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Glycolate Formation and Excretion by *Chlorella pyrenoidosa* and *Netrium digitus*[1]

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ABSTRACT

Conditions are described whereby suspensions of *Chlorella pyrenoidosa* and *Netrium digitus* photosynthetically biosynthesize and excrete glycolate continuously in high yields. Aminooxyacetic acid, an inhibitor of pyridoxal phosphate-linked enzymes, increased the excretion of glycolate approximately 4-fold in 1 hour (8 millimolar) and 20-fold in 4 hours (40 millimolar) in the presence of 0.2% CO_2 in air. The amount of glycolate excreted in the presence of aminooxyacetate and an atmosphere of 0.2% CO_2 in air equaled or exceeded the amount excreted in 0.2% CO_2 in O_2 minus aminooxyacetate. CO_2 and light were required for glycolate excretion. Aminooxyacetate also stimulated photosynthetic glycolate excretion in an atmosphere of 0.2% CO_2 in nitrogen or helium, although the stimulation was not as great as when air or O_2 was present.

The excreted glycolate was converted to H_2 and CO_2 by the combined action of glycolic oxidase and the formic hydrogenlyase complex found in *Escherichia coli* in total conversion yields of 80%.

Glycolate is a well-known photosynthetic product of O_2-evolving forms of photosynthetic life (2, 4) which is excreted by algae (12) and chloroplasts (10). Conditions which favor glycolate formation and excretion are: high O_2 partial pressure, low CO_2 tension, and high light intensity. Under these conditions, the oxygenase activity of ribulose-P_2 carboxylase forms 3-P-glycerate and 2-P-glycolate (6). The latter is dephosphorylated by a specific phosphatase (9). CO_2 and O_2 are competitive substrates for this enzyme activity. Experiments with $^{18}O_2$ have demonstrated that it is incorporated into the carboxyl group of glycolate by the action of the carboxylaseoxygenase enzyme (1).

The goal of the research reported herein was an attempt to find conditions whereby the photosynthetic formation and excretion of glycolate could be obtained in high yields by algae in an atmosphere of air enriched with a low partial pressure of CO_2 rather than a high partial pressure of O_2 as employed by many investigators. The glycolate thus obtained could be enzymically converted to H_2 for an energy source as depicted in the following equations:

$$2CO_2 + 2H_2O \xrightarrow[\textit{Chlorella pyrenoidosa}]{h\nu} \underset{\text{OH}}{\overset{\text{H}}{\text{HC—COOH}}} + 1.5\ O_2 \quad (1)$$

$$\underset{\text{OH}}{\overset{\text{H}}{\text{HC—COOH}}} + O_2 \xrightarrow[\text{oxidase}]{\text{glycolic}} \overset{\text{H}}{\text{O=C—COOH}} + H_2O_2 \quad (2)$$

$$\overset{\text{H}}{\text{O=C—COOH}} + H_2O_2 \xrightarrow{\text{nonenzymic}} \text{HCOOH} + CO_2 + H_2O \quad (3)$$

$$\text{HCOOH} \xrightarrow[\text{hydrogenlyase}]{\text{bacterial formic}} H_2 + CO_2 \quad (4)$$

$$\text{Sum 1 through 4: } H_2O \xrightarrow{h\nu} H_2 + 0.5\ O_2$$

Equation 1 represents the photosynthetic formation of glycolate. In addition to the *Chlorella* species, we also have employed *Netrium digitus*; both are green algae. Equations 2 through 4 represent dark reactions under investigation and the sum of the equations represents the splitting of water to H_2 and O_2.

METHODS AND RESULTS

Chlorella pyrenoidosa strain 1230 and *Netrium digitus* UTEX 1255 were obtained from the University of Texas algal collection. They were cultivated in the medium of Watt and Fogg (14) with the exception that 660 mg urea/l was used as the N source. The composition of the medium was as follows.

Phosphate buffered nutrient medium (pH 6.4) for culture of *C. pyrenoidosa*. NO_3^- and NH_4^+ interfere in glycolate analysis so urea is used as nitrogen source. The ferric ion is chelated with an equimolecular amount of ethylene diaminetetracetic acid (EDTA) to keep it in solution.

Nutrient	mg/l in medium
KH_2PO_4	1,361.0
NaOH	114.0
$CO(NH_2)_2$ (urea)	660.0
$MgSO_4 \cdot 7H_2O$	49.3
$CaCl_2$	20.0
$FeCl_3$ (EDTA)	3.0
$CuSO_4$	0.010
$ZnSO_4$	0.022
$CoCl_2$	0.011
$MnCl_2$	0.180
Na_2MoO_4	0.015

Three L of medium in a 4-L flask were inoculated with 300 ml of a 48-h culture. Growth of the latter was in a 500-ml flask. Conditions for incubation of both cultures were the same. The temperature in the incubator was 30°C and contained 3% CO_2 with the balance as air. The light intensity was 10,000 lux from fluorescent lights. Contents of a culture flask were mixed with a magnetic stirrer at sufficient speed to create a visible vortex. The time of incubation was 48 h and both cultures were maintained on a 48-h cycle with continuous light. The cells from the 3-L

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Table I. *Inhibitory Effects on Glycolate Excretion*

Conditions: Temperature 30°C; the suspensions were continuously gassed with 0.2% CO_2 in air; light, 10,000 lux; pH 8.4. Time, as indicated. Other conditions described in text.

	Chlorella Cells[a] Resuspended in Watt-Fogg-Hepes Medium	Fresh *Chlorella* Cells Suspended in Supernatant Fluid[b]
	mM	
Glycolate excreted 1 h	3.1	0.1
Additional glycoate excreted 2nd h	0.3	0.05

[a] *Chlorella* cells which had previously excreted 3.2 mM glycolate in 1 h. Cells obtained by centrifugation of reaction mixture.

[b] Supernatant fluid obtained by centrifugation of reaction mixture which had excreted 3.2 mM glycolate in 1 h.

Table II. *Glycolate Excreted in 1 Hour by Chlorella in the Presence and Absence of NH_4Cl*

Conditions as in Table I. Continuously gassed with 0.2% CO_2 in air.

NH_4Cl	Glycolate Excreted in 1 h
mM	
0	3.5
1.0	1.7
2.0	0.03

culture were centrifuged and the pellet washed twice with 50 ml of the Watt-Fogg medium minus urea. The final pellet was suspended in double concentrated Watt-Fogg Medium minus urea plus an equal volume of 0.1 M Hepes buffer (pH 8.4) to obtain a 10% wet weight suspension. Two ml of this suspension were placed in 25-ml Warburg flasks that were attached to manometers in an illuminated Warburg bath (10,000 lux incandescent light). The manometers were fitted with a manifold through which the flasks could be flushed continuously with gas mixtures at a positive pressure of 25 mm of Brodie's solution. The temperature was 30°C and the time of incubation was as indicated in the tables. The pH of the incubation mixture remained constant. After incubation, the cells were removed by centrifugation and glycolate in the supernatant fluid was determined by the method of Calkins (3). Controls were examined to determine whether the supernatant fluids contained compounds which interfered with the glycolate determination. None was found.

Despite our efforts to maintain the cultures as constant as possible, the *Chlorella* cells failed to form and excrete glycolate in a consistent manner. For example, with cells from different *C. pyrenoidosa* cultures, the amount of glycolate excreted in 1 h varied from 0.6 to 3.2 mM. This magnitude of glycolate excretion seemed to be somewhat higher than the amounts previously reported. With longer incubation than 1 h under light conditions, the *Chlorella* failed to excrete more glycolate. Time course experiments revealed that the amount of glycolate excretion increased asymptotically during the 1st h achieving a maximum at about 45 min.

Inhibitory Effects on Glycolate Excretion. If the cells from a Warburg flask which had previously formed approximately 3.2 mM glycolate in 1 h were centrifuged and the pellet resuspended in fresh Watt-Fogg-Hepes medium, they again excreted glycolate to the extent of the 1st h of incubation. This process could be repeated several times, *i.e.* up to eight were attempted and each time similar quantities of glycolate were excreted. CO_2 was an absolute requirement for glycolate excretion and darkness or monuron inhibited all glycolate excretion. When the supernatant fluid from cells which had previously formed glycolate during the 1st h was added to fresh cells, no glycolate or only small quantities were excreted (Table I).

Table III. *Effect of KCN and Semicarbazide on Excretion of Glycolate by C. pyrenoidosa*

Conditions as in Table I. Time, 1 h. Continuously gassed with 0.2% CO_2 in air.

Additions	Glycolate Excreted
	mM
0	3.3
KCN, 2 mM	11.0
Semicarbazide, 2 mM	12.2

Table IV. *Reversal of NH_4^+ Inhibition by KCN and Semicarbazide*

Additions	Glycolate Excreted
	mM
Control (0)	3.1
3 mM NH_4Cl	0.3
2 mM KCN plus 3 mM NH_4Cl	8.2
2 mM semicarbazide plus 3 mM NH_4Cl	9.3

Table V. *Stimulatory Effect of AOA on Glycolate Excretion by Buffered Chlorella Cells Gassed with 0.2% CO_2 and Air or 0.2% CO_2 and O_2*

Conditions: Time, 1 h. Others as stated in text.

Additions	Glycolate Excreted in 1 h	
	CO_2 and Air	CO_2 and O_2
	mM	
0	3.2	8.1
2 mM AOA	14.6	14.8

End product inhibition was not the cause of cessation of glycolate formation or excretion, inasmuch as 5 mM glycolate added at the beginning of the experiment did not inhibit glycolate formation as compared to the controls. The possibility that after 1 h glycolate uptake was equal to glycolate excretion could be excluded, because added [^{14}C]glycolate was not taken up by the *Chlorella* at pH over 6.

Analyses of the supernatant fluids for inhibitory substances revealed that some amino acids, namely ornithine of the urea cycle, were present and were inhibitory to glycolate excretion. Inasmuch as NH_3 is involved in the urea cycle, we also tested NH_4^+ and found that it inhibited glycolate excretion (Table II.)

On the hypothesis that NH_4^+ was entering the metabolic pathway of amino acid biosynthesis and by subsequent aminotransferase reactions was removing a keto acid which probably was a precursor of glycolate, we initially employed KCN and semicarbazide as inhibitors of pyridoxal-5-phosphate-linked aminotransferases. The results tabulated in Table III indicated that both compounds stimulated glycolate excretion.

KCN and semicarbazide reversed the inhibitory effects of NH_4^+ ion on glycolate excretion (Table IV). These results are consistent with the hypothesis that the effects of NH_4^+ ion were indirectly involved in pyridoxal phosphate-linked aminotransferase reactions.

Stimulating Effect of AOA[2] on Glycolate Excretion. These results prompted us to try aminooxyacetic acid ($NH_2 \cdot O \cdot CH_2$ COOH) (AOA) as an inhibitor of pyridoxal-5-phosphate-linked aminotransferases (5). Two mM AOA added to *C. pyrenoidosa* cells in buffered medium and gassed continuously with 0.2% CO_2

[2] Abbreviation: AOA, aminooxyacetic acid.

in air under conditions outlined above resulted in the excretion of 14.6 mM glycolate in 1 h as compared to 3.2 mM in controls minus AOA (see Table V).

Combinations of KCN, semicarbazide, and AOA did not increase the stimulation of glycolate excretion. On the average, AOA was more stimulatory than KCN or semicarbazide and perhaps less toxic than KCN, and consequently we have employed AOA routinely.

Stimulatory Effect of AOA on Glycolate Excretion in the Presence of CO_2 and Air and CO_2 in O_2. To establish whether the stimulatory effect of AOA with 0.2% CO_2 in air was related to the stimulatory effect of 99.8% O_2 and 0.2% CO_2, experiments were performed with *Chlorella* suspensions gassed continuously with the CO_2 and air mixture, plus and minus AOA, and a similar suspension with the O_2 and CO_2 mixture plus and minus AOA. The results also tabulated in Table V show that glycolate excretion increased from 3.2 mM in 1 h in CO_2 and air in the absence of AOA to 14.6 mM in the presence of AOA and CO_2 and air. With CO_2 and O_2 in the absence of AOA, 8.1 mM glycolate were excreted in 1 h while in the presence of AOA 14.8 mM were excreted.

Comparing the results obtained with *Chlorella* cells in 0.2% CO_2 in air and 0.2% CO_2 in O_2, the following conclusions can be drawn: AOA stimulates glycolate excretion with cells in CO_2 and air to a level higher than cells in CO_2 and O_2 minus AOA. With cells in CO_2 and O_2, AOA substantially stimulated glycolate excretion over cells in CO_2 and O_2 minus AOA.

Continuous Excretion of Glycolate. To determine whether glycolate excretion was a continuous process for more than 1 h, experiments were performed with *Chlorella* suspensions, plus and minus AOA, with continuous gassing with 0.2% CO_2 in air. These results demonstrated that glycolate was continuously formed and excreted in the presence of 2 mM AOA for a period of 4 h, while in the absence of AOA glycolate excretion was practically nil after 1 h. Control experiments under dark conditions or with light and monuron excreted no glycolate. In the absence of AOA, glycolate was not converted either in the cell or from the supernatant fluid to glycine or serine as judged by amino acid analyses which indicated that they did not accumulate. Fixation of $^{14}CO_2$ photosynthetically into other products is only slightly affected by the presence of AOA (11). The mechanism for AOA stimulation is unknown since it does not alter the ribulose-P_2 carboxylase-oxygenase reaction of the isolated enzyme (11).

After the above experiments were concluded, we became aware of the publication by Vennesland and Jetschmann (13) who demonstrated with *Chlorella* that HCN, semicarbazide, hydroxlamine, and hydrazine stimulated glycolate excretion in an atmosphere of 0.1% CO_2 in air to the level obtained under an atmosphere of 0.1% CO_2 in O_2. Their studies were prompted by the observation of Lorimer *et al.* (7) that cyanide was a component of an inactive form of NO_3^- reductase in *Chlorella*. Pistorous *et al.* (8) found that HCN production was enhanced by high light intensity, high O_2 tension, and low CO_2 tension. The same conditions enhanced glycolate excretion in the absence of carbonyl reagents.

Experiments of Vennseland and Jetschmann (13) were conducted with 50 to 150 μl of packed cells, which had been grown in a mineral salts medium under an atmosphere of 5% CO_2 in air and suspended in 50 mM phosphate buffer (pH 6.8) in a final volume of 3.0 ml in Warburg flasks containing 0.1% CO_2 in air or O_2 with addition of the carbonyl reagents. The results were expressed as total μmol of glycolate excreted, which we have transported to mM in Table VI. In 0.1% CO_2 in air they found 0.066 to 0.1 mM glycolate, and with 0.1% CO_2 in O_2 0.5 to 0.66 mM were excreted in 30 min. Under the conditions we have used, glycolate excretion was greater. They found with HCN a very narrow concentration range in the order of 0.1 μmol that stimulated glycolate excretion. The increase of glycolate excreted in 0.1% CO_2 in air was from 0.1 to 1.0 mM. With semicarbazide, hydroxylamine, and hydrazine, the effective concentration was much broader, yielding glycolate excretions from 0.1 mM in their absence to 0.5, 0.6, and 0.66 mM, respectively, in their presence. While the conditions employed by Vennesland and Jetschmann (13) differed somewhat, *i.e.* density of cells, time of incubation, buffer and pH, the yields obtained in glycolate secretion in our experiments were substantially greater.

In the majority of our experiments, more glycolate was excreted with added AOA in CO_2 in air than with cells under an atmosphere of 0.2% CO_2 in 99.8% O_2 and no AOA. In addition, in the presence of AOA with 0.2% CO_2 in 99.8% O_2, more glycolate was excreted than in the absence of AOA. Furthermore, in the presence of AOA with cells gassed with 0.2% CO_2 in air, glycolate excretion continued to the level of 40 mM in 4 h while in its absence only 3.1 mM was excreted.

Glycolate Excretion under Anaerobic Conditions. The only established mechanism for glycolate formation is the oxygenase activity of ribulose-P_2 carboxylase forming 2-P-glycolate. Glycolate formed after dephosphorylation is metabolized during photorespiration via the glycolate pathway. An attractive hypothesis is that AOA and other carbonyl reagents react with the pyridoxal-5-phosphate cofactor of aminotransferases to prevent conversion of glyoxylate, the oxidation product of glycolate, to glycine, thereby accumulating glycolate for excretion. Because the oxygenase activity requires high O_2 tension and low CO_2 tension, we explored the possibility of glycolate formation under an atmosphere of 99.8% N_2 + 0.2% CO_2 and also under 99.8% helium + 0.2% CO_2 both plus and minus AOA. N_2, He, and CO_2 were of the highest purity obtainable. By GC, the gases contained either undetectable amounts of O_2 or at most no more than 0.05% O_2. The flasks were constantly flushed with these mixtures and the algal suspensions and other conditions as indicated above. The only O_2 present was that in the cells due to photosynthetic activity. The atmosphere above the cells was anaerobic. The results are tabulated in Table VII and indicate that, under these anaerobic conditions in the presence of 2 mM AOA, considerable glycolate was excreted.

Several investigators have found that for optimum glycolate excretion by algae the partial pressure of CO_2 should not exceed 0.2%. Inasmuch as we had obtained glycolate excretion under anaerobic conditions with 0.2% CO_2 and N_2 or He, we tested the effect of increasing the CO_2 concentration to 0.4% in N_2. The flasks were constantly flushed with the gas mixture. Without AOA, 2.1 mM glycolate were excreted, and with (2 mM) AOA, 6.7

Table VI. *Comparison of Glycolate Excretion with HCN (Results in 13) with Those Reported Here with AOA*

	Glycolate Excretion by *Chlorella*			
Vennesland and Jetschmann (30 min)	0.1% CO_2 in air	0.1% CO_2 in O_2	0.1% CO_2 in air + HCN	0.1% CO_2 in O_2 + HCN
	0.1 mM	0.66 mM	1.0 mM	1.0 mM
This report (1 h)	0.2% CO_2 in air	0.2% CO_2 in O_2	0.2% CO_2 in air + AOA	0.2% CO_2 in O_2 + AOA
	3.2 mM	8.2 mM	9.2 mM	14.1 mM

Table VII. *Effect of Anaerobic Conditions on Glycolate Excretion by Chlorella*

Conditions: Time, 1 h. Others as stated in text.

Additions	Glycolate Excreted at Following Gas Phase	
	99.8% N_2 and 0.2% CO_2	99.8% He and 0.2% CO_2
	mM	
0	0.0	0.4
2 mM AOA	4.2	3.2

mM were excreted. CO_2 and light were required for anaerobic excretion of glycolate and monuron inhibited the excretion. The yield of glycolate excretion under these anaerobic conditions in the presence of AOA approximated the level of excretion obtained with 0.2% CO_2 in air without AOA. Because of the general acceptance that the oxygenase activity of the ribulose-P_2 carboxylase is the sole mechanism for glycolate formation, these anaerobic experiments are difficult to explain.

Conversion of Photosynthetically Formed Glycolate to Formate. As stated in the introduction, the goal of this research was to obtain high yields of glycolate excretion over long periods of time in order to convert it to H_2 and CO_2 via glycolic oxidase and the formic hydrogenlyase present in some of the enteric bacteria.

Glycolic oxidase prepared from spinach by the method of Zelitch and Ochoa (16) converted 10 μmol of glycolate, photosynthetically formed to 9.2 μmol of formate. The oxidase was dialyzed to remove the $(NH_4)_2SO_4$ used in its isolation. The oxidation of the glycolate to formate was performed in Warburg flasks containing Watt-Fogg-Hepes buffer (pH 8.4), 3 units of oxidase, total volume 3.0 ml in an atmosphere of air at 30°C. O_2 (9.3 μmol) was consumed. After the conclusion of the oxidation, the mixture was adjusted to pH 7.0 and the formate was determined by following the reduction of NAD^+ by formic dehydrogenase obtained from Boehringer Mannheim.

Conversion of Formate to H_2 and CO_2. After the conversion of glycolate to formate by the procedure described above, the reaction mixture was centrifuged and the formate in the supernatant fluid converted to H_2 and CO_2 by the formic hydrogenlyase complex found in *Escherichia coli* (Wood and Gest [15]). To the supernatant fluid in the main chamber of a Warburg flask, fitted with a serum stopper for removing gas samples for H_2 determination by GC, a suspension of washed *E. coli* cells was tipped in from the side arm to obtain a 10% wet weight of cells. The flask contained NaOH in the center well to absorb the evolved CO_2. Endogenous controls buffered in the same manner as the supernatant fluid but containing no formate were employed simultaneously. H_2 evolution measured manometrically and by GC showed that 8.3 μmol were formed from 9.2 μmol of formate in the supernatant fluid.

CONCLUSIONS

In small scale experiments, *i.e.* total volume of 2.0 ml in 25-ml Warburg flasks, conditions are described whereby suspensions of *C. pyrenoidosa* excreted higher yields of glycolate and over a longer period of time than have previously been reported. AOA significantly increased glycolate excretion. It is known that AOA inhibits pyridoxal-5-P-linked enzymes such as aminotransferases; however, experiments have shown that prevention of glyoxylate (the oxidation product of glycolate) conversion to glycine is not the reason for glycolate accumulation. The mode of action of AOA in these experiments is to be determined.

In an accompanying publication, Tolbert and his colleagues (11) employing *Chlamydomonas* present data on ^{14}C labeling of glycolate during $^{14}CO_2$ photosynthesis in the presence of AOA. Although the methodology differed as well as some of the results, the observation of greater glycolate excretion in the presence of AOA without a noticeable effect on glycine or serine metabolism is comparable to the results reported in this publication.

Most of the experiments reported here were performed interchangeably with cell suspension of *C. pyrenoidosa* and *N. digitus*. With each culture under the same conditions, similar results were obtained.

The conversion of the photosynthetically formed glycolate to H_2 and CO_2 by glycolic oxidase and formic hydrogenlyase was approximately 80%. Escalating the yields of glycolate obtained in small volumes to a pond 100 m × 100 m × 5 cm and assuming a glycolate yield of 30 mM per sunlighted day, calculations show that 3.36×10^5 L of H_2 can be obtained.

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