocitric lactone and U_3, in contrast, contained a consistently increasing percent of the ^{14}C excreted with increasing length of photosynthesis, implying that they

TABLE 3

Amount and % Distribution of ^{14}C in the Compounds Excreted During Different Periods of Photosynthesis in $NaH^{14}CO_3$ by Synchronized Cells of A. braunii. Values are cpm x 10^3/ml 1% cell suspension; in parentheses are the % of the total ^{14}C excreted.

Stage of Algae and Length of Synthesis	Compound Excreted						
	Glycolate	Malate	Glycerate	Isocitric Lactone (U_1)	meso-Tartrate (U_2)	U_3	Total
Dark, 0 hr							
1 min.	0.58 (46.8)	0.21 (16.5)	0.14 (11.4)	0	0.32 (25.3)	0	1.25
2 min.	1.36 (51.4)	0.26 (9.8)	0.22 (8.1)	0	0.81 (30.7)	0	2.65
5 min.	22.40 (80.7)	0.39 (1.4)	0.36 (1.3)	0.14 (0.5)	4.30 (15.5)	0.16 (0.6)	27.75
10 min.	50.63 (71.3)	0.85 (1.2)	0.92 (1.3)	0.92 (1.3)	9.80 (13.8)	7.88 (11.1)	71.00
30 min.	71.38 (13.2)	3.78 (0.7)	2.70 (0.5)	69.76 (12.9)	33.53 (6.2)	359.60 (66.5)	540.75
Dark, 3 hr							
1 min.	0	0.29 (25.0)	0	0	0.86 (75.0)	0	1.15
2 min.	0	0.32 (13.3)	0.19 (8.0)	0	1.73 (72.0)	0.16 (6.7)	2.40
5 min.	0.30 (5.1)	0.38 (6.3)	0.21 (3.6)	0.07 (1.1)	4.14 (69.6)	0.85 (14.3)	5.95
10 min.	0.58 (4.2)	0.46 (3.3)	0.23 (1.7)	0.30 (2.2)	9.54 (69.1)	2.69 (19.5)	13.80
30 min.	1.83 (1.2)	1.67 (1.1)	0.76 (0.5)	18.00 (11.8)	63.29 (41.5)	66.95 (43.9)	152.50

TABLE 3
(Continued)

Stage of Algae and Length of Synthesis	Compound Excreted						
	Glycolate	Malate	Glycerate	Isocitric Lactone (U_1)	meso-Tartrate (U_2)	U_3	Total
Dark, 5 hr							
1 min.	0	0.31 (24.1)	0	0	0.99 (75.9)	0	1.30
2 min.	0.10 (3.3)	0.35 (11.4)	0	0	2.60 (85.3)	0	3.05
5 min.	8.92 (48.2)	1.37 (7.4)	0	0	8.21 (44.4)	0	18.50
10 min.	27.72 (58.6)	2.03 (4.3)	0	0.24 (0.5)	16.51 (34.9)	0.80 (1.7)	47.30
30 min.	143.21 (72.9)	4.52 (2.3)	0.59 (0.3)	7.07 (3.6)	27.90 (14.2)	13.16 (6.7)	196.45
Dark, 8 hr (Light, 0 hr)							
1 min.	2.31 (44.5)	0.40 (7.6)	0	0	2.35 (45.2)	0.14 (2.7)	5.20
2 min.	6.50 (52.2)	0.46 (3.7)	0	0	5.12 (41.1)	0.37 (3.0)	12.45
5 min.	43.85 (61.5)	1.92 (2.7)	0	0.07 (0.1)	22.89 (32.1)	2.57 (3.6)	71.30
10 min.	106.19 (66.7)	3.82 (2.4)	0	0.95 (0.6)	41.55 (26.1)	6.69 (4.2)	159.20
30 min.	524.40 (73.9)	12.77 (1.8)	0	16.32 (2.3)	68.12 (9.6)	87.99 (12.4)	709.60

TABLE 3
(Continued)

Stage of Algae and Length of Synthesis	Compound Excreted						
	Glycolate	Malate	Glycerate	Isocitric Lactone (U_1)	meso-Tartrate (U_2)	U_3	Total
Light, 2 hr							
1 min.	3.12 (98.9)	0	0	0	0.03 (1.1)	0	3.15
2 min.	16.79 (98.2)	0.02 (0.1)	0	0	0.29 (1.7)	0	17.10
5 min.	173.84 (98.8)	0.35 (0.2)	0	0	1.76 (1.0)	0	175.95
10 min.	304.72 (99.0)	0.31 (0.1)	0	0	2.46 (0.8)	0.31 (0.1)	307.80
30 min.	1,041.85 (98.8)	1.05 (0.1)	0	0	4.22 (0.4)	7.38 (0.7)	1,054.50
Light, 8 hr							
1 min.	5.80 (91.4)	0.26 (4.1)	0	0	0.29 (4.5)	0	6.35
2 min.	229.69 (99.8)	0.23 (0.1)	0	0	0.23 (0.1)	0	230.15
5 min.	976.00 (99.8)	0	0	0	1.95 (0.2)	0	977.95
10 min.	1,323.17 (99.7)	0	0	0	3.98 (0.3)	0	1,327.15
30 min.	3,463.45 (99.0)	3.50 (0.1)	7.00 (0.2)	0	20.95 (0.6)	3.50 (0.1)	3,498.40

are later products of carbon metabolism.

The most complicated and probably of most interest were the patterns for the percent distribution of ^{14}C in glycolate during the course of photosynthesis. When the algal cells were rapidly growing and excreting glycolate in a large amount, i.e., at the stages of 2 and 8-hr of the light period (Table 3), glycolate contributed more than 98% of the total excretion throughout the 30-min. photosynthetic course. At the stages of 0 and 3-hr of the dark period, when the algal cells had just started dividing, the maximum percent of ^{14}C excreted in glycolate appeared only after 5-min. photosynthesis. This fact, together with the observation of the earlier appearance of the maximum percent of the ^{14}C-excretion in meso-tartrate and that the excretion of glycolate and meso-tartrate were complementary to each other (Figure 4), implies a possibility that meso-tartrate might be a metabolic precursor of glycolate, or that the two excreted acids might have a common photosynthetic precursor. During the early stages (0 and 3 hr) of the dark period, the maximum percent of ^{14}C excreted as glycolate occurred after 5 min. of photosynthesis. During the later stages (5 and 8 hr) of the dark period, however, it occurred after 10 to 30 min. of photosynthesis. This shift could be due to changes in the algal cells from one stage to the other even during the photosynthetic period, and it could also indicate that the daughter cells were recovering in the dark their capability of normal photosynthesis that the young, growing cells possess.

From the above results, the best condition for the formation of the largest amounts of the three unknown compounds (U_1, U_2 and U_3) was the use of mature or dividing cells of A. braunii at the early stages

(0 to 4 hours) of the dark period. Photosynthesis in $NaH^{14}CO_3$ should continue at least for 30 min. Thus, highest yields of the unknown ^{14}C-compounds with the least accompanying glycolate-^{14}C could be obtained with A. braunii cells from a well synchronized culture at an early stage of the dark period. From many experiments with well synchronized cultures kept for many generations, the percent yield of the unknown ^{14}C-compounds ranged from 30 to 70% of the total ^{14}C-excretion. In other words, even when using the best synchronized cultures at the right time, one to two-thirds of the total ^{14}C excreted was in glycolate-^{14}C. Occasionally, however, the use of a less synchronized culture obtained by dark incubation for one or two days of a dense culture of premature cells followed by dilution and illumination, as described by Stange, et al. (79), resulted in much higher percent yields, sometimes nearly 100%, of the unknown compounds. It seemed, therefore, that newly synchronized cultures gave a higher percent yield of the unknown compounds. Although the reason is not known, it seems reasonable to assume that changes in percent ^{14}C-distribution among the excreted compounds during the algal life cycle were determined by metabolic shifts, which are internally regulated in connection with differentiation.

Preparative separation and partial purification of the compounds excreted:

The procedures described in Methods for large-scale preparation of the unknown organic acids were followed. Photosynthesis, either in $NaH^{14}CO_3$ or in the air for 30 min., were carried out with mature or dividing cells of A. braunii at an early stage (3 to 4 hr) of the dark period. The supernate was separated and analyzed by paper chromatog-

raphy and radioautography. Because the separation of a large volume of supernate by centrifugation followed by filtration was a slow process, more compounds, in addition to those mentioned before, such as succinate, lactate, aspartate, glutamate, serine, glycine and sucrose, tended to leak out of the cells and appeared in the supernates. Only supernates with the highest percent of ^{14}C (more than 40%) in the unknown compounds were saved and used for further separation and purification. When the concentrated supernate was passed through a cation exchange column packed with AG50W resin in H-form, the eluate showed by paper chromatography no loss of any of the unknown compounds, indicating that none of them were positively charged. Any amino acids that might have been present in the supernate were removed by this procedure. When the eluate was subsequently passed through an anion exchange column of AG1 resin in acetate form, the eluate contained according to paper chromatographic analysis only sugars, in most cases sucrose. Glycolate and all of the unknown compounds were retained on the column. It was clear, therefore, that all of the unknown compounds bore only negative charges.

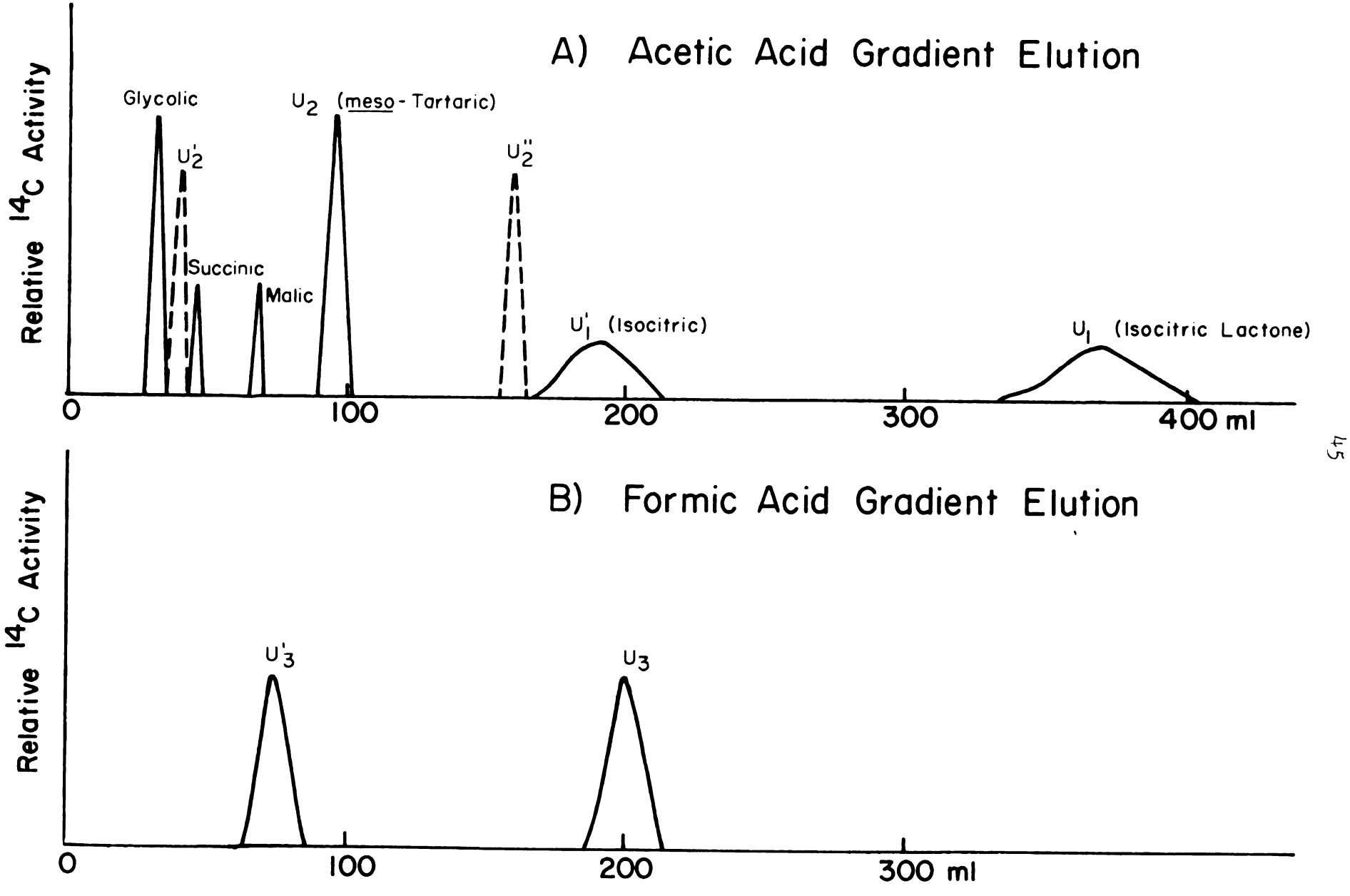
The AG1 column, binding the three unknown organic acids as well as some known organic acids, was then subjected to pH-gradient elution as described in Methods. The radioactive peaks were located by scintillation counting, and their contents and identity were checked by paper chromatography and radioautography. The pattern of separation of those known and unknown organic acids by two-step pH-gradient elution with acetic acid followed by formic acid gradient, is ideally depicted in Figure 5, A and B. Minor components are shown as smaller peaks in the Figure.

FIGURE 5

pH-Gradient Elution of Organic Acids Excreted by A. braunii with an AG1 Resin Column

A) Acetic acid gradient elution:
3N acetic acid was mixed into 200 ml 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.


A) Acetic Acid Gradient Elution
Relative ^{14}C Activity
Glycolic
U_2'
Succinic
Malic
U_2 (meso - Tartaric)
U_2''
U_1' (Isocitric)
U_1 (Isocitric Lactone)
0
100
200
300
400 ml
B) Formic Acid Gradient Elution
Relative ^{14}C Activity
U_3'
U_3
0
100
200
300 ml

Since the pH-gradients were not perfectly reproducible with the use of such a simple apparatus, retention volumes of the organic acids varied from one experiment to the other, especially for those acids with larger retention volumes. Nevertheless, the order of emergence of the acid peaks remained the same, provided that the samples were treated in the same way before chromatography. The range of variation of retention volumes of the acids, together with their mean values which were used to compose Figure 5, are listed in Table 4. Although some characteristics of these acid peaks are shown in this Table, more details about the unknown acids will be given later in the sections concerning their purification and identification.

Since some of the acid peaks were so close to each other, separation and purification of one component from the others usually required repeated passage through the anion exchange columns. After each step of column chromatographic purification, the identity and purity of each peak were checked by paper radioautography. A fast, one-dimensional separation with the butanol-ethyl acetate-formic acid system was found satisfactory for this purpose. The approximate location of the acid spots on such a chromatogram is depicted in Figure 6, together with a typical two-dimensional chromatogram, in which location of some other compounds of interest is added for reference.

Samples of any radioactive compounds, pure in terms of radioactivity, could be obtained by eluting a spot from a paper radioautogram. These samples, however, were not chemically pure, and when they were chromatographed again on paper and sprayed with a bromcresol green solution, several yellow spots not in coincidence with the radioactive spot were visualized. The contaminates were probably from the paper

TABLE 4

Retention Volumes for Organic Acids Excreted by A. braunii on an AG1 Resin Column after pH-gradient Elution

Organic Acid	Retention Volume	
	Range	Mean Value
	Acetic acid gradient elution	
Glycolic	20 - 44 ml	32 ml
Succinic	44 - 48	46
Malic	64 - 72	68
U_2 (meso-Tartaric)	72 - 124	96
U_2' *	28 - 52	40
U_2'' *	124 - 208	160
U_1 (Isocitric lactone)	260 - 480	370
U_1' (Isocitric) *	140 - 240	190
	Formic acid gradient elution	
U_3	44 - 104	74
U_3' *	120 - 280	200

*More detailed classification and description will be given in sections concerning their purification and identification.

FIGURE 6

Paper Chromatographic Maps of Organic Acids Excreted by *A. braunii*

Rf
0.8
0.6
0.4
0.2
Glycolic
Succinic
Malic
meso-Tartaric (U2)
U2' = U2''
Isocitric
U3
U1 (Lactone)
U3
U1' (Free Acid)
U3'
Butanol-Propionic Acid - Water - NaOH
Phenol-Water
Succinic
Glycolic
U2'
Glyceric
Glycine
Malic
U1
Serine
Sucrose
U1'
Citric
U2, U2''
U3, U3'
Origin

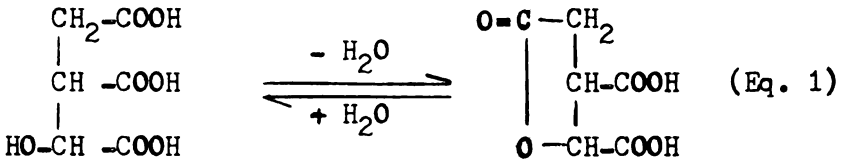
and solvents as well as inorganic anions such as phosphate. Chemically pure samples of radioactive compounds could only be obtained by repeated anion exchange chromatography and collection of samples in the eluates. Even after several repetitions of the procedure, however, paper radioautography showed that samples of U_2 were still contaminated by traces of malate, which were originally present together in the supernates. Furthermore, acid-spray on a paper chromatogram of the partially purified sample of U_2 revealed that it was also contaminated by traces of inorganic phosphate, which came from the photosynthetic buffer. Further purification of these compounds with use of some other procedures was therefore necessary in order to obtain chemically pure samples. Although workable amounts of radioactivity in the unknown compounds were easily obtained from the $NaH^{14}CO_3$ of 50% specific activity, the amounts of the ^{14}C-compounds were generally less than 1 µg per 10 ml cell suspension.

Purification and identification of U_1 as isocitric lactone:

When U_1 was eluted from a paper chromatogram with water, evaporated to dryness with a shaking evaporator at 35-38° under reduced pressure, and then rechromatographed on paper, two spots, which were designated as U_1 and U_1', were observed. The ratio of radioactivity in the two spots varied from time to time. The same two spots were observed when U_1' was eluted, evaporated and rechromatographed by the same way. If the spots as eluted from paper chromatograms were subjected directly to anion exchange chromatography on an AG1-acetate column without evaporation by heating, U_1 gave a peak at a retention volume of 260 to 480 ml, while U_1' gave a peak between 140 and 240 ml. However, when the eluates were collected separately, evaporated to

dryness, and then rechromatographed either on paper or on an AG1 column, again two spots or two peaks were formed. These results showed that U_1 and U_1' were two interchangeable forms of a single compound, that the equilibrium constant between them must be near unity, and that their interchange did not take place to an appreciable extent at room temperature within a short period of time but was accelerated by heating.

On the standard, two-dimensional paper chromatograms the spot U_1' showed R_f values similar to those for citrate. And from laboratory experiences, it was known that evaporation to dryness of either U_1 or U_1' in acetic acid solutions of AG1 eluates favored the formation of U_1, while neutralization of the concentrate prior to chromatography favored the formation of U_1'. All these facts suggested that U_1 and U_1' might be isocitric lactone and the free acid, respectively, since isocitric acid can easily form a stable, five-membered, γ-lactone ring.



(Eq. 1)

Isocitric acid (U_1') Isocitric lactone (U_1)

This hypothesis was further favored by the fact that U_1, which should be less polar, if it is a lactone, than the free acid, actually gave much larger R_f values than U_1' in two of the three solvent systems used (Figure 6). The order of emergence of U_1 and U_1' peaks from an AG1-acetate column, as well as their approximate retention volumes, were also in approximate coincidence with those reported by Palmer (61) for isocitric lactone and the free acid, respectively, from a

Dowex 1-formate column after formic acid gradient elution.

With these facts in mind, further purification of U_1 was performed by repeating anion exchange chromatography, collecting only the U_1 peak after each separation, and rechromatographying the U_1' peak after concentration to complete dryness. By this way any contamination of citric acid in the U_1' peak could be eliminated, because citric acid cannot form a stable lactone. Furthermore, it was known from experimental experiences that the U_1' peak also contained a small amount of a contaminate, which was formed from U_2 for some unknown reasons and was designated as U_2'' (Table 4). The step of purification of U_1 by reconverting U_1' to U_1 and collecting as U_1, therefore, was necessary and effective for getting rid of the U_2'' contamination. Purified U_1, collected from the eluate and concentrated by lyophilyzation instead of heating, gave on paper radioautograms a major spot of U_1 and a minor spot of U_1', but gave no yellow acid spot when the chromatograms were sprayed with a bromcresol green solution.

First direct proof of the identity of U_1 as isocitric lactone was provided by cochromatography on paper. When adequate amounts of authentic DL-isocitric lactone and purified, radioactive U_1 were thoroughly mixed in a pointed test tube, evaporated to dryness with a shaking evaporator, and then chromatographed by one-dimensional and two-dimensional techniques, both radioautography and bromcresol green spray revealed two spots, U_1 and U_1', and the two radioactive spots on the radioautograms perfectly superimposed with the two yellow spots on the paper chromatograms.

The second evidence for the identity of U_1 was obtained by

enzymatic conversion of U_1', which was obtained by alkali hydrolysis of U_1, to glutamate, which was finally identified by cochromatography on paper. Isocitric lactone was first hydrolyzed to the free acid as described by Deutsch and Phillips (16). A 1.8-ml portion of a DL-isocitric lactone solution (10.4 mg/90 ml) was mixed in a test tube with 0.2 ml of N NaOH and an aliquot of U_1 containing approximately 30,000 cpm of ^{14}C-activity. The mixture was heated in a boiling water bath for 10 min., and after cooling the pH was adjusted to 7.5. To this solution was then added a reaction mixture, which was a slight modification of Grafflin and Ochoa (27) and which contained 0.1 ml of 0.1 M TES buffer (pH 7.5), 0.2 ml of 0.02 M $MnCl_2$, and 0.05 ml of 0.005 M $NADP^+$ (in pH 7.5 Tris buffer). To serve as an amino group donor, 0.4 ml of a neutralized, 0.05 M aspartate solution was added (38). When a small aliquot of this mixture was later analyzed by two-dimensional paper chromatography, the radioautogram revealed a single radioactive spot at the R_f of isocitric acid (U_1'), but not in coincidence with the violet spot of aspartate when the paper chromatogram was sprayed with ninhydrin. The enzymes, i.e., suspensions of isocitric dehydrogenase and glutamic aspartic transaminase, were then added to the mixture and the reaction was carried out by incubating it in a water bath at 37° for an hour. The reaction mixture was passed through a small column of AG50W resin in H-form, and the column was thoroughly washed with deionized water and then eluted with N NH_4OH. The radioactive eluate was concentrated and analyzed by two-dimensional paper chromatography. The radioautogram revealed a major radioactive spot at the R_f values for glutamate and a minor spot of unreacted isocitrate (U_1'), while two distinct violet spots were

observed when the paper chromatogram was sprayed with ninhydrin. One of the ninhydrin-positive spots was unreacted aspartate without radioactivity, while the other was located at the R_f of glutamate and perfectly superimposed with the major radioactive spot on the radioautogram. It was therefore concluded that the enzymatically synthesized, radioactive glutamate came from the radioactive isocitrate (U_1'), which in turn had been formed from radioactive isocitric lactone (U_1). Consequently, U_1 must be isocitric lactone, and the spot U_1 on radioautograms of supernates from dividing cells of A. braunii (Figure 2-B) has been designated as isocitric lactone. The citrate spot on the radioautograms, however, could not be definitely assigned as citrate, because of the possibility that it might be isocitrate or a mixture of isocitrate and citrate.

It was considered whether isocitric lactone was excreted by the algae as such or whether it was an artifact formed from isocitrate during the experimental procedures. The only probable steps that might have caused the lactonization of isocitric acid before the supernates were analyzed chromatographically, were the acidification of the supernates with glacial acetic acid and the following concentration at 35-38° with a shaking evaporator. In considering this question, experiments were done in which 2-ml aliquots of the same supernate were subjected to different procedures of acidification and concentration, as described in Table 5. The major modification to minimize lactonization or delactonization was the elimination of acidification and the substitution of lyophilyzation for evaporation by heating. The residues were then analyzed by two-dimensional paper chromatography, and the radioactivities of the spots at the R_f values of isocitric

TABLE 5

Effect of Variations in Methods for Concentration of Supernate from A. braunii on the Amount of Radioactivity Recovered as Isocitric Lactone or Isocitric plus Citric Acids

Experiment	Acidification with Glacial Acetic Acid	$^{12}CO_2$ Aeration	Concentration Procedure	cpm on Chromatogram	
				Isocitric Lactone (U_1)	Isocitric & Citric Acids (U_1')
A	1 drop/2 ml	10 min.	Heating evaporation	6.36 (42%)	8.83 (58%)
B	1 drop/2 ml	10 min.	Lyophilyzation	5.54 (45%)	6.30 (55%)
C	None	30 min.	Heating evaporation	6.24 (44%)	7.96 (56%)
D	None	30 min.	Lyophilyzation	4.43 (45%)	5.40 (55%)

lactone and isocitrate-citrate were counted separately. The data (Table 5) clearly indicated that there was no appreciable change in the ratio of ^{14}C-activity between the isocitric lactone and isocitrate-citrate spots during the concentration procedures prior to the paper chromatographic analysis. Also, algal excretion of isocitric lactone only was sometimes observed. In these cases isocitric lactone was present on the chromatograms without even a trace of accompanying isocitrate or citrate. Throughout the work in an attempt to convert the radioactive isocitrate (U_1') to the lactone (U_1) by acid and heat during the purification of U_1, it was usually observed by counting radioactivity that the lactonization never exceeded 70%. The data reported by Pucher (63), Vickery (91), and Kato and Dickman (42) for the lactonization of isocitric acid by various procedures showed similar yields. It was therefore concluded that isocitric lactone was excreted as such by the algal cells. The occurrence of isocitric lactone in biological sources was first reported by Whiting (97) in blackberry.

Purification and identification of U_2 as meso-tartaric acid:

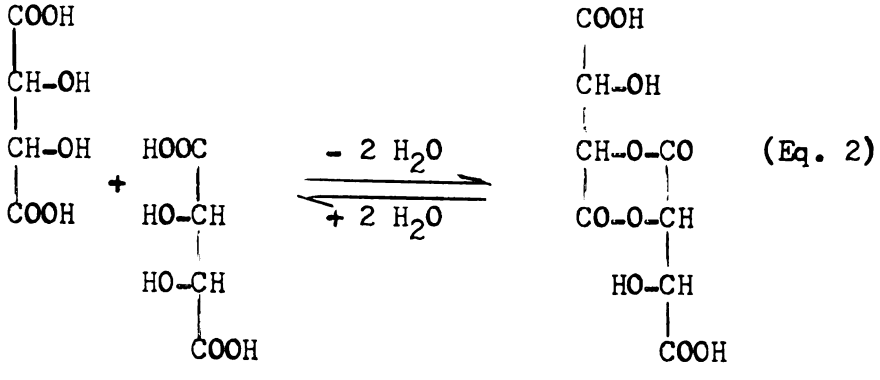
The unknown compound U_2, as shown in Figure 2 B and C, tended to form double spots on two-dimensional paper chromatograms. When either portion of the double spots was eluted and rechromatographed, the same results were usually obtained. When they were run with the butanol-ethyl acetate-formic acid system, however, clear, single spots with the same R_f were obtained. In other words, there was no distinction between the two portions of the double spots. The double spots were therefore considered as a single compound, and designated as U_2.

When U_2 was purified by repeated anion exchange chromatography

with an AG1-acetate column, the fractions containing U_2 in acetic acid solution were usually concentrated with a shaking evaporator at 35-38°. The evaporation was repeated several times on the same sample, with addition of deionized water after each drying, in order to get rid of acetic acid. If the sample, after repeated evaporation, but without neutralization, was again subjected to anion exchange chromatography, generally a tremendous shift of radioactivity from peak U_2 (retention vol. 72-124 ml) to peak U_2' (retention vol. 28-52 ml) was observed (Figure 5 and Table 4). When a fraction containing U_2' in dilute acetic acid solution was concentrated by the shaking evaporator and rechromatographed on an AG1-acetate column, partial or complete conversion of U_2' to either U_2 or U_2'' occurred. The new compound U_2' could also be distinguished from U_2 on paper chromatograms (Figure 6), and therefore was considered as a compound different from U_2. In contrast, there was no clear distinction between U_2'' and U_2 on paper chromatograms with the many solvent systems which were tried. Although the two peaks (U_2 and U_2'') were different in their retention volumes, they could not be clearly distinguished from each other, primarily because their retention volumes varied in such wide ranges (Table 4) that there was no borderline between them, and also because they could never be distinguished by paper chromatography. The formation of the U_2'' peak on anion exchange chromatography was favored either by complete removal of acetic acid by repeated evaporation to dryness, or by neutralization to pH 8-9 prior to chromatography.

The relationship between U_2 and U_2' was much different from that between isocitric lactone (U_1) and the free acid (U_1'). The main difference was that the two forms (U_2 and U_2') were usually not

in an equilibrium. The conversion of U_2 to U_2' was observed only when the former, in a free acid form as eluted off an AG1 column, was heated to dryness in the presence of acetic acid. In contrast, the conversion of U_2' to either U_2 or U_2'' readily took place if it were heated in dilute acetic acid, water, or if neutralized. The only way to save U_2' during the concentration procedure was by lyophilyzation. The lyophilyzed U_2' when analyzed by paper chromatography, was not significantly contaminated with U_2. However, if an aqueous solution of U_2' was stored at -18° for several days, paper chromatographic analysis revealed a considerable increase in the amount of U_2. It was therefore apparent that, while the conversion of U_2 to U_2' was rather difficult to observe, the reversion of U_2' to U_2 was rapid and very likely spontaneous in an aqueous solution. When U_2 was identified as meso-tartaric acid (see below), it seems reasonable from the above observations to assume that the conversion of U_2 (meso-tartaric acid) to U_2' is due to some intermolecular esterification. A logical hypothesis is probably a 6-membered, cyclic ester formed by condensation of two molecules of meso-tartaric acid.



(Eq. 2)

U_2 (meso-tartaric acid) U_2' (an intermolecular ester)

This hypothesis, however, has not been experimentally proven.

As described in the preceding sections, samples of U_2 obtained by repeated anion exchange chromatography were usually contaminated with small amounts of radioactive malate and larger amounts of non-radioactive phosphate. Because of different retention volumes of these acids on an AG1-acetate column (Figure 5 and Table 4), it was apparent that decontamination of malate from U_2 could be accomplished by converting U_2 to U_2' or U_2'' and collecting the latter. In an experiment with ^{32}P-orthophosphate, the phosphate was eluted off the AG1-acetate column by the acetic acid gradient with a retention volume of 160 to 280 ml. So, it was clear that, while peak U_2'' could be contaminated with phosphate, the U_2' peak would be free of phosphate as well as malate. Further purification of U_2 was therefore designed on this basis. Samples of U_2 in acetic acid solution, as eluted off an AG1 column, were concentrated to dryness at 35-38° with a shaking evaporator. The concentration was repeated once with addition of a small amount of deionized water. The completely dried sample of U_2, without neutralization, was dissolved in a small amount of water and immediately introduced onto an AG1-acetate column for acetic acid gradient elution. The U_2' peak, which was the intended product, was collected and evaporated repeatedly to dryness in order to get rid of acetic acid. The acetic acid-free sample was neutralized to pH 7-8 to reconvert it to U_2, and then rechromatographed with an AG1-acetate column. Pure samples of U_2 were thus obtained either as U_2 itself or as U_2''. Paper chromatography and radioautography showed that these samples of U_2 were free from both radioactive malate and nonradioactive orthophosphate.

U_2 was retained on an AG1-acetate column, but not on an AG50W-H^+

column, indicating that U_2 had no positively charged group. It was stable to various chemical and enzymatic treatments, except oxidation. As revealed by paper radioautographic analysis, it was not changed by boiling for an hour either with N HCl or with N NaOH. Treatment with alkaline phosphatase, acid phosphatase, or phosphoglycolate phosphatase did not cause any shift in R_f of the radioactive spot of U_2 on paper radioautograms. It appeared, therefore, that the negative charge on U_2 molecules could not be due to phosphate groups.

On checking with the standard chromatographic maps presented by Bassham and Calvin (6), tartaric acid was found to be the carboxylic acid which was located closest to U_2. The possibility that U_2 was a tartaric acid was further supported by two other facts. First, the radioactive U_2 peak, when eluted from an AGl-acetate column with acetic acid gradient, was located between the peaks of malate-^{14}C and isocitrate-^{14}C (U_1'), which would be the elution pattern for tartaric acid as reported by Palmer (61). Secondly, tartaric acid is a carboxylic acid stable to heat, acid, and alkali treatments, but labile to oxidation. Periodate oxidation of the radioactive U_2 at room temperature liberated, in the first step of the procedure for serine degradation (as described in Methods) as much as 60 to 80% of the total ^{14}C-activity as $^{14}CO_2$ and the remainder as $H^{14}COOH$ (Table 6). Degradation of U_2' and U_2" gave similar results. The significance of this high yield of $^{14}CO_2$ will be discussed later. Since both procedures for periodate degradation yielded 94 to 95% of the total ^{14}C as CO_2 and HCOOH, it is probable that no HCHO or other carbon compounds were produced on periodate oxidation of U_2 at room temperature. This result is in agreement with the mode of periodate

TABLE 6

Degradation of U_2 (meso-tartaric acid) and U_3

A: By degradation procedures for serine (see Methods):

Step of Degradation	Carbons Released as CO_2	U_2 (meso-tartrate)*	U_3**
Periodate oxidation at room temperature	CO_2	70.5% (± 10%)	2.2%
$HgCl_2$ oxidation	HCOOH	23.4% (± 10%)	21.3%
Persulfate oxidation	HCHO Other	6.1% (± 3.5%)	76.5%

* Average value for 11 experiments with different samples.
**Average value for 5 experiments with different samples.

B: By modified degradation procedures for serine (see Methods):

Step of Degradation	Carbons Released as CO_2	U_2 (meso-tartrate)*	U_3**
Periodate oxidation at room temperature	CO_2	80%	2.3%
Periodate oxidation with boiling	HCOOH HCHO	15%	63.4%
Persulfate oxidation	Other	5%	34.6%

* Average value for 2 experiments with different samples.
**Average value for 6 experiments with different samples.

oxidation of d-tartaric acid at room temperature as reported by Sprinson and Chargaff (76).

Direct evidence for the identity of U_2 as meso-tartaric acid was provided by cochromatography both on paper and by gas-liquid partition. When the butanol-ethyl acetate-formic acid solvent system was allowed to flow over the edge of the chromatographic paper, authentic L(+)-tartaric acid gave slightly higher R_f value than authentic meso-tartaric acid. When the purified U_2 was cochromatographed with these two isomeric acids separately, the radioactive spot of U_2 perfectly superimposed with the meso-form, but not with the L(+)-form. The same results were obtained also by the standard, two-dimensional paper chromatography system. By gas-liquid chromatography with a 3% OV-1 column, which was run by linear temperature-programming of 5°/min. starting at 100°, or by isothermal conditions at 148°, TMS-derivatives of the two isomers of tartaric acid were clearly separated. As shown in Table 7, the TMS-derivative of the meso-form preceded that of the L(+)-form. When the purified, radioactive U_2 was cochromatographed with the two isomers separately as their TMS-derivatives, the radioactivity collected at the collection port coincided in location with the mass peak of the TMS-derivative for the meso-form as recorded on the automatic recording chart, but not with that of the L(+)-form. It was, therefore, concluded that the excreted acid, U_2, is meso-tartaric acid, but not L(+)-tartaric acid which is commonly present in various fruits and many higher plants.

Purification and properties of U_3:

When the supernate from dividing cells of A. braunii was paper

TABLE 7

Gas-Liquid Chromatography of TMS-Derivatives of Tartaric Acids and U_2

A: By linear temperature-programming of 5°/min. starting at 100°:

Sample (as TMS-Derivative)	Mass Peak	^{14}C-Activity
meso-Tartaric acid (250 µg)	165 - 172°	None
L(+)-Tartaric acid (200 µg)	170 - 175°	None
meso-Tartaric acid (250 µg) plus ^{14}C-labelled U_2	162 - 170°	162 - 170°

B: Under isothermal conditions at 148°:

Sample (as TMS-Derivative)	Mass Peak	^{14}C-Activity
meso-Tartaric acid (2 µg)	12.0 - 13.7 min.	None
L(+)-Tartaric acid (2 µg)	16.5 - 18.0 min.	None
meso-Tartaric acid (20 µg) plus ^{14}C-labelled U_2	11.5 - 13.2 min.	11.5 - 13.2 min.

chromatographed by the standard, two-dimensional method, without addition of NaOH to the second solvent, U_3 ran to the left of the double spots of U_2 (meso-tartrate). Thus, triple spots were usually observed (Figure 2-C). By the addition of a small amount of NaOH to the second solvent (28), the R_f of U_3 was lowered so that it could be separated from U_2 (Figure 2-B). In this case, however, U_3 fell into the same general location as orthophosphate which came from the buffer for the algae. This coincidence resulted in a roundly diffused spot. Consequently, if U_3 was eluted from such a paper chromatogram, it was usually heavily contaminated with phosphate. This phosphate contamination, however, was easily eliminated by anion exchange chromatography. Orthophosphate was eluted off the AG1-acetate column by acetic acid gradient (retention vol. 160-280 ml), but U_3 was not eluted even by 500 ml of acetic acid. Among the organic acids excreted by dividing cells of A. braunii, only U_3 was eluted by formic acid gradient after 500 ml of acetic acid elution. Therefore, it was easy to obtain samples of U_3 which were pure in terms of radioactivity and also free from phosphate. However, such U_3 samples were not chemically pure. When they were analyzed by paper chromatography and the chromatograms were sprayed with a bromcresol green solution, some yellow spots and violet spots not in coincidence with the radioactive spots were observed. The sources of the nonradioactive contaminates may have been in part from the breakdown products of the resin and partially from the formic acid.

Another difficulty encountered during the purification of U_3 was the relative unstability of U_3', which was formed from and always accompanying U_3. The relationship between U_3 and U_3' was similar to

that between U_1 (isocitric lactone) and U_1' (isocitrate). They were apparently interchangeable, and one was always accompanied by the other. U_3 and U_3' were clearly distinguished when paper chromatographed with the butanol-ethyl acetate-formic acid system, but not by the two-dimensional method. When U_3' was evaporated to dryness and rechromatographed, it was not only partially reversed to U_3, but it also partially decomposed to many minor unknown compounds. Because of this lability, the paper chromatographic analysis of the compounds during the purification procedures was difficult and unclear.

U_3 contained only negatively charged groups. Since U_3 was not eluted off the AG1-acetate column by acetic acid gradient and had low R_f chromatographic values, it probably had more anionic groups or lower K_a values than tartaric acid. Reactions with acid phosphatase, alkaline phosphatase or phosphoglycolate phosphatase did not cause an appreciable shift in the R_f of U_3. Therefore, the negative charge on U_3 molecules seemed to be due to carboxyl groups. Upon periodate degradation, no CO_2 was released at room temperature, but both HCOOH and HCHO were produced, as well as other breakdown compounds (Table 6). Periodate oxidation at room temperature did not oxidize HCOOH and HCHO, but the subsequent $HgCl_2$ oxidation (Method A) converted HCOOH to CO_2. Periodate oxidation at 100° (Method B) oxidized both HCOOH and HCHO to CO_2. By comparing the difference in CO_2 production by the two methods, an estimation of HCHO produced by periodate oxidation at room temperature was obtained. By this estimation about one-third of the ^{14}C in U_3 went to HCHO on periodate oxidation.

According to the standard chromatographic maps (6), gluconic acid was located nearest to U_3, but U_3 and gluconic acid did not

cochromatograph. Like U_3, oxalic-^{14}C acid was found to be eluted from an AG1-acetate column by formic acid gradient following 500 ml of acetic acid elution. However, U_3 also did not cochromatograph with oxalic acid. Other organic acids which did not cochromatograph with U_3 on paper chromatograms were fumaric, maleic, malonic, dihydroxymaleic, dihydroxytartaric, tartronic, and mesoxalic acids. U_3 has not been identified.

Excretion of labeled acids after $^{14}CO_2$ photosynthesis:

It has been established (35, 62, 86) that the formation and excretion of glycolate was light-dependent. Stafford and Loewus (78) have also shown that $^{14}CO_2$ incorporation into tartrate by excised grape leaves was light-dependent. Since photosynthetic CO_2-fixation itself is light-dependent, the question arose whether the light dependency was due to glycolate and tartrate synthesis or whether light played a role in the excretion process. Algal cells were labeled with ^{14}C in a photosynthetic period, washed free of supernate, and then subsequent synthesis and excretion of labeled acids studied in the absence of further $^{14}CO_2$. It has been observed that the amounts of excretion of glycolate and meso-tartrate by synchronized cells of A. braunii during their life cycle were in a reciprocal relationship (Figure 4), and the present experiment were done to search for further metabolic relationships between these two acids.

The algal cells used in these experiments (Table 8) were for experiment A, dividing cells (4 hours in the dark period), for experiment B, growing cells (8 hours in the light period), and for experiment C, growing cells (7 hours in the light period) from synchronized cultures of A. braunii. The cultures for experiments A and

TABLE 8

Amounts of ^{14}C Excreted by ^{14}C-Labeled Cells of A. braunii under Different Conditions. Values are cpm of ^{14}C excreted in one minute by 1 ml of 1% cell suspension.

	Experiment		
	A (dividing cells)	B (growing cells)	C (growing cells)
Photosynthetic $^{14}CO_2$ Fixation			
Supernate	2,707	34,780	10,031
Washing	375	856	601
Cell-extract	21,347	43,953	41,083
Total fixation	24,429	79,589	51,715
Subsequent Excretion			
1) in light	1,275	7,190	3,917
2) in light, + CMU	-	-	2,667
3) in dark	1,110	1,366	1,535
4) in dark, + O_2	-	-	1,593

B had been kept synchronized for many cycles, while that for experiment C had been synchronized for only one cycle. Photosynthesis with 1% cell suspension in $NaH^{14}CO_3$ were run for 18 min. in experiments A and B, and 15 min. in experiment C. The supernates were separated from the ^{14}C-labeled cells by centrifugation followed by Millpore filtration. The ^{14}C-labeled cells were then washed once by suspending in water and centrifuging again. These "washing" (Table 8) were also passed through a Millpore filter to remove any cells. The washed cells were then resuspended in a volume of 0.001 M phosphate buffer at pH 6.0, so that a final suspension of 1% (v/v) algal cells was again obtained. After removing a 1-ml aliquot for ^{14}C-counting and paper chromatographic analysis, the remaining portion of the ^{14}C-labeled cell suspension was divided into 2 (experiments A and B) or 4 (experiment C) parts of equal volumes for different excretion experiments as shown in Table 8.

Light-excretion experiments were carried out in the same way as a normal photosynthesis experiment, except that no further addition of $NaH^{14}CO_3$ or $NaH^{12}CO_3$ was made. In experiment C-2, CMU solution was added to the cell suspension at a final concentration of 8×10^{-6} M at the beginning of illumination. Dark-excretion experiments were done in test tubes wrapped with aluminum foil and shaken occasionally. In experiment C-4, O_2 gas was continuously bubbled into the cell suspension in the darkened test tube. After 18 min. (experiments A and B) or 15 min. (experiment C) of excretion in the light or in the dark, the supernates were separated by centrifugation and filtration. The cells, after washing once with water, were killed and extracted with boiling 80% methanol as usual. Aliquots of the initial supernate,

washing, cell-extract, light-excretions and dark-excretions were counted for ^{14}C-activity, and other portions, as well as the final cell-extracts, were analyzed by paper chromatography and radioautography.

The activity of photosynthetic $^{14}CO_2$-fixation and excretion were much greater for growing cells than for dividing cells (Table 8). The amounts of subsequent excretion by the ^{14}C-labeled cells in the light was likewise greater for the growing cells than the dividing cells. There was little difference between the amounts of ^{14}C excretion in the light and in the dark by dividing cells. For growing cells the amount of ^{14}C excreted in the light was much more than the amount excreted in the dark. In the dark there was an 81 to 61% decrease in excretion. The addition of CMU also reduced the light-excretion by 32%, but O_2-aeration did not show any appreciable effect on the amount of dark-excretion.

These different conditions for excretion by the ^{14}C-labeled algal cells not only affected the amount excreted but also the components in the excretion. Radioactive spots on the paper radioautograms of the initial supernate and the subsequent excretions were counted, and the percent distribution of ^{14}C-activity was calculated. Radioactivities in all other compounds that had leaked out of the cells during the centrifugation and filtration procedures were combined and listed in a separate column designated as "others" in Table 9. The actual amount of radioactivity in each compound was then calculated from the data of total excretion (Table 8) and the percent distribution. In Table 9, the amount of radioactivity in each compound is listed for that found in the initial supernate and that

TABLE 9

Amount and % Distribution of ^{14}C Among Compounds Excreted by A. braunii During $NaH^{14}CO_3$ Fixation and Afterwards During Different Treatments. Values are cpm ^{14}C excreted in one min. by 1 ml of 1% cell suspension; values in parentheses are for % distribution.

Experiment	Total Excretion	Compounds Excreted					
		Glycolate	Malate	Isocitric Lactone, Isocitrate, Citrate	meso-Tartrate	U_3	Others
A. Dividing cells							
Initial supernate	2,707	668.6 (24.7)	138.1 (5.1)	197.6 (7.3)	1,353.5 (50.0)	211.1 (7.8)	138.1 (5.1)
Light excretion	1,275	135.1 (10.6)	90.5 (7.1)	221.9 (17.4)	144.6 (11.5)	228.2 (17.9)	454.7 (35.5)
Dark excretion	1,110	0	62.1 (5.6)	330.8 (29.8)	174.3 (15.7)	230.9 (20.8)	311.9 (28.1)
B. Growing cells							
Initial supernate	34,780	33,980.0 (97.7)	0	0	0	0	800.0 (2.3)
Light excretion	7,190	6,485.4 (90.2)	107.8 (1.5)	0	71.9 (1.0)	28.8 (0.4)	496.1 (6.8)
Dark excretion	1,366	65.6 (4.8)	118.8 (8.7)	0	92.9 (6.8)	32.8 (2.4)	1,055.9 (79.7)

TABLE 9
(Continued)

Experiment	Total Excretion	Compounds Excreted					
		Glycolate	Malate	Isocitric Lactone, Isocitrate, Citrate	meso-Tartrate	U_3	Others
C. Growing cells							
Initial supernatant	10,031	9,469.3 (94.4)	80.2 (0.8)	0	230.7 (2.3)	30.1 (0.3)	220.7 (2.2)
1) Light excretion	3,917	3,117.9 (79.3)	0	62.7 (1.6)	309.4 (7.9)	160.6 (4.1)	266.4 (6.8)
2) Light excretion + CMU	2,667	962.8 (36.1)	0	98.7 (3.7)	757.4 (28.4)	368.0 (13.8)	480.1 (18.0)
3) Dark excretion	1,535	0	0	95.1 (6.2)	508.1 (33.1)	285.5 (18.6)	646.3 (42.1)
4) Dark excretion + O_2	1,593	0	0	0	552.8 (34.7)	366.4 (23.0)	657.9 (41.3)

subsequently excreted. The values are also expressed in Table 9 as percent of that excreted in the designated supernatant fluid.

To further aid in the evaluation of these results, the amount of each compound excreted in the light after $NaH^{14}CO_3$ fixation is expressed in Table 10 as 100, and the change in excretion by darkness or other treatments is given as some percent of the light value. In these experiments the absolute amounts of ^{14}C shown in Table 9 can be compared.

Some general conclusions from these results may be summarized. (A) Excretion of the organic acids after photosynthesis with $NaH^{14}CO_3$ continued during washing of the algae and during the period when they were resuspended in buffer. This excretion of glycolate in the absence of additional $NaH^{14}CO_3$ is similar to that reported by Hess, Tolbert and Pike (35). (B) Growing cells excreted mainly glycolate during photosynthesis and in a subsequent light period. Dividing cells excreted mainly meso-tartrate and some isocitric lactone during photosynthesis and in a subsequent light period. (C) The formation and excretion of glycolate was absent in the dark (Table 10). Light was necessary for glycolate formation and excretion. (D) In the dark the main excretion products were meso-tartrate, isocitric lactone, as well as U_3. The important fact is that both meso-tartrate and isocitric lactone were made and excreted in the dark from ^{14}C-labeled cell constituents made during the light period of photosynthesis. This was not true for glycolate. Consequently both the percent distribution and the actual amounts of ^{14}C in all acids except glycolate increased in the dark. (E) The addition of CMU at a final concentration of 8×10^{-6} M reduced glycolate excretion in the light to 31%,

TABLE 10

Effects of Darkness, CMU and O_2 on the Excretion of Acids by ^{14}C-Labeled Cells of A. braunii

Experiment and Algae	Experimental Condition	Compounds Excreted			
		Glycolate	Isocitric Lactone, Isocitrate, Citrate	meso-Tartrate	U_3
A (dividing cells)	light	100	100	100	100
	dark	0	149	121	111
B (growing cells)	light	100	100	100	100
	dark	1	110	129	114
C (growing cells)	1) light	100	100	100	100
	2) light + CMU	31	157	245	180
	3) dark	0	152	164	242
	4) dark + O_2	0	25	179	247

and there was a concurrent increase in production of ^{14}C-labeled meso-tartrate. CMU treatment was nearly equivalent to an experimental dark condition. (F) O_2-aeration in the dark eliminated the excretion of isocitric lactone and isocitrate and citrate. The reason for this is unknown, but the O_2 could have accelerated the operation of the citric acid cycle or the oxidation of NADPH. (G) U_3 was produced and excreted in larger amounts in the dark than in the light.

These results indicate that glycolate production and excretion is unique to photosynthesis and light conditions. The excretion of isocitric lactone, meso-tartrate and U_3 occurs in the dark and is therefore probably related to some metabolic process.

Distribution of ^{14}C-activity in meso-tartaric acid:

As reported by Sprinson and Chargaff (76), periodate oxidation at room temperature of tartaric acid will give rise first to two molecules of glyoxylic acid, which are then further oxidized to one molecule each of CO_2 and HCOOH. Therefore, periodate oxidation of each meso-tartaric acid molecule results in the formation of two molecules of CO_2 from the carboxyl groups and two HCOOH molecules from carbon atoms 2 and 3 of tartaric acid. These were collected separately in the first and the second steps, respectively, of the normal periodate degradation procedure employed for serine as applied to the degradation of radioactive meso-tartrate (U_2). Due 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 uniformly labeled pattern would be expected in all compounds, and one would expect 50% of the ^{14}C in tartaric acid to be

released as CO_2 and 50% as HCOOH. Such results were observed by Stafford and Loewus (78) for (+) tartaric acid formed by grape leaves. However, as shown in Table 6, the carboxyl groups of meso-tartaric acid excreted by the algae over a 10 min. period had 70.5% of the ^{14}C, leaving only 23.4% in $C_2 + C_3$. The results by method A were confirmed by method B which gave 80% and 15%, respectively, in carboxyls and middle carbons. Thus, the carbon atoms of meso-tartaric acid did not appear to be uniformly labeled even over long time periods. To check this, the specific activity of the carbon atoms of radioactive meso-tartaric acid (U_2) were determined, as described in Method. Authentic meso-tartaric acid was used as carrier in these degradation experiments. Two separate determinations on the same sample of radioactive U_2 gave very similar results (Table 11). Since the dilution factor by the carrier was not determined, the specific activity data from one degradation cannot be compared with the other degradation, but the ratio of specific activity of the carbon atoms from each degradation is directly comparable (Table 11). The ratio of specific activity of carboxyl carbons (C_1 and C_4) to that of middle carbons (C_2 and C_3) was 78:22 in both cases, which was in agreement with the ^{14}C-distribution data obtained by the periodate degradation (Table 6). From the above degradation data, it is concluded that the meso-tartaric acid was labeled predominately in the carboxyl groups.

The carboxyl carbons of meso-tartaric acid molecules were about 4 times as radioactive as the middle carbons. By analogy with oxaloacetic acid formation by carboxylation of pyruvate or P-pyruvate, one of the carboxyl groups could contain most of the label as indicated

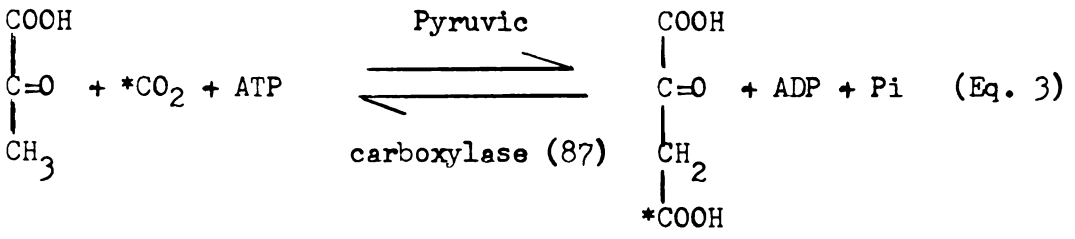
TABLE 11

Specific Activity of the Carbon Atoms of meso-Tartaric Acid (U_2), Expressed in mμc./mg C.

Carbon Atom	Experiment 1 S. A. Ratio*		Experiment 2 S. A. Ratio*		Average Ratio of S. A.*
Carboxyl ($C_1 + C_4$)	3.50	78.2	3.74	77.9	78
Middle ($C_2 + C_3$)	0.98	21.8	1.06	22.1	22

*Ratio of the specific activity for $C_1 + C_4$ to $C_2 + C_3$.

in equation 3.



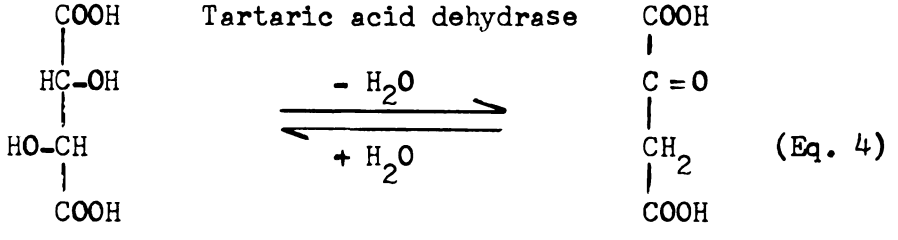
(Eq. 3)

In the case of meso-tartaric acid, the two carboxyl groups could not be differentiated chemically because of the plane of symmetry in the molecule. However, according to the Ogston effect the carboxyl groups of meso-tartaric acid could retain their identity in an enzymatic reaction. Thus, it is possible that most of the ^{14}C in meso-tartaric acid could be in one of the carboxyl groups. On the other hand, in reactions analogous to equation 3, the C_3 compound, pyruvate or phosphoenolpyruvate, may have been derived from carboxyl labeled phosphoglycerate. Upon fixing another molecule of $^{14}CO_2$ at the other end of the molecule, the C_4 product, i.e., malate or oxalacetate, would be labeled in both carboxyl groups. Varner and Burrell (90) have found that 80-90% of the ^{14}C fixed into malate was distributed both in the carboxyl carbons, while Bradbeer, et al. (10) reported that the ^{14}C was mostly in the gamma carboxyl group of malate from crassulacean leaves.

The retention of carboxyl labeling in meso-tartaric acid during the course of 10 min. photosynthesis is unique. This phenomenon could result from the fact that the meso-tartaric acid was excreted by the algae where, outside the cell, no metabolic randomization of the label could occur. If so, the labeling of at least one of the carboxyl groups of meso-tartaric acid must occur near the time of its excretion.

Recently a similar analogy has arisen from the ^{14}C distribution in malate and aspartate formed during photosynthesis by certain plants. Hatch, et al. (29) have established that certain plants incorporate $^{14}CO_2$ first into the gamma carboxyl group of these C_4 compounds before the ^{14}C appears in the carboxyl group of phosphoglycerate. They are at a loss to explain why the label in the gamma carboxyl group does not randomize, even though phosphoglycerate and other constituents of the photosynthetic carbon cycle become uniformly labeled after a few minutes.

A pathway from oxalacetate to meso-tartrate has not been established. An enzyme, "tartaric acid dehydrase," has been reported by Hulbert and Jakoby (37), La Riviere (49), and Shilo (70) for Pseudomonas.



(Eq. 4)

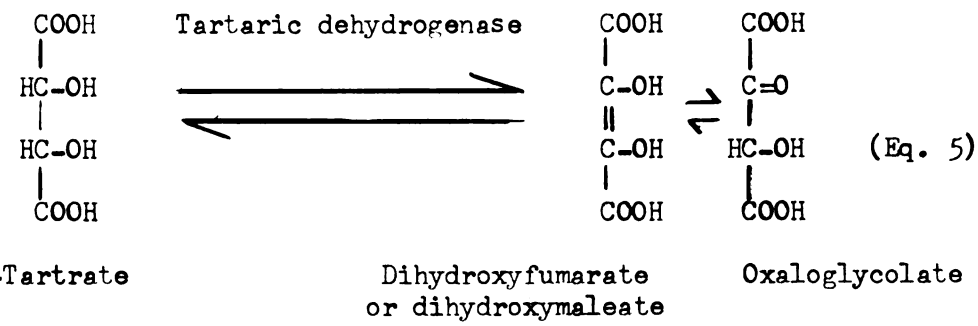
L(+) Tartrate Oxalacetate

However, this enzyme was described to be specific for L(+)-form of tartaric acid, but not for meso-tartrate.

Discussion concerning metabolic relationships between meso-tartaric and glycolic acids:

An NAD-specific dehydrogenase has been found in bacteria (43), mitochondria (47, 48), pigeon-liver extract (67), and higher plants (77), which can catalyze the dehydrogenation of D(-) and meso-forms of tartaric acid. The product, dihydroxyfumarate or dihydroxymaleate

is in equilibrium with the more unstable keto-form, oxaloglycolate (Eq. 5).

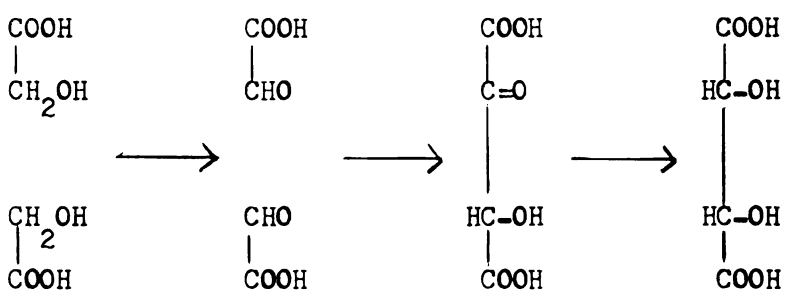


(Eq. 5)

meso-Tartrate — Dihydroxyfumarate or dihydroxymaleate — Oxaloglycolate

Kun and Hernandez (48) have demonstrated the reverse reaction, namely, the reduction of oxaloglycolate to tartrate by this enzyme. However, the pathway between oxaloglycolate and the C_4 acids of the TCA cycle has never been established.

Maroc (52, 53) has recently proposed that meso-tartrate could be formed from two molecules of glycolate via glyoxylate and oxaloglycolate as intermediates (Eq. 6).

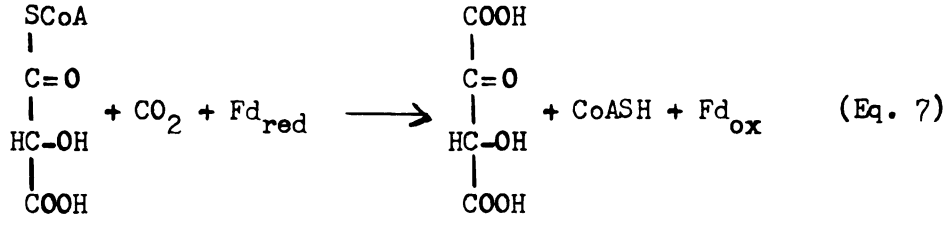


(Eq. 6)

We have considered this scheme for meso-tartaric acid formation, because the biosynthesis and excretion of meso-tartaric acid and glycolate had a reverse relationship and because the excretion of these two acids along with isocitric lactone was unique. The first enzyme of this pathway is glycolate oxidase, the second step has

not been investigated and the third step could be similar to equation 5. Rather from the extensive work of Kornberg's group (45) and Vennesland's group (46) with bacteria, the oxaloglycolate was decarboxylated to hydroxypyruvate which was then reduced to glyceric acid. No evidence has been published for the scheme in plants or algae, and in fact, Tolbert's group has cited extensive data to indicate that this scheme does not function for glycerate formation from glycolate in plants (85).

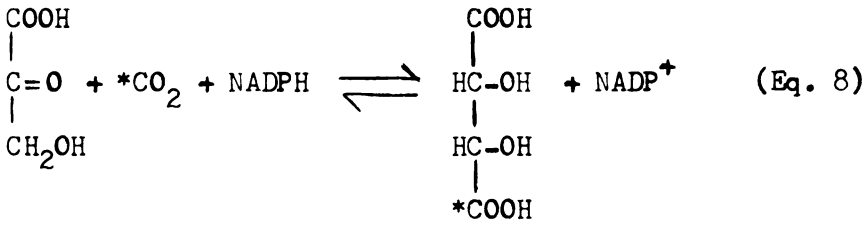
The pathway for meso-tartaric acid biosynthesis as shown in equation 6 could not explain the formation of carboxyl labeled material. Glycolate molecules have always been found to be uniformly labeled in the shortest periods of $^{14}CO_2$ fixation (12, 33, 34, 64, 68). So the meso-tartrate molecule formed from glycolate should consequently be uniformly labeled. Furthermore, Hess and Tolbert (34) have found that algae were devoid of glycolate oxidase, which is responsible for the conversion of glycolate to glyoxylate. Thus, alternative pathways for meso-tartaric acid synthesis must be considered. Oxaloglycolate might be formed, in analogy to the reactions catalyzed by pyruvate synthetase (19) and α-ketoglutarate synthetase (11), by ferredoxin-dependent carboxylation of tartronyl CoA, as proposed in equation 7.



(Eq. 7)

Tartronyl CoA Oxaloglycolate

No evidence exists for this enzyme. Another possible pathway for the formation of carboxyl labeled meso-tartaric acid is analogous to the reaction catalyzed by malic enzyme (59), which would result in the reductive carboxylation of hydroxypyruvate (Eq. 8).

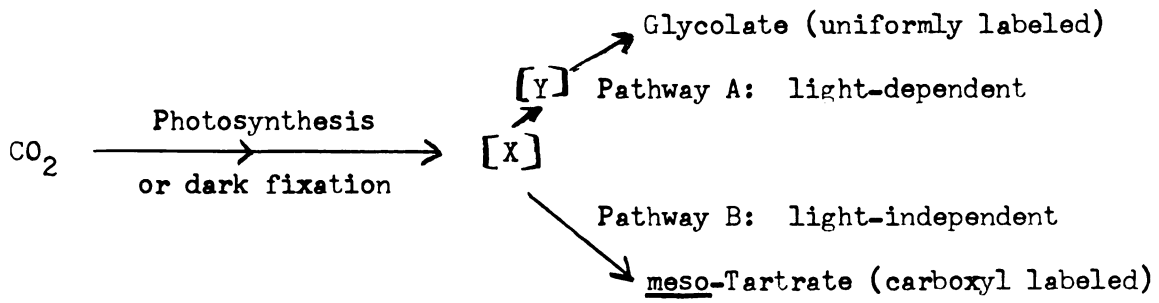


(Eq. 8)

The proposal by Maroc (52, 53) that glycolate is the precursor of meso-tartrate in plants is rendered impossible for the algae by the results from experiments on the excretion of labeled acids after photosynthetic $^{14}CO_2$-fixation. As shown in Tables 9 and 10, the formation and excretion of glycolate were completely inhibited in the dark, but meso-tartrate synthesis in the dark was increased. This fact rather implies that meso-tartrate might be a precursor of glycolate. In such case, the reaction sequence in equation 6 might be reversed. Meso-tartrate would be first dehydrogenated to oxaloglycolate, which, according to Kun and Hernandez (48), is unstable and either rapidly splits into two molecules of glyoxylate or decarboxylates to hydroxypyruvate. The glyoxylate would then be reduced to glycolate by glyoxylate reductase which is present in algae. However, this pathway of glycolate formation from meso-tartrate is unlikely for two reasons. First, the amount of increase in ^{14}C-activity in meso-tartrate in the dark was too small in comparison to the large amount of ^{14}C-glycolate formation which was shut off. Secondly, carboxyl labeled meso-tartrate will give rise to carboxyl labeled glycolate,

rather than uniformly labeled glycolate, which has always been observed. Mention might also be made that the CO_2 condensation hypothesis for glycolate biosynthesis proposed by Stiller (82) and Zelitch (101) could neither be eliminated nor be substantiated by the labeling patterns in meso-tartrate.

A more likely metabolic relationship between meso-tartrate and glycolate is that they are both derived from a common precursor X , and that the pathway A, leading to glycolate, is light-dependent, while pathway B, leading to meso-tartrate, occurs in the dark. The relative activity of the two pathways could be regulated by changes which occur



during cell differentiation. Thus, pathway B would be more active when the cells are dividing, while pathway A is predominant when the cells are growing. When pathway A is shut off in the dark, the formation of meso-tartrate can be increased, but not necessarily in direct proportion to reduced glycolate biosynthesis. For this scheme to confirm with the labeling data, it is necessary to assume the existence of intermediate X which should not be uniformly labeled. However, in pathway A between X and glycolate, there should exist a symmetric intermediate Y , so that the ^{14}C label becomes randomized in glycolate.

The physiological significance of the excretion of meso-tartrate

is not known, just as the significance of glycolate excretion remains unexplained. While glycolate was considered by Hess and Tolbert (34) as an end product of algal carbon metabolism, Vickery and Palmer (92) made a similar speculation concerning the formation of (+)-tartrate in tobacco leaves. In analogy, glycolate and meso-tartrate may also be considered as end products of carbon metabolism by growing and dividing cells, respectively, of A. braunii. Experimental data of this study suggest that the formation and excretion of glycolate and meso-tartrate may be related to each other, and that glycolate excretion must be somehow related to the photosynthetic processes. No relationship could be established. Feeding radioactive meso-tartrate (U_2) to A. braunii cells at various stages of development resulted in negligible uptake by the cells, and no conversion of the radioactive compound was observed by paper chromatography and radio-autography.

Discussion concerning isocitric lactone excretion:

The reason why the algae excrete the lactone rather than the free isocitric acid is unknown. In comparison with the excretion of glycolate and meso-tartrate, isocitric lactone may be excreted because it cannot be further metabolized. Isocitric lactone, though occuring in preparations of isocitric acid, has not previously been thought to be a naturally occuring compound or to be synthesized by plants. The specific excretion of the lactone suggests that an enzymic lactonization reaction may exist which might be associated with the algal membrane. The significance of this work on the lactone and these speculations could be extended by further biological surveys to establish the uniqueness of this compound.

A metabolic relationship between isocitric lactone and glycolate biosynthesis exists in the glyoxylate cycle. In this cycle, isocitric acid is split to succinate and glyoxylate by isocitritase. The glyoxylate could be reduced to glycolate by glyoxylate reductase. Both of these enzymes have been reported in various algae, although no study has been made of them in A. braunii. In the absence of glycolate synthesis and excretion in the dark, isocitrate might accumulate and be converted to the lactone which could be excreted. Much further work would be necessary to establish this hypothesis. This pathway for the synthesis of most of the glycolate by algae has not been favored from kinetic experiments, from $^{14}CO_2$ labeling experiments, and from ^{14}C-acetate feeding experiments (54). However, it is possible that the glyoxylate cycle could account for the formation of isocitric lactone which could be a minor reaction relative to the large amount of glycolate formation by some other route.

SUMMARY

Synchronized cultures of Ankistrodesmus braunii were grown during a 16-hr light and 8-hr dark regimen at 30° with a 1 to 4 dilution at the end of each dark period. The photosynthetic ability, as measured by $^{14}CO_2$ fixation, was the 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.

The ^{14}C compounds excreted during photosynthesis changed during the algal life cycle. Young growing cells excreted glycolate in large amounts, but none of the other acids. Dividing cells excreted only about 4% as much glycolate-^{14}C as young growing cells. Dividing cells also excreted meso-tartrate, isocitric lactone, malate, and an unidentified acid, U_3, and occasionally some citrate and glycerate. Of the acids excreted by dividing cells, glycolate and meso-tartrate were the major ones and they were excreted in comparable amounts. Excretion of meso-tartrate when glycolate excretion decreased implied that the two acids might be metabolically and physiologically related.

Kinetic studies on the excretion of these acids by the synchronized cells were done during 30 minutes of photosynthesis. Growing cells excreted glycolate. With dividing cells, meso-tartrate, malate and glycerate were excreted in relative largest amounts within 1 or 2 minutes of photosynthesis, glycolate in 5 minutes, and isocitric lactone and U_3 in 30 minutes.

Large-scale separation and purification of the excreted acids were carried out by repeated anion exchange chromatography on AG1-

acetate columns with acetic acid gradient elution followed by formic acid gradient elution. The acids were eluted from the column with the acetic acid gradient in the order of glycolic, malic, meso-tartaric, isocitric and citric acids, and isocitric lactone; U_3 was eluted with a subsequent formic acid gradient.

The meso-tartaric acid was identified by cochromatography on paper chromatograms and by gas-liquid cochromatography as the trimethylsilyl derivative. Isocitric lactone was identified by cochromatography on paper, and by hydrolyzing it to isocitrate. The isocitrate was then identified by cochromatography and by converting it to glutamate by a reaction catalyzed by isocitric dehydrogenase and glutamic-aspartic transaminase. Isocitric lactone was experimentally shown to be excreted as such by the dividing cells.

After a period of photosynthesis in $NaH^{14}CO_3$, subsequent excretion of ^{14}C-labeled acids in the light and in the dark was analyzed both for the amount and for the components. In light, excretion of glycolate and the other acids continued. In dark, glycolate excretion was completely stopped, while the excretion of the other acids continued in even larger amounts. Upon addition of CMU during the light, the excretion pattern was similar to that in the dark. Aeration with oxygen during the dark prevented the excretion of isocitric lactone.

The distribution of ^{14}C in meso-tartaric acid was determined. The carboxyl carbons were about 4 times as radioactive as the middle carbons. Since glycolate is known to be uniformly labeled, glycolate could not be the precursor of carboxyl labeled meso-tartrate, nor meso-tartrate a direct precursor of glycolate. The biosynthesis of

meso-tartrate by these algae is unknown, but the carboxyl labeling pattern suggests that a carboxylation to form a C_4-precursor of the tartrate might exist. From the data, it was speculated that glycolate might be formed from a precursor common to both meso-tartrate and glycolate. The reason for the specific excretion of glycolate, meso-tartrate and isocitric lactone is not known, except that all three acids may not be further metabolized by the algae.

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