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EXCRETION OF AN ACID SEMIALDEHYDE BY *GLOEOMONAS**

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Abstract—The major extracellular product liberated by a photoautotrophically grown species of *Gloeomonas* in synchronous axenic culture was not glycollic acid, although it appeared to have similar properties. The product gave a reddish-purple, rather than the typical reddish-violet colour of glycollate with 2,7-dihydroxynaphthalene and an alkali soluble purple coloured 2,4-dinitrophenylhydrazone different from glyoxylic acid. The compound exhibited properties similar to mesoxalic acid semialdehyde (ketomalonic acid semialdehyde) and was excreted by mature cells with high carbohydrate content at the stage of cell division under relatively high light intensity 11,000 lx independent of the CO_2 concentration (0·1 or 5%, v/v in air).

INTRODUCTION

THE EXCRETION of organic compounds has been reported in various cultures of unicellular algae,[1–3] and glycollic acid appears to be the major extracellular product under conditions of high light intensity, low CO_2 concentration and high O_2 pressure.[4–7] In synchronized cultures of *Ankistrodesmus braunii* substantial quantities of mesotartaric acid, isocitrate lactone and another unstable unknown acid have been found to accumulate in the surrounding medium during the photosynthetic fixation of NaH $^{14}CO_3$.[8]

This communication reports the excretion of an acid semialdehyde by a species of *Gloeomonas* in synchronized cultures. The excretory product, tentatively identified as mesoxalic acid semialdehyde, gave a reddish purple colour with 2,7-dihydroxynaphthalene rather than the reddish-violet colour typical of glycollate and it moved to almost the same position as glycollic acid on a Dowex-1 acetate column. Extensive chromatographic studies showed no evidence of the excretion of glycollic acid during the life cycle of this alga.

RESULTS

Culture conditions and excretion during the life cycle. When the daughter cells produced in the dark from synchronized algal cultures were grown under continuous illumination for 24 hr they showed a constant cell number during the first 12 hr whether they were gassed

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[1] J. A. HELLEBUST, *Limno. Ocean.* **10**, 192 (1965).
[2] C. NALEWAJKO, *Limno. Ocean.* **11**, 1 (1966).
[3] C. FORSBERG and Ö. TAUBE, *Physiol. Plantarum* **20**, 200 (1967).
[4] N. E. TOLBERT and L. P. ZILL, *J. Biol. Chem.* **222**, 895 (1956).
[5] J. A. BASSHAM and M. KIRK, *Biochem. Biophys. Res. Commun.* **9**, 376 (1962).
[6] C. P. WHITTINGHAM and G. G. PRITCHARD, *Proc. Roy. Soc. Lond.* **157B**, 366 (1963).
[7] W. D. WATT and G. E. FOGG, *J. Exptl. Botany* **17**, 117 (1966).
[8] W. CHANG, Excretion of organic acids during photosynthesis by synchronized algae, Ph.D. thesis, Michigan State University (1967).

with 0·1% or 5% CO_2 in air (Fig. 1). Thereafter, the mature dividing cells commenced to separate into daughter cells in cultures supplied with low CO_2 concentration and the life cycle was achieved 4 hr earlier than in cultures with 5% CO_2. An average lag period of 4 hr due to the higher CO_2 tension was also observed in the dark and occurred in NO_3^- as well as NH_4^+ cultures. This temporary inhibition of cytokinesis has been observed by Soeder *et al.*[9]

Relatively high CO_2 concentration (5%) promoted the growth of the algal cells during the second 12 hr period in light as indicated by the increase in the protein (Fig. 2). However, this was more pronounced in cultures supplemented with NH_4^+ rather than NO_3^- as the nitrogen source.

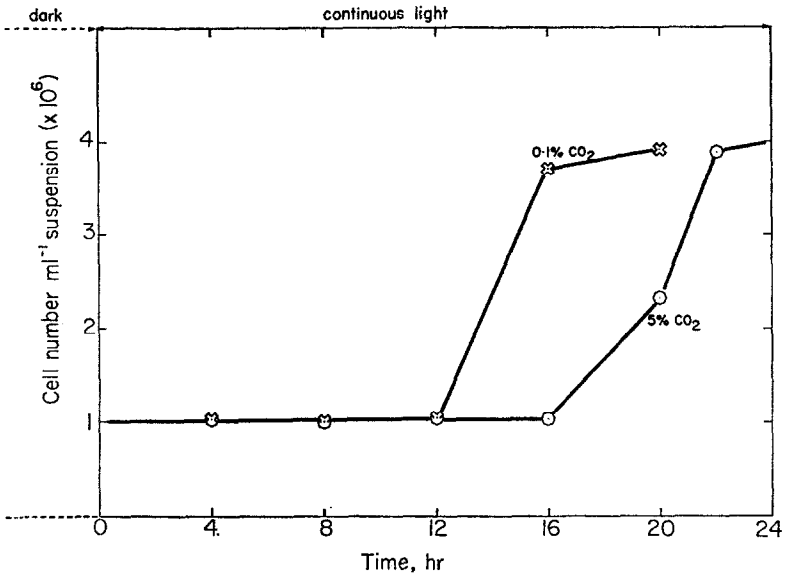

FIG. 1. CELL NUMBER OF AUTOTROPHIC CULTURES OF *Gloeomonas* WHEN THE DAUGHTER CELLS FROM SYNCHRONIZED CULTURES (12:12 hr, L–D-CHANGE) WERE EXPOSED AT THE END OF THE DARK PERIOD TO CONTINUOUS ILLUMINATION (11,000 lx) AND GROWN AT 25° IN THE PRESENCE OF EITHER 0·1% OR 5·0% vol. CO_2 WITH AMMONIUM OR NITRATE AS THE *N* SOURCE.

In an attempt to follow the excretion of glycollate during the life cycle of the alga Calkins'[10] procedure showed the development of a reddish purple colour (540 nm) with a broad absorption band between 475 nm and 575 nm in the supernates obtained from illuminated algal cultures in the second 12 hr period (Fig. 3). As indicated by the increase in absorbancy at 540 nm excretion commenced at the stage of cell division and continued through the stages of daugter cell production. At these developmental stages, the rate of excretion was enhanced by 5% CO_2 and also by the NH_4^+ ion. This excretion occurred only under illumination.

The conditions under which the compound was excreted are shown in Table 1. In these experiments the alga was synchronized as described and grown for 12 hr at 11,000 lx and

[9] J. C. SOEDER, H. STROTMANN and R. A. GALLOWAY, *J. Phycol.* **2**, 117 (1966).
[10] V. P. CALKINS, *Anal. Chem.* **15**, 762 (1943).

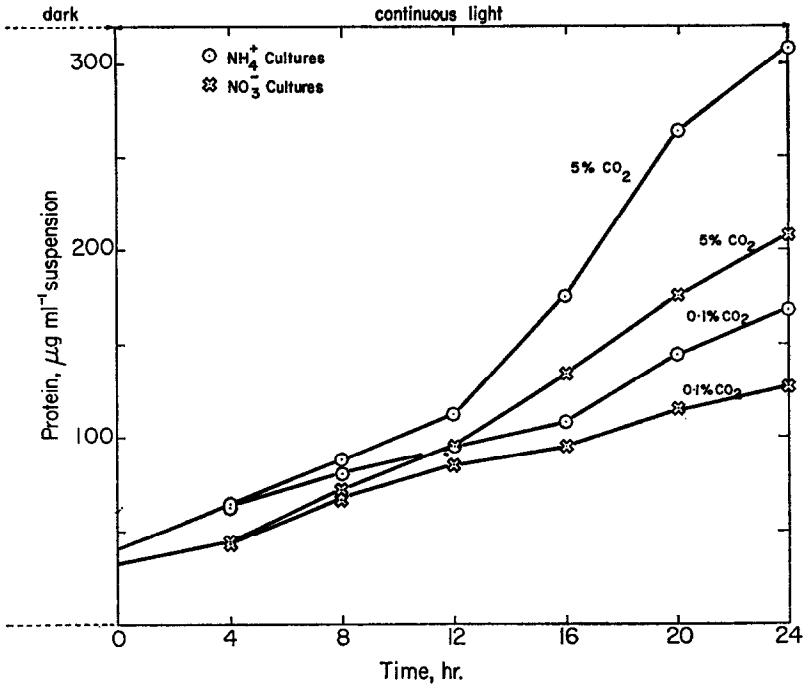

FIG. 2. GROWTH OF AUTOTROPHIC CULTURES OF *Gloeomonas* IN CONTINUOUS LIGHT AS INDICATED BY INCREASES IN PROTEIN/ml SUSPENSION.

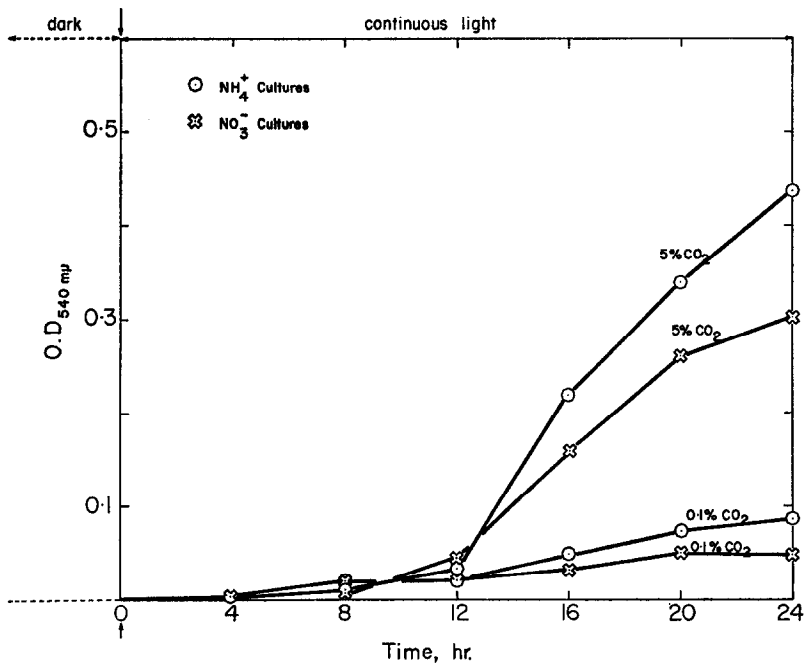

FIG. 3. INFLUENCE OF CO_2 CONCENTRATION AND THE *N* SOURCE OF THE NUTRIENT MEDIA ON THE PRODUCTION OF THE EXTRACELLULAR SUBSTANCE BY *Gloeomonas* GROWN IN CONTINUOUS LIGHT. AN O.D. OF 0·5 IS ROUGHLY EQUIVALENT TO 25 μg EXCRETORY PRODUCT/ml OF ALGAL MEDIUM AT 540 nm.

TABLE 1. COMPARISON OF CARBOHYDRATE CONTENTS OF MATURE CELLS OF *Gloeomonas* WHICH WERE PRODUCED IN SYNCHRONIZED CULTURES AFTER 12 hr GROWTH IN LIGHT AT HIGH OR LOW CO_2 CONCENTRATIONS AND THE EXCRETORY PRODUCT AS INDICATED BY ABSORBANCY OF THE COLOUR PRODUCED WITH CALKINS' REAGENT PER 0·2 ml SUPERNATES UNDER TWO LIGHT INTENSITIES AND IN THE PRESENCE OF 5% OR 0·1% vol. CO_2. NUTRIENT MEDIA CONTAINED NH_4^+ AS THE *N* SOURCE

Growth conditions	Total carbohydrates (μg glucose)		Experimental conditions		Excretion after 4 hr (O.D.) at 540 nm
	Per 10^6 cells	Per μg protein	Light intensity (lux)	CO_2 % in air	
11,000 lx and 5% CO_2 in air	98	1·02	11,000	5·0	0·42
			11,000	0·1	0·54
	120	1·07	3300	5·0	0·00
			3300	0·1	0·00
11,000 lx and 0·1% CO_2 in air	30	0·36	11,000	5·0	0·06
			11,000	0·1	0·00
	42	0·45	3300	5·0	0·00
			3300	0·1	0·00

either 5% or 0·1% CO_2. This was followed by exposure to either the same or lower light intensity (3300 lx) with 0·1% or 5% CO_2 for 4 hr. The excretory product was determined by Calkins' reagent and protein and carbohydrate determinations were made as described in Methods. Table 1 shows that excretion occurred only under conditions of high light intensity. It was independent of the CO_2 concentration, but dependent on previous growth conditions in which high light intensity coupled with relatively high CO_2 concentration favoured the formation of carbohydrates.

Properties and identification of the excretory product. In these experiments, the reddish purple colour produced by the reaction of the compound eluted from a Dowex-1-acetate column with Calkins' reagent was not typical of the reddish violet colour produced by glycollate nor did it give the typical sharp absorption band at 540 nm. Rather it showed a broad absorption between 475 and 575 nm (Fig. 4). Prior to chromatographic analysis, the frozen eluate was lyophilysed overnight to remove the acetic acid used for elution. When the product was chromatographed on paper, two acidic spots consistently appeared on spraying with either bromocresol-green or glucose-aniline solutions. The two acids moved to adjacent areas having R_f values of about 0·45 and 0·55 lower than glycollate (R_f 0·72–0·76) and could not be co-chromatographed with any of the known carboxylic or ketoacids tested. One of the two acids was found to be monobasic orthophosphate. In spite of difficulties encountered during paper chromatographic analysis[4] 5 μg of authentic glycollic acid could be chromatographed on cellulose thin layer without any difficulty within 2 hr. Nevertheless, glycollate was not chromatographically identified either in samples analysed directly from the algal media (1·0 ml) nor in the more concentrated product obtained after column chromatography of more than 1 l. of algal medium and lyophilysed. The resulting

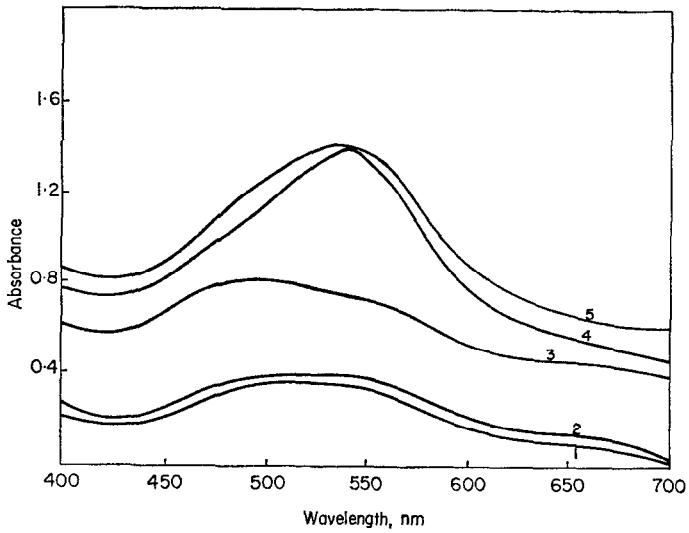

FIG. 4. ABSORPTION SPECTRA OF THE COLOURS PRODUCED BY THE REACTION WITH CALKINS'[10] REAGENT OF THE SYRUPY (1) AND DRIED MESOXALIC ACID SEMIALDEHYDE (3); SYRUPY PRODUCT FROM THE CULTURE MEDIA OF *Gloeomonas* (2); GLYCOLLIC (4) AND TARTRONIC ACIDS (5).

product was a syrupy reddish-brown substance which did not crystallize on cooling. It was associated with some impurities from the Dowex column in the form of a greenish residue, but samples from the freshly prepared syrupy product gave a reddish purple colour with Calkins' reagent.

Of other carboxylic acids tested only tartronic (50 μg) and ketomalonic acid (500 μg) developed a red violet colour at 540 nm with Calkins'[10] reagent at the indicated concentrations, whereas the same colour was developed with only 5 μg of glycollate, but neither of these acids co-chromatographed with the unknown. However, two related compounds, tartronic acid semialdehyde (TSA) and mesoxalic acid semialdehyde (KMSA) both reacted with Calkins' reagent, the former gave a blue or purple colour and the latter a reddish purple colour showing a broad absorption from 475 to 575 nm similar to the unknown syrupy excretory product. On drying either synthetic KMSA or the unknown, the colour reaction was purple with the absorption maximum shifting to shorter wavelengths. Figure 4 illustrates the absorption spectra of some of these compounds after reaction with Calkins' reagent.

The unknown excretory product(s) reacted with 2,4-dinitrophenylhydrazine forming coloured derivatives in NaOH. Freshly treated algal media obtained at the end of the excretion period (cf. Fig. 3) gave a purple hydrazone in alkali with a broad absorption maximum at 560 nm (Fig. 5). On the other hand, brownish or reddish alkaline hydrazone derivatives with absorption maxima at shorter wavelengths were produced by media stored in the cold. Freshly prepared synthetic KMSA and TSA formed orange red crystals after 30 min at room temperature. In N NaOH the *bis*-2,4-dinitrophenylhydrazones of the two acid semialdehydes turned deep purple with absorption maxima at 560 nm similar to that formed by the algal medium (Fig. 5). However, on standing in air in the cold both synthetic KMSA and TSA produced alkali-soluble hydrazone derivatives with absorption maxima at lower wavelengths. Both TSA and KMSA are inherently unstable; the former

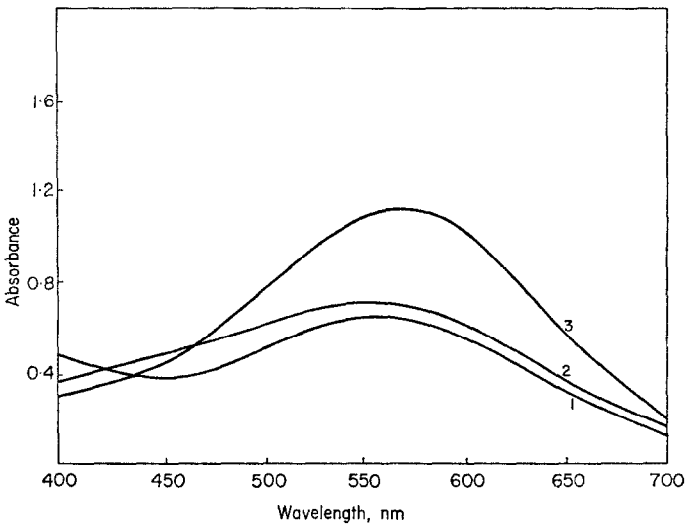

FIG. 5. ABSORPTION SPECTRA OF THE ALKALI-SOLUBLE 2,4-DINITROPHENYLHYDRAZONES OF TARTRONIC ACID SEMIALDEHYDE (1), MESOXALIC ACID SEMIALDEHYDE (3) AND THE CULTURE MEDIA OF *Gloeomonas* (2).

autoxidizes to KMSA or readily decarboxylates to glycolaldehyde. According to Fenton,[11] KMSA is fairly stable in acid solution, but on prolonged evaporation or heating or even on standing it decomposes to glyoxal and CO_2. However, in alkali, KMSA is quickly changed on heating to tartronic acid. The shift in the absorption maxima of the alkaline hydrazone derivatives of KMSA, TSA and the algal media is possibly due to the formation of neutral aldehydes.

Direct evidence for the identity of the extracellular product of *Gloeomonas* as mesoxalic acid semialdehyde (ketomalonic acid semialdehyde) was provided by chromatography. Figure 6 shows that freshly prepared synthetic KMSA has the same R_f and co-chromatographed with the unknown freshly prepared syrupy sample. Glycollic, tartronic and orthophosphoric acids have quite different R_fs, whereas tartronic acid semialdehyde co-chromatographed with KMSA. Its presence as a major component of the excretory product was discounted on the basis of many experiments in which one ml of fresh algal medium was used as a substrate for purified TSA reductase (E.C.1.1.1.g) from *Pseudomonas oxalaticus*. In all determinations, NADH was oxidized only at a very slow rate in comparison to the authentic substrate.

The role of photosynthetic CO_2 fixation in the excretion of mesoxalic acid semialdehyde was examined by $^{14}CO_2$ incorporation experiments in which 50 μc of NaH $^{14}CO_3$ was applied to 25 ml of algal suspensions containing 6×10^6 cells/ml in either the complete nutrient medium or 10^{-3} M phosphate buffer pH 6·2. The experiments were conducted in 125 ml Erlenmeyer flasks with either air or N_2 atmosphere at 13,000 lx in a growth chamber. After 5 or 30 min incubation, the extracellular products were chromatographed. Only one area in *all* 8 treatments on the chromatogram reacted as an acid with either bromocresol green or glucose-aniline. The R_f corresponded to that of (Fig. 7, No. 5) mesoxalic acid

[11] H. J. H. FENTON, *J. Chem. Soc.* **87**, 804 (1905).

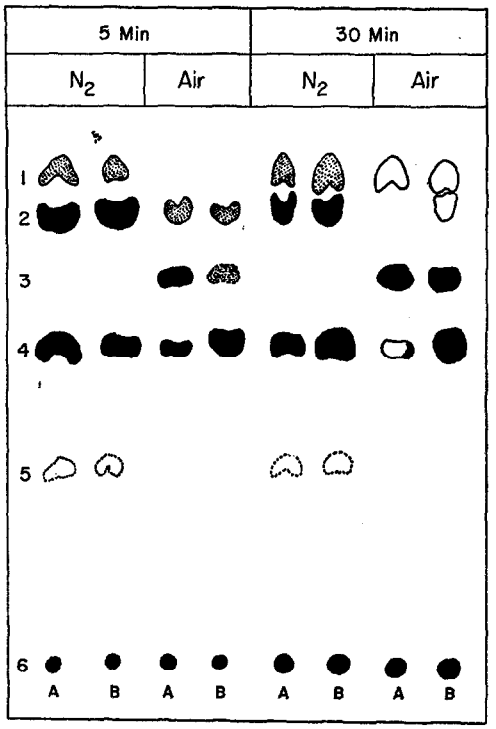

FIG. 7. THIN-LAYER AUTORADIOGRAM OF THE ^{14}C-COMPOUNDS EXCRETED DURING 5 AND 30 min BY *Gloeomonas* SUSPENDED EITHER IN NUTRIENT MEDIUM (A) OR IN PHOSPHATE BUFFER (B) AND BUBBLED WITH AIR OR NITROGEN. SEE TEXT FOR FURTHER EXPLANATION.

semialdehyde and when eluted gave a reddish purple colour with Calkins' reagent. On the other hand, the radioautogram (Fig. 7) showed an array of radioactive areas, but the area corresponding to KMSA showed very low radioactivity and only in nitrogen atmosphere experiments. The radioactive area having the same R_f as glycollic acid (No. 3) did not react with acid indicators and when eluted gave a yellowish-brown colour with Calkins' reagent not typical of glycollate. This unknown radioactive compound was not observed in a nitrogen atmosphere. The other compounds may be neutral aldehydes.

DISCUSSION

The present work has provided evidence that the conventional colorimetric method[10] used for the determination of glycollic acid is non-specific. Substances such as tartronic acid and mesoxalic acid (ketomalonic acid) give a reddish violet colour (absorption maximum at 540 nm) with 2,7-dihydroxynaphthalene identical with the colour produced by glycollic acid. Furthermore, synthetic mesoxalic acid semialdehyde, an impure syrupy substance contaminated with neutral aldehydes (e.g. glyoxal) which is eluted from a Dowex-1-acetate column in almost the same position as glycollate develops a reddish purple colour with Calkins' reagent showing broad absorption from 475–575 nm. Valentine *et al.*[12] in

[12] R. C. VALENTINE, H. DRUCKER and R. S. WOLFE, *J. Bacteriol.* **87**, 241 (1964).

their studies on glyoxylate carboligase and tartronic semialdehyde reductase in *Streptococcus allantoicus* reported an unknown compound which after elution from a Dowex-1-acetate column at the position of glycollate reacted colorimetrically as glycollate. We have confirmed this with the product of glyoxylate carboligase from *Pseudomonas oxalaticus*. Therefore, the development of a reddish violet colour with Calkins' reagent by algal media even after showing the typical glycollate absorption peak at 540 nm may not necessarily indicate the presence of glycollate unless it is confirmed by paper or thin layer chromatographic analysis.

We have shown that the light dependent excretory product from mature cells of *Gloeomonas* with plentiful carbohydrate is a compound which gives a reddish purple colour with Calkins' reagent. It also forms a 2,4-dinitrophenylhydrazone which turns purple in alkali similar to tartronic acid semialdehyde and mesoxalic acid semialdehyde derivatives. TLC studies have indicated that this compound is mesoxalic acid semialdehyde (ketomalonic acid semialdehyde). Under our experimental conditions glycollate could not be detected at either the stage of cell division or any other developmental stage in the life cycle of *Gloeomonas*.

At the stage of cell division during the life cycle of *Ankistrodesmus braunii* Chang[8] and Gimmler *et al.*[13] have shown that the photosynthetic excretion of glycollate using NaH $^{14}CO_3$ was minimal. Instead Chang[8] reported the excretion of mesotartaric acid, isocitrate lactone and an unstable acid. Furthermore, exogenously added glycollate was utilized primarily at the stage of cell division in synchronous cultures of *Scenedesmus* and *Ankistrodesmus*.[14]

In contrast, Hess *et al.*[15] reported that *Scenedesmus* excreted glycollate (55 μg/ml/hr) as determined by Calkins' method solely at the stage of cell division and before the daughter cells separated. Excretion occurred in the light and in the absence of $NaHCO_3$, but increased on its addition. This type of excretion is similar to what we have reported for *Gloeomonas*, but their product was not confirmed by chromatographic analysis.

Mesoxalic acid semialdehyde can be formed by the oxidation of tartronic acid semialdehyde which could arise by the oxidation of glycerate catalysed by tartronic acid semialdehyde reductase or from glyoxylate by isocitrate lyase and glyoxylate carboligase as in bacteria.[16] The former route is ruled out since if this were the pathway it should have become highly radioactive when excreted during the $^{14}CO_2$ incorporation experiment. The other pathway from carbohydrate to glycerate via acetate and isocitrate appears more likely for the following reasons. Excretion occurs only when mature cells, loaded with carbohydrate (Table 1), are subjected to relatively high light intensity during the phase of cell division. At this developmental stage Senger and Bishop[17] have shown that the efficiency of photosystem II decreased in *Scenedesmus* and Tan (unpublished results) in this laboratory has obtained similar results with *Gloeomonas*. In *Gloeomonas* this stage was associated with substantial activity of isocitrate lyase (Badour and Waygood, unpublished results), but we were unable to show the oxidation of DL-glycerate to tartronic semialdehyde by an NAD(P) coupled reaction from extracts at this stage of development. It appears that the decrease in photoreducing power would allow direct utilization of the photosynthetically formed glycerate for protein synthesis (Fig. 2). Whereas the accumulated carbohydrate could be

[13] H. GIMMLER, W. ULLRICH, J. DOMANSKI-KADEN and W. URBACH, *Plant Cell Physiol.* **10**, 103 (1969).
[14] E. B. NELSON, N. E. TOLBERT and J. L. HESS, *Plant Physiol.* **44**, 55 (1969).
[15] J. L. HESS, N. E. TOLBERT and L. M. PIKE, *Planta* **74**, 278 (1967).
[16] H. KALTWASSER, *Arch. Mikrobiol.* **64**, 71 (1968).
[17] H. SENGER and N. I. BISHOP, *Nature* **214**, 140 (1967).

metabolized via acetate partially through isocitrate lyase and glyoxylate carboligase. However, the activity of the latter enzyme in sonic extracts of *Gloeomonas* was relatively low in comparison with bacterial extracts[16,18] as measured manometrically by the anaerobic evolution of CO_2. The product tartronic acid semialdehyde could be excreted *per se* and oxidized non-enzymically to mesoxalic acid semialdehyde or oxidized enzymically by cellular pyridine nucleotide coenzymes and then liberated. This assumption does not exclude the possibility of the formation of dihydroxyfumarate or dihydroxymaleate from two molecules of glyoxylate[19,20] which would be released into the medium and become decarboxylated to tartronic acid semialdehyde.[21]

The authors are not aware of any reports of acid semialdehydes in green algae or higher plants. However, various unknown compounds have been formed during the photometabolism of labelled acetate[22,23] and Krupka and Towers[24] have recorded the presence of an alkali-soluble 2,4-dinitrophenylhydrazone from wheat seedlings with a violet colour absorbing at 570 nm which could be a derivative of an acid semialdehyde. Also, Stinson and Spencer[25] have reported the formation of malonate semialdehyde from β-alanine by β-alanine aminotransferase from etiolated cotyledons of *Phaseolus vulgaris*.

Further studies are being made on the enzymes involved in the excretion of mesoxalic acid semialdehyde.

EXPERIMENTAL

Organism and culture. A unicellular motile green alga identified as a species of *Gleomonas* was isolated from the University Field Station (Delta Marsh). The organism has two anterior flagella, it contains numerous discoid chloroplasts without pyrenoids and has a gelatinous layer of uniform thickness external to the cell wall. The organism was grown aseptically in the mineral nutrient medium of Kuhl[26] in low-form Fernbach culture flasks supplied with continuous current of 5% CO_2 in air. The flasks were placed in growth chambers at 25° under a light intensity of approximately 11,000 lx provided by 'cool white' fluorescent lamps.

Synchronized cultures were obtained by subjecting the cells to alternating periods of 12 hr light and 12 hr dark according to the method of Pirson and Lorenzen.[26] The algal suspension was diluted with fresh medium to a constant cell number of 1×10^6 cell/ml at the beginning of each light period. By this procedure the cultures did not undergo cell division during the light period and all cells divided between the 4th and the 10th hour of the dark period. Under these conditions, the average number of daughter cells formed per mother cell was four. The four daughter cells in this organism are not released through a common mother cell wall and readily separate from one another.

The starting algal material for the experiments to be described was collected at the end of dark period from well synchronized algal cultures and resuspended in fresh sterilized nutrient media containing either nitrate or ammonium ions (10^{-2} M KNO_3 or 10^{-2} M NH_4Cl and 10^{-2} M KCl). In order to maintain the pH value between 6·5 and 7·0 throughout the life cycle of the alga the medium was enriched with 5×10^{-3} M K_2HPO_4 a 10-fold higher phosphate ion concentration than normally supplied in Kuhl's medium. The algal cultures were routinely checked for bacterial contamination.

Analytical methods. Cell number was determined by an Haemocytometer. Total carbohydrate was determined by the anthrone method[27] in which the algal suspension with 75% H_2SO_4 served as blank. The procedure of Lowry *et al.*[28] was used for the estimation of protein content after dissolving the algal cells in

[18] N. K. Gupta and B. Vennesland, *Arch. Biochem. Biophys.* **113,** 255 (1966).
[19] J. Maroc, *Physiol. Végétale* **5,** 37 (1966).
[20] J. Maroc, *Physiol. Végétale* **5,** 47 (1966).
[21] K. Fukunaga, *J. Biochem., Tokyo* **47,** 741 (1960).
[22] M. J. Merrett and K. H. Goulding, *J. Exptl. Botany* **18,** 128 (1967).
[23] K. H. Goulding, M. J. Lord and M. J. Merrett, *J. Exptl. Botany* **20,** 34 (1969).
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[25] R. A. Stinson and M. S. Spencer, *Biochem. Biophys. Res. Commun.* **34,** 120 (1969).
[26] A. Kuhl and H. Lorenzen, in *Methods in Cell Physiology* (edited by D. M. Presscott), Vol. I, p. 159, Academic Press, London (1964).
[27] J. H. Roe, *J. Biol. Chem.* **212,** 335 (1955).
[28] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).

N NaOH for 30 min at 100°. The method of Calkins[10] was employed for the determination of the organic acid excreted by the algal cells using 0·01 % 2,7-dihydroxynaphthalene in conc. H_2SO_4 as the colour reagent. Aliquots of the clear supernatant obtained by centrifugation of the algal suspension were passed through a Dowex AG-1-X-10 acetate column.[29] After washing the column with distilled water the organic acid was eluted with 4 M acetic acid. By this method any nitrate present in the medium was eliminated since concentrations of nitrate above 10^{-3}M interfered significantly with the colour reaction whereas ammonium ions had no effect.

Preparation of metabolites. Mesoxalic acid semialdehyde, ketomalonic acid semialdehyde (KMSA) was prepared from tartaric acid according to Fenton and Ryffel.[30] The product was a reddish brown syrup. However, on extended lyophilysation, it changed to dark brown crystals showing different properties from the original material. The initial product dissolved in ether was a brownish yellow solution which was used as the reference for chromatography. Tartronic acid semialdehyde (TSA) was prepared from dihydroxyfumaric acid according to the procedure of Fukunaga[21] and Valentine *et al.*[12]

Thin layer chromatography. This was carried out on 0·25 mm layers of MN-cellulose using 20 × 20 cm plates. The chromatographic tanks were saturated with solvent vapour from the system EtOAc–HCO_2H–H_2O (11:5:3). The spots on the dried plates were visualized by spraying with glucose–aniline reagent and heating at 125°. A brown colour was produced by acids. Areas of radioactivity on the chromatogram were located by exposure to Kodak No. Screen X-ray film for about 10 days.

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[29] I. ZELITCH, *J. Biol. Chem.* **233,** 1299 (1958).
[30] H. J. H. FENTON and J. H. RYFFEL, *J. Chem. Soc.* **81,** 426 (1902).