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Aminooxyacetate Stimulation of Glycolate Formation and Excretion by *Chlamydomonas*[1]

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ABSTRACT

Aminooxyacetate (1 millimolar) did not inhibit photosynthetic $^{14}CO_2$ fixation by *Chlamydomonas reinhardtii* Dangeard, (−) strain (N.90) but greatly stimulated the biosynthesis and excretion of glycolate. Similar results were obtained from cells grown with 5% CO_2 or low CO_2 (air). After 2 minutes with air-grown cells, [^{14}C]glycolate increased from 0.3% of the total ^{14}C fixed by the control to 11.7% in the presence of aminooxyacetate and after 10 minutes from 3.8% to 41.1%. Ammonium nitrate (0.2 millimolar) in the media blocked the aminooxyacetate stimulation of glycolate excretion. Chromatographic analyses of the labeled products in the cells and supernatant media indicated that aminooxyacetate also completely inhibited the labeling of alanine while some pyruvate accumulated and was excreted. A high percentage (35%) of initial $^{14}CO_2$ fixation was into C_4 acids. Initial products of $^{14}CO_2$ fixation included phosphate esters as well as malate, aspartate, and glutamate in treated or untreated cells. Lactate was also a major early product of photosynthesis, and its labeling was reduced by aminooxyacetate. Inasmuch as lactate was not excreted, glycolate excretion seemed to be specific. When photosynthesis was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, labeled organic and amino acids but not phosphate esters were lost from the cells. Aminooxyacetate did not inhibit the enzymes associated with glycolate synthesis from ribulose bisphosphate.

In 1956 Tolbert and Zill (25) reported that *Chlorella* excreted only glycolate among the products of photosynthetic $^{14}CO_2$ fixation. Glycolate biosynthesis and excretion are increased by high light intensity, high O_2 and low CO_2 concentration, and high pH (20). Excretion normally amounts to 2 to 5% of the total ^{14}C fixation at pH 7 to 8 over a 10-min period. From many subsequent investigations, excretion of glycolate has been recognized as a general but unexplained phenomenon of unicellular algae (27, 28). In higher plants glycolate is also produced in chloroplasts and excreted into the cytoplasm to be metabolized in the peroxisomes (15). There is no evidence that glycolate excretion by algae or chloroplast is an active process but only the unionized acid (pK_a 3.8) diffuses across these membranes (11, 24). Cells grown with saturating levels of CO_2 (1–5%) excrete more glycolate than air-grown cells, when either are tested with low levels of $NaH^{14}CO_3$ (6). At first it was thought that air-grown *Chlorella* did not excrete glycolate, but they will do so at a high O_2/CO_2 ratio (3) or after a short time lag of about 30 min (30), or if glycolate metabolism is blocked by inhibitors (see "Discussion").

The theory has developed that glycolate excretion represents an unnecessary photosynthetic end product that is eliminated by excretion (26). This theory is based upon the fact that those unicellular algae that excrete glycolate do not contain an active peroxisomal glycolate oxidase, as in higher plants, for its conversion to glycine and serine by the glycolate pathway portion of the oxidative photosynthetic carbon cycle (19, 27). Instead these algae contain a mere trace of glycolate dehydrogenase (8, 19) in membranes of their mitochondria or chloroplasts. These algae produce a limited amount of glycine and serine during photosynthesis, and because the serine is carboxyl labeled, the serine may be formed by both the glycolate and glycerate pathways (27). Excess glycolate seems to be excreted, and since algae grown on high CO_2 have half as much glycolate dehydrogenase as when grown on air (19), they should excrete more of the glycolate.

Several inhibitors have been used to block the glycolate pathway and to force glycolate excretion by algae. Krampitz (16) introduced the pyridoxal phosphate inhibitor AOA[2] ($COOH\text{-}CH_2ONH_2$) to inhibit the glyoxylate aminotransferase of the glycolate pathway with the resultant accumulation and excretion of glycolate. In its zwitter ion form AOA apparently rapidly enters cells. Our present investigation was initiated because the amount of glycolate produced in the presence of AOA seemed unusually large, and the mechanism of its involvement in glycolate metabolism is unknown.

MATERIALS AND METHODS

Chlamydomonas reinhardtii, Dangeard, (−) strain (N.90) was obtained from the R.C. Starr algal collection at the University of Texas, and 1-L cultures in 3-L Fernback flasks were grown in a phosphate and NH_4NO_3 medium at pH 7.4 as described previously (20). Fresh cultures were started from the original slant every few months. During growth the algae were continuously mixed on an Eberbach shaker and aerated with about 3 to 5% CO_2 in air or with air alone, corresponding with the experimental designation of algae grown on high CO_2 or on low CO_2. The algae were grown continuously in 150 $\mu E\ m^{-2} \cdot s^{-1}$ of light from fluorescent and incandescent lamps. The temperature of the growth medium was regulated between 21 to 23°C by fans which moved air over the flasks. Growth curves as cells/mm^3 of *Chlamydomonas* were monitored (data not shown) and the algae were harvested during the middle or late part of the log phase of growth, which was in about 48 h for the cultures on high CO_2. An aliquot of cells was counted with a Neubauer cell counter. In general, cultures with approximately 5000 cells $\cdot$ ml^{-1} were used from the log phase of growth, while cultures with 7000 or more cells $\cdot$ ml^{-1} were considered to be in the stationary phase. A fraction of the air-grown cells always appeared motile, while the cells grown on high CO_2 were mostly nonmotile.

Cells were harvested as rapidly as possible (about 10–30 min)

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[2] Abbreviation: AOA, aminooxyacetate.

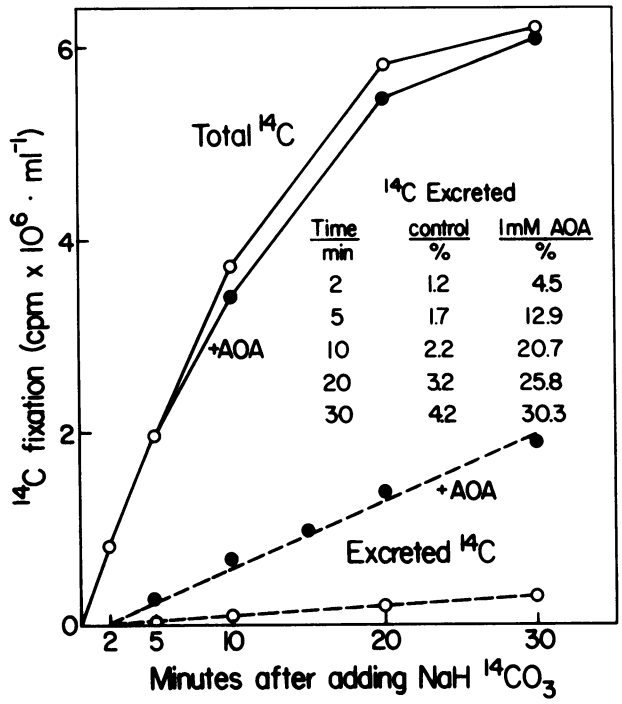

FIG. 1. Rate of $^{14}CO_2$ fixation and glycolate excretion by *Chlamydomonas* grown on air when treated with 1 mM AOA. A 2% suspension containing 4 × 10^4 cells/mm^3 from the log phase of growth were given 1 mM $NaH^{14}CO_3$ at zero time. (——), Total ^{14}C fixed; (- - -), ^{14}C excreted; (○), no treatment; (●), 1 mM AOA was added 2 min prior to addition of $NaH^{14}CO_3$.

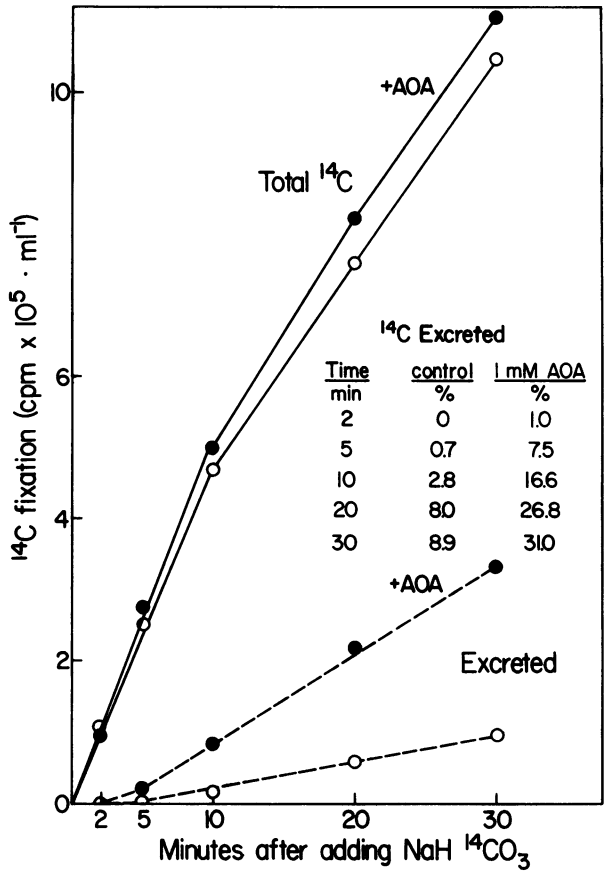

FIG. 2. Rate of $^{14}CO_2$ fixation and glycolate excretion by *Chlamydomonas* grown with 5% CO_2 when treated with 1 mM AOA. Experimental conditions were similar to those in Figure 1 except that the experiments were run at a different periods with $NaH^{14}CO_3$ of lower specific activity.

and tested immediately. The first centrigugation was in 300-ml bottles at 1000*g* for 5 min, and then the algae were washed by resuspension in one-third volume of water and recentrifuged. The pellet was then resuspended in about 20 ml water with a Pasteur pipette and put into a preweighed centrifuge tube and centrifuged at about 10,000*g* for 10 min at 4°C. After decanting the supernatant and drying the tube with a paper towel, the tube was weighed to establish cell wet weight. Approximately 1 ml packed cells weighed 1 g and contained about 1.5 to 2.0 mg Chl. The final algal suspension was prepared in 3 mM K-phosphate at pH 7.5 unless otherwise designated. A range from 1 to 10 g wet weight of cells/100 ml was tested, but in general 1 or 2% suspensions were used. Cell suspensions were used for photosynthesis experiments immediately and never later than 2 h after harvest.

Aliquots of 1 to 3 ml of the algal suspension were placed in a photosynthetic vessel consisting of either flattened test tubes or 2 cm diameter glass vials with a flat bottom so that the light path through the solution was about 2 cm. Most experiments were run with a plastic block which held six vials in a circulating water bath at 25°C, and the contents of each vial were mixed by a magnetic stirrer. For experiments in test tubes, the samples were mixed by a stream of CO_2-free air. Illumination of 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$ from a projector with a built in IR heat filter was used in most experiments. Lower light intensities were obtained by moving the projector or by use of neutral density filters.

After 2 min for light adaptation, 10- to 20-μl aliquots of $NaH^{14}CO_3$ were added to each algal suspension to provide the final bicarbonate concentrations. When AOA or NH_4NO_3 was added, it was present during this 2-min adaptation period. At time intervals, 100- to 500-μl aliquots of the algae suspension were removed and the cells were separated from the supernatant fluid by centrifugation for 10 s in a microfuge or by sedimentation through silicone oil (9). Similar results for the distribution of fixed ^{14}C were obtained by both methods and because centrifugation was simpler, the silicone oil procedure was not used in latter work. Both the supernatant and cell fractions were immediately mixed with 60% methanol and boiled. Aliquots were quantitatively assayed for glycolate by the Calkins procedure (25). Aliquots of the supernatant were added to counting vials containing 200 μl of 2 N HCl. The rest of the supernatant was stored separately in acid for chromatographic analysis. Aliquots of the cell fraction were also put into counting vials with acid. All samples were allowed to stand for at least 2 h in the strong acid for removal of CO_2 and then the fixed ^{14}C was measured with a scintillation counter and a Triton X-100 based scintillant.

Aliquots of the supernatant and of the cells were chromatographed for product identification on Whatman No. 1 paper as described earlier (2). Water-saturated phenol was the first solvent and butanol:proprionic acid:water was the second solvent. Before the paper was completely dry after the second solvent development, it was sprayed with a 1 M $NaHCO_3$ solution over the general area of the acids, to prevent volatilization of glycolic acid. The radioactive compounds were located by exposure to X-ray film for about 2 weeks, and the spots were cut out of the chromatograms and into 5-mm^2 pieces and placed into scintillation vials for counting of each compound. Compound identification was based upon the standard chromatograph map of R_F values (2) and cochromatography with carriers, which were identified on the chromatograms by appropriate spray tests. Several experimental parameters affecting ^{14}C excretion and the time and rate of $^{14}CO_2$ fixation, as discussed in the results, were explored to establish the experimental conditions.

Table I. *^{14}C Distribution among Products from Air-Grown Chlamydomonas with AOA Treatment*

See Figure 1 for total fixation and excretion at other time points.

	No Treatment						1 mM AOA					
	2 min			10 min			2 min			10 min		
Total ^{14}C fixed ml	7.88×10^5 cpm 1.5%			3.72×10^6 cpm 2.2%			7.67×10^5 4.5%			3.39×10^6 20.7%		
	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total
	%											
Products												
Glycolate	0	72.6	0.3	0.9	77.7	3.8	9.2	46.5	11.7	10.6	86.7	41.1
Phosphate esters	25.3	0	25.2	37.9	0	29.2	10.7	0	10.0	17.7	0	10.5
UDPG	0	0	0	2.8	0	2.1	0.4	0	0.4	1.4	0	0.8
Malate	14.9	24.4	14.9	13.0	20.0	30.0	20.5	5.9	19.5	11.5	3.0	8.0
Glutamate	14.8	0	14.7	15.9	2.3	12.3	9.4	0	8.7	19.7	1.1	12.2
Aspartate	6.5	0	6.4	4.7	0	3.6	22.3	0.5	20.8	25.3	2.1	15.9
Lactate	26.3	0	26.2	14.6	0	11.2	16.6	0	15.5	9.2	1.8	6.2
Succinate	5.8	0	5.8	3.8	0	3.0	6.6	0	6.1	1.6	0	0.9
Fumarate	1.1	0	1.1	1.0	0	0.8	1.1	0	1.0	0.4	0	0.2
Glutamine	0	0	0	1.7		1.3	0.5	0	0.5	1.2	0.9	1.1
Alanine	5.4	0	5.4	3.6	0	2.8	0.5	0	0.5	0	0	0
Pyruvate	0	0	0	0	0	0	2.3	47.1	5.2	1.4	4.5	2.7

Table II. *^{14}C Distribution among Products from High-CO_2-Grown Chlamydomonas with AOA Treatment*

See Figure 2 for total fixation and excretion at other time points.

	No Treatment						1 mM AOA					
	2 min			10 min			2 min			10 min		
Total ^{14}C fixed ml	1.04×10^5			4.68×10^5			9.58×10^4			4.97×10^5		
Supernatant	0%			2.8%			1.0%			16.6%		
	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total	Cells	Supernatant	Total
	%											
Products												
Glycolate	0	0	0	3.0	97.8	12.7	0	100	0.9	5.3	99.6	37.3
Phosphate esters	12.0	0	12.0	25.9	0	23.3	12.9	0	12.8	16.0	0	10.5
UDPG	0	0	0	1.8	0	1.7	0	0	0	0	0	0
Malate	16.9	0	16.9	13.9	2.2	12.7	33.5	0	33.2	14.1	0.4	9.4
Glutamate	10.0	0	10.0	5.4	0	4.8	19.2	0	19.0	23.2	0	15.3
Aspartate	4.7	0	4.7	1.2	0	1.1	34.5	0	34.2	21.1	0	13.9
Lactate	51.3	0	51.3	34.9	0	31.3	0	0	0	15.4	0	10.2
Succinate	0	0	0	3.1	0	2.8	0	0	0	3.9	0	2.6
Fumarate	0	0	0	0.4	0	0.3	0	0	0	1.0	0	0.7
Glutamine	0	0	0	0	0	0	0	0	0	0	0	0
Alanine	5.1	0	5.1	10.4	0	9.3	0	0	0	0	0	0
Pyruvate	0	0	0	0	0	0	0	0	0	0	0	0

RESULTS

All experiments were replicated with *C. reinhardtii* grown on air as well as on 5% CO_2. The rates of CO_2 fixation were linear for 10 or 20 min with 1 to 3 mM $NaHCO_3$ in a 2% algae suspension (Figs. 1 and 2). In general, labeling of glycolate from $^{14}CO_2$ had an initial lag period of several minutes relative to ^{14}C incorporation into the phosphate esters and C_4 acids as previously observed (22). This lag in glycolate labeling was more pronounced with cells grown on 5% CO_2. The lag is attributed to the time to label ribulose-P_2, the glycolate precursor, during which time unlabeled glycolate was being formed.

In the accompanying paper (16), Krampitz has noted that a 10% *Chlorella pyrenoidosa* culture aerated with 0.2% CO_2 in air produces enough glycolate in 1 h with AOA to form about a 10 mM solution. Using the Calkins colorimetric assay for glycolate, we observe in our procedure the production of at least 75 μg glycolate/ml · h by 3 to 5% *Chlamydomonas* cells with 1 or 2 mM AOA and the addition of 1 mM $NaHCO_3$ every 15 min. This amount of glycolate is equal to a 1 mM solution after 1 h. The rest of this report deals only with the excretion of photosynthetic $^{14}CO_2$ fixation products.

The distribution of fixed ^{14}C, after a time period of $^{14}CO_2$ photosynthesis, between the cellular fraction and the supernatant media was measured after rapid separation of the two fractions by centrifugation (Figs. 1 and 2). This simple technique was more rapid than filtration of the algae on a celite bed as originally used to study glycolate excretion by algae (25) or chloroplasts (15), but the results were essentially the same. Data in Figures 1 and 2 and Tables I and II are expressed first on the basis of percentage of the total $^{14}CO_2$ fixed photosynthetically that was excreted. Then

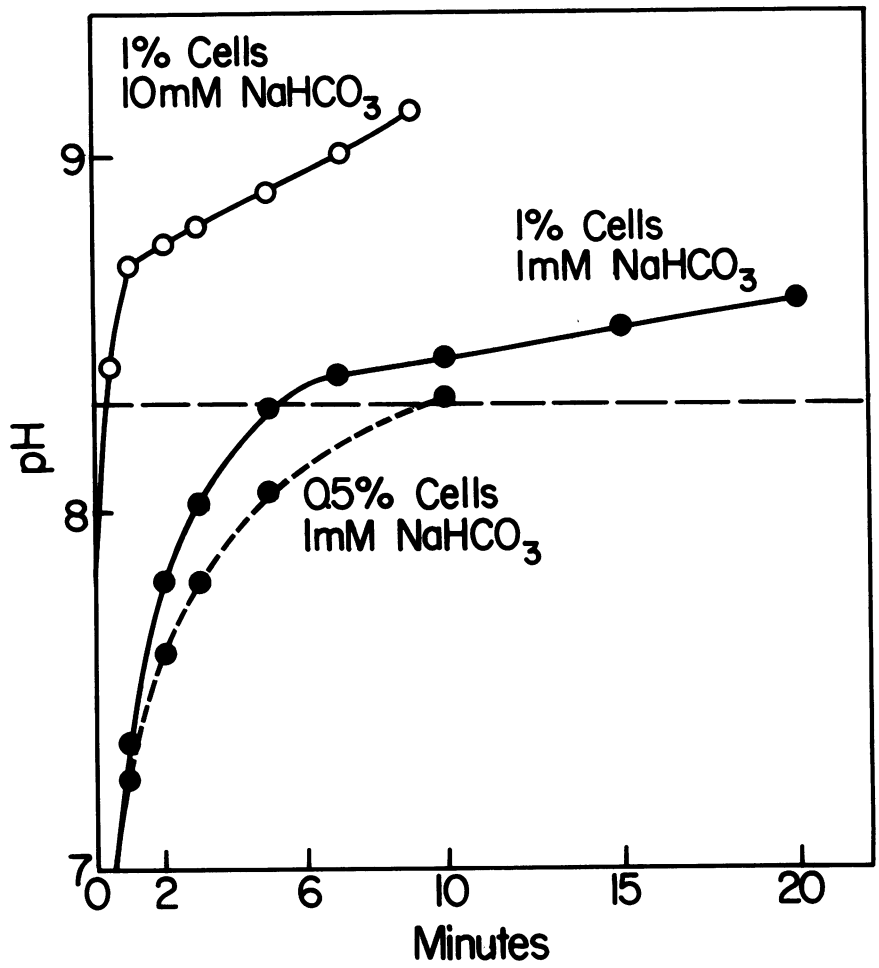

FIG. 3. Increase in pH by air-grown *Chlamydomonas* cells in water in the light upon addition of $NaHCO_3$. (— — —), pH of a $NaHCO_3$ solution. The first pK_a of a bicarbonate solution is 6.3 and the second is 9.4. The pH before adding $NaHCO_3$ was about 5.5 from the natural acidity of the algal suspension. pH was measured by electrodes in the algal suspension. (●- - -●), 0.5% cells in 1 mM $NaHCO_3$; (●——●), 1% cells in 1 mM $NaHCO_3$; (○——○), 1% cells in 10 mM $NaHCO_3$. The pH increase with a 2% cell suspension and 10 mM $NaHCO_3$ was similar to that with 1% cells and 10 mM $NaHCO_3$.

per cent ^{14}C distributions among the different products of both the cell and supernatant fractions are presented. The total percentage of fixed ^{14}C in a compound is the sum of that in the cells plus that in the supernatant. Glycolate represented 98% of the ^{14}C-labeled content in the media after $^{14}CO_2$ fixation periods of longer than 2 min by algae grown on high CO_2. In the media from algae grown on low CO_2, small amounts of malate and other labeled products were also present which reduced the percentage ^{14}C in glycolate to 78 or 87%. In exposures of 2 min or shorter to $^{14}CO_2$, the glycolate pool was not highly labeled and little ^{14}C was excreted although unlabeled glycolate was probably being excreted. Malate, aspartate, glutamate, and phosphate esters contained most of the ^{14}C label in the first 2 min, and the presence of about 1% of these labeled acids and amino acid products in the media was a significant percentage of the small amount of ^{14}C excreted. However, with time, only the amount of [^{14}C]glycolate in the media increased, as if the small pool of the other labeled products represented some background cell leakage or breakage. In subsequent sections, the phrases glycolate excretion or excreted ^{14}C are interchanged, but detailed chromatographic analysis of every sample was not always run. The percentage of ^{14}C in glycolate increased with time for several reasons. (a) The glycolate excreted was an end product that accumulated with time and contained an ever larger percentage of the ^{14}C. In this respect, labeling of glycolate was similar to the label into sucrose, while label into other organic acids and phosphate esters decreased in percentage of total ^{14}C fixed with time. (b) In the first 2 min of $^{14}CO_2$ fixation, there was a lag in the appearance of ^{14}C in glycolate, inasmuch as it is assumed to be formed from ribulose-P_2 which must first be labeled by the reductive photosynthetic carbon cycle. (c) Toward the end of the experiments (*i.e.* after 20 min) when the CO_2 concentration had dropped due to utilization of the $NaHCO_3$ and when the pH had increased, a higher percentage of the ^{14}C appeared in glycolate presumably because there was less CO_2 for the competition between the ribulose-P_2 carboxylase *versus* oxygenase reaction (see next section).

As reviewed in the introduction, glycolate excretion has been reported to occur in much larger amounts with algae grown on high CO_2. Later reports concluded that excretion occurred only from such CO_2-adapted algae. In our current experiments, considerable glycolate excretion occurred by air-grown cultures of *Chlamydomonas*, although more glycolate production in general seemed to occur with CO_2-grown cultures (compare Fig. 1 with Fig. 2).

Bicarbonate Concentration and pH. The pH and $NaH^{14}CO_3$ concentration were two related factors greatly affecting glycolate excretion. Addition of $NaHCO_3$ to an unbuffered 2% algal suspension in the light resulted in a rapid pH increase (Fig. 3) which rose to between 9 and 10 within a few minutes. This has been attributed to HCO_3^- uptake in exchange for OH^- (7). The rate of

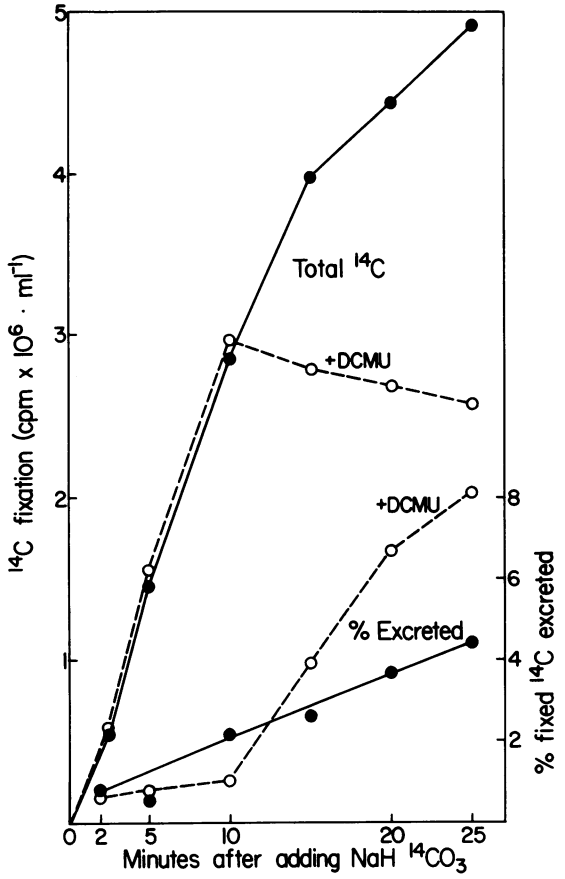

FIG. 4. Effect of DCMU on photosynthetic $^{14}CO_2$ fixation and excretion of ^{14}C by air-grown *Chlamydomonas*. DCMU was added after 10 min to one culture (○); (●), control without DCMU. Total ^{14}C fixation is shown by the two upper lines and the per cent of the fixed ^{14}C that was found in the medium by the two lower lines.

alkalinity increase in the supernatant was dependent on both $NaHCO_3$ concentration and cell density. Upon increasing the cell density from 0.5 to 2%, the rate of increase of pH increased. The rate of pH rise was much greater at 10 mM $NaHCO_3$ than at 1 mM $NaHCO_3$ for a given cell density, and this faster rate of pH increase was against the increased buffer capacity of 10 mM $NaHCO_3$. Because of the HCO_3^-/OH^- exchange, the pH of the algal suspension quickly rose above 8.3 (pH of $NaHCO_3$) into a range which was inhibitory to photosynthesis. This shift with 1 or 2% algae to an unfavorably high pH occurred within about 1 min with 10 mM $NaHCO_3$ and within 5 to 10 min with 1 mM $NaHCO_3$. An initial rate of $^{14}CO_2$ fixation in the 1st min after adding $NaH^{14}CO_3$ was found to be faster than in the buffers we examined. However, in the unbuffered solution the rate of photosynthesis slowed down very quickly, making comparison of rates difficult to interpret.

When the rate of CO_2 fixation decreased due to either a pH increase or to utilization of the added $NaHCO_3$, the percentage of the fixed $^{14}CO_2$ that was excreted as glycolate increased (data not shown) (20). As the $NaH^{14}CO_3$ was used up in a buffered solution after 20 min, the rate of ^{14}C fixation decreased but the per cent ^{14}C excreted increased. The rate of photosynthesis will be linear until CO_2 becomes limiting, but the rate of glycolate excretion is exponential. These results are attributed to preferential ribulose-P_2 oxygenase activity for glycolate synthesis over limited ribulose-P_2 carboxylase activity in the absence of sufficient CO_2. Consequently, it was not possible to study variables effecting glycolate excretion without pH control by either titration with acid, use of $^{14}CO_2$ rather than $NaH^{14}CO_3$, or the use of buffer. In this report, we have used buffers for convenience.

Effect of Buffers on Glycolate Excretion. To investigate glycolate excretion, the pH must be controlled as explained in the previous section. A limited investigation of buffers indicated that they all seemed to inhibit the initial rate of ^{14}C fixation, but that Hepes and Tris at pH 7.4 seemed more inhibitory than phosphate. *Chlamydomonas* in 50 mM Hepes initially fixed ^{14}C at less than 10% of the rate without buffer, but the use of 1 mM Hepes resulted in only a 10 to 20% inhibition. Increased ^{14}C excretion by algae in Hepes buffer was sometimes observed but the products were not analyzed chromatographically. After this limited investigation, we elected to use 3 mM phosphate at pH 7.5 for the photosynthetic media. Higher concentration of around 10 mM would have provided better buffering, but would have limited subsequent chromatographic analyses of the excreted products. During a normal photosynthetic experiment with 1 mM $NaH^{14}CO_3$ added to the phosphate buffer at pH 7.5, about 25% of the total ^{14}C was lost over the 20-min test period, presumably due to CO_2 escaping from the stirred or aerated algal medium. For this reason, experiments were performed in a ventilated hood. Generally, after 20 min most of the ^{14}C had been fixed or lost. Higher concentrations of 3 and 10 mM $NaHCO_3$ did not increase the initial rate and, due to CO_2 loss as a gas from the open flasks, did not greatly prolong the linear period for photosynthesis.

Effect of Cell Age and Storage Condition after Harvest. If the algal cells were harvested and washed as described in the methods section, and then allowed to let stand in water in the dark for 1 to 2 h, the medium became acidic, decreasing to pH 4.5. Cells 2 h or more after harvest had slower rates of CO_2 fixation and were less able to increase the media pH upon addition of $NaHCO_3$. Aerating the cells, storage in light, or use of buffers did not prevent deterioration of their photosynthetic capacity. Rather than investigate this aspect further, only cells freshly harvested within 1 h were used in all experiments.

Production of Acids and Amino Acids during $^{14}CO_2$ Fixation. Benson and Calvin (1) using *Chlorella* first observed a large percentage of the early products of $^{14}CO_2$ fixation to be in organic acids. After 1950 when the phosphate esters of the reductive photosynthetic carbon cycle were considered to be the early product of $^{14}CO_2$ fixation, the rates and the significance of labeling the acids by these algae were largely ignored. These compounds include malate, aspartate, glutamate, glutamine, citrate, succinate, and fumarate (Table I and II). The early ^{14}C labeling of these acids during photosynthesis seems similar to their labeling by C_4 plants. Recently, reinvestigation of $^{14}CO_2$ fixation products by algae has emphasized the large amount of ^{14}C incorporation into the acids as well as into phosphate esters. Our data in Table I and II for *Chlamydomonas* grown on either low or high CO_2 is supportive of this labeling pattern which needs further investigation. The large percentage of 25 to 50% of the total ^{14}C in these acids during initial $^{14}CO_2$ photosynthesis indicates that the acid labeling is not simply due to slow dark respiration superimposed on photosynthesis.

Effect of AOA upon Glycolate Biosynthesis and Excretion. The rate of photosynthetic $^{14}CO_2$ fixation by a 2% air-grown *Chlamydomonas* cell suspension with 1 mM $NaHCO_3$ and in high light intensity was not greatly effected by 1 mM AOA (Fig. 1). In these experiments, the amount of the total ^{14}C fixed that was excreted increased from 4% by the air-grown cells after 30 min to 30% after 30 min with 1 mM AOA. Excretion by the cells grown on 5% CO_2 was also increased by AOA (Fig. 2). Although the untreated cells grown on high CO_2 excreted more of the total ^{14}C as glycolate than the air-grown and untreated cells, both cultures excreted the same percentage of the total ^{14}C as glycolate when treated with AOA. This result might be consistent with the standard hypothesis that the ability to metabolize glycolate by the cells regulates

Table III. *Distribution of ^{14}C among Excreted Products from Chlamydomonas* Treated with DCMU

This experiment is similar to the one in Figure 4 but with half as many algae so that a longer ^{14}C fixation period could be used.

Compound	After 20 min		After 40 min		After 20 min Control plus 20 min with DCMU	
	cpm	*%*	*cpm*	*%*	*cpm*	*%*
Glycolate	29,093	89.60	62,081	94.5	30,271	59.2
Phosphate esters	0		0		3,835	7.5
Malate	2,576	7.9	2,674	4.1	9,205	18.0
Glutamate	376	1.2	478	0.7	3,013	5.9
Aspartate	0		0		350	0.7
Lactate	208	0.6	0		633	1.2
Succinate	0		465	0.7	3,241	6.3
Glutamine	0		0		544	1.1
Other	210	0.6	0		51	0.1
Total in aliquot	32,463	99.9	65,698	100.0	51,143	100

Table IV. *Effect of AOA and Ammonium Nitrate on Glycolate Excretion by Chlamydomonas Grown with Low CO_2*

Algal Media and Time	Treatment							
	0		1 mM AOA		3 mM AOA		10 mM AOA	
	total cpm	*% excreted*	*total cpm*	*% excreted*	*total cpm*	*% excreted*	*total cpm*	*% excreted*
Control								
10 min	6.5×10^4	0.2	8.0×10^4	5.4	7.5×10^4	6.7	6.7×10^4	5.8
20 min	1.3×10^5	0.4	1.4×10^5	13.4	1.4×10^5	13.2	1.2×10^5	12.2
Plus 0.2 mM NH_4NO_3								
10 min	5.5×10^4	0.4	6.4×10^4	0.2	6.6×10^4	0.2	6.3×10^4	2.5
20 min	1.1×10^5	0.5	1.6×10^5	0.3	1.3×10^5	1.2	1.2×10^5	8.6

Table V. *Effect of O_2 and AOA on ^{14}C Excretion by Chlamydomonas*

Air-grown cells were 3 d old and entering the stationary phase. CO_2-grown cells were 5 d old and in the stationary phase. Values are % of total ^{14}C fixed that was excreted after a period of 20 min of photosynthesis with 3 mM $NaH^{14}CO_3$. Inasmuch as the experiments were run in the light and in tubes open to the air, strictly anaerobic conditions were not obtained when gassing with N_2.

O_2 Concn.	Air-Grown Cells		5% CO_2-Grown Cells	
	0	1 mM AOA	0	1 mM AOA
	% ^{14}C excreted			
0 (N_2)	1.3	5.1	2.0	4.8
21% (air)	1.0	8.4	8.0	13.7
100%	3.0	24.9	16.4	22.7

glycolate excretion. When this metabolism was blocked by AOA, which is known to inhibit aminotransferases such as those involved in glycolate metabolism, both cells grown on low or high CO_2 made and excreted the same amount of glycolate. In the "Discussion," reasons will be cited why this hypothesis does not seem to explain all the data.

The products of CO_2 fixation by AOA were altered in two major ways. Most dramatic was that the total ^{14}C in glycolate increased from 0.3% after 2 min to 11.7% with AOA and from 3.8% after 10 min to 41.1% with AOA (Table I). The maximum percentage of the total ^{14}C in glycolate generally varied from 20 to 75% dependent upon unknown factors. In longer term experiments when $NaHCO_3$ became limiting, which increased glycolate production normally, the percentage of fixed ^{14}C in glycolate could reach values of 50 to 75% of the total ^{14}C if AOA were present. Most of this massive production of glycolate was excreted outside the cells into an end product pool. However, glycolate in the cells treated with AOA could amount to 10% of the ^{14}C retained in the cell. Somewhat similar results were obtained with the algae grown on 5% CO_2 (Table II).

Previous literature has indicated that algae grown on high CO_2 produce more [^{14}C]glycolate when tested with low levels of $NaH^{14}CO_3$. A comparison of results in Tables I and II hardly supports this concept. Both the air-grown and CO_2-grown cells produced and excreted about the same percentage of the newly fixed $^{14}CO_2$ as glycolate with or without AOA. Since the two experiments for Tables I and II were done with different ^{14}C specific activity, no comparison between total fixation can be done, but in general both types of algae initially fixed about the same amount of CO_2 per cell per unit of time.

Effect of AOA upon Other Products of Photosynthesis. Lactate was a major product of $^{14}CO_2$ fixation by *Chlamydomonas* (Table I and II). This observation has not been pursued in the past, although Warburg *et al.* (29) reported in 1957 that D-lactate was a major product from CO_2 fixation by *Chlorella*, and lactate dehydrogenase is present in algae (8). AOA reduced the percentage of the total ^{14}C in lactate while increasing the per cent ^{14}C in glycolate. Lactate was not excreted, whereas glycolate, its structural analog, was mostly excreted. These observations suggest a specific translocator mechanism for glycolate.

A definite metabolic change from AOA treatment of air-grown *Chlamydomonas* was the complete blockage of [^{14}C]alanine formation and a nearly corresponding increase of [^{14}C]pyruvate (Table I). Part of the pyruvate was excreted. In control cells, the pool size of pyruvate was too small to be detected by the chromatographic procedure. AOA also blocked alanine formation by *Chlamydomonas* grown on high CO_2, although pyruvate accumulation was not noted. This action of AOA would seem to be an inhibition of the aminotransferase reaction from pyruvate to alanine.

With increased glycolate biosynthesis from AOA, ^{14}C accumulation decreased particularly significantly in the phosphate esters comprising the reductive photosynthetic carbon cycle. This would

be consistent with the biosynthesis of P-glycolate from ribulose-P_2. How the $^{14}CO_2$ fixation rate was maintained under these circumstances is not clear, but it is assumed that the smaller pools of phosphate esters were sufficient to maintain the rate of CO_2 fixation.

The early CO_2 fixation products by *C. reinhardtii* included malate, aspartate, glutamate, and other acids of the tricarboxylic acid cycle (succinate and fumarate). With AOA, an initial larger percentage of ^{14}C incorporation went into these C_4 acids before labeling of glycolate, although AOA did not alter the per cent distribution of ^{14}C into these acids as much as into glycolate and alanine. The per cent ^{14}C incorporated into aspartate was significantly increased by AOA from 6.5 to 22.3% after 2 min of photosynthesis and from 4.7 to 25.3% of the ^{14}C in the cell after 10 min. Aminotransferase reactions are involved in aspartate and glutamate formation and metabolism, so the reason for aspartate accumulation in the presence of AOA is not known. The per cent ^{14}C in glutamate within the cells was probably not altered significantly by AOA.

The presence of a little malate, aspartate, and glutamate in the supernatant from the cells is to be noted. Since the control cells initially excreted only 1 or 2% of the total ^{14}C fixed, the small amount of these excreted acids represents 20 to 24% of the total ^{14}C excreted. In the AOA-treated cells, which excreted large amounts of glycolate, the presence of this small amount of malate represented only a few per cent of the total excretion. In order to simplify the results, the limited amount of these other organic acids and amino acids in the supernatant has been disregarded. However it is to be noted that none of the phosphate esters appeared outside of the cells.

Effect of Light, DCMU, or Darkness on Glycolate Biosynthesis and Excretion. Glycolate biosynthesis is light dependent (26), just as is $^{14}CO_2$ fixation. Assuming that glycolate is formed by ribulose-P_2 oxidation, the dependency on light could be explained by regeneration of ribulose-P_2 and the activation of the carboxylase/oxygenase. Indeed, DCMU or darkness blocks both CO_2 fixation and glycolate biosynthesis (26). In the older DCMU experiments, the algae or plants were poisoned prior or simultaneous with addition of $^{14}CO_2$, and as a consequence no ^{14}C fixation occurred. If glycolate was being formed from endogenous products, it would not have been labeled with ^{14}C. In the present experiments, the *Chlamydomonas* cells with AOA were prelabeled with $^{14}CO_2$ for 10 min and then treated with DCMU or put in darkness. DCMU or darkness stopped $^{14}CO_2$ fixation and in fact the total fixed ^{14}C in the cells suspension decreased, presumably from dark respiration (Fig. 4). DCMU or darkness did not stop ^{14}C excretion into the medium. As a result, the percentage of the ^{14}C in the medium increased with time (Fig. 4). Paper chromatographic analysis of the excreted products indicated that, whereas glycolate was the primary excreted product in the absence of DCMU, in the presence of DCMU the cells lost all of their products labeled with ^{14}C (Table III). In the presence of DCMU, other products, particularly malate, glutamate, and succinate, were lost from the cell and account for the continued excretion. Thus, the loss of total ^{14}C and the excretion of products of respiration are consistent with continued respiratory metabolism, but no photosynthesis or glycolate formation or excretion occurred in the presence of DCMU.

Effect of AOA Concentration, Ammonium Nitrate, and O_2 on Glycolate Excretion. AOA concentrations between 1 and 10 mM had no great effect on the rate of CO_2 fixation by *Chlamydomonas* (Table IV). Because 1 mM AOA was as effective as 10 mM for stimulating glycolate formation and excretion, in all other experiments 1 or 2 mM AOA was used.

As reported by Krampitz (16), addition of 1 to 2 mM NH_4Cl to the algal medium inhibited glycolate excretion. As shown in Table IV, addition of 0.2 mM NH_4NO_3 to the *Chlamydomonas* blocked AOA stimulation of ^{14}C excretion of new fixed $^{14}CO_2$. Higher concentrations of AOA (10 mM), however, did stimulate ^{14}C excretion in the presence of 0.2 mM NH_4NO_3 (Table IV). Similar results were found with *Chlamydomonas* in the log phase of growth or resting phase of development. Paper chromatographic analysis of the ^{14}C fixation products at the end (20 min) of the experiment with or without AOA indicated that the major ^{14}C products of the cells treated with NH_4NO_3 were aspartate and glutamate in both the cells and in the supernatant. Similar accumulation of amino acids by algae and leaves during photosynthesis in the presence of excess NH_4NO_3 has been detailed (17). The results indicate that any increased excretion with higher levels of AOA and NH_4NO_3 is not the result of more glycolate formation and excretion but rather increased cell leakage. AOA stimulation of glycolate production was blocked by NH_4NO_3.

O_2 stimulated ^{14}C excretion by *Chlamydomonas* (Table V) as expected from the increased rate of glycolate biosynthesis by ribulose-P_2 oxygenase in competition with the carboxylase reaction. The old cells grown on 5% CO_2 excreted much more than cells grown on air. The combination of increased O_2 and AOA resulted in more ^{14}C excretion than from either alone. With AOA and O_2, the air-grown algae excreted as much ^{14}C as the CO_2-grown cells, as was also observed in the experiments on the effect of AOA upon glycolate excretion. It appears as if both O_2 and AOA stimulated glycolate biosynthesis and excretion, but that the amount did not exceed similar percentages of fixed $^{14}CO_2$ for both algal cultures.

Carbonic Anhydrase and Glycolate Excretion. Ethoxyzolamide, a carbonic anhydrase inhibitor, causes a 7-fold increase in glycolate excretion while inhibiting photosynthesis 73% by *Chlorella* (13). Diamox, another carbonic anhydrase inhibitor, also stimulated glycolate excretion by the blue-green algae *Coccochloris peniocystis* (12). These results seem similar to the increase in glycolate production at pH over 8 or when the $NaHCO_3$ is near depletion during photosynthesis (previous section), and can be attributed to decreased CO_2 availability relative to O_2. Increased glycolate excretion has been attributed to the absence of carbonic anhydrase in 5% CO_2-grown algae or the inhibition of the anhydrase, which lowers the effective CO_2 level in the cell to favor the ribulose-P_2 oxygenase reaction over the carboxylase. That AOA might be functioning in a similar way was explored by testing it as an inhibitor of carbonic anhydrase. One mM AOA did not significantly inhibit bovine carbonic anhydrase. From a sonicated homogenate of *Chlamydomonas* cells, a $(NH_4)_2SO_4$ fraction for carbonic anhydrase was prepared (21). AOA also did not inhibit this algal carbonic anhydrase. Jahnke (13) has pointed out that the carbonic anhydrase inhibitors have little effect on CO_2-grown *Chlorella* because they contain little carbonic anhydrase. Inasmuch as AOA caused both air-grown and CO_2-grown *Chlamydomonas* to excreted glycolate, it seems that the primary site of action of AOA is not upon carbonic anhydrase.

DISCUSSION

Mode of Action of AOA. The known mode of AOA inhibition is to combine with pyridoxal phosphate to inhibit aminotransferase reactions. Thus, it should inhibit glyoxylate transamination to glycine, a reaction coupled to glutamate, or a serine:glyoxylate aminotransferase in the oxidative photosynthetic carbon cycle (28). The reversal of the AOA stimulation of glycolate excretion by NH_4NO_3 is consistent with a stimulation of the glyoxylate aminotransferase reactions by additional amino groups available from the excess NH_4NO_3. AOA likewise inhibited the formation of alanine, which led to the accumulation and excretion of pyruvate. It is puzzling why AOA did not block the biosynthesis of aspartate or glutamate during photosynthesis by *Chlamydomonas*, which would have involved aminotransferase reactions, but if anything, AOA stimulated the formation of these amino acids. AOA did not inhibit CO_2 photosynthetic fixation by *Chlamydo-*

monas.

Glycolate biosynthesis, excretion, and conversion to glycine and serine combined normally amounted to about 2 to 10% of the ^{14}C fixed in 10 or 20 min from $NaH^{14}CO_3$ by *Chlamydomonas* cells in 3 mM phosphate buffer at pH 7.5. Most of the glycolate would be excreted and in our current experiments so little (<1%) ^{14}C was present in glycine and serine that these compounds were not included in Tables listing the main labeled products of CO_2 fixation. Glycolate excretion has been attributed to an insufficient system for its metabolism; glycolate oxidase is absent and glycolate dehydrogenase activity is very small. *Chlamydomonas* contained adequate ribulose-P_2 carboxylase/oxygenase and P-glycolate phosphatase for glycolate biosynthesis (23). They also contain 1 to 2 $mmol \cdot h^{-1} \cdot mg^{-1}$ Chl of NADPH glyoxylate reductase (23) and an active glutamate or alanine:glyoxylate aminotransferase for glycine formation. Thus, the aminotransferase inhibitor AOA should have inhibited the conversion of glyoxylate to glycine and the glyoxylate could be reduced to glycolate by the NADPH:glyoxylate reductase. We never observed the accumulation of significant quantities of glyoxylate on the chromatograms of *Chlamydomonas* products, even with AOA treatment.

Comparison of AOA with Other Inhibitors of Glycolate Metabolism. Previous experiments with air-grown algae had pointed out that glycolate excretion could be forced by inhibitors of the glycolate pathway, such as isonicotinyl hydrazide or hydroxypyridine methane sulfonate (6, 14, 19). With air-grown cells 0.2 to 1 mM isonicotinyl hydrazide increased glycolate excretion at pH 7.0 from nil to about 30 $nmol \cdot h^{-1} \cdot ml^{-1}$ of 2% (v/v) cells (19). Isonicotinyl hydrazide increased glycolate excretion by *Chlorella* to 17 μg glycolate $\cdot mg^{-1}$ dry weight $\cdot h^{-1}$ (7, 21), but the hydrazide had no effect on glycolate excretion by the blue-green algae *C. peniocystis* (12). α-Hydroxypyridinemethanesulfonate, an inhibitor of glycolate oxidase as well as glycolate dehydrogenase, stimulates glycolate excretion by green algae (13), blue-green algae (12), and *Rhodospirillum rubrum* (5). Thus, the increase in glycolate excretion with AOA would seem to be consistent with the use of other inhibitors of the glycolate pathway to stimulate glycolate excretion. However, the greater magnitude of glycolate excretion from AOA treatment of both CO_2-grown and air-grown algae and the consistency of the results raises questions about this much glycolate excretion which have not been considered previously.

Although AOA ($COOH\text{-}CH_2ONH_2$) is an analog of glycolate, its possible hydrolysis to glycolate cannot account for the large amount of [^{14}C]glycolate produced from $^{14}CO_2$ for two reasons. The AOA was not labeled with ^{14}C, and the amount of excreted glycolate exceeded the amount of AOA added.

Speculation about Stimulated Glycolate Excretion by AOA. Even with the above consistencies for AOA inhibition of the glycolate pathway, the immense increase in glycolate excretion by *Chlamydomonas* cells during photosynthesis in the presence of 1 mM AOA can not be readily explained. In the experiment with cells grown on air (Table II), total production of glycolate after 2 min increased from 0.3% of the total ^{14}C in controls to 11.7% by cells treated with AOA or from 3.8% after 10 min to 41% with AOA treatment. At longer times of 20 to 40 min, 50 to 75% of the total photosynthate was converted into glycolate and excreted. This phenomenon occurs with *Chlamydomonas* cells grown on either air or high CO_2. There is no evidence with these algae for such a large flow of carbon through the glycolate pathway to glycine. Glycine and serine were hardly labeled in controls without AOA. In addition, there is no enzyme of significant activity for converting glycolate to glyoxylate. Upon addition of [^{14}C]glycolate to homogenates of *Chlamydomonas* only trivial amounts were converted to glycine, glyoxylate, or CO_2. [^{14}C]Glycolate was not taken up and metabolized by the whole alga. Therefore, it would appear that there is little glycolate metabolism in this alga and that greatly increased glycolate excretion could hardly be due to AOA inhibition of the aminotransferase for glyoxylate conversion to glycine. *Chlamydomonas* also contain 1 to 2 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl of NADPH:glyoxylate reductase when assayed at its optimum of pH 6.2, but AOA inihbited it with K_i of 1 mM (23). This activity is about 1 to 2% of the rate of photosynthetic CO_2 fixation and seems to be inadequate to account for the fast rate (40% of photosynthesis) of glycolate excretion in the presence of AOA. This argument also tends to exclude biosynthesis of glyoxylate by some unknown pathway, as a precursor for the excreted glycolate. All these facts suggest that AOA might be effecting glycolate biosynthesis rather than its metabolism. However, 1 mM AOA did not inhibit purified ribulose-P_2 carboxylase or oxygenase activities from spinach leaves, or P-glycolate phosphatase, glycolate dehydrogenase, or carbonic anhydrase from the algae. Its point(s) of action is unknown.

The large amount of the total ^{14}C incorporation into glycolate during photosynthesis in the presence of AOA and the lack of evidence for much carbon flow normally from glycolate to glycine in unicellular algae, leads to speculation that the glycolate might be arising from some other source than ribulose-P_2. However, ^{14}C (10) and ^{18}O (18) distribution in glycolate formed by algae has been consistent with its formation by ribulose-P_2 oxygenase from carbohydrates of the photosynthetic carbon cycle (28).

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LITERATURE CITED

1. Benson AA, M Calvin 1950 Carbon dioxide fixation by green plants. Annu Rev Plant Physiol 1: 25–42
2. Benson AA, JA Bassham, M Calvin, M. Goodale, TC Haas, VA Haas, W Stepka 1950 The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J Am Chem Soc 72: 1710–1718
3. Berry J, J Boynton, A Kaplan, M Badger 1976 Growth and photosynthesis of *Chlamydomonas reinhardtii* as a function of CO_2 concentration. Carnegie Inst Year Book 75: 423–432
4. Chang W-H, NE Tolbert 1970 Excretion of glycolate, mesotartrate and isocitrate lactone by synchronized cultures of *Ankistrodesmus braunii.* Plant Physiol 46: 377–385
5. Codd GA, BM Smith 1974 Glycolate formation and excretion by the purple photosynthetic bacterium *Rhodospirillum rubrum.* FEBS Lett 48: 105–108
6. Colman B, AG Miller, B Grodzinski 1974 A study of the control of glycolate excretion in *Chlorella.* Plant Physiol 53: 395–397
7. Findenegg GR 1977 Interaction of glycolate$^-$, HCO_3^-, Cl^- and H^+ balance of *Scenedesmus obliquus.* Planta 135: 33–38
8. Gruber PJ, SE Frederick, NE Tolbert 1974 Enzymes related to lactate metabolism in green algae and lower land plants. Plant Physiol 53: 167–170
9. Heldt HW 1980 Measurement of metabolite movement across the envelope and of the pH in the stroma and the thylakoid space in intact chloroplasts. Methods Enzymol 69: 604–613
10. Hess, JL, NE Tolbert 1967 Glycolate biosynthesis by *Scenedesmus* and *Chlorella* in the presence or absence of $NaHCO_3$. Plant 74: 278–285
11. Howitz KT, RE McCarty 1982 pH dependence and kinetics of glycolate uptake by intact pea chloroplasts. Plant Physiol 70: 949–952
12. Ingle RK, B Coleman 1976 The relationship between carbonic anhydrase activity and glycolate excretion in the blue green algae *Coccochloris peniocystis.* Plant 128: 217–223
13. Jahnke LS 1981 Indirect effects of α-hydroxypyridinemethanesulfonate and cyanide on photosynthesis and glycolate excretion in *Chlorella.* Z Pflanzenphysiol 104: 373–383
14. Kaplan A, JA Berry 1981 Glycolate excretion and the oxygen to carbon dioxide net exchange ratio during photosynthesis in *Chlamydomonas reinhardtii.* Plant Physiol 67: 229–232
15. Kearney PC, NE Tolbert 1962 Appearances of glycolate and related products of photosynthesis outside of chloroplasts. Arch Biochem Biophys 98: 164–171
16. Krampitz LO, CE Yarris 1983 Glycolate formation and excretion by *Chlorella pyrenoidosa* and *Netrum digitus.* Plant Physiol 72: 1084–1087
17. Lawyer AL, KL Cornwell, PO Larsen, JA Bassham 1981 Effects of carbon dioxide and oxygen on the regulation of photosynthetic carbon metabolism by ammonium in spinach mesophyll cells. Plant Physiol 68: 1231–1236
18. Lorimer GH, CB Osmond, T Aakazawa, S Asami 1978 Mechanism of glycolate synthesis by *Chromatium* and *Chlorella.* Arch Biochem Biophys 185: 49–56
19. Nelson EB, NE Tolbert 1969 The regulation of glycolate metabolism in *Chlamydomonas reinhardtii.* Biochim Biophys Acta 184: 263–270
20. Orth GM, NE Tolbert, E Jiminez 1966 Rate of glycolate formation during photosynthesis at high pH. Plant Physiol 41: 143–147

21. RICKLI EE, SAS GHAZANFAR, BH GIBBONS, JT EDSALL 1964 Carbonic anhydrase from human erythrocytes. J Biol Chem 239: 1065–1078
22. SCHOU L, AA BENSON, JA BASSHAM, M CALVIN 1950 The path of carbon in photosynthesis XI. The role of glycolic acid. Physiol Plant 3: 487–495
23. SELPH NL 1982 Enzymes of glycolate metabolism in *Chlamydomonas reinhardtii*. Ph.D. Thesis. Michigan State University
24. TAKABE T, T AKAZAWA 1981 Mechanism of glycolate transport in spinach leaf chloroplasts. Plant Physiol 68: 1093–1097
25. TOLBERT NE, LP ZILL 1956 Excretion of glycolic acid by algae during photosynthesis. J Biol Chem 222: 895–906
26. TOLBERT NE 1963 Glycolate Pathway in Photosynthesis Mechanisms in Green Plants. publication 1145. National Academy of Science—National Research Council, pp 648–662
27. TOLBERT NE 1974 Photorespiration by algae. In SDD Stewart, ed, Algal Physiology and Biochemistry. Blackwell Scientific Publications Ltd, Oxford, England, pp 474–504
28. TOLBERT NE 1980 Photorespiration. In P Stumpf, E Conn, eds, The Biochemistry of Plants Vol 2. Academic Press, NY, pp 488–525
29. WARBURG VO, H-S GEWITZ, W VOLKER 1957 D-(-)-Milchsaure in *Chlorella*. Z Naturforsch 12B: 722–726
30. YOKOTA A, S HAGA, S KITAOKA 1983 Glycolate excretion by air-grown *Euglena gracilis* Z. Photosynth Res. In press