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Photoexcretion and Fate of Glycolate in a Hot Spring Cyanobacterial Mat

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Photosynthesis by *Synechococcus lividus*, the sole oxygenic phototroph inhabiting the surface of the 55°C cyanobacterial mat in Mushroom Spring, Yellowstone National Park, causes superoxic and alkaline conditions which promote glycolate photoexcretion. At O_2 concentrations characteristic of the top 2 mm of mat during the day, up to 11.8% of $NaH^{14}CO_3$ fixed in the light was excreted, and glycolate accounted for up to 58% of the excreted photosynthate. Glycolate was neither incorporated nor metabolized by *S. lividus*, but it was incorporated by filamentous microorganisms in the mat. Incubation of mat samples with $NaH^{14}CO_3$ resulted in labeling of both *S. lividus* and filaments, but the addition of nonradioactive glycolate increased the level of ^{14}C in the aqueous phase and decreased the extent of labeling of filaments. This suggests that cross-feeding of glycolate from *S. lividus* to filamentous heterotrophs occurs and that underestimation of the extent of photoexcretion is probable.

Although carbon flow through decomposition in alkaline silicious hot spring cyanobacterial mats has been well studied (2, 19, 28–31), direct carbon flow from phototrophs to heterotrophs is less well understood. Bauld and Brock (4) have suggested that heterotrophic microorganisms in hot spring microbial mats (Octopus Spring, Yellowstone National Park) derive organic nutrients from products excreted by the cyanobacterium *Synechococcus lividus*. A similar link between phototrophs and heterotrophs has been suggested (6) for *Cyanidium caldarium* algal mats found in hot acid springs.

The results of oxygen and pH microelectrode studies (18) suggest that environmental conditions should favor processes that lead to photoexcretion and subsequent carbon flow to heterotrophic microorganisms. During active photosynthesis, the pH is high (up to 9.4), presumably because of CO_2 consumption, and the photic zone is superoxic. In such an environment, O_2 should compete successfully with CO_2 for the active site of ribulose diphosphate carboxylase-oxygenase, the enzyme responsible for carbon fixation in cyanobacteria (26). Oxygenation of ribulose-1,5-diphosphate results in the production of glycolate (26), an excretion product that is common to many autotrophs (3, 10, 11, 14, 27). In this study, we showed that glycolate is an important photoexcretion product, especially under O_2 conditions characteristic of a hot spring cyanobacterial mat community. We also demonstrated that filamentous bacteria inhabiting the community incorporate glycolate, complicating estimation of the true extent of photoexcretion.

MATERIALS AND METHODS

Study area. Experiments were carried out at Mushroom Spring, an alkaline (pH 8.3) hot spring located about 0.2 km northeast of Great Fountain Geyser along the Howard Eaton Trail in the Lower Geyser Basin of Yellowstone National Park. Experiments were conducted on samples collected from the cyanobacterial mat at 55 to 58°C.

Pure culture methods. An axenic culture of *S. lividus* Y-7c-7b-S, which was isolated from Clearwater Spring in Yellowstone National Park (15), was provided by Richard Castenholz (University of Oregon, Eugene, Oreg.). Stock cultures were maintained in unbuffered, liquid medium D (9) at 50°C. For radiolabeling experiments, cultures were grown in Roux bottles containing 800 ml of liquid medium DG (9), incubated in an aquarium water bath at 50°C, and illuminated with cool white fluorescent lights. To provide aeration, air sterilized by passage through glass wool filters and humidified by passage through sterile distilled water was pumped with an aquarium air compressor into the culture through an air stone. Portions of mid- or late-log-phase cultures (5×10^6 to 9×10^7 cells per ml) were used for radiolabeling experiments. Cell densities were determined with a cell counter (Petroff-Hauser).

Sample preparation. Suspensions of cells from the mat surface were prepared by scraping off portions of the top 0 to 2 mm with a spatula and gently homogenizing these portions in spring water with a Dounce tissue grinder (Wheaton Industries, Millville, N.J.). Mat homogenates ranged from 10^7 to 10^9 *S. lividus* cells per ml, but the cell density was constant within each experiment. Pure cultures of *S. lividus* grew as a homogeneous suspension, so that only gentle mixing was necessary to prepare the cells. For both pure culture and mat experiments, 2- or 5-ml portions of the cell suspension were added to 1- or 2-dram (ca. 3.7- or 7.4-ml) vials. In one case (noted below) whole cores were removed from the mat with a no. 4 brass cork borer (50.3 mm^2 by 5 mm) and transferred directly to 1-dram (ca. 3.7-ml) vials. Vials were capped with recessed butyl rubber stoppers (size 00; A. H. Thomas) and secured with tape. For dark incubations, vials were completely wrapped with two layers of black tape.

Vials were incubated in natural sunlight and were immersed in water that flowed over the mat at a depth of at least 1.5 cm. Experiments were performed between 2 h before and 3 h after solar noon, and light intensities above the water surface ranged from 800 to 2,000 microeinsteins m^{-2} s^{-1}, as measured with a flat quantum sensor (LICOR; Lambda Instruments). Pure culture samples were incubated on racks that were immersed 2-cm deep in a 50°C water bath and were illuminated with cool white fluorescent lights set 10 cm above the vials. The light intensity at the surface of the water was approximately 10% full sunlight.

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The O_2 concentration was increased by running a stream of pure O_2 into the vial headspace for 15 s while the stopper was being secured. The resultant dissolved O_2 concentration was determined by the azide modification of the Winkler assay (1), which was adapted for use on small sample sizes.

In some vials glycolic acid (Sigma Chemical Co., St. Louis, Mo.) adjusted to pH 8.0 was added to a final concentration of 10^{-2} M. Some pure culture samples of *S. lividus* were preincubated with 10^{-4} M glycolate for 4 h before incubation with radiolabeled glycolate (see below).

Radiolabeling. Samples were preincubated for 15 to 30 min before injection of radiolabeled compounds and then they were incubated for 2 h. Photosynthetic uptake of CO_2 and excretion of organic carbon was measured after the addition of 0.2 ml of 10 μCi of $NaH^{14}CO_3$ per ml (50 mCi/mmol; New England Nuclear Corp., Boston, Mass.) prepared in sterile distilled water (pH 8.0). More $NaH^{14}CO_3$ was added (3.6 μCi per vial), and the incubation time was increased to 4 h for microautoradiography experiments. Cells labeled with $NaH^{14}CO_3$ were immediately separated from the supernatant by filtration and centrifugation to minimize cell breakage (21), as described below. The fate of [1-^{14}C]glycolate (50 mCi/mmol; New England Nuclear) was measured after the addition of 0.2 ml from 10-μCi/ml stock solutions. Incubation of cells labeled with [1-^{14}C]glycolate was terminated by injection of 0.1 ml Formalin (37.1%; J. T. Baker Chemical Co., Phillipsburg, Pa.).

$^{14}CO_2$ was determined by gas chromatography-gas proportion analysis of subsamples of the gas headspace of vials by the method of Ward and Olson (30). The radioactivity incorporated into cells was determined by filtering a homogeneous portion (0.1 to 0.5 ml) of each sample through a 0.45-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) and then by rinsing the portions with 3 ml of either medium D or prefiltered Mushroom Spring water, again, to minimize cell breakage (21). The filters were dried and exposed to HCl fumes overnight to remove radioactive carbon dioxide species. The radioactivity on the filters was determined in 10 ml of Aquasol (New England Nuclear) with a liquid scintillation system (Tri Carb 460 CD; Packard Instrument Co., Inc., Rockville, Md.) by the sample channels ratio method to determine disintegrations per minute. Microautoradiograms of cell material labeled with [1-^{14}C]glycolate or $NaH^{14}CO_3$ were prepared by the method of Brock and Brock (8) and exposed for 2 to 3 weeks. Photomicrographs were taken as described by Sandbeck and Ward (19). To determine the extent of labeling of filaments after the addition of $NaH^{14}CO_3$, duplicate slides were counted by two individuals. Because the exposure of cells within whole core samples to added ^{14}C-labeled compounds or metabolites was highly variable, grain densities were determined only for filaments which were obviously labeled. Grains within 5 μm of well-isolated filaments at least 30-μm long were counted. Background grain counts for an equivalent area were subtracted.

The radioactively labeled organic carbon that was present in the aqueous phase was determined after the removal of cells by centrifugation for 2 min at 15,600 × *g* in a centrifuge (5414; Eppendorf; Brinkmann Instruments, Inc., Westbury, N.Y.). The supernatant was acidified to less than pH 2 with 6 N HCl and bubbled with air for 30 min, a period that was sufficient to remove $NaH^{14}CO_3$. Aquasol (2.0 ml) was then added to 1 ml of the bubbled aqueous sample, and radioactivity was determined as described above by using the internal standard method to determine disintegrations per minute. After the radioactivity in cells and the aqueous phase was calculated per milliliter of original homogenate, the percentage of total photosynthate recovered in the aqueous phase was determined by dividing the radioactivity in the aqueous phase alone by that in the cells and the aqueous phase.

TABLE 1. $NaH^{14}CO_3$ fixation by Mushroom Spring mat samples[a]

Condition	Cellular dpm/ml	Aqueous dpm/ml	% ^{14}C in the aqueous phase
Dark	18,600*	23,600*	55.9*
Light	1,020,200	104,800	9.4
Light + O_2	852,100	113,900	11.8*

[a] Dissolved oxygen concentration was measured in samples of the same homogenate used in this experiment; data are presented in Fig. 1. *, Significantly different ($P < 0.05$) value compared with that of the light-incubated controls; $n = 6$.

[^{14}C]glycolate determination. The aqueous phase from radiolabeling experiments was separated into neutral and acidic components with an anion-exchange column (6 mm [inner diameter] by 5 cm; Dowex 2X8-400; Sigma) that was preconditioned with 1 M formate. Samples that were made alkaline by the addition of 1 drop of 6 N NaOH were applied to the column, which was then washed with 4 ml of distilled water. The sample was then eluted with 8 ml of 1 M formate; 1-ml fractions were collected. Neutral compounds were considered to be those that were recovered in the fraction that eluted before glycolate. Organic acid-containing fractions (as determined by the location of [^{14}C]glycolate) were combined, frozen at −70°C, and lyophilized. The sample was suspended in 0.5 to 1 ml of methanol and centrifuged for 2 min (Eppendorf; Brinkmann Instruments) to remove insoluble material. The methanol phase was evaporated under a stream of nitrogen gas, and the sample was resuspended in 50 or 100 μl of methanol.

[^{14}C]glycolate was separated from other radiolabeled compounds in the organic acid fraction by two-dimensional thin-layer chromatography (TLC) (7). Radioactively labeled spots were visualized by autoradiography after TLC sheets were treated with En^{3}Hance (New England Nuclear) and exposed to film (X-OMAT AR; Eastman Kodak Co., Rochester, N.Y.) for 7 days at −70°C. Spots that were visible on the autoradiogram were traced onto the glass microfiber TLC sheet. These areas were cut out and placed in 10 ml of Aquasol for the determination of radioactivity as described above. The position of glycolate in TLC analyses was determined by the comigration of radioactive and nonradioactive glycolate, the latter of which was visualized by spraying the TLC sheet with 0.04% bromophenol blue (ethanolic solution). The radioactivity in the glycolate spot was corrected to a percentage of the radioactivity in either the aqueous fraction or the total fixed ^{14}C (cells plus aqueous fraction).

Statistical methods. A two-sample Student's *t* test was used to compare the means of replicate samples (17).

RESULTS

Production of extracellular photosynthate. Light stimulated the incorporation of $NaH^{14}CO_3$ into cells from the Mushroom Spring mat (Table 1). Fixed ^{14}C was also detected in the aqueous phase of the samples. The amount detected was over 4 times greater in samples incubated in the light than in those incubated in the dark, indicating the production of extracellular photosynthetically derived carbon. However,

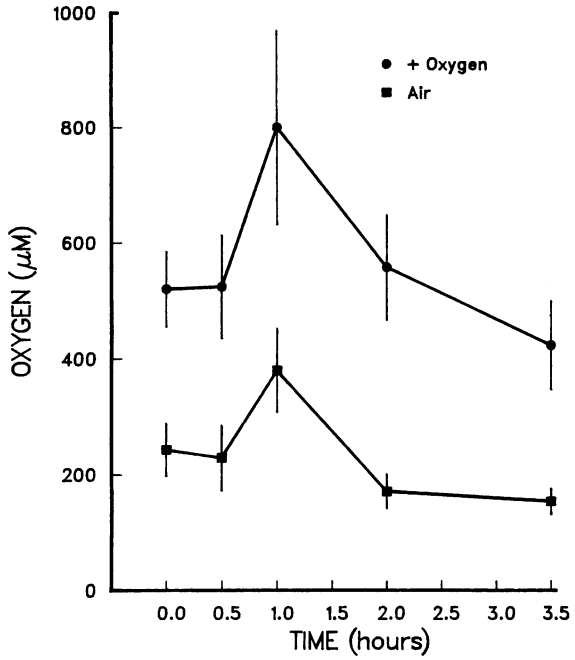

FIG. 1. Dissolved oxygen concentration during incubation of Mushroom Spring mat samples with O_2 or air headspace. Bars indicate standard error (n = 3).

the percentage of $NaH^{14}CO_3$ fixed that was detected in the aqueous phase was much higher in the samples incubated in the dark.

O_2 addition to the vial headspace increased the dissolved O_2 concentration within samples (Fig. 1). Although the O_2 concentration varied with time during the incubation of mat samples contained in closed vials, it was, on average, 2.6 times higher in O_2-enriched samples than in samples incubated with air in the headspace. The peak oxygen concentration in samples with added oxygen was 800 μM, which was 5.3 times that of the spring water overlying the mat (150 μM; ca. 100% saturation at this temperature and atmospheric pressure). O_2 addition resulted in an increase in the percentage of total photosynthate that was detected in the extracellular fraction (Table 1).

TABLE 2. Importance of glycolate as an extracellular photosynthetic product in Mushroom Spring mat samples incubated with and without added O_2[a]

Condition	% ^{14}C in the aqueous phase	Glycolate as % of aqueous photosynthate	Glycolate as % of total photosynthate
Dark	55.9*	0.0*	0.0*
Light	9.4	45.8	4.3
Light + O_2[b]	11.8*	57.9*	6.8*

[a] Includes the example shown in Fig. 2a. *, Significant differences ($P < 0.05$) of dark or oxygenated samples compared with light controls (n = 2).

Identification of glycolate as the major excretion product. The aqueous fraction from $NaH^{14}CO_3$ labeling experiments on Mushroom Spring samples was separated into organic acid and neutral fractions by ion-exchange chromatography. On average, 35% of the radioactivity in the aqueous fraction from samples incubated in the light was recovered as neutral products and 65% was recovered as organic acids. An analysis of organic acids showed that glycolate was a major product, although other compounds in the organic acid fraction (not identified) were also observed (Fig. 2a). The addition of oxygen increased the percentage of glycolate that was produced during photosynthesis (Table 2). Glycolate made up 46% of the excreted photosynthate in the control and 58% of the excreted photosynthate in the presence of added O_2. In the dark, nearly all of the radioactivity was recovered in the organic acid fraction, but glycolate was not detected. Under these conditions one acidic compound accounted for most of the radioactivity in the aqueous fraction (Fig. 2b).

Fate of [1-^{14}C]glycolate. In Mushroom Spring mat samples incubated in the light, [1-^{14}C]glycolate was both incorpo-

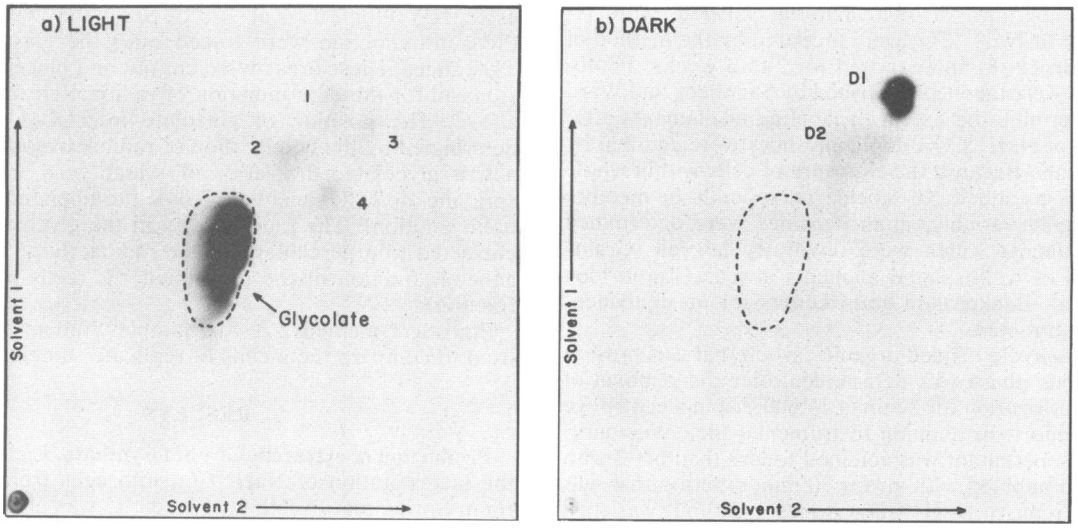

FIG. 2. Autoradiograms of thin-layer chromatograms showing ^{14}C-labeled organic acids excreted during $NaH^{14}CO_3$ labeling of Mushroom Spring mat suspensions. (a) Light conditions (with added O_2); (b) dark conditions. The position of glycolate, as determined by analysis of standards, is indicated by the dashed line. The solvent systems were petroleum ether-anhydrous ether-formic acid (28/12/1) (solvent 1) and chloroform-methanol-formic acid (80/1/1) (solvent 2).

TABLE 3. Fate of [1-^{14}C]glycolate in Mushroom Spring mat samples and pure cultures of *S. lividus*

Sample and condition	% ^{14}C in the following fractions[a]:		
	CO_2	Cells	Aqueous
Mushroom Spring			
Light, 2 h	16.8 ± 1.3	50.3 ± 4.8	32.9 ± 5.6
Formalin[b]	2.4 ± 0.2	0.7 ± 0.1	97.0 ± 0.3
S. lividus			
Light, 2 h	0.2 ± 0.1	0.7 ± 0.3	99.1 ± 0.3
Light, 6 h	0.2 ± 0.0	0.1 ± 0.0	99.7 ± 0.0
Light, induced[c]	0.2 ± 0.0	0.0 ± 0.0	99.8 ± 0.0

[a] A total of 96.5% of the added ^{14}C was recovered. Values are expressed as the mean ± standard error (n = 3).

[b] Samples were injected with Formalin before incubation with [1-^{14}C] glycolate. The biological activity in all other samples was terminated with Formalin following incubation.

[c] Samples were preincubated with 0.1 μM glycolate for 4 h before incubation for 2 h with [1-^{14}C]glycolate.

rated and oxidized to $^{14}CO_2$ (Table 3). In pure cultures of *S. lividus*, incorporation of [1-^{14}C]glycolate into cells or oxidation to $^{14}CO_2$ was negligible. Extension of the incubation time to the doubling time of the culture (6 h) did not increase the uptake or metabolism of glycolate. Preincubation with unlabeled glycolate did not induce metabolism by *S. lividus*.

Autoradiograms prepared from light-incubated samples of the Mushroom Spring mat revealed that filamentous cells, but not *S. lividus*, incorporated [1-^{14}C]glycolate (Fig. 3). Both wide (ca. 1 to 2 μm) and narrow (<1 μm) filaments were labeled (data not shown).

Effects of glycolate addition on the fate of photoexcreted compounds. The addition of nonradioactive glycolate during $NaH^{14}CO_3$ labeling of whole cores or homogenates from Mushroom Spring mat samples increased the amount of ^{14}C-labeled compounds within the aqueous phase, nearly doubling the percentage of total photosynthate recovered in the aqueous phase (Table 4). Microautoradiograms prepared from samples incubated under similar conditions revealed that both *S. lividus* and filamentous cells (of both wide and narrow diameters) incorporated ^{14}C (Fig. 4). The addition of glycolate reduced the amount of labeling of the filaments, with grain densities ranging from 42 to 65% of those for labeled filaments in the control. The differences in mean filament grain densities between controls and samples with added glycolate were significant at $P \leq 0.008$ in four separate comparisons done by two individuals on duplicate sets of autoradiograms.

TABLE 4. Effect of glycolate addition on $NaH^{14}CO_3$ fixation by Mushroom Spring mat whole core or homogenized samples incubated in the light[a]

Sample and condition	Cellular dpm/ml	Aqueous dpm/ml	% ^{14}C in the aqueous phase
Homogenized			
Control	1,551,100	123,500	7.4
Glycolate	1,115,900*	179,000*	13.5*
Whole cores			
Control	1,066,200	36,000	3.3
Glycolate	713,500*	56,100*	7.2*

[a] *, Significant differences ($P < 0.05$) of samples with glycolate compared with controls (n = 6).

DISCUSSION

The responses of Mushroom Spring mat samples to changing O_2 levels were consistent with the hypothesis that

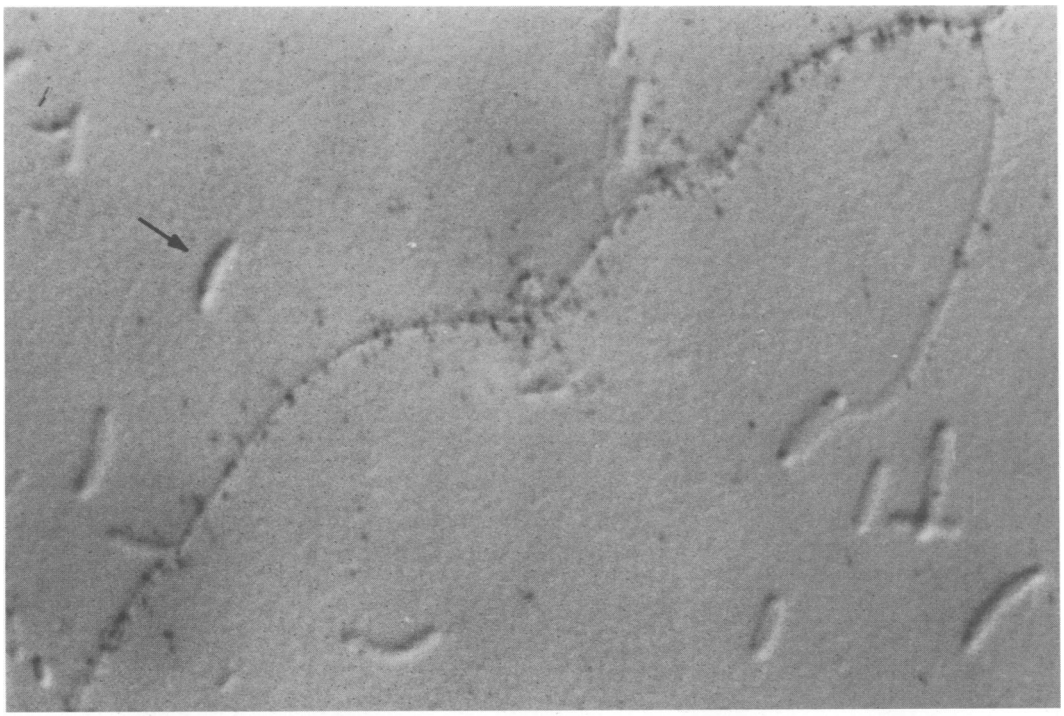

FIG. 3. Microautoradiogram prepared from homogenized cells from a 55°C mat sample from Mushroom Spring that was incubated in the light with [1-^{14}C]glycolate. A typical *S. lividus* cell is indicated by the arrow. Magnification, ×2,900.

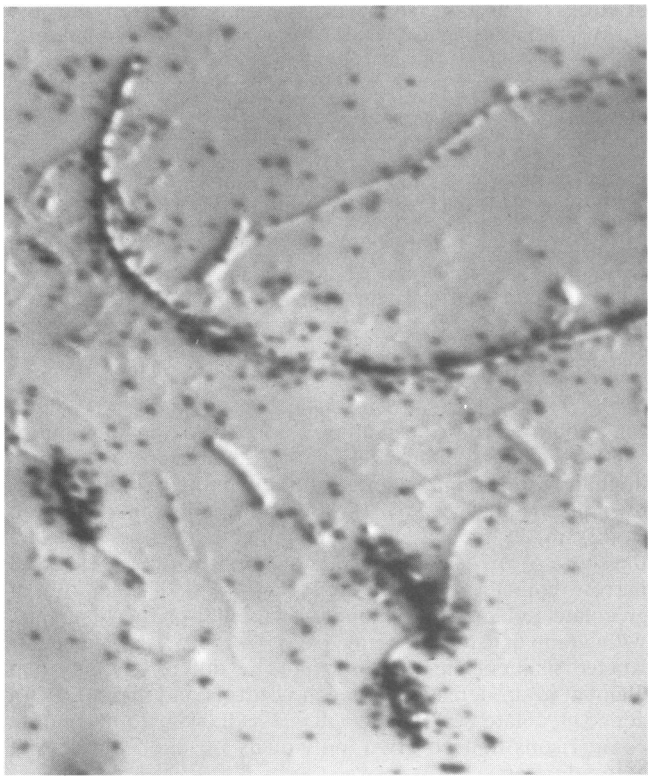

FIG. 4. Microautoradiogram prepared from a whole core sample from the 55°C mat from Mushroom Spring that was incubated in the light with $NaH^{14}CO_3$. Magnification, ×2,900.

glycolate, a product of the oxygenation of the Calvin-Benson cycle intermediate ribulose-1,5-diphosphate, is an important component of the extracellular material detected. A decrease in the CO_2 concentration also increases the amount of excreted photosynthate (M. M. Bateson and D. M. Ward, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I-103, p. 163). Other investigators have demonstrated that high oxygen and low carbon dioxide partial pressures enhance glycolate biosynthesis and excretion of the photoassimilated carbon by a variety of phototrophic organisms (3, 5, 12, 13, 23, 26). The extracellular photosynthate detected in samples from the Mushroom Spring microbial mat was likely produced by the cyanobacterium *S. lividus*, because anoxygenic photosynthesis by the other known mat phototroph, *Chloroflexus aurantiacus*, is probably prevented by oxygen exclusion of sulfide from the photic zone at the light intensities used in our experiments (18).

Analysis of the photosynthate recovered in the aqueous phase revealed that glycolate was a major photoexcretion product in the Mushroom Spring mat sample. Its production was light dependent and increased on the addition of O_2. We estimated (Table 2) that glycolate represents 46 to 58% of the excreted photosynthate, depending on the oxygen concentration. The O_2 concentration varies both with time of day and within the vertical profile of the mat (18). The higher estimate was determined from samples with dissolved O_2 concentrations similar to those of the upper mat layers in full sunlight. The lower estimate was derived from samples in which the O_2 concentration was closer to that of the mat in early morning or late evening.

In the mat glycolate is taken up by filamentous bacteria with diameters corresponding to those of both *C. aurantiacus* and unidentified narrower filaments found in the mat (24). Tayne (T. A., M. S. thesis, Montana State University, Bozeman, 1983) demonstrated that although glycolate uptake is light dependent (15 versus 3.5% of added glycolate recovered in cells during light and dark incubation, respectively), oxidation to CO_2 is not entirely light dependent (27.5% in light versus 19.5% in dark in CO_2). This suggests the possibility that both photo- and chemoheterotrophic organisms are responsible for glycolate metabolism, which is consistent with the results obtained on the microautoradiograms, on which two types of labeled filaments were observed.

Cross-feeding of glycolate (and probably other photoexcretion products) from phototrophs to heterotrophs was demonstrated by microautoradiograms on which labeling of both *S. lividus* and filaments in the mat during incubation with $NaH^{14}CO_3$ were observed. The possibility of autotrophy by *C. aurantiacus* or an unknown filamentous sulfide-oxidizing bacterium that accounts for the labeling of filaments is unlikely due to the oxygen exclusion of sulfide (18) under the experimental conditions. The sensitivity of labeling of filaments to the addition of nonradioactive glycolate strongly suggests that a major portion of ^{14}C in the filaments (if not all ^{14}C in the filaments) was due to secondary labeling from photoexcreted metabolites. The entrapment of excreted [^{14}C]glycolate in a large pool of nonradioactive glycolate was expected. Alternative explanations such as direct inhibition of CO_2 fixation by glycolate seem less plausible, especially as *S. lividus* fixed CO_2 readily in the presence of glycolate. These results suggest that there is a linkage between glycolate excretion by *S. lividus* and consumption by filaments.

The experimental method used to determine percent excretion did not distinguish between the amount of ^{14}C fixed in phototrophs or cross-fed to heterotrophic cells. As a consequence, the percentage of photosynthate in the aqueous phase of control samples was probably an underestimation of the actual photoexcretion by *S. lividus* in the mat. However, our estimates of the importance of glycolate among extracellular products should be realistic, assuming that other excretion products are cross-fed with the same efficiency as glycolate. The reduction in filament labeling during incubation with $NaH^{14}CO_3$, which was caused by addition of glycolate (35 to 58%), is consistent with the percentage of glycolate determined among photoexcretion products (45 to 58%). The observation that labeling of filaments was not totally eliminated by the addition of glycolate supports the hypothesis that other excretion products are also cross-fed.

A high percentage of $^{14}CO_2$ fixed by the Mushroom Spring mat community in the dark was recovered in the aqueous phase, and a single unknown compound represented most of this excreted ^{14}C. In the dark, many mechanisms may account for CO_2 fixation in this community. Thermophilic, chemolithotrophic, and autotrophic bacteria such as *Hydrogenobacter thermophilus* (16, 22) and *Methanobacterium thermoautotrophicum* (20) have been isolated from hot spring environments. *Clostridium thermoautotrophicum*, which, like other homoacetogenic bacteria (25, 33), produces acetate from molecular hydrogen and carbon dioxide, has been isolated from an alkaline silicious hot spring in Yellowstone National Park (32). Because most of the carbon fixed by the mat community in the dark was excreted, it seems likely that catabolic CO_2 reduction by such an organism might occur in the mat under these conditions.

This research has increased our understanding of the

carbon flow within hot spring cyanobacterial mat communities. In the light, a significant proportion of the carbon dioxide fixed by *S. lividus* is excreted as organic carbon, but determination of the actual percentage excreted is complicated by the simultaneous uptake of the excretion products by heterotrophs. Intensive photosynthetic oxygen production (and presumably CO_2 consumption) by *S. lividus* within this benthic community of extremely high population density creates an environment which stimulates the production and excretion of glycolate. This compound constitutes a major portion (up to 58%) of the excreted material when the oxygen concentration is close to that of the top layers of the microbial mat throughout most of the day. The importance of glycolate is somewhat lower in the early morning or late evening, when light intensity and oxygen production are lower. Filamentous bacteria in the mat incorporate the excreted glycolate. In the dark, the fermentation of polymeric organic matter leads to the production of organic acids which supply heterotrophic microorganisms, including *Chloroflexus aurantiacus* (2). Another fermentation product, hydrogen, drives CO_2 reduction to methane (19), but CO_2 reduction to an unknown compound in the dark suggests that other reductive processes may also be important.

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